Medical and Molecular Virology

Medical and Molecular Virology

ERICA SUCHMAN

Contents

Welcome to Medical and Molecular Virology. This book was created for upper division microbiology students studying virology. It will describe the molecular biology and major diseases of virus families that cause significant disease in animals and humans. This book is by no means meant to be exhaustive. In fact, because virology can be so overwhelming, I have tried to keep the book as simple as possible, while still giving the reader a solid understanding of the molecular mechanisms of viral replication and pathogenesis.

This book is different than other virology textbooks in that it is laid out grouping viruses by how they replicate. This should hopefully allow you, the reader, to think about why these viruses replicate similarly, and why replication strategies may be different than those used by other viruses.

Special thanks go to my father Daniel Suchman for his patient and forthright editing.
Erica Suchman, PhD.

Suchman, Medical and Molecular Virology

ERICA SUCHMAN

Medical and Molecular Virology

Medical and Molecular Virology

ERICA SUCHMAN

Contents

Welcome to Medical and Molecular Virology. This book was created for upper division microbiology students studying virology. It will describe the molecular biology and major diseases of virus families that cause significant disease in animals and humans. This book is by no means meant to be exhaustive. In fact, because virology can be so overwhelming, I have tried to keep the book as simple as possible, while still giving the reader a solid understanding of the molecular mechanisms of viral replication and pathogenesis.

This book is different than other virology textbooks in that it is laid out grouping viruses by how they replicate. This should hopefully allow you, the reader, to think about why these viruses replicate similarly, and why replication strategies may be different than those used by other viruses.

Special thanks go to my father Daniel Suchman for his patient and forthright editing.
As well as Ben Greenlee, Deryl Kenney, and Dylan Woods as well as many students
who provided honest and useful feedback. I thank you all.
Erica Suchman, PhD.

Medical and Molecular Virology, Erica Suchman

Suchman, Medical and Molecular Virology

ERICA SUCHMAN

CHAPTER 1

Chapter 1: Introduction To Medical And Molecular Virology.

INTRODUCTION TO VIRUSES

The ability of viruses to infect humans, plants, and animals has been documented in stone tablets as far back as 1580 BC. Even so, viral infection continues to be a problem today. In this chapter we will look at why viruses are so difficult to treat and the best practices for infection prevention. We will also explore the life cycles of the major virus families that cause disease in humans and animals as well as the major diseases associated with these families. Please note this book is not meant to be exhaustive, but rather it is intended to give the reader an introduction to the major families of viruses that cause human and animal diseases as well as a familiarity with the most common diseases.

Vaccination is the best-known way to prevent viral disease. The first vaccines were developed in the 1880s to counter the smallpox virus. The earliest vaccinations involved taking scrapings from smallpox pustules and scratching them into the skin of people who had not contracted smallpox already. Although these people also developed smallpox, their symptoms were less severe and they were less likely to die than those who contracted the disease naturally, where the case-fatality rate was about 30%. Edward Jenner noticed that milk maids who had developed cow pox lesions on their hands after milking infected cows were less likely to contract smallpox. Jenner modified the vaccine, replacing the smallpox lesion scrapings with scrapings from cow pox lesions. This turned out to be far safer, causing only a pox lesion to form at the vaccination site and still providing protection from smallpox. Later, in the 1800s, the virus was changed to Vaccina, the virus that is still used in vaccinations today. Currently, only military personnel and first responders are vaccinated. Vaccination of the general public continued until after the last naturally occurring case of smallpox infection in 1978. Small stocks of smallpox still exist however, but by international agreement, they are not used and are reserved for use in research. They could potentially be used as weapons of bioterrorism, hence the need for vaccination of military personnel and first responders.

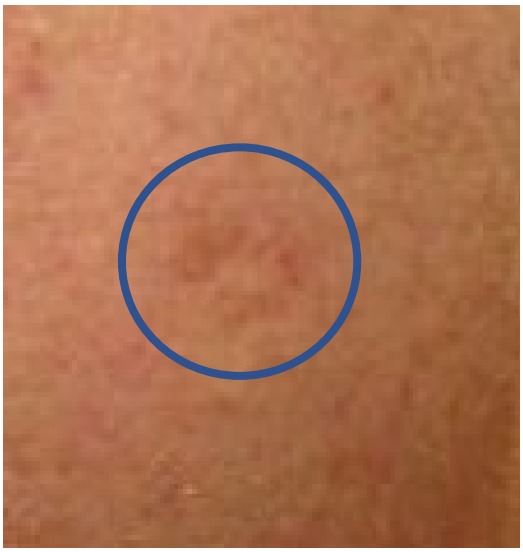


Figure 1-1. Smallpox vaccination scar. Photo courtesy of Erica Suchman, Colorado State University.

Viruses can cause many different types of diseases including cancer. Viruses were first hypothesized to cause cancer in 1911 by Peyton Rous who was trying to determine the cause of his neighbor's chickens' tumors. He ground up the tumors, filtered the solution, and injected the filtrate into apparently healthy chickens. He found that they too developed tumors. It was known in 1911 that bacteria would not be able to pass through the filter into the filtrate, so he hypothesized that the tumors were caused by a virus he named Rous sarcoma virus. This theory was widely disregarded at the time as it was not believed that viruses could cause cancer. He submitted grant requests that were all rejected and eventually, dejected and discouraged, he gave up work on Rous sarcoma virus. Many other scientists over the years theorized the same and were roundly ignored or ridiculed. It wasn't until the 1950s that Howard Temin developed a set of experiments to show that Rous sarcoma virus was indeed causing tumors. Even more shocking was that a retrovirus did not follow the central dogma of molecular biology that DNA creates RNA which creates protein.

Retroviruses create DNA from RNA (using reverse transcriptase) which then makes RNA and then protein thus putting Rous and Temin at direct odds with the Nobel laureate Francis Crick, one of the co-discoverers of the structure of DNA (along with Rosalind Franklin and James Watson) who widely criticized Temin's work. However, Temin was correct, and in 1975 Temin and David Baltimore received the Nobel prize in Physiology/Medicine for their work on Rous sarcoma virus.

HOW DO WE DEFINE A VIRUS?

- They are so small in size that they can pass through a filter into the filtrate.
- They have a viral genome of nucleic acids (either RNA or DNA) that is surrounded by a protein coat.
- They are obligate intracellular parasites.
- Their virions self-assemble from newly synthesized components.

THE STRUCTURE OF VIRUSES

A **virion** is a complete infectious virus particle outside of a cell. The major structural components of a virion are:

- A nucleic acid genome of either RNA or DNA.
- Linear, circular, or segmented nucleic acids. **Segmented** nucleic acids mean there is more than one piece in the genome.
- Double stranded RNA or DNA.
- Single stranded DNA or RNA.
- Single stranded RNA can be either positive sense stranded, or negative sense stranded.
- Positive sense stranded RNA is equivalent to mRNA and can be translated.
- Negative sense stranded is complementary to mRNA and as such cannot be translated and must be used to create mRNA before translating protein.
- A capsid or nucleocapsid. A protein shell surrounding the genome.
- A **capsid** is a protein shell that holds the nucleic acid but is not structured around it.
- A **nucleocapsid** is capsid protein that directly associates with the nucleic acids of the genome. Also can be called a **ribonucleocapsid** or **ribonucleoprotein**.
- It may also have an **envelope**, which is made up of cellular membrane that is stolen from an infected cell as well as viral envelope proteins.
- If a virus has an envelope it is called enveloped.
- If a virus lacks an envelope it is called a naked virus.

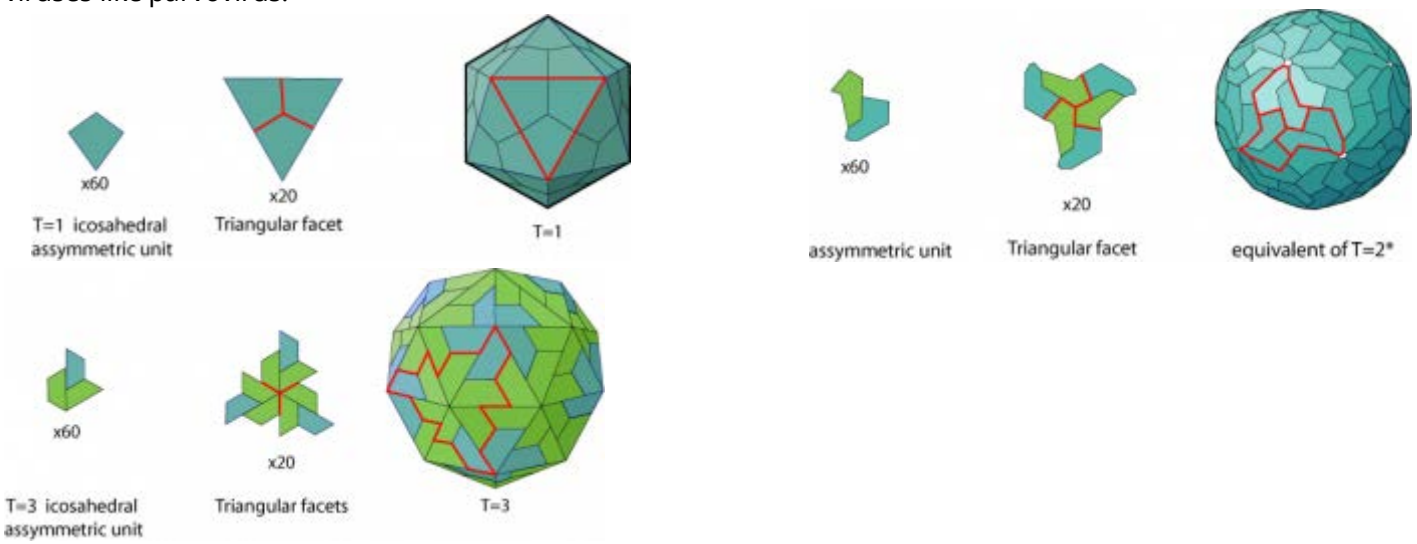
INFORMATION CONTAINED WITHIN VIRAL GENOMES

The viral genome must contain all of the genes to create any proteins required to make up the virus. It must also contain any replication enzymes not provided by the cell, as well as any proteins/enzymes they make to modify the host cell. Which of the cell's enzymes a virus can use is determined by their genome type and site of replication and will be discussed at length in future chapters.

All viruses will have genes for the capsid proteins that make up their capsid or nucleocapsid.

Capsids, which are most commonly icosahedral in animal viruses, are built of identical copies of a small number of proteins. These proteins assemble to form a structural unit. The structural units then arrange symmetrically around the viral genome, as shown in Figures 1-2 & 1-3. These proteins, arranged to provide maximal contact, are held together by non-covalent bonds. Why do you think these bonds need to be non-covalent? The simplest

arrangement contains 60 subunits with 3 units on each triangular face. Parvoviruses are only 18-26 nm in diameter with a genome of only 5kb. As viruses get larger genomes, they require larger more complicated capsids with larger numbers of subunits as shown in Figures 1-3 and 1-4. For efficiency, viruses create a capsid that fits their genomes well, thus viruses cannot package much more nucleic acid than their usual genome size. This limitation becomes important when we start to think about genetically modifying viruses as you cannot add many genes before they become too large to package. Larger viruses by necessity have larger more complicated capsids than the simple viruses like parvovirus.



Figures 1-2 to 1-4. A T=1 icosahedral capsid which is seen in the smallest viruses (Figure 1-2). As viruses get larger, they use more proteins to create their capsids, as shown in Figures 1-3 and 1-4. Courtesy of ViralZone https://viralzone.expasy.org/833

Some viruses such as Adenovirus have structurally complex capsids. Adenovirus has spikes that protrude from the capsid. These are helpful in entering the cell. They also weaken the gaps between cells in the **mucosal membranes** of the nasal passages leading to runny nose with fluids full of virus. How brilliant is that! Figure 1-5.

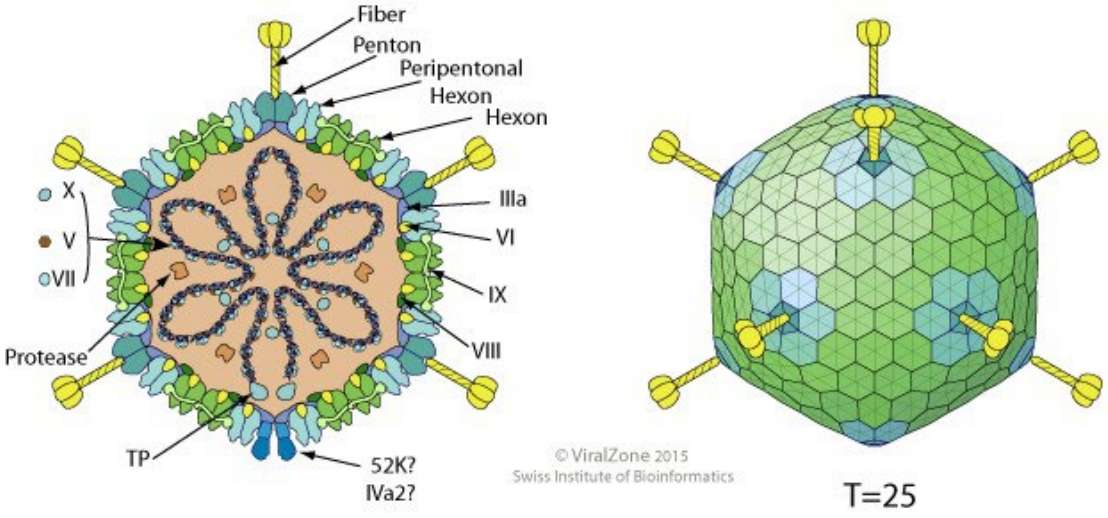
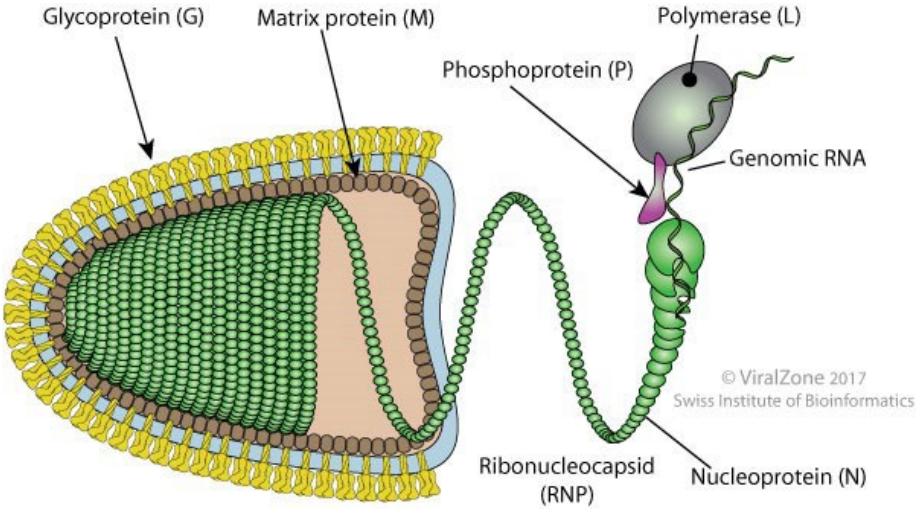
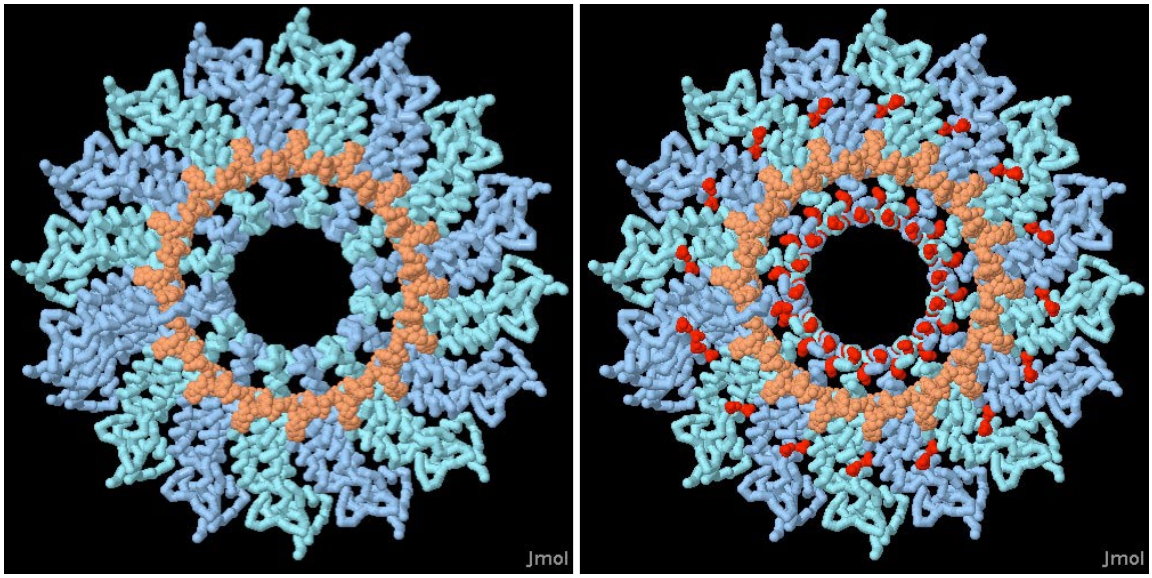


Figure 1-5. Adenovirus capsid with cell-binding fibers. Courtesy of ViralZone www.viralzone.org/2378

Nucleocapsids are seen in **helical viruses** where the capsid proteins accumulate along the nucleic acids. These are also referred to as ribonucleoprotiens (RNP). Each capsid protein binds 3 nucleotides with 16.3 capsid protein molecules per turn making a protective coat of protein around the nucleic acids as is seen in Figure 1-6 and 1-7.





Figures 1-6 and 1-7. The nucleocapsid of rhabdovirus showing a helical structure. courtesy of ViralZone: https://viralzone.expasy.org/2 & https://pdb101.rcsb.org/motm/109

Some viruses will also have an envelope. All enveloped viruses contain genes for envelope proteins. These proteins are integral membrane proteins that insert themselves into the host cell plasma membrane. As you can see in Figures 1-8 and 1-9, the proteins are **integral**, meaning they traverse the entire membrane with portions extruding outside the virion and portions sticking inside the virion, often interacting with the capsid inside.

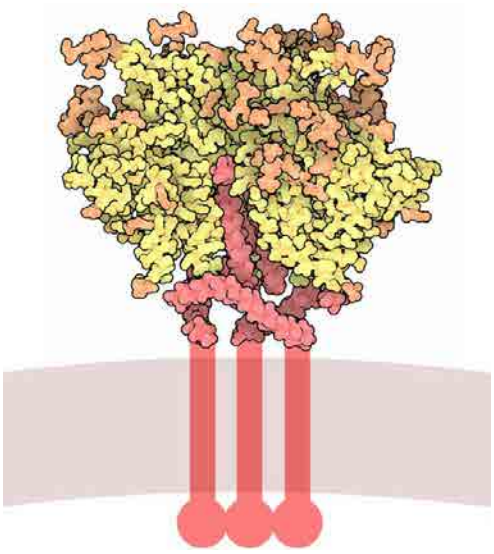


Figure 1-8. The integral envelope protein of HIV traversing the envelope membrane, courtesy of the Protein Data Base https://pdb101.rcsb.org/motm/169

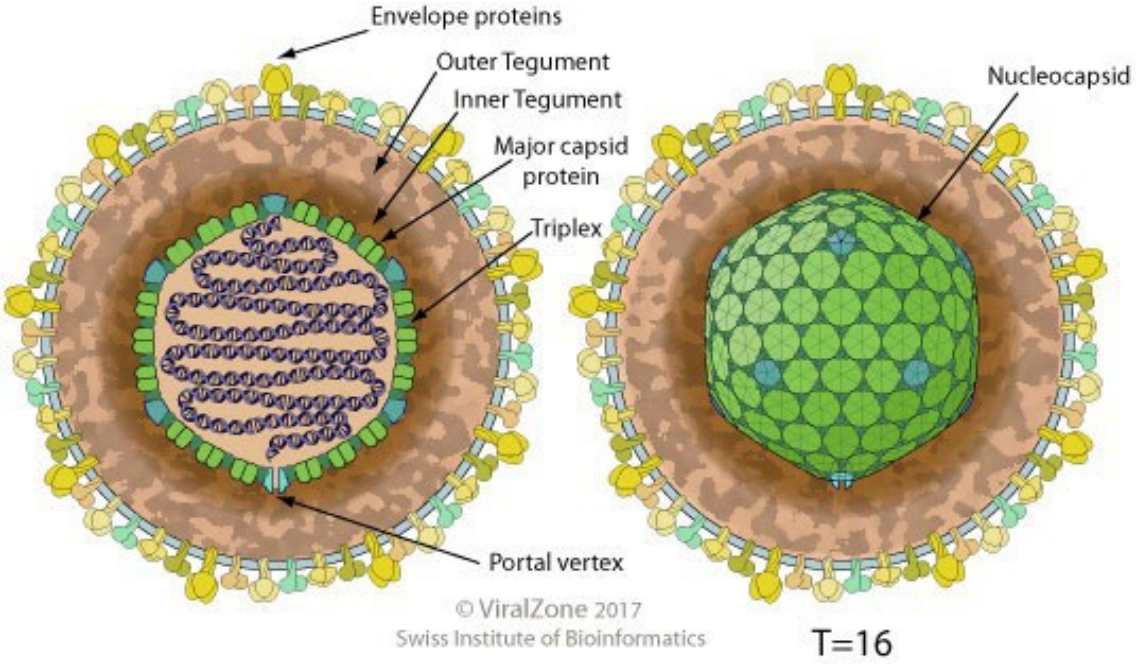


Figure 1-9. The integral envelope protein of Herpesviridae traversing the envelope membrane, courtesy of ViralZone. https://viralzone.expasy.org/176

Along with the structural proteins, there are many replication enzymes. The most common examples are detailed below.

Other genes that may be found in viruses include **RNA-dependent RNA polymerases** (RdRp). All RNA viruses except Retroviruses have genes for RdRp. **RNA-dependent** means the template it reads is RNA, and **RNA polymerase** means it polymerizes (synthesizes) RNA. Most RNA viruses except retroviruses must synthesize RNA from RNA to replicate their genome and make their proteins. This is not a protein that is available to viruses from the host cell, so they must produce their own.

Reverse transcriptase (RT) is used to create double stranded DNA from RNA. This is used by both retroviruses and hepatitis B, although they use RT in different locations. Retroviruses package a single stranded RNA genome that is delivered to a host cell with RT which converts it into double stranded DNA upon entering the cell. Hepatitis B packages single stranded RNA, but RT converts it to a double stranded DNA genome in the virion and this DNA genome is delivered to the cell. All such viruses in the family *Retroviridae* are classified as RNA viruses, although they spend the majority of their life cycle functioning as DNA viruses. Viruses in the family *Hepadnaviridae*, the family to which hepatitis B virus belongs, are classified as DNA viruses. Note that reverse transcriptase is an **RNA-dependent DNA polymerase** (RdDp) that takes RNA and creates DNA, however once it creates the first strand of DNA, it must degrade the template RNA using an **RNAse H** activity leaving the DNA strand to serve as a template for the second strand of DNA using the RT **DNA-dependent DNA polymerase** (DdDp) activity. As such, this one enzyme has 3 functions.

One of the big issues that all animal viruses need to overcome is the fact that eukaryotic cells only make one protein per mRNA. So, how do viruses with very small genomes produce many proteins in animal cells? One mechanism, commonly used by positive sense RNA viruses and some other viruses, is to create one gigantic polypeptide and then cleave this into the many proteins the viruses will need using a virally encoded **protease**. This protease **autocleaves** itself out of the polypeptide and then cleaves out the rest of the necessary proteins. You will see this mechanism used by many of the viral families in later chapters.

Retroviruses always integrate their viral genome into the host cell DNA forming a **provirus** (much like prophage of bacteria). The cell certainly does not want to integrate viral DNA into host cell genomic DNA so the virus needs to carry the genes to produce **integrase**, the enzyme that will integrate the viral genome into the host genome. You will see in future chapters that other DNA animal viruses can sometimes integrate (Chapters 6, 7, 8, 11), but only retroviruses *must,* and therefore have genes for integrase (Chapter 12).

Some of the larger viruses will have other viral proteins such as the **tegument proteins** that occupy the space between the capsid and envelope of herpesviruses, as shown in Figure 1-9. Some of the DNA viruses will wrap their genomes in histone proteins just like the host chromosome does, a brilliant way of convincing the host cell it is replicating its own genome. Polyomaviruses, papillomaviruses, and hepadnaviruses do this. An example of a histone protein associated with DNA is shown in Figure 1-10.

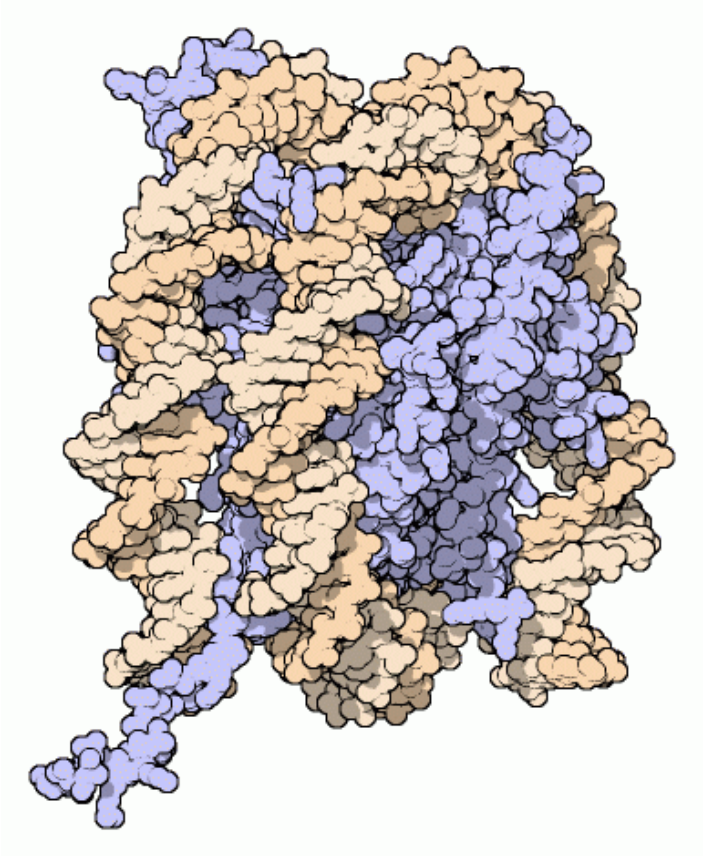


Figure 1-10. A nucleosome with DNA in orange and histone proteins in purple, courtesy of the Protein Data Base https://pdb101.rcsb.org/motm/7

Now that we know the basic structure of viruses and what genes they will contain, let's look at how we classify viruses.

VIRAL CLASSIFICATION

Viruses are simple. As a result, we have little to classify them. Therefore, we use the following viral characteristics:

- Nature of the nucleic acid in the virion.
- Symmetry of the capsid (shape).
- Presence or absence of envelope.
- Nature of diseases they cause.
- Strategy of viral replication as described by the "**Baltimore scheme**". David Baltimore developed this scheme to classify viruses by their replication cycles.
- Viruses are put into families and genera by the International Committee on Taxonomy of Viruses (ICTV). Families have changed over the years and continue to change as we learn more about viruses.

The International Committee on Taxonomy of Viruses (ICTV) nomenclature dictates that:
Order- names end in *virales*
Family- names end in *viridae*
Subfamily- names end in *virinae* Genus- names end in *virus*

Note that order, family, subfamily, genus, and species names are to be italicized. Phylum, class, order, genus, and species names are to be capitalized. It is not necessary to capitalize virus names, although some virus names that are proper nouns, such as "St. Louis encephalitis virus," may be capitalized.

An example for the *Herpesviridae* family is shown below. Ex: *Herpesviridae*
Subfamily*: Alphaherpesvirinae*
Genus: *Simplex virus*
Subfamily: *Betaherpesvirinae*
Genus: Cytomegalovirus
Subfamily *: Gammaherpesvirinae*
Genus: *Epstein-Barr virus*

The one thing that must be memorized in virology is what type of genome does each virus family have (Table 1). I suggest you begin this process soon so that trends in replication styles will become evident to you as you learn about each of the viral families. However, this is where your memorization should turn into looking for trends and articulating how you understand these trends. As you can see, there are many families of viruses. If you only memorize what each virus does without giving thought to why it does that, and, by extension, which other viruses might behave similarly, learning all these viruses will be much more difficult.

DNA viruses					
Polyomaviridae	Papillomaviridae	Adenoviridae	Herpesviridae	Parvoviridae	Poxviridae
Viruses that use Reverse transcriptase DNA					
Hepadnaviridae					
Viruses that use Reverse transcriptase RNA					
Retroviridae					
RNA Viruses double stranded					
Reoviridae					
RNA Viruses negative stranded					
Order: Mononegavirales	Filoviridae	Paramyxoviridae	Pnuenoviridae	Rhabdoviridae	Order: Bunyavirales
Phenuiviridae	Hantaviridae	Nairoviradae	Arenaviridae	Orthomyxoviridae	
RNA Viruses positive stranded					
Flaviviridae	Picornaviridae	Togaviridae	Caliciviridae	Coronaviridae	Matonaviridae

Table 1. Genome types of virus families that infect animals or humans.

Classifying viruses by their replication strategies was proposed by David Baltimore, the Nobel laureate who proved that viruses could cause cancer and identified reverse transcriptase, along with Howard Temin. The Baltimore classification scheme is shown in Figure 1-11. Group I viruses are double stranded DNA viruses that create mRNA that creates protein (many viruses). Group II viruses are single stranded DNA viruses that create mRNA that creates protein (such as parvoviruses). Group III viruses are double stranded RNA viruses that create mRNA that creates protein (such as reoviruses). Group IV viruses are positive sense RNA viruses that use their genome to make proteins but must also make complementary negative sense RNA to use as a template for

creating more positive sense RNA (many virus families). Group V viruses are negative sense RNA viruses that must create mRNA to make proteins and as a template to create more negative sense RNA (many viruses). Group VI viruses are positive sense RNA viruses that use reverse transcriptase (RT) to create a double stranded DNA that integrates into the genome and then creates viral mRNA that can both make proteins and be packaged as genome (retroviruses). Group VII viruses package RNA and convert it to DNA using reverse transcriptase but release double stranded DNA into the cell that will create mRNA which will create protein (hepadnaviruses).

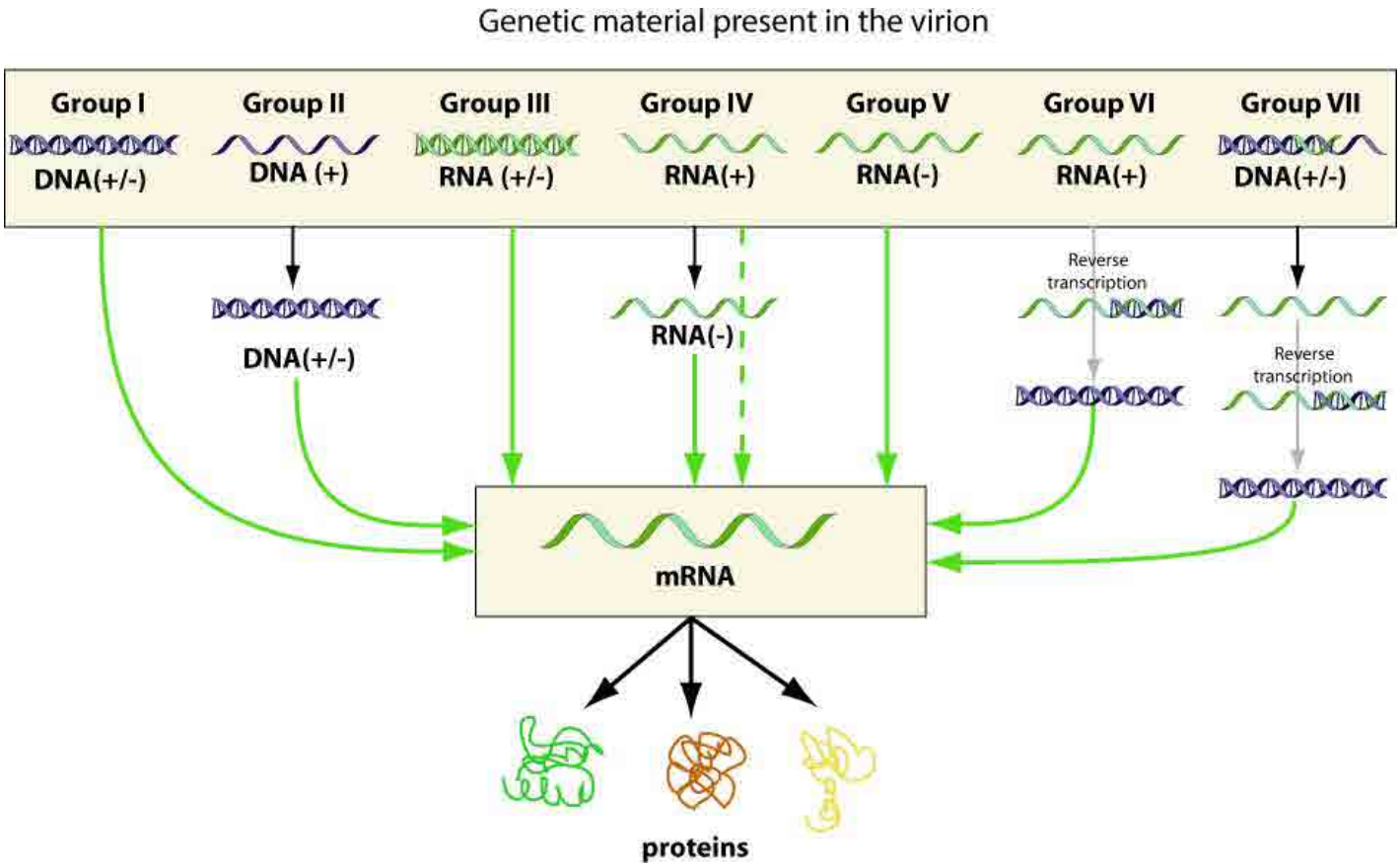
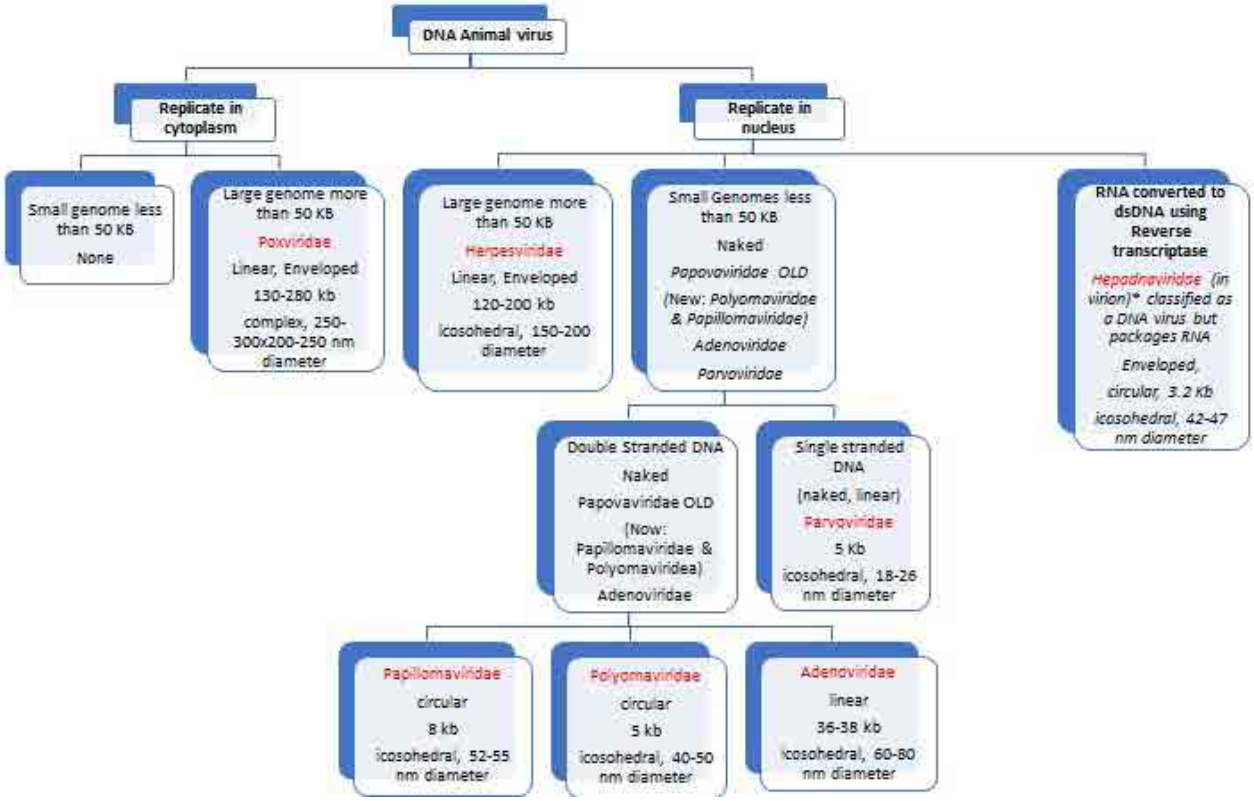
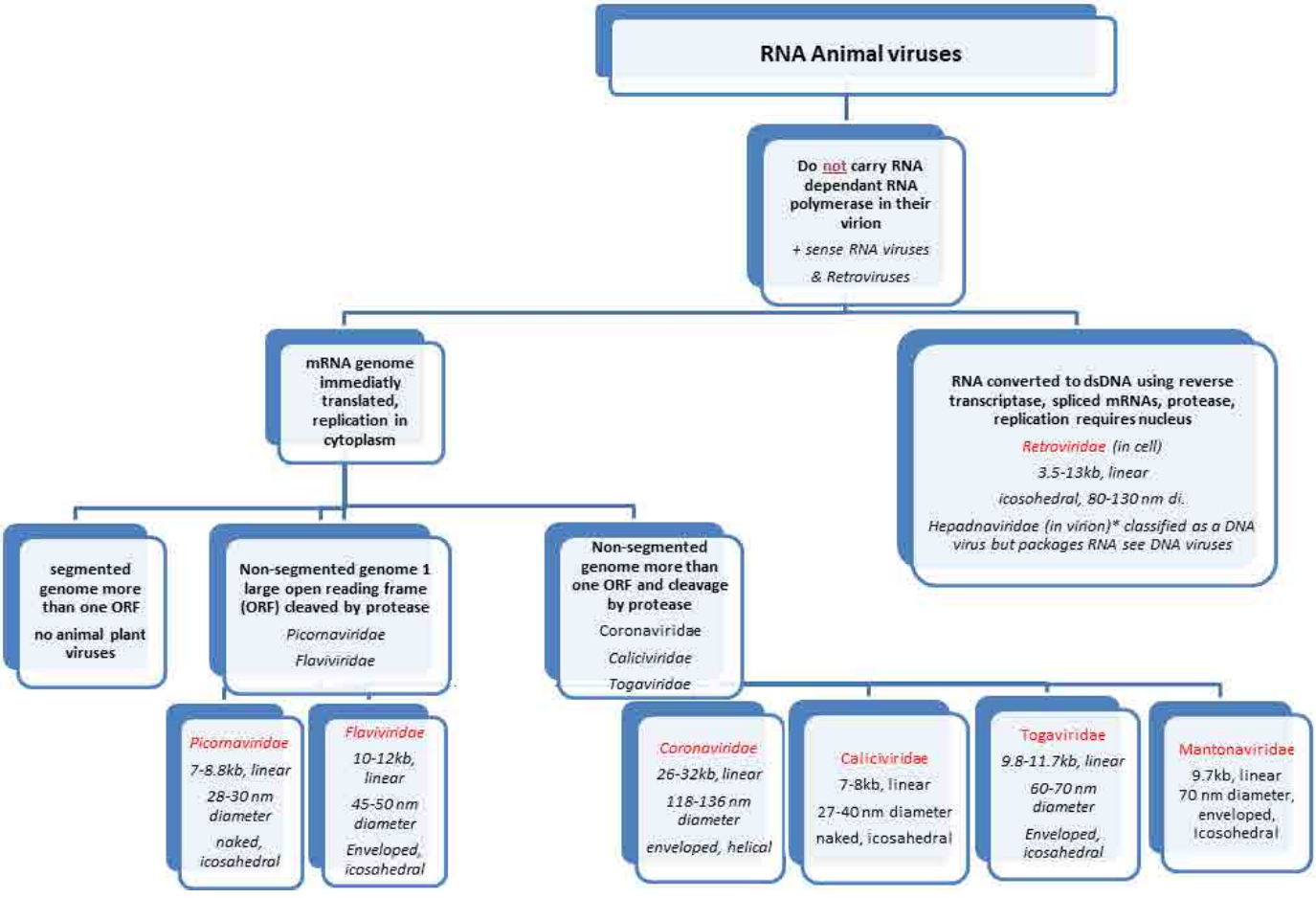


Figure 1-11. The Baltimore viral classification scheme, courtesy of ViralZone https://viralzone.expasy.org/254

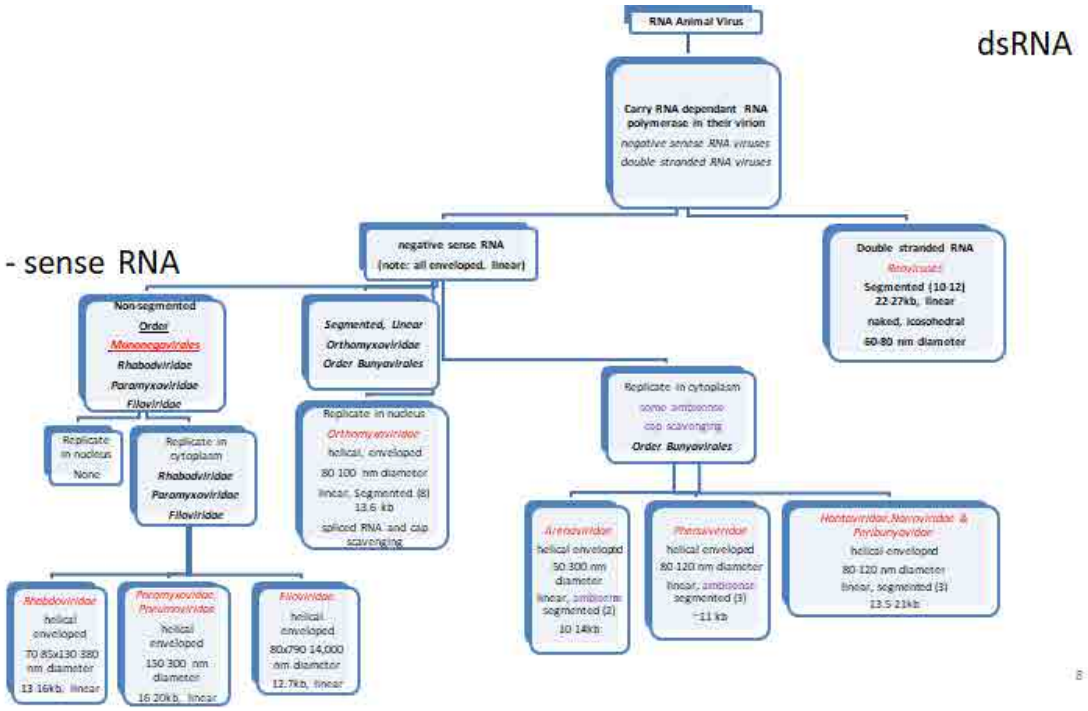
Note: using information about the viruses' many different schemas can be developed allowing you to conceptualize viral replication cycles such as those shown in Figures 1-12 to 1-14. A great way to study is to try to create your own conceptual framework of viral replication cycles or any other topic you can think of. For example, develop your own viral classification schema. We will do concept mapping activities in this course to help you build your own conceptual frameworks. What trends do you notice when you look at these schemes? Can you explain the trends you see? Ask yourself these questions and talk about them with your classmates. This is a great way to learn virology; much more effective than memorizing tons of facts.



Taxonomy of DNA viruses



Taxonomy of positive sense RNA viruses



Figures 1-12 to 1-14. Alternative viral classification schemes based on replication cycles for DNA viruses (Figure 1-12) and RNA viruses (Figures 1-13 to 1-14). Image courtesy of Erica Suchman, Colorado State University.

BOOK SUMMARY

The first 4 chapters of the book will introduce you to basic virology that applies to all viral families.

- Basic virology (Chapter 1).
- The methods we use to study viruses (Chapter 2).
- Basic viral infectious cycles (Chapter 3).
- The host vs virus relationship (chapter 4).

Chapters 5 and 13 will introduce you to challenges unique to DNA and RNA viruses. An introduction to DNA viruses (Chapter 5).

An introduction to RNA viruses will follow the chapters describing the DNA virus families.

At this point we will dive into the viral families. The families will be grouped by genome type and replication style.

Beginning with the DNA viruses in Chapters 6-11,

- Chapter 6 will describe small sized DNA viruses with linear genomes that replicate in the nucleus (*Parvoviridae*).
- Chapter 7 will describe small and medium sized DNA viruses with linear genomes

that replicate in the nucleus (*Adenoviridae*).

- Chapter 8 will describe small DNA viruses with circular genomes that replicate in the nucleus (*Polyomaviridae* and *Papillomaviridae*).
- Chapter 9 will describe large DNA viruses that replicate in the nucleus (*Herpesviridae*).
- Chapter 10 will describe large DNA viruses that replicate in the cytoplasm (*Poxviridae*).
- Chapter 11 will describe viruses that package positive sense RNA and then reverse transcribe it to DNA in the virion and replicate in the nucleus (*Hepadnaviridae*).
- Chapter 12 will describe viruses that package positive sense RNA and then reverse transcribe it to DNA in cytoplasm of newly infected cells replicating partly in the cytoplasm and partly in the nucleus (*Retroviridae*). Note that although these viruses are classified as RNA viruses because they package and deliver RNA to the host cell, in fact they spend the majority of their life cycle as DNA viruses. That is why this chapter is before the introduction to RNA viruses.
- The positive sense RNA viruses (Chapter 14-15).
    - Chapter 14 will describe positive sense RNA viruses that are non-segmented and only have one **open reading frame** (ORF) creating one large polyprotein that is autocleaved by a viral protease and reproduce in the cytoplasm (*Flaviviridae* and *Picornaviridae*).
    - Chapter 15 will describe positive sense RNA viruses that are non-segmented and have more than one ORF creating more than one large polyprotein that is autocleaved by a viral protease, and reproduce in the cytoplasm (*Togaviridae, Caliciviridae, Coronaviridae*).
- The double stranded RNA viruses that are segmented and replicate in the cytoplasm (Chapter 16) (*Reoviridae*).
- The negative sense RNA viruses will be described in Chapters 17-19.
    - Chapter 17 will describe negative sense RNA viruses that are non-segmented that reproduce in the cytoplasm (*Mononegavirales: Rhabdoviridae, Parmyxoviridae, Filoviridae*).
    - Chapter 18 will describe negative sense RNA viruses that are segmented and reproduce in the nucleus (*Orthomyxoviridae*).
    - Chapter 19 will describe negative sense RNA viruses that can sometimes be ambisense segmented and reproduce in the cytoplasm. Note, viruses with ambisense genomes are classified as negative sense RNA viruses as they must carry RdRp into the cell with the virion, but in truth their genome is part negative sense RNA and part positive sense RNA (*Phenuiviridae* and *Arenaviridae*).

We will finish off with Prion biology. When they were first discovered, these diseases were called slow viruses and historically have been studied by virologists. We now know they are not viruses at all, but actually infectious protein particles (Chapter 20).

Now let our journey begin.

CHAPTER 1 END OF CHAPTER QUESTIONS

1. You isolate viruses with a genome of 5kb, 20kb and 100kb. What do you predict will be the relative sizes of the virions of each of these viruses?
2. What is the difference between a capsid and a nucleocapsid?
3. You isolate a new virus with a nucleocapsid. What type of genome would you predict this virus would MOST LIKELY have and why? What genome type would be the next most likely?
4. Looking at the DNA virus taxonomic tree, what trends do you observe?
5. Looking at the positive sense RNA virus taxonomic tree, what trends do you observe?
6. Looking at the negative sense RNA virus taxonomic tree, what trends do you observe?
7. Before we knew what viruses were, they were called filterable agents. Why does it matter that viruses are filterable agents?
8. Compare and contrast peripheral proteins, integral proteins, and glycoproteins. What role do these proteins play in making up virions?
9. Compare and contrast RNA-dependent RNA polymerase (RdRp), DNA-dependent DNA polymerase (DdDp), and reverse transcriptase.
10. What kinds of genome(s) do viruses that utilize RdRp most likely have? Is this enzyme provided by the cell or the virus?
11. What kinds of genome(s) do viruses that utilize DdDp most likely have? Is this enzyme provided by the cell or the virus?
12. What are the functions of reverse transcriptase?
13. Why is this function called reverse transcription and not just transcription?
14. You isolate a new virus. Your colleague says they believe it is a *Mononegavirales*. Is this the virus's species, genus, subfamily, family or order?

REFERENCES:

Center for Disease Control and Prevention. (2021). Smallpox History. https://www.cdc.gov/smallpox/history/history.html

Fields Virology 6th edition David Knipe, Peter Howley, (2013) Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapter 1, 2, 3

Protein Data Base 101. Goodsell, D. (2000). Nucleosomes. https://pdb101.rcsb.org/motm/7

Protein Data Base 101. HIV envelope protein. https://pdb101.rcsb.org/motm/169

Protein Data Base 101. Tobacco Mosaic Virus (PDB entry 2tmv)
https://pdb101.rcsb.org/motm/109

ViralZone. The Baltimore classification Scheme. https://viralzone.expasy.org/254

ViralZone. Herpesviridae. https://viralzone.expasy.org/176

ViralZone. Human Adenovirus type C virion. https://viralzone.expasy.org/237

ViralZone. *Rhabdoviridae*. https://viralzone.expasy.org/2

ViralZone. T1, T2, T3. https://viralzone.expasy.org/833

REVIEWER:

Dr Charles Calisher retired CDC.

CHAPTER 2

Chapter 2: Introduction To Methods Used In Virology.

Methods to study viruses

We will begin our discussion with molecular methods which are now the most common way of analyzing viruses. We will also discuss classical methods as they are still in use as well.

Some assays will be **qualitative**, meaning they can give you a yes or no answer but not quantify how much of the virus is present. Others will be **quantitative** and can provide data about how much virus or virus products such as DNA, RNA, or protein are present.

COMMON MOLECULAR DETECTION METHODS

POLYMERASE CHAIN REACTION

The **qualitative polymerase chain reaction** (PCR) is an extremely useful technique for specific amplification of small amounts of nucleic acids with a large number of applications. One of the more widely known techniques is the use of "DNA fingerprinting" in forensic medicine. The utility of PCR comes from the very small amount of starting material it requires and the ease with which the specificity can be manipulated by varying length, nucleotide sequence of primers, and annealing temperature. In virology, PCR can be of particular importance in diagnosis when a virus is present in low titer such as in HIV infections.

The PCR reaction is set up as follows: two **primer** sequences of approximately 20 nucleotides are annealed to opposite strands of DNA (RNA requires an initial reverse transcription step as DNA polymerase is a DNA-dependent DNA polymerase (DdDp) and will only read a DNA template). Recall that all DNA polymerases require a 3'OH on which to start adding new nucleotides. These primers serve as surrogates for the primers created by primase during cellular DNA replication, hence the name primers. The annealing temperature is determined by the specific set of primers used and must be optimized for each different primer set. The temperature is raised to the optimum for the polymerase from a thermophilic bacterium (72 degrees Celsius), usually *Thermus aquaticus (Taq),* and replication continues from the 3'OH of the primers producing 2 copies of the DNA. The temperature is raised still further (usually around 95 degrees Celsius) causing the DNA strands to separate, and then the temperature is lowered to allow new primers to attach to each of the 4 strands created in the last reaction. The *Taq* polymerase fortunately is stable during the DNA melting step and is able to begin a new cycle of synthesis. The process is repeated for 20-40 cycles in a thermal cycler that automatically changes the temperature so that additional copies arise exponentially, i.e., in a chain reaction. Note that it is important to always run both a positive control and a negative control to be sure your reaction is working (positive control) and that none of your reagents are contaminated and giving you a false positive (negative control). The positive control is usually a sample that you know contains virus and the negative control is a sample you know does not. A set of primers is shown in Figure 2-1. A typical PCR reaction is shown in Figure 2-2. An example of a PCR reaction run on an agarose gel is shown in Figure 2-3.

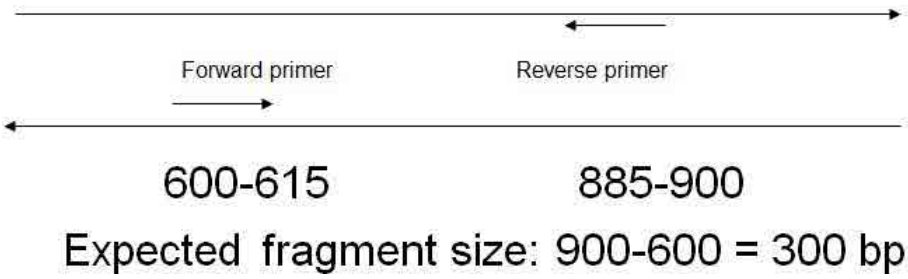


Figure 2-1. An example of a primer set used to amplify a virus. Courtesy of Erica Suchman, Colorado State University.

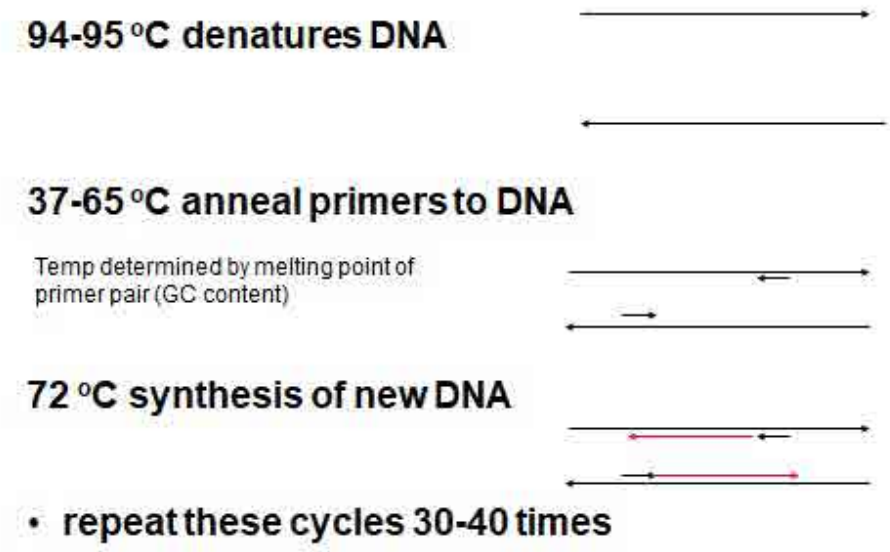


Figure 2-2. An Example of the steps used in PCR reactions. Courtesy of Erica Suchman, Colorado State University.

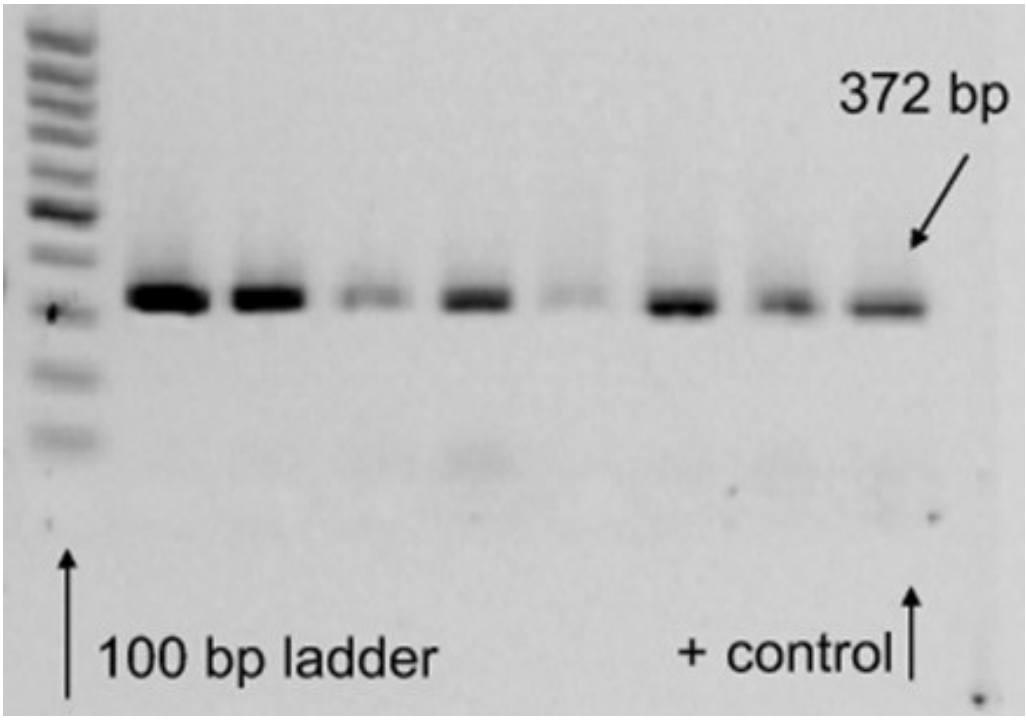


Figure 2-3. An example of a PCR reaction run on an agarose gel via gel electrophoreses. Courtesy of Erica Suchman, Colorado State University.

It is important to note that this is only a qualitative assay. As such, it can only tell you if virus is present, but not how much.

Taq polymerase is a **DNA-dependent DNA polymerase (DdDp)** meaning it can only read a DNA template and as such can only be used directly on DNA viruses. So, can PCR be used on the many RNA viruses? Thanks to **reverse transcriptase** (RT) it can! Remember that reverse transcriptase has 3 functions, an RNA-dependent DNA polymerase (RdDp), RNAase H, and DNA-dependent DNA polymerase (DdDp) activity. As such it takes the RNA genome and converts it into double stranded DNA. It first uses the **RdDp** activity to create a single strand of DNA from the RNA genome. The **RNAase H** activity cleaves the RNA out of the DNA/RNA hybrid created leaving the single stranded DNA to serve as template for the **DdDp** giving rise to a double stranded DNA! This DNA is referred to as complementary DNA or cDNA, as it is complementary to the RNA template. cDNA can be used in PCR reactions. A PCR reaction that starts with reverse transcription of RNA into DNA is called RT PCR for the reverse transcriptase that makes it possible. It should be noted that like all DNA polymerases, reverse transcriptase requires a primer to start replication. The primers most commonly used are either sequence-specific primers for that particular virus, poly T primers that will anneal to the viral poly-A tail, or random hexamers of nucleotides that will prime all over the RNA. Again, this is only a qualitative assay, not a quantitative assay.

QUANTITATIVE PCR: REAL TIME PCR

Quantitative PCR is designed similarly to standard qualitative PCR reactions. The only difference is that fluorescent molecules are included in the reaction. This eliminates the need to run the reaction on a gel. A fluorescence scanner instrument detects amplification in real time as data are generated. Fluorescent dyes are required to be detected by the real time PCR machine. Two principal fluorescence types are used commonly. The first is SYBR Green and the second is a fluorescently labeled hybridization probe (sometimes called **TaqMan real time PCR** or **probe based real time PCR**). Both allow quantification of the amount of starting template but do so by slightly different chemistry. The **SYBR Green** dye selectively binds double stranded DNA as it is a DNA intercalating dye,

meaning it works its way between adjacent nucleotides on a strand of DNA. However, SYBR Green only binds to double stranded DNA and not single stranded DNA. The advantages of using SYBR Green are that it is sequence-independent and cheaper. The disadvantage is poor specificity. It can bind to any dsDNA, so if primers misprime, non-template DNA can be amplified and bound by SYBR Green leading to "false positives".

Probe based real time PCR or TaqMan PCR uses two primers and an internal probe that hybridizes to the target DNA between the two primers. The probe has a fluorescent label on one end and a quencher dye on the other as shown in Figure 2-4. Note that as long as the quencher dye and florescent label are in close proximity, as they are when they are both on the probe, the quencher quenches the fluorescence of the florescent label. Therefore, in probe based PCR, a special Taq polymerase that has a 5'-3' exonuclease activity is used. As the DNA is replicated, the primer degrades, and the fluorescent dye is liberated from the quencher allowing it to fluoresce. Thus, the fluorescence is detected by the real time PCR machine as it is liberated as is shown in Figure 2-5 "in real time". The amount of fluorescence is shown on the Y axis and the cycle number is shown on the X axis. You will note that an orange threshold bar is set horizontally across the reactions. A reaction is said to be positive once it crosses the cycle threshold (C_T) (that can be set by the user or the machine). The faster a reaction reaches the threshold (therefore less cycles) the more target DNA was present in the original sample. Thus, a virus with a high viral copy number has a low C_T value while one with a low viral copy number will have a high C_T value. In general, if a sample has not crossed the threshold by 40 cycles it is considered to be negative for presence of the target DNA. Thus, all of the samples assayed in Figure 2-5 are positive.

It should be noted that both probe based and SYBR Green real time PCRs can only be performed directly on DNA. In order to do real time PCR on an RNA virus you would still need to first do reverse transcription of the RNA virus to cDNA.

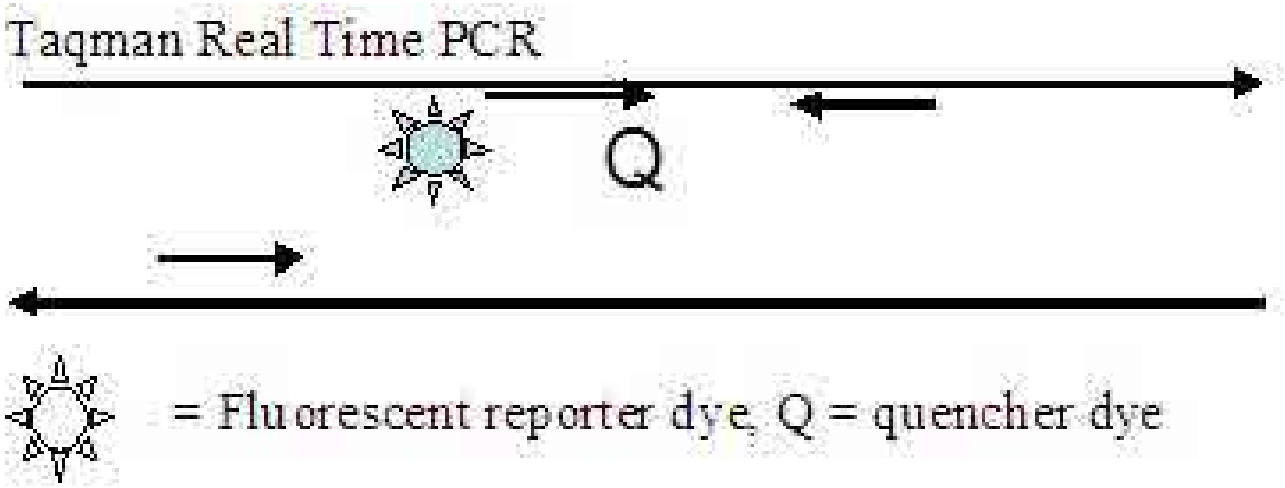


Figure 2-4. A primer and probe used for TaqMan/probe based real time PCR. Courtesy of Erica Suchman, Colorado State University.

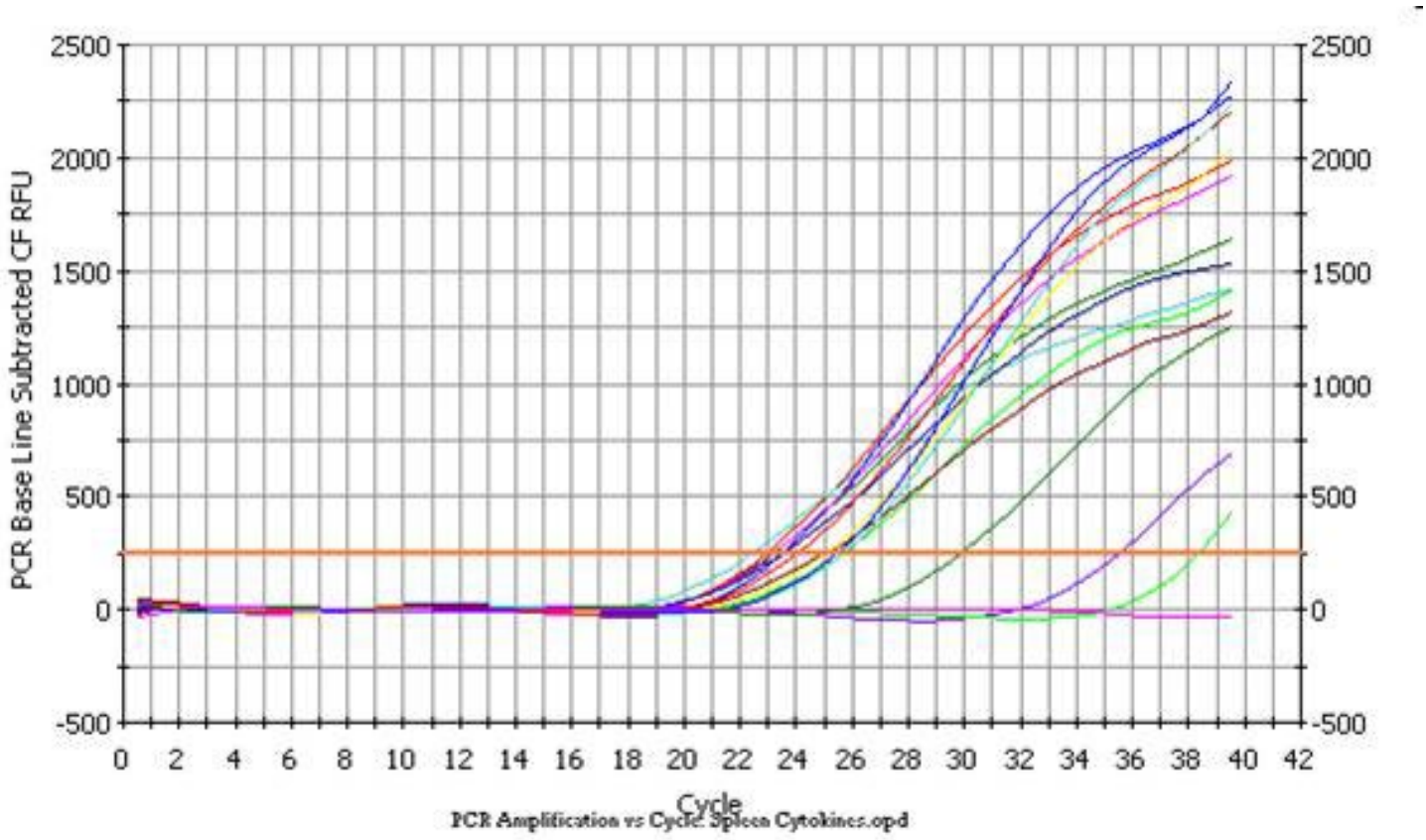


Figure 2-5. Typical data for a real time PCR assay of either SYBR Green or Probe Based Real Time PCR. Note: because all of these amplifications crossed the CT value (orange line) before 40 cycles theses samples would all be positive for virus. Courtesy of Erica Suchman, Colorado State University.

Real time PCR data was used to identify the new variant mutant strains of SARS-Cov-2 in Colorado during the COVID-19 outbreak of 2019-2021. To determine if people were infected while avoiding false positives, nasopharyngeal samples were assayed to determine if amplification of 2 genes, N and S, could be detected. It was noted that if a person had a low amplification (high C_T values) of N that the S gene often failed to amplify, thus allowing the elimination of weak amplification of genes resulting from false positives. Samples were only considered to be positive if a person is positive for amplification of both genes. However, it was found that some people with high levels of amplification (low C_T values) of the N gene failed to amplify the S gene. When samples from these individuals were analyzed by sequencing it was found that these people were in fact infected with SARS-CoV-2; however, the S gene had mutated such that the primers to amplify the S gene were no longer able to bind. Thus, people infected with these mutants were receiving negative test results when in fact they were infected with mutant virus, that was potentially more problematic. As a result, if a sample shows a high N amplification (low Ct) but is negative for amplification of the S gene (C_T higher than 40), the samples are now further analyzed by sequencing.

DNA Sequencing

The **Dideoxynucleotide method, or Sanger method,** is the most commonly used **DNA sequencing** method. It utilizes a DdDp and the addition of **dNTPs,** the nucleotides found in DNA (dATP, dCTP, dTTP, dGTP) in high molar excess, as well as low levels of ddNTPs that are fluorescently labeled, each with a different colored dye (ddATP, ddCTP, ddTTP, ddGTP). ddNTPs lack an OH at both the 2’ and 3’ position of the ribose. The ddNTPs act as terminators. They lack the 3’ OH group required to make phosphodiester bonds with the 5’ phosphate during DNA replication. Hence, when DdDp incorporates a ddNTP, DNA replication is stopped. It is important to note that the ddNTPs are in a low molar concentration relative to dNTP. As such, the DdDp is mostly incorporating dNTPs and

occasionally incorporates ddNTPs allowing polymerization to sporadically terminate as shown in Figure 2-6. The reaction is similar to PCR but only uses one primer (unidirectional). NTPs are added to the 3' end of the primer **hybridized** (bound) to the template, and it requires template DNA, using the 3'OH on the primer to add nucleotides to the growing strand 5' to 3'. Once the primer anneals, then Taq polymerase synthesizes new strands. Thousands of strands are synthesized each cycle and randomly terminated when there is incorporation of ddNTP into DNA as this terminates the chain due to the absence of a 3'OH on which to add the next nucleotide. Therefore, each chain ends with a fluorescently labeled ddNTP, and the different colored fluorescence they release can be detected by the sequencing machine as the sequencing reaction runs through a polyacrylamide gel. The DNA will run through the gel with the smallest fragments, the 5' end of the reaction, because the DdDp (DNA pol) reads 3' to 5' adding 5' to 3' so the fragments closest to the primer will be at the 5' end. This data will present as colored peaks that can be used to determine the sequence of the original template as shown in Figure 2-7. Note that one can also do non fluorescently labeled sequencing reactions. In this case, radio labeled dNTPs are added. Four sequencing reactions are run simultaneously, and each includes a DNA or cDNA template, DNA polymerase, an oligonucleotide primer, and 4 dNTPs, one of which carries a radioactive label. In each reaction there is also a low concentration of one of the nucleotides in dideoxy form. In each synthesis reaction, incorporation of the dideoxynucleotide terminates the nascent DNA chain, therefore every product in the ddATP-containing reaction, for example, will have the base A at its 3' end. After incubation at 37°C for 30-60 min, the 4 reaction mixtures are run side-by-side on a polyacrylamide gel that separates the labeled product DNA by size. The smallest DNA molecules migrate through the gel most rapidly as shown in Figure 2-8, therefore the gel is read from bottom to top, with the 5' end of the DNA creating the smallest fragments that will be found at the bottom of the gel. In Figure 2-8 you can see that the sequence just downstream of the primer on the template strand is TTTCAGGGG… Accordingly, the sequence generated during the sequencing reaction will be AAAGTCCCC… as is observed when reading the gel from the bottom (5' end) to the top (3' end). You will note that the polymerase is reading the template strand 3' to 5' adding nucleotides 5' to 3' so the shortest fragments will be at the 5' end closest to the primer.

The incorporation of the radioactive deoxynucleotide allows visualization of the migration distance of each DNA product after exposure of the gel against X-ray film. The beta-particle emissions result in black bands on the film. See Figure 2-9.

The National Center for Biotechnology Information (NCBI) web page located at http://www.ncbi.nlm.nih.gov/ is maintained by the National Library of Medicine and the National Institutes of Health. It allows you to compare sequences you have obtained to all known sequences in the BLAST search database. Note that data will be reported as % similarity to the top most similar sequences as well as producing a 1) total score – the higher the number the more similar, 2) a query coverage – the percentage of the genome you put in that was homologous to the matching sequence, 3) max identity – what percentage was homologous between your sequence and the matching sequence, and 4) the E value or reliability value – the lower the number the more reliable the matching. Viral mRNAs and RNA genomes cannot be sequenced, however, as sequencing utilizes a DdDp (DNA polymerase). The RNA must first be reverse transcribed to DNA before the sequencing reaction occurs.

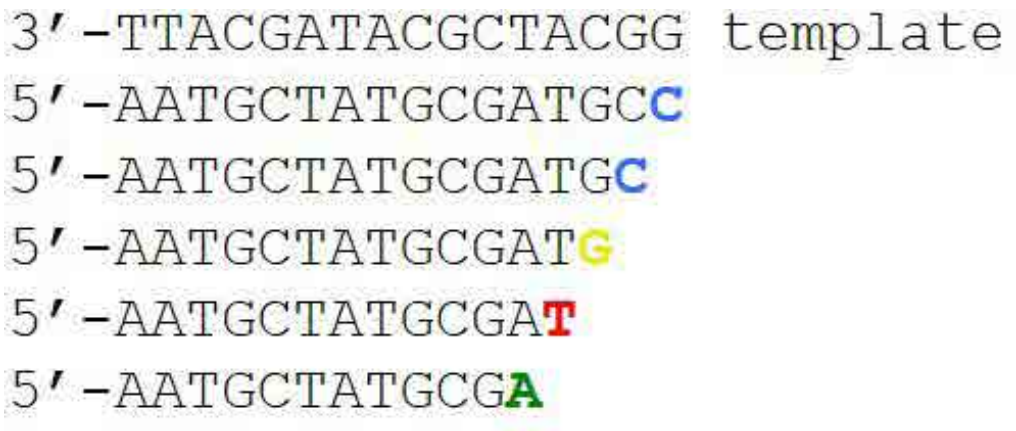


Figure 2-6 shows the fragments generated when ddNTPs are incorporated into the DNA being replicated. Courtesy of Sandra Quackenbush, Colorado State University.

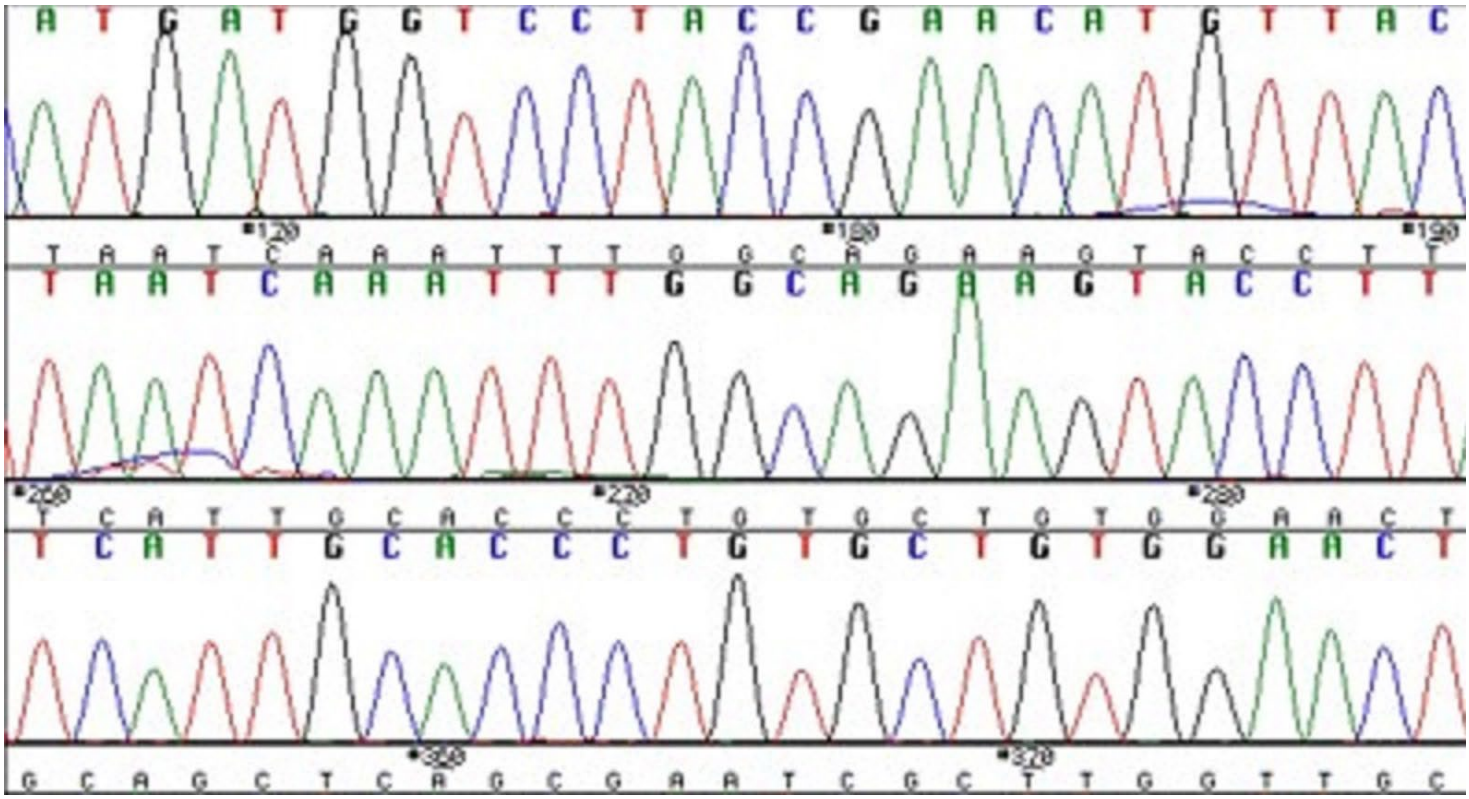


Figure 2-7. An example of sequencing reaction data using fluorescently labeled ddNTPs. Courtesy of Erica Suchman, Colorado State University.

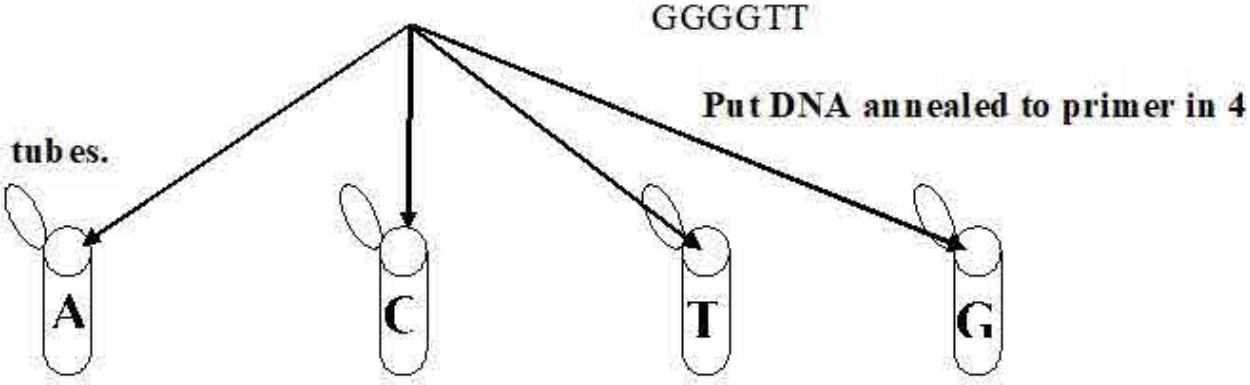
Sequencing:

5' ACGGTTCCAAATTTGGGGACTTTCCCCAA 3' DNA to be sequenced

Add primer, anneal 3' GGGGTT 5'

5' ACGGTTCCAAATTTGGGGACTTTCCCCAA 3'

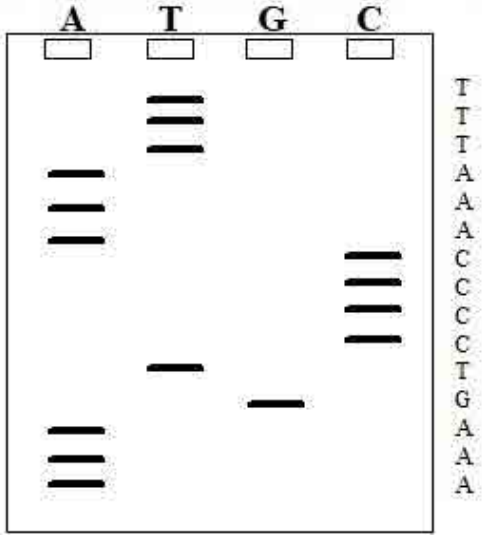
GGGGTT



Add dNTPs	**Add dNTPs**	**Add dNTPs**	**Add dNTPs**
ddATP	ddCTP	ddTTP	ddGTP
And DNA pol	And DNA pol	And DNA pol	And DNA pol

- ddNTP is a di-deoxy NTP which has no OH group on the 3' end available for elongation; therefore, when one of these nucleotides are incorporated, elongation is terminated. Also add radiolabeled $dCTP^{32}$ to each reaction.

Run each of these 4 reactions on its own lane on a polyacrylamide gel.



Put gel under film, read autoradiograph.

Read from bottom to top (5' to 3'). The smaller the fragment the closer it is to the primer (5' end).

Figure 2-8 shows how a Sanger sequencing reaction is performed with radio-labeled ddNTPs. Courtesy of Erica Suchman, Colorado State University.

NEXT GENERATION SEQUENCING

A limitation of Sanger sequencing, whether with radio-labeled or fluorescently labeled ddNTPs, is that you can only sequence one fragment at a time which can lead to bias in what is sequenced. Often it is advantageous to sequence as many virus genomes or mRNAs as possible. Luckily there is now **next generation sequencing,** or **deep sequencing**. This allows the sequencing of a large percentage of the virus present in a sample at one time to look at the range of mutants that have arisen. This is particularly useful when looking at drug susceptibility in viral infections like HIV where mutations lead to reduced susceptibility to drugs. Next generation sequencing typically generates gigabases (10^9) of data in a day or two. This requires massively parallel sequencing reads that are contiguous nucleotides from a sample. Infected cells may contain millions of mRNA and viral RNA molecules which can be analyzed simultaneously. All next generation sequencing platforms perform sequencing of millions of small fragments of DNA in parallel. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to a reference genome. Next generation sequencing, **massively parallel sequencing**, **high-throughput sequencing**, or deep sequencing usually refer to technologies that allow sequencing without the physical separation of individual reactions into separate tubes. Instead, the sequencing reactions occur in parallel on a solid surface (such as glass or beads, depending on the technology) and are only spatially separated. Randomly generated primers are attached to the surface, and as complementary pieces of DNA float by they hybridize to the primer allowing the PCR reaction to start. Thus, billions of sequencing reactions occur and are analyzed simultaneously, dramatically improving the throughput rate and decreasing the labor compared to Sanger sequencing. There are many different platforms for different needs. Some give moderate reads with high fidelity and modest coverage, some give short length reads with high fidelity and very high coverage, and some give very long reads but with high error rates.

You are probably thinking to yourself, "Why would you ever use one with long reads and lots of errors?" A common practice is to use a system that generates very long high error reads in conjunction with one that makes shorter high fidelity reads. The non-error-prone reads are lined up on the long high error reads enhancing the ability to figure out the sequence of the short reads. Once fragments are generated, they have to be assembled, and having the long fragment, even if it contains errors, facilitates the assembly. This is a computationally intensive process that requires a great deal of processing power and RAM. The recent introduction of Graphic Processor Units (GPUs) has accelerated development of this technique. Bioinformaticists often do this work, and this is a rapidly growing field with great job opportunities if you have an interest in biology and computer work. Note that RNAseq is a similar technique that is used to sequence and analyze a large proportion of the mRNA of any virus genome type or genome of an RNA virus simultaneously. Again, the mRNAs must first be reverse transcribed before sequencing because sequencing utilizes DNA polymerase which is a DNA-dependent DNA polymerase (DdDp) which will not read RNA.

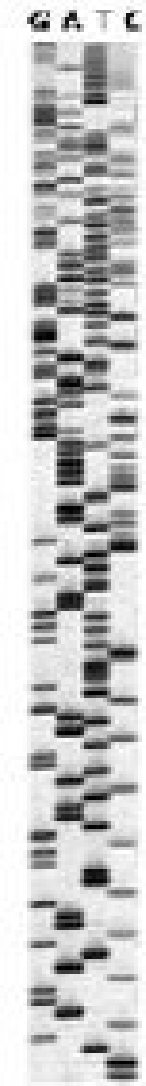


Figure 2-9. Shows an example of sequencing reaction data using radio labeled ddNTPs. Courtesy of Erica Suchman, Colorado State University.

CLASSIC NON-MOLECULAR METHODS OF STUDYING VIRUSES

CELL CULTURE

Viruses are obligate intracellular parasites. They require living cells from a suitable host to replicate. Viruses can be propagated in living animals such as embryonated avian eggs and mice.

Virus propagation in living animals inevitably raises issues such as high cost, time commitment, animal welfare,

and difficulty in handling, maintenance, and uniformity. Scientists motivated by these problems began looking for an alternative method for virus propagation. The development of cell culture was the result and is now the most popular method of virus propagation.

Animal cells were first grown *in vitro* early in the twentieth century. In 1912, **Carrel** began growing bits of chicken heart in drops of horse plasma. The cells at the edge of the explant divided and grew out of the plasma clot. The explants died within a few days, and Carrel reasoned that their death was due to exhaustion of nutrients. He found that cells from a given explant could be maintained indefinitely if they were periodically subdivided and fed with a sterile aqueous extract of whole chick embryos.

In the early 1950s, **Earle** developed a technique for dissociating cells of a whole chick embryo from each other with trypsin. When this suspension of single cells was mixed with plasma and embryo extract and placed in a sterile glass container, the cells adhered to the glass and divided to form a **primary culture**. The primary culture contained a variety of cell types including macrophages, muscle fibers, etc. Some types of cells grew to a **monolayer**, a thin sheet of cells (one layer in thickness), that covered the entire bottom surface of their culture vessel and then stopped dividing. The cells could then be redispersed with trypsin and planted in new culture vessels containing fresh medium. These **secondary cultures** contained fewer cell types than did the primary cell cultures as many of the differentiated primary cells had not divided and were diluted out upon transfer. Secondary cultures were often composed entirely of spindle-shaped cells called **fibroblasts** because of their similarity to cultured connective tissue. Cells derived from kidneys and from certain carcinomas had a polygonal appearance in culture. Because of their tissue of origin, they and other cells with similar morphology are called **epithelial**.

Cells may be grown *in vitro* in several ways. **Organ cultures**, if handled carefully, maintain their original architecture and functions for several days or sometimes weeks. Slices of organs (which are actually tissue cultures) consisting of respiratory epithelium have been used to study the histopathogenesis of infection by certain respiratory viruses that can only be grown outside their natural host in complex, organized tissue. The term **tissue culture** was originally applied to explants of tissue embedded in plasma. The term subsequently became associated with the culture of cells in general and is now obsolete in its original sense. **Cell culture** is the term most commonly used today. It refers to tissue dissociated into a suspension of single cells by both mechanical and enzymatic means. After being washed and counted, the cells are diluted in growth medium and allowed to settle onto the flat bottom surface of a glass or specially treated plastic container. Most types of cells adhere quickly, and under optimum conditions they will undergo mitosis and cell division about once a day until the surface is covered with a confluent cell monolayer. Such cells constitute a primary culture. These cells are usually removed from the surface on which they are growing with a combination of trypsin and a chelating agent such as EDTA (to remove Ca_2^+ which will inhibit trypsin), counted, diluted, and replated in new containers with
fresh medium. This is called a secondary culture.

With the advent of cell culture, many animal viruses have been propagated ***in vitro***, and hundreds of previously unknown viruses have been isolated and identified. The discovery of the adenoviruses, echoviruses, and rhinoviruses, for example, is directly attributable to the use of cultured cells as is the revolution in the diagnosis of viral diseases and the development of poliomyelitis, measles, rubella, and other vaccines.

Three Types of Cultured Cells

Primary cell cultures: When cells are taken freshly from animal tissue and placed in culture, the cultures consist of a wide variety of cell types most of which are capable of very limited growth *in vitro*, usually fewer than ten divisions as shown in Figure 2-10. These cells retain their **diploid karyotype**, i.e., they have the chromosome number and morphology of their tissues of origin. They also retain some of the differentiated characteristics that

they possessed *in vivo.* Because of this, these cells support the replication of a wide range of viruses. Primary

cultures derived from monkey kidneys, mouse fetuses, and chick embryos are commonly used for diagnostic purposes and laboratory experiments.

1. Diploid cell strains: Some primary cells can be passed through secondary and several subsequent subcultures while retaining their original morphological characteristics and karyotype. Subcultures will have fewer cell types than primary cultures. See Figure 2-11. After 20-50 passages *in vitro*, these diploid cell strains usually undergo a crisis in which their growth rate slows and they eventually die out. Diploid strains of fibroblasts derived from human fetal tissue are widely used in diagnostic virology and vaccine production.

Figure 2-10 is a drawing of the multiple cell types observed in primary cell cultures. Courtesy of Jonathan Martino, Colorado State University.

Figure 2-11 is a drawing of the reduced number of cell types observed in secondary cultures when compared to primary cell cultures. Courtesy of Jonathan Martino, Colorado State University.

2. Continuous cell lines**:** Certain cultured cells, notably mouse fetal fibroblasts, kidney cells from various mammalian species, and human carcinoma cells, are able to survive the growth crisis and undergo indefinite propagation *in vitro*. After several passages the growth rate of the culture slows down, isolated

colonies of cells begin to grow more rapidly than diploid cells, their karyotype becomes abnormal (**aneuploid**), their morphology changes, and other poorly understood changes take place that make the cells immortal. The cells are now "de-differentiated" having lost the specialized morphology and biochemical abilities they possessed as differentiated cells *in vivo.* Continuous cell lines such as KB and HeLa, both derived from human carcinomas, support the growth of a number of viruses. These lines and others derived from monkey kidneys (Vero), mouse fetuses (L929), and hamster kidneys (BHK) are widely used in diagnostic and experimental virology as shown in Figure 2-12. Continuous cell lines have been established from many types of vertebrate and invertebrate animal tissues and are available from the American Type Culture Collection.

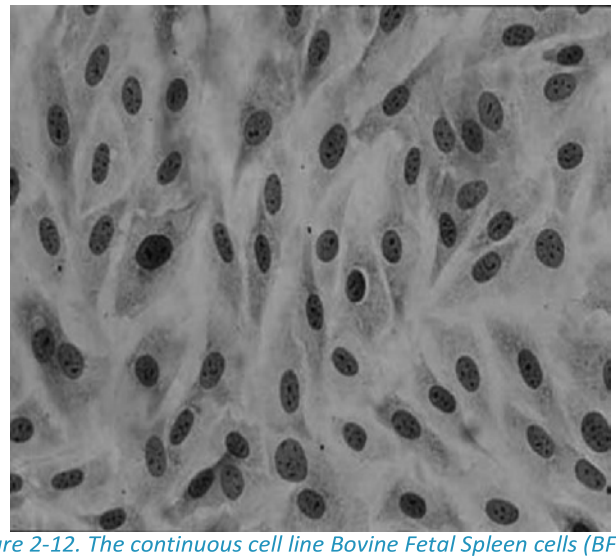


Figure 2-12. The continuous cell line Bovine Fetal Spleen cells (BFS) at 40x magnification. Courtesy of Erica Suchman, Colorado State University.

Development of Continuous Cell Lines

The development of antibiotics during World War II simplified long-term animal cell culture by minimizing the problems of bacterial and fungal contamination. **Eagle** made another important discovery in the 1950s when he determined the minimal nutritional requirements of cultured cells. He began by showing that HeLa and mouse L-cells would grow in a mixture of salts, amino acids, vitamins, co-factors, carbohydrates, and horse serum. By eliminating one component at a time, he then determined which nutrients were essential for cell growth.

His **minimum essential medium (MEM)** contains 13 amino acids (human tissue *in vivo* requires only 8), 8 vitamins and co-factors, glucose as an energy source, and a physiological salt solution that is isotonic to the cell. The pH is maintained at 7.2-7.4 by $NaHCO_3$ in equilibrium with CO_2. The pH indicator **phenol red** is often incorporated into the medium. This pH indicator turns red-purple if the medium is basic, yellow if the medium is acidic, and remains red-orange when the pH is in the **correct range (pH 7.2-7.4).**

Serum in concentrations of 1-10% must be added to the medium to provide the cells with additional poorly defined factors without which most cells will not grow. Most mammalian cells are incubated at 37^oC. Avian, reptilian, and arthropod cells may grow best at higher or lower temperatures. Cell cultures require elevated levels of CO_2 for growth. If cells are grown in vessels open to the atmosphere, their incubator must be humidified and contain an increased CO_2 concentration. Nonvolatile phosphate or substituted sulfonic acid buffers (HEPES, TES) eliminate the requirement for incubators to be gassed with CO_2. Tightly sealed containers also eliminate the need for CO_2.

Animal cells in cell culture also require a surface to grow upon. This surface often takes the form of negatively charged plastic or glass culture flasks. Some types of tumor cells can be adapted to grow in suspension, but most cells require a solid medium to grow on. Cell cultures grow best when the initial concentration of cells used to inoculate the media is between 2×10^5 and 8×10^5 cells per ml of medium or cm^2 of growth area

CYTOPATHIC EFFECT

While synthesis of viral components is occurring in the infected cell, the cell undergoes characteristic biochemical and morphological changes. Progression of these changes is most readily observed in cell culture where infection of cells is more easily synchronized and where the cells can be observed and sampled frequently during the course of infection. Morphological changes in cells caused by viral infection are called **cytopathic effects (CPE**), and the responsible virus is said to be cytopathogenic. The degree of visible damage to cells caused by viral infection varies with type of virus, type of host cells, multiplicity of infection (MOI), and other factors. Some viruses cause very little or no CPE in cells of their natural host. Their presence can be detected visually only by **hemadsorption** or **interference**, in which infected cell cultures showing no CPE inhibit the replication of another virus subsequently introduced into the cultures, or ***in situ* viral antigen or nucleic acid detection**. On the other hand, some viruses cause a complete and rapid destruction of the cell monolayer after infection. The microscopic appearance of the CPE caused by some of these cytocidal viruses may be sufficiently characteristic to allow provisional identification of an unknown virus.

Some CPE can be readily observed in unfixed, unstained cells under low power (10X objective) of the light microscope with the condenser down and the iris diaphragm partly closed to obtain the contrast needed for viewing translucent cells. Several types of CPE are distinguishable in living cultures, but fixation and staining of the cells is necessary to see such manifestations of viral infection as inclusion bodies. Recognizing CPE and using it as a diagnostic tool requires much experience in examining both stained and unstained cultures of many cell types.

Listed below are several general types of CPE. Keep in mind that a given virus may not conform to the norm for its family, or it may produce different CPE in different host cell types. Characteristic CPE is best viewed by daily observation of cultures that have been infected at a low **multiplicity of infection** (MOI) (<0.1). The best knowledge of viral CPE comes from experience. Rate of CPE formation is determined by observing when CPE is seen in the **LOWEST MOI** that shows CPE. Recall that the MOI is the number of viral particles per cell. Total or subtotal destruction of the cell monolayer is the most severe form of CPE.

Figure 2-13 shows BFS cells infected with bovine enterovirus 4 days post infection at 10X demonstrating partial cell destruction. Courtesy of Erica Suchman, Colorado State University.

All or a large portion of cells in the monolayer rapidly shrink and become dense (pyknosis) and detach from the glass within 72 hours. Total cell destruction is typical of most enteroviruses. Sub-total cell destruction is seen in some togaviruses (alphaviruses), some picornaviruses, and some of the paramyxoviruses. Note that these infections are generalized, meaning virus is released into the medium and floats off to infect cells in different parts of the plate as shown in Figure 2-13.

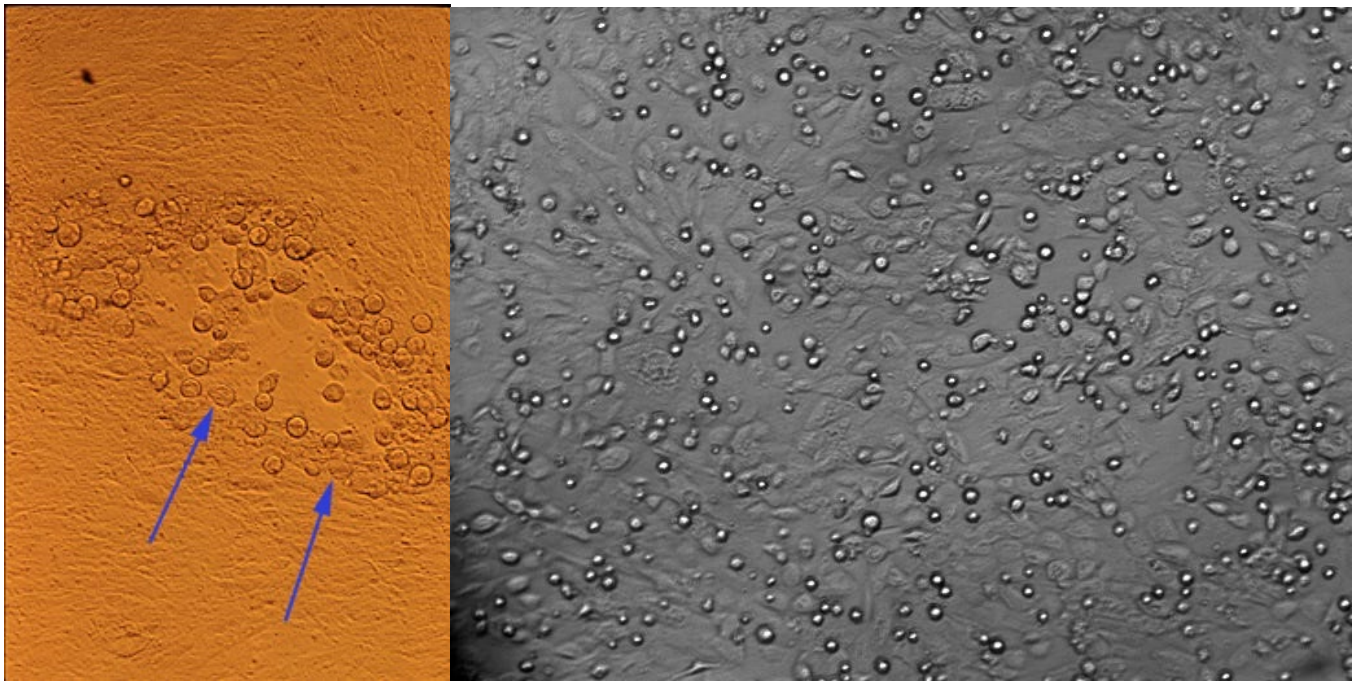
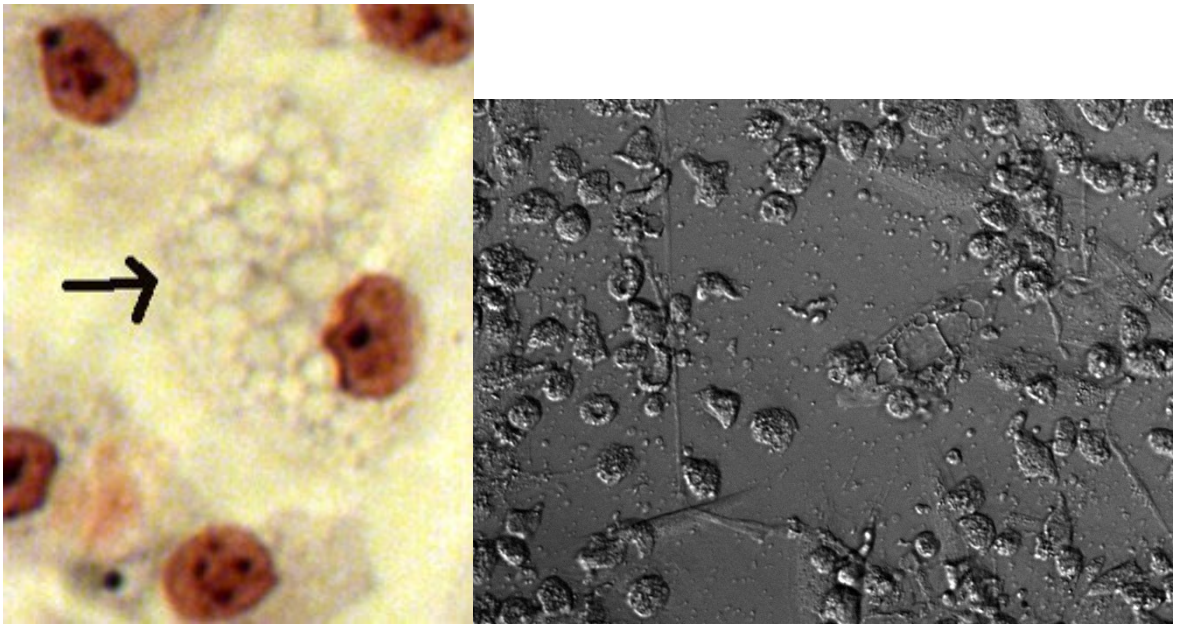


Figure 2-14A shows BFS cells infected with the pox ORF virus 4 days post infection at a low MOI demonstrating focal cell rounding (blue arrows), and in 14B the same virus in the same cell type after the same amount of time using a high MOI demonstrating generalized CPE. Courtesy of Erica Suchman, Colorado State University.

Focal degeneration is characteristic of the herpesviruses and poxviruses. Instead of causing a generalized destruction of the cell monolayer, these viruses produce localized areas (foci) of infection. The focal nature of these lesions is due to direct cell-to-cell transfer of virus rather than diffusion through the extracellular medium. Cells shown here initially become enlarged, rounded, refractile (more easily seen), and eventually detach from the glass leaving cleared areas surrounded by rounded up cells as the infection spreads concentrically. Eventually the entire monolayer may be involved. Note it is important to look at cells infected with a low MOI as at high concentrations all infections appear generalized. Shown in Figure 2-14 A & B.

Foamy degeneration (vacuolization) is due to the production of large and/or numerous cytoplasmic vacuoles. Several RNA virus families including certain retroviruses, paramyxoviruses, and flaviviruses (pestiviruses) may cause vacuolization. Vacuolization is much easier to detect in cells that have been stained but if prominent enough can be detected without staining. Shown in Figure 2-15 A, B, & C.



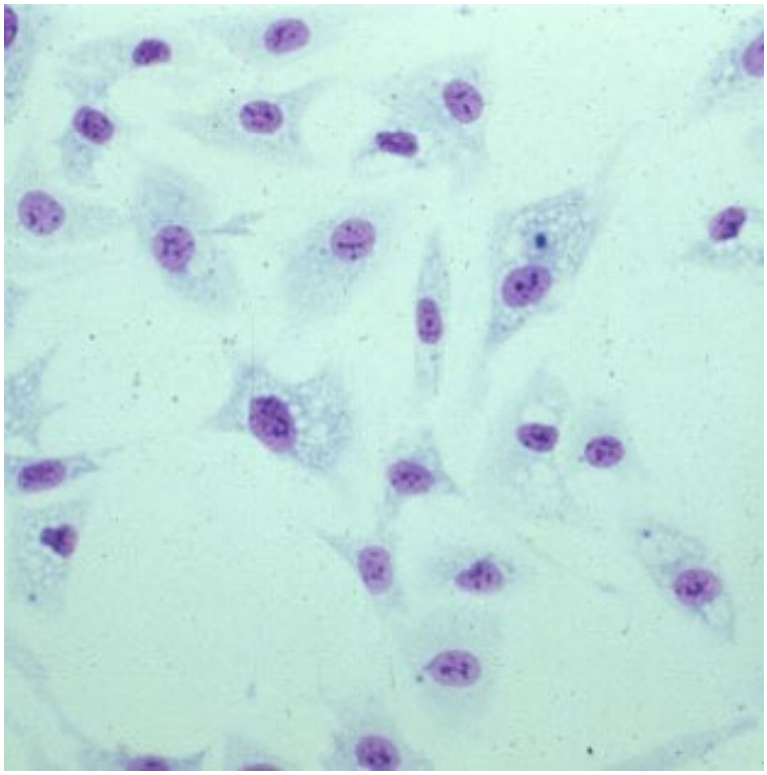


Figure 2-15A shows BFS cells infected with bovine viral diarrhea virus 5 days post infection at 20X demonstrating total cell destruction and vacuoles. Figure 2-15B shows the same cells after Giemsa staining. The arrow points to the vacuoles in the stained cells. Figure 2-15C shows these same cells under 10X magnification. It is much easier to find CPE at 10X and then move to 20X or 40X to observe the CPE in more detail. Image courtesy of Erica Suchman, Colorado State University.

Cell fusion (syncytium or polykaryon formation) involves the fusion of the plasma membranes of 4 or more cells to produce an enlarged cell with 4 or more nuclei. This is carried out by envelop fusion proteins. Small syncytia are readily seen only after staining. Polykaryon formation may be the only detectable CPE of some paramyxoviruses. Herpesviruses may also produce syncytia. It is important to distinguish cell fusion from cell clumping or clustering in which plasma membranes remain distinct. Shown in Figure 2-16 A, B, C; 2-19 & 2-20.

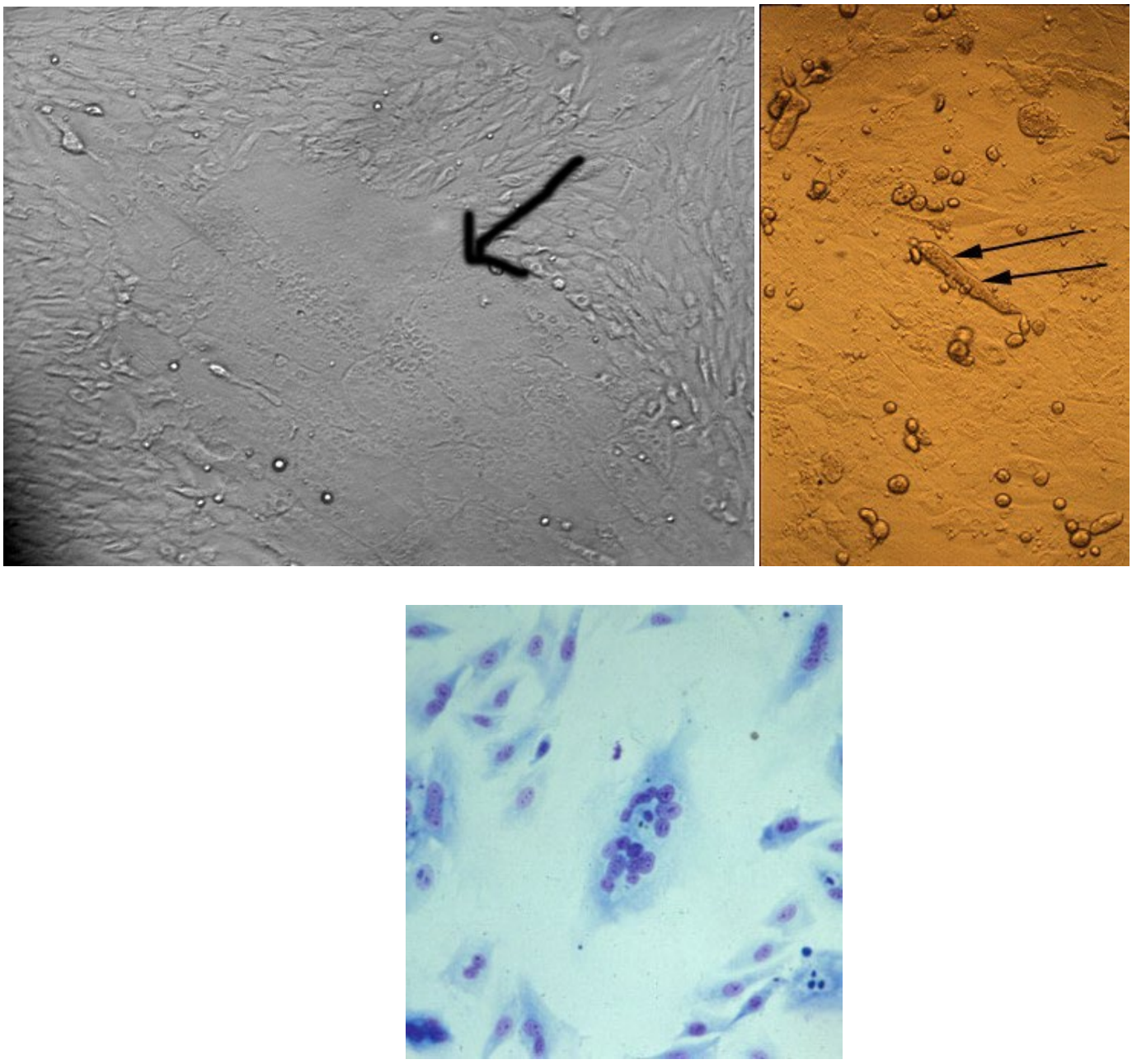


Figure 2-16. An unstained syncytium (arrow shows) in BFS cells infected with bovine herpes virus-2 two days post infection at 10X. 16B shows BFS infected with paramyxovirus 3 (PI_3) 4 days post infection demonstrating different looking more raised syncytium at 10X. Note syncytia can come in many different forms. 16C shows BFS cells infected with bovine syncytial virus 3 days post infection showing a small syncytium demonstrating how much easier it is to see the syncytia when they are stained. Image courtesy of Erica Suchman, Colorado State University.

Cytoplasmic Stranding is when the virus causes the cytoskeleton of infected cells to elongate leading to long, skinny cells. This type of CPE is often seen with particular herpesviruses. Shown in Figure 2-17 A & B.

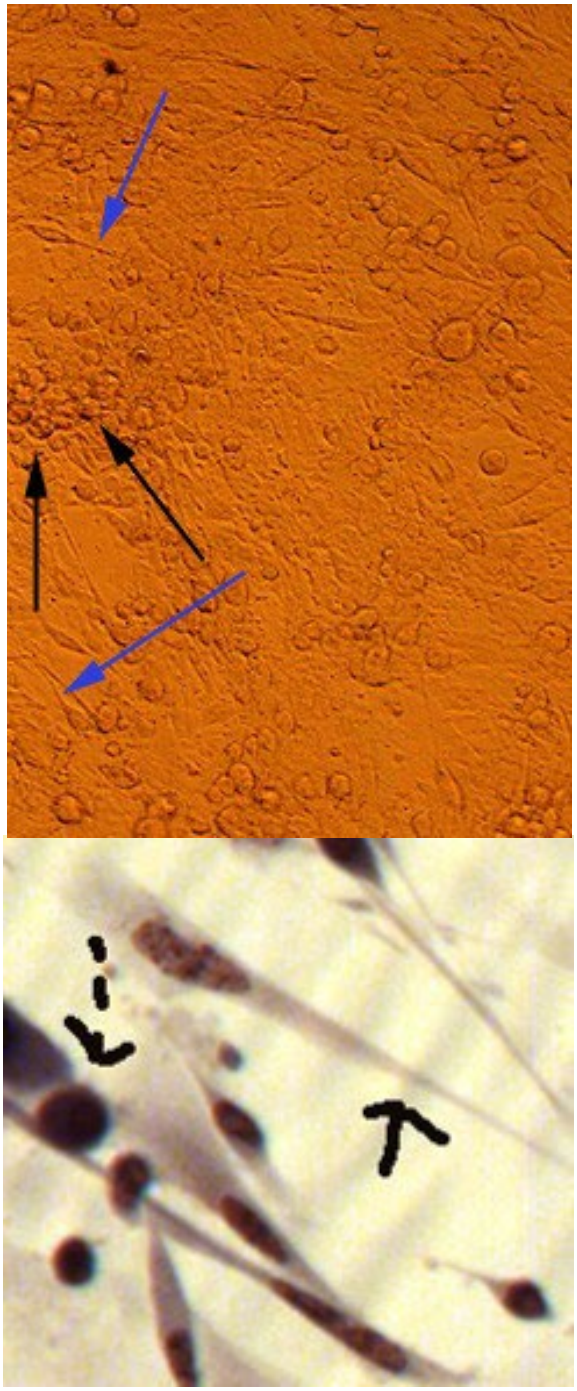


Figure 2-17. BFS cells infected with bovine herpesvirus-1 two days post infection at 10X magnification, unstained. (A) stained under 40X magnification showing cytoplasmic stranding. blue arrows (A) solid arrow (B), and cell rounding black arrows (A), dashed arrows (B). Image courtesy of Erica Suchman, Colorado State University.

Inclusion bodies are areas of altered staining in cells which can't be seen in live or unstained cell cultures. Depending on the causative virus, these inclusions may be single or multiple, large or small, round or irregularly shaped (with a jagged edge) and may have a whitish ring around the inclusion called a halo. Note that it is very important to look at what an uninfected cell looks like to make sure you don't confuse normal nucleoli (regions where the chromosomes reside) with nuclear inclusions. Remember that cell lines have gone through crisis and, as such, have aneuploidy resulting in multiple nucleoli as shown in Figure 2-18. Inclusions can be intranuclear (Figure 2-19 A, B & C), intracytoplasmic (Figure 2-20 A & B), perinuclear (Figure 2-21) (around but **not in** the nucleus), **eosinophilic/acidophilic** (pink staining) (Figure 2-20 A) or **basophilic** (blue-purple staining) (Figure 2-17B, 2-19A, B, 2-20B). Inclusion bodies can be differentiated with the May-Grunwald-Giemsa stain. A simple, rapid Giemsa stain can be performed but will not differentiate eosinophilic and basophilic inclusion bodies. In most cases, they represent areas of the cell where viral protein or nucleic acid is being synthesized or where virions are being assembled, or viral factories, but in some cases no virus is present and the inclusion bodies represent areas of viral scarring. Note that with staining you might also visualize chromatin margination, as shown in Figure 2-22, & 2-19, where the virus disrupts the nucleus so substantially that the chromatin is pushed to the nuclear membrane. At first the chromatin appears in clumps and may look like beads along the nuclear membrane. Later it will smooth out and appear like a thickened darker staining nuclear membrane than seen in uninfected cells. As you might expect, DNA viruses tend to cause nuclear inclusions if they cause them, and RNA viruses tend to cause cytoplasmic inclusions. With the exceptions, of course, of RNA viruses that replicate in the nucleus (orthomyxoviruses) and DNA viruses that replicate in the cytoplasm such as poxviruses.

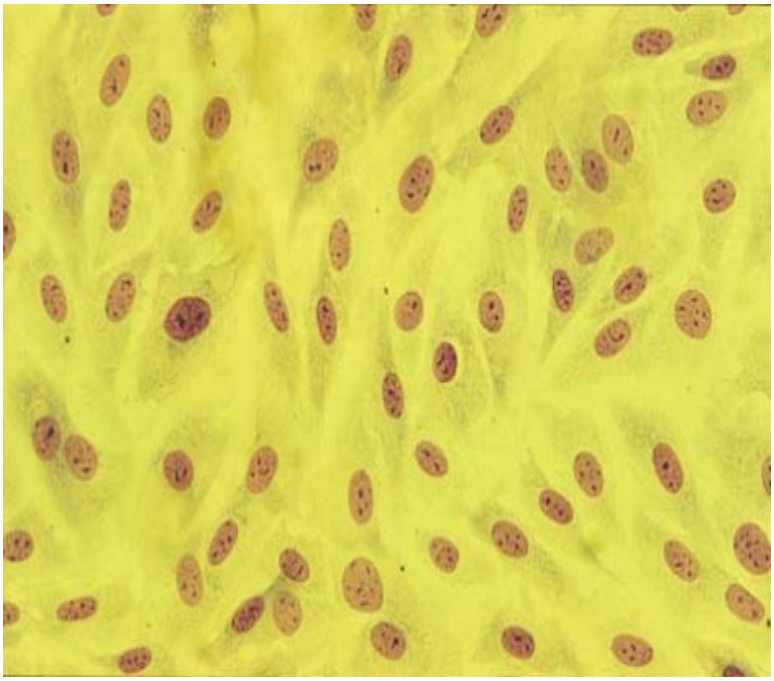
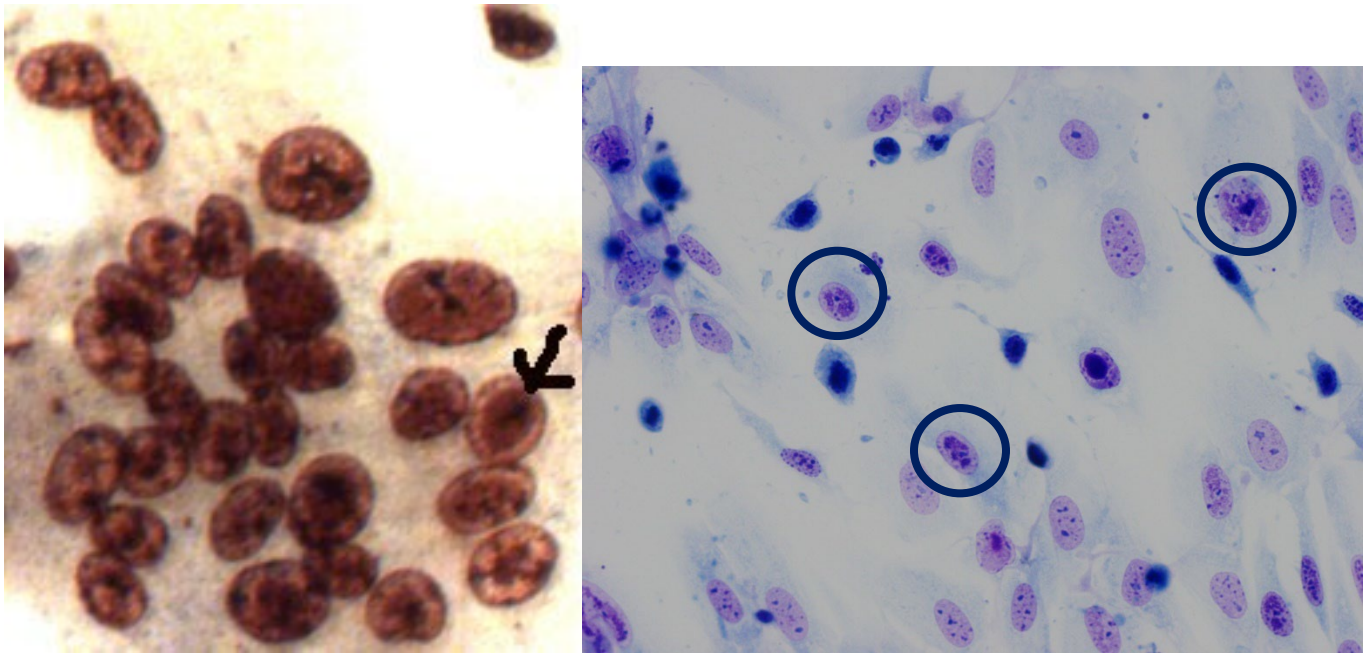


Figure 2-18. Giemsa stained uninfected BFS cells at 20X magnification. Note that each nucleus contains 4-5 nucleoli (pinkish spots within the nucleus). These should not be confused with nuclear inclusions which will be darker and larger and will often obscure the nucleoli. Image courtesy of Erica Suchman, Colorado State University.



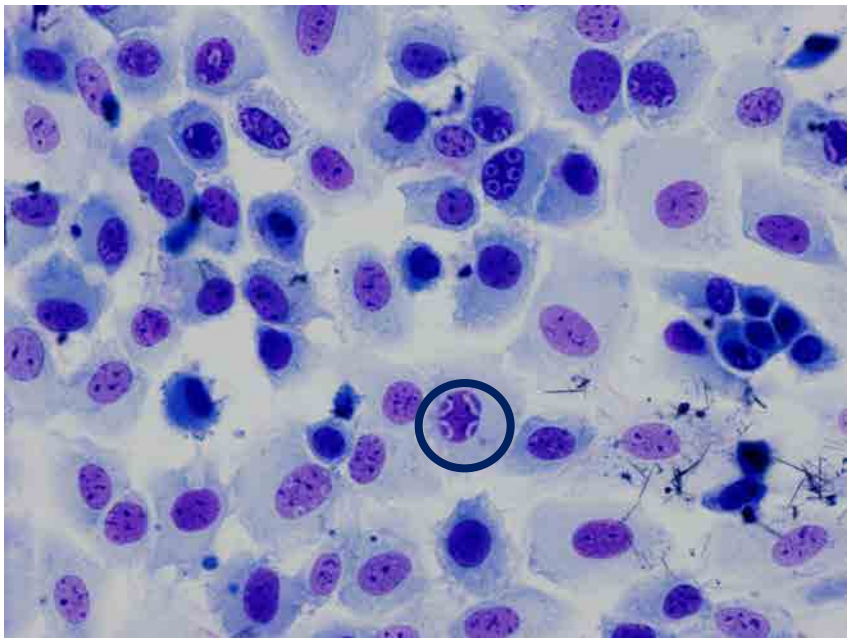


Figure 2-19A & B & C. Nuclear inclusions in BFS cells. A shows the large syncytia and basophilic inclusions with halo and chromatin margination (faint) typical of bovine herpesvirus-2 infection (20X magnification). Note this stain appears somewhat pink but is often a bluer shade typical of basophilic inclusions. B shows basophilic irregular nuclear inclusions with a rough edge typical of Bovine Adenovirus infections (10X magnification). C shows multiple basophilic inclusions with halo that fuse to form irregular nuclear inclusions with a rough edge and halos typical of bovine parvovirus infections (10X magnification). Image courtesy of Erica Suchman, Colorado State University.

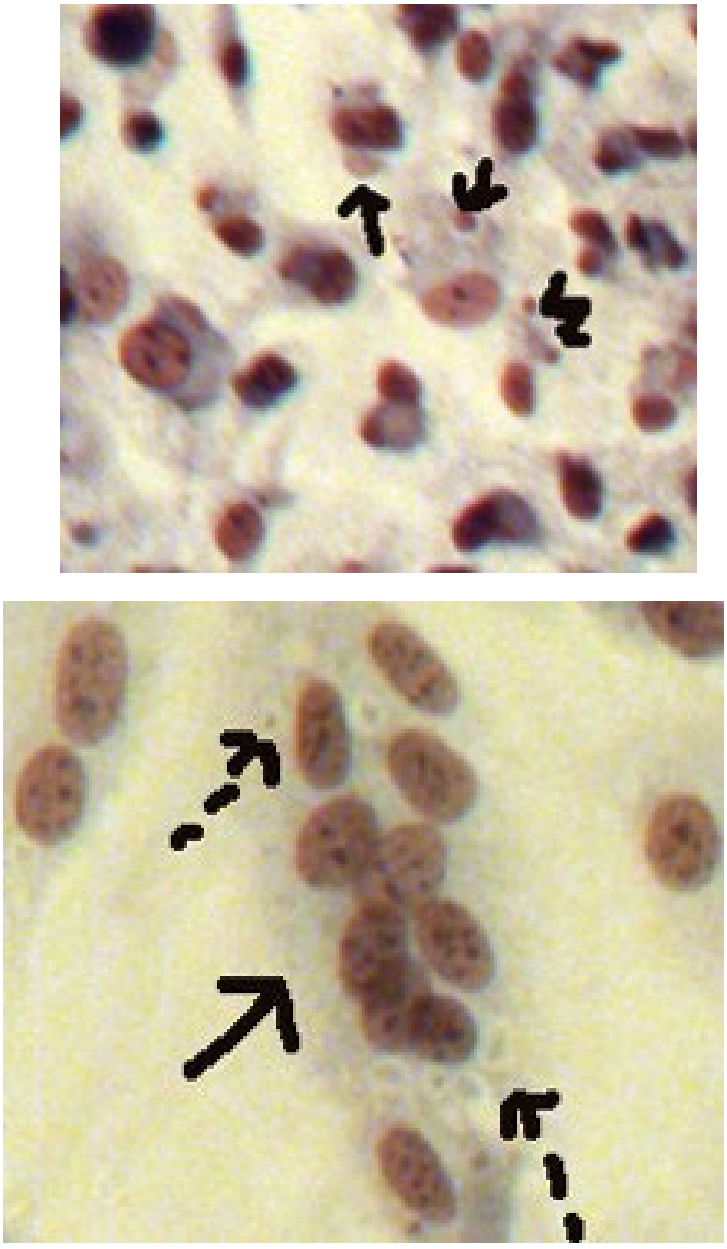
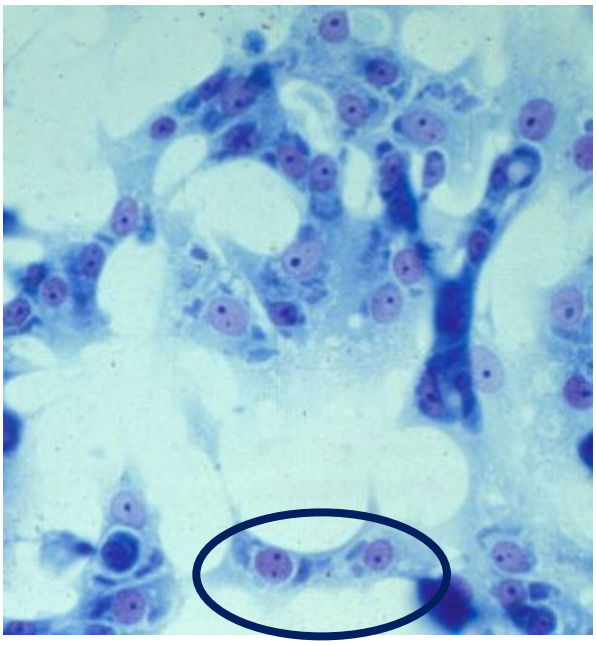
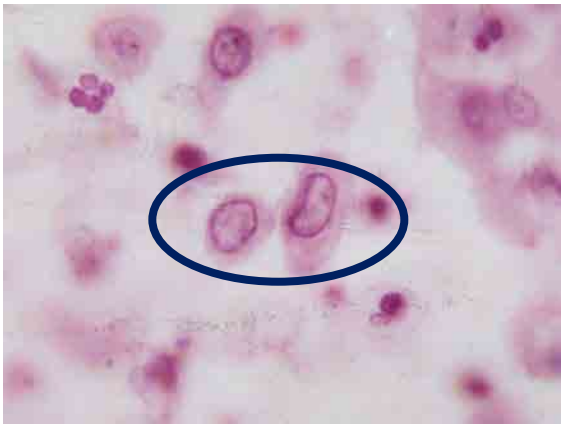


Figure 2-20 A & B. Both show BFS cells demonstrating cytoplasmic inclusions. A shows the eosinophilic cytoplasmic inclusions at 20X magnification of the poxvirus Orf. Although Pox is a DNA virus, it replicates in the cytoplasm, and hence causes cytoplasmic inclusions. B shows the basophilic cytoplasmic inclusions of paramyxovirus infected cells. Paramyxoviruses are RNA viruses that replicate in the cytoplasm. Image courtesy of Erica Suchman, Colorado State University.

Figure 2-21. Vero cells infected with reovirus demonstrating basophilic perinuclear inclusions (Peri means around). The inclusions are near but not in the nucleus as would be expected for the double stranded reoviruses that replicate in the cytoplasm. Image courtesy of Erica Suchman, Colorado State University.





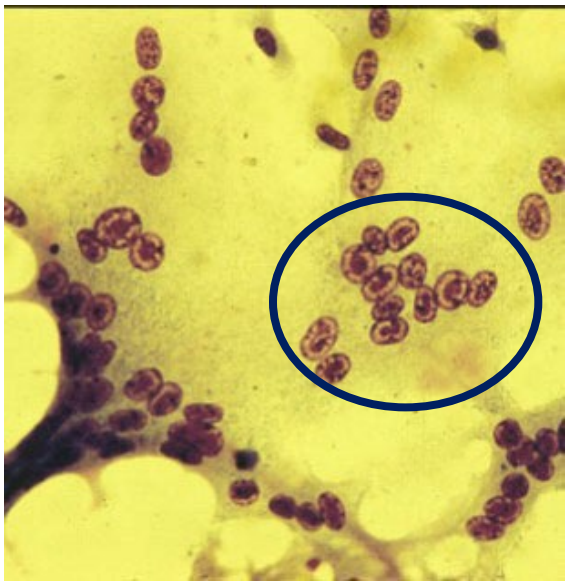


Figure 2- 22 A & B both demonstrate chromatin margination. A shows Feline Nasal Turbinate primary cell culture infected with feline herpes virus 1 at 40X magnification. Courtesy of Tom Walton APHIS (retired). B shows BFS cells infected with bovine herpes virus-2 at 10X magnification, also demonstrating nuclear inclusions with halo and pronounced syncytia formation. Image courtesy of Erica Suchman, Colorado State University.

CULTIVATION OF VIRUS IN EGGS

Viruses can only replicate in living cells. Before cell culture was developed, fertile chicken eggs were used to cultivate viruses in the laboratory. The use of eggs for virus propagation was first demonstrated by Woodruff, Goodpasture, and Burnet in 1930, and much early progress in the field of virology was due to use of this system. Chick embryos continue to have certain uses in virology. Under natural conditions many viruses are relatively host species-specific. Moreover, they may show a marked predilection for certain tissues of the host such as nervous tissue, epithelial tissue, etc.

While a number of viruses display host specificity and tissue affinity, or "**tropism**", the majority can be adapted to foreign hosts by **passage**. The cells and extra-embryonic membranes of the chicken embryo provide varied substrates that allow the growth of many viruses. Because of the ability to alter their tropism and adapt to a new host species, many viruses become capable of growing in chick embryo tissues. They may even attain a higher concentration than in the tissues of the natural host. Figure 2-23 shows the many routes of inoculation that can be used to infect eggs. Note that different viruses prefer different routes.

Viruses can also be cultivated in almost any animal; however, these assays are expensive and detrimental to the animals and as such are only utilized if no other method of cultivation is available.

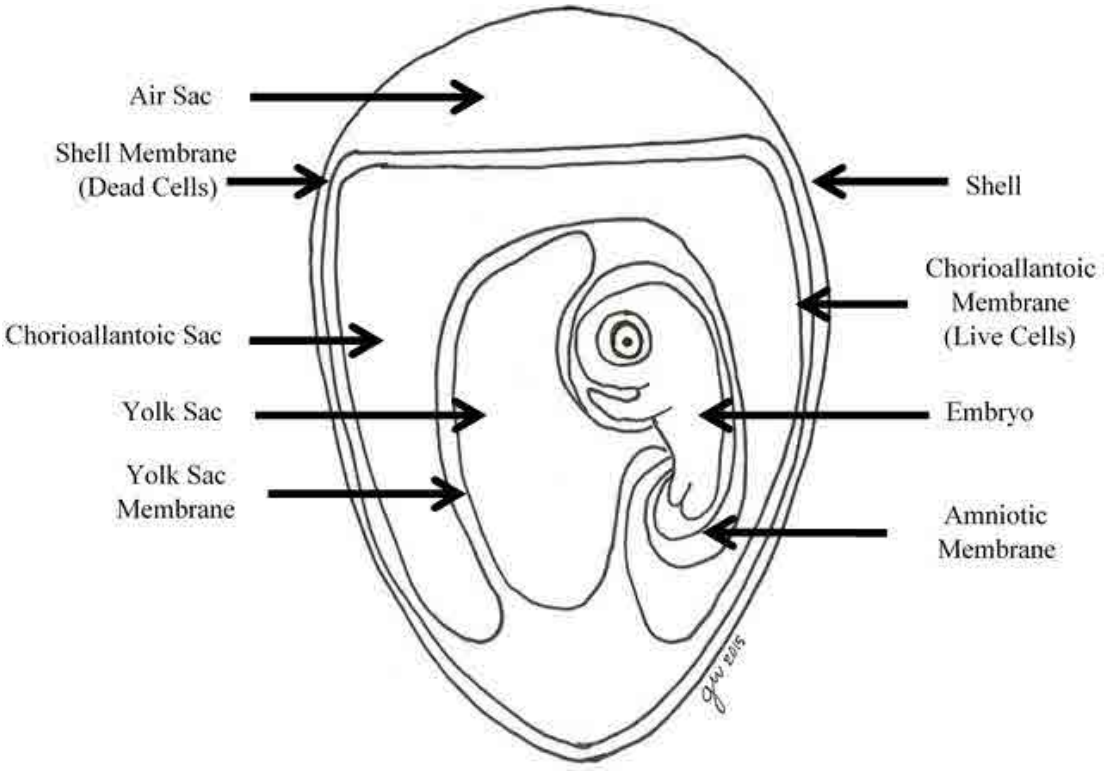


Figure 2-23. A drawing of a 5-day old chicken embryo with various routes of inoculation indicated. Image courtesy of Jonathan Martino, Colorado State University.

ISOLATION OF VIRUS PARTICLES

ISOLATION OF VIRUSES BY DIFFERENTIAL CENTRIFUGATION AND DENSITY GRADIENT PURIFICATION CENTRIFUGATION

The object of any virus purification scheme is to separate and remove from the virus any cellular components from the host system in which the virus was propagated. The techniques employed will depend on the composition and structure of the virus, the degree of cell association of mature virus, and the purpose for which purified virus is intended. Most purification procedures are based on size and density differences between virus particles and cells or cellular components and utilize differential and gradient centrifugation procedures.

Differential centrifugation yields a dirty prep with many cellular components within the virus stock. Often this is sufficient for infecting cells or other basic virology techniques. To do this, cells are centrifuged to a pellet at 5,000 rpm for 10 minutes which will cause the cells and larger cell debris to pellet. The fluid above the pellet, the **supernatant,** is removed leaving the pellet behind, which is discarded. The supernatant contains the viruses which are much lighter than cells and cell debris and cannot pellet except with much higher rpm centrifugation. The virus-containing supernatant is poured off into tubes and centrifuged at 25,000 rpm (~200,000 g) for 2 hours to pellet the virus. The supernatant is then removed and discarded, and the pellet containing the viruses is resuspended in the desired buffer. Figure 2-24.

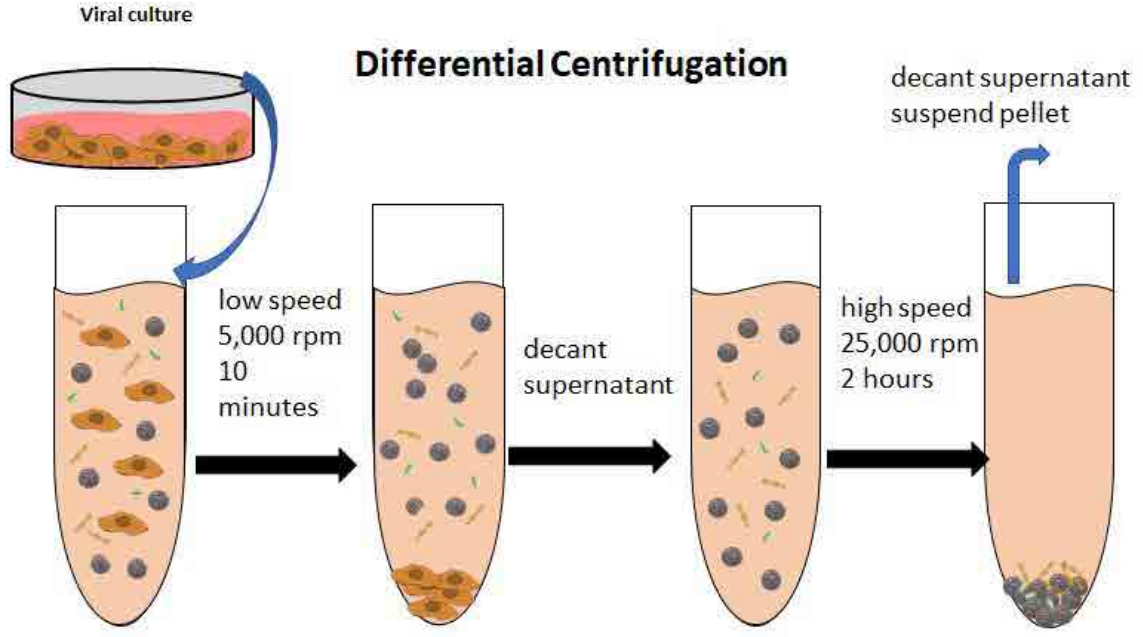


Figure 2-24 shows how differential centrifugation is performed. Image courtesy of Maxwell Drummond, Colorado State University.

VIRUS PURIFICATION GRADIENT CENTRIFUGATION

If cleaner virus preps are required to run a gel or Western blot, for example, or inject an animal to produce antibodies, one must further purify the virus after differential centrifugation. To achieve this, the concentrated virus suspension created by differential centrifugation is layered over a linear gradient of K tartrate, glycerol, or

sucrose and centrifuged to equilibrium buoyant density (12-16 hr at 25,000-35,000 rpm). Fractions of about 0.5 ml are collected by puncturing the bottom of the tube and position of virus band is determined by reading A260. Peak fractions are pooled.

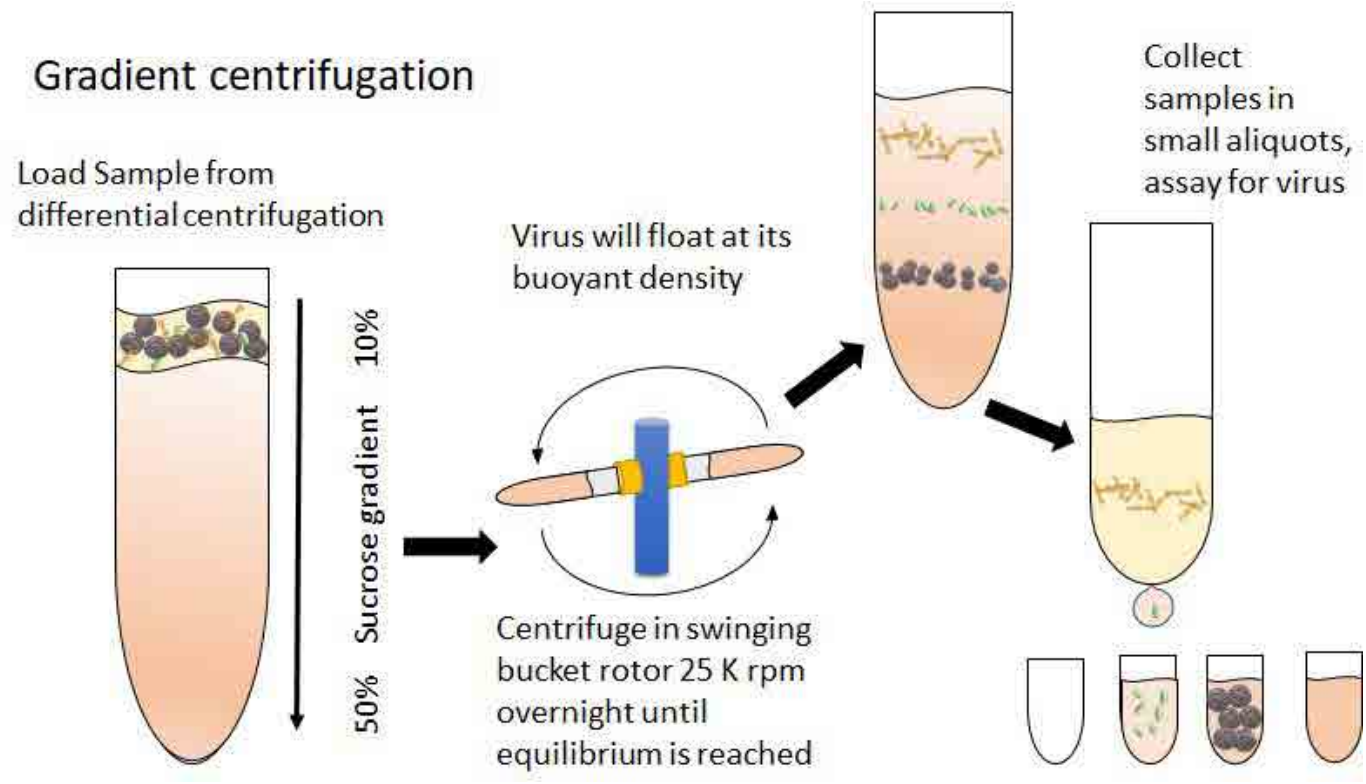


Figure 2-25 shows how gradient centrifugation is performed. Image courtesy of Erica Suchman, Colorado State University.

ANALYSIS OF VIRAL COMPONENTS

DETECTION OF VIRAL PROTEINS

The most commonly used mechanism for determining the size of viral proteins is SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS is a soap that binds to about every 4th amino acid giving proteins a net negative charge. The charge allows the proteins to separate by size by running towards the positive electrode at the bottom of the gel as shown in Figure 2-26.

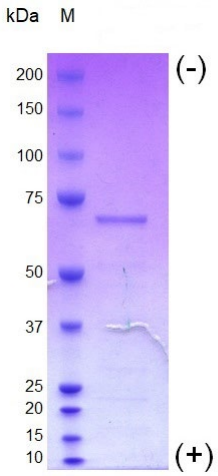


Figure 2-26 shows proteins separated on an SDS page gel by electrophoresis. Image courtesy of Sandra Quackenbush, Colorado State University.

IFA ASSAYS

The immunofluorescent technique is based on an antigen-antibody reaction in which the complex of intracellular antigen with applied antibody is made visible by covalent attachment of a fluorescent dye to the antibody molecule. A fluorescent dye such as rhodamine or fluorescein is conjugated to a specific immunoglobulin to produce a labeled antibody. The reactive form of fluorescein is fluorescein isothiocyanate, FITC. This abbreviation is often used for fluorescein. The labeled antiviral antibody is allowed to bind to viral antigen in infected cells that have been made permeable by treatment with acetone or other fixative. Upon exposure to ultraviolet light of the correct wavelength, the conjugated antibody-antigen complexes will fluoresce and emit light in the visible range. The fluorescein emission is greenish-yellow while rhodamine produces a reddish-orange emission. The fluorescent antibody is used to detect the presence of the corresponding specific viral antigen in cells or tissues. The intracellular location of the fluorescent complexes can give information about virus replication. A nonfluorescent counterstain is frequently used, such as Evan's blue, which will stain uninfected cells a light red.

There are two main techniques for fluorescent antibody staining. In the **direct** method, the fluorescent dye is coupled to the specific antiviral antibody which binds to the antigen in a one-step reaction. The advantages of the direct method are speed, simplicity, and less nonspecific staining. Disadvantages include the necessity to separately label each antiserum to be used. This method might also be less sensitive than the second type of fluorescent antibody staining, the **indirect** method. Indirect staining is a two-step process that can be used for detecting either viral antigen or antibody. In the first step, unlabeled virus-specific (primary) antibody is reacted with the tissue or specimen. In the second step, labeled anti-immunoglobulin (secondary) antibody, produced in a different animal species from the primary antibody, is allowed to bind to the complex. Indirect staining is convenient because only one or a few antisera must be labeled with a fluorescent dye, and these can be purchased. Indirect immunofluorescence is also more sensitive than the direct method, but requires more time and more manipulations, and is more likely to produce nonspecific staining. As shown in Figure 2-27, virus is detected in these cells infected with a DNA virus, showing the expected staining in the nucleus where the virus replicates as well as the cytoplasm where the viral proteins are produced.

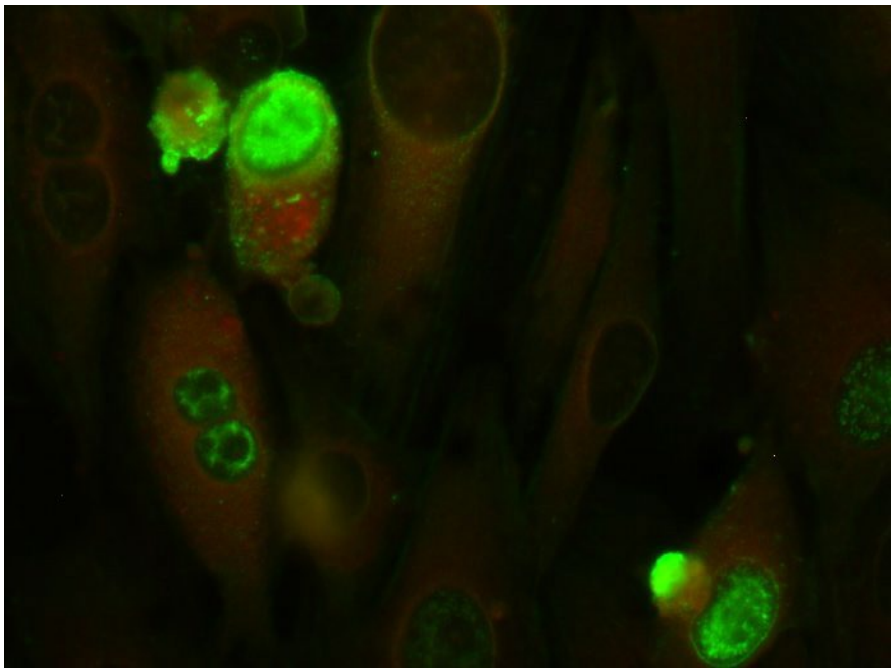


Figure 2-27 shows BFS cells infected with the DNA virus bovine parvovirus stained with fluorescently labeled anti-BPV capsid protein antibodies which give off a green light under UV light. The cells are counter stained with Evan's Blue which stains the cells and they appear red under UV light. Image courtesy of Erica Suchman, Colorado State University.

Remember that there are trends where viruses will replicate within a cell. RNA viruses are more likely to replicate in the cytoplasm, with orthomyxoviruses being the only RNA virus we will discuss in this book that replicates in the nucleus. DNA viruses, with the exception of poxviruses, replicate in the nucleus. You will note that you see staining in the nucleus and cytoplasm in this parvovirus infected cell. Why do you think you see staining in the cytoplasm if this virus replicates in the nucleus? Hint: what viral component does the antibody most likely bind to, and where is this viral component synthesized in the cell?

WESTERN BLOT

Western blots can be used in the study of viruses as well as in detection and diagnosis. Proteins are separated by SDS-Page as described below. When electrophoresis is complete, the proteins are transferred onto a membrane that can be probed with labeled antibodies. If the viral proteins are present, they will bind to the antibodies and a color change will occur at the location of the viral proteins, as shown in Figure 2-28.

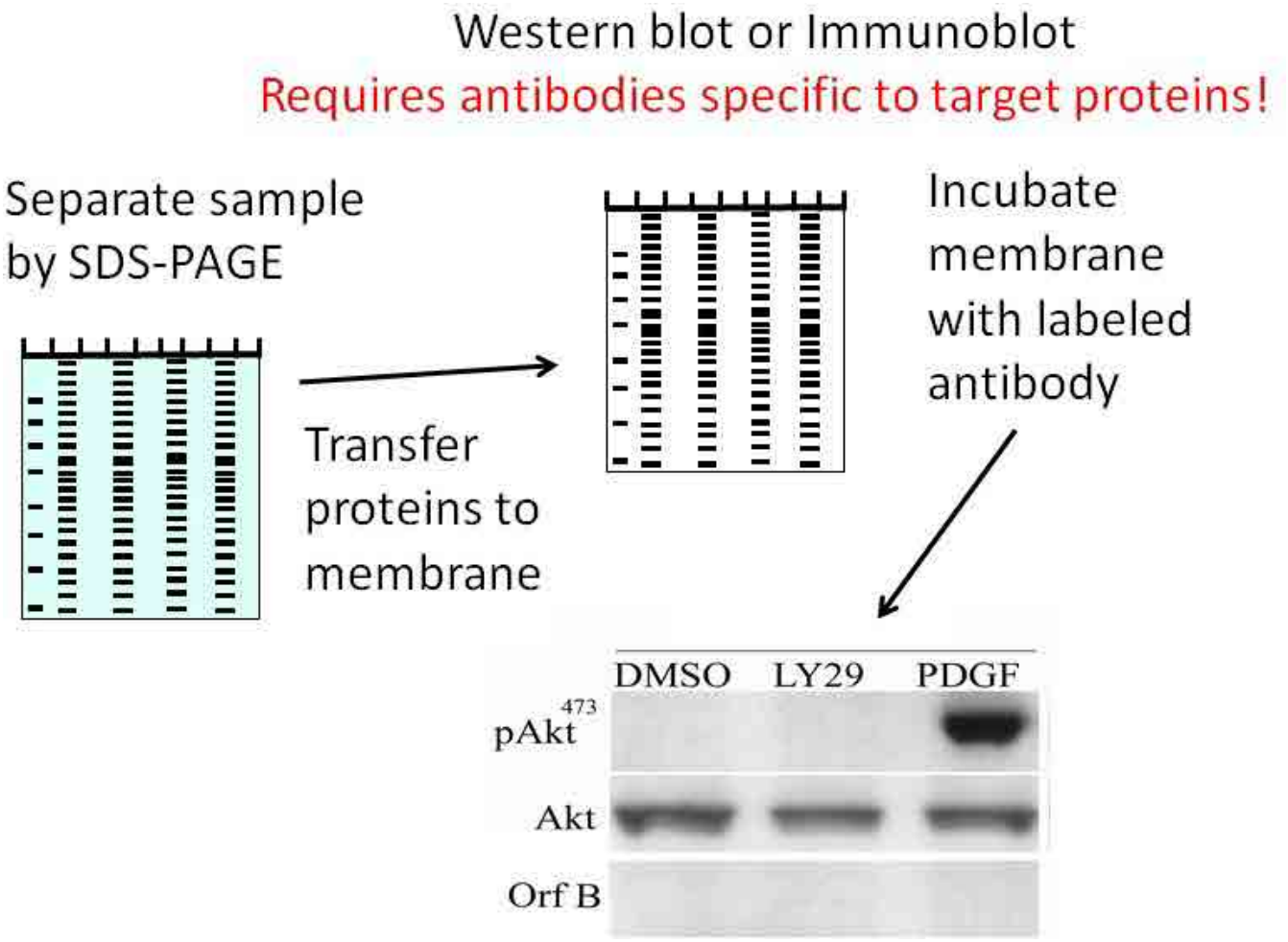


Figure 2-28. The process of creating a western blot for viral proteins. Courtesy of Sandra Quackenbush, Colorado State University.

ELISA ASSAYS

In the ELISA technique, viral antigens or antiviral antibodies are identified by the binding of specific antibody (immunoglobulin) to which an active enzyme has been covalently attached. Subsequently, the enzyme reacts

with a **chromogenic** (color-producing) substrate and the colored product is measured to quantitate the antigen or antibody present. Both IgG and IgM antibodies are routinely detected using ELISA assays. Recall that IgM is the first antibody produced during an immune response and as such can often detect infections more rapidly. However, this antibody is quickly replaced by IgG, so later in the infection IgM may be difficult to detect. Therefore, it is important to think about the timing of the assay relative to the suspected infection.

Purified virus antigen or antibody (frequently monoclonal antibody) is adsorbed to a solid substrate, usually the wells of a microtiter plate. The binding is stable but not covalent. Attachment allows easy separation of bound and free reagents and reactants. Surface areas not covered by antigen or antibody must be blocked with a nonreactive protein to prevent nonspecific sticking of the antigen or antibody in the specimen being assayed. After each step in the procedure, thorough washing of the surface is performed to remove nonspecifically bound material. Instruments may be purchased that add reagents, carry out washes, and finally spectrophotometrically read the absorbance of each reaction in order to automate and standardize the process. The titer of the antiserum is expressed as the ELISA titer and is the reciprocal of the highest dilution that produces the colored product, meaning 1/the highest serum dilution at which there is a color change.

When analyzing serum virus neutralizing IgG antibody, acute and convalescent sera from the same patient are compared in an ELISA with an increase in antibody titer 4-fold or more indicating that the patient was recently (or currently) infected with the test virus. A preexisting high titer of IgG antibodies that will not change over the two weeks may be the result of a previous infection or vaccination with this virus. Some viral infections cannot be cleared; therefore, one IgG ELISA positive serum is sufficient to indicate current infection (i.e., HIV). For the majority of viruses, two samples must be taken at least 2 weeks apart, and at least a 4-fold increase in ELISA IgG titer must be noted to indicate a current viral infection. The reason for a 4-fold difference being the threshold has to do with the fact that these assays are run utilizing a 2-fold serial dilution. Looking for at least a 4-fold increase eliminates the possibility that you are observing a titer that is between the 2-fold serial dilutions. For example, if the titer is 65 it could appear positive or negative at the 40 or 80 dilution and would give you a false positive. See Figures 2-29 A & B. Note that some viral infections can be detected using a single sample if assaying for IgM within the correct time frame. Furthermore, as we saw during the COVID-19 pandemic of 2019, for a brief period of time, infections with a novel virus can be detected with a single serum sample as no previous immunity is possible. However, this ability to sample with only 1 serum sample does not persist over time if the virus establishes within the population and cases are again observed in the future, at which point 2 samples will be required.

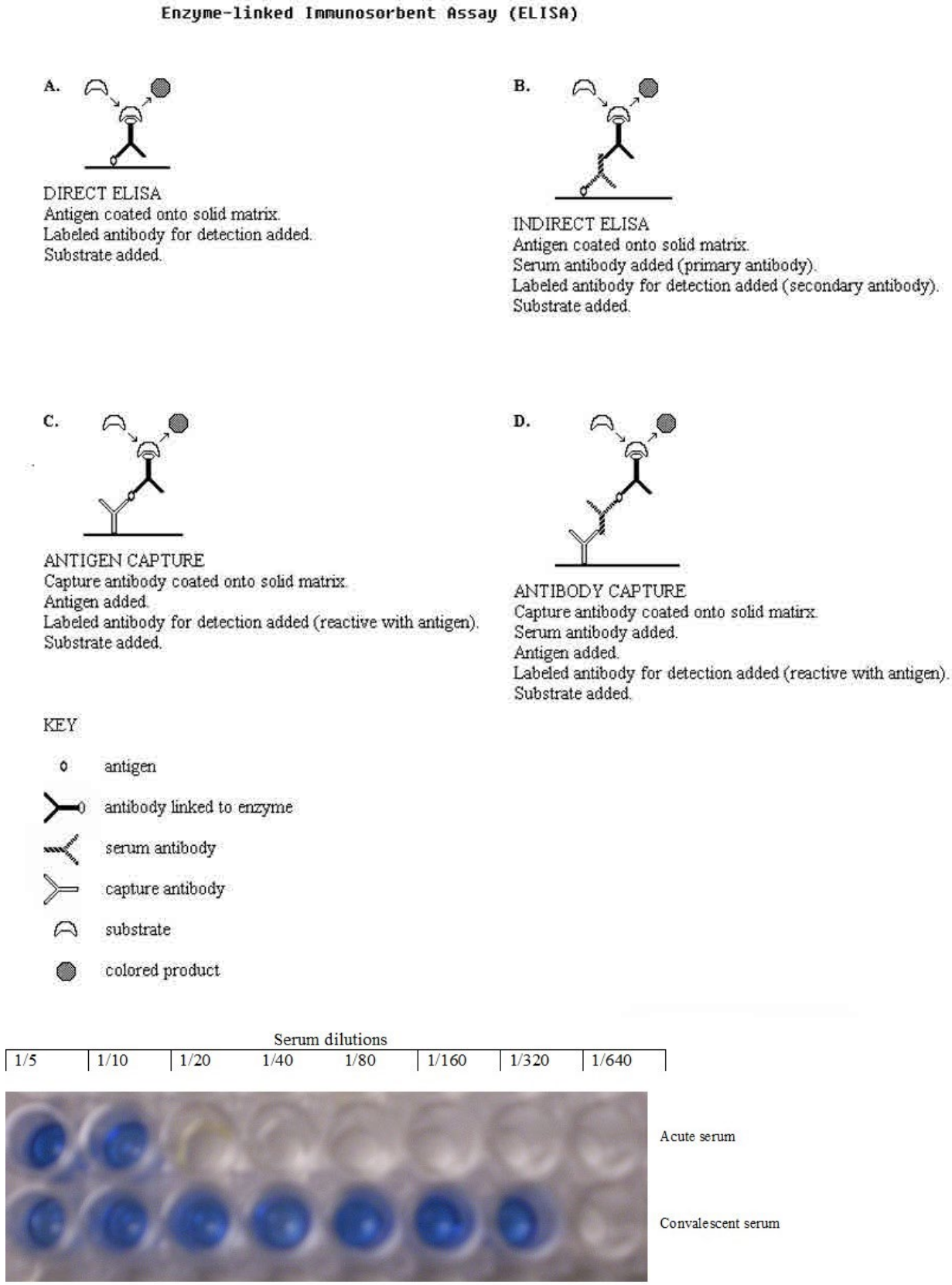


Figure 2-29 A & B ELISAs. The 4 most common ways of setting up an ELISA are demonstrated in Figure 2-29A and expected results are shown in Figure 2-29B. Image courtesy of Erica Suchman, Colorado State University.

Why Do a Capture ELISA? According to Dr Alan Schenkel, Colorado State University

"It's the capture part, greater sensitivity and less noise when the capture antibody is ready to "capture" the antigens. Direct ELISAs tend to be much noisier (non-specific binding and signal where there should not be) and a lot of antigen can be lost/missed. Non-capture ELISAs are probably at least 10-100 fold less sensitive, depending on the antigen."

INFECTIVTY ASSAYS: DETERMINING THE AMOUNT OF INFECTIOUS VIRUS IN A SAMPLE

PLAQUE ASSAYS

This method determines the number of infectious units (infectious virus particles) in a given suspension. This type of enumeration is made from focal lesions such as plaques in cell cultures, pocks on the chorioallantoic membrane of chick embryos, or local necrotic lesions on a plant leaf. The number of infectious units per unit volume can be calculated, and this is referred to as the titer. With plaque assays the titer of the original virus suspension is stated in terms of the number of plaque forming units (PFU) per ml. For a 6 well plate only count the **wells containing 20-100 plaques**. See Figure 2-30 A. Note that individual plaques can be picked to produce viral clones, however these viruses would still require purification by gradient centrifugation. See Figure 2-30 B. If you used a plate with a larger diameter i.e. a 4 well plate or a single well plate the highest countable number of plaques would be larger.

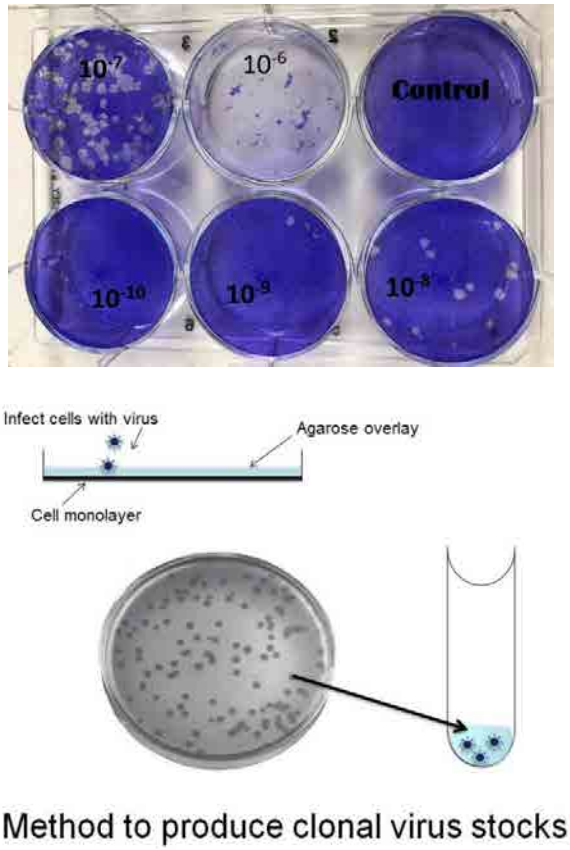


Figure 2-30A demonstrates typical plaque assay data. As expected, an approximately 10-fold difference in the number of plaques is seen in each successive well inoculated with a 10-fold serial dilution. There are too many plaques to count when cells are infected with a dilution of 10^{-6}, 100 at 10^{-7}, 10 (Too few to count) at 10^{-8}, 1 at 10^{-9}, and none at 10^{-10}. 30 B shows how plaques can be isolated to create a viral clone that arose from a single virus particle. Image courtesy of Erica Suchman and Sandra Quackenbush, Colorado State University.

Example Calculation:

You observe three cell culture plates inoculated with 0.1 ml of 10^{-5} dilution of a viral stock. On the first plate you count 47 plaques, on the second 53, and on the third 50 plaques.

1. Find the average number of plaques per plate.

Average # of plaques = Total # of Plaques/Number of Plates
50 = (53+47+50)/3

2. Find the number of **P**laque **F**orming **U**nits/ml (**PFU/ml**) of stocksolution.

PFU/ml = Average # of Plaques/(Dilution)(InoculumVolume)

5.0×10^{7} PFU/ml = 50 plaques/(10^{-5})(0.1 mls)

Note: this can also be expressed as the average # of plaques x 1/(dilution)x 1/(volume)

QUANTAL OR END POINT DILUTION ASSAYS

The end-point assay determines whether or not an inoculum contains infectious particles by allowing them to replicate in a suitable host (test unit) so that the presence of infectious units can be detected by the amplification effect of the infection. The actual number of infectious particles introduced into the test unit is unknown and may vary even between duplicate inoculations from the same dilution.

To determine quantal infectivity titers, multiple replicate test units are inoculated for each dilution of original suspension until the infectivity is diluted out. The result gives the dose necessary to produce a defined response (infection, death, etc). This response is usually based on a 50% endpoint which is the dilution at which 50% of the test animals, eggs, or cell cultures are infected by the virus.

Therefore, the dilutions need to be carried out beyond the point where 50% of the test units are infected. Computation of the 50% endpoint is based on the presence or absence of a predetermined criterion in each test unit, i.e., death (**lethal dose50 or LD50**), infectivity (**tissue culture infective dose50 or TCID50, egg infective dose50 or EID50**), etc. The criterion is binary. It must be either present or absent – either the animal is dead or alive, or the cell culture is infected or not infected. There are no plus/minus or graded reactions. This method does not measure the number of infectious virus particles but only whether or not virus is present at a particular dilution. The titer determined in a quantal assay should not be converted to a quantitative titer (i.e., no direct conversion of $TCID_{50}$/ml to PFU/ml). Figure 2-31.

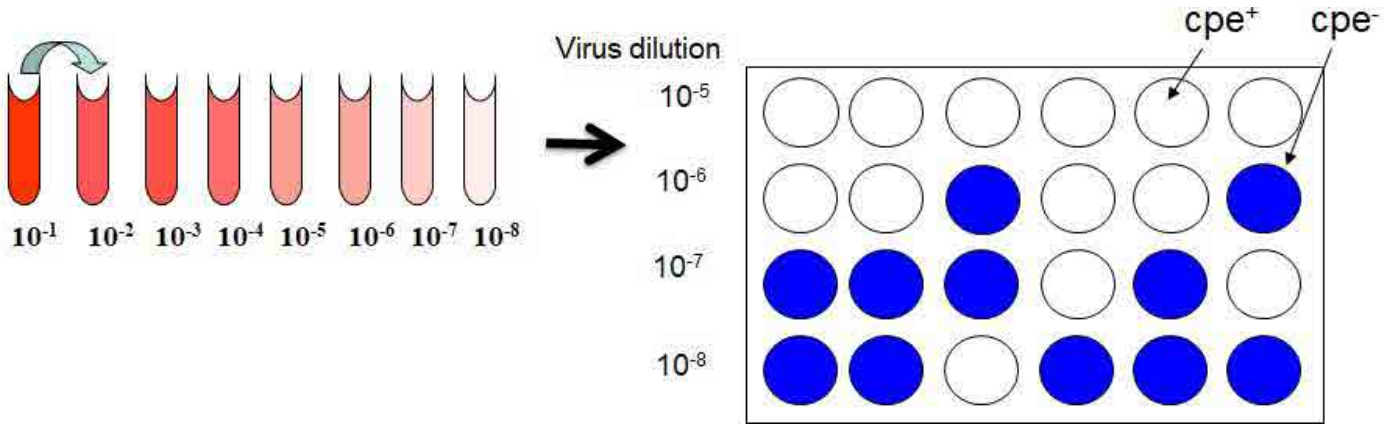


Figure 2-31 demonstrates how to set up an end point assay. Image courtesy of Sandra Quackenbush, Colorado State University.

Methods to determine 50% endpoints

There are two formulas that can be used to determine 50% endpoints: **the Reed-Muench and the Kärber methods**. Both are demonstrated here using the same data. The end point was death of the test units. The inoculum was 0.1 ml/test unit.

Results of Titration

Note that for this set of data you would expect your endpoint (LD_{50}) to fall somewhere between the 10^{-4} and 10^{-5} dilution using both the Kärber and Reed-Muench methods, however they will give slightly different values.

Kärber Method:

Results of titration

Virus Dilution (a)	Died (b)	Survived (c)	***Mortality Ration and Percent*** (For Kärber)		***Accumulated Values*** (for Reed-Muench)			
			Mortality Ratio (d)	**Percent** (e)	Died (f)	Survived (g)	**Mortaliy Ratio** (h)	**Percent** (i)
10^{-2}	6	0	6/6	100	17	0	17/17	100
10^{-3}	6	0	6/6	100	11	0	11/11	100
10^{-4}	4	2	4/6	67	5	2	5/7	71
10^{-5}	1	5	1/6	17	1	7	1/8	13
10^{-6}	0	6	0/6	0	0	13	0/13	0

Kärber Method:

This method uses the data from columns **a-e** to find the 50% endpoint.

Equation:

$$\text{Log}(LD_{50}) = x-[d(P-0.5)]$$

x = log(lowest dilution)

d = log(dilution factor)

Dilution Factor = 1/Dilution

P = Sum % mortality at each dilution/100

Equation explained:

Log(LD_{50}) = (log(lowest dilution)) – log(dilution factor)((sum mortality/100)-0.5)

1. **Identify values:** The lowest dilution used in this experiment is 10^{-2}. The dilution factor is 1/10 because you made 10-fold dilutions. The percent mortality is found in column e. They will be summed to calculate P.
2. Plug in values:

$Log(LD_{50}) = (log(10^{-2})) – log(1/0.1)((100+100+67+17+0/100)-0.5)$

3. Solve: **$Log(LD_{50}) = -2 – 1(2.84-0.5)$**

$Log(LD_{50}) = -4.34$

4. Take the **inverse logarithm** of both sides to find the LD_{50}

$LD_{50} = 10^{-4.34}$

4. Interpret LD_{50}:

- Inoculation of many animals with 0.1 ml of a $10^{-4.34}$ dilution of the virus stock will result in the death of 50% of the animals.

5. **Find Titer:** Titer = the reciprocal of LD_{50}/volume of inoculum

Titer = $10^{4.34}$ LD_{50}/0.1 ml

6. **Find LD_{50}/ml:** Add **log(1/volume of inoculum)** to the exponent of the previous titer.

$10^{5.34}LD_{50}$/ml

To convert titer to LD_{50}/ml add the Log(1/volume of inoculum added)

If you added 0.1 ml, add 1 (1/0.1 = 10; log of 10=1) so $10^{4.34}$ LD_{50}/0.1 ml becomes $10^{5.34}LD_{50}$/ml
If you added 0.2 ml, add 0.7 (1/0.2 = 5; log of 5 = 0.698) so $10^{4.34}$ LD_{50}/0.2 ml becomes $10^{5.04}LD_{50}$/ml
If you added 0.3 ml, add 0.52 (1/0.3 = 3.3; log of 3.3 =0.52 so $10^{4.34}$ LD_{50}/0.3 ml becomes $10^{4.86}LD_{50}$/ml
If you added 0.4 ml, add 0.38 (1/0.4 = 2.5; log of 2.5 =0.38 so $10^{4.34}$ LD_{50}/0.4 ml becomes $10^{4.72}LD_{50}$/ml
If you added 0.5, ml add 0.3 (1/0.5 = 2; log of 2 =0.3 so $10^{4.34}$ LD_{50}/0.5 ml becomes $10^{4.64}LD_{50}$/ml

7. Interpret Titer:

- The original undiluted virus stock contains $10^{5.34}LD_{50}$/ml.

Reed-Muench Method: This method calculates nearly the same value as the Kärber method. It uses the accumulated values (columns f and g) derived from the observed values (columns b and c).

1. **Accumulate Values:** Figures in column (f) are accumulated by adding each successive figure in column (b) starting at the bottom (highest dilution). Figures in column (g) are accumulated from column (c) by adding downward. Figures in column (h) are (f)/(f+g).

2. Using these accumulated values, at the 10^{-4} dilution, 5/7 or 71% of the test animals died, and at the 10^{-5} dilution, 1/8 or 13% died (columns h and i). **The 50% endpoint, therefore, lies somewhere between the 10^{-4} and 10^{-5} dilutions**. The final calculation requires interpolation between these two values.

3. **Find Proportionate Distance:**

(% mortality above 50%)-50%/(% mortality above 50%)-(% mortality below 50%) = Proportionate Distance

4. **Plug in values and solve:**

(71-50)/(71-13) = Proportionate Distance (PD)
21/58 =0.36

- The % mortality values used in this formula **must be the accumulated values**.

5. **Correct for dilution:** Multiply the proportional distance by -1 x log(dilution factor).
   - (2-fold = 0.3, 5-fold = 0.7, 10-fold = 1)

Corrected PD = (PD) (-1)(log(dilution factor))
Corrected PD = (0.36)(-1) = -0.36

6. **Determine 50% Endpoint:**

$$\log (LD_{50}) = \log (10^{-4}) + (-0.36)$$
$$\log (LD_{50}) = -4.36$$

7. **Take inverse logarithm:**

$$LD_{50} = 10^{-4.36}$$

8. **Find titer:** Take the reciprocal of the LD_{50}

Titer = $10^{4.36}$ LD_{50}/0.1 ml or $10^{5.36}$$LD_{50}$/ml

To convert titer to LD_{50}/ml add the Logarithm of the volume factor added

If you added 0.1 ml, add 1 (1/0.1 = 10; log of 10=1) so $10^{4.34}$ LD_{50}/0.1 ml becomes $10^{5.34}$$LD_{50}$/ml
If you added 0.2 ml, add 0.7 (1/0.2 = 5; log of 5 = 0.698) so $10^{4.34}$ LD_{50}/0.2 ml becomes $10^{5.04}$$LD_{50}$/ml
If you added 0.3 ml, add 0.52 (1/0.3 = 3.3; log of 3.3 =0.52 so $10^{4.34}$ LD_{50}/0.3 ml becomes $10^{4.86}$$LD_{50}$/ml If you added 0.4 ml, add 0.38 (1/0.4 = 2.5; log of 2.5 =0.38 so $10^{4.34}$ LD_{50}/0.4 ml becomes $10^{4.72}$$LD_{50}$/ml If you added 0.5 ml, add 0.3 (1/0.5 = 2; log of 2 =0.3 so $10^{4.34}$ LD_{50}/0.5 ml becomes $10^{4.64}$$LD_{50}$/ml

VISUALIZATION AND ENUMERATION OF VIRUS PARTICLES

ELECTRON MICROSCOPY

Remember that not all viral particles will be infectious. See Figure 2-32 for an electron micrograph of densonucleosis virus from the author's lab. You will note some of the particles are dark in the middle meaning they

did not package genome correctly. These particles which do not form correctly are referred to as defective interfering particles because they can bind to receptors blocking infectious particles from doing so. The previously described assays can enumerate infectious particles, but if it is necessary to enumerate both infectious and non-infectious, the only way to count virus particles visually is to use electron microscopy. This can also be used to determine virus capsid shape and morphology as well as detect virus from fluids. There are, however, cheaper and faster ways to count viral particles.

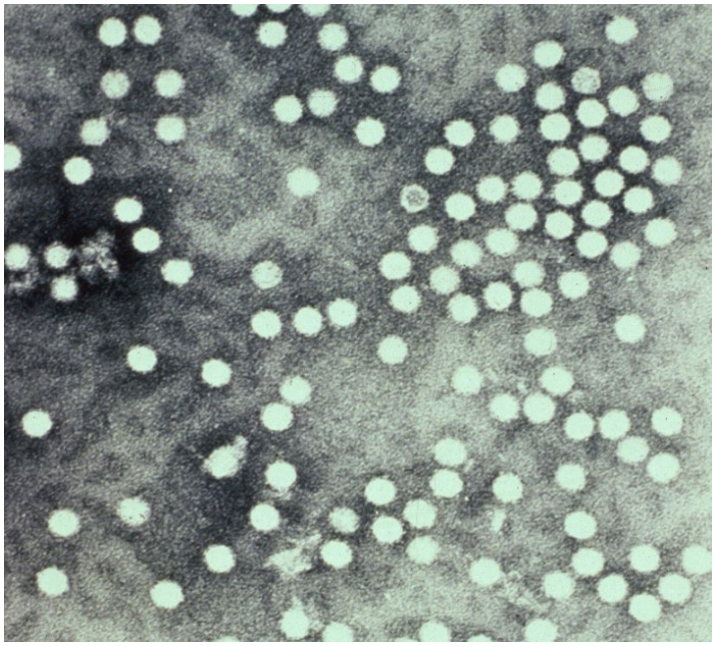


Figure 2-32. Electron micrograph of Densonucleosis virus infecting C636 cells demonstrating both infectious (white centers) and defective interfering (dark centered) viral particles. Image courtesy of Erica Suchman, Colorado State University.

HEMAGGLUTINATION ASSAY

Some virus particles are capable of binding red blood cells together. This process is called **hemagglutination.** The hemagglutination assay capitalizes on this attribute to quantify the concentration of viral stocks. In this assay the viral stock will be diluted to determine the point at which there are not sufficient virus particles to agglutinate the RBCs. This assay is performed in a V-bottomed microtiter plate. With insufficient virus particle concentrations, the RBCs will settle out and form a button on the bottom of the well because the V shape will funnel the RBCs to the bottom. When there is sufficient concentration of virus (generally greater than 10^4 virus particles/ml), the virus will bind the RBCs together along the walls of the well to form a lattice. Note you may see incomplete lattice formation (lattice with a button) when the virus particles are lower than 10^4 particles/ml. The goal of the hemagglutination assay is to determine the greatest dilution that still results in a **complete** lattice formation. This reciprocal of the endpoint dilution is reported as **one H**emagglutination **A**ssay **U**nit or **HAU/ volume** (used in HA assay). An example is shown in Figure 2-33 A & B.

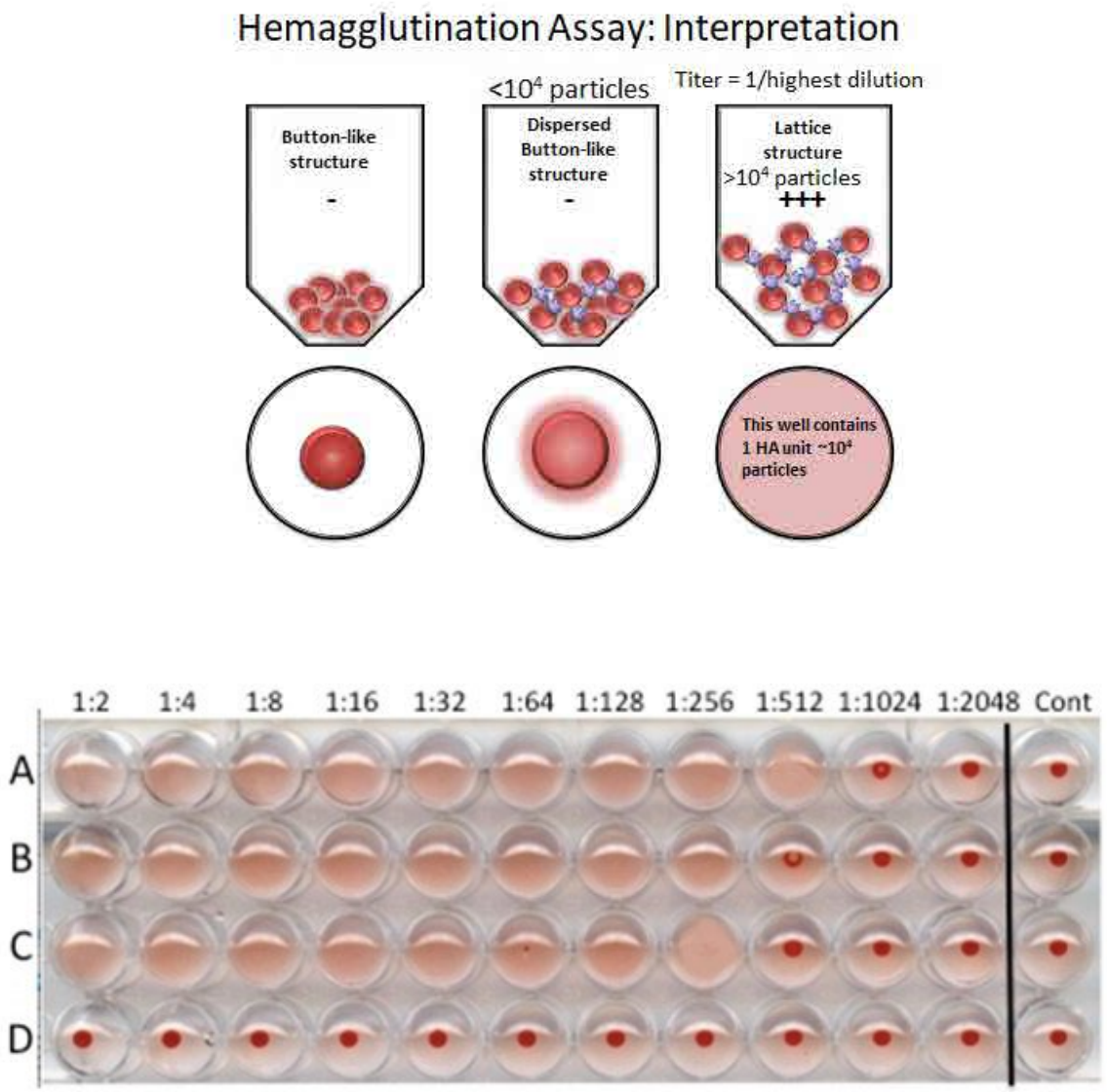


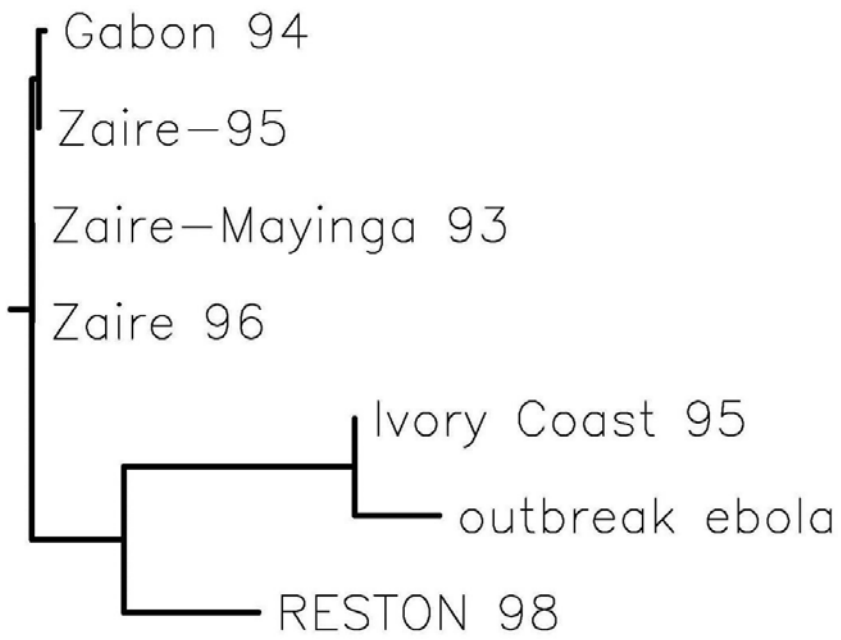
Figure 2-33 A diagrams how to interpret HA assays and 32B shows typical HA assay data. Note the lattice formation in the wells containing a low dilution (high titer) of the virus. As the virus is diluted out (1:512, or 1:1024), buttons form when there is no longer enough virus to hemagglutinate the RBC. In B 1:512 and A 1:1024 bubbles have formed in the buttons as is sometimes seen, these would still be recorded as buttons, and negative for hemagglutination. Image courtesy of Erica Suchman and Traci Kinkel, Colorado State University.

GENETIC RELATIONSHIPS OF VIRUSES: USE OF MOLECULAR SYSTEMATICS IN VIROLOGY

PHYLOGENETICS

Genetic or evolutionary relationships between viruses are often assessed by molecular phylogenetic analysis. Determining molecular relationships between different virus isolates can also be used in epidemiology. When virologists determine nucleotide sequences of DNA or RNA or amino acid sequences of proteins, they usually deposit them in a public database so that they can be accessed by other virologists (discussed above under sequencing). Several programs are available for alignment of sequences, pairwise comparison, and arrangement in a "**phylogenetic tree**". All the programs make particular assumptions about the types and rate of mutations that occur in viral genomes, then construct and compare all possible trees, and use statistical methods to predict relatedness of the viruses being compared coming up with a "best tree". **Bootstrapping** is the process of creating the phylogenetic tree 100-1000 times and seeing what percentage of the time a particular branch is obtained. Generally, a value of over 70 is considered valid and indicates that 70 times out of 100 (or 700 times out of 1000) this branch was obtained.

The tree obtained may vary depending on the region of the viral genome (the gene) or the function of the protein chosen for comparison. It is usually assumed that changes occur more rapidly in structural than in nonstructural proteins. Structural proteins make up the capsid and envelope if present, whereas the non-structural proteins carry out viral replication and control the host cell's antiviral defenses. Genes that mutate more slowly are said to have these sequences "conserved", whereas genes that mutate more rapidly are non-conserved or variable. You can see this in the following trees of Ebola, both rooted (A) and unrooted (B) trees of an Ebola outbreak. Rooted trees indicate the original ancestor, unrooted do not. See Figure 2-34. Both types of trees help you determine where this particular Ebola virus probably originated.



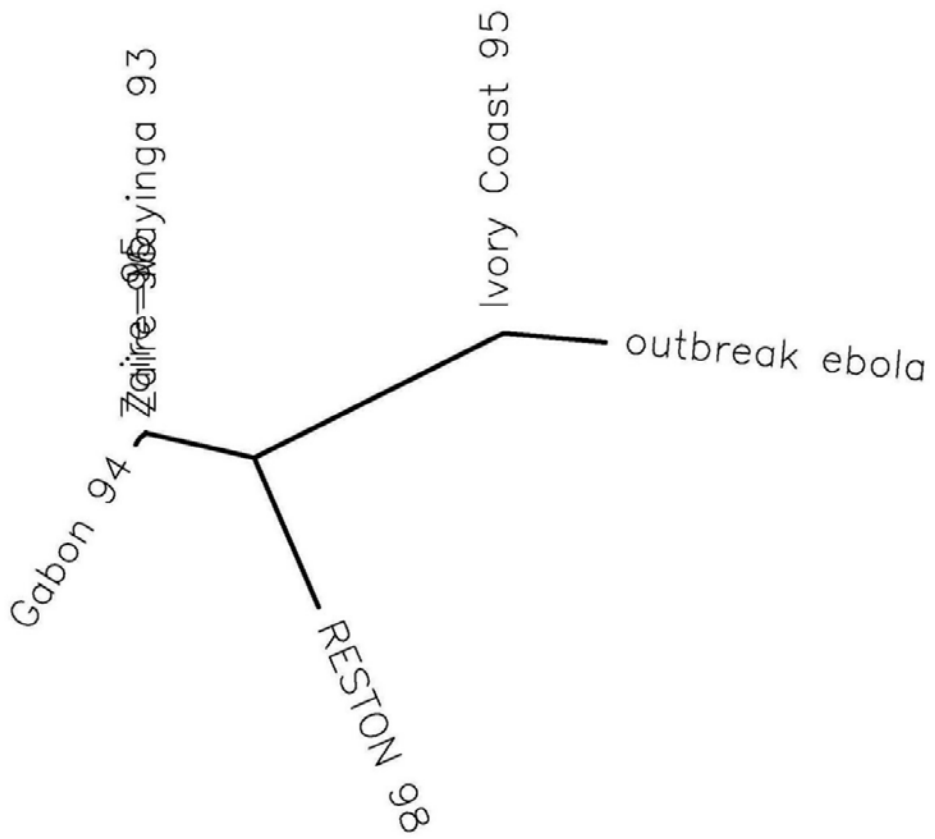


Figure 2-34A B: a rooted (A) and unrooted (B) tree of an Ebola outbreak. Image courtesy of Erica Suchman, Colorado State University.

Note: You can tell from these trees that the Ebola outbreak in Zaire in 1993 and 1996 are of the same virus, however, the outbreak in 1995 was a slightly different virus. Furthermore, the outbreak in Gabon is most closely related to the virus that caused the 1995 outbreak.

METHODS TO MANIPULATE VIRUSES IN THE LAB OR DECREASE REPLICATION

RNA INHIBITION (RNAI)

RNAi is a naturally occurring antiviral defense mechanism found in most eukaryotic cells that is activated by double stranded RNA in stretches longer than 125 base pairs. This is because cells rarely create double stranded RNA that is this long, hence this serves as an excellent signal to the cell that an RNA virus is replicating as it is very difficult for RNA viruses to avoid having stretches of dsRNA longer than 125 base pairs. Although RNA-dependent RNA polymerases (RdRp) usually do not keep the newly synthesized strand and template strand together to avoid activation of interferon responses, it is inevitable that some of these complementary RNAs will find each other and bind in infected cells, hence activating RNAi. Similarly, DNA viruses that express genes from both strands of the virus (which some do in order to fit more genes into their small genomes) must worry about activating RNAi as well. Note, there is much debate about the importance of RNAi in mammalian cells. It is known that mammalian cells contain the machinery to carry out RNAi, however, it is currently believed that the interferon response is much more important and supersedes the RNAi response. However, this is a topic of much debate. Regardless, RNAi can be used to control viral reproduction and cellular gene expression by scientists in the laboratory.

