DISSERTATION

DEVELOPING NEW TOOLS FOR THE STUDY OF VIRUS – VECTOR INTERACTIONS: SINDBIS VIRUS DETERMINANTS OF PRODUCTIVE MIDGUT INFECTION IN *AEDES AEGYPTI* MOSQUITOES

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KEVIN MCDONALD MYLES II ENTITLED "DEVELOPING NEW TOOLS FOR THE STUDY OF VIRUS – VECTOR INTERACTIONS: SINDBIS VIRUS DETERMINANTS OF PRODUCTIVE MIDGUT INFECTION IN *AEDES AEGYPTI* MOSQUITOES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Co-Advisor

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ABSTRACT OF DISSERTATION

DEVELOPING NEW TOOLS FOR THE STUDY OF VIRUS – VECTOR INTERACTIONS: SINDBIS VIRUS DETERMINANTS OF PRODUCTIVE MIDGUT INFECTION IN *AEDES AEGYPTI* MOSQUITOES

Infectious cDNA molecular clones of SIN viruses and expression systems derived from them are powerful tools for *in vivo* studies of virus-vector relationships. Most SIN molecular clones have been developed from the prototype AR339 strain or closely related strains. However, AR339 viruses poorly infect *Aedes aegypti* mosquito midgut epithelial cells following infection by the natural oral route. A Malaysian SIN virus, MRE16, efficiently infects and disseminates from the midgut of *Ae. aegypti* mosquitoes after oral challenge. The observed phenotypic differences in the midgut infection and dissemination potential between the MRE16 SIN and AR339 SIN viruses represent a unique opportunity to investigate viral determinants of vector infectivity and transmissibility. These viruses were developed as a model system for the study arboviral-vector interactions in *Ae. aegypti* mosquitoes.

The midgut infectivity, dissemination, and transmission potential of MRE16 virus and an AR339-derived virus (TR339) were compared in *Ae. aegypti* mosquitoes in order to further characterize observed phenotypic differences. This study determined that the

significantly lower dissemination and transmission potential of TR339 virus resulted from the expression of a classic midgut escape (ME) barrier in this vector species.

To further facilitate studies at the molecular level, the genome of MRE16 virus was sequenced and a full-length MRE16 cDNA infectious clone, designated MRE16ic, was constructed. MRE16ic was identical to the parental virus with regard to its ability to infect and disseminate from the midgut of *Ae. aegypti* mosquitoes. The power of the infectious clone technology was then demonstrated by identifying amino acid residues (E-200 to C-220), in the MRE16 virus E2 envelope glycoprotein, that were an important molecular determinant of midgut infection in *Ae. aegypti* mosquitoes.

From knowledge of MRE16 virus determinants of vector infectivity a double subgenomic SIN (dsSIN) expression system (MRE/3'2J) was constructed in which the structural genes were derived from the MRE16 virus while the remaining genome was derived from laboratory-adapted strains of AR339 SIN virus. The midgut infectivity of MRE/3'2J in *Ae. aegypti* mosquitoes was similar to that of MRE16 virus.

In total, this work provides a basis for additional studies that could lead to better understanding the molecular mechanisms of midgut infection and transmission barriers.

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I would also like to thank my parents for their loving support, and for teaching me that through hard work and persistence anything is possible. To my wife, Niki, thank you for your patience and understanding while I struggled to complete this goal. I also thank my brother for the memories that made me laugh when I was tired and did not want to continue. I also acknowledge my grandparents for their support and inspiration.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Mosquito-borne diseases remain significant threats to global health. Although the latter part of the twentieth century witnessed the emergence of new mosquito-borne diseases, the greater threat as we enter the twenty-first century is from resurgent diseases, once thought to be controlled. The World Health Organization (WHO) estimates that over 275 million cases of malaria each year result in over one million deaths, most of whom are children (WHO, 1999). The morbidity associated with this disease is inestimable. High morbidity and mortality and estimates that more than half of the world's population lives in areas of risk clearly make malaria the most important vector-borne disease (WHO, 1996).

Mosquito-borne viruses constitute another serious public health problem worldwide. Two particularly important examples are dengue (DEN) and yellow fever. An estimated 50-100 million dengue fever (DF) cases, and several hundred thousand cases of the more severe dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) occur annually (Gubler, 2001). DHF results in about a 5% case-fatality rate (Gubler, 2001). WHO estimates that 2.5 billion people, approximately two-fifths of the world's people, are living in areas at risk for DEN transmission (WHO, 1997). Yellow fever virus causes a severe viral hemorrhagic fever (VHF), which despite the existence of a highly effective vaccine, still affects as many as 200,000 persons a year (Monath, 2001). Human populations in the American tropics are especially at risk for yellow fever due to the presence of a large unvaccinated population and the re-infestation of *Aedes* (*Ae.*) *aegypti* (the primary vector of both DEN and yellow fever) that has occurred in the region since the early 1970s (Gubler, 2001; Monath, 2001). Should an outbreak of yellow fever occur

in urban areas of Latin America, disease control would present many difficulties to the local health authorities, not the least of which would be sufficient supply of certified vaccine (Monath, 2001). However, DEN and yellow fever contribute only a part of the total morbidity, mortality, and economic loss associated with all mosquito-borne virus diseases.

The reasons for the resurgence of mosquito-borne diseases are complex and multifaceted. Factors that contribute to resurging disease include changes in public health policy and infrastructure, increasing insecticide resistance, global demographic, economic and societal changes, as well as the genetic variation of pathogens (Gubler, 1998). Not long after the discovery that mosquitoes transmit human disease, it was realized that control of these diseases might be accomplished through the elimination of their arthropod vectors with insecticides. This strategy was used with some success until the 1970s, when it was ultimately proven unsustainable (Gubler, 1998). In many parts of the world complacency led to a lack of financial and political support for insecticide application, in part due to the strategy's own initial success (Gubler, 1998). In many cases, emergency response was emphasized over prevention (Gubler, 1998). This shift in public health policy resulted in the abandonment of many surveillance programs and the demise of training programs for vector-borne disease specialists (Gubler, 1998). Many factors have now complicated the problem, and reinvigoration of vector control strategies alone will probably not be sufficient to reverse the trend.

Resistance of vectors to insecticides (Butler, Maurice, and O'Brien, 1997; Severini et al., 1993), and pathogens to drug treatments, have rendered those solutions less effective (Sibley et al., 2001). The overuse of insecticides contributed to the

development of chemical resistance in the arthropod vector (Gubler, 1998). In addition the use of many chemical insecticides is now considered environmentally unacceptable (United Nations Environment Programme Persistant Organic Pollutants Website; http://www.chem.unep.ch/pops). Population growth has contributed to the overall problem due to changes in agricultural practices (e.g. rice production), the discarding of ready-made breeding containers (consumer product containers, tires, etc.), and the deterioration or lack of public infrastructures in overpopulated urban centers (water and wastewater management, adequate housing, etc.), all of which have resulted in increased mosquito-breeding habitat (Gratz, 1999; Gubler, 1998). New drugs, vaccines, insecticides, and diagnostic tests may not be deployed because of socioeconomic problems in many of the regions affected. Many of the world's poorest citizens are at the highest risk. However, while mosquito-borne diseases most often plague the poorest nations, increased globalization and jet travel now allow both vectors and pathogens to be more widely and rapidly distributed than at any previous time (Gratz, 1999; Gubler, 1998). These factors may be specific to certain pathogens, vectors, the environment, and others may not yet be apparent. The factors listed above are by no means a complete list, but the list illustrates the complexity of the problem.

Mosquito-borne diseases are clearly major threats to human and animal populations throughout much of the world, and will likely present an even larger problem in the future. Solutions for reversing this trend are difficult to identify. Because there is currently no vaccine for either malaria or dengue, two of the most important vector-borne diseases, vector control remains the most viable strategy for reducing disease incidence. However, as mentioned previously traditional methods of vector control may no longer

be sufficient, given the increased complexity of the problem. A multifaceted approach incorporating both traditional and non-traditional strategies may be required in order to achieve sustainable long-term control of mosquito-borne diseases.

The Need to Study Virus-Vector Interactions

Many arthropod-borne viruses are important pathogens of both humans and other animals. These viruses are unique among the animal viruses in that most require a horizontal transmission cycle involving alternating replication in both a vertebrate host and an arthropod vector if they are to persist in nature (Figure 1.1). Birds or small mammals are often reservoirs of infection for mosquitoes, which can then transmit the pathogen to horses, other domestic animals, and humans, which are more frequently tangential hosts. Although domesticated animals and humans usually do not develop a viremia of sufficient intensity and duration to maintain a horizontal transmission cycle, infection may result in disease (typically an asymptomatic or minor non-specific febrile illness but less frequently an encephalitic, arthritic, or hemorrhagic disease).

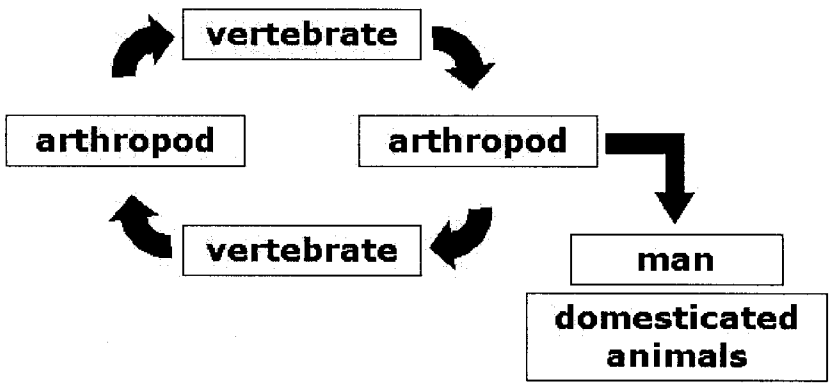

Figure 1.1. A General Transmission Cycle for Arboviruses. The horizontal transmission of most arthropod-borne viruses involves alternating replication in both a vertebrate host and an arthropod vector. Birds or small mammals are often sources of infection for mosquitoes. Humans and domesticated animals are usually tangential or dead end hosts.

In most natural cycles, neither the vertebrate nor the arthropod suffer any adverse effects. The arthropod becomes persistently infected for life, while the vertebrate infection is generally self-limiting. Two notable exceptions are the transmission cycles of dengue and yellow fever viruses, for which humans can serve as definitive hosts for amplifying the viruses during epidemic periods.

While the natural transmission cycles of many arthropod-borne viruses have been determined, our understanding of disease transmission remains largely superficial. Relatively little is known about virus-vector relationships. Studies of virus-vector interactions are essential to understanding the epidemiology of arbovirus diseases. Of particular importance are the molecular processes by which these viruses emerge and are transmitted. The benefits to be gained from such studies are numerous and include: the identification of new methods for assessing the risk associated with arthropod-borne diseases in the field, the identification of new control strategies based on genetic manipulation of vectors, and the design of new transducing systems for gene expression and functional genomics in mosquitoes.

Midgut Infection

The mosquito midgut is largely comprised of a single layer of columnar epithelial cells (Figure 1.2). The midgut has a porous basal lamina surrounding the ablumenal or hemocele side, a microvillar membrane facing the luminal side, and continuous lateral junctions between the cells (Hardy, 1988). Contractile muscle fibers running in both longitudinal and latitudinal directions form a grid-like structure that surrounds the entire organ, and tissues are innervated and permeated by respiratory tracheoles.

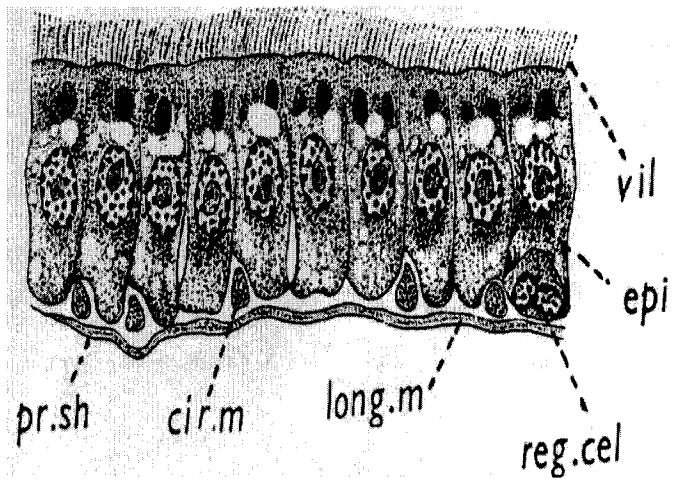

Figure 1.2. The Mosquito Midgut Epithelium. Anatomy of the midgut epithelium. vil=villarium, epi=epithelial cells, reg.cel=regenerative cells, long.m=longitudinal muscles, cir.m=circular muscles, pr.sh=peritoneal sheath (Jobling and Lewis. 1987. *Anatomical Drawings of biting flies*. British Museum of Natural History, London, in association with the Wellcome Trust).

Initial infection of the mosquito midgut by an arbovirus is dependent on the female mosquito ingesting a sufficient concentration of virus to overcome a "threshold of infection" (Chamberlain and Sudia, 1961; Hardy, 1988; McLintock, 1978). The midgut infection (MI) thresholds of numerous arboviruses in many different mosquito species and strains have been determined (Altman, 1963; Barnett, 1956; Collins, Harrison, and Jumper, 1965; Freier and Beier, 1984; Gresser et al., 1958; Hayles, McLintock, and Saunders, 1972; Howard and Wallis, 1974; Hurlbut, 1956; Jupp and McIntosh, 1970a; Jupp and McIntosh, 1970b; Jupp, McIntosh, and Dickinson, 1972; Patrican, DeFoliart, and Yuill, 1985; Scherer et al., 1982; Scherer et al., 1981; Schiefer and Smith, 1974). The concentration of virus necessary to exceed an MI threshold can vary for different viruses within the same mosquito species. Conversely, an MI threshold can also vary for the same virus within different mosquito species and even within different individuals of the same species (Hardy, 1988). One would expect the primary vectors of an arbovirus to possess a low threshold of infection; however, this is not always the case.

Some viruses that are maintained by transovarial transmission or replicate to high titer in their vertebrate hosts (Hardy, 1988). La Crosse (LAC) virus (Bunyaviridae) is efficiently transovarially transmitted by *Ae. triseriatus* mosquitoes (Grimstad, 1988; Miller, Beaty, and Lorenz, 1982; Miller, DeFoliart, and Yuill, 1977; Miller, DeFoliart, and Yuill, 1979; Turell, 1988). LAC analyte can be detected in the ovaries of the mosquito prior to dissemination of virus from the midgut (Chandler, Blair, and Beaty, 1998). This suggests that the sequence of events normally associated with horizontal transmission of virus (Figure 1.2) may not necessarily be required for the maintenance of a vertical transmission cycle. The relationship between the initial efficiency of midgut infection and the downstream sequence of events associated with the movement of an arbovirus in a vector mosquito remains unclear. Nevertheless, that relationship may differ between a vertical transmission cycle and a horizontal transmission cycle.

The molecular mechanisms by which arboviruses gain entry to the cells of the arthropod midgut are unknown but may involve direct fusion of virus to the cells or attachment to specific receptors. While the molecular basis for midgut infection is not known, some evidence does suggest that the environment present in the vector digestive tract (i.e. the presence of proteolytic enzyme activity) may result in the modification of surface proteins and the altering of the surface characteristics of some arboviruses, which may be important for the initiation of infection of vector midgut cells. Protease treatment has a profound effect on the attachment of LAC to *Ae. triseriatus* midgut tissues, suggesting that enzyme treatment might be essential for the attachment and subsequent infection of mosquito midguts in nature (Ludwig et al., 1989). Nonproteolytically processed virus would again be produced following replication of LAC in the midgut but

proteolytic processing is not necessary for infection of secondary target organs. Thus the mechanism by which a virus gains entry to a particular cell type might depend to a large extent on the local environment to which it is subjected (Ludwig et al., 1989).

Ae. aegypti genes conditioning midgut infection and escape for DEN-2 virus have been mapped (Bosio et al., 2000). The results suggested that alleles at two independently segregating loci primarily conditioned midgut infection in the mosquito. Interestingly, one of the loci affecting the expression of this quantitative trait mapped to a location near the early trypsin gene of the mosquito. Early trypsin is a protease found in small amounts within the midgut of female *Ae. aegypti* shortly after ingestion of a blood meal (Noriega et al., 1996b). Ingestion of a blood meal actually induces the synthesis of two trypsins involved in blood protein digestion in the female midgut: early trypsin and late trypsin. Early trypsin is responsible for the first phase of proteolytic digestion of the blood meal, and is also an essential component of the signal transduction system that activates transcription of the more abundant late trypsin, the major endoprotease involved in blood meal digestion (Barillas-Mury, Noriega, and Wells, 1995; Felix et al., 1991; Noriega et al., 1996a). Thus it is possible that proteolytic processing of other arboviruses such as DEN-2 or Sindbis (SIN) viruses may also occur in the midgut environment and be important to initial infection of vector midgut cells.

Once infection of the midgut occurs, the amount of virus replication that occurs in the midgut epithelial cells varies. Some arboviruses propagate rapidly, reaching peak titers within 1 to 3 days post infection. For example Houk et al. (1985) observed peak titers of 10^5 to 10^6 pfu per midgut for western equine encephalitis (WEE) virus (Togaviridae) in *Culex* (*Cx.*) *tarsalis* and *Ae. dorsalis* at 24 to 30 hr after infection.

Thomas (1963) had also reported peak titers for WEE in *Cx. tarsalis* midguts at 24 to 48 hr after ingestion of virus. Eastern equine encephalitis (EEE) virus (Togaviridae) titers were reported to peak in the midguts of *Culiseta* (*Cu.*) *melanura* at 48 to 72 hr by Scott, Hildreth, and Beaty (1984) following peroral infection. LaMotte (1960) found that although the titer of Japanese encephalitis (JE) virus (Flaviviridae) varied within the midguts of *Cx. pipiens*, peak titers were reached in some of the midguts 24 to 48 hr after infection. While these viruses quickly reached maximum titers within the midguts of the mosquito species tested, this pattern cannot be extended to other virus-vector combinations. For example McLean (1955) observed that Murray Valley encephalitis (MVE) virus (Flaviviridae) titers in the midguts of *Cx. annuliostris* mosquitoes failed to increase until 3 days after ingestion, and peak titers were not achieved until 5 days. Titers of California encephalitis (CE) virus (Bunyaviridae) also do not increase in the midguts of *Ae. vexans* until 4 days post infection (Chernesky, 1968). However, *Ae. vexans* is not a natural vector species for this virus.

Immunofluorescent antibody (IFA) analysis has proven to be a valuable technique for visualizing the distribution of arbovirus antigens in the midgut epithelium at various times after peroral infection. Using the technique Doi, Shirasaki, and Sasa (1967) and Doi (1970) observed isolated foci of JE virus infected epithelial cells in the posterior portion of midguts from *Cx. tritaeniorhynchus* and *Cx. pipiens* mosquitoes 4 days after oral infection. At 9 days post-infection JE antigen was detected throughout the entire midgut epithelium. Kuberski (1979) reported similar results using DEN-2 and *Ae. albopictus*. Two days after infection small groups of midgut epithelial cells in the posterior portion of the midgut were positive for virus antigen by IFA. Six days after infection most of the

epithelial cells in the posterior portion of the midgut were infected with DEN-2; however, in contrast to JE in *Cx. tritaeniorhynchus* and *Cx. pipiens* the anterior portion of the midgut was not infected. These results suggest that cell-to-cell spread of virus can occur in the midgut following an initial infection event. Further support for this comes from the fact that a number of arboviruses have been shown capable of infecting and propagating in the mosquito midgut following parenteral infection (Bergold, Suarez, and Munz, 1968; Chernesky, 1968; Liu and Zee, 1976; McLean, 1955; Miles, Pillai, and Maguire, 1973; Ogunbi, 1968; Peers, 1972). Thus the cell-to-cell spread of virus probably occurs by adsorption and penetration at the basolateral-membranes (Hardy, 1988).

Electron microscopy (EM) has also been used to characterize arbovirus infection of the mosquito midgut. Whitfield, Murphy, and Sudia, (1973) employed EM to study infection of *Cx. pipiens* with St Louis encephalitis (SLE) virus (Flaviviridae). Small numbers of SLE virions were observed within an infectious blood meal 1 to 8 hr after ingestion. After 8 hours cytoplasmic vacuoles containing the virions were seen in one cell, possibly suggesting entry by receptor-mediated endocytosis. At 6 days post-infection evidence of virus multiplication was observed in the form of intracytoplasmic masses of convoluted tubules, probably formed by proliferation of endoplasmic reticular membranes, in association with SLE virion maturation and mature virions within the cisternae of the endoplasmic reticulum (ER). In addition, mature virions were also seen between the basal-membrane of the epithelial cells and the basal lamina as well as within the basal lamina, presumably disseminating to the hemocele. Whitfield, Murphy, and Sudia (1973) reported that only one in five midgut epithelial cells became infected with SLE virus during this study. Murphy et al. (1975) conducted a similar study using EEE

and *Ae. triseriatus* and reported similar results with the exception that EEE infected approximately 1 in 3 midgut epithelial cells of this vector species.

The pore size in the basal lamina surrounding the midgut of *Ae. dorsalis* has been determined to be only 10 nm in diameter (Houk, Chiles, and Hardy, 1980), and particles larger than 5 to 8 nm in diameter could not permeate the basal lamina of *Cx. tarsalis* midguts from the hemocele side (Houk, Hardy, and Chiles, 1981). Therefore, it is currently unknown how arboviral particles of 50 to 60 nm in diameter can routinely pass through the basal lamina.

Secondary Amplification

Following dissemination from the infected midgut, arboviruses may infect and multiply in hemocele bathed tissues and organs of the mosquito. A range of organs in which secondary amplification can occur has been determined from studies employing IFA (Beaty and Thompson, 1978; Doi, 1970; Doi, Shirasaki, and Sasa, 1967; Kuberski, 1979; Scott, Hildreth, and Beaty, 1984), EM (Bergold, Suarez, and Munz, 1968; Sriurairatna and Bhamarapravati, 1977; Whitfield, Murphy, and Sudia, 1973), and the determination of viral titers in dissected tissues and organs (Lamotte, 1960). These include the salivary glands, other tissues of the alimentary tract, Malpighian tubules, muscle, neural tissues, reproductive organs, pericardium, and fat body. These studies provide substantial evidence that arboviruses can multiply in many tissues and organs of the mosquito other than the midgut and salivary glands. However, what is not clear from these studies is the extent to which arboviruses differ in their tropisms for the tissues and organs accessible from the hemocele of their mosquito vector species. Furthermore, while

it is evident that after oral infection virus must multiply in the midgut before infecting the salivary glands, it is currently unknown if multiplication in other tissues is also required prior to infection of the salivary glands. It appears that the salivary glands can become infected directly from viremic hemolymph (Beaty and Thompson, 1978; Chamberlain and Sudia, 1961; McLintock, 1978; Murphy et al., 1975). This led Hardy et al. (1983) to suggest that virus escaping from the midgut might directly infect the salivary glands if sufficiently high titers are achieved in the hemolymph, and that secondary amplification might only be necessary when small amounts of virus escape the midgut. LaMotte (1960) reported an increase in JE virus titers in the ganglia, anterior intestine, diverticula, ovaries, and hemolymph of *Cx. pipiens* at approximately the same time that the virus titer in the salivary glands increased (2 to 3 days after ingestion of virus). Similar phenomena were also reported by Scott, Hildreth, and Beaty (1984) and Kubersky (1979) for EEE virus in *Cu. melanura* and DEN-2 virus in *Ae. albopictus,* respectively. However, some have questioned the results of such studies, as it is difficult to avoid contaminating tissues with viremic hemolymph (Hardy et al., 1983). Results reported by Doi, Shirasaki, and Sasa (1967) and Doi (1970) using IFA analysis have indicated amplification of JE occurs in the fat body prior to infection of salivary glands in *Cx. tritaeniorhynchus* and *Cx. pipiens*. Thus additional studies will be required before a generalized picture of the sequential infection of hemocele-associated tissues and organs can emerge.

Salivary Gland Infection

The female mosquito possesses a pair of salivary glands each consisting of two lateral cylindrical lobes, and one median cylindrical lobe (Lanzen and Wright, 1971;

Wright, 1969). Distal and proximal portions of each lobe are typically referred to as the acinus and neck, respectively. Each lobe consists primarily of a single layer of cuboidal epithelial cells and is surrounded by a basal lamina (Hardy, 1988). Each lobe contains a chitinous central duct that converges on the salivary gland duct connecting to the hypopharynx (Hardy, 1988).

