NOTE TO USERS

DISSERTATION

RNA INTERFERENCE AND DENGUE VIRUS REPLICATION IN INSECT CELL CULTURE AND *AEDES AEGYPTI* MOSQUITOES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY EMILY ANNE TRAVANTY ENTITLED RNA INTERFERENCE AND DENGUE VIRUS REPLICATION IN INSECT CELL CULTURE AND *AEDES AEGYPTI* MOSQUITOES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

RNA INTERFERENCE AND DENGUE VIRUS REPLICATION IN INSECT CELL CULTURE AND *AEDES AEGYPTI* MOSQUITOES

RNA interference, or RNAi, can effectively block dengue virus (DEN) replication in mosquito cell culture. The purpose of this study is to examine the mechanism of the RNAi response to DEN-2 virus in mosquito cell culture and to examine the feasibility of RNAi to DEN-2 in the entire mosquito using germ-line transformation. Mosquito cells (C6/36) transformed with a plasmid designed to express double-stranded RNA (dsRNA) derived from the DEN-2 genome do not allow replication of the virus. These cells do not accumulate viral antigen or viral genomic RNA upon infection. The block in DEN-2 replication in these cells was found to be due to an RNAi response, generated by the expression of dsRNA within these cells. The nature of this RNAi response including the dsRNA trigger, the effector molecules (small interfering RNAs or siRNA) and the degradation products of DEN-2 RNA in mosquitoes are defined here. Upon confirmation that the replication silencing observed in mosquito cells was due to RNAi, the process was examined in the mosquito as a whole using a transgenesis approach. Transgenic mosquitoes were engineered to express DEN-2 dsRNA as well as a selectable eye-specific marker. These mosquitoes were tested for their ability to resist DEN-2 infection. None of the transgenic mosquitoes exhibited resistance to DEN-2 infection. The reasons behind the lack of observable DEN-2 resistance in these mosquitoes are discussed along with the pitfalls encountered in the generation of these transgenic mosquitoes.

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DEDICATION

This work is dedicated to my husband, Dr. Brain Kempf, who has loved me, inspired me and supported me in all that I do; and to my parents David and Alice Travanty, who gave me my start in science by naming my first toys after famous scientists, especially the bunny named 'Gregor Mendal McGregor'; and to my brothers Christopher Travanty and Michael Travanty and my sister Constance Travanty, and all of my family; and to Trout...

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Literature Review

Chapter 1 Literature Review.

Molecular Virology of Dengue Viruses.

The four serotypes of dengue viruses (DEN1-4) form a distinct antigenic complex within the *Flavivirus* genus of the family Flaviviridae (Calisher et al., 1989; Westaway et al., 1985). The family Flaviviridae also includes two other genera, (1) the *Pestivirus* genus, represented by the type member bovine viral diarrhea virus and (2) *Hepacivirus*, represented by hepatitis C virus (Kuno et al., 1998). Additionally, Flaviviridae contains a number of unclassified viruses including the hepatitis GB viruses (Kuno et al., 1998). The vast majority of viruses within the *Flavivirus* genus are arthropod-borne viruses, meaning that their transmission is dependent upon the feeding of arthropods, particularly mosquitoes and ticks. The mosquito-borne cluster of flaviviruses can be broken down into serocomplexes named for type members that represent the individual complex members, including the Japanese encephalitis complex (West Nile, Kunjin, Murray Valley encephalitis, and St. Louis encephalitis viruses), the dengue complex (DEN1-4) and yellow fever virus, which forms its own antigenically distinct group (Kuno et al., 1998).

DEN viruses are small (40-60nm), spherical enveloped viruses with a single-stranded RNA genome approximately 11 kilobases (kb) in size (Murphy, 1980). The viral RNA contains a type 1 m^7G5'ppp5'A cap that functions in translation of the viral genomic RNA into a large polyprotein that is co- and post-translationally cleaved by host signalases (furin and other Golgi-localized proteases; Cauchi et al., 1991; Wengler et al., 1990) and the virally encoded serine protease to generate the viral proteins (Thurner et al., 2004). The open reading frame, which is marked by non-coding regions (NCR) on

either end, encodes 3 structural proteins (Capsid, Envelope and (pre)Membrane) and 7 non-structural (NS) proteins in the following order: 5'- Cap-5'NCR-C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS3-3'NCR (Rice et al., 1985). There is no polyadenylated tail at the 3' end of DEN viral genomes. Conserved secondary structure within the 3' non-coding region (NCR) is thought to function in the initiation of (-) strand synthesis (Wengler, 1981; Wengler and Castle, 1986; Zeng et al., 1998; Olsthoom and Bol, 2001). The 5' and 3' NCRs may also play a role, along with sequences within the coding region, in encapsidation of newly generated viral genomic RNAs (Hahn et al., 1987; Hahn et al., 1988) and translation initiation (Brinton and Dispoto, 1988; Holden and Harris, 2004).

DEN viruses replicate in the cytoplasm and the polyprotein is translated by ribosomes at the endoplasmic reticulum (ER) with some portions of the polyprotein extruding into the cytoplasm while others remain within the ER lumen. The three structural proteins are the capsid (C, 11 kilodaltons (kDa)), the premembrane protein (prM, 26kd) and the envelope protein (E, 50 kDa). The C protein is cytoplasmic and has basic residues concentrated at the C- and N- termini that cooperatively bind RNA (Khromykh et al., 2001) and a hydrophobic domain in the central portion that can interact with cellular membranes and functions in virion assembly (Markoff et al., 1997). Efficient cleavage at the C-prM junction by signal peptidase requires determinants in the carboxy terminus of the C protein (Stocks and Lobigs, 1998). The prM protein is generated in its immature form within the ER and remains membrane associated. During egress through the secretory pathway, prM is cleaved by the trans-Golgi resident furin to form the structural protein M (8 kDa) found in the mature virion and the N-terminal pr

segment that is secreted into the extracellular medium (Gollins et al., 1986; Murray et al., 1993; Stadler et al., 1997). Protective immunity can be generated by an antibody response to prM, which is hypothesized to be due to the neutralization of released virions containing residual uncleaved prM (Kaufman et al., 1989). Murray and colleagues (1993) found that unlike in vertebrate cell cultures, in mosquito cells the prM to M cleavage is often incomplete, resulting in some incorporation of the immature prM form into the virions. The E protein also remains in the ER lumen. It is a glycosylated type I membrane protein with a single transmembrane domain and 12 highly conserved cysteine residues that form intramolecular disulfide bonds (Chambers et al., 1990; Nowak and Wengler, 1987; Winkler et al., 1987). The prM and E proteins interact within the ER lumen where the pr portion protects the E protein from triggering pH-dependent cell membrane fusion before virus maturation in both DEN-2 and tick-borne encephalitis viruses (Heinz et al., 1994; Murray et al., 1993). In the mature virion, the E protein occurs as a homodimer and is the major surface protein responsible for cell binding and entry (Chambers et al., 1990; Wang et al., 1999). There are three domains in the E protein; domain II contains the fusion peptide (Zhang et al., 2004), domain III is the receptor binding domain (Yu et al., 2004) and a hinge region occurs between domains I and II (Lee et al., 1997). Modis and colleagues (2004) solved the structure of the E protein after membrane fusion, demonstrating the pH-dependent conformational change that occurs in the E protein within the endosome.

The seven non-structural proteins are found primarily membrane-associated in the cytoplasm of infected cells and are not thought to be incorporated into the virion (Lindenbach and Rice, 2001). The function of NS1 (46kd) is not fully understood, but it

has been associated with RNA replication and can be found within the membranous replication complexes (Mackenzie et al., 1996; Westaway et al., 1997; Uchil and Satchidanandam, 2003). The NS2a (22kd) is cleaved from the viral polyprotein by the viral serine protease at the N-terminus and a host ER protease at the C-terminus indicating that NS2a is a transmembrane protein. NS2a binds to NS3, NS5 and the 3' NCR of the viral RNA transcripts. The membrane association, as well as RNA-protein interactions, of NS2a may play a role in recruitment of RNA templates to the membrane-bound replication complex (Mackenzie et al., 1996). NS2b (14kd) forms a complex with NS3 (70kd) where it acts as a co-factor for the serine-protease function of NS3 (Arias, et al., 1993; Chambers et al., 1991; Chambers et al., 1993; Falgout et al., 1991; Jan et al., 1995). In addition to polyprotein processing, NS3 has also been implicated in RNA replication (Kapoor et al., 1995). The NS2b-3 protease cleaves the viral polypeptide at the NS2a/NS2b, NS2b/NS3, NS3/NS4a and NS4b/NS5 junctions to generate the individual viral proteins and is also responsible for some internal cleavages within NS2a, NS3, and NS4a as well as maturation of C. In addition to its protease activity, NS3 also has NTPase and helicase activities that have been implicated in replication (Cui et al., 1998). Finally, NS3 has been shown to bind caspase-8 and induce apoptosis, possibly to aid in virion release (Prikhod'ko, et al., 2002). The NS4a (16kd) and NS4b (27kd) proteins both contain hydrophobic domains and are membrane anchored (Mackenzie et al., 1998; Westaway et al., 1997). They both localize to the site of RNA replication, and NS4b can also be found dispersed throughout the cytoplasm (Mackenzie et al., 1998). The exact functions of these two non-structural proteins are not known, although NS4a appears to interact with NS1 (Lindenbach and Rice, 1999). The largest and most

conserved non-structural protein, NS5 (103kd), encodes the viral polymerase. It contains regions of sequence homology to the RNA-directed RNA polymerases (RdRPs) of other positive-strand RNA viruses. In particular, NS5 contains the invariant GDD amino acid motif (glycine-aspartate-aspartate) found in common in all RdRPs. Additionally, NS5 has been demonstrated *in vitro* to have RdRP activity in primer extension reactions as well as methyltransferase activity indicating that it probably also functions in methylation of the 5' cap structure (Koonin, 1993; Rice, et al., 1985; Tan et al., 1996).

Dengue viruses and mosquito interactions.

In nature, the DEN viruses are maintained by cycling between *Aedes* species mosquitoes and primates; there are two distinct DEN transmission cycles. The zoonotic or sylvatic cycle in sylvatic habitats of Africa and Malaysia involves nonhuman primate reservoir hosts and several different *Aedes* mosquitoes (Wang, 2000; Wolfe, 2001). The epidemic/endemic or urban cycle is responsible for the majority of DEN disease and involves primarily the mosquito *Aedes aegypti* (Gubler, 1998, Monath 1994). *Ae. aegypti* exhibits anthropophilic behavior in terms of host seeking and adoption of human generated artificial breeding containers. Another mosquito species implicated in the vectoring of DEN viruses in an urban or suburban setting is *Ae. albopictus* (Gubler, 1976). *Ae. albopictus* has become a major concern due to its ability to effectively vector numerous arboviruses and its expanding territorial range (Gratz, 2004).

A mosquito becomes infected with DEN viruses by feeding on an infected human or other primate. After feeding, the virus follows a specific path through the mosquito and the time between ingestion of the blood meal and subsequent transmission of the

virus by an infected mosquito is known as the extrinsic incubation period. The extrinsic incubation period and transmission rates are variable and temperature dependent. DEN viruses can usually be transmitted after 7 days at 32-35°C (Watts, 1987), but can take up to 14 days at colder temperatures. The virus is taken in by a mosquito with an infected blood meal and the first tissue it encounters is the midgut, the site of blood meal digestion. The initial stage of dengue infection in the mosquito is an eclipse phase in which the virus must first establish an infection in the midgut. Here, the virus titers in the lumen of the midgut decline before replication of the virus can be detected in midgut cells. After replicating in this initial tissue, the virus must escape and disseminate through the body to the salivary glands. The virus can readily be followed on this route through the mosquito by dissecting out the individual tissues and staining for viral antigen. DEN is thought to bud from the basolateral membrane of the midgut epithelial cell and accumulate under the basal lamina (Weaver, 1997). From there, DEN enters the hemocoel where it spreads to surrounding tissues such as the fat body and eventually the salivary glands (Weaver, 1997). Once the salivary glands are infected, the mosquito is able to transmit the virus to another host during each blood meal she takes for the rest of her life. In nature, mosquitoes are thought to survive up to 28 days, allowing enough time for 4 complete gonadotrophic cycles, from blood meal to egg deposition (Black and Moore, 1996). *Ae. aegypti* feed frequently and often on multiple hosts per gonadotrophic cycle. An infected mosquito could therefore transmit DEN to 4 or more humans, depending on when during its lifetime it becomes infected, how long it lives, and how many hosts it feeds upon. Most arbovirus replication does not cause any detectable harm in the vector. Virus titer peaks within a week after consumption of the initial infectious

blood meal, followed by a gradual decline 10- to 100-fold over time until a state of persistent infection is entered that is maintained for life (Hardy, 1988; Leake, 1992).

There are a number of naturally occurring roadblocks to DEN infection of mosquitoes, including a midgut infection barrier and midgut escape barrier (Hardy, 1988). These barriers are conditioned by vector genetics, vary geographically, and may explain why mosquitoes of the same species do not transmit DEN to the same degree in all locales (Black et al., 2002; Bennett et al., 2002; Bosio et al., 2000). There is no evidence of a salivary gland infection barrier in *Ae. aegypti,* indicating that if a DEN virus is able to get out of the midgut, the mosquito will become a transmitter (Gubler et al, 1979; Moncayo et al., 2004).

The term vectorial capacity is used to describe the overall ability of a vector species in a given location at a specific time to transmit a given disease agent (Woodring at el., 1996). Vectorial capacity can be described in mathematical terms (Fine, 1981) and incorporates the following factors: vector population size, longevity, length and number of gonadotrophic cycles, feeding behavior (host preference as well as frequency of feeding) and vector competence (the ability of a vector to become infected with and transmit a pathogen) (Black and Moore, 1996). *Ae. aegypti* mosquitoes are extremely efficient vectors, and typically multiple feeders take multiple small blood meals from a number of hosts per gonadotrophic cycle (Scott et al., 1993; Xue et al., 1995). It is the female mosquito that feeds on blood, which is required for egg production, and this is how the mosquito vector encounters the virus (Edman et al., 1992). In fact, the female *Ae. aegypti* mosquito has evolved to depend almost entirely on human blood as the sole energy source, even when other sources are more abundant. The preference has been

shown to increase survivorship in field mosquitoes in both Thailand and Puerto Rico (Costero et al., 1998; Scott et al., 1993; Scott et al., 1997). In summary, *Ae. aegypti* mosquitoes fulfill all of the vectorial capacity requirements necessary to be an extremely efficient vector of DEN viruses. They preferentially live, feed and breed in close association with humans and human dwellings. When the anthropophilic nature of *Ae. aegypti* is combined with its high vectorial capacity and the rapid human population growth, increased urbanization, poor sanitary conditions and hyperendemicity of DEN viruses occurring in most tropical regions of the world, the result is a global public health crisis with 50-100 million cases of dengue fever (DF) and >100,000 cases of life-threatening dengue hemorrhagic fever (DHF) occurring annually worldwide (Gubler, 1988; Gubler, 1996; Gubler, 1997; Halstead, 1988; Monath, 1994, WHO website, 2003).

Dengue and Disease.

Viral Disease Epidemiology.

The four serotypes of dengue virus (DEN1-4) co-circulate throughout the world in a pan-tropical distribution. In the endemic/epidemic cycle, the viruses are vectored primarily by the *Aedes aegypti* mosquito but can also be transmitted by *Ae. albopictus*. Currently, the viruses are hyperendemic, multiple serotypes co-ciruculating within the same community, in many regions of the world including more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific (WHO website). Dengue fever is considered an emerging infectious disease because of the significant increases in both the number and severity of dengue cases over the last 2

decades worldwide: currently greater than 2.5 billion people are at risk for DEN associated disease (Gubler, 1998; Monath, 1994).

Control and Treatment of DEN disease.

DEN disease has multiple clinical manifestations ranging from undetected subclinical infection to severe disease and even death. Disease manifestations depend upon a number of factors including host genetics and immune state, virulence of the viral strain, and previous dengue infection. The mild form of DEN disease, dengue fever (DF), is described as an acute febrile illness characterized by sudden onset fever, intense headache, rash and myalgia (Chin, 2000; Monath, 1994). Typically, symptoms appear after the 3-8 day intrinsic incubation period (Monath, 1994) and last 5-7 days (with recurring fever), followed by complete recovery without sequelae (CDC Website; Chin, 2000). DF is not associated with mortality, but the morbidity costs are significant when lost worker output is also considered.

The more severe form of DEN diseases is dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF/DSS involves the same symptoms of DF, but also causes increased vascular permeability that can result in capillary leakage and hemorrhagic manifestations (Chin, 2000; Gubler 1988; Gubler 1998, CDC website; WHO website). Fatality rates in untreated DHF/DSS can approach 40% (Gubler, 1988). There is no specific treatment for dengue fever or DHF; however, appropriate intensive supportive therapy including fluid management can reduce DHF mortality to less than 1% (WHO website).

Two main hypotheses have emerged to explain why some patients develop DHF/DSS. The first is based on variability between viral strains and proposes that infection by more virulent strains results in more severe disease. This hypothesis is supported by the timing of emergence of DHF in the Americas and the coordination of DHF emergence with the introduction of a new Southeast Asian strain of DEN-2 into Cuba in 1981 (Rico-Hesse, 1998; Rico-Hesse et al., 1997). Since its introduction, this Southeast Asian DEN-2 strain has spread rapidly, causing epidemic DHF in more than 18 countries in South and Central America (CDC Website). There has been an intensive search to identify the regions of the DEN-2 genome responsible for the host interactions resulting in DHF. Viral genome sequence analysis of DHF field samples revealed that several structural differences appear to be consistently associated with either DF or DHF (Leitmeyer et al., 1999). These differences include a total of six encoded amino acid charges in the prM, E, NS4b, and NS5 genes and sequence differences observed within the 5' and 3' NTRs that were predicted to change RNA secondary structures (Leitmeyer et al., 1999). The authors of this study hypothesized that the primary determinants of DHF reside in amino acid 390 of the E protein, which appears to alter virion binding to host cells; in the downstream loop (nucleotides 68 to 80) of the 5' NTR, which may be involved in translation initiation; and in the upstream 300 nucleotides of the 3' NTR, which may regulate viral replication via the formation of replicative intermediates.

The major alternative hypothesis for the cause of DHF is antibody-dependent enhancement (ADE) of DEN infection (Halstead, 1988). In this hypothesis, it is thought that an antibody response to a primary infection with a given DEN serotype, which successfully aided in recovery from the initial infection, is not protective upon

subsequent infection with another serotype, although it would effectively protect against re-infection with the same serotype. Furthermore, the antibodies generated in the primary infection are thought to facilitate the new infection by aiding viral entry into monocytes (Bhakdi and Kazatchkine, 1990). The major flaw with this hypothesis is that DHF is known to occur in primary infections, as well as secondary infections. More recently, alternative hypotheses for DHF have arisen. Host genetic factors such as human leukocyte antigen (HLA) type may play a role (LaFleur et al., 2002; Loke at el., 2001; Stephens et al., 2002;). Ultimately, the underlying cause of DHF is likely to include a variety of components including host factors, viral virulence and ADE. All of these factors must be considered in the design of control and treatment strategies.

Historically, control of dengue has been carried out in the same manner as control of most other mosquito-borne diseases. The goal has been to reduce the vector population, which in turn will reduce transmission rates. Vector control strategies based on an effort to prevent urban yellow fever, which is also transmitted by *Ae. aegypti*, were extremely effective at reducing the vector populations throughout most of the Americas (Gubler, 1998). With the gradual relaxation of *Ae. aegypti* eradication programs throughout the Americas and the official discontinuation of the United States' program in 1970, the mosquitoes began to move back into areas from which they had been eliminated. By 1997, they had expanded their territory to a wider distribution than before the programs had begun (CDC Website). The current range of dengue overlaps but does not fully encompass the range of *Ae. aegypti*, but it is important to note that there is the potential for dengue to move into any area where a competent vector can be found (Gubler, 1988). Vector control programs suffer not only from a lack of governmental

support but also from insecticide resistance (Clark and Quiroz-Martinez, 2001) as well as the continuing problem of re-introduction of vectors and viruses through travel and commerce. Clearly, new methods of control are needed in order to manage dengue worldwide.

One important control strategy that has been long lacking with dengue is an effective vaccine. Vaccination has been used effectively with other flaviviruses, most notably for yellow fever virus using the live-attenuated vaccine strain 17D (Monath, 2001). Although there is currently no effective vaccine routinely in use for DEN viruses, it has long been a global public health goal to generate a highly immunogenic live-attenuated dengue vaccine. Several problems have been encountered while trying to reach this goal. Early attempts at attenuation yielded over-attenuated DEN-4 strains (Eckels et al., 1984) and DEN-2 strains with low immunogenicity (Bancroft et al., 1981; Eckels et al., 1976; Eckels et al., 1980; Innis et al., 1988; Scott et al., 1983;). Work with DEN-1 and DEN-3 yielded poorly attenuated viruses that caused illness (McKee et al., 1987). A major problem that must be avoided in the development of anti-dengue attenuated vaccine strains is ADE, whereby the immunity generated to the vaccine strain acts to enhance infection by a non-targeted serotype. Therefore, the ultimate goal is a tetravalent DEN vaccine that will simultaneously induce immunity to all 4 serotypes (Innis and Eckels, 2003). Recent advances, including the use of serial propagation of the viruses in the unnatural host primary dog kidney (PDK) cells, have resulted in a number of candidate vaccine strains that are currently being tested in phase I studies (Edelman et al., 2003; Eckels et al., 2003; Gwinn et al., 2003; Kanesa-Thasan et al., 2003; Sun et al., 2003).

An alternative vaccine approach based on the well-characterized yellow fever (YF) 17D strain is also being explored. Attempts to generate DEN vaccines using a recombinant YF-17D backbone are promising because the parental virus is known to be effectively attenuated and highly immunogenic. A chimeric flavivirus vaccine can be generated using recombinant DNA technology to replace structural proteins from 17D with those taken from a cDNA clone of another flavivirus (Guirakhoo, et al., 2000; Lai and Monath 2003). This type of recombination would yield a virus with the receptor tropism and antigenicity of the strain that donated the structural proteins and many of the attenuation properties of the virus donating the non-structural protein background, in this case YF-17D. Another concern in generating flavivirus vaccines is vector competence of the vaccine strain; it is undesirable for the vaccine to be replicated within a mosquito following a blood meal on a vaccinated individual. Studies of a commercial tetravalent recombinant DENV vaccine formulation called ChimeraVax-DEN-1,2,3,4 have demonstrated little potential for transmission by *Ae. aegypti* (Johnson et al., 2004). A tetravalent dengue vaccine has also been formulated based on the PDK attenuated DEN-2 virus, incorporating the prM and E genes from each additional serotype (1, 3, and 4) (Kinney and Huang, 2001). The chimeric DEN-2/prM-E DEN-1, DEN-2/prm-E DEN-3, and DEN-2/prM-E DEN-4 viruses, as well as the DEN-2 strain from which these viruses were derived, are immunogenic in mice (Huang et al. 2003). None of these various vaccine approaches has progressed to the stage where it can be routinely used to control DEN disease in a population. It is a very real possibility that problems with the vaccine candidates that are in trials now will arise that will prevent them from becoming the foundation of the DEN control strategy. Research is continuing on alternative vaccine

strains and the problem of insecticide resistance, but these are not the only approaches under investigation. It is often said that control of vector-borne disease will ultimately involve the combination of various strategies and therefore, approaches to alter vector competence through the genetic manipulation of the arthropod are also being pursued.

Genetic manipulation of insects has become an important component of pest management strategies such as sterile insect technique (SIT). SIT is based upon the introduction of large numbers of sterile male insects into a population (Knipling, 1955; Knipling, 1959; Rai, 1996). The introduced sterile males compete with the naturally fertile males for mates and these matings with sterile males do not result in the production of progeny insects, thereby reducing the number of adults in the next generation. Historically, the released males were rendered sterile through the use of radiation or chemical treatment. Recently, there has been a shift towards the use of genetic manipulation of insects to either facilitate the rearing of large numbers of males, or in generating the sexually sterile phenotype. SIT requires extremely large numbers of male insects and in the case of mosquito-borne disease it is undesirable to release additional females along with the males, since it is the females that feed on blood and can therefore transmit disease. In this case, insertion of a conditional female-lethal gene into the strain used to rear the males for release would eliminate the need to sort the insects prior to release. SIT has been used successfully to control the populations of pest species such as the screwworm fly (*Cochliomyia hominivorax*; Knipling, 1959), and the medfly (*Ceratitus capitata*; Robinson et al., 1999). Using SIT to control mosquito populations has been proposed by a number of researchers (Grover and Agarwal, 1980; Benedict and Robinson, 2003) and conditional gene expression in mosquitoes using a tet-on/tet-off

system has also been reported (Lycett et al., 2004). This type of conditional gene expression would allow for expression of a sex-specific lethal gene under certain laboratory-controlled conditions so that both sexes would be available for breeding in the laboratory, but the females could be killed before release.

Genetic manipulation of mosquitoes is a powerful technique that may be used to control insect populations and reduce disease transmission. A mosquito that has been manipulated into a non-competent vector is the ultimate goal of this line of research and may be the result of the application of the concept of pathogen-derived resistance, where a portion of a pathogen's genome is expressed in order to interfere with the pathogen replication (Prins and Goldbach, 1996; Gaines et al., 1996; Blair et al., 2000). Application of the theory of pathogen-derived resistance to mosquito transgenesis along with candidate pathogen molecules are discussed further in the following sections.

RNA Interference.

Background and Significance.

RNA interference (RNAi) is an ancient, highly conserved cellular defense pathway triggered by double-stranded RNA (see Figure 1.1). RNAi, also called post-transcriptional gene silencing or transgene-induced silencing and quelling, was encountered in a number of different experimental systems. Researchers were attempting to increase color pigmentation in the petunia (*Petunia hybrida* VR) by adding additional copies of genes involved in flower pigmentation: dihydroflavonol-4-reductase (DFR) or chalcone synthase (CHS) (Napoli et al., 1990; van der Krol et al., 1990). In most cases the expected result of increased pigment expression was obtained, but in greater than

25% of plants generated there was a dramatic reduction in DFR or CHS gene expression, resulting in decreased pigment intensity. This result is consistent with what would be observed from an antisense RNA silencing experiment. The theory behind antisense silencing is that an RNA of opposite polarity to messenger RNA (mRNA), generated either *in vivo* or *in vitro* and subsequently introduced into a cell, can pair with an mRNA through Watson-Crick base pairing, resulting in an mRNA that is inaccessible to the cellular translation machinery and therefore cannot be translated (Izant and Weintraub, 1985). A major factor in the ability of antisense RNA to block gene expression is concentration, and it has been hypothesized that in order for an antisense mechanism to be highly effective, there must be an excess of the antisense RNA relative to the targeted mRNA to ensure that all mRNAs are bound and unable to be translated (Pestka, 1992). The unexpected reduction in gene expression observed in the petunias occurred with each transcript tested individually and with chimeric transcripts, and was found to be independent of copy number and the promoter used to drive expression of the exogenous gene copy (van der Krol, 1990). Upon examination of these plants it was found that color intensity and mRNA level did not always correlate and in fact some plants displayed color with low levels of mRNA while others had detectable mRNA but little or no color. Based on these observations, the phenomenon was termed post-transcriptional gene silencing (PTGS) because the blockage in gene expression appeared to occur after the step of transcription from the introduced DNA into mRNA. Later efforts showed that PTGS and RNAi are essentially the same phenomenon, although a feedback loop involving additional enzymatic activities and resulting in methylation of the DNA

corresponding to the silenced mRNA occurs in plants to further reduce expression of these genes (Vaucheret, et al., 2001).

In the worm *Caenorhabditis elegans*, an RNA induced silencing phenomenon was also encountered. Sense RNAs were routinely used as negative controls in antisense RNA experiments and some preparations of sense RNAs exhibited silencing effects similar to the antisense RNAs. Researchers hypothesized that contaminating RNA of the opposite polarity was present in what were thought to be preparations of only sense or antisense RNA (Fire et al., 1998). This was a reasonable hypothesis given the known properties of the bacteriophage polymerases (e.g. T3 and T7) used to make RNA *in vitro*. To test this hypothesis, the researchers injected double-stranded RNA and found that dsRNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. Interestingly, the effects of this interference were evident in both the injected animals and their progeny, but this heritable effect seems to be specific to worms. Finally, as was observed in the plant systems, there was no correlation between the amount of injected double-stranded RNA and phenotype, suggesting that there could be a catalytic or amplification component in the interference process (Fire et al., 1998).

Mechanism of RNA Interference.

The enzyme responsible for initiating RNAi, Dicer, was first described by Bernstein and colleagues (2001) in *Drosophila*. Dicer is a dsRNA nonsequence-specific RNAse-III enzyme that cleaves dsRNA into 21-23 base pair small interfering RNAs

(siRNAs; Figure 1.1). There is no requirement for the trigger dsRNA to be the same length as the full-length mRNA target. In fact, dsRNAs as short as 80 bp can efficiently trigger RNAi (Yang et al., 2000). There are two Dicer proteins in *Drosophila*, *dcr-1* and *dcr-2*, and they are large proteins of approximately 220 kDa with an N-terminal DExH/DEAH RNA helicase motif/ATPase domain, a PAZ protein-protein interaction domain (named for homology to the Piwi, Argonaute, and Zwille proteins), two RNAse III-like domains, and a C-terminal RNA binding domain (Provost et al., 2002). Dicer-like proteins are well conserved evolutionarily and have been found in many organisms, including *Drosophila* (Bernstein et al., 2001), *Arabidopsis* (Reinhart et al., 2002), *C. elegans* (Grishok et al., 2001), mosquitoes (Hoa et al., 2003), mice (Nicholson and Nicholson, 2002), and humans (Provost et al., 2002). Dicer activity has been demonstrated *in vitro* in lysates prepared from cultures of human cells (Myres et al., 2003; Kawasaki et al., 2003; Zhang et al., 2002) and *Drosophila* cells (Tuschl et al., 1999; Elbashir et al., 2001a; Elbashir et al., 2001b). These *in vitro* experiments have helped to define the properties of a dicer cleavage product: 21- to 23-bp double-stranded siRNAs with di-nucleotide 3' extensions and a 5' phosphates. Commercially prepared siRNAs with these properties can direct the cleavage step of RNAi *in vitro*.

The siRNAs generated by Dicer cleavage of dsRNA are incorporated into a protein complex, the RNA-induced silencing complex (RISC; Figure 1.1). RISC uses the siRNAs as guide sequences to direct the sequence-specific degradation of mRNA. In addition to the guide siRNA, RISC contains the dsRNA binding protein R2D2 (Liu et al, 2003; Tabara et al., 2002) and members of the Argonaute gene family, some of which have RNA helicase activity (Wang and Carmichael, 2004). The R2D2 protein, along

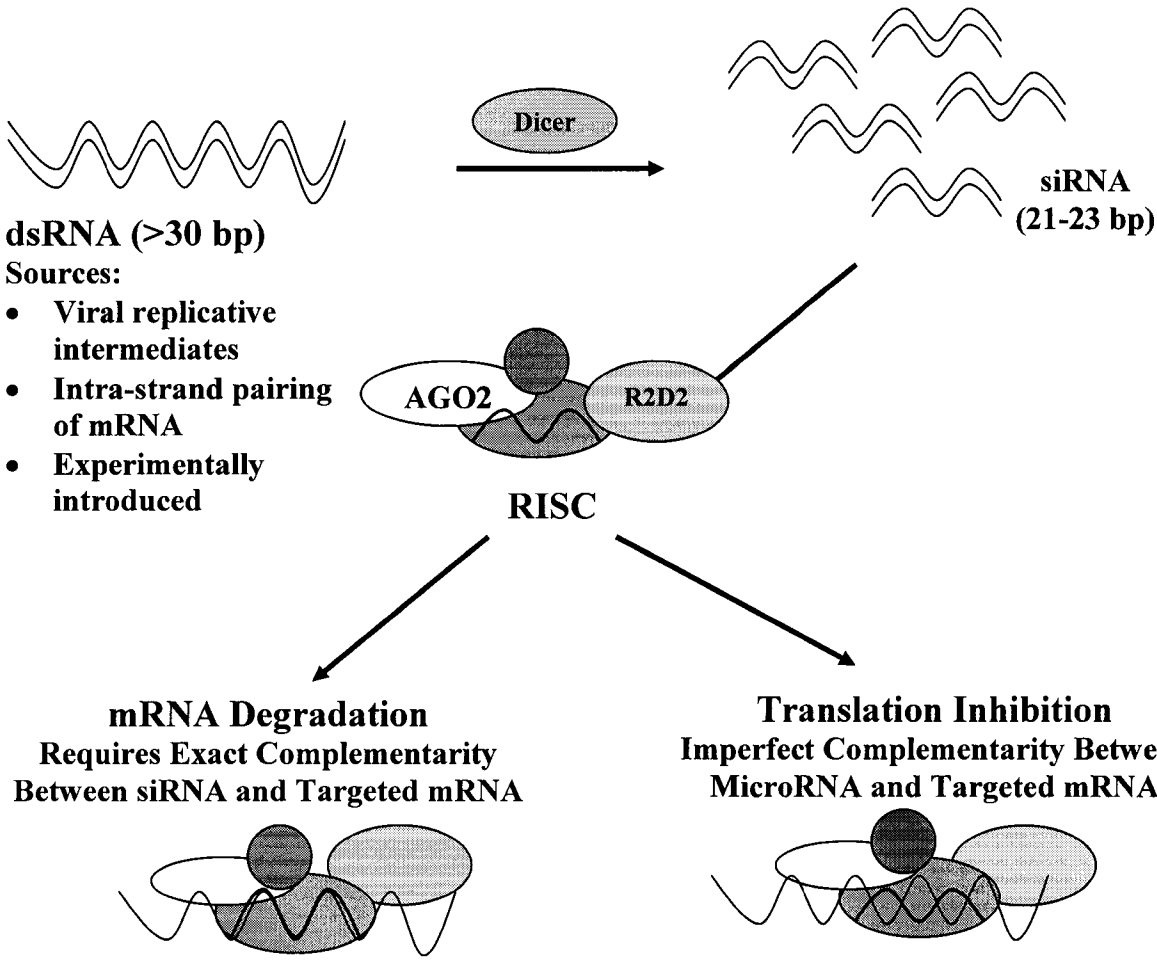
with *dcr-2*, facilitates the loading of the siRNA into RISC in *Drosophila* (Liu et al., 2003). RISC contains the nuclease responsible for mRNA cleavage. The Dicer enzyme is not responsible for this further processing. Instead, Argonaute2 appears to carry out the mRNA degradation, or "Slicer" activity in the effector portion of the pathway (Figure 1.1; Rand et al., 2004; Liu et al., 2004; Sontheimer and Carthew, 2004; Okamura et al., 2004, Meister et al., 2004). The crystal structure of Argonaute2 has been described from the archaebacterium *Pyrococcus furiosus* (Song et al., 2004) and shows the same domain structures, including the PIWI domain that is similar to ribonuclease H. This domain contains a conserved active site aspartate-aspartate-glutamate motif, strongly implicating Argonaute as the enzyme responsible for the mRNA degradation, or "Slicer" activity (Song et al., 2004). Indeed, the architecture of the Argonaue2 molecule and the locations of the PAZ and PIWI domains define a groove for substrate binding and the authors of this study propose a model for the mechanism of siRNA-guided mRNA cleavage within the RISC complex in which the active site in the PIWI domain cleaves the target mRNA in the middle of the siRNA guide binding region. In a separate study, the biochemical characterization of RISC purified from *Drosophila* S2 cells revealed that Argonaute2 is the sole protein component present in enzymatically active fractions and demonstrated that this protein is the only requirment for RNA-induced silencing complex activity (Rand et al., 2004).

Two Dicer proteins (*dcr-1* and *dcr-2*) and five Argonaute family proteins were described in the well-characterized *Drosophila* system (Bernstein et al., 2001; Williams and Rubin, 2002; Hammond et al., 2001). *Dcr-2* is the RNAi initiation enzyme generating siRNAs in *Drosophila*; its functional homolog *dcr-1* appears to be involved in

generating micro-RNAs (miRNAs) that function in translation inhibition, often in a developmentally regulated manner (Lee et al., 2004). Homologs of these genes have been found in the genomic sequence for *An. gambiae* and Dicer can also be found through searching the database for the *Ae. aegypti* sequencing project (Holt et al., 2002; K. Keene unpublished data; *Aedes aegypti* genome project www.tiger.org). Several organisms, including, humans have only one Dicer gene (Zheng et al., 2002) and this gene must encode a protein that is capable of carrying out the functions of both *Drosophila dcr-1* and *dcr-2* or alternatively encode these functions in other proteins.

The microRNA pathway is similar to the RNAi pathway and in some aspects, including the use of Dicer, overlaps with the RNAi pathway. The outcome of both of these two pathways is the inhibition of gene expression. In RNAi, an siRNA usually from an exogenous trigger with exact sequence homology to a target mRNA directs the cleavage of that mRNA and gene expression is inhibited by the destruction of the mRNA, as described above. In the microRNA pathway, however, the mRNA is not destroyed. Instead, there is imperfect pairing between a microRNA derived from an endogenous gene and a target mRNA resulting in a block in translation and subsequently inhibition of expression of that mRNA (see Figure 1.1). The miRNA pathway is important in development and regulation of gene expression.

Figure 1.1. Mechanism of RNA Interference. RNAi is initiated by Dicer recognition and cleavage of dsRNA to generate siRNAs. The siRNAs are incorporated into the RISC complex where they direct sequence specific cleavage of mRNAs with exact sequence complementarity or inhibit translation of mRNAs with imperfect complementarity.



RNA Interference as an Anti-Viral Strategy.

RNAi has been explored as an anti-viral strategy by triggering RNAi response prior to virus infection. It has been demonstrated that an RNA-based strategy for interference with dengue virus replication can work effectively in mosquitoes and mosquito cells. Expression of dsRNA from the DEN genome can trigger RNAi against subsequent DEN challenge (Gaines et al., 1996; Olson et al., 1996; Adelman et al., 2001). In these studies, a double subgenomic Alphavirus expression system based on Sindbis virus (SINV, *Alphavirus*, Togaviridae) was used to express portions of the DEN genome in cultured mosquito cells and adult female mosquitoes. SINV, as a single-stranded RNA virus, must undergo a transient dsRNA form during replication. It is the short-lived dsRNA replicative intermediate expressing the DEN sequence that is believed to induce RNAi. Co-infection of the recombinant Alphavirus containing the DEN virus sequence with a homologous DEN blocks DEN replication in mosquitoes. Interestingly, expression of sequences derived from two DEN serotypes resulted in simultaneous protection against both of these viruses (Adelman et al., 2001). Ultimately, this may provide the basis for generating co-protection against multiple DEN serotypes.

As seen in plants and *C. elegans*, both sense and antisense DEN virus RNA inserts in dsSIN viruses were found to block viral replication in mosquitoes, using dsSIN expression (Gaines et al., 1996; Olson et al., 1996; Adelman et al., 2001), and mosquito cell culture. Mosquito cells were transfected with a plasmid expressing the prM sequence of DEN-2 in either a sense or antisense orientation. Clones of transformed cells were selected and assayed for susceptibility to DEN-2. Some cell lines resulting from

transformation by either the sense or the antisense RNA expressing plasmids demonstrated resistance to DEN-2, while the majority of cell lines derived from these transformations did not show resistance (Adelman et al., 2002). There appeared to be a correlation between plasmid copy number in the various cell lines and their DEN-2 resistance. It was hypothesized that transcription of the high copy plasmids was generating DEN-2 specific dsRNA in these cells that was responsible for the DEN-2 resistance.

Indeed, expression of a dsRNA hairpin structure in mosquito cell culture is a potent inducer of the RNAi response. In order to test the hypothesis that DEN-2 dsRNA can interfere with DEN-2 replication, a plasmid that expressed a hairpin was constructed of 567 nt of the prM sequence from DEN-2 in a sense orientation followed by the first 290 nt repeated in antisense orientation so that transcription made a DEN-2 specific dsRNA (Adelman et al., 2002). The plasmid was used to transform cultured mosquito cells from which clones were selected using the hygromycin B resistance marker. A majority of these cell clones were not permissive to DEN-2 replication. Upon challenge, there was no accumulation of either DEN viral genomic RNA or viral envelope antigen in the cells. Additionally, small DEN-2 prM specific RNAs of approximately 21-23 nucleotides were detected in these transformed cells (Adelman et al., 2002; Sanchez-Vargas et al., 2004). These siRNAs are the specific products of dsRNA cleavage within a cell and are a hallmark of RNAi.

Caplen and colleagues (2002) used a similar approach with Semliki Forest virus (SFV, *Alphavirus*, Togaviridae) and expressed a portion of the DEN-1 viral RNA to interfere with DEN-1 replication in cultured mosquito cells. Another group used adeno-

associated virus (AAV, *Dependovirus*, Parvoviridae) to deliver the effector molecules of RNAi, siRNAs, directly into vertebrate cells, specifically Vero cells and human dendritic cells (DC), demonstrating that an siRNA targeted to the 3' untranslated region common to all DEN serotypes can effectively reduce DEN infection in cell culture and may provide a potential therapy for human DEN disease (Zhang et al., 2004).

RNAi is also effective in attenuating replication of other viruses (Joost Haasnoot et al., 2003, review). RNAi has been used to target RNA viruses such as influenza, using siRNAs directed against NS1, matrix (M1) protein, nucleocapsid or the viral polymerase (Bucher et al., 2004; Ge et al., 2003; Hui et al., 2004; Tompkins et al., 2004) in cell culture and in mice (Ge et al., 2004). Hepatitis C virus (HCV, *Hepacivirus*, Flaviviridae) has been targeted by siRNAs designed against many genomic regions including NS3, NS5B and the 5' NCR (Sen et al., 2003; Takigawa et al., 2004; Randall et al,, 2003; Kronke et al., 2004; Yokota et al., 2003; Wilson et al., 2003; Kapadia et al., 2003). Hepatitis B virus (HBV, *Orthohepadnavirus*, Hepadnaviridae) replication can be blocked by siRNAs in cell culture and *in vivo* in mouse models (Shlomai and Shaul, 2003; McCaffrey et al., 2003; Ying et al., 2003; Giladi et al., 2003).

Retroviruses such as HIV (*Lentivirus*, Retroviridae) can also be targeted by RNAi and there are numerous reports of siRNAs or short-hairpin RNAs (shRNAs) blocking HIV infection and replication upon transfection or expression (Lee and Rossi, 2004). Transfected siRNAs directed against retroviral genes such as *gag* (Novina et al., 2002; Hu et al., 2002; Capodici et al., 2002), *pol* (Hu et al, 2002), *tat* (Coburn et al, 2002; Surabhi et al., 2002; Boden et al., 2004), and *rev* (Coburn et al., 2002,) as well as the LTR (Capodici et al., 2002) have been shown to block HIV replication. Larger pieces of

dsRNA from *gag*, *env* and *nef* have also been shown to trigger RNAi against HIV (Park 2002; Yamamoto et al., 2002). Intracellular expression of siRNAs and of short hairpin RNAs (shRNAs), which are designed to fold back on themselves to make a structure similar to an siRNA, against *tat*, *rev*, *vif*, *nef* and the LTR also work to direct RNAi *in vivo* (Lee at el., 2002; Jacque et al., 2002; Das et al., 2004). Interestingly, RNAi directed against host gene products such as CCR5 and CXCR4, known to function as co-receptors in HIV entry, also block HIV replication (Qin et al., 2003; Novina et al., 2002; Anderson et al., 2003; Martinez et al., 2003; Zhou et al., 2004).

As discussed above, mosquitoes have the necessary genes for the RNAi response and this response can be induced in mosquitoes through virus replication. The ability to generate an RNAi response or the strength of that response may be an important component of vector competence. RNAi generated against an arbovirus in a mosquito may reduce the ability of that mosquito to transmit the virus. This type of resistance could be generated by engineering a transgenic mosquito capable of producing an RNAi response to a virus (Blair et al., 2001). RNAi in mosquitoes represents a natural defense against virus replication, unlike in vertebrate cells where virus replication and dsRNA induces an interferon response. In the mosquito, the RNAi pathway could be induced experimentally in order to control virus infection. Induction of the pathway prior to virus challenge could protect the mosquito from virus replication.

Transgenesis.

Background and significance.

Transgenesis is the process by which foreign DNA is integrated into the genome of a living organism, resulting in a new phenotype (Robinson et al., 2004). The ultimate goal of transgenesis is to engineer an organism that expresses a new phenotype in a stable, heritable manner. Prokaryotic transformation can be accomplished by uptake of plasmids, small circular DNAs with their own origin of replication. A plasmid can be easily manipulated *in vitro* and then once introduced into the cytoplasm of a bacterial cell, either by heat- or electro-shock, the plasmid will be replicated along with the bacterial genome prior to each cell division. While transformation can occur naturally in bacteria via the uptake of DNA from surrounding medium, artificial transformation allows for the specific introduction of desired genes. There is often no advantage conferred upon a cell containing a plasmid and therefore, antibiotic resistance genes are routinely used to provide a selective pressure for the cell to retain the plasmid. In eukaryotic cells, the replication machinery is sequestered within the nucleus, preventing the cytoplasmic introduction of extra-chromosomal DNA from resulting in a permanent phenotypic change. Here, stable integration into the host genome is desirable because it removes the need for selective pressure and self-replication. Barbara McClintock first described a genetic element capable of causing this type of heritable phenotypic change as "jumping genes" (McClintock, 1948). Her studies, conducted during the 1940s in maize, revealed that certain mobile genetic elements could move from one place to another within the maize genome, often interrupting genes. This work was carried out before the development of modern molecular methods such as sequence analysis, but by

using radiation to induce chromosomal breaks McClintock was able to observe structural differences in the maize DNA and correlate the location of certain structures with phenotype (McClintock, 1949).