The presence of dsRNA is recognized by the endoribonuclease enzyme **Dicer** which cuts the dsRNA into 21 bp sequences (called **siRNA,** si = **s**hort **i**nterfering). **Risc (RNA-induced silencing complex)** replaces Dicer and takes only one of the 2 strands of the 21 bp RNA (degrading the other) and uses this to bind to complementary RNA in the cell. Risc then cleaves the RNA to which the siRNA bound. Note that different molecules of Risc will degrade different strands of the dsRNA and hence both strands are represented and can target the virus. See Figure 2-35. RNAi is
being used to target viral replication and gene expression in mosquitoes, plants, and mammalian cells.

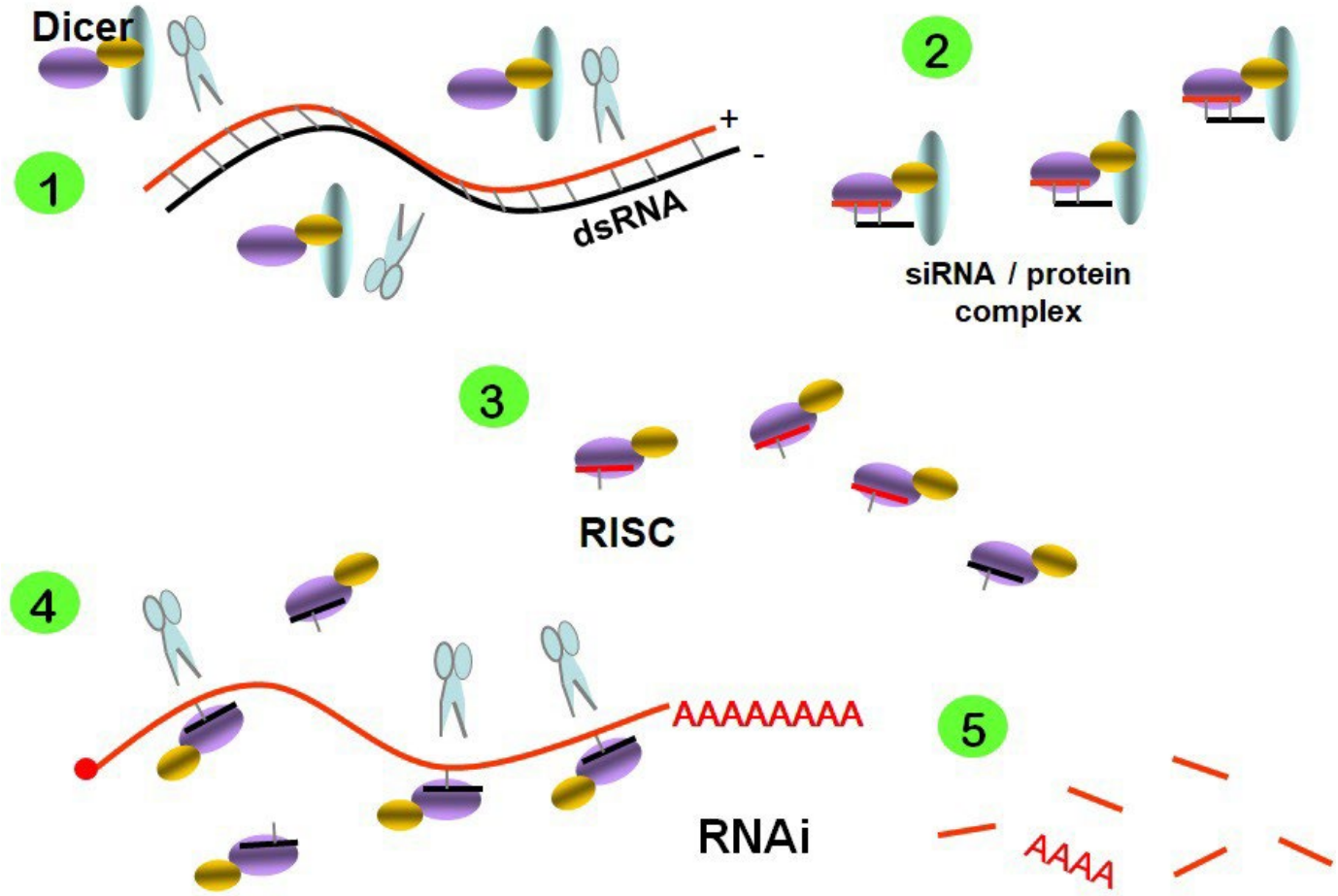


Figure 2-35. How RNA interference (RNAi) is activated and carried out. Courtesy of Alexander Franz Colorado State University.

CRISPER CAS9, CAS13 AND CAS12A

CRISPR stands for **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats. It was originally described in bacteria and is in fact an anti-phage defense. You can think of it as a rudimentary immune response. When a bacterium is infected with a DNA phage, Cas9 cuts it up and puts small bits of the DNA within the CRISPR sequence within the bacterial chromosome. Basically, it stores these bits so it can remember this virus's genome if it ever sees it again. The CRISPR sequences transcribe these bits into short RNAs that can recognize the phage should it ever infect again. This **guide RNA** will "guide" Cas9 to the phage DNA by hybridizing to the complementary sequence in the phage DNA allowing Cas9 to digest the phage DNA. But if these bits are in the chromosome, how is the host DNA not digested? This involves **protospacer adjacent motifs** (PAM) that are NOT part of the CRISPR but are found in the phage genome immediately adjacent to the bits of DNA stored in the CRISPR. When Cas9 originally cuts the phage DNA to put bits into the CRISPR, it cuts right next to the PAM sequences within the viral genome, but the PAMs do not end up in the bits. Hence the PAMs are not included in the bacterial

chromosome. If this is a primitive immune defense in bacteria, why are we interested in this in animal virology? Although CRISPR/Cas9 is not found in eukaryotic cells, scientists have developed mechanisms for introducing Cas9 and target mRNAs with PAMs into cells allowing site specific cleavage of viral or host cell DNA. Then host cell or viral DNA repair mechanisms can be used to either create mutations at the site of cleavage or insert specific DNAs into the cleaved genome. Once the DNA is cut, researchers use the cell's own DNA repair machinery to add or delete pieces of genetic material or to make changes to the DNA by replacing an existing segment with a customized DNA sequence. See Figure 2-36.

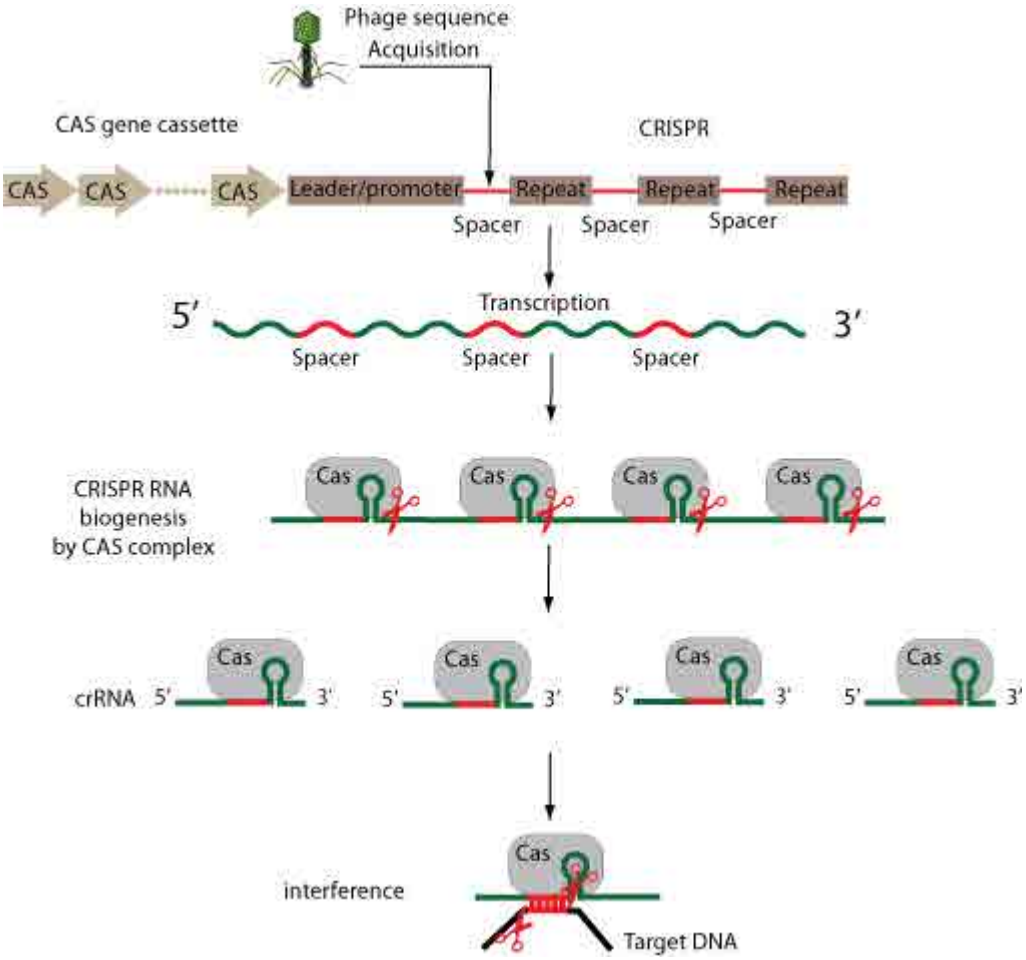


Figure 2-36. How the CRISPR Cas 9 system works. Note this diagram does not indicate the PAM sequences that will be required for CAS9 to cut the target DNA, and the guide RNA is labeled crRNA. Courtesy of the Viralzone https://viralzone.expasy.org/3962

Note that bacterial cells also have to contend with RNA phage, and so, not surprisingly, they have another Cas enzyme, Cas13, that will cleave RNA viruses, also using a guide RNA. Unlike Cas9, which cuts up only the complementary sequence of the guide RNA, Cas13, once activated, cleaves both the host cell RNA and the viral RNA causing infected cells to die (much like Interferon in eukaryotic cells). Note that Cas13 does not require a PAM sequence to cut. However, Cas13 has been genetically modified to make it more specific to cutting the target RNA (and not the host RNA) and is being tested for antiviral therapies for RNA viruses like influenza. In particular, these more specific Cas13 enzymes and guide RNAs can be attached to nanoparticles and inhaled to target influenza viral replication in the respiratory tract.

Recently a new Cas protein, Cas12a, has been found. It functions much like Cas9 using a guide RNA and PAMs to bind the target DNA. Note that there are slight differences in these guide RNAs from those that work for Cas9,

and these are also referred to as crRNAs for **CR**ISPR RNA. This binding activates the cleavage ability of Cas12a, however it, much like Cas13, cuts not just the target mRNA. Although this enzyme is not useful for creating transgenic organisms, it is being developed for detection of microbial infections. The idea is that the guide RNA will bind to the target microorganism near a PAM. Let's say we are creating a diagnostic test for the canine parvovirus that causes disease in dogs. We would create a guide RNA with a sequence that would specifically bind to this parvovirus. We would also add to the sample isolated from a potentially infected dog a dinucleotide probe where one nucleotide has a fluorescent label and the other a quencher dye. As long as these two nucleotides are covalently bonded, the quencher quenches the fluorescence of the other nucleotide. However, if canine parvovirus is present, the crRNA or guide RNA will bind to the virus near a PAM thus activating Cas12a which will cut up the canine parvovirus in the sample as well as the dinucleotide probe added to the test. When the probe is cleaved, the quencher dye and fluorescent dye will be separated, and the fluorescence can be detected. What steps would you need to add to create diagnostics using Cas12a for an RNA virus such as SARS-CoV-2?

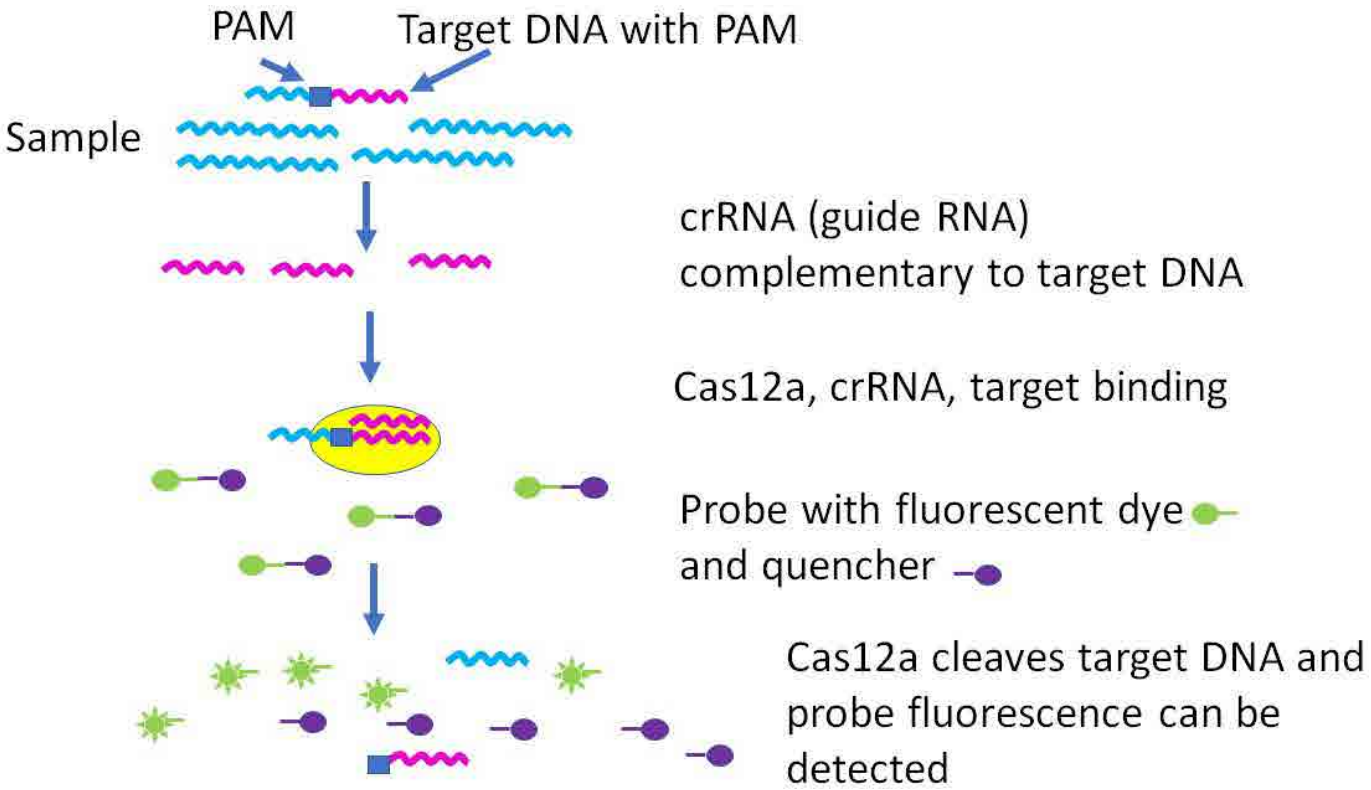


Figure 2-37. CRISPR Cas12a use as a diagnostic tool for viral infections. Note Cas 13 could also be used as a diagnostic for RNA viruses. Courtesy of Erica Suchman, Colorado State University.

As discussed above Cas13 in its natural state also cuts non-specifically. As such Cas13 can also be used to detect RNA viruses or the mRNA of DNA viruses by the exact same mechanism of CAS12a using an added probe with quencher and florescent dye.

CHAPTER 2 END OF CHAPTER QUESTIONS

1. What is the difference between a qualitative and a quantitative assay?
2. What is the difference between a direct and an indirect IFA assay?
3. On what type of sample can you run a PCR reaction? Why only these types of samples?
4. What sample types require a reverse transcriptase PCR analysis, and why?
5. How does real time PCR differ from PCR?
6. You isolate a virus with a CT value of 45 on Real Time PCR and another with a CT value of 24. Which of these samples is/are positive? How do you know?
7. How does next generation sequencing differ from PCR?
8. Give an example of when you would use each of these types of PCR.
9. What are the pros and cons of each type of PCR?
10. You perform an ELISA on a patient you believe has a cytomegalovirus infection. You take a sample when they come to your office and obtain a titer of 200 EU/0.3 ml. Two weeks later you take a second sample and find a titer of 400 EU/0.3 ml, both of which are above the threshold. Why did you take a second sample? What do these test results tell you about this patient and whether they have a cytomegalovirus infection?
11. Give examples of types of CPE and how you will recognize them. Also indicate what characteristics of viruses (not specific families) you might expect viruses showing these types of CPE might have.
12. You are performing a plaque assay. You plate 0.2 ml of a serial dilution in each well. In the 10^{-2} well you observe too many plaques to count. In the 10^{-3} well you count 220, 10^{-4} 21, and 10^{-5} 2. What is the titer of your original viral stock?
13. Compare and contrast growing virus in cell culture vs embryonated eggs.
14. You need to isolate a virus from cell culture. If the virus stock will be used to run a Western blot, what procedure will you use? How will this differ if you want to use the virus to inject into a mouse to isolate anti-viral antibodies?
15. You perform an end point dilution assay where you plated 1 ml of the following serial dilution and obtain the following results. Without doing the math, what do you predict your $TCID_{50}$ will be? Now do the math using either the Kärber or Read-Muench method and see if you get a titer that you expect.

Virus Dilution	CPE	NO CPE
10^{-3}	8	0
10^{-4}	6	2
10^{-5}	3	5
10^{-6}	1	7
10^{-7}	0	8

16. You are creating phylogenetic trees of flaviviruses circulating in the southern United States. Your colleague suggests you perform bootstrapping. What will the bootstrapping analysis tell you?
17. If you obtain a value of 35 for a branch, would this be compelling evidence that this branch is valid?
18. Compare and contrast RNAi and CRISPR Cas13.
19. Compare and contrast CRISPR Cas9 and CRISPR Cas13.

20. You have been asked by your boss to develop a system to knock out a gene of interest in mice using CRISPR Cas9. Explain how you will do this.
21. How is it that the bacterial genome is not targeted by the CRISPR Cas9 system?
22. You isolate a virus that creates nuclear inclusions. What type of genome is this virus MOST LIKELY to possess? Justify your answer.
23. What other assays are Cas12a diagnostic tests similar to, and how?
24. Do you think you could use Cas13 to perform diagnostic tests? Why, or why not?
25. What other steps would you need to perform to run a diagnostic test using Cas12a on an RNA virus?
26. You create a Cas12a diagnostic test for two viruses from different families. Can you use the same crRNA? How about the same fluorescent/quencher dinucleotide probe? Why, or why not?

Use the following data to answer the next 3 questions

	S gene CT value	N gene CT Value
Patient 1	52	15
Patient 2	44	48
Patient 3	20	12
Patient 4	48	34

27. Which patient(s) is/are infected with a non-variant SARS-CoV-2? Explain why you answered as you did.
28. Which patient(s) is are infected with Variant SARS-CoV-2? Explain why you answered as you did.
29. Which patient (s) is/are not infected with SARS-CoV-2? Explain why you answered as you did.

REFERENCES:

Alekseyev, Y et. al. (2018). Next-Generation Sequencing Primer—How Does It Work and What Can It Do? Academic Pathology Volume 5: 1–11. doi: 10.1177/2374289518766521 https://journals.sagepub.com/doi/full/10.1177/2374289518766521

Behjati, S. and Tarpey, P. (2013). What is next generation sequencing? Arch Dis Child Educ Pract Ed. 2013 Dec; 98(6): 236–238. doi: 10.1136/archdischild-2013-304340; https://pubmed.ncbi.nlm.nih.gov/23986538/

Howard Hughes Medical Institute. (2019). https://www.hhmi.org/news/crispr-enzyme-protects-bacteria-turning-infected-cells-themselves/

Maillard, P., et al. (2019). Slicing and dicing viruses: antiviral RNA interference in mammals. EMBO J 38:e100941https://doi.org/10.15252/embj.2018100941

Meeske,A. et. al. (2019). Cas13-induced cellular dormancy prevents the rise of CRISPR-resistant bacteriophage. *Nature*. doi: 10.1038/s41586-019-1257-5

Reed, L. J. and Muench,H. (1938). A simple method of estimating fifty per cent endpoints, Amer. Jour. Hyg., 27, 493-497

Suchman, E., Blair, and C. Martino, J. MIP425 Virology and Cell Culture laboratory manual Colorado State University. (2021)

ViralZone CRISPR-cas system https://viralzone.expasy.org/

CHAPTER 3

Chapter 3: An Introduction To Viral Infectious Cycles

Introduction to viral life cycles

This chapter will introduce you to the viral infectious cycles. This will be a general introduction that will explain to you the mechanisms utilized by viruses in each step of their life cycles. You will note that viruses with different genome types will replicate differently. This chapter will expose you to concepts you will see again as we progress through the book and the viral families that infect and cause significant disease in humans and animals.

A COMMON STRATEGY FOR VIRAL PROPAGATION

The following are common attributes of all viruses:

1. The viral genomes are packaged inside particles.
2. The viral genome contains the information necessary to complete an infectious cycle within an appropriate cell.
3. The viruses are able to establish themselves in a host population to ensure survival.

STEPS IN VIRUS REPLICATION

All viruses replicate in a similar manner. They attach to a cellular receptor protein or cellular receptor membrane glycolipid. They enter the cell via a myriad of methods. They move to their replication location, reproduce their genomes, transcribe their viral mRNA, and translate their viral proteins. After enough viral genome and proteins have accumulated, virions self-assemble and are released from the cell by either lysis, budding, or via exocytic vesicles.

VIRUSES REQUIRE THE HOST CELL FOR PROPAGATION

Viruses are obligate intracellular parasites. In order to replicate, viruses require many cellular components that the viruses cannot produce. As we go through the viral families in future chapters, you will learn which viruses require which cellular components. However, here we will present a basic summary of cellular components a virus may use to replicate.

- Cellular proteins or glycosylations (of proteins or lipids) that are expressed on the outside of the cell and used by the virus as a receptor.
- Endocytic vesicles to enter the cell.
- Endosomes to help the virus uncoat.
- The nucleus to replicate utilizing its DNA replication machinery, transcription machinery, and mRNA modifying proteins (DNA viruses, retroviruses, and orthomyxoviruses). Remember that viruses in the family *Orthomyxoviridae* have negative sense RNA genomes. Note that retroviruses do not need the DNA replication machinery but do need the transcription machinery and mRNA modifying proteins.
- The cytoskeleton to travel through the cell.
- The cytoplasm to replicate (DNA viruses in the family *Poxviridae* and RNA viruses except *Retroviridae* and *Orthomyxoviridae*).
- All viruses use the endoplasmic reticulum and 80s ribosomes to translate viral proteins.
- The Golgi apparatus and endoplasmic reticulum to add glycosylations to viral proteins.
- Plasma membrane, nuclear membrane, or organelle membranes to acquire envelopes in all enveloped viruses or to produce exocytic vesicles that will release virus from the cell. Note that some enveloped viruses will acquire an envelope from the nuclear membrane or an organelle and then leave the cell via exocytic vesicles. Naked viruses also leave the cell by either cell lysis or exocytic vesicles.

PROBLEMS FOR ALL VIRUSES TO OVERCOME

All viruses have issues they must overcome in a host cell. The following is a list of these issues that we will explore in more depth in this chapter and for each of the viral families. As you study the viral families that infect animals, be sure to attempt to answer how each family overcomes these issues.

- How to get into the cell
- How to uncoat
- How to travel to the site of replication
- How to mimic cellular mRNAs
- Eukaryotic translation only monocistronic mRNA
- Viral and cellular mRNA are in competition for translation machinery
- Initiating genome replication
- Replicating the ends of the genome
- Packaging the genome
- How to exit the cell
- The host's immune response inside the cell and outside the cell (Chapter 4)

VIRUS ATTACHMENT TO HOST CELLS

All viruses must find the correct cells to infect. This is in large part determined by the presence of the correct receptor expression on host cells. Receptors found on the cell are recognized by viral attachment proteins found either on the capsid of naked viruses or envelope of enveloped viruses as shown in Figure 3-1. Viruses can use proteins or glycosylations of proteins or lipids. All viruses have specific receptors they will use that determine which cells they can bind, enter, and hence, infect.

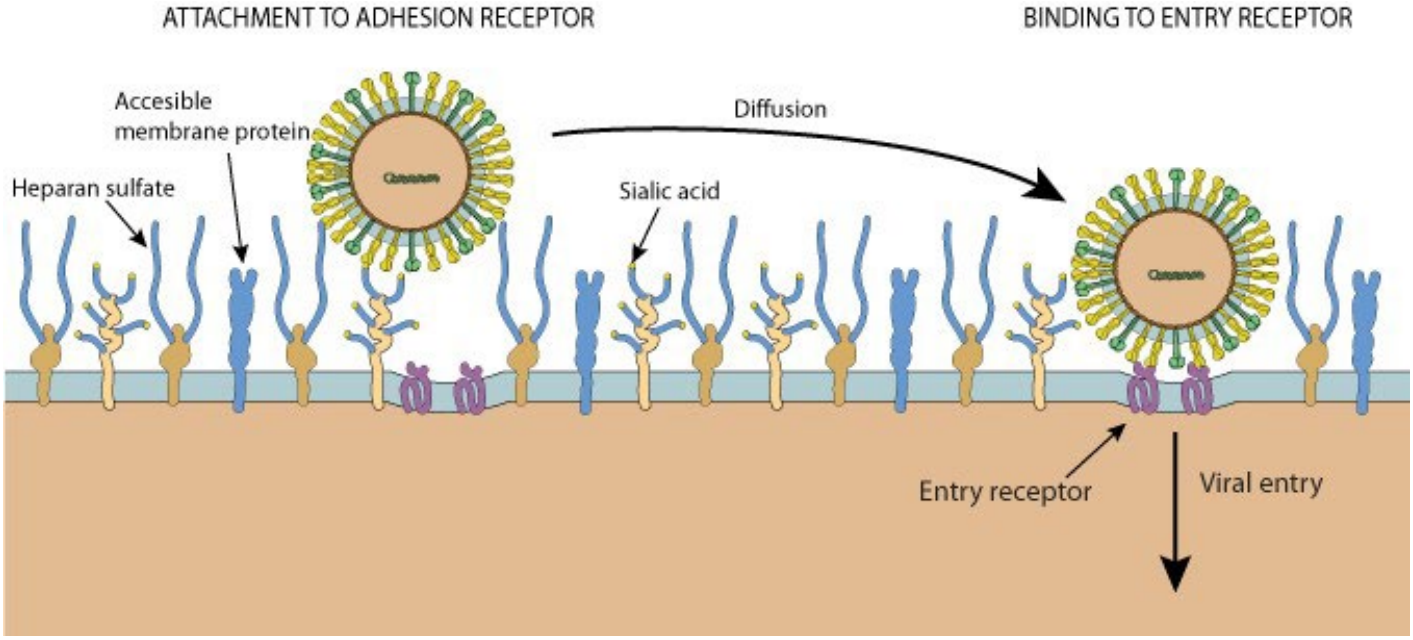


Figure 3-1. Viral attachment proteins on the outside of the virus diffusing close to cells and binding the appropriate receptor (either protein or membrane glycolipid) expressed on the cells. Remember these receptors are different for every virus. Courtesy of ViralZone https://viralzone.expasy.org/956

Viruses that use proteins will use folds in the secondary structure that are accessible to the virus and hence are found on the surface of the protein. Remember, these are proteins that host cells are creating for their own purposes, and the virus is taking advantage of the presence of these proteins to bind to the cell via **adsorption**.

Viruses can also use carbohydrates found in **glycosylations** on the surface of proteins of the cell membrane that are external to the cell. The specificity of these glycosylations can be very important as is demonstrated by the orthomyxovirus influenza A which binds to sialic acid gylcosylations of proteins. In both humans and birds, the final sugar added to this glycosylation is galactose. In humans, however, the bond between the sialic acid molecule and galactose is an α2,6-linkage, and in birds it is an α2,3-linkage. These sialic acids are recognized by the **hemagglutinin** (HA) receptor binding protein of the Influenza A virus envelope. The Influenza A viruses of humans generally prefer the α2,6-linkage found on human cells and cannot bind to the α2,3-linkage of bird's cells. Note there are 18 different HA genes which are expressed by different subtypes of Influenza A viruses. Viruses expressing HA 1, 2, or 3 prefer human sialic acids, while other subtypes prefer other animal's or bird's sialic acids. However, the HA subtype can acquire mutations that allow the virus to bind to other animal or bird sialic acids. This explains why bird Influenza A can sometimes cross from birds to humans and why sometimes it cannot. Note there are also 11 subtypes of **neuraminidase** (N) protein. You may have heard of influenza subtypes being named by their combination of HA and N proteins such as H5N1, the bird flu. H5 does not normally bind to human sialic acids, however, when mutations occur, it can allow influenza infections to cross from birds to humans. See Figure 3-2.

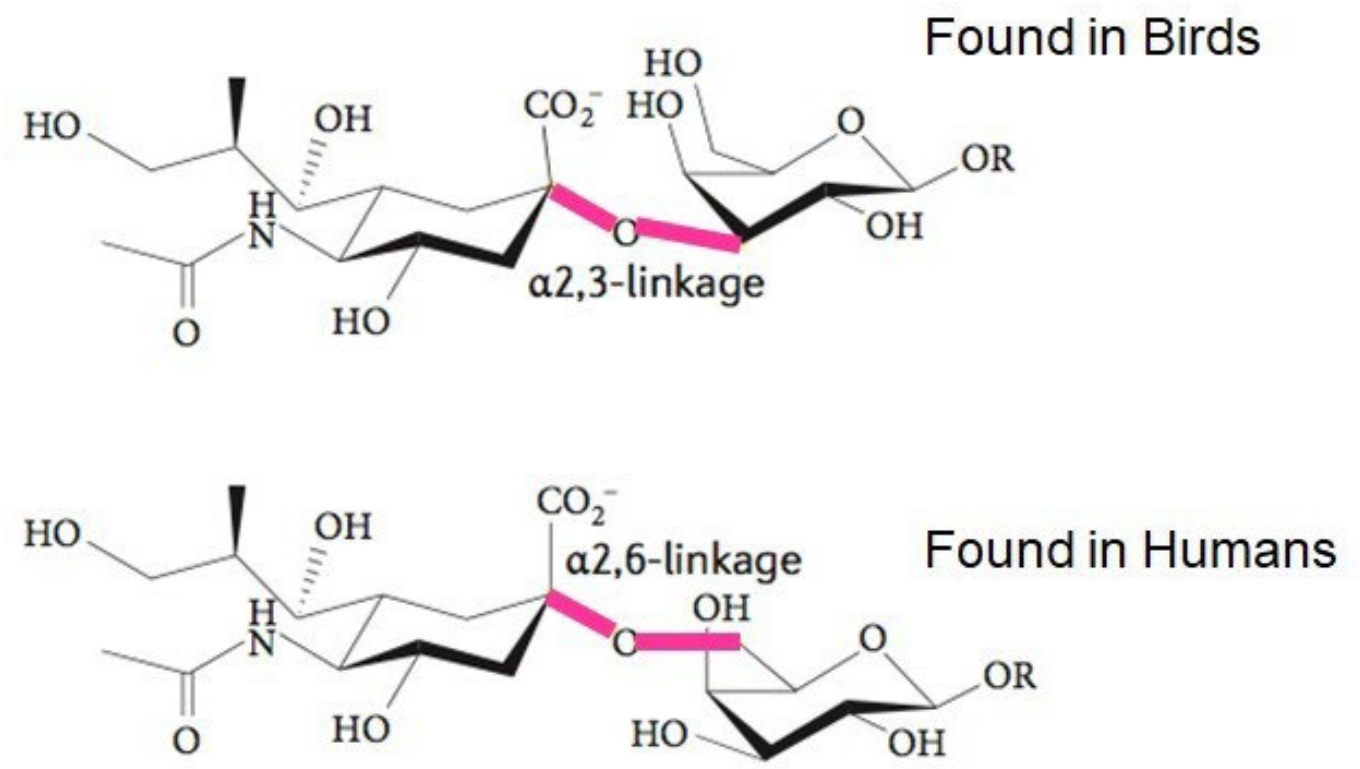


Figure 3-2. Sialic acid alpha 2,3-linkage of birds and alpha 2,6-linkage of humans. Courtesy of ChemDraw

Some viruses bind to only a single receptor while others require both a receptor and a coreceptor to initiate infection. Viruses bind to receptors using a lock and key mechanism similar to antibody epitope binding. The viral receptor binding protein has a shape that fits the shape of the receptor to which it binds. The retrovirus HIV is a classic example of a virus that requires a coreceptor in order to infect. The receptor is CD4, a glycoprotein found on the surface of T-helper cells, macrophages, and dendritic cells. The coreceptor is either the cytokine receptor CCR5 or CXCR4 depending on the stage of infection, as we will see in Chapter 12 describing the family *Retroviridae*. The binding of virus to receptor induces conformational changes to the receptor protein which help the virus enter the cell. You will see this demonstrated in the next section describing viral entry into host cells.

VIRAL ENTRY INTO HOST CELLS

Once a virus has bound to its receptor it must enter into the cell. There are 3 main ways that viruses enter cells, membrane fusion, endocytosis, and pore formation.

Membrane Fusion to Enter Host Cells

Membrane fusion is used by many enveloped viruses. In viruses that only bind one receptor, their receptor binding protein can also function as a **fusion** protein. When the virus binds to the host cell receptor, the fusion protein unfolds and penetrates the host cell membrane facilitating fusion of the two membranes as shown in Figures 3-3, 3-4, and 3-5. In some viruses that bind receptors and coreceptors, the fusion protein is a separate envelope protein. An excellent example is HIV where the envelope protein gp120 serves as the receptor binding protein. HIV binds CD4 with gp120 which induces a conformational change allowing gp120 to bind to the coreceptor, either CCR5 or CXCR4. This binding induces a further conformational change in gp120 which in turn induces a conformational change in the envelope protein gp41, a fusion protein that changes shape and causes the cell membrane and envelope membrane to fuse. Many enveloped viruses contain a fusion protein as an envelope protein that allows the virus to enter the cell via membrane fusion. Naked viruses cannot enter cells via this mechanism.

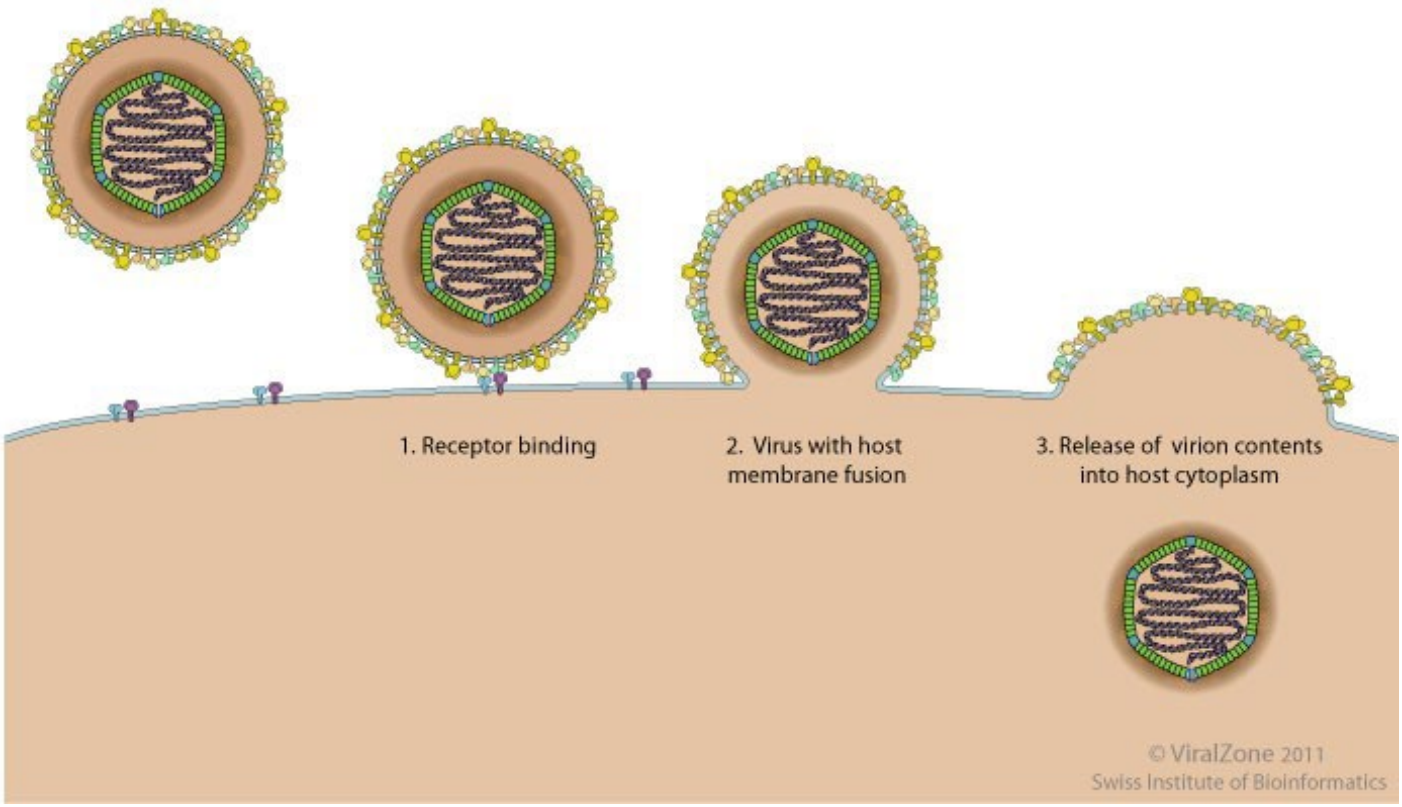


Figure 3-3. Virus entering a cell via membrane fusion. Courtesy of ViralZone https://viralzone.expasy.org/987

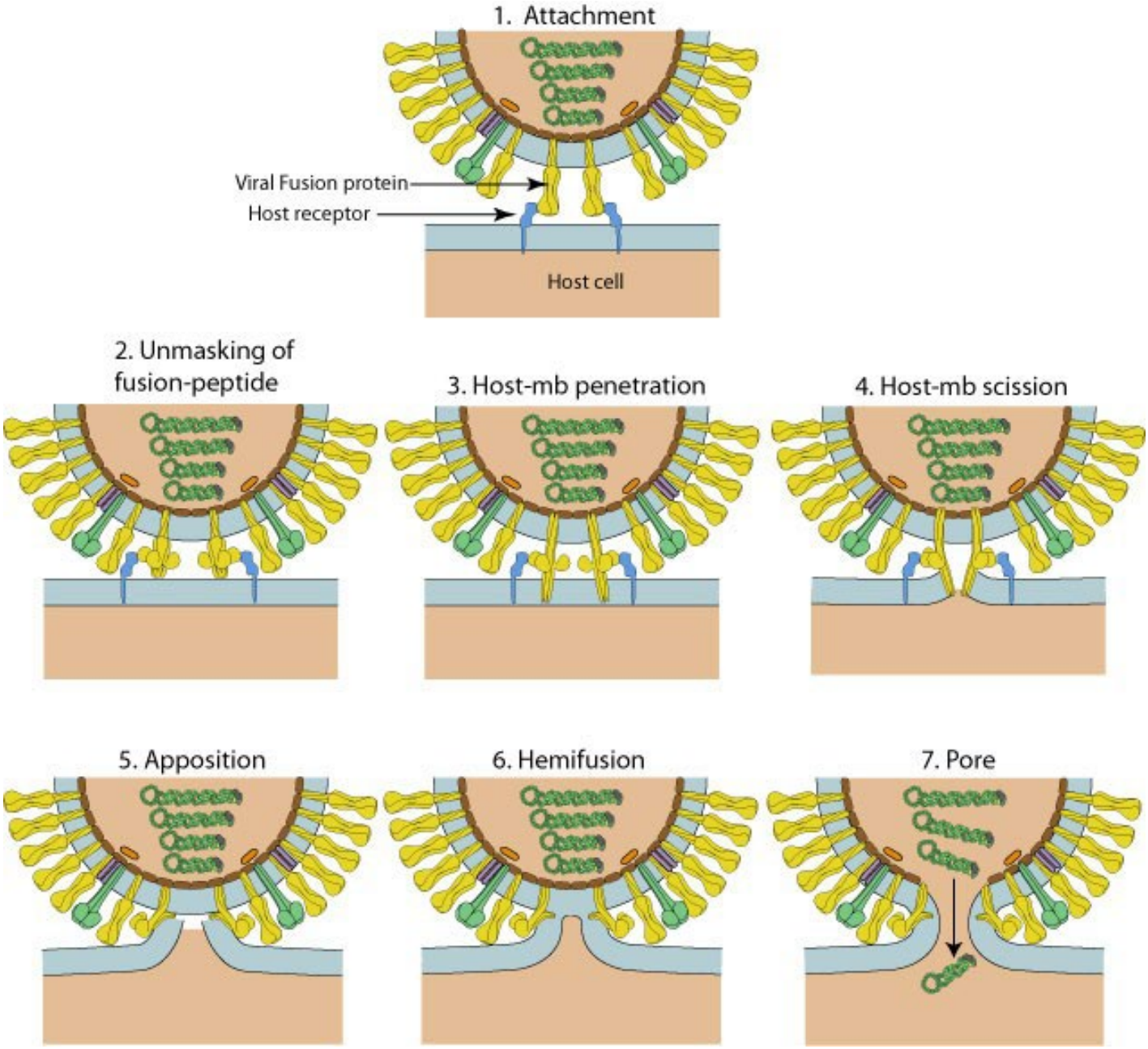


Figure 3-4. Virus inducing fusion of viral envelope and cellular membrane after binding to receptor induces conformational changes in envelope protein. Courtesy of ViralZone https://viralzone.expasy.org/1361

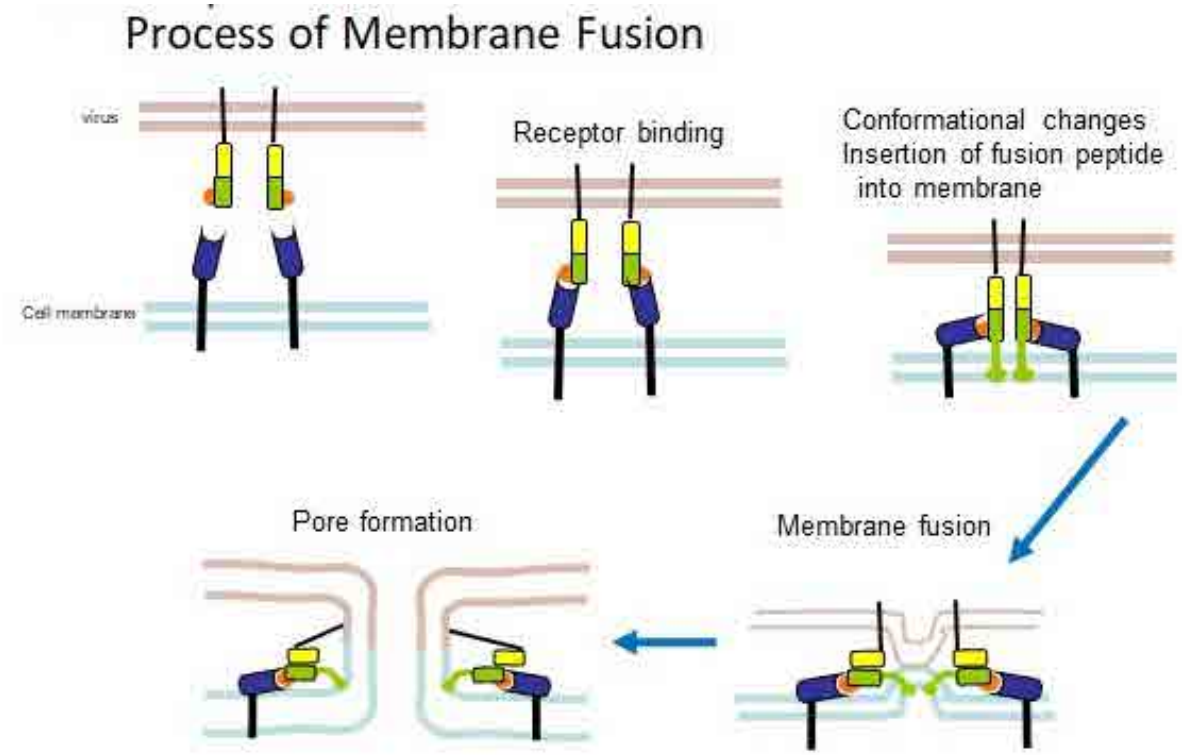


Figure 3-5. Process of membrane fusion demonstrating conformational changes and insertion of fusion peptide into host cell membrane leading to fusion. Image courtesy of Sandra Quackenbush, Colorado State University.

Direct cell to cell transmission via membrane fusion

Once inside cells, viruses can move to other cells without leaving and move directly into adjacent cells, which allows the virus to avoid the extracellular immune response. This is most commonly achieved by fusing the membranes of infected cells with adjacent cells allowing the virus to form syncytia, Figure 3-6. This is achieved using the viral fusion protein; as such, only enveloped viruses can form syncytia. For images of syncytia, please see Chapter 2 viral CPE.

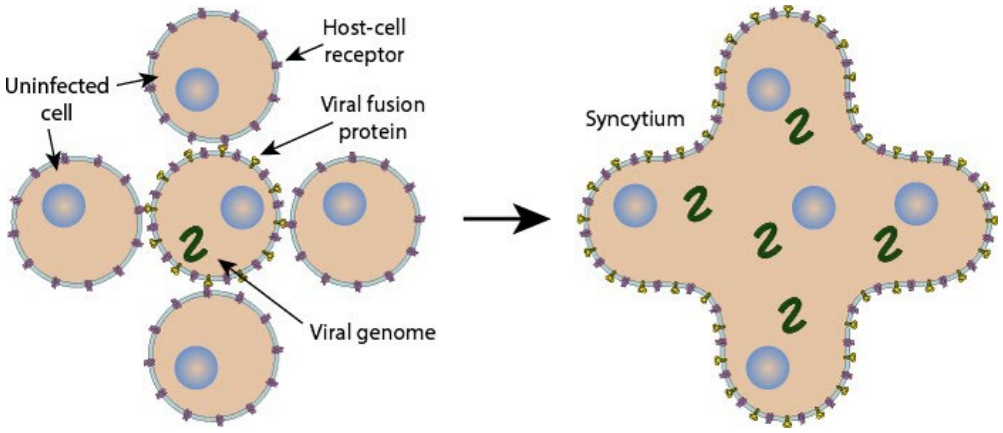


Figure 3-6. Enveloped virus production of a syncytium utilizing the envelope fusion protein. Courtesy of ViralZone https://viralzone.expasy.org/5957

Endocytosis to Enter Cells

Most naked viruses and some enveloped viruses enter the cell via **endocytosis**. There are many types of **endocytic vesicles,** and there are viruses that have adapted to take advantage of almost all of them. The most common is the clathrin-coated vesicle. The virus binds to the cellular receptor and induces the cell to form a clathrin-coated vesicle that invaginates and brings the virus inside the cytoplasm within these endocytic vesicles. Clathrin-mediated endocytosis facilitates the internalization and recycling of receptors engaged in a variety of processes and has been hijacked by viruses that bind to receptors and fool the cell into thinking that there are molecules that need to be recycled. Other types of endocytosis are hijacked in similar ways. Using Clathrin-coated vesicles as an example, once the vesicle enters the cell, the clathrin coat is removed and fusion with **endosomes** (organelles involved in cell transport) occurs. This is how the receptors are recycled to the cell surface. Remember, the vesicle thinks it is carrying receptors that need recycling. If this were true, the receptors would bud off the endosome forming transport vesicles that would carry the receptors to the plasma membrane. The virus remains in the endosome, and as the endosome matures, the pH begins to drop from 7 to 6. This acidification is essential to the virus as it induces structural changes in the proteins of the capsid or envelope allowing the virus to uncoat and/or leave the endosome. Viruses can then leave the endosome directly via fusion with the endosomal membrane. Lysis or permeabilization of the endosome's membrane will then result in the release of the viral genome or capsid into the cytoplasm. See Figure 3-7.

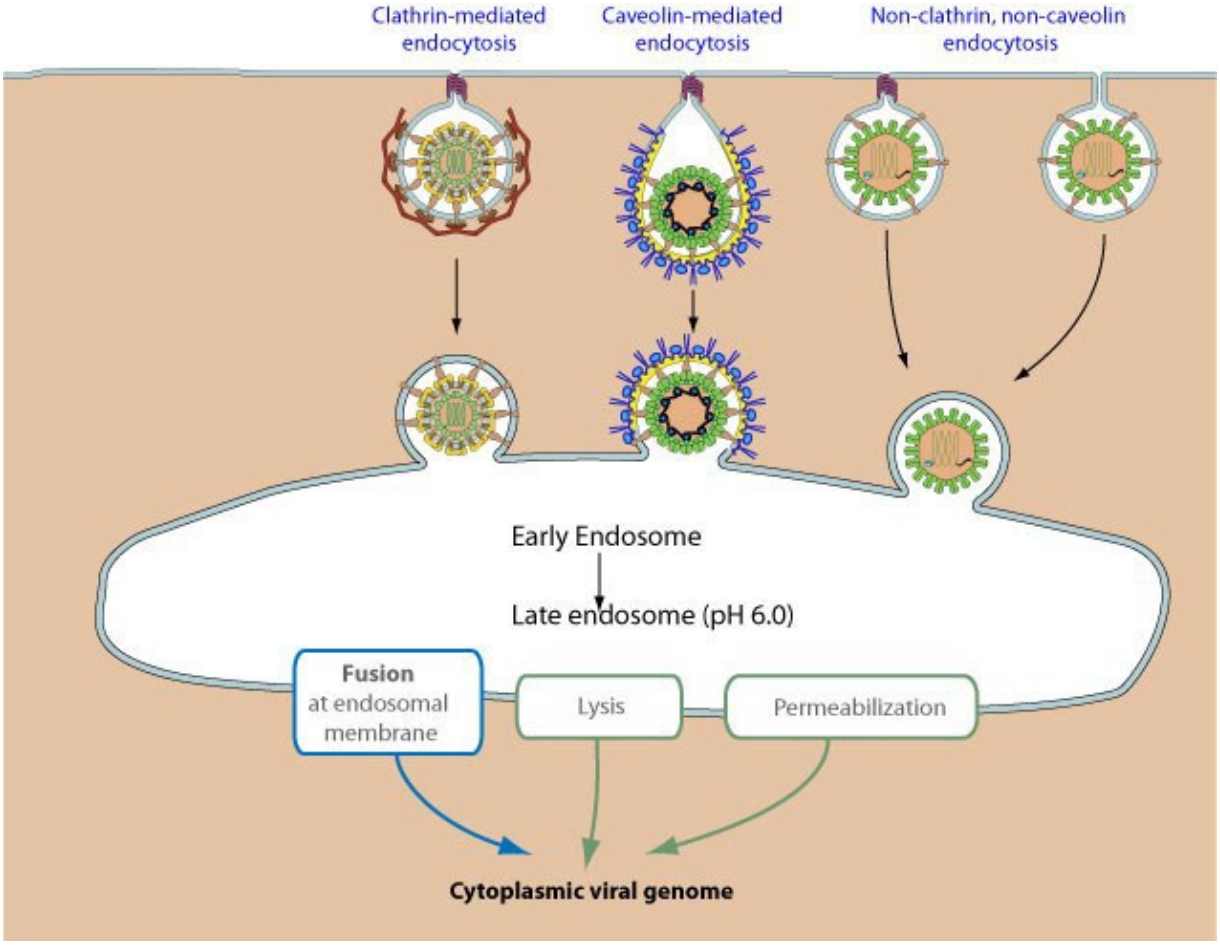


Figure 3-7. The process of endocytosis. Courtesy of ViralZone https://viralzone.expasy.org/977

Formation of a pore in the host cell's plasma membrane to enter cells

Some naked viruses circumvent the need to induce a host cell to endocytose the virus by using their capsid proteins to form a pore in the host cell's plasma membrane. This allows the viruses to inject viral nucleic acids into the cytoplasm. See Figure 3-8. Note that the binding of the viral capsid protein to the host receptor protein induces a conformational change in the capsid protein allowing it to puncture the host cell membrane. This causes the formation of a pore for nucleic acids to travel into the cell.

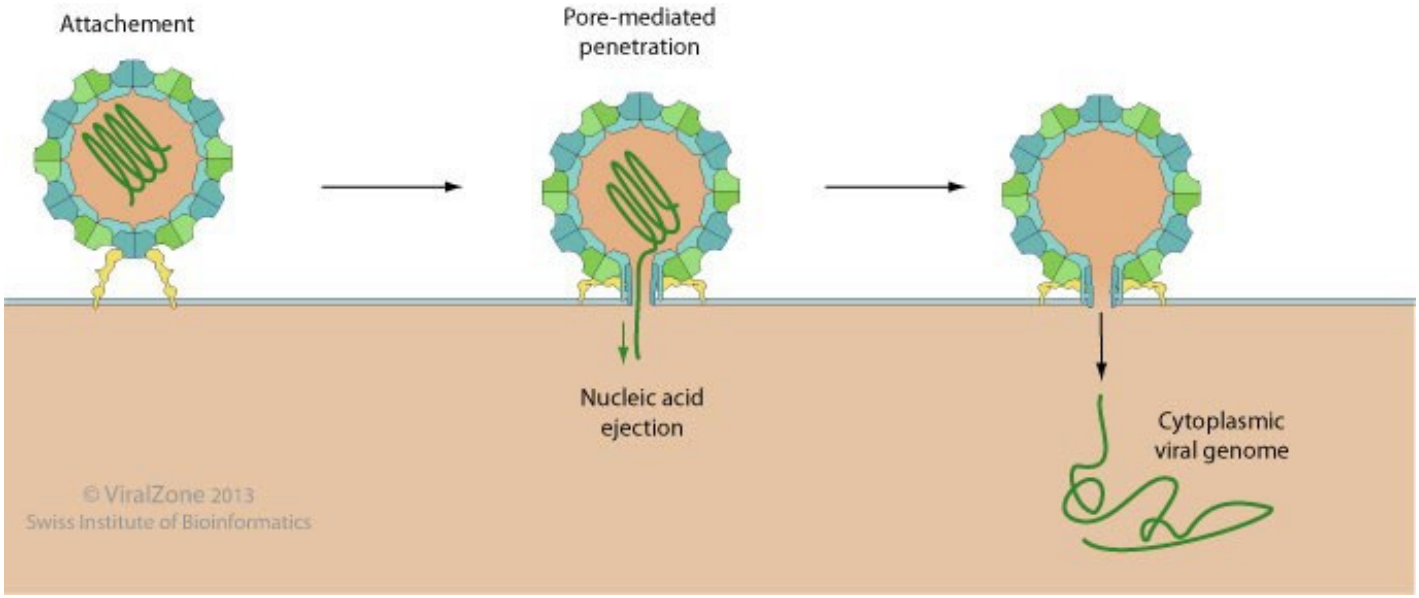


Figure 3-8. Polio viruses creating a pore in the host cell's membrane upon binding to cell receptor protein which induces a conformational change. Courtesy of ViralZone https://viralzone.expasy.org/979

INTRACELLULAR TRANSPORT WITHIN THE CELL

Once viruses enter the host cell, they need to move to the site of viral replication. Viruses require an energy-dependent mechanism to travel through the cytoplasm. Viruses entering the cytoplasm utilize molecular motors to move through the cytosol. The majority of these viruses use microtubules, but some have been described as using actin cytoskeletons. Those moving along microtubules utilize dynein motor proteins to move along the microtubules. See Figure 3-9. RNA viruses, except orthomyxoviruses and retroviruses, will stop somewhere in the cytoplasm. DNA viruses, except poxviruses, will move to the nucleus. Figure 3-10 shows all of the steps we have described thus far.

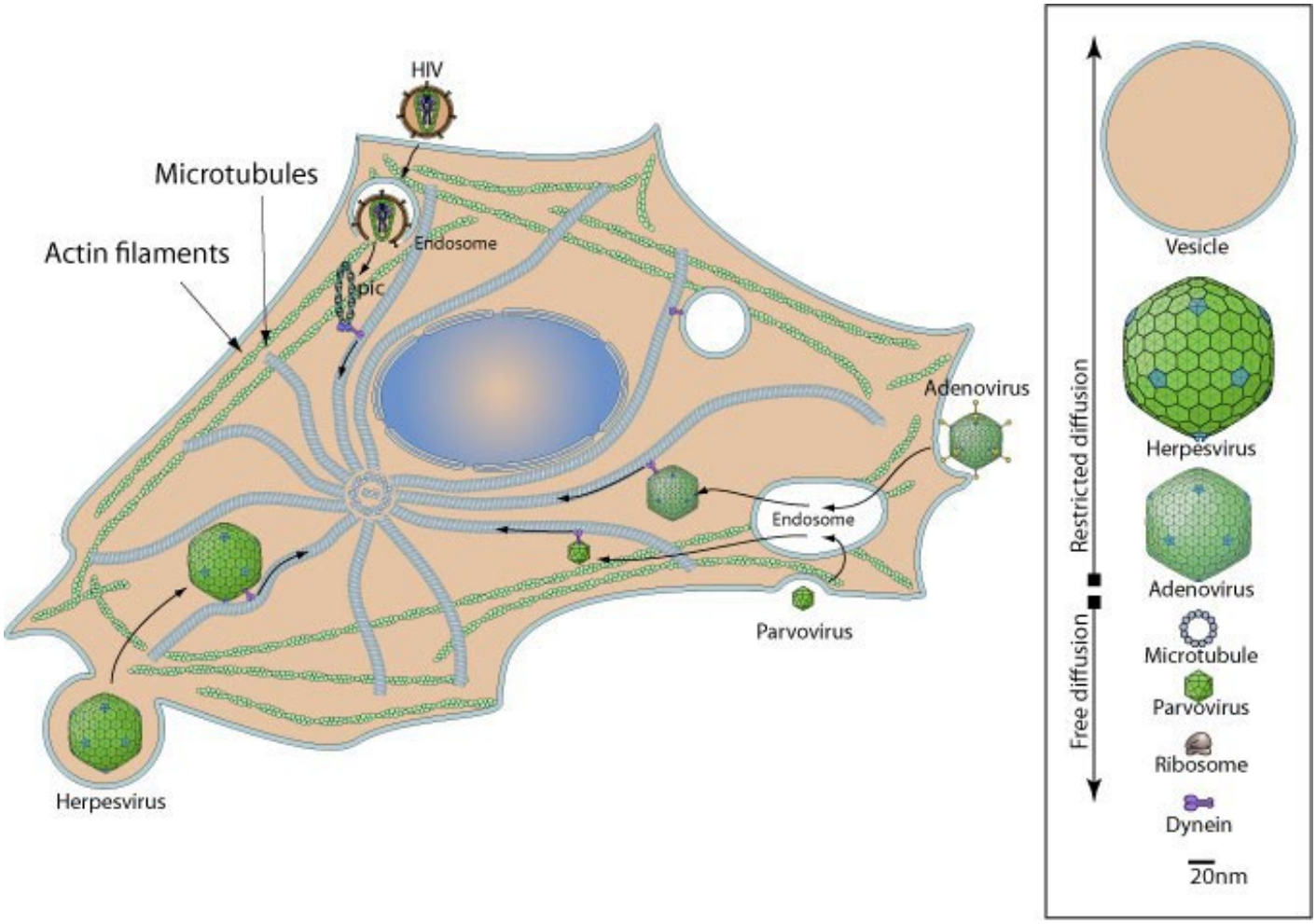


Figure 3-9. Viral intracellular transport using microtubules and actin transport showing that larger viruses have restricted diffusion and must utilize microtubules or actin to move though the cell. Courtesy of ViralZone https://viralzone.expasy.org/990

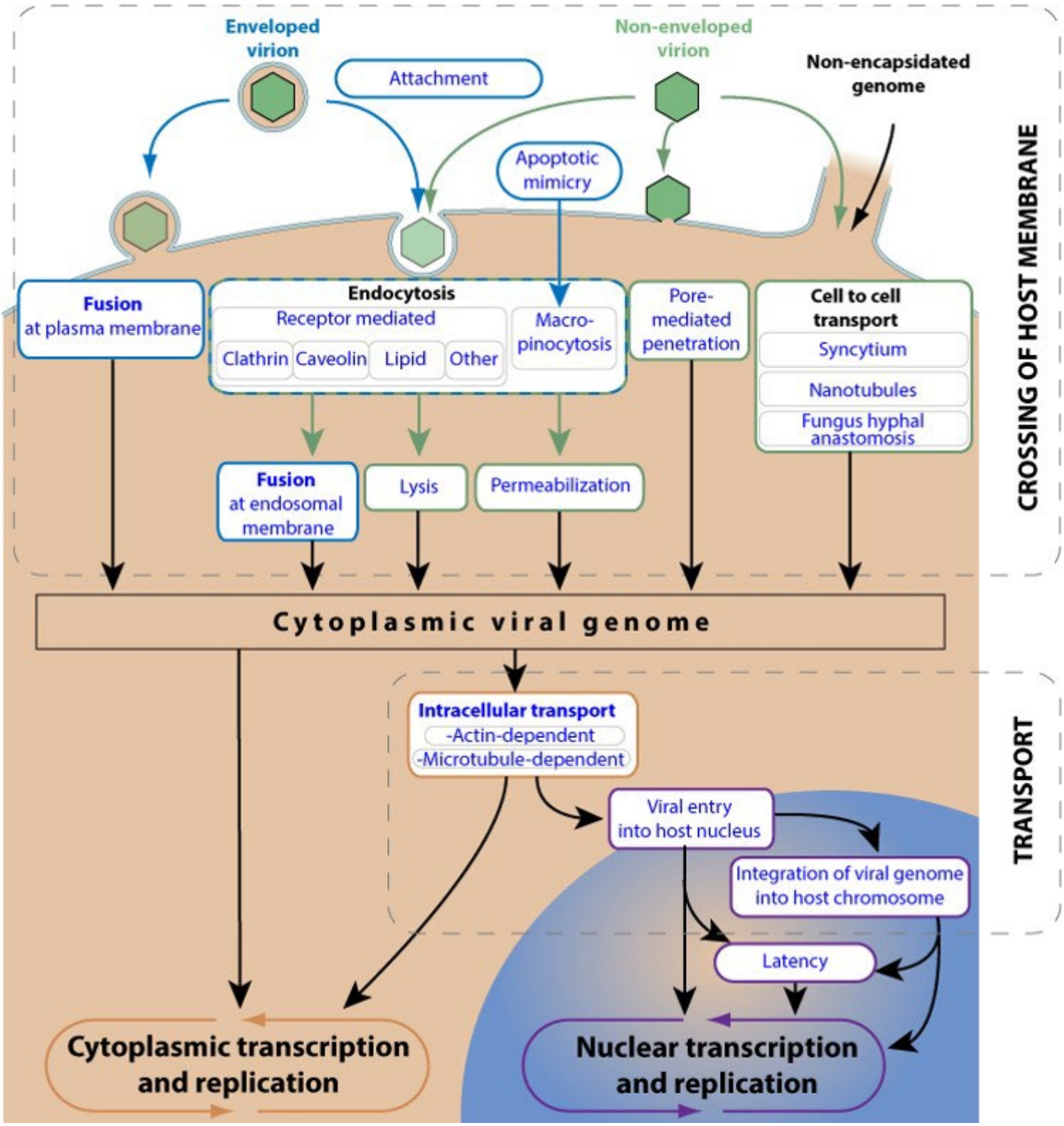


Figure 3-10. A summary image that shows all of the steps from binding to the cell receptor to movement to the site of replication. Courtesy of ViralZone https://viralzone.expasy.org/936

ENTERING THE NUCLEUS

All DNA viruses (except poxviruses) and retrovirus and orthomyxovirus RNA viruses must enter the nucleus. The nuclear membrane is a double membrane that is separated by a nuclear space and is fused to form nuclear pores that contain approximately 60 transmembrane proteins. It is very difficult to cross the nuclear membrane as the nucleus tightly regulates what comes and goes from the nucleus with a complex import mechanism. This may explain why many viruses evolved not to bother with the nucleus and replicate in the cytoplasm instead.

Figure 3-11 shows the different mechanisms that animal viruses have evolved to traverse the nuclear membrane. These mechanisms often have a lot to do with the size of the viral capsid. Let's begin with the RNA virus orthomyxovirus which is one of the two RNA viruses discussed in this book that will replicate in the nucleus. It simply uncoats in the cytoplasm and transports the negative stranded RNA genome through the nuclear pore. Note it must bring **RNA-dependent RNA polymerase** (RdRp) with it into the nucleus to replicate. Some DNA viruses such as polyomaviruses and papillomaviruses do this as well, transporting their uncoated DNA directly into the nucleus, as do the lentiviruses (retroviruses) such as HIV, FIV, and SIV. Note that these viruses do not need to carry RdRp with them into the nucleus, although retroviruses do need to carry integrase with them. You may notice that other retroviruses cannot pass their DNA through the nuclear pore and instead must wait for cells to divide and the nuclear membrane to break down. This is because the more complicated lentiviruses produce proteins that help shepherd the retroviral DNA across the nuclear membrane. The simpler retroviruses lack these proteins. The parvoviruses are small enough to enter the nucleus by simply passing through the pore. You will notice in Figure 3-11 that the proteins coating the pore create a wide space on the cytoplasmic side of the pore and a narrow space inside the nucleus. As such, moderately sized viruses like hepadnaviruses get blocked at the narrow end within their nuclear pore and uncoat at this point pushing their DNA through the membrane into the nucleus. Larger viruses like herpesviruses and adenoviruses that have lost their spikes get stuck at the wide edge of the nuclear pore and must push their DNA out at this point into the nucleus.

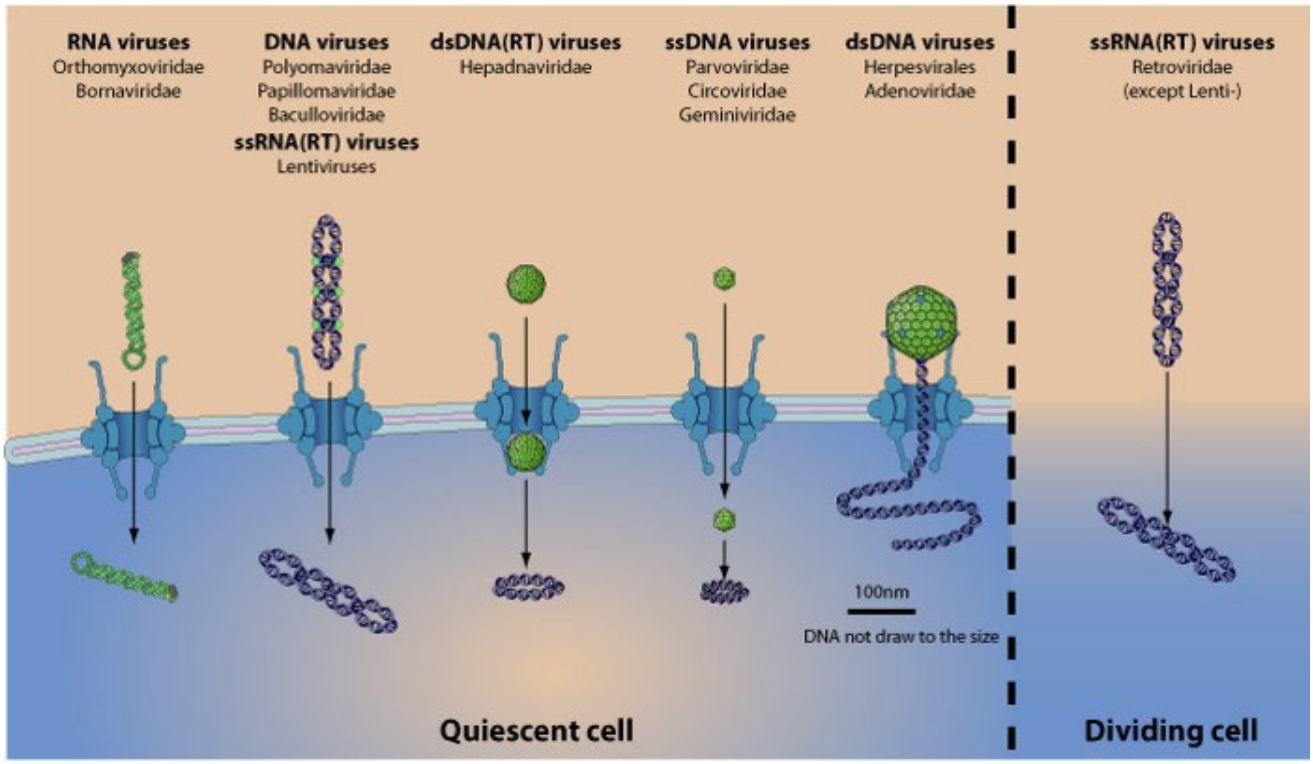


Figure 3-11. Mechanism for animal DNA viruses to enter the nucleus as well as the orthomyxoviruses and retroviruses. Courtesy of ViralZone http://viralzone.expasy.org/all_by_protein/.htm/989l

ENTERING THE NUCLEUS

Most DNA viruses will replicate in the nucleus and form virions within the nucleus. How then do viruses leave the nucleus with complete capsids too large to leave the nucleus? Viruses that use reverse transcriptase such as retroviruses and hepadnavirues will transport their mRNA through the pore and package in the cytoplasm. Small viruses like parvoviruses will simply pass through the pore. But larger viruses like polyomaviruses, papillomaviruses, adenoviruses, and herpesviruses must come up with alternative methods to get out. Many just lyse the nuclear membrane when there is sufficient virus replication, others like herpesviruses bud out of the nuclear membrane in a process known as **nuclear egress.** For many of the viruses, it is not clear which mechanisms they use, and many appear to be able to use different mechanisms under different circumstances. Figure 3-12 shows the different mechanisms viruses can use to leave the nucleus. Figure 3-13 shows nuclear egress.

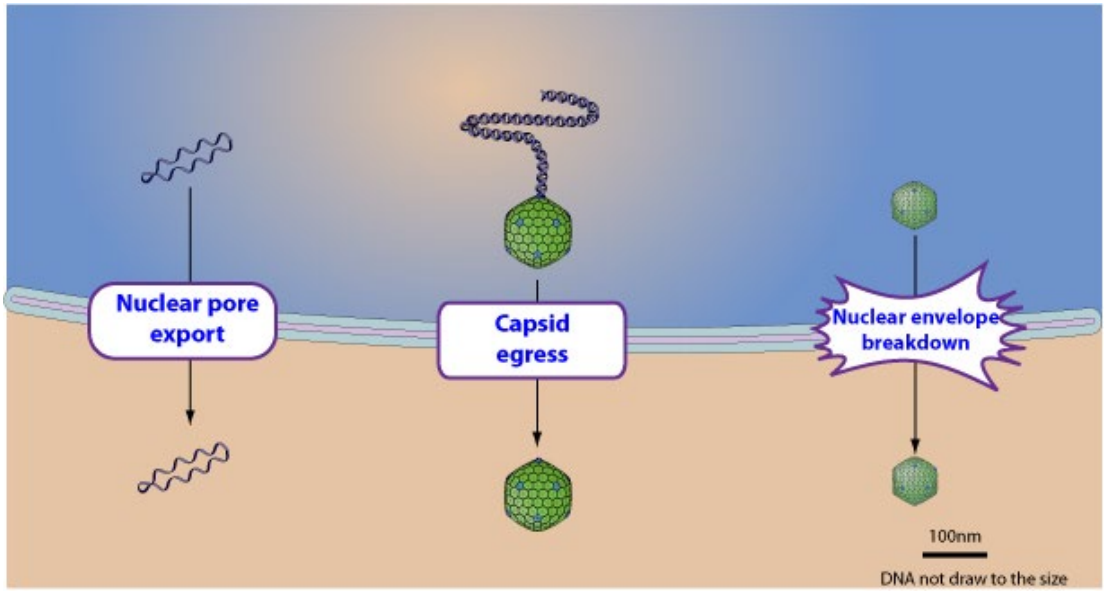


Figure 3-12. Mechanism for animal DNA viruses to exit the nucleus as well as the orthomyxoviruses and retroviruses. Courtesy of ViralZone https://viralzone.expasy.org/2177

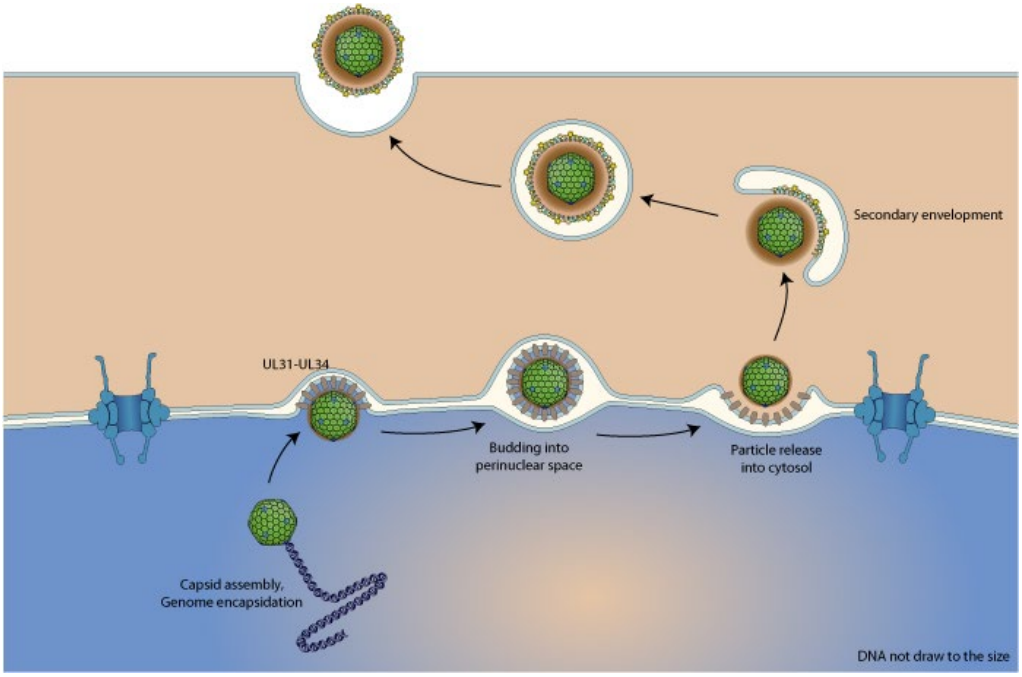


Figure 3-13. Mechanism for animal DNA viruses to exit the nucleus as well as the orthomyxoviruses and retroviruses. Courtesy of ViralZone https://viralzone.expasy.org/1952

BASIC VIRAL REPLICATION CYCLES

DNA VIRUSES

Figure 3-14 shows the basic DNA viral replication cycle. As is true of all viruses, the replication cycle begins with binding a cell receptor protein or glycosylation. The virus then penetrates the cell by one of the 3 mechanisms described above depending on whether the virus is enveloped or naked as there are DNA viruses of both types. The virus must then move to its site of replication most commonly using microtubules and dynein motor proteins. For all DNA viruses except poxviruses this will be the nucleus, and, as discussed above, depending on their size they will use different mechanisms to get through the nuclear membrane. Once inside most will use the host cell's RNA polymerase II, a DNA-dependent RNA polymerase (DdRp), to transcribe the viral mRNA which is then translated by the host cell's ribosomes most often associated with the endoplasmic reticulum. It is necessary to translate viral proteins before DNA replication begins as viral proteins are often involved in regulating DNA replication. Cellular DNA polymerase, a **DNA-dependent DNA polymerase** (DdDp), then replicates the DNA genome of most DNA viruses. Exceptions are poxviruses which replicate in the cytoplasm and as such must have a gene to create their own DNA polymerase; adenoviruses, which prime their DNA with a protein that cannot be recognized by cellular DNA polymerase and as such must create its own; and herpesviruses, which are very large and contain a gene for a viral DNA polymerase in its genome as it replicates in non-replicating cells such as neurons. Note: recall that *Hepadnaviridae* viruses do not package DNA but instead package positive sense RNA that is reverse transcribed once the genome is inside the virion by the enzyme **reverse transcriptase** (RT). But other than this, their replication cycle looks surprisingly like other DNA viruses. See the retroviral replication cycle for a description of the functions of RT. Once the virus has accumulated an adequate amount of viral genome and proteins to form capsids, it will assemble spontaneously. If the virus has an envelope, the viral envelope proteins will accumulate in the plasma membrane (or other internal membranes) and the capsids will push against these proteins causing the virus to bud off the membrane. Note that enveloped viruses that bud off internal structures must leave the cell via **exocytic vesicles**. Viruses that bud from the plasma membrane will exit the cell this way. If the viruses are naked they will usually leave by killing the cell (**lysis**) or by leaving in exocytic vesicles. Note: Because entrance and exit from a cell by a virus is done in the same manner regardless of genome type, the description of this process is repeated for each replication cycle described in this chapter.

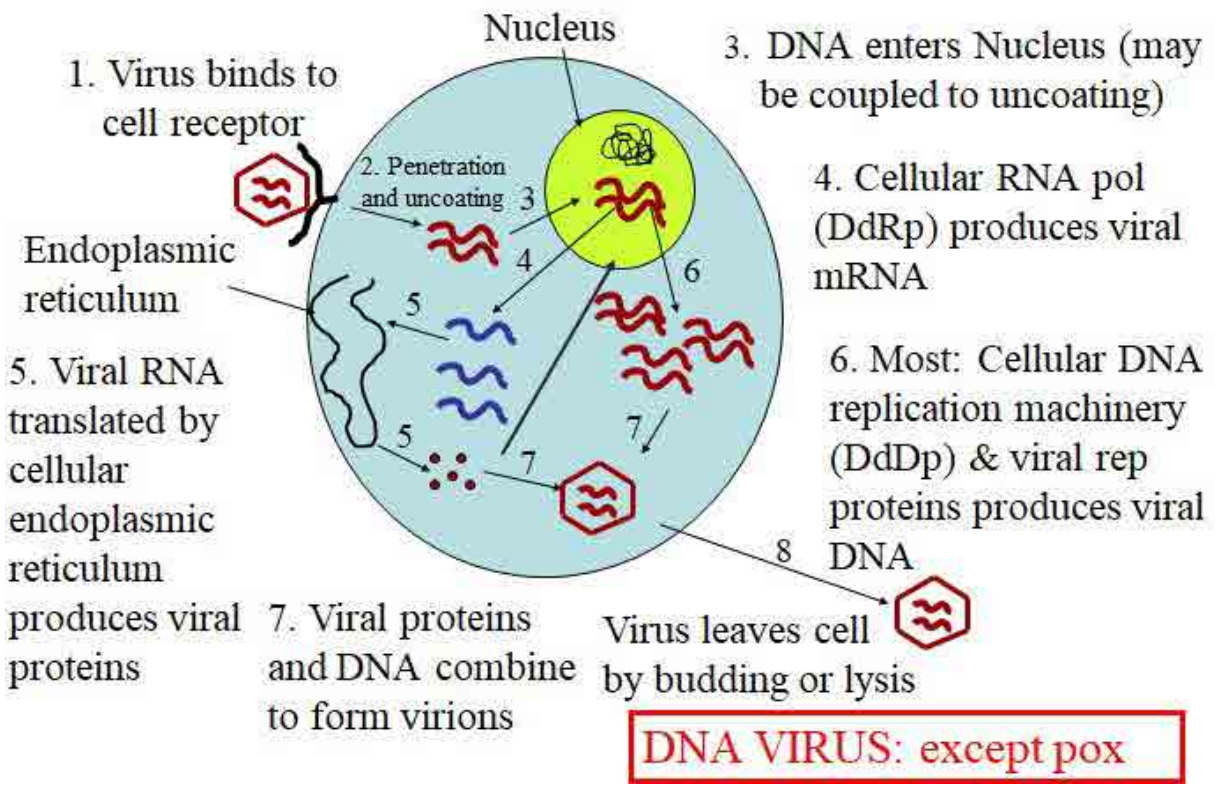


Figure 3-14. Basic DNA viral replication cycle. Courtesy of Erica Suchman, Colorado State University.

As we have discussed, DNA viruses take advantage of the host cell's RNA transcription machinery. This is usually **RNA polymerase II,** but some viruses also utilize RNA polymerase III. Currently there are no known viruses that utilize RNA polymerase I. It makes sense that most use RNA polymerase II as this is the polymerase that eukaryotic cells use to produce their mRNA. Why some viruses choose to use RNA polymerase III which is used by cells to transcribe tRNAs, one of the rRNAs, and SNIPs is most likely explained by the fact that these viruses use RNA polymerase III only to make a few of the viral proteins that are needed in smaller concentrations than the majority of their viral proteins thus allowing the viruses to regulate the production of these few proteins more accurately. The polymerases used by eukaryotic cells and viruses are shown here in Table 1.

RNAs synthesized		
Enzyme	Cellular	Viral
RNA polymerase I	rRNAs (28S, 18S, 5.8S)	None known
RNA polymerase II	mRNAs miRNAs small nuclear RNAs	mRNA (nuclear replicating DNA viruses, Retroviruses)
RNA polymerase III	tRNAs 5S rRNA U6 snRNA	Adenovirus VA-RNAs EBV EBER RNAs

Table 3-1. RNA synthesis enzymes utilized by cells and viruses. Courtesy of Erica Suchman, Colorado State University.

POSITIVE SENSE RNA VIRUSES

Figure 3-15 shows the basic positive sense RNA viral replication cycle. As with all viruses, replication begins with binding a cell receptor protein or glycosylation. The virus then penetrates the cell by one of the 3 mechanisms described above depending on if the virus is enveloped or naked as there are positive sense RNA viruses of both types. The virus must then move to its replication site, most commonly using microtubules and dynein motor proteins. For all positive sense RNA viruses except retroviruses this will be the cytoplasm. All positive sense RNA viruses, except retroviruses, will immediately be translated by the host cell's ribosomes most often associated with the endoplasmic reticulum as their genome is equivalent to mRNA. As all viruses must overcome the one protein per mRNA issue, all the positive sense RNA viruses have evolved to create long **polyproteins** that are cut into individual proteins by the viral enzyme **protease**. Note that protease first **autocleaves** itself from the polyproteins and then cleaves out all of the remaining proteins. Also note that some positive sense RNA viruses will create only one polyprotein while others will create multiple polyproteins from multiple **open reading frames** (ORFs) found within the genome. It is necessary to translate viral proteins before RNA replication because the **RNA-dependent RNA polymerase** (RdRP) that is used to replicate the virus must come from the virus as the host cell does not possess such proteins in its cytoplasm. This activity is called replicase activity. Note that a double stranded intermediate must be formed in this process as a negative sense template must be read to create more positive sense RNA genome. As we saw in the last chapter and will see in the next chapter, this double stranded RNA serves as a signal to the cell that viral infection is occurring as long stretches of dsRNA (longer than 125 base pairs) are rarely seen in eukaryotic cells. To avoid activation of RNAi, and interferon production RNA viruses try not to keep their dsRNA together which causes the polymerases to lose their reproductive editing ability as they have no template to read as the RNA is replicated.

As a result, RNA viruses have very high error rates compared to DNA viruses, and accordingly RNA viruses mutate very rapidly. Once the virus has accumulated sufficient viral genome and proteins to form capsids, they will spontaneously assemble. If the virus has an envelope the viral envelope proteins will accumulate in the plasma membrane (or other internal membranes) and the capsids will push against these proteins causing the virus to bud off the membrane. Note that enveloped viruses that bud off internal structures must leave the cell via exocytic vesicles. Viruses that bud from the plasma membrane will exit the cell this way. If the viruses are naked they will usually leave by killing the cell (lysis) or by leaving in exocytic vesicles.

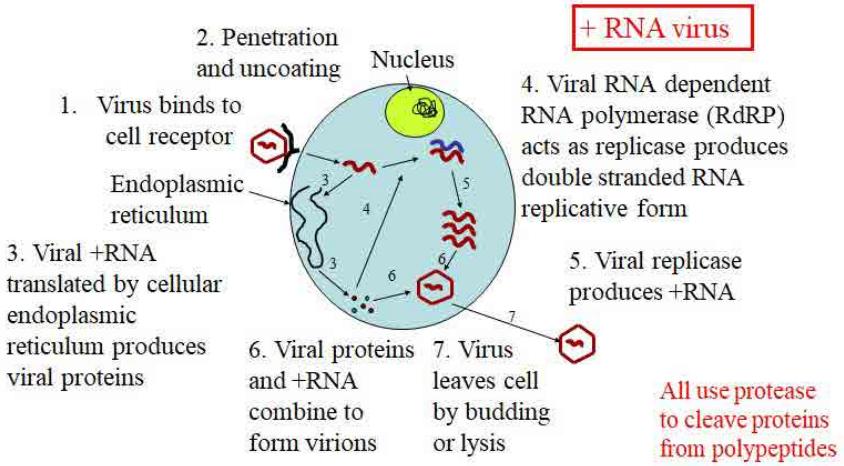


Figure 3-15. Basic positive sense RNA viral replication cycle.

Figure 3-16 demonstrates the functions of RdRp in positive sense RNA viruses.

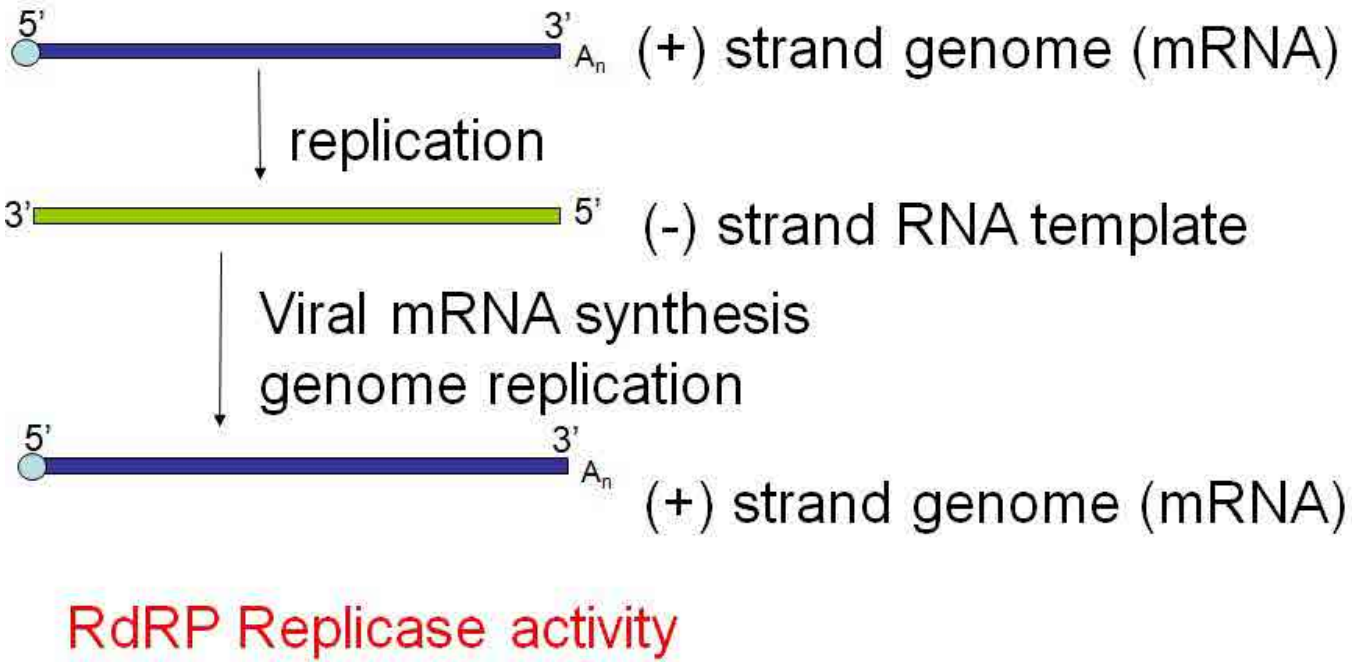


Figure 3-16. Functions of RNA-dependent RNA polymerase in positive sense RNA viruses. Courtesy of Sandra Quackenbush, Colorado State University.

RETROVIRUSES

Figure 3-17 shows the basic retroviral replication cycle. Like all viruses, replication begins with binding a cell receptor protein or glycosylation. The virus then penetrates the cell by either membrane fusion or endocytosis as all retroviruses are enveloped. The virus must then move to its site of replication most commonly using microtubules and dynein motor proteins. For all retroviruses the replication will eventually be in the nucleus, but before going through nuclear pores, the virus must use **reverse transcriptase** (RT) to create viral DNA using the RNA genome as a template. You will note that the virus needs to do this before translation of viral proteins has occurred, therefore the reverse transcriptase enzyme must be carried into the cell along with the retroviral genome.

In order to produce viral DNA from the RNA genome, RT must have all of the following 3 activities: 1) an RNA-dependent DNA polymerase (RdDp) activity to create a negative sense DNA complementary to the positive sense RNA genome, 2) an **RNAse H** activity to cleave the RNA out of the RNA/DNA hybrid leaving the DNA strand to be replicated by 3) a DNA-dependent DNA polymerase (DdDp) activity to create the second strand of DNA. See Figure 3-18.

Once the dsDNA is created, it can move into the nucleus by 1) waiting for the cell to divide and then crossing the nuclear membrane (most retroviruses), or 2) transporting its DNA across the membrane (lentiviruses such as HIV, FIV, SIV only). Once inside the nucleus, all retroviruses integrate into the host cell's genome. This is certainly not something the cell has proteins to help the virus do, and, remember, unlike other positive sense RNA viruses, retroviruses do not immediately use their genome to translate viral proteins. So, by now you might be thinking, "then they must carry the enzyme **integrase** into the cell with the viral genome". And you would be right! Integrase inserts the viral DNA into the host chromosome with no sequence specificity. This integrated virus is

called a **provirus**. The provirus now pretends to be part of the host cell's chromosome, and as the cell replicates, the virus catches a ride. However, note that the virus will never need to make more viral DNA. What it does need is a lot more viral mRNA to be translated and to serve as viral genome. As the provirus is now in the nucleus where cells perform transcription, it takes advantage of the host cell's RNA polymerase II (DdRp) to produce viral mRNA. Once the virus has accumulated a significant amount of viral genome and proteins to form capsids, these will spontaneously assemble. All retroviruses have envelopes, therefore the viral envelope proteins will accumulate in the plasma membrane. The capsids will then push against these proteins causing the virus to bud off the infected cell.

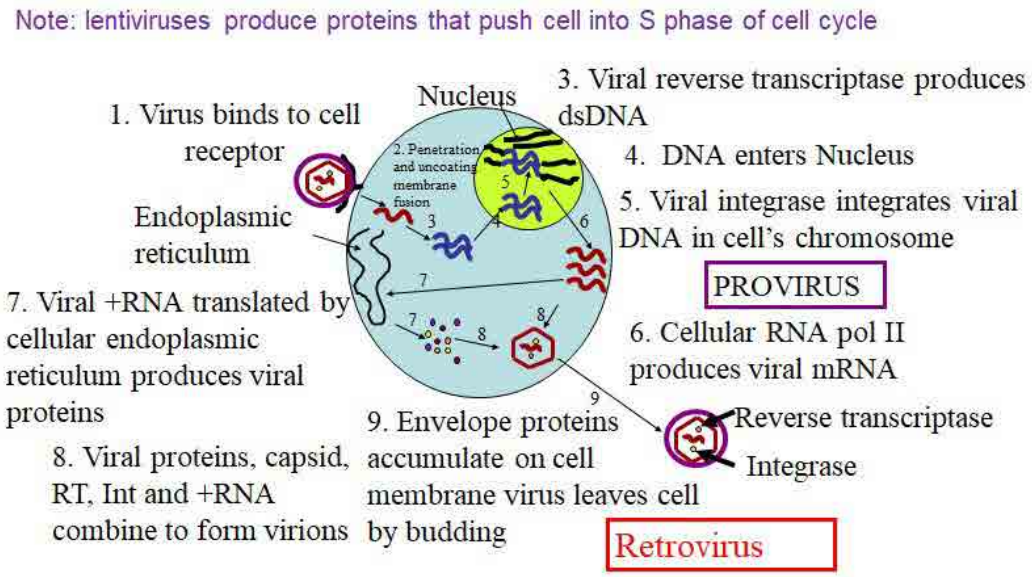


Figure 3-17. Basic retroviral replication cycle. Courtesy of Erica Suchman, Colorado State University.

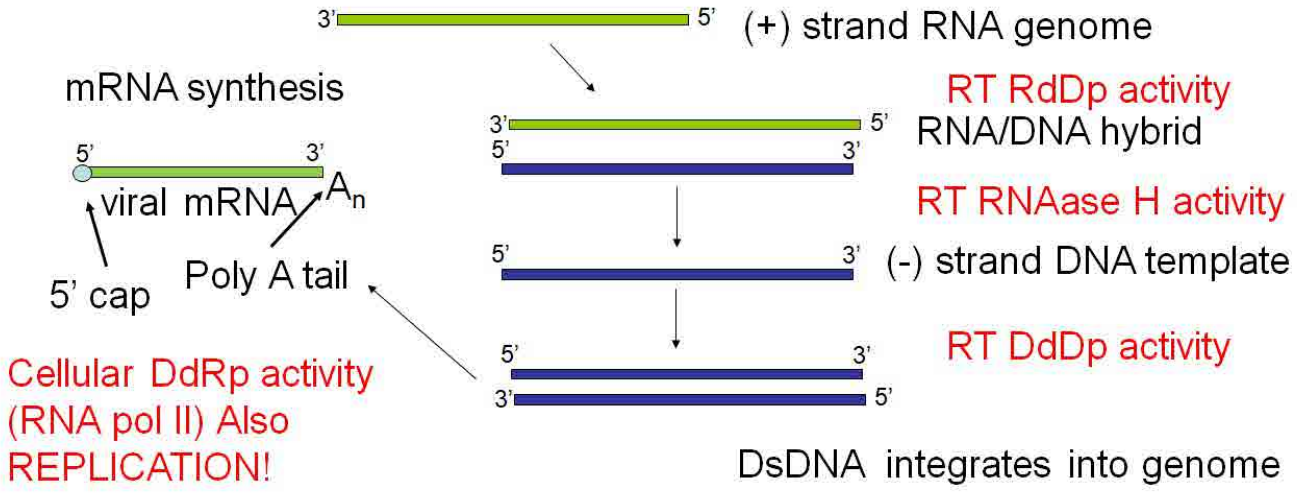


Figure 3-18. The functions of Reverse Transcriptase. Courtesy of Sandra Quackenbush, Colorado State University.

NEGATIVE SENSE RNA VIRUSES AND DOUBLE STRANDED RNA VIRUSES

Figure 3-19 shows the basic negative sense RNA viral replication cycle. Like all viruses, replication begins with binding a cell receptor protein or glycosylation. The virus then penetrates the cell by either membrane fusion or endocytosis as all negative sense RNA viruses are enveloped. The virus must then move to its site of replication most commonly using microtubules and dynein motor proteins. For all negative sense RNA viruses except orthomyxoviruses, this will be the cytoplasm. Unlike positive sense RNA viruses that can immediately be translated by the host cell's ribosomes, negative sense RNA viruses must first create the mRNA required for translation. They need to create mRNA before creating any viral proteins. As such they must carry their RdRp into the cell with the genome so it can be immediately transcribed giving rise to mRNA which will then be read by the host cell's ribosomes. This is called the **transcriptase** activity of RdRp. Now the viral RdRP that has been created in the cell can be used to replicate the virus. This activity is called the **replicase** activity (just like in the positive sense RNA viruses). Note that a double stranded intermediate must be formed in this process as a positive sense template must be read to create more negative sense RNA genome. Much like the positive sense RNA viruses, negative sense RNA viruses try to avoid detection by RNAi and type I interferons by keeping the replicated strand and template strand together for as short a period as possible. This causes the polymerases to lose their reproductive editing ability as they have no template to read as the RNA is replicated. Hence RNA viruses have a very high error rate compared to DNA viruses, and accordingly RNA viruses mutate very rapidly. Note there are a few exceptions including coronaviruses (positive sense RNA) whose RdRp has an editing function thus allowing the virus to proofread and support larger genomes and reoviruses that are double stranded and have developed a mechanism to hide their replication from the host cell (discussed in Chapter 17). Once the viruses have accumulated a significant amount of viral genome and proteins to form capsids, they will spontaneously assemble. All negative sense viruses have envelopes; therefore the viral envelope proteins will accumulate in the plasma membrane (or other internal membranes). The capsids will push against these proteins causing the virus to bud off the infected cell. Note that they may bud off internal membranes and then leave the cell via exocytic vesicles. Note this cycle is identical for the double stranded RNA reoviruses, however these are naked. Therefore, the reoviruses mechanism of entrance is only endocytosis or pore formation, and they will usually leave by killing the cell (lysis) or by leaving in exocytic vesicles. Figure 3-20 demonstrates the functions of RdRp in negative sense and double stranded RNA viruses.

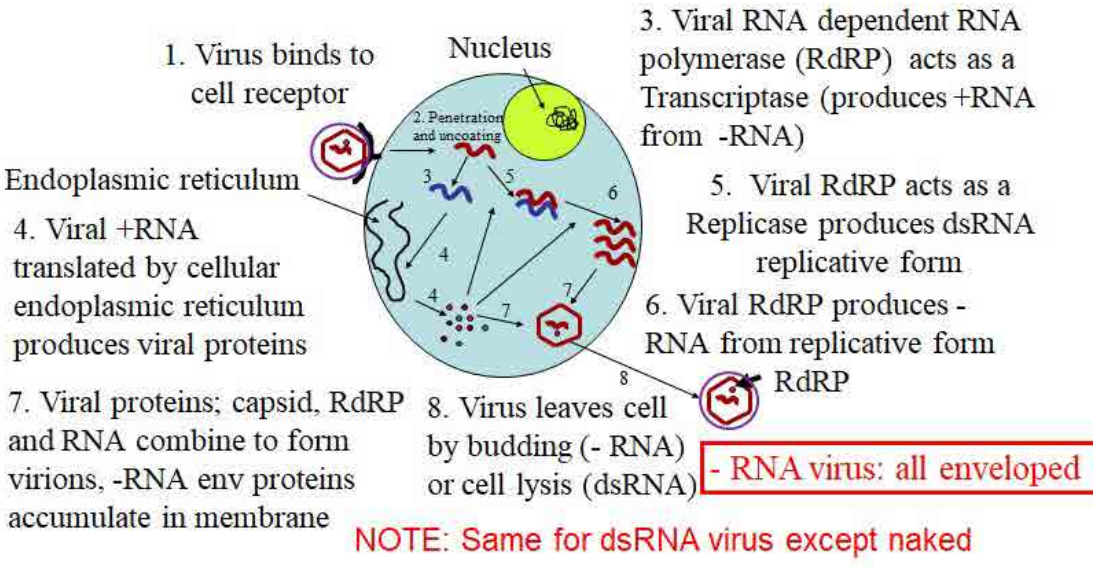


Figure 3-19. Basic negative sense and double stranded RNA viral replication cycle. Courtesy of Erica Suchman, Colorado State University.

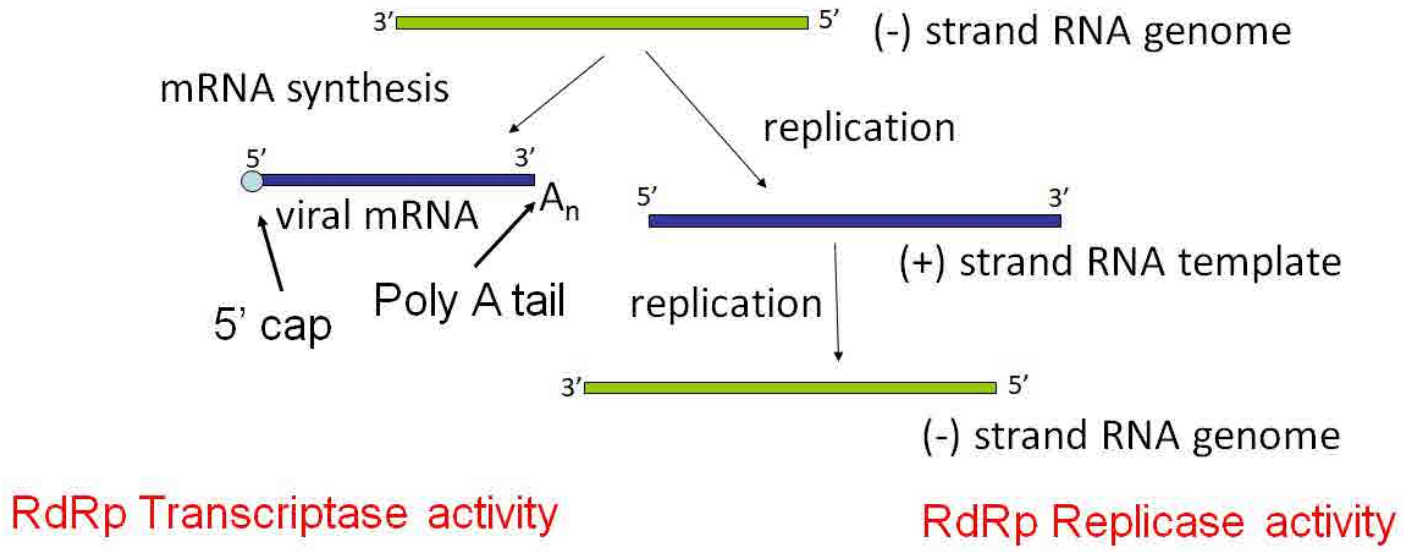


Figure 3-20. RNA-dependent RNA polymerase functions in negative sense and dsRNA viruses. Courtesy of Sandra Quackenbush, Colorado State University.

TRANSLATION OF VIRAL PROTEINS

Now that we have introduced the basic replication cycles, let's look at how viruses can fool host cells into translating their viral mRNAs to produce viral proteins. First it is important to note that, to date, all known viruses utilize the host cell's translation machinery. This includes the ribosomes, amino acids, tRNAs, acetyl transferases, translation initiation, elongation and releasing factors, and poly A tail binding proteins. As you can see, it would be a major task for the virus to carry genes for all of this, so they don't. Instead they depend on the host cell to provide these components for them. Thus, they become obligate intracellular parasites. As such, most viruses try to mimic host cell mRNAs which includes the addition of a 5′ 7-methylguanosine cap (5' cap which will be used to initiate translation as it serves as the ribosome binding site in eukaryotic cells) and a 3′ poly A tail of 50-200 adenosine (A) residues that is required for mRNA export from the nucleus and translation initiation by **binding of poly A binding proteins** required to initiate translation. Eukaryotic ribosomes also usually add the first amino acid (methionine) to the start codon AUG that is closest to a strong **Kozak consensus sequence** (GCCA/ GCC(AUG)G. However, other AUG start sites close to weaker Kozak consensus sequences may be used at lower frequencies, thus allowing cells and viruses to produce some proteins at lower rates.

You will find that DNA viruses that replicate in the nucleus can take advantage of the host cell's post transcriptional machinery to add the 5′ cap and poly A tail. However, RNA viruses and poxviruses must modify mRNA themselves as they do not replicate in the nucleus and as such have no access to the host cell's post transcriptional modification machinery. Figure 3-21 A shows a simplified version of initiation of translation. Note there are more initiation factors (eIF4s) involved but the ones most often manipulated by viruses are shown. Figure 3-21 B shows all initiation factors and the interaction with the poly A binding proteins (PABP). The first step is that the eIF4 elongation factors work in conjunction to bind to the 5′-7 methylguanosine cap, at which point the 40s subunit can be brought to the 5′ 7-methylguanosine cap replacing the eIF4s. However, the 40s subunit requires the elongation factor eIF2α carrying GTP to bind and is released as eIF2α cleaves the GTP to GDP. This GTP must be regenerated in order for more 40s subunits to bind. The poly A binding protein (PABP) is thought to interact with both the poly A tail and eIF4s that are bound to the 5′ 7- methylguanosine cap to help initiate translation. Once the 40s subunit is bound, it will slide along the mRNA until it locates the Kozak consensus sequence. The majority of the time the UAC anti-codon on the initiation tRNA will bind to the AUG found within the Kozak consensus sequence. Occasionally, but at a much lower frequency, it will bind at other AUG sites as

discussed above.

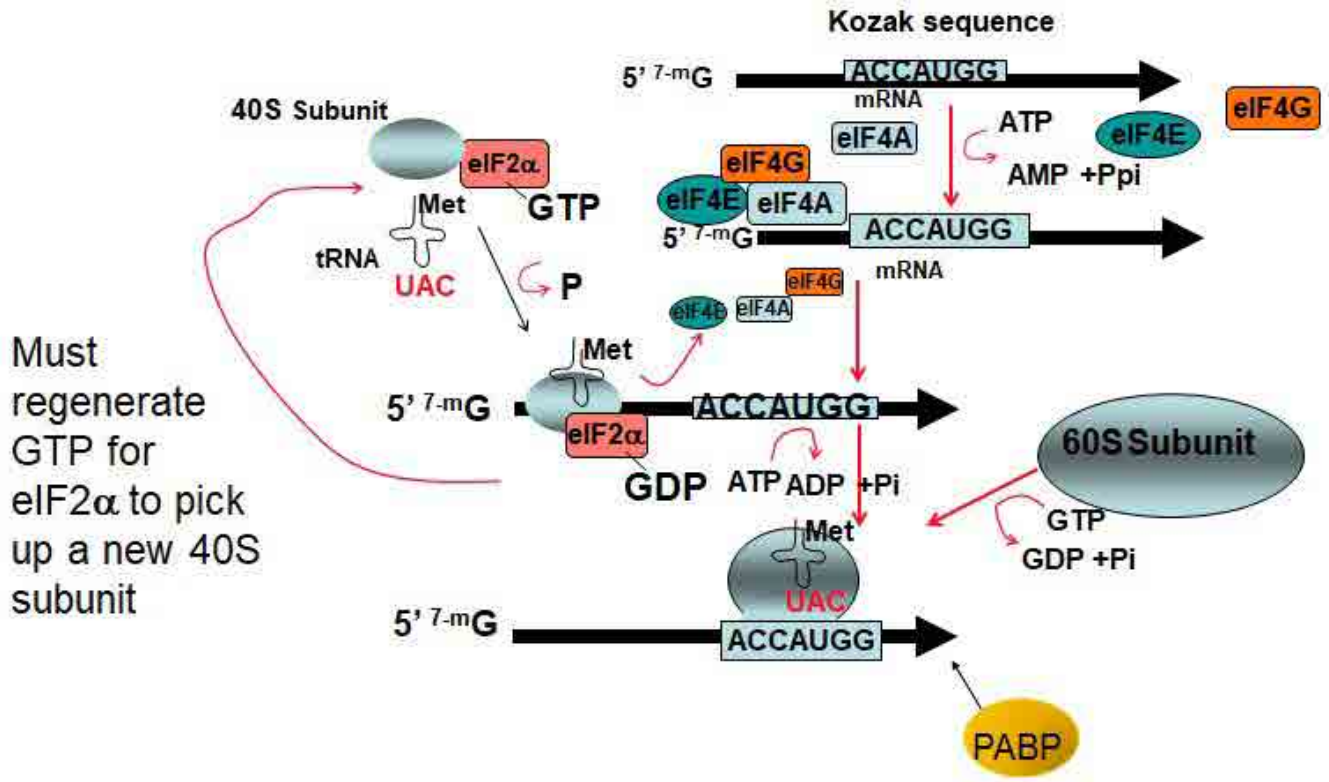


Figure 3-21 A. A simplified diagram of mRNA translation initiation showing the initiation factors most commonly regulated by viruses. Courtesy of Sandra Quackenbush, Colorado State University.

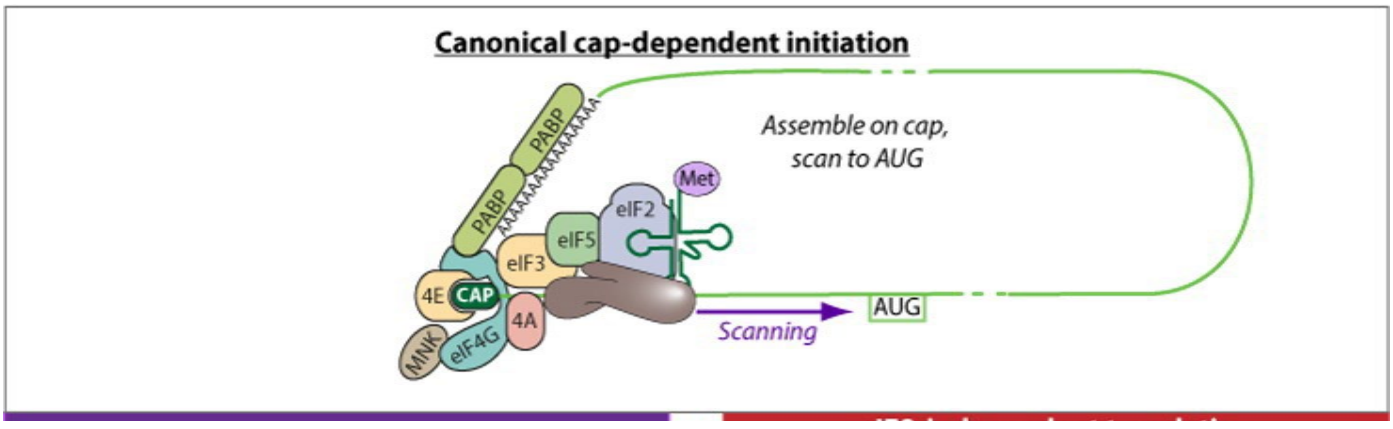


Figure 3-21B. Complete diagram of mRNA translation initiation courtesy of ViralZone. https://viralzone.expasy.org/867

HOW VIRUSES COMPETE WITH HOST CELL MRNAS FOR TRANSLATION

One issue viruses must contend with is that the viral mRNA must compete with host cell mRNA for the translational machinery. Viruses have developed a series of mechanisms to compete with cellular mRNA for translation machinery.

- Inhibition of cellular gene transcription thus reducing the host cell mRNA with which they must compete.
- **Cap snatching –** Stealing the 5' 7-methylguanosine cap from cellular mRNA, thus the cellular mRNA cannot bind to eIF4G to initiate translation.
- Degrading host cell mRNAs.
- Out competing host cell mRNAs.
- Inhibit the function of initiation factors eIF4 or eIF2α by
    - Cleaving these initiation factors.
    - Dephosphorylation of eIF2α.
- Inhibition of Poly A (PABP) protein binding.

OVERCOMING THE ONE PROTEIN PER MRNA IN EUKARYOTIC CELLS ISSUE

Now that viruses have mimicked the host cell mRNA to initiate translation, they must have mechanisms to overcome the fact that they have a very small genome and the host cell only makes 1 protein per mRNA. You will see that the viruses have developed quite a wide range of mechanisms for doing this, and that the type of genome they have (RNA vs DNA) dictates in large part the mechanisms available to them. These include:

- Subgenomic mRNA generated using multiple promoters within the genome: DNA only
- Differential splicing of pre-mRNA to mRNAs: DNA and orthomyxoviruses RNA
- Leaky ribosomal scanning: RNA and DNA
- Segmented genomes: RNA viruses only
- Subgenomic mRNA generated without using multiple promoters within the genome: RNA only
- Transcriptional editing creating novel mRNAs: RNA only
- Polyprotein proteolytic cleavage, protease: RNA & DNA, most common + RNA
- Frame shifting/overlapping ORFs: RNA only
- Termination suppression: RNA only

You will note that **subgenomic mRNA** generated using multiple promoters found within the genome is seen only in DNA viruses. This is because most RNA-dependent RNA polymerases (RdRps) used to transcribe viral mRNA do not require promoters. The very few RNA viruses that create subgenomic mRNAs using promoters do so from their antigenome (RNA complementary to the genome). However, the DNA-dependent RNA polymerases (DdRp) used to transcribe DNA to viral mRNA require promoters. As a result, this is a mechanism that is used by all DNA viruses. Another mechanism used primarily by DNA viruses is differential splicing which only occurs in the nucleus. The only RNA viruses we will discuss, the orthomyxoviruses, and retroviruses replicate in the nucleus and can take advantage of this mechanism. This is the most likely reason that orthomyxoviruses replicate in the nucleus despite the difficulty of getting there. Using proteases to cleave large polyproteins into multiple viral

proteins is found in all positive sense RNA animal viruses and sporadically in DNA and negative sense RNA viruses of animals. Note that the negative sense RNA viruses never use their own proteases; they use cellular ones. Only herpesviruses and adenoviruses use viral proteases, and hepadnaviruses use a cellular protease. Then there is leaky ribosome scanning where the ribosome may bypass the AUG closest to the strong Kozak consensus and initiate at another AUG near a weak Kozak consensus creating small quantities of a second protein. Because leaky scanning occurs during translation by host cell ribosomes, which both DNA and RNA viruses utilize, both can utilize this mechanism. These will be described in greater detail in Chapter 5, introduction to DNA viruses. Then you will note a series of mechanisms that are probably not familiar to you that will be described later in Chapter 12, introduction to RNA viruses. So, when you learn in the viral family chapters that viruses use these mechanisms, you will understand what they are. You may have noticed a trend. DNA viruses tend to do what cells already do normally. RNA viruses use those mechanisms when they can, but if they can't, they have evolved their own mechanisms.

CELLULAR POST TRANSLATIONAL MODIFICATIONS

Once viruses have used the host cell's machinery to translate their proteins, they often also take advantage of cellular post translational mechanisms such as removal of the methionine from the start of proteins. Remember methionine is the amino acid that corresponds to AUG, the start codon, and as such is always the first amino acid and is most often removed via post translational modification. Many proteins are modified by proteases in eukaryotic cells, and there are both RNA and DNA viruses that take advantage of the proteases found in the Golgi apparatus as well as transport vesicles. However, some viruses, particularly all the positive sense RNA viruses and some DNA viruses, create their own proteases as well.

Many cellular proteins are glycosylated as are many of the proteins of viruses. The viruses take advantage of glycosylation pathways found in the endoplasmic reticulum and Golgi apparatus. However, to take advantage of these pathways, the viral proteins must contain a signal sequence that will be recognized by the host cell's glycosylation pathways.

VIRION ASSEMBLY AND RELEASE

Once the virus has reproduced its genome and created all of the viral proteins it needs, it is ready to leave the cell. Animal viruses contain packaging sequences that allow the genome to be packed into the capsid proteins. This will occur spontaneously. As we discussed in Chapter 1, the capsids and nucleocapsids are made of a few viral proteins in predictable numbers and subunits. They can be helical nucleocapsids or icosahedral capsids, and the larger the genome, the bigger the capsid/nucleocapsid. The capsid/nucleocapsid will be held together by non-covalent bonds so that upon entering a new cell, the capsid/nucleocapsid can easily degrade and release the genome. Remember that many of the capsids that form will not contain complete genomes and as such will be defective interfering particles which are not infectious. Some viruses will go on to form an envelope around the capsid and many will not and will remain "naked" or non-enveloped. Capsids must move towards the location of exit and will generally use the same mechanism they used to move to their site of replication, either transport on microtubules or actin.

Non-enveloped viruses tend to assemble in their site of replication, so naked RNA viruses such as picornaviruses assemble in the cytoplasm and naked DNA viruses such as parvovirus assemble in the nucleus. Naked viruses generally lyse the infected cell to be released, but some will also leave in exocytic vesicles (such as polio which can use both mechanisms).

Enveloped viruses will assemble wherever they will bud from a membrane to acquire their envelope. This can occur at the plasma membrane as is seen in the positive sense RNA retroviruses, matonaviruses and togaviruses and most of the negative sense RNA viruses (except some members of *Bunyavirales*) including arenaviruses, all members of the order Mononegavirales (paramyxoviruses, filoviruses, pneumoviruses, rhabdoviruses) and orthomyxoviruses. You will notice that none of the DNA viruses bud from the plasma membranes. Others bud from either the nuclear, ER, or Golgi apparatus membranes as seen in all the enveloped DNA viruses (including herpesviruses, hepadnaviruses and poxviruses), some of the enveloped positive sense RNA viruses (including coronaviruses, flaviviruses), and some members of the negative sense RNA viruses (families in the order *Bunyavirales, Hantaviridae, Phenuiviridae*). Note that enveloped viruses that bud from membranes other than the plasma membrane must use exocytic vesicles to leave the cell after budding into the cytoplasm, or lyse the cell, otherwise they will lose their envelope. The majority will use exocytic vesicles. For all enveloped viruses, the viral envelope proteins will accumulate in the membrane that will be used to form the envelope. The capsids will then push against the membrane until the virus leaves with an envelope that contains cellular membrane lipids with viral envelope integral proteins contained within the membrane. Figure 3-22 is a summary of the ways viruses can exit cells.

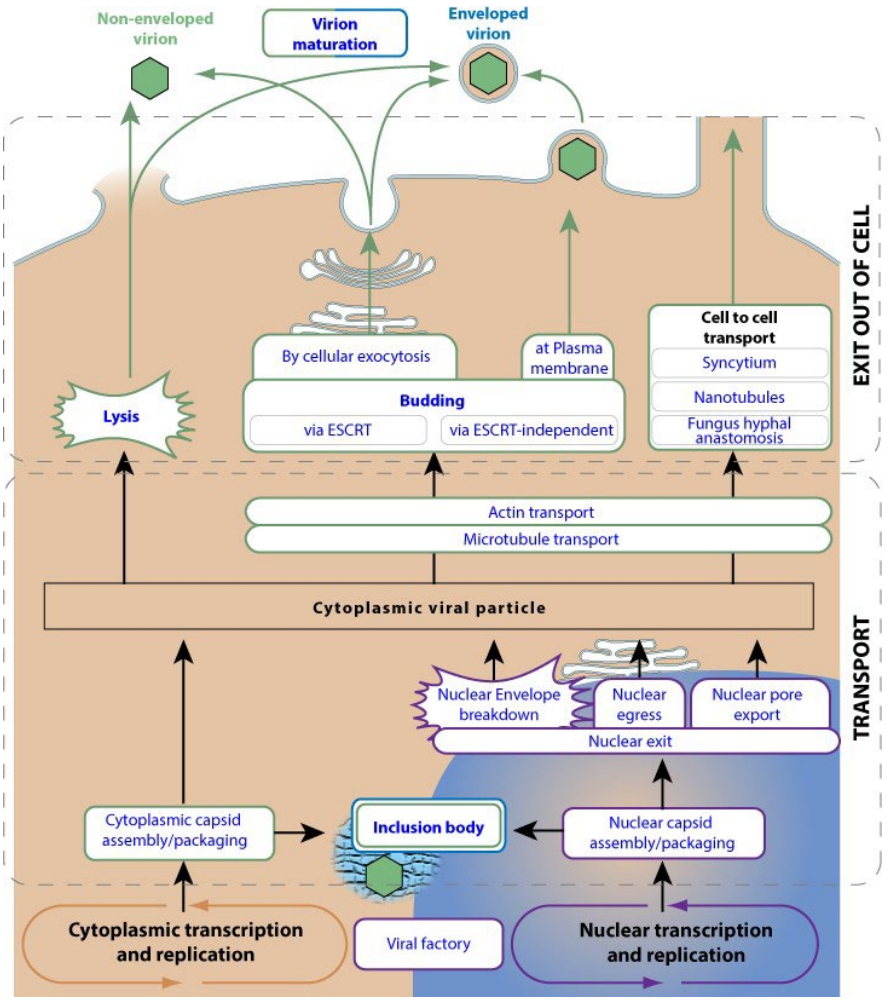


Figure 3-22. Viral exit strategies from cells. Courtesy of ViralZone. https://viralzone.expasy.org/1076

This assembly can often be seen with fluorescently labeled antibodies to viral capsid or envelope proteins. RNA viruses will only show fluorescence in the cytoplasm (with the exception of orthomyxoviruses and retroviruses), while DNA viruses show fluorescence in both the nucleus and cytoplasm (with the exception of poxviruses). Remember that DNA viruses must still translate their proteins in the cytoplasm, and as such, the antibodies to viral capsid or envelope proteins will bind to the proteins both within the cytoplasm and the nucleus.

ONE STEP GROWTH CURVES

One step growth curves are used to look at viral replication cycles. The process involves infecting cells at a given time and allowing them to go through only one round of synchronous replication. It is not done over days but more often hours depending on how long viral replication takes. To start **synchronous replication,** cells are infected with a **multiplicity of infection** (MOI) of usually more than 0.1 as many viruses will fail to take hold at MOIs lower than 0.1. Synchronous replication is where all of the virus is replicating at the same time in the same stage.

To determine the MOI take the number of cells and determine how many virus particles will be added per cell. For example, if you added $5X10^5$ cells on your plate, you would need to add at least 4×10^5 virus particles (a MOI of 0.1) to achieve a successful one step growth curve. If you wanted to add a MOI of 100 you would need to add 5×10^7 virus particles to 5×10^5 cells.

Once the cells have been infected synchronously, the viral cell culture medium is assayed over time to determine how much virus is extruded from the cells. In another separate flask set up at the same time in the same way, cells are lysed and viral titers inside the cell are determined. These two numbers, external virus and internal virus, combine to give a total viral titer that can be compared to both the external and internal concentrations of virus. Some viruses will accumulate most of their virus particles intracellularly. In other viruses, most of the virus will be released extracellularly. An example of a typical one step growth curve is shown in Figure 3-23. As you can see, between the time of infection and the time that virus starts to accumulate internally is called the eclipse period. During the eclipse period, the virus is entering the cells and moving to the site of replication. No virus has yet accumulated either intracellularly or extracellularly. The latent period is the time between the virus infecting the cell and when external virus is accumulating in the culture medium. The period between the eclipse and latent periods is called the intracellular accumulation period (where virus is replicating but no virus particles are leaving the cells yet). In some viruses this period is quite short, and in others it is quite long. You will note that eventually the virus production plateaus as the cells become unable to support additional replication cycles due to damage from the viral infection.

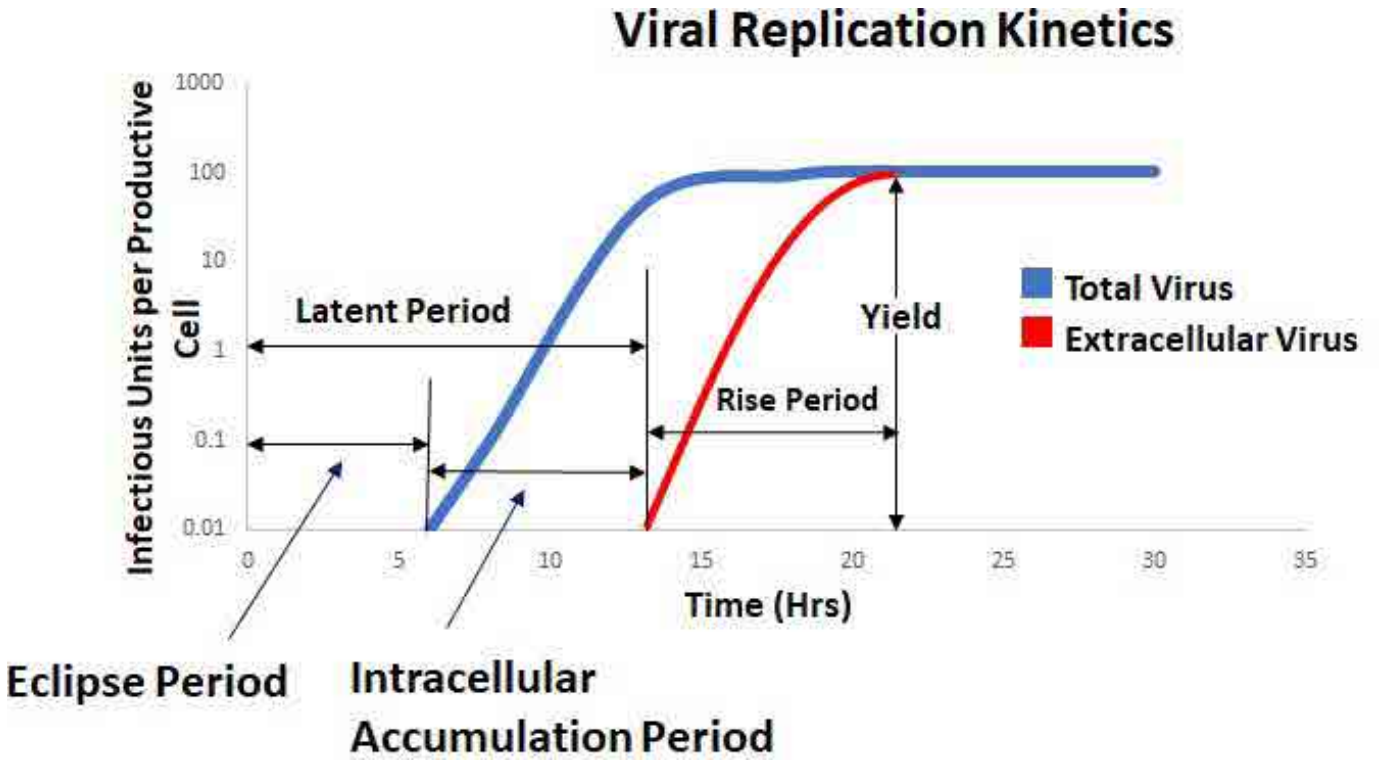


Figure 3-23. Basic one step growth curve. Courtesy of Maxwell Drummond, Colorado State University.

VIRAL GENETICS: MUTATIONS

Viruses, like all organisms, develop spontaneous mutations as they reproduce due to errors in replication. When the spontaneous mutation rates of DNA and RNA viruses are compared, some telling trends emerge.

The mutation rate in number of errors per nucleotides replicated per replication cycle	
DNA viruses	10^{-8} to 10^{-11}
RNA viruses	10^{-3} to 10^{-5}
Retroviruses	10^{-4} to 10^{-6}
Coronaviruses	10^{-6} to 10^{-7}

You will note that the DNA viruses have a much lower rate of mutation (with a mutation of only 1 in every 10^{8}-10^{11} nucleotides replicated) than the RNA viruses (1 in every 1000-100,000 nucleotides replicated), and the retroviruses and coronaviruses are slightly better than the RNA viruses with slightly lower mutation rates. Why is this? RNA-dependent RNA polymerases (RdRp) have higher error rates. There is no long term double stranded form for error repair. RNA viruses are trying to avoid activating RNAi and type 1 interferons, so they keep their RNA double stranded for as short a time as possible. As such their RdRp lacks a proofreading ability. The exception is the coronaviruses whose RdRp is capable of proofreading, and, as you can see, this leads to a lower mutation rate than other RNA viruses. The high mutation rate of RNA viruses leads to another trend you will observe. Most RNA viruses have smaller genomes than DNA viruses. The very high mutation rates make it difficult to maintain long genomes. Some RNA viruses, however, such as coronaviruses, do possess proofreading capabilities. This allows their genomes to become longer. To study the effect of mutations on viral replication, we can induce mutations of nucleic acids in the viral particle by exposure to mutagenic chemicals (base analogs, nucleotide altering chemicals)

or UV light during viral replication. These mutated viruses are now used to infect cells to see what effects these mutations have on viral replication.

Mutations can be **point mutations** where one base is substituted for another. These point mutations can be singles or multiples within a genome. If nucleotides are deleted or inserted, this may lead to frame shift mutations if within the coding region and insertions or deletions do not involve nucleotides in groups divisible by 3. For example, if two nucleotides are inserted or deleted into a genome within the coding region, the codon that has the change may result in a change in the amino acid it codes for as will all codons after the insertion or deletion. However, if 3, 6, 9 etc. nucleotides are inserted, you will have insertion of amino acids, but the downstream codons will be unaffected.

Temperature-sensitive (TS) mutants are often used to study the effect of mutations on viral replication. TS mutants replicate at lower temperatures but poorly or not at all at high temperatures. Often the mutation is a single amino acid substitution that renders the viral protein unstable at the higher temperature. For example, the permissive temperature would be 33^{O}C and the non-permissive temperature would be 39^{O}C. If one were to run a plaque assay on a TS mutant they might observe changes in the plaque size or morphology. In hosts they may observe changes in host range, drug sensitivity, or antigenic variation.

VIRAL GENETICS: ANALYSIS OF MUTANTS

Viral mutants can often be restored to normal function by 4 mechanisms.

1. Complementation where two different non-replicating mutant viruses interact functionally in the same cell permitting viral replication of one or both by providing the lacking component to the other virus or each other.
2. Functional helper viruses can coinfect a cell with a defective virus and provide the components the defective virus lacks allowing it to replicate.
3. If two viruses undergo recombination, a non-replicating virus may receive functional genes from a genotypically different parent virus such that progeny virus contains sequences from both viruses that are covalently bonded.
4. Two different strains of the same virus with a **segmented genome** may infect one cell and exchange segments giving rise to functional **reassortment** progeny, Figure 3-24. Note this can only be carried out by segmented viruses which are some negative sense RNA viruses or double stranded RNA viruses, although not all negative sense RNA viruses have segmented genomes.

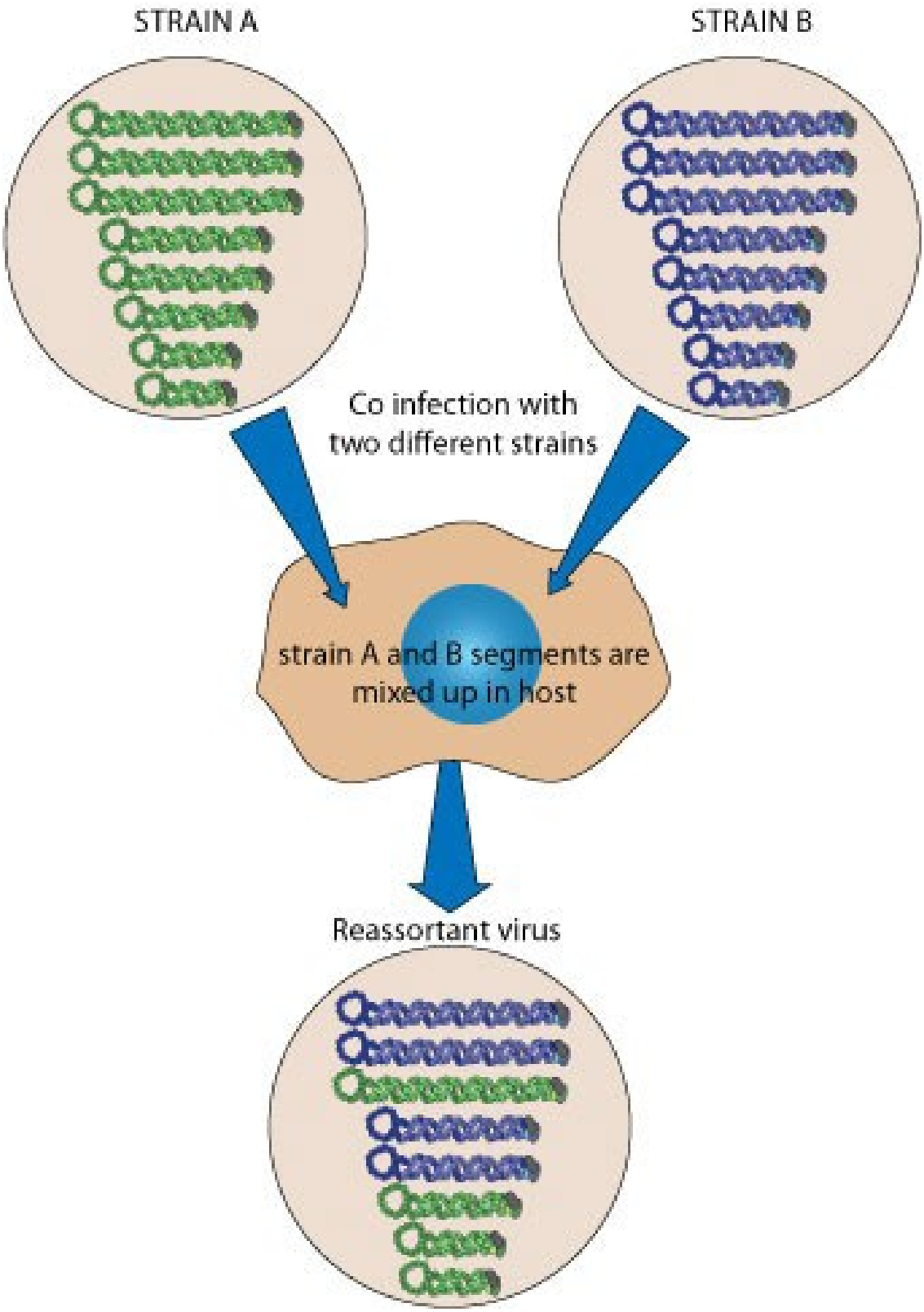


Figure 3-24. Two segmented viruses infecting the same cell resulting in reassortment progeny with segments from both of the parental viruses. Courtesy of The Viralzone https://viralzone.expasy.org/4139

VIRAL GENETICS: INFECTIOUS CLONES

Often it is useful when studying viruses to have infectious clones, for example when genetically modifying viruses in the lab. If working with positive sense RNA viruses, one has the most options for how to get genetic material into the cell. Figure 3-25 shows methods for obtaining **infectious clones** (genetically identical infectious viral particles) with a positive sense RNA virus.

The first is to simply infect cells with virus particles isolated from a single plaque as described in Chapter 2. Note you can use this mechanism for a virus of any genome type. The second is to transfect the positive sense RNA genome directly into cells. Transfection will work for all positive sense RNA viruses except retroviruses as they need reverse transcriptase and integrase to initiate infection, and the transfected RNA will lack that. Similarly, transfection would not work for negative sense or double stranded RNA viruses, both of which require RdRp to create mRNA from the genome to begin making viral proteins.

The third method is to reverse transcribe the RNA into double stranded DNA, clone it into a eukaryotic expression vector, and transfect this vector into cells where the viral mRNA will be expressed by cellular RNA polymerase II (DdRp) from a promoter within the expression vector. This will work for all types of viruses, as even in negative sense RNA viruses, the DNA clone will express viral mRNA that can be translated to give the proteins necessary to replicate the virus from the positive sense RNA (mRNA) produced in the cell.

The fourth mechanism is to use the clone described in method 3 to produce viral mRNA in vitro (in a test tube) and transfect this mRNA into the cell. This mechanism will work for all RNA viruses. This will not work for DNA viruses, however, as they have no way to produce viral DNA from the mRNA.

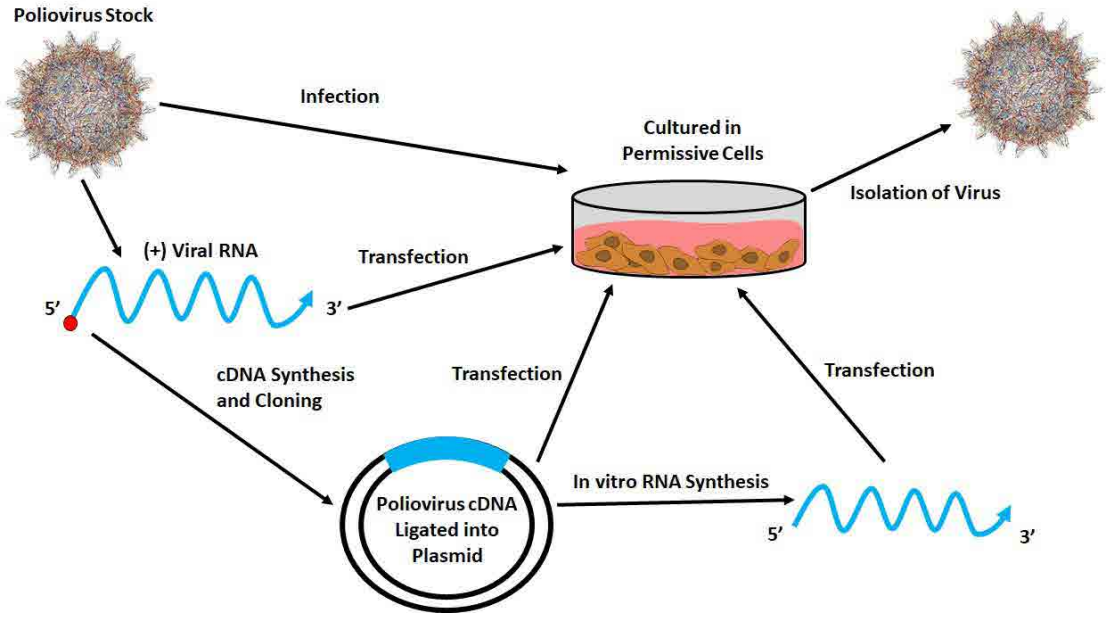


Figure 3-25. Mechanisms for creating infectious clones with positive sense RNA viruses. Courtesy of Maxwell Drummond, Colorado State University.

Which leads us to recent work using reverse genetics to clone negative sense segmented RNA viruses. This has been done to re-create ancient extinct orthomyxoviruses which contain 8 negative sense segments in their genome. To create the desired virus, they take multiple viruses containing different hemagglutinin (H) or neuraminidase (N) genes. They reverse transcribe the desired segments, clone the desired genes into expression vectors, and then transfect cell culture with all 8 segments in the desired arrangement giving rise to a virus with the desired combination of genes that most closely simulates the extinct virus.

CHAPTER 3 END OF CHAPTER QUESTIONS

1. For what purpose do viruses use the Golgi apparatus?
2. What type of genomes do viruses that replicate in the nucleus MOST LIKELY have?
3. What type of genomes do viruses that replicate in the cytoplasm MOST LIKELY have?
4. Are there viruses that create their own translation machinery? If so, why (or why not)?
5. Why can some influenza viruses move from birds to humans when others cannot?
6. Your colleague says in a know it all matter that “only enveloped viruses can form syncytia”. Do you believe this is true? Justify your answer.
7. How do viruses fool cells into endocytosing them?
8. Compare and contrast how naked and enveloped viruses enter cells.
9. Compare and contrast how naked and enveloped viruses exit cells.
10. What is the most common way that viruses carry out intracellular transport within the cell?
11. When analyzing how viruses enter the nucleus, what trends do you observe?
12. How do viruses exit the nucleus?
13. What kinds of genome(s) do viruses that utilize RdRp most likely have? Is this enzyme provided by the cell or the virus?
14. What kinds of genome(s) do viruses that utilize DdDp most likely have? Is this enzyme provided by the cell or the virus?
15. What kinds of genome(s) do viruses that utilize DdRp most likely have? Is this enzyme provided by the cell or the virus?
16. What RNA polymerase is MOST LIKELY to transcribe viral mRNA? Why is this enzyme the most likely one?
17. Your friend wants to know why RNA viruses have a higher mutation rate than DNA viruses. Explain this to your friend.
18. You isolate a virus that creates one large polyprotein that it cleaves into 6 proteins. How many open reading frames (ORF) does this virus have?
19. What types of viruses have integrase? What function does integrase provide?

20. If a virus buds from the endoplasmic reticulum, how will it leave the cell?
21. If you put a negative sense RNA virus genome into a cell free translation system, would you expect translation of viral proteins? Why? (or why not)?
22. If you put a positive sense RNA virus genome into a cell, would you expect the virus to be able to initiate replication? Why, or why not?
23. Do double stranded RNA viruses replicate more like positive sense RNA viruses or negative sense RNA viruses? Justify your answer.
24. What is the importance of the Kozak consensus sequence?
25. What sequences are required for efficient translation of mRNA? Why are they required?
26. Explain how viruses can outcompete cellular mRNAs for translation.
27. What mechanism is used by all DNA viruses to overcome the one protein per mRNA issue in eukaryotic cells? Why is this the one that is used by all?
28. What mechanism is used by all DNA viruses except poxviruses? Why don't poxviruses use this mechanism?
29. If the 5' cap addition and poly A tail addition machinery are in the nucleus, how do most RNA viruses obtain a 5' cap and 3' poly A tail?
30. You want to set up a plate of togavirus infected cells with an MOI of 10. If you plated 5000 cells how many virus particles would you need to add? What if the MOI was 1?
31. Which MOI would you expect to see more CPE?
32. Which plate would you use a higher dilution virus stock, the 10 or 1 MOI plate?
33. What type of genome does a virus need to have to perform reassortment?
34. What type of genome does a virus need to have to perform recombination?
35. What types of viruses can you use to transfer viral mRNA into host cells and establish infections in cells, and why?
36. You isolate a new naked virus. Are you likely to find a fusion protein in the capsid of this virus? Why, or why not?
37. You isolate an RNA virus with a low mutation rate. What type of RNA virus would you suspect? Why would this RNA virus have a lower mutation rate?

REFERENCES:

Fields Virology 6th edition David Knipe, Peter Howley, 2013 Lippincot, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 4, 5, 6.

Kahvejian, A., et. al. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev. 19(1): 104–113. doi: 10.1101/gad.1262905 https://pubmed.ncbi.nlm.nih.gov/15630022/

ViralZone. Fusion of viral membrane to host membranes. https://viralzone.expasy.org/987
ViralZone. HBV membrane fusion. https://viralzone.expasy.org/1361

ViralZone. Nuclear egress https://viralzone.expasy.org/1952

ViralZone. Pore-mediated penetration of viral genome into host cell. https://viralzone.expasy.org/979

ViralZone. Segment reassortment (genetic shift). https://viralzone.expasy.org/4139

ViralZone. Syncytium formation induced by viral infection. https://viralzone.expasy.org/5957

ViralZone. Viral attachment to host cells. https://viralzone.expasy.org/956

ViralZone. Viral cytoplasmic inwards transport. https://viralzone.expasy.org/990

ViralZone. Viral endocytosis into host cells. https://viralzone.expasy.org/977

ViralZone. Viral entry. https://viralzone.expasy.org/936

ViralZone. Viral exit. https://viralzone.expasy.org/1076

ViralZone. Viral exit from the host nucleus. https://viralzone.expasy.org/2177

ViralZone. Viral initiation of translation. https://viralzone.expasy.org/867

ViralZone. Viral penetration into the host nucleus. https://viralzone.expasy.org/989

CHAPTER 4

Chapter 4: Infection of Hosts, Host Defenses and Viral Evasion of Host Defenses.

Host versus virus

INFECTING A HOST

In order to successfully infect a host, viruses require sufficient virions to initiate infection in a host with cells at the site of infection that are physically accessible to the virions. These cells must be both **susceptible** and **permissive** to infection. To be susceptible they must have the viral receptor for entry. To be permissive they must contain cellular proteins needed to support viral replication. Lastly, the host antiviral defense systems must be absent or initially ineffective.

Viruses prefer certain tissues over others. This is called **tropism**. Some classic examples are:

- Enterotropic viruses that replicate in cells of the gut.
- Neurotropic viruses that replicate in cells of the nervous system.

Viruses also prefer certain hosts over others. The **host range** of a virus is the species that the virus can infect.

When a virus infects a host, the virus enters the host by specific routes of entry, follow routes of dissemination, and, lastly, follow routes of viral shedding from the host. We will look at each of these aspects of viral replication in greater detail.

ROUTES OF ENTRY

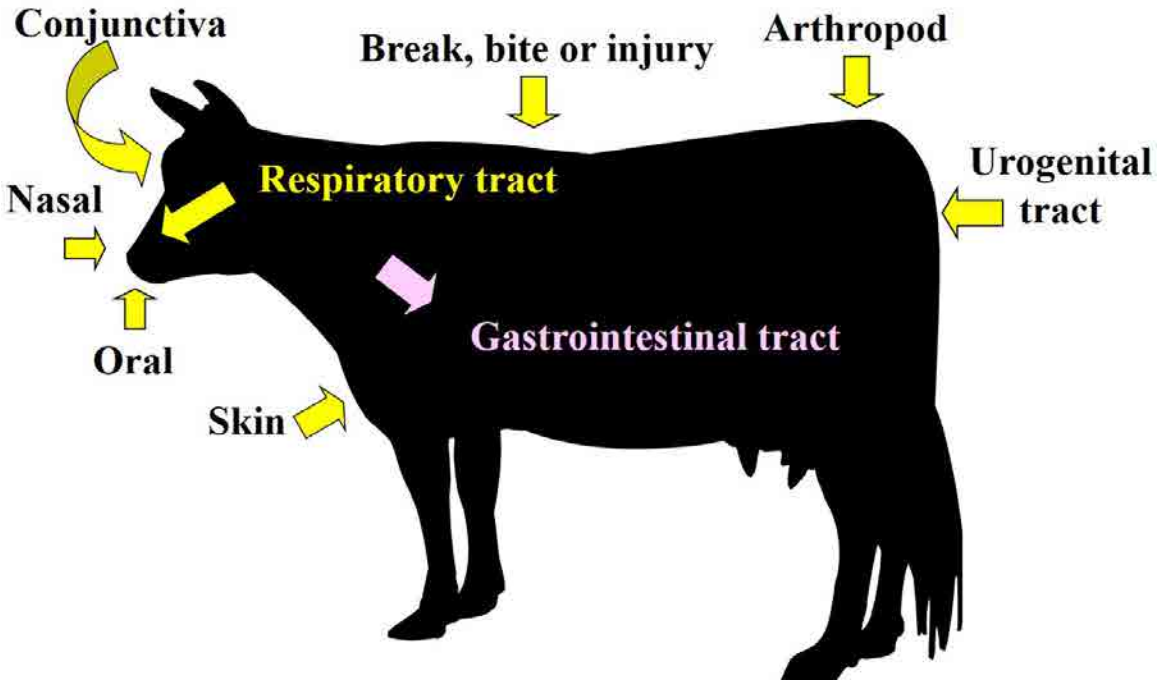


Figure 4-1. The many routes a virus can take to enter a host. Courtesy of Erica Suchman, Colorado State University.

The Skin

The skin has multiple layers. Near the skin's surface is the **epidermis** which is composed of **stratified keratinized epithelial cells** that form a protective barrier as well as a weakly acidic surface. Few viruses can break through the skin barrier; however, many can enter through the eyes, nose, mouth, respiratory tract, and urogenital tract. To get through the skin most viruses will need either a break, bite, needle, surgery, transfusion, injury, or an **arthropod** (mosquito, tick, biting insect or flea) to inoculate the virus past the skin barrier.

Arthropods that infect hosts via the skin have their own cycles, as shown in Figure 14-2. The enzootic, sylvatic or jungle cycle occurs when there is transmission of virus between wild animals (vertebrate hosts) and primary or enzootic arthropod vectors that leads to the amplification of the virus in the vector. This generally occurs in the natural environment in which the vertebrate host and primary/enzootic insect live, such as the jungle, forest, etc. The vertebrate host may also be a reservoir host that can harbor a virus indefinitely without developing symptoms of infection. The reservoir host, therefore, is the primary host of a virus wherein the virus has evolved and infects most effectively. The virus does not kill the host, thus allowing the host to continue infecting other hosts most efficiently. This is shown as A in Figure 14-2. Spillover events into human hosts occur during the epizootic or rural cycle as shown in Figure 14-2 B. The virus is transmitted between non-wild or domestic animals and the primary/enzootic or accessory insect vectors. The primary/enzootic insect vectors may only transmit the virus in the enzootic/sylvatic cycle, or they may also move the virus to new hosts in the epizootic/rural cycle. Furthermore, other insect vectors that do not transmit the virus in the enzootic/sylvatic cycle can transmit the virus to humans in the epizootic cycle. Movement of the virus into domestic animals can lead to an epidemic outbreak of viral disease in a domestic animal population where the virus is amplified (amplifying host). Insect vectors can then transmit the virus to human hosts leading to the spillover event where the virus begins causing disease in humans which are not the evolutionarily favored host. During the urban cycle shown in Figure 4-2C, humans may be the source of infection for arthropod vectors due to high levels of viremia. For some viral diseases, humans are **dead-end hosts** in the infection chain because they do not develop sufficient viremia and do not serve for amplification of

the virus to be transmitted again to arthropod vectors. In an amplifying host the level of virus can become high enough that an arthropod vector, such as a mosquito that feeds on it, will probably become infectious; however the amplifying host succumbs to the disease and therefore does not serve as the reservoir. A dead-end host or **incidental host** is an intermediate host that generally does not allow transmission of the virus to the primary host. They do not develop sufficient viremia to be picked up by the insect vectors. A bridge vector is an arthropod that acquires virus from an infected wild animal and subsequently transmits the agent to human or domestic animal but is not the vector in the enzootic/sylvatic cycle.

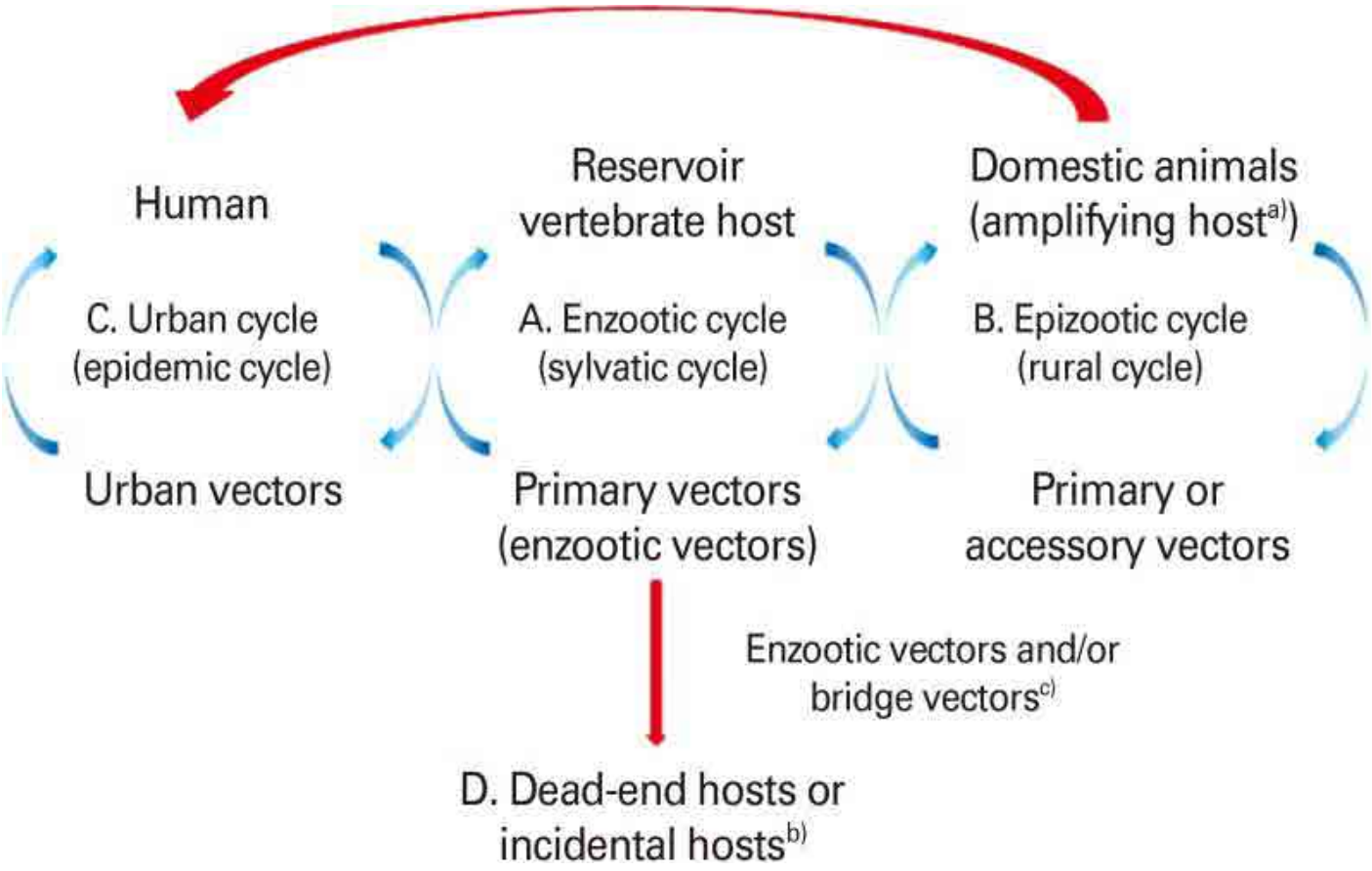


Figure 4-2. Vertebrate host and vector transmission cycles. (A) Enzootic cycle (sylvatic or jungle cycle), (B) Epizootic cycle (rural cycle), (C) Urban cycle, and (D) Human cycle. Humans are dead-end hosts in the infection chain, do not develop sufficient viremia, and do not serve for amplification of the virus to be transmitted again to insect vector. Note: a) Amplifying host is one in which the level of virus can become high enough that an insect vector, such as a mosquito that feeds on it, will probably become infectious. b) Dead-end host, or incidental host, is an intermediate host that does not generally allow transmission of the virus to the definitive host. They do not develop sufficient viremia to be picked up by the insect vectors. c)Bridge vector is an arthropod that acquires virus from an infected wild animal and subsequently transmits the agent to human or secondary host. Courtesy of Zoonotic encephalitides caused by arboviruses: Transmission and epidemiology of alphaviruses and flaviviruses – Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Vertebrate-host-and-vector-transmission-cycles-A-Enzootic-cycle-sylvatic-or-jungle_fig2_259743924.

Note that diseases that are transmitted by **vectors** (arthropods or other animals) have an **extrinsic incubation period**. The extrinsic incubation period is the time between the acquisition of a virus (or other infectious agent) by a vector and the vector's ability to transmit the virus to other susceptible vertebrate hosts. For most viruses this requires that virus infects the vector and reproduces to high enough levels to be transmitted to a new host. The range depends upon the infectious agent but usually takes many days to a few weeks before the vector can transmit the disease to a new host. This is not to be confused with the **intrinsic incubation period** which is the period of time between when a host contracts an infection and begins to show symptoms.

The Respiratory Tract

Viruses can be transmitted via inhalation of aerosolized virus, respiratory droplets containing virus, or close contact. There are many locations along the respiratory tract where viruses can replicate. As you would expect, most of these lead to respiratory symptoms such as runny nose, sore throat, or cough depending upon where they replicate. The upper respiratory tract infections (many viruses) usually lead to **rhinitis** (runny nose), **pharyngitis** (sore throat), and **laryngitis** (swelling of the vocal cords). Those viruses that replicate lower in the respiratory tract, such as influenza, parainfluenza, respiratory syncytial virus (RSV) and adenovirus, can cause symptoms such as **tracheitis** (inflammation of the wind pipe, difficulty breathing), **bronchitis** (inflammation of the bronchia of the lung, difficulty breathing), **and bronchopneumonia** (inflammation of the alveoli or air sacs of the lung, difficulty breathing). Some viruses only replicate in the respiratory epithelium such as rhinoviruses, adenoviruses, and influenza A virus. Others replicate in the respiratory epithelium but then spread systemically throughout the body. Examples of these are measles virus and varicella-zoster virus (chicken pox).

Transmission to the respiratory tract can occur by either droplet or aerosol transmission. Droplet transmission is usually defined as large respiratory droplets (greater than 5 μm) created during coughing, sneezing, exhaling, and talking which transmit infection when they travel directly from the respiratory tract of the infectious individual to susceptible mucosal surfaces of the recipient, generally over short distances. This is because the large droplets do not remain airborne for long periods (several minutes) and rapidly drop to the ground. Close contact involves hand transfer of surface contamination to mouth, nose, or eyes. Airborne transmission is defined as smaller airborne droplet nuclei or small particles, often referred to as aerosols, created during coughing, sneezing, talking, exhaling, and medical procedures that are in the size range that remain airborne (less than 5 μm) and infectious over time and distance and therefore can be inhaled into the lower lung.

The respiratory tract defends itself from viral infection by utilizing a **mucociliary apparatus** where **goblet cells** secrete mucus that is moved up to the esophagus by ciliated respiratory epithelial cells. This mucus traps viruses and other microbes that might try to infect the lungs and moves the mucus up to the esophagus where it is swallowed throughout the day. Furthermore, the alveoli contain macrophages prepared to **phagocytose** (engulf and degrade) invading microbes. Lastly, the lungs have a robust **humoral** immune response (IgA antibody). Remember that the lungs have a very low **normal flora** (resident microbiome) compared to other parts of the body that have access to the outside world. This is why hosts spend a great deal of energy trying to keep the lungs with low numbers of microbes to increase oxygen contact with the alveoli where oxygen exchange takes place.

The Alimentary Tract

Viruses can infect the **alimentary tract** (digestive system, mouth to anus). The gut lining is composed of microvilli involved in protection of the gut by movement of mucus. Interspersed between these microvilli are **microfold (M) cells**, specialized epithelial cells of the lymphoid system which transport antigens from the lumen of the gut to the immune system. Sometimes these cells inadvertently also transport **enteric** (gut) viruses, hence viral entry is usually through M cells in the intestines.

The alimentary tract has many defense mechanisms. In the oral cavity the epithelium of the mouth is difficult for viruses to infect, and saliva helps to wash them away. In the gastrointestinal tract the stomach's acidic environment is difficult for many viruses to survive, although we will see that there are acid resistant viruses that can withstand this defense. In addition, after leaving the stomach the virus enters the intestines where the pH suddenly becomes much more basic. This transition is difficult for many viruses to survive. As we discussed before, the gastrointestinal tract has a ciliated epithelium and mucous layer. Furthermore, the **Peyer's patches** are localized regions of lymphoid nodules found in the small intestines that monitor intestinal pathogen populations. IgA antibody is secreted into the gut. Many of the digestive proteases that are released into the gut can attack

the proteins of viruses. The bile salts released by the gall bladder also help to disable viruses by lysing the viral particles. There are, however, viruses that cause **enteritis** (inflammation of the intestines) that clearly have developed mechanisms to circumvent these defenses.

Urogenital Tract

Viruses can infect the urogenital tract primarily through sexual activity. As you would expect, there are defense mechanisms a virus must overcome to initiate infection here. First, there is the flushing of urine which can help to move viruses out, as well as mucus that can make it difficult to initiate infection. The vagina is very acidic as are vaginal secretions. IgA is also secreted into the genital tract.

Ocular

Viruses can infect through the eyes which defend themselves by producing tears to wash them away, blinking to move them away, the presence of lymphoid tissues to detect them, and the secretion of IgA.

ROUTES OF DISSEMINATION

Viruses utilize 5 main dissemination routes from the initial infection site:

- Lymphatic system
- Infection of other organs
- Neural spread

Viruses that spread by local spread on epithelial surfaces generally do not cause systemic infections and will spread to other hosts by contact with the infected tissue or secretions from infected tissues. Examples of these are papillomaviruses (warts) and herpes simplex viruses.

Other viruses move from the initial infection site by spreading to the blood (**viremia**) and then to a new replication site such as the muscle, liver, spleen, or blood vessels. This is a rapid and effective means of spreading throughout the host organism; however, once the virus leaves the infected tissue's cells, it is no longer protected from the host immune system as it travels through the host via the blood.

Viruses can also travel via neural spread where it is more difficult for the immune system to control them. Rabies virus is a classic example. An animal sustains a bite from an infected animal. The virus then travels along nerve axons to the spinal cord where it continues to travel to the brain with limited contact with the immune system. The virus is then shed in the saliva of an animal that is becoming increasingly more aggressive and more likely to bite due to the brain damage the virus is inducing.

Examples of viruses that disseminate through the lymphatic tissue are the hepatitis viruses. Note that there are multiple families of viruses that have hepatitis viruses. They all cause liver damage and have a common name despite being from very different families and containing different types of genomes. What these viruses do is infect the Kupffer cells of the liver which are macrophages that are found in the liver. These cells become infected, the infection spreads to the local liver cells, and eventually the virus is excreted from the bile ducts.

Lastly, viruses can disseminate by moving from the initial site of infection to other organs where they replicate to high levels and can then be transmitted to other hosts. There are many examples of this such as poliovirus which begins as gastroenteritis that then moves to the brain leading to paralysis.

VIRAL SHEDDING

Viruses can be shed by contact with the following secretions:

- Saliva
- Semen
- Vaginal secretions
- Skin to skin contact
- Vomitus
- Feces
- Blood
- Ocular secretions
- Mucus
- Respiratory droplets
- Breast milk

EPIDEMIOLOGY OF VIRAL TRANSMISSION

REPRODUCTIVE RATE R0

Each virus has a unique **reproductive rate or R0** (pronounced "R naught"). This is the number of people an infected person is expected to infect. For example, an R0 of 2 means an infected person will infect, on average, 2 other people. Clearly the larger the R0 the more rapidly disease cases will be observed. It is important to note that the R0 may not be the same in all populations, with some being more susceptible than others. Things like the size and density of the population, community vaccination rates, general health or access to medical care can all impact the R0. For example, HIV has an R0 of 2-5 in insertive vaginal sex, 4 in anal sex, and 11 in prostitutes in Kenya. Furthermore, it is not a fixed value, and decreases over time as precautions are taken, vaccines are developed and or administered, or herd immunity is achieved. When R0 falls below 1 the disease will usually begin to reduce in the population to levels that don't allow the outbreak to continue. Take, for example, a scenario in which herd immunity is achieved, and on average, one sick individual infects 0.7 other people. If 100,000 people have the virus, they would go on to infect 70,000 more. Those 70,000 will transmit the disease to 49,000 more people, and so on.

It is important to note that each increase in R0 is exponential. So for example if the R0 of a virus is 1 after an infected individual enters a population and 4 rounds of infection 4 new people will be infected; if the R0 is 2, 31 people will be infected (1→2→4→8→16) total = 31; if the R0 is 3, (1→3→9→27→81) 121 people will be infected and so forth.

Some examples of diseases with their most common R0 values are:
Influenza 1.3-2
Mumps 4-7
Smallpox 5-7
Rubella 6-7
Measles 12-18
COVID-19 1.4-5.7; COVID-19 delta variant 5-8; COVID-19 omicron variant higher than delta still unclear how high

From looking at these numbers you can understand why public health professionals get nervous when measles outbreaks occur as the virus has a very high R0 value and as such spread very quickly (after 4 rounds of infection if the R0 is 18, 111,151 people will become infected). As well as why public health professionals were immediately alarmed when initial predictions of the R0 of SARS-CoV-2 the virus that causes COVID-19 were around 2.2-2.4, and are more concerned now that the delta variant is thought to have an R0 of 5-8!

This R0 value is used to predict the level of infection/vaccination required in a population to achieve herd immunity sufficient to protect unvaccinated individuals from infection. This is referred to the herd immunity threshold percentage. To determine this threshold percentage the equation 1-(1/R0) = herd immunity threshold % is used. So, one can see that the higher the R0 the more people need to be vaccinated or infected to achieve herd immunity.

Virus	R0	Herd immunity threshold percentage
Influenza	1.3-2	24-50
COVID-19	2.2-2.4 (note may be up to 5.7 depending upon model used)	54.5 or higher current estimate 70%
Mumps	4-7	75-86
Smallpox	5-7	80-85
COVID-19 Delta variant	5-8	80-87.5
Rubella	6-7	83-85
Measles	12-18	92-94

SERIES INTERVAL

The **series interval** is the time between onset of symptoms in an infected patient (the infector) and the onset of symptoms in a person the infector infects (the infected). The longer the series interval the more slowly the disease will accumulate to large numbers.

The series interval for some example diseases:
Influenza 3-4 days
Coronaviruses:
- SARS 8.4 days
- MERS 14.6 days
- COVID-19 3.96 days

As one might predict we see far more cases of influenza and COVID-19 which have a fairly short series interval when compared to the coronaviral diseases MERS and SARS which have fairly long series intervals.

PATTERNS OF VIRAL INFECTION

Now that we know how viruses can infect, disseminate, and be shed, let's look at the different patterns of viral infection that can be observed in hosts as demonstrated in figure 4-3.

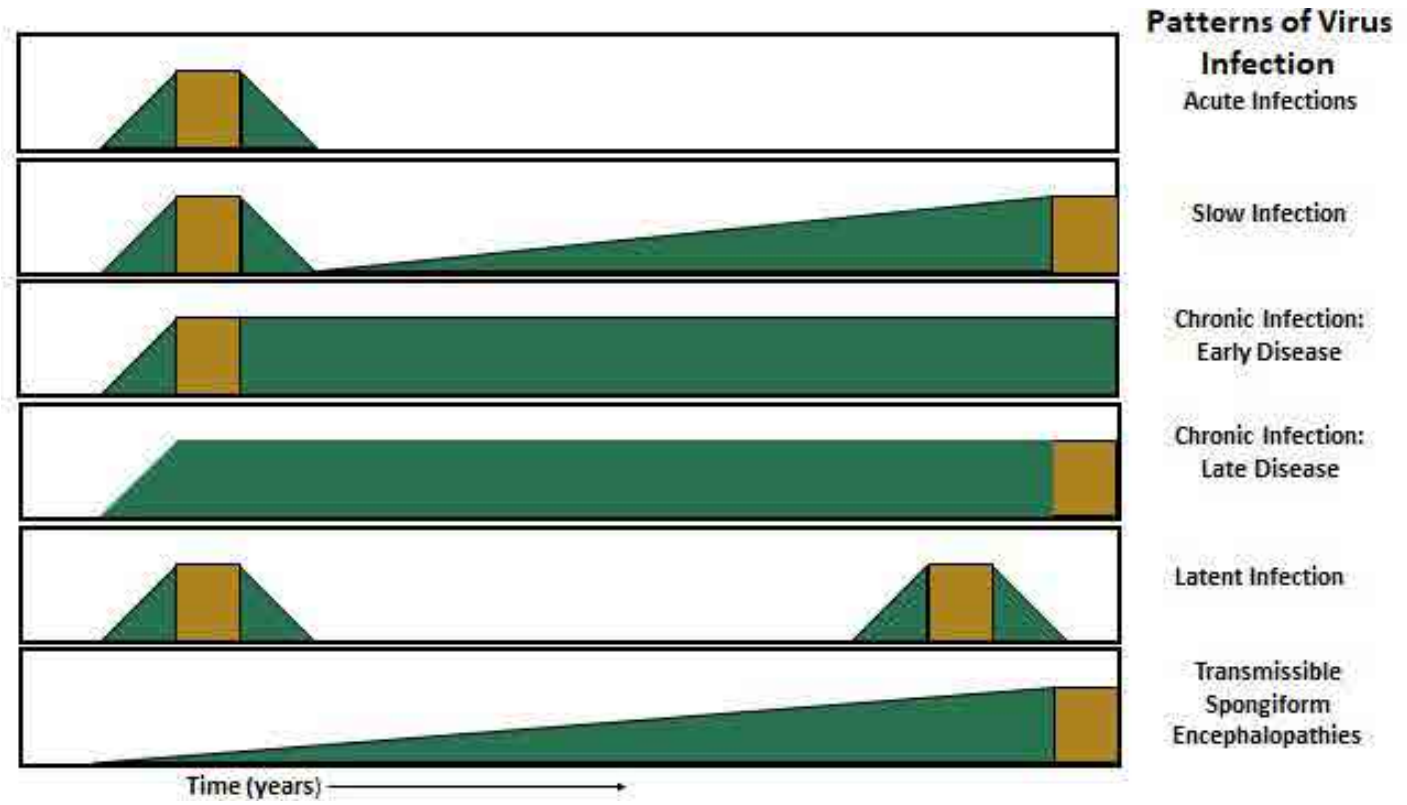


Figure 4-3. Patterns of viral infection. Courtesy of Maxwell Drummond, Colorado State University.

Many common infections are acute in that there is a quick rise in infection with a relatively short symptomatic period followed by a rapid loss of viral infection and symptoms. This acute infection sequence occurs when a virus first infects a susceptible host. Rapid virus replication is followed by rapid onset of symptoms (although many of these are asymptomatic), and then the infection is either resolved by the adaptive immune response or the host progresses to death. The replication can either be localized to the primary infection site, such as we see in rhinoviruses and influenza viruses, or replication can occur at the primary and at secondary sites where the virus has disseminated, such as occurs with the measles virus.

Persistent infections where the primary infection is not cleared efficiently by the adaptive immune response with presence of viral infection in the host for long periods can also occur by multiple mechanisms and result in different patterns of infection. The first is a slow infection. These usually appear to be acute infections, although, after apparent recovery, the virus is slowly replicating to a high level again which may result in symptoms many years later, such as is seen with post-polio syndrome where people who recover from polio develop symptoms again much later in life. The second are chronic infections which have symptoms that will persist from many years to life because the virus continues to replicate within the host. Examples of this are hepatitis B and C infections. The third are latent infections which appear acute and then go away because the virus has stopped replicating but has entered latency, where the virus is present in the host but no longer replicating. The virus, however, can become reactivated later in the life of the host leading to symptoms again. The classic examples of latent infection are the many herpes infections such as herpes simplex 1 or 2 or varicella-zoster (chicken pox). Note that it is important to differentiate between viral latency and clinical latency. Herpesviruses demonstrate viral latency; the viruses stop replicating completely and remain dormant in nervous or lymphoid tissue. They can, however, become reactivated either symptomatically or asymptomatically. Clinical latency is demonstrated by viruses like the retrovirus HIV which

causes initial flu like symptoms but continues to reproduce **asymptomatically** (without symptoms) for many years before a host develops the immunosuppression of AIDS.

It is important to note that the symptoms of viral infections take varying amounts of time to develop. The time between infection and symptoms is called the intrinsic incubation period and can vary greatly depending on the virus, the amount of inoculum, health of the host, and level of susceptibility of the host to infection.

ANIMAL MODELS

Animal models are commonly used to study viral diseases. They allow scientists to recapitulate disease and examine the infectious process, in particular viral kinetics, distribution, pathogenesis, and the host response to infection. It also allows testing of the mechanisms of disease which can be manipulated using experimental alterations. Lastly, it allows testing of interventions such as vaccines and therapeutics. **No animal model is perfect**; however, and some are better than others. Although animal modeling is advancing rapidly, no model can faithfully recapitulate disease processes that occur in humans.

HOST DEFENSE MECHANISMS TO VIRAL INFECTIONS

Now we will explore the rich array of antiviral defenses hosts can deploy to limit replication and spread of viruses involving an innate and adaptive immune response. Viruses must evade these responses long enough to replicate and transmit virus to a new host. To accomplish this, many viruses encode **accessory proteins** which are not necessary to complete a viral replication cycle but that manipulate the host immune response allowing replication to occur.

INTRACELLULAR DEFENSE MECHANISMS

How does an individual cell detect a viral infection? Cells have specific receptor proteins called **pattern recognition receptors** (PRR) which recognize **pathogen-associated molecular patterns** (PAMPs). When a PRR binds to the pattern (PAMP), it recognizes it, and this will result in a cell **signaling transduction cascade pathway** that leads to expression and activation of a number of different antiviral cellular defense systems.

PATTERN RECOGNITION RECEPTORS

There are 3 major classes of pattern recognition receptors (PRR) that recognize viral infections:

1. Toll-like receptors(TLRs)
2. RIG-I like receptors (RLR)
3. Cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS)

In all systems, the binding of the PAMP by the PRR initiates a **signal transduction pathway** that results in activation of **transcription regulatory proteins** (also called **transcription factors**). The transcription regulatory proteins stimulate expression of cytokine genes (interferons and pro-inflammatory cytokines).

Toll-Like Receptor Pattern Recognition Receptors

Figure 4-4 shows the 4 t**oll-like receptors** (TLRs) that respond to viral patterns and the corresponding ligands they respond to. You will note that each receptor has a specific ligand which responds to and activates a different signal transduction cascade pathway. All, however, will ultimately lead to changes in transcription factors binding to genes to induce transcription of cytokines and type I interferons. The transcription factors NFκB and **interferon regulatory factors** (IRFs) are often the transcription factors that activate transcription of the cytokines and type I interferon by binding to sequences on interferon genes that lead to activation of transcription of interferons and other cytokines. Note there are 10 TLRs, but only these 4 respond to patterns (PAMPs) associated with viruses.

The PAMP for each of the TLRs that respond to viruses are as follows:

- TLR3 responds to double-stranded (ds)RNA found within an endosome
- TLR7 & TLR8 respond to single-stranded (ss)RNA found inside an endosome
- TLR9 responds to unmethylated CpG DNA found within an endosome

The figures describing signal transduction cascade pathways of pattern recognition receptors activated by binding their viral pathogen associated molecular patterns (Figures 4-4 to 4-6) and their ultimate activation of the interferon production look daunting at first. The important thing is to observe that they all will activate a signal transduction cascade pathway that will induce transcription of cytokines and interferons by transcription factors like NFκB or IRFs.

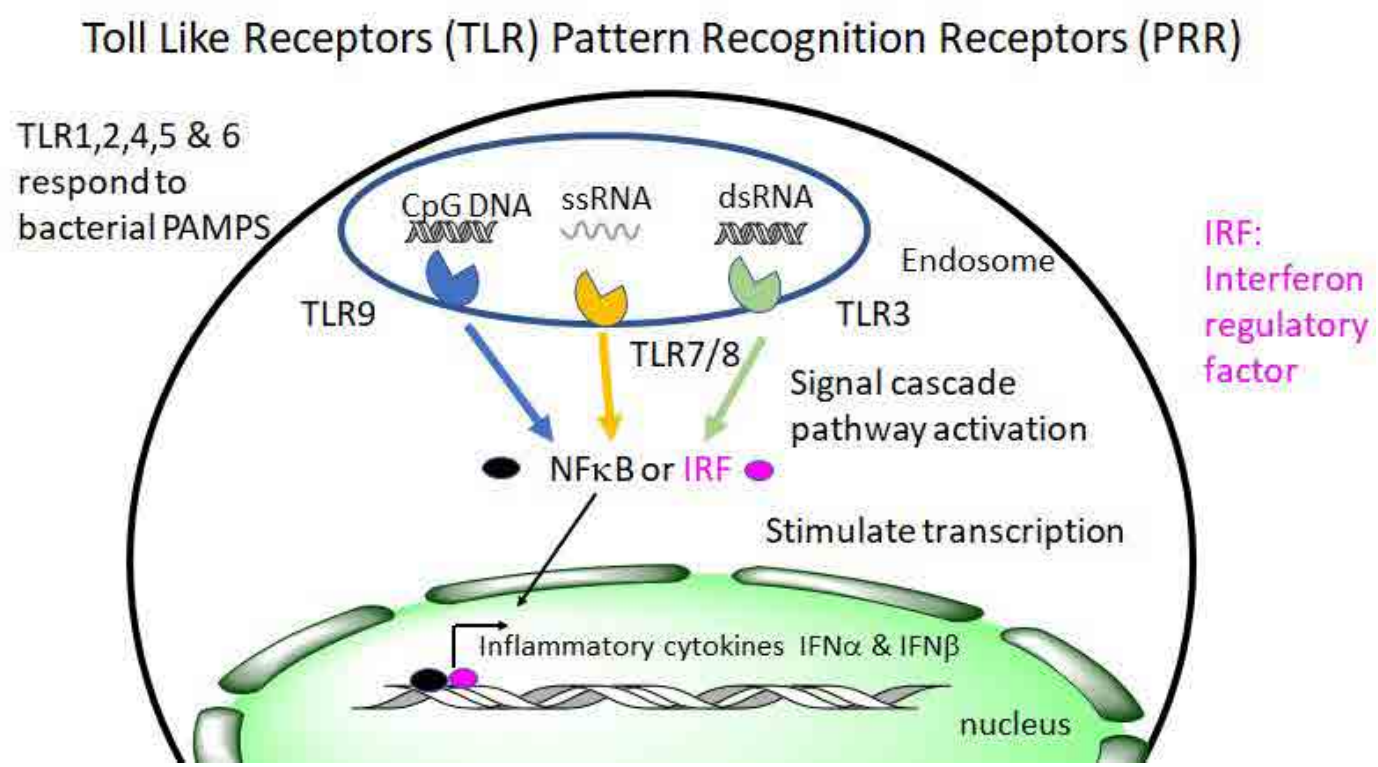


Figure 4-4. The signal transduction cascade pathway activated by the toll-like receptor PRR binding endosomal PAMPs. Courtesy of Erica Suchman, Colorado State University.

You will note that all 4 recognize viral patterns within an endosome. Remember that many viruses enter cells utilizing endosomes. Cellular RNA and DNA will rarely be found within endosomes, hence targeting sequences found within the endosomes increases the likelihood of targeting viruses without being triggered by host cell nucleic acids. CpG stands for C-phosphate-G which is observed when G follows C in a DNA strand. Note the CpG PAMP of TLR9 is rarely unmethylated in eukaryotic cells and not methylated in viral DNA and hence makes a good target. You will also remember that some viruses have evolved mechanisms to avoid using endosomes to enter the cell (such as enveloped viruses that fuse with the host cell membrane, or viruses that cause pores to form within the plasma membrane), but don't fear, cells have ways to detect viral nucleic acids outside of endosomes as well. You will see that there are far more mechanisms for dealing with RNA viruses than DNA viruses. This makes sense as DNA viruses appear so similar to host cell nucleic acids and are therefore more difficult to specifically target.

RIG-I like Receptors (RLR)

Figure 4-5 shows how **RIG-I Like receptors** (RLR) respond to RNA virus PAMPs. Again, RIG-I and MDA-5 recognize different PAMPs and activate signal transduction cascade pathways that induce an antiviral state by inducing secretion of interferon α and β which will lead to transcription activation of **interferon stimulated genes** (ISGs) in other cells.

The cytosolic RNA PAMP recognized by each RIG like receptor are as follows:

- RIG-I responds to single stranded RNA in the cytoplasm that lacks a 5' cap (some viral mRNA lacks a 5' cap, and the genome of negative sense RNA viruses will not have a cap) as identified by triphosphate on the 5' end.
- MDA-5 responds to dsRNA and mRNA that has a 5' cap that is not properly methylated.

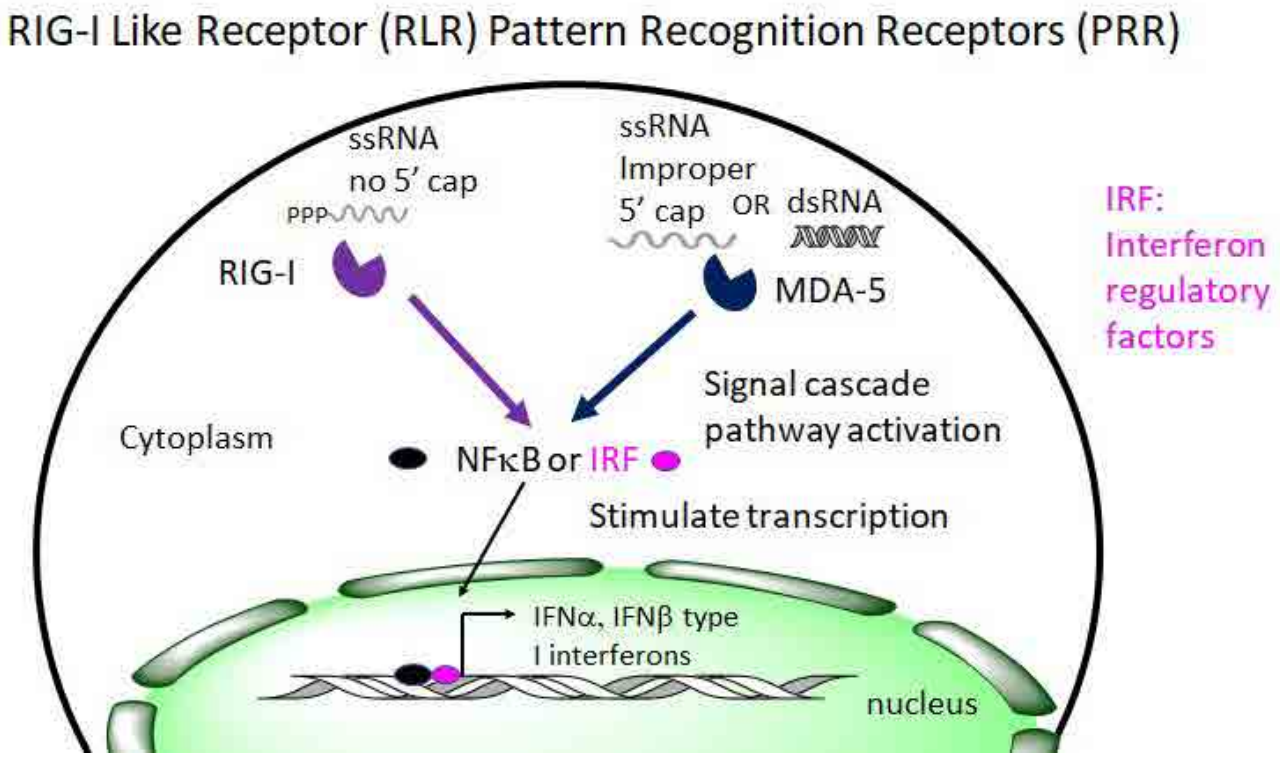


Figure 4-5. The signal transduction cascade pathway activated by the RIG-I like PRRs RIG-1 and MDA-5 binding cytosolic RNA PAMPs. Courtesy of Erica Suchman, Colorado State University.

Cyclic GMP-AMP synthase (cGAS) pattern recognition receptor

cGAS recognizes cytoplasmic DNA as its PAMP because eukaryotic cells do not have DNA in their cytoplasm as shown in Figure 4-6. Binding of cytoplasmic DNA to cGAS PRR causes the activation of a signal transduction cascade pathway and induces **NFκB,** the transcription factor, to activate **type I interferon** production (INF α and β) which will lead to transcription activation of interferon stimulated genes (ISGs) in other cells.

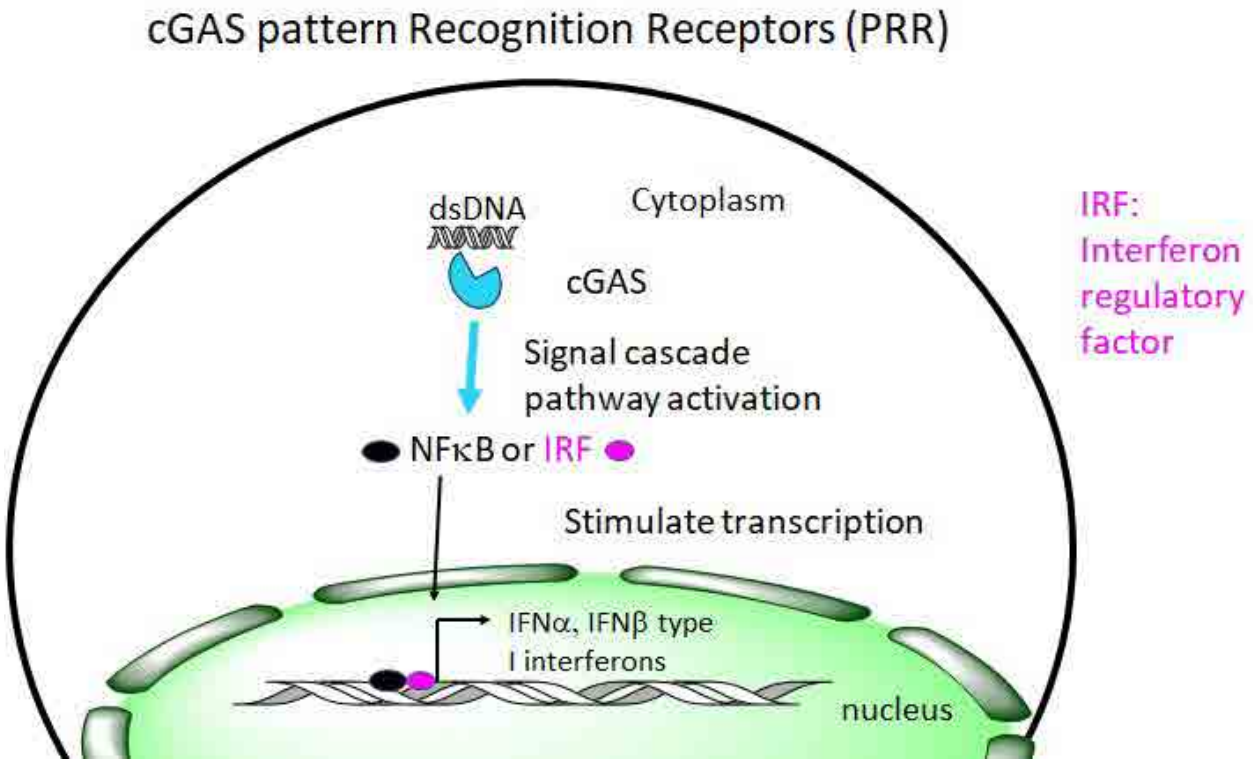


Figure 4-6. The signal transduction cascade pathway activated by the PRR cGAS recognizing the PAMP cytosolic DNA. Courtesy of Erica Suchman, Colorado State University.

CELLULAR CHANGES THAT OCCUR FOLLOWING VIRAL INFECTION

INTERFERONS

Type I interferons are the first line of defense prior to induction of adaptive immunity and lead to an antiviral state. As we saw earlier, PRRs initiate signal transduction cascade pathways that activate the synthesis of type I interferons (INF α, ω [omega] and β). **The type II interferon (IFNγ) receptor** is also constitutively expressed on the surface of all nucleated cells, and its binding by IFNγ leads to an antiviral state in those cells, like the type I interferons. Type I interferons can be produced by any virally infected cell; however, type II interferon (FNγ) is principally expressed by T cells and natural killer cells.

