DISSERTATION

THE USE OF DOUBLE-SUBGENOMIC SINDBIS VIRUS TRANSDUCING SYSTEMS AS TOOLS FOR UNDERSTANDING VIRUS-VECTOR INTERACTIONS

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CHRISTOPHER MICHAEL CIRIMOTICH ENTITLED "THE USE OF DOUBLE-SUBGENOMIC SINDBIS VIRUS TRANSDUCING SYSTEMS AS TOOLS FOR UNDERSTANDING VIRUS-VECTOR INTERACTIONS" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE USE OF DOUBLE-SUBGENOMIC SINDBIS VIRUS TRANSDUCING SYSTEMS AS TOOLS FOR UNDERSTANDING VIRUS-VECTOR INTERACTIONS

Recombinant Sindbis viruses (SINVs) have been developed that express a gene or sequence of interest from an engineered virus-encoded subgenomic promoter. These SINVs are valuable tools for studying virus interactions with mosquito vectors. Transducing systems constructed from the genomes of two distinct Sindbis viruses (TE12 and MRE16) have allowed researchers to study molecular determinants of virus infection in mosquitoes and manipulate immune and reproductive pathways in multiple invertebrate species. However, many aspects of arbovirus transmission cycles including interaction of the infecting virus with the mosquito RNA interference (RNAi) pathway and transmission of virus to the vertebrate host remain incompletely characterized. Here, I have constructed novel double-subgenomic systems to further study SINV interactions with the *Aedes aegypti* RNAi response and develop new tools to analyze aspects of the SINV transmission cycle,

A TE12-based transducing system has been engineered to express the Flock House virus protein B2, a known inhibitor of RNAi. Here I show that RNAi modulates SINV infection of *Ae. aegypti* mosquitoes and that the virus can become pathogenic to the mosquito when the vector's RNAi response is suppressed. These data suggest that RNAi may be necessary to maintain persistent arbovirus infection of the mosquito vector by modulating virus replication.

The mechanism of virus-induced gene silencing was examined in cell culture using dsSINVs expressing sequence of an endogenous mosquito gene (lysozyme) in sense or antisense orientation. TE12-based transducing systems can efficiently mediate silencing of the lysozyme gene but MRE16-based systems do not efficiently silence lysozyme expression. The MRE16 system, which can efficiently infect mosquitoes, does elicit production of siRNAs, the hallmark of RNAi-mediated silencing, but to a much lesser degree than the equivalent TE12 system.

To improve on existing transducing systems, a panel of MRE16 viruses was constructed to express fluorescent and bioluminescent proteins to be used as markers of virus infection. Also, a TE12-based virus was engineered to infect particular mosquito tissues only in the presence of tissue-specific antibody. Although the functionality of the targeted virus system could not be shown, the range of experiments that can be performed using dsSINVs has been expanded.

In toto, this work provides insight into the interactions between SINV and the mosquito RNAi response, characterizes the use of dsSINV for RNAi-mediated silencing studies, and has produced viruses that can be used to better understand the molecular mechanisms of SINV infection of and transmission by the mosquito vector.

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LITERATURE REVIEW

Introduction

More than twenty percent of emergent diseases over the past half century have been vector-borne (Jones et al., 2008). Arthropod-borne viruses (arboviruses) belonging to the virus families *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, and *Rhabdoviridae* have emerged into new areas or re-emerged in areas where transmission was thought to be previously controlled. From the family *Flaviviridae*, Japanese encephalitis virus (JEV) emerged in southeast Asia and Australia and West Nile virus in North America. Each virus may cause encephalitis, and significant morbidity and mortality have been attributed to each emergent virus. Outbreaks of the alphavirus (family *Togaviridae*), chikungunya virus (CHIKV), in the Réunion Islands and Italy and Venezuelan equine encephalitis virus (VEEV) in South America occurred in recent years (Enserink, 2006, Rezza et al., 2007, Weaver et al., 1996). CHIKV, previously known only to Africa and Asia, caused hundreds of cases of fever and arthralgia and one death in Italy (Rezza et al., 2007). Outbreaks of VEEV occurred throughout central and South America during the 1990's, causing thousands of human and equine cases and multiple deaths (Weaver et al., 2004). The phlebovirus Rift Valley fever virus (Family *Bunyaviridae*) re-emerged in nations of Africa and the Middle East, causing a hemorrhagic syndrome and many human deaths (Madani et al., 2003, W.H.O., 2007).

Yellow fever virus (YFV) and the four serotypes of dengue virus (DENV) have emerged or re-emerged in tropical and sub-tropical areas where populations of the major mosquito vector, *Aedes aegypti*, were once controlled (Beaty, 2005). Even though there is a highly efficacious attenuated virus vaccine widely available, YFV has been resurgent over the past 25 years. Unfortunately, there are an estimated 600 million people at risk in

Africa with little chance of receiving the vaccine (Barrett & Higgs, 2007). Dengue viruses have been introduced into most regions of the tropical world, with upwards of two billion people at risk (Halstead, 2007). There is currently no vaccine in wide use for any of the four dengue virus serotypes. Issues arising from enhancement of disease caused by infection with two heterologous dengue virus serotypes require that an effective vaccine produce complete immune coverage of all four serotypes. It is clear that mosquito-borne viruses remain an imminent threat to human health throughout the world. Research of both the virus and the arthropod vector must continue to develop new control strategies and therapies to combat the burden of disease.

Emergence and resurgence of arboviruses are not isolated events and many factors may explain the phenomena. Factors include, but are not limited to, growth and urbanization of the global community and lack of adequate mosquito control programs (Beaty, 2005, Gubler, 1998, Gubler, 2002).

The world population continues to grow, especially in developing areas with minimal resources. As this occurs, urban areas will be expanded, accompanied by necessary societal changes such as land usage and irrigation practices (Gubler, 2001). These trends create new areas for mosquito breeding and egg-laying and bring humans in closer contact with existing mosquito habitats. Many important arbovirus vectors have evolved to use human-produced containers for egg-laying. *Aedes aegypti*, a vector of many viruses including DENV and YFV, uses discarded water-filled containers for larval development.

Expansion of the global economy has necessitated efficient travel across large distances. The advances made in air travel may allow for the introduction of novel

viruses into immunologically-naïve populations. It is hypothesized that a West Nile virus-infected mosquito carried by airplane introduced the virus into the United States for the first time (Gerhardt, 2006). The presence of immunologically-naïve reservoir hosts and competent vectors including *Culex pipiens* and *Culex tarsalis* has allowed the spread of WNV throughout the United States and Canada and into Mexico and Central America.

Throughout the first half of the twentieth century, select vector populations were controlled through pesticide usage and removal of breeding habitats. Infestations of *Anopheles gambiae* and *Ae. aegypti* were controlled in many regions of South America and West Africa (Gubler, 1998). Complacency and the loss of public health infrastructure led to the eventual demise of most control programs through loss of resources (Beaty, 2005, Gubler, 1998). Mosquito populations have since resurged and are even more abundant in many areas, especially in Central and South America where *Ae. aegypti* is considered to be hyperabundant (Beaty, 2005).

Because adequate vaccines are not available for most arboviruses, control of mosquito vector populations and interrupting the arbovirus transmission cycle remain the most plausible solutions to the increasing problem of arboviral disease. Issues with insecticide usage and the appearance of widespread insecticide resistance highlight the need for novel strategies to interrupt the infection of mosquitoes and subsequent arbovirus transmission (Beaty, 2005). Dissection of the molecular mechanisms of vector infection may identify significant details leading to the development of novel and effective control strategies.

Mosquito-virus interactions

Mosquito-borne viruses are maintained in nature principally through the horizontal transmission of virus between vertebrate hosts by the introduction of saliva from an infected mosquito. Vertebrate hosts can develop a high titered viremia capable of infecting a mosquito when a bloodmeal is taken. Small mammals and birds are typical reservoir hosts for arboviruses and disease in these animals is often self-limited with minimal or no deleterious effects. Humans and large animals are typically dead-end hosts for arboviruses because they do not develop a viremia sufficient for mosquito infection. Notable exceptions are YFV and dengue viruses in their respective urban transmission cycles, which strictly use humans as an amplification host.

In some natural arbovirus transmission cycles, a female mosquito becomes infected when a bloodmeal is imbibed from a viremic host. Ingested blood travels to the midgut, a bulbous sac-like organ where primary digestion of the bloodmeal occurs. The first interaction between virus and vector takes place within the alimentary canal, typically at the midgut epithelium. The single-cell thick epithelium is composed of columnar cells with absorptive and secretory functions and specialized endocrine cells (Brown et al., 1985, Hecker, 1977). Time-course studies of eastern equine encephalitis virus (EEEV) infection in the enzootic vector *Culiseta melanura* and VEEV infection in *A. taeniorhyncus* have shown the posterior midgut is the initial infection site for alphaviruses following artificial bloodfeed (Scott et al., 1984, Smith et al., 2007a). However, others have shown that SINV and VEEV may infect the anterior midgut and show random distribution throughout the midgut at early times post-infection (Foy et al.,

2004, Olson et al., 2000, Romoser et al., 2004). Differences in infection kinetics may be virus- and mosquito species-specific.

Barriers to infection of the mosquito midgut can be demonstrated for multiple virus-vector combinations. Collections of *A. aegypti* from Mexico display a midgut infection barrier (MIB) to dengue virus type 2 (DENV2) and Peruvian *Culex spp.* exhibit an MIB for VEEV subtype IIIC (Bennett et al., 2002, Turell et al., 2006). An MIB may be controlled by genetic variation within populations of mosquit vectors. Vector genetic loci controlling for DENV2 infection have been identified in *Ae. aegypti* mosquitoes, but the specific genes are currently unknown (Bosio et al., 2000).

If the correct host cell receptor is present and the midgut epithelial cells are permissive, productive infection may occur. Viral replication occurs within midgut epithelial cells and progeny virions bud into the hemocoel of the mosquito after passing the midgut basal lamina. A single infected midgut epithelial cell may be sufficient for virus to reach the hemocoel (Smith et al., 2008). Midgut- and non-midgut-associated tissues, including musculature, nervous tissue, tracheae, Malphigian tubules, and fat body may become infected, resulting in secondary virus amplification. Some arboviruses are capable of infecting and replicating within midgut epithelial cells but cannot efficiently disseminate into the hemocoel. This phenomenon is termed a midgut escape barrier, and has been demonstrated for a strain of SINV and DENV2 in *A. aegypti* (Bennett et al., 2002, Myles et al., 2004).

Infection of the salivary glands is required for eventual transmission of virus. The salivary glands, located in the mosquito thorax, are composed of secretory cells that produce proteins and enzymes necessary for uptake of a bloodmeal. Like the midgut, the

salivary glands are a single layer of specialized epithelial cells surrounded by a basal lamina and may provide a barrier to successful virus transmission. Salivary gland infection barriers have been described for WEEV infection of *C. tarsalis* and Rift Valley fever virus (RVFV) infection of *C. pipiens* (Kramer et al., 1981, Romoser et al., 2005). Interestingly, RVFV could be detected in adult salivary glands when larval mosquitoes were injected with virus, suggesting that the basal lamina, formed after the molt from pupa to adult, is a physical barrier to salivary gland infection in *C. pipiens* (Romoser et al., 2005). Salivary gland escape (SGE) barriers have been demonstrated for multiple arboviruses (Beaty et al., 1981, Jupp, 1985). A genetic basis for this phenomenon was localized to the middle RNA segment of the California serogroup bunyavirus genome, suggesting a viral genetic component (Beaty et al., 1981). Paulson and Grimstad (1989) demonstrated a genetic basis of SGE barrier. While the salivary glands of both *Ae. triseriatus* and *Ae. hendersoni* mosquitoes are infected with La Crosse virus following oral infectious bloodmeal, only *Ae. triseriatus* is capable of efficient transmission of virus to suckling mice 22 days post infection (Paulson & Grimstad, 1989). Also, Takahashi (1982) described a SGE barrier for Japanese encephalitis virus infection in strains of *C. tritaeniorhyncus*. The barrier was dependent on the geographic location of the mosquito strain, suggesting a role for vector genetics (Takahashi, 1982).

An arbovirus can be transmitted during an infected mosquito's second bloodmeal only after disseminating from the SGE into the vector saliva. Saliva expelled into the bite site may contain a sufficient number of infectious virus particles to infect a susceptible vertebrate host. Estimates of virus titers in collected saliva give a wide range, depending on the method of saliva collection and virus titration (Aitken, 1977, Hurlbut, 1966, Smith

et al., 2005, Styer et al., 2007, Vanlandingham et al., 2004). Although the number of injected virus particles is variable, from an estimated two to more than 1,000 for VEEV, productive infection of the vertebrate may occur (Smith et al., 2005). Factors present in mosquito saliva have been shown to enhance arbovirus infection of vertebrates through the modulation of innate immune responses (Edwards et al., 1998, Gillespie et al., 2000, Limesand et al., 2000, Limesand et al., 2003, Schneider & Higgs, 2008, Schneider et al., 2006, Schneider et al., 2004).

The interval of time from ingestion of a virus by a vector to transmission capability is termed the extrinsic incubation period (EIP). This is not a static number; many factors can influence the EIP for a given vector-virus interaction. Notable among these are genetic factors within the vector and virus and environmental conditions including temperature (Bennett et al., 2002, Black et al., 2002, Bosio et al., 1998, Bosio et al., 2000, Kilpatrick et al., 2008).

EIP is an important aspect of vectorial capacity, a quantitative measure of a vector species' ability to transmit a pathogen to a susceptible host. Other variables taken into account when determining vectorial capacity include the density, daily feeding rate, and daily survival of the vector, all factors that influence the association of pathogen, vector, and vertebrate host. Vector competence is a qualitative assessment of the ability of a vector to transmit a pathogen and is dependent on genetics of the vector. Traits with a genetic component, such as the MEB and other barriers to infection described earlier, influence the vector competence of a mosquito for a specific virus. Both vectorial capacity and vector competence are important factors within the arbovirus transmission

cycle. Efficient control of mosquito-borne viruses relies on decreasing vectorial capacity, eliminating vector competence, or a combination of the two (Beerntsen et al., 2000).

Alphaviruses

Members of the genus *Alphavirus* comprise one of two genera within the virus family *Togaviridae*. The other genus, *Rubivirus*, contains a single virus species, rubella virus, which is related to alphaviruses in genome organization but not sequence or epidemiology. Currently, twenty-nine named species of alphaviruses have been identified that can be antigenically and genetically classified into seven complexes (Calisher & Karabatsos, 1988, Powers et al., 2001).

Alphaviruses have a worldwide distribution excluding Antarctica, but the range of individual virus species within a given geographic area depends on environmental and host range restrictions (Weaver & Barrett, 2004). With the exception of two fish-infecting viruses, all alphaviruses are transmitted by arthropod vectors (Powers et al., 2001). Mosquitoes are the typical vector, although alphaviruses have been isolated from ticks and other arthropods. Cliff swallow bugs (genus *Oeciacus*) have been implicated in the transmission of Fort Morgan virus and the Bijou Bridge strain of Venezuelan equine encephalitis virus (Calisher et al, 1980; Hayes et al, 1977; Monath et al, 1980).

Birds and small mammals are the usual amplification hosts for alphaviruses. Epidemiologic studies show that Old World CHIKV, O'nyong-nyong virus (ONNV), and Ross River virus may use humans as amplification hosts during outbreaks and the New World VEEV is capable of causing sufficient viremia in horses to infect mosquitoes during periodic epidemics in South America (Brault et al., 2004, Weaver & Barrett,

2004). With these exceptions, humans and large mammals such as horses are dead end hosts for alphaviruses, incapable of continuing the transmission cycle.

Systematics analyses suggest alphaviruses evolved from a New World insect-borne plant virus with at least three separate transoceanic introductions between the New and Old World (Levinson et al., 1990, Powers et al., 2001, Weaver et al., 1993). Alphavirus evolution occurs through point mutations attributed to the error-prone RNA-dependent RNA polymerase, a characteristic of all RNA viruses. Estimates from different alphaviruses have established an error rate of approximately 10^{-4} mutations per nucleotide. This rate is much lower than has been described in single-host, non-arthropod-borne RNA viruses such as coronaviruses. The requirement for two hosts from disparate animal phyla (Vertebrata and Invertebrata) is thought to constrain the rate of alphavirus evolution because certain mutations may be deleterious or lethal for the virus in one host (Coffey et al., 2008, Greene et al., 2005, Jerzak et al., 2008). Although the majority of alphavirus evolution occurs through misincorporation of single base pairs and the lack of polymerase proof-reading, there is an example of recombination. SINV- and eastern equine encephalitis-like ancestors recombined to produce the western equine encephalitis virus (WEEV) clade, including WEEV and the related Buggy Creek, Fort Morgan, and Highlands J viruses (Hahn et al., 1988, Weaver et al., 1997).

Old World alphaviruses generally produce a self-limiting illness, causing severe arthralgia and rash in the infected vertebrate host. Examples include CHIKV, Ross River virus, ONNV, and SINV. New World alphaviruses produce more severe disease characterized by an encephalitic syndrome. VEEV, EEEV, and WEEV are examples of New World alphaviruses.

Sindbis virus

The type member of the genus *Alphavirus*, SINV was first isolated from a pool of *C. univittatus* and *C. pipiens* mosquitoes collected in the Sindbis health district, Egypt, in 1952 (Taylor et al., 1955). There are three genetic subtypes of SINV; Paleoarctic-Ethiopian (P-E) found in Europe and Africa, Oriental-Australian (O-A) found in Asia and Oceania, and Southwest genotype found in Western Australia (Olson & Trent, 1985, Rentier-Delrue & Young, 1980, Saleh et al., 2003, Sammels et al., 1999). Genetic subspecies Babanki, Kyzylagach, Ockelbo, and Whataroa viruses are found in Africa, Eurasia, Europe, and New Zealand, respectively (Strauss & Strauss, 1994). The wide distribution is thought to be a result of virus trafficking by avian hosts.

SINV is maintained in a natural transmission cycle between passerine birds and *Culex spp.* mosquitoes. SINV has been isolated from anopheline mosquitoes, but the significance in the natural transmission cycle is unknown (Liang et al., 2000). SINV strains are capable of infection and amplification in laboratory colonies of culicine mosquitoes including *Ae. aegypti*, *Ae. albopictus*, and *C. tritaeniorhynchus* (Bowers et al., 1995, Foy et al., 2004, Jackson et al., 1993). Because it has been extensively studied and can efficiently infect medically important vector mosquitoes, SINV can be a functional model virus for studying arbovirus-vector interactions and specific experiments will be discussed in depth below.

Molecular Biology

SINV contains a positive-sense, single-stranded, nonsegmented RNA genome of approximately 11, 700 nucleotides (Strauss et al., 1984). The genome contains a 5' 7-

methylguanosine cap and 3'-terminal polyadenylate tail. The genomic RNA can be immediately translated when introduced into the cytoplasm of a permissive cell. Four non-structural and five structural proteins are encoded by the genome in the order non-structural protein 1 (nsP1)-nsP2-nsP3-nsP4-capsid-envelope 3(E3)-E2-6K-E1 (Strauss & Strauss, 1994).

The mature SIN virion consists of an inner nucleocapsid surrounded by a host cell-derived lipid membrane containing virus-encoded integral membrane glycoproteins. The entire virion has a diameter of 69 nm, including protruding glycoproteins (Harrison et al., 1971, Paredes et al., 1993). 240 copies of capsid protein in an icosahedral lattice with T=4 symmetry form the nucleocapsid structure that contains a single virus genomic RNA (Coombs & Brown, 1987a, Coombs & Brown, 1987b, Harrison et al., 1992, Paredes et al., 1992). Interactions between the capsid protein and the virus genome have been localized to a packaging signal contained within approximately 100 nucleotides of the nsP1 coding region (Frolova et al., 1997, Geigenmuller-Gnirke et al., 1993, Lee et al., 1996, Owen & Kuhn, 1996, Weiss et al., 1994, Weiss et al., 1989). Each capsid protein is bound to the carboxy-terminal cytoplasmic tail of E2 protein in a 1:1 ratio (Lopez et al., 1994, Owen & Kuhn, 1997). The surface glycoproteins are also at a ratio of 1:1 with single proteins associating to form heterodimers. E1 and E2 glycoproteins have a transmembrane domain near the carboxy terminus (Rice et al., 1982). On the surface of the mature virion, 80 spike complexes consisting of three E1-E2 heterodimers are distributed in an icosahedral lattice with T=4 symmetry (Paredes et al., 1992). Interactions between E1 monomers organize the spike complexes with E1 proteins internal and E2 proteins located peripherally (Anthony & Brown, 1991, Mukhopadhyay

et al., 2006, Phinney et al., 2000). Extra-membrane domains of E1 protein lie parallel to the lipid bilayer in an inner skirt region, while the ectodomains of E2 extrude from the virion in an outer region (Paredes et al., 2004, Pletnev et al., 2001, Zhang et al., 2002).

Replication cycle

The SINV replication cycle is initiated by adsoprtion of an infectious virus particle to a receptor on the surface of a susceptible cell. SINVs require replication in both vertebrate and mosquito cells in order to be propagated in nature and must be able to recognize either a single conserved receptor or multiple receptors. Although no alphavirus receptor has been definitively described, groups have used several different methods to identify a multitude of candidates including histocompatability complex antigens, high-affinity laminin receptor and heparan sulfate (Dubuisson & Rice, 1993, Helenius et al., 1978, Klimstra et al., 2003, Klimstra et al., 1998, Maassen & Terhorst, 1981, Pridgeon et al., 2008, Ubol & Griffin, 1991, Wang et al., 1992). Usage of heparan sulfate as a receptor has been associated with cell culture adaptation (Klimstra et al., 1998). Studies using a recombinant peptide representing the N-terminal portion of the human laminin receptor suggest a role for the protein in SINV infection; SINV infection was inhibited by adsorbing the peptide to virus prior to infection of baby hamster kidney cells (Jamieson et al., 2008). Although a number of different cell receptor proteins have been identified, the promiscuous binding of these receptors to a variety of ligands including SINV E2 may help to explain SINV's ability to infect a wide range of cell types.

Adsorption involves interaction of E2 with a receptor on the cell surface. Determinants of vertebrate and mosquito cell infection have been localized to E2. Amino acids 170-229 of SINV E2 form the cell receptor binding domain important for adsorption to avian, mammalian, and mosquito cells (Strauss & Strauss, 1994). This domain is predicted to be exposed on the surface of the virion (Smith et al., 1995, Stec et al., 1986, Strauss et al., 1991, Strauss & Strauss, 1994) and its importance in binding to target cells has been established (Mendoza et al., 1988, Ubol & Griffin, 1991, Wang et al., 1991, Wang & Strauss, 1991). The same region is important for VEEV binding to its cell receptor (Woodward et al., 1991).

Following adsorption of virus to receptor, SINV enters the cell through receptor-mediated endocytosis or direct penetration of the plasma membrane (Fan & Sefton, 1978, Paredes et al., 2004, Robbins et al., 1983, Wang et al., 2007). During endocytosis, acidic conditions in the endosome cause a conformational change in the virus glycoproteins, leading to formation of E1 homotrimers on the virion surface. The fusion peptide, located in the exposed portion of E1 following trimerization, is inserted into the endosomal membrane, allowing for fusion with the viral membrane. The acidic pH in the endosome also creates changes in the nucleocapsid structure allowing for release of the viral genome into the cell cytoplasm (Strauss & Strauss, 1994).

Cellular ribosomes translate the proximal two-thirds of the virus genome into a nonstructural polyprotein containing three (nsP123) or four (nsP1234) protein units, depending on read-through of an opal termination codon found at the nsP3-nsP4 junction (Strauss et al., 1983). Co- and post-translational cleavages of the nascent polyprotein produce polypeptides involved in viral RNA synthesis. nsP1 is required for negative

strand RNA synthesis and possesses mRNA capping activity, including methyl- and guanylyl-transferase activity. nsP2 is required for subgenomic mRNA production, has RNA helicase activity, and is the virus nonstructural protease responsible for cleavage of the nonstructural polyprotein. nsP3 is a phosphorylated protein that functions in viral RNA synthesis, although the functions are poorly understood. nsP4 is the viral RNA-dependent RNA polymerase responsible for positive and negative RNA synthesis. nsP4 complexed with the nsP123 polyprotein and host cell proteins produces negative strand RNA from the genome template. Cleavage at the nsP1-nsP23 junction produces a complex able to synthesize both negative strand and full-length positive strand genomic copies. Final cleavage of the nsP2-nsP3 bond yields a replicase complex capable of producing positive strand genomic (49S) and subgenomic (26S) mRNA species (Lemm et al., 1994, Shirako & Strauss, 1994, Strauss & Strauss, 1994, van der Heijden & Bol, 2002, Wang et al., 1994).

The 26S mRNA, produced from an internal promoter, is co-linear with the distal one-third of the genome and encodes the virus structural proteins. Translation of the subgenomic mRNA yields a polyprotein that is co- and post-translationally cleaved into capsid protein, two envelope surface glycoproteins (E1 and E2), and two small hydrophobic peptides (E3 and 6K). Capsid, E1, and E2 are incorporated into progeny virions, while E3 and 6K are not essential for infectivity.

Capsid protein has serine protease-like activity that self-cleaves the protein from the nascent polypeptide (Aliperti & Schlesinger, 1978, Choi et al., 1991, Hahn et al., 1985). Following cleavage of capsid protein, the remaining polyprotein is translocated to the endoplasmic reticulum (ER) where host proteases cleave the polyprotein to produce

PE2 (a molecule containing E3 and E2), 6K, and E1 proteins. Carbohydrate side chains are added in the Golgi apparatus. In a *trans* Golgi compartment, PE2 is cleaved by cellular furin protease to form E3 and E2. The E1 and E2 glycoproteins are transported to the plasma membrane where they associate in heterodimers that play an important role in subsequent infection by progeny virions. In the cytoplasm, capsid-bound genome is packaged in a nucleocapsid structure. Progeny virions bud from the plasma membrane after association of the nucleocapsid with the cytoplasmic tail of E2 (Strauss & Strauss, 1994).

Infectious cDNA clones

Infectious complementary DNA (cDNA) clone technology is advantageous because it allows the manipulation of an RNA virus genome by introducing insertions, deletions, or site-specific point mutations into a viral genome without direct RNA manipulation. Infectious clones are constructed by using an RNA-dependent DNA polymerase (reverse transcriptase) to produce a cDNA copy of an RNA virus genome. The cDNA is then inserted into a bacterial plasmid vector to allow efficient propagation. Transcription from an inserted DNA-dependent RNA polymerase promoter directly upstream of the virus genome produces RNA transcripts that are, in the case of many positive-strand RNA viruses, infectious when transfected into a permissive cell line. Transcripts are identical to the manipulated virus genome, providing a platform to examine the effects of individual mutations on virus replication in cell culture and host organisms.

Taniguchi et al (1978) assembled the first RNA virus infectious clone by inserting a cDNA copy of the Q-β bacteriophage genome into a bacterial plasmid. When introduced into bacteria, the phage genome was transcribed and infectious virus was produced (Taniguchi et al., 1978). Poliovirus became the first animal RNA virus produced from an infectious clone when mammalian cells were transfected with a plasmid containing a cDNA copy of the entire genome (Racaniello & Baltimore, 1981). Infectious clones of many plant and animal viruses have since been produced, including many alphaviruses.

First constructed by Rice et al (1987), infectious clones based on the genome of SINV were used to map temperature-sensitive and lethal mutations (Rice et al., 1987). Full-length infectious clones based on the genome of other alphaviruses have been engineered including O'nyong-nyong (Brault et al., 2004), CHIKV (Vanlandingham et al., 2005), Semliki Forest (Liljestrom et al., 1991), Ross River (Kuhn et al., 1991), Venezuelan equine encephalitis (Davis et al., 1989), western equine encephalitis (Schoepp et al., 2002), and Sagiyama viruses (Shirako & Yamaguchi, 2000). Subsequent generations of alphavirus infectious clones have been created to express foreign nucleic acid sequences of interest during virus infection.

The alphavirus replication cycle can be exploited to study virus genetics and genetic components of mosquito-virus interactions through infectious clone technology. The utility of SINV infectious clones in dissecting mosquito infection was demonstrated by Pierro et al (2007, 2008). Through mutational analysis using infectious clones of SINV strains TR339 and MRE16, the determinants of vector midgut infection were

localized to specific amino acid stretches of the E2 glycoprotein (Pierro et al., 2007, Pierro et al., 2008).

SINV-based expression systems

Infectious clones were initially used for mutational analysis of viruses without the need for temperature-sensitive mutant generation (Rice et al., 1987). Derived from infectious clones, expression systems were produced to study both virus infection and exogenous protein expression in cells through cDNA manipulation. First-generation expression systems were based on replacement of SINV defective interfering (DI) genome sequence with foreign sequence (Levis et al., 1987). DI genomes contain *cis*-acting portions of the SINV genome that are mandatory for replication and encapsidation of the genome and portions of sequence from other viral genes. DI genomes are incapable of productive infection because not all necessary virus proteins are encoded. All DI genomes have been shown to contain the extreme 3'-50 nucleotides of the SINV genome, the RNA packaging signal found in nsP1, and one of three separate 5' termini (Monroe et al., 1982, Monroe & Schlesinger, 1983, Tsiang et al., 1985, Tsiang et al., 1988). Interestingly, independent isolates of DI genomes contain cellular transfer RNA sequence at the 5' terminus, suggesting that defined secondary structure is necessary for optimal transcription of the minus strand RNA from the SINV genome (Monroe & Schlesinger, 1983).

Because DI genomes are replication-incompetent, simultaneous introduction of a wild-type helper virus provides the nonstructural proteins necessary for replication and structural proteins required for packaging of the expression system (Levis et al., 1987).

With *cis*-acting elements at the 5'- and 3'-termini in the DI genome, Levis et al (1987) were able to produce packaged DI genomes that expressed detectable levels of chloramphenicol acetyltranferase (CAT). However, expression was inefficient compared to virus structural proteins (Levis et al., 1987).

A second type of expression system is self-replicating (replicon) but incapable of producing infectious particles without a helper virus. In the replicon system, a portion or the entire structural genes are replaced by heterologous sequence (Bredenbeek et al., 1993). Wild-type nonstructural proteins can be produced, allowing for efficient transcription of the minus strand RNA and subsequent subgenomic mRNA production. However, only the sequence of interest will be expressed from the subgenomic mRNA. Structural proteins are supplied *in trans* either by helper virus or a defective helper RNA that is not packaged. Replicon RNA-containing particles are limited to a single round of infection unless another helper virus is subsequently introduced (Bredenbeek et al., 1993).

Viruses containing a bipartite genome are an exception to the rule of replicons not being able to infect subsequent cells (Geigenmuller-Gnirke et al., 1991). A replicon RNA is co-transfected with a DI genome that expresses the virus structural genes. Both RNA species contain sequences required for packaging and, when present in the same cell, will express all proteins necessary for productive infection. Infectious virus particles capable of multiple rounds of replication will contain each of the two RNA species (Bredenbeek et al., 1993, Geigenmuller-Gnirke et al., 1991).

Replicons have a coding capacity for a foreign sequence of more than 5 kilobases, but the level of expression is directly related to the efficiency of transfection (Frolov et

al., 1996, Huang & Summers, 1991). Early methods of transfection use chemicals or liposomes, but are inefficient. The advent of electroporation of RNA molecules into cells produced much higher transfection rates and more efficient generation of replicon particles for study (Liljestrom et al., 1991).

The third SINV-based expression system utilizes an engineered second subgenomic promoter to express a foreign sequence (Figure 1.1) (Foy et al., 2004, Hahn et al., 1992). The engineered promoter is placed within the virus genome, abrogating the need for removal of necessary genes. Therefore, all nonstructural and structural protein genes are present for infectious virus production.

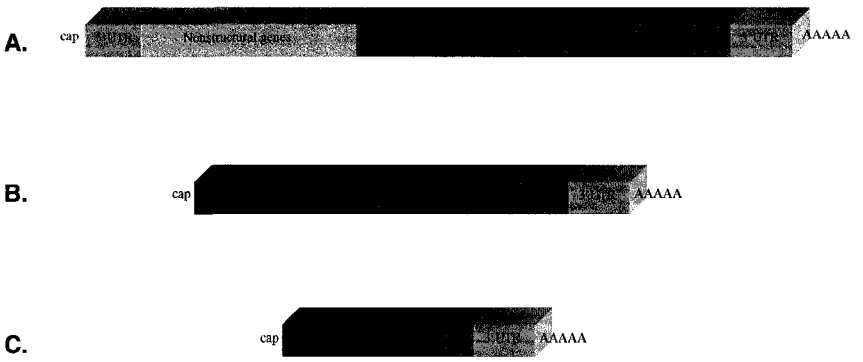

Figure 1.1. 5' double subgenomic SINV transducing system mRNAs. (A.) Representation of entire 5'dsSINV genome. (B.) The gene of interest is expressed from an mRNA transcribed from a subgenomic promoter engineered 5' of the structural genes. (C.) The structural genes are expressed from a second subgenomic mRNA species. In a 3'ds system, the gene of interest and structural genes are reversed.

No helper virus or RNA is necessary to produce multiple rounds of infection but packaging constraints limit insert sizes to less than two kilobases for efficient expression (Hahn et al., 1992). During the replication cycle of the virus, two subgenomic mRNAs are produced. One expresses an inserted gene of interest (Figure 1.1.B.) while the other

will express the virus structural genes (Figure 1.1.C.) (Hahn et al., 1992, Raju & Huang, 1991). Double-subgenomic (ds) SINVs have been constructed to express a gene of interest from a promoter inserted either upstream (5') (Figure 1.1.) or downstream (3') of the structural genes. DS systems based on multiple SINV strains and the genome of ONNV and CHIKV have also been constructed (Brault et al., 2004, Foy et al., 2004, Hahn et al., 1992, Vanlandingham et al., 2005).

SINV-based transducing systems

SINV is capable of infecting a variety of vertebrate and invertebrate cell lines as well as whole organisms (Hurlbut & Thomas, 1960). Non-cytocidal replication and wide host range are desirable characteristics of SINV for expression of a gene or sequence of interest in invertebrate cells. Packaged transducing systems allow for expression of an mRNA after infection of permissive cells but cannot spread to infect neighboring cells. The utility of dsSINV in transduction of vertebrate and invertebrate cells *in vitro* and *in vivo* has been shown. With the publication of the *An. gambiae* (Holt et al., 2002) and *Ae. aegypti* (Nene et al., 2007) genomes and new technologies for germ-line transformation of mosquitoes, it is necessary to have tools to test transgenes prior to mosquito transgenesis. DsSINVs provide an efficient means to study potential transgenes *in vitro* and *in vivo* without germ-line transformation.

Original dsSINV systems were constructed using the genome of strain TE12, a chimeric virus composed of glycoproteins from a mouse-neurovirulent strain and all other genes derived from the prototype AR339 strain, a Paleoarctic-Ethiopian genotype SINV (Lustig et al., 1988). Olson and colleagues (1994) were the first to infect mosquito

cell culture and whole mosquitoes with a dsSINV and show expression of a gene of interest (GOI). Using a TE12 virus that expressed CAT from a second subgenomic promoter located in the 3' non-coding region (TE3'2J), *Ae. albopcitus* cell culture and adult *Ae. triseriatus* mosquitoes were infected. In cell culture, virus titers peaked at four days post-infection, while CAT enzyme activity peaked around day six. Head, midgut, salivary gland, and ovarian tissues showed detectable levels of CAT activity that increased during the first four days post-injection and remained relatively uniform through twelve days (Olson et al., 1994). Kamrud et al (1997) used the same virus and a non-propagating replicon to show expression of CAT in the saliva of *C. pipiens* adult mosquitoes that were intrathoracically inoculated with the virus (Kamrud et al., 1997). The work by Olson et al (1994) and Kamrud et al (1997) were important in showing the utility of a dsSINV system for expression of a foreign gene in multiple organs during virus infection of adult culicine mosquitoes.

Similar experiments performed by Rayms-Keller et al (1995) described the temporal and spatial distribution of virus and showed TE3'2J could express a non-coding RNA sequence in infected mosquito tissues after intrathoracic injection. All hemocoelic tissues became infected following virus injection in *Ae. triseriatus*, including head, midgut, ovary, and salivary gland. Only trace amounts of virus were found in the ovaries and infection of midgut-associated tissue was more likely musculature and tracheae instead of the midgut epithelial cells (Rayms-Keller et al., 1995).

