DISSERTATION

ANALYSIS OF VIRUS-DERIVED SMALL RNAS REVEALS THAT THE RNA SILENCING RESPONSE TO FLAVIVIRUS INFECTION DIFFERS DRAMATICALLY BETWEEN C6/36 AND AAG2 MOSQUITO CELL LINES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JACLYN C. SCOTT ENTITLED ANALYSIS OF VIRUS-DERIVED SMALL RNAS REVEALS THAT THE RNA SILENCING RESPONSE TO FLAVIVIRUS INFECTION DIFFERS DRAMATICALLY BETWEEN C6/36 AND AAG2 MOSQUITO CELL LINES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ANALYSIS OF VIRUS-DERIVED SMALL RNAS REVEALS THAT THE RNA SILENCING RESPONSE TO FLAVIVIRUS INFECTION DIFFERS DRAMATICALLY BETWEEN C6/36 AND AAG2 MOSQUITO CELL LINES

The exogenous small RNA pathway has been shown to be an important antiviral defense in mosquitoes against arboviruses such as dengue virus (DENV), but little is known about how the pathway and the virus interact in the cell. The studies described in this dissertation examine the how small RNA pathways interact with DENV and a mosquito-only flavivirus, cell-fusing agent virus (CFAV), in mosquito cell cultures.

Deep sequencing of virus-specific small RNAs in *Aedes aegypti* Aag2 cells indicates that DENV2 is targeted by the exogenous RNA interference (RNAi) pathway in this cell line, which is consistent with the DENV2-specific small RNAs seen in DENV2-infected *A. aegypti* mosquitoes. When the DENV2-specific small RNAs from the *Aedes albopictus* C6/36 cell line were analyzed, the size and polarity of the small RNAs was not consistent with the exogenous small interfering RNA (siRNA) pathway. Further molecular analysis of the C6/36 cell line indicated that it appears to lack functional Dicer2 processing of long double-stranded RNA (dsRNA).

CFAV small RNAs were also discovered in the Aag2 cell line during the deep sequencing analysis. It appears that this cell line is persistently infected with this

mosquito-only flavivirus, and the virus is also targeted by the exogenous siRNA pathway in the cells. Sequence comparisons between CFAV and DENV2 RNA did not show long regions of sequence identity between the two viruses, indicating that a sequence-specific mechanism for virus-derived small RNAs from one virus to interfere with replication of the other virus during dual infections seems unlikely. The C6/36 cell line was inadvertently infected with CFAV, but the CFAV-specific small RNAs in C6/36 cells did not appear to be generated from the exogenous siRNA pathway, consistent with the DENV2-specific small RNAs in this cell line. The larger sized, mostly positive sense virus-specific small RNAs found in the C6/36 cells suggest that virus infections may be targeted by another small RNA pathway (such as the piwi-interacting pathway) in this cell line.

These studies provide a better understanding of the interactions of DENV2 with the mosquito antiviral RNAi pathway in infected mosquito cells and have revealed a dysfunctional RNAi pathway in the C6/36 cell line. This work also provides a basis for further studies examining the interactions between mosquito-only flaviviruses, arboviruses and the antiviral RNAi pathway.

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CHAPTER 1

LITERATURE REVIEW

Introduction:

The resurgence of mosquito-borne diseases is an important global health concern. Arthropod-borne viruses (arboviruses) cause considerable morbidity and mortality around the world, and the number of cases has gone up dramatically in the last 30 years, due to poor vector control and a lack of effective vaccines or drugs. Previously little-known viruses such as Chikungunya have emerged and caused thousands of new cases. Dengue, arguably the most important arbovirus, causes 50-100 million cases each year of dengue fever in tropical regions of the world. Research into how arboviruses interact with their mosquito vectors will lead to new strategies to reduce virus transmission and arboviral-related disease.

Dengue virus genome:

Dengue viruses are in the family *Flaviviridae*, and have single-stranded positive sense RNA genomes. There are four distinct serotypes of dengue viruses, numbered 1 through 4. The four serotypes are distinguished by the immune response they induce. The four serotypes are spread throughout the tropical regions of the world. Other mosquito-borne flaviviruses include West Nile virus (WNV), which has emerged in the United States in the last decade, Kunjin virus (KUNV), which is a subtype of WNV and is found in Oceania, Japanese encephalitis virus (JEV), which is found mostly in Asia, and yellow fever virus (YFV), which is found in South America and Africa.

The genome of the dengue viruses is approximately 10.7 kilobases (kb) in length and has a 5' cap structure, but lacks a polyA tail. It encodes three structural proteins and

five non-structural proteins, some of which are processed further into shorter proteins (See Table 1.1 for description of genome organization).

Table 1.1: Genome organization of DENV2 from NCBI Reference Sequence NC_001474.2

DENV2 Gene	Proposed Function	Nucleotide Position
5' Untranslated Region (5'UTR)	Cyclization, replication, translation initiation	5-96
Capsid (C)	Encapsidates viral RNA	97-438
Membrane Glycoprotein Precursor (prM)	Blocks envelope fusion with host cell membrane	439-711
Membrane Protein(M)	Viral envelope protein	712-936
Envelope Glycoprotein (E)	Viral binding to all membranes and fusion	937-2421
Non-structural Protein 1 (NS1)	Replication, has a secreted form	2422-3477
Non-structural Protein 2A (NS2A)	Interferon (IFN) inhibition?	3478-4131
Non-structural Protein 2B (NS2B)	NS3 co-factor	4132-4521
Non-structural Protein 3 (NS3)	Triphosphatase (capping), protease, helicase	4522-6375
Non-structural Protein 4A (NS4A)	Membrane modification, replication	6376-6756
2K Peptide	NS4B signal peptide	6757-6825
Non-structural Protein 4B (NS4B)	Replication, IFN inhibition	6826-7569
Non-structural Protein 5 (NS5)	5'-methyltransferase (capping), RNA-dependent RNA Polymerase (RdRP)	7570-10269
3' Untranslated Region (3'UTR)	Cyclization, replication	10270-10723

The order of the genes on the genome from 5'→ 3' is: C-prM-M-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5.

When the virus RNA enters the cell cytoplasm, it is translated to make a large polyprotein, which is cleaved co-translationally into individual viral proteins. Viral RNA replication occurs in the perinuclear region of the cell, most likely in membrane-enclosed replication complexes (Henchal & Putnak, 1990). During replication, full-length double-

stranded RNA (dsRNA) forms from a positive sense genome annealed to a negative sense genome (Henchal & Putnak, 1990).

Dengue clinical disease:

Dengue infection in humans commonly causes dengue fever, but can also cause the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). In the more common dengue fever disease, infected individuals experience a biphasic fever, headache, body aches and rash. Classical dengue fever occurs after an incubation period of 3 to 15 days, with an abrupt fever onset, followed by a retroorbital headache. The fever usually persists for 4 to 6 days, and viremia accompanies the fever. After the fever, a maculopapular or morbilliform rash sometimes appears and lasts from 1-5 days, and a second fever can then appear with the rash. When the fever and rash subside, pinpoint hemorrhagic lesions known as petechiae may appear on the extremities (Halstead, 2007, Henchal & Putnak, 1990).

In the more severe forms of the disease, DHF and DSS, the early stages are similar to dengue fever, but 2-5 days later, the patient rapidly deteriorates and death can occur. There is a rapid onset of capillary leakage due to increased vascular permeability, thrombocytopenia, liver damage and problems with hemostasis. Hypovolemic shock occurs when fluids are lost to tissues and are not adequately replaced in the patient, and can lead to profound shock and loss of blood pressure, pulse and death. Treatment involves supportive care with fluid replacement, and antipyretics and analgesics (Halstead, 2007, Henchal & Putnak, 1990).

There are two main theories for the cause of DHF/DSS. In the first theory, it has been suggested that antibodies from a previous infection with another DENV serotype enhance the ability of virus to infect cells with the second serotype; this is often referred to as antibody-dependent enhancement (ADE). Previously infected people have non-neutralizing, cross-reactive antibodies that may actually help the virus enter immune cells and cause a complex immunological response resulting in DHF/DSS (Holmes & Twiddy, 2003). Epidemiological studies show that there is a higher prevalence of DHF/DSS in secondary infections than in primary infections (Holmes & Twiddy, 2003).

The other theory to explain more severe forms of dengue disease implies that some virus strains are more virulent than others. Some genotypes, such as the American genotype of DENV2, are rarely associated with DHF/DSS, while others such as some of the Southeast Asian genotypes cause more severe disease (Mota & Rico-Hesse, 2009, Rico-Hesse et al., 1997). There are also some cases of DHF/DSS from primary DENV infections (Holmes & Twiddy, 2003, Weaver & Vasilakis, 2009). More research is needed to determine the role of variable virulence of virus genotypes in disease severity.

There are an estimated 50-100 million dengue fever and several hundred thousand DHF cases each year around the world, with a third of the world's population (2.5 billion) at risk for infection. The case fatality ratio varies from <1% up to 15% (Gubler, 2002). Estimates indicate there may be as many as 20,000 deaths per year due to dengue virus diseases (Weaver & Vasilakis, 2009). There are no DENV-specific treatments for those that are infected other than supportive care. Various groups are working on vaccines, although this is a difficult task, as the vaccine will need to provide immunity to all four serotypes simultaneously to avoid any ADE reactions after exposure to the virus.

It is also important for the vaccine to be incapable of transmission by mosquitoes, which might allow for the virus to revert to its more virulent form. The most promising candidates so far have been live attenuated vaccines containing all four of the serotypes or a vaccine using the 17-D yellow fever vaccine backbone to express the DENV envelope and pre-membrane genes from all four serotypes (Guy & Almond, 2008).

Dengue transmission cycles:

DENV is transmitted predominantly between *Aedes* mosquitoes and humans in an urban cycle. The most common mosquito species involved in the transmission of DENV is *Aedes aegypti*. *Aedes albopictus* are also able to transmit the virus, although they are less efficient and produce slow-moving outbreaks compared to the sharp epidemics seen with *A. aegypti* (Halstead, 2007). *A. aegypti* is a peridomestic mosquito that lives in close proximity to humans, lays eggs in water containers, goes into human homes and takes bloodmeals from people multiple times through its gonotrophic cycle, all of which contribute to increased ability to transmit viruses such as DENV between humans. Sylvatic DENV has been found in non-human primates in the forest of West Africa and Malaysia. These viruses are transmitted between primate hosts by various *Aedes* mosquito species, such as *A. furcifer* and *A. luteochephalus*, and are genetically distinct from the viruses found in urban cycles (Weaver & Vasilakis, 2009). Evolutionary analysis showed that the four serotypes of DENV evolved around 1000 years ago, and most likely started causing larger human epidemics in the last several hundred years. After World War II, the more severe form of DHF/DSS began appearing in Southeast Asia and has spread to many areas around the world and has become a significant

problem in the Americas beginning in the 1980's. The exact origin of DENV is still unclear, although it appears that all of the DENV were originally found in primates, and that each serotype independently crossed over to humans (Holmes & Twiddy, 2003).

Dengue and mosquito interactions:

Mosquitoes acquire DENV by imbibing blood from infected humans. The virus first infects the midgut epithelial tissue of the mosquito, disseminates from the midgut (possibly through the tracheal system) into the hemocoel and eventually infects the salivary glands, where the virus can then be injected into a person when a mosquito takes a blood meal. The time from when a mosquito first ingested a virus in a bloodmeal to the time that the mosquito is able to transmit the virus is known as the extrinsic incubation period (EIP) (Salazar et al., 2007). For DENV in *A. aegypti* mosquitoes, this EIP is typically 7-14 days and is affected by environmental factors such as temperature and humidity, and factors such as mosquito vector competence, mosquito and viral genetics (Black et al., 2002). Some strains of *A. aegypti* have an even shorter EIP. For example, some mosquitoes of the Chetumal strain of *A. aegypti*, originally isolated from the Yucatan Peninsula of Mexico, were experimentally shown to have virus in the salivary glands as early as 4 days after taking a DENV infectious bloodmeal (Salazar et al., 2007). A short EIP gives the virus a better chance to be transmitted to a new human host during the mosquito's life span and thus has important epidemiological implications.

Alphaviruses:

Alphaviruses are single-stranded positive sense RNA viruses in the family *Togaviridae*. Their genomes are approximately 11.7 kb in length and have 5' cap and a polyA tail. The genes are arranged 5'-UTR-nonstrutctural protein(nsp)1-nsp2-nsp3-nsp4-capsid-envelope(E)3-E2-6K-E1-UTR-3'. When the virus RNA enters the cell, the ribosomes translate the first two-thirds of the genome containing the non-structural proteins, which form a replicase that generates the negative strand template. The negative strand is then used to generate more full length genomic positive strand RNA (referred to as 49S) and the subgenomic (26S) 3' third of the genome containing the structural genes (capsid, envelope proteins and 6K protein). The 26S RNA is capped and translated into a long polyprotein, which is cleaved to form the structural proteins. The E2 and E1 glycoproteins are present as heterodimers on the surface of the virion (Strauss & Strauss, 1994). E1 protein is involved in fusion of the virus envelope with the intracellular (endosomal) membranes in the host cell and E2 protein is responsible for receptor recognition, host tropism, and virulence (Gardner et al., 2000, Klimstra et al., 1998, Levine et al., 1996, Strauss & Strauss, 1994).

Many alphaviruses are transmitted by mosquito vectors and cause disease ranging from mild arthralgias to encephalitis in their vertebrate hosts. Alphaviruses found in the Old World such as Chikungunya virus (CHIKV), O'nyong nyong virus (ONNV), Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus (SINV) often cause human joint disorders. The New World alphaviruses eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV), as their names imply, cause encephalitis in certain vertebrate hosts.

In recent years, CHIKV has emerged in the southern Indian Ocean region and has spread quickly to other areas including Italy and India as travelers returned from visiting outbreak areas. Chikungunya disease causes fever, skin rash and severe arthralgia in patients (Pialoux et al., 2007). The virus is transmitted by *Aedes* mosquitoes, with *A. albopictus* being the important vector on the island of Reunion in the southern Indian Ocean, and *A. aegypti* being the major vector in India (Pialoux et al., 2007, Reiter et al., 2006, Renault et al., 2007).

SINV has been studied extensively in mosquitoes in the labs at Colorado State University. In nature, the virus is usually transmitted between avian vertebrate hosts and *Culex* mosquitoes, although they have also been isolated *Aedes* species of mosquitoes (Doherty et al., 1977, Doherty et al., 1979). After the virus is ingested in a bloodmeal, the virus replicates in midgut epithelial cells, then reaches the hemolymph and spreads to the salivary glands where it can be transmitted to new hosts (Myles et al., 2003). The prototype SINV strain is AR339, which was originally isolated from *Culex* mosquitoes in Egypt (Taylor et al., 1955). An infectious clone was developed to represent AR339, and viruses made from the infectious clone are termed TR339 (Klimstra et al., 1998, McKnight et al., 1996). Other infectious clones have been engineered based on the mouse neurovirulent SINV strain TE12, and are termed TE3'2J and TE5'2J, with TE3'2J having the second subgenomic promoter for insertion of a gene of interest after the viral structural genes, and TE5'2J having the gene of interest insertion site after the first subgenomic promoter with the viral structural genes after the second subgenomic promoter (Hahn et al., 1992, Pierro et al., 2003). An infectious clone of another SINV strain, MRE16 originally from Malaysia, has also been engineered to express genes both

in front of or after the viral structural genes (Foy et al., 2004, Myles et al., 2003). When virus is given to mosquitoes *per os*, TE3'2J and TR339 will infect *A. aegypti* midguts, but have poor ability to disseminate, while MRE16 both infects midguts and efficiently disseminates into other *A. aegypti* mosquito tissues (Myles et al., 2004, Seabaugh et al., 1998).

These engineered viruses with second subgenomic promoters are referred to alphavirus transducing systems and are used in the laboratory as tools to express genes in insects (Foy et al., 2004). These viruses have been used to express antisense RNA to DENV genes, and to express proteins such as green fluorescent protein, chloramphenicol acetyltransferase enzyme and the Flock House virus (FHV) RNA interference inhibitor B2 (Cirimotich et al., 2009, Foy et al., 2004, Gaines et al., 1996, Myles et al., 2008, Olson et al., 1996, Olson et al., 1994, Olson et al., 2000, Pierro et al., 2003).

Mosquito-only flaviviruses:

Mosquitoes are also infected by flaviviruses that don't appear to be transmitted to vertebrates, but instead are only found in mosquitoes. The viruses found thus far seem to be most closely related to the mosquito-borne flaviviruses, and fall into a separate phylogenetic clade of flaviviruses found only in mosquitoes (Hoshino et al., 2007, Marin et al., 1995). The first described mosquito-only virus was discovered in cell culture, when the media from Peleg's *A. aegypti* embryo cells was transferred to Singh's *A. albopictus* larval cells. The *A. albopictus* cells started to fuse and formed syncytia after approximately 5 days. Scientists weren't sure exactly what was causing this at first and named the causative agent "cell fusing agent" (Stollar & Thomas, 1975). This was later

determined to be a virus and named cell fusing agent virus (CFAV) (Igarashi et al., 1976). It is believed that this virus came from infected mosquitoes that were ground up and used to start Peleg's embryo cell line. Exactly how the virus is transmitted in nature is not completely understood, although it appears it may be a vertical transmission since embryos used to initiate the original cell culture were infected. The virus is approximately 10.6 kb long and has a similar genome structure and genes to other flaviviruses (Seet Table 1.2). The amino acid sequence of the NS5 protein has 45.7% identity with NS5 of DENV2. There is a potential stem loop secondary structure in the 94 3' terminal nucleotides and some secondary structures in the 120 5' nucleotides of the genomic RNA (Cammisa-Parks et al., 1992). It is not clear how commonly this virus is found in mosquitoes in the wild.

Table 1.2. Genome organization of CFAV from NCBI Reference Sequence NC_001564.1

CFAV Gene	Nucleotide Position
5'UTR	1-113
C	114-497
prM	498-770
M	771-938
E	939-2228
NS1	2229-3302
NS2A	3303-4019
NS2B	4020-4466
NS3	4467-6227
NS4A	6228-6632
2K Peptide	6633-6701
NS4B	6702-7472
NS5	7473-10136
3'UTR	10137-10695

In 1999, another mosquito-only virus was found in *Aedes macintoshi* mosquitoes collected in the flooded dambos of Kenya and was named for the nearby Kamiti River. This was the first insect-only flavivirus to be isolated from mosquitoes in nature (Sang et

al., 2003). It was discovered using primers to amplify a wide variety of flaviviruses. Kamiti River virus (KRV) RNA is 11,375 nucleotides long with a single open reading frame that encodes 10 proteins (Crabtree et al., 2003). Laboratory studies showed that KRV could infect *A. aegypti* mosquitoes, and that the virus was transmitted vertically in these mosquitoes at a rate of 3.90% (Lutomiah et al., 2007). KRV does not cause CPE in regular C6/36 cell lines as CFAV does (Crabtree et al., 2003).

In recent years, other mosquito-only flaviviruses have been isolated from *Aedes* and *Culex* mosquitoes. These viruses were also discovered using universal flavivirus primers in reverse transcription polymerase chain reactions (RT-PCR). Culex flavivirus (CXFV) was isolated from *Culex* mosquitoes in Indonesia and Japan (Hoshino et al., 2007). Other strains of CXFV have been isolated from the U.S., Mexico, Guatemala, and Trinidad and Tobago (Farfan-Ale et al., 2009, Kim et al., 2009, Morale-Betoulle et al., 2008). A similar virus, named Quang Binh virus (QBV) was isolated from *Culex tritaeniorhynchus* mosquitoes in Vietnam (Crabtree et al., 2009). Aedes flavivirus (AEFV) was isolated in Japan from *A. albopictus* and *Aedes flavopictus* mosquitoes, and has an RNA genome that is 11,064 nucleotides in length (Hoshino et al., 2009). In Spain, many mosquito pools tested between 2001 and 2005 were positive for unknown flaviviruses related to KRV and CFAV, indicating there may be many more mosquito-only flaviviruses in the wild that have yet to be described (Aranda et al., 2009).

Segments of the genomes of some of these flaviviruses have also integrated into the mosquito genome. Crochu et al. (2004) described various DNA integrations of CFAV-like and KRV-like sequences (Crochu et al., 2004). It is unclear how the virus would integrate its RNA genome into the host DNA genome, but it does suggest that the

viruses have been persistently infecting the mosquito for many years. It is also unclear if there is transcription from these viral-like integrations, and if they provide any defense against infection with other similar viruses. In *Apis mellifera* bees, segments of RNA from a discistrovirus have integrated into the genome of approximately 30% of tested populations. It appears that RNA from this integration is expressed, and bees with the integration are resistant to infection with the homologous virus (Maori et al., 2007). Whether resistance to related viruses occurs in mosquitoes containing viral integrations in their genomes has yet to be determined.

Mosquito Cell Lines:

Various mosquito cell lines were developed in the 1960's from *A. aegypti* and *A. albopictus* mosquitoes. These lines were made from both embryo and larvae life stages. Singh developed lines from larval *A. aegypti* and *A. albopictus* mosquitoes (Singh, 1967). Peleg used *Aedes aegypti* embryos to develop the lines he called '59' and '364' (Peleg, 1966, Peleg, 1968, Peleg, 1969, Peleg & Shahar, 1972).

Singh's *A. albopictus* line is often described as ATC-15, but was cloned and described as LT C-7, and later a subclone of these was named C7-10 cells (Lan & Fallon, 1990, Sarver & Stollar, 1977). The original *A. albopictus* cell line made by Singh supported growth of CHIKV, SINV, JEV, WNV and all four dengue serotype viruses, with cytopathic effects (CPE) seen in JEV, WNV, and DENV-1, DENV-2, DENV-3 and DENV-4 (Singh & Paul, 1968). Singh's *A. aegypti* larval cell line is often referred to as ATC-10. This cell line could support replication of CHIKV and WNV only, with growth of CHIKV being rapid, but to relatively low titers, and WNV growth being slow, but to

somewhat higher titers. SINV growth was very 'erratic' and infectious viruses were only detected on a few days during the growth curve (Singh & Paul, 1968).

Later Igarashi selected clones of Singh's *A. albopictus* mosquito line for their ability to grow arboviruses to high titers (Igarashi, 1978). Twenty clones were isolated from the original Singh line. One known as C6 showed highest yields of DENV and CHIKV and was then re-cloned into 43 more clones. One of these clones, C6/36, grew DENV and CHIKV to significantly higher titers than the original uncloned cells. The uncloned cells had no apparent CPE from infection with the four DENV serotypes, but the C6/36 clone showed 'marked to moderate' CPE for each of the viruses. Igarashi commented "the virus-sensitive C6/36 clone may lack efficient regulatory mechanism for virus RNA synthesis and virus production or may be less demanding metabolically and nutritionally"(Igarashi, 1978). C6/36 cells have since been used routinely in arboviruses studies.

Peleg's *A. aegypti* cell lines have been used less frequently. In 1990, Lan and Fallon resurrected the Peleg '59' (or possibly Peleg '364') line, adapted it to E-5 medium and named this new line Aag-2 (Lan & Fallon, 1990). These cells are fibroblast-like in appearance and grow attached to the flask. As the culture ages, they begin to grow on top of each other and in round aggregates. Karyotype analysis by Lan and Fallon found that most of the cells have three pairs of chromosomes, like *A. aegypti* mosquitoes, although 7% of the cells contained an extra chromosome fragment (Lan & Fallon, 1990). The Aag-2 cells are not from a clonal population and may contain a variety of different cell types from embryonic mosquito tissues, although electron microscopy of the cells showed an ultrastructure similar to secretory cells (Lan & Fallon, 1990, Peleg & Shahar,

1972). The cells that aggregate into spheres that appear to contain melanin, which is released spontaneously into the medium (Peleg & Shahar, 1972). SFV, EEEV and WNV replicate in Peleg's *A. aegypti* cell line (Peleg, 1968).

RNA Interference:

RNA interference (RNAi) is a cellular response triggered by dsRNA in the cell. In the 1990's, a strange phenomenon was observed when experiments were done to try induce petunia flowers to express more of the purple color gene. Genes expressing messenger RNAs (mRNAs) in the petunia pigment production pathway were introduced into the plant to get more pigment production, but this instead caused the plants to lose the pigmentation and also had corresponding decrease in the supplemented gene's mRNA levels in the cell (Napoli et al., 1990, van der Krol et al., 1990). Fire et al. (1998) later observed that injecting long dsRNA from the myofilament gene unc-22 into the nematode *Caenorhabditis elegans* resulted in twitching activity in the animals, indicating a decrease in unc-22 activity. The gene interference was carried on to the progeny, which showed an even stronger knockdown phenotype. They showed that injection of dsRNA to the mex-3 gene into adults reduced mex-3 mRNA levels in their progeny, and that the dramatic reduction in mRNAs was not seen with injection of sense or antisense RNA to the gene alone (Fire et al., 1998).

In *Drosophila* flies, exogenous dsRNA in the cytoplasm is cleaved by the enzyme Dicer-2 (Dcr2) into 21-23 bp small interfering RNAs (siRNAs) (Bernstein et al., 2001, Zamore et al., 2000). Dcr2, approximately 190 kilodaltons (kDa), is an RNase III nuclease and contains a PAZ (Piwi Argonaute Zwille) domain, which binds to RNA

duplex ends with short 2 nt overhangs (Bernstein et al., 2001, Liu et al., 2003). Dcr2 preferentially cleaves longer dsRNA (> 200 bp), although it can cleave dsRNA as short as 80 bp, and does not cleave single-stranded (ssRNA) (Bernstein et al., 2001, Yang et al., 2000). The small-interfering RNAs (siRNAs) generated are between 20-23 bp in length (usually 21 bp), are double-stranded with 2 nt overhangs on the 3' ends and have 5'-phosphate and 3' hydroxyl groups (Elbashir et al., 2001a, Elbashir et al., 2001b, Nykanen et al., 2001, Zamore et al., 2000).

The protein R2D2 then assists Dcr2 to load the siRNAs into a protein complex known as the RNA-induced silencing complex (RISC) (Liu et al., 2003). R2D2 is approximately 36 kDa and contains two dsRNA-binding domains (Liu et al., 2003). R2D2 preferentially binds to the siRNA end that is more thermodynamically stable. Dcr2 binds near the 5' end of the RNA that will become the guide strand, which is retained in the RNA-induced silencing complex (RISC), and R2D2 binds near the 5' end of the RNA passenger strand, which is later destroyed (Tomari et al., 2004). Binding of R2D2 to the end of the passenger strand requires a 5'-phosphate on that end of the siRNA, ensuring that genuine siRNAs are used during the effector stage of the RNAi pathway (Elbashir et al., 2001b, Schwarz et al., 2003, Tomari et al., 2004).

The enzymatic component of the RISC is Argonaute-2 (Ago2) (Okamura et al., 2004, Rand et al., 2004). Members of the Argonaute family have a PAZ domain (as do the Dicer enzymes), and a PIWI (P-element induced wimpy testis) domain that is unique to the Argonaute family (Carthew & Sontheimer, 2009). The PIWI domain of Ago2 has a region that is RNaseH-like and can catalyze the cleavage of its base paired target (Parker et al., 2004, Song et al., 2004). Other proteins such as fragile X mental

retardation protein (dFXR or FMR1), Vasa intronic gene (VIG), and Tudor staphylococcal nuclease (TSN) have been found associated with the RISC, but don't seem to be required for the effector activity of the RISC (Caudy et al., 2003, Caudy et al., 2002, Ishizuka et al., 2002, Rand et al., 2004). Once the double-stranded siRNA is loading into the RISC, Ago2 then cleaves the passenger strand between nucleotides 9 and 10 of the passenger strand and keeps the other guide strand to use in targeting mRNA for degradation (Kim et al., 2007, Matranga et al., 2005, Miyoshi et al., 2005). Dcr2 and R2D2 are required for this cleavage of the passenger siRNA strand, as ovary lysates from Dcr2 and R2D2 knockout flies didn't cleave the strand (Matranga et al., 2005). Guide strands of siRNAs are modified by the enzyme DmHEN1, the *Drosophila* homolog of HEN1, working on ssRNA to give a 2'-O-methylation on the 3' terminus of the siRNA (Horwich et al., 2007).

The RISC then finds long ssRNA complementary to the siRNA in the cell, and Ago2 uses a RNaseH-like 'slicing' activity to cleave the targeted ssRNA (Miyoshi et al., 2005). This cleavage occurs between nts base-paired to nt 10 and 11 of the guide strand, and these cleaved products have a 5'-monophosphate and a 3'-hydroxyl terminus (Schwarz et al., 2004). This 'slicing' activity results in decreased levels of mRNA that are complementary to the dsRNA, resulting in 'knockdown' of genes that are the same sequence as one strand of the dsRNA (Tuschl et al., 1999). After cleavage, the mRNA dissociates from the RISC, the mRNA fragments are further degraded and the RISC is free to cleave more targets. A siRNA/RISC can also bind targets that are partially mismatched and use miRNA-like mechanisms to repress translation, although it is unclear how often this occurs naturally. This phenomenon is probably responsible for

most of the "off-target" effects that are seen in the experimental introduction of siRNAs (Carthew & Sontheimer, 2009). See Figure 1.1 for diagram of pathway.

In plants and *C. elegans*, there is also a mechanism to amplify siRNAs that have been made by Dicer using a cellular RNA-dependent RNA polymerase (RdRP). It appears that most of these 'secondary' siRNAs generated by the RdRP are antisense to the mRNA they target, indicating they probably don't go through a dsRNA/Dicer cleavage pathway as primary siRNAs do. The secondary siRNAs seem to be primary, unprimed RdRP products, and help to greatly increase the potency of the response to dsRNA in the cell. So far, this amplification of siRNAs has not been seen in mammals or insects, although a recent report suggests a role for an RdRP in *Drosophila* RNAi and transposon suppression (Carthew & Sontheimer, 2009, Lipardi & Paterson, 2009). This RdRP, termed D-elp1, makes dsRNA from ssRNA templates with or without a primer initiation step and associates tightly with Dcr2 (Lipardi & Paterson, 2009).

siRNAs can also modify chromatin. In the fission yeast, *Saccharomyces pombe*, an Argonaute family member is part of the RNA-induced transcription silencing (RITS) complex, which is guided to regions on chromosomes by siRNAs it has bound. siRNAs recognize nascent transcripts, and RNA polymerase II and the RITS complex interact to cause histone methyltransferases to methylate histone 3 on lysine 9 (H3K9), resulting in the recruitment of the Swi6 protein and compaction of the chromatin (Buhler et al., 2006, Carthew & Sontheimer, 2009, Djupedal et al., 2005, Kato et al., 2005, Lippman & Martienssen, 2004). This process also activates a RdRP mechanism to create secondary siRNAs to further amplify the effects (Sugiyama et al., 2005).

MicroRNA pathway:

Another small RNA pathway related to the exogenous RNAi pathway is the microRNA (miRNA) pathway. This pathway is also triggered by dsRNA, although this dsRNA is not made of perfect duplexes, but instead is from ssRNA that forms secondary structures on itself, leading to some double-strandedness, but often has mismatches and small 'bumps' in the structure. These precursor molecules are transcribed from either independent miRNA genes or more often from transcription units that encode multiple products, and are usually transcribed by RNA polymerase II and are capped and polyadenylated (Bartel, 2004, Carthew & Sontheimer, 2009, Kim, 2005). The transcripts may encode an miRNA and a protein, with the miRNA being located in an intron, or the transcript may encode multiple distinct miRNAs (Carthew & Sontheimer, 2009). In the *Drosophila* miRNA pathway, these precursor transcripts are called pri-miRNAs and are formed in the nucleus (Lee et al., 2002). The imperfectly paired stem loop is cleaved from the pri-miRNA by the enzyme Drosha and its binding partner Pasha (known as DGCR8 in mammals) forming smaller RNAs known as pre-miRNAs (Denli et al., 2004, Gregory et al., 2004, Landthaler et al., 2004, Lee et al., 2003). Drosha, like Dcr2, is an RNase III enzyme and is approximately 130-160 kDa in size (Filipowicz et al., 2005, Lee et al., 2003). The pre-miRNAs are then sent out of the nucleus through the nuclear pore with the assistance of Ran (Ras-related nuclear protein) GTPase and Exportin-5 (Bohnsack et al., 2004, Cullen, 2004). In the cytoplasm, the pre-miRNA is further cleaved by Dicer-1 (Dcr1) into approximately 22 bp miRNAs (Lee et al., 2004). The miRNAs are loaded in the RISC with the help of the R2D2 miRNA counterpart Loquacious (Loqs), which was also named R3D1-L (Forstemann et al., 2005, Jiang et al.,

2005, Saito et al., 2005). The miRNA RISC (miRISC) complex contains Argonaute-1 (Ago1), an approximately 100 kDa member of the Argonaute/Piwi family (Filipowicz et al., 2005, Okamura et al., 2004). Ago1 contains PAZ and PIWI domains like those found in Ago2. The miRNA strands are unwound and one strand is destroyed (designated the miRNA*), while the other strand (designated the miRNA strand) is kept in the complex (Carthew & Sontheimer, 2009). The miRNA strand selected by the miRISC depends on the thermodynamic stability of miRNA duplex's ends, with the 5' terminus of the kept strand having the less stably based paired end, similar to the siRNA strand retained in the siRNA RISC (siRISC). Unlike siRISC loading, miRISC loading does not seem to be accompanied by cleavage of the discarded strand (Matranga et al., 2005). In *Drosophila*, the structure of the small RNA duplexes appears to aid in the sorting of miRNAs to an Ago1 miRISC, and siRNAs to an Ago2 siRISC, as the Dcr2/R2D2 complex does not bind miRNAs due to the mismatches found in their duplexes (Tomari et al., 2007).

This miRISC then seeks out the 3' UTRs of mRNAs complementary to the miRNA sequence. There are often multiple miRNA binding sites on the 3' UTR of the targeted mRNA and the miRNA usually binds with some bulges and mismatches. The most important region of the miRNA recognition of the mRNA lies in the seed region of the miRNA at nucleotides 2-8. If the complementarity of the miRNA and mRNA is perfect (as is the case with most plant miRNAs) Ago can cleave the mRNA. More often in animals, there are central mismatches in the miRNA and mRNA binding leading to repression of translation rather than mRNA cleavage. The miRISC prevents the mRNA from being translated, either by directly blocking translation, or promoting the degradation of the mRNA (Carthew & Sontheimer, 2009). See Figure 1.1 for diagram.

Exactly how translation is blocked is still debated. Some models propose that the miRISC blocks translation initiation, possibly by competing for cap binding, blocking association of the ribosomal subunits with the pre-initiation complex, or by causing deadenylation of the mRNA tail, resulting in the mRNA being unable to circularize (Carthew & Sontheimer, 2009, Chendrimada et al., 2007, Giraldez et al., 2006, Mathonnet et al., 2007, Wakiyama et al., 2007, Wang et al., 2008a, Wu et al., 2006). Other groups suggest that translation is blocked post-initiation, possibly by causing the ribosome to dissociate from the mRNA (Carthew & Sontheimer, 2009, Nottrott et al., 2006, Petersen et al., 2006).

Once miRNAs have targeted an mRNA for destruction, these target mRNAs, along with the miRISC localize to areas of the cell known as processing bodies (P-bodies), where they are then blocked from translation and may undergo decay. Many of the important components of the cellular mRNA decay machinery are found localized to the P-bodies, including the proteins involved in decapping and deadenylation, GW182, and an RNA helicase (Eulalio et al., 2008). miRNA silencing seems to occur without the presence of P-bodies, indicating that P-bodies are formed in response to miRNA-mediated silencing, and are not the cause of the silencing (Chu & Rana, 2006, Eulalio et al., 2007, Eulalio et al., 2008, Lian et al., 2007).

miRNAs are also linked to increased mRNA degradation, not by Ago cleavage of the mRNA, but by traditional pathways of mRNA decay involving deadenylation, decapping and exonucleolytic digestion (Behm-Ansmant et al., 2006, Carthew & Sontheimer, 2009, Giraldez et al., 2006, Wu et al., 2006). Behm-Ansmant et al. (2006) showed that the mammalian processing body (P-body) component GW182 interacts with

Ago1, linking mRNA degradation to the miRNA machinery, and showed that the decapping and deadenylation machinery were necessary for the miRNA-mediated degradation of mRNAs (Behm-Ansmant et al., 2006). It is still unclear if increased degradation of mRNAs being acted upon by miRNAs is due to miRNA effects on translation, or if the increased decay is an independent mechanism for reducing mRNA transcripts. Some experiments have shown that miRNA-induced deadenylation of transcripts can occur without active translation, indicating that the degradation itself can be the cause of the repression of some targets (Wakiyama et al., 2007).

Virally encoded miRNAs have been described in DNA viruses, but not in RNA viruses to date (Umbach & Cullen, 2009). This is not surprising as many RNA viruses are found in the cytoplasm, where they would be inaccessible to the early processing steps of the miRNA pathway such as Drosha cleavage of the pri-miRNA. This cleavage would also be destructive to RNA viral genomes and could make it difficult for them to complete their normal replication cycle. Some have described a miRNA found encoded in the RNA virus HIV-1, but other groups have disputed this potential miRNA (Klase et al., 2007, Lin & Cullen, 2007, Omoto & Fujii, 2005, Omoto et al., 2004, Ouellet et al., 2008, Umbach & Cullen, 2009). Most of the miRNAs encoded by viruses have been found in the *Herpesviridae* and *Polyomaviridae* families. Viral miRNAs from human viruses have been found from the Herpes simplex 1 and 2 viruses (HSV-1 and HSV-2), human cytomegalovirus (hCMV), Epstein Barr virus (EBV), Kaposi's sarcoma virus (KSHV), BKV and JCV polyomaviruses, and from human adenovirus (hADV) (Aparicio et al., 2006, Cai et al., 2005, Cai et al., 2006, Cui et al., 2006, Grey et al., 2005, Grundhoff et al., 2006, Pfeffer et al., 2005, Pfeffer et al., 2004, Samols et al., 2005, Sano

et al., 2006, Seo et al., 2008, Tang et al., 2008, Tang et al., 2009, Umbach & Cullen, 2009, Umbach et al., 2008, Zhu et al., 2009). So far, only one miRNA has been found from most of the polyomaviruses examined, but the herpesviruses appear to have quite a few miRNAs encoded in each genome, with EBV having at least 25 different pre-miRNAs (Umbach & Cullen, 2009). A miRNA has also been described in an insect virus *Heliothis virescens* ascovirus (HvAc) (Hussain et al., 2008). These viruses are all nuclear DNA viruses, and many of these miRNAs appear to target viral genes, which may be helping the virus to regulate temporal expression of various gene products.

Some of the viral miRNAs target cellular genes, mostly genes that are important for controlling apoptosis or have immunomodulatory functions (Choy et al., 2008, Umbach & Cullen, 2009, Xia et al., 2008). Some viruses also have evolved to work with cellular miRNAs. Hepatitis C virus (HCV) has binding sites for the human liver miR-122 in its 5' UTR. It appears that the binding of the cellular miRNA helps the virus to replicate, and since miR-122 is liver specific, it probably plays a role in the tissue tropism for HCV (Jopling, 2008, Jopling et al., 2005, Umbach et al., 2008). Various groups have also reported changes in cellular miRNA expression after viral infection, yet it is still unclear if these changes are due only to the cellular immune responses to infection, or if the virus is manipulating the cellular miRNAs to its advantage (Cameron et al., 2008, Pedersen et al., 2007, Triboulet et al., 2007, Umbach et al., 2008, Wang et al., 2008b).

Other small RNA pathways:

Other small RNA pathways have been discovered including the Piwi-interacting (piRNA) and endogenous siRNA (endo-siRNA). These pathways are believed to have

important roles in controlling the transcription of transposable elements in the genome and also in development of the reproductive tissues.

piRNAs bind to the members of the Piwi clade of the Argonaute proteins, which include Piwi, Aubergine (Aub) and Argonaute 3 (Ago3) in *Drosophila*. The piRNAs were originally termed repeat-associated small interfering RNAs (rasiRNAs), because of their role in silencing repetitive elements and protecting the germline from transposable elements (Aravin et al., 2001, Saito et al., 2006, Shpiz et al., 2009, Vagin et al., 2006). piRNAs are approximately 24-30 nts in length and are modified by DmHEN1 (also known as Pimet) to have 2'-O-methylation on their 3' terminus (Horwich et al., 2007, Saito et al., 2007). The piRNA trigger appears to be single-stranded RNA since the small RNAs are almost always of the same sense, and the biogenesis is Dcr1 and Dcr2 independent, possibly using the Slicer activity of the Piwi proteins, at least in determining their 5' ends (Gunawardane et al., 2007, Nishida et al., 2007, Saito et al., 2006, Vagin et al., 2006). Piwi and Aub tend to bind antisense transcripts and have a strong preference for a uracil at the 5' end, while Ago3 binds sense transcripts and shows a preference for an adenine at nucleotide 10 (Gunawardane et al., 2007). The first 10 nucleotides of the antisense piRNAs are often complementary to the sense piRNAs that bind Ago3, leading to proposed 'ping-pong' amplification mechanism of piRNAs (Brennecke et al., 2007, Gunawardane et al., 2007). Using Ago3 mutant flies, it was found that Ago3 has a role in amplifying piRNAs and to enforce their antisense bias (Li et al., 2009a). See Figure 1.1 for diagram of pathway.

piRNAs have been mostly studied in the germline where they have a critical role in silencing transposons and controlling germline stem cells (Aravin et al., 2004, Cox et

al., 1998, Cox et al., 2000). Silencing of the *Stellate* locus repetitive element by Aub is necessary to prevent *Drosophila* male sterility (Aravin et al., 2004, Aravin et al., 2001). New evidence also points to roles of piRNAs in somatic tissues. In Ago2 mutant flies, there are small RNAs that are piRNA-like found in the soma (Ghildiyal et al., 2008). piRNAs have also been found in the somatic cells surrounding the germline cells of *Drosophila* ovaries and may have a role in protecting these germline cells from infection from retroviral elements (Malone et al., 2009). It has been proposed that this somatic piRNA pathway functions without Ago3 or Aub, instead loading piRNAs into Piwi and without amplification (Li et al., 2009a).

The endo-siRNA pathway is very similar to the exogenous siRNA pathway. The size of the endo-siRNAs is 21 nts, and Dcr2 and Ago2 appear to be essential for their biogenesis. The trigger for the endo-siRNA pathway is perfect long dsRNA duplexes that are formed in the cell naturally. These transcripts come from regions of the genome containing mobile elements and are believed to have a role in controlling the expression of these elements in the cell. The recently described *Drosophila* RdRP D-elp1 may be involved in converting the ssRNA transposon transcript into dsRNA for processing by Dcr2 (Lipardi & Paterson, 2009). Deep sequencing of *Drosophila* small RNAs associated with Ago2 showed that many endo-siRNAs came from structured loci termed esi-1 and esi-2, that can form 400 bp long dsRNA when the transcripts' 5' and 3' UTRs interact (Czech et al., 2008). The endo-siRNAs derived from these loci are from the same genomic strand, indicating that long ssRNA transcripts fold back to form long dsRNA (Czech et al., 2008). These studies also implicate an important role for Loqs in the generation of endo-siRNAs instead of the expected Dcr2 partner R2D2, as loqs

mutant fly ovaries did not appear to have endo-siRNAs (Czech et al., 2008). Endo-siRNAs also appear to have the same 2'-O-methylation at their 3' end, providing further evidence for use of a similar pathway to the exogenous siRNA pathway (Kawamura et al., 2008). The endo-siRNAs found bound to Ago2 didn't appear to have any nucleotide bias at particular positions, and a large number had single mismatches (Kawamura et al., 2008). Adenosine-to-guanosine mismatches were overrepresented in this population, suggesting adenosine deaminase acting on RNA (ADAR) enzymes may be editing a portion of endo-siRNAs, converting adenosine-to-inosine (Kawamura et al., 2008). When the Ago2 was mutated, piRNA-like small RNAs appeared in somatic tissues, indicating a possible role for the endo-siRNA pathway in repression of piRNA activity in the soma (Ghildiyal et al., 2008, Ghildiyal & Zamore, 2009).

See Figure 1.1 for a diagram of the various small RNA pathways and Table 1.3 for general characteristics of the small RNAs generated from the pathways.

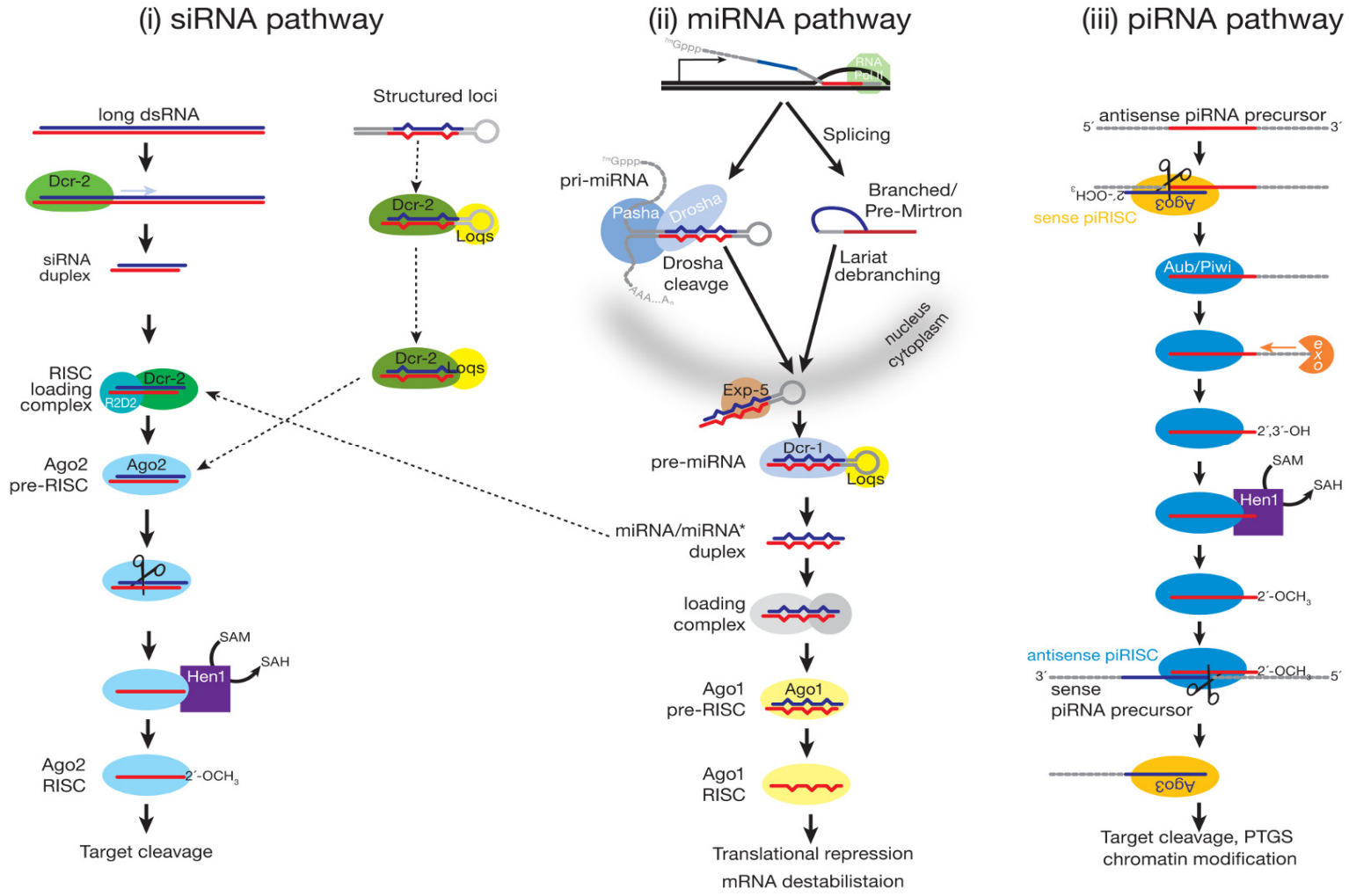


Figure 1.1: Diagram of small RNA pathways from Ghildiyal & Zamore (2009)

Table 1.3. Characteristics of small RNAs in *Drosophila* modified from (Farazi et al., 2008)

Small RNA	**Size (nt)**	**Structure of Precursor**	**3' end modification**	**Mechanism of Action**
miRNA	20-23 (usually 22)	Imperfect hairpin	Unmodified	Translational repression, mRNA cleavage
siRNA (exo- and endo-)	20-23 (usually 21)	dsRNA	2'-O-methylated	mRNA cleavage
piRNA	23-33	Putative ssRNA	2'-O-methylated	Regulation of chromatin structure, mRNA cleavage

Mosquito Immunity:

The sequencing of the *A. aegypti* genome, published in 2007, has aided in the study of mosquito genes and pathways involved in mosquito antiviral immunity (Nene et al., 2007). Mosquitoes have some basic immune responses to bacterial, fungal, parasite and viral threats. Mosquitoes use a peptide-based innate immune response against Gram-positive and Gram-negative bacteria and eukaryotic parasites. These peptides include defensins, cecropins, and transferrins, and have been described in *A. aegypti* mosquitoes (Lowenberger, 2001). Defensins were originally described as important for mosquito immunity to bacterial infections, although more recent experiments seem to indicate that bacterial infections are cleared before defensin is induced, so its exact role in immunity is still unclear (Bartholomay et al., 2004). These antimicrobial peptides are produced in insect fat bodies and are secreted into the hemolymph. The production of these peptides is under the control of the immune pathways immune deficiency (IMD) and Toll in *Drosophila*. The IMD pathway is used to express diptercins and drosocins against Gram-negative bacteria. The Toll pathway controls the systemic expression of the anti-fungal

peptide drosomycin. The induction of defensins, cecropins and attacins in *Drosophila* appears to be controlled by both the IMD and Toll pathways (Osta et al., 2004). The IMD pathway may also have a role in the *Drosophila* antiviral response to SINV (Avadhanula et al., 2009). More recently the Toll and JAK-STAT pathways have been implicated in the antiviral response to DENV2 in *A. aegypti* (Souza-Neto et al., 2009, Xi et al., 2008), although the dsRNA knockdown of the Hop gene, a positive regulator of the pathway, did not result in statistically significant increases in viral titer when compared to the control dsRNA (Souza-Neto et al., 2009).

RNAi as an Antiviral Pathway

Because of its ability to specifically target RNA in a cell after it encounters dsRNA, RNAi has been found to be an essential antiviral pathway in insects. Dcr2 and R2D2 knockout *Drosophila* flies are more susceptible to infection with Flock House virus (FHV) (Family *Nodaviridae*) and with cricket paralysis virus (CrPV) (Family *Dicistroviridae*), and infection of these mutant flies with these viruses caused increased mortality compared to wild type flies (Wang et al., 2006). Similar results were found in other studies using Dcr2 knockout flies and infection with FHV, SINV, and *Drosophila* C virus (DCV) (Family *Dicistroviridae*) (Galiana-Arnoux et al., 2006). SINV infection of wild-type flies did not cause mortality, but there was 70% mortality in the Dcr2 knockout flies infected with SINV, and also a higher viral load in the mutant flies. Transgenic flies expressing FHV constructs were made, and infectious virus was produced in these flies, causing death in these mutants. These studies also highlighted the importance of the B2 protein of FHV to viral RNA amplification in flies. Transgenic flies expressing FHV

constructs with normal B2 died from FHV infection because the they were unable to efficiently control the FHV viral RNA amplification, but flies engineered to express FHV RNA with point mutations disrupting the B2 open reading frame did not die (Galiana-Arnoux et al., 2006). Ago2 was also found to be critical for antiviral immunity in *Drosophila*. When Ago2 knockout flies were infected with DCV or CrPV, they had increased mortality, viral RNA accumulation and viral titers. Studies also indicated that DCV encodes a suppressor of RNAi, DCV-1A, that binds long dsRNA, but does not bind siRNAs or disrupt the miRNA pathway (van Rij et al., 2006). CrPV also appears to encode a suppressor of RNA silencing that maps to a similar genomic region as DCV, although it doesn't seem to have a double-stranded RNA binding domain (dsRBD) that was found in DCV and didn't inhibit dsRNA processing, indicating a different mechanism of action (van Rij et al., 2006, Wang et al., 2006).

This antiviral role for RNAi is not limited to single-stranded RNA viruses, as studies show RNAi as an antiviral immune response against the dsRNA birnavirus *Drosophila* X virus (DXV). This virus will kill wild-type *Drosophila* within two weeks, but kills faster when flies have mutations in their RNAi antiviral pathways. *Drosophila* flies with mutations in Ago2, VIG, R2D2, Aub, Armitage (ARMI), and Piwi all had increased susceptibility to infection and earlier death with DXV (Zambon et al., 2006).

Dcr2 has also been implicated in inducing the gene *Vago* in Drosophila infected with DCV. The *Vago* gene product is an 18 kDa cysteine-rich polypeptide that controlled DCV load in the *Drosophila* fat bodies. This indicates that Dcr2 is a sensor for viral nucleic acids. It is indeed a member of the same DExD/H-box helicase family as the RIG-I-like receptors of mammals that are responsible for detection of viral infection and

induction of interferon. Ago2 nor R2D2 were required to induce the *Vago* expression and the Toll, IMD and Jak-STAT pathways were also not involved in *Vago* expression (Deddouche et al., 2008).

More recently Ars2 has been identified as another component of the antiviral RNAi pathway in Drosophila. The role of Ars2 in antiviral immunity was uncovered using an RNAi based screen following infection with a negative strand RNA virus, vesicular stomatitis virus (VSV). When Ars2 was knocked down using dsRNA, the number of VSV infected cells increased. Knockdown of Ars2 also increased infection of the *Drosophila* cells with DCV, FHV and SINV, but infection with vaccinia virus, which has a double-stranded DNA genome, was not affected by Ars2 depletion. When Ars2 was knocked down in adult *Drosophila* flies, there was an increase in mortality seen with DCV and VSV viruses (Sabin et al., 2009). The researchers suggest Ars2 is involved in the siRNA pathway during siRNA biogenesis, but is not needed for RISC effector function and state that Ars2 is physically interacting with Dcr2. Ars2 also appears to have a role in the endo-siRNA and miRNA pathways (Sabin et al., 2009). In mammals Ars2 interacts with the cap-binding complex (CBC) and shuttles between the nucleus and cytoplasm (Gruber et al., 2009). CBC was also found to be required in *Drosophila* for antiviral defense and siRNA and miRNA silencing and appeared to also act in the upstream steps of the pathways. The authors propose two models for Ars2 function. In one model, Ars2 is a recruitment factor to help guide the miRNA machinery to its proper substrates, while in the other model Ars2 is a cofactor to the dsRNA cleavage enzyme activity of Dcr2 and Drosha which increases cleavage activity and fidelity in the processing of the RNA substrates (Sabin et al., 2009).

Further evidence of the importance of the RNAi pathway in insect antiviral immunity was shown in an evolutionary genetics study indicating that the *Drosophila* siRNA pathway genes are evolving very rapidly due to a molecular arms race between viral pathogens and insect immune responses. Dcr2, Ago2 and R2D2 were evolving faster than the miRNA pathway 'housekeeping' genes Dcr1, Ago1 and R3D1 (also known as Loqs) (Obbard et al., 2006). The siRNA pathway components are also evolving faster than other insect immune response genes such as pathogen-recognition proteins, signal transduction proteins, and antimicrobial peptides. These antiviral siRNA pathway genes are among the fastest evolving 3% of all *Drosophila* genes and the authors suggest that interactions with viruses are driving this rapid evolution (Obbard et al., 2006). Recent research at AIDL suggests that *A. aegypti* siRNA pathway genes are evolving faster than the miRNA pathway genes (Scott Bernhardt, unpublished).

Antiviral immunity to flaviviruses has been examined in *Drosophila*. WNV virus-derived small interfering RNAs (viRNAs) of approximately 25 nts in length were detected in *Drosophila* S2 cells infected with the virus, and knockdown of *Drosophila* Ago2 with siRNAs resulted in earlier WNV detection over controls, but no differences in viral titers were seen with the Ago2 knockdown. Mutant flies with defects in the RNAi genes Ago2, Piwi, Dcr2 and spindle-E were more susceptible to WNV infection than wildtype flies, indicating that RNAi is an antiviral immune response to WNV in *Drosophila*. Surprisingly, C6/36 *A. albopictus* cells infected with WNV did not produce detectable WNV viRNAs (Chotkowski et al., 2008).

Virus Triggers of RNAi

It has always been assumed that RNA viruses must form dsRNA during their replication cycle, which can then be targeted by Dcr enzymes. The presence of dsRNA has been detected using dsRNA-specific antibodies in infection of cells with the alphavirus SINV and the flavivirus KUNV and with various other positive sense ssRNA viruses such as DENV (Sanchez-Vargas et al., 2009, Stollar & Stollar, 1970a, Weber et al., 2006, Westaway et al., 1997). Authors suggested that the dsRNA formed from positive sense ssRNA viruses could be from hybrids of genome and complementary RNA (cRNA) made during genome replication or by secondary RNA structures made by the ssRNA strands themselves. dsRNA was also detected in cells infected with DNA viruses. DNA viruses may make dsRNA from overlapping converging transcription or from structured ssRNA. When the dsRNA antibodies were used in cells infected with negative strand ssRNA viruses such as LACV, there was surprisingly no detection of dsRNA (Weber et al., 2006). The explanation for the lack of dsRNA in negative sense ssRNA viruses is less clear. Possibly the packaging of ssRNA into ribonucleoprotein (RNP) complexes restricts the length of dsRNA in replicative intermediates so it is not detectable with the antibody (Weber et al., 2006). Secondary structures do seem to be dsRNA targets for Dicer enzymes for some positive sense ssRNA viruses. When viRNAs from Cymbidium ringspot tombusvirus (CymRSV) were sequenced, they were not evenly distributed along the genome and 80% were derived from the positive strand, indicating there may be hot spots for Dicer cleavage on the structured viral genome. Northern blotting for viRNAs also detected more with the negative probe, indicating there are more positive sense viRNAs being made during infection (Molnar et al., 2005).

Also, when the viRNAs bound to the viral dsRNA-binding protein p19 were analyzed, the majority of these viRNAs were from the positive strand of the CymRSV viral RNA (Molnar et al., 2005). More studies showed that the RISC targeted ssRNA also has hot spots, and the secondary structure of the target can determine the accessibility of the RNA for RISC cleavage (Overhoff et al., 2005, Pantaleo et al., 2007).

Flaviviruses also have conserved secondary structures that may be targets for Dcr2 cleavage in insect cells. During flavivirus replication, the virus genome cyclizes, with the 5' and 3' ends having regions that are complementary (cyclization sequences) that can bind to form a panhandle-like structure (Hahn et al., 1987). Predicted conserved secondary-structured regions in the DENV genomes based on computational analysis are in the 5' UTR (nts 6-69) and the 3' UTR (nts 10593-10656 and 10710-10762) (Thurner et al., 2004). Some conserved structured regions have also been predicted in the coding sequence for the capsid (nts 122-140, nts 172-192, and nts 270-312) and in the NS3 coding region (nts 4544-4671) (Stadler et al., 2001). Secondary structures in the DENV2 Jamaica strain 3'UTR are proposed to begin at the nt position 10287 (Proutski et al., 1997) (See Figure 1.2.).

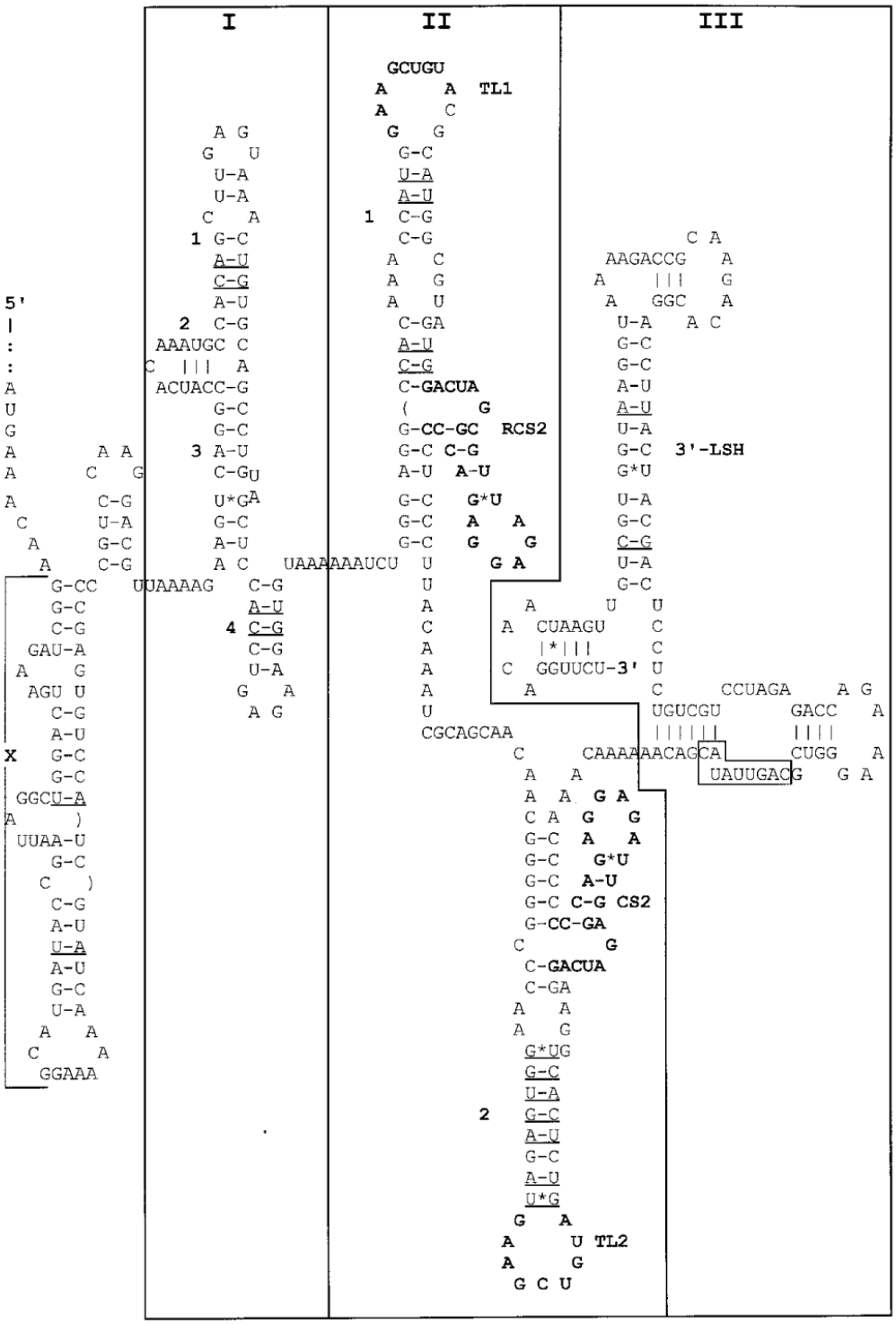


Figure 1.2.: Proposed 3' UTR secondary structure for DENV2 Jamaica 1409 strain (GenBank Accession number M20558) from (Proutski et al., 1997). The first 5' nucleotide corresponds to base 10287 and the last 3' nucleotide corresponds to base 10723 of the genome.

Another highly structured region has been identified in the 3'UTR of flaviviruses, which is approximately 300-500 nts in length and is resistant to ribonucleases. This RNA has been termed subgenomic flavivirus RNA (sfRNA). sfRNAs have been identified from mosquito-borne flaviviruses WNV, YFV, a DENV2 replicon, KUNV and others, along with a tick-borne flavivirus Saumarez Reef virus (SREV) , but were not detected in the pestivirus bovine viral diarrhea virus (BVDV), a hepacivirus hepatitis C (HCV) replicon or the alphavirus SFV (Family Togaviridae). For DENV2, the sfRNA 5' end begins 422 nucleotides from the 3' end of the genome and is approximately 400 nts in length (Pijlman et al., 2008).

