DISSERTATION

DEVELOPMENT OF NOVEL ALPHAVIRUS EXPRESSION AND DETECTION SYSTEMS AND CHARACTERIZATION OF OXIDATION'S EFFECT ON VIRAL REPLICATION

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ABSTRACT

DEVELOPMENT OF NOVEL ALPHAVIRUS EXPRESSION AND DETECTION SYSTEMS AND CHARACTERIZATION OF OXIDATION'S EFFECT ON VIRAL REPLICATION

Infectious diseases cause significant global suffering and death each year. Specifically, arthropod-borne viruses are emerging and re-emerging around the world and infecting millions of people. Mosquitoes that transmit these viruses are spreading to new regions of the world with naïve populations to serve as viral hosts. Climate change and human encroachment of habitat has brought mosquitoes into close proximity with humans. The viruses are evolving and are expanding their vector compatibility to include more than one species of mosquitoes. The combination of these elements results in a serious global need to develop ways to control or prevent arthropod-borne viruses. In order to discover novel antivirals and ways to inhibit these arboviruses, a better understanding of viral infection and replication is needed. This dissertation will describe a combination of projects that all aim to provide enhanced knowledge or tools to prevent, control, or treat arbovirus infection. Specifically, we improved the ability to express recombinant infectious alphaviruses, developed a novel system to detect alphavirus infection in mosquito cell culture and transgenic mosquitoes, and discovered a new role for oxidation during flavivirus replication.

First, we successfully developed and established a method for transcribing infectious alphavirus RNA from a plasmid DNA platform. This approach provides an efficient way for producing high titer infectious recombinant alphavirus in multiple cell types that robustly express foreign proteins. Secondly, we optimized a system for detecting alphavirus infection in mosquito cells using the virus dependent subgenomic promoter to transcribe a reporter gene only during active infection. We demonstrated that mosquito cells can be stably transformed to transcribe an engineered viral reporter RNA that expresses a fluorescent reporter protein (mCherry) only in the presence of wild-type virus infection. The reporter protein is not detected in uninfected controls, but significant expression is readily detected during infection. Transgenic mosquitoes were also developed to transcribe the reporter RNA, which amplifies and expresses the reporter protein during infection. The transgenic mosquitoes are able to express a fluorescent reporter protein only during Sindbis virus (*Alphavirus*) infection, providing a novel mechanism to detect infection of wild-type virus in living mosquitoes. This transgenic reporter system is the first of its kind and demonstrated that a system based on our reporter RNAs could be optimized and used to specifically detect infected mosquitoes. Finally, I was able to study and characterize several aspects of viral RNA replication within the cell. Specifically, we identified that viral RNA replication is dependent on oxidative conditions. We determined individual residues from the flavivirus NS5 capping protein that are specifically involved in the oxidative enhancement of viral replication. Our work provided significant advances to the arbovirus field. We now have a novel method for producing recombinant alphaviruses that is more time, cost, and resource effective. We understand the ability of the

subgenomic promoter to act as a virus inducible promoter to express foreign proteins only during infection to help detect or manipulate infection in mosquitoes. Finally, we have made significant discoveries on how RNA replication works on a molecular level within the cell and better understand the important role of oxidation on virus infection. The work and discoveries described in this dissertation have enhanced multiple aspects of arbovirus research and will hopefully strengthen our ability to fight and control arbovirus infections around the world.

ACKNOWLEDGEMENTS

No one survives graduate school without a lot of help and support. I would like to start by thanking my advisor, Dr. Brian Geiss. He has been the best mentor I could have asked for. There have been many late nights and weekends, when I would not have said that, but his high expectations and commitment to research have inspired me to be a better scientist. He has been understanding and encouraging. He provided necessary guidance while allowing me freedom and flexibility, which has helped me develop and mature as an independent scientist. I would also like to thank the members of my committee for their guidance and suggestions along the way. Dr. Olson, Dr. Schenkel, and Dr. Callan have been pivotal in my research through critical conversations, advising the mosquito work at the arthropod-borne infectious disease laboratories, helping with microscopes, and providing an outside perspective to the work.

Everyone that has come through the Geiss Lab has been influential and helpful. I have met some of my best friends through this lab and I appreciate the friendships that have formed. I couldn't have survived the hard times without occasional pizza trips, cookies, or distractions. Thanks for all the laughs and good times.

Most of all, I would like to thank my family and friends - especially my wife. Natalie has been the biggest support. She has made what seemed impossible at times, not only manageable, but also enjoyable. My kids have been so patient and have made even the worst days seem good. I am thankful for them and their love and support. I am also thankful for my Heavenly Father and His hand in my life. I have so much to be thankful for and I appreciate everyone's help in accomplishing this life-long goal.

DEDICATION

I would like to dedicate this work to all those that have personally suffered from arbovirus infections or have lost friends or family members to the devastating viral diseases transmitted by mosquitoes, including many personal friends in the Philippines.

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Chapter One: Literature Review

Introduction/Impact

Arthropod-borne diseases are responsible for nearly 30% of the emerging infectious disease cases over the last decade (K. E. Jones et al. 2008). For example, dengue viruses alone cause an estimated 350 million infections annually resulting in significant suffering and death worldwide (Bhatt et al. 2013). Currently over 2.5 billion people are at risk of dengue infection, but the mosquitoes that transmit these viruses are spreading across the globe into new countries with naïve human populations and the risk will continue to increase (Porretta et al. 2012). There are few vaccines and limited treatment options available to help those infected with arboviruses. A better understanding of virus replication and enhanced tools to detect and study infection are desperately needed in order to find new ways to prevent or inhibit infection.

Arthropod-borne viruses

Arthropod-borne viruses have caused arthralgic and encephalitic diseases in humans and animals for several centuries. Ancient records describe seasonal fevers and aches that would occur during the summer, suggesting a disease transmitted by an arthropod vector (Scheidel 2009; Altizer et al. 2006; Alma et al. 1981). Chinese encyclopedias describe disease symptoms similar to dengue fever from 265-420 AD (Gubler 1998). Throughout the 1800's there were severe seasonal epidemics of dengue, yellow fever, and other unidentified arthralgic and encephalitic diseases reported throughout the world. In 1881, Carlos Finlay and Walter Reed were the first to

verify that disease transmission could occur through mosquitoes and in 1901, the first arbovirus, yellow fever virus, was isolated and characterized (Strode 1951). The first alphavirus, western equine encephalitis virus (WEEV), was isolated in 1930 (California) from the central nervous system of infected horses (Meyer et al. 1931). Shortly after, eastern equine encephalitis virus (EEEV) was found in brains of infected horses in the eastern United States(Broeck & Merrill 1933). In 1952, Sindbis virus (SINV) was extracted from mosquitoes near Sindbis, Egypt; and in 1953, the alphavirus Chikungunya was found in the blood of people infected with an arthralgic disease in Tanzania (RM et al. 1955; Ross 1956). Over the next several decades, additional arthropod-borne viruses were discovered and classified into three groups based on serological activity- A, B, and C(Porterfield 1986). Group A eventually became further characterized and identified as the alphaviruses (WEEV, EEEV, SINV, -currently about 28 known alphaviruses), group B were the flaviviruses (Dengue virus, Yellow Fever virus, St. Louis Encephalitis virus, Japanese Encephalitis virus, -currently 70-80 known flaviviruses), and group C were orthobunyaviruses and phleboviruses in the bunyaviridae family (La Crosse virus, Rift Valley Fever virus). The work described in this dissertation has focused on alpha- and flavi- viruses.

Alphaviruses

Alphaviruses are positive strand RNA viruses with an ~11 kb genome. Alphavirus genomic RNAs are capped on the 5' terminus and the 3'end has a poly-adenylated tail resembling cellular mRNA (J. Strauss & E. Strauss 1994). Alphaviruses are enveloped and approximately 60-70 nm in diameter with the glycoproteins E1 and E2 forming

heterodimers on the outside of the virion (Knight & Howley 2013). The nucleocapsid is composed of the viral capsid protein and the viral genomic RNA. Alphaviruses have a wide cell tropism and can infect a variety of different cells through the E2 glycoprotein binding cellular receptors (Hurlbut & Thomas 1960; Xiong et al. 1989). The wide cell tropism is likely due to E2 glycoprotein's ability to bind multiple receptors or to recognize ubiquitous cellular surface molecules. The receptor-bound virus is endocytosed into early endosomes. As the pH of the endosome becomes more acidic, a conformational change in the trimers of E1-E2 heterodimers occurs that exposes the fusion peptide on E1. The E1 fusion peptide inserts into the lipid bilayer and fuses the endosome membrane with the viral envelope, releasing the nucleocapsid into the cytoplasm. The nucleocapsid is disassembled and the viral RNA genome is released to begin the replication cycle (Figure 1.1).

The alphaviruses genomic RNA (49S- Svedberg unit) resembles cellular mRNA and is translated by the host's cellular translation machinery. The viral genome is organized so that the non-structural proteins (nsP1, nsP2, nsP3, nsP4) are located at the 5' end of the genome and the structural proteins (C, E3, E2, 6k, E1) are towards the 3' end of the genome (Figure 1.2). A non-structural polyprotein composed of nsP1-3 is translated upon entry into the host cytoplasm. There is an opal stop codon (UGA) after nsP3 that terminates translation, but low level (10-20%) read-through allows the nsP1-4 protein to be produced at low abundance (G. P. Li & Rice 1989). nsP2 contains a protease domain that cleaves nsP4 from the nsP1-4 polyprotein. The nsP4 RNA dependent RNA polymerase interacts with the polyprotein nsP1-3 to transcribe a full-length negative strand copy of the genome, using the positive strand as template. The

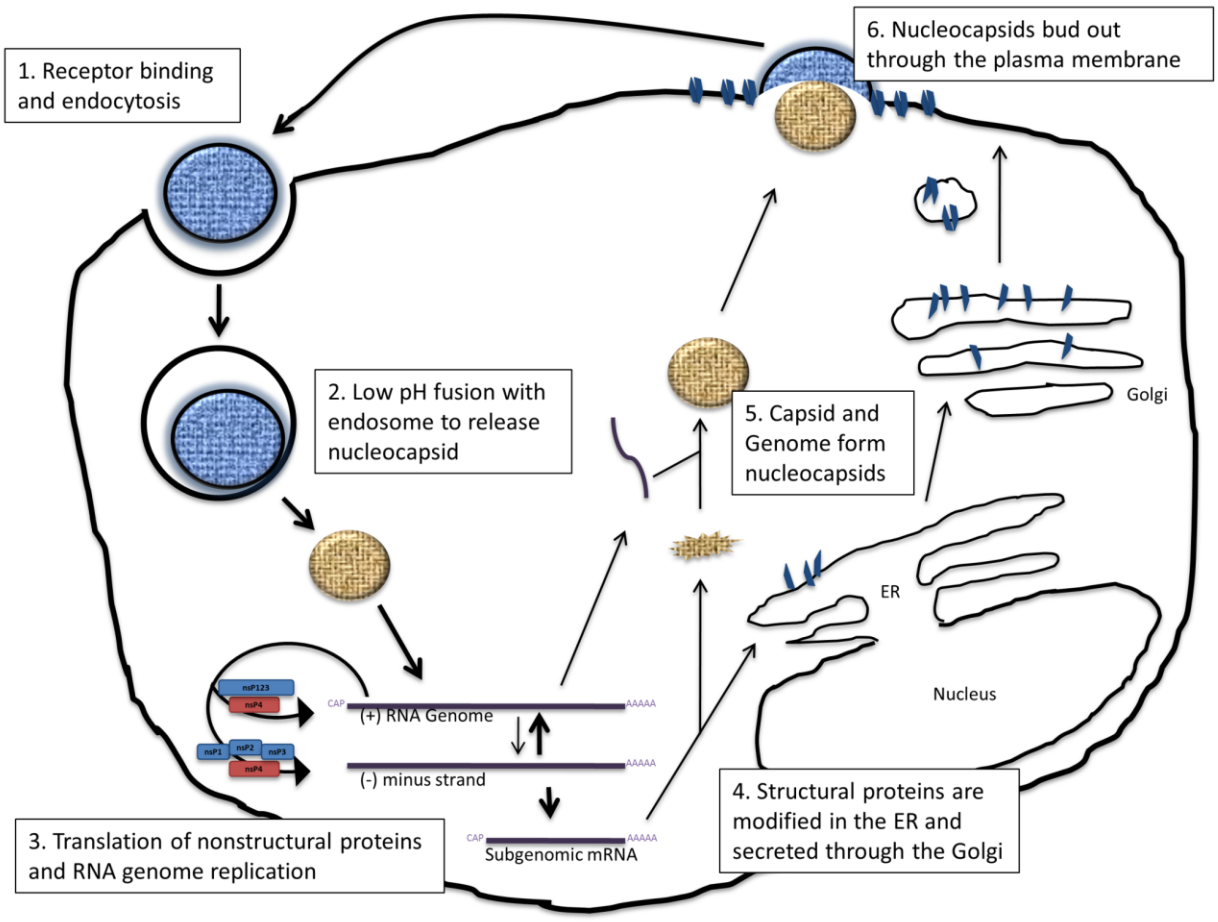

Figure 1.1 Alphavirus life cycle

1) Alphaviruses enter the cell through receptor-mediated endocytosis. **2)** The nucleocapsid is released from the endosome and the positive strand RNA genome enters the cytoplasm. **3)** The RNA is translated into the nonstructural protein replication complex, which transcribes a negative strand copy of the genome. From the negative strand copy, positive strand RNAs and a subgenomic RNA can be synthesized. **4)** The structural proteins are translated from the sgRNA, resulting in cytoplasmic capsid protein and the ER and golgi processing of the envelope glycoproteins. **5)** Capsid proteins bind the positive strand genome and forms nucleocapsids. **6)** The nucleocapsids bud through the plasma membrane, acquiring an envelope and the viral E1 and E2 glycoproteins.

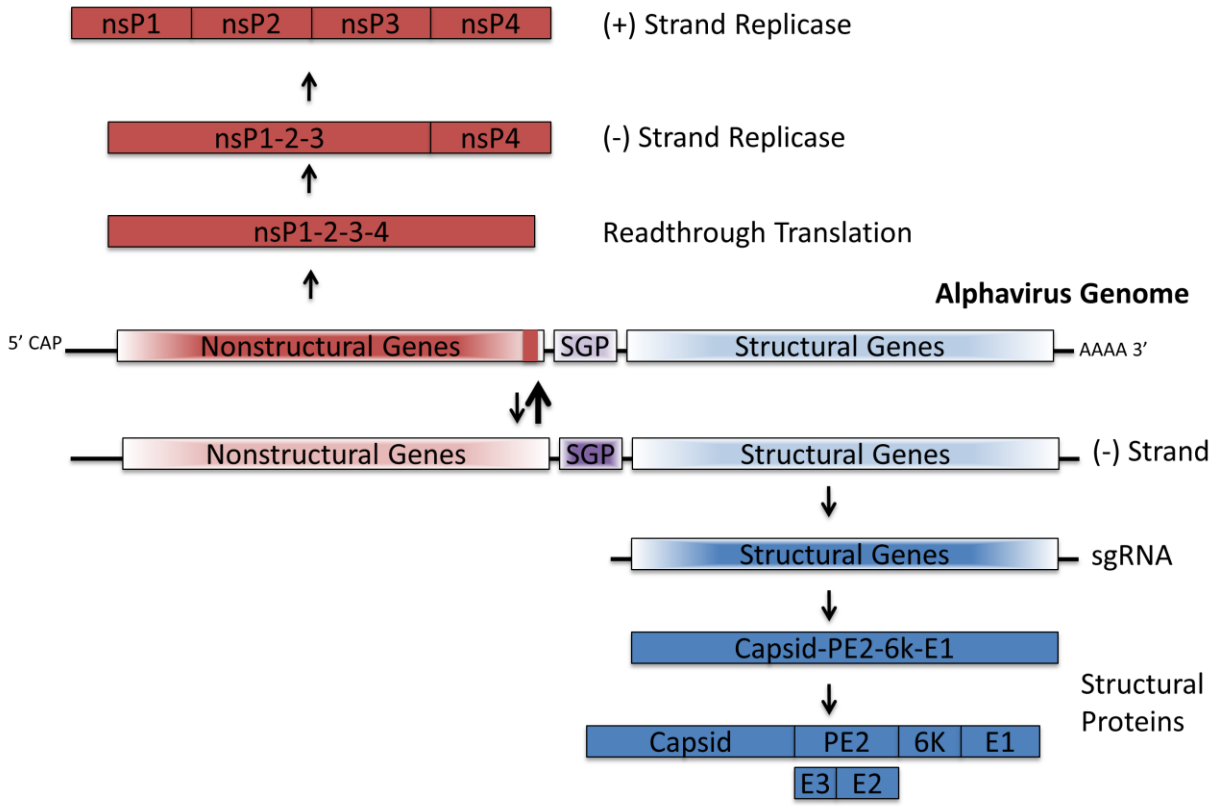

Figure 1.2 Alphavirus genome

The alphavirus positive strand RNA genome is 5' capped and 3' polyadenylated. The 5' end of the genome is translated to form the nonstructural polyprotein (nsP1-2-3-4). nsP4 is cleaved from the polyprotein, resulting in the negative strand replicase, which transcribes negative strand copies of the genome. The nsP1-2-3 is further cleaved to individual proteins, forming the positive strand replicase. The positive strand replicase binds the negative strand RNA and generates new positive strand RNA genomes and a subgenomic RNA (sgRNA) by binding the subgenomic promoter (SGP), which contains the structural proteins. The sgRNA is translated and the structural proteins are cleaved to form capsid, E3, E2, 6K, and E1.

polyprotein nsP1-3 is then further cleaved by nsP2 to produce the four individual non-structural proteins. The cleaved nonstructural proteins (nsP1-4) form a replicase complex that uses the negative strand RNA as a template to transcribe high levels of positive strand genomic RNA. Additionally, the replicase complex can bind to a subgenomic promoter located on the negative strand of the genome and transcribe a 26S subgenomic RNA that encodes the structural protein genes. Alphavirus RNA replication and transcription has been shown to occur at the plasma membrane in cytoplasmic vesicles (Frolova et al. 2010). Within these replication compartments, negative strand/positive strand replicative intermediates are protected and have a specialized environment for replicating RNA. Negative strand RNA is transcribed at only 2-5% of the level of positive strand full length genome, but the subgenomic RNA is transcribed in abundance, with 3x more 26S RNA than the full length 49S RNA (Raju & Huang 1991; Y. F. Wang et al. 1991). The subgenomic RNA is only transcribed when there is a negative strand copy of the genome and the nonstructural protein replicase complex is present to bind the negative strand RNA and transcribe the subgenomic RNA.

The subgenomic RNA is translated into the structural proteins (capsid, PE2 (E3/E2), 6K, E1) necessary for forming infectious virus particles. Capsid contains a protease domain that self-cleaves itself from the polypeptide and is released into the cytoplasm. PE2 (E3/E2), 6K, and E1 are transmembrane proteins that are translocated into the ER membrane, where they are post-translationally modified. 6K is cleaved from the polyprotein by signal peptidase and has been described as functioning in virion formation and budding. The E1 and PE2 (E3/E2) proteins begin to heterodimerize in the

ER membrane and then the structural proteins are transported to the trans-golgi network (Figure 1.1). The E3 protein that is still connected with E2 is believed to stabilize the E1 fusion proteins during transit from the ER and through the Golgi (Lobigs et al. 1990; Lobigs & Garoff 1990). Before the structural proteins arrive at the plasma membrane, PE2 (E3/E2) is cleaved by furin, releasing E3 and allowing the virion to fully mature into an infectious particle (J. Strauss & E. Strauss 1994). The structural proteins reach the plasma membrane and wait for interaction with the nucleocapsid to instigate virion budding. The free cytoplasmic capsid protein interacts with the viral genomic RNA on a specific packaging sequence, which has been identified as nucleotides 945-1076 in SINV (Linger et al. 2004). 240 capsid proteins surround one copy of the viral genome to form a nucleocapsid. The capsid proteins also interact with the cytoplasmic tail of the E2 glycoproteins at a 1:1 ratio. The E2 glycoproteins form heterodimers with E1, which dimers then form trimer spikes on the surface of the viral particle (Paredes et al. 1993; Cheng et al. 1995). The virus particle buds from the plasmid membrane, taking with it a host derived lipid bilayer that contains increased amounts of cholesterol and sphingolipids (Kielian et al. 2000). These newly budded virions can then find a new host cell to infect and continue the cycle.

The alphavirus life cycle has been analyzed for many years because alphaviruses represent significant global human health concerns. Additionally, alphaviruses have been studied because their small genomes are amenable to be used for generating recombinant viruses and viral vectors that express foreign proteins. Alphaviruses have a wide host cell tropism, making them great expression vectors for synthesizing foreign proteins in a wide variety of cells. In order to generate recombinant

viruses, infectious clone cDNA technology was developed to manipulate the RNA genome.

Infectious clone cDNA

To study RNA viruses and determine specific functions, it is essential to generate and mutate infectious virus. Due to the difficulty of directly manipulating RNA genomes, a technique known as infectious complementary DNA cloning was developed to reverse transcribe the viral RNA into complementary DNA. The cDNA copy of the viral genome can then be inserted into a bacterial plasmid for easier propagation, handling, and mutation. Infectious clones were first developed by inserting a cDNA copy of a bacteriophage RNA genome (Qbeta phage) into a bacterial plasmid (Taniguchi et al. 1978). The plasmid containing the viral cDNA was transformed into bacteria and the viral genome was transcribed and translated, resulting in infectious virus (Taniguchi et al. 1978). Vincent Racaniello and David Baltimore were the first to generate an infectious cDNA clone of an animal RNA virus (poliovirus) (Racaniello & Baltimore 1981). The infectious clone cDNA can be mutated through common DNA techniques, which is easier than working directly with the viral RNA. Once mutated, the cDNA was confirmed through sequencing and then used to transcribe RNA *in* v*itro* using a bacteriophage promoter (T7 or SP6) and polymerase resulting in infectious viral RNA. The RNA was then electroporated or transfected into cells to initiate the viral life cycle.

A cDNA infectious clone for Sindbis virus (*Alphavirus*) was first constructed in 1987 (Rice et al. 1987). Additional infectious clones have now been constructed based on a wide variety of arthropod-borne RNA viruses such as Venezuelan Equine

Encephalitis, Semliki Forest, Ross River, Western Equine Encephalitis, O'nyong-nyong, and Chikungunya viruses (Davis et al. 1989; Liljeström et al. 1991; Kuhn et al. 1991; Schoepp et al. 2002; Brault et al. 2004; Vanlandingham et al. 2005). Through this infectious clone technology, researchers have determined exact regions critical for genome packaging signals and specific residues identified on the E2 glycoprotein that are involved in vector midgut infection (Levis et al. 1986; Pierro et al. 2007; Pierro et al. 2008). Infectious clone cDNA continues to be a significant method for rescuing, producing, and manipulating infectious RNA virus.

Double subgenomic alphavirus expression systems

The development of cDNA infectious clones has allowed manipulation of viral genomes using DNA and molecular biology techniques. One significant use of infectious clones has been the ability to remove certain genome segments and replace them with foreign genes, which get co-expressed with the viral proteins. This was first done in alphaviruses by inserting a foreign chloramphenicol acetyltransferase (CAT) gene into an incomplete SINV genome resulting in the expression of an enzymatically active CAT as a method to detect viral replication(S. Schlesinger & Weiss 1986; Levis et al. 1987).

A common technique was to replace the structural proteins with a foreign gene of interest (Bredenbeek et al. 1993). Within these systems the nonstructural proteins and 5' and 3' UTRs were maintained to allow RNA replication to continue. This altered RNA genome can be transfected into a cell and results in translation of the nonstructural proteins. The nonstructural proteins constitute the replicase complex, which can synthesize a negative strand copy of the altered genome and eventually transcribe full

length genomic RNA and subgenomic RNAs. The subgenomic RNA contains the foreign gene instead of the structural proteins. This system is often called a “replicon” and allows for detection of the exogenous gene during RNA replication, but infectious particles are not made or released from the cell unless a helper plasmid is added containing the structural protein genes (Frolov et al. 1996). With the addition of a helper plasmid, virus particles are generated and used for one subsequent round of infection. This replicon system works well, but it is limited to in infection potential and is therefore strictly dependent on transfection efficiencies.

Another method for using the viral genome to express foreign genes is to insert a second subgenomic promoter (SGP) either upstream (5’) or downstream (3’) of the original SGP and structural proteins in the infectious virus cDNA (Hahn et al. 1992; Foy et al. 2004). This technique does not require the removal of any of the viral genes, but instead, simply adds the foreign gene to the viral RNA under the control of a second SGP. pTE3’2J was the first double subgenomic sindbis virus developed from the AR339 strain of sindbis(Hahn et al. 1992; Lustig et al. 1988; Rice et al. 1987; Burge & Pfefferkorn 1966). With this double subgenomic promoter system, all the viral genes are present to create infectious virus and the foreign gene is expressed at the same time as the viral structural proteins. A variety of genes can be inserted into this system for expression during infection, although genes smaller than 2Kb are more efficient and stable (Hahn et al. 1992). Double subgenomic (ds) SINV systems that have the duplicated promoter and foreign gene located in the middle of the viral genome (5’ of the original SGP) tend to be stably expressed, but when the duplicated SGP and gene is inserted at the 3’ end of the genome, higher expression levels can be achieved(Foy et

al. 2004; Pierro et al. 2003; Myles et al. 2004). These dsSINV systems have been successfully used to study alphavirus infection by expressing foreign proteins to track, alter, or inhibit infection and RNA sequences to silence genes during infection (Higgs et al. 1995; Olson et al. 1994; Olson et al. 2000; Pierro et al. 2003; Higgs et al. 1999). These alphaviral expression systems are useful tools for studying alphavirus replication and transmission but require time and resources to generate a double subgenomic virus expression system. The expense and difficulty of making double subgenomic virus expression systems potentially limits their application. Our lab has uses dsSINV expression systems for various applications and we sought to increase their applicability by improving the method to create and use dsSINV recombinant viruses. We have developed a system that skips the *in vitro* transcription step and uses a DNA approach for cloning and transfecting the viral genome into cells. The work performed in our lab has increased the utility of these systems by making them easier, more cost effective, and more efficient to use (see Chapter 2).

Wild-type virus infection detection systems

Recombinant double subgenomic alphaviruses have been especially useful for expressing fluorescent reporter proteins, such as green fluorescent protein (GFP), during infection. These dsSINV-GFP viruses can be used in cell culture or in live mosquitoes as a visible marker of infection. Although engineered dsSINV are important tools for visualizing and tracking infection, they often have slower replication kinetics than wild-type (WT) viruses during infection. The addition of a second subgenomic promoter and an exogenous reporter gene can increase the viral genome size by up to

10%, which significantly reduces viral replication and infection. A comparison between TE3'2J and TE3'2J/GFP (filled box versus open box in Figure 1.3) showed that the dsSINV-GFP produced 2 logs less virus than the dsSINV wild-type virus (Pierro et al. 2003). This represents a 99% reduction in virus production and does not accurately portray wild-type infection. In order to detect infection with wild-type virus, mosquitoes are analyzed post-mortem for viral antigen through immunofluorescence or PCR analysis. RNA has to be extracted and processed or cells have to be prepared and stained for the viral antigen. These current methods to detect wild-type infection require terminal sacrifices and do not allow live, visual detection of wild-type virus infection. The only way to visually detect live infection is to use the attenuated double subgenomic alphavirus expressing a fluorescent reporter.

To improve our ability to monitor wild-type virus infection in living mosquito cells, we have developed a reporter system that detects wild-type virus infection without using attenuated recombinant double subgenomic viruses. We utilized the alphavirus' dependency on the replicase complex to generate subgenomic RNA as a method to express a fluorescent protein only when the virus is actively replicating.

The subgenomic promoter is located on the viral genome between the nonstructural proteins (first 2/3 of the genome) and the structural proteins (last 1/3 of the genome). The nonstructural proteins are translated directly from the genomic 49S RNA early during infection. The structural proteins that form the viral particles are expressed from the 26S subgenomic RNA, which is transcribed later in infection when negative strand RNA is present. The negative strand RNA contains the active subgenomic promoter for transcribing the subgenomic RNA, which is then translated and the

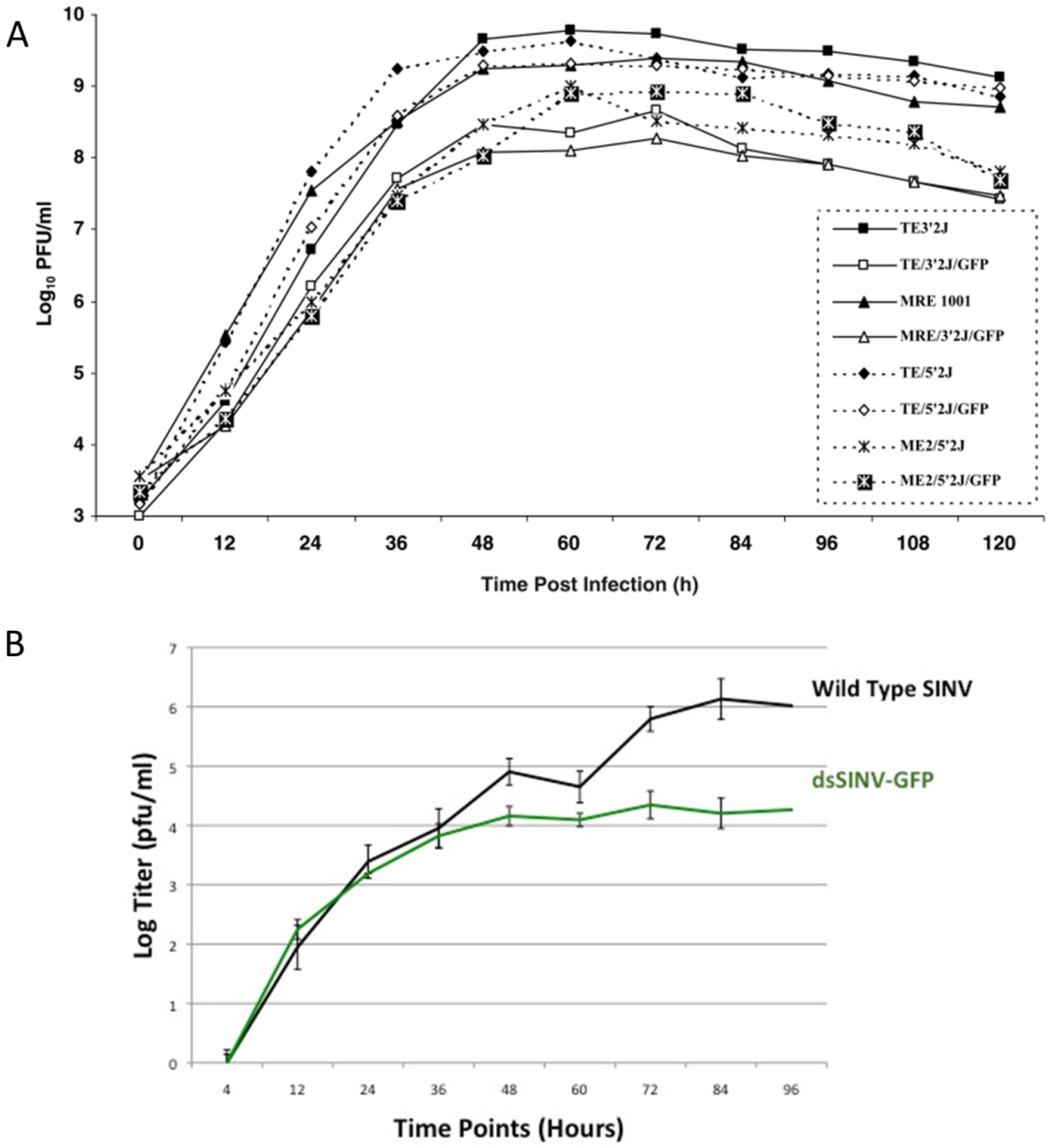

Figure 1.3 Comparison of virus titers with dsSINV-GFP

Replication potential of dsSINV in C636 cells. **A)** The addition of GFP as a reporter reduces the titers of the viruses by nearly two logs in the case of TE3’2J/GFP (boxes) and MRE/3’2J/GFP (triangles)(Pierro et al 2003). **B)** Cultured mosquito cells were infected at an MOI of 0.01 and supernatant was harvested every 12h for 120h. Supernatant was serially diluted and added to Vero cells for plaque assay to determine virus concentration. Significantly lower titers were produced with the dsSINV-GFP

structural proteins are expressed. Host cells generally do not have an RNA-dependent RNA polymerase, so the only way for a negative strand copy of the viral genome to be produced is if the viral RNA replication complex (nsP1-4) is expressed and synthesizes a complementary negative strand. By inserting the subgenomic promoter upstream of a reporter protein, the reporter protein will only be expressed when the replication complex is present and active in a cell. Using this method, we developed a subgenomic reporter RNA system for detecting wild-type alphavirus infection. (See chapter 3).