TE/3'2J has proved to be very useful for studies in cell culture and in mice. The virus infects mosquitoes when injected into the hemocoel but does not disseminate efficiently when fed in an infectious bloodmeal. To improve the ability of virus to infect

mosquitoes orally, a series of chimeric viruses were constructed. Preliminary studies by Seabaugh et al (1998) suggested a different strain of SINV was better able to infect *Ae. aegypti* when fed in a bloodmeal. The strain, MRE16 (Oriental-Australasian genotype), was isolated on a mosquito cell line from pools of *C. tritaeniorhyncus* mosquitoes collected in Malaysia from 1966 to 1969 (Pudney et al., 1979). The virus was passaged exclusively in mosquito cell culture and is highly infectious when given in an oral bloodmeal, infecting nearly 100% of mosquitoes when fed at high titers (Myles et al., 2004, Seabaugh et al., 1998). TE12 and MRE16 viruses share an overall ~75% nucleotide and 86% amino acid homology, with similar amino acid homology in the structural genes (Myles et al., 2004). A chimeric virus containing AR339-derived nonstructural genes and cis-acting sequences (identical to TE3'2J virus sequence) and MRE16 structural genes was produced. The virus, named MRE1001, showed similar infection kinetics to wild-type MRE16 virus in orally-infected mosquitoes. Greater than 90% of mosquitoes that ingested a bloodmeal had SINV antigen in head tissue 14 days post-infection (Seabaugh et al., 1998). This experiment showed determinants of efficient SINV midgut infection lie in the virus glycoproteins and was the first step in development of a SINV that could efficiently infect mosquito midgut epithelial cells and disseminate to non-midgut tissues.

To improve the utility of the MRE1001 system, Olson et al (2000) constructed a double-subgenomic virus that could facilitate exogenous gene expression in infected midgut cells (Olson et al., 2000). Although both MRE1001 and the newer generation virus MRE/3'2J were less efficient than TE3'2J at replication in mosquito cell culture, the viruses containing MRE16-derived structural proteins could infect mosquito midgut

epithelial cells much more efficiently. Expression of enhanced green fluorescent protein (eGFP) from the engineered second subgenomic promoter was found unstable: GFP and viral antigen expression were consistent in infected midgut cells but only viral antigen was found to be expressed in head tissue of most mosquitoes infected from a single infectious bloodmeal (Olson et al., 2000). This phenomenon had been previously described in cell culture infected at a low multiplicity of infection with a TE3'2J virus expressing CAT (Olson et al., 1994). Larval mosquitoes are capable of being infected with MRE/3'2J when virus-infected C6/36 cells are given as a sole food source (Higgs et al., 1999).

Expression of a GOI is only useful if it can be correlated with infection, so Pierro et al (2003) created a generation of dsSINV with the second subgenomic promoter inserted upstream of the structural polyprotein promoter. The virus, designated ME2/5'2J/GFP, was changed from MRE1001 by replacing only the E2 glycoprotein gene of the TE virus with that of MRE16. ME2/5'2J/GFP showed similar infection and dissemination rates as MRE1001 in orally-infected *Ae. aegypti* mosquitoes. After five serial passages in cell culture and in orally-infected mosquitoes, ME2/5'2J/GFP expressed GFP at a level similar to virus from the first passage, showing a more stable genome and less recombination occurring. The authors suggest a decrease in the amount of heterologous virus sequence encoded decreases the potential for incompatible sequence and increases the stability within a single genome (Pierro et al., 2003). Similar incompatibilities have been described in other chimeric alphaviruses (Kuhn et al., 1996, Kuhn et al., 1991). Interaction of the glycoproteins with capsid protein may also explain the incompatibilities, as chimeric SINV-Ross River virus replication efficiency can be

increased by point mutations in the RRV E1 or SINV E2 glycoproteins in the region of capsid-E2 interaction (Kim et al., 2000, Yao et al., 1998).

To circumvent issues associated with chimeric virus production and propagation, a dsSINV based on the entire MRE16 genome was produced from an infectious clone (Foy et al., 2004, Myles et al., 2003). The virus, 5'dsMRE16, infects *Ae. aegypti* female mosquitoes at a rate similar to wild-type MRE16 when fed orally and is capable of infecting *C. tritaeniorhyncus* mosquitoes. In a comparison of infection at early timepoints post-bloodmeal, 5'dsMRE16-eGFP was more efficient than ME2/5'2J-GFP at infection and dissemination from *Ae. aegypti* midguts. The genome of 5'dsMRE16-eGFP was found to be extremely stable; all mosquitoes containing virus E1 glycoprotein antigen in head tissue also expressed eGFP in the same tissue. The authors also used saliva from infected mosquitoes to infect monolayers of Vero cells and showed eGFP-positive foci of infection, demonstrating a high degree of stability upon multiple passages of virus (Foy et al., 2004). A system using a second subgenomic promoter 3' of the structural genes (3'dsMRE16) has also been produced.

Expression of exogenous proteins and over-expresson of endogenous proteins in mosquito cells

As stated above, SINV systems have been used to express reporter proteins and foreign proteins of interest in mosquito cell culture and mosquitoes. Virus infection can be tracked using a reporter protein without the need for fixing tissue samples and other procedures necessary for immunofluorescence or electron microscopy. Reporter proteins including CAT and eGFP have been expressed in adult and larval stages of *Ae. aegypti*

and *Ae. triseriatus* using packaged replicons and dsSINV systems (Higgs et al., 1999, Higgs et al., 1996, Kamrud et al., 1997, Kamrud et al., 1995, Olson et al., 1994, Olson et al., 2000, Pierro et al., 2003). 5'dsMRE16 virus has been used to express eGFP in adult *Ae. aegypti* and *C. tritaeniorhyncus* and larval stages of lepidopteran species *Manduca sexta* and *Bombyx mori* (Foy et al., 2004). Other exogenous non-reporter genes have been expressed in mosquitoes using dsSINV systems including heterologous virus structural proteins and scorpion toxin (Allen-Miura et al., 1999, Higgs et al., 1995).

Native mosquito proteins can be expressed using dsSINV systems. Secretion signals and other protein transport sequences must be engineered into the insert so that proper trafficking of the expressed protein will occur. Inserts intended for protein expression cannot contain an intron because SINV replication is strictly cytoplasmic and RNAs will not be spliced. As an example of endogenous protein expression, Cheng et al (2001) used a MRE/3'2J virus to over-express the immune peptide defensin in *Ae. aegypti* adults and larvae to determine the effects of defensin over-expression (Cheng et al., 2001).

Expression of heterologous RNA sequence in mosquito cells

RNA sequences also can be expressed from the second subgenomic promoter. Expression of an antisense RNA (complement) of an endogenous gene has been shown to inhibit production of the targeted protein. The first evidence that dsSINV could be used to silence endogenous genes in mosquito cells came from experiments performed by Johnson et al (1999) with transgenic mosquitoes. A TE3'2J virus expressing antisense RNA sequence to firefly luciferase significantly decreased expression of a luciferase

transgene under the control of the apyrase salivary gland-specific promoter. Injection of transgenic female *Ae. aegypti* mosquitoes with anti-luciferase virus decreased the relative expression of luciferase in the salivary glands by more than 90% while expression of other salivary gland proteins was unaffected, showing specificity for the transgene (Johnson et al., 1999).

The first demonstration of silencing of a naturally produced mosquito gene using a dsSINV was prophenoloxidase (PPO) knockdown in *Armigeres subalbatus* (Shiao et al., 2001). PPO is the inactive form of an oxidizing enzyme involved in the melanotic immune response to filarial worms (Ashida et al., 1990). Mosquitoes injected with virus containing PPO sequence in the antisense orientation showed a marked decrease in hemolymph PO activity and were less able to melanize *Dirofilaria immitis* microfilariae when compared to mosquitoes injected with a dsSINV expressing PPO gene sequence in the sense orientation. Although PPO mRNA levels were not determined, functional activity assays and determination of the melanization response showed only virus with the antisense PPO sequence could inhibit the function of PPO (Shiao et al., 2001). Subsequent studies have examined the role of another gene, dopa decarboxylase, in the melanotic response of *Ar. subalbatus* to microfilariae (Huang et al., 2005). Knockdown of a GATA factor in the fat body of *Ae. aegypti* has also been demonstrated (Attardo et al., 2003).

To examine the sequence requirements for efficient knockdown of endogenous mosquito genes using a dsSINV system, Tamang et al (2004) engineered TE3'2J viruses with sense and antisense PPO sequence of varying lengths (600, 147, and 36 nucleotides) and determined the melanotic response after injection of virus and microfilariae in *Ar.*

subalbatus. All viruses containing antisense sequence were able to inhibit the melanotic response to the microfilariae to some degree. Larger antisense sequence size showed the highest degree of inhibition, with almost no melanization occurring. PPO-specific siRNAs were detected by northern blot, suggesting a role for RNAi (see below) in gene silencing (Tamang et al., 2004).

The use of dsSINV systems for gene silencing is not exclusive to mosquitoes. Uhlirova et al (2003) were able to silence Broad-Complex (BC), a metamorphosis-induced transcription factor, in the silkmoth *Bombyx mori*. Injection of moth larvae with TE3'2J virus expressing antisense BC sequence or eGFP fused with antisense sequence caused morphological defects in adult structures and arrest at the larva-pupa molting interface. Virus expressing only eGFP or no insert had little effect on larval development. BC-specific siRNAs were isolated, showing the utility of dsSINVs for silencing a gene in non-vector insects by a RNAi mechanism(see below) (Uhlirova et al., 2003).

Expression of anti-pathogen molecules

DsSINVs have been used to express anti-pathogen molecules, including single-chain antibodies (scAb) and pathogen-specific antisense RNA sequences. Jiang et al (1995) expressed a known monoclonal antibody fragment active against louping ill virus (*Flaviviridae*: *Flavivirus*) from a dsSINV. Titers of LIV were significantly lower in porcine cell culture superinfected with the scAb-expressing virus compared to no-insert virus or virus expressing a non-specific antibody fragment. The protection was

incomplete and was not seen in cells infected with an LIV antibody-escape mutant (Jiang et al., 1995).

Because of the persistent nature of SINV infection, the use of scAb expression in mosquitoes is more desirable than in vertebrates. A scAb specific for *P. gallinaceum* has been expressed in mosquitoes using TE/3'2J. The encoded antibody fragment included an upstream signal sequence to allow secretion from infected cells. Adult female *Ae. aegypti* mosquitoes were injected with TE/3'2J-eGFP or TE/3'2J expressing the parasite-specific antibody and were subsequently allowed to feed on *P. gallinaceum*-infected chickens. Mosquitoes infected with the scAb-expressing virus showed a reduction in the number of sporozoites in the salivary glands but not in the midgut compared to control-infected mosquitoes (de Lara Capurro et al., 2000). The lack of effect on parasite infection of the midgut is expected because TE3'2J does not infect the midgut epithelial cells when injected into the mosquito thorax. DsSINV-mediated expression of scAbs is a useful tool for testing the potential of a molecule to block infection or transmission of a pathogen, but is not feasible as a control measure because of the cytotoxic effects in vertebrate cells and the variability of infection seen in invertebrates.

Sequence derived from an RNA virus genome can inhibit viral replication in cells. Pathogen-derived resistance (PDR), coined by Sanford and Johnston (1985), suggests a host expressing a pathogen-encoded gene or gene sequence may be refractory to infection by the pathogen (Sanford & Johnston, 1985). A similar phenomenon known as superinfection exclusion has been described in SINV-infected mosquito cells when challenged with a second homologous virus (Condreay & Brown, 1986, Karpf et al., 1997b), in mosquito cells infected with DEN-1 and later challenged with DEN-3 (Dittmar

et al., 1982), and in mosquitoes orally infected with La Crosse virus (LACV) and subsequently fed LACV or other bunyaviruses (Beaty et al., 1983, Beaty et al., 1985, Sundin & Beaty, 1988). For alphaviruses, the molecular mechanism of superinfection exclusion is thought to be mediated by production of mature viral protease which may prematurely cleave the replication protein complexes of the superinfecting virus (Kim et al., 2004, Sawicki et al., 2006).

DsSINVs have been used to engineer PDR in mosquito cells and mosquitoes by expressing portions of or entire virus gene sequences in infected cells. Powers et al (1996) showed silencing of LACV in cell culture and, for the first time, in orally-challenged mosquitoes. Recombinant TE3'2J viruses expressing LACV S segment sequence in sense or antisense orientation were able to decrease LACV replication in C6/36 cells. DsSINV expressing S segment antisense sequence (anti-S) was able to interfere with replication of the highly homologous snowshoe hare and Tahyna viruses, but not the heterologous trivittatus virus or YFV. *Ae. triseriatus* mosquitoes orally-infected with LACV and intrathoracically injected with anti-S virus showed decreased LACV titers and antigen abundance compared to mosquitoes injected with no virus or TE3'2J (Powers et al., 1996). Higgs et al (1998) used TE3'2J expressing antisense prM and NS5 polymerase sequence to show PDR against YFV in *Ae. aegypti* mosquitoes (Higgs et al., 1998).

The ultimate goal of dsSINV-induced PDR is characterization of sequences that could be used in the generation of mosquitoes that are refractory to arbovirus infection or transmission (Beerntsen et al., 2000, Blair et al., 2000, Olson et al., 2002). To this end, Gaines et al (1996) constructed TE3'2J viruses to express DEN2 virus prM gene

(D2prMs) or prM sequence in antisense orientation (D2prMa). Mosquito cells were infected with the dsSINVs at a high MOI and challenged 48 hours later with DEN2 virus. Immunofluorescence analysis showed D2prMs- and D2prMa-infected cells were resistant while mock and TE3'2J-infected cells were susceptible to DEN2 virus infection. DEN3 and DEN4 viruses were able to infect all samples, showing the specific nature of interference (Gaines et al., 1996).

Olson et al (1996) used the same viruses to expand PDR to DEN2 virus in adult *Ae. aegypti*. Excluding the midgut epithelium, which is infected by DEN2 virus but not SINV after intrathoracic inoculation, tissues infected by D2prMa were refractory to DEN2 virus infection. Biological transmission of DEN2 virus through injection of saliva from infected mosquitoes into naïve mosquitoes was inhibited by D2prMa; a lack of DEN2 virus accumulation in head tissues correlated with an inability to transmit the virus. Although the work of Olson et al (1996) was a breakthrough, the strict specificity for homologous virus inhibition and lack of efficient DNA transposition systems and specific promoters in mosquitoes emphasized the need for continued research into the potential for PDR as an arbovirus control strategy.

To address the issue of specificity, Adelman et al (2001) constructed dsSINVs with hybrid insert sequences derived from multiple DENV serotypes. In mosquito cells, TE3'2J virus expressing a subgenomic RNA containing sequence derived from two separate DENV serotypes conferred resistance to both viruses upon challenge. Varying degrees of cross-protection against heterologous serotypes was observed, potentially because of varying sequence homology between the insert sequences and infecting virus. It was also shown that PDR can be engineered against all four DENV serotypes

individually. Large quantities of dsSINV subgenomic mRNA were not necessary for interference; infection with DENV at timepoints where subgenomic mRNA expression was minimal conferred resistance. Ablation of DENV superinfection exclusion, typically seen within eight hours of initial virus infection, suggested interference was early during infection, prior to viral protein production (Adelman et al., 2001).

Insect innate immunity

In mosquitoes and other insects, the immune response to an invading pathogen is strictly innate. No adaptive immune response has been described in mosquitoes, although immunoglobulin-superfamily proteins have been found in *Drosophila melanogaster* and *Anopheles gambiae* (Dong et al., 2006, Watson et al., 2005). These proteins, produced from the alternatively-spliced Down syndrome cell adhesion molecule gene, are suggested to be pathogen recognition receptors and are considered part of the innate response (Dong et al., 2006). Interestingly, Sadd and Schmid-Hempel (2006) suggest the immune response of the bumblebee *Bombus terrestris* is capable of specific response to a bacteria species when exposed previously to a homologous species. Unfortunately, the immune mechanisms conferring this potential memory response were not examined(Sadd & Schmid-Hempel, 2006). *D. melanogaster* may have similar specific immune responses when challenged with a lethal dose of *Streptococcus pneumoniae* if previously exposed to a non-lethal dose of the same bacterium. This response is specific to the bacterial species and lasts for the life of the fly. It was found that phagocytes may play an important role, suggesting a memory aspect of insect innate immunity (Pham et al., 2007). Currently, no memory response has been described for the insect immune response to virus infection.

The first component of mosquito innate immunity that a pathogen encounters is the chitinous cuticle lining the exoskeleton, tracheae, anterior and posterior midgut, and the peritrophic matrix that is formed after a bloodmeal. In order to efficiently infect the mosquito, a pathogen must traverse this rigid matrix of polymerized sugar molecules. *Plasmodium* and other protozoan parasites encode a chitinase enzyme that digests the peritrophic matrix to allow infection of the mosquito midgut (Huber et al., 1991, Langer & Vinetz, 2001). Prior to peritrophic matrix formation, viruses infect permissive midgut cells that are not protected by chitin. After penetrating the chitinous first layer of insect innate defense, pathogens may encounter multiple mechanisms used by the insect to prevent invasion. Among these are reactive oxygen species produced by the mosquito midgut, which have been shown to modulate bacterial and Plasmodium infection in *Anopheles gambiae* (Molina-Cruz et al., 2008).

Another immune component is a cellular response initiated by hemocytes in the insect fat body, equivalent to the mammalian liver. The hemocytes in the open circulatory system of the insect have two main immune functions, phagocytosis and melanotic encapsulation (Lemaitre & Hoffmann, 2007). Recognized by cell surface molecules called pattern-recognition receptors (PRR), pathogens are engulfed by hemocytes where they are enzymatically destroyed in intracellular compartments. During encapsulation, a multilayered capsule is formed around the pathogen which is eventually killed, potentially by reactive oxygen species. The recognition of parasites prior to encapsulation is not well characterized (Beerntsen et al., 2000).

Described extensively in *Drosophila*, antimicrobial peptides (AMP) comprise the humoral component of the insect innate immune response. AMP expression is regulated

by NF-κB-like transcription factors. Depending on the pathogenic stimulus, AMPs are expressed through either the Imd or Toll signaling pathway. PRR recognition of pathogens in the insect hemolymph induces signaling cascades leading to AMP production. Gram-negative bacteria activate the membrane-bound peptidoglycan-recognition protein that interacts with the Imd pathway. A series of phosphorylation events leads to translocation of the transcription factor Relish into the nucleus and transcription of immune-related genes including cecropins and diptericin. Gram-positive bacteria and fungi induce the Toll signaling cascade after activation of the extracellular cytokine-like molecule, Spaetzle. Up-regulation of different AMPs occurs after translocation of the transcription factors Dif and Dorsal into the nucleus (Lemaitre & Hoffmann, 2007).

Homologues of Relish and Dorsal, *REL1* and *REL2*, have been identified in mosquitoes but little is known about how they regulate AMP expression (Christophides et al., 2002). The AMP families cecropin, defensin, and gambicin have been described in *Ae. aegypti* and *An. gambiae* and gambicin has also been found in *Culex pipiens pipiens* (Bartholomay et al., 2003, Hillyer et al., 2005, Lowenberger et al., 1999a, Lowenberger et al., 1999b, Sun et al., 1999, Vizioli et al., 2001a). Cecropins are active against most Gram-positive bacteria, Gram-negative bacteria, and fungi. Defensins are protective against Gram-positive bacteria. Gambicin, an AMP unique to mosquitoes, is active against bacteria and fungi. Vizioli and colleagues (2001) have shown that both defensin and gambicin are expressed in *An. gambiae* mosquitoes and that gambicin may be active against malaria parasites (Vizioli et al., 2001a, Vizioli et al., 2001b). AMPs active

against viral infection in mammals have been identified, but none have been described in insects, although the Toll pathway may be induced during virus infection (see below).

Insect antiviral immunity

Antiviral mechanisms have been described in non-vector and vector insects. Larvae of the silkworm *Bombyx mori* produce proteins capable of inhibiting replication of the natural pathogen *B. mori* nucleopolyhedrovirus (BmNPV). A serine protease, a lipase, and a NADH-oxidoreductase isolated from the gut juice of larval silkworms have been shown to specifically inhibit BmNPV in cell culture and larval worms (Nakazawa et al., 2004, Ponnuvel et al., 2003, Selot et al., 2007). Selot et al (2007) have recently shown that a reductase enzyme has anti-BmNPV activities *in vitro*; incubation of virus with purified enzyme reduced virus infection in a dose-dependent manner. Interestingly, when levels of reductase were compared across multiple silkworm strains, there was a positive correlation between the enzyme activity level and tolerance of the strain for BmNPV infection (Selot et al., 2007).

Introduction of non-specific dsRNA triggers an antiviral response in shrimp (Robalino et al., 2005, Robalino et al., 2004, Westenberg et al., 2005). More important to vector biology, a similar phenomenon has been described in cell culture from the sandfly *Lutzomyia longipalpis*, vector of important parasitic and viral diseases (Pitaluga et al., 2008). The same may hold true for mosquitoes (see below), but experiments specifically looking at this response have not been performed. Using West Nile virus-like particles, Pitaluga et al (2008) were able to inhibit expression of a virus-encoded reporter gene when viral RNA and a number of non-specific single- or dsRNAs were co-transfected.

Expression was knocked down in mosquito C6/36 (*Ae. albopictus*) cells only when reporter gene-specific dsRNAs were co-transfected, suggesting a difference in antiviral response between the two cell lines. Cell type may explain the differences in response; the sandfly cells are a mixed population of embryonic cells (Tesh & Modi, 1983), while C6/36 cells are derived from a larval homogenate and isolated for efficient arbovirus replication (Igarashi, 1978).

The Toll pathway has been implicated in antiviral immunity in *D. melanogaster* and *Ae. aegypti*. Using transgenic fly lines, Zambon et al (2005) showed that mutant flies with a constitutively active Toll pathway have decreased viral titers while flies deficient in an activator of the Toll pathway have increased viral titers when injected with *Drosophila* X virus (*Birnaviridae*: *Entomobirnavirus*). The authors hypothesized that lysis of infected cells led to activation of the Toll pathway and subsequent phagocytosis of aberrant infected cells by activated hemocytes (Zambon et al., 2005). The effects of Toll pathway in arbovirus infection are discussed below.

Bian et al (2005) used reverse genetics to engineer a transgenic mosquito with an inducible knockdown of Toll pathway transcription factor Rel1. As expected, these mosquitoes showed increased susceptibility to an entomopathogenic fungus but affects during viral infection were not examined (Bian et al., 2005). Using microarray analysis of midgut-specific transcripts following infectious bloodmeal containing SINV, Sanders et al (2005) showed expression of Toll pathway genes decreased at later timepoints post-infection. Downregulation of the *Aedes* homologue of Dif and several ubiquitin ligases suggests inhibition of the Toll pathway (Sanders et al., 2005). Virus-specific inhibition

of a mosquito immune pathway may show involvement in an antiviral response, but there is currently no experimental data supporting this hypothesis.

Mosquito antiviral immunity

Relatively little is known about the antiviral immune response in mosquitoes. Virus infection in vertebrate cells triggers the α/β interferon signaling pathway and subsequent expression of antiviral proteins including protein kinase R, Mx protein, and TRIM5α. Mosquito homologues of these proteins are not present in the *Ae. aegypti* or *An. gambiae* genome. Apoptosis may play a role in mosquito antiviral immunity against virus infection, but the significance of this response has yet to be determined (Vaidyanathan & Scott, 2006).

Protein antiviral factors have been described from mosquito cell lines persistently infected with alphaviruses and flaviviruses (Hommel & Schloemer, 1985, Newton & Dalgarno, 1983, Riedel & Brown, 1979). The peptide produced in SINV-infected cells, first described by Riedel and Brown, has been purified but its identity remains unknown (Luo & Brown, 1993, Riedel & Brown, 1979). Two *Ae. albopictus* cell lines persistently infected with SINV have been shown to produce the inducible peptide (Condreay & Brown, 1988). Characterization of the cellular response to the peptide has shown that viral RNA synthesis is blocked but viral nonstructural proteins are still translated when cells are exposed to the factor prior to virus infection. A cellular protein associated with lysosomes is induced upon exposure to the antiviral factor but direct interaction with viral proteins has not been shown (Luo & Brown, 1994). For each peptide, the antiviral activity was virus- and cell type-specific. There was no protection against other

alphaviruses, flaviviruses , or bunyaviruses (Hommel & Schloemer, 1985, Newton & Dalgarno, 1983, Riedel & Brown, 1979). Unfortunately, the antiviral factors have not been identified in mosquitoes.

Recently, Xi et al (2008) found a potential link between antibacterial and antiviral immunity in mosquitoes. Using microarray analysis of *Ae. aegypti* gene expression in the midgut and remaining carcass, it was found that genes in the Toll and JAK-STAT pathways were significantly up- and down-regulated at ten days post-DEN2 virus infection. Expression of activators and effector molecules of the Toll pathway, including REL1A and AMPs, were significantly up-regulated while expression of the negative regulator Cactus was down-regulated. The JAK-STAT pathway also was up-regulated but to a lesser extent than Toll pathway genes. Removal of the negative regulator of Toll, Cactus, led to activation of the pathway and a significant decrease in midgut virus titers. The converse was also true; mosquitoes with an inactive Toll pathway had significantly higher midgut virus titers. Also, when mosquitoes were rid of the natural flora through injection of antibiotic, a modest but significant increase in DEN2 titer was observed. The authors suggest that a basal level of Toll pathway gene expression stimulated by the consistent presence of natural flora could mediate DEN2 virus infection (Xi et al., 2008). The effect of Toll pathway on virus infection at early times post-bloodmeal was not determined. This immune response may be similar to the JAK-STAT pathway in *Drosophila*; expression of molecules in the pathway may limit virus, but the pathway is not the single modulator of infection (Dostert et al., 2005). In mosquitoes, RNA interference appears to be the strongest modulator of viral infection (Campbell et al., 2008, Keene et al., 2004).

RNA interference

RNAi, post-transcriptional gene silencing, quelling, and RNA silencing are intracellular pathways that encompass a mechanism of targeted RNA degradation stimulated by the presence of dsRNA and mediated by the presence of RNA molecules 21-24 nucleotides in length. First described in plants, the mechanism has been found to be evolutionarily conserved. The mechanism has also been described in mammals, nematodes, fungi, insects, and other arthropods.

Other small RNA-associated pathways have been described in animals. The microRNA pathway has development roles and may act as a defensive response to Plasmodium infection (Winter et al., 2007). The piwi-associated RNA pathway controls transposon activity within the genome (Brennecke et al., 2007). Both the miRNA and piRNA pathways use small RNA molecules derived from the host genome, while the RNAi pathway uses regulatory small RNAs derived from dsRNA.

Mechanism of RNAi

Much of the mechanism of insect RNAi has been described in *Drosophila melanogaster*. Long dsRNA molecules in the cell cytoplasm are the initiators of RNAi. The molecules are recognized by Dicer, a type III endonuclease that will nonspecifically cleave perfectly base-paired dsRNA into small 21-24 dsRNA fragments called small interfering RNAs (siRNAs). Two distinct Dicer molecules have been described in *Drosophila*; Dicer-1 is involved in the miRNA pathway and processing dsRNA molecules produced in the nucleus while Dicer-2 processes dsRNA found in the

cytoplasm of the cell and functions in RNAi (Lee et al., 2004). A single dicer gene has been found in humans and worms while plants encode four separate dicer enzymes.

The second phase of RNAi is the effector phase. Duplex siRNAs are bound by a bi-molecular complex of Dicer-2 and the dsRNA-binding protein R2D2 (Liu et al., 2006). The complex is delivered to the effector Argonaute (AGO) enzyme, which contains a functional domain with similarities to RNaseH. In the insect response to cytoplasmic dsRNA, AGO-2 cleaves the passenger strand from the siRNA duplex. Using the incorporated single-stranded siRNA strand as a guide, the activated RNA-induced silencing complex (RISC), a multi-protein complex including Dicer-2, AGO-2, R2D2 and other proteins, targets complimentary mRNA for cleavage by AGO-2. The mechanism of cleavage, or "slicing", has been shown for *Drosophila* AGO-1 and AGO-2 (Miyoshi et al., 2005, Rand et al., 2005) a mammalian AGO-2 (Liu et al., 2004) and a plant AGO (Baumberger & Baulcombe, 2005). The cleavage products are degraded by the cellular exosome and other degradation machinery (Orban & Izaurralde, 2005).

In the nematode worm *Caenorhabditis elegans,* fungi, and plants primary siRNAs are used by a cellular RNA-dependent RNA polymerase (RdRP) as template to produce secondary dsRNA molecules. Amplification of the RNAi trigger and spread to other cells allows a systemic RNAi response to a specific dsRNA. Plant cells are connected through pores called plasmodesmata that can serve to transfer the dsRNAs to neighboring cells while cells in *C. elegans* encode a transmembrane protein, SID-1, capable of transporting long dsRNA molecules into the cytoplasm from the extracellular environment. The red flour beetle, *Triboleum castaneum*, is the only insect reported to

have a systemic RNAi response but the response has not been characterized (Tomoyasu & Denell, 2004).

RNAi as an antiviral response

RNAi has been described as an antiviral response in plants, fungi, worms, and insects with three lines of supporting evidence. First, virus-specific siRNAs of both positive and negative sense can be detected during positive-strand RNA virus infection, illustrating that RNAi is triggered by the presence of the virus (Campbell et al., 2008).

Second, transgenic organisms deficient in components of the RNAi machinery are more susceptible to virus infection and disease. *Drosophila* mutants deficient in Dicer-2, AGO-2, or R2D2 are more susceptible to infection with the positive-stranded RNA flock house virus (*Nodaviridae*: *Alphanodavirus*), cricket paralysis virus (*Dicistroviridae: Cripavirus*), *Drosophila* C virus (*Dicistroviridae: Cripavirus*), and SINV (Galiana-Arnoux et al., 2006, van Rij et al., 2006, Wang et al., 2006). Interestingly, while AGO-2 and R2D2 mutants are more susceptible than wild-type flies to the dsRNA *Drosophila* X virus, Dicer-2 mutant flies are not (Zambon et al., 2006).

Lastly, some viruses encode proteins that inhibit the RNAi response, suggesting virus evolution to counteract the host innate antiviral response much like animal viruses and the interferon response. Interestingly, components of the RNAi machinery in *Drosophila* evolve rapidly, suggesting co-evolution of the host and pathogen (Obbard et al., 2006). The first virus-encoded inhibitors of RNAi (VIR) were identified in plant viruses. DNA plant viruses encode proteins capable of inhibiting RNAi and some viruses encode multiple VIRs (Li & Ding, 2006).

The insect-pathogenic viruses flock house virus, cricket paralysis virus, and *Drosophila* C virus have been shown to encode VIRs (Li et al., 2002, van Rij et al., 2006, Wang et al., 2006). It is unclear whether arboviruses, which persistently infect their insect vector, encode VIRs. Many animal viruses encode dsRNA-binding proteins, including NS1 from influenza A virus and NsS from La Crosse virus (Li et al., 2004, Soldan et al., 2005). Although these and other proteins interfere with RNAi *in vitro*, the contribution to *in vivo* infection has not been determined. A bacteria-derived dsRNA-binding protein is capable of inhibiting RNAi in a plant system, suggesting RNAi inhibition *in vitro* by dsRNA-binding proteins may be a non-specific experimental artifact (Lichner et al., 2003). Viruses that infect mammals may have evolved dsRNA-binding proteins to combat the interferon response rather than RNAi (Blakqori et al., 2007).

RNAi in mosquitoes

RNAi can be induced by the introduction of exogenous dsRNA molecules into cells by transfection or animals by injection. In *An. gambiae* cell culture, Dicer-2 was shown to be important for RNAi (Hoa et al., 2003). AGO2 and AGO3 have been implicated in the RNAi response to dsRNA in *An. gambiae* mosquitoes and cell culture (Hoa et al., 2003, Keene et al., 2004). Keene et al (2004) used co-injection of *in vitro* synthesized dsRNA and virus to show the involvement of AGO2 and AGO3 in the RNAi response to ONNV. When dsRNA homologous to ONNV nsP3 was injected virus titers significantly decreased, showing RNAi can efficiently target virus infection. However,

virus titers in individual mosquitoes were significantly higher when expression of either AGO2 or AGO3 was simultaneously knocked down via RNAi.

Campbell et al (2008) used a similar approach to show the involvement of RNAi in SINV infection of *Ae. aegypti.* Titers of TR339 virus were significantly higher in individual mosquitoes at four days post-infection when dsRNA derived from AGO2 or Dicer-2 was injected following an infectious bloodmeal. Virus titers were not significantly different from β-gal dsRNA-injected controls at seven days post-infection, showing the transient nature of RNAi toward an endogenous mosquito gene (Campbell et al., 2008). Of note, in both studies virus titers could be decreased by introduction of virus-specific dsRNA but infection could not be completely inhibited. The authors suggest arboviruses may encode suppressors of the RNAi response but no experimental evidence supporting this hypothesis has been presented. A second possibility is the transient nature of dsRNA injection; the lack of an amplification mechanism for RNAi in mosquitoes does not allow for long-lasting knockdown of genes through dsRNA introduction.

Using a transgenic approach, Franz et al (2006) were able to show that stimulation of the RNAi response in a virus-specific manner could down-regulate DEN2 virus infection. *Ae. aegypti* mosquitoes expressing a DEN2 sequence-derived inverted-repeat transgene in the midgut under the control of a bloodmeal-responsive promoter were almost completely refractory to DEN2 virus infection. Viral RNA could not be detected in the transgenic mosquitoes after two days post-bloodmeal. Virus-specific siRNAs were detected and virus infection could be recovered when dsRNA to AGO2 was injected into

bloodfed mosquitoes, showing a role for RNAi in the inhibition of virus infection (Franz et al., 2006).

Summary and goals

As endemic arboviruses continue to circulate and emerge in non-endemic areas, new methods for control must be developed. Moderate success in vector control has been achieved in the past but proven unsustainable. Arboviruses continue to emerge in novel areas and re-emerge in areas that were once controlled. Recent breakthroughs in molecular biology and mosquito transgenesis allow for novel strategies of vector and arbovirus control to be developed. Central to this development is the continued research of virus-vector interactions.

RNAi plays an important role in antiviral immunity in mosquitoes. It is possible that variations in the RNAi response may in part determine vector competence and provide a unique means to control arboviruses.

Sindbis virus can be used as a model for the study of mosquitoes and the interactions that permit transmission of arboviruses because the molecular biology of the virus is well-characterized and SINV is capable of infecting multiple species of medically important mosquitoes. Expression systems based on different lineages of SINV have previously been developed to study the SINV-mosquito interaction. In this body of work, double-subgenomic SINV systems were used to 1.) investigate the interplay between TE3'2J virus and the *Ae. aegypti* RNAi response, to 2.) characterize the ability of different dsSINV lineages to induce RNAi in a mosquito cell culture-based system, to 3.) expand the capabilities of dsSINV use by creating 5'dsMRE16-based systems expressing

novel reporter genes, and to 4.) attempt to target infection of a dsSINV system to specific tissues through an antibody-mediated bridge.

CHAPTER 2

EXPRESSION OF AN ACTIVE VIRAL INHIBITOR OF RNA INTERFERENCE FROM TE/3'2J VIRUS RESULTS IN INCREASED REPLICATION AND VIRUS-ASSOCIATED MORTALITY IN *AEDES AEGYPTI*

Introduction

Arthropod-borne viruses (arboviruses) such as dengue virus and chikungunya virus are transmitted to humans through the bite of an infected mosquito. The viruses exhibit significant morbidity and mortality in the vertebrate host. However, virus persists in the mosquito vector with minimal associated pathology. Examples of arbovirus-induced cytopathology during infection have been described with laboratory-infected mosquitoes, but little is known about the interplay between virus and vector that allows for sustainable arbovirus infection in nature (Bowers et al., 2003, Girard et al., 2007, Moncayo et al., 2000, Weaver et al., 1992, Weaver et al., 1988). Insect-pathogenic viruses produce proteins that inhibit the host immune response (Clem et al., 1991, Johnson et al., 2004, Li et al., 2002, van Rij et al., 2006, Wang et al., 2006). The persistent nature of arbovirus infection of a vector suggests a commensalistic rather than parasitic relationship. Understanding the interactions between arbovirus and mosquito immune system may lead to novel approaches to control these significant human and animal pathogens.