In FHV-infected *Drosophila*, the primary Dcr2 target for generating siRNAs appeared to come from a 400 bp dsRNA region at the 5' terminus of the genome that forms during initiation of replication of the positive strand. Of the viRNAs made from this region, 58% appeared to be from the positive sense and 42% from the negative sense strand of the viral RNA. These viRNAs had 3' methylated modifications and were loaded into Ago2. The production of the viRNAs from the 400 bp dsRNA region was inhibited by the FHV B2 protein, which interacted with the viral dsRNA and the viral RNA replicase (Aliyari et al., 2008). Similarly, nearly equal proportions of positive and negative sense viRNAs were seen in *Drosophila* cells latently infected with FHV (Flynt et al., 2009).

Viral Evasion of RNAi

To combat RNAi, some viruses have evolved proteins to inhibit the pathway at various steps. Many of these inhibitors have been found in plant viruses, but one of the

best characterized is the B2 protein of FHV, which infects both vertebrate and invertebrate hosts. Li et al. (2002) found that FHV was a target of RNAi in *Drosophila* S2 cells and when the B2 gene was deleted, there was a failure of the viral RNA construct to accumulate in cells. When B2 expression was complemented from a plasmid or when dsRNA was used to knockdown Ago2 in the S2 cells, the RNA accumulation was rescued (Li et al., 2002). These studies show evidence of RNAi as an antiviral pathway in insects and that some viruses have evolved proteins to combat the pathway. Further studies using Nodamura virus (NoV) which encodes a B2 protein in a similar genome position to FHV (although <19% sequence identity), showed the antiviral defense pathway in *Anopheles gambiae* was also dependent on Ago2 and was suppressed by B2 (Li et al., 2004). When NoV B2 mutant RNA constructs were introduced into 4a-2s4 *A. gambiae* cells, there was no RNA replication of the construct, but when B2 expression from a plasmid or long dsRNA or siRNAs from *A. gambiae* Ago2 was added, the RNA construct replication was rescued (Li et al., 2004). Studies of the FHV B2 crystal structure, along with biochemical assays, showed that B2 binds dsRNA without a preference for length, suggesting B2 binds long dsRNA, blocking Dicer cleavage, and it also binds shorter double-stranded siRNAs, inhibiting their incorporation into the RISC (Chao et al., 2005, Lingel et al., 2005). More recent work has shown that B2 complexes with FHV siRNAs and viral replicative intermediates, and that B2 also binds the FHV replicase enzyme in infected *Drosophila* cells (Aliyari et al., 2008). It has also been suggested that the B2 protein interacts with the PAZ domain of Dcr1 and Dcr2 in armyworm Sf21 cells (Singh et al., 2009).

Other viral suppressors of RNAi have been identified, including p19 of the plant virus Cymbidium ringspot virus. p19 appears to bind siRNAs and inhibit them from entering the RISC, but does not seem to affect preassembled RISC (Lakatos et al., 2006, Lakatos et al., 2004). The role of dsRNA binding proteins from mammalian viruses in RNAi is less clear. La Crosse virus (LACV) (Family *Bunyaviridae*) encodes a protein on its S segment known as NSs, which has been shown to be an interferon antagonist in mammals in LACV (Blakqori et al., 2007) and in another Bunyavirus, Bunyamwera virus (BUNV) (Kohl et al., 2003, Weber et al., 2002). A study examining the role of LACV NSs in both mammalian and C6/36 mosquito cells demonstrated reduced growth of LACV when siRNAs targeting the genome were introduced by transfection. Using a cDNA plasmid to express LACV NSs in transfected cells, the authors suggested that LACV NSs inhibited siRNA activity in mammalian cells (Soldan et al., 2005). They did not demonstrate inhibition of siRNA activity by NSs in C6/36 cells. Further studies comparing wt LACV with mutant LACV unable to express NSs showed no viral growth advantage due to NSs expression in either insect or interferon-deficient mammalian cells. Virus-specific small RNAs were generated in wt LACV infected C6/36 cells and there appeared to be no suppression of RNAi against a SFV replicon when LACV NSs was co-transfected into *A. albopictus* U4.4 cells. (U4.4 cells were used instead of C6/36 in the co-transfection experiment because SFV causes CPE in C6/36 cells). The authors of this study concluded that LACV NSs was an interferon antagonist in mammalian cells, but did not appear to have a role in RNAi inhibition in insect cells (Blakqori et al., 2007). RNAi against viruses has also been demonstrated in tick cells. When the ISE6 cells from *Ixodes scapularis* were infected with SFV replicons expressing the Hazara virus (HAZV)

(Family *Bunyaviridae*) nucleoprotein gene in sense or antisense orientation, the cells were resistant to HAZV replication. HAZV- and SFV-specific small RNAs between 20-30 nts were detected via RNase protection assays in cells infected with SFV replicon expressing the HAZV nucleoprotein, but were not detected with HAZV infection alone. SFV-specific small RNAs were also detected in cells infected with the SFV replicon without an insert (Garcia et al., 2005). Further work looking at the activity of RNAi suppressors in tick cells was done using a SFV replicon expressing luciferase as a reporter, along with SFV replicons expressing the suppressors NS1 of influenza virus, NSs of tomato spotted wilt virus or HC-Pro of zucchini yellow mosaic virus. When cells were infected with the SFV luciferase replicon alone, luciferase siRNAs between 20-30 nts in length were detected via RNase protection assay, and luciferase activity declined with time, indicating an RNAi response against the SFV luciferase replicon. When the cells were co-infected with the SFV luciferase replicon and with SFV replicons expressing the various suppressor genes, there were increased levels of luciferase expression as compared to the SFV luciferase replicon alone, or co-infection with a SFV replicon with no insert, showing that the viral suppressors of RNAi were able to function in tick cells. Since both the SFV-luciferase construct alone and the SFV-luciferase co-infected with SFV with no insert had the same expression level, there appeared to be no suppressor activity in the SFV replicon itself (Garcia et al., 2006). Interestingly, expression of the NSs protein of Rift Valley fever virus (RVFV) (Family *Bunyaviridae*) in this tick system and did not appear to have any RNAi suppressor activity (Garcia et al., 2006), similar to results with LACV NSs in mosquito cell studies described in Blakquori, et al. (2007).

Viruses may also evade RNAi by sequestering their dsRNA in membrane-bound replication compartments. Replication of most positive-strand RNA viruses occurs in association with membranes in the cytoplasm. Flaviviruses replicate in membrane complexes derived from the endoplasmic reticulum, while togaviruses associate with vesicles derived from endosomes (Salonen et al., 2004). Flavivirus infection of mammalian cells causes membrane proliferation and formation of convoluted membranes and vesicle packets. The replication complex of flaviviruses is located within the vesicle packets, which are enclosed by outer membrane. DENV dsRNA replication forms are believed to be enclosed in the vesicle packet, while the newly formed virus genomes are released to the outside of the vesicle packet, but inside the outer membrane (Miller et al., 2007, Uchil & Satchidanandam, 2003). Other studies looking at the location of the flaviviral replicase proteins NS3 and NS5 of JEV, showed their localization in the nucleus of mammalian cells, suggesting that up to 20% of WNV, JEV, and DENV-2 RdRP activity occurs in the nucleus (Uchil et al., 2006). The location of viral dsRNA structures in the cell could determine their accessibility to the RNAi machinery, and viruses may have evolved a mechanism to induce proliferation of membrane structures to hide their dsRNA from the host innate immune responses.

RNAi in mosquitoes:

In the 1990's a phenomenon know as pathogen derived resistance (PDR) was described in mosquitoes (Blair et al., 2000, Gaines et al., 1996, Olson et al., 1996, Powers et al., 1996). PDR uses artificial expression of a pathogen gene in the host cell to give the host resistance to that specific pathogen (Grumet et al., 1987, Sanford & Johnston,

1985). Double-subgenomic SINV TE3'2J was used to express portions of viral genes in sense or antisense orientation under the control of a second subgenomic promoter following the structural genes. Infecting C6/36 cells with double-subgenomic SINV expressing LACV or DENV structural genes in a sense or antisense orientation gave the cells resistance to challenge from the virus corresponding to the structural genes. Site directed mutagenesis was used to make these viral genes untranslatable, but the effect was the same, indicating that the response was induced by the RNA, not viral protein expression. There also needed to be a high degree of sequence identity between the virus genome and the gene being expressed (Blair et al., 2000, Gaines et al., 1996, Powers et al., 1996). These early experiments were probably triggering RNAi in the cells due to expression of a dsRNA trigger in the double-subgenomic SINV replicative intermediate or possibly the secondary structures of the viral gene RNA was targeted for cleavage by a small RNA pathway.

Similar experiments using TE3'2J SINV expressing the prM coding region of DENV2 showed that injection of the virus into mosquitoes gave resistance to subsequent challenge with DENV from the same serotype as the effector gene (Olson et al., 1996), and infection of C6/36 cells with SINV expressing a DENV2 prM effector gene reduced the accumulation of DENV2 RNA in the cells (Adelman et al., 2001). Later, plasmids were engineered to express inverted repeat RNA, creating a 290 bp dsRNA with the same sequence as DENV2 prM gene and it complement. When C6/36 cells were continuously expressing this RNA foldback, they became resistant to accumulation of DENV envelope protein antigen. Small RNAs 21-25 nts in length were detected from this prM foldback

region, suggesting that an RNAi based mechanism was responsible for the virus resistance seen in the transformed cells (Adelman et al., 2002).

Transgenic mosquitoes were later developed using this concept. *A. aegypti* mosquitoes were engineered to express an inverted repeat transcript from the prM gene of the DENV2 strain Jamaica 1409 in the mosquito midgut under the control of an *A. aegypti* carboxypeptidase A promoter and were named Carb77 mosquitoes. Use of this promoter controlled the expression of the DENV2 prM foldback RNA to times immediately after the mosquito had taken a bloodmeal, which activates the carboxypeptidase promoter. When this transcript was expressed, it made a 578 bp dsRNA, which triggered the RNAi response to target any DENV2 imbibed with the bloodmeal. The Carb77 mosquitoes had significantly lower rates of infection after a DENV2 containing bloodmeal and had no viral antigen in their salivary glands, indicating reduced virus dissemination in the mosquito. These mosquitoes also had reduced ability to transmit virus in an *in vitro* transmission assay (Franz et al., 2006). These results indicate that RNAi can be used in insects against arboviruses to reduce the mosquito infection and ability to transmit. When the RNAi component genes Dcr2 and Ago2 from *A. aegypti* were knocked down prior to DENV2 infection of the Carb77 mosquitoes, the DENV-resistant phenotype was reversed in the transgenic mosquito, further demonstrating the role of RNAi in the transgenic mosquito resistance and in the mosquito antiviral response (Franz et al., 2006). The resistance of Carb77 mosquitoes to DENV2 was lost in later generations of the mosquito, and the effector inverted repeat RNA was no longer being expressed (Franz et al., 2009). This loss of expression may

have been due to a RITS-like mechanism which could have shut off the transcription of the region that was inserted in the mosquito genome.

dsRNA was also used to trigger an RNAi antiviral response to SFV and DENV-1 in C6/36 cells. The RNAi response of these cells was first tested with dsRNA and plasmids expressing an enhanced green fluorescent protein (eGFP) reporter gene co-transfections. dsRNA to eGFP reduced the number of GFP-positive cells by 40-60% when it was co-transfected with the eGFP reporter plasmid. ssRNA was less effective, with sense ssRNA to eGFP having little to no effect on GFP expression. Antisense ssRNA to eGFP inhibited GPF expression by about 40%, whereas dsRNA to eGFP reduced GFP expression by 50%. When the cells were infected with SFV expressing green lantern GFP (glGFP) along with 78 bp dsRNA from the SFV nsp-2 and nsp4 genes, the number of cells expressing GFP was reduced by approximately 50%. When 78 bp dsRNA to DENV1 genes prM, E, NS1 and NS5 was used to transfect cells 18 hours before DENV1 infection, there was a reduction in DENV1 titer compared to ssRNA from the same genes. Sense ssRNA had no effect on the DENV1 replication, but antisense ssRNA did reduce DENV1 replication, although not as much as the dsRNA from the DENV genes did (Caplen et al., 2002).

RNAi pathway genes were discovered in the *A. gambiae* genome, by BLAST searching for sequences similar to *D. melanogaster* RNAi genes. Putative Dcr1, Dcr2 and 5 Argonaute genes were found. Pretreatment of *A gambiae* Sua1B cells with dsRNA from these genes, then treating the cells with dsRNA to luciferase and a luciferase expression plasmid, showed that Dcr2, Ago2 and Ago3 knock-down resulted recovery of luciferase activity to a greater extent than non-specific Beta-galactosidase (β-gal) dsRNA

pretreatment. This indicates their role in the *A. gambiae* RNAi pathway, as using RNAi to knock down Dcr2, Ago2 and Ago3 blocked the ability of the dsRNA from luciferase to knockdown luciferase expression from the plasmid (Hoa et al., 2003).

In experiments with *A. gambiae* mosquitoes, co-injection of ONNV with dsRNA to the nsP3 region of the virus RNA reduced ONNV virus titers and virus spread compared to co-injection of ONNV and control dsRNA from β-gal. Mosquitoes injected with dsRNA to the *A. gambiae* Ago2 gene along with virus showed 16-fold higher viral titers than β-gal dsRNA controls. Injection of dsRNA to *A. gambiae* Ago1, Ago4 and Ago5 did not result in increased viral infection as compared to control β-gal dsRNA, but injection of dsRNA to *A. gambiae* Ago3 with ONNV caused greater virus dissemination and statistically higher virus titers, indicating a possible role for Ago3 in *A. gambiae* antiviral RNAi (Keene et al., 2004). These results show that RNAi is an important antiviral pathway in *A. gambiae* mosquitoes, and that exposure to dsRNA from the virus genome reduces viral infection, while reducing activity of Ago2 (and possibly Ago3) in RNAi increased viral infection.

Using a comparative genomics approach, homologs of various small RNA pathways components were identified in the *A. aegypti* genome (Campbell et al., 2008a). These include the siRNA pathway components Dcr2, Ago2, R2D2, Vig, TSN, and Fmr-1 and miRNA pathway components Drosha, Pasha, Dcr1, Ago1 and Loqs. In the piRNA pathway, there appeared to be an expansion of the genes, with multiple homologs of Armitage, Ago4-like and Ago5-like components, whereas there appeared to be only one homolog of these genes in *D. melanogaster* or *A. gambiae*. One Ago3 and one Spindle-E homolog were found in *A. aegypti* which corresponds to the same number in *A. gambiae*

and *D. melanogaster*. This expansion in the small RNA pathways genes may indicate that these pathways are evolving faster in *A. aegypti* than in *D. melanogaster* flies or *A. gambiae* mosquitoes. Ago2 was also found to be significantly more diverse in mosquito species than Ago1, suggesting that the arbovirus infection of mosquitoes could be driving faster evolution in the antiviral small RNA pathways (Campbell et al., 2008a).

RNAi has also been shown to be an important antiviral pathway in *A. aegypti* against SINV. When dsRNA to *A. aegypti* Ago2, Dcr2 or TSN was co-injected into *A. aegypti* mosquitoes along with double-subgenomic SINV TR339-eGFP, there was an increase in the positive strand viral RNA, in virus titers and in infection rates of mosquitoes. Transcript levels of only TSN among these genes increased significantly in mosquito midguts after and infectious bloodmeal. Virus-specific small RNAs were detected at 2 and 7 days post-infection from TR339 SINV infected mosquitoes, with more small RNAs being detected from an antisense probe, indicating that the positive strand of SINV may be targeted more for Dicer cleavage than dsRNA replicative intermediates. Few virus-specific small RNAs were detected in SINV MRE16 infected mosquitoes (Campbell et al., 2008b).

RNAi also plays an important role in the *A. aegypti* mosquito antiviral response to DENV2. DENV2 infection induces dsRNA and viRNAs in both Aag2 cells and mosquitoes. When mosquitoes were injected with dsRNA to *A. aegypti* Dcr2 or R2D2 and fed a DENV2 infectious bloodmeal two days later, the virus titers in whole mosquitoes increased compared to non-injected or dsRNA β-gal injected mosquitoes. Significant increases in DENV2 titers were not seen with dsRNA to *A. aegypti* Ago2. The percentage of infected mosquitoes was not higher than the non-injected group with

dsRNA to Ago2 or R2D2, but was statistically higher in the Dcr2 dsRNA-injected group. Impairment of RNAi also led to earlier transmission of DENV2 from infected mosquitoes. At 10 days post-infection, mosquitoes injected with dsRNA to Dcr2 had increased titer in a feeding solution over the uninjected mosquitoes, and at 12 days post-infection, mosquitoes injected with dsRNA to Dcr2, R2D2 and Ago2 had increased titers in saliva compared to DENV infected mosquitoes that were not injected with dsRNA. These results showed that inhibiting RNAi can result in a shorter EIP for DENV2 transmission in mosquitoes, further indicating the critical role of RNAi in controlling DENV2 infection of mosquitoes (Sanchez-Vargas et al., 2009).

Additional experiments demonstrated the role of RNAi in mosquito antiviral defense by expressing the FHV B2 protein, which inhibits Dcr2 cleavage of long dsRNA, during SINV infection of *A. aegypti*. SINV TE3'2J was engineered to express the B2 protein under the control of a second subgenomic promoter (Cirimotich et al., 2009, Myles et al., 2008). SINV expressing B2 caused both *A. aegypti* mosquito and cell culture mortality, whereas the original un-engineered virus caused few ill effects in cells or mosquitoes, where it formed persistent non-cytopathic infections (Cirimotich et al., 2009, Myles et al., 2008). Virus titers also increased more rapidly and to higher levels with SINV-B2 compared to SINV alone in both cell culture and mosquitoes. Myles et al. (2008) also expressed the NoV B2 protein in an engineered ONNV, infected *A. gambiae* mosquitoes, and showed decreased survival, with the ONNV-B2 killing all mosquitoes by 11 days, whereas ONNV killed 90% of mosquitoes only after 20 days. These results showed the importance of RNAi in the formation of persistent infections by alphaviruses and that impairing with the pathway led to uncontrolled growth of the viruses and death

in various mosquito species. This highlights the evolution of arboviruses with their mosquito host to form persistent infections that allow the mosquito to survive and further spread the virus to new hosts.

The viRNAs produced during SINV and SINV-B2 infections were analyzed by deep sequencing. There were 453,360 SINV-specific small RNAs 21 nt in length produced in the mosquitoes infected with the wild-type virus (Myles et al., 2008). These were 54% positive sense and 46% negative sense, indicating that many of the small RNAs were probably produced from dsRNA formed by the replicative intermediates made during viral replication. Some areas of the genome appeared to be targeted more, implicating that not only were dsRNA replicative intermediates targets for Dcr2, but the ssRNA virus genome itself was also a Dcr2 target as well (Myles et al., 2008). The viRNAs made up more that 10% of the total cellular small RNAs sequenced in the 18- to 24- nt size range that were sequenced. The authors suggested that number was an underestimate as they only included sequences that were 100% matched to the genome and that total mosquito RNA was used for the libraries, rather than only SINV infected mosquito tissues. Whether or not this is a level that occurs naturally has yet to be determined, as the mosquitoes were not infected through an oral bloodmeal, but instead were injected with high doses of virus. The number of SINV-specific small RNAs in the mosquitoes infected with SINV expressing B2 was greatly reduced to 12,276 viRNAs, indicating that indeed the B2 protein was blocking dsRNA cleavage by Dcr2, reducing the number of viRNAs by a factor of 9.8. These were 63% from the positive strand and 37% from the negative strand (Myles et al., 2008).

ONNV viRNAs from infected *A. gambiae* mosquitoes were examined using high throughput sequencing from two biological replicates. Female mosquitoes were injected with ONNV and RNA was harvested 4 days post injection. There were 15,818 sense and 8945 antisense 21 nt reads that were 100% ONNV genome matches in the first replicate, and 17,230 sense and 9,594 antisense 21 nt 100% match reads in the second replicate. The ratio of sense to antisense viRNAs was 1.8/1 for both replicates. These 21 nt viRNAs represented 0.58% and 0.62%, respectively, of the total number of usable reads sequenced. The viRNAs were spread across the length of the ONNV genome, but there were some 'hot spots' with more hits. There did not appear to be a bias for more viRNAs coming from the subgenomic 26S RNA in either ONNV- or SINV-infected mosquitoes, even though there is only one non-structural gene encoding (full genome) RNA molecule for every 7-10 encoding (subgenomic) RNA molecules (Myles et al., 2009, Myles et al., 2008, Wielgosz & Huang, 1997). If viRNAs were coming from predominately ssRNA secondary structures, one would expect many more viRNAs to be coming from the 26S RNA, implying that the viRNAs are derived from Dcr2 cleavage of long replicative intermediate dsRNA. These deep sequencing results demonstrate that antiviral RNAi is active against ONNV (Myles et al., 2009). When *A. gambiae* mosquitoes were injected with ONNV expressing B2, there was increased mortality in the mosquito, indicating a role of RNAi as an important antiviral pathway for survival of *A. gambiae* infected with ONNV (Myles et al., 2008).

WNV viRNAs have recently been studied in *Culex pipiens quinquefasciatus* mosquitoes. Approximately 2,544 viRNAs were found in mosquitoes at 7 days post infection and 4,419 viRNAs in mosquitoes at 14 days post infection, and approximately

80% of these viRNAs were perfect matches to the WNV genome. These were 74 % positive and 26% negative sense and the majority of them were 21 nts in length, the typical size for products of the siRNA pathway. These viRNAs were spread throughout the genome, with some regions being highly targeted for Dcr2 cleavage and other regions being targeted less frequently. A region coding for the capsid protein was the most targeted area of the WNV genome, and the authors found that areas of the genome that were more intensely targeted by the RNAi machinery were more likely to have point mutations, suggesting that RNAi contributes to WNV evolution and virus diversification (Brackney et al., 2009).

Recent work has examined the ability of mosquito small RNAs to spread between cells. It is unclear if insects have a similar RdRP activity that plants use to amplify their siRNAs for systemic spread, but it has been recently suggested that the *Drosophila* antiviral RNAi pathway requires systemic spread, using a cellular dsRNA uptake mechanisms. When flies had a defective dsRNA uptake pathway, they were hypersensitive to DCV and SINV infections (Saleh et al., 2009). Using *A. albopictus* U4.4 cells and a luciferase reporter system, researchers suggested that dsRNA/siRNAs could also spread over short distance between mosquito cells. They also found that the spread of dsRNA/siRNA between mosquito cells could inhibit SFV replication, but when the p19 siRNA-binding suppressor was expressed, SFV infection other cells was strongly enhanced. They also concluded that SFV does not inhibit dsRNA cleavage, interfere with established RISC, prevent the induction of RNAi, inhibit siRNA-induced antiviral RNAi or inhibit cell-to-cell spread of the RNAi signal between the mosquito cells in their *in vitro* experiments. These experiments show further evidence that arboviruses may not

have evolved encoded suppressors of antiviral RNAi, as high virus loads may have negative impacts on their mosquito host's survival (Attarzadeh-Yazdi et al., 2009).

So far there is no evidence that arboviruses naturally encode protein suppressors of RNAi. In a *Drosophila* screen for viral suppressors of RNAi, no mature DENV protein can rescue FHV infection of flies when the B2 protein of FHV has been deleted, indicating that none of the DENV proteins inhibit the RNAi pathway and allow for normal FHV replication (Li & Ding, 2005).

Summary and Goals:

Arboviruses such as DENV continue to cause disease in millions of people around the world each year and many arboviruses are re-emerging or expanding to new areas. New molecular-based strategies such as genetically modified mosquitoes may help to stop the spread of these viruses, possibly by triggering mosquito immune pathways to target viruses. One of these immune pathways, RNAi, has been identified as a key antiviral pathway in insects, and it plays an important role in mosquito immunity to alphaviruses and flaviviruses.

Small RNA pathways have emerged as major players in gene regulation at the molecular level in cells. The siRNA pathway seems to have co-evolved in insects as an antiviral pathway in a molecular arms race with insect viruses. Viruses have also evolved mechanisms to avoid RNAi such as protein suppressors or hiding from the RNAi machinery. There appears to be a fine balance between producing enough viruses to be transmitted, but not so much to overwhelm the host. How mosquito small RNA pathways and arboviruses are interacting at a molecular level is still unclear. Also, the

role of mosquito-only viruses in the RNA-based immune response to similar arboviruses is poorly understood.

I hypothesize that DENV2 accessible to the RNAi pathway in mosquito cells and is cleaved into siRNAs by Dicer2, and I have used next-generation sequencing technologies to study this hypothesis. This work will examine: 1) the small RNAs produced during DENV2 infection of mosquito cells, 2) how the RNAi activity varies between Aag2 and C6/36 cell lines, and 3) the small RNAs produced during CFAV infection of mosquito cells.

CHAPTER 2

ANALYSIS AND SEQUENCING OF SMALL RNAS FROM DENGUE VIRUS TYPE 2-INFECTED MOSQUITO CELLS

Introduction:

In the last decade, RNA interference-based pathways have emerged as a major component of gene regulation and control of viruses and transposable elements in many organisms. In the pathways, dsRNA acts as a trigger for enzymatic cleavage by Dicer enzymes into small RNAs, which are then loaded into a RNA-induced silencing complex (RISC) containing a member of the Argonaute family. The RISC then uses the small RNA as a guide to identify complementary long ssRNA in the cell for destruction. These triggers may come from hairpins made from the cell's own gene transcription as in the microRNA pathway, from long dsRNA formed during viral replication as in the exogenous small-interfering RNA (siRNA) pathway, or from transcription of repetitive elements or transposons as in the endogenous-siRNA pathway (endo-siRNA) or the PIWI-interacting (piRNA) pathway (Ghildiyal & Zamore, 2009).

These pathways and their small RNA products have been characterized extensively in the insect *Drosophila melanogaster*. Studies found that RNAi was an important antiviral defense in flies against viruses with RNA genomes (Galiana-Arnoux et al., 2006, van Rij et al., 2006, Wang et al., 2006, Zambon et al., 2006), the interactions of viruses and the RNAi pathway seem to drive faster evolution of RNAi genes, and viruses may have also co-evolved mechanisms to get around these pathways, leading to a molecular arms race (Campbell et al., 2008a, Obbard et al., 2006).

Antiviral RNAi activity has also been described in mosquitoes infected with arboviruses (Brackney et al., 2009, Campbell et al., 2008b, Cirimotich et al., 2009, Keene et al., 2004, Myles et al., 2008, Sanchez-Vargas et al., 2009), yet we do not understand why the mosquito RNAi antiviral immune system still allows for arboviruses to survive

and be transmitted by mosquitoes. Arboviruses, particularly the dengue viruses, infect millions of people around the world each year, causing significant morbidity and mortality. New molecular-based methods are needed to help stop the transmission of these viral infections between humans and mosquitoes. RNAi-based technology was used to engineer transgenic mosquitoes that were resistant to DENV2 infection (Franz et al., 2006), yet little is known about how arboviruses interact with RNAi pathways at a molecular level during natural viral infection of mosquitoes.

Mosquito RNAi activity against alphaviruses has been demonstrated with ONNV and SINV (Campbell et al., 2008b, Cirimotich et al., 2009, Keene et al., 2004, Myles et al., 2009, Myles et al., 2008). More recently, RNAi against the flaviviruses DENV2 and WNV was confirmed in mosquitoes (Brackney et al., 2009, Sanchez-Vargas et al., 2009). When the Flock House virus Dcr2 inhibitor B2 protein was expressed in engineered SINV, this greatly increased mosquito mortality from the virus infection, indicating the critical role of RNAi in preventing arboviruses from overwhelming their mosquito hosts (Cirimotich et al., 2009, Myles et al., 2008). The target for RNAi activity on these ssRNA viruses was originally assumed to come from dsRNA replicative intermediates, but that idea was challenged when sequencing of viRNAs from a ssRNA plant virus revealed that the secondary structure of the viral genome itself could be a trigger for Dcr cleavage and RNAi activity (Molnar et al., 2005).

By examining the viral-specific small RNAs that are generated in the mosquito antiviral response, we hope to gain understanding in how the pathway identifies and cleaves viral RNA, which will provide insight into how to manipulate the pathway to reduce mosquito transmission of arboviruses. In this chapter, small RNAs were

sequenced from DENV2-infected Aag2 cells and *A. aegypti* mosquitoes to identify the cleavage pattern of viral RNA in cells, and also to study how other small RNA pathways in the mosquito cell change in response to DENV2 infection.

Materials and Methods:

Cells and Medium

Cultured *A. aegypti* cells (Aag2) were maintained in Schneider's Drosophila Medium (Lonza, Wakersville, MD), modified from manufacturer with L-glutamine, supplemented with 10% heat inactivated (30 minutes at 55°C) fetal bovine serum (FBS) (Colorado Serum), 2 mM L-glutamine (Mediatech, Manassas, VA) and 100 units penicillin and 0.1 mg streptomycin (Sigma, St. Louis, MO), at 28°C in closed flasks with no CO_2 supplementation.

Viruses and Cell Infection

Highly passaged DENV type 2 strain Jamaica 1409 virus was used in infections of Aag2 and C6/36 cells at a multiplicity of infection (MOI) of 0.1 for small RNA sequencing and an MOI 0.05 for small RNA Northern blot timecourse. Infections were done in the same medium as the cells were maintained in, but the FBS concentration was reduced to 2%, and non-essential amino acids were added. The concentrations of L-glutamine and penicillin/streptomycin remained the same as in the maintenance medium. Cells were approximately 90-100% confluent at the time of infection. Before infection, maintenance medium was removed from the cells and a few mLs of the 2% FBS infection medium were added to the cell monolayer along virus at the correct MOI. The

flask was rocked gently at room temperature for one hour, more 2% FBS infection medium was added to reach a final volume of 12 mL in a 75 cm^2 flask, and the flask was incubated at 28°C until samples were harvested. The day of infection was considered day zero in the timecourse.

Mosquitoes and DENV2 Infection

All mosquito samples were generated by Dr. Corey Campbell. *A. aegypti* RexD strain mosquitoes were reared at 28°C with 82% humidity. One week old female mosquitoes were deprived of a sugar source overnight and were then allowed to feed on artificial bloodmeals containing defibrinated sheep blood (40%) (Colorado Serum Company, Boulder, CO) and an infected C6/36 cell suspension (60%) with 1 mM ATP for 1 hour. The bloodmeal was maintained at 37°C in a water-jacketed glass feeder covered with hog gut membrane, and mosquitoes fed on the blood through the membrane. The DENV2 strain Jamaica 1409 titer was approximately 1 X 10^7 pfu/mL in the bloodmeal for the DENV2 infected mosquitoes, while the mock-infected mosquitoes were fed a blood and uninfected C6/36 cell mixture. Bloodfed females were selected and were maintained with water and sugar for 9 days after the infection (or mock infection), when RNA was harvested from 20 whole mosquitoes per group using Trizol.

RNA Extraction

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and quantified using spectrophotometry. Small RNAs used in hybrid selection and cloning were separated from total RNA by gel electrophoresis on a 15% TBE-Urea PAGE, eluted

from the gel slice overnight at room temperature, extracted with phenol/chloroform followed by a chloroform/isoamyl extraction and precipitated in ethanol overnight at -20ºC. Small RNAs used in SOLiD sequencing were separated from total RNA extracted with Trizol reagent using FlashPAGE columns and FlashPAGE apparatus (Applied Biosystems) running at 75 volts for 12 minutes. The RNA was precipitated with linear acrylamide (Applied Biosystems) and ammonium acetate in ethanol overnight at -20°C.

In vitro transcription

ssRNA probes were generated to the 498 nt DENV2 prM gene region in sense or antisense orientation via *in vitro* transcription from a T7 promoter using the MEGAscript kit (Applied Biosystems) with approximately 9% of the UTP in the transcription reaction conjugated to biotin (Applied Biosystems). The templates for transcription were PCR products with a T7 promoter on the 5' end for positive sense probes or a T7 promoter on the 3' end for negative sense probes. The template for PCR was DENV2 Jamaica 1409 infectious clone DNA (Pierro et al., 2006). The biotinylated ssRNA probes used in hybrid selection technique were generated in the same way from DENV2 genome nucleotide regions 1-4995 or 4976-10700, using DENV2 16681 infectious clone DNA (Kinney et al., 1997) as a template for PCR.

Small RNA northern blotting hybridization

Fifty micrograms of total RNA were loaded per lane onto a 15% TBE-Urea denaturing gel (Invitrogen) and separated by electrophoresis. RNA was electrophoretically transferred to a neutrally charged nylon membrane and was

chemically cross-linked to the membrane using carbodiimide (Pall et al., 2007). The membrane was pre-hybridized with UltraHyb (Applied Biosystems) hybridization buffer at 42°C for 30 minutes. Five micrograms of 498 nt probe was reduced to 50-100 nt fragments via hydrolysis in 200 mM carbonate buffer at 60°C for approximately 2.5 hours. The probe was added to the UltraHyb buffer after the pre-hybridization and incubated 16 hr at 42°C. The membrane was washed twice with 2X SSC,0.1% SDS buffer for 5 minutes each, followed by two washes in 0.1X SSC, 0.1% SDS buffer for 15 minutes each. All washes were done at 42°C. RNA was detected with the BrightStar BioDetect Kit (Ambion) and membranes were exposed to autoradiography film for 18 hours.

Small RNA cloning and sequencing using "Hybrid Selection"

Small RNAs were gel-purified from a 15% TBE-Urea gel and hybridized overnight at 42C with biotinylated ssRNA probes from half of the DENV2 genome (strain 16681) (nts 1-4995) in sense or antisense orientation, or with 2 sense probes (nts 1-4995 and 4976-10700) covering the entire genome, or 2 antisense probes (nts 1-4995 and 4976-10700) covering the entire genome. Probes and hybridized small RNAs were mixed with streptavidin-agarose beads and unbound RNA was washed away. Small RNAs were released from the probe by heating, a linker (miRNA cloning linker No. 3, Integrated DNA Technologies, Inc., Coralville, IA) was ligated to the 3' ends and the products were gel-purified. A linker (MRS cloning linker, Integrated DNA Technologies, Inc.) was then ligated to the 5' ends, and reverse transcription, PCR was performed to amplify the products and the PCR band of correct size was extracted from

agarose gel. The PCR products were then ligated together to form concatemers, size selected by agarose gel separation and extraction, cloned into the pTOPO4 cloning vector (Invitrogen) and sequenced by the Proteomics and Metabolomics Facility at Colorado State University. Sequences were analyzed for linker regions using VectorNTI software (Version 10, Invitrogen), and the sequences located between the linkers were used to BLAST search the GenBank database to identify the small RNAs (See Figure 2.1).

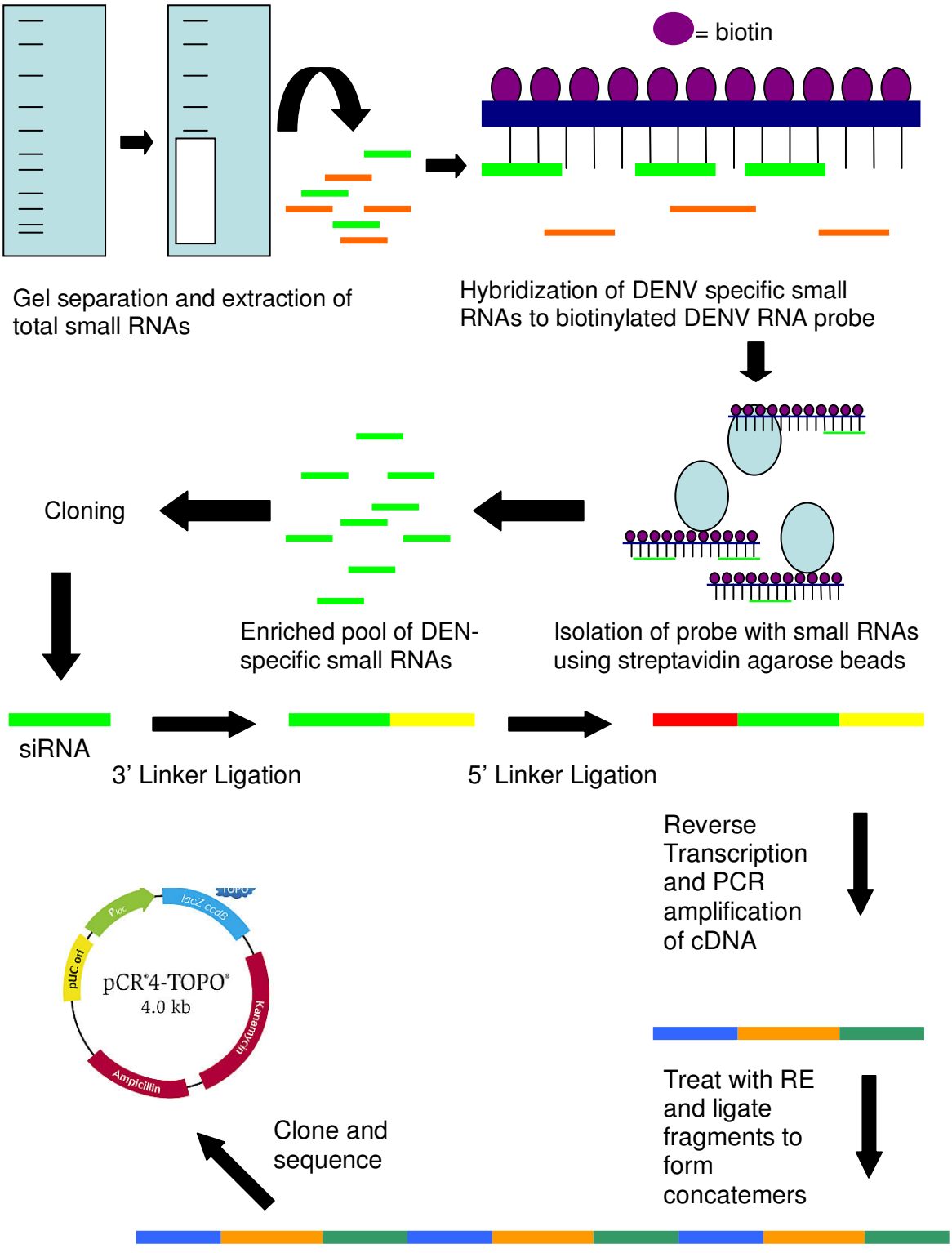


Figure 2.1: Diagram of Hybrid Selection technique and traditional small RNA cloning

Sequencing by oligonucleotide ligation and detection (SOLiD) sequencing

Small RNA libraries were made using the SOLiD Small RNA Expression Kit (Applied Biosystems), November 2008 edition, with Dr. Corey Campbell making the mosquito-based libraries. Ligation reactions were set up with approximately 200 nanograms (ng) of FlashPAGE fractionated small RNAs per cell culture sample (500 ng of FlashPAGE fractionated small RNA per mosquito sample) using Adaptor Mix "A". After a reverse transcription and RNaseH treatment, approximately 20, 50 µl PCR reactions were set up per sample with 1 µl of cDNA used per 50 µl reaction along with 15 cycles of PCR amplification for cell culture samples and 18 cycles of PCR amplification for mosquito samples.

Table 2.1: SOLiD PCR Primer Sets for Aag2 and *A. aegypti* set (See Appendix Table A.2 for primer sequences)

Sample	SOLiD PCR Primer Set
Aag2 Mock	9
Aag2 DENV2 Day 1	5
Aag2 DENV2 Day5	6
A. aegypti mosquito mock	1
A. aegypti mosquito DENV2 Day 9	2

DNA libraries made from the SOLiD small RNA expression kit were sent to the University of Washington (UW) for sequencing using the Applied Biosystems SOLiD 2 sequencing instrument, with each sample running in its own octet (1/8 of a slide).

viRNA Sequencing Analysis

Potential viRNAs were aligned to DENV2 genome using NextGENe (Softgenetics, LLC, State College, PA) software running the transcriptome assembly function with the parameters in Table 2.2 below. Version 1.11 was used for both Aag2

and mosquito samples. CSFASTA (color-space) files from SOLiD sequencing of samples from UW were used as the sample file and a FASTA file of the DENV2 Jamaica 1409 strain RNA from GenBank accession number M20558.1 was used as the reference sequence. Size graphs were adjusted for loss of the 5' most base during length calculation (one nt was added to the length of each small RNA).

Table 2.2: NextGENe software parameters for viRNA alignment

NextGENe parameter	Setting Used	
	Aag2 cell culture	Mosquito
Condensation	Off	Off
Assembly	Off	Off
Alignment	On	On
Alignment Method	Method 1	Method 1
Matching Base Number	12	12
Matching Base Percentage	51	50
Sequence Reads < 1kb	Off	Off
Alignment Memory Ratio	1.0	1.0
Allow Ambiguous Location	Off	Off
Sequence Range Checked	On, 0 to 35	On, 0 to 35
Hide Unmatched Reads	On	On
Mutation Percentage	0.0	0.1
Mutation Coverage	3	1
Allow Deletion of Mutations	Off	Off
FR Balance	Off	Off
Load Assembled Result Files	Off	Off
Load Sage Data	Off	Off
Load Paired End Data	Off	Off
Saved Matched Reads	On	On

viRNAs were also aligned to the DENV2 genome using the ABI SOLiD small RNA Analysis Pipeline Tool v.0.5.0 (RNA2MAP), and the software was set up and run by Dr. Richard Casey at Colorado State University. The DENV2 RNA sequence used as reference was the GenBank accession number M20558.1 for the Jamaica 1409 strain. The primers and adapters were filtered out and the miRbase matching step was turned off.

The following was used as the configuration file:

```
UN_FILTERING	true
RUN_MIRBASE_MATCHING	false
RUN_GENOME_MATCHING	true

tag_length	35
pattern 11111111111111111111111111111111111  #need to be of tag_length size
adaptorCGCCTTGGCCGTACAGCAG

# filtering step
filter_step_reference_file	/projects/jackiescott/primer_filter_reference.fasta
filter_step_number_of_bases_to_use 25
filter_step_number_of_errors 2

# miRBase matching step
miRBase_step_gff_reference_file	/projects/jackiescott/hsa.gff
MAKE_PRECURSOR_FASTA_REFERENCE	no
miRBase_step_reference_fasta_file	/projects/jackiescott/has.fasta
miRBase_step_reference_extenssion 35
miRBase_step_seeds_number_of_bases_to_use	18
miRBase_step_seeds_number_of_errors	3
miRBase_step_extension_max_number_of_errors	6
miRBase_step_output_read_type	unique
miRBase_step_output_countsyes
miRBase_step_output_wiggle	yes
miRBase_step_output_gff_reads	no

# genome matching step
genome_step_reference_fasta_file	/projects/jackiescott/M20558.fasta
genome_step_seeds_number_of_bases_to_use	20
genome_step_seeds_number_of_errors	2
```

```
genome_step_extension_max_number_of_errors      5
genome_step_maximum_number_of_hits_per_tag   5
genome_step_output_read_type         all
genome_step_output_wiggle  yes
genome_step_output_wiggle_coverage_cut   10

# environment information
NAME_OF_QUEUE  secondary       #highmem
FOLDER_FOR_TEMPORARY_FILES_ON_COMPUTE_NODES          /scratch/
NUMBER_OF_PROCESSORS_ALLOCATED_PER_NODE       1
MAX_MEMORY_PER_JOB_IN_BYTES   2e9

# NOT YET IMPLEMENTED

NUMBER_OF_READS_FILE_PARTITIONS        2
```

miRNA Sequencing Analysis

miRNAs were identified using the alignment function of NextGENe (Version 1.11), with the same parameters as Table 2.2 except the matching base percentage was set to 50, the sequence reads <1 kb turned on, and the mutation percentage set to 0.2. The sample files were the CSFASTA files from the SOLiD sequencing for each sample and the reference genome was the complete mature miRNAs from the miRBase website (Ambros et al., 2003, Griffiths-Jones, 2004, Griffiths-Jones et al., 2006, Griffiths-Jones et al., 2008, mirbase.org) downloaded on May 14, 2009, converted to a .fa file format (with the U's converted to T's) by Kevin LaVan at Softgenetics. This reference file is annotated, allowing for identification and counts for each of the various miRNAs in the database. The alignments for miRNAs that were found to increase the most with infection were the manually checked for matching the NextGENe Sequence Alignment viewer to make sure the matches did not overlap miRNA annotations, and those that were not direct matches to a specific miRNA were then not used in the graph.

Other mosquito small RNA analysis

Small RNAs that matched the *Aedes aegypti* Dcr2 and Ago2 mRNA sequences were identified in the mock-Aag2 and DENV2-Aag2 (5 dpi) SOLiD samples by using NextGENe alignment function (Version 1.11) with the same parameters as the miRNA analysis. *A. aegypti* Dcr2 sequence was acquired from GenBank (accession number AY713296), and *A. aegypti* Ago2 sequence was generously provided by Dr. Alexander Franz. The mock-Aag2 SOLiD sample was also used to determine which proportions of the small RNA libraries were derived from *A. aegypti* rRNA/tRNA, the *A. aegypti* transcriptome and from other regions of the genome using the NextGENe transcriptome alignment function (Version 1.92). The *A. aegypti* supercontigs and transcriptome files were acquired from VectorBase.org, the mosquito rRNA/tRNA files were acquired from GenBank and all files were formatted for NextGENe by Dr. Corey Campbell.

Results:

Small RNA northern blot hybridization for DENV2 viRNAs

Northern blotting was performed to look for the presence of DENV2-specific small RNAs in Aag2 cell culture. The prM (premembrane gene) of DENV2 was used as a probe because Emily Travanty had discovered one DENV2-specific small RNA in her small RNA cloning experiment, and it was from the prM gene region (Travanty, 2005). Using a chemical cross-linking technique to bind RNA to a nylon membrane, DENV2 small RNAs were detected by northern blot hybridization with biotin-labeled probes from total RNA from Aag2 cells infected with DENV2 (Figure 2.2). The size of the viRNA band was between 21-25 nt in length. There was no band detected in the mock-infected

samples, indicating that it was viral specific. More DENV small RNAs accumulated at days later after infection, and more DENV2 small RNAs were detected using the antisense prM probe than using the sense probe (blots were exposed to same piece of film for same time), indicating a possible bias towards production of more sense DENV small RNAs. When the combined intensity of the bands on the blot was analyzed with Quantity One Software (Bio-Rad) the antisense probe band at 7 days post infection accounted for approximately 63% of the intensity, while the sense probe band at 7 days post infection accounted for approximately 36% of the combined intensity. This difference may also be due to the antisense prM probe having 32% of the bases as a UTP, while the sense probe has 23% of the bases as UTP, indicating that the antisense probe may be more highly labeled, thus giving a stronger signal. When this difference in the number of UTPs is accounted for, 54% of the signal is from viRNAs from the positive strand and 45% from the negative strand. This blot may indicate that the sense strand is being targeted more by the RNAi machinery, possibly targeting the viral genome's secondary structure, although when the UTP levels are adjusted for, the viRNA levels may be closer to equal from each strand.

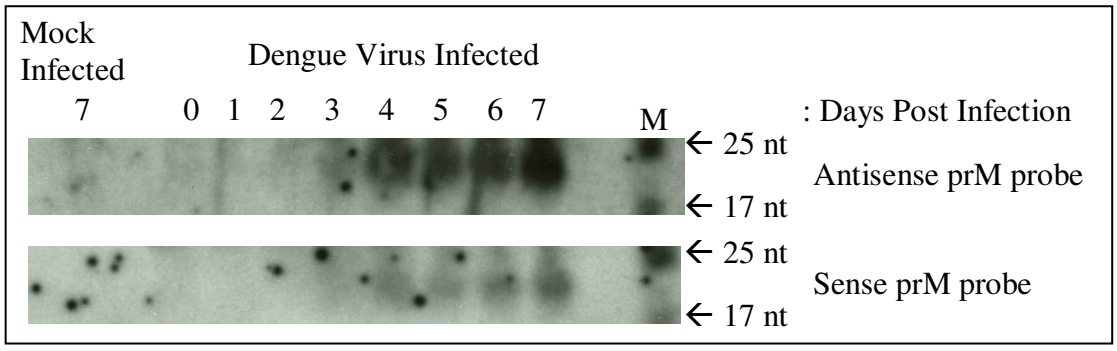


Figure 2.2: Detection of DENV2-specific small RNAs in infected Aag2 cells using sense and antisens probes from the DENV2 prM gene. This figure is published in (Sanchez-Vargas et al., 2009).

Small RNA cloning and sequencing using "Hybrid Selection"

In order to characterize which regions of the DENV2 RNA are being targeted for cleavage, we attempted to clone and sequence virus-specific small RNAs. To enrich for DENV2-specific viRNAs in the small RNA population we employed a "Hybrid Selection" technique. The DENV2 small RNAs identified by hybrid selection and traditional cloning from DENV2-infected Aag2 cells are listed in Table 2.3. Twenty-six DENV2 small RNAs were identified using this technique. Many of the other small RNAs identified were from mosquito ribosomal and transfer RNA, mosquito mRNAs, miRNAs or *Escherichia coli* RNA. These non-specific small RNAs were probably found due in inefficient or incomplete washing, or non-specific binding to the hybrid selection probes. The *E. coli* RNA most likely came from the recombinant RNA ligase that was produced in *E. coli* bacterial cells. In all, 269 small RNAs were cloned and identified, and approximately 10% (26) matched the DENV2 genome. The length of the DENV2 viRNAs identified ranged between 16-25 nt, with most between 20-21 nt in length (Figure 2.3). The small RNAs identified that were less than 20 nts in length may be too short to be generated by Dicer2 cleavage and could be degradation products. Fourteen (54%) matched the sense strand, 12 (46%) matched the antisense strand. The DENV2 small RNAs were found in regions across the DENV2 genome, with slightly more being found in the 5' half of the genome, probably due to more experiments being done with only the 5' half probes (instead of both 5' and 3' half probes together) (Figure 2.4). There were some small RNAs that overlapped, including 3 sense small RNAs from the NS2A gene region covering nt 3909-3940, 2 sense small RNAs from the NS3 gene region covering nt 4966-4995, and 2 sense small RNAs in the 3'UTR covering nt 10453-

10494 (with one nt overlap between the two). Antisense small RNAs that overlapped included 2 from the E gene region covering nt 1079-1102, and 2 from NS2B gene region covering nt 4401-4423.

Table 2.3: Hybrid selected DENV2 viRNAs from Aag2 cells between 16-25 nt in length.

Sequence 5'→ 3'	Sense or Antisense	DENV gene	Nucleotide position	Length (nt)
ggtcctcgtccttgcag	Antisense	C	208-224	17
agtggcactcgttccacatg	Sense	prM/M	714-733	20
ggcaggttgtttggcttctg	Antisense	E	1079-1098	20
gagtggcaggttgtttggcttc	Antisense	E	1081-1102	20
ggaaagggaggcattgtgacc	Sense	E	1261-1281	21
gtagctgtctccgaatgg	Antisense	E	2050-2067	18
acagagccgtccatgccgatat	Sense	NS1	2993-3014	22
ggtagcctggtctgtagttgtg	Antisense	NS1	3181-3202	22
attggcagtgactatca	Sense	NS2A	3909-3925	17
gactatcatggctatcctgtg	Sense	NS2A	3918-3938	21
tatcatggctatcctgtgtg	Sense	NS2A	3921-3940	20
ttgtgcaactcaccttccatgcgtt	Antisense	NS2A	3964-3988	25
atggtcagtgtttgttcttcc	Antisense	NS2B	4401-4421	21
gtatggtcagtgtttg	Antisense	NS2B	4408-4423	16
agaacgggattgttggtg	Sense	NS2B	4429-4446	18
gtgacgtgccacattgtatgg	Antisense	NS3	4659-4679	21
gaaccatcatgggcagacgtcaag	Sense	NS3	4717-4740	24
atctctggacttttcccctgg	Sense	NS3	4899-4919	21
tctttacggtaatggtgttg	Sense	NS3	4966-4984	19
ctttacggtaatggtgttgtcac	Sense	NS3	4966-4988	23
gtgttgtcacaaggagt	Sense	NS3	4979-4995	17
ggctatagcactcacatatg	Antisense	NS3	5000-5019	20
gtctttcctgctcctggatg	Antisense	NS3	5101-5120	20
gctgggtctgtgaagtgggc	Antisense	NS3	5377-5396	20
gggaggccacaaaccatgg	Sense	3' UTR	10453-10471	19
ggaagctgtacgcatggcgtagtgg	Sense	3' UTR	10470-10494	25

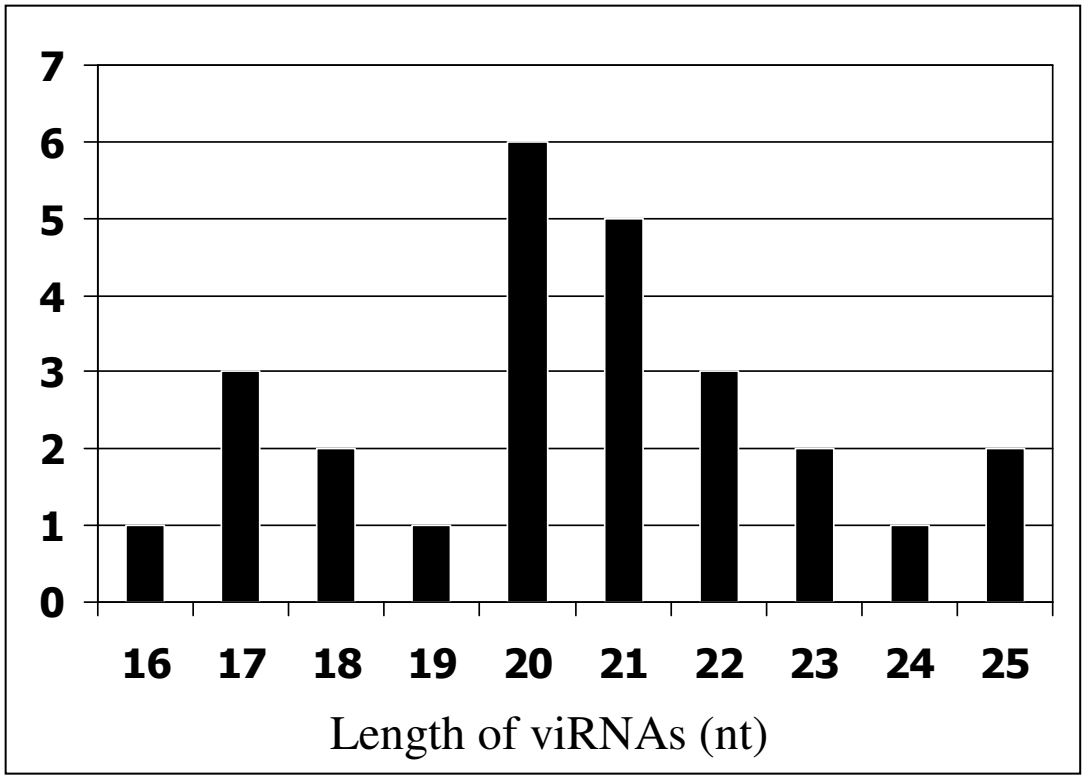


Figure 2.3: Size distribution of DENV viRNAs from Aag2 cells infected for 5 days with DENV2 isolated using hybrid selection technique.

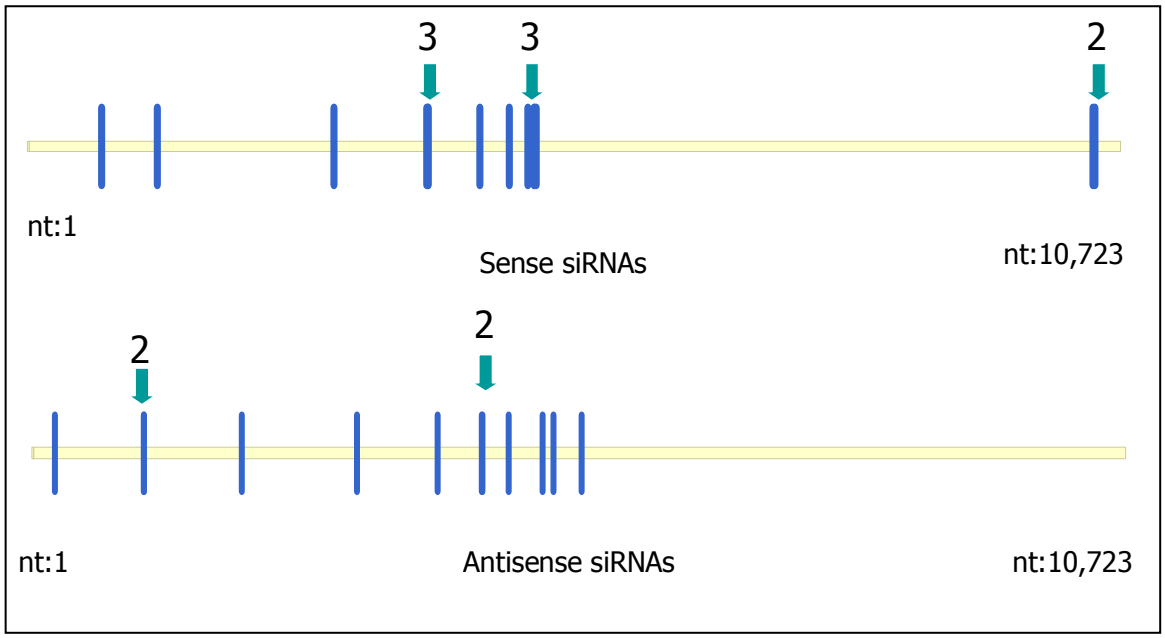


Figure 2.4: Hybrid selected DENV2 viRNA distribution on DENV2 genome. DENV2 viRNAs isolated by hybrid selection technique and cloning, distributed along the length of the DENV genome (5'→ 3' left to right), with arrows indicating where viRNAs overlapped.

Other small RNAs derived from the DENV2 genome longer than 25 nt in length were identified using the hybrid selection technique (Table 2.4). Since they are longer than typical siRNAs, they were probably not generated by Dcr2 cleavage, but may have come from another small RNA pathway or possibly were degradation products. It is unknown if they serve any functions in the cell or would result in any antiviral activity from the mosquito immune response.

Table 2.4: Hybrid selected DENV2 small RNAs from Aag2 cells over 25 nt in length.

Sequence 5'→3'	Sense or Antisense	DENV 2 gene	Nucleotide Position	Length (nt)
cgccacaagggccatgaacagtttcaatggtcctcgtccttgcag	antisense	C	208-252	45
ggaaatgtgtcgttcctatggtgtatgccaggattgctgccattatgg	antisense	M	839-886	48
gtgtcatttccgactgcatgctcttcc	antisense	E	1374-1400	27
gtcaattctgcttctgtgatggaactctgtggtg	antisense	E	1430-1463	34
ggaattgtgacactgtatttgggggtcatg	sense	E	2383-2412	30
catggtgcaggccgatagcggttgcgttgtgagctg	sense	E/NS1	2409-2444	36
actgaccatactcattagaacaggattgctggtgatctcagga	sense	NS2B	4413-4455	43
atcaataccaatcacggcagcagcatggtacctgtg	sense	NS2B	4467-4502	36
tacggtaatggtgttgtcacaaggagt	sense	NS3	4969-4995	27
ggtctgggctatagcactcacatgctccactcc	antisense	NS3	4993-5025	33
gcatgacacattaggtccacgatctcccgcccggtgtg	antisense	NS3	5272-5309	38
ccacgaacgctcagggatttctctttcct	antisense	NS3	5525-5553	29
gaggatggaacgattggacacaagtgcctttc	sense	NS5	9662-9693	32

The hybrid selection technique did not only isolate DENV2-specific small RNAs, but some other small RNAs were also cloned from the enriched population, probably due to inefficient removal of non-specific small RNAs during the washing steps. Some of these small RNAs matched known microRNAs. The miRNAs identified had sequence identities to *D. melanogaster* mir-2a-1, *D. melanogaster* mir-13b-2, and *Mus musculus*

mir-33 (Table 2.5). Their functions are poorly understood, so I will not speculate on their roles in the mosquito cell.

Table 2.5: miRNAs identified from hybrid selection

Sequence (5'→ 3')	**Length (nt)**	**BLAST match (total length of transcript in nt)**	**Percent Identity**
tcacagccagctttgatgagc	21	*Drosophila melanogaster* mir-2a-1 (23 nt)	100%
		Bombyx mori non-coding RNA, ovarian small RNA-23492 (27 nt)	100%
		Culex pipiens quinquefasciatus phosphatase 4 partial mRNA (2955 nt)	100%
tatcacagccattttgacgagt	22	*Drosophila melanogaster* mir-13b-2 (22 nt)	100%
gtgcattgtagttgcattgca	21	*Mus musculus* mir33, microRNA (69 nt)	100%

Interestingly, two mosquito-only flavivirus-like small RNAs were also identified, including a sequence that was a direct match to CFAV and one that matched the Kamiti River virus-like integration described as Cell Silent Agent 2 (CSA2) (Crochu et al., 2004) (Figure 2.5). Both of these sequences matched 100% and were derived from the antisense strand of the virus genomes. The sequence from CFAV was exactly 21 nt in length, suggesting a siRNA pathway origin, while the CSA2 integration sequence was 30 nt in length, suggesting a piRNA pathway (or other non-siRNA pathway) origin.

5’ cgcgcacgatccaaactctgagttcctggc 3’ (30 nt)

```
>  gb|AY347953.1|  Aedes aegypti A20 sequence containing region
similar to NS5 gene
of Kamiti River virus
Length=3001

 Score = 60.0 bits (30),  Expect = 2e-07
 Identities = 30/30 (100%), Gaps = 0/30 (0%)
 Strand=Plus/Minus

Query  1    CGCGCACGATCCAAACTCTGAGTTCCTGGC  30
            ||||||||||||||||||||||||||||||
Sbjct  351  CGCGCACGATCCAAACTCTGAGTTCCTGGC  322
```

5’ tcgtccatcaccgtgccgctgc 3’ (22 nt)

```
>  gb|M91671.1|YFVCFAPP Flavivirus cell fusing agent polyprotein
gene, complete cds
Length=10695

 GENE ID: 1496976 CFA | cell fusing agent polyprotein [Cell fusing
agent virus]
(10 or fewer PubMed links)

 Score = 42.1 bits (21),  Expect = 0.020
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  2     CGTCCATCACCGTGCCGCTGC  22
             ||||||||||||||||||||||
Sbjct  9228  CGTCCATCACCGTGCCGCTGC  9208
```

Figure 2.5: Mosquito-only flavivirus-like small RNAs identified by hybrid selection and alignment to GenBank sequences matching a mosquito DNA integration related to Kamiti River virus and matching cell fusing agent virus.

DENV2 small RNAs identified from SOLiD sequencing using NextGENe analysis

In order to get a more comprehensive look at the DENV2-specific small RNAs produced in infected cells, we used next-generation sequencing technologies to sequence the most of small RNA population in the cell. DENV2-specific small RNAs were analyzed from Aag2 cells and *A. aegypti* mosquito samples either mock- or DENV2-infected using the NextGENe software. The DENV2-infected sample RNA was

harvested 1 and 5 days after virus infection, and the mock-infected sample RNA was harvested 5 days after mock-infection for Aag2 cells and mosquito samples were harvested 9 days after DENV2- or mock-infection. There were only 93 matches to the DENV2 genome in the uninfected Aag2 sample ($7x10^{-4}$ % of the total numbers of reads) suggesting it may be due to non-specific alignment of short reads (Table 2.6). The DENV2-infected sample from 1 day post-infection also had a very low number of DENV2-specific reads (55), indicating that viral replication and/or the dsRNA trigger in the cell was not very prevalent early after infection, although the cells were faintly positive for virus E protein by IFA at this timepoint (data not shown). The RNA harvested from DENV2-infected Aag2 cells at 5 days post-infection had 1612 DENV2-specific viRNAs that aligned to the DENV2 genome using NextGENe (Table 2.6). This is many more than in the uninfected or DENV2-infected 1 dpi, but still only accounted for 0.01% of the total number of small RNAs from the library, indicating possibly low levels of viral replication, or sequestration of the dsRNA trigger in cellular membranes (Uchil & Satchidanandam, 2003). The mock-infected mosquito sample had a few small RNA that matched the DENV2 genome (30), and the DENV2-infected mosquito had 6029 DENV2-specific small RNAs, but this only accounted for 0.05% of the total number of small RNA reads (Table 2.6). Whether or not this level is biologically significant for controlling viral replication in the cells is unknown.