In order to engineer our subgenomic reporter RNA detection systems, we relied on the extensive work and experimentation done by others over the last several decades to elucidate alphavirus RNA replication. Ou et al compared the ends of the alphaviral genome and determined that the subgenomic promoter was located in a 21-nucleotide conserved sequence element (CSE) between the nonstructural and structural proteins (Ou, Rice, et al. 1982a; Ou et al. 1983; Ou et al. 1981; Ou, Trent, et al. 1982b). Levis et al used deletion mapping to identify an exact region that was responsible for subgenomic RNA synthesis. Ou and colleagues determined that the minimal subgenomic promoter required for synthesis was a sequence that extended 19 nucleotides upstream (5') and 5 nucleotides downstream (3') of the transcription start site (Levis et al. 1990). Additional analysis identified regions upstream that increase subgenomic RNA transcription up to six fold (Raju & Huang 1991; Hertz & Huang 1992). The entire promoter sequence is 112 nucleotides long composing of -98 to +14 in relation to the start of the subgenomic mRNA, with -40 to -20 and +6 to +14 being critical regions for increased activity (J. Strauss & E. Strauss 1994; Wielgosz et al. 2001).

Olivo et al (1994) first used the subgenomic promoter to express a foreign protein during infection. They designed a system containing the subgenomic promoter with a downstream luciferase reporter protein. Baby hamster kidney cells were transformed with this construct and during infection luciferase was detected (Olivo et al. 1994). This was the first cell line specifically developed to detect alphavirus infection. However, their system was limited to mammalian cells because of the promoter requirements and the luciferase reporter didn't allow for visual detection and tracking of infection. We have applied the findings from Olivo and colleagues to our system but we have altered the system to allow visual detection (using the red fluorescent reporter- mCherry) and in mosquito cells (the natural vector for most alphaviruses) during wild-type virus infection. This new mosquito subgenomic reporter RNA system allows for specific detection of infection through fluorescence expression in mosquito cell culture (See Chapter 3). With the ability to detect infection in mosquito cell culture, we next developed transgenic mosquitoes that express the fluorescent reporter only during infection (see Chapter 4)

Transgenic mosquitoes expressing the subgenomic reporter RNA

The subgenomic reporter RNA system can effectively be used to detect alphavirus infection in mosquito cell culture. To better understand mosquito infection and transmission of alphaviruses, we generated transgenic mosquitoes containing the subgenomic reporter constructs with expression of the reporter proteins being directly connected to infection. Expression of the fluorescent reporter is only apparent during infection when the replicase complex is supplied in trans by the infecting virus.

The ability to manipulate the mosquito genome has become a powerful tool for studying basic mosquito biology, pest management techniques, and reducing competence for specific pathogens. Mosquito transgenesis was preceeded by the genetic transformation of *Drosophila melanogaster* in the early 1980's using transposable elements(Rubin & Spradling 1982; O'Hare & Rubin 1983).

Transposable elements (TE) are regions of DNA flanked by inverted repeats that can mobilize and re-insert into new locations within the genome, often termed "jumping genes"(Nevers & Saedler 1977; Pray & Zhaurova 2008). Transposable elements are classified into two categories. Class I are the retrotransposons, which create an RNA intermediate that is reverse transcribed into DNA and inserted into the genome. Class II transposable elements are DNA transposons and use a "cut-and-paste" method of inserting into a genome(Muñoz-López & García-Pérez 2010). Class II transposons are commonly used for genetic transformations because of their stability and ease of use. The transposon refers to the segment of DNA including the inverted repeats, which can be excised from the chromosome and moved to a new location. The ability of a transposon to move and insert into new location is dependent on the activity of a protein called transposase. The transposase enzyme recognizes the transposon inverted repeats, cleaves both strands of the DNA, identifies a correct target DNA, and inserts the transposon into the new site (Richardson et al. 2006). Transposable elements integrate at specific sequences in the target DNA; however, the sequence motifs may be as short as two nucleotides, resulting in numerous potential insertion sites (Lidholm et al. 1993). This wide target range allows for insertion into many different sites and selection of the site appears to be random. Insertion may not occur, it may insert once,

or it may be integrated at multiple sites within the genome. Despite the variability of transposable elements, they have successfully been used to generate transgenics. There are several types of Class II transposable elements that have been discovered and developed for insect transgenesis including the P element, mariner *Mos*1, piggyBac, minos, and Hermes. Each transposable element varies with efficiency and stability depending on the species being transformed. The *Mos*I system uses canonical cut-and paste insertions and is highly stable in mosquito species, where as some of the other TE's have more random insertions and seem more likely to remobilize(O'Brochta et al. 2003; Atkinson et al. 2001).

Transposable elements were first used to transform mosquitoes in 1987 using the P element in *Anopheles gambiae*(Miller et al. 1987). A group from Colorado State University successfully integrated foreign DNA into *Aedes triseriatus* shortly following Miller et al(McGrane et al. 1988). The first *Aedes aegypti* (the vector for many Arboviruses) transformation occurred in 1998 using both the *Hermes* and the *Mariner* transposable elements(Coates et al. 1998; Jasinskiene et al. 1998). The mariner element *MosI* is derived from *Drosophila mauritiana,* but has resulted in highly stable integrations in *Ae. aegypti*. *MosI* uses a canonical cut-and-paste technique and integrates 3' of a –TA dinucleotide(Wilson et al. 2003; Coates et al. 2000; Lidholm et al. 1993).

Mosquito transgenesis has become a useful technique by providing ways to express exogenous genes in living mosquitoes. Transgenic mosquitoes have been used to analyze immunologic and pathogen specific pathways within the vector(Wimmer 2003). Transgenic mosquitoes that impair infection, reduce escape from midguts, or

have weakened vector competence have significantly increased our knowledge of arboviruses (Khoo et al. 2010; Bian et al. 2005; Dong et al. 2011). Mariner Mos1 transgenesis of *Ae aegypti* resulted in a genetic family of mosquitoes that are resistant to DENV2 infection(A. Franz et al. 2006). Mosquito transgenesis is an important tool for studying vector-borne pathogens and various applications are being developed. This dissertation describes a transgenic mosquito line that provides a novel mechanism to visually detect alphavirus infected mosquitoes (see chapter 4).

Flavivirus replication

Along with developing new expression systems and methods to detect alphavirus infection, our lab also strives to dissect the intricate mechanisms of viral replication in different classifications of viruses. Flaviviruses (Dengue, West Nile virus, Yellow Fever virus, Japanese encephalitis virus, etc) are also transmitted through arthropod vectors and are responsible for significant infection and disease worldwide. Although both flaviviruses and alphaviruses are small, enveloped RNA viruses transmitted by mosquitoes, the flavivirus proteins, genome, and life cycle are different from alphaviruses. Our lab performs biochemical analyses of the viral replication proteins in order to elucidate their functions and discover ways to inhibit RNA replication.

The flavivirus genome is an ~11Kb positive-sense RNA that is capped at the 5' terminus, but does not have a polyadenylated tail at the 3' end of the genomic RNA (Knight & Howley 2013; Westaway et al. 1985). The 5' and 3' non-coding regions of the viral RNA have been shown to have intricate RNA secondary structures that play vital roles in initiating transcription and translation and help stabilize the RNA from decay

(Holden & Harris 2004; Chiu et al. 2005; Moon et al. 2012). The genome encodes a single open reading frame that results in a polyprotein containing all the viral proteins. The 5' end of the genome encodes the 3 structural proteins (Capsid(C), precursor-Membrane (prM), and Envelope(E)) which are on the N-terminus of the polyprotein, with the nonstructural proteins (NS1, NS2A,NS2B, NS3, NS4A, NS4B, and NS5) on the C terminus(Rice et al. 1985; Castle et al. 1986). The polyprotein is co-translationally inserted into the endoplasmic reticulum (ER) membrane and is co- and post-translationally cleaved to release all 10 of the individual viral proteins (Figure 1.4). Cellular signal peptidase and the viral serine protease NS2B are responsible for the majority of these cleavages. The nonstructural proteins are associated with the endoplasmic reticulum membrane and form invaginations into the ER membrane to create RNA replication compartments(Welsch et al. 2009). NS5 encodes the RNA-dependent RNA polymerase and the RNA capping activity (guanylyltransferase and methyltransferase) on the C- and N- terminal ends respectively(Saeedi & Geiss 2013). NS3 protein is responsible for the helicase and RNA triphosphatase (RTPase) activity. In order to cap the newly synthesized RNA, a phosphate has to be removed from the 5' end of the RNA (NS3-RTPase), then the guanosine cap has to be added to the RNA [NS5-guanylyltransferase (GTPase)], finally, the cap is methylated (NS5-methyltransferase (MTase)) at the N7 position. NS3 and NS5 both contain enzymatic activities that are required to cap the RNA, indicating a close association between these two nonstructural proteins. NS1, NS2A, NS2B, NS4 colocalize in the RNA replication compartments and play crucial roles in viral replication. Replication of the viral genome occurs in specialized compartments located at the endoplasmic reticulum by first

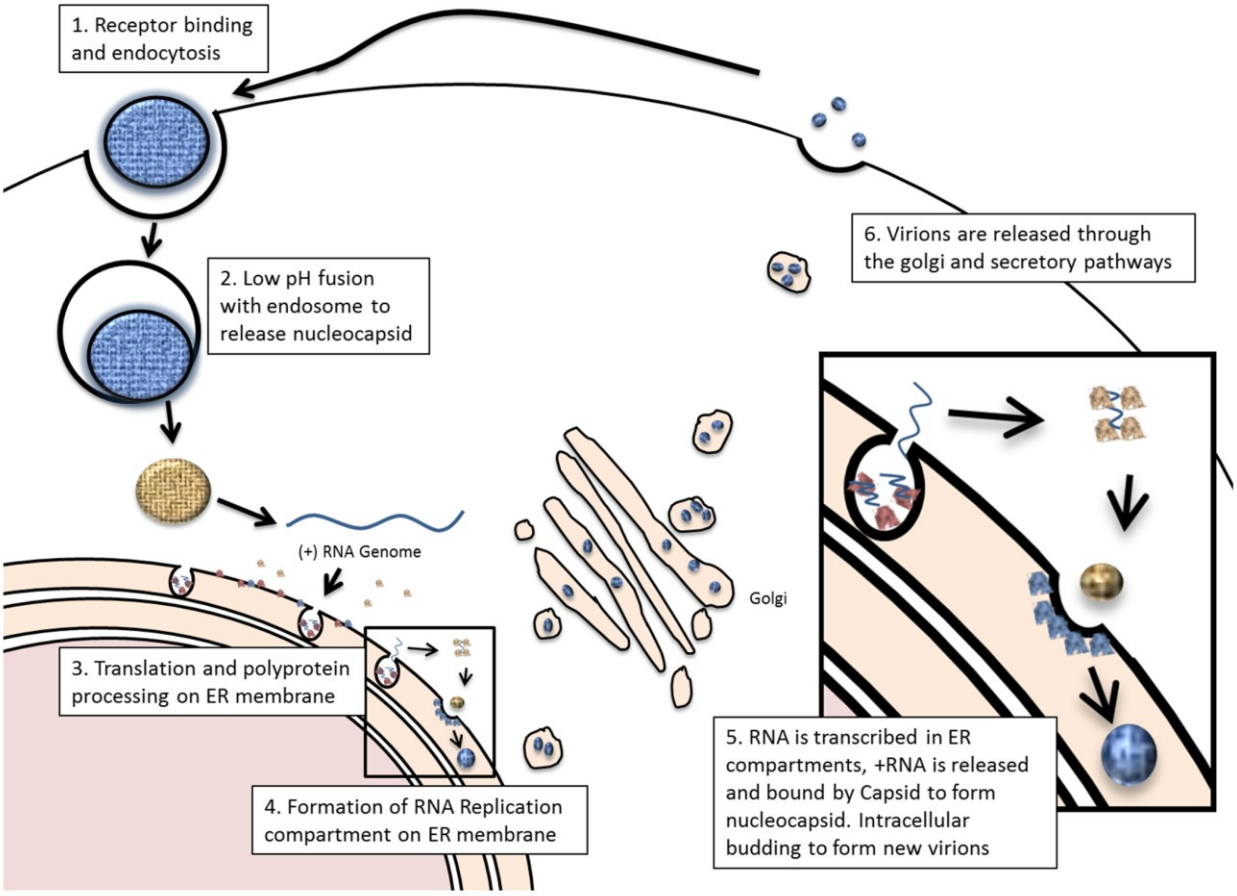

Figure 1.4 Flavivirus life cycle

1) Flaviviruses enter the cell through receptor-mediated endocytosis. **2)** The increasingly acidic endosome triggers a fusion of the virus and the endosome, releasing the nucleocapsid. The nucleocapsid is disassembled and the positive-strand RNA genome enters the cytoplasm. **3)** A polyprotein is translated from the genomic RNA and the polyprotein is co- and post-translationally cleaved into 10 different proteins. These proteins enter the endoplasmic reticulum's membrane and are further processed and modified. **4)** The nonstructural proteins form RNA replication compartments on the ER membrane where a negative strand RNA is synthesized that acts as template for positive-strand genomic RNA transcription. **5)** positive-strand RNA leaves the replication compartments and is either translated or is bound by capsid to form nucleocapsids. The nucleocapsids then bud intracellularly into the ER membrane. **6)** The newly formed virions are transported through the secretory system and are released from the cell.

generating a negative strand copy of the viral RNA. The negative strand genomes serve as templates to synthesize new positive strand genomic copies of the viral RNA. Positive strand RNAs are capped and are used for translation or for packaging into virions. The capsid protein forms dimers and bind with the newly synthesized viral capped- positive strand RNA genomes to form nucleocapsid cores. Nucleocapsids interacts with the envelope (E) and membrane (M) proteins to bud into the endoplasmic reticulum and gain a lipid membrane(J. M. Mackenzie & Westaway 2001; Khromykh et al. 2001). These newly enveloped virions are transported through the cell secretory pathway and are released from the cell to find a new host cell and continue the infection cycle.

Flavivirus replication is completely dependent on the host cell to provide the resources and conditions required to replicate the viral genome. Viral infection alters the normal metabolism and activity of the host cell. This change in cellular metabolism often directly enhances the cellular condition to favor virus replication. Lipid biosynthesis, RNA decay, autophagy, and other pathways are modified during infection(Perera et al. 2012; Moon et al. 2012; McLean et al. 2011). One observed change during flavivirus infection has been the induction of oxidative stress in infected cells (Seet et al. 2009). This altered oxidation state has deleterious effects on the host cell, but it has been unclear what the direct implications are for virus infection. Our lab has investigated oxidation during infection in an effort to determine its effects on virus replication. We have identified a critical role for the oxidative stress to regulate and enhance RNA replication.

Oxidative stress during RNA virus infection

We have determined that antioxidants inhibit viral replication and that virus infection is consistently sensitive to oxidative conditions. We are working to better understand oxidation's role during arbovirus infection.

Oxidative stress is detected in a variety of serious human health conditions including cancer, infectious diseases, neurodegenerative diseases, development, and aging(Kohen & Nyska 2002). In 1954, Daniel Gilbert and Rebecca Gersham published the first paper describing the harmful effects of oxygen to cellular processes(Gilbert 1981). In 1969, cells were discovered to have superoxide dismutase enzymes to reduce radical oxygen species and regulate the toxicity of oxygen. This implied that cells use intricate regulation of redox reactions in order to keep the cell functioning normally(Shapiro 1972; McCord & Fridovich 1969). When there is an abundance of reactive oxygen species (ROS), the condition within the cell is referred to as oxidative stress and causes significant damage to lipid membranes, proteins, and DNA within the cell(Kohen & Nyska 2002).

Arbovirus infection has been described as inducing oxidative stress within host cells. This is true for both flaviviruses and alphaviruses. Patients infected with dengue have significantly higher levels of oxidants and free radicals, and less glutathione and superoxide dismutase (antioxidants)(Seet et al. 2009; GIL et al. 2004). Japanese encephalitis virus (JEV) reduces thioredoxin (reductant) and increases radical oxygen species (ROS) activation, indicating that viruses are inducing a specific oxidative environment within the host cell (Yang et al. 2010).

Inhibiting the induced oxidative stress directly affects viral infection. The addition of antioxidants during infection has been reported to have a positive outcome during dengue infection (J. Wang et al. 2013). Antioxidants have been shown to reduce alphaviral infections or prevent the infection from inducing apoptosis (Yoshinaka et al. 1999; Joubert et al. 2012). Additionally, mosquitoes use antioxidants as a natural mechanism for controlling infection(Chen et al. 2011; Chen et al. 2012; Molina-Cruz et al. 2008). Recent work indicates that flaviviruses and alphaviruses induce oxidative stress during infection, which can be inhibited by adding antioxidants. Our lab has data demonstrating that antioxidants are potent inhibitors of viral replication in both flaviviruses and alphaviruses and in multiple cell lines (See Chapter 5). We hypothesize that oxidative conditions are critical for viral RNA replication and that the oxidative stress is carefully regulated within the cytoplasm and replication compartments to provide optimal conditions for infection to proceed.

The cytoplasm of cells is maintained as a reducing environment while the mitochondria and endoplasmic reticulum are sites of reactive oxygen generation (G. C. Brown & Borutaite 2012; Csala et al. 2010). Flaviviruses replicate in membrane compartments that bud from the cytoplasm into the ER. These modified membrane complexes function to spatially provide a region for RNA replication to occur. Interestingly, these compartments are localized between a reducing (cytoplasm) and an oxidizing (ER) environment. Viral infection has been shown to induce oxidative stress in host cells and we hypothesize that the oxidative stress, despite its deleterious effect on the host cell, may be intentionally induced to benefit viral RNA replication. We have

discovered that oxidation enhances RNA replication and is crucial for positive and capped RNA formation during infection (see chapter 5).

Summary and specific aims

The work presented in this dissertation represents significant advancements in the field of arbovirology that could only have been accomplished because of the years of research and discoveries that have paved the way for our lab's work. This literature review and background chapter has described only a portion of the work that has been done to allow our lab to continue making progress and better understand arboviruses.

Arboviruses continue to cause significant global morbidity and mortality due to the lack of tools and resources to stop or prevent infection. Over the last 5 years, I have been able to work on a variety of projects that have helped increase our understanding of arbovirus infection and replication. Specifically, we have developed and optimized alphavirus expression systems derived from plasmid DNA to allow more efficient generation of recombinant viruses for various applications and tools in virology. We developed a novel method for detecting infection in mosquito cells, allowing for wild-type virus detection and visual monitoring/tracking of infection with a fluorescent reporter. That system was successfully introduced into transformed *Aedes aegypti* mosquitoes, resulting in transgenic mosquitoes that express a fluorescent reporter protein during infection. We have also identified an essential advantage for RNA viruses to induce oxidative stress during infection to enhance RNA replication. We are the first to report a specific role for oxidation during viral replication and have found it to be consistent with multiple RNA virus species and families. We have identified a potential

mechanism that RNA viruses use to regulate RNA synthesis and capping, utilizing oxidation conditions within the host cell. The work described in this dissertation has increased our ability to use alphavirus expression systems, enhanced our methods and tools for detecting infection, and has improved our knowledge and understanding of infection-induced oxidative stress. All of these advancements will potentially help in controlling arbovirus infection and reducing the global disease burden.

Chapter Two: Production of DNA-based Alphavirus Infection Systems

Introduction

Alphaviruses (Family: *Togaviridae)* are used extensively in molecular biology as tools for gene expression and delivery(Lundstrom 2009). Alphaviruses can infect a wide range of species and have small manipulable genomes that can encode and express heterologous genes (Huang et al. 1989; Xiong et al. 1989). Alphaviruses possess a positive sense capped RNA that is approximately 11.7 kilobases in length. The 5' end of the viral RNA is translated into 4 nonstructural proteins (nsP 1-4), which are involved in replicating the viral genome. A negative strand RNA is synthesized from the full-length positive strand viral RNA that contains a subgenomic promoter (SGP) to drive transcription of the 26S subgenomic RNA. The subgenomic RNA encodes the viral structural proteins (Capsid, E3, E2, 6K, and E1) necessary for virion assembly(J. Strauss & E. Strauss 1994). The SGP has previously been duplicated in alphavirus double subgenomic systems, allowing for foreign genes to be expressed during infection similar to the expression of the viral structural proteins (Frolov et al. 1997; Hahn et al. 1992). Exogenous genes that have been engineered into alphavirus genomes include fluorescent proteins, luciferases, cellular proteins, antisense RNAs, and ribozymes (Cirimotich et al. 2009; Olson et al. 1994; Pierro et al. 2003; Cook & Griffin 2003; Smith et al. 1997; Uhlirova et al. 2003; Travanty et al. 2004). Engineering an exogenous gene or RNA behind the second subgenomic promoter allows for the production of a fully infectious virus simultaneous with the expression of the foreign gene in a wide range of species.

The current method used to create a recombinant double-subgenomic virus that expresses a foreign gene is relatively inefficient. To insert the gene of interest (GOI) into the virus, the plasmid containing the viral infectious clone cDNA is digested with a unique restriction enzyme and the PCR-amplified GOI is digested with the same restriction enzyme, which can then be ligated into the virus infectious clone plasmid. This approach usually results in the GOI ligating in either the sense or antisense orientation, requiring screening of the resulting clones for the orientation of the insert. Of additional concern with single-site restriction cloning is vector re-ligation or multiple copies of the GOI ligating into the virus infectious clone plasmid if small inserts are used with single restriction site cloning. Once a clone with the GOI in the correct orientation has been identified and sequenced, the plasmid is linearized using a unique restriction site at the end of the viral genome to allow for run-off in vitro RNA transcription. Several micrograms of extracted and purified plasmid DNA is used for *in vitro* RNA transcription reactions with a nucleotide cap analog to generate capped viral RNAs. The RNA is then either electroporated into cells or transfected with chemical or liposomal RNA transfection reagents and virus is collected from the cell culture media 24-72 hours later.

Several points in this process reduce efficiency and increase the time for virus production. Insertion of a GOI into the viral genome by restriction cloning is relatively inefficient due to vector self-ligation and from the need to screen for insert orientation. Linearizing the infectious clone plasmid adds additional manipulation to the process and generally requires that at least several micrograms of the infectious clone plasmid be prepared. *In vitro* T7 transcription kits that are commonly used are expensive

(~$15/transcription reaction) and generally result in low yields of long capped RNAs. Additionally, phage DNA-dependent RNA polymerases (such as T7 and SP6) have low fidelity and can result in quasi-species from the *in vitro* transcription reaction (Pugachev et al. 2004). Electroporation of cells with RNA requires large numbers of cells (1-5x10^6cells/electroporation), is sensitive to salt concentration that can damage cells during electroporation, and requires specialized equipment, which is not always available in laboratories.

To make alphavirus expression systems easier to use and more accessible to researchers, we have developed virus expression plasmids that are simple to manipulate and can rapidly and inexpensively produce infectious virus. Building on previous work done in the lab with Sindbis virus replicon expression plasmids (Geiss et al. 2007), we generated a double-subgenomic Sindbis virus expression plasmid that transcribes RNA from a cytomegalovirus (CMV) PolII promoter and cleaves the RNA at the 3' end of the viral genome similar to plasmid-based replicon expression systems (Geiss et al. 2007; Ivanova et al. 1999; Dubensky et al. 1996). This allows for infectious viral RNA to be transcribed directly from the plasmid constructs in the transfected cells. In addition, we have developed variants of this system that utilize recent recombination technology to rapidly and efficiently insert a Gene of interest (GOI) into the virus in the desired orientation. The negative and positive selection capability of the Gateway cloning system makes it attractive for rapid GOI cloning. Using this system we have produced several reporter-gene expressing viruses and demonstrate their use in cell culture.

Materials and Methods

Plasmid construction

The base TE/3'2J Sindbis virus expression plasmid (pBG167) was constructed by digesting a TE/3'2J replicon expression plasmid pBG68 (Geiss et al. 2007) with HpaI and XbaI restriction enzymes and ligating the vector with T4 DNA ligase to a 4631bp XbaI/HpaI fragment from the pTE/3'2J infectious clone(Hahn et al. 1992). pBG218 was created by ligating NheI flanked GFP open reading frame into the unique XbaI site in pBG167. The orientation of the GFP insert was verified by sequencing with BG626 (5' cacctctagaccatggatcc) and BG583 (5' CTAGATAAATGGTTAATATAGT). pBG167-based recombination ready plasmids were generated by ligating a PCR amplified attR1/attR2 recombination cassette from Gateway pDEST32 (Invitrogen) into pBG167. BG121 (5' catggctagcacaagtttgtacaaaaaagctgaacg) and BG122 (5' catggctagcACCACTTTGTACAAGAAAGCTGAACG) contain NheI restriction sites, and were used to ligate the recombination cassette into XbaI digested pBG167 and were transformed into ccdB resistant DB 3.1 E. coli cells (Invitrogen). Forward and reverse attR1/attR2 recombination cassettes were identified by DNA sequencing and resulted in pBG210 and pBG211, respectively. pBG440 (pENTR-D/Topo-GFP) and pBG403 (pENTR-D/Topo-Renilla Luciferase) were constructed by PCR amplifying the eGFP gene from pIE-GFP (Clontech) with primers BG518 (5' caccgctagcatggggatgcatggtaccatgg) and BG519 (5' aaGTgctagcTTACTTGTACAGCTCGTCCATGCC) or the Renilla luciferase gene from pWN5'RucPur (Geiss et al. 2005) with primers BG556 (5' caccatggctagcaaggtgtacgacc) and BG557 (5' CTACTGCTCGTTCTTCAGCACG) and incubating the gel extracted

PCR products with pENTR-D/Topo. pBG212 was generated by performing a LR Clonase II reaction between pBG440 and pBG210, and pBG451 was generated from a LR Clonase II Reaction between pBG403 and pBG210.

All viral sequences in pBG167 plasmid were verified with a panel of Sindbis-specific primers. Inserts ligated into the unique XbaI site in pBG167 were sequenced with primers BG 583 (5' CTAGATAAATGGTTAATATAGT) and BG626 (5' cacctctagaccatggatcc) to verify sequence and orientation. PCR products that were Topo-cloned into pENTR-D/Topo were sequenced with M13-20 and M13 Reverse primers. All Gateway attR1/attR2 containing plasmids were grown on ccdB resistant DB3.1 E. coli cells (Invitrogen), and all other plasmids were grown in DH5a E. coli cells. Diagrams of each virus expression construct are provided in Figure 2.1.

Cell culture and transfection

Baby Hamster Kidney (BHK-21) and Vero cells were maintained in Hyclone DMEM supplemented with 10% fetal bovine serum (FBS), 5% Pen/Strep, and 5% L-Glutamine (Geiss et al. 2005). These mammalian cells were kept in a 37°C incubator with 5% CO2. BHK-21 cells were plated in 6 well plates for transfection with Lipofectamine 2000. Cells were transfected at 60% confluency with 125ng DNA per well, using the manufacturer's recommendations. Transfection media was removed and replaced with fresh media 6-8 hours post transfection. *Aedes albopictus* C6/36 cells were cultured in L-15 insect medium with 10% FBS, 5% Pen/Strep, and 5% L-Glutamine and were maintained at 28°C (Zach N Adelman et al. 2002a).

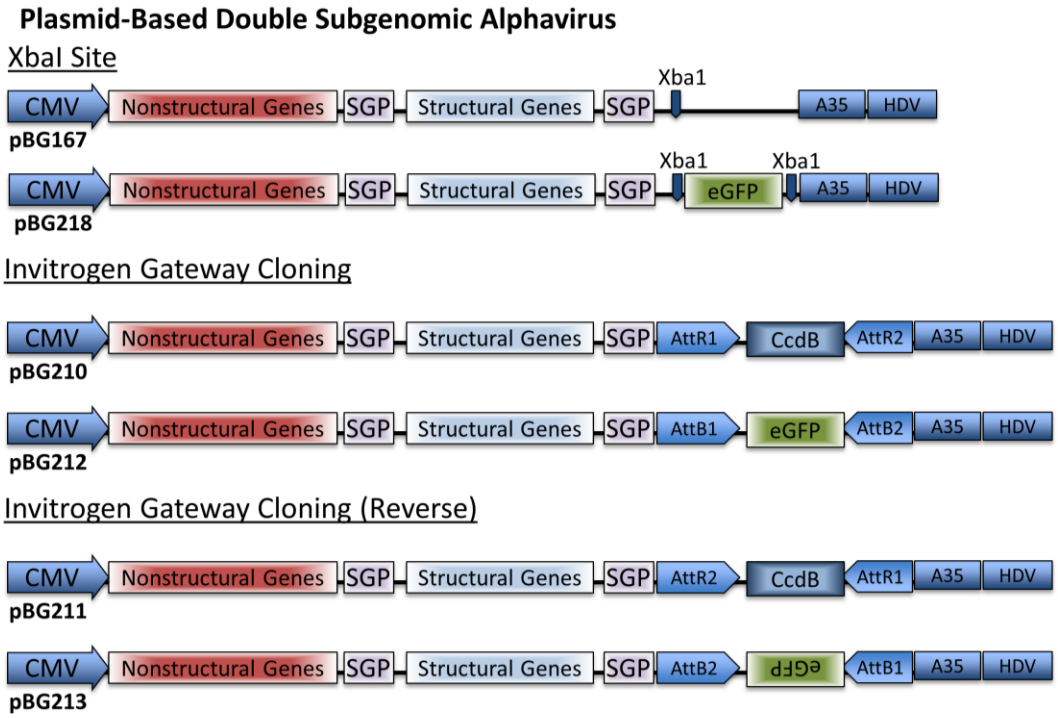

Figure 2.1 Diagram of plasmid constructs

Constructs were designed and generated to contain the full-length RNA genome with a 5' cytomegalovirus (CMV) promoter and a second subgenomic promoter (SGP) at the 3' end. Downstream of the 2nd SGP is a unique XbaI site or Invitrogen's Gateway cloning cassettes for insertion of foreign genes of interest. pBG 167, 210, and 211 are the original constructs and pBG 218, 212, and 213 are the constructs with GFP inserted behind the 2nd SGP. All sequences have a poly adenine sequence and a hepatitis delta virus ribozyme at the 3' end.