Since the early work in maize, mobile genetic elements, or transposable elements (TEs), have been found in most organisms from bacteria to humans. There are two classes of eukaryotic TEs based upon structural organization and mechanism of transposition. Class I elements, also called the retrotransposons, transpose via an RNA-mediated replication scheme in which an RNA copy of the element is transcribed, converted to DNA by the TE-encoded reverse transcriptase (RT) activity, and re-inserted at a new location, while the original insertion remains in the original location. The retroposons are extremely common in eukaryotic genomes. SINES (short interspersed elements) are short DNA sequences that represent reverse-transcribed RNA molecules originally transcribed from DNA by RNA polymerase III into tRNA and other small nuclear RNAs. The most common SINE in the human genome, the 300 base pair Alu element, named for its sensitivity to the Alu I restriction enzyme, is present in approximately one million copies and is thought to account for up to 10% of the genome (Weiner, 2002). Other retroposon elements found in humans are the LINES (long interspersed elements). These long DNA sequences represent reverse-transcribed RNA molecules originally transcribed by RNA polymerase II into mRNA to be translated into proteins. The reverse transcribed copies are also called a pseudogenes because they are often not fully functional copies, they do not contain introns or promoters, but code for the reverse transcriptase or integrase, enabling them to copy both themselves and other, noncoding LINES. Because LINES move by copying themselves, they enlarge the

genome. The human genome, for example, contains about 500,000 LINES, and the LINE L-1 represents more than 20% of the human genome (Weiner, 2002). Weiner (2002) and others have hypothesized that repetitive elements such as LINES and SINES are important in driving genome evolution (Okada et al., 2004). Viruses such as HIV-1 behave like retrotransposons and contain similar enzymes, reverse transcriptase and integrase, which function similar to transposase (Nelson et al., 2004).

Class II elements use a DNA-based mode of transposition. Some class II TEs use a "copy and paste" mechanism similar to class I but with a DNA intermediate, while others use a conservative "cut and paste" method in which the transposase enzyme excises the transposon from the genome, repairs the excision site, and catalyses the insertion at a new location. The basic structure of a class II TE contains insertion site duplications, called the left and right long terminal repeats (LTRs), on either side of the transposase and resolvase genes. The terminal repeats function in transposon excision in a mechanism in which the transposon folds back on itself, allowing the transposase and resolvase activities to catalyse strand break and repair at this point; a similar reaction occurs at insertion to generate the target site duplication. In nature, TEs that replicate by the "cut and paste" excision method can occasionally pick up host genes. This occurs when there is imperfect pairing of the terminal repeats, resulting in the looping out of intervening host DNA. In the laboratory, the ability of a TE to integrate exogenous genes can be manipulated to generate TEs carrying a marker gene such as green fluorescent protein (GFP) or β-galactosidase (β-gal) between the LTRs. Additionally, the ability of the transposase enzyme to function in *trans* allows the *in vitro* generation of non-

autonomous replicating transposons that can be stably integrated into a genome and cannot move unless the transposase is supplied externally.

Examples of class II TEs include the P element originally discovered in *Drosophila melanogaster* (Kidwell et al, 1977), *Minos* isolated from *D. hydei* (Franz and Savakis, 1991), *Hermes* from *Musca domestica* (Atkinson et al., 1993; Warren et al., 1994), *mariner* found in *D. mauritiana* (Haymer and Marsh, 1986; Jacobson and Hartl, 1985; Medhora et al., 1991) and piggyBAC, discovered within a baculovirus genome (Fraser et al., 1995). Not all class II elements are found in insects, they can be found in diverse species from fish to mice. For example, the Sleeping Beauty element (SB), originally isolated from fish and found to be a member of the mariner family of TEs, has been found to efficiently transpose many other vertebrate cell lines, and also will transform *in vivo* systems including zebrafish and mouse germlines (Ivics and Izsvak, 2004; Ivics et al., 2004). Insertion sites for class II TEs vary with the different elements: some integrate many times within a host genome and there are so called insertion "hot spots" within genomes. Insertion sites may be distinct sequences such as TTAA, which is targeted by the family of TTAA-specific TEs including piggyBAC (Bauser et al., 1999), or may be structural features such as for the P element, a class II TE from *Drosophila*, that prefers a particular palindromic arrangement of hydrogen bonding sites over a 14-bp region centered on its insertion site (Liao et al., 2000). Interestingly, TE insertions are often found within genes, possibly indicating that accessibility within the chromatin structure is important in determining insertion site (Blumenstiel et al., 2000).

Finally, it should be noted that in bacteria there is an additional class of TEs, which may be the simplest form; these consist of two insertion sequences, a right IS and a

left IS, flanking the transposase (Bennett, 2004). There are also composite insertion elements that are made up of two complete IS units on either side of a selectable marker such as an antibiotic resistance gene.

Insect Transgenesis.

The development of tools for the genetic manipulation of insects has important implications for the basic study of genetics as well as for control of insect vectors of human disease. P-element transgenesis revolutionized the study of gene function by mutation and gene importation in *Drosophila* (Searles et al., 1982; Sentry and Kaiser, 1992), and this element has also been used in enhancer trapping and GAL4/UAS experiments designed to elucidate the mechanisms of spatial and temporal regulation of gene expression (Bellen et al., 1989; Wilson et al., 1989; Smith et al., 1993; Halder et al., 1995; Gustafson and Boulianne, 1996).

In addition to genetic studies, transgenesis provides an opportunity to alter vector competence and reduce transmission of vector-borne diseases such as malaria and dengue (Crampton et al., 1994). Successful engineered resistance to pathogens will rely on a number of factors, including the availability of TEs that successfully transform the insect species, anti-pathogen targets that can be expressed within an insect, and promoter systems that direct transcription of the anti-pathogen gene. Another factor is the site of TE integration. This is perhaps the most variable factor in that it cannot be directly controlled, and the so-called position effect can up- or down-regulate expression of the transgene based upon signals found in the regions of the host genome surrounding the TE integration site.

Other factors to consider are re-mobilization of the element after integration and drive mechanisms to move a transgene through a population. In the laboratory, it is easy to perform transgenesis experiments using non-autonomous elements to generate families of transgenic organisms, each based upon a single transposition event. In this case, the transposon would remain integrated in the same location within each family member across each generation. This condition is desirable for initial laboratory characterization but will not allow a newly introduced transgene to spread within a population. In order for a transgenic organism to become established within a population of wild-type organisms, there must be a mechanism besides simple mating to confer an advantage upon that transgene. Evidence to date suggests that a transgenic mosquito is at a significant disadvantage in terms of fitness compared to wild-type, and any mechanism that can increase abundance will help to drive the transgene into the population (Irvin et al., 2004). Perhaps the simplest drive mechanism relies completely on the properties of replicative transposition in which the transgene is coupled to a functional transposase activity, which will move the transgene throughout the genome, leaving behind a copy and increasing the number of copies within a genome so that all progeny will carry the transgene (James, 2000). Other drive mechanisms include meiotic drive, in which there is selective pressure to determine which parental chromosome is passed to the next generation (Robbins et al., 1996; Wood and Ouda, 1987). Using symbionts such as the maternally inherited obligate intracellular bacterium *Wolbachia* (Sinkins and O'Neill, 2000) can affect mating via inducing a state of cytoplasmic incompatibility, whereby successful mating can only occur between individuals that have the same *Wolbachia*. Addionally, successful mating can also occur between a female with *Wolbachia* and a

non-infected male (Yen and Barr 1971; Turelli and Hoffmann, 1991; Pettigrew and O'Neill, 1997; Turelli and Hoffman, 1999). There are many strategies under examination for driving transgenes through mosquito populations, but this is still an unresolved issue and area for more research.

There is a large body of work that addresses some of the requirements necessary for insect transgenesis; the following discussion will focus on mosquito transgenesis as that is the insect that transmits the most vector-borne human pathogens and is the most relevant to this work.

Mosquito Transgenesis.

The first requirement for mosquito transgenesis is a transposable element capable of jumping into the mosquito genome. Unfortunately the *Drosophila* P element, the most well characterized insect TE, does not function in true transposon-mediated integration in the mosquito, although there is some evidence for low-frequency random integration at recombination events between P-element carrying an antibiotic resistance marker and the mosquito genome (Miller et al., 1987; McGrane et al., 1988; Handler, 2000). There are a number of other TEs that have been found to actively facilitate transposition in the mosquito. Some members of the hAT superfamily (named for *hobo* from *D. melanogaster*, *Ac* from corn, and *Tam3* from snapdragons) such as Hermes, are active in mosquitoes (Atkinson et al., 1993; Warren et al., 1994). The mariner element MosI (Garza et al., 1991; Coates et al., 1998) and piggyBac (Handler et al., 1998; Grossman et al., 2000) are also capable of transforming mosquitoes.

Transgenesis of mosquitoes has been successful on a number of occasions in the dengue vector *Ae. aegypti* (Jasinskiene et al., 1998; Coates et al, 1998; Coates et al., 1999; Pinkerton et al., 2000). Specific examples of transgene expression in *Ae. aegypti* include the gut-specific expression of luciferase, using the *Ae. aegypti* carboxypeptidase promoter to drive luciferase expression in both Hermes and mariner (MosI) constructs (Moreira et al., 2000). The piggyBac vector has been used to generate *Ae. aegypti* that express the *Drosophila cinnabar* (cn) gene and restore eye color to the kh^{w} mosquito strain (Lobo et al., 2001). These data indicate that transformation of *Ae. aegypti*, while far from routine as in *Drosophila*, is possible.

The next hurdle is expression of the anti-pathogen gene and this depends upon mosquito-active promoters with defined expression profiles. There are two types of promoters that can be used for transgenesis: constitutive promoters such as the actin5C promoter from *Drosophila* and spatially and/or temporally regulated promoters such as the carboxypeptidase promoter from *Ae. aegypti*. Muscle-associated GFP expression was engineered into *Ae. aegypti* using the *Drosophila* actin5C promoter, generating stably transformed mosquitoes that glow green in muscle tissue under UV irradiation, with GFP expression detectable at all life stages (Pinkerton et al., 2000). Coordinated expression of the luciferase transgene upon blood feeding was achieved using the *Ae. aegypti* carboxypeptidase promoter, a female midgut-specific gene that is induced during blood feeding (Edwards et al. 2000; Moriera et al., 2000). Expression from the carboxypeptidase promoter begins by 8 hours post blood meal, peaks at 20-24 hours, and decreases by 30 hours post feeding, and this is the same expression profile for the luciferase transgene in these transgenic mosquitoes (Moriera et al., 2000). There are

additional mosquito-active promoters available for use in transgenesis, including other midgut specific promoters such as those for glutamine synthetase (Smartt et al., 2001), glucosamine: fructose-6-phosphate aminotransferase (GFAT; Kato et al., 2002) and ferritin heavy-chain (Pham et al., 2003). Salivary gland specific promoters such as D7 (Arca et al, 1999) and apyrase (Johnson et al., 1999; Coates et al., 1999; Lombardo et al., 2000) have also been described.

The final piece of the transgenesis puzzle is a transgene with anti-pathogen, in this case specifically anti-dengue, activity. There are a number of types of molecules that have anti-pathogen properties, including proteins, non-coding nucleic acids such as antisense RNAs and coding nucleic acids such as mRNAs. An example of a protein based anti-pathogen molecule that has been incorporated into a transgenic mosquito would be the anti-malarial SM1 peptide generated by multiple rounds of selection from phage display; SM1 binds to *Anopheles gambiae* midguts and salivary glands, interfering with plasmodium infection *in vitro* and in transgenic mosquitoes expressing SM1 (Ghosh et al., 2001, Moreira et al., 2004).

What type of anti-DEN effector molecule may affect vector competence? It is now known that *Drosophila melanogaster*, *Caenorhabditis elegans*, many plants, and even humans have the ancient anti-viral pathway, termed RNA interference (RNAi), triggered by the presence of intracellular double-stranded RNA (dsRNA) (Bernstein et al., 2001; Montgomery et al., 1998a; 1998b; van der Krol et al., 1990; Chiu and Rana, 2002). The RNAi pathway was described earlier. Briefly, dsRNA is an early intracellular warning signal of RNA virus invasion that initiates an RNAi cascade resulting in destruction of mRNA having sequence identity with the dsRNA. Many RNA

viruses normally generate dsRNA in infected cells as a byproduct of replication and these replicative intermediates serve as potent inducers of the RNAi intracellular response. We have evidence from our laboratory that at least two mosquito species, *Ae. aegypti* and *Anopheles gambiae,* develop an RNAi response very similar to that found in *D. melanogaster*, because they can silence endogenous gene expression or virus replication after introduction of dsRNA targeted to a specific gene (Sanchez-Vargas et al., 2004). Additionally, induction of an RNAi response against an exogenous gene has recently been demonstrated in mosquitoes: *defensin* and a complement-like protein *factor C3* in *An. gambiae* (Levashina et al., 2001; Blandin et al., 2002) and eGFP in *An. stephensi* (Brown et al., 2003a; 2003b).

Replication of several arboviruses appears to trigger the RNAi response in mosquito cells, and we now have evidence for some of the genes involved in the *An. gambiae* antiviral response to the arbovirus O'nyong-nyong (ONNV; *Alphavirus*, Togaviridae; Keene et al., 2004).

If RNA viruses trigger RNAi, why are mosquitoes such efficient vectors of arboviruses? This is not known for certain, but DEN may escape the antiviral effects of RNAi in competent mosquitoes either by failing to present the threshold concentration of dsRNA molecules required for triggering the response or by encoding a viral protein that suppresses the RNAi response. Uchil and Satchidanandam (2003a; 2003b) suggest that the dsRNA replicative form of DEN is sequestered in double-membrane structures in the cytoplasm of infected mammalian cells to possibly limit its exposure to the RNAi or interferon pathways. It may be possible, however, to induce an RNAi response to DEN in the midgut as a transgenic mosquito ingests an infected blood meal, thus resulting in

destruction of the virus genome before virus replication begins and the virus can evade the RNAi response. The midgut is a likely target for mounting this line of defense, because it is the first tissue the virus encounters in the vector and is the major determinant of vector competence in the mosquito (Weaver, 1997). DEN is ingested during blood feeding and first encounters the midgut. The virus must infect and replicate in the midgut epithelium, escape the midgut and replicate at secondary sites within the body before ultimately infecting the salivary gland. Once the salivary gland is infected, the female mosquito is competent for transmission for the duration of her life.

Evidence for RNA-based interference to dengue viruses in mosquitoes.

Previous work has demonstrated that an RNA-based strategy for interference with dengue virus replication can work effectively in mosquitoes and mosquito cells. Expression of dsRNA from the DEN-2 genome can trigger RNAi against subsequent DEN-2 challenge (Gaines et al., 1996; Olson et al., 1996; Adelman et al., 2001). As described earlier, a dsSIN expression system was used to express a 290-567 nt portion of the DEN-2 prM gene in adult female mosquitoes. Replication of the dsSIN involves a transient dsRNA intermediate and it is believed that this intermediate may induce RNAi. Co-infection of the recombinant Alphavirus containing the dengue sequence and a DEN blocks DEN replication in mosquitoes.

RNAi to DEN is also effective in cultured mosquito cells expressing a dsRNA hairpin of DEN-2 sequence. As discussed earlier, cells expressing the DEN-2 prM hairpin RNA make a DEN-2 specific dsRNA (Adelman et al., 2002). These cells were

not permissive to DEN-2 replication. Upon challenge, there was no accumulation of either DEN viral genomic RNA or viral envelope antigen in the cells. Additionally, small DEN-2 specific RNAs of approximately 21-23 nucleotides were detected in these transformed cells (Adelman et al., 2002; Sanchez-Vargas et al., 2004). These RNAs, termed small interfering RNAs (siRNAs), are the RNAi breakdown products of dsRNA within a cell and are a hallmark of RNAi.

There are several lines of evidence supporting the notion that this observed resistance is RNAi. First, the RNA species found in the RNAi pathway, the dsRNA trigger and the siRNA product, are detectable in our experimental system. Additionally, the interference is sequence specific, extending to closely related dengue serotypes (DEN-3) but not to a more distantly related flavivirus (West Nile virus, WNV). Second, the enzyme responsible for initiating the RNAi response, Dicer, was discovered and extensively studied in *Drosophila* (Bernstein et al., 2001), and its homolog is present in mosquitoes (Holt et al., 2002; Keene et al, 2004). Taken together, these data indicate that RNAi is active in the mosquito and it can be used to interfere with DEN replication.

Harnessing RNAi to alter vector competence for dengue viruses in mosquitoes.

After validating the anti-DEN effector molecule and the feasibility of an RNAi-based approach in the mosquito, the next step was to move this mechanism into a stable, heritable interference strategy. Mosquito transgenesis, while more laborious than *Drosophila* transgenesis, has become routine. This approach was harnessed to generate mosquitoes that constitutively express an anti-DEN effecter RNAi construct (Travanty et al., 2004). The first generation anti-DEN-2 transforming construct was based on the

hairpin construct used successfully in cell culture. This construct was cloned into the mosquito active piggyBAC transposable element (Lobo et al., 2001; Kokoza et al., 2001). No resistance to DEN-2 was observed in mosquitoes transformed with this piggyBAC construct. Interestingly, the piggyBAC transposable element was shown to be unstable in *Ae. aegypti* mosquitoes (Adelman et al., 2004) as the number of transgenic individuals in each generation did not follow a mendelian pattern. Upon further examination of these transgenic families, the formation and subsequent loss of large tandem array copies of the piggyBAC element were demonstrated by *in situ* hybridization (Adelman et al., 2004). The piggyBAC element was not pursued further for the generation of new lines of transgenic *Ae. aegypti* mosquitoes.

Evidence from studies of anti-viral RNAi constructs in plants showed that using an intron as a spacer between the sense and antisense sequences instead of a hairpin loop yielded a more efficient RNAi response (Smith et al., 2000). This is likely due to splicing of the intron to give a perfect dsRNA instead of relying on a hairpin to form the intended secondary structure. In addition to constructs based on the hairpin used in the cell culture experiments, second generation constructs were developed to include an intron from the *Ae. aegypti sialokinin* gene (major intron; 840 bp; Beerntsen et al., 1999) between 2 copies of the 290 bp DEN-2 prM, one in sense orientation and the other in antisense, followed by an 800 bp 3' untranslated region (3' UTR) from baculovirus *immediate-early 1* (Ie1) gene containing a polyadenylation signal (Travanty et al., 2004). This intron-spaced inverted-repeat, abbreviated 2-i-2 for dengue-2 prM sense-intron-dengue-2 prM antisense, was cloned into the mosquito active, *mariner*-based transposable element pMos under the control of a constitutive promoter. Tests *in vitro*

using HeLa cell extracts demonstrated that the construct was efficiently spliced (Adelman, unpublished data).

The promoters used to drive expression of the anti-DEN RNAi construct were the *Drosophila melanogaster polyubiquitin* promoter (either an 800bp version, piggyBAC-ubD2FB and pMosUb800/2-i-2 or a 1300bp version, pMosUb1300/2-i-2) and the baculovirus Ie1 promoter (pMosIE/2-i-2). Both promoters were expected to direct constitutive expression of the transgene and were highly active when tested in mosquito cell culture. Additionally, the piggyBAC and pMos backbones both contain the 3xP3-eGFP eye-specific selection marker for easy screening of green fluorescent protein expression in transgenic individuals at all life stages (larva, pupa and adult; Horn et al, 2000; Shin et al, 2003). The piggyBAC plasmid was co-injected into the K^{hw} DEN-2 competent strain of *Ae. aegypti* along with a helper plasmid supplying the transposase *in trans*. The pMos plasmid was co-injected into the Higgs white-eye variant (HWE) of the Puerto-Rican Rexville D, DEN-2 competent strain of *Ae. aegypti,* along with a helper plasmid supplying the transposase *in trans*.

Statement of aims.

The studies described in this dissertation were designed to build upon previous work in the field and to investigate the RNAi phenomenon as an antiviral response to DEN in mosquitoes and mosquito cells.

A major goal was to characterize the RNAi pathway in mosquito cells by examining the role of each portion of the pathway. Previously characterized cells, FB9.1 cells (Adelman, 2000; Adelman et al., 2002), that are silenced for DEN-2 replication

were re-evaluated and used for studies that examined the nature of the silencing, to demonstrate that the response was directed by RNA and not protein and to explore the sequence specificity requirements of RNAi in mosquitoes. Additionally, the role of the siRNA was investigated by direct transfection of anti-DEN siRNAs and at the pool of siRNAs that result from the expression of a hairpin RNA or from viral replication.

Additionally, the knowledge, reagents, and techniques developed previously to silence DEN-2 in cell culture and transiently in dsSIN-infected mosquitoes was exploited to generate stable, heritable resistance in mosquitoes. The basic approach investigated in this dissertation used molecular biological and transgenesis techniques to engineer strains of *Ae. aegypti* that transcribed an anti-DEN effector molecule that formed dsRNA in mosquito cells and triggered the RNAi response. Upon challenge with DEN-2, the RNAi response was expected to target the incoming viral genomic mRNA, destroy it, and thereby prevent translation of viral proteins and replication of viral RNA.

Chapter 2

RNA interference to DEN-2 in mosquito cell culture

Chapter 2. RNA Interference to DEN-2 In Mosquito Cell Culture.

Introduction

The hypothesis examined in this section is that DEN-2 replication can be inhibited in mosquito cell culture by RNA interference (RNAi). Previous data from this laboratory indicated that DEN-2 dsRNA can trigger RNAi against DEN-2, but this work sought to characterize the response and add to the evidence that the observed replication block is indeed due to RNAi. RNAi (discussed in Chapter 1) is a highly conserved cellular defense pathway triggered by double-stranded RNA that results in the degradation of target mRNAs with sequence homology to the triggering dsRNA. The RNAi pathway is initiated by Dicer, which cleaves the dsRNA into 21-23 nucleotide (nt) small-interfering RNAs (siRNAs). These siRNAs act as guide sequences within the RNA-induced silencing complex (RISC) to direct sequence specific cleavage of mRNA. In addition to the siRNAs, RISC also contains a dsRNA binding protein R2D2 and Argonaute2, the enzyme responsible for mRNA degradation. In nature, the RNAi pathway has been implicated as an anti-viral strategy used to degrade viral RNA and as a mechanism to control endogenous gene expression by post-transcriptional regulation. There are a number of experimental examples reported that use RNAi to block virus replication in diverse organisms ranging from plants to mice (see Chapter 1, Literature Review for discussion of these experiments).

Previous work in this laboratory demonstrated the ability to block dengue virus (DEN) replication using an RNA based strategy (Gaines et al., 1996; Olson et al., 1996; Adelman et al., 2001). In the initial experiments, replication of double-subgenomic

Sindbis (dsSIN) viruses with an insert derived from the DEN genome was shown to interfere with DEN replication during sequential infection in mosquito cell culture and co-infection experiments in adult mosquitoes. The observation of interference with DEN-2 replication when either sense or antisense RNA was used in the dsSIN virus led to the hypothesis that the interference was not due to the dosage dependant antisense mechanism, but rather was similar to the phenomenon of post-transcriptional gene silencing that had recently been described in plant RNA. To test this hypothesis, mosquito cell lines were engineered to express DEN-2 RNA in sense, antisense or double-stranded configurations (Adelman et al., 2002). Interestingly, some degree of resistance was observed with all three of these approaches, but the cells transformed to express dsRNA led to the greatest proportion of colonies exhibiting DEN-2 inhibition. It is now known that post-transcriptional gene silencing and RNA interference are actually the same pathway, described in different experimental systems.

The work described in this section of the dissertation sought to investigate the nature of the silencing phenomenon in the mosquito cells expressing DEN-2 dsRNA and to characterize the RNAi pathway in mosquitoes. The underlying hypothesis examined here was that silencing of DEN-2 replication in mosquitoes and mosquito cells can be accomplished via an RNAi based mechanism. To further characterize the dsRNA expressing cell line, the number of integrated copies of the plasmid in transformed DEN-resistant cells was examined. Additionally, the accumulation of DEN-2, and DEN-3, viral RNA was observed over time in these cells. Each step in the RNAi pathway was examined in mosquito cells. In order to examine the initiation of RNAi in mosquitoes, an *in vitro* RNAi assay was developed from mosquito cell extracts using techniques

described for *in vitro* RNAi in *Drosophila* cell extracts. RNAi was initiated in these extracts by exposure to a dsRNA trigger and the effect of this trigger was examined by studying the stability of with single-stranded (ss)RNA with sequence homology to the trigger. The effector stage of RNAi was examined in mosquito cell culture through the direct transfection of synthetic and *in vitro* prepared siRNAs. The ability of these siRNAs to by-pass the initiation step of RNAi and to directly block DEN-2 replication was investigated. Finally, the degradation products of RNAi in mosquito cells were studied by cloning and sequencing the pool of small RNAs present in virus infected and control un-infected mosquito cells. This pool of small RNAs within a mosquito cell was hypothesized to contain small RNAs such as microRNAs involved in regulation of expression of endogenous mosquito transcripts and in the case of infected cells, may contain degraded viral RNAs. Sequencing and analysis of these RNAs will result in a better understanding of the role of RNAi in controlling virus replication and gene expression in mosquito cells.

The experiments outlined here sought to examine the RNAi response to DEN-2 in mosquito cells by examining the RNAi pathway at each step from initiation by dsRNA trigger, to the siRNA effector phase, and ultimately to the degradation of mRNA. The data presented here clearly demonstrate that RNAi can be directed against DEN-2 in mosquitoes by DEN-2 specific dsRNA and DEN-2 specific siRNAs.

Materials and Methods

Cells. Cell lines were obtained from stocks maintained at AIDL/CSU or ordered from American Type Culture Collection (ATCC; Manassas, VA).

Insect cells: C6/36 *Aedes albopictus* cells (Singh, 1967; Igarashi, 1978; American Type Culture Collection (ATCC) CRL-1660) were maintained in Lieboweitz 15 (L-15; Cellgro) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 mM L-glutamine at 28°C. FB9.1 cells are a clonally selected cell line derived from C6/36 cells transformed with a plasmid expressing the hygromycin B resistance gene selectable marker as well as an anti-DEN-2 RNAi construct (Adelman et al., 2002). Expression of the RNAi effector is directed from the baculovirus *Immediate Early 1* gene promoter (*Ie1*) and the transcript is a hairpin dsRNA derived from the DEN-2 (New Guinea C; GenBank accession #M29095; Putnak et al., 1998) genome. This genome fragment, called Mnp (np = no protein), has been previously described as a PCR product generated from position 400-996 of the DEN-2 viral genome and corresponding to the 3'end of the C gene, the entire prM-M gene (with stop codon mutations that preclude translation) and the 5' end of the E gene in sense orientation (Gaines et al., 1996). The final construct used to generate dsRNA conatins the Mnp sequence followed by 290 nt of the prM gene repeated in antisense orientation (Adelman, 2000). Upon transcription, the RNA from this construct folds back upon itself to generate a 290 base pair (bp) region of dsRNA that acts as a trigger of RNAi. H9.1 cells are a clonally selected cell line derived from C6/36 cells transformed with the

hygromycin B resistance gene selectable marker but without any DEN-2 sequence (Adelman et al., 2002). These cells were used as a control for growth under the hygromycin B selection conditions. FB9.1 and H9.1 cells were grown in L15 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 mM L-glutamine and 300 U/mL of hygromycin B (Calbiochem, EMD Biosciences, Inc., San Diego, CA) at 28°C. Cells under hygromycin B selection had their medium changed every three days to ensure there was no breakdown of the hygromycin B and were therefore under constant selective pressure to retain the transforming plasmid. *Drosophila* S2 cells (Schneider, 1972; ATCC CRL-1963) were maintained in DS2 Insectagro cell culture medium (Cellgro) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 mM L-glutamine at 28°C.

Mammalian Cells: LLC-MK2 cells (rhesus monkey, *Macaca mulatta* kidney cells; ATCC CCL-7) and BHK-21 cells (Syrian golden hamster, *Mesocricetus auratus* kidney fibroblast cells; ATCC CCL-10) were maintained in Dulbecco's modification of Eagle's medium with 4.5 g/L glucose and without L-glutamine or sodium pyruvate (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% MEM vitamin solution (Cellgro, MediaTech, Inc, Herndon, VA), and 1% MEM non-essential amino-acid solution (Cellgro) at 37°C in the presence of 5 % CO_2.

Viruses. Dengue viruses. Stocks of the prototypic isolate of each of the four DEN serotypes were obtained from laboratory stocks maintained at AIDL/CSU: DEN-1

Hawaii, DEN-2 Jamaica/83 1409 (Genbank accession #M20558, Deubel et al., 1988), DEN-2 New Guinea C (GenBank accession #M29095; Putnak et al., 1998), DEN-3 H-87, DEN-4 H-241 (GenBank accession #U18433; Lanciotti et al., 1997). Working stocks were prepared as follows: an 80% confluent 150-cm^2 tissue culture flask of C6/36 cells was infected at a multiplicity of infection (MOI) of 0.01 infectious virus particles per cell for one hour in 5 mL L15 medium with gentle rocking. The virus suspension was then removed and the cells were incubated at 28°C for 14 days in L15 medium supplemented with 2.5% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 mM L-glutamine; at 7 days post infection the medium was changed. At 14 days post infection, the cells were harvested using a cell scraper and transferred along with the supernatant to a 50mL conical tube in which the cells were pelleted by centrifugation at 2000 rpm in a Beckman GS-6R centrifuge for 5 minutes at 4°C. The supernatant was supplemented with 1 mL of freezing solution per 10 mL of infected cell supernatant (freezing solution: 25ml FBS, 1ml 7.5% sodium bicarbonate) and frozen at -70°C in 0.5 mL aliquots. Titer of the working stock for each virus was determined by plaque assay as described below.

West Nile Virus. A vial of working stock of West Nile virus, New York 1999 isolate (WNV; GenBank accession #AF260967) was obtained from colleagues at AIDL/CSU. The titer of this stock was 3.08 x 10^6 PFU/mL as determined by plaque assay. This virus was used to infect C6/36, FB9.1 and H9.1 cells at an MOI of 0.01 and infection was determined 7 days post-infection by immunofluorescent assay using the flavivirus-complex envelope specific monoclonal antibody 4G2.

Sindbis viruses. Double-subgenomic Sindbis virus (dsSIN) infectious clones have been described based on TE/3'2J (Gaines et al., 1996; Hahn et al., 1992) and MRE16 (Myles et al., 2003; Foy et al., 2004). To generate template for transcription, the plasmid containing the infectious clone was linearized with the restriction enzyme *Xho* I and RNA was transcribed *in vitro* from the SP6 promoter of the linearized plasmid in a reaction containing 1X transcription buffer (40mM Tris-HCl, pH7.9, 6mM $MgCl_2$, 10mM NaCl; Ambion), 40 units of SP6 RNA polymerase, 1 mM cap analog (Ambion), 1 mM rNTPs, 5 mM dithiothreitol (Ambion), 0.1% bovine serum albumin (BSA) and 50 U RNasin RNAse inhibitor (Promega). The *in vitro* transcribed RNA was electroporated into BHK-21 cells using a BTX electro cell manipulator 600 at 450 V, 100 μF, and 720 Ohms. Cells in cuvettes were pulsed twice with a pulse length of 0.8-1.0 millisecond per pulse, 0.5 ml of growth medium was added to the cuvette and cells were immediately seeded into 25-cm^2 tissue culture flasks with 5 mL of growth medium. After 24 hours of incubation at 37°C, there were no significant cytopathic effects (CPE) in the control samples electroporated without RNA and, cell death was visible in the flasks electroporated with viral RNA. The culture medium was harvested and centrifuged to remove cellular debris, and aliquots were stored at –70°C. Viruses were titrated using the end-point titration method described below and BHK-21 cells were scored for infection by dsSIN viruses by counting the number of wells with CPE for each replicate of the serial dilution and the titer was calculated using the Karber method as described below (Karber, 1931). All dsSIN constructs were prepared by the above method, including TE/3'3J, 5'dsMRE16ic-eGFP and 5'dsMRE16ic-eGFP/D2prM.

Virus titration. Viruses were titrated by either end-point or plaque assays.

End-point titration: Serial 10-fold dilutions of the sample were made in L15 medium and 6 replicates of each dilution were inoculated into a row of wells containing C6/36 cells in 96 well plates. The C6/36 cells were maintained in L15 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 mM L-glutamine. Plates were incubated at 28°C for 7 days, and then fixed in acetone: phosphate-buffered saline (PBS), 3:1. Fixed cells were stained for DEN-2 antigen using either the DEN-2 specific 3H5 mouse monoclonal antibody or the 4G2 anti-flavivirus envelope mouse monoclonal antibody. These monoclonal antibodies were used interchangeably as primary antibody in an immunofluorescence assay. The primary antibody was diluted 1:200 in PBS, 40 μL applied to each well and incubated at 37°C for 40 min. Primary antibody was removed; plates were washed 3 times with PBS and incubated at 37°C for 40 min with 40 μL per well of a 1:200 dilution in PBS of secondary antibody, sheep anti-mouse IgG biotinylated (Amersham Biosciences, Piscataway, NJ) and 1:200 dilution of 5% Evan's Blue counterstain. Secondary antibody was removed, plates washed 3 times with PBS for 40 minutes and then incubated with 40 μL of a 1:200 dilution in PBS of fluorescein-streptavadin conjugate (Amersham Biosciences) at 37°C for 10 min. Following a final PBS wash, samples were preserved with 3:1 ratio of glycerol to PBS and plates were viewed using an Olympus BH-2 fluorescent microscope. Samples were scored for fluorescence and 50% tissue culture infectious dose titers ($TCID_{50}$) were calculated using the Karber method (1931) using the following equation: $\log_{10}$ of $TCID_{50}$ = X-d(P-0.5), where X = $\log_{10}$ of the highest

concentration (lowest dilution) used, d = $\log_{10}$ of dilution factor; P = (sum of percent cell death at each dilution)/100.

Plaque assay: Serial 10-fold dilutions of the sample were made in L15 media and inoculated onto confluent monolayers of LLC-MK2 cells in 6 well tissue culture plates. Plates were gently rocked for 1 hour at 37°C a 5% CO_2 incubator and then covered with 4 mL of initial overlay per well. The overlay is made by mixing equal volumes of 2% Seakem LE agarose (prepared in ddH_20 and autoclaved prior to use; ISC Bioexpress, Kaysville, UT) and 2X nutrient medium. One liter of 2X nutrient medium contains 196 mL 10x Earl's BSS (Sigma), 66 mL Ye-Lah (Solution A = 2g yeast extract per 100mL, Solution B = 10g lactalbumin hydrolysate per 100 ml, combine solution A and B, and autoclave for 55 min), 40 mL FBS, 60 mL 7.5% sodium bicarbonate, 2 ml gentamycin (50 mg/mL; Invitrogen), 4 mL Fungizone (1 mg/mL; Sigma), and 632 mL deionized water. Plates were inverted and incubated 8 days at 37°C in a 5% CO_2 incubator before the second overlay was applied. Second overlay was the same as the first overlay except that it also contained 16 mL/L of 1% neutral red (Sigma) and only 2 mL of overlay was added per well. Plaques were counted the next day and counting was repeated over the next 3-4 days, until plaque counts remained constant with no new plaques developing for 24 h. Titers were calculated in plaque forming units (PFU) per mL using the following formula: Titer (PFU/mL) = (# plaques counted) x (reciprocal of the dilution counted) x (volume inoculated in mL) x (dilution factor of original sample at time of harvest) (Miller and Mitchell, 1986).

Replication enhancement assay. C6/36 cells were seeded into 12 well tissue culture plates containing 18mm circular glass coverslips at 1.9 x 10^6 cells per well and grown in L15 growth medium as described above. Once confluent, growth medium was removed and cells were infected with TE3'/2J at an MOI of 10 in 300 μL of L-15. Virus was allowed to adsorb to the cells at room temperature for 30 minutes with gentle rocking, was then removed, and cells were maintained on L15 supplemented with 2.5% FBS to prevent overgrowth of the cell monolayer during the incubation period. At 48 hours post infection, a sample coverslip was removed, fixed in acetone and stained with fluorescent antibody to detect SIN E1 antigen using the monoclonal antibody 30.11a. The immunofluorescence assay was performed as described above in the end point titration method with the following changes, using 400 μL of the 1:200 dilutions of primary antibody, secondary antibody/Evan's Blue counterstain, and fluorescein-streptavidin per coverslip/well and incubations were performed at 37°C within a humid chamber to prevent drying. Coverslips were mounted cell side down in glycerol:PBS (3:1) on microscope slides. If cells were all shown to be positive for SIN antigen at 48 hours post infection, the remaining, unfixed TE3'/2J infected cells in the 6 well tissue culture plates, as well as control uninfected cells, were challenged with DEN-2 New Guinea C virus at an MOI of 0.025. The protocol for DEN-2 challenge was the same as that used for TE3'/2J infection, except that virus was allowed to adsorb for 1 hour. Coverslips were collected daily from 1 to 5 days post DEN-2 challenge and fixed and stained for DEN-2 antigen using the DEN-2 specific monoclonal antibody 3H5 in the previously described immunofluorescence assay. Three fields of view from each coverslip were photographed using an Olympus BH-2 fluorescent microscope and an

Olympus DP10 digital camera. All digital files were modified in Adobe Photoshop (Adobe Systems Inc., San Jose, CA) by adjusting the brightness (+20) and the contrast (+75). Antigen positive cells were counted and expressed as a ratio to the total number of cells counted. Experiments were duplicated, with two coverslips and three fields per coverslip analyzed per treatment for each replicate. The experiment was also repeated using another Sindbis virus, MRE16, but this virus caused significant cell death in the C6/36 cells at the MOI (10) investigated here and the experiment was terminated due to the low cell survival rate.

RNA extraction. Total RNA was extracted using a modified version of the guanidinium thiocyanate-phenol-chloroform extraction method (GTC; Chomczynski and Sacchi, 1987). Cell pellets were disrupted in 400 μL of RNA lysis buffer (4M guanidinium-thiocyanate), 40 μL of 2M sodium acetate was added to each sample prior to acid phenol extraction (400 μL) and chloroform extraction (400 μL). Total RNA was precipitated with 500 μL isopropanol, resuspended in 180 μL RNase-free deionized water, and re-precipitated with 20 μL 3M sodium acetate and 500 μL ethanol. Alternatively, total RNA was rapidly extracted using the QiaShredder and RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer-provided protocol. RNA extracted by either method was used for RT-PCR and northern blot analysis, but siRNA northern blot analysis was only performed on RNA extracted by the GTC method because these small RNAs are not efficiently bound by the column used in the RNeasy method (RNeasy protocol, Qiagen) and would have been lost prior to collection during the binding and column washing steps.

RNA labeling. The following plasmid were obtained from colleagues at AIDL/CSU: pBlue/D2prM290, pBlue/D2ns2b-3, pBlue/D3capsid, and pTE/3'2J-D2prM(antisense). All plasmids, except dsSIN infectious clones, containing sequences inserted into the multiple cloning site of the pBluescript II KS(+) cloning vector (Stratagene). The pBlue/D2prM290, pBlue/D2ns2b-3, pBlue/D3capsid, and pBlue/actin plasmids were linearized with either *Nhe* I (for sense) or *Xba* I (for antisense), while the pTE3'/2J plasmids were cut with *Xba* I.

Non-isotopic labeling: RNA was transcribed *in vitro* using the T3 (pBlue/D2prM290, pBlue/D2ns2b-3, pBlue/D3capsid, pBlue/D3GDD, pBlue/actin antisense), T7 (pBlue/D2prM290, pBlue/D2ns2b-3, pBlue/D3capsid, pBlue/D3GDD, pBlue/actin sense) or SP6 (pTE/3'2J) RNA polymerase in a 20 μL reaction using the MAXIscript *In Vitro* Transcription kit (Ambion, Austin, TX) according to manufacturer protocol at 39°C for 1 hour. RNA was precipitated using 3 volumes 100% ethanol and 0.1 volume 3M sodium acetate and centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting RNA pellet was resuspended in 20 μL RNase-free water and quantified by spectrophotometery. RNA (0.5 μg per sample) was labeled in a 10 μL reaction with the BrightStar Psoralen-Biotin Non-Isotopic Labeling Kit (Ambion) to cross-link biotin to the RNA by 45 minutes exposure to 345 nm UV light. Resulting labeled RNA had a concentration of ~ 0.5 ng/μL after purification.

Isotopic ^{32}P labeling: Radioactive labeling was used to generate probes for northern blot analyses. These ^{32}P labeled RNAs were transcribed *in vitro* from linearized plasmid templates (described above) using the MAXIscript *In Vitro* Transcription kit (Ambion) in

20 μL reactions including 10 mCuries(mCi)/mL of α-^{32}P-labeled UTP with a specific activity of 800 Ci/mmol. Specific activity of probes was determined by comparison of a sample of the total transcription reaction to a sample taken after removal of unincorporated nucleotides by purification over Microspin G-25 columns (Amersham Biosciences). Both samples were subjected to quantitation of counts per minute (cpm) on a liquid scintillation counter and values were used to calculate percent incorporation and specific activity for each probe. Specific activity value for individual probe preparations ranged between 1.0×10^9 cpm/μg and 1.0×10^{11} cpm/μg.

Enrichment for siRNAs. Total RNA was enriched for small RNAs using a method described by Baulcombe and colleagues (1999; Sanchez-Vargas et al., 2004; Travanty et al., 2004). The total RNA pellet was dissolved in water, heated to 65°C for 5 to 10 min, and then placed on ice. Polyethylene glycol (molecular weight 800; Sigma) was added to a final concentration of 5% and NaCl to a final concentration of 0.5 M. After a 30 minute incubation on ice, the RNA samples were centrifuged at 10,000 x g for 10 min. Three volumes of ethanol were added to the supernatant and the RNA was precipitated at –20°C for at least 2 hours. The low molecular weight RNAs were pelleted by centrifugation for 20 min at 10,000 x g. The pellet was dissolved in gel loading dye II (Ambion, Austin TX) and heated for 5 min at 65°C before loading on an 8M urea 15% polyacrylamide 1X TAE sequencing gel. Gels were pre-run for 30 min at 400 volts prior to sample loading and run for ~2.5 hours at 400 volts after loading. Gels were stained with SYBR green II RNA stain (Molecular Probes, Eugene, OR) and RNA was

transferred to BrightStar positively charged nylon membrane (Ambion) by electrotrasfer in 1X TAE for 45 min at 100 volts.

Northern blot analysis. Blots were pre-hybridized in 10 mL ULTRAHyb buffer (Ambion) at 68°C (42°C for siRNA blots) and probed with ^{32}P labeled RNA generated by *in vitro* transcription using the MAXIscript kit (Ambion). Probes for siRNA blots were digested with 300 μL carbonate buffer (80 mM $NaHCO_3$, 120mM Na_2CO_3) per 20 μL probe for 2 to 3 hours at 60°C. Digestion was stopped by the addition of 20 μL of 3M sodium acetate, pH 5.0. All other probes were full length and were used without digestion. Hybridization was allowed to proceed overnight at 68°C (42°C for siRNA blots), and then membranes were washed twice for 20 minutes each in 50 mL of 2X SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate) + 0.2% SDS at 50°C and twice for 20 minutes each in 50 mL of 0.2X SSC + 0.2% SDS at 50°C. Blots were then exposed to Kodak ML film at -80°C for 7-14 days or to phosphor storage screens for between 20 min and 14 days (depending on signal intensity) and were analyzed by development of the autoradiograph or by using a STORM phosphorimager (Molecular Dynamics, Amersham Biosciences, GE Healthcare, Piscataway, NJ).

***In vitro* RNAi assay.** *In vitro* RNAi assays have been developed for *Drosophila* cells (Elbashir et al., 2001a; Elbashir et al., 2001b; Tuschl et al., 1999) and embryo lysates (Haley et al., 2003). Based on these published methods, an *in vitro* assay for RNAi in mosquitoes was examined. Crude cell extracts were prepared from uninfected C6/36 cells and C6/36 cells 5 days post infection with TE3'/2J/D2prM-antisense. Cells

in a 150-cm^2 tissue culture flask were washed 3X in PBS and spun down at 2,000 x g for 5 min. Hypotonic buffer (10mM HEPES pH 7.3, 6mM β-mercapthoethanol) was used to wash the cell pellet. The cell pellet was resuspended in 0.7 packed cell volumes of hypotonic buffer containing Complete Protease Inhibitors (1 tablet/10 mL; Roche Applied Sciences, Indianapolis, IN) and 0.5 unit/mL RNasin (Promega, Madison, WI). Cells were disrupted using 5 strokes of a Dounce homogenizer with a type B pestle and the lysate was centrifuged at 21,000 x g for 20 minutes at 4°C. The supernatant was used to investigate RNAi activity. RNAi was analyzed by examining the stability of added non-isotopic labeled single-stranded RNA over time under the following reaction conditions: 10 μL cell lysate, 2 mM $CaCl_2$, 1 mM DTT, 20 mM HEPES pH 7.3, 110 mM potassium acetate, 3 mM EGTA at 28°C. Samples were removed at predetermined times and electrophoresed on a 1.25% agarose denaturing gel (1X MOPS, 0.66 M formaldehyde). Samples were blotted by capillary transfer in 10X SSC to BrightStar-Plus positively charged nylon membrane (Ambion). Labeled RNA was visualized after treatment with the BrightStar Non-Isotopic Detection Kit (Ambion) and exposure to Kodak ML light film.