The DENV2-specific small RNAs from DENV2-infected Aag2 cells (5 dpi) were 59% positive sense, and the small RNAs from DENV2-infected mosquitoes were 55% positive sense. The ratios of positive to negative sense DENV2 small RNAs are were

close to equal, indicating most small RNAs may be derived from dsRNA replicative intermediates, rather than ssRNA secondary structures.

Table 2.6: Numbers of DENV2 viRNAs identified with NextGENe software

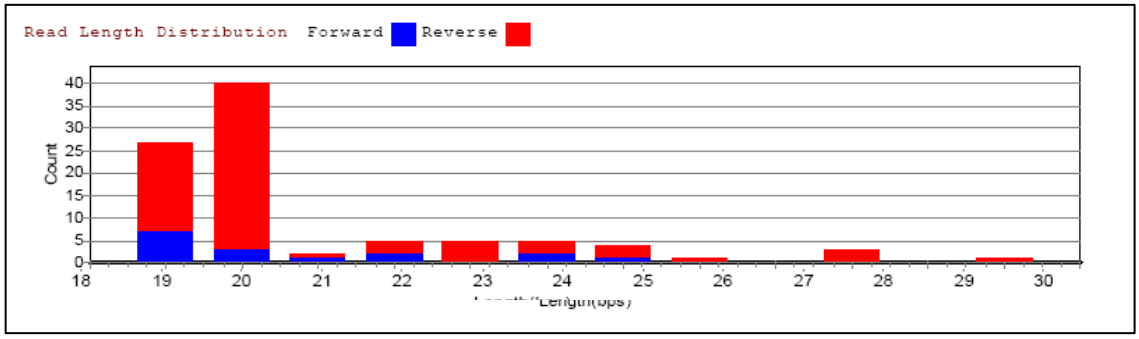
Sample (Total number of reads)	Number of DENV small RNAs (Percentage of total)	Percent from positive sense strand	Percent from negative sense strand
Aag2 Mock (14,087,714)	93 ($7x10^{-4}$ %)	17%	83%
Aag2 DENV Day 1 (12,615,439)	55 ($4x10^{-4}$ %)	41%	59%
Aag2 DENV Day 5 (12,131,018)	1612 (0.01%)	59%	41%
Mosquito Mock (7,687,058)	30 ($4x10^{-4}$ %)	60%	40%
Mosquito DENV2 Day 9 (12,267,708)	6029 (0.05%)	55%	45%

The predominant size of the DENV2-specific small RNAs in the DENV2-infected Aag2 sample (5 dpi), was 21 nts in length, which is the expected size for Dcr2 products, indicating that the exogenous-siRNA pathway was the most likely pathway used by the cell to target DENV2 dsRNA (Figure 2.6). The sizes in the mock- and DENV2-infected (1 dpi) Aag2 samples were smaller, indicating they may be due to non-specific matching in the alignment.

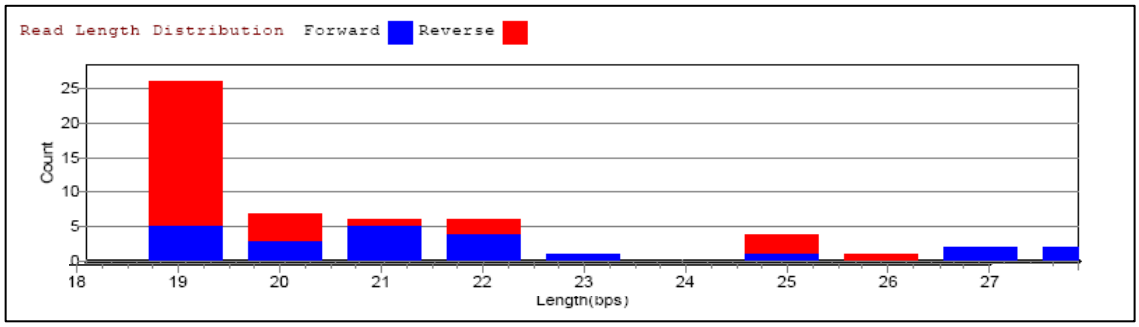
The distribution pattern of the DENV2-specific small RNAs in the DENV2-infected Aag2 sample (5 dpi) was across the entire DENV2 genome (Figure 2.7). There appeared to be one hot spot for cleavage from nt 10033-10061, located in the NS5 gene, although there are no specific secondary structures known for this region, and it is difficult to perform Mfold analysis (Zuker, 2003) on the large DENV2 genome. When the viRNA sequences are not allowed to 'overlap' and form 'shoulders', but instead are

counted only as one read matching their starting nucleotide, their distribution is very even across the genome (Figure 2.7), with only one read per starting nucleotide location, except at the hot spot in the NS5 gene. The even distribution of the viRNAs across the genome is another indication that long dsRNA replicative intermediates were the target of the Dcr2 cleavage.

A)



B)



C)

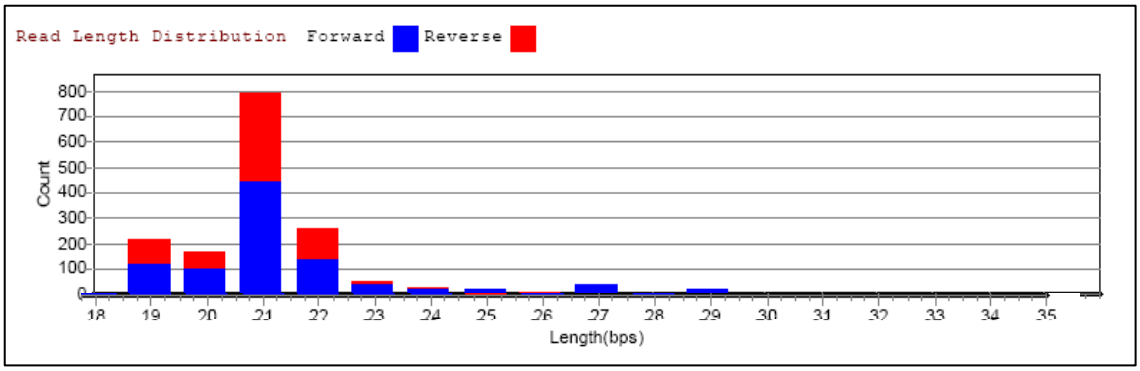


Figure 2.6: Size distribution of DENV2 viRNAs identified from NextGENe alignment from Aag2 cells Mock-DENV2-infected (A), DENV2-infected 1 dpi (B) and DENV2-infected 5 dpi. (C). Blue indicates the viRNA was from the positive strand and red indicates the viRNA was from the negative strand of the RNA. (Note different scales on x-axis)

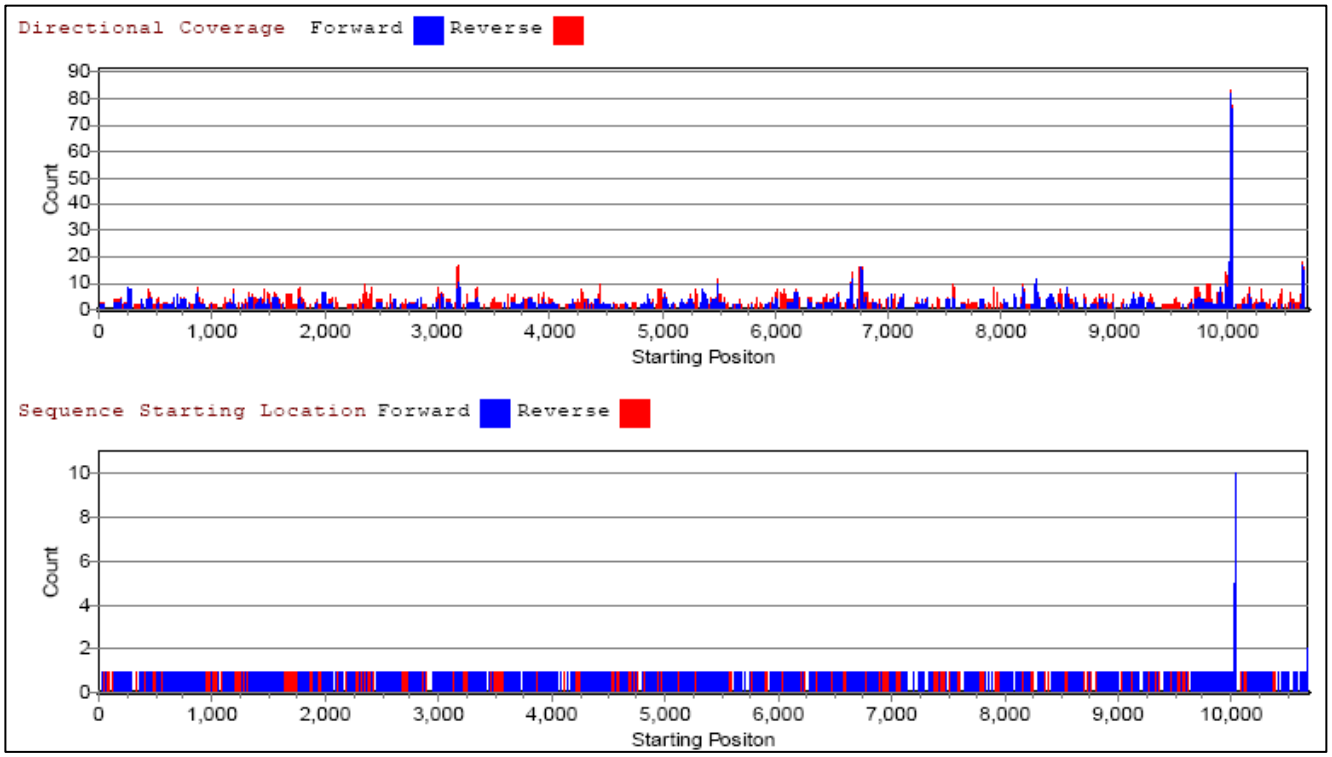


Figure 2.7: Location of DENV2 viRNAs on DENV2 genome in DENV2-infected Aag2 cells 5 dpi. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

The DENV2-specific small RNAs from DENV2-infected mosquitoes showed a similar size distribution when examined with the NextGENE software. The predominant size of the DENV2-specific small RNAs was 21 nt in length, the size expected from Dcr2 cleavage in the exogenous siRNA pathway (Figure 2.8).

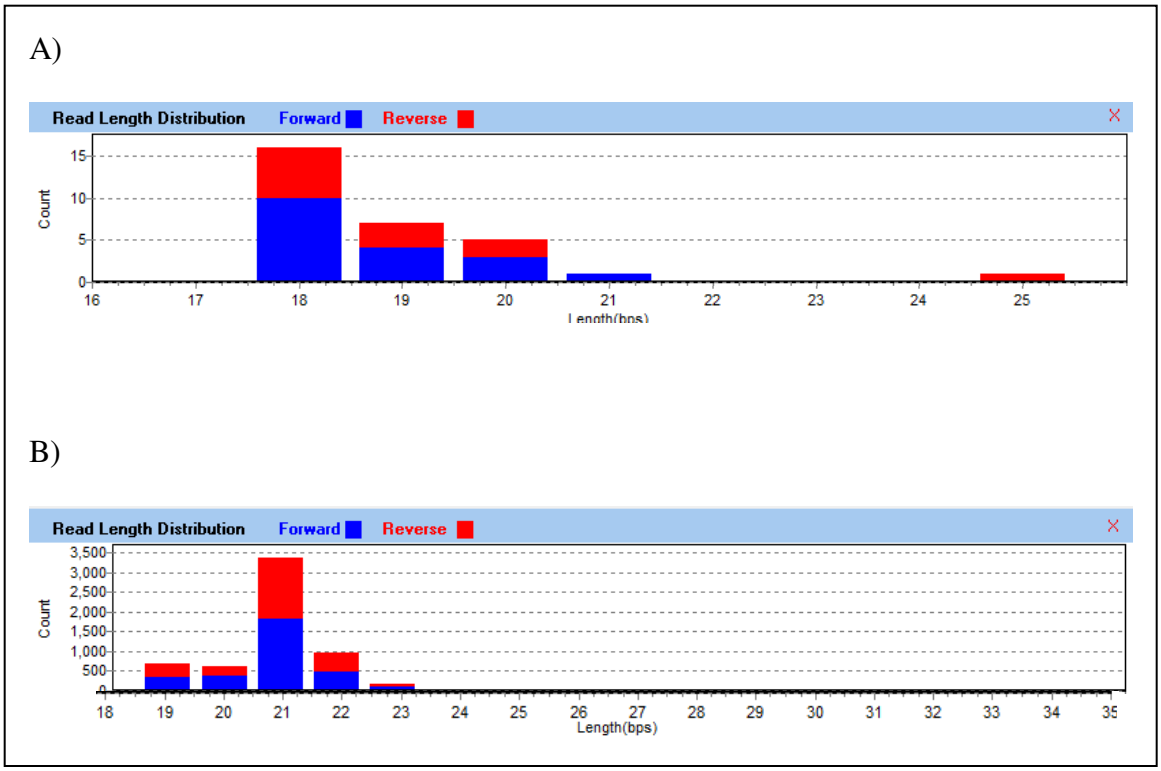


Figure 2.8: Size distribution of DENV2 viRNAs identified by NextGENe alignment from *A. aegypti* mosquitoes Mock-DENV2-infected (A), and DENV2-infected 9 dpi (B). Blue indicates the viRNA was from the positive strand and red indicates the viRNA was from the negative strand of the RNA. (Note scale differences)

The distribution of the DENV2 viRNAs from infected mosquitoes along the DENV2 genome was somewhat different than was seen in the DENV2-infected Aag2 cells (Figure 2.9). The DENV2 viRNAs were located in all regions of the gnome, but there seemed to be more 'hot spots' for cleavage, indicating that ssRNA secondary structures of the DENV2 genome may have been targeted more by RNAi in the mosquito than in cell culture.

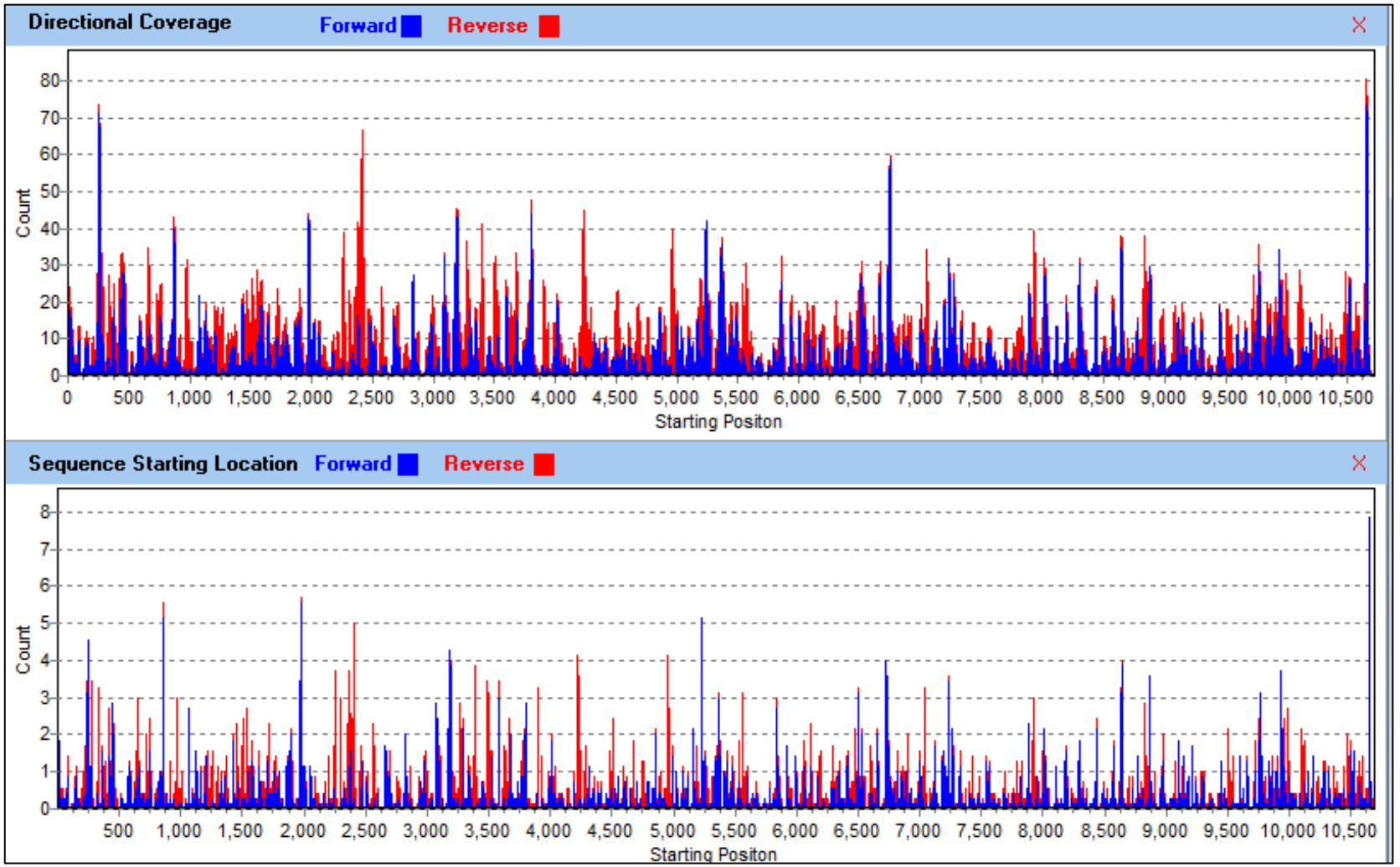


Figure 2.9: Location of DENV2 viRNAs on DENV2 genome in DENV2-infected *A. aegypti* mosquitoes 9 dpi. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

SOLiD pipeline analysis

The Aag2 cell small RNA libraries were also aligned to the DENV2 genome using the SOLiD small RNA analysis pipeline to compare to the results seen with the NextGENe alignment software. The SOLiD software sorts the counts by the number of mismatches, but does not give the size of the aligned small RNA and does not map the aligned sequences on the genome. With the SOLiD analysis there were less DENV2-specific small RNAs found in the mock- and DENV2-infected 1 dpi Aag2 samples. NextGENe had identified 93 potential DENV2 small RNAs in the mock-infected Aag2 samples and 55 potential DENV2 small RNAs in the DENV2-infected 1 dpi Aag2 samples, while the SOLiD software found 33 in the mock-infected and 17 in the DENV2-infected 1 dpi Aag2 samples (Table 2.7). More DENV2-specific small RNAs were found in the DENV2-infected 5 dpi sample using the SOLiD software pipeline (1,853 viRNAs) compared to the NextGENe software (1,612 viRNAs). When the SOLiD software allowed for one mismatch, the number of DENV2 viRNAs increased for all samples, up to 7863 hits in the Aag2 Mock sample. Since these viRNAs are so small, allowing for one mismatch may allow for many more non-specific alignments, as is probably the case in the mock-infected Aag2 samples.

The number of matches in the DENV2-infected mosquito sample was 3,880 (with no mismatches) with the SOLiD pipeline alignment, compared to 6,020 matches with the NextGENe alignment (Table 2.7). The differences are probably due to the alignment algorithms, but we are not clear at this point which numbers are more accurate.

Table 2.7: DENV2-specific small RNAs from SOLiD pipeline

	Total number of reads	Number of DENV2 viRNAs with zero mismatches
Aag2 Mock	14087714	33 (2 x 10^{-4}%)
Aag2 DENV2 Day 1	12615439	17 (1 x 10^{-4}%)
Aag2 DENV2 Day 5	12131018	1853 (0.02%)
Mosquito DENV2 Day 9	12,267,708	3880 (0.03%)

Changes in miRNA levels after DENV2 infection

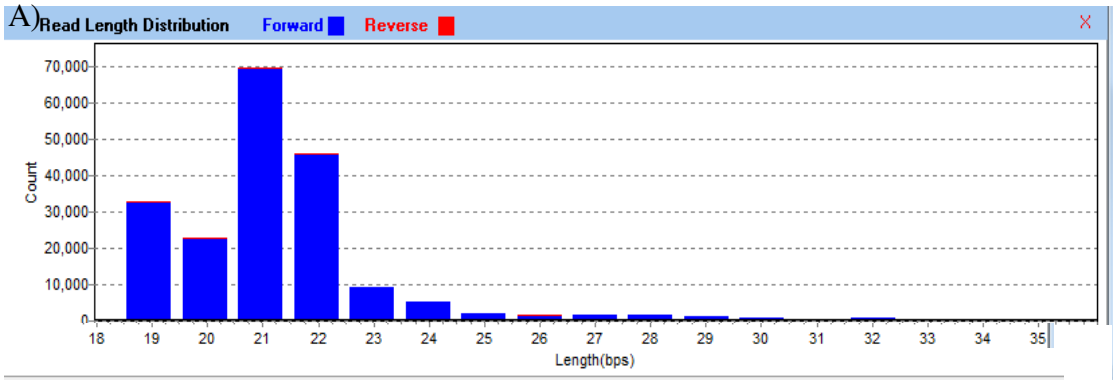
The levels of known miRNAs in the small RNA libraries were analyzed to see if changes occurred during infection of Aag2 cells. The percent of the total small RNA library made up of miRNAs increased with infection at both one day and 5 days post-DENV2 infection. The miRNA percentage in uninfected Aag2 cells was 1.4%, while the miRNA percentage increased to 2.3% in DENV2-infected cells at 5 days post-infection (Table 2.8). Although this is only one dataset, this increase may indicate an overall increase in miRNA gene regulation after infection.

Table 2.8: Numbers of potential miRNAs identified in Aag2 cells using the whole miRBase database as the reference sequence

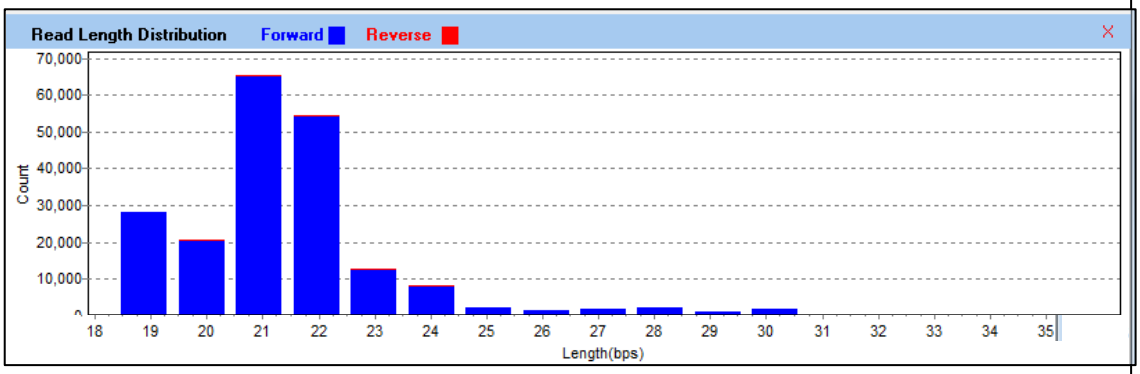
Sample	Mature miRNA hits	Percent of total reads
Aag2 Mock	196,252	1.4%
Aag2 DENV2 Day 1	202,106	1.6%
Aag2 DENV2 Day 5	276,018	2.3%

Interestingly, the most prominent miRNA size in all three samples was 21 nt, followed by 22 nt (Figure 2.10), while in *Drosophila*, most miRNAs are 22 nt in length. This may due to increased Dcr2 activity (which makes 21 nt small RNAs) in Aag2 cells, or is possibly due to loss of a base in the NextGENe alignment or trimming applications.

A)



B)



C)

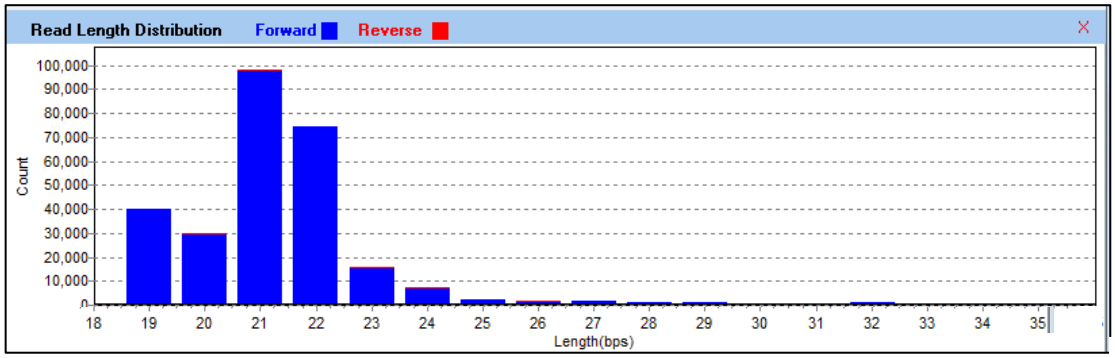


Figure 2.10: miRNAs read length distribution in Aag2 cells mock-infected (A), DENV2-infected 1 dpi (B), and DENV2-infected 5 dpi (C). Blue indicates the small RNA matches the positive sense of the reference sequence, red indicates the small RNA matches the negative sense of the reference sequence.

The miRNAs were analyzed to see which miRNA species increased with DENV2 infection over the uninfected cells (Figure 2.11). The miRNA counts were adjusted to compensate for the differences in the total small RNA library size and then compared to each other for miRNA increases over the uninfected cells at both one and five days post-DENV2 infection. Matches to both insect and non-insect miRNAs were found to increase with infection, including an *A. gambiae* miRNA and some *Drosophila* species miRNAs.

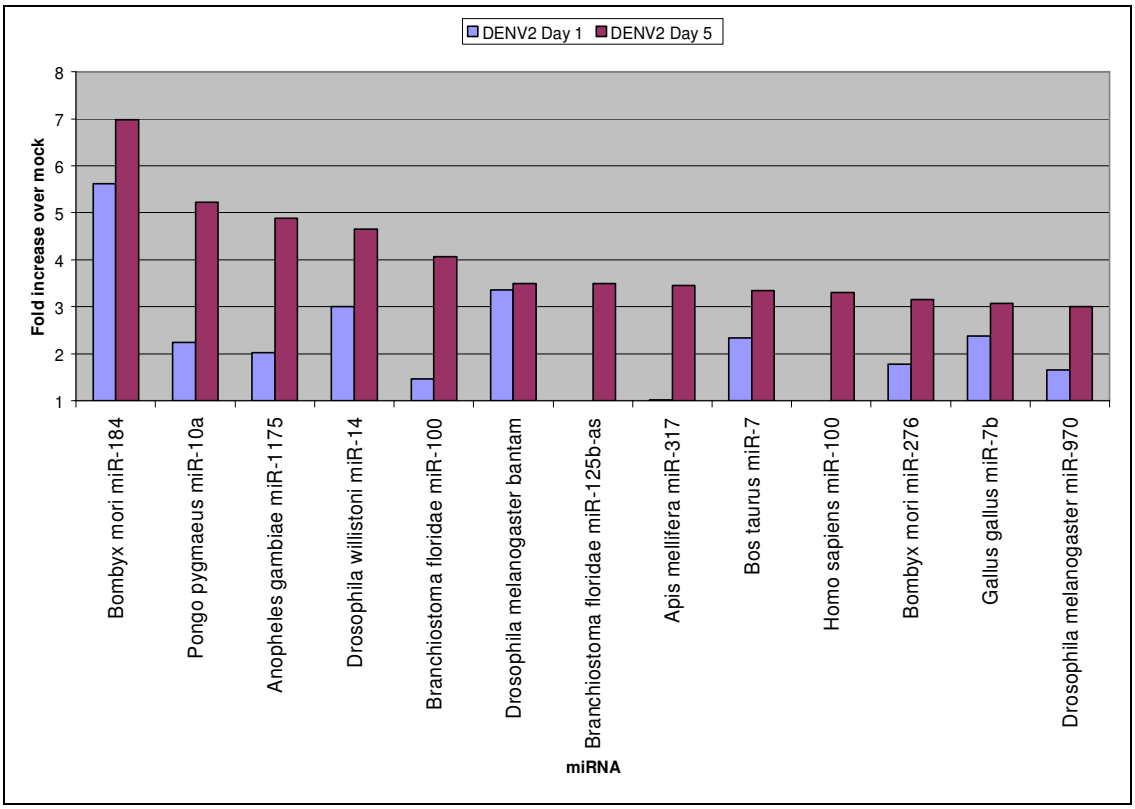


Figure 2.11: Fold-increase of Aag2 cell miRNAs over mock-infected Aag2 miRNA levels at one and five days after DENV2 infection. The blue bars indicate the fold increase in the 1 dpi sample and the red bars indicate the fold increase in the 5 dpi sample.

Comparison of Dcr2 and Ago2 small RNAs before and after DENV2 infection

The levels of small RNAs from the coding regions of Dcr2 and Ago2 genes were analyzed to see if any changes occurred in the small RNAs produced from these transcripts in response to DENV2 infection of Aag2 cells, as they are important in the mosquito antiviral response, and levels small RNAs derived from their sequences may be modulated to change the RNAi antiviral pathway gene expression levels in the cell.

In mock-infected Aag2 cells, there were 227 small RNA hits to the Ago2 mRNA, while in the DENV2-infected Aag2 cells (5 dpi), there were 226 small RNA hits. The small RNAs mostly matched the positive strand and the most common small RNA size in both samples was 27 nt (Figures 2.12 and 2.13). Most of the hits matched the 5' 1/3 of the gene, with the strongest peak between nt 600-800. This region does not contain a known functional domain (Campbell et al., 2008a, Campbell et al., 2008b), but has some secondary structure when analyzed with an online version of the M-fold software program (Zuker, 2003, Zuker & Markham, 1995-2009) (data not shown). There were few small RNAs that matched the 3' end of the mRNA, where most miRNAs would be expected to act. Since the small RNAs were mostly from the sense strand, and 27 nt was the most common length, this suggests a possible role for the piRNA pathway in generation of these small RNAs.

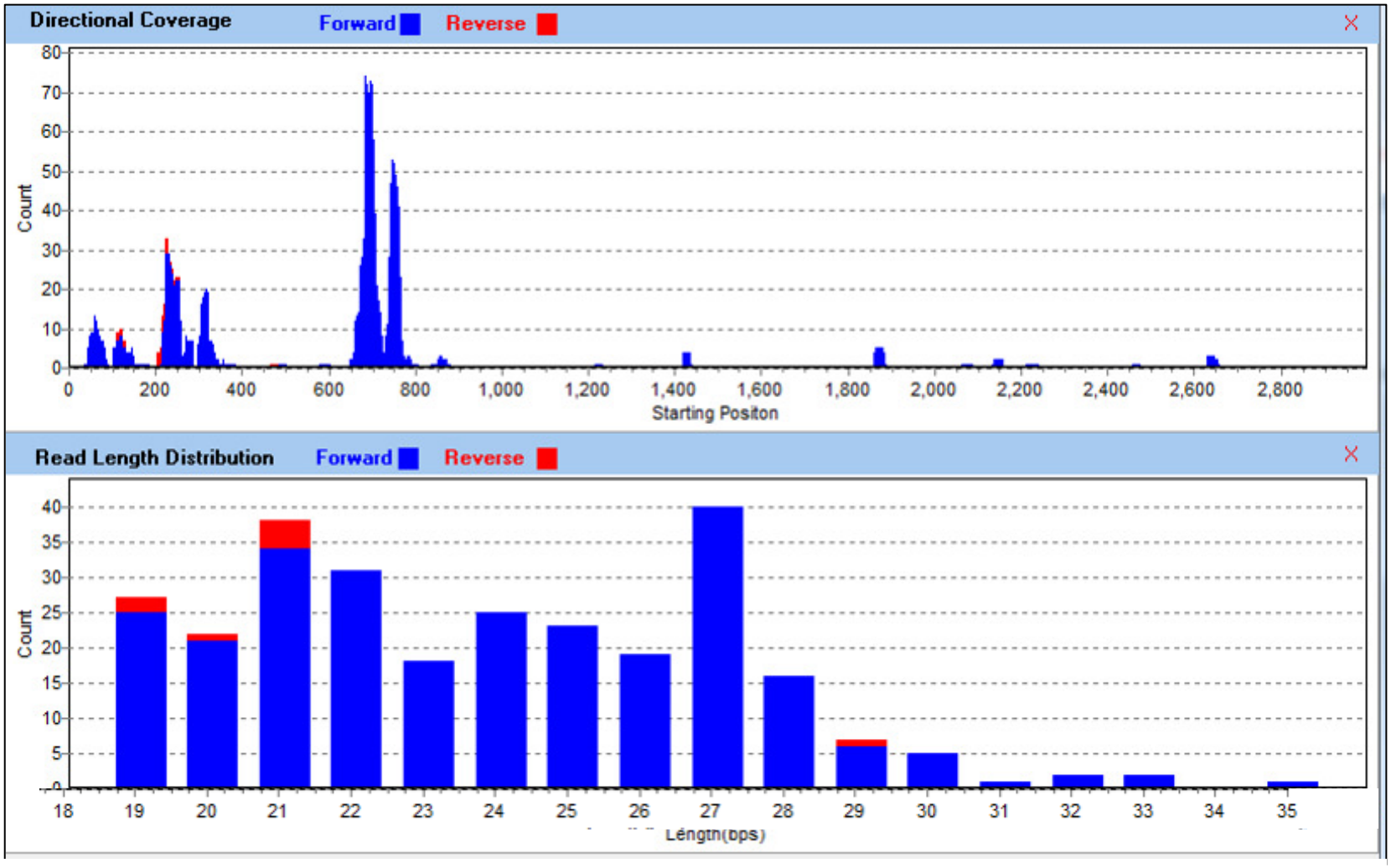


Figure 2.12: Ago2 mRNA-derived small RNAs from mock-infected Aag2 RNA sample. The top graph shows the small RNA size distrubtion across the gene sequence, and the bottom graph shows the size distribution of these small RNAs. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

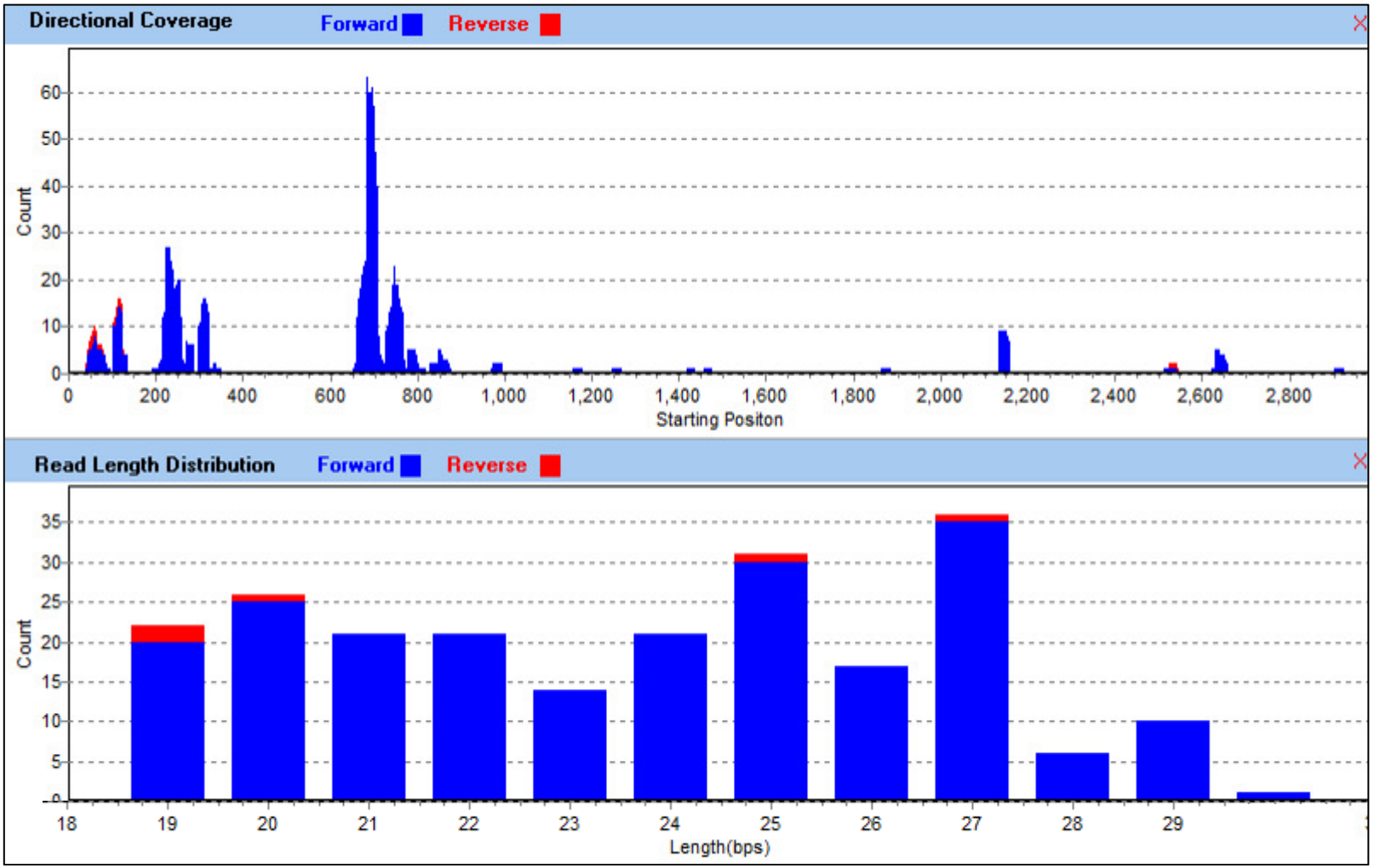


Figure 2.13: Ago2 mRNA-derived small RNAs from DENV2-infected Aag2 RNA sample (5 days post-infection). The top graph shows the small RNA size distrubtion across the gene sequence, and the bottom graph shows the size distribution of these small RNAs. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the

The small RNAs that matched Dcr2 mRNA had a similar pattern to the Ago2 mRNA-derived small RNAs. The Dcr2 small RNAs were also mostly from positive sense strand (Figures 2.14 and 2.15). There were 141 hits in the mock-infected Aag2 sample, and 97 hits in the DENV2-infected Aag2 (5 dpi) sample. There was a slight difference between the mock-infected and DENV2-infected samples, but the number of matches was so low it is difficult to make any conclusions on the control of this gene during infection. The pattern of positive sense small RNAs with a propensity toward a 27 nt length may suggest a piRNA-like mechanism for their generation.

Since neither the Dcr2 and Ago2 genes produce very many small RNAs, and their numbers do not appear to change dramatically during infection, it is unlikely that they have a large role in modulating the RNAi pathway gene expression. Their size and sense orientation suggest they are not made from a siRNA or miRNA pathway, but instead are possibly generated by a piRNA-like pathway. Similar analysis was performed with the *A. aegypti* R2D2 mRNA (sequence kindly provided by Dr. Alexander Franz), but there were very few small RNAs that matched perfectly, and the NextGENe program appeared to be forcing 'matches' even when the sequences were obviously not the same (data not shown).

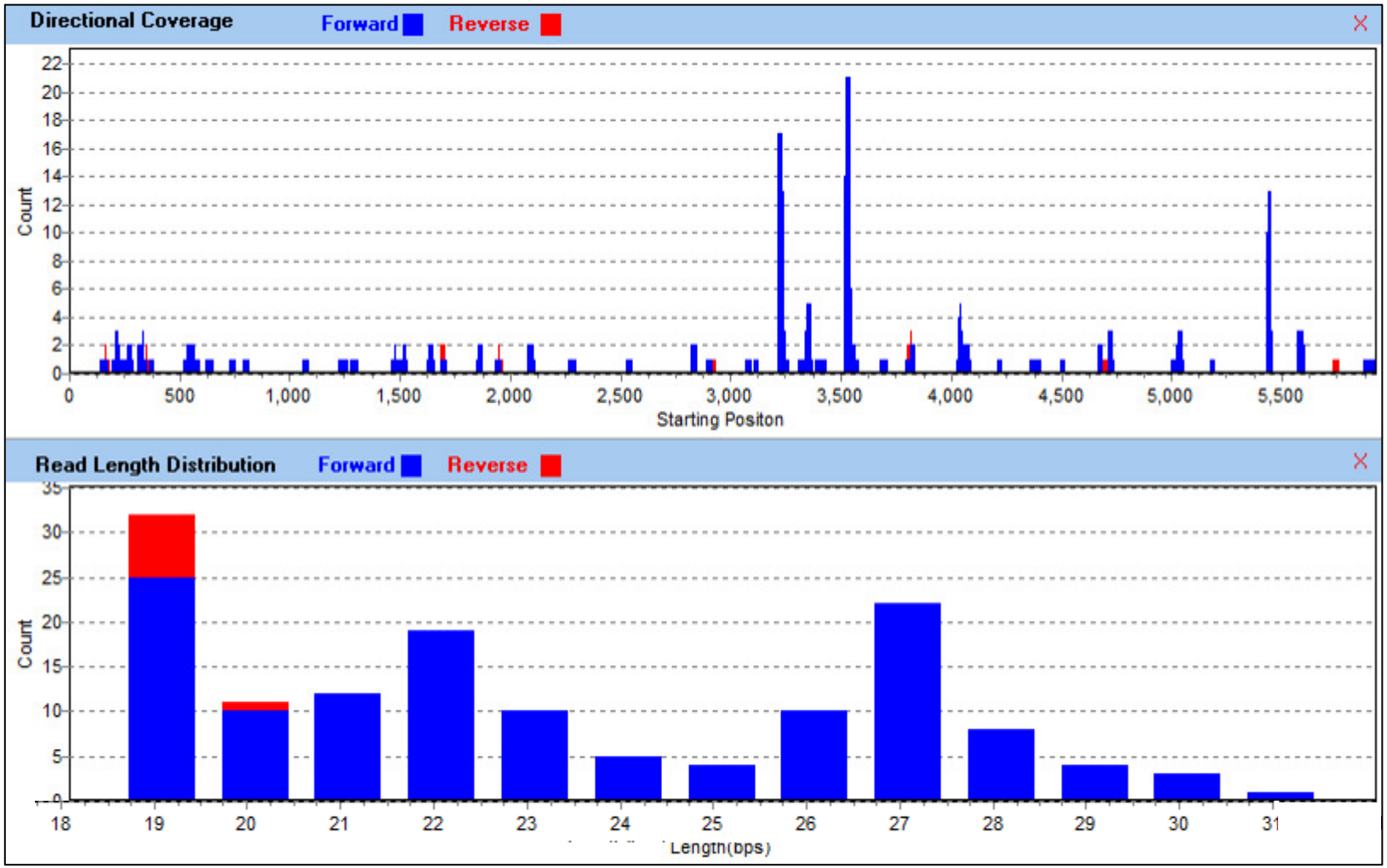


Figure 2.14: Dcr2 mRNA-derived small RNAs from mock-infected Aag2 RNA sample. The top graph shows the small RNA size distrubtion across the gene sequence, and the bottom graph shows the size distribution of these small RNAs. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

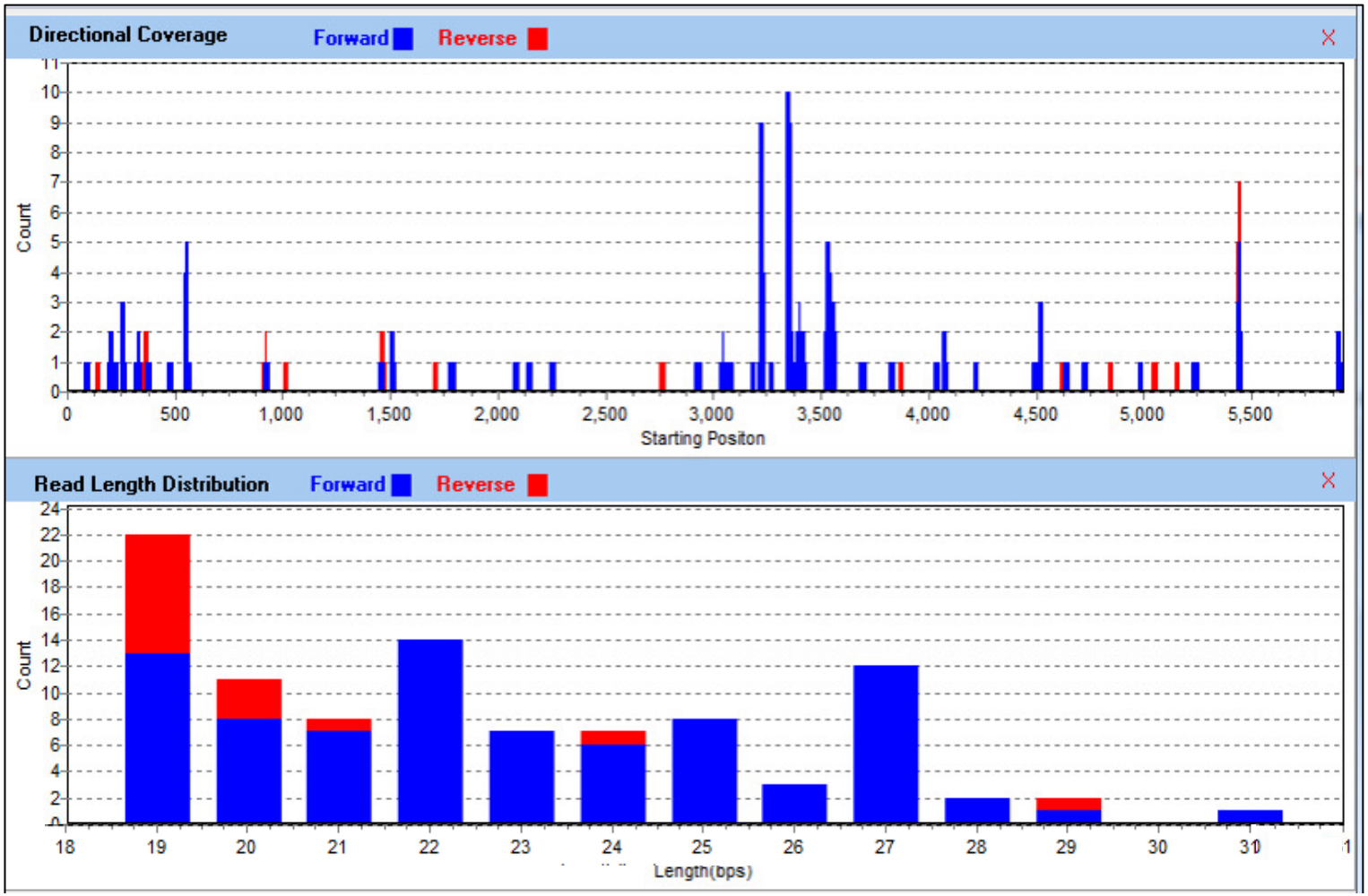


Figure 2.15: Dcr2 mRNA-derived small RNAs from DENV2-infected Aag2 RNA sample (5 days post-infection). The top graph shows the small RNA size distrubtion across the gene sequence, and the bottom graph shows the size distribution of these small RNAs. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

Other components of mock-infected Aag2 small RNA library

The small RNA libraries were also analyzed to determine the source of the other small RNAs in the libraries. Alignment to known mosquito genome and transcriptome data from the Vectorbase.org website, along with matching the small RNAs to the primers used in small RNA library kit and known mosquito rRNA and tRNA species, resulted in only 37% of the library matching any of these sequences (Table 2.9). Possibly the algorithm for matching used by NextGENe is too strict, the data in Vectorbase.org is incomplete, there are contaminating organisms in the cell culture (such as mycoplasma or fungi), there is contaminating RNA in the samples, or there are a lot of artifacts generated from the SOLiD sequencing.

Table 2.9: Alignment of small RNAs from mock-DENV2-infected Aag2 cells to primers and *A. aegypti* genome sequences

	Mock-infected Aag2 small RNA library	**% of total reads**
Primers	473,731	3.3%
Genome Supercontigs	3,395,977	24%
rRNA/tRNAs	22,911	0.16%
Transcriptome	1,048,270	7.4%
miRNAs	249,679	1.7%
Total reads accounted for:	5,190,568	37%
Total reads in library	14,087,714	

Discussion:

The purpose of this study was to examine how the antiviral RNAi immune response in mosquitoes interacts with the ssRNA virus, DENV2. There is little known about what the RNA trigger of the RNAi antiviral pathway in mosquitoes is, how many viral-specific siRNAs are made, how the miRNA pathway responds to infection and if small RNA pathways control RNAi genes during infection. Small RNA Northern

blotting, new methods for virus-specific small RNA isolation, along with next generation deep sequencing technologies were used to provide insight into these areas.

Since DENV2 makes a dsRNA replicative intermediate during its replication cycle (Stollar et al., 1967, Stollar & Stollar, 1970b), this would be the most obvious trigger for Dcr2 cleavage and activation of an RNAi response. Some analyses of small RNAs from cells infected with positive-strand ssRNA viruses have found that more siRNAs appear to come from the positive sense strand, indicating that secondary structure of the ssRNA genome alone may be a trigger for RNAi (Molnar et al., 2005). Results from small RNA northern blot hybridization for DENV2 small RNAs with DENV2-prM probes in sense and antisense orientations seemed to indicate that there were more small RNAs being made from the positive strand than the negative strand (Figure 2.2 and (Sanchez-Vargas et al., 2009). Further analysis using traditional small RNA sequencing and next generation deep sequencing seemed to show that the there were approximately 54-59% positive sense DENV2-small RNAs in DENV2-infected Aag2 cells, closer to the 1:1 ratio that one would expect if the trigger was a true double-stranded intermediate made of long strands of positive genomic RNA annealed to a long, complementary negative sense strand. The distribution pattern seen with the DENV2-small RNAs in Aag2 cells at 5 days post DENV2 infection is very even across the genome, also implicating a long dsRNA replicative intermediate as the main source of DENV2-specific small RNAs in the Aag2 mosquito cells. In DENV2-infected mosquitoes, this ratio was even closer to 1:1, with 55% of the DENV2-specific small RNAs being derivatives from the positive sense strand.

Previous studies of another flavivirus, WNV, in *Culex quinquefasciatus* mosquitoes found that approximately 74% of the virus-specific small RNAs were from the positive sense RNA strand (Brackney et al., 2009). These differences may be due to the virus itself and how it replicates in the cell, or possibly due to a different RNAi response in the *Culex* mosquito when compared to *A. aegypti*. Small RNA sequencing studies done with the positive sense ssRNA alphavirus SINV in *A. aegypti* mosquitoes found that 54% of the virus-specific small RNAs were from the positive sense strand of the virus RNA (Myles et al., 2008), a very similar proportion to what we found in the DENV2-infected *A. aegypti.* When the alphavirus ONNV was studied in *A. gambiae* mosquitoes, the proportion of positive sense virus-specific small RNAs was slightly higher at 64% (Myles et al., 2009). The difference seen in the alphaviruses examined in mosquitoes may be due to differences in the viral replication between SINV and ONNV or possibly due to different mosquito genera. In the studies of DENV2 and SINV infected *A. aegypti* mosquitoes, the ratios were quite similar.

The number of DENV2-specific small RNAs in our total RNA samples was very low. This was not very surprising as previous attempts to clone DENV2 small RNAs from mosquito cells yielded very few matches (Travanty, 2005). Deep sequencing analysis revealed that that less than 0.02% of the small RNAs in the DENV2-infected Aag2 cell (5 dpi) sample and less than 0.05% of the small RNAs in the DENV2-infected *A. aegypti* mosquito (9 dpi) were DENV2-specific. These results do not seem to be uncommon in flavivirus-infected mosquitoes, as *Culex* mosquitoes infected with WNV had less than 0.05% WNV-specific small RNAs in the total small RNA population at 7 days post-infection and 0.12% WNV-specific small RNAs at 14 days post-infection

(Brackney et al., 2009). This may be due to flavivirus replication occurring in membrane vesicles, that may prevent Dcr2 access to the dsRNA replicative intermediates (Uchil & Satchidanandam, 2003). Alphaviruses appear to make more virus-specific small RNAs. Approximately 10% of the 18-24 nt RNAs sequenced from SINV-infected *A. aegypti* mosquitoes were SINV-specific, and the percentage may be higher as only SINV small RNAs with no mismatches were counted (Myles et al., 2008). In ONNV-infected *A. gambiae* mosquitoes, the percentage was lower, with 1.2% of the total useable small RNA reads matching the ONNV genome (Myles et al., 2009). These higher percentages of alphavirus small RNAs when compared to flavivirus small RNAs are not that surprising, and may be due to differences in accessibility of the replicative intermediate RNA to RNAi machinery during viral replication, or possibly because of higher titers in alphavirus infected mosquitoes. These higher percentages of alphavirus viRNAs in the total small RNA population is consistent with the results I found with small RNA northern blot hybridization, where it was much easier to detect SINV-specific viRNAs in cell culture and mosquito samples than it was to detect DENV2-specific viRNAs (Cirimotich et al., 2009, Sanchez-Vargas et al., 2009). The SINV-specific viRNAs appear to play an important role in mosquito antiviral defense, as when SINVs were engineered to express the B2 suppressor of Dcr cleavage, the number of SINV-specific viRNAs was drastically reduced and mosquito mortality was increased, while there was little mortality in mosquitoes infected with the wild-type SINV (Cirimotich et al., 2009, Myles et al., 2008). If the RNAi response is knocked down in mosquitoes using dsRNA to Dcr2, Ago2 or R2D2, viral replication in mosquitoes increases and the EIP decreases, indicating that RNAi does play an antiviral role against DENV2 (Sanchez-Vargas et al.,

2009), but since the levels of DENV2 viRNAs are so low in infected cells and mosquitoes, it is unclear if the viRNAs themselves have an important role in the RNAi response. Possibly the Dcr2 cleavage of viral RNA alone helps to keep the DENV2 infection from overwhelming mosquito cells and causing pathology in the insect, as one cleavage event could destroy a viral RNA. In latent FHV infection of *Drosophila* cells, the majority of the FHV-specific small RNAs in the cell were not loaded into an Argonaute protein, and luciferase reporters with sequences that could be targeted by the viral RNAs were not repressed (Flynt et al., 2009). This suggests that the Dcr2 cleavage of the FHV replication intermediates alone may be important for forming a persistent infection of these cells (Flynt et al., 2009). A similar phenomenon could be occurring in mosquito cells persistently infected with arboviruses.

The DENV2 viRNAs in both the DENV2-infected Aag2 cells (5 dpi) and DENV2-infected *A. aegypti* mosquitoes had a predominant size of 21 nts and similar proportions of sense and antisense viRNAs, indicating that the RNAi pathway mechanism must be similar between the Aag2 cell line and whole *A. aegypti* mosquitoes. The mosquitoes had more viRNAs overall and as a larger proportion of the total small RNA population, which may be due to a stronger viral infection in the mosquito possibly because of a longer infection time or due to the presence of a mosquito-only flavivirus in the Aag2 cell line (to be discussed in Chapter 4) that may reduce DENV2 replication or possibly tie up the RNAi machinery, resulting in less viRNAs.

Levels of certain miRNAs increased in DENV2-infected Aag2 cells compared to uninfected cells. The percentage of known miRNAs in the total small RNA population also increased in the DENV2-infected samples, with 2.3% of the total small RNA

population from DENV2-infected Aag2 cells (5 dpi) consisting of miRNAs, while in the uninfected Aag2 had only 1.4% miRNAs in the population. Since both samples were taken at 5 days post infection (or mock-infection), this increase shouldn't be due to the age of the cell culture. The virus infection itself may trigger an increase in miRNA activity, or possibly the infection itself may upregulate all of the small RNA pathways. The majority of the miRNAs in all of the samples were 21 nt in length, with the second highest numbers at 22 nt in length. Typically miRNAs in insect cells are 22 nt in length, so possibly a different small RNA pathway may be involved in generating these 21 nt miRNA species, or the apparent size may be due to base loss in the sequencing technology or in the software alignment. The vast majority of the miRNAs are 'positive' sense, but this was expected as the reference genome from miRBase designated the miRNAs as 'positive' sense.

Some of miRNAs that increased most after DENV2 infection of Aag2 cells have functions related to the control of cellular growth. *Drosophila* mir-14 and bantam miRNAs have roles in the suppression of cell death (Brennecke et al., 2003, Xu et al., 2003). As these roles have been described in another insect species, they may have quite similar roles in the mosquito, and these anti-apoptotic roles would not be surprising during a DENV2 infection. These miRNAs may be upregulated to keep the cells from undergoing apoptosis that may be triggered from the viral infection. The functions of the other miRNAs that were upregulated are poorly understood, and many are from species evolutionarily very far from mosquitoes, so it is difficult to say that their functions may be similar in mosquitoes to those seen in the species where they were described. Also

this was only one 'replicate', and more samples would need to be analyzed to make any statistically significant conclusions.

A small RNA identical to *A. gambiae* mosquito miRNA, *A. gambiae* mir-1175 (aga-mir-1175), was found to be upregulated. An ortholog of the *A. gambiae* mir-1175 was identified in the *A. aegypti* genome, but was not found in *D. melanogaster*, suggesting a mosquito-only function, and its expression was found only in the midgut tissues of *A. gambiae* mosquitoes (Winter et al., 2007). Not much is known about its function in the cell, but it has been studied with regard to *Plasmodium* invasion. aga-mir-1175 levels decreased with *Plasmodium* infection of the mosquito compared to the levels of the miRNA in uninfected *A. gambiae* (Winter et al., 2007). It is interesting that this miRNA would increase with DENV2 viral infection, but decrease with *Plasmodium* infection, suggesting a viral immune response function.

The small RNAs that aligned with Ago2 and Dcr2 mRNAs were also examined to see if they changed in response to infection as a way to control their levels. There were no changes in the pattern or levels of the Ago2- or Dcr2-derived small RNAs in Aag2 cells between the uninfected and DENV2-infected (5 dpi) cells, thus it appears that a small RNA pathway does not control these mRNAs during DENV2 infection. There were few hits to either of the genes (<300) in the samples. The largest peak of small RNA matches to the Ago2 mRNA was between nt 600-800, and this peak did not change with infection. M-fold analysis revealed some longer hairpin-like structures in this area, which could possibly be a trigger for cleavage by a small RNA pathway.

The composition of the total small RNA population that was sequenced was also analyzed for the uninfected Aag2 cell sample. The small RNAs were compared to known

mosquito rRNAs and tRNAs, the *A. aegypti* genome supercontigs and transcriptome from Vectorbase, and SOLiD primers used in making the libraries. Surprisingly, only 37% of the library could be accounted for using these reference sequences. Possibly the sample may have had some cell culture contaminants, the alignments were too strict, or the Vectorbase sequences were incomplete, possibly not including repetitive regions of the genome that could account for many small RNA species.

Overall the DENV2 small RNA sequencing studies gave us more insight into how the virus interacts with the mosquito antiviral immune response. It appears that not very many small RNAs are made during DENV2 infection, giving more support for the idea that the replication complexes formed during infection may be hiding the dsRNA from the RNAi machinery. It appears that the virus-specific small RNAs are made from all regions of the genome, with few hot spots in cell culture, indicating that the majority of the small RNAs may come from long dsRNA replicative intermediates, rather than ssRNA viral genomes in Aag2 cells. The low levels of DENV2 small RNAs are consistent with the low levels small RNAs in mosquito infections with the flavivirus WNV (Brackney et al., 2009), and seem to be much lower than the levels seen in alphavirus infected mosquitoes (Myles et al., 2009, Myles et al., 2008). This may be due to differences in how the two different virus families replicate in mosquito cells, with regard to access of RNAi to viral dsRNA, location of viral replication and levels of viral replication. It appears that mosquitoes may be more dependent on RNAi machinery to keep the alphaviruses in a state of persistent infection than flaviviruses, since the alphaviruses make so many more virus-specific small RNAs and it has been shown that blocking Dcr2 activity with the B2 inhibitor of RNAi will lead to mosquito death from

alphaviral infection (Cirimotich et al., 2009, Myles et al., 2008). Whether introducing a potent inhibitor of RNAi during flavivirus infection would lead to mosquito death has not been studied.

It is also unclear if the DENV2 small RNAs are being loaded into the RISC or are at a high enough concentration in the cell to have a biological relevance. Further studies need to be done to determine that the viRNAs are genuine Dcr2 products and if they are being loaded into RISC. More research into the molecular interactions of arboviruses with the antiviral RNAi machinery will give us better understanding of the mechanisms of persistent arboviral infections of mosquitoes, and how we might block these persistent infections or use the pathway against the virus or against the mosquito itself to reduce the transmission of arboviruses to people.

CHAPTER 3

COMPARISON OF RNA INTERFERENCE ACTIVITY IN TWO MOSQUITO CELL LINES

Introduction:

Mosquito cell cultures have been used routinely in arbovirology studies to grow viruses and to elucidate aspects of viral infection and replication in mosquitoes. Many of these cell lines were established by Peleg and Singh in the 1960's (Peleg, 1968, Singh, 1967). One of the most commonly used mosquito cell lines, known as C6/36, is a clone of Singh's *Aedes albopictus* larval line selected by Igarashi for its ability to grow dengue and chikungunya viruses to high titers (Igarashi, 1978). This cell line has been used for many arbovirology studies. (Reigel, 1980, Sasao et al., 1980, White, 1987). Since *Aedes aegypti* is the most important vector for arboviruses such as dengue, another cell line, derived from *A. aegypti* embryos and known as Aag2, has been used in several recent studies (Cirimotich et al., 2009, Sanchez-Vargas et al., 2009). This cell line was originally established by Peleg in 1968 and was further characterized by Lan and Fallon in 1990 (Lan & Fallon, 1990).

RNA interference (RNAi) has been shown to play an important role in insect antiviral immunity (Galiana-Arnoux et al., 2006, van Rij et al., 2006, Wang et al., 2006). RNAi is a molecular pathway that is triggered by long exogenous double-stranded RNA (dsRNA) in the cell. Much of what we know about RNAi in insects has been elucidated in *Drosophila* flies and cultured cells. Dicer-2 (Dcr2) is an RNase III that recognizes and cleaves the dsRNA into small interfering RNAs (siRNAs) to initiate the RNAi pathway. The siRNAs are usually 21 bp in length with 5' phosphates and two nt overhangs on the 3' hydroxyl ends (Bernstein et al., 2001, Elbashir et al., 2001a, Elbashir et al., 2001b, Nykanen et al., 2001, Zamore et al., 2000). siRNAs, in association with Dcr2 and the protein R2D2, are loaded into a multi-protein RNA-induced silencing complex (RISC),

which contains the endonuclease Argonaute-2 (Ago2) (Liu et al., 2003, Okamura et al., 2004, Rand et al., 2004). The RISC unwinds and degrades one of the siRNA strands, and uses the other strand to guide RISC to long single-stranded RNA (ssRNA) complementary to the siRNA. Ago2 cleaves the long ssRNA at the point of complementarity, leading to its further destruction (Miyoshi et al., 2005, Schwarz et al., 2002, Schwarz et al., 2004).