Evidence primarily supports the hemolymph as the primary source of infection for the salivary glands (Doi, 1970; Doi, Shirasaki, and Sasa, 1967; Lamotte, 1960; Scott, Hildreth, and Beaty, 1984), although there is some evidence to indicate that Whataroa (WHA) virus (Togaviridae) may be able to infect by a neural pathway in *Ae. australis* (Miles, Pillai, and Maguire, 1973) [Maguire, unpublished data referenced in Murphy et al., 1975]. However, other pathways of infection were not specifically ruled out in these studies and it remains to be determined if a neural pathway of infection is common to other viruses as well.

Chamberlain and Sudia (1961) have postulated that an infection threshold, similar to that described for midgut infection, might also exist for salivary gland infection but this has not been proven. The molecular mechanisms by which arboviruses infect the cells of the salivary glands are unknown but virus must again pass through a basal lamina in order to gain access to the acinar cell plasma membrane. In general only the lateral lobes of the salivary glands are infected, with relatively few cells in each lobe becoming infected (Hardy et al., 1983; Murphy et al., 1975; Whitfield, Murphy, and Sudia, 1973). However, the tropisms displayed by some arboviruses for different lobes of the salivary glands is of interest as the medial and lateral lobes apparently differ in their secretions (Orr, Hudson, and West, 1961).

Gaidamovich et al. (1973) examined by IFA the infection of salivary glands by SIN virus (Togaviridae) and Venezuelan equine encephalitis (VEE) virus (Togaviridae) in *Ae. aegypti.* Virus antigen was first observed in the cells of the lateral acinus and subsequently spread to the cells of the lateral neck, but the cells of the median lobe apparently never became infected. Similar results were also reported for JE and *Cx. tritaeniorhynchus* using both EM and IFA (Takahashi and Suzuki, 1979). Gubler and Rosen (1976a) also reported similar results using DEN-2 virus and *Ae. albopictus*, but did observe infection of the median lobe following longer incubation periods. A correlation between transmission of virus and site of replication within the lateral lobes was not observed in this study. However, a second more recent IFA study by Takahashi (1982) did report observing JE infection of the median lobes in strains of *Cx. tritaeniorhynchus* considered to be efficient transmitters.

In contrast to some plant viruses that are merely concentrated by the salivary glands of their insect vectors, arboviruses actually multiply in the salivary glands of the mosquito. Definitive evidence of this came from a number of ultrastructural studies using EM, which observed the maturation of virus particles within infected cells (Sriurairatna and Bhamarapravati, 1977; Takahashi and Suzuki, 1979; Whitfield, Murphy, and Sudia, 1971; Whitfield, Murphy, and Sudia, 1973). The estimated amount of virus transmitted by a mosquito during the feeding process ranges from a few infectious units to as high as 100,000 infectious units depending the method of estimation used and length of extrinsic incubation (EI) period (Chamberlain, Kissling, and Sikes, 1954; Gubler and Rosen, 1976a; Hurlbut, 1966; Mellink, 1982). In some cases virion concentrations in the salivary glands are so great that crystalline aggregates can be seen (Hardy et al., 1983).

Extrinsic Incubation

The time interval from ingestion of virus by a vector to transmission capability is known as the extrinsic incubation period (Hardy, 1988; Turell, 1988). However, a more epidemiologically relevant definition would be based on a 50% transmission endpoint (Hardy, 1988). Nevertheless, EI periods of arboviruses are quite variable, and numerous factors, both genetic and nongenetic, can affect arbovirus infection, multiplication, and dissemination within the mosquito (Hardy et al., 1983). Notable among these are environmental temperature, infecting dose, the vector competence of the mosquito for a particular virus, as well as the genetics of the virus.

Barriers

After ingestion of an infectious blood meal the movement of an arbovirus through the vector mosquito occurs in a step-wise fashion (Figure 1.3). However, an arbovirus must overcome several potential barriers to infection and dissemination to be transmitted by a mosquito vector (Figure 1.3).

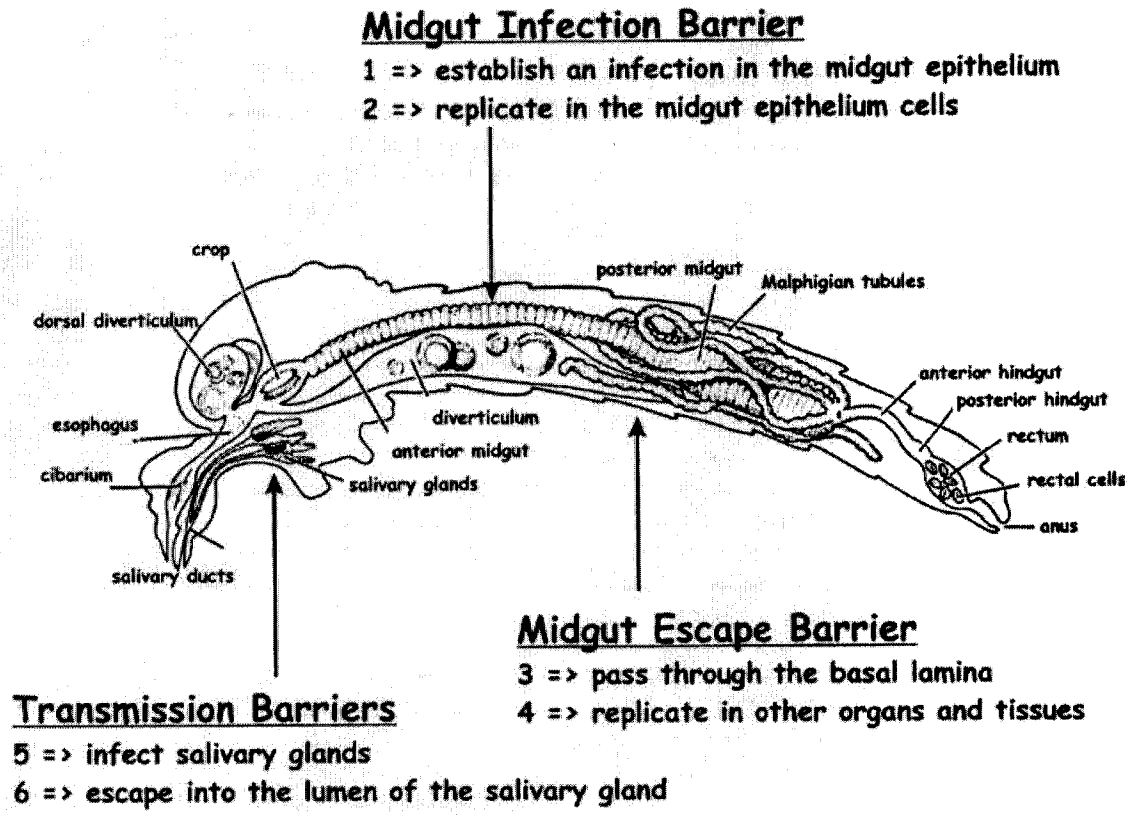

Figure 1.3. Potential Barriers to the Sequential Movement of Arboviruses. A generalized diagram of the sequential movement of an arbovirus within the vector mosquito, and potential barriers associated with that movement. An arbovirus must overcome each of these barriers before being transmitted by a female mosquito (Black et al., 2002).

Dissemination of arboviruses in competent mosquito vectors following *per os* (or oral) infection begins with an initial, unidirectional, productive infection of the midgut epithelium. This requirement is restrictive to non-compatible or low titer viruses and represents a midgut infection (MI) barrier (Hardy, 1988; Woodring, Higgs, and Beaty, 1996). Viruses that fail to overcome this barrier will not disseminate to other mosquito organs, such as the salivary glands, thus interrupting virus transmission and the virus maintenance cycle. The molecular nature of the MI barrier is currently unknown. However, there is evidence to suggest that it may be associated with at least two mechanisms genetically influenced by both virus and vector: specific mosquito-pathogen

interactions that determine susceptibility of midgut epithelial cells to infection and modulation of virus multiplication in cells that become infected [Reviewed in Hardy, 1988].

Infection and replication in the midgut is not the only requirement for dissemination and subsequent transmission of an arbovirus by a mosquito; other barriers are also present. Kramer et al. (1981) first demonstrated that the inability of infected *Cx. tarsalis* mosquitoes to transmit western equine encephalitis (WEE) virus was associated with a midgut escape (ME) barrier. In female mosquitoes exhibiting a ME barrier, virus infected the midgut but was unable to disseminate to other organs and remained sequestered in the infected midgut cells. Kramer et al. (1981) found virus concentrations in the midguts of females exhibiting an ME barrier to be lower than those found in the midguts of fully competent females. This suggests that the ME barrier may also be associated with a genetically controlled mechanism of modulating replication of the arbovirus in the midgut. The ME barrier was found to be dose dependent and occurred only when low doses of virus were ingested. A ME barrier was also demonstrated as the primary determinant of transmission potential in *Cx. taeniopis* mosquitoes for an epizootic strain of VEE virus (Togaviridae) (Weaver et al., 1984) and in *Cx. pipiens* for Rift Valley fever (RVF) virus (*Bunyaviridae*) (Turell, Gargan, and Bailey, 1984). Neither of these was dose dependent.

Other barriers to transmission of an arbovirus have been postulated. For example, a salivary gland infection (SGI) barrier has been demonstrated for WEE in *Cx. tarsalis* mosquitoes (Kramer et al., 1981). A salivary gland escape (SGE) barrier has also been definitively demonstrated for several arbovirus-vector combinations (Beaty et al., 1981;

Grimstad, Paulson, and Craig, 1985; Jupp, 1985; Takahashi, 1982). Jupp and Philips (1998) observed arrays of SIN SAAR 86 virus particles associated with endoplasmic reticulum in salivary gland cells of *Ae. aegypti* but not in surrounding vacuoles. However, transmission potential was not tested.

Sindbis Viruses as a Model System

A SIN virus model system for the study of arbovirus-vector interactions has great potential. SIN virus is one of the most well studied arboviruses. The genome structure and replication strategy of the virus have been determined and molecular clones of the virus can be constructed that are easily manipulated. In addition, SIN virus expression systems have been developed from some of the available molecular clones for use in mosquitoes. In addition, the biosafety level required to work with the virus is lower (BSL2) than that for other positive-strand arboviruses, such as certain flaviviruses and other alphaviruses. This continues to be a benefit in the study of virus-vector interactions because work with arboviruses becomes more dangerous when vectors are used. *Ae. aegypti* is a good vector model for these types of studies. It is a medically important vector species, the molecular biology of the vector is one of the best understood of any vector species, and it is easy to rear and manipulate in the laboratory.

Host Range

SIN is the prototype member of the genus *Alphavirus* in the family *Togaviridae*. Members of the *Alphavirus* genus have been grouped together on the basis of serological cross-reactivity (Calisher and Karabatsos, 1988; Porterfield, 1980). SIN is arthropod-

borne (as are all alphaviruses), and mosquitoes are the primary or exclusive vectors (Calisher and Karabatsos, 1988; Niklasson, 1988).

The prototype SIN strain AR339 was originally isolated from a pool of *Cx. pipiens* and *Cx. univittatus* mosquitoes collected from the Sindbis health district of Egypt in 1952 (Taylor et al., 1955). However, subsequent isolations have been made from *Culex, Culiseta, Aedes, Anopheles,* and *Mansonia* mosquito species (Chamberlain, 1980; Karabatsos, 1985). SIN also has a broad vertebrate host range, and numerous isolates have been made from a wide range of avian and mammalian species as well (Karabatsos, 1985).

Not surprisingly given its host range, SIN has a very wide geographic distribution, with isolates having been made in Europe, Africa, Asia (including India and the Philippines), and Australia (Strauss and Strauss, 1994) covering four of the world's six zoogeographic regions, Paleoarctic, Oriental, Australian, and Ethiopian (Calisher and Karabatsos, 1988). SIN-like viruses have also been isolated from New Zealand (Whataroa) and South America (Aura) (Strauss and Strauss, 1994). Birds, which are the primary vertebrate hosts for SIN viruses, are postulated to have distributed these viruses widely. However, once introduced into a new environment the viruses typically evolve into an endemic strain that remains in a localized area. For example, despite near worldwide distribution, SIN-like viruses tend to genetically segregate by location rather than year of isolation (Shirako et al., 1991). On the basis of RNA-RNA hybridizations and RNase T1 mapping, SIN viruses segregate into one of two larger genetic groupings, Paleoarctic/Ethiopian and Oriental/Australian (Olson and Trent, 1985; Rentier-Delrue

and Young, 1980). Thus far limited nucleotide sequence analysis has confirmed these earlier genetic groupings (Sammels et al., 1999; Shirako et al., 1991).

Molecular Biology

The Sindbis genome consists of a positive-sense, non-segmented, single-stranded RNA molecule of about 11.7 kb (Strauss and Strauss, 1994). The viral RNA has a 5′-terminal cap and a 3′-terminal poly (A) tract. The 5′ two thirds of the genome encodes the nonstructural or replicase proteins. This genome region is translated to form polyproteins (P123 or P1234) that contain the viral nonstructural proteins (nsP1- nsP3 or nsP1- nsP4). Post-translational processing of these polyproteins produces a number of cleavage intermediates that form the viral replicase complexes which synthesize positive or negative sense RNAs (Strauss and Strauss, 1994). A subgenomic mRNA (26S RNA) also is transcribed from an internal subgenomic promoter present in the full-length negative sense RNA. The 26S RNA is translated to form a polyprotein that is co-and post-translationally cleaved to form capsid (C), two envelope glycoproteins (E1 and E2), and two small peptides, E3 and 6K. The negative-sense strand also functions as the template for production of new genomic RNA (49S RNA). Four conserved sequence elements (CSEs) have been identified in the alphavirus genome (Strauss and Strauss, 1994). Mutations in CSEs have different effects on virus growth in different cell types, suggesting they play a role in host specificity, possibly through the binding of host proteins (Kuhn et al., 1992).

Virion Structure

The SIN virion, including glycoproteins, is 69 nm in total diameter (Harrison et al., 1971; Paredes et al., 1993). Two hundred and forty glycoprotein heterodimers

assemble into 80 spikes on the surface of the virus. Each spike consists of three heterodimers twisted around one another in an anticlockwise direction comprising a stalk with a tripartite head (Strauss and Strauss, 1994). Heterodimers are composed of the two glycoproteins of the virus, E1 and E2, both of which are glycosylated (Harrison, 1986; Rice and Strauss, 1982). Evidence suggests that E1-E1 interactions maintain the homotrimer conformation of the spike (Anthony and Brown, 1991). The finding that fusion of the viral envelope and endosomal membrane during viral infection results in disassembly of the E1-E2 heterodimer, yielding E1 trimers and E2 monomers, lends additional support to the hypothesis (Wahlberg et al., 1992). The virion envelope is derived upon budding from the plasma membrane of the infected host cell and has a thickness of 4.8 nm (Strauss and Strauss, 1994). The virus glycoproteins are anchored in the membrane by conventional membrane-spanning domains in their C-terminal regions (Gahmberg, Utermann, and Simons, 1972; Garoff and Simons, 1974; Harrison, 1986; Rice et al., 1982; Schlesinger and Schlesinger, 1986). Glycoprotein heterodimers emerging from the lipid bilayer envelope interact with the virus' capsid protein subunits in a one to one ratio (Strauss and Strauss, 1994). The nucleocapsid of the virus is composed of 240 copies of a single virus-encoded capsid protein arranged in an icosahedral lattice with T=4 symmetry (34-41 nm in diameter) (Coombs and Brown, 1987; Harrison, 1986; Harrison et al., 1992; Paredes, Simon, and Brown, 1992; Stubbs et al., 1991). The SIN nucleocapsid contains a single-stranded, positive-sense RNA genome of approximately 11.7 kb (Strauss and Strauss, 1994).

Cellular Virogenesis

SIN entry into a susceptible cell is presumably initiated by specific interaction between the viral glycoprotein spike and a cellular receptor. The maintenance of SIN viruses in nature involves a transmission cycle that requires alternating replication in both an invertebrate (mosquitoes) and vertebrate host (numerous species of birds and mammals). In addition, within each of these hosts the virus replicates in a wide variety of tissues and cell types. How the virus achieves such a broad host range is unknown but could conceivably result in one of two ways: (i) the virus could use a single highly conserved receptor common to many different cell types over a diverse range of species; or (ii) the virus could use more than one receptor to facilitate entry into different cell types. Nevertheless, many studies have now provided enough evidence to indicate that attachment to the cell surface is likely mediated through virus interaction with a host protein.

Studies involving anti-receptor antibodies (Ludwig, Kondig, and Smith, 1996; Wang et al., 1992), anti-idiotypic antibodies (Ubol and Griffin, 1991; Wang et al., 1991), chemical crosslinking (Maassen and Terhorst, 1981), enzymatic inactivation of cellular receptors (Smith and Tignor, 1980; Ubol and Griffin, 1991), and co-purification of virus receptor complexes (Duda and Berencsi, 1980) have all provided support for this hypothesis. Although a cellular receptor has not been definitively identified for any alphavirus (Helenius et al., 1978; Oldstone et al., 1980; Strauss et al., 1994), a number of putative receptors of different molecular weights have been identified on different cell types (Duda and Berencsi, 1980; Ludwig, Kondig, and Smith, 1996; Maassen and Terhorst, 1981; Ubol and Griffin, 1991; Wang et al., 1992; Wang et al., 1991). This has

led some researchers to suggest that multiple receptors may be used, even in the same cell type (Smith and Tignor, 1980; Ubol and Griffin, 1991; Wang et al., 1992). Wang et al. (1992) identified an antireceptor antibody that effectively blocked SIN binding to BHK cells. The antibody bound to a cell surface protein referred to as the high-affinity laminin receptor. However, this protein is somewhat controversial as it was originally isolated as a 67-kDa protein with a high affinity for laminin. However, what has been identified as the open reading frame (ORF) encodes a protein of only 295 amino acids [Reviewed in Strauss and Strauss, 1994]. This has led to speculation that the 295 residue protein encoded in the ORF might a precursor of the 67-kDa protein (Castronovo et al., 1991; Castronovo, Taraboletti, and Sobel, 1991) or that the ORF has been incorrectly identified as that of the 67-kDa protein (Grosso, Park, and Mecham, 1991). Ludwig, Kondig, and Smith (1996) identified a 32-kDa protein expressed on the surface of mosquito cells that appears to be a receptor for VEE virus. The protein also binds laminin with high affinity. Although the relationship between the 67-kDa protein identified by Wang et al. (1992), the protein encoded by the 295-residue ORF, and the 32-kDa protein remains unclear, the 32- and 67-kDa proteins do appear to share sequence identity (Ludwig, Kondig, and Smith, 1996). The laminin receptor, or at least the 295 residue ORF, is highly conserved across a diverse range of vertebrate species and may be conserved in all multi-cellular animals (Strauss and Strauss, 1994; Wang et al., 1992). Thus, the wide host range of alphaviruses may result at least in part from the ability of the virus to use this conserved receptor.

Charge might also be important. The treatment of SIN virus, uniformly distributed and attached to fixed cell surfaces, with a buffer of high-ionic-strength results in the

elution of most of the bound virus particles (Pierce, Strauss, and Strauss, 1974). Several studies have suggested that sulfated polyanions, such as cell surface glycosaminoglycans (GAGs), in particular, are involved. Symington and Schlesinger (1978) isolated a negatively charged SIN variant able to bind to and infect mouse plasmocytoma cells better than the wild-type virus. Pretreatment of the mouse plasmacytoma cells with heparin resulted in a large increase in the ability of the wild-type virus to bind. Mastromarino et al. (1991) demonstrated that a sulfated molecule may be a component of the receptor site. They were able to at least partially compete SIN infectivity with several sulfated polyanions, and were able to diminish infectivity using a heparinase to digest heparin sulfate (HS) on the cell surface. Polyionic compounds found to interfere with enveloped viruses from other families have also been found to inhibit the infectivity of alphaviruses, including SIN (Schols et al., 1990). However, Klimstra, Ryman, and Johnston (1998) suggested that HS may play little or no role in the infection of cells by natural virus isolates *in vivo*. The study found that a SIN consensus sequence virus, devoid of cell-culture adaptive mutations and thus representative of the original natural isolate, bound to baby hamster kidney (BHK) cells very poorly and infected the cells predominantly through an HS-independent mechanism. The attachment of cell culture-adapted viruses, possessing mutations in the E2 gene, was found to occur through an HS-dependent mechanism. Upon passage in cell culture, mutations (in some cases single amino acid changes) conferring HS attachment were found to occur rapidly in the SIN consensus sequence virus and may represent a common adaptive mechanism among alphaviruses. Interestingly, the consensus sequence virus proved to be more virulent in neonatal mice when compared with two cell-culture adapted viruses. Each of the three

viruses exhibited identical tissue tropisms in the neonatal mice, differing by only rate and extent of spread. The authors offered possible explanations for this by proposing that HS attachment might result in the binding of virus to unproductive receptor structures *in vivo* (Klimstra, Ryman, and Johnston, 1998). In addition, an inverse relationship between HS attachment efficiency and virion production was observed in individual infected BHK cells, suggesting HS attachment may interfere with particle release. The results reported by Klimstra, Ryman, and Johnston (1998) may also have implications for studies that have identified putative receptors of different molecular weights for SIN on different cell types. HS can exhibit structural heterogeneity between cell types by being presented through attachment to different core proteins or in different protein-proteoglycan-GAG complexes. This raises the possibility that previously reported virus interactions with different cellular core proteins may have been mediated through the expression of HS and GAG chains.

The E2 glycoprotein contains important epitopes for host tropism, receptor recognition, virus neutralization, and virulence (Davis et al., 1987; Dubuisson et al., 1997; Dubuisson and Rice, 1993; Gardner et al., 2000; Klimstra, Ryman, and Johnston, 1998; Levine et al., 1996; Lustig et al., 1988; Pence, Davis, and Johnston, 1990; Russell, Dalrymple, and Johnston, 1989; Schoepp and Johnston, 1993a; Schoepp and Johnston, 1993b; Strauss et al., 1991; Tucker and Griffin, 1991; Tucker et al., 1993; Ubol and Griffin, 1991; Wang et al., 1991; Wang and Strauss, 1991). A domain comprising residues 170-220 of the SIN E2 glycoprotein appears to be particularly important for cell receptor binding (Strauss and Strauss, 1994). Even single amino acid changes in this domain can have a dramatic effect on alphavirus infectivity and/or virulence (Kerr, Weir,

and Dalgarno, 1993; Tucker and Griffin, 1991; Woodward et al., 1991). However, Omar and Koblet (1988) engineered Semliki forest (SF) virus lacking E2, and although the resulting virus lost 90% of its infectivity, it remained capable of binding and penetrating cells. These results suggest that E2 may not be solely responsible for viral interaction with cellular receptors, and that E1 may also be involved. The E1 glycoprotein has also been shown to be important in fusion of the virus envelope with host intracellular membranes (Garoff et al., 1980; Kondor-Koch, Burke, and Garoff, 1983; Kondor-Koch et al., 1982; Rice and Strauss, 1981).

Following binding to a cellular receptor, virus enters the cell by an endocytic pathway. After entry of the alphavirus particle in the clathrin-coated vesicle, it is transferred to an endosome where the low pH exposes the fusion domain of E1. Fusion of the virus envelope with the endosomal membrane results in the release of the capsid into the cytoplasm.

Disassembly of the nucleocapsid occurs in the cytoplasm, making the viral RNA available for translation by cellular ribosomes. A putative ribosomal binding site has been identified on the capsid protein and may be involved in uncoating (Singh and Helenius, 1992; Wengler, 1984; Wengler and Wurkner, 1992). Replicated viral genomic RNA and capsid subunits self-assemble in the cytoplasm to form the nucleocapsid. The nucleocapsid is then transported to the plasma membrane, where it acquires an envelope from the host cell by budding through the lipid bilayer at locations where viral glycoproteins have accumulated. The envelope proteins are processed through the endoplasmic reticulum (ER) and golgi apparatus of the cell. The glycosylated envelope proteins are then transported to the surface of the cell.

Alphavirus Interactions within the Mosquito Vector

The virogenesis of many alphaviruses within the mosquito host has been delineated by numerous studies (Bowers, Abell, and Brown, 1995; Houk et al., 1985; Jackson, Bowen, and Downe, 1993; Scott, Hildreth, and Beaty, 1984; Scott, Lorenz, and Weaver, 1990; Weaver, 1986; Weaver, Lorenz, and Scott, 1992; Weaver, Lorenz, and Scott, 1993; Weaver, Scott, and Lorenz, 1990; Weaver et al., 1988; Weaver et al., 1991). Eastern equine encephalitis (EEE) virus has been reported to preferentially infect the posterior midgut epithelium of *Cu. melanura* following oral infection (Scott, Hildreth, and Beaty, 1984). Within 3 days post-infection virus had disseminated from the midgut and infected most other mosquito tissues. Within several days after infection, alphaviruses typically disseminate from midguts to infect fat body, nerves, muscle tissue, hindgut, oviducts, and many other tissues. Salivary glands typically become infected 2-3 days after infection. Weaver (1986) employed electron microscopy (EM) to visualize VEE infection and maturation at the cellular level in the mosquito *Cx. taeniopus* following oral infection. Virions were observed budding from the basal membranes of midgut epithelial cells, and on both sides of the basal lamina 3-4 hours post-infection. By 2 days post-infection, VEE virus could be detected in the hindgut and abdominal nerve ganglia. Dissemination from the midgut to other organs, such as salivary glands and neural tissues, also occurred from 2 to 4 days post-infection.