The job of type I interferons is to induce an antiviral state in non-infected cells after interferons bind to **interferon receptors** (**INFR)**. There are receptors that bind specifically to type I and type II interferons, but both will lead to transcriptional activation of genes. As is shown in Figure 4-7, uninfected cells bind type I and II interferon (INF) with IFN receptors (IFNR) which activate the JAK/STAT signal transduction cascade pathway. JAK1 phosphorylates multiple copies of STATs 1 and 2, which then dimerize in the cytoplasm, enter the nucleus and bind to **interferon stimulated response elements** (**ISRE)** (sequences of DNA) that are found in the promoter regions of many genes. This binding leads to expression of over 200 proteins called **interferon stimulated genes** (**ISGs**) involved in creating an antiviral state within the cell, as shown in Figures 4-7 & 4-8. You will notice in this figure that viruses have developed a wide range of mechanisms to disrupt the JAK/STAT pathway.

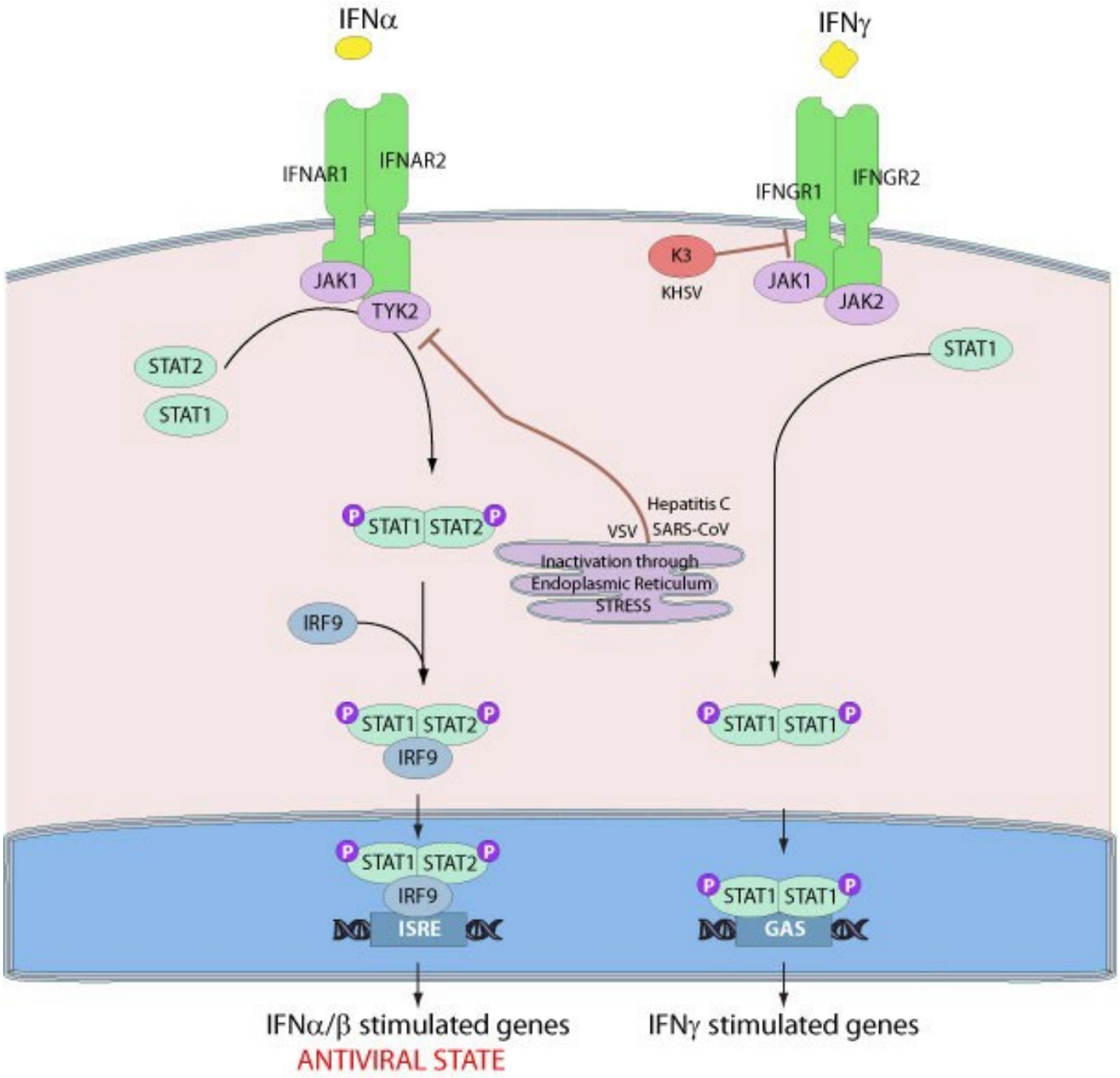


Figure 4-7. Uninfected cells bind type I and II interferon (INF) with INFR which activates the JAK/STAT signal transduction cascade pathway. JAK1 phosphorylates STATs, which dimerize in the cytoplasm (pink), enters the nucleus (blue) and binds to ISRE. This binding leads to expression of over 200 proteins (ISG) from ISRE involved in creating an antiviral state within the cell. Red circles indicate viral accessory proteins that interfere with the JAK/STAT pathways. Courtesy of ViralZone. https://viralzone.expasy.org/883

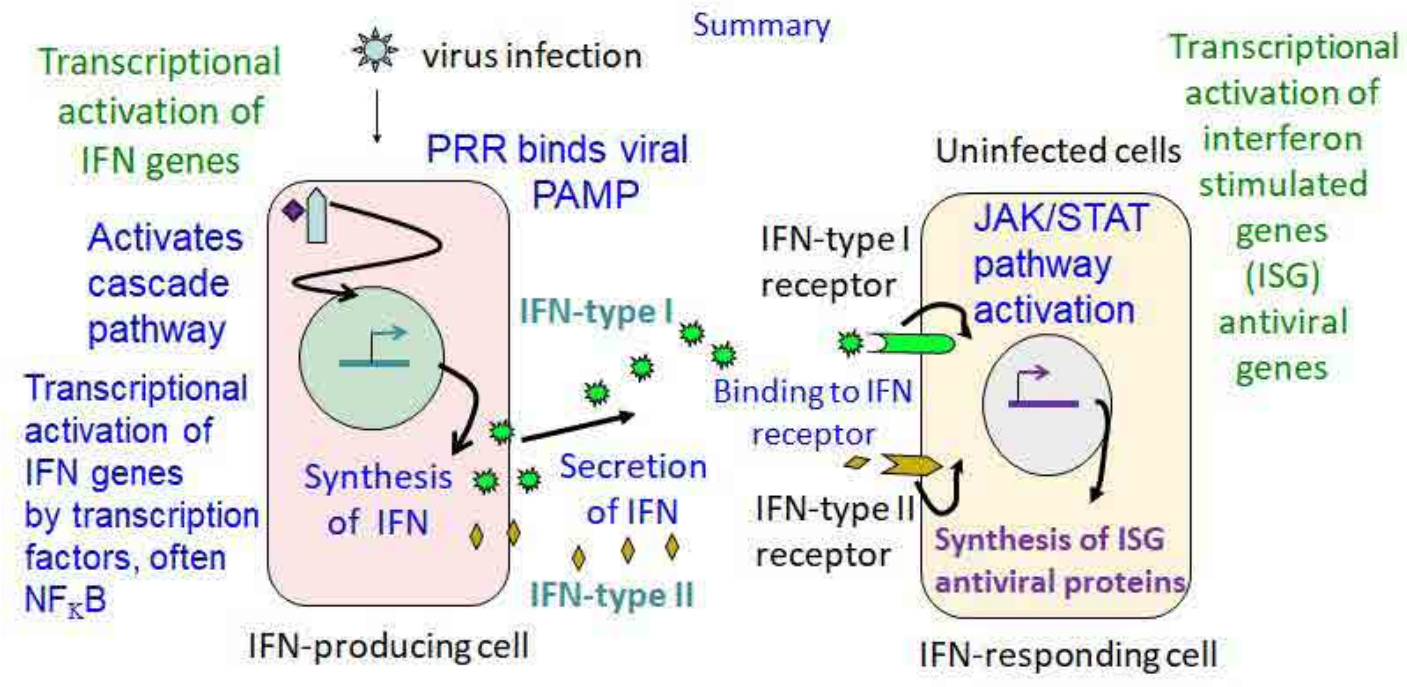


Figure 4-8. Virally infected cells produce type I (α & β) and II interferons (γ) in response to PRR binding viral PAMPs (via signal transduction cascade pathways and transcription activation of IFN genes by transcription factors, usually NFκB). Type I interferons activate antiviral states in uninfected cells after binding to interferon receptors (INFR) by binding the Interferon Stimulated Response Element (ISRE) to induce expression of ~200 interferon stimulated genes (ISG). Courtesy of Sandra Quackenbush, Colorado State University.

TYPE I IFN-INDUCED ANTIVIRAL MECHANISM

As discussed above, transcriptional activation of over 200 antiviral proteins from interferon stimulated genes are activated by the JAK/STAT pathway following the binding of type I interferons to IFN I receptors. We will describe a few of the most important **interferon stimulated genes (ISG)** here, as shown in Figures 4-9 A & B.

Protein Kinase R (**PKR**) is activated by dsRNA in the cytoplasm. In response to binding dsRNA, PKR phosphorylates the translation initiation factor eIF2α which inhibits translation of all proteins, both cellular and viral. Note, the cell is willing to die to rid the host of viral infection.

Oligoadenylate synthetase (**OAS)** is activated by dsRNA in the cytoplasm. Binding of dsRNA causes OAS to synthesize adenylic acid which activates **RNaseL,** and RNaseL degrades both cellular and viral mRNA, again leading to cell death.

Mx is activated by viral **ribonucleoprotein** (RNP, helical viruses with their nucleocapsid also called ribonucleocapsid) which inhibits the replication of RNA viruses in both the cytoplasm and nucleus. Note: orthomyxoviruses have ribonucleoproteins and as such are inhibited by Mx as they replicate in the nucleus. The majority of animal viruses that Mx inhibits will replicate in the cytoplasm.

Viperin/Teherin are thought to recognize changes in the plasma membrane associated with accumulation of envelope proteins membranes of enveloped virus-infected cells and will inhibit budding from either the plasma

membrane or organelles.

These are only a few of the antiviral proteins that are transcribed in response to type I interferons. As you can see, cells have a plethora of ways to inhibit viruses, and, as you can imagine, viruses have a plethora of ways of countering these defenses.

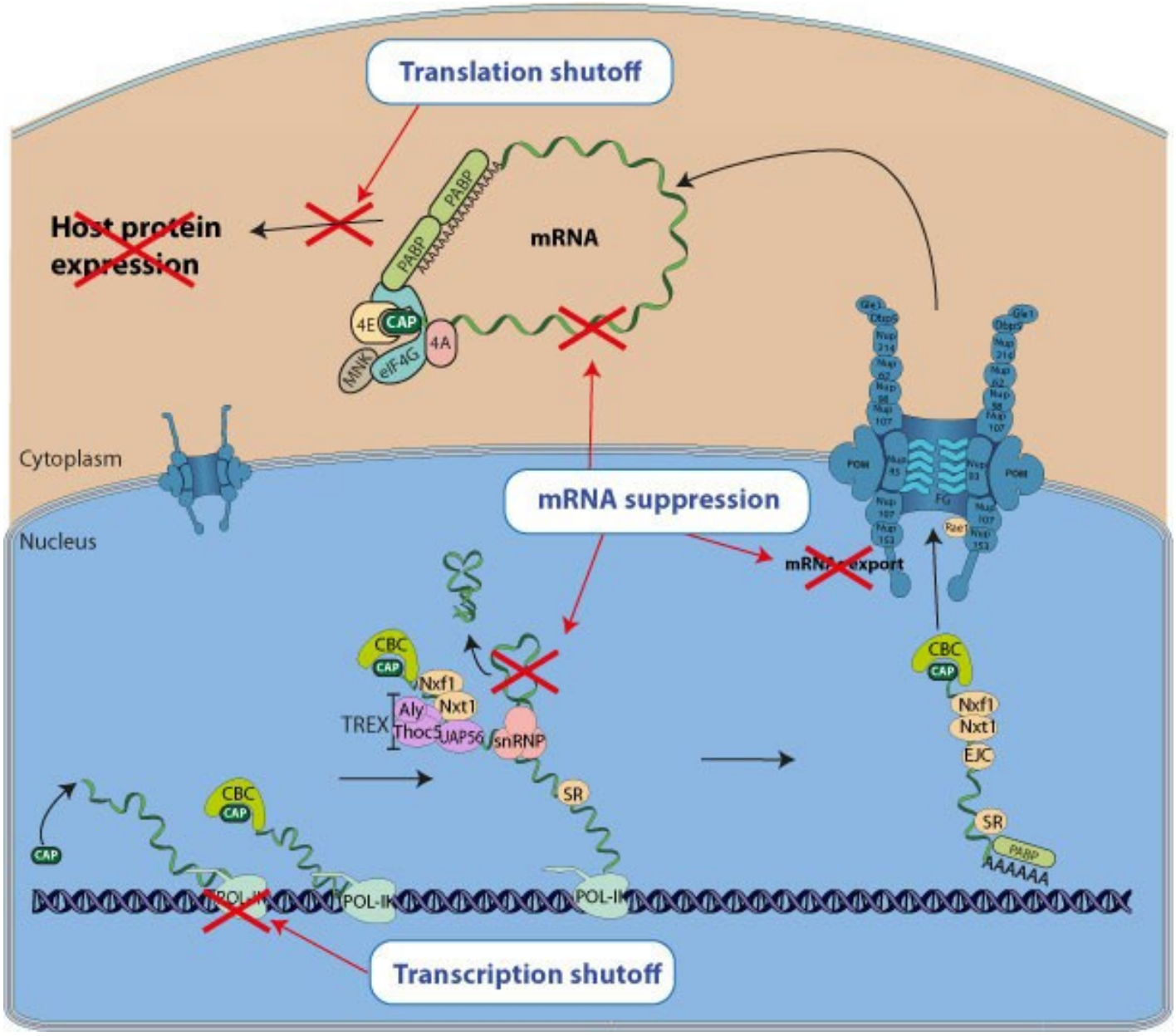


Figure 4-9A. Cell shut off mechanisms induced by type I interferons used to reduce viral replication including transcription shutoff, mRNA suppression, and translation shutoff. Note, inhibition of budding is not shown. Courtesy of ViralZone https://viralzone.expasy.org/1582

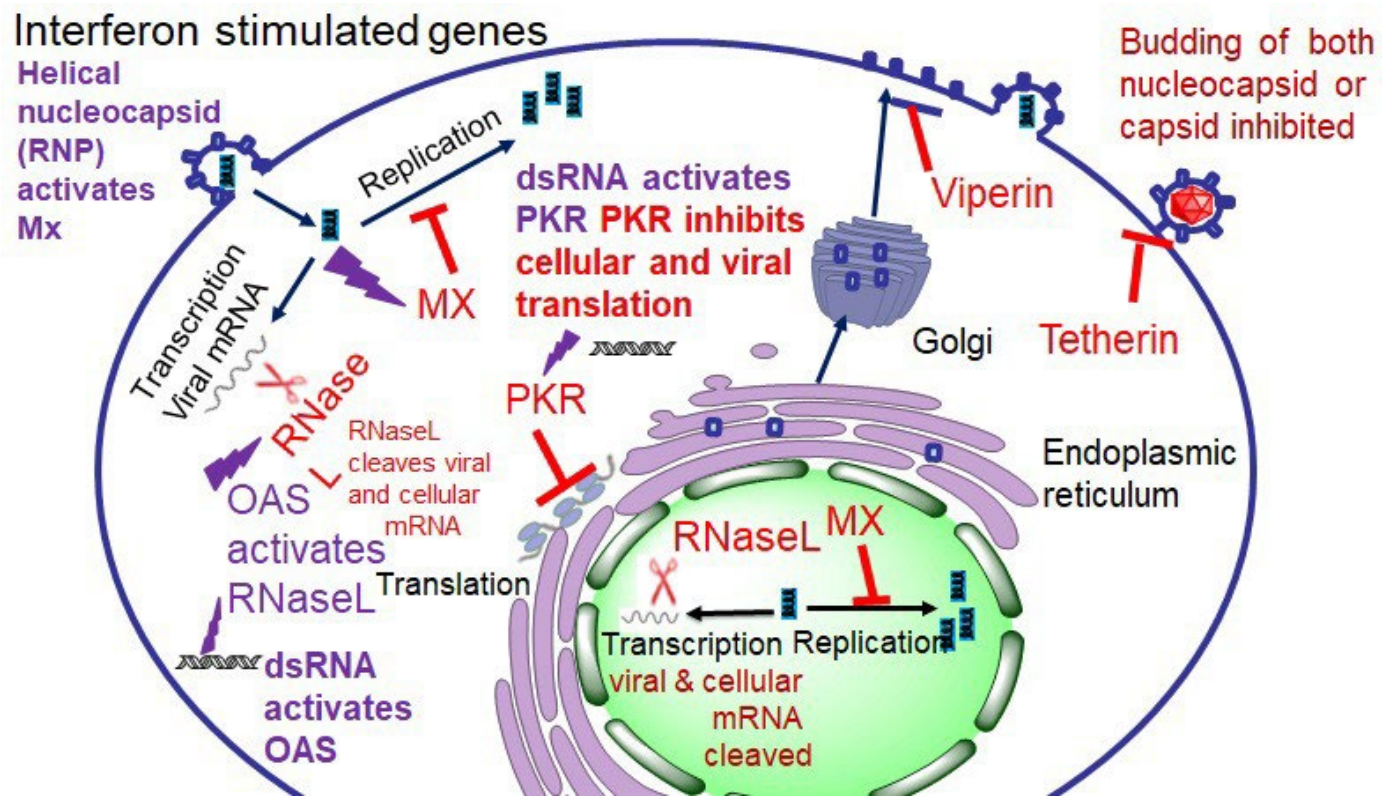


Figure 4-9B. Important antiviral proteins induced by type I Interferons and how they contribute to creating an antiviral state in infected cells via inhibition of transcription, translation, mRNA stability, or budding . Courtesy of Erica Suchman, Colorado State University.

Now that we know how cells defend themselves from viral infection, let's look at how viruses counter these defenses.

VIRAL STRATEGIES FOR COUNTERACTING PATTERN RECEPTOR RECOGNITION AND INTERFERON RESPONSE

The following mechanisms are utilized by viruses to evade PRR induced interferon responses. You will see example viruses disrupting these pathways in the figures indicated.

- Disruption of pattern recognition receptor (PRR) signal transduction cascade pathway signaling.
- Inhibition of transcription factor activation. Figure 4-10
- Inhibition of interferon signaling via the JAK/STAT pathway. Figure 4-7
- Inhibition of interferon stimulated gene function. Figure 4-11

Many viruses have developed mechanisms to inhibit the signal transduction cascade pathways of the pattern recognition receptors (PRR). Many viruses have accessory proteins. Note that **accessory proteins** are not required for viral replication but are used to control antiviral responses.

The signal transduction cascade pathways activated by both PRR binding of their respective PAMPs and type I interferon binding to interferon receptor proteins lead to transcription factor activation that binds to specific promoters. These transcription factors are common targets of antiviral accessory proteins as shown in Figure 4-10.

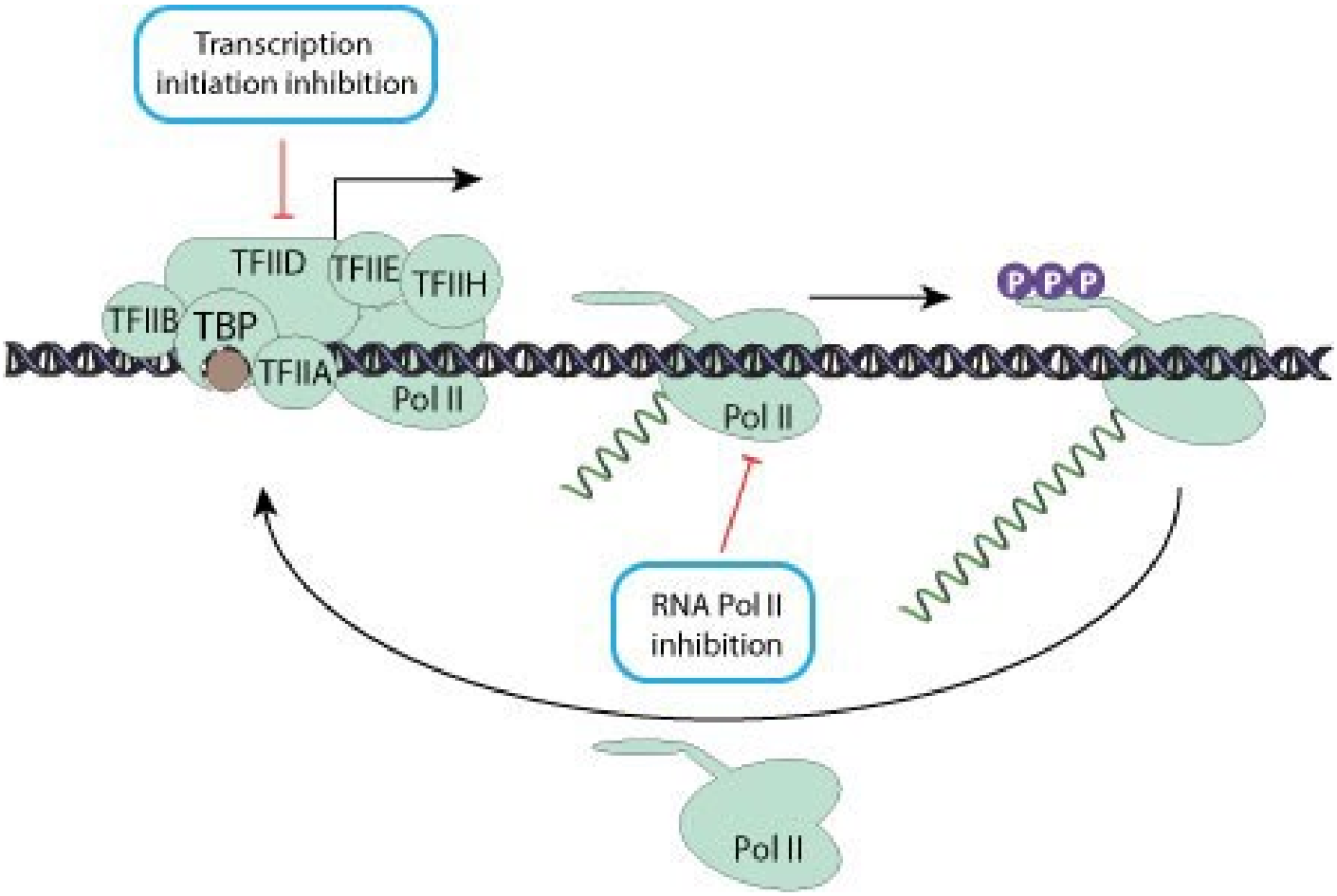


Figure 4-10. Transcription activation requires transcription factors that allow the RNA polymerase II, a DdRp, to begin transcription. Transcription factors are common targets of viral accessory proteins produced to evade the cell's antiviral response. Courtesy of ViralZone https://viralzone.expasy.org/1577

When interferon binds to its interferon receptor, it activates the JAK/STAT pathway that leads to the activation of interferon stimulated genes (ISG). The JAK and STAT proteins are common targets of viral accessory proteins as shown in Figure 4-8.

The interferon stimulated gene products are also common targets of viral accessory proteins. Figure 4-11 shows all of the viral accessory proteins that work against PKR, one of the major antiviral proteins created when cells are stimulated by type I interferons. Remember that there are over 200 ISG products, and each of them would probably have a diagram that looks similar to this!

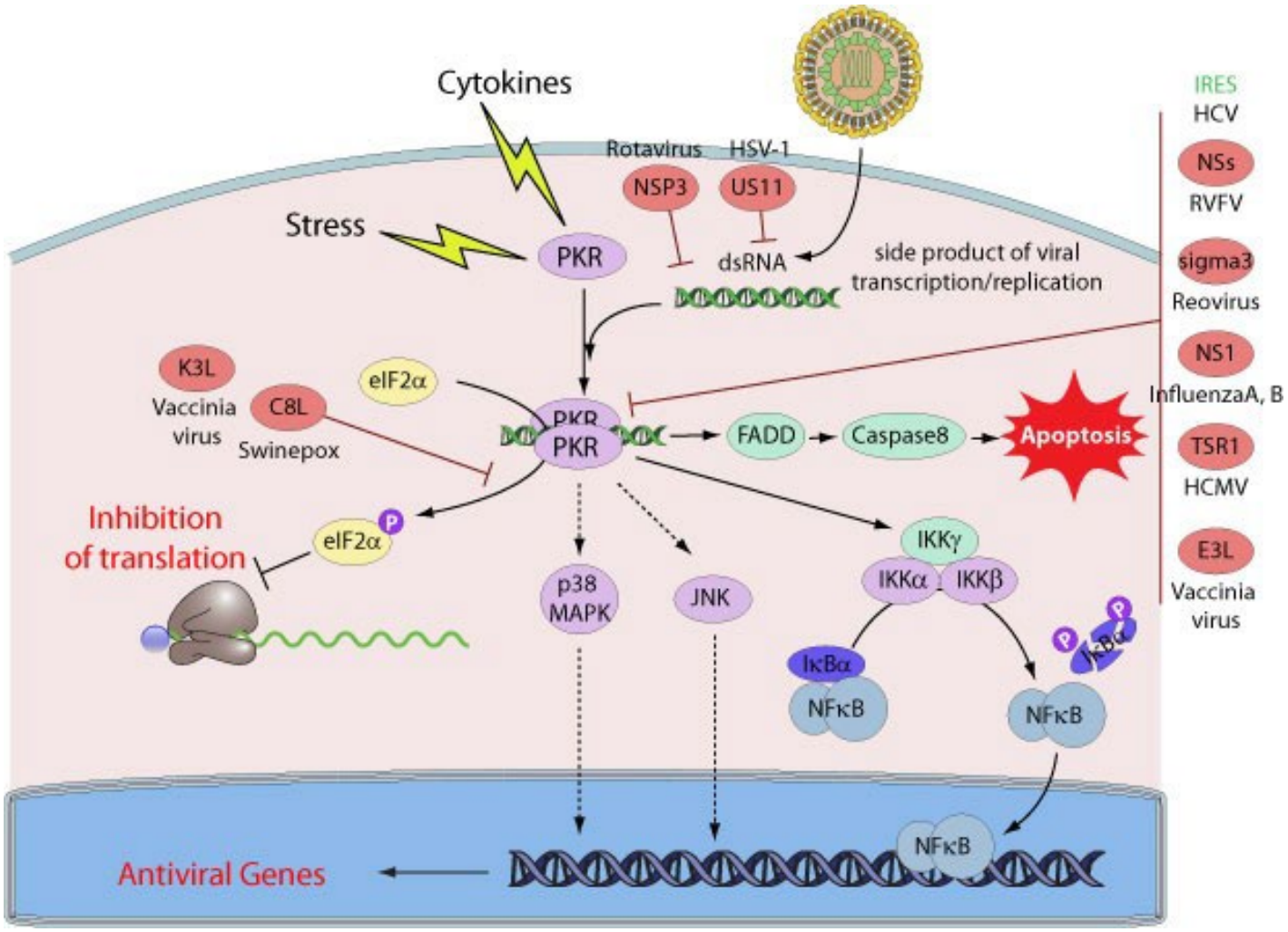


Figure 4-11. Many antiviral responses are regulated by interfering with interferon induced antiviral proteins such as PKR as shown here. Note similar responses are mounted against the other (~200) interferon induced antiviral proteins described. PKR serves as an example. Courtesy of ViralZone https://viralzone.expasy.org/554

APOPTOSIS

Apoptosis is programmed cell death. It occurs naturally in cells as they become old, is mediated by the mitochondria, and regulated by the tumor suppressor gene **p53**. The process begins with DNA fragmentation and chromatin condensing followed by fragmentation of the nucleus and, lastly, fragmentation of the cell. The cell fragments are engulfed by macrophages. Cells can induce apoptosis to rid a viral infection. This is not great for that cell but is an altruistic act that saves other cells from becoming infected and ultimately may save the host, because if the cell dies the virus can no longer propagate and infect other cells. Apoptosis is triggered by biochemical changes in the cell induced by the virus infection, in particular, increased production of the tumor suppressor protein **p53** and the production of the pro-inflammatory cytokine **tumor necrosis factor** (**TNF**), as well as stimulation by FasL, perforin, or granzyme B which are released by **cytotoxic T-lymphocytes** (**CTLs)**. All of these can result in induction of apoptosis. TNFα, FasL, granzyme B, and perforin do so by inducing an **extrinsic** signal transduction cascade pathway involving a series of **caspase enzymes**. Note there are also intrinsic non-caspase dependent pathways that are activated by the mitochondria due to stress and old age.

p53 is a transcription factor that up-regulates many proapoptotic genes during the intrinsic apoptosis pathway. Not surprisingly, viruses have developed many different mechanisms to inhibit the extrinsic caspase signal transduction cascade pathway and the intrinsic pathway to inhibit apoptosis thus allowing the cells to live while continuing to replicate the virus. Some viruses have developed mechanisms to activate apoptosis by activating the caspase activation pathway, as was shown in Figure 4-12. For example, parvoviruses are known to activate the caspase signal transduction cascade leading to apoptosis. Inducing apoptosis is thought to help the virus leave the nucleus and cell. Note that cells killed by apoptosis induce less inflammation than cells killed by lysis, however, most work focuses on viral inhibition of apoptosis because induction of apoptosis is the predominant effect of viral replication on infected cells. Note, although not shown on this diagram, many viruses produce proteins to inhibit the function of p53 to inhibit apoptosis.

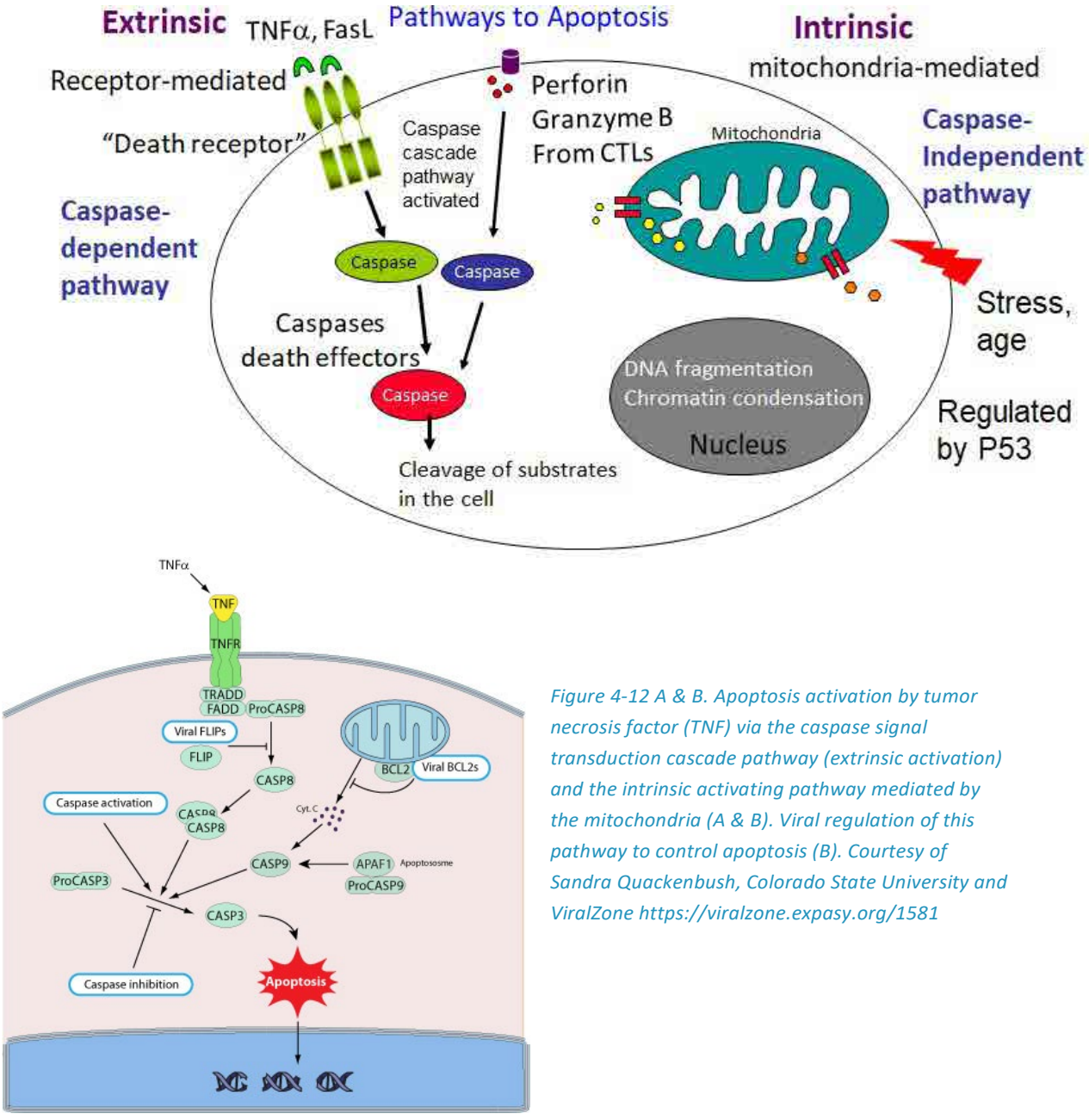


Figure 4-12 A & B. Apoptosis activation by tumor necrosis factor (TNF) via the caspase signal transduction cascade pathway (extrinsic activation) and the intrinsic activating pathway mediated by the mitochondria (A & B). Viral regulation of this pathway to control apoptosis (B). Courtesy of Sandra Quackenbush, Colorado State University and ViralZone https://viralzone.expasy.org/1581

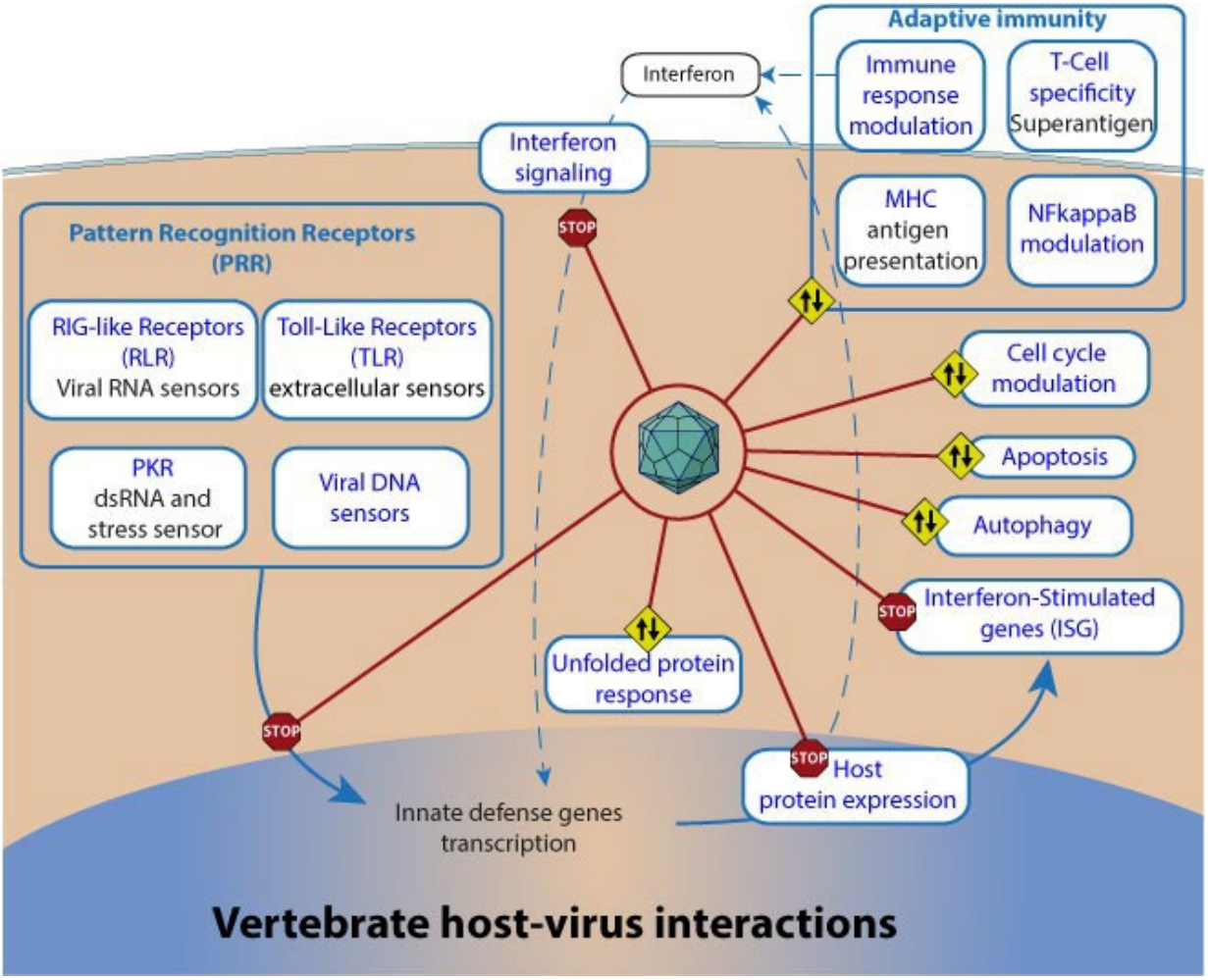


Figure 4-13. Summary of host cell-virus interactions. Note in some cases the virus is always inhibitory, such as inhibition of host protein expression, and in others the virus can turn up or down a cellular response to fit its needs. Courtesy of ViralZone https://viralzone.expasy.org/886

Now that we have an understanding of the cellular defenses a virus must overcome; it is time to look at the host's immune response to viral infection. We will begin with the first line of defense, the innate immune response. Recall that the **innate immune response** is non-specific and does not require activation like the adaptive immune response does. As such it responds while the **adaptive immune response** is being activated.

INNATE IMMUNE RESPONSE: NATURAL KILLER (NK) CELLS

Natural Killer cells (**NK**) are part of the innate immune response and are the first line of the immune response against viral infections. Natural killer cells stimulated by type I interferons (IFN α & β) released from infected cells causes them to release Type II interferons (IFNγ). IFNγ stimulates macrophage to produce IL-12 which stimulates the NK cells to become activated such that they can kill virally infected cells in the absence of antigen presentation and recognition. As shown in Figure 4-14, NK cells will recognize ligands that are expressed when cells are under stress or pathogen infected (**stress ligands**). These ligands, however, are often also expressed in normal cells. This leads to a dilemma. How do NK cells know which cells to kill and which to leave alone? Fortunately, viruses

often leave NK cells a clue. Viruses, when they infect cells, down-regulate MHC class I to avoid detection by $CD8^+$ **cytotoxic T lymphocytes** (CTLs), which we will talk about in the next section. So, NK cells bind to the stress ligand, and they have a second receptor that must bind to MHC class I concurrently to **stop** the NK cell from releasing cytotoxic granules to kill the cell as shown in Figure 4-15A. Conversely, NK cells need a positive stimulation from the stress ligand binding receptor and a negative response from the MHC class I binding receptor (failure to bind) to induce apoptosis killing the cell as shown in Figure 4-15 B. Therefore, the virus's downregulation of MHC class I serves as a signal to the NK cells that the cell is virally infected allowing the NK cell to induce apoptosis to kill the cell. Additionally, if the cell is getting very stressed, it can overexpress the stress ligand, and the increased stimulation can induce apoptosis and killing even when the MHC class I molecule is present, Figure 4-15C. As such, it is the ratio of the MHC class I molecules to the stress ligands that determines if the cell will be killed with higher amounts of stress ligand than MHC class I favoring cell killing.

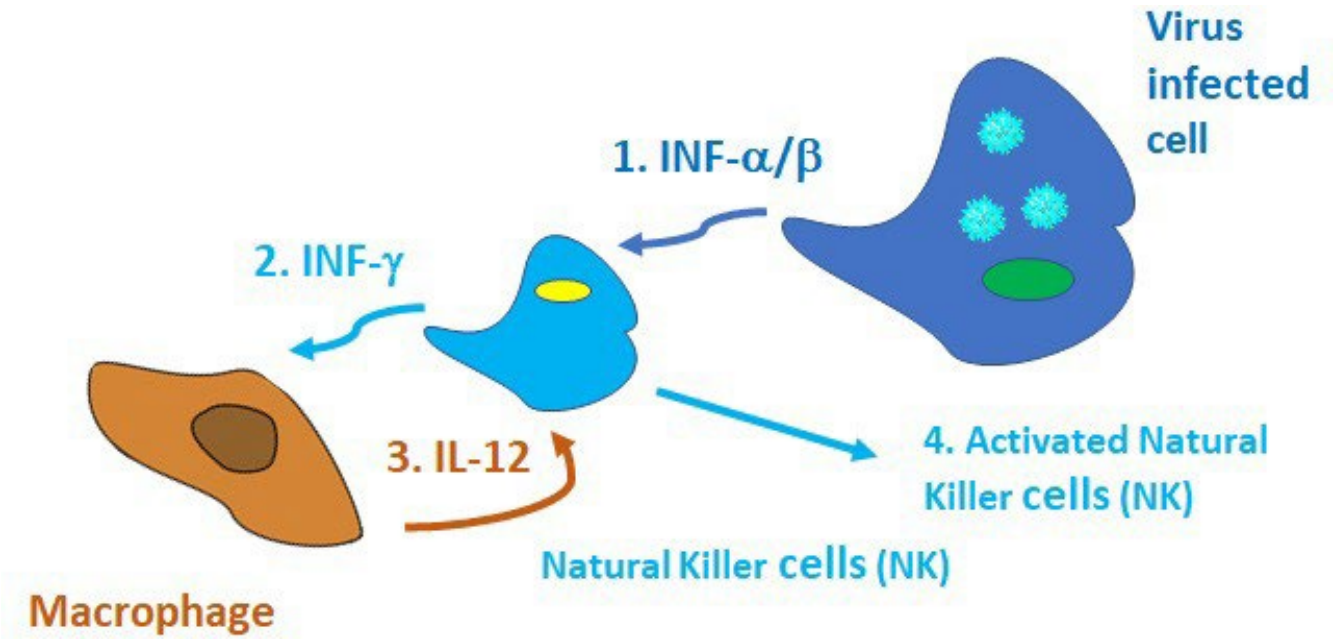


*Figure 4-14 . Natural killer cell stimulated by type I interferons released from infected cells causes them to release type II interferon (*IFNγ*) that stimulate macrophage to produce IL-12 which stimulates the NK cells to become activated such that they can kill virally infected cells. Courtesy of Erica Suchman, Colorado State University.*

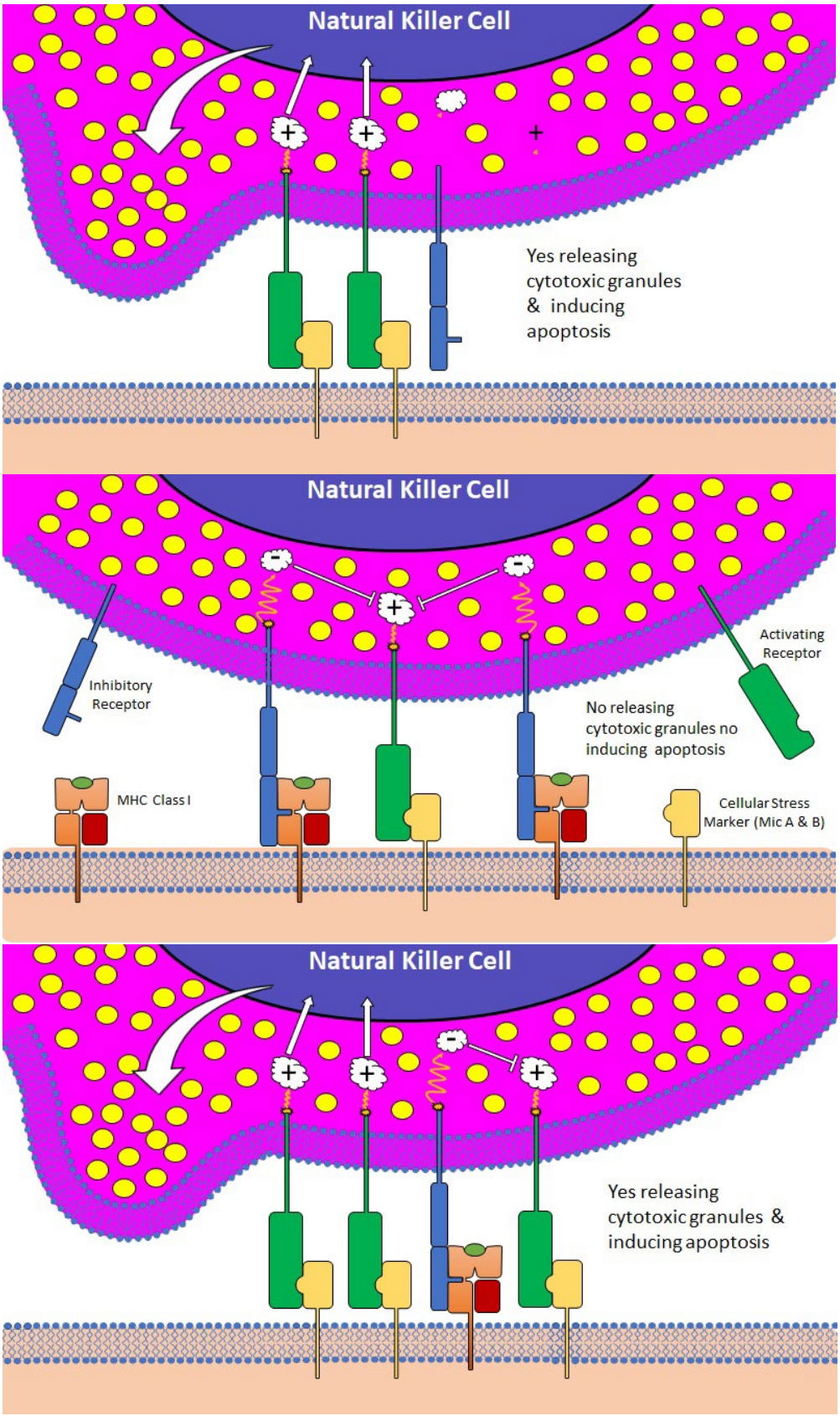


Figure 4-15. NK cells need a positive stimulation from the stress ligand binding receptor and a negative response from the MHC class I binding receptor to induce apoptosis leading to cell death (A). If the MHC class I is present, binding to the MHC class I receptor will trigger a negative response and inhibit release of cytotoxic granulocytes and cell killing (B). If too many stress ligands are present, NK cells will still release granulocytes and induce apoptosis killing infected cells even if MHC class I is present (C). Courtesy of Maxwell Drummond, Colorado State University.

HOW VIRUSES COUNTER NK CELL-MEDIATED EFFECTS

Remember that virally infected cells downregulate MHC class I to avoid detection and destruction by $CD8^+$ CTLs. This loss of MHC class I serves as a signal to NK cells that the cell is virally infected and targets it for destruction. So, what can a virus do to fool the NK cell that it is not infected?

- Produce a protein with homology to MHC class I that is expressed on the surface of infected cells that will bind to the MHC class I receptor of NK cells and fool the NK cell that the cell is not infected.
- Upregulate expression of non-classical MHC I (HLA-E) that will bind to the MHC I receptor of NK cells and fool the NK cell that the cell is not infected.
- Produce viral proteins that can block the activating ligand binding receptor.
- Infect the NK cell itself and kill it.

ADAPTIVE IMMUNE RESPONSE: T AND B LYMPHOCYTES

CELL MEDIATED IMMUNITY, THE T LYMPHOCYTES

Unlike the natural killer cells of innate immunity, adaptive immunity requires presentation by viral antigens to respond. Three major cell types play a role in controlling viral infection, $CD4^+$ T-helper (Th) cells, $CD8^+$ cytotoxic T cells (CTL), and B-cells. Each of these cells have a receptor protein specific to an **epitope** of an antigen found on an infectious agent. These receptors are generated during the cell's development, and the ones that will respond to a current infection must be selected from the millions of lymphocytes that reside within the lymphatic system. The process of selecting the ones that will respond is called **clonal selection** and this selection is driven by the pathogen. For a review of this topic any immunology textbook will be helpful, but here is a quick overview. CTLs are activated by any infected cell (except red blood cells) that presents short viral peptides (~8-10 amino acids) on MHC class I proteins. Th cells are activated by **professional antigen presenting cells** (e.g., dendritic cells and macrophages) that have engulfed viral particles, degraded their proteins, and expressed these peptides on their surface in conjunction with MHC class II proteins. Figure 4-16 shows these interactions. Note that $CD8^+$ CLTs also require activation "help" by T-helper cells (that have been activated by APCs) activated by antigen presented by MHC II. Recall from immunology that some of these activated T-cells will respond now, and some will become memory cells that will be ready to respond to any future infections with this virus.

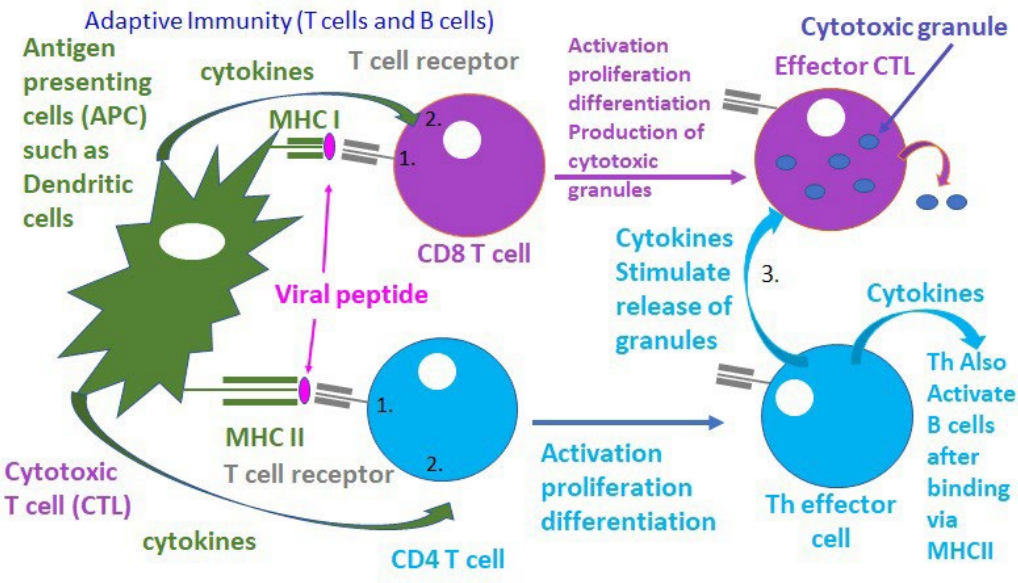


Figure 4-16. Antigen presenting cells activate $CD4^+$ T-helper cells by presenting antigen peptides in conjunction with MHC class II. Antigen presenting cells activate $CD8^+$ CTLs by presenting antigen peptides in conjunction with MHC I. Note that $CD8^+$ CLTs also require activation by T-helper cells. Courtesy of Erica Suchman, Colorado State University.

Antigen presentation by MHC class I occurs when viral proteins made on cytoplasmic ribosomes are digested into peptides by a tubular molecule called the **proteosome**, then translocated into the lumen of the endoplasmic reticulum by the **transporter associated with antigen processing** (TAP), where they are loaded onto MHC class I. These antigen peptides/MHC class I molecule complexes will be transported to the cell surface where they can be recognized by activated CTLs that express a **T-cell receptor** (TCR) specific for the epitope being expressed. This process is shown in Figure 4-17. When a CTL recognizes an infected cell, **it kills it**, thus depriving the virus of its ability to replicate. Note as shown on this diagram, viruses have multiple mechanisms for downregulating MHC class I in order to avoid detection by $CD8^+$ CTLs. These MHC class I downregulation methods include inhibition of:

- The proteases that process the viral antigens.
- TAP-mediated transport of the peptides to MHC class I.
- Transport of MHC class I/antigen complex to the plasma membrane.
- Degradation of MHC class I molecules.

For a great review on T-cells and coronavirus infections please listen to This week in virology, TWiV #736
https://www.microbe.tv/twiv/twiv-736/

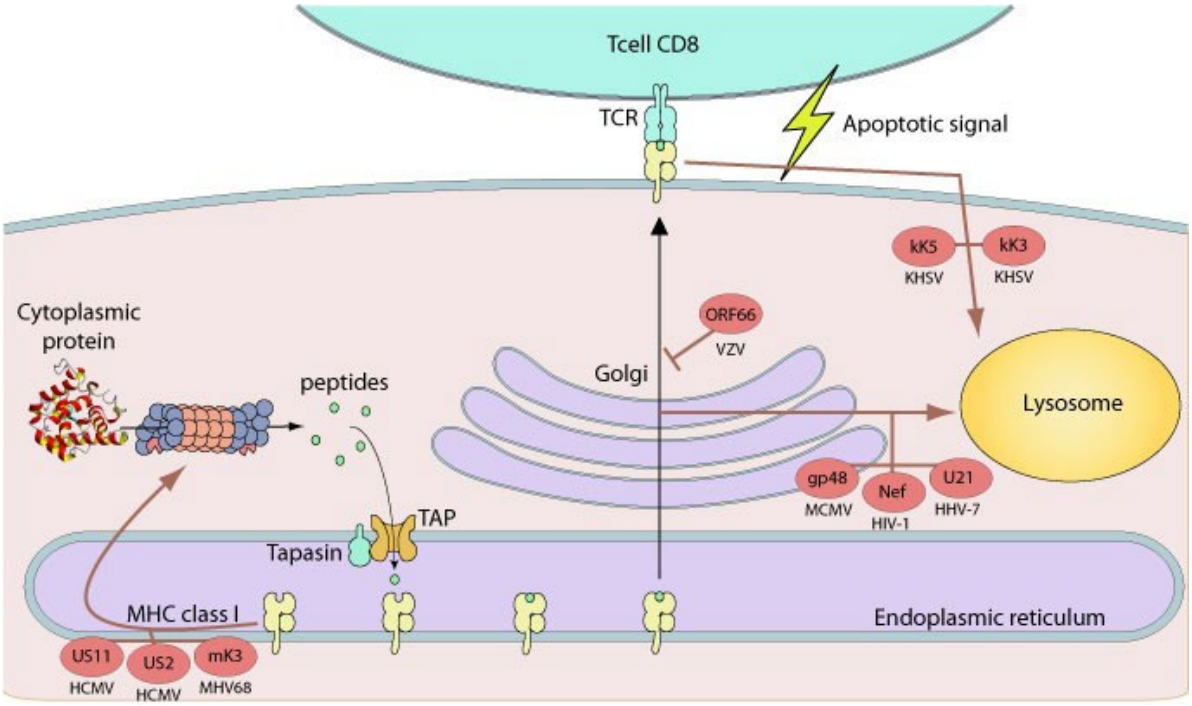


Figure 4-17. Antigen presentation with MHC class I mediated by TAP and viral mechanisms to inhibit MHC class I presentation of viral antigens. The red circles indicate viral accessory proteins used to inhibit the function. Courtesy of ViralZone https://viralzone.expasy.org/819

Antigen presentation by MHC class II occurs when viral proteins are digested into peptides (~10-12 amino acids) inside the endosome as they enter the cell. These endosomes then fuse with vesicles from the Golgi apparatus that contain newly-made MHC class II molecules, and the peptides are loaded onto MHC class II. These antigen peptides/MHC class II molecule complexes will be transported to the cell surface where they can be recognized by naïve $CD4^+$ T-helper cells that express a T-cell receptor (TCR) specific for the epitope being expressed, as shown in Figure 4-18. When a Th cell recognizes a peptide on a professional APC, **it responds by secreting cytokines that modulate the local immune response**. It does not kill the professional APC (because it's not infected), This is a critical distinction between Th cells and CTLs.

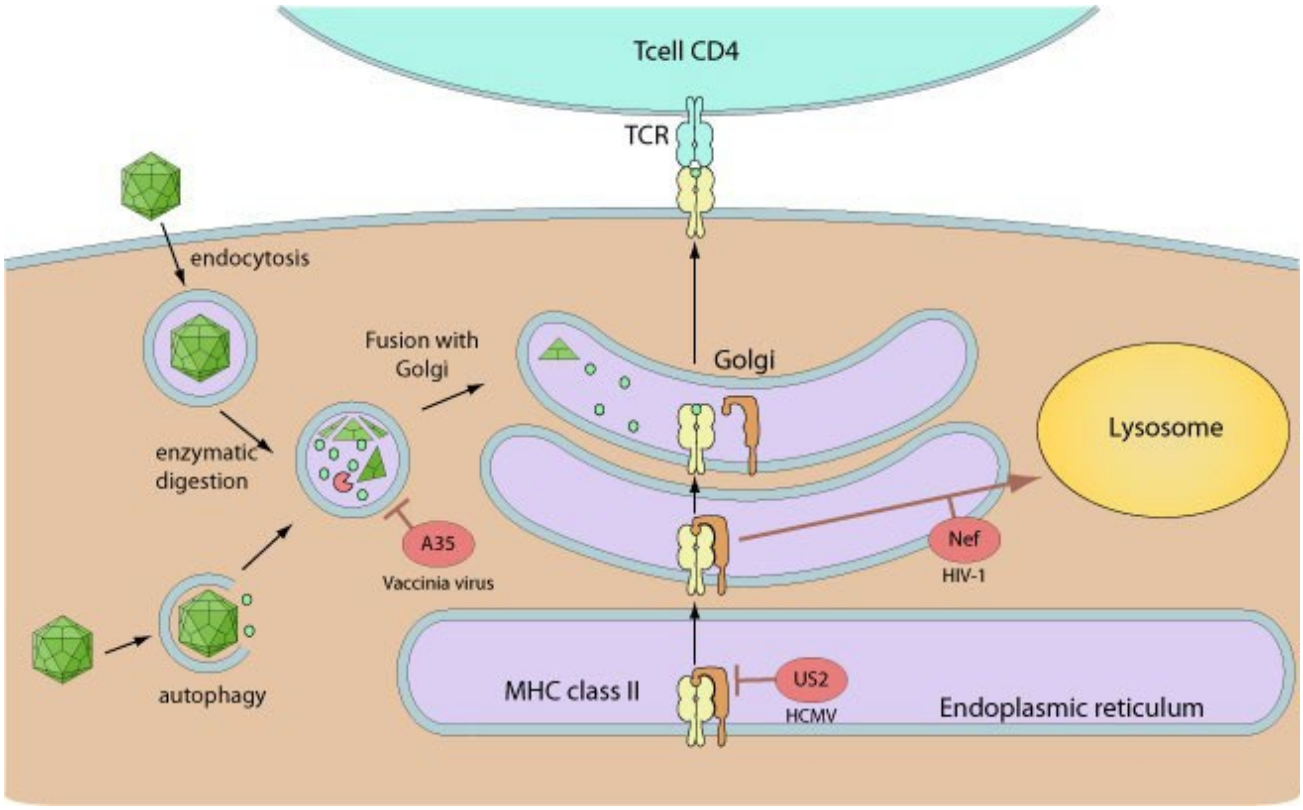


Figure 4-18. Antigen presentation with MHC class II mediated by viral protein degradation within the endosome and viral mechanisms to avoid MHC class II presentation. The red circles indicate viral accessory proteins used to inhibit the function. Courtesy of ViralZone https://viralzone.expasy.org/820

Cell-mediated immunity is performed when activated CTLs kill virus-infected cells by inducing apoptosis. Killing infected cells facilitates recovery from viral infection by eliminating virus-infected cells without damaging uninfected cells. CTLs induce apoptosis by releasing vesicles by exocytosis upon binding to target cells via MHC class I molecules expressing epitopes for which the CTL has a receptor protein. The granules contain perforin, which forms pores in the plasma membrane, as well as granzyme B and FasL. Both granzyme B and FasL activate the caspase pathway which leads to apoptosis. FasL does so by binding to a death receptor on the cell's surface. granzyme B enters the cell through the pores created by perforin and then induces a caspase signal transduction cascade leading to apoptosis.

HUMORAL-MEDIATED IMMUNITY, THE B LYMPHOCYTES

Humoral immunity is mediated by antibodies that help to clear viral infection and provide protection against new infections. Naïve B cells require activation by activated T-helper cells via MHC class II on the B cell's surface loaded with viral peptides (~10-12 amino acids in length). Antibody secreting B cells are called plasma cells. Plasma cells can secrete 4 classes of antibody (IgA, IgG, IgE, and IgM). IgA, IgG, and IgM will respond to viral infections. During a primary

immune response (the first time a virus is encountered) IgM is produced first (3-7 days) and is rapidly replaced after class switching occurs, by IgG (7-10 days) the primary serum antibody and generally peaks around 10-15 days and the strength of response will determine how long titers will remain high. IgA is found in mucosal tissues and secretions, including nasal, oral, gastrointestinal, and genital as well as breast milk. The antibody produced can be either neutralizing or non-neutralizing. Non-neutralizing antibodies bind to infected cells and induce opsonization of viral particles targeting them for phagocytosis or cell lysis by either the complement cascade or by cell mediated cytotoxicity by antibody-dependent cytotoxic cells. They can also target enveloped viral particles for lysis via the complement pathway. Neutralizing antibodies bind to viral particles and lead to loss of infectivity because the binding interferes with the virus' ability to bind to its cellular receptor to initiate infection. The binding can simply cover the virus so it can't bind, or it can cause the virus particles to aggregate into clumps that are unable to bind.

Two critical events in B cells that are mediated by T cells are **class switching** (to IgG, IgA) and **affinity maturation**. Class switching results in different effector functions of antibodies (e.g., complement fixation, antibody-dependent cell cytotoxicity) and exponentially greater binding affinities to antigens. Without these helper T cell-mediated events, antibody responses are poor and can lead to premature death of the person. One such example of this importance is **hyper-IgM syndrome**, in which genetic mutations in either CD40 (on the B cell) or CD154 (on the helper T cell, formerly CD40L) prevent these two cell surface proteins from interacting during antigen presentation. The result is **an inability for the person's B cells to switch to IgG or IgA, and they cannot generate high affinity antibodies**. Consequently, they suffer from recurrent infections that often lead to high mortality in childhood.

During a secondary immune response (all subsequent infections with a given virus) memory B-cells will rapidly produce IgG or IgA within hours allowing the infection to usually be cleared without symptoms.

A summary of all of the host-virus interactions is discussed including cellular and immune mediated as shown in Figure 4-13. You will note that viruses can upregulate or downregulate some functions such as apoptosis and MHC antigen presentation or only inhibit some functions like PKR activity or other interferon-stimulated genes (ISG).

Now that we are familiar with neutralizing antibodies, it is time to discuss the diagnostic assays that have been developed to determine if a particular virus is present.

Serological Assays to Quantify Neutralizing Antibody Concentrations and Their Use in Diagnostic Labs

Serological Assays can be used to diagnose viral infections. Presence of high levels of IgG in acute serum generally indicates past exposure or immunization. If IgG is absent, it indicates that past exposure is unlikely. IgG levels increase as seroconversion occurs. In an acute infection, presence of IgM indicates a primary or recent infection. If assaying for IgG, samples must be taken at least 2 weeks apart. The first sample is an acute sample and the second the convalescent when presumably the host should be getting better. A greater than 4-fold increase in IgG titer between the acute and convalescent titers must be observed to indicate a recent infection. If the titer is high and does not increase more than 4-fold, the IgG is from a past infection or vaccination.

IgA is also useful for some viruses; however, again a 4-fold increase in titer must be observed to call an infection current or recent. Common serological assays will be described below.

You may be thinking to yourself, "if IgM detects current infections and IgG requires 2 samples, why not always use IgM?" The reason for this is that IgM is not as specific as IgG. As the immune response progresses, antibody specificity improves. Recall that IgM is made first during the immune response and then class switching occurs changing to IgG (or IgA, IgE) expression. Because IgG, A, and E are made later in the immune response, the specificity is improved. Therefore, if there are many viruses within a viral family, IgM often cross reacts with other members of the family causing false positives. A good example of this was seen with SARS-CoV-2 the virus that causes COVID-19. Some IgM also binds the 4 coronaviruses that cause the common cold as well as SARS and MERS, and as such was not very useful for diagnosing infection. Therefore, COVID-19 testing is done with real time PCR to determine if a person is currently infected, and IgG is used to determine only if they have ever been infected.
IgM testing is usually done in conjunction with IgG allowing diagnosis without waiting 2 weeks as if the person is both IgM and IgG positive it is a current infection.

Virus Neutralization Assay or Serum Neutralization Assay

Neutralization tests measure the capacity of a specific antibody to neutralize viral infectivity. These antibodies may either be purchased or isolated from the serum of animals suspected to be infected with a specific virus. Major uses for this test include the identification of or diagnosis of unknown virus or antibody, the determination of antibody levels, the comparison of antigenically related viruses, and the study of the kinetics of antigen- antibody reactions.

When performing the neutralization test, two systems are used. The reaction system is the virus incubated with specific antiserum until equilibrium is reached. The indicator system is the susceptible host or host cells inoculated with the virus-serum mixture. If neutralizing antibodies are not present, lesions such as cytopathic effects, pocks, plaques, disease, or death will be seen in the indicator system. In a plaque neutralizing assay if neutralizing antibodies are present, there will be no lesions or their number will be reduced below a predetermined level (e.g., 80% plaque reduction).

The beta serum plaque neutralization test is used to determine antibody titer. In the beta procedure a constant virus concentration is incubated in serial two-fold dilutions of serum before inoculation into the host system. The titer of the antiserum is the reciprocal of the greatest dilution that neutralizes the virus, which is the inverse of the highest serum dilution at which there is no viral CPE (expressed as NU/volume). This type of assay, which is called an **endpoint dilution assay**, relies on dilution to extinction of antibody in a sample. Importantly, a "high titer" antibody response is principally a **function of affinity maturation** – the higher the affinity, the greater the dilution that can be made before endpoint is reached. It is essential for you to understand that endpoint dilution assays do not measure the amount of antibody, but instead the high affinity of antibodies because of T cell help during B cell antibody production. For example, if the well containing a 1:240 dilution of serum was the last well in which neutralization was observed, the titer would be 1 over 1:240 or 240 NU/volume of serum added to the well. When acute and convalescent sera from the same patient are

compared in a neutralization assay, a titer increase of 4-fold or more indicates that the patient was recently infected with the test virus. A preexisting high titer of antibodies will not change over the two weeks and may be the result of a previous infection or vaccination with this virus. Figure 4-19 shows a typical data set for a serum neutralization assay determining if the serum of a cow contains a sufficient titer of neutralizing antibody to bovine herpes virus 1 (BHV-I) to indicate a current infection. Let's examine how we will interpret these results.

We expect:

1. The cell culture control (no virus, no serum). Will see happy monolayers of cells in every well. (These look healthy).
2. The BHV-1 virus titration to cause CPE that destroys the monolayer leading to clearing of the cells in all dilutions that contain infectious virus. Our virus has a titer of 40 $TCID_{50}$/0.025 ml, which is an acceptable level that should show CPE.
3. The acute and convalescent serum controls which have not had virus added. There will be no virus- caused damage to the monolayers. Note, however, that some sera at high concentrations can cause cell toxicity, as is seen in the 1/10 dilution wells of both acute and convalescent sera. This tells us that we should disregard the 1/10 dilutions of sera in the neutralization test as the cell killing is most likely due to the serum and not the virus.

Now let's analyze our data when virus and serum are incubated together before addition to BFS cell culture.

1. We have 4 replicates of each serum dilution, and, as expected, all 4 wells look the same at all dilutions.
    - The titer for the acute serum is less than 20. Remember we can't say it is zero as we did not plate any lower dilutions, and we can't be sure of the results in the 1/10 dilutions (see below). In this case there are no neutralizing antibodies at dilutions of 1/20 and higher. Therefore, the added virus has caused CPE in all of the wells, clearing the monolayers of BFS cells.
    - The titer for the convalescent serum is 320, as the 1/320 dilution is the last well where neutralization of the virus has occurred causing the virus to be unable to infect the BFS cells and resulting in an intact monolayer of cells. At the 1/640 dilution the antibodies are too dilute to neutralize the virus and viral CPE causes a clearing of the monolayer.
2. We can conclude that this patient was recently infected (shortly before the acute serum sample was collected) with BHV-1 as there was greater than a 4-fold increase in neutralizing antibody titer.
3. We can conclude that there is something toxic in the serum at low dilutions (1/10), that is diluted out by the 1:20 dilution. This assay is however still accurate, and titers can still be determined.

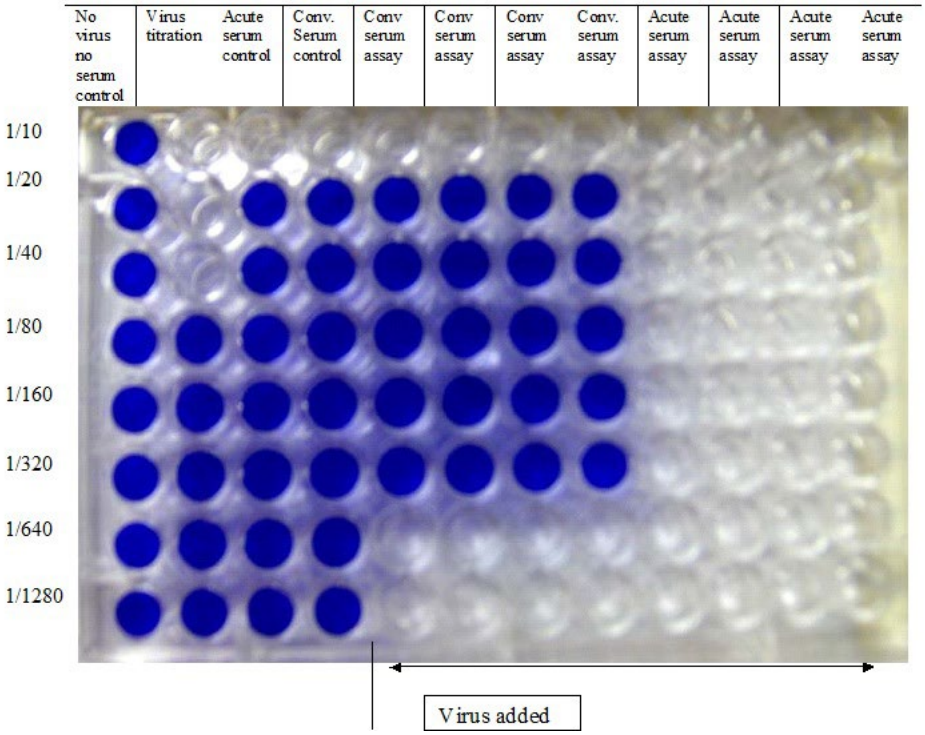


Figure 4-19. Typical set-up of beta neutralization assay demonstrating current infection with a virus. Courtesy of Erica Suchman, Colorado State University.

Note: plaque neutralization assays can also be carried out in larger welled plates and plaques actually counted. In this case a threshold level of plaque reduction is often set for a sample to be called positive for example 50% plaque reduction. If the virus would cause 100 plaques without antibody, you would need to see 50 or fewer plaques to call the dilution positive for serum neutralization. As usual a 4-fold increase in neutralizing antibody must be observed to call a sample positive.

Hemagglutination Inhibition Assay

Viral hemagglutination may be inhibited in several ways. By combining with viral antigens that normally interact with RBC receptors, specific anti-viral antibodies can prevent the virus-RBC interaction that normally brings about hemagglutination. Since infection with a virus will elicit production by the host animal of antibodies directed against each virus-induced protein, including the **hemagglutinin**, specific inhibition of hemagglutination by an animal's serum indicates that the animal has been infected at some time by the virus, and **hemagglutination inhibition** (HI) is a convenient test to detect antiviral antibodies. A 4-fold or greater rise in titer between two serum samples taken a few weeks apart (as during acute and convalescent phases of a disease) indicates that infection progressed during the period between the sampling times and that the infection was recent. A HI titer may also indicate that an animal is immune to reinfection. A preexisting high HI titer that does not change over the two weeks may be the result of a previous infection or vaccination with this virus. You may have noticed that no exact number was given for how one decides if an antibody titer is "high". This is because every virus

has a threshold level of antibody that has been determined to be indicative of a positive antibody response. This is true for all the serological assays (ELISA, hemagglutination assay, and neutralization assay).

Although HI uses similar reagents to HA, the HI test measures serum **antibodies** rather than hemagglutinin (virus). The **serum** is diluted in microtiter plates, and each dilution is allowed to react with a constant dose of virus (usually four HAU) for an interval of 15 min to one hour before RBCs are added. The reciprocal of the highest serum dilution that inhibits HA is the HI titer. Express the titer as the HI units/volume.
Figure 4-20 shows typical HI data. Let's examine how we will interpret these results. The titer of the acute serum would be 160 HI units (HIU)/25 µl. The titer of the convalescent serum would be 80 HIU/25µl. This would constitute a 2-fold decreased difference in titer which would indicate that this person was not recently infected but probably was infected with this virus at some time in the past and retained an antibody titer or was previously vaccinated. In order to call this individual currently infected they would need to have a convalescent titer of 640 HIU/25 µl. or higher. Note the reason these assays require at least a 4-fold increase in titer is that if the acute and convalescent titers are very close (for example 1.5-fold difference) there is a statistical probability that a button may be observed in the 2-fold greater well. This is seen here where the acute titer is actually higher than the convalescent titer because the titer is probably the same for the acute and convalescent serums and is somewhere between 80 and 160 HIU/25 µl.

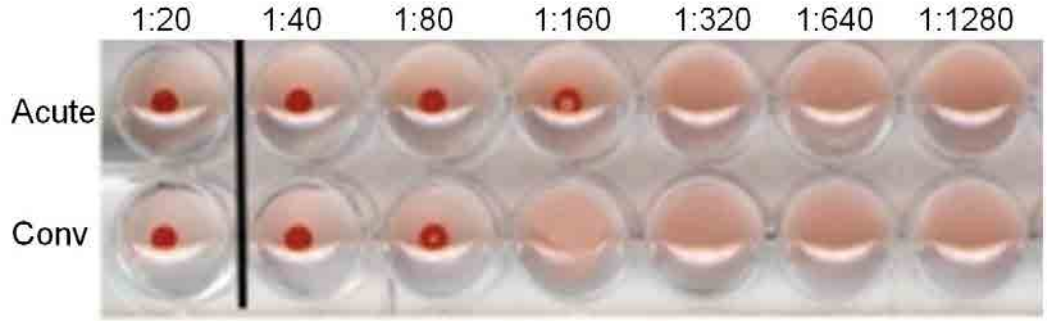


Figure 4-20. Typical data for a hemagglutination inhibition assay with low dilutions of the antibody demonstrating a button formation due to presence of viral neutralizing antibodies that eventually dilute out allowing the virus to agglutinate the red blood cells resulting in a lattice formation. Note: at the 1:160 dilution in the acute serum a small bubble has formed in the button, this is still considered a button. Courtesy of Erica Suchman, Colorado State University.

VIRAL EVASION OF THE HUMORAL RESPONSE

Viruses can evade the **humoral** (antibody) response by changing the antigens to which neutralizing antibodies bind. Mechanisms that aid in this evasion are high mutation rates, as those seen in RNA viruses where the RNA-dependent RNA polymerases (RdRp) have a high mutation rate (**antigenic drift**). Some viruses such as adenoviruses have many antigenically distinct serotypes such that one adenovirus infection confers little memory immunity to future adenovirus infections. This is the reason we can catch adenovirus colds repetitively. Recall also that segmented viruses can perform **antigenic shift** by exchanging segments with coinfected viruses of the same species. (See Chapter 3).

Now that we have explored basic viral replication cycles, how we study viruses, and how the

host-pathogen reaction works in viruses, we are ready for the part you all have been waiting for, learning the viral families, their diseases, diagnostics, treatments, and prevention. We will begin with an introduction to the DNA viruses in Chapter 5.

END OF CHAPTER QUESTIONS

1. Compare and contrast enzootic, epizootic, and urban cycles of a vector born disease.
2. What role do amplifying hosts play in spillover events?
3. You find that 8 days after being fed upon by infected mosquitoes, humans develop symptoms of a new virus. What is the scientific terminology for this time period? How would your answer differ if 8 days was the time period between when a mosquito took an infected blood meal and when it was able to transmit disease to a new host?
4. You develop West Nile disease. Your physician says, “You are a dead-end host”. What does this mean?
5. Explain the role of bridge vectors in outbreaks of vector borne diseases.
6. What type of symptoms would most likely be associated with a virus that is transported by M cells?
7. Compare and contrast the antiviral defense mechanisms of the alimentary and respiratory tracts.
8. What are advantages and disadvantages of a virus causing viremia?
9. Compare and contrast the TLR, RLR, and cGAS pattern recognition receptors.
10. You isolate a cell line that cannot control viral infections because it has mutations in the JAK/STAT pathway. Why would a poorly functioning JAK/STAT pathway lead to failure to control viral infections?
11. You are studying a new virus that does not create a 5’ cap on its viral mRNA. What PRR will recognize this? What other disadvantage might lacking a 5’ cap have for the virus?
12. What is the significance of NFκB in antiviral defenses?
13. What does the production of type I interferon lead to in a host?
14. You take a genetic test, and they inform you that you have a mutation in the OAS gene. What do you predict will be the result of this deficiency?
15. Which ISG proteins require activation by double stranded RNA?
16. Why do many viruses produce accessory proteins?
17. What is the importance of p53?
18. Some viruses turn off apoptosis while others turn it on. How can this be explained?

19. Explain how when natural killer cells are not specific for epitopes on antigens, they know what cells are virally infected and should be killed.
20. You isolate a new virus and find that it inhibits the Tap pathway. Why might a virus want to do this?
21. Compare and contrast how naïve B and T-helper cells are activated during the adaptive immune response.
22. You develop gastroenteritis. What antibody is most likely to neutralize the virus in your gut?
23. Are there any viral infections that will activate IgE production? If so, what types? If not, why not?
24. What does viral neutralization mean?
25. What does viral opsonization mean?
26. You perform a serum plaque neutralization assay to determine if your friend is infected with dengue fever virus. The test indicates that to be called positive a well must reduce plaques by at least 75%. Your untreated control has 200 plaques. What is the maximum number of plaques you can observe to call a well positive for neutralization?
27. If you find a neutralizing IgG antibody titer of 200 NU/0.2 ml, which is above the threshold value, can you conclude that your friend has dengue fever currently? If not, why? What tests would you do to conclude that they are in fact currently infected?
28. You decide to be extra cautious and perform a hemagglutination inhibition assay on your friend as well. If your friend has neutralizing antibody, what do you expect to observe in the well?
29. If your friend is recently or currently infected, what results would you have to observe on the HI test overall?
30. Why can you diagnose someone with a viral disease with only 1 sample if assaying for IgM, but must use two if assaying for IgG?
31. If you must assay two samples if assaying IgG, why not always assayIgM?

Use the following data to answer the next 3 questions

	S gene CT value	N gene CT Value
Patient 1	52	15
Patient 2	44	48
Patient 3	20	12
Patient 4	48	34

32. Which patient(s) is/are infected with a non-variant SARS-CoV-2? Explain why you answered as you did.

33. Which patient(s) is are infected with Variant SARS-CoV-2? Explain why you answered as you did.

34. Which patient (s) is/are not infected with SARS-CoV-2? Explain why you answered as you did.

REFERENCES:

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 8, 9 10, 12.

Suchman, E., Blair, and C. Martino, J. MIP425 Virology and Cell Culture laboratory manual Colorado State University. (2021)

ViralZone. Apoptosis modulation. https://viralzone.expasy.org/1581

ViralZone. Eukaryotic host gene expression shutoff by virus. https://viralzone.expasy.org/1582

ViralZone. Eukaryotic host transcription shutoff by virus. https://viralzone.expasy.org/1577

ViralZone. Host-virus interactions. https://viralzone.expasy.org/886

ViralZone. Inhibition of host interferon signaling pathway by virus. https://viralzone.expasy.org/883

ViralZone. Inhibition of host MHC class I molecule presentation by virus. https://viralzone.expasy.org/819

ViralZone. Inhibition of host MHC class II molecule presentation by virus. https://viralzone.expasy.org/820

ViralZone. Inhibition of host PKR by virus. https://viralzone.expasy.org/554

Reviewed by:

Tony Shountz, PhD. Department of Microbiology, Immunology and Pathology, Colorado State University Fort Collins, CO.

CHAPTER 5

Chapter 5: An Introduction To DNA Viruses.

INTRODUCTION TO THE CHAPTER

Finally, we can start discussing specific viruses. As we have learned there are both DNA and RNA viruses. We will examine these separately with Chapters 6-10 covering different DNA viruses and Chapter 12 covering Retroviridae, an RNA virus that converts itself to DNA and spends the rest of its life cycle functioning like a DNA virus. This Chapter will serve as an introduction to DNA viruses. Chapter 13 will be an introduction to RNA viruses. Chapters 14-19 will cover the RNA virus families. You should notice that chapters are set up with virus families that replicate similarly which will hopefully help you to observe the trends.

You will discover that I will discuss retroviruses in both the chapters introducing DNA viruses and RNA viruses as a retrovirus truly spends part of its life cycle as each. This is different than the hepadnaviruses which only live as DNA viruses despite packaging RNA. This RNA, however, is reverse transcribed to DNA within the capsid, and the virus then functions entirely as a DNA virus.

As you study the viruses, I hope that you will begin to look for and see the trends that occur among them. Trying to understand the reasons for these trends is a more fruitful study technique than trying to remember the many facts that make up virology. I for one was not capable of memorizing all the facts of virology, and, only after much thought, reading, and practice, did I start to understand the beauty that is virology. I hope you will come to appreciate the complexity yet simplicity of these tiny invaders as well. So, let's get started!

PROBLEMS FOR ALL VIRUSES TO OVERCOME

In Chapter 3 we discussed that all viruses must overcome the following issues in order to survive:

- How to get into the cell
- How to uncoat
- Cellular defense systems
- How to get to site of replication
- How to mimic cellular mRNAs
- Eukaryotic translation only monocistronic mRNA
- Viral and cellular mRNA are in competition for translation machinery
- Initiating genome replication
- Replicating the ends of the genome
- Packaging the genome
- How to get out of the cell
- The host's immune response

In this Chapter we will focus on how DNA viruses tend to overcome these issues.

We discussed how viruses get into cells, uncoat, and get to their site of replication in Chapter 3. These mechanisms are common to both DNA and RNA viruses. We learned how viruses deal with cellular anti-viral defenses in Chapter 4. As such, we will begin here with a discussion of where DNA viruses replicate. As we indicated in Chapter 3, all DNA animal viruses discussed in this book replicate in the nucleus with the exception of poxviruses that replicate in the cytoplasm. Poxviruses are extremely large, so large they have genes for their own DNA polymerase (DdDp) and RNA polymerase (DdRp) which allows the virus to replicate in the cytoplasm away from the nucleus where DNA replication and transcription machinery are found. Note that although retroviruses are classified as RNA viruses, as that is what they package in their virion and carry to the cell, they use reverse transcriptase to create a double stranded DNA intermediate that enters the nucleus, integrates into the host cell chromosomes, and becomes a DNA virus from this point. However, they will create RNA genome that will be packaged in the cytoplasm, so retroviruses actually replicate in both locations.

We also discussed in Chapter 3 the fact that all animal DNA viruses, including the DNA portion of the retroviral life cycle, with the exception of poxviruses, replicate in the nucleus. This means they have to develop a mechanism to interact with the complex nuclear transport pathway. One of the driving factors of the evolution of RNA viruses may well have been escaping the need to enter the nucleus. This topic was discussed extensively in Chapter 3 so we will not review it again here.

USING HOST CELL MACHINERY TO TRANSCRIBE MRNA

Once the virus has entered the nucleus, its first job is to create viral mRNA so it can begin creating viral proteins to assist in DNA replication and accessory proteins to decrease cellular antiviral defense mechanisms and immune responses. With the exception of poxviruses, all of the DNA viruses and retroviruses will utilize the host cell's transcription machinery, in particular the DNA dependent RNA polymerase RNA pol II. Note there are exceptions to this rule. A few viruses make only a few of their proteins using RNA pol III. This also was discussed in Chapter 3.

In order to be transcribed by host cell RNA pol II, the virus must mimic the promoter and enhancers that serve as the transcription signals. They must also mimic the host cell's transcription termination signals as well as polyadenylation signals. Other than poxviruses, all animal viruses that bring DNA into the nucleus follow these rules. Because these viruses are creating mRNA in the nucleus, they can also take advantage of the host cell's 5' capping machinery including the methyltransferase, as well as the poly A polymerase that will add the poly A tail to the 3' end of the mRNA. Remember that all polymerases, both DNA and RNA, read the template from 3' to 5' adding 5' to 3'. This is also true for all viral polymerases.

The only DNA virus that does not make use of the host cell's machinery is the poxvirus which replicates in the cytoplasm. As such, poxviruses must have genes for quite a few of these functions including a DdRp (RNA pol II), and DdDp (DNA pol III), a 5' capping enzyme with methyltransferase, and poly A polymerase. Note: that enzymes for transcription and mRNA modification must be carried in the virion, while the DdDp can be made after mRNA is produced and as such does not need to be carried in the virion.

PROBLEM TO BE OVERCOME: EUKARYOTIC TRANSLATION OF ONLY MONOCISTRONIC MRNA

Viruses with their very small genomes have an issue to deal with, eukaryotic cells only want to make one protein per mRNA. So how can a virus with a small genome create the many proteins it needs to replicate under these circumstances? Viruses have evolved the following mechanisms to overcome the problem of being able to create only one protein per mRNA in eukaryotic cells. Some of these involved making multiple mRNAs, while others allow a virus to create multiple proteins from one mRNA using clever mechanisms to get the cellular ribosomes to accomplish what they do not normally want to do. Those that are underlined below are utilized by DNA viruses. Those that are not purple and underlined are only used by RNA viruses. We will focus on the first4.

1. mRNA generated using multiple promoters within the genome: DNA viruses only
2. Differential Splicing of pre-mRNA to mRNA: DNA and Orthomyxo RNA viruses
3. Polyprotein Proteolytic cleavage; protease: All + RNA, Herpesviruses, Hepadnaviruses and Retroviruses
4. Leaky Ribosomal Scanning, overlapping ORFs: DNA and RNA viruses
5. Segmented genomes: RNA viruses only
6. Subgenomic mRNA generated without using multiple genomic promoters: RNA viruses only
7. RNA editing by RdRP, overlapping ORFs: RNA viruses only
8. Frame shifting/overlapping ORFs: RNA viruses only
9. Termination suppression: RNA viruses only

MECHANISMS TO GENERATE MULTIPLE MRNAS

MRNA GENERATED USING MULTIPLE GENOMIC PROMOTERS

DNA viruses can create multiple proteins by utilizing more than one promoter found within the genome to create multiple mRNAs, each of which will translate different proteins. Very few RNA viruses can do this. There is an important reason for the fact that RNA viruses do not utilize multiple promoters within their genome. Most RNA dependent RNA polymerases (RdRp) that RNA viruses utilize to transcribe their mRNA do not recognize or utilize promoters. They simply begin transcription **de novo** reading the first 3' nucleotide. Those RNA viruses that cannot initiate RNA synthesis de novo utilize promoters found within the antigenome. Note that all DNA viruses including poxviruses utilize multiple promoters with the DNA genome to create multiple mRNAs. Although the retroviruses function as DNA viruses after reverse transcription, they only create mRNA from one promoter. The reasons for this will become clear when we learn the retroviral life cycle. A typical DNA virus utilizing multiple promoters is shown in Figure 5-1. The polyomavirus SV40 demonstrates multiple promoters. Note that the arrows demonstrate the direction of transcription. Two promoters are utilized, one transcribing the mRNAs that point to the right and one transcribing the mRNAs that point to the left from different DNA strands. Note that further processing of these mRNAs via splicing explains the shorter versions observed.

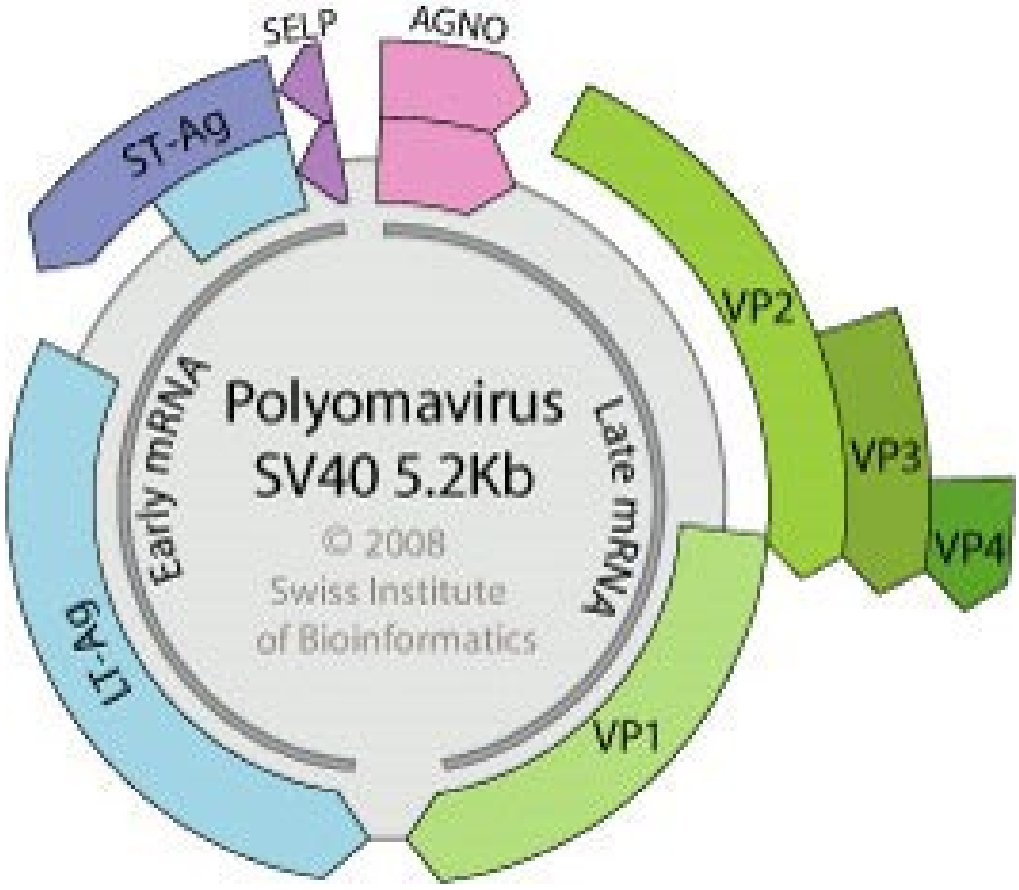


Figure 5-1. The polyomavirus SV40 demonstrating multiple promoters; note the arrows demonstrate the direction of transcription. Two promoters are utilized, one transcribing the mRNAs that point to the right and one transcribing the mRNAs that point to the left. Note further processing of these mRNAs via splicing explains the shorter versions observed. Courtesy of ViralZone https://viralzone.expasy.org/148

DIFFERENTIAL SPLICING OF PRE-MRNA TO MRNA

Differential splicing of a pre-mRNA to multiple mRNAs, as shown in Figures 5-1 and 5-2, is used by all animal DNA viruses except poxvirus because it replicates in the cytoplasm and does not have access to the extensive machinery required to perform splicing. Note that retroviruses, while acting like DNA viruses, do utilize splicing, and one of the rare RNA viruses that replicates as an RNA virus in the nucleus, orthomyxovirus, probably evolved to replicate in the nucleus to get access to the splicing machinery that it utilizes during its replication. Each of these spliced mRNAs will give rise to a different protein allowing a very small genome to give rise to many proteins.

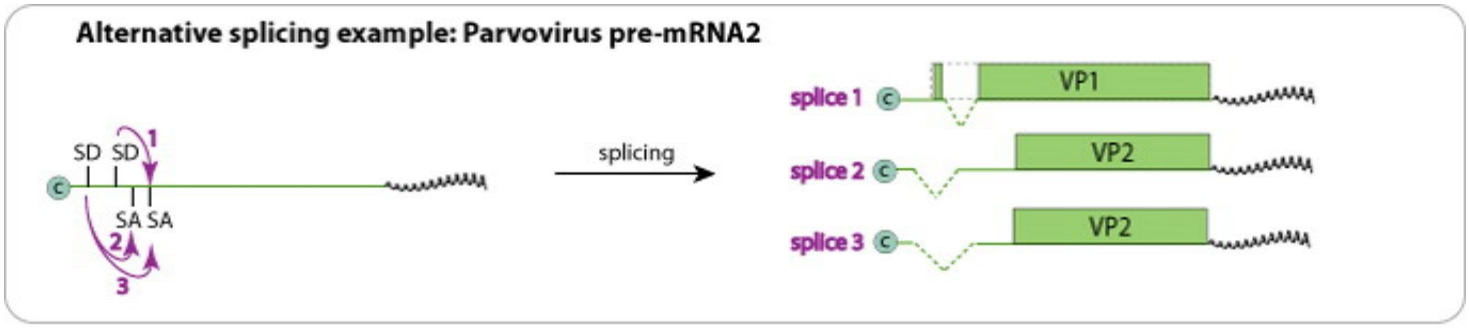


Figure 5-2. Parvovirus demonstrating multiple mRNAs generated by splicing a pre-mRNA. Courtesy of ViralZone https://viralzone.expasy.org/1943

MECHANISMS TO GENERATE MULTIPLE PROTEINS PER MRNA.

PROTEOLYTIC CLEAVAGE OF LARGE POLYPEPTIDES INTO INDIVIDUAL PROTEINS: PROTEASE

Although this mechanism is utilized heavily by positive sense RNA viruses, sporadic DNA viruses utilize this mechanism as well. It should be noted that some viruses such as hepadnaviruses and negative sense RNA viruses also take advantage of host cell proteases. The ones that utilize viral proteases are herpesviruses, and retroviruses (which, remember, are positive sense RNA viruses that live part of their lives as a DNA virus). The protease is **autocatalytic** meaning it can cleave itself out of the polyprotein before or after cleaving out all of the other proteins. Figure 5-3 shows the picornaviruses (positive sense RNA virus) that we will use as a classic example of protease activity. The protease (3Cpro) first cleaves the polyprotein into 4 proteins which have a specific function. When the virus needs different proteins, the first 4 are further cleaved to 6, which are further cleaved to 10, and so on. It is not until this third round of cleavage that the protease actually cleaves itself out of the polyprotein. This coordinated sequential cleavage allows the viruses to regulate their proteins over time with enzymes being cleaved out exactly when they are needed, as precursor proteins may have different functions than the mature forms.

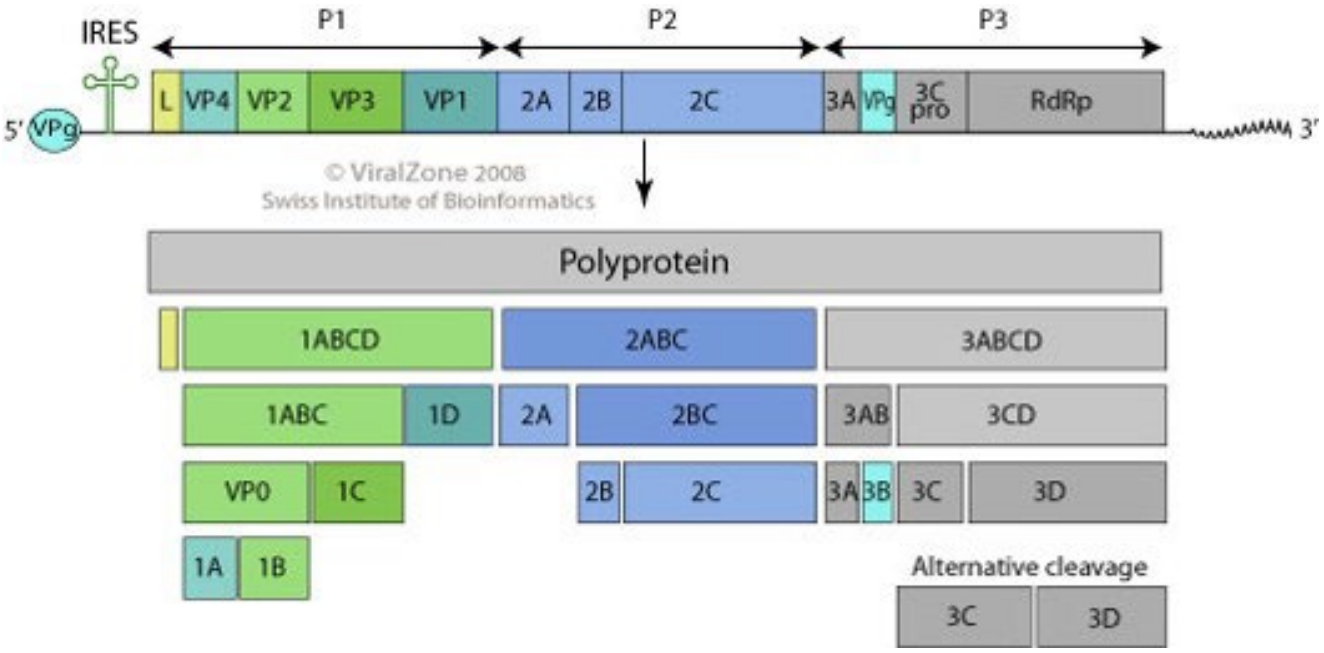


Figure 5-3. Picornavirus demonstrating proteolytic cleavage of polyprotein. Courtesy of ViralZone https://viralzone.expasy.org/33

LEAKY RIBOSOMAL SCANNING

Leaky ribosomal scanning, which is often called **leaky scanning,** is a rarely used mechanism for obtaining more than one protein from an mRNA in eukaryotic cells. Leaky ribosomal scanning is used by some members of the families *Herpesviridae, Hepadnaviridae, Polyomaviridae, Papillomaviridae* and *Retroviridae.* It is used on **overlapping open reading frames** (ORF), meaning there are multiple AUG start sites that are out of frame of one another. The first AUG is generally associated with a weak Kozak consensus and often the ribosome scans past it and initiates at the second AUG associated with a stronger Kozak consensus sequence, (Figure 5-4). Often the proteins created from the first ORF are used to help overcome host cell anti-viral defenses and are made in times of stress, such as when PKR is present. Remember PKR phosphorylates the translation initiation factor eIF2α shutting off translation. When the virus senses that it has a need for the proteins produced from ORF 1, leaky scanning allows that ORF to be expressed thus allowing expression of these proteins only when necessary.

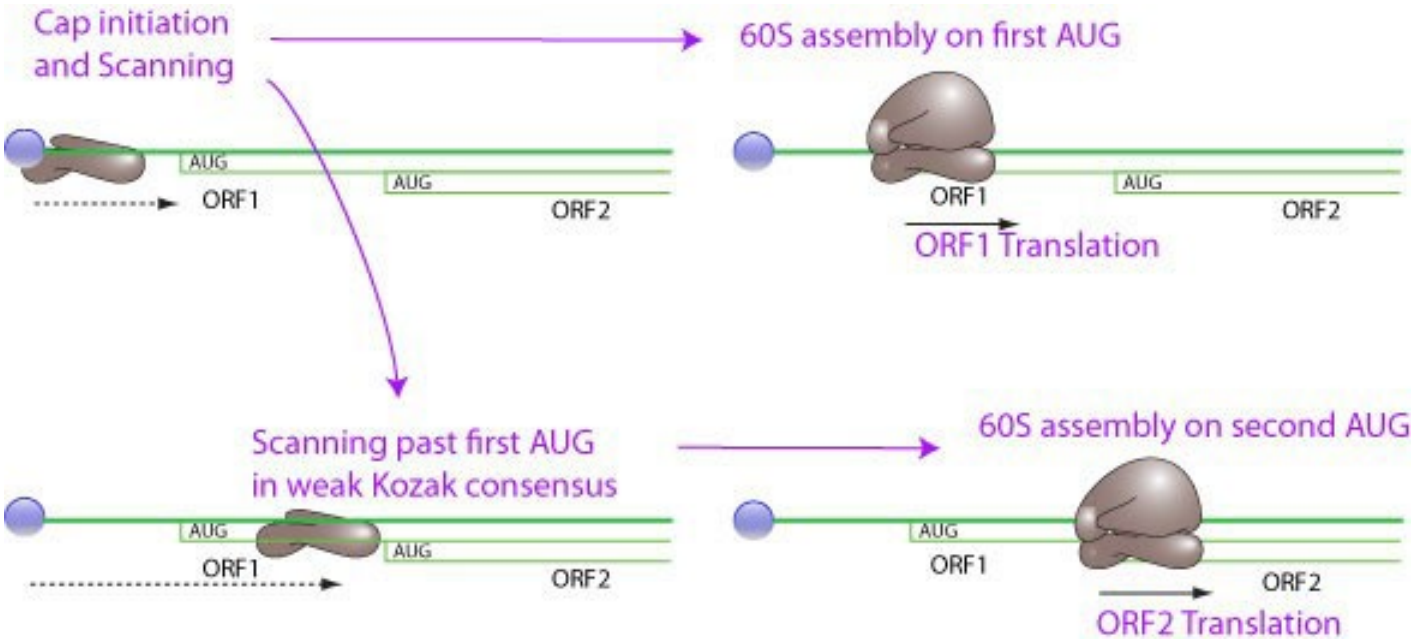


Figure 5- 4. Polyomavirus demonstrating leaky scanning. Courtesy of ViralZone https://viralzone.expasy.org/1976

VIRAL AND CELLULAR MRNA ARE IN COMPETITION FOR TRANSLATION MACHINERY

Now that we have shown how viral mRNA is able to make multiple proteins despite the limitation that eukaryotic host cells only want to make one protein per mRNA, let us examine how DNA viruses compete for host cell translation machinery. Again, many mechanisms have evolved for this purpose. Some are specific to RNA or DNA viruses and some are shared.

Mechanisms to outcompete cellular mRNAs for translation include:

- Inhibit cellular gene transcription
- Degrade host cell mRNAs

- Outcompete host cell mRNA
- Inhibit function of initiation factors used for cellular mRNA translation
    - Cleavage: eIF4G, eIF2α
    - Dephosphorylation: eIF2α
- Inhibit function of poly A binding proteins used for cellular mRNA translation (PABP)
- Cap snatching removes 5' 7-methylguanosine cap of cellular mRNA

Note that all these mechanisms except cap snatching and inhibiting the function of PABP are seen in DNA viruses.

DNA VIRUSES: WHAT STARTS DNA REPLICATION?

The first thing to consider is that most DNA viruses are going to use the host's DNA-dependent DNA polymerase (DdDp) (DNA pol III) to replicate their DNA, and DNA pol III, like all DdDp, needs a 3' OH to start adding nucleotides (a **primer**). DNA viruses have evolved multiple mechanisms of providing a primer for the initiation of DNA replication.

The mechanism most similar to host cell DNA replication is to replicate a circular genome utilizing an origin of replication which is opened by helicase, and primase adds RNA primers to provide the 3' OH, as is done by *Polyomaviridae*, *Papillomaviridae*, and *Herpesviridae*. Now wait a moment. You're thinking, "Didn't I learn that *Herpesviridae* has a linear genome?" Well, my smart friend, you would be correct. However, herpesviruses circularize their genome just before replication taking advantage of the opportunity to utilize all of the host cell's replication machinery. And we will see later in this Chapter that circularization also has the added benefit of making sure the ends of the genome are properly replicated.

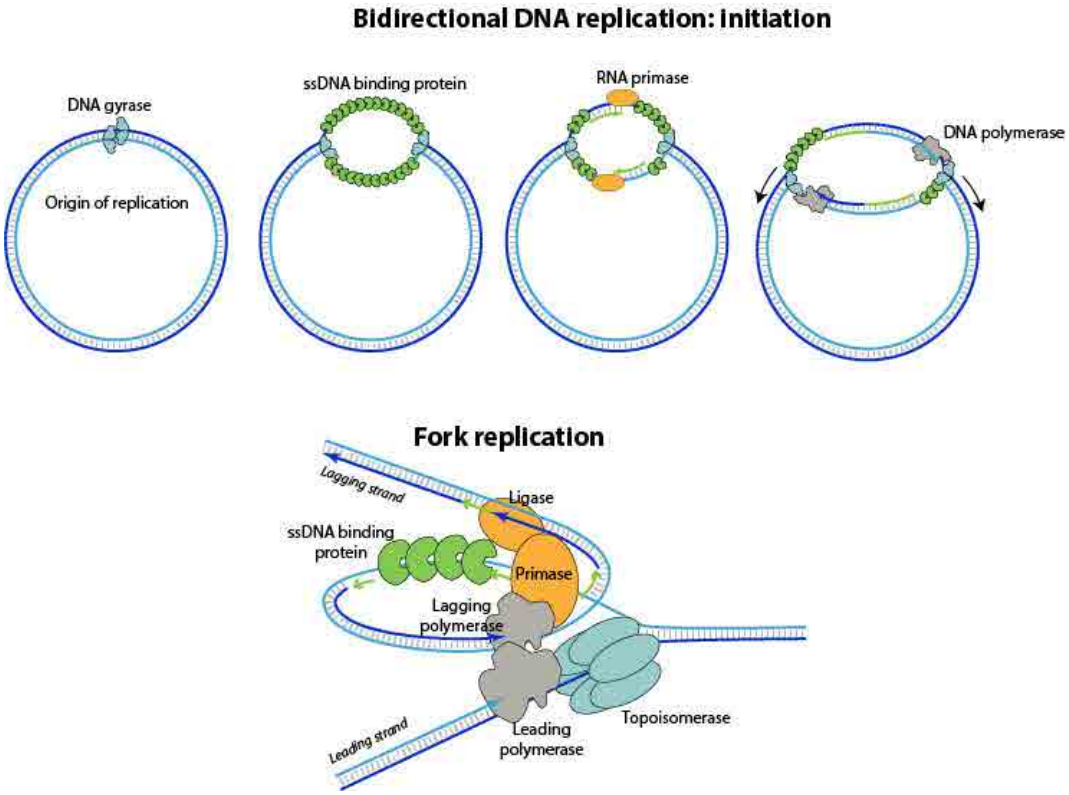


Figure 5-5. Circular DNA virus replication. The origin of replication is opened by helicase, and primase adds RNA primers to provide 3' OH necessary for DNA pol III to start adding NTPs. Courtesy of Erica ViralZone. https://viralzone.expasy.org/1939.

Some viruses create their own protein primer that serves as the 5’ end of the genome segment being produced and has the amino acid serine, threonine or tyrosine as the final amino acid that provides the 3’ OH group on which DNA polymerase can add nucleotides, as shown in Figure 5-6. Use of a protein primer is seen in *Hepadnaviridae* (RT in capsid), *Adenoviridae*, and many phages.

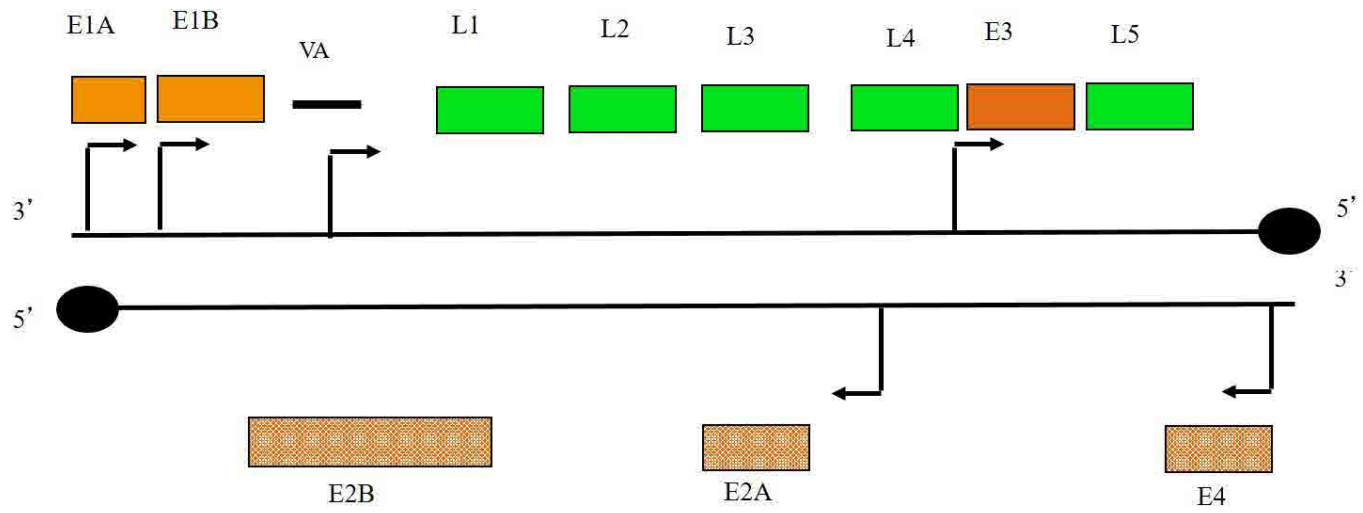


Figure 5-6. Adenovirus 5’ protein primers to provide 3’ OH necessary for DNA pol III to start adding NTPs. Courtesy of Sandra Quackenbush, Colorado State University.

Some viruses have inverted terminal repeat sequences at the ends of the genome that fold over due to complementarity between the inverted repeats. The 3’ end of this **T structure** (or **hairpin loop**, or **stem loop** structure) is used to initiate DNA replication with the 3’ end of the folded piece serving as the primer for syntheses of the opposite stand, as shown in Figure 5-7. Both parvoviruses and poxviruses have terminal repeats at the end of their genomes that they can use to prime DNA synthesis. The use of terminal repeats to initiate DNA replication is also referred to as “**self-priming**” as the viruses’ own genome is used as the primer.

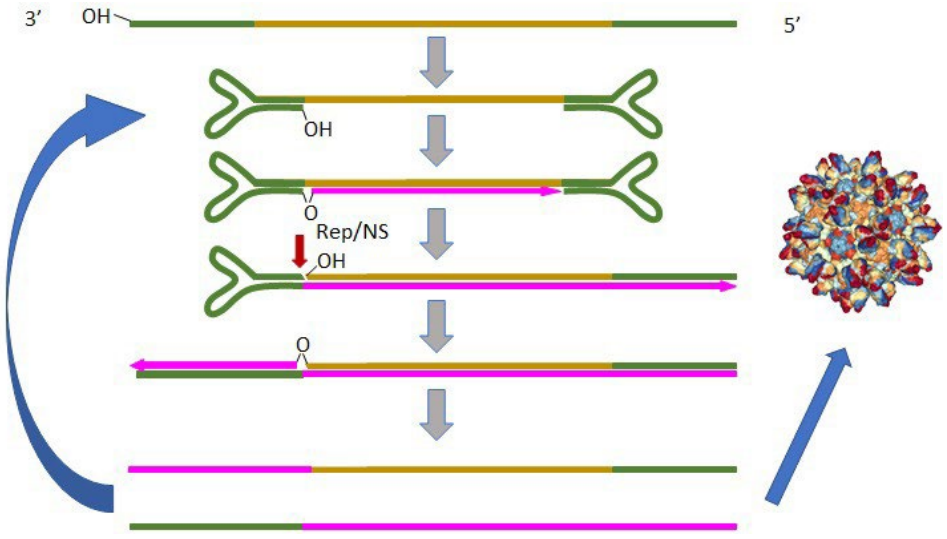


Figure 5-7. Parvoviral DNA replication initiation at inverted terminal repeats. The 3’ OH is used to initiate DNA replication. Courtesy of Maxwell Drummond, Colorado State University

Here is another instance where we must look at retroviruses as DNA viruses. Retroviruses need to make double

stranded DNA from their positive sense RNA genome. As there is no cellular protein to carry out this function, retroviruses must carry the reverse transcriptase enzyme into the cell with the genome (as well as integrase). Furthermore, retroviruses carry a specific tRNA molecule with the genome that is complementary to the primer binding site in the viral genome. The tRNA's 3'OH is used as the primer by reverse transcriptase to initiate reverse transcription of the RNA genome into double stranded DNA.

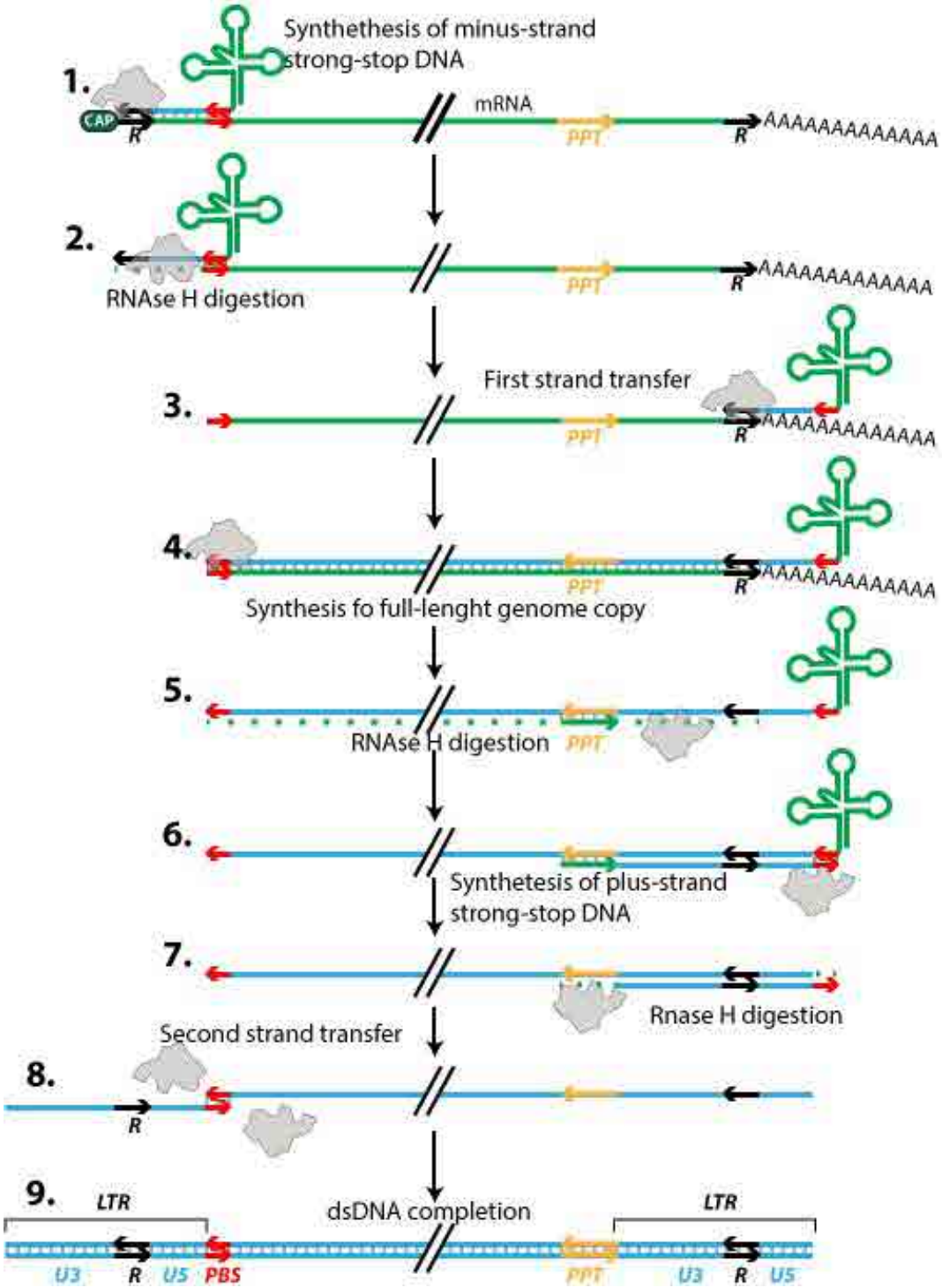


Figure 5-8. Retroviral tRNA primers to provide 3' OH necessary for reverse transcriptase to start adding NTPs. Note: Focus on the first two images to see the tRNA binding to the primer binding site (pbs). We will discuss the steps after that in Chapter 12. Courtesy of ViralZone https://viralzone.expasy.org/5061

DNA VIRUSES: HOW TO GET THE ENDS REPLICATED?

Now that we know how the viruses initiate replication, i.e., what they use as their primers, we need to answer another important question. How do they make sure their ends are replicated? Just as this is a major issue in our cells where we must have mechanisms to replicate the ends of our chromosomes as our DNA replication requires primers, so too must viruses evolve mechanisms for assuring the replication of their ends.

As discussed above, having a circular genome means your genome has no ends to be replicated! Polyomaviruses, papillomaviruses and hepadnaviruses have circular genomes. Recall that early viral taxonomic charts had polyomaviruses and papillomaviruses in the same family. As molecular techniques improved, virologists noted that although appearing very similar with small, circular dsDNA genomes and naked icosahedral capsids, their mechanisms for overcoming the 1 protein per mRNA issue were very different.

The vast majority, however, of animal DNA viruses have linear genomes, so, how do they assure their ends are replicated?

As discussed above, herpesviruses circularize their linear genomes just before DNA replication, as is shown in Figure 5-9. This ensures that the ends will be reproduced right before packaging the re-linearized genome.

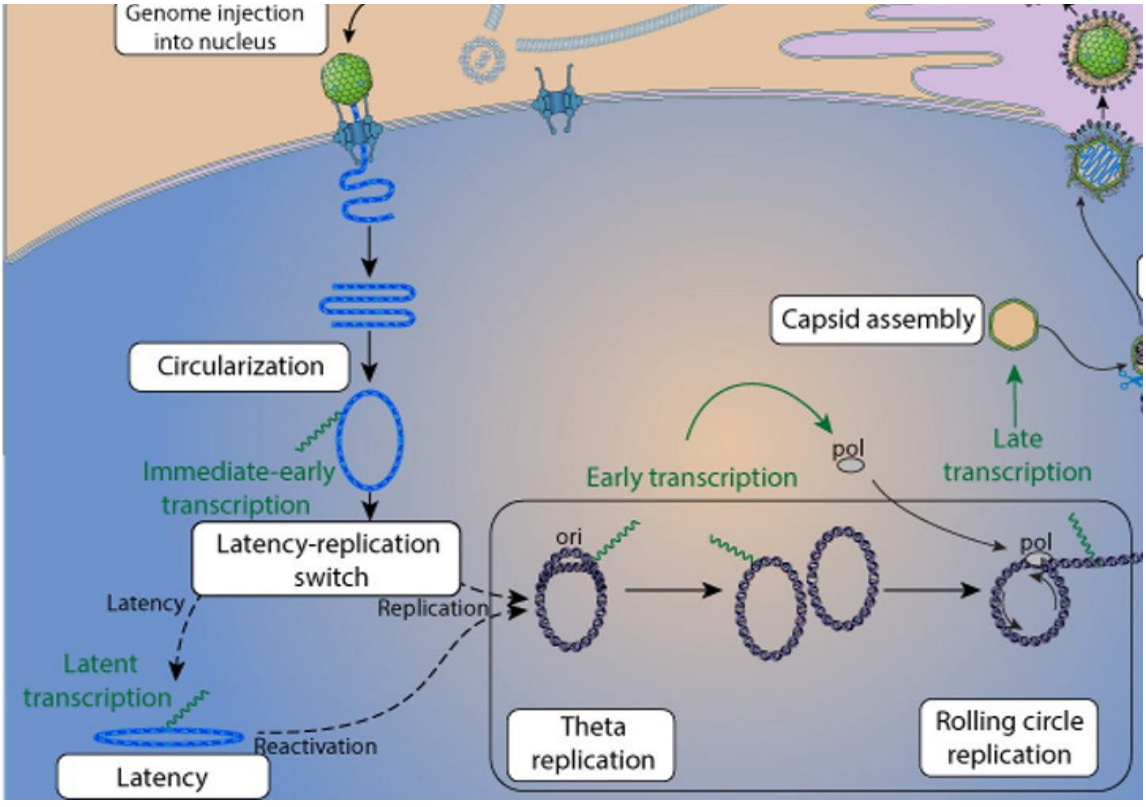


Figure 5-9. Herpesvirus circularization of a linear genome to ensure replication of the viral ends. Courtesy of ViralZone https://viralzone.expasy.org/5836

Remember, as discussed above, that adenovirus uses a protein to prime replication of the viral genome (See Figure 5-6). This protein is physically attached to the 5’ ends of the virus and provides a 3’ OH for synthesis of the complementary strand, also guaranteeing that the ends are replicated. Although the virus must have a gene to create its own DdDP (DNA pol), it is worth it to easily initiate replication and ensure the ends are completed correctly.

Remember that parvoviruses have an inverted terminal repeat sequence that folds over to form a T structure (or stem loop, hairpin loop) that leaves a 3’ open to serve as a primer to initiate DNA replication. This T structure also provides the virus a fairly simple mechanism for replicating the ends of the genome. As parvoviruses replicate, the T structures unfold and serve as a template for synthesis. The issue arises when the virus needs to replicate the priming end. In order to replicate the priming end, a viral enzyme is required that nicks (cuts) the DNA right by the inverted terminal repeat. The 3’ OH from that nick is used to replicate the priming inverted repeat, hence the entire virus, with both ends, has now been reproduced, as shown in Figure 5-10.

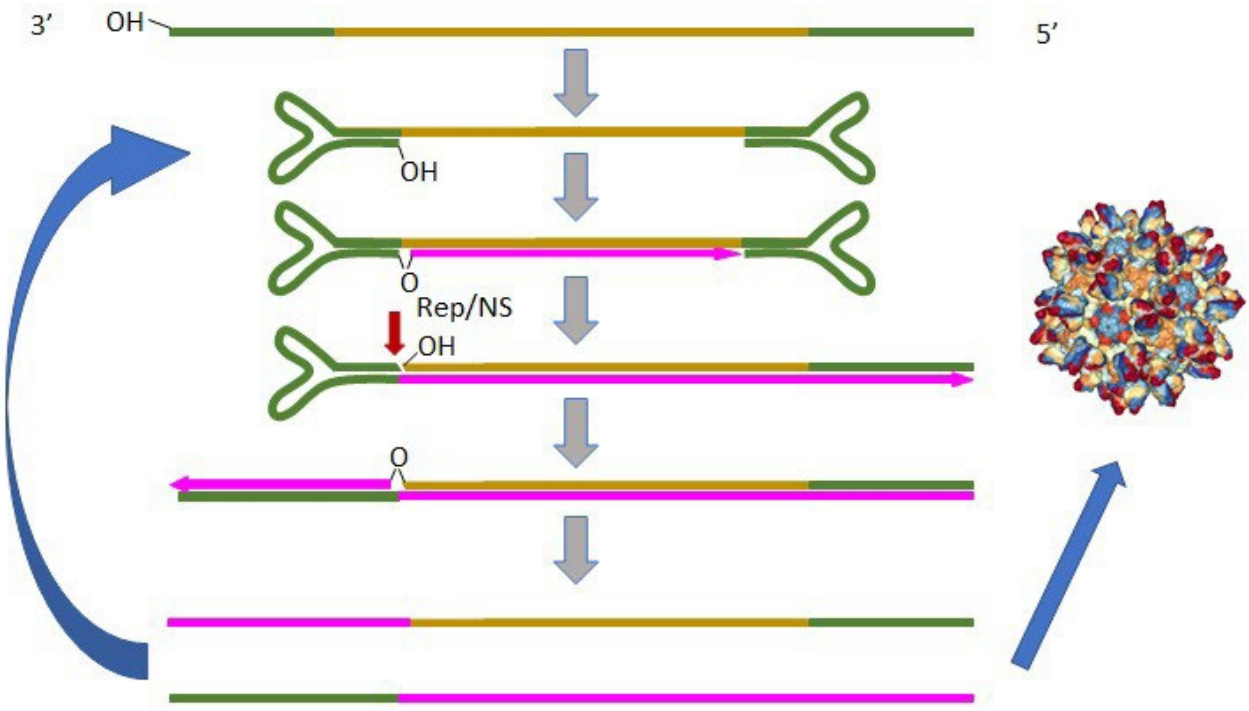


Figure 5-10. Parvovirus DNA replication from inverted terminal repeat ends. Note the 3’ end of one terminal repeat is used to prime synthesis of a complementary strand of DNA, but a viral enzyme is required to nick the priming terminal repeat so it can also be replicated. Courtesy of Maxwell Drummond, Colorado State University.

Although poxviruses have inverted repeats on their ends, they do not form a T structure as parvoviruses do. Instead, they form a linear structure with covalently-linked ends. Think of it as a single stranded DNA circle that is complementary to itself. As such, the 2 strands bind to each other leaving small covalently-linked loops at the ends. See Figure 5-11. One of the ends will be nicked by a viral enzyme leaving a loop-shaped structure, similar to the one seen in parvovirus replication, that will now unwind. The free 3' OH of the nicked DNA will be used as the primer to synthesize a strand complementary to the other strand. Replication will continue all the way around the circle leading to **concatemer** formation (2 genomes back to back) that will be cut into 2 by a viral enzyme and ligated to reform the genome with linked ends by viral ligase (as poxviruses replicate in the cytoplasm).

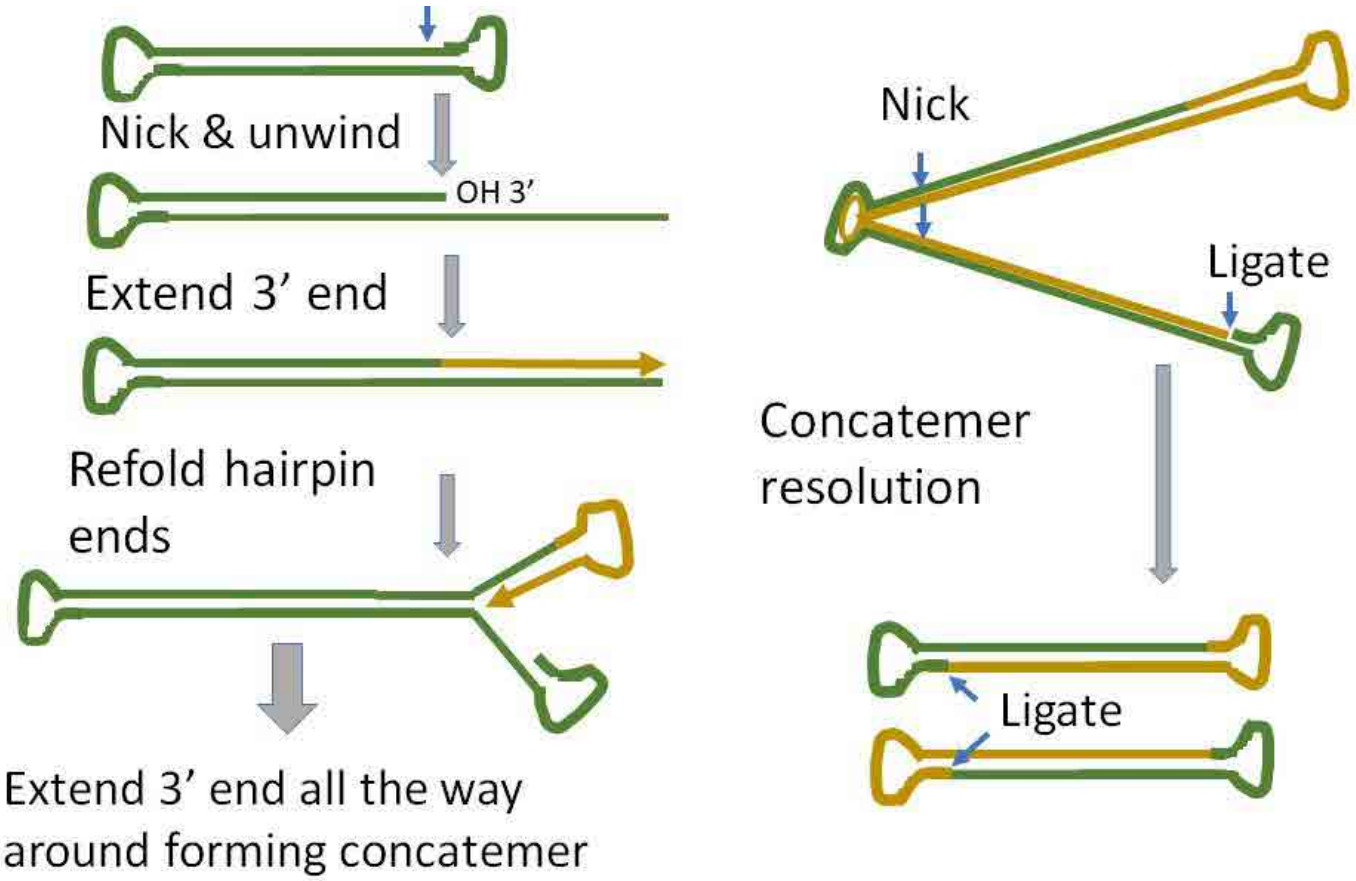


Figure 5-11. Poxvirus DNA replication from covalently linked ends. Courtesy of Erica Suchman, Colorado State University.

Recall in Figure 5-8 that retroviruses use a tRNA to prime DNA production from the RNA genome by reverse transriptase. Retroviruses and hepadnaviruses both use template switching mechanisms combined with terminal repeats to ensure that both ends are reproduced. This is not surprising. Their mechanisms are similar as they both utilize reverse transcriptase in their life cycle albeit at different times. Also note they use different primers. Retroviruses reverse transcribe their RNA genome to dsDNA inside infected cells using a tRNA primer. Hepadnaviruses reverse transcribe an RNA intermediate they package to the DNA genome that they bring to the cell using a protein primer, with reverse transcriptase serving as the primer.

Let's look at retroviruses as an example of how this works. As you will see in Figure 5-8, the tRNA binds to a specific tRNA binding site in the genome. The 3' OH of the tRNA is then used to start DNA synthesis. This tRNA binding site, however, is very close to the end of the genome. As the reverse transcriptase RdRp activity hits the end of the genome, it turns on its RNase H activity and cleaves out the RNA from the DNA/RNA hybrid. Part of the sequence that has been synthesized is the R (repeat) sequence found at both ends of the virus. As such, this piece of DNA jumps to the other side of the genome by binding the R site found on the other end. The 3' on this fragment allows synthesis to start from the other end. Note that the reverse transcriptase RNase H activity continues to cleave out RNA leaving only small bits to serve as primer for the second strand.

DNA VIRUSES: HOST CELLS THAT ARE DIFFERENTIATED DO NOT PRODUCE DNA POLYMERASE IF THEY ARE NOT REPLICATING. HOW TO OVERCOME THIS?

We have discussed that DNA viruses usually go to the nucleus where host cells create and utilize the machinery for DNA replication and transcription. However, differentiated non-duplicating cells do not need to create DNA replication machinery. This machinery is only needed when cells are dividing and need to copy their DNA to create new cells. Again, not requiring the host cell and DNA replication machinery might be another reason RNA viruses evolved and have been so successful, as they do not need to worry about this issue. As you might expect by now, DNA viruses have evolved many different ways to overcome this issue. The following is a list:

- Produce enzymes to push cells into **S phase** of **cell cycle** where DNA replication machinery are produced. S phase is the **synthesis phase** where DNA doubles in preparation for **mitosis** (cell reproduction). You will notice papillomavirus is a virus that can push a cell into S phase, so it should not come as no surprise to you that this is the virus that causes warts and can also cause cancer.
    - Ex: *Polyomaviridae, Adenoviridae, Papillomaviridae, Herpesviridae*
- Stay in the cytoplasm and make their own DNA replication machinery.
    - Ex: *Poxviridae* only
- Reproduce only in highly replicative cells such as the lining of the gut, bone marrow, or in developing fetuses of pregnant females.

    - Ex: *Parvoviridae*
- Make their own DdDp but still replicate in the nucleus and use parts of the host cell replication machinery other than the DNA-dependent DNA polymerase (DdDp, or DNA pol).
    - Ex: *Herpesviridae, Adenoviridae*
- Package RNA and reverse transcribe to DNA. Note: neither needs to reproduce the DNA, just make the mRNA that will be packaged, therefore the constantly present DdRp (RNA pol II) will be used.
    - Ex: *Retroviridae* (inside newly infected cell)
    - *Ex: Hepadnaviridae* (inside virion)

MECHANISMS TO OVERCOME TYPE I INTERFERON AND RNAI ACTIVATED BY LONG STRETCHES OF DOUBLE STRANDED RNA (>125 BP)

Remember that although cells have some mechanisms for detecting viral DNA, such as DNA in endosomes or cytoplasmic dsDNA, most viruses replicate in a manner to try and avoid this. Examples would be having a membrane that fuses with the plasma membrane so it does not enter an endosome or remaining inside its capsid until it reaches the nuclear membrane and uncapping there (as most of them do). However, this does not mean they needn't worry about activating RNAi or type I interferon responses. If viruses produce double stranded RNAs longer than 125 bases, the double stranded RNA can trigger RNAi or interferon responses. So how do viruses avoid triggering dsRNA induced antiviral responses?

The first way is to express all their mRNAs from one strand, as papillomaviruses, hepadnaviruses and parvoviruses do. Then there will be no complementarity between the mRNAs (even if they are overlapping), and they cannot bind to each other and form long stretches of double stranded RNA that can be detected by the many antiviral defenses that detect double stranded RNA. In Figure 5-12 you will see that papillomaviruses utilize 2 promoters; however, these are on the same strand, and differential splicing is used to produce many mRNAs from these 2 promoters. We have already looked at the polyomavirus transcriptome in Figure 5-1. If you refer to that figure again, you will note that although there are multiple promoters and they are transcribing off opposite strands (hence the different directions of the arrows on the mRNAs produced), these mRNAs never overlap. About ½ of the genome is used by one promoter to transcribe mRNAs, and the other ½ is used by a second promoter on the other strand. Remember that for many years they were in one family, *Papovaviridae*. This difference in gene expression was one of the main reasons these 2 very similar groups of viruses were separated into different families.

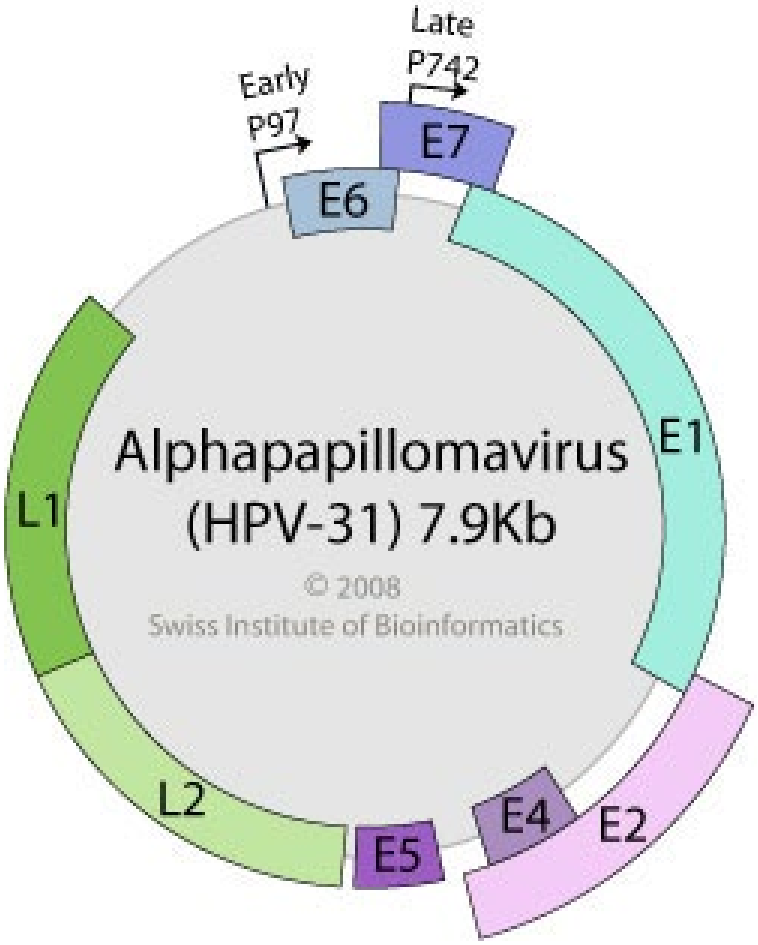


Figure 5-12. Papillomavirus genome showing gene expression from 2 promoters found on the same strand of the genome to avoid producing overlapping mRNAs that could be detected by RNAi or Type I interferon response. Courtesy of ViralZone https://viralzone.expasy.org/187

Remember that the **pathogen associated molecular patterns** (PAMPs) of viruses are detected by **pathogen recognition receptor** proteins (PRR) in virally infected cells, which then leads to activation of a signal transduction cascade response and ultimately to type I interferon expression and secretion. Type I interferons bind to type I interferon receptor proteins on local cells. This binding will activate the JAK/STAT pathway which will activate transcription activators to turn on interferon stimulated genes (ISG) which will produce approximately 200 antiviral proteins. One of the main antiviral proteins is PKR which phosphorylates the translation initiation factor eIF2α inhibiting its function and thereby shutting down translation. The following are the mechanisms DNA viruses have used to shut down the antiviral responses of type I interferons.

- Secreted IFN receptor mimicking protein expression
    - Binds up interferon so it cannot bind the actual receptor protein
- Inhibit IFN activation of JAK phosphorylation of STAT via accessory proteins
    - Inhibits STAT's dimerization and transcription activation

- Inhibit IFN transcriptional activations via accessory proteins
- Inhibit Protein Kinase R (PKR) inactivation of translation via accessory proteins
    - Interferon activates PKR expression
    - Double stranded RNA activates PKR that has been expressed
    - PKR phosphorylates translation initiation factor (eIF2α)
    - Decreases translation of cellular mRNAs
    - Inhibiting PKR leads to increased protein translation
- Produce dsRNA-like analogs
    - PKR and OAS are activated by presence of dsRNA
    - The analogs bind and inactivate PKR and OAS
    - OAS activates RNAse L which cleaves mRNA
    - Inhibiting OAS leads to increased viral and cellular mRNA half life

CHAPTER 5 END OF CHAPTER QUESTIONS

1. Do DNA viruses utilize RdRp? If yes, for what purpose? Is this enzyme provided by the cell or the virus?
2. Do DNA viruses utilize DdRp? If yes, for what purpose? Is this enzyme provided by the cell or the virus?
3. Do DNA viruses utilize DdDp? If yes, for what purpose? Is this enzyme provided by the cell or the virus?
4. What RNA polymerase is MOST LIKELY to transcribe viral mRNA? Why is this enzyme the most likely one?
5. What mechanism is used by all DNA viruses to overcome the one protein per mRNA issue in eukaryotic cells? Why is this the one that is used by all?
6. What mechanism is used by all DNA viruses except poxviruses? Why don't poxviruses use this mechanism?
7. How do DNA viruses acquire the 5' cap addition and poly A tail addition machinery?
8. How do DNA viruses get into the nucleus? Do they all use the same mechanism?
9. Are there any DNA viruses that you would predict can carry out reassortment? Justify your answer.
10. Are there any DNA viruses that you would predict can carry out recombination? Justify your answer.
11. If you performed an IFA assay on cells infected with most DNA viruses, where would you expect to see staining, and why?
12. If you performed an IFA assay on cells infected with a poxvirus, where would you expect to see staining, and why?

13. Do all DNA viruses splice their mRNA? If so, why do they do so? If not, why not?
14. Why do some viruses have genes to produce a viral protease?
15. What is the importance of Kozak consensus sequences to DNA viruses?
16. What are overlapping reading frames? What does having them accomplish for viruses that have them?
17. Some viruses can dephosphorylate eIF2α. What does this accomplish?
18. DNA viruses must use a primer to initiate DNA replication. Different viral families have evolved different mechanisms to accomplish this. Describe at least 4 of these different mechanisms.
19. Linear DNA viruses must have a mechanism for ensuring the ends of the genome are reproduced. Describe at least 4 different mechanisms that different viruses have evolved.
20. Why is it important to DNA viruses that the host cell be in the S phase?
21. Do RNA viruses require a host cell that is in the S phase? If yes, explain why? If no, why not?
22. Many tissues lack cells in the S phase. How do viruses overcome this issue?
23. What activates RNAi?
24. How do DNA viruses avoid activating RNAi?
25. What is the most common activator of type I interferon production?
26. What are some mechanisms used by DNA viruses to avoid activating type I interferon production?
27. Which PRR are most likely activated by DNA viruses? What PAMPs are they responding to?

REFERENCES:

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 53-67.

ViralZone. Alphapapillomavirus. https://viralzone.expasy.org/187

ViralZone. Alternative splicing. https://viralzone.expasy.org/1943

ViralZone. dsDNA bidirectional replication. https://viralzone.expasy.org/19439

ViralZone. Herpesvirus replication cycle. https://viralzone.expasy.org/5836

ViralZone. HIV-1 reverse transcriptase (RT) https://viralzone.expasy.org/5061

ViralZone. Leaky scanning. https://viralzone.expasy.org/1976

ViralZone. *Picornaviridae*. https://viralzone.expasy.org/33

ViralZone. *Polyomaviridae.* https://viralzone.expasy.org/148

CHAPTER 6

Chapter 6: Small Linear Single Stranded DNA Viruses That Replicate In The Nucleus: Parvoviridae

INTRODUCTION TO THE FAMILY *PARVOVIRIDAE*

You will notice in Figure 6-1 that parvoviruses are among the smallest virions because they have a single-stranded DNA genome of only 4-5 kb that can fit into the very small 16-26 nm diameter naked icosahedral capsid. Like most DNA viruses they replicate within the nucleus.

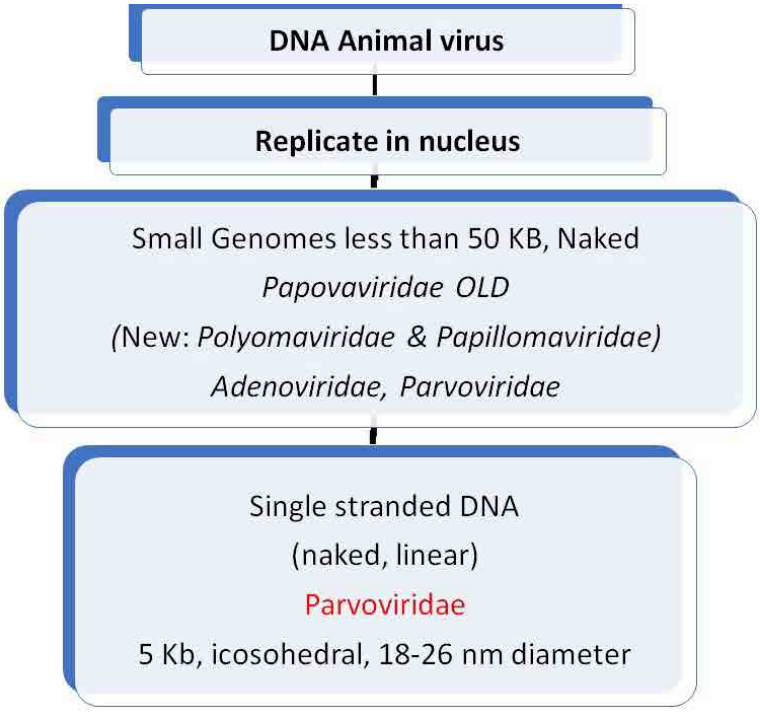


Figure 6-1. Taxonomy of DNA parvoviruses. Courtesy of Erica Suchman, Colorado State University.

There are two major parvovirus subfamilies:

Densovirinae which infect insects such as mosquitoes and are currently explored as a possible biological control mechanism for mosquito-borne viral diseases.

Parvovirinae which infect mammals. There are 3 genera:

Parvovirus infects mammals and birds,

Erythrovirus infects humans,

Dependovirus infects humans. *Dependovirus* is also called adeno associated virus (AAV) as it requires an adenovirus helper to establish disease. There are no diseases associated with AAV, but it is being explored as a possible gene therapy vector.

Most parvoviruses can replicate autonomously, meaning that they do not require a helper virus to establish or maintain infection. However, because they need the host cell to provide **DNA-dependent DNA polymerase** (DdDp called **DNA pol III**) and other DNA replication machinery, parvoviruses require the host cell to be in the S phase of the cell cycle. The S phase is the synthesis phase when the cell is reproducing its DNA in preparation for mitosis. Note, however, that *Dependoviruses* (AAV) require a helper virus such as a herpesvirus or adenovirus that is capable of pushing a host cell into S phase.

AAV can establish a latent infection. The latency involves integration of the viral genome into the host cell's genome. Once integrated the virus is inactive and transcribes no viral mRNA. However, if the cell containing a latent AAV virus is co-infected with a helper virus (herpesvirus or adenovirus), the helper virus pushes the host cell into S phase and activates the integrated AAV virus. The fact that the virus integrates into the genome and is not associated with disease has caused it to be a popular vector for gene replacement therapy. As we will see, the integration event is sequence specific and always integrates into the same place in the genome furthering its ability to safely deliver replacement genes to a host without the risk of activating **oncogenes** associated with tumor formation by integration within the oncogene.

GENOME STRUCTURE

The 4-5 kb single stranded DNA genome contains **palindromic** sequences on the 5' and 3' ends that fold back onto themselves due to the complementary sequences found in a palindromic sequence. These palindromes are 100-399 bp in length and differ between individual viruses. They form **stem loop** or **hairpin loop** or **T** structures (all names for the same thing) when the complementary sequences fold back and bind to themselves. This folding over provides a 3' end on which cellular DNA pol III can begin adding dNTPs to replicate the DNA genome. The genome is very simple. It contains only capsid proteins (sometimes called VP proteins) and enzymes that assist cellular DNA pol III with DNA replication, called either **replicase** (rep) or **nonstructural** (NS) proteins depending on the parvovirus.

VIRAL REPLICATION CYCLE

As shown in Figure 6-2, the parvovirus replication cycle begins when

1. The naked capsid proteins bind to receptors that contain sialic acid glycosylation.
2. They are endocytosed into clatherin-coated vesicles. It is unclear how the virus escapes the endocytic vesicle, but no conformational changes occur in acid, and they leave the vesicles still within a complete capsid.
3. The virus travels to the nucleus along microtubules.
4. The capsid is small enough that it can traverse the nuclear pore, and the virus uncoats within the nucleus.
5. Like all DNA viruses, the first thing it must do is transcribe viral mRNA to produce viral proteins. In particular, it requires the rep/non-structural proteins to assist in DNA replication. Host cell **RNA pol II,** a **DNA-dependent RNA polymerase** (DdRp), will transcribe all of the viral proteins.
6. Like all viruses, the host cell's translation machinery will translate the viral proteins.
7. Once rep/NS protein has accumulated, if the parvovirus is in a replicative cell, the cell will contain all of the components necessary to replicate the parvovirus DNA. Note: this requires a replicative cell that is in the S phase of the cell cycle. Parvoviruses do NOT have the ability to push cells into the S phase, and as such have a preference for infecting highly replicative cells. We will see that this requirement for replicative cells has a dramatic influence on the diseases associated with parvoviruses.

8. Once large quantities of viral DNA and capsid/VP proteins have accumulated, the virus will self-assemble in the nucleus and will leave the cell by lysis.

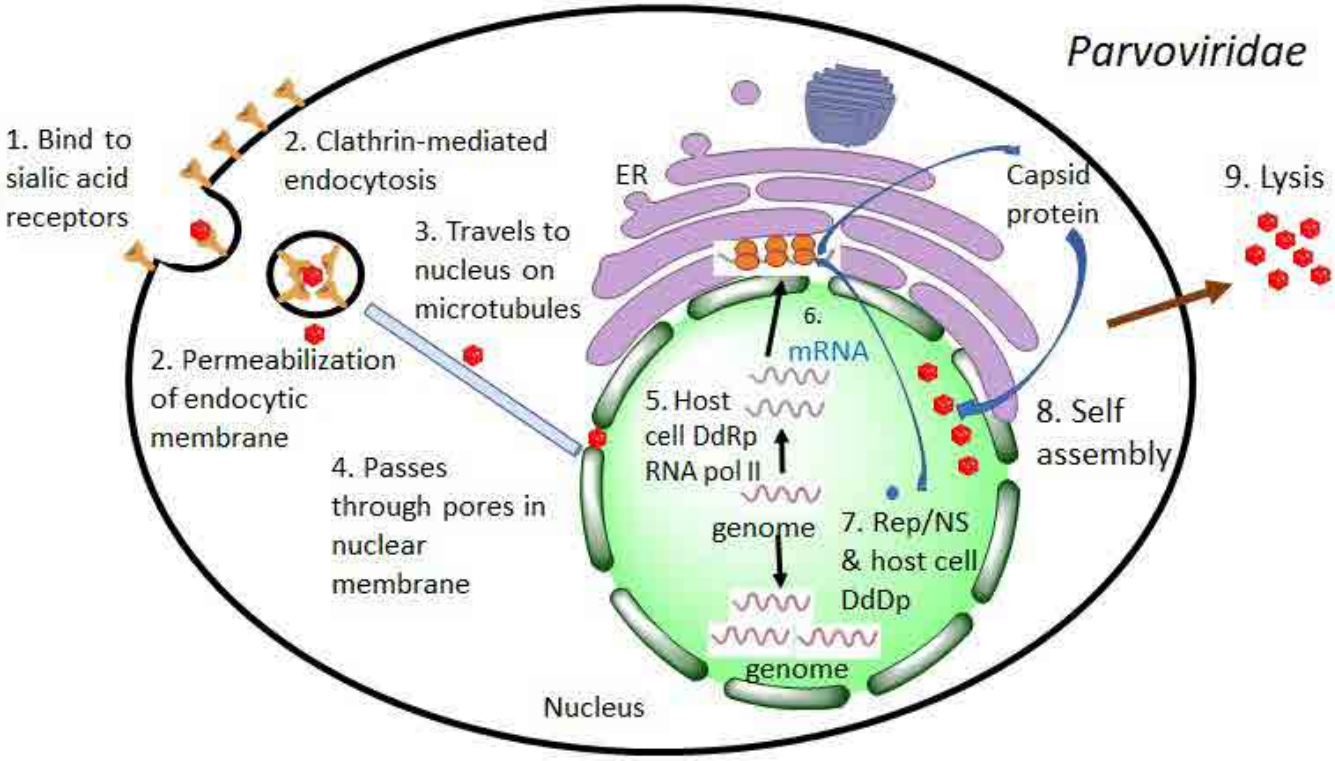


Figure 6-2. Parvovirus replication cycle. Courtesy of Erica Suchman, Colorado State University.

HOW DO PARVOVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all nuclear replicating DNA viruses of animals, parvoviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells, as shown in Figure 6-3. In particular:

1. Using multiple promoters to create many mRNAs. Most parvoviruses have more than 1 promoter, although B19, the human parvovirus, only has 1. For most parvoviruses each mRNA will create one protein. Some parvoviruses such as members of the insect infecting subfamily Densovirinae, and genus Dependoparvovirus can perform leaky scanning to produce overlapping reading frames producing multiple proteins per mRNA.
2. Using alternative splicing to create many smaller versions of a pre-mRNA, all of which produce different proteins.

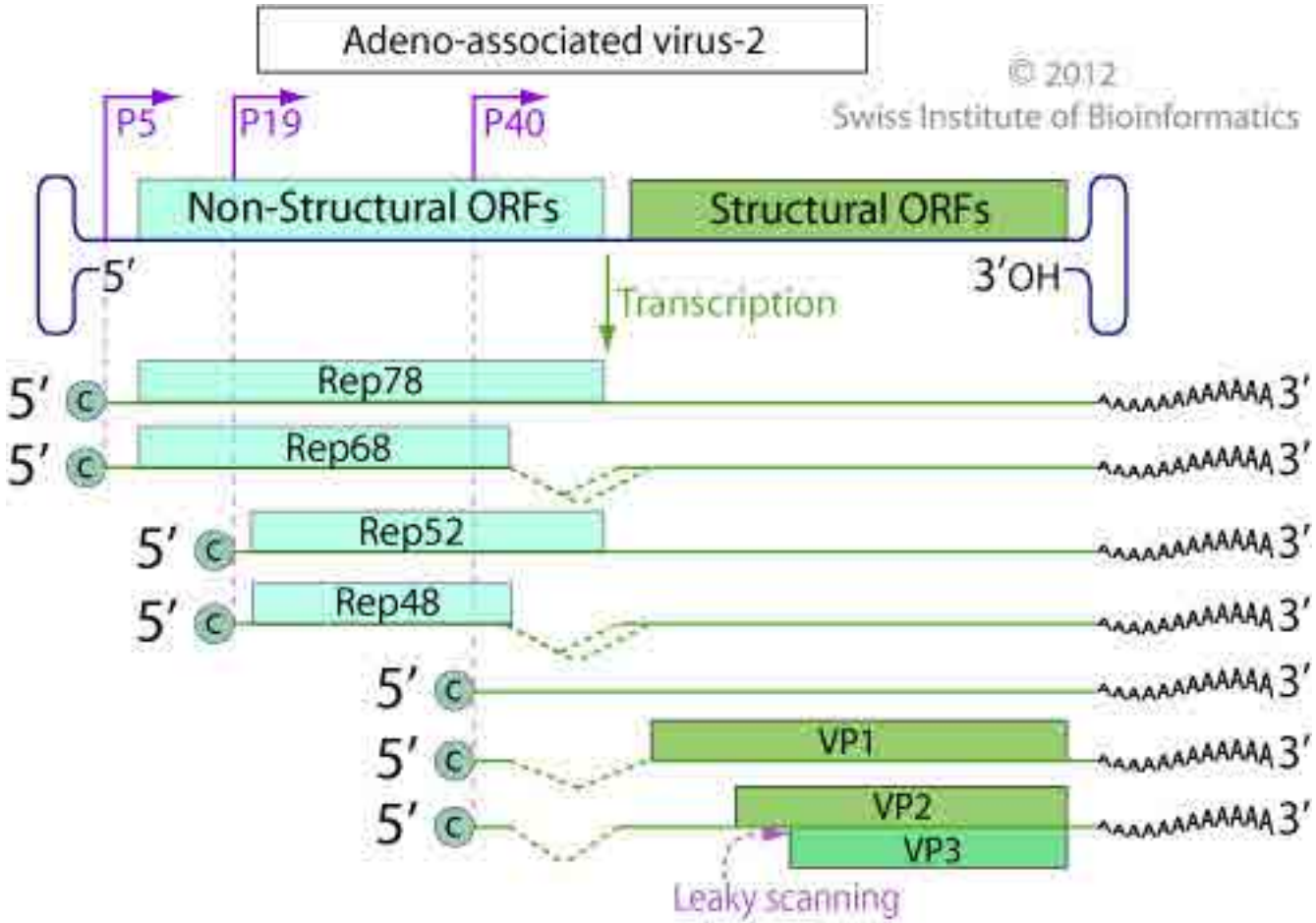


Figure 6-3. Mechanism used to overcome one protein per mRNA issue in eukaryotic cells by parvoviruses. Courtesy of ViralZone https://viralzone.expasy.org/226

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that will be recognized by the necessary initiation factors (eIF4E &G and PABP as discussed in Chapter 3). Only 2 types of proteins will be produced: capsid or VP proteins and the non-structural rep/NS proteins that will help the virus replicate. Furthermore, the non-structural proteins will interfere with cellular DNA replication assuring that the host cell's DNA replication machinery focuses on viral replication.

DNA REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA:

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Parvoviruses overcome this issue by only replicating in highly replicative cells.
2. How to prime their DNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes.

Note that parvoviruses like many viruses take care of the problem of replicating the ends and priming replication via the same mechanism as will be described below.

Figure 6-4 shows parvovirus DNA replication using the stem loop structures at the 3' end. Base pairing at the 3' end of the genome template-primer initiates viral DNA synthesis by cellular DNA polymerase III (DdDp). Note that no RNA primers are required nor are Okazaki fragments created which is different than DNA replication of host cell DNA. This is accomplished by continuous synthesis from the 3' OH group of the hairpin loop to the 5' end of the parental strand which is analogous to leading-strand synthesis during replication of double-stranded DNA templates. To completely copy the parental strand, a nick must be introduced to form a new 3' OH. Nicking occurs at a specific site (**terminal resolution site**) using viral Rep or NS1 proteins which are site-specific **endonucleases**. Endonucleases cut with a piece of DNA. Elongation from the nick is carried out by cellular DNA pol III (DdDp). The newly replicated 3' end forms a hairpin structure to prime a new cycle of DNA synthesis.

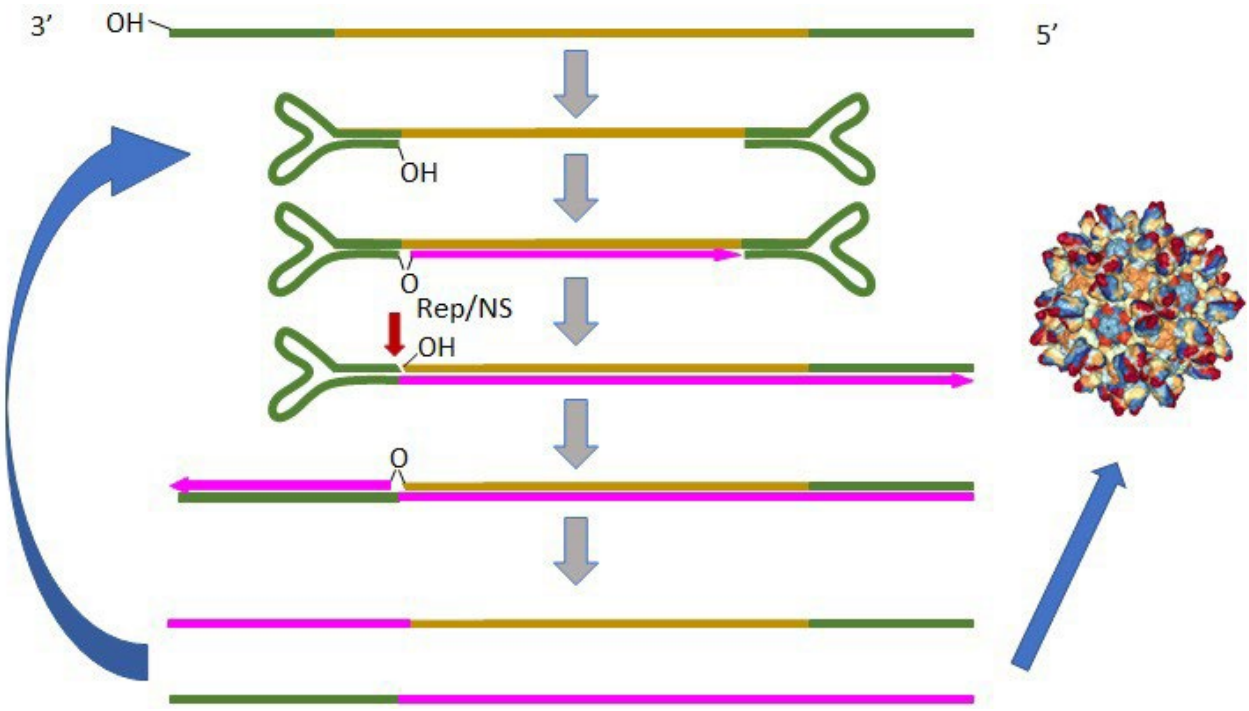


Figure 6-4. Parvovirus DNA replication using the stem loop structures at the 3' end. Courtesy of Max Drummond, Colorado State University.

VIRION FORMATION

Viral capsids form spontaneously in the nucleus. The virus leaves the nucleus by diffusion through the nuclear pore and leaves the cell via cell lysis like most naked viruses.

AVOIDING THE HOST IMMUNE RESPONSE

Parvoviruses probably do not induce a significant type I interferon response primarily because they do not uncoat in the endosome thus avoiding TLR 9 pathogen recognition receptor (PRR). Nor is the viral genome DNA ever in the cytoplasm thus avoiding cGAS PRR. The virus is transcribed, capped, polyadenylated, and translated by the host cell's machinery and can avoid detection by RIG-1, the PRR that recognizes non-capped mRNAs, and MDA-5, the PRR that recognizes incorrectly capped mRNAs. Furthermore, most parvoviruses only transcribe mRNA from one strand thus avoiding creating complementary overlapping mRNAs. They are single stranded DNA viruses and do not form dsRNA. It is unclear how parvoviruses avoid the host's innate immune response. The disease is generally cleared by acquired immunity if the animal survives.

PARVOVIRAL DISEASES

The first thing to note is that parvoviruses require highly dividing cells to replicate. As a result, they target tissues that are constantly replicating, such as:

1. Stem cells in the bone marrow and lymphoid organs
2. Stem cells in the intestines (crypts of Lieberkühn)

3. Cells in the external layer of the cerebellum
4. Fetal tissues

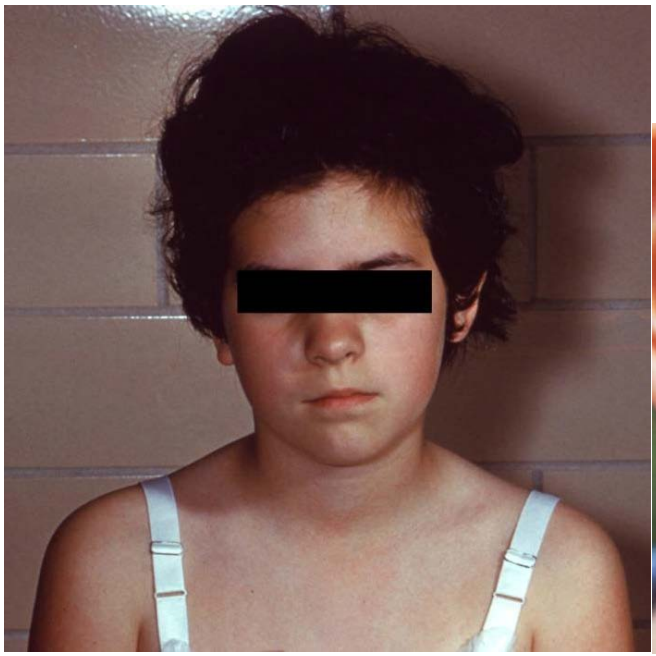
PARVOVIRAL DISEASE OF THE BLOOD

B19 is the sole parvovirus disease of humans. B19 is also called fifth disease, and it is one of the 5 very common viruses of childhood. It is also often called erythema infectiosum. B19 causes a non-specific flu like syndrome with a very specific rash that occurs due to the immunopathology of the infection. The rash is called a slapped cheek rash as the cheeks get very red, and the body can have a rash all over. See Figures 6-5 A & B. It can also cause inflammation of the joints, although this is more common in infected adults than children. This is a highly contagious and common disease, and 50-90% of adults have antibodies against this virus depending upon the geographic region.

The tropism for B19 is human **erythroid progenitor** cells (**stem cells**) which are actively dividing to create all the cell types of the blood. The infection is cytolytic and causes a decrease in erythropoiesis (creation of RBCs) for 5-7 days post infection. A sharp decline in **packed cell volume** (PCV) is seen during this 5-7 day period. When blood is spun, the packed cell volume is the number of cells that are measured. In healthy children this will cause an acute, short lived, non-life-threatening hemolytic crisis and anemia.

In patients with preexisting hemolytic anemias, such as sickle cell anemia or thalassemia, the hemolytic crisis caused by B19 exacerbates the already present anemia and can cause a complete disappearance of RBC precursors from the bone marrow as well as sudden severe and life-threatening anemia.

In patients with severe immunosuppression, such as cancer, AIDs, or transplant recipients taking immunosuppressive drugs, their inability to clear the B19 infection can lead to chronic anemia.



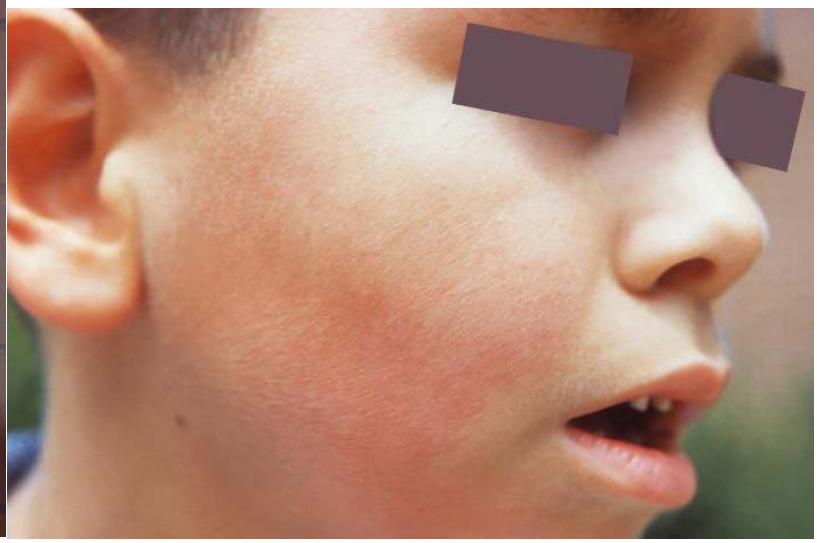


Figure 6-5 A & B. A. A view of a child's face and upper chest which displays signs of a condition known as erythema infectiosum, or fifth disease, caused by the human parvovirus B19. Note the blotchy red rash that has manifested over these areas. B. A close up of the face of a child showing the classic slap-cheeked rash of B19 infection. Courtesy of https://phil.cdc.gov/Details.aspx?pid=4507

As shown in Figure 6-6, a few days after infection viremia becomes high and is brought under control by serum IgM that is then replaced by IgG via **class switching**. As the antibody titers are increasing, the non-specific symptoms begin, most notably **pyrexia** (fever). Concurrently white blood cell (WBC) and red blood cell (RBC) numbers are dropping significantly. These numbers will recover and in fact will become elevated. As the WBC count becomes elevated, the rash and **arthralgia** (joint pain) develop and clear up as WBC counts return to normal.

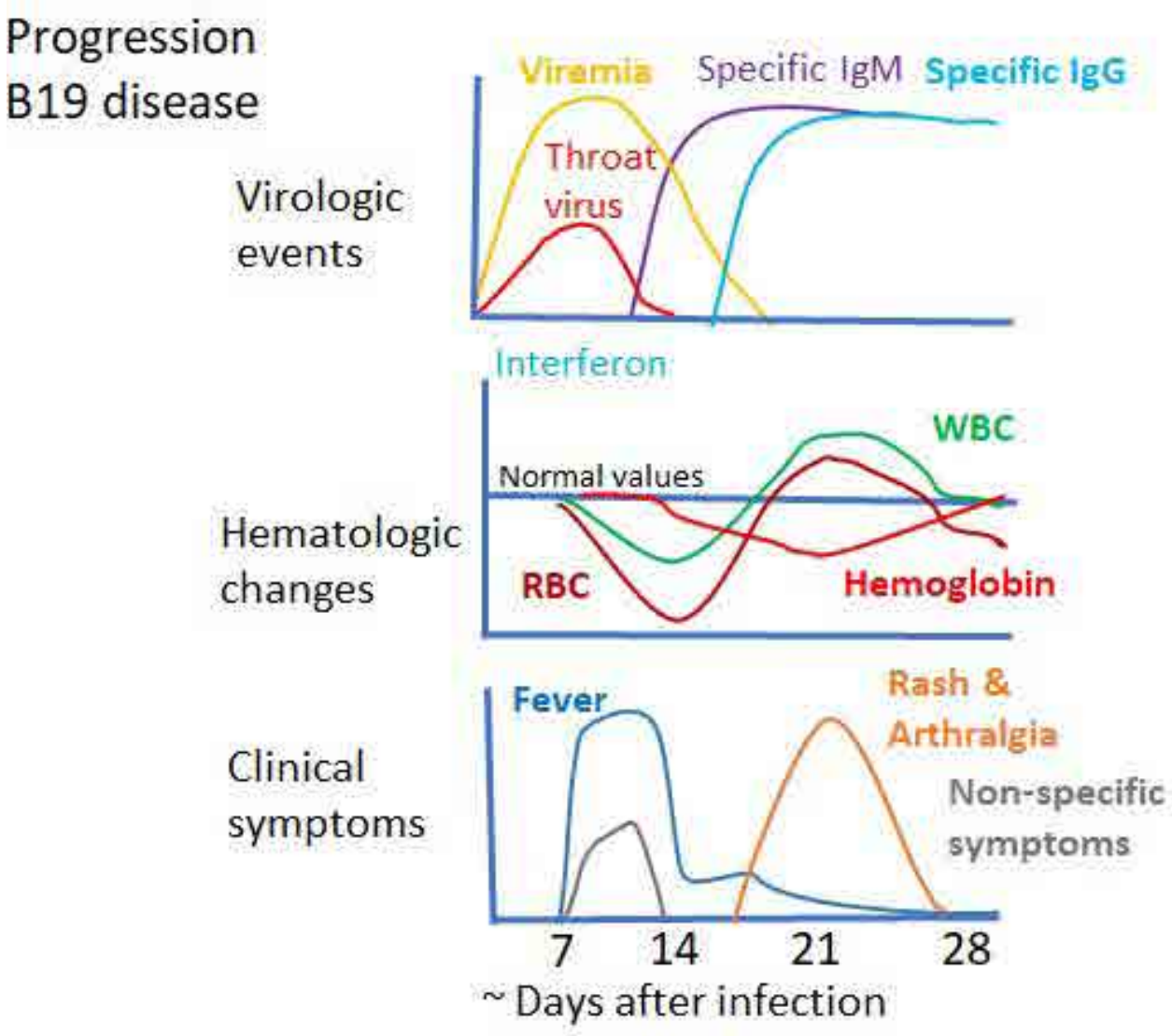


Figure 6-6. Disease progression in B19 infections. Courtesy of Erica Suchman, Colorado State University.

PARVOVIRAL INFECTIONS OF THE FETUS

Parvoviruses have the ability to cross the placenta and infect the fetus in many animals including humans, dogs, cats and pigs. This infection causes significant fetal mortality. We will begin by discussing B19 infection in pregnant mothers. If the mother gets infected for the first time, the virus can cross the placenta and infect the fetus. Note this is only an issue in primary infections, because if it is a secondary infection the mother will have memory cells that will respond to the viral infection before it can infect the fetus. The effects on the fetus are noted a few weeks

following maternal infection and will result in **hydrops fetalis**, where the fetus develops severe fetal anemia and **cardiac insufficiency** (improperly functioning heart) leading to **edema** (fluid accumulation in tissues) and fetal death.

As mentioned, a similar syndrome is seen in pigs, cats, and dogs. These can be economically devastating infections in hog farm operations or dog and cat breeding kennels. In pigs it is called **SMEDI,** for **s**tillbirth, **m**ummification, **e**mbryonic **d**eath and **i**nfertility. It is transmitted by oral-nasal contact, venereal infection (sexual), and by crossing the placenta to fetuses. Depending upon how far along the mother is when she develops an infection will determine the outcome to the fetuses.

If the mother is less than 30 days pregnant, the fetuses will die and be reabsorbed which will lead the mothers to fail to give birth and appear infertile.

If the mother is 30-70 days pregnant, the fetuses may die in utero, become mummified and aborted (often found in their pens), or be born as still births.

If the mother is more than 70 days pregnant the fetuses will normally live but will be small or stunted and as such will make poor meat animals. These animals will, however, develop an immune response and immunotolerance to future infection.

A similar syndrome is seen with canine parvovirus type 1 (CPV-1) infection with fetal death, abortions, and resorption. However, CPV-1 also causes pneumonia in neonatal puppies and mild enteritis. Note there are two genetically distinct canine parvoviruses, CPV-1 and CPV-2. Approximately 50% of adult dogs have antibodies to CPV-1.

GASTROINTESTINAL TRACT INFECTIONS BY PARVOVIRUSES

We will begin with canine parvovirus type 2 (CPV-2), but you will see that this disease is found in both dogs and cats. CPV-2 emerged in 1978 as a virgin-soil pandemic (i.e. never seen in this place before) and quickly become a worldwide disease (pandemic).

The clinical presentations are slightly different depending upon the age of the puppies when first infected. If puppies are infected as neonates (after birth) 8 to 12 weeks of age, it causes predominantly hemorrhagic enteritis as well as **leukopenia** (reduced neutrophils), **lymphopenia** (reduced WBC), fever, vomiting, anorexia, lethargy, depression, bloody diarrhea, dehydration, secondary **septicemia** (bacterial blood infections), and death. If the infections occur earlier in neonates 4 to 8 weeks of age, sudden death, **myocarditis** (swelling of heart muscles), and pulmonary edema (fluid in the lungs) are more common.

As seen in Figure 6-7A, dogs become infected when they ingest infected material contaminated with fecal material or vomitus. Within 1-2 days the virus is replicating in the local lymph nodes of the oral cavity, by days 2-4 it is replicating in the lymph nodes of the intestines, and by day 4-7 virus is being shed in the feces. Beginning at day 2 the virus begins to replicate in the **crypts of Lieberkühn** of the intestines that are found at the base of and between each villus as shown in Figure 6-7B. The crypts contain the stem cells that are constantly replicating to regenerate the mucosal layer of the gut. Remember the virus only replicates in highly replicative cells, and as such, targets these highly replicative cells at the base of the villi in the intestines. However, as these cells are killed by the lytic infection, the villi are damaged, exposing the capillaries within the villi which then bleed into the gut leading to the predominant feature, hemorrhagic diarrhea.

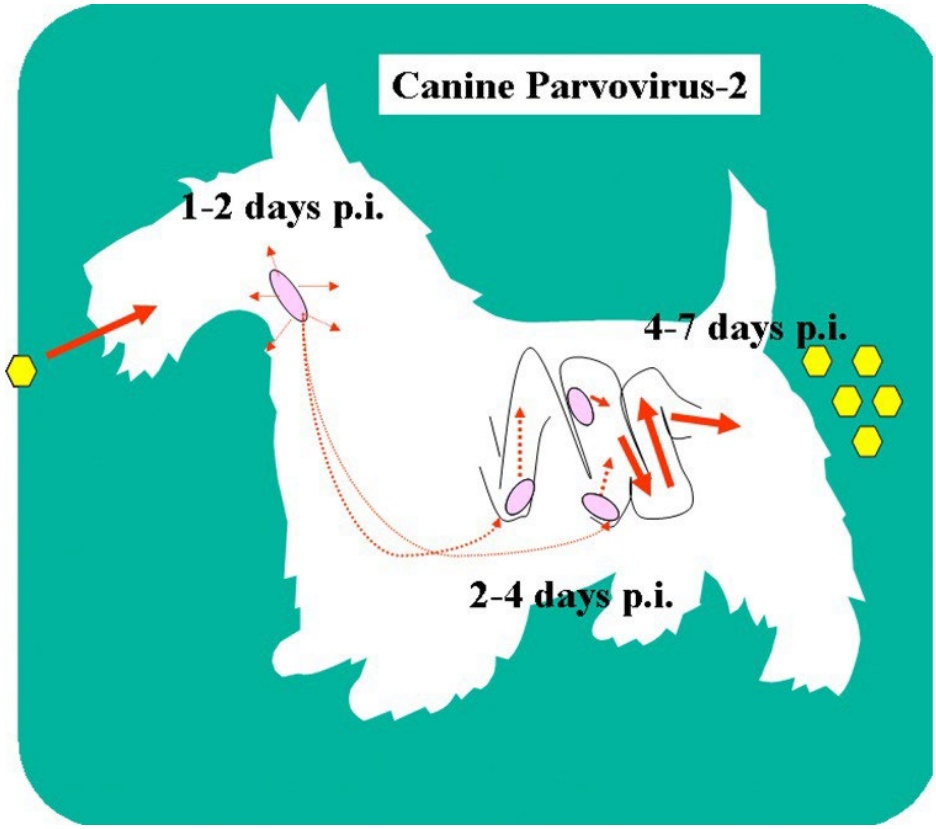


Figure 6-7A. CVP-2 infection's clinical progression. Courtesy of Erica Suchman, Colorado State University.

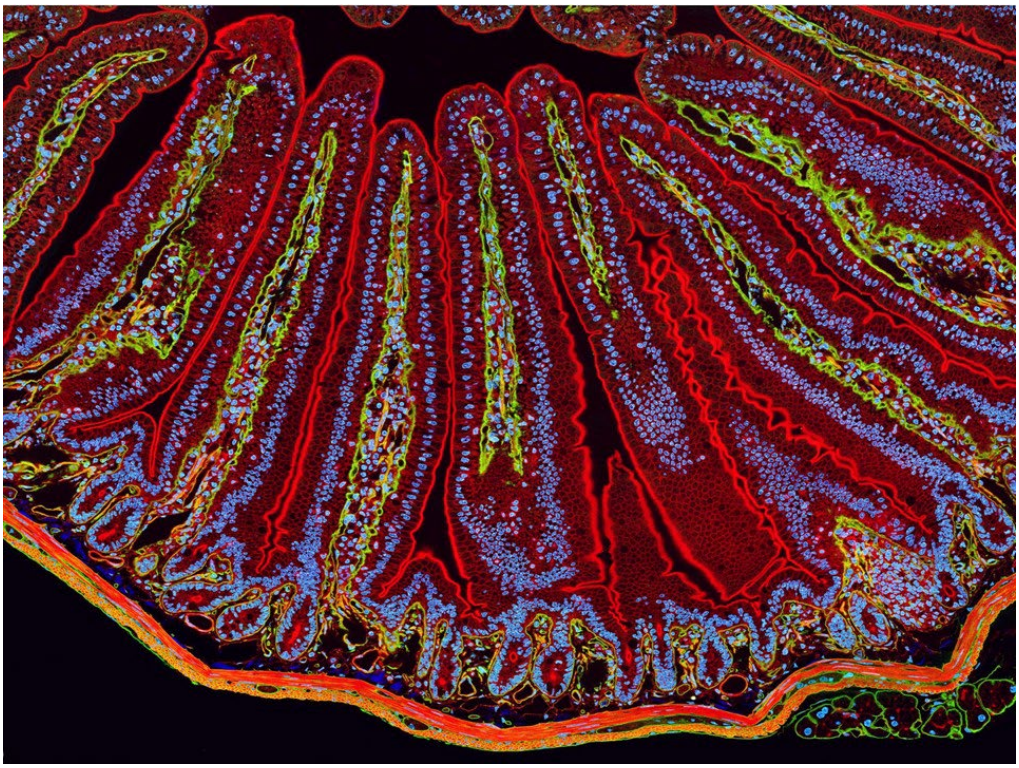


Figure 6-7B. Microscopic images of intestinal villi showing the crypts of Lieberkühn at the base between each villus. Courtesy of the National Center for Microscopy and Imaging Research https://ncmir.ucsd.edu/site/media/images/gallery/intestine-1.png

Figure 6-8 shows the immune response to the CPV-2 virus. As you can see virus shows up in the feces of infected dogs around 3-4 days post infection as the circulating WBCs decline in response to lytic infection of parvovirus in lymphoid tissue. The clinical disease starts on day 2 and if the animal survives, antibody develops and clears the infection (about 20% of untreated dogs will live). This antibody can be detected in the serum using a hemagglutination inhibition assay for diagnostic purposes. If the antibody is present, it will bind to the constant level of virus that is added to the V-bottomed plates and neutralize the virus such that it cannot bind to the red blood cells. Hence the RBCs will fall to the bottom of the well causing a button to form as described in Chapter 4.

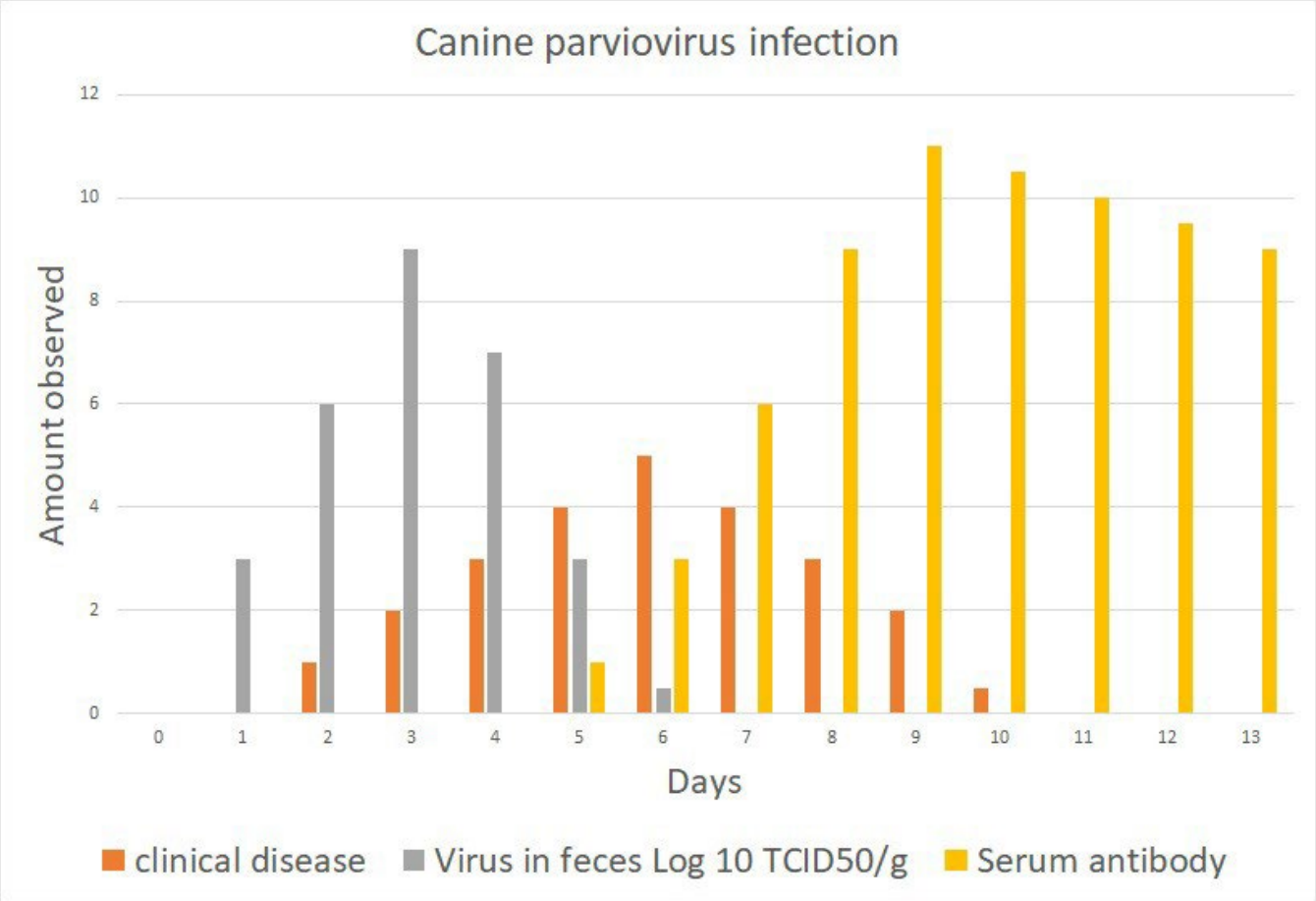
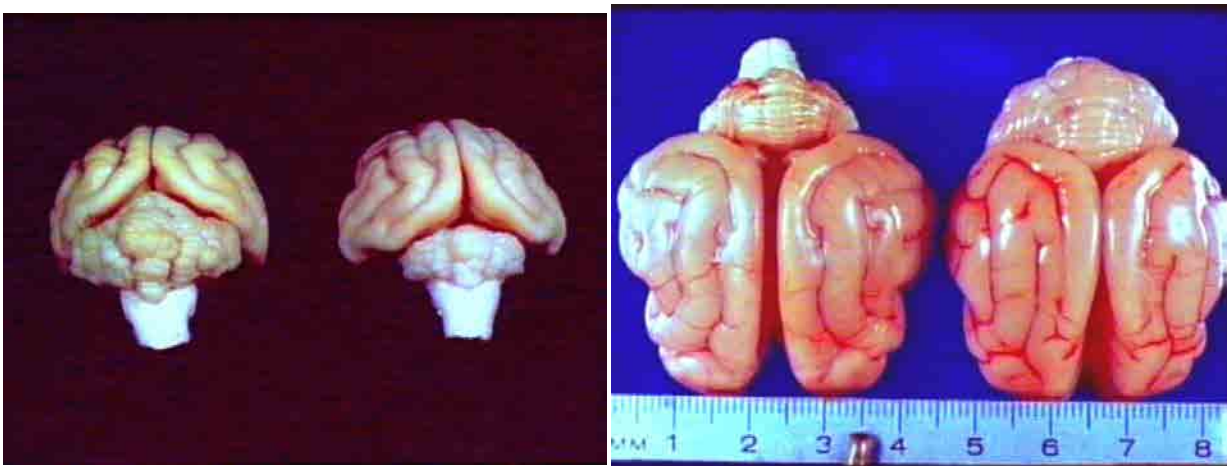


Figure 6-8. Immune response to CPV-2. Courtesy of Erica Suchman, Colorado State University.