Other small RNA pathways have been discovered in *Drosophila* including the Piwi-interacting (piRNA) and endogenous siRNA (endo-siRNA) pathways (Aravin et al., 2001, Chung et al., 2008, Ghildiyal et al., 2008, Okamura et al., 2008, Saito et al., 2006, Shpiz et al., 2009, Vagin et al., 2006). These pathways are believed to have important roles in controlling the transcription of transposable elements in the genome and also in development of the reproductive tissues.

piRNAs bind to the members of the Piwi clade of the Argonaute proteins, which include Piwi, Aubergine (Aub) and Argonaute 3 (Ago3) in *Drosophila*. piRNAs are approximately 24-30 nt in length and are modified by DmHEN1 (also known as Pimet) to have 2'-O-methylation on their 3' terminus (Horwich et al., 2007, Saito et al., 2007). The piRNA trigger appears to be single-stranded RNA since the small RNAs are almost always of the same sense, and the biogenesis is Dcr1 and Dcr2 independent, possibly using the Slicer activity of the Piwi proteins, at least in determining their 5' ends (Gunawardane et al., 2007, Nishida et al., 2007, Saito et al., 2006, Vagin et al., 2006).

Genomic analyses show that mosquitoes have orthologous RNAi pathway components including Dcr2, Ago2, and R2D2 as well as paralogous microRNA pathway (miRNA) pathway components Drosha, Pasha, Dicer-1 (Dcr1), Argonaute-1 (Ago1) and

Loquacious (Loqs; R3D1) (Campbell et al., 2008a). We recently showed that injection of *A. aegypti* mosquitoes with dsRNA derived from *A. aegypti* Dcr2 or R2D2 mRNA followed by oral challenge with DENV2 two days later resulted in increased virus titers in whole mosquitoes compared to non-injected or unrelated dsRNA (β-gal)- injected mosquitoes (Sanchez-Vargas et al., 2009), indicating a role for Dcr2 in the mosquito antiviral response. DENV2-related siRNA-like RNAs (viRNAs) were also detected in DENV2-infected *A. aegypti* and Aag2 cells in this study (Sanchez-Vargas et al., 2009). Small RNAs derived from West Nile virus (WNV) RNA were not detectable in WNV-infected C6/36 cells (Chotkowski et al., 2008), but WNV-derived viRNAs were detected in WNV-infected *Culex* mosquitoes via deep sequencing (Brackney et al., 2009).

RNAi has also been shown to be an important antiviral pathway against Sindbis virus (SINV) in *A. aegypti*. When dsRNA derived from *A. aegypti* Ago2 or Dcr2 mRNA was co-injected into *A. aegypti* mosquitoes with SINV TR339-eGFP, there were increases in detectable viral RNA, infectious virus titers and infection rates of mosquitoes (Campbell et al., 2008b). When SINV were engineered to express the B2 protein, an inhibitor of RNAi, the viruses replicated to higher titers in mosquitoes and C6/36 mosquito cell cultures, and caused cytopathic effects in cell cultures and mortality in mosquitoes, indicating the importance of the RNAi pathway in maintaining persistent, non-pathogenic arboviral infections of the mosquito host (Cirimotich et al., 2009, Myles et al., 2008). RNAi as an antiviral defense was also demonstrated in *Anopheles gambiae* mosquitoes, in which injection of dsRNA to knock-down expression of Ago2 resulted in increased replication and dissemination of another alphavirus, O'nyong-nyong virus (ONNV) (Keene et al., 2004).

Studies in our lab examining the *in vitro* dicing activity of the C6/36 cell line indicated that it did not seem to cleave long dsRNA into siRNAs (Figure 3.5), and SINV-specific small RNAs were difficult to detect in C6/36 cells (data not shown). Another lab studing WNV infection of C6/36 cells was unable to detect WNV-specific small RNAs in the C6/36 cell line (Chotkowski et al., 2008). The findings were unexpected when compared to the results seen in the Aag2 cell line, so we decided to analyze the DENV2-specifc small RNAs made during DENV2 infection of C6/36 cells using next generation sequencing technologies.

In this study we describe DENV2-specific small RNAs made during infection of C6/36 mosquito cells, and present evidence that C6/36 cells appear to have an aberrant antiviral RNAi pathway.

Materials and Methods:

Cells and Medium

A. aegypti cells (Aag2) were grown and maintained as described in Chapter 2. *A. albopictus* cells (C6/36) were grown in Leibovitz's L-15 medium (Mediatech), modified from manufacturer with L-glutamine, supplemented with 10% heat inactivated (30 minutes at 55°C) fetal bovine serum (FBS) (Colorado Serum), 2 mM L-glutamine (Mediatech) and 100 units penicillin and 0.1 mg streptomycin (Sigma) at 28°C in closed flasks without CO_2 supplementation.

Viruses and Infection

DEN type 2 strain Jamaica 1409 highly passaged virus was used in infections of Aag2 and C6/36 cells at a multiplicity of infection (MOI) of 0.1. Infections were done in the same medium as the cells were grown in, but the FBS concentration was lowered to 2%, and non-essential amino acids were added. The concentrations of L-glutamine and penicillin/streptomycin remained the same as in the growth medium. Cells were approximately 90-100% confluent at the time of infection. Before infection, medium was removed from the cells and a few ml of the 2% FBS (maintenance) medium was added to the cell monolayer along with the correct volume of virus to get the desired MOI. The flask was rocked gently at room temperature for one hour, maintenance medium was added to reach a final volume of 12 mL in a 75 cm^2 flask, and the flask was incubated at 28°C until samples were harvested. The day of infection was considered day zero in the timecourse.

RNA Extraction

Total RNA was extracted from cells and mosquitoes using TRIZOL reagent (Invitrogen) and quantified using spectrophotometry. Small RNAs were isolated from total RNA using FlashPAGE columns and FlashPAGE apparatus (Applied Biosystems) running at 75 volts for 12 minutes. The RNA was precipitated with linear acrylamide (Applied Biosystems) and ammonium acetate in ethanol overnight at -20°C.

Sequencing by oligonucleotide ligation and detection (SOLiD) sequencing

Small RNA libraries were made using the SOLiD Small RNA Expression Kit (Applied Biosystems), November 2008 edition. Ligation reactions were set up with approximately 200 nanograms (ng) of FlashPAGE fractionated small RNAs per sample using Adaptor Mix "A". After a reverse transcription and RNaseH treatment, approximately 20 50 µl PCR reactions were set up per sample with 1 µl of cDNA used per 50 µl reaction along with 15 cycles of PCR amplification.

Table 3.1: SOLiD PCR Primer Sets for C6/36 samples (See Appendix for primer sequences and Chapter 2, Table 2.1 for Aag2 primer sets)

Sample	SOLiD PCR Primer Set
C6/36 Mock	3
C6/36 DENV2 Day 5	4

DNA libraries made from the SOLiD small RNA expression kit were sent to the University of Washington (UW) for sequencing using the Applied Biosystems SOLiD 2 sequencing instrument, with each sample running in its own octet (1/8) of a slide.

viRNA Sequencing Analysis

Potential viRNAs were aligned to DENV2 genome using NextGENe (Softgenetics, LLC, State College, PA) software running the transcriptome assembly function with the same parameters listed in Table 2.2 (Chapter 2). CSFASTA (color-space) files from SOLiD sequencing of samples from UW were used as the sample file and a FASTA file of the DENV2 Jamaica 1409 strain from Genbank accession number M20558.1 was used as the reference sequence.

viRNAs were also aligned to the DENV2 genome using the ABI SOLiD Small RNA Analysis Pipeline Tool v.0.5.0, and the software was set up and run by Dr. Richard Casey at Colorado State University. The primers and adapters were filtered out, the miRBase matching step was turned off and the genome matching step used the accession number M20558 DENV2 sequences from GenBank as the reference.

Logo Analysis

Logo analysis was performed using WebLogo 3 located at http://weblogo.threeplusone.com in March, 2010 (Crooks et al., 2004, Schneider & Stephens, 1990). Reads that matched DENV2 and CFAV viral genomes were identified with NextGENe alignment, converted to base-space with NextGENe, and used in the WebLogo. The full length (35 bp) of the matched read was used to allow comparison of all of the viRNAs at once as all reads must be the same length when analyzed with the WebLogo program. The program default settings were used, except the Y-axis scale was set to 1 bit.

In vitro Transcription

dsRNA was prepared by *in vitro* transcription of a 498 bp region of the *Escherichia coli* beta-galactosidase (β-gal) gene from a PCR product with T7 RNA polymerase promoters on both strands with approximately 9% of the UTP conjugated to biotin (Applied Biosystems, Foster City, CA) using the MEGAscript T7 Kit (Applied Biosystems) for approximately 16 hours at 37°C. The reaction was treated with Turbo DNase (Applied Biosystems) for 30 minutes, followed by a phenol/chloroform extraction

and an overnight ethanol precipitation. The RNA was purified on a 6% polyacrylamide TBE-Urea gel (Invitrogen, Carlsbad, CA) and eluted overnight at room temperature in gel elution buffer. RNA was extracted using phenol/chloroform (5:1), followed by a chloroform/isoamyl alcohol (24:1) extraction, and precipitated overnight at -20°C in ethanol. The RNA was quantified by spectrophotometry.

Non-biotinylated Dcr2 dsRNA was made from *A. aegypti* and *A. albopictus* Dcr2 nt1211-1715 sequences from using the MEGAscript T7 transcription kit (Applied Biosystems) and agarose gel-extracted RT-PCR products amplified from Aag2 or C6/36 cells as templates. Non-biotinylated dsRNA from β-gal PCR amplicons was made as in the previous paragraph, without using biotinylated UTP. 20 µl reactions were set up according to manufacturer's protocols, incubating at 37°C for 16 hours. Reactions were treated with TurboDNase following the manufacturer's instructions, and RNA was purified with a phenol/chloroform extraction, followed by a chloroform/isoamyl extraction and ammonium acetate/ethanol overnight precipitation at -20°C.

Biotinylated antisense ssRNA probes were transcribed from the *A. aegypti* or *A. albopictus* Dcr2 nt 4919-5116 PCR product made with a T7 RNA polymerase promoter on the 3' end of the PCR primer using the MEGAscript T7 Kit (Applied Biosystems) with approximately 9% of the UTP conjugated to biotin (Applied Biosystems) for approximately 16 hours at 37°C. The reaction was treated with TurboDNase (Applied Biosystems) for 30 minutes, followed by a phenol/chloroform extraction and an overnight ammonium acetate/ethanol precipitation.

In vitro Dicing Assay

Cell-free lysates were generated from Aag2 cells and C6/36 cells using a protocol similar to (Haley et al., 2003). Briefly, cells are washed in PBS three times, and then resuspended in 1X lysis buffer with protease inhibitors and 5mM DTT. The cells are disrupted in a Dounce homogenizer, and then centrifuged at 14,000xg for 25 minutes at 4°C. The supernatant is flash frozen in a dry ice/ethanol bath and stored at -80°C. Protein concentrations were determined with the DC Protein Assay (Bio-Rad) and samples were equilibrated to the same protein concentration using lysis buffer immediately before the dicing assay was set up. Dicing activity reactions were set up according to Haley, et al. (2003) and contained 1/2 volume lysate, 1/3 volume 40X reaction mix and approximately 70 nanograms (for two timepoint experiments) or 300 nanograms (for 6 timepoint experiments) of 498 bp biotinylated β-gal dsRNA, with the lysate being added last. Reactions were incubated at 28°C in a thermocycler block with a 28°C lid. At each timepoint, 10 microliters (µl) of the reaction was removed, added to 2X PK buffer and flash frozen. RNA was extracted with phenol/chloroform (5:1), followed by a chloroform/isoamyl alcohol (24:1) extraction, and precipitated overnight at -20°C in ethanol. RNA was electrophoresed on a 20% polyacrylamide TBE non-denaturing gel (Invitrogen, Carlsbad, CA), stained with ethidium bromide, electrophoretically transferred to a positively charged nylon BrightStar-Plus membrane (Applied Biosystems) and UV-crosslinked (2 runs of the autocrosslink function of Stratagene UV Stratalinker 2400 crosslinker) to the membrane. Biotinylated RNA was detected with the BrightStar BioDetect Kit (Applied Biosystems) and exposed to autoradiography film. In some reactions, 1 µl (0.5 units) of human recombinant dicer

enzyme (Genlantis Inc., San Diego, CA) was added to the 10 µl reaction just before addition of the lysate.

Plasmid Construction

These experiments were designed and performed by Dr. Doug Brackney (at the University of New Mexico). The enhanced green fluorescent protein (EGFP) gene was amplified from the pEGFP-1 plasmid (Clontech, Mountain View, CA) using the forward primer EGFP-*Nco* I F and reverse primer EGFP-*Xho* I R. The amplicon was digested with *Nco* I and *Xho* I and cloned into the insect specific expression plasmid pIEx (Novagen, Madison, WI) to generate our pIEx-EGFP vector.

Small interfering RNAs (siRNA) and double stranded RNAs (dsRNA) production

These experiments were designed and performed by Dr. Doug Brackney (at the University of New Mexico). The synthetic Accell EGFP siRNA was used in these experiments (Dharmacon, Lafayette, CO) and the control WNV siRNA was synthetically produced by Dharmacon and was complementary to a 21 nt region of the WNV genome starting at position 85 in the capsid gene.

Approximately 500 bp DNA fragments corresponding to EGFP or the WNV capsid genes were amplified using primers that included a T7 RNA polymerase promoter sequence in both the forward and reverse primers. The amplicons were PCR purified and subsequently used as templates for dsRNA transcription. Synthesis of dsRNA molecules was carried out using the T7 MEGAscript kit (Applied Biosystems) as described above

with omission of biotinylated UTP. The dsRNA was re-suspended in 50 µl of PBS, quantified and brought to a final concentration of 1 µg/µl.

Transfection Conditions

These experiments were designed and performed by Dr. Doug Brackney (at the University of New Mexico). The day prior to transfection, Aag2 or C6/36 cells were seeded in 24-well tissue culture plates at a density of 5 x 10^5 cells/well. For the transfections, 250 ng/well of the pIEx-EGFP plasmid were combined with either EGFP or WNV siRNA (to a final concentration of 50 nM), or 1 µg/ well of EGFP or WNV dsRNA in Opti-MEM medium. Subsequently, the Attractene Transfection Reagent (Qiagen, Valencia, CA) was added and lipid-nucleic acid complexes were allowed to form for 15 min. at room temperature. The medium on the cells was discarded and 440 µl of Opti-MEM were added to each well followed by dropwise addition of 60 µl of the complexes. The cells remained in the presence of the transfection reagent for four hours, after which appropriate medium for each cell line was replaced. Cell viability was monitored for 48 hours post transfection, when cell images were acquired and the cells harvested. The cell pellets were re-suspended in 500 µl Trizol (Invitrogen) and total protein for immunoblots precipitated according the manufacturer's instructions.

Microscopy

These experiments were designed and performed by Dr. Doug Brackney (at the University of New Mexico). Images were acquired using a Nikon TE2000 inverted microscope with a Hamamatsu Orca camera and Wasabi software (Hamamatsu

Photonics, Japan). Representative areas as determined by cell density were photographed under 10X magnification. Fluorescent images were acquired using a 222 ms exposure without gain and the light images were acquired using a 30 ms exposure without gain. The monochrome images were subsequently pseudo-colored using the Slidebook software (Intelligent Imaging Innovations, Denver, CO).

Immunoblots

These experiments were designed and performed by Dr. Doug Brackney, Virginie Bondu-Hawkins, and Dr. Brian Hjelle (at the University of New Mexico). Total protein recovered from transfected cell cultures was quantified using the Bradford Kit on the Bio-Rad SmarSpec Plus spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA). Fifteen micrograms of total protein were separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. The presence of EGFP was detected using a primary mouse anti-*Aequorea victoria* EGFP monoclonal antibody (Clontech) at a dilution of 1:1000 in TBST + 5% non-fat dry milk. The blot was subsequently probed with phosphatase labeled goat anti-mouse IgG at a 1:1000 dilution (KPL Inc., Gaithersburg, MD). Detection of actin was performed with primary rabbit polyclonal antibodies at a 1:1000 dilution in TBST + 5% BSA (Abcam, Cambridge, MA) and phosphatase labeled goat anti-rabbit IgG secondary antibody at a 1:1000 dilution (KPL Inc.). Membranes were developed with the 1-Step NBT/ BCIP reagent for 5-10 minutes at room temperature (Pierce, Rockford, IL).

dsRNA soaking into cells

The dsRNA soaking into Aag2 cells protocol was generously provided by Dr. Rollie Clem at Kansas State University, and the medium was modified from Schneider's Drosophila medium to L-15 for C6/36 cells. Aag2 and C6/36 cells were plated into 6-well plates and when they reached approximately 80% confluency, medium was removed and replaced with 1 ml/well of incomplete medium lacking FBS. This medium was removed immediately and replaced with 1 ml fresh incomplete medium followed by 20 µg/well of non-biotinylated Dcr2 dsRNA (nt 1211-1715) to 5 wells (*A. aegypti* Dcr2 dsRNA was added to Aag2 cells and *A. albopictus* dsRNA was added to C6/36 cells). Non-biotinylated β-gal dsRNA was added to the last well of both the Aag2 and C6/36 plates as a control. Plates were briefly rotated vigorously to spread the dsRNA, were wrapped in parafilm and incubated at 28°C for 5 hours. 1 ml of medium with 20% FBS was added to the wells. RNA was extracted from cells in one Dcr2 dsRNA-treated well on days 0, 1, 2, 3 and 5 after addition of dsRNA. RNA was extracted from cells in the β-gal dsRNA-treated well on day 5 after dsRNA addition. To extract RNA, the cells were scraped into the 2 mls of medium, transferred to a microcentrifuge tube and centrifuged at 3,500 rpm for 3 minutes at room temperature. Supernatant was removed and cell pellet was resuspended in 1 mL of Trizol reagent (Invitrogen). The tube was incubated at room temperature for 5 minutes and placed at -80°C until the end of the timecourse. All RNA was then extracted at the same time using the Trizol (Invitrogen) manufacturer's instructions and quantified via UV spectrophotometry.

Dicer-2 Northern Blotting

5 µg of total RNA from the dsRNA soaking experiments was heated at 95°C for 5 minutes, then placed on ice, then loaded onto a 1.25% agarose denaturing formaldehyde gel, and electrophoresed in a MOPS/formaldehyde buffer. The RNA was then passively transferred from the gel overnight to a BrightStar-Plus positively charged nylon membrane (Applied Biosystems) with 10X SSC buffer. The membrane was autocrosslinked two times and pre-hybridized in 5 mL of UltraHyb Hybridization Buffer (Applied Biosystems) for 1 hour at 68°C. Biotinylated Dcr2 antisense ssRNA probes (nt 4919-5116) were added to the hybridization buffer to a final concentration of 0.1 nM of probe (approximately 80 ng of probe in 5 ml buffer). *A. aegypti* Dcr2 probe was added to the membrane with the Aag2 RNA bound, and *A. albopictus* Dcr2 probe was added to the membrane with C6/36 RNA bound and the hybridizations took place in separate tubes. Membranes and probes hybridized for 18 hours at 68°C. Membranes were then washed twice for 30 minutes in 2X SSC, 0.1% SDS buffer and twice for 60 minutes in 0.1X SSC, 0.1% SDS buffer. All washes were done at 68°C and *A. aegypti* and *A. albopictus* membranes remained in separate tubes. The biotinylated probes attached to the membrane were then detected with the BrightStar BioDetect Kit (Applied Biosystems), following manufacturer's instructions, with all washes performed for maximum recommended times. The membranes were then exposed to autoradiography film for various times and the film was developed in an automatic autoradiography developer machine.

Dicer-2 sequencing

dcr2 cDNA was sequenced from Aag2 cells using *A. aegypti* primers designed by Scott Bernhardt. The initial cDNA was generated with reverse transcription reactions with an OligodT primer and the Superscript II Reverse Transcriptase (Invitrogen) following manufacturer's instructions. Additional PCR reactions were carried out with the *A. aegypti* primers. The PCR products were sent to the Proteomics and Metabolomics Facility at Colorado State University for sequencing. Amplification of dcr2 cDNA from C6/36 cells was attempted with the *A. aegypti* primer set was and most primers sets did not generate products, but the cDNAs that were amplified were sent for sequencing. Some degenerate primers were designed using conserved areas of dcr2 sequences from *A. aegypti* and *A. gambiae* mosquito genomes, but very few of these generated products in PCR reactions with the C6/36 cDNA. Sequences were assembled into contigs and alignments were compared between the two species using VectorNTI Version 10 and Version 11 software (Invitrogen).

A. aegypti mosquito (GenBank Accession number AY713296) Aag2, and C6/36 dcr2 sequences were compared for their percent sequence identity using the EMBOSS pairwise alignment algorithm program located at http://www.ebi.ac.uk/Tools/emboss/align/index.html using the settings: EMBOSS:needle (global), Matrix: EBLOSUM62, open gap penalty of 10.0 and a gap extension penalty of 0.5 (Rice et al., 2000). The sequences were also compared for their percent sequence identity using BLASTN (Altschul et al., 1997) located at http://blast.ncbi.nlm.nih.gov/Blast.cgi with the settings: Max target sequences of 100, Expect threshold of 10, Word size of 16, Match score of 2, Mismatch score of -3, Gap

costs of Existence 5 and Extension 2, filtering for low complexity regions and masking for lookup table only.

miRNA Sequencing Analysis

miRNAs in the small RNA libraries were identified using the alignment function of NextGENe, using the same parameters as Table 2.2 (Chapter 2) except the matching base percentage was set to 50, the sequence reads <1 kb turned on, and the mutation percentage set to 0.2. The sample files were the CSFASTA files from the SOLiD sequencing for each sample and the reference genome was the complete mature miRNAs from the miRBase website (Ambros et al., 2003, Griffiths-Jones, 2004, Griffiths-Jones et al., 2006, Griffiths-Jones et al., 2008, mirbase.org) downloaded on May 14, 2009 (miRBase Release 13), converted to a .fa file format (with the U's converted to T's) by Kevin LaVan at Softgenetics. This reference file is annotated, allowing for identification and counts for each of the various miRNAs in the database. The alignments for miRNAs that were found to increase the most with infection were manually checked for matching with the NextGENe Sequence Alignment viewer to make sure the matches did not overlap miRNA annotations, and those that were not direct matches to a specific miRNA were then not used in the graph.

Results:

DENV2 small RNAs from SOLiD sequencing analyzed with NextGENe

DENV2-specific small RNAs were examined in DENV2-infected C6/36 cells to see if this cell line had different levels of RNAi activity when compared to Aag2 cells.

Many more DENV2-specific small RNAs (24,938 from over 12 x 10^6 reads) were found in the C6/36 cells at 5 dpi compared to Aag2 cells (1,612), possibly related to the ability of the virus to grow to higher titers in these cells than in Aag2 cells (Table 3.2). In C6/36 cells, DENV2-specific small RNAs were 95% positive sense, suggesting that they were derived from ssRNA, and were not generated by Dcr2 cleavage as in *A. aegypti* cell cultures and mosquitoes, where the DENV2-specific small RNAs from Aag2 cells at 5 days post DENV2-infection were 59% positive (genome) sense, and the small RNAs from DENV2-infected mosquitoes were 55% positive sense (Table 3.2). The most common size of DENV2 small RNAs in the C6/36 cell library was 27 nt, which is not expected from the exogenous siRNA pathway (Figure 3.1B). Furthermore, the few 21 nt DENV2-derived RNAs in the C6/36 cell library were predominantly positive sense, unlike the more nearly equal sense to antisense ratio found in the Aag2 cell library. The DENV2-specific small RNAs in C6/36 cells were not equally distributed along the genome as was seen in DENV2-infected Aag2 cells, but instead were derived from a few specific regions of the genome, which might represent intrastrand secondary structure in the positive-strand virus genome (Figures 3.2 and 3.3).

Table 3.2: viRNAs from C6/36 and Aag2 cell and *A. aegypti* mosquito samples aligned to DENV2 RNA with NextGENe

Sample (Total number of reads)	Number of DENV small RNAs (Percentage of total)	Percent from positive sense strand	Percent from negative sense strand
C6/36 Uninfected (11,915,311)	57 (5x10^{-4} %)	61%	39%
C6/36 DENV2 Day 5 (12,558,261)	24938 (0.2%)	96%	4%
Aag2 Mock (14,087,714)	93 (7x10^{-4} %)	17%	83%

Aag2 DENV Day 1 (12,615,439)	55 (4x10^{-4} %)	41%	59%
Aag2 DENV Day 5 (12,131,018)	1612 (0.01%)	59%	41%
Mosquito Mock (7,687,058)	30 (4x10-4 %)	60%	40%
Mosquito DENV2 Day 9 (12,267,708)	6029 (0.05%)	55%	45%

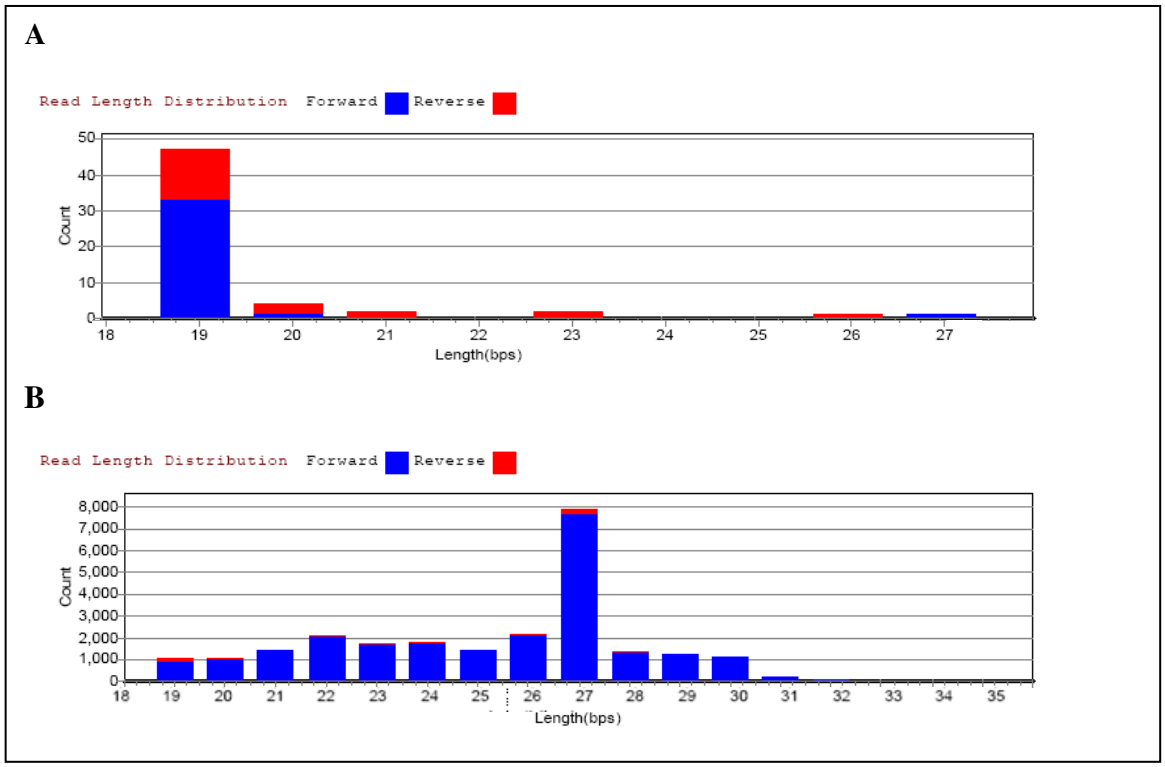


Figure 3.1: Size distribution of DENV2 viRNAs identified from NextGENe alignment from Mock-infected (A) and DENV2-infected (5 dpi) (B) C6/36 cells. Blue indicates the viRNA was from the positive strand and red indicates the viRNA was from the negative strand of the virus RNA.

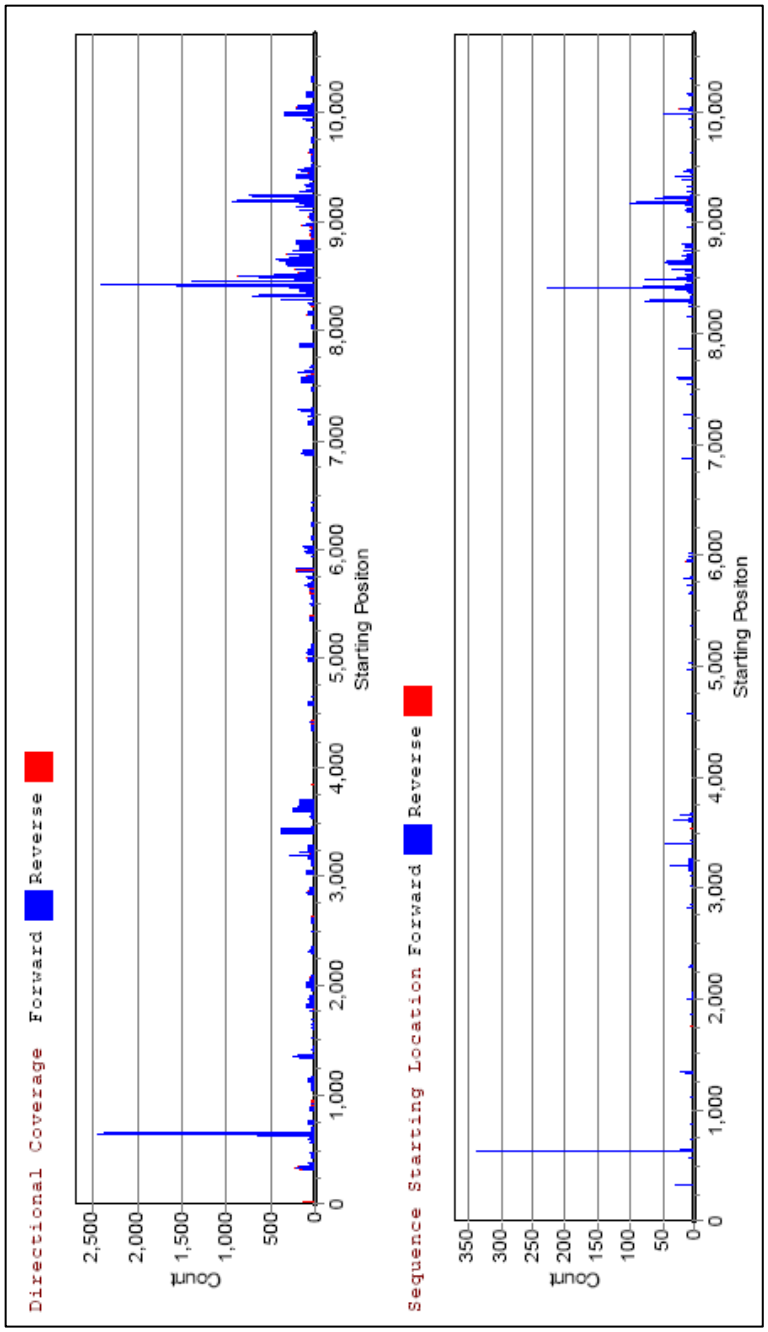


Figure 3.2: Location of viRNAs on DENV2 genome from DENV2-infected C6/36 cells. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

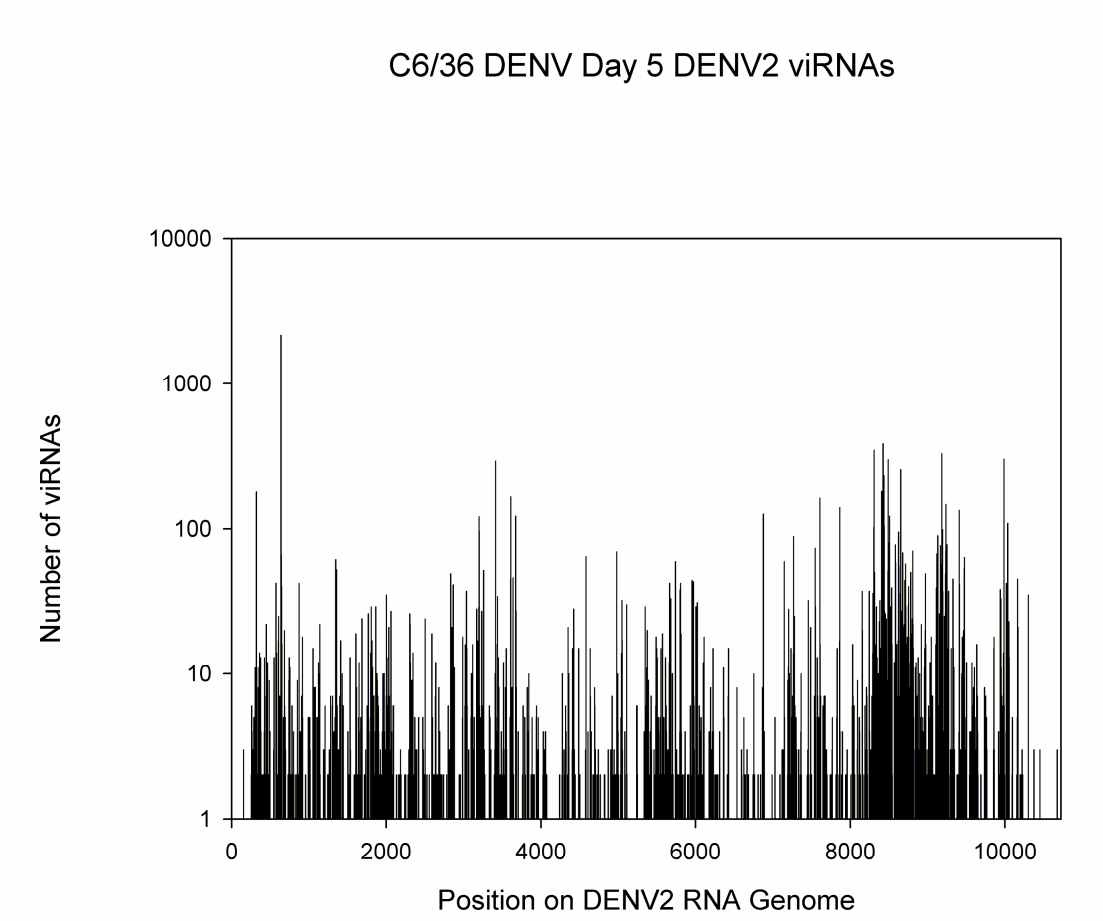


Figure 3.3 Distribution of DENV2 viRNAs from C6/36 infected with DENV2 (5 dpi) graphed on a log scale to better visualize the smaller peaks.

SOLiD pipeline analysis

The SOLiD small RNA software pipeline was used to analyze DENV2 viRNAs from C6/36 cells to compare the results seen with the NextGENe software. The SOLiD software pipeline analysis results were very similar to the NextGENe software analysis. There were fewer DENV2 small RNAs with zero mismatches found in the uninfected cells (18 with SOLiD vs. 57 with NextGENe) and approximately 900 fewer DENV2 viRNAs with zero mismatches in DENV2-infected cells found in with SOLiD software analysis (Table 3.3). These differences are most likely due to differences in the softwares' matching algorithms. When the SOLiD small RNA software was allowed up

to one mismatch in the viRNAs, the total more than doubled in the C6/36 DENV2 Day 5 sample. This could be due to the presence of virus genome quasispecies in the cells, which would have some differences in sequences when compared to the reference sequence and may also be due to allowing for one mismatch in these short sequence allows for more non-specific matching to the genome to occur (which is probably why the numbers of DENV2 viRNAs with one mismatch also increased in the C6/36 Mock sample).

Table 3.3: DENV2 viRNAs identified from C6/36 samples with SOLiD pipeline analysis

	Total number of reads	**Number of DENV2 viRNAs with zero mismatches**	**Number of DENV2 viRNAs with one mismatch**
C6/36 Mock	11,915,311	18 (1×10^{-4}%)	409 (3×10^{-3}%)
C6/36 DENV2 Day 5	12,558,261	24027 (0.19%)	50705 (0.40%)

Logo Analysis

The DENV2-small RNAs from both cell types were analyzed with the WebLogo 3 program (http://weblogo.threeplusone.com) to determine if there were preferences for specific nucleotides at certain positions. The total untrimmed 35 nt length of virus RNA-matching reads was analyzed in the program; therefore, the sequences for the six 3'-terminal nucleotides match the linker attached to the small RNAs in preparation of libraries. In the DENV2 viRNAs from Aag2 cells or *A. aegypti* mosquitoes, there were no apparent preferences for specific nucleotides at any positions in the 5' 21 nt (Figure 3.4 and 3.4B). However, in the C6/36 cell libraries, there appeared to be a bias for

adenine on the nucleotide at position 10 in the DENV2-specific small RNAs (Figure 3.4C). Ago3-associated Piwi-interacting RNAs (piRNAs) often have an adenine at the 10th position, hinting at a possible mechanism for generation of these small RNAs in C6/36 cells (Brennecke et al., 2007, Gunawardane et al., 2007).

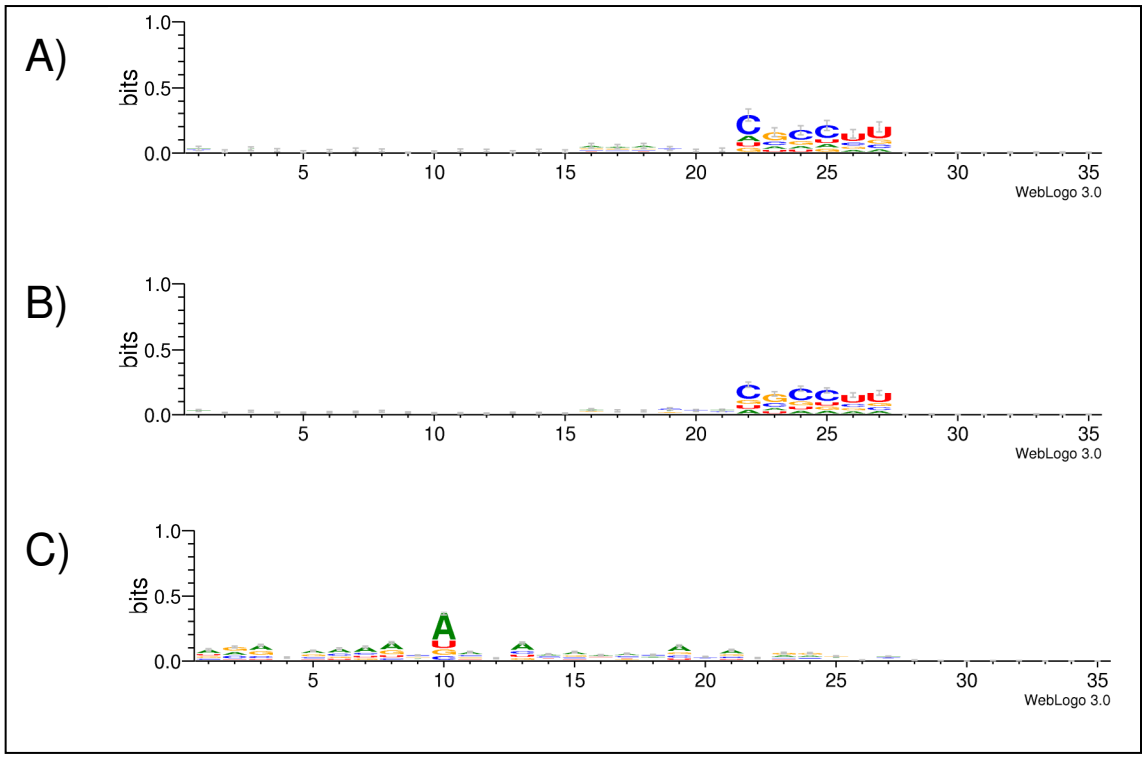


Figure 3.4. Logo analysis of DENV2 viRNA from mosquitoes and cell culture and CFAV viRNAs from cell culture. Logo analysis was performed on the viral viRNAs using WebLogo 3. (A) Aag2 DENV2 5 dpi viRNA logo. (B) *A. aegypti* DENV2 9 dpi viRNA logo. (C) C6/36 DENV2 5 dpi viRNA logo.

In vitro dicing assay with both cell lines

The dicing acivity of the cell lines was examined in the preliminary stages of this project to develop a way to study changes in Dcr2 activity in cells after arbovirus infection. Later this assay was used to determine if the differences seen in the virus-specific small RNA populations in the Aag2 and C6/36 cell lines was due to differences in Dcr2 activity. An *in vitro* dicing activity assay for cultured mosquito cells based on

similar methods used to prepare *Drosophila* cell lysates (Haley et al., 2003) was developed. This assay was used to compare the ability of cell lysates from Aag2 and C6/36 cells to cleave a long exogenous dsRNA into 21 bp small RNAs, indicative of Dcr2 activity. Aag2 cell lysates produced the appropriate size product (matching the recombinant human dicer control product) within 18 hr after 500 bp long dsRNA was added to the lysate. C6/36 cell lysates did not make a 21 bp small RNA product after overnight incubation (the slight band seen in the C6/36 overnight timepoint lane is spillover from the human recombinant Dcr reaction lane to the right) (Figure 3.5). There was a faint band in the 2 hour timepoint in the C6/36 cells, but it is not well defined and had mostly disappeared after 4 hours, whereas the 21 bp product appears after 4 hours in the Aag2 cells and continues to accumulate more at each timepoint thereafter (Figure 3.5). The bands larger than 21 nt also are degraded faster in all timepoints (except zero) in the C6/36 lysate samples when compared to the Aag2 lysate samples, suggesting there may be more RNase activity in the C6/36 cells. The C6/36 cell lysates also produce distinct bands between 100 and 200 nt in length, which are not seen (or are masked) in the Aag2 lanes. There may be some ssRNA in the β-gal dsRNA preparation that is being targeted by an RNase in the C6/36 cell lysate (Figure 3.5).

These lysate experiments were repeated to include mixing of the two lysates and introduction of human recombinant Dcr into the lysates to show that there are not agents in the C6/36 lysate that inhibit the Dcr2 reaction. When the C6/36 and Aag2 lysates were combined in equal protein concentrations and the labeled β-gal dsRNA was added, a Dcr2 product still appeared in this mixed lysate reaction, although somewhat less than in the Aag2 lysate alone (Figure 3.6A). When human recombinant dicer was added to the

lysates, a siRNA-like product was made in the C6/36 cell lysate, indicating that the C6/36 lysate lacks endogenous Dcr2 activity, but does not inhibit exogenously provided enzyme (Figure 3.6B).

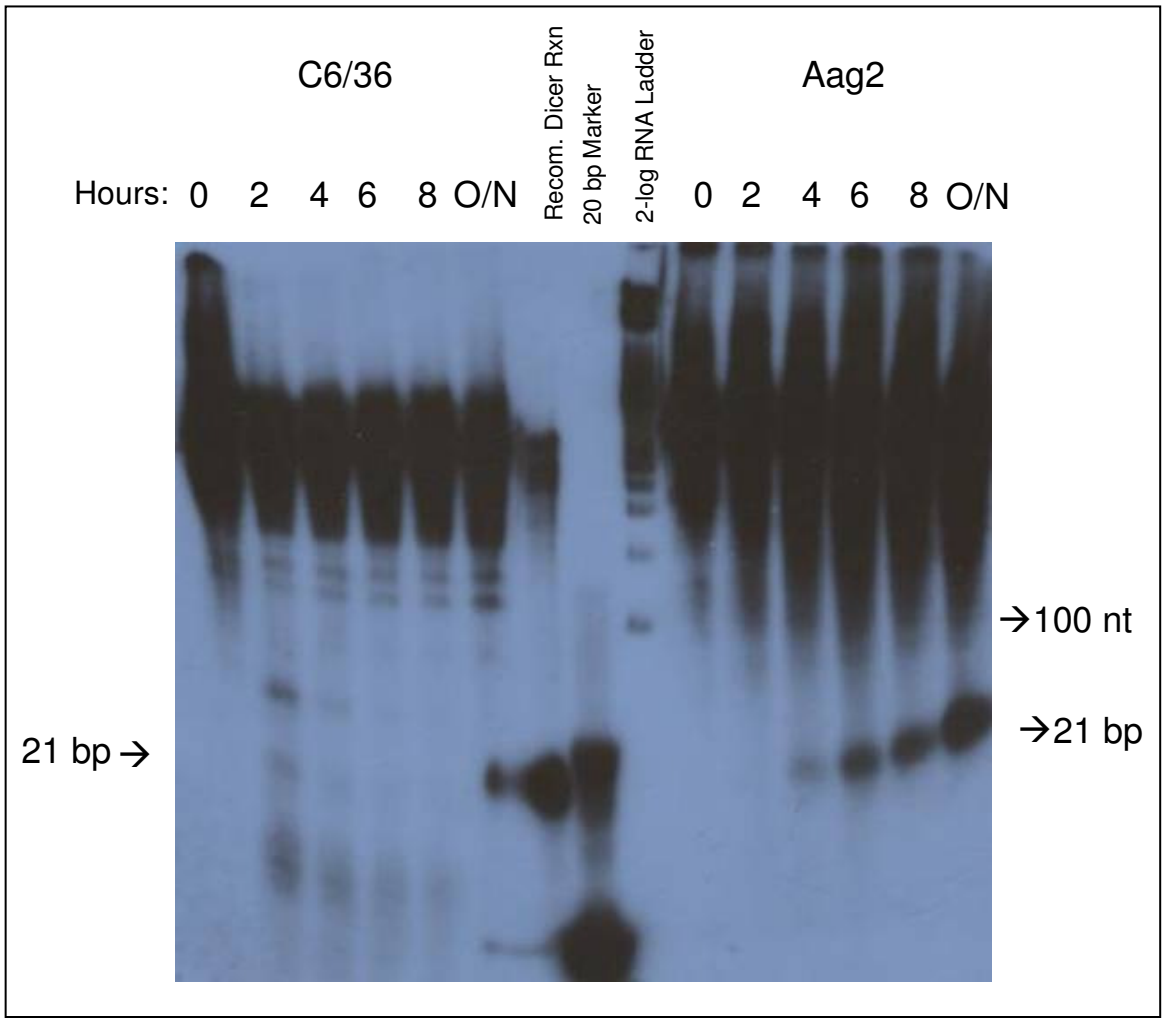


Figure 3.5: Comparison of dicing activity in C6/36 and Aag2 lysates over time after the addition of labeled dsRNA. Time is listed in hours after addition of dsRNA. A recombinant Dicer reaction is used as a size marker for 21 bp RNAs.

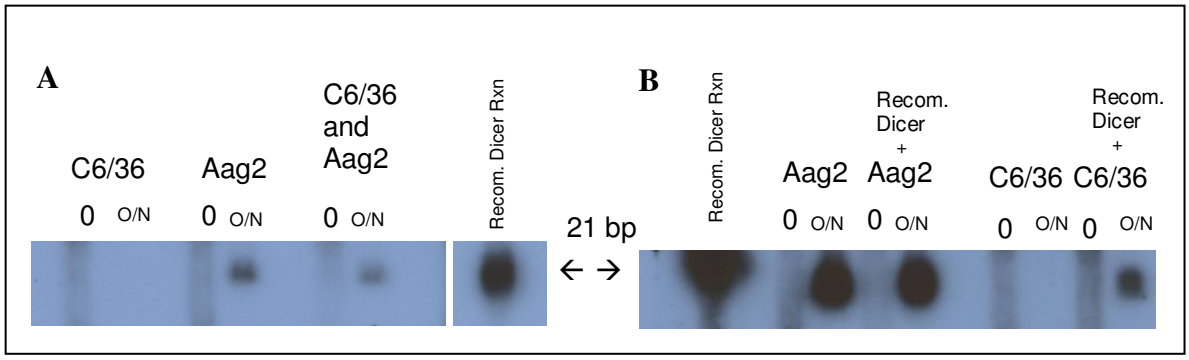


Figure 3.6: Comparison dicer activity after addition of labeled dsRNA in C6/36, Aag2 and both lysates combined (A) and with supplemented human recombinant Dcr (B). Recombinant Dicer reaction used as a size marker for 21 bp RNAs.

Assay of RNAi activity by measurement of GFP expression in whole Aag2 and C6/36 cells

Intact C6/36 and Aag2 cells were also tested for the ability of their RNAi pathways to inhibit EGFP expression from a transfected plasmid to elucidate the step in the siRNA pathway where the C6/36 cells are not functioning properly. Each cell line was transiently transformed with a plasmid expressing EGFP along with siRNAs or long dsRNA derived from the EGFP sequence or control RNAs derived from the WNV genome sequence. Transfection of EGFP-derived-siRNAs into either cell type resulted in knock-down of EGFP expression, indicating that both have a functional RNAi pathway if pre-formed siRNA is loaded into RISC. However, transfection of cognate long dsRNA resulted in knock-down of EGFP expression only in Aag2 cells, suggesting that only this cell line was able to efficiently carry out Dcr2-mediated cleavage of dsRNA (Figure 3.7A). Immunoblotting for EGFP expression in the cells confirmed these results (Figure 3.7B). These results provide further evidence that C6/36 cells are defective in Dcr2 activity and suggest that both cell lines are able to form a functional RISC.

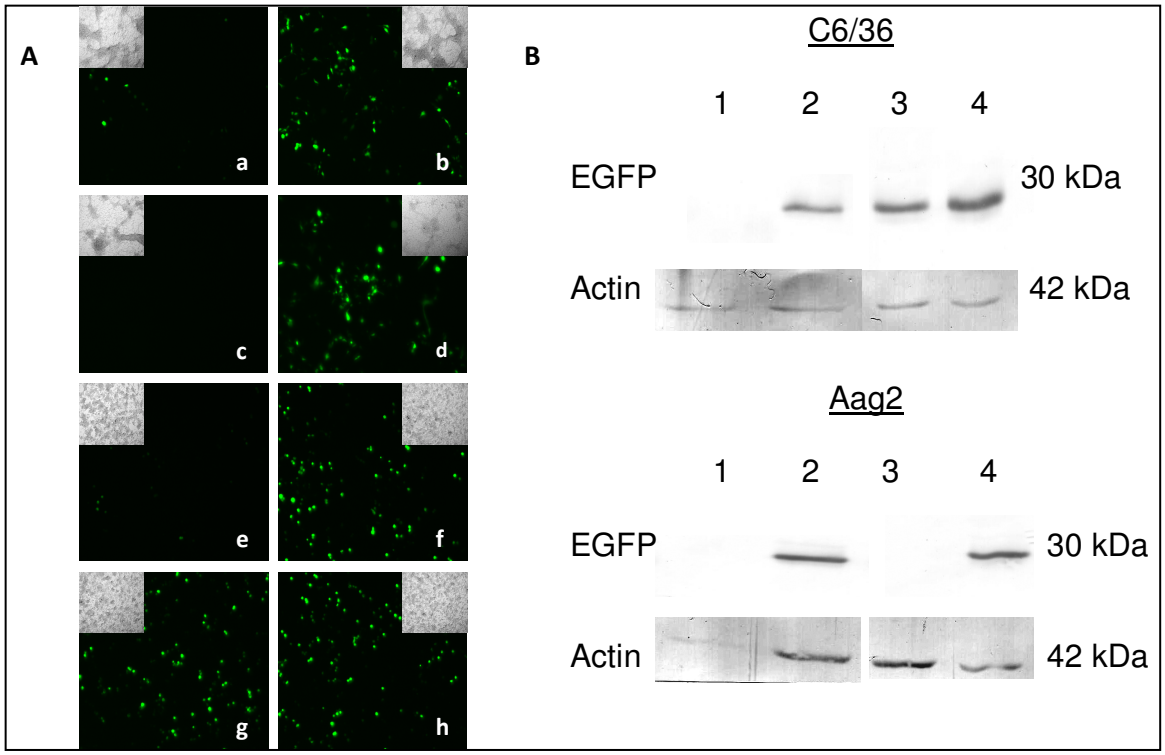


Figure 3.7. C6/36 cells can not knockdown GFP expression with dsRNA. A) Analysis of dicing activity in Aag2 (a-d) and C6/36 (e-h) cell cultures. Aag2 cells were transfected with (a) pEGFP + EGFP siRNA, (b) pEGFP + WNV siRNA, (c) pEGFP + EGFP dsRNA, (d) pEGFP + WNV dsRNA. C6/36 cells were transfected with (e) pEGFP + EGFP siRNA, (f) pEGFP + WNV siRNA, (g) pEGFP + EGFP dsRNA, (h) pEGFP + WNV dsRNA. All cells were analyzed by fluorescent microscopy at 48 hr after transfection. (B) Immunoblot shows EGFP protein silencing. 1, pEGFP + EGFP siRNA, 2, pEGFP + WNV siRNA, 3, pEGFP + EGFP dsRNA, 4, pEGFP + WNV dsRNA. (Images courtesy of Dr. Doug Brackney)

Dcr2 Northern blotting

To determine if dcr2 mRNA is being expressed in the C6/3 cell line, levels of dcr2 mRNA were analyzed from total RNA from C6/36 cells, *A. albopictus* mosquitoes, Aag2 cells and *A. aegypti* mosquitoes using northern blotting with C6/36-dcr2 and Aag2-dcr2 sequence probes (Figure 3.8). dcr2 messenger was detected in both Aag2 cells and C6/36 cells using their respective probes (both probes had same number of UTPs, so their intensity should be the same). When the band intensity compared with the BioRad

Quantity One software, the Aag2 band was approximately 1.7 fold stronger than the C6/36 band (after adjustment for background). A faint band was also seen in the *A. aegypti* and *A. albopictus* (band running slightly smaller) lanes. When the dcr2 band from cells and the dcr2 band from mosquitoes were compared, the Aag2 cell band was approximately 5 fold stronger than the *A. aegypti* dcr2 mosquito band and the C6/36 cell dcr2 band was approximately 3 fold stronger than the *A. albopictus* band. The lower levels of dcr2 seen in the whole mosquito RNA is probably due variability in gene expression levels in the various mosquito tissues.

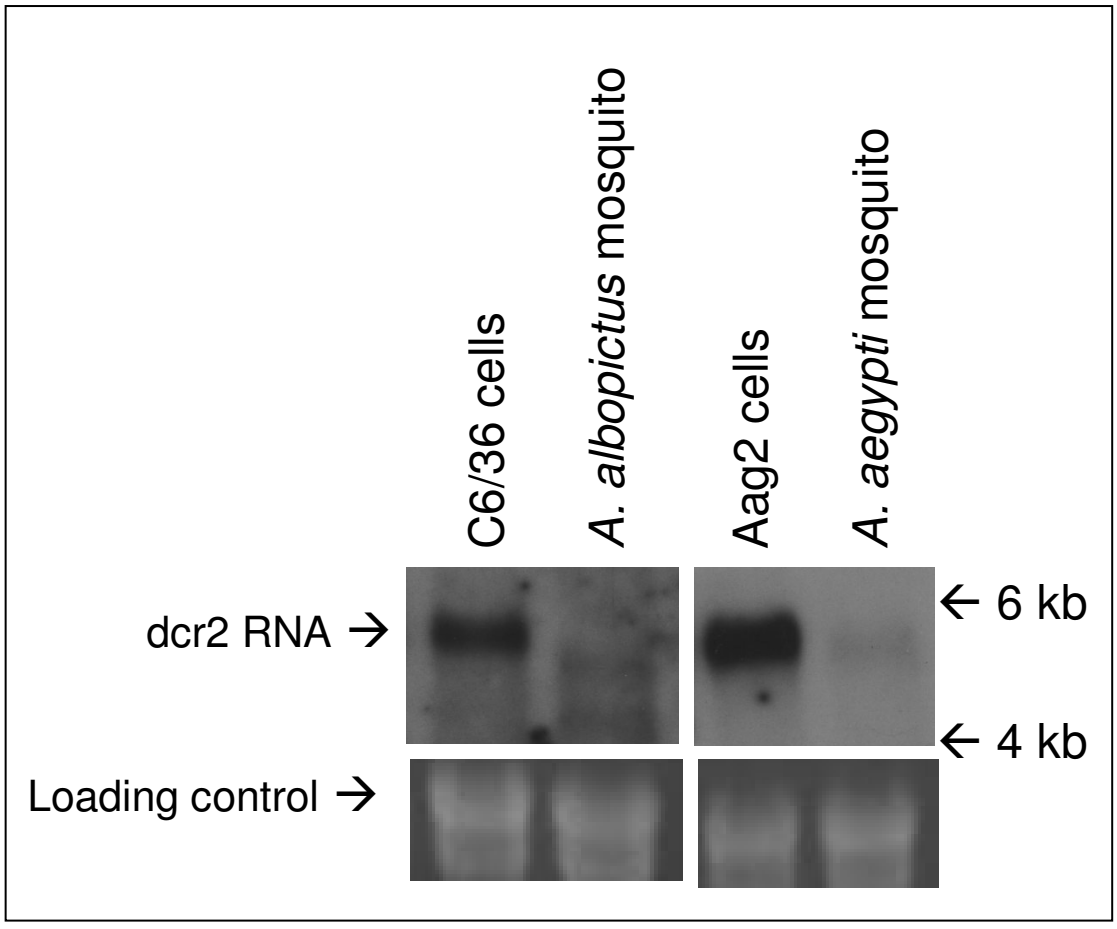


Figure 3.8: Northern blot analysis of dcr2 mRNA levels in mosquito cell cultures and whole mosquitoes. Total RNA was used for each sample and an antisense probe to dcr2 nt 4919-5116 was used for detection.

dsRNA knockdown of Dcr2 in cells

The ability of dsRNA to knockdown Dcr2 expression was tested in the Aag2 and C6/36 cells to test if Dcr2 could knockdown its own mRNA expression as another way to test Dcr2 activity in the cells. dsRNA specific to Dcr2 sequence from both *A. aegypti* and *A. albopictus* (from the same region of the gene) was soaked into the cells and then total RNA was extracted at various days following dsRNA addition. This RNA was then probed for dcr2 messenger RNA using *A. aegypti* or *A. albopictus* antisense ssRNA probes from a region further downstream of the sequence of the dsRNA used to knockdown the gene. In the Aag2 cells, the Dcr2 messenger appeared to decrease somewhat on Days 2, 3 and 5 after addition of dsRNA (Figure 3.9, lighter exposure), and this may also have occurred in the C6/36 cells, although the bands are somewhat too faint to really make any conclusions. No differences are seen in the darker exposure in the C6/36 samples. This 'lack' of dcr2 knockdown by dsRNA in C6/36 cells could be due to the inability of dsRNA to knockdown of gene expression due to ineffective Dcr2 processing in the C6/36 cells, or could also be attributed to a lack of dsRNA uptake by the C6/36 cells during the dsRNA soaking. It is interesting to note that the bands for dcr2 mRNA are much stronger in all of the Aag2 samples compared to the C6/36 samples, which may indicate that there is less dcr2 messenger present in the C6/36 cells (Figure 3.9). It should be noted that the C6/36 and Aag2 samples were run on the same gel, probed with the same amount of antisense dcr2 probe (although the sequences were specific to *A. albopictus* or *A. aegypti* dcr2) and the blots were exposed to the same sheet of film. Also, when this experiment was first attempted, the *A. albopictus* dcr2 probe was inadvertently used on the Aag2 sample membrane and the *A. aegypti* dcr2 probe was used

on the C6/36 sample membrane and no bands appeared in either sample (data not shown), indicating the sequence differences in this region of the dcr2 gene are enough for the probes to not cross-react between the species.

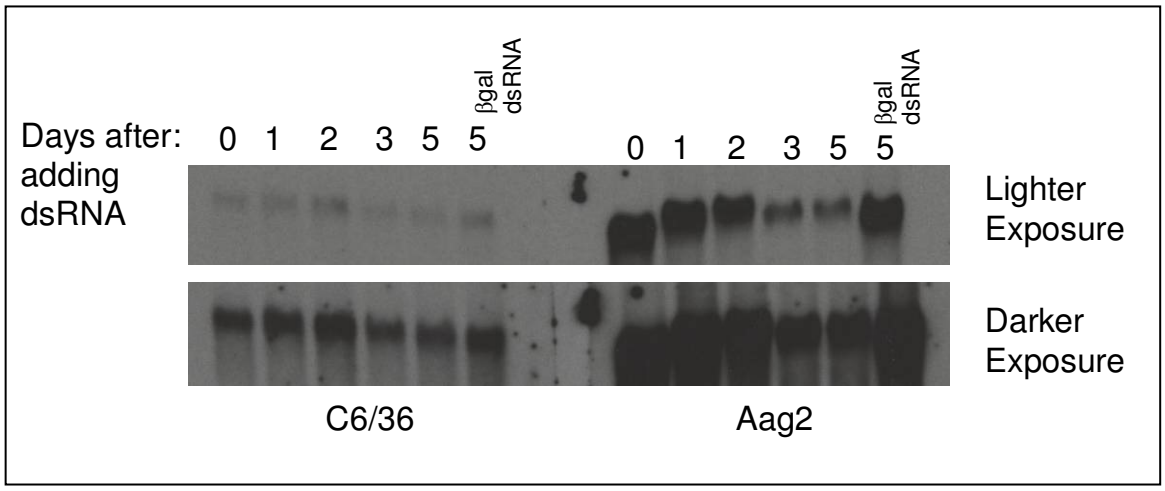


Figure 3.9. Northern blot for dsRNA knockdown of dcr2 in C6/36 cells (on left) and Aag2 cells (on right). RNA was extracted for 5 days after addition of dsRNA. βgal dsRNA was used as control dsRNA. The upper picture is a lighter blot exposure and the lower picture is a darker exposure to show the Dcr2 bands in the C6/36 sample.

Dicer-2 cDNA sequences from Aag2 and C6/36 cells

dcr2 cDNA amplicons from Aag2 and C6/36 cells were sequenced in both the forward and reverse directions to determine if there were mutations in the C6/36 *dcr2* sequence that could explain why it may not be functioning properly. The nucleotide sequences were aligned using VectorBase (see Appendix for alignment) and were compared to each other and the *A. aegypti* mosquito *dcr2* sequence from GenBank using EMBOSS. *dcr2* sequence from nt 1200 to 5120 was recovered from C6/36 cells, while an almost complete sequence was recovered from Aag2 cells. The Aag2 cell and *A. aegypti dcr2* sequences had 99% identity, while the C6/36 *dcr2* had only 52.7% identity with the *A. aegypti* mosquito and Aag2 cells (Table 3.4). This lower number is most likely due to a lack of full C6/36 dcr2 sequence as well as to comparing different

mosquito species. When the sequences were compared using BLASTN, a region of 3897 nt (from nt 1211 to 5104 of Aag2 sequence), had approximately 79% identity, which is probably a more accurate comparison between the two cell line genes since the regions where no C3/36 *dcr2* sequence was available were eliminated (Table 3.5).

This sequencing revealed a single nucleotide deletion in the C6/36 gene at the equivalent position to nt 2688 of *A. aegypti* mosquito *dcr2* sequence (position 1508 of the incomplete C6/36 sequence) (See Figure A.1 in the Appendix for the nucleotide sequence alignment). This deletion would result in either a stop codon, a completely altered amino acid sequence by comparison to *A. aegypti* Dcr2, or a frame shift downstream. Any of these would greatly affect the structure and probable function of the protein. When the C6/36 nucleotide sequence was translated into amino acid sequence, the +1 (from first nucleotide in known C6/36 sequence) reading frame amino acid sequence aligned to the *A. aegypti* amino acid sequence from amino acids 825 to 1632, and the +2 reading frame translation aligned to the *A. aegypti* amino acid sequence from amino acids 333 to 824 (See Figure A.2 in Appendix for the amino acid sequence alignment). The region where the frame shift occurred in the amino acid sequence corresponds to the region of the deleted nucleotide. This region of the C6/36 cell cDNA was sequenced 3 separate times (2 times with one primer set one cDNA library, once with a different primer set and a second cDNA library) using a high-fidelity DNA polymerase, so to the best of my knowledge this deletion is genuine. The protein resulting from complete translation of either the +1 or +2 reading frame would probably function quite poorly as either the PAZ and RNase III domains (located in the amino acid 825-1632 segment) would be translated correctly or the helicase and dsRNA binding domains (located in the amino acid 333 to

824 segment) would be translated correctly, but not both, making binding and cleavage of long dsRNA faulty for the enzyme. Even if this deletion is an artifact of sequencing, there are many non-conservative changes in functional domains of the amino acid sequence of C6/36 cell Dcr2 that could result in changes in protein structure/function (See Figures A.3-A.7 in the appendix for the regions of the functional domains). It is difficult to speculate which of these changes could affect protein structure and function without knowledge of the *A. albopictus* mosquito Dcr2 sequence.