A number of mosquito species can be infected with SIN virus if midgut barriers are circumvented by injection of virus into the thorax. The spatial and temporal progression of SIN infection following intrathoracic inoculation has been described in the mosquito *Ae. albopictus* by Bowers, Abell, and Brown (1995). SIN antigen was detected

in fat bodies, hemolymph, tracheoles, gut visceral muscles, salivary glands, thoracic muscle, and neural tissues during the acute phase of the infection (48 to 72 hours post-infection). The onset of a persistent phase of the infection (approximately 72 hours post-infection) was characterized by clearance of antigen from many of the previously infected tissues. However, antigen continued to be detected in the fat body, hindgut visceral muscles, and tracheoblasts, purportedly for the life of the mosquito. Notably, viral antigen was never detected in the midgut epithelial cells, although the gut-associated musculature and tracheoles were infected during the acute phase of infection.

Manipulation of the Genome

The genome structure and replication strategy of SIN virus is amenable to the construction of infectious cDNA clones. The genomes of many RNA viruses can be cloned. The cDNA of a viral genome can be easily manipulated by the insertion or deletion of sequence, or through site-specific mutation. It is possible to transcribe RNA *in vitro* from the cDNA (modified or unmodified) through the use of a DNA-dependent RNA polymerase (e.g., bacteriophage SP6 polymerase) if a suitable promoter has been inserted upstream of the genomic sequence. The viral RNA produced can then be transfected into a permissive cell type allowing the virus to complete its replication cycle. Other than modifications introduced at the cDNA level, the virus produced from the *in vitro* transcripts will be identical to the source virus used and will produce a strain-specific phenotype reflecting the source RNA used for cDNA synthesis. Rice et al. (1987) first constructed a full-length cDNA SIN clone. Expression systems have been developed from SIN-based cDNA infectious clones exploiting the capacity of the SIN

genome to accept additional sequences without compromising the ability of the virus to replicate.

Three types of expression systems have been developed from the SIN genome, and all utilize the viral subgenomic promoter to achieve high-level expression of an inserted sequence. The first expression system to be developed was based on defective interfering (DI) genomes. The genome contains the essential *cis*-acting sequences required for replication and may or may not also contain one or more promoters for expression of a foreign sequence. Because the genome is defective, it cannot replicate, be packaged, or express the foreign sequence. Therefore, wild-type helper virus must be present in the same cell to provide these functions. The helper virus serves as a source of the nonstructural proteins required for replication and expression and the structural proteins required for packaging. Although expression of a foreign gene can be achieved, packaging of the DI RNA is very inefficient (Levis, Huang, and Schlesinger, 1987).

A second type of expression system utilizes a self-replicating vector. In this case the viral genome is not intact, usually because a heterologous sequence has been inserted downstream of the subgenomic promoter in place of the virus' structural gene sequences (Xiong et al., 1989). However, because the nonstructural genes are present the virus is self-sufficient for RNA replication and gene expression, but a helper genome is still required for packaging progeny RNA into infectious particles. The structural proteins are often provided in trans by co-transfection with a defective helper RNA, which cannot itself be packaged (Bredenbeek et al., 1993). Without co-transfection of the helper RNA, these types of particles will be infectious but self-limited (unable to initiate a second round of infection). An exception to this occurs if the defective helper RNA possesses a

packaging signal derived from the full viral genome. This creates a particle with a bipartite genome that can be amplified though passage (Bredenbeek et al., 1993; Geigenmuller-Gnirke et al., 1991). An advantage inherent to this type of vector is that by removing the structural genes of the virus, the cloning capacity is increased to greater than 6 kb (Huang and Summers, 1991).

A third type of expression system uses a self-replicating, self-packaging double promoter vector. In this type of expression vector the subgenomic promoter of the virus has been duplicated, with one expressing the structural genes and one expressing a heterologous sequence cloned downstream (Hahn et al., 1992; Raju and Huang, 1991). Because the vector contains both nonstructural and structural gene sequences, it is completely self-sufficient for replication, expression, and packaging. The size of the foreign sequence that can be inserted into this type of vector is limited to between 2-3 kb in length by packaging constraints.

Transduction

SIN virus-based expression systems have proven successful in transiently transducing mosquito cells both *in vivo* and *in vitro* (Carlson et al., 1995; Higgs et al., 1995; Higgs, Powers, and Olson, 1993; Kamrud et al., 1995; Olson, Carlson, and Beaty, 1992; Olson et al., 1994; Rayms-Keller et al., 1995). Thus they offer a viable alternative to germ-line transformation methods for introducing and evaluating the effects of heterologous genetic material in the laboratory. SIN-based expression systems possess a number of desirable properties that make them well suited for this purpose. Of particular importance is the ability to establish a persistent, noncytocidal infection in many different tissues in a wide variety of mosquito species. The ability of the virus to replicate

efficiently in a wide range of cell types makes it possible to evaluate the effects of exogenous sequences introduced into multiple tissues of the mosquito over an extended period of time.

The value of SIN expression systems for *in vivo* studies in mosquito vectors has now been clearly demonstrated using TE/3´2J double subgenomic SIN (dsSIN) viruses (Hahn et al., 1992).

Expressing reporter genes

TE/3´2J has been successfully employed to express reporter genes such as chloramphenicol acetyltransferase (CAT) and GFP in salivary glands, neural ganglia, ommatidia, and midgut-associated nerves, muscles and tracheoles of adult *Ae. aegypti, Ae. albopictus, Ae. triseriatus, Cx. pipiens,* and *An. gambiae* mosquitoes following intrathoracic injection (Higgs et al., 1995; Higgs et al., 1996; Kamrud et al., 1997; Olson, Beaty, and Higgs, 1998; Olson et al., 1994). The TE/3'2J expression system has also been used effectively to express GFP in larval and pupal tissues following parenteral infection (Higgs et al., 1996; Olson, Beaty, and Higgs, 1998).

Evaluating anti-pathogen effector molecules

Double subgenomic SIN viruses have also been used to successfully evaluate various anti-pathogen strategies including the expression of antisense RNAs and single chain antibodies in the mosquito. TE/3´2J was used to express antisense viral RNA sequences effectively blocking LaCrosse virus infection in *Ae. triseriatus* (Powers et al., 1996), and the transmission of dengue type 2 and yellow fever viruses by *Ae. aegypti* mosquitoes (Higgs et al., 1998; Olson et al., 1996). The potential of this system for evaluating anti-parasite genes was also recently demonstrated, when a TE/3´2J virus was

used to express a recombinant single-chain antibody, which effectively blocked salivary gland infection by *Plasmodium gallinaceum* sporozoites in *Ae. aegypti* mosquitoes (de Lara Capurro et al., 2000).

Down regulation of endogenous genes

The transcription of effector RNAs from double subgenomic SIN viruses provides a rapid and reproducible method for inhibiting the expression of specific genes in the mosquito. In this way dsSIN expression systems are furthering our understanding of gene function and also may lead to the identification of genes that determine vector competence or are involved in pathogen transmission. Proof of concept was first provided when a TE/3´2J virus was used to transcribe RNA complementary to the 5´ end of luciferase (LUC) mRNA in mosquitoes germ-line transformed to express LUC from an apyrase promoter (Johnson et al., 1999). LUC expression was reduced by 90% in mosquitoes infected with the anti-LUC virus when compared with control mosquitoes (Johnson et al., 1999). In a later study a dsSIN virus was used to express antisense RNA targeted to a highly conserved region of a phenol oxidase (PO) gene in *Armigeres subalbatus* mosquitoes, resulting in a significant reduction in hemolymph PO activity (Shiao et al., 2001). When mosquitoes transduced with SIN viruses expressing the antisense RNA were challenged with filarial parasites, the normal melanization response was almost completely inhibited. The results provide the first direct evidence that PO is a component of parasitic melanization pathways. These studies demonstrated the power SIN expression systems possess for analysis of gene function within the mosquito. The genome sequence of the major malaria vector *An. gambiae* has recently been completed, and undoubtedly the genomes of other major vectors will soon follow. These important

achievements will now require tools to facilitate functional genomic analysis in the mosquito.

It is important to point out that in each of these studies the TE/3´2J constructs were introduced into the mosquito through intrathoracic inoculation. This was necessary because these SIN expression systems poorly infected the mosquito midgut epithelium, and efficient use of the constructs mandated this route of infection. For many mosquito-borne pathogens, the midgut is the first organ system encountered following ingestion of an infectious blood meal by a potential vector. Infection and escape from the midgut is essential if transmission is to occur, and thus the midgut is a primary determinant of vector competence (Hardy, 1988; Turell, 1988). Therefore, a successful strategy for abrogating mosquito vector competence may require the expression and characterization of exogenous genetic sequences in the mosquito midgut.

Summary and Goals

Although somewhat successful, previous strategies for the control of arthropod-borne virus diseases have thus far proven unsustainable or unsuccessful. Currently arthropod-borne viruses are a resurging threat to worldwide public health. Changes in public health policy and infrastructure, increasing insecticide resistance, global demographic, economic and societal changes, as well as the genetic variation of pathogens (Gubler, 1998) have all contributed to this resurgence. Relatively little is known about the transmission of these viruses as few studies of virus-vector interactions have been done. However, additional research in this area will be required if the ecology and epidemiology of arthropod-borne viruses are to be understood. Of particular

importance are the molecular processes by which these viruses emerge and are transmitted. Information gained from such studies will be invaluable in the development of new control strategies and possibly the prediction of conditions favorable to new outbreaks.

A SIN virus model system for the study of arbovirus-vector interactions has many advantages; among them are: 1) The genome structure and replication strategy of the virus have been well studied and are amenable to the construction of molecular clones that are easily manipulated. 2) The use of SIN viruses presents fewer biosafety problems than other positive strand viruses. 3) SIN virus expression systems have been developed from some of the available molecular clones for use in mosquitoes. 4) Information gained from studies of virus-vector interactions using a SIN model should lead to the development of new expression systems. Additionally, *Ae. aegypti* is also well suited to these types of studies as it is a medically important vector species, the molecular biology of the vector is one of the best understood of any vector species, and it is easy to rear and manipulate in the laboratory.

This work developed new tools and approaches for the study of virus-vector interactions in the SIN-*Ae. aegypti* system in several different ways: 1) New SIN-based double subgenomic expression systems were developed that have improved mosquito infection potential following oral infection. 2) Genetically distinct SIN viruses differing greatly in vector infectivity were exploited to develop model systems for further molecular investigations. 3) A SIN infectious clone capable of efficiently infecting *Ae. aegypti* was developed as a molecular tool for the elucidation of viral determinants of

midgut infectivity. 4) The power of such a clone was demonstrated in the identification of a region of the E2 glycoprotein of SIN viruses involved with mosquito infectivity.

CHAPTER 2

DEVELOPMENT OF A SINDBIS VIRUS EXPRESSION SYSTEM THAT EFFICIENTLY EXPRESSES GREEN FLUORESCENT PROTEIN IN MIDGUTS OF *AEDES AEGYPTI* FOLLOWING *PER OS* INFECTION

Introduction

Critical to the maintenance and transmission of most vector-borne pathogens is productive infection of the arthropod-midgut, which is a primary determinant of vector competence (Hardy, 1988; Woodring, Higgs, and Beaty, 1996). Unfortunately, knowledge of the anatomic, molecular, and genetic determinants conditioning the infection of this organ is limited. Molecular tools for the expression and characterization of both endogenous and exogenous genes in the arthropod midgut should greatly facilitate studies to further characterize the nature of these determinants. Sindbis (SIN) virus-based expression systems offer great potential in this regard.

Infectious cDNA clones have been developed for the alphaviruses SIN (Rice et al., 1987), SF (Liljestrom et al., 1991), Ross River (RR) (Kuhn et al., 1991), and VEE (Davis et al., 1989; Kinney et al., 1993), and have permitted genetic manipulation of the RNA genomes of these viruses. Alphavirus molecular clones have facilitated the identification of virus gene function and determinants of host range, virulence, replication, transcription, assembly and packaging (Davis et al., 1989; Kuhn et al., 1991; Liljestrom et al., 1991; Rice, 1996; Rice et al., 1987; Seabaugh et al., 1998; Strauss and Strauss, 1994). In addition, this technology has led to the development of expression systems that can be used in both vertebrate and invertebrate host systems (Bredenbeek et al., 1993; Hahn et al., 1992; Olson, Beaty, and Higgs, 1998).

The double subgenomic SIN virus (dsSIN) TE/3′2J was constructed from infectious clones derived from a heat resistant small plaque (HRSP) and neurovirulent strain of AR339 SIN virus (Hahn et al., 1992; Lustig et al., 1988). TE/3′2J is a self-replicating, self-packaging double promoter vector. The subgenomic promoter of the

virus has been duplicated, with one expressing the structural genes and one expressing a heterologous sequence (Hahn et al., 1992; Raju and Huang, 1991). The duplicated promoter was inserted between the structural genes and the 322 nucleotides (nt) that are non-coding at the 3′ terminus of the SIN virus genome (3′NCR). In the invertebrate host stable long-term efficient expression can be achieved, while efficient expression in vertebrate systems is transient. TE/3′2J has been successfully employed to express reporter genes such as chloramphenicol acetyltransferase (CAT) and GFP in salivary glands, neural ganglia, ommatidia, and midgut-associated nerves, muscles and tracheoles of adult *Ae. aegypti, Ae. albopictus, Ae. triseriatus, Cx. pipiens,* and *An. gambiae* mosquitoes following intrathoracic injection (Higgs et al., 1995; Higgs et al., 1996; Kamrud et al., 1997; Olson, Beaty, and Higgs, 1998; Olson et al., 1994). The TE/3'2J expression system has also been used effectively to express GFP in larval and pupal tissues following parenteral infection (Higgs et al., 1996; Olson, Beaty, and Higgs, 1998). Parenteral infection with TE/3'2J also has been used to express antisense viral RNA sequences effectively blocking LaCrosse virus infection in *Ae. triseriatus* (Powers et al., 1996) and the transmission of dengue type 2 and yellow fever viruses by *Ae. aegypti* mosquitoes (Higgs et al., 1998; Olson et al., 1996).

However, midgut epithelial cells are poorly infected with TE/3'2J viruses by both parenteral and oral routes of infection. This has limited the use of this expression system in studies of pathogen-vector interactions at these cells, which are the initial sites of pathogen infection and replication following ingestion of the blood meal.

Seabaugh et al. (1998) constructed a chimeric SIN infectious clone, pMRE1001, containing cDNA encoding the structural genes of the Malaysian SIN virus, MRE16.

Seabaugh et al. (1998) derived the nonstructural genes (nsP1-nsP4), *cis*-acting sequences, as well as the 59 nucleotide 5′ NCR and 3′ NCR of the clone from TE/3'2J. The MRE16 virus infects midgut cells and disseminates in nearly 100% of *Ae. aegypti* (Rexville D strain) mosquitoes following *per os* exposure. Seabaugh et al. (1998) demonstrated that chimeric MRE1001 virus disseminated from the midgut in >90% of orally infected mosquitoes. These results suggested that MRE16 determinants of midgut infectivity and dissemination co-segregate with the structural proteins of the virus.

This chapter describes the development of a dsSIN expression system from the chimeric infectious clone pMRE1001 that efficiently infects midgut tissues of *Ae. aegypti* mosquitoes following infection *per os*. The utility of the MRE/3′2J expression system for transducing midgut cells was demonstrated by expressing GFP from the dsSIN virus.

Materials and Methods

Cells and Medium

Baby hamster kidney cells (BHK-21) were grown in Leibovitz (L-15) medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY) at 37°C. *Ae. albopictus* cells (C6/36) (Igarashi, 1978) were grown in L-15 medium containing 10% FBS plus antibiotics at 28°C.

Viruses

MRE16 SIN virus was isolated between 1966 and 1969 from a pool of *Cx. tritaeniorhynchus* mosquitoes in Malaysia (Pudney, Leake, and Varma, 1979). The virus was isolated in *Ae. pseudoscutellaris* cells (AP61) (Varma, Pudney, and Leake, 1974) and

passaged six times in AP61 cells and at least three times in C6/36 cells. A low but unspecified number of additional passages were also done in C6/36 cells. The prototype SIN AR339 virus (Taylor et al., 1955) used in the current study was obtained from the CDC, Division of Vector-borne Infectious Diseases Laboratory (Fort Collins, CO). The virus had been passed an unknown number of times, extensively in BHK-21 and Vero cells.

Infectious cDNA Chimeric Clones of SIN and dsSIN Viruses

The construction of pTE/3'2J, pTE/3'2J/GFP, and pMRE1001 have been previously described (Hahn et al., 1992; Higgs et al., 1996; Seabaugh et al., 1998). The MRE/3'2J/GFP, dsSIN infectious cDNA clone, was derived from pTE/3'2J/GFP and pMRE1001. Initially, a 1200 base pair (bp) fragment of pTE/3'2J/GFP containing the SIN second subgenomic promoter element, GFP coding sequence, and SIN 3' NCR was amplified by polymerase chain reaction (PCR) using forward (5' gaaggccttcaggtagacaatattacac 3') and reverse (5' cttaccgctgttgagatc 3') primers. The PCR product was digested with Stu I and Xho I restriction endonucleases and ligated into the Stu I and Xho I sites of pMRE1001 (Figure 2.1). While a Stu I site is present in the forward primer, the Xho I site is not present in either primer and was amplified from pTE/3'2J/GFP.

in vitro Transcription

The MRE/3'2J/GFP and TE/3'2J/GFP plasmids were linearized at the 3'-terminal Xho I site. After cutting with XhoI, self–digested proteinase K was added to 100 μg/ml. Reactions were incubated at 37°C for 1 hour. Linearized plasmid DNA was phenol/chloroform extracted, chloroform extracted, and then ethanol precipitated

(Sambrook, Fritsch, and Maniatis, 1989). Transcription reactions (50 μl) contained 40 mM Tris-HCL (pH 7.9); 6 mM $MgCl_2$; 4 mM; 2 mM spermidine; 10mM NaCl; 50 units RNase inhibitor (Ambion, Inc., Austin, TX); 1mM each of ATP, GTP, UTP, and CTP (Ambion, Inc., Austin, TX); 1mM cap analog [m^7G(5´)ppp(5´)G (Ambion, Inc., Austin, TX)]; 5 μg ultrapure BSA (Ambion, Inc., Austin, TX); 1-2μg linearized DNA template; and 30 units SP6 polymerase (Ambion, Inc., Austin, TX). Reactions were incubated at 39°C for 1 hour and then placed on ice.

Electroporation

Transcribed RNAs were electroporated into BHK-21 cells using a BTX Electro Cell Manipulator 600 apparatus (BTX Inc., San Diego, CA). BHK-21 Cells grown to 70-80% confluency were trypsinized, washed twice with cold phosphate buffered saline (PBS) (pH 7.4), and resuspended in PBS at a concentration of 10^7 cells/ml. For each electroporation, 0.4 ml of cell suspension and 12 μl of transcription reaction were transferred to a 2-mm-gap cuvette and pulsed twice at room temperature (rt). Electro Cell Manipulator settings were 500 V, 100 μF, and 720 ohms with a pulse length between 0.7-0.9 ms. Immediately after pulsing, 0.6 ml L-15 (10% FBS) was added to the electroporated cells, and the entire volume was transferred to a 25-cm^2 flask containing 4 ml L-15 (10% FBS). The flask was incubated at 37°C for approximately 24-30 hours or until >70% of the cells exhibited cytopathic effects (CPE). Supernatant was harvested and clarified by centrifugation at 1,000 g at room temperature for 3 minutes. The virus containing medium was aliquoted and stored at -70°C.

Endpoint Titrations

Titers of virus culture fluid were determined by endpoint dilution assays. BHK-21 cells were seeded into 96-well plates and infected with 10-fold serial dilutions of virus-culture fluid. Cells were grown in L-15 (5% FBS) for 2-4 days after infection. Endpoints were determined by observation of virus-induced CPE, relative to sample dilution. All titrations were performed in triplicate. Virus titers were determined by the Karber method (Karber, 1931) and expressed as $\log_{10}$ $TCID_{50}$/ml.

Virus Growth Curves

Rates of virus growth for AR339, MRE16, TE/3'2J/GFP, and MRE/3'2J/GFP were compared in C6/36 cells. C6/36 cell monolayers were infected at a multiplicity of infection (MOI) of 0.1 with each virus. Medium was removed from confluent monolayers of cells in 25-cm^2 flasks, virus stocks were diluted to the appropriate titer in 1 ml of L-15 (10% FBS), and added to each flask. Virus was absorbed to the cells for 1 hour while rocking at room temperature. An additional 4 ml of L-15 (10% FBS) was added to each flask and the cells placed at 28°C. One ml of supernatant was removed from each flask for virus titration and replaced with 1 ml of L-15 (10% FBS) at timed intervals (t = 0, 6, 12, 24, 48, 72, 120, and 192 hours) and stored at -70°C. Virus titers were determined for each sample as described previously.

RNA Detection

Total cellular RNA was extracted from C6/36 cells infected with dsSIN virus using the Qiashredder and RNeasy mini kits (Qiagen Inc., CA) according to the manufacturer's specifications. For Northern blot analysis, 3 to 5 μg of total RNA were electrophoresed in a 1% agarose/1X MOPS/2% formaldehyde gel (Sambrook, Fritsch,

and Maniatis, 1989) and transferred to a positively charged Brightstar nylon membrane (Ambion). For slot blot analysis, 5 μg of total RNA were serially diluted, denatured in 150 μl of 6X SSC (Ausubel et al., 1994), 7% formaldehyde at 65°C for 10 min and loaded onto a Brightstar nylon membrane (Ambion) using a Minifold II slot blot apparatus (Schleicher +Shuell, Keane, NH). Wells were washed with 100 μl of 6X SSC, 7% formaldehyde. RNA was UV-crosslinked (Stratagene, La Jolla, CA) to the blots prior to hybridization. An oligonucleotide (5' gctggtcggatcattggggcg 3') that was complementary to a sequence in the 3'NCR of SIN RNA was used as a probe for both Northern and slot blot hybridizations (Olson et al., 1996). Probes were labeled with a psoralen-biotin conjugate according to the manufacturer's specifications (Ambion). Probe was added to Church hybridization buffer (1 mM EDTA, 0.5 M NaPO4 pH 7.5, 7% SDS) and hybridized overnight at 42°C. Blots were washed 2x with 2X SSC, 0.1%SDS, followed by 2 washes in 0.2X SSC, 0.1% SDS. Hybridization was detected using the Brightstar Non-Isotopic Detection Kit (Ambion).

IFA Analysis of Mosquito Head Tissue Preparations

Dissected tissues from infected or control mosquitoes were placed on pre-cleaned superfrost® plus slides (VWR Scientific, West Chester, Pennsylvania). Mosquito tissues were fixed and permeabilized by immersion in cold acetone for 15 minutes. Tissues were incubated in mouse anti-SIN E1 Mab 30.11a (Chanas et al., 1982) (1:200) for 40 minutes (37°), washed twice by immersing in PBS, and then incubated with biotinylated sheep anti-mouse antibody (Amersham Corp., Arlington Heights, Illinois) (1:200) for 40 minutes (37°C). Tissues were washed again and incubated with fluorescein isothiocynate (FITC)-conjugated streptavidin (Amersham) (1:200) for 10 minutes (37°C). Following

three final washes (2 x PBS, 1 x ddH_2O), a drop of glycerol/PBS (9:1) containing 2.5% 1.4. diazobicyclo (2,2,2) octane (DABCO) was applied to each tissue, and a glass coverslip overlaid on the slide. Fluorescence analysis was carried out using a fluorescent microscope (Olympus BH2, with 10X, 20X and 40X objectives).

GFP Detection

The GFP gene used in this study encoded a red-shifted mutant, designated GFP-S65T (Clonetech, Palo Alto, CA). Wild type GFP is a 27 kDa monomeric protein derived from *Aquorea victoria* jellyfish (Prasher et al., 1992). GFP-S65T was analyzed in mosquito tissues by excitation ($\lambda = 488$) of the protein during fluorescence microscopy with an Olympus BH2 microscope (Higgs et al., 1996). GFP-S65T emits a red-shifted green light ($\lambda = 507$). GFP expression in infected C6/36 cells was observed using an Olympus IMT-2 inverted epifluorescent microscope.