Plaque assays and growth curves

Viral titers were determined using plaque assay titrations on BHK-21 cells as described previously (Hernandez et al. 2005). BHK-21 cells seeded on 24 well tissue culture plates were infected with serial dilutions of virus samples for 1 hour at 37°C, and then an agarose nutrient overlay was added. Cells were maintained at 37°C for 3 days for visible plaques to develop. On day 3, Thiazolyl Blue Tetrazolium Blu (MTT) at 5 mg/ml in PBS was added to the overlay to visualize plaques and incubated at 37°C for 12 hours. Viral plaques were counted and titers determined as plaque forming units (PFU)/ml.

P0 (transfection initiated) and P1 (virus initiated) growth curves were performed in 6-well plates. For P0 growth curves, BHK-21 cells were transfected as described above. The cells were washed with media to remove excess transfection complexes, and 500 ul samples were collected at 4, 8, 12, 24, 36, 48, 60, and 72 hours post transfection. Sample volumes collected were replaced with fresh media to maintain a total volume of 2 ml. Aliquots of samples were stored at -80°C until titration or infection. For P1 growth curves, titered P0 derived virus (48 hr post-transfection) was added to BHK-21 or Vero cells at MOI=0.1 or 0.01 as indicated. Cells were incubated in 37°C incubator for specified times post infection to allow the virus to infect and replicate within the cell cultures. Samples were collected by taking media from the infected cells and were analyzed as described for P0 growth curves. Each growth curve was replicated three times and average titers and standard error calculated. Data was graphed using Microsoft Excel.

GFP and Renilla luciferase expression

GFP expressing cells were imaged on an inverted Nikon Diaphot 200 fluorescence microscope with a CoolSnap CCD camera using either 488nM/535nM filters for GFP detection or phase contrast for cell imaging. Image contrast was adjusted for all images equally using ImageJ software. Luciferase assays were performed using Viviren Live Cell Renilla Luciferase Reagent (Promega) in white opaque 96-well plates. Briefly, at the indicated times media was removed from the infected wells and replaced with 25 mL DMEM supplemented with 37 mg/ul Viviren reagent. The plates were incubated at 37°C for 10 minutes and then relative light units (RLU) in each well were determined on a Victor 3V Multimode plate reader (Perkin Elmer). All experiments were performed at least 3 times, and averages and standard errors are reported.

Results

Developing infectious DNA plasmid constructs

Double subgenomic recombinant alphaviruses have been generated for over two decades. However, the production of these viruses has always required the manipulation of the plasmid cDNA, followed by *in vitro* transcription to generate viral RNA, which is then transfected into cells. The *in vitro* transcription step requires significant resources and careful handling of the RNA. In 2007, our lab developed a system containing the cytomegalovirus promoter to drive transcription of a SINV replicon RNA directly in cells, without the need for the *in vitro* transcription. The cytomegalovirus promoter worked well at expressing RNA from a plasmid backbone directly in the transfected cell. However, the previous system was a replicon and didn't

have the structural protein genes required for virus maturation and budding. It was therefore incapable of making fully infectious virus. We have further applied this concept to generate fully infectious RNA from a plasmid containing the full-length RNA genome (SINV TE3'2J). To establish this system, we used the cytomegalovirus promoter and replicon construct previously described and added the structural proteins behind the subgenomic promoter. A second subgenomic promoter was maintained that had a unique XbaI site for inserting a gene of interest (pBG167). The 3' ends of the constructs have a poly adenine sequence and a hepatitis delta virus ribozyme to cleave the RNA (Figure 2.1). Additional constructs were engineered to contain the Invitrogen Gateway Cloning cassettes in either the forward or reverse orientation, to allow for quick and easy directional insertion of a gene of interest (pBG210 and pBG211).

Production of infectious Sindbis viruses from plasmid transfections

To test if infectious Sindbis virus could be produced by simply transfecting our DNA plasmid into cells, 125ng of pBG167 plasmid was transfected with Lipofectamine 2000 reagent into a single well of BHK-21 cells in a 6-well plate. Four hours post transfection the cells were washed with media to remove excess transfection complexes, and media samples were collected at the indicated times post transfection (Figure 2.2A). Viral growth kinetics and maximal viral titers are similar to TE3'2J produced from RNA transcripts (Pierro et al. 2003; Heise et al. 2003), indicating that our newly designed DNA-plasmid based system produces similar amounts of virus as traditional RNA transfection production methods.

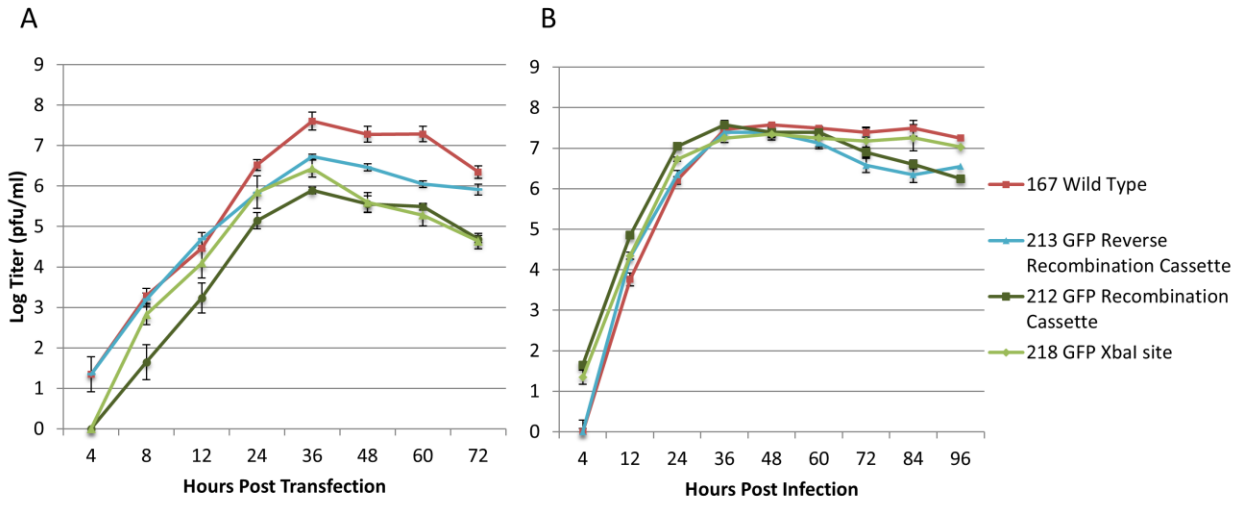

Figure 2.2 Production of infectious Sindbis viruses from plasmid transfection

BHK cells were transfected with 125ng of construct DNA and samples were collected at the given time points post transfection **(A).** Samples were titered through plaque assay to find virus concentration. Titered virus samples were then used to infect BHK cells for round of infection using the plasmid derived virus **(B).** Samples were also collected and titered for growth curve analysis.

Once we determined that infectious Sindbis virus could be produced directly from transfected pBG167 plasmid, we generated a series of constructs with GFP inserted behind the 3' SGP either by ligation into the XbaI site (pBG218) or by recombination into a Gateway attR1/attR2 recombination cassette in plasmids pBG210 or pBG211 (pBG212, and pBG213). These plasmids were stably propagated in *E. coli* in the pcDNA3.1 vector backbone with average plasmid miniprep yields of 100 ng/ml. Following transfection into BHK-21 cells with the plasmid construct, infectious virus was collected and tittered, resulting in passage 0 (P0) growth curves (Figure 2.2A). We observed that the maximal titers for each GOI-containing clone was lower than pBG167-derived virus, likely due to the additional size and replication burden of the GOI viral constructs, which has been reported previously from transcription-derived TE3'2J viruses (Pierro et al. 2003).

There was a high amount of variability between the constructs and the viral titers they produced (Figure 2.2A), which is most likely due to differences in transfection efficiencies or the differing molar amounts of plasmid being transfected due to the various plasmid sizes. To determine if the plasmid derived viruses were able to replicate in a second passage (P1), we collected and titered virus from the initial transfection cultures. BHK-21 cells were infected with each P0 virus at MOI=0.01 and growth kinetics were determined (Figure 2.2B). The P1 viral growth kinetics and maximal titers were much more uniform than the P0. Through these experiments, we were able to show that our constructs make virus directly from plasmid transfection and virus is infectious and more consistent with subsequent rounds of infection.

Reporter gene expression

We next tested reporter gene expression from the different virus constructs. pBG212 and pBG218 derived virus both produced GFP expression as visualized by fluorescence microscopy, whereas pBG213 derived virus with GFP inserted in reverse orientation and pBG167 did not express GFP (Figure 2.3A). In order to get a more quantitative understanding of the reporter expression, we inserted luciferase genes behind the subgenomic promoter in place of the GFP gene. pBG445 contained a firefly Luciferase gene and pBG451 has a Renilla Luciferase. We also inserted mCherry in place of the GFP to have a 'red' SINV. All of these constructs were made using the gateway att-recombination cassettes and efficiently express the reporter during infection (Figure 2.4). We transfected these plasmids and collected virus for subsequent infections. We then compared the growth kinetics (viral titers) with the expression of the luciferase over time. The kinetics of Renilla luciferase expression in the P1 infection closely followed the kinetics of virus production in growth curve titration assays (Figure 2.3B), indicating that viral replication and reporter gene expression are closely linked. To verify that reporter gene expression is sensitive to a known viral replication inhibitor, we treated cells with the cellular calmodulin kinase inhibitor W-7 (N- (6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) that had previously been shown to interfere with viral packaging(Liu & D. T. Brown 1993). W-7 treatment (20 mM) reduced reporter gene expression by about 1 log in P1 infections as compared to untreated samples (Figure 2.3C), demonstrating that Renilla luciferase expression was linked to viral replication.

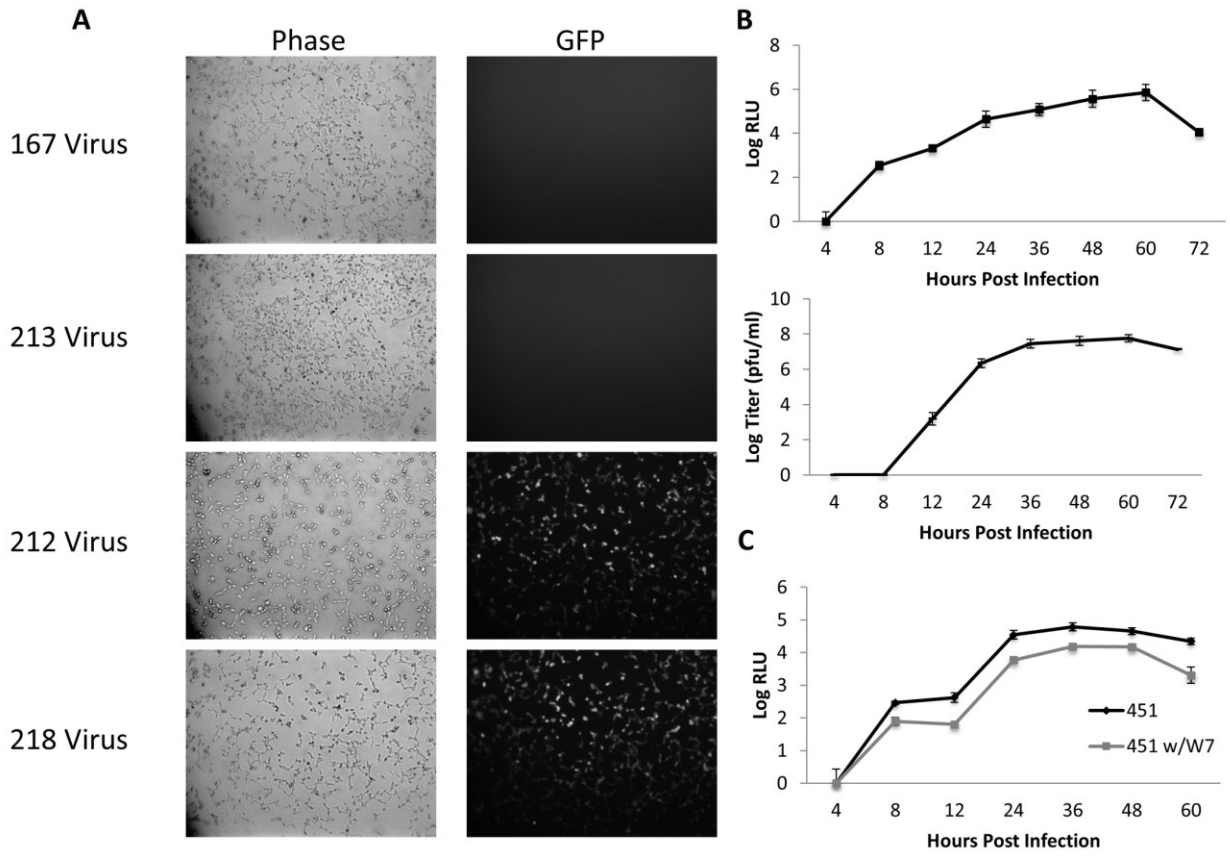

Figure 2.3 Gene of interest expression during infection with recombinant dsSINV constructs

A) BHK cells transfected with constructs display visual detection of GFP expression in pBG212 and pBG218, but not in pBG167 and pBG213. **B)** Viral growth curve titers are directly linked to the expression of the foreign inserted gene over time. **C)** Inhibiting the viral replication also inhibits the reporter expression.

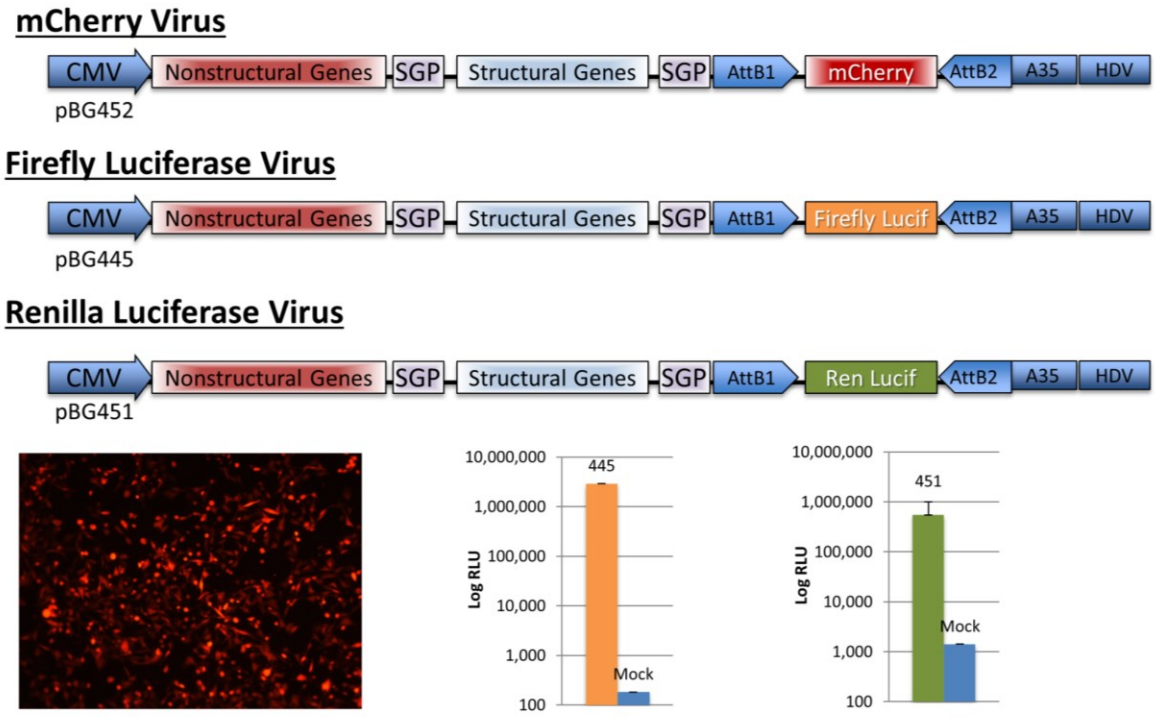

Figure 2.4 Additional foreign reporter genes expressed in dsSINV constructs

mCherry, firefly luciferase, and renilla luciferase were inserted into the gateway recombination cassettes and result in significant expression of the foreign protein following infection. Infection of BHK cells with MOI=0.1 and analyzed at 48 hours post infection.

Replication of plasmid derived virus in various cell lines

Sindbis is known for broad host cell tropism and is able to infect different species with relatively similar efficiencies. We determined if plasmid-derived virus was able to effectively replicate in cell lines other than BHK-21 cells. Renilla luciferase expressing pBG451 virus (P0) was used to infect BHK-21 (murine), Vero (primate), and C6/36 (mosquito) cell lines at MOI=0.1, and viral titers and Renilla luciferase activity were assessed at 48 hours post infection (P1) (Figure 2.5). BHK-21 cells and Vero cells produced similar titers 48 hours post infection (~$1x10^6$ PFU/ml), and showed similar Renilla luciferase signals at 48 hours (~$1x10^5$ RLU). C6/36 cells produced lower titers and Renilla luciferase signal than BHK-21 and Vero cells at 48 hours post infection, though this difference may be due to slower growth kinetics for invertebrate cell lines. These data indicate that the P0 virus was able to effectively infect and replicate in murine, primate, and mosquito cell lines, and that the Renilla luciferase reporter gene was expressed in each cell line during the P1 infection.

The stability of reporter gene expression

To test the stability of the att-containing viruses, we passaged the mCherry virus (pBG452) and the GFP virus (pBG212) three times in either BHK-21 or C6/36 cells using MOI = 0.01 for each infection. For each cell line we monitored the cells for visual expression of the fluorescent reporters and also assayed for viral titer. We observed that each cell type showed equal fluorescence with each passage and had similar titers for each passage, indicating that the att-flanked GOI remained stable over the course of at least 3 passages in both BHK-21 and C6/36 cells (Figure 2.6)

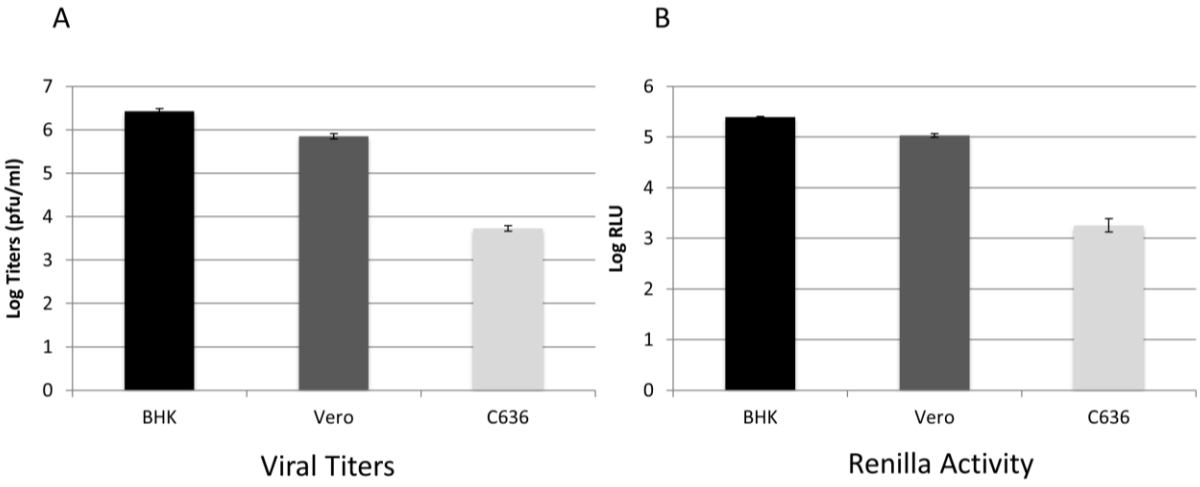

Figure 2.5 Replication and foreign gene expression in cell lines from different species

BHK (murine), Vero (Primate), and C636 (mosquito) cell lines were infected with P0 derived pBG451 virus (dsSINV-REN) at MOI=0.1 **A)** Plaque assay titrations and **B)** Renilla luciferase assays were performed in triplicate on each cell line at 48 hours post infection.

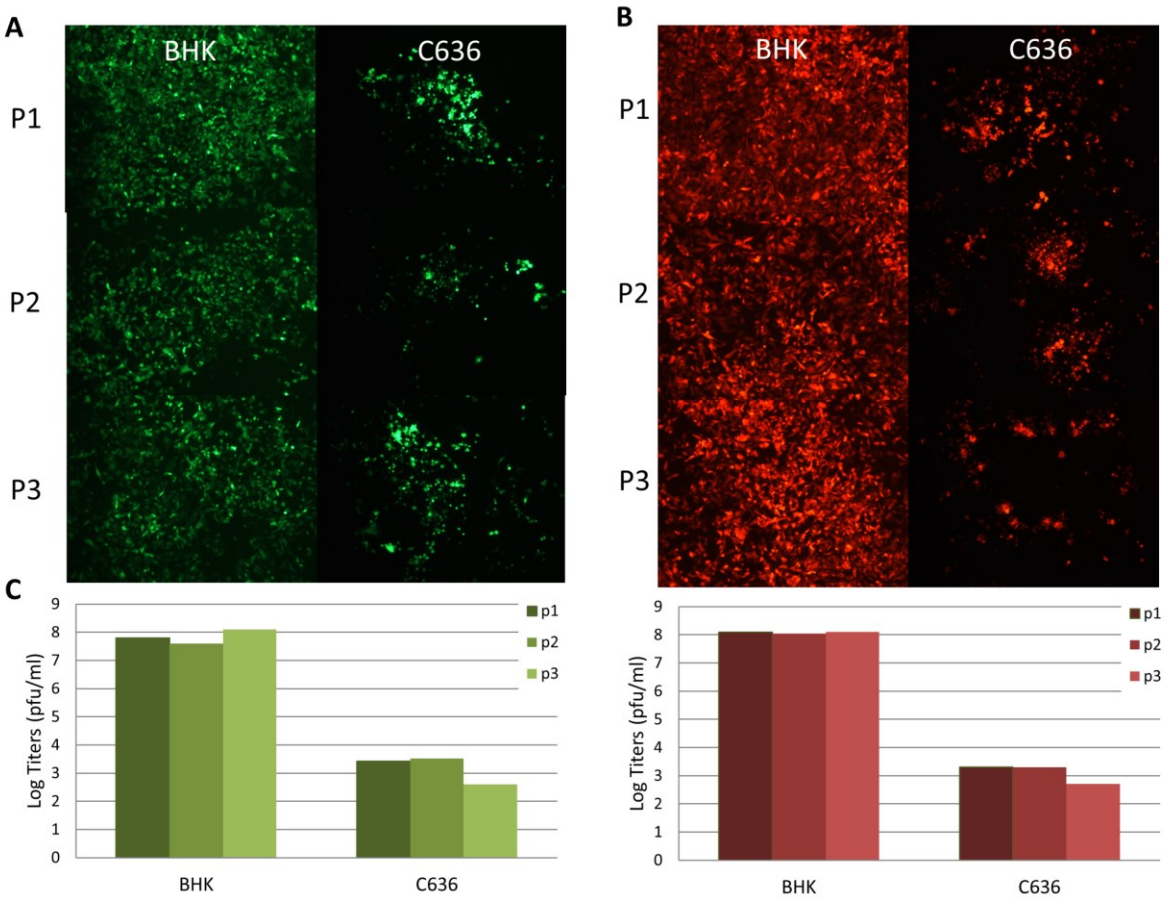

Figure 2.6 Stability of reporter gene expression.

BHK and C636 cells were used to passage either dsSINV-GFP(212)(**A)** or dsSINV-mCherry (452)(**B)** for 3 passages. Expression of the fluorescent reporter is stable through all the passages **(A and B)** and the titers remain constant over time **(C).**

Modification of expression plasmids for mosquito transcription

The work described previously within this chapter was published in 2011 in the Virology Journal(Steel et al. 2011). We have continued to work with these constructs to further enhance and optimize their utility. The constructs previously developed contained only the cytomegalovirus (CMV) pol II promoter, which works well in mammalian cell culture, but does not function in insect cells. The recombinant viruses described above were transfected directly into BHK-21 (mammalian) cell culture and then infectious virus was collected and proved to be infectious in different cell lines (murine, primate, and mosquito) (See Figure 2.5). In an effort to simplify this process and broaden the applicability of this system, we developed a plasmid that could be transfected directly into mosquito cells and produce infectious virus. To accomplish this, we PCR amplified the baculovirus immediate early promoter (IE3) and inserted it downstream of the CMV promoter (Jarvis et al. 1990).This new hybrid CMV/IE3 promoter construct initiated infection in both mammalian and insect cell culture (Figure 2.7). The mosquito cells grow slower and show less dramatic expression of the reporter, but GFP fluorescence is seen in cells transfected with the pBG505 CMV/IE hybrid and not in mosquito cells transfected with pBG212 CMV alone. Virus can be collected from the transfected C6/36 cells and is infectious for subsequent rounds of infection in both mammalian and insect cell lines. This new plasmid allows researchers to quickly and efficiently make recombinant virus directly in mosquito cells and shows that the plasmid launched infection systems can be modified to be species specific through promoter variations.

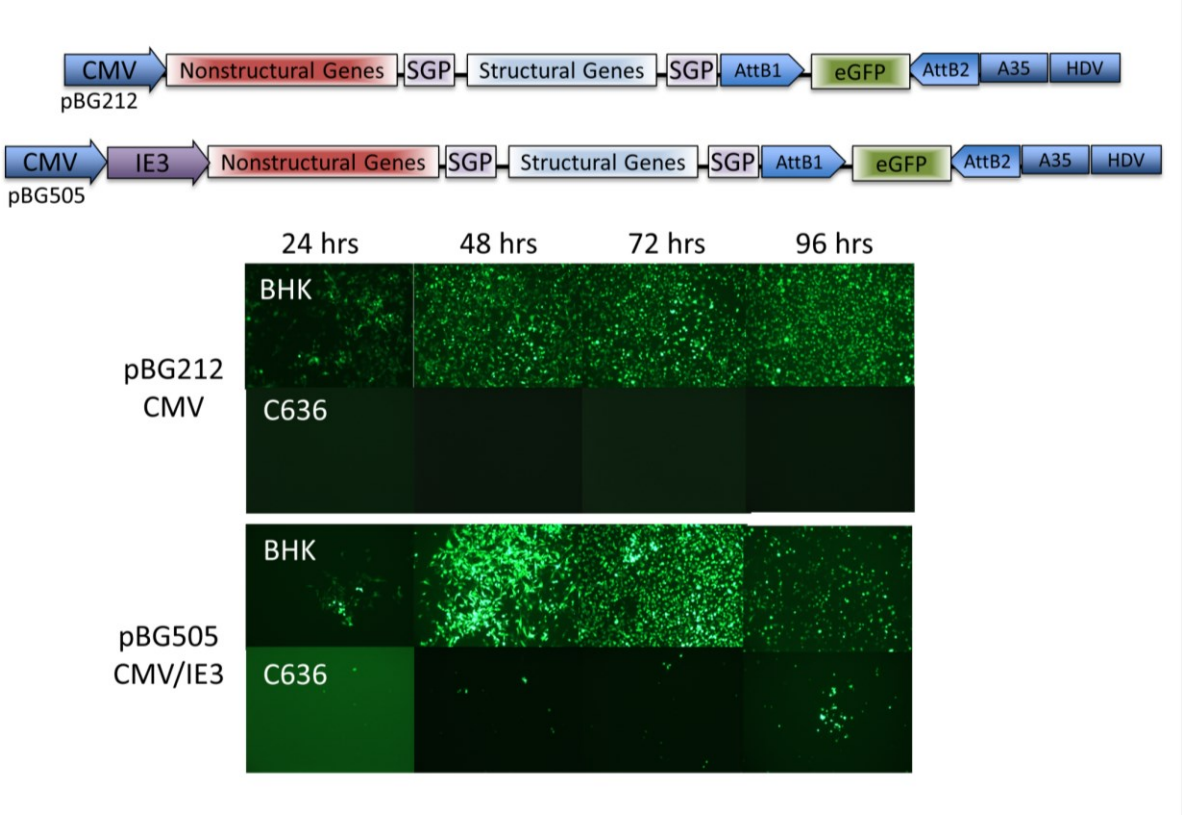

Figure 2.7 CMV/IE promoter hybrid allows transcription to begin in both mammalian and insect cell lines.

Diagram of plasmid constructs containing either the CMV promoter (pBG212) or both the CMV and the IE3 promoter (pBG505). pBG212 (CMV promoter) is only effective at transcribing the RNA and expressing GFP in the BHK cells. pBG505 (CMV/IE3 hybrid) has both the CMV promoter and the baculovirus IE3 promoter, which allows transcription to occur in both BHK and C636 cells once they are transfected with the plasmid.

Discussion

In this chapter we describe the construction and characterization of a new double subgenomic Sindbis (TE3'2J) virus DNA production system. The virus expression plasmids we describe have several features that make them useful for rapidly generating recombinant Sindbis viruses that express genes of interest. We have developed a system in which reporter-gene expressing and fully infectious dsSINV virus can be produced by simply transfecting a small amount of plasmid DNA into cultured mammalian or insect cells. Genes of interest can be rapidly incorporated into viruses in specific orientations via Gateway recombination. High-titer virus produced from our system can infect multiple species of cultured cells and maintain reporter gene expression.

The typical sequence of events for production of a GOI expressing TE'32J virus takes approximately 3-4 weeks. Cloning and inserting the GOI 3' of the viral subgenomic promoter usually takes ~2 weeks from the PCR amplification step and requires screening clones for insert orientation. Once a clone has been identified and the GOI sequenced, the production of infectious RNA takes 2-3 additional days due to preparation of linearized maxiprepped plasmid DNA for *in vitro* transcription and electroporation of RNA transcripts into ~2-10x10^6 cells using expensive electroporation systems. Additionally, because RNA is being used, extra precautions to reduce RNase degradation of transcripts must be used. Therefore, the traditional production of a recombinant GOI-expressing TE3'2J is time consuming and relatively expensive.

Our system circumvents many of the problems associated with production of a recombinant GOI-expressing TE3'2J virus. GOI PCR products can be rapidly cloned

into attL containing pENTR-D/Topo vectors, then the GOI can be recombined into pBG210 (forward) or pBG211 (reverse) TE3'2J expression plasmids with high efficiency. In our hands this process takes about 1 week and is highly efficient. The pENTR-D/Topo vector and LR Clonase II systems from Invitrogen are relatively expensive, but each reaction can be scaled down to reduce costs and extend the number of reactions that can be performed. We have successfully recombined short hammerhead ribozymes (67 bp) and the large Firefly luciferase gene (1.6 Kb) into pBG210 (Figure 2.4), indicating that a wide range of insert sizes can be accommodated. Once the GOI containing virus expression plasmid has been constructed, virus is produced by transfecting a small amount of the plasmid into BHK-21 cells using common transfection agents and $2x10^5$ cells in a single well. Virus is produced within a few days with minimal effort. The format for transfection can be adjusted from 96-well plates to T_{150} flasks as needed, making virus production very flexible and rapid. GOI expression was verified from several viruses, including viruses that produce GFP, mCherry, and Firefly or Renilla luciferase (Figures 2.3 and 2.4). The kinetics of Renilla luciferase expression closely mirrored viral replication kinetics, indicating that the GOI is stable and can be used as a readout for viral replication as previously described (Cook & Griffin 2003; Olson et al. 1994; Pierro et al. 2003). The ease of cloning and specificity of insert orientation would make this system ideal for generating libraries of infectious viruses expressing randomized trans-cleaving ribozymes or inverted cDNA libraries to screen for host genes that are involved in viral replication or antiviral responses. In addition, the ability to launch virus production directly in cells with stable plasmid DNA may open up the possibility of using these constructs for stable Sindbis virus vaccines

that can be launched via plasmid injection that may provide more robust immune responses than non-spreading replicon vaccines.