Table 3.4: Results of EMBOSS needle Pairwise Alignment Algorithm comparison of dicer2 sequences.

***A. aegypti* mosquito vs. Aag2 dicer2 Sequence**	
Parameter	**Score**
Identity	5869/5927 (99.0%)
Similarity	5869/5927 (99.0%)
Gaps	30/5927 (0.5%)
Length	5927
Score	33865.5
***A. aegypti* mosquito vs. C6/36 dicer2 Sequence**	
Identity	3121/5919 (52.7%)
Similarity	3121/5919 (52.7%)
Gaps	2005/5919 (33.9%)
Length	5919
Score	17343.0
Aag2 vs. C6/36 dicer2 Sequence	
Identity	3119/5919 (52.7%)
Similarity	3119/5919 (52.7%)
Gaps	2005/5919 (33.9%)
Length	5919
Score	17342.0

Table 3.5: Comparison of dcr2 Sequences from Aag2 and C6/36 cells using BLASTN

Parameter	**Score**
Identity	3088/3897 (79%)
Gaps	27/3897
Score	3333 bits
Expect	0.0

miRNA analysis

The number of probable miRNA sequences in the C6/36 cell samples from the SOLiD sequencing was analyzed with NextGENe software using the miRBase database to determine which miRNAs increased with DENV2 infection of the cells. Approximately 2% of the total number of reads was miRNAs in the C6/36 Mock sample, while 1.5% of the reads were miRNAs in the C6/36 DENV2 Day 5 post infection sample (Table 3.6). Most of the miRNAs in the samples were 21-22 nt in length. In the C6/36 Mock sample, 22 nt was the most common size, while 21 nt was the most common size in the DENV2 infected C6/36 sample (Figure 3.10 and 3.11). This slight change from 22 nt to 21 nt might be related to the change in small RNA pathways during infection of the cells such as proteins from one pathway being shared with another, but more replicates of the sequencing would need to be done to make any conclusions.

The change in levels of miRNAs during DENV2 infection was also analyzed by determining which miRNA increased most in the DENV2 infected sample compared to the mock sample (Figure 3.12). miR-77a was the miRNA found to increase most after infection, although its target and function have not been determined yet. miRNAs matching both *Drosophila erecta* and *Bombyx mori* mir-277 were also found to be increased (Figure 3.12). In *Drosophila*, miR-277 is believed to possibly target genes for protein GalNAc transferase, valine leucine and isoleucine degradation, cell polarity and membrane proteins. This miRNA is also conserved in *Anopheles* (Stark et al., 2003). The degradation of amino acids is regulated when the cells have excessive dietary intake or are undergoing starvation, so this miRNA may be responsible for regulating a metabolic response (Stark et al., 2003). miR-277 and miR-7 have also been identified in

A. aegypti and *C. quinquefasciatus* mosquitoes and *A. albopictus* C7/10 cells (Li et al., 2009b, Skalsky et al., 2010), although there is little data on targets for miR-7 in invertebrates. It is unclear why the best matches are to *Drosophila*, *Capitella* (polychaete worm) and *B. mori* miRNAs when a mosquito equivalent miRNA has been found, possibly due to how the NextGENe software picks which sequence it should align to first when matching small RNAs to the reference sequence (miRBase in this case). miR-216b was also found to be increased, but its gene target is still unknown, and no *Aedes* or *Culex* counterpart has been described in miRBase yet. miRNAs from the miR-276 and miR-7 families were also increased after DENV2 infection of Aag2 cells (see Chapter 2).

Table 3.6: Potential miRNAs identified from C6/36 samples with NextGENe analysis

	Mature miRNA hits	**Percent of total reads**
C6/36 Mock	238,454	2.0%
C6/36 DENV2 Day 5	182,374	1.5%

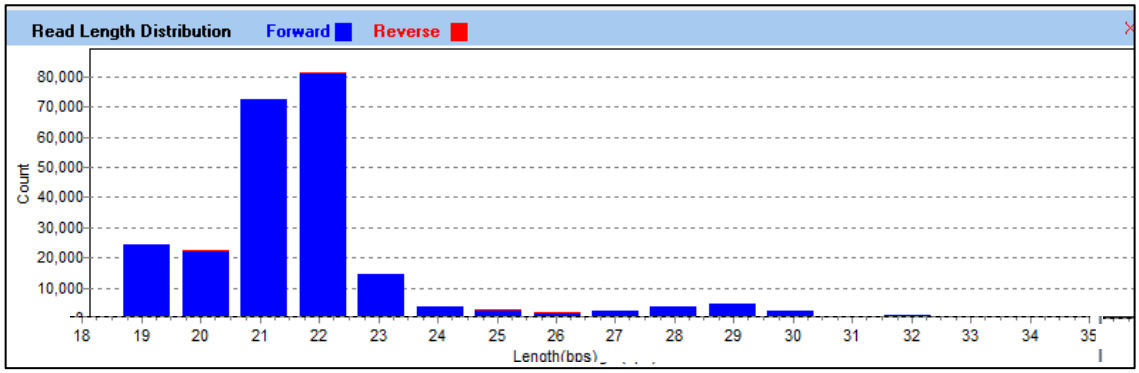


Figure 3.10: miRNA size distribution from C6/36 Mock sample. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

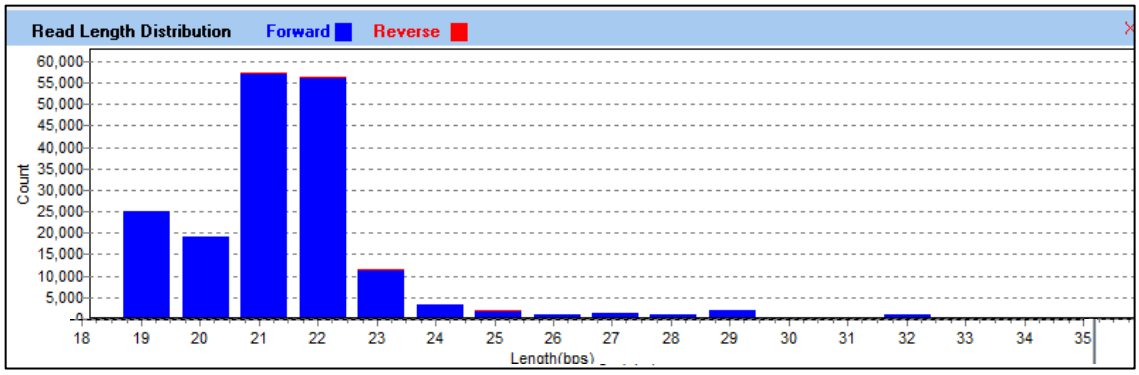


Figure 3.11: miRNA size distribution from C6/36 DENV2-infected 5 dpi sample. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

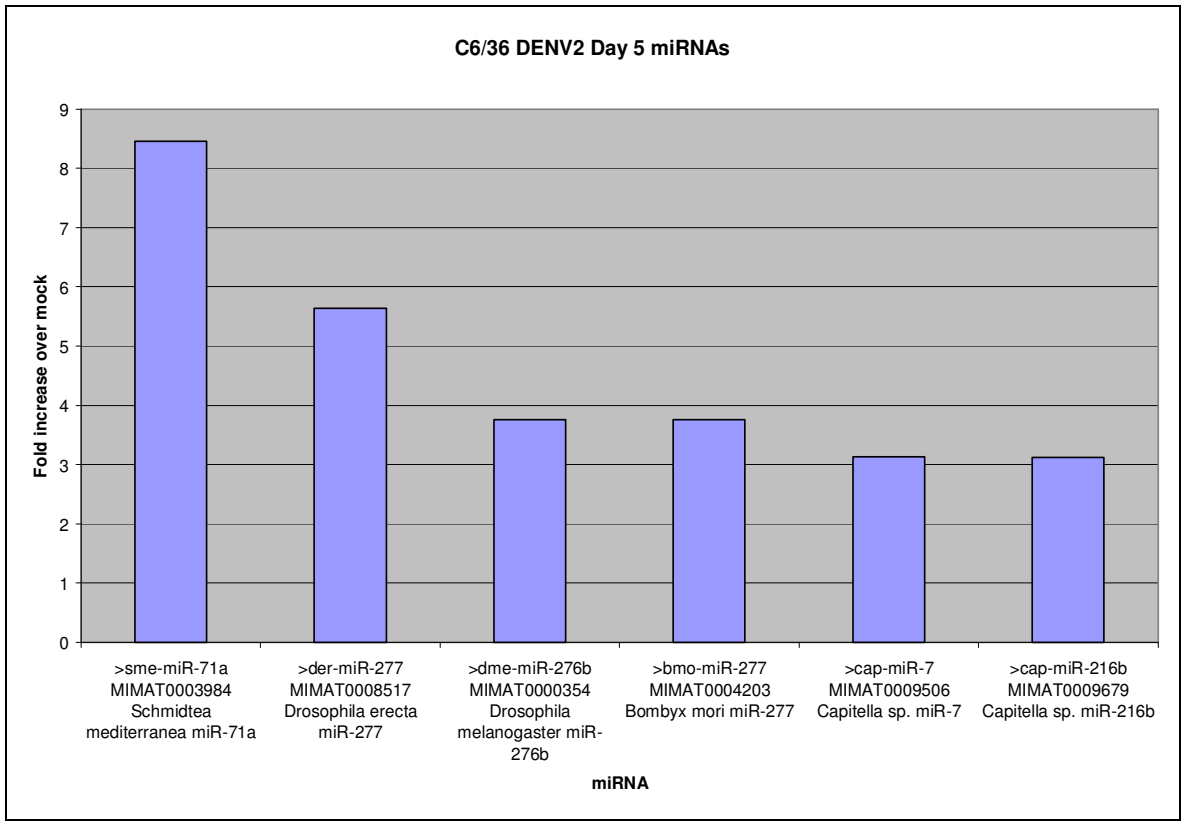


Figure 3.12: Fold-increase of C6/36 cell miRNAs over mock-infected C6/36 miRNA levels at five days after DENV2 infection.

Discussion:

The purpose of this study was to compare the antiviral RNAi responses to infection with the positive-sense RNA arbovirus DENV2 in two mosquito cell lines by characterization of the virus-specific small RNAs (viRNAs) produced after infection. In previous studies, when the *A. aegypti* RNAi response was knocked down by injection of dsRNA derived from the dcr2, ago2 or r2d2 genes, viral replication in mosquitoes increased, indicating that RNAi plays an antiviral role against DENV2 (Sanchez-Vargas et al., 2009). Little is known about the nature of the DENV2 RNA trigger of the RNAi antiviral pathway and the resulting DENV2-specific siRNAs in mosquitoes and mosquito cell cultures. Since DENV2 induces the production of dsRNA during its replication cycle (Stollar et al., 1967, Stollar & Stollar, 1970b), this would be the most obvious trigger for Dcr2 cleavage and activation of an RNAi response. However, some analyses of small RNAs from cells infected with positive-strand RNA plant viruses have shown that a much higher proportion of siRNAs are derived from the positive sense strand than from the antisense strand, indicating that secondary structure of the ssRNA genome may be a trigger for RNAi (Molnar et al., 2005). Analysis of our deep sequencing data showed that approximately 54-59% of the DENV2-small RNAs in Aag2 cells were positive sense, close to the 1:1 ratio that would be expected if the trigger were a double-stranded intermediate composed of long strands of positive genomic RNA annealed to a long, complementary negative sense strand (Chapter 2). The distribution pattern of viRNAs along the DENV2 genome in Aag2 cells at 5 days post DENV2 infection is relatively uniform, also implicating a long dsRNA replicative intermediate as the main source of DENV2-specific small RNAs in Aag2 mosquito cells (Chapter 2). In DENV2-infected

mosquitoes, the positive strand:negative strand ratio was even closer to 1:1, with 55% of the DENV2-specific small RNAs being derived from the positive sense strand (Chapter 2). Surprisingly, the C6/36 DENV2-specific small RNAs were almost all positive sense, and seemed to be generated from specific regions of the genome. When data were analyzed with the SOLiD pipeline software, similar numbers of DENV2-viRNAs from the C6/36 samples were found as with the NextGENe software analysis.

The DENV2-specific viRNAs in both Aag2 cells (5 dpi) and *A. aegypti* mosquitoes were predominantly 21 nt long with similar proportions of sense and antisense polarities, indicating that their RNAi mechanism must be similar (Chapter 2). The predominant length of DENV2-derived small RNAs in C6/36 cells was 27 nt, a size characteristic of piRNAs. The C6/36 cells also did not produce typical 21 nt viRNAs in response to infection by WNV, SINV or LACV, as is shown in Brackney, et al., 2010 (Brackney et al., 2010).

The presence of unusual DENV-specific small RNAs in C6/36 cells suggested that they used a compensating mechanism for generation of viral-specific small RNAs. The larger size and predominance of positive sense small RNAs in C6/36 cells suggested a piRNA-like mechanism like that mediated by Ago3 (Brennecke et al., 2007). Virus-specific piRNAs were recently described in *Drosophila* Wu et al., 2010 and Zambon et al., 2010 showed that *piwi*-family mutants of *Drosophila* were more susceptible to Drosophila virus X infection (Wu et al., 2010, Zambon et al., 2006). Logo analysis of DENV2- small RNAs from C6/36 cells showed a bias for adenine at the 10^{th} position from the 5' end, which is also characteristic of piRNAs (Gunawardane et al., 2007). The overwhelming predominance of genome-sense small RNAs, if they are loaded into a

RISC, would result in inefficient cleavage of newly-synthesized viral genomes and a comparatively weak innate immune response.

The piRNA pathway may serve as a backup mechanism when the exogenous siRNA pathway is not functioning correctly. Evidence for this hypothesis was seen when the endo-siRNA pathway was disrupted by mutation of Ago2 in *Drosophila*, causing appearance of somatic cell piRNAs, possibly serving as a backup for transposon surveillance (Ghildiyal et al., 2008, Ghildiyal & Zamore, 2009). In our examination of RNAi in *A. gambiae* mosquitoes we found that co-injection into mosquitoes of dsRNA derived from Ago3 with ONNV resulted in increased ONNV titers, hinting at a possible role for Ago3 in antiviral immunity in mosquitoes (Keene et al., 2004).

In a previous study, northern blot hybridization failed to detect WNV-specific siRNAs in C6/36 cells (Chotkowski et al., 2008). In this study, *in vitro* lysate analysis indicated that C6/36 cells may lack the ability to cleave long dsRNA into siRNAs. Only transfected siRNAs could be used to knock-down GFP expression from a plasmid in the cells, and long dsRNA did not. Deep sequencing analysis of small RNA in DENV2-infected C6/36 cells revealed abundant DENV2-specific small RNA that did not have the size and polarity patterns expected of Dcr2 generated siRNAs. These results all suggest a lack of Dicer cleavage activity in C6/36 cells. However, since addition of recombinant Dicer to the lysate resulted in production of siRNAs, it appeared that the lack of Dcr2 activity was not due to its inhibition in C6/36 cells.

dcr2 mRNA levels were analyzed in the two cell lines and in whole mosquitoes. It appears that there is more dcr2 mRNA produced in the Aag2 cells than in the C6/36 cells. There was also more dcr2 mRNA in both mosquito cell lines than in the whole

mosquitoes, which is probably a result of more varied dcr2 expression in different tissues of the mosquito. When dsRNA derived from dcr2 was soaked into cells, only Aag2 cells showed a decrease in dcr2 messenger RNA. This may be due to lack of Dcr2 protein cleavage activity in C6/36 cells, which would prevent dcr2 dsRNA from being effective against reducing dcr2 messenger RNA, or could also be due to lack of dsRNA uptake into the C6/36 cells during dsRNA soaking.

Sequencing of major regions of the Aag2 and C6/36 *dcr2* cDNAs showed that the mRNAs have approximately 79% nucleotide sequence identity. Sequencing also revealed a single nucleotide deletion in the C6/36 *dcr2* sequence that would result in a translational stop or frame shift or complete alteration of the amino acid sequence, which would severely disable the protein's ability to function correctly and may explain why there appears to be dysfunctional Dcr2 cleavage in the C6/36 cell line.

In *Drosophila*, missense mutations in the DExH helicase domain resulted in reduced dsRNA processing ability and a missense mutation in the RNase III domain caused a loss of dsRNA processing comparable to a null dcr2 mutation (P1496L) (Lim et al., 2008). The P1496L dcr2 mutation still allowed for functional RISC activity, which is comparable to the results seen in the EGFP knockdown experiments in C6/36 cells showing a lack of dsRNA processing, but functional siRNA activity in the C6/36 cells (Figure 3.7). Neither of the P1496L or A1453T mutations was seen in the C6/36 dcr2 sequence, but any one of the mutations seen in the RNaseIII domains of the C6/36 dcr2 sequence could have a similar effect (See Figures A.6 and A.7 in the Appendix).

miRNA analysis to determine increased miRNA expression after DENV2 analysis did not show any surprising results, although miRNAs from the miR-276 and miR-7

families increased in both the C6/36 cells and the Aag2 cells after DENV2 infection. Further investigation is needed to determine if these miRNAs are involved in antiviral defense during DENV2 infection.

The lack of functional Dcr2 activity in C6/36 cells and production of predominantly positive-sense piRNA may play a role in their increased ability to support the replication of arboviruses such as DENV and chikungunya, and may account for Igarashi's speculation that "the virus-sensitive C6/36 clone may lack efficient regulatory mechanism for virus RNA synthesis and virus production" (Igarashi, 1978).

Future studies could examine how Dcr2 functions in other *A. albopictus* cell lines to determine if this phenomenon is specific to C6/36 cells. Dcr2 wild-type sequence could also be expressed in the C6/36 cells and virus-specific small RNAs could be analyzed by deep sequencing to see if the Dcr2 activity could be rescued in the cell line.

In summary, deep sequencing analysis and various *in vitro* studies have indicated that C6/36 cells exhibit inefficient Dcr2 cleavage of long dsRNA. The C6/36 *A. albopictus* cell line produces more abundant DENV2-specific small RNAs, although they appear to be generated by a different small RNA pathway, possibly through a piRNA-like mechanism, and this aberrant pattern of viral small RNA production extends to other flaviviruses, alphaviruses and bunyaviruses (Brackney et al., 2010). Caution should be used when analyzing the molecular interactions of arboviruses with the C6/36 cell line, as they do not appear to have a similar antiviral defense mechanism as *A. aegypti* mosquitoes.

CHAPTER 4

ANALYSIS OF CELL FUSING AGENT VIRUS-SPECIFIC SMALL RNAS IN MOSQUITO CELLS

Introduction:

Some flaviviruses are found only in mosquitoes and are not transmitted to humans. These viruses are related to the arboviruses in the *Flaviviridae* family but fall into their own phylogenetic clade (Hoshino et al., 2007, Marin et al., 1995), and are referred to as mosquito-only flaviviruses. The first mosquito-only virus was described as an agent in *A. aegypti* embryo cell culture that resulted in fusion of *A. albopictus* larval cell cultures when medium from the *A. aegypti* cells was transferred to the *A. albopictus* cells. This agent was first described as Cell Fusing Agent (Stollar & Thomas, 1975) and later characterized as a virus (Igarashi et al., 1976). The prevalence of the virus in natural mosquito populations and how it is transmitted between mosquitoes is unknown, although a vertical transmission route seems plausible, as mosquito embryos were used to start the original cell culture (Peleg *A. aegypti* cells) in which CFAV was found.

Other mosquito-only flaviviruses have been found in the wild including Kamiti River virus (KRV) (Sang et al., 2003) and other *Culex* and *Aedes* flaviviruses (Bolling et al., 2010 (Submitted), Crabtree et al., 2009, Farfan-Ale et al., 2009, Hoshino et al., 2009, Hoshino et al., 2007, Kim et al., 2009, Morale-Betoulle et al., 2008). KRV is vertically transmitted in mosquitoes in the laboratory at rate of 3.9% (Lutomiah et al., 2007), and it is suspected that the other mosquito-only flaviviruses are also transmitted vertically.

DNA integrations with similar sequences to CFAV and KRV have also been described in the mosquito genome (Crochu et al., 2004). It is unclear how these were integrated into the genome, but some of the flanking sequences are related to *Drosophila* transposons, suggesting a mechanism for integration (Crochu et al., 2004). The presence of these integrations indicates that these viruses have been persistently infecting

mosquitoes for a long time (Crochu et al., 2004). Whether transcription occurs from these integrated regions, and if that transcription would have any effect on the replication of other viruses in mosquitoes (such as arboviruses) is still unknown. An integration of RNA from a dicistrovirus into the genome of the bee *Apis mellifera*, is present in 30% of the bee populations and has been shown to provide the bees with resistance to infection with the homologous virus (Maori et al., 2007). Whether the mosquito flaviviral DNA integrations provide resistance to other viruses in the mosquito needs further investigation.

Upon examination of the deep-sequencing results from the Aag2 small RNA libraries, many small RNAs from the CFAV genome were discovered. This was not a complete surprise, as the Aag2 cell line was derived from the original Peleg *A. aegypti* embryo cell line where CFAV was first described. In this chapter the small RNAs derived from CFAV infection of mosquito cells are analyzed, the possibility for interactions between CFAV small RNAs and the DENV2 genome is described, the cell lines are examined for mosquito-only flaviviral DNA integrations and the effect of CFAV infection on Dcr2 activity of C6/36 cells is studied.

Materials and Methods:

Cells and Medium

Aag2 and C6/36 cell lines were maintained as described in previous chapters.

Development of a C6/36 cell line persistently infected with CFAV

A CFAV-persistently infected C6/36 cell line was made by centrifuging medium from Aag2 cells at 3,500 rpm for 5 minutes, filtering 2 ml of supernatant from centrifuged medium through a 0.1 µm filter, then through another 0.1 µm filter. Approximately 1 ml of this filtered medium was added to a T150 flask of confluent C6/36 cells. The cells showed CPE of syncytia formation and most died, but some cells remained to regenerate the culture.

SOLiD sequencing

The same small RNA libraries and SOLiD sequencing used for the Aag2 and C6/36 cell samples from previous chapters are analyzed here. The small RNA sequences were aligned to the CFAV genome from GenBank Accession number NC_001564.1 using the NextGENe and ABI SOLiD small RNA analysis pipeline.

ABI SOLiD small RNA analysis pipeline config file:

```
RUN_FILTERING     true
RUN_MIRBASE_MATCHING    false
RUN_GENOME_MATCHING     true

tag_length  35
pattern     11111111111111111111111111111111 #need to be of
tag_length size
adaptor     CGCCTTGGCCGTACAGCAG

# filtering step
filter_step_reference_file
      /projects/jackiescott/primer_filter_reference.fasta
filter_step_number_of_bases_to_use  25
filter_step_number_of_errors  2

# miRBase matching step
miRBase_step_gff_reference_file     /projects/jackiescott/hsa.gff
MAKE_PRECURSOR_FASTA_REFERENCE      no
miRBase_step_reference_fasta_file   /projects/jackiescott/has.fasta
miRBase_step_reference_extenssion   35
miRBase_step_seeds_number_of_bases_to_use 18
miRBase_step_seeds_number_of_errors 3
miRBase_step_extension_max_number_of_errors     6
```

```
miRBase_step_output_read_type unique
miRBase_step_output_counts    yes
miRBase_step_output_wiggle    yes
miRBase_step_output_gff_reads no

# genome matching step
genome_step_reference_fasta_file
      /projects/jackiescott/CFAVNC001564.fasta
genome_step_seeds_number_of_bases_to_use  20
genome_step_seeds_number_of_errors  2
genome_step_extension_max_number_of_errors      5
genome_step_maximum_number_of_hits_per_tag      5
genome_step_output_read_type  all
genome_step_output_wiggle     yes
genome_step_output_wiggle_coverage_cut    10

# environment information
NAME_OF_QUEUE     secondary   #highmem
FOLDER_FOR_TEMPORARY_FILES_ON_COMPUTE_NODES     /scratch/
NUMBER_OF_PROCESSORS_ALLOCATED_PER_NODE   1
MAX_MEMORY_PER_JOB_IN_BYTES   2e9

# NOT YET IMPLEMENTED

NUMBER_OF_READS_FILE_PARTITIONS     2
```

RT-PCR for CFAV RNA

Reverse transcriptase PCR (RT-PCR) was performed on cellular RNA extracted with Trizol following the manufacturer's protocol. The Superscript II Reverse Transcriptase kit (Invitrogen) was used according to the kit's instructions using primers CFAV-3720 REV for the RT reaction and CFAV-3500 FWD and CFAV-3720 REV for the PCR reaction (see Primers Table in Appendix for primer sequences).

Alignment of CFAV genome to DENV2 genome

The CFAV RNA sequence accession number NC_001564.1 and the DENV2 RNA sequence accession number M20558.1 were aligned to each other using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (http://blast.ncbi.nlm.nih.gov/) using blastn, with an expect threshold of 10, a word size

of 7, 2 match and -3 mismatch scores, existence of 5 and extension of 2, filtering low complexity regions, and masking for look up table only. These parameters were the default parameters set by BLAST.

CFAV and DENV2 RNAs were also compared for their percent sequence identity using the EMBOSS pairwise alignment algorithm program located at http://www.ebi.ac.uk/Tools/emboss/align/index.html using the settings: EMBOSS:needle (global), Matrix: DNAfull, open gap penalty of 10.0 and a gap extension penalty of 0.5 (Rice et al., 2000).

Search of Vectorbase for CFAV-like integrations

Vectorbase.org *A. aegypti* nucleotide sequences (Assembly AaegL1 Supercontigs - Liverpool Strain, Transcripts AaegL1.2 Gene Build, ESTs September 2009 GenBank, Trace Reads Liverpool Strain September 2009) were searched for CFAV (accession number NC_001564.1) sequences using blastn, with a maximum e-value of 1, word size 11, scoring matrix BLOSUM62, and complex masking low on the website: http://aaegypti.vectorbase.org/Tools/BLAST/.

PCR for CFAV-like integrations

DNA was extracted from 1 ml of Aag2 or C6/36 cells (scraped into their growth medium) with the DNeasy DNA extraction kit (Qiagen) following the manufacturer's instructions including an RNase A treatment. PCR was performed using primers described in (Crochu et al., 2004): CSA NS3, CSA seq#2 (NS5) and PF1, with the

following PCR cycle: 94°C for 2 minutes, followed by 35 cycles of [94°C for 1 minute, 50°C for 30 seconds,72°C for 1 minute] followed by a 10 minute extension at 72°C.

Analysis of small RNAs from CFAV-like DNA integration

The DNA integration resembling KRV RNA sequence that was previously described in *A. aegypti* A20 cells (GenBank accession number AY347953.1), referred to as Cell Silent Agent 2 by Crochu, et al. (2004), was used as reference sequences in NextGENe to analyze the small RNAs transcribed from these regions in the Aag2 Mock and Aag2 DENV2 Day 5 SOLiD small RNA libraries.

In vitro dicing activity assay with CFAV-persistently infected cells

dsRNA was prepared by *in vitro* transcription of a 498 bp region of the beta-galactosidase (β-gal) gene as described in Chapter 3. Cell-free lysates were generated from Aag2 cells, C6/36 cells and C6/36 cells persistently infected with CFAV, using the protocol described in Chapter 3 with 50 ng of β-gal dsRNA per lysate (25 ng/lane).

Results:

CFAV viRNAs from SOLiD sequencing

During preliminary small RNA analysis, we detected cell fusing agent virus (CFAV) small RNA in Aag2 cells, suggesting persistent infection by this insect-only flavivirus. The sequences of small RNA libraries prepared from Aag2 cells were aligned with a CFAV genome sequence using NextGENe software to investigate this original small RNA finding. Surprisingly, there were many more small RNAs in all Aag2

libraries that aligned to CFAV RNA than to DENV2 RNA (approximately 80,000 CFAV viRNAs (DENV2 5 dpi sample) vs. 1,612 DENV2 viRNAs (DENV2 5 dpi sample) (Table 4.1). CFAV was first described in the precursor cell line to Aag2 cells (Brennecke et al., 2007, Cammisa-Parks et al., 1992, Gunawardane et al., 2007, Stollar & Thomas, 1975). Neither the mock-infected nor DENV2-infected *A. aegypti* mosquitoes appeared to have a CFAV infection, as only a small number of reads (< 60) from those libraries matched the CFAV genome (data not shown). The mock infected C6/36 cells also had <60 CFAV-specific small RNAs, but > 21,000 CFAV small RNAs were detected in the DENV2 5 dpi C6/36 cell culture sample (Table 4.1). Since the DENV2 stock used to infect the C6/36 cells was grown in Aag2 cells, this was probably the source of the CFAV, which was introduced to the C6/36 cells during the DENV2 infection.

Interestingly, the patterns of size and polarity of the CFAV-derived small RNAs were very similar to those of the DENV2-derived small RNA in both cell lines. Aag2 cell CFAV-specific small RNAs were predominantly 21 nt in length and were 54-63% positive sense (Table 4.1 and Figure 4.1). The numbers of CFAV-specific small RNAs were lower in the Aag2 DENV2 Day 1 library than in the other samples (Table 4.1), and this is most likely due to the RNA from these samples being harvested at 1 day after infection, whereas the other samples were taken at 5 days after infection or mock-infection, so these later samples probably had more cells and possibly a higher titer of CFAV. C6/36 cell CFAV-specific small RNAs were mostly 27 nt in length and 99% were derived from the positive sense strand, similar to the characteristics observed for DENV2-specific small RNAs, indicating that the defect in Dicer activity was not limited

to production of DENV2 small RNAs (Table 4.1 and Figure 4.1). The distribution of viRNAs along the CFAV genome was similar in all three of the Aag2 samples, with the small RNAs spread across the genome with peaks around nt 2400, 3600-3800, 8000 and near the 3' end (Figures 4.2, 4.3 and 4.4).

The distribution of CFAV viRNAs along the CFAV genome in the C6/36 DENV2 day 5 library was mostly confined to two peaks around nt 8800 and 9800 (Figure 4.5). Since these are from such specific regions and are mostly positive sense, this indicates they may be coming from secondary structures of the CFAV positive sense RNA, and their predominant size of 27 nt could indicate that they are generated by a piRNA-like mechanism, although it is still unknown whether the piRNA pathway targets ssRNA or dsRNA.

Table 4.1: CFAV small RNAs from NextGENe analysis

Sample (Total number of reads)	**Number of CFAV small RNAs (Percentage of total)**	**Percent from positive sense strand**	**Percent from negative sense strand**
Aag2 Mock (14087714)	79732 (0.5%)	57	43
Aag2 DENV2 Day 1 (12615439)	25663 (0.2%)	63	37
Aag2 DENV2 Day 5 (12131018)	88863 (0.7%)	54	46
C6/36 Mock (11915311)	59 (<0.00%)	54	46
C6/36 DENV2 Day 5 (12558261)	21807 (0.17%)	99	1

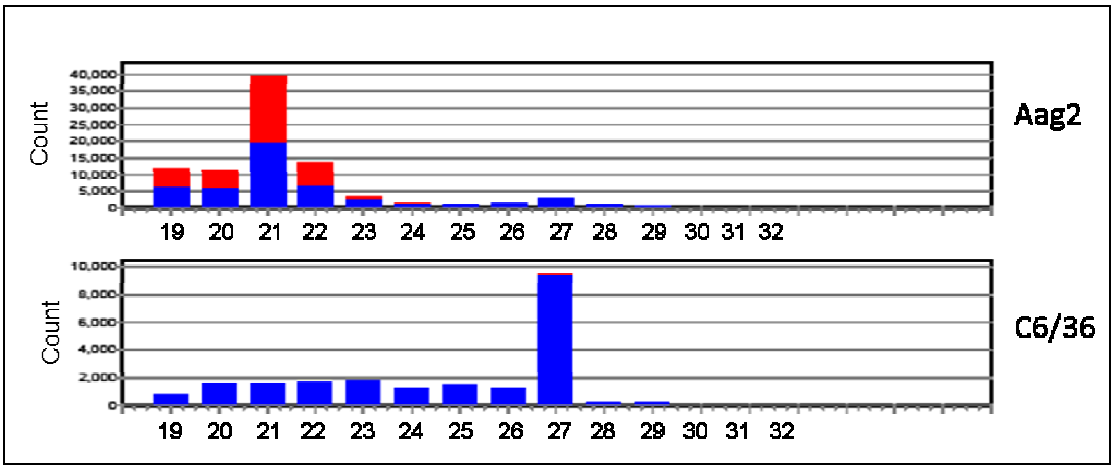


Figure 4.1: Size distribution of CFAV specific small RNAs from Aag2 cells DENV2 day 5 sample and C6/36 DENV day 5 sample.

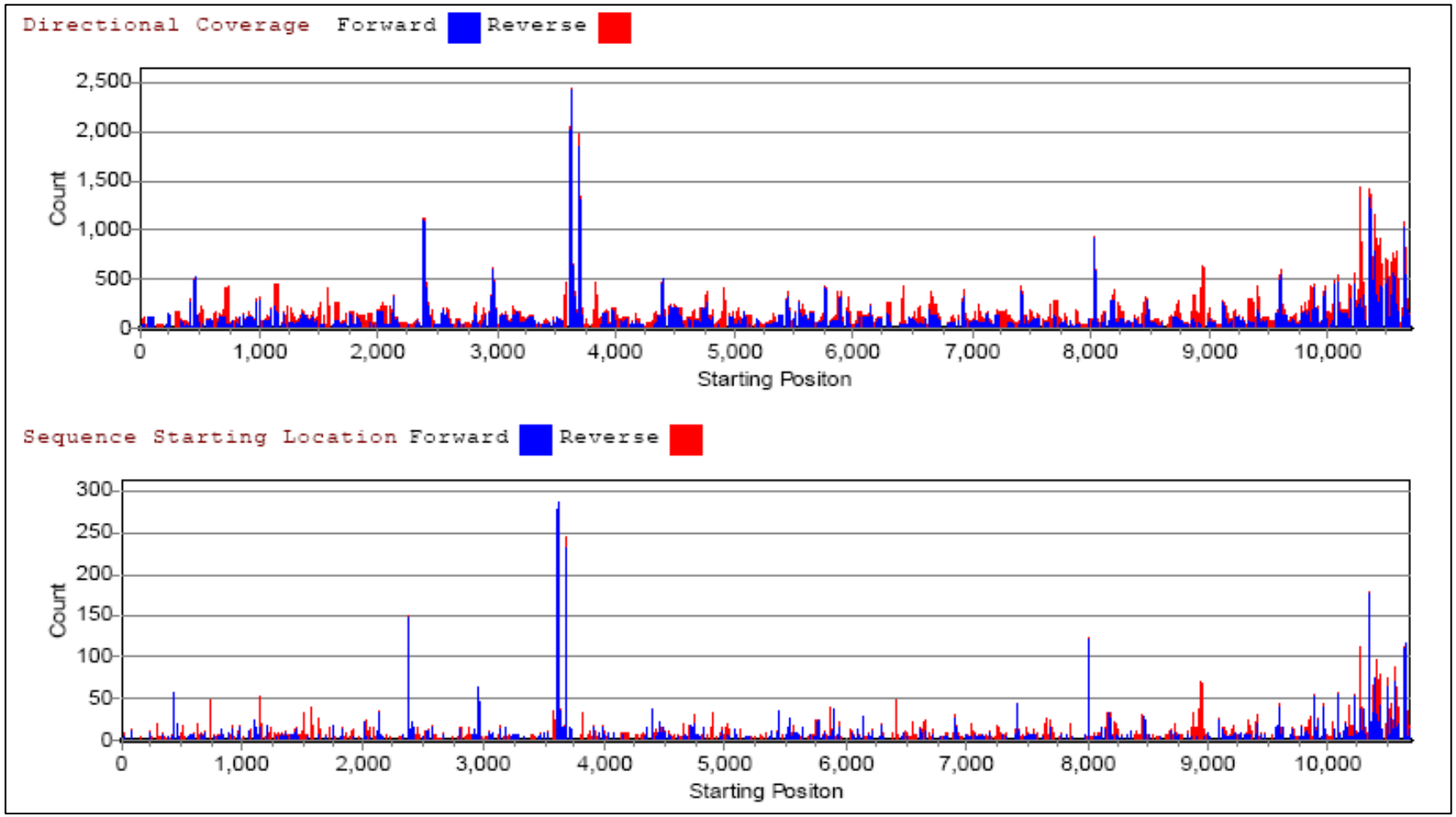


Figure 4.2: Alignment of CFAV viRNAs from Aag2 mock-(DENV)infected along the CFAV genome. Blue indicates the viRNA matches the positive sense strand and red indicates the viRNA matches the negative sense strand. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

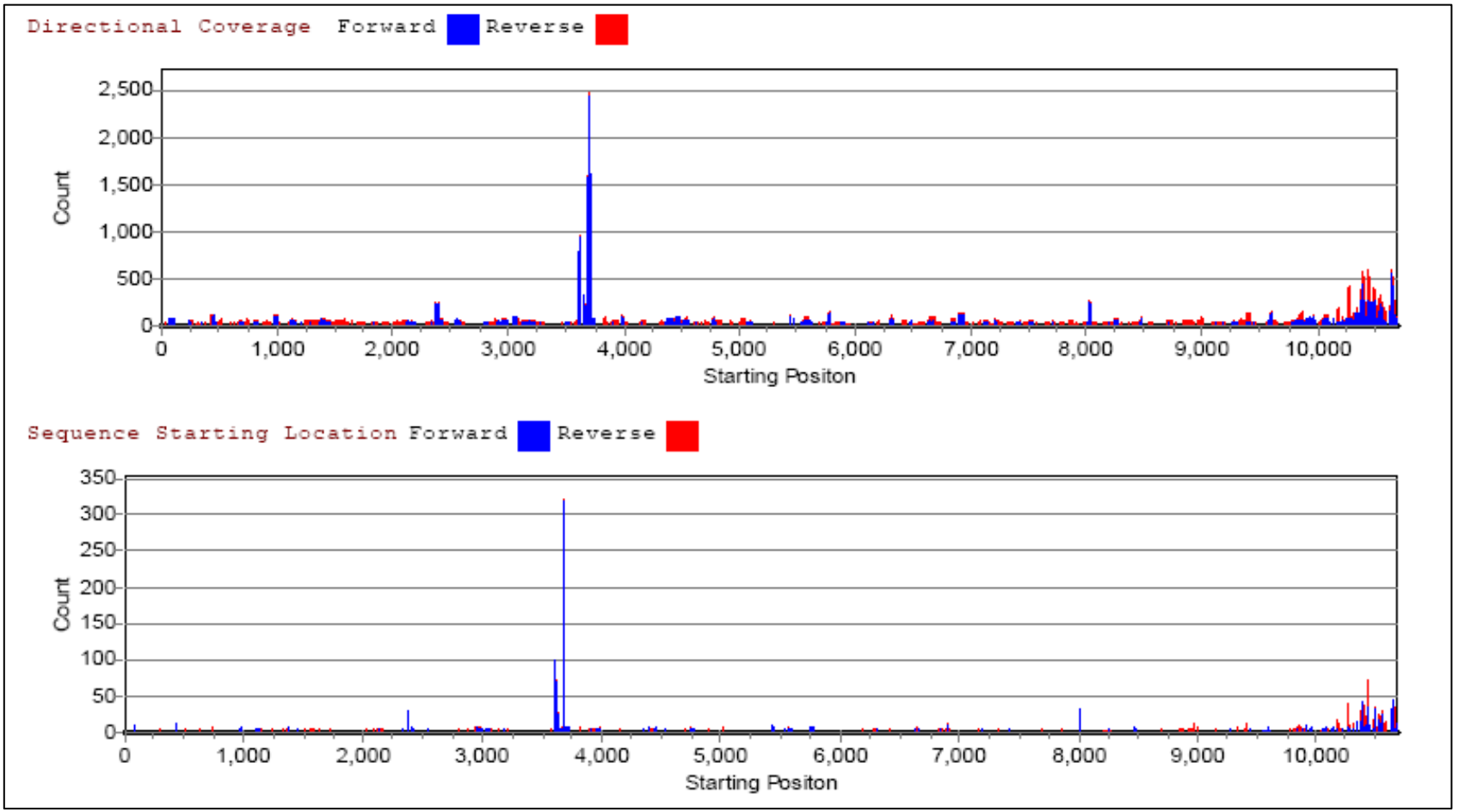


Figure 4.3: Alignment of CFAV viRNAs from DENV2-infected (one day post-infection) Aag2 cells along the CFAV genome. Blue indicates the viRNA matches the positive sense strand and red indicates the viRNA matches the negative sense strand. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

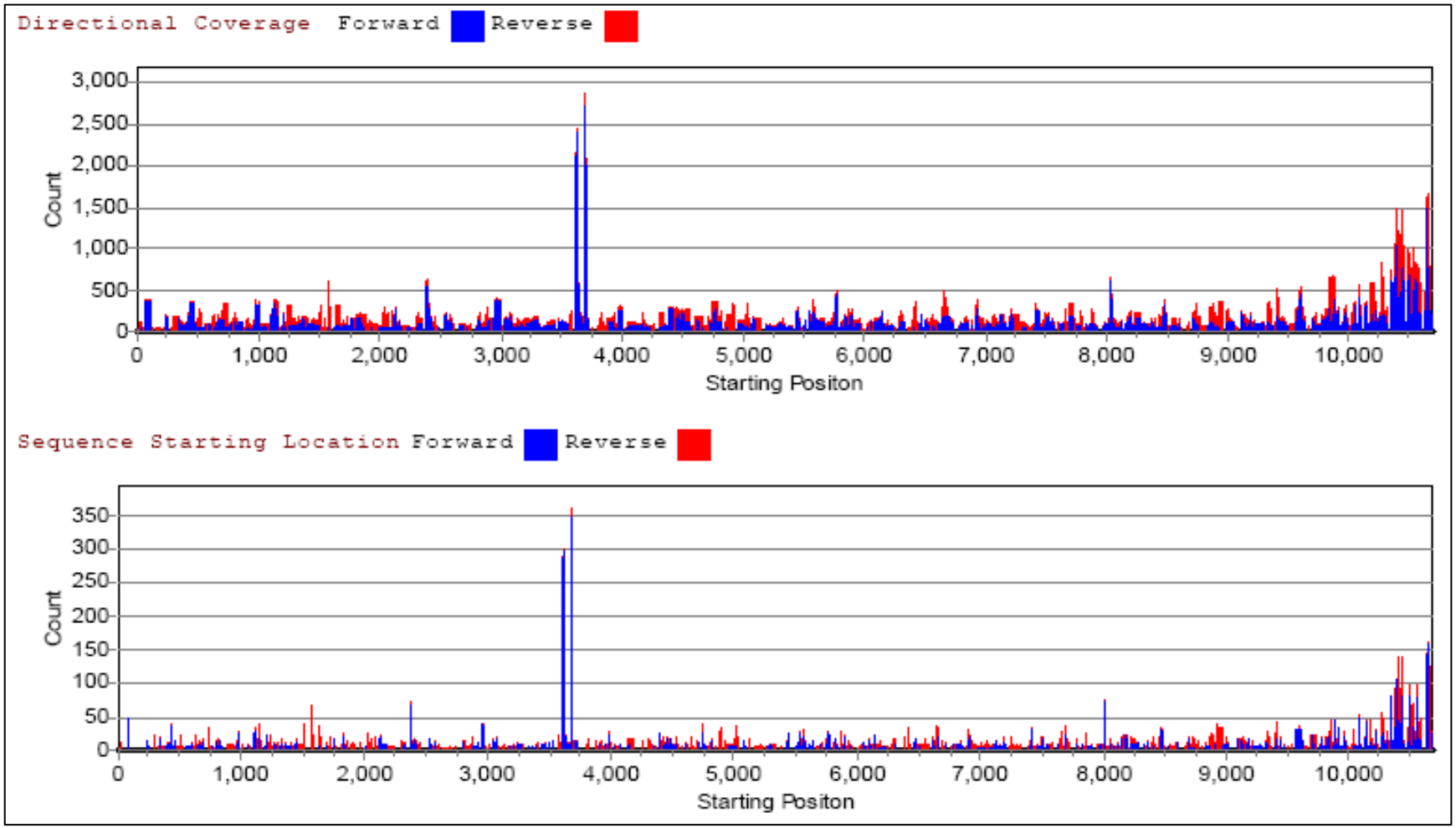


Figure 4.4: Alignment of CFAV viRNAs from DENV2-infected (five days post-infection) Aag2 cells along the CFAV genome. Blue indicates the viRNA matches the positive sense strand and red indicates the viRNA matches the negative sense strand. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

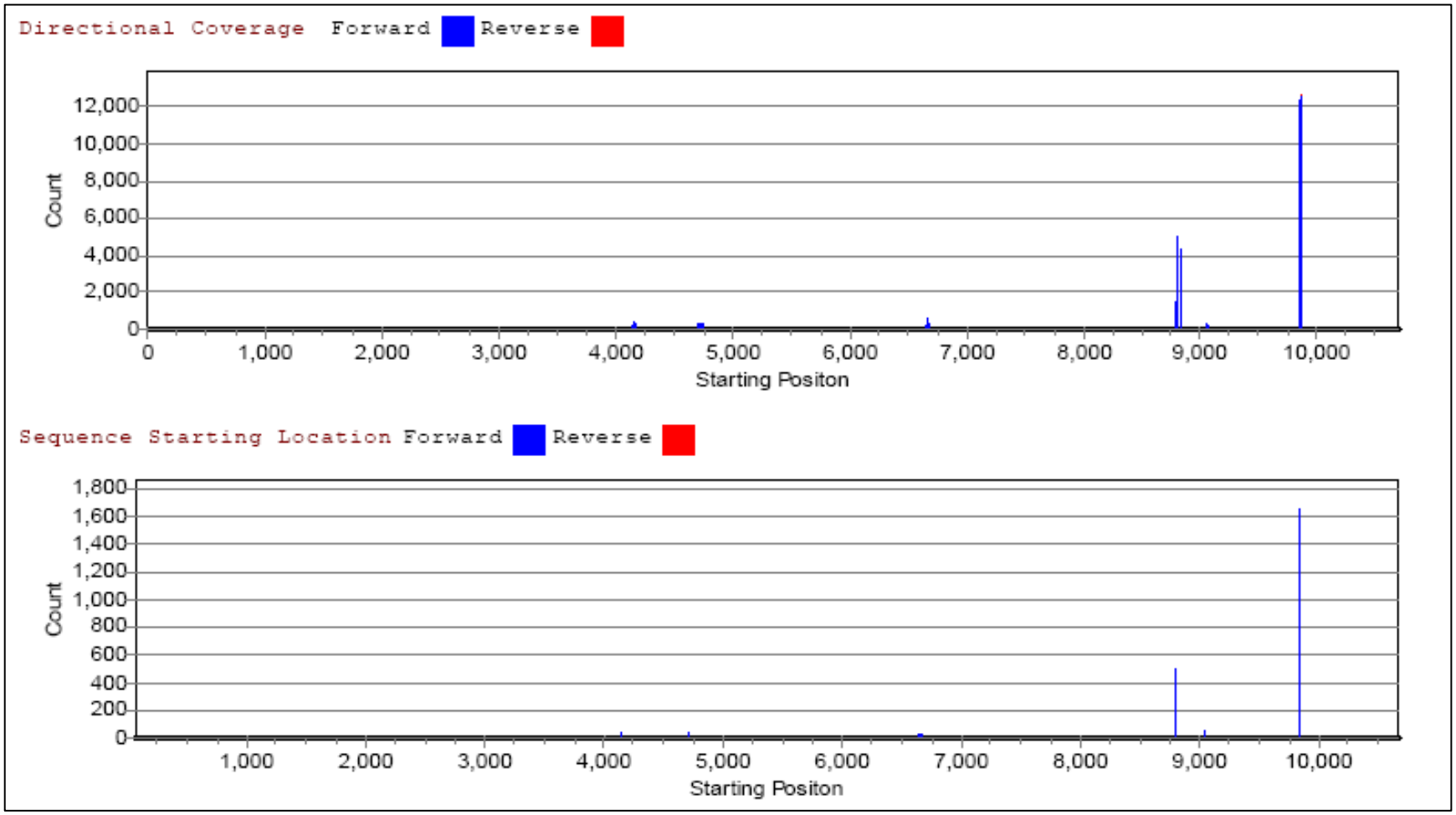


Figure 4.5: Alignment of CFAV viRNAs from DENV2-infected (five days post-infection) C6/36 cells along the CFAV genome. Blue indicates the viRNA matches the positive sense strand and red indicates the viRNA matches the negative sense strand. The top graph shows the distribution when the small RNAs are allowed to overlap (shouldering), and the bottom graph shows the distribution of each siRNA based on its starting nucleotide postion and does not allow for overlapping viRNAs to increase the peak size.

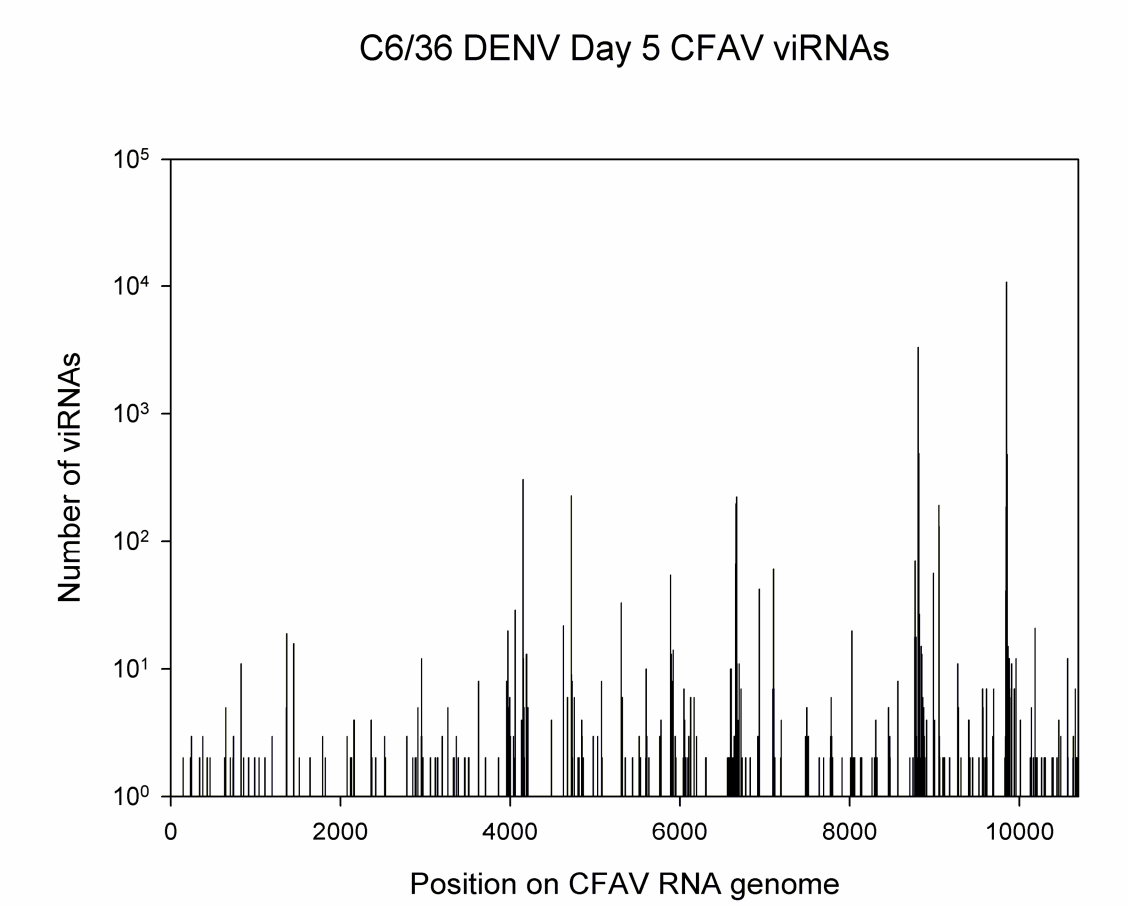


Figure 4.6: Distribution of CFAV viRNAs from C6/36 infected with DENV2 (5 dpi) graphed on a log scale.

SOLiD Pipeline Analysis of CFAV viRNAs

SOLiD Pipeline Analysis of CFAV viRNAs was performed to compare with the number of CFAV viRNAs found with the NextGENe software. The numbers of CFAV viRNAs with no mismatches were lower with the SOLiD pipeline than with the NextGENe software (Table 4.1 and Table 4.2). This is probably due to the NextGENe software including small RNAs with mismatches.

Table 4.2: Numbers of CFAV viRNAs found in Aag2 and C6/36 samples from SOLiD sequencing using SOLiD small RNA analysis pipeline.

	Total number of reads	Number of CFAV viRNAs with zero mismatches	Number of CFAV viRNAs with one mismatch
Aag2 Mock	14087714	64655 (0.46%)	38259 (0.27%)
Aag2 DENV2 Day 1	12615439	20627 (0.16%)	11399 (0.09%)
Aag2 DENV2 Day 5	12131018	71882 (0.59%)	33759 (0.28%)
C6/36 Mock	11915311	2 (0.00%)	9 (0.00%)
C6/36 DENV2 Day 5	12558261	18787 (0.15%)	8617 (0.07%)

Logo Analysis of CFAV-specific small RNAs

The DENV2- and CFAV-specific small RNAs from both cell types were analyzed with the WebLogo 3 program (http://weblogo.threeplusone.com) to determine if there were preferences for specific nucleotides at certain positions. The total untrimmed 35 nt length of virus RNA-matching reads was analyzed in the program; therefore, the sequences for the six 3'-terminal nucleotides match the linker attached to the small RNAs in preparation of libraries. In both the DENV2 and CFAV viRNAs from Aag2 cells, there were no apparent preferences for specific nucleotides at any positions in the 5' 21 nt (Figure 4.7A and Figure 3.4A). However, in the C6/36 cell libraries, there appeared to be a bias for adenine on the nucleotide at position 10 in the CFAV-specific small RNAs (Figure 4.7B). Ago3-associated Piwi-interacting RNAs (piRNAs) often have an adenine at the 10^{th} position, hinting at a possible mechanism for generation of these small RNAs in C6/36 cells (Brennecke et al., 2007, Gunawardane et al., 2007).

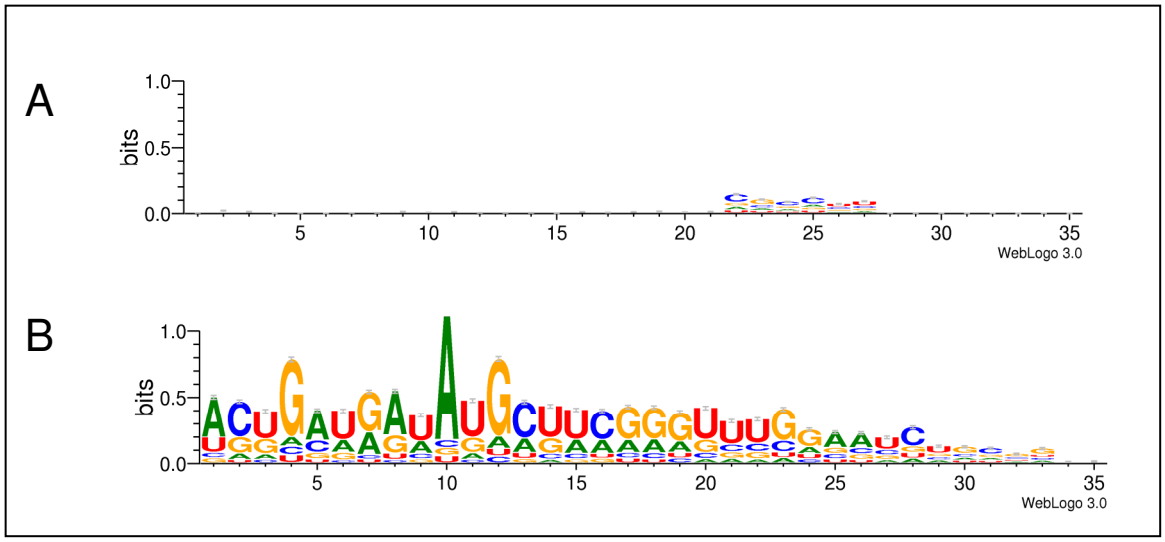


Figure 4.7. Logo analysis of CFAV viRNAs from mosquito cell cultures. Logo analysis was performed on the viRNAs using WebLogo 3. (A) Aag2 CFAV viRNA logo. (B) C6/36 CFAV viRNA logo.

RT-PCR for CFAV RNA

RT-PCR was used to confirm if CFAV RNA was present in the small RNA libraries, as well as in our cell cultures. RT-PCR to detect CFAV RNA was performed on RNA used in SOLiD sequencing analysis (prepared in October 2008), as well as freshly prepared RNA from uninfected C6/36 cells, persistently-CFAV infected C6/36 cells and Aag2 cells (grown in April 2009). All of the samples from Aag2 cells, as well as from the C6/36 cells infected with DENV2 and harvested at 5 dpi were positive (Figure 4.8A). The C6/36 cells that were not infected before preparation of the SOLiD sequencing samples (October 2008) were not positive for CFAV via RT-PCR (Figure 4.8A). It is not surprising that the C6/36 cells infected with DENV2 for the SOLiD small RNA sequencing were positive for CFAV RNA, since the DENV2 used to infect these cells was grown in Aag2 cells, and thus the CFAV was introduced to the C6/36 cells during the DENV2 infection. The C6/36 cells that were growing in April 2009 did have

a faint band in the RT-PCR reaction (Figure 4.8A and B). This may mean they had a low level of contamination with CFAV (although this probably would have produced more CFAV RNA) or the RT-PCR reaction had a slight contamination with Aag2 RNA.

The C6/36 cells that were persistently infected with CFAV by adding cell culture medium from Aag2 cells (filtered through 0.1 µm filter) were strongly positive for CFAV RNA by RT-PCR (Figure 4.8B). This indicates that the CFAV from persistently infected Aag2 cells is shed into the cell culture medium and can infect C6/36 cells. The control reactions where no reverse transcriptase (RT) was added were all negative, indicating that the CFAV sequence was amplified from viral RNA, not DNA integrations.

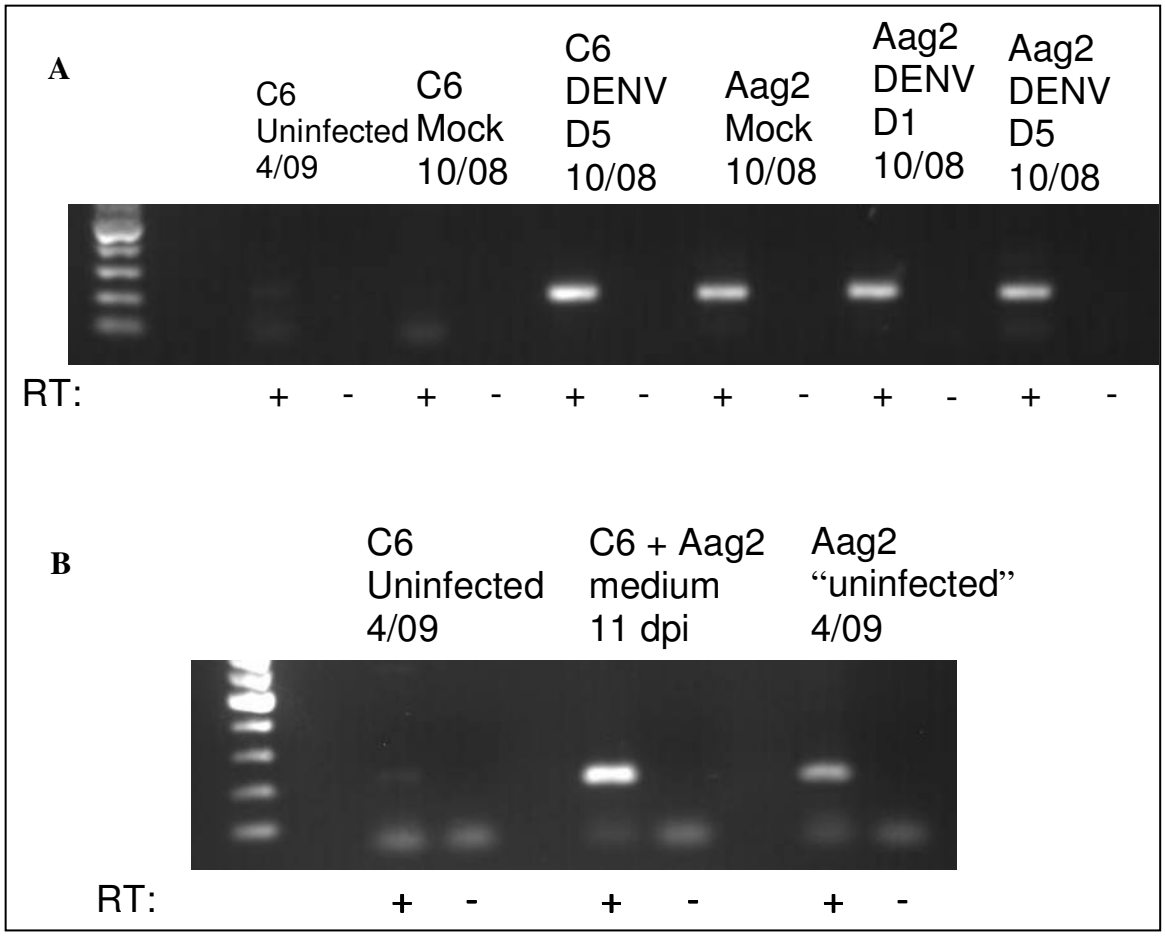


Figure 4.8: RT-PCR for CFAV RNA. Template was total RNA used to make SOLiD libraries (A) and RNA from April 2009 cell cultures of C6/36 uninfected, C6/36-CFAV infected and Aag2 cells. Reactions were set up with and without reverse transcriptase (RT) enzyme to demonstrate amplification was from RNA only.

Alignment of CFAV genome to DENV2 genome

The CFAV and DENV2 (Jamaica 1409) genome sequences were aligned with BLAST to identify regions of sequence similarity that may provide small RNAs from one virus to target the other. The two longest homologous sequence alignments are shown in Figure 4.9. A region 176 nt in length from CFAV nt 8763-8936 (NS5 gene) and DENV2 nt 8890-9063 (NS5) had 72% sequence identity, and another 399 nt region from CFAV nt 9555-9950 (NS5) and DENV2 nt 9692-10086 (NS5) had 65% sequence identity. Even in these regions, there are no areas where the sequences matched for 18 or more

nucleotides. This suggests that small RNAs made from the genome of one virus are not able to target the other virus in a RISC-based sequence specific mechanism.

The sequence identity of the two virus genomes was compared using the EMBOSS (needle) Pairwise Alignment Algorithm. The virus RNAs are approximately 47% identical at the nucleotide level when the full length genomes are aligned using the EMBOSS program (Table 4.3). This is further indication that small RNAs from one virus genome are probably not able to target the genome of the other virus by RNAi in mosquito cells.