Determination of DNA plasmid copy number per cell. The FB9.1 cell line has been previously described (Adelman, 2000; Adelman et al., 2002). The determination of plasmid copy number was repeated here using the STORM phosphorimager instead of film as was done previously because a phosphorimager collects data in a linear fashion across a broader range. The plasmid, pIe-Mnp-D2prM-AS/Hyg #8, that was originally used to transform C6/36 cells, leading to the generation of FB9.1 cells (Adelman, 2000;

Adelman et al., 2002) was purified and digested with *Eco*RI (New England Biolabs (NEB), Beverly, MA) at 37°C for 4 hours. DNA was extracted from FB9.1 cells at passage 16 using the DNeasy extraction kit (Qiagen) and digested with *Eco*RI. Both DNA samples were precipitated with 0.1 volume of 3M sodium acetate, pH 5.5 and 3 volumes of 100% ethanol at –20°C for 20 minutes and centrifuged at 16,000 x g for 20 minutes at 4°C. Pellets were washed with 70% ethanol and centrifuged at 16,000 x g for 5 minutes, dried and resuspended in nuclease-free water and quantified by spectrophotometery. A dilution series of the *Eco*RI digested pIe-Mnp-D2prM-AS/Hyg #8 plasmid (100ng, 10ng, 1ng, 100pg, 10pg, and 1pg) was generated. Three samples of *Eco*RI-digested FB9.1 cell total DNA representing 1 x 10^6 cells per sample (cells were counted by hemocytometer prior to DNA extraction) were precipitated, washed, and resuspended in 90 μL of nuclease-free water. All 9 samples were heat denatured at 60°C for 1 hour in the presence of 1/10 volume 3M NaOH. Samples were diluted in one volume 6X SSC and vacuum blotted to BrightStar-Plus positively charged nylon membrane (Ambion) using a Minifold II slot blot apparatus (Schleicher and Schuel, Keane, NH). Each slot used was first prepared for sample with 500 μL of 6X SSC and after the samples were run through, each slot was washed with 6X SSC. The membrane was UV crosslinked using the autocrosslink function of a Stratalinker (Stratagene, La Jolla, CA). A DNA probe was prepared from the pIe-Mnp-D2prM-AS/Hyg #8 plasmid with a specific activity of 4.6 x 10^9 cpm/μg using random primers included in the Megaprime DNA labeling system (Amersham Biosciences). Hybridization was performed in ULTRAHyb buffer (Ambion) at 65°C overnight and blots were washed twice for 20 minutes each in 25 mL of 2X SSC + 0.2% SDS at 65°C and twice for 20

minutes each in 25 mL of 0.2X SSC + 0.2% SDS at 65°C. Blots were then exposed to phosphor storage screens for 1 hour and analyzed using a STORM phosphorimager. The intensity of signal in each slot was measured using ImageQuant software (Amersham Biosciences) and a linear regression was performed on the pIe-Mnp-D2prM-AS/Hyg #8 plasmid dilution series using Microsoft Excel. The average intensity of the three FB9.1 samples was then used in the linear regression equation to determine the amount (in μg) of plasmid in the FB9.1 cells. One copy of the plasmid is 11.2 kilobases (or 11200 bp). Using the conversion of 1bp = 660 g/mol the plasmid is 73,920,000 g/mol, or 1.23×10^{-17} g/plasmid. This number was used to convert the μg of plasmid per cell into plasmid copy number.

RNA slot blot. Total RNA was extracted from cells using the GTC method described above. Each sample (5 μg) was denatured in 150 μL of denaturation buffer (6X SSC, 7% formaldehyde) at 65°C for 10 minutes. RNA was vacuum blotted to BrightStar-Plus positively charged nylon membrane using a Minifold II slot blot apparatus as described for DNA above. The blots were washed and cross-linked as previously outlined for DNA slot blots. RNA blots were probed with ^{32}P labeled riboprobes at 65°C, washed with SSC as described and analyzed by phosphorimager.

siRNA Transfection. Single siRNA experiments: Commercially prepared siRNAs were obtained in duplex form from Dharmacon, Inc. (Layfette, CO) or Qiagen, Inc. (Valencia, CA). Custom siRNA duplex was designed from a region of similarity between DEN-2 prM and DEN-3 prM genes: 5'- ACA UGG GUA ACU UAU GGG -

dTdT -3'. Control was luciferse GL2 siRNA duplex sequence: 5'- CGT ACG CGG AAT ACT TCG A - dTdT -3' with a 5' fluorescein label. C6/36 cells for transfection were grown in 12 well tissue culture plates on 18mm circular glass coverslips until they were approximately 70% confluent. Transfection complexes were prepared by combining L15 medium, transfection reagent and siRNA duplex (20 μM) in 12 x 75 mm polystyrene tubes. Samples were mixed briefly by gentle vortex and incubated for 20 minutes at room temperature. Growth medium was removed from the cells and 0.5 mL of fresh medium without supplements was added. The siRNA/transfection reagent complexes (120 μL) were added drop-wise to each well and plates were rocked to mix. Experiments were set up in triplicate: one plate for anti-DEN siRNA transfection, one for control labeled siRNA transfection and one for mock transfection (treatment with transfection reagent alone) and all complexes were incubated on the cells for 48 hours at 28°C. Transfection reagents, TransIT-TKO siRNA (Mirus Bio Corporation, Madison, WI) and RNAifect (Qiagen) were used according to manufacturer recommended protocols. Wells that were transfected with the control labeled duplex RNA were observed for cellular uptake of the fluorescein labeled siRNA by UV microscopy and once it was determined that the cells had taken up the siRNAs, DEN-2 challenge of the mock-, control siRNA-, and anti-DEN-2 siRNA cells was performed. Fluorescent antibody staining was performed at 7 days post viral challenge.

Pool siRNA experiments: Double-stranded RNA corresponding to the DEN-2 M gene was made by *in vitro* transcription from PCR products. The following primer pair was used to amplify a region of the DEN-2 genome (PCR product represents bases 406-843 of the DEN-2 genome spanning the 3'end of C into prM amplified using the

pBlue/Mnp plasmid containing the entire Mnp region described above as template) adding the T7 polymerase promoter sequence to each 5' end (underlined region): T7MnpFwd, 5'-TAA TAC GAC TCA CTA TAG GCA GGC GTG ATT ATT ATG TTG AT-3': T7MnpRev, 5 '-TAA TAC GAC TCA CTA TAG GTA TGG TAA AGC CTG GAT GTC TC-3'. PCR amplification was carried out under the following conditions: 94°C 3 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 1 minute followed by a final 5 minute incubation at 68°C. PCR product size (438 bp) was confirmed by agarose gel electrophoresis and the PCR product was purified using QiaQuick PCR purification columns (Qiagen). Purified PCR products were then used as templates in a high yield *in vitro* transcription reaction (MEGAscript, Ambion) and template was removed after transcription by treatment with DNase I. The dsRNA was generated by *in vitro* transcription, annealed by heating to 72°C for 10 minutes and slowly cooled to room temperature, and digested into siRNAs using the recombinant human Dicer enzyme (Gene Therapy Systems, Inc., San Diego, CA). Control siRNA corresponding to double-stranded GFP RNA was generated using templates and primers provided in the recombinant human Dicer kit. Digested siRNAs were precipitated with 3 volumes 100 % ethanol and resuspended in nuclease-free water. These Dicer generated siRNAs (d-siRNAs) were used in transfection experiments as outlined above for siRNA experiments.

siRNA cloning. The pool of small RNAs from mock-, DEN-2 infected-, and DEN-3-infected C6/36 and FB9.1 cells were cloned using a protocol adapted from Bartel and colleagues (Lau et al., 2001; see Figure 2.1 for diagram of cloning protocol). Total

RNA was extracted from mock-infected cells and from the DEN-2 and DEN-3 infected cells at 3, 7, 10, and 14 days post infection as described above. Infections were performed at an MOI of 0.01 and pooled cell pellets from four confluent 150-cm^2 tissue culture flasks were used for each time point of each sample. Samples were enriched for low molecular weight RNA using the polyethylene glycol precipitation protocol described above. Low molecular weight RNA was separated on 1X TBE, 8M urea, 15% polyacrylamide sequencing gels. Gels were pre-run for 30 min at 400 volts to heat the gel and buffer, and after loading, run for ~2.5 hours at 400 volts. RNA was visualized by staining gels with SYBR green II RNA stain (Molecular Probes, Eugene, OR) and size was determined based on the position of sample bands relative to the 10bp DNA step ladder (Promega, Madison, WI). Bands in the size range of 18 to 30 bp, expected to include the siRNAs, were excised and eluted overnight in 0.3 M NaCl at 4°C in non-stick micro-centrifuge tubes. From this point on, all manipulations were performed under RNAse-free conditions in non-stick microfuge tubes to prevent loss of the small RNAs. Eluted siRNAs were precipitated by the addition of 3 volumes of 100% EtOH and 20 μg of glycogen (Ambion, Austin, TX) as a carrier, incubated at –70°C for at least 20 minutes, and centrifuged at 16,000 x g for 20 minutes at 4°C.

The siRNA were ligated to the following 3' adaptor made of DNA with a phosphate at the 5' terminus and blocked at the 3' terminus with the amino modifier C6 (6 carbon amino group): 5'-phosphate-CTG TAG GAT CCA TCA AT 3aminoModC6-3' (400μM stock; underlined region is *Bam*HI restriction site; Integrated DNA Technologies (IDT), Inc., Coralville, IA) with T4 RNA ligase (Amersham) overnight at 16°C. The 3'adapted siRNAs were separated from un-adapted siRNAs and unused adaptor by size

selection using the same gel and excision method described. Purified 3' adapted siRNAs were then ligated to the following 5' adaptor made of both DNA and RNA: 5'-ATC GTrA rGrGrA rUrCrC rUrGrA rArA-3' (400μM stock; underlined region is *Bam*HI restriction site; RNA residues are denotes with an r; IDT) using T4 RNA ligase overnight at 16°C. The doubly adapted siRNAs were again separated from un-adapted siRNAs and unused adaptor by size selection after gel electrophoresis.

RT-PCR was used to amplify the adapted siRNA using primers that bind to the adaptor sequences: RT primer 5'-ATT GAT GGA TCC TAC AG-3', forward primer 5'-ATC GTA GGA TCC TGA AA-3' (underlined region is *Bam*HI restriction site). RT-PCR products were digested with *Bam*HI and ligated into concatamers using T4 DNA ligase at 16°C overnight. Concatamers were separated by size on precast 10% polyacrylamide/TBE gels (NOVEX, Invitrogen, Carlsbad, CA). Bands, or usually a smear representing a size range of concatamers rather than distinct bands, larger than 300 bp were excised and eluted as described. The DNA was then concentrated by ethanol precipitation using a glycogen carrier and cloned into the TOPO-TA vector for sequencing (pCR®4-TOPO; Invitrogen). Colonies were screened for inserts using the M13 forward (-20) primer 5'-GTA AAA CGA CGG CCA G-3' and M13 reverse primer 5'-CAG GAA ACA GCT ATG AC-3' with binding sites that flank the pCR®4-TOPO multiple cloning site. Positive M13 PCR products were sequenced using the T3 primer 5'-ATT AAC CCT CAC TTA AGG GA-3' and T7 primer 5' TAA TAC GAC TCA CTA TAG GG-3', with binding sites internal to the M13 binding sites in pCR®4-TOPO. Sequencing was performed using the Big Dye® version 3.1 termination chemistry and analyzed using an ABI PRISM® 377 DNA sequencer (Applied Biosystems, Foster City,

CA). Sequence data were compared to DEN-2 and DEN-3 viral genomic sequence and subjected to blastn NCBI BLAST searches for short, nearly exact matches to sequences in GenBank (http://www.ncbi.nlm.nih.gov/; National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD).

Figure 2.1. Flow Chart of siRNA Cloning Procedure.

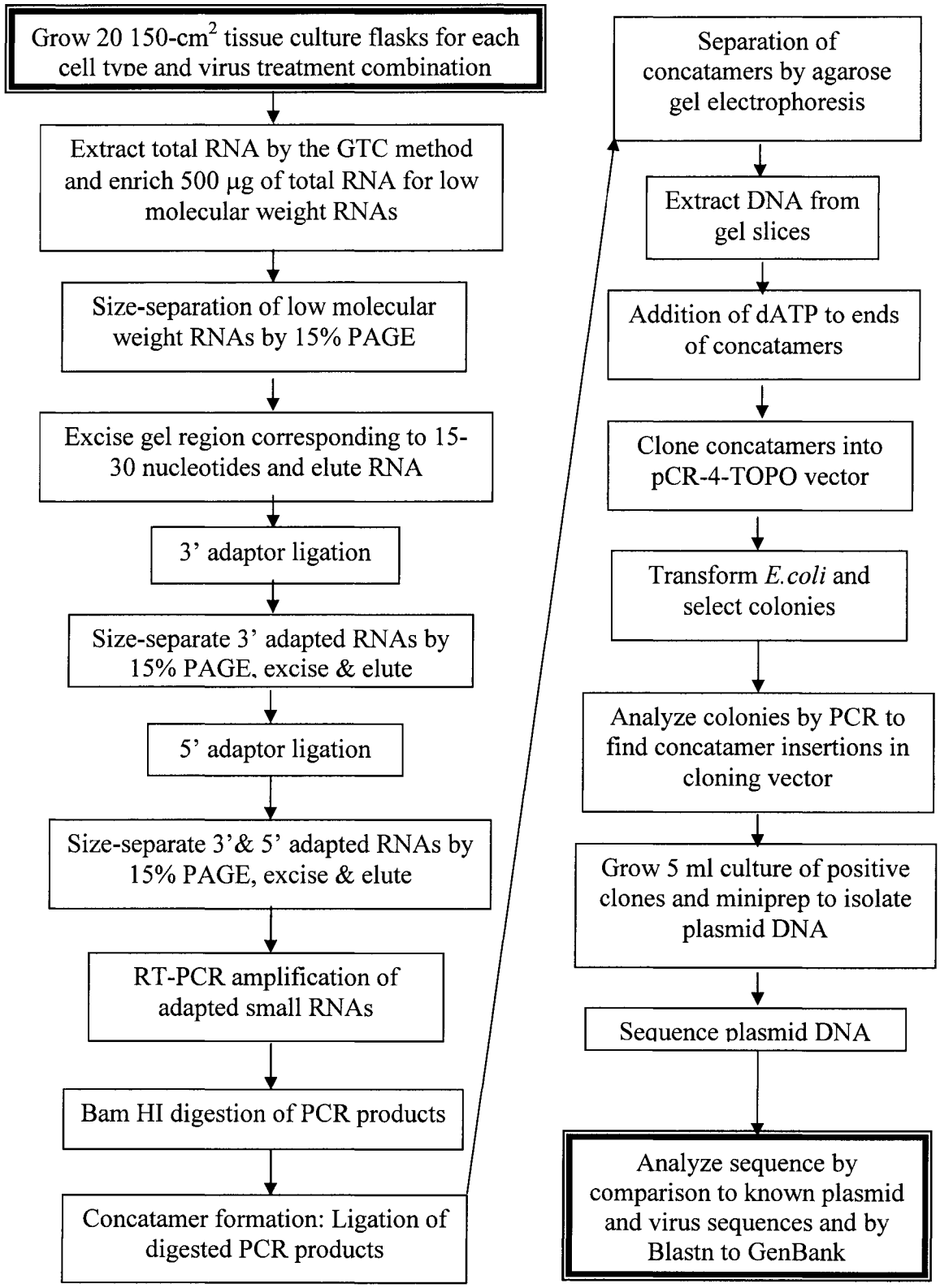


Table 2.1. Cultured Cell Lines.

Type	Cell Line	Source
Insect	C6/36	*Aedes albopictus* mosquito (Singh, 1967; Igarashi, 1978)
	FB9.1	Transfected with plasmid containing hygromycin B resistance gene and DEN2-RNA hairpin (Adelman et al., 2002)
	H9.1	Transfected with plasmid containing hygromycin B resistance gene (Adelman et al., 2002)
	S2	*Drosophila melanogaster* (Schneider, 1972)
Mammal	BHK-21	*Mesocricetus auratus* Golden Syrian hamster kidney cells
	LLC-MK2	*Macaca mulatta* Rhesus monkey kidney cells

Table 2.2. Viruses.

Virus Family	Virus Name	Description
Flaviviruses	DEN-1 (Dengue-1 virus)	Hawaii
	DEN-2 (Dengue-2 virus)	Jamaica 1409/83 and New Guinea C strains
	DEN-3 (Dengue-3 virus)	H-87
	DEN-4 (Dengue-4 virus)	H-241
	WNV (West Nile virus)	New York 1999
Alphaviruses	TE3'/2J	Sindbis virus (SIN) infectious clone (Hahn et al., 1992)
	TE3'/2J/D2prM-antisense	SIN infectious clone with DEN-2 prM insert antisense orientation under control of 3' second subgenomic promoter (Adelman et al., 2001)
	MRE16	(Myles et al., 2003)
	5'dsMRE16ic-eGFP	SIN-MRE16 infectious clone with eGFP insert under control of 5' subgenomic promoter (B. Foy, unpublished)
	5'dsMRE16ic-eGFP/ D2prM-antisense	SIN-MRE16 infectious clone with eGFP and DEN-2 prM antisense insert under control of 5' subgenomic promoter (B. Foy, unpublished)

Table 2.3. Antibodies.

Antibody	Type	Species	Specificity
3H5	Monoclonal IgG1	Mouse	DEN-2 envelope (Henchal et al., 1985)
2H2	Monoclonal IgG2a	Mouse	DEN 1-4 prM (Falconar, 1999)
4G2	Monoclonal IgG2a	Mouse	Flavivirus-complex envelope (Henchal et al., 1985)
30.11a	Monoclonal	Mouse	SIN E1 (Chanas et al., 1982)

Results

I. Co-infection of DEN-2 and TE3'2J.

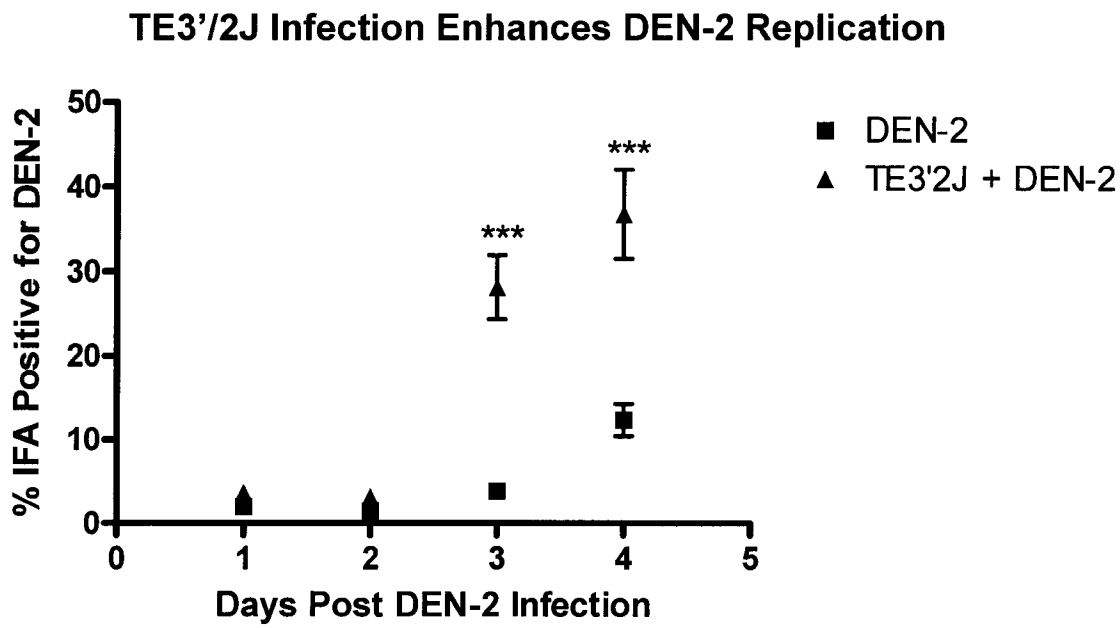
Replication Enhancement. Replication of most RNA viruses alters the host cellular environment, usually in a manner favorable to replication of the virus. In some cases, replication of one virus can serve to enhance the replication of another virus. The purpose of this study was to examine the ability of TE3'/2J, a double-subgenomic Sindbis virus (dsSIN; *Alphavirus*, Togaviridae) to affect the replication of a genetically unrelated flavivirus, DEN-2. TE3'/2J is based on a cDNA clone of Sindbis virus (Lustig et al., 1988; Hahn et al., 1992), with no insert in the 3' second subgenomic promoter. Cells were infected with DEN-2 alone or after TE3'/2J was allowed to establish an infection, and the replication of DEN-2 was monitored over a four day time course by assaying for the accumulation of DEN-2 antigen. TE3'/2J can establish a persistent infection in mosquito cells with no cytopathic effects detectable for at least 6 days when an MOI of 10 is used for infection. Indeed, through the course of the 6-day incubation period used in this assay (2 days for SIN to establish infection plus the 4 day DEN-2 time course) no cytopathic effects were observed. DEN-2 virus replicated slowly in mosquito cell culture during the first 4 days following infection at a multiplicity (MOI) of 0.025; only 13.5% of cells expressed antigen detectable by IFA at that MOI (See Figure 2.2). When the MOI was increased to 0.1, all cells were DEN antigen positive by 7 days post infection (data from generation of working stock of virus, not shown). When 100% of the cells were infected with SIN, the replication rate of DEN-2 remained low over the first two days,

but increased rapidly by day 3 and day 4 post-infection, resulting in almost 40% of cells positive for DEN antigen (39.1% by 4 days post infection). These results indicated that the replication of TE3'2J altered the environment within the mosquito cell, allowing DEN-2 to replicate faster. Indeed, statistical analysis by 2-way analysis of variance (ANOVA) found the difference between DEN-2 antigen positive cells between the two treatments (DEN-2 alone and SIN infection prior to DEN-2 challenge) to be statistically significant with a p-value of <0.001.

Repetition of these results was attempted using another SIN virus, MRE16, but this virus caused significant cytopathology (CPE) at the MOI tested (10) and there were insufficient cells remaining alive at each time point to perform the necessary cell counts. Confirmation of these results may be possible using a lower MOI (perhaps 0.1), but in this case, the time needed to establish a persistent SIN infection in which all cells are positive for SIN antigen by need to be extended.

This study demonstrated a relationship between SIN replication and DEN-2 replication that appears to result in the enhancement of DEN-2. The next logical steps in the characterization of this relationship would be to examine the kinetics of the enhancement by varying the time of DEN-2 challenge, including co-infection with both viruses at the same time. Additionally, the enhancement phenomenon could be mapped to the effect of specific SIN proteins by expressing each SIN protein individually in mosquito cells and then challenging the cells with DEN-2 and examining the level and rate of DEN-2 by assaying viral antigen accumulation.

Figure 2.2. DEN-2 replication in C6/36 *Aedes albopictus* cells is enhanced by co-infection with TE3'/2J. Cells were either mock infected or infected with TE3'/2J and superinfected 48 hours later with DEN-2. Results represent data from two trials (6 replicates per trial) combined and are expressed as the mean ratio of antigen positive cells to total cells counted (as determined by immunofluorescence assay). Data are shown as percentage of cells containing DEN-2 antigen after infection with DEN-2 alone (■) or TE3'/2J followed by DEN-2 (▲). Error bars represent standard error of the mean.
*** P<0.001 by 2-Way ANOVA and Bonferroni test (Prism 4, GraphPad Software, Inc.).



II. *In vitro* RNAi assays.

Cell Culture Based *In Vitro* RNAi Assay. In order to examine the initiation of RNAi in mosquito cells, an *in vitro* assay was developed based on assays used to study RNAi in lysates from *Drosophila* cells and embryos (Elbashir et al., 2001a; Elbashir et al., 2001b; Tuschl et al., 1999; Haley et al., 2003). Lysates prepared from mosquito cells were used hypothesized to be able to replicate the RNAi pathway *in vitro*. The stability of a labeled DEN-2 prM RNA was studied in lysates prepared from uninfected C6/36 cells and C6/36 cells infected with TE/3'2J-D2prM(antisense). The RNA was expected to be stable in lysates prepared from control un-infected C6/36 cells, and unstable in the lysate prepared from the dsSIN infected cells. The difference in RNA stability between the two lysates was hypothesized to be a result of an RNAi response in the dsSIN infected cell lysates that would not be present in the un-triggered control cell lysates. The trigger in the dsSIN infected cells would be dsRNA produced during replication of dsSIN. For these experiments, the dsRNA was the intermediate in replication of TE3'/2J-D2prM(antisense), a dsSIN virus (*Alphavirus*, Togaviridae) with a 290 nt insert from the prM region of DEN-2 RNA. The hypothesis tested was that replication of this dsSIN would trigger RNAi towards the DEN bearing the cognate prM sequence in its genome.

The lysate made from virus infected cells and a control, uninfected cell lysate were both used to test stability of an added labeled prM antisense single-stranded RNA. In the infected cell lysate, the labeled RNA was unstable and was degraded over the hour of incubation (See Figure 2.3). There was no detectable full-length 290 nt prM RNA remaining in the samples containing lysate from the virus-infected cells by 50 minutes.

The uninfected lysate did not efficiently degrade the labeled RNA to the same extent, although some degradation was detected. The degradation in the C6/36 cell lysate was not complete and there was still detectable full length (290 nt) RNA at the end of the 60-minute incubation period.

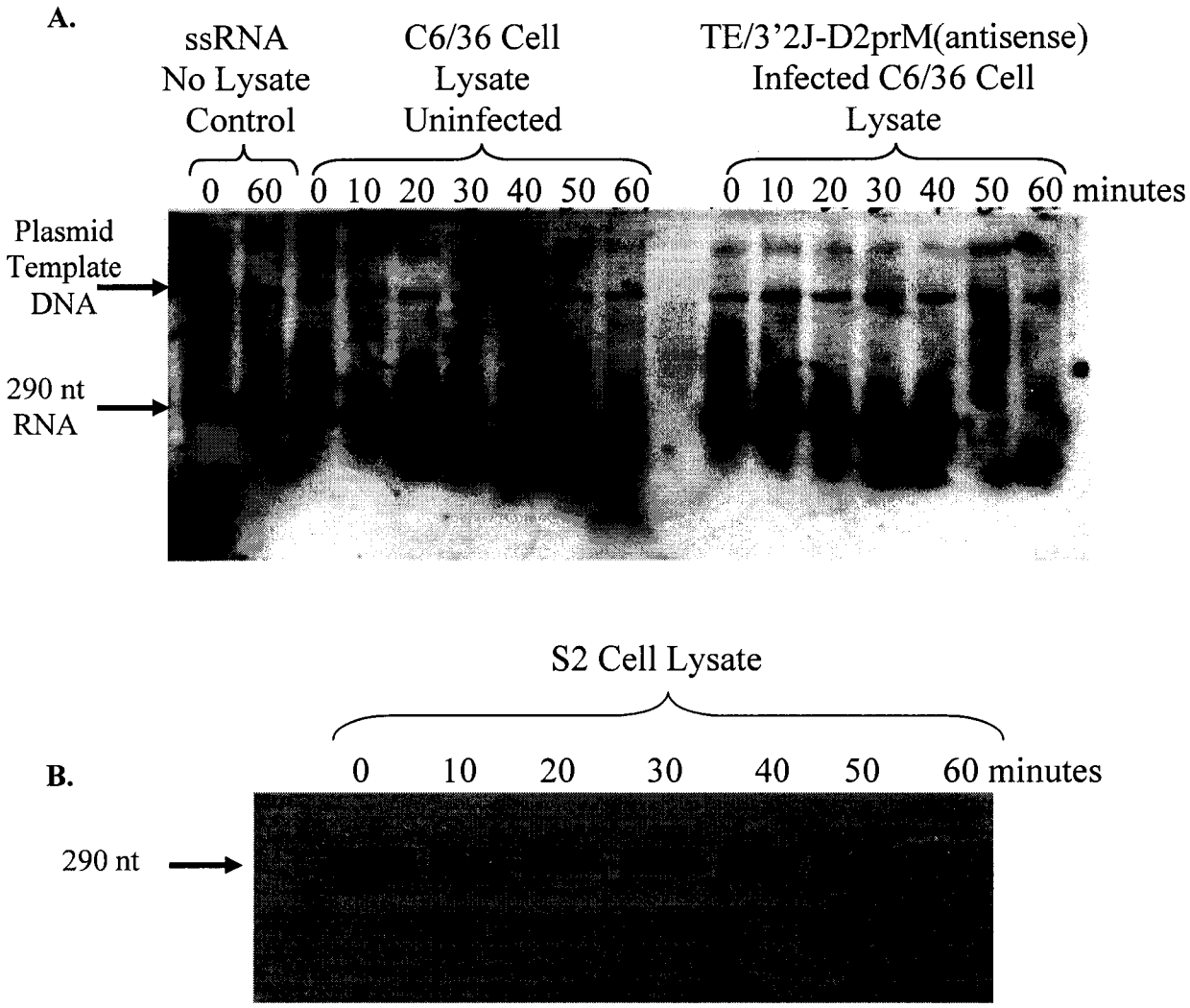
Additional studies were performed in order to determine if this non-specific degradation in the control lysate would effect RNAs of a different sequence than the DEN-2 prM region. Testing was performed on the stability of an unrelated RNA from the DEN-3 non-structural protein 5 (D3GDD) in both the control lysate and the lysate that was triggered for RNAi against DEN-2 prM by TE3'/2J-D2prM(antisense) replication. In this case, no rapid specific degradation of the RNA was observed, as had been observed with the DEN-2 prM RNA (data not shown). Again, there was some non-specific degradation, but the RNA was not completely degraded and full-length RNA was still present at the end of the incubation period (data not shown).

The observed degradation in the non-infected C6/36 control lysate appeared to be due to non-specific RNA instability and not RNAi because of the smearing effect visible in the gel. The degradation products are variable in size and are not the specific ~22 nt RNA that would be produced by RNAi. Altering the amount and type of RNase inhibitor in the lysate reaction was examined. First, the concentration of RNasin (Promega) was doubled, but the same degree of non-specific degradation was still observed. Next, the RNAse inhibitor was changed to SUPERaseIn (Ambion), but again the non-specific degradation was still observed. Finally, the labeling method may have been the cause of the non-specific degradation. In the non-radioactive labeling method used, a conjugated biotin molecule was psoralin cross-linked to bases of the RNA. This process may alter

the structure of the RNA and block access to bases, which could in turn affect the recognition of the RNA by the RNAi initiating enzyme dicer or digestion of the single-stranded RNA by RISC.

To rule out effects of the labeling method on RNA stability, the labeled single-stranded RNA was tested in lysates prepared from *Drosophila* S2 cells. Since the protocol was originally developed in the S2 cells, it was hypothesized that non-specific RNA degradation would not be observed and that the labeled DEN-2 prM RNA would remain intact during the course of the incubation. Figure 3.2B demonstrates stability of the labeled RNA in S2 cell lysates, indicating that the non-specific degradation of this RNA in the C6/36 cell lysates is not due to RNAse contamination or a problem with the labeled RNA itself, but instead is due to the environment of the mosquito cell. The high level of non-specific RNase activity in mosquito cells may represent a defense system used by the mosquito to control the expression of non-mosquito messages, including those from viruses. Since mosquitoes lack the adaptive antibody response present in mammals, it is reasonable to speculate that mosquitoes may contain antiviral pathways in addition to RNAi that can control the replication of invading viruses and this RNAse activity may be an example of this type of defense mechanism.

Figure 2.3. C6/36 cell culture based *in vitro* RNAi assay. A. C6/36 cells were either mock infected or infected with TE/3'2J-D2prM(antisense) and used to prepare lysates at 5 days post infection. Lysates were assayed for their ability to degrade a labeled single-stranded 290 nt RNA from the prM region of DEN-2 RNA. B. The same labeled D2prM RNA was stable in an S2 cell lysate prepared by the same method as the C6/36 cell lysates, demonstrating that the non-specific RNA degradation observed in uninfected C6/36 cell lysates is a property unique to this mosquito cell line.



III. Characterization of the RNAi response in FB9.1 DEN-2 silenced cells.

Determination of DNA plasmid copy number per cell. The FB9.1 cell line has been previously characterized (Adelman et al., 2002) and shown to be refractory to DEN-2 replication by an RNAi response to the anti-DEN-2 dsRNA expressed within these cells. The cells were originally generated by transfection of a plasmid that expressed a DEN-2 dsRNA. We hypothesized that the number of plasmids incorporated into the FB9.1 cell was important for the generation of the RNAi response and that ore plasmids would result in more DEN-2 dsRNA and therefore a strong anti-DEN-2 RNAi response. Indeed, these cells were shown to have a strong RNAi response (Adelman et al., 2002). Here, the plasmid copy number in the FB9.1 cells was determined using sensitive phosphorimager quantitation. A slot blot containing a dilution series of the original transforming plasmid, pIe-Mnp-D2prM-AS/Hyg, from 1pg to 100ng, was probed along with 3 replicates of DNA extracted from the FB9.1 cells at passage 16. The resulting blot was analyzed by phosphorimager scan and the phosporimage was quantified by measuring the intensity of the signal in each slot.

In Figure 2.4, boxes #4 through #9 represent the plasmid dilution series and boxes #1 through #3 are the FB9.1 cell replicates. Linear regression was performed (Microsoft Excel) on the dilution series resulting in the equation $y = 71.335x\text{-}114586$, $R^2 = 0.9926$. The average (304817.32) intensity for the 3 FB9.1 samples (270053.67, 318175.87, and 326222.43) showed that there were 5897.35 pg of plasmid in each FB9.1 sample. There were 1×10^6 cells per sample, corresponding to 2.4×10^{-14} g of plasmid/cell. Conversion from grams of plasmid to plasmid copy number was accomplished by division by 1.23×10^{-17} g/plasmid resulting in a plasmid copy number of 1951 plasmids per cell.

These results were then compared to estimates made previously from these same cells at passage 5 using scans of exposed film (Adelman, 2000). Data on plasmid copy number obtained on early passage cells (passage 5) shortly after the transformed cell line was established demonstrated that each cell contained approximately one copy of the plasmid (Adelman, 2000). However, further analysis of these cells investigated the physical state of the plasmid within the FB9.1 cells and found multiple copies of the plasmid (Adelman et al., 2002). Total DNA from FB9.1 cells between passage 4 and 10 after establishment was restriction digested with *Eco*RI, which recognizes nine sites within the transforming plasmid, and *Kpn* I, which does not cut the plasmid but recognizes sites within C6/36 genomic DNA (Adelman et al., 2002). These restriction digests were subjected to Southern blot analysis that revealed multiple junction fragments between the plasmid and genomic DNA as well as a strong signal for the intact plasmid (Adelman et al., 2002). Interestingly, it was hypothesized that the plasmid may be ordered into extra-chromosomal plasmid arrays as have been observed by others working with transformed C6/36 cells. (Adelman et al., 2002; Monroe et al., 1992).

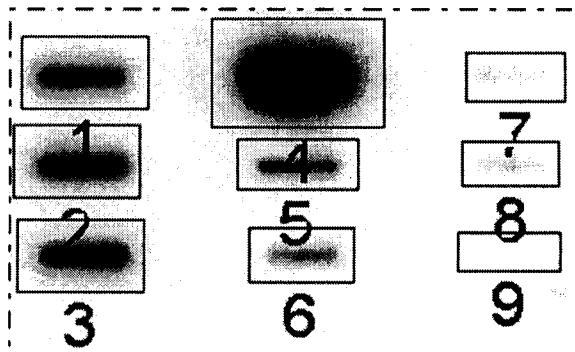
Finally, in this study we demonstrated almost 2000 copies per cell of the plasmid, indicating that either the plasmid copy number had increased with passage, that the phosphorimager quantification was more sensitive than the scanned film method, or a combination of these factors. Importantly, detection a high plasmid copy number confirms the hypothesis that plasmid copy number is an important factor in the RNAi response directed against DEN-2 in these cells.

Figure 2.4. FB9.1 cells contain large amounts of pIe-Mnp-D2prM-AS/Hyg plasmid.

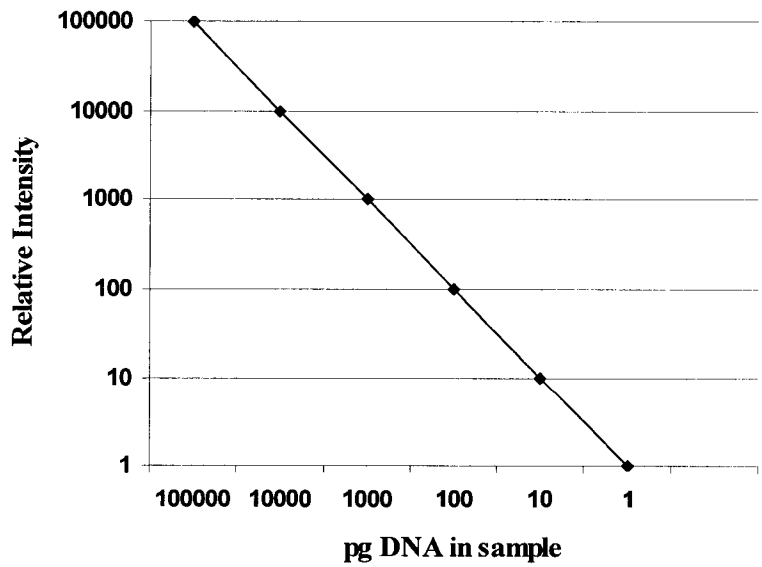
A. Analysis of the phosporimage, volume represents signal intensity within box. Boxes 4-9 are the plasmid dilution series, 1-3 are the three FB9.1 cell replicates. Background was determined for each box individually, normalizing the data to the line drawn around the slot using the local median setting in ImageQuant. B. Logarithmic plot of standard curve.

A. Quantification of digested pIe-Mnp-D2prM-AS/Hyg plasmid DNA in 3 replicates of FB9.1 cell extracts (samples 1-3) and dilution series of purified plasmid (samples 4-9) by slot blot hybridized with random primed ^{32}P labeled pIe-Mnp-D2prM-AS/Hyg plasmid DNA.

Sample	Relative Intensity
1. FB9.1	270053.67
2. FB9.1	318175.87
3. FB9.1	326222.43
4. 100 ng	7067959.2
5. 10 ng	97693.09
6. 1 ng	52688.69
7. 100 pg	10018.92
8. 10 pg	9462.86
9. 1 pg	793.22



B. Logarithmic plot of the standard curve generated from dilution series of pIe-Mnp-D2prM-AS/Hyg plasmid DNA.



Accumulation of dengue viral RNA in FB9.1 cells. To further study the RNAi response in the FB9.1 cell line, the rate of viral genomic RNA accumulation after infection was examined in the transformed FB9.1 and H9.1 cells and C6/36 cells. The plasmid expressed by the FB9.1 cells was designed to direct an RNAi response to DEN-2 by transcription of a DEN-2 prM specific dsRNA in these cells. The cells were shown to be refractory fro DEN-2 replication by the lack of DEN-2 antigen in these cells post challenge with DEN-2 (Adelman et al., 2002). The basis of the block in DEN-2 replication in these cells was thought to be RNAi and to confirm this was an example of RNAi, the accumulation of viral RNA was examined after challenge with DEN-2.

FB9.1, H9.1 and C6/36 cells were infected with DEN-2 Jamaica 1409 strain at an MOI of 0.1. Cells were harvested daily until 6 days post infection and total RNA was extracted. A total of 5 μg of total RNA from each sample, as determined by spectophotometric quantification of the RNA, was used for a slot blot. The blot was hybridized to detect DEN-2 RNA using a ^{32}P-labeled riboprobe corresponding to the complement of the ns2b-3 region of the DEN-2 genome. This genomic region was chosen for the probe because a portion of prM RNA was expressed from the plasmid construct by transformed FB9.1 cells. Results from this analysis indicated that viral genomic RNA accumulated from day 1 in the C6/36 cells as expected (See Figure 2.5). In the H9.1 cells, transformed with the hygromycin B resistance gene but without any DEN-2 sequence, DEN-2 RNA accumulated but was delayed by 2 days as compared to the C6/36 cells. DEN-2 RNA was not detected until day post infection in the H9.1 cells (Figure 2.5). The delayed accumulation observed in the H9.1 cells could have been due

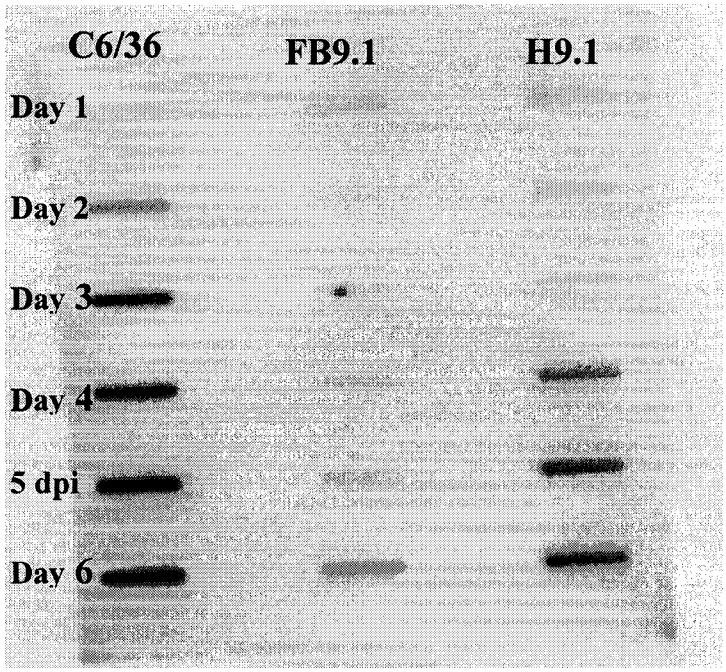
to the decreased growth rate observed in cells undergoing continuous hygromycin B selection. The FB9.1 cells transformed with the hygromycin B resistance gene and transcribing dsRNA corresponding to the DEN-2 prM gene, did not show accumulation of DEN-2 RNA. The final time point examined in this experiment, 6 days post infection, revealed there may be a low level of DEN-2 replication in the FB9.1 cells.

The experiment was repeated and RNA was extracted from days 1-14 after infection to ensure that the lack of accumulation observed in the FB9.1 cells continued beyond the first 6 days post infection and was not simply a longer delay than that observed in the H9.1 (See Figures 2.6 and 2.7). Based on the observation of signal for RNA accumulation at 6 days post infection in FB9.1 cells in the first experiment, it was necessary to extend the experiment to 14 days post infection in order to observe that DEN-2 viral RNA accumulation was indeed inhibited in these cells.

Additionally, the blot was stripped after hybridization to the DEN-2 ns2b-3 riboprobe and re-probed for actin mRNA to demonstrate relatively equivalent sample loading (See Figure 2.8). Based on the actin mRNA blot it was assumed that the spectrophotometric quantification of total RNA resulted in relatively equivalent sample loading in the previous 6-day experiment and in future experiments. These data demonstrated that the FB9.1 cells fail to accumulate DEN-2 RNA up to 14 days post-infection (Figures 2.6 and 2.7). The control H9.1 cells did support virus replication, although at a lower rate than C6/36 cells (Figure 2.5B).

Figure 2.5. DEN-2 RNA does not accumulate 6 days after DEN-2 infection of FB9.1 cells. A. RNA slot blot probed for the ns2b-3 region of DEN-2 RNA. B. Quantitation of the intensity of hybridization signal for each sample.

A.



B.

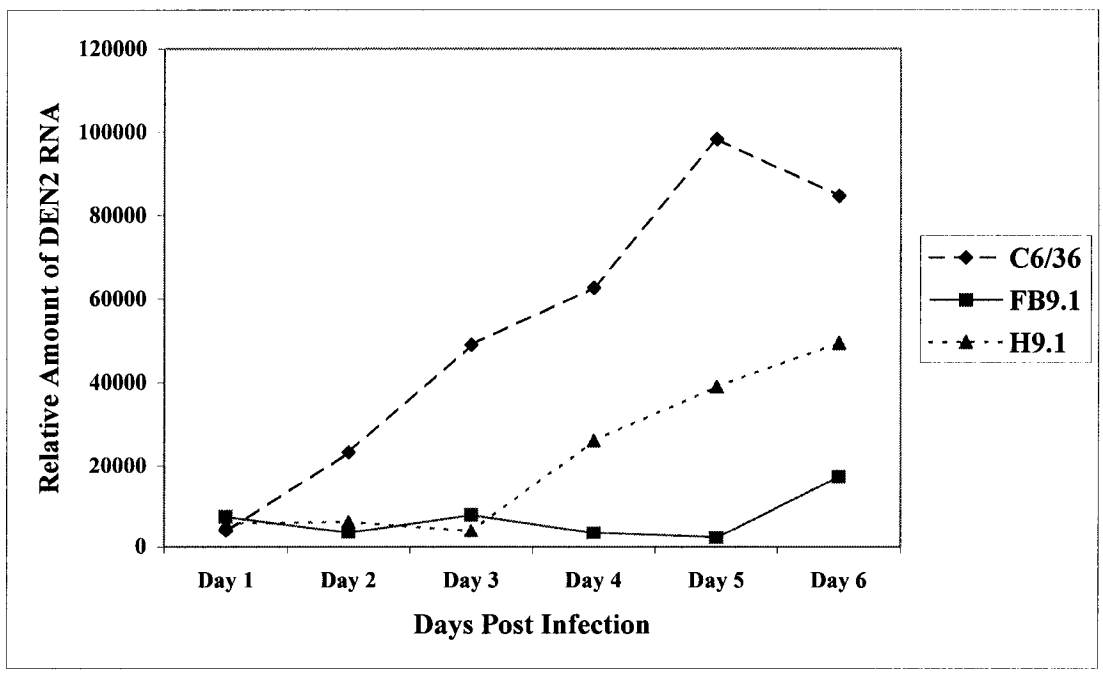


Figure 2.6. Accumulation of DEN-2 RNA is inhibited in FB9.1 cells and not in H9.1 cells: RNA slot blot analysis. FB9.1 cells express a hairpin dsRNA corresponding to the prM region of DEN-2 as well as the hygromycin B resistance gene, while H9.1 express only the resistance gene. RNA slot blot was probed for the ns2b-3 region of DEN-2. The day 11 sample for the H9.1 cells was lost during extraction due to breakage of the microfuge tube.