In cats we see a similar disease called **feline panleukopenia virus**, or **feline distemper virus**. The clinical presentation of feline distemper, or panleukopenia, manifests as an acute infection with fever (>104^{O}F), lethargy, depression, anorexia and **mucopurulent** (mucus puss mix) oculonasal discharge, panleukopenia, leukopenia, lymphopenia (reduced number of different types of white blood cells), and the predominant symptom enteritis that results in bloody diarrhea and severe dehydration as shown in Figures 6-9 A & B. If a pregnant mother is infected and the kittens become infected in utero, the virus replicates in the brain and results in **cerebellar hypoplasia** (small underdeveloped cerebellum) as shown in Figures 6-10 A and B. This leads to **ataxia** (uncoordinated), **hypermetria** (over estimation of movement required for adjusting) and ultimately may cause death.

Figures 6-9 A & B. Autopsies on dogs and cats that died due to extensive damage of the intestines from parvovirus infection. Images courtesy of Tom Walton APHIS retired.



Figures 6-10 A & B. Autopsies on cats that died due to severe cerebellum hypoplasia from parvovirus infections in utero. Images courtesy of Tom Walton APHIS retired.

Figure 6-11 shows blast search data performed using the feline panleukopenia virus VP2 capsid protein gene. You will note that the feline panleukopenia virus is equally related to mink enteritis virus as it is to other strains of feline panleukopenia viruses and that only a small change in the total score occurs. There is another group of feline panleukopenia viruses that is equally related to canine parvovirus 2 (CVP-2) boosting the hypothesis that CPV-2 most likely arose from an infection of a dog with feline panleukopenia virus. Note this jump from cats to dogs occurred sometime in the late 1970s and this canine adapted virus quickly spread to other dogs and then across the globe leading to a pandemic. The original infection was called a **virgin soil infection**

as it had never been observed before. It is believed that this jump occurred in a small animal veterinary practice where cats with parvovirus were housed in close proximity to dogs. Remember these dogs were most likely at the vet for other illnesses, and many of them could have been immunosuppressed. Circumstance had created the perfect storm for the virus to make the jump from cats to dogs. Sequence data indicates that only 1 amino acid difference is seen between the parental feline panleukopenia virus and early strains of CPV-2. Thus, the new strain of parvovirus that made the jump from cats to dogs occurred by **antigenic drift** (mutations in the VP capsid proteins).

Blast search Data

Description	Total score	Query coverage	E Value
Feline panleukopenia virus capsid protein (VP2) gene, complete	3169	100	0.0
Feline panleukopenia virus gene for capsid protein complete cds, isolate: TU4	3169	100	0.0
Mink enteritis virus coat protein VP2 gene, complete cds	3169	100	0.0
Feline panleukopenia virus gene for capsid protein 2, complete cds, isolate: Fukagawa	3164	100	0.0
Canine parvovirus isolate CPV-04/08/CN-1 capsid protein VP2 gene, complete cds	3164	100	0.0
Feline panleukopenia virus strain 42/06-12 capsid protein gene, complete cds	3164	100	0.0
Feline panleukopenia virus strain 42/06-G4 capsid protein gene, complete cds	3164	100	0.0

Figure 6-11. Blast search results using feline panleukopenia virus VP2 capsid gene generated using https://blast.ncbi.nlm.nih.gov/. Courtesy of Erica Suchman, Colorado State, University.

DIAGNOSIS OF PARVOVIRAL DISEASES

Definitive diagnosis is often not necessary as B19 is usually a mild self-limiting disease of children. If diagnosis is necessary, however (for example in pregnant mothers, children with sickle cell anemia, thalassemia or severely immunosuppressed individuals), it is usually done by

using serological assays, such as an ELISA, looking for neutralizing antibodies. For veterinary diagnosis an ELISA is the common method of diagnosis. Like all serological assays, if they are assaying for neutralizing IgG antibodies, samples must be taken 2 weeks apart (**acute** and then **convalescent**) and the titer must rise at least 4-fold over the period of time to indicate a current or recent infection (Chapter 4).

PREVENTION OF PARVOVIRUS INFECTIONS

Prevention of parvovirus infections is very difficult. This is a naked virus, and, like many naked viruses, it is very hardy and can survive for long periods of time (weeks!) outside the body and on surfaces. This is different from enveloped viruses which are generally more sensitive to heat, drying, and UV light and don't last long outside the body unless protected by fluids. Fortunately, there are effective vaccines for canines, cats, and pigs. Note, however, that there is no vaccine for B19 virus as the vast majority of infections are acute and non-life threatening. In dogs there are live attenuated and killed versions of CPV-2 vaccines. The live vaccine is the recommended version as it has better immunity and fewer side effects. The adjuvants added in the killed vaccine sometimes cause an adverse response. For cats there are both live attenuated and killed vaccines that are thought to work equally well.

Have you ever wondered why you have to give infant animals (including humans) so many vaccine **boosters**? This is because maternal antibodies interfere with vaccine action. The virus components are neutralized by the maternal IgG (that came across the placenta) and IgA that came from breast feeding, and as such cannot activate a memory immune response. You must therefore give multiple vaccines because you do not know when maternal antibodies will be gone. Thus the vaccine effectiveness differs from animal to animal. For example, for most vaccines if we assay when babies will lose maternal antibodies:

- 25% of animals the antibody is gone by 6 weeks,
- 40% of animals the antibody is gone by 9 weeks,
- 60% of animals the antibody is gone by 13 weeks,
- 80% of animals the antibody is gone by 16 weeks

The recommended vaccine schedule for dogs is at 8, 12, 16, 20 weeks, and then annually. For cats, it is 8, 12, 16 weeks, and then annually. This is done so that if a puppy or kitten loses the antibody at 10 weeks, they won't go a long time before being vaccinated and being able to produce their own antibodies. It is far cheaper to vaccinate repetitively than isolate antibodies from the blood over and over to determine the optimal time to vaccinate young animals and human babies.
You will note that these vaccines are then given annually. Why might this be? Some viruses can undergo **antigenic drift** (mutations of antigens that change them and reduce the effectiveness of the memory response) or **antigenic shift** which in viruses occurs when the virus has a segmented genome and undergoes reassortment. As a result, new vaccines will need to be developed to account for these changes. Influenza is a great example of this moving target. Its

constant mutation and reassortment necessitates new vaccines each year. However, parvoviruses, which are DNA viruses, do not mutate that rapidly when compared to an RNA virus like the orthomyxovirus that causes influenza. So why vaccinate every year? The vaccine is not that strongly antigenic and as such requires boosters as the memory cells that are created have been shown to not last for long periods of time. They need booster vaccines to keep them stimulated and maintain adequate levels of blood antibody.

So, if an animal or child gets a parvovirus infection, what are the best ways to prevent the spread of the virus? This is very difficult to achieve and accordingly these highly contagious viruses tend to spread to many other animals from a single infected animal. They have a high reproductive rate (R0). R0 is the number of other animals an infected animal is likely to infect. The best control methods are to quarantine sick animals, clean up feces or vomit quickly, disinfect areas where they animal has been with a 3% bleach solution (allow to stay in contact with area for approximately 10 minutes), and be very careful about **fomites** (inanimate objects) that can transmit the virus. Be sure these are cleaned as well. And, lastly, the virus can be transmitted on clothing and moved to new areas on shoes. So, changing clothes and shoes frequently may be required to get outbreaks under control.

TREATMENT OF PARVOVIRUS INFECTIONS

Treatment of human infections is just supportive care until the infection clears. In pigs, there is no treatment; they simply wait for the virus to run its course. In dogs and cats treatment is very expensive, so prevention is a much better option. If vaccination has not been performed, then the treatment will be IV fluid replacement for 3-7 days in a hospital. With treatment in dogs, 80% recovery is achievable, however many owners opt not to pay for this expensive treatment. Without treatment in dogs, an 80% case-fatality rate is normally seen. In cats without treatment, a slightly different mortality rate is observed with kittens experiencing a 75-90% case-fatality rate and adult cats showing a 50% case-fatality rate. Unlike dogs, in cats with treatment, the survival rate in kittens is still poor and only slightly better in adults. Veterinarians indicate that kittens and cats often lose the will to live, stop eating, and pass away. For both dogs and cats, it is important to note that the general health of the animal at time of infection affects outcome as does the nutritional status with better nutrition and less infections with parasites leading to better outcomes.

END OF CHAPTER QUESTIONS

1. What enzymes does parvovirus use to reproduce the genome and transcribe mRNA? Are these enzymes cellular or viral?
2. How do parvoviruses initiate replication of their genome and ensure replication of the ends of the genome?
3. Does this virus create subgenomic mRNAs? Why, or why not? If so, how?
4. How do parvoviruses obtain a 5’ cap and poly A tail?

5. Do parvoviruses need to have mechanisms for overcoming RNA interference? If so, why? (and what are they)? If not, why not?
6. Can parvoviruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
7. How do parvoviruses deal with needing host cells to be in the S phase in order to reproduce?
8. What PRR are most likely to recognize parvoviral infections? Do parvoviruses have mechanisms to avoid theses PRR? If so, what are they?
9. Why are parvovirus infections difficult to control?
10. Are there effective treatments for parvovirus infections? If not, why not?
11. What do parvoviral diseases have in common?
12. If most children easily clear B19 infections, why is the infection often lethal in children with sickle cell anemia?
13. What about children with AIDs?
14. What is a virgin soil infection?
15. What is responsible for the rash and arthralgia often associated with B19 infections?
16. What is the best protection for avoiding kittens born with hypocerebellum?
17. Why do canine and feline parvoviral infections cause bloody diarrhea?
18. Why do kittens born of mothers who have feline parvovirus exhibit hypermetria?
19. What is the relationship between CPV-2 and feline panleukopenia virus?

REFERENCES:

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapter 57

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1975). B19 rash. https://phil.cdc.gov/Details.aspx?pid=4507

National Center for Microscopy and Imaging Research. https://ncmir.ucsd.edu/site/media/images/gallery/intestine-1.png

U.S. National Library of Medicine, National Center for Biotechnology Information. Blast. https://blast.ncbi.nlm.nih.gov/

ViralZone. Dependoparovirus. https://viralzone.expasy.org/226

Chapter 7: Medium-Sized Linear Double Stranded DNA Viruses That Replicate In The Nucleus: Adenoviridae

INTRODUCTION TO THE FAMILY *ADENOVIRIDAE*

You will notice in Figure 7-1 that adenoviruses are medium sized (36-38 kb), linear, double stranded DNA viruses that are naked, icosahedral viruses (Figure 7-2) between 60-80 nm in size that replicate in the nucleus.

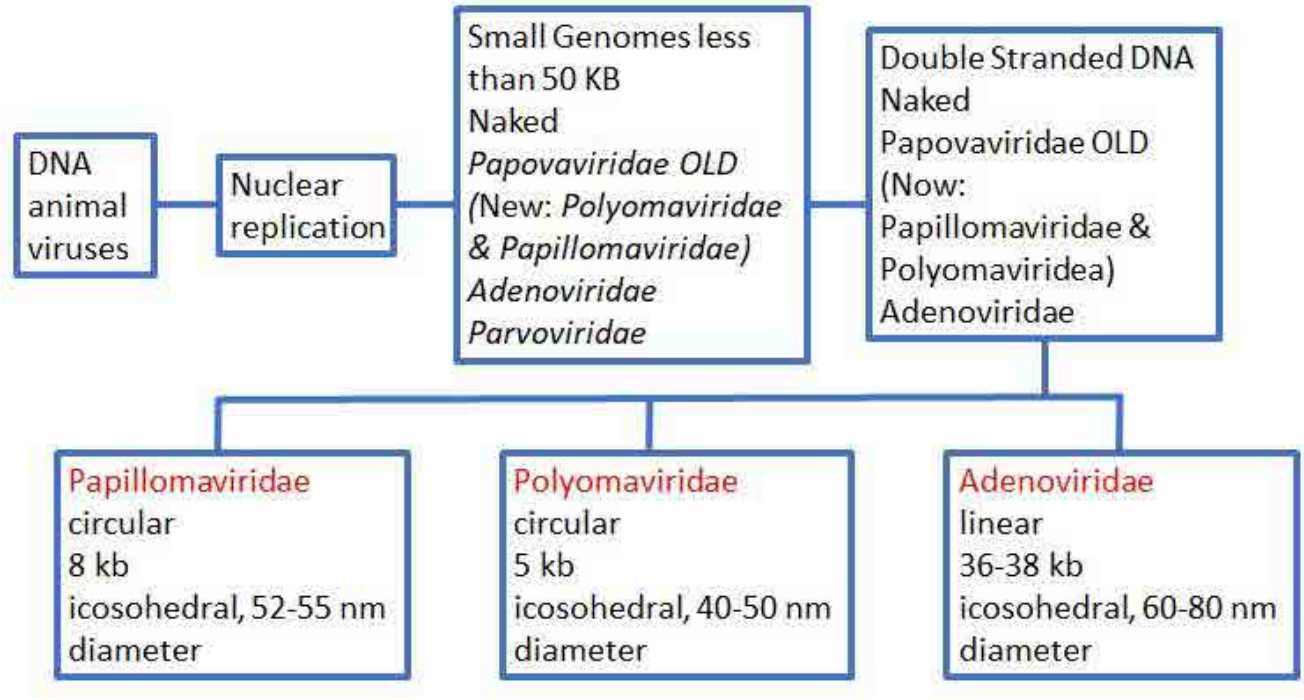


Figure 7-1. Taxonomy of DNA viruses emphasizing adenoviruses. Courtesy of Erica Suchman, Colorado State University.

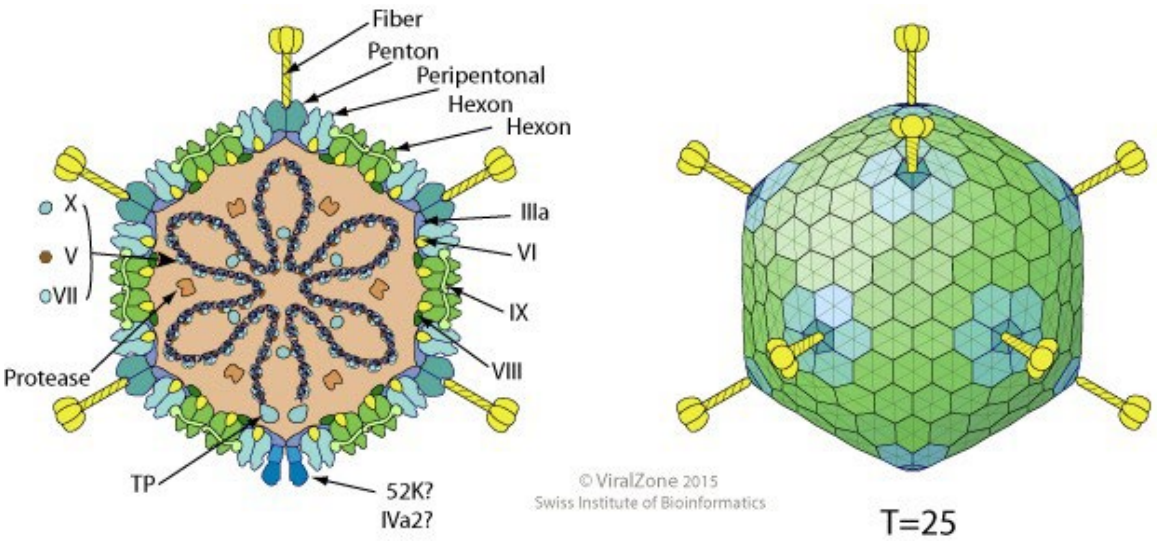


Figure 7-2. A typical adenovirus virion. Courtesy of ViralZone https://viralzone.expasy.org/4

These viruses were first isolated from adenoid tissue. They are pathogens that infect the respiratory, gastrointestinal, ocular, and urogenital tracts. They are highly species specific.

There are *Adenoviridae* genera with *Mastoadenovirus* infecting mammals including humans. There are 7 species of adenoviruses (A-G) with 57 serotypes that infect humans. The serotypes are defined by the amino acid sequence of the major capsid protein which contains the neutralizing epitopes.

The four other genera (*Aviadenovirus, Atadenovirus, Siadenovirus, Ichtadenovirus*) infect birds, reptiles, marsupials, fish, and mammals but not humans.

GENOME STRUCTURE

The 36-38 kb genome is linear, double stranded DNA with a terminal protein covalently attached to the 5‘ end of both strands. Six different promoters transcribe early and late genes using both strands, and alternative splicing creates multiple mRNAs from each promoter.

VIRAL REPLICATION CYCLE

As shown in Figure 7-3, the adenovirus replication cycle begins when

1. The naked capsid proteins bind to adhesion cellular receptor proteins.
2. They are endocytosed into clatherin-coated vesicles. The virus leaves the endocytic vesicle by lysis, still within its capsid.
3. The virus is transported via microtubular by dynein towards the nucleus, the viral genome is still protected by the core proteins .
4. The capsid is large enough that it gets stuck on the wider cytoplasmic side of the nuclear pore. The virus then uncoats, releasing its genome within the nucleus.
5. Like all DNA viruses, the first thing it must do is transcribe viral mRNA to produce viral proteins. The host cell RNA pol II will transcribe most of the viral proteins with the exception of VA (a PKR regulator) which is required to block anti-viral cellular defenses in small quantities and is transcribed by RNA pol III at low levels. Note that VA blocks PKR activity (PKR shuts down translation and inhibits interferon activity) thus allowing translation of viral proteins.
6. Again, like all viruses, the host cell's translation machinery will translate the viral proteins. These proteins will need to have signals to be transported to the nucleus where the virus replicates.
7. Like all DNA viruses, adenoviruses require a replicative cell that is in the S phase of the cell cycle. One of the early proteins of adenovirus, E1A, has the ability to push cells into the S phase thus allowing the virus to replicate in many tissues that normally do not undergo cell replication. Furthermore, E1A activates transcription of other early genes. Adenoviruses express many other early genes predominantly involved in suppressing the innate immune response, interferon response, and apoptosis as well as activating transcription and carrying out DNA replication. Adenoviruses need to carry genes for producing a viral DNA-dependent DNA polymerase (DdDp). The viral DdDp is used to replicate their DNA as they use a 5' pTP protein to prime DNA replication and ensure the ends are replicated completely. The late genes will be involved in packaging and exit from the cell via lysis.
8. Once large quantities of viral DNA and capsid proteins have accumulated, the virus will self-assemble in the nucleus and will leave the cell by lysis.

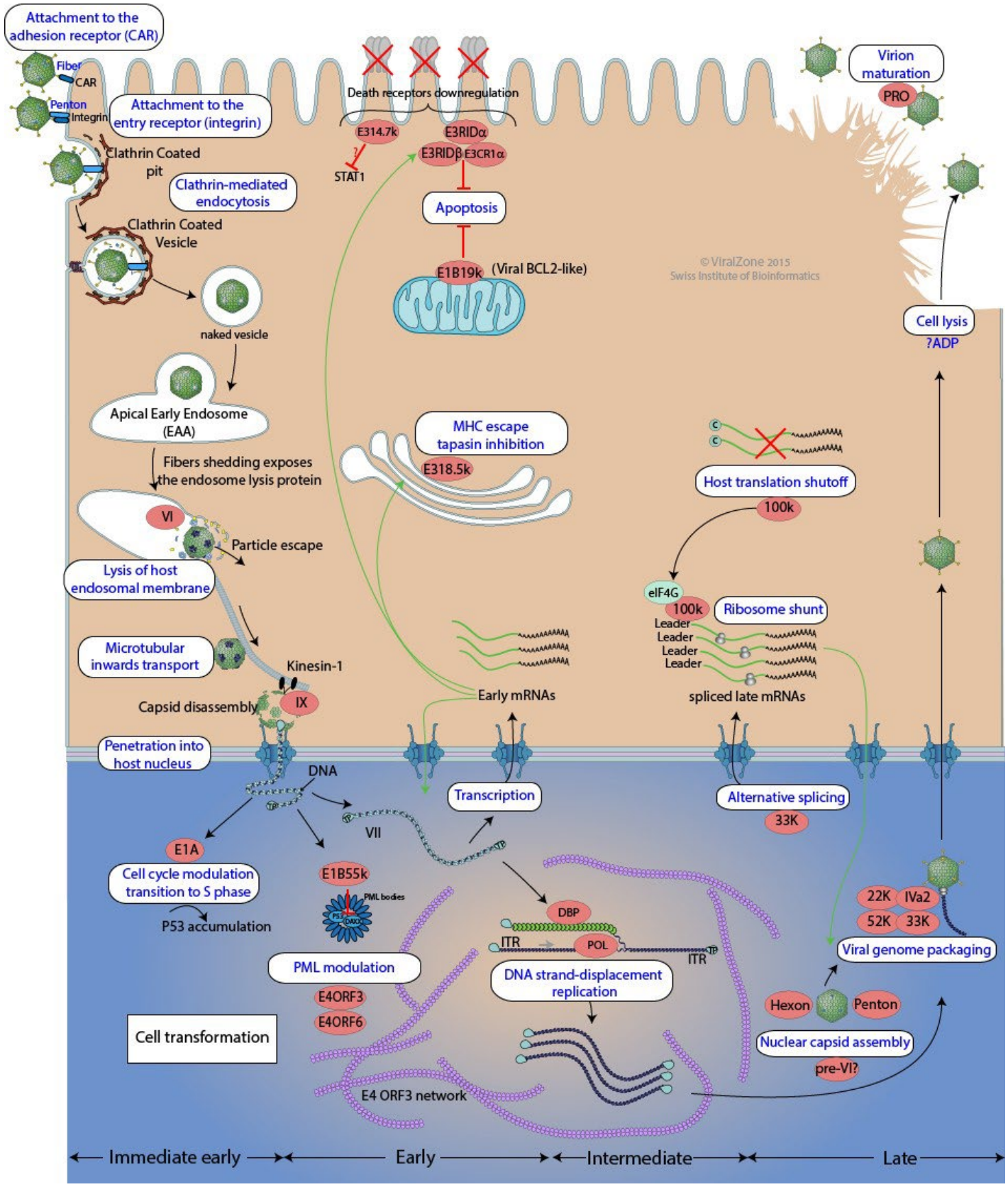


Figure 7-3. Adenovirus replication cycle. Courtesy of ViralZone https://viralzone.expasy.org/4378

HOW DO ADENOVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all nuclear replicating DNA viruses of animals, adenoviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells (Figure 7-4). In particular:

1. Using multiple promoters to create many mRNAs. Adenoviruses have more than 1 promoter like most DNA viruses. Adenoviruses have 6.
2. Using alternative splicing to create many smaller versions of a pre-mRNA (subgenomic mRNA) all of which produce different proteins.
3. Expressing mRNA from both strands of the DNA. You will note that figure 7-4 shows promoters expressing mRNAs from both strands as indicated by arrows pointing in opposite directions.

Adenoviruses create over 30 proteins using these 3 mechanisms.

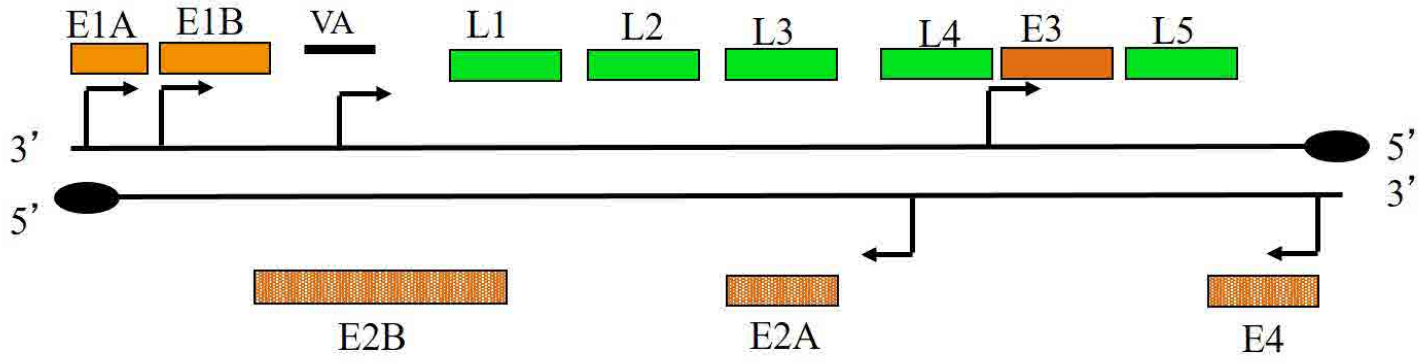


Figure 7-4. Mechanisms used by adenoviruses to overcome the one protein per mRNA issue in eukaryotic cells. These include transcription from multiple promoters on both strands, and splicing. Courtesy of Sandra Quackenbush, Colorado State University.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that will be recognized by the necessary initiation factors (such as eIF4E and G). Over 30 different proteins will be produced, some of which will be discussed below. Adenoviruses, like many viruses, regulate the timing of expression of their genes. There are early (E) genes and late (L) genes that are transcribed by cellular RNA pol II. RNA pol III is used to transcribe VA. The early genes are involved in **a**) pushing the host cell into the S phase so the virus can replicate, **b**) inhibiting the interferon response, **c**) inhibiting killing by CTL cells by decreasing MHC class 1 expression, **d**) inhibiting apoptosis, **e**) the terminal protein pTP needed for priming DNA replication, **f**) the viral DdDp that carries out replication, and **g**) DBP, a single-stranded DNA binding protein that helps facilitate DNA replication. VA binds to the interferon α induced PKR and blocks its ability to phosphorylate the translation initiation factor eIF2α to turn off translation of host and viral proteins. The late gene products are the capsid proteins and capsid spikes as well as a death protein that will lyse the cell releasing the virus. Note the spikes of the capsid cause epithelial cells to loosen their contacts thus allowing the virus to be shed into the mucus.

DNA REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA:

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Adenoviruses overcome this issue by pushing the cell into the S phase, so they are replicating. Note that although adenoviruses produce their own DdDp, they still use some of the host cell's replication machinery and hence still require the host cell to be in the S phase to replicate. The early gene products of adenoviruses induce the cells to enter the S phase by binding to and inactivating p53, a tumor suppressor gene that helps regulate the cell cycle, causing it to pause and not continue into the S phase. p53 also induces apoptosis, hence the cell will not die in response to infection allowing the virus to continue replication.
2. How to prime their DNA replication. Note that adenoviruses, like many other viruses, solve the problem of replicating the ends and priming replication via the same mechanism. They use a viral pTP protein as the primer, Figure 7-5. Because they use pTP to prime DNA replication they must carry genes to create their own DdDp as the host cell DNA pol II (DdDp) will not recognize the pTP primer.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes. Figure 7-5 shows adenovirus DNA replication using the 5' pTP protein as the primer thus solving both the priming and end replication issue.

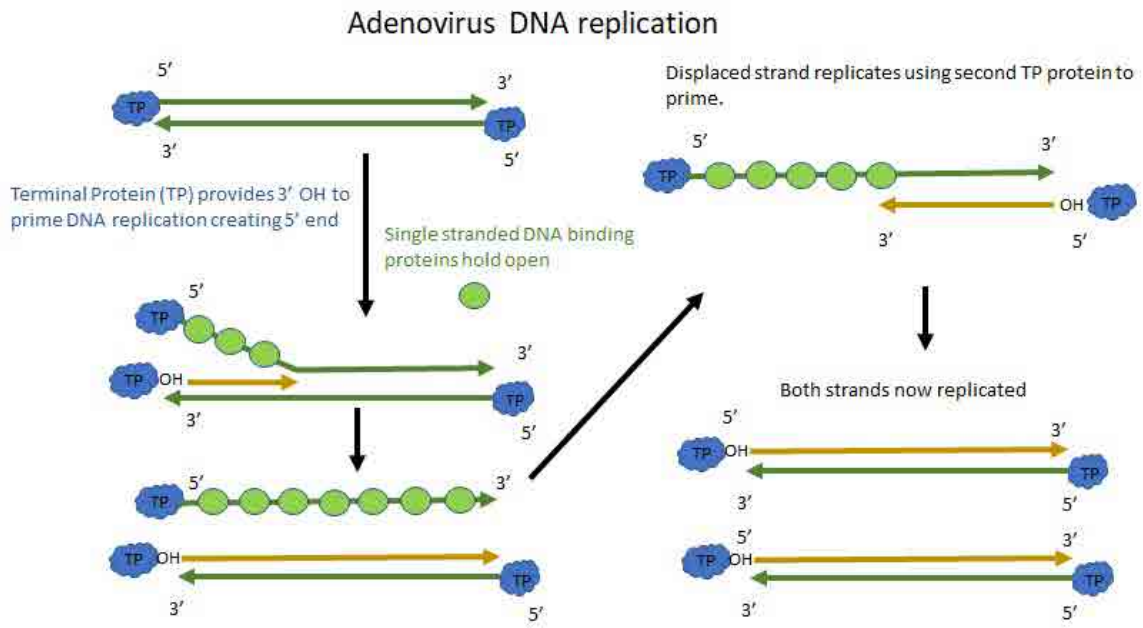


Figure 7-5. Adenovirus DNA replication using the 5′ TP protein as the primer thus solving both the priming and end replication issues. Courtesy of Dr Erica Suchman, Colorado State University.

VIRION FORMATION

Viral capsids form spontaneously in the nucleus, and the virus leaves the cell via cell lysis as is true for most naked viruses. Adenoviruses create a late protein called the death protein that lyses the cell.

AVOIDING THE HOST IMMUNE RESPONSE

Adenoviruses avoid detection by the pathogen recognition receptor (PRR) TLR9 by remaining in their capsids in the endosome as TLR9 recognizes endosomal DNA. They avoid detection by the PRR cGAS by remaining in their capsids as they travel through the cell cytoplasm as cGAS recognizes cytoplasmic DNA. They uncoat after getting stuck at the cytoplasmic side of the nuclear pore sending the dsDNA genome into the nucleus. Note that because the virus transcribes genes from both strands that overlap, mRNAs are created that are complementary and can bind each other thus activating type 1 interferon production. The early accessory genes and VA protein helps to overcome this response. Figure 7-6 shows the typical infectious adenovirus cycle.

You will note that the virus begins making early proteins within 5 hours, and that during this time the virus begins to shut down the host cell's DNA replication. This allows it to compete with

the host cell DNA for necessary replication machinery. Also, the virus begins shutting down the host cell's antiviral defenses. The late proteins begin to appear at about hour 10. By hour 15, completed infectious virus begins to appear, at which point the virus stops inhibiting PKR allowing inhibition of host cell translation. The virus no longer needs the host cell's translation machinery since it is no longer making viral proteins and instead is focused on creating new virus particles and leaving the cell.

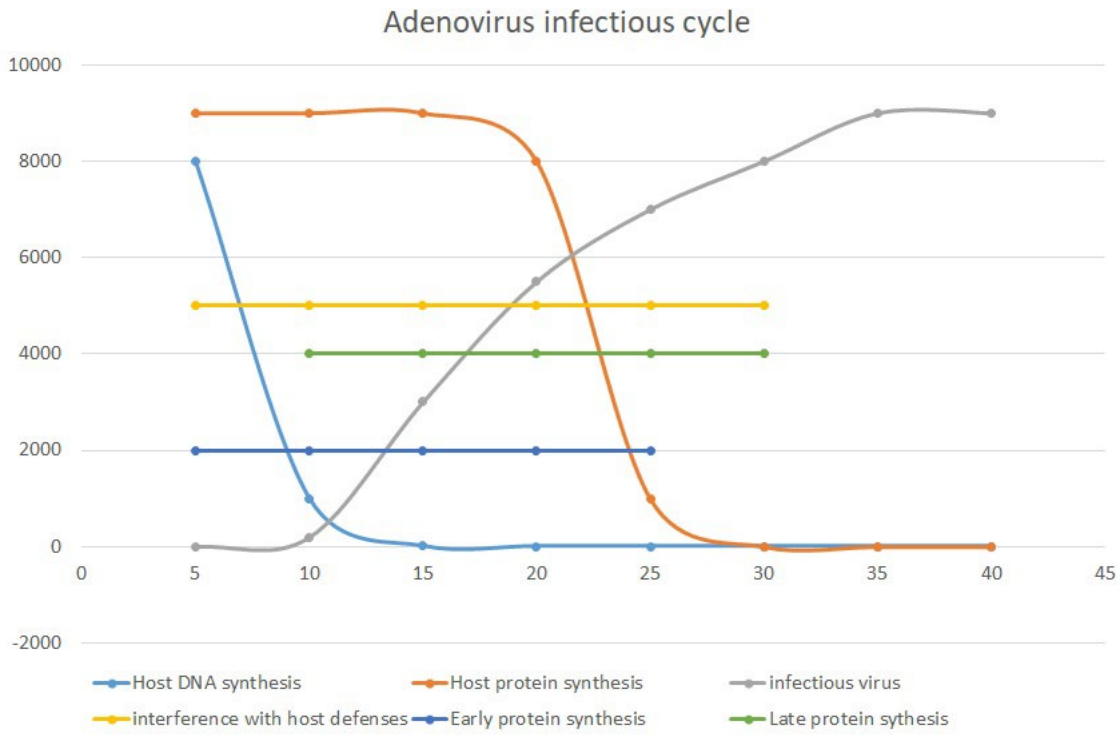


Figure 7-6. The adenovirus infectious cycle. Courtesy of Erica Suchman, Colorado State University.

ADENOVIRAL DISEASES

Remember there are 57 serotypes of adenovirus that infect humans. These occur in 7 subfamilies (A-G). Each subfamily has a different part of the body where infections are generally found, although there is much overlap between the locations. In general, they are transmitted by the respiratory route, fecal oral route, or direct contact. They infect the upper respiratory tract (colds) or lower respiratory tract (pneumonia), gastrointestinal tract (**enteritis**), eye (**conjunctivitis**), and less frequently the bladder (**cystitis**). The virus can establish persistent infections in the tonsils, adenoids, and intestines, and an infected person may shed virus for months to years.

DIAGNOSIS OF ADENOVIRAL DISEASE

Adenovirus infections are very difficult to distinguish from other respiratory viruses and are often diagnosed solely on their symptoms rather than the virus family (for example, cold, cystitis, enteritis, conjunctivitis) as there is no real treatment. Definitive diagnosis is of limited use clinically. If deemed necessary, for example in a major outbreak in a hospital, nursing home or armed forces facility, they can be diagnosed by:

1. Inoculation of cell cultures looking for classical CPE or nuclear inclusions.
2. Looking for viral nucleic acids via PCR reaction.
3. Looking for presence of neutralizing antibodies by either hemagglutination inhibition or serum neutralization. Like all IgG antibody tests, a 4-fold increase in neutralizing antibody titer must be observed in samples taken at least 2 weeks apart, as described in Chapter 4. Or an IgM and IgG titer above the threshold must be observed.

PREVENTION OF ADENOVIRUS INFECTIONS

Prevention of infection is very difficult. This is a naked virus, and, like many naked viruses, it is very hardy in the environment and can survive for long periods of time (weeks!) outside the body unlike enveloped viruses which are more sensitive to heat, drying, and UV light, and don't last long outside the body unless protected by fluids. There is no vaccine for the general public. There is, however, a live oral adenovirus (types 4 and 7) vaccine that is used on military personnel who are susceptible to outbreaks due to their high-density and stressful living conditions.

TREATMENT OF ADENOVIRUS INFECTIONS

Treatment of human infections is limited to supportive care until the infection clears. No other treatments are generally required because the infections are usually not life threatening and are self-limiting.

ADENOVIRUSES AS VACCINE VECTORS

Adenoviral vectors have been researched as possible vaccine vectors for diseases such as Ebola, AIDS, Zika, Malaria and Tuberculosis and COVID-19 due to their ability to infect a broad array of tissues (**broad tissue trophism**). Furthermore, they are not known to integrate into the host genome and deletion of the E replication proteins and replacement with genes of vaccine proteins can render the virus unable to replicate while still being able to deliver expressible vaccine genes to host cells. Vaccine virus is produced in cell lines that produce the missing E proteins allowing the virus to reproduce producing the vaccine which can infect new cells and create vaccine proteins but not reproduce.

END OF CHAPTER QUESTIONS

1. What enzymes do adenoviruses use to reproduce the genome and transcribe mRNA? Are these enzymes cellular or viral?
2. How do adenoviruses initiate replication of their genome and ensure replication of the ends of the genome?
3. Does this virus create subgenomic mRNAs? Why, or why not? If so, how?
4. Why do parvoviruses and adenoviruses utilize different mechanisms for entering the nucleus?
5. How do adenoviruses avoid the host antiviral responses?
6. How do adenoviruses obtain a 5' cap and poly A tail?
7. Must adenoviruses have mechanisms for overcoming RNA interference? If yes, why, and what are they? If not, why not?
8. Can adenoviruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
9. Compare and contrast the mechanisms parvoviruses and adenoviruses utilize for avoiding the antiviral response.
10. PKR is activated by ds RNA. Adenovirus creates VA that inhibits PKR activity. Why do adenoviruses need to produce VA when they are DNA viruses?
11. Compare and contrast how parvoviruses and adenoviruses deal with needing host cells to be in the S phase to reproduce.
12. What types of gene products are likely to be expressed by early genes and late genes?
13. How does VA expression differ from most DNA virus genes? Why might it be expressed in this manner?
14. What PRR are most likely to recognize adenoviral infections? Do adenoviruses have mechanisms to avoid these PRR? If so, what are they?
15. There are 57 serotypes of adenovirus. Do they all cause the same symptoms? If so, what are they? If not, what are the types of symptoms observed in different infections?
16. You perform a hemagglutination inhibition assay to determine if an army recruit is suffering from an adenovirus infection. You find that the recruit has a titer of 400 HI units/0.1 ml, which is above the threshold. Two weeks later you take blood samples from the recruit again. This time, the titer is 400 HI units/0.1 ml. Do you believe this recruit is currently suffering from an adenovirus infection?
17. Why are adenovirus infections difficult to control?
18. Are there effective treatments for adenovirus infections?
19. What is the significance of p53? Why do adenoviruses want to regulate it?

REFERENCES

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, chapter 56

Mendonca, S.A., et al. 2021 Adenoviral vector vaccine platforms in the SARS-CoV-2 pandemic. NPJ Vaccines vol 6:97. doi: 10.1038/s41541-021-00356-x
https://www.nature.com/articles/s41541-021-00356-x

ViralZone. *Adenoviridae*. https://viralzone.expasy.org/4

ViralZone. Human adenovirus type C replication cycle. https://viralzone.expasy.org/4378

CHAPTER 8

Chapter 8: Small Circular DNA Viruses That Replicate In The Nucleus: Polyomaviridae and Papilomaviridae

INTRODUCTION TO THE FAMILIES *POLYOMAVIRIDAE* AND *PAPILLOMAVIRIDAE*

You will notice in Figure 8-1 A & B that polyomaviruses and papillomaviruses are small sized, naked, icosahedral DNA viruses with circular, double stranded DNA that replicate in the nucleus.

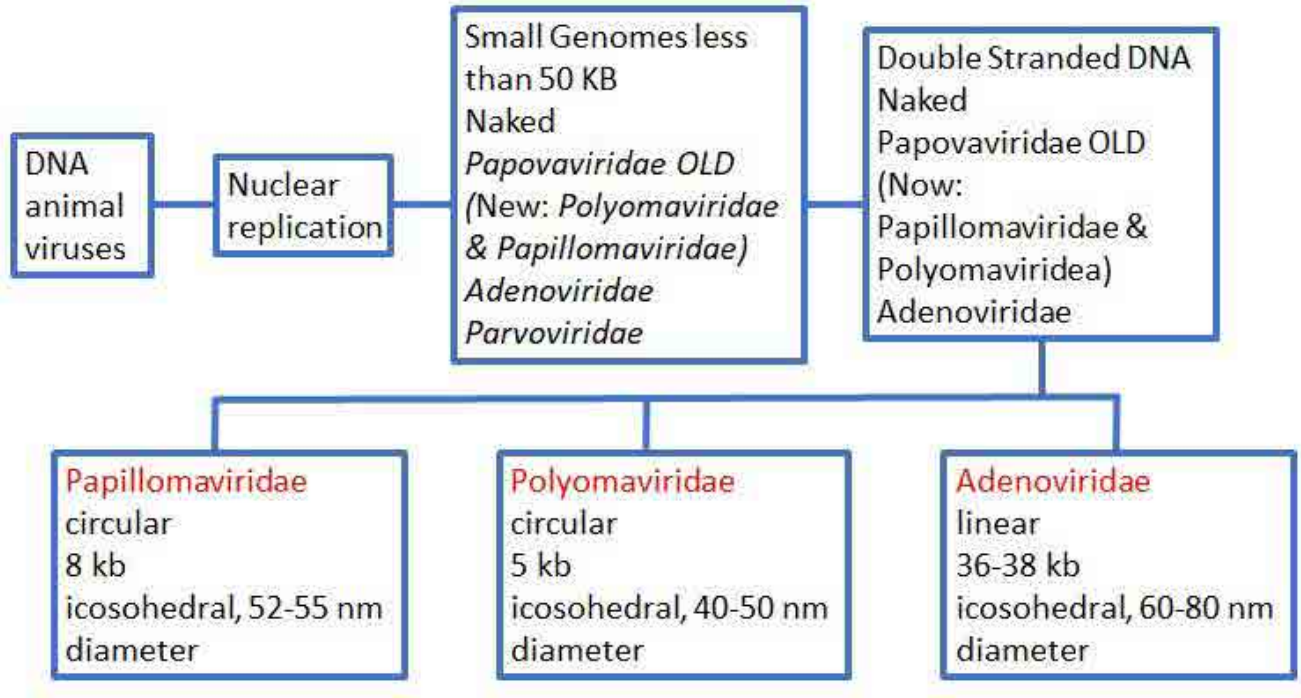


Figure 8-1A. Taxonomy of DNA viruses emphasizing polyomaviruses and papillomaviruses. Courtesy of Erica Suchman, Colorado State University.

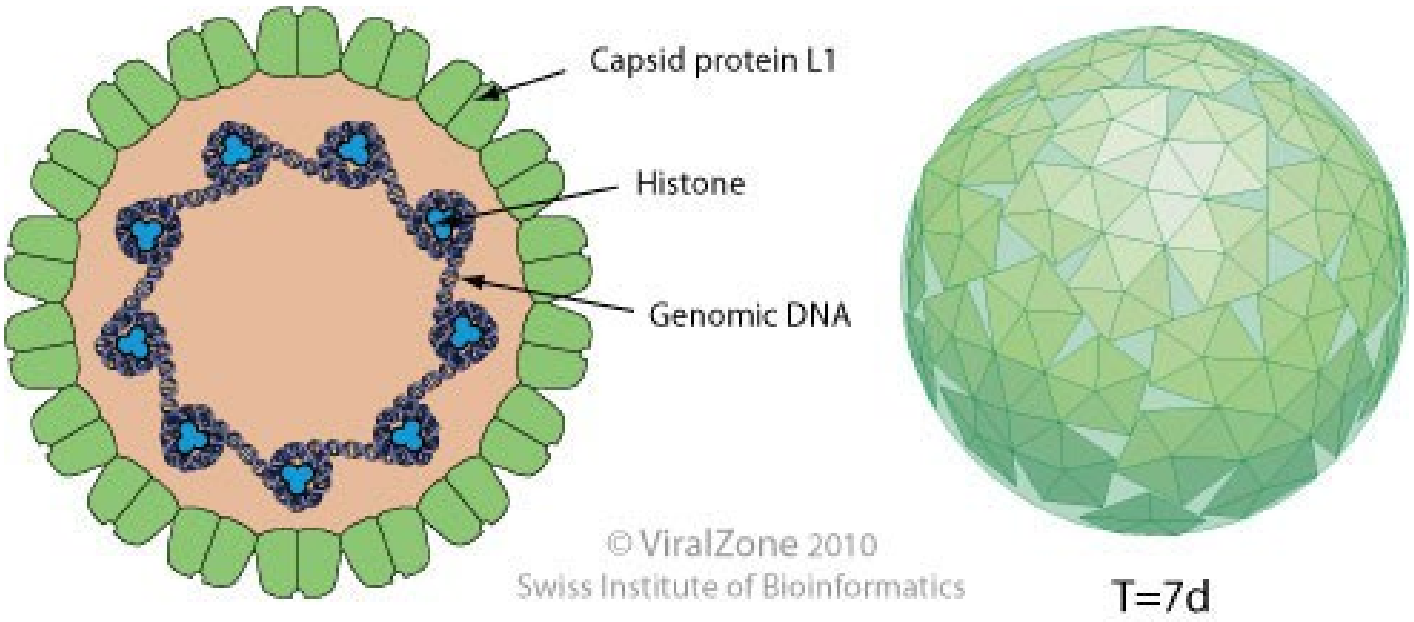


Figure 8-1B. A typical polyomavirus or papillomavirus virion. Courtesy of ViralZone https://viralzone.expasy.org/148

POLYOMAVIRIDAE

The virions are non-enveloped and 40-50 nm in diameter. They are made up of the capsid proteins VP1, VP2, VP3 and contain an approximately 5 kb double stranded circular DNA genome that is associated with cellular histone proteins. These viral particles are relatively resistant to heat, formalin inactivation, and lipid solvents. The family has one genus, *Polyomavirus,* that contains many viruses that infect animals and humans. These viruses have a narrow host range due to presence or absence of necessary receptors and host factors that permit viral gene expression and replication. These viruses frequently cause persistent asymptomatic latent infections.

GENOME STRUCTURE

Polyomaviruses possess a circular dsDNA genome about 5 kb in size and are associated with cellular histones in a chromatin-like complex. It encodes 5-9 proteins. Upon rare non-specific recombination, the viral genome can be integrated in the host chromosome. Integration inactivates the virus but can give the host cell a replicative advantage sometimes leading to malignant tumors. Transcription is nuclear in two phases: early, where the early genes are expressed (those required for replication and controlling host cell antiviral responses) and late, where the late genes are expressed (those involved in virion assembly and exit). The 5-9 proteins are expressed from the two pre-mRNAs by alternative splicing. All genes are transcribed by host RNA pol II (DdRp).

VIRAL REPLICATION CYCLE

As shown in Figure 8-2, during the polyomavirus replication cycle:

1. The naked capsid proteins bind to cellular receptors that contain glycolipids or sialic acid.

2. The capsids are endocytosed into either caveolae or clatherin-coated vesicles. The virus escapes the endocytic vesicle when the pH drops, and they leave the vesicles still within a complete capsid.
3. The capsid is disassembled in the ER, and the nucleic acids enter the nucleus.
4. Like all DNA viruses, the first thing it must do is transcribe viral mRNA to produce viral proteins. In particular it requires the early proteins to assist in DNA replication and control of the host anti-viral response. Host cell RNA pol II (a DNA-dependent RNA polymerase, DdRp) will transcribe all of the mRNAs to produce the viral proteins including the late proteins that will be translated after DNA replication occurs and will create the capsid proteins.
5. Like all viruses, the host cell's translation machinery will translate the viral proteins.
6. Once early gene proteins have accumulated, the polyomavirus pushes the host cell to replicate, at which point the cell will contain all of the DNA replication components necessary to replicate the polyomavirus DNA. Note: this requires a replicative cell that is in the S phase of the cell cycle.
    - The early proteins include the large T-antigen that inactivates the tumor suppressor proteins Rb and p53. Without Rb and P53 the cell will move into the S phase due to loss of suppression.
    - Another early gene product, small T-antigen, regulates the cell signaling pathways that stimulate cell proliferation.
7. The DNA is replicated using a viral origin of replication that is recognized by the host cell primase that produces an RNA primer that provides the 3' OH for replication by DNA pol III (a cellular DNA-dependent DNA polymerase, DdDp). Large T-antigen (LT) also helps initiate DNA replication.
8. After the DNA is replicated, late genes are expressed including the capsid (VP) proteins and agnoprotein, a viral protein that increases permeability of the host cell membrane allowing the virus to exit the cell.
9. Newly synthesized DNA molecules will associate with host cell histone proteins.
10. Once large quantities of viral DNA is wrapped around histone proteins and capsid/VP proteins have accumulated, the virus will self-assemble in the nucleus and will leave the cell by lysis induced by the agnoprotein.

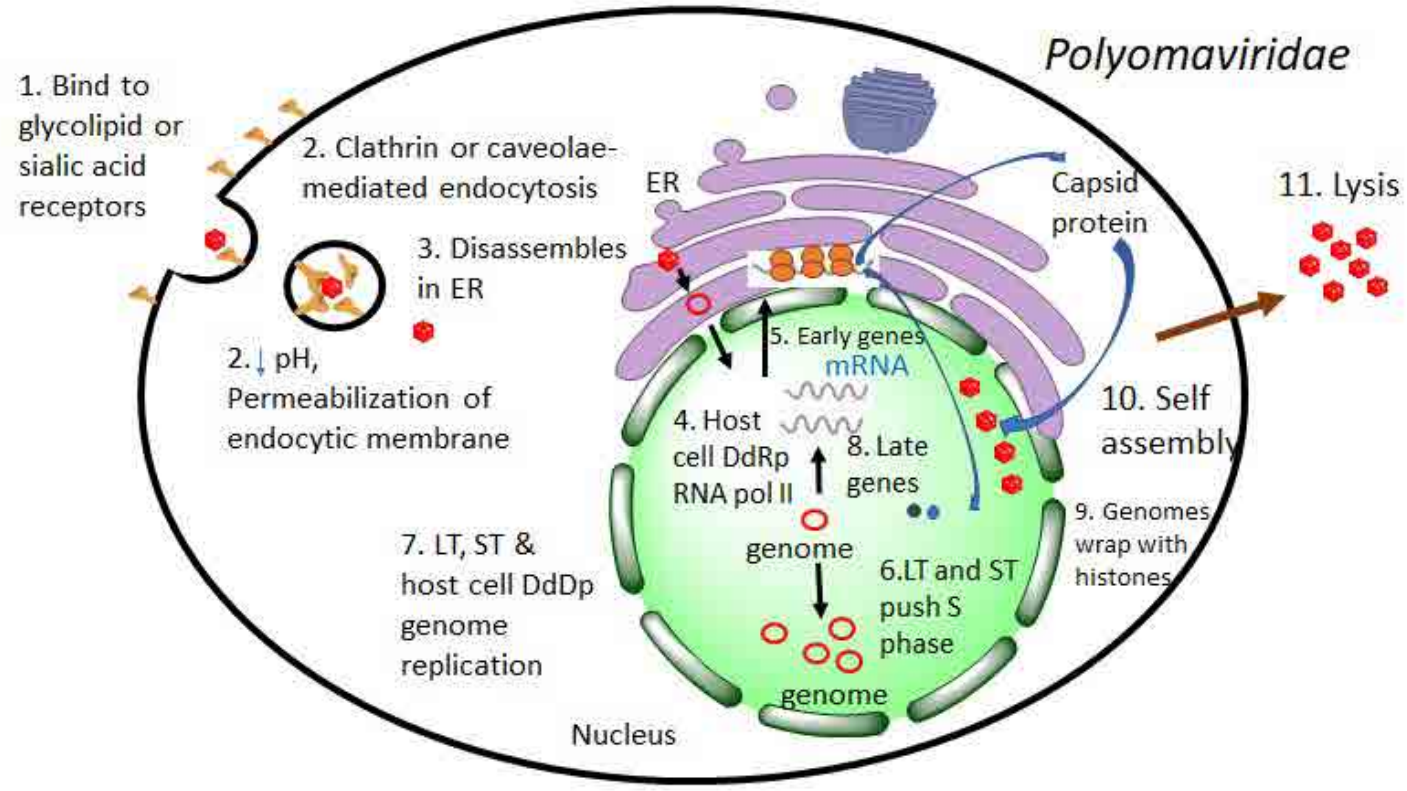


Figure 8-2. Polyomavirus replication cycle Courtesy of Erica Suchman, Colorado State University.

HOW DO POLYOMAVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

As is true for all nuclear replication of DNA viruses of animals, polyomaviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells, as shown in Figure 8-3. In particular:

1. Using multiple promoters to create many mRNAs. Polyomaviruses have more than 1 promoter. These promoters are found on both strands of the DNA, however the mRNAs created from different strands never overlap thus avoiding complementary mRNAs that might for dsRNA. This is important as this eliminates the formation of complementary mRNA that can bind and induce expression of type I interferon genes or possibly RNAi.
2. Using alternative splicing to create many smaller versions of a pre-mRNA all of which produce different proteins.

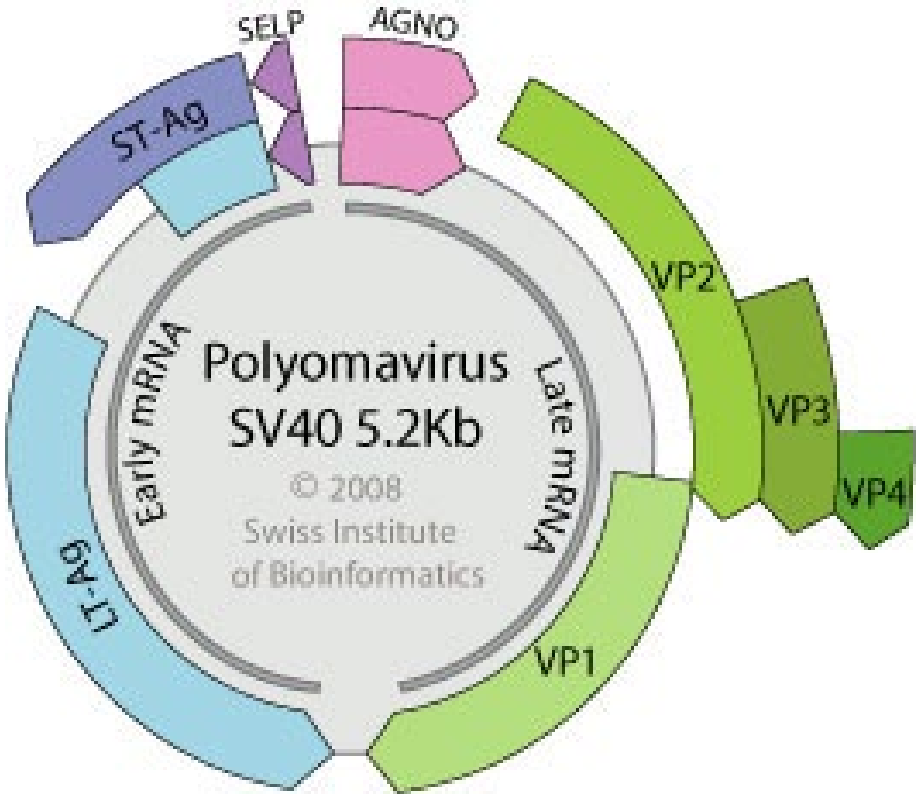


Figure 8-3. Mechanism used to overcome one protein per mRNA issue in eukaryotic cells by polyomaviruses. Courtesy of ViralZone https://viralzone.expasy.org/148

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral proteins. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that will be recognized by the necessary initiation factors (eIF4E &G and PABP, see Chapter 3). The virus will regulate production of viral proteins first synthesizing the early genes including large-T antigen and small T-antigen. Small and large T-antigen will work together to push the cell into the S phase. Remember the S phase is required for viral replication as this is a DNA virus that utilizes cellular DNA replication machinery to reproduce, and cells only produce these enzymes during the S phase of mitosis. Pushing the host cells into S phase allows polyomaviruses to replicate in non-replicative cells. Large T-antigen does this by inhibition of Rb, a protein that negatively regulates transcription of genes necessary for DNA synthesis and S phase progress. The large T-antigen also inhibits the ability of p53 to activate transcription of p21 which blocks cell cycle progression. The small T-antigen regulates kinases that are involved in cell cycle control. The late genes produce the capsid proteins and are expressed after viral DNA replication.

DNA GENOME REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA:

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Polyomaviruses overcome this issue by producing early gene products (large and small T-antigen) that push the host cell into S phase. The Large T antigen serves as a helicase opening the origin or replication as shown in Figure 8-4.

2. How to prime their DNA replication. Polyomaviruses, like other circular DNA viruses, utilize an origin of replication to replicate the viral genome and depend on the host cell's primase enzyme to provide RNA primers with a 3' OH for DNA synthesis.
3. How to replicate the ends of their genome. Because polyomaviruses are not linear, this is not an issue.

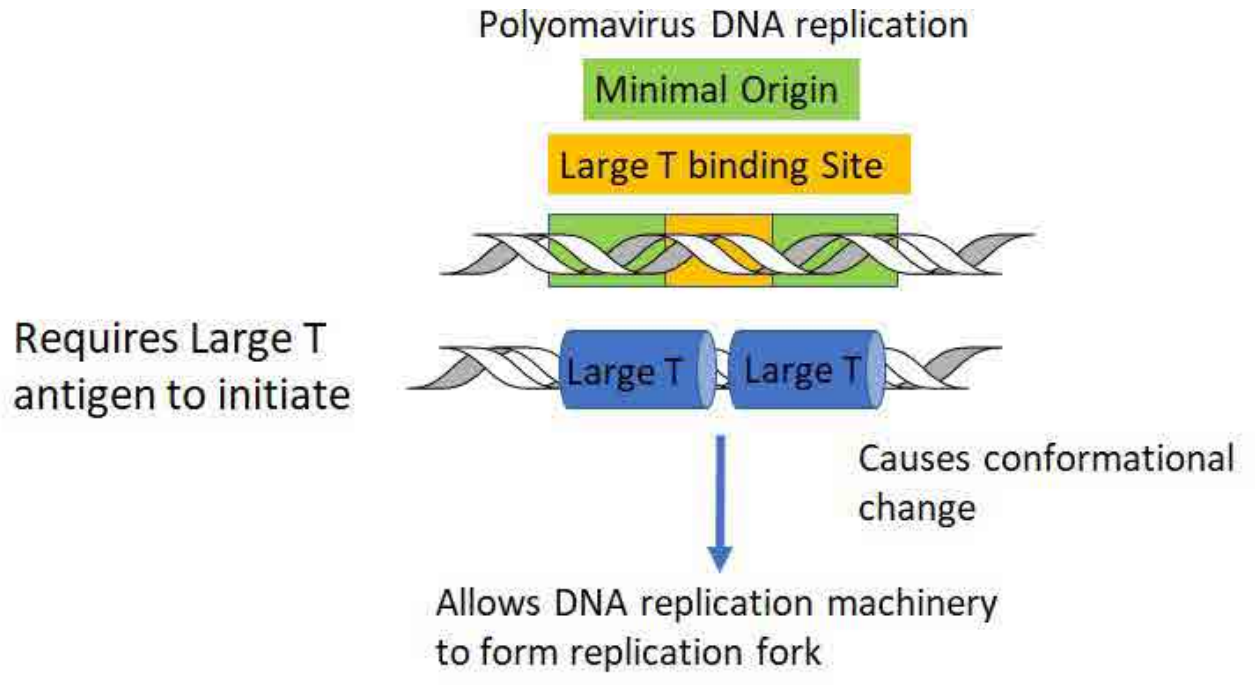


Figure 8-4. Polyomavirus DNA replication using an origin of replication and host cell DNA replication machinery with the help of the large T-antigen. Courtesy of Erica Suchman, Colorado State University.

VIRION FORMATION

Viral capsids form spontaneously in the nucleus. The virus leaves the cell when the agnoprotein causes the membrane to become permeable leading to lysis allowing the virus to escape.

AVOIDING THE HOST IMMUNE RESPONSE

Remaining in the capsid until the virus leaves the endosome avoids recognition of the **pattern recognition receptor** (PRR) TLR9 which recognizes the **pathogen associated molecular pattern** (PAMP) of endosomal DNA containing CpG. In addition, disassembly in the ER helps protect the virus from detection by the PRR cGAS which detects cytosolic double stranded DNA PAMPs. As mentioned above polyomaviruses never create overlapping complimenatry mRNAs from opposite strands. As a result, they do not produce the double stranded RNA intermediates that can activate type I interferon production and possibly RNAi as shown in Figure 8-3. The virus is transcribed, capped, poly- adenylated, and translated by the host cell's machinery and can avoid detection by RIG-1 and MDA-5 the PRRs that recognizes non-capped or improperly capped RNA PAMPs.

POLYOMAVIRUS DISEASES

Because polyomaviruses push host cells into S phase, some have the ability to cause tumors. In particular, SV40 has been studied extensively. SV40 is a virus that infects monkeys but is not associated with disease. However, SV40 was introduced into the human population in the late 1950s or early 1960s in polio vaccine produced in monkey cell lines that had an inapparent SV40 infection. In permissive cells, SV40 pushes the cell into S phase allowing the virus to replicate. The production of agnoprotein, however, leads to cell lysis. In nonpermissive cells, for example from other species of animal (such as rodents), the virus lacks the replication factors necessary for efficient replication, and the large T-antigen does not bind to DNA polymerase/primase. The viral genome can be integrated into the host cell's chromosome causing chromosomal damage and leading to the production of tumors.

JC and BK are polyomavirus infections of humans, both transmitted by fecal-oral, respiratory, blood transfusion, organ transplantation, trans placental, seminal fluid, and urine. Both persist in the kidneys and are excreted in the urine, which is thought to be a major route of transmission. Infection typically occurs in childhood. Studies show that 50% of children seroconvert for BK virus by 3-4 years of age, and JC virus by 10-15 years of age. Viral replication is restricted by the immune responses including humoral immune response IgG and cell mediated immune response when CD8+ T cells (CTLs) recognize epitopes on the capsid protein VP1. The vast majority of infections are self-limiting, although the virus often remains as a persistent latent asymptomatic infection. The disease is due to lytic infection of target cells. It is most closely associated with kidney disease in transplant recipients. Viral inclusions and inflammation can be observed in kidney transplant recipients undergoing viral re-activation. Although BK, JC, and SV40 push cells into S phase, they are not commonly associated with tumor formation. There is some data that indicates they may be associated with tumors in humans, but this is an area still under investigation, and much of the literature is contradictory or inconclusive. However, there are well documented cases where tumors have been induced in rodents by the mechanism described for SV40.

Immunosuppressed people with JC infections can develop **progressive multifocal leukoencephalopathy** (PLM). This occurs when JC virus reactivates, infects the brain, and causes a lytic infection of the myelin producing **oligodendrocytes** of the brain. Prognosis is very poor when patients develop PML with substantial mortality within 6-15 months of diagnosis. Despite the high seroprevalence of JC infections, thankfully PML is fairly rare.

Although it is unclear if SV40, BK, and JC virus can cause tumors, merkel cell polyomavirus causes merkel cell carcinoma. And, similarly to JC virus, the tumors are more frequently observed when persistent infections reactivate and push the host cells into S phase so aggressively that tumors form. As discussed with SV40, the virus is found integrated into the host cell's chromosomes in tumor cells. In merkel cell virus, it is known that the T-antigen is often truncated turning the T-cell antigen into a greatly overexpressed **oncogene** that leads to host cell transformation and skin cancer. Oncogenes are genes that when overexpressed can cause tumor formation.

PREVENTION OF POLYOMAVIRUS INFECTIONS

Unfortunately, there are no vaccines against polyomavirus infections. Most people develop infections in early childhood or adolescence and carry these viruses for life as persistent latent infections that can reactivate if we become severely immuno-suppressed.

DIAGNOSIS OF POLYOMAVIRUS INFECTIONS

Polyomavirus infections can be detected with serological assays including hemagglutination inhibition and ELISA. Like all serological assays if they are assaying for neutralizing IgG antibody, samples must be taken 2 weeks apart (acute and then convalescent), and the titer must rise at least 4-fold over the period of time to indicate a current or recent infection. They can also be confirmed with tissue biopsy and staining looking for intranuclear basophilic (blue) inclusions, or PCR of viral DNA.

TREATMENT OF POLYOMAVIRUS INFECTIONS

Treatment of human infections is supportive care until the infection clears as most of the infections are usually not life threatening and are self-limiting. However, in immunosuppressed individuals dialysis may become necessary. Note there are currently no drug treatments for polyomavirus infections.

PAPILLOMAVIRIDAE

The virions are non enveloped and 52-55 nm in diameter. They are made up of the capsid proteins L1 and L2 and contain an approximately 8 kb double stranded circular DNA genome that is associated with cellular histone proteins. These viral particles are extremely resistant to drying and lipid solvents and, as such, can survive in the environment for long periods of time. The family contains 240 distinct types classified into 37 genera that infect animals and humans. These viruses are classified on the basis of the nucleotide sequence. Some are highly associated with tumor formation, and others are not, and are grouped based on their associated tumor risk. These viruses frequently cause persistent asymptomatic latent infections.

GENOME STRUCTURE

The genome is a circular double stranded DNA about 8 kb in size and is associated with cellular histone proteins in a chromatin-like complex. Upon rare non-specific recombination, the viral genome can be integrated in host chromosome. Integration inactivates the virus (in particular the E1 and E2 genes) and can give the host cell a replicative advantage that sometimes leads to warts or malignant tumors. Only one strand of the genome is transcribed and yields two classes of proteins expressed by alternative splicing:

a) Early Proteins: non-structural regulatory proteins (Early Proteins 1-7 or E1-7).
b) Late Proteins: the structural capsid proteins (Late 1 & 2, or L1 & L2).

All genes are transcribed by host RNA pol II.

VIRAL REPLICATION CYCLE

As shown in Figure 8-5, the papillomavirus replication cycle begins when

1. The naked capsid proteins bind to **heparin sulfate proteoglycan** (HSPG) receptors found on **epithelial precursor basal cells** at the lower levels of the skin. Binding leads to a conformational change in the virion which exposes a **furin** cleavage site on the capsid protein L2. Furin is a cellular protease. Once the L2 capsid protein is cleaved, the virus will interact with one of many **coreceptors** to enter basal cells.
2. They are endocytosed into caveolin-coated vesicles. The virus escapes the endocytic vesicle when the pH drops, and they leave the vesicles still within a complete capsid.
3. The capsid is disassembled in the cytoplasm, and the nucleic acids enter the nucleus.
4. Like all DNA viruses, the first thing papillomaviruses must do is transcribe viral mRNA to produce viral proteins. In particular it requires the early proteins to assist in DNA replication and control of the host anti-viral response. Host cell RNA pol II, a DdRp, will transcribe all of the viral mRNAs to produce proteins including the late proteins that will be translated after DNA replication occurs and will create the capsid proteins.
5. Like all viruses, the host cell's translation machinery will translate the viral proteins.
6. Once early gene proteins have accumulated, the papillomavirus pushes the host cell to replicate at which point the cell will contain all of the DNA replication components necessary to replicate the papillomavirus DNA. Note: this requires a replicative cell that is in the S phase of the cell cycle. The early proteins include the regulatory proteins that force the cell into the S phase. The DNA is replicated using a viral origin of replication that is recognized by the host cell primase that produces an RNA primer that provides the 3' OH for replication by DNA pol III. The early proteins E1 and E2 (early 1 and 2) help recruit host cell replication machinery to the origin of replication.
7. After the DNA is replicated, late genes are expressed including the capsid (L) proteins.
8. Newly synthesized DNA molecules will associate with host cell histone proteins.
9. Once sufficient quantities of viral DNA is wrapped around histone proteins and capsid/L proteins have accumulated, the virus will self-assemble in the nucleus and will leave the cell by lysis.

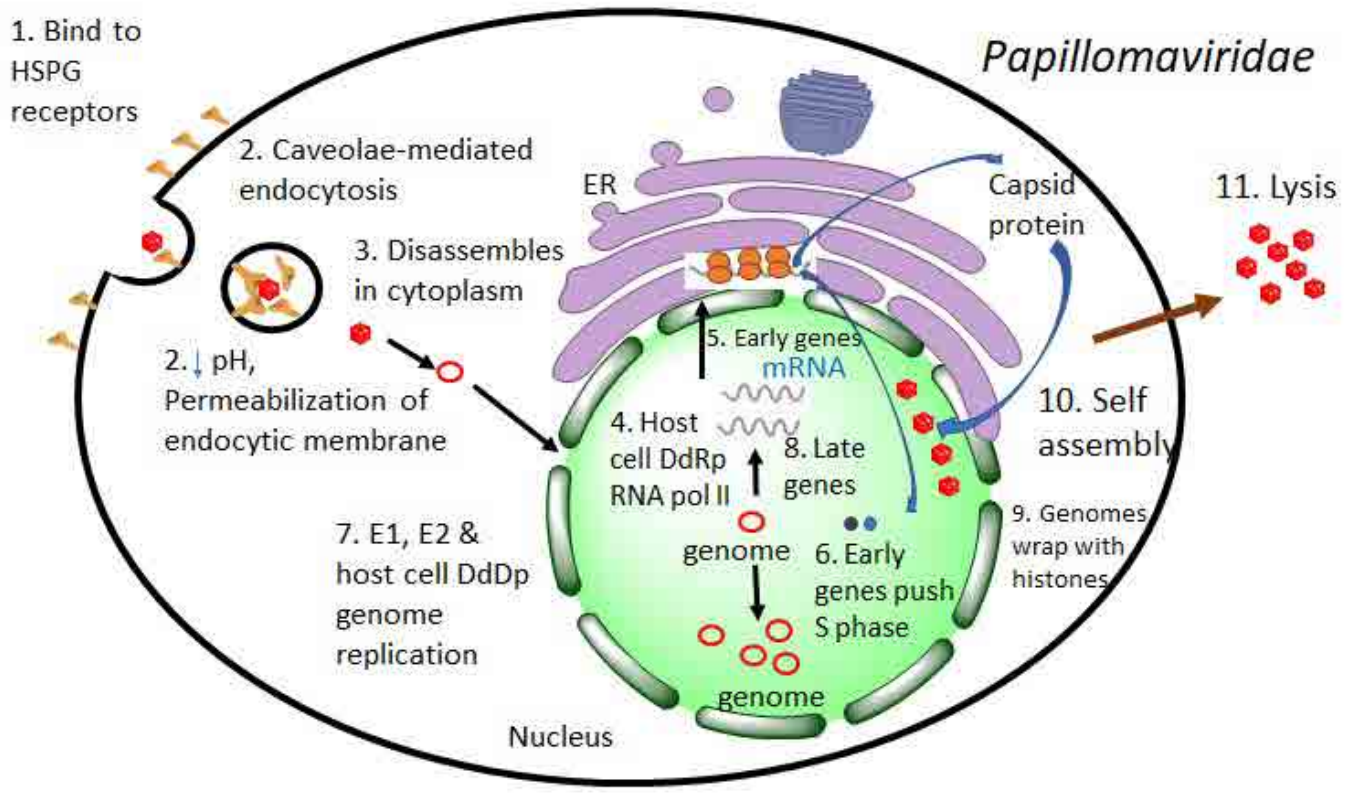


Figure 8-5. A typical papillomavirus replication cycle. Courtesy of Erica Suchman, Colorado State University.

HOW DO PAPILLOMAVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all nuclear replicating DNA viruses of animals, papillomaviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells as shown in Figure 8-6. In particular:

1. Using multiple promoters to create many mRNAs. Papillomaviruses have more than 1 promoter. These promoters are found on only one strand of the DNA ensuring the mRNAs are never complementary to each other. Many of these transcripts are overlapping, but because they are from the same strand, they are not complementary. This is important as this eliminates the formation of complementary mRNA that can bind and induce expression of type I interferon genes, activation of interferon stimulated genes or possibly RNAi.
2. Using alternative splicing to create many smaller versions of a pre-mRNA all of which produce different proteins.

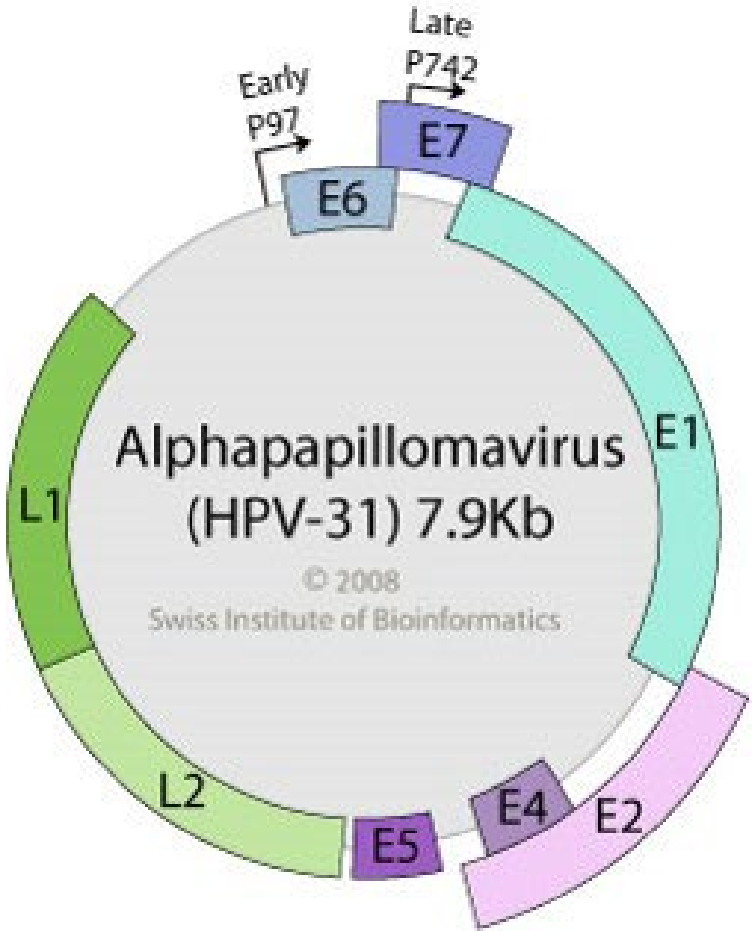


Figure 8-6. Mechanism used to overcome one protein per mRNA issue in eukaryotic cells by papillomaviruses. Courtesy of ViralZone https://viralzone.expasy.org/187

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that will be recognized by the necessary initiation factors (eIF4E &G and PABP). The virus will regulate production of viral proteins first synthesizing the early genes including E1-E7. E6 and 7 will work together to push the cell into the S phase. Remember, cells in the S phase are required for viral replication as this is a DNA virus that utilizes cellular DNA replication machinery to reproduce, and cells only produce these enzymes during the S phase of mitosis, and papillomavirus replicates in non-dividing cells. E7 pushes cells into the S phase by inhibition of Rb, a protein that negatively regulates transcription of genes necessary for DNA synthesis and S phase progress. E7 is necessary for stable maintenance of HPV episome. E6 targets p53 for degradation. Note that p53 blocks cell cycle progression. E6 also induces **telomerase** activity. In normal cell aging as the telomeres get shorter it induces the cell to enter **apoptosis**. By activating the telomerase, which adds back the telomeres E6 prevents the cell from entering apoptosis. The late genes produce the capsid proteins and are expressed after viral DNA replication.

DNA GENOME REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA.

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Papillomaviruses overcome this issue by producing early gene products (E6 and E7) that push the cell into S phase.
2. How to prime their DNA replication. Papillomaviruses, like other circular DNA viruses, utilize an origin of replication to replicate the viral genome and depend on the host cell's primase enzyme to provide RNA primers with a 3' OH for DNA synthesis. The early protein E1 helps the origin bind the host cell DNA replication machinery, while E2 forms a complex with E1 and tethers the genome to the host cell chromosome, as shown in Figure 8-7.
3. How to replicate the ends of their genome. Because papillomaviruses are not linear, this is not an issue.

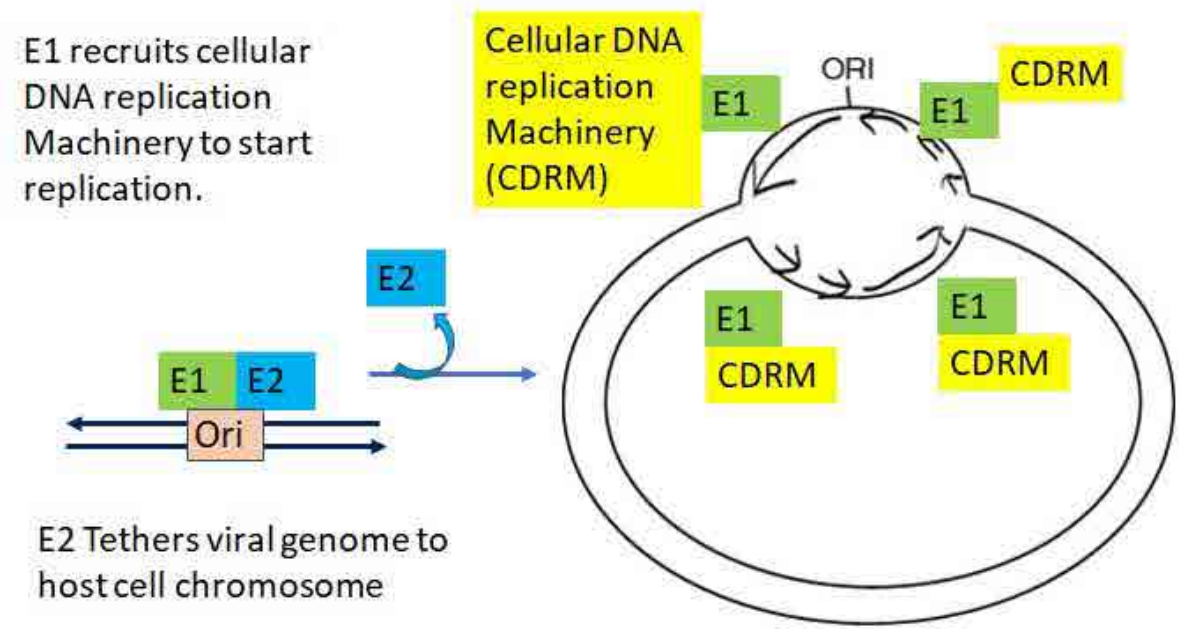


Figure 8-7. Papillomavirus DNA replication using an origin of replication and host cell DNA replication machinery. Courtesy of Erica Suchman, Colorado State University.

VIRION FORMATION

Viral capsids form spontaneously in the nucleus. The cell then lyses allowing the virus to escape.

AVOIDING THE HOST IMMUNE RESPONSE

Remaining in the capsid until the virus leaves the endosome avoids recognition of the PRR TLR9 which recognizes the PAMP of endosomal DNA containing CpG. As mentioned above, papillomaviruses never create complimentary overlapping mRNAs from opposite strands. As a result, they do not produce the double stranded RNA intermediates that can activate type I interferon production as shown in Figure 8-6 and possibly RNAi. The virus is transcribed, capped, polyadenylated, and translated by the host cell's machinery and can avoid detection by RIG-1, and MDA-5 the PRRs that recognizes non-capped or improperly-capped RNA PAMPs. Despite the fact that papillomaviruses uncoat in the cytoplasm leaving it open to recognition by cGAS a unique transport pathway protects the DNA from detection and the early gene protein E7 inhibits the cGAS activation pathway allowing the virus shut down the interferon response quickly.

PAPILLOMAVIRUS DISEASES

Because papillomaviruses push host cells into S phase, they cause warts to form on the skin, and some have the ability to cause tumors. Similar to SV40, papillomaviruses push cells into S phase allowing the virus to replicate which eventually leads to cell lysis. In some cells, however, the viral genome can be integrated into the host cell's chromosome causing deletions in E1 or E2 that lead to **dysregulated** expression of **E6 and E7,** proteins which lead to tumors. Some viral genotypes (high risk) are more likely to undergo integration and dysregulation.

However, the cell types that are infected also play a role depending on the cellular transcription factors available to the virus. Some viral types cause tumors in mucosa, others cause cutaneous tumors, and others can cause tumors in both. The types of tumors observed is in large part controlled at the level of viral gene expression rather than at the level of viral entry into the cell, and regulatory elements in the long control region are important in determining the tissue range of each viral type. Furthermore, the level of the host's immunity plays an important role. Warts occur more often in older people and women undergoing menopause. This spike in warts with age is due to reactivation of long latent infections as warts or genital warts.

People who are immunosuppressed also often have papillomavirus reactivations, some so severe that they become covered with warts and require surgery to have them removed. In very severe cases, they will become unable to use their hands or walk. As you might have guessed HPV infections in people with HIV can be quite extensive.

There are over 180 different human papillomaviruses (HPV). Papillomaviruses follow a very consistent infection progression. The virus first infects the basal epithelial cells and then enters the **establishment phase** with early gene expression and viral DNA replication resulting in 50-100 copies of viral DNA per infected cell. Note that late gene expression is very low at this point. The virus then enters the **maintenance phase** with strong E6 and E7 expression with reduced viral genome replication to once per cell cycle as the cell progresses towards the surface of the skin and becomes an epidermal epithelial cell. As the cells become more differentiated as they near the skin surface, the virus enters a **highly productive phase,** often called the **genome amplification phase** or **vegetative replication phase,** with high levels of viral DNA synthesized (in the 1000's) and late gene expression. It is during

this phase that viral particles are released from the surface of the skin or mucous membranes and can be transmitted by skin to skin contact with abrasions, both sexually and non-sexually, as shown in Figure 8-8. These infections often result in warts of the skin or mucous membranes as shown in Figure 8-9, and in certain HPV types can lead to tumor formation. The majority of HPV infections will regress due to immune responses of the CTLs which are the most important response for regression and the humoral response which helps prevent reinfection. However, it should be noted that regression and resistance to reinfection are type-specific with very little cross reactivity between viruses. Different HPV types are associated with specific tumor locations. The highest risk types for human tumors of the cervix, penis, anus, and mouth are HPV-16, 18, 31, 33, 45, 52, and 59. Note that 99% of cervical tumors are found to have one of these 7 strains, and HPV 16 and 18 account for 70% of cases. HPV 6 and 11 are commonly found in genital warts, recurrent respiratory papillomas, and conjunctival warts (of the eye) but are lower risk for tumor formation. Production of infectious virions is restricted in HPV- associated neoplasias (due to lack of E1 and E2) and accompanied by elevated E6/E7 expression. Cervical carcinoma is associated with the integration of HPV DNA genome by non-homologous recombination. Integration results in deletion of portions of the viral genome, and disruption of E1 and E2 leads to high level expression of E6 and E7, as shown in Figure 8-10.

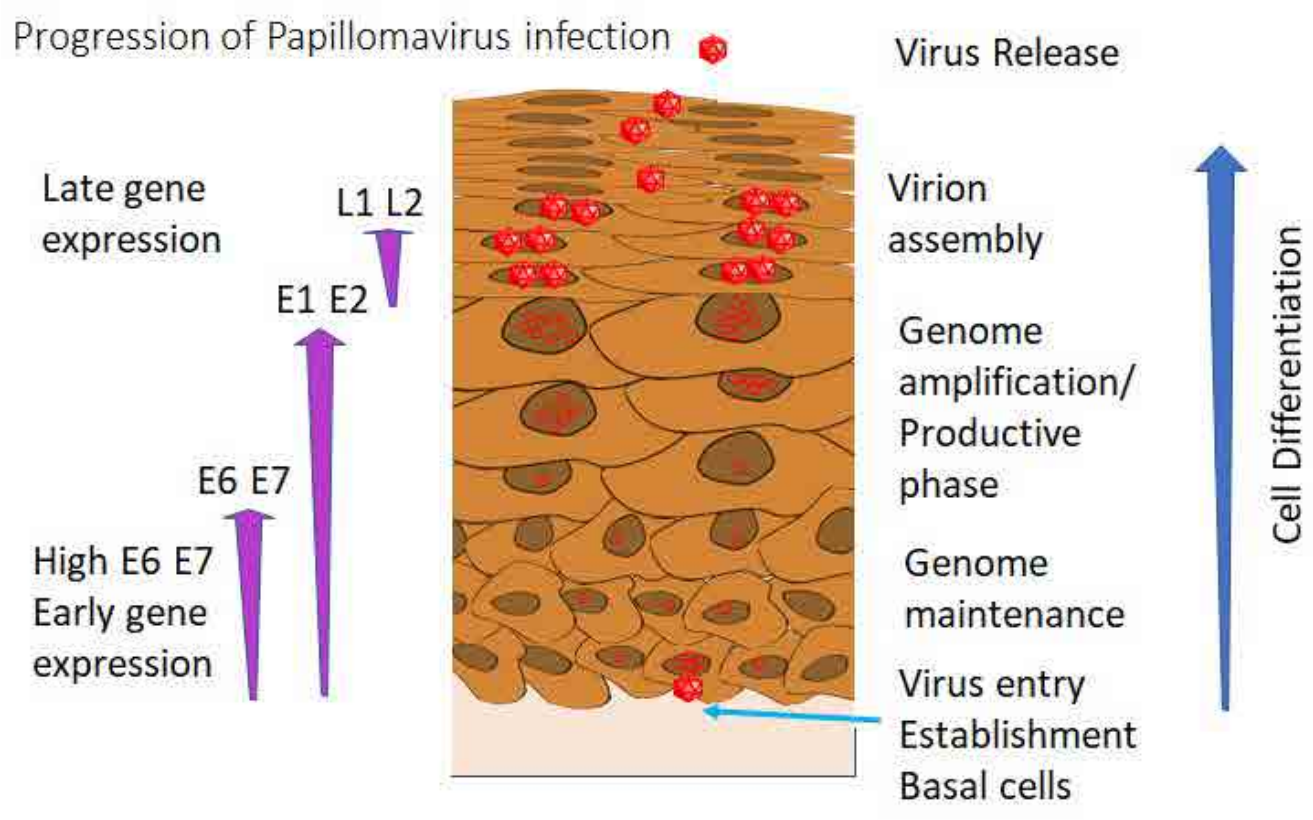


Figure 8-8. HPV infection of the epidermis showing the establishment, maintenance, and highly productive phases. Courtesy of Erica Suchman, Colorado State University.

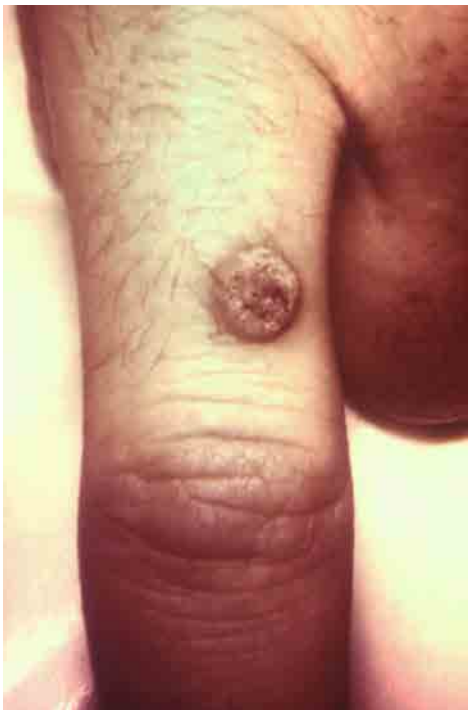


Figure 8-9. A typical wart induced by HPV. Courtesy of https://phil.cdc.gov/Details.aspx?pid=19094

Figure 8-10. HPV integration into the host cell genome with loss of E1 and E2. Courtesy of Sandra Quackenbush, Colorado State University.

According to the CDC, as shown in Figure 8-11, the average number of cases of cancer attributed to HPV infection is highest in the cervix of people with vaginas followed by the anus, vulva, oral cavity, and vagina with approximately 10,000 cases of HPV related cervical cancer per year and 2000 anus and vulva tumors and slightly fewer oral cancers. In people with penises, it is predominantly oral tumors with approximately 7000 cases per year, approximately 1800 cases of anal cancer, and less than 1000 cases of penile cancer per year.

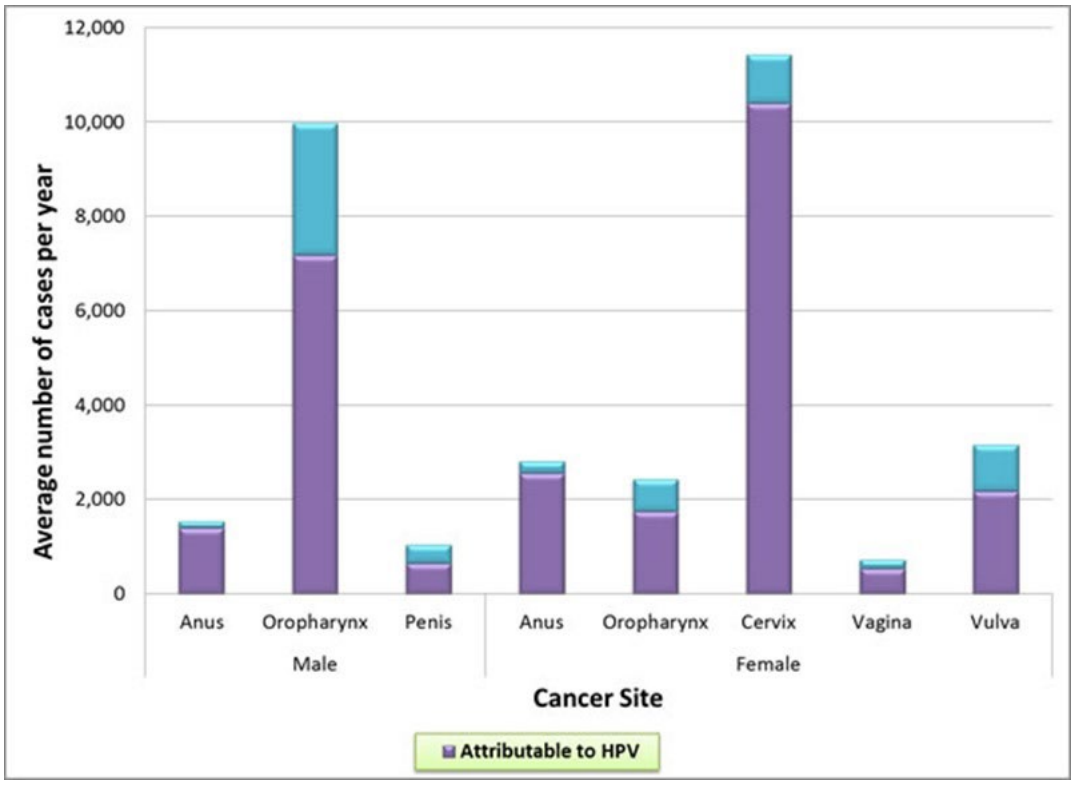


Figure 8-11. CDC estimates of average cancer types attributable to HPV infection per year. The purple bars are cases attributable to HPV infection, the blue bars are not. As shown, the majority of the anogenital and oral cancers are attributable to HPV infection. Courtesy of www.cdc.gov/cancer/hpv/statistics/cases.htm.

Many animals suffer from papillomavirus infection. The list includes cattle, rabbits, dogs, and horses to name only a few. Horses can contract 2 strains of **bovine** (cattle) papilloma that can lead to sarcoid tumors (tumors of the connective tissue of skin). These tumors are rarely life threatening, but also rarely heal on their own, and can be disfiguring.

PREVENTION OF PAPILLOMAVIRUS INFECTIONS

Very effective vaccines are available to prevent HPV infection by the high-risk types. Gardasil 9 covers the 7 HPV types most commonly associated with tumors as well as HPV 6 and 11 which cause 90% of genital warts, respiratory warts, and conjunctival warts. There have been few adverse responses reported to these vaccines. In a study of 21,000 women, the vaccine was 90-100% effective at preventing cancers and warts of the genital tract if vaccination occurred before sexual activity started (current recommendations are to vaccinate starting at age 9). Adverse responses that were reported were exactly the same between the placebo and vaccinated group indicating the vaccine is safe and effective.

Note that the vaccine must cover all of the strains because there is so little cross reactivity of the immune response between the HPV types. Therefore, even if you have been diagnosed with HPV, it is still recommended to get the vaccine as you are not protected from the other 8 strains that can cause warts and tumors.

Note that earlier vaccines Gardasil and Cervarix protected against 4 and 2 types, respectively, predominantly protecting against types 16 and 18, as they are associated with 70% of tumors. The most current vaccine, Gardasil 9, covers the viruses that result in the majority of the other 30% of tumors as well as genital warts. All 3 vaccines

contain the L1 capsid protein which contains the immunodominant neutralizing epitopes and induces high titers of neutralizing antibodies. Note, however, that there are 14 HPV types that are associated with tumor formation, so all types are not covered, and condom use is recommended to help reduce transmission. However, as these infections can often be **perigenital** (around the genitals) condom use is not 100% effective at preventing infections.

The current CDC recommendations are that children of all sexes get vaccinated between 11-14, although vaccination may begin at the age of 9. If initiated before age 15 only 2 doses are necessary assuming they are administered between 6-12 months apart. If the 2 doses are given less than 6 months apart a 3rd dose is necessary. Furthermore, a third dose is recommended for people aged 14 with a weakened immune system.

Three doses are recommended for people between the ages of 15-26. After the age of 26 vaccination should be discussed with a health care provider and may not be covered by insurance.

DIAGNOSIS OF PAPILLOMAVIRUS INFECTIONS

HPV infections can progress from benign to invasive cervical carcinoma, and these changes can be picked up by routine pap smears. As such it is recommended that people with vaginas who are sexually active should get an annual pap smear where cervical cells are taken with a swab and viewed under the microscope looking for changes indicative of changes to the cervix. It should be noted that some of these changes warrant a careful watch and more frequent pap smears to watch for further changes that indicate the need for interventions such as surgical procedures to remove involved tissues. Unfortunately, there is no such annual screening available for tumors outside the cervix, vulva, and vagina, so prompt medical attention for potential tumors of the penis, anus, or oral cavity should be sought as they often require chemotherapy. Furthermore, there is an HPV DNA test available for people with vaginas, however there is no such test for people with penises as there is no good location from which to collect samples. Routine HPV testing is not recommended unless suspected, as approximately 80% of infections will resolve on their own, and it is currently not possible to predict which patients will go on to develop tumors. Therefore, it is instead recommended that people get vaccinated and have regular pap smears.

TREATMENT OF PAPILLOMAVIRUS INFECTIONS

There are many over the counter treatments for common warts, however they are not recommended for genital warts. If tissue is showing histological changes, **cryotherapy** (freezing), **electrocautery** (burning off), surgical removal, or laser therapy may be performed by your health care provider. For recurrent genital warts, Imiquimod, an immune response modifier that is also used to treat skin cancer, can be used. Imiquimod is a toll-like receptor 7 (TLR7) **pattern recognition receptor** (PRR) **agonist** (activator). It activates TLR7 leading to the cascade response that activates transcription of type I interferon genes leading to a very strong type I interferon response. This, in turn, leads to inflammation and degradation of the warts by the infiltrate. This is interesting as HPV is a DNA virus and TLR7 recognizes single stranded RNA in an endosome as its **pathogen associated molecular pattern** (PAMP) thus showing that activation of the interferon response can affect other viruses than the intended target as these responses are not specific to any virus type. This concept is well illuminated by the use of Imiquimod to treat skin cancer, a tumor that is not caused by viral infections.

END OF CHAPTER QUESTIONS

1. Why do you think these 2 viruses are grouped together in a single chapter?
2. What enzymes do polyomaviruses and papillomaviruses use to reproduce their genome and transcribe their mRNA? Are these enzymes cellular or viral? Identify these enzymes. Why does the virus use these particular enzymes?
3. Compare and contrast how polyomaviruses and papillomaviruses initiate replication of their genome and ensure replication of the ends of the genome.
4. Do these viruses create subgenomic mRNAs? Why, or why not? If so, how?
5. Compare and contrast how parvoviruses, polyomaviruses, and papillomaviruses avoid the host's antiviral responses.
6. Compare and contrast how polyomaviruses and papillomaviruses push cells into the S phase.
7. How do these viruses obtain a 5' cap and poly A tail?
8. Do these two viruses need to have mechanisms for overcoming RNA interference? If so, why (and what are they)? If not, why not?
9. Can these viruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
10. What types of gene products are likely to be expressed by early and late genes of these 2 viruses?
11. What PRR are most likely to recognize polyomavirus and papillomavirus infections? Do these viruses have mechanisms to avoid these PRR? If so, what are they?
12. Do these viruses create accessory proteins to deal with the host anti-viral defenses? If so, what are they? If not, why not?
13. Who are most at risk of contracting polyomavirus diseases?
14. You perform an ELISA assay to determine if a patient who comes to your clinic has a polyomavirus infection. You find that the patient has a titer of 120 EU/0.1 ml which is above the threshold. Two weeks later you take blood samples from the patient again. This time the titer is 240 EU/0.1. Do you believe this patient is currently suffering from a polyomavirus infection?
15. You are analyzing CPE in polyomavirus infected cells. What type of inclusion bodies do you expect to observe, and why?
16. When do papillomavirus infections lead to cancer? Where are these tumors predominantly observed in people with vaginas and people with penises?
17. Are there effective treatments for polyomavirus or papillomavirus infections? If so, what are they? If not, why not?
18. What are the best ways to prevent polyomavirus infections in humans?
19. What are the best ways to prevent papillomavirus infections in humans?
20. What causes people to develop progressive multifocal leukoencephalopathy?

21. What is the significance of p53? How do viruses manipulate it and for what purposes? Which viruses we have learned about so far regulate p53?
22. What is the significance of Rb? How do viruses manipulate it and for what purposes? Which viruses we have learned about so far regulate Rb?

REFERENCES:

Barbanti-Brodano G, et al. BK Virus, JC Virus and Simian Virus 40 Infection in Humans, and Association with Human Tumors. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6100/

Center for Disease Control and Prevention. HPV and Cancer. https://www.cdc.gov/cancer/hpv/statistics/cases.htm

Center for Disease Control and Prevention. Public Health Image Library (PHIL). R. S. Hibbits. (1971). Warts. https://phil.cdc.gov/Details.aspx?pid=19094

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, chapters 53 and 54.

Shaikh, MH, Bortnik, V., et al. (2019). cGAS-STING responses are dampened in high-risk HPV type 16 positive head and neck squamous cell carcinoma cells. Microbial Pathology. 132:162-165. doi: 10.1016/j.micpath.2019.05.004. https://pubmed.ncbi.nlm.nih.gov/31054871/

Uhlorn, B., et al. (2020). Vesicular trafficking permits evasion of cGAS/STING surveillance during initial human papillomavirus infection. PLOS pathogens https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1009028

Viral Zone. Alphapapillomavirus. https://viralzone.expasy.org/187

Viral Zone. *Polyomamaviridae*. https://viralzone.expasy.org/148

Chapter 9: Large Linear DNA Viruses That Replicate In The Nucleus: Herpesviridae

INTRODUCTION TO THE FAMILY *HERPESVIRIDAE*

You will notice in Figure 9-1 that herpesviruses are large, linear, double stranded DNA viruses that are enveloped icosahedral viruses with a virion size of 120-200 nm and that replicate in the nucleus. The genome is a non-segmented, linear, dsDNA which is 150-200 kb and contains terminal and internal reiterated sequences. Due to its large size, the viruses encode a large number of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins. Although herpesviruses replicate and assemble their capsids in the nucleus, the virion acquires tegument proteins in the cytoplasm. This requires a complicated process where the virus buds multiple times to acquire the final envelope (described below). Herpesviruses employ **latency** to establish life-long persistent infections in their hosts. Recall that latency is when a virus stops replicating for long periods of time without leaving the cell as described in Chapter 3.

The family contains 3 subfamilies, each with multiple genera.

The subfamily *Alphaherpesvirinae* contains the genera *Simplexvirus* (i.e., herpes simplex type 1 (HHV-1) and 2 (HHV-2), feline herpesvirus 1 (FHV-1), bovine herpesvirus 1 (BHV-1), *Varicellovirus* (i.e., varicella-zoster (HHV-3 chicken pox).

The subfamily *Betaherpesvirinae* contains the genera *Cytomegalovirus,* i.e., cytomegalovirus (HHV-5) and *Roseolovirus,* i.e., human herpesvirus 6 and 7.

The subfamily *Gammaherpesvirinae* contains two genera, *Lymphocryptovirus,* i.e., Epstein-Barr virus (HHV-4) and *Rhadinovirus,* i.e., human herpesvirus 8.

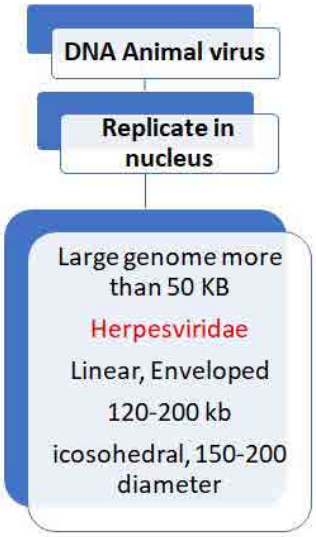


Figure 9-1. The taxonomy of DNA viruses focusing on herpesviruses. Courtesy of Erica Suchman, Colorado State University.

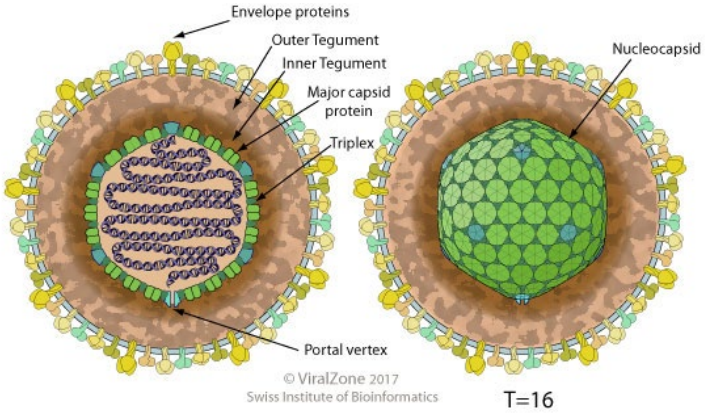


Figure 9-2A. A typical herpesvirus virion. Courtesy of ViralZone https://viralzone.expasy.org/

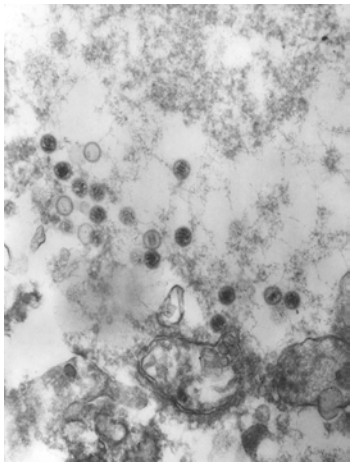


Figure 9-2B. A transmission electron microscopic (TEM) image of numerous Epstein-Barr virus (EBV) virions, members of the Herpesviridae virus family. EBV is also known as human herpesvirus-4 (HHV-4). Courtesy of https://phil.cdc.gov/Details.aspx?pid=10234 Dr. Fred Murphy

GENOME STRUCTURE

Herpesvirus genomes are non segmented, linear, dsDNA genomes of 120-200 kb. The genome contains terminal and internal reiterated sequences and encodes greater than 100 gene products (proteins).

NUCLEAR REPLICATION

Lytic replication:

1. Attachment of the viral envelope proteins to host receptors (different ones used by each herpesvirus) and of the fusion peptide to different host receptors.
2. Most, but not all, enter by fusion with the plasma membrane to release the core and the tegument proteins into the host cytoplasm. The tegument proteins stop interferon transcription activation.
3. The capsid is transported to the nuclear pore via microtubules where the viral DNA and some tegument proteins are released into the nucleus when it becomes trapped on the cytoplasmic side of the nuclear pore.
4. Transcription of immediate early gene mRNAs by host RNA polymerase II (DNA-dependent RNA polymerase, DdRp) which promote transcription of early genes and protect the virus against innate host immunity.
5. Transcription of early viral mRNA by cellular RNA polymerase II encoding regulatory proteins as well as proteins involved in replication of the **viral** DNA, including DNA-dependent DNA polymerase (DdDp) and primase. Note that they must make their own DNA replication enzymes because they replicate in cells that are not undergoing mitosis (non-replicative) such as neurons. Recall that non-replicative cells are not producing the enzymes to reproduce cellular DNA. DNA only replicates when cells are replicating. However, herpesviruses can push cells into S phase as well to acquire other components of DNA replication from the cell.
6. The linear genome undergoes circularization and genome amplification occurs by bidirectional replication using viral DdDp.
7. Transcription of late mRNAs by host RNA polymerase II encoding structural proteins that make up the capsid and envelope.
8. Assembly of the virus in nuclear viral factories and budding through the nuclear membrane occurs. This particle then fuses with the endoplasmic reticulum releasing the virion into the cytoplasm. As it passes through the cytoplasm to the Golgi, it acquires tegument protein and then buds into the Golgi apparatus. The virus then leaves the Golgi in an exocytic vesicle and the final release at the plasma membrane is mediated by exocytosis.

Note: Herpesviruses all become permanent lifelong infections and enter a **latent state** where the virus restricts expression of lytic cycle genes and upregulates expression of latency genes. This causes viral replication to cease. The virus can then either remain latent or occasionally leave the latent state by **reactivation** and enter the **lytic replication cycle.** During the lytic cycle, production of infectious virions that can infect other cells and hosts occurs. There is a regulated cascade of lytic gene expression that leads to genome replication, assembly, egress, and transmission.

Latent cycle: There is replication of circular viral **episome** (outside of the chromosome) without the production of infectious virus particles.

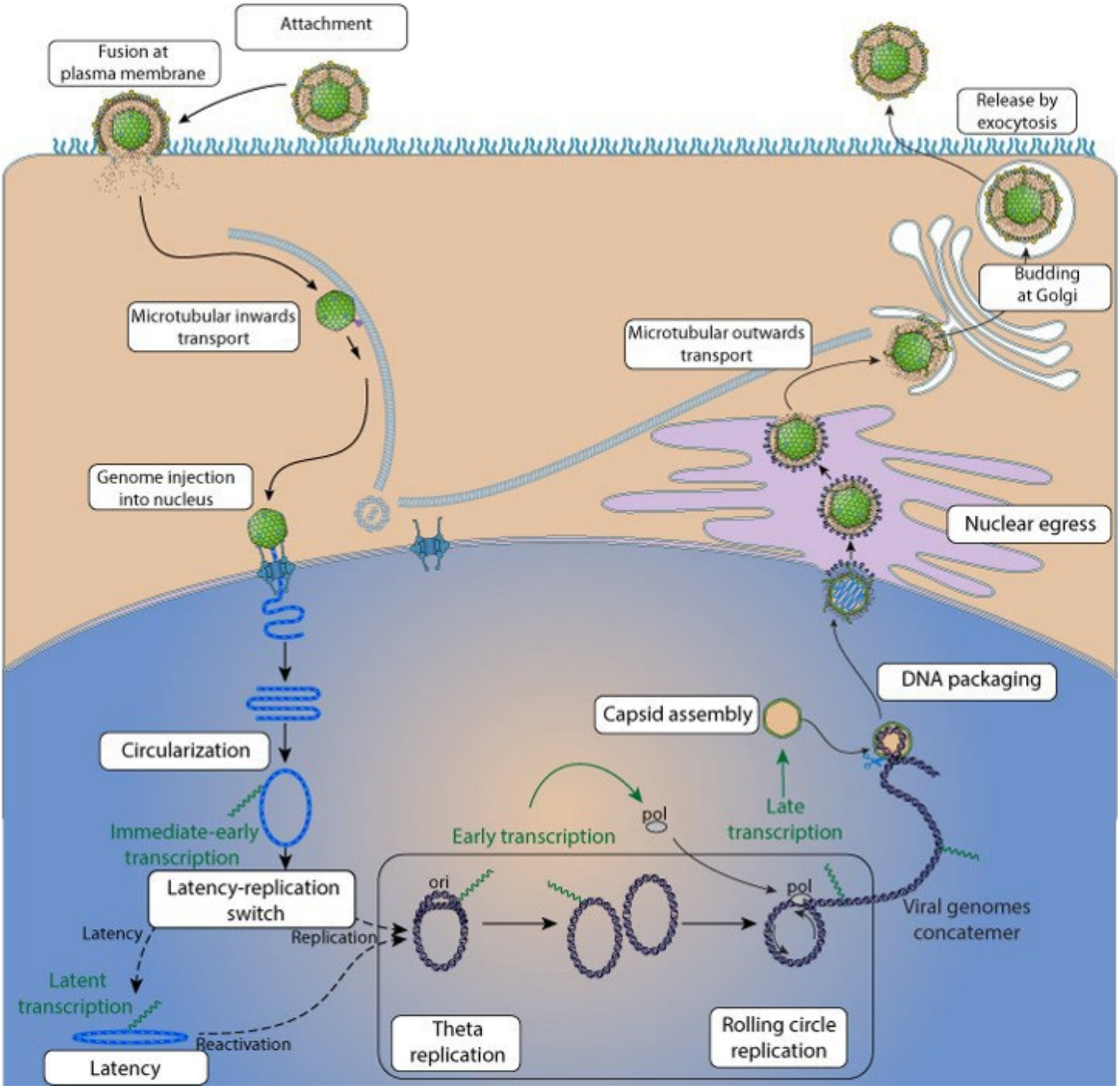


Figure 9-3. Herpesvirus replication cycle. Courtesy of ViralZone https://viralzone.expasy.org/5839

HOW DO HERPESVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all nuclear replicating DNA viruses of animals, herpesviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells (Figure 9-3). In particular:

1. Using multiple promoters to create many mRNAs.
2. Expressing genes from both strands or overlapping genes. Notice that this leads to production of complementary mRNAs that can bind and activate type I interferon responses, interferon stimulated genes such as PKR, OAS, and possibly RNAi. As such, herpesviruses must create multiple accessory proteins to inactivate these responses. It is not a problem to produce many extra accessory proteins because herpesviruses have very large genomes and capsids.
3. Using alternative splicing to create smaller versions of a pre-mRNA all of which produce different proteins. Note that most of the mRNA of herpesviruses are not spliced.
4. Viral protease cleavage of polyproteins. Again, most of the proteins produced by herpesviruses do not require proteolytic processing (but some do).
5. **Leaky ribosomal scanning**. Recall from Chapter 3 that leaky scanning occurs when the ribosome can initiate translation a low percentage of time at a second AUG start codon in context with a weak Kozak consensus sequence.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that will be recognized by the necessary initiation factors (eIF4E &G, PABP). Over 100 proteins will be produced including all of the structural proteins that make up the virion, the enzymes required to replicate the viral DNA (allowing the virus to replicate in non-replicating cells), and a whole host of viral accessory proteins to overcome the host's cellular immune response as well as the adaptive and innate immune responses. Herpesviruses also have multiple mechanisms to outcompete host cell mRNA translation including degradation of cellular mRNAs, inhibition of host gene transcription, inhibition of splicing of host cell mRNAs, degradation of cellular proteins, blocking the activation of PKR, and mediating dephosphorylation of eIF2α. Recall that PKR is one of the proteins produced in response to type I interferon in infected cells. PKR binds dsRNA (which herpesviruses make in low levels) and phosphorylates eIF2α inactivating it, such that eIF2α cannot activate translation of protein. Therefore, herpesviruses use a 2-pronged approach, both inactivating PKR prohibiting the phosphorylation of eIF2α as well as actively dephosphorylating eIF2α.

DNA GENOME REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA:

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Herpesviruses overcome this issue by producing their own DNA replication enzymes.
2. How to prime their DNA replication. Herpesviruses circularize their linear genomes and then, like other circular DNA viruses, utilize an origin of replication to replicate the viral genome and then use their own primase enzyme to provide RNA primers with a 3' OH for DNA synthesis.
3. How to replicate the ends of their genome. Herpesviruses circularize to replicate to assure their ends are replicated.

VIRION FORMATION

Assembly of the virus capsids occurs spontaneously in the **nucleus inside viral factories** (protected regions within the cell), and then the viral particles bud through the nuclear membrane and fuse with the endoplasmic reticulum. During transport they acquire tegument proteins and they then bud into the Golgi apparatus obtaining their envelope with viral envelope proteins. They leave the Golgi in an exocytic vesicle and the final release at the plasma membrane occurs by exocytosis.

AVOIDING THE HOST IMMUNE RESPONSE

Herpesviruses have so many antiviral defenses we will not be able to expand on all of them. We will, however, hit on a few of the major ones. First, the viruses can enter a latent state where they stop replicating and therefore can effectively hide from the host cell's antiviral defenses as well as innate and adaptive immunity. While in the lytic cycle, the virus remains in the capsid until it reaches the nucleus thus avoiding detection by pathogen recognition receptors (PRR) such as TLR9 that detect viral DNA in an endosome and cGAS which detects viral DNA in the cytoplasm. Herpesviruses also replicate in viral factories protecting them from detection by host cellular immunity. Although most herpesvirus genes do not overlap creating possible dsRNA due to complementarity, some do. Herpesviruses make many accessory proteins (over 200 gene products) to deal with the interferon responses. They also have the ability to downregulate MHCI production by inhibiting TAP-mediated transport of MHCI to the plasma membrane thus avoiding detection by CD8+ Cytotoxic T-cells (CTLs). However, recall from Chapter 4 that the downregulation of MHCI serves as an activating signal to natural killer cells which utilize binding of MHCI as a "do not kill" signal. Therefore, to overcome natural killer cell detection of infected cells due to the downregulation of MHCI, some herpesviruses can produce MHCI homologs that provide the "do not kill" signal to natural killer cells but cannot present antigen as normal MHCI would. Furthermore, many herpesviruses are capable of moving from cell to cell by **cell fusion** forming syncytia, giant multi-nucleated cells (as described in Chapter 2). By fusing the cell membranes of adjacent cells, the virus is able to move from cell to cell without leaving the cell and exposing itself to the innate and adaptive immune responses. Lastly, some herpesviruses can inhibit apoptosis (cell death) thus allowing the virus to continue replicating and inhibiting the ability of the host cell to die to kill off the virus.

HERPESVIRAL DISEASES

ALPHAHERPESVIRINAE SIMPLEXVIRUS, HUMAN HERPESVIRUS 1 (HHV1 OR HSV-1) HUMAN HERPESVIRUS 2 (HHV2 OR HSV-2)

Human herpes simplex viruses 1 and 2, also called Human herpesvirus 1 and 2, replicate rapidly in cell culture and demonstrate pronounced cytopathic effect (CPE) such as cell fusion and nuclear inclusions as shown in Figures 9-4. HSV viruses establish latent infections in primary sensory neurons where the virus exists as a non- replicating episome and lytic infections in epidermal cells of the skin which are the site of primary infection and recurrent infections. Herpesviruses infect mucosal epithelial cells in a lytic replication cycle that leads to the formation of vesicles due to inflammatory responses that rupture and then heal. Transmission occurs via contact

with mucosal surfaces or abraded skin. Following the primary infection, the virus establishes a latent infection in sensory neurons. HSV-1 generally causes fever blisters, or cold sores, that are most commonly found on the lips or inside the mouth as shown in Figure 9-5. HSV-2 generally causes genital lesions as shown in Figure 9-6. It is important to note that HSV-1 can also cause genital lesions, however it is not common (although it does happen) for HSV-2 to cause oral lesions. The presence of HSV in the birth canal can lead to neonatal HSV infections that can be serious and sometimes fatal. Cesarean delivery is recommended if maternal genital HSV lesions are present at the time of labor. Also, antiviral suppressive therapy is recommended for women with known genital herpes without current lesions to decrease HSV shedding from the genital tract at the time of vaginal delivery. However, most neonatal infections occur in infants born to women without a history of genital HSV. HSV-1 infections are not limited to the oral and genital mucosal membranes and can cause ocular infections that can lead to blindness as shown in Figure 9-7. HSV-1 is also the most common cause of sporadic fatal encephalitis in the United States with a 70% case-fatality rate in untreated patients. It is also important to note that infected individuals often become asymptomatic viral shedders when low level reactivations occur without sufficient tissue damage to cause symptoms. As such, it is impossible to tell if a person can transmit HSV infections solely by looking for symptoms of blisters. This is true for most herpesvirus infections which can also result in asymptomatic shedding as not all reactivations lead to symptoms. The most recent prevalence data from the CDC indicates that approximately 48% of the population in the US is infected with HSV-1 and 12% with HSV-2. These percentages vary depending on demographics with geographic location, socioeconomic status, age, and having a vagina as the primary factors that influence acquisition of HSV infection. It is interesting to note that these percentages have significantly decreased from the 1990's and seem to be continuing to decline. HSV-1 & HSV-2 have a worldwide distribution, and it is estimated by the World Health Organization that worldwide approximately 67% of the population has HSV-1 and 25% of the population has HSV-2. This is a dramatic reduction for HSV-1 as estimates used to be a 90% infection rate worldwide. The R0 for HSV infections is estimated to be similar to influenza with each infected person expected to infect 1.3-2 other people.

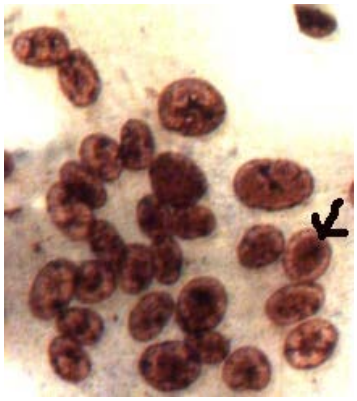


Figure 9-4. Cytopathic effect of bovine herpes virus 2 infections in BFS cells demonstrating syncytia and nuclear inclusions with halos and chromatin margination. Courtesy of Erica Suchman, Colorado State University.

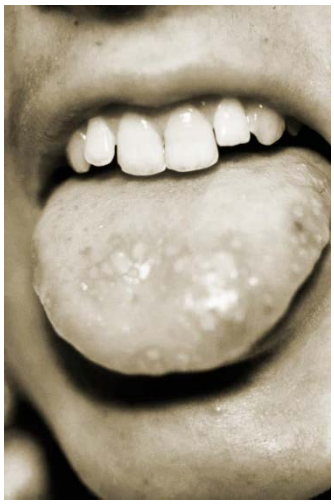


Figure 9-5. The tongue of this patient reveals numerous blister-like lesions on the tongue's dorsal surface which have manifested as a result of a herpes simplex virus-1 (HSV-1) infection, also known as an oral herpes infection, and which is referred to as herpes stomatitis. Courtesy of https://phil.cdc.gov/Details.aspx?pid=22130

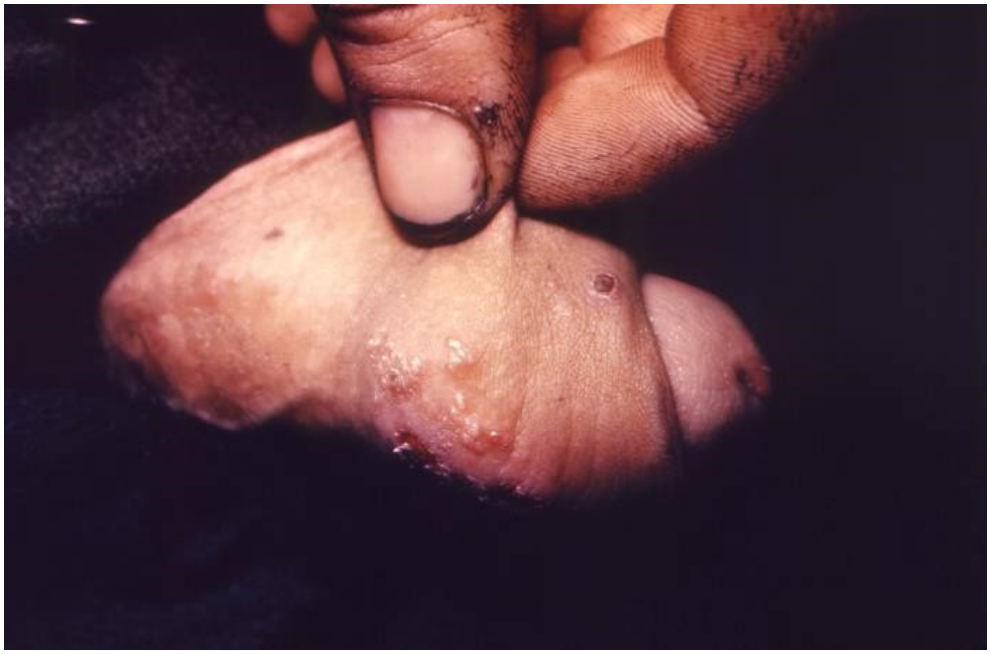


Figure 9-6. A close view of a patient's penis highlighting an erosive maculopapular rash that was at the time thought to have been caused by a syphilis infection. After performing a differential diagnostic study, however, the rash proved to be a herpes infection caused by the HSV-2 virus. Courtesy of https://phil.cdc.gov/Details.aspx?pid=16783 CDC and Dr. Dancewicz

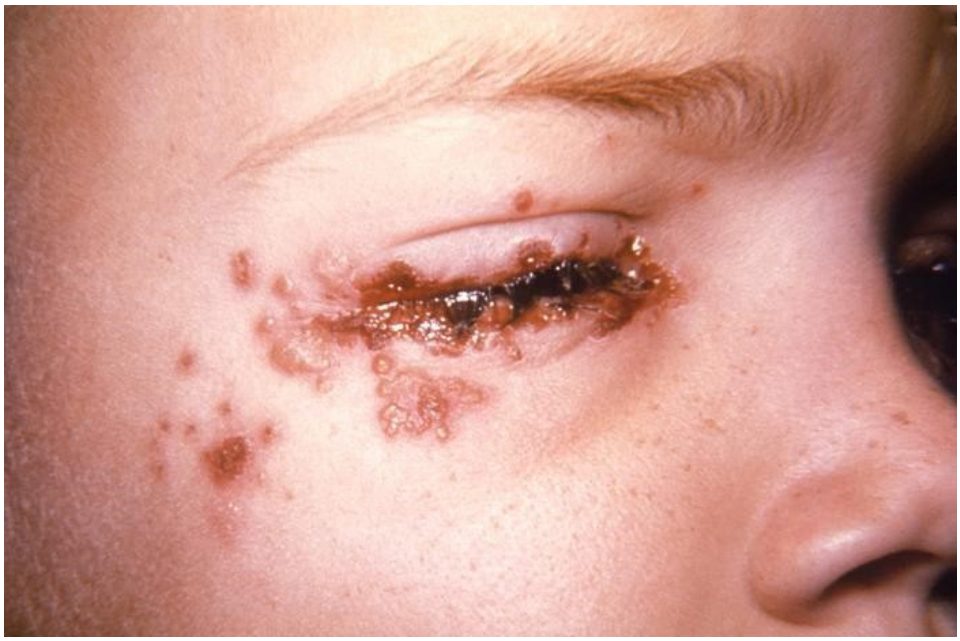


Figure 9-7. A 7-year-old child's face which reveals the presence of a periocular herpes simplex virus (HSV) vesicular outbreak. This patient had a history of recurrent herpes labialis (HSV-1). Courtesy of https://phil.cdc.gov/details.aspx?pid=6492 CDC and Dr. K.L. Hermann

ALPHAHERPESVIRINAE SIMPLEXVIRUS: FELINE HERPES VIRUS

Feline herpesvirus (FHV-1, feline viral rhinotracheitis) is caused by a virus in the family *Herpesviridae* and the genus *Vericellovirus* (subfamily *Alphaherpesvirinae*). FHV-1 is found worldwide and can infect members of the family *Felidae* of any breed or age. However, kittens, immunosuppressed cats, cats infected with feline T-cell leukemia virus or feline immunodeficiency virus, or cats under stress or with poor nutrition are more susceptible. The virus was first identified in 1957 and is one of two viruses that cause acute respiratory disease of kittens (the other is calicivirus). About half of all kittens with acute respiratory disease have FHV-1. Transmission is via contact with infected materials either by direct contact, contact with infected fomites, or via respiratory droplets. It is known that the sneeze of FHV-1 infected cats can spread the virus up to 4 feet. It is thought that most cats (~70%) develop FHV-1 infections at an early age. Due to poor immune responses, cats can suffer multiple infections, and this virus is a problem in animal care facilities and catteries that house many cats in high density.

Most infections are mild causing nasal discharge, swelling of the conjunctiva of the eye (Figure 9-8), sneezing, coughing, fever, and anorexia beginning 2 to 5 days after exposure. However, in some cats the virus can infect the eye causing blindness. Oral lesions are also often noted but are more common in calicivirus infections. In young kittens and senior cats, pneumonia may result in death. Case-fatality rates may reach as high as 30% in young kittens and older cats. Pregnant females may abort their fetuses. Like all herpesviruses, the viruses' ability to establish a lifelong latent state causes some cats to become latent asymptomatic shedders of the virus, probably serving as a reservoir for infection of other cats. Cats are known to increase shedding when under stress. All cats that recover from these infections should be considered potential carriers. There is a vaccine available, however it reduces disease but does not prevent infection.

Figure 9-8. Feline demonstrating ocular lesions of feline herpes virus. Courtesy of ViralZone https://viralzone.expasy.org/ Courtesy of Tom Walton, APHIS retired.

ALPHAHERPESVIRINAE VARICELLOVIRUS: CHICKEN POX VARICELLA ZOSTER VIRUS (VZV)

Varicella Zoster virus (VZV) is the causative agent of chicken pox as well as the reactivation syndrome **shingles** (also called **herpes zoster)**. VZV has a worldwide distribution. Transmission is via the respiratory route when infected respiratory droplets or aerosols are inhaled or by contact with fluid in blisters. The reproductive rate R0 is 10-12, so an infected person is expected to infect 10-12 other people. The virus has tropism for T-cells, cutaneous epithelial cells, and trigeminal and dorsal root ganglia of the nervous system. The virus first replicates in the mucosal epithelium of the respiratory system and then the T-cells of the tonsils, at which point it moves to the epithelium and rash is observed. The virus also moves to the central nervous system where it establishes latency and can later reactivate.

Initial symptoms develop after 7-21 days (average 14-16 days) and include fever and a **centripetal** rash that occurs predominantly on the trunk and face and has many different stages of the rash present at any given time (Figure 9-9 A & B). The rash begins as **macules** (flat discolorations of the skin), and then progresses to **papules** (raised discolorations of the skin), **vesicles** (blisters), pustules (pus filled vesicle), and finally crusted scabs. The rash usually demonstrates many of the phases concurrently. This rash distribution is very different than smallpox which has a **centrifugal rash** that appears densely on the palms, soles of the feet, and face, with all of the rash being at the same stage at the same time. These differences were long used to differentiate between the two diseases that began with fever and rash. Figure 9-9C shows a child with leukemia and chickenpox showing an increased severity of disease due to immunosuppression. Before widespread vaccination the mortality rate for chickenpox in children was very low with ~ 1 death/100,000 cases in children aged 1-14. Case mortality rates increase with age, with 2.7 deaths/100,000 cases in 15-19 year-olds, and 25.2 deaths/100,000 cases in 30-49 year-olds. Before vaccination ~11,000 people a year would be hospitalized with VZV, the vaccine has reduced this by 70%, and case fatality by 88%. Varicella is highly contagious and spreads via coughing or sneezing. Complications include bacterial infection of the skin, swelling of the brain, and pneumonia. Furthermore, in people over the age of 14, the virus often causes an extremely robust immune response that is responsible for the increased mortality rate. Maternal infection can lead to congenital neurological abnormalities and neonatal death.

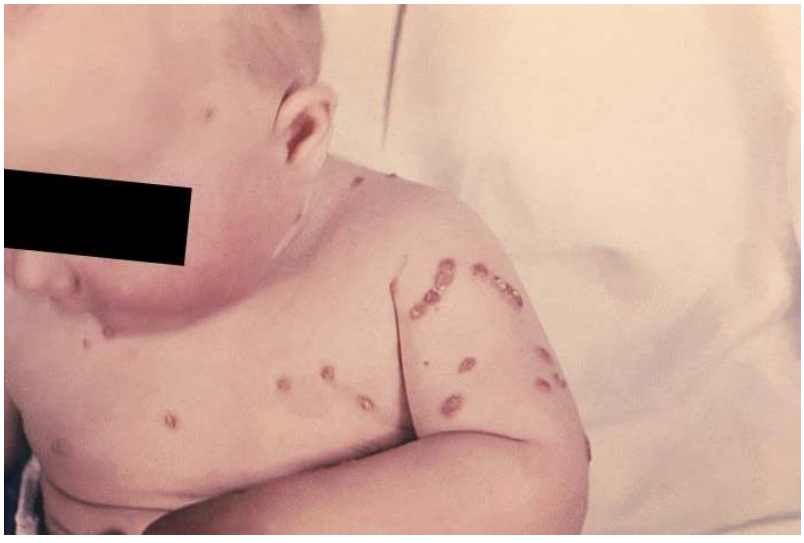


Figure 9-9A. An infant whose upper torso displays the characteristic maculopapular lesions in various stages of development, i.e., papular vesicles and crusts dispersed in a region indicative of an early onset of chickenpox, a disease caused by the varicella-zoster virus (VZV). Courtesy of https://phil.cdc.gov/Details.aspx?pid=16783 *Susan Lindsley.*

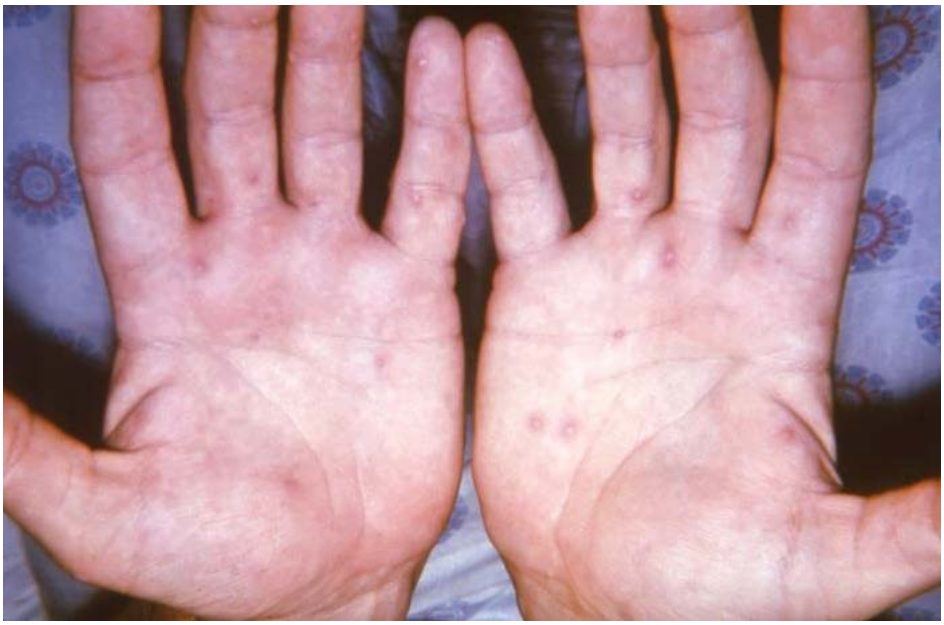


Figure 9-9B. A close view of a patient's palms with the presence of a maculopapular rash that turned out to be lesions caused by a case of chickenpox due to the varicella zoster virus (VZV). Note that this crop of lesions appears to be predominantly in what is referred to as the vesicular stage, in which the lesions are fluid-filled. These will eventually mature, rupture, liberate their highly contagious fluid contents, and subsequently scab over to enter their crusty stage of development. Courtesy of https://phil.cdc.gov/Details.aspx?pid=21514 K.L. Herrmann.

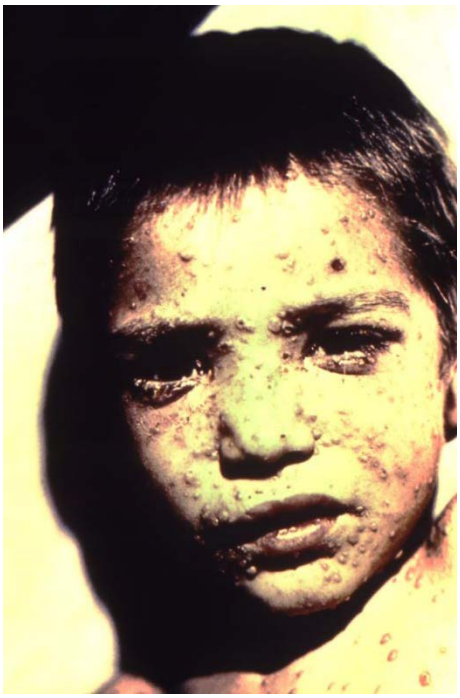


Figure 9-9C. The face and chest of a child with leukemia who has secondarily contracted chickenpox which manifests as this severe, generalized maculopapular rash. The severity of this patient's outbreak was greatly increased due to his leukemia-induced lowered immune response to the varicella zoster virus (VZV). Courtesy of https://phil.cdc.gov/Details.aspx?pid=18904 Dr. Joel D. Meyers.

VZV, like other herpesviruses, establishes latency in the nervous system. VZV establishes latency in the spinal cord after the virus undergoes **retrograde transport** from the skin, where the virus establishes the primary infection, to the spinal cord. Upon reactivation, the virus undergoes **aneterograde transport** from the spinal cord back to the skin where the typical shingles maculopapular rash is seen along the neuron that harbors the infection. Note that this will not be the disseminated rash of primary chickenpox infections as is shown in Figure 9-10. In this patient the T-10 and T-11 thoracic nerves are infected, and the rash follows the route of these nerves. Reactivation occurs

when VZV specific T cells fall below the **Zoster Threshold** (the level of T cells necessary to keep the varicella zoster infection in check) thus repressing herpes zoster symptoms. As shown in Figure 9-11, after infection VZV specific T cells accumulate to above the threshold level and remain high over a person's life with increases resulting from varicella exposure, or possibly silent reactivations. These cells then begin to decline with age eventually falling below the threshold, at which point the virus can leave latency and reactivate resulting in **shingles**/**herpes zoster** symptoms. As you might expect, stress and immunosuppression are also correlated with VZV reactivations resulting in shingles outbreaks. However, effective vaccines exist, and, if given during the period of time when a person's immunity is waning (over 50), can keep the VZV specific T-cells above the zoster threshold.

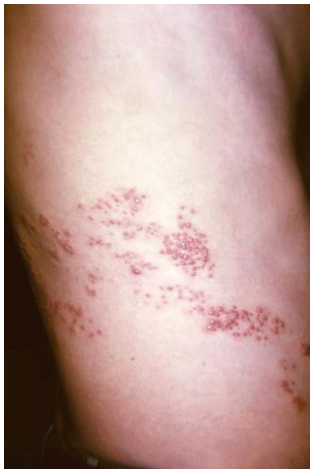


Figure 9-10. A patient's skin revealing a maculopapular rash due to an outbreak of shingles caused by the varicella zoster virus (VZV) demonstrating that the rash follows the T10 – T11 thoracic nerves emanating from the spinal cord at these two levels. Courtesy of https://phil.cdc.gov/Details.aspx?pid=21506 K.L. Herrmann.

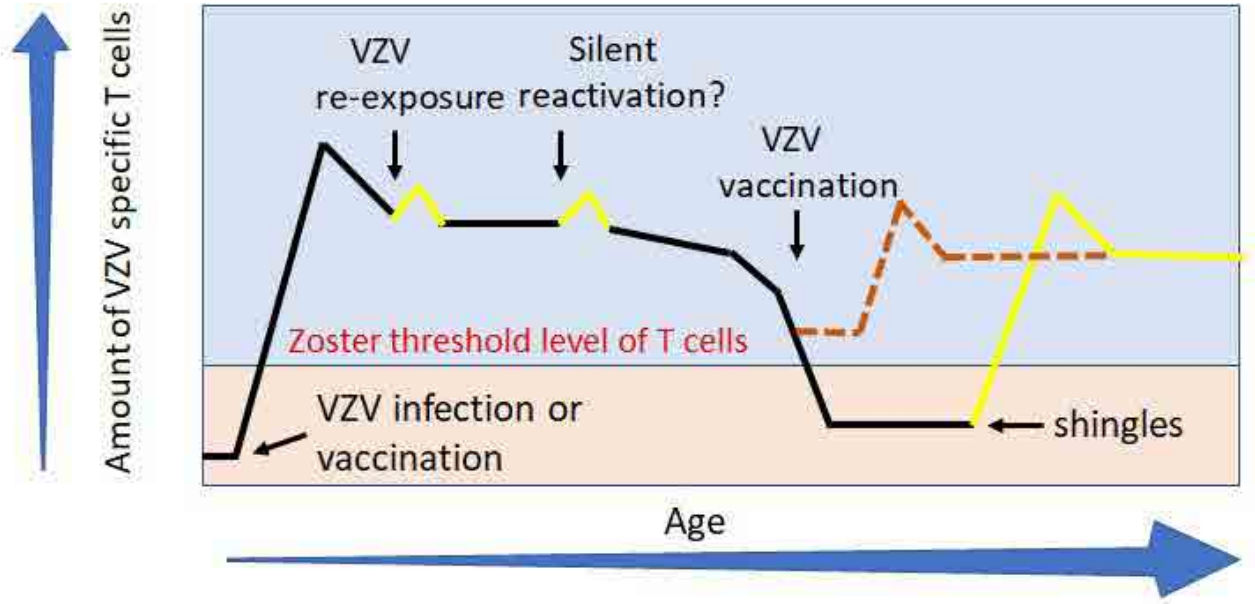


Figure 9-11. VZV specific T-cells accumulate to above the threshold level and remain high over a person's life with increases resulting from varicella exposure, or possibly silent reactivations, but begin to decline with age eventually falling below the threshold at which point the virus can leave latency and reactivate resulting in shingles/herpes zoster symptoms. Courtesy of Erica Suchman, Colorado State University.

BETAHERPESVIRINAE CYTOMEGALOVIRUS: HUMAN CYTOMEGALOVIRUS (CMV) OR HUMAN HERPESVIRUS 5 (HHV-5)

CMV/HHV-5 replicates slowly in cells leading to **cytomegaly** (enlarged cells). The virus has a restricted host range with a tropism for differentiated hemopoietic and epithelial cells, and latency is established in secretory glands and the kidneys. The virus is ubiquitous with virus infections worldwide with a 50-80% infection rate in the US. Transmission occurs horizontally by direct contact, sexual transmission, virus shed in saliva, urine, breast milk, tissue transplantation, and by blood transfusions. It can also occur vertically via trans placental infection of the fetus. The R0 predicts that an infected individual is expected to infect 2.5 other people. The incubation period is 4-6 weeks. In an immunocompetent host, infection is usually subclinical with a mild self-limiting cold-like infection. However, CMV can cause **congenital** infection (of the fetus) and is seen in 1 in every 150 babies born to mothers who contract the infection during pregnancy as shown in Figure 9-12. This can result in severe disease and is the leading cause of viral-induced birth defects which include hearing loss, impaired vision, and severe damage to the central nervous system. Furthermore, in an immunocompromised host, reactivation of latent infections occurs which can lead to reinfection of patients. Viral reactivation is a major issue in transplantation where primary infection through infected tissues and organs can be fatal, as can reactivations due to the immunosuppressive drugs patients must take for life to avoid tissue rejection by the immune system. The symptoms include pneumonia, gastrointestinal lesions, renal impairment, hepatitis, myocarditis, pancreatitis, and graft rejection.

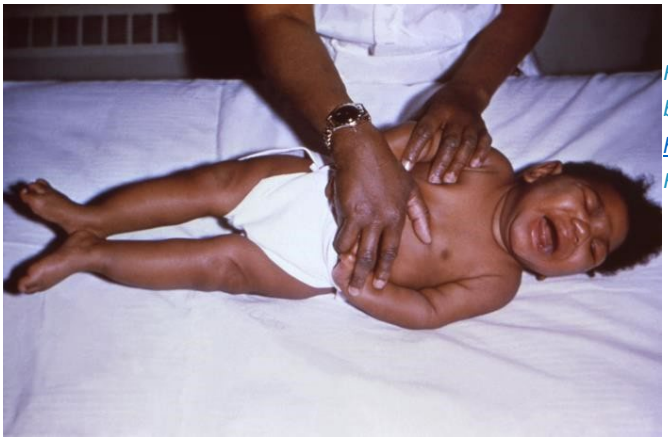


Figure 9-12. This image depicts a young child who was born with a congenital cytomegalovirus (CMV) infection. https://phil.cdc.gov/Details.aspx?pid=17976 CDC and Rodger Feldman.

Infection initiates with replication in mucosal epithelium leading to a leukocyte associated viremia where immature monocytes disseminate virus to other organs where virus infects epithelial cells from which virus is shed and transmitted to new hosts. The virus also establishes a persistent infection with replication in epithelial cells of the salivary glands and kidneys leading to sporadic shedding throughout life. The persistence of active primary infection may be due to viral proteins that interfere with host response. CMV is known to block TAP-mediated transport of MHCI as well as transport of MHCI to the plasma membrane and degradation of MHCI. CMV avoids the natural killer cell response by expressing MHCI-like homologs that bind to natural killer cells but don't present antigens to CD8+ cells thus fooling natural killer cells into thinking no downregulation of MHCI has occurred thus allowing a persistent infective state to occur.

A primary infection is brought under control by the innate and adaptive immune response, in particular the interferon natural killer cell response, and cell-mediated immune response (CD8+ CTLs). The adaptive immune response is important for life-long suppression of virus replication and maintenance of latency. Latency is established in hematopoietic progenitors, myeloid progenitor cells, monocyte-derived macrophages, and dendritic cells. Differentiation and activation of these cells drive maturation and contribute to viral reactivation.

GAMMAHERPESVIRINAE LYMPHOCRYPTOVIRUS: EPSTEIN BARR VIRUS (EBV) MONONUCLEOSIS

EBV replication occurs in lymphoblastoid (T or B) cells, and latent infection is in lymphoid tissue. The lytic infection is in epithelial cells, fibroblasts, and lymphocytes. EBV was first isolated from cultured tumor cells and is known to cause the B lymphocyte Burkitt's lymphoma. Burkitt's cases occur only in regions with malaria in Africa, and, as such, it is thought that a coinfection of EBV and malaria results in Burkitt's lymphoma. However, EBV infections occur outside of Africa, and in fact are endemic worldwide with a 95% prevalence in adults. Outside of Africa, EBV causes infectious mononucleosis which leads to **lymphadenopathy** (enlarged lymph nodes), **tonsillitis** (enlarged tonsils) (Figure 9-13), and necrotic **exudate** (discharge). It can also cause an enlarged fragile spleen that can burst due to inflammation. Other possible symptoms include hemorrhaging in the eyes (Figure 9-14).

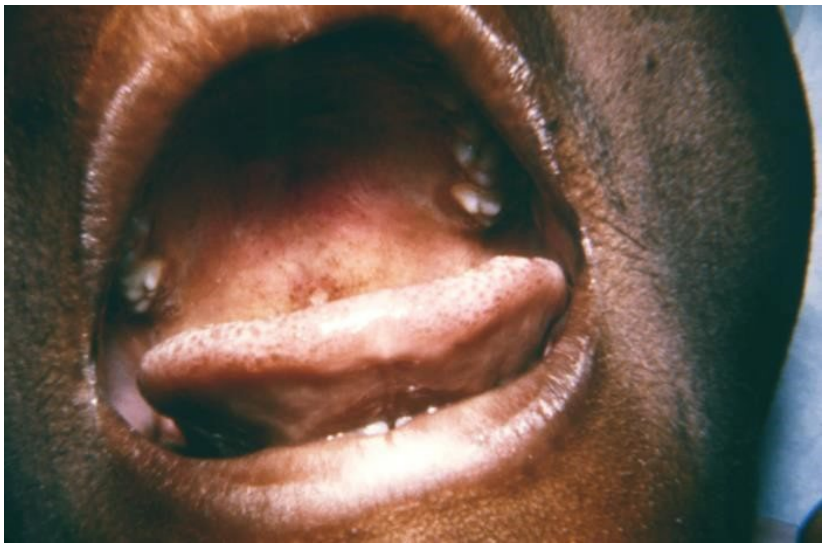


Figure 9-13. A close view of a patient's open mouth highlighting the mouth's interior showing inflammation of the tongue and back of the mouth due to a mononucleosis infection caused by the Epstein-Barr virus. Note the maculopapular rash on the hard palate. Courtesy of https://phil.cdc.gov/Details.aspx?pid=1620 CDC and Dr. Thomas F. Sellers, Emory University.

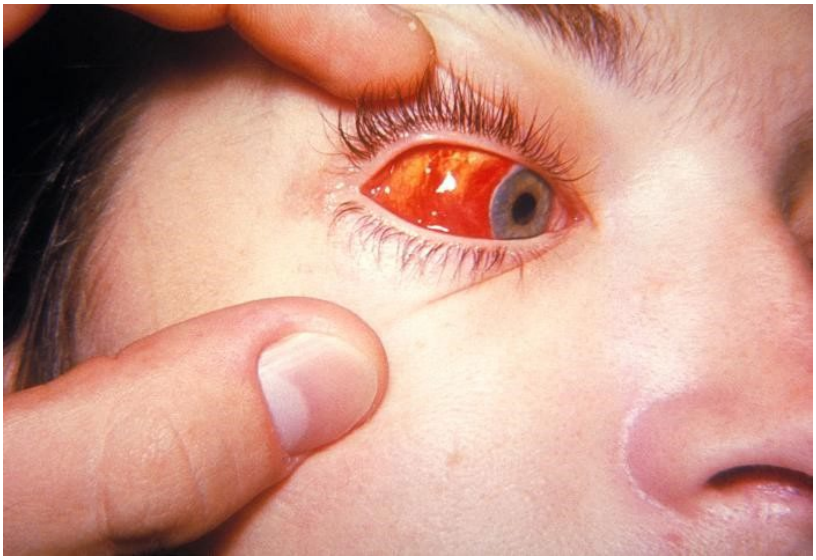


Figure 9-14. A conjunctival hemorrhage of the right eye of a patient with infectious mononucleosis caused by the Epstein-Barr virus. Courtesy of https://phil.cdc.gov/Details.aspx?pid=2862 CDC and Dr. Thomas F. Sellers, Emory University.

Primary infection occurs in resting B-cells causing a lytic infection in these infected B-cells. The virus causes a latent infection in resting memory B-cells and the virus reactivates and enters the lytic cycle as the memory cell becomes activated. These cells can have an altered appearance on histopathology, as shown in Figure 9-15.

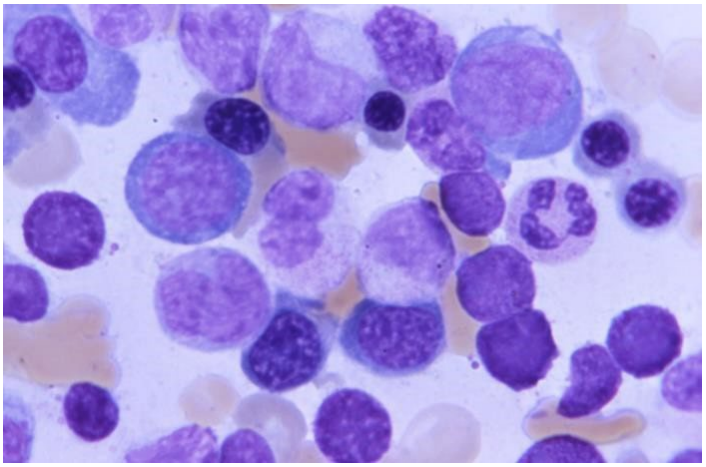


Figure 9-15. Photomicrograph reveals some of the histopathology seen in a peripheral blood smear obtained from a patient with infectious mononucleosis showing numerous white blood cells (WBCs) and primarily lymphocytes, some of which are atypical in appearance. Courtesy of https://phil.cdc.gov/Details.aspx?pid=19470 CDC and Carl Flint; Dr. Volger, Emory University Hospital.

EBV is transmitted by contact with saliva, blood, semen, and via organ transplants. The severe

immunosuppression associated with organ transplantation allows EBV to cause B-cell lymphoproliferative disorders or "post-transplant lymphoproliferative disease" (PTLD) that often occurs within the first year following transplantation. As such, like CMV, both the donor and recipient must be tested, and a CMV negative recipient must receive a CMV negative organ. However, PTLD is still a risk due to the life-long need for immunosuppressive treatments to prevent organ rejection.

GAMMAHERPESVIRINAE RHADINOVIRUS HERPESVIRUSES THAT CAUSE OPPORTUNISTIC INFECTIONS HHV8

Human Herpes Virus 8 (HHV8) is associated with Kaposi sarcoma in people with HIV infection. Kaposi sarcoma is a tumor of the lining of the blood or lymph vessels that is normally seen in about 1 in 1 million Haitian men over the age of 65. Kaposi sarcoma was one of two diseases that was noticed in a group of young homosexuals in San Francisco and Los Angeles in the 1980s that led to the discovery of HIV. The first disease noticed of AIDs was pneumocystis pneumonia (PCP) caused by the protozoa *Pneumocystis carinii* which was known to only cause pneumonia in immunocompromised hosts. Kaposi sarcoma is a malignant tumor of the lymphatic endothelium, also known as Kaposi's sarcoma-associated herpesvirus (KSHV) and arises from a cancer of the lymphatic endothelial lining. It is characterized by bluish-red cutaneous nodules. Kaposi sarcoma is thought of as an opportunistic infection affecting patients whose immune systems have been compromised, as in the case of patients with HIV/AIDS as shown in Figure 9-16.

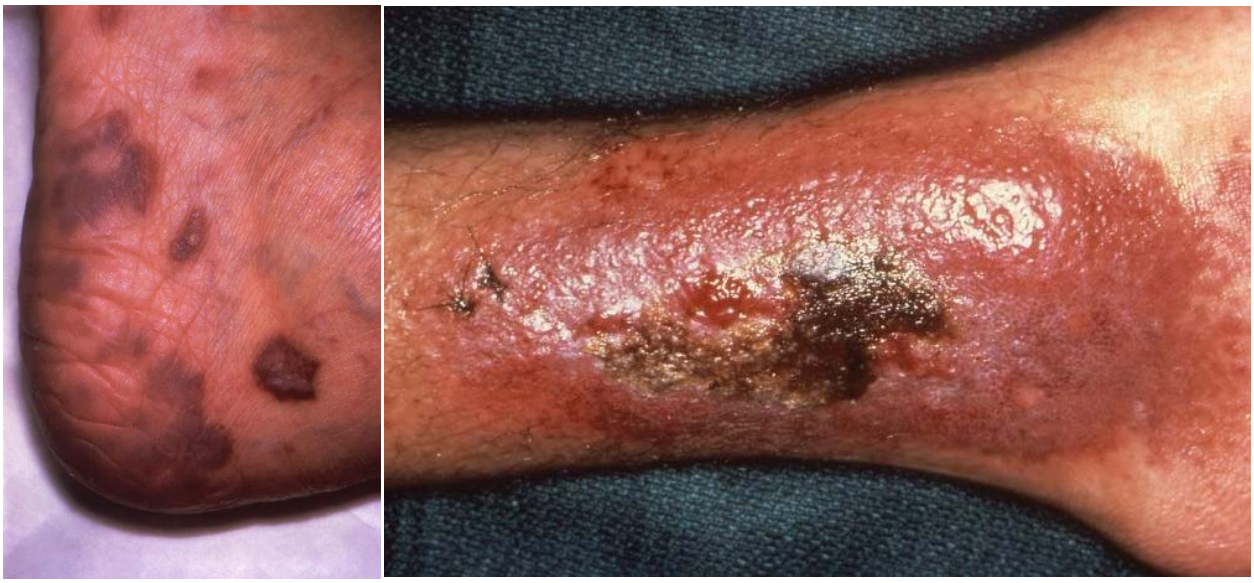


Figure 9-16 A & B. Kaposi sarcoma on a patient's right heel (A) and a more advanced tumor on the left leg (B). Kaposi sarcoma is a malignant tumor of the lymphatic endothelium caused by the human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV) and arises from a cancer of the lymphatic endothelial lining. It is characterized by bluish-red cutaneous nodules. Kaposi sarcoma is thought of as an opportunistic infection affecting patients whose immune systems have been compromised, as in the case of patients with HIV/AIDS. Courtesy of https://phil.cdc.gov/Details.aspx?pid=14430

DIAGNOSIS OF HERPESVIRUS INFECTIONS

HSV-1 & HSV-2

Virus Isolation, PCR, and serological assays such as ELISA.

VZV CHICKEN POX AND SHINGLES

Appearance of the distinctive vesicular rash is often sufficient; however PCR and virus isolation can also be useful. VZV IgG antibodies are useful for determining immune status of people over 14 to determine if vaccination is recommended.

CMV

Serologic tests for antibody to CMV can be used and are important for transplantation and blood donors as CMV negative recipients cannot receive organs from CMV positive donors. Virus can also be detected by viral isolation and PCR.

EBV

Serologic tests for antibody to EBV can be used. The test consists of an IgM test and IgG test. If only the IgG test is positive, a second sample must be obtained in no less than 2 weeks, and then at least a 4-fold increase in IgG titer must be observed for an individual to be having a primary infection. Serology tests can be used and are important for transplantation and blood donors as EBV negative recipients cannot receive organs from EBV positive donors. Virus can also be detected by viral isolation and PCR.

PREVENTION OF HERPESVIRUS INFECTIONS

HSV-1 AND HSV-2 INFECTIONS

To prevent HSV-1 and HSV-2 infections, safe sex including condom use is recommended, although condom use is not 100% effective as HSV infections are often **perigenital** (meaning around but not in the genitals), and many people are asymptomatic shedders. There is currently no HSV-1 or HSV-2 vaccine.

VZV CHICKEN POX AND SHINGLES

Multiple vaccines are available for chicken pox.

Vaccines that are recommended for children are:

Varivax (chicken pox vaccine) is a live attenuated vaccine.
ProQuad, combination with mumps, measles, rubella, a live-attenuated vaccine.

Note that these vaccines are not recommended for pregnant people due to the potential of the attenuated virus to cross the placenta and cause infections in the fetus.

Vaccines that are approved for people older than 60 yrs:

Zostavax, which is a live-attenuated vaccine that increases numbers of CD4 and CD8 effector and memory T cells and reduces the incidence of zoster by 50%. This vaccine, however, could be problematic in the immunosuppressed.

In 2017 the RZV/Shingrix Vaccine was approved for adults 50 and over. This is a recombinant Zoster vaccine consisting of the cloned mass-produced Varicella Zoster glycoprotein E. Glycoprotein E was chosen because it is the most abundant viral protein on viral envelope membrane and induces neutralizing antibody as well as CD4 T-cell responses. Multiple adjuvants are utilized to increase the cytokine and interferon response. Two doses are given 2-6 months apart.

CMV INFECTIONS

CMV is best prevented by avoiding exposure to saliva and urine of infected individuals. For organ transplantation, CMV negative patients cannot be given organs from CMV positive donors, so both donors and recipients are always screened. There is currently no CMV vaccine.

EBV INFECTIONS

Like CMV, EBV is best prevented by avoiding exposure to saliva, blood, and semen of infected individuals. For organ transplantation, EBV-negative patients cannot be given organs from EBV positive donors, so both donors and recipients are always screened. There is currently no EBV vaccine.

TREATMENT OF HERPESVIRUS INFECTIONS

Many herpesvirus infections can be treated (but not cured) with antiviral drugs that are derivatives of acyclovir (valacyclovir, penicyclovir, famciclovir, ganciclovir). These therapies will reduce the length and severity of outbreaks. Acyclovir and its derivatives are guanosine analogs which are converted by viral thymidine kinase to acyclovir monophosphate and then converted by **host cell kinases** to acyclovir triphosphate (ACV-TP). It is important to note that acyclovir lacks a 3'OH that would be required to create a phosphodiester bond when incorporated into a growing viral DNA strand by herpes-encoded DdDp (DNA polymerase). As such, ACV-TP results in chain terminations when the herpes-encoded DNA polymerase tries to incorporate it into the viral DNA during replication. Note, acyclovir and its derivatives are not effective against EBV. As such, there is no treatment for EBV.

END OF CHAPTER QUESTIONS

1. Compare and contrast why adenoviruses and herpesviruses provide their own DdDp.
2. Compare and contrast how herpesviruses, polyomaviruses, and papillomaviruses initiate replication of their genome and ensure replication of the ends of the genome.
3. Do herpesviruses create subgenomic mRNAs? If so, how and why? If not, why not?
4. Compare and contrast how parvoviruses and herpesviruses avoid the host antiviral responses.
5. Compare and contrast how parvoviruses, papillomaviruses, and herpesviruses deal with the need for replication components that are only present in cells during the S phase.
6. Do herpesviruses need to have mechanisms for overcoming RNA interference? If so, why, and what are they? If not, why not?
7. Can herpesviruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
8. What types of gene products are likely to be expressed by early, intermediate, and late genes of these herpesviruses?
9. What PRR are most likely to recognize herpesvirus infections? Do herpesviruses have mechanisms to avoid these PRR? If so, what are they?
10. Do herpesviruses create accessory proteins to deal with the host anti-viral defenses? If so, what are they? If not, why not?
11. How do herpesviruses leave the cells they infect?
12. Why are herpesviruses able to form syncytia?
13. You perform an ELISA assay to determine if a patient who comes to your clinic has a CMV infection. You find that the patient has a titer of 120 HI units/0.1 ml which is above the threshold. Two weeks later you take blood samples from the patient again. This time the titer is 240 HI units/0.1 ml. Do you believe this patient is currently suffering from a CMV infection? What led you to this conclusion?
14. What is thought to be the cause of VZV (chickenpox virus) reactivations? What disease is observed when this occurs?
15. How does vaccination prevent VZV reactivations?
16. Are there effective treatments that cure herpesvirus infections? If so, what are they? If not, why not?
17. How does acyclovir function? Does it only interact with viral enzymes or both viral and cellular enzymes to be activated?
18. What population subgroup is at greatest risk for complications of EBV infections?

19. Do herpesvirus infections ever lead to tumor formation? If so, which ones?

REFERENCES:

Centers for Disease Control and Prevention. Varicella Zoster. https://www.cdc.gov/vaccines/pubs/pinkbook/varicella.html

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). Dr. Joel D Meyers. (1973). Chickenpox in child with leukemia. https://phil.cdc.gov/Details.aspx?pid=18904

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1963) Rodger Feldman Congenital CMV. https://phil.cdc.gov/Details.aspx?pid=17976

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). Carl Flint, Dr. Volger, Emory University. (1969). EBV blood smear. https://phil.cdc.gov/Details.aspx?pid=19470

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1964). Emory Dr. Sellers. EBV Mono oral. https://phil.cdc.gov/Details.aspx?pid=1620

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). Emory Dr. Sellers. (1964). EBV ocular. https://phil.cdc.gov/Details.aspx?pid=2862

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1975). HHV-4. https://phil.cdc.gov/Details.aspx?pid=10234

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). HSV-1. https://phil.cdc.gov/Details.aspx?pid=22130

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1966). HSV-1 ocular. https://phil.cdc.gov/details.aspx?pid=6492

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1972). HSV-2. https://phil.cdc.gov/Details.aspx?pid=16783

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1986). Kaposi sarcoma. https://phil.cdc.gov/Details.aspx?pid=14430

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). K.L. Herrmann. (1966). Singles. https://phil.cdc.gov/Details.aspx?pid=21506

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). K.L. Herrmann. (1966). VZV hands. https://phil.cdc.gov/Details.aspx?pid=21514

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). (1977). Susan Lindsley. VZV infant. https://phil.cdc.gov/Details.aspx?pid=14823

Fenner, F., et al. 1987. Veterinary virology, p. 360. Academic Press, Inc., Orlando, Fla.

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 59-65.

Roberts, W. A., and G. A. Carter. (1976). Essentials of veterinary virology, p. 130. Michigan State University Press, East Lansing, Mich.

Roizman, R., and P. E. Pellet. (2001). The family *Herpesviridae*: a brief introduction, p. 1679–1728. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott Williams and Wilkins, Philadelphia, Pa.

ViralZone. Herpesvirus replication cycle. https://viralzone.expasy.org/5836

Chapter 10: Large Linear DNA Viruses That Replicate In The Cytoplasm: Poxviridae

INTRODUCTION TO THE FAMILY *POXVIRIDAE*

You will notice in Figure 10-1 that poxviruses are large, linear, double stranded DNA viruses that are enveloped with a brick-shaped or ovoid virion 250-300 nm long and 200-250 nm wide as shown in Figure 10-2. The surface envelope membrane displays surface tubules or surface filaments obtained from the cell via exit from the cell. The virion contains a core particle within a core wall made up of 3 different proteins. The core contains the genome wrapped in nucleocapsid proteins, although this not a helical structure. Surrounding the core is an envelope membrane. Assembly involves the acquisition and shedding of multiple lipid bilayers. Between the core wall and the membranes is the lateral body which is composed of proteins of unclear function. Because more than 70 proteins are known to assemble in the mature virion, the functions of these proteins have yet to be identified. Two distinct infectious virus particles exist. These are the internal mature virion and the extracellular enveloped virion. There are 3 subfamilies. Of these *Chordopoxvirnae* contains viruses which infect humans and animals and is divided into 9 genera. The genera that infect animals and humans are Orthopoxvirus (which includes vaccinia (cow pox)), Variola (smallpox), Ectromelia (mice), Monkeypox (monkeys, rodents and humans), *Parapoxvirus* (which includes Orf virus (sheep), Pseudocowpox, *Capripoxvirus* (which includes sheep poxvirus), *Leporipoxvirus* (which includes myxomavirus (rabbits), and *Molluscipoxvirus* (which contains molluscum contagiosum (humans)).

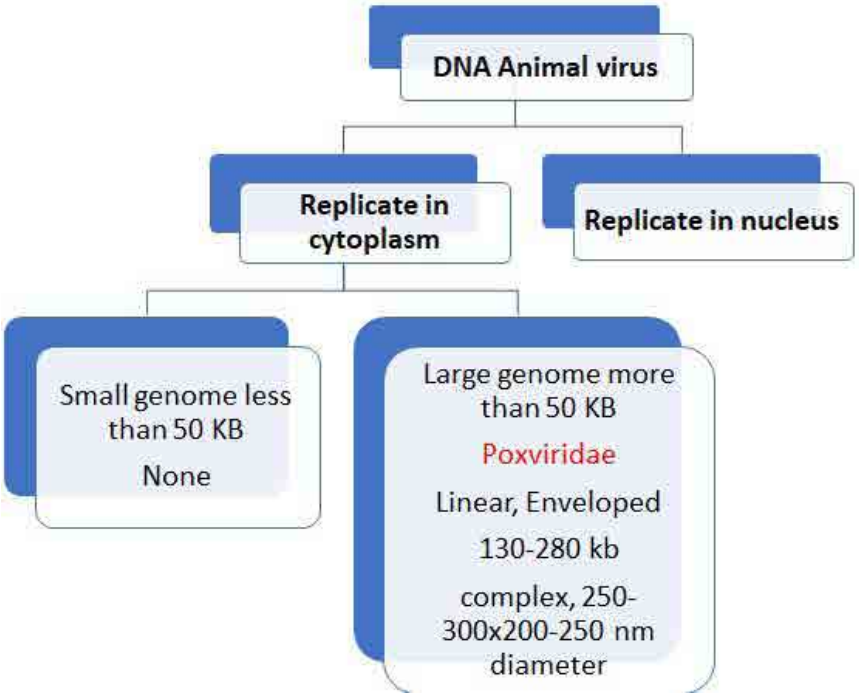


Figure 10-1. Taxonomy of DNA viruses. Courtesy of Erica Suchman, Colorado State University.

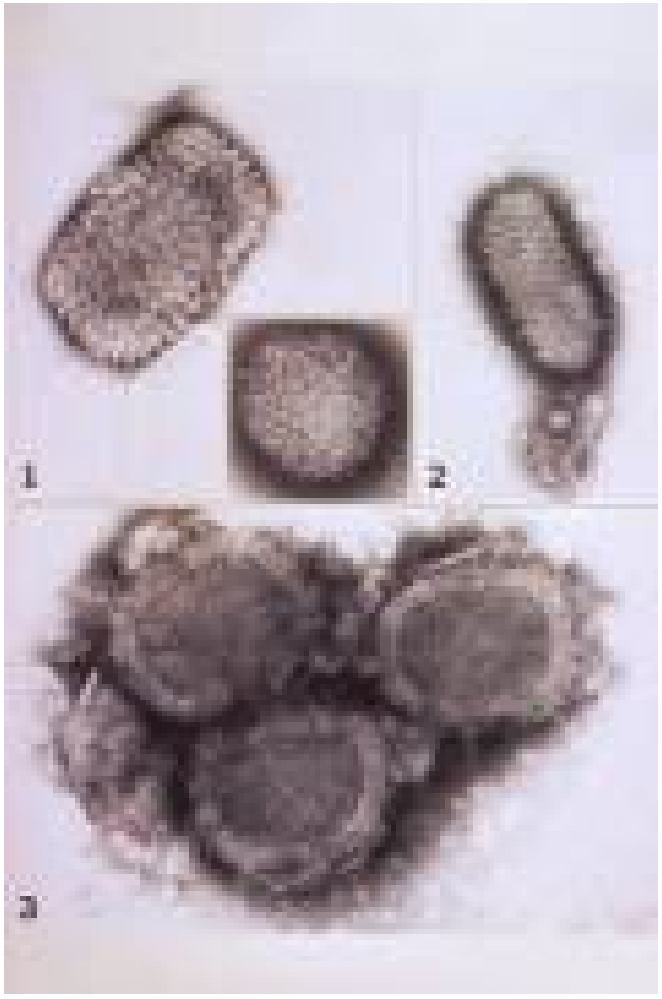


Figure 10-2. Electron microscopy images of different types of Pox viruses. Courtesy of https://phil.cdc.gov/

It is worth discussing the history of smallpox before we dive into the virus and its replication cycles. Animals are the only host for variola virus, the cause of smallpox. There is no animal reservoir. There exist two species, variola major (20% fatality) and variola minor (1-2% fatality). Smallpox has played a major role in shaping civilization many times. The earliest evidence of smallpox infection was in Egyptian mummies, in particular the Egyptian king Ramses V (1157 BC). When his tomb was uncovered, his mummy was found to be covered in scars indicative of a smallpox infection.

The virus was introduced to the Americas by European explorers. The British army used smallpox as a biological weapon against the Pontiac Indians in 1763. Overall, 40 million native Americans died from European diseases. It has been shown that Native Americans have limited MHC polymorphisms, and, as such, were particularly susceptible to fulminant disease and death. Smallpox is thought to have killed 300 million people in the 20th century alone with total fatalities probably near 1 billion. It was once a highly feared disease. It became a great success story when it was eradicated worldwide by vaccination. In fact, it was the first documented successful vaccination of humans.

GENOME STRUCTURE

The variola genome is a linear, dsDNA genome of 130-280 kb. The linear genome is flanked by highly AT-rich **inverted terminal repeat** (ITR) sequences which are covalently closed at the extremities. The ITR sequences are important in replication of the genome as will be discussed later in the chapter. Over 200 mRNAs are expressed from many promoters on both strands that are occasionally overlapping by a virally encoded DNA-dependent RNA polymerase (DdRp), none of which are spliced as the virus replicates in the cytoplasm and does not have access to the splicing machinery in the nucleus.

CYTOPLASMIC REPLICATION

1. Attachment of the viral envelope proteins to host glycosaminoglycans (GAGs) mediates endocytosis of the external enveloped virus (EEV) into the host cell. The other form of the poxvirus, the intracellular mature virus (IMV), is thought to enter the cells by fusion of the envelope to the plasma membrane. EEV is thought to mediate cell to cell infection within the host while IMV mediates infection of new hosts.
2. EEV then mediates fusion with the endosomal membrane to release the core into the host cytoplasm. Note that both mechanisms allow the virus to avoid detection by endoplasmic PRR TLR 9 which would detect endosomal DNA.
3. **Early phase**: early genes are transcribed in the cytoplasm by viral **DNA-dependent RNA polymerase** (DdRp), **capping enzyme,** poly A polymerase, and **early transcription factors** that enter the cell along with the genome. Early expression begins at 30 minutes post-infection and predominantly creates proteins to counteract cellular antiviral defenses.
4. The core is completely uncoated as early expression ends. The viral genome is now free in the cytoplasm.
5. **Intermediate phase:** Intermediate genes are expressed triggering genomic DNA replication at approximately 100 minutes post-infection by viral **DNA-dependent DNA polymerase** (DdDp). Transcription occurs in viral factories created in the cytoplasm sequestering the replication from cellular antiviral responses such as cGAS that will detect double stranded cytoplasmic DNA.
6. **Late phase:** Late genes are expressed from 140 min to 48 hours post-infection producing all structural capsid and envelope proteins, as well as transcription factors, to be packaged in the virion to facilitate early transcription upon infection.
7. Assembly of progeny virions starts in cytoplasmic viral factories producing a spherical immature particle. This virus particle matures into brick-shaped intracellular mature virion (IMV) after acquiring a membrane from membrane crescents that are formed by a still unknown mechanism.
8. IMV virion can be released upon cell lysis or can acquire a second double membrane from the *trans*-Golgi or endoplasmic reticulum via wrapping (wrapped virion, WV). It then travels along actin filaments and leaves the cell when the membrane fuses with the plasma membrane thus removing one layer. It is projected from the cell on actin tails as external enveloped virion (EEV) as shown in Figures 10-3 & 10-4.

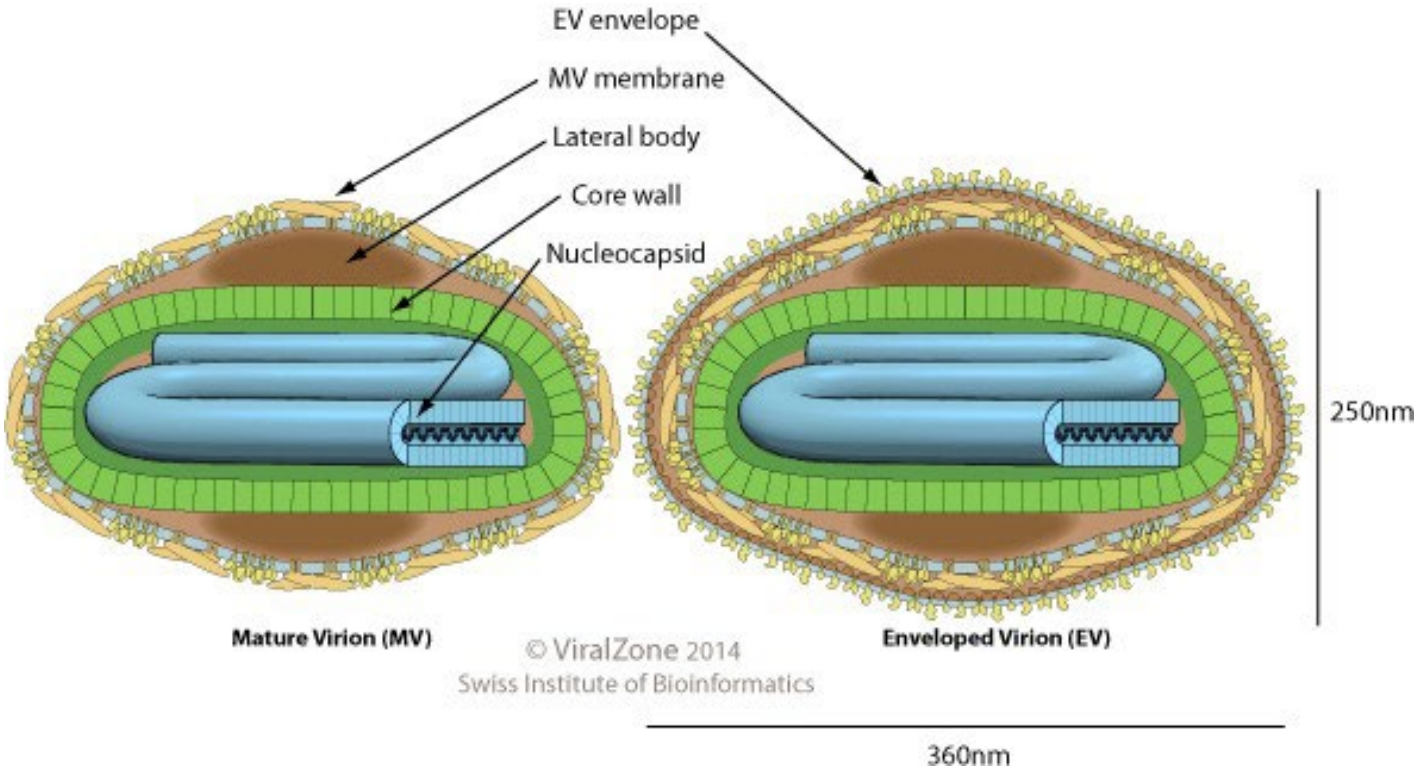


Figure 10-3. Poxvirus infectious virions: internal mature virions and external enveloped virions. Note that both particles contain an envelope membrane, but the external enveloped virion contains a second envelope membrane. Courtesy of the ViralZone https://viralzone.expasy.org/174

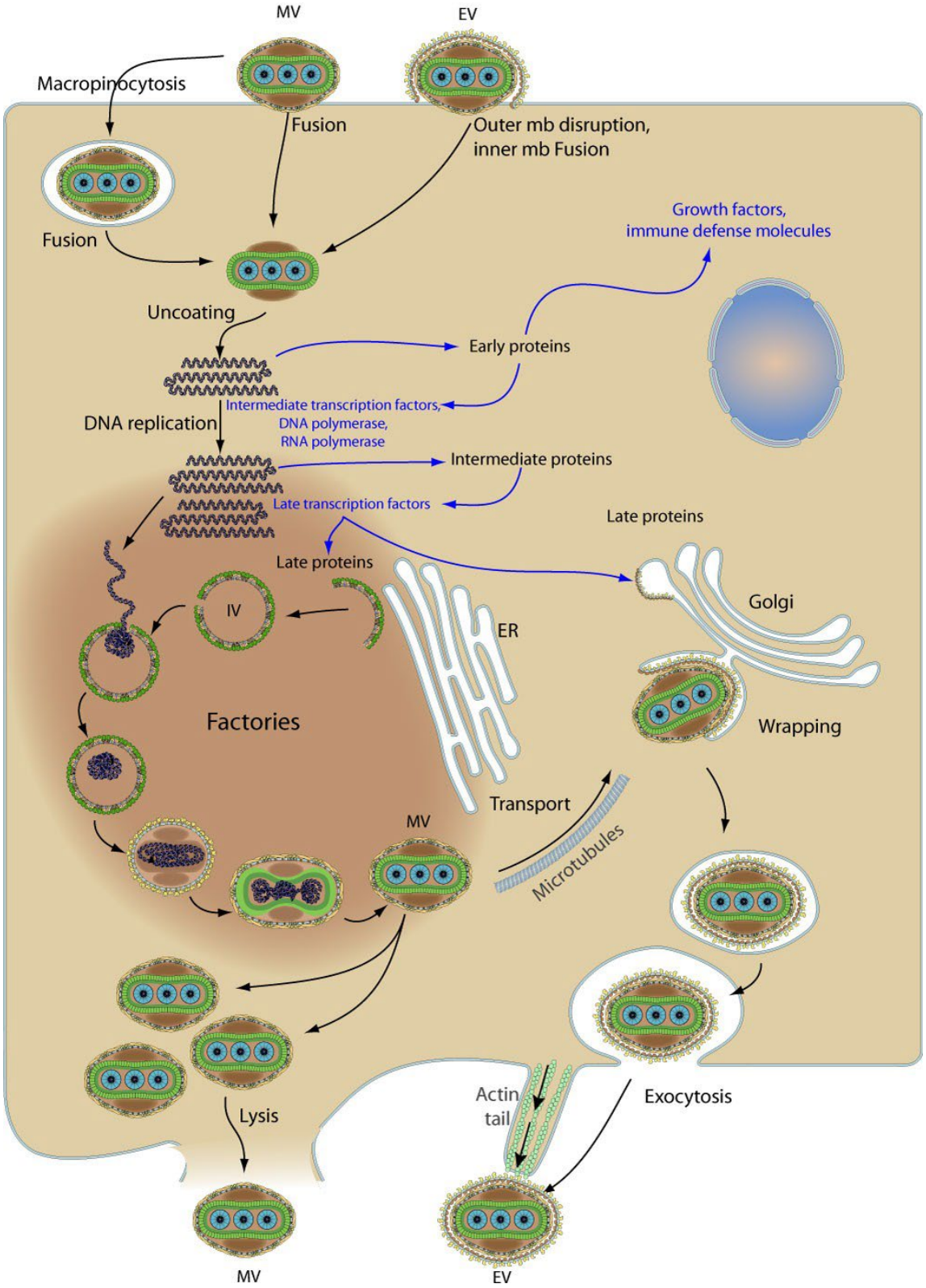


Figure 10-4. Poxvirus replication cycle. Courtesy of ViralZone https://viralzone.expasy.org/4399

HOW DO POXVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Unlike all nuclear replicating DNA viruses of animals, poxviruses cannot take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells due to their replication in the cytoplasm.

Poxviruses can use multiple promoters to create many mRNAs. There are thought to be approximately 200 promoters in poxviruses. This is their sole mechanism for producing many proteins. They must, however, create their own DdRp to carry out transcription as cellular RNA pol II is only found in the nucleus.

Poxviruses cannot use alternative splicing to create many smaller versions of a pre-mRNA, all of which produce different proteins, because the splicing machinery is in the nucleus.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral proteins. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that will be recognized by the necessary initiation factors (eIF4E &G and PABP, Chapter 3). However, unlike all other DNA viruses, the 5' cap and 3' poly A tail will be added by viral proteins. Proteins will be translated in a temporal fashion with early, intermediate, and late transcription and translation events. All of the products to carry out transcription and DNA replication must be produced by the virus.

DNA REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA:

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Poxviruses overcome this issue by replicating in the cytoplasm and creating their own DNA-dependent DNA polymerase (DdDp, DNA pol).
2. How to primer their DNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome.

Note that poxviruses, like many other viruses, solve the problem of replicating the ends and priming replication via the same mechanism described below.

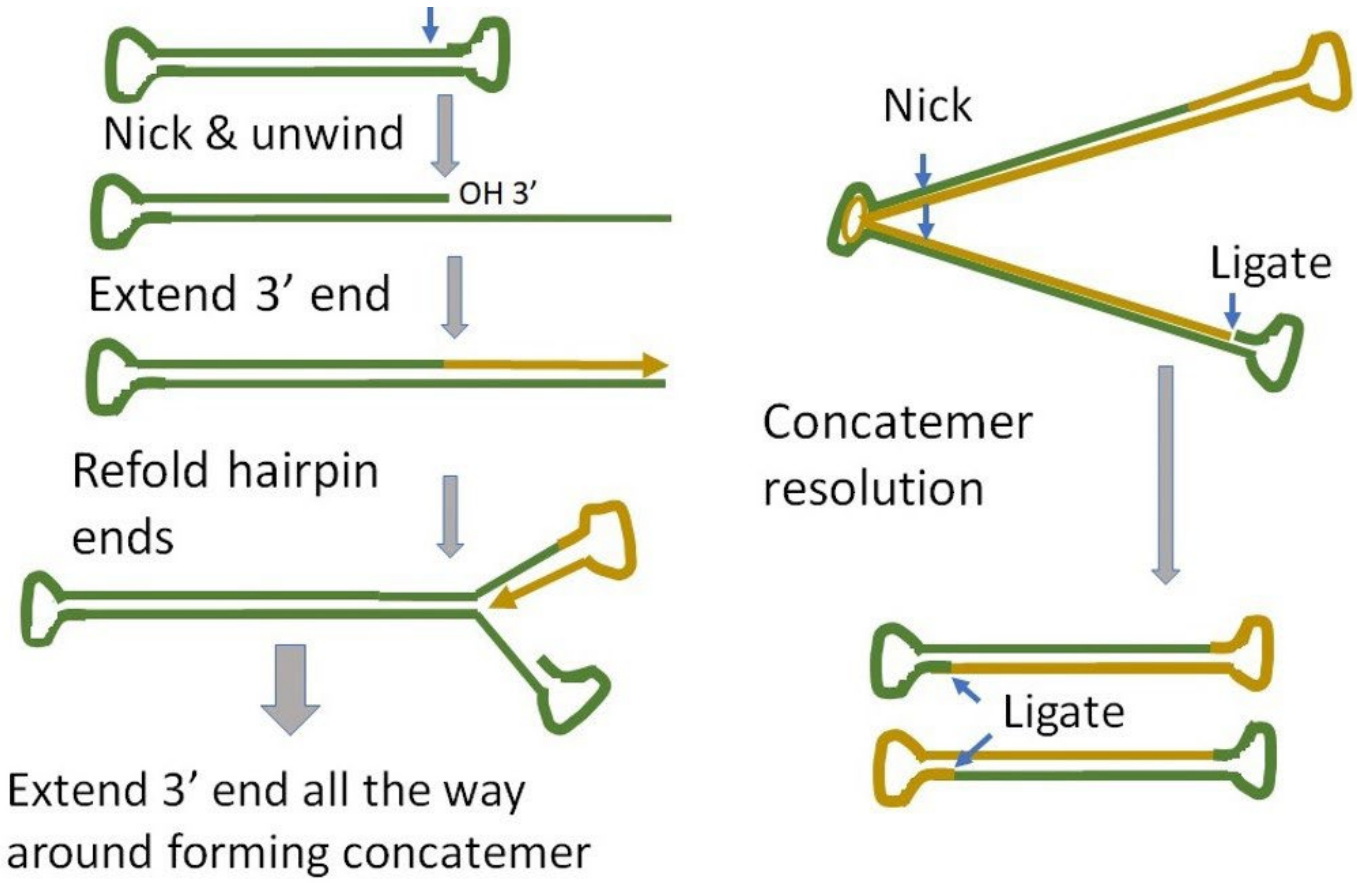


Figure 10-5. Poxvirus DNA replication using the inverted terminal repeats. Courtesy of Erica Suchman, Colorado State University.

The covalent linkage at the end of the virus is nicked by a viral replication enzyme, and the inverted terminal repeat at the end of the genome template unwinds leaving a 3' OH that serves as the primer to initiate viral DNA synthesis by viral DdDp. Note that no RNA primers are required nor are **Okazaki fragments** created. This method of priming is different than DNA replication of host cell DNA. Instead, continuous synthesis continues from the 3'OH group of the loop to the end of the parental strand. Once the inverted terminal repeat has been copied, it folds back upon itself allowing the DdDp to continue replicating the rest of the strand. Note that when the DdDp reaches the other covalently linked end of the virus, it continues replicating creating 2 end-to-end covalently linked **concatemer** copies of the genome. A viral enzyme then cuts the phosphodiester bonds between the 2 copies creating 2 individual genomes. Viral ligase then creates the covalent bonds at the end of each genome.

VIRION FORMATION

Immature virions form within the viral factory after the formation of crescents of membrane that are formed by an unknown mechanism. The immature virions then become the brick-shaped intracellular mature virus (IMV) which are the most abundant form of the virus. IMV are retained within the cell and only released when the cell lyses. They are very stable in the environment and are therefore most important in transmission between different hosts. The IMV can also be wrapped in membrane fragments from either the Golgi apparatus or endoplasmic reticulum forming 2 envelope membranes and becoming a wrapped virus (WV). These WV travel to the cell surface via microtubules. The EEV can now leave the cell by membrane fusion of WV forming EEV leaving a particle which

contains only one envelope membrane. Both forms are infectious. IMVs are more important for transmission from one host to another, while EEVs are more important for transmission within a host via cell-to-cell spread as the second membrane makes them more susceptible to drying. IMVs are released when cells lyse, and EEVs are released by membrane fusion and then is projected from the cell on actin tails. Poxviruses are unique as there are multiple infectious forms that both enter and leave cells by different mechanisms as shown in Figure 10-4.

AVOIDING THE HOST IMMUNE RESPONSE

Pox viruses, having more than 200 genes, have many defenses against both the cellular antiviral defenses as well as the innate and adaptive immune responses. In particular, they have mechanisms to inactivate cascade pathways activated by **pathogen recognition receptors** (PRRs), such as cGAS, that recognize cytosolic double stranded DNA, or Rig I and MDA-5 that recognize improperly capped mRNAs as **pathogen associated molecular patterns** (PAMP). They enter cells in a way to avoid the endosomal PRR TLR 9 that detects endocytic DNA. Some forms enter by membrane fusion avoiding entering endosomes and others enter cells via endocytosis but remain in their capsid until they leave the endosome.

Furthermore, poxviruses create dense viral factories that protect the virus from detection by PRRs. They can also inactivate interferon responses by creating secreted proteins with homology to interferon receptors which can bind and sequester interferon inhibiting its ability to activate production of antiviral cellular defenses. However, they also inactivate the main **interferon stimulated gene** products **OAS** and **PKR**. In addition, they also secrete a **tumor necrosis factor receptor** (TNFR) homolog as well as many **interleukin receptors,** they sequester interleukins as well. Also, poxviruses possess anti-apoptosis as well as anti-cellular protease mechanisms. Studies of monkeypox infections have shown that poxviruses have anti-complement defenses that, if lost, make the virus weakly pathogenic. Recall from Chapter 4 that **complement** can form holes in enveloped viruses, infected cells and induce **opsonization**. So, as you can see, they have many accessory proteins that help overcome the host immune response. Unfortunately, very little is known about how smallpox virus (variola) evades the immune system as it is too dangerous to work with, and there are no good animal model systems.

POXVIRUS DISEASES

SMALLPOX

The three main poxvirus diseases contracted by humans are smallpox and monkeypox (an infection humans can contract from rodents) and ORF (an infection of ruminants that humans can catch). Note that there are many other poxviruses of animals and birds (sheep, goats, pigs, mice, rabbits) all with similar rash and disease progression.

Smallpox (variola) is spread by droplet aerosol or contact with material from pustules of infected individuals. Initial symptoms develop after 7-17 days and include fever and a **centrifugal rash** that appears densely on the palms, soles of the feet, and face with all of the rash being at the same stage at the same time. They begin as **macules** (flat discolorations of the skin), then progress to **papules** (raised discolorations of the skin), **vesicles** (blisters), pustules (pus filled vesicle), and finally crusted scabs as shown in Figure 10-6. This rash distribution is very different from chicken pox which has a **centripetal** rash that occurs predominantly on the trunk and face and has many different stages of the rash present at any given time. These differences were long used to differentiate between the two diseases that began with fever and rash. Death occurred in approximately 10-30% of people infected with variola major and almost never in chickenpox (unless the infection occurred in an adult).