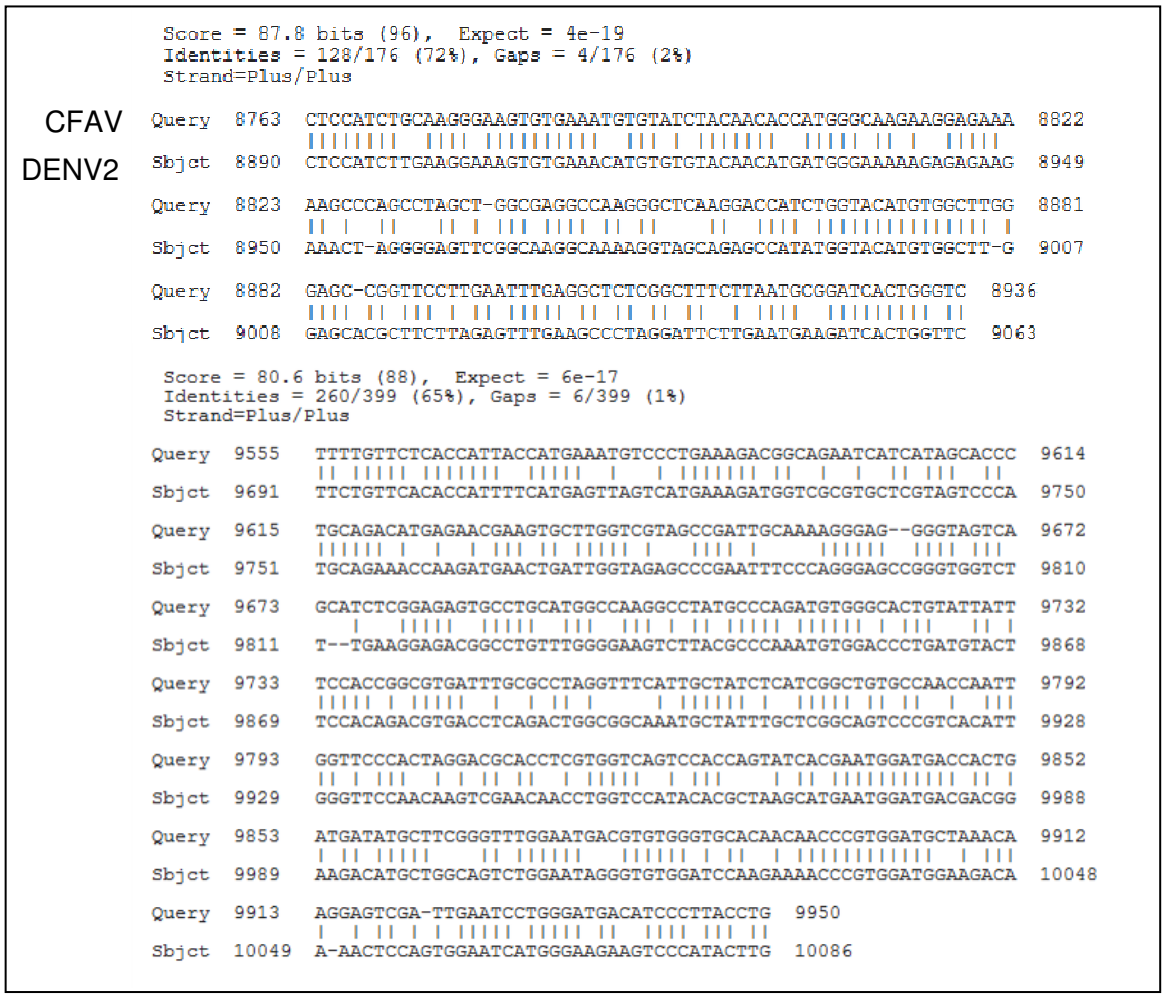


Figure 4.9: Top two results of BLAST alignment of CFAV and DENV2 Jamaica 1409 strain RNAs. CFAV is the Query sequence and DENV2 is the Subject sequence.

Table 4.3: Results of EMBOSS needle Pairwise Alignment Algorithm comparison of DENV2 and CFAV sequences.

Parameter	Score
Identity	6104/12875 (47.4%)
Similarity	6104/12875 (47.4%)
Gaps	4332/12875 (33.6%)
Length	12875
Score	10243.5

Examination of CFAV derived small RNA peak in Aag2 cells

A similar group of peaks of CFAV viRNAs was found in all of the Aag2 cell samples (but not C6/36) when viRNAs were aligned to the CFAV genome. These peaks were located between nt 3600-3800 of the CFAV genome (Figure 4.10). This corresponds to the NS2A gene of the virus. The small RNAs from these peaks were mostly from the positive strand of the genome, indicating they may generate from the secondary structure of the genomic RNA.

When looking at the alignment of CFAV and DENV2 RNAs, only one similar sequence 19 nt in length falls into this region (although this small sequence actually falls between the two peaks), is only 19 nt in length and the match only occurs if one nucleotide from the DENV2 genome is deleted (Figure 4.10). Since there are not many matches from these abundant CFAV small RNAs to the DENV2 genome, it seems unlikely that these small RNAs have any role in reducing DENV2 replication in of Aag2 cells.

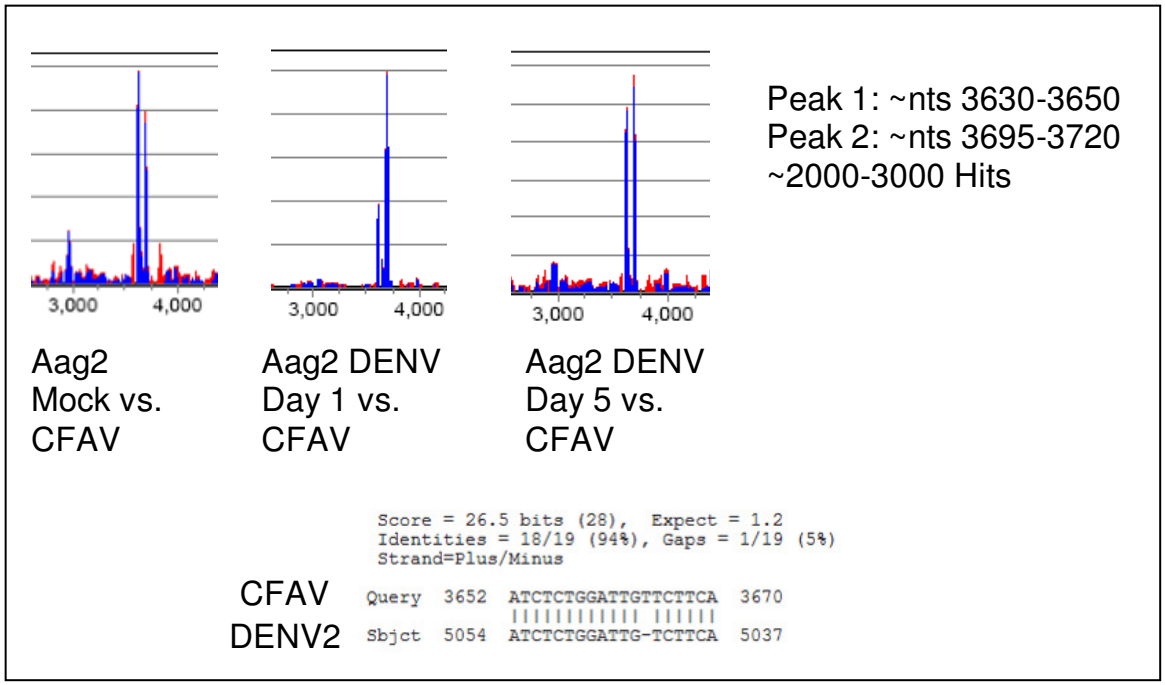
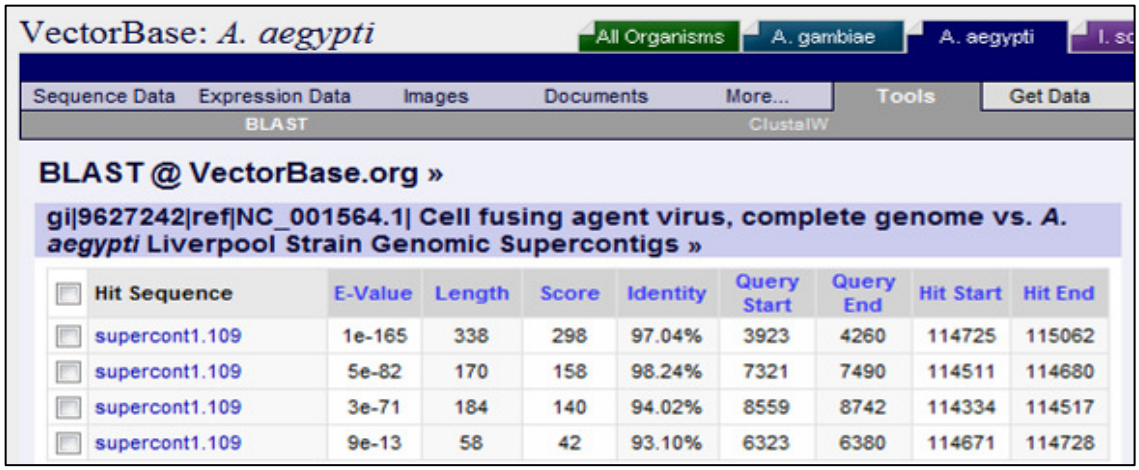


Figure 4.10: Examination of similar CFAV and DENV2 RNA sequences aligning with nt 3000-4000 of CFAV RNA. Region from BLAST alignment that had nucleotide identity between CFAV and DENV2 in this 3000-4000 nt region shown.

Search of VectorBase for CFAV-like integrations

The mosquito genome database, VectorBase, was searched for CFAV-like integrations in the *A. aegypti* genome to see if any larger segments of the virus may have been integrated. Various mosquito-only flaviviral-like sequences have been described in mosquito genomic DNA previously (Crochu et al., 2004). A few short segments (58-338 nts in length) closely matching CFAV sequences were found in the *A. aegypti* database (Figure 4.11). All of these sequences are found in the same supercontig, and some of them overlap with each other. How these sequences were integrated into the genome and what role they may play in mosquito immunity to mosquito-only flaviviruses is not understood.



Hit Sequence	E-Value	Length	Score	Identity	Query Start	Query End	Hit Start	Hit End
supercont1.109	1e-165	338	298	97.04%	3923	4260	114725	115062
supercont1.109	5e-82	170	158	98.24%	7321	7490	114511	114680
supercont1.109	3e-71	184	140	94.02%	8559	8742	114334	114517
supercont1.109	9e-13	58	42	93.10%	6323	6380	114671	114728

Figure 4.11: *A. aegypti* genomic sequences in VectorBase that match CFAV genome.

PCR for CFAV DNA Integrations

PCR was performed on DNA extracted from Aag2 and C6/36 cells to determine if the sequences described by Crochu *et al*., (2004) were present in our cell cultures. The same PCR primers described in Crochu *et al*., (2004) were used in the PCR reactions with DNA from Aag2 and C6/36 cell cultures. The sequence described as Cell Silent Agent 2 (CSA2) was found in Aag2 cells, but not in C6/36 cells (Figure 4.12), which was consistent with the results of Crochu *et. al*, (2004), as they found this sequence in *A. aegypti*. Since this sequence insert was found in both *A. aegypti* mosquitoes and in the Aag2 cell line, this suggests that the sequence could have integrated into the genome a long time ago and may be found in many *A. aegypti* populations. Both the Cell Silent Agent (CSA) sequence inserts that were detected by Crochu *et al.* (2004) in C6/36 cells were detected in our C6/36 cells, but not in the Aag2 cells using the same primers (Figure 4.12). The sequence identity between the CSA integration and CFAV is approximately 68%, and the sequence identity between the CSA2 integration and CFAV is approximately 65% (data not shown).

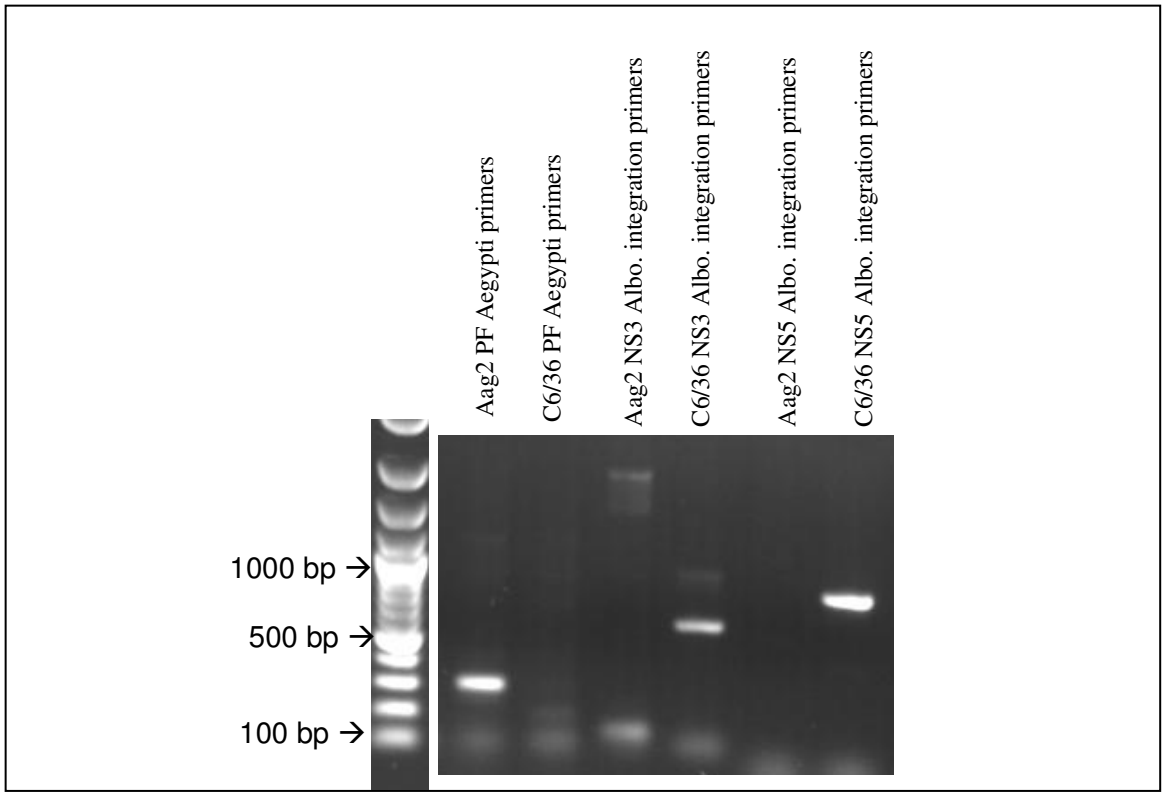


Figure 4.12: PCR for Cell Silent Agent DNA integrations in Aag2 and C6/36 cells. DNA from either Aag2 or C6/36 cells was used in PCR for the integrations describe in Crochu, et. al. (2004), using the same primer sequences. PF stands for the Pan-flavi primers, NS3 and NS5 Albo. stand for the primers used to detect the NS3 and NS5-like sequences found in the *A. albopictus* cells.

Small RNAs from CFAV DNA Integration

Small RNAs potentially transcribed from the Cell Silent Agent 2 (CSA2) genome integration found in Aag2 cells were searched for in the small RNA libraries prepared from both the mock-(DENV2)-infected and DENV2-infected (5 dpi) Aag2 cells for SOLiD sequencing. The purpose was to see if there was a change in small RNA production from these regions during DENV2 infection of the Aag2 cells, to investigate if the CSA2 integrations had a role in small RNA antiviral defense.

The total number of small RNAs that matched this region was 3,286 for the mock-infected and 2,798 for the DENV2 infected cells. Thus, there did not seem to be a

dramatic change in levels of small RNA generated from this region during DENV2 infection (Figures 4.13 and 4.14). All of the small RNAs matched from the negative sense strand of potential RNA transcripts. The most common sizes were 22 and 27 nt in length, possibly hinting at a role for endogenous-siRNA and piRNA type responses. The distribution of the small RNAs along this region was not even, with peaks of small RNAs at the 5' and 3' ends. Nt 548-2026 of CSA2 are similar to the NS5 gene of Kamiti River virus, and nt 2699-3001 are similar to agCP11637 of *A. gambiae*, according to its GenBank entry AY347953. This agCP11637 from *A. gambiae* is related to a transposon from *D. melanogaster*. The presence of small RNAs derived from a transposon-like region is not surprising and these could be generated by an endo-siRNA or piRNA mechanism, as they have roles in protecting the genome from transposons. The presence of a transposable element flanking the virus-like sequence may also explain how the viral sequence may have been integrated into the mosquito genome.

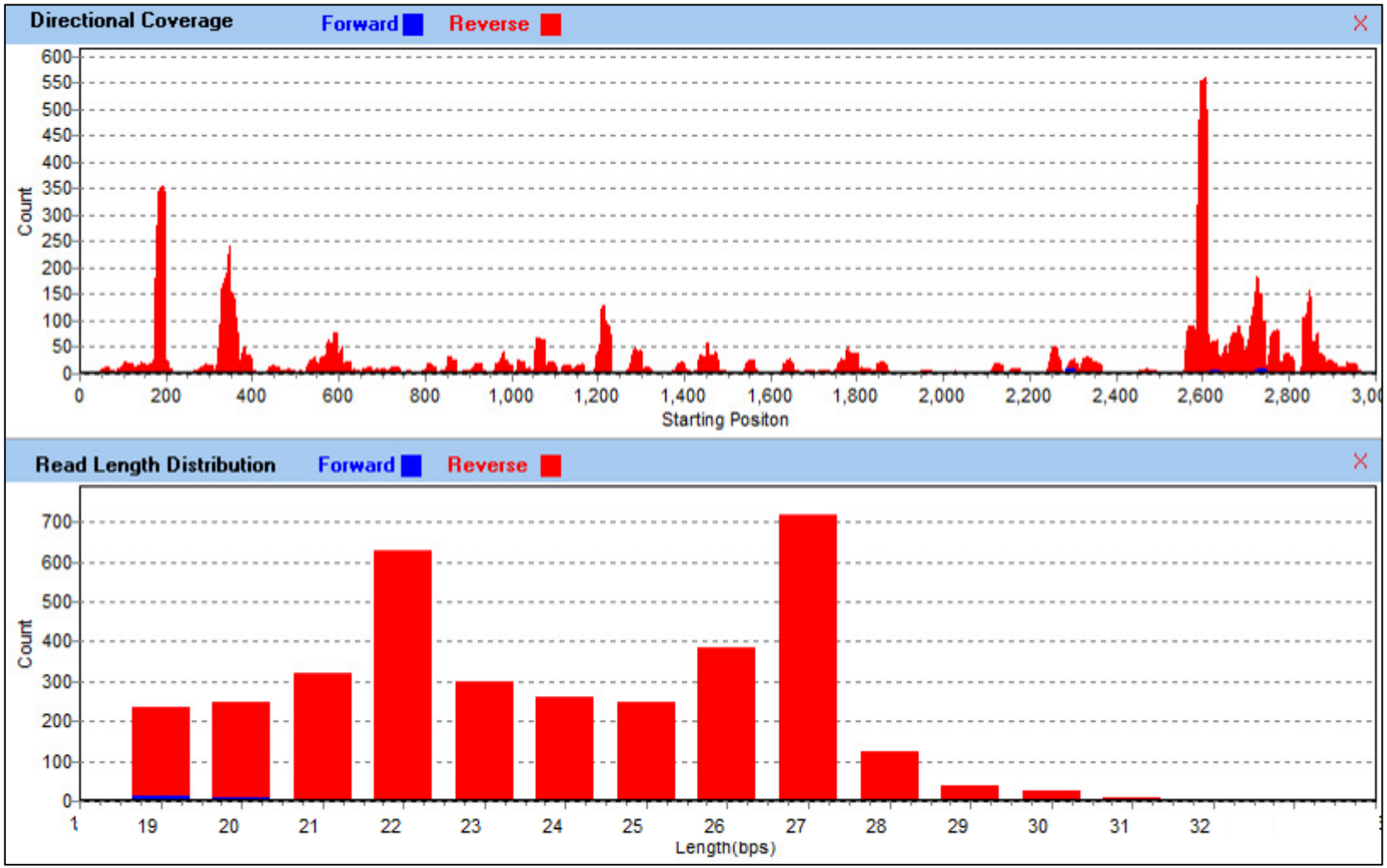


Figure 4.13: Small RNAs matching the Cell Silent Agent 2 sequence described in Crochu *et al.* (2004) in mock infected Aag2 cells. The top graph shows the small RNA size distrubtion across the gene sequence, and the bottom graph shows the size distribution of these small RNAs. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

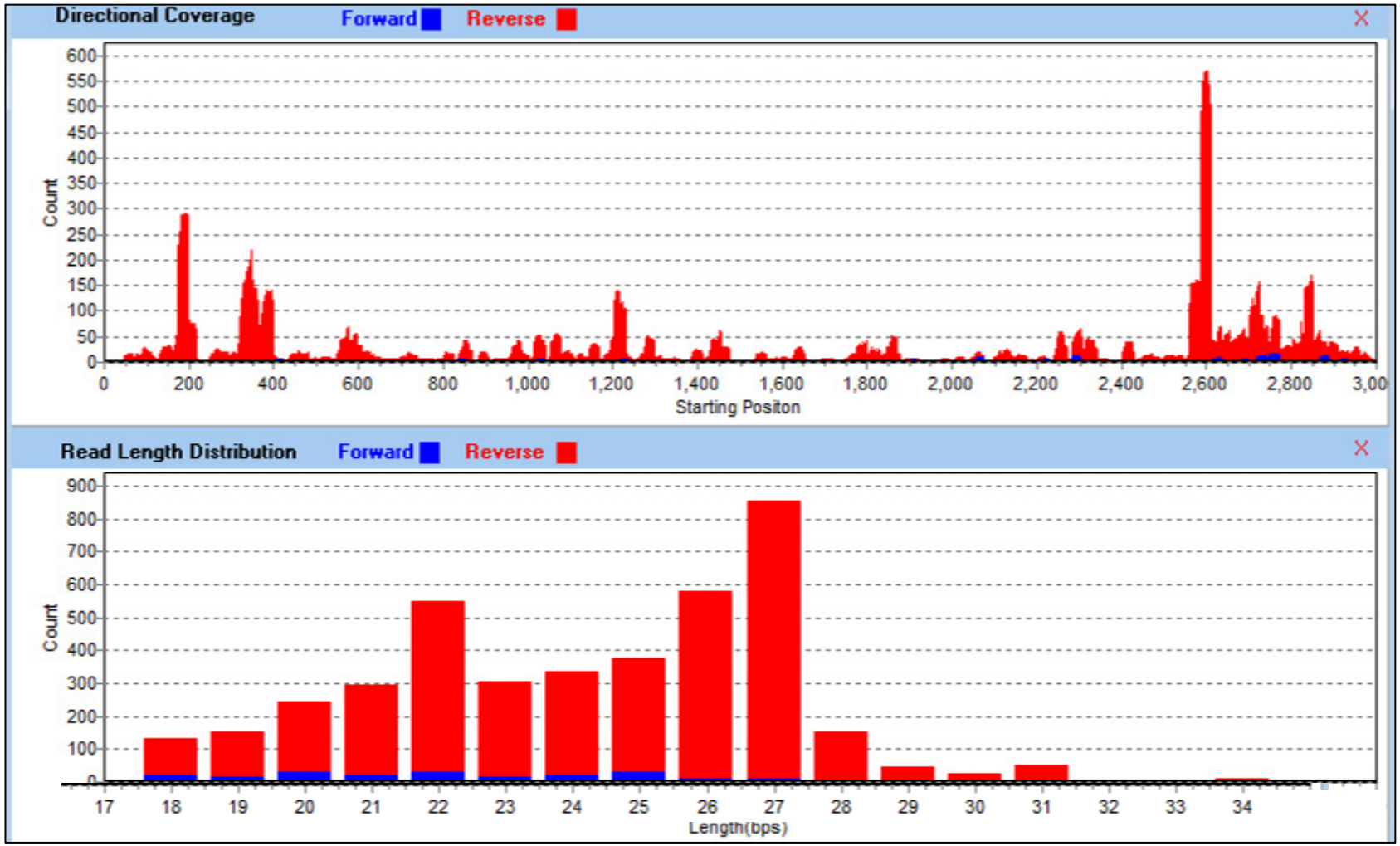


Figure 4.14: Small RNAs matching the Cell Silent Agent 2 sequence described in Crochu *et al.* (2004) in Aag2 cells 5 days post DENV2 infection. The top graph shows the small RNA size distrubtion across the gene sequence, and the bottom graph shows the size distribution of these small RNAs. Blue indicates the small RNA was derived from the positive sense strand, and red indicates the small RNA was derived from the negative sense strand.

Dicing Activity of C6/36 cells Persistently Infected with CFAV

Dicing activity was assayed in CFAV-persistently infected C6/36 cells to determine if the presence of CFAV was the reason Aag2 cells appeard to have more Dcr2 activity in the *in vitro* dicing assay (in Chapter 3). A lysate was made from a C6/36 cell line that was persistently infected with CFAV and compared to Aag2 cell lysate (which are naturally persistently infected with CFAV) and the non-CFAV infected C6/36 cell line (using the same protein concentration in each lysate). The dsRNA added to the lysate was a biotinylated 500 bp segment from the β-gal gene. Neither the uninfected C6/36 cells nor the CFAV-persistently infected C6/36 cell line produced a 21 bp dsRNA cleavage product, while the Aag2 cell line was able to produce a product that was the same size as the human recombinant Dcr product (used as the marker) (Figure 4.15). This indicated that a persistent infection with CFAV did not affect the innate ability of either mosquito cell line to produce Dcr2-like products. The observed differences are most likely due to a deficiency in Dcr2 activity of C6/36 cells (as described in Chapter 3).

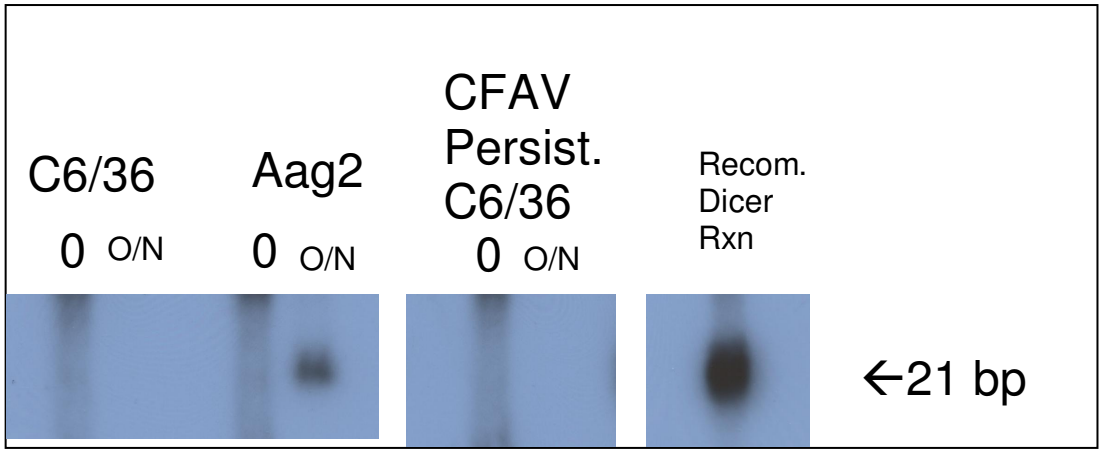


Figure 4.15: *In vitro* dicing assay with C6/36 cells persistently infected with CFAV compared to uninfected C6/36 cells and Aag2 cells. Recominant Dicer reaction shows size of 21 bp RNAs.

Discussion:

CFAV persistent and acute infection presented an opportunity to examine small RNAs of another flavivirus. During our small RNA analysis we discovered many CFAV-specific small RNAs in the Aag2 cell culture samples, but only a few CFAV-matching reads in the *A. aegypti* mosquitoes. The Aag2 cell line is persistently infected with this insect-only flavivirus, and it appears to be recognized by the antiviral RNAi pathway. Although the CFAV RNA-specific proportion of small RNAs was higher (0.2-0.7%), the size distribution and polarity of the CFAV-specific small RNAs in Aag2 cells were similar to the DENV2-specific small RNAs found after DENV2-infection, and these characteristics suggest that they are products of the exogenous siRNA pathway.

We inadvertently co-infected the C6/36 cells with CFAV contained in the DENV2 virus stock, and the CFAV-specific small RNAs produced had similar properties to the DENV2-specific small RNAs, but were uncharacteristic of an exogenous siRNA pathway. Logo analysis of CFAV-specific small RNAs from C6/36 cells showed a bias for adenine at the 10^{th} position from the 5' end, which is also characteristic of piRNAs bound by Ago3 (Brennecke et al., 2007, Gunawardane et al., 2007).

The possibility that CFAV-derived small RNAs could affect replication of DENV2 in the Aag2 cells was examined. NextGENe analysis identified a large peak of positive-sense small RNAs between CFAV RNA nt 3600-3800 in all three of the Aag2 samples. This region was also compared to the sequences of the DENV2 genome to examine if small RNAs from this peak could target DENV2 RNA. A 19 nt match, with one gap was found, so it is most likely too small to function in targeting of DENV2 RNA in the cell.

The CFAV and DENV2 genomes were compared to determine if there is enough sequence similarities for small RNAs from one virus to target the other virus. These alignments did not show very many areas of the viral genomes with exactly matched sequences, so a sequence-specific response to DENV2 infection in CFAV-persistently infection of Aag2 cells seems unlikely, although an increase the in level of RNAi activity due to persistent CFAV may have a non-specific effect on DENV2 replication in these cells. Possible effects of CFAV persistent infection on DENV2 replication in the Aag2 cells are unknown and need further study.

The cell lines were also examined for the DNA integrations of mosquito-only flavivirus sequences that were previously found by Crochu *et al.* (2004). The *A. aegypti* integration (CSA2) was found in the Aag2 cells, and the integration in C6/36 cells described by Crochu *et al.* (2004) was also found in our C6/36 cell line. The small RNAs that matched the CSA2 integration in Aag2 cells were analyzed to examine if they changed in response to DENV2 infection. The number of CSA2 small RNAs actually went down slightly after DENV2 infection (5 dpi), although the numbers were fairly low overall for both the DENV2-infected and DENV2-mock infected Aag2 samples. Interestingly, all of the small RNAs from this region were derived from the negative strand of the mosquito genome, indicating they may be derived from an endo-siRNA or piRNA pathway-like small RNA mechanisms. A large peak of CSA2-derived small RNAs matched the area of the CSA2 integration around nt 2600 that corresponds to a known *Drosophila* transposon (which may have been responsible for the viral sequence integration in the DNA) (Crochu et al., 2004), further implicating a role for the endo-

siRNA or piRNA pathways in defending the integrity of the mosquito genome from areas with transposons.

C6/36 cells were also persistently infected with CFAV by 'infecting' them with medium from Aag2 cells. A cell-free lysate was made from these persistently CFAV-infected C6/36 cells to test if the presence of CFAV was the reason that Aag2 cells had better dicing activity in the cell-free lysate than non-CFAV infected C6/36 cells (Chapter 3). The CFAV-persistently infected C6/36 cell line did not cleave the β-gal dsRNA into 21 bp small RNAs, which is the same result seen in uninfected C6/36 cells, showing that the presence of CFAV is not the reason that Aag2 cells have better dicing activity than C6/36 cells.

Overall the analysis of CFAV small RNAs in Aag2 cells showed a similar pattern of small RNA sizes and distributions along the genome as DENV2 small RNAs in the Aag2 cells, indicating that both virus infections seem to be targeted by the mosquito cells' antiviral exogenous siRNA pathway. The inadvertent infection of C6/36 cells with CFAV demonstrated that the small RNAs from CFAV were similar in polarity, sense, and genome distribution as the DENV2-specifc small RNAs in C6/36 cells, showing further indication of the lack of a *Drosophila*-like exogenous siRNA antiviral pathway in these cells. The size and polarity, along with Logo analysis of CFAV-derived viRNAs suggest that CFAV is possibly targeted by a piRNA-like mechanism, which is similar to the results seen in C6/36 cells with DENV2.

There does not appear to be enough similarity between the viral RNAs to result in sequence specific targeting of viRNAs from one virus to the other virus genome, but does not rule out the possibility that the presence of a persistent infection with one of the

viruses many increase the overall amount of antiviral RNA activity in the cell and may explain why DENV2 grows relatively poorly in Aag2 cell cultures. The dynamics of the interaction of mosquito-only flaviviruses and arboviruses, and their effects on the antiviral RNAi activity in the cell are an exciting area for further research in arbovirology.

CHAPTER 5

SUMMARY

In the last decade, small RNA pathways have emerged as a predominant antiviral defense in insects (Galiana-Arnoux et al., 2006, van Rij et al., 2006, Wang et al., 2006). The work described in this dissertation examined the small RNAs produced in mosquito cell lines from both *A. aegypti* and *A. albopictus* mosquitoes during infection with DENV2 and an insect-only flavivirus, CFAV, and also described the apparent dysfunctional exogenous siRNA pathway in the *A. albopictus* cell line C6/36.

Components of the exogenous RNAi pathway in mosquitoes have been shown to play a role in the antiviral response to arboviruses such as DENV, SINV, WNV and ONNV (Brackney et al., 2009, Campbell et al., 2008b, Cirimotich et al., 2009, Keene et al., 2004, Myles et al., 2008, Sanchez-Vargas et al., 2009), as knockdown of RNAi genes resulted in increased viral titers in infected mosquitoes or expression of an inhibitor of the RNAi pathway caused morbidity and mortality from a typically non-pathogenic virus. Although RNAi was shown to modulate arboviral infection of mosquitoes, little was known about how the virus was being targeted by the mosquito's small RNA pathways. Massively parallel sequencing was used in this work to examine how DENV2 interacts with the antiviral RNAi pathways of mosquito cell lines. DENV2 small RNAs from the *A. aegypti* Aag2 cell line had the characteristics of exogenous siRNA pathway products such as a length of 21 nt and a close to equal sense to antisense ratio. The DENV2 small RNAs from the Aag2 cells appeared to be generated from almost all regions of the genome, with few 'hot spots' for Dcr2 cleavage. These results were consistent with the exogenous siRNA antiviral pathway first described in *Drosophila*, and the Aag2 cell DENV2 small RNA characteristics matched those seen in DENV2 infected *A. aegypti* mosquitoes.

When DENV2 small RNAs produced during infection of the *A. albopictus* cell line C6/36 were examined, a very different small RNA size and polarity pattern emerged. DENV2 small RNAs from C6/36 cells were almost all from the positive sense stand and were much longer in length, with the majority of the DENV2 small RNAs being 27 nt in length. The distribution pattern along the DENV2 genome also showed that most of these DENV2 small RNAs from infected C6/36 cells were being generated from specific regions of the genome. These results were consistent with *in vitro* lysate analysis of Dcr2 activity in both C6/36 cells and Aag2 cells, showing that C6/36 cells had a poor ability to cleave exogenous long dsRNA. Further *in vitro* work showed that C6/36 cells did not recognize long dsRNA as a trigger to knockdown GFP expression in the cells, although exogenously provided siRNAs targeting GFP were functional in the C6/36 cells. These *in vitro* results combined with the deep sequencing data indicate that the C6/36 cells have a dysfunctional exogenous siRNA pathway, mostly at the step of Dcr2 cleavage of long dsRNA. Northern blotting for dcr2 mRNA expression indicated that there may be lower levels of Dcr2 expression in the C6/36 cells than in the Aag2 cells. The size and sense of the C6/36-derived DENV2 viRNAs implicated a possible role for the piRNA pathway in the cleavage of viral RNA in the cell. Logo analysis of the DENV2-specific small RNAs from C6/36 cells also implicated the piRNA pathway in their generation. Virus-derived piRNAs were recently described in *Drosophila* (Wu et al., 2010), and could possibly function as a backup system when the exogenous siRNA antiviral pathway is not functioning properly or is overwhelmed.

During our deep sequencing studies, we also determined that the Aag2 cell line was infected with the mosquito-only flavivirus CFAV. There were many more CFAV-

derived small RNAs in our Aag2 samples than DENV2 small RNAs, although their size and polarity suggest that they are also derived from the same exogenous siRNA pathway as the DENV2 small RNAs. C6/36 cells were also inadvertently infected with CFAV, and the CFAV-derived small RNA size, polarity and genome distribution was similar to the DENV2-derived small RNAs from C6/36 cells, showing that this abnormal dicing of viral RNA was not just restricted to DENV2. DENV2 and CFAV sequences were compared to examine if infection with one virus may result in reduced ability to infect the cells with another flavivirus, and it seems there would be little opportunity for siRNA derived from one virus to target the other virus in a sequence-specific fashion, although a persistent infection with a mosquito-only flavivirus could result in an overall increased antiviral RNAi response, and could lead to increased antiviral surveillance by the cellular machinery. Further work studying the dynamics of mosquito-only flaviviruses and arboviral interactions with the antiviral RNAi pathway could provide some valuable insight into mosquito-arboviral infections in the wild.

In summary the work presented in this dissertation showed that the exogenous antiviral RNA pathway appears to target DENV2 during infection of Aag2 cells and *A. aegypti* mosquitoes. Interestingly the DENV2-derived small RNAs from the *A. albopictus* C6/36 cell line appear to be derived from a different small RNA pathway, such as the piRNA pathway, and this cell line seems to have dysfunctional Dcr2 activity. Since this cell line grows arboviruses very well, it has been used extensively for cell culture-based arbovirology studies, and it seems that caution should be used when using the C6/36 cell line for studying the interactions of arboviruses with the cells' antiviral defenses. This work has expanded our knowledge of the interactions of DENV2 and

CFAV with the small RNA pathways of Aag2 and C6/36 mosquito cell cultures, and has opened up the field for further investigation into the small RNA pathway used for antiviral defense in C6/36 cells and how small RNAs and antiviral small RNA pathways may interact in dual infections of mosquito cells.

APPENDIX

Table A.1: Primers used in experiments

Primer name	**Primer sequence 5'→ 3'**
Chapter 2:	
DENV2 M F	ttc cat tta acc aca cgt aat gg
DENV2 M T7 F	taa tac gac tca cta tag ggt tcc att taa cca gac gta atg g
DENV2 M R	tgt cat tga agg agc gac ag
DENV2 M T7 R	taa tac gac tca cta tag ggt gtc att gaa gga gcg aca g
DENV2 nt 1 F	agt tgt tag tct acg tgg acc gac aa
DENV2 nt 1 T7 F	taa tac gac tca cta tag gga gtt gtt agt cta cgt gga ccg aca a
DENV2 nt 4995 R	act cct tgt gac aac acc att acc g
DENV2 nt 4995 T7 R	taa tac gac tca cta tag gga ctc ctt gtg aca aca cca tta ccg
DENV2 nt 4976 F	atg gtg ttg tca caa gga gtg gag
DENV2 nt 4976 T7 F	taa tac gac tca cta tag gga tgg tgt tgt cac aag gag tgg ag
DENV2 nt 10700 R	cat tcc att ttc tgg cgt tct gt
DENV2 nt 10700 T7 R	taa tac gac tca cta tag ggc att cca ttt tct ggc gtt ctg t
5' MRS cloning linker primer	tgg aat tct cgg gca cca agg t
3' cloning linker primer	ctg gaa ttc gcg gtt aaa
BGAL F	gtc gcc agc ggc acc gcg cgc ctt tc
BGAL T7 F	taa tac gac tca cta tag ggg tcg cca gcg gca ccg cgc gcc ttt c
BGAL R	ccg gta gcc agc gcg gat cat cgg
BGAL T7 R	taa tac gac tca cta tag ggc cgg tag cca gcg cgg atc atc gg
For SOLiD library PCR primers see Appendix Table A.2	
Chapter 3:	
T7 Aegypti/Albopictus Dicer2 1211 F	taa tac gac tca cta tag ggc ttt ttg cga aag cct gag gca tc
T7 Aegypti Dicer2 1715 R	taa tac gac tc cta tag gga tta cca tat act gac tgt tct tc
T7 Albopictus Dicer2 1715 R	taa tac gac tca cta tag gga tca cca tat act ggc tgt cct tc
Aegypti Dicer2 4619 F	ccg ggc agt taa cag atc tgc gct cg
T7 Aegypti Dicer2 5116 R	taa tac gac tca cta tag ggc ata cgc ttc gtg ttg ttg gtt cc
Albopictus Dicer2 4619 F	ccg gac agt tga ccg atc ttc gtt cg
Albopictus Dicer2 5116 R	taa tac gac tca cta tag ggc ata cgc ctc atg ctg ctg att cc
For Dicer2 sequencing primers, see Appendix tables A.3 and A.4	
Chapter 4:	
CFAV-3500 FWD	cta gaa cat cga cac taa ctc g
CFAV-3720 REV	gct tcc tcc taa tca tat acc

CSA_NS3 F	gat cat cgt gcg cag ctt tat gg
CSA-NS3 R	cct tgg ttt cag aaa caa tga cc
CSA-seq#2 (NS5) F	aat tag caa gga aga ctt gc
CSA-seq#2 (NS5) R	gtg agg ttc ttt cct caa ga
PF1 F	tgy rtb tay aac atg atg gg
PF1 R	gtg tcc cad ccd gcd gtr tc

Table A.2: Primer sets used in SOLiD library PCR reactions (provided by the SOLiD Small RNA Expression Kit)

5' SOLiD Primer Set	**Sequence 5'→ 3'**
Same 5' set for all reactions	cca cta cga ctc cgc ttt cct ctc tat ggg cag tcg gtg at
3' SOLiD PCR Primers:	**Sequence 5'→ 3'**
SOLiD PCR Primer Set 1	ctg ccc cgg gtt cct cat tct ct**a agc cc**c tgc tgt acg gcc aag gcg
SOLiD PCR Primer Set 2	ctg ccc cgg gtt cct cat tct ct**c aca cc**c tgc tgt acg gcc aag gcg
SOLiD PCR Primer Set 3	ctg ccc cgg gtt cct cat tct ct**c ccc tt**c tgc tgt acg gcc aag gcg
SOLiD PCR Primer Set 4	ctg ccc cgg gtt cct cat tct ct**c atc gg**c tgc tgt acg gcc aag gcg
SOLiD PCR Primer Set 5	ctg ccc cgg gtt cct cat tct ct**t cgt tg**c tgc tgt acg gcc aag gcg
SOLiD PCR Primer Set 6	ctg ccc cgg gtt cct cat tct ct**g ggc ac**c tgc tgt acg gcc aag gcg
SOLiD PCR Primer Set 9	ctg ccc cgg gtt cct cat tct ct**c cct tc**c tgc tgt acg gcc aag gcg

Table A.3: Primers used in Dicer-2 mRNA gene sequencing from Aag2 cells

Primer	Sequence 5'→3'
Aegypti Dicer2-1 F	ggt tag caa aat tta atc gtg
Aegypti Dicer2-395 R	tta tcc aga tct ttg ccc att tcc
Aegypti Dicer2-396 F	gcc att aac tga agg tgg
Aegypti Dicer2-743 F	gtg taa tcg gtc ttt ctg
Aegypti Dicer2-774 R	ata gag taa cat tcc aga aag acc g
Aegypti Dicer2-1225 R	aag taa tac acg acc tca acg
Aegypti Dicer2-1226 F	ttt gcg aaa gcc tga ggc atc
Aegypti Dicer2-1539 F	gga tag aag ggt tct cg
Aegypti Dicer2-1548 R	cct tct atc ctt ttt agc ac
Aegypti Dicer2-1879 F	att ccg ccg ttt ttc ac
Aegypti Dicer2-1903 R	cct tgg cgg tga aaa acg gcg
Aegypti Dicer2-2251 R	ctg cat caa aat cct tcc aat g
Aegypti Dicer2-2252 F	acc tcg gca aac tag cag
Aegypti Dicer2-2567 F	ccg aaa tag aac ttg ctc
Aegypti Dicer2-2645 R	aga aat tcc ttc cac agt
Aegypti Dicer2-2924 R	ccg tact cc gga ttt ggg aaa g
Aegypti Dicer2-2925 F	ttc cta cgc gaa tta ttt c
Aegypti Dicer2-3283 F	gag cgg aaa gga aag cag
Aegypti Dicer2-3299 R	tgc ttt cct ttc cgc tcc ttg
Aegypti Dicer2-3656 F	caa tct tgg atg tac ccg tag
Aegypti Dicer2-3676 R	cta cgg gta cat cca aga
Aegypti Dicer2-4003 F	caa cca cct cta gca acg
Aegypti Dicer2-4012 R	gag gtg gtt gcc agt cgt
Aegypti Dicer2-4334 F	cgc aca atg tcc tga agc
Aegypti Dicer2-4413 R	atc tac ttc acg aat atc aa
Aegypti Dicer2-4781 F	tta ccg atc agg tga ac
Aegypti Dicer2-4789 R	gat cgg taa ttt cgt gtt
Aegypti Dicer2-5100 F	aca aca cga agc gta tgg
Aegypti Dicer2-5215 R	aaa cga aac att act tag cac
Aegypti Dicer2-5557 F	gac tgt tta cat gaa acc
Aegypti Dicer2-5660 R	aga agc taa taa caa agc
Aegypti Dicer2-5909 R	aaa ggt aat ata aaa cgt gc

Table A.4: Primers used in dcr2 mRNA gene sequencing from C6/36 cells

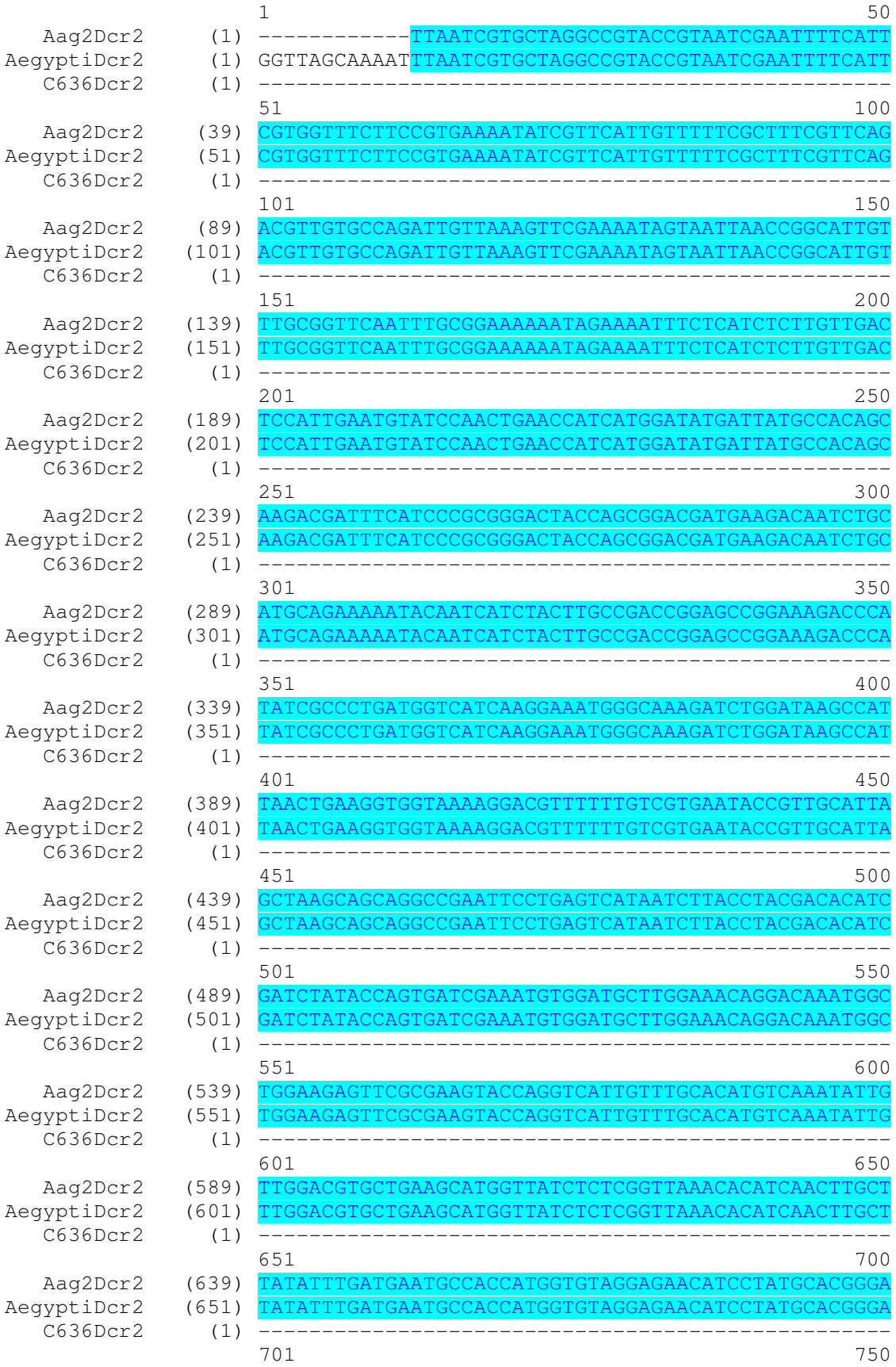
Primer	**Sequence 5'→3'**
Aegypti Dicer2-1226 F	ttt gcg aaa gcc tga ggc atc
C6/36 "1" F	gac tta caa tag ggc gat tga t
C6/36 "501" F	cag tcg aag gga agg gcc agg a
C6/36 "700" R	aat atc tca ata tat aac tcc t
C6/36 "1001" F	ttg act gca gaa atc agc tga a
C6/36 "1200" R	gcc ggc tcc aat gcg caa aac g
C6/36 "1501" F	gta gtc ctt tga aga aat cga c
C6/36 "1700" R	ctc atg cac cat ggt aac gac g
C6/36 "2001" F	ctt ctt ttg gcg gag aag att c
C6/36 "2200" R	gcg acc ttt tcg ctt cct cca a
C6/36 "2501" F	agt ttg cca taa aac tgc tgc a
C6/36 "2700" R	ttc gtg cca ttc ctt gtg ata c
C6/36 "2901" F	gtt gac aat gac gga tga gga g
C6/36 "3200" R	taa tcg atg tgt gtt cta agc c
C6/36 "3423" F	aca gtt gac cga tct tcg tt
C6/36 "3600" R	cag gtt gac ctg atc ggt gat t
C6/36 "3921" R	tgg cga cat acg cct cat gct g
Albopictus Dicer2-2412F	caa cat ttt cca ctc gtt gta c
Albopictus Dicer2-2532 F	tgt gga gac ccc cat cgt gtt gc
Albopictus Dicer2-2592F	gca att cca cat tac ggt att c
Albopictus Dicer2-4203F	tct ttc aca gcg gac caa gg
Albopictus Dicer2-4223R	cct tgg tcc gct gtg aaa ga
Albopictus Dicer2-4397F	ggc tag gaa cac aca tcg att atc g
Albopictus Dicer2-4619F	ccg gac agt tga ccg atc ttc gtt cg
Albopictus Dicer2-4404R	cga taa tcg atg tgt gtt cct agc c
Albopictus Dicer2-5006 F	acg agt tta agc cac cat gtt cg
Albopictus Dicer2-5039F	cgc gcg cca ttc cgg acg atg
Albopictus Dicer2-5060R	cat cgt ccg gaa tgg cgc gac
Albopictus Dicer2-5116 R	cat acg cct cat gct gct gat tcc
Dicer2 Degenerate 3186F	tty ccr gck gac tac tgg ytg aag gc
Dicer2 Degenerate 3583R	tcc cag ttk cgy tcm asa tcw syc g
Dicer2 Degenerate 4956F	gct vga cgt kcc saa ggy gct ggg cg
Dicer2 Degenerate 5280R	ttg gcn gck gcm ckc ttn gcr tck tcc

Nucleotide Code:

A: Adenine
C: Cytosine
G: Guanine
T: Thymine
R: A or G
Y: C or T
S: G or C

Nucleotide Code Contiuned:

W: A or T
K: G or T
M: A or C
B: C or G or T
D: A or G or T
H: A or C or T
V: A or C or G
N: any base


1 50
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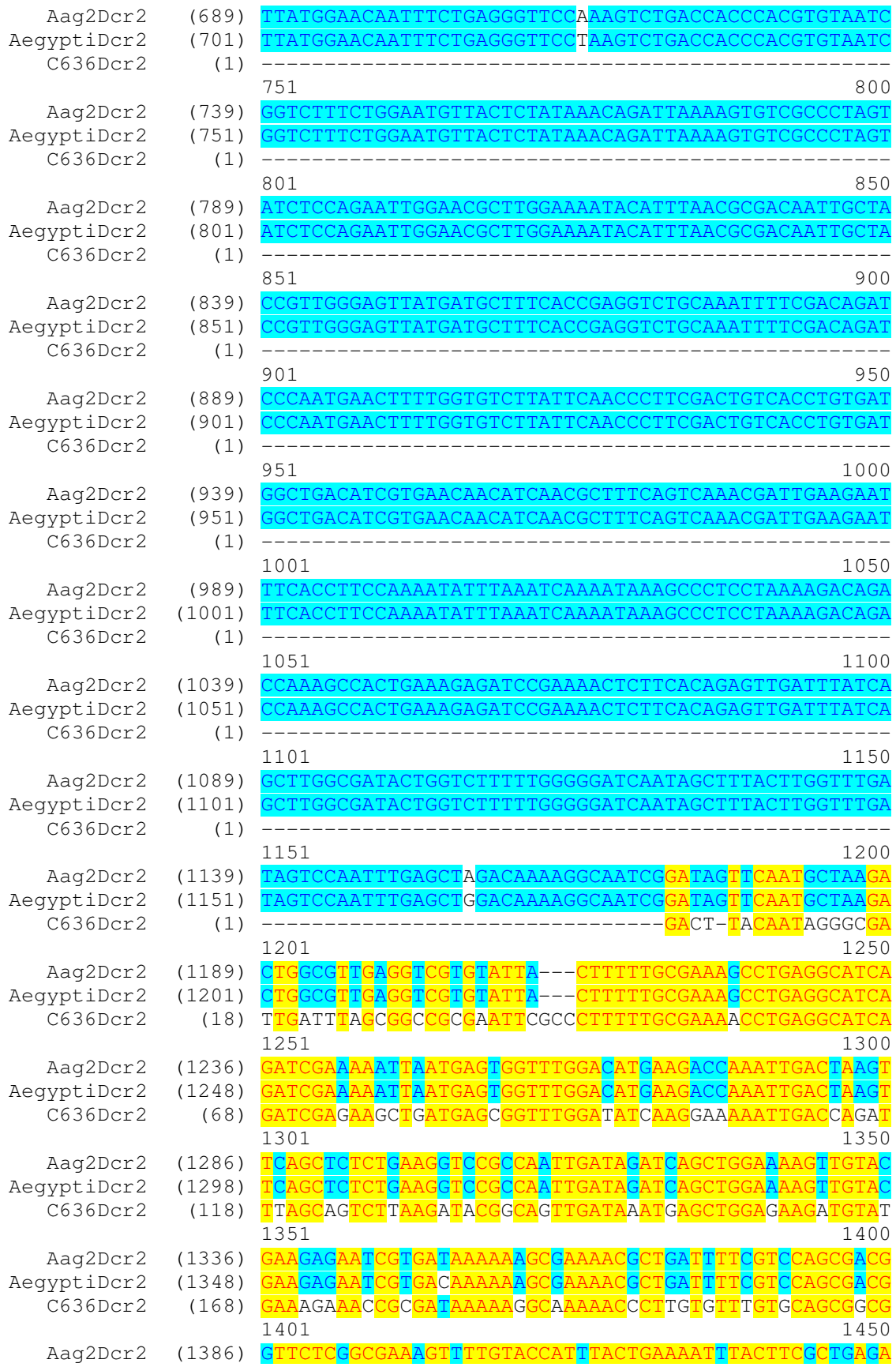
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1201 1250
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1301 1350
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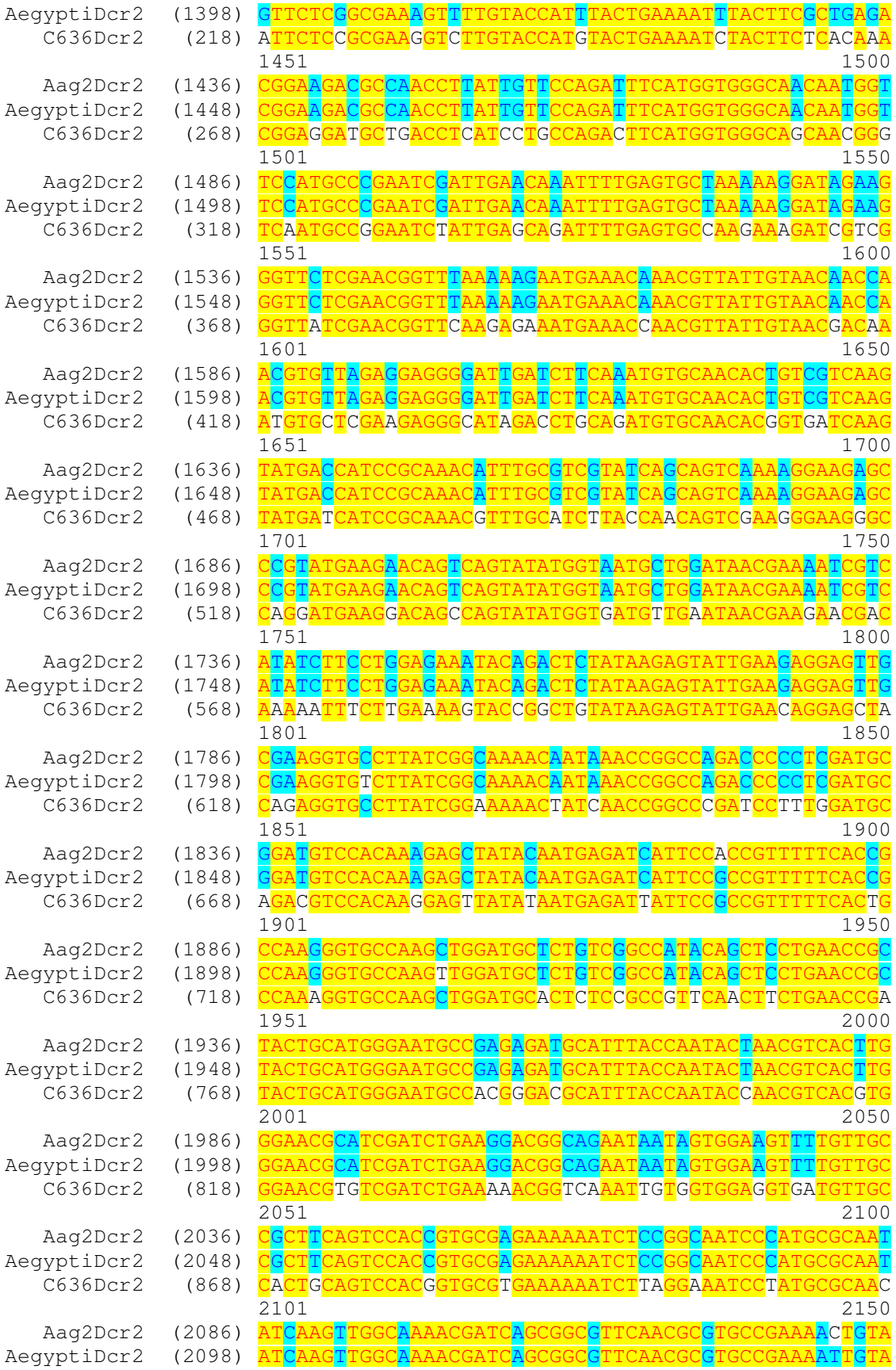
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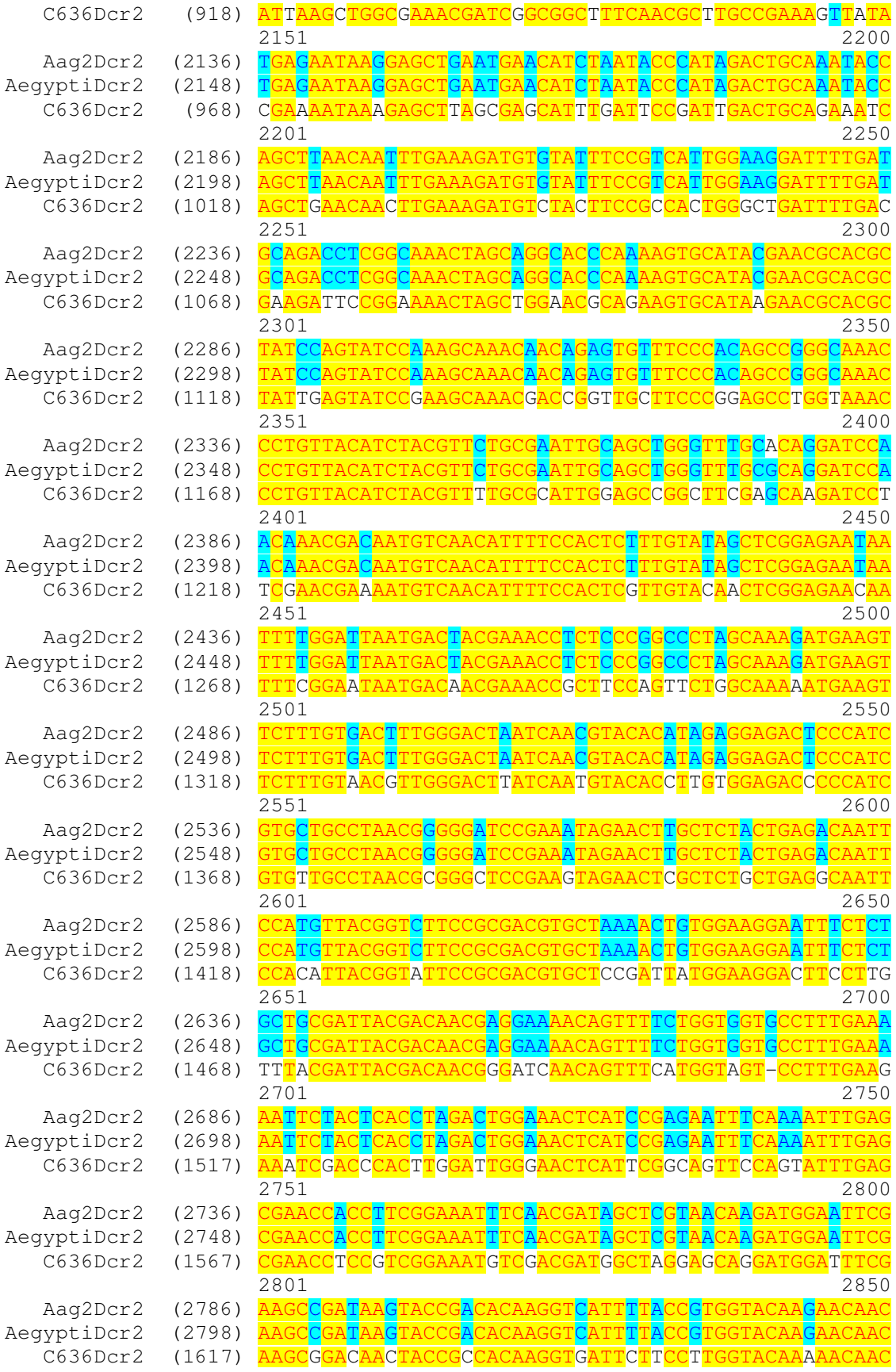
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2251 2300
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   C636Dcr2  (2417) TCGGCATACACATCGGCCATTTACAGACGGGCCACGAGCAGTCCGAAGAG
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AegyptiDcr2  (3645) AGAACCTATGGCAATCCTGGATGTACCCGTAGACCAGAAATTTGCCATTA
   C636Dcr2  (2467) GGAACCAATGGCAATTTTGGACGTTCCGGTAGAGCAAAAGTTTGCCATAA
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   C636Dcr2  (2917) GGATGAGGAGATAAAAACCGGAGTGGTGGATCCGAAGACTAGCGATGAAT
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   Aag2Dcr2  (4133) TTATTGCTCAGCTTGAGCTGCATGGTAACATGCCTGATCCCTCGCCAATG
AegyptiDcr2  (4145) TTATTGCTCAGCTTGAGCTGCATGGTAACATGCCTGATCCCTCGCCAATG
   C636Dcr2  (2967) TCATCGCGCAACTTGAGATGCACGGGAATACGCCCGATCCGTCTCCGATG
                    4201                                             4250
   Aag2Dcr2  (4183) GCAAATTATCTCTCACAGCAGACCATGGGGGACAAGACACCGGCGGATGC
AegyptiDcr2  (4195) GCAAATTATCTCTCACAGCAGACCATGGGGGACAAGACACCGGCGGATGC
   C636Dcr2  (3017) GCCAACTATCTTTCACAGCAGACCATGGGGGACAAGACGCCGGCGGATGC
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   C636Dcr2  (3317) ACCCATGCCTCCTATCCGACTAATCGGATAACCGGAAGCTATCAGCAGTT
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AegyptiDcr2  (4545) GGAATTCCTCGGCGATGCGGTATTAGATTTCCTCATCTCGATGTACATCT
   C636Dcr2  (3367) GGAATTCCTCGGAGATGCGGTCCTGGACTTCCTCATCTCAATGTACATCT
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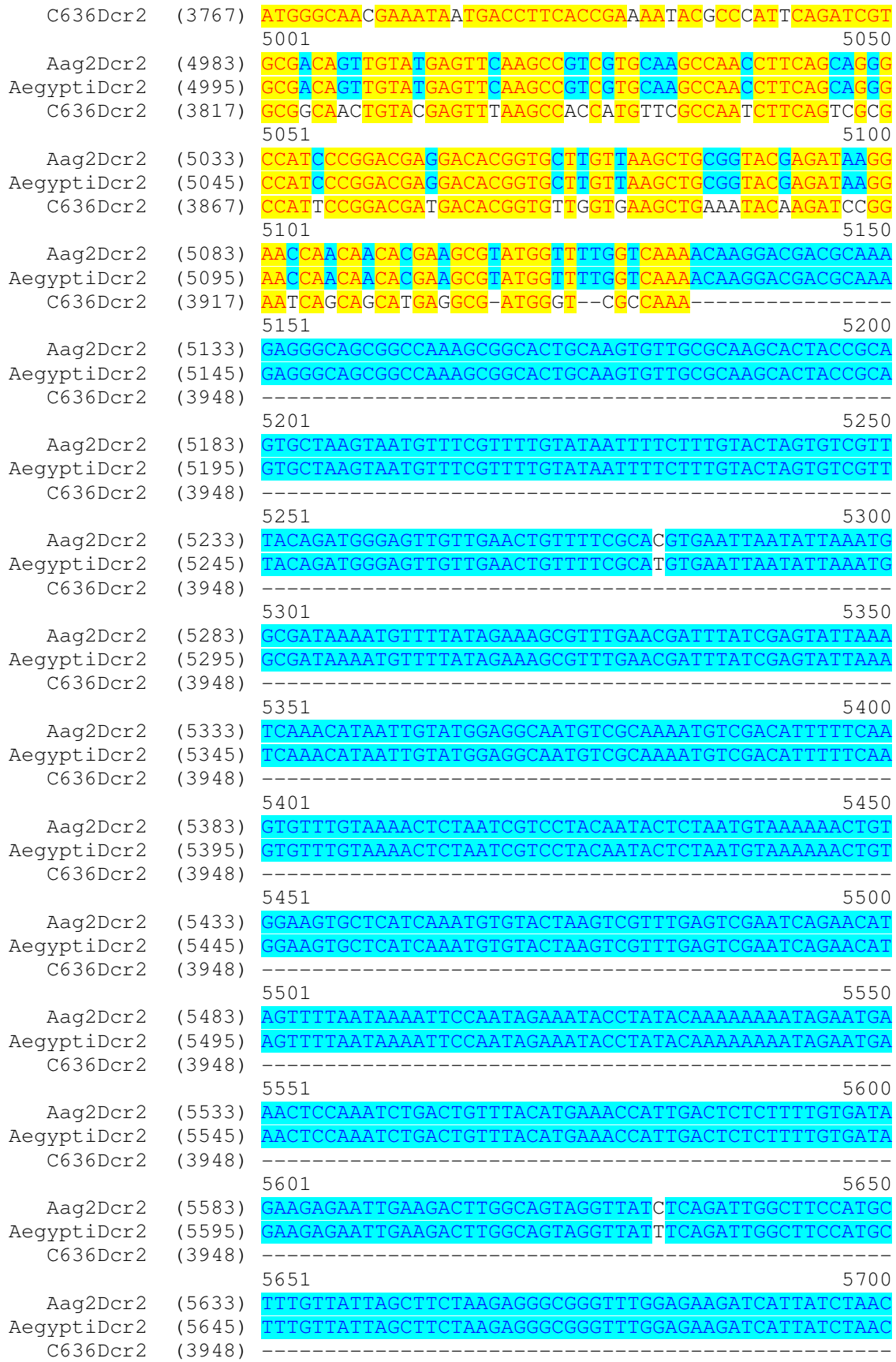
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5051 5100
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5101 5150
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Aag2Dcr2 (5133) GAGGGCAGCGGCCAAAGCGGCACTGCAAGTGTTGCGCAAGCACTACCGCA
AegyptiDcr2 (5145) GAGGGCAGCGGCCAAAGCGGCACTGCAAGTGTTGCGCAAGCACTACCGCA
C636Dcr2 (3948) ----------
5201 5250
Aag2Dcr2 (5183) GTGCTAAGTAATGTTTCGTTTTGTATAATTTTCTTTGTACTAGTGTCGTT
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C636Dcr2 (3948) ----------
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Aag2Dcr2 (5233) TACAGATGGGAGTTGTTGAACTGTTTTCGCACGTGAATTAATATTAAATG
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C636Dcr2 (3948) ----------
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Aag2Dcr2 (5283) GCGATAAAATGTTTTATAGAAAGCGTTTGAACGATTTATCGAGTATTAAA
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C636Dcr2 (3948) ----------
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Aag2Dcr2 (5333) TCAAACATAATTGTATGGAGGCAATGTCGCAAAATGTCGACATTTTTCAA
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C636Dcr2 (3948) ----------
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C636Dcr2 (3948) ----------
5451 5500
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C636Dcr2 (3948) ----------
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C636Dcr2 (3948) ----------
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AegyptiDcr2 (5595) GAAGAGAATTGAAGACTTGGCAGTAGGTTATTTCAGATTGGCTTCCATGC
C636Dcr2 (3948) ----------
5651 5700
Aag2Dcr2 (5633) TTTGTTATTAGCTTCTAAGAGGGCGGGTTTGGAGAAGATCATTATCTAAC
AegyptiDcr2 (5645) TTTGTTATTAGCTTCTAAGAGGGCGGGTTTGGAGAAGATCATTATCTAAC
C636Dcr2 (3948) ----------