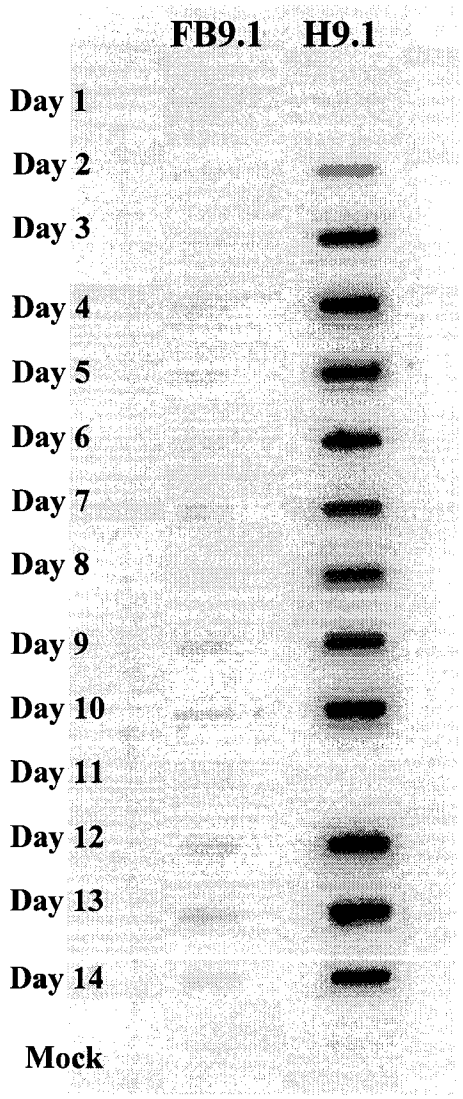


Figure 2.7. Accumulation of DEN-2 RNA is inhibited in FB9.1 cells and not in H9.1 cells: Quantitation of signal intensity. The RNA slot blot in Figure 2.6 was subjected to quantitation of signal intensity (ImageQuant). Results are displayed graphically as relative amount of DEN-2 RNA over time.

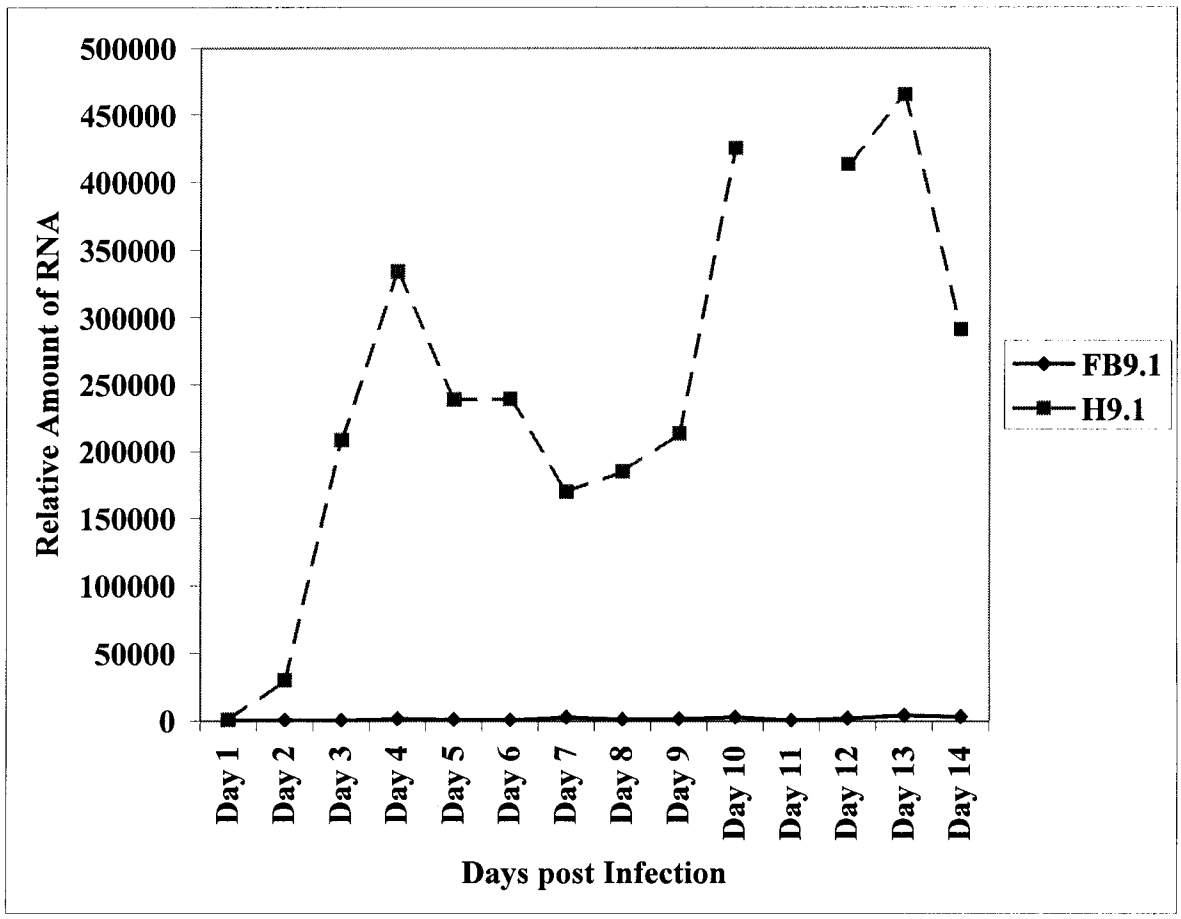
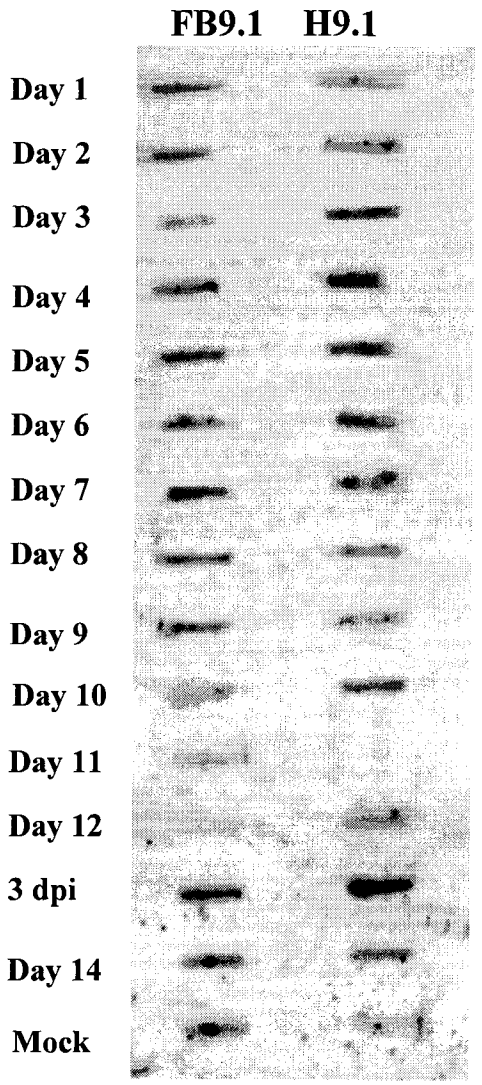


Figure 2.8. Actin mRNA probe demonstrates relatively equal expression after DEN-2 infection in FB9.1 and H9.1 cells. Blot from Figure 2.6 was stripped and re-probed for actin mRNA expression.



DEN-3 RNA accumulation was also investigated in these cells. The working hypothesis was that the mechanism by which DEN-2 replication was inhibited in the FB9.1 cells was RNAi. RNAi is a sequence specific pathway and should only result in interference with expression of RNAs of identical sequence as the trigger dsRNA. In the FB9.1 cells, the RNAi trigger was directed against DEN-2 and DEN-2 was shown to be targeted by RNAi in these cells (Figures 2.5, 2.7 and 2.7; Adelman et al., 2002). DEN-3 replication was examined over 14 days in the FB9.1 and H9.1 cell lines and was expected to accumulate to similar levels in both cell lines.

Surprisingly, DEN-3 RNA exhibited similar accumulation profiles in the two cell lines as DEN-2 (See Figures 2.9 and 2.10). DEN-3 viral RNA was detected beginning at day 2 post infection in the H9.1 cells and the amount of DEN-3 viral RNA increased over the 14 day time course in these cells. In the FB9.1 cells, however, DEN-3 viral RNA did not accumulate (Figure 2.9). A nucleotide alignment of the prM regions from DEN-2 and DEN-3 RNA revealed a high degree of similarity in small regions between the two sequences (See Figure 2.11). In one region, shown boxed, 21 out of 23 bases match. Mismatches between the target, in this case DEN-3, and the triggering dsRNA, in this case DEN-2, are known to be tolerated in RNAi (Elbashier et al., 2001). There is also another dsRNA directed pathway that controls gene expression. This pathway, like the RNAi directed degradation of mRNA described previously, involves a Dicer enzyme. The effector molecules in this pathway are endogenous microRNAs (miRNAs), which bind to mRNAs with similar but not identical sequences, resulting in characteristic bulges. This type of binding results in translation inhibition instead of mRNA degradation observed with siRNA binding (Doench et al., 2003). It is possible that the

miRNA pathway acts on the DEN-3 mRNA and inhibition of translation ultimately prevents accumulation of virus RNA within an infected cell. In this case, the DEN-2 specific small RNAs may be binding to the DEN-3 viral genomic mRNA. Since there is not perfect sequence homology between these two sequences, the bulges that would occur in a duplex of these two sequences may signal the microRNA pathway. This would result in inhibition of translation of DEN-3 message. This could be tested for by examining the stability of DEN-3 viral genomic RNA in the FB9.1 cells and by looking for production of DEN-3 specific viral proteins within these cells.

DEN protein expression in FB9.1 cells is detectable after viral replication. FB9.1 cells were assayed for expression of the prM protein to ensure that there was no expression of DEN-2 proteins from the plasmid construct in the FB9.1 cells. Broad antiviral effects of virus-encoded proteins have been previously reported for Banzi virus (Flavivirus, Yellow fever virus group; Lee and Schloemer, 1981a; 1981b). The original construct used to transform these cells was designed to express the prM gene without the start codon, and cells were examined by fluorescent antibody staining for prM using the monoclonal antibody 2H2, which is specific for dengue virus prM (Falconar, 1999).

Results from this assay revealed that uninfected FB9.1 cells did not express any prM antigen (See Figure 2.12, upper and middle panels). When FB9.1 cells were infected with DEN-2, prM antigen could be detected at a low level at 7 days post infection. Therefore, prM antigen was not produced in the FB9.1 cells as a result of expression from the transforming construct but was synthesized upon DEN-2 infection in a small number of cells, presumably resulting from expression directed by the incoming

viral genome. The intensity of DEN-2 infection of FB9.1 cells revealed by anti-prM 2H2 antibody staining mirrored what was previously demonstrated by anti-DEN-2 envelope 3H5 antibody staining (Adelman et al., 2002). This supports the hypothesis that the interference with DEN-2 replication in the FB9.1 cells is due to RNA expression and an RNAi response, and not due to expression of viral proteins within the cells prior to infection.

The FB9.1 cells demonstrate resistance to DEN-2, but they are under constant selection based on the Hygromycin B resistance gene expressed from the same plasmid as the anti-DEN-2 effector. Is constant selection necessary for DEN-2 resistance? Selection pressure due to hygromycin B treatment was removed from samples of FB9.1 and H9.1 cells. Cells were grown in the absence of hygromycin B for approximately one month (4 passages) with fresh L15 medium containing 10% FBS added every three days. At the end of this non-selection period, the cells were challenged with DEN-2 and assayed for DEN-2 antigen at 7 days post infection. All of the FB9.1 cells tested were positive for DEN-2 antigen, just as were the control H9.1 and C6/36 cells tested at the same time (data not shown). Removal of the hygromycin B treatment removed the selective pressure on the cells to retain the plasmid and, consequently the plasmid or its expression was presumably lost. These results indicate that constant production of siRNAs from the hairpin dsRNA construct are required to interfere with DEN-2 replication.

Figure 2.9. Accumulation of DEN-3 RNA is inhibited in FB9.1 cells and not in H9.1 cells: RNA slot blot analysis. FB9.1 cells express a hairpin dsRNA corresponding to the prM region of DEN-2 as well as the hygromycin B resistance gene, while H9.1 express only the resistance gene. RNA slot blot was probed for expression of the capsid (C) region of DEN-3 RNA after challenge with DEN-3.

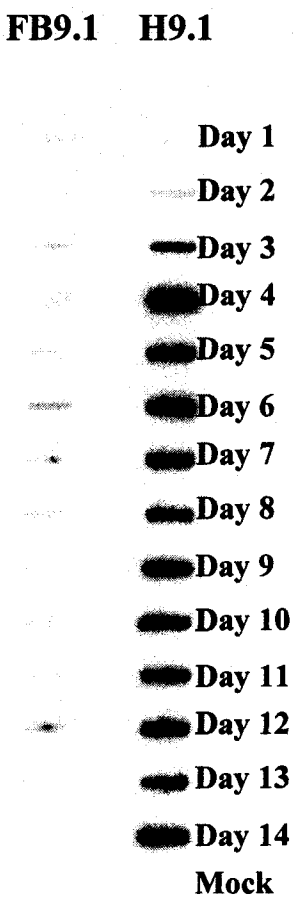


Figure 2.10. Accumulation of DEN-3 RNA is inhibited in FB9.1 cells and not in H9.1 cells: Quantitation of signal intensity. The RNA slot blot in Figure 2.9 was subjected to quantitation of signal intensity (ImageQuant). Results are displayed graphically as relative amount of DEN-3 capsid RNA over time.

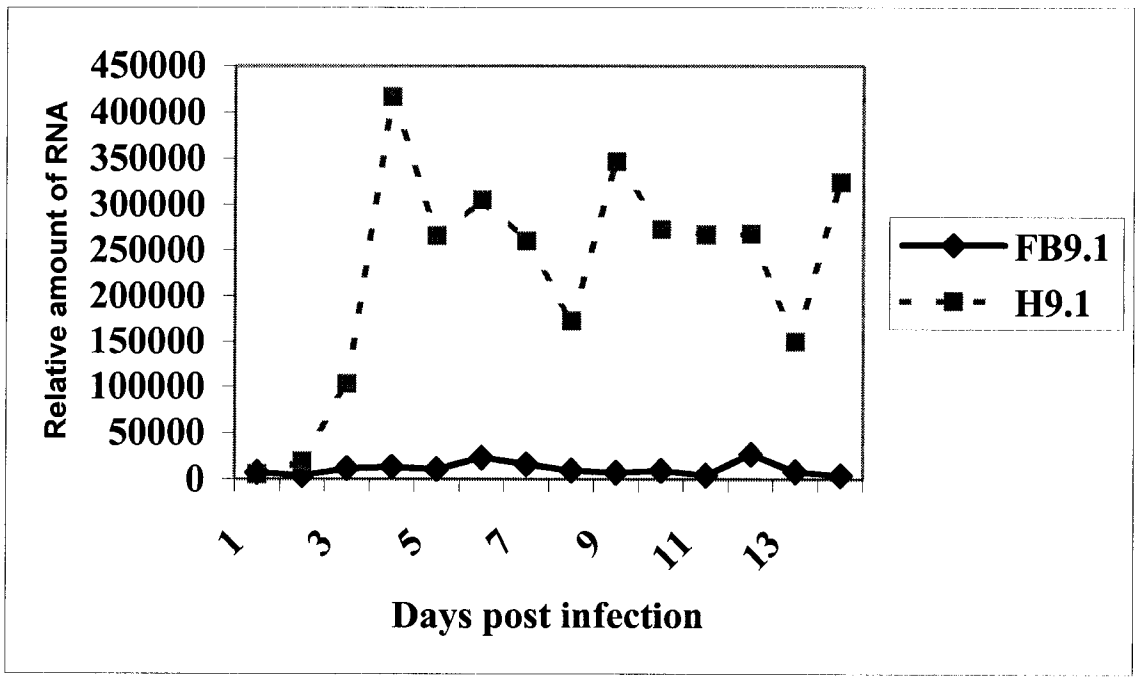


Figure 2.11. ClustalW alignment of the prM-M regions of DEN-2, DEN-3 and WNV reveals areas of sequence homology. Similar residues are shown in uppercase and identical residues are blue. DEN3 H87, nt #437-934 (GenBank accession #M931340); DEN2 Jamaica 1409, nt #437-934 (GenBank accession #M20558); WNV NY99-eqhs, nt #466-966 (GenBank accession #AF260967). Nucleotide numbers are relative to the viral genomic RNA sequence. (ClustalW, San Diego Supercomputer Center Biology Workbench http://workbench.sdsc.edu). Boxed region represents area of homology between DEN-2 and DEN-3 selected for siRNA sequence (* represents the mismatched base at position 18 of the siRNA sequence). Note that the area of homology between the 3 sequences located 5' to the boxed region was not selected for siRNA synthesis due to mismatched bases in the middle of the 21 base sequence.

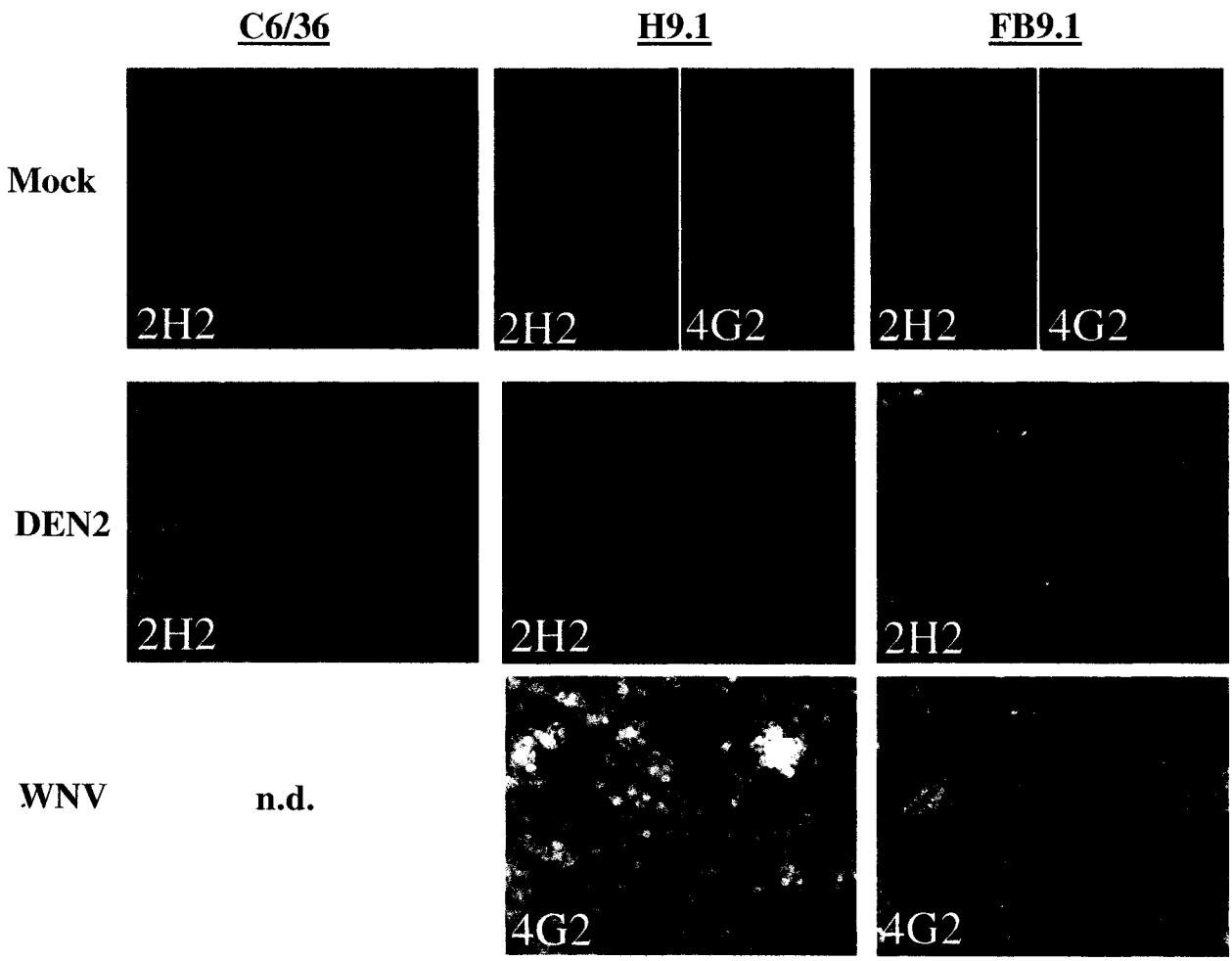
```
DEN3              1 -TTCCACTTAACTtCACGaGATGGAGAGCCGCGCATGATTGTGGGgAAGAATGAAAGAGG
DEN2-JAM1409      1 -TTCCATTTAACCACACGTAATGGAGAACCACACATGATCGTtGGTAGGcAaGAGAAAGG
WNV-NY99          1 gTTaCcCTCtcTaACtTcCAAgGGAAGGTgATG-ATGACgGTAAATGctAcTGAcGtcAc
consensus         1 -TTcCacTtaactaCacgtaAtGGAgagccacgcATGAttGTgggtaagaatGAaagagg

DEN3             57 AAAAtCCCTaCTtTTTAAGACAGCCtCTGGAATCAACATGTGCACaCTCATAGCCATGGA
DEN2-JAM1409     57 GAAAAgTCTTCTGTTCAAAACAGagGaTGGtGTTAACATGTGTACCCTCATGGCCATAGA
WNV-NY99         42 AGAtGTCaTCaCGaTTccAACAGCTGCTGGAAagAACcTATGCATTgTCAgAGCaATGGA
consensus        61 aaAaacccTtctgtTtaaaACAGccgcTGGaatcAACaTgTGcAcccTCAtaGCcATgGA

DEN3            112 TTTGGGAGAGATGTGTGATGACACGGTCACTTACAAATGCCCC---CaCaTTACCGAAGt
DEN2-JAM1409    112 TCTtGGtGAAtTGTGTGAaGATACAATCACgTACAAGTGTCCC---CTCCTCAggcAAAA
WNV-NY99         90 TgTGGGAtAcATGTGCGATGATACtATCACTTATGAATGCCCagtgCTgTCgGCTGGtAA
consensus       121 TtTgGGagAgaTGTGtGAtGAtACgaTCACtTAcaAaTGcCCc---Ctccttaccgaaaa

DEN3            166 gGAGCCtGAAGACATTGACTGTTGGTGCAACCTTACATCGACATGGGTGACTTATGGAAC
DEN2-JAM1409    161 TGAACCAGAAGACATaGATTGTTGGTGCAACTCTACGTCGACATGGGTAACTTATGGGAC
WNV-NY99        139 TGAtCCAGAAGACATCGACTGTTGGTGCAcaaagtCAgCAGTcTAcGTcAggTATGGAAg
consensus       181 tGAgCCaGAAGACATtGAcTGTTGGTGCAaccttaCatCgacaTggGTgActTATGGaAc

DEN3            224 ATGCAaTcAAGCtGGAGAGCATAGACGCGATAAGAGATCAGTGGCGTTAGCTCCcCATGT
DEN2-JAM1409    219 ATGTGCCAccACAGGAGAACACAGAaGaGAaAAAAGATCAGTGGCACTcGTTCCACATGT
WNV-NY99        185 ATGCACCAAGACAcGccAcTcaAGACGCAGTcGGAGGTCAcTGACAgTGcagaCACACGg
consensus       241 ATGcaccaaaaCagGagAgcatAGAcGcgataagAGaTCAgTGgCatTagctcCaCAtGt

DEN3            280 cGGCATGGGACTGGAcACACGcACTcAAACCTGGATGTCGGCTGAAGGAGCTTGGAGACA
DEN2-JAM1409    273 GGGTATGGGACTGGAGACACGAACTGAAACaTGGATGTCAtCaGAAGGGCCTGGAAACA
WNV-NY99        231 AGAaAgcActCTAGcGAacaAGAagGGGGCTTGGATGgacAgCAccAAGGCCacAAGGTA
consensus       301 gGgcAtgggaCTgGagAcacgaActgaaaCcTGGATGtcggctgaagggGCctggAgacA

DEN3            336 aGTCGAGAAGGTAGAGACATGGGCCCTtAGGCACCCAGGGTTTACCATAcTAGCc-CTAT
DEN2-JAM1409    330 TGTTcAGAGAATTtGAAACcTGGATCTTGAGACATCCAGGcTTTACCATAATGGCA-gCAa
WNV-NY99        272 TtTgGtAAAAACAGAAtCATGGATCTTGAGGaACCCtGGATaTGCCcTGGTGGCAgCCGT
consensus       361 tgTcgagAaaataGAaaCaTGGatCtTgAGgcAcCCaGGgTtTaCCaTaaTgGCa-ccat

DEN3            391 TTCTtGCCCATTACATAGGcACttCcTTgACCCAGAAAGTgGTtATTTTTATACTATTAA
DEN2-JAM1409    383 TCCTGGCaTACacCATAGGAACGACACattTCCAAAGGGCCtTGATTTTCATcTTACTGA
WNV-NY99        323 CaTTGGTTgGaTGCtTGGGAGCAACACC-ATgCAGAGAGTTGTGtTTgTCGTGCTATTGc
consensus       421 ttcTgGcccattaCaTaGGaaCgaCact-atcCAgAgaGtcgTgaTTtTcaTacTAtTga

DEN3            443 TGCTGGTTACCCCATCC-ATGACA
DEN2-JAM1409    435 CAgCtGTCGCTCCtTCa-ATGACA
WNV-NY99        374 TtTTGGTgGCCCCAgCTtACAGC-
consensus       481 tgctgGTtgCcCCatCc-AtgaCa
```

The RNAi response directed against DEN-2 in the FB9.1 cells also extended to DEN-3 (Figures 2.9 and 2.10). Since RNAi is a sequence specific phenomenon and inhibition of virus replication due to prM protein expression had been ruled out (Figure 2.12, upper ad middle panel), these cells were tested by infection with a more distantly related flavivirus. West Nile virus, New York 1999 isolate, (WNV) was used for infection (MOI of 0.01). The alignment of the prM and M regions from DEN-2, DEN-3 and WNV demonstrate that DEN-2 and DEN-3 share short stretches of sequence homology (Figure 2.11) that may account for the cross-reactive RNAi response to both DEN-2 and DEN-3. There is also similarity between DEN-2 and WNV in this region, although not as much as between DEN-2 and DEN-3 (Figure 2.11).

At 7 days post infection, both FB9.1 cells and the control H9.1 cells contained significant amounts of WNV antigen as indicated by staining with the flavivirus-complex envelope specific antibody 4G2 (See Figure 2.12, lower panel). These data show WNV replicated in both cell types and that the effect of the anti-DEN-2 RNAi did not extend to WNV. This further supports the notion that the replication block in the FB9.1 cells is a specific RNAi response based on the DEN-2 dsRNA expressed within these cells.

Figure 2.12. FB9.1 cells do not express the prM protein prior to challenge with a DEN virus and are not resistant to WNV challenge. Cells were infected at an MOI of 0.01 with either DEN-2, WNV or mock infected. At 7 days post infection they were assayed for either DEN-2 prM antigen using the prM specific 2H2 antibody or envelope antigen using the flavivirus E specific 4G2 antibody.

n.d. not determined.



Double-subgenomic SIN replication assay. A dsSIN virus carrying the prM sequence from DEN-2 should be sensitive to targeting by the anti-DEN-2 prM RNAi response demonstrated in the FB9.1 cells (Adelman et al., 2002). SIN viruses replicate faster than DEN viruses in mosquitoes and mosquito cell culture, therefore earlier time points (hours post infection for SIN and days post infection for DEN) are necessary to observe the kinetics of their replication. This difference in kinetics is important in designing assays in order to rapidly screen for resistance to DEN-2. The replication of 5'dsMRE16-eGFP/D2prM may be able to function as a surrogate for DEN-2 replication in replication assays when RNAi is initiated DEN-2. In the next chapter, transgenic mosquitoes are tested for their ability to block replication of DEN-2 and using a dsSIN construct would allow for more rapid screening for inhibition of viral replication.

In order to determine if the DEN-2 prM sequence is sensitive to RNAi when it is presented in the context of dsSIN replication, the replication of a dsSIN with an insert encoding the visual marker gene GFP fused to the prM region of DEN-2, 5'dsMRE16-eGFP/D2prM, was compared to the replication of the parental virus with only the GFP marker gene inserted (see Table 2.2 for description of the virus and Figures 2.13 and 2.14). FB9.1 and H9.1 cells were infected at two different MOIs, 0.01 and 0.1, with each virus separately, and virus replication was followed by visually inspecting the cells for GFP expression at 3 and 5 days post infection. Figure 2.13 shows representative images of the replication, by GFP expression, of both viruses in control H9.1 cells (part A) and DEN-2 silenced FB9.1 cells (part B).

Both dsMRE16 viruses replicated well at both the lower and higher MOI in the control H9.1 cells, although the virus with the eGFP-prM insert appeared to replicate

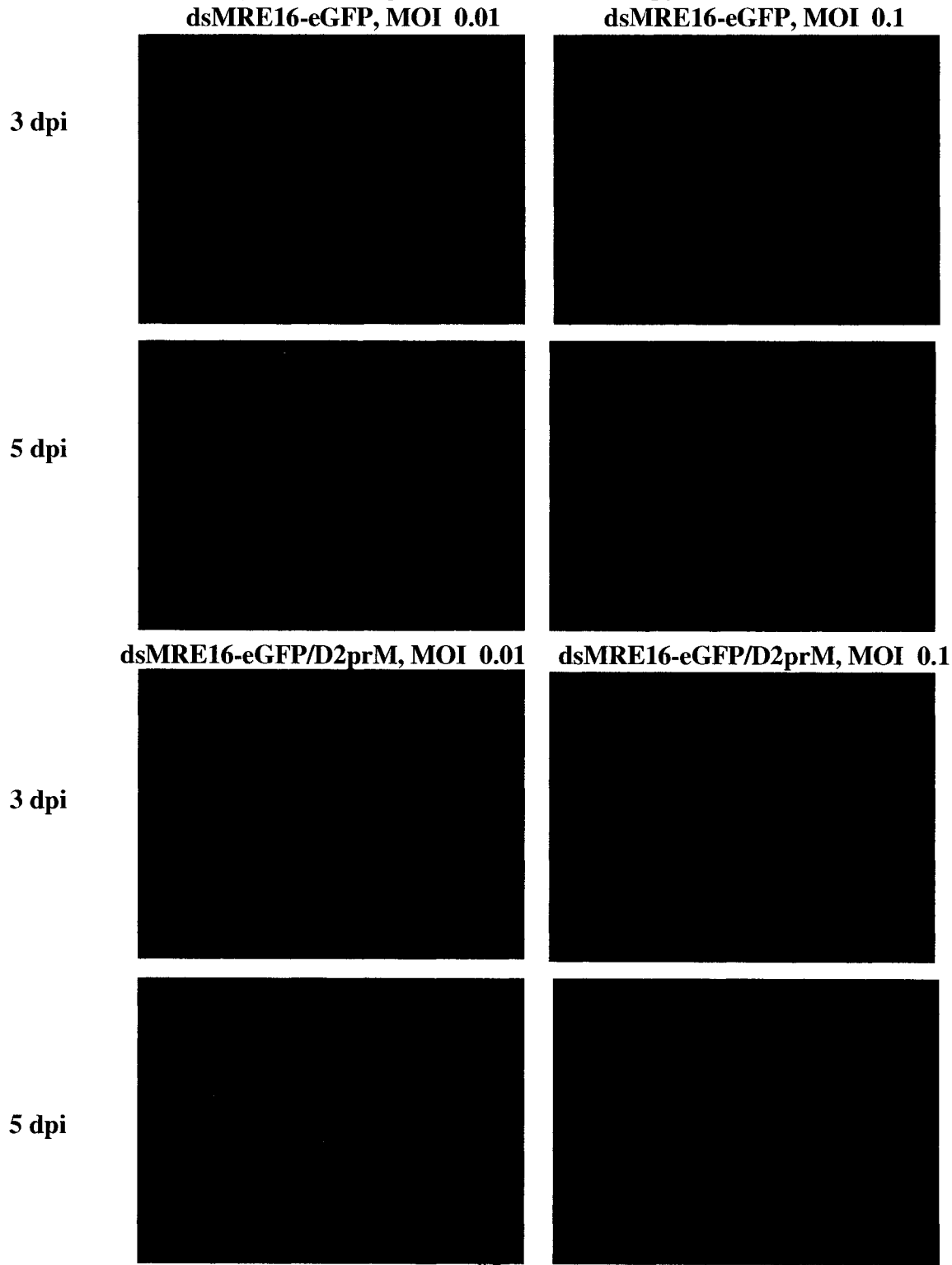
more slowly than the virus with the eGFP insert alone, perhaps due to the increased sequence length. The dsMRE16-eGFP virus replicated in the FB9.1 cells at a lower rate than in the H9.1 cells; however, there was little or no replication of the dsMRE16-eGFP/D2prM virus as demonstrated by observation of GFP in the infected cells.

Medium was removed from these infected cells at 9 days post infection and titrated to examine production of infectious virus in each cell type. Figure 2.14 shows the titer of each virus obtained from the two cell types. The dsMRE16-eGFP virus grew to similar titers in both the FB9.1 and H9.1 cells for both MOIs tested. Infection at an MOI of 0.01 resulted in 4 x 10^6 PFU/mL in H9.1 cells and 4.5 x 10^6 PFU/mL in FB9.1 cells and infection with an MOI of 0.1 yielded 3.5 x 10^7 PFU/mL in H9.1 cells and 4 x 10^6 PFU/mL in FB91 cells. Importantly, the level of dsMRE16-eGFP/D2prM produced by the FB9.1 cells was significantly ($\geq$ 2 logs) lower than that produced by the H9.1 cells. Specifically, the FB9.1 cells produced 2.5 x 10^5 PFU/mL (MOI 0.01) or 3.5 x 10^5 PFU/mL (MOI 0.1) while the H9.1 cells generated significantly more virus, 2 x 10^8 PFU/mL (MOI 0.01) and 3.5 x 10^7 PFU/mL (MOI 0.1).

These data indicate that the prM sequence in the dsMRE16-eGFP/D2prm virus genome is a target of the RNAi maintained in the FB9.1 cells by expression of the hairpin DEN-2 prM RNA.

Figure 2.13. FB9.1 cells do not replicate dsSIN with a DEN-2 prM genome insert. FB9.1 and H9.1 cells were infected with dsMRE16-eGFP or dsMRE16-eGFP/D2prM and examined for GFP expression at 3 and 5 dpi.

A. H9.1 cells (Photographed using fluorescence microscopy).



B. FB9.1 Cells (Photographed using visible light and/or fluorescence), arrows indicate GFP positive cells.

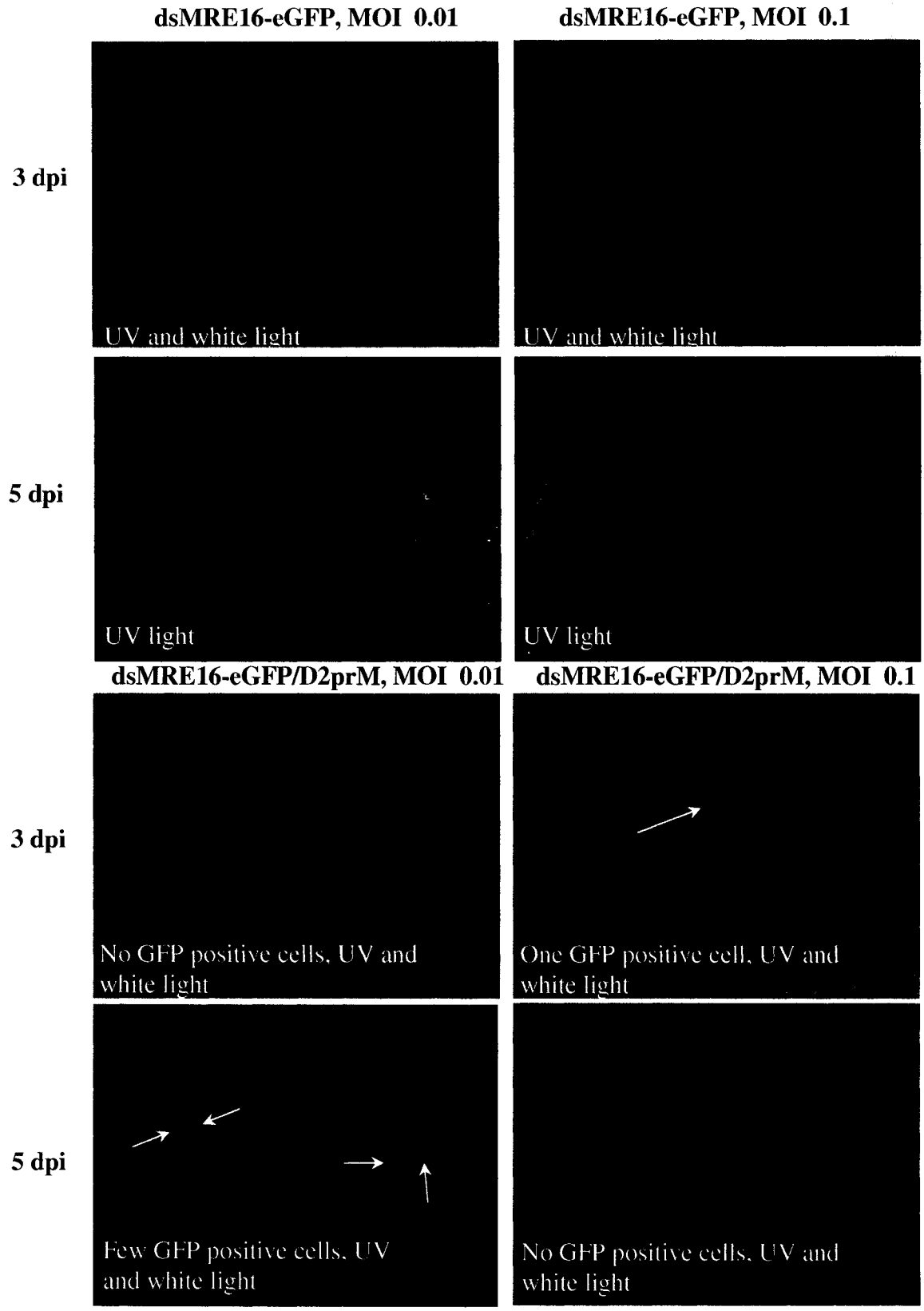
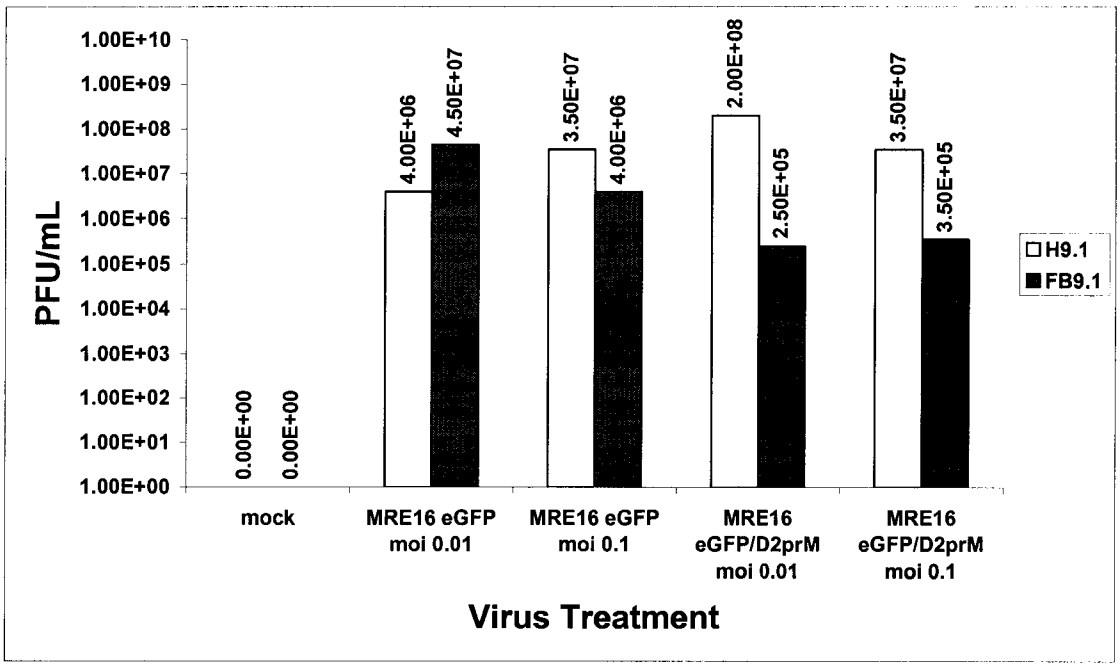


Figure 2.14. Production of dsSIN with DEN-2 prM sequence genome insert is reduced in FB9.1 cells. Medium from cell cultures shown in Figure 2.13 was harvested and titrated by plaque assay at 9 days post infection. Titers of virus generated in the H9.1 cells were compared to titers from the FB9.1 cells. Each bar represents a single replicate for each cell type and infecting virus.



IV. RNAi induction by transfected siRNAs.

Induction of RNAi by transfection of synthetic siRNA. The experiments described above, including the inhibition of dsSIN with a DEN-2 prM genome insert in FB9.1 cells, indicate that DEN-2 virus is susceptible to RNAi in mosquito cells. In other to confirm this hypothesis, experiments to dissect the RNAi pathway within these cells were performed. The dsRNA trigger of RNAi has been shown using hairpin RNA and viral replicative intermediates. The next step was to demonstrate that the trigger could be by-passed by the direct addition of the effector molecules, siRNAs.

Transfection of a commercially prepared anti-DEN-2 siRNA into C6/36 cells resulted in interference with DEN-2 replication in those cells (See Figures 2.15 lanes 8-10, 2.16A, and 2.17). The sequence of the siRNA was designed from nucleotides 204 to 226 in the DEN-2 prM gene (119 to 221 in the DEN-3 prM gene) where 22 of 23 bases are identical between DEN-2 and DEN-3 (See Figure 2.11, boxed region). Cells that were transfected with a control siRNA directed against luciferase or treated with transfection reagent alone were not resistant to DEN-2 infection (Figure 2.15, lanes 1-7, Figure 2.16B-C, Figure 2.17).

Total RNA extracted from siRNA-transfected cells at 3, 5, and 7 days post challenge was subjected to northern blot analysis. The blot was hybridized using a ^{32}P-labeled riboprobe corresponding to the DEN-2 RNA prM genomic region. Over time, concentration of DEN-2 viral RNA increased in cells treated only with transfection reagent and in cells transfected with the luciferase-specific siRNA 48 hours prior to infection (See Figure 2.15). The amount of viral RNA present in the cells that were

transfected with the anti-DEN-2 siRNA was significantly decreased at all three time points examined (see Figure 2.15).

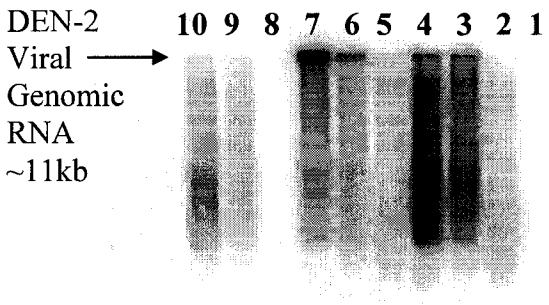
Viral antigen was not detected in cells transfected with anti-DEN-2 siRNA 48 hours prior to DEN-2 viral challenge. Cells from 3-, 5- and 7- days post challenge from each transfection treatment group were assayed for DEN-2 antigen by fluorescent staining using the DEN-2-specific monoclonal antibody 3H5. Figure 2.16 shows the results of this staining. Part D shows the mock-infected cells treated with transfection reagent but no siRNAs. Part C demonstrates the increase in viral antigen over time in cells that were not siRNA transfected. The control siRNA transfected cells, shown in part B of the same figure, also expressed increasing viral antigen over time. The amount of viral antigen present in the control siRNA transfected cells was less than what was observed in the un-transfected controls. This may be due in part to the upregulation of components of the RNAi pathway in response to the presence of any siRNA. Finally, the anti-DEN-2 siRNA transfected cells shown in part A did not express DEN-2 antigen, indicating that DEN-2 replication was inhibited in these cells. Interestingly, the RNAi response appears to be somewhat cytopathic in these cells as the cells receiving both the anti-dengue siRNA and the control siRNA demonstrate CPE upon infection with DEN-2. These data correlate with the northern blot analysis of RNA expression shown in Figure 2.15.

At each time point examined above by immunofluorescent staining, cell culture medium was titrated by plaque assay to examine virus production in the transfected cells. The data, shown in Figure 2.17, correlated with RNA and protein expression as shown by northern analysis and antigen staining. No virus was produced in the mock-infected

controls. No virus was detected in the 3 dpi samples (data not shown), perhaps because this time point was simply too early in the infection process to detect newly formed virus. The 3 dpi time point samples were also negative by antigen staining, indicating that there was little or no viral antigen being produced at this time. However, the northern blot was positive indicating the presence of viral RNA. The 5 dpi and 7 dpi time point samples both contained infectious virus in the medium. The data represent the combined results from two trials, with three replicates per treatment per trial. At day 5 post infection, the un-transfected cells had an average titer of 1.43×10^3 PFU/mL, which increased to 2.33×10^5 PFU/ml by 7 days post infection. Cells transfected with the control siRNA had 2.06×10^2 PFU/mL of virus in their cell culture supernatant at 5 dpi and 1.27×10^5 PFU/ml by 7 dpi. The titer in the anti-DEN2 siRNA transfected cells remained low, 70 PFU/ml at 5dpi and 8.45×10^2 PFU/ml at 7dpi.

These results demonstrate that the initiation step in the RNAi pathway, the dsRNA trigger, can be by-passed by the direct addition of the effector molecule, siRNA, in mosquito cell culture. This means that the steps needed to recognize dsRNA and cleave it into the 21-23 nt effector siRNAs that were demonstrated to function in RNAi in mosquito cell culture by the earlier lysate, FB9.1 cell, and dsSIN replication experiments are not required in order for downstream activities. In other words, RNAi does not be initiated in order for the effectors to function. Direct addition of DEN-2 siRNA blocked DEN-2 replication without need for longer DEN-2 specific siRNA. This may have a role in a potential therapeutic approach that uses anti-DEN-2 siRNAs to control an active DEN-2 infection and this could be tested in cell culture by varying the timing of siRNA transfection and DEN-2 challenge.