Mosquitoes

Aedes aegypti Rexville D (AaRexD) strain mosquitoes originating from Rexville, Puerto Rico (Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO) were reared at 28°C, 80% relative humidity, and photoperiod of 16 hours light (L): 8 hours dark (D). Adult mosquitoes were provided with sugar cubes and water.

Per Os Infection of Mosquitoes by Artificial Blood Meal

Clone derived virus was passaged once in C6/36 cells prior to blood feeding. This was done because recombinant SIN viruses, used directly from transfected BHK-21 cells, inefficiently infected mosquito midguts following *per os* infection (Cheng et al., 2001). Confluent monolayers of C6/36 cells in a 25cm^2 flask were infected at an MOI of 0.01.

Infected cells were maintained at 28°C in 4 ml of L-15 (10% FBS). Approximately 48 hours post-infection, the infected cells were scraped into the medium. The infected cell suspension was harvested, and clarified by centrifugation at 1000 x g for 3 minutes. Aliquots of the supernatant were stored at –70°C. Virus titers were determined for one ml of the infected cell suspension in BHK-21 cells as described previously. One ml of the virus suspension was mixed with 1.0 ml of defibrinated sheep blood (Colorado Serum Co., Boulder, CO). The blood meal-virus mixture was warmed to 37°C and pipetted into the chamber of a water-jacketed (37°C) glass membrane feeder (Seabaugh et al., 1998). Mosquitoes (5-7 days post-eclosion) were allowed to feed for approximately one hour through a mouse skin membrane. Sugar cubes were removed from the mosquitoes three days prior to blood feeding. Blood meal samples were collected post-feeding for virus titration. Following the blood meal, mosquitoes were cold-anesthetized, and only individuals that had ingested the blood meal were retained for further analysis. Blood fed mosquitoes were housed at the species-specific insectary conditions described previously and provided water and sugar until analyzed (Higgs and Beaty, 1996). Additionally, control *Ae. aegypti* mosquitoes were intrathoracically inoculated with 1.0 μl of virus (positive control) or L-15 medium without virus (negative control), and maintained at insectary conditions until analyzed (Gubler and Rosen, 1976a).

Results

MRE/3′2J/GFP Virus

A plasmid, designated pMRE/3′2J/GFP, was constructed by replacing the 3′ NTR of pMRE1001 with the second subgenomic promoter, GFP reporter gene, and 3′NTR of

pTE/3´2J/GFP (Figure 2.1; see Materials and Methods). dsSIN genomic RNA was transcribed *in vitro* from pMRE/3´2J/GFP. The RNA was then transfected into BHK-21 cells. Virus was harvested at 24 hours post-transfection, and virus concentration was determined using an end point dilution assay. Transfected BHK-21 cells produced approximately 10^7 $TCID_{50}$/ml of MRE/3´2J/GFP virus and 10^{8-9} $TCID_{50}$/ml of TE/3´2J/GFP virus.

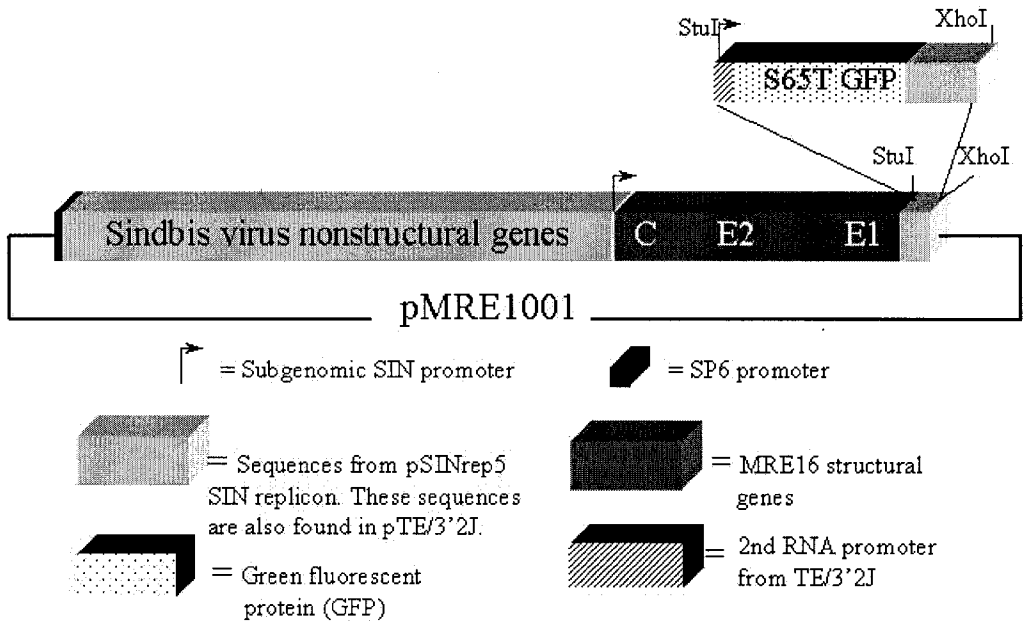

Figure 2.1. Chimeric dsSIN cDNA infectious clone, pMRE/3´2J/GFP. pMRE/3´2J/GFP was constructed from pMRE1001 and pTE/3´2J/GFP. A PCR fragment containing the second subgenomic promoter, GFP, and 3´ NTR of pTE/3´2J/GFP was inserted into the StuI and XhoI restriction endonuclease sites of pMRE1001.

Growth of Viruses in C6/36 Cells

MRE/3´2J/GFP virus growth in C6/36 cells was compared with that of AR339, MRE16, and TE/3´2J/GFP viruses. C6/36 cells were infected with each virus at an MOI of 0.1, and virus was harvested at specified time points. AR339 and TE/3´2J/GFP virus titers increased to a maximum of $10^{9.5}$ $TCID_{50}$/ml by 48 hours post-infection; MRE16 and MRE3´2J/GFP viruses also attained maximum titer at 48 hours post-infection with $10^{8.2}$

and $10^{6.5}$ $TCID_{50}$/ml, respectively (Figure 2.2). The MRE/3´2J/GFP virus titers were more similar to that of MRE16 virus than to either AR339 or TE/3´2J/GFP virus.

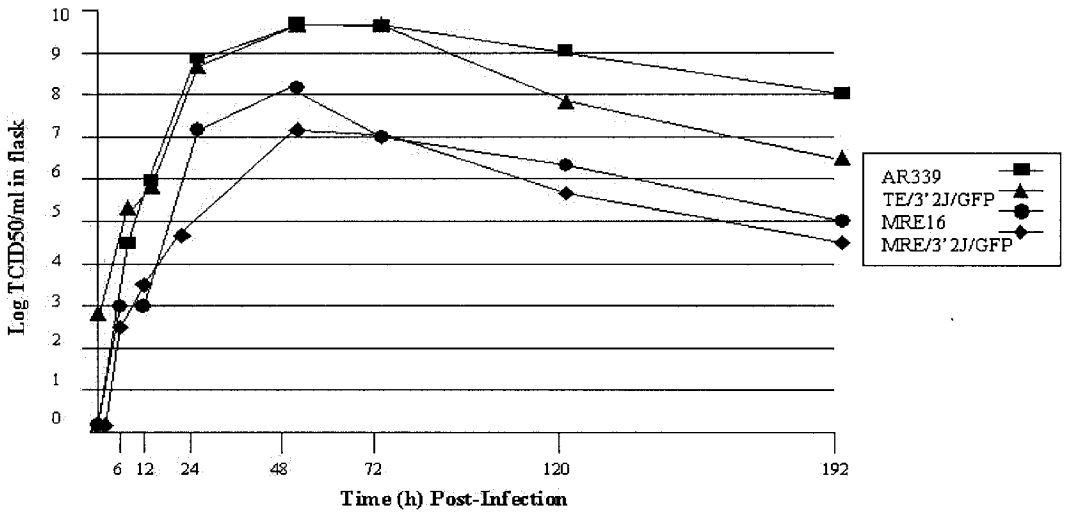

Figure 2.2. MRE/3´2J/GFP virus growth in C6/36 cells. C6/36 cells were infected with each virus at 0.1 MOI, and virus titer ($TCID_{50}$/ml) was determined at 0, 6, 12, 24, 48, 72, 120, and 192 hours post-infection in BHK-21 cells by determining the end point dilution of cytopathology.

RNA Analysis

The viral RNA species produced by both TE/3´2J/GFP and MRE/3´2J/GFP viruses were analyzed by infecting C6/36 cells at 0.1 MOI for 48 hours, isolating total cellular RNA, and detecting virus-specific RNA by Northern blot. The dsSIN viruses transcribe three virus-specific RNA species in infected mosquito cells (Figure 2.3 A). Viral RNA derived from MRE/3´2J/GFP virus infection was compared with RNA from TE/3´2J/GFP and TE/3´2J virus infections. All three predicted RNA species were detected for each virus by using an oligonucleotide probe complementary to the 3´ NCR of the SIN viral RNA. The size of each RNA species was increased by the size of the inserted S65T GFP gene (Figure 2.3 B).

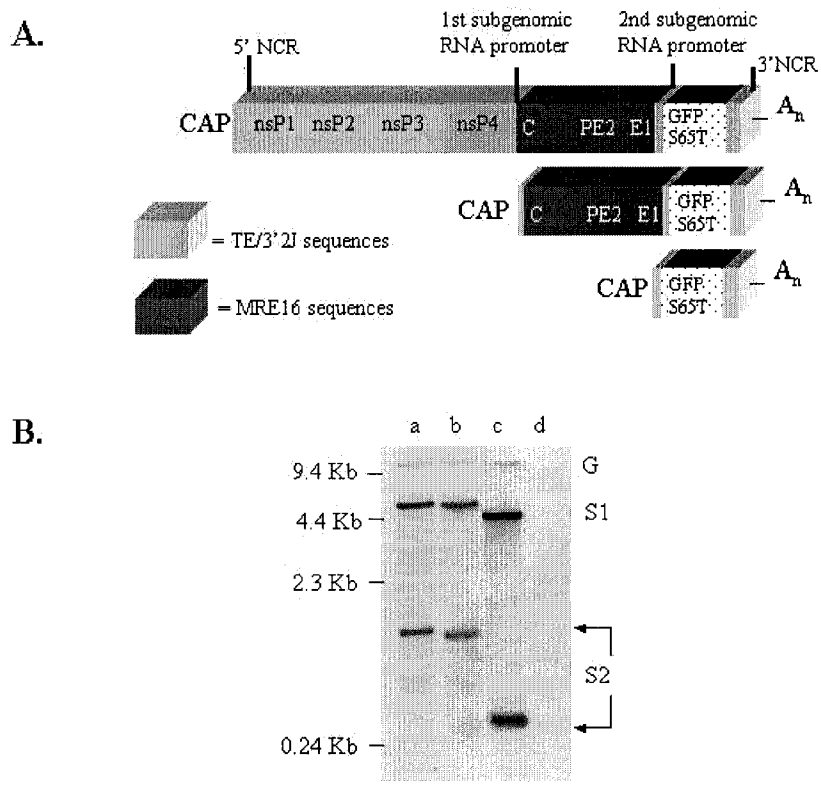

Figure 2.3. Characterization of MRE/3´2J/GFP virus-specific RNA and GFP expression in C6/36 cells. (A) Diagram of predicted virus-specific RNA species in C6/36 cells infected with MRE/3´2J/GFP. (B) Northern blot analysis of actual RNA species found in infected C6/36 cells. Lanes contain RNA isolated from cells infected with (a) MRE/3´2J/GFP, (b) TE/3´2J/GFP, or (c) TE/3´2J control virus. Lane (d) contains RNA from uninfected C6/36 cells.

Relative amounts of viral RNA transcription in infected C6/36 cells were analyzed for both TE/3´2J/GFP and MRE/3´2J/GFP viruses. C6/36 cells were infected with each virus at an MOI of 0.4 and incubated at 28°C for 48 hours, total cellular RNA was isolated, and virus-specific RNA was detected in serial sample dilutions by slot blot analysis. The relative amount of viral RNA present in the MRE/3´2J/GFP infected cells was comparable to that of TE/3´2J/GFP infected cells at 48 hours post-infection (Figure 2.4 A). Viral RNA species were detected using the same SIN 3´ NCR specific oligonucleotide primer used in the northern blot analysis. A second identical slot blot membrane, prepared in parallel with the one used in the analysis of viral RNA, was

hybridized in the presence of a probe specific for a cellular actin. The relative amounts of actin RNA present in the total cellular RNA isolated from TE/3´2J/GFP or MRE/3´2J/GFP infected C6/36 cells were also comparable (Figure 2.4 B). Thus approximately equal amounts of total cellular RNA had been detected using the SIN specific primer.

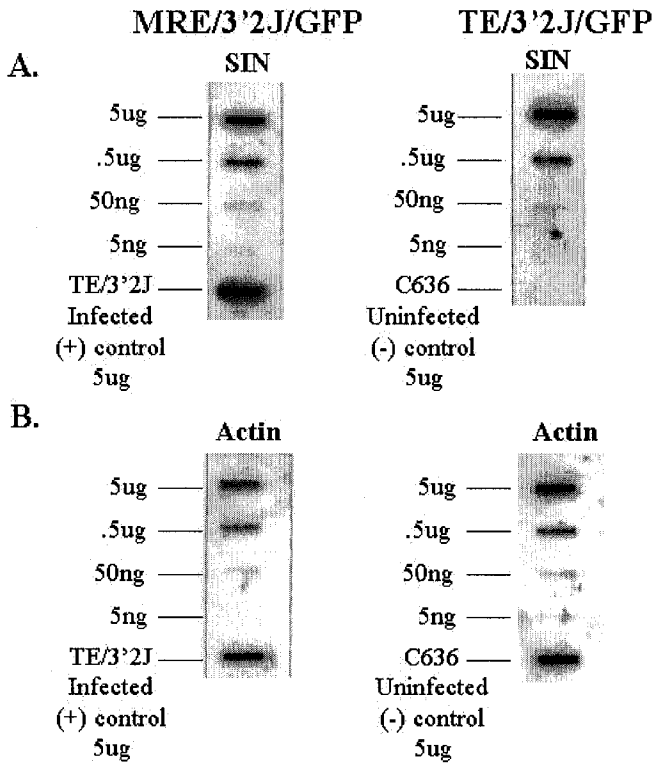

Figure 2.4. Slot blot analysis of relative viral RNA amounts in C6/36 cells infected with MRE/3´2J/GFP or TE/3´2J/GFP. Serial sample dilutions of total cellular RNA from uninfected, TE/3´2J-infected, TE/3´2J/GFP-infected, or MRE/3´2J/GFP infected cells was blotted on a nylon membrane and then hybridized with a (A.) probe specific to the 3´ NCR of TE/3´2J/GFP, MRE/3´2J/GFP, and TE/3´2J or a (B.) actin specific probe.

GFP Expression in C6/36 Cells

GFP expression was monitored in C6/36 cells that were infected with MRE3´2J/GFP virus at an MOI of 0.1. At 24 hours post-infection, less than 20% of cells

infected with MRE3′2J/GFP virus expressed GFP (Figure 2.5 A). At 48 hours post-infection, approximately 80% of cells expressed GFP (Figure 2.5 B). In contrast 24 hours after cells were infected with TE/3′2J/GFP virus at the same MOI, approximately 100% of the cells expressed GFP (data not shown).

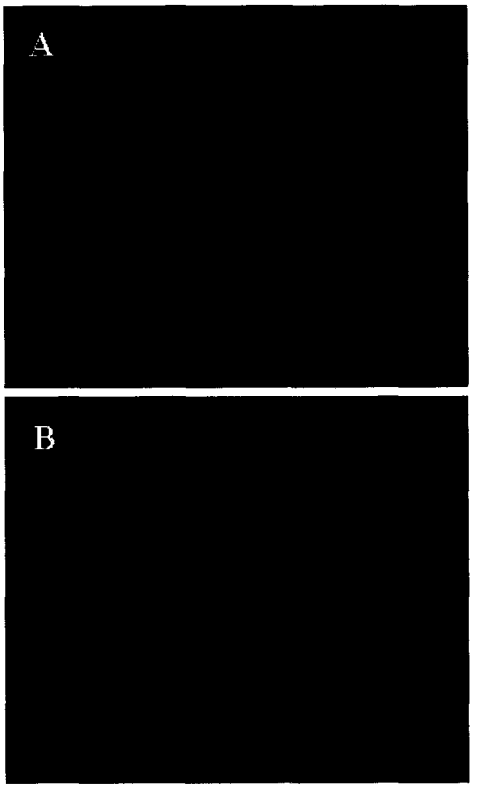

Figure 2.5. GFP expression in C6/36 cells infected with MRE/3′2J/GFP virus. GFP expression at 24 hours (A) and 48 hours (B) post-infection. (Original magnification 40X).

Comparison of GFP Expression in Mosquitoes Orally Infected with MRE/3′2J/GFP or TE/3′2J/GFP Virus

AaRexD mosquitoes were infected orally with either TE/3′2J/GFP or MRE/3′2J/GFP virus, and expression of GFP was monitored in the mosquitoes over a 12 day time course. Each virus was amplified in C6/36 cells for 48 hours prior to preparing the blood meal. Mosquitoes were allowed to ingest an infected blood meal for 1 hour.

The post-blood meal titer of MRE/3´2J/GFP and TE/3´2J/GFP viruses were $10^{6.8}$ and $10^{9.5}$ $TCID_{50}$/ml, respectively. Most of the midguts dissected from mosquitoes infected with either MRE/3´2J/GFP or TE/3´2J/GFP virus showed trace amounts of GFP expression from the hemocele side at 1 to 2 days post-infection (Figure 2.6 A). However, when a longitudinal incision was made in the midgut of mosquitoes orally infected with MRE/3´2J/GFP virus, and the organ was inverted revealing the lumenal side of the midgut, numerous foci of expression were observed at 2 days post infection (Figure 2.6 B). These foci of expression presumably represented initial cells infected with virus from the blood meal.

The midguts from five infected mosquitoes were dissected each day for 12 days after infection, and the level of GFP expression was scored as either trace, low, moderate, heavy, or no expression. A midgut displayed trace expression if one or a few foci of expression were observed from the hemocele side of the midgut. A midgut dissected from a mosquito infected orally with MRE/3´2J/GFP virus, and displaying a trace amount of GFP expression 2 days after infection is shown in Figure 2.6 A. A midgut was scored as having low expression if several foci of expression fused to form one or more larger regions of expression; however, these regions made up less than ¼ of the midgut. A midgut of a mosquito infected with MRE/3´2J/GFP virus, and displaying low GFP expression at 3 days post-infection is shown in Figure 2.6 C. Midguts were scored as having moderate and heavy GFP expression if they had ¼ to ½ or greater than ½ of their midgut expressing GFP, respectively. Figures 2.6 D and 2.6 E show moderate and heavy GFP expression in a midgut infected with MRE/3´2J/GFP virus at 4 and 5 days post-infection, respectively.

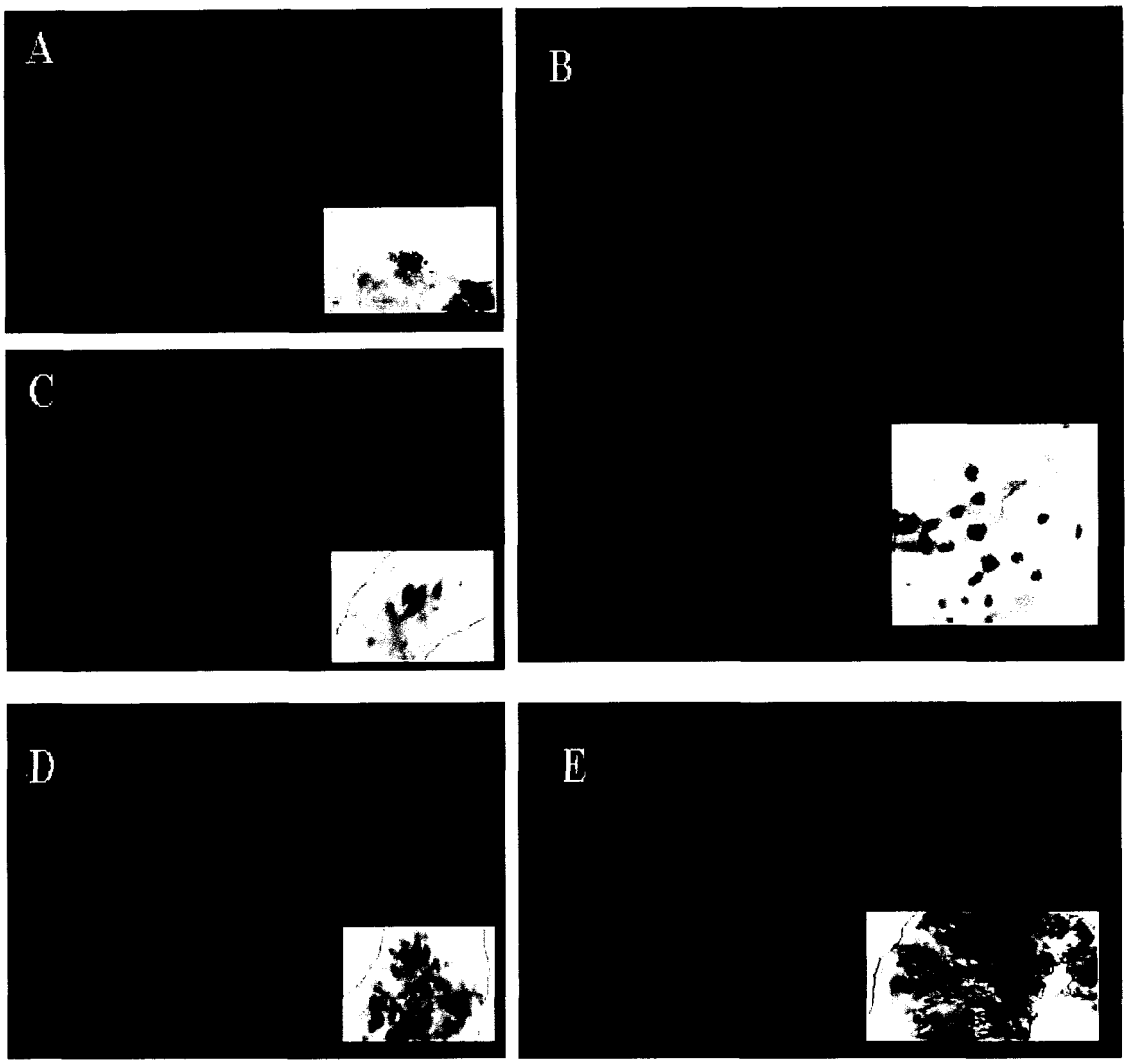

Figure 2.6. GFP expression in *Ae. aegypti* mosquitoes infected orally with MRE/3′2J/GFP virus. Mosquitoes were infected *per os* with a blood meal containing approximately 10^7 $TCID_{50}$/ml of MRE/3′2J/GFP virus. The midguts of selected mosquitoes were dissected and observed for GFP expression. (A) Abdominal midgut at 2 days post infection showing a single site of GFP expression from the hemocele-side. (B) Abdominal midgut 2 days post-infection that has been dissected and inverted to reveal GFP expression in the lumen-side of the midgut. (C, D, E) hemocele-side of abdominal midguts from selected mosquitoes showing low, moderate, and heavy expression of GFP at 3, 4, and 5 days post-infection, respectively. (Panel A original magnification 250X; Panels B, C, D, and E original magnification 125X). An inset that shows a false color inverted image to outline the midgut was added to each panel.