This system has been well received by the research community and we have sent requested plasmids to research institutions throughout the world (France, Belgium, Canada, Wisconsin, Maryland, Indiana, Texas, etc).

Chapter Three: Subgenomic Reporter RNA System for Detection of Alphavirus Infection in Mosquito Cells

Introduction

Alphaviruses are mosquito-borne pathogens that can cause severe human and veterinary disease, several of which are considered potential biological weapons (Atkins 2013; Weaver et al. 2004). Alphaviruses are a major global health concern due to the widespread prevalence of arthropod vectors and limited prevention and treatment options for infection(Porretta et al. 2012). Alphavirus infection results in a wide range of clinical symptoms, including fatal encephalitis or long-term arthritis (Atkins 2013; Porretta et al. 2012; Marimoutou et al. 2012).

Defining how alphaviruses infect the mosquito vector and transmit to mammalian hosts is an active area of study, but the tools for monitoring alphavirus infection in mosquitoes have largely relied on postmortem analysis or using recombinant viruses engineered to express fluorescent or luminescent proteins from duplicated subgenomic promoters (SGP) (Phillips et al. 2010). Although recombinant alphaviruses are useful tools in various applications and our previous work enhanced their utility, generating a recombinant alphavirus still requires an infectious clone of the particular strain of virus be available and that the clone be engineered to express a reporter protein suitable for use in the study. Another complicating factor is that modifications to the viral genome can increase the viral genome size by over 10% and often leads to a reduction in viral replication kinetics that attenuates virulence in the mammalian and/or arthropod hosts (Figure 1.3)(Wiley et al. 2010; Phillips et al. 2010; Steel et al. 2011) . These issues

demonstrate that alternative approaches for detection of wild-type alphavirus infection in mosquito cells need to be developed to provide more physiologically relevant data. A system that would allow live visual detection of SINV infection in the natural mosquito vector would be a valuable tool for further understanding the transmission and infection of alphaviruses.

Alphaviruses (Family *Togaviridae, genus alphavirus*) are positive strand RNA viruses with a genome size of ~12Kb containing a 5' RNA cap and a 3' polyadenylated tail (J. Strauss & E. Strauss 1994). Sindbis virus is considered a prototypical alphavirus and has been used extensively to understand alphavirus replication. The 5' two-thirds of the genome encodes a polyprotein of nonstructural proteins (nsP) 1 through 4 that are required for viral RNA replication. The nsP1-4 polyprotein is initially translated from the viral genomic RNA to form the nsP1-2-3 / nsP4 complex that produces a negative strand copy of the genomic RNA. The nonstructural polyprotein is cleaved to nsP1/nsP2/nsP3/nsP4 to form the positive strand replicase complex, which produces new genomic RNAs. The positive strand replicase complex also produces 26S RNAs from a subgenomic promoter present on the negative strand RNA later in infection (J. Strauss & E. Strauss 1994). The structural proteins are translated from the 26S subgenomic RNA to express capsid, E2, and E1 glycoproteins that form virus particles.

Production of the subgenomic 26S RNA is dependent on the viral replicase complex binding to the 3' end of the viral RNA and synthesizing a negative sense RNA (J. Strauss & E. Strauss 1994) . The requirement for the viral nonstructural protein replication complex to transcribe a subgenomic RNA and subsequently express proteins encoded on the subgenomic RNA provides a mechanism for expressing foreign genes

only during infection (when the replication complex is present). By inserting the subgenomic promoter sequence upstream of a reporter protein (such as fluorescent proteins or luciferase enzymes), the reporter will only be expressed when virus is actively replicating within a cell, providing the replication complex *in trans*. The alphavirus subgenomic promoter has been used in alphavirus expression systems, which utilize a duplicated subgenomic promoter to express a gene of interest concurrent with virus replication (Foy & Olson 2008; Phillips et al. 2010; Hahn et al. 1992). These double subgenomic recombinant alphaviruses are efficient at expressing reporters, but because the reporter is inserted directly into the viral genome, this approach is limited to virus strains with infectious clones and applies an extra genetic load to the recombinant virus replication. Instead of inserting the reporter into the viral genome (11.7 kb), we inserted the reporter into the mosquito genome (1.38 billion bp), which applies a reduced genetic burden and allows detection of unlabeled, non-recombinant wild-type viruses (Nene et al. 2007). Inserting the reporter RNA into the cell genome instead of the viral genome allows mosquito cells to express the reporter RNA constitutively and upon virus infection, the subgenomic RNA can be synthesized and the reporter protein translated. Olivo et al previously used a similar system to express a luciferase protein in BHK-21 cells during Sindbis virus infection; however, their system was not designed for mosquito cells and the use of luciferase did not allow for visual detection of real-time infection (Olivo et al. 1994; J. Li et al. 2012; Olivo 1996). Alphaviruses are transmitted mainly by mosquito vectors and better tools are needed to monitor transmission between mosquitoes and vertebrate hosts. We have adapted the system described by Olivo et al to function in mosquito cells. Although the luciferase reporter functioned well

in BHK-21 cell culture, we sought to develop a system that would produce fluorescence within mosquitoes when infected by alphaviruses. A fluorescent reporter was used instead of the luciferase to provide real-time visual detection and avoid the difficulty of injecting mosquitoes with luciferin in order to detect the luciferase reporter. The fluorescent reporter protein provides a convenient way to monitor infection as it progresses through mosquito cells.

To visually track alphavirus infection in mosquitoes, we used insect specific promoters to constitutively transcribe reporter RNA constructs in mosquito cells. The reporter RNAs can be replicated by transcomplementing viral proteins and produce a fluorescent reporter protein only during infection. Here we show the ability of our subgenomic reporter constructs to detect alphavirus infection in mosquito cell culture. These results represent the first time an alphavirus subgenomic reporter RNA has been launched from DNA in mosquito cells and demonstrates a new method for detecting alphavirus infection in mosquitoes.

Materials and Method

Plasmid constructs

Reporter RNA constructs were engineered with the baculovirus immediate early promoter (IE3) (Jarvis et al. 1990) for transcription of the reporter RNA in C6/36 cell culture. The reporter constructs were developed from SINV sequences using the TE3'2J/TR339 strain of Sindbis and from SINV replicon pBG254 and pBG60 previously described (Hahn et al. 1992; Lustig et al. 1988; Geiss et al. 2007). The 5' and 3' UTR sequences were included from SINV (5' end to the start of the nonstructural protein and

the 3’ end from the c-terminus of E1 through the poly A tail). The first 143 residues of nsP1 were inserted in frame with an enhanced green fluorescent protein (eGFP) gene followed by stop codons used to identify transfected cells and stop translation initiated from the 5’ end of the reporter RNA. We then inserted the subgenomic promoter sequence followed by the mCherry gene, which will produce a subgenomic RNA that mCherry can be translated from in the presence of transcomplemented nsP1-4 proteins. 50 adenosine residues were added downstream of the 3’ UTR to produce a polyadenylated end (pBG426) as previously described (Avadhanula et al. 2009).

eGFP was removed from pBG426 by BglII restriction enzyme digest and the plasmid was re-ligated to produce plasmid pBG446. Alternatively, eGFP was removed from pBG426 with BglII and replaced with the antibiotic resistance gene puromycin acetyltransferase (PAC) (Geiss et al. 2007), which had been amplified with primers containing BglII sites (BG661- ATGCAGATCTTTCGTGAAGACCC and BG662-CCTGAGATCTGGCACCGGGCTTGC). The PAC gene was ligated into the BglII site, producing plasmid pBG461.

pBG460 was designed using the West African strain of Chikungunya virus (CHIKV) 37997 (pCHIK-37997–5GFP, GenBank accession number EU224271) (Tsetsarkin et al. 2007). The CHIKV sequence included identical regions (5’ UTR, nsP1, SGP) as the SINV sequence (pBG426) and was synthesized by GenScript. The CHIKV reporter sequence was amplified from the synthesized plasmid using a reverse primer with a 5’ end XhoI site and a forward primer with the 5’ end having 20bp overlap with the 3’ end of the IE promoter (BG657-GTTCATGTTGGATATTGTTTCATGGCTGCGTGAGACACACG and BG658-

CGGGCCCTCAAGACTCGAG). The IE promoter was amplified with a forward primer containing a 5' NheI site and reverse primer with 5' overlap of the 5' CHIKV UTR (BG655-GTCGGGTCCATTGTCCGTGTG and BG656-CGTGTGTCTCACGCAGCCATGAAACAATATCCAACATGAAC). The two PCR products were the templates for fusion PCR for 10 rounds followed by addition of primers BG655 and BG658 for 30 more cycles. The resulting full-length fragment was ligated into the NheI and XhoI sites in pBG426.

Additional subgenomic promoters from Western equine encephalitis virus (WEEV)) (pBG447) or CHIKV (pBG448) were inserted into pBG446 by ligating a virus specific SGP/mCherry PCR product into the XbaI site. The WEEV and CHIKV SGPs were amplified with forward primers containing 5' XbaI sites (BG582-TACATCTAGACTCTACGGCTGACCTAAATAGG and BG581-TACATCTAGACTGTACGGTGGTCCTAAATAGG). The reverse primer annealed to the 3' end of mCherry and had a 5' XbaI site (BG576-ATATTCTAGACTACTTGTACAGCTCGTCCATGC).

Cell culture, transfection, and viruses

C6/36 *Aedes albopictus* cells were grown in 6-well cell culture plates with L-15 media containing 10% FBS, 100U/ml penicillin/streptomycin, and 5% NaHCO3. Cells were maintained in a 28°C incubator. C6/36 cells were transfected with Mirus 293T transfection reagent following manufacturer's protocols for 1µg DNA. 12 hours post transfection; the cells were infected with SINV at a Multiplicity of Infection (MOI) of 10. At 36-48 hours post infection, the cells were examined for mCherry expression. Stable

reporter expressing cells were selected with 2μg/ml of Puromycin and drug was replaced every 3-4 days.

TE3'2J SINV virus stocks for *in vitro* infections were produced from plasmid transfection into Baby Hamster Kidney (BHK-21) cells (Steel et al. 2011) . MRE16 5'double subgenomic (ds) GFP and MRE16 5'ds were generated from infectious clones(Pierro et al. 2008). Chikungunya virus (La Reunion strain LR2006-OPY-1) and Western Equine Encephalitis virus (McMillian strain) infections were performed in biosafety level 3 (Tsetsarkin et al. 2006; Mossel et al. 2013). The CHIKV LR strain was used because we had access to an infectious clone containing a 5' duplicated subgenomic promoter and GFP gene for visual detection of infection. Sindbis and West Nile virus (Kunjin subtype) (KUNV) infections were performed at biosafety level 2 (Stahla-Beek et al. 2012). 24 hours after plating cells or 12 hours post transfection, cells were infected by replacing media and adding in sufficient amounts of virus for desired MOI.

Fluorescence quantification

Fluorescent images were acquired with a Nikon Diaphot 200 inverted fluorescent microscope. Images were analyzed and fluorescence was quantified using Image J software (Collins 2007; J. Y. Kim et al. 2013). Specifically, images were separated to RGB color channels. The red or green stack was selected and the threshold was adjusted to detect only fluorescent cells that were brighter than negative controls. Following the threshold adjustment, fluorescent cells were analyzed for pixel counts, total area, average size, area fraction, and integrated density. Relative fluorescence

corresponds to integrated density (intDen) value. Fold change in fluorescence was determined by dividing intDen values of infected images by the uninfected control.

RNA and protein quantification

RNA was extracted using Trizol reagent as previously described (Khoo et al. 2010) . Northern Blots to detect RNAs were performed with DIG-labeled RNA probes (Roche DIG Northern kit) specific for the mCherry gene in the reporter and subgenomic RNA (S. W. Kim et al. 2010). qRT-PCR was performed using the Brilliant III Ultra-Fast SYBR Green QPCR kit (Stratagene, Agilent Technologies) with primers corresponding to the mCherry gene. Total protein was extracted from triturated mosquitoes in PBS. Primary rabbit anti-GFP antibody (Cat#ab290, Abcam, Cambridge, MA) or mouse anti-mCherry antibody (ab125096, Abcam, Cambridge, MA) were used to detect GFP and mCherry in western blot assays. Secondary goat polyclonal antibodies conjugated to horseradish peroxidase that are anti-rabbit (anti-rabbit, Cat#ab97051; anti-mouse; Cat#ab97023 Abcam, Cambridge, MA) were used to detect primary antibodies. Northern and Western blots were imaged using a Chemidoc XRS for chemiluminescent detection with HRP peroxide/luminol (Thermo-Scientific, Rockford IL) and CDP-Star (Sigma-Aldrich, St. Louis MO).

Results

Rationale for design of Sindbis virus subgenomic reporter constructs

Sindbis virus infection was detected through virus-specific transcription of a subgenomic RNA containing a fluorescent mCherry reporter gene. Plasmids were

engineered to contain alphavirus RNA elements (5’ UTR, SGP, 3’ UTR) with reporter proteins replacing the viral proteins. The 5’ end of the reporter genome was aligned with the transcription start site of the DNA pol II baculovirus IE promoter to allow full-length reporter RNAs to be transcribed in mosquito cells (Steel et al. 2011). Alternatively, as a confirmatory control in mammalian cells, the cytomegalovirus (CMV) promoter was used in some constructs to initiate transcription in BHK-21 cells, similar to the work done by Olivo et al (Data not shown). The 5’ and 3’ UTRs were maintained to allow the reporter RNA to be replicated in the presence of viral non-structural proteins (Hardy & Rice 2005; Frolov et al. 2001). The nonstructural proteins were removed from the 5’ open reading frame, except for the first 143 amino acids of nsP1 which contain conserved sequence elements required for RNA replication (J. Strauss & E. Strauss 1994) (Figure 3.1). Initial constructs contained an eGFP gene fused to the nsP1 fragment that allowed for visual detection of cells expressing the full-length reporter RNA (Figure 3.1 and 3.2). The structural proteins, which are encoded from a subgenomic RNA at the 3’ end of the genome, were replaced with a mCherry fluorescent protein under control of the subgenomic promoter. The IE promoter drives expression of the reporter RNA in transfected mosquito cells which was evident by visual detection of eGFP expression (Figure 3.2A). Similarly, constructs containing the CMV promoter allowed for transcription and detection of eGFP in BHK-21 cells (Data not shown). Open reading frames located at the 3’ end of the genome cannot be translated from the full length reporter RNA due to stop codons upstream of the subgenomic promoter. Transcription from the subgenomic promoter, which is initiated by the nonstructural protein replication complex, generates a short subgenomic RNA that is competent for mCherry translation.

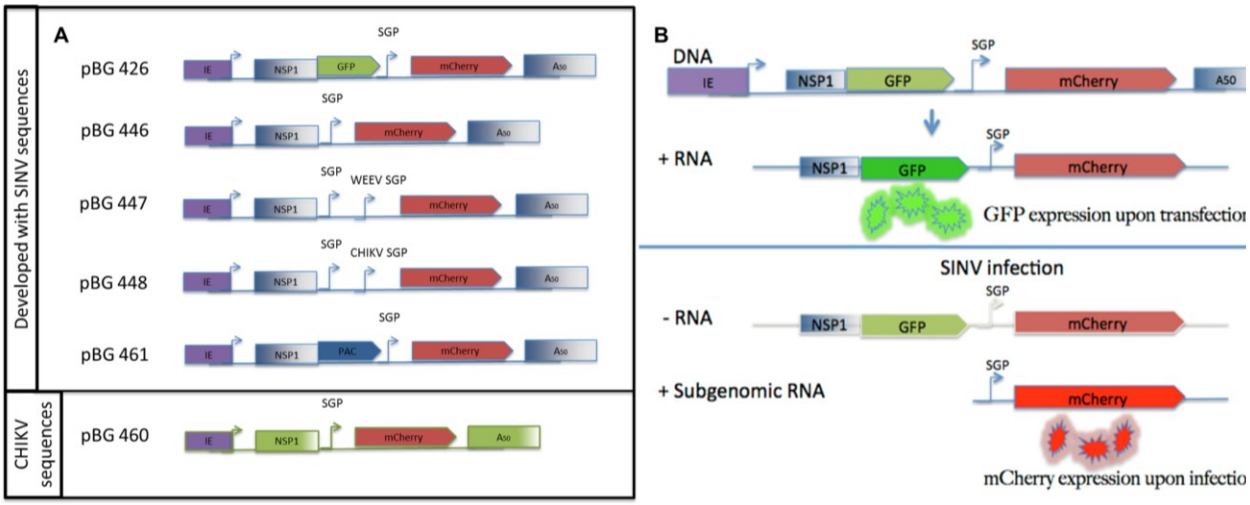

Figure 3.1 Diagram of subgenomic reporter RNA constructs

A) Diagram of the individual constructs. They all contain the IE promoter and a segment of nsP1 which has CSE. Various reporters and subgenomic promoters have been inserted as indicated by the figure. All of the constructs contain a 50 adenosine tail at the 3' end. **B)** Diagram of Reporter RNA. Plasmid DNA with reporter constructs can be transfected into cells and RNA is transcribed from the baculovirus IE promoter. The resulting positive strand reporter RNA can be translated from the 5' end and GFP is expressed. The cells continue transcribing the reporter RNA and expressing the 5' gene with or without virus. During alphavirus infection, the replication complex would bind the reporter RNA, transcribe a negative strand copy of the reporter, which would then provide an active subgenomic promoter to initiate transcription of the subgenomic reporter RNA and expression of the reporter protein (mCherry)

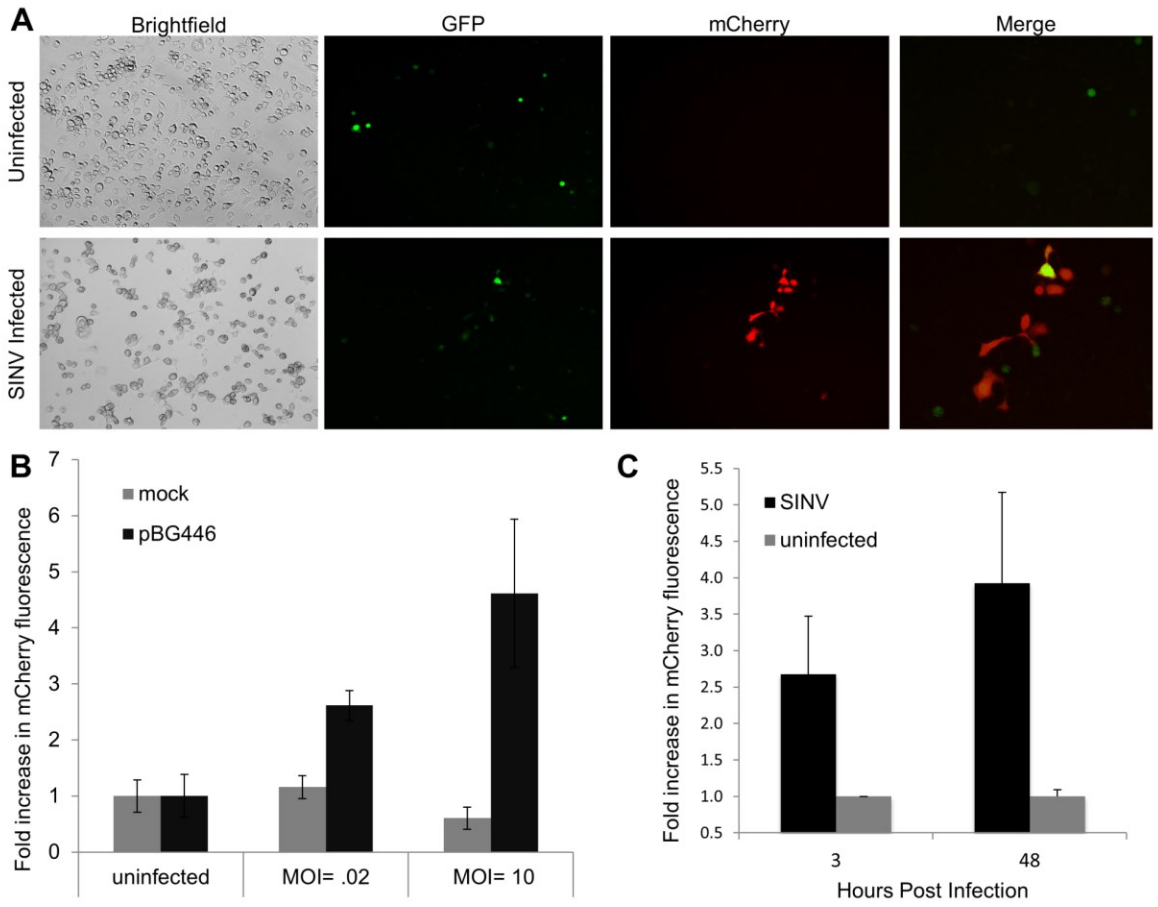

Figure 3.2 Expression of mCherry reporter during SINV infection

C6/36 cells were transfected with pBG426 and subsequently infected with pBG167 TE3'2J SINV for 36 hrs. **A)** Brightfield, GFP, and mCherry fluorescence was determined for each sample. Uninfected (top) and infected (bottom). **B)** Fluorescence increase with different MOIs of SINV. C6/36 cells were transfected with pBG446 and infected for 36 hrs at the indicated MOI. Images were collected from each experimental well and the mCherry fluorescence was quantified. **C)** Increased fluorescence with increased infection times. C6/36 cells were transfected with pBG446 and infected at MOI = 10. Images were collected at the indicated times and fluorescence quantified.

The full-length reporter RNAs do not contain the open reading frames for the nonstructural proteins, so the reporter RNA can only be replicated and produce a subgenomic RNA when replication-competent virus infects the cell and provides the nsP1-4 proteins *in trans*. The infecting virus translates its own nonstructural replication complexes, which bind to the full-length reporter RNAs already present in the cell and replicates the reporter RNA. The subgenomic RNA is transcribed from the negative strand copy of the replicating full-length reporter RNA, and the fluorescent mCherry protein is translated from the newly synthesized subgenomic RNA (Figure 3.1B).

Reporter RNA constructs can detect infection in mosquito cells

Reporter RNA expressing plasmids transiently transfected into *Aedes albopictus* C6/36 cells show eGFP expression within 6 hours after transfection, indicating transcription of the reporter RNA is occurring within the transfected cells. The low number of eGFP positive cells reflects the low transfection efficiencies commonly observed with C6/36 cell transfection. mCherry expression is not observed in uninfected cells, whereas mCherry fluorescence can be observed following infection with Sindbis virus (Figure 3.2A). Interestingly, we observed that cells that strongly expressed eGFP tend to have reduced mCherry expression upon infection, and we conversely observed that cells expressing low levels of eGFP tend to display higher levels of mCherry following infection. mCherry fluorescence was quantified from images collected at given time points and multiplicity of infections. Significant expression of the reporter was detected with a virus MOI of as little as 0.02 at 36 hours post infection and infection with an MOI of 10 provided significant mCherry fluorescence at 3 hours post infection

(Figure 3.2B and 3.2C). These results indicate that infectious Sindbis virus can provide the replication complex in trans to activate the subgenomic promoter on reporter RNAs and express subgenomic RNA encoded proteins. We removed the eGFP to simplify the system (pBG446), and consistently observed mCherry expression during SINV infection but not in the absence of SINV (data not shown). Therefore, this system is able to visually detect SINV infection in cultured mosquito cells.

Reporter RNA expression is virus specific

To determine if activation of the subgenomic promoter on the reporter constructs is virus specific, we tested if related alphaviruses or unrelated flaviviruses could activate the Sindbis virus subgenomic promoter. C6/36 cells were transfected with pBG446 and infected with different viruses. mCherry expression was visually detected by fluorescence microscopy at 36 hours post infection (MOI 10) and fluorescence intensity was calculated. Infection with two strains of SINV (TE and MRE) resulted in a significant increase in mCherry expression, indicating that the detection was not strain specific (Figure 3.3A). However, infection with a flavivirus (KUNV) or a New World alphavirus (WEEV) (Weaver et al. 1993) did not result in detectible mCherry expression (Figure 3.3A). Old World Chikungunya virus (CHIKV) was able to induce expression of mCherry, although to a lesser extent than Sindbis virus infection. This confirms that the viral replication complex has specificity for binding to the reporter RNA sequences, likely the 5’ or 3’ UTR in conjunction with the SGP, and that some cross-reactivity can be observed between related alphaviruses (Hardy & Rice 2005; Frolov et al. 2001).

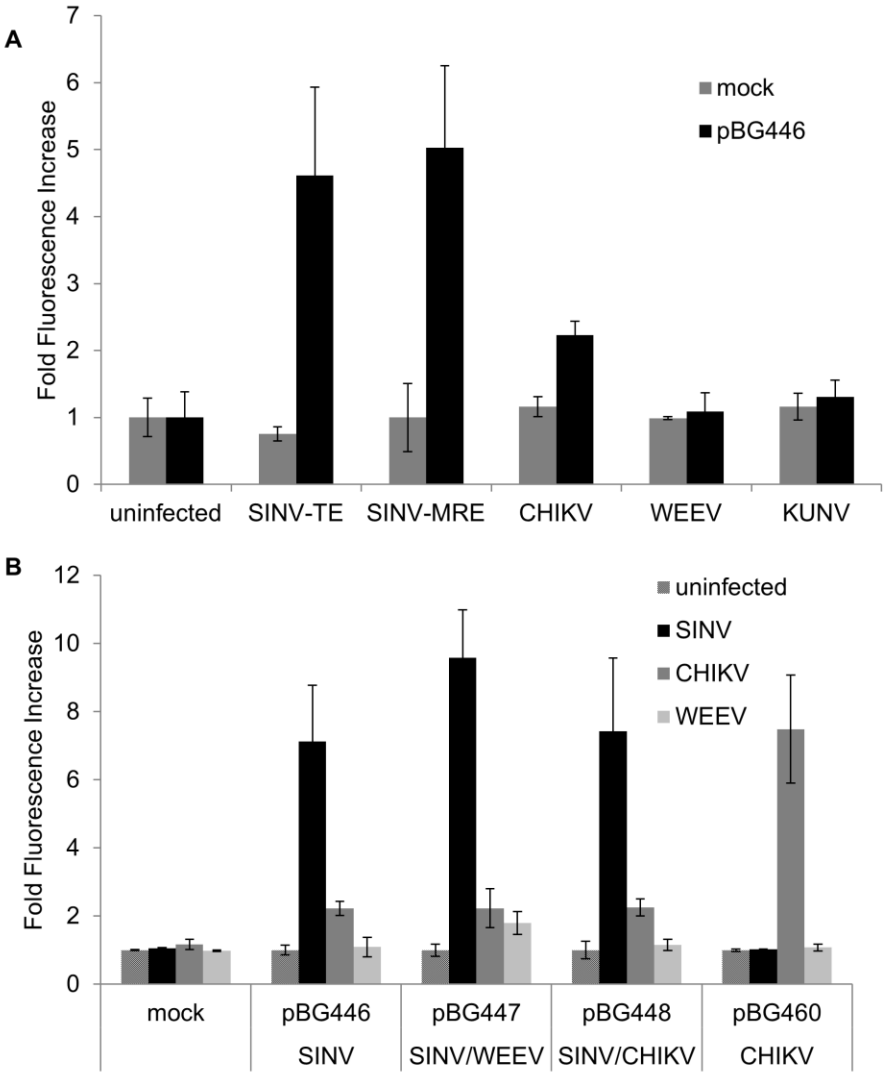

Figure 3.3 Fluorescent reporter expression is virus specific

A) C6/36 cells were transfected with pBG446 and then infected with different viruses (SINV (TE3'2J strain), SINV (MRE16 strain), CHIKV (La Reunion strain), WEEV (McMillan strain), and West Nile (Kunjin subtype). **B)** Virus-specific subgenomic promoter constructs for WEEV and CHIKV (pBG447 and pBG448), pBG446 (SINV specific), and pBG460 (CHIKV specific) were transfected into C6/36 cells and subsequently infected with SINV, CHIKV, WEEV, or uninfected mock control. All Images were taken at 36hrs post infection and mCherry fluorescence was quantified.

The subgenomic promoter is not sufficient to induce subgenomic RNA synthesis

To determine if this system could detect a broad range of alphaviruses, an additional subgenomic promoter was inserted behind the SINV SGP. Theoretically, a reporter system with multiple SGPs from different viruses would be able to detect infection of any of the viruses. The WEEV or CHIKV subgenomic promoter sequence was added 3' to the SINV subgenomic promoter in the SINV construct (pBG447 and pBG448- Figure 3.1). C6/36 cells transfected with pBG447 or pBG448 and infected with WEEV or CHIKV respectively, did not result in significant expression of mCherry (Figure 3.3B). However, a construct that was engineered and developed entirely based on CHIKV sequences showed detectable amounts of mCherry fluorescence during CHIKV infection but not with other viruses (Figure 3.3B). These results indicate that virus specific 5' UTR, 3' UTR, and subgenomic promoters are all required for the production of the 26S RNA from the reporter RNA and subsequent protein expression.