Figure 2.15. Transfection of C6/36 cells with DEN-2 specific siRNAs blocks DEN-2 viral RNA accumulation after virus infection. Cells were transfected with control siRNA, anti-DEN2 siRNA or no siRNA and challenged with DEN-2 at an MOI of 0.01 48 hours post transfection. Total RNA was harvested from cells at 3, 5, and 7 days post challenges, subjected to northern blot analysis with a DEN-2 prM riboprobe.

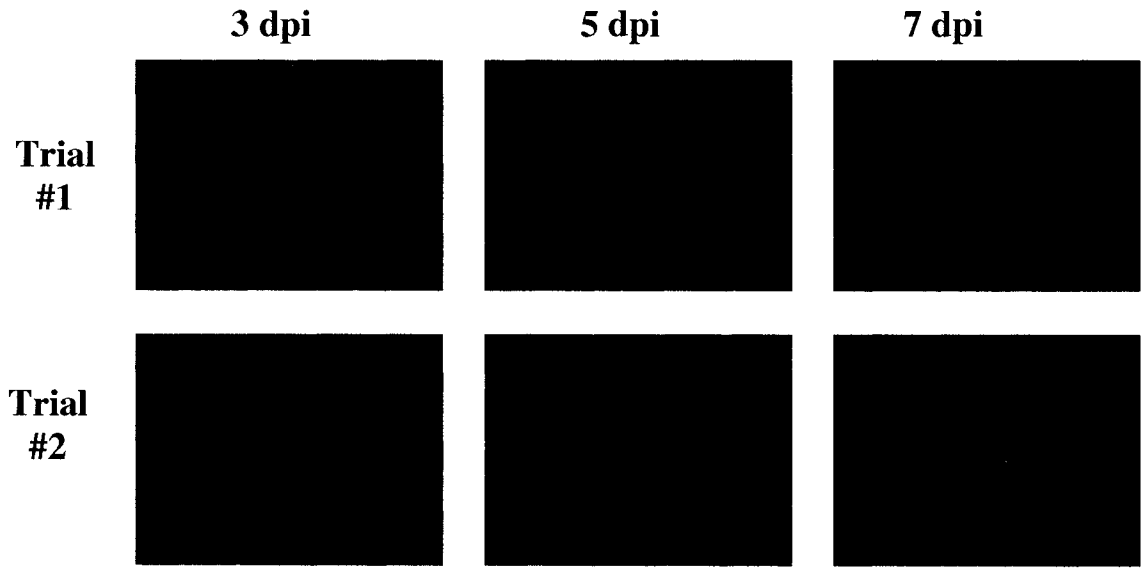


Sample order:

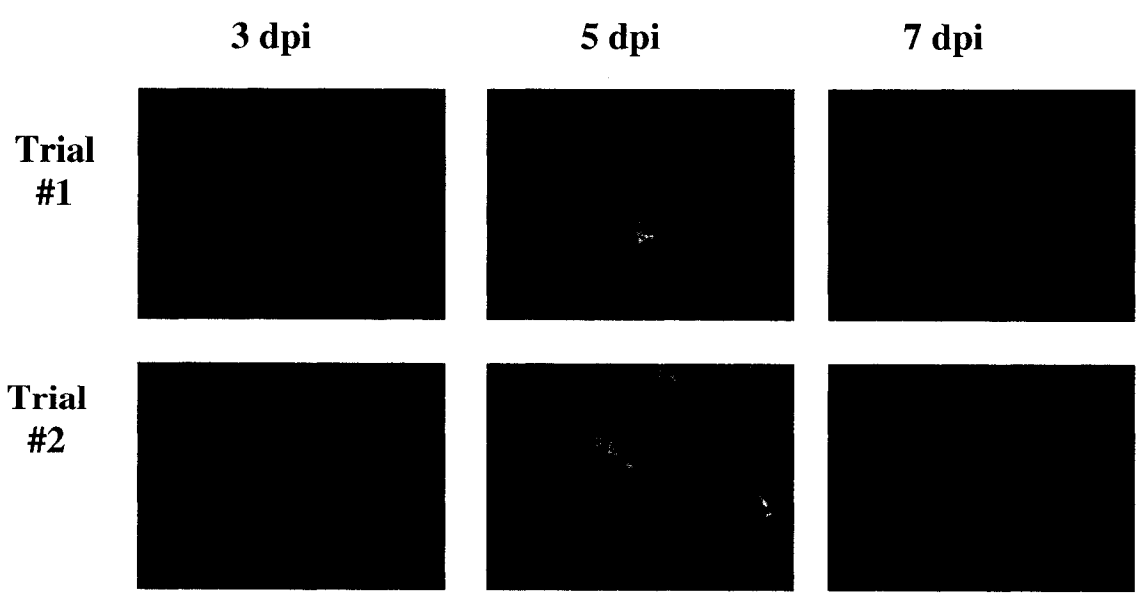
1. No siRNA, Mock Infected.
2. No siRNA, DEN-2 3dpi.
3. No siRNA, DEN-2 5dpi.
4. No siRNA, DEN-2 7dpi.
5. Control siRNA, DEN-2 3dpi
6. Control siRNA, DEN-2 5dpi
7. Control siRNA, DEN-2 7dpi
8. Anti-DEN2 siRNA, DEN-2 3dpi
9. Anti-DEN2 siRNA, DEN-2 5dpi
10. Anti-DEN2 siRNA, DEN-2 7dpi

Figure 2.16. DEN-2 virus antigen accumulation is inhibited by transfection of anti-DEN-2 synthetic siRNAs in C6/36 cells. Cells were transfected with control siRNA, anti-DEN-2 siRNA or no siRNA and challenged with DEN-2 at an MOI of 0.01 48 hours post transfection. Cells were fixed and stained for DEN2 antigen at 3, 5, and 7 days post challenge.

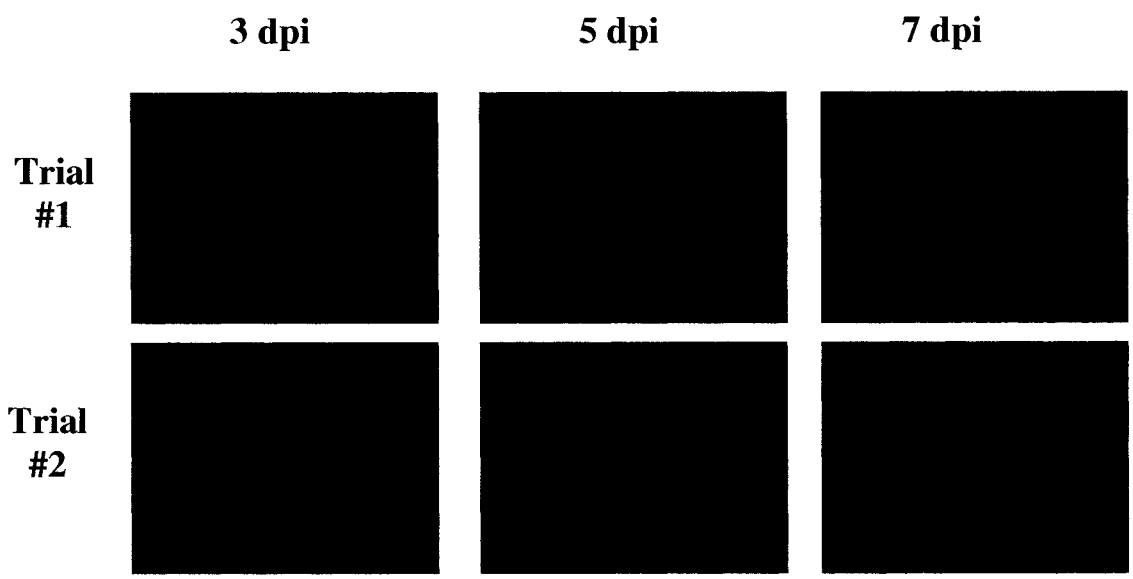
A. Anti-DEN-2 siRNA, DEN-2 Infected.



B. Control siRNA, DEN-2 Infected.



C. No siRNA, DEN-2 Infected.



D. No siRNA, Mock Infected.

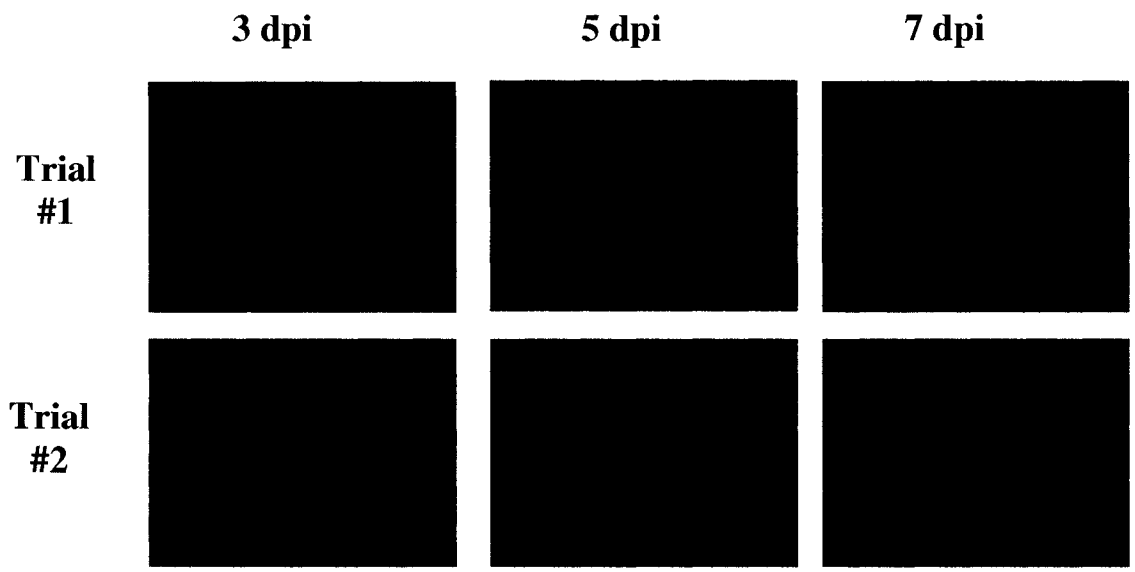
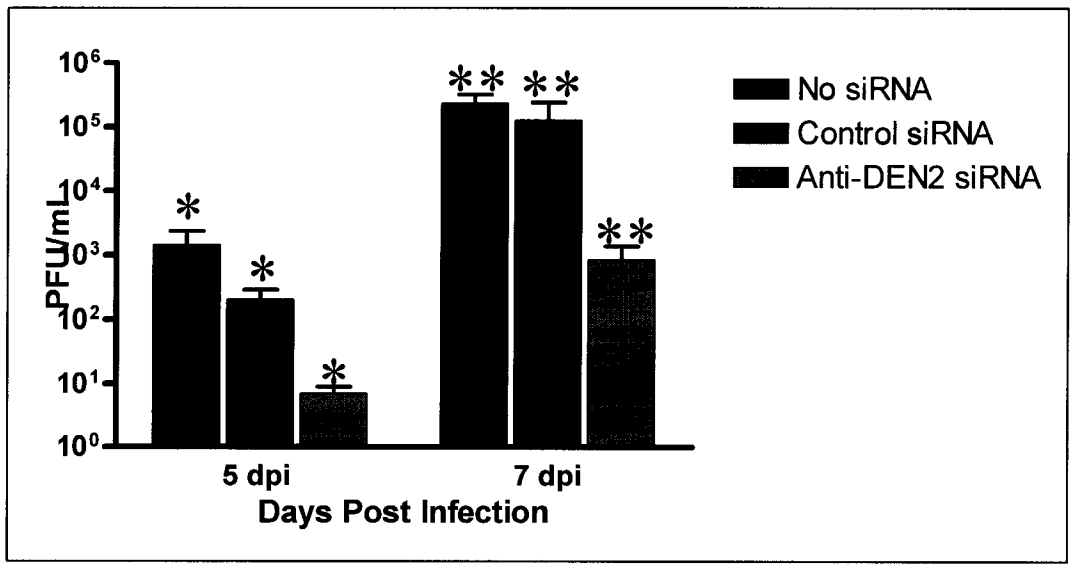


Figure 2.17. DEN-2 virus production following siRNA transfection of C6/36 cells. Cells were transfected with control siRNA, anti-DEN-2 siRNA or no siRNA and challenged with DEN-2 at an MOI of 0.01 48 hours post transfection. Cell culture supernatants were collected at 3, 5, and 7 days post challenge and subjected to plaque assay to determine virus production (Titer is expressed as PFU/mL). The mock infected and 3dpi samples did not produce any plaques. Combined data from two trials with triplicate samples per trial. *P=0.0001, **P=0.0017 One-way ANOVA Friedman test (Prism 4 GraphPad Software, Inc.)



Induction of RNAi by transfection of pooled siRNAs. In a natural infection, any accessible viral RNA represents a potential target for RNAi. In order to more closely approximate the RNAi response in a natural infection, we investigated the total pools of siRNAs that could be generated from the DEN-2 M protein coding sequence by the Dicer enzyme. Pools of siRNAs were generated *in vitro* from 438 bp dsRNA corresponding to the Mnp sequence using the recombinant human dicer enzyme as described above in the methods section. Each pool represents all possible siRNA that can be generated by dicer from dsRNA corresponding to Mnp region (M d-siRNAs) or a control GFP mRNA transcript provided along with the enzyme kit (GFP d-siRNAs). Each of these siRNA pools was transfected into C6/36 cells that were challenged with DEN-2 48 hours later. The cells were assayed for DEN-2 replication by northern blot analysis for the accumulation of DEN-2 viral RNA at 2, 4, and 6 days post infection (See Figure 2.18). When the transfection was performed with the M d-siRNA pool in the presence of the transfection reagent, there was little or no detectable DEN-2 viral RNA present, but when transfection reagent was not used the virus was able to replicate normally. The GFP d-siRNA pool did not alter viral replication. Interestingly, there were breakdown products detectable in the total RNA from cells transfected with the M d-siRNA pool in the presence of the transfection reagent prior to DEN-2 challenge. Quantification of the 6 dpi time point demonstrates that the d-siRNA generated *in vitro* was similarly capable of generating RNAi against DEN-2 virus as the commercially prepared siRNAs examined earlier (See Figure 2.19).

Figure 2.18. DEN-2 viral RNA accumulation decreases after transfection with pooled d-siRNA derived from DEN-2 M dsRNA. C6/36 cells were transfected with pooled d-siRNAs prepared by digestion of DEN-2 M dsRNA or GFP dsRNA with human Dicer and challenged with DEN-2 (MOI 0.01) 48 hours later. Total RNA from 2, 4, and 6 days post infection was subjected to northern blot analysis with a ^{32}P labeled DEN-2 prM riboprobe. Lane: 1. Cells transfected with 1 μg M d-siRNAs + transfection reagent 2. Cells transfected with 500 ng M d-siRNAs + transfection reagent 3. Cells exposed to 1 μg M d-siRNAs, no transfection reagent 4. Cells not d-siRNAs 5. 1 μg GFP d-siRNAs + transfection reagent 6. Cells not exposed to d-siRNAs, mock infected

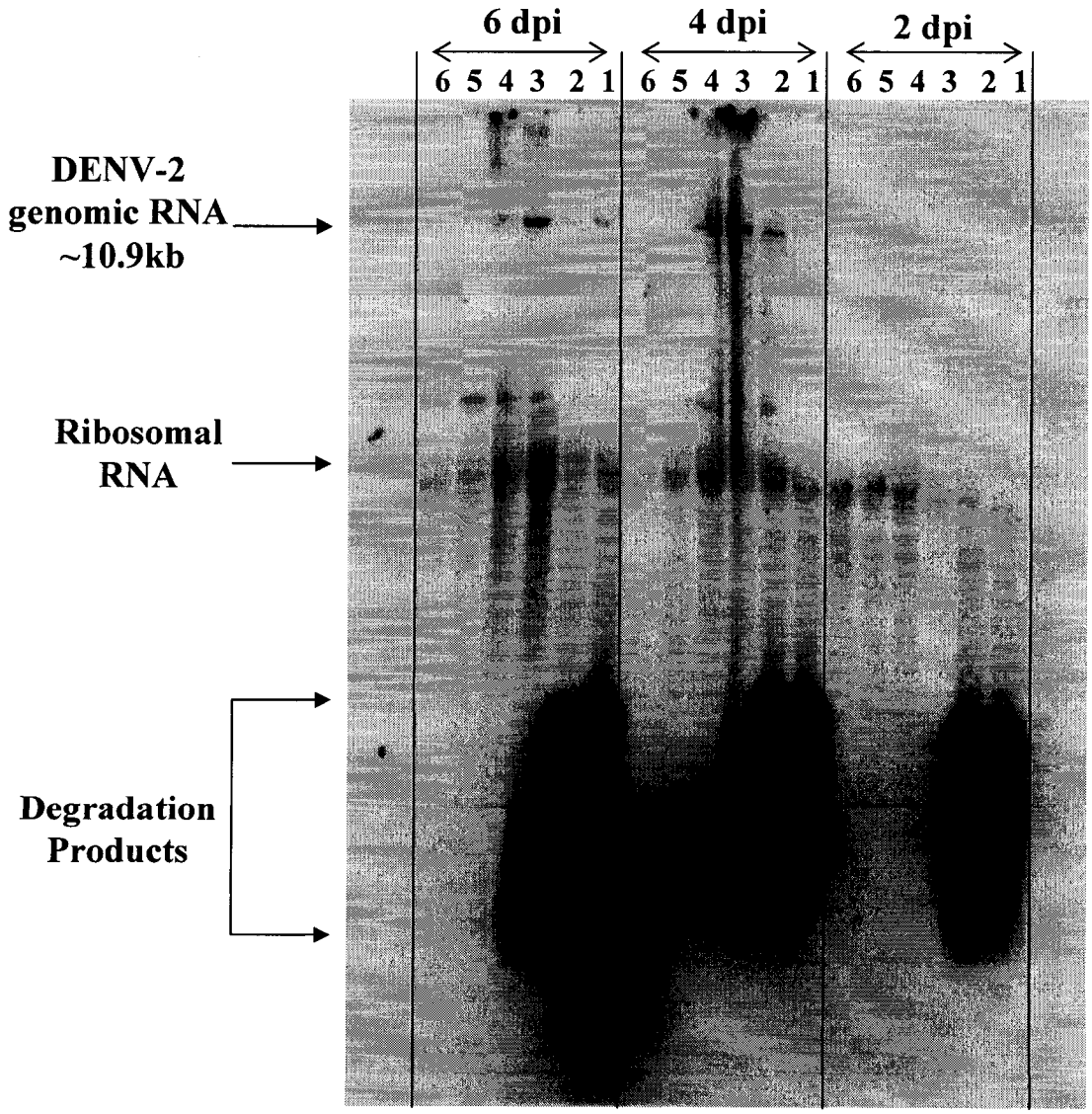
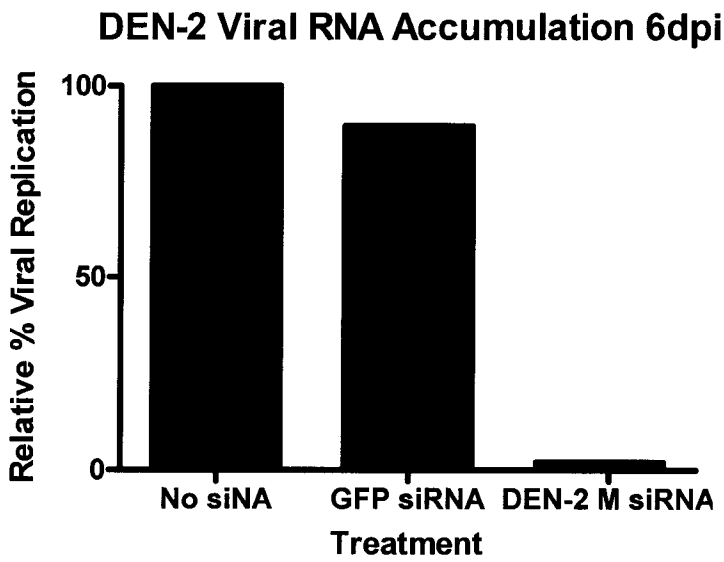


Figure 2.19. DEN-2 viral RNA accumulation decreases after DEN-2 M pooled d-siRNA transfection: Quantitation of signal intensity. The 6 dpi time point on the Northern Blot in Figure 2.18 was subjected to quantitation of signal intensity (ImageQuant). Results are displayed graphically as percentage of maximal viral RNA replication relative to the no siRNA treatment control (set at 100%).



V. Cloning of siRNAs from FB9.1 and C6/36 cells.

In order to examine the result of RNAi against DEN viruses in mosquito cells, the end point of the RNAi pathway, the degradation products, were examined. The pool of low molecular weight RNAs, including the siRNAs, was isolated and cDNAs were cloned from FB9.1 and C6/36 cells that were either mock infected or infected with DEN-2 or DEN-3. Samples from 3, 7, 10, and 14 days post infection (dpi) were used from each cell type. This approach was designed to include endogenous small RNAs resulting from normal gene expression in cultured C6/36 cells as well as any siRNAs produced in response to transformation or dengue virus replication in these cells. In addition to endogenous mosquito siRNAs, the uninfected FB9.1 cells should also contain DEN-2 prM specific siRNAs resulting from the RNAi pathway processing of the hairpin dsRNA expressed in these cells. Finally, DEN-2 and DEN-3 infection is predicted to result in siRNA generated by the cellular response to DEN virus infection.

Over 280 cDNA segments in clones from the various samples described above were sequenced and analyzed for siRNAs by comparison to the genome database (GenBank). Only two clones (113 and 116) were found to contain DEN-2 RNA sequences. They both contained the same DEN-2 prM sequence, but were determined to be independant clones because of surrounding sequence differences. Both of these clones were from FB9.1 cells at 3 days post infection with DEN-2. The sequence of both clones corresponds to bases 443-466 of DEN-2 DNA, within the prM region.

There were a number of cloned cDNA sequences that are significantly larger than a 21- to 23- bp siRNA; the longer sequences range from 40 to 300 bp. These are most likely the result of carry over in the extraction process or contamination during excision

from gels. Also, there were a number of matches to Bunyavirus sequences, particularly La Crosse virus (LAC; *Bunyavirus*, Bunyaviridae), which most likely represent a contamination of either DEN virus stock, cell culture stock or biosafety cabinets used for cell culture manipulation. LAC virus was not used during this research, but could have caused undetected persistent infection of the cell cultures used, as this virus does not cause any CPE in C6/36 cells. All LAC sequences were obtained from cells infected with DEN-3, indicating that the most likely source of the contamination was from the stock of DEN-3 used in these experiments. This could have been confirmed by immunofluorescence assay for LAC virus antigen in the DEN-3 infected cells, but this was not performed since the sequences were obtained after all live cell cultures used had been disposed of.

Interestingly, all LAC sequences obtained ranged between 21- and 23- bp in length, the size expected for siRNA. There are a number of potential hypotheses for the presence of siRNA sized LAC RNA within these cells. First, the genome of LAC virus is coated with nucleocapsid protein for much of its life cycle, thereby limiting exposure of the viral RNA to host defenses, including Dicer. The small non-structural protein NSs of bunyaviruses has been implicated in perturbing the RNAi pathway. NSs from LAC virus has been shown to counteract the effects of siRNAs (Soldan et al., 2005) and NSs from tomato spotted wilt virus (*Tospovirus*, Bunyaviridae) has been shown to suppress RNAi (Takeda et al., 2002; Bucher et al., 2003). Based on these published studies and the observation of LAC virus specific siRNA sized sequences in these studies it is possible to hypothesize that there is a relationship between the RNAi pathway and LAC.

Tables 2.4-2.16 contain a summary of all the sequences analyzed that showed matches to sequences in GenBank that are considered significant. Blast searches with short sequences often do not result in matches that are by definition significant (high E value) but all matches to either virus or mosquito sequences were recorded. Many matches returned in the Blast searches were stretches of one or two nucleotides; these data are not included in the summary tables because they are unlikely to be true sequences and may be artifacts from the sequencing process. Sequences that matched to interesting targets, including known mosquito and viral sequences, are indicated in the notes column. Any sequence with a match within the expected size range of siRNAs is denoted as a potential siRNA sequence based solely on its size and the sequence of the potential siRNA from the clone is given in Table 2.17. Some samples (C6/36 cells with DEN-2 at 10 dpi, C6/36 cells with DEN-3 at 3 dpi, FB9.1 cells with DEN-2 at 10dpi, FB9.1 cells with DEN-3 at 3dpi and 10dpi) did not result in any sequences, presumably due to the low abundance of small RNAs and the possibility of incomplete sample recovery at each step in the cloning.

The siRNA cloning results tables are organized as follows: Part A of each table contains sequences within the expected size range of siRNAs (≤40 bases) and part B of each table contains sequences larger than expected for siRNAs. Sequences not described in the following tables resulted in no significant matches upon analysis and GenBank searches.

C6/36: mock (Table 2.4)

DEN-2 3dpi (Table 2.5)

7dpi (Table 2.6)

10dpi (No clones obtained)

14dpi (Table 2.7)

DEN-3 3dpi (No clones obtained)

7dpi (Table 2.8)

10dpi (Table 2.9)

14dpi (Table 2.10)

FB9.1: mock (Table 2.11)

DEN-2 3dpi (Table 2.12)

7dpi (Table 2.13)

10dpi (No clones obtained)

14dpi (Table 2.14)

DEN-3 3dpi (No clones obtained)

7dpi (Table 2.15)

10dpi (No clones obtained)

14dpi (Table 2.16)

Table 2.4. Results of siRNA cloning from mock infected C6/36 cells.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
57	Human DNA clone (AL161430.19)	22/22	**Potential siRNA**
86	*Ae. aegypti* 35707 unknown mRNA (AY431561.1)	20/21	Mosquito sequence **Potential siRNA**
99	*Mus musculus* chromosome 18 (AC101740.7)	18/18	
100	Bovine herpesvirus 2 major capsid protein (AY3557736.1)	17/17	Virus sequence
105	*Rickettsia prowazekii* (RPXX02)	18/18	
110	*Mus musculus* BAC clone (AC129219.3); *An. gambiae* (XM_316967.1)	24/25	Mosquito sequence
204	*Homo sapiens* AW nonfunctional immunoglobulin (AY640495.1)	37/37	
218	*Zea mays* CL5575_3 (AY109346.1)	17/17	

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
58	*An. bwambae* cytochrome oxidase I, mitochondrial product (AF222328.1)	109/129	Mosquito sequence
84	*Ae. aegypti* cytosolic large ribosomal mRNA (AY431452.1)	245/270	Mosquito sequence
202, 207	*Bos taurus* LN1 mRNA (AFO11925)	68/71	
203, 209	*Rattus norvigicus* cnt-1 (RNO566901)	57/58	
205	*Chionodraco hamatus* partial MT gene for metallothionein-I (CHA308478)	57/58	
214	*Escllonia sp.* Oxelman largest subunit rpb2 (ESC557244)	57/59	
223	*Vipera aspis aspis* ammodytin I1 (VAS580157)	39/44	

Table 2.5. Results of siRNA cloning from C6/36 cells infected with DEN-2, 3dpi. A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
1	*Mus Musclus* synaptotagmin-like homolog (NM_176846.2)	21/22	**Potential siRNA**
2	Human DNA clone (HSJ300G12)	19/19	
4	*Homo sapiens* BAC clone (AC012593.8)	21/22	**Potential siRNA**
7	Kashmir bee virus, *Cripavirus*, Dicistroviridae (AY452696.1)	15/15	Viral sequence
8	Mouse DNA clone (AL844591.6)	21/21	**Potential siRNA**
9	*Anopheles gambiae* clone (CNS0Z57)	24/24	Mosquito sequence
11	Broad bean necrosis virus gene for triple-gene-block (contains RNA helicase domain) (D86638.1)	22/22	Viral sequence
12	*C. elegans* cosmid (CEF17E5)	19/19	
16	*Lotus corniculatus* DNA clone (AP005564.1)	18/18	
17	*D. melanogaster* (AE003710.2; AC010122; CG6624) *An. gambiae* (XM_308194.1) sequences associated with transposable elements	21/21	**Potential siRNA** Mosquito sequence
18	*Mus musculus* BAC clone (AC126940.4)	19/19	
19	*Mus musculus* BAC clone (AC123844.4)	19/19	
21	*An. gambiae* (XM_308385.1)	19/19	Mosquito sequence
25	*Yarrowia lipolytica* CllB99 (X_504422.1)	15/15	
27	*An. gambiae* DNA from 2R (AGA439060)	21/21	Mosquito sequence **Potential siRNA**
29	*An. gambiae* (XM_312199.1)	32/34	Mosquito sequence
31	*Mus musculus* similar to marapsin 2 (XM_137602.2); *An gambiae* (CNS08Z57)	21/21	**Potential siRNA** Mosquito sequence
32	Mouse DNA clone (AL731800.11)	19/20	
34	*Homo sapiens* lin-10 protein homolog (NM_025187.3)	20/21	**Potential siRNA or miRNA**

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
24	*An gambiae* (XM_320687.1)	72/88	Mosquito sequence
22	*Ae. aegypti* cytosolic large ribosomal mRNA (AY431452.1)	273/289	Mosquito sequence
28	*Ae. aegypti* cytosolic large ribosomal subunit L5 (AY431452.1)	273/288	Mosquito sequence
30	*Ae. aegypti* cytosolic large ribosomal mRNA (AY431452.1)	273/289	Mosquito sequence
35	*Ae. aegypti* cytosolic large ribosomal mRNA (AY431452.1)	269/289	Mosquito sequence

Table 2.6. Results of siRNA cloning from C6/36 cells infected with DEN-2, 7dpi.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
39	*Kluyveromyces lactis* NRRL Y-1140 (XM_453635.1)	18/18	
40	Zebrafish DNA clone (AL831758.22)	20/20	
42, 43	Soybean glycine max (AC121763.1)	16/16	
44	Homo sapiens BAC (AC005911.6)	16/17	
51	Zebrafish novel gene similar to TFPI (Tissue factor pathway inhibitor, lipoprotein-associated coagulation inhibitor) and a CpG island (AL732481.4)	21/21	**Potential siRNA**

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
52	*Ae. aegypti* cytosolic large ribosomal mRNA (AY431452.1)	245/270	Mosquito sequence

Table 2.7. Results of siRNA cloning from C6/36 cells infected with DEN-2, 14 dpi.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
66	*An. gambiae* (XM_312199.1)	33/34	Mosquito sequence
67	*An. gambiae* (XM_312199.1)	32/34	Mosquito sequence
68	Rhizobium ATP sulfurylase subunit (RBU59507)	23/23	
81	*An. gambiae* (CNS08WNK)	20/20	Mosquito sequence **Potential siRNA**
82	*Mus musculus* similar to marapsin 2 (XM_137602.2)	21/21	**Potential siRNA**
88	*Lagothrix lagotricha* Alu insertion locus (AY620716.1)	23/24	**Potential siRNA**
94	*Ae. aegypti* mRNA for GATA transcription factor (AAE400338)	18/18	Mosquito sequence
97	*Homo sapiens* clone (AC009554.14)	18/18	
103	*Mus musculus* clone (AC129313.4)	17/17	
109	*An. gambiae* clone (CNS09MRB)	20/21	Mosquito sequence **Potential siRNA**

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
76	*An. gambiae* (XM_309483.1)	65/78	Mosquito sequence
92	*An. gambiae* (XM_312199.1)	79/82	Mosquito sequence
93	*An. gambiae* (XM_312199.1)	73/85	Mosquito sequence

Table 2.8. Results of siRNA cloning from C6/36 cells infected with DEN-3, 7 dpi. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
261	*Homo sapiens* (AC006003.4)	18/18	
262	La Crosse Virus (AF528167.1)	23/23	Viral sequence **Potential siRNA**
263	*Homo sapiens* (AC006003.4) 18/18	18/18	

Table 2.9. Results of siRNA cloning from C6/36 cells infected with DEN-3, 10 dpi. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
264	*Homo sapiens* clone (HSM807534)	22/23	**Potential siRNA**
266	*D. melanogaster* (AE003774.4) 19/19	19/19	
267	La Crosse Virus (AF528167.1)	22/22	Viral sequence **Potential siRNA**
277	La Crosse Virus (AF528167.1)	22/22	Viral sequence **Potential siRNA**

Table 2.10. Results of siRNA cloning from C6/36 cells infected with DEN-3, 14 dpi. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
279	La Crosse Virus (AF528167.1)	22/22	Viral sequence **Potential siRNA**
281	La Crosse Virus (AF528167.1)	22/22	Viral sequence **Potential siRNA**
268	Zebrafish clone (BX072563.7)	19/20	
269	*Homo sapiens* clone (AC103726.5)	17/17	
270	*Kairi virus* (BKNNSA)	21/21	Viral sequence **Potential siRNA**
272	*Mus musculus* (AC115302.4)	17/17	
273	*D. yakuba* CG10911 (AF531949.1)	19/19	
274	*Homo sapiens* paraneolastic antigen MA1 (BC014926.2)	18/18	
275	*Homo sapiens* clone (AC006003.4)	18/18	
276	*Mus musculus* clone (AC136456.3)	18/18	
280	Human DNA part of C2H2 type zinc finger similar to *Drosophila* Scratch (HSDJ850E9)	22/23	**Potential siRNA**
282	*Homo sapiens* clone (AC087641.5)	18/18	

Table 2.11. Results of siRNA cloning from mock infected FB9.1 cells.

Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases
193	*Chionodraco hamatus* partial MT gene for metallothionein-I (CHA308478)	57/58
195	*Mus musculus* mRNA for suppressor of actin mutations (SAC1) (MMU245720)	56/62

Table 2.12. Results of siRNA cloning from FB9.1 cells infected with DEN-2, 3 dpi.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
111	Human DNA (AL6737.9)	18/18	
112	Human DNA (AL390959.12)	19/19	
113	**DEN2 JAM 1409 bases 443-466**	**23/24**	**Virus sequence siRNA**
116	**DEN2 JAM 1409 bases 443-466**	**24/24**	**Virus sequence siRNA**
120	*An. gambiae* (XM_371179.1)	16/17	Mosquito sequence

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
117	*Armigeres subalbatus* 43879 transcription factor mRNA (Ay439887.1)	83/101	Mosquito sequence
119	*Armigeres subalbatus* 43879 transcription factor mRNA (Ay439887.1)	93/105	Mosquito sequence

Table 2.13. Results of siRNA cloning from FB9.1 cells infected with DEN-2, 7 dpi.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
121	*An. gambiae* (XM_371179.1)	16/17	Mosquito sequence
126	*An. gambiae* (XM_314962.1)	16/16	Mosquito sequence

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
122	*Armigeres subalbatus* 43879 transcription factor mRNA (Ay439887.1)	93/105	Mosquito sequence

Table 2.14. Results of siRNA cloning from FB9.1 cells infected with DEN-2, 14 dpi.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
134	dsRNA expressing P-element vector (AB186054.1)	30/32	Potential transposon sequence
136	*D. melanogaster* 3L BAC (AC011905.4)	15/15	
140	Mouse DNA (AL670399.4)	22/22	**Potential siRNA**

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
137	*Ae. aegypti* unknown mRNA (AY431509.1)	45/51	Mosquito sequence
138	*Armigeres subalbatus* 43879 transcription factor mRNA (Ay439887.1)	98/111	Mosquito sequence
142	*Ae. aegypti* 37989 conserved unknown mRNA (AY431521.1)	287/342	Mosquito sequence

Table 2.15. Results of siRNA cloning from FB9.1 cells infected with DEN-3, 7 dpi.

A. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
146	*An. gambiae* mRNA for PROSAg25 protein (AGA416109)	22/22	Mosquito sequence **Potential siRNA**
148	*Rattus norvigicus* paplemmin (Palm) mRNA (NM_130829.1)	22/22	**Potential siRNA**
151	*Siniperca kneri* growth hormone (AY155227.3)	17/17	
152	*Barbula unguiculata* mRNA for chloroplastic copper dismutase (AB066500.1)	25/25	
163	*Mus musculus* Chm3 clone (AC113293.16)	21/21	**Potential siRNA**
165	*Homo sapiens* paraneoplastic antigen MA1 (NM_006029.3)	17/18	
167	*Mus musculus* BAC clone (AC122340.4)	27/29	

B. Sequence matches larger than the expected size range for siRNAs (>40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
149	*Armigeres subalbatus* 43879 transcription factor mRNA (Ay439887.1)	86/103	Mosquito sequence
150	*Armigeres subalbatus* 43879 transcription factor mRNA (Ay439887.1)	45/50	Mosquito sequence
159	*An. gambiae* (XM_319186.1)	103/129	Mosquito sequence
162	Feldmannia irregularis virus strain FirrC-1 (AY225135.1)	37/41	Virus sequence
168	DEN3 (DVU93302)	84/85	Potential virus sequence
169	*An. gambiae* (XM_319572.1)	101/132	Mosquito sequence

Table 2.16. Results of siRNA cloning from FB9.1 cells infected with DEN-3, 14 dpi. Sequence matches within expected size range for siRNAs (≤40 bases).

Sequence #	GenBank Match	Matching Bases	Notes
171	*Rattus norvegicus* similar to KIAA0843 protein (XM_225889.2)	16/16	
173	*Engraulis japomicus* aCat2 mRNA for cathespsin L-like (AB081844.1); *Sus scrofa* PKR (AB104654.1)	21/21	**Potential siRNA**
174	Machupo virus (AY358021.2)	18/18	Virus sequence
176	*Sus scrofa* PKR (AB104654.1)	22/25	
177	Zebrafish clone (BX000465.12)	23/25	
179	Zebrafish similar to AP4, STRN, COCH (AL596026.13)	23/24	**Potential siRNA**
182	*Barbula ungriculata* copper dismutase (AB066500.1)	22/22	**Potential siRNAs**

Table 2.17. Sequences of potential siRNAs

Clone #	Source of RNA	Potential siRNA sequence
1	C6/36 DEN-2 3dpi	TCCATGCTGNCTCTCCCACAGC
4	C6/36 DEN-2 3dpi	TCTCNTACATTCTGACTCTTA
8	C6/36 DEN-2 3dpi	TCACCTCATTGCTCTCTCTTG
17	C6/36 DEN-2 3dpi	AACGAATTCGTTTCTAGTGAG
27	C6/36 DEN-2 3dpi	GACTACCGCTACTACTACTAC
31	C6/36 DEN-2 3dpi	AGCGGAAACAGCAGGTATCCC
34	C6/36 DEN-2 3dpi	CTGCAGGTTTACGAATTCGCC
51	C6/36 DEN-2 7dpi	GATGTACGATGTGATGTCGCT
57	C6/36 mock	CCTTTTAAAAATGGTTTTCC
81	C6/36 DEN-2 14dpi	GCTTCTTGCCGTTTTTGCGG
82	C6/36 DEN-2 14dpi	AGCGGAAACAGCAGGTATCCC
86	C6/36 mock	TCACTGGGCCGTCGTTTTACA
88	C6/36 DEN-2 14dpi	GGGCGAATTCCCGGCCNCTAAATT
109	C6/36 DEN-2 14dpi	CGGNCGGTTCGGTTTTTTACA
113	FB9.1 DEN-2 3dpi	GTTCTCCATTACNTGTGGTTAAAT
116	FB9.1 DEN-2 3dpi	GTTCTCCATTACGTGTGGTTAAAT
140	FB9.1 DEN-2 14dpi	AGGGGAGGATTTGTCTTCATTC
146	FB9.1 DEN-3 7dpi	CCGTACTAGTCGACGCGTGGCC
148	FB9.1 DEN-3 7dpi	CCGTACTAGTCGACGCGGGCC
163	FB9.1 DEN-3 7dpi	TAAATTTTGAAGATTCAGAAA
173	FB9.1 DEN-3 14dpi	GGTACTAGTCGACGCGTGGCC
179	FB9.1 DEN-3 14dpi	ATCATAAGCTGNTTCCTGGAAAA
182	FB9.1 DEN-3 14dpi	CAGTACTAGTCGACGCGTGGCC
262	C6/36 DEN-3 7dpi	CTAAATGGGTGGGTGGTAGGGGA
264	C6/36 DEN-3 10dpi	AGTTTACCGATCCTTGAATTCC
267	C6/36 DEN-3 10dpi	CTAAATGGGTGGGTGGTAGGG
270	C6/36 DEN-3 14dpi	TTTGTTTGNTTTAACATGNCAC
277	C6/36 DEN-3 10dpi	CCCCTACCACCCACCCATTAG
279	C6/36 DEN-3 14dpi	CTAAATGGGTGGGTGGTAGGGG
280	C6/36 DEN-3 14dpi	GGATTCATCGACTCACTAAATTT
281	C6/36 DEN-3 14dpi	CCCCTACCACCCACCCATTTAG

Discussion

The interaction between dengue virus and *Aedes albopictus* C6/36 mosquito cells was studied in order to better understand the interaction between Flaviviruses and their mosquito host. Mosquitoes have an ancient anti-viral defense pathway known as RNAi and this pathway can be used to control DEN-2 replication mosquito cells. This chapter presented a collection of experiments designed to examine the RNAi response to DEN-2. The interaction of two arboviruses replicating within the same cell was studied. Cells that were silenced for DEN-2 by expression of an RNAi anti-DEN-2 dsRNA were studied for further evidence of the RNAi response and to determine if the RNAi response could extend to silence sequences that were related to DEN-2. Initiation of RNAi was examined in mosquito cell lysates and the effector phase of RNAi was studied in siRNA transfection experiments. Finally, the resulting degradation products generated by an RNAi response were studied by cloning and sequencing the pool of small RNAs DEN-2 silenced and control mosquito cells before and after virus infection.

Co-infection of C6/36 cells with a Sindbis virus and a dengue virus resulted in an increase in DEN replication as shown in Figure 2.2. The number of DEN antigen positive cells increased more rapidly and to a greater proportion in samples treated with both viruses than in samples infected with dengue alone. In these studies, the Sindbis virus was allowed to infect all cells in the culture and establish a persistent-infection before challenge with dengue. Under these conditions, the Sindbis virus appeared to alter the host cell in a manner that was favorable to dengue infection. Speculation as to the nature of these alterations seems to point to the ability of this Sindbis virus to down-

regulate the host innate immune response, including RNAi, in order to make the cellular environment more permissive to dengue replication. An alternative hypothesis for the observed results, since viral replication depletes the host of macromolecules, is that there is some direct interaction between the two viruses. In addition, SIN may directly impact RNAi in these cells by encoding an RNAi suppressor or replication of SIN may generate siRNAs and overload the RNAi machinery so that when the challenge DEN-2 entered, it could not be subjected to RNAi. Finally, replication of SIN may have triggered an antiviral response in the mosquito cell and this response may have a lag time after triggering by SIN before it can respond to another infection with the challenge virus.

RNAi is a highly conserved innate immune pathway triggered by dsRNA. It has been extensively studied in *Drosophila* and is only beginning to be understood in mosquitoes. Recent advances such as the *Anopheles gambiae* and *Aedes aegypti* sequencing projects have revealed many of the components of RNAi in mosquitoes. Using methods described in *Drosophila*, development of an *in vitro* RNAi system for mosquitoes was attempted. The results were mixed in that there was sequence specific degradation of mRNA in lysates primed for RNAi, but there was a significant amount of non-specific RNAse activity present in extracts prepared from mosquito cells. The non-specific degradation appeared to be specific to the mosquito cells since *Drosophila* cell extracts were also tested and the same RNAs were stable in *Drosophila* cell extracts. This mosquito *in vitro* RNAi system could be improved by size or affinity fractionating the crude cell extract in order to purify components that are necessary for RNAi without the inclusion of the non-specific nuclease activity.

Dengue viruses are susceptible to RNAi in cell culture. An in depth analysis of the transformed cell line FB9.1 was performed to further characterize the RNAi response in these cells. The cells have been previously described (Adelman et al., 2002) as a marker-selected clonal cell line derived from C6/36 *Aedes albopictus* cells. They were transformed with a plasmid designed to express a hairpin dsRNA derived from the DEN-2 prM gene. Expression of a dsRNA hairpin structure in mosquito cell culture is a potent inducer of the RNAi response. The hairpin was constructed of 567 nt of the prM sequence from DEN-2 in a sense orientation followed by the first 290 nt repeated in antisense orientation and cloned into a plasmid that transcribed the inverted repeat RNA from the baculovirus Ie1 promoter (Adelman et al., 2002). The plasmid expressed the hygromycin B antibiotic resistance marker that was used to select transformed cells. These cells were not permissive to DEN-2 replication. Upon challenge, there was no accumulation of either DEN viral genomic RNA or viral envelope antigen in the cells. Additionally, small DEN-2 prM specific RNAs of approximately 21-23 nucleotides were detected in these transformed cells (Adelman et al., 2002; Sanchez-Vargas et al., 2004). These siRNAs are the specific products of dsRNA cleavage within a cell and are a hallmark of RNAi.

This work demonstrates that viral RNA does not accumulate in the FB9.1 cells after challenge with DEN-2, even at 14 days post challenge. Removal of the hygromycin B from the cell culture medium was shown to reverse this phenotype making the cells again susceptible to DEN-2 infection, presumably through the loss of expression of copies of the transgene. Interestingly, the RNA accumulation data demonstrate that there

is no delayed replication effect. This means that once the initial replication is blocked, the virus is unable to recover.

The FB9.1 cells were also found to be resistant to DEN-3 replication. This can be explained by aligning the nucleotide sequences of DEN-2 prM and DEN-3 prM. These two sequences have many regions of identity, including a highly conserved stretch of 23 bases in which 21 are the same in both sequences. The regions of homology seem to explain the cross-resistance observed with DEN-3 in the FB9.1 cells.

Further evidence that a cross-reactive RNAi response results in both DEN-2 and DEN-3 resistance in the FB9.1 cells came from the assay for WNV replication in these cells. WNV, a more distantly related flavivirus, was found to replicate in the FB9.1 cells. The cross-reactive resistance phenomenon did not extend to WNV. Additionally, the cells were screened to ensure they were not expressing any portion of the M protein since it has been shown in the literature (Lee and Schloemer, 1981a; 1981b) that the M protein can have anti-viral properties. Antibody staining for DEN-2 prM in uninfected FB9.1 cells confirmed that they were not producing any protein from the transforming plasmid construct. This result was expected since the construct was engineered to prevent prM expression by removal of the initiation codon (Gaines et al., 1996). Importantly, the cells were able to produce M at a low level only after infection with DEN-2. Here the prM protein production was presumably directed by the infecting virus itself.