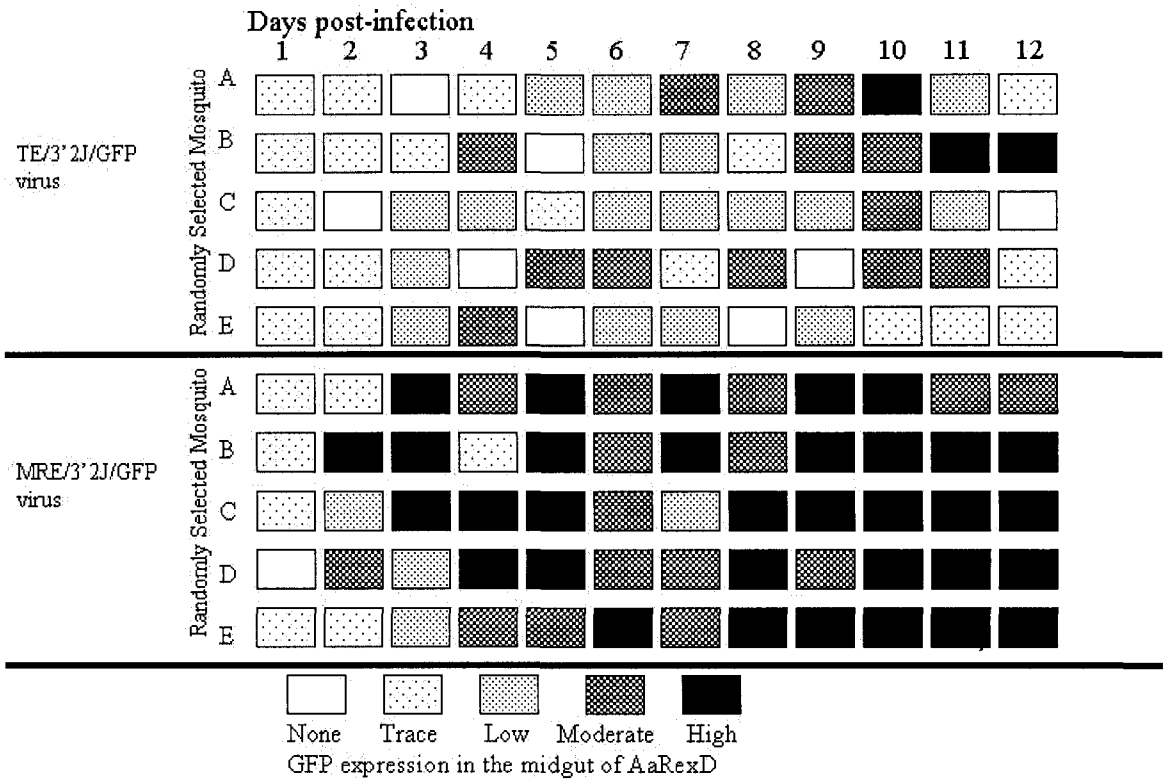

Figure 2.7. Temporal expression of GFP in *Ae. aegypti* mosquitoes infected orally with MRE/3´2J/GFP and TE/3´2J/GFP viruses. Mosquitoes were infected orally with blood meals containing approximately 10^7 or $10^{9.5}$ $TCID_{50}$/ml of MRE/3´2J/GFP or TE/3´2J/GFP virus, respectively. Five mosquito midguts were assayed for GFP expression each day for 12 days.

Mosquitoes infected orally with the higher titered TE/3´2J/GFP virus generally had trace to low expression levels of GFP. At 1 to 9 days post-infection, many of the mosquitoes had only one or a few areas of GFP expression in the midgut (Figure 2.7). Only at 10-12 days post-infection did 3 of 15 mosquitoes show heavy GFP expression in the midgut. MRE/3´2J/GFP virus began showing heavy levels of GFP expression as early as 3 days post infection. At 4-12 days post-infection, 43 of 45 mosquitoes orally infected with MRE/3´2J/GFP had moderate to heavy expression of GFP in the midgut and at 10-12 days post-infection, 13 of 15 mosquitoes had heavy GFP expression (Figure 2.7).

Despite high levels of GFP expression in the midguts of most mosquitoes orally infected

with MRE/3´2J/GFP, only one mosquito in 40 analyzed showed GFP expression in the head of the mosquito. We then examined the heads of these mosquitoes by IFA for the presence of SIN E1 antigen. Twenty-six of the 28 mosquitoes tested had disseminated infections at 18 days post-infection, suggesting that selection of viral genomes with deletions or other mutations in the GFP coding region occurred during replication of the virus in the midgut and secondary target organs of the mosquito. Thus MRE/3´2J viruses efficiently express genes of interest in midgut epithelial cells, but the route of infection has limitations for the expression of heterologous genes in other mosquito tissues.

Discussion

Seabaugh et al. (1998) previously used the chimeric SIN virus MRE1001 to map viral determinants of midgut infectivity and dissemination in *Ae. aegypti* to the structural genes. That work provided the basis for possible improvement of oral infectivity of dsSIN expression systems in a medically important arthropod vector. In the current study, a chimeric SIN expression system, designated MRE/3´2J/GFP, was constructed by inserting the second subgenomic promoter-GFP gene of the double promoter vector TE/3´2J/GFP into the chimeric pMRE1001 infectious cDNA clone. Virus derived from the MRE/3´2J/GFP clone was shown to effectively and efficiently transduce *Ae. aegypti* midgut epithelial cells with the GFP gene following infection *per os*.

Replication of MRE/3´2J/GFP in C6/36 cells was similar to that of MRE16, and like the Malaysian SIN virus, MRE/3´2J/GFP replicated less efficiently than either TE/3´2J/GFP or a laboratory strain of AR339. Given this, the observed similarity in the relative amounts of viral RNA transcription for both TE/3´2J/GFP and MRE/3´2J/GFP

viruses in C6/36 at 48 hours post-infection is difficult to explain (Figure 2.4). Endpoint dilution assays estimate the titer of biologically relevant virus particles whereas the slot blot analysis estimated the relative amount of total viral RNA present in the cells. Presumably only genomic length RNA is present in a biologically relevant virus particle, the amount of which is determined in estimations of titer. The virus RNA species detected in the slot blot analysis using the SIN specific probe which hybridizes to the 3´ NCR present in each include genomic, 1st subgenomic, and 2nd subgenomic mRNAs (figure 2.3). Thus the production of 1st and 2nd subgenomic mRNAs by both viruses may be roughly equivalent at 48 hours post-infection and obscure differences present in the amount of genomic length RNA when measured with this type of assay. It is also possible that the production of infectious MRE/3´2J/GFP virus particles in C6/36 cells is not equivalent to that of TE/3´2J/GFP despite near equal levels of viral RNA synthesis, as a number of events remain in the life cycle of an alphavirus following synthesis of viral RNAs (Strauss and Strauss, 1994). One possibility could be that differences in RNA packaging efficiency exist between the two viruses.

Following peroral challenge of *Aedes aegypti*, infection and expression of GFP in midgut epithelial cells was more efficient with MRE/3´2J/GFP than with TE/3´2J/GFP. While virus derived from TE/3´2J/GFP also infected midguts, GFP expression was only rarely observed to be widely distributed in a dissected midgut. This was observed despite the fact that mosquitoes were challenged with titers of TE/3´2J/GFP more than 100 times greater than those of MRE/3´2J/GFP.

An orally infectious MRE/3´2J expression system provides specific advantages for use in certain types of studies and is a valuable tool for the expression of exogenous

genes in the midgut of *Ae. aegypti* mosquitoes following oral infection. For example, several groups have suggested that antibacterial immune system peptides such as defensins and cecropins may adversely affect filarial worms and malaria parasites in the mosquito host (Gwadz et al., 1989; Lowenberger et al., 1999). The importance of defensins has been suggested from studies in which the mosquito immune system was activated by parenteral bacterial challenge prior to infection with *B.malayi, P.gallinaceum,* or *P.berghei* (Lowenberger et al., 1996; Lowenberger et al., 1999). However, immune system activation by injection of bacteria can elicit the expression of numerous immune system peptides (Beerntsen, James, and Christensen, 2000). Since intrathoracic inoculation of TE/3´2J viruses expressing these peptides also induce an immune response, it has been difficult to rule out an as yet unidentified immune system protein as being responsible for the observed anti-pathogen effects. MRE/3´2J provides the potential for specific expression in the midgut of a single immune peptide, which might then be secreted into the hemolymph of *Ae. aegypti.* Hence if tissues are infected naturally (orally), problems associated with bacterial challenge and intrathoracic inoculation are precluded. The utility of the chimeric expression system for this purpose has been demonstrated (Cheng et al., 2001). MRE/3'2J viruses were engineered to express *Ae. aegypti* defensin genes A or C. *Ae. aegypti* mosquitoes were then exposed to the viruses either in an infectious blood meal as adults or in tissue culture flasks of infected C6/36 cells as larvae. Infection of *Ae. aegypti* larvae with dsSIN viruses by the "swimmer method" was first described by Higgs et al. (1999). Mosquitoes that ingested the recombinant viruses by either method became infected and expressed defensin (Cheng et al., 2001). In addition, the transcription of other immune peptides was not

induced by either method of infection (Cheng et al., 2001). However, mosquitoes exposed as larvae to the dsSIN viruses demonstrated higher levels of infectivity than those exposed at the adult stage (Cheng et al., 2001). The exposure of mosquitoes in the larval stage also resulted in the expression of defensin genes in the adult mosquito at a location (midgut) and time (3-5 days post-eclosion) ideal for assessing its effect on pathogens taken up with a blood meal in a laboratory setting (Cheng et al., 2001).

Cheng et al. (2001) reported that the MOI used to infect C6/36 cells played a critical role in the expression of defensin genes in adult mosquitoes. Cheng et al. (2001) described experiments to determine critical conditions for efficient gene expression in mosquitoes. These experiments revealed consistently higher levels of defensin expression when mosquitoes were infected with virus supernatant from C6/36 cells infected at an MOI of 0.01 than with virus passaged at an MOI of 0.1 (Cheng et al., 2001). The *in vitro* transcription reaction may generate incomplete transcripts from the recombinant viral cDNAs lacking a portion of the 3' end of the genome. Such transcripts would be replication defective but could possibly be packaged in the presence of helper virus. Thus it may be necessary to passage the resulting progeny virus at a low MOI to eliminate the infectious but replication defective virion particles.

Transformation of *Ae. aegypti* has been successfully achieved (Coates et al., 1998; Jasinskiene et al., 1998; Lobo et al., 2002). However, germ-line transformation in mosquitoes remains an expensive and labor-intensive process. Sindbis virus systems represent a powerful tool for the rapid *in vivo* evaluation of the effectiveness of antipathogen candidate genes. The development of MRE/3´2J complements TE/3´2J expression systems and effectively expands the usefulness dsSIN viruses for this purpose

by extending the number of tissues in the mosquito that can now be efficiently transduced. In addition, MRE/3´2J will facilitate studies of pathogen-vector interactions in the midgut, an organ of great importance to vector competence.

Detection of SIN E1 in head tissues indicated that MRE/3´2J virus disseminated efficiently from the midguts of infected mosquitoes (Olson et al., 1994). However, expression of GFP was only rarely observed in the heads of the MRE/3´2J/GFP virus infected mosquitoes, indicating dsSIN genome instability. It has been previously demonstrated that continuous passaging of dsSIN viruses in cell culture leads to the selection of viruses that have deleted the foreign gene of interest (Higgs et al., 1995). In the present study, a deletion spanning the second subgenomic promoter and gene of interest in an MRE/3´2J virus was shown to occur during amplification in cell culture prior to blood feeding (data not shown). The GFP gene and second subgenomic promoter constitute extra RNA sequence in the viral genome. Thus there is undoubtedly selection to package genomes of normal length. Viruses in which the additional sequences were deleted would probably have a selective advantage in replication. Alternatively an inherent incompatibility may exist between the structural genes of MRE16 virus and the nonstructural genes and *cis*-acting regions of TE/3´2J/GFP virus. Other chimeric alphaviruses have been shown to have a lower fitness than the parental virus (Kuhn et al., 1996; Yao, Strauss, and Strauss, 1996). A dsSIN virus based entirely on the MRE16 genome sequence may lead to improved dissemination of the heterologous gene product. However, the work described here illustrates that if care is taken during cell culture passages prior to blood feeding (optimization of the infecting MOI, minimization of the

number and duration of passages), the MRE/3´2J virus expression system is an effective tool for achieving efficient gene expression in midgut epithelial cells of *Ae. aegypti.*

CHAPTER 3

A COMPARISON OF THE TRANSMISSION POTENTIAL OF TWO GENETICALLY DISTINCT SINDBIS VIRUSES FOLLOWING *PER OS* INFECTION OF *AEDES AEGYPTI*

Introduction

The biological transmission of an arbovirus depends on its ability to infect, replicate and disseminate through target organs of the vector. After ingestion in the infectious blood meal, the arbovirus must first infect the midgut of the mosquito. Entry into a susceptible cell is presumably initiated by specific interaction with a cellular receptor followed by penetration of the cell. Maturation of new virions occurs after uncoating, transcription, and translation of the virus genome. Infectious virions then must disseminate from the midgut to infect other target organs. Barriers to infection and dissemination of an arbovirus within the mosquito host are particularly important aspects of vector competence (Woodring, Higgs, and Beaty, 1996). The arbovirus may be blocked at early stages of midgut infection (e.g. receptor binding, uncoating, transcription, or translation), at a MI barrier. Infectious virions may also be prevented from disseminating to the hemocele (and other target organs) if a ME barrier is present. Finally, the arbovirus must infect, replicate and be shed into the lumen of the salivary gland before transmission can occur in a subsequent bite. Transmission can be prevented by an SGI or SGE barrier. The mechanisms responsible for these barriers have yet to be elucidated. Intrinsic genetic factors of the mosquito host no doubt have an influence. However, the genetic characteristics of the arbovirus also may determine, at least in part, the phenotypic expression of the mechanisms involved.

SIN viruses (Togaviridae) are single-stranded, positive-sense RNA viruses that are cycled principally between *Culex* species of mosquitoes and avian vertebrate hosts (Doherty et al., 1977; Doherty et al., 1979; Taylor et al., 1955). However, SIN viruses are infectious for a number of other vertebrate (including humans) and arthropod species

(Hurlbut and Thomas, 1960; Malherbe and Strickland-Cholmley, 1963). SIN viruses have primarily an Old World distribution and can readily be separated into distinct Paleoarctic/Ethiopian and Oriental/Australian antigenic and genetic groups (Olson and Trent, 1985; Rentier-Delrue and Young, 1980; Sammels et al., 1999; Seabaugh et al., 1998; Shirako et al., 1991).

The prototype SIN strain AR339, was originally isolated from a pool of *Cx. pipiens* and *Cx. univittatus* mosquitoes collected from the Sindbis health district of Egypt (Taylor et al., 1955). The prototype strain and viruses derived from this strain have limited dissemination and transmission potential in *Ae. aegypti* following *per os* infection (Jackson, Bowen, and Downe, 1993; Seabaugh et al., 1998). The AR339 SIN strain has been shown to produce only limited infection of midguts following oral or parenteral infection of *Ae. aegypti* mosquitoes (unpublished data). In contrast, a SIN virus strain designated MRE16, from the Oriental/Australian zoogeographic region, has been previously demonstrated to disseminate in nearly 100% of *Ae. aegypti* mosquitoes ingesting an infectious blood meal (Seabaugh et al., 1998). In addition, a chimeric cDNA infectious clone (pMRE1001) based on the nonstructural and *cis*-acting sequences of an AR339 variant and the structural genes of MRE16 SIN virus produced virus that efficiently and rapidly infected midgut tissues of *Ae. aegypti* and disseminated in greater than 90% of the mosquitoes within 14 days (Olson et al., 2000; Seabaugh et al., 1998).

Almost all of the available information on the molecular biology, structure, and pathogenesis of SIN virus has been obtained using a biologically cloned strain of AR339 (Pfefferkorn and Hunter, 1963) or the heat-resistant (HR) strain derived from it (Burge and Pfefferkorn, 1966). A small-plaque variant of HR, HR_{sp}, was the first complete

sequence of the SIN virus genome to be published (Strauss, Rice, and Strauss, 1984). Viral cDNAs amplified from the genome of HR_{sp} are also major constituents of the first complete cDNA clones of SIN (Rice et al., 1987). HR_{sp} and many other laboratory strains of AR339 have undergone considerable passage and biological cloning in cell culture. Sequences of many AR339 laboratory strains and those of the cDNA clones derived from them likely contain significant changes from the sequence of the original AR339 isolate as a result of selection for efficient growth in cell culture (McKnight et al., 1996). Such changes may be of considerable significance in studies of infectivity, as even single nucleotide differences can have profound effects on the *in vivo* phenotype of an alphavirus (Davis et al., 1986; Davis et al., 1991; Kinney et al., 1993; Lustig et al., 1988; Polo et al., 1988; Polo and Johnston, 1991; Schoepp and Johnston, 1993a; Tucker and Griffin, 1991; Tucker et al., 1993; Woodward et al., 1991). The modified genetic backgrounds present in cell culture adapted strains may also complicate the interpretation of genetic studies to elucidate the molecular determinants of an expressed phenotype *in vivo*. For example, McKnight et al. (1996) found the virulence of AR339 strains, isogenic except at nt 5 (A or G) in the 5' NCR, did not differ significantly as measured by neonatal mice mortality. However, the presence of an A at nt 5 greatly enhanced the effect of a second attenuating mutation in the E2 gene (McKnight et al., 1996). The results suggest the even minimal changes to the "wild-type" genetic background into which an additional mutation is introduced can have a dramatic effect on the apparent *in vivo* phenotype. Given the potential magnitude of mutations introduced by cell culture passage, the appropriate genetic background for the examination of mutations affecting AR339 mosquito infectivity would be that of the original isolate. The SIN AR339 virus

consensus sequence has been deduced and represents the closest approximation of the sequence of the "wild-type" isolate (McKnight et al., 1996). For the reasons mentioned a cDNA clone of the consensus sequence, designated TR339 (Klimstra, Ryman, and Johnston, 1998), was used in the present study. By using TR339 laboratory-induced variation was controlled, and the genetic background stabilized, which may prove valuable in future studies to identify genetic determinants.

Many aspects of the arbovirus-vector relationship are poorly understood. For example, arbovirus genetic determinants of differences observed in tissue susceptibility, extrinsic incubation periods, and transmission efficiencies within a single mosquito species following peroral infection are not well studied. The MRE16 and AR339 strains of SIN virus represent an intriguing opportunity to explore viral determinants of infectivity and transmissibility in a medically important arthropod vector. However, prior to undertaking studies at the molecular level, a more extensive characterization of the two SIN strains in the vector mosquito is required.

This chapter, describes the development of a SIN virus-*Ae. aegypti* model to determine the viral genetic determinants of virus-vector interactions. A comparison in *Ae. aegypti* of the midgut infection, dissemination, and transmission potential of MRE16 (Oriental/Australian genotype), and virus derived from a cDNA clone, TR339, engineered to reflect the sequence of AR339 virus (Paleoarctic/Ethiopian genotype) at the time of its isolation, was done. These studies provided information on the mechanisms of arboviral infection barriers in vector mosquitoes. In addition, SIN-based expression systems have been derived from both viruses and are now used extensively for gene expression and functional genomic analyses (unpublished; McKnight et al., 1996).

Characterization of the temporal and spatial infection patterns generated by these viruses in *Ae. aegypti* establishes a baseline, which will be of value to those using these expression systems.

Materials and Methods

Cells and Medium

BHK-21 cells were grown in minimal essential medium (MEM) containing 10% FBS, 1X non-essential amino acids (NEAA) for MEM (Mediatech, Inc., Herndon, VA), 292 μg/ml L-glutamine (Life Technologies, Inc., Grand Island, NY), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY), at 37°C. C6/36 cells were grown in MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics at 28°C. African green monkey kidney cells (Vero) were grown MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics at 37°C.

Viruses

MRE16 virus was isolated in AP61 cells (Varma, Pudney, and Leake, 1974), passaged six times in AP61 cells and five times in C6/36 cells. The consensus sequence of the SIN virus AR339 isolate, the prototype alphavirus, was deduced (McKnight et al., 1996) and cloned as a cDNA (pTR339) (Klimstra, Ryman, and Johnston, 1998) from which infectious virus (TR339) was derived. The cDNA clone pTR339 was generously provided by Dr. Robert Johnston (Division of Infectious Diseases, Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7030). The consensus sequence was deduced from the complete sequences of the AR339 laboratory derivatives TRSB (McKnight et al., 1996) and HR_{sp} (Strauss, Rice, and

Strauss, 1984), from partial sequences of the glycoprotein genes of the three other AR339 laboratory strains SV1A (Lustig et al., 1988), NSV (Griffin and Johnson, 1977; Lustig et al., 1988) and SIN (Davis et al., 1987; Strauss et al., 1991), and by comparison with the sequences of the four closely related alphaviruses S.A.AR86 (Russell, Dalrymple, and Johnston, 1989; Simpson et al., 1996), Girdwood S.A. (Simpson et al., 1996), Ockelbo82 (Shirako et al., 1991) and Aura (Rumenapf, Strauss, and Strauss, 1995). A total of 8 coding differences and a difference at nt 5 in the 5' NCR were found between the published sequence of HR_{sp} and the consensus sequence (McKnight et al., 1996). Coding differences within open reading frames were at nucleotides 1380 U→A, 1381 G→U, 2992 C→U, 3579 A→G, 8638 U→C, 8698 U→A, 8838 A→G, 9144 A→G and 10773 U→G, using SIN HR_{sp} numbering (McKnight et al., 1996). In addition an A was present at nt 5 in the 5' NCR of the HR_{sp} genome, while the consensus sequence has a G at this position (McKnight et al., 1996). Clone-derived virus was produced as described previously in Chapter 2 (*in vitro Transcription* and *Electroporation*). Clone-derived virus was passaged once in C636 cells. The MRE16 and TR339 virus working stocks contained 4.0×10^9 and 2.0×10^9 plaque forming units (PFU)/ml, respectively.

Plaque Titrations

Plaque titrations were performed in Vero cells grown in six- or twelve-well plates as described by Miller and Mitchell (1986) with minor modifications. Cells were infected with 150 μl of serially diluted virus in MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics, and rocked every 5 minutes for 1 hour at 37°C and 5% CO_2 before adding the first agarose overlay. Four (six-wells) or two (twelve-wells) ml per well of the first agarose overlay (42°C) was applied. The first overlay contained 1% SeaKem®

LE agarose (Cambrex corp., East Rutherford, NJ) 1X Earle's Balanced Salt Solution (BSS) (Life Technologies, Inc., Grand Island, NY), 0.33 mg/ml of Yeast Extract, 1.65 mg/ml of Lactalbumin hydrolysate, 2% FBS, 0.23% $NaHCO_3$, 50 μg/ml of Gentamicin, and 2 μg/ml of Fungizone® (Amphotericin B) (Life Technologies, Inc., Grand Island, NY). After setting the first overlay for 30 minutes at room temperature (rt), plates were inverted and incubated at 37°C with 5% CO_2 for 2 days. On day 2, two (six-wells) or one (twelve-wells) ml per well of the second agarose overlay (42°C) was applied. The composition of the second agarose overlay was identical to that of the first except that it contained 198 μg/ml of Neutral Red (Sigma Chemical Co., St. Louis, Missouri). Plaques were counted on the next day and subsequent days for a total of 3 days. PFU/ml were calculated using the formula: # of plaques x dilution x 6.66 (for .15 ml of inoculum).

Virus Growth Curves

Rates of virus growth for TR339 and MRE16 were compared in BHK-21 and C6/36 cells. Electroporation supernatants were used for infection of cells. Cell monolayers were infected at a MOI of 0.01 with each virus. Medium was removed from confluent monolayers of cells in 25-cm^2 flasks, virus stocks were diluted to the appropriate titer in 1 ml of MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics, and added to each flask. Virus was absorbed to the cells for 1 hour while rocking at rt. An additional 6 ml of MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics was added to each flask of BHK-21 or C6/36 cells and the flasks placed at 37°C or 28°C, respectively. One hundred μl of supernatant was removed from each flask for virus titration at timed intervals (t = 0, 12, 24, 36, 48, 60, 72, 84, 96 and 108 hours). The supernatant was added to 100 μl of MEM containing 20% FBS plus NEAA, L-

glutamine and antibiotics and stored at -70°C. Virus titers were determined for each sample as described previously.

IFA Analysis of Mosquito Tissues

IFA was used to detect SIN antigen in dissected organs and mosquito tissue preparations. Several protocols were used, depending on the IFA analyses being done. The protocol used for IFA analysis of mosquito head tissue preparations has been described in Chapter 2 (*Immunofluorescent Antibody Analysis of Mosquito Head Tissue Preparations*). The same protocol was also used for IFA analysis of mosquito leg tissue preparations in the current chapter.

Dissection and IFA Analysis of Mosquito Midgut Sheets

Distended midguts were dissected from infected or control mosquitoes in cold 1X Ashburners PBS (130 mM NaCl, 7 mM Na_2HPO_4, 10 mM $NaH_2PO_4 \cdot H_2O$). Midguts were placed in the protein cross-linking reagent paraformaldehyde (4%, 1X PBS) for 30 seconds. An incision spanning the length of the midgut was made, yielding flat sheets of tissue. The blood meal was removed from each midgut by peeling the epithelium away and then using surface tension to force the bolus out. Following removal of the bolus, tissues were placed in a microtube containing paraformaldehyde (4%, 1X PBS) for a longer fixation step (at least 2 hours). Fixation of tissues was followed by permeabilization and blocking in a solution containing the mild nonionic detergent Triton X-100 (Sigma Chemical Co., St. Louis, Missouri) (PBT, 1X Ashburners PBS, 1% BSA, 0.2% Triton X-100). The paraformaldehyde solution was removed and discarded. However, midguts were always immersed in approximately 100-200 µl of paraformaldehyde solution to prevent the midguts from drying out before PBS rinsing.