Stable cell line expressing reporter RNA

To determine if mosquito cells could be stably transformed to express alphavirus reporter RNAs, C6/36 cells were transfected with reporter constructs containing a puromycin acetyltransferase (PAC) gene (pBG461- Figure 3.1) and transfected cells were selected with puromycin treatment. A bulk stable cell line was established that expressed the reporter RNA constitutively at low levels (Figure 3.4B) Once the cells were infected with SINV, the subgenomic reporter RNA was synthesized and the mCherry reporter could be detected through visual fluorescence, western blot, and RNA analysis (Figure 3.4). mCherry reporter protein was detected during infection, but not in

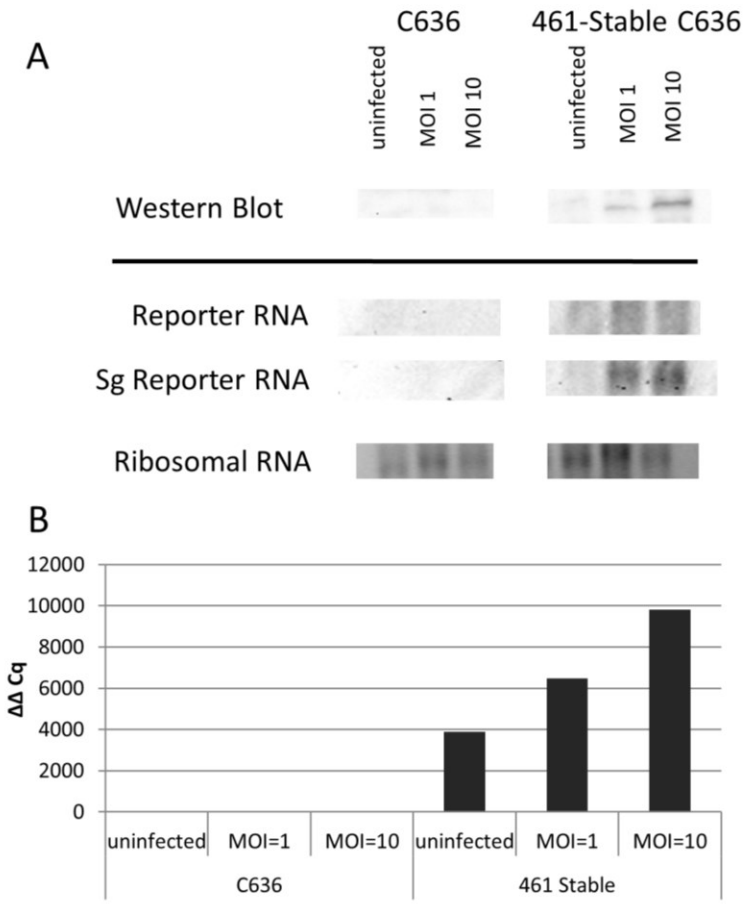

Figure 3.4 Stable C636 cell line with reporter RNA construct

A) Western blot (top) with IgG directed to mCherry. Protein is only detected in infected table C636 cells (461). Northern Blot analysis (below) with a probe targeted to the mcherry gene. No binding is seen in wild type C636 cells, but significant reporter RNA in 461 stably transformed cells and subgenomic RNA during infection**. B)** qRTPCR with primers targeting the mCherry reporter show no reporter RNA in C636 wild type cells, but significant levels in the transformed cells (reporter RNA) and an increase during infection (reporter and subgenomic RNA).

untransfected C6/36 or uninfected control cells (Figure 3.4). Reporter detection was significantly higher with the stable cell line (pBG461) than transient transfection (pBG446) during SINV infection (Figure 3.5A and 3.5B). Infections were performed with a recombinant double subgenomic Sindbis virus that expresses eGFP (SINV-GFP) to visually track infection (green) and confirm the reporter (red). Interestingly, cells that are not highly infected (low amounts of SINV-GFP) tend to have higher reporter mCherry expression. Although the entire stable cell line is resistant to puromycin, indicating that all cells are transformed with our construct, only 5-6% of infected cells expressed detectable amounts of mCherry (Figure 3.5C). This implies that there is an intricate balance of reporter RNA and infection that needs to be achieved in order for the reporter to be detected. The cell line has been maintained for over 18 months, with consistent ability to detect SINV infection. These results indicate that reporter RNAs can be stably expressed in mosquito cells and detect alphavirus RNA replication.

Induction of an inhibitor protein during infection

The reporter RNAs used in this study express fluorescent proteins during infection and allow visual confirmation of Sindbis virus infection. However, a system similar to this could be engineered to express other proteins only during active virus replication. An interesting application for this technology would be to engineer reporter RNAs that encode a cytotoxic or mosquitocidal gene which would only be expressed concurrent with virus infection. Mosquito cells could constitutively express the reporter RNA and once a virus infected the cell, it would induce the expression of an inhibitory or

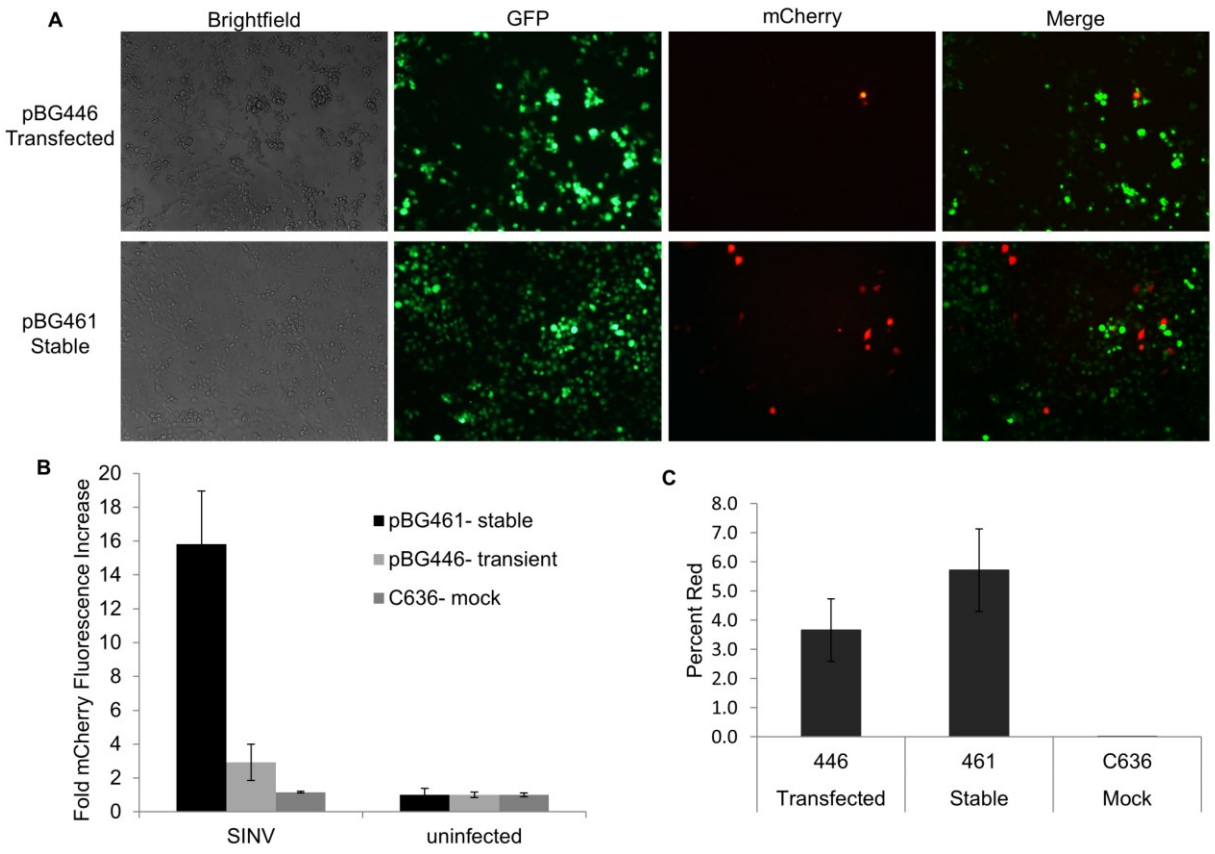

Figure 3.5 Stably transformed C636 cells express reporter mCherry during SINV infection

A) Brightfield, GFP, mCherry, and overlay images are shown from SINV-GFP infected C636 cells that are pBG446 transiently transfected (top) or pBG461 stably selected cells (bottom). Infected with an MOI of 10. Images collected at 48hours post infection. **B)** Average mCherry fluorescence is significantly higher in pBG461 stably transformed C6/36 cells than transiently transfected cells. **C)** Averages of total red fluorescence was calculated as a percent of total green fluorescence and is displayed for pBG446

detrimental protein, causing the cell or mosquito to die and prevent the infection from continuing.

To test this potential application, we acquired a Saporin ribosomal toxin gene that has been shown to arrest protein synthesis and results in cell death(Fabbrini et al. 1997; Puri et al. 2012; Kaur et al. 2011). We inserted this Saporin gene in place of the mCherry reporter protein, resulting in new reporter constructs containing the saporin reporter instead of a fluorescent marker. Transfection of cells with this construct reduced the subsequent infection in all MOI's tested (Figure 3.6A). Analysis of virus inhibition was monitored using a dsSINV expressing Renilla Luciferase, which provided quantitative values for viral replication. Cell viability was monitored using the Cell Titer Glo assay (Promega), which measures cellular ATP as an indication of the metabolic activity within the cell. Specifically, cells that are alive and healthy can generate ATP, but cells that are dead or altered metabolically cannot replenish ATP and have a lower Cell Titer Glo result. Cells that had the saporin construct reduced virus replication significantly and maintained cell viability as compared to mock-transfected cells that had high viral infection and low cell viability (Figure 3.6B and 3.6C). The ability of this death-upon-infection system to inhibit viral replication appeared to be promising; however the efficacy was limited to early during infection. Later time points post infection resulted in less obvious of an effect, possibly due to the death of the transfected cells containing saporin. This is most likely due to low transfection efficiencies, which results in a sub-optimal population of cells containing the saporin construct, leaving many cells available to be infected, especially during high concentrations of virus or later once the transfected cells have died. In order to circumvent the transient transfection issue, we

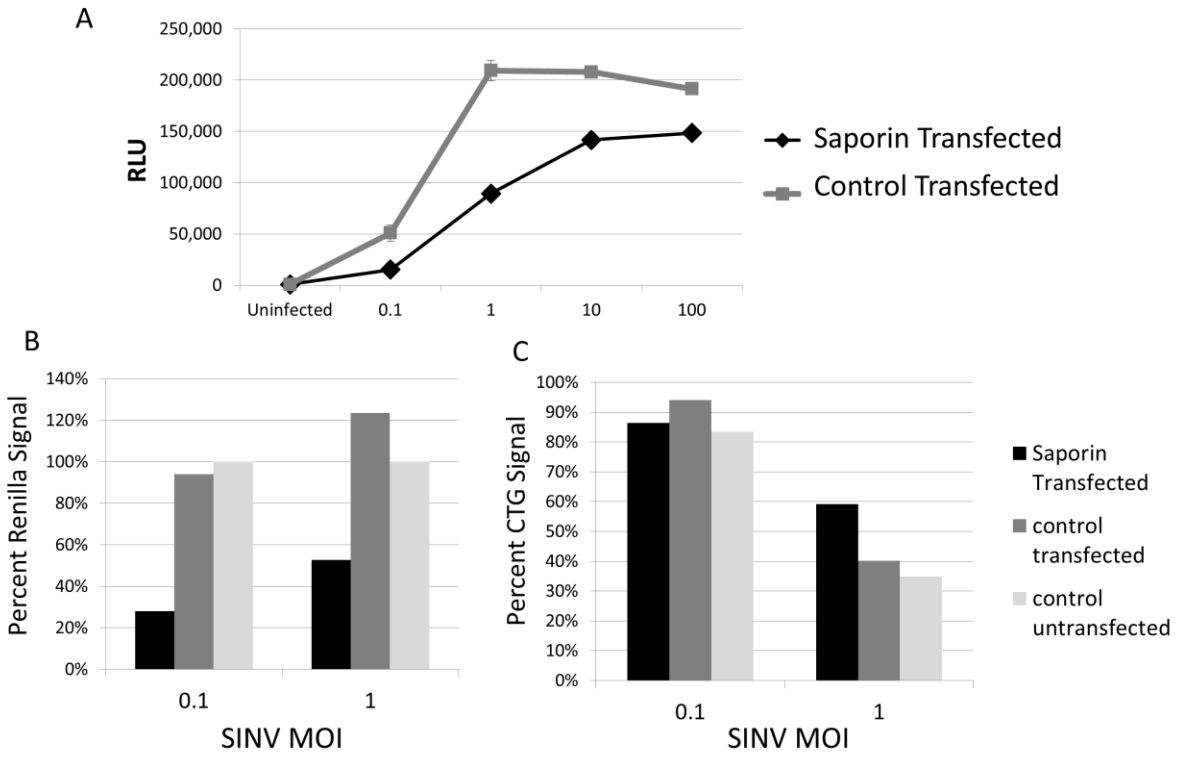

Figure 3.6 Cytotoxic saporin induced expression during infection

A) Cells transfected with a saporin reporter RNA construct show reduced virus based on Renilla Luciferase detection from a dsSINV-REN. Control transfected cells have higher levels of renilla luciferase and implied virus replication. **B)** Viral Replication. At two given MOIs, 0.1 and 1, the saporin construct significantly inhibits viral replication. Mock or untransfected cells have high levels of viral replication. **C)** Cell Viability. Due to the inhibition of the virus infection, cells containing the saporin construct have higher Cell Titer Glo values than cells that have no saporin and uninhibited viral replication. Cells were infected 12 hours post transfection and luciferase signal was detected 12 hours post infection.

sought to establish a stable cell line that contains the saporin reporter RNA construct. Efforts to generate this stable cell line have not been successful. This is potentially due to the selection of highly active transformed cells with the puromycin treatment, resulting in survival of cells that have high levels of the reporter RNA. With high amounts of reporter RNA, there is a potential for leaky or low levels of expression of saporin, which may lead to cell death overtime and inhibit the ability to establish a stable cell line. Despite the difficulty of generating a stable cell line that would express the cytotoxin saporin during infection, the transient transfection data is encouraging. This suggests that the subgenomic reporter RNA system could be used for other applications besides expressing a reporter protein to help detect or visually track infection. Cytotoxic, insecticidal, or other inhibitory proteins could be inserted behind the SGP to allow specific virus-induced expression.

Discussion

This chapter describes a novel approach for detecting and monitoring Sindbis virus infection in mosquito cell culture. We have developed a reporter RNA system that expresses an engineered alphavirus RNA in mosquito cells that only produces a subgenomic RNA and reporter protein in the presence of actively replicating alphaviruses. Initial work by Olivo et al designed a reporter RNA system that expressed luciferase in BHK-21 cells during infection. We have modified their method to be more applicable for studying alphaviruses in the natural mosquito vector and provide a way to visually track infection with a fluorescent reporter. This new system allows for rapid and simple visual detection of wild-type alphaviruses in mosquito cells. Transiently

transfected or stably selected C6/36 cells transcribing reporter RNAs expressed significant levels of reporter genes during infection and were only activated by related alphaviruses. These results indicate that a subgenomic reporter RNA system can be used to diagnose and detect alphavirus infection in mosquito cells.

The sensitivity and quick detection of the fluorescent subgenomic reporter RNA system in cell culture provides potential applications for diagnostic tests to quickly and accurately identify virus infection. Standard diagnostics require significant time and resources to identify an infecting virus. With the subgenomic reporter RNA system, virus specific constructs (SINV, CHIKV, WEEV) could be developed that would express a visibly detectable fluorescent reporter during specific viral infections within hours and with small samples, providing a novel method for diagnosing alphavirus infections

The species of alphavirus that activated the reporter RNAs was relatively narrow. The SINV reporter RNAs expressed significant levels of mCherry during infection by two different strains of SINV and a lower level of mCherry during CHIKV infection, but the SINV reporter RNAs were not able to produce mCherry when infected by WEEV. SINV and CHIKV are both Old World alphaviruses, indicating some level of functional conservation between the SINV and CHIKV replicase complexes and RNA elements. New World alphaviruses such as WEEV (whose nonstructural replicase proteins are derived from Eastern Equine Encephalitis virus) were not able to induce mCherry expression, indicating that Old and New World alphavirus replicase complexes are not interchangeable. The observation that addition of a virus-specific subgenomic promoter to the SINV reporter construct did not result in mCherry expression indicates that the virus specific 5’ and 3’ UTRs are required for negative strand RNA and subgenomic

RNA synthesis. A CHIKV specific reporter RNA showed similar specificity as the SINV system, demonstrating that this approach is applicable to different types of alphaviruses and that species specificity can be achieved with this approach. A potential application in future systems is to integrate multiple species-specific reporter RNAs with different fluorescent proteins into mosquito genomes that would be able to detect multiple alphavirus species in the same mosquito. Because the RNA expressed in this system is small compared to the full-length alphavirus genome, it will prove a useful tool for dissecting the RNA and protein requirements for alphavirus RNA replication in mosquito cells in addition to its utility in identifying viral species.

Interestingly, when we expressed reporter RNAs in C6/36 cells by transient transfection, the cells with the highest levels of eGFP expression did not express the reporter mCherry well when infected. Cells that displayed low amounts of eGFP expression (and by extension lower amounts of reporter RNA) expressed higher levels of mCherry upon infection. We have not yet determined the reason for this dichotomy, but there are several possibilities for this effect. The Sindbis virus 5' and 3' UTR present on the reporter RNA bind to the viral non-structural proteins, and an excess of the reporter RNA may sequester the non-structural proteins away from the full length RNA genome and reduce viral RNA replication. However, our stable cell line does not show a reduction in virus replication when compared to control C6/36 cells. Alternatively, high levels of the reporter RNA may reduce the overall level of translation in cells and reduce translation of the viral genomes to produce nonstructural proteins (Patel et al. 2013). It may prove beneficial in subsequent versions of this system to use a less robust promoter to drive reporter RNA transcription in mosquito cells and decrease the level of

subgenomic reporter RNA. Regardless of the variability, we consistently see activation of the subgenomic promoter and expression of the reporter protein during infection in cell culture.

Instead of a fluorescent reporter protein being expressed during infection, we also tested the ability to insert a cytotoxic or cellular inhibitory protein behind the subgenomic promoter to allow expression only during infection. We hypothesized that the cytotoxic gene would not be expressed in uninfected cells, but if the cell became infected, the toxic protein would be expressed and the infected cell would then die, making the infecting virus unable to escape and continue infection. We tested this idea using a ribosomal inhibitor protein called saporin. Transient transfection worked well to inhibit viral infection, with significant inhibition being seen at early time points of infection. Other toxic or inhibitory proteins could be inserted for similar purposes. A mosquito-cidal gene could potentially be inserted into the subgenomic reporter RNA system and transgenic mosquitoes could be made to express these constructs. When the mosquito would get infected, it would express the mosquito-cidal gene and the mosquito would die, preventing the mosquito from taking a bloodmeal and transmitting the virus. Multiple applications could benefit from this subgenomic reporter RNA system to express specific proteins only during infection. The ability to regulate and limit expression of a foreign protein to only during infection could be very useful in studying viral infection in more ways than just visually detecting infection.

This subgenomic reporter RNA system successfully detects alphavirus infection in mosquito cell culture(Steel et al. 2013). The use of the baculovirus promoter and fluorescent reporter protein allows for visual detection of alphavirus infection directly in

mosquito cells, representing the first time this detection ability has been described. Infection can be visibly identified due to the fluorescent protein expression during infection, whereas uninfected and mock-infected controls have no fluorescence. Mosquito cells can be stably transformed with the constructs and expression of the reporter protein is only detected during infection. Because activation of the subgenomic reporter RNA is species specific and sensitive to low amounts of virus, the expression of reporter proteins through the subgenomic reporter RNA system can potentially be used as a diagnostic tool. This system could further be modified and used to limit gene expression of other foreign proteins to only during infection. Overall, the mosquito-specific subgenomic reporter RNA system is efficient at expressing a reporter protein only during infection and can be used for further applications, including generating transgenic subgenomic reporter RNA mosquitoes (See Chapter Four).

Chapter Four: Transgenesis of *Aedes aegypti* with Subgenomic Reporter RNA Constructs

Introduction

Mosquitoes have been genetically modified over the last decade to express foreign proteins or RNAs which have helped increase our understanding of arbovirus infection. Transgenic mosquitoes have been established that impair infection, reduce escape from midguts, or have weakened vector competence, but a transgenic mosquito has never been generated to detect wild-type virus infection (Khoo et al. 2010; Bian et al. 2005; Dong et al. 2011). Current methods to detect infection in mosquitoes either require the sacrifice of the insect and post-mortem analysis for infection, or the use of double subgenomic recombinant viruses for detecting infection in live mosquitoes. Post-mortem analysis is accurate and virus can readily be detected using immunofluorescence, plaque assays, or qRT-PCR. However, post-mortem analysis does not allow identification of infected mosquitoes for studying downstream infection consequences or transmission studies. The double subgenomic viruses provide a mechanism for expressing a fluorescent reporter protein with the virus, allowing for visual tracking of infection, but recombinant viruses are highly modified and do not accurately represent wild-type virus infection.

With our positive results from the subgenomic reporter RNA system in mosquito cell culture, we sought to establish a transgenic line of mosquitoes that would express a fluorescent reporter during infection and provide an effective detection system for wild-type alphavirus infection in living mosquitoes. A transgenic mosquito line that would

express a visibly detectable fluorescent reporter only during infection would be a valuable tool for studying the whole virus life cycle and witnessing how mosquitoes get infected and how they transmit the virus to subsequent hosts. This represents the first time a transgenic mosquito has been established to visually detect infection in live mosquitoes.

Materials and Method

Plasmid constructs

The IE3 promoter was replaced with the *Ae. aegypti* poly ubiquitin promoter (PUb) (Anderson et al. 2010) and inserted into the transposon backbone containing the 3xP3 promoter and an eGFP gene for mosquito transgenesis(Berghammer et al. 1999). The IE3 promoter was replaced with the PUb promoter through overlapping PCR amplification. The SINV reporter construct was PCR amplified with a forward primer containing 5' 19bp overlap with the PUb and a reverse primer with a terminal AscI site (BG 671- GCAAAGGCAAAACCAGCTCATTGACGGCGTAGTACACAC and BG 675- CTGGCGCGCCGCCCTCAAGACTCGAG). The PUb fragment was PCR amplified with a forward primer containing a 5' AscI site and a reverse primer with 5' 20bp overlap with the beginning of the reporter construct. (BG669- CTGGCGCGCCTATCTTTACATGTAGC and BG670- GTGTGTACTACGCCGTCAATGAGCTGGTTTTGCCTTTGC).The two PCR fragments were fused for 10 rounds and then primers BG669 and BG675 were added to amplify the fusion PCR product. The PCR product and destination plasmid were digested with

AscI and ligated into the transposon backbone (pMos[3xP3-eGFPaf]) resulting in pBG471(Berghammer et al. 1999). All clones were verified with sequencing.

Aedes aegypti transformation

Aedes aegypti mosquitoes from the Higgs White Eye strain (HWE)(Zach N Adelman et al. 2004) were hatched and allowed to mature to adulthood. Females were given a bloodmeal consisting of defibrinated sheep's blood (Colorado Serum Company, Denver, CO) 4 days before oviposition. On the day of injection, females were presented with an oviposition paper inside a 50ml conical tube and freshly laid eggs were collected for injection (Zachary N Adelman et al. 2002b). 1,736 eggs were injected at the posterior pole with plasmid pBG471 (Mariner Mos1 and reporter construct) and a helper plasmid containing the mariner transposase gene using an Eppendorf FemptoJet injector (A. Franz et al. 2006). Following injection, eggs were returned to oviposition papers for 4-5 days to mature. The eggs were then placed in water to allow larvae to hatch. The 327-hatched larvae (18% survival) were grown and separated into individual containers as pupae. The emerged adults were pooled together into 65 families depending on gender (1 male with 15 HWE wild-type (WT) females, or 12 female with 3 HWE WT males). Each of the 65 families was bloodfed separately three times and eggs were collected on oviposition papers. The eggs were hatched and the larvae were screened at 2nd or 3rd instar stages for eGFP expression in the eyes of the larvae on a fluorescent dissecting microscope. Nine families contained green eye positive larvae and were outcrossed with HWE for 3 generations, then were intercrossed for subsequent generations. Two families did not produce viable offspring, but the

remaining 7 lines were stable through at least 10 generations. Each generation is screened for positive GFP eye expression as a marker for the presence of the transposon.

Mosquito infections

7-14 day old mosquitoes were orally infected with a blood meal containing GFP-expressing SINV (MRE16 5'dsGFP) (Foy et al. 2004). Virus for bloodfeeds was prepared by infecting Vero cells with the respective virus 36-48 hours prior to bloodfeeds at an MOI or 1.0 (Seabaugh et al. 1998; Pierro et al. 2008). The virus is collected prior to feeding by scraping the cultured vero cells, centrifuging the cells and media to pellet the cells, removing media to desired volume (5mls) and then mixing the infected media and vero cells with 5 mls of sheep's blood and 1 ml Adenosine triphosphate (ATP) (A. W. E. Franz et al. 2009). Mosquitoes were fed through an artificial glass feeder with hog's gut as the membrane. Bloodfed mosquitoes were separated and maintained following the feeding and the bloodmeal was titered to verify virus titers. Mosquitoes engorged with a bloodmeal were assumed infected and at 4dpi, 7dpi, and 14dpi the mosquitoes were screened for GFP (infection) and mCherry (reporter of infection) using a dissecting fluorescence microscope. Midguts were dissected from whole mosquitoes to examine reporter protein expression within internal tissues. Alternatively, whole mosquitoes were triturated, supernatant was filtered through 0.2uM syringe filter, and samples were assayed for virus titers by plaque assay (Khoo et al. 2010).

Results

Development of reporter RNA expressing transgenic *Aedes aegypti* Mosquitoes

Based on our *in vitro* results with the subgenomic reporter RNA detecting infection in cell culture (See Chapter 3), we sought to establish transgenic mosquitoes that could be used to visually detect specific alphavirus infections. Sindbis virus is mainly vectored by *Culex* mosquitoes, but transgenic *Culex* species of mosquitoes have only been successfully generated in one lab, despite several labs attempts(Wilke et al. 2013). *Aedes aegypti* also transmits alphaviruses and has been used many times for generating transgenic mosquitoes. *Aedes aegypti* transgenesis has been well characterized and is routinely performed at Colorado State University by researchers at the arthropod-borne infectious disease laboratories (AIDL), so we decided to make transgenic *Aedes aegypti* mosquitoes to express our reporter RNA constructs and detect infection in living mosquitoes.

In order to develop transgenic *Ae aegypti* mosquitoes that express SINV reporter RNAs and can express mCherry in response to infection *in vivo,* we utilized a transposable element system to insert our construct into the mosquito genome. There are multiple transposon and integration techniques that have been used in mosquito transgenesis, but recent data suggests that although the bacteriophage integrase systems are location specific, they require docking strains and are not more efficient than transposable element systems. Through collaboration with Dr. Alexander Franz, we decided to use the Mariner transposon system (Mos1) as the mechanism for transformation (A. Franz et al. 2006; A. W. E. Franz et al. 2011; Coates et al. 2000; Atkinson et al. 2001). To prepare the constructs for transgenesis and working within the

mosquito, we replaced the baculovirus IE3 promoter with an *Ae. aegypti* poly ubiquitin promoter (PUb) to provide more stable expression throughout the mosquito midgut than has been observed with the IE3 promoter (Anderson et al. 2010). The poly ubiquitin promoter construct (pBG471) worked *in vitro* similar to the previous IE3 construct (pBG446) (Figure 4.1). The transposon contains an eye-specific 3xp3 promoter that expresses eGFP in eyes to identify transgenic mosquitoes (Berghammer et al. 1999) . The reporter RNA sequence was inserted into the mariner transposon with the mariner inverted repeat sequences flanking the reporter RNA construct and the green eye marker. This Mos1 transposon containing the reporter RNA construct was co-injected with a helper plasmid expressing the mariner transposase into 1,736 pre-dermoblast mosquito embryos (Jasinskiene et al. 2007). 327 (18%) of the injected eggs hatched and were outcrossed with wild-type Higgs White Eye (HWE) *Ae. aegypti* mosquitoes to establish 65 families (Figure 4.2). Each family was bloodfed three times and eggs collected. The G1 offspring were screened for the eGFP green eye marker for transgenesis, resulting in 7 viable transgenic families being founded. Each of the Sindbis-induced mCherry (SIM) transgenic mosquito lines was separately hatched, screened, and females were bloodfed to maintain transgenic populations. Transgenic lines were verified each generation through screening for the eye specific eGFP expression from our inserted constructs. eGFP was consistently seen and GFP protein was detected through western blot (Figure 4.3). Initial transgenic families were also screened for the reporter RNA integrated into the mosquito's genomic DNA through PCR analysis (Figure 4.3).

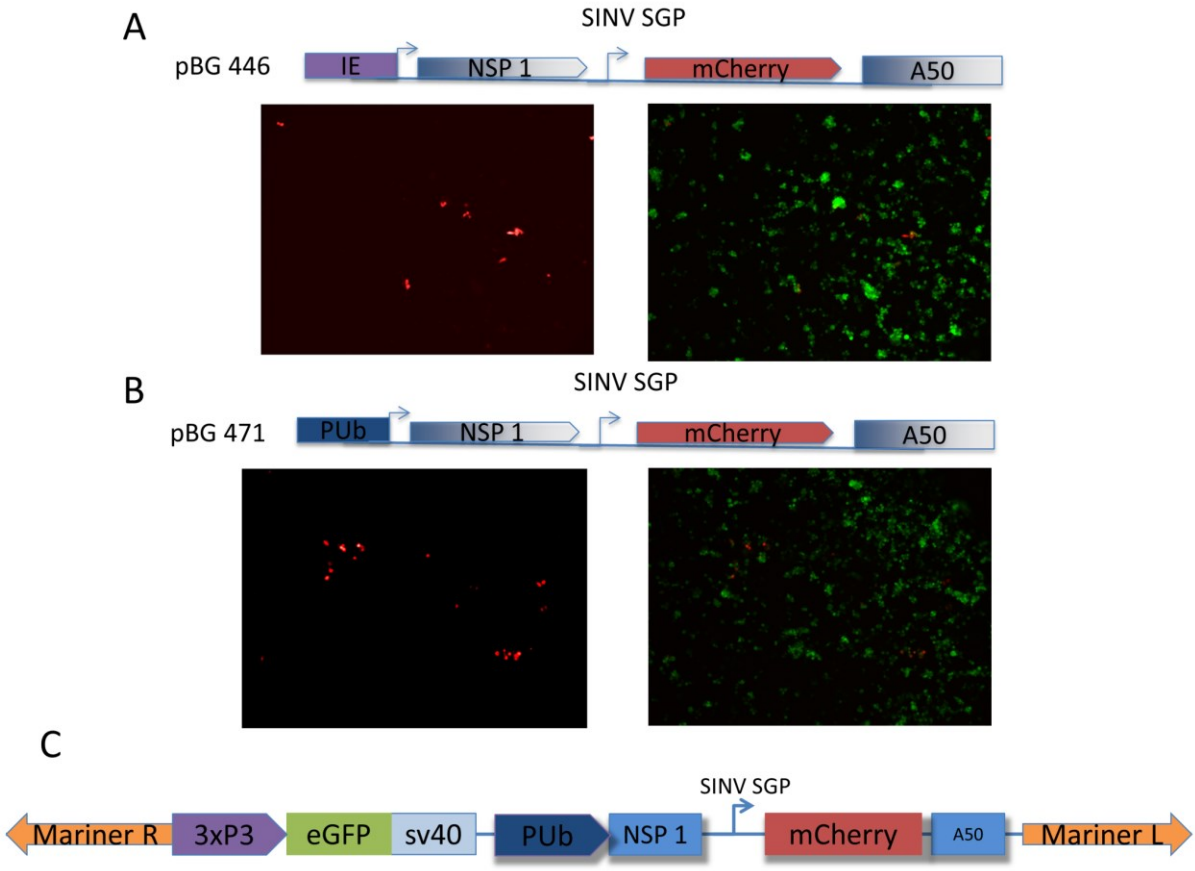

Figure 4.1 Preparing subgenomic reporter RNA constructs for transgenesis

A) The immediate early baculovirus (IE) promoter was replaced with the Aedes aegypti polu ubiquitin promoter (PUb) to provide more robust expression in the mosquito. Both the IE and the PUb constructs detect infection at similar levels in cell culture **(A and B). C)** The PUb construct was inserted into the mariner transposon for transgenesis.