Very little is known of the progression of the viral infection in a host, the immune response, or viral counter measures as there has not been a smallpox infection in a human since the late 1970's.

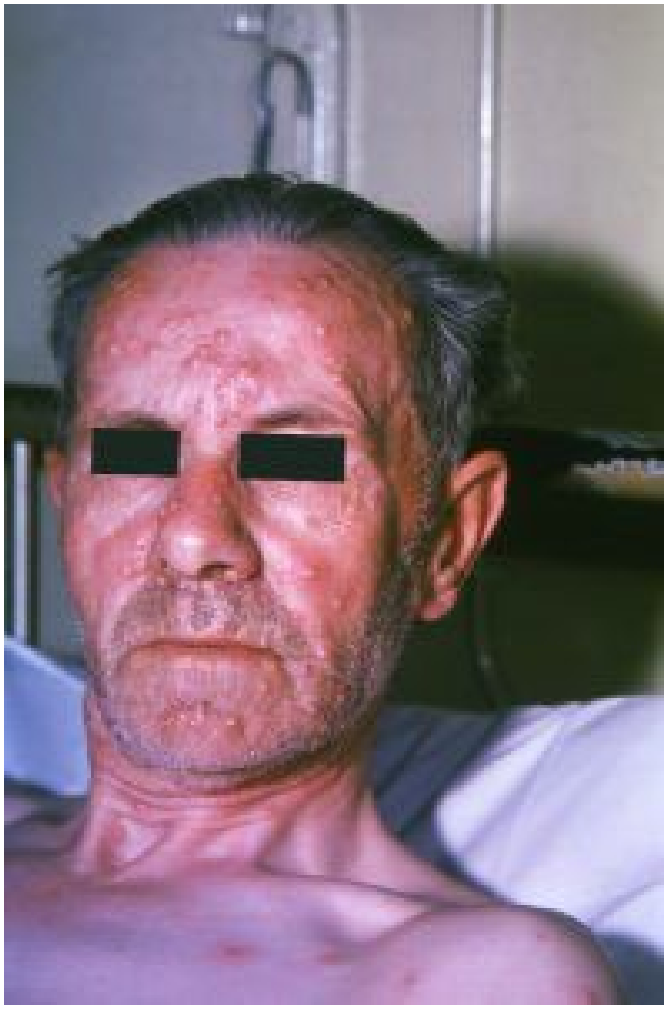


Figure 10-6. Patient in the 1960's with smallpox rash. Courtesy of https://phil.cdc.gov/Details.aspx?pid=19561

Since smallpox virus was only known to infect humans, scientists wanted to know whether an animal model could be developed to study pathogenesis. What could science learn from studying the course of infection and immune response in an animal model? Because of 9/11, CDC and USAMRIID Scientists were granted permission by the WHO to infect cynomolgus macaques with smallpox in an attempt to assess pathogenesis using contemporary laboratory techniques. This required highest containment. BSL-4 (biosafety level 4 lab) and greater than 10^9 pfu had to be injected IV for infection to take hold. This resulted in fulminant disease that was fatal in most macaques and allowed scientists to use more modern techniques to study the relationship between the virus and the host.

MONKEYPOX

In 2003, the US had its first ever outbreak of monkeypox. There were 72 documented cases in Wisconsin, Illinois, Indiana, Kansas, Missouri, and Ohio. The virus was introduced from Africa to a US pet supplier via Gambian giant rats and prairie dogs. The infection was not fatal to humans. In Africa the disease is fatal in 1-10% of cases. In the Democratic Republic of Congo, fatality is approximately 10%. In West Africa, however, it is only 1-2%. Fortunately, the US strain was from West Africa. Genomic comparisons between the 2 strains revealed 5 genes mutant or missing in West African strains compared to the Central African strains. The West African strains do not have a protein that disables complement enzyme activity, whereas the Central African strain does. This inability to inactivate complement's ability to pop holes in the envelope of the monkeypoxvirus caused the virus to lose its virulence. We also know that during a primary immune response, IFNs are responsible for containment of primary infection, and that macrophages and NK cells lead to clearance of virus, whereas CTLs are responsible for clearance of virus from infected cells. The virus encodes many proteins that function to evade or modulate the host's response to infection.

In 2022, monkeypox virus began to infect and spread, and in late 2022 the WHO declared an emergency, and several US states have also declared emergencies. At this writing, more than 13,000 monkeypox infections have occurred in the United States during the current outbreak. There is concern that the virus may become endemic in many countries, including the United States. Although most cases have been among gay and bisexual men, other groups have also had infections. The virus is of the west African clade and case fatality rates have been low. It is thought that monkeypox transmission is through close or direct contact and that abrasions may be necessary for transmission to occur. A new vaccine has been developed for smallpox that confers protection against monkeypox due to their antigenic similarity. It is a replication defective vaccinia virus, modified vaccinia Ankara (MVA), that has fewer risks of adverse events relative to the original vaccinia Ankara virus used for immunization.

ORF VIRUS

Orf virus is a member of the *Parapoxvirus* genus in the *Poxviridae* family which is associated with viral infection of herbivores such as sheep and goats but can be transmitted to people through cuts or lesions in the skin. Infections of animals with orf virus occur throughout the world wherever small ruminants exist. Diagnosis can be confirmed when goats and sheep typically develop scabby sores (lesions) around lips, muzzle and inside the mouth. Early in the infection sores appear as blisters and turn into crusty scabs shown in Figure 10-7. Orf virus is spread through direct contact with infected animals or virus-contaminated fomites. The infection will only establish at sites where the skin is traumatized. Orf virus has been found to survive up to 17 years in environments with dry climates. Additionally, it can remain viable on the wool of animals and other contaminated fomites for significant periods. In cold, wet environments the virus has been found to lose its infectivity after a few years.

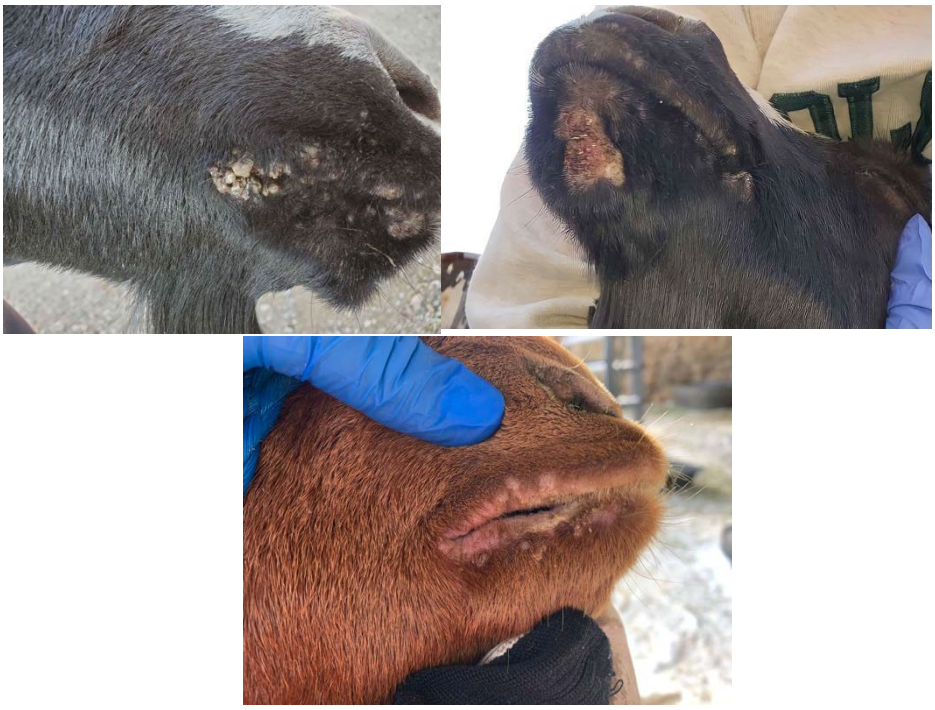


Figure 10-7 A-C: Case appearances of orf in goat. Source- Anna Gongaware, student Colorado State University.

DIAGNOSIS OF POXVIRUS INFECTIONS

Pox virus infections can be detected using PCR amplification of specific poxvirus genes. Serological assays such as ELISA and serum neutralization assays can also be performed. Like all serological assays, if assaying for neutralizing antibody, samples must be taken 2 weeks apart (**acute** and then **convalescent**), and the titer must rise at least 4-fold over the period of time to indicate a current or recent infection.

PREVENTION OF POXVIRUS INFECTIONS

The best prevention of smallpox infections today is vaccination. This vaccine has a long and interesting history. It was one of the first vaccinations ever used in humans and led to a great public outcry from individuals who feared they would develop bovine traits from being infected with a cow virus. Edward Jenner, the inventor of vaccination, observed that milkmaids rarely contracted smallpox, and that all had recalled earlier cowpox infections which were nearly universal in milkmaids. Cowpox usually only causes a mild disease in humans with lesions on the hands. Jenner hypothesized that the infectious agent of cowpox protected against smallpox. He inoculated a nephew by scarification with cowpox crusts, termed variolation. This led to formation of vesicles at the site of inoculation that would heal and leave a scar as shown in Figure 10-8. His nephew never developed smallpox which helped convince others to try the vaccine to protect themselves and their children from possible death from an infection that had a 10-30% case-fatality rate. For many years the vaccine was a live, attenuated vaccinia virus that was manufactured by infecting calves or rabbits and collecting lymph. Today's vaccine is a replication incompetent vaccinia virus, thus eliminating the issues of using an attenuated virus that could cause issues in the immunosuppressed. Vaccinia's genome is similar to cowpox, but it is not identical. Smallpox had been killing millions of people per year for millennia. In the 20th century, it is estimated that 300-500 million people died from smallpox.

Eradication was proposed by the Soviet Union in 1958, and an Intensified Smallpox Eradication Program was initiated in 1967 by the World Health Organization (WHO) using a ring immunization strategy. Whenever an outbreak was reported, intense vaccination of surrounding communities was conducted moving inward toward the epicenter (in a ring) as shown in Figure 10-9. Thirteen years after initiation, smallpox was declared eradicated in 1980. The last reported case of smallpox was in 1977. This was the first infectious disease agent to be eradicated. This was able to be accomplished in large part because there are no reservoir hosts other than humans.

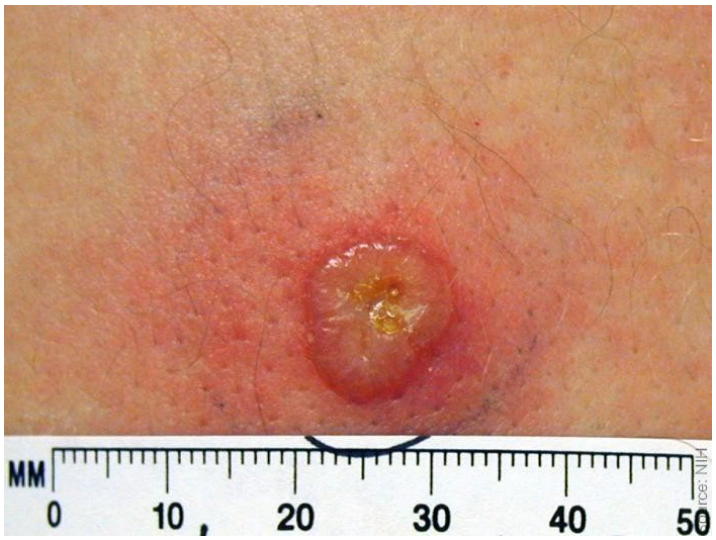


Figure 10-8. Normal primary, 8 days post vaccination. Large vesicle on erythematous base. Source: NIH, digital enhancement © Logical Images. Image courtesy of https://www.cdc.gov/smallpox/ clinicians/major-take-reactions-images.html

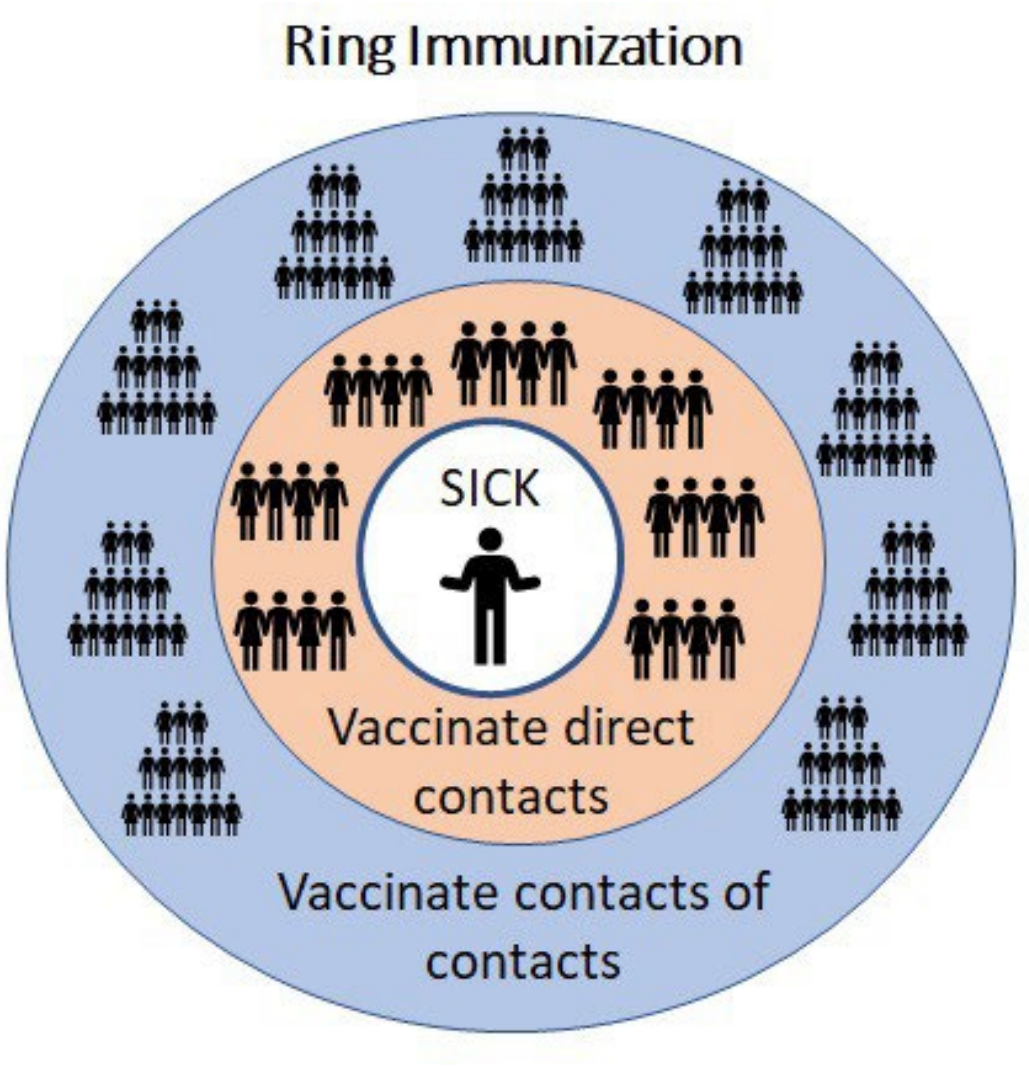


Figure 10-9. Ring immunization of contacts and contacts of contacts of smallpox that was used to eradicate the disease. First vaccinate the surrounding community and work your way towards the outbreak. Courtesy of Erica Suchman, Colorado State University.

The best prevention of monkeypox infections is to not handle rodents such as prairie dogs or Gambian giant rats from Africa. Also people suffering from monkeypox should quarantine and refrain from having sex until the virus has cleared (~21 days).

ORF virus infection is difficult to prevent because it can survive in the environment for long periods of time. However, the possibility of exposure can be decreased if various steps are taken:

1. Remove all harsh brush from pastures or stalls in order to reduce cuts or abrasions around the mouth because animals with cuts are most susceptible.
2. Disinfect all common living and feed areas
3. Wash and disinfect hands and any tools used when working between infected and non-infected animals
4. Quarantine any animals that are infected with orf virus and any new animals in order to rule out sore mouth

Additionally, vaccines can be used for secondary prevention mechanisms. There are live attenuated vaccines available for use by farmers. If a producer considers using the vaccine in their herd, they should consult a veterinarian. The orf vaccine is a preparation of live virus, so it is only suggested for use in herds that have experienced orf virus infection before or herds in which the vaccine has already been used. Vaccinating a clean herd will introduce the disease into the herd, so the producer should consider this fact prior to vaccinating. The live attenuated virus vaccine may not provide long-term protection because the live virus from the vaccine must replicate (grow) in the animal in order to induce an immune response. If anything either interferes with the replication or damages the live organism then the vaccine can become ineffective, so it is recommended that farmers vaccinate each year.

TREATMENT OF POXVIRUS INFECTIONS

Unfortunately, there are no successful drug treatments for poxvirus infections, so prevention is of the utmost importance.

TREATMENT OF ORF VIRUS INFECTIONS

In mild cases of an infection, treatment may not be necessary. Sores will go away on their own within 24 to 48 days after the infection. In more severe cases, softening ointments may help. There are no successful drug treatments for an orf virus infection, therefore prevention is important in order to avoid infection of a goat or sheep herd with this virus. If a secondary bacterial infection of orf virus does occur, it can be treated with topical or systemic antibiotics.

END OF CHAPTER QUESTIONS

1. What enzymes do poxviruses use to reproduce the genome and transcribe mRNA? Are these enzymes cellular or viral? Why does it use these particular enzymes?
2. How do poxviruses initiate replication of their genome and ensure replication of the ends of the genome?
3. How does poxvirus DNA replication differ from host cell DNA replication?
4. Does this virus create subgenomic mRNAs? Why, or why not? If so, how? If it does, how do the mechanisms it uses compare to other DNA viruses?
5. How do poxviruses avoid the host antiviral responses?
6. How do poxviruses obtain a 5’ cap and poly A tail?
7. Do poxviruses need to have mechanisms for overcoming RNA interference? If so, why, and what are they? If not, why not?

8. Can poxviruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
9. What types of gene products are likely to be expressed by early, intermediate and late genes of poxviruses?
10. What PRR are most likely to recognize poxvirus infections? Do poxviruses have mechanisms to avoid these PRR? If so, what are they?
11. What are some host anti-viral evasion mechanisms that are found in poxviruses?
12. What symptoms do most poxvirus infections have in common?
13. You perform a serum plaque neutralization assay to determine if an army recruit is suffering from a poxvirus infection. You find that the recruit has a titer of 400 NU/0.1 ml which is above the threshold. Two weeks later you take blood samples from the recruit again., The titer is 400 NU/0.1 ml again. Do you believe this recruit is currently suffering from a poxvirus infection?
14. You perform an IFA assay on poxvirus infected cells. Where do you expect to see staining, and why?
15. You are analyzing CPE in poxvirus infected cells. What type of inclusion bodies do you expect to observe, and why? What other type of CPE are you likely to observe in pox infected cells?
16. Compare and contrast poxviral EEV and IMVs.
17. Are there effective treatments for poxvirus infections? If so, what are they? If not, why not?
18. Monkeypox viruses are found to vary greatly in their pathogenicity. What major difference was found between the lethal strains from the Congo and the less lethal strains from West Africa?
19. How were smallpox virus infections and chicken pox infections differentiated historically?
20. What are the best ways to prevent smallpox infections?
21. What are the best ways to prevent monkeypox infections in humans?

REFERENCES:

Centers for Disease Control and Prevention. Smallpox diagnosis skin test. https://www.cdc.gov/smallpox/clinicians/major-take-reactions-images.html/

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). Dr. B. Challenor. (1966). Smallpox. https://phil.cdc.gov/Details.aspx?pid=19561

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, chapters 66, 67.

Harrison, S. C., et al. (2004). 101 (31) 11178-11192. Discovery of antivirals against smallpox PNAS; https://doi.org/10.1073/pnas.040360010

Jahrling B., et al. (2004). Exploring the potential of variola virus infection of cynomolgus macaques as a model for human smallpox PNAS. 101:15196-15200. https://www.pnas.org/content/101/42/15196.full

Moussatche, N. and Condit, R.C. (2015). Fine structure of the vaccinia virion determined by controlled degradation and immunolocalization Virology. 475: 204–218. DOI:10.1016/j.virol.2014.11.020

Rubins, K. H., et al. (2004). The host response to smallpox: analysis of the gene expression program in peripheral blood cells in a nonhuman primate model. PNAS. 101:15190-15195, http://www.pnas.org/content/101/42/15190.full/

ViralZone. *Poxviridae.* https://viralzone.expasy.org/174

ViralZone. Poxvirus replication cycle. https://viralzone.expasy.org/4399

Acknowledgment:

Anna Gongaware Colorado State University, Fort Collins, CO authored the orf disease sections.

Reviewed by:

Dr Tony Schountz, Colorado State University, Fort Collins, CO.

CHAPTER 11

Chapter 11: DNA Viruses That Use Reverse Transcriptase And Replicate In The Nucleus: Hepadnaviridae

INTRODUCTION TO THE FAMILY *HEPADNAVIRIDAE*

You will notice in Figure 11-1 that hepadnaviruses are DNA viruses that are enveloped, have an icosahedral capsid, replicate in the nucleus, and use reverse transcriptase to create double stranded DNA from their **RNA pregenome** inside their virion. These viruses have a small genome of only 3200 base pairs and a virion of 42-47 nm. Although hepadnaviruses have a smaller genome than parvoviruses, they have a larger virion. Can you think of any reasons why this might be? The virion contains an icosahedral capsid surrounded by core proteins that is enclosed within an envelope that contains 3 viral envelope proteins. It is partially double stranded when the virion enters the cell, however you will see that it packages mRNA that is reverse transcribed in the virion. This is different than retroviruses which also package mRNA but carry this to a new host cell and then reverse transcribe in the cytoplasm. There are 2 genera in the family: *Orthohepadnavirus,* which includes hepatitis B and hepatitis viruses of many other mammals (woodchucks, ground squirrels, and monkeys) and *Avihepadnavirus,* hepatitis viruses of birds.

Although there are many hepatitis viruses (A, B, C, D, and E), hepatitis B is the only one in the *Hepadnaviridae* family. All of the others are RNA viruses that do not use reverse transcriptase to make DNA from RNA. Then why are they all hepatitis viruses you might ask? Because they all cause damage to hepatocytes leading to jaundice (hepatitis). Long before we could analyze the molecular biology of hepatitis viruses, these viruses caused similar symptoms yet were known to transmit by different routes, and thus were all named hepatitis.

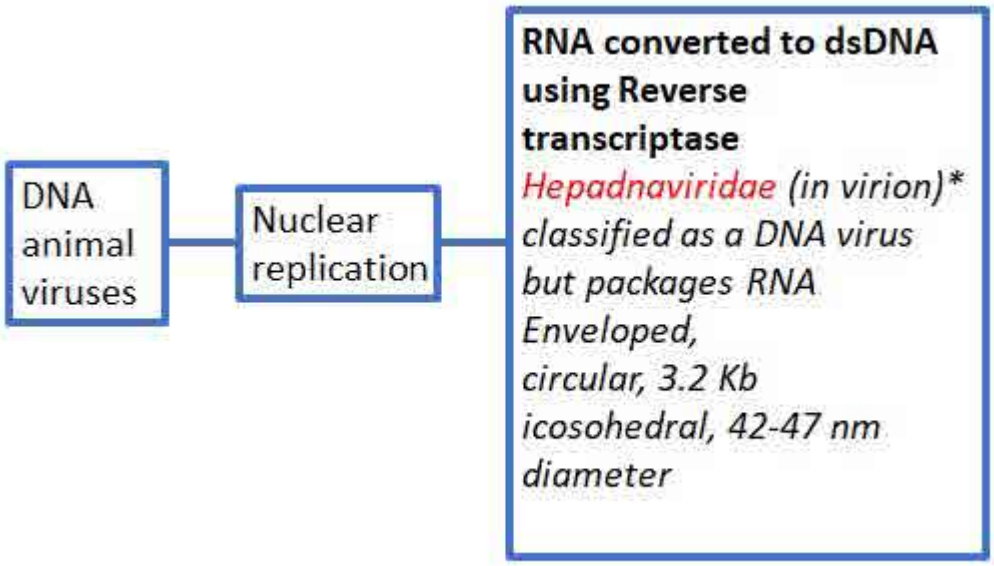


Figure 11-1. The taxonomy of DNA viruses focusing on hepadnaviruses. Courtesy of Erica Suchman, Colorado State University.

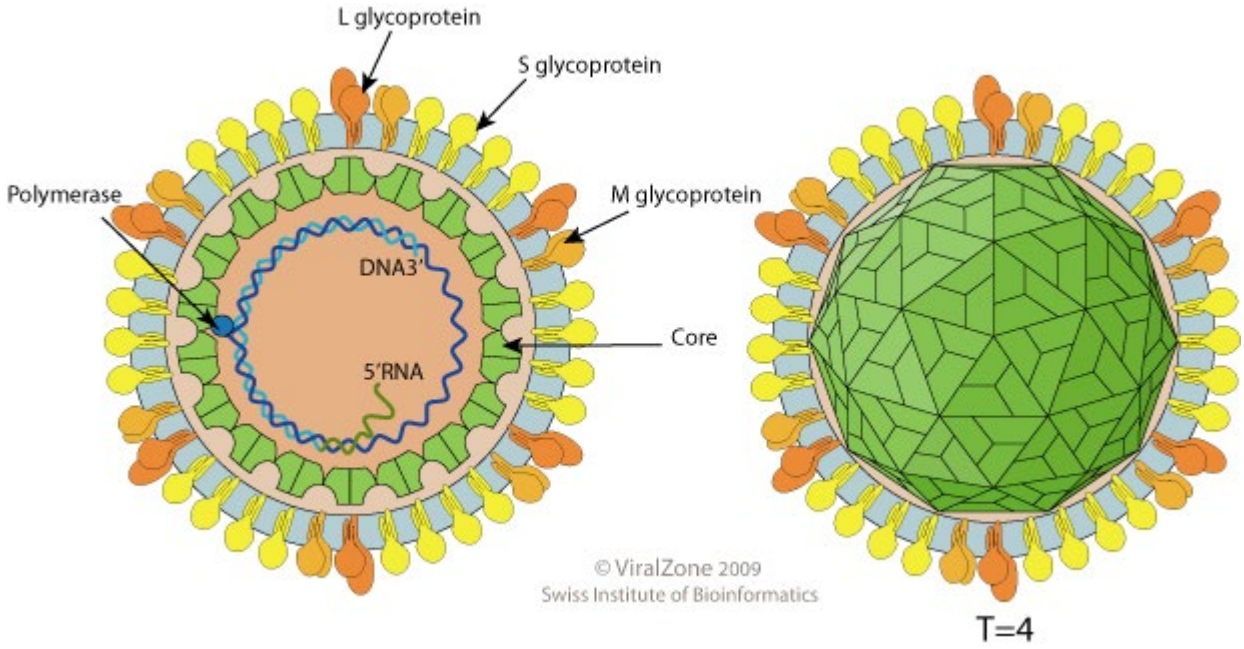


Figure 11-2. A hepatitis B particle. Courtesy of ViralZone https://viralzone.expasy.org/9

GENOME STRUCTURE

Hepadnaviruses have a partially double stranded DNA genome that is 3.2 kb in size and contains multiple promoters which produce mRNAs. These mRNAs undergo splicing like all other nuclear replicating DNA viruses and contain a 5' cap and 3' poly A tail. Transcripts are created from 4 different promoters, all on the same genome strand. As a result, although the mRNAs are overlapping, they are not complementary, and the virus avoids activating type I interferons, interferon stimulated genes and possibly RNAi via double stranded RNA.

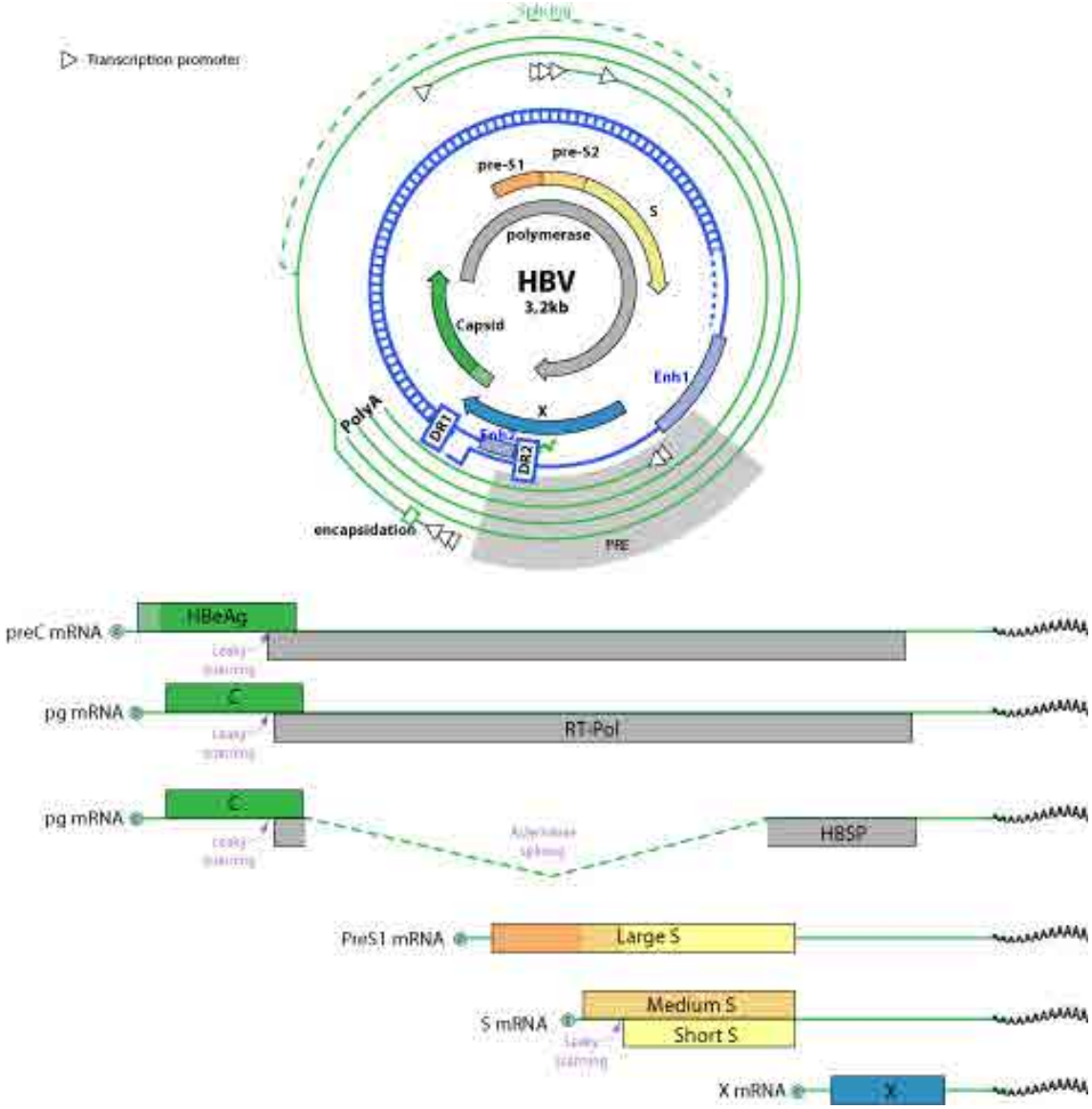


Figure 11-3. Hepadnaviridae genome structure. Courtesy of ViralZone https://viralzone.expasy.org/7

VIRAL REPLICATION CYCLE

As shown in Figure 11-4, the hepadnavirus replication cycle begins when

1. The virus attaches to host receptors through major surface antigen and enters the cell by endocytosis. It leaves the endocytic vesicle by fusing its membrane with the membrane of the vesicle.
2. **Relaxed circular partially double stranded DNA** (RC-DNA) and capsid are transported via microtubules to the nucleus where DNA is released through the nuclear pore. The capsid is trapped on the smaller nuclear side of the pore the DNA is injected through the pore into the nucleus. Now the DNA is repaired to form **covalently closed circular DNA** (cccDNA). This repair is necessary for the DNA to be transcribed by the host cell RNA Pol II. The circular genome also now associates with histone proteins.
3. Transcription is carried out by cellular RNA polymerase II of the **pregenomic RNA** (pgRNA) that will be packaged into the virion and subgenomic mRNAs (created by splicing) that are used for synthesis of all the viral proteins. pgRNA is encapsulated together with the P protein (reverse transcriptase which also serves as the primer) and reverse-transcribed inside the nucleocapsid by reverse transcriptase (P).
4. The (-) DNA is covalently linked to P protein (RT).
5. (+) DNA synthesis from the (-) DNA template generates new RC-DNA. RC-DNA containing nucleocapsids are enveloped at the ER-Golgi compartment, and new virions are released by exocytosis. Note: occasionally the DNA can integrate into the host genome leading to liver tumors. Hepadnaviruses, however, do not create an integrase protein.

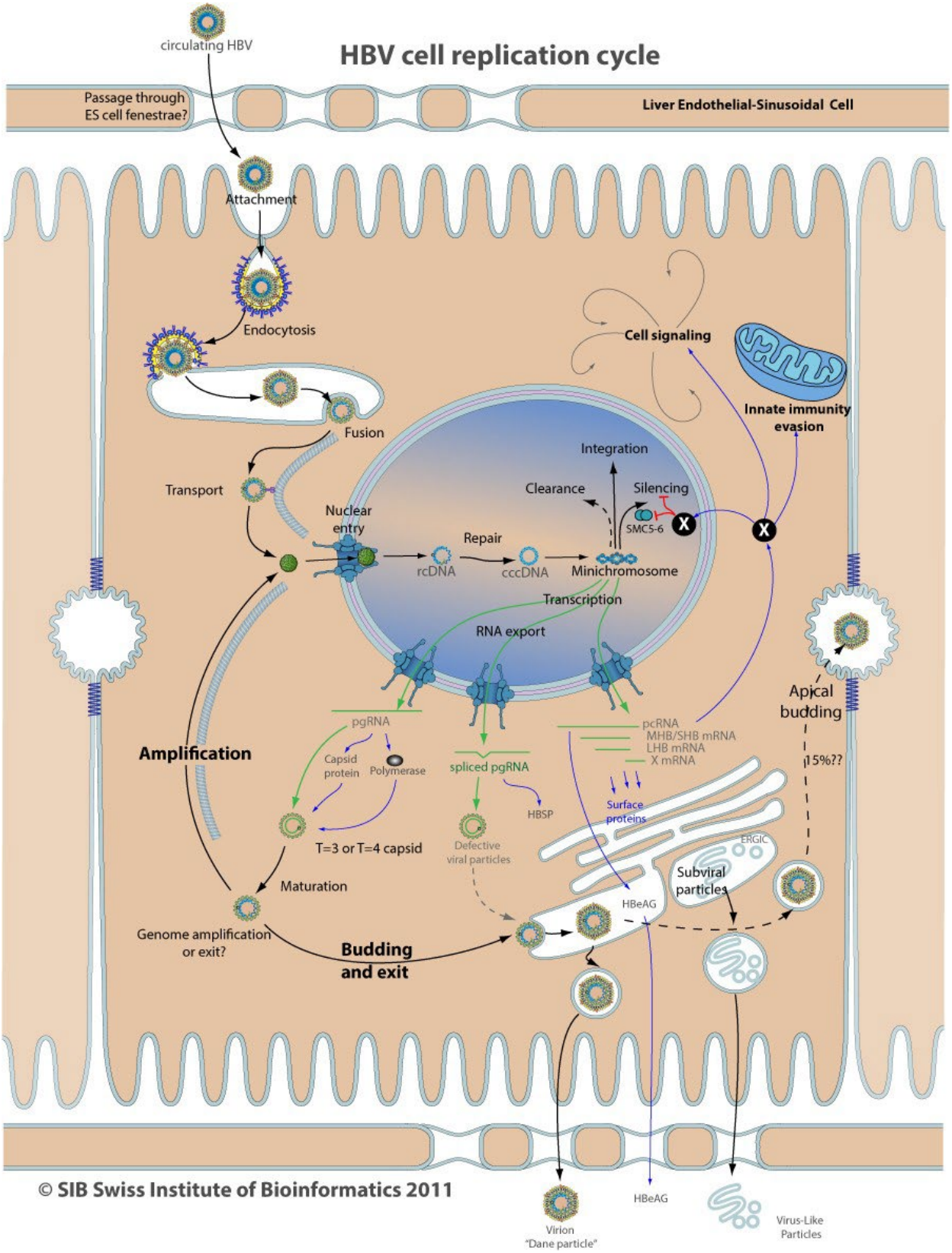


Figure 11-4. Hepadnavirus replication cycle. Courtesy of ViralZone https://viralzone.expasy.org/1280

HOW DO HEPADNAVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all nuclear replicating DNA viruses of animals, hepadnaviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells (Figure 11-3). In particular:

1. Using multiple promoters to create many different pre-mRNAs. Remember, these promoters are all on only one strand of the DNA genome.
2. Using alternative splicing to create many smaller versions of a pre-mRNA, all of which produce different proteins. Simple hepadnaviruses create many spliced mRNAs allowing them to get many proteins from one of the smallest genomes.
3. Hepadnaviruses also use host cell proteases to cleave large polyproteins similarly to all other positive sense RNA viruses. Note: unlike some other viruses where the proteases make a nice anti-viral drug target, these proteases are cellular and are therefore not good drug targets as they cause host cell mortality as well as inhibiting viral replication.
4. Leaky ribosomal scanning is used to create proteins from most of the viral mRNAs. Recall that leaky ribosomal scanning is when the ribosome initiates translation at an AUG start codon associated with a weak Kozak consensus sequence, as described in more detail in Chapter 5.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes are used to translate the viral protein. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that is added by the host cell machinery and recognized by the necessary initiation factors (eIF4E &G). Four promoters are used to make all of the mRNAs, some of which are spliced. The major products are the pre C (pre-core) protein HBeAg, Core protein (HbcAg), and P protein (polymerase/ reverse transcriptase) as well as the envelope proteins (HBsAg). The proteins that serve as antigens in the immune response are noted, i.e., HBcAg, HBeAg, HBsAg. The pre-proteins are cleaved by *cellular* proteases as described above. Note that HBs stand for surface antigen, as the envelope proteins are found on the external surface of the virus. As noted above, many hepatitis B proteins will be created by ribosomal leaky scanning leading to expression of proteins from overlapping open reading frames (ORFs).

DNA REPLICATION

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA.

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Hepadnaviruses overcome this issue by circumventing the need for DNA replication machinery. Hepadnaviruses package a positive sense RNA pre genome that is reverse transcribed to DNA. This DNA never needs to be replicated. Instead it will use the host cell's transcription machinery (RNA pol II, transcription factors, methyl transferase to make the 5' cap, and poly A polymerase to produce poly A tail) to create a positive sense RNA that will be packaged into the virion and then reverse transcribed to DNA within the virion. Thus, this mRNA serves both as a transcript for protein production and as the pre genome for future viral particles.
2. How to prime their DNA replication. Note that hepadnaviruses will not need to replicate their DNA once the initial double stranded DNA is created by reverse transcriptase. However, reverse transcriptase does require primers and will serve as the protein primer of DNA synthesis from the mRNA pre

genome.

3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome. Note that hepadnaviruses have a circular genome and as such have no ends to be replicated.

Reverse transcription of the viral pre genome within the virion requires the 3 functions of RT:

1. The RT protein primer will prime synthesis of a DNA copy of the mRNA pre genome using an RNA-dependent DNA polymerase (RdDp) activity.
2. The RNAse H activity of RT will cleave the RNA out of the hetero duplex of RNA and DNA.
3. The DNA-dependent DNA polymerase (DdDp) activity of RT will replicate the DNA using the DNA template left behind after RNAse H activity.

Note, the reverse transcription of hepadnaviruses requires a complicated mix of inverted repeats and a partially double stranded genome that will eventually be covalently linked. However, we will not elaborate all of the details of this very complicated replication pathway here.

VIRION FORMATION

Viral capsids form spontaneously in the cytoplasm encapsulating the transcribed pre genome mRNA that will serve as the template for the reverse transcription reaction that will form the partially double stranded DNA genome. This process occurs before the virus even leaves the cell. The virus leaves the cell via budding from the ER then goes through the Golgi compartment where it acquires an exocytic vesicle by which it leaves the cell. As in all enveloped viruses, the viral envelope proteins accumulate on the membrane and the viral capsid pushes against these enzymes until they leave the cell/organelle taking the membrane containing the viral enzymes with them. Recall that the virus must also package the RT enzyme.

AVOIDING THE HOST IMMUNE RESPONSE

Since hepadnaviruses enter the cell by endocytosis but do not uncoat until they reach the nuclear membrane, they avoid detection by TRL 9 **pattern recognition receptors** (PRR) which recognize the **pathogen associated molecular pattern** (PAMP) of double stranded DNA in an endosome, and cGAS, the PRR that detects cytosolic DNA PAMPs. Hepadnaviruses do not create mRNAs from both strands of the DNA genome, because they express mRNA from only one strand of the genome, thus avoiding creating complementary mRNAs that can induce type I interferons or RNAi responses to double stranded RNA. The virus is transcribed, capped, poly- adenylated and translated by the host cell's machinery and can avoid detection by RIG-1 & MDA-5, the PRRs that recognizes non-capped and improperly-capped RNA PAMPs. Furthermore, the HBeAg the pre-core is excreted from infected cells and helps to dampen host immune responses to the virus.

HEPADNAVIRAL DISEASES

We will focus this chapter on hepatitis B, the only member of the family that infects humans. Hepadnaviruses are contracted through contact with infectious blood or body fluids. The primary modes of transmission are infected mothers to neonates, sexual transmission, injection drug use, needle stick injuries, and sharing of razors or toothbrushes contaminated with blood. Figure 11-5 shows the progression of the disease. Once a person is infected, the virus travels through the blood to the liver and infects hepatocytes which leads to viremia. Infection of hepatocytes is not cytopathic. Instead, damage is mediated by the vigorous adaptive CLT response to the virus killing infected hepatocytes and mononuclear inflammatory cell infiltrate which causes hepatocytes to undergo

necrosis (death).

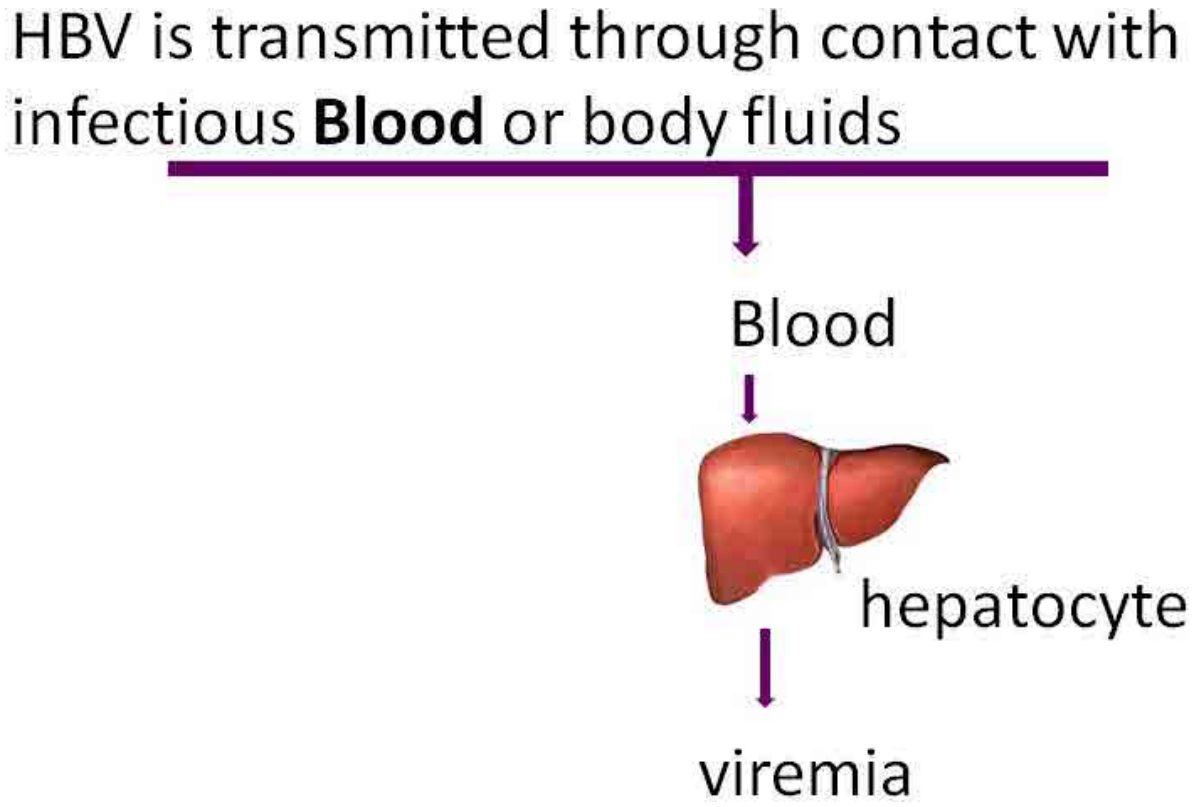


Figure 11-5. Progression of hepatitis B within an infected host. Courtesy of Sandra Quackenbush, Colorado State University.

The outcome of infection in adults is described in Figure 11-6. After an incubation period of 45-120 days, people develop symptoms. In 90-95% of infected adults the infection is acute and can be asymptomatic, or symptomatic including jaundice, nausea, and liver (flank/lower back) pain. These symptoms are often due to acute inflammation of the liver (hepatitis). This infection will resolve when a strong T cell CTL response and antibody response are mounted. However, in 5-10% of infected individuals, the infection will become chronic. These individuals will express high levels of virus that replicates in more than 95% of hepatocytes and causes high blood viremia. In some of these patients there will be resolution of symptoms, however they become asymptomatic carriers. The remainder of chronic infections remain active with either mild chronic or chronic active hepatitis and can lead to **cirrhosis** (liver damage) and **hepatocellular carcinoma** (HCC, liver cancer). Note that in infants infected at birth, 95% will develop chronic infections. As a result, babies in the US are now vaccinated right after delivery. For poorly understood reasons, chronic infections are characterized by absent or weak $CD4^+$ and $CD8^+$ T-cell responses to viral antigens.

It has also been noted that people who are infected with hepatitis B and subsequently become superinfected with hepatitis D (but oddly not if they are co-infected at the same time) become chronically infected 80% of the time and the progression to cirrhosis and HCC are much more rapid and severe. It is unclear why this is the case, as hepatitis D is a replication deficient virus that can only replicate if hepatitis B. If the infections happen as a co-infection at the same time, 95% of patients clear both infections. It is of note that in superinfections people must be treated with RT inhibitors to clear the hepatitis B infection but this will not treat the hepatitis D infection so they must also undergo IFN-a treatment as that is currently the only approved treatment in the US. Fortunately, hepatitis D is rare in the US and is seen mostly in immigrants from South East Asia and parts of the Middle East where the virus is much more prevalent, and different strains are circulating that may be more likely to cause superinfections.

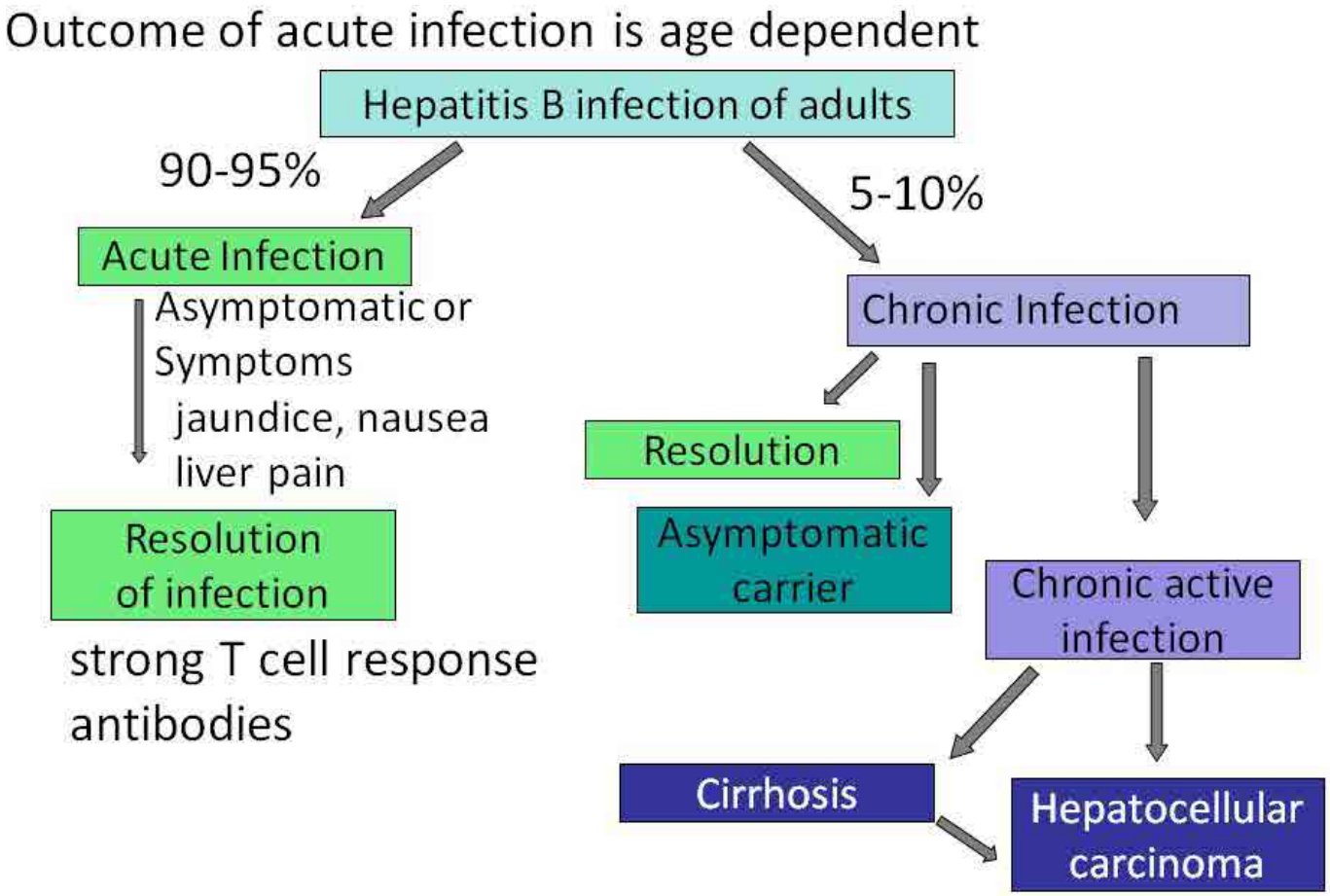


Figure 11-6. Outcomes of hepatitis B infection in adults. Courtesy of Sandra Quackenbush, Colorado State University.

TUMOR INDUCING HEPADNAVIRUS INFECTIONS

Hepatitis B (HBV) can cause hepatocellular carcinoma (HCC) in individuals that have inflammation of the liver when HBV DNA integrates into the host cell's genome without the use of an integrase protein. This integration, when combined with mutations that result in decreased production of p53, can cause cirrhosis and HCC. Remember that p53 controls cell cycle and apoptosis, so low levels of p53 can cause increased cell cycles and decreased apoptosis allowing tumors to form.

DIAGNOSIS OF HEPATITIS B DISEASES

If we analyze the types of antibodies produced specific to the 3 hepatitis B antigens (HBcAg, HBeAg, and HBsAg) and levels of the antigens in blood, there are consistently observed differences between acute hepatitis B and chronic hepatitis B infections. In acute infections, HBsAg (envelope protein antigen) and HBeAg (pre-core protein antigen) rise approximately 4 weeks after infection and become undetectable about 24 weeks after exposure. Total anti-HBc (core protein) antibodies and anti-HBe antibodies (first IgM and then IgG) develop beginning at about 6 weeks reaching high levels as the HBsAg and HBeAg levels reduce dramatically along with HBV DNA in the blood. During the time that viremia, HBeAg, and HBsAg are high, there are elevated liver enzymes such as ALT indicating liver inflammation. These high levels quickly reduce to normal as the antibody titers become elevated.

In chronic hepatitis B infections, despite similar timing, levels of HBsAg and HBeAg do not fall, and neither does viremia nor the liver enzyme ALT indicating that the liver remains inflamed. Furthermore, anti HBe and HBs antibodies do not form, and both HBeAg and HBsAg levels remain elevated as shown in Figure 11-7, which has led to the development of the following serologic profiles of HBV infections.

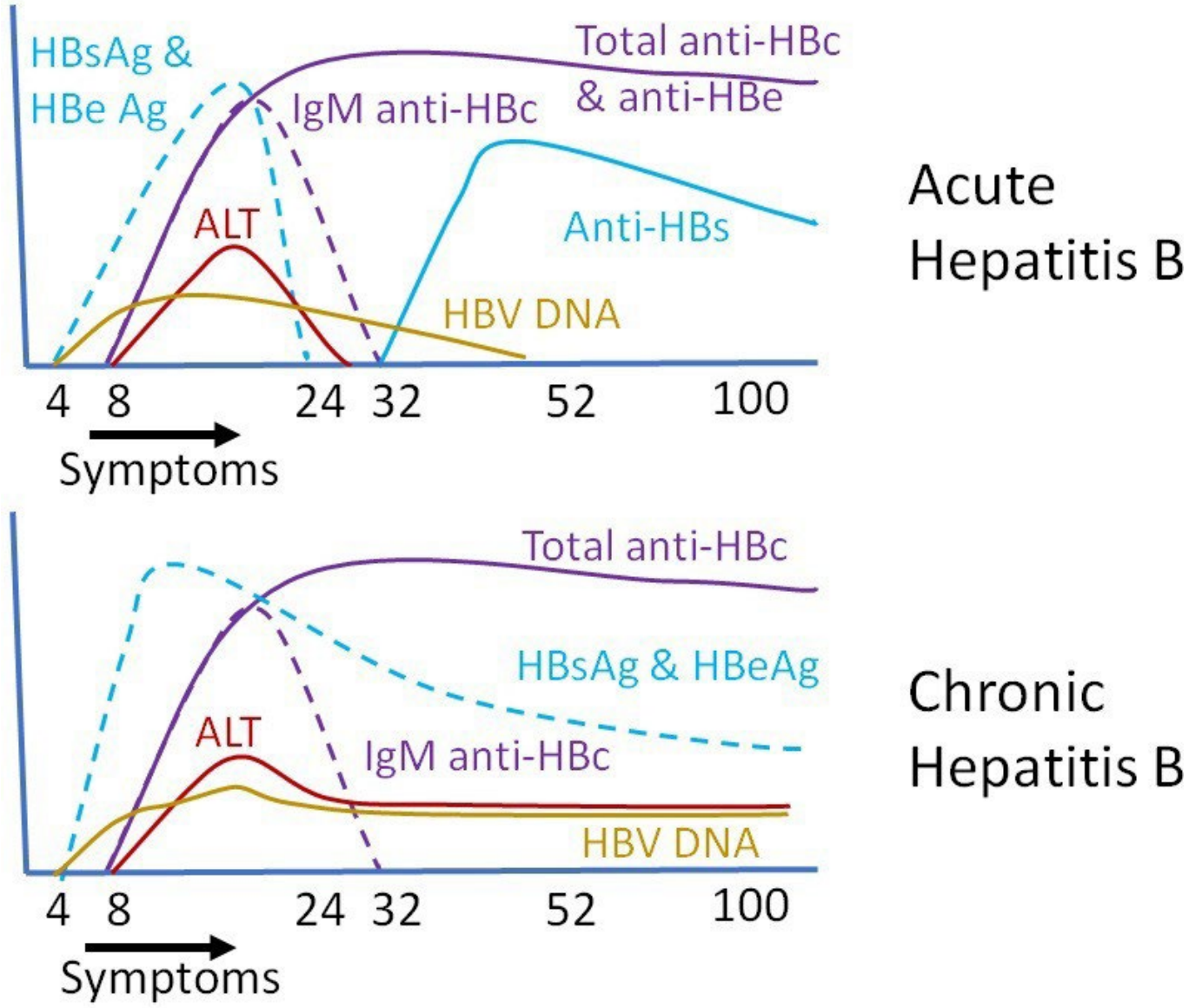


Figure 11-7. Comparison of antibody and antigens in acute and chronic hepatitis B infection. Courtesy of Erica Suchman, Colorado State University.

HBsAg: surface antigen (env). Indicates person is infectious, detected in serum during acute and chronic infection.

Anti-HBs: Indicates recovery and immunity, or successful vaccination as the vaccine contains only the HBs antigens, fails to develop in chronic infections.

HBeAg: secreted protein. Indicates that the virus is replicating, found in serum during acute and chronic

infection.

Anti-HBe: Because anti-HBe is only created in acute infections, it indicates seroconversion, predictor of long-term clearance.

Anti-HBc: Core antibody appears at the onset of symptoms in acute infection and persists for life. Indicates previous or ongoing infection.

Anti-HBc IgM: Indicates recent infection (≤6 months), acute infection.

The following table shows how to interpret hepatitis B tests.

HBsAg anti-HBc anti-HBs	negative negative negative	Susceptible
HBsAg anti-HBc anti-HBs	negative positive positive	Immune due to natural infection
HBsAg anti-HBc anti-HBs	negative negative positive	Immune due to hepatitis B vaccination
HBsAg anti-HBc IgM anti-HBc anti-HBs	positive positive positive negative	Acutely infected
HBsAg anti-HBc IgM anti-HBc anti-HBs	positive positive negative negative	Chronically infected

PREVENTION OF HEPADNAVIRUS INFECTIONS

Vaccines provide the best protection from hepatitis B infections. Additionally, one should practice safe sex (condom use), not sharing needles, razors, and toothbrushes with infected individuals, and using self-capping needles to avoid accidental needle sticks in hospital settings.

The vaccine is made from recombinant DNA that expresses HBsAg. There are 2 on the market, Recombivax and Engerix. The vaccine is routinely administered intramuscularly in the thigh within 24 hours after birth. The recommended dosing is within 24 hours after birth, 1 to 2 months booster, and 6 to 18 months booster. Remember that babies that contract infection from infected mothers during birth have a 95% chance of developing chronic hepatitis B. The vaccine, therefore, is given within 24 hours after birth. This has resulted in a 90% reduction of HepB disease in the United States. The practice of routinely vaccinating newborns began in the United States in 1992. If you were born before that, you may want to consider getting vaccinated for hepatitis B, particularly if you plan to work in a medical field.

TREATMENT OF HEPATITIS B INFECTIONS

Anti-hepatitis B drugs come in 2 classes:

1) Reverse transcriptase inhibitors. Note these are also effective against retroviral infections.

2) Interferon α will bind to interferon receptors and activate JAK to phosphorylate STAT which will bind to interferon stimulated response elements (ISRE) and activate transcription of ~200 interferon stimulated genes (ISG), such as OAS, RNAseL, PKR, etc., involved in the cellular antiviral response.

END OF CHAPTER QUESTIONS

1. What enzymes do hepadnaviruses use to reproduce their genomes and transcribe mRNAs? Are these enzymes cellular or viral?
2. Do you find the size of the hepadnavirus virion surprising? If so, why? If not, why not?
3. Does this virus create subgenomic mRNAs? Why, or why not? If it does, how? How do these mechanisms compare to other DNA viruses?
4. Why do parvoviruses and hepadnaviruses utilize different mechanisms for entering the nucleus?
5. Do hepadnaviruses require the host cells to be in the S phase? If so, why? If not, why not?
6. Where do hepadnaviruses undergo reverse transcription? How is this different from retroviruses? How does this effect their classification in the Baltimore scheme?

7. How is p53 involved in hepatitis B pathogenesis?
8. How do hepadnaviruses avoid the host antiviral responses?
9. How do hepadnaviruses obtain a 5' cap and poly A tail?
10. Do hepadnaviruses need to have mechanisms for overcoming RNA interference? If so, why, and what are they? If not, why not?
11. Can hepadnaviruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
12. Drugs have been developed against many viruses that use proteases to cleave their viral proteins. Although hepatitis B utilizes a protease to cleave a viral polyprotein, no drug has been created to target this protease. Why might this be?
13. Do hepadnaviruses reproduce their DNA genome? If so, why? If not, why not?
14. Compare and contrast the parvovirus and hepadnavirus mechanisms for avoiding the antiviral response.
15. What PRR are most likely to recognize hepadnavirus infections? Do hepadnaviruses have mechanisms to avoid these PRR? If so, what are they?
16. Why do hepatitis B infections sometimes lead to tumor formation?
17. Do all people clear hepatitis B infections?
18. You go to the physician for a possible hepatitis B infection. They find that you are chronically infected with hepatitis B. How can they determine this from only one blood test?
19. Compare and contrast your test results if you were found to be acutely infected or immune due to natural infection or vaccination.
20. Are there effective treatments for hepadnavirus infections? If so, what are they?
21. Compare and contrast how hepadnaviruses leave the cell to how other enveloped DNA viruses leave the cell. What trends do you observe?
22. Compare and contrast how hepadnaviruses, polyomaviruses, papillomaviruses, and retroviruses cause tumors.
23. Are there any major arthropod borne DNA virus diseases?
24. What trends do you observe in how DNA viruses enter cells?
25. How is hepatitis B virus different from other hepatitis viruses?
26. What do hepadnaviruses have in common with polyomaviruses and papillomaviruses?
27. Why are all babies vaccinated within 24 hours of birth against hepatitis B infections? Why not wait a few months as is done for most viral vaccinations?
28. Why must these vaccines be boostered at 1-2 months and 6-18 months?

REFERENCES:

American Academy of Pediatrics. Policy Statement. (2017) Elimination of perinatal Hepatitis B: Providing the first vaccine dose within 24 hours of birth. http://pediatrics.aappublications.org/content/140/3/e20171870

Centers for Disease Control and Prevention. Hepatitis B. https://www.cdc.gov/hepatitis/hbv/pdfs/serologicchartv8.pdf/

Fields Virology 6th edition David Knipe, Peter Howley, 2013 Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, chapter 68

Kramvis A, et al. (2018). Immunomodulatory Function of HBeAg Related to Short-Sighted Evolution, Transmissibility, and Clinical Manifestation of Hepatitis B Virus. *Front Microbiol*. 2018;9:2521. Published doi:10.3389/fmicb.2018.02521 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6207641/

Milich DR. (2019). Is the function of the HBeAg really unknown? Hum Vaccin Immunother. 15(9):2187-2191. doi: 10.1080/21645515.2019.1607132. https://pubmed.ncbi.nlm.nih.gov/31063442/

ViralZone. *Hepadnaviridae*. https://viralzone.expasy.org/7

ViralZone. Hepatitis B virus resource. https://viralzone.expasy.org/1280

CHAPTER 12

Chapter 12: RNA Viruses That Use Reverse Transcriptase And Replicate Partly In The Cytoplasm And The Nucleus: Retroviridae

INTRODUCTION TO THE FAMILY *RETROVIRIDAE*

You will notice in Figure 12-1 that retroviruses are positive sense RNA viruses that are enveloped icosahedral viruses that replicate partially in the cytoplasm and partially in the nucleus. They use a viral **reverse transcriptase** enzyme to create double stranded DNA from their RNA genome inside the cell's cytoplasm and the viral enzyme **integrase** to integrate their genome into the host cell chromosome forming a **provirus**. Many retroviruses are able to transform cells in cell culture and cause tumor formation by transforming cells in infected hosts. **Transformed** cells are immortal. They can grow indefinitely in culture, they have reduced need for growth factors in the media, there is loss of contact inhibition and loss of anchorage dependency, altered cell morphology, and, if injected into a host, will cause tumors. There are two subfamilies, *Orthoretrovirinae and Spumaretrovirinae*. The majority of disease-causing retroviruses are in the subfamily *Orthoretrovirinae which* contains 6 genera with *Alpharetrovirus, Betaretrovirus, gammaretrovirus, Deltaretrovirus and Epsilonretorivus* containing tumor inducing viruses, and Lentiviruses which contain immunodeficiency viruses such as HIV, FIV and SIV.

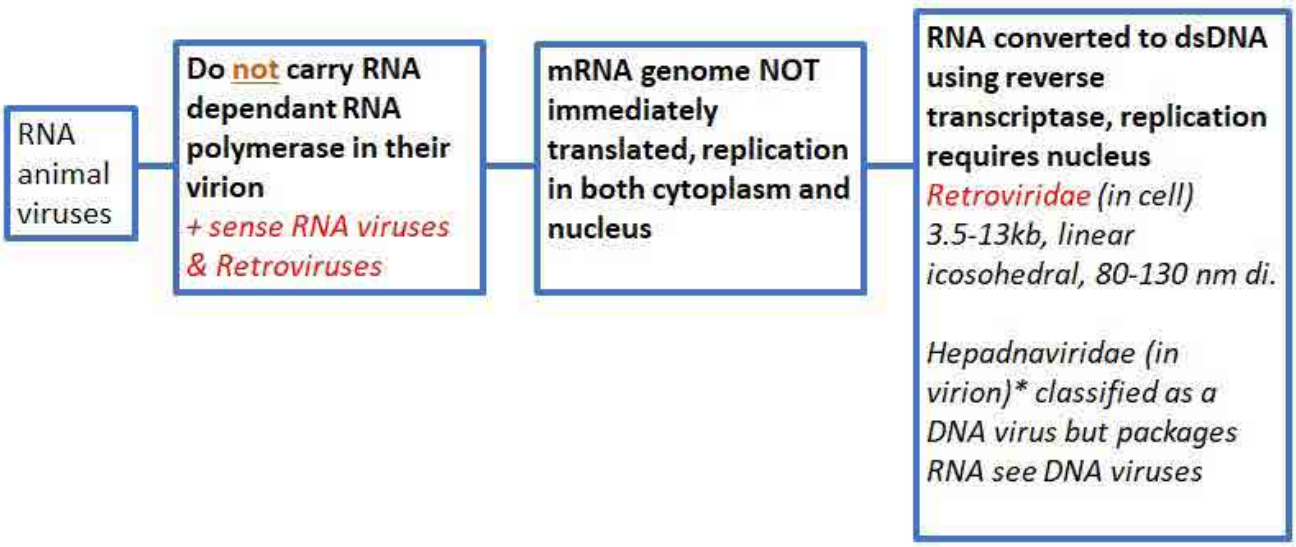
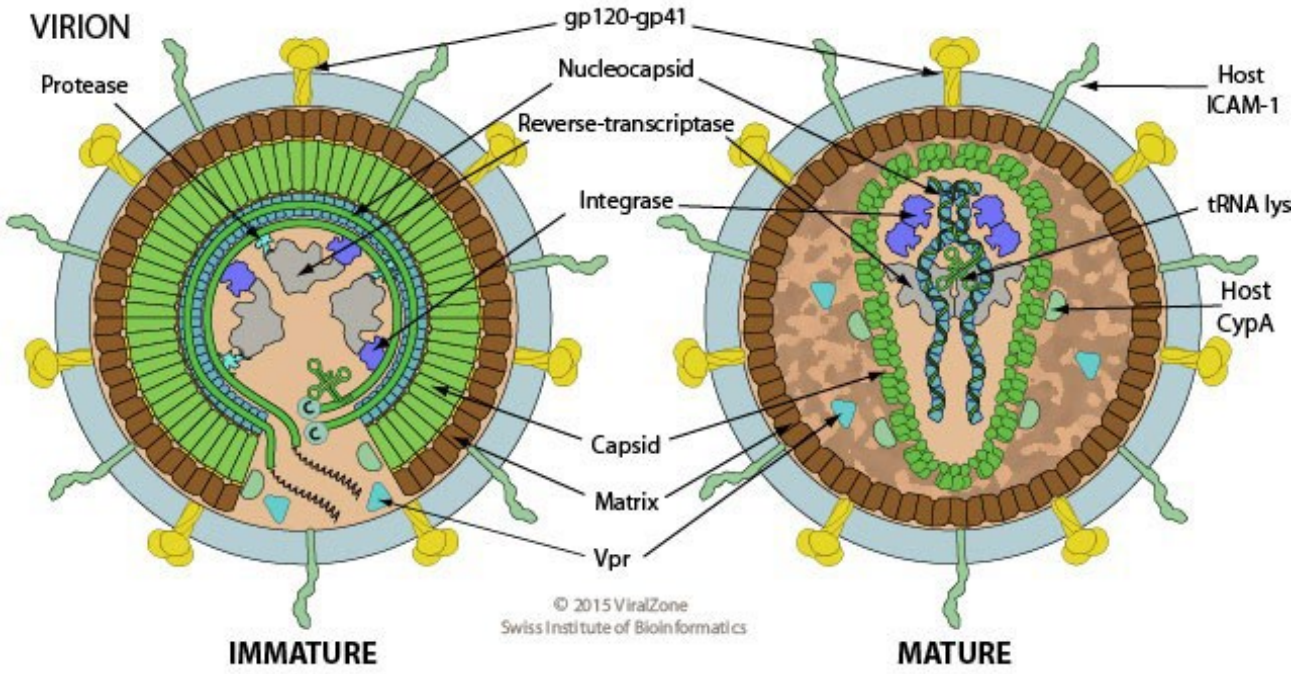


Figure 12-1. Taxonomy of positive sense RNA viruses focusing on retroviruses. Courtesy of Erica Suchman, Colorado State University.



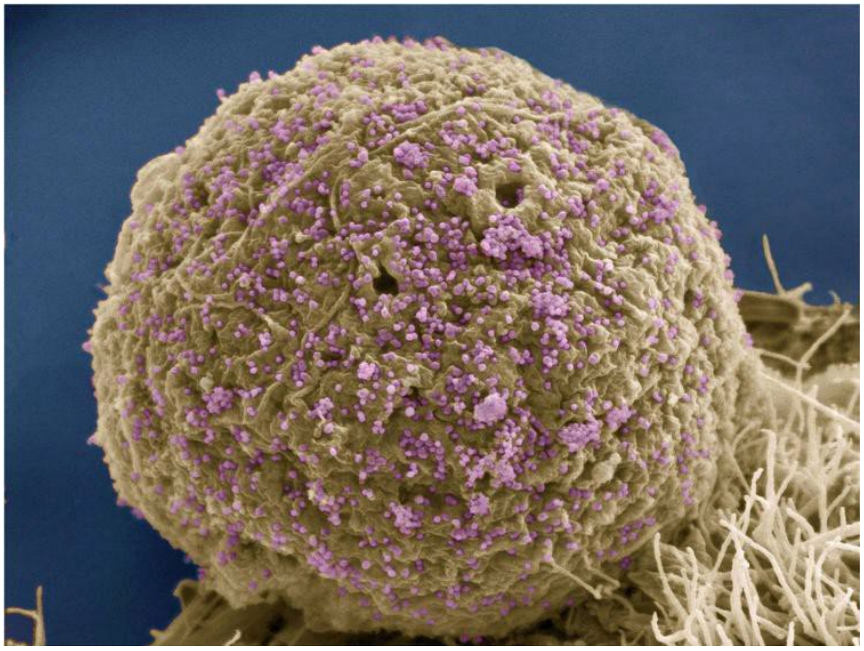


Figure 12-2A. An HIV particle as a representative retroviral particle. Courtesy of ViralZone https://viralzone.expasy.org/5182. Figure 12-2B. An electron micrograph of a HIV particle budding from a cell. Courtesy of The National Center for Microscopy and Imaging Research https://ncmir.ucsd.edu/site/media/images/gallery/hiv_infected_cells-1.png.

GENOME STRUCTURE

Retroviruses are unique in being RNA viruses that carry 2 pieces of their positive sense RNA genome in the virion. Furthermore, they are the only positive sense RNA viruses that do not immediately get translated by the host cell ribosomes. This is because the virus enters the cell with a tRNA primer attached to the mRNA that immediately serves as the primer for reverse transcriptase to begin converting the mRNA into double stranded DNA. The structure of the mRNA genome is not the same as the structure of the **proviral DNA** that will integrate into the host cell's chromosome, as shown in Figure 12-3. Simple retroviruses are composed of 4 genes: **gag** which produces the capsid proteins, **pro** which creates the protease, **pol** which creates reverse transcriptase and integrase, and **env** which codes for the envelope proteins gp41 and gp120. More complex retroviruses such as HTLV I and II and the lentiviruses have many more genes that produce **accessory proteins**.

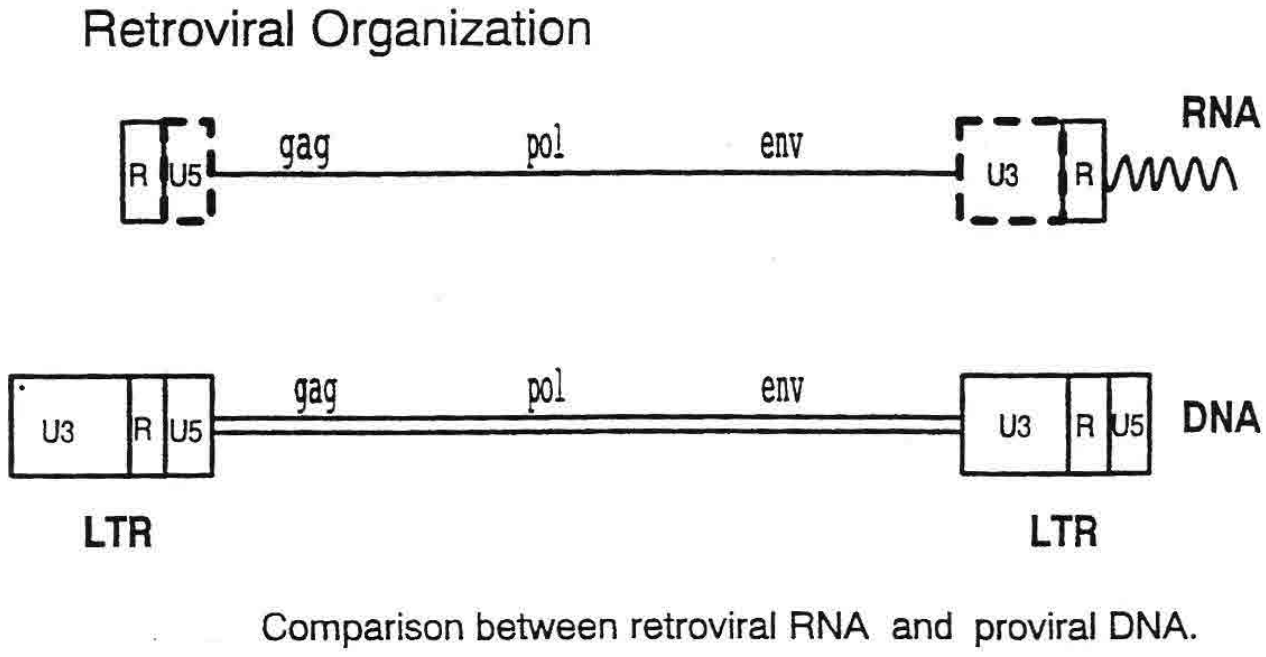


Figure 12- 3. Retroviral mRNA and provirus comparison in a simple retrovirus (note pro is often part of the pol gene and as such is not shown here). Courtesy of Erica Suchman, Colorado State University.

VIRAL REPLICATION CYCLE

As shown in Figure 12-4, the retrovirus replication cycle begins when:

1. The enveloped capsid proteins bind to receptors. The diagram shows HIV which uses CD4 as a receptor and CCR5 or CXCR4 as a coreceptor. Note other retroviruses will use different recptors/coreceptors.
2. Most retroviruses fuse their envelope with the plasma membrane using a fusion peptide in the gp120 envelope protein. When gp41 binds the receptor, gp41 undergoes a conformational change that allows gp41 to bind the coreceptor. Gp120 then unfolds, and the fusion peptide causes the retroviral envelope and the cell's plasma membrane to fuse releasing the capsid inside the cell. See Chapter 3 for a review of this process.
3. The retroviruses are unique among the positive sense RNA viruses because they are the only positive sense RNA viruses that do not immediately get translated upon uncoating.
4. The virus is reverse transcribed by the viral enzyme reverse transcriptase (RT) that is carried within the viral capsid along with the genome, a tRNA primer, integrase, and protease. RT creates a double

stranded DNA with **long terminal repeats** (LTR) on either end by a mechanism that will be described later in the chapter.

5. The virus travels to the nucleus via microtubule transport using dynein proteins.
6. The virus then moves into the nucleus. Lentiviruses such as HIV, SIV, and FIV can move through the nuclear pores. Accessory proteins only found in more complicated retroviruses facilitate the movement of these viruses through the nuclear pore. All other retroviruses must wait for the host cell to replicate and enter the nucleus when the nuclear membrane breaks down.
7. Now that the DNA is in the nucleus, the viral enzyme integrase that was carried within the virion will integrate the viral DNA into the host cell's chromosome. This integrated virus is called a **provirus**. There is no specificity as to where the virus will integrate, however there are hallmarks of retroviral integration based on how integrase functions. First, it cuts 2 nucleotides off the end of each LTR and duplicates 4 bases of the insertion site, so they end up repeated on either end of the integrated provirus.
8. Retroviruses are now basically DNA viruses. The host cell's transcription machinery (RNA pol II and transcription factors) will transcribe the viral mRNA from the one promoter found within the U3 sequence of the LTR. Note the LTRs also contain enhancer sequences to increase transcription from the retroviral promoter and enhancers found within the LTR. Transcription will begin at the +1 that is the start of the repeated R sequences of the LTR. Termination and polyadenylation will occur at the last nucleotide of the R sequence on the 3' end. As such, the mRNA will have an R and unique 5' sequence on the 5' end of the mRNA and a unique 3' (U3) and R sequence at the 3' end. The polyadenylation sequence is found within the U5 sequence and will add a poly A sequence as the mRNA terminates at the end of the R sequence on the 3' end. See Figures 12-4 and 12-5. The mRNA does not contain a long terminal repeat but instead has a short terminal repeat in the R sequence. The long terminal repeat duplicates the U3 and U5 sequences on each end. This is called a long terminal repeat as both ends will have U3-R-U5 duplicated on each end. We will describe the process of creating a long terminal repeat on each end below under replication. Note: the mRNA will serve as the genome for new viral particles.
9. Like all viruses, the host cell's translation machinery will translate the viral proteins.
10. Once large quantities of viral RNA and capsid proteins, envelope proteins, RT, and integrase have accumulated, the virus will self-assemble in the cytoplasm and will leave the cell by budding from the plasma membrane.

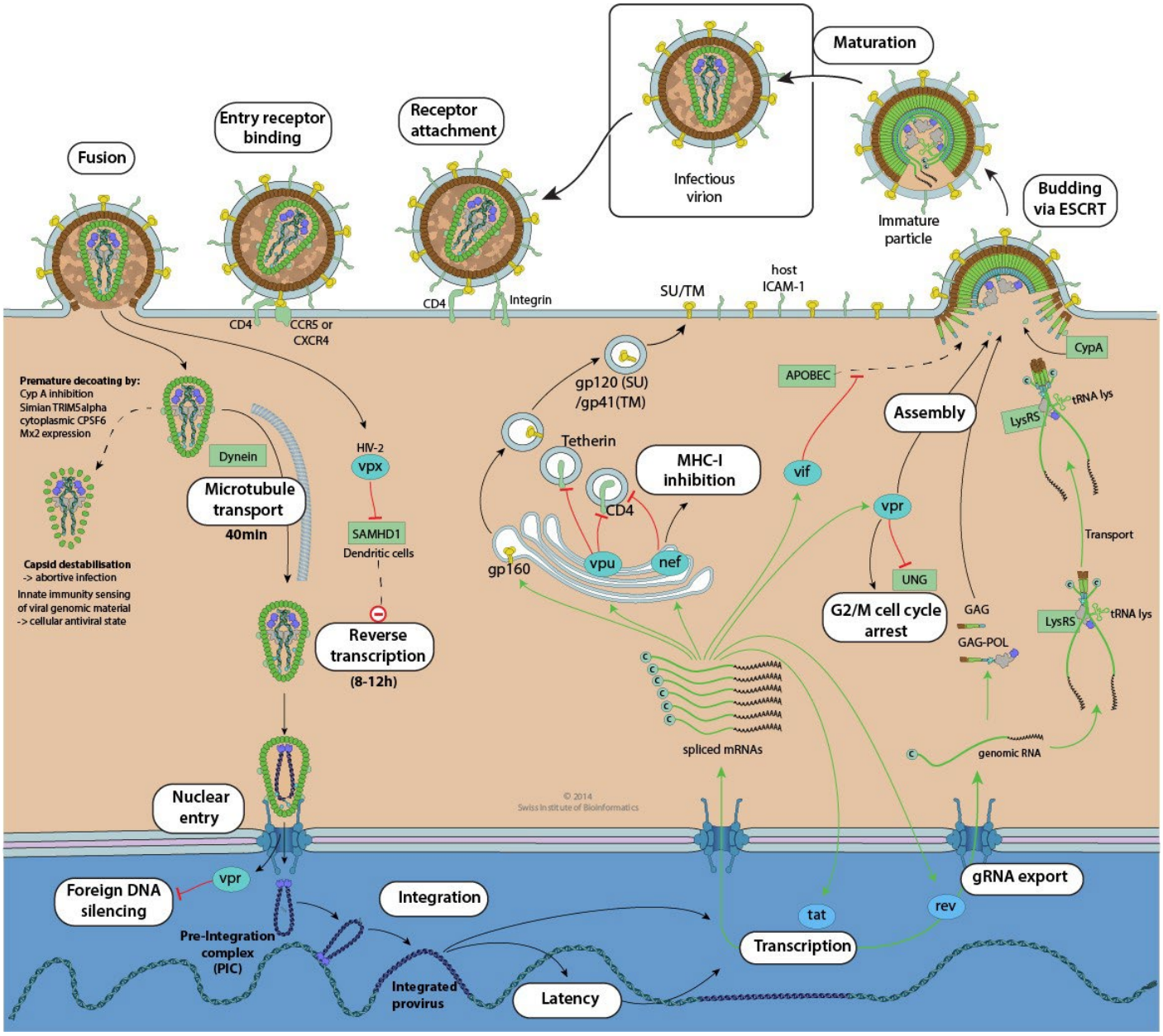


Figure 12-4. Retrovirus replication cycle. Courtesy of ViralZone https://viralzone.expasy.org/5096

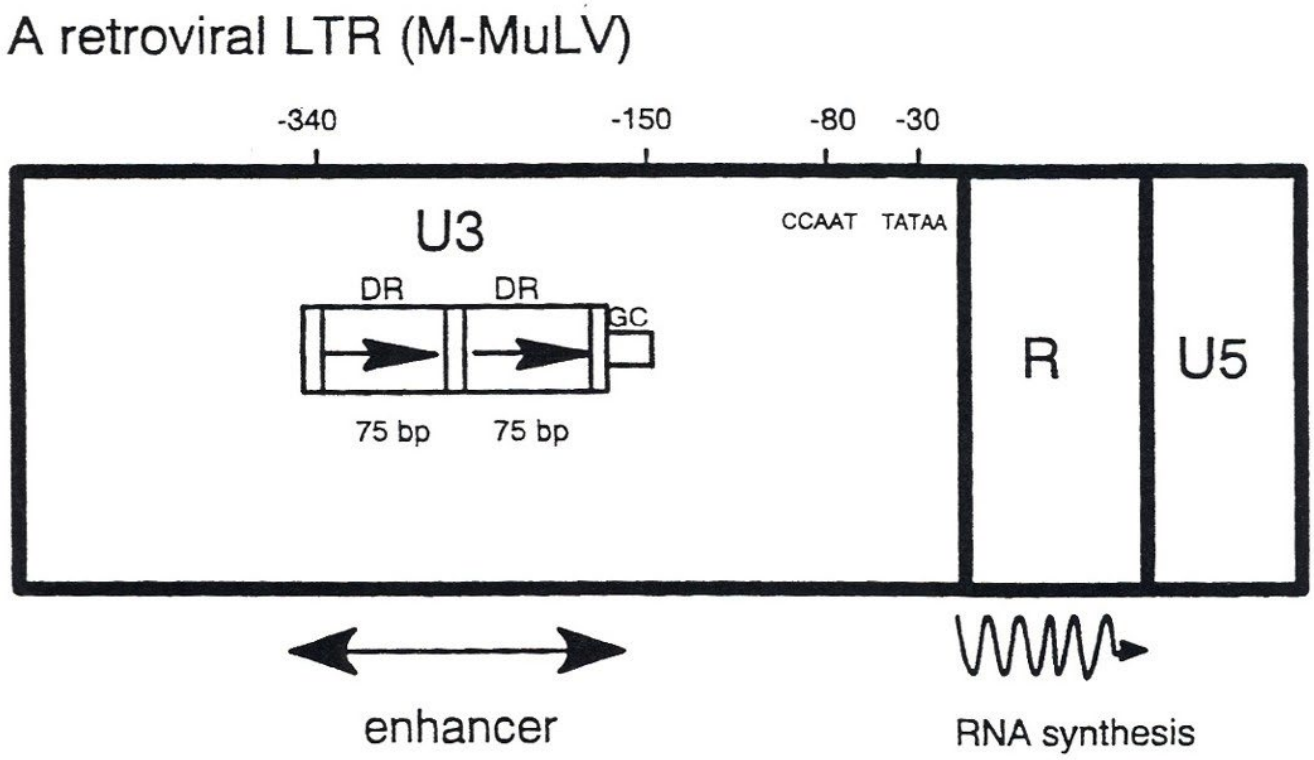


Figure 12-5. Retroviral LTR showing transcription regulation signals. The promoter sequences include the -80 and -30 sequences, and the enhancer includes the 75 bp direct repeats between base pairs -340 and -150. Courtesy of Erica Suchman, Colorado State University.

HOW DO RETROVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Once becoming a provirus, retroviruses essentially behave like DNA viruses. Like all nuclear replicating DNA viruses of animals, retroviruses take advantage of mechanisms for creating multiple proteins from small amounts of DNA commonly used in the host cells as shown in Figure 12-6 A & B. In particular:

1. Using alternative splicing to create many smaller versions of a pre-mRNA all of which produce different proteins. Simple retroviruses only produce one spliced mRNA that will produce the Env genes (envelope proteins). Lentiviruses and other more complex retroviruses, such as HTLV-I and HTLV-II, create many more spliced mRNAs that produce accessory proteins not found in the simpler retroviruses.
2. Retroviruses also use proteases to cleave large polyproteins similarly to all other positive sense RNA viruses.
3. In order to express more gag proteins (capsids) than the protease and pol gene products (RT and integrase), retroviruses use leaky scanning (labeled in Figure 12-6 as alternative initiation), termination suppression, and frameshifting.

- Leaky scanning occurs when the ribosome initiates translation at an AUG that is associated with a weak Kozak consensus sequence a small percentage of the time.
- Termination suppression occurs when an amino acid bearing tRNA binds the stop codon during translation allowing the ribosome to continue reading into another open reading frame thus producing the pro and pol gene products.
- Frameshifting occurs when the ribosome reaches a secondary structure called the pseudoknot in the mRNA being translated. The majority of the time the ribosome is able to unwind the pseudoknot and will translate the gag proteins. A small percentage of the time, however, the ribosome will slip back 1 nucleotide causing it to read all future codons in another frame leading to the production of the pro and pol gene products.

Note: for a review of these mechanisms please see Chapters 3, 5 and 13.

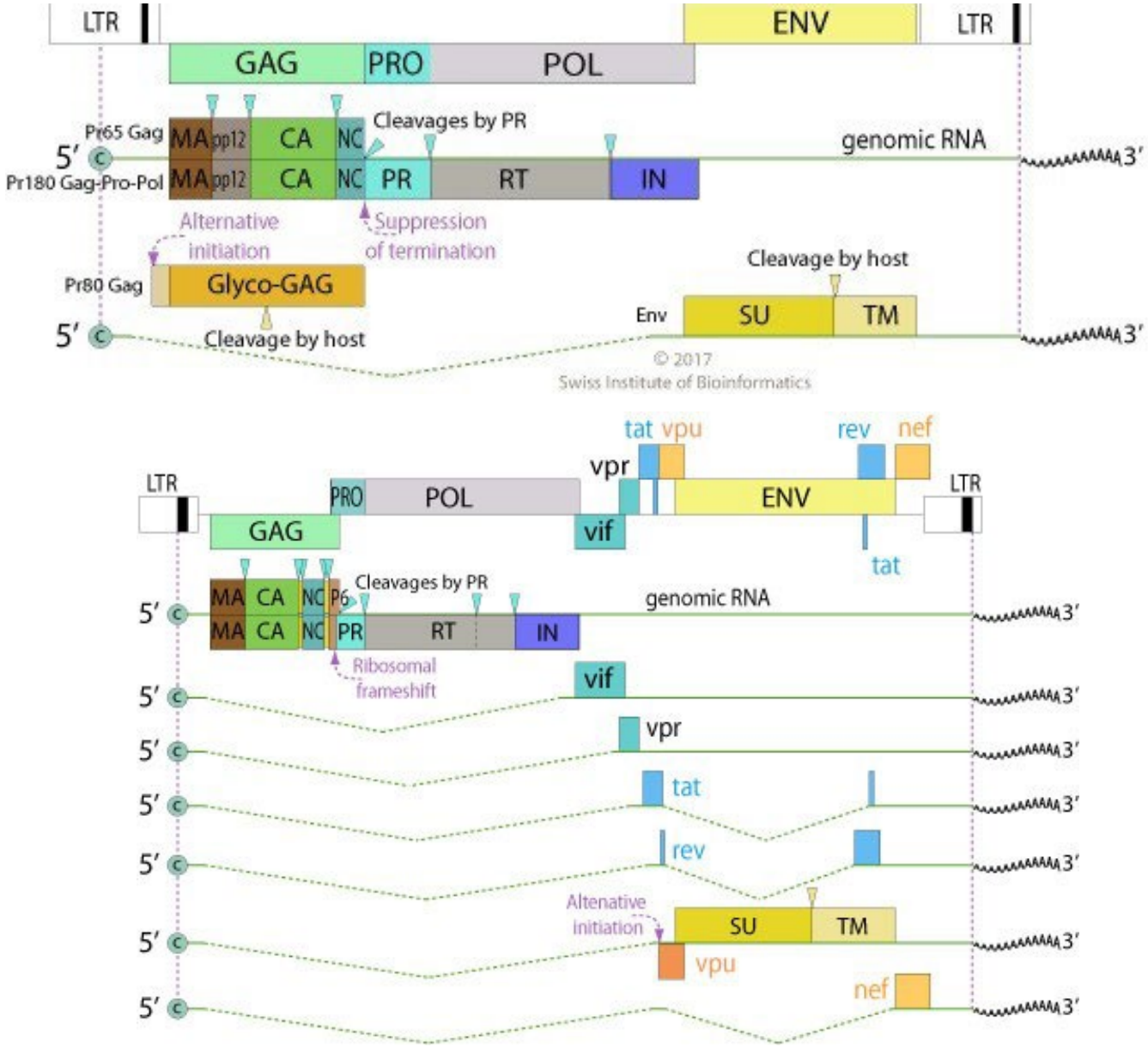


Figure 12-6. Mechanisms used to overcome the one protein per mRNA issue in eukaryotic cells by retroviruses. Figure 12-6A shows a non-Lentivirus retrovirus which is smaller and simpler, lacking the many accessory proteins, and has fewer splicing events. Figure 12-6B shows a typical Lentivirus with many accessory proteins and more splicing events. Courtesy of ViralZone https://viralzone.expasy.org/65 *and* https://viralzone.expasy.org/264

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like all DNA viruses, the mRNA will have a 5' 7-methylguanosine cap and 3' poly A tail that is added by the host cell machinery and will be recognized by the necessary initiation factors (eIF4E &G and PABP, as described in Chapter 3). As discussed above, retroviruses have developed many mechanisms to create differing levels of proteins from the mRNA transcript that is transcribed from the single promoter within the LTR. Retroviruses want the most capsid protein and hence alternative mechanisms are used to get smaller amounts of the pro and pol gene products, such as frameshifting, termination suppression, and leaky ribosome scanning. The env gen products are produced from an mRNA created from a splicing event. All of the gene products are created as polyproteins that are autocleaved by the viral protease protein.

DNA PRODUCTION FROM MRNA GENOME

Recall that there are three major issues for DNA viruses to overcome when replicating their DNA.

1. How to get the DNA replication machinery they need when host cells only produce these enzymes when the host cell is going to divide. Retroviruses overcome this issue by circumventing the need for DNA replication machinery. Retroviruses carry a positive sense RNA genome that is reverse transcribed to DNA that integrates into the host cell chromosome as a provirus. Once integrated it need never reproduce this DNA. Instead, it will use the host cell's transcription machinery: RNA pol II, transcription factors, methyl transferase to make the 5' cap, and poly A polymerase to produce poly A tail. This mRNA will be used both to serve as a transcript for protein production and as genome for future viral particles.
2. How to prime their DNA replication. Note that retroviruses will not replicate their DNA once the initial double stranded DNA is created by reverse transcriptase. However, reverse transcriptase does require primers, a tRNA is packaged in the virion and will initiate reverse transcription as described below.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome. Note that retroviruses use a mechanism involving the repeated terminal sequences of the mRNA and jumping to replicate the ends and create the long terminal repeats. This is described below.

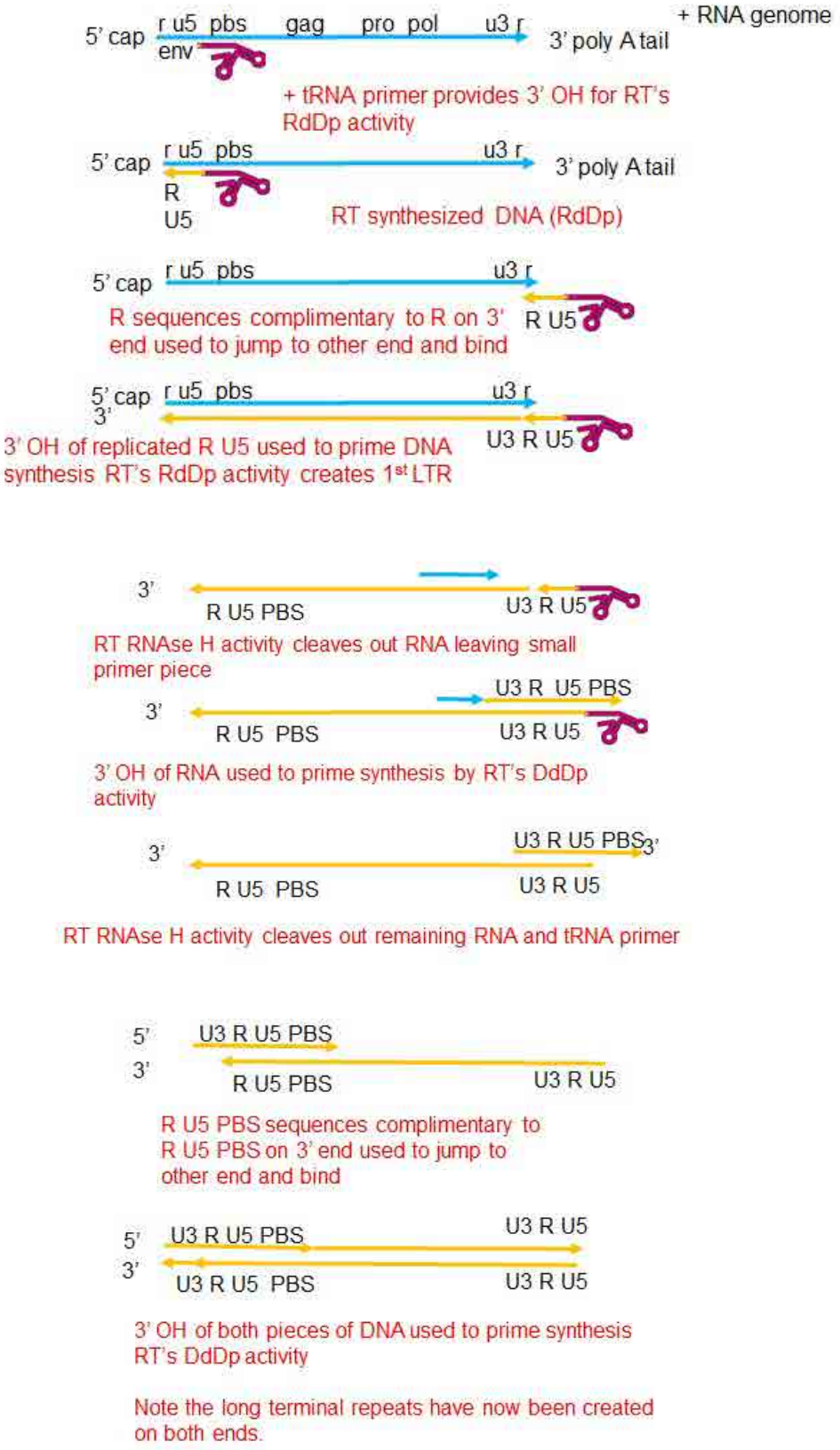


Figure 12-7. Shows retrovirus DNA replication using terminal repeats and fragment jumping to replicate the ends during reverse transcription. Note PBS is the primer binding site. Courtesy of Erica Suchman, Colorado State University.

Retroviruses have a complicated mechanism for taking an mRNA that has a short repeated sequence on each end (R) and a unique sequence on both the 3' and 5' ends of the mRNA (U3 and U5 respectively, see Figure 12-3 & 12-7) and creating a **long terminal repeat** (LTR) which has U3-R-U5 on each end.

1. The diploid copies of the positive sense RNA genome are packaged along with a tRNA molecule that is complementary to a **primer binding site** (PBS) within the 5' end of the viral genome. The 3' OH of tRNA is used by the RNA-dependent DNA polymerase (RdDp) activity of the Reverse Transcriptase (RT) to create a short segment of DNA that is complementary to the U5 and R sequences.
2. The newly synthesized R sequence is complementary to the R sequence at the 3' end of the virus, and RT will help the R sequence to jump to the other end and bind using the complementary R sequences, when the genome circularizes. Note the circularization is not shown in Figure 12-7.
3. This 3' OH is now used by the RdDp activity of the RT to replicate the negative strand of DNA from the positive sense RNA genome.
4. As this is being replicated, the RNAse H activity of RT will cleave the RNA out of the heteroduplex of RNA and DNA, however it will leave one small patch of RNA close to the U3-R sequences at the 3' end of the mRNA. You will note that at this point the first LTR has been created at the 3' end of the original mRNA.
5. The 3' OH on the small patch of RNA is used as the primer for the DNA-dependent DNA polymerase (DdDp) activity of RT. This will replicate the U3-R-U5 and PBS.
6. Now the RNAse H activity of RT will cleave out the final RNA and tRNA primer.
7. At this point the U3 R U5-PBS sequence will bind to the complementary R U5-PBS sequences on the other end of the replicated DNA molecule, again because the genome is circularized. The circularization is not shown in Figure 12-7.
8. This provides the 3' OH primers and template for the completion of the second LTR.

   For a nice video of this process Provided by Dr Vincent Racaniello host of This Week in Virology podcast (TWiV https://www.youtube.com/playlist?list=PL55B28888146FAD78) please click https://www.youtube.com/watch?v=RYwVnzYf4V8 for the retroviral replication video.

VIRION FORMATION

Viral capsids form spontaneously in the cytoplasm, and the virus leaves the cell via budding from the plasma membrane. The viral envelope proteins will accumulate on the plasma membrane and the viral capsid will push against these enzymes until they leave the cell taking the membrane containing the viral enzymes with it. Recall that the virus must also package the tRNA primer, RT, integrase, and protease within the virion. One interesting feature of HIV budding is that it occurs only on surfaces of the infected cell that are in very close proximity to other cells thus minimizing their exposure to the immune response.

AVOIDING THE HOST IMMUNE RESPONSE

As retroviruses enter the cell without using an endosome, they avoid detection by TRL 7 **pattern recognition receptors** (PRR) which recognize the **pathogen associated molecular pattern** (PAMP) of single stranded RNA in an endosome. The virus, however, is reverse transcribed in the cytoplasm, but it most likely stays associated with the capsid to avoid detection by cGAS, the PRR that detects cytosolic DNA PAMPs. Simple retroviruses do not have a great deal of anti-viral defenses other than the ones listed above. The more complex retroviruses and the lentiviruses have a whole host of accessory proteins devoted to controlling the immune response. Once the virus has been reverse transcribed and integrates into the host cell's genome, it essentially becomes part of the host cell and is transcribed, capped, polyadenylated, and translated by the host cell's machinery and, as such, can avoid

detection by RIG-1 and MDA-5, the PRRs that recognize non-capped and improperly capped RNA PAMPs.

RETROVIRAL DISEASES

Retroviruses are associated with proliferative diseases, immunodeficiency syndromes, and neurologic disorders. The proliferative diseases lead to cancers, which are caused by most non-lentiviral retroviruses, and immunosuppression leading to opportunistic infections, which are caused by lentiviruses. We will begin with the tumor causing viruses and then move on to the more complicated immunodeficiency viruses at the end of this chapter in a special section for HIV infections.

TUMOR INDUCING RETROVIRUSES

Typical retroviral cancers are shown below.

Hematopoietic neoplasms	Examples
Lymphoma, leukemia	MLV, ALV, FeLV, GaLV, BLV, HTLV-1, HTLV-II, (mice, chickens, cats, monkeys, cows, and humans)
Myeloblastosis, erythroblastosis	AMV, SFFV (chickens and mice)
Sarcomas	
Fibrosarcoma	RSV, FeSV, SSV (chickens, cats and monkeys)
Carcinomas	
Mammary	MMTV (mice)
Pulmonary	JSRV (sheep)

There are 4 major mechanisms of retroviral oncogenesis, each of which will be discussed below.

1. Insertional mutagenesis
    - Enhancer activation
    - Promoter insertion
2. Oncogene capture by acute transforming retroviruses
3. Regulatory/accessory gene
4. Deregulation of cell signaling

INSERTIONAL MUTAGENESIS

Hallmarks of tumors induced by insertional mutagenesis include viral replication during a long latent period where the virus integrates in the vicinity of a cellular **proto-oncogene** and alters the expression of that gene by LTR activation. Many cellular genes have been identified as potential targets, i.e., c-myc, c-erb, c-myb. Proto-oncogenes are genes involved in controlling the cell cycle that, when not properly regulated, become **oncogenes** and cause dysregulated cell growth and cellular transformation. When this occurs, the proto-oncogene is now referred to as an oncogene. A proto-oncogene has the potential to induce tumors but is not currently doing so as it is properly regulated. An oncogene is a proto-oncogene that is no longer properly regulated or has deletions in regulatory portions such that it induces tumors. These genes include growth factors, growth factor receptors, protein kinases, and G proteins involved in signal transduction and cell cycling. Increased or improperly regulated

expression of these proteins that are involved in the cell cycle control causes the cell to reproduce uncontrollably leading to tumor formation. Transformation is carried out by replication-competent retroviruses. The transformation is due to the effect of the LTR upon expression of host genes.

The first type of insertional mutagenesis we will discuss is **enhancer activation** of local proto-oncogene expression. When a provirus integrates **upstream** (before) or **downstream** (after) of a cellular gene, increased cellular gene expression can occur due to influence of the enhancers within the U3 region of the LTR (Figure 12-5) on the promoter-controlling expression of the proto-oncogene. Note that these can be upstream or downstream because **enhancer sequences** can be quite a distance from a promoter and still lead to increased transcription via the transcription factors recruited by the enhancer, as shown in Figure 12-8.

The second type is **promoter insertion** which occurs when a provirus integrates upstream or within a cellular gene which is a proto-oncogene. The viral promoter can initiate transcription that reads into the cellular gene. The 3' LTR is often used as the promoter and continues to read into adjacent oncogenes. If the provirus is integrated within the oncogene, it can lead to truncation of the oncogene's protein causing loss of regulatory regions. This loss leads to dysregulation of the oncogene protein and hence loss of cell cycle control that can lead to tumor formation. Note: if the provirus integrates upstream of the oncogene, no truncation will occur, however the oncogene will no longer be under normal cellular control of expression and will lead to tumors when it is overexpressed. Each retrovirus is commonly found integrated near a specific set of oncogenes despite the fact that there is no sequence specificity to these integration events. For example, when the integration sites of avian leukosis viruses are analyzed in tumors, the integration sites cluster within the non-coding exon 1 and intron 1 of the *myc* gene, and most integrated proviruses are oriented in the direction of *myc* transcription, toward the right. A few, however, are oriented in the opposite direction as shown in Figure 12-9, although different patterns may be observed with other proto-oncogenes.

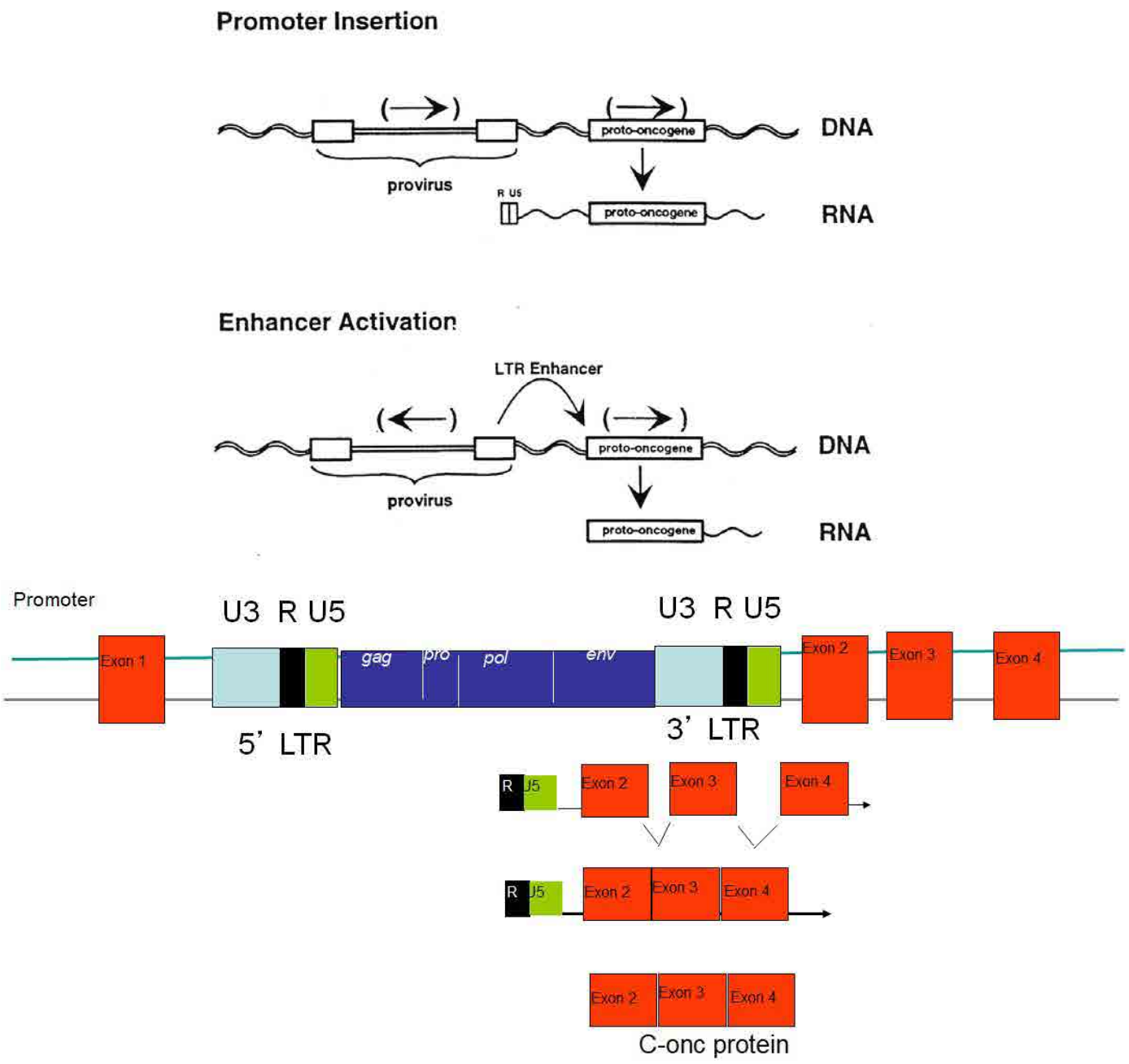


Figure 12-8 A. Retroviral activation of local proto-oncogenes by promoter insertion and enhancer activation. B. Oncogene truncation that can occur if the provirus integrates within the oncogene. Courtesy of Erica Suchman, Colorado State University.

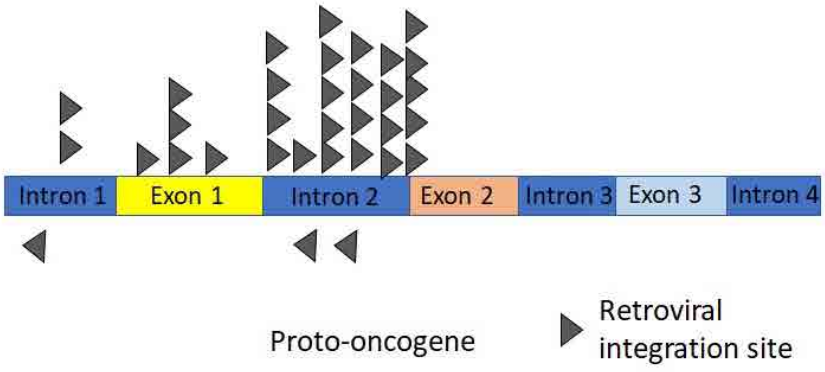


Figure 12-9. Insertion patterns of ALV in c-myc oncogene. Courtesy of Erica Suchman, Colorado State University.

ONCOGENE CAPTURE BY ACUTE TRANSFORMING RETROVIRUSES

In **acute transforming retroviruses,** the viruses contain a **viral oncogene** (v-*onc*) within their genome. These viruses induce tumors rapidly within several weeks of infection. Each virus arises spontaneously in the host due to recombination between viral and cellular sequences. Most viruses are replication defective because the oncogene has replaced viral genes. Remember that virus capsids can only accommodate a certain amount of genetic material, so if the genome gets too large, it will not package. The exception is Rous Sarcoma Virus (RSV) which contains a full replication competent genome AND a captured cellular oncogene. These viruses cause rapid cell transformation in cell culture and in hosts. Note that even though most of these viruses are replication incompetent, they still induce tumors because they cause an oncogene to be expressed at high levels without the normal control mechanisms.

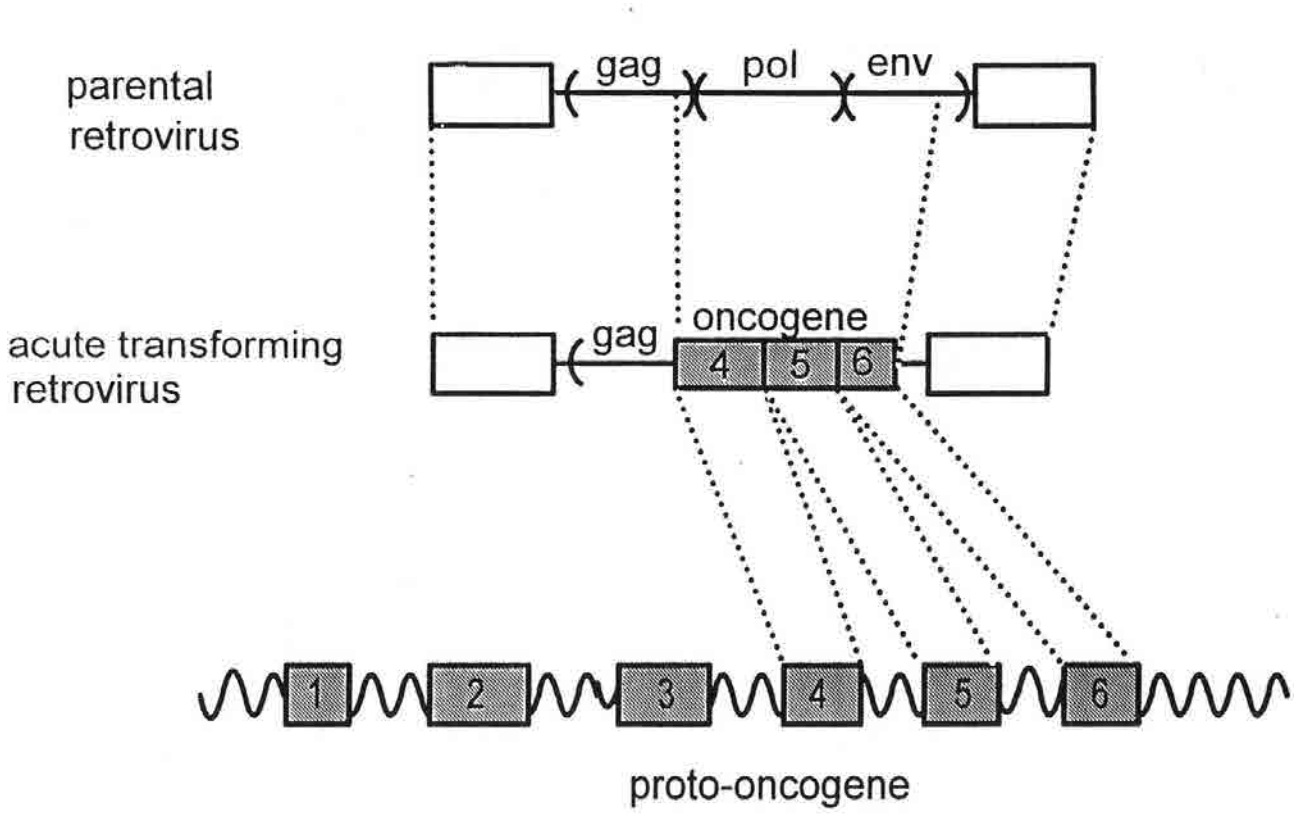


Figure 12-10. Acute transforming retroviruses that contain oncogenes. Courtesy of Erica Suchman, Colorado State University.

REGULATORY OR ACCESSORY GENE TRANSFORMATION OF CELLS

In viruses such as **human T lymphotropic viruses** (HTLV-1) and bovine leukemia virus, viral-encoded proteins are responsible for oncogenesis. These viruses have a long latency period from time of infection to development of disease. These viruses are much more complex than the simple retroviruses and, as such, have many accessory proteins. In HTLV-1, multiple accessory proteins are involved in oncogenesis.

HTLV-1 infects 15-25 million people worldwide. Transmission is predominantly infected mother to child, sexual contact, and spread through contact with blood. It causes **adult T cell leukemia** (ATL), a $CD4^+$ T cell malignancy. It can also lead to HTLV-1 associated **myelopathy/tropical spastic paraparesis** (HAM/TSP), a neurodegenerative disorder that is seen after many years. In this US almost all people who have ATL are HTLV-1 positive.

HTLV-2 infected individuals sporadically develop neurologic disorders and may develop **lymphocytosis** (over proliferation) in $CD8^+$ T cells but HTLV-2 is not associated with leukemia.

How do HTLV-1 & HTLV-2 cause neurological symptoms when they cannot infect neurons because they are not replicating? Remember: Only Lentivirus retroviruses have accessory proteins to replicate in non-replicating cells. HTLV-I and HTLV- II are not lentiviruses. Both of these viruses infect **astrocytes** (macrophages of the brain) in the nervous system, and their Tax protein leads to inflammation which causes neurological damage. Astrocytes increase adult **neurogenesis** (nerve growth). Although they replicate slowly they do replicate, and hence HTLV-I and HTLV-II can cause damage in non-dividing nervous tissue.

In bovine leukemia virus the accessory protein Tax has been implicated in oncogenes.

DEREGULATION OF CELL SIGNALING

Some retroviruses' accessory proteins can control the cell cycle and downregulate cell cycle signal pathways leading to tumors such as JSRV in sheep, where it induces lung tumors, or walleye dermal sarcoma virus of fish.

THE DISEASES OF *LENTIVIRUSES*

NON-IMMUNODEFICIENCY LENTIVIRUSES

Although most lentiviruses cause immunodeficiency, it is important to note that a few animal models cause other diseases such as **Maedi/visna** viruses which cause disease in Icelandic sheep. Maedi causes pneumonia, visna causes wasting, depression, and paralysis. Like most retroviruses, these viruses have a long incubation period between 3 to 8 years. **Equine infectious anemia virus** causes recurrent cycles of viremia and associated clinical symptoms (fever, anorexia, anemia, **thrombocytopenia** (low platelet count), and weight loss). In **Caprine arthritis encephalitis virus** of goats, the target cells for infection are monocytes and macrophages, and infection leads to chronic inflammation. This can lead to **encephalomyelitis** in young goats (swelling of brain due to infiltration of the infected macrophages) and chronic inflammation of the joints (**arthritis**) in adult goats.

IMMUNODEFICIENCY LENTIVIRUSES

We will devote the rest of this chapter on the immunodeficiency viruses (HIV I and II, SIV, and FIV). We will describe HIV I which has similar disease mechanisms as the viruses seen in monkeys and cats. All of the immunodeficiency viruses have long latency periods between infection and the opportunistic infections associated with the onset of **acquired immunodeficiency syndrome** (AIDS).

To give you an indication of the scope of devastation this disease has caused worldwide, as of 2020 approximately 40 million people have died of HIV, approximately 36.7 million people are living with HIV infection, and approximately 40,000 people contract HIV in the US each year.

To begin we must look at how *Lentivirus* genomes differ from simple retroviruses, see Figure 12-6B. Recall that although simple retroviruses use strong promoters to constantly express viral gene products, lentiviruses have much better control over their expression so we must discuss some of the important accessory proteins they use to accomplish this control.

ACCESSORY PROTEINS OF HIV

There are two important accessory proteins required for HIV replication, Rev and Tat. Rev regulates mRNA splicing. Initial mRNA transcripts from the provirus are multiply spliced and lead to production of the early genes, such as Rev and Tat, and multiple other accessory proteins. Once Rev accumulates, Rev covers a splice site contained within the **Rev Responsive Element** (RRE) by binding to the RRE on the mRNA, thus allowing the virus to reduce the amount and type of spliced mRNAs being produced. Tat allows the virus to produce unspliced full length mRNA. It does this by binding to the **Tat Activated Region** (TAR). The TAR sequence in the mRNA covers another splice site. When Tat is abundant it binds to the TAR and inhibits splicing of the mRNA. Note that Tat is also a transcription factor that increases transcription from the HIV promoter. The full-length mRNA will be used to produce the gag, pol, pro, and env proteins. Note that HIV has 4 other accessory proteins that are involved in a host of activities such as helping the virus cross the nuclear membrane and fighting anti-viral responses of the host cell.

DISEASE COURSE IN HIV 1 (SIMILAR TO SIV AND FIV)

The virus establishes the initial infection with adsorption to the cellular receptor CD4 found on macrophage and T-helper cells, but requires coreceptors for fusion. There are two coreceptors that HIV can use, CCR5 or CXCR4. The virus goes through two phases, the **M-trophic phase** (macrophage and memory $CD4^+$ T-cell infecting) and **T-trophic phase** (naïve $CD4^+$ T-cell infecting), and it uses different receptors during these two phases. During the M-tropic phase it uses **Fusin/CCR5 or CCR3** (which we will refer to as CCR5 for the remainder of the chapter). During the T-tropic phase it switches to the coreceptor **CXCR4**. The initial viral infection is with an M-trophic strain (CCR5 co-receptor) that will mutate and become a T-trophic strain (CXCR4 coreceptor) during the course of infection within the individual. The M-trophic strain that initiates the infection in a new host infects predominantly macrophages but is not **cytopathic** meaning the cells do not die. CCR5 is expressed primarily on memory T cells, activated T-cells, and macrophages, whereas CXCR4 is expressed primarily on naïve T-cells. Activated T-cells and memory cells better support viral reproduction than naïve T-cells, therefore if the virus begins infection in these cells, it is better able to establish a robust infection. Furthermore, there are a great deal of resident macrophage and memory $CD4^+$ cells in the genital tract but not many naïve $CD4^+$ cells. Therefore, beginning the infection using the CCR5 coreceptor allows infection to get a hold, and then the virus switches to less supportive cells over time once the virus is well established.

Note: People with mutations in CCR5 (**CCR5 Δ 32 mutations** pronounced delta 32) have been found to be resistant to HIV infection. This is because this coreceptor is necessary to establish the initial infection and demonstrates that the T-trophic strain is very poor at establishing the initial infection. A patient in Berlin was the first person to be cured of HIV infection when his bone marrow was completely eliminated with chemotherapy in order to cure him of acute myeloid leukemia which required a stem cell transplant. His doctor had read papers implying that people with the Δ32 mutation were not contracting HIV despite admitting to poor condom use with long term HIV positive partners. The physicians searched for a stem cell donor who had the Δ32 mutation in the CCR5 gene. Of the 267 people who matched him for a transfusion, only 1 had the Δ32 mutation! Although he was cured for 1 year the leukemia returned and he had to have a second transfusion. This second round of intensive chemotherapy almost killed him. He ended up with extensive brain injury but was still free of HIV. Unfortunately, the leukima returned again and he succumbed to his disease. Although this can be a curative treatment, it is very expensive, dangerous (survival from complete bone marrow depletion is about 50%), and time consuming. Also, people must allow their bone marrow to repopulate and their immunity to return which requires long periods of quarantine. Furthermore, all memory cells are depleted, and as such their immune system is like a baby's, so they are quite prone to infections for many years and must re-do all their vaccinations. Furthermore, drug treatment can now

achieve viral loads that are undetectable allowing infected people to live normal life spans and not risk infecting sexual partners.

If we follow an infection in an untreated host, it will follow a very predictable pattern.

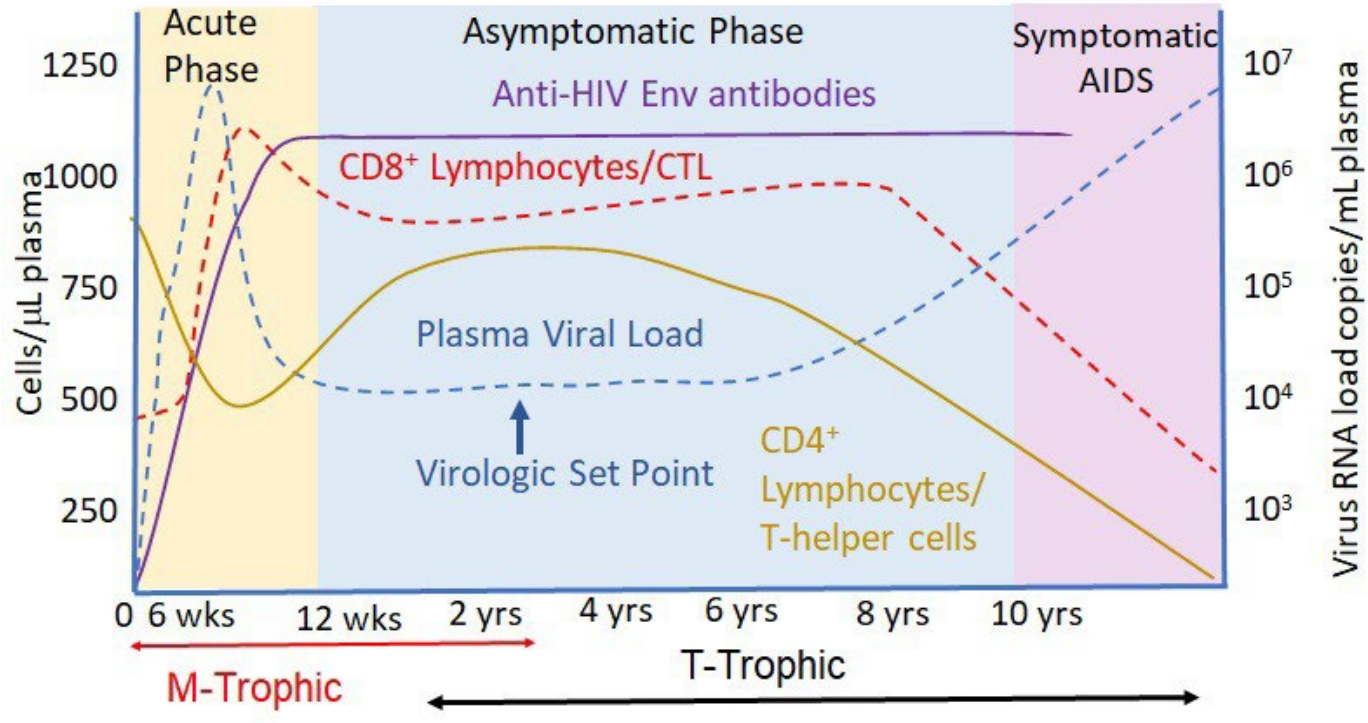


Figure 12-11. Progression of HIV infections if untreated. Courtesy of Erica Suchman, Colorado State University.

Acute Phase

As shown in Figure 12-11, an acute phase begins a few days post infection and lasts 3-4 months. This is characterized by the following symptoms: flu-like syndrome caused by massive proliferation of virus in the lymph nodes including fever, malaise, swollen lymph nodes, headache, diarrhea, and rash, but nothing that would lead an individual to believe that they have contracted HIV. As many as 25% of people in the US who have HIV are thought not to be aware of their status. During this phase high titers of virus can be detected in serum. During this time $CD4^+$ T-cell numbers decline but then rebound. This phase lasts only a few months when symptoms resolve as $CD8^+$ CTLs begin clearing the virus and viral load is decreased.

Asymptomatic Phase

The infection now enters the asymptomatic phase. This phase begins as the acute phase ends between 3-4 months post infection. During this time there are generally no symptoms, however the patient may experience sporadic episodes of fatigue, weight loss, swollen lymph nodes, thrush, shingles, or **oral hairy leukoplakia** (oral cancer associated with EBV herpesvirus infections). During this phase continuous replication of HIV continues to occur in all infected individuals, however the rates of virus production vary by up to 70-fold in different individuals. Differences in viremia (**virologic set point**) correlate closely with progression to onset of active AIDS with higher viremia resulting in more rapid onset of clinical disease. During asymptomatic phase, $CD4^+$ cells decrease approximately 60,000 cells/ml/year (60/µl/yr). This is due to: i) killing of $CD4^+$ cells by virus, ii) apoptosis due to inappropriate cytokine production, and iii) $CD8^+$ CTL killing of infected $CD4^+$ cells. The M-tropic virus population

also accumulates mutations which results in a more heterogeneous population made up of both M- and T-tropic strains.

The Symptomatic Phase

In the US the **symptomatic Phase** (historically **AIDS Related Complex, ARC**) and AIDS begins on average 8-10 years after infection and lasts on average 2-4 years, 1-2 without treatment, and eventually ends with death. Note that ARC is an older term describing a series of opportunistic infections with weak pathogens that is observed before the onset of full-blown AIDS. This term is not often used anymore, but we will use it in this text as I believe it is a useful way to think about the progression of the disease.

As infection progresses and mutations accumulate, virus becomes a more homogeneous T-tropic strain. T- tropic strains differ from M-tropic strains in that T-tropic strains i) infect naïve T-helper cells using the CXCR4 coreceptor, ii) cause the formation of **syncytia** (cell fusion) in infected cells, iii) cause T-helper cell **cytopathogenicity,** iv) have decreased sensitivity to neutralizing antibodies, v) increased cell range including neurons, and vi) increased pathogenicity in other organs & neurons.

There is a paradox in that many people obtain viral infections from individuals producing primarily T-tropic strains yet are being infected primarily with M-tropic strains that exist as a minority component of the viral load. However, remember that activated T-cells and memory cells better support viral reproduction than naïve T-cells, therefore beginning the infection in more receptive cells allows infection to get established, then the virus switches to less supportive cells. M-tropic strains are able to initiate infection in mature T-cells in the genital tract and once established switch cell types to less supportive naïve T cells.

The increase in T-tropic virus causes a decrease in $CD4^+$ T-helper cells. Remember that the functions of $CD4^+$ T-helper cells include i) activation of $CD8^+$ CTL function, ii) activation of naïve B-cells to produce plasma cells that produce antibodies, and iii) release of IL2 that stimulates natural killer cells. Note: Decreased antibodies lead to decreased **neutralization**, **opsonization**, and decreased **complement fixation**. For an in-depth description of the immune system, please see Chapter 4. Remaining infected $CD4^+$ cells become less responsive, including reduced production of IL2 and reduced proliferative response to antigens probably due to downregulated CD4 production. The lymph node architecture also begins to degrade for unknown reasons thus eliminating the site of antigen presentation and further reducing the immune response.

At the point that $CD4^+$ cells drop to 200-500 cells/μl (normal is 1×10^3/μl), the patient enters ARC. The symptoms observed are now just called the initial symptoms in the symptomatic phase. These include swollen lymph nodes, oral lesions, thrush, hairy leukoplakia, ulcers, shingles, **basal cell carcinoma** (skin cancer), headache, TB reactivations, HSV I and HSV II reactivations, skin lesions, and the viral infection *Molluscum contagiosum.* These are all general symptoms of infections with weak pathogens that can cause infections in people who are run down or whose immune systems are not functioning optimally for many reasons. So again, many people go undiagnosed during this phase.

As their $CD4^+$ cells drop below 200 cells/μl, the infected individual enters full blown AIDS, where infections with extremely weak pathogens or non-pathogenic organisms result in opportunistic symptom-causing infections. There are many different opportunistic infections of AIDS. This array of infections is one of the big issues in treating AIDS patients. They are developing infections with organisms that are not pathogenic in healthy hosts, hence there are limited drugs to treat many of these infections. There are protozoal, bacterial, viral, and fungal opportunistic infections, many of which can be fatal. Many of the viral infections are associated with an increased risk of cancer seen in people with HIV in the symptomatic phase, in particular, lymphoma associated with EBV, human herpes virus 8 (HHV-8) infections, **Kaposi sarcoma** associated with HHV8 infections, and anogenital carcinoma associated with HPV infection. During full blown AIDS, a wasting syndrome commonly occurs, the cause

of which is not well understood, as patients with and without chronic diarrhea develop wasting syndrome. Two thirds of patients will develop neurological symptoms including numbness, tingling, uneven gait, AIDS dementia complex (neurotoxic HIV), behavioral changes, and seizures. The opportunistic infections eventually result in death.

Great variability in length of time between infection and AIDS onset has been observed and is due to differences in virologic set point, genetic variation in humans, viral genetic variation, and the number of activated T cells the patient has at the time of infection. We know that HIV replicates best in activated T cells. People chronically infected with parasites have more activated T-cells and produce more virus and as such have a higher virologic set point. Furthermore, we know that there are different strains of HIV circulating on earth. Those on the African continent, where the disease in humans is older, are different than the strains circulating in the US, and unfortunately the strains in Africa cause disease that tends to progress more rapidly.

Along these lines, there is another HIV that is found predominantly in Africa called HIV- 2 which has a longer clinical latency, where infected people more frequently remain non-progressors, and does not spread as rapidly.

HIV was first identified as a disease in the US in 1981 when 2 unusual illnesses were noted in previously healthy young gay men in Los Angeles and San Francisco, CA. The first was Pneumocystis pneumonia (PCP) caused by the protozoa *Pneumocystis carinii,* which was known to only cause pneumonia in immunocompromised hosts. The second was Kaposi sarcoma, a tumor of the lining of the blood or lymph vessels that is normally seen in about 1 in 1 million Haitian men over the age of 65. These were both highly unexpected findings. Fortunately, the physicians treating the men in San Francisco and Los Angeles met at a meeting and discussed these unusual cases and decided to write a paper to warn other physicians to keep an eye out for similar patients. It was at this point that they realized these patients were not limited to San Francisco and Los Angels. In fact, they realized this was a disease that has been described in Africa for quite a few years. When frozen blood samples from Africa were analyzed, virus was isolated from a sample from 1959 and antibodies from samples in 1963, so the virus has been around in Africa for a long time.

By 1992 AIDS was the number one killer of 25-44-year-olds in the US. In 1988, after 4 years of requesting permission, C Everett Koop sent flyers to American homes to warn people of the new disease and urge them to protect themselves with condoms, and numbers dropped significantly. On the African continent, the disease spread rapidly, and in southern African nations up to 36 percent of the adults were infected with HIV. For reference, in the United States and Europe less than 1% of the population is infected, yet we have approximately 40,000 new cases documented per year in the US.

So where did the HIV-1 virus come from? Phylogenetic trees have been very helpful where HIV-1 branches were more closely related to local simian virus isolates than other HIV-1 viruses or HIV-2 viruses. This indicates that HIV may have made multiple jumps from monkeys to humans, most likely when the monkeys were hunted and eaten. A major effort is underway in Africa to educate people of the dangers of consuming primate bush meat. Although the practice of selling bush meat has been prohibited in many markets, the practice is known to be thriving in black market trade, as well as by individual hunters.

If the virus has been in the population since at least 1959 with multiple introductions, why did we all of a sudden see a pandemic emerge in the 1980s? This was most likely due to changes in travel, in social behavior, more sex and IV drugs use, increases in the human population allowing more opportunities for transmission, and possibly mutations in the virus making it more readily spread. Or perhaps we just finally noticed a long-standing problem.

TRANSMISSION OF DISEASE

When analyzed, the following quantities of virus were isolated from the fluids of HIV infected people: $1\text{-}5\times10^6$ infectious particles/ml in plasma, 10-10,000 infectious particles/ml in cerebrospinal fluid, and very low levels in tears, ear wax, saliva, sweat, feces, urine, vaginal secretions, semen, and breast milk. Note: Quantity here does not include infected cells that may be present in fluids such as semen, vaginal secretions, saliva, and breast milk. When these components are included, the titers of these four secretions go up dramatically.

Transmission is predominantly sexual and shared IV drug use in the US, but also includes childbirth, health care workers, and blood transfusions in the developing world. Sexually active people with penises have less risk of contracting infection than people with vaginas during penetrative vaginal sex. Receptive partners in anal intercourse have the highest risk. People with vaginas having sex with people with vaginas have the lowest risk. During all types of sex, the risk of infection is higher if the person has other sexually transmitted diseases because there will be more memory-T cells in the genital tract for the virus to infect. The recipient partner is always at more risk, although insertive partners still have significant risk. It has been documented that uncircumcised penises have a 2X higher risk of infection than circumcised penises as the foreskin is susceptible to infection. In the US, transmission most commonly occurs through people with penises having sex with people with penises. It should be noted, however, that this is not true in the developing world, where the vast majority of transmission is occurring by penetrative vaginal sex and both people with penises and vaginas are contracting the disease. In fact, the vast majority of HIV transmission worldwide is via penetrative vaginal sex.

People who share needles while injecting drugs have a very high risk of contracting HIV, as do hospital workers or researchers who accidentally stick themselves with contaminated needles. Needle exchange programs have been instituted in many areas and are shown to dramatically reduce HIV transmission among IV drug users. Self-capping needles have greatly reduced health care workers' accidental sticks.

During birth and while breast feeding mothers can transmit the virus to their newborn. On average 25% of babies born to HIV infected mothers will contract the disease, and the range is between 11% -60% depending upon viral load of mother and prevalence of breast feeding. If the mother is given RT inhibitors during labor, this greatly reduces transmission. However, in developing nations only 30% of infected mothers are receiving drug treatments. Programs to educate HIV infected mothers to reduce breast feeding greatly reduces transmission. However, as mothers in the developing world have been encouraged to breast feed as long as possible to reduce their **fecundity** (birth rate) and increase the health of their babies, it is often difficult to convince them that long learned mothering patterns should be reversed. It is estimated that approximately 160,000 babies a year contract HIV, a number that has been steady for many years.

PREVENTION OF RETROVIRUS INFECTIONS

There are currently no effective vaccines for retroviral diseases in humans. Although scientists have been working on finding a vaccine since the 1980s, it might be particularly difficult to achieve sufficient immunity to HIV. First, it has a high mutation rate. Second, infected people mount a quite vigorous immune response that initially overcomes the virus, but the virus then overcomes the immune response. Also, the virus can move from cell to cell by cell fusion and by release of virus only from the surfaces close to other susceptible cells, both of which help the virus avoid the immune response. It is theorized that to achieve sufficient immunity, the vaccine may need to induce sterilizing immunity, which is not the case for most viruses. Normally, reducing the viral load will allow the immune system to clear the remaining virus. There was a vaccine for cats against FIV, but it was taken off the market in 2017 due to low usage by pet owners as it was only advised in outdoor cats and not indoor cats. The

best prevention therefore for most of these diseases is safe sex involving consistent use of condoms, barrier nursing, caution handling blood products, not sharing needles during IV drug use, not sharing materials that might be contaminated with blood such as toothbrushes and razors, screening and heating of blood transfusion materials, reduced breast feeding in HIV infected mothers, anti-HIV drug treatment of HIV infected mothers prior to birth, anti-HIV drugs to partners of HIV infected individuals (PREP), and quarantine of infected animals. There is an effective vaccine for Feline Leukemia Virus (FeLV) that needs a booster every 2 years.

TREATMENT OF RETROVIRAL DISEASES

Anti-retroviral drugs come in 6 classes. Figure 12-12 shows an overview of the majority of them.

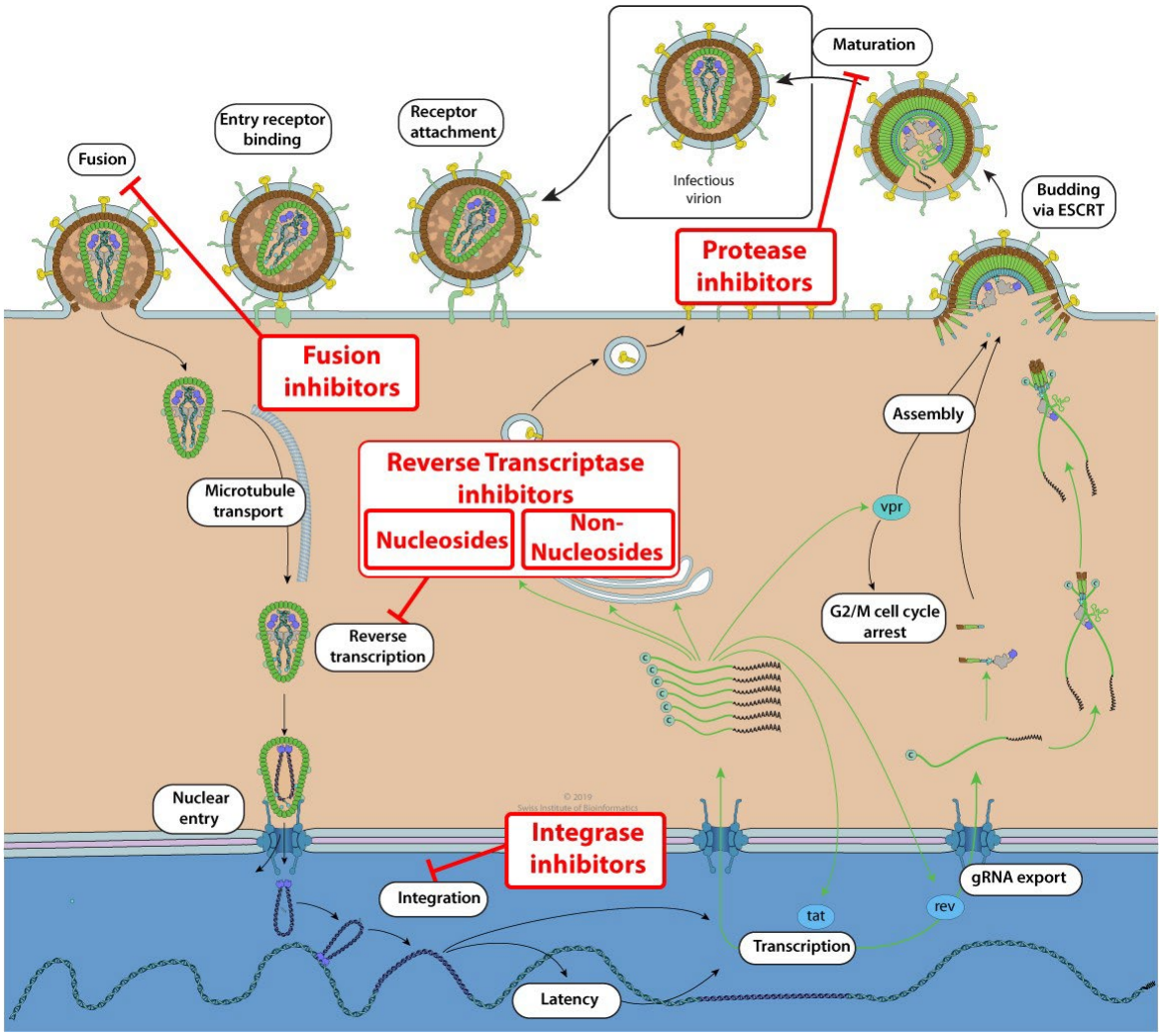


Figure 12-12. Overview of anti-HIV drugs. Note anti CCR5 binding drugs and anti-capsid protein association drugs are not shown. Courtesy of ViralZone https://viralzone.expasy.org/5180

1) Reverse transcriptase inhibitors: there are 2 different types of **competitive inhibitors** that bind to the active site of RT inhibiting the ability of the active site to bind nucleotides and carry out reverse transcription as shown in Figure 12-13.

- Nucleotide RTinhibitors
- Nucleoside RTinhibitors

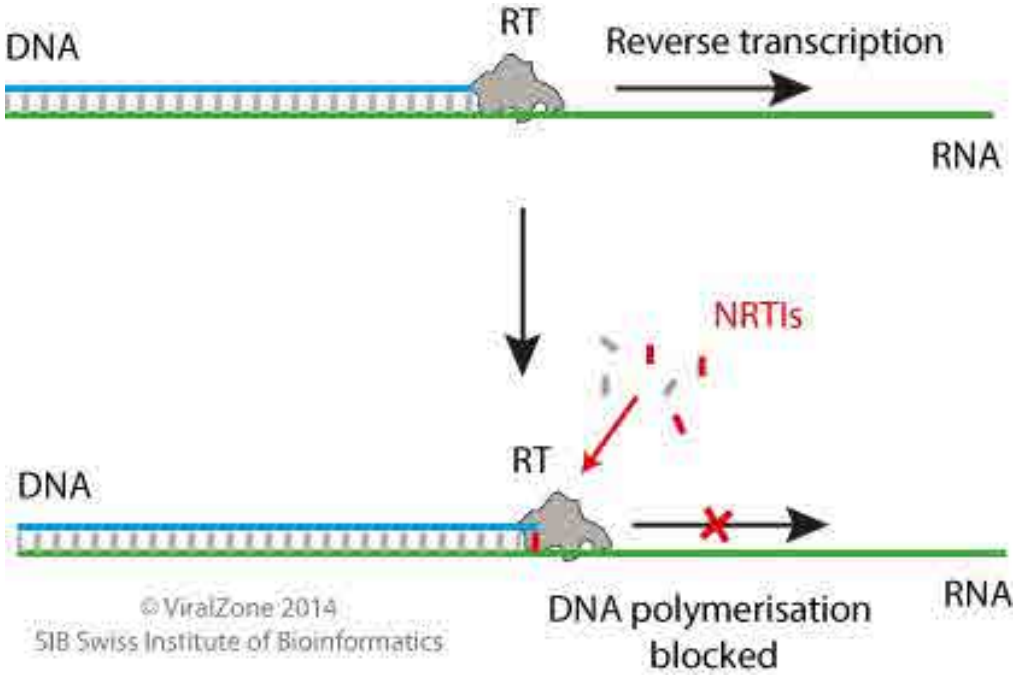


Figure 12-13. Competitive inhibitors of reverse transcriptase (Nucleoside or Nucleotide RT inhibitors NRTI). Courtesy of ViralZone https://viralzone.expasy.org/5136

There are also **non-competitive reverse transcriptase inhibitors** that bind to a regulatory site of RT which causes a conformational change in RT's active site inhibiting the ability to bind nucleotides and carry out reverse transcription, as shown in Figure 12-14.

- Non-nucleoside RT inhibitors

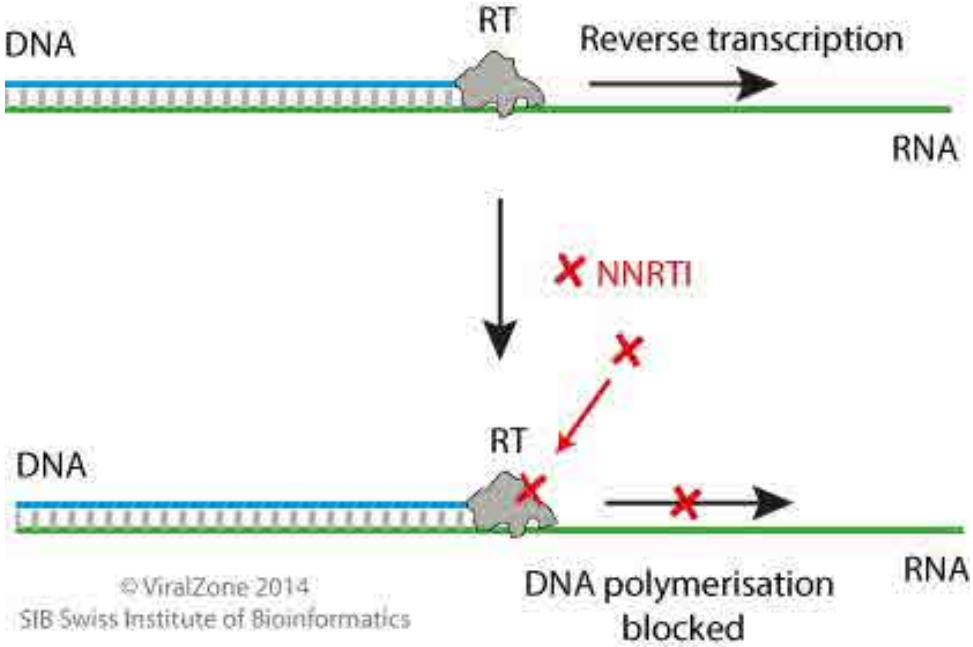


Figure 12-14. Non-competitive inhibitors of reverse transcriptase (Non-nucleoside RT inhibitors or NNRTI). Courtesy of ViralZone https://viralzone.expasy.org/5179

There are many competitive inhibitors binding to the active site of the following viral enzymes inhibiting binding of the substrate.

2) Protease inhibitors, as shown in Figure 12-15.

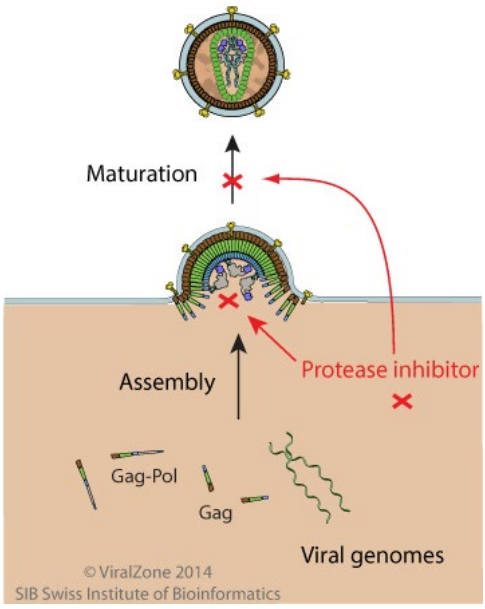


Figure 12-15. Competitive inhibitors of protease. Courtesy of ViralZone https://viralzone.expasy.org/5176

3) CCR5 antagonists stop binding and fusion, as shown in Figures 12-16 & 12-17.

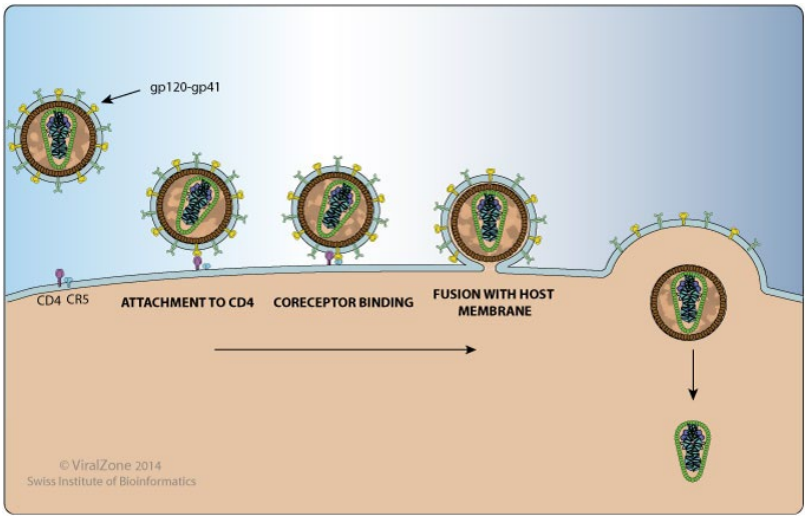


Figure 12-16. Inhibitors of HIV binding to CD4 and CCR5 activating fusion. Courtesy of ViralZone https://viralzone.expasy.org/5117

4) Entry (fusion) inhibitors, as shown in Figure 12-16 & 12-17.

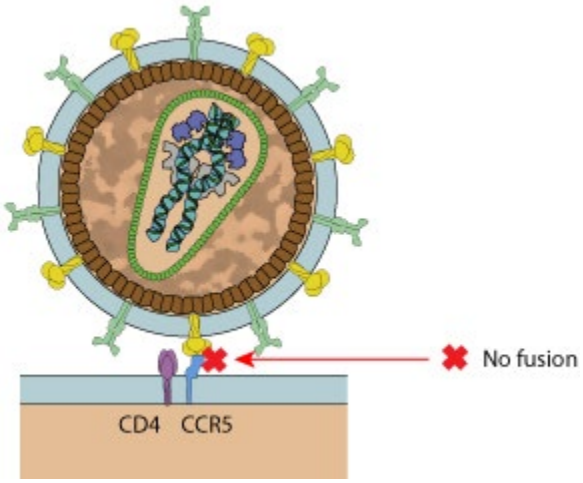


Figure 12-17. Competitive inhibitors of fusion by inhibiting fusion peptide or blocking CCR5. Courtesy of ViralZone https://viralzone.expasy.org/5178

5) Integrase Inhibitors, as shown in Figure 12-18.

- Integrase inhibitors were approved in Oct 2007 and are currently only used in people with drug resistant HIV.

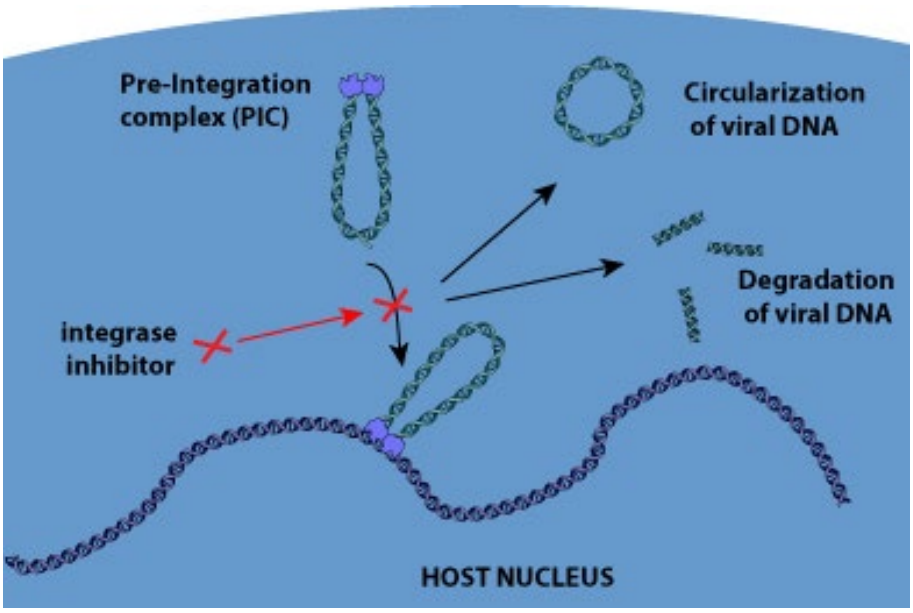


Figure 12-18. Integrase inhibitors inhibiting integration of viral provirus into host cell chromosome. Courtesy of ViralZone https://viralzone.expasy.org/5156

6) Capsid protein association inhibitors. (not shown on figure).

   These drugs were developed based on x-ray crystallography data of capsid protein interactions and inhibit the ability to form capsids. These were approved in August of 2022 by the FDA. They last 6 months and as such treatments involve every 6-month injections.

Unfortunately, due to the high mutation rate of retroviruses, the problem all of them share is rapid resistance development. Early in HIV history, patients were only given AZT, a nucleotide inhibitor, however they almost always developed resistance within a few years and succumbed to their disease. However, physicians now use **combined therapy** with multiple drugs from multiple classes**.** Combined therapy has the advantage of inducing little resistance, and combined therapy can dramatically increase life span, although the sooner the drugs are started, the better the outcome. The CDC estimates that an infected 25-year-old on combined therapy has an approximately 39-year life expectancy (64 years) with treatment. This is only 12 years less than uninfected individuals. However, for people whom the treatment is started early in the infection and where viral titers become undetectable will have a life expectancy equivalent to the general population and cannot transmit the virus to sexual partners. The problems are that it is very expensive, and one must take it for life. This is why HIV screening is so important as HIV is asymptomatic on average 7-10 years. Many people who would have better outcomes if they were on anti-HIV treatment miss the opportunity to increase their lifespan as they don't begin treatment until they become symptomatic. **Next generation sequencing** can be used to sequence the majority of mutants circulating in a host at any given time. This allows physicians to predict what drugs a patient will develop resistance to and to tailor the cocktail to maximize efficiency. For a review of next generation sequencing please see Chapter 2.

Note that **pre-exposure prophylaxis** (PrEP) can be used to prevent infections of partners of HIV infected individuals. This is a once daily pill containing 2 drugs (Truvada). One is a nucleotide RT inhibitor (competitive inhibitor) that also works on HepB, as HepB uses a reverse transcriptase to create a DNA copy of the mRNA it packages in its capsid. The other is a nucleoside RT inhibitor (competitive inhibitor) that also works on HepB but is not currently FDA approved as of 2021. This is given to people at risk of HIV infection and is 92% effective if taken correctly. Note: people undergoing PrEP need to be tested every 3 months to ensure they have not become infected, as PrEP can allow the reproduction of HIV drug resistant mutants in people who are unaware of their HIV infections.

As a last resort, total bone marrow depletion with irradiation or chemotherapy followed by a bone marrow transfusion with stem cells with the CCR5 Δ32 mutation can be used if a suitable donor can be found (which is rare).

A new treatment may soon be available called **LASER-ART** which combines CRISPR with **long-acting slow- effective release** (LASER) antiretroviral therapy (ART). LASER involves guide RNAs and CAS 9 cleavage of the viral genome at highly conserved regions within the LTRs and Gag gene. This has been shown in studies to eliminate replication-competent HIV DNA from genomes of ~ 30% of infected humanized mice.

DIAGNOSIS OF RETROVIRAL DISEASES

Diagnosis can be performed using PCR (called the **nucleic acid test,** or **NAT**) on blood but requires reverse transcription of samples as the test assays for the viral mRNA not the provirus. Serological assays are also available utilizing ELISAs and are the preferred mechanism of diagnosis as PCR is expensive and can lead to false positives. However, PCR is the preferred mechanism on newborns of infected mothers as the babies will have the mother's antibodies due to passive immunity. The PCR test is also used on partners of people who have had risky sexual encounters as they can detect the virus much earlier than the serologic tests.

Serological tests can detect the virus within 18-90 days, and blood drawn from a vein is more accurate than a test on blood drawn by a finger prick. Rapid blood tests from a vein draw can detect within 23-90 days, and home antibody saliva tests are not reliable until after 90 days, whereas the NAT PCR test can reliably detect virus within 13-42 days of infection. Note that unlike other viruses where serum antibody diagnosis requires acute and convalescent samples, HIV tests can utilize only 1 sample as people do not clear the infection. Therefore, if they are positive for HIV antibodies, they are positive for the virus.

HIV AS A THERAPEUTIC AGENT (CAR T-CELL THERAPY)

CAR T-cell therapy is a form of chemotherapy that uses recombinant HIV altered T cells to kill cancer cells. A sample of a patient's T cells are collected from the blood then modified with a recombinant HIV virus that produces **chimeric antigen receptors** (CARs) on their surface. CARs are recombinant molecules which consist of antigen-binding variable regions of a monoclonal antibody. The activation and co-stimulatory domains of the T cell receptor are linked to these CARs. They are composed of an **extracellular single-chain variable fragment** (scFv) derived from an antibody and joined to a transmembrane domain which is linked to the intracellular T cell signaling domains of the T cell receptor. When these CAR expressing T-cells are reinfused into the patient, the new receptors (specific for tumor antigens) enable them to latch onto a specific antigen on the patient's tumor cells and kill them. HIV is used because it integrates into the chromosome of the T-cells it infects causing them to express the CAR. However, as essential viral genes have been removed and replaced with the CAR, the virus is replication deficient and cannot cause AIDs. Because of the danger of this therapy, only people who have failed chemotherapy multiple times are eligible for this treatment that was approved in 2017. CAR T-cell therapy is also being explored as a potential way to clear people of HIV infections.

END OF CHAPTER QUESTIONS

1. Compare and contrast how retroviruses and hepadnaviruses produce the nucleic acids they will package in their virions. Will they use similar or different enzymes? Will the nucleic acids be produced in the same or a different location in the cell?

2. Compare and contrast how retroviruses and hepadnaviruses produce the nucleic acids they will utilize as their genome, i.e., the nucleic acids that they will deliver to a new cell. Will they use similar or different enzymes to produce this nucleic acid? Will the nucleic acids be produced in the same or different locations?
3. List as many things that you can think of that retroviruses and hepadnaviruses have in common, and as many that you can think of that are different between the two. Explain why they might be the same, or different.
4. Do retroviruses have multiple promoters?
5. How do retroviruses create more than one mRNA?
6. Do retroviruses have overlapping open reading frames (ORF)? If so, what mechanism do they use to express alternative ORF?
7. Explain the importance of the different coreceptors that HIV uses to infect cells.
8. HIV requires a coreceptor as well as a receptor. Why is this? How do the two work together?
9. Many retroviruses induce tumors. Do they do this like DNA viruses by pushing the host cell into S phase with accessory proteins? If not, why not?
10. Describe at least 4 mechanisms that retroviruses use to induce tumors.
11. Explain the difference between a proto-oncogene and an oncogene.
12. Explain the difference between enhancer and promoter insertional activation of proto-oncogenes by retroviruses. How would you tell the difference in a lab?
13. Why can lentiviruses enter the nucleus without the cells undergoing cellular replication while other non- lentiviral retroviruses cannot?
14. How do the diseases of simple retroviruses like Moloney murine leukemia virus differ from the more complicated retroviruses like the lentiviruses?
15. Explain the difference between clinical and viral latency. Which is observed with HIV, and why?
16. Do retroviruses behave more like RNA viruses in their mechanisms for producing proteins or DNA viruses? Support your answer.
17. Do DNA viruses need to create more DNA once they have created a provirus? If so, why, and for what purpose?
18. You are performing a blood test on a horse to determine if it has infectious equine anemia using PCR. Would you use reverse transcriptase PCR or standard PCR, or could you use either? Explain your answer.
19. How does HIV cause immunodeficiency?
20. What is the difference between T-tropic and M-tropic HIV? How do these two sub types play a role in HIV progression?
21. How is next generation sequencing used in HIV treatment?

22. Explain the current treatment recommendations for HIV. What is recommended, and why?
23. How do each of the classes of anti-HIV treatments on the market work?
24. Some of the anti-HIV drug treatments are also successfully used on hepatitis B infections. Which classes of drugs do you think would work for this, and why?
25. What is PREP, and what is it used for?
26. How are they proposing to use CRISPR to treat HIV infections?
27. In what ways do retroviruses behave like DNA viruses? RNA viruses?
28. Why do you think HIV we chosen for CAR T therapies? How do you think they made the virus safe?

REFERENCES:

Avert, Global information and education on HIV and AIDS. (2021). http://www.avert.org/worldstats.htm/

Brown, T., R. I am the Berlin patient: a personal reflection. (2015). *AIDS research and human retroviruses* vol. 31,1: 2-3. doi:10.1089/AID.2014.0224 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4287108/

Centers for Disease Control and Prevention. HIV. https://www.cdc.gov/hiv/basics/testing.html#how-soon-after-an-exposure

Creative Biogene. (2021). Lentiviral vectors- the application for CAR-T therapies. https://www.creative-biogene.com/support/Lentiviral-Vectors-the-Application-for-CAR-T-Therapies

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapter 7, 13, 47, 48, 49, 50.

Levy, J. HIV and the Pathogenesis of AIDS (2nd) Second Edition (1998). ASM Press; 1752 N Street NW, Washington, DC 20036-2804

Link, JO., et al. (2022) Clinical targeting of HIV capsid protein with a long-acting small molecule. Nature. 584(7822):614-618. doi: 10.1038/s41586-020-2443-1

National Center for Microscopy and Imaging Research. https://ncmir.ucsd.edu/site/media/images/gallery/hiv_infected_cells-1.png

Shanmugasundaram, U., et al. (2014) Phenotype and functionality of $CD4^+$ and $CD8^+$ T cells in the upper reproductive tract of healthy premenopausal women. Am J Reprod Immunol. 71(2): 95–108. Published online 2013 Dec 6. doi: 10.1111/aji.12182. https://pubmed.ncbi.nlm.nih.gov/24313954/

ViralZone. Alpharetrovirus https://viralzone.expasy.org/65

ViralZone. Fusion inhibitors. https://viralzone.expasy.org/5178

ViralZone. HIV antiviral drugs.. https://viralzone.expasy.org/5180

ViralZone. HIV-1 envelope proteins (ENV) https://viralzone.expasy.org/5117

ViralZone. HIV viral particles. https://viralzone.expasy.org/5182

ViralZone. HIV replication cycle. https://viralzone.expasy.org/5096

ViralZone. Integrase inhibitors. https://viralzone.expasy.org/5156

ViralZone. Lentivirus. https://viralzone.expasy.org/264

ViralZone. Non-nucleoside reverse transcriptase inhibitors. https://viralzone.expasy.org/5179

ViralZone. Nucleoside reverse transcriptase inhibitors. https://viralzone.expasy.org/5136

ViralZone. Protease inhibitors. https://viralzone.expasy.org/5176

Chapter 13: An introduction To RNA Viruses

AN INTRODUCTION TO RNA VIRUSES

As we have learned, both DNA and RNA viruses exist. We examine these separately, with Chapters 6-10 covering different DNA viruses and Chapters 11 and 12 covering *Hepadnaviridae* and *Retroviridae,* viruses that package RNA that is transcribed to DNA and spend the rest of their life cycles functioning like DNA viruses. Note, however, that retroviruses are classified as RNA viruses as they carry out reverse transcription in the host cell cytoplasm, and hepadnaviruses are classified as DNA viruses as they carry out reverse transcription in the virion and carry DNA to new cells to establish infection.

Chapters 14-19 will cover the RNA virus families. This chapter will serve as an introduction to RNA viruses as Chapter 5 was an introduction to DNA viruses. You will note that I discuss retroviruses in both the chapters as retroviruses truly spend part of their life cycle as both. Retroviruses are different than the hepadnaviruses which truly only function as DNA viruses despite initially packaging RNA.

As you study the viruses, I will remind you to begin looking for and seeing the trends that occur among the viruses. Trying to understand the reasons for these trends is a more fruitful study technique than trying to remember the many facts that make up virology. There are problems for all viruses to overcome. You may want to return to Chapter 1 and refamiliarize yourself with the taxonomic trees for the RNA viruses.

In Chapter 3 we discussed that all viruses must overcome the following issues:

- How to get into the cell
- How to uncoat
- Cellular defense systems
- How to get to site of replication
- How to mimic cellular mRNAs
- Eukaryotic translation only monocistronic mRNA
- Viral and cellular mRNA are in competition for translation machinery
- Initiating genome replication
- Replicating the ends of the genome
- Packaging the genome
- How to get out of the cell
- The host's immune response

In this chapter we will focus on how RNA viruses tend to overcome these issues.

We discussed how viruses get into cells, uncoat, and get to their sites of replication in Chapter 3. These mechanisms do not vary between DNA and RNA viruses. We learned how viruses deal with cellular anti-viral defenses in Chapter 4, so we will begin with where RNA viruses replicate.

As we indicated in Chapter 3, all RNA animal viruses covered in this book replicate in the cytoplasm with the exception of orthomyxoviruses that replicate in the nucleus and retroviruses that replicate partly in the nucleus and partly in the cytoplasm. Note that although retroviruses are classified as RNA viruses (as that is what they package in their virion and carry to the cell), they use reverse transcriptase to create a double stranded DNA intermediate that enters the nucleus and integrates into the host cell chromosomes and as such become DNA viruses from this point. However, they will transcribe an RNA genome that will be packaged in the cytoplasm, so retroviruses actually replicate in both locations.

TRANSCRIPTION OF mRNA FROM RNA VIRUSES

RNA viruses, with the exception of retroviruses, cannot use the host cell's transcription machinery DNA-dependent RNA polymerase (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As such, with the exception of retroviruses, they must all have genes to produce an RNA-dependent RNA polymerase (RdRp). Positive sense RNA viruses whose genomes are equivalent to mRNA can be translated immediately upon entry into the cell. However, negative sense and double stranded RNA viruses cannot, and must therefore package their RdRp in their virion with the genome so that mRNA can be transcribed by RdRp via a transcriptase activity. Remember that all cellular polymerases, both DNA and RNA, read the template from 3' to 5', synthesizing the new strand 5' to 3'. This is also true for all viral polymerases.

PROBLEM TO OVERCOME: EUKARYOTIC TRANSLATION OF ONLY MONOCISTRONIC mRNA

Viruses with their very small genomes have an issue to deal with; eukaryotic cells only make one protein per mRNA. So how can a virus with a small genome create the many proteins it needs to replicate under these circumstances? Viruses have evolved the following mechanisms to overcome the problem of creating only one protein per mRNA in eukaryotic cells. Some of these involved making multiple mRNAs, whereas others allow a virus to create multiple proteins from one mRNA using clever mechanisms to get the cellular ribosomes to accomplish what they do not normally want to do. The following are mechanisms evolved by viruses to create many viral proteins. Those that are underlined are utilized by only DNA viruses. Those that are not underlined are only used by RNA viruses. We will focus on the ones used by RNA viruses only as those used by DNA viruses were described in depth with figures in Chapter 5. Note: viruses can overcome this issue by either 1. creating multiple mRNAs, or 2. creating more than one protein per mRNA through mechanisms such as proteolytic cleavage of polyproteins, ribosomal frameshifting, RNA editing, or termination suppression. DNA viruses create multiple mRNAs the same way that host cells do by using multiple genomic promoters and mRNA splicing. However, very few RNA viruses have splicing mechanisms available to them, and none of the RNA viruses utilize multiple genomic promoters, although a few utilize promoters found on the anti-genome. They don't use genomic promoters because RNA virus RdRps most often start synthesis de novo (at the first nucleotide). This chapter will explore the alternative mechanisms the RNA viruses have evolved to transcribe more than one mRNA from the genome. We will also observe trends in mechanisms used by positive sense RNA viruses and negative sense RNA viruses.

1. mRNA generated using multiple genomic DdRp promoters: DNA only.

2. Differential splicing of pre-mRNA to mRNA: DNA viruses (except poxviruses) plus retroviruses and orthomyxovirus: RNA viruses.

3. Segmented genomes: RNA viruses only.

4. Subgenomic length mRNA generated without using multiple genomic promoters: RNA viruses only.
    A. Positive sense RNA viruses:
        - Creating a short mRNA from negative sense anti-genome using an internal RdRp promoter.
        - Nested mRNA created by polymerase jumping.
    B. Negative sense RNA viruses:
        - Segmented genomes All members of the order Bunyavirales (Hantaviridae, Phenuiviridae, Nairoviridae, Arenaviridae; Orthomyxoviridae, and Reoviridae double stranded RNA viruses, too).
        - Ambisense genomes (*Phenuiviridae, Arenaviridae)* .
        - RdRp start stop (viruses in the order *Mononegavirales, Paramixoviridae, Filoviridae, Rhabdoviridae, Pneumoviridae*).
5. Polyprotein proteolytic cleavage, protease: RNA and DNA, ALL positive sense RNA viruses.
6. Leaky ribosomal scanning (overlapping ORFs): DNA and RNA viruses.
7. RNA editing by RdRp (overlapping ORFs): non-segmented negative sense RNA viruses only (*Paramyxoviridae, Filoviridae*).
8. Frame shifting/overlapping ORFs: mostly positive sense RNA viruses.
9. Termination suppression: positive sense RNA viruses.

Note: that mechanisms 1-4 are ways to create more than one mRNA (blue), and that only mechanisms 2-4 are available to RNA viruses, with splicing only being available to RNA viruses that replicate in the nucleus. Mechanisms 5-9 are ways to create more than one protein from one mRNA when the ribosome will only read one protein per mRNA in eukaryotes (purple). We will describe the clever workarounds RNA viruses have evolved to create multiple proteins in this chapter.

CREATING MULTIPLE mRNAS

mRNA Generated Using Multiple Genomic Promoters

DNA viruses can create multiple proteins by utilizing multiple DNA-dependent RNA polymerase (DdRp) promoters to create multiple mRNAs each of which will translate different proteins. Most RNA viruses cannot utilize multiple promoters, and there is an important reason for this. Most RNA-dependent RNA polymerases (RdRp) that RNA viruses utilize to transcribe their mRNA do not recognize or utilize traditional promoters that are used by DdRp in DNA viruses. Note that most DNA viruses, including poxviruses, utilize multiple promoters to create multiple mRNAs. The exceptions to this rule are retroviruses which only create mRNA from one promoter. The reason for this is the promoter exists in the **long terminal repeat** (LTR), and there is only one. A few members of the family *Parvoviridae* only use one promoter, but the majority of the parvoviruses also use multiple promoters. We will see that 3 families of positive sense RNA viruses will use one RNA-dependent RNA polymerase (RdRp) promoter found on the negative sense anti-genome to create one subgenomic mRNA from the genome.

Differential Splicing of Pre-mRNA to mRNA

Differential splicing of a pre-mRNA to multiple mRNAs is used by all animal DNA viruses except poxviruses because poxviruses replicate in the cytoplasm, do not have access to the extensive machinery required to perform splicing, and have not developed the ability to facilitate their own splicing. Note: retroviruses while acting like DNA viruses do utilize splicing. The only RNA viruses that replicate as an RNA virus in the nucleus, orthomyxoviruses, probably evolved to replicate in the nucleus to get access to the cell's splicing machinery that it requires during its gene expression. Retroviruses undergo splicing when their mRNA is produced by cellular RNA pol II in the nucleus but recall that they have been reversed transcribed to DNA and as such are functioning as DNA viruses at this point. See previous Chapters (5-12) for figures describing splicing.

Segmented Genomes: RNA Viruses

Segmented viral genomes in animal viruses are found only in negative sense and double stranded RNA viruses. The number of segments varies from 2 (arenaviruses) to 12 (reoviruses 10-12). The virus translates one or a few proteins per segment. The following families produce segmented genomes: *all members of the order Bunyavirales (Arenavirdiae, Phenuiviridae, Nairoviridae, Hantaviridae and others),* and all members of the families *Orthomyxoviridae* and *Reoviridae.* Remember that only viruses with segmented genomes can undergo **reassortment** where coinfection of a cell can result in an exchange segment, an important mechanism for developing antigenic variation by **antigenic shift**. You will see that this is the only mechanism used by reoviruses to create multiple mRNAs and proteins, but the negative sense segmented RNA viruses can also use cellular proteases to cleave polyproteins to multiple proteins. The arenaviruses and phenuiviruses have what are referred to as **ambisense genomes** that will help them create subgenomic length mRNAs – as will be described below.

Subgenomic Length mRNA Generated Using RNA-Dependent RNA Polymerase

Subgenomic length mRNAs are shorter than the entire genome, and as such comprise only a small portion of the genome. They can be generated by a variety of mechanisms which we will describe in this chapter. Very few RNA viruses create subgenomic mRNA using multiple promoters or splicing while the majority of DNA viruses do.

Ambisense Transcription of Subgenomic mRNAs

Phenuiviruses and arenaviruses have segmented genomes where some of the segments can be **ambisense**. We discussed earlier that **segmented genomes** allow negative sense and ds RNA viruses to produce multiple mRNAs one from each segment that each gives rise to a separate protein. However, the ambisense segments also have genomes that contain a positive protein-producing and negative non-protein-producing section within one piece of their RNA genome separated by a hair pin loop that separates the coding from non-coding portions. These viruses are classified as negative sense RNA viruses because most, and sometimes all the segments are negative sense. The ambisense segments have a section that is capable of producing proteins, but because their genomes do not have the 5' cap necessary to produce proteins immediately they must package RdRp in the virion. It is not until the RdRp creates a reverse-complementary copy of the genome, called the **anti-genome**, from the genome that the RdRp's transcriptase activity will create an mRNA from the complementary negative strand that has a 5' cap added and can be translated. It is important to note that the RdRp will also produce an mRNA from the negative sense portion of the genome, as shown in figure 13-1. Hence these viruses can create multiple (2) mRNA from each segment, although in phenuiviruses only one segment is ambisense. You may also have noticed that only the families of viruses with a small number of segments (*Arenaviridae,* 2; *Phenuiviridae,* 3) have ambisense genomes that allow them to produce more proteins from their limited number of segments. *Orthomyxoviridae* and *Reoviridae,* which have far more segments, do not need to be ambisense to create a sufficient number of viral proteins. The other members of order *Bunyavirales,* although having only 3 segments have slightly larger genomes than the phenuiviruses, do not have ambisense segments.

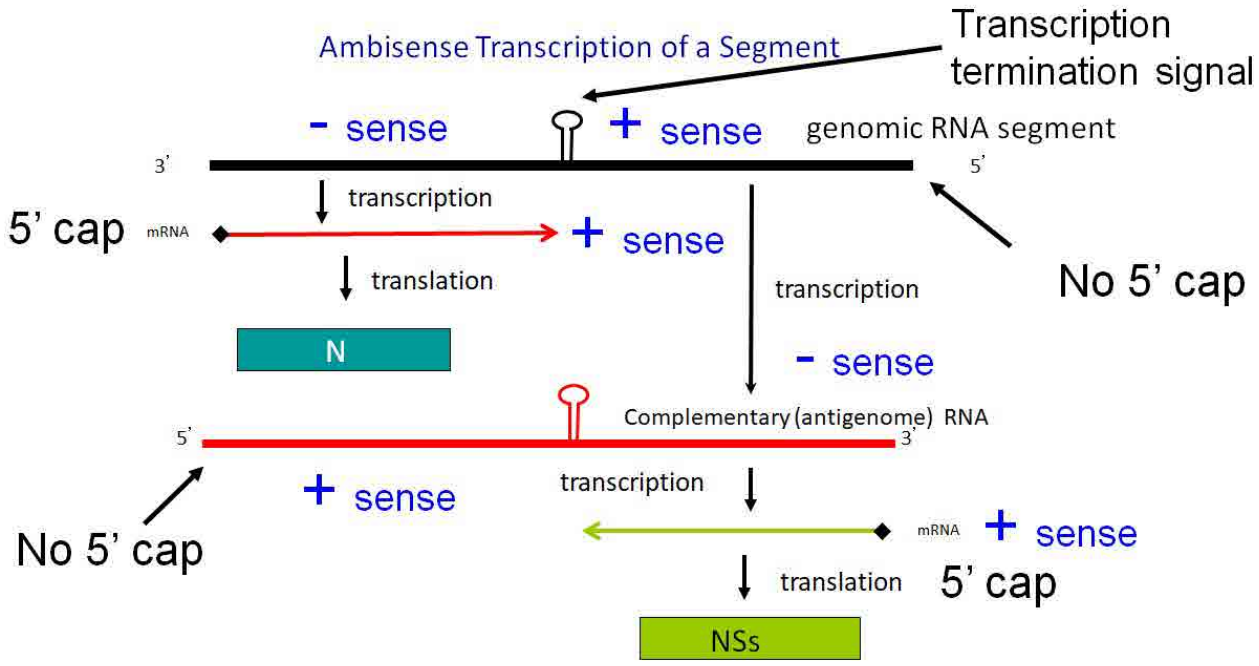


Figure 13-1. Subgenomic length mRNAs produced by ambisense genomes. Note that the hairpin separates the coding from non-coding sections of the segment. Courtesy of Erica Suchman, Colorado State University

Subgenomic Length mRNA Produced by RdRp Start Stop Mechanism

You will note that the viruses in the super family *Mononegavirales* (*Rhabdoviridae*, *Paramyxoviridae*, *Pneumoviridae* and *Filoviridae*) use the RdRp start stop mechanism to create their viral mRNAs. In these viruses, the RdRp pauses at the end of each gene, and the mRNA gains a poly A tail and falls off as the polymerase begins again and caps the next segment that will be synthesized, as shown in Figure 13-2. Note this is not true of the nuclear replicating *Mononegavirales* viral family *Bornaviridae*, however, as these viruses are not associated with a major disease they are not discussed in this book.

Furthermore, the subgenomic mRNAs made from the 5' end of the genome are made in lower and lower quantities than those transcribed from the 3' end allowing these viruses to regulate how much of each protein is produced.

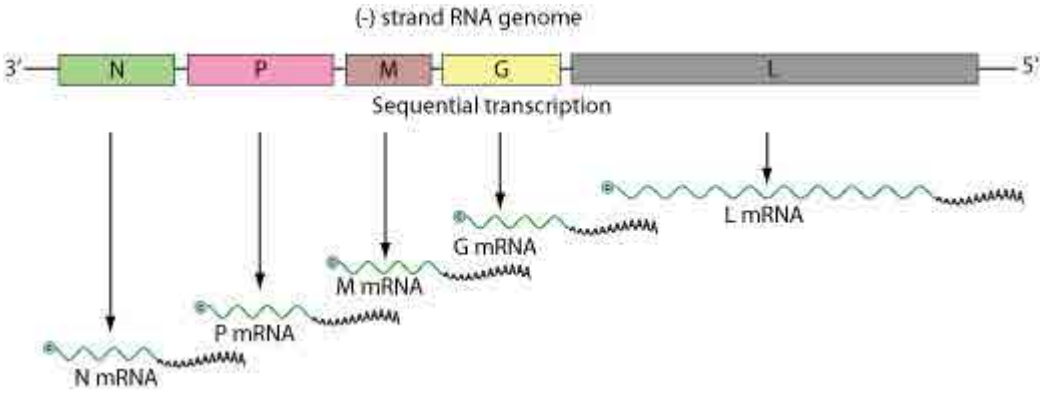


Figure 13-2. Subgenomic length mRNAs produced by negative sense RNA viruses by the RdRp start stop mechanism. Courtesy of ViralZone https://viralzone.expasy.org/2

Subgenomic Length mRNA Read from an Internal Promoter in the Negative Strand in a Positive Sense RNA Virus with More Than One Open Reading Frame (ORF)

This mechanism is seen in togaviruses, matonaviruses and caliciviruses, three positive sense RNA viruses that can function as mRNA right upon entrance into the cell. One of the proteins made will be RNA-dependent RNA polymerase (RdRp) which will create a full-length negative sense strand (**anti-genome**) that can serve as the template for RdRp to produce more positive sense genome. However, the anti-genome can also serve as template to produce a smaller subgenomic length mRNA from an internal promoter that is used to produce other proteins. Note: the mechanism by which RdRp initiates transcription of this subgenomic mRNA is not well understood but is similar to using multiple promoters. You will note that unlike all of the other RNA viruses, RdRp is not initiating production of the mRNA de novo from the 3' end of the genome. This mechanism of initiating RNA transcription upstream of the 3' end (within the middle of the genome) is only seen in togaviruses, motonaviruses and caliciviruses of the viruses we cover in this book. Furthermore, the full-length transcript is not created using a promoter but is created via de novo synthesis at the 3' end, as shown in Figure 13-3. Remember that viruses must develop mechanisms to regulate how much of each protein they make. This smaller subgenomic length mRNA will create proteins that are needed in much lower concentrations than the proteins created from the full-length genomic mRNA.

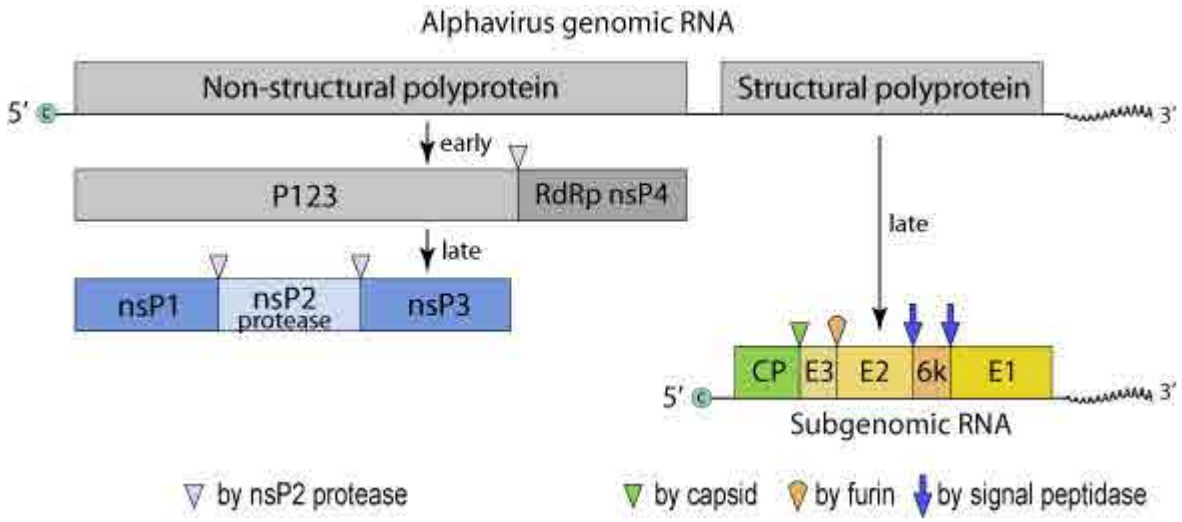


Figure 13-3. Subgenomic length mRNA created when shorter mRNA is produced from the negative sense (anti-genome) RNA template of a positive sense RNA virus. Courtesy of ViralZone https://viralzone.expasy.org/625

Nested Subgenomic Length mRNAs Positive Sense RNA Viruses That Create More Than One ORF

The positive sense RNA coronaviruses also produce subgenomic length mRNAs although by a different mechanism than togaviruses, matonaviruses and caliciviruses, the other 3 positive sense RNA viruses that create more than one **open reading frame (ORF)**. See Figure 13-4. Coronavirus genomes have multiple **transcription regulation sequences (TRS)** throughout the genome. As RdRp replicates the RNA it can either read through the TRS or can jump to the identical TRS sequence further towards the 5' end. The jump is always to the same TRS on the 5' end of the genome. Thus, all of the mRNA created have the same 5' end but are **nested** in that portions of the middle of the genome are missing. This allows cytoplasmic replicating virus to produce mRNAs that look much like mRNAs produced by splicing but without utilizing the splicing mechanisms that are found in the nucleus. The smaller subgenomic mRNAs are made in higher quantities, with each subsequently longer mRNA in slightly less quantity, allowing coronaviruses to regulate how much of each protein is produced.