The natural antiviral activity of RNA interference (RNAi) against RNA viruses has been described in plants, fungi, nematodes, and insects (Keene et al., 2004, Schott et al., 2005, Segers et al., 2007, Wang et al., 2006, Zambon et al., 2006). RNAi is a highly conserved molecular pathway triggered by the presence of intracytoplasmic double-stranded RNA (dsRNA) that results in the cleavage of RNA molecules with sequence homologous to the dsRNA. In insects, the mechanism of RNAi has been thoroughly studied in *Drosophila melanogaster*. dsRNA is recognized by the RNase III enzyme Dicer-2 and cleaved into 21-24 nucleotide short interfering RNAs (siRNAs). The

siRNAs are initially double-stranded in nature and one strand of the duplex is used by the RNA-induced silencing complex (RISC) to target degradation of RNA with cognate sequence.

Using genetic knock-out or mutant flies, the importance of RNAi in limiting RNA virus replication has been established in *Drosophila* (Galiana-Arnoux et al., 2006, van Rij et al., 2006, Wang et al., 2006). Transgenic mosquito technology is advancing rapidly but remains a laborious process. Therefore, more indirect techniques have been used to dissect the role that RNAi plays during arbovirus infection of the mosquito vector. Keene et al (2004) and Campbell et al (2008) used dsRNA injection to show that knockdown of key RNAi components transiently increases viral loads in individual mosquitoes. Titers of O'nyong-nyong virus in *Anopheles gambiae* and Sindbis virus in *Aedes aegypti* were higher if Argonaute 2 or Dicer 2 expression was silenced (Campbell et al., 2008, Keene et al., 2004). These studies show that RNAi is restricting replication of an arbovirus in the mosquito.

Essential virulence factors of plant and animal viruses have been shown to functionally interfere with the host's RNA silencing pathways. The plant potyvirus helper component-proteinase (Hc-Pro) was the first described viral suppressor of RNA silencing (VSR). The protein was capable of inhibiting both transgene- and virus-induced gene silencing in transgenic tobacco plants (Anandalakshmi et al., 1998). Proteins from a number of positive- and negative-sense RNA plant viruses are now characterized as VSRs, most of which bind to dsRNA to varying degrees (Li & Ding, 2006).

The B2 protein from the insect-pathogenic Flock House virus is a potent VSR that binds to dsRNA as a dimer interacting with one major and one minor groove of the molecule in a sequence-independent manner and can bind a range of dsRNA sizes (Chao et al., 2005, Lingel et al., 2005). The generic and promiscuous nature of dsRNA binding by B2, evidenced by its ability to inhibit RNAi in plants, nematodes, and insects, makes it an excellent candidate to study the effects of RNAi suppression in mosquitoes (Galiana-Arnoux et al., 2006, Li et al., 2002, Lu et al., 2005, van Rij et al., 2006).

SINV is the prototypic member of the *Alphavirus* genus and the molecular biology is well understood. In the cytoplasm of infected cells, viral nonstructural proteins are translated from genomic RNA while structural proteins are subsequently produced from a subgenomic mRNA. During replication of the viral genome, positive and negative sense RNAs base-pair to form dsRNA intermediates that could be recognized by Dicer-2. Alternatively, secondary structure of the plus sense RNA genome may be targeted by the RNAi machinery, as has been shown in plants infected with positive-sense, ssRNA viruses (Molnar et al., 2005, Szittya et al., 2002). SINV-specific siRNAs of both polarities can be detected in infected mosquitoes. However, more sense siRNAs were detected, suggesting secondary structure is the primary source, but not the only molecular RNAi target (Campbell et al., 2008).

Infectious cDNA clones based on the SINV genome have been engineered to express heterologous sequence from an inserted second subgenomic promoter. A double-subgenomic SINV constructed using the genome of strain TE12 has been used to knock down gene expression in mosquito cells and mosquitoes by an RNAi-specific manner through expression of heterologous RNA (Adelman et al., 2001, Attardo et al., 2003,

Higgs et al., 1998, Huang et al., 2005, Johnson et al., 1999, Olson et al., 1996, Powers et al., 1995, Powers et al., 1996). Heterologous proteins have also been expressed using this system (de Lara Capurro et al., 2000, Higgs et al., 1995, Higgs et al., 1996).

This chapter describes the production of a recombinant SINV that expresses a VSR protein and use of the virus to study the direct effects of RNAi on vector infection. A TE/3'2J virus was engineered to express the B2 protein. We hypothesize that B2 protein expressed during SINV infection will inhibit the RNAi response in infected mosquito cells and that this inhibition will lead to increased virus replication within the mosquito.

The VSR was functional in mosquito cells and affected the replication of TE/3'2J virus in *Ae. aegpti* cell culture. Mosquito infection experiments show that not only are rates of infection and dissemination of SINV in *Ae. aegypti* increased if RNAi is inhibited, but that the virus may become pathogenic in the mosquito vector. These studies highlight the potential necessity for RNAi from both the standpoint of the mosquito and the arbovirus.

Material and methods

Cells and medium

African green monkey kidney (Vero) and baby hamster kidney (BHK-21) cells were maintained in minimal essential medium (MEM) (Mediatech, Inc., Herndon, VA) supplemented with 7% fetal bovine serum (FBS), 1x nonessential amino acids for MEM (NEAA) (Mediatech, Inc.), 2 mM L-glutamine (Mediatech, Inc.), 100 units/ml penicillin and 100μg/ml streptomycin (Mediatech, Inc.). Human cervical carcinoma (Hela) cells

were maintained in Dulbecco's MEM (Mediatech, Inc.) supplemented with 7% FBS plus NEAA, L-glutamine, and antibiotics. *Ae. albopictus* C6/36 cells were maintained in Leibovitz-15 (L-15) medium (Mediatech, Inc.) supplemented with 10% FBS plus NEAA, L-glutamine, and antibiotics. *Ae. aegypti* Aag2 cells (a generous gift of Dr. Alexander Raikhel, University of California, Riverside) were maintained in modified Schneider's *Drosophila* medium (Lonza Walkersville, Inc., Walkersville, MD) supplemented with 10% FBS plus NEAA, L-glutamine, and antibiotics. Mammalian cells were grown at 37°C, 5% CO_2 and mosquito cells were grown at 28°C, ambient CO_2.

Construction of TE/3'2J/B2

Construction of the plasmid infectious cDNA clones pTE/3'2J and pTE/3'2J encoding green fluorescent protein (GFP) have been previously described (Hahn et al., 1992, Higgs et al., 1996). pTE/3'2J was kindly provided by Dr. Charles M. Rice (Rockefeller University, New York, NY). To construct pTE/3'2J/B2, the 321 base pair B2 gene was amplified by polymerase chain reaction (PCR) from an expression plasmid containing the entire B2 gene (). The forward primer contained sequence of V5 epitope, encoding the C-terminal 14 amino acids (GKPIPNPLLGLDST) of V protein from simian virus 5 (family *Paramyxoviridae*), a widely used protein epitope tag (Southern et al., 1991). The PCR product was digested with Xba I restriction endonuclease and ligated into the Xba I site of pTE/3'2J. Proper insertion of V5-B2 was verified through orientation PCR and sequencing.

Infectious virus production

Clones pTE/3'2J, pTE/3'2J/GFP, and pTE/3'2J/B2 were digested with Xho I enzyme to linearize the plasmids immediately downstream of the virus genome.

Digested plasmid DNA was extracted once with phenol:chloroform:isoamyl alchohol (25:24:1 volume ratio), twice with chloroform, and ethanol precipitated.

Pure linear DNA was used as a template for *in vitro* transcription using the MAXIscript® kit containing bacteriophage Sp6 DNA-dependent RNA polymerase (Ambion, Inc., Austin, TX). Transcription reactions contained 1x transcription buffer; at least 1.5 μg DNA template; 500 μM each of ATP, CTP, and UTP; 50μM GTP; 500μM cap analog [m7G(5')ppp(5')G (New England Biolabs, Ipswich, MA)]; and 100 units of Sp6 polymerase. Transcription reactions were incubated at 37°C for at least 1 hour.

Cell monolayers of BHK-21 cells were washed once with phosphate-buffered saline (PBS) without magnesium or calcium ions (Mediatech, Inc.), trypsinized, washed twice with ice cold PBS, and resuspended in ice cold PBS to a final concentration of $1x10^7$ cells per ml. Twenty-five microliters of transcription reaction were mixed with 0.4 ml of cells and the entire suspension was transferred to a 2 mm gap cuvette. Using a BTX Electro Cell Manipulator 630 apparatus (Harvard Apparatus, Inc., Holliston, MA), cells were pulsed twice at 450 volts (electroporator low-voltage setting), 125 ohms, and 25 μF. Immediately following electroporation, reactions were transferred to a 25 cm^2 flask containing 5 ml MEM (7% FBS) and incubated at 37°C, 5% CO_2 until greater than 80% of the cells exhibited cytopathic effects (typically 36-48 hours). At this time, supernatant containing passage 1 (P1) virus was aliquoted and stored at -70°C. Passage 2 (P2) virus was produced by infecting confluent monolayers of Vero cells with P1 virus at a multiplicity of infection (MOI) ~ 0.01. Supernatants for P2 virus were collected and stored similar to P1.

Plaque titrations

Infectious virus titers were determined by plaque formation on Vero cells. In a 24-well plate, confluent Vero cell monolayers were infected with 150 µl of virus suspension serially diluted 10-fold in supplemented MEM. Plates were incubated at 37°C, 5% CO_2 for greater than 1 hour before the addition of 1 ml of an overlay solution containing 1x molecular-grade agar (Sigma-Aldrich, St. Louis, MO), 1x Earl's balanced salt solution (Invitrogen Corporation, Carlsbad, CA), 3.3% YE-LAH (1% yeast extract plus 5% hydrolyzed lactalbumin in dH_20), 2% FBS, 0.11% $NaHCO_3$, 1 unit/ml penicillin, and 1µg/ml streptomycin. The overlay set for 30 minutes before plates were inverted and incubated at 37°C, 5% CO_2 for four days. At this time, 150µl of a solution containing 3.5 mg/ml of thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) in PBS was added to each well to visualize individual plaques. Plates were returned to incubation conditions for four hours and then placed at 4°C overnight before plaques were counted. Plaque-forming units (PFU)/ml were calculated according to the formula : # of plaques x $(\text{dilution})^{-1}$ x $(\text{inoculum volume in ml})^{-1}$.

Identification of V5 epitope-tagged B2 protein in mosquito cells

The presence of V5-B2 protein in virus-infected mosquito cells was determined by western blot. Cell monolayers were infected with TE/3'2J (designated TE/3'), TE/3'2J/GFP (designated GFP), and TE/3'2J/B2 (designated B2) virus at a MOI~0.01, or mock-infected with medium by rocking at room temperature for one hour. Forty-eight hours post-infection, medium was removed and cells were scraped into PBS containing Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Cell suspensions were sonicated and stored at -20°C.

After quantifying total protein using the Micro BCA Protein Assay kit (Thermo Fisher Scientific), ten micrograms of total protein were separated by SDS-polyacrylamide gel electrophoresis in a 10% NuPAGE Bis-Tris gel (Invitrogen Corp.). Proteins were blotted onto a nitrocellulose membrane at 30 volts for 1 hour. Membranes were blocked for 1 hour at room temperature in PBS plus 0.05% Tween-20 (PBS-T) and 5% lowfat dry milk (blocking buffer). V5- B2 protein was detected by blotting membranes at 4°C overnight with a mouse anti-V5 IgG antibody (Invitrogen Corp.) diluted 1:5,000 in blocking buffer followed by a room temperature incubation with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (KPL, Inc., Gaithersburg, MD) diluted 1:1,000 in blocking buffer for 30 minutes. The Pierce ECL western detection kit (Thermo Fisher Scientific, Inc., Rockford, IL) was used to develop the membranes according to manufacturer's protocols. Chemiluminescence was detected using the Storm 860 phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Virus growth curves

The ability of TE/3', GFP, and B2 viruses to replicate in cell culture was examined through growth curve analysis. Triplicate monolayers of Aag2, C6/36, BHK-21, Vero, and Hela cells in 25 cm^2 flasks were infected with virus at an MOI ~0.01. Medium was removed from the cells and P2 virus diluted in the corresponding growth medium supplemented with 3% FBS plus NEAA, L-glutamine, and antibiotics was added to each flask. Cultures were rocked at room temperature for 1 hour and medium was added to 5 ml total. Immediately following medium addition, a 500 µl sample was taken to determine input virus and an additional 500µl of fresh growth medium was reintroduced. The removal and addition of medium procedures were used every 12 hours

post-infection for a total of 48 hours for mammalian cells or 84 hours for mosquito cells. Samples were immediately stored at -80°C until determination of titers by plaque titration as described previously.

To observe differences in cytopathic effect between virus treatments, TE/3', GFP, and B2 virus-infected Aag2 cells were visualized using an Olympus IMT-2 inverted epifluorescent microscope using visible light. Pictures were obtained using an Olympus MicroFire camera (model S99809) and accompanying software.

Mosquitoes

Aedes aegypti Higgs white eye (HWE) mosquitoes, a variant of the Rexville D strain originating from Puerto Rico (Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO) (Adelman et al., 2004, Miller & Mitchell, 1991, Travanty et al., 2004) were reared at 28°C, 80% relative humidity, with a 16:8 light:dark photoperiod. *Aedes albopictus* mosquitoes originating from Saint Charles, Louisiana (Centers for Disease Control and Prevention) and *Culex tritaeniorhynchus* mosquitoes originating from Thailand (provided by Dr. Barry Miller at the Centers for Disease Control and Prevention) were reared at 27°C, 80% humidity, 14:10 photoperiod. *Aedes triseriatus* mosquitoes originating from La Crosse, Wisconsin and colonized at the Arthropod-borne and Infectious Diseases Laboratories (Fort Collins, CO) were reared at 25°C, 75% humidity, with a 16:8 photoperiod All adult mosquitoes were provided water and sugar *ad libitum*.

Northern blot for detection of virus-specific RNA

Virus-specific RNA species (genomic, subgenomic, and siRNAs) in cell culture and whole mosquitoes were detected by Northern blot analysis. For the detection of viral

RNA from Aag2 cells, 75 cm^2 flasks of cells (~80% confluency) were infected with TE/3', GFP, B2, or mock-infected as described for virus growth curves. At 0, 24, 48, and 72 hours post-infection, total RNA was extracted from cells using Trizol reagent (Invitrogen Corp.) following the manufacturer's recommended protocols. For viral RNA detection from infected mosquitoes, 3 to 5 day old female HWE mosquitoes were injected with 69 nl of $1x10^7$ PFU/ml TE/3', GFP, or B2 virus, or mock-injected with medium as described previously. Immediately following injection, at day two and four post-infection, ten individual mosquitoes from each experimental group were triturated in 500 µl of Trizol reagent and total RNA was extracted according to manufacturer's protocols.

Twenty micrograms (for Aag2 cells siRNA detection) or 40 µg (for mosquito siRNA detection) of RNA per sample were used for SINV-specific siRNA detection. Low molecular weight RNAs were separated by electrophoresis in a 15% TBE-Urea denaturing gel that was stained with ethidium bromide to visualize concentrations of RNA as a loading control. RNA was transferred to a neutral-charged nylon membrane and chemically cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Pall et al., 2007). Membranes were pre-hybridized in Ultrahyb buffer (Ambion, Inc.) at 42°C for 30 minutes.

An antisense biotinylated single-stranded RNA probe corresponding to nucleotides 10,765 to 11,264 (E1 gene) of TE/3'2J was generated from a PCR template containing a 3' T7 promoter. The MEGAscript *in vitro* transcription kit (Ambion, Inc.) was used according to manufacturer's recommended protocols with 9% of the total UTP conjugated to biotin. Five micrograms of riboprobe were reduced to 50-100 nt fragments

by hydrolysis in 200 mM carbonate buffer at 60°C for approximately two hours. Digested riboprobe was added to the hybridization buffer and incubated at 42°C for 16 hours. Following two washes with 2x SSC-0.1% SDS (5 minutes each) and two washes with 0.1x SSC-0.1% SDS (15 minutes each) RNA was detected using the BrightStar BioDetect kit (Ambion, Inc.) and exposed to autoradiography film for approximately 16 hours.

To detect SINV genomic and subgenomic RNA species, 5μg of the same RNA isolated from infected Aag2 and HWE was separated on a 1.25% agarose gel containing 0.6M formaldehyde. The RNA was transferred to a positively-charged Brightstar nylon membrane (Ambion, Inc.) and cross-linked using ultraviolet light. Methods similar to those used for siRNA detection were used here except that all hybridization and wash steps were carried out at 68°C. A labeled riboprobe corresponding to the 3' portion of the SINV genome (nt 11,148-11,320) capable of recognizing all three dsSIN viral RNA species was used to detect viral genomic and subgenomic RNAs.

In vitro dicing assay

This experimental procedure was developed at AIDL by Jaclyn Scott. Cell-free lysates were generated from Aag2 cells that were mock-infected or infected with TE/3', GFP, or B2 virus (MOI: 0.01). Lysates were formed 36 hours post-infection using a protocol similar to Haley et al. (2003). Briefly, cells were washed three times in PBS and resuspended in 1X lysis buffer (100mM potassium acetate; 30mM Hepes-KOH, pH 7.4; 2mM magnesium acetate) with protease inhibitors and 5mM DTT. The cells were disrupted in a Dounce homogenizer and centrifuged at 14,000xg for 25 minutes at 4°C. The supernatant was flash frozen in a dry ice/ethanol bath and stored at -80°C (Haley et

al., 2003). Protein concentrations were determined using a DC protein assay (Bio-Rad) and equilibrated prior to setting up the dicing activity reaction.

Dicing activity reactions were generated similar to that described by Haley et al. (2003) and incubated at 25°C. The reaction contained 1/2 volume of cell lysate, 1/3 volume of 40X reaction mix (50µl water; 20µl 500mM creatine monophosphate; 20µl amino acid stock at 1mM each) and ~450 ng of 500 bp biotinylated β-gal dsRNA (Haley et al., 2003).

The biotinylated dsRNA was produced by *in vitro* transcription of a 500 base pair region of the β-galactosidase gene from *Escherichia coli* using forward and reverse primers containing a bacteriophage T7 DNA-dependent RNA polymerase promoter. The transcription reaction was incubated at 37°C for one hour and was treated with DNase for one hour at 37°C. Transcribed RNA products were extracted once with phenol: chloroform, once with chloroform, and precipitated in ethanol.

At each timepoint, 10 µl were removed, added to 2X proteinase K buffer (200mM Tris-Cl, pH 7.5; 25mM EDTA, pH 8.0; 300mM NaCl; 2% weight/volume sodium dodecyl sulfate) and flash frozen. RNA was extracted using phenol chloroform, followed by a chloroform isoamyl alcohol extraction, and precipitated in ethanol. RNA was electrophoresed on a 20% TBE non-denaturing gel, stained with ethidium bromide, electrophoretically transferred to a BrightStar membrane (Ambion) and UV-crosslinked. Biotinylated RNA was detected with the BrightStar BioDetect Kit (Ambion) and exposed to autoradiography film for approximately 1.5 hours.

Per os infection of mosquitoes via artificial bloodmeal

P2 virus derived from Vero cells was used for bloodfeeding experiments. Aliquots of virus stocks with pre-determined titers were diluted to 10^7 PFU/ml in MEM containing 3% FBS plus NEAA, L-glutamine, and antibiotics. Virus was mixed with warmed defibrinated sheep's blood (Colorado Serum Co., Boulder, CO) and 10mM adenosine triphosphate (ATP) (45:45:10 v/v) and placed into the central chamber of a water-jacketed glass feeding apparatus using stretched Parafilm (Pechiney Plastic Packaging Inc., Neenah, WI) as an artificial membrane. Mosquitoes that had eclosed five to seven day earlier were allowed to feed for approximately 45 minutes before feeders were removed. Sugar was removed two days prior and water six hours prior to bloodfeeding. Bloodmeal samples were taken post-bloodfeed for virus titration as previously described. Mosquitoes were cold-anesthetized and engorged females were separated and kept at normal rearing conditions until analysis. All mosquitoes were provided sugar and water *ad libitum.*

Infectious virus titers in individual mosquitoes

Female HWE mosquitoes were given an infectious bloodmeal containing $1x10^7$ PFU/ml of TE/3', GFP, or B2 virus. At four and seven days post-infection, 48 individual mosquitoes from each group were randomly selected. Midguts were dissected from each mosquito and kept in individual tubes. The remaining carcass was placed in a separate tube and paired tubes for each mosquito were kept at -80°C until processing.

Using a motor-powered pestle, individual mosquito tissues were triturated in 500 µl DMEM supplemented with 7% FBS, NEAA, L-glutamine, antibiotics, and 1% HEPES buffer until no mosquito tissues were obviously discernible. Medium was added to 1ml

total and passed through a sterile 0.22μm filter (Pall Life Sciences, Ann Arbor, MI). Infectious virus titers were determined by plaque titration as previously described.

Mortality assays in adult mosquitoes

Mortality of mosquitoes following oral infection or intrathoracic injection with TE/3', GFP, and B2 virus was determined. For oral infections, five to seven day old female HWE mosquitoes were given a bloodmeal containing $1x10^7$ PFU/ml of virus or mock-infected cell culture supernatant. One hundred fully-engorged mosquitoes were randomly selected and kept at optimal rearing conditions for 21 days. Dead mosquitoes were counted daily for the duration of the experiment.

Because TR339-derived viruses efficiently infect *Aedes* and *Culex* mosquitoes following intrathoracic injection (Higgs et al., 1995, Olson et al., 1994, Rayms-Keller et al., 1995), 100 *Ae. aegypti* HWE, *Ae. albopictus*, *Ae. triseriatus*, and *Cu. tritaeniorhynchus* mosquitoes were injected with virus or mock-infected culture supernatant using a Nanoject II auto-nanoliter injector (Drummond Scientific Company, Broomall, PA). Sixty-nine nanoliters of virus ($1x10^7$ PFU/ml) or mock supernatant were injected into individual adult female mosquitoes that were cold-anesthetized. Injected mosquitoes were kept at optimal rearing conditions and dead mosquitoes were counted daily for the duration of the experiment.

To determine a 50% lethal dose (LD_{50}) for B2 virus in *Ae. aegypti*, female mosquitoes were injected with a serial 10-fold dilution series of B2 virus diluted in cell culture medium. Using the Nanoject II, groups of 50 female HWE mosquitoes were injected with 69 nl of virus diluent beginning with a stock virus titer of $1x10^7$ PFU/ml

and ending with $1x10^2$ PFU/ml. Injected mosquitoes were maintained and counted daily as previously described.

Immunohistochemical staining of infected mosquito sections

Female HWE mosquitoes were injected with $7x10^2$ PFU of GFP or B2 virus or mock-infected cell culture supernatant as previously described. Five days post virus injection, legs and wings were removed and 4% paraformaldehyde (PF) in PBS was injected into three individual mosquitoes from each group. Injected mosquitoes were dipped briefly in 70% ethanol and submerged in 4% PF overnight at 4°C. Mosquitoes were then removed from the PF, oriented in embedding cassettes and placed in 70% ethanol until processing. Mosquitoes were paraffin-embedded and sectioned (5 μm thick) by Colorado Histo-prep (Fort Collins, CO).

Tissues were fixed to the slide by heating at 60°C for 1 hour. Paraffin was removed by washing slides twice in xylenes (Sigma-Aldrich) for 30 minutes and 5 minutes. Tissues were rehydrated through a graded ethyl alcohol series (100%, 95%, 70%, 50%; five minutes each) and a final rehydration in PBS.

For immunohistochemistry (IHC), viral antigen detection was performed essentially as described by Olson et al (1994) with minor modifications. Slides were washed three times in PBS (15 minutes each) before incubation at 4°C overnight with mouse anti-SINV polyclonal serum MB92 (produced by Dr. Stephen Higgs at AIDL) diluted 1:100 in PBS. After three washes in PBS (15 minutes each), slides were incubated at 37°C for 1 hour with secondary alkaline phosphatase-conjugated horse anti-mouse Ig antibody (Vector Laboratories, Inc.) diluted 1:500 in PBS. Following three

additional washes in PBS, the Vector Red kit (Vector Laboratories, Inc.) was used to develop a bright red color where virus antigen was present.

Next, using the TACS 2 TdT *In situ* apoptosis detection kit and manufacturer's recommended protocols (Trevigen, Inc., Gaithersburg, MD) with cobalt ion as a cofactor for the terminal deoxynucleotide transferase enzyme, slides were stained for cell death by the covalent attachment of biotinylated deoxynucleotides to the ends of nicked DNA. Cells were permeabilized with Cytonin (Trevigen, Inc.) at room temperature for two hours prior to staining. A streptavidin-horseradish peroxidase conjugate (Vector Laboratories, Inc., Burlingame, CA) was applied and color was developed using the 3,3'-Diaminobenzidine (DAB) substrate kit (Vector Laboratories, Inc.) with the addition of nickel to produce a gray-black color. After a final wash in deionized water, slides were counterstained with hematoxylin and dehydrated in a graded ethyl alcohol series (50%, 70%, 95%, 100%; five minutes each). Slides were washed twice in xylenes and a coverslip was mounted with Permount (Thermo Fisher Scientific Inc.). Sections were visualized using a Leica DM4500B microscope (Meyer Instruments, Houston, TX). Images were taken using a Retiga 2000R camera and QCapture Pro software from Q Imaging (Surrey, British Columbia, Canada).

Results

Production of V5-B2 protein and virus replication in cell culture

After rescue of infectious virus from cDNA-derived RNA, TE/3', GFP, and B2 viruses were characterized in invertebrate and vertebrate cells. Production of V5-B2 protein from the second subgenomic promoter was verified by western blot analysis of

total protein from infected mosquito cell cultures. Using a commercial antibody against the V5 epitope, a single band of approximately 12 kilodaltons (kDa) was detected in B2-infected Aag2 and C6/36 cells (Figure 2.1). This agrees with the predicted size of B2 protein (11 kDa) with a V5 epitope tag (1.4 kDa).

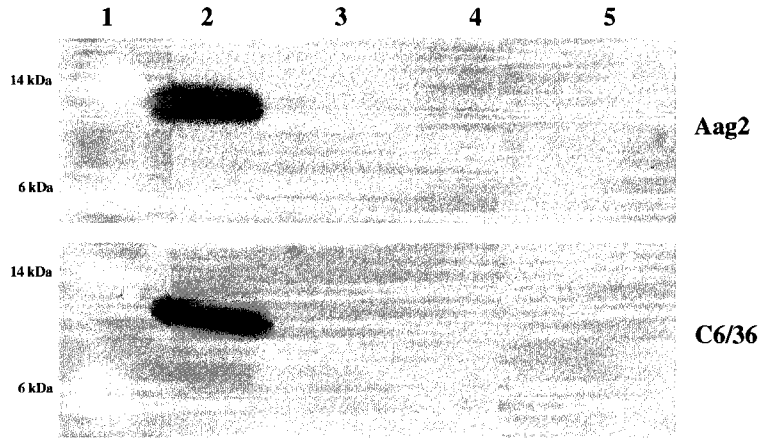

Figure 2.1. Detection of V5-B2 protein in mosquito cell culture. V5-B2 protein was detected in total protein from mosquito cell culture using an anti-V5 antibody. Molecular weights are indicated on the left side of each panel. Top panel is total protein from Aag2 cells, bottom panel from C6/36 cells. 1. Protein molecular weight marker 2. TE/3'2J/B2 3. TE/3'2J/GFP 4. TE/3'2J 5. Mock-infected cells.

To identify possible differences in replication kinetics between viruses, two-step growth curves were performed in vertebrate cells (Hela, Vero, and BHK-21) (Figure 2.2) and invertebrate cells (C6/36 and Aag2) (Figure 2.3). Virus replication was least efficient in Hela cells and was nearly identical among the three viruses. Maximal titers of 6.9-7.1 $\log_{10}$ PFU/ml were reached at 24-36 hours post infection (hpi). Peak titers of virus in BHK-21 cells appeared to be dependent on the size of heterologous sequence inserted into the virus genome. TE/3' (no insert) reached the highest maximal titer of 7.3 $\log_{10}$ PFU/ml at 24 hpi, followed by B2 virus (369 bp insert; 6.8 $\log_{10}$ PFU/ml at 24 hpi) and GFP virus (719 bp insert; 6.5 $\log_{10}$ PFU/ml at 12 hpi). Replication of all three

viruses was similar in Vero cells; peak titers of 7.1, 7.0, and 6.7 $\log_{10}$ PFU/ml were reached at 48 hpi for TE/3', GFP, and B2 viruses, respectively. No differences were observed in vertebrate cell CPE during virus infections.

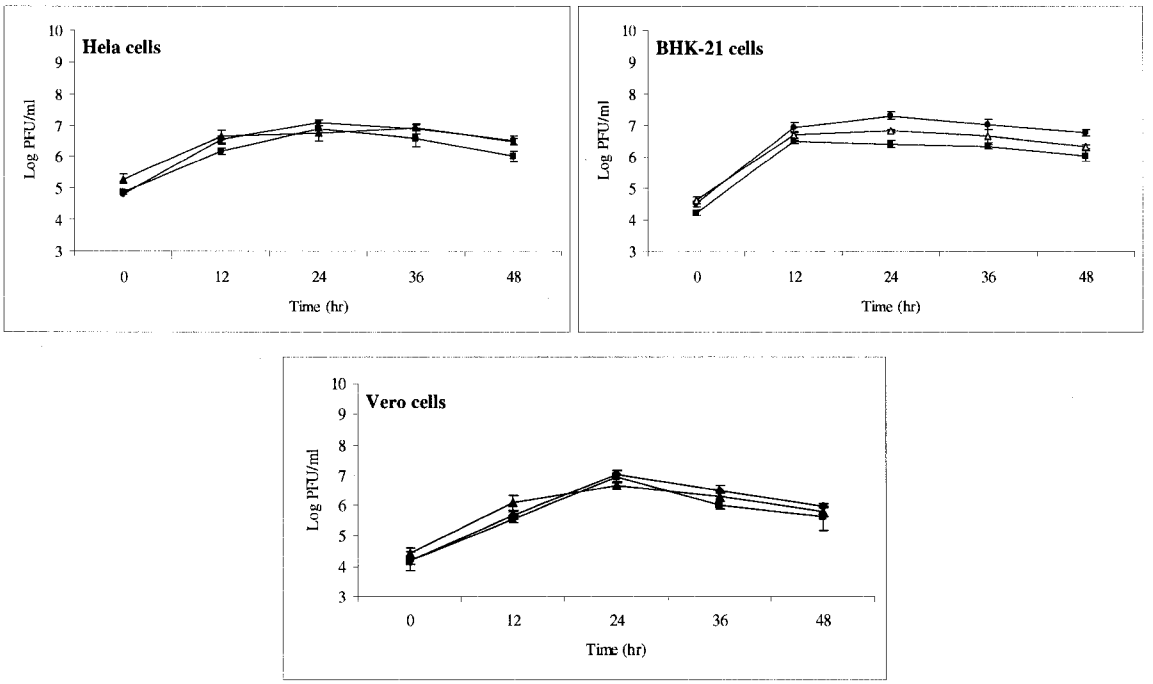

Figure 2.2. Growth of TE/3', GFP, and B2 viruses in vertebrate cells. Triplicate flasks of cell monolayers were infected at MOI~0.01. Titers were determined by plaque formation on Vero cells. ● = TE/3'2J, ■ = TE/3'2J-GFP, ▲ = TE/3'2J-B2.

All three viruses replicated with similar efficiency in C6/36 cells, reaching maximal titers of 8.1-8.5 $\log_{10}$ PFU/ml at 48-60 hpi. Similar to BHK cells, the relative maximal titer was virus genome-size dependent. However, virus replication in Aag2 cells was affected by the presence of B2 protein. B2 virus replicated to a maximum titer of 8.8 $\log_{10}$ PFU/ml at 48 hpi. This was more than 10-fold higher than TE/3' (7.4 $\log_{10}$ PFU/ml) and 100-fold higher than GFP (6.6 $\log_{10}$ PFU/ml). A marked decrease in titer was observed at later timepoints during B2 virus infection, coinciding with the presence of cytopathic effect that was less obvious during TE/3' or GFP virus infection of Aag2

cells (Figure 2.4). Notwithstanding, the titer of B2 virus was greater than the titers of TE/3' and GFP at all time points tested in this cell line.

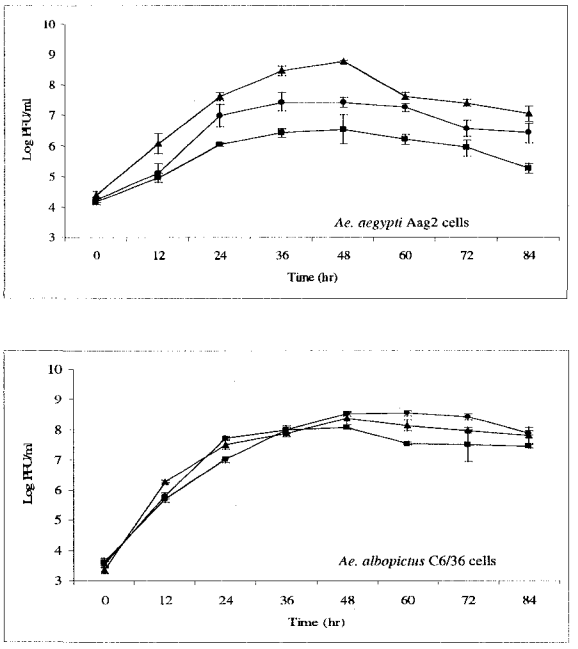

Figure 2.3. Growth of TE/3', GFP, and B2 viruses in invertebrate cells. Triplicate flasks of cell monolayers were infected at MOI~0.01. Titers were determined by plaque formation on Vero cells. ● = TE/3'2J, ■ = TE/3'2J-GFP, ▲ = TE/3'2J-B2.

Detection of virus-specific RNA in infected Aag2 cells

Virus-specific RNA was detected in total RNA from Aag2 cells by Northern blot using a biotinylated riboprobe derived from the E1 gene of TE/3'2J (Figure 2.5). Starting at 24 hpi, three viral RNA species were detected in cells infected with TE/3', GFP, and B2 viruses (Figure 2.5a). These bands represent the genomic, first subgenomic, and second subgenomic RNAs produced during virus infection. The second subgenomic RNA, expressed from the most 3' virus promoter, is the most highly transcribed RNA species. The relative amount of viral RNA reflects the infectious virus titers determined in growth curve analysis (Figure 2.3).

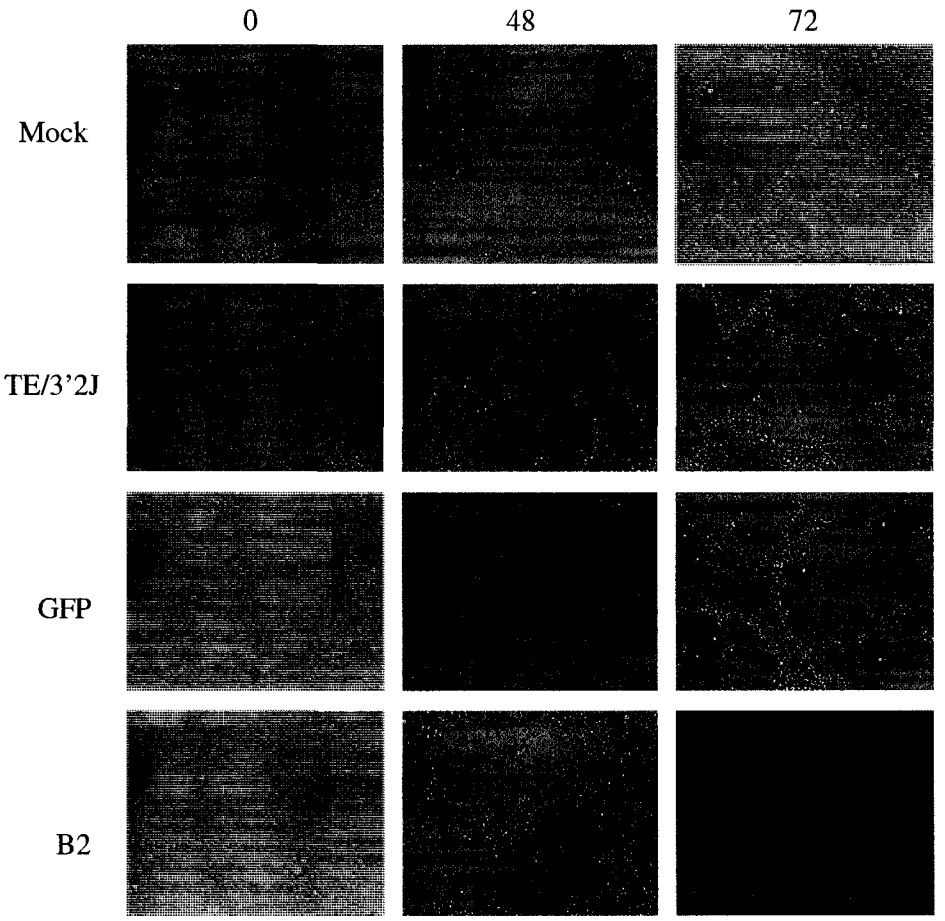

Figure 2.4. Virus-induced cytopathic effect in Aag2 cells. Subconfluent monolayers of Aag2 cells were infected at MOI~0.01. Images were taken using an inverted microscope and Microfire camera system. Times post infection are indicated to the left and infecting virus above the panels.