The number of copies of the transforming plasmid was determined for passage 16 of the FB9.1 cells. While speculation about the stability of plasmid copy number over multiple passages cannot be addressed by the assays performed here, it was demonstrated that there are close to one thousand copies per cell present at passage 16. Additionally,

nothing can be said from these slot blot data about the form in which the copies are present or their integration status with respect to the host genome. The major conclusion from these experiments was that there are many copies of the transforming plasmid present in the FB9.1 cells; therefore there are many templates for DEN-2 prM dsRNA transcription and these transcripts represent many dsRNA targets for Dicer to initiate RNAi against DEN-2. Copy number could be further characterized using *in situ* hybridization techniques to directly visualize the plasmid within cells. The high copy number of the plasmid may explain why the FB9.1 cells so efficiently inhibit DEN-2 replication.

The ability of the FB9.1 cell line to replicate a dsSIN with an insert from DEN-2 genome was tested. FB9.1 cells were found to be resistant to infection with dsMRE16-eGFP/D2prM, presumably due to the RNAi response triggered by DEN-2 prM hairpin dsRNA. These results have important implications for the experiments discussed in Chapter 3. Mosquito transgenesis is a long, laborious process and challenge experiments of mosquitoes *per os* with a dengue virus takes up to one month (rearing the mosquitoes and producing fresh virus takes approximately 2 weeks and the incubation period of DEN in the mosquito is 14 days) to complete and analyze, from rearing the mosquitoes to completing the 14-day extrinsic incubation period for the virus. Sindbis viruses replicate much faster than dengue virus, and the ability to include a marker gene such as GFP allows for real time tracking of the virus as it moves through the mosquito without having to sacrifice mosquitoes. This dsMRE16-eGFP/D2prM virus represents an excellent tool to rapidly screen transgenic mosquitoes engineered to perform RNAi against DEN-2 for resistance to DEN-2. It must be noted that any resistance observed with a dsSIN must be

confirmed by an actual *per os* DEN-2 challenge due to potential differences in replication kinetics between the two viruses.

The dsRNA-triggered Dicer-dependent initiator step of RNAi can be by-passed and RNAi to DEN-2 can still be achieved in cultured cells. Direct transfection of either a commercially prepared anti-DEN-2 siRNA or a pool of DEN-2 specific siRNAs generated *in vitro* resulted in inhibition of dengue replication. The replication inhibition was complete, as observed for RNAi triggered from a dsRNA previously, and there was no accumulation of DEN-2 viral antigen, genomic RNA, or production of new virus upon challenge of the transfected cells. Interestingly, there was a slight decrease in all of these factors in cells transfected with control siRNAs when compared to the untransfected controls. This is presumably due to the introduction of exogenous RNA into the cell.

The pool of siRNAs present in C6/36 and FB9.1 cells was examined before and after infection with either DEN-2 or DEN-3 virus. The siRNAs were cloned using a highly inefficient protocol involving at least four gel elution steps. Extremely large quantities (> 500 μg) of total RNA were required for each sample tested. In the end, over 280 sequences were examined for evidence of siRNAs. Two siRNAs with sequence derived from DEN-2 prM DNA were detected from FB9.1 cells infected with DEN2. Many candidate siRNAs or miRNAs were identified on the basis of their size and homology to sequences from mosquitoes, mice and humans. The ability of these sequences to function as siRNAs remains to be determined and this could be assayed using transfection of synthetic copies of each of these potential siRNA and by RNAse protection assay in which targeted mRNAs could be identified based on their ability to bind to the synthetic siRNAs. These data represent some of the siRNA present in

mosquitoes and may help to understand control of gene expression in mosquitoes. The sequences, although they do not match any miRNAs known to date, may represent as yet uncharacterized mosquito miRNAs. As more data on the *Aedes aegypti* genome and its annotation become available, it may be possible to revisit the analysis of these clones to determine if they have homology to any sequences in the *Aedes aegypti* genome.

The data presented in this chapter represent additional evidence that RNAi is an effective strategy to control DEN-2 replication in mosquito cells. In addition to demonstrating that the response is RNA-based and characterized by sequence similarity requirements, these data clearly demonstrate that the effector molecule, siRNA, can direct silencing in the absence of long dsRNA. This is important because it demonstrates that a small effector can be used to achieve the same degree of silencing observed in the FB9.1 cells expressing a 290 nt dsRNA. This information can be used to design an RNAi construct targeting all four serotypes of DEN-2 with a small piece directed against each serotype. Finally, the effectiveness of siRNAs in controlling DEN-2 replication may have implications as a potential therapeutic agent if siRNA can be shown to control an active DEN-2 infection.

Chapter 3

Transgenesis of *Aedes aegypti* mosquitoes: towards generation of DEN resistant mosquitoes

Chapter 3. Transgenesis of *Aedes aegypti* mosquitoes: towards generation of DEN resistant mosquitoes

Introduction

New approaches to DEN control are needed to complement traditional disease control strategies. The underlying hypothesis of this research is that one method of achieving DEN disease control is to introduce an anti-DEN gene or sequence into mosquito populations competent for transmission of DEN viruses and profoundly alter the vector competence of the population. A key step in testing the validity of this hypothesis is to develop transgenic *Ae. aegypti* mosquitoes in which replication and transmission of the viruses is specifically blocked. This block would occur by expression of specific anti-DEN effector molecules throughout the whole mosquito, or preferentially in the salivary glands or midgut of the transgenic mosquito, preventing replication and transmission of the viruses.

The effector RNA molecules tested in this work were generated from a region of dsRNA with sequence identity to part of the DEN-2 genome. The virus derived dsRNA sequence is expressed upon transcription in the transformed mosquito. The dsRNA trigger the endogenous RNAi response, resulting in the generation of DEN-2 specific siRNAs and a mosquito that is pre-sensitized to destroy DEN-2 mRNA.

The transgenesis approach discussed here used a binary system of transformation that involves co-injection of two plasmids into mosquito embryos. One plasmid encoded the anti-DEN-2 sequence and an eye color selection marker flanked by the transposable

element inverted-repeats (IRs) and the second plasmid encoded the transposase. This allowed stable integration of the anti-DEN sequence and prevented mobilization of the transposon. The following chapter will discuss a number of transgenic mosquito families that were generated in collaboration with Dr. Anthony James and colleagues at the University of California-Irvine. These transgenic *Ae. aegypti* mosquitoes were shown to contain the anti-DEN-2 sequence stably inserted in the mosquito genome and to transcribe the anti-DEN RNA. The results of challenge of the transgenic mosquitoes with DEN-2 (Jamaica 1409) are discussed.

Materials and Methods

Mosquito Strains: The following is a description of mosquito strains used in this study. Rex-D is the *Aedes aegypti* laboratory strain Rexville-D originating from Rexville, Puerto Rico (Miller and Mitchell, 1991) was used as a control, non-transgenic mosquito strain. Mosquito strains lacking eye pigmentation were used as the genetic background strain for transgenesis in order to facilitate the observation of an eye-specific marker of transgenesis. The HWE strain is a white-eye colored derivative of the Rex-D *Ae. aegypti* strain arising from mutation in the laboratory colony; however, the genetic basis of white-eye mutation is unknown in this strain (Higgs strain). The K^{hw} strain is an

Ae. aegypti strain containing a mutation in the kynurenine hydroxylase (kh) gene involved in tryptophan catabolism and synthesis of ommochrome pigments resulting in white eyes, and this mutation can be complemented by the *Drosophila* cinnabar gene (cn) resulting in a reddish colored eye pigment (Han et al., 2003; Cornel et al., 1997). The 3xP3-eGFP, transgenic strain of *Ae. aegypti* is a positive control for expression of the eye-specific transgenesis marker gene based on the K^{hw} strain. 3xP3-eGFP mosquitoes express the enhanced green fluorescent protein (eGFP) under control of an eye-specific synthetic promoter construct containing 3 copies of the eye-specific enhancer Pax6 (Kokoza t al., 2001; Singh et al., 2000; Kim et al., 2001; Hauck et al., 1999; Horn and Wimmer, 2000).

Mosquito Rearing: *Ae. aegypti* strains were reared in insectaries at the Arthropod-borne and Infectious Diseases Laboratory (AIDL) in the Department of Microbiology, Immunology & Pathology on the foothills campus of Colorado State University (CSU) Fort Collins, CO, or in McGaugh Hall at the Department of Molecular Biology & Biochemistry of the University of California at Irvine (UCI) Irvine, CA. Environmental conditions at both locations were held constant at 28°C and 80% humidity with a photoperiod of 12h light and 12h darkness (Higgs and Beaty, 1996). Adult colonies were maintained on sugar cubes and raisins as a sugar source, while oviposition was generated through blood feeding on mice and embryos were collected on moist paper towel or filter paper egg liners. Egg liners were dried and maintained under insectary conditions for up to 3 months or could be hatched as soon as 2 days post deposition depending upon experimental needs. Eggs were hatched by placing the egg liner in ~700

mL deionized water containing 2-3 drops of brain-heart infusion (BFI), and emerging larvae were reared at 80-100 individuals per pan until pupation. Larvae were maintained on a mixture of ground fish and mouse feed. Pupae were collected and allowed to emerge as adults in cages or cartons. For challenge experiments and controlled mating, pupae were selected for sex based on size; large pupae were classified as female and smaller pupae as male. Classification was confirmed upon emergence as adults, and any females that were inadvertently reared with males were not used for the controlled mating experiments.

Transforming Constructs. Constructs used for transformation were generated by Dr. Zachary N. Adelman (UCI) and Dr. Alexander W. E. Franz (AIDL/CSU) and injections were performed by Dr. Adelman, Dr. Franz and me in the laboratory of Dr. Anthony A. James at UCI. Embryos were transformed with a dual plasmid system as described by Jasinskiene and others (1998). One plasmid contained the right and left arms of the transposable element, selectable screening marker, and the anti-DEN effector gene. The second plasmid supplied the appropriate transposable element-specific transposase driven by the promoter from the *Drosophila* heat responsive *hsp70* gene. The initial transgenesis experiment involved injection of the same anti-DEN-2 hairpin dsRNA construct that was successfully used in transformed cell culture experiments (Adelman et al., 2002) inserted into a piggyBAC transposable element (see Table 3.1). The DEN effector region of this construct, referred to as fold-back, consisted of the 567 nucleotides (nt) of the DEN-2 genome corresponding to the 3'end of the C gene, the entire prM-M gene and the 5' end of the E gene in sense orientation followed by 290 nt

of the prM gene repeated in antisense orientation and upon transcription folds back upon itself to generate a 290 base pair (bp) region of dsRNA that acts as a trigger of RNAi (Adelman, 2000; Adelman et al., 2002; Travanty et al, 2004). The construct also incorporated the 3xP3-eGFP eye specific selectable marker and used the *polyubiquitin* gene promoter (Ub), 800 bp in length, from *D. melanogaster* to drive transcription of the anti-DEN-2 hairpin. The later transgenesis experiments used the *MosI* (*Mariner*) transposable element, the 3xP3-eGFP marker gene, and an anti-DEN-2 dsRNA trigger derived from repeats of 290 nt of the DEN-2 prM gene in sense and antisense separated by the *Ae. aegypti sialokinin* gene major intron, designed to generate a 290 bp dsRNA without a hairpin upon expression *in vivo* (see Table 3.1). The promoters used in the *MosI* construct are the *D. melanogaster polyubiquitin* gene promoter (Ub; 800bp and 1300bp versions), the baculovirus *Immediate early 1* gene promoter (*Ie1*), and the female midgut specific, blood feeding induced *Ae. aegypti carboxypeptidase* gene promoter (Carb).

Table 3.1. Constructs used for transgenesis of *Aedes aegypti* mosquitoes.

TE[a]	Selectable marker	Promoter for DEN effector expression	Promoter expression profile	Anti-DEN dsRNA effector	Name
piggyBAC	3xP3-eGFP	*Drosophila* Ubiquitin 800bp	Constitutive	Hairpin[b]	piggyBac-3xP3eGFP/ UbD2FB
MosI	3xP3-eGFP	Baculovirus Immediate Early 1(Ie1)	Constitutive	Intron[c]	pMosIe/2-i-2
MosI	3xP3-eGFP	*Drosophila* Ubiquitin 800bp	Constitutive	Intron	pMosUb800/2-i-2
MosI	3xP3-eGFP	*Drosophila* Ubiquitin 1300bp	Constitutive	Intron	pMosUb1300/2-i-2
MosI	3xP3-eGFP	*Aedes aegypti* Carboxypeptidase	Female midgut specific, induced by blood feeding	Intron	pMos/Carb2-i-2

[a] TE, Transposable element

[b] Hairpin is 567 nucleotides from the start of the DEN-2 M protein (Mnp = modified to produce no protein) in sense orientation followed by the first 290 nucleotides repeated in antisense orientation. (Adelman et al., 2002).

[c] Intron is the *Aedes aegypti* sialokinin major intron flanked by 290 nucleotides from DEN-2 M protein in sense orientation on the left side and 290 nt from DEN-2 M protein repeated in antisense orientation on the right side of the intron (Travanty et al., 2004).

Transformation. Mosquitoes with white eyes of the appropriate genetic background, either K^{hw} or HWE, were reared to adulthood. The females were isolated and allowed to take a blood meal from a mouse. They were held in cartons without an oviposition site and after 2 days were ready to deposit eggs. Twenty-five to thirty females were removed to either a 50 ml conical tube or a *Drosophila* rearing tube, which contained damp cotton in the bottom third covered by a circle of Whatman filter paper large enough to cover the cotton and turn up slightly at the edges, contacting the sides of the tube. The opening of the tube was blocked with dry cotton. The egg-laying chambers were then placed in a dark place in the insectary to stimulate coordinated egg laying, ensuring that all of the embryos were approximately the same age. Timing of embryo injection was crucial in mosquito transgenesis because, unlike *Drosophila*, there was no way to stop chorion formation or dechorionate an *Ae. aegypti* embryo. Therefore, injection must take place during chorion formation when the embryo was not too soft or too hard. This prevented breaking or plugging the microinjection needle. Fortunately, as the embryo hardens it darkens in color from white through shades of gray to black. The ideal texture for injection was during the lighter gray stages where the egg will yield under pressure from a sharp, properly tapered needle and will not break. After 45 minutes, the females were removed and the pre-blastoderm embryos on the filter papers were aligned in a vertical row with for injection into the pole plasm. The filter paper was dried and when the embryos reached the correct color, they were attached to double-stick tape on a square plastic coverslip where they were allowed to desiccate, briefly. Halocarbon-27 oil (Sigma, St. Louis, MO) was used to cover the embryos and stop

desiccation. Embryos were injected with an amount of fluid that restored their original pre-desiccation volume with each plasmid at a final concentration of 0.5 mg/ml for the non-autonomous transposable element plasmid and 0.3 mg/ml for the transposase plasmid in 5 mM KCl and 0.1 mM NaH_2PO_4, pH 6.8 (Jasinskiene, et al., 1998). Needles for embryo injection were prepared from glass capillary tubes using a needle puller and grinding wheel to generate the necessary fine tip. Microinjected embryos were exposed to a heat shock (24 hours post injection) of 39-41°C for 1 h to induce the expression of the transposase, which was under the control of the *D. melanogaster hsp*70 gene promoter. Embryos were then held under insectary conditions for 5 days before hatching.

Mating and Selection of Transgenic Mosquitoes. After transformation, all viable individuals surviving to adulthood were reared individually and given a unique identifying number. Once adults emerged, they were sexed and the males were maintained separately, while females were pooled in groups of 5-10 mosquitoes. Adults resulting directly from the injected embryos were called generation 0 or G_0. Mosquitoes were not screened for GFP expression at this stage because the goal of these experiments was germ line transgenesis for stable, heritable expression of the transgene. Instead, the progeny of the surviving injected individuals, the G_1 individuals, were screened. Individual males founded families through mating with approximately 25 virgin un-transformed females from the background genetic strain, either HWE or K^{hw}. Each of the female pools was out-crossed by mating to approximately 5 untransformed males. Larvae in the next generation, G_1, were screened for expression of the eye-specific marker using the GFP specific filter for ultraviolet light on an Olympus SZX12 dissecting

microscope. Results were recorded and GFP positive larvae were out-crossed and re-selected until large transgenic families resulted (usually by G_3) at which point each family was allowed to interbreed towards homozygosity. Each surviving individual was given a number (#), each pool was given a pool number (P#), and after each round of injections the numbering was started over again with number 1, but was coded with a separate color tape to ensure clarity in labeling.

Viruses. The virus used was the DEN-2 Jamaica 1409 strain, which was originally isolated by from a case of dengue hemorrhagic fever (DHF) in Jamaica in 1983 (Deubel et al., 1986). The virus was passed in C6/36 *Ae. albopictus* cells as well as LLC-MK2 rhesus monkey (*Macaca mulatta*) kidney cells following primary isolation and has been subsequently passed multiple times in C6/36 cells. The isolate was made available by the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Fort Collins, CO, and then further passed in our laboratory. To prepare the working stock from the initial C6/36 passage-5 stock, an 80% confluent 150-cm^2 tissue culture flask of C6/36 cells was infected at a multiplicity of infection (MOI) of 0.1 virus particles per cell for one hour in 5 ml Liebowitz-15 (L15) medium with gentle rocking. The virus suspension was removed and the cells were incubated at 28°C for 14 days in L15 medium supplemented with 2.5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 mM L-glutamine; at day 7 post infection the medium was changed. At 14 days post infection, the cells were harvested using a cell scraper and transferred along with the medium to a 50 mL conical tube in which the cells were pelleted by centrifugation at 2000 rpm in a Beckman GS-6R

centrifuge for 5 minutes at 4°C. The supernatant was supplemented with 1 ml of freezing solution (25 ml FBS, 1 ml 7.5% sodium bicarbonate) per 10 ml of viral supernatant and frozen at -70°C in 0.5 ml aliquots. The infectivity of the working stock was titrated by plaque assay and found to be 6 x 10^7 PFU/mL (Miller and Mitchell, 1986).

Interference Assays: All challenge experiments were performed under biosafety level-3 (BSL3) containment. Virus for *per os* (oral) blood feeding challenge experiments was made fresh for each challenge. Virus was propagated in the same manner as previously described except that upon harvest, the cells and medium were mixed 1:1 with defibrinated sheep blood and placed in membrane feeders covered with either hog gut or human scented parafilm (Higgs and Beaty, 1996).

Transgenic mosquitoes were prepared for virus challenge by removal of sugar and water for approximately 24 hours before blood feeding. The membrane feeders were attached to a circulating water bath that provided a constant 37°C temperature in the outer jacket, warming the blood meal to simulate the host from which the female mosquito naturally feeds. Mosquitoes were allowed to feed for approximately 1 hour and pre- and post-feed samples of the blood meal, as well as a sample of the undiluted virus, were saved for titration. Intrathoracic inoculation with 0.5 µl containing approximately 3 $\log_{10}TCID_{50}$ of stock DEN-2 per adult female mosquito was also performed to provide positive controls. The titers of the virus and blood meal samples were determined by the end point titration method (Karber, 1931; see Materials and Methods, Chapter 2). Titers ranged from 7.2 to 7.7 $\log_{10}TCID_{50}$, shown to be optimal for DEN-2 virus disseminated infection in mosquitoes (Bennett et al., 2002).

After feeding, the mosquitoes were anesthetized with CO_2 and cold, and fully engorged females were selected. Sorting GFP-positive transgenic mosquitoes and GFP-negative siblings was performed at this time using the UV illumination goggles (Figure 3.1; GF-sPectacles, Biological Laboratory Equipment, Maintenance and Service, LTD., Budapest, Hungary). Adults were screened for GFP expression in the eyes, except in a few cases in which they were sorted at the larval or pupal stage prior to challenge. Sorting was no longer necessary at the post challenge stage once a family was intercrossed for multiple generations and every individual was GFP-positive, but visual inspection for GFP expression was continued to ensure they had not lost the transgene.

Post challenge mosquitoes were held under insectary conditions for 14 days, the extrinsic incubation period for DEN-2. At the end of the incubation, head tissue was acetone fixed to pre-washed slides and assayed by immunofluorescence for the presence of DEN-2 antigen with the 3H5 anti-DEN-2 envelope protein specific monoclonal antibody or the more broadly reactive 4G2 anti-flavivirus envelope monoclonal antibody. Samples were assayed using the immunofluorescence assay described in Chapter 2 Materials and Methods, except that head tissue was first encircled with a tech pen (VWR International, West Chester, PA) and 100 μL of each diluted antibody was applied to each head tissue sample. Specimens were mounted in glycerol: PBS (3:1), and covered with a coverslip prior to microscopic evaluation. Time points of 3, 7, 10, and 14 days post infectious blood meal were assayed in most experiments, depending on availability of sufficient numbers of mosquitoes that were fully engorged. Additional tissues such as midgut and salivary gland were also examined in some of the transgenic families.

Figure 3.1. Screening transgenic mosquitoes for eye-specific GFP expression. Transgenic mosquitoes were anesthetized with CO_2 and kept on chill table or ice during sorting using the GFP viewing goggles, GF-sPectacles, (Biological Laboratory Equipment).

RNA analysis. Total RNA was extracted from individual mosquitoes or mosquito pools by a modified version of the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) as described in Chapter 2. Alternatively, total RNA was rapidly extracted from mosquitoes using the QiaShredder and RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer-provided protocol. RNA extracted by either method was used for RT-PCR and northern blot analysis, but northern blot analysis of siRNA was only performed on RNA extracted by the GTC method because these small RNAs were not efficiently bound by the column used in the RNeasy method (RNeasy protocol, Qiagen) and would have been lost in the column wash steps. Enrichment for low molecular weight RNA was performed by polyethylene glycol precipitation of high molecular weight RNA as described in Chapter 2, Materials and Methods.

RT-PCR. RT was performed on extracted total RNA samples using the SuperScript II reverse transcriptase enzyme according to manufacturer protocol (Invitrogen, Carlsbad, CA). RNA and reverse primer were denatured and 10 mM dNTPs, 5x first-strand synthesis buffer and RNAse inhibitor were added for a 1 hour incubation at 42°C followed by an inactivation step. Custom oligonucleotide primers were designed using PrimerSelect (LaserGene software package, DNAstar, Inc., Madison, WI) and obtained from Invitrogen. Primer pairs (reverse from each pair was also used in RT step): DEN-2 M primers: (based on DEN2 JAM 1409 sequence GenBank Accession # M20558 (Deubel et al., 1988) Forward 5'-GTAGGCAAGAGAAAGGGAAAG-3',

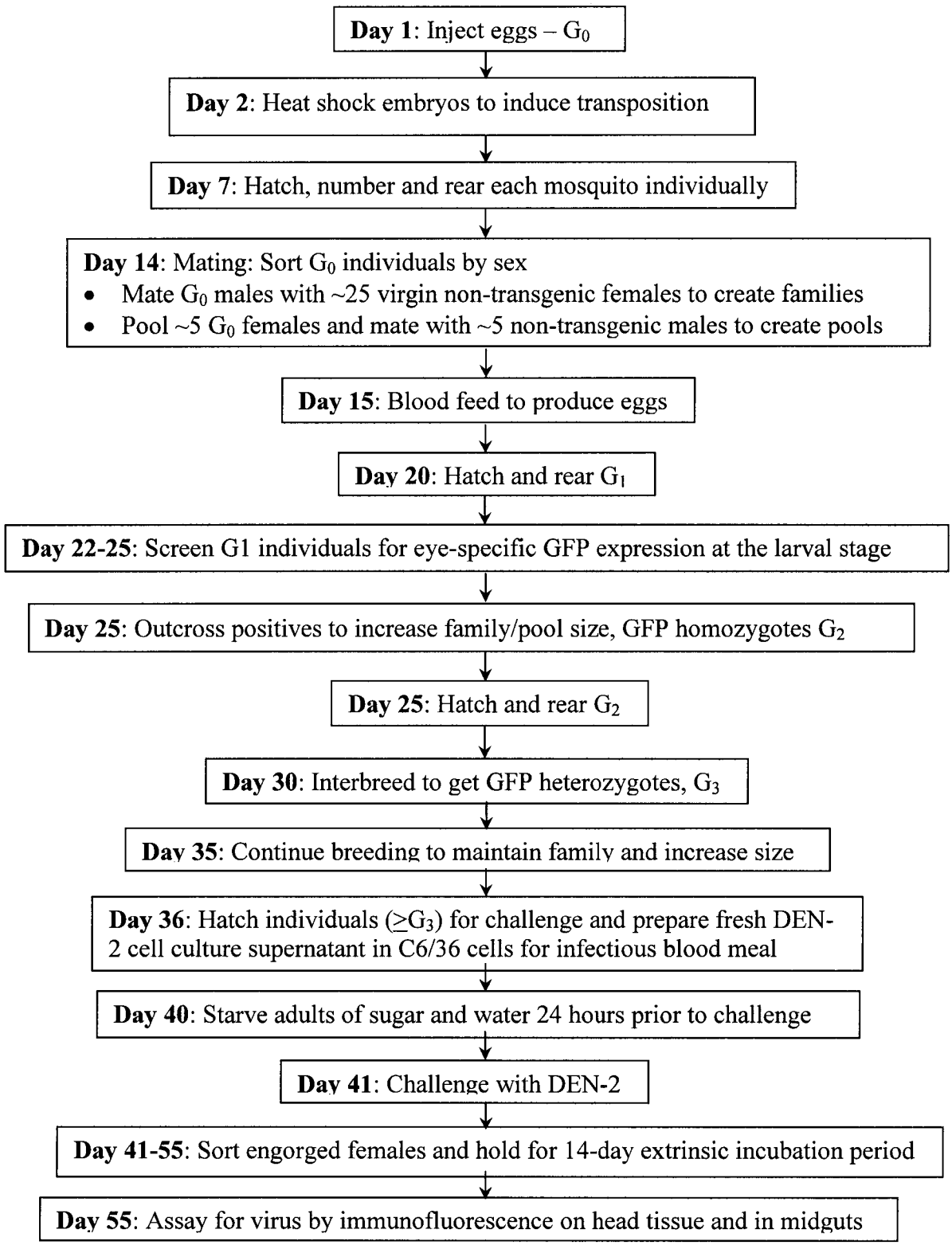
Reverse 5'TCATTGAAGGA GCGACAGC-3'; Actin primers: (based on sequence from B. Kempf, Ph.D. dissertation, Colorado State University, 2003) Forward 5'-TCCAGAGCAAGAGGTA-3', Reverse 5'-TCAGGTAGTCGGTCAGAT-3'. PCR was performed using 3 μL of the RT reaction, Taq polymerase and 1x Taq reaction buffer (Promega, Madison, WI) with 50 pmol of each primer, 250 pmol each dNTP and 2.5 mM $MgCl_2$ per 50 μL reaction. Reactions were cycled as follows: 94°C 5 min, 25 cycles of 94°C 30 sec, 55°C 1 min, 72°C 1 min, and 72°C 7 min.

Results

Selection of GFP-positive transformants from piggyBac-3xP3eGFP/UbD2FB injected embryos. Two approaches to generating a DEN-2 resistant transgenic mosquito were examined in this work. In the first approach described above, the piggyBAC transposable element was used to express a fold-back (FB) hairpin dsRNA-expressing construct driven by the *Drosophila* ubiquitin promoter, 800 bp version. This was the same insert that was previously used to successfully transform cell culture, although the promoter used in the cells was the baculovirus *immediate early 1* gene promoter (*Ie1*; Adelman et al., 2002). Microinjection of this construct, piggyBac-3xP3eGFP-UbD2FB (see Tables 3.1, 3.2 and 3.3), into K^{hw} strain *Ae. aegypti* embryos was performed during a visit to the collaborating laboratory at UCI and, after heat shock treatment that induced expression of the transposase, embryos were transported back to AIDL/CSU for rearing. A total of 1789 *Ae. aegypti* K^{hw} embryos were injected with the piggyBAC-3xP3eGFP/UbD2FB at UCI (see Figure 3.2 for flow chart of transgenesis experiments). During transit, the embryos were housed within the passenger cabin of a commercial airplane and although they passed through x-ray security screening, they were not subjected to the harsh conditions of checked baggage and were not allowed to dry out. They were held in the AIDL insectary for 5 days post injection and then hatched, reared and mated as described in Materials and Methods section. Large numbers of the parental K^{hw} strain were also reared at the same time for use in mating of resulting transgenic adults. After two weeks, 234 G_0 individuals survived to pupation (13.1% of injected embryos) and 209 of these survived eclosion and emerged as adults (89.3% of pupae;

11.7% of total). Of these G_0 adults, 95 were male (45.5%) and 114 were female (54.5%). Many, 37%, of the males (n = 33) were sterile and did not produce any offspring when out-crossed to virgin K^{hw} females. The 114 females were divided into 23 pools containing between 3 and 8 individual G_0 female mosquitoes; 10 pools failed to produce any eggs. Three egg liners, each resulting from one round of blood feeding and egg laying, were generated from each fertile family (families are based on a founding injected male) and pool (pools are based on 3-5 founding injected females) and these G_1 liners were hatched and screened for GFP expression in the eyes. Some families and pools did not produce any eggs; they may have been sterile or may not have blood fed. The goal was to screen $\geq$300 individuals from a family or pool founded by an surviving injected individual without finding a GFP positive before making the determination that the family/pool was not transgenic. However, in some cases (9 of 11 pools and 38 of 47 families) 300 viable offspring were not generated. Mosquitoes that produced eggs that did not hatch were labeled sterile. All larvae that hatched were screened, but number of offspring screened from each group was variable. The numbers of individuals screened are shown in Tables 3.2 for the families and 3.3 for the pools. None of the female-based pools contained any GFP-positive larvae, indicating that there was no germ line transposition in these mosquitoes. One family, 111, was found to contain 19 GFP positive larvae (see Figure 3.3 for examples of GFP expression at each life stage). These larvae were pooled and out-crossed to the K^{hw} parental strain over two additional generations to increase the size of the family before challenging the family with DEN-2.

Figure 3.2. Flow Chart of *Aedes aegypti* transgenesis.



Total time: approximately 55 days from embryo injection to assay for DEN-2 infection.

Table 3.2. piggyBac-3xP3eGFP/UbD2FB Generation 1 Families. The total number of G_1 offspring for each family is given.

Family #	GFP expression in G_1	Family #	GFP expression in G_1
4	434 negative	87	237 negative
6	Sterile**	91	lst liner no eggs*, sterile
9	Sterile	92	2nd liner 227 negative
12	Sterile	93	21 negative
14	Sterile	95	lst liner no eggs, sterile
16	Sterile	97	396neg
17	1st & 2nd liners, no eggs	99	9neg
20	1st & 2nd liner-no eggs, 210 negative	100	sterile
21	1st liner-no eggs, 2nd liner-120 negative, 3rd liner-no hatch	102	sterile
22	1st liner no eggs, 2nd liner 47 negative	105	sterile
23	1st liner-no eggs, 342 negative	106	1st & 2nd liners, no eggs
24	Sterile	108	sterile
25	1st & 2nd liners, no eggs	109	1st liner no eggs, sterile
26	218 negative	**111**	**19 positive, 284 negative, 3 did not emerge from pupal stage, 2 died as adults: 14 GFP (+) surviving adults**
27	Sterile	112	42 negative,
30	1st & 2nd liners, no eggs	116	109 negative
31	1st liner no eggs, 37 negative	120	Sterile
33	1st liner no eggs, sterile	121	160 negative
37	Sterile	124	Sterile
38	Sterile	127	lst liner no eggs, sterile
40	270 negative	129	157 negative

Table 3.2. piggyBac-3xP3eGFP/UbD2FB Generation 1 Families, continued.

Family #	GFP expression in G_1	Family #	GFP expression in G_1
41	Sterile	130	215 negative
45	48 negative	131	197 negative
46	Sterile	132	121 negative, 2nd liner didn't hatch
50	lst & 2nd liners, no eggs	133	185neg
52	Sterile	139	30neg, 2nd liner didn't hatch
54	2nd liner 110 negative	142	2nd liner 45 negative
55	108 negative	143	Sterile
63	Sterile	155	2nd liner 160 negative
66	2nd liner 387 negative g	164	Sterile
69	46 negative	166	Sterile
70	lst, 2nd & 3rd liners, no eggs	169	115 negative
71	lst, 2nd & 3rd liners, no eggs	179	110 negative
75	lst, 2nd & 3rd liners, no eggs	181	241 negative
77	lst, 2nd & 3rd liners, no eggs	184	82 negative
78	Sterile	185	127negative
81	lst, 2nd & 3rd liners, no eggs	191	155 negative
84	321 negative	197	Sterile
198	Sterile	217	525 negative
200	3rd liner no eggs, 57 negative	218	59 negative
202	152 negative	219	1st liner no hatch, 2nd liner 23 negative

Table 3.2. piggyBac-3xP3eGFP/UbD2FB Generation 1 Families, continued.

Family #	GFP expression in G_1	Family #	GFP expression in G_1
203	Sterile	224	424 negative
204	No eggs	225	Sterile
206	312 negative	227	174 negative
208	1st liner 67 negative, 2nd liner no eggs	228	235 negative
214	Sterile	232	Sterile
215	1st liner 127 negative, 2 nd liner no eggs		

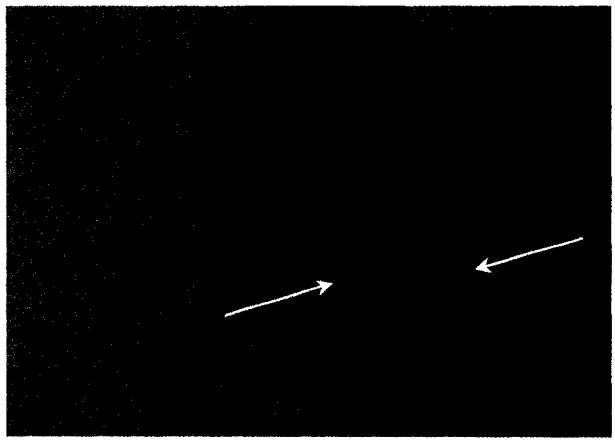
*No eggs, no eggs were produced after blood feeding.
**Sterile, eggs were produced, but the eggs did not contain embryos and did not hatch.

Table 3.3. piggyBac-3xP3eGFP/UbD2FB Generation 1 Pools. The G_0 individuals in each pool and total number of G_1 GFP (-) offspring for each pool is given.

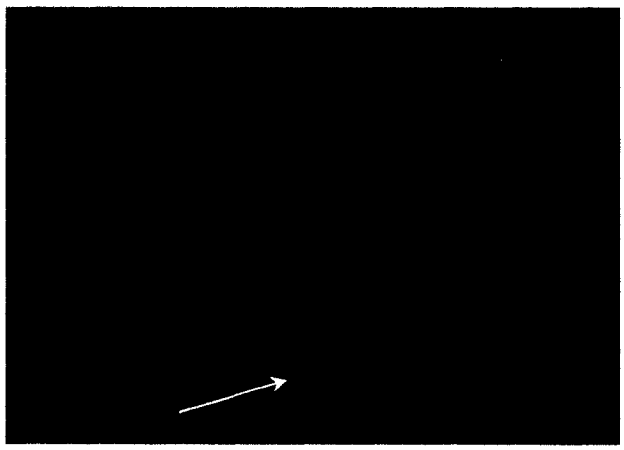
Pool #	G_0 Females	GFP expression in G_1
1	1,2,3,5,7	130 negative, 3rd liner no eggs
2	8,10,11,15,18	197 negative
3	19,28,29,32,35	96 negative
4	39, 42,43,44,47,49,51,53	313 negative, 3rd liner no eggs
5	56,57,60,62,76	34 negative
6	61,64,67,68,80	110 negative, 2nd liner no eggs
7	59,65,73,88,103	133 negative, 2nd liner no eggs
8	79,83,86,94,101	100 negative
9	74,85,90,96,98	No eggs
10	104,107,117,118,123	1st, 2nd & 3rd liners no eggs
11	113,114,115,119,122	1st liner hatched but larvae died, 194 negative
12	125,126,128,135,136	1st, 2nd & 3rd liners no eggs
13	134,138,140,146,147	80 negative, 3rd liner no eggs
14	141,144,148,149	1st & 2nd liner, no eggs
15	153,158,161,162,	1st & 2nd liners no eggs
16	151,159,163,165,168,	1st & 2nd liners hatched but larvae died
17	150,152,156,157,160	1st & 2nd liner no eggs
18	177,180,190,192,193	1st liner no eggs, 2nd liner-no hatch
19	170,171,172,174,188	1st, 2nd & 3rd liners, no eggs
20	173,175,176,182,189	1st, 2nd & 3rd liners no eggs
21	194,196,207,210,211	1st liner no eggs, 2nd liner-no hatch
22	226,229,230,233,234	1st, 2nd & 3rd liners no eggs
23	213,216,220	743 negative

Figure 3.3. Eye-specific GFP expression from the 3xP3-eGFP marker gene is detectable at all life stages in transgenic *Aedes aegypti* mosquitoes. Viewed under dissecting microscope with white and fluorescent light. A. Larva. B. Pupa (viewed from the side, only one eye is visible). C. Adult (head only). Arrows indicate eye-specific GFP expression.

A.



B.



C.

DEN-2 challenge of piggyBac-3xP3eGFP/UbD2FB transgenic mosquitoes.

Family #111 was the only transgenic family generated from embryo injection of the piggyBAC-3xP3eGFP/UbD2FB construct. There were 14 GFP (+) G_1 individuals (see Table 3.2) and these mosquitoes were out-crossed to the parental non-transgenic K^{hw} strain to establish Family #111. Mosquitoes from family #111 (G_3; n = 122 individuals, 49 transgenic and 73 non-transgenic) were challenged twice *per os* with a freshly prepared blood meal containing 8 $\log_{10}TCID_{50}$ of DEN-2. At 14 days post challenge, head tissue was analyzed as described in the Material and Methods section and scored positive if any portion of the tissue contained DEN-2 antigen (see Figure 3.4 for examples of antigen stained head tissues). The combined challenge data showed that 73.5% of the transgenic individuals (36 out of 49) were positive for DEN-2 antigen in the head tissue at 14 days post challenge. This was not significantly different from the 78% (57/73) positive observed in the control, non-transgenic mosquitoes. Of the mosquitoes that were intrathoracically inoculated (n = 126 individuals, 47 transgenic and 79 non-transgenic), the results were essentially identical between the two groups: 97.8% (46/47) of injected transgenic mosquitoes were positive and 98.7% (78/79) of controls were positive (see Tables 3.4 and 3.5). Therefore, no resistance to DEN-2 replication was observed in piggyBac-3xP3eGFP/UbD2FB family #111.

Total RNA extractions were performed on pools of 10 midguts and 10 bodies from family #111, as well as from the K^{hw} and 3xP3eGFP control mosquito strains. This RNA was subjected to RT-PCR analysis to examine expression of the DEN-2 fold-back construct in the transgenic mosquito line. RT-PCR analysis using primers designed to detect a 453 bp region of the M sequence demonstrated expression of the fold-back

construct in piggyBAC-3xP3GFP-UbD2FB family #111 (see Figure 3.5). Additional families (#114 and #93) generated at UCI using the *Hermes* transposable element and the *Drosophila cn* selectable eye color marker were also analyzed by RT-PCR as negative controls and RNA from the FB9.1 cell line containing the fold-back hairpin was analyzed as positive control (Adelman et al., 2002). The RNA used here was DNAse I treated using the DNA-free kit (Ambion) to remove any contaminating genomic DNA that may still have been present after RNA extraction and could have given a falsely positive signal. Figure 3.5 demonstrates expression of the anti-DEN-2 RNA effector in RNA extracted from FB9.1 cells, but not in the transgenic mosquitoes. The control actin transcript, however, is detectable in all of the transgenic and control mosquito samples as well as in RNA from the FB9.1 cell line. These data suggest that there was little or no transcription of the anti-DEN-2 effector RNA construct in family piggyBAC-3xP3GFP-UbD2FB #111. This lack of detectable anti-DEN-2 RNA transcription in family #111 likely accounts for the lack of resistance to DEN-2 replication observed in the *per os* and intrathoracic inoculation challenge of these mosquitoes.

Figure 3.4. DEN-2 antigen in head tissue of transgenic and mosquitoes after DEN-2 challenge. #111, 3xP3eGFP/UbD2FB family #111 transgenic mosquitoes; K^{hw}, control mosquitoes. A. Intrathoracic injection. B. Oral blood feed.

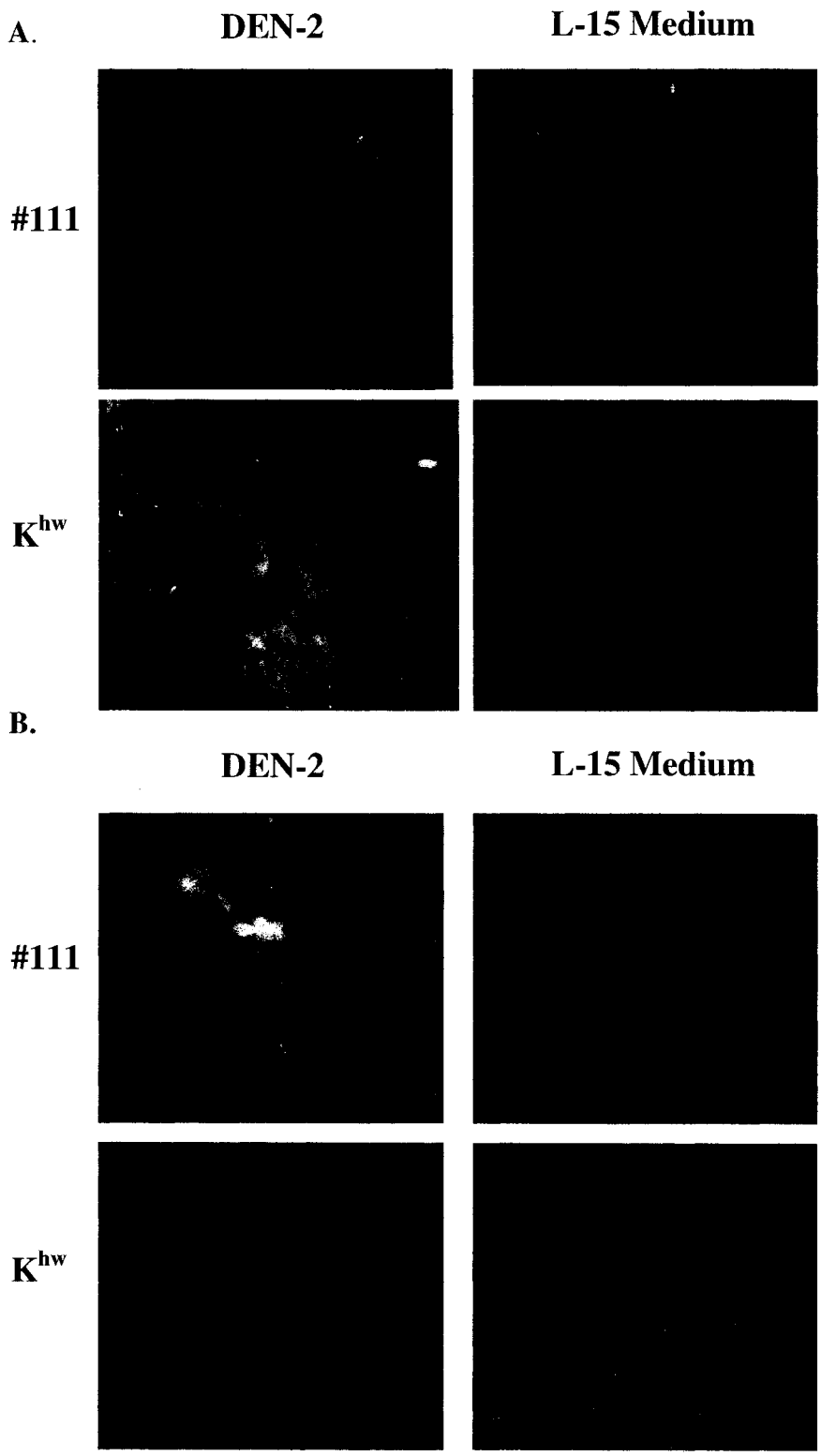
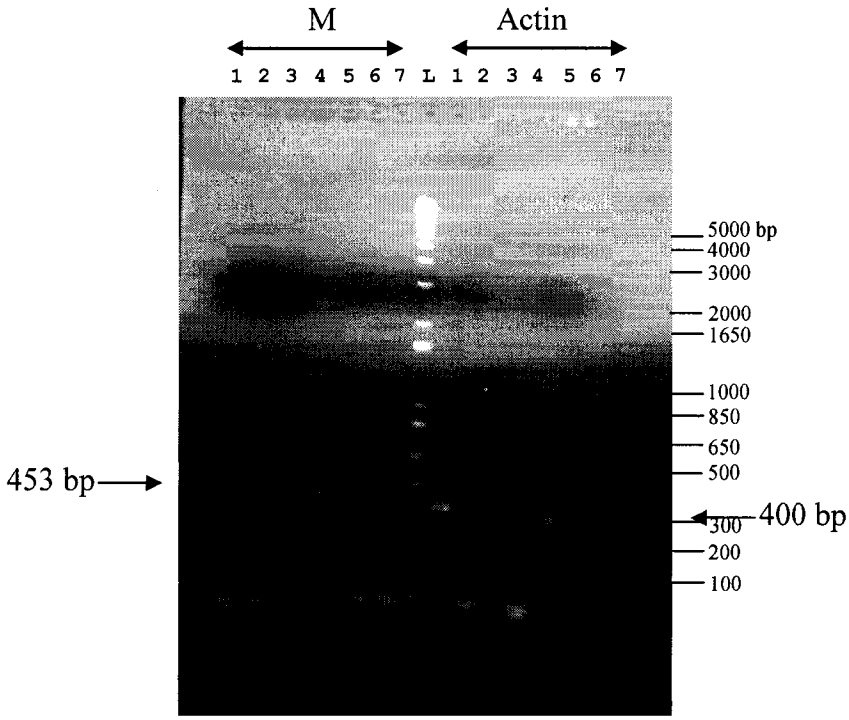


Figure 3.5. RT-PCR analysis of fold-back transgene expression in transgenic mosquito family piggyBAC-3xP3eGFP/UbD2FB #111. Samples were extracted and amplified as outlined in the materials and methods section.