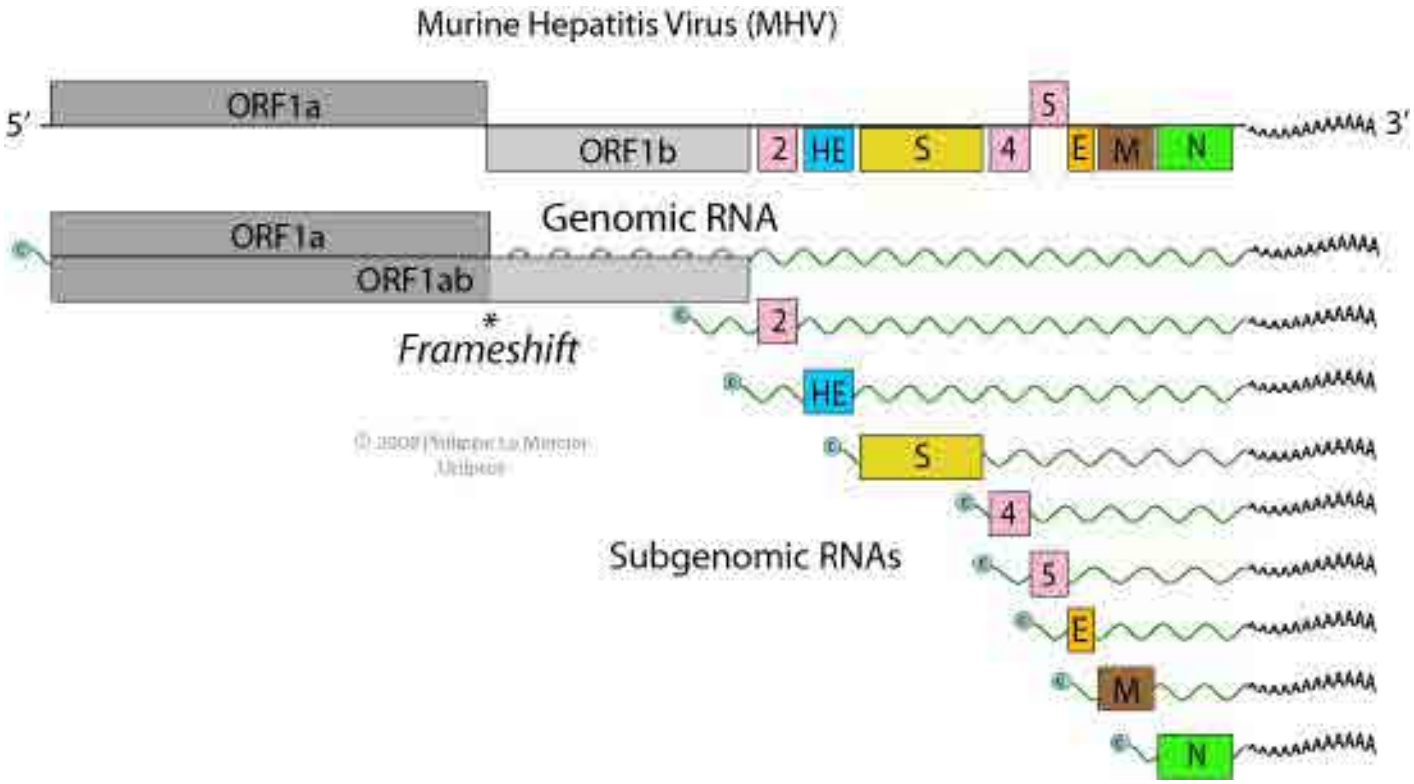


Figure 13-4. Subgenomic length mRNAs created when the RdRp jumps from the first TRS to downstream TRSs during transcription. Courtesy of ViralZone https://viralzone.expasy.org/764

MECHANISMS USED BY RNA VIRUSES TO CREATE MULTIPLE PROTEINS FROM ONE mRNA

Proteolytic Cleavage of Large Polypeptides into Individual Proteins: Protease

Although proteolytic cleavage is a mechanism always used by positive sense RNA viruses, sporadic DNA viruses and RNA viruses with negative sense genomes or ds RNA genomes have been found to use this mechanism as well. A close analysis allows certain trends to emerge. Positive sense RNA viruses all use their own viral proteases, including retroviruses. The protease is **autocatalytic**, meaning it can cleave itself out of the polyprotein before or after cleaving out all the other proteins. A classic example that was described in Chapter 5 (Figure 5-3) are picornaviruses (positive sense RNA virus). The protease first cleaves the polyprotein into 4 proteins each having a specific function. When the virus needs different proteins, the first 4 are further cleaved to 6, which are further cleaved to 10, and so on. It is not until this third round of cleavage that the protease actually cleaves itself out of the polyprotein. This coordinated sequential cleavage allows the virus to regulate its proteins over time, with enzymes cleaved out only when they are needed, as precursor proteins may have different functions than the mature forms. See Chapter 5 for figures. Unlike the positive sense RNA viruses that all use their own viral proteases, DNA viruses can either use their own protease, as herpesviruses and adenoviruses do, or use host cell proteases, as hepadnaviruses do. Negative sense RNA viruses only use host cell proteases, viruses that use cellular proteases include all members of *Bunyravirales:* nairoviruses, hantaviruses, phenuiviruses, and areanviruses.

Ribosomal Leaky Scanning Overlapping reading frames DNA and RNA viruses

This mechanism is not very commonly used by cellular mRNAs but allows viruses from many families (both RNA and DNA) to obtain more than one protein from a mRNA. It is used on **overlapping open reading frames (ORF),** meaning there are multiple AUG start sites that are out of frame of one another. The first AUG is generally associated with a **weak Kozak consensus,** and often the ribosome scans passed it and initiates at the second AUG associated with a **stronger Kozak consensus** sequence. Often, the proteins created from the first ORF are to help overcome host cell anti-viral defenses and are made in times of stress, such as when PKR is present. Remember PKR phosphorylates the translation initiation factor eIF2α, shutting off translation. When the virus senses this, it has a need for the proteins produced from ORF 1. Leaky scanning allows that ORF to be expressed when necessary. See Chapters 3 and 5 for figures.

RNA Editing by RdRp Overlapping reading frames Negative Sense RNA Only

RNA editing is carried out by some negative sense RNA viruses in the families *Paramyxoviridae* and *Filoviridae* (order *Mononegavirales*), see Figure 13-5. The vast majority of the time RdRp reads the negative template strand correctly and results in a transcript that will be translated into most of the viral proteins. However, there are a few proteins that are needed in much lower concentrations. A low percentage of the time the RdRp will perform RNA editing where it inserts one nucleotide triphosphate (NTP) into the mRNA. This leads to a frame shift that allows production of a protein from a second ORF in an overlapping reading frame needed in low concentrations.

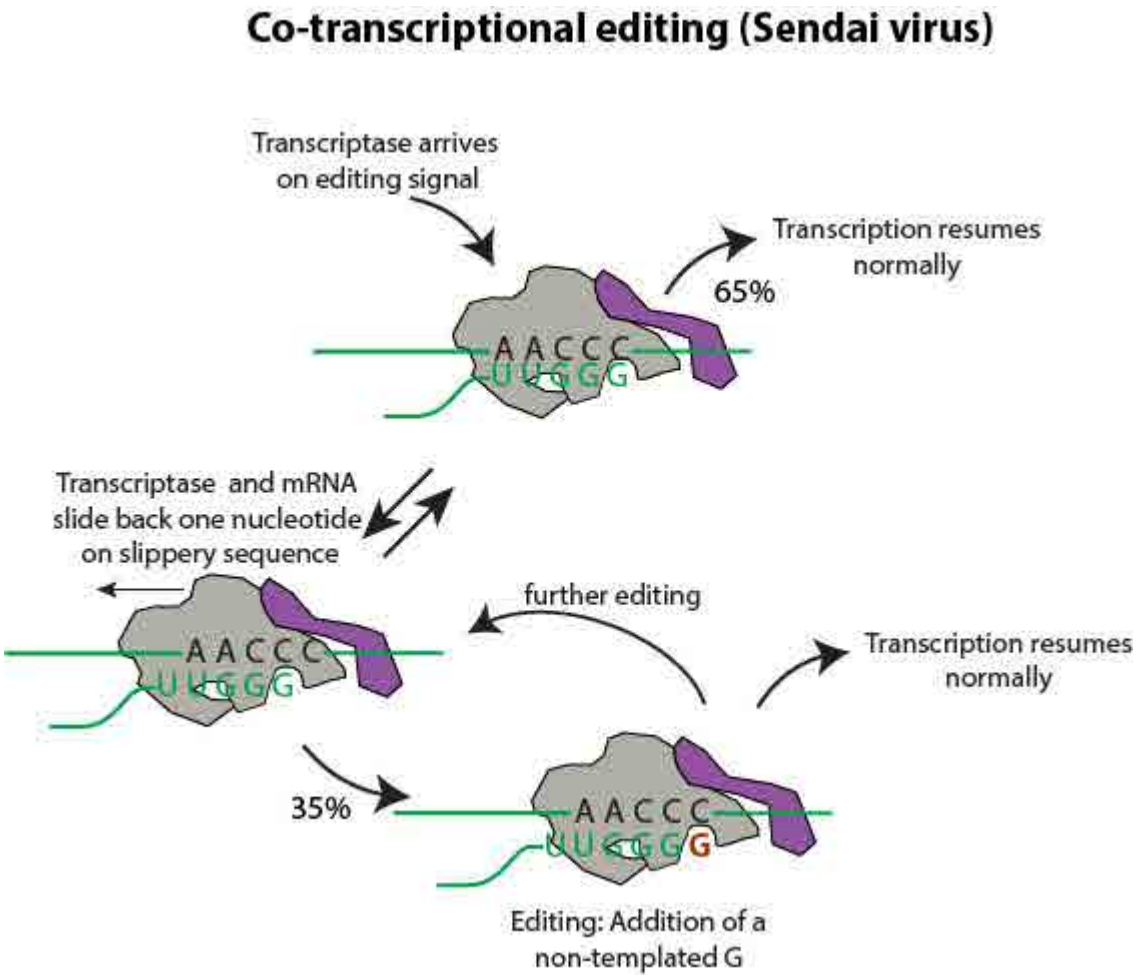


Figure 13-5. RNA editing occurs when RdRp inserts an extra nucleotide a small percentage of the time and creates a second open reading frame that creates proteins in a small concentration. Courtesy of ViralZone https://viralzone.expasy.org/857

Frame Shifting/Overlapping ORFs: RNA Only

Ribosomal frame shifting is used more often by the positive sense RNA viruses and a few negative sense RNA viruses. See Figure 13.6. Again, viruses do this for two reasons: to increase the number of proteins they can make from a small genome while also controlling the amount of proteins produced. The mRNA of these viruses creates a secondary structure called a **pseudoknot**. When the mRNA is translated to protein, the ribosome normally unwinds this pseudoknot and creates large quantities of proteins in one open reading frame. However, a small percentage of time, the ribosome hits the pseudoknot and slips back one nucleotide resulting in a frame shift and producing proteins in a second overlapping open reading frame. This benefits the virus because it can make the proteins in the second open reading frame at much lower concentrations than the primary open reading frame proteins, which are needed in much greater concentrations.

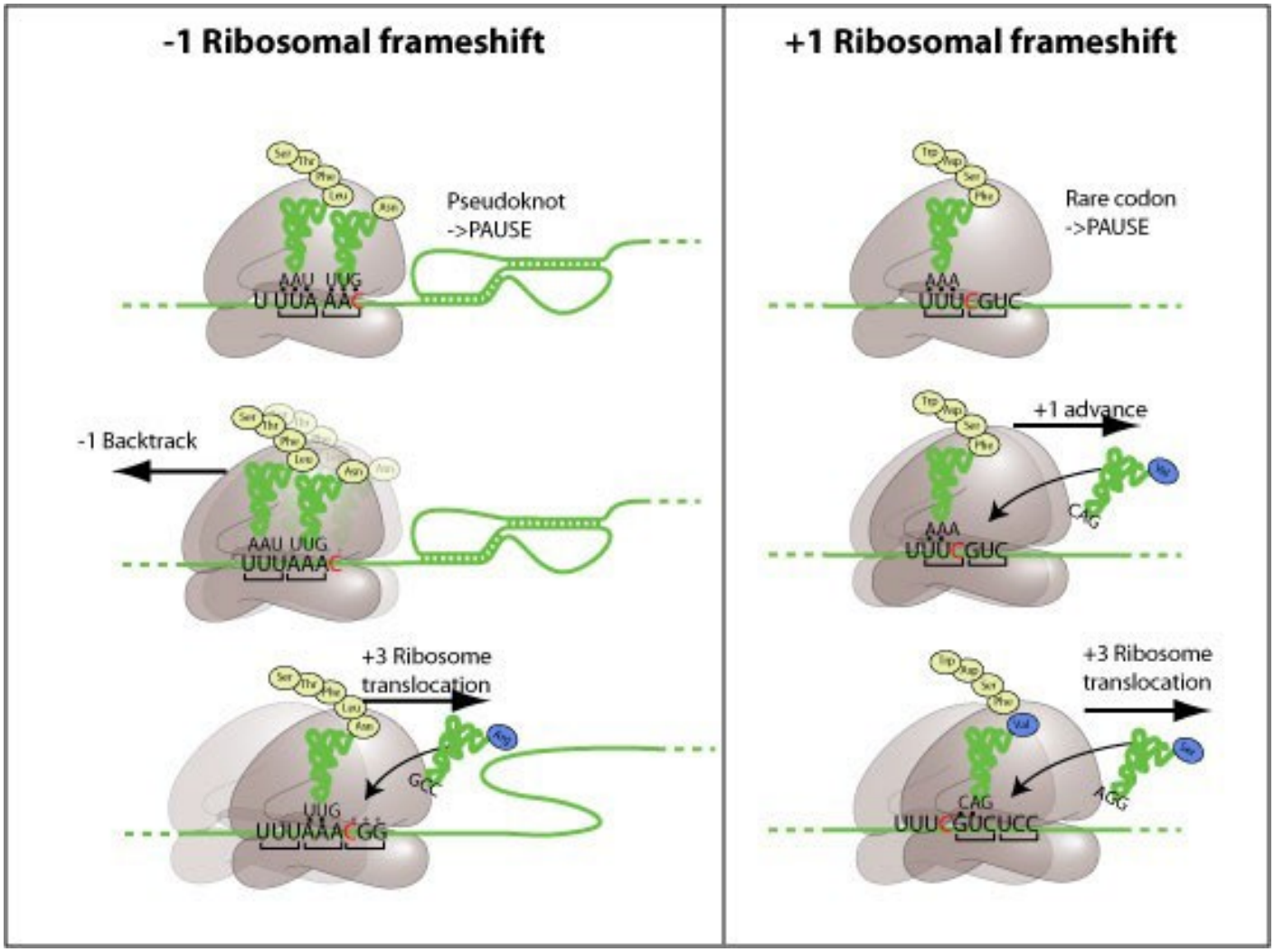


Figure 13-6. Ribosomal frameshifting is shown on the left side of this figure where the ribosome encounters a pseudoknot and slips back one nucleotide leading to a shift in the frame that allows the low-level reading of a second open reading frame. The right side shows a very infrequently used mechanism that we will not discuss in this text. Courtesy of ViralZone https://viralzone.expasy.org/860

Termination Suppression: Positive Sense RNA Only

Termination suppression is carried out by the positive sense RNA viruses in the families *Retroviridae* as well as sporadic viruses in other families such as *Togaviridae.* Again, viruses do this for two reasons: to increase the number of proteins they can make from a small genome while also controlling the amount of proteins produced. Normally, as the mRNA is translated, the termination will occur when a stop codon is reached. However, a small percentage of the time, a tRNA carrying an amino acid will bind to the stop codon adding another amino acid and allowing reading of a second open reading frame which is in frame with the first. Thus, when termination suppression occurs, this second open reading frame's proteins are made in much smaller amounts.

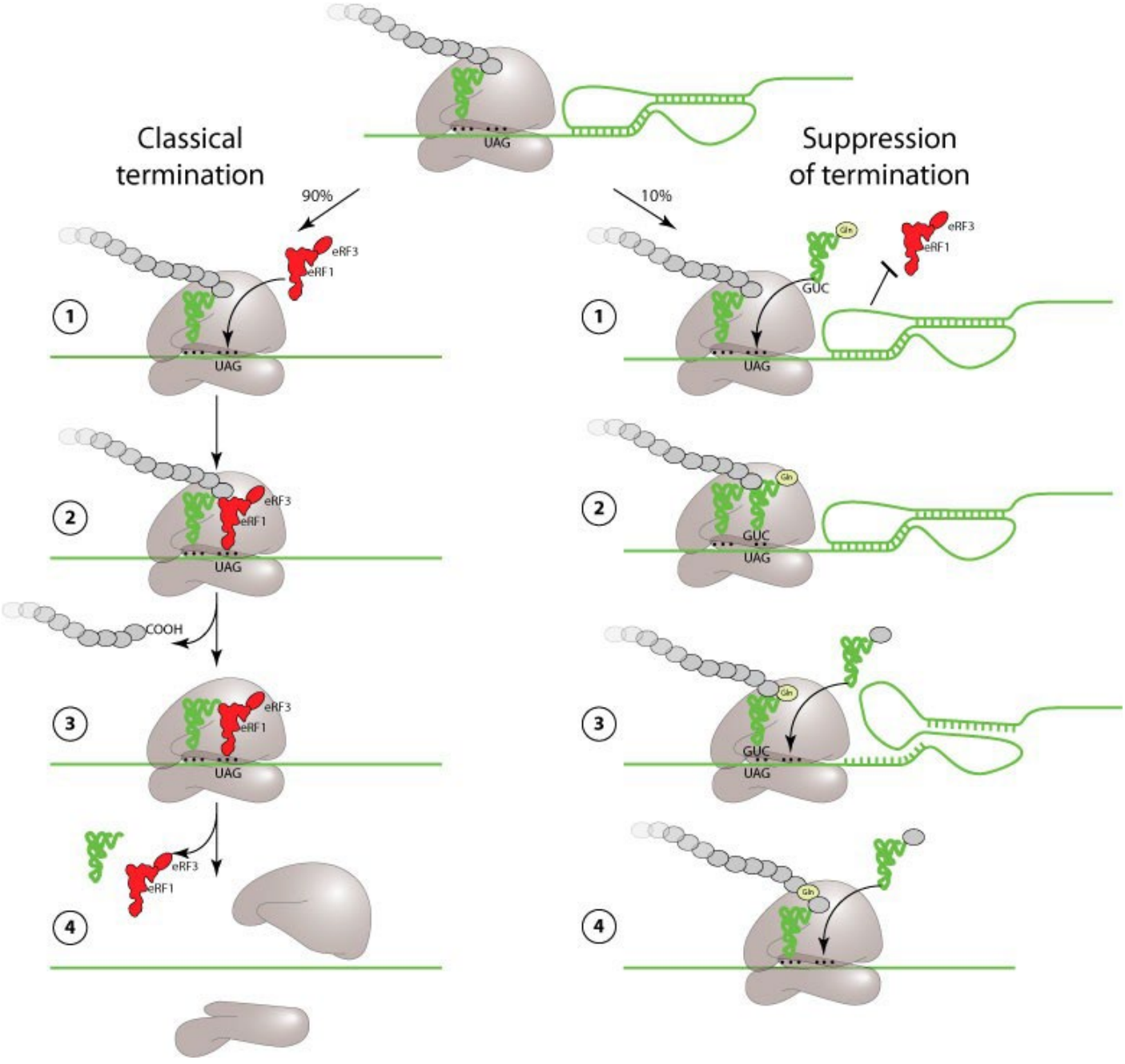


Figure 13-7. Translation termination suppression when a tRNA binds to the stop codon inserting an amino acid that allows read through to a second open reading frame as shown on the right. Courtesy of ViralZone https://viralzone.expasy.org/859

VIRAL mRNA ACQUISITION OF 5' CAP AND 3' POLY A TAIL

Recall that cellular mRNA has a 5' 7-methylguanosine cap on the 5' end of the mRNA as well as a long stretch of adenines called the poly A tail on the 3' end. The **5' 7-methylguanosine cap** (henceforth referred to as the 5' cap) is used for ribosome translation initiation. The 3' poly A tail (50-300 adenines) has multiple functions including: 1. required for mRNA export from nucleus, 2. involved in translation initiation, 3. determines half-life of mRNA (as the poly A tail gets shorter it signals the cell to degrade the mRNA), and 4. the poly-A binding proteins (PABP) bind poly-A tail and 5' cap during translation initiation.

Now recall that DNA viruses (except poxviruses) replicate in the nucleus, and as such have access to the machinery that adds the 5' cap and 3' poly A tail. However, RNA viruses tend to replicate in the cytoplasm and cannot access this machinery. So how do they get a 5' cap and 3' poly A tail?

We will start with acquisition of the poly A tail which is simpler to understand. The vast majority of RNA viruses acquire a poly A tail when they have a poly U sequence that is being read in the 5' end of the template RNA. This poly U sequence may create enough As, or when RdRp reads this poly U sequence, it can stutter and create a long poly A tail. However, flaviviruses lack a poly U sequence and therefore lack a poly A tail. Despite this, their mRNAs are still able to be recognized by **poly A binding proteins** (PABPs) because they form a secondary structure in the 3' end of their mRNAs that binds PABPs allowing the PABPs to interact with the ribosome initiation complex to start translation. Other RNA viruses, like reoviruses, and viruses in the order *Bunyavirales* that lack a poly A tails, have evolved PABP independent translation initiation mechanisms. Note: using a PABP independent mechanism allows these viruses to outcompete host cell translation be disabling PABP.

Now we will describe how RNA viruses can acquire a 5' cap. Retroviruses become DNA, integrate into the host genome, and are transcribed by host cell RNA polymerases II while the host cell capping machinery and poly A tail addition machinery modify the ends. Many viruses' RdRp can perform the capping function. However, some viruses' RdRp lacks the ability to add a 5' cap. The negative sense segmented RNA viruses (orthomyxoviruses, viruses in the order *Bunyavirales)* perform what is called **cap snatching**. The 5' cap is snatched from host cell mRNAs either in the nucleus (orthomyxoviruses) or cytoplasm (viruses in the order *Bunyavirales*). The cap and approximately 10 base pairs are used to prime transcription of the viral mRNA, as shown in Figure 13-8. Note: cap snatching also allows these viruses to compete with host cell mRNA for translation machinery as host cell mRNAs now lack the 5' cap necessary to initiate translation.

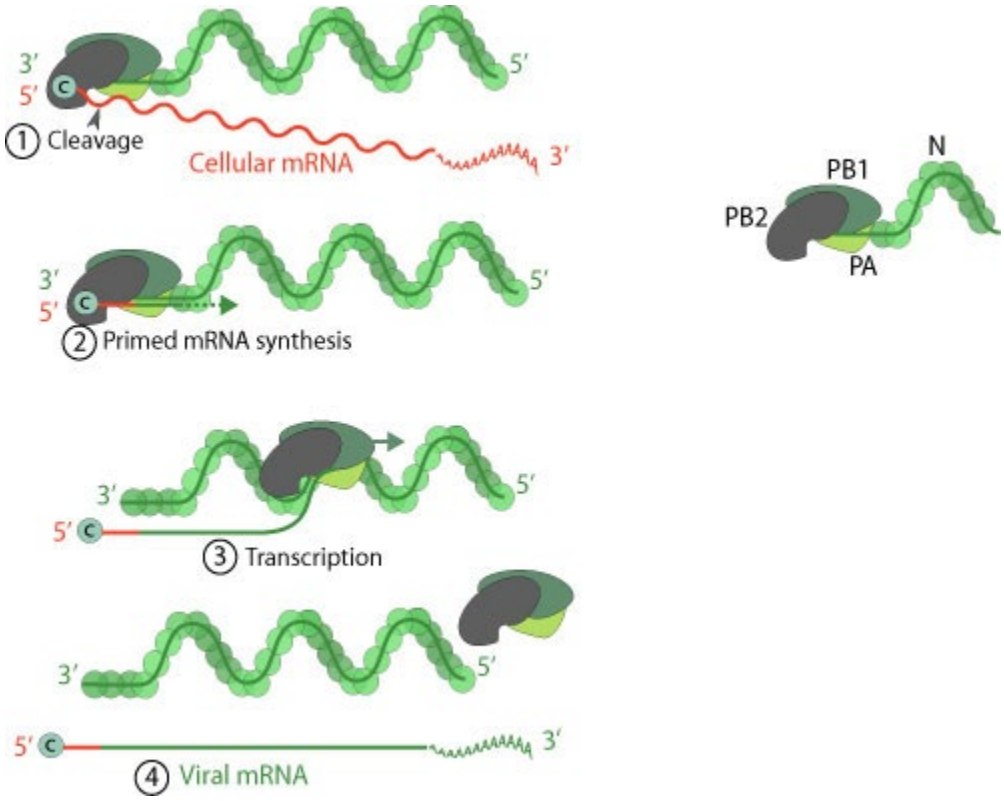


Figure 13-8. Cap snatching of host cell mRNA 5′ cap in either the cytoplasm or nucleus. The cap and approximately 10 base pairs are cleaved off random host cell mRNAs. These are used to prime transcription of viral mRNAs thus giving the viral mRNA a 5′ cap and depriving host cell mRNA of the cap necessary for translation and allowing the virus to out compete host cell mRNA for translation.
Courtesy of ViralZone https://viralzone.expasy.org/839

Some viruses have evolved a mechanism to initiate translation without the addition of the 5′ cap. In particular, picornaviruses prime their RNA replication with a 5′ terminal primer protein but are unable to have a 5′ cap added due to the terminal protein blocking the 5′ end. Although most flaviviruses' RdRp can add a 5′ cap, hepatitis C virus evolved to function without adding a 5′ cap.

We learned in Chapter 3 that translation initiation requires many initiation factors to bind the 5′ cap. In particular eIF4E binds to the cap and eIF4G binds to eIF4E and then other initiation factors. So then, how do viruses without a 5′ cap initiate translation? They can contain **internal ribosome entry sites (IRES)**. IRES are secondary structures that form within the 5′ end of the mRNA, as shown in Figure 13-9. However, eIF4G cannot bind the other initiation factors efficiently if eIF4E is not bound to the 5′ cap. Therefore, these viruses all produce a viral protease that cleaves the eIF4E binding site off eIF4G allowing it to bind to the IRES and the other initiation factors without the need for eIF4E biding to the 5′ cap, as shown in Figure 13-10. Note: cleaving eIF4G also allows the viruses to out compete the cellular mRNA for translation as cellular RNA requires the now cleaved eIF4G to bind eIF4E on the 5′ cap. You may be thinking, "if picornaviruses and the flavivirus hepatitis C must use a protease to cleave eIF4G, do they need to carry their protease in their capsid"? The answer is no. They do not need to package their protease. Although initiation occurs most efficiently with a 5′ cap present, it can still initiate at very low levels without it. Thus,

these viruses can produce small amounts of protease before cleavage of eIF4G is achieved. Furthermore, recall that each protein will perform its function over and over before it is degraded.

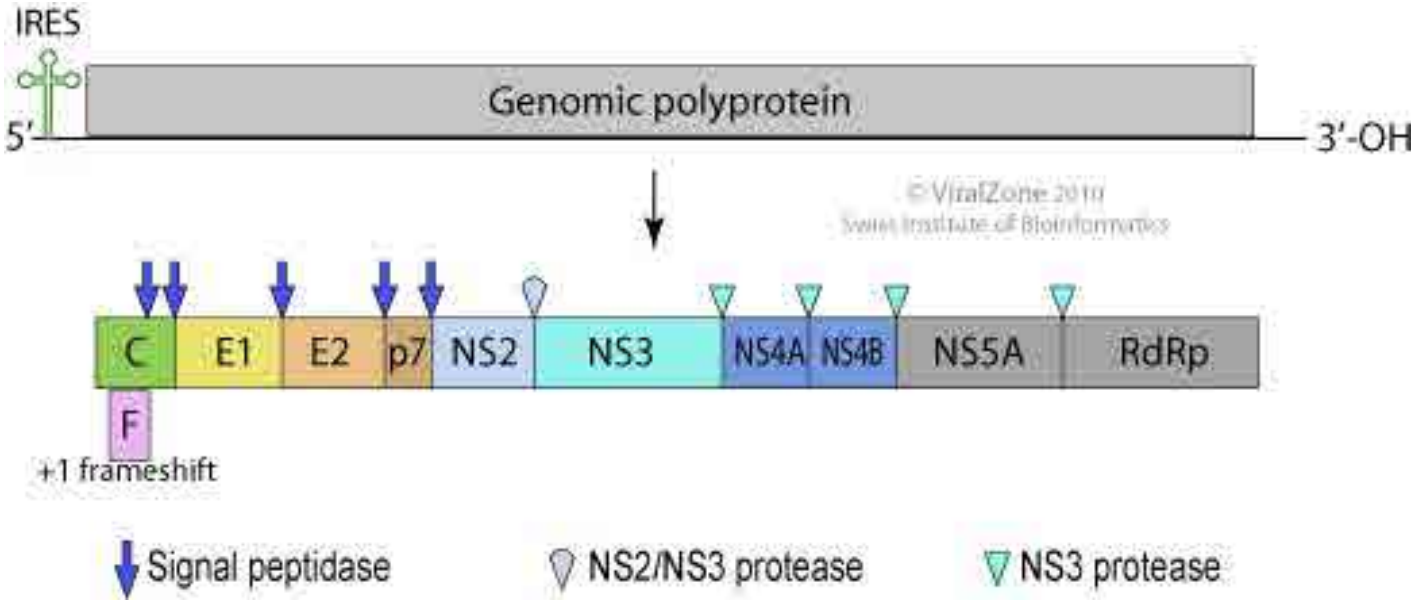


Figure 13.9. The flavivirus hepatitis C lacks a 5' cap and poly A tail which are replaced with secondary structures that allow the 5' end to bind initiation factors at the IRES, and the 3' end that allows poly A binding proteins to bind the secondary structure at the 3' end. Note: this image shows the lack of poly A tail but not the 3' secondary structure. Courtesy of ViralZone https://viralzone.expasy.org/37

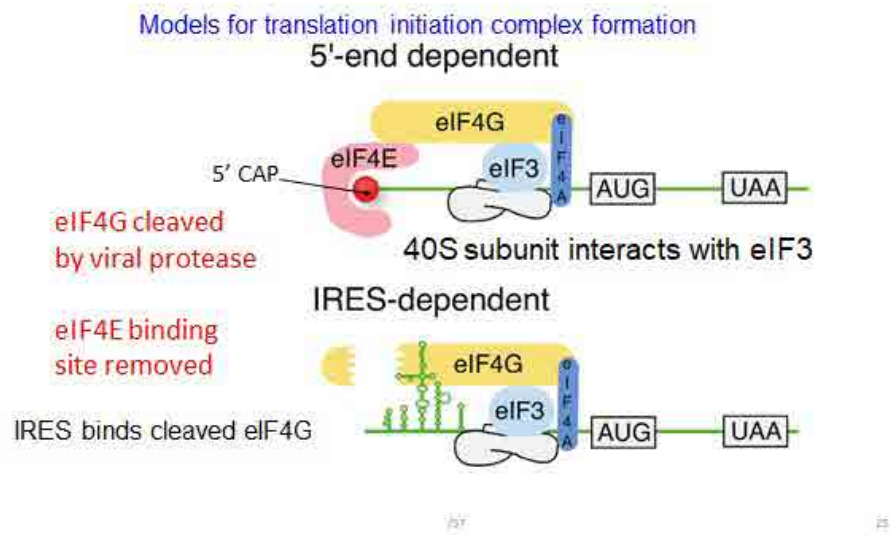


Figure 13-10. 5'-end dependent initiation (top) where eIF4E binds the 5' 7 methyl G cap (most viral mRNA) or the 5' viral capping protein VpG (caliciviruses). The internal Ribosome Entry Sites (IRES) and viral protease that cleaves the eIF4E binding site off eIF4G allowing it to bind to the IRES and the other initiation factors without the need for eIF4E binding to the 5' cap (bottom) as seen in picornaviruses and Hepatitis C virus. Courtesy of Sandra Quackenbush, Colorado State University.

The caliciviruses also have a 5’ protein that is used to initiate RNA replication, however, caliciviruses do not use an IRES but instead have evolved a mechanism for translation initiation where the calicivirus VPg protein serves the function the 5’ cap normally does by binding the cap-binding protein eIF4E to initiate translation. If you refer to figure 13-10 eIF4E would bind the VpG protein on the 5’ end of the positive sense genome rather than the 5’ cap.

VIRAL AND CELLULAR mRNA ARE IN COMPETITION FOR TRANSLATION MACHINERY

Now that we have shown how viral mRNA is able to make multiple proteins despite the limitation that eukaryotic host cells only want to make one protein per mRNA, let us examine how RNA viruses compete for host cell translation machinery. Again, there have been many mechanisms that have evolved, some specific to RNA or DNA viruses and some shared.

The mechanisms viruses use to compete with host cell mRNAs for translation machinery include:

- Inhibit cellular gene transcription
- Degrade host cell mRNAs
- Out compete host cell mRNA
- Inhibit function of initiation factors used for cellular mRNA translation
    - Cleavage: eIF4G, eIF2α
    - Dephosphorylation eIF2α
- Inhibit function of poly-A binding proteins used for cellular mRNA translation (PABP)
- Cap snatching removes 5’ 7-methylguanosine cap of cellular mRNA

Note: that all these mechanisms, except cap snatching and inhibiting the function of PABP, are also seen in DNA viruses.

RNA VIRUSES: WHAT STARTS RNA REPLICATION?

Note: all RNA viruses of animals have linear genomes and as such must have mechanisms to make sure the ends of their genome are replicated.

Most RNA viruses overcome this easily as RdRp starts synthesizing at the end of the genome without a primer; this is referred to as de novo synthesis. De novo initiation by RdRP occurs at the 1st nucleotide. However, a few virus families (*Picornaviridae* and *Caliciviridae*) have RdRps that lack the ability to perform de novo synthesis. These viruses use a mechanism we saw in the DNA viruses with adenoviruses. They use a protein primer that binds to the 5’ end and contains the amino acids serine, threonine, or tyrosine which have R groups containing an OH and as such provide a 3’ OH group on which RdRp can add nucleotides. Remember, retroviruses do not use an RdRp. Instead their mRNA genome is reverse transcribed to double stranded DNA using a

packaged tRNA that binds to the tRNA binding site and provides the 3’ OH used by reverse transcriptase to initiate DNA synthesis.

For almost all RNA viruses, these methods of replication ensure that the ends are replicated. The only exception is the family *Retroviridae* which uses terminal repeats and DNA jumping to replicate the ends. Please see Chapter 12 which describes retroviruses for a further explanation. Note: a similar method is also used by viruses in the family *Hepadnaviridae,* however, their reverse transcription occurs in the capsid, and as such hepadnaviruses are classified as DNA viruses.

MECHANISMS TO OVERCOME TYPE I INTERFERON AND RNAi ACTIVATED BY LONG STRETCHES OF DOUBLE STRANDED RNA (>125 BP)

All RNA viruses, except retroviruses, must contend with activation of RNAi and type I interferon responses because in order to replicate their genome, they must create double stranded RNA. One mechanism most RNA viruses use is by not keeping the two strands of RNA together during replication or transcription. Although this has the benefit of not having long stretches of double stranded RNA, it comes at a cost. Error repair is performed when polymerases look for abnormalities in the double stranded nucleic acids caused by miss-matched base pairs (for example G base pairing with A). Because the two strands are kept together as briefly as possible, it is not possible to **proofread** the newly synthesized RNA molecules which leads to very high mutation rates. As a result, RNA viruses generally have very small genomes compared to DNA viruses. However, some RNA viruses such as coronavirus the RdRp dose have a proofreading function, and as such the genomes can get longer than most RNA viruses. Note that although this helps avoid RNAi and interferon production, complementary strands of RNA that are made can still find each other and hybridize. Some RNA viruses sequester their replication in vesicles, endosomes, lysosomes, **inclusion bodies** (viral factories), or inside their virion to avoid detection by PRRs. The following are the mechanisms RNA viruses have used to shut down the antiviral responses of type I interferons once activated.

RNA viruses can inhibit the pathways for production of IFN

- Inhibit the cascade responses that are activated when pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRR)

They can inhibit the pathways activated by IFN

- Inhibit IFN transcriptional activations
- Secreted IFN receptor mimicking protein expression
    - Binds up interferon so it cannot bind the actual receptor protein
- Inhibit IFN activation of JAK phosphorylation of STAT

- Inhibits STAT's dimerization and transcription activation

They can inhibit the antiviral responses induced by IFN

- Inhibit protein kinase R (PKR) inactivation of translation
  - Interferon activates PKR expression
  - Phosphorylates translation initiation factor (eIF2α)
  - decreases translation of cellular mRNAs
  - Inhibition of PKR leads to increased translation of viral proteins
- Produce dsRNA-like analogs
  - PKR activated by presence of dsRNA
  - The analogs bind and inactivate PKR
  - OAS is activated by presence of dsRNA
  - The analogs bind and inactivate OAS
    - OAS activates RNAse L which digests viral and cellular RNA
    - Reduced OAS function leads to reduced cleavage of mRNA

END OF CHAPTER QUESTIONS

1. Do RNA viruses utilize RdRp? Why, or why not? Is this enzyme provided by the cell or the virus?
2. Do RNA viruses utilize DdRp? Why, or why not? Is this enzyme provided by the cell or the virus?
3. Do RNA viruses utilize DdDp? Why, or why not? Is this enzyme provided by the cell or the virus?
4. What RNA polymerase is MOST LIKELY to transcribe viral mRNA in RNA viruses? Why is this enzyme the most likely one?
5. What is the function of an mRNA pseudoknot? Why would viruses want to have them?
6. What is a subgenomic mRNA?
7. Do MOST RNA viruses utilize more than one promoter within their genome to produce subgenomic mRNAs? If they do, how do they do so? If they do not, why not?
8. For RNA viruses that utilize more than one promoter, where is this promoter generally found?
9. How do MOST RNA viruses acquire the 5' cap addition and poly A tail addition machinery?
10. Are there any RNA viruses that you would predict can carry out reassortment? Justify your answer.

11. Are there any RNA viruses that you would predict can carry out recombination? Justify your answer.
12. If you performed an IFA assay on cells infected with most RNA viruses, where would you expect to see staining, and why? Are there any outliers? If so, who and what would you expect to see?
13. Do all RNA viruses splice their mRNA? If so, why do they do so? If not, why not? If only some do so, which ones do, and which ones do not?
14. What is RNA editing and why would RNA viruses utilize it?
15. What is termination suppression, and why would RNA viruses utilize it?
16. Why do some viruses have genes to produce a viral protease? Are these proteins equally likely to be found in positive and negative sensed RNA viruses?
17. How does leaky scanning work?
18. What are overlapping open reading frames (ORF)? What does having overlapping ORF accomplish for viruses that have them?
19. Some mechanisms to compete with host cell mRNA for translation are unique to RNA viruses. Which mechanisms are these, and why don't DNA viruses use these mechanisms?
20. Compare and contrast subgenomic mRNA production from the antigenome and ambisence genome production of subgenomic mRNA.
21. Linear RNA viruses must have a mechanism to ensure that the ends of the genome are reproduced. Describe at least 3 different mechanisms that different viruses have evolved for this purpose.
22. Do RNA viruses require a host cell that is in the S phase? If so, why? If not, why not?
23. What activates RNAi?
24. How do RNA viruses avoid activating RNAi?
25. What is the most common activator of type I interferon production?
26. What are some mechanisms used by RNA viruses to avoid activating type I interferon production?
27. Which PRR are most likely activated by RNA viruses? To what PAMPs are they responding?
28. Are there any RNA viruses that can perform reassortment? If so what types of genomes do they have?
29. What does it mean to have an ambisense genome? What might be an advantage of having an ambisense genome?
30. What are the advantages of having a segmented genome?
31. What type of genome do the viruses that use the start stop mechanism to produce subgenomic mRNAs possess?
32. What are nested subgenomic mRNAs and how are they produced?
33. What is the difference between creating subgenomic mRNAs and expressing multiple open reading frames?

34. What is an IRES? What might be an advantage of having one?
35. How do MOST RNA viruses initiate genome replication and ensure that the ends are replicated?
36. What mechanism do RNA viruses that cannot utilize this mechanism use to initiate genome replication and ensure that the ends are replicated?

REFERENCES:

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapter 5.

Goodfellow, I. et al. (2005). Calicivirus translation initiation requires an interaction between VPg and eIF4E. EMBO Rep. Oct; 6(10): 968–972. https://pubmed.ncbi.nlm.nih.gov/16142217/

ViralZone. Alphavirus. https://viralzone.expasy.org/625

ViralZone. Cap snatching. https://viralzone.expasy.org/839

ViralZone. Coronaviridae. https://viralzone.expasy.org/764

ViralZone. Hepacivirus. https://viralzone.expasy.org/37

ViralZone. Rhabdoviridae. https://viralzone.expasy.org/2

ViralZone. Ribosomal frameshifting. https://viralzone.expasy.org/860

ViralZone. RNA editing. https://viralzone.expasy.org/857

ViralZone. RNA suppression of termination. https://viralzone.expasy.org/859

REVIEWED BY:

Tony Schountz, Department of Microbiology, Immunology and pathology, Colorado State University, Fort Collins, CO

CHAPTER 14

Chapter 14: Positive Sense RNA Viruses With One Open Reading Frame That Use A Viral Protease And Replicate In The Cytoplasm: Flaviviridae and Picornaviridae

INTRODUCTION TO THE FAMILIES *FLAVIVIRIDAE* AND *PICORNAVIRIDAE*

You will notice in Figure 14-1 that flaviviruses and picornaviruses are positive sense stranded RNA viruses that are icosahedral and replicate in the cytoplasm. While picornaviruses are naked, flaviviruses are enveloped. You will further note that there are no segmented positive sense RNA viruses, and all RNA viruses contain a linear genome, and, as such, they both contain only one piece of linear RNA. The virion RNA is infectious (as it is for all positive sense RNA viruses except retroviruses) and serves as both genome and viral messenger RNA. Like all RNA viruses (except retroviruses), these viruses have genes to produce an **RNA-dependent RNA polymerase** (RdRp) that will replicate the virus. However, as these viruses' genomes are equivalent to mRNA, the virus genome can be translated immediately upon entering the cell. These viruses do not package their RdRp in the virion as they can create RdRp upon entrance into the cell and then utilize the RdRp to create more viral mRNA after translation is completed.

Flaviviridae and *Picornaviridae* are the only two families of positive sense RNA viruses that contain one open reading frame (ORF) that creates only one mRNA and one large polyprotein that is cleaved into individual proteins by a virally encoded protease. The other 4 families of positive sense RNA viruses (*Coronaviridae*, *Caliciviridae*, *Matonaviridae* and *Togaviridae*) all have more than one mRNA and open frame and, as a result, create more than one polyprotein that will be cleaved to individual proteins by a virally encoded protease. As a result, this chapter will focus on the 2 positive sense RNA viruses of animals that contain only one open reading frame. We will begin with *Picornaviridae*.

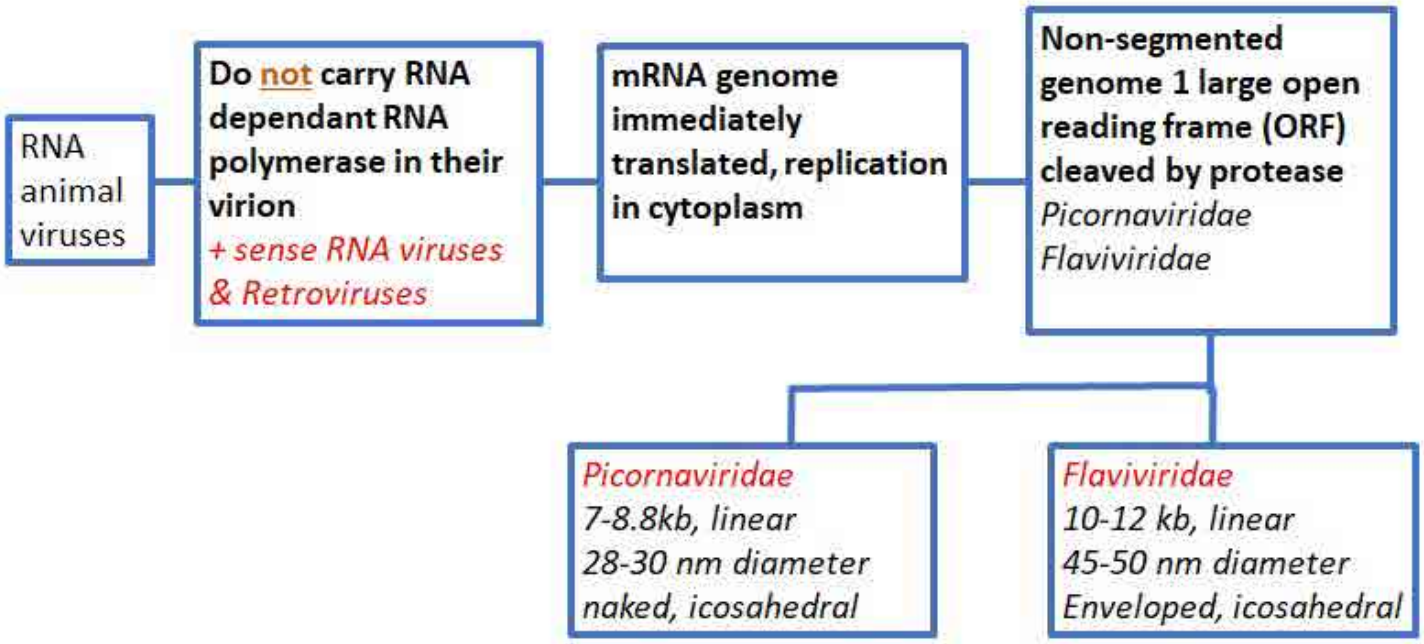


Figure 14-1. Taxonomy of positive sense RNA viruses focusing on picornaviruses and flaviviruses. Courtesy of Erica Suchman, Colorado State University.

INTRODUCTION TO THE FAMILY *PICORNAVIRIDAE*

Picornaviruses include many medically important viruses in many different genera including poliovirus and human rhinovirus A which are in the genus *Enterovirus*, hepatitis A which is in the genus *Hepatovirus*, encephalomyocarditis virus which is in the genus *Cardiovirus*, and hand foot and mouth disease which is in the genus *Aphthovirus*. Picornaviruses have a linear positive sense RNA genome of 7-8.8 kb that is packaged into a naked icosahedral capsid of 28-30 nm in diameter, as shown in figure 14-2 A & B.

GENOME STRUCTURE

The genome, and mRNAs, lack a 5' cap but they do have a 3' poly A tail. The presence of the covalently attached 5' VPg protein that is used to primer RdRp synthesis of viral proteins prohibits the addition of a 5' cap.

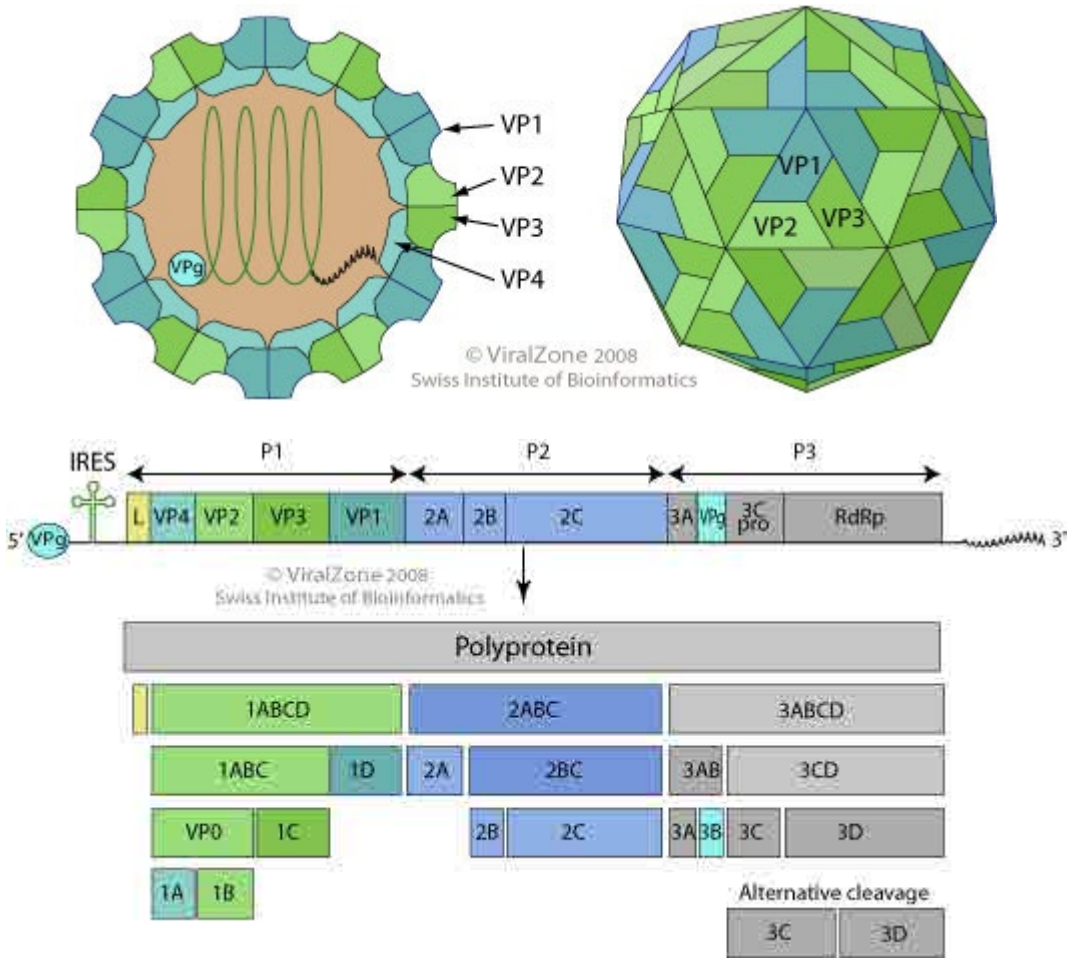


Figure 14-2A. A typical picornavirus virion. Figure 14-2B. A typical picornavirus genome with a 5' VPg protein and 3' poly A tail. Courtesy of ViralZone https://viralzone.expasy.org/33

VIRAL REPLICATION CYCLE

As shown in Figure 14-3, the picornavirus replication cycle begins with

1. Attachment of the virus to a variety of host receptors mediates endocytosis of the virus into the host cell.
2. The capsid undergoes a conformational change and releases the capsid protein VP4. The VP4 opens a pore in the host's endosomal membrane, and the viral genomic RNA penetrates into the host cell cytoplasm.
1. VPg is removed from the viral mRNA which is then translated into a polyprotein. The polyprotein is cleaved into all structural and non-structural proteins (to yield the replication proteins) by the virally encoded protease by autocatalysis.
3. In enteroviruses, rhinoviruses, and aphthoviruses, shutoff of cellular cap-dependent translation through the cleavage of translation initiation factor (eIF4G) by viral protease occurs.
4. Replication occurs in viral factories made of membrane vesicles derived from the ER or Golgi. A dsRNA genome is synthesized from the genomic positive sense RNA using RNA-dependent RNA polymerase (RdRp).
5. The RNA genome is transcribed/replicated thereby providing viral mRNAs/new positive sense RNA genomes by RdRp.
6. New genomic RNA is believed to be packaged into pre-assembled procapsids.
7. Cell lysis and virus release occurs when the cell undergoes apoptosis.
8. Maturation of virions occurs by an unknown host protease.

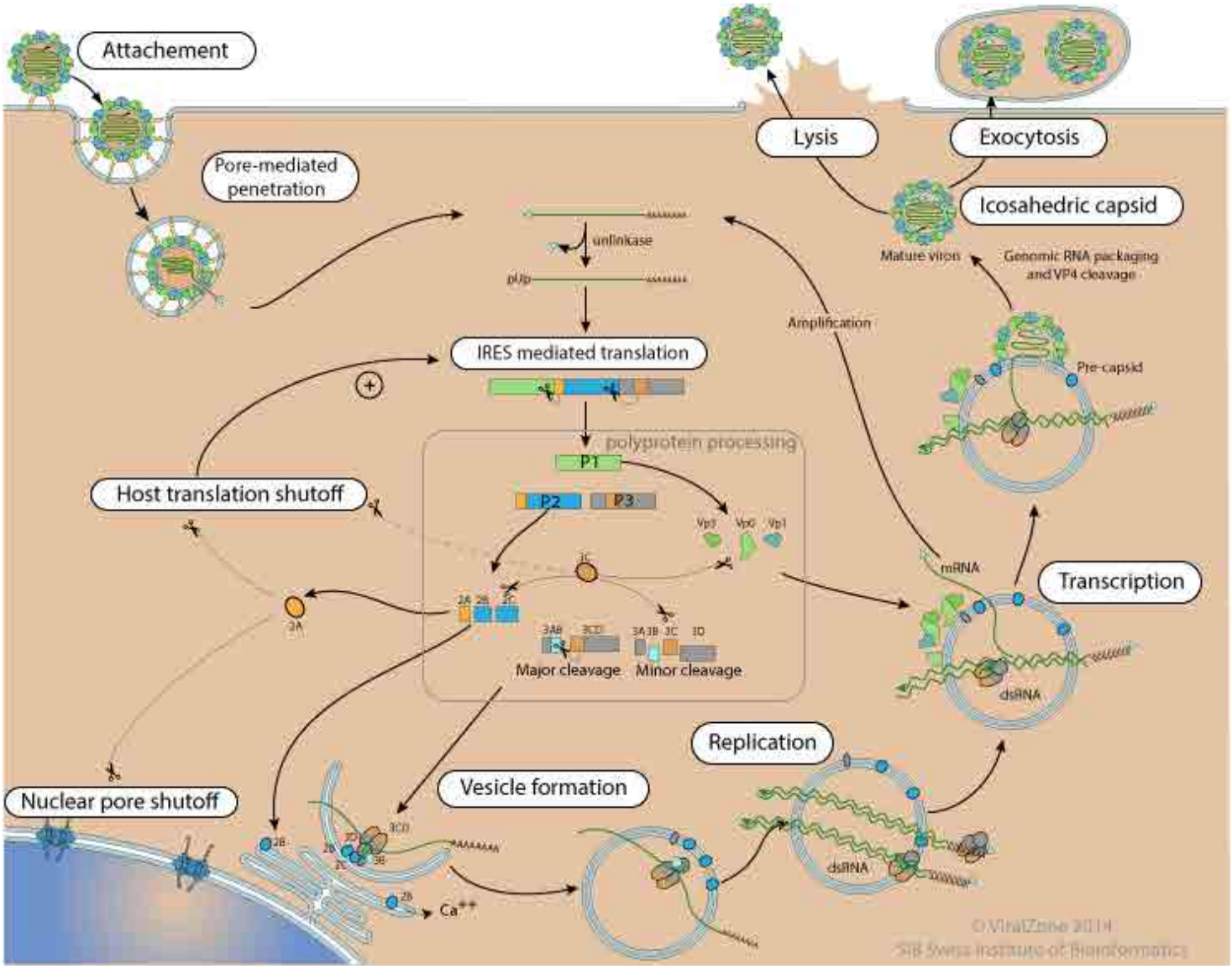


Figure 14-3. Picornavirus replication cycle. Courtesy of ViralZone https://viralzone.expasy.org/3276

HOW DO PICORNAVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS AND IT REPLICATES IN THE CYTOPLASM?

Most RNA viruses' RdRp have a poly U tract in the 5' end of the template (negative sense) strand of RNA. This will result in the addition of a long poly A tail when the RdRp stutters adding far more A's than exist in the tract. However, picornaviruses' RdRp lacks the ability to perform de novo RNA synthesis and requires a primer to initiate replication and transcription. To prime, picornaviruses use a protein called VPg that provides the 3' OH on which RdRp can begin adding NTPs. As such, there is a protein covalently attached to the 5' end of the mRNA, and the 5' end cannot accommodate a 5' cap which necessitates the use of an **internal ribosome binding site (IRES)**. The RdRp does, however, add a poly A tail.

HOW DO PICORNAVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all cytoplasmic replicating positive sense RNA viruses of animals, picornaviruses utilize a viral protease to cleave up a large viral polyprotein, as shown in Figure 14-2. As discussed above, picornaviruses and flaviviruses only create one large polypeptide that is cleaved to form all of the proteins necessary for replication.

TRANSLATION OF VIRAL PROTEINS

We learned in chapters 3 and 13 that translation initiation requires a number of initiation factors to bind the 5' cap. In particular, eIF4E binds to the cap and eIF4G binds to eIF4E and then other initiation factors. So then, how do viruses without a 5' cap initiate translation? They contain Internal Ribosome Entry Sites (IRES). IRES are secondary structures that form within the 5' end of the mRNA due to complementary sequences. However, eIF4G cannot bind the other initiation factors efficiently if eIF4E is not bound to the 5' cap. Therefore, these viruses produce a viral protease that cleaves the eIF4E binding site off eIF4G thus allowing the cleaved eIF4G to bind to the IRES and the other initiation factors without the need for eIF4E biding to the 5' cap. You might be wondering, "do picornaviruses need to carry the protease in their virion to cleave eIF4G to initiate translation of the mRNA genome?" They do not. Translation can begin at low levels without the cleavage thus allowing the protease to be translated leading to increased translation.

Note that cleaving eIF4G also allows the viruses to out compete the cellular mRNA for translation as it requires the now cleaved eIF4G to bind eIF4E on the 5' cap. See Chapters 3 and 13 for Figures and a more robust description. Using poliovirus as an example, inhibition of host cell translation begins about 1 hour after infection. Increased overall translation begins at 3 hours after infection primarily due to synthesis of viral proteins. Host cell translation is markedly inhibited by 5 hours after infection.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome.

Note: picornaviruses like many viruses take care of the problem of replicating the ends and priming replication via the same mechanism.

RNA viruses, with the exception of retroviruses, cannot use the host cell's transcription machinery **DNA-dependent RNA polymerase** (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As such, with the exception of retroviruses, they must all have genes to produce an **RNA-dependent RNA polymerase** (RdRp). Positive sense RNA viruses, whose genomes are equivalent to mRNA, can be translated immediately upon entrance into the cell and do not need to carry RdRp into the host cell with the genome. They must, however, produce it immediately in order to begin replicating. Note: all RNA viruses of animals have linear genomes and therefore must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses including picornaviruses overcome this easily as RdRp starts synthesizing at the end of the genome without a

primer. Beginning replication without the use of a primer is referred to as de novo synthesis. De novo initiation by RdRP occurs at the 1st nucleotide. For mostRNA viruses this method of replication ensures that the ends are replicated. However, as discussed above, picornaviruses' RdRp lacks the ability to perform de novo RNA synthesis and requires a primer to initiate replication and transcription. To prime it uses a protein called VPg that binds the 5' end and provides the 3' OH on which RdRp can begin adding NTPs. VPg both serves as the primer and ensures that the ends of the genome are replicated.

VIRION FORMATION

Viral capsids form spontaneously in the viral factory. The virus leaves the cell when it undergoes apoptosis, lysing the cell, and allowing the virus to leave.

AVOIDING THE HOST IMMUNE RESPONSE

Picornaviruses avoid detection of pathogen recognition receptor (PRR) TLR 7, which recognizes the pathogen associated molecular pattern (PAMP) of single stranded endosomal RNA, by remaining in their capsids until they leave the endosome. They avoid the PRRs RIG-I (cytoplasmic RNA lacking a 5' cap), MDA-5 (cytoplasmic dsRNA, and improper cap), and RNAi (ds RNA) by replicating within viral factories. Furthermore, recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication to avoid activating the interferon response and possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi), as well as the interferon stimulated genes (ISG) dsRNA-dependent PKR, dsRNA-dependent OAS and the PRR MDA-5. As such, RdRp is unable to proofread as it replicates the genome which leads to a high mutation rate allowing antigens to change rapidly. Some picornaviruses also produce accessory proteins that help inactivate the type I interferon response.

PICORNAVIRAL DISEASES

HEPATITIS A (HAV)

Note that hepatitis A is not in the same virus family as Hepatitis B, C, or E. All of the hepatitis viruses cause liver damage and jaundice and similar general symptoms, so they are all named hepatitis. Transmission of HAV is fecal-oral as large amounts of virus (up to 10^9 infectious virions per gram of stool) are shed in the feces of infected individuals. The source is fecal contamination of food, water, or eating raw filter-feeding shellfish. HAV is stable at low pH and is resistant to heat, detergents, and organic solvents. There is only one serotype. The tissue tropism is for the liver which serves as the primary site of replication for the virus. Figure 14-4 shows the typical clinical course of events in HAV infection. The virus has an incubation period of about 2 ½ weeks followed by the **prodromal** period where the person is beginning to feel ill but has not yet developed full blown symptoms. During the incubation period, at about the 2nd week, virus can be isolated from the blood and feces in large quantities, and transmission can occur despite the fact that symptoms may not develop for 2 more weeks. During the **prodrome,** before symptoms develop, the person will have elevated **transaminase enzyme** in blood samples, which is a marker for liver damage. At about 4 weeks IgM is found in the serum but is rapidly replaced by IgG by week 6-7. This IgG will provide lifelong immunity as there is only one serotype of HAV. As IgM accumulates the person enters the **icteric** or symptomatic phase and develops symptoms including fever, malaise, muscle pain (**myalgia**), nausea, vomiting, and **jaundice** (yellow tinge to skin and whites of the eyes) due to

liver damage. As the IgM and IgG titers increase, virus particles in the blood and feces diminish and only viral RNA can be detected. Infected individuals can transmit the virus beginning in the incubation period and throughout the symptomatic phase, but the antibody prohibits transmission as the person enters the **convalescent** (recovery) phase. Interestingly, HAV does not cause damage to the hepatocytes directly as it is non-cytopathic. However, the liver damage is immune mediated by $CD8^+$ CTLs. $CD4^+$ T-helper cells produce antiviral cytokines that are involved in control of the virus replication.

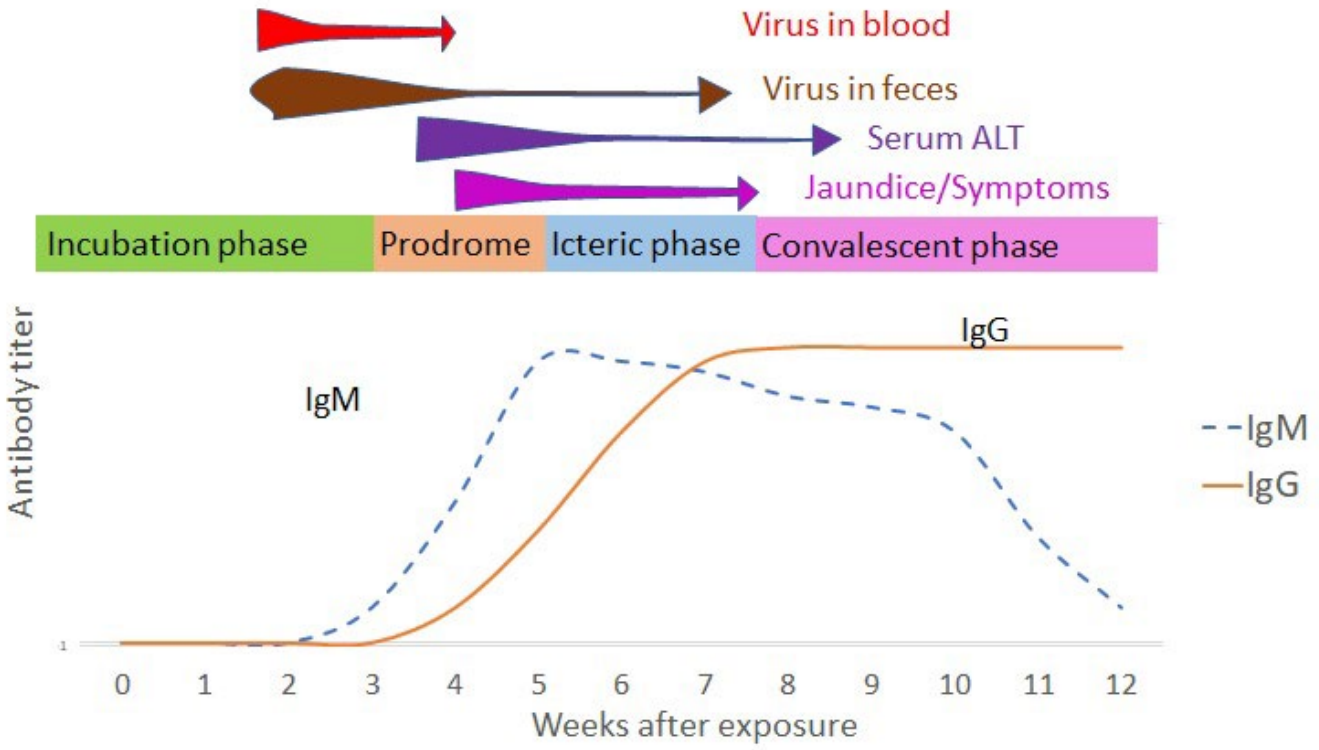


Figure 14- 4. Course of infection in HAV infection. Courtesy of Erica Suchman, Colorado State University.

Note that HAV evokes a minimal type I interferon response in the liver. It does so by trying to avoid detection by PRRs by not leaving the capsid in the endosome and replicating in viral factories, but the virus also produces accessory proteins that inactivate the cascade pathways of PRRs that detect double stranded RNA.

ENTEROVIRUSES

The e*nteroviruses* are an important genus within *Picornaviridae*. They include many medically important viruses including poliovirus, coxsackievirus, echovirus, human rhinovirus A and enterovirus.

Polio

Although polio has been eradicated in the US, cases still occur in the developing world. In the 1930s, 80-90% of adults were seropositive for polio due to naturally occurring infections (the vaccine did not exist yet), and approximately 35,000 people a year developed paralytic polio. Infants were naturally exposed during early childhood while they were protected by maternal antibodies resulting in a mostly asymptomatic disease or mild febrile illness with or without gastrointestinal signs. The development of flush toilets, sewers, and improved

hygiene in the early 20th century reduced transmission to infants but created an increase in the susceptible population which led to increased cases of paralytic disease. The virus has a fairly high reproductive rate (R0) with one infected person infecting 5-7 other people. This is much higher than influenza which has an R0 of 1.3-2 depending upon the year. If you consider that on average 9-45 million Americans contract influenza, you can imagine the number of people who would develop polio before the advent of vaccination. Transmission is predominantly the fecal-oral route as infected individuals have large amounts of the virus in their feces, and people were not as diligent as necessary about hand washing after using the restroom. Replication occurs in the lymphoid tissue in tonsils and Peyer's patches as well as the intestinal epithelium. Virus then replicates in draining lymph nodes which leads to viremia and dissemination to target organs in the central nervous system (CNS). One of the target organs includes the anterior horn cells of the spinal cord where the virus replicates causing **myelitis** which is inflammation of the gray (**polio**) matter. The myelitis can result in paralysis of certain motor nerves in the limbs in approximately 1 % of infected individuals. Note that although the percentage of infected people who get limb paralysis is low, the virus is highly contagious, and before development of the vaccine, most individuals contracted this infection, so numbers of paralyzed individuals was very high.

Coxsackievirus Group A and B

Coxsackievirus was first isolated from feces of paralyzed children during a poliomyelitis outbreak in Coxsackie, NY. Coxsackievirus group A (CVA) causes hand foot and mouth disease. The symptoms include acute fever, rash, and blisters which occur on the soles of the feet, palms, and in the mouth, and can progress to viral meningitis and encephalitis. It is transmitted by the fecal-oral route and through contact with nasal and throat secretions.

Coxsackievirus group B (CVB3) is transmitted by the fecal-oral route and most frequently causes a self-limiting and sub-clinical infection which can become persistent and induce inflammation that can lead to cardiomyopathy causing impaired heart function and mortality. The peak age group is 20-39, and there is a higher prevalence in men. Virus infection of cardiomyocytes (heart muscle cells) and infiltration of macrophages, CD8+ CTL cells and NK cells can be observed upon histopathology.

Enterovirus D68

Enterovirus D68 was first identified in California in 1962 and is transmitted by respiratory secretions. It causes a mild to severe respiratory illness which includes fever, runny nose, sneezing, cough, wheezing, and difficulty breathing. Infants, children, and teenagers are most likely to be infected and become ill. A nationwide outbreak occurred in the US in 2014 with severe respiratory illness in children. 998 individuals were confirmed to have infections in 47 states with respiratory illness caused by EV D68 and causing 8 deaths. Only a few cases since the 2014 outbreak have been reported, and the cause of the sudden occurrence and disappearance of this disease are unexplained.

Rhinovirus

Rhinoviruses are one of the causes (approximately 50%) of rhinitis, otherwise known as the common cold. The virus is well adapted to grow at 33^{O}C, which is the temperature of nasal passages which run cooler than the rest of the body which is approximately 37^{O}C. Recent studies suggest that the virus can replicate in lower airways where temperature is 35°C. The replication is greatest in upper airways but in some individuals may replicate in medium and larger airways. There are more than 108 serotypes of rhinoviruses. Unfortunately, there is no immunologic

cross protection. That is the reason we can get the common cold repeatedly. Transmission is via aerosol and direct contact with mucus.

PREVENTION OF PICORNAVIRUS INFECTIONS

For hepatitis A, prevention involves ensuring adequate sanitation, sewage and water treatment, enforcing standards of hygiene in food preparation, and hepatitis A immunization (inactivated whole virus vaccines) to high risk cohorts. Hepatitis A vaccines are recommended for children 12-23 months of age or for unvaccinated individuals traveling to areas that have high rates of hepatitis A.

For polio there are two vaccines. Dr. Jonas Salk developed a formalin-inactivated poliovirus vaccine (IPV) licensed in 1954, and Dr. Albert Sabin developed a live attenuated poliovirus vaccine licensed 1961-62 (OPV). It is estimated that over 80% of individuals must be vaccinated to achieve the **threshold vaccination level** for a population to develop herd immunity to polio (where non-vaccinated individuals are less likely to become infected). Note that the threshold vaccination level is different for every infection.

The vaccines must be serotype specific, and there are three serotypes; acquired immunity is lifelong. Mucosal immunity (IgA) prevents infection and reduces replication in the GI tract; systemic immunity (IgG) neutralizes virus present in blood and prevents infection of the CNS. Both versions of the vaccine lead to high levels of IgG, but only the live attenuated vaccine induces significant nasal and duodenal IgA that is necessary to inhibit transmission and, as such, creates a more robust immunity and decreases transmission, as shown in Figure 14-5. In the US in the 1960s, it was the original public health policy to vaccinate with the live attenuated vaccine which offered better immunity but had the risk of the attenuated vaccine mutating and becoming pathogenic again, or of symptomatic infections occurring in immunosuppressed individuals. However, as cases in the US became less prevalent in the 1990s, the recommendation changed to 2 doses IPV then 2 doses of OPV thus providing the increased intestinal immunity of the OPV vaccine and less risk as IPV initiated a strong IgG response before administering the live vaccine. By 1999 the recommendation changed again and now calls for 4 doses of IPV only as the risk of contact was much lower, and the risk associated with the attenuated vaccine was no longer warranted. The US had not seen a case of polio for many years, including traveler polio, due to better vaccination rates among travelers.

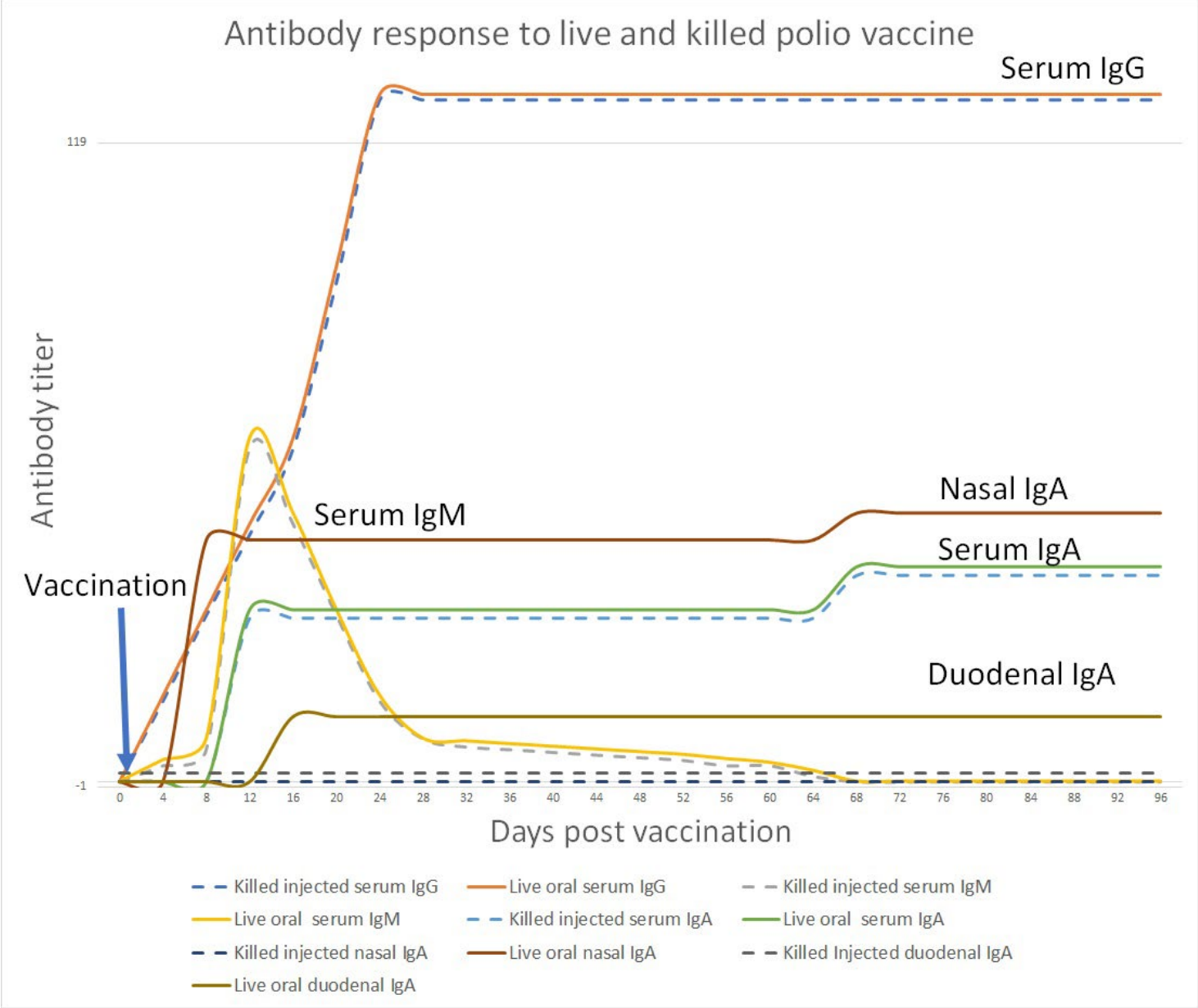


Figure 14-5. Example antibody responses following the inactivated IPV vaccine and attenuated oral OPV vaccine showing similar IgG response but OPV (live attenuated oral) only activating a significant mucosal IgA response. Erica Suchman, Colorado State University.

DIAGNOSIS OF PICORNAVIRUS INFECTIONS

HEPATITIS A

Hepatitis A can be diagnosed with either a positive IgM & IgG serology test or nucleic acid amplification tests (NAAT) using reverse transcriptase PCR to detect viral genome or mRNA. If you test positive to IgG but not IgM it is a past infection or due to vaccination, if IgG and IgM are positive it is a current infection.

POLIO

Virus isolation from stool samples and grown in cell culture is the most sensitive method to diagnose poliovirus infection. To increase the probability of isolating poliovirus, at least two stool specimens 24 hours apart are collected from patients. These should be collected within 14 days after onset of symptoms to maximize likelihood

of isolation. Real-time reverse transcription PCR is used to differentiate possible wild strains from vaccine-like strains called **intratypic differentiation** using virus isolated in culture as the starting material. Partial genome sequencing is used to confirm the poliovirus genotype and determine its likely geographic origin. Serology may support the diagnosis of paralytic poliomyelitis particularly if a patient is known or suspected to not be vaccinated. An acute serum specimen should be obtained as early in the course of disease as possible, and a convalescent specimen should be obtained at least 3 weeks later. A 4-fold increase in IgG titer must be observed for a current or recent infection.

COXSACKIE

Symptoms of childhood coxsackievirus A infections are usually sufficient for diagnosis of these infections. Coxsackievirus B infections are definitively diagnosed by viral isolation from stool samples taken early in the course of disease. Serology can be difficult to interpret but can be achieved if a greater than 4-fold increase in IgG titer is observed between acute and convalescent samples. PCR is also available, although the sensitivity can be quite variable ranging from 66-90%.

RHINOVIRUS

Rhinovirus infections are usually mild and self-limiting and do not require laboratory diagnosis.

TREATMENT OF PICORNAVIRUS INFECTIONS

There are not many successful treatments for picornavirus infections. As a result, prevention is the best method of protection. If someone develops polio and develops paralysis of the rib muscles they may require ventilation to aid with breathing. For all other picornavirus infections treatments include supportive care, but there are no antiviral treatments currently.

INTRODUCTION TO THE FAMILY *FLAVIVIRIDAE*

As discussed above, *Flaviviridae* and *Picornaviridae* are the only two families of positive sense RNA viruses that contain only one **open reading frame** (ORF) which creates one large polyprotein that is cleaved into individual proteins by the viral protease. Flaviviruses contain multiple genera with members that cause significant diseases in animals and humans, in particular, the genera *Pestiviruses* (diarrheal disease of cattle), *Flaviviruses* (yellow fever, West Nile, Japanese encephalitis, dengue fever), and *Hepaciviruses* (hepatitis C).

GENOME STRUCTURE

Flaviviruses are enveloped positive sense RNA viruses with a linear genome of 10-12 kb in a 45-50 nm diameter virion containing an icosahedral capsid, as shown in Figures 14-6 A & B. The virus contains 3 structural proteins: two envelope proteins (envelope glycoprotein (E) that mediates binding and fusion and is the major antigenic determinant and membrane glycoprotein (M) that is a proteolytic fragment of a precursor protein) and one capsid protein (C). The genome, and therefore mRNA, has a 5’ cap but lacks a poly A tail. Note: Hepatitis C is the sole exception. It lacks both a 5’ cap and poly A tail, as shown in Figures 14-7 A &B.

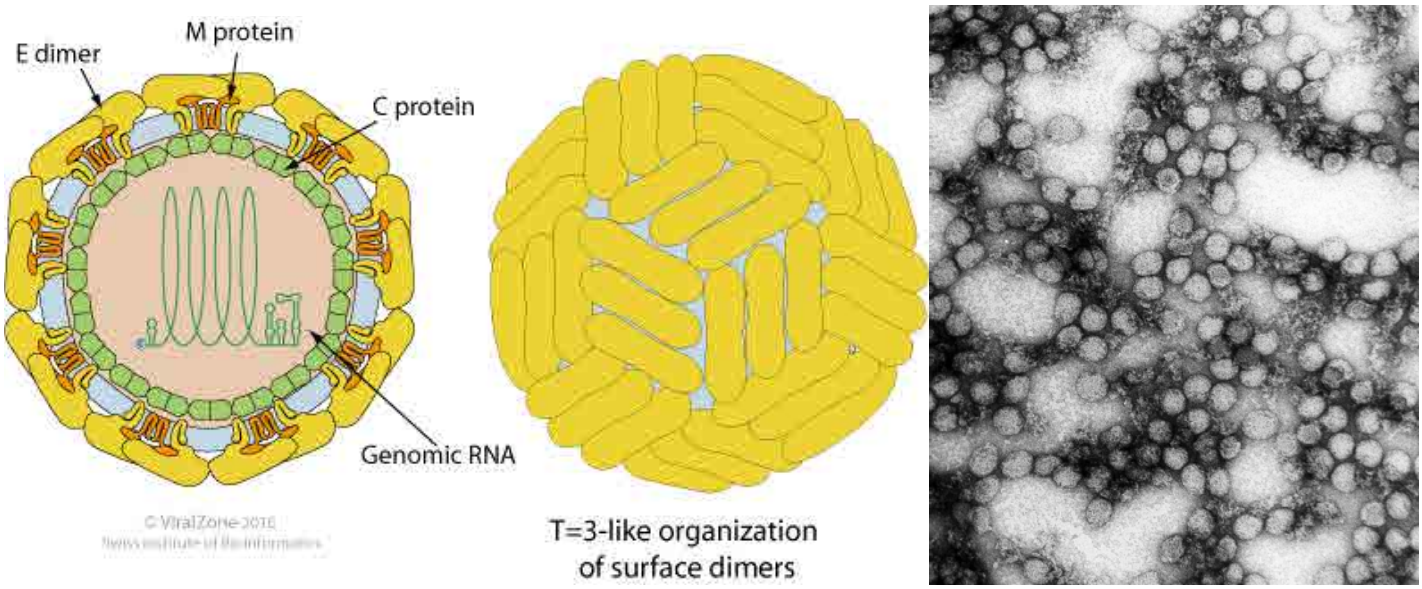


Figure 14-6 A. A flavivirus virion. Courtesy of ViralZone https://viralzone.expasy.org/43 B. Electron microscopy image of yellow fever virus. Courtesy of https://phil.cdc.gov/Details.aspx?pid=8239 Dr E. Palmer

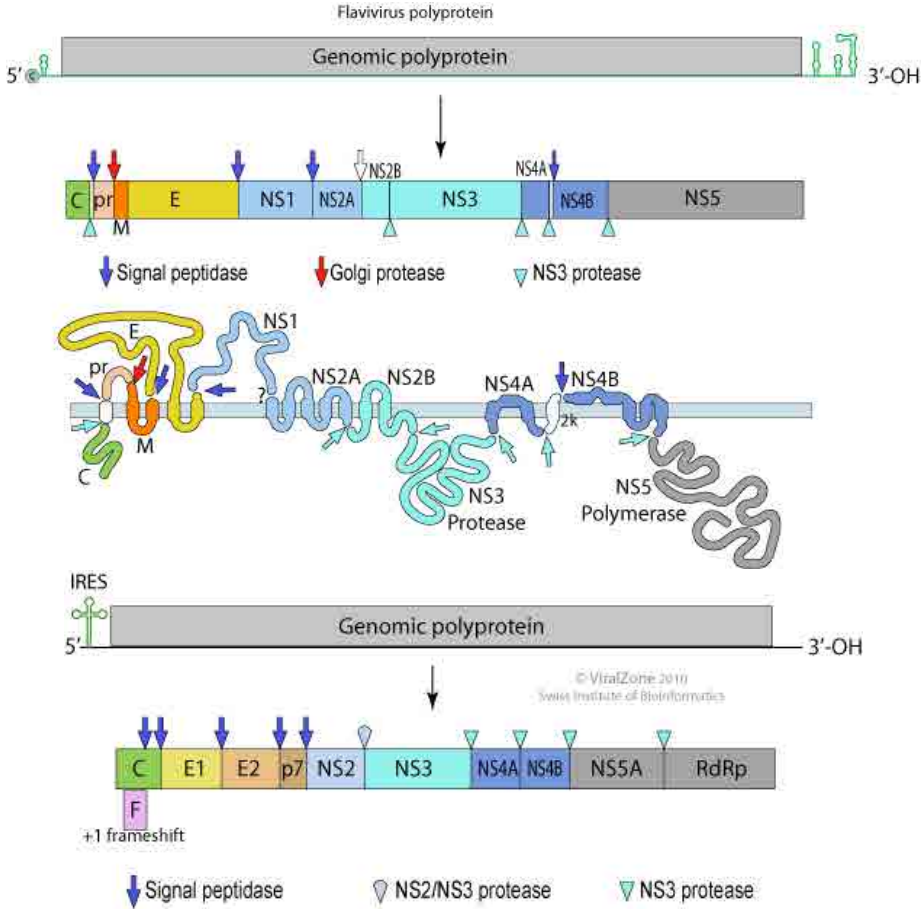


Figure 14-7 A. A typical flavivirus genome showing a 5' cap but no poly A tail with a secondary structure for PABP binding. Figure 14-7B. The hepatitis C genome lacking the 5' cap and poly A tail with an IRES at the 5'end to facilitate ribosome binding. Note: the hepatitis C genome 3' end would contain a secondary structure for PABP binding. Courtesy of ViralZone https://viralzone.expasy.org/43

VIRAL REPLICATION CYCLE

As shown in Figure 14-8, during the flavivirus replication cycle:

2. Attachment of the viral envelope protein E to host receptors mediates internalization into the host cell by clathrin-mediated endocytosis.
3. Fusion of virus membrane with host endosomal membrane occurs when the endosome acidifies and the envelope fuses with the endosomal membrane. RNA genome within the capsid is released into the cytoplasm. The capsid degrades as it enters the endoplasmic reticulum.
4. The positive sense genomic RNA is translated into a polyprotein which is cleaved into all structural and non-structural proteins (to yield the replication proteins) by the virally encoded protease by autocatalysis.
5. Replication takes place at the surface of endoplasmic reticulum in cytoplasmic viral factories protected by vesicles by RdRp. It is not clear how the virus is transported to the site of replication.
6. A negative stranded RNA is synthesized from the genomic positive sense RNA by RdRp replicase activity. The negative stranded RNA anti-genome is transcribed/replicated thereby providing viral mRNAs/new positive sense RNA genomes by RdRp replicase activity.
7. Virus assembly occurs at the endoplasmic reticulum and seems to be facilitated by the viral ionic channel. The virion buds at the endoplasmic reticulum, is transported to the Golgi apparatus and engulfed in exocytic vesicle.
8. Release of new virions by exocytosis.

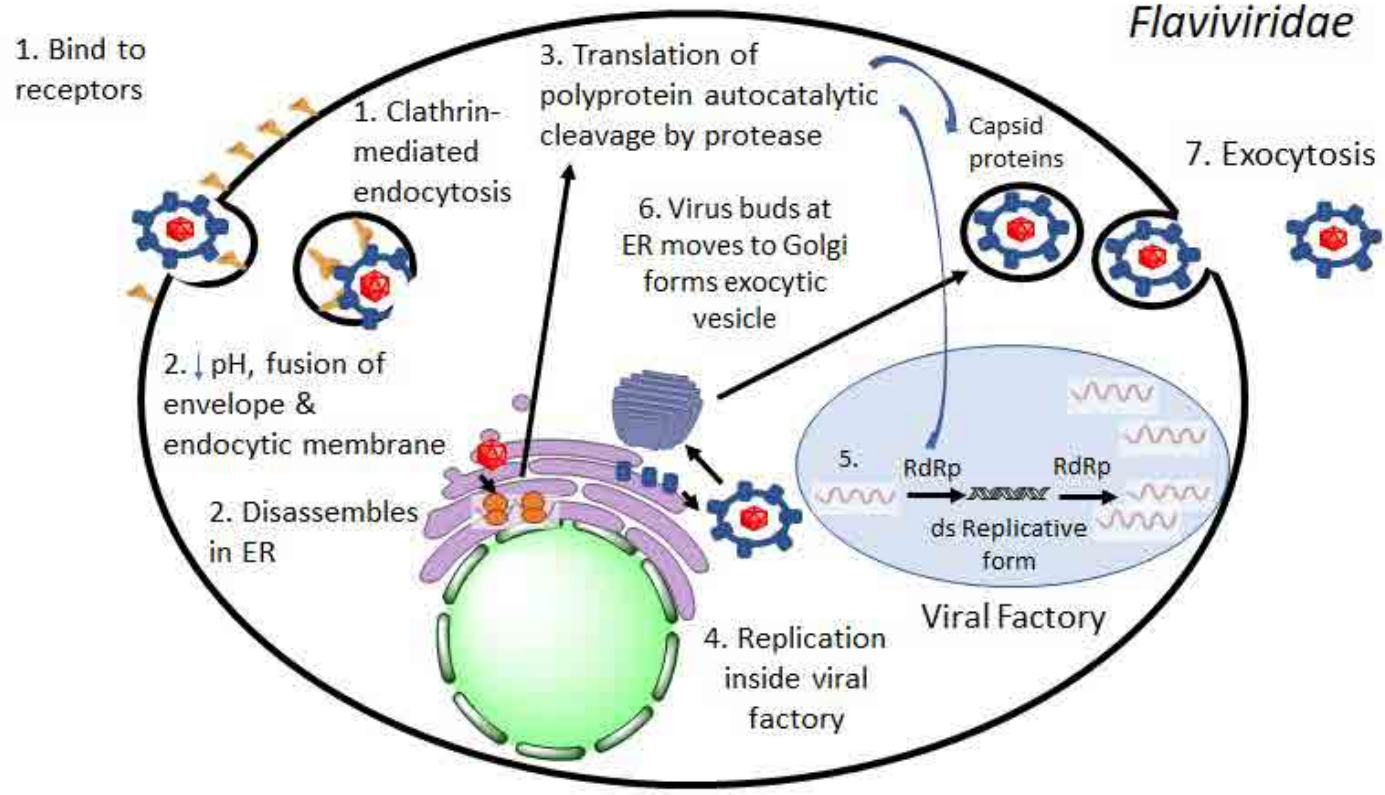


Figure 14-8. Flavivirus replication cycle. Courtesy of Erica Suchman, Colorado State University.

HOW DO FLAVIVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS AND IT REPLICATES IN THE CYTOPLASM?

Most RNA viruses' RdRp have the ability to create the 5' cap and have a poly U tract in the 5' end of the template (negative sense) strand of RNA that will result in the addition of a long poly A tail when the RdRp stutters adding many more A's than exist in the tract. However, flaviviruses do not have the ability to add a poly A tail, and as a result, they lack the 3' poly A tail. As the poly A tail binding proteins (PABP) are essential to initiating translation, flaviviruses contain a secondary structure in their 3' end that forms due to complementarity within the mRNA. This secondary structure is able to bind the PABPs allowing them to interact with the initiation factors that have bound the cap and initiate translation. The RdRp of most flaviviruses, except Hepatitis C, can produce a 5' cap.

HOW DO FLAVIVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all cytoplasmic replicating positive sense RNA viruses of animals, flaviviruses utilize a viral protease to cleave a large viral polyprotein, Figure 14-7 A & B. As discussed above, flaviviruses, like picornaviruses, only create one large polypeptide that is cleaved to form all the proteins necessary for replication. Note, however, that unlike picornaviruses, flaviviruses utilize both their own protease and proteases from the Golgi apparatus, Figure 14-7A. Also, although Hep C is a flavivirus, it does have one small second open reading frame that is expressed via ribosomal frame shifting when the ribosome hits the pseudoknot that forms in the viral mRNA. But again, this is an outlier for the family. All other members only create one open reading frame.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like most RNA viruses, the mRNA will have a 5' 7-methylguanosine cap (except members of the genus *Hepacivirus* including hepatitis C), but it will lack the usual 3' poly A tail. The 5' cap will be recognized by the necessary initiation factors (eIF4E &G). However, as described above, a secondary structure must form within the 3' end of the mRNA allowing the PABPs, thus translation initiation can proceed as normal. The structural proteins consist of a capsid or C protein, 2 envelope proteins, a protease, RdRp, and multiple non-structural accessory proteins involved in overcoming the host cell's antiviral defenses.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA.

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome.

Note that flaviviruses, like many viruses, take care of the problem of replicating the ends and priming replication via the same mechanism.

As discussed above flaviviruses must have genes to produce a RdRp. Positive sense RNA viruses whose genomes are equivalent to mRNA can be translated immediately upon entrance into the cell. As a result, they do not need to carry RdRp into the host cell with the genome. They must, however, produce it immediately in order to begin replicating. Note that all RNA viruses of animals have linear genomes and, as such, must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses, including flaviviruses, replicate their ends easily. RdRp starts synthesizing at the end of the genome without a primer. This is referred to as de novo synthesis. De novo initiation by RdRP occurs at the 1st nucleotide. For most RNA viruses, this method of replication ensures that the ends are replicated.

VIRION FORMATION

Viral capsids form spontaneously in the viral factory. The virus leaves the cell via budding from the endoplasmic reticulum and then into the Golgi apparatus, where it gains an exocytic vesicle, and leaves the cell by exocytosis.

AVOIDING THE HOST IMMUNE RESPONSE

Recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication to avoid activating the interfereon response and possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi), as well as the interferon stimulated genes (ISG) dsRNA-dependent PKR, dsRNA-dependent OAS and the PRR MDA-5. As such, RdRp is unable to proofread as it replicates the genome which leads to a high mutation rate which allows antigens to change rapidly. As well as trying to avoid detection by PRRs by remaining in the viral capsid until leaving the endocytic vesicle and replication in viral factories, many flaviviruses create accessory proteins to avoid the immune response. For example, HCV creates a viral protease that cleaves intermediates in the cascade response of PRR that recognize dsRNA (MDA-5 and TLR 3). Flaviviruses also inhibit the JAK-STAT signal transduction pathway activated by type I interferons and inhibit PKR and OAS once they are produced in response to type I interferons. You might think it is odd that HCV would need to inactivate PKR when it has no 5’ cap. Recall, however, that PKR phosphorylates the eIF2α required to bind the 40S subunit. This step is still necessary even though the virus is initiating translation at an IRES. Consequently, if eIF2α is phosphorylated, HCV cannot translate its proteins either! So, it makes accessory proteins to inhibit PKR’s ability to shut down both host cell and viral translation.

FLAVIVIRAL DISEASE *PESTIVIRUS*

BOVINE VIRAL DIARRHEA

Bovine viral diarrhea virus is an economically important disease of cattle that causes severe diarrhea leading to reduced weight gain and milk production, and as such, can have significant economic ramifications when herds become infected. It is transmitted by the fecal-oral route. Do you find anything odd about a flavivirus causing a diarrheal disease? It is an enveloped virus. Most viruses that can infect the intestines are acid resistant and naked as envelopes generally do not do well with environmental changes. The pH of the stomach is quite acidic (pH 2), the intestines quite basic, and bile salts released from the gall bladder lyse many viral envelopes. The envelope

of these flaviviruses must be abnormally resilient in the environment. This resilience has been demonstrated in studies but is not well understood. In discussion with virologists, we speculate that the vast majority of the virus particles are inactivated by the stomach acid (as is observed in the literature) but that a few virus particles are sufficient to initiate the infection.

FLAVIVIRIDAE HEPACIVIRUSES

HEPATITIS C (HCV)

Hepatitis C, like hepatitis B, A and E, causes symptoms of the disease hepatitis, but all 4 are in different families. As of 2019 there are thought to be 131-150 million people worldwide with chronic HCV infection, and approximately 3 million of these reside in the US. There are 6 genotypes (genotype 1 is most common in US). The virus is transmitted by injection drug use (most common in US), transfusions, needle stick injuries in health care settings, birth to an HCV-infected mother, sexual transmission, and sharing of blood contaminated razors or toothbrushes. The virus, like HAV and HepB, targets hepatocytes. Progression of HCV infections are shown in Figure 14-9A. The infection is contracted via blood or sex and usually begins with an asymptomatic acute infection that may develop into mild symptoms including fatigue, nausea, anorexia, **jaundice** (yellow tinge to skin and whites of the eyes), dark urine, and abdominal pain. Approximately 15-40% of infected individuals will clear their infection within 6 months. Between 60-85% will develop a chronic infection. They may remain stable without progressing, but 5-20% of those infected will develop **cirrhosis** (severe liver damage) after 25 years after which point, they will slowly progress, or approximately 7% per year will develop **hepatic carcinoma** (HCC). You will note in Figure 14-9B that in acute infections viremia is detectable within days, and the decrease in viremia is associated with increase in the liver enzyme **alanine amino transferase** (ALT), which is due to immune-mediated **cytolysis** of infected liver cells. ALT is a commonly tested indicator of liver health that is included in many yearly blood tests. In chronic infection high levels of viremia are also detected but at slightly lower levels than acute infections with varying degrees of chronic inflammation.

$CD4^+$ and $CD8^+$ T-cell responses are critical for the control of HCV infection and are detectable 5 to 10 weeks after infection. HCV-specific $CD8^+$ T cells become dysfunctional in individuals who progress to chronic infection, exhibit poor *ex vivo* proliferation and IFNγ production, and a decreased ability to lyse target cells.

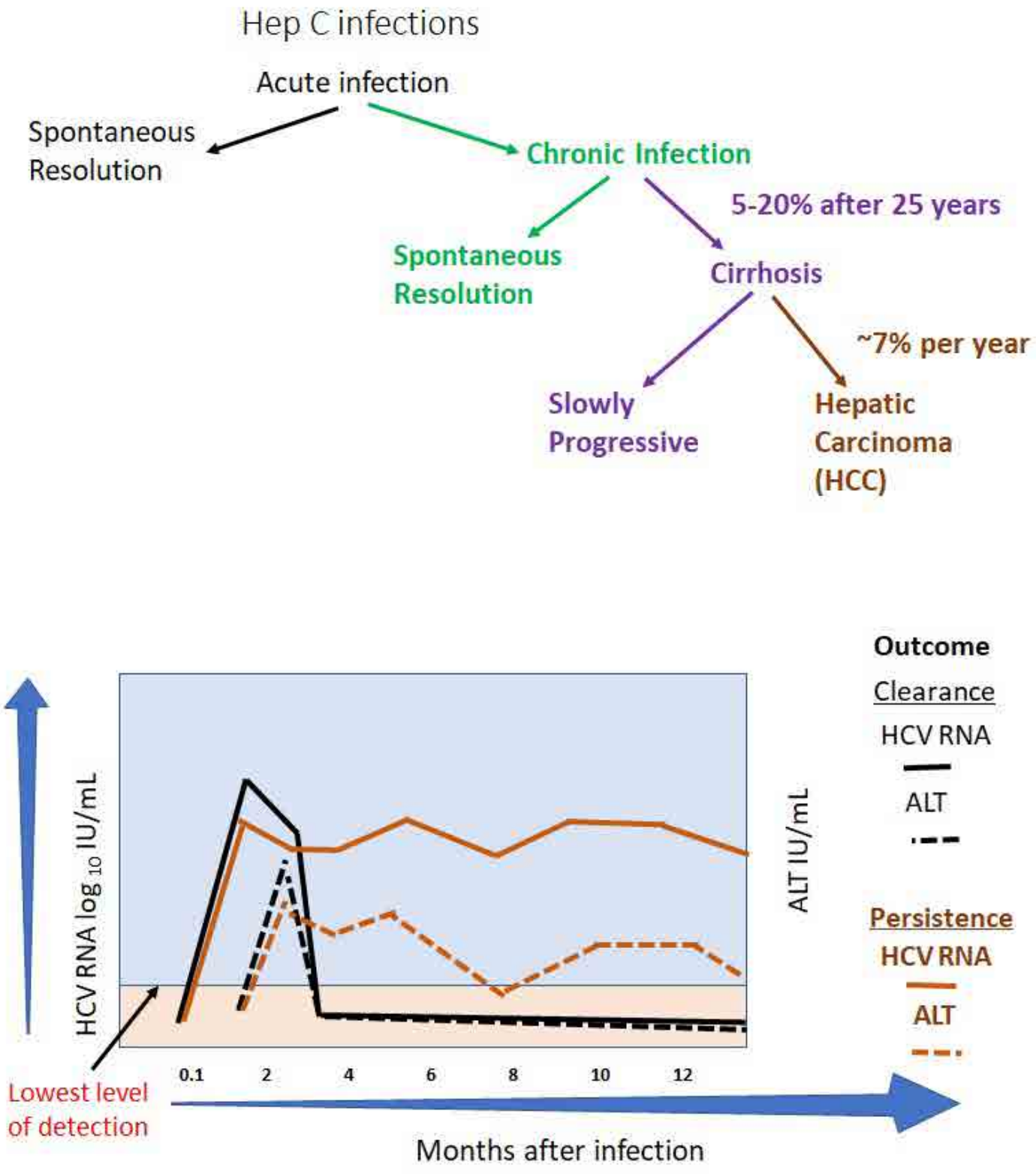


Figure 14-9 A & B. Progression of HCV infections. B. An example of expected HCV blood test results in a person who clears the infection and a persistently infected patient. Courtesy of Erica Suchman, Colorado State University.

FLAVIVIRIDAE, GENUS, *FLAVIVIRUSES* DISEASES: YELLOW FEVER, WEST NILE, DENGUE FEVER, ZIKA VIRUS, ST LOUIS ENCEPHALITIS, JAPANESE ENCEPHALITIS, TICK-BORNE ENCEPHALITIS

As you can see there are quite a few viruses in the genus *Flavivirus* that cause human disease. They fall into three groups that are defined by their mode of transmission: i) tick-borne flaviviruses, ii) mosquito-borne flaviviruses, iii) no known vector.

Arthropod borne viruses must be able to replicate in the arthropod vector and in a vertebrate host which is only seen in RNA viruses as DNA virus promoters are very species specific. So, you will notice that all the arthropod borne viruses will be RNA viruses. However, despite being able to transfer between arthropod vectors and human or animal hosts, the virus does not infect just any mosquito or tick. Each disease has a narrow range of arthropods that will serve as vectors.

Figure 14-10A & B. Arthropods that can transmit disease, A ticks, B mosquitoes. Courtesy of https://phil.cdc.gov/Details.aspx?pid=22871, J. Gathany, https://phil.cdc.gov/Details.aspx?pid=23157 Pablo Cabrera

As discussed in Chapter 4, many of these diseases, such as yellow fever virus and dengue virus, have a **sylvatic or enzootic** (jungle) cycle that involves non-human primates and one set of mosquitoes. Another set of mosquitoes is involved in moving the virus out of the sylvatic cycle into a **spillover cycle** (moving into human hosts) involving the **zone of emergence,** which is often in rural communities, and then into the **urban cycle,** where a very narrow set of mosquitoes are transmitting the virus to human hosts. It is important to note that many of these viruses can be transmitting from one generation of mosquitoes to the next by **transovarial transmission** when the virus is deposited into the eggs. For example, as shown in Figure 14-11, yellow fever virus in the sylvatic cycle is carried by multiple primate species and transmitted by mosquitoes in the genus *Haemagogus,* and, as yellow fever virus moves into urban centers, yellow fever is transmitted primarily by *Aedes aegypti* mosquitoes to humans. It is also important to note that the virus cannot immediately be transmitted by the mosquito after it takes an infected blood meal. The virus must get to the correct tissues and replicate, eventually infecting the salivary glands to be transmitted as the female mosquito takes a blood meal to get protein to produce eggs. The time period between when a mosquito takes a blood meal and when it can transmit the disease is called the **extrinsic incubation period.** This usually takes at least 10 days for yellow fever with optimal temperature and access to food but can take longer. Note that an **intrinsic incubation period** also exists, which is the time between a host getting infected and when they develop symptoms.

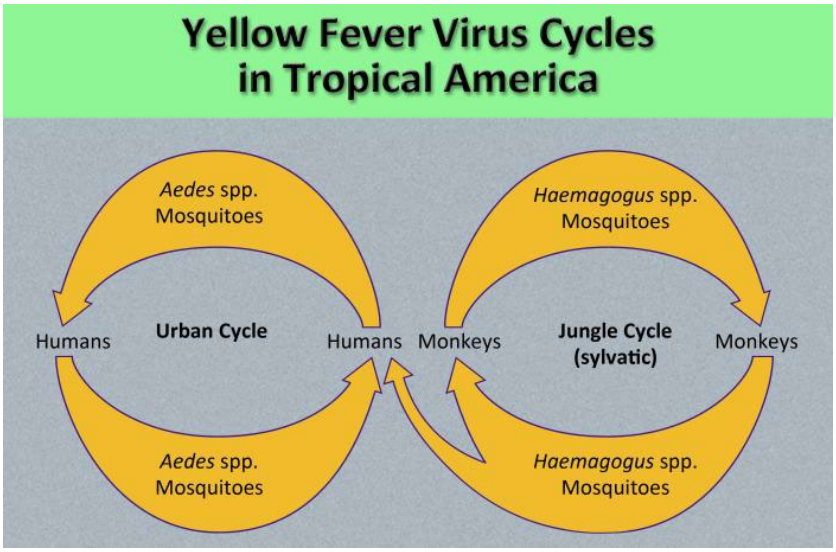


Figure 14-11. Yellow fever virus cycles in tropical America. Courtesy of https://phil.cdc.gov/Details.aspx?pid=14997

YELLOW FEVER VIRUS

The virus is inoculated via skin by mosquitoes during blood feeding. These are most commonly *Aedes aegypti* mosquitoes. The intrinsic incubation period (how long it takes a host to get ill after infection) is between 3 and 6 days. Replication occurs first in regional lymph nodes and leads to a period of **peak viremia**. Symptoms include fever, chills, headache, back pain, and myalgia. During this time frame, individuals are infectious to mosquitoes who blood feed on them. This is followed by a period of **remission** with improved clinical symptoms, and then a period of **intoxication** when the virus is replicating in the liver. During the period of intoxication, the symptoms include vomiting, epigastric pain, jaundice, multi-organ failure, and hemorrhage. The virus causes many of the infected cells, particularly in the liver, to undergo apoptosis which leads to organ failure. Furthermore, **Kupffer cells**, macrophages of the liver, are killed by the infection which leads to hepatocyte damage. **Disseminated intravascular clotting** (DIC) begins which depletes the platelets as they are all taken up in the DIC leading to hemorrhaging. The jaundice can become quite pronounced causing the whites of the eyes to yellow, as well as the skin, leading to the name yellow fever, as shown in Figure 14-12 A and B.

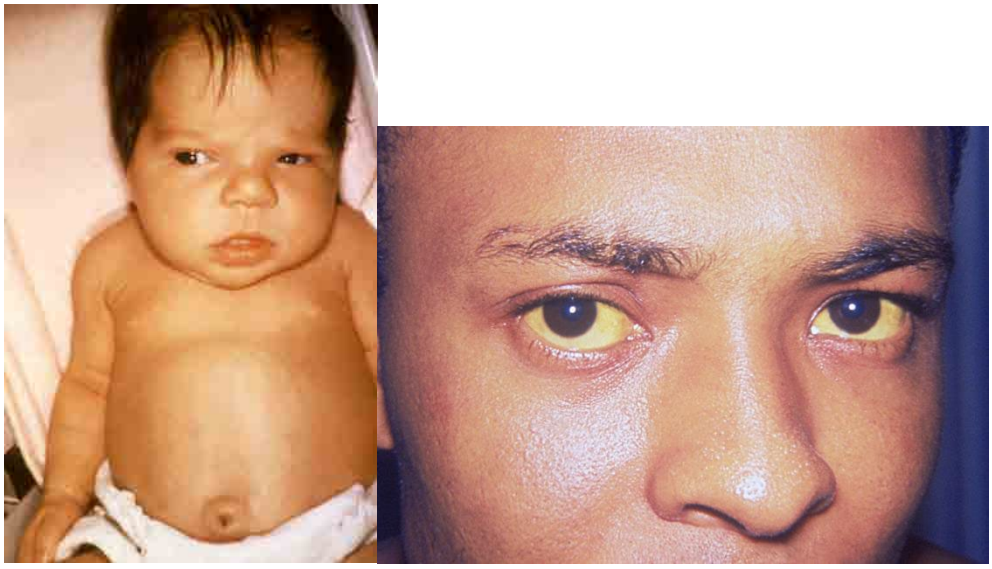


Figure 14-12A and B. Images of a baby with yellow fever showing a yellow tinge to the skin and a patient with yellowing of the whites of the eyes. Courtesy of https://phil.cdc.gov/Details.aspx?pid=5604 Dr. Hudson, https://phil.cdc.gov/Details.aspx?pid=2860 Dr. T Sellers

In 1937, Theiler & Smith developed an attenuated yellow fever vaccine called 17D. It is quite effective. Immunity is developed in 95% of vaccinates, and it triggers long-term protective adaptive immunity. The vaccine may cause mild symptoms in people who receive it, particularly those who are immunosuppressed (as unfortunately occurred for the author). Rarely, vaccine-induced deaths can occur, but mosquito control programs are also necessary which involve reducing standing water (in planters, water cisterns, or discarded trash) and potentially spraying for mosquitoes. See Figure-14-13 for the mosquito life cycle. Mosquitoes breed in standing water; they don't need much, and they don't need it for long. Many mosquitoes go from larva instar to adults in only a few days if the temperatures are warm and there is plenty of food.

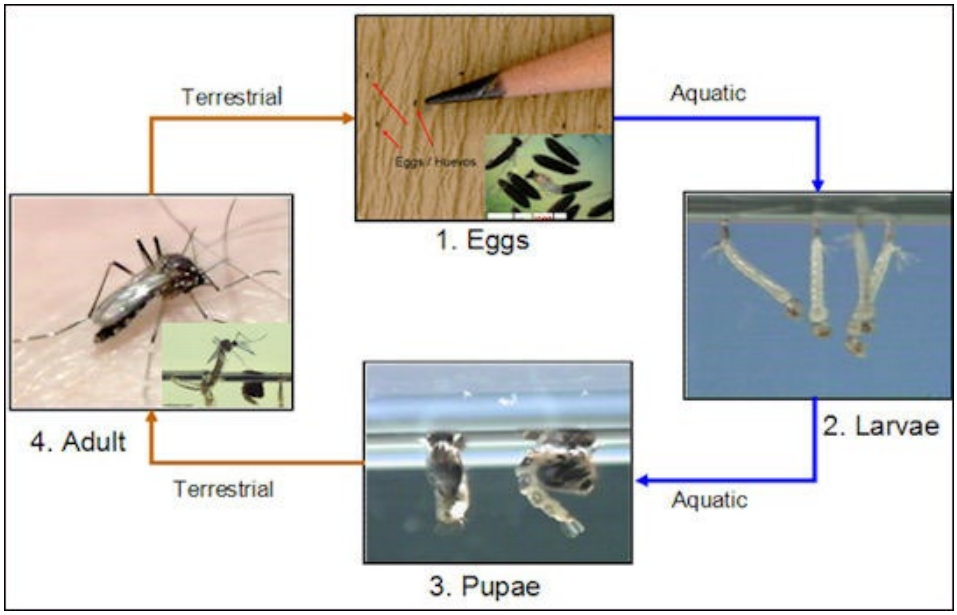


Figure 14-13. Typical mosquito life cycle. Courtesy of https://www.cdc.gov/mosquitoes/about/life-cycles/aedes.html

DENGUE FEVER (DENV)

Dengue fever, like yellow fever, is transmitted by *Aedes* mosquitoes, primarily *Aedes aegypti*. It also has a sylvatic jungle cycle that involves non-human primates and an urban cycle where the disease is primarily spread between humans by infected mosquitoes, as shown in Figure 14-11. The extrinsic incubation period for dengue virus is approximately 8-12 days and the intrinsic incubation period is between 3 and 14 days, as shown in Figure 14-14.

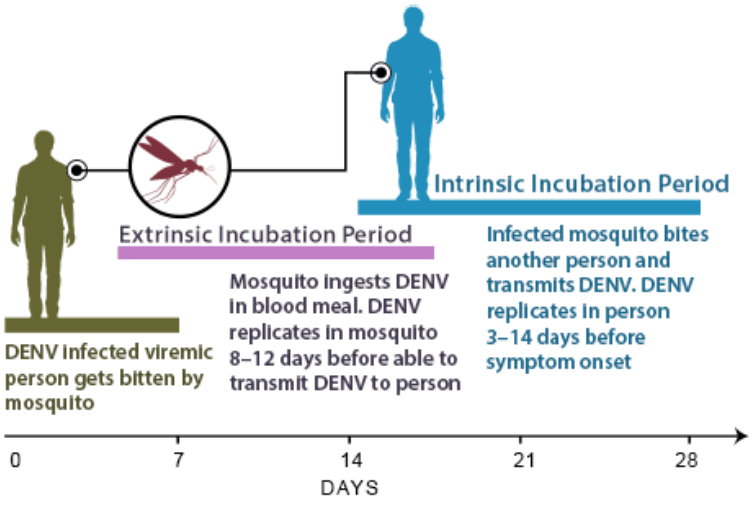


Figure 14-14. The extrinsic and intrinsic incubation periods of dengue virus. Courtesy of https://www.cdc.gov/dengue/training/cme/ccm/page45915.html

DENV infection causes a spectrum of clinical disease from self-limiting dengue fever to life-threatening hemorrhagic and capillary leak syndrome [dengue hemorrhagic fever (DHF)/shock syndrome (DSS)]. There are four distinct serotypes of DENV (1-4). They differ at the amino acid level in viral envelope proteins by 25-40%. DENV2 infections are most commonly associated with DHF/DSS and result when a person previously infected with one serotype is exposed to a different serotype. This is thought to be due to antibody-dependent enhancement (ADE). The symptoms of DHF/DSS are fever followed by rapid onset of capillary leakage accompanied by **thrombocytopenia** (low platelet count) due to **disseminated intravascular clotting** (**DIC**), and mild to moderate liver damage. Multiple facets of immune response cause damage including: i) innate immunity cells killing of infected cells, ii) complement system causing **vasculitis** (inflammation of the vascular system) and DIC, and iii) cytokine release (T cells, innate cells) leading to fever, endothelial cell apoptosis and dysfunction, and hemorrhage. Without treatment DHF/DSS has a case-fatality rate as high as 20%, but with supportive care this can be dropped to 2-5%.

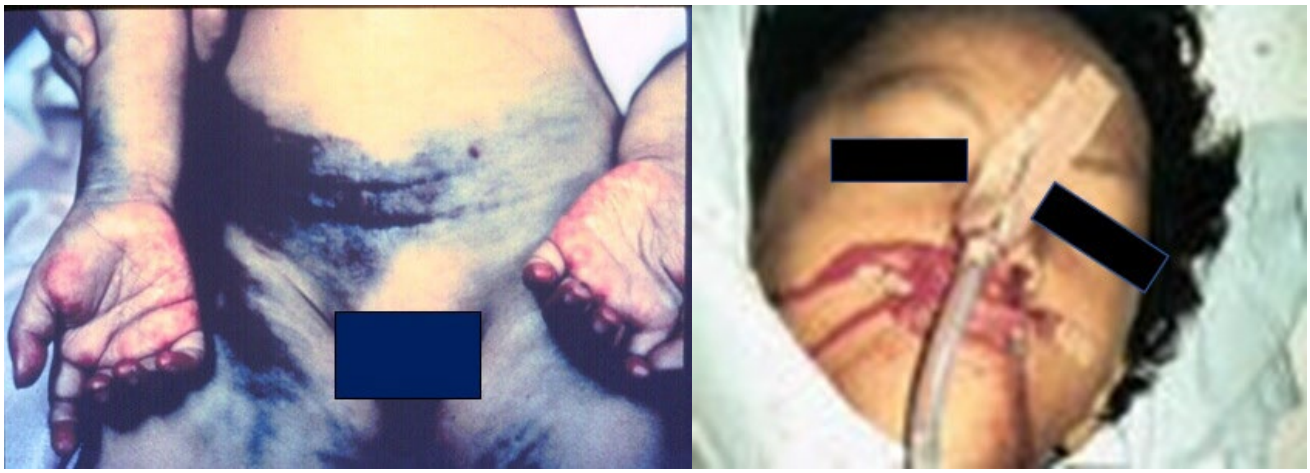


Figure 14-15 A & B. Images of people suffering from dengue hemorrhagic syndrome. Courtesy of the phil.CDC.gov

WEST NILE VIRUS

West Nile virus is another arbovirus carried by *Culex* mosquito species in the US. It was first isolated from a Ugandan woman in 1937 and first appeared in the U.S. in 1999. It reached Colorado in 2003 when Fort Collins, CO had the most cases in the United States. It is now endemic in North America. The life cycle of West Nile virus is predominantly in birds. Infected birds transmit the virus to mosquitoes in the *Culex* genus who then transmit the virus back to birds, their preferred food source, as shown in Figure 14-16. The mosquitoes are opportunistic feeders, however, and if birds are not available, the mosquitoes will feed on many other animals. There are more than 75 free-ranging avian species that *Culex* feeds on, and, if they are not available, they feed on primates, rodents, bats, camels, horses, donkeys, dogs, cats, cattle, goats, sheep, pigs, amphibians, and reptiles.

Humans and horses are dead-end incidental hosts that do not reach sufficient viremia to transmit the virus to new mosquitoes. In horses, **encephalomyelitis** (swelling of the brain) is common with a case-fatality rate of

40-45%. Although the majority of human infections are asymptomatic, some patients will develop a rash and febrile illness, as shown in Figure 14-17, and **meningoencephalitis** (swelling of the brain and membranes around the CNS **meninges**) can occur with a case-fatality rate of 4-11%. Current estimates are that 1 in 150-250 infected people will develop neurological disease including meningitis, encephalitis or flaccid paralysis. West Nile has also been shown to be transmitted by organ transplants, blood transfusions, from mother to baby during pregnancy or during birth, and by breast feeding.

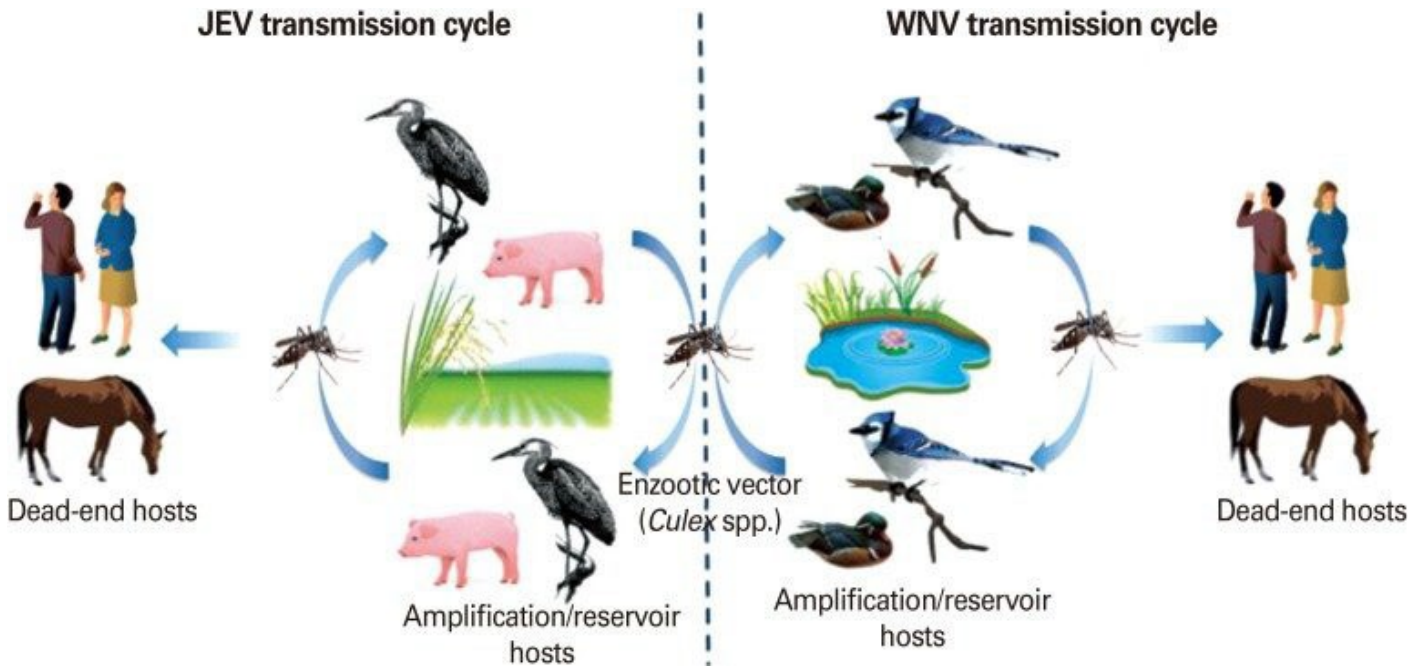


Figure 14-16. Enzootic and epizootic/epidemic transmission cycles of Japanese encephalitis virus (JEV) and West Nile virus (WNV). Left: JEV is transmitted by primarily Culex tritaeniorhynchus. Pigs and aquatic birds are the efficient amplification and reservoir hosts of JEV that develop high-titered viremia. Humans and horses are dead-end hosts since the level of viremia is insufficient for mosquito transmission. Right: WNV maintains an enzootic transmission cycle between Culex mosquitoes and birds (reservoir host). Horses, humans, and other mammals infected in a spillover transmission are "dead-end" hosts. Courtesy of Zoonotic encephalitides caused by arboviruses: Transmission and epidemiology of alphaviruses and flaviviruses – Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Enzootic-and-epizootic-epidemic-transmission-cycles-of-Japanese-encephalitis-virus-JEV_fig7_259743924

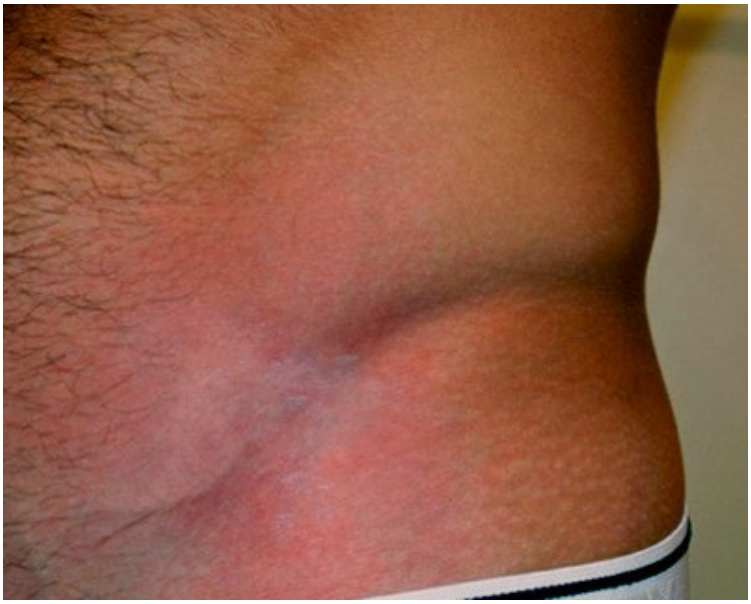
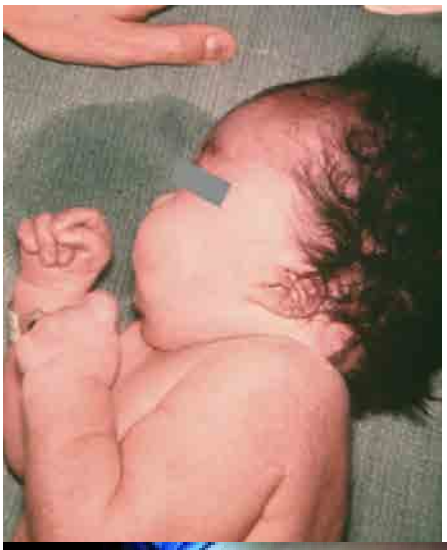


Figure 14-17. The rash seen with West Nile virus infection. Courtesy of the patient Dan Warro.

ZIKA VIRUS

Zika virus was first identified in humans in Uganda in the 1950's. The first large outbreak was on the island of Yap (Micronesia) in 2007. In the 2000s a faculty member at CSU wrote a paper suggesting sexual transmission of Zika virus. He contracted Zika doing field work in Africa, and his wife caught it from him when he returned home. This theory was widely disregarded, however, until 2015 when an outbreak occurred in Brazil, and babies began to be born at abnormally high rates with **microcephaly** (abnormally small brains), as shown in Figure 14-18. It was found that men secrete virus long-term in their semen. The Brazilian strain of Zika causes other neurological manifestations in fetuses and adults that have not been observed with other strains. Like other arboviral diseases, Zika virus is transmitted between humans by mosquitoes that get infected when taking a blood meal from an infected host. The incubation period is 1-2 weeks in the host and while the extrinsic incubation period for Zika virus in mosquitoes is not yet well characterized, it appears to be around 16 days. The virus is best transmitted by the mosquitoes *Aedes aegypti and Aedes albopictus.* A big question remains unresolved, "Will Zika virus become endemic in USA like West Nile did?" In most people Zika causes a fever, rash, malaise, and temporary arthralgia (painful joints). Figure 14-19 shows the rash.



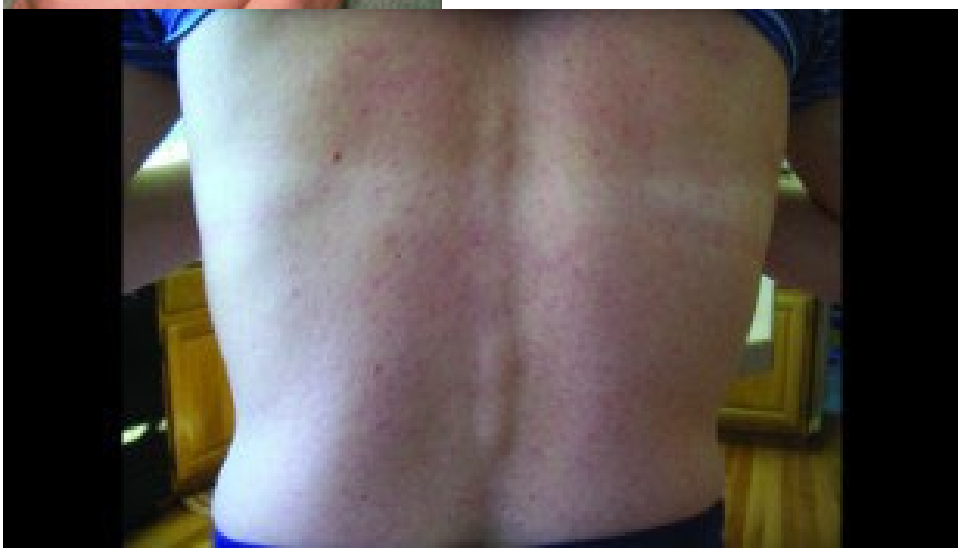


Figure 14-18. A baby with microcephaly. Courtesy of the https://phil.cdc.gov/Details.aspx?pid=15403 & Figure 14-19. The rash associated with Zika infection. Courtesy of the patient.

TICK-BORN ENCEPHALITIS VIRUS (TBEV)

TBEV causes a fatal neurologic syndrome in humans and livestock. The disease is primarily observed in Northern Europe, Russia, China, and Japan. The vector is I*xodes ricinus and Ixodes persulcatus* ticks. The transmission cycle of tick-born encephalitis is shown in Figure 14-20. The disease is usually transmitted between rodents and ticks who may feed on livestock and humans in a dead-end infection.

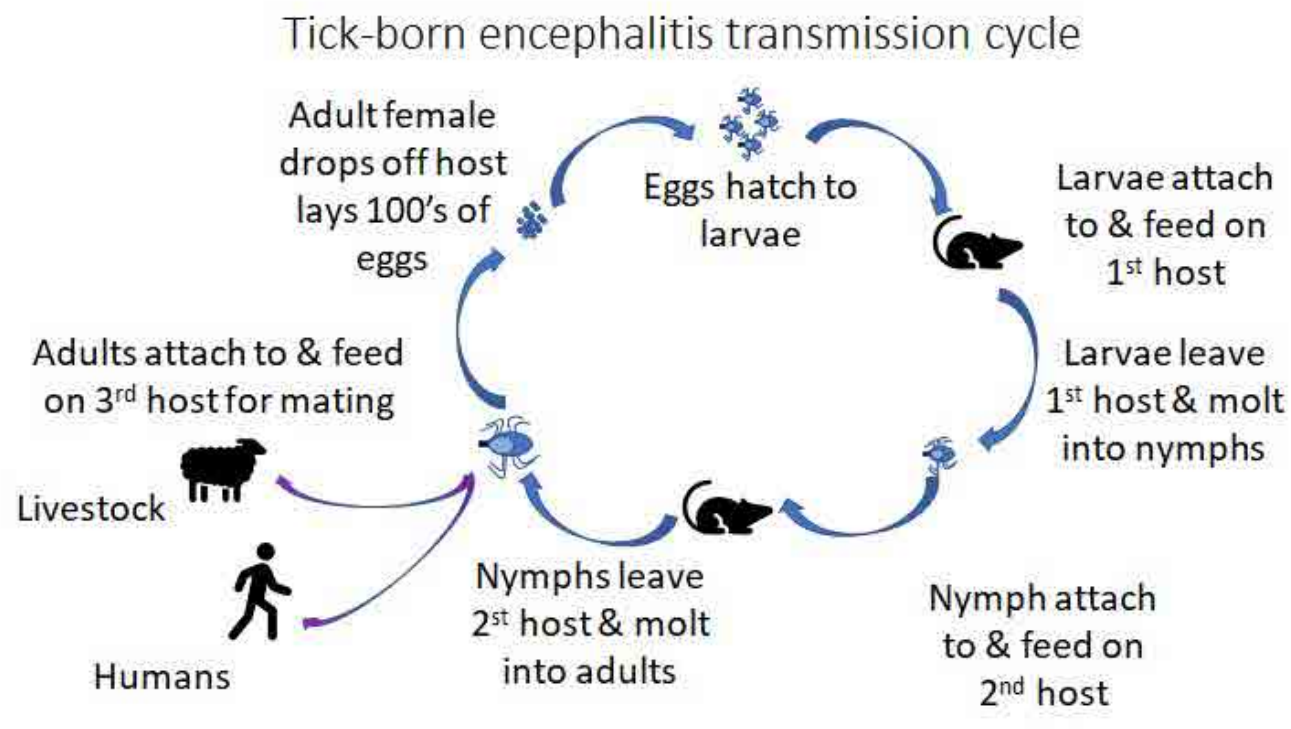


Figure 14-F20. The life cycle of tick-born encephalitis. Courtesy of Erica Suchman, Colorado State University.

DIAGNOSIS OF FLAVIVIRUS INFECTIONS

HEPATITIS C INFECTIONS

Hepatitis C is diagnosed using a serological test for antibodies to HCV (past exposure) or detection of the viral genome in plasma or serum for acute infections. If a person has no antibodies, no further action is required. If they are positive for serum antibodies, they are then tested for HCV RNA using **reverse transcriptase PCR**. If the person is antibody positive but HCV RNA negative, it is presumed to be a past cleared acute infection and no further treatment is needed. If they are antibody and HCV RNA positive they require drug treatment.

YELLOW FEVER

Reverse transcriptase polymerase chain reaction (PCR) testing of blood or urine can sometimes detect the virus in early stages of the disease. In later stages, serological assays for a 4-fold increase in IgG titer are better.

DENGUE FEVER

Depending on the time of patient presentation determines the optimal sampling method. Patient samples collected during the first week of illness should be tested by both IgM/IgG ELISA and reverse transcriptase PCR (RT PCR). Although RT PCR can detect virus earlier in the course of disease, it requires expensive equipment that is often not available in the regions that experience dengue fever outbreaks. Rapid diagnostic tests for the NS1 protein can give results in 20 minutes; however, the test is only reliable for the first 7 days of infection and cannot determine the serotype of infection. It is recommended that the NS1 rapid test be run concurrently with an IgM antibody ELISA. ELISA testing for both IgM and IgG are used and can detect IgM within a week of infection, and IgG takes a few weeks. If a patient is positive for IgM only, or IgM and IgG, they are currently infected. If only IgG is detected, it is not a current infection; it is a past infection.

WEST NILE

West Nile virus can be detected using an IgG antibody ELISA demonstrating a 4-fold increase in samples taken 2 weeks apart, by a positive IgM antibody capture ELISA, or via a 4-fold titer increase in serum plaque neutralization assays with samples taken 2 weeks apart. Viral RNA can be detected by reverse transcriptase PCR, or the virus can be isolated by growth in cell culture. IgM can be detected in cerebrospinal fluid (CSF) and serum specimens. Serum IgM antibody may persist for more than a year.

ZIKA

Reverse transcriptase PCR (**Nucleic Acid Testing** or **NAT** assay) are preferred for patients showing symptoms for less than 7 days. Serology tests are used for patients showing symptoms for more than 7 days. Negative results on NAT assays does not rule out infection as viremia drops rapidly 7 days after onset of symptoms and may not be detected by the test as virus drops to the lower end of sensitivity.

TREATMENT OF *FLAVIVIRIDAE* INFECTIONS

There are multiple drugs to treat HCV infections including type I interferon injections and antiviral drugs such as Ribavirin, a nucleoside inhibitor that mimics guanosine, protease inhibitors, replication complex inhibitors, and RdRp inhibitors. In the summer of 2016, Epclusa received approval from the FDA to treat adults with all 6 genotypes of HCV. The treatment is a combination of two drugs that is found to have 100% efficacy and is curative! It is a 12-16 week therapy (depending on serotype) but has a very high cost of approximately $75,000 to $100,000! Note that Epclusa contains an RdRp inhibitor and a replication complex inhibitor. Sadly, many young people are dying of opioid overdoses that have HCV infections (due to IV drug use) making their livers unusable for transplantation. Now that there is a 100% effective way to cure people of HCV infections, there is discussion of using their livers for transplantation and then treating the recipient with Epclusa to cure them of their HCV infection.

Unfortunately for the majority of infections by viruses in the family *Flaviviridae,* there are few treatment options outside of supportive care. As a result, prevention is the primary mechanism of dealing with these infections.

PREVENTION OF *FLAVIVIRIDAE* INFECTIONS

There is only one vaccine available for flavivirus infections in the US. Only yellow fever has an effective attenuated vaccine. In 2022 a dengue fever vaccine was approved for children aged 9-16 who have already had dengue fever and live in a dengue virus endemic area. So, this vaccine is not available to children in the US at the time of this writing. All the others are best prevented by safe sex, safe handling of blood products, not sharing needles or products such as razors and toothbrushes that may be contaminated with blood and taking precautions to avoid mosquito or tick bites. Prevention also includes clearing potential mosquito breeding grounds of standing water and avoiding being outside uncovered at dusk and dawn, which are the prime mosquito breeding times. You should wear long clothes when outside and, if there are heavy mosquito populations, use DEET on your skin and permethrin on your clothes to prevent mosquitoes from feeding on you. Lastly, perform frequent tick checks and remove ticks promptly with a pair of tweezers firmly grabbing only the head and pulling them out in one swift movement to avoid leaving the head parts in your skin.

END OF CHAPTER QUESTIONS

1. Why do you think that these 2 virus families were grouped together in this chapter?
2. Do both of these viruses use the same or different enzymes to reproduce their genome and transcribe their mRNA? Are these enzymes cellular or viral? Identify these enzymes.
3. Compare and contrast how these 2 viruses start their genome replication.

4. Compare and contrast how these 2 viruses start protein translation.
5. Do these 2 viruses create subgenomic mRNAs? Why or why not?
6. Compare and contrast how these 2 viruses avoid the host immune response.
7. Compare and contrast how these 2 viruses obtain a 5' cap and poly A tail.
8. These viruses are all viruses with single stranded RNA genomes. Do they need to have mechanisms for overcoming RNA interference? If so, why? If not, why not?
9. Do both of these families use viral proteases? If so, for what purpose? If not, which ones do not?
10. What is an IRES? Which of these viral families utilize IRES? For what purpose?
11. How do viruses that lack a 3' poly A tail initiate translation?
12. What advantage might lacking a 5' cap provide a virus?
13. Can a virus create more than one ORF from an mRNA? If so, do either of these 2 virus families do so? If so, explain how they do this. If not, explain why.
14. What is a viral factory? Why might a virus utilize them? How do the viral factories of most RNA viruses differ from those of most DNA viruses?
15. Compare and contrast the Sabin and Salk polio vaccines.
16. Compare and contrast the picornavirus diseases.
17. What is an amplification host? What role does it play in the diseases described in this chapter?
18. Compare and contrast how mosquitoes and ticks become infected and can transmit viruses to new hosts.
19. What is ALT? For what disease is it significant, and why?
20. Explain the difference between an extrinsic and intrinsic incubation period.
21. What is a dead-end host? For which diseases described in this chapter are humans dead-end hosts?
22. What is DIC and what role does it play in viral diseases in the family *Flaviviridae?* In which diseases is this an issue?
23. Explain the difference between an enzootic/sylvatic cycle and an epizootic transmission cycle. For which of the families discussed in this chapter would you expect to observe these different cycles, and why?
24. What are the predominant diseases caused by viruses in the family *Flaviviridae*? What do they have in common?
25. You are tracking a new disease that is transmitted by mosquitoes. Is it most likely caused by a picornavirus or flavivirus? Explain your answer.
26. Compare and contrast hepatitis A, B, and C. Are they all caused by viruses in the same family? If not, why are they all named hepatitis?

REFERENCES:

Centers for Disease Control and Prevention. Life cycle of Aedes aegypti and Ae. albopictus Mosquitoes. https://www.cdc.gov/mosquitoes/about/life-cycles/aedes.html

Centers for disease control and Prevention. Mosquito-borne Transmission. https://www.cdc.gov/dengue/training/cme/ccm/page45915.html

Centers for Disease Control and Prevention. Polio. https://www.cdc.gov/polio/what-is-polio/polio-us.html

Centers for Disease Control. Public Health Images Library (PHIL) Erskine Palmer Ph.D. https://phil.cdc.gov/Details.aspx?pid=8239

Centers for Disease Control. Public Health Images Library (PHIL). J. Gathany. (2018) .https://phil.cdc.gov/Details.aspx?pid=22871

Centers for Disease Control. Public Health Images Library (PHIL) P. Cabrera. (2018). https://phil.cdc.gov/Details.aspx?pid=23157

Centers for Disease Control. Public Health Images Library (PHIL) Dr. T.F. Sellers Emory. (1963). *https://phil.cdc.gov/Details.aspx?pid=2860*

Centers for Disease Control. Public Health Images Library (PHIL). Dr.Hudson. (1967). *https://phil.cdc.gov/Details.aspx?pid=5604*

Centers for Disease Control. Public Health Images Library (PHIL). (1969) . https://phil.cdc.gov/Details.aspx?pid=14997

Centers for Disease Control. Public Health Images Library (PHIL). (1968). https://phil.cdc.gov/Details.aspx?pid=15403

Centers for Disease Control
https://www.cdc.gov/dengue/vaccine/index.html

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 16, 17, 18 19 (Picornaviridae) 25, 26, 27 Flaviviridae.

Go, Yun et al. (2014). Zoonotic encephalitides caused by arboviruses: Transmission and epidemiology of alphaviruses and flaviviruses. Clinical and experimental vaccine research. 3. 58-77. 10.7774/cevr.2014.3.1.58. https://www.researchgate.net/figure/Enzootic-and-epizootic-epidemic-transmission-cycles-of-Japanese-encephalitis-virus-JEV_fig7_259743924

Petersen LR, et. al. (2013). West Nile Virus: Review of the Literature. *JAMA.* 310(3):308–315. doi:10.1001/jama.2013.8042 https://pubmed.ncbi.nlm.nih.gov/23860989/

The College of Physicians of Philadelphia. The History of Vaccines. (2018). https://www.historyofvaccines.org/content/articles/history-polio-poliomyelitis

ViralZone. *Flaviviridae.* https://viralzone.expasy.org/43

ViralZone. *Picornaviridae.* https://viralzone.expasy.org/33

ViralZone. Poliovirus replication cycle. https://viralzone.expasy.org/3276

CHAPTER 15

Chapter 15: Non-Segmented Positive Sense RNA Viruses With More Than One Open Reading Frame That Use A Viral Protease And Replicate In The Cytoplasm: Togaviridae, Mantonaviridae, Caliciviridae, Coronaviridae

INTRODUCTION TO POSITIVE SENSE RNA VIRUSES WITH MORE THAN ONE OPEN READING FRAME

You will notice in Figure 15-1 that viruses in the family *Togaviridae*, *Matonaviridae, Caliciviridae*, and *Coronaviridae* replicate in the cytoplasm and are non-segmented, positive sense RNA viruses that contain more than one **open reading frame** (ORF) and use a virally encoded protease to cleave the viral polyproteins created by these ORFs. Recall that in Chapter 14 we discussed *Flaviviridae* and *Picornaviridae,* families that replicate in the cytoplasm and are non-segmented positive sense RNA viruses that contain only one ORF and use a virally encoded protease to cleave the one viral polyprotein that is produced. So, the major difference between the viruses discussed in this chapter and the viruses discussed in Chapter 14 are that these need a mechanism to produce a second open reading frame, whereas the viruses in Chapter 14 do not as they only produce one polyprotein. We will see that viruses in the *Togaviridae, Matonaviridae* and *Caliciviridae* family create their second ORF by the same mechanism, and viruses in the family *Coronaviridae* use a unique mechanism. The virion RNA is infectious (as it is for all positive sense RNA viruses, except retroviruses) and serves as both genome and viral messenger RNA. Like all RNA viruses (except retroviruses), these viruses have genes to produce an **RNA-dependent RNA polymerase** (RdRp) that will replicate the virus. However, as these viruses' genomes are equivalent to mRNA, the virus genome can be translated immediately upon entering the cell, and as such these viruses do not package their RdRp in the virion as they can create RdRp when they enter the cell and then utilize the RdRp to create more viral mRNA after translation is completed.

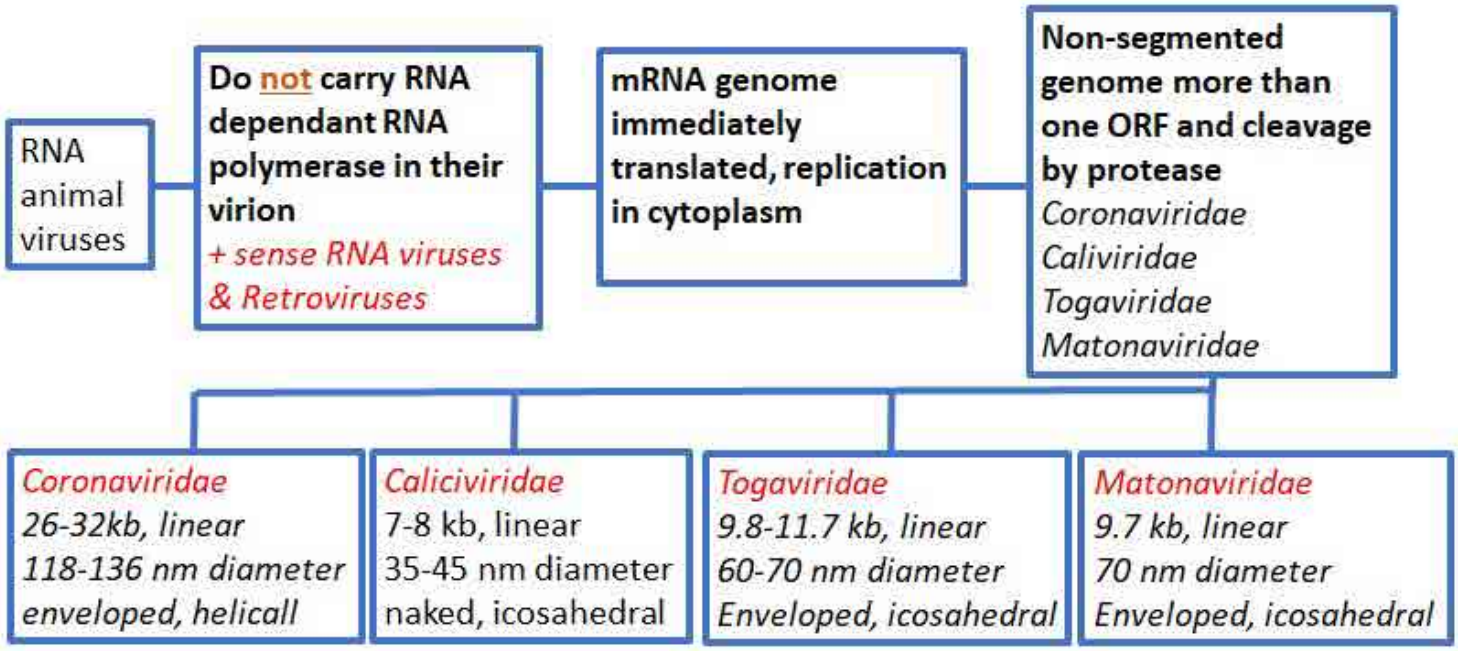


Figure 15-1. Taxonomy of positive sense RNA viruses with more than one open reading frame that use protease to cleave viral proteins. Courtesy of Dr. Erica Suchman, Colorado State University.

INTRODUCTION TO *TOGAVIRIDAE & MATONAVIRIDAE*

Viruses in the family *Togaviridae* are approximately 60-70 nm in diameter, are enveloped with an icosahedral capsid, and include a genome that is 9.8 to 11.7 kb in length, as shown in Figures 15-2 A, B, 15-3. The family contains 1 genera. *Alphavirus*, which contains many mosquito-borne diseases including Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), Venezuelan Equine Encephalitis virus (VEEV), chikungunya, O'nyong nyong, Ross River virus, Mayaro virus, Fort Morgan virus, Highlands J virus, and Sindbis virus. The family *Matonaviridae* was historically a genus *Rubivirus* in the family *Togaviridae,* which includes the rubella virus.

However, as you will see, rubella virus is transmitted by a very different mechanism (human to human) than all of the alphaviruses that are transmitted via insects. As such in 2018 the International Taxonomy of Virus Society (ITVS) reclassified *Rubivirus* into a family of its own, *Matonaviridae*. However, the replication and general mechanisms of *Matonaviridae* and *Togaviridae* are so similar that they will be covered together in this section.

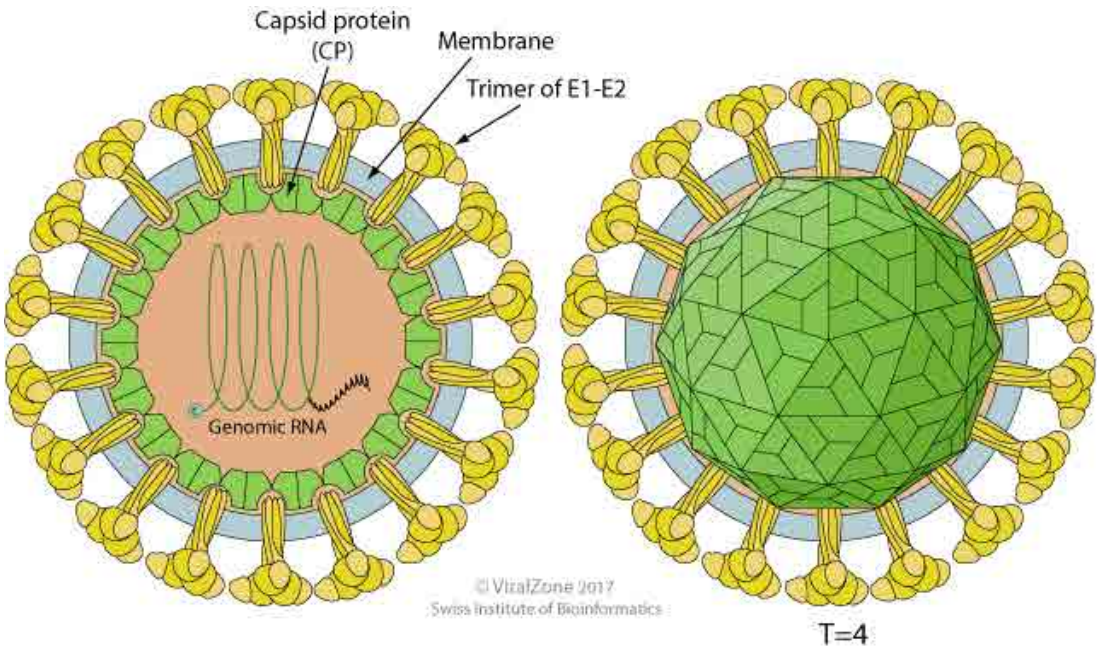


Figure 15-2 A. Typical Togaviridae or Matonaviridae viral particle. Courtesy of ViralZone https://viralzone.expasy.org/3

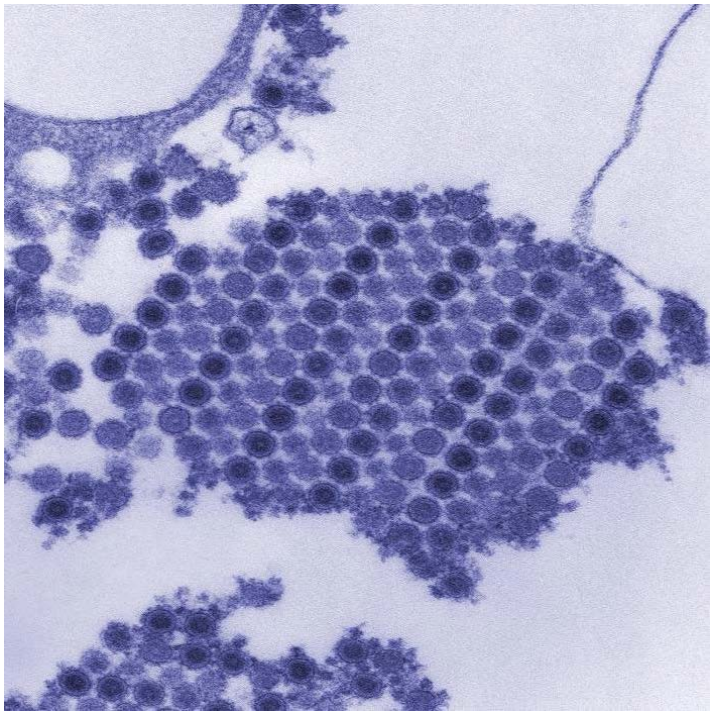


Figure 15- 2B. Digitally colorized transmission electron microscopic (TEM) image of numerous chikungunya virus particles which are composed of a central dense core that is surrounded by a viral envelope. Courtesy of https://phil.cdc.gov/Details.aspx?pid=17550 Cynthia Goldsmith, James A. Comer, and Barbara Johnson.

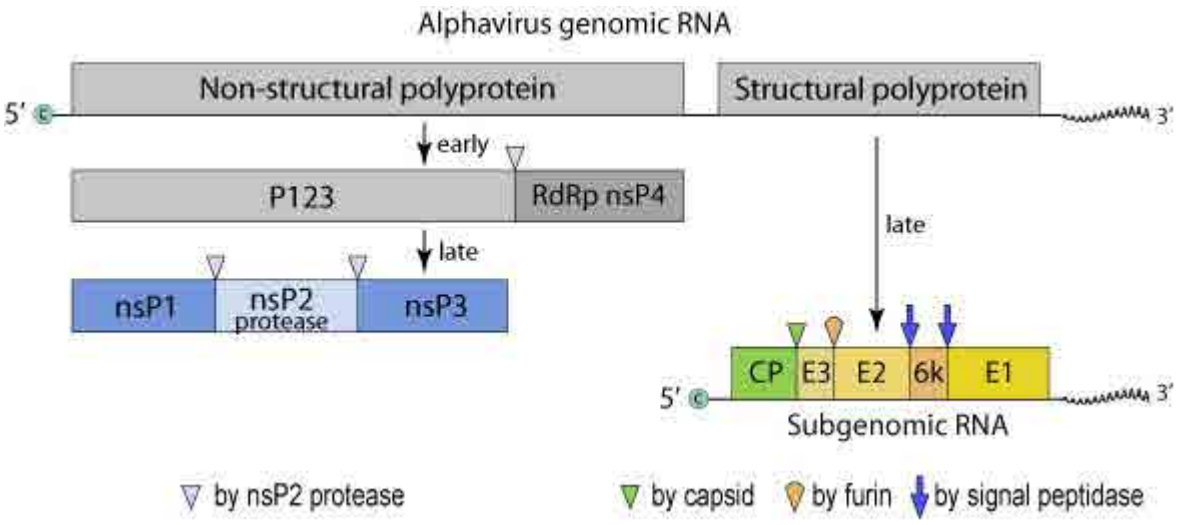


Figure 15-3. Typical Togaviridae/Matonaviridae genome showing two open reading frames and cleavage sites for viral protease. Note that furin, a cellular protease, is also involved in cleaving viral proteins from the large polyproteins produced during translation. The first open reading frame is read from the full-length genome mRNA and encodes the non-structural proteins including the RdRp and protease. The second is created from a subgenomic mRNA transcribed from the negative sense antigenome and encodes the structural proteins including the capsid and envelope proteins. Courtesy of ViralZone https://viralzone.expasy.org/3

REPLICATION CYTOPLASMIC

As shown in Figure 15-4, the replication cycle includes:

1. Attachment of the viral glycoprotein to host receptors mediates clathrin-mediated endocytosis of virus into the host cell.
2. Fusion of virus membrane with host endosomal membrane. The capsid containing the RNA genome is released into the cytoplasm.
3. The positive-sense genomic ssRNA is translated into a polyprotein which is cleaved into non-structural proteins necessary for RNA synthesis (replication and transcription).
4. Replication takes place in cytoplasmic viral factories at the surface of endosomes using viral RdRp replicase activity.
5. The RNA genome is transcribed/replicated thereby providing viral mRNAs/new positive sense RNA genomes and a negative sense antigenome by the viral RdRp.
6. Expression of the subgenomic RNA (sgRNA) from the negative sense antigenome by RdRp gives rise to the structural proteins.
7. Capsid assembly occurs in cytoplasm.
8. The capsid is enveloped by budding at the plasma membrane where the virion exits the cell.

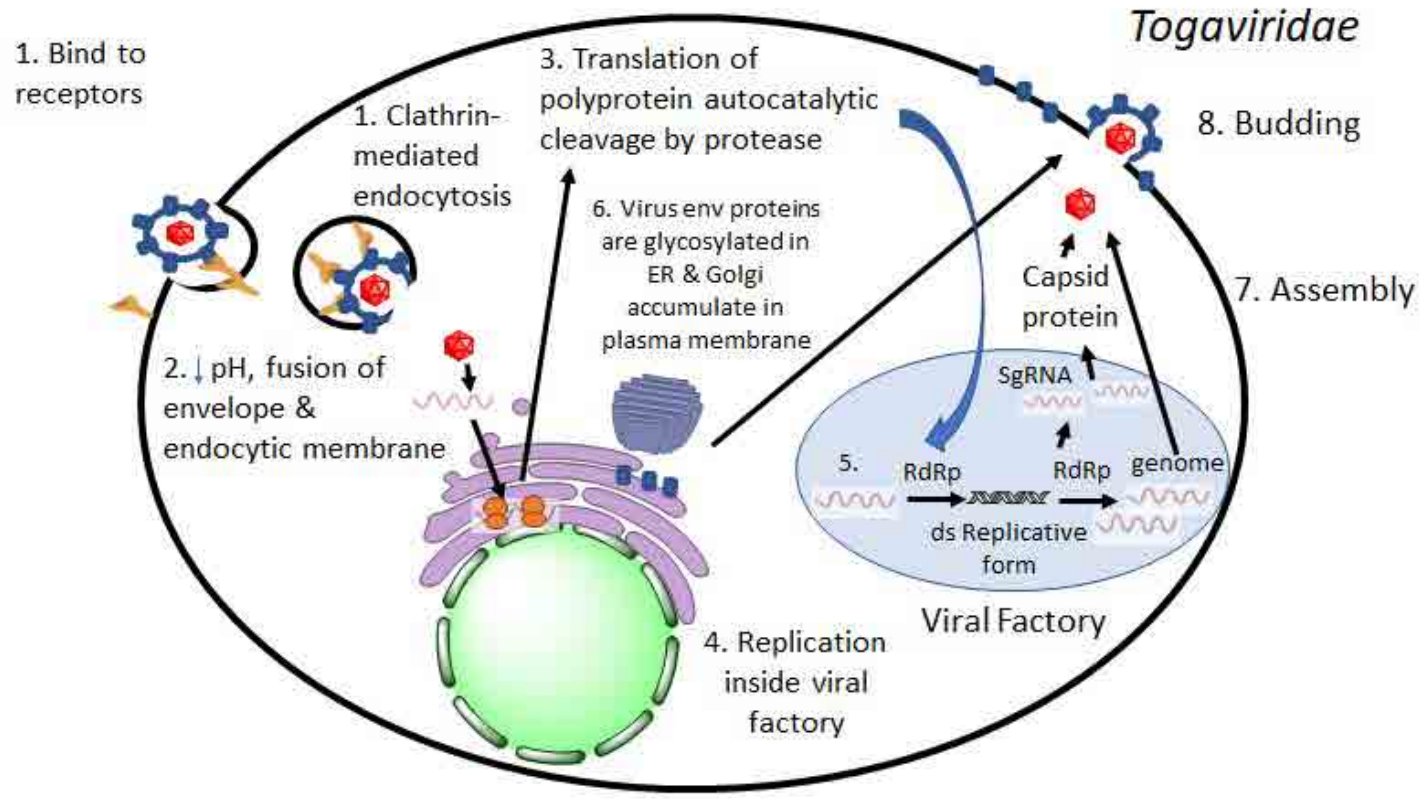


Figure 15-4. Typical viral replication cycle of a togavirus/matonavirus. Courtesy of Dr. Erica Suchman, Colorado State University.

HOW DO TOGAVIRUSES & MATONAVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS AND THE VIRUS REPLICATES IN THE CYTOPLASM?

Like most RNA viruses, the togavirus RdRp has the ability to create the 5' cap and have a poly U tract in the 5' end of the template (negative sense) strand of RNA. This results in the addition of a long poly A tail when the RdRp stutters adding many more A's than exist in the tract.

HOW DO TOGAVIRUSES & MATONAVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all cytoplasmic replicating positive sense RNA viruses of animals, togaviruses use a viral protease to cleave large viral polyproteins into their functional subunits as shown in Figure 15-3. As discussed in Chapter 14, flaviviruses and picornaviruses only create one large polypeptide that is cleaved to produce all the proteins necessary for replication. Togaviruses, matonaviruses and caliciviruses, translate one large polyprotein from the full-length genomic mRNA and one smaller sub-genomic mRNA transcribed from the negative sense antigenome thus creating a second open reading frame and polyprotein. Furthermore, the alphaviruses can use termination suppression to extend an open reading frame giving rise to more proteins, and leaky scanning and frame shifting to read overlapping open reading frames.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes are used to translate the viral protein. Like most RNA viruses, the mRNA will have a 5' 7-methylguanosine cap, and the usual 3' poly A tail. The 5' cap will be recognized by the necessary initiation factors (eIF4E &G), 3' end of the mRNA will bind the PABPs thus, allowing translation initiation to proceed. The structural proteins consist of capsid, envelope proteins, and a protease. The non-structural proteins are RdRp and multiple accessory proteins involved in overcoming the host cell's antiviral defenses.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome.

Note that togaviruses, like many viruses, take care of the problem of replicating the ends and priming replication via the same mechanism.

As discussed above, togaviruses must have genes to produce an RdRp. Positive sense RNA viruses whose genomes are equivalent to mRNA can be translated immediately upon entrance into the cell. As a result, they do not need to carry RdRp into the host cell with the genome. They must, however, produce it immediately in order to begin replicating. Note: all RNA viruses of animals have linear genomes and as such must have mechanisms to make sure the ends of their genomes are replicated. Most RNA viruses, including togaviruses, overcome the need to replicate their ends easily. RdRp starts synthesizing at the end of the genome without a primer. Starting without utilizing a primer is referred to as **de novo synthesis**. De novo initiation by RdRP occurs at the first nucleotide. For most RNA viruses, this method of replication ensures that the ends are replicated.

VIRION FORMATION

Viral capsids form spontaneously in the viral factory, and the virus leaves the cell via budding from the plasma membrane.

AVOIDING THE HOST IMMUNE RESPONSE

Togaviruses and matonaviruses avoid detection of pathogen recognition receptor (PRR) TLR7 which recognizes the pathogen associated molecular pattern (PAMP) of single stranded endosomal RNA by remaining in their capsids until they leave the endosome. They avoid the PRRs RIG-I (cytoplasmic RNA lacking a 5' cap), MDA-5 (cytoplasmic dsRNA, no proper 5' cap on RNA), PKR and OAS and possibly RNAi (ds RNA) by replicating within viral factories. Recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication to avoid activation of type I interferons, as well as PKR, OAS, and MDA-5 and possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi) although this is still under debate. As such, RdRp is unable to proofread as it replicates the genome. The lack of RdRp proofreading leads to a high mutation rate which allows antigens to change rapidly.

TOGAVIRUS DISEASES

The alphaviruses are all transmitted by the bite of an infected mosquito. Recall that the viruses in the genus *Alphavirus* include the equine encephalitis viruses (EEEV, WEEV, and VEEV), chikungunya, O'nyong nyong, Ross River virus, Mayaro virus, Fort Morgan virus, Highlands J virus, and Sindbis virus. The virus is inoculated into the blood when the female mosquito spits anticoagulant from her salivary glands to keep the blood flowing thus allowing her to continue taking the meal that will provide protein for the eggs she is producing. Infection occurs in fibroblast, muscle, and Langerhans cells. Langerhans cells are dendritic cells that reside in the epidermis and in the epithelia of the respiratory, digestive, and urogenital tracts. They specialize in antigen presentation and belong to the skin immune system. Langerhans cells or other dendritic cells transport the virus to lymph nodes. The virus then spreads via viremia through the blood to target tissues. These diseases all include a rash and arthritis when the virus affects the skin and joints as well as possible encephalomyelitis, inflammation of the brain and spinal cord, when the virus infects these tissues.

These infections follow a predictable pattern, as shown in Figure 15-5. Shortly after infection, the virus replicates to high levels in the site of infection and then shortly thereafter is seen in high levels in the blood. After around 4 days, the virus disseminates to target tissues, the muscle, joints, brain and skin of the body beyond the site of the initial infection. Viral titers peak approximately 7 days after infection and then begin to fall. If we follow the immune response during this time, a rapid interferon response is detected by day 2 that peaks at day 3 and leads to inflammation that begins on day 3 and is mediated by T-cells, B-cells, and monocytes as well as a robust antibody response that peaks at about day 7. The clinical symptoms correlate with the viral replication and immune response. Fever and joint pain begin about 2 days after infection when viral titers and interferon levels are high and begin to drop as the interferon levels decrease. Fevers rise again, however, as inflammation becomes prominent and rash, encephalomyelitis, and myositis (swelling and degeneration of muscle tissue) begin.

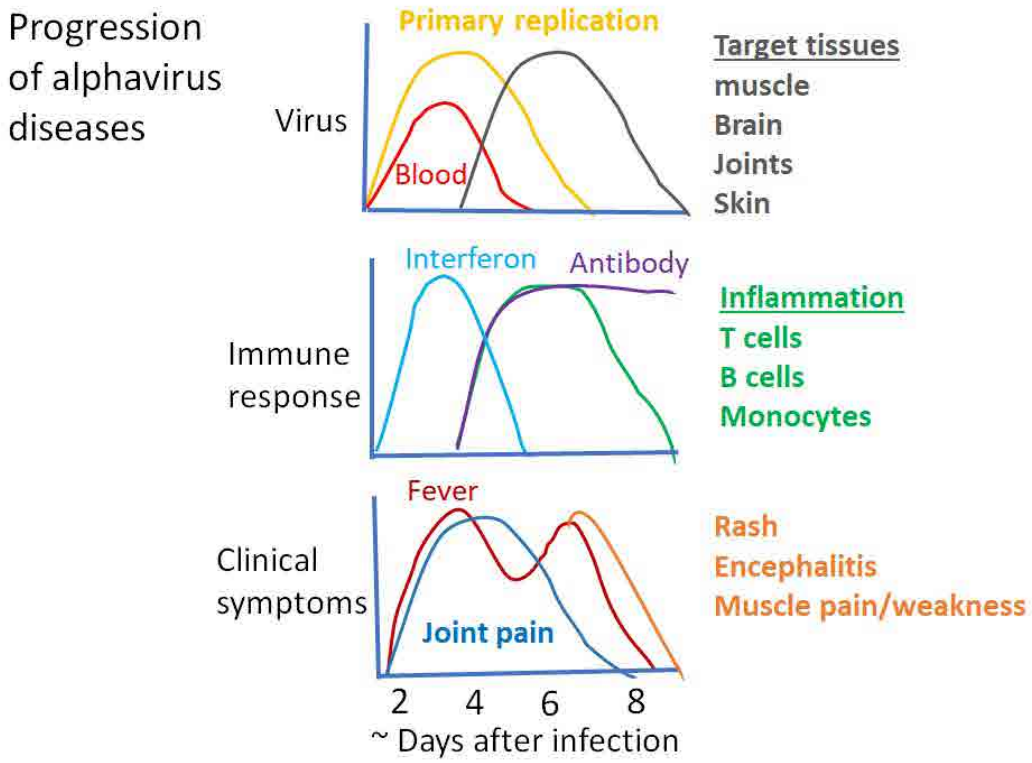


Figure 15-5. Progression of Alphavirus infections. Courtesy of Dr. Erica Suchman, Colorado State University.

EQUINE ENCEPHALITIS VIRUSES

The equine encephalitis viruses are associated primarily with encephalitis. They each have birds as their reservoir hosts, although VEE also has small mammal reservoirs and WEE has been proposed to have rodent and rabbit reservoirs as well. Although they all cause disease in horses and humans, they have distinct patterns that differ. WEEV causes a very low viremia in horses with a case-fatality rate of 20-40% and only has a 3-10% case-fatality rate in humans. EEEV also usually has a low viremia in horses with a case-fatality rate in horses of 50-90% and a high 50-70% case-fatality rate in humans. VEEV has a high viremia in horses with a 50-80% case-fatality rate and causes 1-30% case-fatality rate in humans. Each virus also has different bird reservoirs as well as mosquito vectors. Recall from Chapters 4 and 14 that vector-borne diseases have a reservoir host (the intended host) where the virus reaches high viremia but does not usually kill the host. The viral cycle usually involves an enzootic or sylvatic cycle (jungle) that occurs between animals (birds for the alphaviruses) and the vector, for example mosquito. Bridge vectors will lead to a spillover event where the virus begins infecting humans when they transmit the virus to humans in a new rural environment, called the **zone of emergence**, and then to urban environments leading to establishment of the disease in these areas, and an epizootic or epidemic transmission cycle often involving a new set of vectors. The cycle might also involve an amplifying host that achieves high levels of viremia that can infect more vectors but is not the reservoir, as it often succumbs to disease. It is important to remind yourself that every mosquito-borne viral disease has a specific set of mosquitoes that are capable by both biology and lifestyle (for example living in or near homes) of transmitting the virus, and most diseases are transmitted by different mosquitoes in the natural sylvatic cycle (in the jungle) than those that transmit the virus in rural and urban settings. Figures 15-6 A & B show a few examples of mosquitoes capable of transmitting alphavirus infections. You will see in Figure 15-7, 15-8, and 15-9 that each of the equine encephalitis viruses has a slightly different cycle that involves multiple mosquitoes and reservoirs. You will also notice that only in VEEV outbreaks do horses serve as an amplifying host. This is because only VEEV induces high viremia in horses allowing them to serve as sources of infection for mosquitoes that then transmit the virus to new horses and humans. However, as horses have a high case-fatality rate, they cannot serve as the reservoir for the virus between outbreaks. As one might expect, EEEV is found in the Eastern coast of the United States of America, whereas WEEV is found more on the Western region of the United States but is found all the way to the Midwest. All three are found in North, Central, and South America.

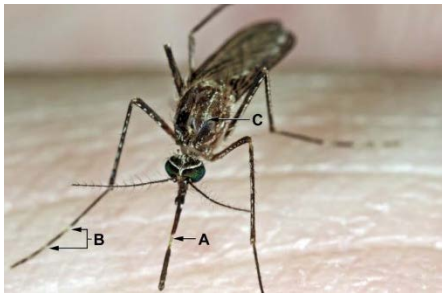


Figure 15-6A. A female Culex tarsalis mosquito as it is about to begin feeding after having landed on the skin of what will become its human host. Note the light-colored band wrapped around its dark-scaled proboscis (A), and the multiple, similarly light-colored bands wrapped around its distal appendages, i.e., the tibia and femur, of its forelegs, and middle pair of legs (B), and the presence of one of the two silver markings on its dorsal scutum (C). The epidemiologic importance of C. tarsalis lies in its ability to spread western Equine Encephalitis (WEE), St. Louis encephalitis (SLE), and California encephalitis and, at the time when this image was created, was the main vector of West Nile virus (WNV) in the western United States. Courtesy of https://phil.cdc.gov/Details.aspx?pid=7957 James Gathany

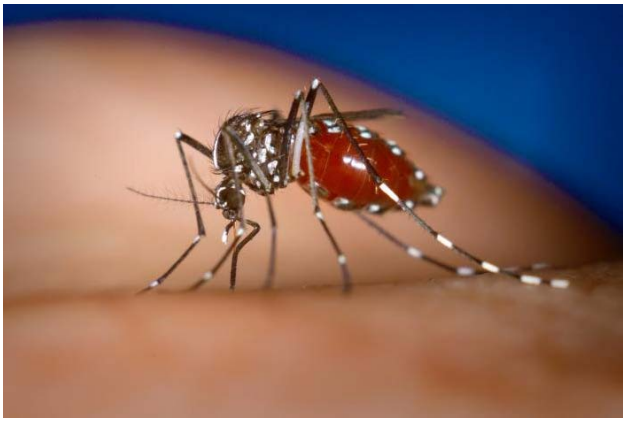


Figure 15-6B. A female Aedes albopictus mosquito as she is feeding on a human host. You can see the red, needle-like proboscis that has penetrated the skin and is filled with the host's blood, which has filled her abdomen. A. albopictus is known to be a vector for a number of arboviral diseases, including yellow fever, dengue fever, and chikungunya fever. Courtesy of https://phil.cdc.gov/Details.aspx?pid=4490 James Gathany CDC.

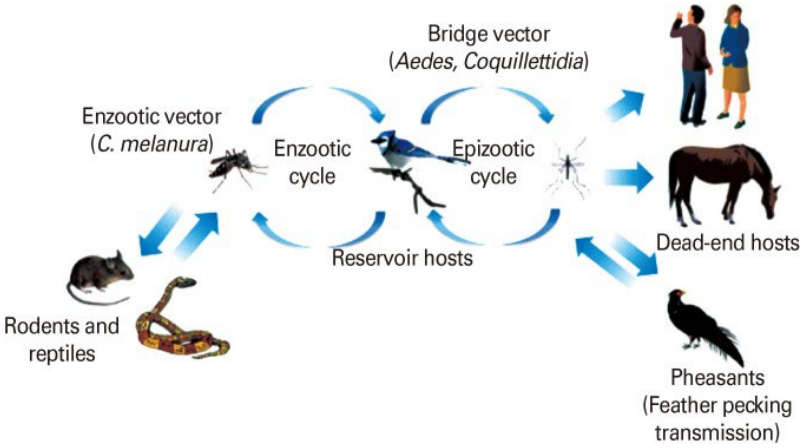


Figure 15-7. Enzootic and epizootic/epidemic transmission cycles of eastern Equine Encephalitis virus (EEEV). The enzootic EEEV transmission cycle is maintained between passerine birds as reservoir/amplification hosts and Culiseta melanura, as the main enzootic vector in swamp habitats. Rodents/marsupials may serve as principal enzootic vectors and reservoirs in South America. Songbirds develop extremely high levels of viremia, enough to infect both enzootic vectors as well as a variety of bridge vectors. Humans and horses are dead-end hosts since they do not develop sufficient viremia to transmit the virus. Courtesy of Zoonotic encephalitides caused by arboviruses: Transmission and epidemiology of alphaviruses and flaviviruses – Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Enzootic-and-epizootic-epidemic-transmission-cycles-of-Eastern-equine-encephalitis-virus_fig4_259743924

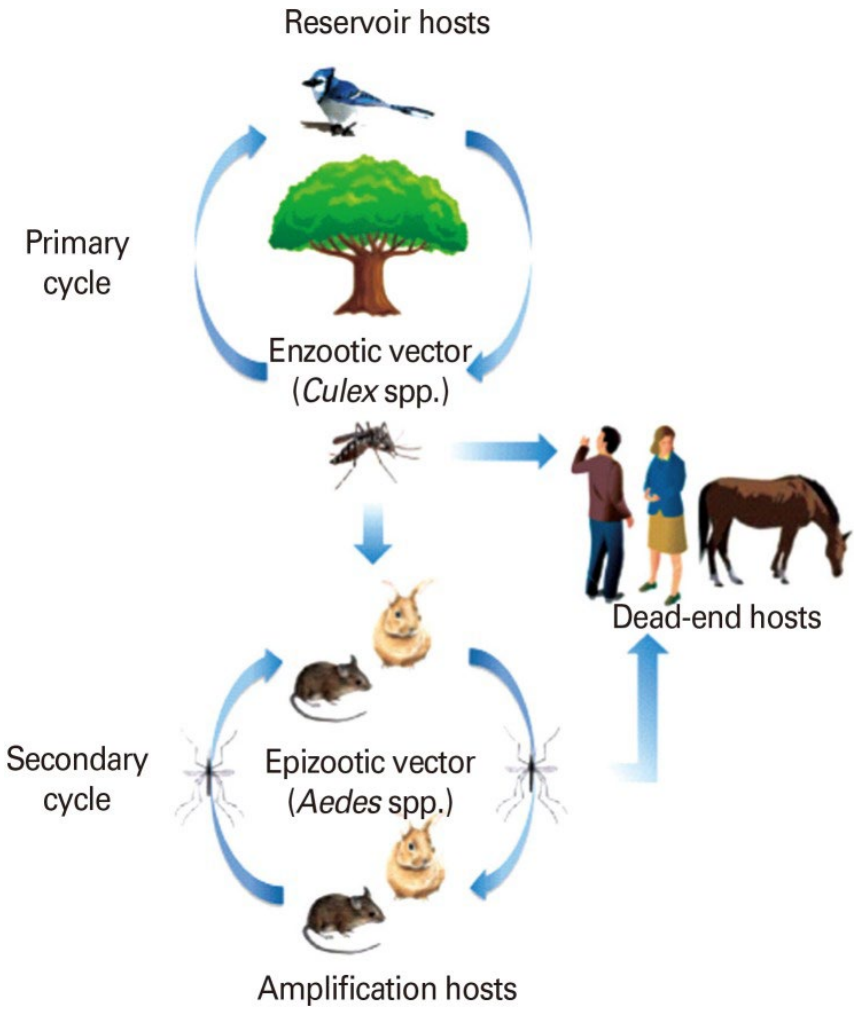


Figure 15-8. Enzootic and epizootic/epidemic transmission cycles of western Equine Encephalitis virus (WEEV). WEEV is maintained in an enzootic cycle between songbirds as reservoirs and its specific mosquito vector, C. tarsalis. Domestic and wild birds are considered important reservoir and epizootic amplifying hosts. It has also been suggested that rabbits and rodents can serve as amplification hosts when they are infected with WEEV by Aedes mosquitoes. Courtesy of Zoonotic encephalitides caused by arboviruses: Transmission and epidemiology of alphaviruses and flaviviruses – Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Enzootic-and-epizootic-epidemic-transmission-cycles-of-Western-equine-encephalitis-virus_fig5_259743924

Prevention of Equine Encephalitis Viruses

The best method of protection of horses is vaccination. Inactivated EEEV and WEEV vaccines exist, and both an inactivated and attenuated VEEV vaccine are available. However, there are not human vaccines. Mosquito control by reducing standing water is also recommended, as well as restricted movement of infected horses.

Diagnosis of Equine Encephalitis Viruses

Diagnosis can be performed by virus isolation, viral RNA (RT-PCR), antibody (IgM/IgG in serum) in neutralization assays demonstrating a 4-fold increase in antibody titers between acute and convalescent samples taken at least 2 weeks apart for IgG tests.

ALPHAVIRUSES ASSOCIATED PRIMARILY WITH POLYARTHRITIS AND RASH

Chikungunya virus (CHIKV), O'nyong nyong virus, Ross River virus and sindbis are all mosquito borne viruses that cause rash and **arthralgias** (joint pain). Chikungunya has been identified in over 60 countries in Asia, Africa, Europe, and the Americas. It was first identified in Africa in 1952, then in Asia in the late 1950s and early 1960s, and in 2013 it was found in North, Central, and South America. O'nyong nyong virus is found primarily in Africa. Ross river virus is found primarily in Australia, and Sindbis is found throughout Africa, Asia, Australia, and the Middle East. We will focus on chikungunya in this chapter as an example.

Chikungunya is transmitted primarily by *Aedes aegypti or Aedes albopictus* mosquitoes, as shown in Figure 15-10. The reservoir hosts are humans and monkeys. Although the disease is very painful and can often become a debilitating illness, it is rarely fatal. The clinical symptoms include fever, headache, lethargy, nausea, vomiting, rash, muscle aches, and prolonged joint pain. The fever usually begins 2-6 days after infection as the viremia begins and lasts the same time period as the viremia, about 1 week, as do the **myalgia** (muscle aches) and rash, although rash usually starts a few days after fever and myalgia. The **polyarthraligia** and **arthritis** (bone pain and stiffness), however, can last weeks to months in 95% of patients. The joint pain and stiffness coincide with the development of IgM (3-8 days after symptom onset lasting 1-3 months) and IgG (4-10 days after symptoms and persisting for years).

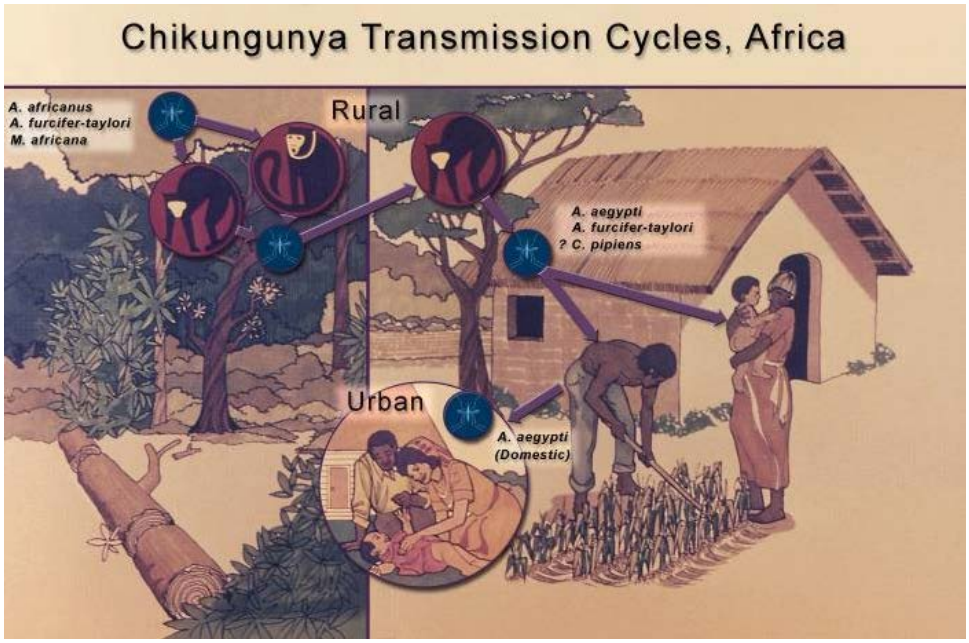


Figure 15-10. The transmission cycles implemented by the disease known as chikungunya fever. The virus responsible for the disease is a member of the genus Alphavirus in the family Togaviridae. This diagram lays out the modes of possible transmission in Africa. Chikungunya virus is spread by the bite of an infected mosquito. Mosquitoes become infected when they feed on a person infected with chikungunya virus. Infected mosquitoes can then spread the virus to other humans when they bite. Monkeys and possibly other wild animals may also serve as reservoirs of the virus. The mosquito involved in transmission is Aedes aegypti, the yellow fever mosquito, a species of mosquito that breeds in household containers and is attracted to humans for blood meals. Courtesy of https://phil.cdc.gov/Details.aspx?pid=14764 Dr. Thomas Monath.

Prevention of Chikungunya Virus Infections

There is no vaccine for chikungunya virus, therefore the best prevention is mosquito control including elimination of standing water, wearing long pants and shirts, avoiding being outside at dusk and dawn (prime mosquito feeding times), using DEET to repel mosquitoes and coating your clothing with permethrin as shown in Figure 15-11 which explains how to avoid mosquito born Zika virus infections.

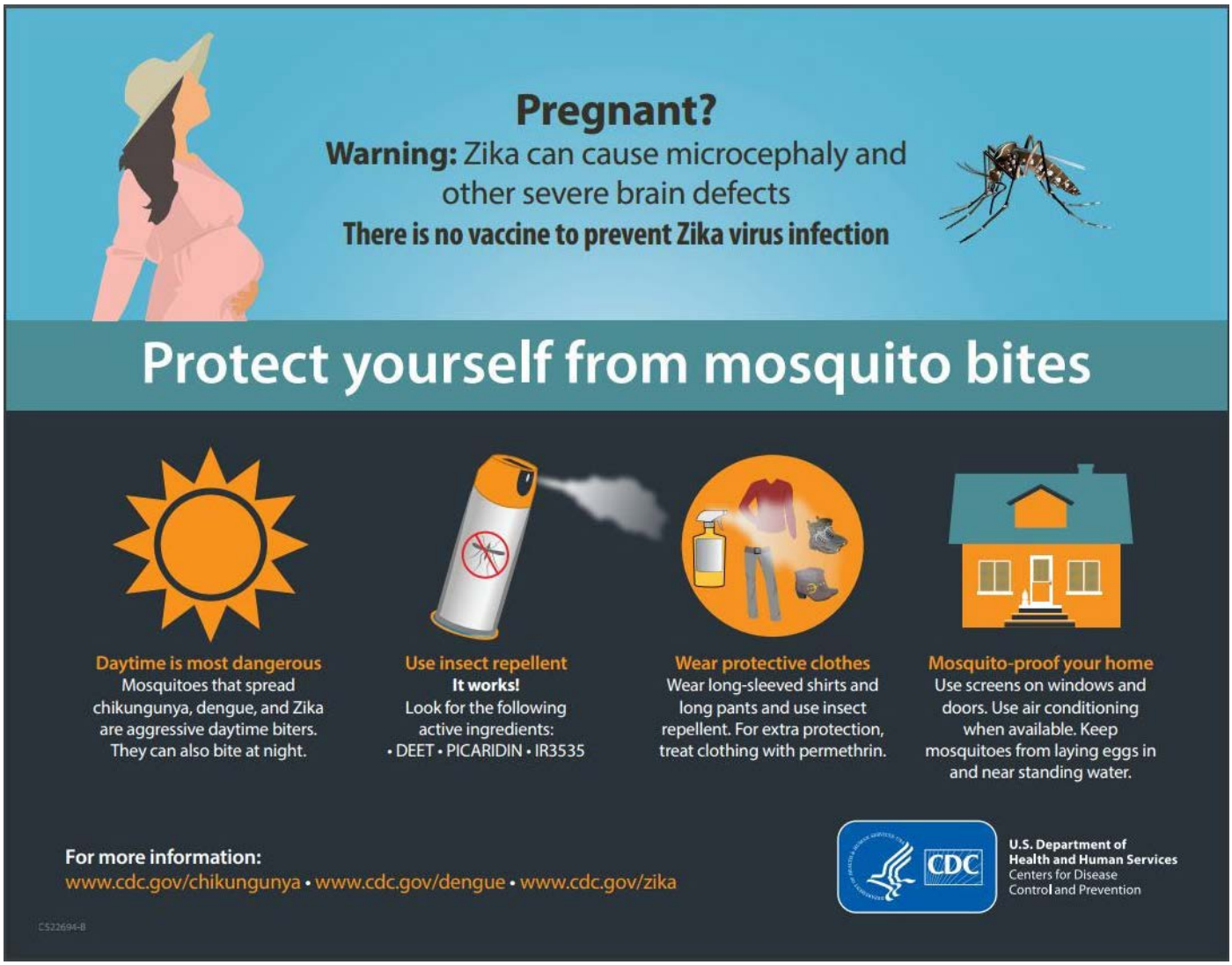


Figure 15-11. Centers for Disease Control recommended precautions for avoiding mosquito borne diseases. Courtesy of https://cdc.gov/Chikungunya.

Treatment of chikungunya

There are no specific treatments other than anti-inflammatory drugs to control the pain.

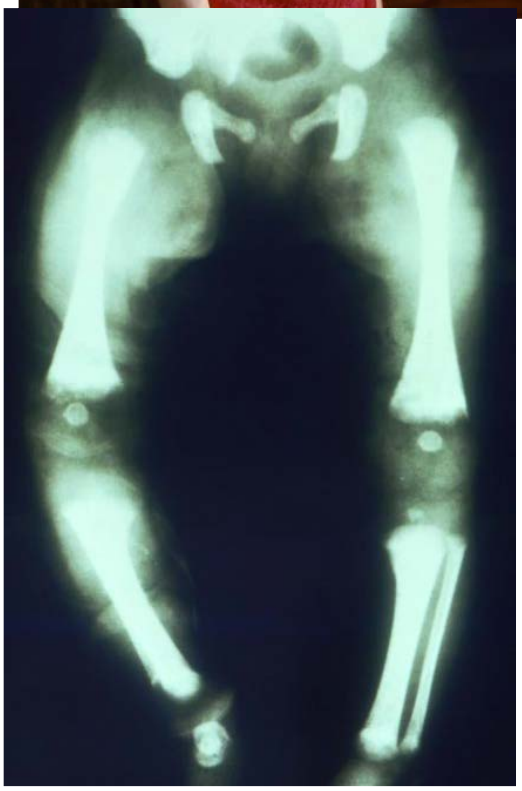
Diagnosis of chikungunya

Diagnosis can be performed by virus isolation, viral RNA (RT-PCR), antibody (IgM/IgG in serum) in neutralization assays demonstrating a 4-fold increase in antibody titers between acute and convalescent samples taken at least 2 weeks apart if assaying for IgG.

MATONAVIRIDAE RUBIVIRUS DISEASE: RUBELLA

There are ten genotypes and one serotype of the genera rubivirus found in the family *Matonaviridae*. Humans are the only natural host and reservoir. Transmission is by respiratory secretions. It is predominately a childhood disease with rash, as shown in Figure 15-12A. **Lymphadenopathy** (swollen lymph nodes) and arthritis is sometimes seen in adolescent and adult women, however, in rare instances the virus can cause **encephalomyelitis** (swelling of the brain and spinal cord). Additionally, congenital rubella syndrome can occur when the virus crosses the placenta and infects the fetus. Transmission occurs 90% of time during the first 8 weeks of gestation, 25-35% during second trimester, and increases again near term. Symptoms include deafness, skeletal abnormalities (Figure 15-12B), **cataracts** (cloudy lenses of the eye, Figure 15-12C), glaucoma, heart disease (Figure 15-12D), intellectual disability, delayed endocrine abnormalities such as Type I diabetes, and thyroid disease.

Figure 15-12A. Two young siblings both exhibiting a rash on their faces, arms, and torsos. Both had been infected with the German measles virus, also known as rubella. Courtesy of https://phil.cdc.gov/Details.aspx?pid=22144



15-12B. This X-ray depicts a view of the lower extremities of a 3-day-old infant who had been born with skeletal abnormalities due to a case of congenital rubella having been born to a mother ill with the disease while pregnant. Note the skeletal demineralization and cartilaginous irregularities surrounding the metaphyseal regions, both knee joints, which is typical in cases of congenital rubella. The round shape in the center of each knee represents the patella, or kneecap. Courtesy of https://phil.cdc.gov/Details.aspx?pid=17935 Aaron Rausen, MD.