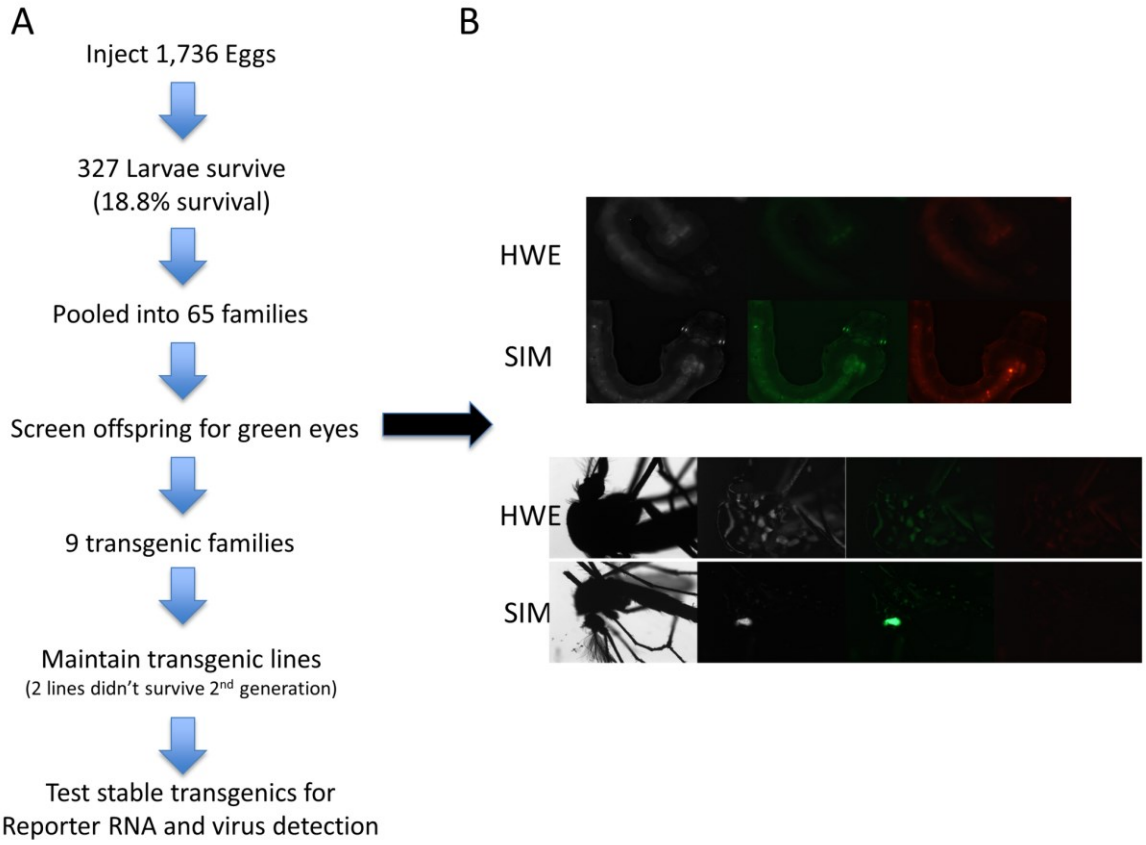

Figure 4.2 *Aedes aegypti* transgenesis with subgenomic reporter RNA construct

A) Flow-chart diagram of A*edes aegypti* transgenesis. **B)** Surviving embryos were screened for the green eye transgenesis marker at the larval and adult stages (B top and bottom, respectively). Higgs White Eyer (HWE) are wild type, non-transformed controls and Sindbis Induced Mcherry (SIM) mosquitoes are transgenics generated and described.

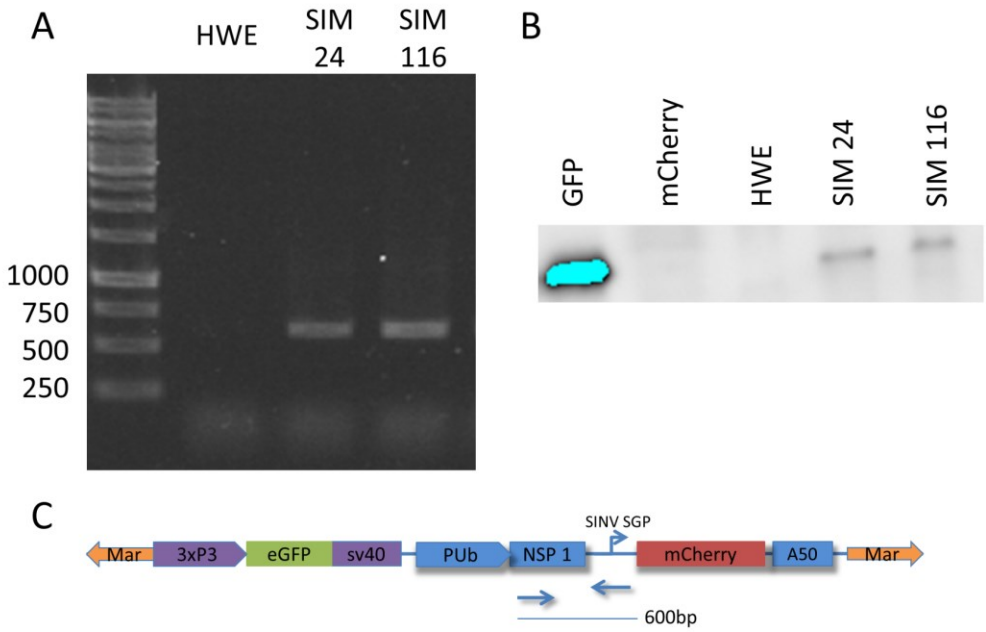

Figure 4.3 Verification of reporter RNA integration in transgenic mosquitoes

A) DNA was extracted from whole transgenic mosquitoes and purified using TriZOL reagent. The DNA was then used in PCR reactions with forward and reverse primers designed from the reporter RNA region of nsP1 and the SGP. Only SIM transgenic mosquitoes showed the positive band for the reporter RNA, but not in the wild type Higgs White Eye (HWE). **B)** Whole mosquitoes were also triturated and protein was used in a western blot to detect GFP (transgenesis eye marker). GFP was detected in the SIM transgenics, but not in the HWE**. C)** Map of primer location for PCR reaction.

Sindbis virus can activate reporter RNAs in transgenic mosquitoes

To test if Sindbis infection could activate the subgenomic reporter RNA in the transgenic lines, we infected the transgenic mosquitoes with an eGFP expressing SINV (MRE16 5'dsGFP) by feeding female mosquitoes a bloodmeal containing 7 logs of infectious virus (Pierro et al. 2003; Seabaugh et al. 1998). Visibly engorged mosquitoes were collected after the bloodmeal and kept for 4, 7, or 14 days post-infection. At the indicated times, mosquitoes were cold anesthetized and eGFP and mCherry expression was assessed under a dissecting fluorescence microscope. Infected mosquitoes were apparent by the eGFP fluorescence and several infected transgenic SIM mosquitoes displayed mCherry fluorescence located in the abdomen (Figure 4.4A). Whole mosquitoes were collected and triturated. Protein was extracted and subjected to western blot analysis using an anti-mCherry antibody to detect mCherry expression. HWE wild-type mosquitoes never showed mCherry expression in either uninfected or infected samples. However, infected SIM transgenic mosquitoes resulted in mCherry expression being detected through western blot. mCherry was not detected in uninfected mosquitoes (Figure 4.4B). Mosquitoes that were visibly infected with the SINV-GFP were sacrificed and dissected midguts were imaged. Midguts provided more obvious detection of mCherry expression in the infected SIM mosquitoes. Uninfected SIM and HWE did not show mCherry expression (Figure 4.4C).

Varying levels of infection occurred throughout the bloodfed mosquitoes and mCherry expression was found to be independent of infection titers. Multiple rounds of infection with each transgenic family were performed using SINV MRE16 5'dsGFP, SINV TE 3'dsGFP, and wild-type SINV MRE16, and mosquitoes were monitored at 4, 7

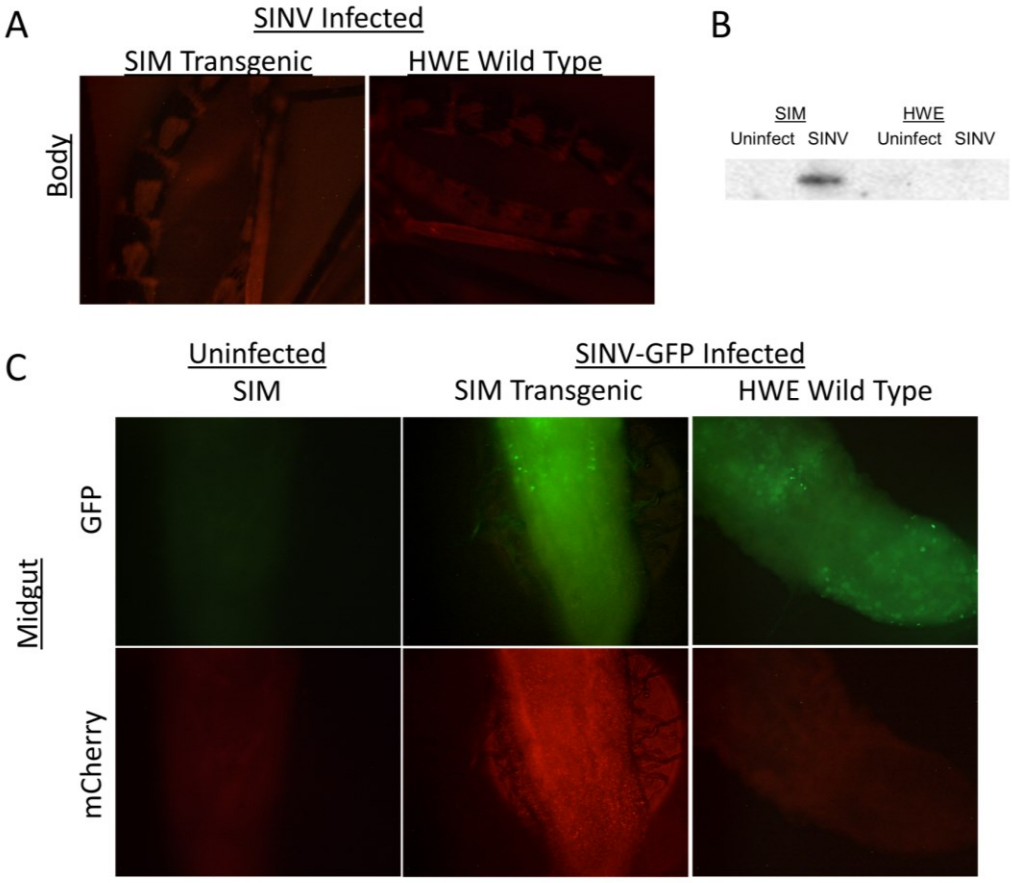

Figure 4.4 Transgenic SIM mosquitoes express mCherry reporter during Infection.

A) Whole bodies of living infected mosquitoes can be screened for mCherry expression. SIM transgenic mosquitoes display a band of mCherry fluorescence, but the wild type HWE do not. **B)** Mosquitoes were collected 7 days post blood meal and were triturated. Protein was extracted and used in a western blot detecting mCherry protein. HWE wild type mosquitoes did not express mCherry. The SIM transgenic mosquitoes did not express mCherry when they were uninfected, but mCherry was expressed following SINV infection. **C)** Midguts can be dissected from the whole bodies and show more obvious expression of the reporter. When infected with a dsSINV-GFP virus, infection in the mosquitoes can easily be seen by GFP fluorescence. Infected HWE and uninfected SIM mosquitoes do not show mCherry fluorescence, but infected SIM mosquitoes show obvious mCherry expression.

and 14 days post-infection for fluorescence. Some mosquitoes had obvious expression and loci of mCherry fluorescence, while other similarly infected mosquitoes did not. Attempts to optimize detection by changing virus titers, strains, or time post bloodmeal, did not seem to increase or affect the detectable amounts of mCherry during SINV infection. Despite inconsistent detection abilities of these transgenic mosquitoes, mCherry was never identified in uninfected mosquitoes, while obvious mCherry expression could be detected in certain transgenic Sindbis-infected mosquitoes.

Approximately 2-4% of the SINV-infected transgenic mosquitoes displayed detectable mCherry expression. Similar low levels of reporter fluorescence was detected in the stable C6/36 cell line, indicating that this system needs to be further optimized to be effective at sensitive diagnostics and modeling of infection. However, despite the low level of reporter expression, mCherry fluorescence was positively detected in bodies and midguts of a subset of mosquitoes (Figure 4.4). mCherry expression by fluorescence or Western blot was never observed in uninfected mosquitoes, indicating that reporter RNA activation and mCherry expression was specific to SINV infection.

Optimization of reporter RNA System

In order to increase the expression of reporter during infection, we attempted several techniques to optimize the reporter detection. Based on *in vitro* data with the subgenomic reporter RNA, we had observed that some cells expressed high amounts of fluorescent reporter, while others had limited to no expression. It seemed highly variable on a cell-to-cell basis. We hypothesized that the expression reporter could be

dependent on different stages of the cell cycle or the virus replication. In order to test this hypothesis, we synchronized the infection by adding virus (MOI=0.3) to cells at 4°C, this would allow the virus to bind the cells but endocytosis has been shown to be temperature dependent and the virions wouldn't be able to enter the cells until the temperature was increased (Lippincott-Schwartz et al. 2000; G. Wang et al. 2007; Vancini et al. 2013). After allowing the virions to bind the cells for 30 minutes at 4°C, the cells were incubated at 37°C to allow endocytosis of the virions and synchronized replication to begin. The cells were checked for fluorescent reporter expression every 2 hours for the first 8 hours, then subsequently at 12, 18, 24, 48, and 72 hours post infection. The attempts to synchronize the infection did not have a significant effect on increasing reporter expression during infection (Figure 4.5). Reporter mCherry expression was still visible within the cells, but the variability persisted.

It appeared that the stage of the virus replication cycle was not critical for detection of infection. We then sought to coordinate the stage of the cell cycle in order to determine if cell division affected the reporter detection. It has been shown that during dengue infection, the S-phase of the cell cycle enhances infection in mosquito cells(Helt & Harris 2005). Hydroxyurea has been used to synchronize cells by inhibiting DNA synthesis during the S-phase of cell cycle(Gerenday et al. 1997). Cells being treated with hydroxyurea are paused in S-phase until removal of the compound. Once hydroxyurea is removed the cells quickly recover and proceed synchronized to go through each stage of the cell cycle. We treated cells with 0.2 mM hydroxyurea for 18 hours to synchronize the cells in S-phase, then following the removal of the drug, infected with Sindbis virus (MOI= 0.3). The infected cells were monitored for mCherry

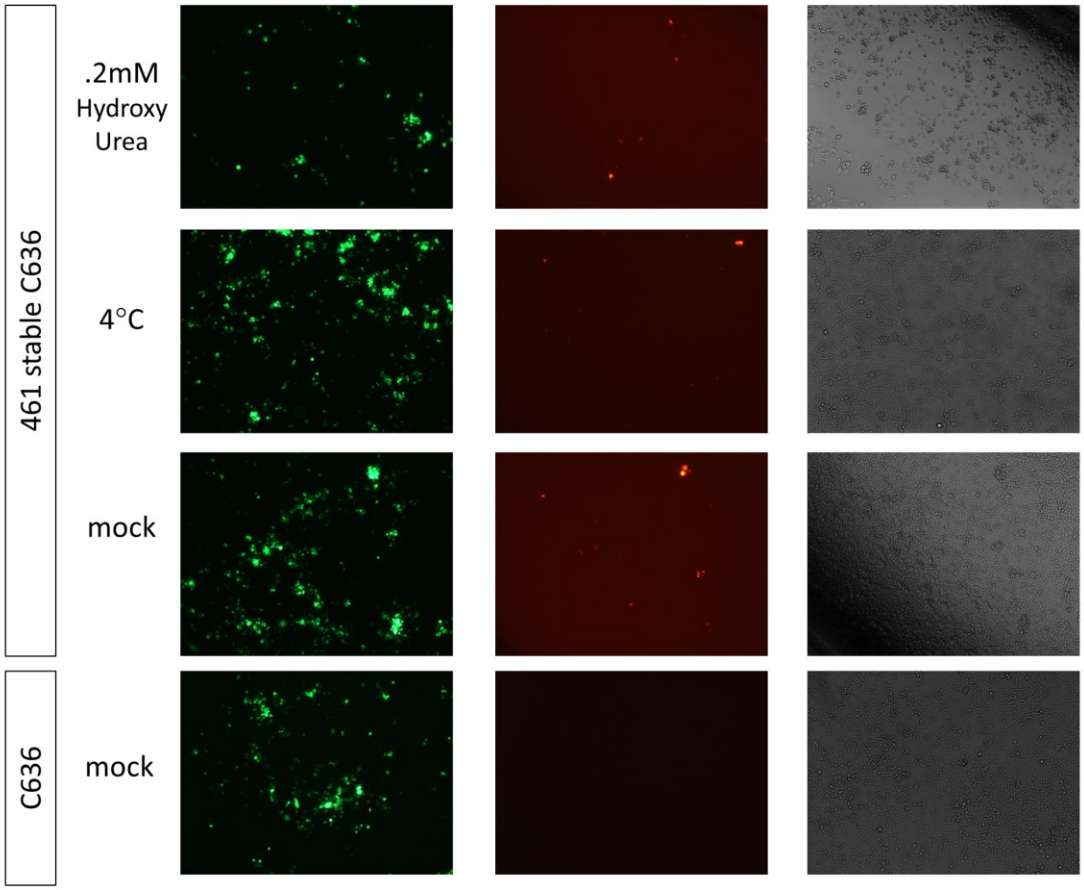

Figure 4.5 Synchronizing the cell cycle and infection in attempt to increase reporter RNA activity.

C636 and C636 stably transformed with pBG461 were treated with 0.2mM Hydroxyurea or left untreated. One plate was incubated at 4C in order to inhibit endocytosis. The cells were infected with dsSINV-GFP (212 virus) at an MOI=0.3. The plate at 4C was incubated for 30 minutes post infection, then was returned to 28C with the other plates. Fluorescence was monitored every 12-24 hours and pictures collected at 48 hours post infection. Treating with 0.2mM Hydroxyurea or incubating at 4C to synchronize infection did not have a significant effect on increasing mCherry reporter fluorescence.

reporter fluorescence every 2 hours for the first 8 hours, then at 12, 28, 24, 48, and 72 hours post infection. mCherry was detected in infected cells, but the synchronization of the cells did not seem to increase the reporter expression as compared to untreated cells (Figure 4.5).

Additionally, we had observed that cells expressing the highest amounts of fluorescent reporter appeared to colocalize with cells that had lower levels of RNA expression. This led to the prediction that less reporter RNA may result in higher expression levels. In order to control RNA levels, we first attempted to use the inducible *Aedes aegypti* heat-shock promoter (AaHSP70 promoter). It has been shown to have controllable levels of transcription based on specific incubation temperatures(Anderson et al. 2010; Gross 2011; Carpenetti et al. 2012). This promoter was acquired from Dr. Zach Adelman's group, but successful expression has not yet been achieved using this promoter due to technical sequence issues.

To quickly determine if RNA levels were affecting reporter expression and detection ability, we decided to attenuate a promoter we already had inserted into a construct instead of trying to completely replace the promoter. To do this, we utilized our mammalian reporter RNA constructs containing a CMV promoter. The mammalian subgenomic reporter RNA constructs are very similar to the insect constructs, but the promoters are different. The insect system has a baculovirus immediate early (IE3) promoter and the mammalian constructs have a cytomegalovirus (CMV) promoter. It has been reported that simply mutating the TATATA box in the CMV promoter to a TCTATA box will reduce transcription by 4 fold(Patwardhan et al. 2009). We performed quick-change mutagenesis to attenuate the CMV promoter and verified the mutation

through sequence analysis. The mammalian constructs were similar to the first insect constructs containing eGFP on the RNA as a marker of transfection and transcription (similar to pBG426 in Figure 3.1). The eGFP provided a mechanism to determine relative levels of transcription. Transfections were performed identically for the wildtype CMV promoter and the mutated CMV promoter constructs, but transfection with the plasmid containing the mutant promoter resulted in less RNA (as indicated by GFP fluorescence), but the percent of cells expressing detectable amounts of mCherry fluorescence was similar to the full strength CMV promoter (Figure 4.6A and 4.6B). These results indicated that the promoter and RNA levels are not solely crucial for the variability of reporter expression and detection.

We hypothesized that the RNA interference response may play a role in our reporter RNA's ability to detect infection, due to the potential formation of double stranded RNA during infection and subgenomic reporter RNA synthesis. It is known that the RNAi pathway is a major innate response to control viral infections in mosquitoes(Blair 2011). Our reporter constructs were potentially forming double stranded RNA and triggering the RNAi response within the cell and silencing the reporter RNA in the mosquito. However, C6/36 cells are known to have a non-functional RNAi response to viral infection, so it was likely that RNAi wasn't the sole reason for the variability in reporter expression(Brackney et al. 2010). To further test the involvement of RNAi in our reporters, we infected cells containing our reporter RNA with a double subgenomic Sindbis virus expressing B2, which is a potent inhibitor of the RNAi pathway (Cirimotich et al. 2009). Cells infected with the SINV-B2 and a control MRE16 SINV did not show significantly different fluorescence of the mCherry reporter in

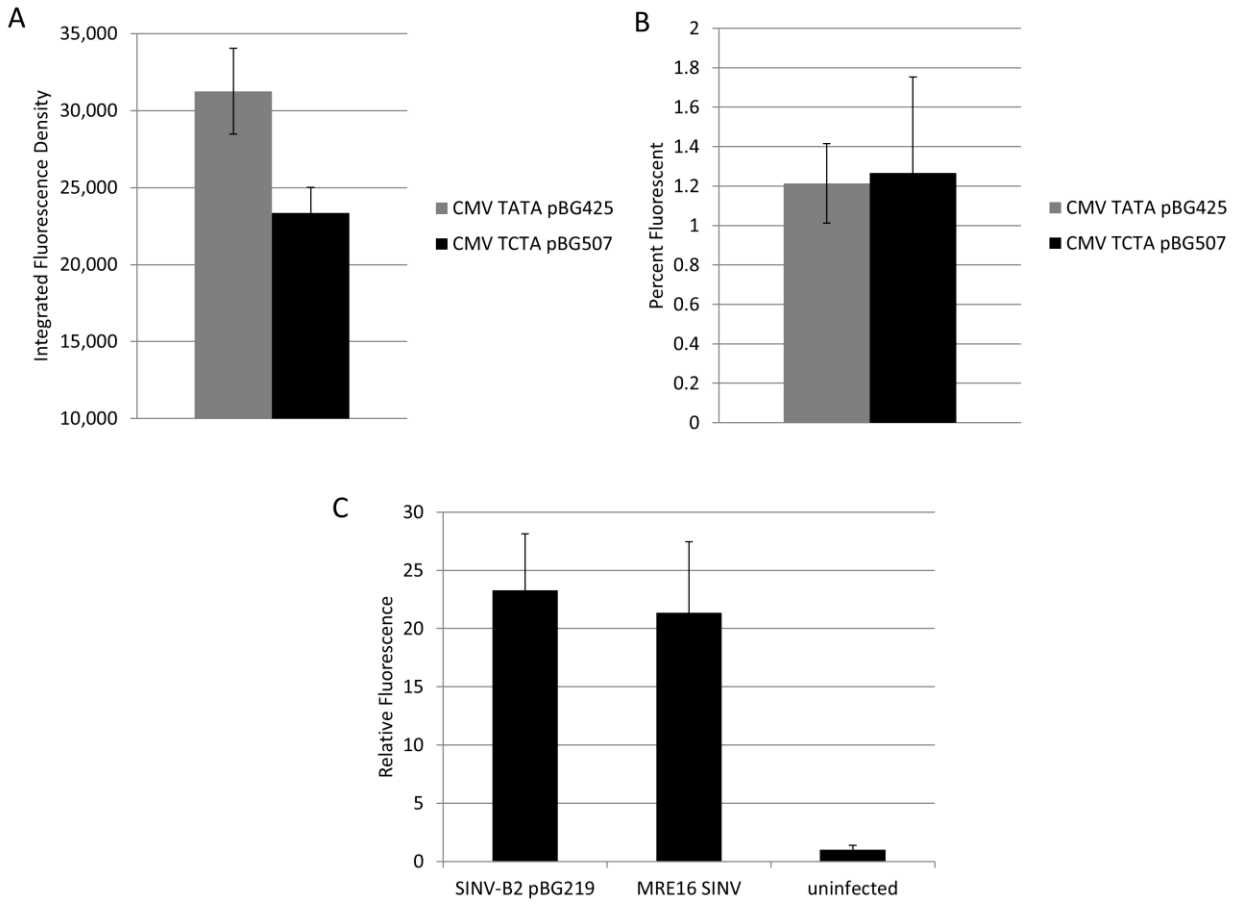

Figure 4.6 Mutating the Promoter mutation to reduce transcription and RNAi inhibition to increase mCherry reporter activity.

BHK cells were transfected with constructs (A/B) or C636 cells containing the reporter constructs (C) were infected with SINV. Fluorescence was monitored and fluorescence was quantified using ImageJ software. **A)** The CMV promoter's TATATA box was mutated to TCTATA to reduce transcriptional levels. Transcription is reduced in the CMV TCTA pBG507 constructs, as measured by GFP fluorescence that is transcribed on the reporter RNA. **B)** Percent expressing mCherry reporter is shown for CMV TATA (pBG425) and for CMV TCTA (pBG507). The crippled promoter does not have an increase in reporter activity. **C)** Infection with a dsSINV-B2 to impair the RNAi response did not have a significant change on mCherry reporter fluorescence when compared to wildtype MRE16 SINV in C636 cells stably transformed with pBG461 reporter RNA construct.

response to infection, indicating that RNAi does not play a significant role inhibiting the reporter RNA (Figure 4.6C).

Through many efforts to optimize and increase the efficiency of our reporter system to detect infection, it appeared that the variability was independent of virus species, viral load, time post infection, cell cycle, viral cycle, and RNA levels. Despite this variability, the reporter RNA system works well as an overall indicator of infection. Fluorescent reporter was not found in uninfected cells, but significant expression was detected during infection in cell culture. Similarly, transgenic mosquitoes expressed the reporter only during infection, representing the first time a transgenic mosquito line has been established that detects infection. Making transgenic mosquitoes is still a developing field and the use of the mariner transposase system results in unspecified insertion events. This results in mosquito families with many unknown changes and variables.

Discussion

Transformation of *Aedes aegypti* using the Mariner Mos1 transposase system with our reporter constructs inserted into the transposon resulted in transgenic lines that transcribe the SINV reporter RNA. We detected mCherry expression in a subset of infected transgenic mosquitoes, but did not detect mCherry expression in all infected transgenic mosquitoes. mCherry reporter expression was never detected in wild-type mosquitoes or uninfected transgenic mosquitoes, indicating that activation of the reporter RNA was specific to virus infection. However, because mCherry expression was detected in some but not all of the infected transgenic mosquitoes, it indicates that

the reporter RNAs may not be active in all transgenic mosquitoes. In the stable cell lines we observed a similar effect, with some stable cells expressing mCherry during infection and others not expressing mCherry. These *in vitro* and *in vivo* findings indicate that there are mechanisms at play in mosquito cells limiting the ability of the reporter RNAs to be replicated in the presence of infecting alphaviruses. These transgenic mosquitoes we describe should be considered a proof-of-concept system, which demonstrates that subgenomic reporter RNA expression is a viable approach for detecting wild-type alphavirus infection in mosquitoes, but this system will need to be further optimized for more efficient detection of SINV infection in live mosquitoes. Future optimization approaches could include the use of site-directed transgenesis systems to more precisely insert the reporter constructs into the mosquito genome. Additionally, different promoters should be tested and used to regulate RNA levels and more specifically control the expression of the reporter RNA.

Despite the variability of the transgenic mosquitoes in expressing mCherry during infection, only infected transgenic mosquitoes expressed mCherry. mCherry was not detected in wild-type mosquitoes or in uninfected transgenic mosquitoes. This system is virus specific and is an accurate marker of infection. Through future work, this subgenomic reporter RNA system can be optimized to express a fluorescent reporter in all of the infected transgenic mosquitoes. The resulting transgenic line of mosquitoes would be an essential tool that accurately reports wild-type alphavirus infection through a visible fluorescent reporter in living mosquitoes.

Chapter Five: Oxidative Stress Enhances RNA Synthesis and Capping During Viral Infection

Introduction

Flaviviruses, including dengue, West Nile Virus, and yellow fever viruses, cause significant disease in humans worldwide. It is estimated that approximately 2.5 billion people (40% of the world's population) live in an area with endemic dengue fever, resulting in an estimated 350 million cases every year (Guzman et al. 2010; Bhatt et al. 2013). The recent emergence of WNV in the Americas (Pesko & Ebel 2012) and the re-emergence of dengue in North America make flaviviruses a significant domestic public health issue. There are currently no therapeutics to treat flavivirus infections, making it imperative to delineate their intricate replication mechanisms so effective anti-viral strategies can be developed.

Flaviviruses are small, positive strand RNA viruses with a 5' cap and an approximate 11Kb genome. The viral RNA encodes a single polyprotein which is co-translationally cleaved by cellular and viral proteases into three structural proteins (C, prM and E) and eight nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B and NS5) (Lindenbach et al. 2007). The nonstructural proteins have various roles during infection, but in particular, NS3 and NS5 interact to form a replicase complex needed for duplicating the genome(Knight & Howley 2013). These nonstructural proteins intricately work and associate with each other to form the replication complex and synthesize negative and positive strand copies of the genome.

NS5 is highly conserved across the *Flavivirus* genus and encodes three enzymatic functions: N7/2′-*O*-methyltransferase (Egloff et al. 2002; Zhou et al. 2007) and guanylyltransferase (Issur et al. 2009) in the ~265 amino acid N-terminal capping enzyme domain and a RNA-dependent RNA polymerase (RdRp) in the C-terminal 2/3 of NS5 (Grun & Brinton 1986). NS3 contains the helicase-NTPase enzymatic activity needed to unwind the template RNA and cleave the phosphates from the nascent RNA for capping. The nascent RNA is synthesized by the C terminus of NS5 (RdRP) and then the NS5 N terminus (capping enzyme) caps the RNA (guanylyltransferase) and methylates the guanosine cap at the N7 position (methyltransferase) to allow for translation and protection from cellular mRNA decay. Capped RNAs are also used for assembly into new virions. Viral RNA capping plays a crucial role in RNA stability and also alters the host cell's RNA decay machinery and mRNA stability (Silva et al. 2010; Moon et al. 2012; Hussain et al. 2012).

The flavivirus 5' RNA cap ($^{m7}GpppA_{2'OMe}$) is critical for genomic RNA stability and translation(Cleaves & Dubin 1979; Wengler et al. 1978). The 5' cap structure is formed by the combined action of the NS3 RNA triphosphatase domain(Wengler & Wengler 1993) and the NS5 N-terminal RNA capping enzyme. Specifically, the RNA triphosphatase activity of NS3 will hydrolyze the gamma phosphate from a new mRNA. Guanosine monophosphate (GMP) is then transferred to the RNA (guanylyltransferase domain of NS5). Finally, the guanosine cap is methylated at the N7 and 2'O positions (NS5 methyltransferase). The guanylyltransferase reaction occurs in two steps, formation of the covalent enzyme-GMP intermediate and subsequent transfer of the

GMP from NS5 to the diphosphorylated RNA forming the GpppN structure (Ghosh & Lima 2010).