Lane	Sample
1	Family #111 piggyBAC-3xP3GFP-UbD2FB
2	Family #114 piggyBAC-cn
3	Family #93 piggyBAC-cn
4	FB9.1cells
5	3xP3eGFP
6	K^{hw}
7	Negative control, no template
L	1KB plus ladder (3 μL, Invitrogen)

Table 3.4. *Per os* DEN-2 challenge of transgenic mosquitoes.

Transforming Construct	Family	IFA Result GFP (+) Transgenic	IFA Result GFP (-) Non-Transgenic
pMos/Ub800-2-i-2	P3 purple, G_7	75% (12/16)	46.7% (7/15)
	18 green, G_6	78.6% (11/14)	66.7% (2/3)
	52 green, G_7	50% (2/4)	n.d.*
pMos/Ie-2-i-2	P1 blue, G_6	25.7 % (9/35)	50% (4/8)
	P3 blue, G_6	74.3% (26/35)	100% (5/5)
	P5 blue, G_6	90.1% (10/11)	100% (4/4)
	35 blue, G_5	83.3% (5/6)	100% (4/4)
	45 blue	80% (4/5)	80% (4/5)
	57 blue	n.d.	n.d.
	P2 green	7.8% (42/54)	70% 7/10
	P3 green, G_6	81.8% (9/11)	66.7% (6/9)
	P5 green	65.2% (15/23)	63.6% (7/11)
	8 green, G_7	51.9% (14/27)	55.6% (10/18)
	44 green	59.4% (19/32)	56.3% (9/16)
	31 red, G_5	85% (34/40)	68.2% (15/22)
pMos/Ub1300-2-i-2	10 orange, G_3	86.9% (20/23)	100% (5/5)
	13 orange, G_3	100% (14/14)	100% (4/4)
	47 orange, G_4	100% (7/7)	90% (18/20)
	100 orange, G_4	42% (5/12)	68.4% (13/19)
pMos/Carb-2-i-2	P3 gray, G_3	50% (2/4)	78.6% (11/14)
	31 gray, G_3	66.7% (2/3)	100% (5/5)
	81 gray, $G_{3/4}$	71.9% (23/32)	90% (37/41)
	87 gray, G_3	60% (6/10)	55.6% (5/9)
piggyBAC-3xP3GFP UbD2FB	111 white, $G_{4/5}$	73.5% (36/49)	78% (57/73)
Control Strains	K^{hw}	n.a.**	78.6% (33/42)
	HWE	n.a.	74.3% (52/70)
	Rex-D	n.a.	81.8% (36/44)
	3xP3-eGFP	n.a.	77.3% (34/44)

*n.d., not done; **n.a., not applicable; colors represent tracking of each round of injection since numbering was re-started each time.

Table 3.5. Intrathoracic injection DEN-2 challenge of transgenic mosquitoes.

Transforming Construct	Family	IFA Result GFP (+) Transgenic	IFA Result GFP (-) Non-Transgenic
pMos/Ub800-2-i-2	P3 purple, G_2	100% (4/4)	n.d.
	18 green, G_4	70% (35/50)	n.d.
	52 green, G_3	80% (8/10)	n.d.
pMos/Ie-2-i-2	P1 blue, G_2	100% (15/15)	n.d.
	P3 blue	93.3% (14/15)	n.d.
	P5 blue	100% (7/7)	n.d.
	35 blue, G_2	100% (3/3)	n.d.
	45 blue	n.d.	n.d.
	57 blue, G_2	88.4% (38/43)	n.d.
	P2 green	100% (84/84)	n.d.
	P3 green, G_2	87.5% (7/8)	n.d.
	P5 green	n.d.	n.d.
	8 green	100% (13/13)	n.d.
	44 green		n.d.
	31 red	n.d.	n.d.
pMos/Ub1300-2-i-2	10 orange	n.d.	n.d.
	13 orange	n.d.	n.d.
	47 orange	n.d.	n.d.
	100 orange	n.d.	n.d.
piggyBAC-3xP3GFP UbD2FB	111 white, $G_{4/5}$	97.8% (46/47)	98.7% (78/79)
Control Strains	K^{hw}	n.a.	100% (25/25)
	HWE	n.a.	98.1% (53/54)
	Rex-D	n.a.	100% (23/23)
	3xP3-eGFP	n.a.	100% (25/25)

*n.d., not done; **n.a., not applicable; colors represent tracking of each round of injection since numbering was re-started each time.

DEN-2 challenge of pMosUb800/2-i-2, pMosUb1300/2-i-2, and pMos/Ie2-i-2 transgenic mosquitoes. The second approach to generating DEN2 resistant transgenic mosquitoes involved using the *MosI* (*Mariner*) transposable element containing the inverted repeat DEN2 prM 290 nt separated by the sialokinin major intron, abbreviated 2-i-2. The piggyBAC element described earlier in the first approach, exhibited instability and appeared to move multiple times per generation in experiments designed to pinpoint integration site (Adelman et al., 2004). The *MosI* element has been successfully used by other groups to generate transgenic *Ae. aegypti* (Coates et al., 1998, Coates at al., 2000). Generation of these families by embryo injection was performed as described in the Materials and Methods section, and dried egg liners were sent to AIDL/CSU as G_3 or higher for challenge. Briefly, three different promoters were tested; two versions of the *D. melanogaster polyubiquitin* promoter (Ub800 and Ub1300) and the baculovirus *immediate early 1* promoter (Ie1). The anti-DEN-2 construct tested in the *MosI* element transgenesis experiments contained an intron spaced anti-DEN-2 dsRNA (see Table 3.1).

Three transgenic families were generated by transformation with the pMosUb800/2-i-2 construct (P3 purple, 18, and 52 green), four with the pMosUb1300/2-i-2 construct (10, 13, 47, and 100 orange) and 12 with the pMosIE/2-i-2 construct (P1, P3, P5, 35, 45, and 57 blue, P2, P3, P5, 8, and 44 green, 31 red). All families (except one pMosIE/2-i-2 family that was lost due to insufficient egg production) were analyzed for resistance to DEN-2 infection. Each family was challenged *per os* (Table 3.4) with an infectious blood meal prepared from cell culture propagated DEN-2 (Jamaica 1409) virus containing ~7.5 $\log_{10}TCID_{50}$ per ml of infectious virus (Bennett et al., 2002). After challenge, mosquitoes were held and assayed as described previously. Additionally,

DEN-2 was intrathoracically injected (Table 3.5) into any transgenic individuals that were not engorged after blood feeding. In all cases, there was no observable difference in the percentage of mosquitoes with a disseminated infection in the transgenic group when compared to the parental HWE control strain challenged at the same time.

Integration and expression of the transgene sequence was assayed at UCI and Southern blot analysis demonstrated integration of the entire transforming construct in all families (Z. Adelman, unpublished data not shown; Travanty et al., 2004). Northern blot analysis detected DEN-2 prM RNA in whole body RNA preparations (See Figure 3.6) but not in extracts from the midguts of transformed mosquitoes (Z. Adelman, unpublished data not shown; Travanty et al., 2004). Specifically, all 3 pMosUb800/2-i-2 families, two of the four pMosUb1300/2-i-2 families (10 and 100) and 8 of the 12 pMosIe/2-i-2 families (P5, P3, P1, 35 and 57 blue, P3 and 8 green, and 31 red) gave positive signals on the northern blots on preparations from whole mosquitoes. The lack of signal in midgut preparations indicated that RNAi against DEN-2 was not induced in every tissue of the mosquito, possibly because expression may have been limited. It is possible that RNAi against DEN functioned in certain tissues of these transgenic mosquitoes, but we were unable to detect it by simply looking at virus dissemination.

In at least one transgenic line, pMosIe/2-i-2 P1 blue, production DEN-2 specific-small RNAs of the same size class as siRNAs was detected in whole mosquitoes (see Figure 3.7). This suggested that RNAi was induced in that transgenic line in some tissues, but silencing of DEN-2 replication was not observed on the level of the whole mosquito. Therefore, siRNA expression was likely not present in all relevant tissues for virus replication and transmission (Figure 3.7).

Figure 3.6. Northern analysis of representative transgenic families generated from the pMosUb800/2-i-2, pMosUb1300/2-i-2, and pMosIe1/2-i-2 constructs demonstrates expression of the anti-DEN-2 RNAi effector RNA. Each family is denoted by generation number (G#) followed by family or pool number, and homozygous families are marked (hom). Arrows indicate position of the DEN-2 specific RNA. Larger non-specific bands represent ribosomal RNA (rRNA).

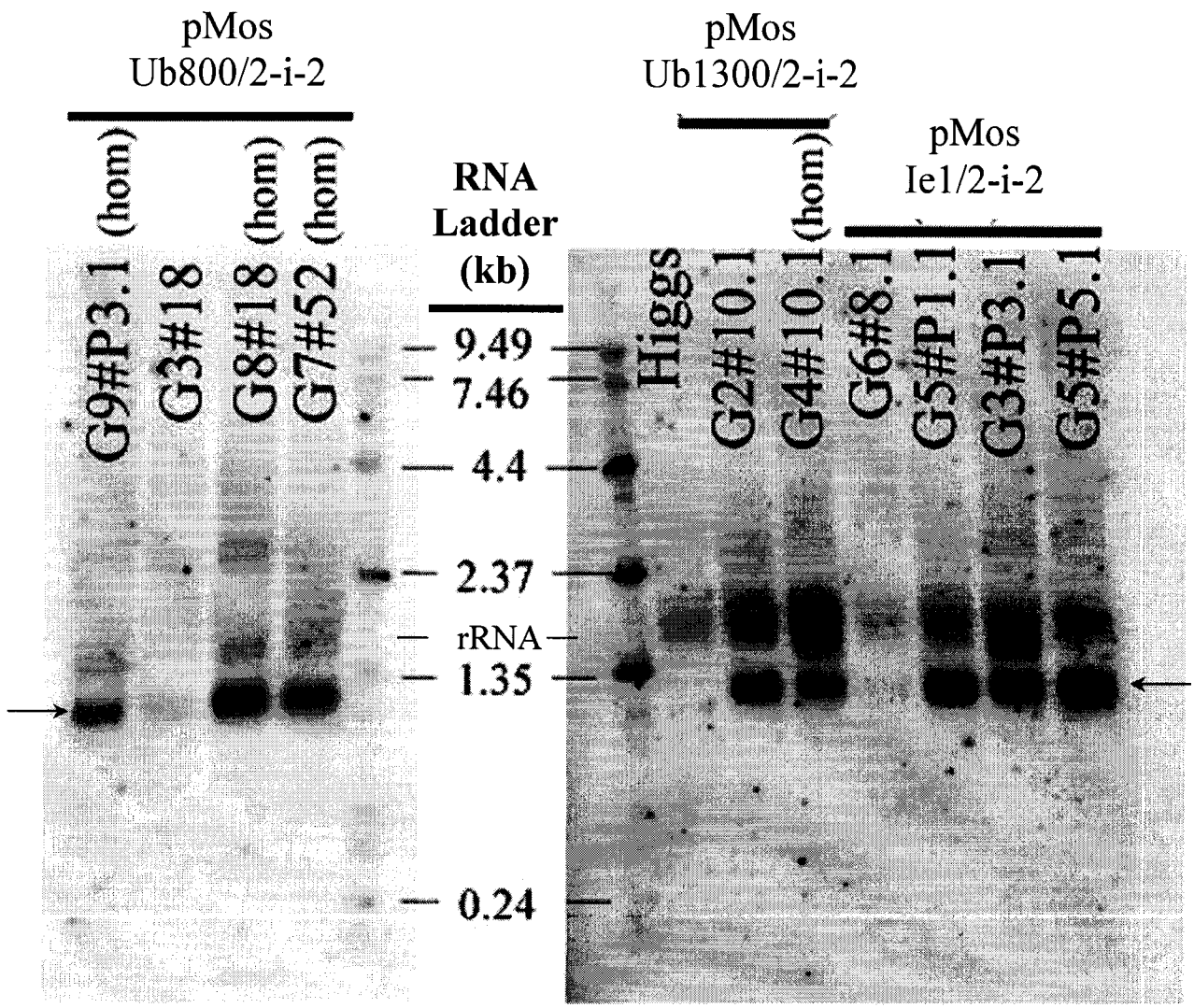
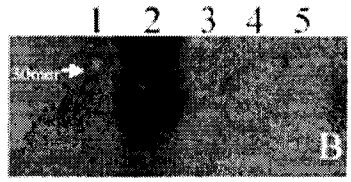


Figure 3.7. Detection of DEN-2 siRNAs in pooled RNA from a transgenic line of *Aedes aegypti.* Total RNA extracted from 80 whole mosquitoes per transgenic family. Sample order: 1) Transgenic Family pMosUb800/2-i-2 P3; 2) Transgenic Family pMosIE/2-i-2 -1 P1; 3) 20 nt oligo (50uM); 4) 10 bp step ladder; 5) Transgenic Family pMosUb800/2-i-2 #18. **A. SYBR-green II-stained 12% polyacrylamide/8M urea/1X TBE gel. B. Northern blot of RNA gel from part A.** α -P32-UTP labeled RNA probe derived from the prM region of DEN-2 virus RNA.

A.

B.



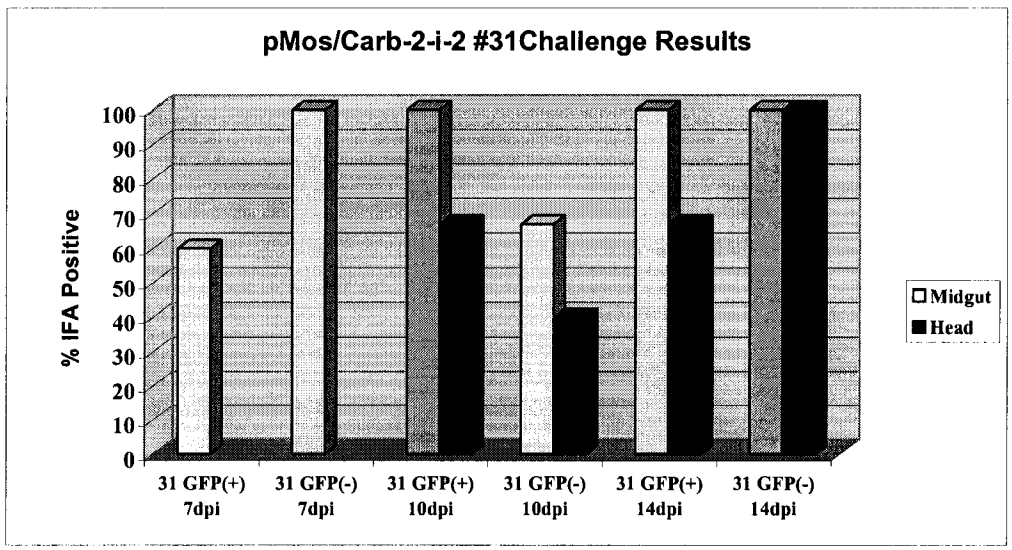
DEN-2 challenge of pMos/Carb2-i-2 transgenic mosquitoes. Anti-DEN-2 dsRNA was detected in a number of the transgenic families generated from the *Ub* and *Ie1* promoter constructs, and small DEN-2 specific RNAs in the size range of siRNAs were detected in one of these families. Despite detection of the effector, no resistance to DEN-2 was observed in any of these families. It was hypothesized that the lack of anti-DEN-2 expression in the midgut resulted in the lack of resistance. In order to address the issue of midgut expression, a new transforming construct that directed expression in the midgut in response to blood feeding was developed. This new construct utilized the *Ae. aegypti carboxypeptidase* promoter to drive transcription of the anti-DEN-2 effector (see Figure 3.10, part A). Four transgenic families were derived from this transformation experiment (pMosCarb/2-i-2; P3, 31, 81 and 87; Table 3.1). All four families were challenged as described previously. Midguts and head tissues were analyzed at 7, 10, and 14 days post challenge and none showed any resistance to DEN-2 (Tables 3.6, Figure 3.8 and 3.9). Figure 3.9 shows representative stained midguts from transgenic (panels A-C, GFP(+)) and non-transgenic (panel D, GFP(-)) pMosCarb/2-i-2 #81 siblings 14 days after *per os* DEN-2 challenge. Challenges were performed *per os* only. No intrathoracic injections were done with these lines because the injection route allows the virus to by-pass the midgut and establish infection in other tissues. The midgut was the only tissue in which RNAi was targeted, therefore only the midgut infection route was analyzed. Southern blots performed at UCI confirmed integration of the transgene construct in all the families (see Figure 3.10, part B), but the northern blot analysis could not detect transcription of the inverted repeat RNA in preparations from the midguts 24 hours post feeding on a non-infectious blood meal (see Figure 3.10, part C).

Table 3.6. ***Per os*** **DEN-2 challenge of transgenic mosquitoes with blood meal inducible promoter, pMos/Carb-2-i-2.** Head tissue was assayed for DEN-2 antigen at 14 days post challenge.

Transforming Construct	Family	DEN-2 Antigen GFP (+) Transgenic	DEN-2 Antigen GFP (-) Non-Transgenic
pMos/Carb2-i-2	P3, G_3	80% (12/15)	80% (4/5)
	31, G_4	66.7% (2/3)	100% (5/5)
	81, $G_{3/4}$	76.9% (20/26)	88.9% (24/27)
	87, G_3	70.6% (12/17)	86.7% (13/15)

Figure 3.8. ***Per os*** **DEN-2 challenge of transgenic mosquitoes with blood meal inducible promoter, pMos/Carb-2-i-2, reveals no difference between transgenic and non-transgenic mosquitoes.** Midguts and head tissue were assayed for DEN-2 antigen at 7, 10 and 14 days post infection and infection of transgenic mosquitoes (GFP(+)) was compared to non-transgenic mosquitoes (GFP(-)). **A.** Family # 31. **B.** Family #81

A.



B.

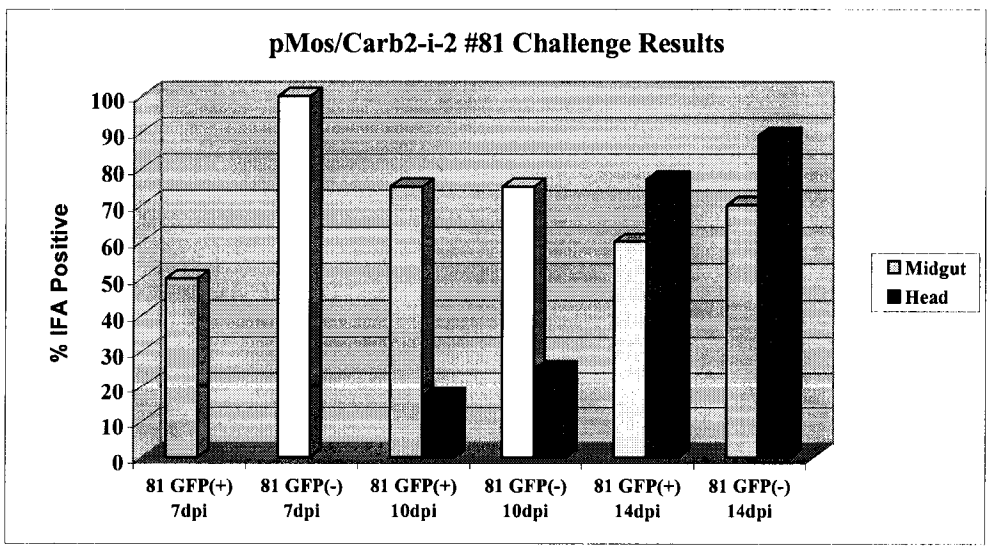


Figure 3.9. Midgut staining of pMos/Carb2-i-2 transgenic mosquitoes after oral challenge with DEN-2. A-C. GFP (+) transgenic. D. GFP (-) sibling.

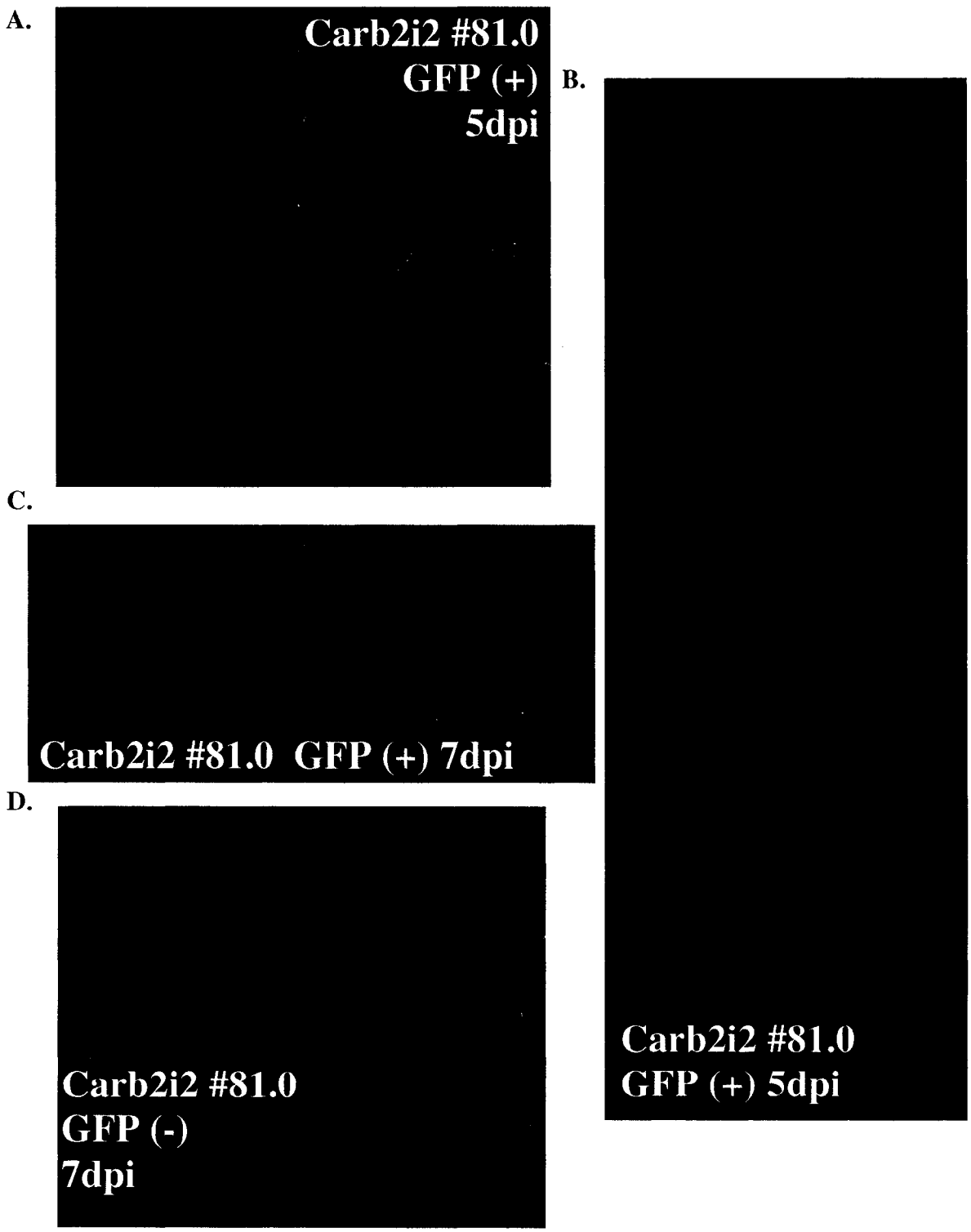
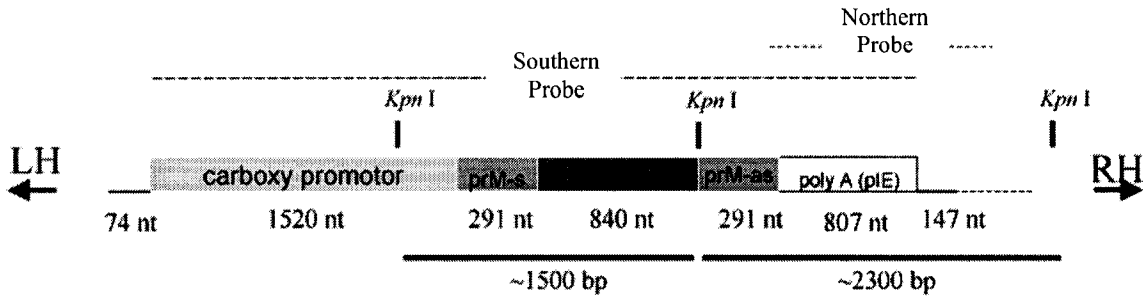


Figure 3.10 Transgenic mosquitoes carrying the pMosCarb/2-i-2 construct do not express DEN-2 specific RNA.

A. Diagram of pMosCarb/2-i-2 transforming construct showing the left hand (LH) and right hand (RH) ends of the *MosI* transposable element, *carboxypeptidase* promoter, anti-DEN-2 dsRNA effector, *Kpn* I sites and the position of probed used for Southern and northern analysis.



B. Southern analysis of pMosCarb/2-i-2 genomic DNA demonstrated integration of the transgene in families. Genomic DNA was digested with *Knp* I and probed for integration of the transforming construct using probe indicated in part A. H, HWE control, non-transgenic mosquitoes; pMosCarb/2-I-2 families #31, 81, 87 and Pool #3.

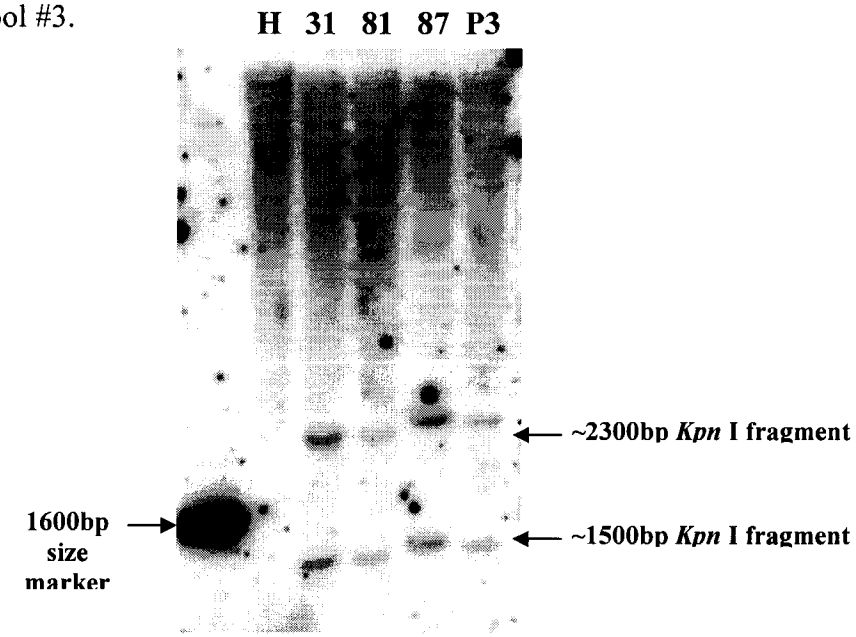
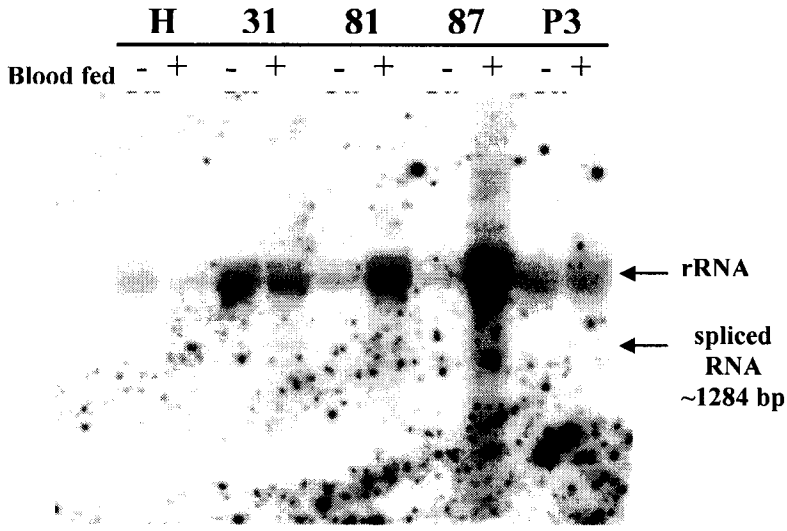


Figure 3.10, continued.

C. Northern analysis reveals that blood-feeding failed to induce transcription of the anti-DEN-2 RNA. Mosquitoes were fed a non-infectious blood meal and midguts were dissected 24 h post-feeding from both the blood fed (+) and non-fed controls (-). Total RNA was etracted and probed for expression of the spliced anti-DEN-2 effector RNA using the probe indicated in part A. Unspliced RNA is ~2142 bp and spliced RNA is ~1284 bp, just below the ribosomal RNA (rRNA) band as indicated by the arrows. H, HWE control, non-transgenic mosquitoes; pMosCarb/2-I-2 families #31, 81, 87 and Pool #3.



Discussion

In the course of this work, transgenic *Aedes aegypti* mosquitoes were successfully generated. Embryos were injected and surviving adults were used to found families that were intercrossed in order to obtain homozygosity for the transgene. These mosquitoes were selected based on expression of the eye-specific eGFP marker gene and subjected to challenge with DEN-2 virus.

Two different transposable elements were used to direct integration in these mosquitoes. First, the piggyBAC transposable element was used successfully to generate family piggyBAC-3xP3eGFP-UbD2FB #111 (Tables 3.2 and 3.3). As described in Table 3.1, this family contains the same anti-DEN-2 dsRNA construct that had previously been used to generate RNAi against DEN-2 in cell culture (Adelman et al., 2002). Upon challenge with DEN-2, there was no observable difference in DEN-2 infection and dissemination between the transgenic mosquitoes and mosquitoes from the non-transgenic parental strain, K^{hw} (Tables 3.4 and 3.5). Expression of the anti-DEN-2 construct was examined by RT-PCR, but no expression was detected in the transgenic family #111 mosquitoes (Figure 3.5). Ultimately, however, this element was found to be unstable and resulted in transposon hopping during each subsequent generation (Adelman et al., 2004).

Next, the *MosI* transposable element was used to generate additional families of transgenic *Ae. aegypti* mosquitoes. Transformation with the *MosI* element resulted in numerous transposition events and in the generation of multiple transgenic families. *MosI* was stable in these mosquitoes since the number of transgenic individuals within a

given family followed predictions based on a mendelian pattern of heredity, with the number of transgenic offspring increasing during out-crossing and inter-crossings resulted in homozygosity. In this set of experiments, three different promoters were tested for their ability to drive transcription of the same anti-DEN-2 intron construct (Table 3.1). Three transgenic families were made using pMosUb800/2-i-2 and all three were found to contain DEN-2 effector RNA (Figure 3.6). Four transgenic families were derived from the pMosUb1300/2-i-2 construct and two of these families were positive by northern blot for DEN-2 effector RNA (Figure 3.6). Embryo injection of pMosIE/2-i-2 resulted in 12 transgenic families, of which 8 expressed the DEN-2 effector RNA (Figure 3.6). Importantly, one of these effector RNA expressing families was also shown to contain DEN-2 specific small RNAs of the appropriate size (~21 nt) for siRNAs (Figure 3.7). Despite the demonstrated transcription of anti-DEN-2 effector RNA, and siRNAs in one family, none of these mosquitoes demonstrated any resistance to DEN-2 upon *per os* or able 3.4) intrathoracic challenge (Tables 3.5).

The results of these transgenesis experiments can be explained in several ways. One hypothesis is that the virus may overcome the interference by an initial burst of midgut replication that overwhelms the RNAi response. An alternative, and more likely, explanation is that the RNAi initiated against DEN-2 in these transgenic mosquitoes occurs in tissues other than the midgut and that the RNAi response and the DEN-2 replication may not be co-localized in the same tissues. Both of these possibilities are currently under investigation in the continuation of this collaborative research effort between UCI and AIDL/CSU.

An alternative hypothesis to account for the lack of resistance to DEN-2 observed in transgenic mosquitoes that express the anti-DEN-2 effector RNA involves concentration. Perhaps there simply was not enough of the effector siRNAs present within the cells of the transgenic mosquitoes to alter DEN-2 replication. All of the challenge experiments were conducted using heterozygous mosquitoes, or a mixed population of heterozygous and homozygous mosquitoes. Once G_3 was obtained, GFP-positive individuals from a specific family or pool were selected as either larvae or pupae and combined for mating with females from the parental strain. This type of mating would ensure that all progeny receive at least one copy of the GFP transgene (and presumably the linked anti-DEN-2 construct), as would result from the mating of a transgenic individual with a non-transgenic individual. There was not, however, any attempt made to select for or against the mating of two transgenic individuals at this stage, because it was not possible to discriminate between expression in the eyes of one or two copies of the eGFP gene using microscopy. Therefore, populations from generations beyond G_3 most likely contained a mixed population of heterozygotes and homozygotes. Once the colonies of each family or pool became large enough to sustain themselves, the out-crossing to parental mosquitoes was eliminated and the transgenic individuals were allowed to interbreed. This interbreeding, with GFP-negative individuals removed as larvae each generation, usually resulted in colonies in which all individuals expressed the eGFP marker by G_7. Again, there was no way to know if all individuals were homozygous using the microscopic screening method, but it was assumed to be highly likely. There may have been, however, unknown selective pressures resulting in decreased the rates of homozygosity in these populations. This

may have had an impact on the DEN-2 resistance phenotypes scored here, since as generation number increased resistance level may also change.

Finally, the effect of transcription of the anti-DEN-2 dsRNA within the mosquito's germ line over multiple generations is unknown. The RNAi-related phenomenon in plants, post-transcriptional gene silencing or PTGS, is known to use a feed back regulation loop in addition to mRNA degradation. In some cases, PTGS of a transgene homologous to an endogenous gene resulted in down regulation of the endogenous gene copy due to methylation of the DNA. This step is not thought to occur in mosquitoes, but it is possible that large amounts of dsRNA irrelevant to the mosquito's own life cycle may not be tolerated, particularly during development where RNAi plays a major role in regulated insect gene expression through the microRNA pathway.

In order to inhibit the initial step in infection of the mosquito, another transforming construct was made in which the *Ae. aegypti carboxypeptidase* gene promoter was used for expression of the DEN-2 RNA to specifically target the first tissue encountered by DEN on its path through the mosquito, the midgut epithelium. *Carboxypeptidase* is a female midgut-specific gene, and expression from the *carboxypeptidase* promoter is induced by blood feeding (Edwards et al., 2000). Expression begins by eight hours after the female takes a blood meal, peaks at 20-24 hours and decreases by 30 hours post feeding. This promoter was used in these studies to drive the same *sialokinin* intron-spaced DEN-2 prM inverted repeat construct used with the constitutive *Ub* and *Ie1* promoters. Unfortunately, no resistance was observed in any of the transgenic mosquito families generated using this promoter (Table 3.6). It was demonstrated by northern blot analysis (Figure 3.10, panel C) that expression of the anti-

DEN-2 effector RNA was not induced upon blood feeding. This result was unexpected, especially since the Southern analysis shown in Figure 3.10 (part B) confirmed integration of the transgene within each of these families.

There are a number of possible explanations for these observations. The anti-DEN effector RNA may not have been transcribed in these transgenic families using the *carboxypeptidase* promoter construct. All transposition events are subject to position effects due to where the transgene integrates relative to other signals within the host genome. However, the lack of expression due to position effects seems unlikely due to the detectable expression of the eGFP eye marker. Additionally, perhaps if the RNAi effector was transcribed in these mosquitoes, the transcripts were unstable in the environment of the mosquito midgut epithelial cell. It is likely that signals within a 3'UTR play a role in the stability and turnover of mRNAs. The 3'UTR used in this construct is derived from the baculovirus *Ie1* polyadenylation sequence and this sequence is foreign to a mosquito midgut. Using a midgut-specific 3'UTR (e.g., *carboxypeptidase*) containing tissue specific signals may stabilize the transcript within the environment of the midgut. Yet another alternative explanation is that mRNA turnover in a midgut may occur at an elevated rate, especially after a blood meal, due to the amount of processing that must occur to digest the blood meal. In this case, the anti-DEN-2 transcript may be rapidly degraded as a consequence of blood meal digestion.

As work towards the ultimate goal of a mosquito engineered to be refractory for DEN replication continues, additional new transforming constructs engineered to address the issue of RNA expression and stability in different tissues also under investigation. The new constructs under development at AIDL and UCI seek to address each one of the

problems observed in the first sets of constructs discussed above, as well as a number of potential problems that can be foreseen. Replacing the 840 bp *sialokinin* gene major intron with the shorter 62 bp *sialokinin* gene minor intron, in the construct pMosCarboxy/2-short(s)i-2, may increase efficiency of intron processing as well as decrease the amount of insert devoted to non-dsRNA forming sequence. To address the problem of tissue-specific mRNA instability in the *carboxypeptidase* based promoter constructs, the 3'UTR from the baculovirus *ie1* transcript used previously will be replaced with the gene-specific *carboxypeptidase* 3'UTR. Using the 3'UTR that matches the promoter used to drive the anti-DEN effector RNA should result in a dsRNA expression profile that more closely mirrors that seen with the endogenous *carboxypeptidase* gene.

New tissue-specific promoters that are active in the tissues most important for DEN replication and transmission, the midgut and the salivary gland, are also under investigation for potential to drive the anti-DEN RNAi response. These include additional midgut promoters such as those for *glutamine synthetase* (Smartt et al., 2001), *glucosamine: fructose-6-phosphate aminotransferase* (GFAT; Kato et al., 2002) and *ferritin heavy-chain* (Pham et al., 2003) that are induced or upregulated upon blood feeding, and salivary gland promoters such as D7 (Arca et al., 1999) and *apyrase* (Johnson et al., 1999; Coates et al., 1999; Lombardo et al., 2000). In addition, new target regions in the DEN genome, including the *nonstructural 5* (*NS5*) gene may provide a more accessible sequence target for RNAi. Increasing the size of the dsRNA from the 290 bp currently in use to ~580 bp would result in an increase in the amount and variety of anti-DEN specific siRNAs and could make the RNAi response more efficient. RNAi in

mosquitoes has been demonstrated to be a dosage dependent mechanism (Brown et al., 2003) therefore it will be important to determine the number of insertions in each line and select those with multiple copies to examine the correlation between transgene expression and silencing of DEN.

Since the completion of this work, further characterization of the pMosIe1/2-i-2 P1 blue family by Dr. Irma Sanchez-Vargas has revealed a decreased rate of DEN-2 infection in the head, thorax and abdomen, but not in the midgut, compared to the HWE parental strain. Also, the *carboxypeptidase* promoter has been re-engineered by Dr. Alexander Franz, shortened by removing sequence from the 5' end that may have contained transcriptional inhibitors. This new *carboxypeptidase* construct has been used to generate additional transgenic lines and shown to effectively drive transcription of the anti-DEN2 dsRNA within the transgenic mosquitoes. The ability of these lines to resist DEN-2 replication is currently under investigation. The re-engineering of the *carboxypeptidase* promoter underscores the importance of promoters in the generation of transgenic mosquitoes. This new data also demonstrates that the hypothesis underlying this work is still valid and that the ultimate goal of a transgenic mosquito resistant to DEN-2 using an RNAi based approach is still a very real possibility. The choice of promoter used to drive transcription of the anti-DEN-2 effector RNA within a transgenic mosquito is extremely important in achieving an RNAi-based resistance to DEN replication. The promoter conditions the time, place and level of transcription of the transgene, thereby directing the RNAi response. Choosing the appropriate promoter may allow the RNAi response to be specifically targeted to the appropriate time (concurrent with, or just prior to, blood feeding since this is the route of viral entry into the

mosquito), tissues (midgut and salivary glands since these are the key tissues for virus replication and transmission), and level (strong promoter activity will result in a high level of effector RNA and consequently, a stranger RNAi response). Ultimately, a combination of midgut and salivary gland promoters driving RNAi constructs directed against multiple DEN genes in the same transgenic mosquito family may give the best chance at developing a resistant organism.

Chapter 4

Summary

Chapter 4 Summary

The underlying goal of this research was to understand to relationship between dengue virus and its host and host factors that condition DEN infection at the molecular level and the host antiviral RNAi response. A complex interaction between two arboviruses, dsSIN and DEN-2, was uncovered in which persistent infection by one virus, TE3'2J, increased the replication of a second unrelated virus, DEN-2. These data demonstrate that infection with a dsSIN may alter the mosquito's ability to mount an anti-viral response to another virus. The dsSIN virus may contain an RNAi inhibitor or may overload the RNAi pathway rendering it unable to respond to infection with a second virus.

RNAi is a highly conserved innate immune pathway triggered by dsRNA. It has been extensively studied in *Drosophila* and is only beginning to be understood in mosquitoes. Recent advances such as the *Anopheles gambiae* and *Aedes aegypti* genome sequencing projects have revealed that many components of RNAi are present in mosquitoes, including Dicer and Argonaute2. At the time this dissertation research began, this information was not yet available. The phenomenon of RNAi was investigated as a potential control strategy for the replication of DEN-2. Evidence for RNAi against DEN-2 in mosquito cell culture and cell extracts was presented in chapter 2. RNAi was initiated in cultured cells by either the expression of a dsRNA trigger from a plasmid within the cell, or by the introduction of the siRNA effector molecules directly to the cell culture.

Using methods described for *Drosophila*, an *in vitro* RNAi system for mosquitoes was developed. The results were mixed in that there was sequence evidence for specific degradation of mRNA in mosquito cell extracts that had been primed or triggered for RNAi by homologous virus replication, but there was a significant amount of non-specific RNAse activity present in both the infected and control extracts prepared from mosquito cells. This non-specific degradation was not observed in the extracts prepared from *Drosophila* cells. The nature of the non-specific RNA degradation remains to be investigated and may have important implications for virus replication in mosquitoes.

Cells expressing an anti-DEN-2 hairpin dsRNA derived from the prM sequence, FB.1 cells, are resistant to DEN-2 challenge. They do not accumulate any sign of virus infection as assayed by detection of viral antigen, viral genomic RNA or production of new virus upon challenge with DEN-2. The hairpin dsRNA expressing cells were also found to be resistant to DEN-3 replication. This observation was explained by examining an alignment of the nucleotide sequences of DEN-2 prM and DEN-3 prM. These two sequences have many regions in common, including at least one highly conserved stretch in which 21 of 23 bases are identical in both sequences.

The hypothesis of a cross re-active RNAi response was responsible for DEN-2 and DEN-3 resistance in the cells came from studies with WNV. As a more distantly related flavivirus, WNV has lower sequence homology to DEN-2. WNV was found to replicate equally well in the DEN2-specific hairpin RNA expressing cells as in the control cells, as assayed by detection of viral antigen. The cross-reactive resistance phenomenon observed with DEN-2 and DEN-3 did not extend to WNV. Cells were tested to ensure they were not expressing any portion of the M protein since it has been

shown in the literature that the M protein can have anti-viral properties (Lee and Schloeder, 1981a; 1981b). Antibody staining for prM in uninfected FB9.1 cells confirmed that they were not producing any protein from the transforming plasmid construct. Importantly, the cells were able to produce prM at a low level only after infection with DEN-2. Here the prM protein production was presumably directed by the infecting virus itself and not by the plasmid sequence since this sequence did not include an initiation codon for M protein expression (Adelman et al., 2002).

An important outcome of these studies was the observation that a dsSIN that expressed a portion of the DEN-2 genome, dsMRE16-eGFP/D2prM, was subjected to the same RNAi response as an entire DEN virus. These results have important implications for the mosquito transgenesis experiments. Sindbis viruses replicate much faster than dengue virus, and the inclusion a marker gene such as GFP allows for real time tracking of the represents an important screening tool for the generation of transgenic mosquitoes silenced for DEN by RNAi.

Examination of the pool of small RNAs present in C6/36 and FB9.1 cells before and after infection with either DEN-2 or DEN-3 virus provided a window into the role of RNAi in controlling DEN-2 virus replication as well as its role in routine gene expression. Two of the small RNAs from FB9.1 cells infected with DEN-2 were found to be specific for DEN-2. Candidate siRNAs were identified on the basis of their size and homology to sequence from mosquitoes, mice and humans. The function of these potential siRNAs remains to be confirmed but they may help to understand control of gene expression in mosquitoes and the limits of RNAi in controlling virus replication. siRNA function could be confirmed by transfection of synthetic siRNAs with each of the

small RNA sequences and observation of the effect on cells and on the stability of homologous RNAs, perhaps using an RNAse-protection assay. The availability of the *Aedes aegypti* genome sequence will greatly enhance the validation of the function of these potential siRNAs. As mosquito mRNAs are assigned a function it may be possible to determine, based first on sequence homology and later functionally, if these potential siRNAs function in the regulation of gene expression in mosquitoes.

RNAi was shown to be responsible for inhibition of dengue viruses in cell culture. The next step was to translate these results into the entire mosquito using transgenesis to express dsRNA derived from DEN-2 genome in the whole mosquito. A number of transformed lines were developed and examined for resistance to DEN-2. The first attempt at transgenesis-based interference used a construct that expressed the same hairpin dsRNA that effectively blocked DEN-2 replication in cell culture. The dsRNA was introduced into mosquitoes using the piggyBAC transposable element and transcription was directed by the *Drosophila polyubiquitin* promoter (Ub) that had been shown to be active in mosquito cell culture. Although transformation of *Aedes aegypti* was observed with this construct, as demonstrated by expression of the eye-specific eGFP marker gene and analysis of the genomic DNA, no resistance to DEN-2 was observed. That these experiments did, however, yield some interesting results. Data from transgenic families made with the piggyBAC transposable element revealed that this element is not stable in *Aedes aegypti* and its use for transformation results in the generation and loss of arrays of the transposable element with each subsequent generation.