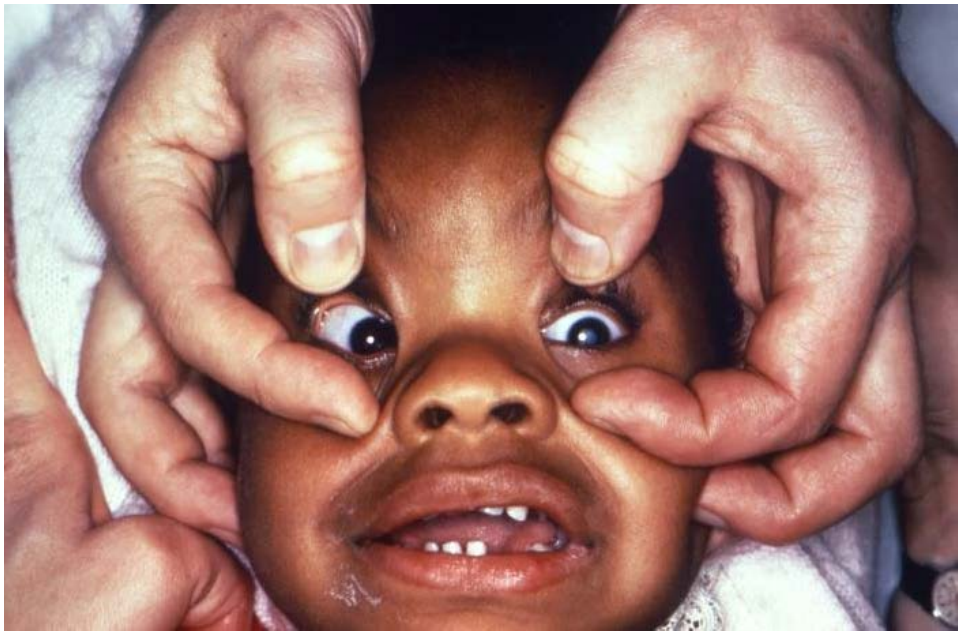


Figure 15-12C. This image depicts the hands of a clinician retracting the lids of both eyes of a 7-month-old child who had been operated on at age 3-days for the purpose of correcting a condition known as congenital glaucoma due to a case of congenital rubella. Note that the baby's left eye also displays the cloudiness associated with a congenital rubella cataract and can be compared to the normal appearance of the infant's right eye. Courtesy of https://phil.cdc.gov/Details.aspx?pid=17455 Dr. Andre J. Lebrun, CDC

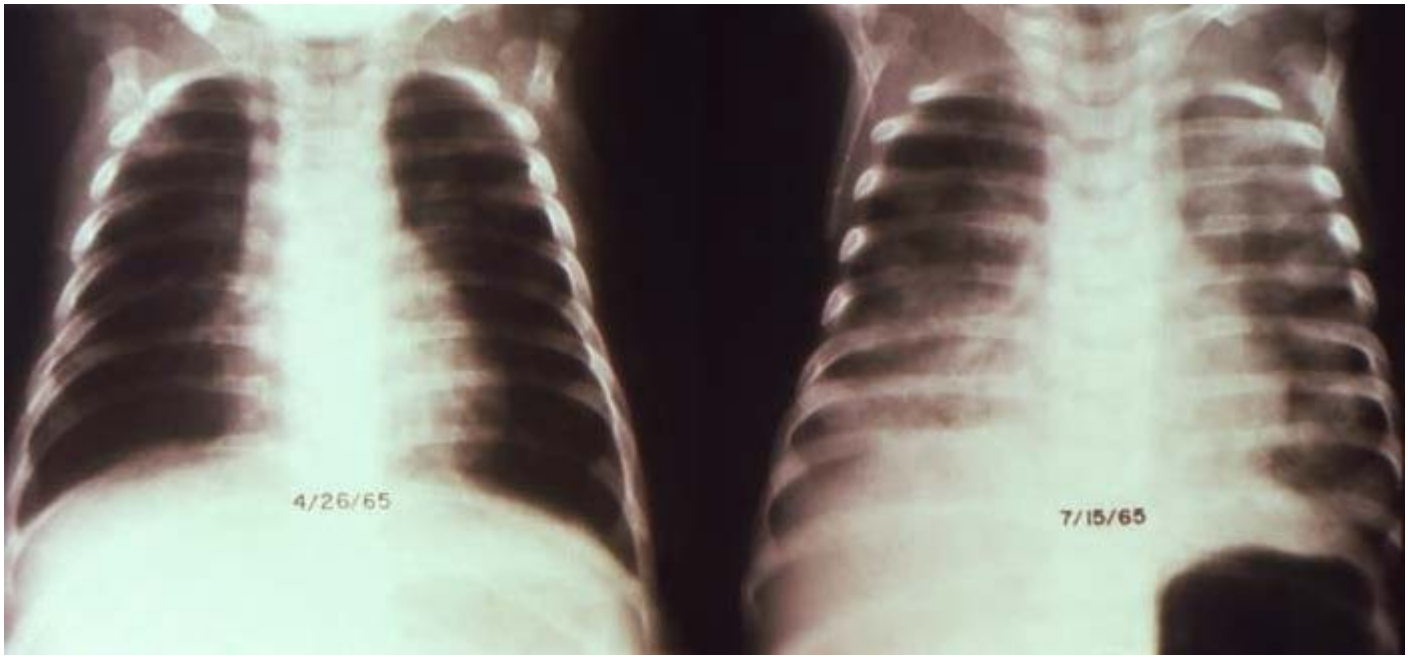


Figure 15-12D. Placed side-by-side for comparative purposes, these two chest x-rays of an infant's chest cavity which had been taken almost 3-months apart reveals the progressive worsening of a condition known as rubella pneumonitis as well as the onset of congestive heart failure. This infant had been born with congenital rubella. Note the increased radiopacity (whitish appearance) in the x-ray on the right due to an accompanying increase in pulmonary infiltrates. Courtesy of https://phil.cdc.gov/Details.aspx?pid=17782 Dr. Andre J. Lebrun, CDC

Rubella infections cause a fairly rapid viremia that peaks at about 2 weeks with virus shedding for up to 4 weeks. Rubella elicits a robust IgM response that is quickly replaced by the IgG response which brings the infection under control in most children, as shown in Figure 15-13.

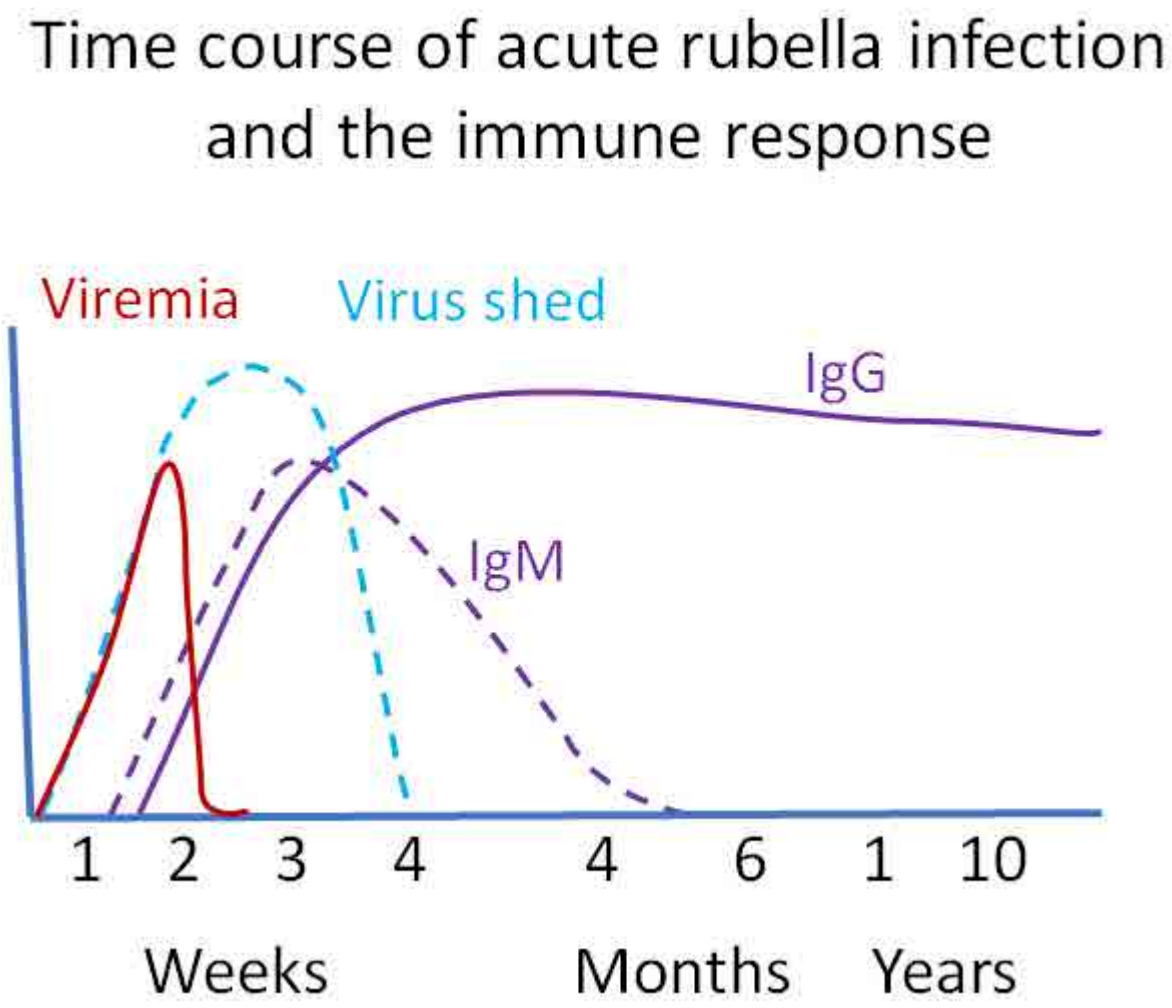


Figure 15-13. The time course of acute rubella infections and the accompanying immune response. Courtesy of Dr. Erica Suchman, Colorado State University.

PREVENTION OF RUBELLA

The best prevention is proper vaccination and boostering with the live-attenuated measles mumps rubella vaccine (MMR) or MMRV vaccine which also contains the Varicella Zoster virus that causes chicken pox and shingles. The vaccine should be administered at 12-15 months of age with a booster at 4 to 6 years of age. Figure 15-14 shows a 2013 flyer produced on behalf of the Measles & Rubella Initiative (M&RI) containing information that was intended to educate the viewer regarding the facts about the rubella virus and the need to vaccinate children against this viral disease. The rubella virus is a major cause of birth defects. Since it costs only $1 to vaccinate a child against measles and rubella, there is no excuse for measles, mumps, and rubella to continue to be problems. Both measles and rubella move fast, therefore M&RI has committed to moving faster.

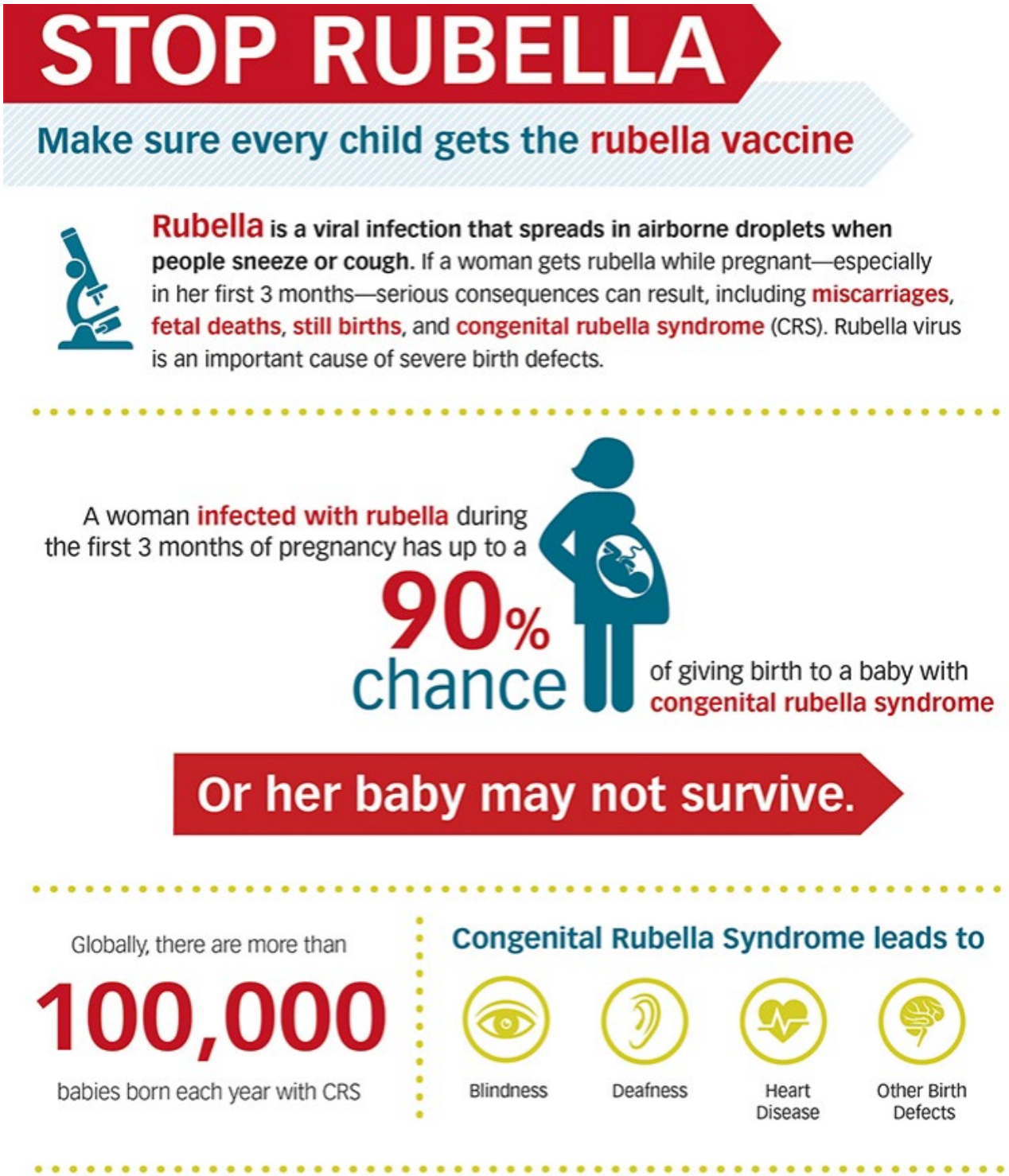


Figure 15-14. This 2013 flyer produced on behalf of the Measles & Rubella Initiative (M&RI) contains information that was intended to educate the reader about the rubella virus and the need to vaccinate children against this viral disease. The rubella virus is a major cause of birth defects, and yet it costs only $1 to vaccinate a child against measles and rubella. Both measles and rubella move fast, therefore M&RI has committed to moving faster. M&RI's main goal is that every child must get the rubella vaccine. Courtesy of https://www.cdc.gov/globalhealth/immunization/infographic/stop_rubella.htm Molly Kurnit; Melanie Jankun CDC

CALICIVIRIDAE

Caliciviruses have a naked icosahedral virion that is 27-40 nm in diameter with a genome of approximately 7-8 kb that is polyadenylated on the 3' end but lacks a 5' cap because the virus uses a viral capping protein to prime RNA replication as calicivirus RdRp lacks the ability to initiate replication de novo (without a primer), as shown in Figures 15-15A and B. The virus is named calici because of cup-shaped depressions on virus surface, and, in Latin, "*calyx*" = cup.

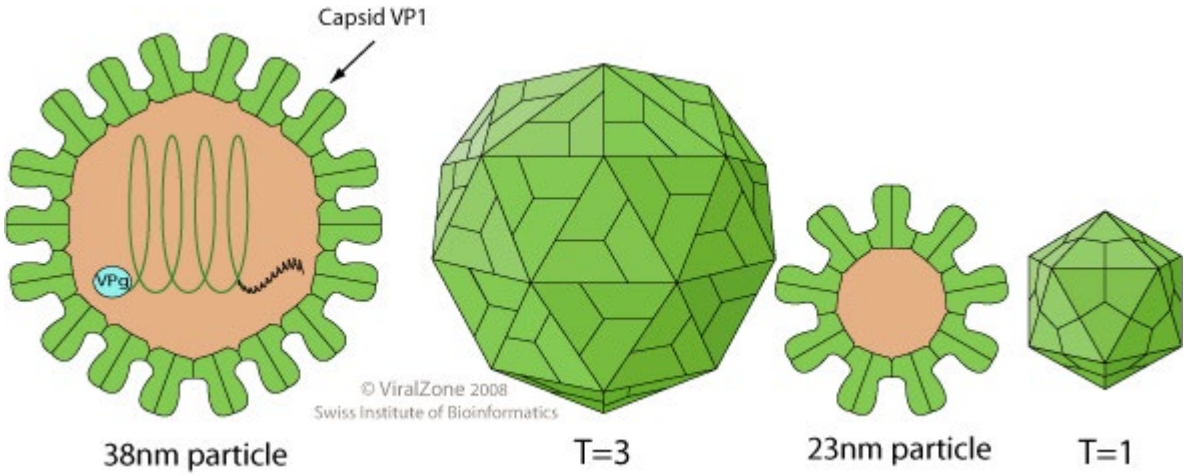


Figure 15-15A. Typical calicivirus virion. Courtesy of ViralZone https://viralzone.expasy.org/32

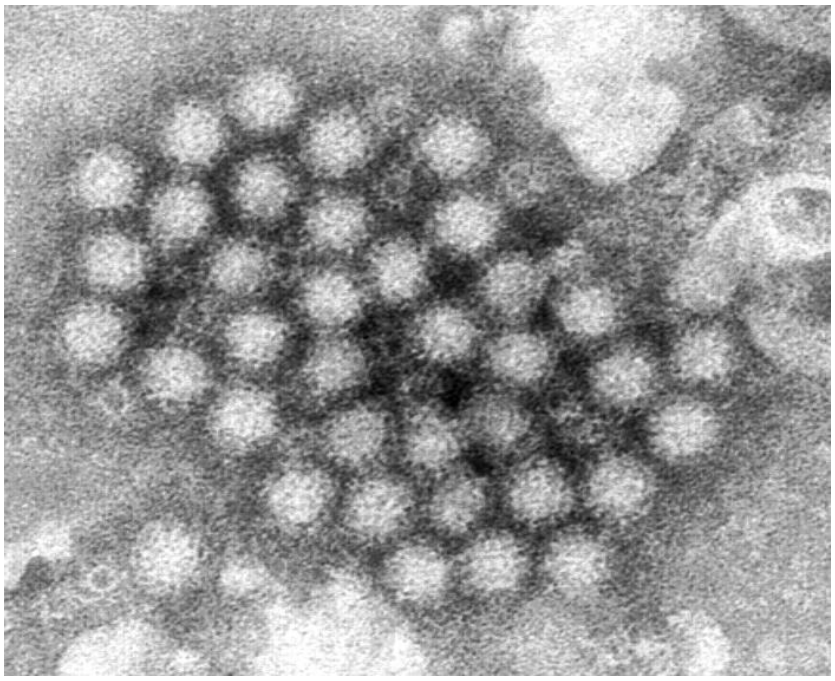


Figure 15-15B. Transmission electron microscopic (TEM) image revealing some of the ultrastructural morphology displayed by a cluster of norovirus virions, or virus particles. Courtesy of https://phil.cdc.gov/Details.aspx?pid=2172 Charles D. Humphrey.

The genomic RNA is infectious and serves as both the genome and viral messenger RNA. The full-length genomic mRNA encodes a polyprotein (ORF1) that produces the nonstructural replication enzymes including RdRp, the viral protease, and VPg 5' cap protein. One or two smaller ORFs are expressed from a subgenomic RNA and create the structural capsid proteins, as shown in Figure 15-16. Cleavage of ORF1 polyprotein by the virus-encoded protease yields the mature nonstructural proteins. Some noroviruses express an alternative ORF by leaky scanning.

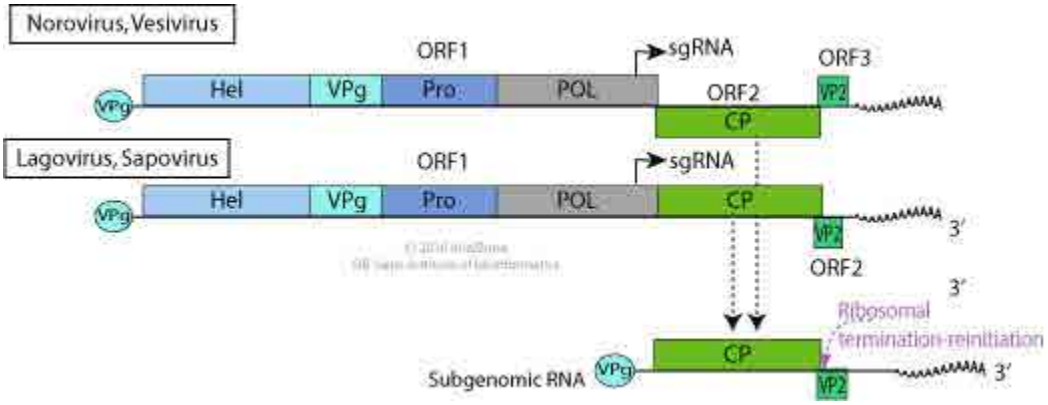


Figure 15-16. A typical Caliciviridae genome showing two open reading frames and cleavage sites for viral protease. The first ORF is read from the full-length genomic mRNA and encodes the non-structural proteins, including the RdRp, protease, and VPg 5' cap protein. The second is created from a subgenomic mRNA transcribed from the negative sense antigenome and encodes the structural proteins including the capsid proteins. Note that the 5' end of the genome has a viral protein cap (VPg) that is used to initiate RNA genome replication. Courtesy of ViralZone https://viralzone.expasy.org/32

CALICIVIRUS CYTOPLASMIC REPLICATION

As shown in Figure 15-17 the virus replication includes:

1. Attachment to host receptors mediates endocytosis of the virus into the host cell.
2. Uncoating and release of the viral genomic RNA into the cytoplasm by an as yet still unknown mechanism.
3. Viral RNA is translated into a processed ORF1 polyprotein to yield the replication proteins. The replication proteins are autocleaved from the polyprotein by the viral protease.
4. Replication occurs in viral factories. A negative sense stranded RNA anti-genome is synthesized from the genomic positive sense RNA using the viral RdRp.
5. The negative sense RNA anti-genome is transcribed and replicated by RdRp thereby providing viral mRNAs and new positive sense RNA genomes as well as subgenomic mRNAs created from the negative sense antigenome.
6. Subgenomic mRNA translation gives rise to the capsid proteins.
7. Assembly of new virus particles and release by cell lysis.

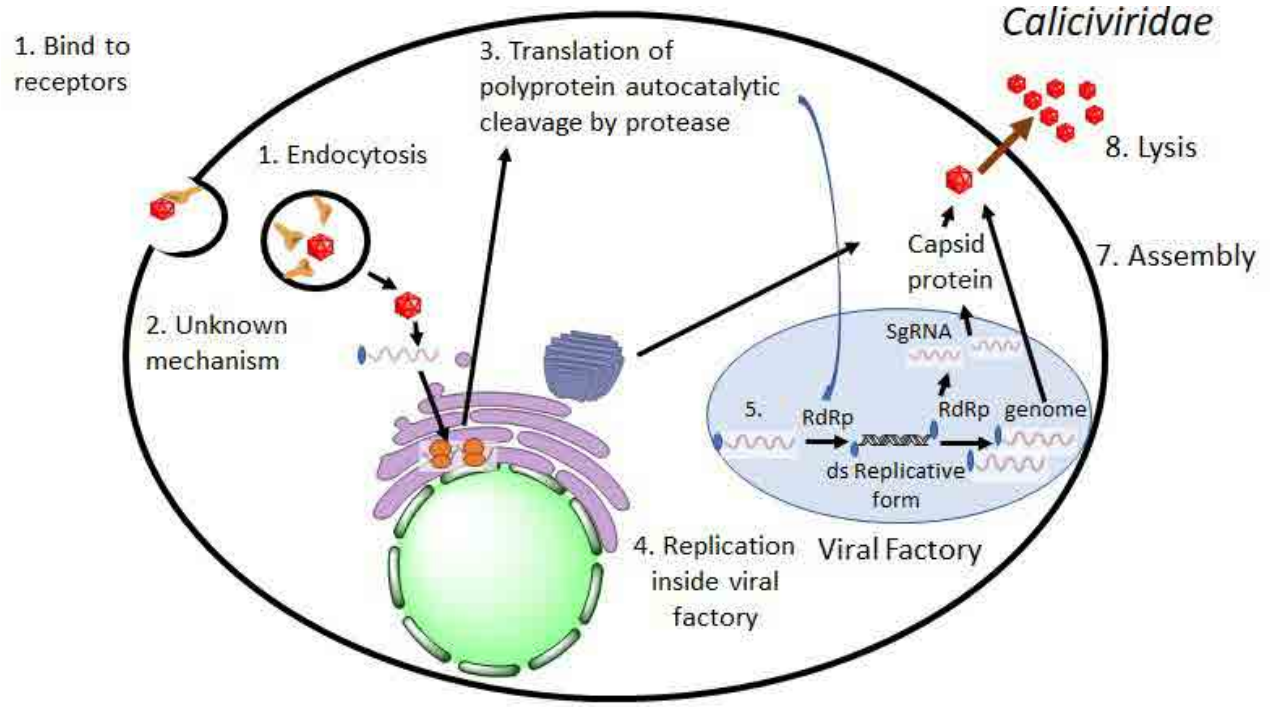


Figure 15-17. Calicivirus replication cycle. Courtesy of Dr, Erica Suchman, Colorado State University.

HOW DO CALICIVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS WHILE IT REPLICATES IN THE CYTOPLASM?

Most RNA viruses RdRp's have the ability to create the 5' cap and have a poly U tract in the 5' end of the template (negative sense) strand of RNA that will result in the addition of a long poly A tail when the RdRp stutters adding far more A's than exist in the tract. However, caliciviruses' RdRp lacks the ability to perform de novo RNA synthesis and therefore requires a primer to initiate replication and transcription. To prime it uses a protein called VPg that provides the 3' OH on which RdRp can begin adding NTPs. As such, there is a protein covalently attached to the 5' end of the mRNA and the 5' end cannot accommodate a 5' cap.

HOW DO CALICIVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all cytoplasmic replicating positive sense RNA viruses of animals, caliciviruses utilize a viral protease to cleave up a large viral polyprotein, as shown in Figure 15-2. Caliciviruses, like togaviruses and matonaviruses, create one large polyprotein from the full-length genomic mRNA and one smaller sub-genomic mRNA from the negative sense antigenome that creates a second open reading frame and polyprotein.

TRANSLATION OF VIRAL PROTEINS

We learned in Chapters 3 and 13 that translation initiation requires a whole host of initiation factors to bind the 5' cap. In particular, eIF4E binds to the cap and eIF4G binds to eIF4E and then to other initiation factors. So how do viruses without a 5' cap initiate translation? Recall that in caliciviruses there is a protein covalently attached to the 5' end of the mRNA and the 5' end cannot accommodate a 5' cap. However, this VPg protein will substitute for the 5' cap for ribosomal binding and thus translation initiation. Because VPg can substitute for the 5' cap to initiate translation caliciviruses do not have an IRES.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with a linear genome.

Note that caliciviruses like many viruses solve the problem of replicating the ends and priming replication via the same mechanism.

RNA viruses, with the exception of retroviruses, cannot use the host cell's transcription machinery DNA-dependent RNA polymerase (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As such, with the exception of retroviruses, they must all have genes to produce an RNA-dependent RNA polymerase (RdRp). Positive sense RNA viruses whose genomes are equivalent to mRNA can be translated immediately upon entering the cell and, as such, do not need to carry RdRp into the host cell with the genome but must produce it immediately in order to begin replicating. Note: all RNA viruses of animals have linear genomes and as such must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses replicate their ends easily as RdRp starts synthesizing at the end of the genome without a primer. This is referred to as **de novo synthesis**. De novo initiation by RdRP occurs at the 1st nucleotide. For most RNA viruses this method of replication ensures that the ends are replicated. However, as discussed above, caliciviruses' RdRp lacks the ability to perform de novo RNA synthesis and requires a primer to initiate replication and transcription. To prime it uses a protein called VPg that binds to the 5' end of the genome and provides the 3' OH on which RdRp can begin adding NTPs. This both serves as the primer and ensures that the ends of the genome are replicated.

VIRION FORMATION

Viral capsids form spontaneously in the viral factory. The virus leaves the cell when the cell lyses allowing the virus to leave.

AVOIDING THE HOST IMMUNE RESPONSE

Caliciviruses avoid detection of pathogen recognition receptor (PRR) TLR7 which recognizes the pathogen associated molecular pattern (PAMP) of single stranded endosomal RNA by remaining in their capsids until they leave the endosome. Caliciviruses avoid the PRRs RIG-I (cytoplasmic RNA lacking a 5' cap), MDA-5 (cytoplasmic dsRNA, improper cap), PKR, OAS and RNAi (ds RNA) by replicating within viral factories. Furthermore, recall from Chapter 13 that RdRp does not keep the two RNA strands together during replication to avoid activation of type I interferons, PKR, OAS, and MDA-5 as well as possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi), although this is debated. As such, RdRp is unable to proofread as it replicates the genome which leads to a high mutation rate which allows antigens to change rapidly.

CALICIVIRIDAE DISEASES: NOROVIRUS

The main *Caliciviridae* disease is caused by the Norwalk virus, otherwise known as norovirus, that causes outbreaks of **gastroenteritis**, inflammation of the gastrointestinal track that can lead to abdominal pain, cramps, diarrhea, nausea, and vomiting. The virus is transmitted primarily by contaminated raw foods and shellfish. Outbreaks are commonly associated with schools, nursing homes, restaurants, and cruise ships. It is a pathogen with a fecal-oral transmission cycle due to poor hygiene practices. The virus replicates in intestinal epithelial cells, and it impairs intestinal absorption of water and nutrients. Symptoms usually resolve in about 24 hours.

CORONAVIRIDAE

Although all of the virus families discussed in this chapter have more than one ORF, viruses in the *Togaviridae, Matonaviridae* and *Caliciviridae* families create their second ORF by the same mechanism, while viruses in the family *Coronaviridae* use a unique mechanism. Coronaviruses are enveloped with a helically symmetric nucleocapsid and are between 118 to 136 nm in diameter containing a positive sense RNA genome of 26 to 32 kb (the largest of all RNA virus genomes) as shown in Figures 15-18 A & B. The genomes are capped and polyadenylated. The leader RNA (65-89 bp) at the 5' end of the genome is also present at the ends of each subgenomic RNA. The prominent envelope proteins are spiked glycoproteins that give coronaviruses a unique appearance of a sun or crown (Latin *corona*) as shown in Figures 15-18 A, B, & C.

The family contains two subfamilies, *Orthocoronavirinae* and *Torovirinae*. Within *Torovirinae* are multiple toroviruses such as human torovirus, bovine torovirus, Chinook salmon torovirus. Human toroviruses cause nosoncomial gastroenteritis. Within *Orthocoronavirinae* there are 4 genera, *Alphacoronavirus*, *Betacoronavirus,* G*ammacoronavirus* and *Deltacoronavirus*. The most significant public health threats are from the betacoronaviruses, including **Middle East respiratory syndrome coronavirus** (MERS-CoV) (*Merbecovius* subgenus), **severe acute respiratory syndrome coronavirus** (SARS-CoV) (*Sarbecovirus* subgenome), and, as of this writing, the current **coronavirus disease 2019** (COVID-19) caused by the virus SARS-CoV-2 (*Sarbecovirus* subgenome). *Alphacoronavirus* and *Betacoronavirus* also contain four common cold viruses (229E, NL63, OC43, HKU1), as well. Taxonomic analysis indicates that bats likely the original sources of all mammalian coronaviruses including MERS-CoV, SARS-CoV, and SARS-CoV-2. Phylogenetic trees show that these viruses share genetic similarity to currently circulating bat viruses. SARS-CoV and possibly SARS-CoV-2, in particular, appear to have emerged from individuals who contracted the disease from live animal markets. However, people did not seem to catch the disease directly from the bats, but rather from **amplifying intermediate** (sometimes called "**bridge**") **hosts.**

Recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication for most RNA viruses to avoid detection by type I interferons, PKR, OAS, and MDA-5, and possibly dicer, the enzyme that

recognizes long stretches of double stranded RNA to activate RNA interference (RNAi) as well as, although is heavily debated. As such, RdRp is unable to proofread as it replicates the genome which leads to a high mutation rate that allows antigens to change rapidly. This is not the case for coronaviruses, which do keep the two strands of RNA together during replication allowing the viral enzyme non-structural protein 14 (nsp14; also called ExoN, an exonuclease that removes mismatched nucleotides) that is part of the **replicase complex** (also called the **replisome**) that includes the RdRp and ExoN to proofread the newly synthesized RNA. This ability to proofread replication allows coronaviruses to achieve much larger genomes as their mutation rates are not nearly as high as other RNA viruses. However, having the two strands remain together facilitating proofreading leaves them more vulnerable to the host's antiviral defenses such as activation of type interferon responses and possibly RNAi. Note the replicase complexes with even more enzymes and other proteins to form the **replisome** that carries out replication and proofreading.

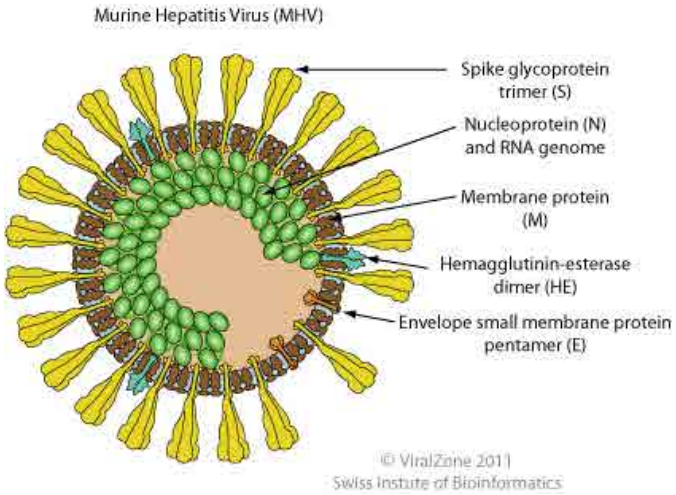


Figure 15-18A. Typical coronavirus virion. Courtesy of https://viralzone.expasy.org/30

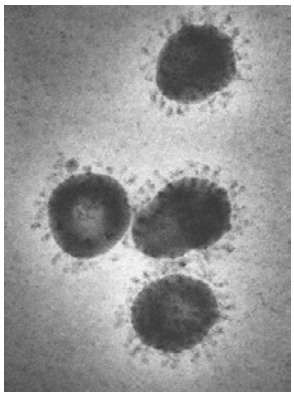


Figure 15-18B. 1975 Transmission electron microscopic (TEM) image revealing the presence of a number of infectious bronchitis virus (IBV) virions, which are Coronaviridae family members and members of the genus Coronavirus. IBV is a highly contagious pathogen which infects poultry of all ages affecting a number of organ systems including the respiratory and urogenital organs. Courtesy of https://phil.cdc.gov/Details.aspx?pid=10270 Fred Murphy; Sylvia Whitfield CDC

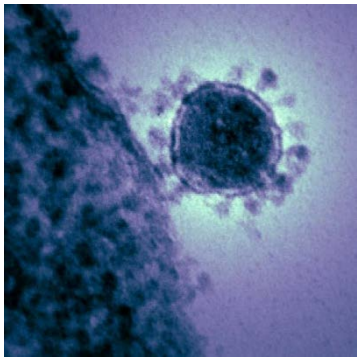


Figure 15-18C. Produced by the National Institute of Allergy and Infectious Diseases (NIAID), this highly magnified, digitally colorized transmission electron microscopic (TEM) image reveals ultrastructural details exhibited by a single, spherical shaped, Middle East respiratory syndrome coronavirus (MERS-CoV) virion with the spike proteins giving the virion a sun or crown like appearance. Courtesy of https://phil.cdc.gov/Details.aspx?pid=18114 NIAID

GENOME

The genomic RNA is infectious and serves as both the genome and viral messenger RNA. Genomic RNA encodes ORF1a. As for ORF1b, it is translated by ribosomal frameshifting. The resulting polyproteins are processed by viral proteases into the replisome, including an RdRp, that are required for subsequent RNA synthesis. Structural proteins are expressed as subgenomic RNAs from the negative sense subgenomic antigenomes. Each RNA (genomic and subgenomic) is translated to yield only the polypeptide encoded by the 5'-most ORF, as shown in Figure 15-19A and the entire replisome in Figure 15-19B.

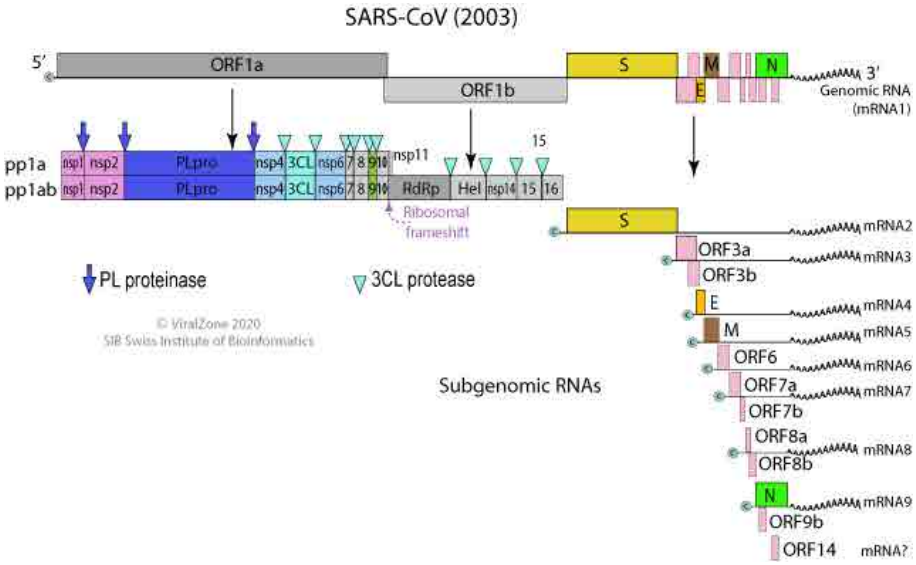


Figure 15-19A. Typical coronavirus production of nested subgenomic mRNAs by polymerase jumping such that all mRNAs have the same 5' end but are sequentially smaller in length. Courtesy of https://viralzone.expasy.org/30

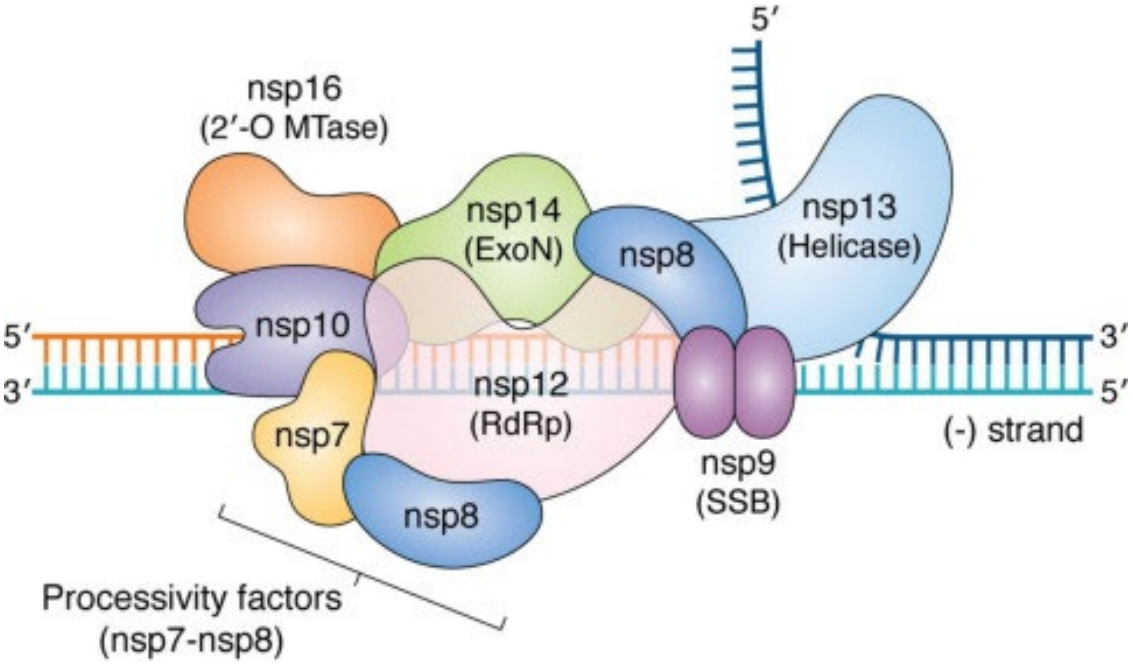


Figure 15-19B. ORF-1a encodes the different proteins in the coronavirus replisome that come together on the viral negative strand during synthesis of the positive-strand RNA. The core replicase is predicted to consist of the RdRp (nsp12), processivity factors (nsp7-8), and ExoN complex (nsp14, nsp10). A helicase (hel) that has been shown to unwind the dsRNA ahead of the replisome, and single stranded binding proteins (SSB, nsp9) protect single-stranded regions of the RNA. Additionally, nsp16 which is predicted to be involved in RNA capping. https://www.jbc.org/article/S0021-9258(17)49954-6/fulltext

CORONAVIRIDAE CYTOPLASMIC REPLICATION CYCLE

As shown in Figure 15-20, the replication cycle includes:

1. Attachment of the viral envelope spike protein to host receptors mediates entry of the virus into the host cell. This often requires cleavage of the S protein by the cellular protease into S1 and S2. S1 will bind to the host cell receptor. For SARS-CoV(-1) and SARS-CoV-2 this receptor is the ACE2 protein and the S1/S2 cleavage is performed by the cellular surface enzyme TMPRSS2. Subsequently, a second cleavage event occurs at a site called S2' and a number of cellular enzymes have been implicated in this event, including TMPRSS2. Although SARS-CoV and SARS-CoV-2 directly penetrate the plasma membrane of lung cells, in other cells they enter the cytoplasm via the endocytic pathway, where the low pH activates cellular cathepsins that perform the S2' cleavage. This can also occur with other coronaviruses, including MERS-CoV.
2. Fusion of the virus membrane with the cellular membrane (probably mediated by S2). The positive sense RNA genome is released into the cytoplasm where it can immediately serve as an mRNA for ORF1a and ORF1b, leading to synthesis of the replisome complex. **Importantly, only the ORF1 gene serves as an mRNA; all other genes must be synthesized into subgenomic mRNAs.**
3. Translation and viral protease cleavage of the Orf1a/Orf1b polyprotein into the functional replisome, including the RdRp and ExoN (Figure 15-19B).
4. Replication occurs in viral factories. A negative stranded (anti-sense) RNA genome is synthesized from the genomic positive sense RNA by viral RdRp as part of a replisome that contains an nsp14 exonuclease for proofreading.
5. Subgenomic anti-sense strands are generated when RdRp starts reading at the 3' end but occasionally jumps to identical **transcription regulatory sequences** (TRS) that are found at the 5' end of each gene (**polymerase jumping**) creating nested subgenomic antigenomes that are transcribed giving rise to subgenomic mRNAs. Note RdRp will only jump to the TRS found at the 5' in the leader (untranslated) sequence from the TRS found in the body of the RNA; never within the body. As such all the subgenomic mRNAs produced will have the same 5' end.
6. Assembly and budding at membranes of the endoplasmic reticulum (ER) and/or the Golgi complex.
7. Release of new virions by exocytosis. Prior to release from the cell, some coronaviruses, such as MERS-CoV have their spike proteins cleaved at the S1/S2 junction by the cell's **furin protease**. SARS-CoV-2 also has a weak furin cleavage site in its spike S1/S2 junction and it may be cleaved by cellular furin before viral release from the producer cell.

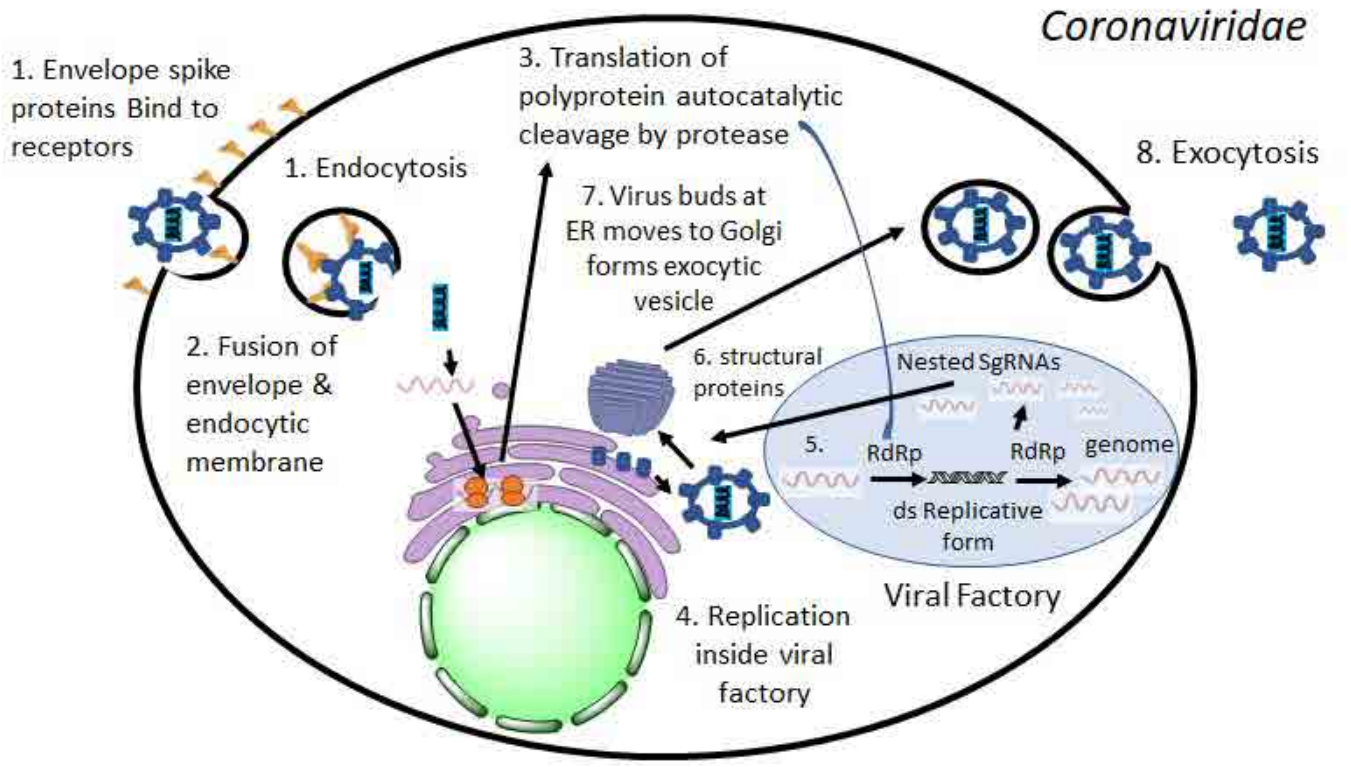


Figure 15-20. A typical coronavirus replication cycle. Note, this illustration is for endocytic entry of a prototypical coronavirus; however, SARS-CoV and SARS-CoV-2 can directly enter via the plasma membrane if the cellular protease TMPRSS2 is present (such as on lung cells). Courtesy of Dr. Erica Suchman, Colorado State University.

HOW DO CORONAVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS AND IT REPLICATES IN THE CYTOPLASM?

Like most RNA viruses, the coronavirus nsp16 of the replisome (Figure 15-19B) has the ability to create the 5' cap and have a poly U tract in the 5' end of the template (negative sense) strand of RNA. This results is the addition of a long poly A tail when the RdRp stutters adding many more A's than exist in the tract.

HOW DO CORONAVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

Like all cytoplasmic replicating positive sense RNA viruses of animals, coronaviruses use a viral protease to cleave up a large viral polyprotein as shown in Figure 15-19. Coronaviruses, like caliciviruses, matonaviruses and togaviruses, must create **subgenomic** mRNA (shorter than the full-length mRNA) to create more than one open reading frame. However, unlike togaviruses, matonaviruses and caliciviruses, coronaviruses do not create only one subgenomic mRNA from the antigenome. Instead, they create many by an entirely different mechanism involving polymerase jumping that leads to **nested subgenomic** mRNAs. Although RdRp begins genome replication at the 3' end of the genome to produce antigenome, the RdRp jumps to a TRS at the 5' end of each gene to transcribe the many subgenomic antigenomes that will be transcribed giving rise to subgenomic mRNAs, each with its own TRS. This transcription mechanism allows the virus to create a gradient of mRNAs with the full-length mRNAs that produce the RdRp and protease being produced least abundantly. Each successively smaller mRNA is produced in higher quantity until the smallest gene's mRNA (nucleocapsid protein) is produced in the highest abundance. This allows the virus to control how much of each protein it produces.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes are used to translate the viral protein. Like most RNA viruses, the mRNA will have a 5' 7-methylguanosine cap, and 3' poly A tail. The 5' cap will be recognized by the necessary initiation factors (eIF4E &G). Translation initiation can thus proceed as normal. The nonstructural proteins are translated from the full-length mRNA. Each of the other genes is translated from a subgenomic mRNA. The full-length mRNA also undergoes a **ribosomal frameshift** where the ribosome slips back one nucleotide after encountering a **pseudoknot** in the mRNA causing the ribosome to change frames and produce the proteins of a second open reading frame. **Leaky scanning** can also be used to create proteins in overlapping reading frames.

The structural proteins consist of nucleocapsid and envelope proteins as well as a spike protein that gives the virus its distinctive **corona** (sun) shape and is involved in binding and fusion. The non-structural proteins include protease, all of the components of the replisome including RdRp, and Nsp14 ExoN (the **proofreading exonuclease**), and multiple accessory proteins involved in overcoming the host cell's antiviral defenses.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes.

Note that coronaviruses, like many viruses, take care of the problem of replicating the ends and priming replication via the same mechanism.

As discussed above, coronaviruses must have genes to produce an RdRp. Positive sense RNA viruses, whose genomes are equivalent to mRNA, can be translated immediately upon entrance into the cell. As a result, they do not need to carry RdRp into the host cell with the genome. They must, however, produce RdRp immediately upon cellular entry to begin replicating. Note: all RNA viruses of animals have linear genomes and as such must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses, including coronaviruses, replicate their ends easily. RdRp starts synthesizing at the end of the genome without a primer. This is referred to as de novo synthesis. De novo initiation by RdRP occurs at the first nucleotide. For most RNA viruses this method of replication ensures that the ends are replicated.

VIRION FORMATION

Viral nucleocapsid proteins spontaneously package the viral genomic RNA in the viral factory. The virus leaves the cell via budding from the endoplasmic reticulum and then into the Golgi apparatus where it gains an exocytic vesicle and leaves the cell by exocytosis. For some coronaviruses, such as MERS-CoV, cellular furin cleaves the S1/S2 site so the virus is ready to infect the next cell that it encounters that has the appropriate receptor (dipeptyl peptidase-4 for MERS-CoV).

AVOIDING THE HOST IMMUNE RESPONSE

Many coronaviruses create accessory proteins to evade the immune response, including avoiding detection by PRRs and disabling PKR and OAS. For example, MERS-CoV has multiple open reading frames that are predicted to create anti-viral accessory proteins including its ns4a and ns4b proteins that inhibit OAS, one of the major antiviral cellular defenses activated by type I interferons. Recall that OAS activates RNAseL which cleaves up both cellular and host cell mRNA in response to binding double stranded RNA.

Although coronaviruses do not avoid the host immune response by rapid mutation of antigens as efficiently as other RNA viruses (due to the presence of the viral exonuclease that performs proofreading), genetic evidence indicates coronaviruses undergo mutation as well as recombination leading to variants. The mechanism of recombination is unclear. Some suggested mechanisms involve RdRp template switching or the exoribonuclease involved in proofreading. It requires coinfection of a cell with two distinct coronaviruses. At least 3 distinct recombinants of MERS-CoV exist in camels in Saudi Arabia, and one of these was responsible for the South Korean outbreak of 2015.

For a fantastic review of the T-cell response to SARS-CoV-2 infections please listen to TWiV #736 it is wonderful and really helped me better wrap my head around T-cell responses.

CORONAVIRUS DISEASES

Since the 1960s, four coronaviruses have been associated with the common cold, although none are known to cause lethal infections in most people. Two are alphacoronaviruses 229E and NL63, and two are betacoronaviruses OC43 and HKU1. All cause mild to moderate disease of the upper respiratory tract. The origin of these viruses is unknown, but their ancestral viruses are likely bat viruses. OC-43 is descendent of bovine coronavirus (BCoV) and likely spilled over into humans when cattle were domesticated. In 2002 SARS-CoV emerged with a 10% case-fatality rate due to pneumonia. In 2012 MERS-CoV emerged with a 36% case-fatality rate. In 2019 SARS-CoV-2, which causes COVID-19, emerged and to date appears to have a 1.7% case-fatality rate; although this varies significantly by region, access to health care, vaccination coverage and population's recovery from infection. All three of the recent emergences appear to be zoonotic having spilled over from animals into humans. Interestingly, it has been found that 3 of these common cold viruses can cause reinfections despite robust production of neutralizing antibody.

SARS

The SARS outbreak began in November of 2002 and was first identified in Hong Kong and continued until July of 2003 at which point it had spread to more than 26 countries on 5 different continents. There were 8,098 diagnosed cases and more than 774 deaths (9.6% case-fatality rate). SARS was characterized by infection of the upper respiratory tract that then spread to alveoli and resulted in destruction of pneumocytes, alveolar edema, and inflammatory cell infiltration. Infection of the airway epithelium when people inhaled respiratory droplets led to proinflammatory cytokine and chemokine release. In people who recovered, a decrease in proinflammatory mediators occurred as the SARS-CoV antibody response and anti-viral T cell responses increased. The opposite was seen in people with progressive disease. The proinflammatory chemokines and cytokines increased over time with poor antibody and anti-viral T cell response. This inflammation caused severe damage to the lungs and pneumonia that either led to death or recovery. However, recovery still resulted in **pulmonary fibrosis** (scarring of the lung tissue) and residual lung damage.

Why did the virus disappear in July and never return? Partially because quarantines of places with many infections (China, Hong Kong, Canada) helped to reduce transmission, partly because **patients showed signs of disease**

prior to shedding of virus (thus were easily identified) and partially because the virus did not transmit from person to person by casual contact requiring sustained, close contact such as occurs in a hospital or households.

Field studies showed that the reservoir host may have been the Chinese horseshoe bat (*Rhinolophus sinicus*). However, people were most likely not getting SARS virus directly from the bat. Instead they were getting it from civets, wild animals prized for their meat and commonly sold at live animal markets throughout Asia, that served as the amplifying hosts infecting people at the market. It is theorized that the stressful conditions of the markets made the civets immunosuppressed causing them to be more susceptible to infection with SARS-CoV and to shed more virus than they might in the wild.

Although SARS-CoV has not been found in bats, much of its genome has been discovered in a single cave in Yunnan Province, China. Several SARS-related coronavirus (SARSr-CoV) sequences were detected in multiple species of horseshoe bats in this cave, including 11 complete genomes. Many of the genes in these viruses had high similarity to SARS-CoV, although none of the viruses was clear direct ancestor or descendent of SARS-CoV. This suggests that the progenitor of SARS-CoV was likely a recombinant virus that was generated by the co-infection of bats with multiple coronaviruses over time. How much time is unknown. It also suggests that because of the high frequency of recombination that the ancestral virus of SARS-CoV will never be found; by now, it has undergone additional recombination with other SARSr-CoV, such that it no longer exists. Notably, in bats, all coronaviruses appear to be **intestinal viruses**; there is little evidence that other organs, such as their lungs, become infected with their coronaviruses. It is also important to note that **bats do not develop conspicuous disease** when infected with coronaviruses, which likely is because the viruses have adapted to infect bats without causing disease.

We now know that there are, at least, **hundreds of sarbecoviruses circulating in bats**, and likely *hundreds or thousands more*. Each of these viruses are **mosaic viruses** (i.e., recombinants) and they are continuing to recombine as they coinfect the hundreds of millions of horseshoe bats that are found in Asia and Europe. This raises the possibility of future spillover events, some of which may lead to another pandemic.

MERS

Middle East respiratory syndrome (caused by MERS-CoV) emerged in 2012 in Saudi Arabia. As of mid-2020, there have been more than 2,200 diagnosed cases and more than 800 deaths in 27 countries (36.4% case-fatality rate). However, this virus has not disappeared as SARS-CoV did and is still ongoing with new cases each year. In 2015 there was a dramatic spike in cases when an outbreak occurred in Korea, brought back by a traveler to Kuwait. When cases were analyzed, it was found that 12% were in health care workers, 33% were acquired in a health care facility, 14% resulted from human-to-human transmission, with the rest being undetermined. MERS-CoV has a slightly larger genome than other coronaviruses (30,119 bases). It contains 10 ORFs and 25 punitive polypeptides including 5 possible accessory proteins which may explain its higher case-fatality rate in humans (these may encode proteins to control the host immune response). The NS4a accessory protein inhibits IFN lambda activity and it and NS4b antagonize OAS-RNaseL. The infection ranges from asymptomatic to severe pneumonia and kidney failure. Fatalities and severe disease are associated with comorbidities including diabetes, cancer, and chronic lung, heart, or kidney disease. The virus is transmitted from camels to camels, camels to humans, and humans to humans by respiratory droplets. However, phylogenetic studies indicate that MERS-CoV is closely related to bat coronaviruses. This led to the hypothesis that both bats (primary reservoir) and dromedary camels (secondary reservoir) serve as the reservoirs from MERS-CoV and both may be capable of infecting humans. Figure 15-21 shows scientists sampling camels during a MERS outbreak.

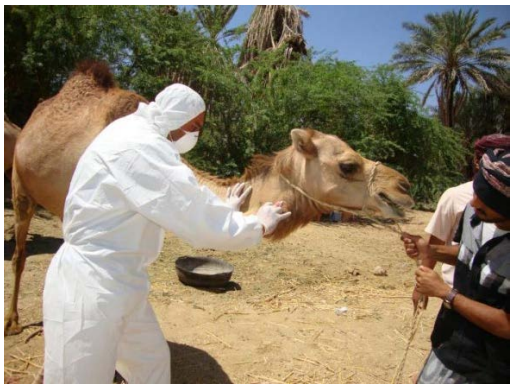


Figure 15-21. This image was captured during an investigation into the first reported Middle East Respiratory Syndrome Coronavirus (MERS-CoV) case in Haramout, Yemen. The study was led by Field Epidemiology Training Program (FETP) residents. In this particular view, veterinarian Hasan Alkaf, DVM is shown extracting blood samples from a camel's neck as the animal was held in check by an assistant. The FETP trains workers on the ground to help countries build sustainable capacity for detecting and responding to health threats. The program develops in-country expertise so that disease outbreaks can be detected locally and prevented from spreading. Courtesy of https://phil.cdc.gov/Details.aspx?pid=19622 Awadh Mohammed Ba Saleh, Yemen.

SADS-CoV

In 2016 a new alphacoronavirus emerged in Guangdong, China called swine acute diarrhea syndrome (SADS-CoV). The virus has a high 50% case-fatality rate in piglets in experimental infections due to intestinal villi atrophy. The virus has 98% identity to a Chinese horseshoe bat coronavirus. Recall that Chinese horseshoe bats were also found to be the reservoir for SARS-CoV. Do you find anything odd about a coronavirus causing a diarrheal disease? It is an enveloped virus. Most viruses that can infect the intestines are acid resistant and naked, as envelopes generally do not do well with environmental changes. The pH of the stomach is quite acidic (pH 2), the intestines quite basic, and bile salts released from the gall bladder lyse many viral envelopes. The envelope of these coronaviruses must be unusually resilient in this environment. This has been found in studies but is not yet well understood. In discussion with virologists, we speculate that the vast majority of the virus particles are inactivated by the stomach acid (as is observed in the literature) but that a few virus particles are sufficient to initiate the infection.

COVID-19 SARS-CoV-2

In December of 2019, a new coronaviral disease emerged in Wuhan China, and again the outbreak is most likely linked to live animal markets. The phylogenetic evidence strongly suggests the reservoir host was a horseshoe bat (genus *Rhinolophus*); however, just like SARS, people most likely did not contract the infection from bats directly instead getting it from an amplifying host. It is likely the bats do not have close enough contact to infect humans and instead infect an amplification host on wildlife farms, via their feces, that are then transported to live animal (wet) markets that leads to the spillover into humans, although this remains to be proven. The symptoms if present develop 2-14 days post infection and include fever, dry cough, shortness of breath, sore throat, and loss of taste or smell. The disease ranges from asymptomatic to severe pneumonia and vascular inflammation that can lead to heart attacks or strokes and can lead to death. Symptoms can last many weeks, although in some individuals symptoms persist for many months and permanent lung damage has been observed due to fibrosis, much like SARS. Pneumonia is more common in people with comorbidities like obesity, heart and lung disease, smokers, and the elderly. Strokes are seen in people under 65, and vascular inflammation is seen in children. The receptor for SARS-CoV-2 is ACE2. ACE2 is expressed in the lungs, GI tract, heart, liver, gallbladder, kidney, urinary tract/bladder, testes, and low levels in endocrine tissues and adipose tissues, which explains the wide array of symptoms that different people experience. When ACE-2 expression is compared between healthy people and

people with comorbidities associated with an increase in severity of SARS-CoV-2 infections, it is noted that ACE-2 is expressed at low levels in the lungs of most people but that this level is dramatically increased in people with the comorbidities associated with severe COVID-19 disease.

Currently the case-fatality rate appears to be around 1.7% among the immunologically-naïve (e.g., unvaccinated or never infected), although this varies greatly by location and access to medical care, this is much lower than SARS or MERS. However, SARS-CoV-2 that causes COVID-19 appears to be transmissible during the incubation period and by asymptomatic carriers, which SARS and MERS are not, which helps to explain why SARS-CoV-2 infections are harder to contain. In addition, SARS-CoV and MERS-CoV principally replicate in the lower respiratory tract (i.e., lungs), whereas SARS-CoV can also replicate to high titers in the upper respiratory tract (i.e., oral and nasal cavities). Transmission can occur via droplet as well as airborne.

Droplet transmission is usually defined as large respiratory droplets (greater than 5 mm) created during coughing, sneezing, and talking which transmit infection when they travel directly from the respiratory tract of the infectious individual to susceptible mucosal surfaces of the recipient, generally over short distances. This is because the large droplets do not remain airborne for long periods (several minutes max) and rapidly drop to the ground. Close contact involves hand transfer of surface contamination to mouth, nose, or eyes. Airborne transmission is defined as smaller airborne droplet nuclei or small particles, often referred to as aerosols, created during coughing, sneezing, talking, and medical procedures that are in the size range that remain airborne (less than 5 mM) and infective over time and distance and therefore can be inhaled into the lower lung.

Outbreaks of SARS-CoV infections (SARS) were associated with improperly functioning sewage systems in hotels. Thus, feces of patients with COVID-19 were analyzed for live virus. Virus has been isolated and cultivated in feces, and as such recommendations are to use caution in public restrooms and ensure proper hand hygiene after using public restrooms. Furthermore, testing waste waters became an excellent way to look for SARS-CoV-2 infection outbreaks in community living spaces such as dormitories, and Greek houses, as well as in communities to predict when cases were on the rise.

The case-fatality rate of seasonal influenza which kills approximately 30,000 people a year in US (250,000-950,000 worldwide) is estimated to be around 0.1%. For comparison, approximately 20-40 million people a year contract seasonal flu in the US. See Figure 15-22 for influenza statistics. The **R0** (number of people an infected person will infect) of SARS-CoV-2 is currently thought to be 1.4-8 depending upon the variant. Note that R0 it is difficult number to determine, and different models come up with different numbers. Also, recall that R0 is not the same in all populations, and changes over time. For comparison the R0 of influenza is usually 1.3-2 while the 1918 flu pandemic R0 was 1.8. The incubation period of COVID-19 appears to be 2-14 days between infection and symptoms. It should be noted that as of this writing the outbreak is currently ongoing, and numbers are changing rapidly.

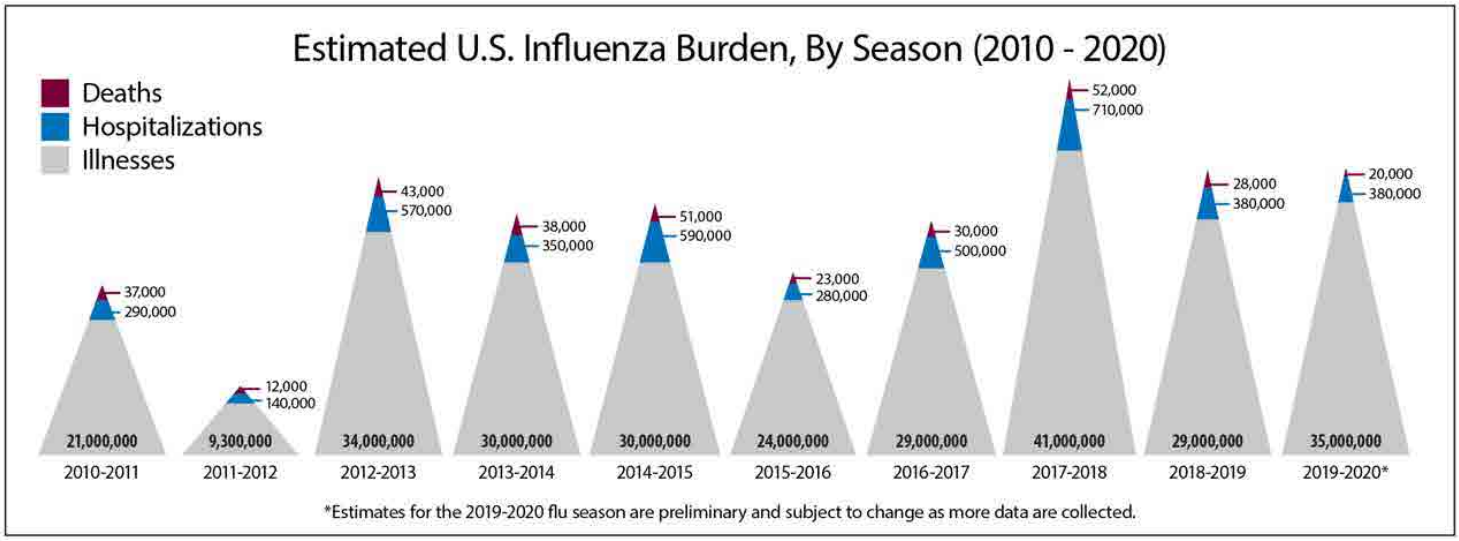


Figure 15-22. Estimated US influenza burden by season, 2010-2020. The case-fatality rate of the virus is on average 0.1% with some years being higher than others. Courtesy of https://www.cdc.gov/flu/about/burden/index.html

The following section was written in early 2020 to demonstrate the potential impact the pandemic might have in the US. You will note that my cowboy math predictions ended up being quite close to what actually happened. This demonstrates that these numbers are useful to epidemiologists and were the reason they were sounding the alarm when it seemed that there were very few cases. How can what we know about influenza help us make predictions about COVID-19? Let's start with the fact that, on average, between 9-45 million people a year develop influenza infections resulting in 140,000-810,000 hospitalizations and 12,000-61,000 deaths a year. Recall that influenza has a lower R0 than COVID-19 and a much lower apparent case-fatality rate, although this number will most likely end up being lower than it appears in the heat of the outbreak when asymptomatic infection rates are still unknown. Current predictions are that the case-fatality rate of COVID-19 is ~1.7% among unvaccinated. Compare this to the case-fatality rate of an average influenza A outbreak of 0.1%. If on average approximately 30,000 people a year die of influenza and COVID-19 has a case-fatality rate that may be 20X higher than influenza and is spread more readily, it is not difficult to see how, if severe control mechanisms are not put in place, upwards of 600,000 people could die of COVID-19 (30,000 X 20). Which not surprisingly is very close to the number of deaths we observed in the first year of the pandemic. Furthermore, the large numbers of cases entering the hospital could, and in many states and countries did, overwhelm the hospital capacity. Although the US has a population of over 332 million, we have only approximately 100,000 ICU beds. Thus, the survival rate of COVID-19 decreases due to an overburdened health care system. In fact, in Italy which had severely overwhelmed medical facilities, the case fatality rate was much higher than anywhere else as of this writing. It is important to realize that this could also cause people to die more readily of other medical emergencies that require hospitalization such as heart attacks, cancer, etc. As such, the number of people who die if populations are unable to flatten the curve of seriously infected patients entering the hospital could be substantially higher. In fact, when deaths in the US were compared to expected averages, in almost every state, when analyzing number of deaths per month, correcting for known COVID-19 deaths, and comparing the deaths to the average expected value, the deaths were higher than expected even without COVID-19. It is important to note that this might be undiagnosed/misdiagnosed COVID-19 deaths or people not getting timely health care for fear of going to the doctor and contracting COVID-19.

What does flatten the curve mean? When we think about viruses entering a naive population, the virus will be able to infect almost everyone because theoretically nobody has immunity. As such, there will be a very rapid rise in the number of cases that will eventually plateau as people die or develop immunity and recover and the population develops herd immunity. If no measures are put in place, this rise in cases will be very fast and the seriously ill will enter the hospitals in rapid succession. If, however, we practice social distancing, we can slow the spread of the virus thus allowing cases to enter the hospital over a longer time period but at much lower numbers at a time. This allows hospitals to better care for those who enter the hospital as well as retain the capacity to deal with other

medical emergencies effectively.

Comparison of SARS-CoV and SARS-CoV-2

If SARS and COVID-19 are caused by closely related viruses (SARS-CoV and SARS-CoV-2) why do they behave so differently in humans? SARS has a high case-fatality rate of approximately 10% while COVID-19 has a case-fatality rate of only 1-2%. SARS-CoV only infected 8000 people before cases stopped. SARS-CoV-2 has infected over 5 million people to date. Studies show that SARS-CoV-2 accumulates virus in the upper respiratory tract before symptoms develop, thus asymptomatic patients transmit SARS-CoV-2 much more efficiently than SARS-CoV which does not accumulate to significant levels until after symptoms begin. The differences between SARS-CoV and SARS-CoV-2's ability to transmit during the asymptomatic phase may help explain why quarantine was such an effective mechanism of control for SARS-CoV but has not been as successful for SARS-CoV-2. For SARS-CoV, it was obvious who needed to be quarantined because only the symptomatic could transmit the disease. For SARS-CoV-2, it is far more difficult to know who needs to be quarantined as many of the people spreading the virus are asymptomatic and have no idea they ever had contact with an infected person.

SARS-CoV-2 in domestic animals

As SARS-CoV-2 is most likely a zoonotic infection, it is natural to wonder if people need to worry about transmitting the disease to their pets or catching the disease from their pets. As of this writing, although a very small number of pets, including dogs and cats, have been reported to be infected with the virus that causes COVID-19 after close contact with people with COVID-19, it is not believed that these animals develop sufficient viremia to infect humans. The CDC has not received many reports of pets becoming sick with COVID-19 in the United States.

In April of 2020, a tiger with a respiratory illness at a zoo in New York City became ill. Samples from this tiger were taken and tested after several other lions and tigers at the zoo showed signs of respiratory illness. It is currently believed that these large cats became sick after being exposed to a zoo employee who was actively shedding virus, although this is still under investigation. In June of 2020, it was documented that workers at a mink farm had contracted COVID-19 from infected American mink (*Neogale vison*), which was then documented at other farms leading to culling of many mink. In Utah, tens of thousands of mink were infected after spillover from human workers and thousands of them died. Notably, American mink were sold at live animal markets in Wuhan when the virus emerged, suggesting a potential role for mink as amplifying hosts from bats or other intermediate host. Further studies are needed to understand if and how different domesticated and wild animals could be affected by COVID-19. Therefore, at the current time the CDC recommends that if you are sick with COVID-19, you should restrict contact with pets and other animals just like you would around other people, although there have been very few reports of pets becoming sick with COVID-19 in the United States. When possible, have another member of your household care for your animals while you are sick. If you must care for your pet or be around animals while you are sick, wash your hands before and after you interact with them. Pets are not considered to be a source of infection for humans, so they do not need to be abandoned.

The psychology of a new disease

At this point I think it would be of interest to analyze the psychological effects of an outbreak of a new virus. Let's start by looking at the story that unfolded worldwide. The virus was first noted in China in late December of 2019. The first death was reported in early January. By February 9th more people had died of COVID-19 than had died of SARS. Recall, however, that fewer than 9000 people were confirmed to have contracted SARS worldwide. On Feb 14th the first case outside China was identified in Egypt. This led to strict quarantines in China as well as travel restrictions to and from China in February of 2020. This method of control was extremely successful in

containing the SARS outbreak, and it was hoped that it would work for the COVID-19 outbreak as well. However, by late February it was clear the virus had spread outside of China with major outbreaks in Iran, Italy, South Korea, Hong Kong, and cases were increasing in the US. The US reported its first case on February 26th and first death on February 29th in Washington state in a nursing home where many residents developed COVID-19. On February 24th the stock market began a rapid downfall. Many major conferences and music shows were cancelled for fears that large gatherings of people might cause the virus to spread. The week of March 10th brought news of multiple universities moving all classes online for unknown periods of time. Many states declared states of emergency such as New York, Washington, California, and Colorado. Multiple cruise ships had passengers develop the disease causing all passengers to be quarantined on the ships and in military installations after disembarking. By March 12 the NBA ended its season and NCAA's basketball tournament was cancelled, baseball's spring training was cancelled, and multiple universities, including Colorado State University where the author works, moved all classes online and prohibited faculty and staff from travelling internationally or domestically. The US banned travel to and from Europe due to the large outbreak in Italy. By March 14th major ski resorts in California and Colorado closed for the season despite record amounts of snow. On March 16th the city of San Francisco became the first city in the US to enact mandatory home isolation. By March 18 multiple states were on mandatory home isolation with all restaurants, bars, gyms etc. shutting down. People were only allowed to leave their homes to get food and exercise (walk, run, bike riding, or other outdoor activities) and were required to stay 6 feet from other people. By March 23rd 13 states had similar orders in place and many others requested but did not mandate shelter in place rules. The outbreak led to a shortage of face masks, hand wipes, toilet paper, and paper towels while panic set in among the population. People were reported to be waiting in lines as long as 4 hours to get into Costco to purchase supplies. It should be noted that the CDC did **NOT originally** recommend face masks for the general public as they had not been shown to be an effective mechanism of protection, and the fiddling around with them that is done with hands might actually increase the risk of infection. However, they did recommend masks for people who are sick to potentially reduce the spread of the virus to others. Their suggestions include staying away from people who are ill, social distancing (avoiding crowds), staying at least 6 feet from other people (the travel distance of respiratory droplets,) proper hand washing, avoiding touching one's face, eyes, nose and mouth, and avoiding shaking hands. On April 2nd the CDC revised these recommendations indicating that people might want to start wearing masks, not only to protect themselves, but to protect others by preventing them from asymptomatically spreading the virus to others. This link is to an excellent video demonstrating why one should wear masks. As shown in Figure 15-23, the author and her husband are shown wearing masks while shopping. As panic grew food ran out at grocery stores, as shown in Figure 15-24, a grocery store visited by the author while she was writing this chapter.

Figure 15-23. The author and her husband wearing masks while shopping during the COVID-19 outbreak, 2020.

Figure 15-24. Image of empty shelves at a grocery store on March 16, 2020. Courtesy of Erica Suchman, Colorado State University.

One positive outcome of the outbreak was a renewed focus on proper hand washing and other techniques that help people to avoid many viral infections. Figure 15-25 shows a World Health Organization (WHO) handout taped to the bathroom wall in Ventura, California, an area that at the time was not yet showing any COVID-19 cases. This resulted in far fewer cases than expected of seasonal flu in 2020 and 2021.

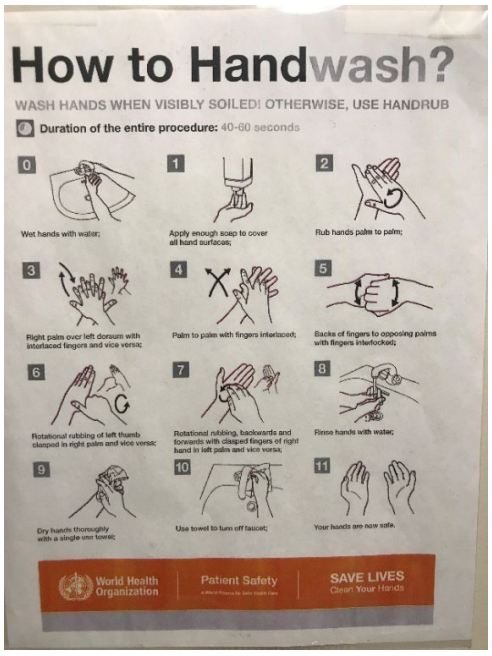


Figure 15-25. Image of WHO recommendations for proper hand washing on the wall of a delicatessen in Ventura, California during the COVID-19 outbreak, March of 2020. Courtesy of the World Health Organization and Erica Suchman, Colorado State University.

By March 27th the US surpassed China in the total number of cases and had one of the fastest rates of increases in cases on par with Spain. New York was the epicenter of this outbreak and had the vast majority of cases in the US. New Orleans, however, failed to shut down Mardi Gras which is believed to have contributed to a major outbreak there as well. In early May people began to tire of the restrictions on business/work and began demonstrating, often with guns and often without wearing masks. In late May many states began loosening the social distancing requirements and allowed business to begin opening. This led to major increases in cases over the summer and many states reinstating stay at home orders. As people continued to push back against public health measures cases soared in November of 2020 and the surge continued well into 2021. By spring of 2020 variants of the original infecting virus began to develop. The viruses were originally named by the location they were first noted; however, just because a virus is first noted in a place does not mean it arose there, so that was not optimal. Unfortunately the naming systems that were developed based on lineages such as B.1.1.7 were very difficult for the public to understand, so the World Health Organization proposed changing the names of variants of concern to alpha for one noticed in September of 2020 in the UK (B.1.1.7), beta for the one noticed in May of 2020 (B.1.351), gamma for a variant noticed in November 2020 in Brazil (P.1) and delta for a variant (B.1.617.2) noticed in October of 2020 in India. This last variant has been the most problematic with a much greater R0 (estimated to be a 97% increase in R0 from the original infecting virus). Thus, it was recommended that people get a booster vaccine at least 6 months after completing the first 2 shot regiment.

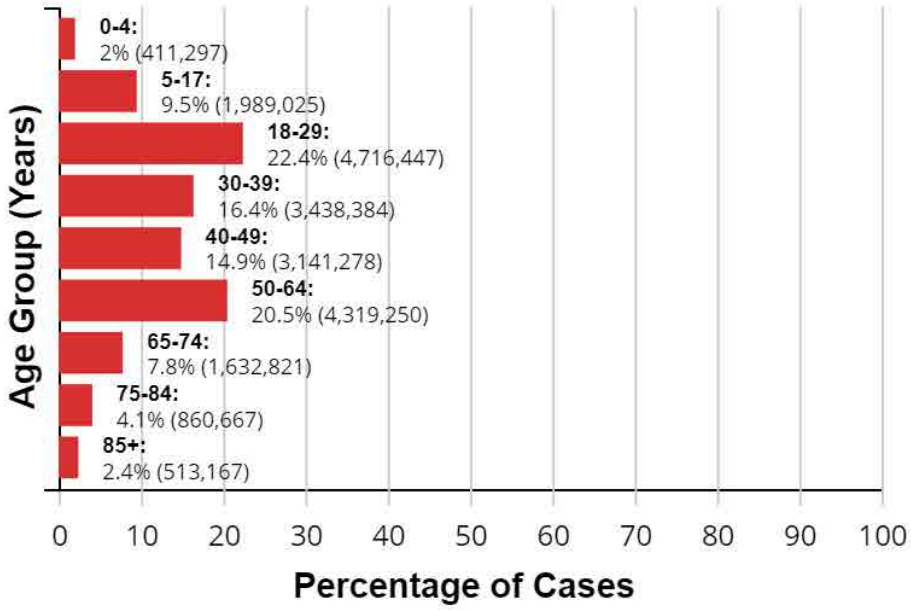
In November of 2021 a new variant, named omicron, was first noted in South Africa that had an even higher R0 (8.2) than the delta variant, as expected this variant spread very rapidly. Fortunately, this variant had a slightly reduced pathogenic potential and infected people were less likely to be hospitalized or admitted to the ICU. However, the substantially increased R0 meant that hospitalizations were still quite high due to the dramatic spike in infections. Although vaccinated people developed infections, it was at a much-reduced rate, and hospitalization was significantly lower.

The public saw firsthand the importance of understanding small nuances of viral infections, such as, “What is the incubation period? Who can transmit the disease? How?” and, “How long will surfaces contaminated with virus in the environment remain infectious?” For COVID-19, it appears to be a few days depending upon the surface type and how moist the virus is on the surface. Original reporting on the news indicated that the virus would last up to 9 days. This was reported in a paper, but that was for virus in growth medium which is very moist and has a lot of protective protein. Samples from hosts do not appear to last that long. Then the CDC did a study to look at transmissions in cruise ships entitled “Public Health Responses to COVID-19 Outbreaks on Cruise Ships Worldwide”, February-March 2020. The paper had one line that stated “SARS-CoV-2 RNA was identified on a variety of surfaces in cabins of both symptomatic and asymptomatic infected passengers up to 17 days after cabins were vacated on the Diamond Princess but before disinfection procedures had been conducted”, (Takuya Yamagishi, National Institute of Infectious Diseases, personal communication, 2020). “Although these data cannot be used to determine whether transmission occurred from contaminated surfaces, further study of fomite transmission of SARS-CoV-2 aboard cruise ships is warranted.” The newspapers reported this as “CDC report: Novel coronavirus survived up to 17 days on Diamond Princess cruise ship”. As one might expect this reporting led to increased panic in an

already nervous population. However, let's look at what this paper actually said. First, the CDC was not studying how long the virus can remain infectious on surfaces, it was just examining cases and how they were handled. The statement above was a brief mention in the discussion of issues to think about in handling of future outbreaks. Second, they did not analyze whether the virus SURVIVED. They only looked for evidence of its presence, i.e., they could find viral RNA which demonstrated that the virus was landing on surfaces. Third, they never did any kind of assay to determine if live virus was still present. This should remind us to make sure we double check what we read in the news for ourselves as the journalists clearly did not understand the paper they were referencing.

DEMOGRAPHICS OF COVID-19 DEATHS

COVID-19 was much worse for older populations then younger ones, in part because as people age, they tend to develop many of the co-morbidities linked to poor outcomes. Figure 15-26 shows demographic data by age of infection (A) and deaths (B) in early 2021. As you can see people of all ages could get infected but those who were older were more likely to die. However, it is important to note that younger people can still die and are prone to develop debilitating long term COVID symptoms sometimes referred to long hauler COVID-19.



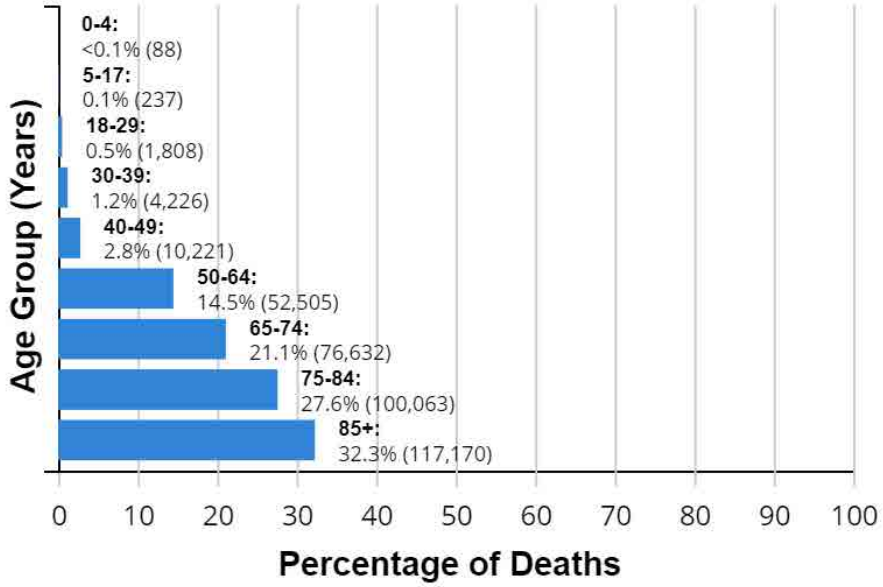
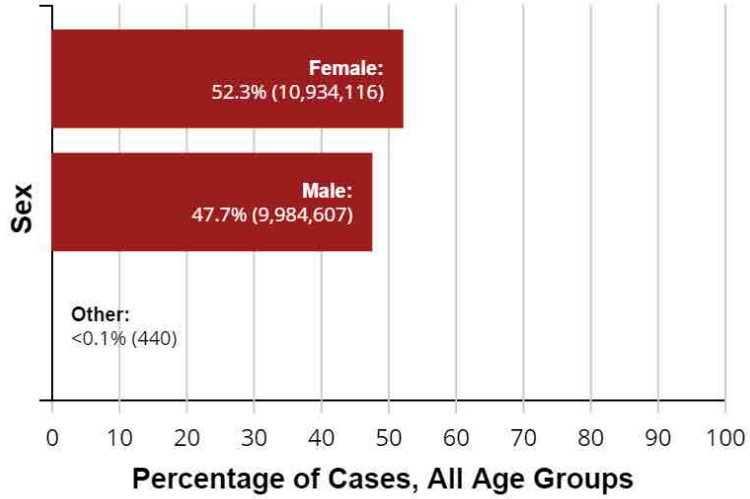


Figure 15-26. Demographic data of SARS-CoV-2 infections (A) and COVID-19 deaths (B) by age. Courtesy of https://covid.cdc.gov/covid-data-tracker/#demographics

When infections are analyzed by gender slightly higher rates of infection are seen in women (Figure 15-27A), but men are more likely to die (Figure 15-27 B)



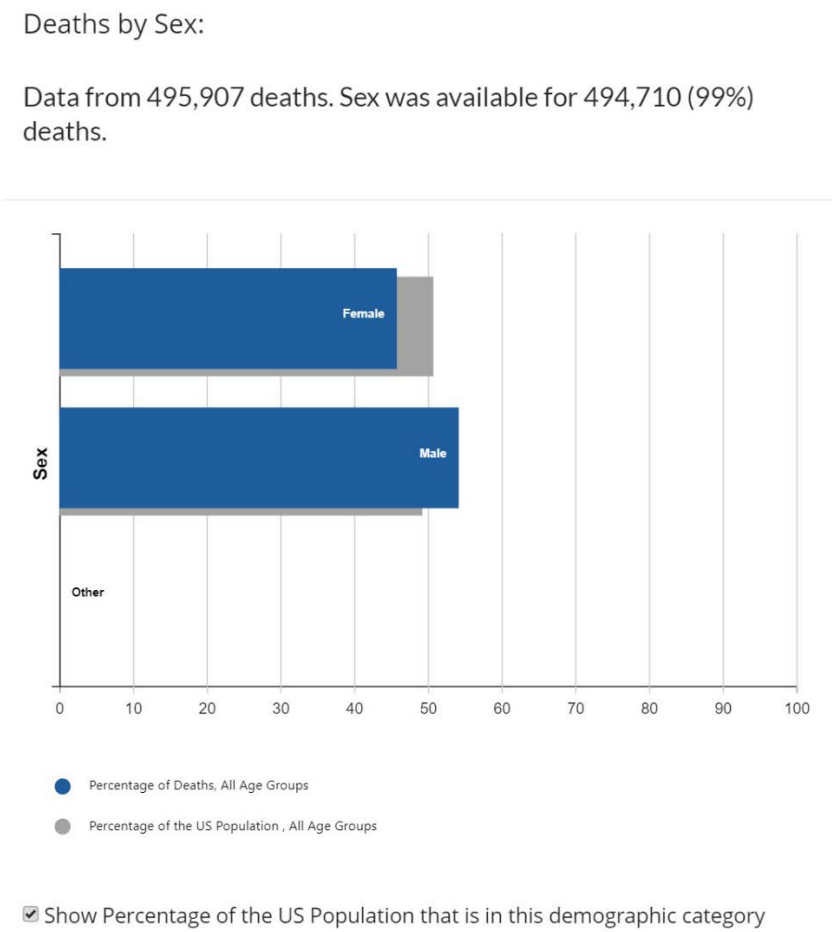


Figure 15-27. Demographic data of SARS-CoV-2 infections (A) and COVID-19 deaths (B) by sex July 2021. Courtesy of https://covid.cdc.gov/covid-data-tracker/#demographics

One of the outcomes of the pandemic is that the US saw firsthand the health disparity that exists in the United States. Figure 15-28 show that when deaths from COVID-19 are analyzed you can see that minoritized populations represent a higher proportion of the deaths due to COVID-19 (blue bars) than their representation in the population shown in grey.

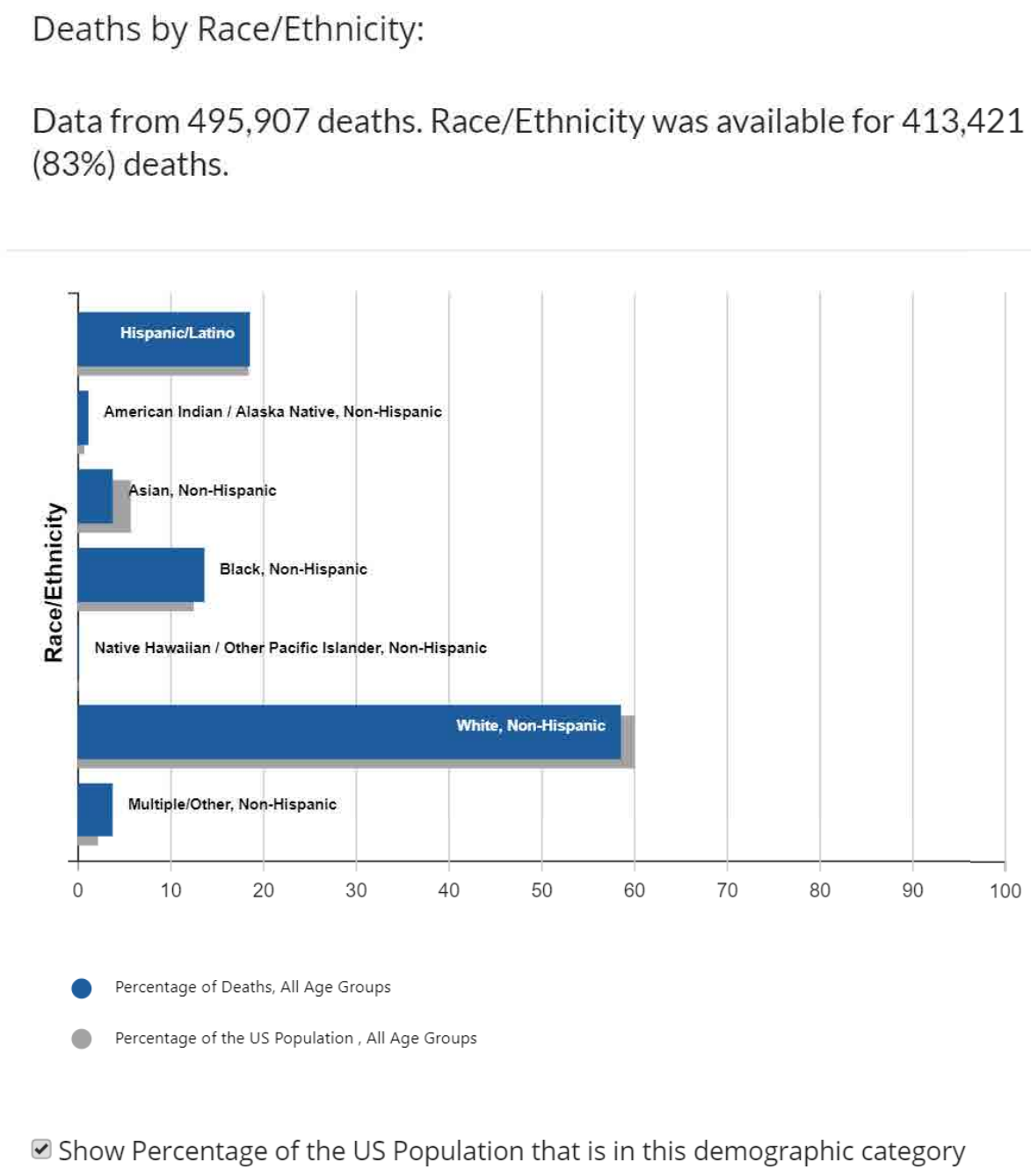


Figure 15-28. Demographic data of SARS-CoV-2 deaths by ethnicity (blue bars) as compared to the percentage of the population grey bars. Courtesy of https://covid.cdc.gov/covid-data-tracker/#demographics

PREVENTING CORONAVIRUS INFECTIONS

Masking, social distancing and handwashing are the most effective non-medical interventions. The efficacy of these methods can be witnessed indirectly by comparing the flu season of October 2020-

April 2021to all previous years. Note that if you look at the data for the 2019-2020 season you will see it looks like previous years, as the flu season was already done by the time control measures were put in place. This data can be viewed using this interactive map (click play and it will show you when cases show up by week).

When the pandemic began, no vaccines were available for coronavirus infections as until recently, they only caused the common cold, and thus vaccines were not necessary. Although attempts were made to develop vaccines against SARS-CoV, the virus disappeared due to effective quarantine and tracing negating the need to develop a vaccine.

The production of effective SARS-CoV-2 vaccines is the fastest in history and was made possible in large part by the large amounts of money committed to tackling this serious problem allowing it to move through approval more rapidly than in the past. Furthermore, mRNA vaccine technology had long been studied and was at a critical point of being ready for production of new vaccines. The first 2 vaccines were granted emergency use authorization (EUA) in December 2020 were mRNA-based vaccines that contained the spike protein (S) mRNA surrounded by lipid nanoparticles that cells engulf into endocytic vesicles bringing the mRNA into the cell's cytoplasm where it is translated by cellular ribosomes (Pfizer and Moderna). The vaccines activate both the innate immune response by activation of pathogen recognition receptors (PRR) when the mRNA as well as contaminants bind PRR and activate production of interferon and pro-inflammatory cytokines. These vaccines required booster shots at 21 and 28 days respectively and required a level of cold chain that was difficult to achieve, limiting their ability to be distributed quickly. However, eventually these issues were overcome. Note: again, we must talk about misinformation, many people became convinced that mRNA vaccines would somehow alter their DNA, which is not true, the mRNA never enters the nucleus. In March of 2021 the Johnson and Johnson adenovirus vectored one dose vaccine became available. This vaccine also expresses the S protein. Vaccination began with people over 80 in January of 2021, by April 19 of 2021 all people over 16 were eligible to receive the vaccine and approximately 4 million people a day were receiving the vaccine. Which is an amazing feat in slightly more than a year. However, vaccine hesitancy and disinformation is a major issue hindering the ability to get the pandemic under control and allowing the variants to evolve and thrive. In October of 2021 booster vaccines were approved for people who received the Pfizer vaccine who are over 50, have medical issues that make them more susceptible, or had occupational exposer. This included teachers which allowed the author to get a booster at 6 months post vaccination. The reason for boosters was an observed waning of immunity by 6 months. Boosters for people who received Moderna and J&J vaccines followed shortly thereafter. In addition, new omicron boosters are now available and can be administered 3 months after a previous COVID-19 immunization.

It should be noted that vaccination was found to induce superior immunity to infection. Studies by the CDC found that the odds of contracting SARS-CoV-2 infections were 5.49-fold higher for people who had been infected than those who had been vaccinated,

Another thing people learned about during the pandemic is vaccine efficacy, as people tried to decide which vaccine was best for them. Let's pretend that we created a new vaccine and we say that it has a 60% efficacy. Most people think this means that 100-60, or 40% of people will get sick despite getting vaccinated. This

would be a large over estimation of the number of people who would get disease. Let's look at data from a hypothetical vaccine.

	# people sick/5000	# people severe disease/5000
Unvaccinated placebo group	50/5000 x 100 = 1.0%	5/5000 x 100 = 0.10
vaccinated	20/5000 x 100 = 0.4%	1/5000 x 100 = 0.02
Efficacy at preventing illness	(1.0-0.4)/1.0 X100 = 60% efficacy or 60% fewer cases	
Efficacy at preventing severe illness		(0.1-0.02)/0.1 x 100 = 80% efficacy or 80% reduction in severe disease

As you can see this is far fewer than 40% of 5000 people (2,000) who got sick.

TREATING CORONAVIRUS INFECTIONS

There was no curative treatment for any of the coronavirus infections. As it is extremely difficult, expensive, and time consuming to bring new drugs on the market, the best hope for a rapid drug treatment was to test current antivirals against COVID-19 as their safety has already been assessed thus allowing them to come to market more rapidly if they are found to work. One of the first experimental treatments of COVID-19 patients in Seoul, South Korea, was with Kaletra, a protease inhibitor used as a treatment against HIV, a retroviral infection. Kaletra appeared to reduce the viral load and severity of COVID-19 disease in early trials. Sadly, the drug did not reduce severity or timeline of infection when tested on 200 patients.

Also tested was an anti-malarial drug, hydroxychloroquine, which alkalizes the endosome hampering the low-pH-dependent steps of fusion and uncoating as well as reducing inflammation by inhibiting cells involved in the inflammatory response. Chloroquine is traditionally used to treat malaria as well as auto-immune diseases due to the decreased inflammation. Chloroquine was tested against SARS-CoV infections and was thought to show promise, but the epidemic ended without being able to test the drug sufficiently. Chloroquine has been tested against SARS-CoV-2 infections in Vero cell culture and was found to reduce viral replication; however, SARS-CoV-2 enters lung cells directly through the plasma membrane (because of cellular TMPRSS2 that cleaves the spike protein, thus activating it), bypassing endosomal processing and, thus, has no effect on SARS-CoV-2 infection of lung cells. It should be noted, however, that similar findings were found for other viral infections in vitro that did not show similar inhibition in hosts in vivo. It should be noted that cardiologists have long associated the drug with a rare increase in arrhythmias that can cause heart attacks. A large-scale trial in

Brazil was halted as they found that the death rates were higher in those taking hydroxyquinoline, and a study in the US of 368 veterans who took the drug found that their risk of death was higher than for those who didn't. Sadly, those with the most severe symptoms are more likely to already have heart problems and are most at risk from adverse responses to this drug. France and the World Health Organization stopped studies on the drug because of the adverse response and no demonstrated benefit. Several double-blinded, randomized clinical trials examined the efficacy of hydroxychloroquine and in each no benefit was found.

It has been noted that SARS-CoV-2 seems to initiate a cytokine storm similar to some strains of influenza A, such as the H1N1 swine flu. Therefore, drugs that are used for rheumatoid arthritis, such as tocilizumab and anakinra, have been approved for trials in patients who appear to have elevated cytokine responses. The immunosuppressant dexamethasone has modest efficacy in reducing deaths in severe COVID-19 patients. However, it is only given during the second (immunopathologic) phase of COVID-19.

Remdesivir an RdRp inhibitor was found to decrease the mean number of symptomatic days from 15 to 11, however it did not show any significant decrease in mortality for severely ill patients. However, further studies where the drug was **only** administered early in the infection did show that remdesivir decreased mortality. Many patients do not seek treatment early enough for remdesivir to be effective. Furthermore, the treatment could only be administered by transfusion.

Studies were also conducted on Ivermectin. Those of you with horses and dogs may recognize this as an anti-worm medication. You may be thinking, why on earth would this work on viruses? Ivermectin works by inhibiting the transport of certain proteins across the nuclear membrane. So, now you may be thinking, but coronaviruses are RNA viruses that replicate in the cytoplasm. Why would that work? This is where virologists obsessing on every detail of viral replication becomes important. Many RNA viruses create what are called accessory proteins to shut down host cell's antiviral defenses, and these often must be transported to the nucleus to function. Ivermectin inhibits the ability of these proteins to shut down host cell anti-viral defenses which then clear the viral infection. Unfortunately, this drug is also not thought to work, except possibly at dangerously high doses. However, this did not stop people from purchasing this drug from veterinary supply stores and dosing themselves.

Early trials of Molnupiravir an oral antiviral agent that is converted in cells to the nucleoside derivative N4-hydroxycytidine and elicits antiviral effects by introducing copying errors during viral RNA replication, on small numbers of patients appear promising, however; far more data is required. Molnupiriavir has been found to incorporate into DNA so concerns about mutations associated with use need to be evaluated.

Studies were conducted giving convalescent sera to hospitalized CVOID-19 patients. This application of artificial passive immunity was thought to help neutralize the virus and reduce infections. Results of these studies have been inconsistent, and although may slightly reduce death better treatments are needed.

Monoclonal antibodies against the virus have shown great efficacy. This is the treatment that was given to President Trump when he contracted COVID-19. The limitation of this treatment is that it is administered via transfusion and as such cannot be administered at home. Unfortunately, with the emergence of omicron variants, these monoclonal antibodies now have little efficacy.

In November of 2021 Pfizer announced a protease inhibitor combined with a drug that slows the metabolism of protease inhibitors prolonging the activity of the protease inhibitor called Paxlovid, in trials that were halted early due to promising data appear to decrease hospitalizations by 85% and deaths by 100% if treatment is initiated within 7 days of symptoms, however, results were better (88%) if initiated within 3 days.

DETECTING CORONAVIRUS INFECTIONS

The CDC recommends diagnosing coronavirus diseases (MERS, SARS, COVID-19) using a reverse transcriptase real time PCR assay. However, in March 2020 there were insufficient tests available (mostly due to lack of machines to read the tests and reagents to run the tests) which dramatically reduced the ability to assess who was infected. This lack of data hindered our ability to control the spread as well as scientist's ability to get an accurate analysis of the case-fatality rate. To address the issues of PCR testing shortages, newer more rapid antibody ELISA assays were approved in March of 2020 that can detect infections in minutes rather than days. However, they were not as sensitive or accurate and as such real time PCR assays are still considered the gold standard. Although IgM is made first (and mostly during a primary immune response), it is not as specific for epitopes as is the later IgG antibody. Recall that there are 4 coronaviruses that can cause the common cold. Apparently, the tests that have been developed against IgM have not been shown to be specific enough to differentiate SARS-CoV-2 from these less pathogenic viruses. As time has passed and we now have millions of people in the US who have recovered from COVID-19, we can no longer use a single IgG test to determine if a person is currently infected as the antibody might be from an infection a person has recovered from. Thus, to use IgG as a diagnostic test would require an acute sample (when symptoms are noticed), a convalescent sample (at least 2 weeks later when they should be recovering), and a 4-fold increase in IgG antibody titer indicating the patient is responding now and indicating a current infection. As such, unless they can find a more reliable IgM test, the CDC's current recommendation that serology be used to determine who **has** been infected rather than who is **currently** infected (which will be done by real time PCR) will remain the recommended testing procedure. However, the real time PCR test, also called **nucleic acid test** (NAT), is only sensitive for the first 5 days of symptoms after which point false negatives are common. Recall from chapter 2 that the real time PCR test now includes 2 genes N and S and that amplification of both genes is required to call a sample positive. Unless, amplification of the N genes are strong (low CT values) and the S gene is negative, as this may indicate that mutations have occurred in the S gene that prohibit S gene PCR primer binding. This is an indication that the person may be infected with a variant of the original historical virus.

This would be a good place to think about why not having accurate numbers of infections would affect our ability to determine the **case-fatality rate** and **mortality rate**. Case-fatality rate is the number of people who die divided by the number of people who are infected with an organism. Early in an outbreak the case-fatality rate can appear quite high but later those numbers are usually revised and lowered. Why might this be? Outbreaks of lethal viral diseases are usually noticed at a regional hospital. Many cases of a new disease show up at the hospital with similar symptoms that cannot be explained by any previously identified viruses. Let's say 1000 people are admitted to the hospital with this new disease and 200 die. This would be an apparent case-fatality rate of 200/1000 = 0.2 which we multiple by 100 giving us a case-fatality rate of 20%. This rate seems very high and very scary! This rate gets reported in the news. However, these are the people with the most severe symptoms who come to the hospital. What we don't know right now is how many people are sick but did not get sick enough to come to a hospital? How many just went to their primary care doctor? How many didn't seek medical care at all? And most importantly, how many developed very mild symptoms or have **asymptomatic infections**? Asymptomatic infections do not have any symptoms of the disease but are infected. It takes time and lots of testing to figure out these actual numbers. Using SARS-CoV as an example, when cases first began in Hong Kong, estimates were that the case-fatality rate was 50-80%. However, with time and more testing, it was determined the case-fatality rate was actually only 9-10%, still a very high rate, and scary, but not nearly as terrifying as 50-80%. Therefore, our initial case-fatality rate estimates of 2-3% for SARS-CoV-2 (COVID-19) may fall as our capacity to test people improves. The case-fatality rate should not be confused with the mortality rate which is the number of deaths from a disease as a fraction of the total population. For example, if 5000 people die of a new disease and the current population of the country is 10,000,000 people, the mortality rate is 5000/10,000,000 or 0.0005. However, these rates are usually expressed in deaths per 100,000 so the mortality rate would be 50/100,000 people. Assuming this new disease would have the same mortality rate in the US, we would predict 164,000 deaths in the US with a population of approximately 328 million people. As of this writing there are 560,000 deaths in the US this would mean, the mortality rate for SARS-CoV-2 is approximately 170/100,000.

END OF CHAPTER QUESTIONS

1. Why do you think that these 4 virus families were grouped together in one chapter?
2. How do the viruses in this chapter differ from the viruses in chapter 14?
3. Do all 4 of these virus families use the same or different enzymes to reproduce their genome and transcribe their mRNA? Are these enzymes cellular or viral? Identify these enzymes.
4. Compare and contrast how these 4 viruses start their genome replication.
5. Compare and contrast how these 4 viruses start protein translation.
6. Compare and contrast how these 4 viruses create subgenomic mRNAs.
7. Compare and contrast how these 4 viruses avoid the host immune response.
8. These viruses all have single stranded RNA genomes. Do they need to have mechanisms for overcoming RNA interference? If so, why? If not, why not?

9. Do all 4 of these families use viral proteases? If so for what purpose? If not, which ones do not?
10. What is an IRES? Which of these viral families utilize IRES, and why?
11. Can a virus create more than one ORF from an mRNA? If so, do any of these 3 virus families do so? If so, explain how.
12. What is a viral factory? Why might a virus utilize them? How do the viral factories of most RNA viruses differ from those of most DNA viruses?
13. Compare and contrast the *Togaviridae* alphavirus and rubivirus diseases.
14. Explain the pathogenesis of alphavirus diseases. Is it the viral replication that causes the symptoms? Is it tissue destruction? Or is it the anti-viral immune response? Justify your answer.
15. Explain the difference between an enzootic/sylvatic cycle and an epizootic transmission cycle. For which of the families discussed in this chapter would you expect to observe these different cycles, and why?
16. Rubella is usually a mild childhood disease that rarely causes encephalomyelitis. Why then, in many states, was it policy that women must prove rubella vaccination before obtaining a marriage license?
17. What does the MMR vaccine cover, and why must it be boostered?
18. What is the predominant disease caused by viruses in the family *Caliciviridae?*
19. What are the predominant symptoms caused by viruses in the family *Coronaviridae*? What is the difference between most of the recently emerged coronavirus infections and long known coronavirus infections?
20. During the COVID-19 outbreak an anti-HIV drug was proposed as a potential treatment for COVID-19. What viral enzyme do you think this drug most likely targeted? Hint: what do these two viruses have in common?
21. During the COVID-19 outbreak the anti-malarial drug chloroquine was also tried as a potential treatment. Chloroquine increases the pH of cellular endosomes. How might this inhibit COVID-19 replication?
22. SARS-CoV and SARS-CoV-2 both cause gastroenteritis. Do you find this odd? If so, why?
23. The first approved test for COVID-19 was a real time reverse transcriptase PCR. Why did it have to be a reverse transcriptase PCR? What would a positive test result look like?
24. What reservoir is thought to be utilized by most if not all coronaviruses?
25. Is this reservoir generally thought to be the animal that transmits coronaviruses to humans? If not, why not?
26. You are tracking a new disease that is transmitted by mosquitoes. Is it most likely caused by a calicivirus, picornavirus, or togavirus? Explain your answer.
27. What other family of viruses is a likely cause of a novel disease transmitted by mosquitoes?

Use the following data to answer the next 3 questions

	S gene CT value	N gene CT Value
Patient 1	46	50
Patient 2	44	11
Patient 3	43	32
Patient 4	22	20

28. Which patient(s) is/are infected with a non-variant SARS-CoV-2? Explain why you answered as you did.
29. Which patient(s) is are infected with Variant SARS-CoV-2? Explain why you answered as you did.
30. Which patient (s) is/are not infected with SARS-CoV-2? Explain why you answered as you did.
31. You are testing a new vaccine. You find that of 1000 control patients 20 get infected with the virus in question and in the vaccinated group 5 get infected. What is the efficacy of the vaccine at preventing infections?

REFERENCES:

KEY REFERENCE: Worobey et al. The Huanan Seafood Wholesale Market in Wuhan was the early epicenter of the COVID-19 pandemic. Science. 2022.

KEY REFERENCE: Pekar et al. The molecular epidemiology of multiple zoonotic origins of SARS-CoV-2. Science. 2022.

KEY REFERENCE: Hartenian et al. The molecular virology of coronaviruses. J Bio Chem. 2020.

Andersen, K. et al., 2020 The proximal origin of SARS-CoV-2. Nature Medicine. https://doi.org/10.1038/ s41591-020-0820-9 https://www.nature.com/articles/s41591-020-0820-9

Aynaud, J-M., et al. (1985) Transmissible Gastoenteritis (TGE) of Swine: survivor Selection of TGE Virus Mutants in Stomach Juice of Adult Pigs. J. gen. Virology (66) 1911-1917. https://www.sciencedirect.com/science/article/pii/0378113587900265

Bosio, C., et al. (2021) Laboratory-Confirmed COVID-19 Among Adults Hospitalized with COVID-19–Like Illness with Infection-Induced or mRNA Vaccine-Induced SARS-CoV-2 Immunity — Nine States, January–September 2021 MMWR *70(44);1539–1544.* *https://pubmed.ncbi.nlm.nih.gov/34735425/*

Buschmann, M.D., et al. (2021). Nanomaterial Delivery Systems for mRNA Vaccines. Vaccines 9, 65. https://doi.org/10.3390/vaccines 9010065 https://www.mdpi.com/2076-393X/9/1/65

Cao B, et al., 2020, A Trial of Lopinavir-Ritonavir in Adults Hospitalized with Severe Covid-19. N Engl J Med. . doi: 10.1056/NEJMoa2001282. https://www.nejm.org/doi/full/10.1056/NEJMoa2001282

Carlson, P., et. al. 2020, Chloroquine for the 2019 novel coronavirus SARS-CoV-2. International Journal of Antimicrobial Agents, Volume 55, Issue 3, 105923 https://doi.org/10.1016/j.ijantimicag.2020.105923

Centers for Disease Control. Chikungunya. https://cdc.gov/Chikungunya

Centers for Disease Control. COVID-19. https://www.cdc.gov/media/releases/2020/a0229-COVID-19-update.html

Centers for Disease Control. COVID-19 data. https://covid.cdc.gov/covid-data-tracker/#demographics

Centers for Disease Control. Influenza. https://www.cdc.gov/flu/about/burden/index.html

Centers for Disease Control. Stop Rubella. (2017) M. Kurnit; M. Jankun https://www.cdc.gov/globalhealth/immunization/infographic/stop_rubella.htm
Centers for Disease Control. Public Health Image Library (PHIL). (2003) J. Gathany. *Aedes albopictus.* https://phil.cdc.gov/Details.aspx?pid=4490

Centers for Disease Control. Public Health Image Library (PHIL). (1976) Chikungunya fever. https://phil.cdc.gov/Details.aspx?pid=14764

Centers for Disease Control. Public Health Image Library (PHIL). Chikungunya TEM image. C. Goldsmith, J. Comer, B. Johnson. (2014) https://phil.cdc.gov/Details.aspx?pid=17550

Centers for Disease Control. Public Health Image Library (PHIL). Dr. A. Rausen. (1969) Congenital Rubella x-rays. https://phil.cdc.gov/Details.aspx?pid=17935

Centers for Disease Control. Public Health Image Library (PHIL). Dr. A. J. Lebrum. (1969) Congenital rubella glaucoma. https://phil.cdc.gov/Details.aspx?pid=17455

Centers for Disease Control. Public Health Image Library (PHIL). Dr. A. J. Lebrum (1969) Congenital rubella pneumonia. https://phil.cdc.gov/Details.aspx?pid=17782

Centers for Disease Control. Public Health Image Library (PHIL). NAID. (2014) Coronavirus EM. https://phil.cdc.gov/Details.aspx?pid=18114

Centers for Disease Control. Public Health Image Library (PHIL). Dr. F Murphy, S. Whitfield. (1975) Coronavirus TEM. https://phil.cdc.gov/Details.aspx?pid=10270

Centers for Disease Control. Public Health Image Library (PHIL). (2005) J. Gathany. *Culex tarsalis.* https://phil.cdc.gov/Details.aspx?pid=7957

Centers for Disease Control. Public Health Image Library (PHIL). A. Mohammed Ba Saleh, Yemen. MERS (2014) https://phil.cdc.gov/Details.aspx?pid=19622

Centers for Disease Control. Public Health Image Library (PHIL). E.L. Palmer. (1981) Norovirus. https://phil.cdc.gov/Details.aspx?pid=2172

Centers for Disease Control. Public Health Image Library (PHIL). (1966) Rubella. https://phil.cdc.gov/Details.aspx?pid=22144

Fields Virology 6th edition David Knipe, Peter Howley, 2013 Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 20, 22, 23, 24, 28.

Go, Y. et al. (2014). Zoonotic encephalitides caused by arboviruses: Transmission and epidemiology of alphaviruses and flaviviruses. Clinical and experimental vaccine research. 3. 58-77. 10.7774/cevr.2014.3.1.58. https://www.researchgate.net/figure/Enzootic-and-epizootic-epidemic-transmission-cycles-of-Western-equine-encephalitis-virus_fig5_259743924

Hamming,I et al. (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis J Pathol. 203(2): 631–637.doi: 10.1002/path.1570 https://pubmed.ncbi.nlm.nih.gov/15141377/

Hartenian et al., (2020) The molecular virology of coronaviruses. 295:12910. J Biol Chem. https://www.jbc.org/article/S0021-9258(17)49954-6/fulltext

Jia, H.P., et al. (2005). ACE2 Receptor Expression and Severe Acute Respiratory Syndrome Coronavirus Infection Depend on Differentiation of Human Airway Epithelia. J Virol. 79(23): 14614–14621. doi: 10.1128/ JVI.79.23.14614-14621.2005 https://pubmed.ncbi.nlm.nih.gov/16282461/

Kirchdoerfer, R.N., et al. (2018). Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. *Sci Rep* **8,** 15701. https://doi.org/10.1038/s41598-018-34171-7

Lu, R., et al. (2020). Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet, Vol 395:10224, 565-574. https://www.thelancet.com/article/S0140-6736(20)30251-8/fulltext

National Institutes of Health Directors blog. COVID-19 pill early data. F. Collins. (2021). https://directorsblog.nih.gov/2021/11/16/early-data-suggest-pfizer-pill-may-prevent-severe-covid-19/

Pardi, N., et al. mRNA vaccines — a new era in vaccinology. *Nat Rev Drug Discov* **17,** 261–279 (2018). https://doi.org/10.1038/nrd.2017.243

Syed AM, et al. (2021). Rapid assessment of SARS-CoV-2 evolved variants using virus-like particles. T Science. :eabl6184. https://pubmed.ncbi.nlm.nih.gov/34735219/

Tang, X. et al. (2020). On the origin and continuing evolution of SARS-CoV-2. *National Science Review*, https://doi.org/10.1093/nsr/nwaa036

Touret, F., and Lamballerie, X. (2020). Of chloroquine and COVID-19. Antiviral Research 177:104762 https://doi.org/10.1016/j.antiviral.2020.104762

Verveke,R., et al. (2021). The down of mRNA vaccines the COVID-19 case. The journal of Controlled release. https://www.sciencedirect.com/science/article/pii/S0168365921001541?via%3Dihub

ViralZone. Flaviviridae. *https://viralzone.expasy.org/43*

ViralZone. Caliciviridae. *https://phil.cdc.gov/Details.aspx?pid=2172*

ViralZone. Coronaviridae. https://viralzone.expasy.org/30

Xiao, X. et al, (2021). Animals for Whuan wet markets immediately prior to the COVID=19 pandemic. Scientific Reports, 11 article # 11898 https://www.nature.com/articles/s41598-021-91470-2

Xu,J. et al. (2020). Systematic comparison of two animal-to-human transmitted human coronaviruses: SARS-CoV-2 and SARS-COV. Viruses. *12*(2), 244; https://doi.org/10.3390/v12020244

Ying L, and J Rocklöv (2022). The effective reproductive number of the Omicron variant of SARS-CoV-2 is several times relative to Delta. J Travel Med. May 31;29(3):taac037. *doi: 10.1093/jtm/taac037. https://pubmed.ncbi.nlm.nih.gov/35262737/*

REVIEWED BY:

Dr Tony Schountz Colorado State University, Department of Microbiology, Immunology and Pathology, Fort Collins CO, 80523

CHAPTER 16

Chapter 16: Segmented Double Stranded RNA Viruses That Replicate In The Cytoplasm: Reoviridae

INTRODUCTION TO THE FAMILY

You will notice in Figure 16-1 that reoviruses are segmented, linear, double stranded RNA viruses with 10-12 segments, and that they are naked icosahedral viruses that replicate in the cytoplasm. This is the largest and most diverse family of dsRNA viruses, although there are many dsRNA viruses that infect plants and insects. Reoviridae is the only virus family that infects animals and humans. The genera that cause disease in animals and humans include: *Orbivirus* which infects mammals, birds, and arthropods; *Rotavirus* that infects mammals and birds; *Coltivirus* that infects mammals and arthropods; and *Orthoreovirus* that infects mammals, birds, and reptiles. Those that infect both mammals and arthropods are transmitted by infected arthropods. They are 65-80 nm in diameter, non enveloped, and contain 1, 2, or 3 capsid layers (i.e., they can have a capsid inside a capsid). The virion contains all the enzymes needed for mRNA production and replication (RNA-dependent RNA polymerase, RdRp, and capping enzymes). Reoviruses, like negative sense RNA viruses, must carry their RdRp in their virion with their genome to produce mRNA once inside the cell. See Figures 16-2 & 16-3.

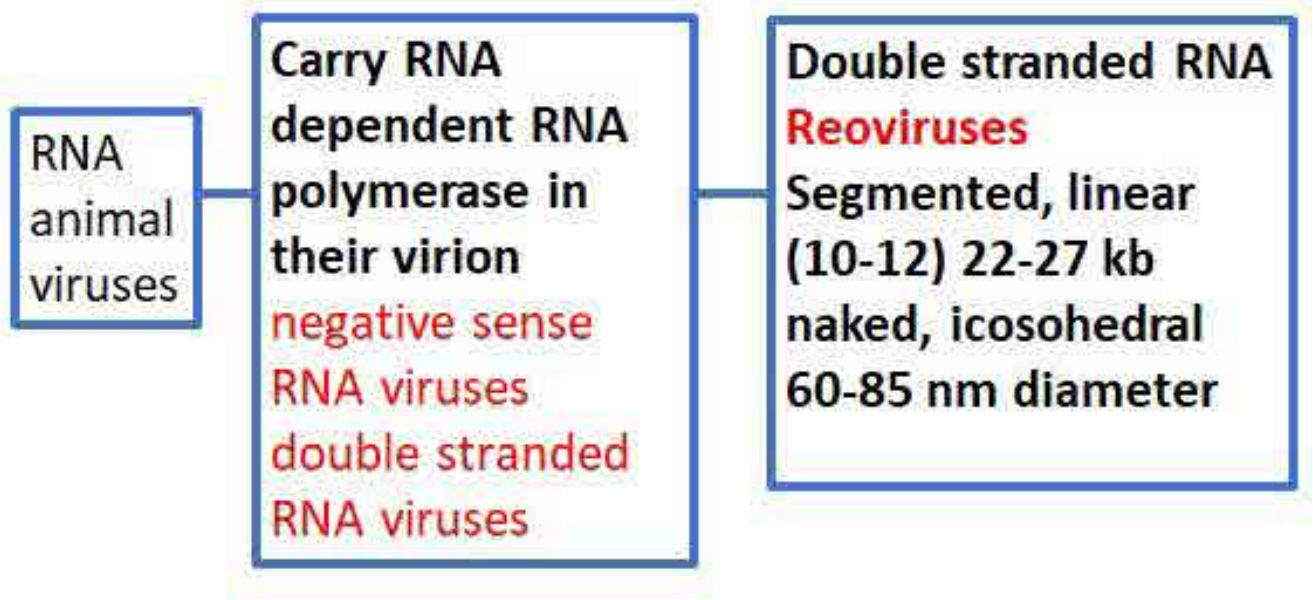


Figure 16-1. The taxonomy of RNA viruses with an emphasis on reoviruses. Courtesy of Erica Suchman, Colorado State University.

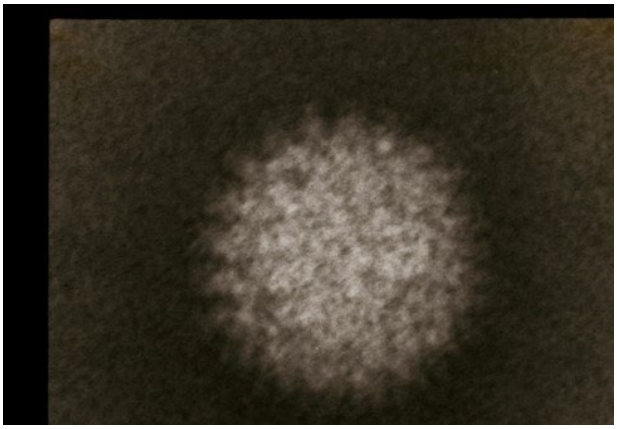


Figure 16-2. An electron micrograph of blue tongue virus, a disease of sheep in the genus Orbivirus. Courtesy of Tom Walton, APHIS (retired).

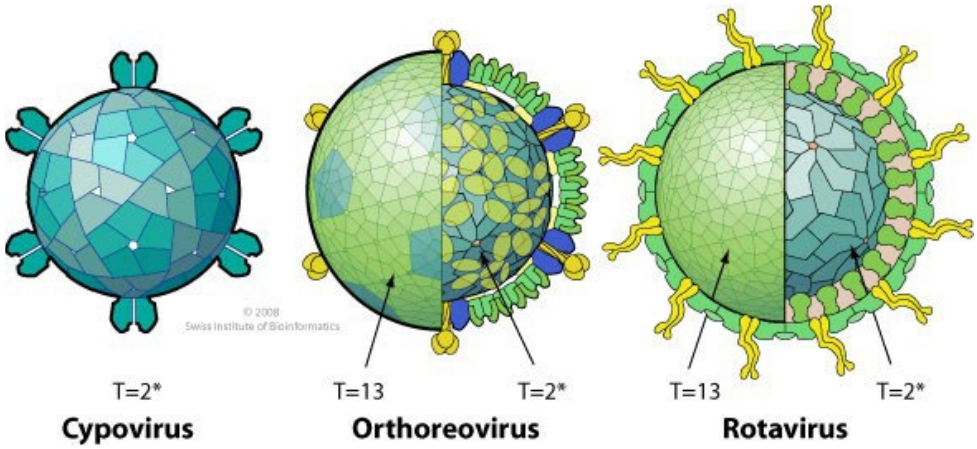


Figure 16-3. A typical Reovirus virion with multiple capsids and no envelope containing 10-12 segments of double stranded RNA. Courtesy of ViralZone https://viralzone.expasy.org/104

GENOME STRUCTURE

Reoviruses have 10-12 segments, each of which produces a separate mRNA and at least one open reading frame (ORF). 1 or 2 of the segments have more than one overlapping ORF that can be expressed via leaky scanning as shown in Figure 16-4. The total genome size is 22-27 kb.

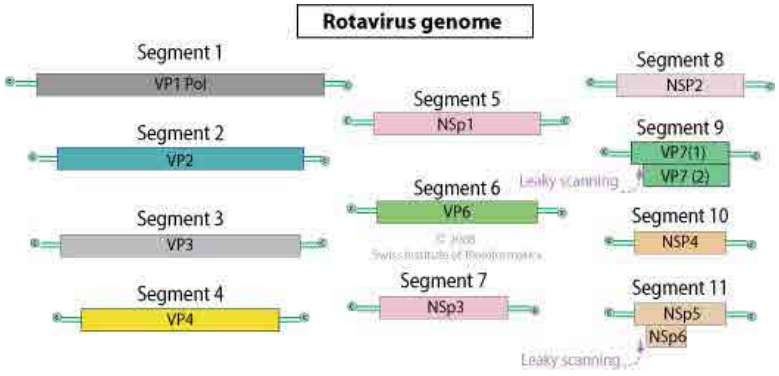


Figure 16-4. Typical reovirus genome structure with 10-12 segments of double stranded RNA. Courtesy of ViralZone https://viralzone.expasy.org/104

REPLICATION CYTOPLASMIC

1. Attachment to a surface carbohydrate and junctional adhesion molecule A (Jam-A) host receptors (the carbohydrates vary between the genera) mediates endocytosis of virus into host cell.
2. Particles are partially uncoated in endosome when cellular endocytic proteases cleave away some capsid proteins. This leads to formation of the **infectious subvirion particles** (ISVP). Cleavage of viral capsid proteins in the endosome also leads to pore formation on the endosome membrane and penetration into the cytoplasm. The ISVP capsid proteins are further cleaved giving rise to **non-infectious core particles**. It has been shown that the ISVP form is required for replication in host cells. See Figure 16-5.
3. Early transcription of the double stranded RNA genome by viral RdRP polymerase occurs inside core particle so that dsRNA is never exposed to the cytoplasm thus avoiding detection by pathogen recognition receptors (PRR).
4. Transcription from each of the dsRNA segments by viral RdRp transcriptase activity produces mRNA templates for translation. Reovirus capping enzymes add a 5’ cap but no poly A tail as shown in Figure 16-6.
5. Viral proteins and genomic RNAs aggregate in cytoplasmic viral factories close to the nuclear membrane that can be observed as **perinuclear inclusions** (in the cytoplasm but near the nucleus).
6. Positive sense RNAs are encapsidated in an infectious subviral particle in which they are replicated to give negative sense RNA molecules thus producing dsRNA genomes.
7. The capsid is assembled in the subviral particle. The virus leaves the viral factory (called **viroplasm**) by budding out of the viroplasm through the ER and then removes the temporary envelope as it gains its third capsid layer.
8. Mature virions are released after cell death when the virus induces the cell to enter apoptosis and the associated breakdown of host plasma membrane occurs as shown in Figure 16-7.

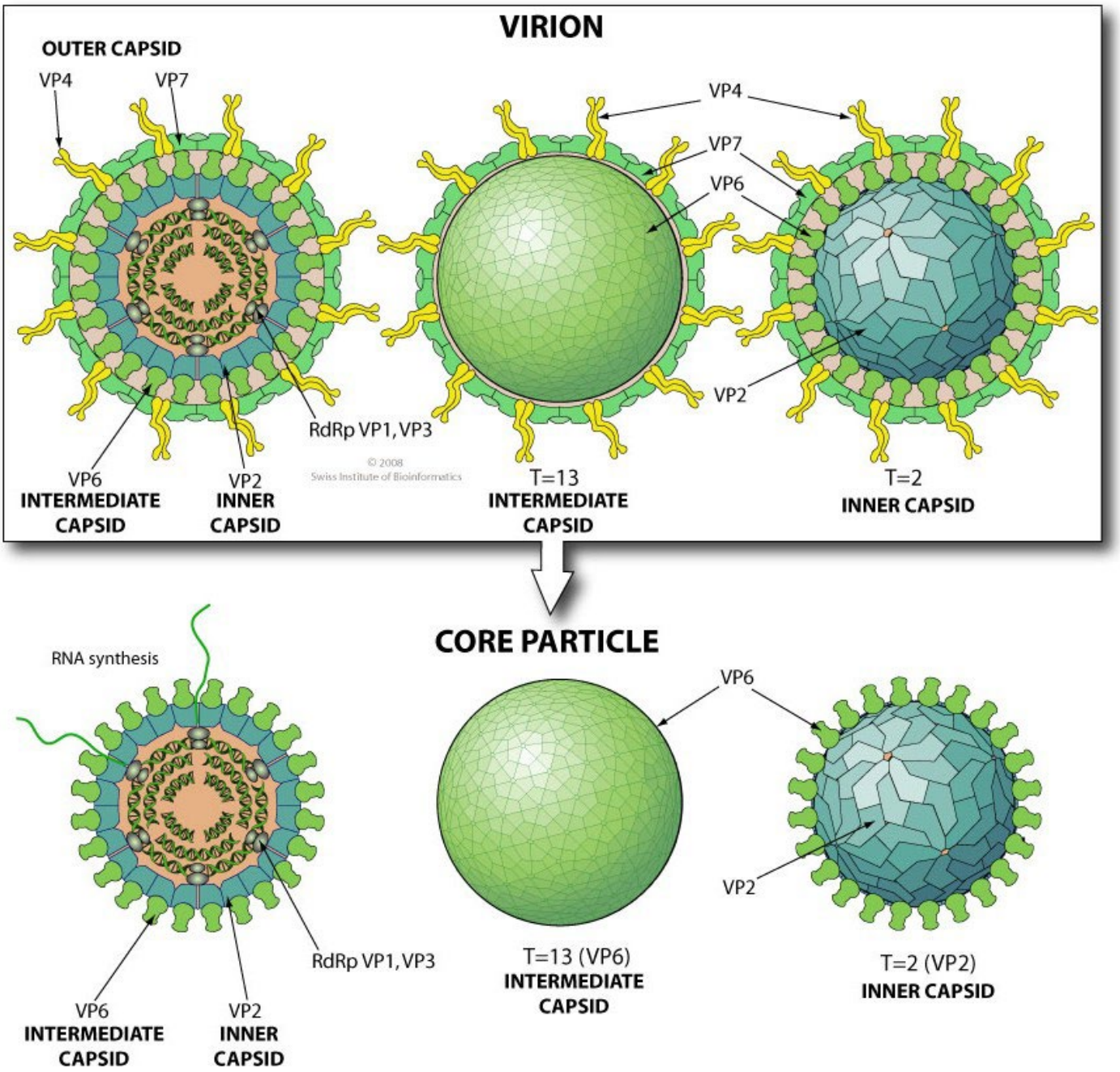


Figure 16-5. Reovirus maturation as cellular proteases cleave viral capsid proteins away from the infectious viral capsid revealing the infectious subviral partial and then further to reveal the noninfectious core viral particle where viral RNA will be transcribed and extruded from the capsid. Courtesy of ViralZone https://viralzone.expasy.org/resources/Rotavirus_maturation.jpg

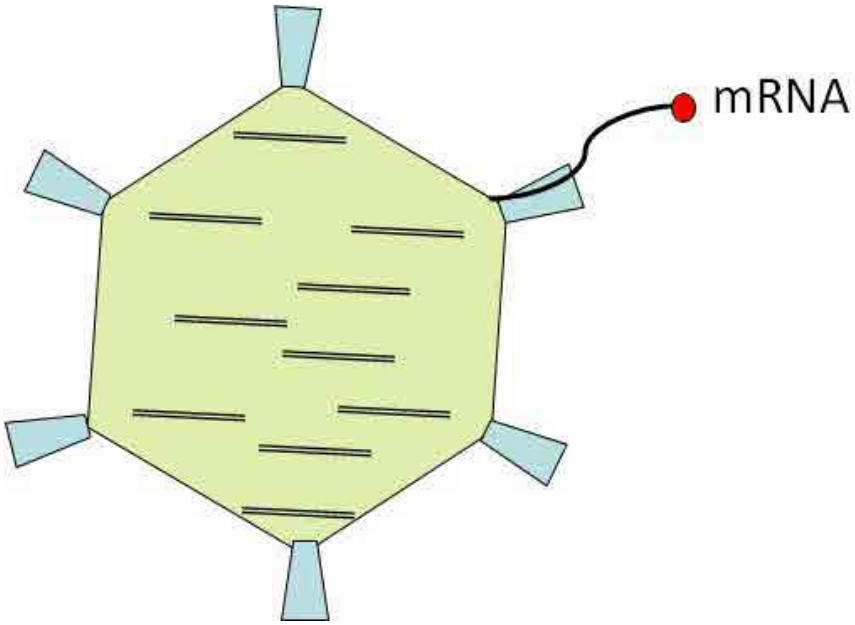


Figure 16-6. Reovirus transcription occurs within the inner core particle after the removal of the outer core proteins by cellular proteases. Courtesy of Sandra Quackenbush, Colorado State University.

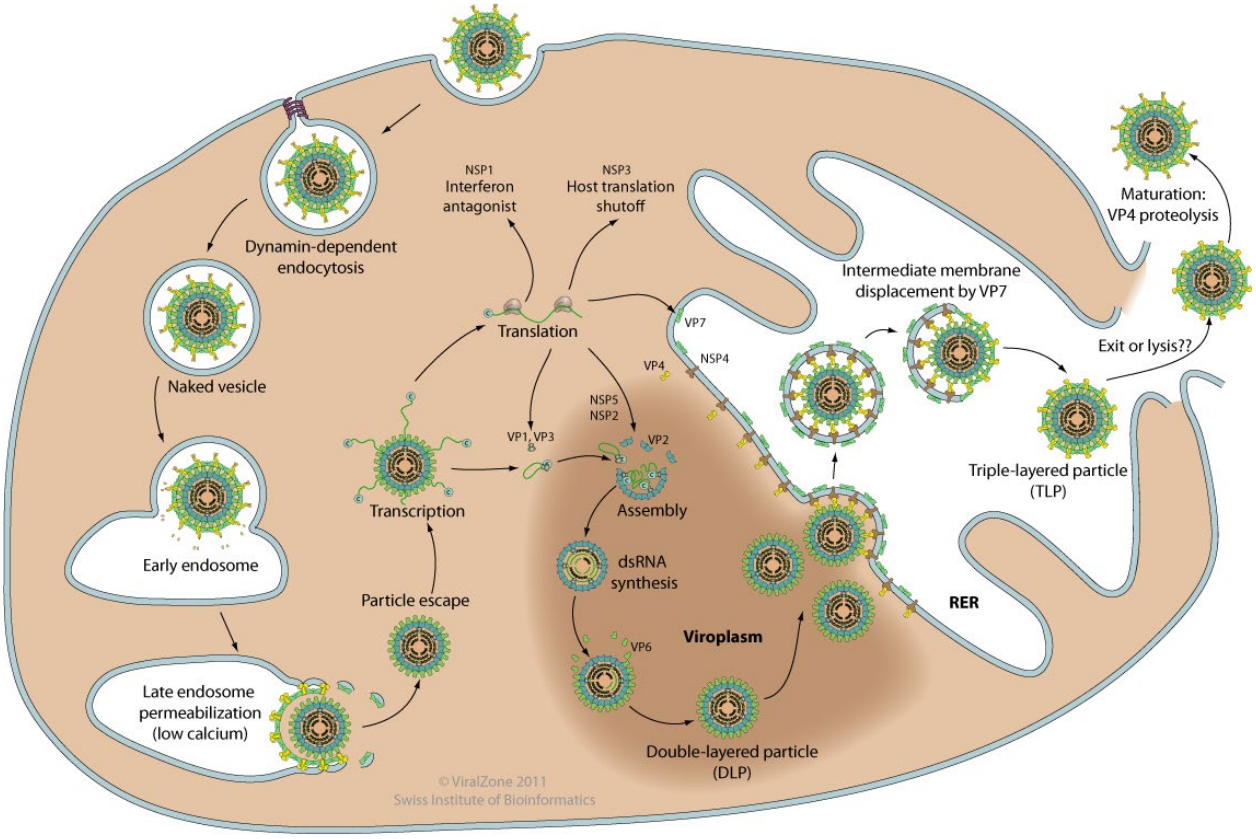


Figure 16-7. A typical reovirus life cycle. Courtesy of ViralZone
https://viralzone.expasy.org/107?outline=complete_by_species#tab5

HOW DO REOVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS WHEN IT REPLICATES IN THE CYTOPLASM?

Reoviruses, like most RNA viruses, have the ability to create the 5' cap, however, unlike many other RNA viruses, it is not the RdRp which performs this function. It creates multiple proteins to add the 5' cap. Also, it cannot create a 3' poly A tail. As such it must either have a secondary structure within the 3' end to associate with poly A binding proteins (PABP) or have evolved PABP independent translation initiation mechanisms. Reoviruses have evolved alternative mechanisms.

HOW DO REOVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

1. Reoviruses have multiple segments that each make separate mRNAs. Recall that having segmented genomes means that these viruses can undergo **reassortment** when multiple viruses infect one cell and exchange segments as new viral particles are formed.
2. Additional proteins are produced by leaky scanning when the ribosome initiates translation at an AUG associated with a poor Kozak consensus sequence allowing expression of overlapping reading frames.
3. Note: Although reoviruses use cellular proteases to cleave the viral capsid proteins to mature the viral particle as it enters the cell, they **do not** use viral or cellular proteases to create multiple proteins from large polyproteins created from one ORF. All of the negative sense stranded segmented RNA viruses discussed in this book do this.

TRANSLATION OF VIRAL PROTEINS

We learned in Chapters 3 and 13 that translation initiation requires a whole host of initiation factors to bind the 5' cap; in particular, eIF4E binds to the cap, and eIF4G binds first to eIF4E and then to other initiation factors. Because reoviruses create a 5' cap, it is able to interact with these initiation factors, but because it lacks a 3' poly A tail, it must either have a secondary structure in the 3' region that will interact with the PABP to initiate translation or use a PABP independent mechanism. It appears that reoviruses do the latter. Not needing PABP allows these viruses to inhibit PABP to outcompete cellular mRNAs during translation.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes.

Note that reoviruses, like many viruses, take care of the problem of replicating the ends and priming replication via the same mechanism.

RNA viruses, with the exception of retroviruses, cannot use the host cell's transcription machinery DNA-dependent RNA polymerase (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As a result, with the exception of retroviruses, they must all have genes to produce an RNA-dependent RNA polymerase (RdRp). Negative sense RNA virus genomes are not equivalent to mRNA (they are complementary to mRNA), cannot be translated immediately upon entrance into the cell, and, as such, must carry RdRp into the host cell with the genome to make mRNA from the negative sense RNA genome that can undergo translation. Double stranded RNA viruses, such as the reoviruses, do not have a single stranded positive sense RNA available for translation, and as such they must also carry an RdRp, just like a negative sense RNA virus would. Note that all RNA viruses of animals have linear genomes and must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses including reoviruses ensure the replication of their genome's ends easily as RdRp starts synthesizing at the end of the genome without a primer. This is referred to as **de novo synthesis**. De novo initiation by RdRP occurs at the 1st nucleotide. Reoviruses transcribe their genome within their core capsid shielding the double stranded RNA genome from cellular antiviral responses as shown in Figure 16-6, and the genome is replicated in cytoplasmic viral factories to shield from cellular antiviral responses as shown in Figure 16-7.

VIRION FORMATION

Viral capsids form spontaneously in cytoplasmic viral factories. First the core particle forms, and then the secondary particle (ISVP) forms around the core particle. The cell leaves the viral factory when it buds out of the ER obtaining a temporary envelope that is soon lost as it is replaced by the 3rd layer of the capsid forming a mature virion. The virus then leaves the cell when the cell undergoes apoptosis that is activated by the viral infection.

AVOIDING THE HOST IMMUNE RESPONSE

Reoviruses avoid detection of pathogen recognition receptor (PRR) TLR 3 which recognizes the pathogen associated molecular pattern (PAMP) of double stranded endosomal RNA by remaining in their capsids until they leave the endosome. They avoid the PRRs RIG-I and MDA-5 (cytoplasmic RNA lacking a 5' cap) by creating enzymes to produce a 5' cap, MDA-5 RNAi, PKR, and OAS (cytoplasmic dsRNA), by transcribing their double stranded RNA genome within the core capsid and replicating their genomes within viral factories. Some also produce accessory proteins that help inactivate the type I interferon response. Furthermore, due to their segmented genomes, reoviruses can perform gene reassortment to avoid the host immune response by changing their external antigens.

REOVIRAL DISEASES IN ANIMALS

AFRICAN HORSE SICKNESS

African horse sickness (AHS, virus AHSV) is an infectious, noncontagious (does not spread from horse to horse), lethal virus in the family *Reoviridae* and genus *Orbivirus* that is transmitted to domestic and wild equids (horses, mules, donkeys) by biting midges (no-see-ums) in the genus *Culicoides*. Horses often die after an illness that includes red swollen eye lids, frothy nasal discharge, and hemorrhaging in internal organs.

Figure 16.8 A horse suffering from AHS showing late stages of infection including frothy nasal discharge and red swollen eye lids. Courtesy of Tom Walton APHIS (retired).

BLUETONGUE DISEASE

Bluetongue viruses (BTV) are infectious, noncontagious viruses in the family *Reoviridae* and genus *Orbivirus* that are transmitted to domestic and wild ruminants (sheep, cows, goats, deer) by biting midges (no-see-ums or tiny flies) in the genus *Culicoides.* The disease is most problematic in sheep where case-fatality rates can approach 80%. Factors such as severe weather, infection with parasites, poor diet, and stress are known to exacerbate mortality. Mild infections often go unnoticed. Disease signs include fever (usually 104-106^{O}C) and depression beginning 6-8 days

post infection. The disease progresses to salivation and redness and swelling of the mucous membranes of the mouth. It also eventually leads to ulceration of the mouth and nose often giving a burnt appearance (Figure 16-9A). Vomiting may result which results in bacterial pneumonia due to common aspiration and is the most common cause of BTV mortality. **Coronitis** (swelling of the tissue at the top of the hoof) is also common and may lead to lameness (Figure 16-8B) and sloughing of the hooves (Figure 16-8C). The bluish appearance of the tongue, from which the virus gets its name, is observed only occasionally. The case-fatality rate approaches 80% in sheep. The case-fatality rate in cattle is much lower, only 5%.

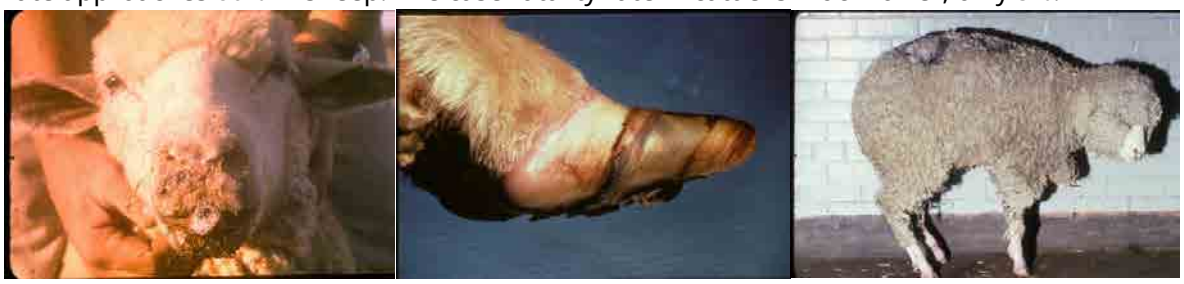


Figure 16.9A-C. A sheep suffering from blue tongue showing late stages of infection including ulcers on the muzzle (A) coronitis (B) and commonly observed hunch back appearance due to pain when weight is placed on the hooves. Courtesy of Tom Walton APHIS (retired).

REOVIRAL DISEASES IN HUMANS:

ROTAVIRUS

Rotavirus is the single most important cause of severe diarrhea in children worldwide and accounts for 30%-50% of diarrheal illness worldwide. It is estimated that approximately 500,000 children under the age of 5 die of diarrhea each year, thus rotavirus infections are a major source of childhood mortality. Estimates are that there are 2.39 billion episodes of diarrheal disease globally each year with 957.5 million of these being in children, so rotaviruses also cause a great deal of illness as well and have huge economic impacts. There are 3 serotypes that are classified based on their capsid protein antigens. Most individuals have serologic evidence of past reovirus infection. These infections occur in early childhood. Most rotavirus infections are asymptomatic or cause a mild respiratory or gastrointestinal illness. Rotaviruses are one of the few viruses that carry genes for and create a viral toxin. Rotaviruses create an enterotoxin viroporin (NSP4) that modulates intracellular calcium levels in intestinal epithelium leading to poor water reabsorption and thus diarrhea. Inducing diarrhea is a brilliant mechanism for viral transmission as the feces carry abundant viral particles with up to 10^{11} particles/ml of feces. Furthermore, the virus is very stable in the environment and can remain on **fomites** (inanimate objects) for several days. As one might expect for a virus that remains stable in the environment and infects the intestines, this virus is transmitted by the **fecal-oral route**. When illness does occur the intrinsic incubation period is usually less than 48 hours. The symptoms include sudden onset of vomiting and diarrhea resulting in dehydration and hospitalization. The duration of illness is 3-5 days. The virus replicates in the villi of the intestines in enterocytes. By 12 hours post infection viral antigen can be detected in the villi, and by 24-72 hours post infection villus atrophy and blunting are observed with mononuclear infiltration and vacuolation of the epithelial cells. NSP4 is the viral **enterotoxin.** As it is expressed, it disrupts tight junctions in the villi, perturbs protein signaling,

induces a chemokine response, and leads to agitation of the nervous system of the gut. This agitation causes the vomiting and diarrhea. By 7 days the villi are regenerated and symptoms pass. Young children without previous immunity and the elderly with declining immunity are most at risk. Infection generates robust serum IgG and intestinal IgA as well as serum T-cell response. The T-cell and IgA response have been found to be important for virus clearance. By one year of age most children have a strong **memory response** (see Chapter 4) that is protective. In old age, however, this response declines significantly, and an increase in disease is observed in these populations.

DIAGNOSIS OF REOVIRUS INFECTIONS

Reovirus infections can be diagnosed by isolation of virus, however this is a very slow method and rarely used. Faster methods involve detection of viral protein via Western blots or viral RNA via reverse transcriptase PCR. Serologic assays such as ELISAs showing a 4-fold increase in IgG antibody titer between an acute sample and a convalescent sample taken at least 2 weeks later are also used.

PREVENTION OF REOVIRUS INFECTIONS

To prevent animal disease, reduction of *Culicoides* breeding sites by reducing standing water or using insecticides or moving animals inside during the vector's peak activity time (dusk and dawn) have been found to be helpful. Due to the seasonal increase in the vector, disease is most prevalent midsummer to early fall.

For rotavirus the best prevention is vaccination and proper hand hygiene involving washing hands thoroughly and often.

The rotavirus vaccine history has a checkered past. Early versions of the vaccine caused severe intestinal blockages. Newer safer vaccines have been developed and have been shown to have high efficacy.

Rotarix is live-attenuated human rotavirus. Vaccination is given at 2 and 4 months of age, administered orally.

RotaTeq is a live-attenuated reassortant vaccine. The vaccine contains five reassortant viruses. In particular, the bovine rotavirus strain WC-3 was reassorted with VP7 surface proteins of human rotaviruses G1, G2, G3 and G4 and one reassortant between bovine and human VP4. This bovine segment causes the vaccine to lose the ability to cause disease. This vaccine is administered orally at 2, 4 and 6 months of age. Studies conducted in Mexico tracking childhood diarrhea deaths before and after RotaTeq introduction show a dramatic reduction in diarrheal deaths after the vaccine was introduced.

TREATMENT OF REOVIRUS INFECTIONS

There is no treatment for blue tongue or African horse sickness.

Rotavirus is a self-limiting disease lasting a few days. The treatment generally involves oral rehydration therapy (Pedialyte), and 1 in 70 children require hospitalization for IV fluids.

END OF CHAPTER QUESTIONS

1. What is unusual about the reovirus capsid?
2. Compare and contrast reoviruses to togaviruses and flaviviruses.
3. Compare and contrast reoviruses to orthomyxoviruses and filoviruses.
4. Compare and contrast reoviruses and bunyaviruses.
5. Do reoviruses create subgenomic mRNAs? If they do, why do they do so? If they do not, why not?
6. Compare and contrast how these 2 viruses avoid the host immune response.
7. Do reoviruses need to have mechanisms for overcoming RNA interference? If so, why? If not, why not?
8. Do reoviruses use viral proteases to cleave polyproteins? If not, do they use proteases (viral or cellular) for any other reason? If so, for what purpose?
9. How do reoviruses that lack a 3' poly A tail initiate translation?
10. What advantage might lacking a 3' poly A tail provide reoviruses?
11. Can reoviruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
12. Why do reoviruses undergo transcription within the core particle of their capsid?
13. Do you find anything unusual about reoviruses undergoing budding? If so, what?
14. If you stained reovirus-infected cells to view their viral factories, where would you expect to observe staining?
15. You perform a reverse transcriptase PCR to determine if your child has a rotavirus infection. You use a primer that is complementary to bases 100-115 and another that is complementary to the opposite strand bases 655-670. What will your results look like if your child is positive? What if they are negative? What controls will you need to run?
16. Reoviruses undergo reassortment. Why are they able to do this?
17. What advantages does undergoing reassortment confer on reoviruses?
18. What do reoviral diseases of domestic animals have in common?
19. What is unusual about rotavirus?

20. What facet of the immune response is most important for clearing rotavirus infections?
21. How are severe rotavirus infections treated?
22. Explain why the rotavirus vaccine uses reassortants?

REFERENCES:

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, chapters 44-46.

International Health Metrix Evaluation (IHME) E. Head. (2017). Rotavirus deaths. http://www.healthdata.org/news-release/despite-substantial-global-reduction-diarrhea-deaths-half-million-children-still-die

Parashar and Patel. (2012). Fulfilling the promise of rotavirus vaccines: how far have we come since licensure? N Engl J Med. 365:773 https://pubmed.ncbi.nlm.nih.gov/22742639/

ViralZone. *Reoviridae.* https://viralzone.expasy.org/104

ViralZone. Rotavirus maturation. https://viralzone.expasy.org/resources/Rotavirus_maturation.jpg

ViralZone. Rotavirus replication cycle. https://viralzone.expasy.org/107?outline=complete_by_species#tab5

CHAPTER 17

Chapter 17: Non-Segmented Negative Sense Stranded RNA Viruses That Replicate In The Cytoplasm: Rhabdoviridae, Paramyxoviridae, Filoviridae, Pneumoviridae (Mononegavirales).

INTRODUCTION TO THE ORDER *MONONEGAVIRALES*

You will notice in Figure 17-1 that viruses in the family *Rhabdoviridae*, *Paramyxoviridae*, F*iloviridae* and *Pneumoviridae* are all cytoplasmic replicating viruses with non-segmented negative sense RNA genomes, helical, enveloped virions, and are classified within the order *Mononegavirales*. This order is different than all other negative sense RNA viruses which all have segmented genomes. Negative sense RNA viruses package RNA-dependent RNA polymerase (RdRp) in their virions. The viruses encode a similar set of genes, and the order of the genes is conserved, as shown in Figure 17-2. Note that conserved gene order is also true for the segmented negative sense RNA viruses as well. Although the genes are found on different segments, they are still conserved in their order. *Mononegavirales* also has shared replication pathways. As such, we will only provide one diagram of viral replication (Ebola, a filovirus) as a representative for members of the order *Mononegavirales* as it will be very similar for paramyxoviruses, pneumoviruses and rhabdoviruses, with the exception of how the viruses enter the cell. You will notice that all negative sense RNA viruses discussed in this book except for orthymyxoviruses replicate within the cytoplasm. Note: we will not discuss *Bornaviridae* in this chapter due to the limited diseases associated with these infections.

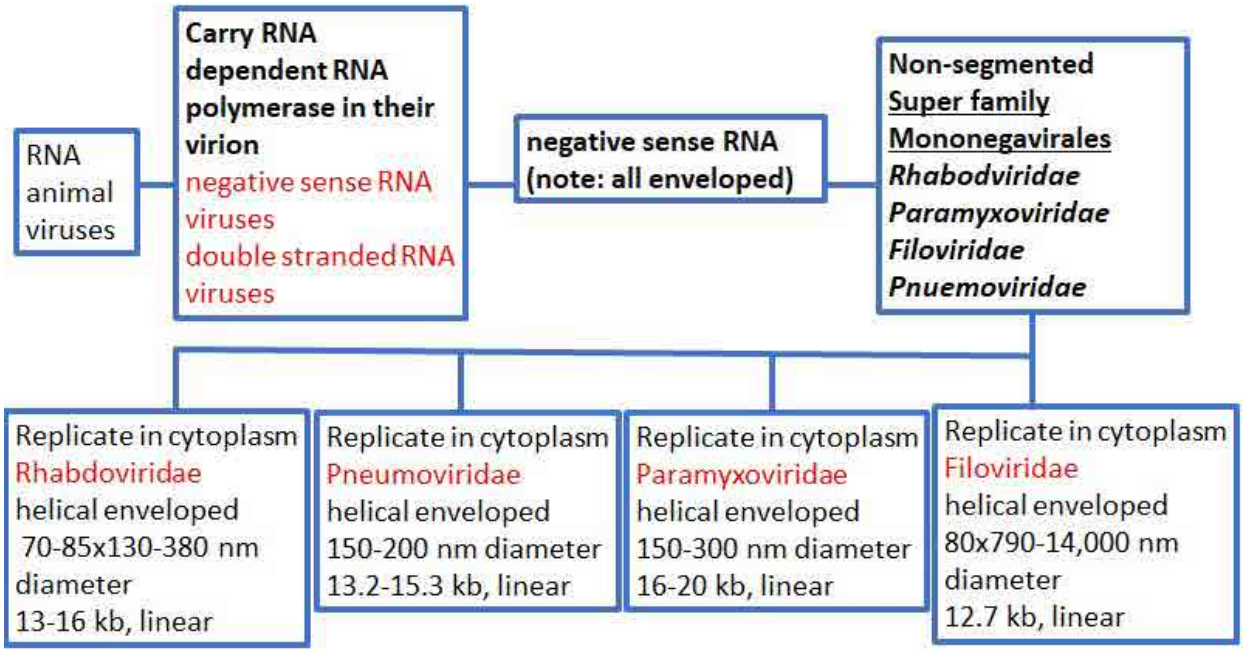


Figure 17-1. The taxonomy of RNA viruses focusing on Mononegavirales. Courtesy of Erica Suchman, Colorado State University.

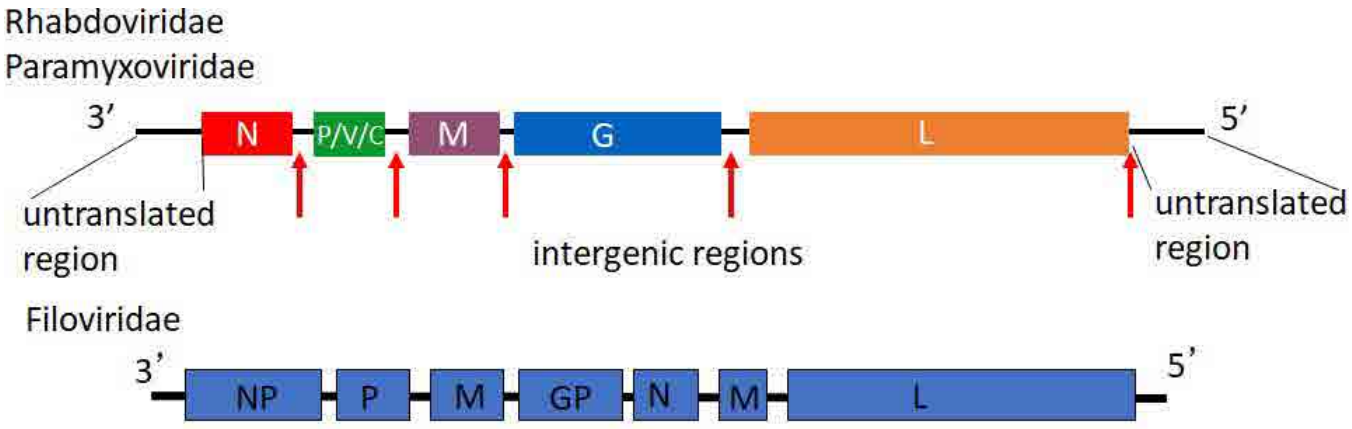


Figure 17-2. Genome structure of rhabdoviruses, paramyxoviruses, pneumovirues and filoviruses. Note that the conservation of gene order is also observed in all other negative sense RNA viruses. Also Pneumoviridea genome is the same as Rhabdoviridae. Courtesy of Sandra Quackenbush, Colorado State University.

The conserved gene order for negative sense RNA viruses including the *Mononegavirales* is shown in Figure 17-2. The genes are as follows: N/NP which codes for the nucleocapsid nucleoprotein; P/V/C which codes for co-factors for viral RNA synthesis, virus assembly and release, and for counteracting antiviral responses; M which codes for the matrix protein that is found between the helical nucleocapsid and envelope; G which codes for the envelope glycoprotein; and L which codes for the RNA-dependent RNA polymerase (RdRp).

GENE EXPRESSION

The viral RdRp binds the still encapsidated genome at the 3' end of the leader region to begin **de novo syntheses** without a promoter (Chapter 13) then **sequentially transcribes** each of the 5 genes by recognizing start and stop signals flanking viral genes. The mRNAs are capped and polyadenylated during synthesis when RdRp stutters on a short poly U track at the 5' end of each gene. Transcription attenuation results in a gradient of mRNAs and protein expression with more N protein being created than L protein allowing the virus to regulate how much of each protein it creates with each subsequent gene being expressed at a lower level, as shown in Figures 17-3 and 17-4.

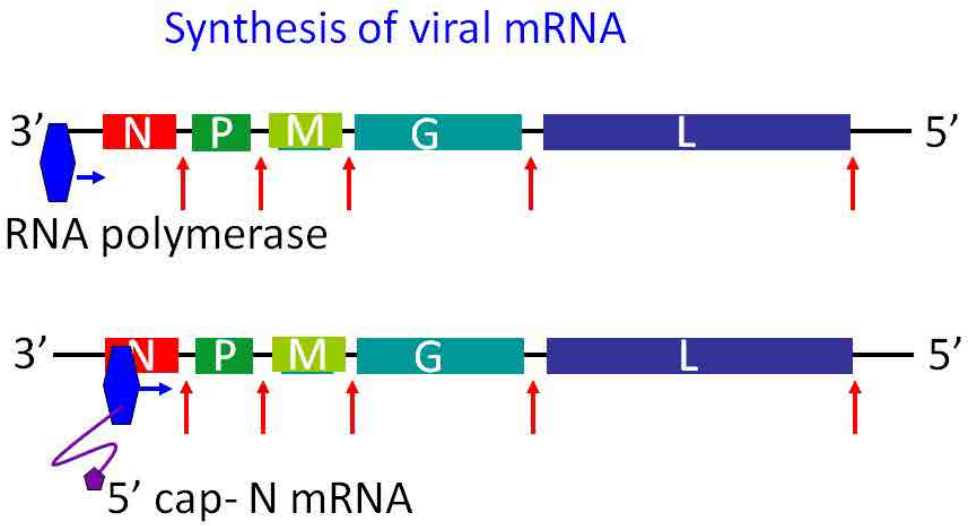


Figure 17-3. Initiation of mRNA synthesis rhabdoviruses, paramyxoviruses, pneumovirses and filoviruses. Courtesy of Sandra Quackenbush, Colorado State University.

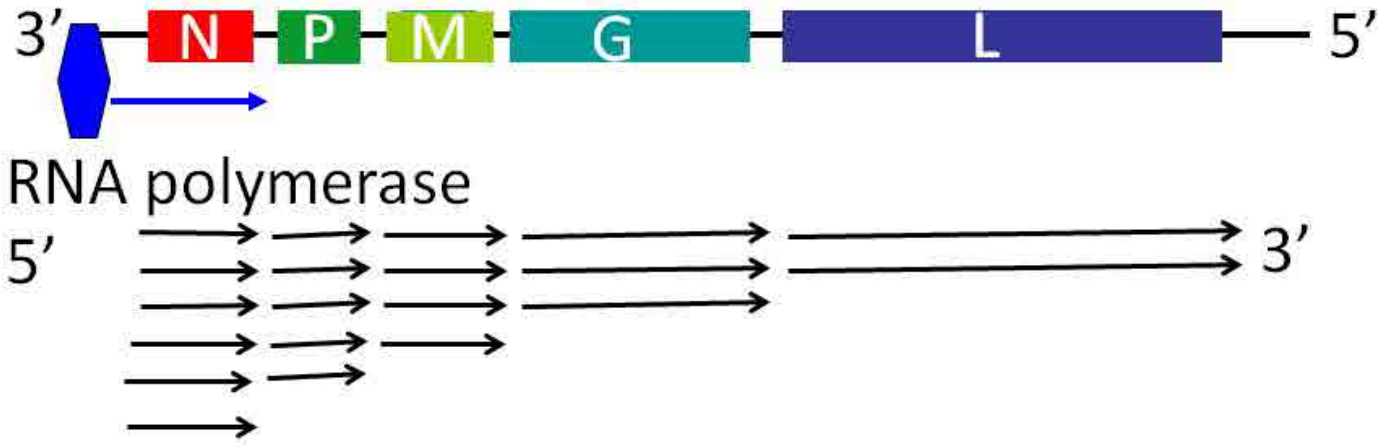


Figure 17-4. mRNA synthesis transcription attenuation resulting in a gradient of mRNAs and protein expression in rhabdoviruses, paramyxoviruses, pneumoviruses and filoviruses. Courtesy of Sandra Quackenbush, Colorado State University.

CYTOPLASMIC REPLICATION OF VIRUSES IN *MONONEGAVIRALES*

As shown in Figure 17-5 & 17-6, the replication cycle of *Mononegavirale*s viruses begins when

1. Attachment of the viral envelope glycoproteins to host receptors mediates endocytosis of the virus into the host cell. Note: in filoviruses pinocytosis creates the endocytic vesicle, in rhabdoviruses the viruses are endocytosed into clatherin coated vesicles, and in paramyxoviruses and pneuoviruses the virus fuses with the plasma membrane and is not endocytosed.
2. Fusion of virus membrane with the vesicle membrane (rhabdoviruses and filoviruses) and fusion of the viral envelope and plasma membrane (paramyxoviruses and pneumoviruses) leads to the helical ribonucleocapsid release into the cytoplasm. Note Filoviruses require cellular proteases to cleave envelope proteins to initiate this process.
3. Sequential transcription occurs within the nucleocapsid, and viral mRNAs are capped by RdRp and polyadenylated by RdRp stuttering in a poly U track, this occurs in the cytoplasm.
4. Replication presumably starts when enough nucleoprotein is present to encapsidate synthesized genomes. As nucleocapsid protein accumulates, the virus switches from transcription to genome replication as shown in Figure 17-5.
5. The helical ribonucleocapsid binds to the matrix protein and buds at the plasma membrane releasing new virions.

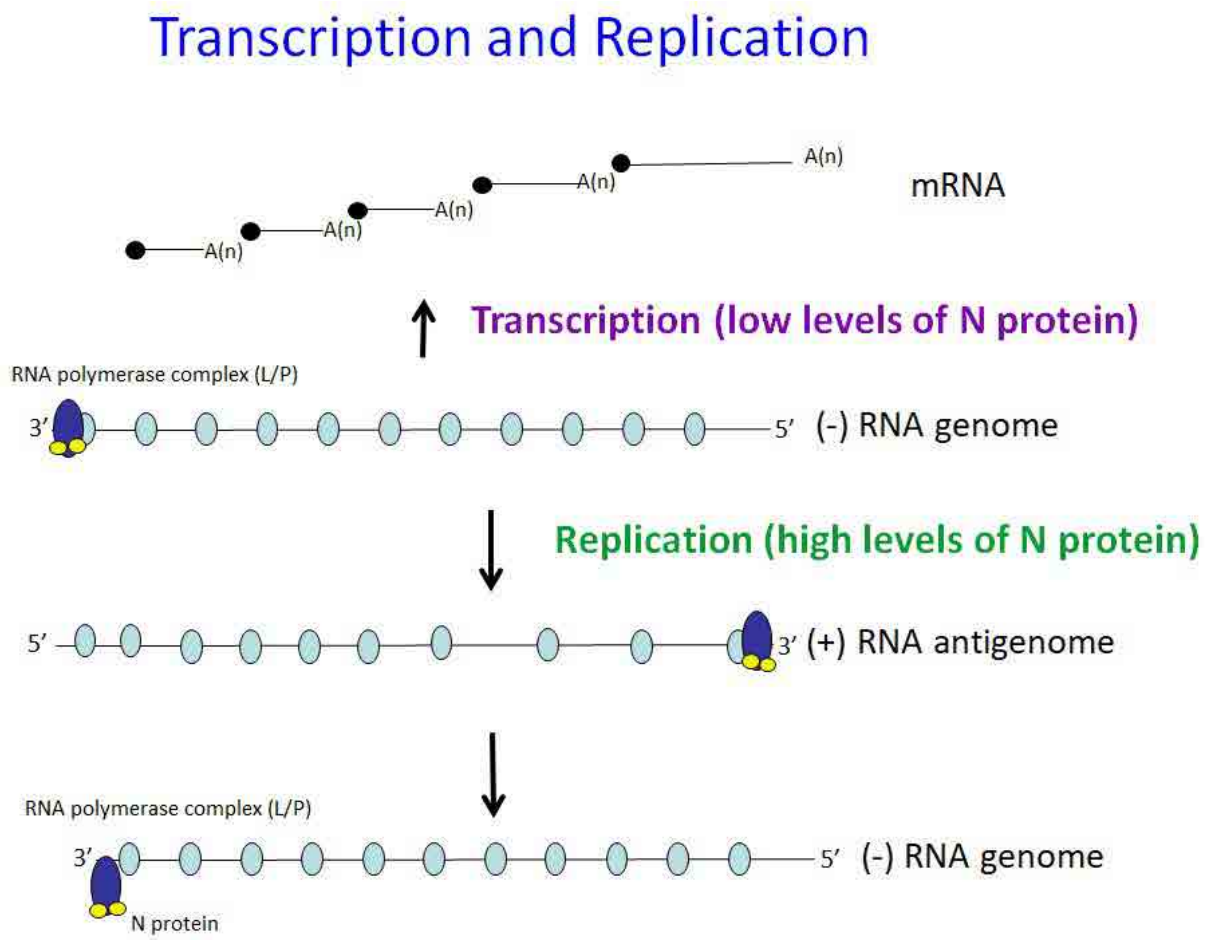


Figure 17-5. Mononegavirales switching from transcription to genome replication when high levels of nucleocapsid protein (N) accumulate. Courtesy of Sandra Quackenbush, Colorado State University.

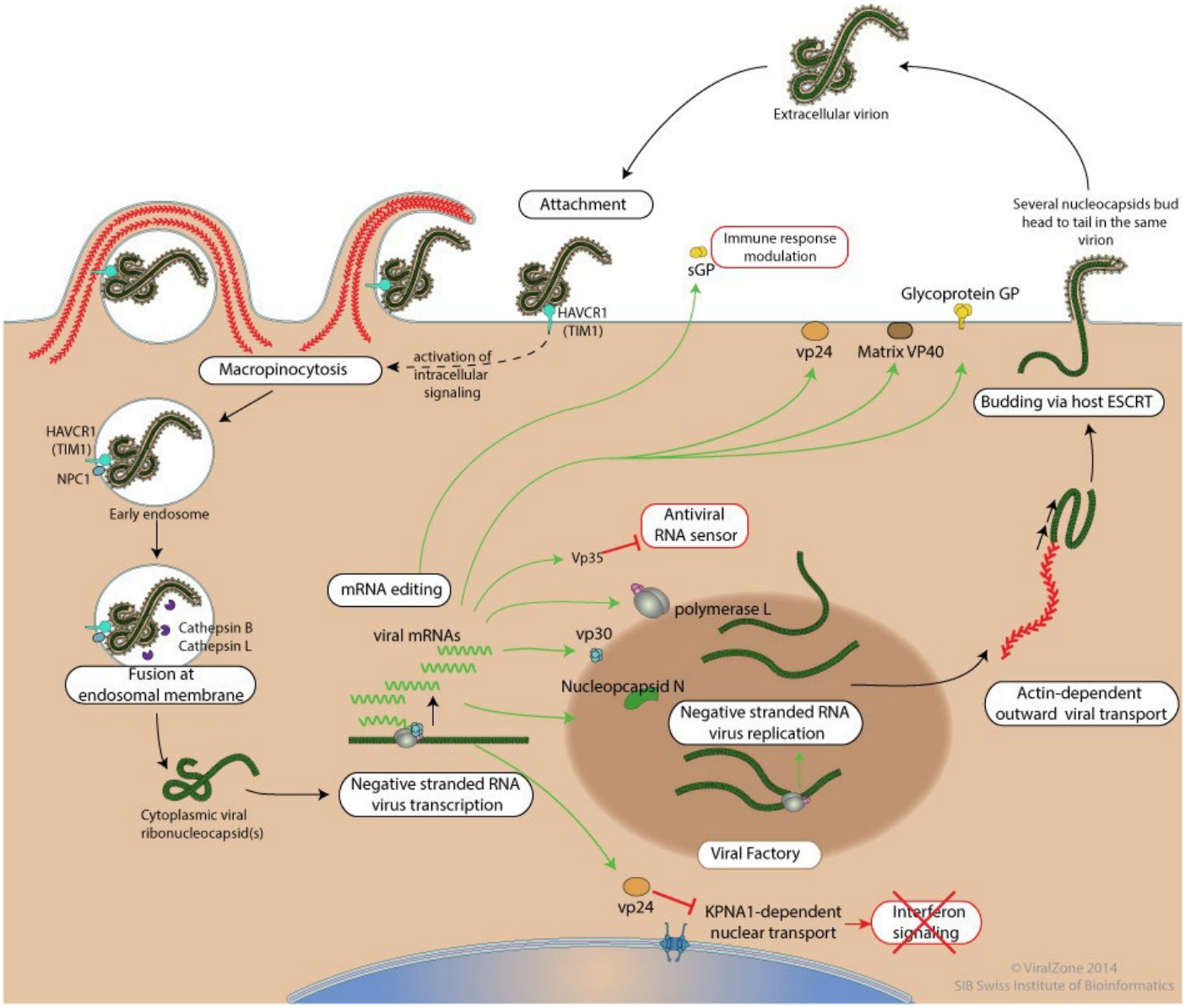


Figure 17-6. Replication of the filovirus Ebola as a representative of Mononegavirales replication. Note only the mechanism for entering cells (fusion with the plasma membrane or endocytic vesicles) varies between the families. Courtesy of ViralZone https://viralzone.expasy.org/5016

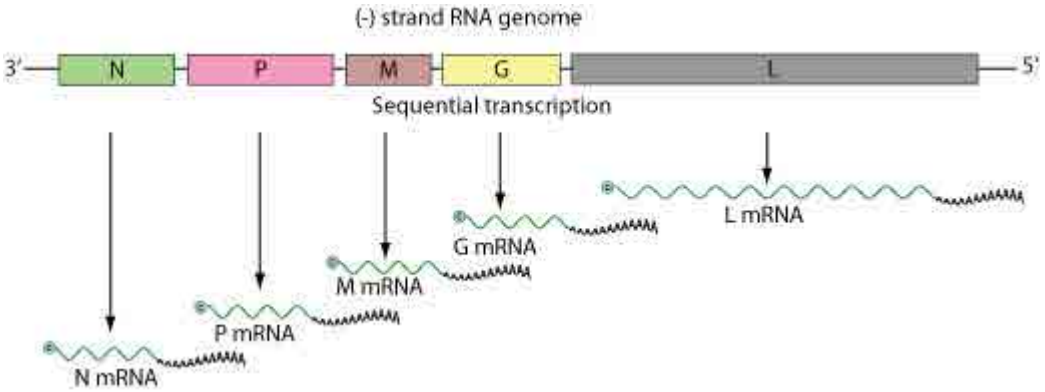
HOW DO *MONONEGAVIRALES* VIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS WHEN THE VIRUSES REPLICATE IN THE CYTOPLASM?

Like most RNA viruses, the RdRp of *Mononegavirales* viruses have the ability to create the 5' cap and have a poly U tract in the 5' end of the template (negative sense) strand of RNA that will result in the addition of a long poly A tail when the RdRp stutters adding far more A's than exist in the tract.

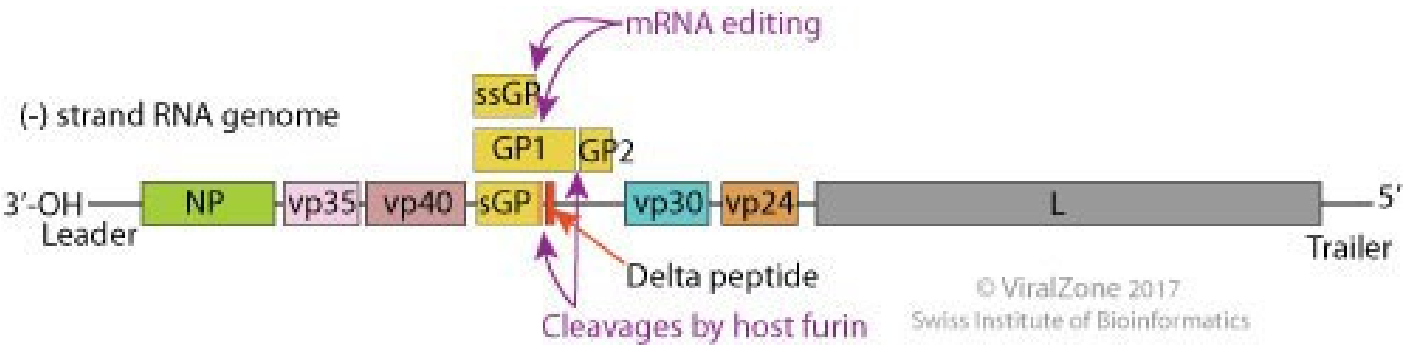
How do *Mononegavirales* viruses overcome the one protein per mRNA problem in eukaryotic cells?

1. Expressing multiple mRNAs from a single genome using the start stop mechanism of transcription (rhabdoviruses, paramyxoviruses, pneumoviruses and filoviruses).
2. RNA editing (paramyxoviruses, and filoviruses but NOT pneumoviruses and rhabdoviruses). In **RNA editing** 1 or more extra G nucleotides are added to the mRNA during transcription by RdRp a low percentage of the time leading to frame shifting and expression of a second overlapping open reading frame from the 2nd mRNA type produced.
3. **Leaky ribosomal scanning** (paramyxoviruses, pneumoviruses). Recall from Chapter 3 that leaky scanning occurs when the ribosome can initiate translation a low percentage of time at a first AUG start codon in context with a weak Kozak consensus sequence leading to translation of an overlapping reading frame.
4. Note: Although filoviruses use cellular proteases to cleave the viral envelope proteins as they enter the cell, they **do not** use viral or cellular proteases to create multiple proteins from large polyproteins created from one ORF. All of the segmented negative sense stranded RNA viruses covered in this book do cleave polypeptides into multiple proteins using cellular proteases.

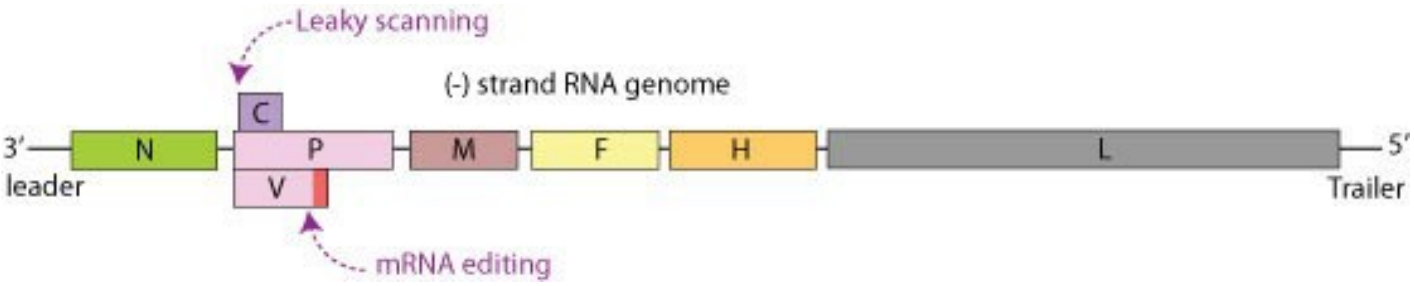
Although all the *Mononegavirales* viruses create their mRNAs via a start stop mechanism, they use different mechanisms to create multiple proteins from some of the mRNAs as shown in figures 17-7A, B, C & D. You will notice that rhabdoviruses only create multiple mRNAs via the start stop mechanism (Figure 17-7A), and that filoviruses utilize RNA editing to create more than one protein from the PVC gene. As shown in Figure 17-7B there is a site for proteolytic cleavage by a cellular protease named furin, this cleavage occurs when the virus is in the endosome and exposes the fusion peptide allowing the virus to leave the endosome, this is not producing more proteins, it is activation of a protein. Protein expression in the paramyxoviruses, such as the *Morbillivirus* measles virus shown in Figure 17-7 C & D, utilizes both leaky scanning of multiple start sites and RNA editing.



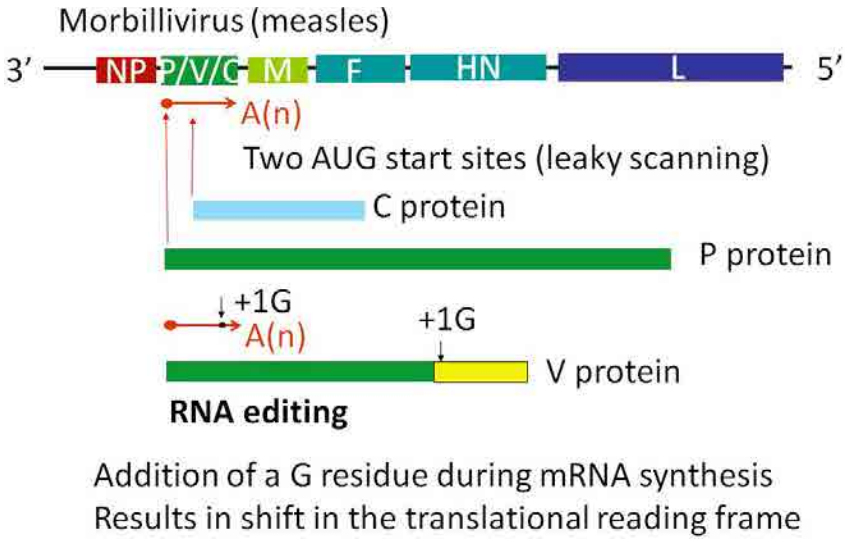
17-7A. Protein expression in the rhabdoviruses demonstrating only the production of multiple mRNAs and no further mechanisms. Courtesy of ViralZone https://viralzone.expasy.org/2



17-7B. Protein expression in the filoviruses demonstrating RNA editing. The furin cleavage sites are used to modify the envelope proteins exposing the fusion peptide allowing the virus to exit the endosome not to create more proteins. Courtesy of ViralZone https://viralzone.expasy.org/23



17-7C. Protein expression in the paramyxoviruses demonstrating leaky scanning and RNA editing. Note pnuemoviruses used to be in the family paramyxoviridae and also use mRNA editing and leaky scanning. Courtesy of ViralZone https://viralzone.expasy.org/556



17-7D. Protein expression in the paramyxoviruses demonstrating leaky scanning and RNA editing. Courtesy of Sandra Quackenbush, Colorado State University.

TRANSLATION OF VIRAL PROTEINS

We learned in Chapter 3 and 13 that translation initiation requires a whole host of initiation factors to bind the 5' cap. In particular, eIF4E binds to the cap and eIF4G binds to eIF4E and then other initiation factors. Poly A binding proteins (PABPs) bond to the poly A tail and interact with the initiation factors in order to begin translation. All the viruses of *Mononegavirales* create mRNAs with a poly A tail and 5' cap and as such can utilize these cellular translation factors.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes.

RNA viruses except for retroviruses cannot use the host cell's transcription machinery DNA-dependent RNA polymerase (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As such, except for retroviruses, they must all have genes to produce an RNA-dependent RNA polymerase (RdRp). Negative sense RNA viruses, whose genomes are not equivalent to mRNA, cannot be translated immediately upon entrance into the cell, and as such must carry RdRp into the host cell with the genome in order to begin replicating. Note: all RNA viruses of animals have linear

genomes and as such must have mechanisms to make sure the ends of their genomes are replicated. Most RNA viruses including those in *Mononegavirales* accomplish end replication easily as RdRp starts synthesizing at the end of the genome without a primer. Starting without using a primer is referred to as **de novo synthesis**. De novo initiation by RdRP occurs at the 1st nucleotide. For most RNA viruses this method of replication ensures that the ends are replicated.

VIRION FORMATION

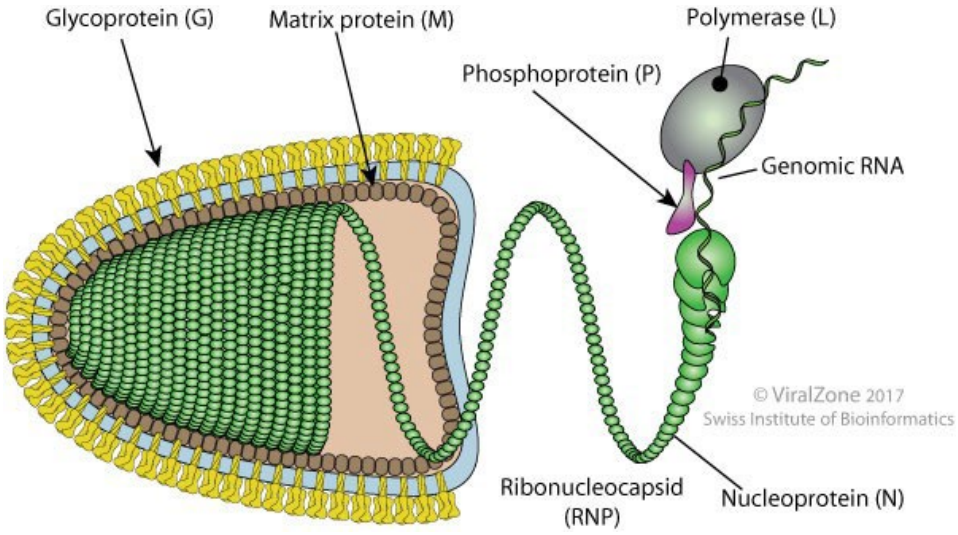
Viral capsids form around newly created full-length positive sense RNAs (**antigenomes**) and production of the negative sense genome occurs within the nucleocapsid. All leave the cell by budding from the plasma membrane.

AVOIDING THE HOST IMMUNE RESPONSE

Mononegavirales viruses avoid detection of the pathogen recognition receptor (PRR) TLR 7 which recognizes the pathogen associated molecular pattern (PAMP) of single stranded endosomal RNA by remaining in their capsids after they leave the endosome. *Mononegavirales* viruses avoid the PRRs RIG-I (cytoplasmic RNA lacking a 5' cap), MDA-5 (cytoplasmic dsRNA and an improper cap), that will initiate the interferon response and possibly RNAi (ds RNA) by replicating within their nucleocapsids and in viral factories. Furthermore, recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication to avoid activating the interferon response and possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi), as well as the interferon stimulated genes (ISG) dsRNA-dependent PKR, dsRNA-dependent OAS and the PRR MDA-5. As such, RdRp is unable to proofread as it replicates the genome which leads to a high mutation rate allowing antigens to change rapidly. Some *Mononegavirales* viruses also produce accessory proteins that help inactivate the type I interferon response by inhibiting the JAK/STAT pathway, decreasing NF-κB transcription activation, and inhibiting dsRNA-dependent Protein Kinase R (PKR). Remember that NF-κB is activated during the cascade pathways activated by multiple pattern recognition receptors and in turn activates transcription of type 1 interferons, β interferons and chemokines in infected cells (see Chapter 4). JAK phosphorylation of STAT1 leads to STAT1/STAT2 dimerization which binds to IFN-stimulated response sites (ISRE) to activate transcription of over 200 IFN stimulated genes (ISG). Recall that PKR binds viral ds RNA and then phosphorylates eIF2α inactivating its ability to initiate translation which inhibits translation (Chapter 4).

RHABDOVIRIDAE

Rhabdoviruses have bullet-shaped enveloped virions of 70-85 x 130-380 nm containing a single stranded RNA genome in a helical nucleocapsid of 13-16 kb, as shown in Figure 17-8A & B. There are many viruses with broad host ranges. Classification of the Family *Rhabdoviridae* includes two genera, the genus *Lyssavirus* (which includes rabies virus) found in vertebrates, invertebrates and plants, and the genus *Vesiculovirus* (vesicular stomatitis virus).