Peak levels of RNA accumulation coincide with peak virus titers at 48 hpi and an observed drop in both RNA and infectious virus production occurs by 72 hpi. Considerably more viral RNA accumulates in cells infected with B2 virus beginning at 24 hpi and continues throughout all time points tested. No bands were observed in RNA from mock-infected cells.

The presence of virus-derived siRNAs in Aag2 cells was also determined by Northern blot. Beginning at 24 hpi, a band of approximately 21-23 nucleotides, consistent with siRNA size from *Ae. aegypti* cells (Adelman et al., 2002, Adelman et al.,

2008, Campbell et al., 2008, Olson et al., 2002, Sanchez-Vargas et al., 2004, Travanty et al., 2004), could be detected from cells infected with TE/3' and GFP virus (Figure 2.5b). The relative abundance of siRNAs increased at each time point, with maximum abundance at 72 hpi. No bands were present in mock or B2-infected cells.

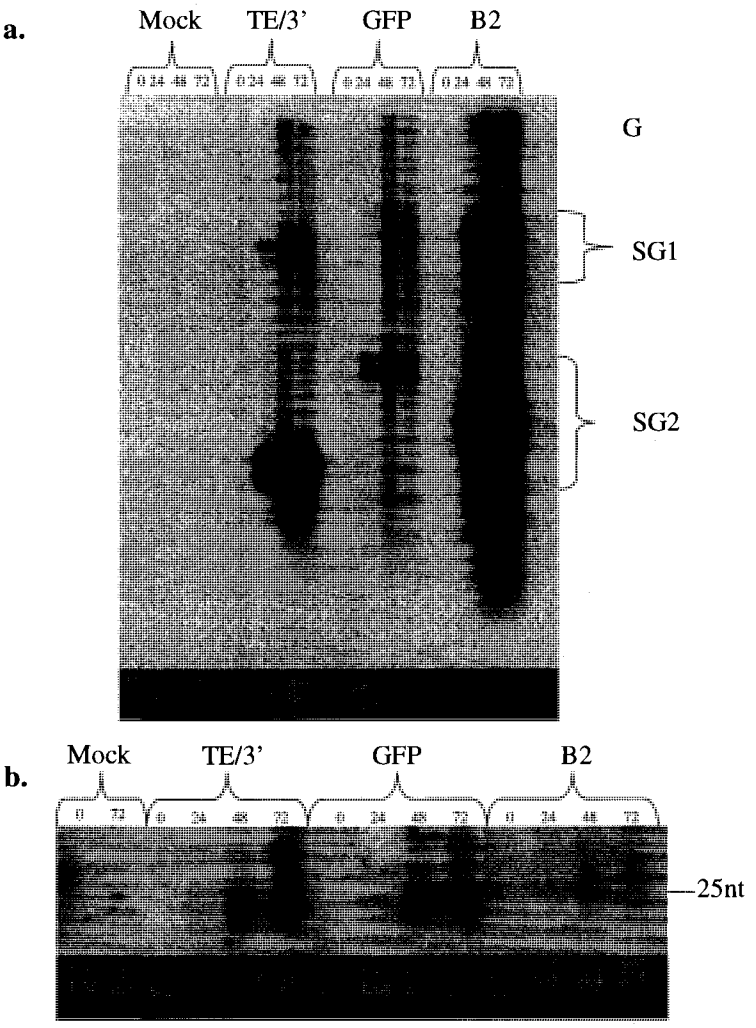

Figure 2.5. Detection of virus-specific RNAs in Aag2 cells. Monolayers of Aag2 cells were mock-infected or infected with TE/3', GFP, or B2 virus at MOI=0.01. At indicated times post infection, total RNA was isolated and probed using an E1-specific riboprobe for (a) virus RNA or (b) virus-derived siRNA. Ethidium bromide-stained ribosomal RNA below each blot serves as a loading control. Time in hours post infection. G=genomic; 1SG=first subgenomic; 2SG=second subgenomic.

In vitro *dicing assay in Aag2 cell lysates*

An *in vitro* dicing assay was performed to determine the ability of B2 virus to inhibit the accumulation of siRNAs. A synthetic 500 bp biotinylated dsRNA derived from the bacterial gene β-galactosidase was introduced into cell lysates derived from cells mock-infected or infected with GFP or B2 virus. The presence of siRNA product suggests that Dicer-2 is functional in the cell lysate. Biotinylated RNA approximately 21-23 nucleotides in length accumulated in mock- and GFP-infected cell lysates (Figure 2.6). No biotinylated RNA was detected in the expected size range at any time points tested during incubation with B2-infected cell lysates.

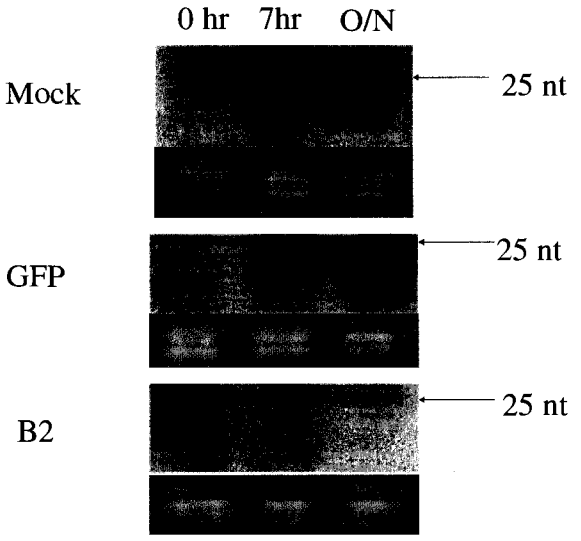

Figure 2.6. Accumulation of Dicer cleavage products in cells infected with TE/3' or B2 virus. Cell lysates were generated from Aag2 cells 36 hours post mock-, GFP-, or B2 virus-infection (MOI=0.01) (indicated to left of each panel). A synthetic 500 bp biotinylated dsRNA product was introduced into the lysates and, at indicated time points, samples were taken and the presence of small RNAs was determined by Northern analysis. Ethidium bromide-stained ribosomal RNAs located below each blot serve as loading controls. Arrows indicate position of 25 nucleotide marker. O/N=overnight.

Infection and dissemination of viruses in Aedes aegypti following per os bloodmeal

Infectious virus titers were determined for dissected midguts and mosquito carcasses following an infectious bloodmeal. At four and seven days post infection (dpi),

midguts were dissected from 48 mosquitoes per group and, along with remaining mosquito carcass, were titrated on Vero cells. Titers of infectious virus represent the extent to which individual mosquitoes were infected while the total number of infected midguts and carcasses represent the infection and dissemination rates, respectively. *Aedes aegypti* (HWE strain) mosquitoes ingested a bloodmeal containing $1x10^7$ PFU/ml of TE/3', GFP, or B2 virus. Because electroporation-derived recombinant SINV's and invertebrate cell-derived viruses based on SINV strain TE12 inefficiently infect mosquito midguts following oral infection, virus passaged once in Vero cells was used for all bloodfeeds (Cheng et al., 2001, Pierro et al., 2007).

B2 virus exhibited the highest rates of infection and dissemination and the highest average titers at both time points (Figure 2.7). Of 48 mosquitoes tested, 12 had detectable B2 virus in the midgut at four dpi, significantly more compared to TE/3' and GFP (P=0.0074 for both comparisons, Fisher's exact test). Infection rates increased in all three groups at seven dpi, but the number of B2 virus-infected mosquitoes remained significantly higher than TE/3' (P=0.0094) and GFP (P=0.0020). TE/3' and GFP virus infection rates were not different at 4 or 7 dpi.

All mosquitoes exhibiting a disseminated infection had detectable virus in the midgut. Four of 12 (25%) mosquitoes with detectable B2 virus in the midgut exhibited disseminated infection at day four while no virus was detected in carcasses of mosquitoes infected with TE/3' or GFP virus. At seven dpi, 60.9% (14 of 23) of B2 virus-infected mosquitoes had disseminated infections compared to 40% (4 of 10) for TE/3' and 37.5% (3 of 8) for GFP.

Significantly higher average B2 virus titers were found in the midgut at seven dpi (P=0.0446 TE/3':B2; P=0.0439 GFP:B2; unpaired Student's t test) and in mosquito carcasses at seven dpi (P=0.0043 TE/3':B2; P=0.0038 GFP:B2) compared to TE3'- and GFP-infected mosquitoes. Average B2 titers in the midgut at four dpi was higher, but not significantly (P=0.1023 TE/3':B2, P=0.1115 GFP:B2). At four and seven dpi, infection and dissemination titers were not statistically significant between TE/3' and GFP viruses.

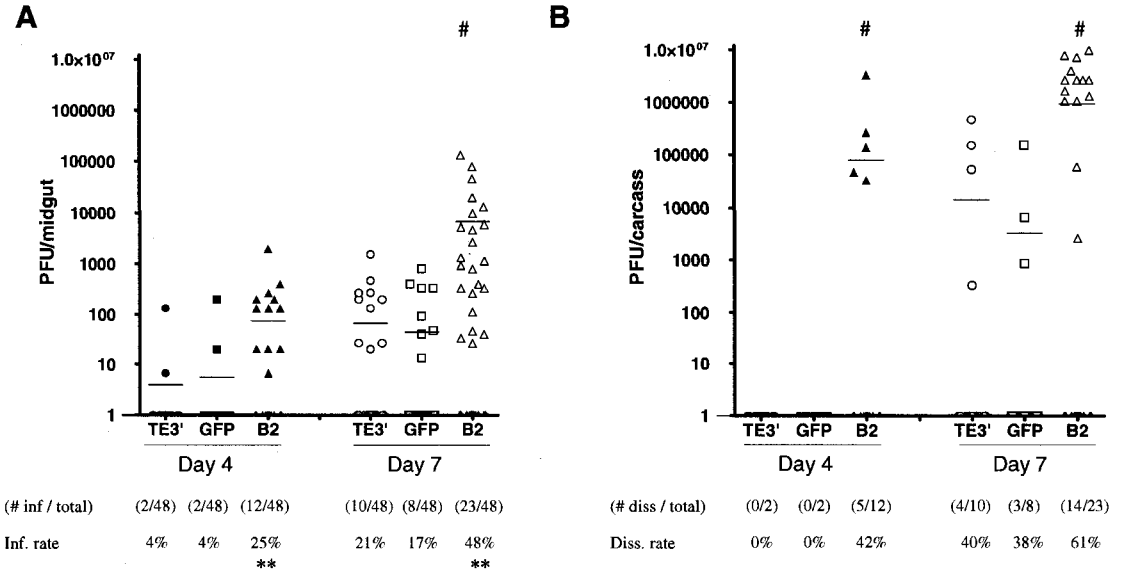

Figure 2.7. Infection and dissemination of TE/3',GFP, and B2 viruses in *Ae. aegypti* mosquitoes following oral bloodmeal. At the indicated days post-bloodmeal, viral titers were determined for midguts (A) and remaining mosquito carcass (B). n=48 per group. "TE/3'"=TE/3'2J, "GFP"=TE/3'2J-GFP, "B2"=TE/3'2J-B2. Horizontal line represents the mean for each data set. (*) indicates mean titer significantly higher than TE/3'2J and GFP infections.

Detection of virus-specific RNA in infected HWE mosquitoes

Groups of intrathoracically-injected HWE mosquitoes were assayed for the presence of SINV-specific RNAs. At zero, two, and four days post injection, total RNA was isolated from groups of ten mosquitoes and analyzed for viral RNA by Northern blot (Figure 2.8).

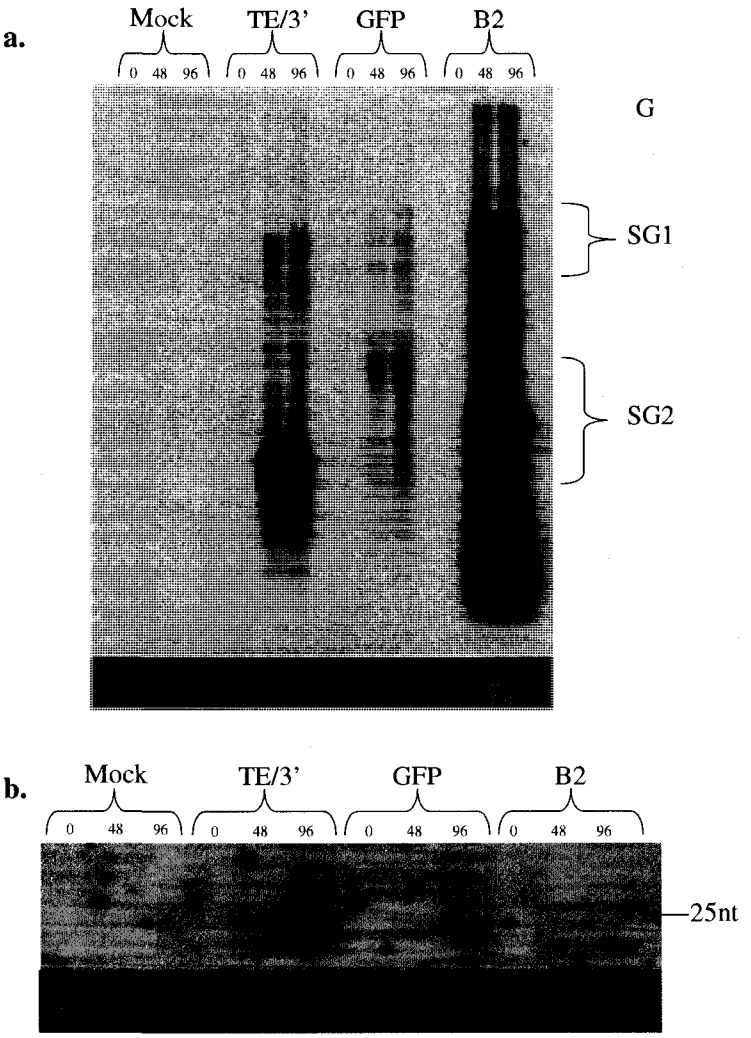

Figure 2.8. Virus-specific RNA detection in HWE mosquitoes. Total RNA was isolated from mosquitoes intrathoracically-inoculated with cell culture medium, TE/3', GFP, or B2 virus. Virus-specific RNAs (a) or virus-derived siRNAs (b) were detected using an E1-specific probe. Ethidium bromide-stained ribosomal RNAs seen under each blot serve as a loading control. G=genomic; 1SG=first subgenomic; 2SG=second subgenomic

By 48 hpi, three RNA species were transcribed in mosquitoes infected with TE/3', GFP, and B2 virus (Figure 2.8a). These RNAs correspond to the genomic, first, and second subgenomic RNAs. Similar to virus RNAs in cell culture (Figure 2.5a), the second subgenomic RNA is the most abundant transcript, followed by the first subgenomic and genomic RNAs. B2 virus produces considerably more RNA than either TE/3' or GFP virus. TE/3' produces more RNA than GFP virus. Using the same RNA samples, virus-

specific siRNAs were also detected by Northern blot (Figure 2.8b). In TE/3' and GFP-injected mosquitoes, siRNAs (21-23nt in size) began to accumulate by two days post infection and noticeably more are present by four days. No virus-specific RNAs in this size range accumulate in mock- or B2-injected mosquitoes.

Mosquito mortality associated with B2 virus infection

Mosquito mortality assays were performed to determine the effects of virus infection on mosquito survival. Female HWE mosquitoes were given a bloodmeal containing $1x10^7$ PFU of TE/3', GFP, B2, or cell culture medium only. Engorged females were separated and kept at optimal rearing conditions, including fresh sugar and water every day, for 21 days and individual mortality was monitored daily. Beginning at four dpi, more mosquitoes infected with B2 virus died than mock-infected mosquitoes or those infected with TE3' and GFP viruses (Figure 2.9). Eighty-three percent of the mosquitoes ingesting a bloodmeal containing B2 were dead by day 21 versus 21% for mock, 11% for TE/3', and 30% for GFP. Daily survival for mosquitoes that ingested B2 virus was significantly lower than mock, TE3', or GFP-infected mosquitoes ($P<0.0001$ for each comparison, Logrank test). Survival of TE/3'-infected mosquitoes was significantly different from GFP-infected mosquitoes ($P=0.0030$). TE/3' and GFP were not significantly different from mock-infected mosquitoes ($P=0.0623$ and 0.2496, respectively).

Because some mosquitoes that ingested a bloodmeal did not become infected, individual mosquitoes were intrathoracically-injected with virus to correlate infection with mortality. Female HWE mosquitoes were injected with approximately 700 PFU of virus or cell culture medium and were monitored daily for mortality.

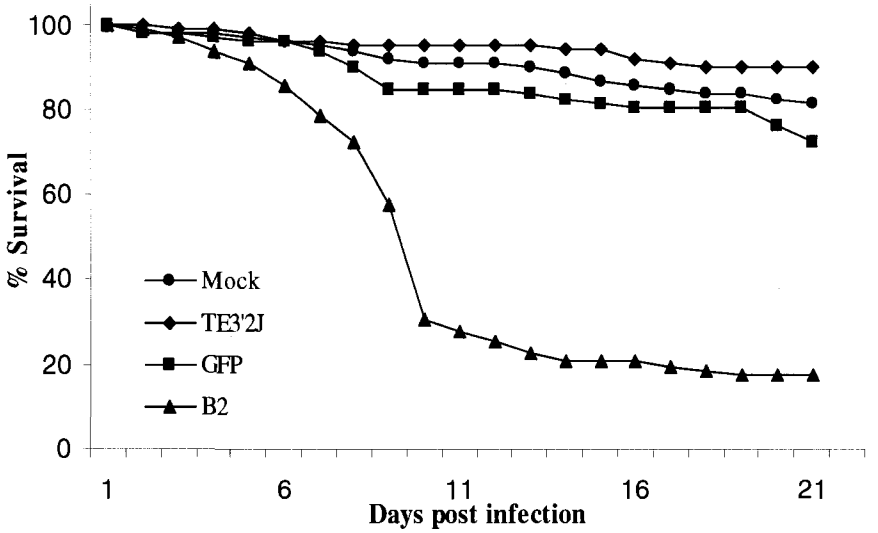

Figure 2.9. Virus associated mortality of *Ae. aegypti* HWE mosquitoes following ingestion of TE/3', GFP, or B2 virus. Mosquitoes were given an infectious oral bloodmeal containing $1x10^7$ PFU of virus and kept at optimal rearing conditions. Mortality was monitored daily for a total of 21 days. n=200 mosquitoes per group.

At ten dpi, all mosquitoes injected with B2 virus were dead (Figure 2.10a). By day 13, at least 70% of mock-, TE/3'-, and GFP-injected mosquitoes survived, suggesting B2 virus infection leads to death in *Ae. aegypti* mosquitoes.

B2 virus-associated mortality was mosquito species-independent as 100% of *Ae. albopictus*, *Ae. triseriatus*, and *Cu. tritaeniorhynchus* mosquitoes injected with B2 virus succumbed to infection by 11, 13, and 12 dpi, respectively (Figure 2.10b, c, and d). *Cu. tritaeniorhynchus* mosquitoes showed considerable mortality at one day post-injection, a phenomenon previously described for intrathoracic injection of this mosquito species (Foy et al., 2004).

TE/3'2J-B2 virus-associated mortality in different mosquito species after intrathoracic injection

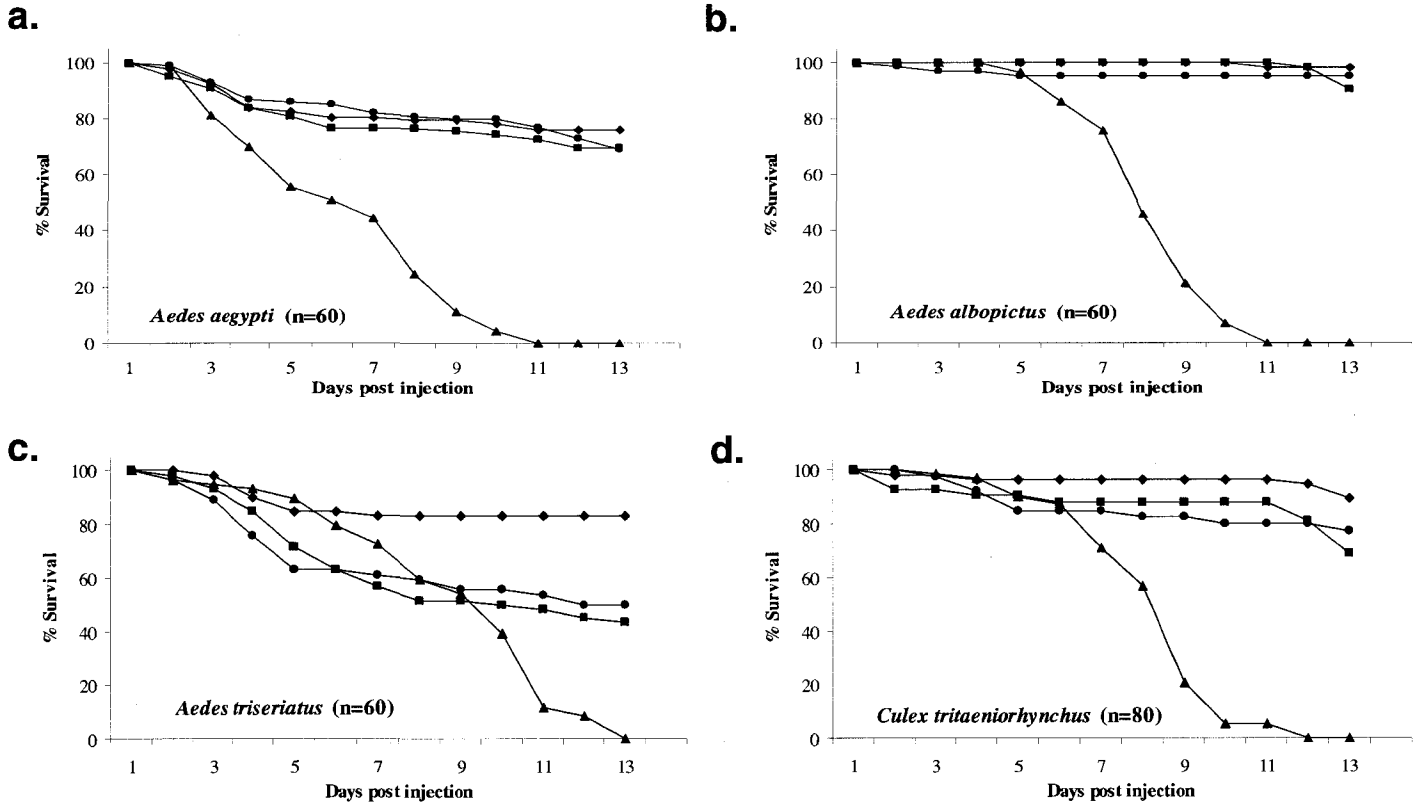

Figure 2.10. Mortality of different mosquito species following intrathoracic injection of TE3', GFP, and B2 virus. Mosquitoes were injected with virus stock diluted to $1x10^7$ PFU/ml and mortality was monitored daily. Day one mortality was not included. ♦=Mock; ●=TE/3'2J; ■=TE/3'2J-GFP; ▲=TE/3'2J-B2.

To determine if B2-associated mortality was dose-dependent, a 50% lethal dose at seven days post-injection was determined by mosquito intrathoracic injection. Groups of 50 *Ae. aegypti* (HWE) mosquitoes were injected with B2 virus diluted 10-fold in cell culture medium and monitored for mortality. Although extremely lethal, needing less than one PFU per mosquito to cause more than 50% mortality, B2 virus mortality was dose-dependent (Figure 2.11). The median survival time for mosquitoes was five days at the highest dose (10^7 PFU/ml) and seven days at the lowest dose that caused more than 50% mortality (10^3).

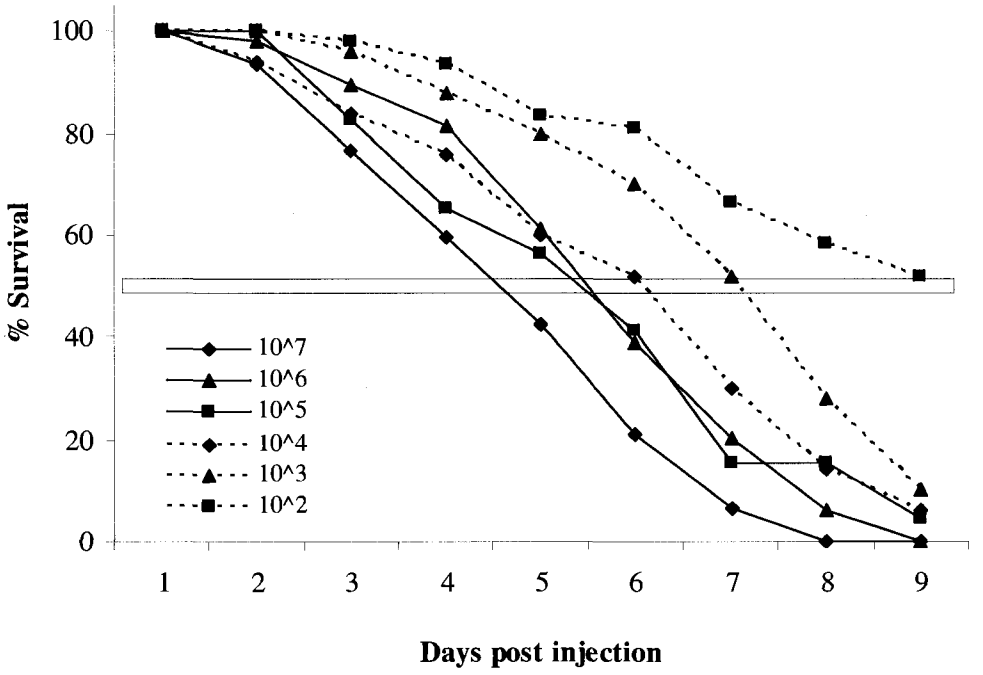

Figure 2.11. Determination of a mosquito 50 percent lethal dose for TE/3'2J-B2 infection. Groups of mosquitoes were intrathoracically injected with TE/3'2J-B2 virus diluted ten-fold and mortality was monitored daily. N=50 mosquitoes/group. White bar indicates 50 % mortality.

Immunohistochemical staining of infected mosquito sections

HWE mosquitoes were mock-injected or injected with GFP or B2 virus, kept at optimal insectary conditions for five days, and paraffin-embedded. Sectioned mosquitoes

were first stained for the presence of virus antigen using MB92 polyclonal mouse serum. After the addition of secondary antibody and substrate deposition, virus-infected cells should stain red in the cytoplasm.

No red staining was observed in mock-injected mosquito sections or GFP-injected mosquito sections that were incubated without primary antibody (Figure 2.12, data not shown). Virus antigen was detectable in sections of GFP and B2-injected mosquitoes (Figure 2.12). Antigen was distributed throughout the abdomen, thorax, and head of infected mosquitoes. In multiple mosquitoes from each virus-infected group, antigen was detected in the ommatidia, brain, Johnston's organ, salivary gland epithelium, thoracic ganglion, and throughout the abdominal and thoracic fat body. No matter the organ, more viral staining was observed in B2-infected mosquitoes and was consistently more intense than in GFP sections, in line with the higher virus titers and more viral RNA previously observed. GFP virus antigen was detected in the brain neuropile and glial cells, but to a much lesser extent than B2 virus. In many of the mosquito sensory organs, including Johnston's organ and ommatidia, more cells were infected and more antigen was detected in B2 sections (Figure 2.12). Malphigian tubules showed non-specific staining, a phenomenon previously described for immunohistochemistry in mosquito sections (Linthicum et al., 1996, McElroy et al., 2008, Olson et al., 1994, Romoser et al., 1992). No staining was observed in the midgut epithelium or ovarian tissue, consistent with previous reports for intrathoracic injection of SINV (Olson et al., 1996, Rayms-Keller et al., 1995).

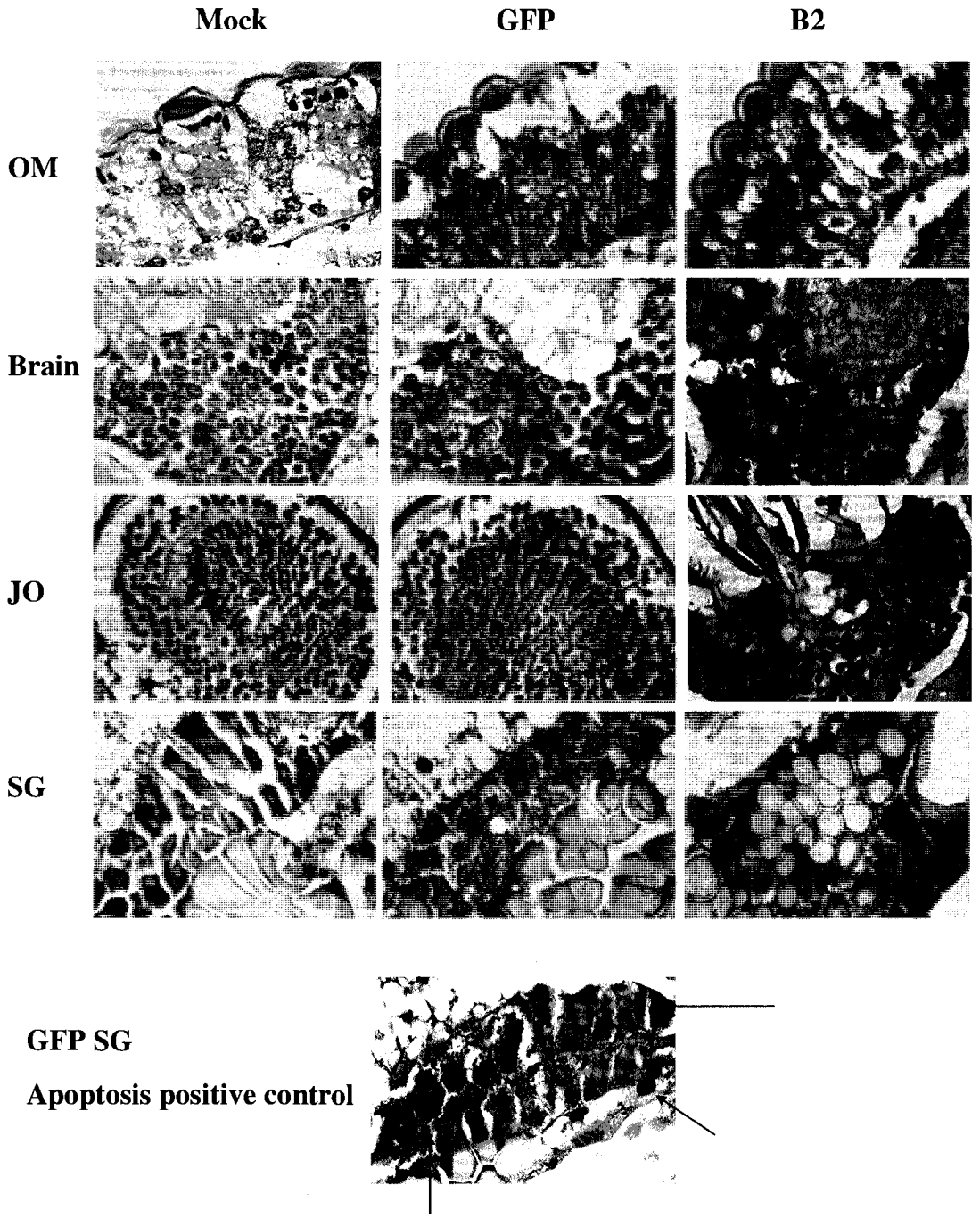

Figure 2.12. Immunohistochemical staining of sectioned mosquitoes showing GFP and B2 virus antigen distribution. Sections of HWE mosquitoes five days post injection with culture medium, GFP or B2 virus were stained for SINV antigen. A polyclonal mouse anti-SINV serum was used to detect SINV (red) and hematoxylin was used to stain nuclei (blue). OM=ommatidia, JO=Johnston's organ, SG=salivary gland. The GFP SG apoptosis postive control slide was nuclease-treated prior to staining. Arrows point to nuclei stained for apoptosis. All images were taken at 1000x magnification using a Leica DM4500B microscope with Retiga 2000R camera and QCapture Pro software.

Nucleus-associated grey-black staining was observed throughout a GFP-injected mosquito section that was treated with nuclease as a positive control for apoptotic staining (data not shown). Apoptosis staining was variable throughout all mosquito sections. Individual nuclei did stain a grey-black color in the nuclease-treated GFP-injected section, but even then staining was not uniform. In experimental slides, single cells within the midgut epithelium and throughout the fat body of GFP and B2-injected mosquitoes stained for apoptosis, suggestive of normal cellular death and regeneration. No cells stained for both virus antigen and DNA fragmentation (data not shown).

Discussion

RNAi is a major antiviral response in mosquitoes. The only other described mosquito immune response to arbovirus infection is the Toll antimicrobial pathway (Xi et al., 2008). RNAi is a highly conserved mechanism that is stimulated by the presence of an invading virus and controls viral replication through the sequence-specific degradation of the virus genome. To study RNAi during SINV infection of *Ae. aegypti*, we have engineered a dsSINV to express B2 protein, a potent VSR (Li et al., 2002). In our system, the B2 protein is translated only in infected cells, avoiding potential off-target effects associated with transient dsRNA-mediated silencing of the RNAi pathway. Tschuch et al (2008) found that introduction of siRNA specific for green fluorescent protein (GFP), a commonly used negative control and commercially available product, into human cell culture that did not express GFP could regulate more than 200 genes non-specifically (Tschuch et al., 2008). A similar non-specific dsRNA-mediated regulation of gene expression has been described in sandfly, *Lutzomyia longipalpis*, cell culture and the

marine shrimp, *Litopenaeus vannamei* (Pitaluga et al., 2008, Robalino et al., 2005). Although no such experiments have been performed in mosquito cells, introduction of dsRNA could have a similar effect. Detectable yet not statistically-significant increases in viral titer have been observed when control experiments injecting β-gal dsRNA and virus into mosquitoes have been performed (Franz et al., 2006, Keene et al., 2004). While the potential for non-specific response of the mosquito immune system to dsRNA is intriguing and should be studied further, examining the RNAi response using a SINV expressing a viral RNAi suppressor is superior to dsRNA-mediated interference of the RNAi pathway.

Three lines of experimental evidence suggest V5 epitope-tagged B2 protein is functional in RNAi suppression when expressed during TE/3'2J/B2 virus infection. First, significantly less SINV-specific siRNAs accumulate in cell culture and mosquitoes infected with B2 virus compared to TE/3' and GFP virus infection. The dsRNA formed by viral replicative intermediates may be bound by B2 protein, protecting the dsRNA from detection by the RNAi machinery. However, this may interfere with replication of the genome and translation of virus proteins. During the early phases of SINV infection of individual cells, dsRNA intermediates are formed prior to B2 protein expression, producing a potential elicitor of RNAi before the VSR is made in high enough concentration for efficient silencing. Also, because viral RNAs accumulate to such high amounts in B2-infected cells, the VSR may not be in sufficient stoichiometric concentrations for complete protection. Because B2 virus siRNAs do not accumulate, viral replicative intermediates are not the only RNAi trigger. B2 protein might also bind long stretches of dsRNA within secondary structures formed by the SINV genome to

protect the virus from RNAi. Accumulation of siRNAs in TE/3'- and GFP-infected Aag2 cells shows that the cell line has a functional RNAi response, the first description of RNAi from *Ae. aegypti* cell culture.