Flavivirus genomic replication occurs on modified membranes on the endoplasmic reticulum (ER). Invaginations of the ER membrane form structures referred to as vesicle packets or replication compartments in which NS3 and NS5 replication complexes assemble to replicate viral RNA (Chu & Westaway 1992; Gillespie et al. 2010). The outer surface of the replication compartments is exposed to the oxidizing ER lumen (Csala et al. 2010). The interior of the replication compartments is continuous with the cytoplasm, which is generally accepted to be a reducing environment (Figure 5.1). Cellular proteins such as glutathione and superoxide dismutase are responsible for keeping the cytoplasm from becoming too oxidizing and forming aberrant disulfide bonds (Saaranen & Ruddock 2013). Oxidative stress occurs when insults to the ER, such as induction of the unfolded protein response during infection or disruption of ER membrane integrity causes and imbalance of intracellular reactive oxygen species (ROS)(Ambrose & J. M. Mackenzie 2011). Reactive oxygen species (ROS) such as oxygen ions and peroxides escape the ER and enter the cytoplasm, resulting in oxidative stress. Maintaining this homeostatic balance between the oxidizing ER and reducing cytoplasm is crucial for the well-being of the cells. Oxidative stress or changes in cellular redox conditions can lead to cell death due to damage of cytoplasmic components and induction of apoptosis.

Flaviviruses are known to induce oxidative stress during infection in both cell culture and *in vivo* (Y. L. Lin et al. 2000; Raung et al. 2001; Liao et al. 2002; R.-J. Lin et al. 2004; Verma et al. 2008; Kumar et al. 2009; Yang et al. 2010). Treatment of infected

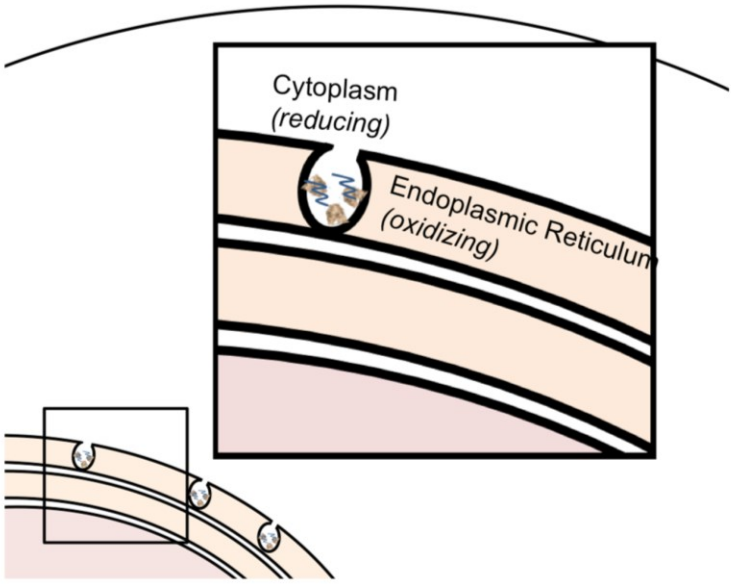

Figure 5.1 Diagram of flavivirus replication compartments within the endoplasmic reticulum.

Flaviviruses form replication compartments by invaginating into the endoplasmic reticulum. The ER is an oxidizing environment and the cytoplasm is assumed to be a reducing environment. The location of the replication compartments occurs between such drastic environments and the sensitivity to oxidative conditions indicates that viral replication may play a critical role in virus-induced oxidative stress.

cells with antioxidants to relieve oxidative stress in mammals or mosquitoes seems to improve disease outcomes (Nazmi et al. 2010; Chen et al. 2011; Pan et al. 2012; Chen et al. 2012). Conversely, oxidation by the nitric oxide donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) attenuates infection and appears to suppress negative strand RNA strand synthesis *in vitro* (Charnsilpa et al. 2005; Takhampunya et al. 2006). These data suggest that the intracellular redox balance is critical for viral RNA replication. However, it was previously unknown if the oxidative stress induced by flavivirus infection plays an active role in viral RNA replication.

Here we show that oxidative stress affects RNA replication during flavivirus infection. We demonstrate that antioxidant treatment can significantly impair viral RNA replication and alters the amount of capped viral RNA present in cells. Oxidation significantly enhances the activity of the flavivirus NS5 RNA capping enzyme and that enhancement appears to be partially attributable to a conserved methionine in the capping enzyme. Mutation of the conserved methionine in a replicon system significantly affected RNA replication. We have also developed intriguing preliminary data that alphavirus RNA capping may also be sensitive to oxidation. These findings provide the first demonstration that flaviviruses use oxidative stress in infected cells to help regulate their genomic replication.

Materials and Methods:

Cells, chemicals, and viral assays

Baby Hamster Kidney (BHK-21) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5%

Pen/Strep, 5% Sodium Pyruvate and 5% L-Glutamine. West Nile virus (subtype Kunjin) was used for viral infection studies (Generously provided Dr. Alexander Khromykh). Butylated hydroxyanisole (BHA) or S-Nitroso-N-acetyl-DL-penicillamine (SNAP) was obtained from Sigma-Aldrich (St. Louis, MO); an equal volume of DMSO was used as a control in experiments with BHA or SNAP. Kunjin RNA was collected from culture media and extracted using Trizol LS (Invitrogen, La Jolla, CA). Released Kunjin RNA was quantified using real-time quantitative reverse transcriptase PCR (qRT-PCR) (Stahla-Beek et al. 2012). Dengue replicon assays were performed using persistent dengue 2 replicon cells expressing Renilla luciferase (Whitby et al. 2005). Renilla luciferase activity and cellular viability were determined using Viviren Live Cell Renilla Luciferase substrate and Cell Titer-Glo (Promega), respectively, on a Victor X5 Multi-Mode platereader. Renilla and cell viability curves were generated using the Prism Graphpad software.

A plasmid-launched West Nile virus replicon(pBG438) expressing firefly luciferase was previously described (Pierson et al. 2006). Site-directed mutagenesis of the replicon expression plasmid was performed using Quick Change mutagenesis (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. To test the effect of mutations of replicon replication, wildtype or mutant replicon plasmids were transfected into Baby Hamster Kidney (BHK-21) cells using Lipofectamine 2000 (Invitrogen). 24 hours post-transfection the cells were lysed with Glo Lysis buffer (Promega, Madison, WI) and 20 µl of the lysates were transferred to an opaque 96 well plate. Firefly luciferase signal was detected using the Victor Multi-Mode plate reader

(Perkin-Elmer) by injecting 100 µl of firefly luciferase substrate (Promega, Madison, WI) into each well and measuring relative light units.

Intracellular oxidative stress was detected in BHK-21 cells 24 hours after chemical treatment or virus infection. Cells were washed with PBS and incubated with CM-H2DCFDA according to the manufacturer's recommendations (Life Technologies, Carlsbad CA). Images were collected using a digital camera attached to a Nikon Diaphot 200 inverted fluorescence microscope.

Expression and purification of the flavivirus NS5 capping enzymes

Expression and purification of the wild-type dengue virus NS5 protein for oxidation studies was performed as previously described (Geiss et al. 2009; Henderson et al. 2011). Briefly, the recombinant NS5 capping enzyme domain from dengue virus type 2 (strain 16681, amino acids 1–267) was expressed from inducible T7 expression plasmids that contain a carboxy-terminal 6-histidine tag. The plasmids were transformed into BL21 DE3 pLysS *E. coli* and cultures were induced with 400 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 24°C. The bacterial pellets were collected, homogenized using a micro-fluidizer, and the histidine-tagged proteins were purified from clarified lysates by nickel affinity chromatography. The eluted proteins were further purified through a size exclusion gel filtration column. Small-batch purification was used to prepare cysteine and methionine mutant-NS5 capping enzymes. Mutants were generated using Quick Change mutagenesis and verified by sequencing. Mutant and wild-type plasmids were transformed into BL21 DE3 pLysS *E. coli* cells and induced with 400uM IPTG overnight. Bacterial pellets were collected in

low imidazole buffer, disrupted by sonication, and clarified lysates collected. Clarified lysates were incubated with nickel beads to bind the histidine (HIS)-tagged protein using HIS-select High Flow (HF) Nickel Affinity beads (Sigma-Aldrich, St. Louis, MO) at 4°C for 30 minutes, then the lysate was separated from the beads on Micro Bio-spin columns (Bio-Rad, Hercules, CA) and eluted in 250mM imidazole buffer. Protein concentrations for gel filtration purified and small batch purified proteins were determined using A_{280} absorbance and extinction coefficients obtained from the ExPASy website. Recombinant VEEV nsP1 protein (Trinidad Donkey Strain) was expressed and purified as above.

Protein guanylation assay

Protein guanylation assays were performed as previously described (Geiss et al. 2011). Briefly, the enzyme (3 μM) was incubated with 1 μM GTP-ATTO 680 (Jena Bioscience, Germany) in 5 mM Tris-base (pH 7.5), 500 nM $MgCl_2$ and 0.1% NP-40 for 120 minutes at 37°C. The reactions were resolved on 12.5% polyacrylamide gels, and the extent of protein guanylation was quantified using an Odyssey UV Imaging system (Li-Cor, Lincoln, NE). Gels were stained with Coomassie Blue and Atto-680 signals were normalized for protein loading.

Cap immunoprecipitation and qRT-PCR analysis

Total RNA was extracted from Kunjin virus infected BHK-21 cells treated with BHA, SNAP, or DMSO at 24 hours post infection using TRIzol (Invitrogen). A mouse monoclonal antibody to the m^3G/m^7G RNA cap structure (Synaptic Systems) was used

to fractionate 5 µg of total RNA into capped and uncapped portions as previously described(Moon et al. 2012) . An uncapped, 5' tri-phosphorylated spike RNA was prepared by *in vitro* transcription from linearized pGEM-4 plasmid using a MEGAscript SP6 kit (Invitrogen) followed by gel purification. Equal volumes of each RNA fraction were reverse transcribed using Improm-II reverse transcriptase (Promega) and qRT-PCR was performed with SYBR Green Supermix (BioRad) on a BioRad CFX96 Real-Time PCR detection system. The relative amount of uncapped Kunjin virus RNA in each treatment was determined by comparing the amount of Kunjin RNA in the unbound fraction to the 10% input fraction using the ΔΔCt method with pGEM set as a reference gene.

Fluorescence polarization assays

GTP displacement assays were performed as described (Geiss et al. 2009). Fluorescence polarization (FP) values were determined on a Victor X5 Multi-Mode plate reader (Perkin-Elmer). Nonlinear regression curve (variable slope) were generated with the Prism Graphpad software package to determine K_i values. The dissociation constant (K_D) of small-batch purified proteins was determined as previously described (Geiss et al. 2009). Briefly, mutant proteins were serially diluted 1.5X in 50mM Tris pH7.5 from 100 µM. The mutant proteins were incubated with 50mM Tris pH 7.5, 0.01%NP-40, 2mM TCEP, and 10nM GTP-Bodipy (Guanosine 5'-*O*- (3-Thiotriphosphate), BODIPY-FL Thioester, Invitrogen) for 60 minutes at 28°C. The fluorescence polarization (FP) values were averaged and nonlinear regression curve analyses were used to calculate K_D values with the Kalidagraph software.

Results:

Antioxidants reduce viral replication

To explore whether changes in the oxidative environment during infection affects viral replication, we assessed if blocking oxidative stress with antioxidants alters virion production. We utilized a fluorescent molecular probe that is sensitive to oxidation conditions to determine when oxidative stress can be detected in BHK-21 cells. Uninfected and mock cells did not show significant oxidative stress (indicated by green fluorescence from the probe) (Figure 5.2A). However, in cells infected with Kunjin virus or treated with hydrogen peroxide, obvious fluorescence was detected, indicating an induced oxidative stress. Interestingly, when infected cells were treated with an antioxidant agent [butylated hydroxylamine (BHA)], the oxidation fluorescence was drastically reduced. The release of viral RNA in media at increasing concentrations of BHA was monitored by qRT-RT-PCR analysis at 48 hours post infection. Treatment of Kunjin virus infected BHK-21 cells with increasing concentrations of BHA resulted in a one log reduction of viral RNA in media at 200 μM BHA and a three log reduction of viral RNA in media at 300 μM BHA (Figure 5.2B).

The flavivirus E glycoprotein is known to contain several disulfide bonds (Nybakken et al. 2006), and BHA could affect formation of these bonds and release of virion particles. To decouple virion assembly and release from viral RNA replication, we tested the effect of BHA on the replication of a dengue virus replicon in BHK-21 cells (Whitby et al. 2005). After 48 hours of treatment with different concentrations of BHA *Renilla* luciferase expression from the replicon and cellular viability were measured (Figure 5.2C). We observed a strong antiviral effect with minimal cell toxicity with BHA

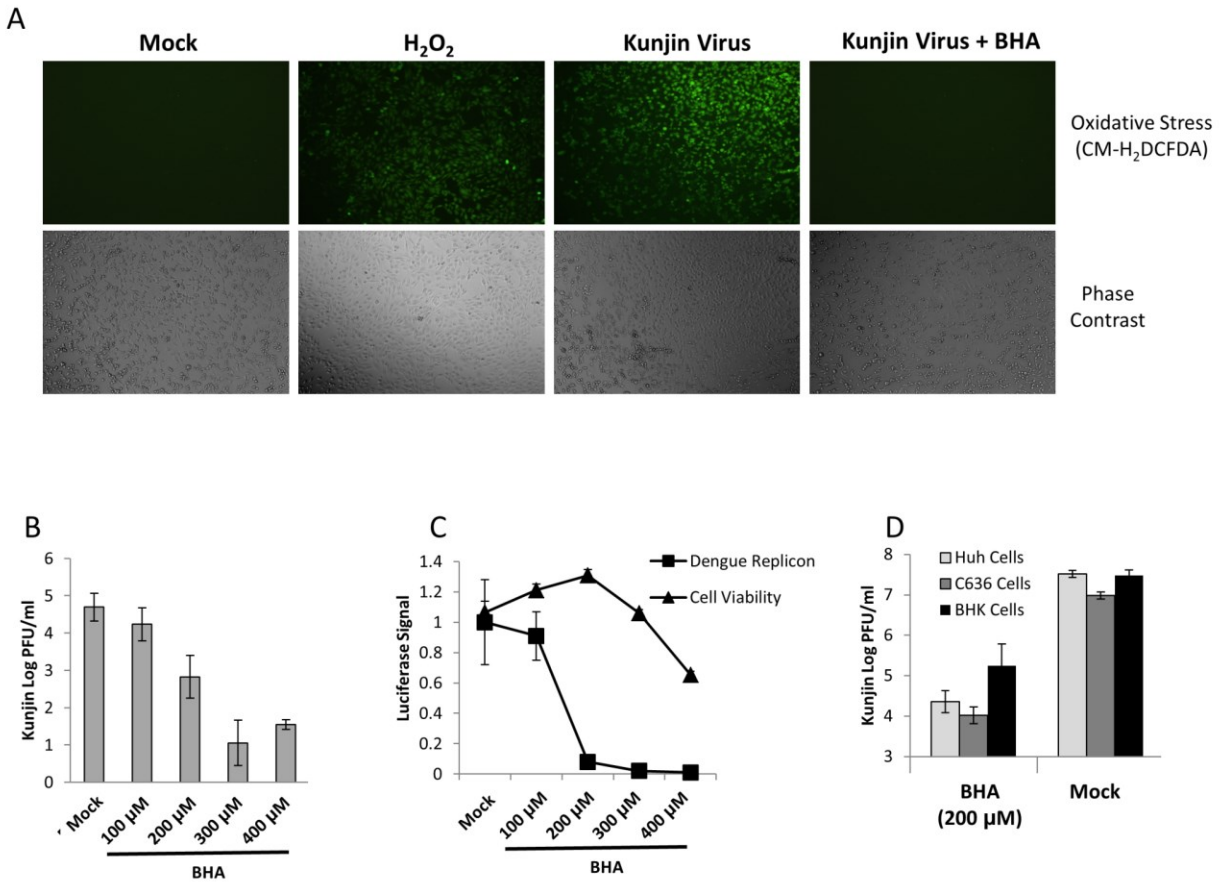

Figure 5.2 Antioxidants reduce virus replication

A) Cells were plated out and either infected with kunjin virus or left uninfected. Following infection the cells were either treated with 200uM hydrogen peroxide (oxidant) or 200uM BHA (antioxidant) or left as mock. An oxidation sensitive fluorescent probe was added to the cells and monitored for fluorescence. H_2O_2 and kunjin infection had activated oxidative stress, but uninfected or BHA treated did not demonstrate oxidative fluorescence of the probe. **B)** BHK cells were infected with Kunjin and treated with BHA. RNA was collected and titers were determined through qRT-PCR. **C)** Using a dengue replicon, luciferase was measured with different amounts of BHA treatment. Cell viability was measured with Cell Titer Glo. **D)** Comparison of different cell lines that were infected with Kunjin virus and either treated with BHA or left as a control. BHA antioxidant reduced infection in all three cell lines.

concentrations less than 400uM, indicating that BHA was affecting viral RNA replication and not solely virion production. To verify that reduction of viral replication is not cell-line specific, we determined viral titers in BHK-21 (hamster), Huh7 (human), and C6/36 (mosquito) cells at 200 μM BHA and observed similar effects in each cell line (Figure 5.2D). The results demonstrated the potent effect of antioxidants on reducing virus replication, specifically the nonstructural proteins and RNA replication, within multiple cell types.

Antioxidants affect the proportion of capped positive strand RNAs.

We then assessed the effects of BHA on accumulation of negative and positive strand RNAs during infection to determine if blocking oxidation affected either strand preferentially. Using a Kunjin strand-specific qRT-PCR protocol (Ambrose & J. M. Mackenzie 2013) we observed that the normal positive-to-negative strand RNA ratio was approximately 25:1, whereas treating cells with 200 μM BHA reduced the ratio to 5:1 (Figure 5.3A). We then examined how BHA affected the accumulation of capped viral RNAs during infection. We immunoprecipitated capped RNA from infected cells using a cap-specific antibody as previously described (Moon et al. 2012) and compared the amount of positive strand RNA in the capped and uncapped fractions. We observed no significant change of the proportion of uncapped RNA in the presence of SNAP, whereas we observed approximately a 60% increase in the uncapped RNA fraction in the presence of BHA (Figure 5.3B), indicating that blocking intracellular oxidation increases the amount of uncapped viral RNAs in cells. The virus needs capped-positive strand RNA for forming new virions. These results demonstrate that interfering with the

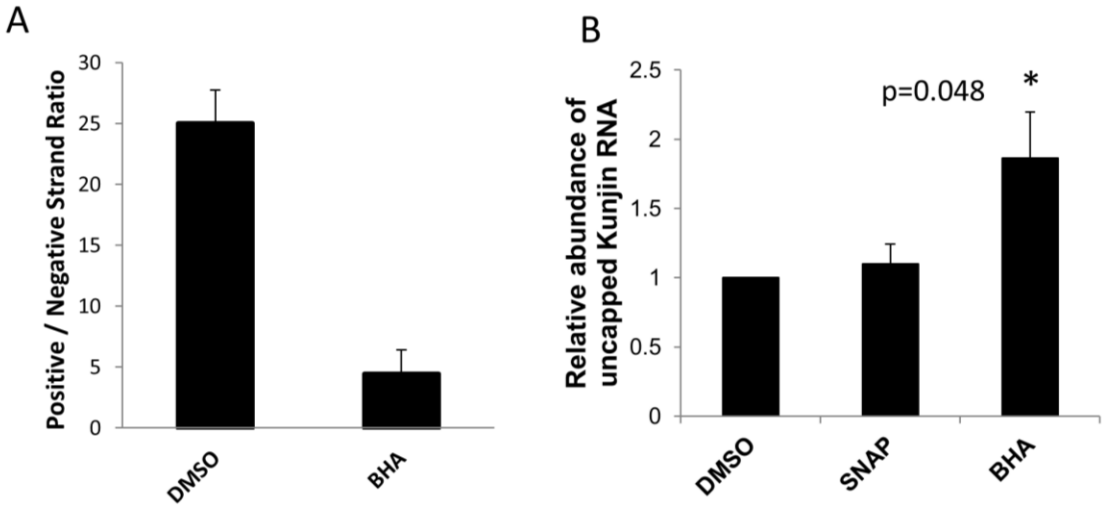

Figure 5.3 Antioxidant effect on capped positive-strand RNA.

A) Total RNA was extracted from Kunjin infected (MOI =0.1) BHK cells and RNA was quantified using strand specific qRT-PCR. Cells infected and treated with BHA have significantly less positive strand RNA than mock (DMSO) treated infected cells. **B)** Treatment with BHA increases the amount of uncapped RNA. Total RNA was extracted from Kunjin infected cells and capped RNA was immunoprecipitated from the samples. Positive and negative-strand Kunjin RNAs were detected from the precipitated and soluble fractions by strand specific qRT-PCR. GAPDH mRNA was detected in immunoprecipitated and soluble fractions as a control.

oxidative conditions by treating with an antioxidant has a significant negative effect on positive strand RNA and capped RNA synthesis.

Oxidation upregulates NS5 guanylyltransferase activity

The finding that BHA reduced the amount of capped RNA during infection suggested that oxidation affects viral RNA capping. Capping of flavivirus genomic RNAs by NS5 is crucial for replication(Stahla-Beek et al. 2012), so we investigated if altering of redox conditions affected the guanylyltransferase activity of the NS5 capping enzyme. Protein guanylation (formation of a covalent protein:GMP adduct) is considered a rate-limiting step in the guanylyltransferase reaction and is used as a measure of guanylyltransferase activity(Issur et al. 2009; Ahola & Kääriäinen 1995; Guarino et al. 1998). Guanylation reactions with recombinant dengue capping enzyme (Geiss et al. 2011) were performed with two different oxidizing agents, hydrogen peroxide (H_2O_2) and diamide. We observed that increasing concentrations of either oxidizing agent resulted in increased levels of protein guanylation (Figure 5.4A). Calf intestinal phosphatase (CIP) was used as a negative control and did not incorporate label even at high concentrations of oxidizing agent. We next tested the effects of reducing agents on the guanylation reaction. Increasing concentrations of TCEP resulted in a drastic reduction of protein guanylation (Figure 5.4B), indicating that reducing conditions inhibited the guanylation reaction.

A possible mechanism for modulating guanylyltransferase activity is altering GTP binding by the capping enzyme. To test this possibility, we assessed the effect of reducing or oxidizing agents on the ability of dengue capping enzyme to bind to a

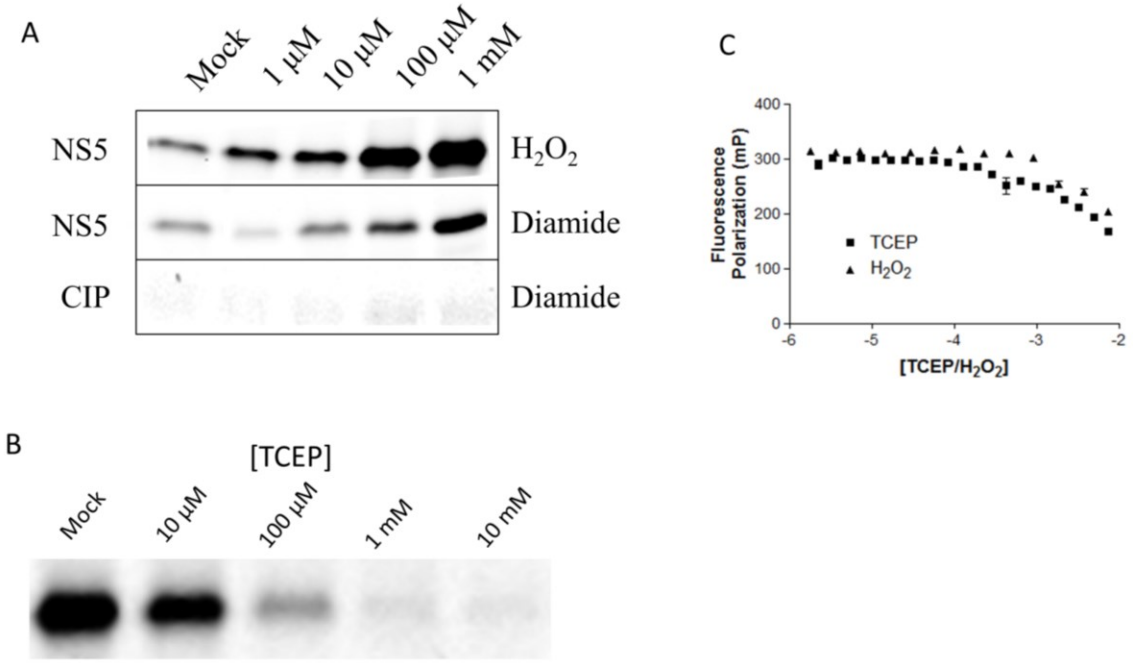

Figure 5.4 Oxidation increases the activity of NS5 capping enzyme guanylyltransferase

A) Recombinant dengue capping enzyme NS5 was used to measure the activity of the guanylyltransferase using an Att0-680 labeled GTP. In the presence of H_2O_2 and diamide (oxidants), NS5 was able to more efficiently be guanylated, indicating higher guanylyltransferase activity. CIP was used as a control and did not show an increase in guanylation with oxidation. **B)** A reducing agent (TCEP) had the opposite effect of an oxidant and significantly inhibited the guanylation of the NS5 enzyme. **C)** Fluorescence polarization assays show that H2O2 and TCEP do not alter binding ability of the enzyme and substrate until concentrations that are higher than the ones tested with the protein.

fluorescently labeled GTP substrate in fluorescence polarization assays (Geiss et al. 2009). We observed that TCEP and H_2O_2 reduced GTP binding by the capping enzyme only at concentrations higher than were used for the guanylation reactions (Figure 5.4C). Therefore, modulating the oxidative conditions during protein guanylation affects the enzymatic activity of the capping enzyme but does not significantly affect the ability of the enzyme to bind GTP.

Conserved oxidative sensitive NS5 residues

We hypothesized that oxidation of amino acids in the NS5 capping enzyme could be responsible for the increased activity during oxidation conditions with infection. Cysteine and methionine residues are considered readily oxidizable, so we examined the contribution of conserved cysteine and methionine residues to oxidative activation. Two cysteine residues (Cys82 and Cys145) and one methionine residue (Met219) are completely conserved across the flavivirus genus (Figure 5.5). We mutated Cys82 and Cys145 to serine residues and Met219 to an isoleucine to block oxidation. Activity of the NS5 mutants was reduced by about 50% for all of the mutants, indicating that Met219, Cys82, and Cys145 play critical roles in oxidative activation, but they are not individually the sole residue responsible for the oxidation sensitivity phenotype (Figure 5.5).

Dimerization enhances NS5 guanylyltransferase activity

Cysteine residues are known to form disulfide bonds and oxidation can induce disulfide bond formation. We hypothesized that the oxidation during infection could be acting on the conserved cysteines 82 and 145 to form disulfide bonds and increase

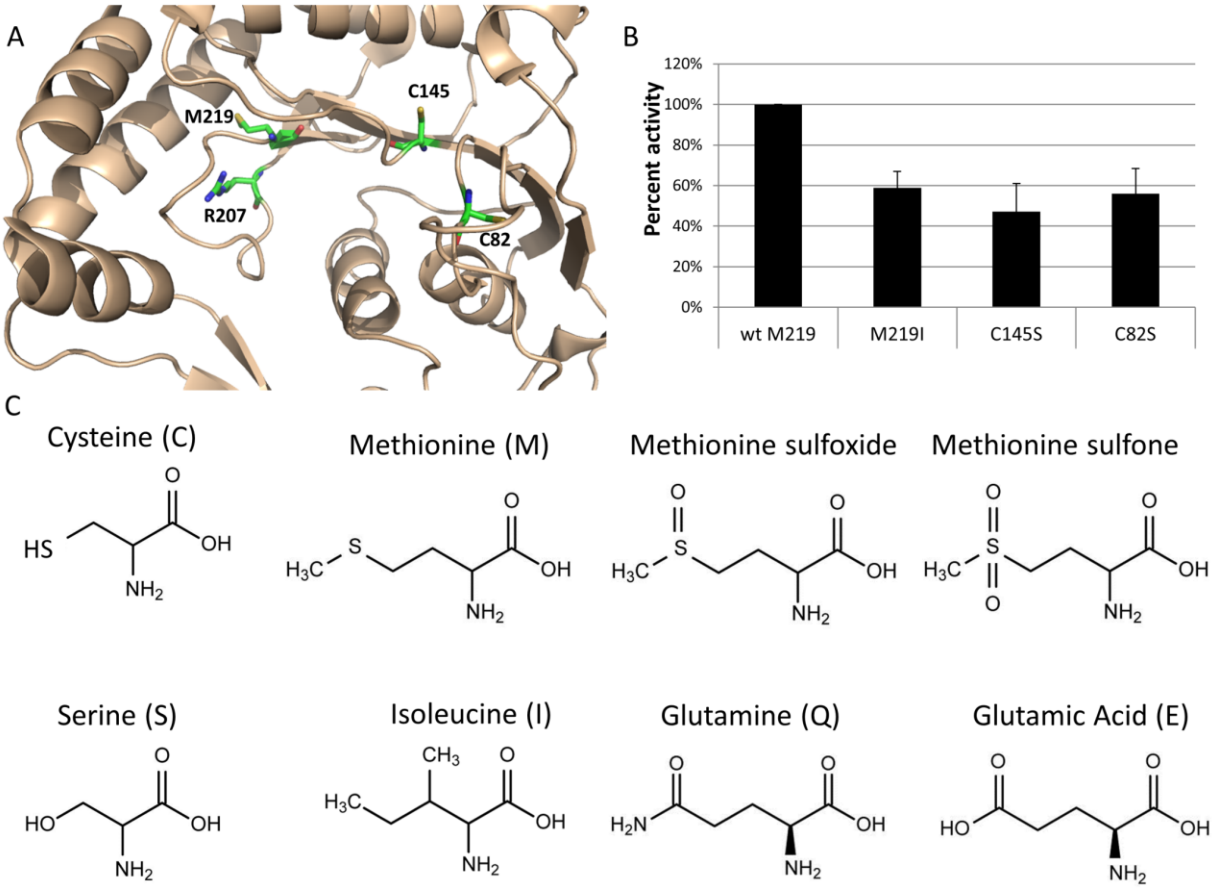

Figure 5.5 Conserved oxidation sensitive NS5 residues

A) Crystal structure of DENV NS5 capping enzyme. Methionine 219, Cysteine 82, and Cysteine 145 are conserved in the flavivirus genus. **B)** Mutation of the conserved residues to unoxidizable serines and isoleucines results in significant reduction in guanylation activity. **C)** Diagram of mutated residues. Cysteines were mutated to Serines. Methionine was mutated to Isoleucine. Glutamine was used to mimic methionine sulfoxide, and glutamic acid was used to mimic methionine sulfone.

activity. To test if disulfide bonds were occurring, we resolved oxidized and un-oxidized proteins on SDS-PAGE gels in the presence or absence of β-mercaptoethanol. We observed that diamide treatment caused the capping enzyme to run as a dimer based on guanylation signal (Figure 5.6A) and treatment of the samples with β-mercaptoethanol resolved the dimer. C82S and C145S mutants continued to form dimers indicating that the NS5 protein forms dimers under oxidative conditions, but C82 and C145 are not completely responsible for the dimerization. Next we examined if protein concentration, which would be increased by dimerization, affects guanylyltransferase activity. 30 pMol of NS5 was incubated in volumes of 10, 30, and 100 µl (0.3 µM, 1 µM, and 3 µM NS5 concentrations) in standard guanylation conditions for 4 hours, then the volume of each reaction was adjusted to 100 µl and equal volumes were resolved on SDS-PAGE gels (Figure 5.6B). 0.3 µM NS5 showed significant reduction in activity compared to 3 µM NS5, indicating that NS5's activity was positively affected by protein concentration. The increased activity of the NS5 capping enzyme during oxidative stress may be due to the dimerization and increased concentration of protein interactions within the replication complex.