Midguts were rinsed by inverting several times in PBS. This was repeated using PBT. Between rinses and incubations midguts always remained in 100-200 µl of solution to prevent drying. Midguts were then incubated, through gentle rocking, in PBT for 1 hour. The choice of reagents used reflects an emphasis on the preservation of cell structure. Tissues were then ready to be prepared for fluorescence analysis by using a variety of stains and antibodies for the visualization of pathogens as well as cellular structures.

Midguts were incubated overnight at 4°C in mouse anti-SIN E1 Mab 30.11a (Chanas et al., 1982) diluted 1:400 in PBT and filtered through a .45 µm acetate filter (Pall Corp., Ann Arbor, Michigan). Following incubations in both primary and secondary antibodies, tissues were washed several times in another PBT solution containing less Triton X-100 (1X Ashburners PBS, 1% BSA, 0.1% Triton X-100). Midguts were washed twice by gently rocking for 20 minutes at rt, and then a third wash was done overnight at 4°C. The tissues were then incubated in Alexa Fluor goat anti-mouse IgG (Molecular Probes Inc., Eugene, Oregon) (1:400) for 3-4 hours at rt. This was followed by incubation in Alexa Fluor phalloidin F-actin probe (Molecular Probes) (1:40 in PBS + 1% BSA) for 20 minutes at rt. Midguts were washed twice at rt and then incubated in TO-PRO-3 nucleic acid dye (Molecular Probes) (1:500 in PBS), which was used as a nuclear counter stain, for 5 minutes. Midguts were given a final rinse in PBS. Finally, the midgut sheets were mounted in Mowiol (Aldrich Chemical Company Inc., Milwaukee, Wisconsin) mounting medium [prepared as described in Lane and Harlow, 1990], which was allowed to polymerize overnight. Fluorescence analysis and imaging were carried out using a fluorescent microscope (Olympus F-IX70, with 10X, 20X and 60X objectives), as well as an integrated confocal imaging system [Olympus FVX-IHRT Fluoview confocal laser

scanning microscope (LSM)] with the necessary software to perform data acquisitions and manipulations such as three dimensional reconstruction, stereo imaging, and quantitative analysis.

IFA Analysis of Whole Mosquito Midgut Dissections

Infected or control mosquito midguts were dissected. Midguts were placed in a microtube containing paraformaldehyde (4%, 1X PBS) for at least 2 hours. The paraformaldehyde solution was removed and the midguts were rinsed once in a PBS solution containing Triton X-100 (1X Ashburners PBS, 0.005% Triton X-100). The amount of detergent added to the PBS was not sufficient to permeabilize the midgut tissue, but was added to prevent the midguts from sticking to the walls of the microtube and drying out. This PBS-detergent solution was used for all subsequent incubations and rinsing. Midguts were incubated in mouse anti-SIN E1 Mab 30.11a (1:200) for 40 minutes at 37°. Tissues were then rinsed twice, and incubated with biotinylated sheep anti-mouse antibody (Amersham Corp., Arlington Heights, IL) (1:200) for 40 minutes at 37°C. The midgut tissues were again rinsed twice and incubated with fluorescein isothiocynate (FITC)-conjugated streptavidin (Amersham) (1:200) for 20 minutes at 37°C. After two final rinses, midguts were mounted in Mowiol mounting medium, which was allowed to polymerize overnight. Fluorescence analysis and imaging were carried out using a fluorescent microscope (Olympus BH2, with 10X, 20X and 40X objectives).

Mosquitoes

Ae. aegypti Rexville D (AaRexD) strain mosquitoes originating from Rexville, Puerto Rico (Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO) were reared at 28°C, 80% relative humidity, and

photoperiod of 16 hours L: 8 hours D. Adult mosquitoes were provided with sugar cubes and water.

Per Os Infection of Mosquitoes by Artificial Blood Meal

Confluent monolayers of C6/36 cells were infected with TR339 electroporation supernatant from BHK-21 cells at an MOI of 0.01. Infected cells were maintained at 28°C (5% CO_2) in MEM containing 10% fetal bovine serum, non-essential amino acids, L-glutamine, and antibiotics. Approximately 60 hours post-infection, the infected cells were scraped into the medium. The infected cell suspension was harvested, and clarified by centrifugation at 1000 g for 3 minutes. Aliquots of the supernatant were stored at –70°C. Virus working stock was plaque titrated on Vero cell monolayers as previously described to determine virus concentration. *Per os* infection of mosquitoes was described in Chapter 2 (*Per Os Infection of Mosquitoes by Artificial Blood Meal*). Mosquitoes (5-7 days post-eclosion) were allowed to feed for approximately 1 hour through a previously frozen (-20°C) hog gut membrane, rinsed in distilled water. Blood meal samples were collected post-feeding for virus titration. Following the blood meal, mosquitoes were cold-anesthetized, and only individuals that had ingested the blood meal were retained for further analysis. Blood fed mosquitoes were housed at the species-specific insectary conditions described previously and provided water and sugar until analyzed (Higgs and Beaty, 1996). Additionally, control *Ae. aegypti* mosquitoes were intrathoracically inoculated with 1.0 μl of virus (positive control) or MEM containing 10% fetal bovine serum, non-essential amino acids, L-glutamine, and antibiotics, without virus (negative control), and maintained at insectary conditions until analyzed (Gubler and Rosen, 1976a).

Transmission Studies

Five- to seven-day old female mosquitoes were given an artificial blood meal containing either MRE16 or TR339 virus as described previously. Control mosquitoes were fed on defibrinated sheep blood (Colorado Serum Co)/MEM containing 10% fetal bovine serum, non-essential amino acids, L-glutamine, and antibiotics (1:1). Male mosquitoes were introduced into the cages containing the separated blood-fed females. Mosquitoes were provided with sugar, water, and oviposition containers. Several days prior to feeding single female mosquitoes were separated from male mosquitoes and oviposition containers, but were again provided with sugar and water. The sugar was removed 48 hours before feeding. Twelve days after ingestion of the infected blood meal, females were fed individually on 2-3 day old mice (ICR (CD-1) strain, Charles River Laboratories, St. Constant, Quebec). Individual mice were placed in the cages of single mosquitoes. Mice that were observed being probed by the mosquito were returned to their cages. Controls consisted of (a) neonatal mice injected subcutaneously with virus and exposed to uninfected mosquitoes, and (b) uninfected non-exposed neonatal mice. Immediately following the transmission attempts, mosquitoes that had been observed probing were cold-anesthetized, and leg squashes were prepared. The remainder of the mosquito was frozen at –70°C. Frozen mosquitoes were later triturated separately in 1 ml of diluent and assayed for virus as described below in *Trituration of Whole Mosquitoes.* Two days after the transmission attempts, mice were sacrificed and placed at –70°C. The brains of the mice were later homogenized by aspiration in 20% (wt:vol) of diluent (MEM containing 2% FBS plus NEAA, L-glutamine and antibiotics) (Gould and Clegg,

1985). Presence of virus in the mouse brains was determined by plaque assay in Vero cells as previously described.

Isolation of virus from a mouse brain indicated transmission. Recovery of virus from a mosquito body tissue suspension indicated infection. Transmission rates were calculated based on transmission to mice from infected mosquitoes. IFA assay of leg squash preparations indicated the number of mosquitoes from each group considered to have infections limited to the midgut (non-disseminated).

Trituration of Whole Mosquitoes

Mosquitoes were triturated with a pestle in 100 µl of diluent (MEM containing 10% fetal bovine serum, non-essential amino acids, L-glutamine, and antibiotics) until no recognizable parts remained. Additional diluent was added to a total volume of 1 ml. The mosquito triturate was passed through a sterile (0.2 µm pore-size) filter (Pall Corp., Ann Arbor, Michigan), and the PFU/ml was determined by plaque assay as previously described.

Virus Assay in Orally Infected Mosquitoes

Adult *Ae. aegypti* (5-7 days post eclosion) female mosquitoes were given an artificial blood meal containing either MRE16 or TR339 virus as described previously. Control mosquitoes were fed on defibrinated sheep blood (Colorado Serum Co)/MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics (1:1). The oral infectivity of each virus was scored as the ability of the virus to infect and disseminate from the mosquito midgut. At least 30 midguts were dissected from each group of mosquitoes ingesting an infectious blood meal on days 2, 4, 6, 8, 10, 12, and 14. In addition, seventeen midguts were dissected from each group of mosquitoes ingesting an infectious

blood meal at 24 hours post-infection. Mosquito midguts were prepared and analyzed by IFA for SIN antigen. Beginning at 2 days post-infection, the distribution of antigen in each posterior midgut examined was arbitrarily scored as light, moderate, or heavy. The presence or absence of SIN antigen in other tissues was also recorded. Flat sheets of midgut tissue (24 hour post-infection) were prepared in order to characterize early infection of the midgut with each virus. Mosquito head squashes were prepared from at least 30 mosquitoes infected with each virus in a separate feed on days 2, 4, 6, 8, 10, 12, and 14. Head squash preparations were analyzed by IFA for the presence of SIN antigen and dissemination rates were determined as the percentage of infected mosquitoes displaying SIN antigen in head tissues.

Results

Growth of MRE16 and TR339 Viruses in BHK-21 and C636 Cells

Growth curve experiments were performed to examine the differences in virus replication between MRE16 and TR339 viruses in different host cell types. Growth curves for the MRE16 and TR339 viruses indicated that both viruses replicated with nearly equal efficiency in both BHK-21 and C636 cells (Fig. 3.1 and 3.2). In BHK-21 cells maximum titers of 8.8-8.9 $\log_{10}$ PFU/ml were reached between 36-48 hours post infection. For C6/36 cells titers of 9.5 – 10.2 $\log_{10}$ PFU/ml were achieved at 48 –60 hours post infection.

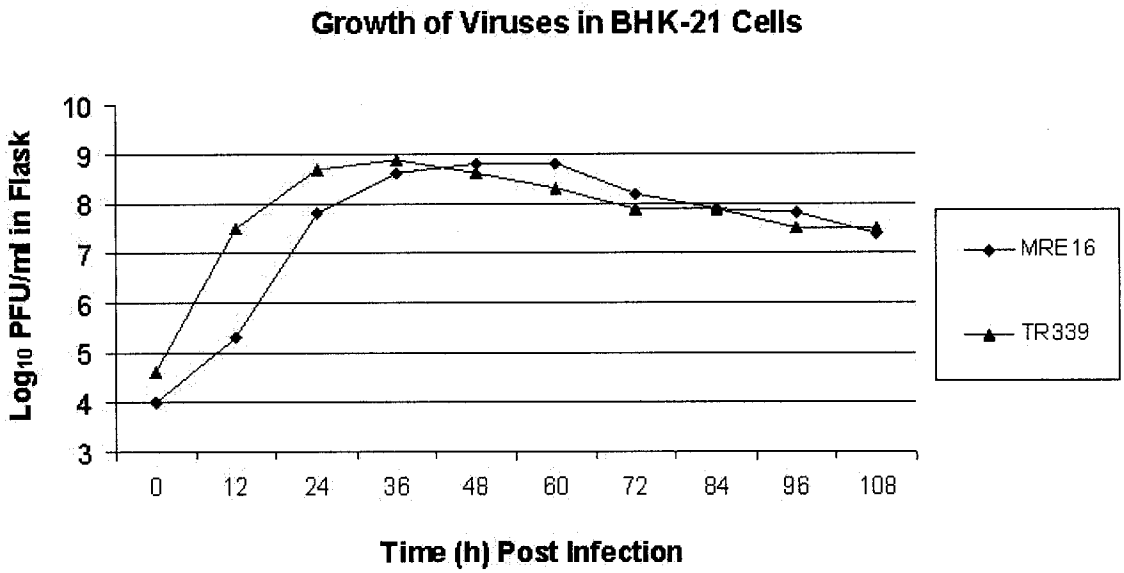

Figure 3.1. Comparative growth rates of MRE16 and TR339 viruses in BHK-21 cells. Viruses were grown in 25-cm^2 tissue culture flasks of BHK-21 cells. Multiplicity of infection was approximately 0.01 PFU/cell.

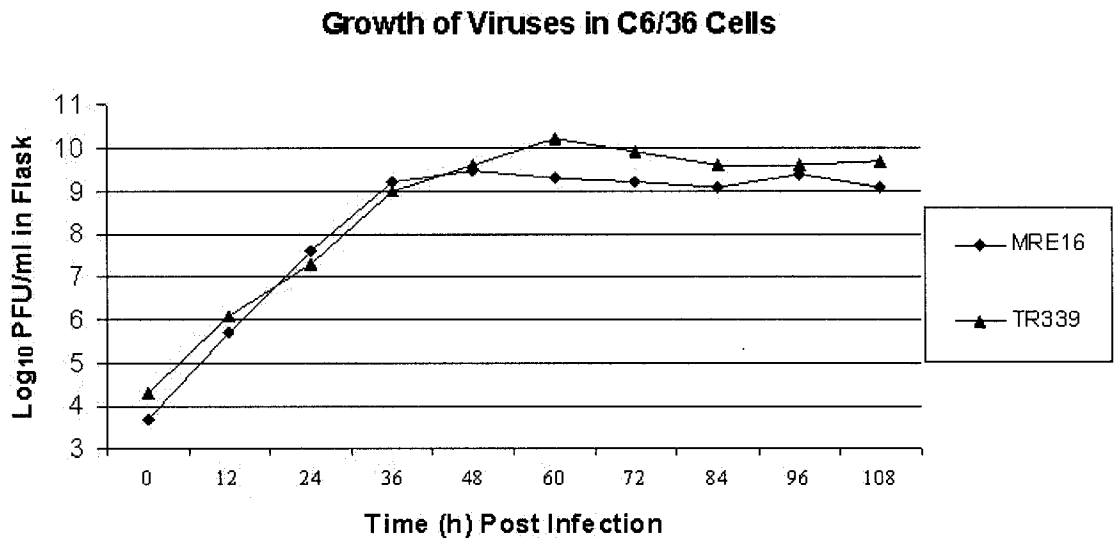

Figure 3.2. Comparative growth rates of MRE16 and TR339 viruses in C6/36 cells. Viruses were grown in 25-cm^2 tissue culture flasks of C6/36 cells. Multiplicity of infection was approximately 0.01 PFU/cell.

Immunofluorescent Analysis of Mosquito Midgut Tissues

Mosquito midguts were assayed for the presence of MRE16 or TR339 virus. Midguts were dissected from mosquitoes that ingested a blood meal containing either $10^{9.6}$ PFU/ml of MRE16 virus or $10^{9.3}$ PFU/ml of TR339 virus and analyzed by IFA. Significant fluorescence was not observed in any of the negative controls. Seventeen midguts were examined from both groups at 24 hours post-infection and each exhibited some level of infection. For both viruses, multi-focal infection of the posterior midgut epithelial cells was observed from the lumenal side (Figure 3.3 A, B, C, and D) of each midgut. However, infection with MRE16 virus appeared to be more efficient. The number of foci observed on each midgut was counted and the mean and standard deviation (SD) was calculated. The mean number of foci ± SD observed in a midgut infected with MRE16 virus was 42.1 ± 30.4 and ranged from 4-110. In TR339 virus infected midguts, the number of foci observed ranged from 3-33 with a mean of 12.4 ± 8.4. Early infection with both viruses appeared to be nearly always confined to the posterior midgut. Rarely, a focus of infection could also be seen in the anterior midgut. The distribution of foci within the posterior midgut appeared to be random with no discernable pattern or polarity.

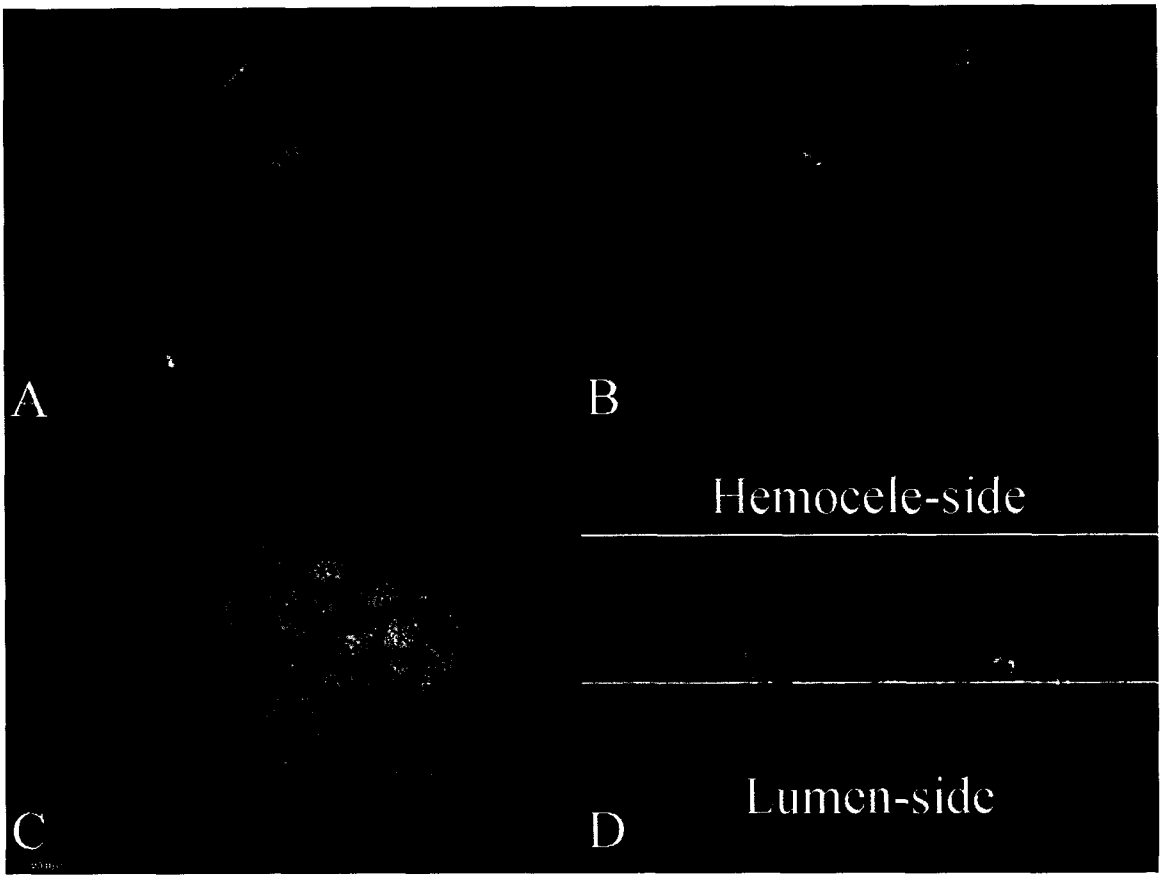

Figure 3.3. Sheets of mosquito midgut epithelium assayed for the presence of MRE16 or TR339 virus at 24 hours post-infection. An infection of the midgut begins the same way for both MRE16 virus (A) and TR339 virus (B), with discrete foci of infection, visible from the luminal side. However, a greater number of foci are seen in MRE16 infected midguts. (C) A discrete focus of MRE16 infected midgut cells. The view is looking down from the basal side of the midgut. The musculature is not shown in this image. (D) A lateral view of the same MRE16 virus infected midgut cells shown in panel C (Color key: Cell nuclei are shown in red, the musculature surrounding the midgut is shown in blue, and SIN E1 antigen is shown in green.). (Panel A and B Olympus F-IX70 fluorescent microscope; original magnification 125X; Panel C and D Olympus FVX-IHRT Fluoview confocal LSM).

The distribution of SIN virus-specific antigen in midgut tissues was analyzed at 48-hour intervals, and the results are summarized in Figures 3.4 and 3.5.

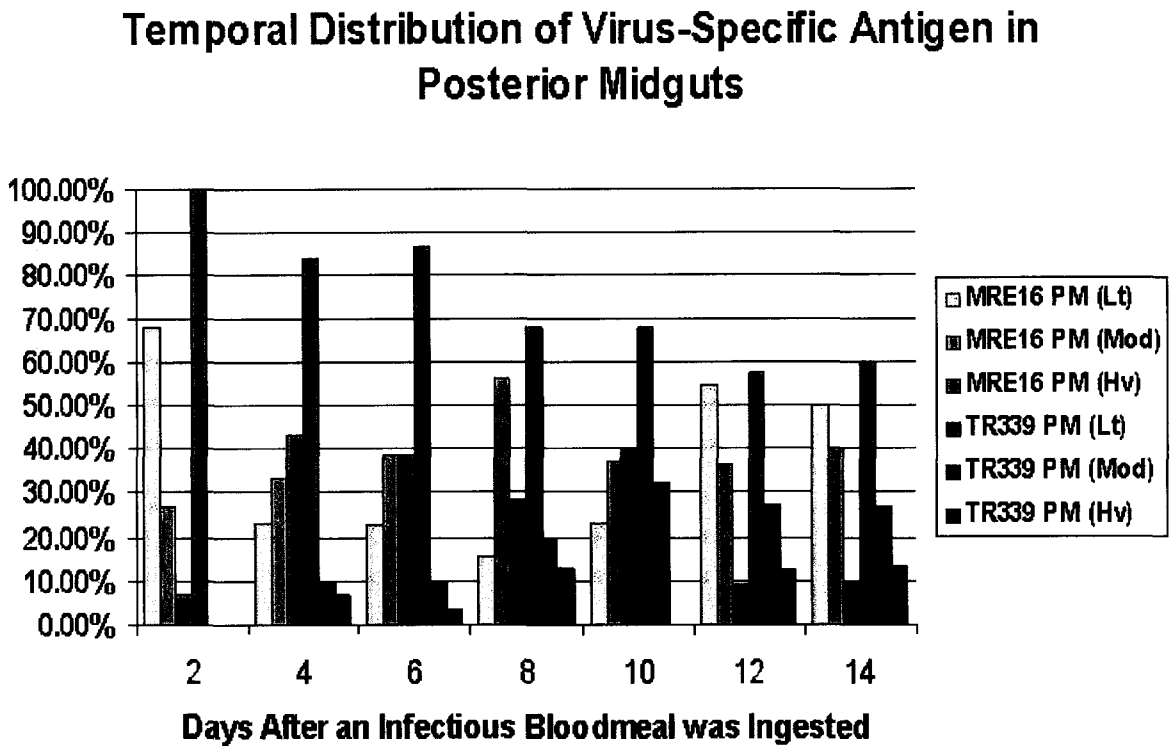

Figure 3.4. Temporal distribution of SIN virus-specific antigen in posterior midguts from MRE16 or TR339 virus infected *Ae. aegypti.* The course of MRE16 or TR339 virus infection in posterior midguts (PM) of *Ae. aegypti* mosquitoes following oral exposure. The distribution of SIN virus-specific antigen observed in the posterior midgut epithelial cells was scored as being either light (Lt), moderate (Mod), or heavy (Hv). The total number of mosquito midguts with a score of Lt, Mod, or Hv at a given time point after oral exposure is expressed as a percentage of the total midguts examined ($n \geq 30$).