The second line of evidence that V5-B2 protein is functional comes from *in vitro* dicing experiments, which show inhibition of dicing activity in cell lysates derived from infected Aag2 cells. As was seen in cell culture and mosquitoes, the presence of B2 inhibits the accumulation of siRNAs, presumably by binding to and sequestering from Dicer2 the synthetic dsRNA. The presence of siRNAs in mock- and GFP-infected lysates provides further evidence that Aag2 cells have a functional RNAi mechanism and that the inhibition of siRNA accumulation is B2 virus-specific.

Finally, titers observed in Aag2 cells and adult *Ae. aegypti* mosquitoes were significantly higher when V5-B2 protein was expressed during infection. This agrees with the work of Campbell et al (2008) and Keene et al (2004) showing that inhibition of the RNAi pathway allows for arboviruses to replicate more efficiently in mosquitoes. By injecting mosquitoes with dsRNA to Dicer2 or Argonaute-2 following an infectious bloodmeal, Campbell et al (2008) were able to show that SINV titers in individual mosquitoes increased significantly by day four as compared to β-gal dsRNA injected controls. The same effect was not seen at day seven and the authors suggest this may be due to a stimulation of the antiviral response by this time point or degradation of the dsRNA mediators via decay (Campbell et al., 2008). Keene et al (2004) showed a similar general phenomenon with ONNV infection of *An. gambiae* mosquitoes, but could detect an increase in virus titer up to six days post infection. This difference may be explained by the inoculation route as both dsRNA and ONNV were administered intrathoracically,

bypassing any infection barriers associated with the midgut and ensuring introduction of virus and dsRNA into the hemocoel (Keene et al., 2004). A significant increase in SINV titers was observed at both four and seven days post infectious bloodmeal in our experiments B2 virus. The RNAi response is continuously inhibited by B2 protein as it is produced in infected mosquito cells. dsRNA intermediates or secondary structure of the virus genome will not be recognized by the RNAi machinery, allowing virus replication to continue unabated.

The first evidence that SINV may become pathogenic when RNAi is suppressed comes from cell culture CPE. Aag2 cells show significant CPE as little as 48 hpi with B2 virus. Minimal effects are seen during TE/3' or GFP virus infection compared to mock-infected cells, suggesting that it is a B2-specific phenomenon. Surprisingly, the same trend was not as apparent in C6/36 cells. RNAi is functional in this cell line; viral siRNAs accumulate during SINV and DENV2 infection of C6/36 cells (Adelman et al., 2001, Caplen et al., 2002, Sanchez-Vargas et al., 2004, Travanty et al., 2004). It is interesting to note that West Nile virus-specific siRNAs were not detected in C6/36 cells (Chotkowski et al., 2008). The authors suggest that the virus may be able to evade the cellular immune response through the formation of cytoplasmic membrane-bound compartments. In insect cells, alphaviruses have been shown to produce similar compartments, yet SINV-specific siRNAs can be detected in this cell line (Miller & Brown, 1992, Sanchez-Vargas et al., 2004). *Ae. aegypti* mosquitoes or the Aag2 cell line may have a more robust virus-specific RNAi response compared to *Ae. albopictus* and derived cell culture. Although this has not been directly studied, mortality assays showing no obvious difference in B2 virus-associated mortality between the two

mosquito species suggests that RNAi efficiency in these mosquitoes is not the confounding factor. A more likely scenario involves the selection of C6/36 cells for their ability to efficiently replicate arboviruses, which may have selected for a clonal population of cells that are less capable of inducing a sufficient RNAi response to suppress virus replication (Igarashi, 1978).

Pathology and mortality have been associated with alphavirus infection of mosquitoes, but the pathology is specific to the midgut or salivary glands and the mortality can, in some cases, be attributed to intrathoracic inoculation of large amounts of virus (Bowers et al., 2003, Moncayo et al., 2000, Weaver et al., 1992, Weaver et al., 1988). No matter the infection route, B2 virus killed *Ae. aegypti* mosquitoes and we hypothesized that the death was caused by massive apoptotic death of infected cells. FHV has been shown to induce apoptosis in *Drosophila* cell culture through the depletion of an intracellular inhibitor of apoptosis (Settles & Friesen, 2008). Apoptosis in alphavirus-infected mosquito cell lines is dependent on the amount of viral RNA and infectious virus produced during infection (Karpf et al., 1997a, Karpf et al., 1997b, Miller & Brown, 1992, Tatem & Stollar, 1986). We show that ten times the amount of SINV subgenomic RNA and 100-fold more infectious virus are produced in mosquitoes when B2 protein is expressed during infection. Although co-localization of virus antigen and apoptosis was not seen in immuno-stained sections, this possibility cannot be ruled out. We attempted to optimize the apoptosis stain and our positive controls showed nuclear staining, but the assay itself may not have been sensitive enough to detect DNA fragmentation in the infected cells. Also, the time at which samples were taken (five

days post injection) may have been too early to visualize apoptotic cell death in the mosquitoes.

It is possible that cell death caused by B2 virus is a non-apoptotic mechanism that can not be detected using TUNEL assays. Necrosis has been observed in midgut epithelial cells of *Culiseta melanura* mosquitoes orally-infected with EEEV at times corresponding to peak midgut virus titers (Weaver et al., 1988). Electron microscopy of infected cell morphology and detailed analysis of infected mosquito gene expression using microarray analysis may help to more clearly define the mechanism of B2 virus-associated mortality.

Significantly more virus antigen can be detected immunohistochemically in B2 compared to GFP-infected virus. Large portions of B2-injected mosquito brains showed intense virus staining that was not seen in GFP-injected mosquitoes. Sensory organs including Johnston's organ and ommatidia showed much more infection during B2 virus infection. Behavioral changes have been suggested as a direct result of arbovirus infection . B2 virus infection of the brain and sensory organs may lead to changes in mosquito behavior that eventually lead to death such as decreased nutrient and water uptake or inability to oviposit. Although not looked at here, quantitative observation of behaviors such as bloodfeeding and oviposition may provide evidence for neurologic effects associated with virus infection (Huang et al., 2005).

The salivary glands are an important organ for successful transmission of an arbovirus. If B2 virus infection does lead to cytopathology, transmission of the virus may be more efficient or could be hindered. Bowers et al (2003) suggest that SINV-associated pathology in *Ae. albopictus* midgut-associated musculature and salivary glands

could lead to a decrease in feeding success. If this is true, then transmission of B2 virus could be more efficient as mosquitoes take a longer time to probe the skin prior to imbibing blood. On the contrary, if salivation is compromised by virus-induced cytopathology, transmission would be less efficient.

B2 protein alone is likely not the mosquito mortality-associated factor. FHV was capable of replication to high titers when injected into *Ae. aegypti* mosquitoes, but no mortality was associated with the infection (Dasgupta et al., 2007). Also, transgenic *Drosophila* flies that express B2 protein have been shown to be deficient in siRNA-mediated but not microRNA-mediated RNA silencing and are more susceptible to RNA virus infection and virus-associated mortality (van Rij et al., 2006). This suggests that B2 protein by itself is not capable of causing mortality in dipterans, but that B2 protein in combination with an infecting RNA virus is capable of shifting the virus away from the influence of RNAi. To experimentally determine whether B2 protein is capable of mosquito mortality, a B2-expressing transgenic mosquito line would need to be created and tested for mortality before and during SINV infection.

The implications of B2 virus-associated mortality are two-fold. First, unlike pathogenic viruses that do not require persistent infection of the host, arboviruses may not encode true suppressors of RNAi. B2 protein and many proteins produced by pathogenic plant viruses are dsRNA binding proteins and potent suppressors of the RNAi response. The NSs protein of La Crosse virus, an arbovirus transmitted by *Ae. triseriatus* mosquitoes, is a dsRNA-binding protein suggested as a VSR in mammalian cells, but was later shown to be an interferon antagonist that could not interfere with RNAi in mosquito cells (Blakqori et al., 2007, Soldan et al., 2005). Similar conclusions were made with the

NS1 protein of influenza A virus, a non-vectored virus (Kok & Jin, 2006, Li et al., 2004). To our knowledge, there is no description of an arbovirus-produced protein that is a VSR in mosquito cells.

Second, mortality of B2 virus-infected mosquitoes suggests there may be a natural balance between mosquito immune response and virus replication that allows for the persistent nature of arbovirus infection in the vector. In the model of Semliki Forest virus (genus *Alphavirus*) regulation of RNA replication suggested by Kim et al (2004), production of minus-strand RNA, serving as a template for full-length virus genome and subgenomic RNA, is restricted to the early phase of replication. Limiting the production of minus-strand RNA may allow for more efficient allocation of cellular resources to progeny virus production and may have evolved to exclude subsequent viruses from establishing infection. The authors propose that regulation of minus strand RNA synthesis, in turn regulating full length and subgenomic positive strand RNA, evolved to moderate virus-associated virulence in the mosquito vector (Kim et al., 2004). Our experiments with B2 virus suggest that the replicase proteins of SINV, which control the amounts of viral RNA through sequential cleavage of polyprotein complexes, may not be the sole regulators of virus RNA quantities. Rather, there is a coordinated effort from both the virus and the mosquito immune system to regulate virus RNA replication to a point where both can persist. Further studies could compare virus RNA accumulation at early times during TE/3' and B2 virus infection and may shed light on the potential cooperation of viral replicase complexes and mosquito RNAi response in regulation of virus RNA production that may be responsible for persistent infection of mosquito cells. Identifying key mosquito factors necessary for the viral RNA regulation may lead to

novel transgenic mosquitoes that over-express these factors and are, therefore, refractory to arbovirus infection.

Chapter 3

COMPARISON OF DOUBLE SUBGENOMIC SINDBIS VIRUSES FOR THEIR EFFICIENCY IN INDUCING RNA SILENCING IN CULTURED MOSQUITO CELLS

Introduction

Plant viruses have been used to silence endogenous genes by a mechanism known as virus-induced gene silencing (VIGS). Endogenous genes of a biosynthetic pathway could be silenced in tobacco plants in a virus-dependent manner. The silencing was mediated by the expression of sense or antisense sequence homologous to the endogenous mRNA from recombinant virus or from a virus subgenomic promoter-containing transcript supplied to infected plants in *trans* (Kumagai et al., 1995, Ruiz et al., 1998). VIGS has also been described in mosquitoes. Johnson et al (1999) were able to silence a transgene in *Ae. aegypti* mosquitoes using a recombinant SINV. Salivary gland-specific transgenic expression of luciferase enzyme was inhibited in *Ae. aegypti* mosquitoes that were intrathoracically injected with a TE/3'2J virus transcribing antisense luciferase sequence. At five and nine days post infection, mosquitoes injected with the anti-luciferase virus showed a 90% reduction in salivary gland luciferase activity compared to mosquitoes that were not injected or injected with virus expressing a nonspecific antisense sequence (Johnson et al., 1999).

Endogenous mosquito and lepidopteran genes have been studied using TE/3'2J virus-mediated VIGS. In *Ae. aegypti*, dsSINV-mediated silencing of a known transcription factor regulating expression of yolk protein precursor provided evidence for a mechanism by which female mosquitoes regulate egg production (Attardo et al., 2003). In this study, RNAi was suggested to be the mechanism of action, but no gene-specific siRNAs were detected. Uhlirova et al (2003) showed a developmental role for the Broad-Complex gene in *Bombyx mori* larvae using a TE/3'2J virus expressing homologous sequence in antisense orientation. Broad-Complex-specific siRNAs were detected,

providing direct evidence for an RNAi-mediated silencing mechanism (Uhlirova et al., 2003). These experiments and others have shown the utility of TE/3'2J systems in studying endogenous protein function in mosquitoes through virus-induced RNAi-mediated gene silencing. dsSINVs are an important tool for studying gene function without the need to produce transgenic knockout lines of mosquitoes.

Tamang et al (2004) began to study the efficiency of RNAi-mediated silencing in mosquitoes using virus constructs expressing different lengths of sense and antisense sequence. Prophenoloxidase is a key enzyme in the melanotic cascade of invertebrate immunity and silencing of this gene had previously been established using a dsSINV expressing 600 bp of antisense sequence to the highly conserved copper-binding region of the protein (Shiao et al., 2001). Viruses expressing 600, 147, or 36 base pairs of sense or antisense sequence derived from the *Armigeres subalbatus* prophenoloxidase I gene were used to transduce adult mosquitoes. The efficiency of silencing was measured by the degree to which injected microfilariae were melanized: the higher the degree of melanization, the less efficient dsSINV-mediated VIGS of prophenoloxidase. TE/3'2J viruses expressing sense sequence were unable to interfere with melanization at any day tested. However, all three lengths of antisense sequence could mediate silencing and resulted in a decrease in the total degree of melanization. The 600 bp sequence was most efficient, almost completely inhibiting the melanization response and significantly inhibited total phenoloxidase activity in the mosquito by 15 days post infection. The virus expressing 36 bp of antisense sequence could silence the targeted gene, but the efficiency was much reduced. Importantly, prophenoloxidase-specific siRNAs were detected in mosquitoes at later times post infection with virus expressing 600 bp of

antisense sequence (Tamang et al., 2004). The work presented by Tamang et al (2004) was important to describe the requirements for VIGS in mosquitoes using dsSINVs.

Antisense-mediated silencing of a target gene by dsSINVs has also proved to be a useful tool for studying pathogen-derived resistance in mosquitoes. Heterologous viruses including dengue virus type 2 (DENV2), yellow fever virus, and La Crosse virus have been targeted in cell culture and mosquitoes by expressing virus-specific antisense sequence from a dsSINV vector (Adelman et al., 2001, Blair et al., 2000, Gaines et al., 1996, Higgs et al., 1998, Olson et al., 1996, Powers et al., 1995, Powers et al., 1996). Studies of DENV2 interference mediated by dsSINV have shown the power of such an approach in mosquitoes and set the groundwork that led to the construction of a transgenic *Ae. aegypti* mosquito refractory to oral DENV2 infection (Franz et al., 2006).

The size requirements necessary for efficient dsSINV-induced RNAi-mediated virus interference were determined in a TE/3'2J-DENV system similar to the prophenoloxidase system of Tamang et al (2004). Original constructs expressing 240 nucleotides of antisense sequence derived from the dengue virus type 1 (DENV1) NS5 gene were able to completely inhibit DENV1 infection in cultured mosquito cells. Similar constructs containing 183 and 160 nucleotides also efficiently interfered with DENV1 infection. However, truncation of the antisense sequence to 105 nucleotides significantly decreased the degree of interference. Similar to Tamang et al (2004), all viruses were capable of silencing their target genes with the most efficient silencing produced by larger heterologous sequence sizes (Blair et al., 2000).

To date, virus-induced mosquito gene silencing experiments have been performed exclusively with TE/3'2J or derivatives of this virus. Because TE/3'2J virus does not

efficiently infect midgut epithelial cells following oral administration, intrathoracic inoculation is required to mediate silencing and midgut-specific genes cannot be targeted with this virus. MRE/3'2J virus, a chimeric virus composed of TE/3'2J-derived nonstructural proteins and MRE16 structural proteins, has been used to successfully target *Ae. aegypti* midgut-specific gene expression, but stability of the virus is variable (Pierro et al., 2003, Sanchez-Vargas et al., 2004). Because of efficient mosquito midgut infection and high virus stability, a dsMRE16 virus capable of targeted silencing would be a powerful tool to study mosquito midgut genes using a stable virus platform (Foy et al., 2004, Myles et al., 2004).

We hypothesized that dsSINV systems derived from the TE12 and MRE16 strains of SINV are different in their abilities to mediate VIGS in mosquito cells. To test this, dsSINV-mediated silencing of the inducible *Ae. albopictus* lysozyme gene was examined in C6/36 cell culture using TE12- and MRE16-based systems. Lysozyme is a small enzyme characterized by its ability to cleave glycosidic bonds within certain bacterial cell walls and is thought to play an important role in the mosquito immune response to bacteria within the hemocoel and the midgut, but no effect on virus infection has been described (Ursic Bedoya et al., 2005). Lysozyme expression has been characterized in cell lines derived from multiple mosquito species (Gao & Fallon, 2000, Hernandez et al., 2003, Nasr & Fallon, 2003, Ursic Bedoya et al., 2005).

From our studies and previous data generated at AIDL, it became apparent that the two distinct SINV genotypes are very different in RNAi-mediated gene silencing. TE12 systems were capable of inducing a specific RNAi response when expressing antisense lysozyme sequence in C6/36 cells whereas MRE16-based systems were not.

The 3'ds systems expressing antisense gene sequence were then used to more closely examine differences between the two viruses. No variance in replication for TE/3'2J and 3'ds/MRE16 viruses was observed in C6/36 cells.

Aag2 cells appear to be a more useful system to investigate RNAi in mosquito cells, so viruses expressing *Ae aegypti* lysozyme sequence were also constructed. Virus strain-specific replication differences were observed in this cell line. Contrary to what was observed in C6/36 cells, lysozyme-specific siRNAs were detected in Aag2 cells infected with TE/3'2J and 3'ds/MRE16 viruses expressing antisense lysozyme sequence, suggesting that MRE16 does not completely inhibit mosquito RNAi. Defining the differences between MRE16 and TE12-based RNA silencing may help identify important determinants of alphavirus escape from the RNAi pathway and virus-vector interactions that could lead to novel vector-based arbovirus control strategies.

Materials and Methods

Cells and medium

C6/36 (*Ae. albopictus*) cells were grown in Leibovitz-15 medium containing 10% FBS plus L-glutamine and antibiotics. *Ae. aegypti* Aag2 cells (developed by Dr. Ann Fallon, University of Minnesota, and kindly provided by Dr. Alexander Raikhel) were grown in Schneider's *Drosophila* medium containing 10% FBS plus NEAA, L-glutamine, and antibiotics. Both cell lines were maintained at 28°C. Vero cells were grown in MEM containing 7% FBS plus NEAA, L-glutamine, and antibiotics at 37°C, 5% CO_2.

Virus construction

Plasmid clones pTE/5'2J, pTE/5'2J/GFP, pTE/3'2J, p5'ds/MRE16, p5'ds/MRE16/GFP and p3'ds/MRE16 have been described elsewhere (Foy et al., 2004, Hahn et al., 1992, Pierro et al., 2003). pTE/5'2J and pTE/3'2J were provided by Dr. Charles Rice. Viruses were constructed to express sequence corresponding to the 3' portion of the lysozyme gene of *Ae. albopictus* and *Ae. aegypti*. The entire lysozyme mRNA was cloned from either C6/36 cells or Aag2 cells using primers (Table 3.1) designed from the published sequences (*Ae. albopictus* accession #AY089957; *Ae. aegypti* accession #AJ290428) (Gao & Fallon, 2000, Hernandez et al., 2003) and inserted into the cloning vector pCR4 (Invitrogen Corp.). The plasmids were used as template in a PCR reaction to produce the lysozyme sequences inserted into the dsSINV vectors using internal forward and reverse primers TE-LYSO or MRE-LYSO (Table 3.1).

Viruses containing *Ae. albopictus* lysozyme sequence were constructed to encode a region of the sequence in either sense or antisense orientation. Using forward and reverse primers containing infectious clone multiple cloning site-specific restriction sites (NotI for dsMRE16, XbaI for dsTE12) (Table 3.1), nucleotides 237-549 of the published sequence were amplified. The amplicon was purified, digested with the appropriate restriction enzyme, and ligated into the corresponding SINV vector. Because ligation using a single restriction site is not orientation-specific, plasmids containing sense and antisense inserts were obtained from the same ligation reaction. Insert sequence and orientation were verified by sequencing (Proteomics and Metabolomics Facility, Colorado State University). A similar strategy was used to produce viruses encoding a portion of the *Ae. aegypti* lysozyme gene. Nucleotides 172-538 were amplified with

primers containing Not I or Xba I restriction sites (Table 3.1) and ligated into the equivalent SINV vector.

Table 3.1

Primers used in current study

Primer name	Sequence (5' to 3')
ALBO LYSOZYME FOR	ATCAGTTAGACAGCTACCG
ALBO LYSOZYME REV	CGCTTGGCATTTATAATTTATTAG
AEGYPTI LYSOZYME FOR	ATCAGTTAAACAAGCAGCTTGAAAG
AEGYPTI LYSOZYME REV	ATTAGGGCTATGAATTCAATCTATTTAG
TE-LYSO-AAl-FOR	CCTCTAGAGCGGCATGGATACCACC
TE-LYSO-AAl-REV	CCTCTAGAGGGCTTACGATTACC
MRE-LYSO-AAl-FOR	AAGCGGCCGCGCGGCATGGATACCACC
MRE-LYSO-AAl-REV	AAGCGGCCGCGGGCTTACGATTACC
TE-LYSO-AAe-FOR	AATTCTAGACAAGGATTCAAGAAAGC
TE-LYSO-AAe-REV	AATTCTAGAGTTACCAAAATTATCTGC
MRE-LYSO-AAe-FOR	AATGCGGCCGCCAAGGATTCAACAAAGC
MRE-LYSO-AAe-REV	AATGCGGCCGCGTTACCAAAATTATCTGC
AAl-LYSO-QPCR-FOR	GTGGGATTTATGGGCCTC
AAl-LYSO-QPCR-REV	ACCAGGCATACCCAGTTCC
RPS7-QPCR-FOR	GACCCCAACAAGCAGAAGCG
RPS7-QPCR-REV	TTGATCAGCTGCGATCCGTC
AAl-LYSO-NORTHERN-FOR	CCTTTGATCGTACTTGTGG
AAl-LYSO-NORTHERN-REV	TAATACGACTCACTATAGGTTAGCTTCATGGGGCTTACG
AAe-LYSO-NORTHERN-FOR	ATTGTACTGGTGGGTCTTTTTGC
AAe-LYSO-NORTHERN-REV	TAATACGACTCACTATAGGAGGGCTATGAATTCAATC

Virus production

The same procedures as described in Chapter 2 (Virus production) were used to produce infectious virus from linearized plasmids except that C6/36 cells were used for production and propagation of all virus stocks. *In vitro*-transcribed RNA was electroporated into $4x10^6$ C6/36 cells by pulsing once with 200 V, 25 Ω, and 550 μF. Passage 1 virus was harvested three to four days post-transfection. Monolayers of C6/36 cells were infected at an MOI ~0.01 with Passage 1 virus to produce passage 2, which was harvested similarly. Only passage 2 virus was used for cell culture experiments. The viruses included in these studies are listed in Table 3.2.

Table 3.2

Double-subgenomic Sindbis viruses used in current study*

Virus name	Inserted sequence
TE/5'2J	None
TE/5'2J/GFP	Green fluorescent protein coding sequence
TE/5'2J/Aal/LYSOs	*Ae. albopictus* lysozyme in sense orientation
TE/5'2J/Aal/LYSOas	*Ae. albopictus* lysozyme in antisense orientation
TE/3'2J	None
TE/3'2J/Aal/LYSOs	*Ae. albopictus* lysozyme in sense orientation
TE/3'2J/Aal/LYSOas	*Ae. albopictus* lysozyme in antisense orientation
5'ds/MRE16	None
5'ds/mRE16/GFP	Green fluorescent protein coding sequence
5'ds/MRE16/Aal/LYSOs	*Ae. albopictus* lysozyme in sense orientation
5'ds/MRE16/Aal/LYSOas	*Ae. albopictus* lysozyme in antisense orientation
3'ds/MRE16	None
3'ds/MRE16/Aal/LYSOs	*Ae. albopictus* lysozyme in sense orientation
3'ds/MRE16/Aal/LYSOas	*Ae. albopictus* lysozyme in antisense orientation
TE/3'2J/Aae/LYSOas	*Ae. aegypti* lysozyme in antisense orientation
3'ds/MRE16/Aae/LYSOas	*Ae. aegypti* lysozyme in antisense orientation

* Viruses expressing *Ae. aegypti* lysozyme in sense orientation were also produced, but were not used in the following experiments.

Induction of lysozyme expression in cell culture

C6/36 cells were split into a 12-well plate at $1x10^5$ cells per well and allowed to attach. Twenty-four hours later, duplicate wells were infected with MRE16- and TE12-based viruses containing no insert or *Ae. albopictus* lysozyme sequence (Table 3.2) diluted to MOI~10 in medium containing 3% FBS plus NEAA, L-glutamine, and antibiotics by rocking for one hour at room temperature. Duplicate wells were also mock-infected as a control for lysozyme induction. Medium was supplemented to 1.5 ml total and 500µl of phosphate-buffered saline (PBS) or heat-killed *Escherichia coli* cells in PBS (A_{260}=1) was added to each well. Infected cells were incubated at 28°C for 24 hours.

At 24 hours post infection, virus-infected C6/36 cells were scraped into the existing medium, transferred to microcentrifuge tubes and harvested by centrifugation at 3,000 rpm for 5 minutes. RNA was extracted using the RNeasy Mini kit and the

manufacturer's recommended protocols (Qiagen, Inc., Valencia, CA). Contaminating genomic DNA was digested with Turbo DNase (Ambion, Inc.) for thirty minutes at 37°C. DNase was removed using the DNase inactivation reagent (Ambion, Inc.). One-tenth volume of sodium acetate and three volumes of absolute ethanol were added and RNA was stored at -80°C until use.

To test induction of lysozyme expression in Aag2 cells, a similar procedure was used. However, because Aag2 cells did not efficiently adhere to 12-well plates after overnight incubation, heat-killed bacteria were added 72 hours after splitting the cells. To determine the amount of bacteria necessary for optimal lysozyme induction in Aag2 cells, various quantities of bacteria solution were added to the cells. RNA was treated and stored as described above.

Detection of lysozyme mRNA using quantitative reverse transcriptase PCR

RNA samples were centrifuged at 14,000 rpm for 15 minutes at 4°C to remove the ethanol/salt mixture and samples were resuspended in RNase-free water. The RNA was used as template for quantitative reverse transcriptase-PCR (qRT-PCR) determination of lysozyme mRNA expression using the Quantitect SYBR Green RT-PCR kit (Qiagen, Inc.) and a BioRad iQ5 Real-Time detection system (Hercules, CA). Reactions were performed in duplicate for each RNA sample using primers to detect lysozyme mRNA and ribosomal protein S7 mRNA as an internal standard (Table 3.1). One hundred nanograms of RNA was included in each 20µl reaction containing 1x Master mix, 300nM forward primer, 300nM reverse primer, 0.2µl of Quantitect RT mix (Qiagen, Inc.), and RNase-free water. For each qRT-PCR run, a no template-control and no RT-control for each primer set were also included. Cycling parameters were: 50°C for

30 minutes; 95°C for 15 minutes; 40 cycles of [95°C for 15 seconds, 57°C for 30 seconds, 72°C for 30 seconds, 77°C for 15 seconds, plate read]. Total light emitted by SYBR green binding to DNA in each reaction was detected at the "plate read" step. A melting curve, consisting of a plate read at 0.5°C intervals from 60°C-95°C with a six second dwell time, was performed at the end of the PCR reaction to verify the production of a distinct product. The total protocol is represented pictorially in Figure 3.1.

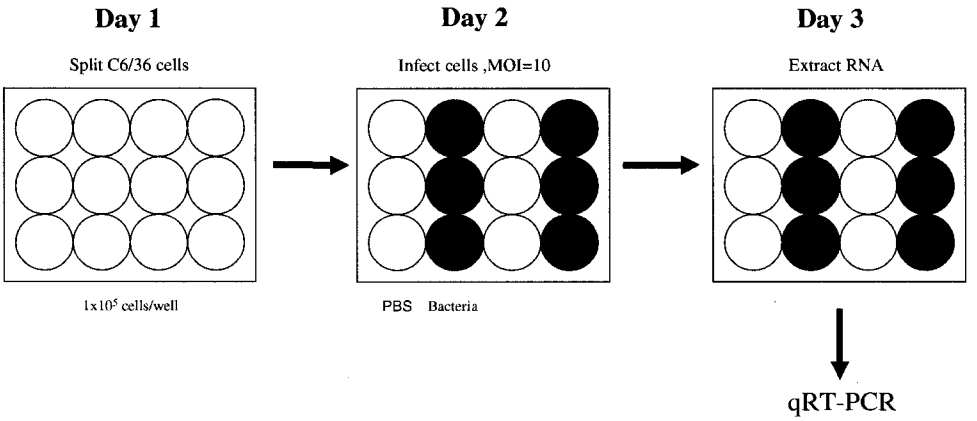

Figure 3.1. Outline of protocol for lysozyme induction. Diagram of lysozyme induction in C6/36 cells, beginning with splitting of cells and ending with qRT-PCR analysis of lysozyme mRNA. Black wells represent cells that are induced for lysozyme expression by the addition of heat-killed bacteria.

For each treatment, a cycle threshold (Ct) was determined by the amount of primer-specific template amplified in the reaction. The Ct values for each replicate of lysozyme and rpS7 were averaged and the difference between the two was calculated to produce a ΔCt value, representing the difference between the amount of experimental and control mRNA in a given sample. Calculating the difference in ΔCt values between PBS- and bacteria-treated cells for each virus treatment produced a ΔΔCt value representing the change in lysozyme mRNA levels between the two treatments. The fold-induction of lysozyme mRNA between PBS- and bacteria-treated cells was calculated as $2^{\Delta\Delta Ct}$.

Virus growth curves

Replication kinetics of TE/3'2J, TE/3'2J /Aal/LYSOas, 3'ds/MRE16, and 3'ds/MRE16/Aal/LYSOas viruses in C6/36 cells and the corresponding *Ae. aegypti* mutants in Aag2 cells were determined as described in Chapter 2 (Virus growth curves). Virus titers were determined by serial dilution of virus supernatant on Vero cell monolayers exactly as described in Chapter 2 (Plaque titrations).

Detection of lysozyme-specific siRNAs

The presence of lysozyme-specific siRNAs was determined in total RNA extracted from infected C6/36 and Aag2 cells. Matching 75 cm^2 flasks of cells were infected with TE/3'2J, 3'ds/MRE16, or the corresponding mosquito species-matched LYSOas mutant at an MOI~10 or mock-infected with cell culture medium by rocking for one hour at room temperature. Medium was supplemented to 10 ml total and 3 ml of bacteria solution was added to one of the flasks for each virus group. Cells were incubated at 28°C overnight. Twenty-four hours post infection, cells were scraped into the existing medium and harvested by centrifugation at 3,000 rpm for five minutes. Supernatant was removed and the cell pellet was resuspended in 3 ml of Trizol solution (Invtrogen Corp.). RNA was extracted from mosquito cells following the manufacturer's recommended protocols and stored at -80°C until use.

Samples were electrophoresed, transferred to a nylon membrane, and probed similar to methods described for SINV-specific siRNA detection in Chapter 2 (Northern blot for detection of virus-specific RNA) except that a carbonate buffer-hydrolyzed biotinylated riboprobe of the entire *Ae. albopictus* or *Ae. aegypti* lysozyme gene in antisense orientation was used to detect lysozyme-specific siRNAs. The probe was

produced by *in vitro* transcription from a PCR product amplified with gene-specific forward primer and a reverse primer containing an engineered T7 RNA polymerase promoter (Table 3.1).

Results

Induction of lysozyme in C6/36 cells and efficiency of dsSINV-mediated silencing

Double-subgenomic SINVs based on TE12 and MRE16 were engineered to express a portion of the *Ae. albopictus* lysozyme gene in either sense or antisense orientation. Infectious virus was produced by electroporating C6/36 cells with *in vitro*-transcribed RNA. After a second passage in C6/36 cells, more than 10^6 PFU of each virus was produced.

To determine the efficiency of RNAi-mediated knock down of an endogenous gene, a cell culture-based assay was used. Expression of the *Ae. albopictus* lysozyme gene was induced by the addition of heat-killed *E. coli* bacteria and levels of lysozyme-specific mRNA production were determined by qRT-PCR. When bacteria were added, cells became rounded and clumped together in small aggregates (data not shown), similar to what has been described after the introduction of heat-killed bacteria to other mosquito cell lines (Fallon & Sun, 2001).

The addition of bacteria stimulated expression of the lysozyme gene. The fold induction of lysozyme mRNA between uninfected, uninduced cells and uninfected, induced cells had a wide range, from less than 20-fold to more than 120-fold (Figure 3.2 and 3.3, Bac. Only columns). The average fold induction of lysozyme mRNA for all trials was 68 ± 32 (data not shown).

The efficiency of virus-mediated silencing of lysozyme mRNA in C6/36 cells was determined by calculating the fold-induction of lysozyme mRNA in the presence of dsSINVs and comparing this to the fold-induction observed in mock-infected cells. The level of lysozyme expression and efficiency of virus-induced RNAi are inversely related; the lower the induction of lysozyme mRNA, the more efficient a given virus is at silencing the gene. In all virus treatments, a large variance was observed between trials, evidenced by the large error bars in Figures 3.2 and 3.3. Because trials between the different dsSINV backbones were performed separately, the data for each is presented individually.

Although the induction of lysozyme was variable, the difference in fold-induction between uninfected cells and virus-infected cells was consistent between trials (data not shown). dsTE12-based viruses containing no insert or expressing green fluorescent protein did not significantly decrease lysozyme induction (Figure 3.2). For TE/5'2J virus trials, the fold-induction of lysozyme in uninfected cells was 42, compared to 33 for GFP-expressing virus (P=0.59, Student's t-test with unequal variance) and 60.0 for virus containing no insert (P=0.29). Lysozyme was induced 56-fold in uninfected cells for the TE/3'2J virus trials, compared to 57-fold for TE/3'2J (P=0.95).

TE/5'2J and TE/3'2J viruses expressing lysozyme sequence in the antisense orientation were able to significantly decrease overall lysozyme mRNA induction (Figure 3.2). During TE/5'2J/Aal/LYSOas virus infection, lysozyme mRNA was induced 17-fold compared to 42-fold for uninfected cells, a significant reduction (P=0.024). TE/3'2J virus expressing the same insert was able to efficiently decrease the levels of lysozyme from 56-fold induction for uninfected cells to 23-fold induction for virus infected cells

(P=0.049) (Figure 3.2). Lysozyme sequence expressed in sense orientation from TE/3'2J was able to decrease the levels of induced lysozyme mRNA, but the difference was not significant (P=0.097).

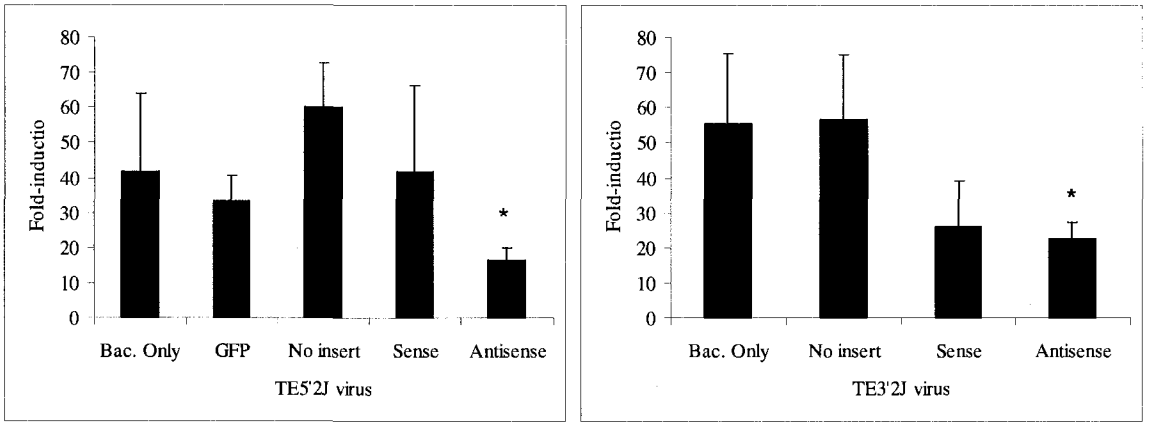

Figure 3.2. Induction of lysozyme mRNA in dsTE12 virus-infected C6/36 cells following bacteria treatment. C6/36 cells were infected with the indicated viruses at an MOI~10 and lysozyme mRNA was induced by the addition of heat-killed bacteria. After 24 hour incubation, total RNA was isolated and levels of lysozyme mRNA were determined by qRT-PCR. Each column is an average and standard deviation calculated from three individual trials. "Sense"=virus expressing lysozyme sequence in sense orientation. "Antisense"=virus expressing lysozyme sequence in antisense orientation. *=significant decrease from Bacteria only (P<0.05).