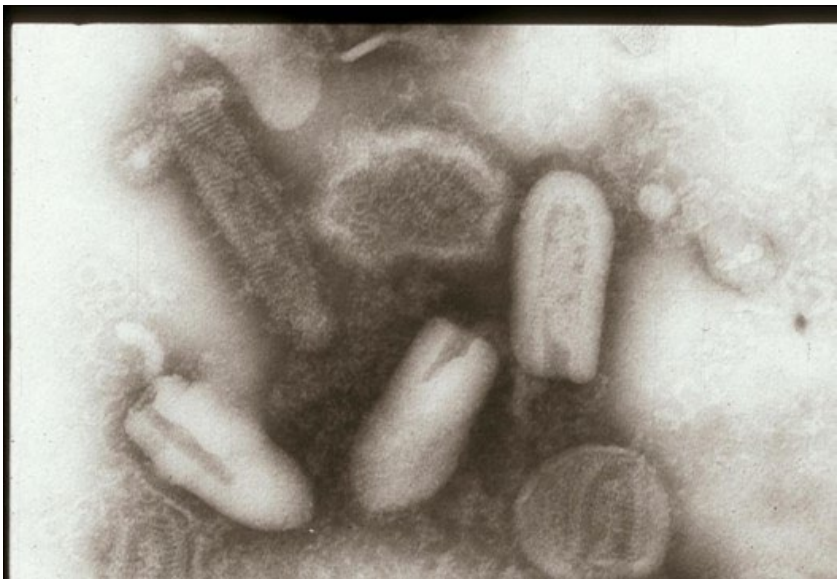


Figure 17-8A & B. A Typical rhabdovirus virion with a bullet shape. Courtesy of ViralZone https://viralzone.expasy.org/2 B. Electron micrograph of Rabies virus. Courtesy of Tom Walton APHIS (retired).

RHABDOVIRAL DISEASES: RABIES

All warm-blooded animals can be infected with rabies with varying susceptibility. Those that are considered highly susceptible are wolves, coyotes, foxes, and dogs. Those with intermediate susceptibility include skunks, raccoons, and bats, and low susceptibility includes opossums. Virus occurs in saliva, nervous system, urine, lymph, and milk. Recovery only occurs in bats and is fatal in nearly all other mammals (99.99% fatality rate). Vampire bats can transmit rabies virus for months. Pathogenesis of rabies infections requires several weeks for infection to become apparent. Transmission usually occurs through the bite or scratch of an infected animal.

Replication occurs in muscle and connective tissues at site of inoculation and then enters the peripheral nervous system at neuromuscular junctions. It then spreads up the peripheral nerves to the central nervous system causing **encephalitis** (swelling of the brain). The virus then replicates to high titers in the salivary glands. Rabies patients must be restrained due to becoming aggressive as the disease progresses and can transmit the disease in their saliva. **Negri bodies** appear in neuron cell bodies as shown in Figure 17-9. Negri bodies are **eosinophilic** (pink), sharply outlined **inclusion bodies** (2–10 µm in diameter) found in the cytoplasm of rabies virus infected nerve cells, which can be used to help diagnose infections **postmortem** (after death). The clinical spectrum begins with a **prodrome** before the onset of classical disease which includes nausea, headaches, fever, sore throat, and **photophobia** (aversion to light) and progresses to an acute neurologic phase that includes apprehension, nervousness, hallucinations, behavioral anomalies, salivation, perspiration, **hydrophobia** (inability to drink), photophobia, and, finally followed by coma, seizures, and death.

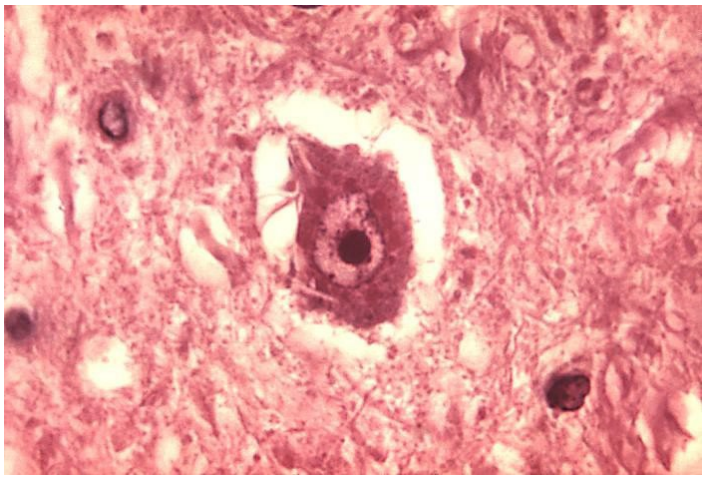


Figure 17-9. Negri bodies in the brain of an infected animal. Courtesy of https://phil.cdc.gov/Details.aspx?pid=3408 Dr. D. P. Perl.

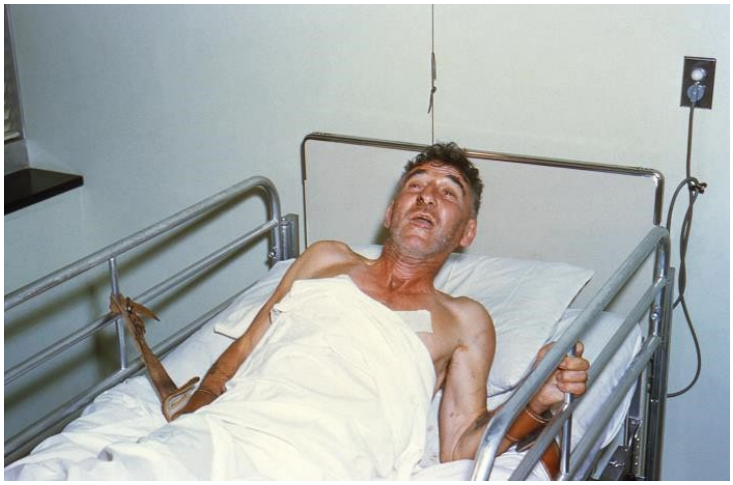


Figure 17-10. Patient from 1958 confirmed as having Rabies. Note that the patient had to be restrained to the bed for the safety of the medical staff. Courtesy of https://phil.cdc.gov/Details.aspx?pid=17348.

DIAGNOSIS OF RHABDOVIRUS INFECTIONS

Rabies infections are most often diagnosed using reverse transcriptase PCR, serology using IFAs looking for Rabies antigens, and histological analysis of brain tissue for the presence of Negri bodies. Note IFAs and histological analysis are most often done postmortem, but RT PCR can be done on live animals.

PREVENTION OF RHABDOVIRUS RABIES INFECTIONS

Animal control is the most important way to control rabies infections and has been used very successfully in the United States which has fairly low rabies fatalities in pets and humans. Rabid or suspected rabid animals are killed and examined by histopathology for Negri bodies and viral antigen by IFA. If positive, all known contacts will be contacted and encouraged to receive vaccination.

Vaccination is also very important to help prevent Rabies infections. Vaccination of pets is required by law in most states with boosters being administered every 3 years. The first rabies vaccine was developed by Pasteur using spinal cords from infected dogs. Today there are 2 principal vaccines 1. is the human diploid cell vaccine (HDCV), which is produced in cell culture, and 2. the Purified Chick Embryo Cell Vaccine (PCECV) both of which the virus is inactivated chemically. It is recommended that high risk individuals including veterinarians, wildlife animal workers, and virologists studying rabies should be vaccinated. The American Veterinary Medical Association (AVMA) recommends viral titers be analyzed every 2 years and boosters administered when antibody titers fall below the recommended threshold. In non-vaccinated individuals who are thought to be exposed to rabies virus, **post-exposure prophylaxis** should be performed as soon as possible after contact with a potentially infected animal or sample. This includes one dose of **hyperimmune antiserum** in the wound (containing anti-rabies virus antibodies) followed by four immunizations over 14 days. The antibody in the wound neutralizes the virus before it can establish infection in the neurons. Note: there have been documented cases of patients who were

vaccinated but did not have their wound treated with the antiserum that still contracted the disease and died. So, the two, vaccination and hyperimmune antiserum, really must be done together.

TREATMENT OF RHABDOVIRUS RABIES INFECTIONS

Rare survival has been observed using a novel medical treatment developed in 2005 when 15-year-old Jeanna Giese was bitten by a bat. She was not taken to the hospital for rabies vaccination and presented with clinical rabies after one month. The treatment included induced coma. She was administered high doses of ketamine to suppress brain activity (this required mechanical ventilation to allow her to breath), administered heparin (an anticoagulant), and ribavirin (an antiviral to protect the heart from rabies-induced cardiomyopathy). At days 8-10 she showed

improvement in cardiovascular and neurological functions. By day 23 she could sit up in bed, but neurological manifestations persisted. She required prolonged physical therapy but continued to recover although with permanent **neurological sequelae** (cognitive, sensory, and motor deficits). It is important to note that this treatment failed for most others it has been attempted on. It is called the **Milwaukee Protocol**. Therefore, prevention and proper administration of vaccines and gamma globulin antiserum in the wounds of people exposed to rabies virus is the best course of treatment to prevent rabies disease. It should also be noted that there are documented cases of multiple organ recipients of a patient who had an inapparent rabies infection (died in an accident) developing rabies up to a year after receiving the organs.

PARAMYXOVIRIDAE

Paramyoviruses have genomes that range from 16-20 kb that contain 6-9 genes, and have a helical ribonucleocapsid within an envelope that contains a hemagglutinin glycoprotein as well as a fusion glycoprotein on a round virion that is 150-300 nm in diameter, as shown in Figures 17-11 A & B. It is **labile** (easily destroyed in the environment) but highly infectious, and the family contains many medically important viruses found in 4 genera. These include *Respirovirus (*parainfluenza viruses), *Rubulavirus* (mumps, parainfluenza viruses), *Morbillivirus* (measles), *Henipavirus* (Hendra and Nipah viruses).

The genome is slightly more complicated than rhabdoviruses with a few more accessory genes, however the gene order remains consistent with all other negative sense RNA viruses.

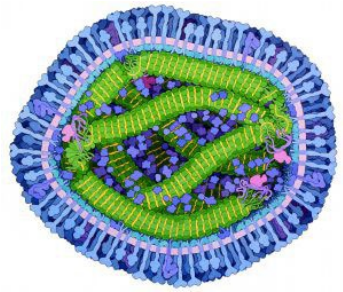


Figure 17-11 A. Typical paramyxovirus virion. Courtesy of the Protein Data Base: https://pdb101.rcsb.org/sci-art/goodsell-gallery/measles-virus-proteins. (doi: 10.2210/rcsb_pdb/goodsell-gallery-018).

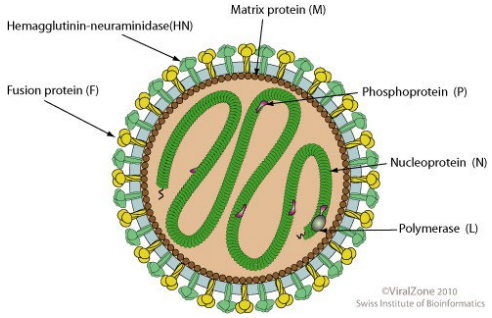


Figure 17-11 B. Typical paramyxovirus genome Courtesy of ViralZone https://viralzone.expasy.org/556.

PARAMYXOVIRAL DISEASES

MEASLES

Measles is derived from the Latin word "misery". Symptoms begin with fever, runny nose, cough, red weepy eyes, and a fine rash appears within a few days, first on the forehead then spreads to rest of body as shown in Figures 17-12 A & B. Symptoms generally disappear within 1 week. Many cases, however, are complicated by secondary bacterial infections. In particular, pneumonia and earaches are most common secondary conditions. Less common complications include encephalitis and **subacute sclerosing panencephalitis** (SSPE) which can occur decades after the initial infection and are often fatal. Measles usually does not kill but instead leads to secondary infections that do kill due to virally induced **immunosuppression**.

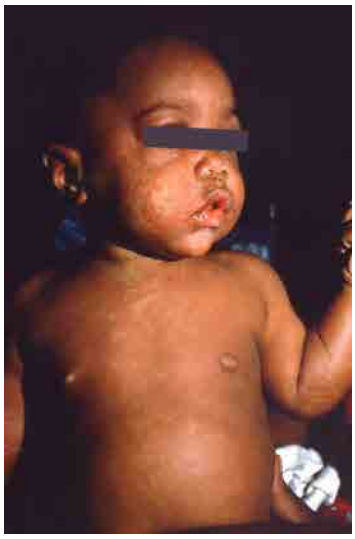


Figure 17-12 A. A child demonstrating typical rash of measles. Courtesy of https://phil.cdc.gov/Details.aspx?pid=17639 Betty Partin CDC.

Figure 17-12 B. A child demonstrating typical rash of measles. Courtesy of https://phil.cdc.gov/Details.aspx?pid=19434 Jim Goodson M.P.H CDC.

Measles virus is spread via the respiratory route when the virus becomes airborne in respiratory droplets from coughs, sneezing, talking, or blowing the nose. Measles is one of the most contagious viral diseases with an **R0** (number of people an infected person will infect) of 12-18

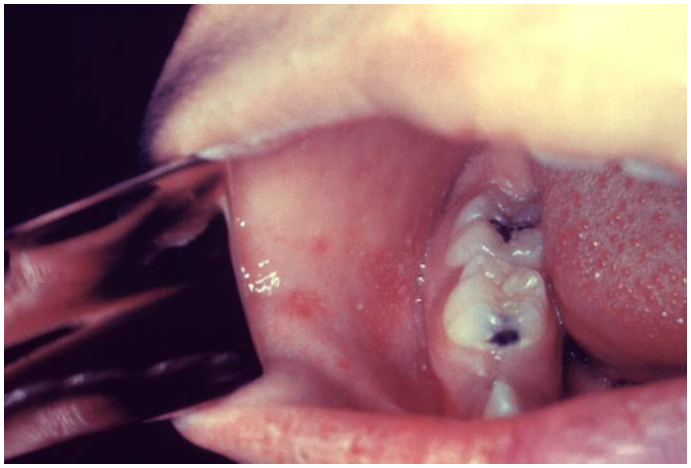


Figure 17-13. A patient demonstrating typical Koplik spot oral rash of measles as demonstrated by the white spots surrounded by red in the side of the cheek. Courtesy of https://phil.cdc.gov/Details.aspx?pid=6111 Dr. H. F. Eichenwald

which is incredibly high. For comparison, the R0 of influenza, which infects between 9-45 million people a year in the US, is 1.4-2. R0 is pronounced R-naught. This is also often called the **reproductive rate** of the disease. The Measles virus replicates in epithelium of upper respiratory tract and then spreads to lymph nodes where further replication occurs. It then spreads to all parts of the body. Infected mucous membranes are an important diagnostic sign as well as oral membranes covered with **Koplik spots** (Figure 17-13) which appear as white spots seen in back of throat or opposite the molars. The infected membranes may explain increased susceptibility to secondary infection particularly to middle ear and lung infections. The skin rash is due to effects of virus replication within skin cells as well as the cellular immune response to viral antigens in the skin.
Humans are the only (known) natural host. Before routine immunization, over 99% of the population would become infected at some point in their lives. The vaccine resulted in decline of annual cases. As such, measles is no longer endemic in the United States. Outbreaks still occur and are due to infected individuals having contact with susceptible populations including children too young to be vaccinated, preschool children never vaccinated, children and adults inadequately vaccinated, and persons not vaccinated for religious or medical reasons.

Measles has multiple virulence factors that increase its pathogenicity. It expresses 3 accessory proteins that regulate the immune response. P protein is a transcription factor that increases the production of the cellular enzyme A20. A20 negatively regulates NF-κB transcription. Remember that NF-κB is activated during the cascade pathways activated by multiple pattern recognition receptors and in turn activates transcription of type 1 interferons, β interferons, and chemokines in infected cells (see Chapter 4). Also, V protein blocks JAK phosphorylation of STAT1 and blocks STAT1/STAT2 dimerization, therefore, type I interferons cannot activate the interferon stimulated genes that express the more than 200 antiviral proteins. Lastly, C protein interferes with double stranded RNA-dependent PKR thus inhibiting PKR's ability to stop translation.

Measles is a global health issue. Measles occurs predominantly in Africa and Asia, although North America has an outbreak every few years including a significant one in 2019 in unvaccinated populations. In 2000 there were more than 700,000 deaths per year from measles, and the great majority of these deaths were in children. In 2001 the Measles Initiative was started by the **World Health Organization** (WHO), and this initiative seems to have been successful as in 2005 there were 454,000 deaths from measles worldwide and that number dropped to less than 100,000 in 2016. It is estimated that from 2001 to 2016 more than 20,000,000 measles deaths were prevented by the vaccination program. This program has been vigorously supported by Rotary International with funding and volunteers.

MUMPS

The symptoms of mumps begin with fever, loss of appetite, headache, and then progresses to painful swelling of one or both **parotid** (salivary) glands and spasms. This usually makes it difficult to chew and swallow. The symptoms disappear in about a week, however symptoms are much more severe in individuals past puberty.

Post-pubertal people with testicles can suffer painful swelling of testicles. Ovarian involvement occurs in about 20% of cases causing pregnant people to often miscarry.

Humans were thought to be the only natural host of mumps virus until the recent discovery of a very similar bat virus. It is very likely the mumps virus **spilled over** into human populations from bats. Mumps virus is so similar to a newly discovered bat virus that they are considered the same species (**conspecific**).

Mumps is transmitted by inhalation of infected droplets and has a long intrinsic **incubation period** of 15 to 20 days. Recall that the intrinsic incubation period is the time between infection and onset of disease in a host. During this time the virus replicates in the upper respiratory tract and then spreads throughout the body via the bloodstream, at which point Mumps virus produces symptoms after infecting salivary gland tissues. The virus multiplies in the epithelium of salivary ducts destroying the epithelium which leads to inflammation and release of the virus into the saliva. The inflammation is responsible for the symptoms and pain. Natural infection confers a lifelong immunity. The virus is spread by asymptomatic individuals in high numbers as the virus can be present in saliva of asymptomatic persons. The R0 of mumps, like measles, is very high with each infected person estimated to infect 4-7 other people.

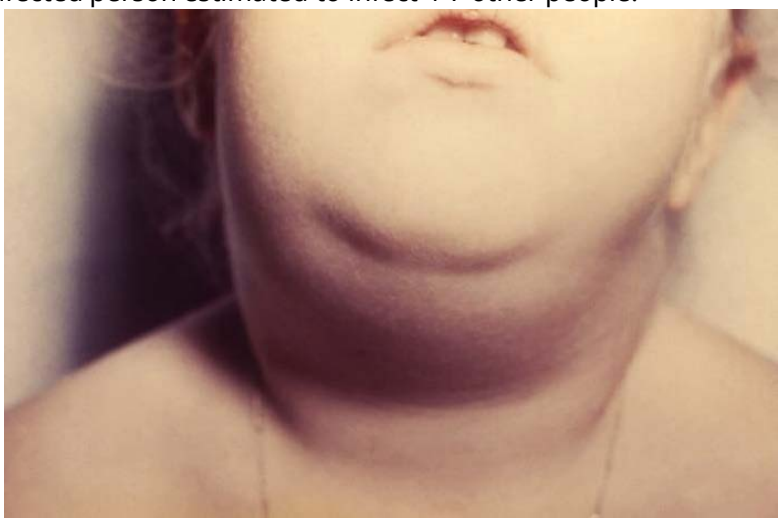


Figure 17-14. Patient demonstrating swollen parotid (salivary) glands of mumps. Courtesy of https://phil.cdc.gov/Details.aspx?pid=14488.

NIPAH AND HENDRA VIRUSES

A mysterious new disease began in 1998 with an outbreak of human encephalitis near Ipoh, Malaysia. Deaths in local swine populations were also observed. All totaled, 265 people become ill, and 105 of these died. The Malaysian government declared it a Japanese encephalitis (JE) virus outbreak and started a vaccination campaign. Remember that JE is a mosquito-borne flavivirus, so mosquito control programs were also instituted. However, there were clear problems with this theory. First, the patients were almost all males which would be unusual for a mosquito-

borne disease. Second, the patients all had ethnic Chinese names. Malaysia is 60% Malay, 25% Chinese, and 15% Indian, which indicated the disease was occurring in family clusters which would also be odd for a mosquito-borne disease. Lastly, the control programs did not seem to curb the outbreak. It turned out the virus responsible for this outbreak was a paramyxovirus that was named the Nipah virus. The outbreak in Malaysia and a similar outbreak in Australia where humans and horses were getting ill (Hendra virus) led to the discovery of a new genus of paramyxoviruses: the *Henipaviruses* (**He** for Hendra, **nipa** for Nipah viruses). Henipaviruses currently include 3 major viruses: the Nipah virus (NiV) of Asia, the Hendra virus (HeV), and the Cedar virus (CedPV), which are found in Australia are transmitted through a reservoir of bats—in particular in the genus Pteropus (flying foxes). Natural infections are found to occur in bats; however, they show no symptoms of disease. Humans and horses, however, develop encephalitis with high case-fatality rates, while pigs develop respiratory disease with low case-fatality rates. Scientists have also demonstrated experimental infections in dogs, ferrets, and hamsters. In 2004 Bangladesh had another outbreak of Nipah virus with a 75% case-fatality rate. As such, the *Henipaviruses* are now classified as biosafety level-4 (BSL-4) pathogens and select agents that must be monitored by the government.

It turned out that another *Henipavirus* (Hendra virus) was responsible for horse and human deaths in Queensland, Australia in 1994. In this case it was found that bats infected the horses when they roosted above the horses' living spaces, and the horses infected the humans who worked with them in the end phases of their disease when a frothy, bloody discharge was coming out of their nostrils and mouths. However, not everyone who worked with the animals become sick, and not everyone who contracted it died. Sadly, some people who contracted Hendra and then recovered, mysteriously relapsed with encephalitis a year later and died. Australia has had many outbreaks of Hendra virus in the years since. Interestingly, people who serve as bat rehabilitators (commonly known as bat carers) were not found to be infected with the virus despite handling infected bats. It is hypothesized that humans require the horse to serve as an **amplification host** in order to become infected because bats do not achieve a high enough viremia to cause disease in humans. Horses must require a smaller dose to contract the disease and, as such, can contract Hendra from infected bats.

What we currently know is that *Henipavirus* infections occur asymptomatically in the bat *Pteropus alecto* with no apparent pathology for all 3 viruses. In horses (HeV) and pigs (NiV), however, infections do show symptoms. Respiratory transmission occurs between these animals. Both cause neurological manifestations, facial swelling, and nasal discharge. Respiratory transmission also occurs in humans, and the primary symptom is severe acute encephalitis. It is important to note that the NiV manifestations can occur up to 4 years post infection and, for both NiV and HeV, relapses in encephalitis are known to occur.

The molecular biology of *Henipavirus* infection is interesting. In Nipah and Hendra viruses the envelope protein
(G) provides **broad species tropism**, meaning it can infect many species of animals. Similarly, the fusion protein
(F) can induce fusion of the envelope with cells from different species. The cellular receptors used for viral adsorption are ephrin B2 and B3 protein. These are found on neurons, smooth muscle, and capillary endothelial cells. The P gene, which expresses accessory proteins to control

the immune response, undergoes RNA editing. Hendra and Nipah viruses edit the P gene by inserting up to 11 G residues. This results in 4 proteins (P, V, W, C) each of which attenuates the innate response. P and V interact with and disable STAT1 transcription factor (JAK/STAT pathway of interferon) while W interacts with and disables IRF-3 transcription factor involved in the cascade pathway induced by multiple pattern recognition receptors. These events disable the type I IFN pathway of infected cells.

You are probably wondering now, what about the Cedar *Henipavirus?* Doesn't it cause disease? Although this virus was isolated from bats in Australia, it has not been associated with disease outbreaks and did not cause disease in any of the experimental models tested. Interestingly, Cedar virus does not have the RNA editing site in the P gene and cannot produce V or W proteins and, as such, does not produce some of the accessory proteins to inhibit the immune response. Thus, animals appear to mount a successful immune response that clears the viral infection before symptoms occur. Furthermore, Cedar virus only binds to ephrin B2 as a host cell receptor. It does not bind to ephrin B3 which prevents brain stem infection and probably explains the lack of encephalitis.

DIAGNOSIS OF PARAMYXOVIRUS INFECTIONS

The predominant testing is reverse transcriptase real time or quantitative PCR called **RT-qPCR**. Measles and Henipaviruses are also diagnosed using serum neutralization assays called plaque reduction neutralization (PNR) assays. This requires 2 samples for Henipaviruses. IgM ELISAs can also be used for each. Recall that with an IgM assay a single sample can be used as IgM is made predominantly in the primary immune response. The mumps IgM test is a capture ELISA.

PREVENTION OF PARAMYXOVIRUS INFECTIONS

Prevention is best achieved by vaccination, if available. The measles vaccine is usually given in conjunction with mumps and rubella and may also include varicella (chicken pox) and MMR(V) vaccine. Children should get two doses of MMR vaccine, with the first dose at 12 to 15 months of age and the second dose at 4 through 6 years of age. If not vaccinated as a child, adolescents and adults can still achieve immunity by receiving two doses of the vaccine. Immunization prevents latent recurrent measles infections that can cause SSPE and is effective because there is only one viral serotype.

The best way to prevent mumps is immunization along with the measles, rubella, and varicella zoster (see above). Immunity wanes, however, in about 10 years, thus boosters may become routine.

In order for a vaccine to induce herd immunity (enough immunity that non-vaccinated people are unlikely to encounter infectious people), a threshold vaccination level must be achieved. For mumps this is estimated to be 75-86 percent of the population, and for measles with the higher R0 it is estimated that 83-94 percent of the population needs to be vaccinated to achieve herd immunity. As these two viruses are vaccinated in the same vaccine, it is public health policy to

try to maintain vaccination rates sufficient to control Measles outbreaks. In 2019 low vaccination rates in residents of New York city who did not believe in vaccinations led to a major outbreak of measles.

There are no vaccines yet for any of the *Henipaviruses*. As such, the best protection is avoiding contact with bat droppings and infected individuals and animals. For veterinarians, this means the use of proper barrier nursing techniques and personal protective equipment (PPE) to protect oneself.

TREATMENT OF PARAMYXOVIRUS INFECTIONS

There are no effective treatments for any of the paramyxovirus infections. Therefore, prevention with vaccines if available is advised, as is hand washing, avoiding touching one's mouth nose and eyes with unclean hands, shaking hands, and coughing and sneezing into one's elbow to avoid transmitting virus to other people if infected.

PNEUMOVIRIDAE

Pneumoviruses were in the family Paramyxoviridae until 2016 when they were move into their own family. Thus, they are very similar to paramyxoviruses. They have a helical ribonucleocapsid with an envelop and a diameter of 150-200 nm and a genome of 13.2-15.3 kb. Pneumoviridae contains two genera *Pneumovirus* (respiratory syncytial virus), and *Metapneumovirus* (metapneumovirus). As one might guess these are pneumonia causing viruses. Their virions are identical to paramyxoviruses as shown in Figures 17-11 A & B.

PNEUMOVIRAL DISEASES

RESPIRATORY SYNCYTIAL VIRUS (RSV)

In most children RSV causes cold like symptoms or bronchitis and are often not diagnosed. However, RSV can cause serve pneumonia infections and is a significant cause of hospitalizations and death in children and older adults and the immunosuppressed. The CDC estimates there are over 235,000 hospitalizations a year in the USA, with approximately 100-500 deaths among children under 5, and approximately 14,000 deaths among adults over 65 years of age. These infections are diagnosed primarily in the fall and winter concurrent with the cold and flu season.

DIAGNOSIS OF PNEUMOVIRUS INFECTIONS

In most cases diagnosis is not necessary as the virus is an acute infection that resembles a cold and goes away on its own. If severe infections occur diagnosis is done by a rapid antigen detection test (RADT) however, false positives are common and should be double checked with

PCR. Direct fluorescent antibody testing is reliable in children but not older adults.

PREVENTION OF PNEUMOVIRUS INFECTIONS

There are no vaccines for pnuemoviruses so the best protection is proper hygiene and keeping newborns away from people with respiratory illness symptoms. Like the flu RSV levels dropped dramatically in 2020 when public health measures were instituted to control SARS-Cov-2 (COVID 19) infections demonstrating that hygiene, social distancing and masks are effective methods for avoiding RSV infections. In 2021 when these public health measures were relaxed, an increase in RSV hospitalizations was observed.

FILOVIRIDAE

Filoviruses have genomes of approximately 12.7 kb that contain 7-9 genes and have a helical ribonucleocapsid within an envelope that contains a hemagglutinin glycoprotein as well as a fusion glycoprotein on a filamentous virion that is 80 x 790-14,000 nm in diameter that often takes on a **shepherd's crook structure** where the helical virion winds over on itself and creates loops, as shown in Figures 17-15 A, B, and C. The NP gene encodes the nucleoprotein that wraps around the viral RNA forming the helical nucleocapsid. The P gene creates a nonstructural accessory protein that acts as a type 1 interferon **antagonist** (inhibitor) by inhibiting **interferon response elements** found in the promoters of many antiviral genes. It also suppresses translation inhibition by the double stranded RNA-dependent PKR. The M1 gene encodes the matrix protein. The GP gene encodes the envelope protein glycoprotein spike which binds to lectin-containing receptors and serves as a fusion protein that causes the viral envelope to fuse with the endosome to release the viral nucleocapsid into the cytoplasm. The N gene encodes a transcription factor that increases transcription of viral genes. The M2 gene encodes a protein involved in viral assembly and serves as a STAT1 inhibitor. The L gene encodes the RdRp. The filamentous RNA viruses in the family *Filoviridae* are predominantly found in Africa, the Philippines, and Europe. They are found in 3 genera: *Ebolavirus* (Africa, Philippines), *Marburgvirus* (Africa only) and *Cuevavirus* (Europe only).

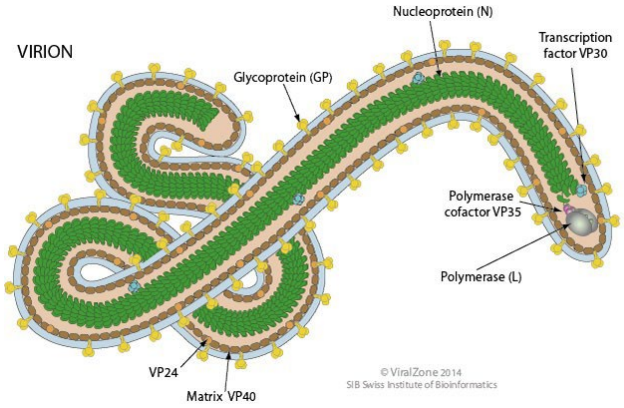


Figure 17-15A. Typical filovirus virion. Courtesy of ViralZone https://viralzone.expasy.org/23.

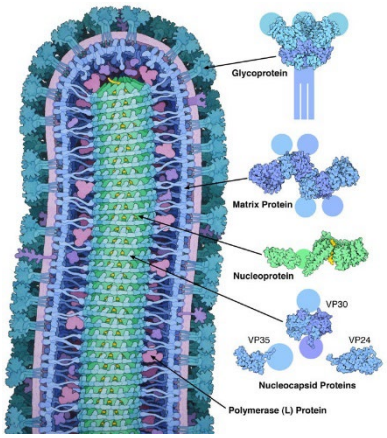


Figure 17-5B. Typical filovirus virion. Courtesy of the Protein Data Base: https://pdb101.rcsb.org/motm/178 D. Goodsell.

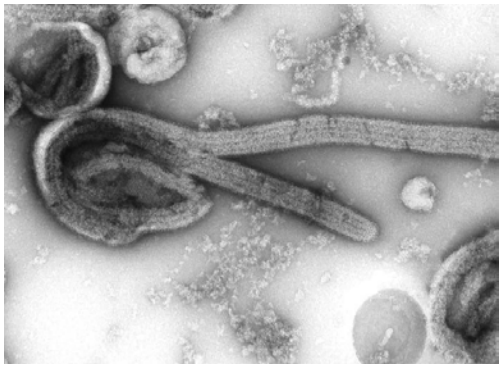


Figure 17-5C. Electron microscope image of Ebola virus from 1976. Courtesy of https://phil.cdc.gov/Details.aspx?pid=23185 Dr Frederick Murphy CDC.

FILOVIRAL DISEASES: MARBURG AND EBOLA

Filoviruses can cause hemorrhagic fevers with high fatality rates (up to 90%). Infection appears to be transmitted by close contact with infected persons, and these viruses are highly contagious. The first ever recorded filovirus outbreak was a Marburg outbreak in 1967 in Germany and Yugoslavia. A vaccine company was processing primary kidney cells from African green monkeys when several workers developed a hemorrhagic fever, and several dozen others were infected by person-to-person transmission. Fewer than half of those infected died.

The first ebola outbreak occurred in 1976 in Zaire and the Sudan. Hundreds were infected with a 70%-90% fatality rate. Sporadic outbreaks still occur in Africa. There are currently 6 ebola virus species all of which require biosafety level 4 (BSL-4), the highest level of biosafety. These are: Zaire ebola virus, Sudan ebola virus, Tai Forest ebola virus (formerly Ivory Coast ebola virus), Bombali virus (not known to cause disease in humans), Bundibugyo ebola virus, and Reston ebola virus (Virginia, USA) (not known to cause disease in humans). The Reston ebola virus occurred in a monkey quarantine facility at the company JRH Biosciences in Reston, Virginia. Monkeys imported from the Philippines began dying from a hemorrhagic fever. Samples were sent to the nearby military lab, the **United States Army Medical Research Institute for Infectious Diseases** (USAMRID), for identification. Electron micrographs showed shepherd's crook particles. The JRH Biosciences facility was secured by the Army. Although some of the workers were shown to be infected with the virus, fortunately none ended up becoming symptomatic. It has been shown that the Reston ebola virus is nonpathogenic in humans, however, due to the high mutation rate of RNA viruses, the Reston ebola virus is still only studied under BSL-4 conditions.

In the 5 ebola virus species that cause disease in humans, 80% to 90% of patients that die do so because of dehydration caused by vomiting and diarrhea. Only 10% to 20% develop hemorrhagic

disease because the hemorrhaging is usually late onset and patients usually die before hemorrhaging begins. When hemorrhaging is noted, it is caused by vascular leakage and is associated with a high titer viremia while bleeding from orifices (eyes, ears, nose etc.) is common as is **disseminated intravascular coagulation** (DIC). A major clinical feature is an inflammatory response resembling septic shock. In nonhuman primate models, initial replication is found in monocytes, macrophages, and dendritic cells thus blocking maturation to antigen presenting cells (APC). Some of these cells disseminate virus throughout the body and a systemic cytokine and chemokine inflammatory response occurs. This leads to multi system organ failure when **cell surface tissue factors** trigger **extrinsic coagulation pathways** and disseminated intravascular coagulation occurs. Note: endothelial cell infection that leads to vascular leakage appears late in disease, and, as such, hemorrhaging occurs late in the course of disease if the patient survives the initial phase. Lymphocytic death occurs by apoptosis and not viral infection and can lead to **lymphopenia** (low lymphocyte counts) and subsequent immunosuppression. Two viral accessory proteins suppress the type I interferon response: the P gene VP35 protein inhibits activation of **interferon regulatory factor 3** (IRF3) required for transcription activation by PRR induced cascades, and the M2 gene VP24 protein blocks STAT1 localization to the nucleus inhibiting interferon activation of interferon stimulated genes.

In 2014 West Africa experienced a significant outbreak with an unprecedented number of infections. The disease had not been previously reported in West Africa. The cause was Zaire ebola virus. The suspected index case was a 1-year old girl who died in December 2013 in the village of Meliandou, Guinea. The World Health Organization (WHO) was criticized for its slow response to the outbreak. In total the disease spread to three other countries (Liberia, Sierra Leone, and Nigeria) with 28,652 suspected cases, 15,261 laboratory-confirmed cases, and 11,325 deaths. The disease did not only impact Africa. Four medical facilities in USA, Atlanta, GA, Bethesda, MD, Omaha, NE, and Missoula, MT, treated 11 Patients. Seven of these were US health care workers evacuated from West Africa, and all survived. Two were US health care workers treating an ebola patient from Liberia; both survived. One was a Sierra Leone physician who died, and one was a Liberian residing in USA who died. Interestingly it has been anecdotally noted that hiccups are a prognostic indicator of a poor outcome, though it is not entirely clear why.

There has been a long search for the reservoir of ebola virus infections in humans. Viral and epidemiologic data suggest that ebola virus existed long before these recorded outbreaks occurred. Factors like population growth, encroachment into forested areas, and direct interaction with wildlife (such as bushmeat consumption) may have contributed to the spread of the ebola virus. Although it is known that gorilla and chimpanzee die-off events often coincide with human outbreaks, they are dead-end hosts, meaning these animals die quickly when infected and are therefore unlikely to serve as the reservoir (much like humans). So, where does the virus reside between the sporadic outbreaks? The fact that the reservoir host did not appear to develop symptoms of infection made it very difficult to determine what the reservoir host was. Large scale trapping and testing of local bats near outbreaks indicate that bats may play a role in outbreaks and may serve as the reservoir, although this has not been conclusively proven yet. The 1980 and 1987 Marburg outbreaks were associated with visits to the Kitum Cave at Mount Elgon in Kenya. Marburg virus was isolated from fruit bats in this cave demonstrating that bats are the reservoir for these infections as well. Furthermore, with all the diseases discussed in this chapter, the bats that are thought to transmit the viruses do not themselves become ill from the viral infections they harbor.

DIAGNOSIS OF FILOVIRUS INFECTIONS

The most common test used is reverse transcriptase PCR because it can detect low levels of the ebola virus, however, when viremia is low, PCR becomes less effective at which point ELISAs are used.

PREVENTION OF FILOVIRUS EBOLA VIRUS INFECTIONS

A vaccine using the Zaire ebola virus envelope glycoprotein (Ervebo) was developed in 2005 using an experimental vesicular stomatitis virus to produce the glycoprotein. In December of 2019 the vaccine was approved by the US Food and Drug Administration (FDA) for use in humans in the US over the age of 18.

Outside of vaccination, proper use of personal protective equipment (PPE) is essential. During outbreaks, health care workers who are wearing PPE have been known to get infected which is thought to occur when the equipment is not properly **donned** (put on) or **doffed** (removed); improper glove removal is particularly prone to causing infections. Family members touching infected individuals and contact with infected bodily fluids is likely to transmit the disease which fortunately is not known to be transmitted by respiratory droplets. However, decreasing these practices requires educating the public about risks associated with long held burial practices and overcoming a distrust of health care workers who are often accused of bringing the disease they are there to help alleviate, thus leading to fear and mistrust. Direct contact with the bodies of those who died from ebola viruses proved to be one of the highest risk activities associated with transmission. Furthermore, a spike in cases after an ebola virus outbreak was thought to be over was linked to sexual activity. It was found that men secrete ebola virus in their semen for up to 8 weeks after recovering from Ebola. Changes in behaviors related to mourning and burial, along with the adoption of safe burial practices, is critical in controlling Ebola epidemics.

TREATMENT OF FILOVIRUS EBOLA VIRUS INFECTION

Recall that ebola virus disease is about fluid loss, and as such fluid and electrolyte balance management are the primary treatment and, if begun early in the course of the disease, can greatly improve the odds of survival. Hemorrhagic disease only occurs in about 15% of patients and, unfortunately, supportive care is all that can be offered to help patients survive.

WHY BATS?

You might well be thinking, “Why do so many viral diseases seem to be transmitted by bats? Why do the bats not get symptoms of these highly pathogenic viral infections?” One simple answer is they make up a major percentage (~20%) of mammals, so there are many bats capable of transmitting the virus. Furthermore, the practice of eating bats and having them available in live animal markets where bats are stored along with many other animals makes the transmission of

bat viruses to other animals and humans more likely. Work at Colorado State University on bats by Dr. Tony Schountz, as well as at other universities, hopes to answer the question, "Is there anything unique about bats that makes them more likely to serve as reservoirs, in particular their immune systems?" Further work is also required to determine if they are in fact the reservoir of some of these infections or if there is a different reservoir. Bats, with their ability to fly, simply move the viruses from the reservoir to new susceptible populations. There is evidence that aspects of bat immune systems may play a role. Research suggests that bat immune systems are down regulated to compensate for the metabolic stresses of being a mammal that flies. This down regulating of the immune system to avoid overreaction to those stresses may also create an environment in which viruses are more tolerated in bats than in other mammals. Furthermore, when bats fly their body temperatures become extremely elevated which may keep the viruses from developing viremia high enough to cause disease and death in the bats allowing them to live and transmit the virus. The following images, Figure 17-16 A & B, show a researcher in Uganda catching Egyptian fruit bats, the Marburg virus reservoir.

Figure 17-16 A & B. Bats are a major reservoir or presumptive reservoir of many zoonotic diseases including coronaviruses, paramyxoviruses, rhabdoviruses, and filoviruses. These images show field studies of bat populations for viruses. Images Courtesy of Tony Schountz PhD Colorado State University, Fort Collins, CO.

END OF CHAPTER QUESTIONS

1. Why do you think these 3 viruses are presented in the same chapter?
2. Compare and contrast *Mononegavirales* to orthomyxoviruses.
3. Compare the gene order of the viruses in *Mononegavirales*.
4. Compare and contrast *Mononegavirales* to flaviviruses.
5. Do *Mononegavirales* create subgenomic mRNAs? If they do, why? If they don't, why not? If they do create subgenomic mRNAs, do they use similar or different mechanisms to do so?
6. Do *Mononegavirales* viruses need to have mechanisms for overcoming RNA

interference? If so, why? If not, why not?

7. Do *Mononegavirales* viruses use viral proteases to cleave polyproteins?
8. Can *Mononegavirales* viruses create more than one ORF from an mRNA? If so, explain how. If not, explain why.
9. If you stained the Mononegavirales virus-infected cells to view their viral factories, where would you expect to observe staining?
10. How do *Mononegavirales* viruses avoid the host immune response?
11. Paramyxovirus infections are most often diagnosed using RT-qPCR. How does this differ from standard PCR?
12. Hendra virus infections can be diagnosed using IgM ELISAs. You take a sample from a horse and find it has a titer well above the threshold of 300 EU/0.1 ml. Is this sufficient information to determine that this horse has Hendra virus? If not, what other data would be needed?
13. Can *Mononegavirales* viruses undergo reassortment?
14. Your friend was bitten by a skunk. She wants to know if she should get a rabies vaccine now or wait to see if she develops symptoms and then get treatment. What advise will you give her?
15. If she waits, can she count on the Milwaukee protocol? Justify your answer.
16. What role does hyperimmune antiserum play in rabies treatment?
17. Why do measles virus infections often lead to secondary infections?
18. How does inhibiting NFkB help measles virus?
19. How does inhibiting PKR help measles virus?
20. How does inhibiting the JAK/STAT pathway help measles virus?
21. From what animal did mumps most likely spillover into humans?
22. From what animal did other paramyxoviruses spillover into humans? How do these spillover events differ from the mumps spillover event?
23. Some viruses can only be transmitted when a person is symptomatic. Is this true of Mumps?
24. Why does the Cedar henipavirus not cause disease when Nipah and Hendra do?
25. What is the treatment for paramyxovirus infections?
26. What do henipavirus and filovirus viruses have in common?
27. What role does DIC play in ebola disease?
28. Do most patients with ebola die as a result of hemorrhaging? If not, why?
29. Chimpanzees and gorillas can transmit ebola virus to people who hunt them and eat them. Are they reservoir hosts for ebola virus? Why, or why not? Justify your answer.
30. Is ebola virus likely transmitted by respiratory droplets?

31. What was an unexpected source of new ebola virus transmission during an ebola virus outbreak?
32. Many of the diseases in this chapter can be diagnosed with a RT-qPCR. What is this test? What would a positive result look like?
33. What is the significance of Negri bodies?

REFERENCES:

Center for Disease Control and Prevention. Ebola history. (2021). https://www.cdc.gov/vhf/ebola/history/summaries.html

Center for Disease Control. and Prevention (2020). Marburg. https://www.cdc.gov/media/releases/2020/s0124-marburg-virus.html/

Center for Disease Control and Prevention. (2021). RSV. https://emergency.cdc.gov/han/2021/han00443.asp

Center for Disease Control and Prevention. Public Health Image Library (PHIL). Dr. F. Murphy. (1977). Ebola virus. https://phil.cdc.gov/Details.aspx?pid=23185

Center for Disease Control and Prevention. Public Health Image Library (PHIL). (1958). Man with rabies. https://phil.cdc.gov/Details.aspx?pid=17348

Center for Disease Control. Public Health Image Library (PHIL). H.F. Eichenwald. (1958). Measles Koplik spots. https://phil.cdc.gov/Details.aspx?pid=6111

Center for Disease Control and Prevention. Public Health Image Library (PHIL). J. Goodson M.P.H. (2014). Measles rash. https://phil.cdc.gov/Details.aspx?pid=19434

Center for Disease Control and Prevention. Public Health Image Library (PHIL). B. G. Partin. Measles rash. (1976). https://phil.cdc.gov/Details.aspx?pid=17639

Center for Disease Control and Prevention. Public Health Image Library (PHIL). (1975). Mumps. https://phil.cdc.gov/Details.aspx?pid=14488

Center for Disease Control and Prevention. Public Health Image Library (PHIL). Dr. D. P. Perl (1971). Negri bodies. https://phil.cdc.gov/Details.aspx?pid=3408

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 30-38.Kuzmin, I.V. et.al, 2010. Marburg Virus in Fruit Bat, Kenya. Emerg Infect Dis. Feb; 16(2): 352–354. doi: 10.3201/ eid1602.091269

Food and Drug Administration. (2019). Ebola vaccine. https://www.fda.gov/news-events/press-announcements/first-fda-approved-vaccine-prevention-ebola-virus-disease-marking-critical-milestone-public-health

Henrickson KJ, Hall CB. (2017). Diagnostic assays for respiratory syncytial virus disease. Pediatr Infect Dis J. 26(11 Suppl):S36-40. https://pubmed.ncbi.nlm.nih.gov/15630022/

Protein Data Base: D. S. Goodsell. (2014). Ebola virus proteins. https://pdb101.rcsb.org/motm/178

Protein Data Base: D. S. Goodsell. (2019). Measles virus proteins. https://pdb101.rcsb.org/sci-art/goodsell-gallery/measles-virus-proteins & doi: 10.2210/rcsb_pdb/goodsell-gallery-018

Willoughby, R. et al. (2005). Survival after Treatment of Rabies with Induction of Coma. New England Journal of Medicine. 352:2508-2514. https://www.nejm.org/doi/full/10.1056/nejmoa050382

ViralZone. Ebolavirus. https://viralzone.expasy.org/5016

ViralZone. *Filoviridae.* https://viralzone.expasy.org/23

ViralZone. *Rhabdoviridae.* https://viralzone.expasy.org/

REVIEWED BY:

This chapter was edited and reviewed by Dr Tony Schountz, Arboviral Infectious Disease Center Colorado State University

CHAPTER 18

Chapter 18: Segmented negative sense stranded RNA viruses that replicate in the nucleus: Orthomyxoviridae

INTRODUCTION TO THE FAMILY *ORTHOMYXOVIRIDAE*

You will notice in figure 18-1 that orthomyxoviruses are segmented negative stranded enveloped RNA viruses with 7-8 segments each contained in a helical nucleocapsid (also called **ribonucleocapsid** or **ribonucleoprotein**) that replicate in the nucleus. 3 different types of influenza viruses infect humans: influenza A, B, and C, with A being the most pathogenic and medically important. Influenza C has only 7 segments while influenza A & B have 8. There are 18 different **hemagglutinin** (HA) genes and 11 different **neuraminidase** (NA) genes for influenza A both of which determine the serotype of the virus (i.e., H1N1, etc.). Viruses are named according to their genus, species from which the virus was isolated, location of the isolate, number of the isolate, and year of isolation. For example, the 220th isolate of an H5N1 subtype virus isolated from chickens in Hong Kong in 1997 is designated influenza A/chicken/Hong Kong/220/97 (H5N1). The capsid is surrounded by a layer of matrix proteins that line the envelope. The envelope contains an M2 ion channel that facilitates fusion of the endocytic vesicle and envelope membrane as well as a nuclear export protein that helps the nucleocapsid get in and out of the nuclear pores. The virion is 80-100 nm in diameter.

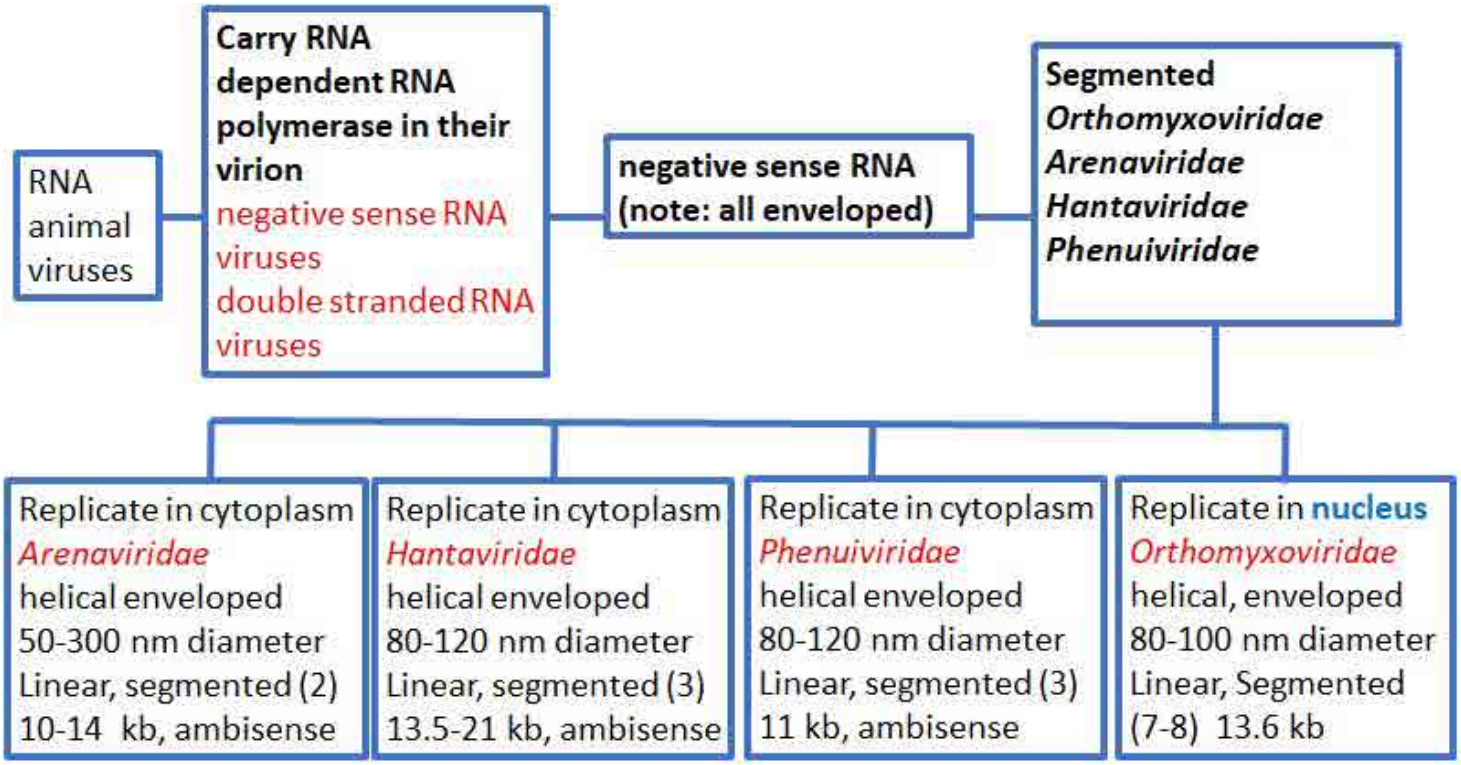
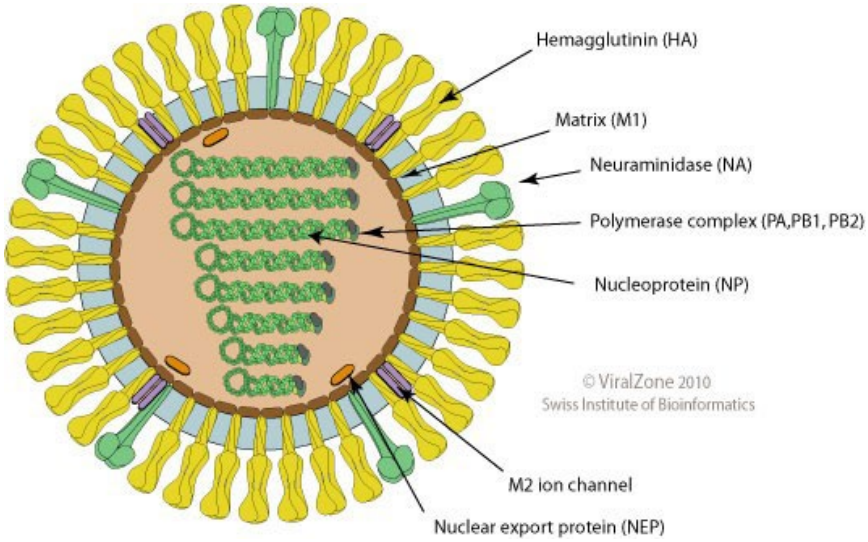


Figure 18-1. Taxonomy of negative sense RNA viruses focusing on segmented viruses. Courtesy of Erica Suchman, Colorado State University

GENOME STRUCTURE

Influenza viruses contain 7 (influenza C) or 8 (influenza A & B) segments of negative sense RNA with a total size of approximately 13.6 kb. Each segment is packaged into its own helical nucleocapsid (or ribonucleoprotein) with a molecule of RNA-dependent RNA polymerase (RdRp) as shown in Figures 18-2 A and B. Recall that the gene order is consistent within all the negative sense RNA viruses (Chapter 17).



18.2 A. Influenza A virion. Courtesy of ViralZone https://viralzone.expasy.org/223

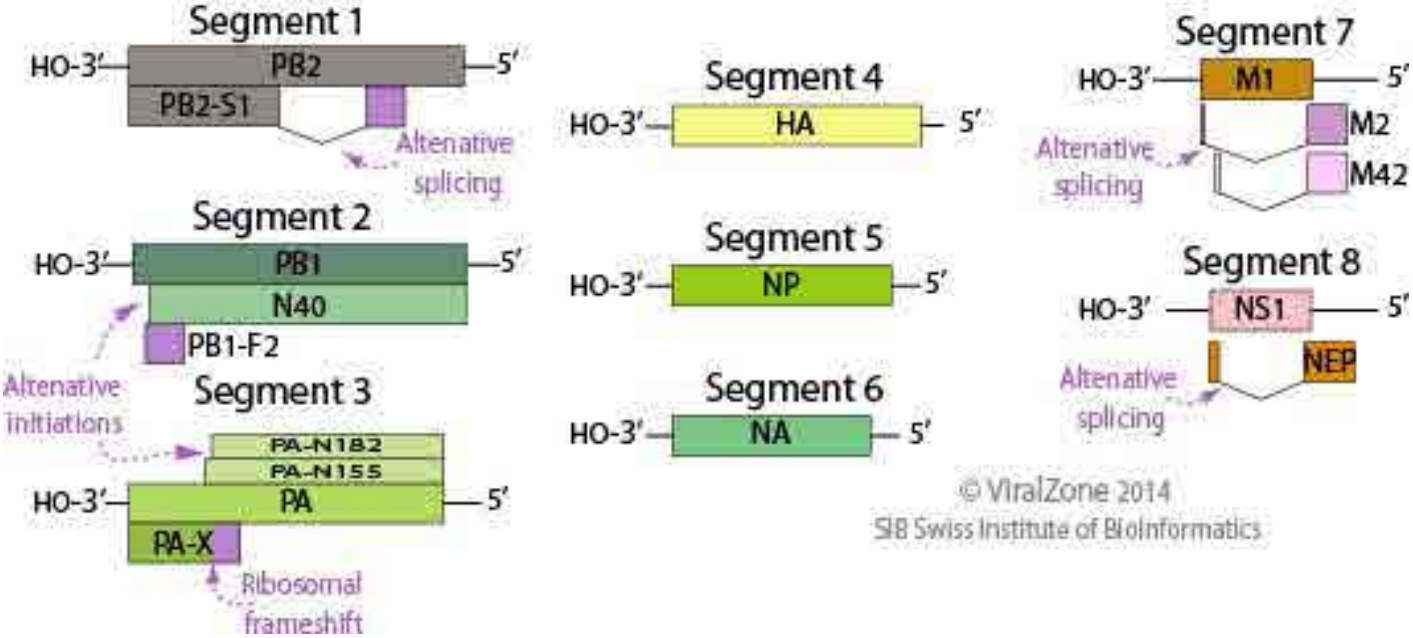


Figure 18-2B. Influenza genome. Courtesy of ViralZone https://viralzone.expasy.org/223.

VIRAL REPLICATION CYCLE

As shown in Figure 18-3, during the replication cycle:

1. The virus attaches to the sialic acid receptor through HA protein and is endocytosed into clathrin coated vesicles into the host cell.
2. Endosome acidification induces fusion of the virus membrane with the vesicle membrane when the fusion peptide of HA changes shape in response to the low pH and requires activation of the M2 envelope protein channel.
3. The encapsidated RNA segments migrate to the nucleus via microtubules. A viral accessory protein transports the viral nucleocapsid through the nuclear pore.
4. Transcription of mRNA using the genomic negative sense segments as template by the viral RNA-dependent RNA polymerase (RdRp) attached to each segment produces mRNAs that are capped via **cap snatching** (where it steals the cap and 10-13 base pairs of cellular mRNAs to prime transcription), and polyadenylated by the viral RdRP when it stutters at a poly U track in the 5' end of the template. Some of the segments produce mRNAs that are created via splicing which they can take advantage of because they replicate in the nucleus.
5. Translation of viral proteins by cellular ribosomes on endoplasmic reticulum.
6. Replication of genomic segments is carried out by viral RdRp in the nucleus by first creating a positive sense RNA replicative form (RF) that provides the template for creating more negative sense genomic RNA. This is carried out in the ribonucleocapsid. Newly formed segments associate with nucleocapsid proteins in the nucleus.
7. High level of viral M1 matrix protein induces genome segments' export from nucleus by viral NP protein through nucelopores.
8. Virus assembly of the segments associated with nucleocapsid proteins and budding occurs at the plasma membrane.
9. Neuraminidase (NA) enzyme cleaves sialic acid residues on the cell surface and in mucus. On the surface, NA helps the virus leave the cell, and in the mucus, NA helps the virus move through the mucus to infect the cell. Note: although NA cleaves sialic acid, the virus is still able to bind and enter the cell because entrance happens rapidly (before NA has a chance to cleave the sialic acid receptors).

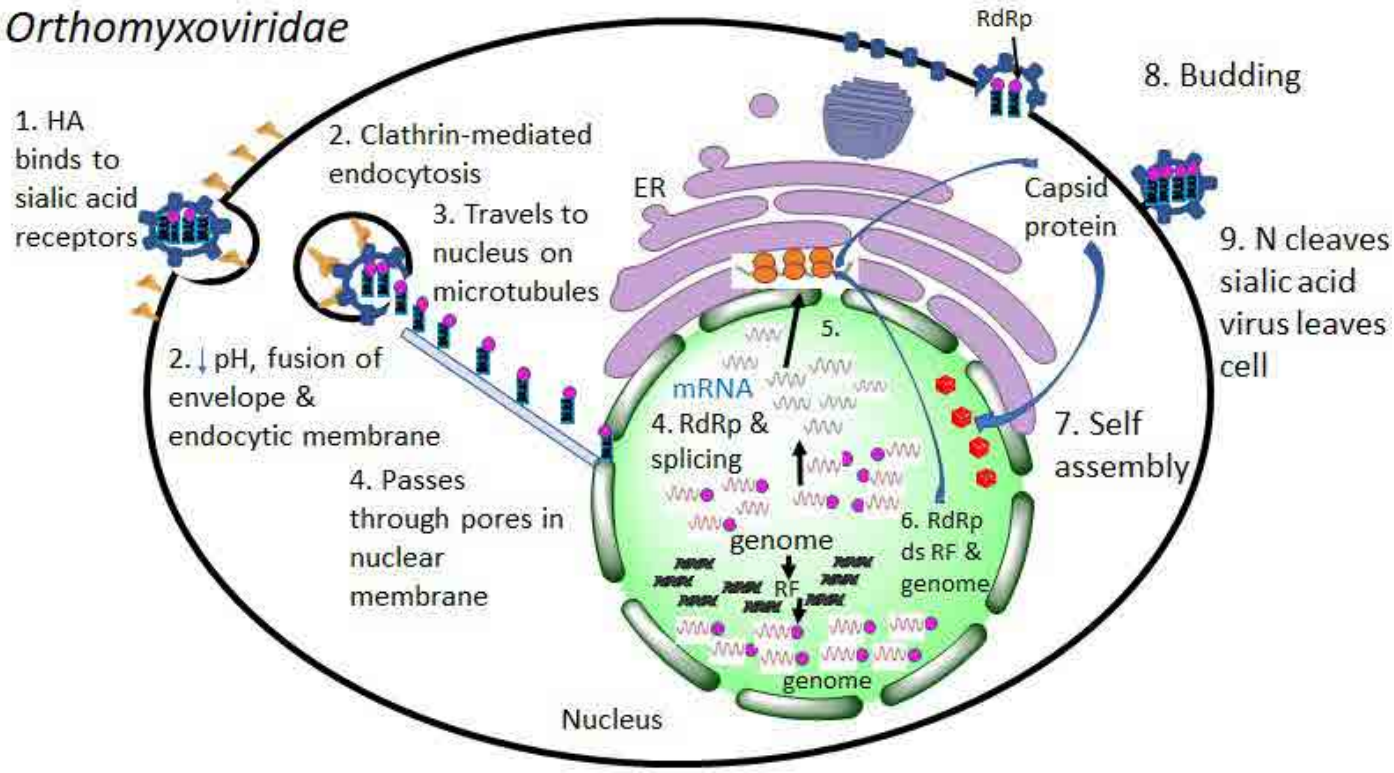


Figure 18-3. Influenza replication cycle. Courtesy of Erica Suchman, Colorado State University

HOW DO ORTHOMYXOVIRUSES OVERCOME THE ISSUE THAT THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY ARE IN THE NUCLEUS?

Most RNA viruses' RdRp has the ability to create the 5' cap and use a poly U tract in the 5' end of the template (negative sense) strand of RNA that will result in the addition of a long poly A tail when the RdRp stutters adding far more A's than exist in the tract. However, orthomyxoviruses' RdRp lacks the ability to add a 5' cap, and as such it performs cap snatching where it steals the cap and 10-13 base pairs of cellular mRNAs to prime transcription. Note that the RdRp replicase activity does have the ability to perform de novo synthesis while replicating the viral genome. Remember that orthomyxoviruses perform all these activities in the nucleus unlike all other RNA viruses discussed in this book. The other RNA viruses that carry out part of their replication cycle in the nucleus are retroviruses, but recall they are functioning as DNA viruses when they are in the nucleus.

HOW DO ORTHOMYXOVIRUSES OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

1. They do this by using alternative splicing to create many smaller versions of a pre-mRNA all of which produce different proteins.
2. In order to express more polymerase proteins (RdRP) which are created from products from 3 different segments (PB1,2, PA) than other accessory proteins, **ribosomal frameshifting** or **leaky scanning** is utilized (Chapter 13).

3. Leaky scanning (labeled "alternative initiations" on Figure 18-2B) occurs when the ribosome initiates translation at an AUG that is associated with a weak Kozak consensus sequence. This occurs a small percentage of the time.
4. Frameshifting occurs when the ribosome reaches a secondary structure called the **pseudoknot** in the mRNA being translated. The majority of the time the ribosome is able to unwind the pseudoknot and translate one set of proteins. A small percentage of the time, however, the ribosome will hit the pseudoknot and hop back 1 nucleotide causing it to read all future codons in another frame leading to the production of another set of gene products in an overlapping reading frame (Chapter 13).

TRANSLATION OF VIRAL PROTEINS

We learned in Chapters 3 and 13 that translation initiation requires a whole host of initiation factors to bind the 5' cap. In particular, eIF4E binds to the cap and eIF4G binds to eIF4E and then other initiation factors. Furthermore, poly A binding proteins (PABPs) must bind to the 3' poly A tail and interact with the initiation complex to begin translation. The RdRp of orthomyxoviruses do not have the ability to add a 5' cap. This is why orthomyxoviruses perform cap snatching to provide the necessary 5' cap. Orthomyxovirus RdRp does add a poly A tail.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes.

Note that orthomyxoviruses, like many viruses, take care of the problem of replicating the ends and priming replication via the same mechanism.

RNA viruses, with the exception of retroviruses, cannot use the host cell's transcription machinery DNA-dependent RNA polymerase (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As such, with the exception of retroviruses, they must all have genes to produce an RNA-dependent RNA polymerase (RdRp). Negative sense RNA viruses whose genomes are not equivalent to mRNA cannot be translated immediately upon entrance into the cell and as such must carry RdRp into the host cell with the genome in order to begin replicating. Note that all RNA viruses of animals have linear genomes and therefore must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses including orthomyxoviruses overcome this easily as RdRp starts synthesizing at the end of the genome without a primer. This is referred to as de novo synthesis. De novo initiation by RdRP occurs at the 1st nucleotide. For most RNA viruses this method of replication ensures that the ends are replicated.

VIRION FORMATION

Viral capsids form spontaneously in the nucleus are exported through nuclear pores and the virus leaves the cell by budding from the plasma membrane.

AVOIDING THE HOST IMMUNE RESPONSE

Orthomyxoviruses avoid detection by pathogen recognition receptor (PRR) TLR 7, which recognizes the pathogen associated molecular pattern (PAMP) of single stranded endosomal RNA, by remaining in their capsids until they leave the endosome. They avoid the PRRs RIG-I (cytoplasmic RNA lacking a 5' cap), MDA-5 (cytoplasmic dsRNA and improper cap), that will initiate the interferon response and possibly RNAi (ds RNA) by replicating within the nucleus. Furthermore, recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication to avoid activating the interferon response and possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi), as well as the interferon stimulated genes (ISG) dsRNA-dependent PKR, dsRNA-dependent OAS and the PRR MDA-5. Because the two stands are not kept together, no proofreading can occur which leads to a high mutation rate which allows antigens to change rapidly. However, RIG-I can recognize the virus due to the lack of a 5' cap on the negative sense RNA genome when it exits the nucleus and is not yet surrounded by nucleocapsid proteins. Influenza A creates an accessory protein that inhibits the RIG-I cascade pathway that would activate type I interferon expression. Furthermore, due to their segmented genomes, influenza viruses can perform gene reassortment where segments from two or more viruses are swapped to avoid the host's immune response by changing their external antigens as shown in Figure 18-4.

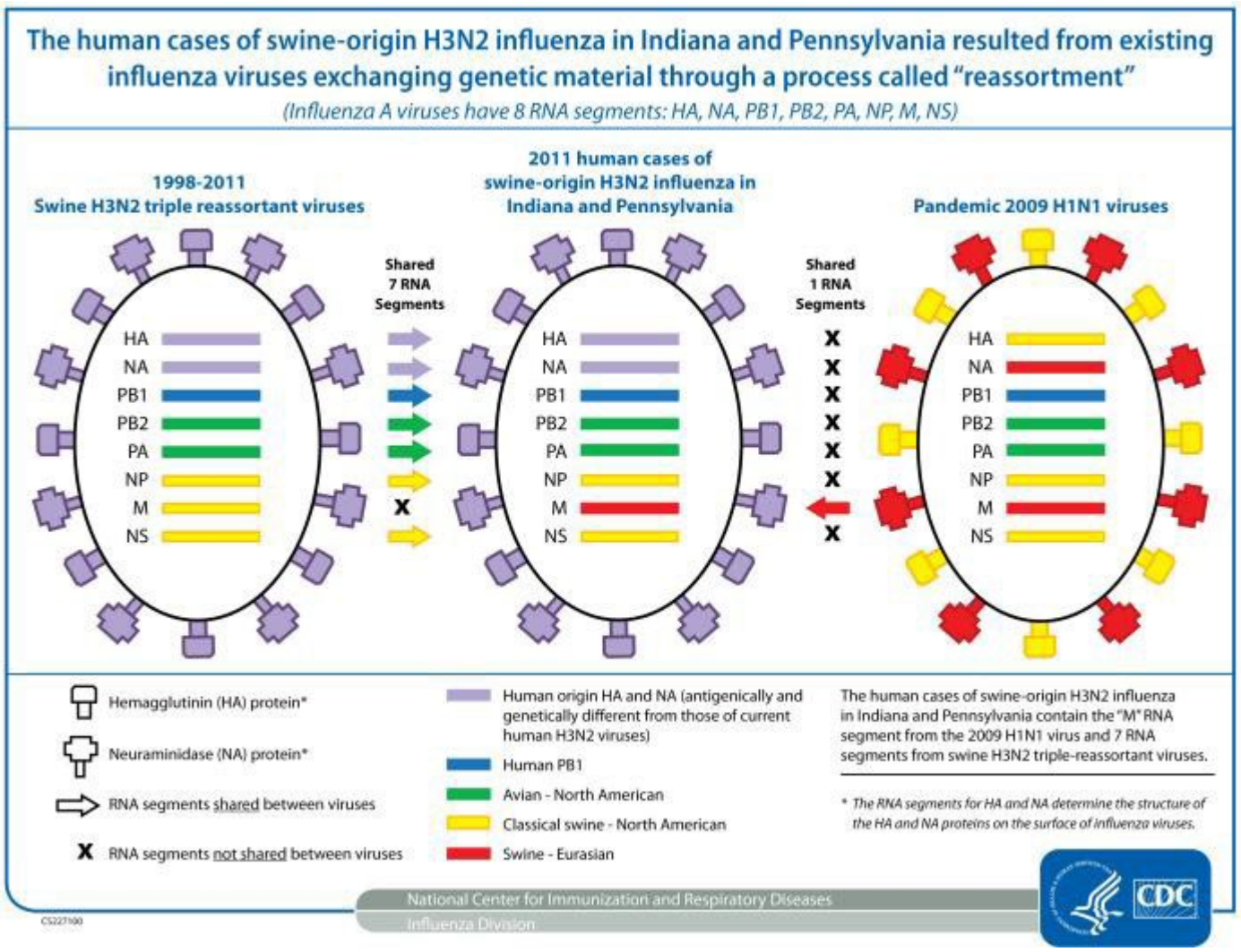


Figure 18-4. Influenza virus reassortment via exchange of segments. Courtesy of https://phil.cdc.gov/Details.aspx?pid=13469

ORTHOMYXOVIRUS DISEASES

Influenza C viruses cause respiratory disease in humans. It is the least severe influenza infection. Influenza B viruses cause moderately severe respiratory disease in humans. Influenza A infects a wide range of species, humans, horses, swine, poultry, waterfowl, whales, seals, bats, etc., causing a respiratory disease. Influenza A has been known to cross species. All 3 have a worldwide distribution. Influenza A virus infections in humans are generally acute and self-limiting but can be quite severe.

Only H1, H2 and H3 viruses routinely circulate in humans. H1-H16 viruses primarily circulate in waterfowl (e.g., ducks, geese). They can cause seasonal epidemic outbreaks, often referred to as "**seasonal flu**", and occasional pandemics, referred to as "**pandemic flu**". There are 9 to 35 million cases in USA each year and 12,000 to 56,000 deaths (approximately 30,000 average) depending upon the strains that are circulating. Human to human transmission occurs via aerosol droplets, large droplets, and direct contact with contaminated surfaces that occurs during the symptomatic phase. Although shedding occurs before symptoms during the **prodromal** stage (12 hours before symptom onset), there is little evidence that this leads to transmission. Transmission appears to require sustained contact, as studies of school children who rode on school buses while symptomatic failed to demonstrate transmission to school mates despite rides of over 50 minutes. Currently it is thought that large respiratory droplets that do not stay afloat in the air for long periods of time are the primary route of transmission. These large respiratory droplets land on mucosal membranes such as the mouth, eyes, or nose, and may also be inhaled into the lungs. Symptoms include fever, headache, cough, sore throat, nasal congestion, sneezing, and body aches. In the lungs, cell stress, necrosis, and death are observed followed by decreased basal cells and decreased tissue repair leading to **hypoxia** (decreased oxygen) and then inflammation of the lungs. Until the COVID-19 pandemic, influenza A was the leading cause of virally induced **acute respiratory distress syndrome (ARDS)**. Death is usually a result of ARDS or organ failure due to inflammation induced by the immune response. The variability in the number of cases and case-fatality rates each year is a result of differences between the influenza A strains that are circulating.

Influenza viruses accumulate mutations over time leading to **antigenic drift,** and reassortment of viral RNA segments in cells infected with two or more different viruses leads to **antigenic shift**. Antigenic drift occurs because, in seasonal influenza, there is rapid mutation in the RNA genome and selection pressure exerted by immunity stimulated by previous infections. Successful replication occurs in mutants that can "escape" immunity of human populations by accumulation of point mutations in the HA gene and gradual changes in the antigenicity of the HA molecule.

Antigenic shift results in a sudden appearance of a new subtype of influenza A virus in humans. This usually occurs when a new subtype from an avian species (**reservoir**) transfers to humans. This occurs because of reassortment of the avian virus with circulating human influenza A virus and leads to the introduction of the new subtype in an immunologically naïve human population as shown in Figure 18-4 and 18-5. Note that coinfection of cells with two different influenza A viruses can theoretically result in 256 genotypes. Reassortment is a major mechanism for the generation of pandemic influenza viruses. Wild aquatic birds are the principal reservoirs of H1-H16 influenza A viruses. The virus is then transmitted from wild waterfowl to domestic poultry, pigs, horses, sea mammals and humans.

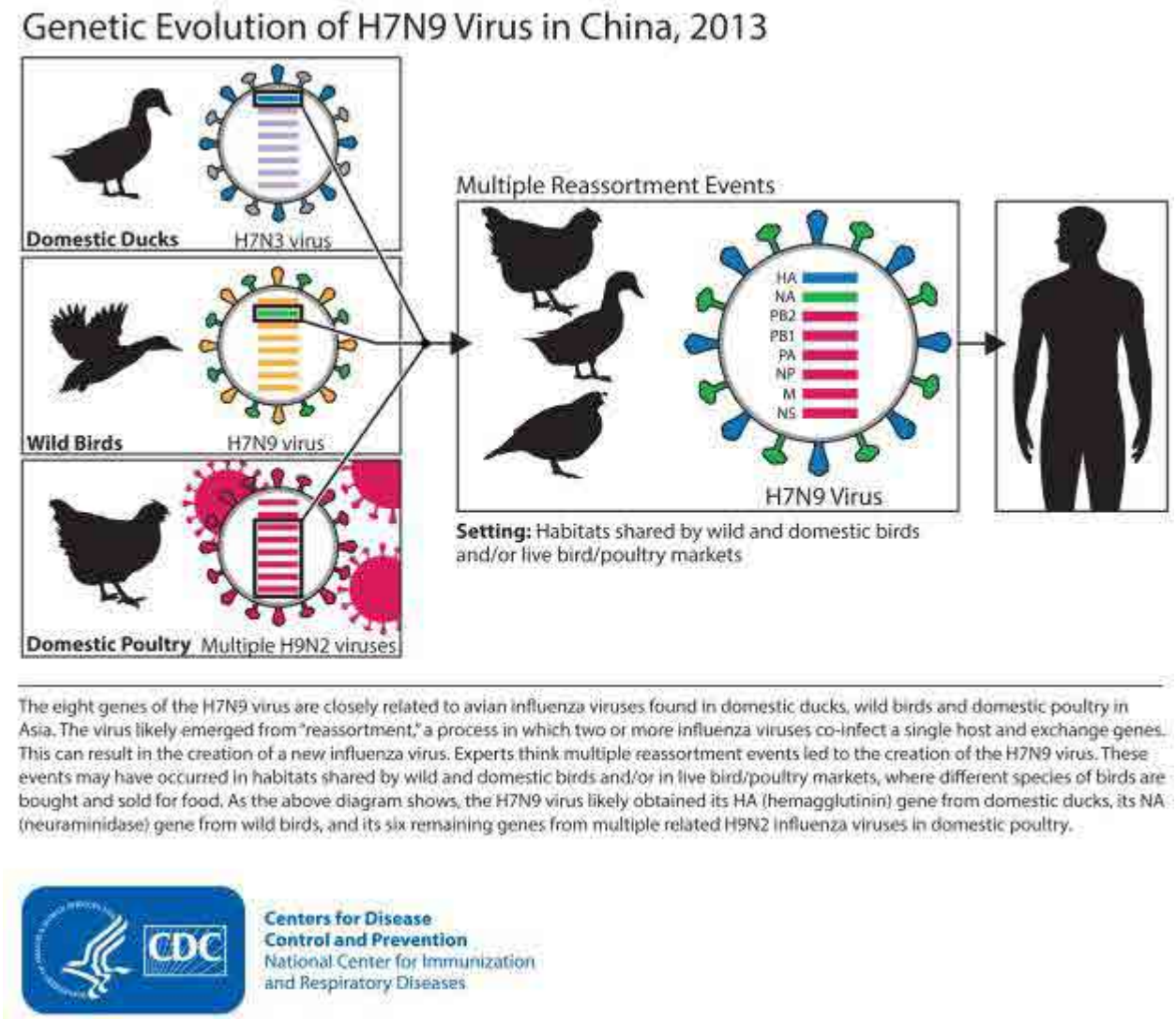


Figure 18-5. Influenza transmission cycle. Courtesy of https://phil.cdc.gov/Details.aspx?pid=15798 D. E. Jordan

In 1918-1919 there was a particularly devastating outbreak of H1N1 influenza A. It may have started at Fort Riley, Kansas, and resulted in 50-100 million deaths worldwide and 80% of U.S. Army deaths during WWI. The particularly frightening thing about this outbreak was that primary mortality was seen in young, healthy adults. In normal seasonal flu the deaths are primarily in infants, young children, the elderly, and the immunosuppressed due to their reduced ability to fight these infections. Furthermore, people with comorbidities such as obesity, **chronic obstructive pulmonary disease** (COPD), or asthma were at increased risk. It is thought that these deaths in young healthy populations resulted from the massive amounts of cytokines created to reduce the infection resulting in a "**cytokine storm**" that overwhelmed infected individuals and led to death. The 1918 pandemic had a first wave that ran from March of 1918 to the summer of 1918 when cases dropped to very low levels. At this point social distancing and masking practices that were in place were relaxed. Then a second and much larger wave occurred in the fall of 1918 when mutant variants emerged that subsided by the end of 1918 at which time a third wave occurred in the winter of 1919 that was smaller than the 2nd wave but larger than the first. Note that this was during World War I. The stressful conditions under which people in the army were living may have contributed to the high case-fatality rate. Furthermore, this was before the development of antibiotics to control the secondary bacterial pneumonia that is common in influenza A induced disease.

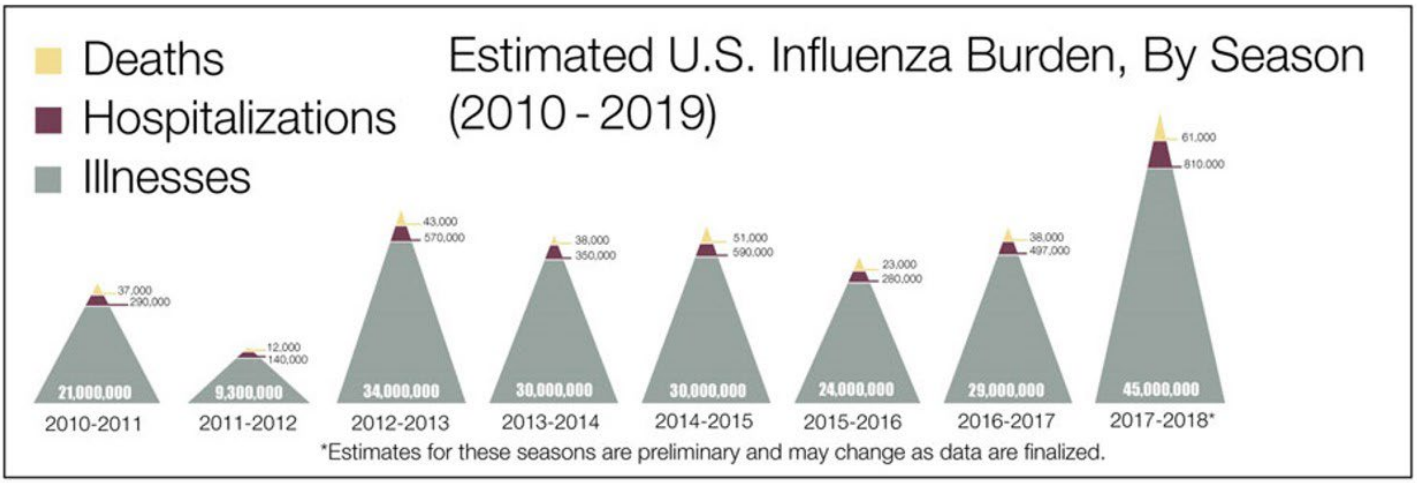
H1N1 subtype Influenza type A circulated from 1918-1957, with occasional outbreaks, and disappeared from 1957-1977 when the virus reemerged and continues to circulate today. It is thought that reassortments between different strains of H1N1 subtype caused the changes in viral pathogenicity over time.

In 2009 another less devastating outbreak of H1N1 that originated from swine occurred. This one began in Oaxaca, Mexico, and resulted in 684 deaths out of 126,148 confirmed cases for a case-fatality rate of 0.6%. The median age of fatal cases was 37 years with 51% of deaths in 20-49 years of age, again much different than the epidemiology of seasonal flu. The following underlying risks were found to increase the likelihood of death: pregnancy, obesity, diabetes, heart disease, and respiratory disease. In August 2010 the WHO declared H1N1 2009 to be post-pandemic (seasonal) influenza. This zoonotic **spillover** event occurred because pigs can be naturally infected with avian or human influenza because the epithelial cells in pig's trachea contain receptors for both avian and human influenza viruses. This allowed **reassortment** of segments to occur in pig cells coinfected with multiple strains of influenza virus. The reassortments found in this epidemic are shown in Figure 18-4. This outbreak led to school closures across the country.

Based on their pathogenicity in chickens, avian influenza viruses are classified as either low pathogenic (LPAI) viruses which cause mild respiratory disease and decrease egg production, or as high pathogenic (HPAI) with high case-fatality rate. Avian influenza viruses kill chicken embryos in eggs in as little as 4 days with lower titers than seasonal flu. This limits the global capacity for vaccine production, and as such we are not able to make enough vaccine to control an avian influenza pandemic.

JUST HOW LARGE IS THE INFLUENZA BURDEN?

It is estimated that in an average year in the US, 9-45 million people will develop the flu, 140-810 thousand people will have symptoms so severe they will require hospitalization, and 12,000-61,000 will die with 250-950 thousand deaths per year worldwide. The average case-fatality rate is 0.1%, although this can vary from year to year depending upon the strains circulating. The **RO** (number of people that one person will infect, an indication of how contagious the virus is) is usually between 1.3-2.0 This is low compared to viruses such as mumps and measles with ROs of ~7 and ~18 respectively. Figure 18-6 A & B shows the burden of influenza infections from 2010-2020. Note the last year of data given is always an estimate, and often changes once the final data analysis is complete. This data take a long time to analyze as they have to receive data from all states which takes time.



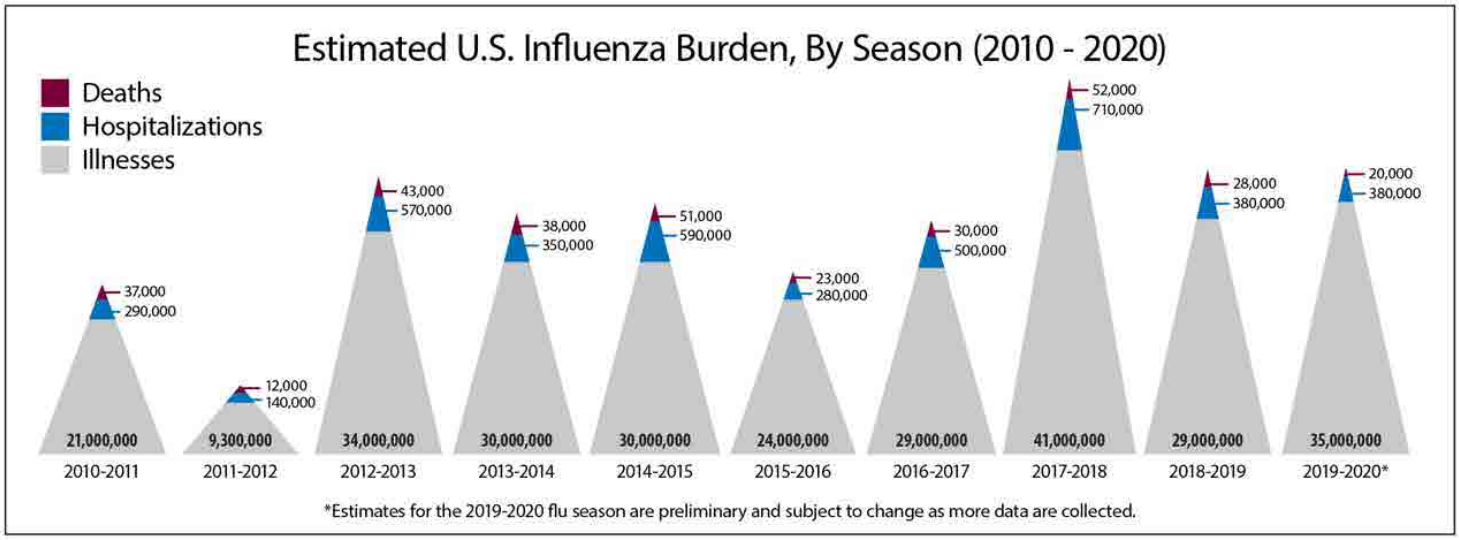


Figure 18-6 A & B. A. Estimated US influenza burden by season, 2010-2019. ***B.*** *A. Estimated US influenza burden by season, 2010-2020. The case-fatality rate of the virus is on average 0.1% with some years being higher than others. Note that in the first diagram 2017-2018 was an estimate that was later decreased when all of the final analysis was completed Courtesy of https://www.cdc.gov/flu/about/burden/index.html*

DIAGNOSIS OF ORTHOMYXOVIRUS INFECTIONS

There are multiple rapid diagnostic tests for influenza that detect viral nucleoprotein antigens in respiratory samples. These tests use monoclonal antibodies that target the viral nucleoprotein and employ an **enzyme immunoassay and immunochromato-graphic techniques**, and as such can be read similarly to a pregnancy test. EIA is an ELISA that uses an enzyme attached to the monoclonal antibody attached to a substrate and causes a color change if the antibody binds the influenza nucleoprotein antigens. These tests can be run in 15-30 minutes; however these tests have limited **sensitivity** leading to a high rate of false negatives. Negative samples should therefore be confirmed with reverse transcriptase PCR which takes 1-8 hours depending upon the assay used. The rapid tests also are often poor at detecting Influenza B infections and cannot differentiate between seasonal flu and novel influenza infections. They are, however, useful for allowing people who have positive test results to start treatment rapidly, which is required as influenza treatments only reduce symptoms if they are initiated within 24-48 hours of developing symptoms. Direct and indirect IFA on nasal or throat swabs or washings takes 1-4 hours and can also be used.

PREVENTION OF ORTHOMYXOVIRUS INFECTIONS

Every year a new seasonal flu vaccine is released. They are either egg-based or cell culture-based vaccines or recombinant vaccines that are produced in insect cells. Every year epidemiologists with the CDC predict what viruses are most likely to be circulating each year and include those strains in the vaccine which often contains both influenza A and B strains. The vaccine changes every year, and as such it is recommended to get the vaccine early each fall before the start of flu season in November. The predicted necessary **vaccine threshold** to generate herd immunity to seasonal flu in the United States is 80% in healthy people and 90% in high risk people. Currently the vaccination rates in the US are not sufficient to achieve herd immunity hence approximately 30,000 people a year die of seasonal flu. The effectiveness of masking and social distancing was evidenced by the COVID-19 outbreak of 2020. In the 2020-2021 flu season a dramatic reduction in cases was observed when compared to previous seasons (https://www.cdc.gov/flu/weekly/index.htm#ILIActivityMap).

TREATMENT OF ORTHOMYXOVIRUS INFECTIONS

There are 3 types of drugs that inhibit influenza replication. The first are neuraminidase inhibitors which include oseltamivir (Tamiflu), zanamivir (Relenza), and peramivir (Rapivab). These are active against influenza A and B viruses. Neuraminidase inhibitors prohibit the virus from leaving the cell by budding.

The second are M2 ion channel inhibitors which include Amantadine and Rimantadine and are only active against influenza A virus. There are high levels of resistance to these M2 channel-inhibiting antivirals among influenza A viruses currently circulating. M2 channel inhibitors inhibit the ability of the virus to fuse the viral envelope with the membrane of the endocytic vesicle.

The third is a cap-snatch inhibitor (approved Oct 2018) called Xofluza. Inhibiting cap snatching inhibits the ability of the virus to produce proteins.

Most of these drugs must be administered shortly after infection to have much effect (usually within 24 hours) and only shorten the duration of the infection by a few days. They do not stop the symptoms in their tracks. Most people do not seek medical care until much too late for these drugs to be effective.

END OF CHAPTER QUESTIONS

1. Compare and contrast orthomyxoviruses to togaviruses and flaviviruses.
2. Compare and contrast reoviruses to orthomyxoviruses and filoviruses.
3. Compare and contrast orthomyxoviruses and arenaviruses.
4. Do orthomyxoviruses create subgenomic mRNAs? If they do, why do they do so? If they do not, why?
5. Do orthomyxoviruses need to have mechanisms for overcoming RNA interference? If so, why? If not, why not?
6. Do orthomyxoviruses use viral proteases to cleave polyproteins? If not, do they use proteases (viral or cellular) for any other reason? If so, for what purpose?
7. What PRR are most likely to detect orthomyxovirus infections? How do they avoid them?
8. What ISG will inhibit orthomyxovirus replication?
9. What is the significance of the HA and NA proteins of influenza viruses?
10. Compare how orthomyxoviruses obtain a 5’ cap to how the viruses discussed in Chapters 14-17 obtain their 5 cap.
11. Can orthomyxoviruses read more than one ORF from an mRNA? If so, explain how.
12. Why do orthomyxoviruses replicate in the nucleus?
13. You perform a reverse transcriptase real time PCR on multiple tissues from an infected child. You use a primer that is complementary to bases 100-115 and another that is complementary to the opposite strand bases 655-670. What will your results look like for the tissue that has the highest viral load? What about tissues that fail to replicate the virus?
14. What types of changes occur in influenza viruses that are likely to result in pandemics?
15. Are pandemics more lethal than epidemics?
16. What do reoviral diseases of domestic animals have in common?
17. What is the significance of Kozak consensus sequences to influenza viruses?
18. What is the significance of mRNA pseudoknots to influenza viruses?
19. What allows influenza viruses to spillover from animals or birds into humans?

20. How do orthomyxoviruses outcompete host cell mRNA for translation machinery?
21. What facet of the immune response is most important for clearing rotavirus infections?
22. Compare and contrast influenza exit from the cell to enveloped DNA viruses.
23. Explain why we must get influenza vaccines each year.
24. How did the 2009 swine flu differ from seasonal flu?
25. What are the 3 major mechanisms of drugs that inhibit influenza viruses?
26. What is a major weakness of the drugs that are currently available?
27. What is the significance of an RO value? How does the RO of seasonal influenza compare to the measles or mumps virus? How does it compare to COVID-19?
28. There is a rapid diagnostic test for influenza. What are the pros and cons of this test?

REFERENCES:

Centers for Disease Control and Prevention. (2021). Flu data. https://www.cdc.gov/flu/weekly/index.htm#ILIActivityMap

Centers for Disease Control and Prevention.. Public Health Image Library (PHIL) (2013). D. Jordan, M.A. Evolution of influenza H7N9 in China. https://phil.cdc.gov/Details.aspx?pid=15798

Centers for Disease Control. and Prevention. Public Health Image Library (PHIL) (2011). D. Jordan, M.A. Influenza reassortment. https://phil.cdc.gov/Details.aspx?pid=13469

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 40, 41.

Glatman-Freedman,A, et al. (2012). Attack rates assessment of the 2009 pandemic of H1N1 influenza A in children and their contacts: A systematic review and meta-analysis. PLoS One. 2012; 7(11): doi: 10.1371/ journal.pone.0050228 https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0050228&type=printable

Killbourne, E. (2006). Influenza Pandemics of the 20th Century. Emerging Infectious Diseases. 12(1):9-14. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3291411/

Nelson, MI, et al. (2008). Multiple Reassortment Events in the Evolutionary History of H1N1 Influenza A virus since 1918. PLoS Pathog 4(2):e1000012. doi.10.1371/journal,ppat.1000012 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2262849/

Plans-Rubió, P. (2012). The vaccination coverage required to establish herd immunity against influenza viruses. Preventive Medicine Volume 55, Issue 1, Pages 72-77. https://pubmed.ncbi.nlm.nih.gov/22414740/

Rossman JS, and Lamb RA. (2011). Influenza virus assembly and budding. *Virology*. 411(2):229-236. doi:10.1016/j.virol.2010.12.003. https://pubmed.ncbi.nlm.nih.gov/21237476/

ViralZone. *Orthomyxoviridae*. https://viralzone.expasy.org/223

CHAPTER 19

Chapter 19: Segmented Negative Sense RNA Viruses That May Have Ambisense Segments That Replicate In The Cytoplasm: Order Bunyavirinae, Families Hantaviridae, Phenuiviridae, Nairoviridae, Arenaviridae.

INTRODUCTION TO THE FAMILIES IN ORDER *BUNYAVIRALES*

You will notice in Figure 19-1 that viruses in the order *Bunyavirales* are segmented negative-stranded RNA viruses that are enveloped and have helical nucleocapsids with 2 (*Arenaviridae*) or 3 (*Hantaviridae, Nairoviridae, Phenuiviridae*) segments that replicate in the cytoplasm. Arenaviruses have a virion of 50-300 nm in diameter, phenuiviruses, nairoviruses and hantaviruses have a virion of 80-120 nm as shown in Figures 19-2A-C. All are classified as negative sense RNA viruses because they must carry their RNA-dependent RNA polymerase (RdRp) in their virion with their genome to produce mRNA once inside the cell. As we will see, arenavirus and phenuivirus genomes can also contain **ambisense** segments meaning they are partially positive sense RNA and partially negative sense RNA within one segment.

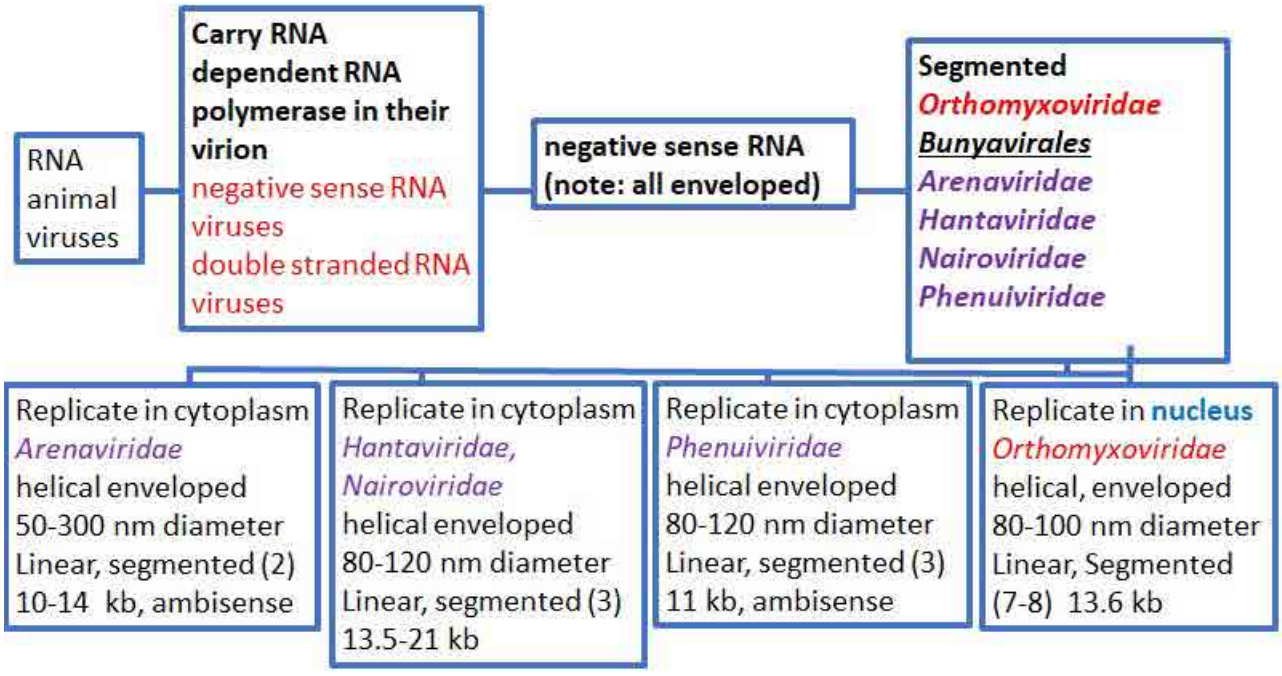


Figure 19-1. Taxonomy of negative sense RNA viruses focusing on segmented viruses. Courtesy of Erica Suchman, Colorado State University

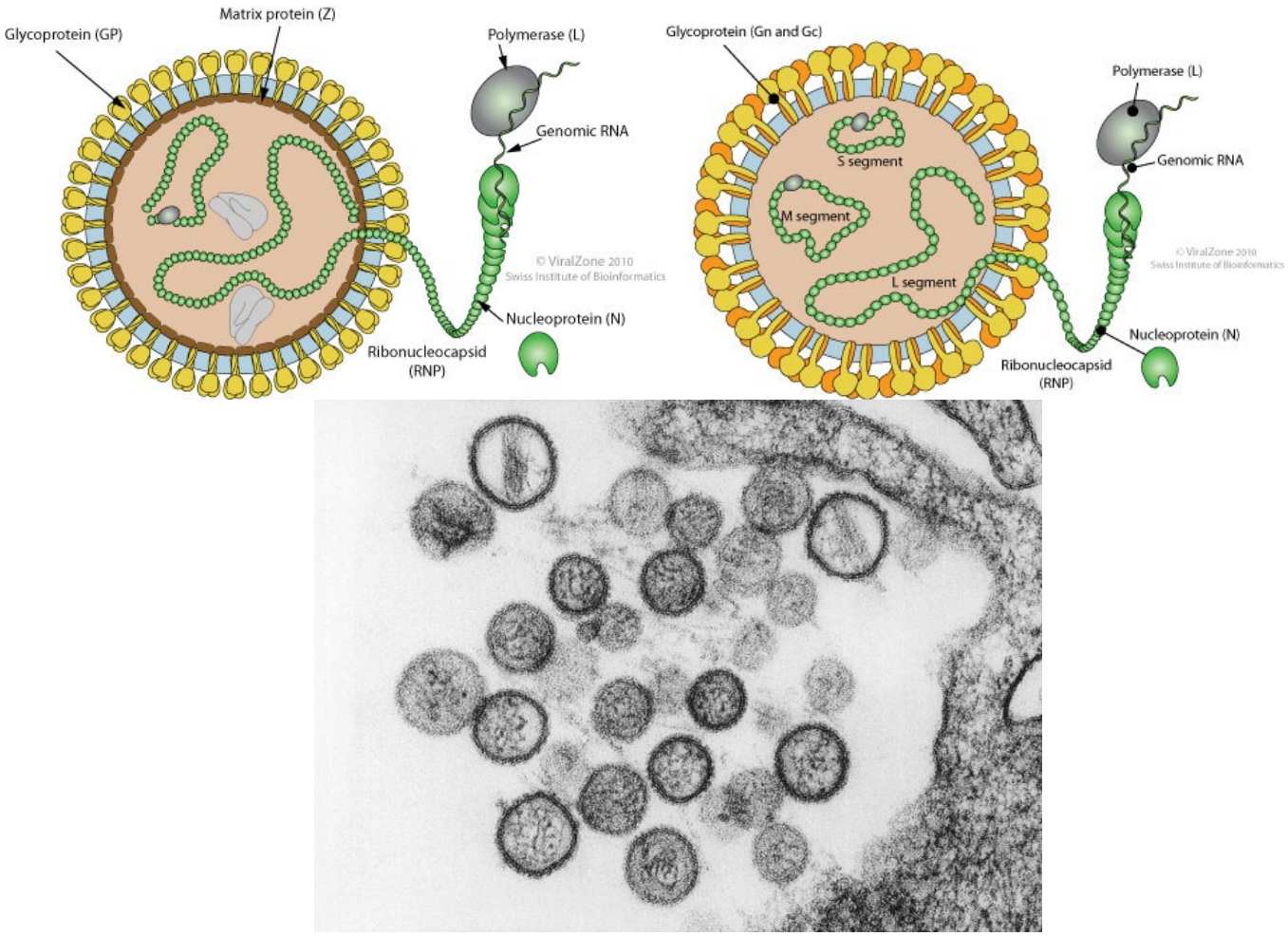


Figure 19-2 A-C. A typical Arenaviridae virion with 2 helical segments in an envelope each with a molecule of RdRp. ViralZone https://viralzone.expasy.org/501. B. Typical Hantaviridae, Nairoviridae or Phenuiviridae virion with 3 helical segments in an envelope each with a molecule of RdRp. Courtesy of ViralZone https://viralzone.expasy.org/7101. C. Electron microscope image of hantavirus. https://phil.cdc.gov/Details.aspx?pid=12734 C. Goldsmith.

Bunyavirales has 5 families that infect animals and humans and 1 that infects plants. We will cover those that are marked with a * as they are the most medically important.

Family	Vector	Examples	Disease Host
Peribunyaviridae	Mosquito	LaCrosse encephalitis virus	Human
*Nairoviridae**	Tick	Crimean Congo Hemorrhagic	Human
*Phenuiviridae**	Mosquito	Rift Valley fever virus	Cattle, Human
	Sandflies	Sandfly fever, Naples virus	Human
	Tick	Uukuniemi virus	Seabirds
*Hantaviridae**	Rodent	Hantaan virus, Sin Nombre virus	Human
Fimoviridae	Thrips	Tomato spotted wilt virus	Plant
*Arenaviridae**	Rodents	Lassa fever	Humans

Arenaviridae has 2 genera:

Genus *Mammarenavirus* has 27 mammalian arenaviruses 10 of which are zoonotic. The genus *Reptarenavirus* has 3 reptile arenaviruses none of which are known to be zoonotic. More viruses are found in the **New World** (the Americas including both North, Central, and South America), but there are ones such as lymphocytic choriomeningitis virus (**LCMV**) and Lassa fever that are found in the **Old World** (Africa, Asia, and Europe).

GENOME STRUCTURE

We will describe the genome structure *Bunyavirales* viruses together in this chapter as they are extremely similar. In fact, they are so similar that the International Committee on Viral Taxonomy had proposed putting them into one family. As discussed earlier, all are classified as negative sense RNA viruses because they must carry their RdRp in their virion with their genome in order to produce mRNA once inside the cell. Two families of viruses (*Arenaviridae* and *Phenuiviridae)*, however, actually also possess some ambisense segments, in which each segment is part negative sense (non coding) and part positive sense (coding). You will notice that *Bunyavirales* viruses have far fewer segments (2-3) than orthomyxoviruses (7-8) or reoviruses (10-12). Having ambisense segments allows some of them to produce more than one mRNA from each segment and increases the number of proteins they can produce. Figure 19-3 shows the typical genome of an arenavirus showing multiple ambisense segments. Recall that the gene order is consistent within all the negative sense RNA viruses (Chapters 17 and 18). Arenaviruses have a genome of 10-14 kb while other members of *Bunyavirales* have a genome of 13.5-21 kb. You should note that 2 mRNAs are created thus giving rise to 2 different non overlapping open reading frames. One is transcribed from the negative sense portion, and the positive sense portion serves as the 2nd.

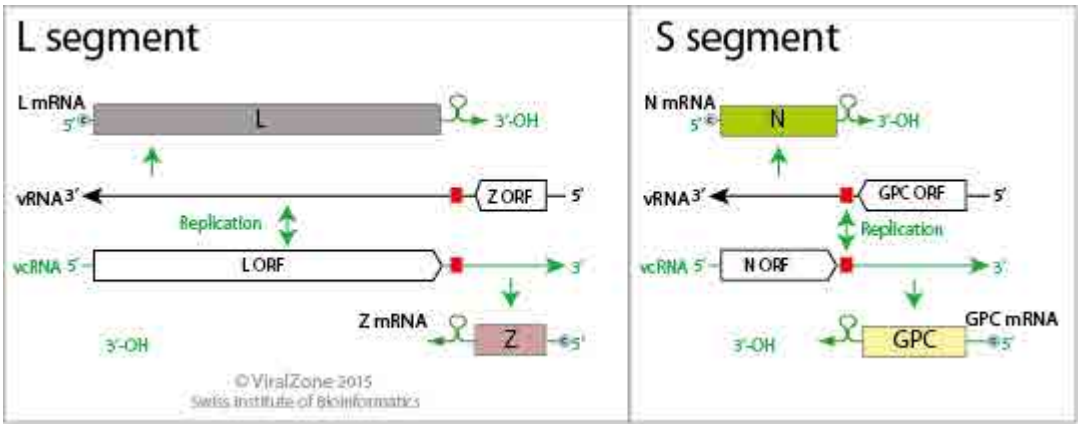


Figure 19-3. The typical genome of an arenavirus showing multiple ambisense segments. Note that both the L and S segments contain one ORF that is read directly from the genome (the + sense portion) as well as creating a subgenomic mRNA from the negative sense portion that will create a second protein. Courtesy of ViralZone https://viralzone.expasy.org/501.

VIRAL REPLICATION CYCLE

Figure 19-4 shows a typical replication cycle of a virus in the *Bunyavirales* order all of which will be extremely similar except how the arenaviruses leaves the cell.

PHENUIVIRIDAE, NAIROVIRIDAE & HANTAVIRIDAE

During the phenuivirus, nairovirus and hantavirus replication cycle

1. The virus attaches to host receptors through envelope glycoproteins and is endocytosed into vesicles in the host cell.
2. Helical ribonucleocapsid segments are released in the cytoplasm when the virus membrane fuses with the vesicle membrane.
3. Transcription is carried out by the RdRp that is carried into the cell with the genome. The viral mRNAs are capped in the cytoplasm via cap snatching, but the mRNAs lack a poly A tail. Note in *Phenuiviridae* that mRNA is made directly from the genome and due to the ambisense nature of the genome also from the anti- genome creating 2 mRNAs from some segments.
4. Replication is presumed to start when enough nucleoprotein is present to encapsidate synthesized genomes.
5. The helical ribonucleocapsid buds at Golgi apparatus releasing the virion by exocytosis.

ARENAVIRIDAE

During the arenavirus replication cycle

1. The virus attaches to host receptors through envelope glycoproteins and is endocytosed into vesicles in the host cell.
2. Helical ribonucleocapsid segments are released in the cytoplasm when the virus membrane fuses with the vesicle membrane.
3. Transcription is carried out by RdRp that is carried into the cell with the genome. The viral mRNAs are capped in the cytoplasm via cap snatching, but the mRNAs lack a poly A tail. Note that mRNA is made directly from the genome and due to the ambisense nature of the genome also from the anti-genome creating 2 mRNAs from some segments.
4. Replication is presumed to start when enough nucleoprotein is present to encapsidate synthesized genomes.
5. The helical ribonucleocapsid interacts with the viral Z protein that accumulates under the plasma membrane and buds releasing the virion.

As you can see, the genome types and replication cycles of these families are quite similar other than the number of segments as well as number that are ambisense and the mechanism for leaving the cell. This has led many scientists to argue that these viruses should be in one family. What do you think? In 2020 the international committee on the Taxonomy of Viruses (ICTV) decided instead to put all of these viruses into a new order *Bunyavirales*, and break *Bunyaviridae* into 5 new families. This was done in part because there were members of the family *Bunyaviridae* that had ambisense genomes, but most did not; furthermore, most were vectored by ticks and mosquitoes except the hantaviruses. The new families created in 2020 separated viruses to reflect these characteristics. The life cycle of phenuiviruses is shown in Figure 19-4. Note that this cycle is very similar to the life cycle of arenaviruses other than the fact that the virus would leave the cell by budding from the plasma membrane. You should use this diagram to understand the replication cycle of both families.

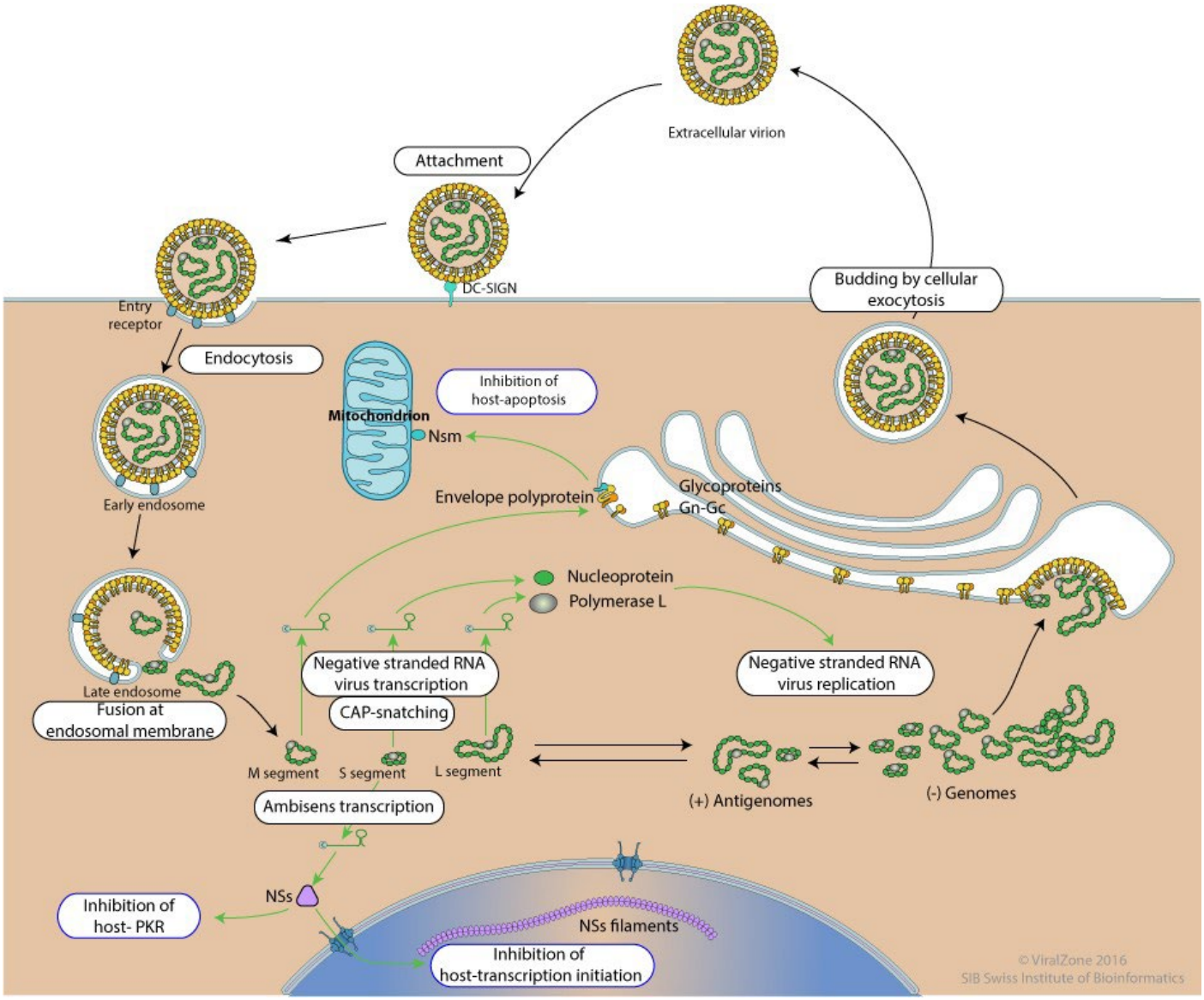


Figure 19-4. A typical replication cycle for a Bunyavirales virus. Note this is very similar to the replication cycle of an arenavirus other than the mechanism for leaving the cell. Courtesy of ViralZone https://viralzone.expasy.org/6776.

HOW DO VIRUSES IN THE ORDER *BUNYAVIRALES* OVERCOME THE ISSUE OF THE 5' CAPPING MACHINERY AND POLY A TAIL ADDITION MACHINERY BEING IN THE NUCLEUS AND VIRUS REPLICATION OCCURRING IN THE CYTOPLASM?

Most RNA viruses' RdRp have the ability to create the 5' cap, and they have a poly U tract in the 5' end of the template (negative sense) strand of RNA that will result in the addition of a long poly A tail. However, viruses in the order *Bunyavirales* the RdRp lacks the ability to add a 5' cap, and as such it performs cap snatching, where it steals the cap and 10-13 base pairs of cellular mRNAs in the cytoplasm to prime transcription. Note that the RdRp replicase activity does have the ability to do **de novo synthesis** while replicating the viral genome. De novo initiation by RdRP occurs at the 1st nucleotide. Both of these viruses' RdRp also lacks the ability to add a poly A tail, and as such they both must have secondary structures in their 3' end that will bind poly A binding proteins allowing translation to be initiated or have alternative PABP independent translation mechanisms. Viruses in the order *Bunyavirales* use an alternative PABP independent translation mechanism.

HOW DO VIRUSES IN THE ORDER *BUNYAVIRALES* OVERCOME THE ONE PROTEIN PER MRNA PROBLEM IN EUKARYOTIC CELLS?

1. Both phenuiviruses and arenaviruses have multiple segments that make separate mRNAs and ambisense segments that will allow more than one mRNA to be produced from some segments. Recall that having segmented genomes means that these viruses can undergo **reassortment**.
2. Viruses in the order *Bunyavirales* will utilize *cellular* proteases to cleave polyproteins into individual proteins.
3. Viruses in the order *Bunyavirales* utilize leaky scanning to create more proteins in overlapping reading frames from some of the segments. Leaky scanning occurs when the ribosome initiates translation at an AUG that is associated with a weak Kozak consensus sequence a small percentage of the time allowing translation of an overlapping reading frame.

TRANSLATION OF VIRAL PROTEINS

The host cell's ribosomes will be used to translate the viral protein. Like most RNA viruses, the mRNA will have a 5' 7-methylguanosine cap, but it will lack the usual 3' poly A tail. The 5' cap will be recognized by the necessary initiation factors (eIF4E &G). Most RNA viruses' RdRp have the ability to create the 5' cap, and the genomes have a poly U tract in the 5' end of the template (negative sense) strand of RNA that will result in the addition of a long poly A tail when the RdRp stutters adding far more A's than exist in the tract. However, arenaviruses and bunyaviruses' RdRp lack the ability to add a 5' cap, and as such they perform cap snatching where their RdRp steals the cap and 10-13 base pairs of cellular mRNAs to prime transcription. Unlike viruses in the *Orthomyxoviridae*, the other family of viruses that performs cap snatching to obtain a 5' cap, arenaviruses and bunyaviruses perform cap snatching in the cytoplasm where they replicate. Furthermore, cap snatching allows viruses in these families to outcompete cellular mRNAs for translation as the 5' cap is required to bind initiation factors. The RdRp of these families also lacks the ability to create a poly A tail. This is why these viruses must either have a secondary structure in the 3' region that will interact with the PABP to initiate translation or use a PABP independent mechanism. It appears that these viruses do the latter. Not needing PABP allows these viruses to inhibit PABP to outcompete cellular mRNAs during translation.

RNA GENOME REPLICATION

Recall that there are three major issues for RNA viruses to overcome when replicating their RNA:

1. How to get the RNA replication machinery they need when host cells don't provide the necessary activities.
2. How to prime their RNA replication.
3. How to replicate the ends of their genome. This is particularly an issue for viruses with linear genomes.

Note that viruses in the order *Bunyavirales,* like many other viruses, take care of the problem of replicating the ends and priming replication via the same mechanism.

RNA viruses, with the exception of retroviruses, cannot use the host cell's transcription machinery DNA-dependent RNA polymerase (DdRp, RNA pol II) to produce their viral mRNA from an RNA template. As such, with

the exception of retroviruses, they must all have genes to produce an RdRp. Negative sense RNA viruses whose genomes are not equivalent to mRNA cannot be translated immediately upon entrance into the cell and must carry RdRp into the host cell with the genome in order to begin replicating. Note that all RNA viruses of animals have linear genomes and as such must have mechanisms to make sure the ends of their genome are replicated. Most RNA viruses including viruses in the order *Bunyavirales* overcome this easily as RdRp starts synthesizing at the end of the genome without a primer via de novo synthesis. For most RNA viruses this method of replication ensures that the ends are replicated.

VIRION FORMATION

Viral capsids form spontaneously in the cytoplasm, and genome replication does not begin until large amounts of viral nucleocapsid proteins are created and can immediately wrap the genome in an attempt to avoid the non-capped RNA detecting pathogen recognition receptors (PRR) RIG-I and MDA-5. The viruses leave the cell when arenaviruses bud from the plasma membrane; phenuiviruses and hantaviruses bud from the Golgi apparatus and leave the cell via exocytic vesicles.

AVOIDING THE HOST IMMUNE RESPONSE

Viruses in the order *Bunyavirales* avoid detection by the PRR TLR 7 which recognizes the pathogen associated molecular pattern (PAMP) of single stranded endosomal RNA by remaining in their capsids until they leave the endosome. Furthermore, recall from Chapter 13 that RdRp does not keep the two strands of RNA together during replication to avoid activating the interferon response and possibly detection by Dicer, the enzyme that recognizes long stretches of double stranded RNA to activate RNA interference (RNAi), as well as the interferon stimulated genes (ISG) dsRNA-dependent PKR, dsRNA-dependent OAS and the PRR MDA-5. As such, RdRp is unable to proofread as it replicates the genome which leads to a high mutation rate which allows antigens to change rapidly. However, viruses in the order *Bunyavirales* do not completely avoid the PRRs RIG-I (cytoplasmic RNA lacking a 5' cap) and MDA-5 (cytoplasmic dsRNA, improperly capped RNA) so these viruses create accessory proteins to interfere with the cascade pathways these PRR activate. Furthermore, due to their segmented genomes, viruses in the order *Bunyavirales* can perform gene reassortment to avoid the host immune response by changing their external antigens.

HANTAVIRIDAE: HANTAVIRUS DISEASES

Probably the most famous disease caused by a virus in the order *Bunyavirales* in the US is hantavirus. This virus in the family *Hantaviridae,* genus *Hantavirus,* is transmitted to humans when they inhale urine, feces, or saliva of infected deer mice (US predominantly), pygmy rice rats, and white footed mice who are asymptomatic carriers. The mice spread the virus among themselves during fighting. Figure 19-5 shows a deer mouse which can be distinguished by its white abdomen. Figure 19-6 shows the hantavirus transmission cycle. Hantavirus transmission by deer mice was unusual in the original family *Bunyaviridae* (split into 5 families in 2020) where the majority were transmitted by ticks or mosquitoes, and may have been one of the reasons this family was broken up into many families. This virus infects the mucosal surfaces of the respiratory tract leading to viremia, whereupon the virus spreads to target tissues including central nervous system, various organs, vascular endothelium, dendritic cells, and macrophages. The dendritic cells or alveolar macrophages become infected and travel to the lungs causing

infection of lung endothelial cells and an increase in proinflammatory cytokines which leads to endothelial cell permeability. As such this is an immune mediated pathology from inflammatory cytokine response and not a direct viral cytopathology. A vigorous antibody response is essential to control the infection because the virus infects the vascular endothelial cells. The immune response to infected cells can cause damage to the vascular system, thus leading to serum leakage from damaged vesicles. Hantaviruses can cause hantavirus cardiopulmonary syndrome (HCPS) or hemorrhagic fever with renal syndrome (HFRS) and results in 200,000 cases and 10,000 deaths per year worldwide. This disease is endemic in mice in the US and particularly the west of North America, including Colorado. The virus is also endemic in mice in South America, and a related Hantaan virus is found in Asia. The incubation period is 9-33 days. The symptoms include fever, **myalgia** (body aches), headache, abdominal pain, vomiting, diarrhea, **pulmonary edema** (fluid in the lungs), **hypotension** (low blood pressure), and **shock** (severely low blood pressure). In the US, Sin Nombre virus has a case-fatality rate of 30%, and in South America the Andes virus has a case-fatality rate of 40-50%. Figure 19-7A shows a typical distribution pattern for the deer mouse (*Peromyscus maniculatus*) reservoir and Sin Nombre cases from the year 1997. Note that the distribution of the mouse reservoir has remained stable for many years, but disease cases fluctuate. Figure 19-7B shows mouse trapping and hantavirus analysis at the author's home in Ault, Colorado, where a 4% positive rate was found in the micetrapped.

Figure 19-5. Deer mouse (Peromyscus maniculatus). Courtesy of https://phil.cdc.gov/Details.aspx?pid=1138

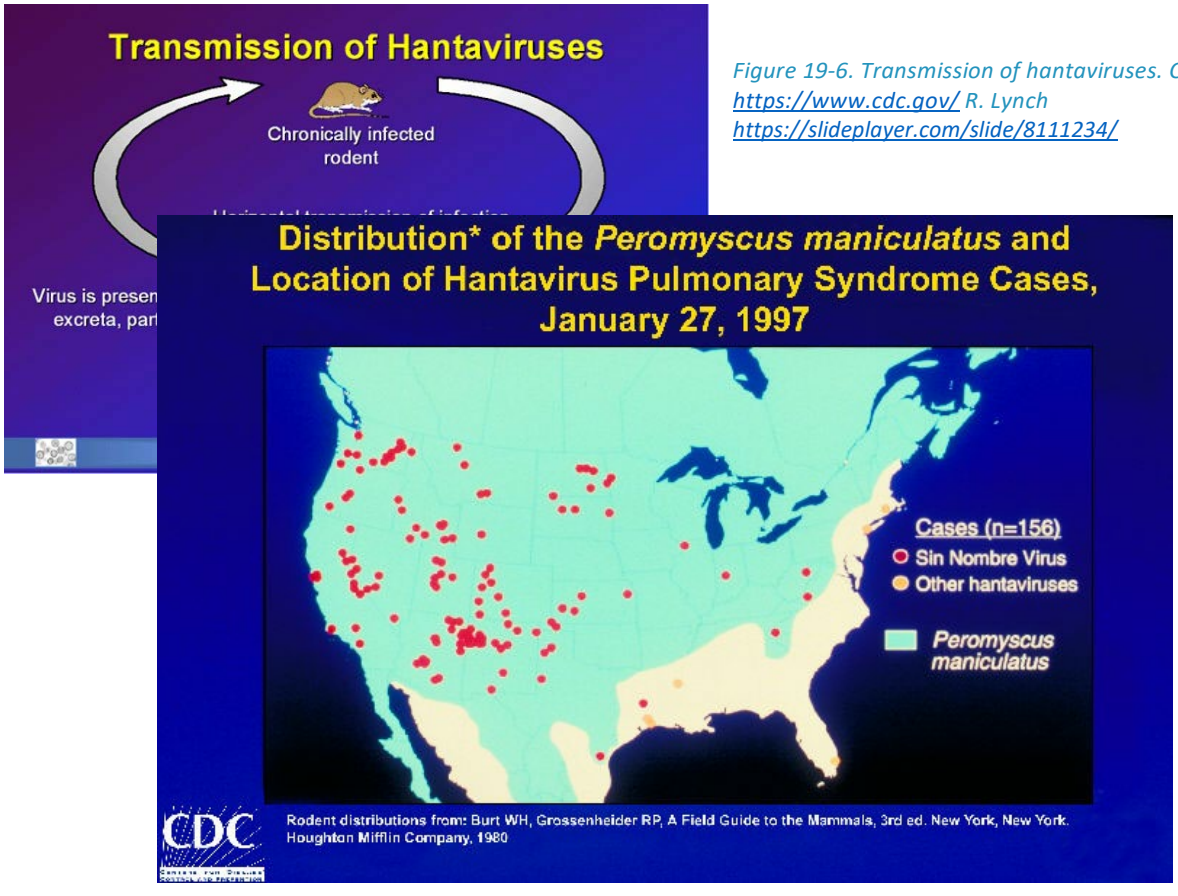


Figure 19-6. Transmission of hantaviruses. Courtesy of https://www.cdc.gov/ R. Lynch https://slideplayer.com/slide/8111234/

Figure 19-7B. Dr. Charlie Calisher trapping mice to determine hantavirus infection levels in deer mice in the eastern plains region of Weld County Colorado, which was determined to be 4% in 2006. Courtesy of Erica Suchman, Colorado State University.

In mice, the infection is persistent and asymptomatic. The virus can replicate in mice despite an initial inflammatory response, because a vigorous **regulatory T cell** (Treg) response predominates during persistent infection and allows the virus to continue to replicate when the inflammatory response is brought under control by the Treg cells as shown in Figure 19-8.

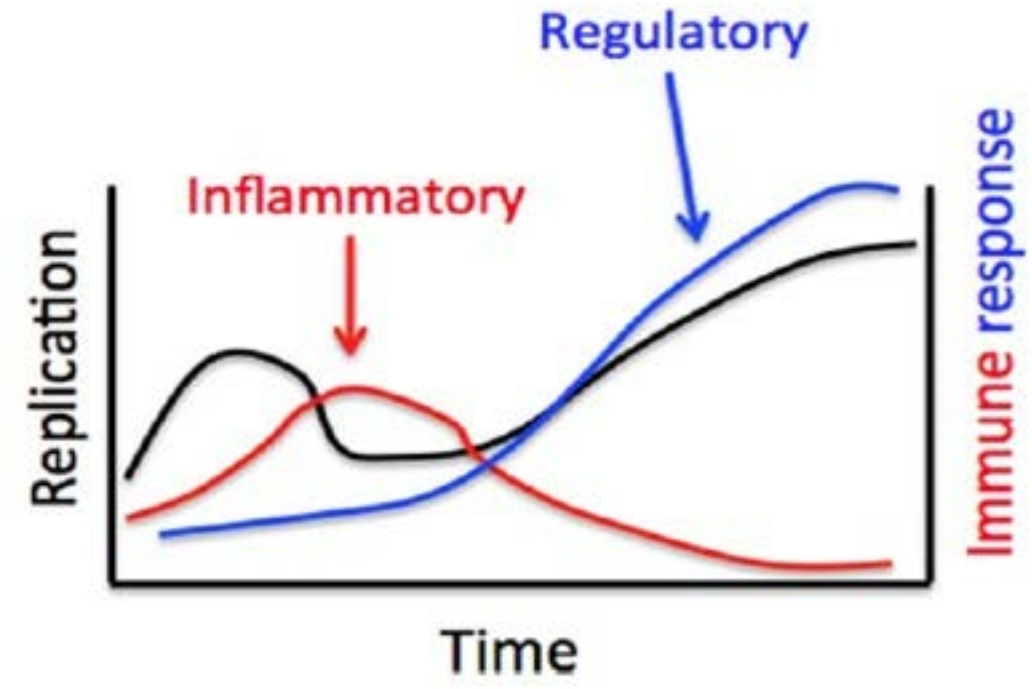


Figure 19- 8. Immune response to hantavirus infection that allows them to maintain a persistent infection. Courtesy of Tony Schountz, Colorado State University.

The disease in humans runs the following course. After an incubation period of 9-33 days, the patient enters a **febrile** (fever) phase and then a cardiopulmonary phase which goes from a respiratory infection to respiratory distress and then cardiac complications. This is an immune-mediated disease. A lack of a Treg response leads to elevated inflammatory cytokines and increased activation of $CD8^+$ CTLs which kill infected endothelial cells leading to fluid leakage from the vascular system. At this point in about 30-40% of cases, the patient will go into cardiac arrest resulting in death. In the rest of patients, they will enter a **diuretic phase** where they must eliminate all the fluid that has accumulated in their tissues, and they will then enter a **convalescence phase** and recover. Figure 19-9 shows a chest X-ray of a hantavirus patient showing extensive fluid buildup, the white in the lungs, which, if healthy, should appear black.

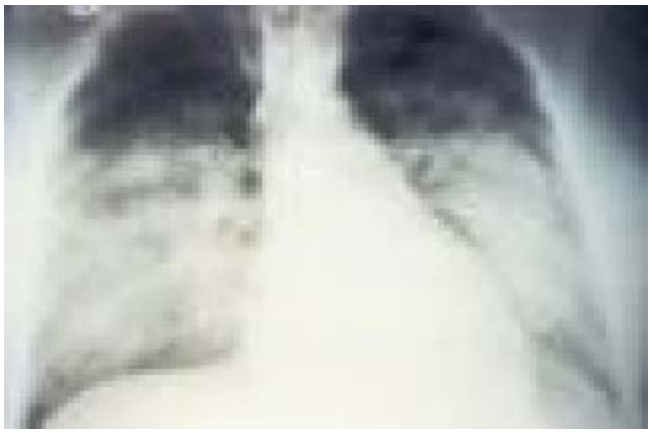


Figure 19-9. Chest X-ray of a hantavirus patient. Courtesy of https://phil.cdc.gov/Details.aspx?pid=6076 Dr. D. L. Ketai.

MOSQUITO BORN *PHENUIVIRIDAE* VIRUSES

RIFT VALLEY FEVER

Rift Valley fever virus is a phenuivirus that is transmitted by mosquitoes. Unlike many viral diseases of mosquitoes, taking an infected blood meal to become infected is not the sole route of infection in mosquitoes. The Rift Valley fever virus is transmitted by transovarial transmission of virus from the mother to the embryos in the eggs. Virus replication in livestock, mostly goats and cattle, results in high case-fatality rates and spontaneous abortion. See Figures 19-9 A and B. The livestock act as **amplifying hosts** that transmit virus to other mosquitoes. **Spillover** infection of humans usually occurs after the appearance of spontaneous abortions and disease in livestock (Figures 19-10A & B). This primarily causes a self-limiting febrile illness but is also associated with severe disease including hemorrhagic fever, neurological disorders, blindness, and **thrombosis** (blood clots) leading to death. The disease is found primarily in West and Sub-Saharan Africa as well as Madagascar. Symptoms begin 2-6 days after being bitten by an infected mosquito with a self-limiting febrile illness that lasts 3-4 days. This is followed by a temporary recovery that lasts only 1-3 days and then return of the fever, headaches, body aches, etc. that lasts 1-10 days, at which point the fever ends. The convalescent phase lasts 2 or more weeks with abdominal discomfort, weakness, and headaches. During the convalescent phase, however, some people develop vision loss which can result in complete or partial blindness, while others develop blood clots that can result in death. Note that during the initial febrile phase some patients develop **hemorrhagic fever** which can include **macular rash** (flat red with bump in center), bleeding, and **disseminated intravascular coagulation** (DIC) which can lead to death. The virus replicates in many tissues including lymph node, spleen, lung, kidney, and liver, and replication is cytopathic.

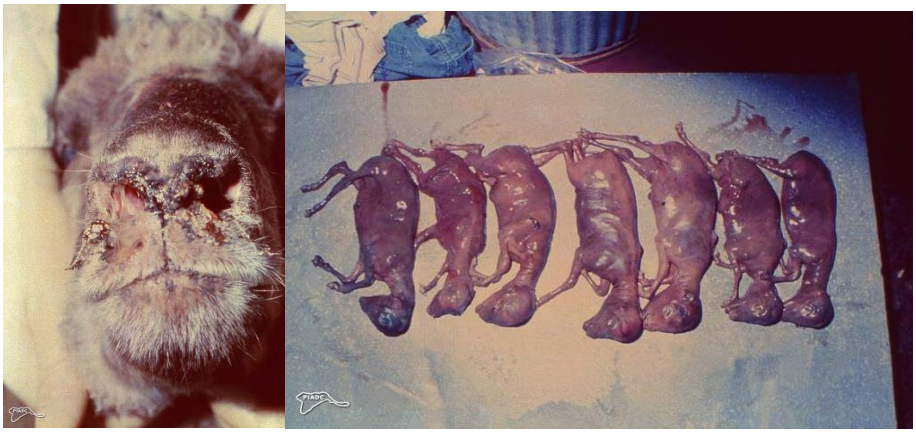


Figure 19-10A. An adult goat with Rift Valley fever. B. Stillborn goat fetuses due to maternal infection. Courtesy of https://phil.cdc.gov/Details.aspx?pid=12950 & https://phil.cdc.gov/Details.aspx?pid=12951.

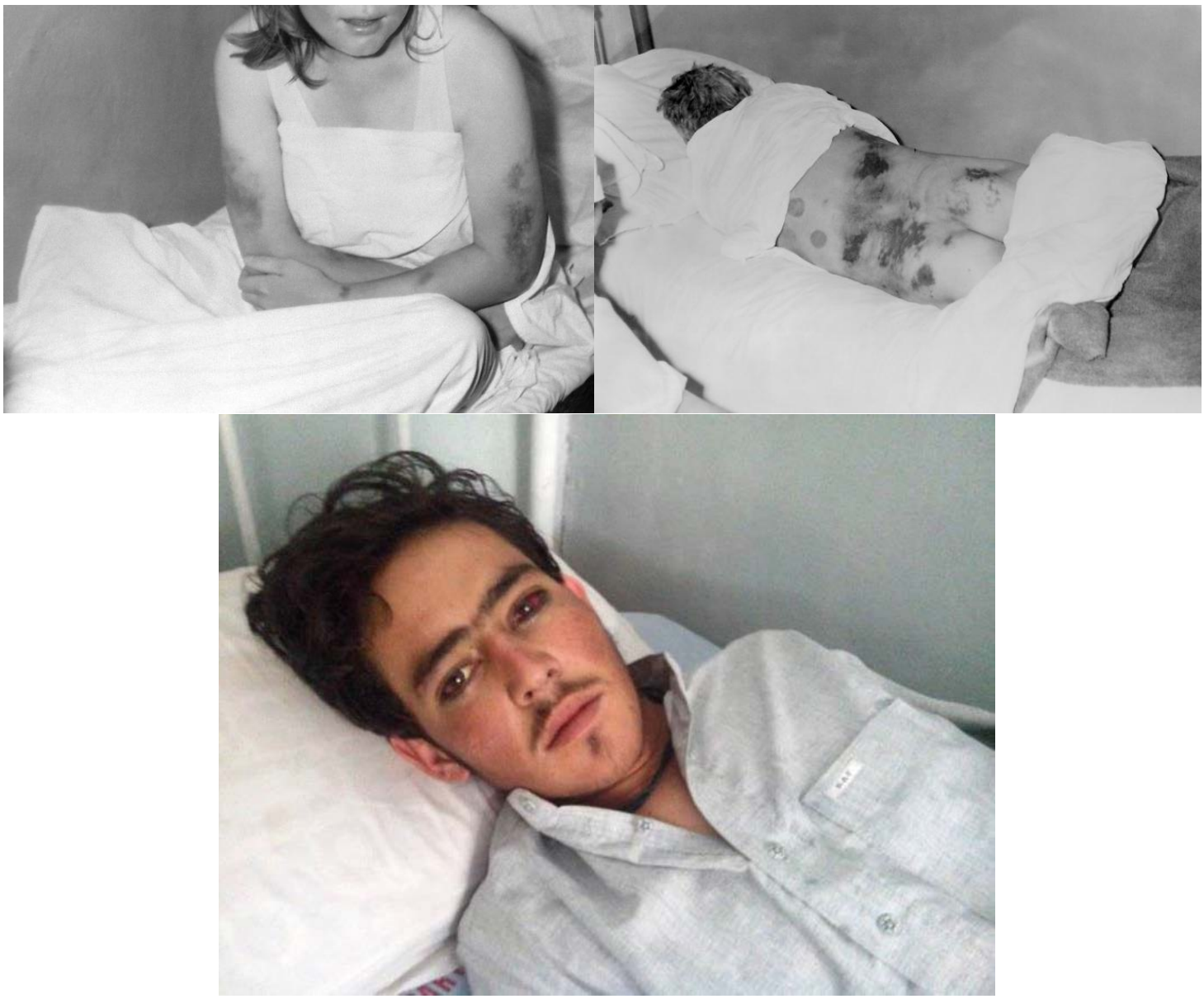
THE HEARTLAND VIRUS

The heartland virus, (genus *Phlebovirus*) is found in Missouri which is in the heartland of the USA. It causes a febrile (fever) illness with **leukopenia** and **thrombocytopenia** (decreased white blood cells and platelets). It is transmitted by ticks and can be fatal.

TICK BORN *NAIROVIRIDAE* VIRUSES

CRIMEAN-CONGO HEMORRHAGIC FEVER

Crimean-Congo hemorrhagic fever virus is transmitted by ticks and causes severe hemorrhagic fever with high case-fatality rates (up to 40%) in regions of Asia, Europe, and Africa. Figures 19-11 A-C.



Figures 19-11 A-C. Images of Crimean-Congo hemorrhagic fever patients. Courtesy of https://phil.cdc.gov/Details.aspx?pid=2317, https://phil.cdc.gov/Details.aspx?pid=2315, https://phil.cdc.gov/Details.aspx?pid=19900

RODENT BORN *ARENAVIRIDAE* VIRUSES

Arenaviral diseases are generally rodent-transmitted diseases. Each virus is usually associated with one rodent species or a few closely related rodents. The natural reservoir is the rodents that are chronically infected with virus. The viruses do not appear to cause disease in the rodents (**asymptomatic** infections), but virus is shed into the environment mostly through urine and feces. Human infection occurs when they have contact with excretions, ingest contaminated food, have direct contact with abraded skin, or through aerosols that are inhaled.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS

The natural rodent host and reservoir for lymphocytic choriomeningitis virus (LCMV) is the house mouse (*Mus musculus, Mus domesticus*). LCMV is maintained in the reservoir rodents, mice and hamsters primarily by vertical transmission from mother to fetus in the uterus, producing asymptomatic infections in the offspring who can then transmit the virus to humans or uninfected offspring as the virus is shed in nasal secretions, milk, semen, urine, and feces. Other rodents such as hamsters can be infected. Mice on all continents except Antarctica have been found to carry LCMV. Human infection occurs primarily from contact with excretions (saliva, nasal secretions, urine, feces) of house mice or from receiving an organ transplant from someone with an inapparent LCMV infection. Serological evidence of LCMV infection is found in approximately 5% of adults in the US. In immunocompetent individuals, it generally causes an asymptomatic infection or mild self-limiting illness, but a minority of patients can progress to **meningitis** (swelling of the meninges that line the nervous system). Infection has been documented in transplant patients who died of multisystem organ failure. Also, infection in first trimester of pregnancy has been associated with fetal death. Congenital infection is likely under- or misdiagnosed and is associated with **retinal** and **neurotropism** causing permanent and severe nerve dysfunction. The clinical manifestations depend on the developmental stage of the fetus at time of infection but can result in the following issues: vision impairment due to **chorioretinal scarring**, learning disabilities and ataxia due to **cerebellar hypoplasia** (small cerebellum), or severe mental deficiencies, **spastic quadriparesis** (all 4 limbs are stiff and difficult to move), or **epilepsy** (seizures) due to **microencephaly** (small brain frontal cortex) with **periventricular calcification** (calcification of the ventricles of the brain).

HEMORRHAGIC FEVERS OF ARENAVIRUSES

Quite a few arenaviruses cause hemorrhagic diseases including Lassa fever, Lujo hemorrhagic fever, Argentine hemorrhagic fever, Bolivian hemorrhagic fever, Venezuelan hemorrhagic fever, Brazilian hemorrhagic fever, and Machupo. All of these are transmitted by contact with rodent excretions. Lassa fever virus and Machupo virus have been associated with secondary human-to-human transmission.

LASSA FEVER

Lassa fever virus (LASV) is carried by the host species multimammate mouse *(Mastomys natalensis)* and is endemic in West Africa with estimates of up to 500,000 annual human infections and

approximately 5,000 deaths per year. It is estimated that 20% of infected people have severe disease which can have complications and lead to deafness.

THE NEW WORLD HEMORRHAGIC FEVERS

The New World hemorrhagic fevers (those found in North, Central, and South America) have similar clinical presentations which include early non-specific signs including headache, chills, **myalgia** (body aches), anorexia, and moderate fever (38-39^{O}C). They then develop more progressive disease symptoms including nausea and or vomiting, **retro-orbital pain** (eye pain), **petechiae** (flat red rash), as shown in Figures 19-12, and seizures. This can progress to terminal hemorrhagic shock syndrome including multiorgan failure with hemorrhage and extensive **necrosis** (tissue death). The liver and lymphoid organs are particularly targeted, and the lungs can develop **interstitial pneumonitis** and hemorrhage. These infections generally have 15-30% case-fatality rate. Furthermore, late neurological syndromes can occur in about 10% of survivors.

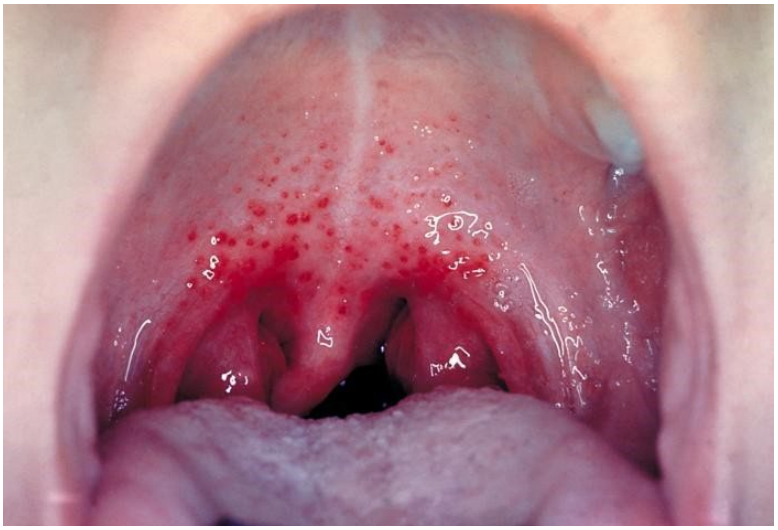


Figure 19-12. Petechiae, small flat blood spot rash on the back of upper pallet as is seen in hemorrhagic fevers. Courtesy of https://phil.cdc.gov/Details.aspx?pid=3184

DIAGNOSIS OF *BUNYAVIRALES* INFECTIONS

Diagnosis of these diseases is generally by viral isolation or is based on serological assays, such as ELISA, showing a 4-fold or higher increase in neutralizing IgG antibody titer as well as the presence of IgM.

PREVENTION OF *BUNYAVIRALES* INFECTIONS

For all of the arenaviruses and hantaviruses the best protection is to use precaution in areas that have been infested with rodents, keeping food well contained, and trapping and carefully removing mice. Note: live traps are not recommended. Spraying areas with visible rodent excrement with bleach and allowing it to sit for 10-20 minutes and cleaning to minimize aerosolization of excrement are advised. Note that vacuum cleaners lead to a tremendous amount of aerosol and are not a safe way of removing excrement unless it has been properly treated with bleach.

For the viruses in the order *Bunyavirales* other than hantavirus, the best protection is protecting one's self from mosquito bites by avoiding being outdoors at dusk and dawn unless wearing protective clothing, using DEET on unprotected skin, permethrin applied to clothing, and sleeping under a permethrin coated bed net. For the tick born virus the best protection is avoiding tick bites by wearing protective clothing, using DEET on unprotected skin, permethrin applied to clothing, and daily tick checks. Ticks should be removed using tweezers to grab the head (not the body) close to the skin and pulling out in one quick movement to remove the head and mouth parts along with the body. Matches, Vaseline, and other folk remedies may allow the tick to spit out large amounts of virus and lead to disease.

TREATMENT OF *BUNYAVIRALES* INFECTIONS

Unfortunately, there are no antiviral drug treatments or vaccines for any of the hantavirus, nairoviruses or phenuivirus diseases. Treatment is primarily supportive care, oxygen therapy, and IV fluids to maintain proper electrolyte balance and help to get patients past the symptoms and into the convalescent phase if more severe symptoms develop. The earlier the treatments are started the better.

For the arenaviral hemorrhagic fever infections, Ribavirin, a nucleoside inhibitor of RdRp, has been shown to be effective for some of the viruses. It must be administered intravenously early in disease course to be effective, but, because the disease begins with flu like symptoms, people often do not receive medical care in time for these treatments to be effective. The side effects include headache, and **myalgia** (body aches) that lead to mood swings, nightmares, and insomnia. Transfusion of convalescent plasma (serum from a recovered previously infected patient) has also been used, however this comes with the risk for transfusion-borne disease.

Only one vaccine has been developed against the arenaviruses. It is a live attenuated vaccine against Argentine hemorrhagic fever (AHF). This vaccine is not FDA approved in the USA but is used in areas with high AHF levels outside the US. Prior to introduction of the vaccine, annual AHF cases were routinely between 300 and 1,000 per year. The vaccine has a 95% efficacy reported against development of hemorrhagic fever. High-risk populations (agricultural workers) are now routinely vaccinated in Argentina.

END OF CHAPTER QUESTIONS

1. Why do you think these viruses were grouped together in this chapter?
2. Compare and contrast how negative sense RNA viruses leave cells to how positive sense RNA viruses leave cells. Do you notice any trends? If so, what are they?
3. Compare and contrast how negative sense RNA viruses leave cells to how enveloped DNA viruses leave cells. Do you notice any trends? If so, what are they?
4. Compare and contrast how RNA viruses that must carry RdRP into the host cell produce multiple proteins in eukaryotic cells that only want to produce one protein per mRNA. What trends do you see?
5. How do these trends in mechanisms for producing multiple proteins compare to the trends in positive sense RNA viruses that do not utilize reverse transcriptase?
6. How do most RNA viruses initiate genome replication and transcription? What viral families are outliers?
7. Compare and contrast filoviruses and arenaviruses.
8. What do arenaviruses have in common with other negative sense RNA viruses?
9. What does it mean to be ambisense?
10. Why is it advantageous for phenuiviruses and arenaviruses to have ambisense segments?
11. Compare and contrast RNA viruses that contain ambisense segments to other negative sense segmented RNA viruses.
12. How do viruses in the order *Bunyavirales* initiate translation if they lack a 3’ poly A tail? What trends do you notice when observing segmented viruses?
13. What PRR are most likely to detect infections with viruses in the order Bunyavirales? How do they avoid them?
14. You isolate a new RNA viral disease virus that contains ambisense segments that is transmitted to humans by the urine of rodents. In what virus family is this virus MOST LIKELY in? Justify your answer. Are there any other families this virus might be in? If so, which ones?
15. If the virus was just an RNA virus, what other virus families might the virus described in question 14 belong to?
16. If the virus was just an DNA virus, what other virus families might the virus described in question 14 belong to?
17. You isolate a virus that is transmitted by arthropods (flies, ticks, or mosquitoes). What DNA viruses and dsRNA, positive sense RNA, or negative sense RNA viruses can be transmitted by arthropods? Do you notice any trends?
18. Compare how viruses in the order *Bunyavirales* obtain a 5’ cap to how the viruses discussed in Chapters 14-17 obtain their 5' cap.
19. Compare how orthomyxoviruses obtain a 5’ cap to how viruses in the order *Bunyavirales* obtain their 5' cap.

20. Why, when testing for viruses in the order *Bunyavirales* infections, do they test for both IgG and IgM at the same time? What is the advantage of this technique over testing for just IgG?
21. Can these virus families create more than one ORF from an mRNA? If so, explain how.
22. Why can you run a diagnostic test with just an acute sample for IgM but need both an acute and convalescent sample for IgG?
23. Compare and contrast New and Old-World arenaviruses.
24. What causes the pathology of hantavirus disease?
25. What is the extrinsic incubation period of Rift Valley fever?
26. What demographic of people are most at risk from adverse response to lymphocytic choriomeningitis infections?
27. What are the best ways to protect yourself against most infections with viruses in the family *Phenuiviridae?*
28. What are the best ways to protect yourself against most arenavirus infections?
29. Are there any drug treatments for arenavirus, hantavirus, or phenuivirus diseases? If so, how effective are they?

REFERENCES:

Centers for Disease Control and Prevention. Public Health Image Library. Dr. B. Henderson. (1969). Crimean-Congo Hemorrhagic Fever patient 1. https://phil.cdc.gov/Details.aspx?pid=2317

Centers for Disease Control and Prevention. Public Health Image Library. Dr. B. Henderson. (1969). Crimean-Congo Hemorrhagic Fever patient 2. https://phil.cdc.gov/Details.aspx?pid=2315

Centers for Disease Control and Prevention. Public Health Image Library. (2013). Crimean-Congo Hemorrhagic Fever patient 3. https://phil.cdc.gov/Details.aspx?pid=19900

Centers for Disease Control and Prevention. Public Health Image Library. Deer Mouse. https://phil.cdc.gov/Details.aspx?pid=1138

Centers for Disease Control and Prevention. Public Health Image Library. C. Goldsmith. Hantavirus particles. https://phil.cdc.gov/Details.aspx?pid=12734

Centers for Disease Control and Prevention. Special Pathogens Branch DVRD/NCID/CDC Hantavirus Pulmonary Syndrome (HPS). R. Lynch. https://slideplayer.com/slide/8111234/

Centers for Disease Control and Prevention. Public Health Image Library. (1997). Hantavirus distribution. https://phil.cdc.gov/Details.aspx?pid=90

Centers for Disease Control and Prevention. Public Health Image Library. (1994). Hantavirus lung x-rays. Dr. L. Ketai. https://phil.cdc.gov/Details.aspx?pid=6076

Centers for Disease Control and Prevention. Public Health Image Library. (1958). Hemorrhagic Fever oral petechia. https://phil.cdc.gov/Details.aspx?pid=3184

Centers for Disease Control and Prevention. Public Health Image Library. (1982). Drs. J. Callis, B. Mahy. Rift Valley Fever goat. https://phil.cdc.gov/Details.aspx?pid=12950

Centers for Disease Control and Prevention. Public Health Image Library. (1982). Drs. J. Callis, B. Mahy. Rift Valley Fever aborted fetuses. https://phil.cdc.gov/Details.aspx?pid=12951

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 42, 43.

ViralZone. *Arenaviridae*. https://viralzone.expasy.org/501

ViralZone. *Bunyavirales.* replication. https://viralzone.expasy.org/6776

ViralZone. *Phenuiviridae.* https://viralzone.expasy.org/7101

REVIEWED BY:

This chapter was edited and reviewed by Dr Tony Schountz, Arboviral Infectious Disease Center Colorado State University

CHAPTER 20

Chapter 20: Prions

INTRODUCTION TO PRION BIOLOGY

This chapter will explore prions and their diseases. Prions are **proteinaceous infectious agents** that contain no nucleic acids and do not replicate per se. So, you might be wondering, "Why include this topic in a virology textbook or course?" The answer is that when scientists first began to study diseases caused by prions, they could find no viruses. It was assumed that the diseases were caused by as yet unclassified "slow viruses". As such, these prion hunters were housed in the virology section of most departments with these scientists, so it has been included as a topic in virology ever since.

Prions cause **protein misfolding disorders** (PMDs), and it is estimated that 36 million people worldwide suffer from one of these disorders which include diabetes, **Parkinson's disease**, **amyotrophic lateral sclerosis** (disease that the famous physicist Steven Hawking and the baseball player Lou Gehrig suffered from), **Huntington's disease, Alzheimer's**, **chronic brain injury** (as seen in war veterans), **chronic traumatic encephalopathy** (as seen in football players, boxers, and soccer players), and **transmissible spongiform encephalopathy** (famous examples include mad cow disease and chronic wasting disease). The last one is the one we will focus on as TSEs are caused by prions.

There are many examples of prion diseases in both animals and humans. The first one identified in animals was **scrapie** of sheep and goats, and then others such as **bovine spongiform encephalopathy** (BSE), otherwise known as mad cow disease, and chronic wasting disease which is seen in deer, elk, moose and reindeer. Unfortunately, cows that died of BSE were used to feed both large (lions, tigers, etc.) and domestic cats causing **feline spongiform encephalopathy**. Lastly, **transmissible mink encephalopathy** has also been identified. In humans there has long been known a disease that was seen in very low numbers in people over the age of 65 called sporadic **Creutzfeldt-Jakob Disease** (sCJD). There are also unfortunate families that carry the gene for **Fatal Familial Insomnia** (FFI) which contains a mutation in the prion gene PRNP and **Gerstmann Straussler Scheinker** (GGS), another fatal neurological disorder caused by mutations in the naturally occurring prions found in the nervous system that cause symptoms of neurological disease in a person's mid-thirties. Fortunately, these diseases are not naturally transmitted to other people. However, there are prion diseases that have been shown to be transmissible.

Of all the protein misfolding disorders, only CWD, scrapie, BSE, and vCJD have been found to be both **infectious and transmissible**. All the rest are **infectious but not transmissible**, meaning the disease, although it causes issues, are not known to be transmitted to others. They all have similar protracted **neurodegenerative** disorders that are caused by a similar mechanism. A

protein such as a prion misfolds, and as it contacts other prion proteins that are found in all tissues, it causes them to also misfold causing oligomers and fibers to form which eventually aggregate and create an amyloid fold as shown in Figures 20-1 A-C. Note although prion protein is found in all tissues, it is found at very different concentrations, and the highest expression of the prion proteins is in the central nervous system.

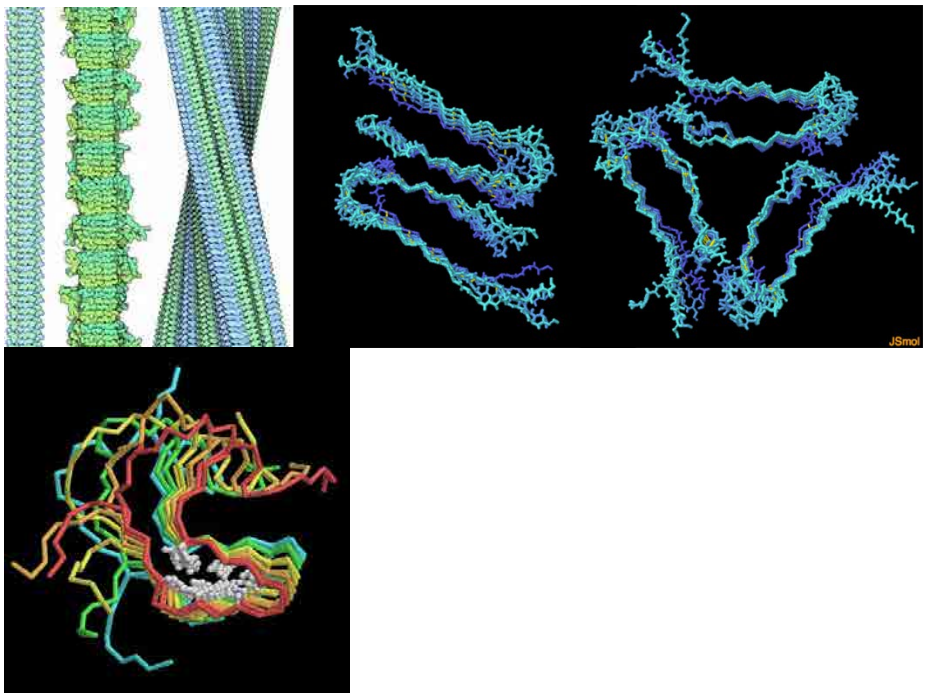


Figure 20-1 A-C. The fibrils (A) and amyloids (B, C) that form when the protein misfolds resulting in an increase in β sheets. Courtesy of the Protein Data Base-101 https://pdb101.rcsb.org/motm/189. D. Goosell.

This same mechanism is seen in all of the PMDs, however these result from the misfolding of other proteins such as **β amyloid** (chronic traumatic encphalopathy and **Tau** (both of which are involved in Alzheimer's, and CTE is associated with Tau), **α synuclein** (Parkinson's disease), or **transthyretin** (Transthyretin amyloidosis) and subsequent amyloid formation. Again, these proteins are found in all tissues. What is not quite clear yet in most of these diseases (with the exception of the genetically inherited mutated genes) is how the conversion from normal to misfolded occurs. What we do know is that the conversion does not require nucleic acids.

Expression of normal prion proteins (**PrP^C**) is found in neurons (brain/CNS) as well as ALL cells of the body. The physiological function is still unknown, but hypotheses include involvement in cell signaling, synaptic function, or response to oxidative stress. What is clear is that they are essential as all animals have them. However, when PrP^C is knocked out in mice (by deleting the *PRNP* gene), the mice develop normally. The pathological function is clear: they are required to generate TSEs, and **protease K resistant** forms correlate with disease.

The normal form is **α helical rich**, **soluble** (meaning it does not precipitate out of solution), **proteinase K sensitive** (it can be digested), and does not form **aggregates**. The mutated form,

however, is **β sheet rich**, **insoluble**, partially **proteinase K resistant,** forms aggregates, and is a biological marker of disease. It is important to note that not all mutations will lead to the formation of β sheet rich prion proteins, and that the β sheet rich forms can occur without the presence of mutations. As such, it is the β sheet rich conversion that causes aggregates more so than mutations.

You should now see that prions are proteins that can adopt two different forms, a normal form heavy in α helices and a misfolded form that is heavy in β pleated sheets. This may not seem unusual since many proteins are flexible and adopt different shapes. Remember, however, that prions have another unusual characteristic: the misfolded form of the prion can force normal prions to change into the misfolded shape. In this way a few misfolded prions can corrupt a whole population of normal prions converting them one-by-one into the misfolded β sheet heavy shape. This conversion process is referred to as **templating**. This can have deadly consequences as the levels of misfolded proteins build up, however it takes many years for this process to develop to the point of being symptomatic, so these diseases take many years to develop. However, we know that in animal prion diseases, asymptomatic animals appear to be able to transmit the disease to other animals before showing signs of disease. We also know that people who have died of inapparent prion diseases have transmitted the disease to others via organ transplants and blood transfusions. Furthermore, disease can occur **sporadically** due to mutations that occur in a person's lifetime and can be acquired by eating contaminated animals or through inheritance (**familial**). But what is not clear, and may never be, is if the disease originated by a sporadic mutation, through familial inheritance, or via infection.

The normal form of the prion protein PrP is found on the surface of nerve cells. When it changes into its misfolded form, it aggregates into long fibrils that clog up the normal functioning of the brain. Furthermore, this is also true for the other protein misfolding diseases.

What is unique to the prion diseases is the spongiform encephalopathy and neurodegeneration that results. **Spongiform encephalopathy** means the brain becomes spongy due to many areas of tissue death caused by the amyloid deposits, Figures 20-2 and 20-3.

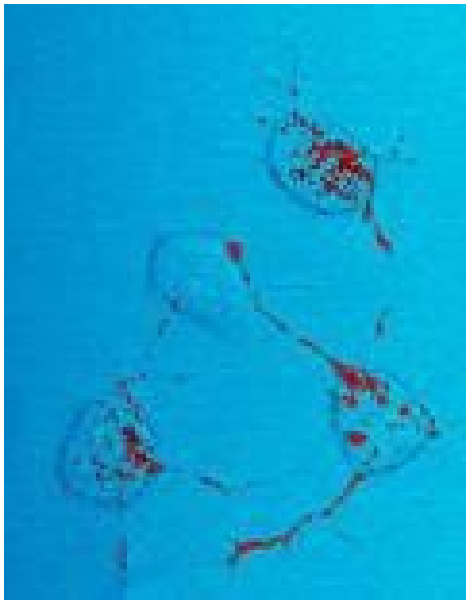


Figure 20-2. Histological staining of mice that have been infected with the PrP^{SC} scrapie prion. Accumulation of the protein aggregates can be seen as they stain red. Courtesy of https://phil.cdc.gov/Details.aspx?pid=18131 image by National Institute of Allergy and Infectious Disease (NIAID).

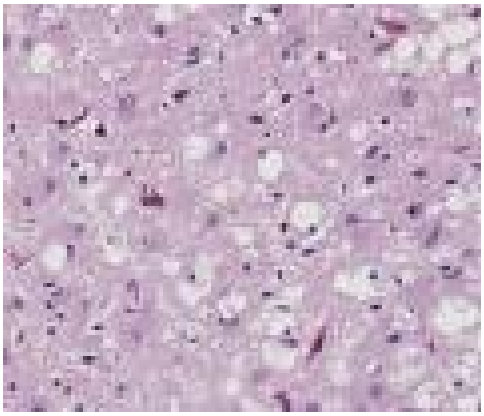


Figure 20-3. Stained section of brain from a patient who died of variant Creutzfeldt-Jakob disease. Note the white areas are foci of cell death giving the brain a "spongiform" appearance. Courtesy of https://phil.cdc.gov/ image by Sherif Zaki; MD, PhD; Wun-Ju Shieh; MD, PhD, MPH.

It is beneficial to review the history of prion diseases. As early as the 1700s a disease of sheep and goats called **Trotters disease** (now called s**crapie**) was described in Europe. In 1930 this disease was classified as a slow virus. In 1947 it was imported to the USA in sheep from Scotland. It is now an endemic disease of sheep and goats in the USA and is found worldwide (except in Australia and New Zealand) under many different names. In the 1950s Drs. Carlton Gajdusek and Michael Alpers traveled to Papua New Guinea to study a disease that had been documented in the Fore Tribe, an isolated tribe that carried out cannibalistic funeral rituals meant to keep the spirit of their loved ones alive after death. The epidemic probably started when one person developed the disease spontaneously. The misfolded prions then spread through the community when the infected person was eaten. In the 1960s a veterinary pathologist named Dr. Bill Hadlow characterized the spongiform encephalopathy of scrapie after creating animal bioassays in mice and sheep. Dr. Tikvah Alper determined that the infectious agent of scrapie was not sensitive to UV inactivation suggesting that it did not contain any nucleic acids and was probably therefore not a virus. In 1967 Drs. Gajdusek and Alpers demonstrated that if they took brain tissue of people who died of Kuru and injected it into primates' brains, they developed the same disease. Dr.

Gajdusek received the Nobel prize for this work in 1976. This led to a greatly reduced Kuru disease incidence when he was able to convince the Fore people to stop cannibalism. Unfortunately, Kuru has a 50-year incubation period, so it took until very recently for the disease to completely disappear. Dr. Stan Prusiner worked on CJD and developed the Prion = **pr**oteinaceous **i**nfectious **on**ly particle lacking nucleic acid theory in the 1980s in which posttranslational conversion of PrP^{c} α-helix structures to predominately β sheet PrP^{res} structures caused disease. In the 1990s PrP^{c} knockout mice were developed by Dr. Bueler by deleting the PRNP gene that codes for PrP^{c} and showing that they developed normally and were resistant to infection with PrP^{res}.

PRION DISEASES

BSE MAD COW DISEASE AND NEW VARIANT CREUTZFELDT-JAKOB

In 1986 the UK first described mad cow disease (BSE). It is thought to have been transmitted to cattle that were fed feed containing offal (internal organs) from scrapie infected sheep or cattle that had died of BSE. This offal was rendered improperly and added to cattle feed as a source of protein. The cattle developed neurological symptoms, **ataxia** (inability to walk), aggression, weight loss, and then died. Animals suffering from mad cow disease are shown in Figures 20-4 A & B. This resulted in the slaughter of more than 4.4 million cows in the UK during the epidemic. Even with these measures, approximately 450,000 BSE-infected cows entered the human food chain, peaking in 1987 when the disease was recognized as an epidemic. Cases continued until the early 2000s. Human variant CJD (vCJD) cases first developed in the mid-1990s, peaked around 2002, and then slowly declined as infected cattle were removed from the food supply and new food safety regulations went into effect. However, there are concerns that the disease may reemerge due to people with subclinical disease serving as blood and organ donors. This will be discussed later in the chapter.

Figure 20-4 A& B. Cattle demonstrating mad cow disease. Courtesy of https://phil.cdc.gov/Details.aspx?pid=5438 & https://phil.cdc.gov/Details.aspx?pid=5437 images provided by U.S. Dept. of Agriculture – Animal and Plant Health Inspection Service, APHIS, Dr. Art Davis.

CJD in the sporadic form has long been observed, so what made scientists think people were getting CJD from eating cows with BSE? Sporadic CJD was seen in 1-2 in 1 million people over the age of 60 worldwide. Variant CJD by comparison began in 1995 and was only observed in the UK in people under the age of 40. Was this **causation** or **correlation**? Just because two things happen at the same time does not mean one CAUSED the other. It may just mean there is a correlation between the two. To determine whether BSE was in fact the cause of vCJD, scientists turned to molecular biology.

Prion proteins are glycosylated. It turns out the glycosylation patterns of prion proteins are different between sporadic CJD with more than one type, and this was different than **iatrogenic** CJD (that is observed when people contract the disease medically), and this was different still from the pattern observed in people with vCJD. When the pattern of vCJD was compared to the pattern in BSE-infected cows using Western blots, these patterns matched, as shown in Figure 20-5.

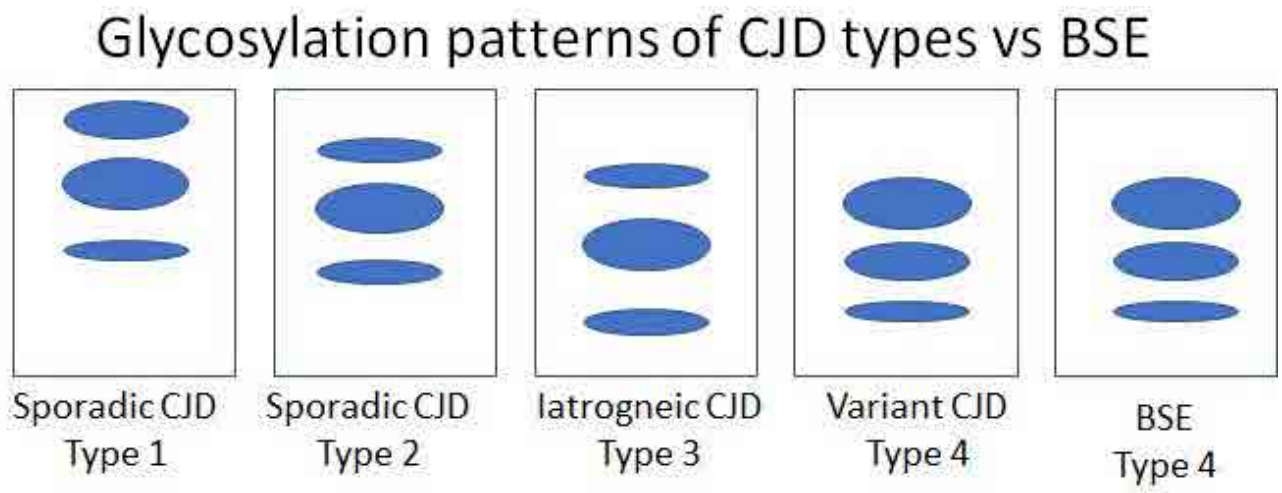


Figure 20-5. Glycosylation patterns of PrP comparison between sporadic CJD (sCJD), iatrogenic CJD (iCJD contracted from a medical procedure) variant CJD (vCJD), and BSE. It was found that the pattern of vCJD matches BSE but not the other forms of human CJD. Courtesy of Dr. Candace Mathiason, Colorado State University Prion Center adapted by Dr. Erica Suchman.

It should be noted however, that these changes are very subtle and Western blots are not sufficient evidence to prove this is the pattern of transmission and as such animal bioassays are also run, but Western blots can be used as a piece of evidence, but not the sole evidence. Extensive transgenic mouse bioassay studies also provided even more rubust evidence.

As mentioned above there is concern about subclinical carriers of vCJD, therefore scientists have been analyzing people in the UK to determine the likelihood that transmission might still occur. In a 2009 retrospective study analyzing frozen appendix and tonsil samples, 1 in 10,000 tonsils analyzed were found to be carriers and estimates in 2013 were that 1 in 2000 people in the UK may be subclinical carriers. Assays repeated in 2016 found the same results. This data indicates

that subclinical infection is not cleared and probably remains for life. We currently have documented **iatrogenic transmission** (from a medical procedure) and transmission by blood transfusions. We do not know yet if it is transmitted in saliva or from mother to child. Epidemiological studies predict that the initial primary infections should be ending soon, but, with a subclinical carrier rate of 1 in 2000, secondary cases contracted from medical procedures and blood transfusions will begin in the 2020s and may last well into the 2080s. Only time will tell if that will become an issue. Luckily for the author, when she attended school at Cambridge in England in 1984, she was a strict vegetarian.

BSE MAD COW DISEASE AND SPONGIFORM ENCEPHALOPATHIES IN OTHER ANIMALS

Unfortunately, the mad cow issues did not stop with human transmission. Cows that died of BSE were often quartered and fed to large cats at zoos (lions, tigers etc.) or turned into cat food for domestic pets. In the late 1990s, approximately 100 cases of feline spongiform encephalopathy (FSE) developed in the UK, Italy, France, and Spain in both domestic and non-domestic felines. To determine if BSE was in fact the cause, the glycosylation patterns of PrP were again analyzed, and, once again, the glycosylation patterns of the PrP proteins from afflicted felines matched BSE, as shown in Figure 20-6.

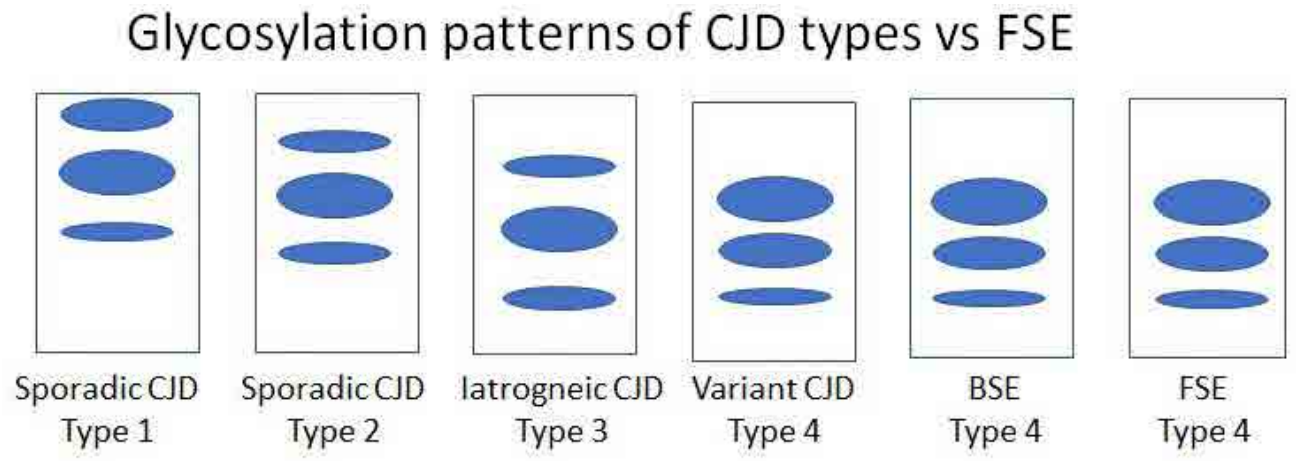


Figure 20-6. Glycosylation patterns of PrP comparison between sporadic CJD (sCJD), iatrogenic CJD (iCJD, contracted from a medical procedure), variant CJD (vCJD), BSE, and FSE (cats). It was found that the pattern of FSE matched BSE but not the other forms of human CJD. Courtesy of Dr. Candace Mathiason, Colorado State University Prion Center adapted by Dr. Erica Suchman.

All of this new knowledge led scientists to revisit an old disease of mink which had similar neurological disorders and had been documented since 1965 in farmed mink in Wisconsin. Sure enough, PrP that was resistant to proteinase K was found. When the glycosylation patterns of PrP from mink suffering from what they now termed **Transmissible Mink Encephalopathy** (TME) were compared to other forms of TSEs, it was again the same as the PrP from cows with BSE, as shown in Figure 20-7. The data is not shown here, however the

glycosylation pattern also did not match scrapie PrP. This is an enigma. How did mink in Wisconsin contract a disease of cattle that was not observed in this country? The original theory was that the mink had been fed carcasses of sheep that died of scrapie, but the glycosylation patterns showed that this was not true. The current theory is that mink were fed carcasses of old cows who contracted spontaneous BSE (much like spontaneous CJD is observed in humans).

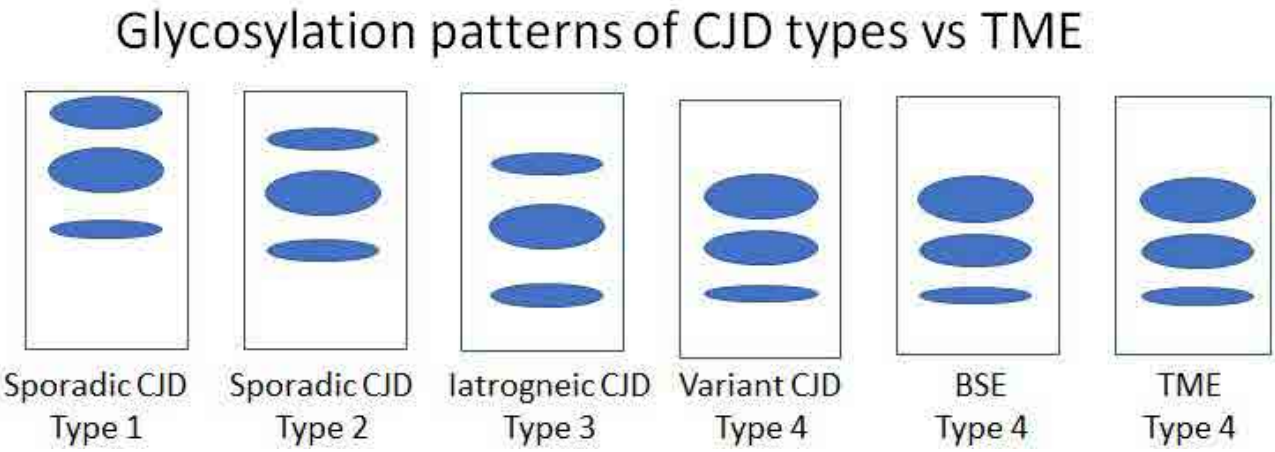


Figure 20-7. Glycosylation patterns of PrP comparison between sporadic CJD (sCJD), iatrogenic CJD (iCJD contracted from a medical procedure), variant CJD (vCJD), BSE, and TME (mink). It was found that the pattern of FSE matched BSE but not the other forms of human CJD. Courtesy of Dr. Candace Mathiason, Colorado State University Prion Center adapted by Dr. Erica Suchman.

CHRONIC WASTING DISEASE

We will end with an animal disease that is most prevalent in North America, particularly in the western USA, chronic wasting disease (CWD). It was first observed in the late 1960s in captive herds of deer at Colorado State University and then in wild populations in Colorado and Wyoming. It is now found in 26 states in the USA, 3 Canadian provinces, South Korea (2002), Norway (2016), Finland (2018) and Sweden (2019). It is the only TSE in a free-range population of animals. Up to 30-50% of free-range animals can be infected depending on the density of the herd, the higher the density the higher the infection rate. However, in captive populations, the infection rate can reach 100%. This demonstrates that the disease has a high transmission rate between animals. What is not yet known is whether CWD can be transmitted to humans, or if it can be transmitted to cattle as they often come into contact while grazing.

Gifted scientists, like the ones at the Colorado State University Prion Research Center and all over the world, are exploring these questions and hopefully one day we will have an answer.

PRION GENETICS

As we discussed earlier in the chapter, prion diseases are caused when prions mutate causing misfolding or when the host has contact with already misfolded prions that cause their PrP to misfold. The questions are, “Are all people equally susceptible to diseases from misfolded prions?” or, “Are there genotypes that are more likely to cause amyloid formation?” To analyze

these questions scientists again turned to molecular biology.

The genes that produce prion proteins were sequenced for many different prion diseases. When CJD PrP sequences were analyzed, interesting trends emerged: susceptibility to developing disease is controlled by codon 129. The genotype at codon 129 can be **methionine homozygous** (MM), **valine homozygous** (VV), or **heterozygous** (MV). People who are susceptible to vCJD infection are predominantly methionine homozygous 129 M/M with only one case of 129 M/V transmission via a blood transfusion documented. The same is found in people with spontaneous CJD. They are mostly 129 M/M as are all cases of iatrogenic CJD. However, familial CJD is caused by a point mutation at codon 200, and point mutations in other codons are also responsible for the other two prion diseases, FFI and GSS.

In animals the codons are different. For example, in scrapie, if the animals are homozygous R/R at codon 171, they do not develop the disease. But if they are homozygous Q/Q, they are susceptible, and all known cases analyzed to date have been Q/Q.

In BSE the same mutation is observed to be important as in fCJD where a point mutation at codon 200 makes an animal susceptible to developing the disease. In Germany, cattle with a 12 bp deletion of a regulatory region of PrP were also found to be susceptible. Furthermore, for CWD, deer have partial resistance if codon 96 is heterozygous G/S but are susceptible if they have either of the homozygous genotypes.

Thus, it appears that those who contracted the disease had the right combination of amino acids at specific codons to develop the disease.

PRION TRANSMISSION

The transmissions for each of the prion diseases discussed are slightly different, so we will elaborate on each individually. The prevalence is based solely on terminal identified cases which does not account for subclinical infections.

Scrapie is transmitted across the placenta, by blood, saliva, milk, and the environment and has a prevalence of 1-7%.

CWD is transmitted via saliva, blood, urine, feces, the environment, and vertically from mother to offspring and has a prevalence of 2-50% in free ranging herds and up to 100% in captive herds.

BSE, FSE, and TME are transmitted by eating contaminated meat and bone meal from either sheep or cattle. In 1993 BSE had a prevalence rate of 0.3%. These cattle, however, were culled.

vCJD is transmitted via consumption of BSE-infected cattle and blood transfusion. All forms of CJD combined only have a prevalence rate of 0.0001%.

Kuru was transmitted by ritualistic cannibalism and had a prevalence rate of 1-20% before the tribe stopped cannibalization.

iCJD (iatrogenic) is contracted by exposure to contaminated surgical instruments or tissue. These infections are lumped in with all CJD for a rate of 0.0001%.

Note: Recall that there are inherited forms of protein misfolding diseases such as FFI and GGS. They are very rare and not known to be transmitted by horizontal transmission from one host to another.

DIAGNOSING PRION DISEASES

Unfortunately, these diseases can only be currently diagnosed **postmortem** (after death). This is because the diagnosis requires sampling brain tissue. Diagnosis is currently achieved using histochemistry which involves staining tissues and looking for the spongiform vacuolization as shown in Figure 20-2. However, this process can only pick up the disease once it has highly progressed.

Immunohistochemistry using labeled antibodies can detect these diseases a bit earlier. See Figure 20-3. Western blots looking for proteinase K resistant PrP are also useful once enough of the resistant prions have developed. Bioassays in animals such as mice or primates, where potentially infectious prions are injected into animals to see if they develop disease, is time consuming and expensive and not a realistic diagnostic tool. Bioassay has, however, been helpful in research. Two quick ELISA assays are also currently available but again can only be utilized postmortem.

Researchers are working diligently on developing diagnostics that can detect the disease antemortem (before death) and when the disease is subclinical. A promising research tool that may eventually be diagnostic is the protein misfolding cyclic amplification (PMCA). This is analogous to PCR amplification of DNA only PMCA amplifies misfolded prions. The assay is conducted when normal brain PrP^{c} is added to a tube with a small amount of sample that potentially contains PrP^{res} and incubated allowing the PrP^{res} to convert the PrP^{c} to PrP^{res}. The tube is then sonicated to dissociate the aggregates and incubated again allowing the PrP^{res} to convert PrP^{c} to PrP^{res}. This sequence is repeated over and over until large amounts of aggregate have developed that can be identified by western blot analysis. If aggregates are formed the sample is positive for containing the misshapen form of prion protein capable of converting PrP^{c} to PrP^{res}.

A new version of this technique, real time quaking induced conversion (RT QuIC), has been developed recently. It is the equivalent to next generation PMCA. RT QuIC uses a recombinant protein (PrP^{c}) and adds sample that may contain PrP^{res} to induce the conversion which can be detected only when amyloids form and a thioflavin T fluorescent dye binds to the aggregates. When thioflavin T binds to β sheet rich structures such as amyloid fibrils, it displays enhanced fluorescence and a characteristic blue shift in the emission spectrum. Soluble proteins in folded, unfolded, or partially folded states do not enhance thioflavin T fluorescence. As such, aggregate formation can be tracked by monitoring the change in fluorescence over time. The goal is to be able to detect aggregates in urine or blood before clinical manifestations of the disease develop.

Hopefully these tests will evolve to a point where they can be used for early detection and diagnosis before death.

END OF CHAPTER QUESTIONS

1. Why, historically, are prions studied by virologists?
2. What are diseases thought to be related to prion diseases? How are they thought to be related?
3. What does it mean when we say that some protein misfolding disorders are infectious but not transmissible, and others are both infectious and transmissible? Give examples of each.
4. Compare and contrast chronic wasting disease and chronic traumatic encephalopathy.
5. Compare and contrast scrapie and feline spongiform encephalopathy.
6. Compare and contrast mad cow disease and Kuru.
7. Explain the pathology of TSEs.
8. Why does it take many years for TSEs to develop symptoms?
9. What are 3 ways people can develop TSE diseases?
10. What is spongiform encephalopathy?
11. What is the theory for how cattle developed Mad Cow Disease?
12. What is the difference between correlation and causation?
13. Explain how glycosylation patterns can be used to help identify the cause of prion disease.
14. Since feeding practices for cattle have changed, are we most likely done with seeing cases of vCJD? If not, why not?
15. What genotype makes people most susceptible to TSEs?
16. When can most TSEs be detected?
17. What is PMCA?
18. Compare and contrast PMCA and RT QuIC.
19. What criteria must a Western blot for TSEs satisfy in order to call a sample positive?

REFERENCES:

Bruce, M.E. et al. (1997). Transmission to mice indicate that 'new variant' CJD is caused by BSE agent. Nature. 389(6650):498-501. doi: 10.1038/39057 https://pubmed.ncbi.nlm.nih.gov/9333239/

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). NIAID. (2011). Scrapie brain staining. https://phil.cdc.gov/Details.aspx?pid=18131

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). S. Zaki; MD, PhD; W. Shieh; MD, PhD, MPH. (2004). https://phil.cdc.gov/details.aspx?pid=10130

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). U.S. Dept. of Agriculture – Animal and Plant Health Inspection. Dr. A. Davis. (2003). https://phil.cdc.gov/Details.aspx?pid=5438

Centers for Disease Control and Prevention. Public Health Image Library (PHIL). U.S. Dept. of Agriculture – Animal and Plant Health Inspection. Dr. A. Davis. (2003). https://phil.cdc.gov/Details.aspx?pid=5437

Fields Virology 6th edition David Knipe, Peter Howley, (2013). Lippincott, Williams & Wilkins, 530 Walnut Street, Philadelphia PA, 19106, Chapters 76.

Kobayashi, A., et al. (2016). Neuropathological and biochemical criteria to identify acquired Creutzfeldt-Jakob disease among presumed sporadic cases. *Neuropathology*, 36: 305– 310. doi: 10.1111/neup.12270.https://onlinelibrary.wiley.com/doi/full/10.1111/neup.12270

Mackenzie G., and Will, R. (2017). Creutzfeldt-Jakob disease: recent developments, F1000Res 6: 2053, doi: 10.12688/f1000research.12681.1, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5710312/

Malmos, K.G. et al. (2017) ThT 101: a primer on the use of thioflavin T to investigate amyloid formation, Amyloid, 24:1, 1-16. DOI: 10.1080/13506129.2017.1304905 https://www.tandfonline.com/doi/full/10.1080/13506129.2017.1304905

Mathiason, C. (2017). Chapter 12- Scrapie, CWD, and Transmissible Mink Encephalopathy. Progress in Molecular Biology and translational science. Volume 150. Pages 267-292 Elsevier. https://doi.org/10.1016/bs.pmbts.2017.07.009
For many articles on Prions see.
https://www.sciencedirect.com/science/article/abs/pii/S1877117317301199

Nonno R., et al. (2020). Studies in bank voles reveal strain differences between chronic wasting disease prions from Norway and North America. Proc Natl Acad Sci U S A. 117(49):31417-31426. doi: 10.1073/pnas.2013237117 https://pubmed.ncbi.nlm.nih.gov/33229531/

Protein Data Base-101. D. Goodsell (2015). https://pdb101.rcsb.org/motm/189 & doi:10.2210/rcsb_pdb/mom_2015_9

Race, R. E., et al. (2002). Comparison of abnormal prion protein glycoform patterns from transmissible spongiform encephalopathy agent-infected deer, elk, sheep, and cattle. *Journal of virology*, *76*(23), 12365–12368. https://doi.org/10.1128/jvi.76.23.12365-12368.2002 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC136873/

REVIEWED BY:

This chapter was edited and reviewed by Dr Candace Mathiason, Prion Research Center Colorado State University