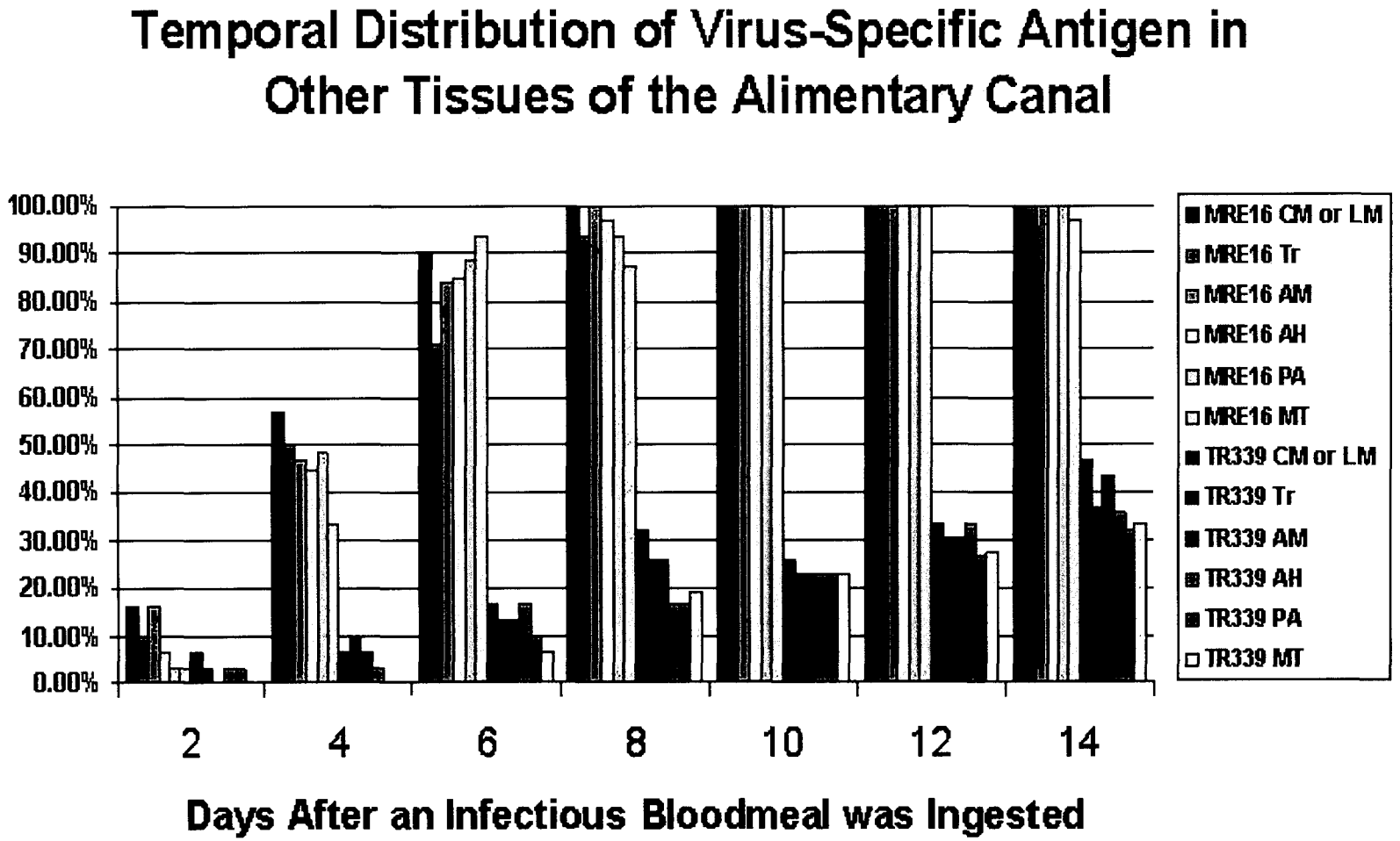

Figure 3.5. Temporal distribution of SIN virus-specific antigen in other tissues of the alimentary canal from MRE16 or TR339 virus infected *Ae. aegypti.* The course of MRE16 or TR339 virus infection in other tissues of the alimentary canal of *Ae. aegypti* mosquitoes following oral exposure. Other tissues of the alimentary canal such as the circular or longitudinal musculature (CM or LM), trachea (Tr), anterior midgut (AM), anterior hindgut (AH), pyloric ampulla (PA), and malpighian tubules (MT) were scored as being either positive or negative for the presence of SIN virus-specific antigen. The total number of positives according to tissue at a given time point after oral exposure is expressed as a percentage of the total number of tissues examined ($n \geq 30$).

The distribution of SIN virus-specific antigen observed in the posterior midgut epithelial cells was scored as light, moderate, or heavy. A score of light was given if antigen was detected in less than half of the visible surface area of the midgut (Fig 3.6 A). Midguts were assigned a score of moderate if antigen could be seen in one-half to three-quarters of the visible surface area (Figure 3.6 B), and heavy if antigen distribution was greater than three-quarters (Figure 3.6 C). Midguts in which the distribution of observed antigen was borderline were assigned the lower of the two scores. SIN virus-specific antigen appeared to radiate from initial foci of infection in midguts infected with either MRE16 or TR339 virus. When foci could be distinguished at time points later than 24 hours post-infection, they were generally larger than those observed earlier. Virus appeared to radiate more rapidly in the MRE16 virus-infected midguts, whereas infection with TR339 virus appeared to be more self-limited. Two days after infection, distinct foci were discernable only rarely in MRE16 virus infected midguts but were frequently discernable in midguts infected with TR339 virus. Most mosquitoes that ingested either MRE16 (21 of 30 or 67.7%) or TR339 (30 of 30 or 100%) virus displayed viral antigen that was only lightly distributed in the midgut at 2 days post-infection (Figure 3.4). However, the number of TR339 virus infected midguts displaying light distributions of antigen was significantly greater than the number of MRE16 virus infected midguts displaying light distributions of antigen at this early time point ($P = 0.002$, Fisher exact test). Four days after infection only 23.3% (7 of 30) of the midguts from mosquitoes that ingested MRE16 virus were lightly infected as compared to 83.9% (26 of 31) for TR339 virus (Figure 3.4). Eight days after infection a maximum of 84% (27 of 32) of the MRE16 virus infected midguts exhibited moderate to high distributions of viral antigen, as

compared to a maximum of only 40% (13 of 33) at 12 days for TR339 virus (Figure 3.4). Viral antigen began to wane in MRE16 virus infected midguts by day 12 post-infection, suggesting that infection of the midgut epithelial cells may be transient (Figure 3.4). The distribution of virus-specific antigen was increasing or relatively constant in TR339 virus infected midguts throughout the time course (Figure 3.4).

Other organs of the alimentary canal also were found to contain viral antigen at various time points (Figure 3.5). These organs included musculature (Figure 3.6 E), trachea (Figure 3.6 F), foregut, hindgut, pyloric ampulla, and malpighian tubules (Figure 3.6 D). Infection of the trachea and musculature indicated that the virus had escaped the midgut epithelial cells. Midgut escape rates can be determined by analysis of these tissues and roughly correlate with dissemination rates determined from IFA analysis of head tissues at similar time points (Figures 3.5 and 3.7). Infection of the foregut, hindgut, pyloric ampulla, and malpighian tubules also roughly correlated with dissemination to the head, although not as well as infection of the trachea and musculature. Infection of the foregut and hindgut was characterized by the presence of antigen in the associated musculature, epithelial cells, or both for each of the viruses. Both uninfected and infected malpighian tubules, sometimes adjacent to each other, were commonly observed attached to the same midgut. Antigen was also frequently seen in just part of a tubule, sometimes with uninfected tissues flanking either side, but most often near the tubules attachment at an infected pyloric ampulla.

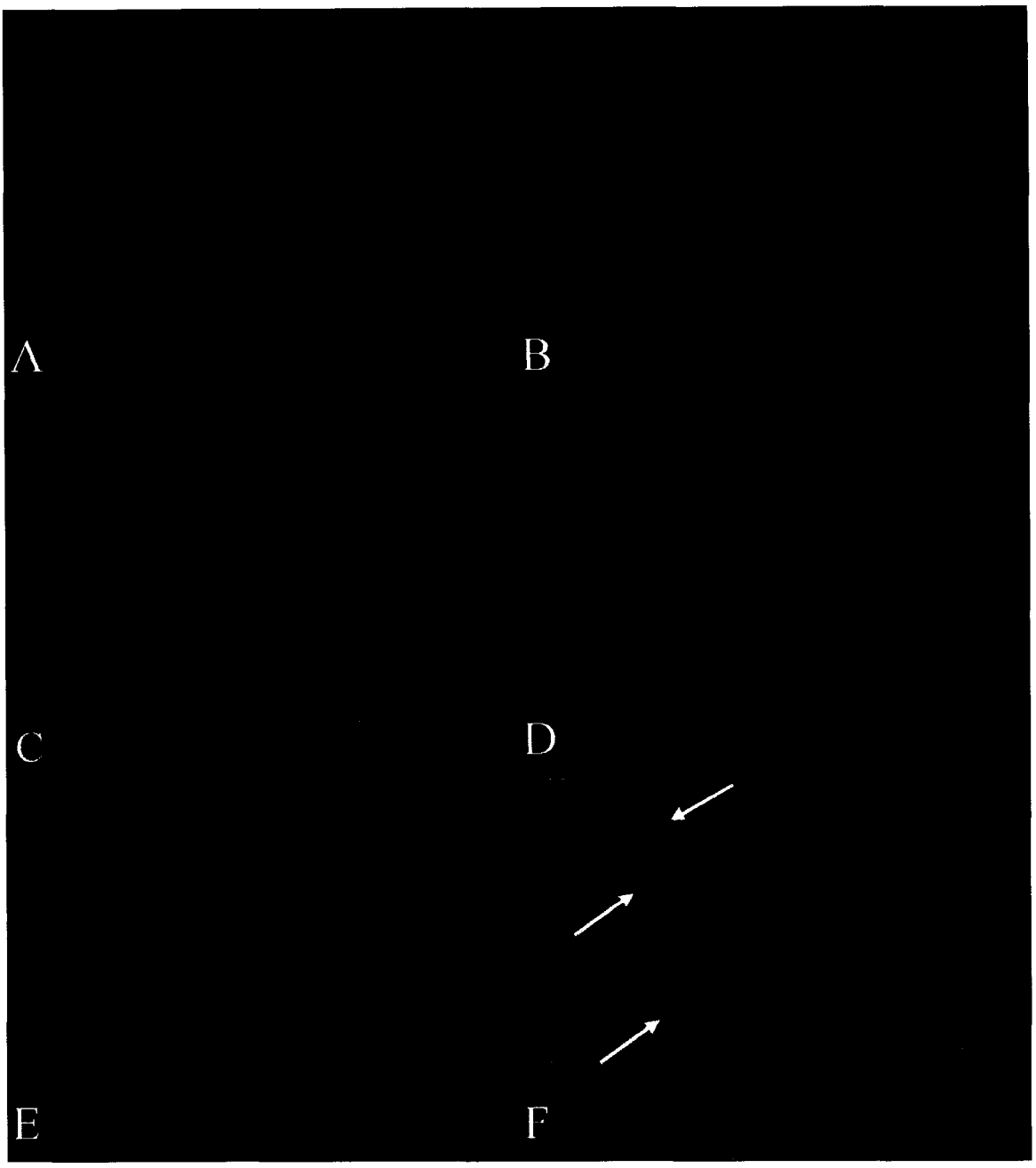

Figure 3.6. Intact mosquito midguts assayed for the presence of MRE16 or TR339 virus at various times post-infection (Panels A, B, C, and D). Sheets of mosquito midgut epithelium assayed for the presence of MRE16 or TR339 virus at 60 hours post-infection (Panels E and F). Composite images of mosquito midguts infected with either MRE16 (A and C) or TR339 (B) virus and demonstrating low (A), moderate (B), or heavy distributions (C) of SIN E1 antigen. (D). A composite image showing antigen distribution in a MRE16 virus infected midgut displaying a disseminated infection. MRE16 or TR339 virus-specific antigen was typically detected in trachea, musculature, anterior midgut, hindgut, pyloric ampulla, and malpighian tubules in midguts displaying an infection that had disseminated from midgut epithelial cells. (SIN E1 antigen shown in green). (E) TR339 infected circular and longitudinal musculature associated with posterior midgut. (F) Numarsky image of infected trachea associated with MRE16 infected posterior midgut. (Color key: Cell nuclei are shown in blue, the musculature surrounding the midgut is shown in red, and SIN E1 antigen shown in green.). (Panel A, B, C, and D Olympus F-IX70 fluorescent microscope; original magnification 125X; Panel E and F Olympus FVX-IHRT Fluoview confocal LSM).

Immunofluorescent Analysis of Mosquito Head Tissues

A preliminary oral infection trial of the AeRexD strain showed that MRE16 virus disseminated from the midgut of these mosquitoes with greater efficiency than did TR339 virus (Figure 3.7). For MRE16 virus, greater than 90% (28 of 30) of mosquitoes assayed were positive for a disseminated infection by 8 days post-infection and 100% (30 of 30) by 12 days. A maximum of 37% (11 of 30) of AeRexD mosquitoes had a disseminated TR339 virus infection at 12 days post-infection. Disseminated virus infections were detected as soon as 2 days after oral exposure to MRE16 virus. Disseminated TR339 virus was first detected at 6 days post infection. Therefore, dissemination of virus from MRE16 virus infected *Ae. aegypti* midguts is more rapid, and the maximum number of MRE16 virus infected mosquitoes developing disseminated infections is significantly greater than the maximum number of TR339 virus infected mosquitoes that develop disseminated infections ($P < 0.001$, Fisher exact test).

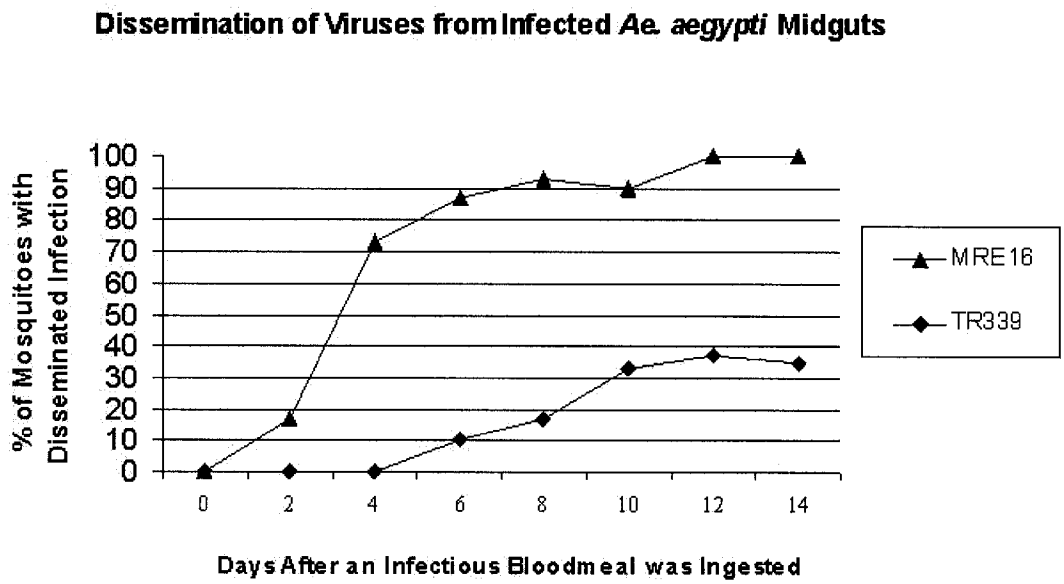

Figure 3.7. Comparative disseminated infection rates of MRE16 and TR339 viruses in *Ae. aegypti* mosquitoes. The percentage of mosquitoes orally exposed to either MRE16 or TR339 viruses with disseminated infections (e.g. positive for SIN-specific antigen by IFA of head tissues) at timed intervals ($n \geq 30$).

Transmission to Mice

The transmission potential of AaRexD mosquitoes for MRE16 and TR339 virus was analyzed using neonatal mice. Mosquitoes ingested a blood meal containing either $10^{9.6}$ PFU/ml of MRE16 virus or $10^{9.3}$ PFU/ml of TR339 virus. Each of the *Ae. aegypti* used in the transmission experiments developed at least a midgut infection. The titer of virus recovered from each mosquito is given in Table A.3 (Appendix A). A significantly greater number of mosquitoes exposed to MRE16 virus (14/14 or 100%) had disseminated infections compared with mosquitoes exposed to TR339 virus (10/16 or 62.5%) ($P = 0.019$, Fisher exact test). Furthermore, 86% (12/14) of mosquitoes infected with MRE16 virus transmitted virus to mice compared with 37.5% (6/16) of mosquitoes infected with TR339 virus. The transmission of virus to newborn mice was significantly higher by MRE16 virus infected *Ae. aegypti* than by TR339 virus infected mosquitoes ($P = 0.011$, Fisher exact test), despite the presence of nearly equivalent MRE16 and TR339 viral titers in the respective blood meals ingested. Mosquitoes exhibiting a ME barrier were infected, but did not develop a disseminated infection as determined by IFA analysis of leg tissues for SIN-specific antigen. ME barriers were observed only in TR339 virus infected *Ae. aegypti* (6 of 16), and not in any of the MRE16 virus infected mosquitoes (0 of 14). A second barrier was also present at the salivary glands (SGI or SGE barrier), as not all of the mosquitoes with disseminated infections transmitted virus. Excluding mosquitoes with a ME barrier, the salivary gland barrier was present in 14.3% (2 of 14) of MRE16 virus infected *Ae. aegypti* and 40% (4 of 10) of TR339 virus infected mosquitoes, not a significantly different result ($P = 0.192$, Fisher exact test). Therefore, excluding mosquitoes with a salivary gland barrier, the different transmission potentials

of AeRexD infected with either MRE16 virus (12 of 12) or TR339 virus (6 of 12) can be primarily attributed to a significant difference in the expression of a ME barrier in these mosquitoes ($P = 0.014$, Fisher exact test).

Discussion

The transmission potential or rate determined here for TR339 virus infected *Ae. aegypti* (RexD strain) was similar to that determined previously by Jackson, Bowen, and Downe (1993) using the AR339 strain (40% transmission) and a neuroadapted SIN (NSIN) strain (52.4% transmission) in *Ae. aegypti* (N.I.H. strain). Jackson, Bowen, and Downe (1993) also described the sequential infection of organs following peroral infection of *Ae. aegypti* with the AR339 and NSIN strains. We have further characterized infection of the *Ae. aegypti* midgut with SIN viruses following peroral infection because differences observed in the susceptibility of this tissue appeared to have the largest influence on the dissemination and transmission efficiencies of the two SIN viruses used in the present study. The *Ae. aegypti* used in the transmission studies (Table A.3, Appendix A), and nearly all of those used in the IFA analysis of midgut tissues (Tables A.1 and A.2, Appendix A), were infected after ingesting a blood meal containing either MRE16 or TR339 virus using an artificial membrane feeder. The dissemination and transmission potential of these two viruses in *Ae. aegypti* differed significantly. The data suggested that the expression of a classic ME barrier is primarily responsible for the differences observed in dissemination and transmission potential. A second barrier is probably also present in the salivary glands but appears to affect the transmission potential of both viruses nearly equally.

Kramer et al. (1981) were the first to demonstrate the existence of a dose-dependent escape barrier in the midgut by showing that *Cx. tarsalis* were unable to transmit WEE virus, in part due to the inability of the virus to escape the infected midgut. The ME barrier described by Kramer et al. (1981) was found to be dose dependent and occurred only when low doses of virus were ingested. However, ME barriers which are not dose dependent have also been demonstrated for other virus-vector combinations (Turell, Gargan, and Bailey, 1984; Weaver et al., 1984). The molecular nature of the ME barrier is currently unknown. Some have suggested that the ME barrier may be associated with modulation of virus multiplication in infected cells. Kramer et al. (1981) found that WEE viral titers were usually lower in the midguts of female mosquitoes exhibiting a ME barrier than in midguts from competent females or females exhibiting a salivary gland infection barrier. However, this observation does not extend to all virus-vector systems. Miller and Mitchell (1991) found YF virus -susceptible and -resistant lines of *Ae. aegypti* were similar in virus titer for the first five days after oral infection with YF virus. After five days the titer of YF virus continued to increase in virus-susceptible mosquitoes, presumably due to replication in other tissues after dissemination from the midgut, but did not in the resistant line. This led them to conclude that equal levels of virus replication occurred in the midguts of both lines. Gubler and Rosen (1976b) found similar titers of DEN-2 virus in mosquitoes that varied in their susceptibility to the virus after oral infection. Consistent with both of these findings, Bosio, Beaty, and Black (1998) found that the amount of DEN-2 virus present in the midguts of two strains of *Ae. aegypti*, differing markedly in DEN-2 virus susceptibility, did not influence if or how much virus disseminated from the midgut. It was not possible to determine if the amount

of virus in the midgut influenced escape of virus from the midgut in the present study. A more quantitative analysis of this relationship represents a future area of research. However, IFA analysis did reveal that a number of mosquitoes with lightly infected posterior midguts also had disseminated infections (Table A.1 and A.2) suggesting that widespread infection of midgut epithelial cells is not necessarily required for dissemination from the midgut. Nevertheless, the modulation of alphavirus titers has been described in mosquito cell cultures (Brown and Condreay, 1986) and in mosquito midgut cells. Some aspects of infection in mosquito cell cultures with SIN virus and SF virus appear similar to midgut infection in susceptible mosquitoes (Hardy et al., 1983). Both infections begin with an initial acute phase, characterized by high viral titers, which is then somehow modulated, resulting in lower titers and the establishment of a persistent infection (Hardy et al., 1983). Kramer et al. (1981) reported mean WEE virus titers in *Cx. tarsalis* mosquitoes after oral infection with high concentrations of virus. An initial increase in titer was followed by a decrease, and then leveled off at a titer ten-fold lower than peak titer. The same pattern was not observed in mosquitoes fed on lower titers of the same virus but equivalent titers were eventually reached. This could be explained by differences in the number of midgut epithelial cells initially infected. Gradual decreases in the number of mature virions in mosquito midguts infected with EEE or SLE virus has been observed over time in studies using EM (Murphy et al., 1975; Whitfield, Murphy, and Sudia, 1971; Whitfield, Murphy, and Sudia, 1973). Results obtained in the current study are consistent with the concept that alphavirus titers are somehow modulated in the mosquito midgut. IFA analysis of midgut tissues revealed that MRE16 virus was usually associated with a more widespread infection of the epithelial cells than TR339 virus.

Virus appeared to radiate more rapidly from initial foci of infection in the MRE16 virus infected midguts than in TR339 virus infected midguts. The observation that the levels of viral antigen began to decrease in MRE16 virus infected midguts at 12 days post infection also suggests that a mechanism of modulation exists in the midgut cells of AaRexD mosquitoes. Several hypotheses have been proposed to explain the mechanism of viral modulation in mosquito cells (Murphy, 1975; Murphy et al., 1975; Stollar, 1980). Riedel and Brown (1979) recovered a virus specific, low molecular weight material from the extracellular fluids of SIN virus infected *Ae. albopictus* cells that interfered with virus multiplication. Similarly Lee and Schloemer (1981a; 1981b) reported that *Ae. albopictus* cells infected with Banzi virus (*Flaviviridae*) produced a protein possessing anti-viral properties specific to the virus. The protein was found to be identical to a viral polypeptide and was postulated to act by regulating viral RNA synthesis. However, attempts to find similar substances using other viruses and in other mosquito cell types have been unsuccessful (Hardy et al., 1983; Newton, Short, and Dalgarno, 1981). Other studies using SIN virus have shown a reduction in viral RNA synthesis in persistently infected mosquito cells (Stollar, 1980; Tooker and Kennedy, 1981). Indirect evidence supports a hypothesis in which the proposed "modulating factors" are under the control of the mosquito and not the virus [reviewed in Hardy et al., 1983].

The phenomenon of RNA interference (RNAi) was first described in *Caenorhabditis elegans* and resulted in the silencing of a specific gene sequence following the introduction of homologous double-stranded RNA (dsRNA) (Fire et al., 1998). It was quickly realized that RNAi might be related to the homology dependent gene silencing mechanisms described in plants several years earlier. The phenomenon,

called co-suppression or post transcriptional gene silencing (PTGS) in plants, had been observed both following the introduction of transgenes homologous to endogenous loci (Jorgensen, 1990; Napoli, Lemieux, and Jorgensen, 1990; van der Krol et al., 1990), and in response to infection with RNA viruses (Angell and Baulcombe, 1997; Dougherty et al., 1994; Ruiz, Voinnet, and Baulcombe, 1998). The observation of virally induced gene silencing suggested that the mechanism might play a central role in pathogen resistance. This hypothesis was supported by the identification of suppressors of gene silencing in many plant viruses (Baulcombe, 1999; Voinnet, Lederer, and Baulcombe, 2000), and the discovery that mutations in the silencing machinery of the host can also influence viral pathogenesis (Baulcombe, 1999; Mourrain et al., 2000). Similar gene silencing phenomena have also been described in *Neurospora* (Romano and Macino, 1992), and *Drosophila* (Pal-Bhadra, Bhadra, and Birchler, 1997). Genetic and biochemical studies are only just beginning to reveal the similarities and differences of the individual phenomena. However, results would seem to indicate that each is mechanistically related, and that dsRNA appears to be a common trigger. The discovery of similar mechanisms in such a diverse range of organisms may indicate that each stems from a single ancient evolutionary origin, and may in fact be present in some form in many, if not all, eukaryotes. Both DEN-2 derived sense and anti-sense premembrane (prM) RNA expressed from dsSIN viruses have been shown to interfere with DEN-2 virus replication in mosquito cells (Gaines et al., 1996). The interference was specific because the replication of DEN-3 virus and DEN-4 virus were not inhibited (Gaines et al., 1996). SIN viruses expressing DEN-2 derived anti-sense prM RNA have also been used to transduce female *Ae. aegypti* (Olson et al., 1996). These mosquitoes were unable to support

replication of DEN-2 virus in their salivary glands when challenged with the virus (Olson et al., 1996). It is possible that the interference observed in these studies was mediated by an RNAi-like mechanism, as the replication of SIN viruses includes double stranded intermediates. Studies by Caplen et al. (2002) and Adelman et al. (2001) have recently demonstrated the presence of an RNAi mechanism in both mosquitoes (*Ae. aegypti*) and mosquito cells (C6/36). One can then speculate that an RNAi mechanism might be responsible for the modulation of alphaviruses that has been observed in mosquito cell cultures and in mosquito midgut cells.