MRE16 viruses containing no insert or expressing GFP did not significantly decrease the induction of lysozyme after the addition of bacteria to C6/36 cells (Figure 3.3). The average fold-induction of lysozyme for viruses containing no insert increased dramatically, from 70-fold to 130-fold for 5'ds/MRE16 (P=0.13) and from 100-fold to 130-fold for 3'ds/MRE16 (P=0.42) (Figure 3.3).

A slight decrease in fold-induction was observed in induced C6/36 cells infected with 5'ds/MRE16/Aal/LYSOas virus, from 70-fold for uninfected cells to 50-fold for infected cells (P=0.377) (Figure 3.3). In cells infected with all other 5'ds/MRE16 viruses, the fold-induction was either the same or increased compared to uninfected cells. Lysozyme mRNA levels were consistently higher between 3'ds/MRE16 virus infections

and uninfected controls, with observed 100-, 140-, and 120-fold increases for bacteria only, LYSOs, and LYSOas virus infections.

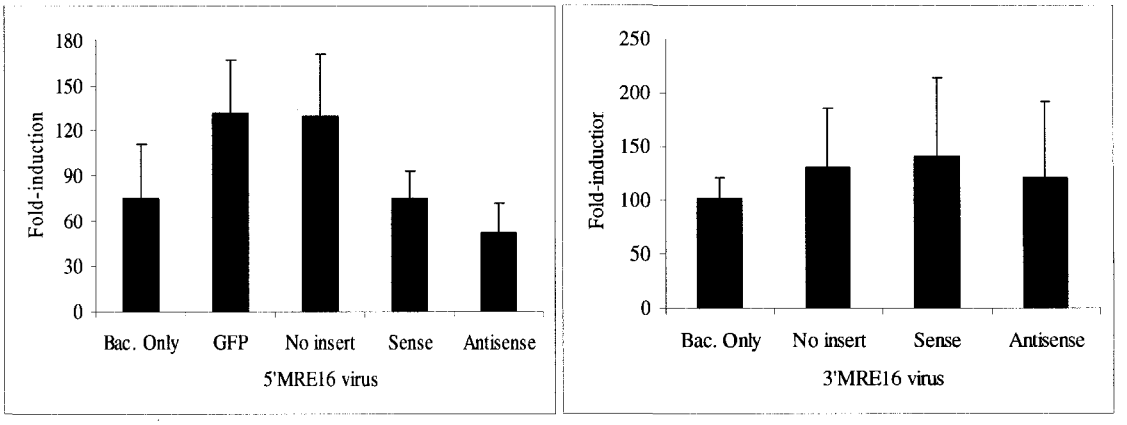

Figure 3.3. Induction of lysozyme mRNA in dsMRE16 virus-infected C6/36 cells following bacteria treatment. C6/36 cells were infected with the indicated viruses at an MOI~10 and lysozyme mRNA was induced by the addition of heat-killed bacteria. After 24 hour incubation, total RNA was isolated and levels of lysozyme mRNA were determined by qRT-PCR. Each column is an average and standard deviation calculated from three individual trials. "Sense"=virus expressing lysozyme sequence in sense orientation. "Antisense"=virus expressing lysozyme sequence in antisense orientation. Note the difference in y-axis values between the two graphs.

Replication kinetics of dsSINVs in C6/36 cells

The 3'ds systems of TE12 and MRE16 viruses were used to determine any strain-specific replication differences in C6/36 cells (Figure 3.4). C6/36 cells were infected with TE/3'2J/Aal/LYSOas, 3'ds/MRE16/Aal/LYSOas, and the corresponding parental viruses at an MOI=0.01. Samples were taken every 12 hours and virus titers were determined by plaque formation on Vero cell monolayers. TE/3'2J and TE/3'2J/Aal/LYSOas virus replication curves were nearly identical, reaching peak titers at 36 hpi of 8.2 and 8.0 $\log_{10}$PFU/ml. Although 3'ds/MRE16 reached high titers at 24 hpi, maximal titers of 8.3 $\log_{10}$PFU/ml were not reached until 60 hpi. Similarly, 3'ds/MRE16/Aal/LYSOas reached peak titer (8.0 $\log_{10}$PFU/ml) at 60 hpi. TE/3'2J

viruses maintained high titers throughout the 84 hour time course, while 3'ds/MRE16-derived virus titers decreased at later time points.

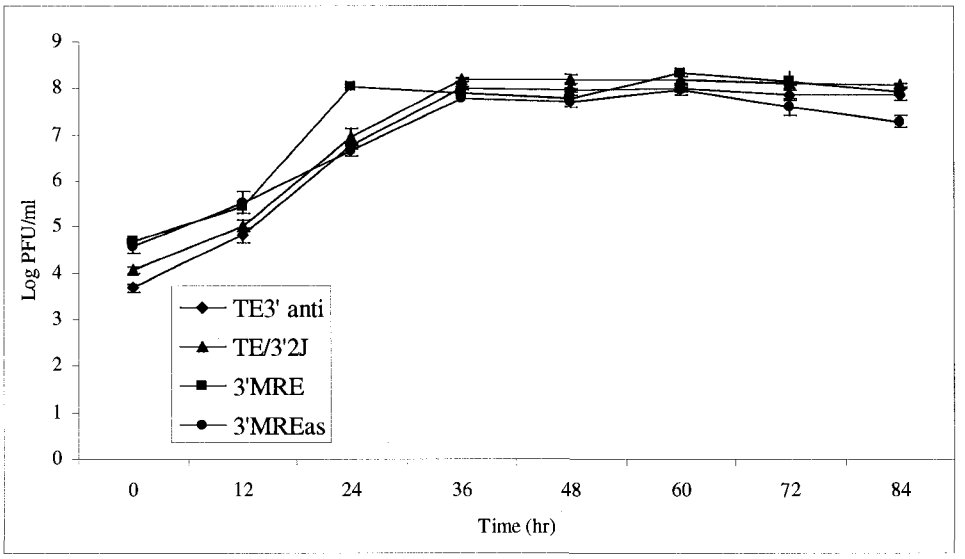

Figure 3.4. Replication kinetics of dsSINVs in C6/36 cells. Triplicate monolayers of cells were infected with the corresponding viruses at an MOI~0.01. Virus titers of samples taken every 12 hours were determined by titration on Vero cell monolayers. "TE3' anti"=TE/3'2J/Aae/LYSOas; "3'MREas"= 3'ds/MRE16/Aae/LYSOas.

Induction of lysozyme mRNA in Aag2 cells

Aag2 cells appear to have a more efficient RNAi response. Therefore, we began to develop the cells as a system to examine mosquito RNAi. Aag2 cells did not attach efficiently following an overnight incubation (data not shown). However, after incubation for three days, the cells were sufficiently attached to observe lysozyme induction mediated by the addition of heat-killed bacteria. Various volumes of bacteria were added to the cells and total RNA was extracted 24 hours later. Lysozyme mRNA levels were determined using *Ae. aegypti* gene-specific primers in a qRT-PCR exactly as described for *Ae. albopictus*.

Lysozyme induction was observed in Aag2 cells when bacteria were added (Figure 3.5). *Ae. aegypti* lysozyme mRNA expression was induced more than 100-fold when 500 and 750µl of heat-killed bacteria solution was added. Lysozyme induction was dose-dependent; the fold-induction increased with the addition of increasing amounts of bacteria. As was observed in C6/36 cells, lysozyme induction was highly variable between experiments.

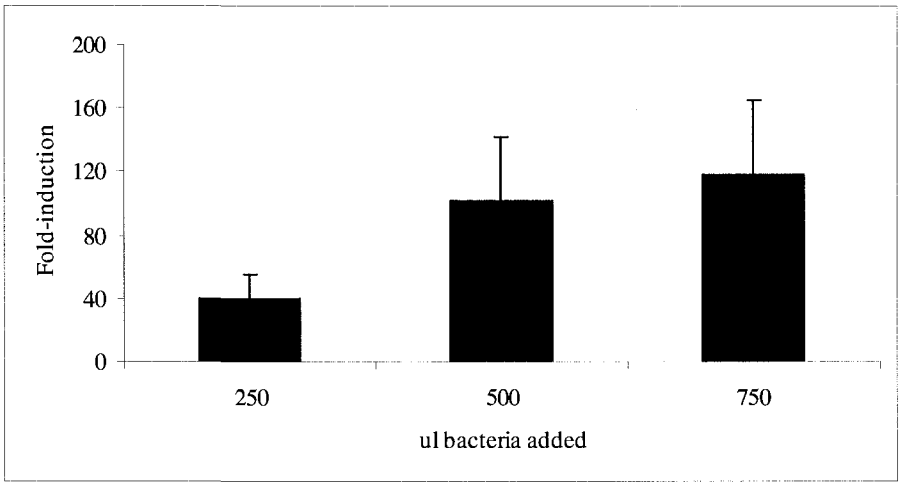

Figure 3.5. Induction of lysozyme mRNA in Aag2 cells. The induction of lysozyme mRNA 24 hours following heat-killed bacteria induction was analyzed by qRT-PCR. Each column represents the average fold-induction and error bars represent the standard deviation of two experiments for each treatment.

Replication kinetics of dsSINVs in Aag2 cells

The replication kinetics of recombinant dsSINVs expressing *Ae. aegypti* lysozyme antisense sequence and parental viruses were determined in Aag2 cells (Figure 3.6). Triplicate monolayers of cells were infected with virus at an MOI=0.01 and virus titers were determined at 12 hour timepoints. Both parental viruses replicated more efficiently than the viruses expressing LYSOas sequence. Beginning at 24 hpi, TE/3'2J replicated to titers more than 0.5 $\log_{10}$PFU/ml higher than TE/3'2J/Aae/LYSOas. Peak titers of 7.7 and 6.8 $\log_{10}$PFU/ml were obtained for parental and insert-containing virus, respectively.

The parental 3'ds/MRE16 efficiently replicated in Aag2 cells with similar kinetics to the TE/3'2J virus; a maximal titer of 7.5 $\log_{10}$PFU/ml was reached by 72 hpi. However, 3'ds/MRE16/Aae/LYSOas did not replicate efficiently in the mosquito cells. After a modest increase in titer at 12 hpi, 3'ds/MRE16/Aae/LYSOas titers were below the input titer of 4.8 $\log_{10}$PFU/ml.

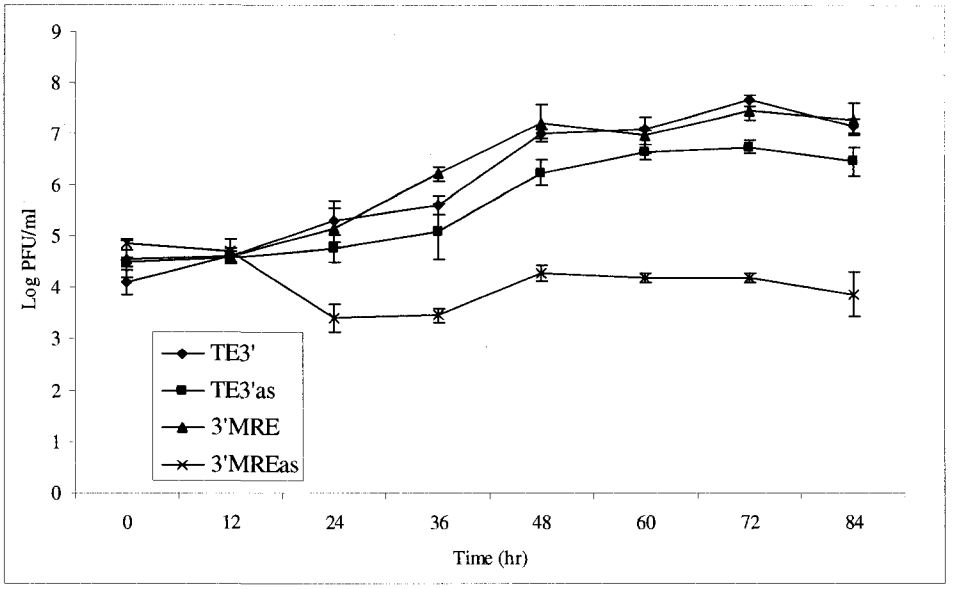

Figure 3.6. Replication kinetics of dsSINVs in Aag2 cells. Triplicate monolayers of cells were infected with the corresponding viruses at an MOI~0.01. Virus titers of samples taken every 12 hours were determined by titration on Vero cell monolayers. "TE3' anti"=TE/3'2J/Aae/LYSOas; "3'MREas"= 3'ds/MRE16/Aae/LYSOas.

Detection of lysozyme-specific RNA in infected mosquito cells

Lysozyme mRNA could be detected in uninfected and dsSINV-infected Aag2 cells and gene-specific siRNAs could be detected in LYSOas-infected cells (Figure 3.7). Messsenger RNA was detected in both un-induced and bacteria-induced cells, with a strong accumulation following bacteria exposure. RNA species 21-23 nucleotides in size, consistent with siRNAs produced in mosquito cells, did accumulate in cells infected with TE/3'2J/Aae/LYSOas and 3'ds/MRE16/Aae/LYSOas viruses, suggestive of a

targeted RNAi response against the endogenous mRNA. Interestingly, even though lysozyme expression is induced in the cells, no difference in siRNA accumulation was observed between un-induced and bacteria-induced treatments. No bands 21-23 nucleotides in size are present in mock-, TE/3'2J-, or 3'ds/MRE16-infected Aag2 cells.

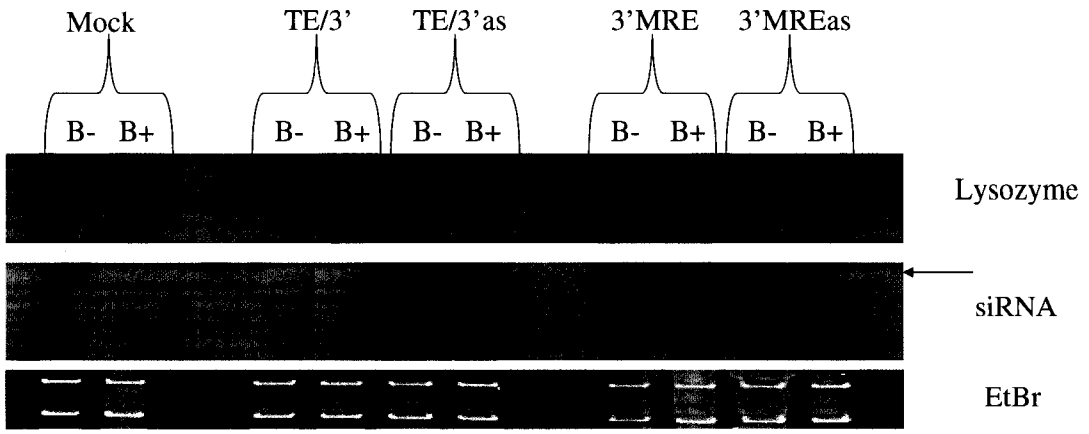

Figure 3.7. Accumulation of lysozyme-specific RNAs following dsSINV infection of Aag2 cells. Aag2 cells were infected at MOI=10 and lysozyme expression was induced by the addition of heat-killed bacteria. Total RNA was extracted 24 hours later and RNAs were detected using an *Ae. aegypti* lysozyme riboprobe. The position of a 25 nt marker is indicated by an arrow. B-=uninduced, B+=bacteria-induced. "Lysozyme" represents mRNA, "siRNA" represents small RNA, "EtBr" represents ethidium bromide-stained gel.

Discussion

The original purpose of this study was to characterize the efficiency of different dsSINVs to induce targeted RNAi against an endogenous mosquito gene with the hopes of improving our current systems. RNAi is a potent inhibitor of virus replication in plant and invertebrate cells and plays an important role in the antiviral response to RNA virus infection. SINV has been shown to be a target of RNAi and the expression of heterologous sequence by a dsSINV can mediate RNAi against other genes.

An inducible cell culture system was utilized to show the effectiveness of TE12- and MRE16-based dsSINVs in RNA silencing. Mosquito (C6/36) cells were exposed to

heat-killed *E. coli* bacteria, inducing the expression of the immune peptide lysozyme. We chose this inducible system because lysozyme from *Ae. albopictus* cell culture has previously been described, providing a cell culture-based platform to study RNAi induction by our dsSINV systems (Hernandez et al., 2003). Also, the induction of lysozyme mRNA in C6/36 cells could be detected via quantitative PCR, serving as an efficient marker for silencing (Figure 3.2 and 3.3).

dsSINV systems expressing sense and antisense sequence to the *Ae. albopictus* lysozyme gene were generated and used in the inducible C6/36 system to observe RNA silencing activity against the induced lysozyme mRNA. A comparison of bacteria-treated and untreated samples yields a relative efficiency of RNA silencing for a given dsSINV. It immediately became apparent that TE12-based systems expressing antisense lysozyme sequence were able to knock down induction of lysozyme mRNA. TE/3'2J with an antisense lysozyme insert could efficiently mediate lysozyme-specific VIGS in mosquito cells, in line with previous results using this virus and other derivatives (Attardo et al., 2003, Johnson et al., 1999, Olson et al., 2002, Olson et al., 1996, Sanchez-Vargas et al., 2004). Association of the viral RNA with lysozyme mRNA in a dsRNA molecule may trigger the RNAi machinery within the cell, degrading the cellular transcript in a Dicer2-dependent mechanism. A second hypothesis is that replicative dsRNA intermediates formed during virus replication are the trigger for RNAi and RISC-loaded lysozyme siRNAs mediate the decrease in mRNA levels.

Constitutive GFP expression in transgenic tobacco plants can be silenced when plants are infected with potato X virus encoding a translatable GFP gene, suggesting that sense insert orientation can also mediate silencing (Ruiz et al., 1998) . *Ae. aegypti* early

trypsin was silenced in mosquitoes infected with MRE/3'2J expressing the early trypsin gene in sense orientation (Olson et al., 2002). In the TE/3'2J/Aal/ LYSOs virus used here, lysozyme induction was decreased to a level similar to antisense virus, but was not significant because of a greater variance. Replicative forms of the virus or base pairing of the viral negative strand RNA with endogenous mRNA probably mediates silencing of lysozyme by this system. Targeting constitutively expressed genes or genes of high abundance, such as the luciferase transgene investigated by Johnson et al (1999), may be less effective because of the number of mRNA molecules already present in the infected cell.

It is interesting that TE/5'2J/Aal/LYSOs is incapable of decreasing lysozyme induction. This suggests that an antisense-target mRNA pairing is essential for efficient RNAi-mediated silencing using the 5' double-subgenomic system. The observed difference between the 5' and 3' TE12-based systems could be explained by the amount of lysozyme transcripts produced from the virus. As mentioned previously, the 3' SINV promoter has the highest transcription rates (Raju & Huang, 1991). Because the structural genes of TE/5'2J, and not the lysozyme insert, have the highest rate of transcription, the amount of lysozyme-specific dsRNA would be much less than would be produced during TE/3'2J infection. There may be a threshold level of dsRNA necessary to induce a specific RNAi response toward lysozyme mRNA. However, 3'dsSINVs containing no insert can trigger a RNAi response producing virus-specific siRNAs detected using an E1-specific probe, suggesting that the threshold would be overcome during transcription of either subgenomic RNA or from the virus genome (Olson et al., 2002, Sanchez-Vargas et al., 2004).

Unlike our TE12 systems, dsMRE16 viruses inefficiently silenced lysozyme expression in C6/36 cells, showing modest or no decrease in expression levels compared to uninfected cells. From previous work, it has been hypothesized that MRE16 is capable of inhibiting or evading the RNAi response in mosquito cells, potentially by expressing a viral suppressor of RNAi (VSR) (Campbell et al., 2008). Data described in Chapter 2 showed that SINV expressing a known inhibitor of RNAi becomes pathogenic in the mosquito and no VSRs have been described from arboviruses, suggesting that MRE16 does not encode a true RNAi suppressor.

The efficient infection of mosquito vectors following oral bloodmeal may, in part be determined by the efficiency of targeted RNAi against the infecting virus. TE12 viruses are capable of VIGS and virus-specific siRNAs are detected in infected mosquitoes. On the other hand, MRE16 viruses do not appear to mediate VIGS and minimal siRNAs are detected during mosquito infection (Campbell et al., 2008). We have previously shown that RNAi inhibition allows TE/3'2J to more efficiently infect and replicate within orally-infected mosquitoes. The efficient replication and dissemination of MRE16 viruses in mosquitoes suggests that the virus is capable of evading the mosquito RNAi response during infection.

Adelman et al (2008) have produced a transgenic *Ae. aegypti* mosquito line that would be useful for examining both dsSINV-mediated gene silencing and the potential ability of MRE16 to suppress RNAi. Eye-specific expression of an enhanced GFP (eGFP) mRNA and an inverted repeat sequence specific to eGFP silences expression of the protein. Injection with TE/3'2J viruses expressing antisense sequence to Dicer2 and AGO2 rescued eGFP expression in the eyes, showing functional inhibition of the RNAi

pathway through dsSINV-mediated silencing (Adelman et al., 2008). The transgenic mosquito strain could be used to characterize the potential RNAi suppression mediated by MRE16. If eGFP expression is recovered during MRE16 infection, the virus is capable of functionally inhibiting the RNAi response in *Ae. aegypti.*

Lysozyme-specific siRNAs were not detected in C6/36 cells even though lysozyme mRNA expression levels decreased (data not shown). In the same cell line, virus- and gene-specific siRNAs were generated during TE/3'2J and MRE/3'2J infection when virus was infected at a low MOI. Accumulation of the siRNAs began at 48 hours post infection, with the largest amounts accumulating between three and five days post infection (Adelman et al., 2001, Olson et al., 2002, Sanchez-Vargas et al., 2004). Even though our cells were infected at a much higher MOI, the 24 hour time point may be too early during infection for detectable levels of siRNAs to form in C6/36 cells. Along the same lines, the Northern analysis that was used may not be sensitive enough to detect the small amount of siRNAs present.

It was unexpected that lysozyme siRNAs were detected in Aag2 cells infected with 3'ds/MRE16 viruses. Previous attempts in *Ae. aegypti* mosquitoes identified minimal but detectable virus-specific siRNA production following oral infectious bloodmeal containing MRE16 (Campbell et al., 2008). In our study and theirs, TR339-derived viruses produced significantly more siRNAs than did MRE16. The use of pyrosequencing in both cell culture and mosquitoes could be beneficial to quantify true amounts of siRNAs produced. It would also be interesting to look at virus-specific siRNAs in Aag2 cells using Northern analysis or high-throughput sequencing projects to

determine if the MRE16 genome can trigger a robust RNAi response, which is not seen in C6/36 cells.

It would be informative to look at dsSINV-mediated reduction of lysozyme mRNA induction following bacterial treatment of Aag2 cells. The viruses are available and lysozyme expression can be induced in this cell line, but original attempts to perform such experiments were unsuccessful because the cells do not efficiently adhere to welled plates (data not shown). In order to precisely monitor dsSINV-mediated silencing between experiments, cell numbers and infection MOIs must be constant. Because the Aag2 cells do not adhere following an overnight incubation, standardization is difficult. More expensive alternatives include coating plates or growing cells on glass coverslips to improve adherence, or using tissue culture flasks instead of plates. Additional steps to optimize cell seeding density and quantity of bacteria solution to add for lysozyme induction would have to be performed.

The efficiency of RNAi-mediated silencing may be a potential reason for the observed difference in cell lines. In Chapter 2, we describe that Aag2 cells have what appears to be a more robust RNAi response than C6/36 cells and potential reasons for this phenomenon were presented. Detection of lysozyme siRNAs in Aag2, but not C6/36 cells infected with similar viruses strengthens this hypothesis. However, this is circumstantial evidence and RNAi efficiency between the two cell lines requires direct experimental validation. Cloning and expression profiling of key RNAi components within C6/36 and Aag2 cells may give insight into the observed efficiency disparity.

From our cumulative results and the results of others, it is obvious that TE12-derived viruses are efficiently recognized by cellular RNAi machinery and can induce a

targeted silencing response against endogenous mosquito genes when an antisense sequence is expressed. No dsMRE16 systems have been shown to induce gene silencing in mosquito cells, although siRNA production suggests that VIGS may occur in Aag2 cells.

As described previously, MRE/3'2J virus shows an RNAi silencing phenotype in mosquitoes. Infection of mosquitoes with MRE/3'2J expressing a 500 bp sequence of early trypsin gene led to silencing of early trypsin expression, degradation of the mRNA in the midgut, and the production of early trypsin-specific siRNAs (Olson et al., 2002, Sanchez-Vargas et al., 2004). Taken together, these data provide evidence that MRE16 structural genes are not responsible for the reduced RNAi induction phenotype we have observed. Instead, the nonstructural genes of MRE16 may be responsible, as is the case with many other RNA viruses that are capable of evading RNAi in plants and invertebrates (Li & Ding, 2006). To this end, we have begun to develop a reciprocal chimeric virus to MRE/3'2J, encoding the nonstructural genes of MRE16 and the structural genes of TE12, that will be informative for determining whether the nonstructural genes play a role in RNAi sensitivity. If it is determined that the RNAi silencing phenotype can be localized to the nonstructural proteins, chimeric viruses switching specific individual proteins from MRE16 and TE12 may be constructed and examined, similar to what has been done for midgut infection determinants of SINV in *Ae. aegypti* (Pierro et al., 2007, Pierro et al., 2008).

It is also possible that the untranslated regions (UTR) of SINV may also play a role in evading or inhibiting RNAi. Both the 5' and 3' UTRs of alphavirus genomes contain secondary structures involved in virus replication. Because genomic RNA

secondary structure may be a target for RNAi, it could be hypothesized that TE12 viruses contain more structure within the UTRs and could more efficiently trigger RNAi. Computer software programs could be used to predict secondary structure within the UTRs of TE12 and MRE16 virus genomes to determine if this hypothesis may be true. If more secondary structure is formed by TE12, mutational analysis of selected structures may be used to show potential involvement with RNAi induction.

Mutations destabilizing secondary structures within a 51-nucleotide conserved region of the SINV 5' UTR have been found to decrease virus replication in mosquito, but not mammalian cells (Fayzulin & Frolov, 2004, Niesters & Strauss, 1990). However, compensatory mutations in the nsP2 gene were found to recover virus replication to near-wild type levels (Fayzulin & Frolov, 2004). It could be hypothesized that MRE16 has evolved to contain less secondary structure within the UTRs that would allow the virus genome to evade the RNAi machinery yet replicate efficiently because of compensatory mutations within the nonstructural proteins.

Current experimental evidence suggests that dsMRE16-based transducing systems will not function as inducers of RNAi in mosquitoes, an unfortunate finding because of the efficient vector infection of MRE16 virus. Also, because of the observed differences in RNAi between the two mosquito cell lines used in these studies, it may be necessary to conduct RNAi experiments in Aag2 cells instead of the often-used C6/36 cells. Dissecting the differences between MRE16 and TE12 virus-induced RNAi will provide a deeper understanding of the role of RNAi in arbovirus infection of the mosquito vector and may help to identify specific alphavirus determinants affecting RNAi.

Chapter 4

EXPANDING THE CURRENT CAPABILITIES OF DOUBLE SUBGENOMIC SINDBIS VIRUS TRANSDUCING SYSTEMS: ANTIBODY-MEDIATED TARGETING OF TE/5'2J INFECTION TO MOSQUITO TISSUES AND THE DEVELOPMENT OF MRE16-BASED SYSTEMS EXPRESSING DIVERSE REPORTER PROTEINS

Introduction

SINV vectors are desirable candidates for gene expression because of the relative ease in generating high titer viral stocks and the high efficiency of transgene expression in nearly 100% of cultured cells. Because SINV replication is strictly cytoplasmic with no DNA intermediate, splicing machinery is not required and chromosomal integration is not of concern. Important to many aspects of SINV vector usage, cells of diverse organisms can be infected by the virus. The list of cell types that SINV can infect includes mammalian, avian, amphibian, reptilian, and invertebrate species (Xiong et al., 1989). SINV is an excellent tool for studying mosquito-virus interactions because the virus can persistently infect many species of culicine mosquitoes either by oral or intrathoracic inoculation and provide efficient, long-term expression of the transgene (Olson et al., 1994). Also, in terms of biosafety, SINV is much safer for use in mosquito infection compared to other arboviruses including other members of the *Alphavirus* genus, especially when anthropophilic vector species are used.

Engineered SINV vectors produced from cDNA clones are used to express heterologous genes or sequences of interest in infected cells. Structural proteins encoded by the virus are dispensable for both transcription and replication of the genome and have been replaced by heterologous coding sequence to facilitate transgene expression in some SINV expression systems. Defective interfering (DI) SINV vectors contain a transgene flanked by *cis*-acting elements necessary for replication (Levis et al., 1987). Replicon vectors encode the replicase genes and the structural genes are replaced with a transgene on the same RNA molecule (Bredenbeek et al., 1993, Xiong et al., 1989). Both DI and replicon vectors can be packaged and efficiently express large transgenes but require a

helper virus to infect neighboring cells because the structural proteins necessary for progeny virus production are not encoded.

Other avenues have been taken to produce infectious virus capable of transgene expression. These vectors demonstrate the plasticity of the SINV genome that allows the incorporation of heterologous sequence without severe fitness constraints on virus replication. Reporter genes have been expressed using recombinant SINVs containing either two or three separate packaged RNA species (Fayzulin et al., 2005, Geigenmuller-Gnirke et al., 1991).

Marker gene expression can be temporally controlled based on the genomic position of the insert. Green fluorescent protein (GFP) has been successfully expressed by either inserting the gene within nsP coding regions or at junctions between two nsPs, relegating expression to the early events post-infection when the nonstructural polyprotein is translated (Atasheva et al., 2007, Cristea et al., 2006, Frolova et al., 2006). Thomas et al (2003) were able to limit GFP expression to a later stage of infection by expressing the protein within the structural polyprotein and regulating cleavage with a SINV-encoded heterologous virus protease (Thomas et al., 2003).

Double subgenomic SINV (dsSINV)-based transducing systems facilitate the expression of a gene or sequence of interest in infected cells from a second internal initiation site within a fully infectious virus genome where the protein coding regions of the virus genome are not interrupted. The transgene is highly expressed in infected cells from its own subgenomic RNA species. dsSINVs are an important tool for studying the over-expression of biologically-active proteins including reporter proteins, single-chain antibodies, toxins, and endogenous proteins in mosquito vectors and non-vector

arthropods (Cheng et al., 2001, de Lara Capurro et al., 2000, Foy et al., 2004, Higgs et al., 1995, Higgs et al., 1996, Kamrud et al., 1995, Lewis et al., 1999, Olson et al., 1994, Olson et al., 2000, Pierro et al., 2003).

The E2 glycoprotein of SINV is a viral membrane protein important for virus attachment to susceptible cells and contains potential neutralization epitopes. The protein is 423 amino acids long; the first 260 amino acids constitute the ectodomain followed by an approximately 100 amino acid stem region with the final 60 amino acids making up the transmembrane domain and cytoplasmic tail (Mukhopadhyay et al., 2006, Pletnev et al., 2001). In vertebrates, domains responsible for receptor binding, human cell infection, virus neutralization, and mouse neurovirulence have been localized to E2 (Davis et al., 1986, Davis et al., 1987, Gardner et al., 2000, Hurtado et al., 2005, Lee et al., 2002, Mendoza et al., 1988, Smith et al., 1995, Suthar et al., 2005, Tucker et al., 1993). SINV determinants of mosquito infection have also been localized to the ectodomain of the protein (Myles et al., 2003, Olson et al., 2000, Pierro et al., 2003, Pierro et al., 2007, Pierro et al., 2008, Seabaugh et al., 1998). Through transposon-mediated insertion of a 19 amino acid linker into E2, groups have shown that the glycoprotein can manage interruptions within its coding sequence at multiple amino acid positions to produce viable progeny virus, suggesting that the protein in its native conformation is not required for virus infection (London et al., 1992, Navaratnarajah & Kuhn, 2007).

SINV was developed as a potential cancer therapeutic because the virus may use the high-affinity laminin receptor, a highly up-regulated cell surface receptor in some human cancers, as a major cellular receptor for infection (Menard et al., 1998, Wang et al., 1992). Unfortunately, this targeted infection is not exclusively restricted to cancerous

cells because the laminin receptor is a highly conserved protein expressed in many different tissues in all five kingdoms of organisms (Nelson et al., 2008). To more specifically target SINV infection, Ohno et al (1997) inserted an antibody binding domain into the E2 glycoprotein of a SINV replicon. A synthetic domain (ZZ domain) modeled from protein A of *Staphylococcus aureus* was inserted into a region of E2 previously shown to be surface-exposed and interfere with SINV attachment but not virion assembly or release (Dubuisson & Rice, 1993, Phinney et al., 2000). Under normal conditions, the mutated E2-containing replicon was unable to efficiently infect cells permissive to wild type replicon infection. When a cell surface antigen-specific antibody was adsorbed prior to infection, the replicon was capable of infection and high-level expression of a bacterial β-galactosidase reporter transgene (Ohno et al., 1997) (Figure 4.1).

Hela cells expressing a human CD4 surface receptor have been used previously as a model cell line to show inhibition of ZZ-containing SINV vector infection without the presence of receptor-specific antibody. Expression of a β-galactosidase transgene is below the limit of detection when CD4 positive cells are infected with electroporation-derived ZZ replicon but almost reaches wild type levels when less than 500ng of anti-CD4 antibody is adsorbed prior to infection (Iijima et al., 1999, Ohno et al., 1997, Sawai & Meruelo, 1998). Cytotoxic genes can also be transferred to target cells using the same vector, showing a proof of principle for targeted gene therapy using SINV vectors (Iijima et al., 1999).

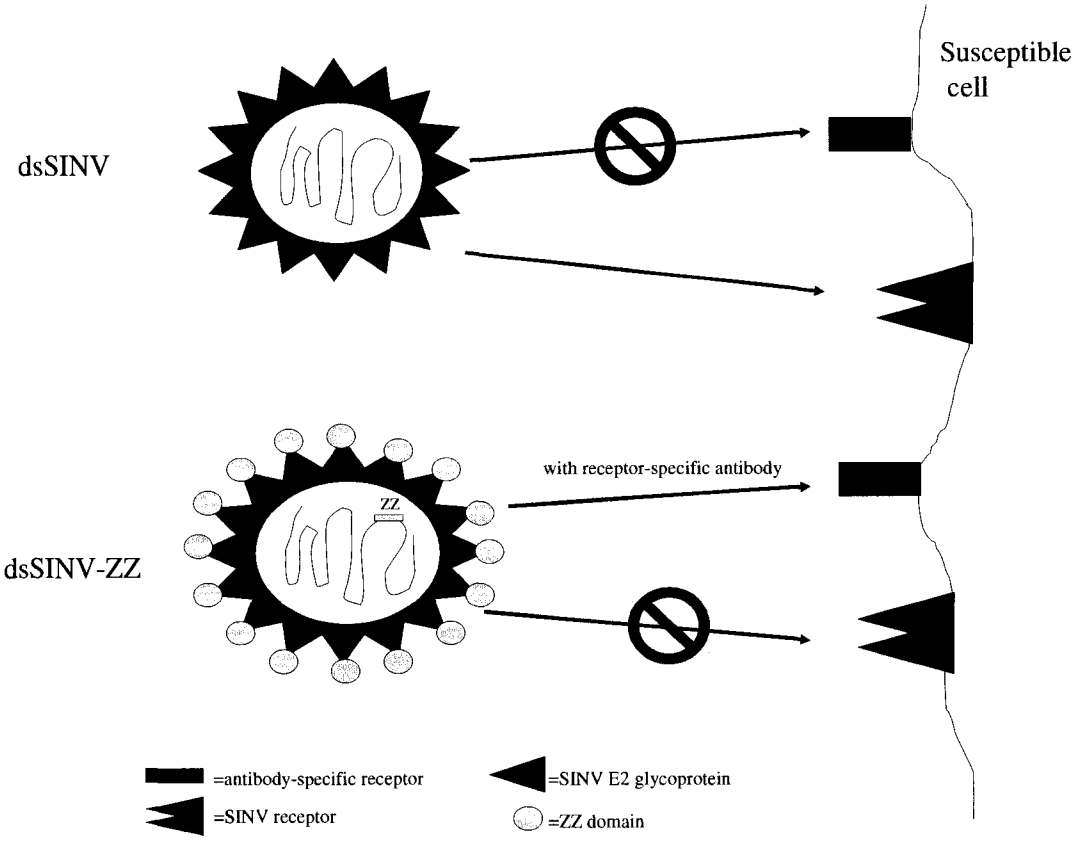

Figure 4.1. Diagram of targeted infection strategy for dsSINV. Introduction of the ZZ domain into SINV E2 should ablate cell infection via the typical SINV receptor. Infection can be targeted by adsorbing a receptor-specific antibody to ZZ virus prior to infection.