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   C636Dcr2  (3948) --------------------------------------------------
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   Aag2Dcr2  (5733) TTGTCTACGTGTGCTGGACGTTTATGATCTTCGGAACTGCGAATATGCAT
AegyptiDcr2  (5745) TTGTCTACGTGTGCTGGACGTTTATGATCTTCGGAACTGCGAATATGCAT
   C636Dcr2  (3948) --------------------------------------------------
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   Aag2Dcr2  (5783) TTAGGCAAAGTGTGCTTAAGATTGTCAAAGAAATGTTTTCCTTCTCAGAA
AegyptiDcr2  (5795) TTAGGCAAAGTGTGCTTAAGATTGTCAAAGAAATGTTTTCCTTCTCAGAA
   C636Dcr2  (3948) --------------------------------------------------
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AegyptiDcr2  (5845) GGAAAACATGAACAATAATAAGAAAAGCTATTTGATTGAATATATGCACG
   C636Dcr2  (3948) --------------------------------------------------
                    5901                      5930
   Aag2Dcr2  (5883) TTTTATATTACCTTTAAAAAAAAAAAAAAA
AegyptiDcr2  (5895) TTTTATATTACCTTTAAAAAAAAAAAAAAA
   C636Dcr2  (3948) ------------------------------
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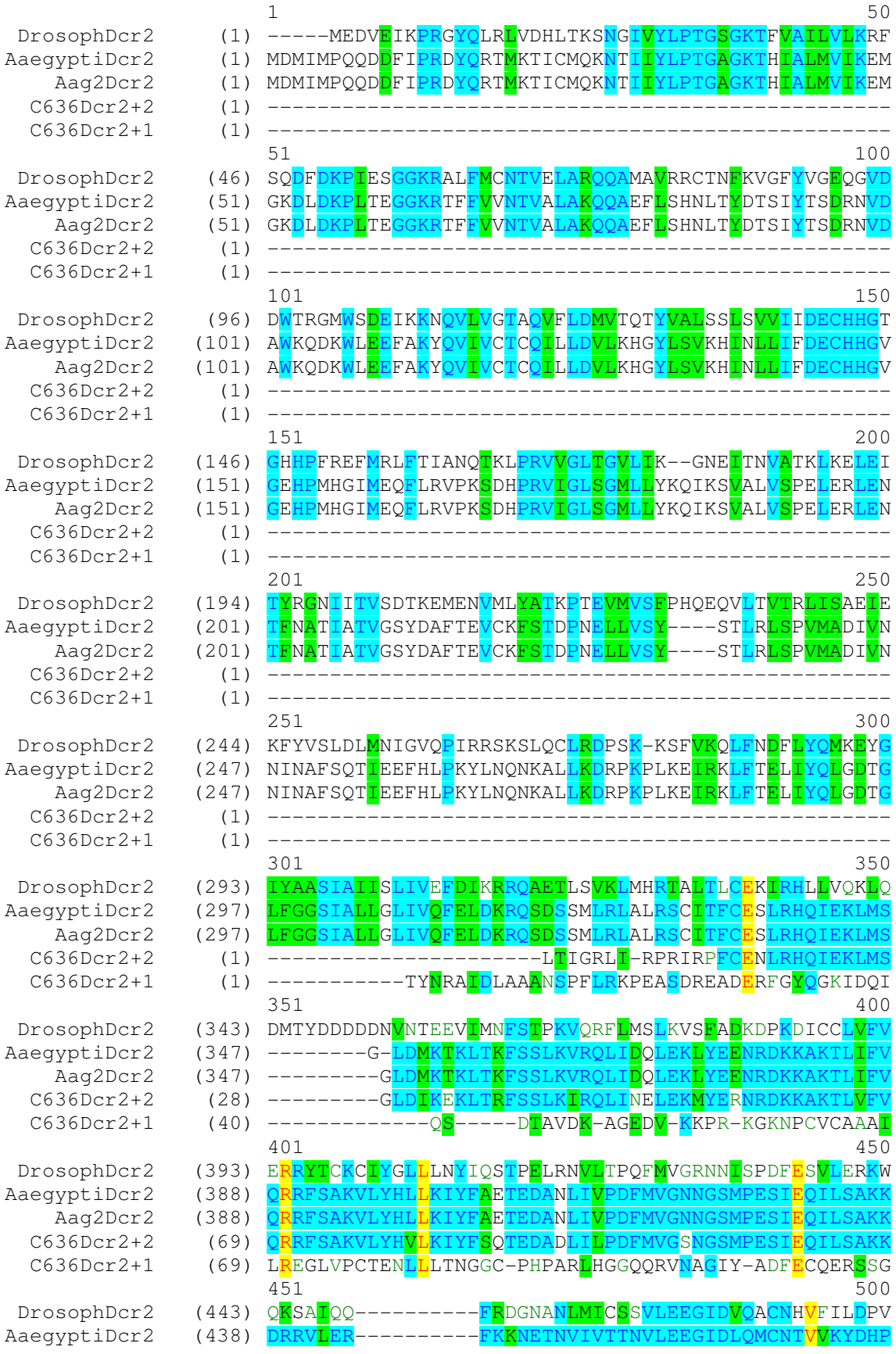
Figure A.1: Alignment of dcr2 nucleotide sequences from *A. aegypti* mosquitoes (from GenBank), Aag2 and C6/36 using VectorNTI AlignX described in Chapter 3.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Aag2Dcr2 (1) MDMIMPQQDDFIPRDYQRTMKTICMQKNTIIYLPTGAGKTHIALMVIKEM
C636Dcr2+2 (1) ----------
C636Dcr2+1 (1) ----------
51 100
DrosophDcr2 (46) SQDFDKPIESGGKRALFMCNTVELARQQAMAVRRCTNFKVGFYVGEQGVD
AaegyptiDcr2 (51) GKDLDKPLTEGGKRTFFVVNTVALAKQQAEFLSHNLTYDTSIYTSDRNVD
Aag2Dcr2 (51) GKDLDKPLTEGGKRTFFVVNTVALAKQQAEFLSHNLTYDTSIYTSDRNVD
C636Dcr2+2 (1) ----------
C636Dcr2+1 (1) ----------
101 150
DrosophDcr2 (96) DWTRGMWSDEIKKNQVLVGTAQVFLDMVTQTYVALSSLSVVIIDECHHGT
AaegyptiDcr2 (101) AWKQDKWLEEFAKYQVIVCTCQILLDVLKHGYLSVKHINLLIFDECHHGV
Aag2Dcr2 (101) AWKQDKWLEEFAKYQVIVCTCQILLDVLKHGYLSVKHINLLIFDECHHGV
C636Dcr2+2 (1) ----------
C636Dcr2+1 (1) ----------
151 200
DrosophDcr2 (146) GHHPFREFMRLFTIANQTKLPRVVGLTGVLIK--GNEITNVATKLKELEI
AaegyptiDcr2 (151) GEHPMHGIMEQFLRVPKSDHPRVIGLSGMLLYKQIKSVALVSPELERLEN
Aag2Dcr2 (151) GEHPMHGIMEQFLRVPKSDHPRVIGLSGMLLYKQIKSVALVSPELERLEN
C636Dcr2+2 (1) ----------
C636Dcr2+1 (1) ----------
201 250
DrosophDcr2 (194) TYRGNIITVSDTKEMENVMLYATKPTEVMVSFPHQEQVLTVTRLISAEIE
AaegyptiDcr2 (201) TFNATIATVGSYDAFTEVCKFSTDPNELLVSY----STLRLSPVMADIVN
Aag2Dcr2 (201) TFNATIATVGSYDAFTEVCKFSTDPNELLVSY----STLRLSPVMADIVN
C636Dcr2+2 (1) ----------
C636Dcr2+1 (1) ----------
251 300
DrosophDcr2 (244) KFYVSLDLMNIGVQPIRRSKSLQCLRDPSK-KSFVKQLFNDFLYQMKEYG
AaegyptiDcr2 (247) NINAFSQTIEEFHLPKYLNQNKALLKDRPKPLKEIRKLFTELIYQLGDTG
Aag2Dcr2 (247) NINAFSQTIEEFHLPKYLNQNKALLKDRPKPLKEIRKLFTELIYQLGDTG
C636Dcr2+2 (1) ----------
C636Dcr2+1 (1) ----------
301 350
DrosophDcr2 (293) IYAASIAIISLIVEFDIKRRQAETLSVKLMHRTALTLCEKIRHLLVQKLQ
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Aag2Dcr2 (297) LFGGSIALLGLIVQFELDKRQSDSSMLRLALRSCITFCESLRHQIEKLMS
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C636Dcr2+1 (1) ----------TYNRAIDLAAANSPFLRKPEASDREADERFGYQGKIDQI
351 400
DrosophDcr2 (343) DMTYDDDDDNVNTEEVIMNFSTPKVQRFLMSLKVSFADKDPKDICCLVFV
AaegyptiDcr2 (347) --------G-LDMKTKLTKFSSLKVRQLIDQLEKLYEENRDKKAKTLIFV
Aag2Dcr2 (347) ---------GLDMKTKLTKFSSLKVRQLIDQLEKLYEENRDKKAKTLIFV
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401 450
DrosophDcr2 (393) ERRYTCKCIYGLLLNYIQSTPELRNVLTPQFMVGRNNISPDFESVLERKW
AaegyptiDcr2 (388) QRRFSAKVLYHLLKIYFAETEDANLIVPDFMVGNNGSMPESIEQILSAKK
Aag2Dcr2 (388) QRRFSAKVLYHLLKIYFAETEDANLIVPDFMVGNNGSMPESIEQILSAKK
C636Dcr2+2 (69) QRRFSAKVLYHVLKIYFSQTEDADLILPDFMVGSNGSMPESIEQILSAKK
C636Dcr2+1 (69) LREGLVPCTENLLLTNGGC-PHPARLHGGQQRVNAGIY-ADFECQERSSG
451 500
DrosophDcr2 (443) QKSAIQQ----------FRDGNANLMICSSVLEEGIDVQACNHVFILDPV
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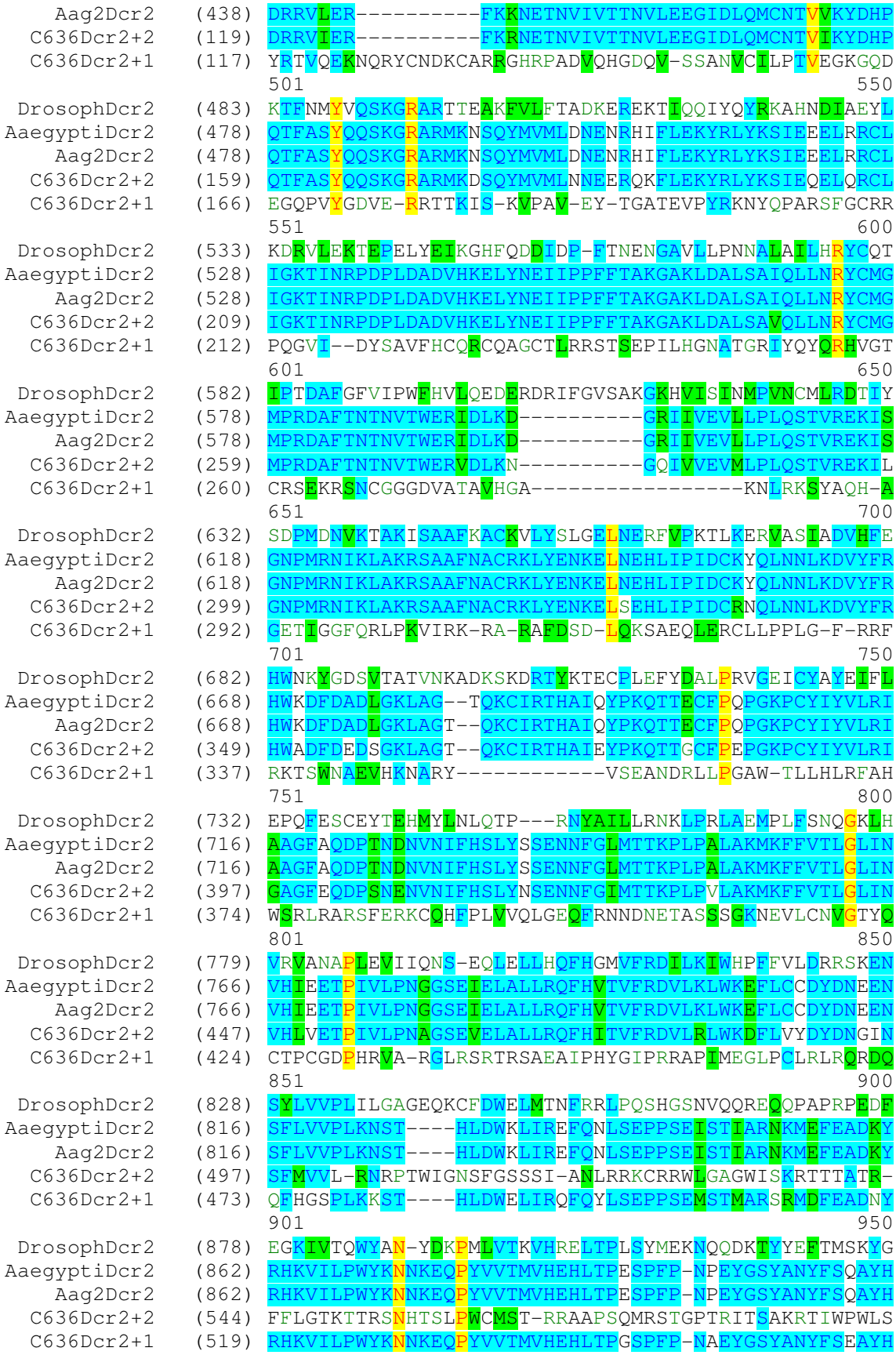
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C636Dcr2+1 (117) YRTVQEKNQRYCNDKCARRGHRPADVQHGDQV-SSANVCILPTVEGKGQD
501 550
DrosophDcr2 (483) KTFNMYVQSKGRARTTEAKFVLFTADKERKTIQQIYQYRKAHNDIAEYL
AaegyptiDcr2 (478) QTFASYQQSKGRARMKNSQYMVMLDNENRHIFLEKYRLYKSIEEELRRCL
Aag2Dcr2 (478) QTFASYQQSKGRARMKNSQYMVMLDNENRHIFLEKYRLYKSIEEELRRCL
C636Dcr2+2 (159) QTFASYQQSKGRARMKDSQYMVMLNNEERQKFLEKYRLYKSIEQELQRCL
C636Dcr2+1 (166) EGQPVYGDVE-RRTTKIS-KVPAV-EY-TGATEVPYRKNYQPARSFGCRR
551 600
DrosophDcr2 (533) KDRVLEKTEPELYEIKGHFQDDIDP-FTNENGAVLLPNNALAILHRYCQT
AaegyptiDcr2 (528) IGKTINRPDPLDADVHKELYNEIIPPFFTAKGAKLDALSAIQLLNRYCMG
Aag2Dcr2 (528) IGKTINRPDPLDADVHKELYNEIIPPFFTAKGAKLDALSAIQLLNRYCMG
C636Dcr2+2 (209) IGKTINRPDPLDADVHKELYNEIIPPFFTAKGAKLDALSAVQLLNRYCMG
C636Dcr2+1 (212) PQGVI--DYSAVFHCQRCQAGCTLRRSTSEPILHGNATGRIYQYQRHVGT
601 650
DrosophDcr2 (582) IPTDAFGFVIPWFHVLQEDERDRIFGVSAKGKHVISINMPVNCMLRDTIY
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651 700
DrosophDcr2 (632) SDPMDNVKTAKISAAFKACKVLYSLGELNERFVPKTLKERVASIADVHFE
AaegyptiDcr2 (618) GNPMRNIKLAKRSAAFNACRKLYENKELNEHLIPIDCKYQLNNLKDVYFR
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C636Dcr2+2 (299) GNPMRNIKLAKRSAAFNACRKLYENKELSEHLIPIDCRNQLNNLKDVYFR
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701 750
DrosophDcr2 (682) HWNKYGDSVTATVNKADKSKDRTYKTECPLEFYDALPRVGEICYAYEIFL
AaegyptiDcr2 (668) HWKDFDADLGKLAG--TQKCIRTHAIQYPKQTTECFPQPGKPCYIYVLRI
Aag2Dcr2 (668) HWKDFDADLGKLAGT--QKCIRTHAIQYPKQTTECFPQPGKPCYIYVLRI
C636Dcr2+2 (349) HWADFDEDSGKLAGT--QKCIRTHAIEYPKQTTGCFPEPGKPCYIYVLRI
C636Dcr2+1 (337) RKTSWNAEVHKNARY----------VSEANDRLLPGAW-TLLHLRFAH
751 800
DrosophDcr2 (732) EPQFESCEYTEHMYLNLQTP---RNYAILLRNKLPRLAEMPLFSNQGKLH
AaegyptiDcr2 (716) AAGFAQDPTNDNVNIFHSLYSSENNFGLMTTKPLPALAKMKFFVTLGLIN
Aag2Dcr2 (716) AAGFAQDPTNDNVNIFHSLYSSENNFGLMTTKPLPALAKMKFFVTLGLIN
C636Dcr2+2 (397) GAGFEQDPSNENVNIFHSLYNSENNFGIMTTKPLPVLAKMKFFVTLGLIN
C636Dcr2+1 (374) WSRLRARSFERKCQHFPLVVQLGEQFRNNDNETASSSGKNEVLCNVGTYQ
801 850
DrosophDcr2 (779) VRVANAPLEVIIQNS-EQLELLHQFHGMVFRDILKIWHPFFVLDRRSKEN
AaegyptiDcr2 (766) VHIEETPIVLPNGGSEIELALLRQFHVTVFRDVLKLWKEFLCCDYDNEEN
Aag2Dcr2 (766) VHIEETPIVLPNGGSEIELALLRQFHVTVFRDVLKLWKEFLCCDYDNEEN
C636Dcr2+2 (447) VHLVETPIVLPNAGSEVELALLRQFHITVFRDVLRLWKDFLVYDYDNGIN
C636Dcr2+1 (424) CTPCGDPHRVA-RGLRSRTRSAEAIPHYGIPRRAPIMEGLPCLRLRQRDQ
851 900
DrosophDcr2 (828) SYLVVPLILGAGEQKCFDWELMTNFRRLPQSHGSNVQQREQQPAPRPEDF
AaegyptiDcr2 (816) SFLVVPLKNST----HLDWKLIREFQNLSEPPSEISTIARNKMEFEADKY
Aag2Dcr2 (816) SFLVVPLKNST----HLDWKLIREFQNLSEPPSEISTIARNKMEFEADKY
C636Dcr2+2 (497) SFMVVL-RNRPTWIGNSFGSSSI-ANLRRKCRRWLGAGWISKRTTTATR-
C636Dcr2+1 (473) QFHGSPLKKST----HLDWELIRQFQYLSEPPSEMSTMARSRMDFEADNY
901 950
DrosophDcr2 (878) EGKIVTQWYAN-YDKPMLVTKVHRELTPLSYMEKNQQDKTYYEFTMSKYG
AaegyptiDcr2 (862) RHKVILPWYKNNKEQPYVVTMVHEHLTPESPFP-NPEYGSYANYFSQAYH
Aag2Dcr2 (862) RHKVILPWYKNNKEQPYVVTMVHEHLTPESPFP-NPEYGSYANYFSQAYH
C636Dcr2+2 (544) FFLGTKTTRSNHTSLPWCMST-RRAAPSQMRSTGPTRITSAKRTIWPWLS
C636Dcr2+1 (519) RHKVILPWYKNNKEQPYVVTMVHEHLTPGSPFP-NAEYGSYANYFSEAYH

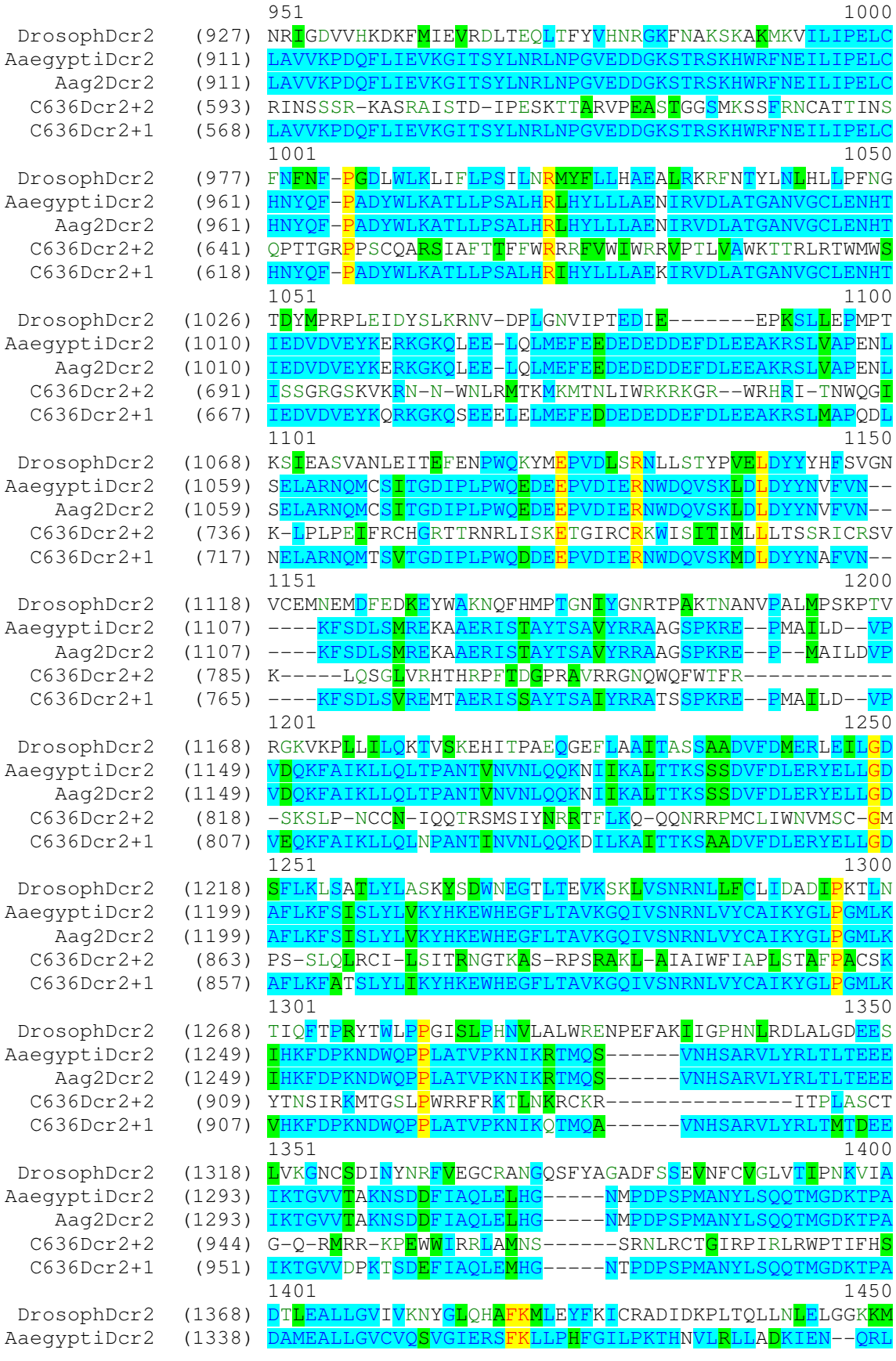
951 1000
DrosophDcr2 (927) NRIGDVVHKDKFMIEVRDLTEQLTFYVHNRGKFNAKSKAKMKVILIPELC
AaegyptiDcr2 (911) LAVVKPDQFLIEVKGITSYLNRLNPGVEDDGKSTRSKHWRFNEILIPELC
Aag2Dcr2 (911) LAVVKPDQFLIEVKGITSYLNRLNPGVEDDGKSTRSKHWRFNEILIPELC
C636Dcr2+2 (593) RINSSSR-KASRAISTD-IPESKTTARVPEASTGGSMKSSFRNCATTINS
C636Dcr2+1 (568) LAVVKPDQFLIEVKGITSYLNRLNPGVEDDGKSTRSKHWRFNEILIPELC
1001 1050
DrosophDcr2 (977) FNFNF-PGDLWLKLIFLPSILNRMYFLLHAEALRKRFNTYLNLHLLPFNG
AaegyptiDcr2 (961) HNYQF-PADYWLKATLLPSALHRLHYLLLAENIRVDLATGANVGCLENHT
Aag2Dcr2 (961) HNYQF-PADYWLKATLLPSALHRLHYLLLAENIRVDLATGANVGCLENHT
C636Dcr2+2 (641) QPTTGRPPSCQARSIAFTTFFWRRFVWIWRRVPTLVAWKTTRLRTWMWS
C636Dcr2+1 (618) HNYQF-PADYWLKATLLPSALHRIHYLLLAEKIRVDLATGANVGCLENHT
1051 1100
DrosophDcr2 (1026) TDYMPRPLEIDYSLKRNV-DPLGNVIPTEDIE-------EPKSLLEPMPT
AaegyptiDcr2 (1010) IEDVDVEYKERKGKQLEE-LQLMEFEEDEDEDDEFDLEEAKRSLVAPENL
Aag2Dcr2 (1010) IEDVDVEYKERKGKQLEE-LQLMEFEEDEDEDDEFDLEEAKRSLVAPENL
C636Dcr2+2 (691) ISSGRGSKVKRN-N-WNLRMTKMKMTNLIWRKRKGR--WRHRI-TNWQGI
C636Dcr2+1 (667) IEDVDVEYKQRKGKQSEEELELMEFEDDEDEDDEFDLEEAKRSLMAPQDL
1101 1150
DrosophDcr2 (1068) KSIEASVANLEITEFENPWQKYMEPVDLSRNLLSTYPVELDYYYHFSVGN
AaegyptiDcr2 (1059) SELARNQMCSITGDIPLPWQEDEEPVDIERNWDQVSKLDLDYYNVFVN--
Aag2Dcr2 (1059) SELARNQMCSITGDIPLPWQEDEEPVDIERNWDQVSKLDLDYYNVFVN--
C636Dcr2+2 (736) K-LPLPEIFRCHGRTTRNRLISKETGIRCRKWISITIMLLLTSSRICRSV
C636Dcr2+1 (717) NELARNQMTSVTGDIPLPWQDDEEPVDIERNWDQVSKMDLDYYNAFVN--
1151 1200
DrosophDcr2 (1118) VCEMNEMDFEDKEYWAKNQFHMPTGNIYGNRTPAKTNANVPALMPSKPTV
AaegyptiDcr2 (1107) ----KFSDLSMREKAAERISTAYTSAVYRRAAGSPKRE--PMAILD--VP
Aag2Dcr2 (1107) ----KFSDLSMREKAAERISTAYTSAVYRRAAGSPKRE--P--MAILDVP
C636Dcr2+2 (785) K-----LQSGLVRHTHRPFTDGPRAVRRGNQWQFWTFR------------
C636Dcr2+1 (765) ----KFSDLSVREMTAERISSAYTSAIYRRATSSPKRE--PMAILD--VP
1201 1250
DrosophDcr2 (1168) RGKVKPLLILQKTVSKEHITPAEQGEFLAAITASSAADVFDMERLEILGD
AaegyptiDcr2 (1149) VDQKFAIKLLQLTPANTVNVNLQQKNIIKALTTKSSSDVFDLERYELLGD
Aag2Dcr2 (1149) VDQKFAIKLLQLTPANTVNVNLQQKNIIKALTTKSSSDVFDLERYELLGD
C636Dcr2+2 (818) -SKSLP-NCCN-IQQTRSMSIYNRRTFLKQ-QQNRRPMCLIWNVMSC-GM
C636Dcr2+1 (807) VEQKFAIKLLQLNPANTINVNLQQKDILKAITTKSAADVFDLERYELLGD
1251 1300
DrosophDcr2 (1218) SFLKLSATLYLASKYSDWNEGTLTEVKSKLVSNRNLLFCLIDADIPKTLN
AaegyptiDcr2 (1199) AFLKFSISLYLVKYHKEWHEGFLTAVKGQIVSNRNLVYCAIKYGLPGMLK
Aag2Dcr2 (1199) AFLKFSISLYLVKYHKEWHEGFLTAVKGQIVSNRNLVYCAIKYGLPGMLK
C636Dcr2+2 (863) PS-SLQLRCI-LSITRNGTKAS-RPSRAKL-AIAIWFIAPLSTAFPACSK
C636Dcr2+1 (857) AFLKFATSLYLIKYHKEWHEGFLTAVKGQIVSNRNLVYCAIKYGLPGMLK
1301 1350
DrosophDcr2 (1268) TIQFTPRYTWLPPGISLPHNVLALWRENPEFAKIIGPHNLRDLALGDEES
AaegyptiDcr2 (1249) IHKFDPKNDWQPPLATVPKNIKRTMQS------VNHSARVLYRLTLTEEE
Aag2Dcr2 (1249) IHKFDPKNDWQPPLATVPKNIKRTMQS------VNHSARVLYRLTLTEEE
C636Dcr2+2 (909) YTNSIRKMTGSLPWRRFRKTLNKRCKR---------------ITPLASCT
C636Dcr2+1 (907) VHKFDPKNDWQPPLATVPKNIKQTMQA------VNHSARVLYRLTMTDEE
1351 1400
DrosophDcr2 (1318) LVKGNCSDINYNRFVEGCRANGQSFYAGADFSSEVNFCVGLVTIPNKVIA
AaegyptiDcr2 (1293) IKTGVVTAKNSDDFIAQLELHG-----NMPDPSPMANYLSQQTMGDKTPA
Aag2Dcr2 (1293) IKTGVVTAKNSDDFIAQLELHG-----NMPDPSPMANYLSQQTMGDKTPA
C636Dcr2+2 (944) G-Q-RMRR-KPEWWIRRLAMNS------SRNLRCTGIRPIRLRWPTIFHS
C636Dcr2+1 (951) IKTGVVDPKTSDEFIAQLEMHG-----NTPDPSPMANYLSQQTMGDKTPA
1401 1450
DrosophDcr2 (1368) DTLEALLGVIVKNYGLQHAFKMLEYFKICRADIDKPLTQLLNLELGGKKM
AaegyptiDcr2 (1338) DAMEALLGVCVQSVGIERSFKLLPHFGILPKTHNVLRLLADKIEN--QRL

Figure A.2: Alignment of dcr2 amino acid sequences from *Drosophila melanogaster* (GenBank NP_523778) *A. aegypti* mosquitoes (GenBank AAW48725), Aag2 and C6/36 using VectorNTI AlignX described in Chapter 3. Two different reading frames (+1 and +2 from first nucleotide in C6/36 sequence) were translated for C6/36 dcr2, and both are aligned here.

Vector NTI AlignX Amino Acid alignment color scheme:
black on window default color - non-homologous residues

blue on cyan - consensus residue derived from a block of similar residues at a given position

black on green - consensus residue derived from a single conservative residue at a given position

red on yellow - consensus residue derived from completely conservative residues at a given position

green on window default color - residue weakly homologous to consensus residue at given position

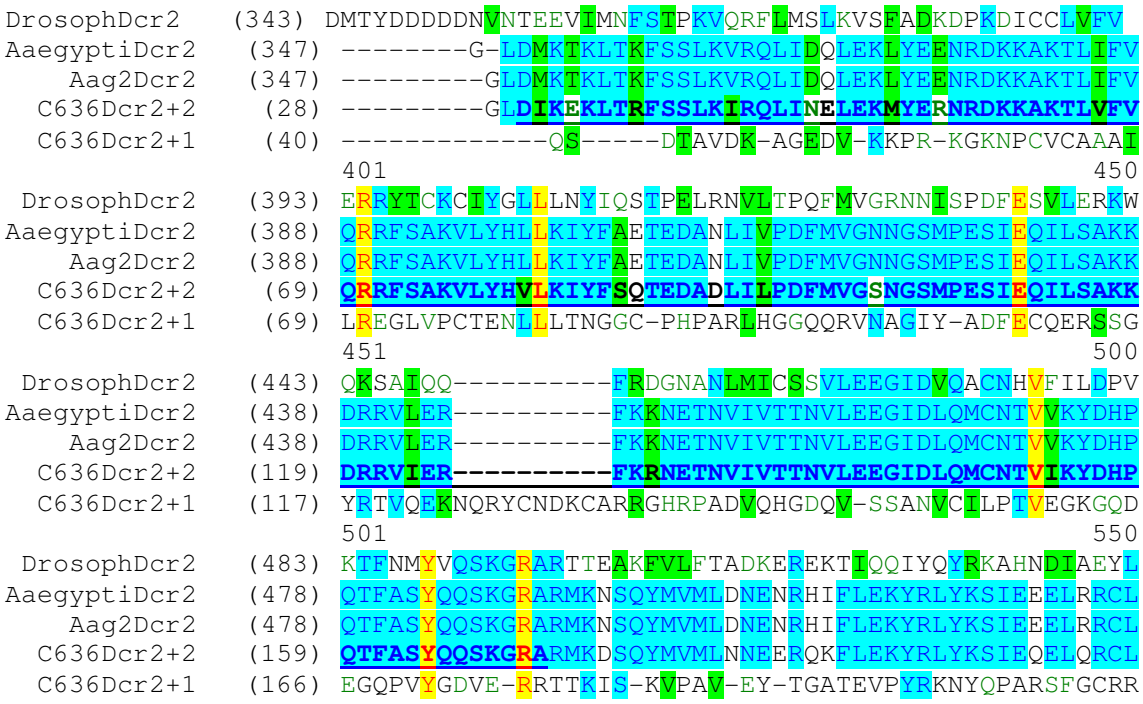

Figure A.3: Predicted Helicase domain for C6/36 Dcr2 +2 translation from NCBI BLAST conserved domains finder. The domain region is underlined and in bold type.

Figure A.4: Predicted dsRNA binding domain for C6/36 Dcr2 +2 translation from NCBI BLAST conserved domains finder. The domain region is underlined and in bold type.

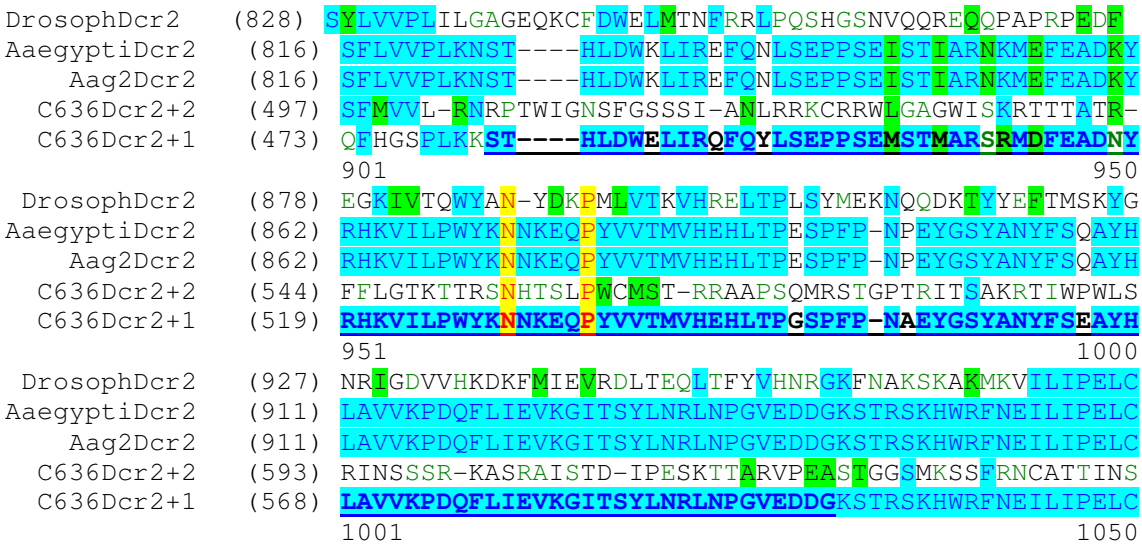

Figure A.5: Predicted PAZ domain of C6/36 Dcr2 +1 translation from NCBI BLAST conserved domains finder. The domain region is underlined and in bold type.

Figure A.6: Predicted partial region of RNaseIIIa domain of C6/36 Dcr2 +1 translation from NCBI BLAST conserved domains finder. The domain region is underlined and in bold type.

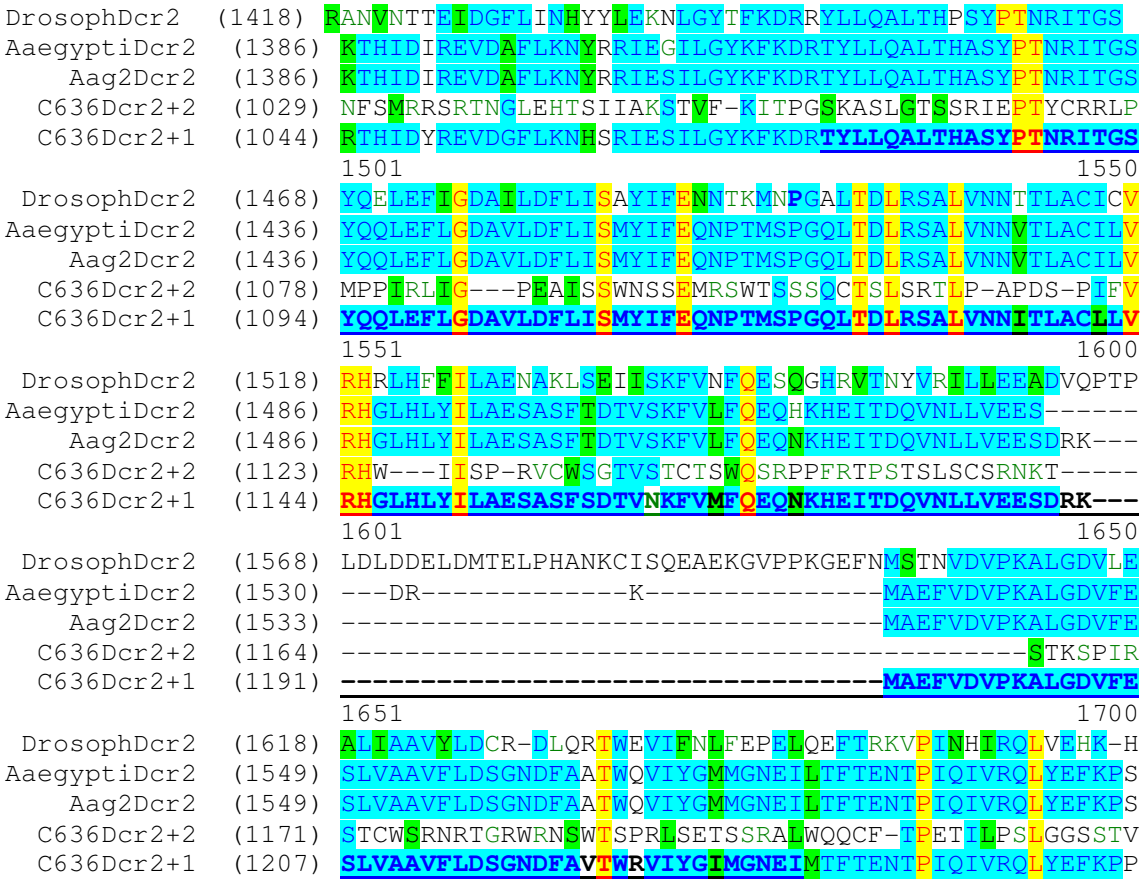

Figure A.7: Predicted RNaseIIIb domain of C6/36 Dcr2 +1 translation from NCBI BLAST conserved domains finder. The domain region is underlined and in bold type.

In vitro lysate analysis with infected cells

Introduction:

Cells were examined for changes in Dcr2 activity after infection with DENV2, TE3'2J SINV, or MRE16 SINV using the *in vitro* lysate system described in Chapter 3.

Methods:

C6/36 and Aag2 cells were grown and maintained as previously described in Chapters 2 and 3. Cells were infected at an MOI of 0.1 for DENV2 and 1 for the SINV (MRE16 and TE3'2J). Cells were harvested for lysate preparation at 2 dpi for SINV infections and 4 dpi for DENV2 infections. Lysates were equilibrated for protein concentration. *In vitro* dicing assays were preformed as described in Chapter 3, with biotinylated dsRNA derived from the β-gal gene.

Results:

These in vitro dicing assay experiments were performed to help analyze if Dcr2 activity changed during infection with DENV2 and two SINVs. There appeared to be an increase in Dcr2 activity after DENV2 infection, as the Dcr2 products appeared at earlier time points than in the mock-infected lysate (Figure A.8). No Dcr2 product was seen in the C6/36 cells mock- or DENV2-infected (Figure A.9), although this is not surprising as these cells did not produce 21 bp products in experiments shown in Chapter 3. Infection with the MRE16 strain of SINV seemed to increase Dcr2 activity (Figure A.10), while TE3'2J strain of SINV seemed to reduce the Dcr2 activity (Figure A.12). No Dcr2

cleavage products were seen in the C6/36 cells mock-, TE3'2J or MRE16-infected cells (Figures A.11 and A.13).

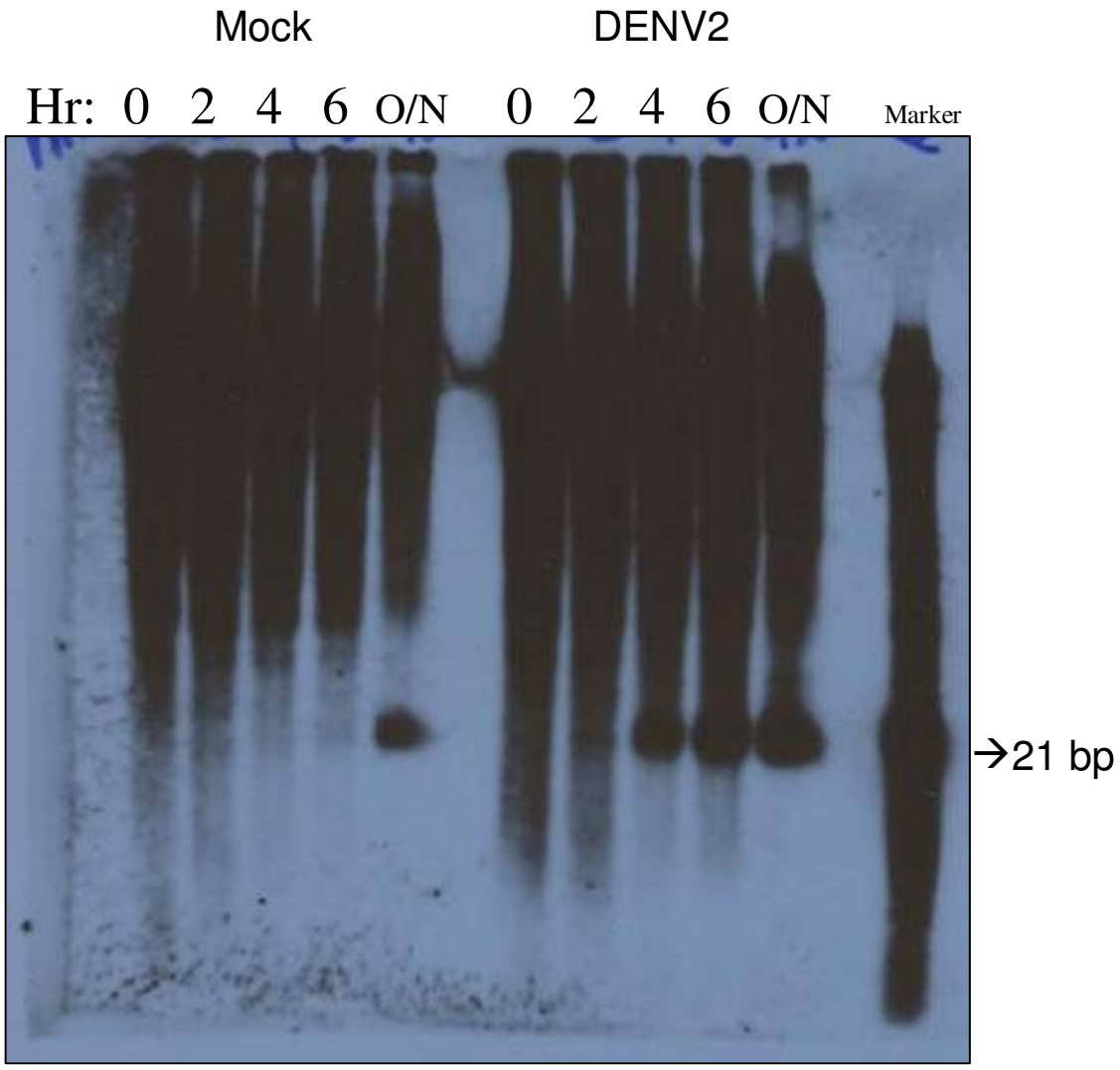

Figure A.8: Aag2 cells mock- and DENV2- infected in vitro dicing assay using biotinylated β-gal dsRNA, take timepoints at 0, 2, 4, 6 hours and overnight. The marker is biotinylated β-gal dsRNA incubated with human recombinant Dicer.

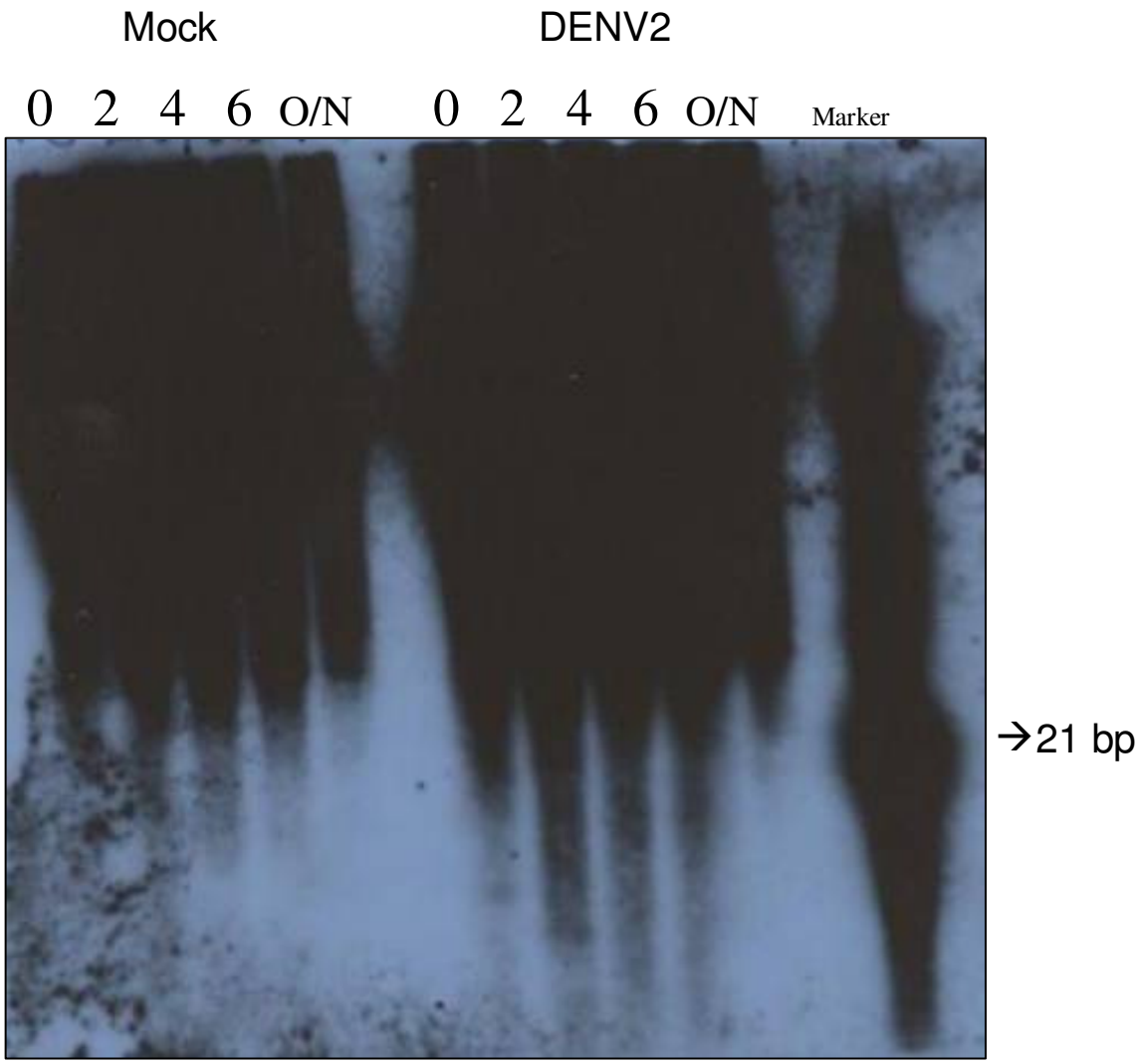

Figure A.9: C6/36 cells mock- and DENV2- infected in vitro dicing assay using biotinylated β-gal dsRNA, take timepoints at 0, 2, 4, 6 hours and overnight. The marker is biotinylated β-gal dsRNA incubated with human recombinant Dicer.

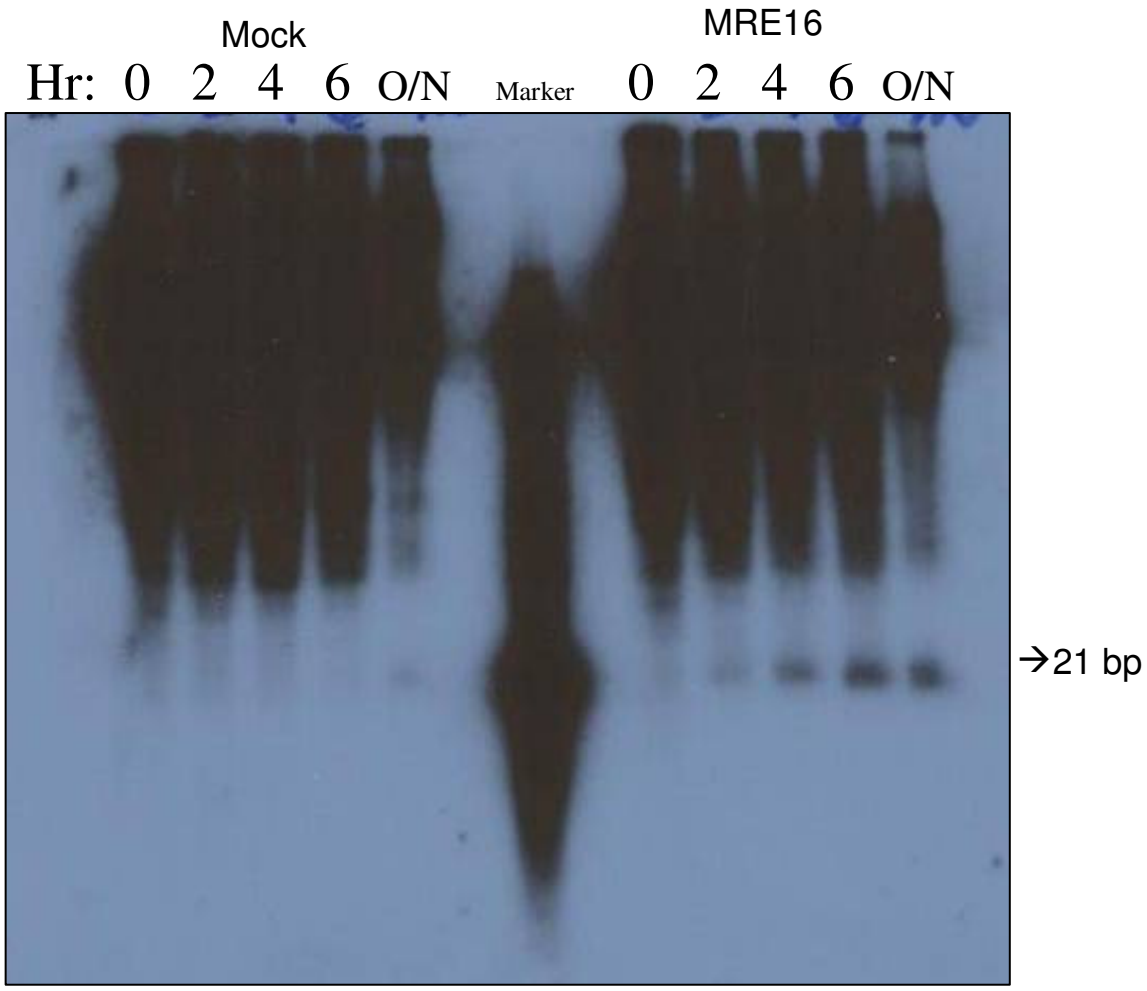

Figure A.10: Aag2 cells mock- and SINV MRE16- infected in vitro dicing assay using biotinylated β-gal dsRNA, take timepoints at 0, 2, 4, 6 hours and overnight. The marker is biotinylated β-gal dsRNA incubated with human recombinant Dicer.

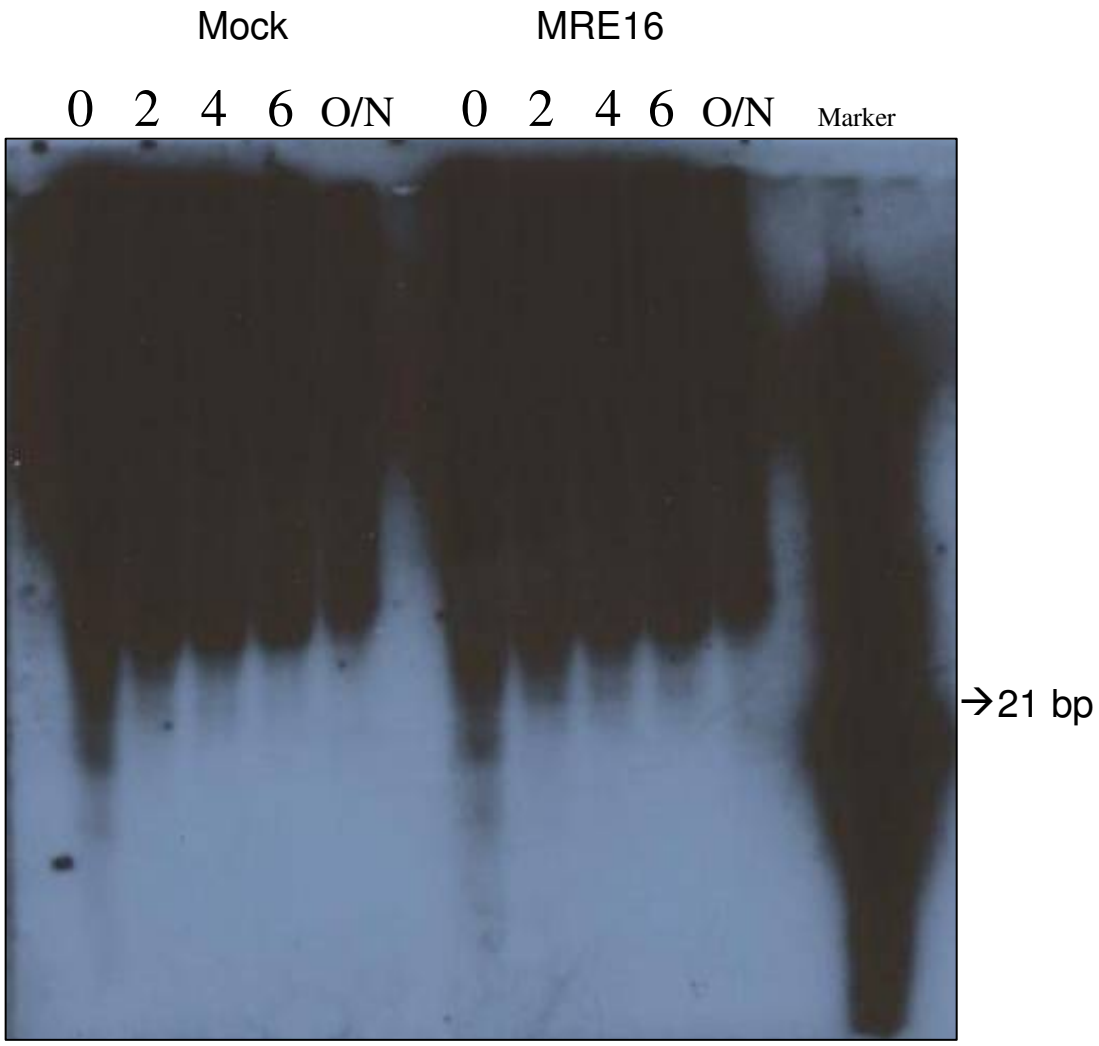

Figure A.11: C6/36 cells mock- and SINV MRE16- infected in vitro dicing assay using biotinylated β-gal dsRNA, take timepoints at 0, 2, 4, 6 hours and overnight. The marker is biotinylated β-gal dsRNA incubated with human recombinant Dicer.