Methionine 219 is important for oxidative activation of NS5 guanylyltransferase activity

Methionines are oxidized to a hemi-oxidized and reversible sulfoxide, with the addition of one oxygen, or oxidized to a completely and committed oxidation modification can result in a methionine sulfone, with the addition of two oxygen's (Figure 5.5C). To mimic these oxidative forms of methionine, we mutated Met219 to a glutamine

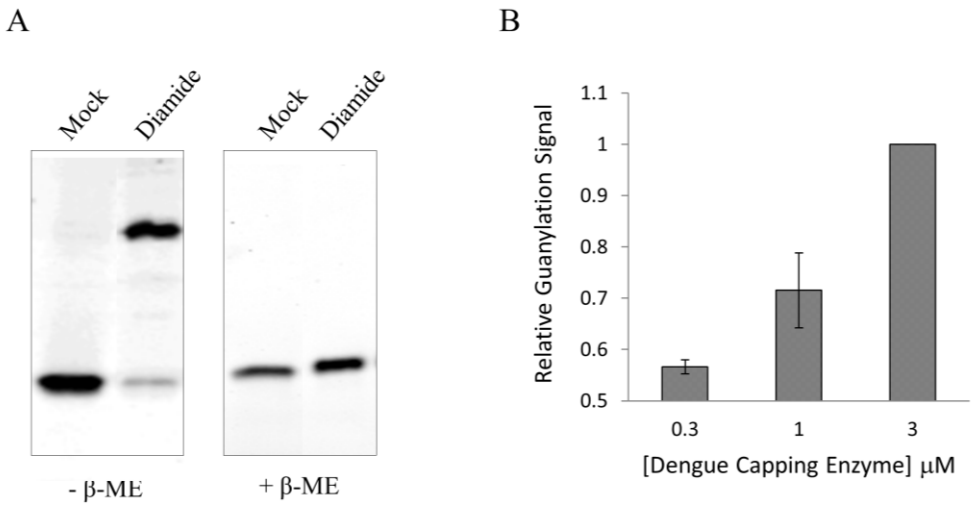

Figure 5.6 Dimerization of NS5 during oxidation conditions.

A) NS5 resolved on an SDS-PAGE gel. Treatment of NS5 with diamide (oxidant) caused the formation of dimers. Those dimers could be reduced to monomers by the addition of Beta Mercaptoethanol, indicating that the disuflide bonds are involved in the dimerization. **B)** Capping enzyme activity is increased at higher concentrations. Disulfide bond formation with oxidation may result in increased activity because of the induced dimerization and close association.

(Gln/Q) to resemble the methionine sulfoxide, and a glutamic acid (Glu/E) to resemble methionine sulfone. M219I reduced the activity of the NS5 capping enzyme. M219Q restored guanylation activity to wild-type levels and M219E increased the activity to above wild-type (Figure 5.7A). The addition of an oxidant (H_2O_2) to the mutants resulted in significant activation for the M219E mutant and very little activation for the M219I mutant (Figure 5.7B). These results indicate that oxidation status of Met219 influences oxidation activation of NS5 guanylyltransferase activity.

Methionine 219 is critical for viral replication

The suppressive effect of mutating methionine 219 to an isoleucine residue on oxidative activation of guanylyltransferase activity suggested that Met219 may be important during replication. To test if Met219 is important for viral replication we used a plasmid launched Firefly luciferase expressing West Nile virus replicon (Pierson et al. 2006; Pierson et al. 2005) . BHK-21 cells were transfected with the wild-type replicon expression plasmid, M219I, M219Q, M219E, C82S, C145S, or a replication defective polymerase active site mutant (GDD>GVD) (Khromykh et al. 1998). C82S and C145S showed about 60% and 40% activity, respectively, indicating that the residues are not crucial for replication (Figure 5.7C). The M219I was significantly reduced with replication levels similar to the GVD mutant. To confirm this inhibition, we mutated Met219 to the small unoxidizable residue Alanine (A). This M219A mutant also showed significant reduction in RNA replication. The hemi-oxidized methionine mutant M219Q was able to replicate but was not as strong as wildtype. M219E, representing a fully oxidized methionine residue, was severely inhibited and did not allow RNA replication (Figure

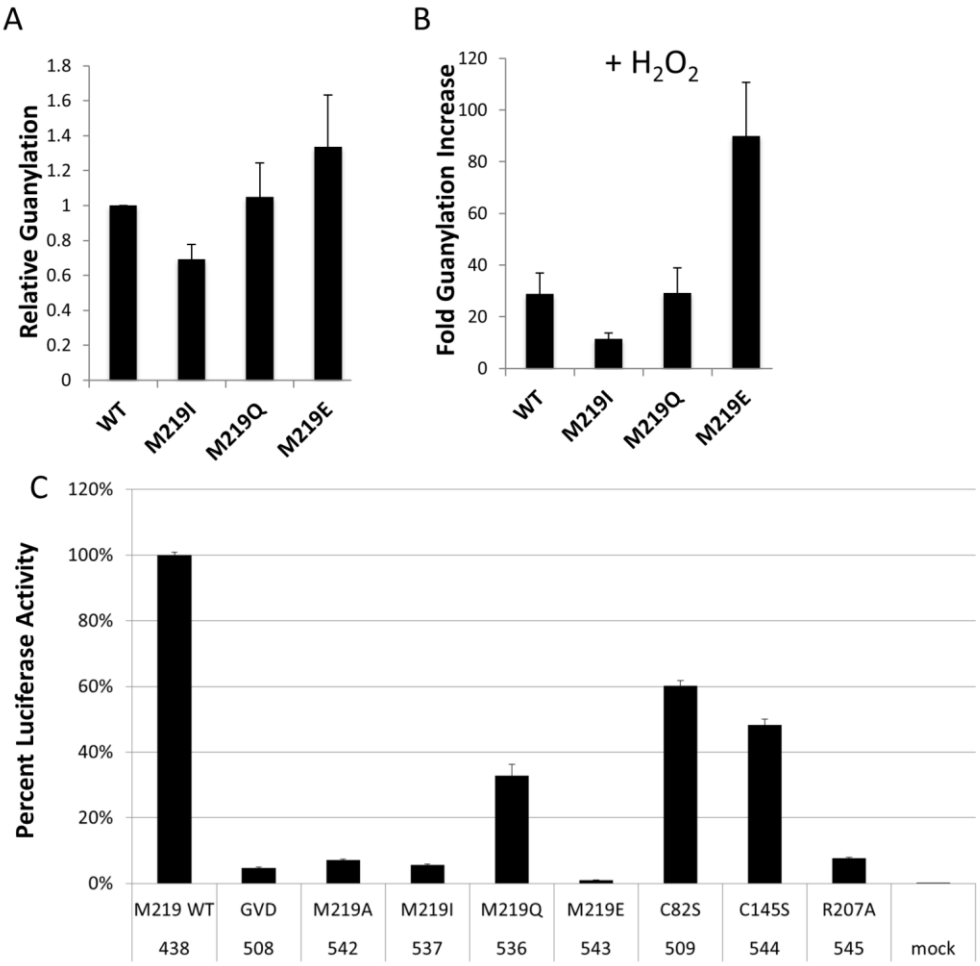

Figure 5.7 Mutation of conserved NS5 residues affect enzyme activity and virus replication

A) Mutant NS5 proteins were made with Methionine 219 mutated to isoleucine (I), glutamine (Q), or glutamic acid (E). Guanylation assay performance shows a reduction in the unoxidized M219I, but the hemi-oxidized M219Q restores activity to wild-type. The fully oxidized mimic M219E has an increased activity. **B)** The addition of H2O2 to the guanylation assay results in oxidation activation of the enzyme. M219I has the least activation phenotype, while M219E is significantly enhanced with the addition of an oxidant. **C)** Corresponding mutations were made in the west nile replicon containing luciferase. BHK cells were transfected with a specific mutant replicon and luciferase activity was measured to indicate virus replication. The cysteine mutants inhibited, but did not kill the activity of the replicon. However, M219I and M219E had significant reduction of virus replication, similar to the GVD dead enzyme control.

5.7C). These results demonstrate that M219 is essential for viral replication and that mutations that mimic unoxidized, or fully oxidized methionine are not tolerated whereas a mutation that mimics a partially oxidized methionine is tolerated.

The arginine(R) residue at position 207 is also conserved and is located adjacent to the methionine 219. Arginines have not been reported to be sensitive to oxidation, but protein structure modeling has shown that R207 forms critical hydrogen bonds within the enzyme that help hold the structure of the protein. Interestingly, mutating the arginine (R) 207 to a small alanine (A) residue significantly reduced the activity. Further work needs to be done to understand R207's role in enzyme activity, but we hypothesize that mutating the R207 to an alanine breaks crucial hydrogen bonds required for optimal activity. The alanine at position 207 would increase the flexibility in the protein, which may help NS5 form dimers or other intermolecular bonds and complexes within the replication complex and enhance RNA replication.

Alphavirus RNA capping is also sensitive to oxidation

If flaviviruses use oxidation to aid their replication, could other RNA viruses do the same? Alphaviruses are capped RNA viruses that replicate in replication vesicles formed from the plasma membrane with a reducing environment on the cytoplasmic side and a potentially oxidizing environment on the extracellular side(Kujala et al. 2001). We tested the sensitivity of Sindbis virus, an alphavirus, to BHA to determine if blocking oxidative stress altered viral replication. We found that the replication of a luciferase expressing Sindbis virus (dsSINV-Renilla Luciferase) (Steel et al. 2011) was significantly affected by BHA at similar concentrations that affected flavivirus replication

(Figure 5.8A). We then tested if oxidation affected the activity of the alphavirus nonstructural protein (nsP1) capping enzyme. nsP1 is different than the flavivirus capping enzyme, but nsP1 contains the guanylyltransferase activity similar to the flavivirus NS5 capping enzyme. We expressed and purified histidine-tagged Venezuelan Equine Encephalitis Virus (VEEV) nsP1 protein in *E. coli* and purified the protein to homogeneity. Adding increasing concentrations of Diamide enhanced the guanylation of nsP1 (Figure 5.8B), similar to what we observed with the flavivirus NS5 capping enzyme. These data suggest that alphavirus RNA capping may also be sensitive to oxidation.

Discussion

Oxidative stress is common during RNA virus infection and our results indicate that flaviviruses and alphaviruses may actively induce oxidative stress as a mechanism to regulate their RNA replication. Many positive strand RNA viruses (Flaviviruses, Togaviruses, Caliciviruses, Coronaviruses, etc.) replicate their genomes in induced compartments derived from cellular membranes with differential environmental conditions on either side, including the ER, mitochondria, plasma membrane, and endosomes. The prevailing rationale for forming these membranous replication compartments is protection of their dsRNA intermediates from cellular antiviral defense mechanisms such as TLRs and RNAi. Formation of these compartments would also provide a unique microenvironment that could be modulated during the course of infection to tailor replication towards either negative or positive strand RNA synthesis. During flavivirus infection, negative strand RNA synthesis is thought to occur early and

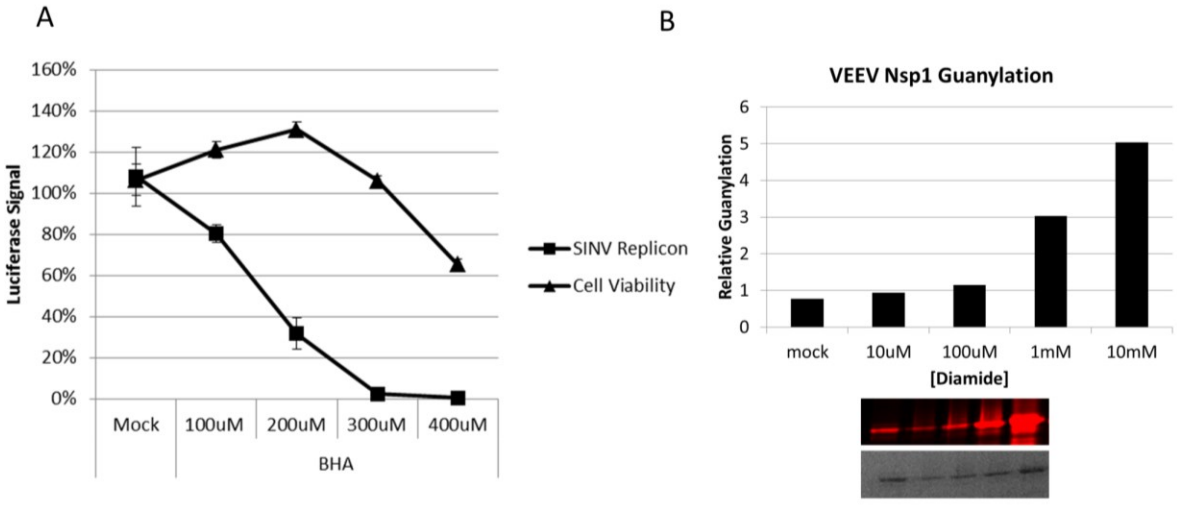

Figure 5.8 Alphavirus RNA replication and capping is also sensitive to oxidative conditions.

A) Sindbis replicons in BHK cells demonstrate a sensitivity to antioxidant (BHA) treatment, similar to flaviviruses. At 200uM BHA, the virus is significantly inhibited, while cell viability is maintained. **B)** Purified VEEV nsP1, which is the capping enzyme for alphaviruses, also shows an increase in guanylation activity with the addition of oxidants (Diamide).

may not require replication compartment formation. The cytoplasmic environment would be reducing at this point. Once the replication compartments are formed, reactive oxygen species could enter the replication compartment, modify enzymes, and switch replication from negative to positive strand mode.

Oxidative activation of the NS5 guanylyltransferase is reminiscent of an oxidative switch, where redox sensitive cytoplasmic proteins are activated by ROS-mediated oxidation of amino acid residues. An example of this is the ataxia-telangiectasia mutated (ATM) protein kinase, which is activated by oxidative stress through ROS-induced disulfide-bonded dimers(Guo et al. 2010) . Methionine oxidation has also been recently shown to activate the *E. coli* HypT transcription factor(Drazic et al. 2013), and our data with Met219 suggest that flaviviruses may use methionine oxidation as a mechanism to activate their enzymes. Met219Ile showed reduced oxidative activation, indicating that other residues may be involved in the process, which we are actively pursuing. There are other potential redox switches in NS5 that could influence enzymatic activity. The NS5 polymerase contains a conserved cysteine pair with a very similar structure to the CxxC redox switch found in thioredoxin(Malet et al. 2007; Yoshihara et al. 2014). Additionally, a disulfide bonded cysteine pair is observed between conserved residues Cys733 and Cys852 in the 2HFZ West Nile virus RdRP crystal structure near the GDD active site loop, suggesting that disulfide bonding could take place within the NS5 RdRP and affect enzymatic activity. There have been no studies of intramolecular disulfide bonding or methionine oxidation of any flavivirus nonstructural protein besides NS1 during infection, and such data would be critical for further definition of the effects of oxidation on flavivirus RNA replication.

In our current model, a reducing environment early in infection biases towards negative strand RNA synthesis whereas increasing cytoplasmic oxidation later in infection biases replication towards positive strand synthesis. Our data with BHA indicates that blocking intracellular oxidation significantly reduces viral replication, suggesting that oxidation is necessary for efficient RNA replication. It is possible that part of the effect observed with BHA is due to changes in disulfide bonding within NS1, which is a luminal ER protein and known to contain several disulfide bonds(Blitvich et al. 2001). However, it is unlikely that virus-induced oxidative stress significantly affects NS1 oxidation unless ROS levels within the ER decline later in infection. It has previously been reported that treatment of cells and *in vitro* RNA replication assays with the oxidant SNAP reduces negative strand dengue virus RNA replication(Charnsilpa et al. 2005; Takhampunya et al. 2006), indicating that negative strand synthesis is more active in a reducing environment. Therefore, limiting oxidative stress with antioxidants will likely block positive strand RNA synthesis and bias replication towards negative strand synthesis. These effects would manifest as changes in the ratio of RNA species produced during infection. We observed that treatment of infected cells with BHA reduced the levels of both positive and negative strand RNAs, but positive strand RNA accumulation was affected to a greater extent than negative strand accumulation. The change in proportions of capped RNAs suggests that blocking oxidative stress interferes with RNA capping, which is very similar to our *in vitro* results. Oxidation of NS5 Met219 appears to enhance guanylation activity as demonstrated by decreased *in vitro* activity with the Met219Ile mutant and increased activity with the Met219Glu mutation. Mutating M219 to either Ile or Glu completely disrupts replication, whereas mutating the

residue to Gln partially restores replication, suggesting that the Ile mutation biases towards negative strand synthesis while the Glu mutation biases towards positive strand synthesis. If the Met219Ile mutant is strongly biased towards negative strand, positive strand synthesis would not be able to occur, and vice versa with Met219Glu. Met219Gln, which shows similar oxidative activation as wild-type protein, may be able to switch between negative and positive strand synthesis more effectively and allow the replication cycle to proceed, although less efficiently than the wild-type protein. These data suggest that modulating oxidation of Met219 may significantly contribute to the flavivirus RNA replication cycle. We are currently pursuing experiments to ascertain the oxidation status of Met219 and other residues at different points during infection by mass spectrometry.

How do flaviviruses induce oxidative stress during infection? There is currently no definitive data on this topic, but several tantalizing possibilities exist. Japanese encephalitis infection down-regulates thioredoxin expression(Yang et al. 2010), which would increase cytoplasmic oxidation and interfere with homeostatic redox balance during infection. Dengue virus infection can induce autophagy, which is used to support virus-induced membrane remodeling(McLean et al. 2011). Autophagy can induce a release of ROS, although this may not be a common mechanism as West Nile virus does not appear to utilize autophagy during replication(Vandergaast & Fredericksen 2012). An interesting candidate for inducing oxidative stress is NS2A. NS2A is a transmembrane ER protein and is involved in RNA replication and evasion of the interferon response(Muñoz-Jordan et al. 2003; M. Jones et al. 2005). NS2A is partially responsible for the cytopathic effect produced during viral infection (Rossi et al. 2007).

NS2A has also been demonstrated to possess pore-like activity when expressed in *E. coli*(Chang et al. 1999), supporting the hypothesis that NS2A may act as a pore during infection. NS2A can induce interferon-independent apoptosis(Melian et al. 2013), and oxidative stress is well known to induce apoptosis. Two cell-adapted mutants in the NS2A protein, D73H and M108K, increase cellular viability and reduce RNA replication during infection (Rossi et al. 2007). A recent topology study of NS2A localizes D73H and M108K to transmembrane regions of NS2A(Xie et al. 2013), which raises the possibility that NS2A possesses channel or pore-like activity that allows ROS into the cytoplasmic side of the ER membrane. These results support the hypothesis that NS2A may alter the cellular redox balance late in infection and induce a switch from negative strand to positive strand RNA synthesis.

Virus-controlled use of oxidative, pH, or ionic potential differences on either side of a replication membrane would provide an elegant mechanism for flaviviruses to regulate their genomic replication. Many RNA viruses replicate their RNA on intracellular membranes with different environmental conditions on either side and our findings with flaviviruses and alphaviruses may have broad implications for many different types of positive strand RNA viruses. Further analysis of how RNA viruses use oxidation and other environmental conditions to control RNA replication will help increase our understanding of how these pathogens usurp cellular resources to replicate their genomes and provide novel points of intervention that may be exploited for antiviral or vaccine development.

Chapter Six: Summary, Future Directions, and Impact

Summary

Arthropod-borne infectious diseases cause a significant global health burden due to limited prevention and treatment options. Dengue virus alone is responsible for over 350 million infections each year and is a leading cause of death among children in some asian and latin American countries (Bhatt et al. 2013; WHO 2014). These arboviruses are spreading into new regions of the world with naïve populations. With the increased distribution of competent mosquitoes, arboviruses are reaching new areas of the world. Recent reports show that Chikungunya virus has established infection in the Caribbean (Epstein 2007; De Valk et al. 2013; D. Mackenzie 2013), representing the first cases of this alphavirus in the Americas. Arboviruses are emerging and re-emerging all over the world. The global threat caused by arboviruses continues to increase and poses a significant health concern.

Substantial advancements over the last several decades have enhanced our understanding of virus infection and transmission, but there has not been significant success to prevent, control, or treat these viral infections. We still do not have an effective drug or treatment strategy to productively fight and inhibit virus infection. The work presented in this dissertation significantly advances the field of arbovirology and provides novel mechanisms to express recombinant alphaviruses, detect wild-type infection using a subgenomic reporter RNA, and increases our comprehension of the virus-induced oxidative stress to regulate RNA replication.

Future Directions

Double subgenomic alphavirus expression and infection systems

Double subgenomic alphavirus (dsSINV) expression vectors were improved through the engineering of DNA plasmids containing a promoter to directly transcribe the viral genome in transfected cells. Our DNA plasmid platforms allow for quick, easy, and efficient generation of recombinant alphaviruses that can be designed to stably express foreign proteins concurrent with infection in a wide variety of cell types. Double subgenomic recombinant alphaviruses are essential tools to study viral infection and the newly optimized systems will significantly increase their utility. We have used the dsSINV-luciferase as a critical tool in screening drug compounds through a quick and high-throughput method using the luciferase as a reporter of infection. Other groups around the world have requested our system to be used in a wide variety of applications.

Similar systems could be engineered and created for various RNA viruses to allow more efficient tools for generating recombinant RNA viruses. Multiple people have expressed interest in a Chikungunya system that could be designed similar to the constructs we have developed with dsSINV. This DNA plasmid launched double subgenomic expression and infection system could be used for other alphaviruses or modified for potentially any RNA virus.

These improved dsSINV expression systems could also be used in vaccine development. Alphavirus RNA and cDNA have been used as vectors for driving immunization. Traditionally, incomplete replicon systems are used to express an antigen in the cells transfected with the viral RNA/DNA(Liljeström & Smerdou 2000; JM et al.

2000). Our infectious dsSINV plasmids could be used to transfect cells and not only express the antigen in the transfected cell, but the virus could leave the cell and infect other cells to achieve a higher immune response. The dsSINV could be mutated to make it less robust, allowing the immune system to clear the SINV infection and also gain immunity to the antigen being expressed from the subgenomic promoter(Ivanova & M. J. Schlesinger 1993; Nivitchanyong et al. 2009). A paper was recently published that cited our work and used the CMV promoter/alphavirus expression systems to develop a Venezuelan Equine Encephalitis virus expression system that has successfully been used in vaccine studies to induce an immune response and protect mice from infection(Tretyakova et al. 2013). This system could be modified for other viruses and be used as a significant tool for vaccine development.

Infecting mosquitoes in the lab has traditionally been performed by feeding adult mosquitoes an infectious bloodmeal or intrathoracicly injecting virus. However, to infect mosquitoes at an earlier stage, larvae can be infected by allowing the larvae to feed on infected cells (L L Cheng 2001). It has been described that highly active virus infection in larval stages impairs their ability to emerge as adults(Higgs et al. 1999). Through our plasmid launched infection, the larvae could be transfected and the infection would be slower (See figure 2.7), potentially allowing the larvae to pupate and emerge as adults before infection reaches high levels. This system would allow male and female mosquitoes to be infected at a young and developmentally immature stage, which may be useful for studying trans-ovarian transmission, venereal transmission, or dsSINV expression of foreign genes at earlier mosquito life stages.

Wild-type alphavirus infection detection systems

We also developed a novel method for detecting wild-type virus infection using the alphavirus subgenomic promoter. This promoter is only active on a negative strand copy of the viral genome and is completely dependent on the viral replication complex. We utilized this strict dependence to engineer a subgenomic RNA containing a reporter that would only be expressed when the virus is present. For the first time, we were able to create a subgenomic reporter system that works directly in mosquito cells. Significant expression of the reporter protein was detected only during infection. This subgenomic reporter RNA technique was then used to generate transgenic *Aedes aegypti* mosquitoes that constitutively express our construct in cells, but only during infection does the reporter protein get detected. This provides the first time a transgenic mosquito has been developed to detect wild-type infection in living mosquitoes. There are many potential applications that this improved detection system can be used for. Through optimization of this system, it would provide the ability to visually monitor the complete transmission cycle of a virus infection from various mosquitoes and hosts. Living infected mosquitoes could be identified and used in subsequent studies pertaining to transmission, progression of the virus infection within the mosquito, or treatments to control or impair infection in the mosquito. This fluorescent detection system can provide a simple and visual system for detecting infection.

Additionally, the subgenomic reporter RNA system can be used as a diagnostic tool to detect infectious virus. The subgenomic reporter RNA system was sensitive at detecting small amounts of infectious virus in relatively short periods of time (See Figure 3.4). This sensitivity and quick results present a potentially important way to identify

infectious virus. The stable C6/36 cell line expressing our reporter construct could readily be used to detect infection in samples collected from patients or field studies. The sample would simply be applied to the cell line and fluorescence would be monitored to indicate infection. Our constructs are currently engineered for either detecting Sindbis virus or Chikungunya virus, but additional virus detection systems could be developed to provide a wide array of constructs that can be used to diagnose different viral infections.

Alternatively, the subgenomic reporter RNA constructs can be used as a mechanism to inhibit or control infection. Instead of a fluorescent reporter protein being expressed during infection, a cytotoxic or other inhibitory protein can be turned on during infection. Virus specific induction of the ribosomal inhibitory protein saporin resulted in significant reduction in virus replication (See Figure 3.6). Future application of this system would be to use different inhibitory proteins that may allow for more specific control or regulation during infection. Instead of a toxic protein, a protein or antisense RNA could be used to alter normal mosquito physiology resulting in a post infection mosquito that is impaired at transmitting the virus. Hypothetically, a protein/RNA that prevents midgut escape or inhibits the mosquitoes' ability to fly or find a blood meal would significantly reduce viral transmission.

Oxidative stress during flavivirus infection

We also discovered an essential role for the induction of oxidative stress during infection. It has been known for many years that infection causes an increase in oxidation within infected cells, but we present the first explanation for why viruses

induce this oxidative stress. The ability to synthesize capped positive-strand genomic RNA is significantly inhibited in the presence of antioxidants, whereas the addition of oxidants results in enhanced capping activity. This indicates that the virus is intentionally inducing oxidative stress to aid its replication and this is the first described role for oxidative stress during infection. Additionally, this oxidative manipulation may be conserved among a wide variety of RNA viruses. Both flaviviruses and alphaviruses demonstrate an increased activity of RNA capping during oxidative conditions. This novel discovery significantly helps enlighten how viral RNA replication is coordinated and regulated within the cell and may lead to potential targets for antivirals.

We are actively working to define how antioxidants inhibit replication. Data indicates that the NS5 capping enzyme is directly influenced by oxidation conditions, but other potential oxidation sensitive proteins and viral functions may be identified. Do the other nonstructural proteins in the replication compartments also benefit from oxidation? What all is being inhibited with the addition of an antioxidant? How effective are antioxidants at treating infection in a mammalian system? There is a lot of work that needs to be done to identify exactly how antioxidants function to inhibit viral replication and characterize the effects of oxidation on other viral proteins and functions.

The balance and regulation of oxidation appears to be critical for viral replication and work needs to be done to understand the temporal and spatial induction of the oxidative stress. When does the oxidation get induced? Is it initially localized in the replication compartments? A better understanding is needed of the induction of oxidative stress and its implications with the different stages of viral replication. Specific details regarding negative strand and positive strand synthesis needs to be generated

based on different times post infection and the induction of oxidation. Is oxidation really the switch from negative to positive strand synthesis?

It is also unknown how flaviviruses induce oxidative stress. It would be valuable to identify how the viruses are triggering oxidative stress during infection. We have hypothesized that one of the nonstructural proteins in the replication compartment may be responsible for releasing reactive oxygen species from the endoplasmic reticulum. However, a number of experiments are needed to evaluate this idea. There are several transmembrane and membrane associated viral proteins such as NS2A that may play critical roles in inducing oxidative stress. It would be beneficial to identify which proteins are involved because they can be targets for developing antiviral drugs.

The oxidation activation detected in flavivirus and alphavirus infection is important knowledge regarding virus replication and may be conserved in a wide variety of RNA viruses. Further tests will need to be performed with other RNA viruses to identify how conserved this phenotype is among other viruses. We anticipate oxidation to be an increasingly important part of viral infection that will continue to provide insight and potential targets for virus control.

Impact

Over the last 5 years, we have made significant progress with the projects described in this dissertation. Presentations, talks, and posters have been presented and were well received at conferences around the nation. Papers have been published in Virology Journal and the Public Library of Science (PLoS)(Steel et al. 2011; Steel et al. 2013). We have sent our plasmids to labs around the world and have collaborated

with others in efforts to better understand virus infection and make significant improvements on our projects. We have made important contributions and advancements to the field of arbovirology that will hopefully lead to finding ways to prevent, control, or treat arbovirus infections. Although these alphaviruses and flaviviruses continue to cause serious health concerns worldwide, significant advancements are being made through scientific research every year. We describe several projects in this dissertation that have successfully enhanced our understanding of virus infection.

We have optimized a DNA-launched system for generating double subgenomic alphaviruses that increases their ease of use and will make it more efficient for researchers to utilize the double subgenomic alphaviruses for virus infection and expression systems and for vaccine development. Just recently a paper described the success of a VEEV vaccine based on the DNA-launched plasmid system described in chapter 2.

The alphavirus subgenomic reporter RNA system that we have described in chapter 3 and chapter 4 can effectively be used to detect infection. The novel application of the subgenomic promoter in transgenic mosquitoes provides evidence that this system can be used in future studies to selectively express a foreign protein during infection. This system provided researchers the ability to track and monitor infection to better comprehend the complete transmission cycle of arboviruses. The subgenomic reporter RNA also provides a mechanism that may be used to express toxic proteins to limit or control infection or kill the infected mosquito. This system has

potential applications that could significantly enhance our ability to prevent or control mosquito virus infections.

The oxidation enhancement observed with flavivirus RNA replication represents a critical increase in our knowledge of viral replication and how viruses alter the cellular state of the host cell. The oxidation sensitivity has been observed in two different classifications of viruses (flavivirus and alphavirus) and may have extended application to other RNA viruses. The change in oxidation conditions within the cell enhances our knowledge of the intricate mechanisms of replication and may lead to potential drug targets that could be used to treat infection. We observe that viral replication requires oxidation and that antioxidant treatment significantly reduces virus infection. We are actively pursuing antioxidants as a method to control infection and will elucidate the effect of oxidation during infection. Characterization of oxidative stress's role during infection described in Chapter 5 is a novel finding and the ramifications are yet to be fully understood. A more complete understanding of RNA replication and the requirements for oxidation may lead to potent anti-viral therapies that could be used to treat millions of those infected each year.

Despite the devastation caused by these arboviruses, we are making significant advances. This dissertation has described new methods that can be used to generate recombinant alphaviruses for expression or vaccine studies, novel applications of the alphavirus subgenomic promoter to detect wild type infection or express foreign proteins during infection, and a new discovery of oxidation sensitivity and oxidative enhancement of RNA virus replication during infection. All of these advancements will help

researchers better understand arbovirus infections and will hopefully lead to improved methods to prevent, control, or treat these serious viral infections worldwide.

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