This chapter describes the production of SINV transducing systems expressing fluorescent and bioluminescent marker genes and the extension of targeted SINV infection to mosquito cells. We believe that engineering dsSINVs with different marker genes or targeted infection will facilitate the research of all aspects of the arbovirus transmission cycle. Using a double-subgenomic virus system based on the Malaysian SINV strain MRE16, we have produced a panel of constructs that can be used for various molecular and vector biology applications and will aid in studies of vector infection and arbovirus transmission. Also, using the same strategy as Ohno et al (1997) to produce mutated E2 glycoprotein, a fully-infectious TE/5'2J virus was engineered to express the

antibody-binding domain of protein A with the intention of targeting virus infection to specific mosquito tissues. Although the utility of targeted TE/5'2J virus infection in the mosquito could not be shown, we have produced a plasmid construct that shows promise as a means to display different proteins on the virus surface.

Materials and Methods

Cells and Medium

Vero and BHK-21 cells were grown at 37°C, 5% CO_2 in MEM containing 10% FBS plus NEAA, L-glutamine, and antibiotics. Hela cells (kindly provided by Dr. Ramesh Akkina, Colorado State University) were grown at 37°C, 5% CO_2 in DMEM containing 10% FBS plus NEAA, L-glutamine, and antibiotics. Hela cells that express human CD4 receptor on the surface via integration of a retrovirus vector (Chesebro & Wehrly, 1988) (kindly provided by Dr. Akkina) were maintained as described for Hela cells. To select for CD4 expression, cells were grown in maintenance medium containing G418 antibiotic (Mediatech, Inc., Herndon, VA) at a final concentration of 1μg/ml. C6/36 cells were grown at 28°C, 5% CO_2 in MEM containing 10% FBS plus NEAA, L-glutamine, and antibiotics.

TE/5'2J/ZZ virus construction

Construction and use of infectious clone pTE/5'2J/GFP have been described previously (Hahn et al., 1992, Pierro et al., 2003). To construct virus containing an antibody-binding domain, an intermediate mutant was created. A BstEII restriction endonuclease recognition site was inserted into the E2 protein gene at amino acid 71 similar to Ohno et al (1997). pTE/5'2J-GFP was mutagenized with the forward primer

5'-atgtcgcttaagca**ggtaacc**accgttaaagaaggc-3' and reverse primer 5'-gccttctttaacggt**ggttacc**tgcttaagcgacat-3' (BstEII restriction site in **bold**) in a reaction using the entire plasmid DNA as template and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Thermocycling parameters were: 95°C for 30 seconds; 20 cycles of (95°C for 30 s, 54°C for 1 minute, 68°C for 14 minutes); and a final 12°C hold. To remove template DNA, 10U of the methylation-dependent restriction enzyme DpnI was added and the reaction was incubated at 37°C overnight to ensure complete digestion. Introduction of the BstEII site was confirmed by sequencing. The plasmid containing a BstEII site was named pTE/5'2J/GFP/Bst.

A PCR fragment corresponding to two synthetic immunoglobulin G (IgG) binding domains, ZZ domain based on B domain of protein A from *Staphylococcus aureus* (Nilsson et al., 1987), was amplified from plasmid pEZZ 18 (Amersham Biosciences, Piscataway, NJ) with forward and reverse primers containing BstEII restriction sites at their 5' ends. PCR amplicon and pTE/5'2J/GFP/Bst were digested with BstEII and ligated together. The resulting plasmid, pTE/5'2J/GFP/ZZ, was sequenced in the E2 gene to verify insert orientation.

Construction of 5'dsMRE16 viruses expressing different reporter genes

The construction and use of p5'ds/MRE16 and p5'ds/MRE16/GFP have been described elsewhere (Foy et al., 2004). Fluorescent and bioluminescent reporter genes were cloned into the multiple cloning site of p5'ds/MRE16 using the NotI restriction site. Reporter genes and plasmids from which they originated are listed in Table 4.1. Insert orientation was verified by sequencing through the multiple cloning site (Proteomics and Metabolomics Facility, CSU).

Table 4.1

5'ds/MRE16 viruses		
Virus*	**Reporter**	**Source#**
eGFP	enhanced green fluorescent protein	Foy et al (2004)
eCFP	enhanced cyan fluorescent protein[a]	pECFP[$]
eYFP	enhanced yellow fluorescent protein[b]	pEYFP[$]
DsRed	DsRed (drFP5843) fluorescent protein[c]	pSinrep5-DsRed
AcGFP	Monomeric form of GFP[d]	pAcGFP1[$]
mCherry	Monomeric cherry fluorescent protein[e]	pmCherry[$]
Timer	Mutant DsRed fluorescent protein[f]	pTimer-1[$]
RLUC	Renilla luciferase[g]	pRL[**]
FLUC	Firefly luciferase[h]	pGL3-Basic[**]

* all viruses were engineered in a 5'ds/MRE16 background

plasmid from which reporter gene was amplified

$ plasmid purchased from Clontech Laboratories, Inc. (Mountain View, CA)

** plasmid purchased from Promega (Madison, WI)

a=Heim et al (1994)
b=Labas et al (2002)
c=Matz et al (1999)
d=Matz et al (1999)
e=Shaner et al (2004)
f=Terskikh et al (2000)
g=Lorenz et al (1991)
h=de Wet et al (1985)

Infectious virus production

Infectious virus was derived from pTE/5'2J cDNA clones as described in Chapter 2 (*Infectious virus production*) with modifications. Passage 1 virus was produced by electroporation of transcription products into Vero cells. Virus was passaged once in C6/36 cells at MOI=0.01 to produce passage 2 virus.

5'ds/MRE16 viruses were produced using the same procedures as described in Chapter 2 (*Infectious virus production*) except that AscI was used to linearize the infectious clone plasmid prior to *in vitro* transcription.

Plaque titrations

Virus titers were determined by plaque formation of serially-diluted viruses on Vero cell monolayers in 24-well plates as previously described in Chapter 2 (*Plaque titrations*).

Reverse transcriptase PCR

After harvesting supernatant for P2 virus stocks, RNA was isolated from infected C6/36 cells for reverse transcriptase-polymerase chain reaction (RT-PCR). The remaining cells from mock-infected, GFP, Bst, and ZZ virus-infected C6/36 cells were scraped into 1 ml of Trizol® reagent (Invitrogen Corp.). RNA was extracted according to manufacturer's protocols. Similar reactions were performed using DNase-treated *in vitro* transcription reactions. cDNA of all polyadenylate tail-containing RNA was synthesized in a 20μl reaction using an oligo $dT_{12\text{-}18}$ primer and Superscript II RT enzyme (Invitrogen Corp.) according to manufacturer's recommended protocols. A region spanning the ZZ domain insertion site (nucleotides 8750 to 9500 of pTE/5'2J) was amplified using 1 μl of cDNA as template and Platinum Taq DNA polymerase (Invitrogen Corp.).

Western blot analysis of E2 protein

To detect SINV E2 protein, antibody was commercially-generated in rabbits by Biosource (Hopkinton, MA). Animals were immunized with a peptide corresponding to SINV E2 amino acids 127-143 (ARKIKPKFVGREKYDLPP) conjugated to the carrier protein keyhole limpet hemocyanin. Antibody was tested by enzyme-linked immunosorbent assay and affinity-purified at Biosource prior to being received. Proper antibody dilution for western blot analysis was determined empirically and was found to be 1:1000.

E2 protein from infectious viruses was detected in cell culture by western blot analysis using virus-containing supernatant from infected C6/36 cells. GFP, Bst, and ZZ suspensions were diluted in phosphate-buffered saline (PBS) and separated by SDS-

PAGE as previously described in Chapter 2 (*Identification of V5 epitope-tagged B2 protein in cells*). Proteins were transferred to a nitrocellulose membrane and E2 was detected using the rabbit anti-E2 IgG primary antibody (1:1000) and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:500) (Kierkegaard and Perry Laboratories, Gaithersburg, MD). Detection was performed similar to methods described in Chapter 2 (*Identification of V5 epitope-tagged B2 protein in cells*).

Virus growth curves

The ability of GFP, Bst, and ZZ viruses to replicate in cell culture was determined by performing growth curve analysis in multiple cell lines. Triplicate monolayers of Vero, C6/36, Hela, and Hela-CD4 cells in six well plates were infected by rocking for one hour at room temperature with virus diluted to MOI~0.01 in the appropriate medium type supplemented with 3% FBS plus NEAA, L-glutamine, and antibiotics. Medium was added to 3 ml total and cells were incubated at the corresponding cell type growth conditions. Samples were taken every six hours (Hela and Hela-CD4) or 12 hours (Vero and C6/36) and frozen immediately at -80°C. Titers were determined by plaque assay on Vero cell monolayers.

Infection of Hela cells after pre-incubation of virus with cell receptor-specific antibody

Because ZZ virus should be able to infect cells only when a bridging antibody is present, a Hela cell culture-based assay was performed to show targeted infection by the ZZ virus. Stock virus was diluted to $4x10^4$ PFU/ml (MOI~0.01) in DMEM containing 3% FBS plus NEAA, L-glutamine, and antibiotics and increasing amounts (0 ng, 100 ng, 500 ng, 1 μg) of mouse anti-human CD4 antibody (BD Biosciences, San Jose, CA) were added. Antibody was adsorbed to virus by rocking the suspension for one hour at 4°C.

Monolayers of Hela and Hela-CD4 cells were infected with antibody-adsorbed virus for one hour at room temperature before medium was supplemented to 2 ml total. Infected cells were incubated for 18 hours at 37°C, 5% CO_2. Supernatants were collected and virus titers were determined by plaque assay on Vero cell monolayers.

Intrathoracic injection of mosquitoes

Female mosquitoes five to seven days post-eclosion were injected intrathoracically with electroporation- and C6/36 cell –derived GFP, Bst, and ZZ viruses. Mosquitoes were injected with 69 nl of suspension containing $1x10^7$ PFU/ml of virus using the Nanoject II nanoliter injector (Drummond Scientific) and placed under optimal rearing conditions. At seven days post-injection, mosquitoes were assayed for virus infection by visualizing GFP expression in the ommatidia using an inverted fluorescent dissecting microscope.

Per os infection of mosquitoes via artificial bloodmeal

Ae. aegypti Higgs' white eye (HWE) strain mosquitoes were reared as previously described in Chapter 2 (*Mosquitoes*). Mosquitoes were given an artificial bloodmeal containing virus stock diluted to $1x10^7$ PFU/ml or less, defibrinated sheep's blood, and ATP. Stock titers ranged from $6.0x10^6$ for 5'ds/MRE16/FLUC to $6.0x10^8$ PFU/ml for 5'ds/MRE16/EGFP. Mosquitoes were sampled at various times post infection depending on the experimental procedure. Specific virus titers and assay time points are identified within figure headings.

Visualization of reporter gene expression in 5'ds/MRE16-infected mosquitoes

Orally- and intrathoracically-infected mosquitoes were assayed for reported gene expression by visualization using standard epifluorescent microscopes or the IVIS200

imaging system (Caliper Life Sciences, Hopkinton, MA). Fluorescent protein expression was examined using either the fluorescein isothiocyanate (EGFP, AcGFP, ECFP, EYFP, Timer) or red fluorescence wavelength (DsRed, mCherry, Timer). FITC is not optimal for ECFP visualization but was used to determine whether the protein was expressed. Mosquitoes were cold-anesthetized prior to fluorescence visualization.

For bioluminescence assays, female mosquitoes were injected with Vero cell-derived FLUC and RLUC viruses using the Nanoject II system. Five days post injection, luciferase activity was assayed by bioluminescence detection using the IVIS200 imaging system. Mosquitoes were injected with luciferase substrate (luciferin for FLUC- and coelenterazine for RLUC-infected mosquitoes) and visualized using the Living Image software package.

Detection of mosquito-transmitted 5'ds/MRE16/DsRed virus in mice

A preliminary study was performed to detect mosquito bite transmission of virus to mice using a fluorescent protein-expressing virus. Forty to sixty HWE mosquitoes were intrathoracically injected with ~ $3x10^3$ PFU of 5'ds/MRE16/DsRed virus or mock-injected with cell culture medium. Eight days later, mock- and virus-injected mosquitoes were allowed to separately feed on a single albino B6 mouse (The Jackson Laboratory, Bar Harbor, ME) for one hour. During the mosquito feeding, a third mouse was injected in the right rear footpad with 25 µl of 10^4 PFU of 5'ds/MRE16/DsRed virus diluted in cell culture medium.

Following mosquito feeding, engorged mosquitoes were removed and counted. At various times post-mosquito exposure, mice were anesthetized with isofluorane and visualized under fluorescent light (DsRed filter) with the IVIS200 system and a one

minute exposure. Seventy-two hours post-exposure, all mice were sacrificed according to guidelines set by the Animal Care and Usage Committee (Colorado State University). Mosquito and mouse infection experiments were performed by Aaron Phillips and Dr. Eric Mossel (Colorado State University).

Results

Verification of ZZ domain insertion into TE/5'2J/GFP virus

Virus containing an antibody-binding domain in the E2 ectodomain was constructed for targeting infection using a mosquito tissue-specific bridging antibody. After constructing pTE/5'2J/GFP/ZZ, infectious virus was produce by electroporation of *in vitro*-transcribed RNA into Vero cells. Supernatants from the original transfection produced plaques on Vero cell monolayers. To increase working titers of virus, a single passage on C6/36 cells was performed. Again, supernatant from infected cells produced plaques on Vero cell monolayers, suggesting infectious virus had been produced.

To confirm that the antibody-binding ZZ domain was successfully inserted into the E2 gene of TE/5'2J/GFP, a RT-PCR was performed with primers flanking the E2 insertion site. cDNA synthesized from either DNase-treated *in vitro* transcription reaction or RNA extracted from infected C6/36 cells was used in a PCR reaction to amplify the region that should contain the ZZ domain. A band of approximately 860 base pairs (bp) was amplified from pTE/5'2J/GFP and cDNA from GFP and Bst viruses, corresponding to the SINV E2 region (Figure 4.2). In the transcription reaction of ZZ virus, a fragment of approximately 1,110 bp was amplified, the size of the E2 region (860 bp) with ZZ domain insert (350 bp). The same band was seen when cDNA from C6/36-

passaged virus was amplified. However, additional smaller bands not seen in any other reactions were also amplified.

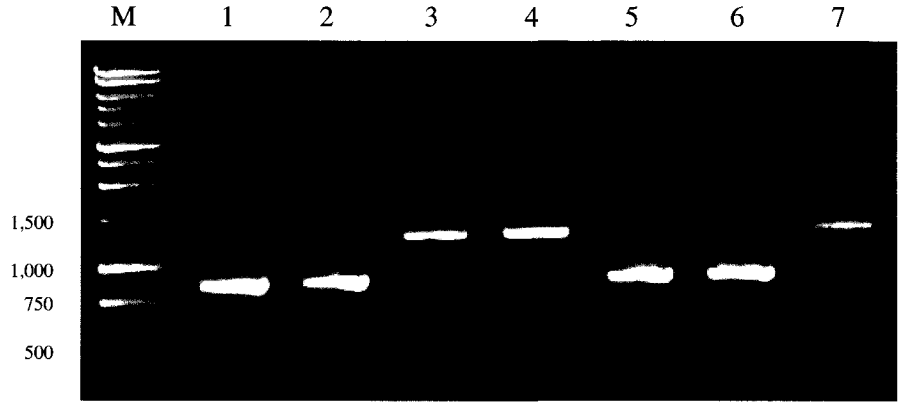

Figure 4.2. RT-PCR of TE/5'2J viruses. Viral RNA was used to make cDNA that was used as template to amplify a region corresponding to nucleotides 9040-9900 of pTE/5'2J. M=Molecular weight marker; 1= GFP transcription; 2=Bst transcription; 3= ZZ transcription; 4=ZZ passage 1 virus; 5=GFP passage 2 virus; 6=Bst passage 2 virus; 7=ZZ passage 2 virus. Molecular weights for seleced marker bands are presented to the left in base pairs.

Insertion of the ZZ domain into the E2 gene of TE/5'2J/GFP should increase the overall molecular weight of the translated E2 protein. To show the shift in size of E2 after ZZ domain insertion, a western blot detecting the E2 protein was performed. Antibody specific for SINV E2 protein was generated in rabbits using a peptide immunogen corresponding to amino acids 127-143 of E2. This region was chosen because it was predicted by calculations performed by Biosource to be surface-exposed and a potentially highly-immunogenic epitope.

Total protein from infected C6/36 cells was separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was probed using the E2-specific antibody. A single band of approximately 47 kilodaltons (kDa) was visualized in total protein from GFP and Bst virus-infected cells (Figure 4.3, lanes 3 and 4). This molecular

weight corresponds to wild-type SINV E2 protein. A double band of higher molecular weight, approximately 60 kDa, was observed in total protein from ZZ virus-infected cells (Lane 5). The predicted size of E2 protein plus ZZ domain is 61 kDa. No bands were present in total protein from mock-infected cells (Lane 2).

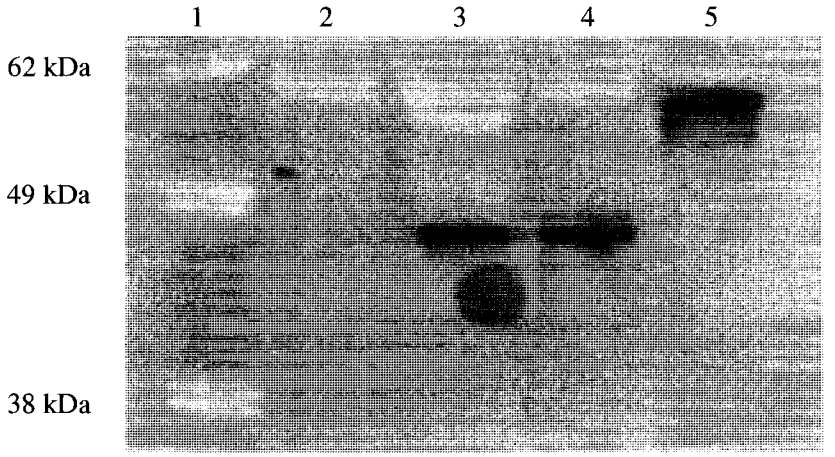

Figure 4.3. Identification of E2 protein from C6/36 cells infected with GFP, Bst, and ZZ viruses. Total protein from infected cells was probed using a SINV E2-specific antibody. Predicted size of E2 and E2-ZZ proteins are 47 and 61 kDa, respectively. Molecular weight markers are indicated to the left of the panel. 1=Molecular weight marker; 2=Mock; 3= GFP; 4=Bst; 5= ZZ.

Replication of GFP and ZZ viruses in cell culture

Replication of GFP and ZZ viruses were compared in Vero and C6/36 cells. After infecting cell monolayers with passage 1 or 2 virus at MOI=0.01, supernatants were sampled every 12 hours and virus was titered. Virus with interrupted E2 protein was capable of producing progeny virus over time, an unexpected finding as the ZZ insert has been shown to severely cripple virus infection of mammalian cells (Iijima et al., 1999, Ohno et al., 1997). However, it is apparent that the ZZ virus replicates much less efficiently than GFP virus at early times post-infection (Figure 4.4).

In both cell lines, passage 1 ZZ virus could replicate to high titers, but required more time to do so (Figure 4.4). At 48 hours post infection, peaks titers in Vero cells

were 6.9 and 5.6 $\log_{10}$ PFU/ml for GFP and ZZ viruses, respectively. In C6/36 cells, peak titers were 6.5 and 6.4 $\log_{10}$ PFU/ml at 60 and 72 hours. These titers are low for published growth of GFP virus in C6/36 cells, suggesting that ZZ virus titers may also be repressed (Pierro et al., 2003). In Vero cells, ZZ virus did not produce detectable infectious virus until 36 hours post-infection, but was able to reach titers similar to those of GFP by 60 hours. No virus was detectable in C6/36 cells until 24 hours post-infection, but by 72 hours the viruses replicate identically. Of note, cytopathic effects seen in Vero cells were titer-dependent; GFP virus induced CPE by 24 hours while ZZ virus CPE was not visualized until 48 hours.

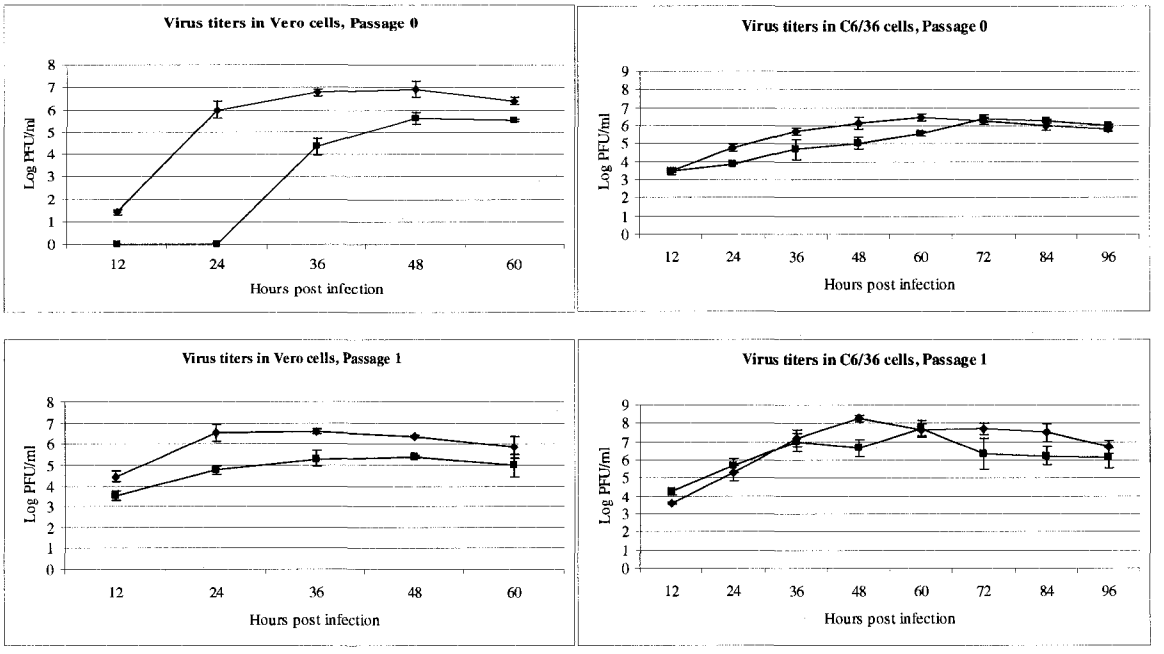

Figure 4.4. Replication kinetics of GFP and ZZ virus passages in Vero and C6/36 cells. Cell monolayers were infected with virus at MOI=0.01. Samples were obtained every 12 hours and titered on Vero cells. ♦=GFP; ■=ZZ.

When cells were infected with virus that had been passaged once after electroporation, ZZ-containing virus remained less able to replicate in Vero cells (Figure

4.4). ZZ virus consistently grew to titers one log less than GFP virus. However, ZZ virus replication kinetics in C6/36 cells were almost identical to GFP during the first 60 hours of infection except for a drop in titer at 48 hours post infection.

When comparing passage 1 and 2 virus growth, titers in Vero cells were similar. Titers in C6/36 cells were lower for GFP virus during passage 1 infection (Figure 4.4, left panels), and much more ZZ virus was produced during infection by virus passage 2 (Figure 4.4, right panels).

Targeted infection of Hela-CD4 cells using an anti-CD4 antibody bridge

Before attempting to show targeted infection, growth curve analysis was performed in Hela and Hela-CD4 cells (Figure 4.5). The difference in virus growth was most evident in Hela cells, where ZZ virus was apparently incapable of efficiently replicating to produce infectious progeny virus (Figure 4.5). This agrees well with previous findings using replicon vectors (Iijima et al., 1999, Ohno et al., 1997, Sawai & Meruelo, 1998). Without prior antibody adsorption, viral replication was not completely inhibited in Hela-CD4; after an initial lag period virus titers reached the same levels as GFP by 36 hours (Figure 4.5). GFP reached a peak titer of 5.5 $\log_{10}$ PFU/ml at 36 hours post infection of Hela cells and 5.9 $\log_{10}$ PFU/ml in CD4 positive cells at 30 hours. ZZ virus reached a peak of 6.0 $\log_{10}$ PFU/ml at 36 hours post infection of Hela-CD4 cells.

To provide evidence that infection by our ZZ virus system could be targeted using a receptor-specific bridging antibody, we attempted to show targeted infection of Hela-CD4 cells. Using increasing amounts of anti-CD4 antibody adsorbed to passage 2 virus prior to infection, infectious virus output was used as a marker for infection. Titers were determined at 18 hours post infection, a time at which the difference in titers between the

control viruses and ZZ virus was most evident in growth curve analysis (Figure 4.5, bottom panel).

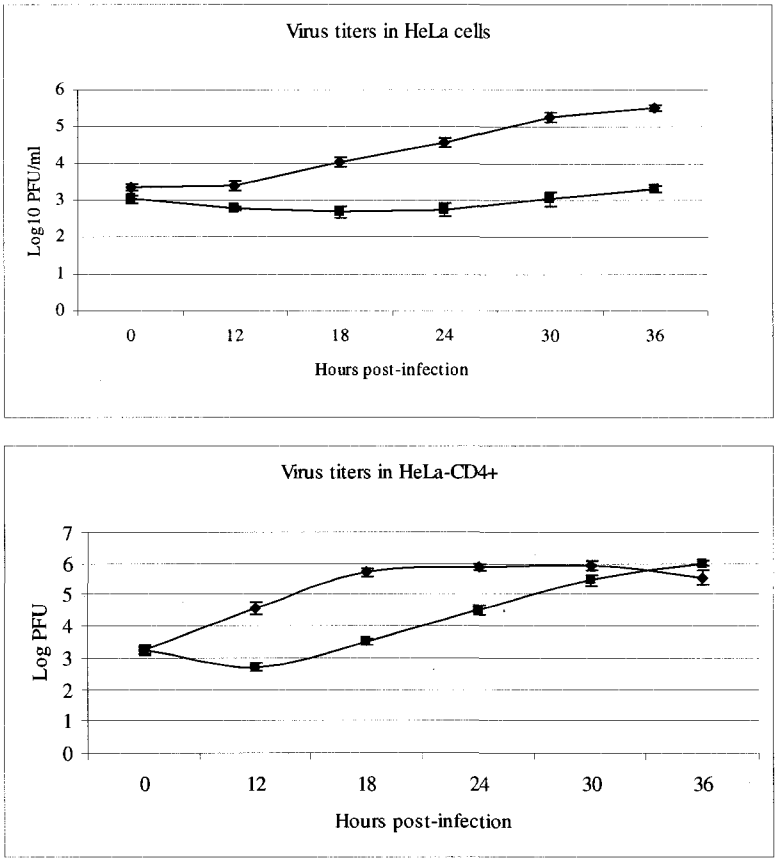

Figure 4.5. Replication kinetics of GFP and ZZ viruses in Hela cells. Monolayers of Hela and Hela-CD4 cells were infected with passage 2 virus at MOI=0.01. Samples taken every six hours were titered on Vero cell monolayers. ♦=GFP; ■=ZZ.

GFP and Bst viruses did not differ in infectious virus output at any antibody amount tested (Figure 4.6). Peak titers of approximately 4 $\log_{10}$ PFU/ml were achieved for both viruses when no antibody was present. There was a noticeable increase in infectious virus output for ZZ virus as the amount of CD4 antibody was increased. However, the difference between ZZ virus output with no antibody and the maximum amount of antibody tested (1 μg) was 1.5 $\log_{10}$ PFU/ml (1.8 vs 3.3 $\log_{10}$ PFU/ml, respectively). Previous differences of more than five-fold change between infection with no antibody

and the highest antibody amounts have been reported (Ohno et al., 1997). The titer of Bst and ZZ virus was equivalent when 1μg of anti-CD4 antibody was adsorbed, suggesting that equal amounts of each virus were able to infect cells.

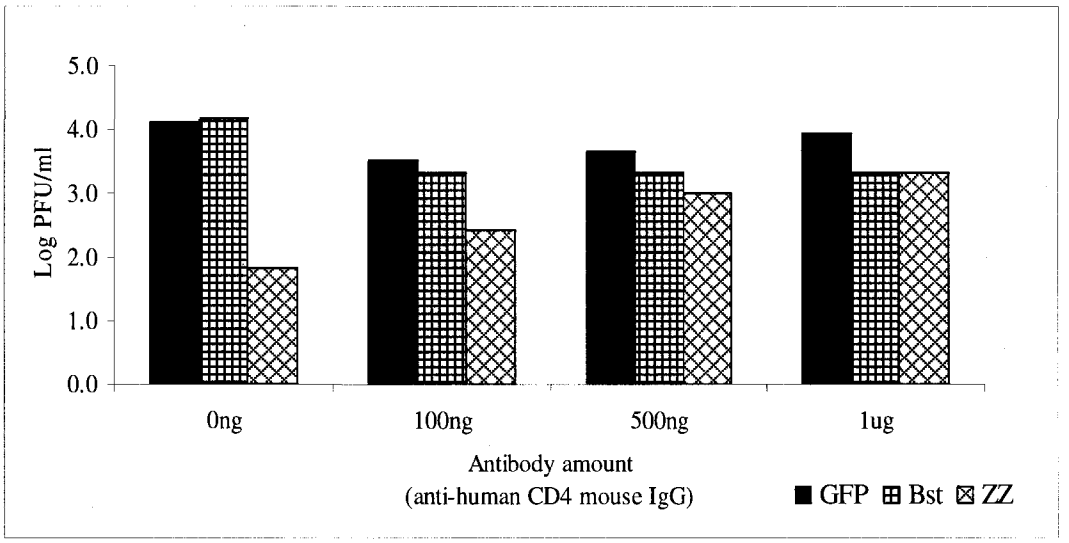

Figure 4.6. Infectious virus release from Hela-CD4 cells infected with virus pre-adsorbed to CD4 antibody. Viruses were pre-adsorbed with anti-human CD4 antibody prior to infection of Hela-CD4 cells. Virus titers were determined at 18 hours post infection.

Replication of ZZ virus in Aedes aegypti mosquitoes

The utility of ZZ virus as a targeted SINV vector relies on the ability of virus to infect only those mosquito tissues targeted through an antibody bridge. To determine whether ZZ virus could replicate in *Ae. aegypti* mosquitoes without an antibody present, intrathoracic injection of virus alone was performed. Mosquitoes were injected with stock virus and GFP expression was visualized after seven day incubation. All three viruses from both passages were able to efficiently infect mosquitoes via intrathoracic inoculation (Table 4.2).

Table 4.2

Detection of GFP expression in intrathoracically injected mosquitoes*

		GFP expression in ommatidia			
SIN virus	**Passage**	**# positive**	**Total**	**% positive**	**P-value#**
TE5'2J/GFP	V1	28	28	100.0	0.4918
TE5'2J/GFP	C2	26	26	100.0	0.2425
TE5'2J/GFP/Bst	V1	28	32	87.5	0.6724
TE5'2J/GFP/Bst	C2	31	33	93.9	0.6673
TE5'2J/GFP/ZZ	V1	28	30	93.3	N/A
TE5'2J/GFP/ZZ	C2	28	31	90.3	N/A

*HWE were injected with either passage 1 or 2 virus and GFP expression in the ommatidia was observed seven days post infection
P-values generated using Fisher's exact test comparing infection characteristics to ZZ virus from the same passage

Fluorescence was observed in the ommatidia of all mosquitoes injected with GFP virus and most mosquitoes injected with Bst virus (87.5% and 93.9% for passage 1 and 2 virus, respectively), agreeing with previous data that TE5'2J viruses efficiently infect mosquitoes after intrathoracic inoculation (Pierro et al., 2003). Interestingly, and unfortunately, a similar percentage of mosquitoes injected with ZZ virus expressed GFP in their ommatidia seven days after injection (93.3% and 90.3%). Qualitatively, there were no differences in the level of GFP expression; infected mosquitoes expressed GFP throughout their ommatidia (data not shown).

Mosquito infection with 5'ds/MRE16 viruses

HWE mosquitoes were infected with 5'ds/MRE16 viruses expressing various reported genes orally by infectious bloodmeal or intrathoracically by injection and assayed qualitatively for reporter gene expression. Mosquitoes ingesting a bloodmeal containing virus were assayed at later times post infection to show the utility of different reporter proteins during mosquito infection. A representative image of a mosquito with a

fully disseminated 5'ds/MRE16/DsRed infection is shown in Figure 4.7. DsRed protein is expressed highly in the midgut and can be detected throughout the abdomen and thorax, in the legs, ommatidia, antennae, and proboscis. Salivary glands dissected from this mosquito expressed the red protein as well. All viruses expressing fluorescent reporter proteins were capable of infecting the midgut and disseminating to hemocoelic tissues when fed orally to *Ae. aegypti* mosquitoes.

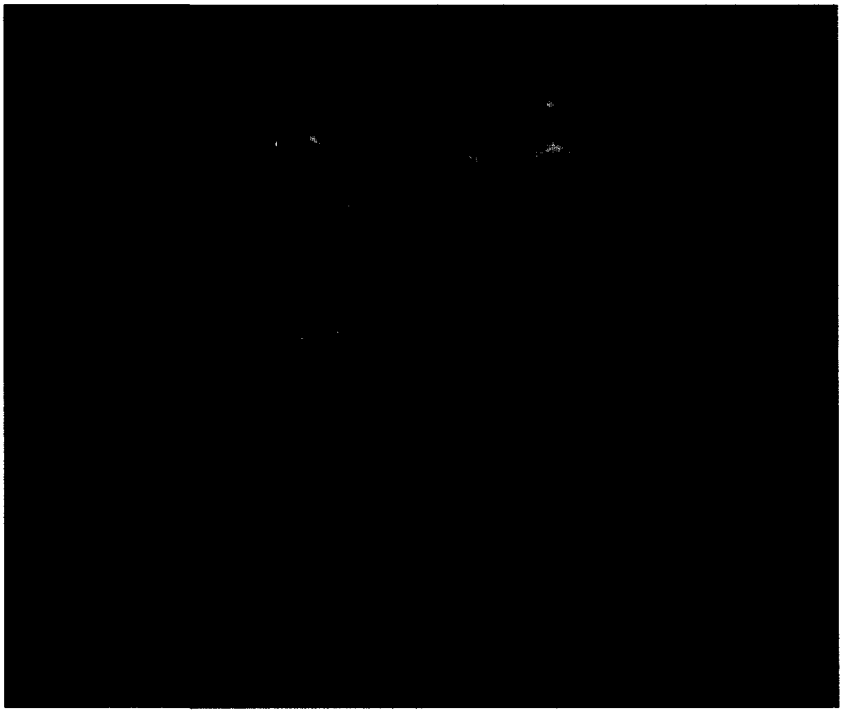

Figure 4.7. DsRed expression in HWE mosquito infected orally with 5'ds/MRE16/DsRed. Mosquitoes were infected orally with 7 logs of 5'ds/MRE16/DsRed. At 13 days post infection, red fluorescence was detected using an epifluorescent microscope.

Dual infection of mosquitoes following per os infectious bloodmeal

Mosquitoes ingested blood containing 7 $\log_{10}$ PFU of 5'ds/MRE16 viruses expressing EGFP and DsRed in the same bloodmeal. Both viruses were able to infect the midgut and disseminate to non midgut tissues. The green and red fluorescent proteins could be detected in the midgut at eight days post infection and in the salivary glands at

day ten (Figure 4.8). Areas of the salivary glands contain cells expressing EGFP and DsRed. The medial lobe of both individual glands is not infected by either virus, consistent with previous findings that SINV infects only the two lateral lobes of the salivary gland.