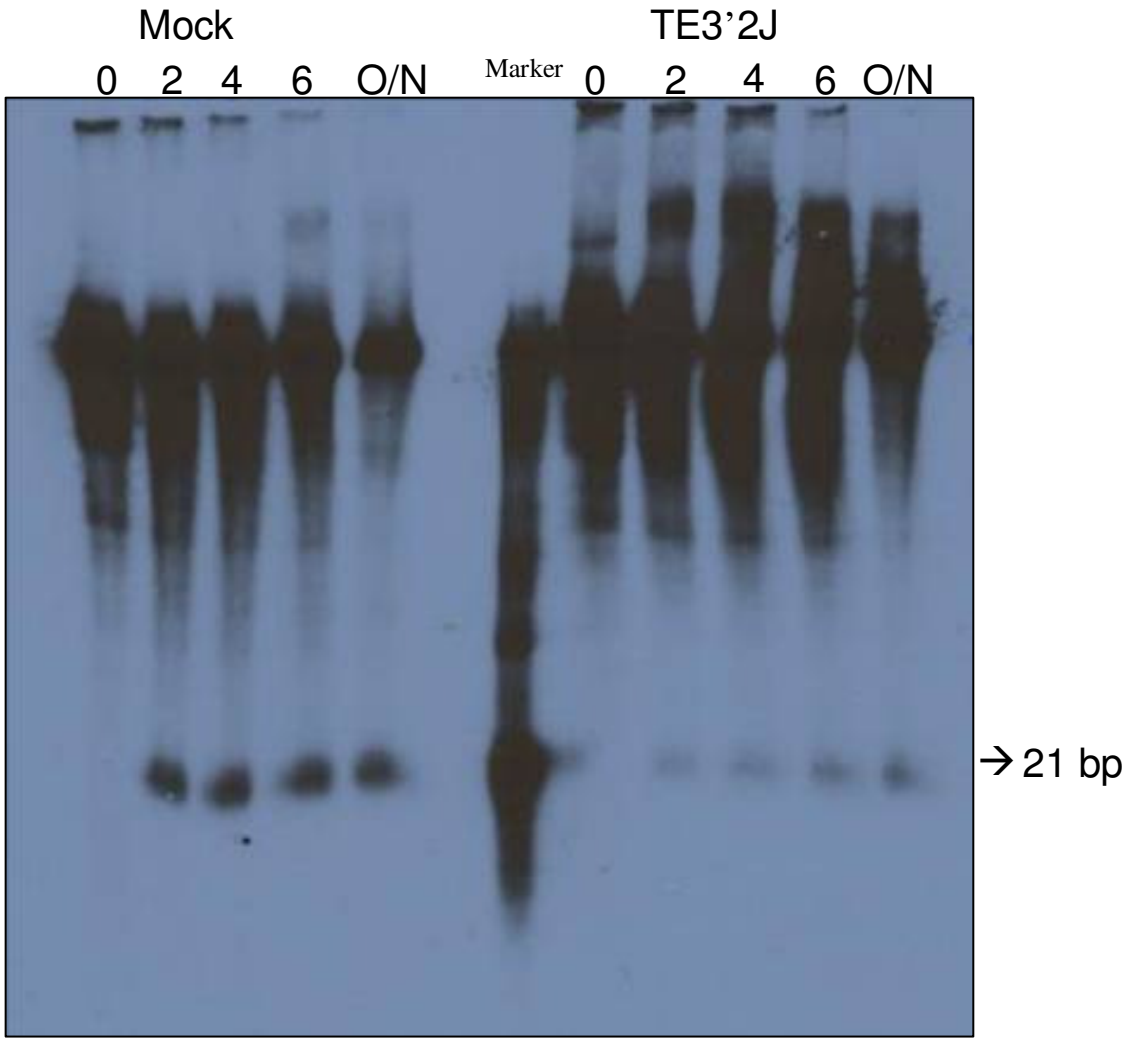

Figure A.12: Aag2 cells mock- and SINV TE3'2J- infected in vitro dicing assay using biotinylated β-gal dsRNA, take timepoints at 0, 2, 4, 6 hours and overnight. The marker is biotinylated β-gal dsRNA incubated with human recombinant Dicer.

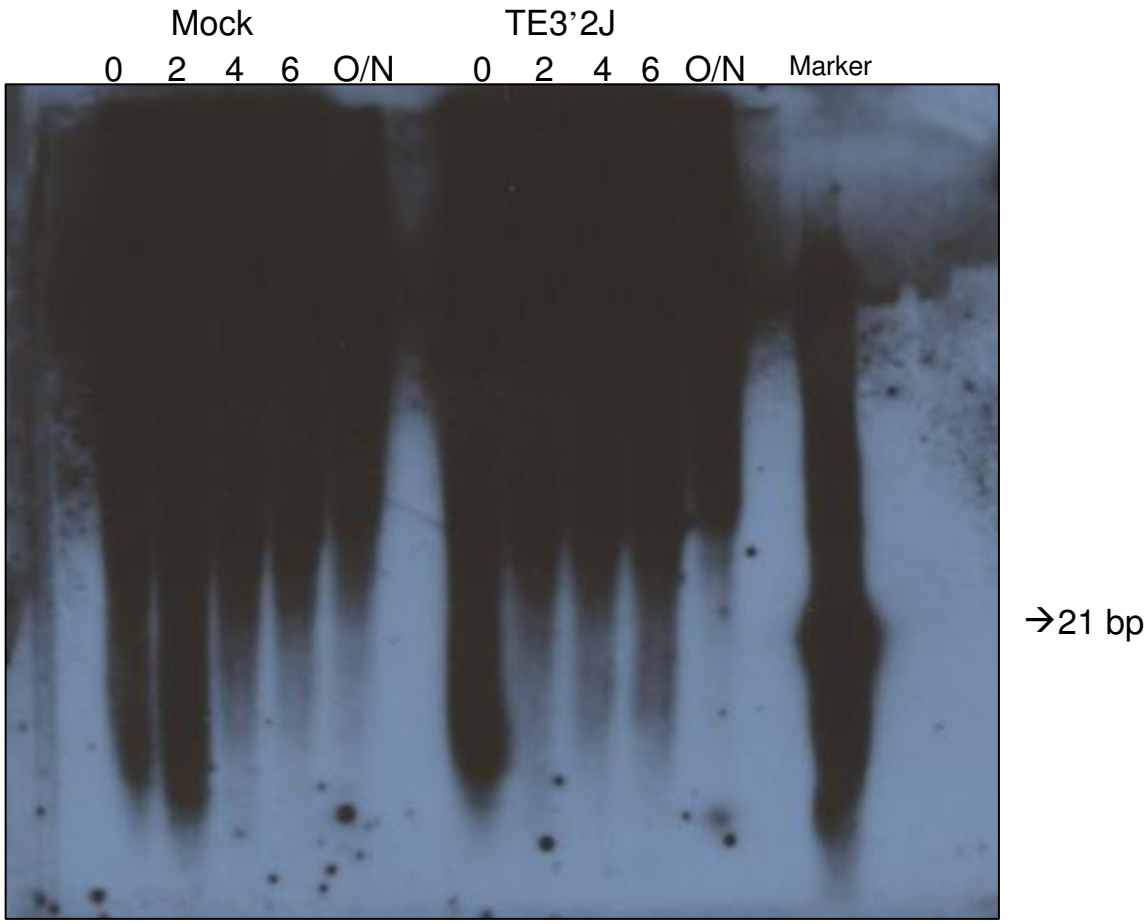

Figure A.13: C6/36 cells mock- and SINV TE3'2J- infected in vitro dicing assay using biotinylated β-gal dsRNA, take time points at 0, 2, 4, 6 hours and overnight. The marker is biotinylated β-gal dsRNA incubated with human recombinant Dicer.

Discussion

A cell-free lysate assay was used to analyze the effect of DENV or SINV infection of the Aag2 and C6/36 cell lines. It appears that infection with DENV and the MRE16 strain of SINV led to increased levels of Dcr2 activity in Aag2 cells, while infection with the TE3'2J strain of SINV resulted in less Dcr2 activity. Campbell et al. (2008a) showed that there seem to be less viRNAs made from the MRE16 strain than the TE3'2J strain. Combined with the results from these *in vitro* lysate assays, it may suggest that in TE3'2J infected Aag2 cells, Dcr2 is busy processing the viral RNA, and

thus is not active to participate in cleavage of the exogenous β-gal RNA that is supplemented into the lysate.

REFERENCES

Adelman, Z. N., Blair, C. D., Carlson, J. O., Beaty, B. J. & Olson, K. E. (2001). Sindbis virus-induced silencing of dengue viruses in mosquitoes. *Insect Molecular Biology* **10**, 265-273.

Adelman, Z. N., Sanchez-Vargas, I., Travanty, E. A., Carlson, J. O., Beaty, B. J., Blair, C. D. & Olson, K. E. (2002). RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. *Journal of Virology* **76**, 12925-12933.

Aliyari, R., Wu, Q. F., Li, H. W., Wang, X. H., Li, F., Green, L. D., Han, C. S., Li, W. X. & Ding, S. W. (2008). Mechanism of Induction and Suppression of Antiviral Immunity Directed by Virus-Derived Small RNAs in Drosophila. *Cell Host & Microbe* **4**, 387-397.

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs
10.1093/nar/25.17.3389. *Nucl. Acids Res.* **25**, 3389-3402.

Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., Dreyfuss, G., Eddy, S. R., Griffiths-Jones, S., Marshall, M., Matzke, M., Ruvkun, G. & Tuschl, T. (2003). A uniform system for microRNA annotation. *RNA* **9**, 277-279.

Aparicio, O., Razquin, N., Zaratiegui, M., Narvaiza, I. & Fortes, P. (2006). Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production. *Journal of Virology* **80**, 1376-1384.

Aranda, C., Sanchez-Seco, M. P., Caceres, F., Escosa, R., Galvez, J. C., Masia, M., Marques, E., Ruiz, S., Alba, A., Busquets, N., Vazquez, A., Castella, J. & Tenorio, A. (2009). Detection and Monitoring of Mosquito Flaviviruses in Spain between 2001 and 2005. *Vector-Borne and Zoonotic Diseases* **9**, 171-178.

Aravin, A. A., Klenov, M. S., Vagin, V. V., Bantignies, F., Cavalli, G. & Gvozdev, V. A. (2004). Dissection of a natural RNA silencing process in the Drosophila melanogaster germ line. *Molecular and Cellular Biology* **24**, 6742-6750.

Aravin, A. A., Naumova, N. M., Tulin, A. V., Vagin, V. V., Rozovsky, Y. M. & Gvozdev, V. A. (2001). Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. *Current Biology* **11**, 1017-1027.

Attarzadeh-Yazdi, G., Fragkoudis, R., Chi, Y., Siu, R. W. C., Ulper, L., Barry, G., Rodriguez-Andres, J., Nash, A. A., Bouloy, M., Merits, A., Fazakerley, J. K. & Kohl, A. (2009). Cell-to-Cell Spread of the RNA Interference Response Suppresses Semliki Forest Virus (SFV) Infection of Mosquito Cell Cultures and Cannot Be Antagonized by SFV. *Journal of Virology* **83**, 5735-5748.

Avadhanula, V., Weasner, B. P., Hardy, G. G., Kumar, J. P. & Hardy, R. W. (2009). A Novel System for the Launch of Alphavirus RNA Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response. *PLoS Pathog* **5**, e1000582.

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297.

Bartholomay, L. C., Fuchs, J. F., Cheng, L. L., Beck, E. T., Vizioli, J., Lowenberger, C. & Christensen, B. M. (2004). Reassessing the role of defensin in the innate immune response of the mosquito, Aedes aegypti. *Insect Molecular Biology* **13**, 125-132.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P. & Izaurralde, E. (2006). MRNA degradation by miRNAs and GW182 requires both CCR4 : NOT deadenylase and DCP1 : DCP2 decapping complexes. *Genes & Development* **20**, 1885-1898.

Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Black, W. C., Bennett, K. E., Gorrochotegui-Escalante, N., Barillas-Mury, C. V., Fernandez-Salas, I., Munoz, M. D., Farfan-Ale, J. A., Olson, K. E. & Beaty, B. J. (2002). Flavivirus susceptibility in Aedes aegypti. *Archives of Medical Research* **33**, 379-388.

Blair, C. D., Adelman, Z. N. & Olson, K. E. (2000). Molecular strategies for interrupting arthropod-borne virus transmission by mosquitoes. *Clinical Microbiology Reviews* **13**, 651-+.

Blakqori, G., Delhaye, S., Habjan, M., Blair, C. D., Sanchez-Vargas, I., Olson, K. E., Attarzadeh-Yazdi, G., Fragkoudis, R., Kohl, A., Kalinke, U., Weiss, S., Michiels, T., Staeheli, P. & Weber, F. (2007). La Crosse bunyavirus nonstructural protein NSs serves to suppress the type I interferon system of mammalian hosts. *Journal of Virology* **81**, 4991-4999.

Bohnsack, M. T., Czaplinski, K. & Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *Rna-a Publication of the Rna Society* **10**, 185-191.

Bolling, B. G., Eisen, L., Moore, C. G. & Blair, C. B. (2010 (Submitted)). Insect-specific Flaviviruses from Culex Mosquitoes in Colorado, with Evidence of Vertical Transmission.

Brackney, D. E., Beane, J. E. & Ebel, G. D. (2009). RNAi Targeting of West Nile Virus in Mosquito Midguts Promotes Virus Diversification. *PLoS Pathog* **5**, e1000502.

Brackney, D. E., Scott, J. C., Sagawa, F., Woodward, J. E., Miller, N. A., Schilkey, F. D., Mudge, J., Wilusz, J., Olson, K. E., Blair, C. D. & Ebel, G. D. (2010). C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response. *Submitted*.

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R. & Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* **128**, 1089-1103.

Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. & Cohen, S. M. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113**, 25-36.

Buhler, M., Verdel, A. & Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. *Cell* **125**, 873-886.

Cai, X. Z., Lu, S. H., Zhang, Z. H., Gonzalez, C. M., Damania, B. & Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5570-5575.

Cai, X. Z., Schafer, A., Lu, S. H., Bilello, J. P., Desrosiers, R. C., Edwards, R., Raab-Traub, N. & Cullen, B. R. (2006). Epstein-Barr virus MicroRNAs are evolutionarily conserved and differentially expressed. *Plos Pathogens* **2**, 236-247.

Cameron, J. E., Fewell, C., Yin, Q. Y., McBride, J., Wang, X., Lin, Z. & Flemington, E. K. (2008). Epstein-Barr virus growth/latency III program alters cellular microRNA expression. *Virology* **382**, 257-266.

Cammisa-Parks, H., Cisar, L. A., Kane, A. & Stollar, V. (1992). The Complete Nucleotide-Sequence of Cell Fusing Agent (CFA) - Homology between the Nonstructural Proteins Encoded by CFA and the Nonstructural Proteins Encoded by Arthropod-Borne Flaviviruses. *Virology* **189**, 511-524.

Campbell, C., Black, W., Hess, A. & Foy, B. (2008a). Comparative genomics of small RNA regulatory pathway components in vector mosquitoes. *BMC Genomics* **9**, 425.

Campbell, C. L., Keene, K. M., Brackney, D. E., Olson, K. E., Blair, C. D., Wilusz, J. & Foy, B. D. (2008b). *Aedes aegypti* uses RNA interference in defense against Sindbis virus infection. *BMC Microbiology* **8**, 47.

Caplen, N. J., Zheng, Z. L., Falgout, B. & Morgan, R. A. (2002). Inhibition of viral gene expression and replication in mosquito cells by dsRNA-triggered RNA interference. *Molecular Therapy* **6**, 243-251.

Carthew, R. W. & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**, 642-655.

Caudy, A. A., Ketting, R. F., Hammond, S. M., Denli, A. M., Bathoorn, A. M. P., Tops, B. B. J., Silva, J. M., Myers, M. M., Hannon, G. J. & Plasterk, R. H. A. (2003). A micrococcal nuclease homologue in RNAi effector complexes. *Nature* **425**, 411-414.

Caudy, A. A., Myers, M., Hannon, G. J. & Hammond, S. M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes & Development* **16**, 2491-2496.

Chao, J. A., Lee, J. H., Chapados, B. R., Debler, E. W., Schneemann, A. & Williamson, J. R. (2005). Dual modes of RNA-silencing suppression by flock house virus protein B2. *Nature Structural & Molecular Biology* **12**, 952-957.

Chendrimada, T. P., Finn, K. J., Ji, X. J., Baillat, D., Gregory, R. I., Liebhaber, S. A., Pasquinelli, A. E. & Shiekhattar, R. (2007). MicroRNA silencing through RISC recruitment of eIF6. *Nature* **447**, 823-U1.

Chotkowski, H. L., Ciota, A. T., Jia, Y., Puig-Basagoiti, F., Kramer, L. D., Shi, P. Y. & Glaser, R. L. (2008). West Nile virus infection of Drosophila melanogaster induces a protective RNAi response. *Virology* **377**, 197-206.

Choy, E. Y. W., Siu, K. L., Kok, K. H., Lung, R. W. M., Tsang, C. M., To, K. F., Kwong, D. L. W., Tsao, S. W. & Jin, D. Y. (2008). An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival. *Journal of Experimental Medicine* **205**, 2551-2560.

Chu, C. Y. & Rana, T. M. (2006). Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *Plos Biology* **4**, 1122-1136.

Chung, W.-J., Okamura, K., Martin, R. & Lai, E. C. (2008). Endogenous RNA Interference Provides a Somatic Defense against Drosophila Transposons. **18**, 795-802.

Cirimotich, C. M., Scott, J. C., Phillips, A. T., Geiss, B. J. & Olson, K. E. (2009). Suppression of RNA interference increases alphavirus replication and virus-associated mortality in *Aedes aegypti* mosquitoes. *BMC Microbiology* **9**, 49.

Cox, D. N., Chao, A., Baker, J., Chang, L., Qiao, D. & Lin, H. F. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes & Development* **12**, 3715-3727.

Cox, D. N., Chao, A. & Lin, H. F. (2000). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503-514.

Crabtree, M. B., Nga, P. T. & Miller, B. R. (2009). Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. *Archives of Virology* **154**, 857-860.

Crabtree, M. B., Sang, R. C., Stollar, V., Dunster, L. M. & Miller, B. R. (2003). Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. *Archives of Virology* **148**, 1095-1118.

Crochu, S., Cook, S., Attoui, H., Charrel, R. N., De Chesse, R., Belhouchet, M., Lemasson, J. J., de Micco, P. & de Lamballerie, X. (2004). Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of Aedes spp. mosquitoes. *Journal of General Virology* **85**, 1971-1980.

Crooks, G. E., Hon, G., Chandonia, J.-M. & Brenner, S. E. (2004). WebLogo: A Sequence Logo Generator. *Genome Research* **14**, 1188-1190.

Cui, C., Griffiths, A., Li, G. L., Silva, L. M., Kramer, M. F., Gaasterland, T., Wang, X. J. & Coen, D. M. (2006). Prediction and identification of herpes simplex virus 1-encoded MicroRNAs. *Journal of Virology* **80**, 5499-5508.

Cullen, B. R. (2004). Transcription and processing of human microRNA precursors. *Molecular Cell* **16**, 861-865.

Czech, B., Malone, C. D., Zhou, R., Stark, A., Schlingeheyde, C., Dus, M., Perrimon, N., Kellis, M., Wohlschlegel, J. A., Sachidanandam, R., Hannon, G. J. & Brennecke, J. (2008). An endogenous small interfering RNA pathway in Drosophila. *Nature* **453**, 798-U7.

Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., Galiana-Arnoux, D., Dostert, C., Antoniewski, C., Hoffmann, J. A. & Imler, J. L. (2008). The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. *Nature Immunology* **9**, 1425-1432.

Denli, A. M., Tops, B. B. J., Plasterk, R. H. A., Ketting, R. F. & Hannon, G. J. (2004). Processing of primary microRNAs by the Microprocessor complex. *Nature* **432**, 231-235.

Djupedal, I., Portoso, M., Spahr, H., Bonilla, C., Gustafsson, C. M., Allshire, R. C. & Ekwall, K. (2005). RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. *Genes & Development* **19**, 2301-2306.

Doherty, R. L., Carley, J. G., Filippich, C., Kay, B. H., Gorman, B. M. & Rajapaksa, N. (1977). Isolation of Sindbis (Alphavirus) and Leanyer Viruses from Mosquitos Collected in Northern-Territory of Australia, 1974. *Australian Journal of Experimental Biology and Medical Science* **55**, 485-489.

Doherty, R. L., Carley, J. G., Kay, B. H., Filippich, C., Marks, E. N. & Frazier, C. L. (1979). Isolation of Virus-Strains from Mosquitos Collected in Queensland, 1972-1976. *Australian Journal of Experimental Biology and Medical Science* **57**, 509-520.

Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001a). RNA interference is mediated by 21-and 22-nucleotide RNAs. *Genes & Development* **15**, 188-200.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. (2001b). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *Embo Journal* **20**, 6877-6888.

Eulalio, A., Behm-Ansmant, I., Schweizer, D. & Izaurralde, E. (2007). P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Molecular and Cellular Biology* **27**, 3970-3981.

Eulalio, A., Huntzinger, E. & Izaurralde, E. (2008). Getting to the root of miRNA-Mediated gene silencing. *Cell* **132**, 9-14.

Farazi, T. A., Juranek, S. A. & Tuschl, T. (2008). The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. *Development* **135**, 1201-1214.

Farfan-Ale, J. A., Lorono-Pino, M. A., Garcia-Rejon, J. E., Hovav, E., Powers, A. M., Lin, M., Dorman, K. S., Platt, K. B., Bartholomay, L. C., Soto, V., Beaty, B. J., Lanciotti, R. S. & Blitvich, B. J. (2009). Detection of RNA from a Novel West Nile-like Virus and High Prevalence of an Insect-specific Flavivirus in Mosquitoes in the Yucatan Peninsula of Mexico. *American Journal of Tropical Medicine and Hygiene* **80**, 85-95.

Filipowicz, W., Jaskiewicz, L., Kolb, F. A. & Pillai, R. S. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. *Current Opinion in Structural Biology* **15**, 331-341.

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811.

Flynt, A., Liu, N., Martin, R. & Lai, E. C. (2009). Dicing of viral replication intermediates during silencing of latent Drosophila viruses
10.1073/pnas.0813412106. *Proceedings of the National Academy of Sciences* **106**, 5270-5275.

Forstemann, K., Tomari, Y., Du, T. T., Vagin, V. V., Denli, A. M., Bratu, D. P., Klattenhoff, C., Theurkauf, W. E. & Zamore, P. D. (2005). Normal microRNA maturation and germ-line stem cell maintenance requires loquacious, a double-stranded RNA-binding domain protein. *Plos Biology* **3**, 1187-1201.

Foy, B. D., Myles, K. M., Pierro, D. J., Sanchez-Vargas, I., Uhlirova, M., Jindra, M., Beaty, B. J. & Olson, K. E. (2004). Development of a new Sindbis virus transducing system and its characterization in three Culicine mosquitoes and two Lepidopteran species. *Insect Molecular Biology* **13**, 89-100.

Franz, A. W. E., Sanchez-Vargas, I., Adelman, Z. N., Blair, C. D., Beaty, B. J., James, A. A. & Olson, K. E. (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4198-4203.

Franz, A. W. E., Sanchez-Vargas, I., Piper, J., Smith, M. R., Khoo, C. C. H., James, A. A. & Olson, K. E. (2009). Stability and loss of a virus resistance phenotype over time in transgenic mosquitoes harbouring an antiviral effector gene. *Insect Molecular Biology* **18**, 661-672.

Gaines, P. J., Olson, K. E., Higgs, S., Powers, A. M., Beaty, B. J. & Blair, C. D. (1996). Pathogen-derived resistance to dengue type virus 2 in mosquito cells by expression of the premembrane coding region of the viral genome. *Journal of Virology* **70**, 2132-2137.

Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J. A. & Imler, J. L. (2006). Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nature Immunology* **7**, 590-597.

Garcia, S., Billecocq, A., Crance, J. M., Munderloh, U., Garin, D. & Bouloy, M. (2005). Nairovirus RNA sequences expressed by a Semliki Forest virus replicon induce RNA interference in tick cells. *Journal of Virology* **79**, 8942-8947.

Garcia, S., Billecocq, A., Crance, J. M., Prins, M., Garin, D. & Bouloy, M. (2006). Viral suppressors of RNA interference impair RNA silencing induced by a Semliki Forest virus replicon in tick cells. *Journal of General Virology* **87**, 1985-1989.

Gardner, J. P., Frolov, I., Perri, S., Ji, Y. Y., MacKichan, M. L., Megede, J. Z., Chen, M. C., Belli, B. A., Driver, D. A., Sherrill, S., Greer, C. E., Otten, G. R., Barnett, S. W., Liu, M. A., Dubensky, T. W. & Polo, J. M. (2000). Infection of human dendritic cells by a Sindbis virus replicon vector is determined by a single amino acid substitution in the E2 glycoprotein. *Journal of Virology* **74**, 11849-11857.

Ghildiyal, M., Seitz, H., Horwich, M. D., Li, C. J., Du, T. T., Lee, S., Xu, J., Kittler, E. L. W., Zapp, M. L., Weng, Z. P. & Zamore, P. D. (2008). Endogenous siRNAs

derived from transposons and mRNAs in *Drosophila* somatic cells. *Science* **320**, 1077-1081.

Ghildiyal, M. & Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nature Reviews Genetics* **10**, 94-108.

Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J. & Schier, A. F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75-79.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**, 235-240.

Grey, F., Antoniewicz, A., Allen, E., Saugstad, J., McShea, A., Carrington, J. C. & Nelson, J. (2005). Identification and characterization of human cytomegalovirus-encoded microRNAs. *Journal of Virology* **79**, 12095-12099.

Griffiths-Jones, S. (2004). The microRNA Registry. *Nucl. Acids Res.* **32**, D109-111.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A. & Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucl. Acids Res.* **34**, D140-144.

Griffiths-Jones, S., Saini, H. K., van Dongen, S. & Enright, A. J. (2008). miRBase: tools for microRNA genomics. *Nucl. Acids Res.* **36**, D154-158.

Gruber, J. J., Zatechka, D. S., Sabin, L. R., Yong, J., Lum, J. J., Kong, M., Zong, W. X., Zhang, Z. X., Lau, C. K., Rawlings, J., Cherry, S., Ihle, J. N., Dreyfuss, G. & Thompson, C. B. (2009). Ars2 Links the Nuclear Cap-Binding Complex to RNA Interference and Cell Proliferation. *Cell* **138**, 328-339.

Grumet, R., Sanford, J. C. & Johnston, S. A. (1987). Pathogen-Derived Resistance to Viral-Infection Using a Negative Regulatory Molecule. *Virology* **161**, 561-569.

Grundhoff, A., Sullivan, C. S. & Ganem, D. (2006). A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. *Rna-a Publication of the Rna Society* **12**, 733-750.

Gubler, D. J. (2002). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends in Microbiology* **10**, 100-103.

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H. & Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5 ' end formation in *Drosophila*. *Science* **315**, 1587-1590.

Guy, B. & Almond, J. W. (2008). Towards a dengue vaccine: Progress to date and remaining challenges. *Comparative Immunology, Microbiology and Infectious Diseases*
Aspects of vaccine development **31**, 239-252.

Hahn, C. S., Hahn, Y. S., Braciale, T. J. & Rice, C. M. (1992). Infectious Sindbis Virus Transient Expression Vectors for Studying Antigen Processing and Presentation. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 2679-2683.

Hahn, C. S., Hahn, Y. S., Rice, C. M., Lee, E., Dalgarno, L., Strauss, E. G. & Strauss, J. H. (1987). Conserved Elements in the 3' Untranslated Region of Flavivirus Rnas and Potential Cyclization Sequences. *Journal of Molecular Biology* **198**, 33-41.

Haley, B., Tang, G. L. & Zamore, P. D. (2003). *In vitro* analysis of RNA interference in *Drosophila melanogaster*. *Methods* **30**, 330-336.

Halstead, S. B. (2007). Dengue. *Lancet* **370**, 1644-1652.

Henchal, E. A. & Putnak, J. R. (1990). The Dengue Viruses. *Clinical Microbiology Reviews* **3**, 376-396.

Hoa, N. T., Keene, K. M., Olson, K. E. & Zheng, L. (2003). Characterization of RNA interference in an Anopheles gambiae cell line. *Insect Biochemistry and Molecular Biology* **33**, 949-957.

Holmes, E. C. & Twiddy, S. S. (2003). The origin, emergence and evolutionary genetics of dengue virus. *Infection Genetics and Evolution* **3**, 19-28.

Horwich, M. D., Li, C. J., Matranga, C., Vagin, V., Farley, G., Wang, P. & Zamore, P. D. (2007). The Drosophila RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Current Biology* **17**, 1265-1272.

Hoshino, K., Isawa, H., Tsuda, Y., Sawabe, K. & Kobayashi, M. (2009). Isolation and characterization of a new insect flavivirus from Aedes albopictus and Aedes flavopictus mosquitoes in Japan. *Virology* **391**, 119-129.

Hoshino, K., Isawa, H., Tsuda, Y., Yano, K., Sasaki, T., Yuda, M., Takasaki, T., Kobayashi, M. & Sawabe, K. (2007). Genetic characterization of a new insect flavivirus isolated from Culex pipiens mosquito in Japan. *Virology* **359**, 405-414.

Hussain, M., Taft, R. J. & Asgari, S. (2008). An insect virus-encoded microRNA regulates viral replication. *Journal of Virology* **82**, 9164-9170.

Igarashi, A. (1978). Isolation of a Singhs *Aedes Albopictus* Cell Clone Sensitive to Dengue and Chikungunya Viruses. *Journal of General Virology* **40**, 531-544.

Igarashi, A., Harrap, K. A., Casals, J. & Stollar, V. (1976). Morphological, Biochemical, and Serological Studies on a Viral Agent (Cfa) Which Replicates in and Causes Fusion of Aedes-Albopictus (Singh) Cells. *Virology* **74**, 174-187.

Ishizuka, A., Siomi, M. C. & Siomi, H. (2002). A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes & Development* **16**, 2497-2508.

Jiang, F., Ye, X. C., Liu, X., Fincher, L., McKearin, D. & Liu, Q. H. (2005). Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. *Genes & Development* **19**, 1674-1679.

Jopling, C. L. (2008). Regulation of hepatitis C virus by microRNA-122. *Biochemical Society Transactions* **36**, 1220-1223.

Jopling, C. L., Yi, M. K., Lancaster, A. M., Lemon, S. M. & Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* **309**, 1577-1581.

Kato, H., Goto, D. B., Martienssen, R. A., Urano, T., Furukawa, K. & Murakami, Y. (2005). RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* **309**, 467-469.

Kawamura, Y., Saito, K., Kin, T., Ono, Y., Asai, K., Sunohara, T., Okada, T. N., Siomi, M. C. & Siomi, H. (2008). Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. *Nature* **453**, 793-U5.

Keene, K. M., Foy, B. D., Sanchez-Vargas, I., Beaty, B. J., Blair, C. D. & Olson, K. E. (2004). RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; *Togaviridae*) infection of *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17240-17245.

Kim, D. Y., Guzman, H., Bueno, R., Dennett, J. A., Auguste, A. J., Carrington, C. V. F., Popov, V. L., Weaver, S. C., Beasley, D. W. C. & Tesh, R. B. (2009). Characterization of Culex Flavivirus (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad. *Virology* **386**, 154-159.

Kim, K., Lee, Y. S. & Carthew, R. W. (2007). Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes. *Rna-a Publication of the Rna Society* **13**, 22-29.

Kim, V. N. (2005). MicroRNA biogenesis: Coordinated cropping and dicing. *Nature Reviews Molecular Cell Biology* **6**, 376-385.

Kinney, R. M., Butrapet, S., Chang, G. J. J., Tsuchiya, K. R., Roehrig, J. T., Bhamarapravati, N. & Gubler, D. J. (1997). Construction of infectious cDNA clones for dengue 2 virus: Strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* **230**, 300-308.

Klase, Z., Kale, P., Winograd, R., Gupta, M. V., Heydarian, M., Berro, R., McCaffrey, T. & Kashanchi, F. (2007). HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. *Bmc Molecular Biology* **8**.

Klimstra, W. B., Ryman, K. D. & Johnston, R. E. (1998). Adaptation of sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *Journal of Virology* **72**, 7357-7366.

Kohl, A., Clayton, R. F., Weber, F., Bridgen, A., Randall, R. E. & Elliott, R. M. (2003). Bunyamwera Virus Nonstructural Protein NSs Counteracts Interferon Regulatory Factor 3-Mediated Induction of Early Cell Death. *J. Virol.* **77**, 7999-8008.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E. J., Carrington, J. C., Liu, Y. P., Dolja, V. V., Calvino, L. F., Lopez-Moya, J. J. & Burgyan, J. (2006). Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *Embo Journal* **25**, 2768-2780.

Lakatos, L., Szittya, G., Silhavy, D. & Burgyan, J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *Embo Journal* **23**, 876-884.

Lan, Q. & Fallon, A. M. (1990). Small Heat-Shock Proteins Distinguish between 2 Mosquito Species and Confirm Identity of Their Cell-Lines. *American Journal of Tropical Medicine and Hygiene* **43**, 669-676.

Landthaler, M., Yalcin, A. & Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and its D-melanogaster homolog are required for miRNA biogenesis. *Current Biology* **14**, 2162-2167.

Lee, Y., Ahn, C., Han, J. J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. & Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-419.

Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. *Embo Journal* **21**, 4663-4670.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z. Y., Sontheimer, E. J. & Carthew, R. W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69-81.

Levine, B., Jiang, H. H., Kleeman, L. & Yang, G. (1996). Effect of E2 envelope glycoprotein cytoplasmic domain mutations on Sindbis virus pathogenesis. *Journal of Virology* **70**, 1255-1260.

Li, C. J., Vagin, V. V., Lee, S. H., Xu, J., Ma, S. M., Xi, H. L., Seitz, H., Horwich, M. D., Syrzycka, M., Honda, B. M., Kittler, E. L. W., Zapp, M. L., Klattenhoff, C., Schulz, N., Theurkauf, W. E., Weng, Z. P. & Zamore, P. D. (2009a). Collapse of

Germline piRNAs in the Absence of Argonaute3 Reveals Somatic piRNAs in Flies. *Cell* **137**, 509-521.

Li, H. W. & Ding, S. W. (2005). Antiviral silencing in animals. *Febs Letters* **579**, 5965-5973.

Li, H. W., Li, W. X. & Ding, S. W. (2002). Induction and suppression of RNA silencing by an animal virus. *Science* **296**, 1319-1321.

Li, S., Mead, E., Liang, S. & Tu, Z. (2009b). Direct sequencing and expression analysis of a large number of miRNAs in Aedes aegypti and a multi-species survey of novel mosquito miRNAs. *BMC Genomics* **10**, 581.

Li, W. X., Li, H. W., Lu, R., Li, F., Dus, M., Atkinson, P., Brydon, E. W. A., Johnson, K. L., Garcia-Sastre, A., Ball, L. A., Palese, P. & Ding, S. W. (2004). Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1350-1355.

Lian, S., Fritzler, M. J., Katz, J., Hamazaki, T., Terada, N., Satoh, M. & Chan, E. K. L. (2007). Small interfering RNA-mediated silencing induces target-dependent assembly of GW/P bodies. *Molecular Biology of the Cell* **18**, 3375-3387.

Lim, D. H., Kim, J., Kim, S., Carthew, R. W. & Lee, Y. S. (2008). Functional analysis of dicer-2 missense mutations in the siRNA pathway of Drosophila. *Biochemical and Biophysical Research Communications* **371**, 525-530.

Lin, J. & Cullen, B. R. (2007). Analysis of the interaction of primate retroviruses with the human RNA interference machinery. *Journal of Virology* **81**, 12218-12226.

Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. (2005). The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. *Embo Reports* **6**, 1149-1155.

Lipardi, C. & Paterson, B. M. (2009). Identification of an RNA-dependent RNA polymerase in Drosophila involved in RNAi and transposon suppression. *Proceedings of the National Academy of Sciences* **106**, 15645-15650.

Lippman, Z. & Martienssen, R. (2004). The role of RNA interference in heterochromatic silencing. *Nature* **431**, 364-370.

Liu, Q. H., Rand, T. A., Kalidas, S., Du, F. H., Kim, H. E., Smith, D. P. & Wang, X. D. (2003). R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science* **301**, 1921-1925.

Lowenberger, C. (2001). Innate immune response of Aedes aegypti. *Insect Biochemistry and Molecular Biology* **31**, 219-229.

Lutomiah, J. J. L., Mwandawiro, C., Magambo, J. & Sang, R. C. (2007). Infection and vertical transmission of Kamiti river virus in laboratory bred Aedes aegypti mosquitoes. *Journal of Insect Science* **7**.

Malone, C. D., Brennecke, J., Dus, M., Stark, A., McCombie, W. R., Sachidanandam, R. & Hannon, G. J. (2009). Specialized piRNA Pathways Act in Germline and Somatic Tissues of the Drosophila Ovary. *Cell* **137**, 522-535.

Maori, E., Tanne, E. & Sela, I. (2007). Reciprocal sequence exchange between non-retro viruses and hosts leading to the appearance of new host phenotypes. *Virology* **362**, 342-349.

Marin, M. S., Zanotto, P. M. D., Gritsun, T. S. & Gould, E. A. (1995). Phylogeny of Tyu, Sre, and Cfa Virus - Different Evolutionary Rates in the Genus Flavivirus. *Virology* **206**, 1133-1139.

Mathonnet, G., Fabian, M. R., Svitkin, Y. V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W. C., Darzynkiewicz, E., Pillai, R. S., Filipowicz, W., Duchaine, T. F. & Sonenberg, N. (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex elF4F. *Science* **317**, 1764-1767.

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P. & Zamore, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**, 607-620.

McKnight, K. L., Simpson, D. A., Lin, S. C., Knott, T. A., Polo, J. M., Pence, D. F., Johannsen, D. B., Heidner, H. W., Davis, N. L. & Johnston, R. E. (1996). Deduced consensus sequence of Sindbis virus strain AR339: Mutations contained in laboratory strains which affect cell culture and in vivo phenotypes. *Journal of Virology* **70**, 1981-1989.

Miller, S., Kastner, S., Krijnse-Locker, J., Buhler, S. & Bartenschlager, R. (2007). The Non-structural Protein 4A of Dengue Virus Is an Integral Membrane Protein Inducing Membrane Alterations in a 2K-regulated Manner. *Journal of Biological Chemistry* **282**, 8873-8882.

mirbase.org miRBase: The microRNA Database, Release 13 edn.

Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. & Siomi, M. C. (2005). Slicer function of Drosophila Argonautes and its involvement in RISC formation. *Genes & Development* **19**, 2837-2848.

Molnar, A., Csorba, T., Lakatos, L., Varallyay, E., Lacomme, C. & Burgyan, J. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *Journal of Virology* **79**, 7812-7818.

Morale-Betoulle, M. E., Pineda, M. L. M., Sosa, S. M., Panella, N., Lopez, M. R., Cordon-Rosales, C., Komar, N., Powers, A. & Johnson, B. W. (2008). Culex Flavivirus Isolates from Mosquitoes in Guatemala. *Journal of Medical Entomology* **45**, 1187-1190.

Mota, J. & Rico-Hesse, R. (2009). Humanized Mice Show Clinical Signs of Dengue Fever according to Infecting Virus Genotype. *Journal of Virology* **83**, 8638-8645.

Myles, K. M., Morazzani, E. M. & Adelman, Z. N. (2009). Origins of viRNAs in mosquitoes. *RNA Biology* **6**, 1-5.

Myles, K. M., Pierro, D. J. & Olson, K. E. (2003). Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in Aedes aegypti. *Journal of Virology* **77**, 8872-8881.

Myles, K. M., Pierro, D. J. & Olson, K. E. (2004). Comparison of the transmission potential of two genetically distinct Sindbis viruses after oral infection of Aedes aegypti (Diptera : Culicidae). *Journal of Medical Entomology* **41**, 95-106.

Myles, K. M., Wiley, M. R., Morazzani, E. M. & Adelman, Z. N. (2008). Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 19938-19943.

Napoli, C., Lemieux, C. & Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans
10.1105/tpc.2.4.279. *Plant Cell* **2**, 279-289.

Nene, V., Wortman, J. R., Lawson, D., Haas, B., Kodira, C., Tu, Z. J., Loftus, B., Xi, Z. Y., Megy, K., Grabherr, M., Ren, Q. H., Zdobnov, E. M., Lobo, N. F., Campbell, K. S., Brown, S. E., Bonaldo, M. F., Zhu, J. S., Sinkins, S. P., Hogenkamp, D. G., Amedeo, P., Arensburger, P., Atkinson, P. W., Bidwell, S., Biedler, J., Birney, E., Bruggner, R. V., Costas, J., Coy, M. R., Crabtree, J., Crawford, M., deBruyn, B., DeCaprio, D., Eiglmeier, K., Eisenstadt, E., El-Dorry, H., Gelbart, W. M., Gomes, S. L., Hammond, M., Hannick, L. I., Hogan, J. R., Holmes, M. H., Jaffe, D., Johnston, J. S., Kennedy, R. C., Koo, H., Kravitz, S., Kriventseva, E. V., Kulp, D., LaButti, K., Lee, E., Li, S., Lovin, D. D., Mao, C. H., Mauceli, E., Menck, C. F. M., Miller, J. R., Montgomery, P., Mori, A., Nascimento, A. L., Naveira, H. F., Nusbaum, C., O'Leary, S., Orvis, J., Pertea, M., Quesneville, H., Reidenbach, K. R., Rogers, Y. H., Roth, C. W., Schneider, J. R., Schatz, M., Shumway, M., Stanke, M., Stinson, E. O., Tubio, J. M. C., VanZee, J. P., Verjovski-Almeida, S., Werner, D., White, O., Wyder, S., Zeng, Q. D., Zhao, Q., Zhao, Y. M., Hill, C. A., Raikhel, A. S., Soares, M. B., Knudson, D. L., Lee, N. H., Galagan, J., Salzberg, S. L., Paulsen, I. T., Dimopoulos, G., Collins, F. H., Birren, B., Fraser-Liggett, C. M. & Severson, D. W. (2007). Genome sequence of Aedes aegypti, a major arbovirus vector. *Science* **316**, 1718-1723.

Nishida, K. M., Saito, K., Mori, T., Kawamura, Y., Nagami-Okada, T., Inagaki, S., Siomi, H. & Siomi, M. C. (2007). Gene silencing mechanisms mediated by Aubergine-piRNA complexes in *Drosophila* male gonad. *RNA* **13**, 1911-1922.

Nottrott, S., Simard, M. J. & Richter, J. D. (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nature Structural & Molecular Biology* **13**, 1108-1114.

Nykanen, A., Haley, B. & Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309-321.

Obbard, D. J., Jiggins, F. M., Halligan, D. L. & Little, T. J. (2006). Natural selection drives extremely rapid evolution in antiviral RNAi genes. *Current Biology* **16**, 580-585.

Okamura, K., Balla, S., Martin, R., Liu, N. & Lai, E. C. (2008). Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in *Drosophila melanogaster*. *Nature Structural & Molecular Biology* **15**, 581-590.

Okamura, K., Ishizuka, A., Siomi, H. & Siomi, M. C. (2004). Distinct roles for argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes & Development* **18**, 1655-1666.

Olson, K. E., Higgs, S., Gaines, P. J., Powers, A. M., Davis, B. S., Kamrud, K. I., Carlson, J. O., Blair, C. D. & Beaty, B. J. (1996). Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. *Science* **272**, 884-886.

Olson, K. E., Higgs, S., Hahn, C. S., Rice, C. M., Carlson, J. O. & Beaty, B. J. (1994). The Expression of Chloramphenicol Acetyltransferase in Aedes-Albopictus (C6/36) Cells and Aedes-Triseriatus Mosquitos Using a Double Subgenomic Recombinant Sindbis Virus. *Insect Biochemistry and Molecular Biology* **24**, 39-48.

Olson, K. E., Myles, K. M., Seabaugh, R. C., Higgs, S., Carlson, J. O. & Beaty, B. J. (2000). Development of a Sindbis virus expression system that efficiently expresses green fluorescent protein in midguts of Aedes aegypti following per os infection. *Insect Molecular Biology* **9**, 57-65.

Omoto, S. & Fujii, Y. R. (2005). Regulation of human immunodeficiency virus 1 transcription by nef microRNA. *Journal of General Virology* **86**, 751-755.

Omoto, S., Ito, M., Tsutsumi, Y., Ichikawa, Y., Okuyama, H., Brisibe, E., Saksena, N. & Fujii, Y. (2004). HIV-1 nef suppression by virally encoded microRNA. *Retrovirology* **1**, 44.

Osta, M. A., Christophides, G. K., Vlachou, D. & Kafatos, F. C. (2004). Innate immunity in the malaria vector Anopheles gambiae: comparative and functional genomics. *Journal of Experimental Biology* **207**, 2551-2563.

Ouellet, D. L., Plante, I., Landry, P., Barat, C., Janelle, M. E., Flamand, L., Tremblay, M. J. & Provost, P. (2008). Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element. *Nucleic Acids Research* **36**, 2353-2365.

Overhoff, M., Alken, M., Far, R. K. K., Lemaitre, M., Lebleu, B., Sczakiel, G. & Robbins, I. (2005). Local RNA target structure influences siRNA efficacy: A systematic global analysis. *Journal of Molecular Biology* **348**, 871-881.

Pall, G. S., Codony-Servat, C., Byrne, J., Ritchie, L. & Hamilton, A. (2007). Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic Acids Research* **35**.

Pantaleo, V., Szittya, G. & Burgyan, J. (2007). Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. *Journal of Virology* **81**, 3797-3806.

Parker, J. S., Roe, S. M. & Barford, D. (2004). Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *Embo Journal* **23**, 4727-4737.

Pedersen, I. M., Cheng, G., Wieland, S., Volinia, S., Croce, C. M., Chisari, F. V. & David, M. (2007). Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* **449**, 919-U13.

Peleg, J. (1966). Growth of Aedes Aegypti Embryonic Cells and Tissues in Vitro. *Experientia* **22**, 555-556.

Peleg, J. (1968). Growth of Arboviruses in Monolayers from Subcultured Mosquito Embryo Cells. *Virology* **35**, 617-619.

Peleg, J. (1969). Inapparent Persistent Virus Infection in Continuously Grown Aedes-Aegypti Mosquito Cells. *Journal of General Virology* **5**, 463-471.

Peleg, J. & Shahar, A. (1972). Morphology and Behavior of Cultured Aedes-Aegypti Mosquito Cells. *Tissue & Cell* **4**, 55-62.

Petersen, C. P., Bordeleau, M. E., Pelletier, J. & Sharp, P. A. (2006). Short RNAs repress translation after initiation in mammalian cells. *Molecular Cell* **21**, 533-542.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M. C., Russo, J. J., Ju, J. Y., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M. & Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nature Methods* **2**, 269-276.

Pfeffer, S., Zavolan, M., Grasser, F. A., Chien, M. C., Russo, J. J., Ju, J. Y., John, B., Enright, A. J., Marks, D., Sander, C. & Tuschl, T. (2004). Identification of virus-encoded microRNAs. *Science* **304**, 734-736.

Pialoux, G., Gauzere, B. A., Jaureguiberry, S. & Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. *Lancet Infectious Diseases* **7**, 319-327.

Pierro, D. J., Myles, K. M., Foy, B. D., Beaty, B. J. & Olson, K. E. (2003). Development of an orally infectious Sindbis virus transducing system that efficiently disseminates and expresses green fluorescent protein in Aedes aegypti. *Insect Molecular Biology* **12**, 107-116.

Pierro, D. J., Salazar, M. I., Beaty, B. J. & Olson, K. E. (2006). Infectious clone construction of dengue virus type 2, strain Jamaican 1409, and characterization of a conditional E6 mutation. *Journal of General Virology* **87**, 2263-2268.

Pijlman, G. P., Funk, A., Kondratieva, N., Leung, J., Torres, S., van der Aa, L., Liu, W. J., Palmenberg, A. C., Shi, P. Y., Hall, R. A. & Khromykh, A. A. (2008). A Highly Structured, Nuclease-Resistant, Noncoding RNA Produced by Flaviviruses Is Required for Pathogenicity. *Cell Host & Microbe* **4**, 579-591.

Powers, A. M., Kamrud, K. I., Olson, K. E., Higgs, S., Carlson, J. O. & Beaty, B. J. (1996). Molecularly engineered resistance to California serogroup virus replication in mosquito cells and mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 4187-4191.

Proutski, V., Gould, E. A. & Holmes, E. C. (1997). Secondary structure of the 3' untranslated region of flaviviruses: Similarities and differences. *Nucleic Acids Research* **25**, 1194-1202.

Rand, T. A., Ginalski, K., Grishin, N. V. & Wang, X. D. (2004). Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14385-14389.

Reigel, F. (1980). Studies on Igarashi's *Aedes Albopictus* Cell Clone C6/36. *Experientia* **36**, 1450-1450.

Reiter, P., Fontenille, D. & Paupy, C. (2006). Aedes albopictus as an epidemic vector of chikungunya virus: another emerging problem? *Lancet Infectious Diseases* **6**, 463-464.

Renault, P., Solet, J. L., Sissoko, D., Balleydier, E., Larrieu, S., Filleul, L., Lassalle, C., Thiria, J., Rachou, E., de Valk, H., Ilef, D., Ledrans, M., Quatresous, I., Quenel, P. & Pierre, V. (2007). A major epidemic of Chikungunya virus infection on Reunion Island, France, 2005-2006. *American Journal of Tropical Medicine and Hygiene* **77**, 727-731.

Rice, P., Longden, I. & Bleasby, A. (2000). EMBOSS: The European Molecular Biology Open Software Suite. *Trends in Genetics* **16**, 276-277.

Rico-Hesse, R., Harrison, L. M., Salas, R. A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J., deMesa, M. T. R., Nogueira, R. M. R. & daRosa, A. T. (1997). Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology* **230**, 244-251.

Sabin, L. R., Zhou, R., Gruber, J. J., Lukinova, N., Bambina, S., Berman, A., Lau, C. K., Thompson, C. B. & Cherry, S. (2009). Ars2 Regulates Both miRNA- and siRNA-Dependent Silencing and Suppresses RNA Virus Infection in Drosophila. *Cell* **138**, 340-351.

Saito, K., Ishizuka, A., Siomi, H. & Siomi, M. C. (2005). Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. *Plos Biology* **3**, 1202-1212.

Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. & Siomi, M. C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. *Genes & Development* **20**, 2214-2222.

Saito, K., Sakaguchi, Y., Suzuki, T., Siomi, H. & Siomi, M. C. (2007). Pimet, the *Drosophila* homolog of HEN1, mediates 2 '-O-methylation of PIWI-interacting RNAs at their 3 ' ends. *Genes & Development* **21**, 1603-1608.

Salazar, M. I., Richardson, J. H., Sanchez-Vargas, I., Olson, K. E. & Beaty, B. J. (2007). Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes. *Bmc Microbiology* **7**.

Saleh, M. C., Tassetto, M., van Rij, R. P., Goic, B., Gausson, V., Berry, B., Jacquier, C., Antoniewski, C. & Andino, R. (2009). Antiviral immunity in Drosophila requires systemic RNA interference spread. *Nature* **458**, 346-U109.

Salonen, A., Ahola, T. & Kaariainen, L. (2004). Viral RNA replication in association with cellular membranes. In *Membrane Trafficking in Viral Replication*, pp. 139-173.

Samols, M. A., Hu, J. H., Skalsky, R. L. & Renne, R. (2005). Cloning and identification of a MicroRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *Journal of Virology* **79**, 9301-9305.

Sanchez-Vargas, I., Scott, J. C., Poole-Smith, B. K., Franz, A. W. E., Barbosa-Solomieu, V., Wilusz, J., Olson, K. E. & Blair, C. D. (2009). Dengue Virus Type 2 Infections of *Aedes aegypti* Are Modulated by the Mosquito's RNA Interference Pathway. *PLoS Pathogens* **5**, e1000299.

Sanford, J. C. & Johnston, S. A. (1985). The Concept of Parasite-Derived Resistance - Deriving Resistance Genes from the Parasites Own Genome. *Journal of Theoretical Biology* **113**, 395-405.

Sang, R. C., Gichogo, A., Gachoya, J., Dunster, M. D., Ofula, V., Hunt, A. R., Crabtree, M. B., Miller, B. R. & Dunster, L. M. (2003). Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field-collected flood-water Aedes mosquitoes sampled from a dambo in central Kenya. *Archives of Virology* **148**, 1085-1093.

Sano, M., Kato, Y. & Taira, K. (2006). Sequence-specific interference by small RNAs derived from adenovirus VAI RNA. *Febs Letters* **580**, 1553-1564.

Sarver, N. & Stollar, V. (1977). Sindbis Virus-Induced Cytopathic Effect in Clones of Aedes-Albopictus (Singh) Cells. *Virology* **80**, 390-400.

Sasao, F., Igarashi, A. & Fukai, K. (1980). Amino-Acid-Requirements for the Growth of *Aedes Albopictus* Clone C6/36 Cells and for the Production of Dengue and Chikungunya Viruses in the Infected-Cells. *Microbiology and Immunology* **24**, 915-924.

Schneider, T. D. & Stephens, R. M. (1990). Sequence logos: a new way to display consensus sequences. *Nucl. Acids Res.* **18**, 6097-6100.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z. S., Aronin, N. & Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199-208.

Schwarz, D. S., Hutvagner, G., Haley, B. & Zamore, P. D. (2002). Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Molecular Cell* **10**, 537-548.

Schwarz, D. S., Tomari, Y. & Zamore, P. D. (2004). The RNA-induced silencing complex is a Mg2+-dependent endonuclease. *Current Biology* **14**, 787-791.

Seabaugh, R. C., Olson, K. E., Higgs, S., Carlson, J. O. & Beaty, B. J. (1998). Development of a chimeric sindbis virus with enhanced per Os infection of Aedes aegypti. *Virology* **243**, 99-112.

Seo, G. J., Fink, L. H. L., O'Hara, B., Atwood, W. J. & Sullivan, C. S. (2008). Evolutionarily Conserved Function of a Viral MicroRNA. *Journal of Virology* **82**, 9823-9828.

Shpiz, S., Kwon, D., Rozovsky, Y. & Kalmykova, A. (2009). rasiRNA pathway controls antisense expression of *Drosophila* telomeric retrotransposons in the nucleus. *Nucleic Acids Research* **37**, 268-278.

Singh, G., Popli, S., Hari, Y., Malhotra, P., Mukherjee, S. & Bhatnagar, R. K. (2009). Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. *FASEB J.* **23**, 1845-1857.

Singh, K. R. P. (1967). Cell Cultures Derived from Larvae of *Aedes Albopictus* (Skuse) and *Aedes Aegypti* (L). *Current Science* **36**, 506-508.

Singh, K. R. P. & Paul, S. D. (1968). Multiplication of Arboviruses in Cell Lines from Aedes Albopictus and Aedes Aegypti. *Current Science* **37**, 65-67.

Skalsky, R., Vanlandingham, D., Scholle, F., Higgs, S. & Cullen, B. (2010). Identification of microRNAs expressed in two mosquito vectors, Aedes albopictus and Culex quinquefasciatus. *BMC Genomics* **11**, 119.

Soldan, S. S., Plassmeyer, M. L., Matukonis, M. K. & Gonzalez-Scarano, F. (2005). La Crosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. *Journal of Virology* **79**, 234-244.

Song, J. J., Smith, S. K., Hannon, G. J. & Joshua-Tor, L. (2004). Crystal structure of argonaute and its implications for RISC slicer activity. *Science* **305**, 1434-1437.

Souza-Neto, J. A., Sim, S. & Dimopoulos, G. (2009). An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense 10.1073/pnas.0905006106. *Proceedings of the National Academy of Sciences*, -.

Stadler, P. F., Hofacker, I. L., Witwer, C., Thurner, C. & Stocsits, R. (2001). Vienna Atlas of RNA Structures: http://rna.tbi.univie.ac.at/virus/.

Stark, A., Brennecke, J., Russell, R. B. & Cohen, S. M. (2003). Identification of Drosophila MicroRNA Targets. *PLoS Biol* **1**, e60.

Stollar, B. D. & Stollar, V. (1970a). Immunofluorescent Demonstration of Double-Stranded Rna in Cytoplasm of Sindbis Virus-Infected Cells. *Virology* **42**, 276-&.

Stollar, V., Schlesin.Rw & Stevens, T. M. (1967). Studies on Nature of Dengue Viruses: III. RNA Synthesis in Cells Infected with Type 2 Dengue Virus. *Virology* **33**, 650-658.

Stollar, V. & Stollar, B. D. (1970b). Immunochemical Measurement of Double-Stranded RNA of Uninfected and Arbovirus-Infected Mammalian Cells. *Proceedings of the National Academy of Sciences of the United States of America* **65**, 993-1000.

Stollar, V. & Thomas, V. L. (1975). Agent in *Aedes Aegypti* Cell Line (Peleg) Which Causes Fusion of *Aedes Albopictus* Cells. *Virology* **64**, 367-377.

Strauss, J. H. & Strauss, E. G. (1994). The Alphaviruses - Gene-Expression, Replication, and Evolution. *Microbiological Reviews* **58**, 491-562.

Sugiyama, T., Cam, H., Verdel, A., Moazed, D. & Grewal, S. I. S. (2005). RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 152-157.

Tang, S., Bertke, A. S., Patel, A., Wang, K., Cohen, J. I. & Krause, P. R. (2008). An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 10931-10936.

Tang, S., Patel, A. & Krause, P. R. (2009). Novel Less-Abundant Viral MicroRNAs Encoded by Herpes Simplex Virus 2 Latency-Associated Transcript and Their Roles in Regulating ICP34.5 and ICP0 mRNAs. *Journal of Virology* **83**, 1433-1442.

Taylor, R. M., Hurlbut, H. S., Work, T. H., Kingston, J. R. & Frothingham, T. E. (1955). Sindbis Virus - a Newly Recognized Arthropod-Transmitted Virus. *American Journal of Tropical Medicine and Hygiene* **4**, 844-862.

Thurner, C., Witwer, C., Hofacker, I. L. & Stadler, P. F. (2004). Conserved RNA secondary structures in Flaviviridae genomes. *Journal of General Virology* **85**, 1113-1124.

Tomari, Y., Du, T. & Zamore, P. D. (2007). Sorting of Drosophila small silencing RNAs. *Cell* **130**, 299-308.

Tomari, Y., Matranga, C., Haley, B., Martinez, N. & Zamore, P. D. (2004). A protein sensor for siRNA asymmetry. *Science* **306**, 1377-1380.

Travanty, E. A. (2005). RNA Interference and Dengue Virus Replication In Insect Cell Culture and *Aedes aegypti* Mosquitoes. In *Department of Microbiology, Immunology and Pathology*, pp. 216. Fort Collins, CO: Colorado State University.

Triboulet, R., Mari, B., Lin, Y. L., Chable-Bessia, C., Bennasser, Y., Lebrigand, K., Cardinaud, B., Maurin, T., Barbry, P., Baillat, V., Reynes, J., Corbeau, P., Jeang, K. T. & Benkirane, M. (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315**, 1579-1582.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. & Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. *Genes & Development* **13**, 3191-3197.

Uchil, P. D., Kumar, A. V. A. & Satchidanandam, V. (2006). Nuclear localization of flavivirus RNA synthesis in infected cells. *Journal of Virology* **80**, 5451-5464.

Uchil, P. D. & Satchidanandam, V. (2003). Architecture of the flaviviral replication complex - Protease, nuclease, and detergents reveal encasement within double-layered membrane compartments. *Journal of Biological Chemistry* **278**, 24388-24398.

Umbach, J. L. & Cullen, B. R. (2009). The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Genes & Development* **23**, 1151-1164.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**, 780-U108.

Vagin, V. V., Sigova, A., Li, C. J., Seitz, H., Gvozdev, V. & Zamore, P. D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320-324.

van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. & Stuitje, A. R. (1990). Flavonoid Genes in Petunia: Addition of a Limited Number of Gene Copies May Lead to a Suppression of Gene Expression
10.1105/tpc.2.4.291. *Plant Cell* **2**, 291-299.

van Rij, R. P., Saleh, M. C., Berry, B., Foo, C., Houk, A., Antoniewski, C. & Andino, R. (2006). The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes & Development* **20**, 2985-2995.

Wakiyama, M., Takimoto, K., Ohara, O. & Yokoyama, S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes & Development* **21**, 1857-1862.

Wang, B. B., Yanaz, A. & Novina, C. D. (2008a). MicroRNA-repressed mRNAs contain 40S but not 60S components. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5343-5348.

Wang, F. Z., Weber, F., Croce, C., Liu, C. G., Liao, X. D. & Pellett, P. E. (2008b). Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. *Journal of Virology* **82**, 9065-9074.

Wang, X. H., Aliyari, R., Li, W. X., Li, H. W., Kim, K., Carthew, R., Atkinson, P. & Ding, S. W. (2006). RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science* **312**, 452-454.

Weaver, S. C. & Vasilakis, N. (2009). Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infection Genetics and Evolution* **9**, 523-540.

Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Kessler, N., Randall, R. E. & Elliott, R. M. (2002). Bunyamwera Bunyavirus Nonstructural Protein NSs Counteracts the Induction of Alpha/Beta Interferon. *J. Virol.* **76**, 7949-7955.

Weber, F., Wagner, V., Rasmussen, S. B., Hartmann, R. & Paludan, S. R. (2006). Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *Journal of Virology* **80**, 5059-5064.

Westaway, E. G., MacKenzie, J. M., Kenney, M. T., Jones, M. K. & Khromykh, A. A. (1997). Ultrastructure of Kunjin virus-infected cells: Colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *Journal of Virology* **71**, 6650-6661.

White, L. A. (1987). Susceptibility of *Aedes Albopictus* C6/36 Cells to Viral-Infection. *Journal of Clinical Microbiology* **25**, 1221-1224.

Wielgosz, M. & Huang, H. (1997). A novel viral RNA species in Sindbis virus-infected cells. *J. Virol.* **71**, 9108-9117.

Winter, F., Edaye, S., Huttenhofer, A. & Brunel, C. (2007). Anopheles gambiae miRNAs as actors of defence reaction against Plasmodium invasion. *Nucl. Acids Res.*, gkm686.

Wu, L. G., Fan, J. H. & Belasco, J. G. (2006). MicroRNAs direct rapid deadenylation of mRNA. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4034-4039.

Wu, Q. F., Luo, Y. J., Lu, R., Lau, N., Lai, E. C., Li, W. X. & Ding, S. W. (2010). Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 1606-1611.

Xi, Z., Ramirez, J. L. & Dimopoulos, G. (2008). The Aedes aegypti Toll Pathway Controls Dengue Virus Infection. *PLoS Pathog* **4**, e1000098.

Xia, T., O'Hara, A., Araujo, I., Barreto, J., Carvalho, E., Sapucaia, J. B., Ramos, J. C., Luz, E., Pedroso, C., Manrique, M., Toomey, N. L., Brites, C., Dittmer, D. P. & Harrington, W. J. (2008). EBV MicroRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3. *Cancer Research* **68**, 1436-1442.

Xu, P., Vernooy, S. Y., Guo, M. & Hay, B. A. (2003). The Drosophila MicroRNA Mir-14 Suppresses Cell Death and Is Required for Normal Fat Metabolism. **13**, 790-795.

Yang, D., Lu, H. & Erickson, J. W. (2000). Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in Drosophila embryos. *Current Biology* **10**, 1191-1200.

Zambon, R. A., Vakharia, V. N. & Wu, L. P. (2006). RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. *Cellular Microbiology* **8**, 880-889.

Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.

Zhu, J. Y., Pfuhl, T., Motsch, N., Barth, S., Nicholls, J., Grasser, F. & Meister, G. (2009). Identification of Novel Epstein-Barr Virus MicroRNA Genes from Nasopharyngeal Carcinomas. *Journal of Virology* **83**, 3333-3341.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**, 3406 - 3415.

Zuker, M. & Markham, N. (1995-2009). mfold server.