The second attempts at a transgenic approach to silencing DEN-2 replication incorporated two major changes in the transforming construct. First, the transposable

element was changed to the mariner element, *MosI*, which appeared to be stable in *Aedes aegypti*. Second, the anti-DEN-2 dsRNA was generated not from a hairpin as previously described, but from a sense sequence and an antisense sequence of exact reciprocal homology separated by an intron. This new sequence was used in order to generate a more perfect stretch of dsRNA without an intervening hairpin by splicing out the intron. The use of introns to generate dsRNA had been effectively employed in RNAi-based interference strategies for plant viruses. In the *MosI* transposable element constructs, three constitutively active promoters were tested to drive transcription of the anti-DEN-2 dsRNA. Two different forms of the Drosophila Ub promoter (an 800 bp version previously tested in the piggyBAC constructs and a 1300 bp version that included more of the upstream regulatory sequences) were tested. Additionally, the baculovirus *immediate-early 1* gene promoter was also tested since this was the same promoter that effectively drove transcription of the dsRNA hairpin in the DEN-2 resistant FB9.1 cells. A large number of families were generated with the *MosI* element and these various constitutively active promoters. None of the transgenic mosquitoes showed any significant resistance to DEN-2 challenge, despite the presence of DEN-2 specific RNA in many of the families. One family expressed anti-DEN-2 siRNAs, but the siRNAs did not appear to be expressed in the tissues that are critical for DEN infection, specifically the midgut. The results of these transgenesis experiments can be explained by the hypothesis that RNAi occurred in tissues other than the midgut or that the RNAi response and the DEN replication may not be co-localized in the same tissues. Perhaps the issue is one of concentration and there is simply not enough of the effector siRNAs present within these transgenic mosquitoes to alter DEN-2 replication. This hypothesis is

supported by the efficient silencing of DEN-2 observed in the FB9.1 cell line. This cell line was found to contain close to one thousand copies of the plasmid directing transcription of DEN-2-specific siRNAs. With that amount of trigger, it seems likely that the concentration of DEN-2 specific siRNAs would be high in a cell prior to DEN-2 infection, resulting in the strong replication inhibition phenotype observed.

In order to address some of these issues, a third transforming construct was generated based on the *MosI* transposable element. In this case, however, a tissue specific inducible promoter was investigated. The *Aedes aegypti carboxypeptidase* promoter was used to drive transcription of the intron-based dsRNA construct. It was hypothesized that using a promoter from the same species would result in efficient transgene expression and work from other labs using this same promoter had resulted in transgenic mosquitoes that could express the transgene with the same kinetics as the natural mosquito gene. In the mosquito, the endogenous carboxypeptidase promoter is female specific and up regulates expression within the midgut tissue in response to blood feeding. Since blood feeding is the natural route of DEN-2 infection, this promoter seemed the ideal choice. The transgenic families generated with this conditional promoter all expressed the marker gene, eGFP, in the eye tissue. None of these mosquitoes demonstrated resistance to DEN-2 and transcription of the anti-DEN-2 dsRNA was not detected in the midgut tissues. It was hypothesized that this was due to position effects in which the genomic sequence surrounding the site of transposable element integration altered the expression pattern of the transgene. There were, however, four separate transgenic families, each with its own unique integration event, derived from these experiments and it seemed unlikely that position effects could account for the

negative silencing results in all four families. Indeed, further examination of the *carboxypeptidase* promoter construct by other researcher revealed problems that have subsequently been addressed and the new *carboxypeptidase* promoter constructs are currently in use to generate transgenic mosquitoes that appear to have reduced DEN-2 replication.

Perhaps the most important finding described in this work is the importance of promoter choice and functionality in the generation of transgenic *Aedes aegypti* mosquitoes. The hypothesis that RNAi silencing of DEN-2 in mosquitoes could be used to control DEN infection in mosquitoes was first demonstrated in cell culture. Each level of the RNAi pathway from initiation with dsRNA, to the siRNA effector, to the small RNA degradation products was described. Demonstration that transgenic *Aedes aegypti* mosquitoes could be generated was also successfully accomplished using selection of the eGFP marker gene. The final step was to show resistance to DEN-2 in these mosquitoes. This stage of the research was not effectively accomplished during the course of this dissertation work, but the data generated here helped to develop protocols for the screening, rearing and infectious blood meal DEN-2 challenge of transgenic *Aedes aegypti*. This work clearly demonstrates the importance of the promoter in mosquito transgenesis and provides support for the ultimate goal of interruption of the DEN transmission cycle by a transgenic mosquito that is incapable of supporting DEN replication due to an RNAi response to the viral genomic sequence.

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Appendix I: Summary of small RNA clones (*denotes no match to sequences in GenBank)

Sequence Number	Cell line RNA Source	Virus Treatment	BLAST results: Best match (GenBank Accession Number)	E-value	Length of GenBank Match (nt)	Blast results: Other interesting matches (GenBank Accession Number)	E-value
1	C6/36	DEN-2, 3dpi	*Mus musculus* synaptotagmin-like homologue (NM_176846.2)	87	22		
2	C6/36	DEN-2, 3dpi	Human DNA clone RP1-300G3 (HSJ300G12)	88	19		
3	C6/36	DEN-2, 3dpi	No significant homology*				
4	C6/36	DEN-2, 3dpi	*Homo sapiens* BAC clone RP11-511B24 (AC012593.8)	81	21		
5	C6/36	DEN-2, 3dpi	*Mus musculus* chromosome 18 clone RP24-182H11 (AC102255.10)	643	15		
6	C6/36	DEN-2, 3dpi	Human sequence from clone RP11-439J5 (AL445190.9)	544	17		
7	C6/36	DEN-2, 3dpi	*Homo sapiens* BAC clone RP11-472B (AC021148.8)	55	15	Kashmir bee virus structural polyprotein (AY452696.1); Kashmir bee viruscomplete genome AY275710.1)	218
8	C6/36	DEN-2, 3dpi	Mouse DNA from clone RP23-414J11 (AL844591.6)	3.9	21		
9	C6/36	DEN-2, 3dpi	Mouse DNA from clone R23-418D21 (AL840635.6)	3.00E-04	27	*Anopheles gambiae* clone (CNS08Z57)	0.019
10	C6/36	DEN-2, 3dpi	No significant homology*				
11	C6/36	DEN-2, 3dpi	Broad Bean necrosis virus gene for triple-gene-block (contains RNA helicase domain)(D86638.1)	0.58	22		
12	C6/36	DEN-2, 3dpi	*Caenorhabditis elegans* cosmid F17E5 (CEF17E5)	60	19		
13	C6/36	DEN-2, 3dpi	*Homo sapiens* BAC clone R11-466G (AC083829.5)	68	19		
14	C6/36	DEN-2, 3dpi	*Homo sapiens* chromosome 8 clone (AC104580.5)	90	25		
15	C6/36	DEN-2, 3dpi	No significant homology*				
16	C6/36	DEN-2, 3dpi	*Lotus corniculatus var. japonicus* genomic DNA clone	164	18		
17	C6/36	DEN-2, 3dpi	*Oryza sativa* cDNA clone (AK058563.1)	0.24	21	*Drosophila melanogaster* genomic DNA clone (AE003710.2) and (AC010122) and (RE13477); *Anopheles gambiae* sequence associated with transposable elements (XM_308194.1)	228
18	C6/36	DEN-2, 3dpi	*Mus musculus* BAC clone RP23-77E (AC126940.4)	74	19		
19	C6/36	DEN-2, 3dpi	*Mus musculus* BAC clone RP23-416P14 (AC123844.4)	92	19		
20	C6/36	DEN-2, 3dpi	No significant homology*				
21	C6/36	DEN-2, 3dpi	*Brugia malayi* Snowski mRNA (AY389813.1)	26	22	*Anopheles gambiae* clone (XM_308385.1)	26
22	C6/36	DEN-2, 3dpi	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	E-121	289	Kashmir bee virus structural polyprotein (AY452696.1); Kashmir bee viruscomplete genome AY275710.1)	73
23	C6/36	DEN-2, 3dpi	Mouse DNA clone RP23-37B4 (AL808126.10)	63	22	*Drosophila melanogaste* r clone (AC022347)	991
24	C6/36	DEN-2, 3dpi	*Anopheles gambiae* partil cds (XM_320687.1)	1.00E-09	88		
25	C6/36	DEN-2, 3dpi	*Yarrowia lipolytica* CIIB99 (X_504422.1)	129	15		
26	C6/36	DEN-2, 3dpi	*Mus musculus* chromosome 5 clone (AC122782.8)	204	18		
27	C6/36	DEN-2, 3dpi	*Xenopus laevis* cDNA MGC:83504 (BC078099.1)	1.6	21	*Anopheles gambiae* DNA fro 2R chromosome clone 11N17(AGA439060); *Drosophila melanogaster* clones (AY129452.1)(AY119490.1)(AC010047.8)(AY75272.1)(NM_079601.2)	*An. gambiae* 1.6; *D. mel* 0.0026
28	C6/36	DEN-2, 3dpi	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	E-125	288		
29	C6/36	DEN-2, 3dpi	*Ralstonia solanacearum* GMI100 chromosome (AL646059.1)	8.00E-06	288	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	5.00E-04
30	C6/36	DEN-2, 3dpi	Aedes aegypti ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	E-125	289		
31	C6/36	DEN-2, 3dpi	*Mus musculus* similar to marapsin 2 (XM_137602.2)	1.7	21		
32	C6/36	DEN-2, 3dpi	Mouse DNA sequence from clone RP23-385E7 (AL731800.11)	429	20		
33	C6/36	DEN-2, 3dpi	No significant homology*				
34	C6/36	DEN-2, 3dpi	*Homo sapiens* lin-10 protein homolog (NM_025187.3)	513	21		
35	C6/36	DEN-2, 3dpi	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	E-144	289		
36	C6/36	DEN-2, 7dpi	*Xylella fastidiosa* 9a5c (AE004068.1)	135	21		
37	C6/36	DEN-2, 7dpi	*Caenorhabditis elegans* cosmid T08D2 (CET08D2)	6.2	20		
38	C6/36	DEN-2, 7dpi	*Plasmodium falciparum* 3D7 chromosome (AE014821.1)	0.001	27		
39	C6/36	DEN-2, 7dpi	*Kluyveromyces lactis* NRRL Y-1140 (XM_452635.1)	134	18		
40	C6/36	DEN-2, 7dpi	Zebrafish DNA clone (AL8311758.22)	7.5	20		
41	C6/36	DEN-2, 7dpi	Zebrafish DNA clone from DKEY-18C8 (BX510324.7)	6.4	20	*Drosophila melanogaster* chromsome (AE003670.4)	6.4
42	C6/36	DEN-2, 7dpi	Genomic sequence for Glycine max (soybean) (AC121763.1)	28	16		
43	C6/36	DEN-2, 7dpi	Genomic sequence for Glycine max (soybean) (AC121763.1)	129	16		
44	C6/36	DEN-2, 7dpi	*Homo sapiens* 12 BAC RP11-372B4 (AC005911.6)	109	17		
45	C6/36	DEN-2, 7dpi	*Homo sapiens* clone RP11-343P23 (AL355612.8)	0.47	26		
46	C6/36	DEN-2, 7dpi	No significant homology*				
47	C6/36	DEN-2, 7dpi	No significant homology*				
48	C6/36	DEN-2, 7dpi	No significant homology*				
49	C6/36	DEN-2, 7dpi	No significant homology*				

Sequence Number	Cell line RNA Source	Virus Treatment	BLAST results: Best match (GenBank Accession Number)	E-value	Length of GenBank Match (nt)	Blast results: Other interesting matches (GenBank Accession Number)	E-value
50	C6/36	DEN-2, 7dpi	No significant homology*				
51	C6/36	DEN-2, 7dpi	Zebrafish DNA sequence from clone BUSM1-44F8 contains novel gene similar to TFPI (tissue factor pathway inhibitor, lipoprotein-associated coagulation inhibitor)	1.8	21		
52	C6/36	DEN-2, 7dpi	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	E-112	427		
53	C6/36	mock	No significant homology*				
54	C6/36	mock	*Rattus novegicus* hypothetical gene (XM_346775.11)	1.8	21		
55	C6/36	mock	No significant homology*				
56	C6/36	mock	No significant homology*				
57	C6/36	mock	Human DNA sequence from clone RP11-419D4 (AL161430.19)	0.5	22		
58	C6/36	mock	*Anopheles bwambae* cytochrome oxidase I (COI) mitochondrial gene product (AF222328.1)	2.00E-19	257		
59	C6/36	mock	Zebrafish DNE sequence from clone DKEY-18C8 (BX510324.7)	6.8	20		
60	C6/36	mock	No significant homology*				
61	C6/36	mock	No significant homology*				
62	C6/36	mock	No significant homology*				
63	C6/36	DEN-2, 14dpi	No significant homology*				
64	C6/36	DEN-2, 10dpi	No significant homology*				
65	C6/36	DEN-2, 10dpi	*Aplysia californica* NMDA-like glutamate receptor protein (AY163562.1)	9.6	43		
66	C6/36	DEN-2, 10dpi	*Ralstonia solanacearum* GMI100 chromosome (AL646059.1)	5.00E-10	85	*Anopheles gambiae* cDNA (XM_312199.1)	8.00E-06
67	C6/36	DEN-2, 10dpi	*Ralstonia solanacearum* GMI100 chromosome (AL646059.1)	3.00E-05	48	Anopheles gambiae cDNA (XM_312199.1)	0.002
68	C6/36	DEN-2, 10dpi	*Rhizobium* BR816 ATP sulfurylase subsunits (nodP) (RBU59507)	0.14	23		
69	C6/36	DEN-2, 10dpi	No significant homology*				
70	C6/36	DEN-2, 10dpi	*Homo sapiens* 3 BAC RP11-451B8 (AC117513.4)	2	25		
71	C6/36	DEN-2, 10dpi	*Mus musculus* BAC clone RP24-554F1 (AC124370.3)	47	22	*Drosphila melanogaster* chromosomal DNA clone (AC010668.7)	185
72	C6/36	DEN-2, 14dpi	Mouse DNA clone RP23-395H4 (AI928577.21)	0.75	22		
73	C6/36	DEN-2, 10dpi	No significant homology*	148	21		
74	C6/36	DEN-2, 10dpi	No significant homology*				
75	C6/36	DEN-2, 14dpi	No significant homology*				
76	C6/36	DEN-2, 10dpi	*Lepimer dorsalis* genomic fragment RAPD(LDO244007)	1.00E-05	30	*Anopheles gambiae* cDNA (XM_309483.1)	6.00E-04
77	C6/36	DEN-2, 10dpi	No significant homology*				
78	C6/36	DEN-2, 10dpi	No significant homology*				
79	C6/36	DEN-2, 10dpi	No significant homology*				
80	C6/36	DEN-2, 10dpi	*Pan troglodytes* BAC clone CH251-5712 from Y (AC146268.2)	1.9	21		
81	C6/36	DEN-2, 10dpi	Single read from an extremity of a full clone from *Anopholes gambiae* total adult females (CNS08WNK)	13	20		
82	C6/36	DEN-2, 10dpi	*Mus musculus* similar to marapsin 2 (XM_137602.2)	2.1	21	*Anopheles gambiae* cDNA (XM_312612.1)	8.2
83	C6/36	DEN-2, 10dpi	*Atropa belladonna* partial mRNA 3'UTR(ABE309394)	1.00E-09	62		
84	C6/36	mock	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	4.00E-85	270		
85	C6/36	mock	No significant homology*				
86	C6/36	mock	*Lepimer dorsalis* genomic fragment RAPD(LDO244007)	0.2	34	*Aedes aegypti* ID: 35707 unknown mRNA (AY431561.1)	193
87	C6/36	DEN-2, 14dpi	No significant homology*				
88	C6/36	DEN-2, 14dpi	*Lagothrix lagotricha* Alu insertion locus 885_2_1 La (AY620716.1)	3.7	24		
89	C6/36	DEN-2, 14dpi	No significant homology*				
90	C6/36	mock	No significant homology*				
91	C6/36	mock	No significant homology*				
92	C6/36	mock	*Ralstonia solanacearum* GMI100 chromosome (AL646059.1)	1.00E-12	92	*Anopheles gambiae* cDNA (AM_312199.1)	4.00E-06
93	C6/36	DEN-2, 14dpi	*Ralstonia solanacearum* GMI100 chromosome (AL646059.1)	1.00E-12	92	*Anopheles gambiae* cDNA (AM_312199.1)	4.00E-06
94	C6/36	DEN-2, 14dpi	*Ostertagia ostertagi* mRNA clone heat shock (OOS3100811)	3.8		*Aedes aegypti* mRNA for GATA transcription factor (AAE00338)	3.8
95	C6/36	mock	No significant homology*				
96	C6/36	DEN-2, 14dpi	No significant homology*				
97	C6/36	DEN-2, 14dpi	*Homo sapiens* clone RP11-16B9 (AC009554.14)	150	18		
98	C6/36	DEN-2, 14dpi	No significant homology*				
99	C6/36	mock	*Mus musculus* chromosome 18 clone RP23-478L7 (AC101740.7)	151	18	*Drosphila melanogaster* chromosomal DNA clone (AY118626.1)	597
100	C6/36	mock	Bovine herpesvirus 2 major capsid (AY35773.1)	365	17		
101	C6/36	DEN-2, 14dpi	*Mus musculus* BAC clone RP24-383N (AE003757.2)	22		*Drosophila melanogaster* cDNA (AC008215 and AC008214); *Anopheles gambiae* cDNA (XM_310417.1 and XM_310416.1)	*D. mel* 22; *An. gambiae* 326
102	C6/36	mock	*Mus musculus* BAC clone RP23-342K4 (AC129313.4)	629	17		
103	C6/36	DEN-2, 14dpi	No significant homology*				
104	C6/36	DEN-2, 14dpi	No significant homology*				
105	C6/36	mock	*Rickettsia prowazekii* strain Madrid E (RPXX02)	133	18		
106	C6/36	mock	No significant homology*				
107	C6/36	mock	No significant homology*				

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108	C6/36	mock	Zebrafish DNA from clone CH211-106B10 (BX84689.6)	399	17	*Drosophila melanogaster* chromosome (AC005788.8, AE003624.4, AC005889)	399
109	C6/36	DEN-2, 14dpi	Single read from an extremity of a full clone from *Anopholes gambiae* total adult females (CNS09MRB)	127	21		
110	C6/36	mock	*Mus musculus* BAC clone RP24-474C15 (AC129219.3)	1.9	25	*Drosophila caudal* (cad) mRNA fragment (DMCAD and DROCADA1); *Anopheles ambiae* cDNA (XM_316967.1, XM_316963.1, and XM_307445.1)	*Dros.* 29; *An. gambiae* 115
111	FB9.1	DEN-2, 3dpi	Human DNA sequence from clone RP11-118A1 (AL356737.9)	121	18		
112	FB9.1	DEN-2, 3dpi	*Aedes aegpti* ASAP ID: 35679 unknown mRNA (AY431509.1	3.00E-13	74		
113	FB9.1	DEN-2, 3dpi	Dengue virus type 2 Jamaica 1409 (323449)	0	24	*Anopheles gambiae* mRNA (AGAJ675)	2.00E-12
114	FB9.1	DEN-2, 3dpi	*Aedes aegypti* ID: 36917 cytosolic large ribosomal mRNA (AY431452.1)	3.00E-13	74		
115	FB9.1	DEN-2, 3dpi	*Papio anubis* clone RP41-335G18	8.3	20		
116	FB9.1	DEN-2, 3dpi	Dengue virus type 2 Jamaica 1409 (323449)	0	24	*Anopheles gambiae* mRNA (AGAJ675)	2.00E-37
117	FB9.1	DEN-2, 3dpi	*Armigeres subalbatus* ASAP ID: 43879 transcription factor mRNA (AY639887.1)	9.00E-06	101		
118	FB9.1	DEN-2, 3dpi	No significant homology*				
119	FB9.1	DEN-2, 3dpi	*Armigeres subalbatus* ASAP ID: 43879 transcription factor mRNA (AY639887.1)	3.00E-20	105	*Aedes aegypti* ASAP ID: 35597 transcription factor mRNA (AY431139.1)	4.00E-04
120	FB9.1	DEN-2, 3dpi	*Leifsonia xyli subsp.xyl* i str CTCB07 (AE016822.1)	14	24	*Anopleles gambiae* cDNA (XM_321179.1)	56
121	FB9.1	DEN-2, 3dpi	*Leifsonia xyli subsp.xyl* i str CTCB07 (AE016822.1)	12	24	*Anopleles gambiae* cDNA (XM_321179.1)	49
122	FB9.1	DEN-2, 7dpi	*Feldmannia irregularis* virus a strain Firr-1 (AY225135.1)	5.00E-23	90	*Armigeres subalbatus* ASAP ID: 43879 transciption factor mRNA (AY439887.1)	2.00E-19
123	FB9.1	DEN-2, 7dpi	No significant homology*				
124	FB9.1	DEN-2, 7dpi	*Mycoplasma pulmonis* (strain UAB CTIP) (MPULM03)	2.6	21		
125	FB9.1	DEN-2, 7dpi	*Agrobacterium tumefaciens* str. C58 liner chromosome (AE009383.1)	82	19		
126	FB9.1	DEN-2, 7dpi	*Anopheles gambiae* cDNA (XM_314962.1)	69	16		
127	FB9.1	DEN-2, 7dpi	No significant homology*				
128	FB9.1	DEN-2, 7dpi	Mus musculus BAC clone RP24-472I15 (AC132255.3)	3.7	18	*Drosophila melanogaster* BAC clone (AC009744.8 and AC093196.2)	57
129	FB9.1	DEN-2, 7dpi	No significant homology*				
130	FB9.1	DEN-2, 7dpi	No significant homology*				
131	FB9.1	DEN-2, 7dpi	No significant homology*				
132	FB9.1	DEN-2, 14dpi	No significant homology*				
133	FB9.1	DEN-2, 14dpi	No significant homology*				
134	FB9.1	DEN-2, 14dpi	Unidentified bacterium partial 16s rRNA (UBA518182)	3.00E-36	85	Dengue virus type 3 SLMC (DVU93302 and DVU93301) and Dengue virus type 3 DOH (DVU93295); dsRNA expressing P-element vector (AB186054.1)	1E-22; 9E 20
135	FB9.1	DEN-2, 14dpi	No significant homology*				
136	FB9.1	DEN-2, 14dpi	*Sinorhizobium meliloti* 1021 (SME91792)	4.2	18	*Drosophila melanogaster* BAC clone (AC011905.4 and AC010006.8); *Armigeres subalbatus* ASAP ID: 43	*D. mel* 256; *A. sub* 9E-16
137	FB9.1	DEN-2, 14dpi	*Aedes aegypti* ASAP ID: 35679 unknown mRNA (AY431509.1)	8.00E-04	51		
138	FB9.1	DEN-2, 14dpi	*Armigeres subalbatus* ASAP ID: 43879 transcription factor mRNA (AY639887.1)	3.00E-19	111		
139	FB9.1	DEN-2, 14dpi	No significant homology*				
140	FB9.1	DEN-2, 14dpi	Mouse DNA sequence from clone RP23-263M10 (AL670399.4)	0.68	22		
141	FB9.1	DEN-2, 14dpi	No significant homology*				
142	FB9.1	DEN-2, 14dpi	*Aedes aegypti* ASAP ID: 37989 unknown mRNA (AY431521.1)	6.00E-81	342		
143	FB9.1	DEN-2, 14dpi	*Mus musculus* BAC clone RP24-336G3 (AC122003.2)	9.5	20		
144	FB9.1	DEN-3, 7dpi	No significant homology*				
145	FB9.1	DEN-3, 7dpi	*Mus musculus* chromosome 3 clone (AC103604.4)	0.69	21		
146	FB9.1	DEN-3, 7dpi	*Rattus norvegicus* paralemmin (Palm) mRNA (NM_130829.1)	0.027	22	*Anopheles gambiae* mRNA (AGA416109); *Aedes aegypti* mRNA (AAE00338)	*An. gambiae* 0.027; *Ae. aegypti* 0.11
147	FB9.1	DEN-3, 7dpi	*Barbula unguiculata* mRNA for chloroplastic copper dismutase (AB066500.1)	1.5	22		
148	FB9.1	DEN-3, 7dpi	*Rattus norvegicus* paralemmin (Palm) mRNA (NM_130829.1)	0.69	22	*Anopheles gambiae* mRNA (XM_309317.1, XM_309316.1 and XM_308649.1)	100
149	FB9.1	DEN-3, 7dpi	*Armigeres subalbatus* ASAP ID: 43879 transcription factor mRNA (AY639887.1)	3.00E-09	103		
150	FB9.1	DEN-3, 7dpi	*Armigeres subalbatus* ASAP ID: 43879 transcription factor mRNA (AY639887.1)	7.00E-07	50	*Drosophila melanogaster* chromsome X (AE003451.4), Chromosome X BAC CH221-17A11	2.7
151	FB9.1	DEN-3, 7dpi	*Siniperca kneri* growth hormone (GH) mRNA (AY155227.3)	21	17		

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152	FB9.1	DEN-3, 7dpi	Barbula unguiculata mRNA for chloroplastic copper dismutase (AB066500.1)	7.00E-04	25		
153	FB9.1	DEN-3, 7dpi	*Caenorhabditis elegans* cosmid M03F8 (AF016441.1)	5	18		
154	FB9.1	DEN-3, 7dpi	No significant homology*				
155	FB9.1	DEN-3, 7dpi	Mouse DNA from clone RP23-402H11 (AL929409.5)	0.13	22	*Drosophila melanogaster* SD05937 (AY122251.1)	2.00E-04
156	FB9.1	DEN-3, 7dpi	*Mus musculus* chromosome X clone RP23-124C16(AC091452.2)	5.6	20		
157	FB9.1	DEN-3, 7dpi	*Mus musculus* BAC clone RP23-20H6 (AC122936.3)	159	18	Mouse DNA for the otholoh of human PRP4 pre-mRNA processing factor 4 hololog (AL732594.5)	159
158	FB9.1	DEN-3, 7dpi	*Rhododendron metternichii var. hondoense* CT repseats (AF022900.1)	7.1	20	*Drosophila melanogaster* cDNA clone (AC011072.9 and AC099033.2)	436
159	FB9.1	DEN-3, 7dpi	*Anopheles gambiae* cDNA (XM_319186.1)	0.005	129		
160	FB9.1	DEN-3, 7dpi	Zebrafish DNA from clone DKEY-202I10 (BX511267.6)	6.52			
161	FB9.1	DEN-3, 7dpi	Human DNA sequence from clone RP3-455H14 (HS455H14)	24	19	*Drosophila melanogaster* cDNA (AE003702.2)	93
162	FB9.1	DEN-3, 7dpi	Feldmannia irregularis virus a strain Firr-1 (AY225135.1)	3.00E-04	41		
163	FB9.1	DEN-3, 7dpi	*Mus musculus* chromosome 3 clone RP23-418O15 (AC113293.16)	1.5	21		
164	FB9.1	DEN-3, 7dpi	No significant homology*				
165	FB9.1	DEN-3, 7dpi	*Homo sapiens* paraneoplastic antigen MA1 (NM_006029.3)	192	18	*Anopheles gambiae* cDNA (XM_309655.1)	759
166	FB9.1	DEN-3, 7dpi	No significant homology*				
167	FB9.1	DEN-3, 7dpi	*Actinetobacter radioresistens* strain SW2-37 16s ribosomal mRNA (AY568479.1)	6.00E-06	51		
168	FB9.1	DEN-3, 7dpi	Unidentified bacterium partial 16s rRNA (UBA518182)	5.00E-37	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	3.00E-23
169	FB9.1	DEN-3, 7dpi	*Chlaydomonas reinhardtii* NIMA-related kinase 4 mRNA (AY364646.1)	0.009	24	*Anopleles gambiae* cDNA (XM_319572.1); *Aedes aegypti* mRNA (CNS09PY0, CNS09IWP)	0.57
170	FB9.1	DEN-3, 14dpi	No significant homology*				
171	FB9.1	DEN-3, 14dpi	*Rattus norvegicus* similar to KIAA0843 protein (XM_225889.2)	79	16		
172	FB9.1	DEN-3, 14dpi	No significant homology*				
173	FB9.1	DEN-3, 14dpi	*Engraulis japonicus* aCat2 mRNA for cathepsin L-like protein (AB081844.1)	0.23	21	*Sus scrofa* PKR mRNA (AB104654.1)	91
174	FB9.1	DEN-3, 14dpi	Muchupo virus strain Carvallo segment L (AY358021.2)	8	18		
175	FB9.1	DEN-3, 14dpi	No significant homology*				
176	FB9.1	DEN-3, 14dpi	*Barbula unguiculata* mRNA for chloroplastic copper dismutase (AB066500.1)	4.00E-04	25	*Sus scrofa* PKR mRNA (AB104654.1)	0.087
177	FB9.1	DEN-3, 14dpi	Zebrafish DNA sequence from clone DKEY-30A6 (BX000465.12)	5.9	25		
178	FB9.1	DEN-3, 14dpi	*Bacillus sp.* 34hs1 16s ribosomal RNA (rrn) gene (AF526913.2)	2.00E-18	51		
179	FB9.1	DEN-3, 14dpi	Zebrafish DNA sequence from clone BUSM1-234G15; contains nocel gene similar to AP4 (adpator-related protein complex 4, sigma subunit), part of a novel gene similar to STRN (striatin), a novel gene similar to COCH (coagulation factor C homolog, cochlin), a gene for a novel 7 transmembrane receptor (rhodopsin family), a gene for a novel vescile-transport related procin and a novel gene similar to KIAA1333 (AL596026.13)	1.3	23		
180	FB9.1	DEN-3, 14dpi	*Mus musculus* chromosome 19 cDNA clone RP23-321D22 (AC102790.7)	82	18		
181	FB9.1	DEN-3, 14dpi	No significant homology*				
182	FB9.1	DEN-3, 14dpi	*Barbula unguiculata* mRNA for chloroplastic copper dismutase (AB066500.1)	0.022	22	*Aedes aegypti* mRNA (AAE400338)	1.4
183	FB9.1	DEN-3, 14dpi	No significant homology*				
184	FB9.1	DEN-3, 14dpi	*Mus musculus* chromosome 9 clone RP23-128A11 (AC120869.12)	27	23		
185	FB9.1	DEN-3, 14dpi	*Barbula unguiculata* mRNA for chloroplastic copper dismutase (AB066500.1)	5.5	26	Nipah virus isolate UMMC2 (AY029768.1) Nipah virus isolate UMMC1 (AY029767.1)	338
186	FB9.1	DEN-3, 14dpi	*Barbula unguiculata* mRNA for chloroplastic copper dismutase (AB066500.1)	1.4	22	*Anopheles gambia* c mRNA (AGA416109); Aedes aegypti mRNA (AAE00338)	*An. gambiae* 22; *Ae. aegypti* 86
187	FB9.1	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	5.00E-37	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	3.00E-23
188	FB9.1	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	5.00E-37	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	3.00E-23
189	FB9.1	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	8.00E-39	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	4.00E-25
190	FB9.1	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	3.00E-35	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	2.00E-21

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191	FB9.1	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	3.00E-35	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	2.00E-21
192	FB9.1	mock	No significant homology*				
193	FB9.1	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	2.00E-21	58	dsRNA expressing P-element (AB186054.1)	1.00E-19
194	FB9.1	mock	Uncultured bacterium Dr1 partial nhs gene alpha chain (UBA306166)	1.2	21		
195	FB9.1	mock	*Vipera ammodytes ruffoi* mRNA for ammodytin (VAM580214)	5.00E-28	80	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295); *Mus musculus* mRNA for suppressor (MMU245720)	DEN3 3E-17; *M. musculus* 2E-20
196	FB9.1	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	5.00E-37	85	Dengue virus type 3 SLMC (DVU93302) and Dengue virus type 3 DOH (DVU93295)	3.00E-23
197	FB9.1	mock	*Mus musculus* mRNA for suppressor (MMU245720)	5.00E-06	36		
198	C6/36	mock	No significant homology*				
199	C6/36	mock	Vipera ammodytes ruffoi mRNA for ammodytin (VAM580214)	4.00E-28	84	*Anopheles albimanus* clone (AAU60880 and AAU60877)	2.00E-20
200	C6/36	mock	No significant homology*				
201	C6/36	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	3.00E-35	85	Dengue virus type 3 SLMC (DVU93302) Dengue virus type 3 DOH (DVU03295) Dengue virus type 3 SLM (DVU93301)	3.00E-23
202	C6/36	mock	*Bos taurus* LN1 mRNA (AF011925)	4.00E-22	71	Dengue virus type 3 SLMC (DVU93302) Dengue virus type 3 DOH (DVU03295) Dengue virus type 3 SLM (DVU93301)	2.00E-17
203	C6/36	mock	*Rattus norvegicus* mRNA (RNO566901)	2.00E-21	58	Dengue virus type 3 SLMC (DVU93302) Dengue virus type 3 DOH (DVU03295) Dengue virus type 3 SLM (DVU93301)	2.00E-17
204	C6/36	mock	*Homo sapiens* clone AW nonfunctional immunoglobulin gene (AY640495.1)	4.00E-10			
205	C6/36	mock	Chionodraco hamatus partial MT gene (CHA308478)	4.00E-22	59		
206	C6/36	mock	No significant homology*				
207	C6/36	mock	*Bos taurus* LN1 mRNA (AF011925)	4.00E-22	71	*Anopheles albimanus* clone (AAU60880 and AAU60877)	2.00E-17
208	C6/36	mock	No significant homology*				
209	C6/36	mock	*Mus musculus* cDNA (MMU245720)	1.00E-22	61		
210	C6/36	mock	*Bos taurus* LN1 mRNA (AF011925)	2.00E-20	71		
211	C6/36	mock	*Mus musculus* cDNA (MMU245720)	3.00E-17	67	*Anopheles albimanus* clone (AAU60880 , AAU60877and AAU60876)	2.00E-11
212	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	6.00E-21	58		
213	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	2.00E-17	59		
214	C6/36	mock	*Escallonia sp. Oxelman* 2340 partial mRNA for second largest subunit (rpd2 gene), d paralogue (ESC557244)	1.00E-19	59		
215	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	6.00E-21	58		
216	C6/36	mock	*Oryza sativa* (japonica cultivar-group) (NM_193001.1)	139	18		
217	C6/36	mock	No significant homology*				
218	C6/36	mock	*Zea mayes* CL5575_3 mRNA sequence (AY109346.1)	615	17		
219	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	6.00E-21	58		
220	C6/36	mock	Unidentified bacterium partial 16s rRNA (UBA518182)	2.00E-37	85		
221	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	1.00E-21	58		
222	C6/36	mock	No significant homology*				
223	C6/36	mock	*Vipera aspis aspis* mRNA for ammodytin I1 (VAS580157)	0.007	44	*Anopheles gambiae* mRNA (AGAJ675)	1.8
224	C6/36	mock	No significant homology*				
225	C6/36	mock	No significant homology*				
226	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	4.00E-19	58		
227	C6/36	mock	*Chionodraco hamatus* partial MT gene (CHA308478)	2.00E-21	58		
228	C6/36	mock	No significant homology*				
229	C6/36	mock	*Bos taurus* LN1 mRNA (AF011925)	1.00E-19	71		
230	C6/36	mock	No significant homology*				
231	C6/36	DEN-2, 3dpi	No significant homology*				
232	C6/36	DEN-2, 3dpi	*Anopheles gambiae* cDNA (XM_316107.1)	142	18		
233	C6/36	DEN-2, 3dpi	*Drosphila melanogaster* chromosome 2L region BACE42J13(AC009392.6)	202	18		
234	C6/36	DEN-2, 3dpi	*Mus musculus* BAC clone RP24-165o21 (AC132339.3)	808	17		
235	C6/36	DEN-2, 3dpi	*Mus musculus* chromosome 17 clone RP23-478J10 (AC118608.7)	673	17		
236	C6/36	DEN-2, 3dpi	*Arabidopsis thaliana* Full-length cDNA GSLTFB84ZC12 of flowers and buds od strain col-0 (Thale cress) (CNS0AC38)	232	18		
237	C6/36	DEN-2, 3dpi	*Homo sapiens* chromosome 3 clone RP11-697B5 (AC112525.4)	2.5	21		

Sequence Number	Cell line RNA Source	Virus Treatment	BLAST results: Best match (GenBank Accession Number)	E-value	Length of GenBank Match (nt)	Blast results: Other interesting matches (GenBank Accession Number)	E-value
238	C6/36	DEN-2, 3dpi	*Helicobacter hepaticus* ATCC 5144 (AE017148.1)	65	26		
239	C6/36	DEN-2, 3dpi	*Homo sapiens* chromosome 1 clone RP4-683M8(AC099679.3)	730	17		
240	C6/36	DEN-2, 7dpi	Uncultured bacterium gene for 16s rRNA clone (AB128887.1)	4.00E-16	59	La Crosse strain Human/78 segment S (AF528167.1) Lumbo virus genomic RNA for N and NSs proteins (BLNNSA), Tahyna virus RNA for N protein and RNA for NS protein (TAHYNAS), Tahyna cirus prototype Bardos 92 nucleocapsid (TVU47142) Morro Bay virus S and NSs (MBU31989), California encephalitis virus E6071(CEU12800)	0.14
241	C6/36	DEN-2, 7dpi	chromosome IV of strain GB-M1(CNS07EGB)	2.3	19		
242	C6/36	DEN-3, 7dpi	Lumbo virus (BLNNSA), Kairi virus (BKNNSA), Inkoo virus (INKOOVS, IVU47138, IVU47137), South River virus (SRU47141), Trivittatus virus (TVU12803), La Crosse virus (AF528167.1)	0.18	21		
243	C6/36	DEN-3, 7dpi	La Crosse virus (AF528167.1), Lumbo virus (BLNNSA), Tahyna virus (TAHYNAS, TVU47142), Morro Bay virus (MBU31989), California encephalitis virus (CEU12800, CEU12797), Snowshoe hare virus (SSH3VC, SSHS), La Crosse virus (BLCSRNA)	0.01	23		
244	C6/36	DEN-2, 7dpi	Mouse DNA from clone RP23-28O9 (AL732503.8)	104	24		
245	C6/36	DEN-2, 7dpi	*Zea mayes* PCO118979 mRNA sequence (AY106361.1)	20	18		
246	C6/36	DEN-2, 7dpi	Uncultured *Chloroflexi* bacterium S02F19 16s rRNA (AY149077.1)	0.75	21	*Armigeres subalbatus* ASAP ID: 42297 unknown mRNA (AY440287.1, AY440281.1); *Aedes aegypti* ASAP ID: 35007 unknown mRNA (AY432384.1, AY433205.1)	3
247	C6/36	DEN-2, 10dpi	Predicted: *Gallus gallus* similar to general transcription III C 1(XM_424339.1)	6.7	18	*Drosophila melanogaster* 3L BAC clone (AC104508.4, AC091208.4)	25
248	C6/36	DEN-3, 10dpi	La Crosse genome molecule S cDNA (BLCNCNP)	0.14	21		
249	C6/36	DEN-2, 10dpi	Mouse DNA sequence from clone RP23-383L13 (AL844849.13)	1.5	21		
250	C6/36	DEN-2,14dpi	*Tetraodon nigroviridis* cDNA (CE659628.1)	7	20		
251	C6/36	DEN-2,14dpi	*Mus musculus* chromosome 7 clone RP23-184M3 (AC107761.9)	154	18		
252	C6/36	DEN-2,14dpi	*Tetraodon nigroviridis* cDNA (CE659628.1)	7	20		
253	C6/36	DEN-2,14dpi	*Bacillus thuringiensis* serovar konkukian str 97-2(AE017355.1)	45	17		
254	C6/36	DEN-2,14dpi	*Bacillus cereus* ATCC 14579 (AE017009.1)	3.1	34	*Drosophila melanogaster* GH01006 (AY118458.1)	49
255	C6/36	DEN-2,14dpi	*Brandyrhizobium japonicum* USDA 110 DNA (AP005961.1)	5.5	19	*Drosophila melanogaste* r mRNA clone (DROHAYWIRA, CMERCC3A)	86
256	C6/36	DEN-2,14dpi	*Homo sapiens* T cell receptor beta locus (TRB@) (NG_001333.1)	21	21		
257	C6/36	DEN-2,14dpi	*Mus musculus* chromosome 16 RP23-198M10 (AC087799.43)	13		*Drosophila melanogaster* clone (AC010003.8)	197
258	C6/36	DEN-2,14dpi	*Leishmania donovani* iron superoxide dismutase B1 (AF312585.1)	9.7	19		
259	C6/36	DEN-3,14dpi	*Fugu rubripes* U2 small nuclar riboprotein subunit-related protein (U2AF1-RS2), 19 kDa Golgi adaptor protein adaptin (AP19), and phosphorylase kinase alpha 2 subunit (PHKA2) genes, complete cds, kelch protein (KELCH1 and KELCH2) genes, and protein phosphatase 1 (PPEF-1), X-linked juvenile retinoschisis precursor protein (XLRS1) and serine-threonine kinase 9 (STK9)(FRNINE1)	71	17		
260	C6/36	DEN-3, 7dpi	*Homo sapiens* chromosome 15 clone RP11-427)16 (AC023844.6)	0.59	24	*Drosophila melanogaster* X BAC clone (AC023721.3)	36
261	C6/36	DEN-3, 7dpi	*Homo sapiens* PAC clone RP4-782K2 (AC006003.4)	8.5	18	*Drosophila pseudoobscura* FOSMID (AC134177.1)	132
262	C6/36	DEN-3, 7dpi	La Crosse virus (AF528167.1), Lumbo virus (BLNNSA), Tahyna virus (TAHYNAS, TVU47142), Morro Bay virus (MBU31989), California encephalitis virus (CEU12800, CEU12797), Snowshoe hare virus (SSH3VC, SSHS), La Crosse virus (BLCSRNA)	0.12	23		
263	C6/36	DEN-3, 7dpi	*Homo sapiens* PAC clone RP4-782K2 (AC006003.4)	6	18	*Drosophila melanogaster* clone (AC006414)	
264	C6/36	DEN-3, 10dpi	*Homo sapiens* mRNA cDNA DKFZp686G03144(HSM807534)	2.6	23		
265	C6/36	DEN-3, 10dpi	*Mus musculus* BAC clone RP23-121L4 (AC098734.3)	23	22		
266	C6/36	DEN-3, 10dpi	*Drosophila melanogaster* chromosome 3R clones (AE003774.3, AC007820, AC008299)	2.7	19		
267	C6/36	DEN-3, 10dpi	La Crosse virus (AF528167.1), Lumbo virus (BLNNSA), Tahyna virus (TAHYNAS, TVU47142), Morro Bay virus (MBU31989), California encephalitis virus (CEU12800, CEU12797), Snowshoe hare virus (SSH3VC, SSHS), La Crosse virus (BLCSRNA)	0.045	22		
268	C6/36	DEN-3, 14dpi	Zebrafish DNA sequence from clone CH211-277G22 (BX072563.7)	32	20	*Drosophila melanogaster* RE12569 (AY094852.1)	
269	C6/36	DEN-3, 14dpi	*Homo sapiens* chromosome 8 clone RP11-737F9 (AC103726.5)	34	17	*Anopheles gambiae* clone (XM_313256.1)	532
270	C6/36	DEN-3, 14dpi	*Mus musculus* BAC clone RP23-398C14 (AC121789.2)	170	24		

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271	C6/36	DEN-3, 14dpi	No significant homology*				
272	C6/36	DEN-3, 14dpi	Kairi virus genomic RNA (BKNNSA)	0.18	21		
273	C6/36	DEN-3, 14dpi	*Oryza sativa* (japonica cultivar-group) (AP003210.3)	2.2	19	*Drosophila yakuba* CG10911 mRNA (AF531949.1)	35
274	C6/36	DEN-3, 14dpi	*Homo sapiens* paraneoplastic antigen MA1 (BC014926.2)	12	18	Serra do Navio virus (SNU47140), Kaystone virus (KVU128010 Melao virus (MVU12802)	12
275	C6/36	DEN-3, 14dpi	*Homo sapiens* PAC clone RP4-782K2 (AC006003.4)	9.8	18	*Drosophila melanogaster* cDNA (AC006414 and AE003710.2)	153
276	C6/36	DEN-3, 14dpi	*Mus musculus* BAC clone RP24-135C6 (AC136456.3)	10	18	*Drosophila melanogaster* cRNA (AE003836.4 and AC007085)	40
277	C6/36	DEN-3, 14dpi	La Crosse virus (AF528167.1), Lumbo virus (BLNNSA), Tahyna virus (TAHYNAS, TVU47142), Morro Bay virus (MBU31989), California encephalitis virus (CEU12800, CEU12797), Snowshoe hare virus (SSH3VC, SSHS), La Crosse virus (BLCSRNA)	0.046	22		
278	C6/36	DEN-3, 14dpi	No significant homology*				
279	C6/36	DEN-3, 14dpi	La Crosse virus (AF528167.1), Lumbo virus (BLNNSA), Tahyna virus (TAHYNAS, TVU47142), Morro Bay virus (MBU31989), California encephalitis virus (CEU12800, CEU12797), Snowshoe hare virus (SSH3VC, SSHS), La Crosse virus (BLCSRNA)	0.047	22		
280	C6/36	DEN-3, 14dpi	Human DNA sequence from clone RP5-85 part of the gene for a novel C2H2 type zinc finger protein simlar to *Drosophila* Scratch (SCrt), Slug and *Xenopus* Snail, a novel gene simlar to*Drosophila* CG6762, STSs, GSSs, and five CpG islands (HSDJ850E9)	2.1	23		
281	C6/36	DEN-3, 14dpi	La Crosse virus (AF528167.1), Lumbo virus (BLNNSA), Tahyna virus (TAHYNAS, TVU47142), Morro Bay virus (MBU31989), California encephalitis virus (CEU12800, CEU12797), Snowshoe hare virus (SSH3VC, SSHS), La Crosse virus (BLCSRNA)	0.037	22		
282	C6/36	DEN-3, 14dpi	*Homo sapiens* chromosome 15 clone clone RP11-368J22 (AC087641.5)	69	18		