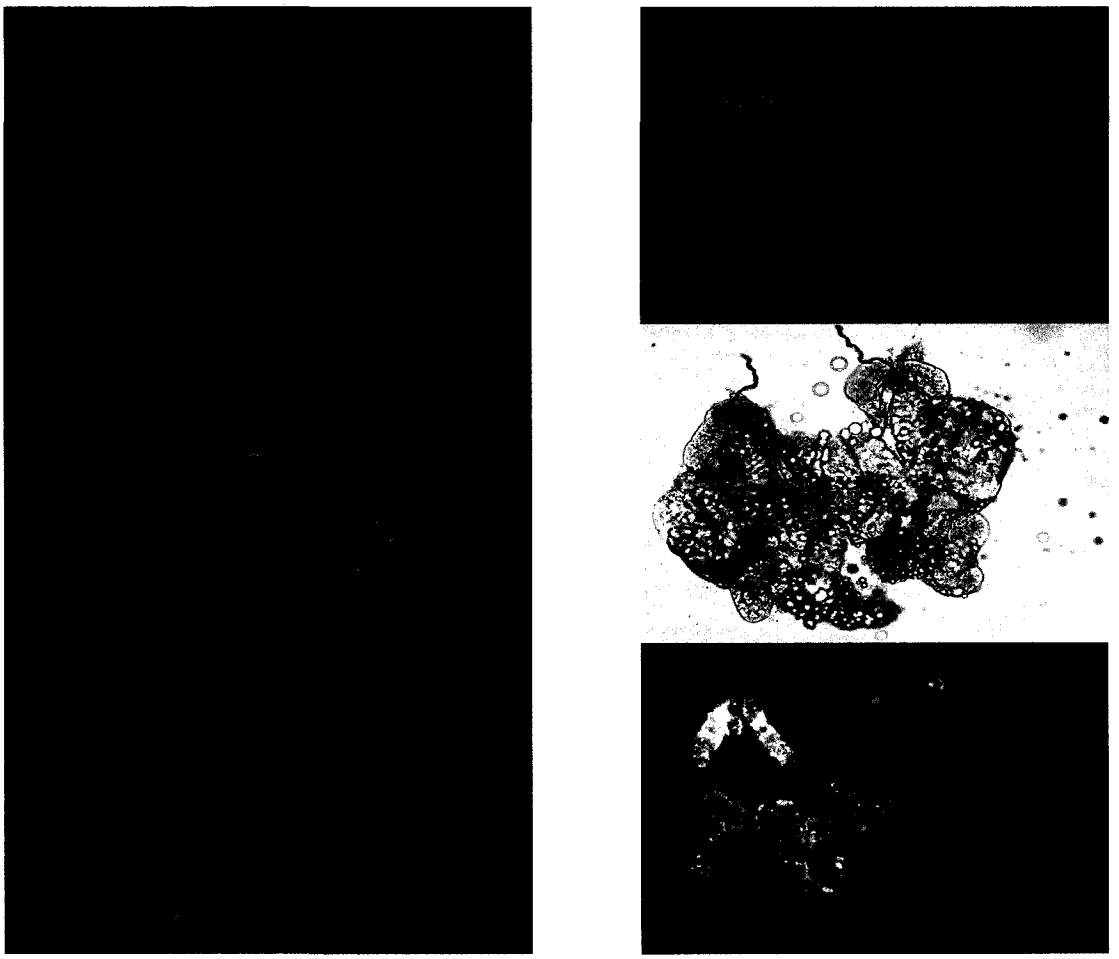

Figure 4.8. Expression of EGFP and DsRed in a mosquito infected simultaneously by oral bloodmeal. Mosquitoes were given a bloodmeal containing 7 logs of 5'ds/MRE16/EGFP and DsRed. Midgut was dissected eight days post infection and salivary glands at day ten from separate mosquitoes. Fluorescence was detected using the FITC and red fluorescence filters. Midgut photo is composite of overlaid images.

Detection of virus-expressed fluorescence and bioluminescence in cell culture and mosquitoes

The utility of identifying infected mosquitoes using the IVIS200 imaging system was determined by visualizing cold-anesthetized injected mosquitoes that were previously visualized for infection using a standard epifluorescent microscope. Individual mosquitoes infected with 5'dsMRE16, EGFP, ECFP, and DsRed viruses were visualized using both the FITC and red fluorescent wavelengths. Both EGFP and DsRed can be visualized in infected mosquitoes with the most intense signal emerging from the midgut/abdomen region (Figure 4.9).

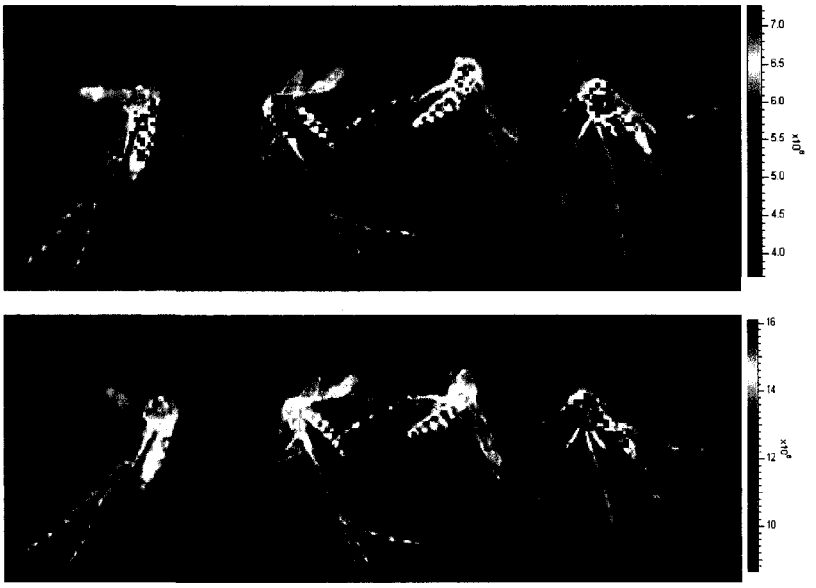

Figure 4.9. Whole mosquito fluorescence using IVIS200 imaging system. Mosquitoes were orally infected with 5'ds/MRE16 viruses and observed at nine days post infection. From left to right- MRE16 (no insert), MRE-EGFP, MRE-ECFP, MRE-DsRed. Top panel visualized using a FITC filter. Bottom panel visualized using a DsRed filter. Bloodmeal titers were $1x10^7$ PFU of each virus. Scales to right give relative signal intensity.

Using the FITC filter, background signal is detected in all mosquitoes including one infected with virus expressing no reporter protein. When mosquitoes are visualized with

the red fluorescence filter, only the DsRed-infected mosquito emits a strong signal in the abdomen and thorax. Other infected mosquitoes do not show signal using the same filter.

Vero cells and mosquitoes were infected with 5'ds/MRE16/FLUC and RLUC viruses. At two days (Vero cells) or seven days (mosquitoes) post infection, the corresponding substrate was added to cell culture suspension or intrathoracically-injected into mosquitoes and bioluminescence was detected using the IVIS200 system in a 96 well plate format (Figure 4.10). Infected Vero cells were scraped into one ml of culture medium, serially-diluted ten-fold and substrate was added to all wells simultaneously. Sixteen mosquitoes were randomly selected, injected with substrate, and imaged two minutes later.

Signal was detected from cells infected with both FLUC and RLUC expressing viruses (Figure 4.10A). The emitted signal was more intense from RLUC-infected cells than FLUC but both infection signals could be titrated by serial dilution. The substrate used for RLUC detection, coelenterazine, does show background bioluminescence when added to mock-infected cells whereas FLUC substrate, luciferin, does not (Figure 4.10B).

Signal was detected in orally-infected *Ae. aegypti* mosquitoes that had been cold-anesthetized, injected with substrate, and placed into individual wells of a 96-well plate. Just as in cell culture, activity of both types of luciferase enzyme could be detected in infected mosquitoes (Figure 4.10 C and D). Four of 16 randomly-selected mosquitoes in each group emitted detectable signal. The signal was strongest in the abdomen and thorax of each mosquito. No background was detected in mock-infected mosquitoes injected with either substrate.

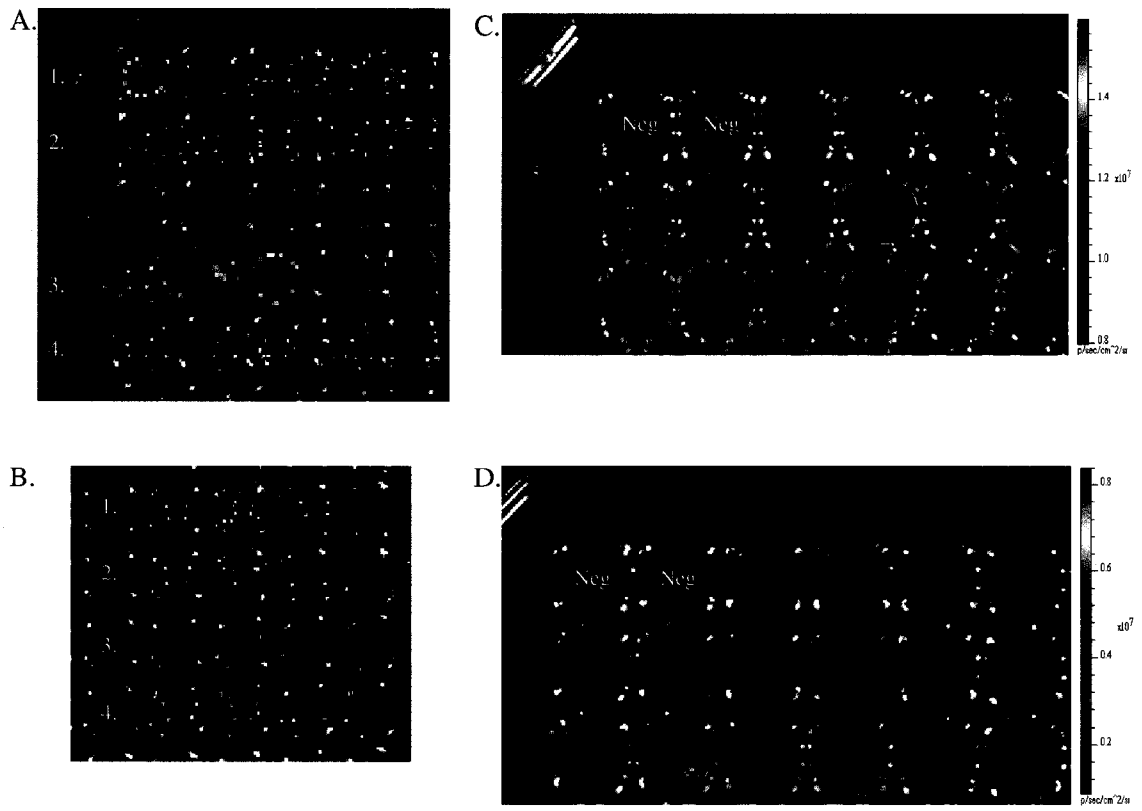

Figure 4.10. Detection of FLUC and RLUC expression in MRE16-infected Vero cells and HWE mosquitoes. **A**. Monolayers of Vero cells infected with 5'ds/MRE16/FLUC (Lanes 1 and 2) or 5'ds/MRE16/RLUC (Lanes 3 and 4) were scraped into culture medium and diluted ten-fold (from left to right). Luciferase substrates were added to lanes 1 and 3 and bioluminescence was visualized after two minutes using the IVIS200 imaging system with Living Image 3.0 software and a two minute exposure. **B.** Mock-infected Vero cells were incubated with luciferase substrate and visualized the same as (A.). 1=Luciferin substrate. 2=No substrate. 3=Coelenterazine substrate. 4=No substrate. **C.** HWE mosquitoes were given an oral bloodmeal containing $6.0x10^6$ PFU of 5'ds/MRE16/FLUC virus. Seven days post infection, sixteen mosquitoes were injected with luciferin (30μg/ml) and visualized the same as (A.). Infected mosquitoes are circled in white. Neg=mock-infected mosquito injected with substrate. **D.** HWE mosquitoes seven days post oral infection with $1.0x10^7$ PFU of 5'ds/MRE16/RLUC were injected with coelenterazine (50μg/ml) and visualized the same as (A.). Infected mosquitoes are circled in white. Neg=mock-infected mosquito injected with substrate. In all frames, red color or clearing of color represents pixels saturated with bioluminescent signal.

Detection of 5'ds/MRE16/DsRed transmission to mice

The IVIS200 imaging system was used to detect the transmission of 5'ds/MRE16/DsRed to mice by the bite of infected mosquitoes (Figure 4.11). Injected HWE mosquitoes seven days post-infection were allowed to feed on a single albino B6

mouse for one hour. In parallel, one mouse was exposed to uninfected mosquitoes and another was injected in the right rear footpad with 10^4 PFU of virus. At various times post-infection, mice were anesthetized with isofluorane and visualized using the IVIS200 imaging system with the DsRed filter.

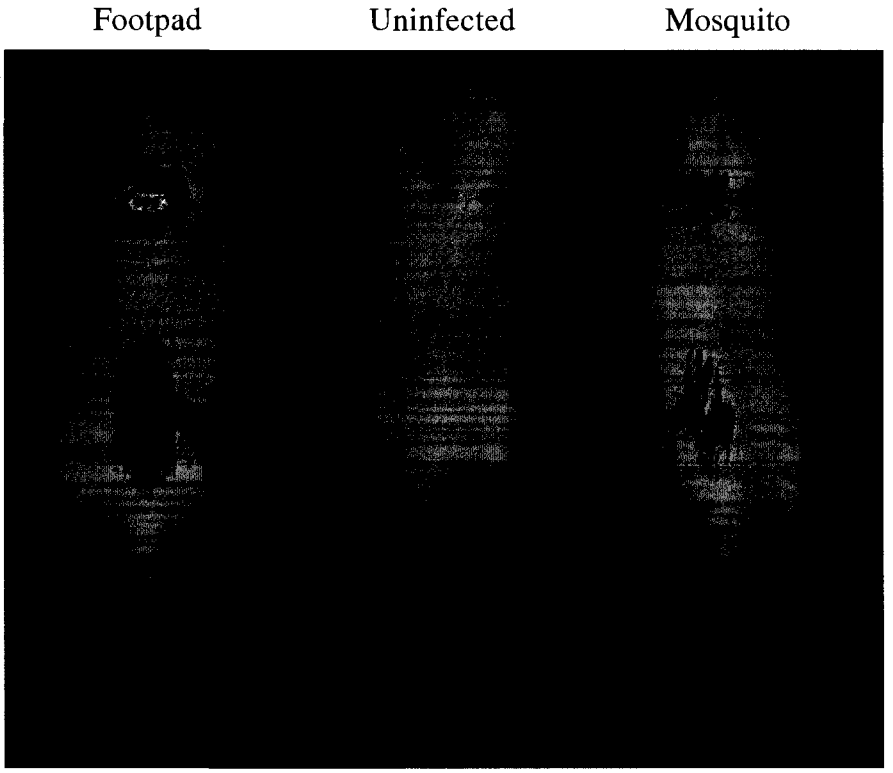

Figure 4.11. Mosquito transmission of 5'ds/MRE16/DsRed virus to mice. Albino B6 mice were infected with 5'ds/MRE16/DsRed virus by needle injection or mosquito bite. A single mouse was injected with virus in the right rear footpad (Footpad), fed on by uninfected mosquitoes (Uninfected), or fed on by mosquitoes intrathoracically injected with virus (Mosquito). Mice were observed 16 hours post mosquito exposure using the IVIS200 system with the DsRed fluorescent filter. Images provided by Aaron Phillips and Dr. Eric Mossel, Colorado State University.

At 16 hours post-infection, fluorescence was detected in the footpad-injected and mosquito-infected mice, showing that DsRed produced during virus infection can be detected using the IVIS200 system (Figure 4.11). Importantly, the pattern of fluorescence was nearly identical between the two infected mice but was not detected in the mouse exposed to uninfected mosquitoes. The most intense signal was seen near the

base of the brain and lesser signal in the basal spinal cord of infected mice. Signal was more intense in the mouse infected via footpad injection, especially in the brain. No signal was detected in the injection site for the needle-inoculated mouse. Similar patterns of fluorescence were observed up to 72 hours post infection (data not shown).

Discussion

As described previously, the infectious clones MRE16 and TE12 are derived from the genomes of SINVs belonging to distinct phylogeographic virus genotypes. Double-subgenomic virus systems based on the two separate virus lineages differ in their mosquito infection kinetics and RNAi induction (Myles et al., 2004) (Chapter 3). This chapter sought to expand the current capabilities that we and others have with the dsSINV systems.

SINV replicons mutated by insertion of an antibody-binding domain into a surface-exposed region of E2 glycoprotein could be efficiently targeted to mammalian cells only when a bridging antibody was first adsorbed to the virus surface (Iijima et al., 1999, Ohno et al., 1997, Phinney et al., 2000, Sawai & Meruelo, 1998). Our engineered virus, mutated in the same manner as the replicon from Ohno et al (1997), is capable of infection without prior antibody adsorption, although inefficiently.

The virus strain used in this study is not the same as was previously used (TE/5'2J is a chimeric virus including AR339 and mouse-adapted HRsp strain sequence while TOTO1101 is derived from HRsp only) and may play a minor role in the observed differences. Another strain of SINV, S.A.AR86, is capable of efficient infection of mice when an E3-E2 cleavage null mutation, producing a E2 larger than normal, is

incorporated (Russell et al., 1989). The infection of mosquito cells was not examined in this study. Similar mutants based on strain AR339, the parent strain for TE/5'2J, are capable of efficient BHK-21, but not C6/36 cell infection (Heidner et al., 1996). Although the mutations are not similar to ours, it is interesting that AR339 viruses with mutated E2 have the opposite replication kinetics to our ZZ virus.

dsSINVs are capable of multiple rounds of infection because the virus possesses all of the nonstructural and structural proteins necessary for replication. A drawback to double-subgenomic systems is that packaging constraints limit the amount of foreign gene sequence that can be encoded by the virus (Hahn et al., 1992). From analysis of viral RNA by RT-PCR (Figure 4.2) and E2 protein detection in cell culture (Figure 4.3) it appears that our virus containing an interrupted E2 gene has recombined with a second virus transcript to delete the insert from the virus genome after multiple rounds of replication, potentially as a mechanism to manage the inclusion of an expressed transgene and excess sequence within the structural genes. Kinetics of replication in cell culture also suggests that some event rendering the virus more capable of infection has occurred.

Because interruption of the SINV E2 protein may disrupt virus binding but not replication and assembly (Dubuisson & Rice, 1993, Ohno et al., 1997), it is logical that a recombinant or deletion mutant without the E2 insert would have a selective advantage over ZZ domain-containing virus for infection of susceptible cells. Two separate RNA molecules produced during ZZ virus infection may have recombined to produce a virus genome that, when packaged, was more readily able to productively infect cells. Significantly more subgenomic RNA is produced during SINV infection, producing a

large amount of viral RNAs that could be used for a recombination event with the genomic RNA that may have occurred in the E2 gene.

The ZZ domain was apparently deleted from some of the TE/5'2J virus genomes. Deletion of large portions of the SINV genome give rise to DI genomes that cannot produce infectious virus (Dohner et al., 1979, Levis et al., 1986, Monroe et al., 1982, Monroe & Schlesinger, 1983, Sanz et al., 2003, Weiss et al., 1974, Weiss & Schlesinger, 1973). Viable deletion mutants of SINV and RRV have been isolated from cell culture (Leone et al., 1980, Myles et al., 2003, Vrati et al., 1986). Leone et al (1980) isolated a SINV clone containing a deletion in the E1 glycoprotein that was able to infect mammalian and avian cells as efficiently as the parental virus strain (Leone et al., 1980). Myles et al (2003) isolated a small plaque mutant of SINV strain MRE16 with a deletion in E2 that could inefficiently replicate in mammalian cells but had replication kinetics similar to wild-type virus in mosquito cells. The virus was able to efficiently replicate in mosquitoes after intrathoracic injection but was much less able to infect and disseminate from the mosquito midgut following oral bloodmeal (Myles et al., 2003).

Deletion mutants that have been isolated from cell culture show replication kinetics similar to parental virus in either mammalian or mosquito cell culture and are attenuated in vector or host infection (Leone et al., 1980, Myles et al., 2003, Vrati et al., 1986). Similar to the described deletion mutants, ZZ virus is capable of replicating in C6/36 cells as efficiently as GFP after a single passage in the same cell type. Also, like the MRE16 deletion mutant, GFP and ZZ viruses are capable of efficient mosquito infection following intrathoracic injection. This circumstantial evidence along with the

presence of smaller bands in the RT-PCR suggests that a deletion may have occurred in the ZZ virus during passage.

It is apparent that ZZ virus is capable of replicating in cell culture and mosquitoes and will not be useful to target infection of specific mosquito tissues. Although minimal, ZZ domain-containing replicon particles are able to infect cells without an antibody bridge present (Iijima et al., 1999, Ohno et al., 1997, Sawai & Meruelo, 1998). TE/5'2J is a fully infectious virus capable of progeny virus infection and, therefore, could select for viruses capable of efficient infection during each passage. Rapid adaptation of SINV has been shown previously in mosquito cells, suggesting that production of a recombinant virus after a single passage is possible (Fayzulin & Frolov, 2004). The similar growth kinetics of GFP and ZZ virus in C6/36 cells after a single virus passage in the same cell line suggests that ZZ virus may be adapting to and selecting for virus that efficiently replicates in mosquito cells. The ability of ZZ virus to replicate in mosquitoes after intrathoracic injection may be related to the rapid adaptability of SINV to mosquitoes. The lag in virus replication in mammalian cells after the C6/36 passage could indicate that the adaptation is cell type-specific.

Infectious SINV has been used for targeted infection by inserting multiple antibody-binding domains from protein L of *Peptostreptococcus magnus* into the genome upstream of the E2 gene (Klimstra et al., 2005). Placed between the E3 and E2 genes, the domain was fused to E2 with a 17 amino acid linker sequence. Infection of unsusceptible mouse and human cells expressing antibody Fc receptors was minimal unless a species-matched IgG molecule was first adsorbed. However, the virus remained able to efficiently infect susceptible cells, suggesting that this is not a viable option for targeted

infection of susceptible mosquito tissues (Klimstra et al., 2005). Gorchakov et al (2007) used a similar linker-based approach to engineer SINV to express the envelope glycoprotein Gn of Rift Valley fever virus. The entire glycoprotein gene (318 amino acid) could be expressed on the surface of infected cells and was efficiently incorporated into progeny virions (Gorchakov et al., 2007). The experiments did not investigate the effects of multiple passages or mosquito cell infection of these viruses so stability of the linker insert viruses remains unknown.

A potential option for targeting SINV infection to mosquito tissues would be the incorporation of a single-chain antibody into the E2 protein. Single-chain antibodies maintaining antigen specificity have been expressed in dsSINV systems from the second subgenomic RNA (Aires Da Silva et al., 2005, de Lara Capurro et al., 2000, Jiang et al., 1995). The antibodies would need to be cloned from hybridoma cell lines that are available, but individual viruses would be required for each tissue instead of a single virus with multiple antibodies (Barreau et al., 1999).

ZZ virus was constructed as a tool for the study of mosquitoes in a tissue-specific manner. The hope was to induce infection of specific mosquito tissues such as the midgut or salivary glands using *Ae. aegypti* tissue-specific antibodies (Barreau et al., 1999) and use engineered viruses to study gene expression through an RNA interference mechanism (Attardo et al., 2003, Johnson et al., 1999, Olson et al., 1996, Uhlirova et al., 2003). The ability of ZZ virus to infect mosquitoes with no antibody present minimized the use of this construct in this endeavor. With the advent of efficient transformation systems and mosquito tissue-specific promoters, the utility of a ZZ virus is somewhat obsolete. The germlines of numerous arbovirus- and malaria-transmitting mosquito

species have been successfully transformed using transposable elements of other dipterans (Allen et al., 2001, Catteruccia et al., 2000, Coates et al., 1998, Jasinskiene et al., 1998, Lobo et al., 2002, McGrane et al., 1988, Miller et al., 1987, Morris et al., 1989, Perera et al., 2002, Rodrigues et al., 2006). In *Ae. aegypti* alone, tissue-specific transgene expression has been demonstrated in the salivary glands, midgut, testes, ovary, and fat body (Bian et al., 2005, Cho et al., 2006, Coates et al., 1999, Franz et al., 2006, Johnson et al., 1999, Kokoza et al., 2000, Moreira et al., 2000, Smith et al., 2007b). New genetic engineering systems using bacteriophage site-specific integration mechanisms have been developed that allow the efficient production of transgenic mosquito strains with the capability of inserting extremely large transgenes (Nimmo et al., 2006). Also, with the publication of the *Anopheles gambiae*, *Ae. aegypti*, and *Culex pipiens* genomes and the ongoing sequencing of other important disease vectors, the identification of genes and promoters of interest will increase the capabilities of mosquito transgenesis (Holt et al., 2002, Nene et al., 2007). The limitations of coding capacity of dsSINVs and required engineering and production of virus prior to transgenesis are less appealing steps with the novel technologies that have recently been developed.

Using a similar cloning approach to what was described in this chapter, the enhanced GFP gene has been inserted into E2 of pTE/5'2J/Bst (Dr. Brian Foy, unpublished data). This virus, in which the GFP expressed from the second subgenomic promoter has been removed, contains less foreign sequence and may prove to be sufficiently stable for multiple rounds of replication. If so, a fluorescently-tagged integral viral membrane protein may be useful for tracking of single virus particles. Individual DENV2 particles labeled with a fluorescent probe in the viral membrane have been used

to examine binding and fusion events of virus infection (van der Schaar et al., 2007). Using various microscopy techniques, similar events of SINV infection, along with processing and trafficking of the E2 protein in mammalian or insect cells could be visualized. Also, fluorescence can be detected in tobacco plants infected with a filamentous virus expressing a coat protein-GFP fusion (Santa Cruz et al., 1996).

Engineered viruses capable of targeted infection are a useful tool for potential development of gene and cancer therapies. The same may have been true for targeted infection of mosquitoes just a few years ago. With the advent of new efficicent technologies that allow for tissue-specific expression in mosquitoes, targeted SINV infection may not be as useful. We have shown that targeted infection using a fully infectious engineered dsSINV may not be practical in mosquitoes because of rapid adaptation of SINV, but that intermediates created during the original engineering procedures may become useful tools to study SINV biology.

We have also created 5'ds/MRE16 viruses that may prove useful to study SINV and mosquito biology. Viruses expressing a multitude of fluorescent and bioluminescent proteins provide a panel of candidates for use in numerous experimental procedures. Because the viruses are derived from cDNA clones, the virus genome can be manipulated with molecular techniques to study determinants of many aspects of arbovirus infection (Pierro et al., 2007, Pierro et al., 2008). The utility of marker gene expression during SINV infection of mosquitoes has been thoroughly studied using MRE16, TE12, and chimeric viruses constructed from the two (Foy et al., 2004, Higgs et al., 1996, Olson et al., 2000, Pierro et al., 2003). To date, the only marker genes used for dsSINV-mediated expression in mosquitoes are GFP-derived proteins. We have expanded the panel of

5'ds/MRE16 viruses to include other GFP-derived proteins (ECFP, EYFP, AcGFP), red fluorescent proteins (DsRed and mCherry), a fluorescent protein that has green and red fluorescent intermediates (Timer), and two enzymes capable of producing a bioluminescent reaction product (FLUC and RLUC).

Fluorescent proteins have proved to be a useful marker of virus infection in a number of different virus-host systems (Baulcombe et al., 1995, Higgs et al., 1996, Lee et al., 1997). GFP has been the gold standard for fluorescent protein expression over the last decade. We have shown here that fluorescent proteins with emission wavelengths different than GFP and bioluminescent proteins can be expressed from 5'ds/MRE16 to track infection of the mosquito and potentially virus transmission to animals.

GFP has an emission spectrum that overlaps with many endogenous proteins and commonly used detection fluorophores. Using the FITC filter to detect GFP also produces background levels of fluorescence, which we have shown using the IVIS 200 imaging system (Figure 4.8). By producing viruses expressing proteins with a longer emission wavelength, such as DsRed and mCherry, we can now detect mosquito infection without background expression. This may be extremely useful when performing experiments requiring large numbers of infected mosquitoes. Anesthetized mosquitoes can be placed in a 96-well plate and screened using the IVIS 200 imaging system, allowing for selection of infected mosquitoes in a matter of seconds. Activities including microarray analysis of gene transcription in infected mosquitoes can be expedited using this selection procedure (Sanders et al., 2005).

By infecting mosquitoes with viruses expressing EGFP and DsRed and detecting green and red fluorescence in multiple tissues, we have also shown the utility of using

various fluorescent proteins in dual-infection experiments (Figure 4.7). It may be interesting to infect mosquitoes with 5'ds/MRE16 and at later time points infect with another naturally co-circulating arbovirus to determine the effects that previous infection may have on infection and dissemination. The high efficiency of MRE16 infection of the mosquito may either interfere with or enhance infection by the second virus.

Timer is a novel fluorescent protein that matures from a green to red-emitting fluorophore over a matter of hours. This provides a unique opportunity to study the infection characteristics of virus over time. Following an oral bloodmeal, virus infects individual cells of the mosquito midgut and spreads to neighboring cells over time. There is the potential to visualize and corroborate this phenomenon using the Timer-expressing virus. As protein matures, the primary cells infected would predominantly express red fluorescence whereas secondarily infected cells would appear green, presenting as an infection focus with a bull's-eye like pattern. Timer could also prove useful in studying potential virus quiescence in infected mosquitoes. A clearance phenomenon may occur in the midgut of infected mosquitoes over time. This may be caused by the removal of virus-infected cells or a "latent" virus infection that could be reestablished when a second bloodmeal is taken and nutrients are replenished. Renewal of Timer protein expression would produce an observed green fluorescence in infected cells.

Aside from vector infection, another important aspect of arboviruses is transmission to the vertebrate host. Using a mouse model of SINV infection and the IVIS 200 system for real-time imaging, transmission from mosquito to mouse was visualized over a time course within individual animals. Fluorescence was detected in mice infected

with 5'ds/MRE16/DsRed via needle injection and mosquito bite (Figure 4.10). This provides direct evidence that virus-expressed proteins can be detected following mosquito transmission to a mouse.

Luciferase reporter proteins may provide a better platform for visualization of virus transmission because bioluminescence can be detected in deep tissues that cannot be penetrated by fluorescent wavelengths of light. FLUC has been shown to be detected in mice during infection by neurotropic SINV infection and protocols for mosquito transmission of SINV to mice have been described (Cook & Griffin, 2003, Jackson et al., 1993, Myles et al., 2004, Ryman et al., 2007). There would be no need to sacrifice numerous mice per time point because the mice can be temporarily anesthetized within the imaging system, decreasing the total number of experimental animals needed. Also, after initial testing to correlate relative light units with virus titers, quantitative analysis of virus infection could be carried out without organ harvesting (Cook & Griffin, 2003). Viruses are produced from cDNA, allowing manipulation of the genome to help identify sequences that enhance or inhibit SINV transmission. Detection of marker proteins expressed during virus infection may facilitate the identification of viral determinants of virulence and infection following mosquito inoculation of the vertebrate. Studies have shown that mosquitoes are not just "flying syringes" and that factors within vector saliva may contribute to and facilitate arbovirus pathogenesis in the vertebrate host (Limesand et al., 2000, Limesand et al., 2003, Schneider & Higgs, 2008, Schneider et al., 2007, Schneider et al., 2004). Along with modeling mosquito-borne virus transmission, the LUC-expressing viruses could be used in antiviral treatment studies to evaluate the

efficacy of antiviral approaches after a natural virus challenge from the bite of an infected mosquito rather than needle inoculation.

Genome instability associated with the incorporation of larger transgenes such as FLUC has been shown and could make such viruses less useful. Having a panel of viruses will ensure that where one virus may fail, another may succeed. Developing new tools like those described here are imperative to understanding vector infection and mosquito-borne virus transmission. We may now have the tools necessary to study the molecular aspects of the arbovirus transmission cycle, from vector infection to transmission to a vertebrate host.

Chapter 5

SUMMARY

Double-subgenomic Sindbis virus transducing systems are effective tools for the study of arbovirus-mosquito interactions at the molecular level. The work described in this dissertation has developed new systems to study important events of the arbovirus transmission cycle, from initial infection of the mosquito midgut to eventual transmission to a vertebrate host.

RNA interference is a major immune response to virus infection in mosquitoes and has been shown to modulate infection of arboviruses in *Aedes aegypti* and *Anopheles gambiae* mosquitoes (Campbell et al., 2008, Keene et al., 2004). However, these studies have relied on transient silencing of effector molecules within the RNAi pathway following parenteral injection of double-stranded RNA into mosquitoes. The potential off-target effects associated with dsRNA-mediated silencing have recently been shown (Kenderell & Carthew, 1998, Tschuch et al., 2008). To avoid these off-target effects and link virus infection with RNAi suppression, a dsSINV was engineered to express during its replication cycle a known viral suppressor of RNAi, the B2 protein of Flock House virus. The virus, designated TE/3'2J/B2, efficiently inhibited RNAi in infected cell culture and *Ae. aegypti* mosquitoes. Significantly higher titers and more accumulation of viral RNA in *Ae. aegypti* show that RNAi does modulate SINV infection in this vector. Virus-associated mortality was observed in multiple species of mosquitoes following infection, providing evidence for a role of the mosquito immune response in persistent arbovirus infection. TE/3'2J/B2 provides a fully-infectious virus that should facilitate a better understanding of the molecular mechanisms of arbovirus persistence in relation to the mosquito RNAi response.

SINVs have been used to study gene function in mosquitoes through a virus-induced gene silencing mechanism by expressing antisense sequence derived from the gene of interest (Adelman et al., 2008, Attardo et al., 2003, Higgs et al., 1998, Johnson et al., 1999, Shiao et al., 2001). The virus expression systems can be produced using standard cloning procedures and provide a means to study gene function prior to the more laborious methods of mosquito transgenesis. Gene silencing using the TE12-based systems has proven effective in mosquitoes following parenteral injection. A second system based on the MRE16 strain efficiently infects mosquitoes by the oral route but its gene silencing capabilities have not been fully characterized (Myles et al., 2004, Seabaugh et al., 1998). To further characterize the efficiency of virus-induced gene silencing mediated by the two SINV systems, an inducible cell culture-based approach was used. TE12 viruses were capable of efficiently silencing targeted RNAs using our methodologies but MRE16 transducing systems were not able to silence. Gene-specific siRNAs for both systems were identified in an *Ae. aegypti* cell line, suggesting that RNAi is induced by both viruses, but to a much lesser extent during MRE16 virus infection. Our results show that a TE12 system should be used when performing SINV-mediated gene silencing experiments. MRE16 and TE12 transducing systems should be important tools for better understanding the molecular determinants of RNAi induction in the mosquito vector has also been provided.

The arbovirus transmission cycle is a complex series of molecular interactions between virus, vector, and vertebrate host. The successful transmission of an arbovirus is dependent on genetic and environmental factors that are not well understood. dsSINVs provide a platform to study these factors within the mosquito vector. Systems based on

TE12 and MRE16 were engineered to target virus infection to specific mosquito tissues and express various reporter proteins to expand our current capabilities. An antibody-binding domain was engineered into TE/5'2J for display on the virion surface, but virus stability was variable and targeted infection could not be shown. However, a plasmid vector that may facilitate the display of genes of interest on the virus surface has been produced. A panel of 5'ds/MRE16 viruses expressing various marker proteins was also created. The utility of individual viruses in tracking infection of the mosquito vector and transmission to a vertebrate host were shown. The viruses produced provide an important collection of SINVs that should facilitate the study of virus-vector interactions.

In total, the work presented here shows the power of dsSINVs for studying interactions between the mosquito vector and infecting virus. A role for the vector immune response in arbovirus infection has been clearly shown and this response may be induced differently by distinct SINV genotypes. New tools are now available to study the relationship between RNAi and persistent arbovirus infection. Other SINVs have been engineered that should be useful for monitoring virus infection in mosquio cells, track virus dissemination in mosquitoes, and detect SINV transmission to an animal model.

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