If modulation of the TR339 SIN strain occurs in the midguts of AaRexD mosquitoes, our results indicate that the MRE16 SIN strain may not be as susceptible to the effects of the mosquito's "modulating factors". It is not clear if the efficiency of initial infection of the midgut epithelium, which appears to be more efficient with MRE16 virus than with TR339 virus, contributes to differences in dissemination potential. Perhaps more efficient infection by MRE16 virus allows the virus to initially overwhelm the ME barrier mechanism before the infection can be brought under control. This might also explain how the expression of some dissemination barriers can be overcome by a high dose of virus (Kramer et al., 1981).

Detection of viral antigen in midgut-associated musculature and trachea indicated that virus had escaped the midgut and disseminated to other tissues, and correlated well with the dissemination rates determined for the two viruses. We have previously observed infection of these tissues following intrathoracic inoculation of *Ae. aegypti* with both viruses (Myles and Olson, unpublished data). The spatial and temporal progression of SIN virus infection following intrathoracic inoculation has also been described by

Bowers, Abell, and Brown (1995) in the mosquito *Ae. albopictus*. Infection of gut visceral muscles and trachea were also observed in that study. Prior to dissemination of virus from the midgut, SIN virus-specific antigen was nearly always confined to the epithelial cells of the posterior midgut. Detection of SIN antigen in other tissues of the midgut correlated well with its appearance in the trachea and musculature. Whether the trachea or gut-associated musculature was infected after dissemination of virus from the midgut or was involved in facilitating virus dissemination remains ambiguous.

A significant ME barrier does appear to exist for TR339 virus in this strain of *Ae. aegypti,* as the detection of viral antigen indicated that nearly 100% (232/233) (Table A.1, appendix A) of mosquitoes ingesting an infectious blood meal were infected, although the level of the infection in the midgut epithelium was typically lower than that observed with MRE16 virus. It is clear that dissemination, and thus transmission, of this virus within AaRexD mosquitoes is largely influenced by virus genetics.

Studies by Beaty et al. (1981) showed that the failure of an arbovirus to be transmitted from infected mosquito salivary glands may be largely determined by the genetics of the virus. Studies such as these demonstrate that viral determinants can have a significant influence on the infectivity and transmissibility of arboviruses by mosquitoes. The epidemiological significance of this is that the genetics of the arbovirus may play a large role in determining which species and strains of mosquito will act as competent vectors. Studies by Beaty et al. (1982) found that the determinants of dissemination and horizontal transmission for the arboviruses Lacrosse (LAC) (*Bunyaviridae*) and snow shoe hare (SSH) (*Bunyaviridae*) likely reside in the M segment RNA encoding the envelope glycoproteins. Both LAC and SSH viruses efficiently infected the midgut of *Ae.*

triseriatus mosquitoes. However, LAC virus disseminated from 100% of midguts, whereas SSH virus disseminated from only 17%. Reassortant SSH viruses containing the M segment of LAC virus were found to disseminate efficiently from the midgut, whereas reassortant LAC viruses with the M segment of SSH did not (Beaty et al., 1982). In another study, intrathoracically inoculated reassortant viruses containing the M segment of LAC virus were transmitted efficiently by *Ae. triseriatus* mosquitoes, whereas reassortants with the M segment of SSH were not, despite the fact that the salivary glands became infected (Beaty et al., 1981).

We have also shown that the primary determinants of MRE16 virus dissemination from the midgut of *Ae. aegypti* appear to be encoded in the E2 glycoprotein gene of the virus. A chimeric cDNA infectious clone (MRE1004) based on the non-structural, structural and *cis*-acting sequences of an AR339 variant and the E2 glycoprotein gene of SIN MRE16 virus produced virus that efficiently and rapidly infected midgut tissues and disseminated in greater than 80% of *Ae. aegypti* mosquitoes within 14 days (Olson, unpublished data). In addition, Brault, Powers, and Weaver (2002) recently used chimeric Venezuelan equine encephalitis (VEE) viruses to demonstrate the importance of the E2 envelope glycoprotein gene for infection of the epizootic mosquito vector *Ae. taeniorhynchus* with epizootic subtypes of VEE virus. The model system we have developed now represents an unprecedented opportunity to further explore how viral genetic determinants influence the expression of a ME barrier in a mosquito vector.

Another objective of this study was to determine the infection patterns and transmission potentials of both MRE16 and TR339 viruses, which have now been developed into SIN virus expression systems (Olson, unpublished data; Klimstra, Ryman,

and Johnston, 1998). Non-heritable SIN expression systems have been used to complement transposon-based DNA transformation systems in mosquitoes. SIN expression systems have been used to express anti-sporozoite/single chain antibodies, anti-mosquito neurotoxins, and anti-bacterial peptides (Cheng et al., 2001; de Lara Capurro et al., 2000; Higgs et al., 1995). These systems have been successfully used to transcribe RNA derived from genetically unrelated arboviruses such as DEN or YF virus to interfere with replication of the homologous virus through a mechanism similar to RNA interference (RNAi) (Adelman et al., 2001; Blair, Adelman, and Olson, 2000; Higgs et al., 1998; Olson et al., 1996). Thus, the dsSIN viruses have become powerful tools for the post-transcriptional silencing of endogenous genes in adult mosquitoes (Shiao et al., 2001).

The present work provides a baseline for the temporal and spatial infection/expression patterns in *Ae. aegypti* of viruses from which these expression systems have been derived. This is knowledge that will certainly aid those using these types of expression systems.

CHAPTER 4

VIRUS PRODUCED FROM A FULL-GENOME LENGTH cDNA CLONE OF THE MALAYSIAN SINDBIS VIRUS, MRE16, EFFICIENTLY INFECTS *AEDES AEGYPTI* AND *CULEX TRITAENIORHYNCHUS* BY THE ORAL ROUTE: IDENTIFICATION AND CHARACTERIZATION OF A VIABLE MRE16 DELETION MUTANT

Introduction

Most medically important arboviruses are transmitted to vertebrate hosts by species of *Aedes* and *Culex* mosquitoes (Karabatsos, 1985; Mackenzie et al., 1994). The general features of arbovirus infection of mosquitoes have been described (Hardy, 1988). The virus enters the lumen of the midgut with ingestion of a blood meal and replicates in the midgut epithelial cells. Virus then escapes the midgut, enters the hemolymph, and disseminates to other tissues, including head and salivary glands. Following multiplication in the salivary glands, virus is transmitted through saliva to a susceptible vertebrate host. However, our understanding of the molecular determinants of vector-pathogen interaction is minimal.

SIN viruses are cycled principally between *Culex* species of mosquitoes and avian vertebrate hosts (Doherty et al., 1977; Doherty et al., 1979; Taylor et al., 1955). However, SIN viruses also have been isolated from *Aedes* species of mosquitoes (Doherty et al., 1979). SIN viruses have a wide distribution in four of the world's six zoogeographic regions, Paleoarctic, Oriental, Australian, and Ethiopian (Calisher and Karabatsos, 1988). The nucleotide sequence of the genome has been determined for several SIN and SIN-like viruses from the Paleoarctic/Ethiopian zoogeographic regions, and several strains have been developed as full-length infectious clones (McKnight et al., 1996; Rice et al., 1987; Simpson et al., 1996).

Infectious cDNA clones based on the alphavirus, SIN, are important tools for identifying virus determinants of infection in *Aedes* and *Culex* mosquitoes. *Ae. aegypti* and *Cx. tritaeniorhynchus* were selected for these studies because they are medically relevant vector species, and they are easy to rear and manipulate in the laboratory. SIN

viruses offer several important advantages in vector studies. The molecular biology and virogenesis of SIN viruses are well understood, SIN virus molecular clones are easy to construct and manipulate and generally present fewer biosafety problems than other arboviruses, including some other alphaviruses, especially when studying virus interactions with an anthropophilic vector. In addition, infectious clones can be manipulated for development of new generations of SIN virus expression systems, which have become increasingly important tools for studying the molecular biology of mosquito vectors (Adelman et al., 2001; de Lara Capurro et al., 2000; Johnson et al., 1999; Shiao et al., 2001).

The glycoproteins of an alphavirus are inserted into a host-derived lipid envelope and are present on the surface of the virion in the form of homotrimeric spikes composed of three E1-E2 heterodimers. The E2 glycoprotein has been shown to contain epitopes important for host tropism, receptor recognition, virus neutralization, and virulence (Dubuisson et al., 1997; Gardner et al., 2000; Klimstra, Ryman, and Johnston, 1998; Levine et al., 1996; Strauss and Strauss, 1994), while the E1 glycoprotein is important in fusion of the virus envelope with host intracellular membranes (Strauss and Strauss, 1994). Residues 170-220 (SIN AR339 numbering) of E2 are postulated to be particularly important for cell binding, and appear to constitute a cell-receptor binding domain in the glycoprotein. Physical properties of the domain and the results of several studies predict that it is exposed on the surface of the viral particle (Davis et al., 1987; Smith et al., 1995; Stec et al., 1986; Strauss et al., 1990; Strauss et al., 1991; Strauss and Strauss, 1994; Wang and Strauss, 1991). The importance of this region to cell binding has been established through anti-idiotypic antibody and virus mutant studies (Mendoza, Stanley,

and Griffin, 1988; Strauss et al., 1991; Ubol and Griffin, 1991; Wang et al., 1991; Wang and Strauss, 1991). Additional studies have demonstrated that residue changes within this domain can affect the ability of an alphavirus to infect different vertebrate cell types (Burness et al., 1988; Strauss and Strauss, 1994; Tucker and Griffin, 1991). Importantly the domain might also be important for productive alphavirus infection of arthropod vectors. Woodward et al. (1991) demonstrated that the infectivity and dissemination of VEE in *Ae. aegypti* mosquitoes could be altered as the result of a single amino acid change within the domain.

The prototype SIN strain, AR339, and viruses derived from this strain have limited infection and transmission potential in *Ae. aegypti*, the vector of YF and DEN viruses. Approximately 40% of *Ae. aegypti* mosquitoes orally infected with AR339 virus developed disseminated infections in head tissues by 14 days post-infection (Seabaugh, 1998). Jackson, Bowen, and Downe (1993) showed only 40% of *Ae. aegypti* mosquitoes orally infected with AR339 transmitted virus to newborn mice. In addition, AR339 virus generally only produces a limited infection of midguts following oral or parenteral infection of *Ae. aegypti* mosquitoes (unpublished data). A SIN virus strain based on the mouse neurovirulent TE12 virus (Lustig et al., 1988) also shows restricted midgut infection in *Ae. aegypti* mosquitoes, with less than 20% dissemination at 14 days post-infection (Seabaugh et al., 1998). These characteristics complicate the use of virus expression vectors based on the AR339 strain of SIN or its derivatives for molecular studies in *Ae. aegypti*. However, a SIN virus strain designated MRE16, from the Oriental/Australian zoogeographic region, readily infects midguts of *Ae. aegypti* and disseminates in nearly 100% of the mosquitoes by 14 days post-feeding (Seabaugh et al.,

1998). A chimeric cDNA infectious clone (pMRE1001) based on the nonstructural and *cis*-acting sequences of an AR339 variant and the structural genes of SIN MRE16 virus produced virus that efficiently and rapidly infected midgut tissues of *Ae. aegypti* and disseminated in greater than 90% of the mosquitoes within 14 days (Olson et al., 2000; Seabaugh et al., 1998).

MRE16 virus represents an intriguing opportunity to explore viral determinants of midgut infection and dissemination in medically important arthropod vectors. The present study reports data that complete the first full genomic sequence of a SIN virus from the Oriental/Australian genetic group, and describes the construction of a full-length MRE16 cDNA infectious clone (MRE16ic) which generates virus that is identical to wild-type MRE16 in its interactions with *Ae. aegypti*. The MRE16 clone was then used to further define the importance of the proposed E2 cell-receptor binding domain to alphavirus midgut infectivity and dissemination in the mosquito vector, as well as viral replication in vertebrate and invertebrate cells.

Materials and Methods

Cells and Medium

BHK-21 cells were grown in MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics at 37°C. C6/36 cells were grown in MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics at 28°C. African green monkey kidney cells (Vero) were grown MEM containing 10% FBS plus NEAA, L-glutamine and antibiotics at 37°C.

Viruses

MRE16 virus was isolated in AP61 cells (Varma, Pudney, and Leake, 1974), passaged six times in AP61 cells and five times in C6/36 cells. The MRE16 virus working stock used was the same as that used in Chapter 3 (*Viruses*). MRE16 virus exhibiting a small plaque phenotype (spMRE16) in plaque assays in Vero cells was plaque purified one time in Vero cell monolayers and then grown in C6/36 cells. The construction of pMRE16ic is described below. The construction of deletion mutants are described below. Briefly contiguous nucleotide base pairs were removed from pMRE16ic in order to generate two cDNA infectious clones, pMRE16ic ΔE200-Y229 and pMRE16ic ΔE200-C220, containing deletions. Clone-derived virus was produced as described previously in Chapter 2 (*in vitro Transcription* and *Electroporation*). Clone-derived virus was passaged once in C636 cells. The MRE16, MRE16ic, MRE16ic ΔE200-Y229, and MRE16ic ΔE200-C220 virus working stocks contained 4.0 X 10^9, 2.5 X 10^9, 4.0 X 10^8, and 1.3 X 10^8 plaque forming units (PFU)/ml, respectively. The spMRE16 virus C6/36-1 seed contained 1.0 X 10^9 PFU/ml. Plaque titrations were performed in Vero cells grown in six- or twelve-well plates as described previously in Chapter 3 (*PlaqueTitrations*).

Virus Growth Curves

Rates of virus growth for MRE16, MRE16ic, MRE16ic ΔE200-Y229, and MRE16ic ΔE200-C220 were compared in BHK-21 and C6/36 cells as described previously in Chapter 3 (*Virus Growth Curves*).

IFA Analysis of Mosquito Tissues

The protocol used for IFA analysis of mosquito head tissue preparations has been described in Chapter 2 (*Immunofluorescent Antibody Analysis of Mosquito Head Tissue Preparation*). The protocol used for IFA analysis of Whole Mosquito Midgut Dissections has been described in Chapter 3 (*IFA Analysis of Whole Mosquito Midgut Dissections*).

Mosquitoes

Ae. aegypti Rexville D (AaRexD) strain mosquitoes originating from Rexville, Puerto Rico (Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO) were reared at 28°C, 80% relative humidity, and photoperiod of 16 hours L: 8 hours D. Adult mosquitoes were provided with sugar cubes and water.

Per Os Infection of Mosquitoes by Artificial Blood Meal

Confluent monolayers of C6/36 cells were infected with MRE16ic, MRE16ic ΔE200-Y229, or MRE16ic ΔE200-C220 electroporation supernatant at an MOI of 0.01. Infected cells were maintained at 28°C (5% CO_2) in MEM containing 10% FBS plus NEAA, L-glutamine, and antibiotics. Approximately 60 hours post-infection, the infected cells were scraped into the medium. The infected cell suspension was harvested, and clarified by centrifugation at 1000 g for 3 minutes. Aliquots of the supernatant were stored at –70°C. Virus working stock was plaque titrated on Vero cell monolayers as previously described to determine virus concentration. *Per os* infection of mosquitoes was described in Chapter 2 (*Per Os Infection of Mosquitoes by Artificial Blood Meal*). Mosquitoes (5-7 days post-eclosion) were allowed to feed for approximately 1 hour through a previously frozen (-20°C) hog gut membrane, rinsed in distilled water. Blood

meal samples were collected post-feeding for virus titration. Following the blood meal, mosquitoes were cold-anesthetized, and only individuals that had ingested the blood meal were retained for further analysis. Blood fed mosquitoes were housed at the species-specific insectary conditions described previously and provided water and sugar until analyzed (Higgs and Beaty, 1996). Additionally, control *Ae. aegypti* mosquitoes were intrathoracically inoculated with 1.0 μl of virus (positive control) or MEM containing 10% FBS plus NEAA, L-glutamine, and antibiotics without virus (negative control), and maintained at insectary conditions until analyzed (Gubler and Rosen, 1976a).

Growth of Viruses in Mosquitoes

Stock viruses were diluted in MEM containing 10% FBS plus NEAA, L-glutamine, and antibiotics to equal titers, prior to intrathoracic inoculation into mosquitoes. *Ae. aegypti* mosquitoes were intrathoracically inoculated with 10-15 PFU of MRE16ic, MRE16ic ΔE200-Y229, or MRE16ic ΔE200-C220 virus. Control *Ae. aegypti* mosquitoes were intrathoracically inoculated with MEM containing 10% FBS, NEAA, L-glutamine, and antibiotics, without virus (negative control). Ten mosquitoes from each group were cold-anesthetized and placed at –70C at timed intervals of 0, 24, 48, and 96 hours post infection. Mosquitoes were taken at time zero in order to confirm that the approximate amount of virus initially inoculated was similar to that estimated from dilution of virus. Mosquitoes were housed at the species-specific insectary conditions described previously and provided water and sugar until frozen (Higgs and Beaty, 1996). Frozen mosquitoes were later triturated separately in 1 ml of diluent and assayed for virus as described previously in Chapter 3 (*Trituration of Whole Mosquitoes*).

Sequencing of the MRE16 Virus Genome

Primers for reverse transcriptase- polymerase chain reaction (RT-PCR) were designed from a consensus sequence generated from six previously published SIN virus or SIN-like virus genome sequences and a previously published MRE16 sequence (Seabaugh et al., 1998). Genomic RNA was isolated from MRE-16 virus seed using a QIAamp® viral RNA mini kit as recommended by the manufacturer (Qiagen, Valencia, CA). Primers were used to transcribe and amplify six overlapping cDNA fragments from the nonstructural gene region (nsP1-nsP4) and the 5′ NCR. RNA was transcribed into cDNA using Roche Biochemicals' Titan™ one tube RT-PCR system. The RT-PCR products were TA cloned® into the pCR®2.1 vector (Invitrogen, Carlsbad, CA). Chemically Competent INVαF′ cells (Invitrogen, Carlsbad, CA) were transformed and plated on terrific broth (TB) plates containing 100μg/ml of ampicillin (Sambrook, Fritsch, and Maniatis, 1989). DNA was purified by maxiprep (Qiagen, Valencia, CA). Automated DNA sequencing was performed as recommended by Applied Biosystems 310 Gene Analyzer (Foster City, CA) / Perkin-Elmer (Shelton, CT). The 5' and 3' ends of each insert were sequenced using pCR®2.1 specific primers. Primers for succeeding reactions were designed from the previously determined sequence.

Construction of the Full-Length MRE16 Infectious cDNA Clone

New sequencing primers efficiently spaced to span the entire genome were designed based on the MRE16 sequence and previously published data (Seabaugh et al., 1998). The 5′ terminus of the genome was cloned and sequenced using Ambion's First Choice™ RLM-RACE kit as recommended. The 3′-terminal sequence had been previously reported (Seabaugh et al., 1998). Primers flanking the restriction sites to be

used in the final assembly of the clone were designed from the MRE16 sequence. Additional restriction sites were also engineered into the primers to facilitate cloning, including unique HindIII and PacI restriction sites, as well as an SP6 promoter sequence upstream of the 5′ genomic terminus. Moreover, an additional guanosine nucleotide was inserted between the SP6 promoter sequence and the 5′-terminal nucleotide of the genome. Unique restriction sites AscI and SacI were engineered downstream of the 3′ terminus and polyA tail. The unique AscI site was engineered to permit linearization prior to transcription of RNA. These primers were used in RT-PCR reactions to amplify 6 overlapping cDNA fragments containing genome regions (nt) 1 – 1446 (EcoRV), 1446 – 3388 (BclI), 3388 – 5196 (FseI), 5196 – 7489 (AgeI), 7489 – 9757 (AvrII), 9757 – 11693. The fragment encoding sequences for nsP1-nsP4 and the 5′ NCR were directly sequenced without cloning in both directions with the new sequencing primer set.

Each of the amplified fragments was subcloned into the multiple cloning site (MCS) of the pBRUC vector. The construction of pBRUC was performed as has been described previously (Kinney et al., 1997). Plasmid DNA was amplified in electroporation competent XL-1 blue cells (Stratagene, La Jolla, CA) cultured in 2X yeast tryptone (YT) broth containing 100μg/ml of ampicillin (Sambrook, Fritsch, and Maniatis, 1989) following electroporation (BTX ECM 630 electroporator set to 2.5kV, 200ohms, and 25μF) and plating on 2X YT plates containing Carbenicillin (80μg/ml) (Sambrook, Fritsch, and Maniatis, 1989). DNA was purified by the Miniprep procedure using Qiagen spin columns (Qiagen, Valencia, CA). At least one strand of at least two independent subclones was sequenced for each amplified region of the complete genome. Subcloned fragments found to contain non-silent errors were not used in the construction of the full-

length cDNA clone. The full-genome-length cDNA clone was constructed by sequentially ligating the fragments from the six subclones into the MCS of a single pBRUC plasmid (Figure 4.1). Plasmid DNA was purified from bacterial culture by the Maxiprep procedure (Qiagen, Valencia, CA).

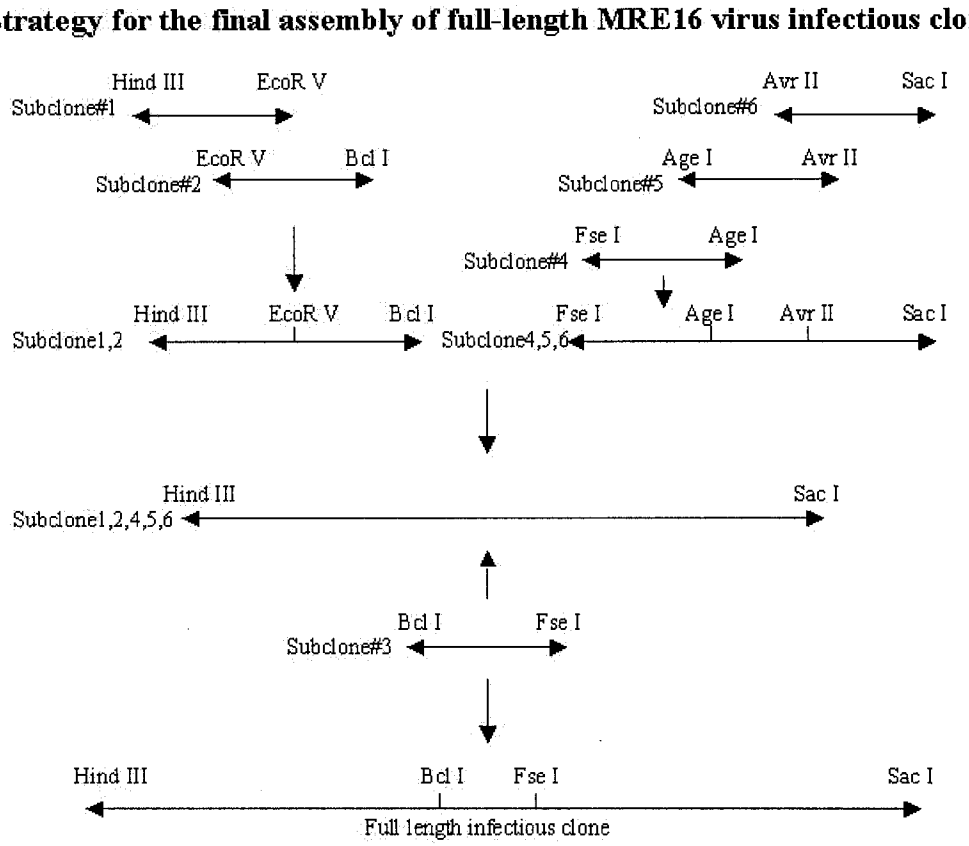

Figure 4.1. Strategy for the final assembly of the full-length infectious cDNA clone MRE16ic. Six subclones contained the complete 11,693-nt-long genome, SP6 promoter, 5' cap, and a polyA tail as overlapping amplified cDNA regions. The pBRUC vector was used for all cloning steps.

Site-Directed Mutagenesis and Construction of MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220

Genomic RNA was isolated from spMRE16 virus seed stock as described previously. The primers previously used in the construction of the MRE16 infectious clone were used in RT-PCR reactions to amplify overlapping cDNA fragments

containing the region of the genome encoding the structural genes. The cDNA fragments were sequenced directly without cloning as previously described. Using phosphorylated forward (5' P-aaaagtgaccagacaaagtgggtc 3') and reverse (5' P-gtacgtgacgttctttccagaaggggc 3') primers and the ExSite™ PCR-based Site-directed mutagenesis kit (Stratagene, La Jolla, CA), as recommended, a deletion mutant was generated lacking 30 contiguous amino acid residues from E2-200-Glu through E2-229-Tyr in the MRE16ic cDNA subclone #5 (Figure 4.1) (AgeI, 7489 – 9757, AvrII). Another deletion mutant was generated lacking 21 contiguous amino acid residues from E2-200-Glu through E2-220-Cys in the MRE16ic subclone #5 using phosphorylated forward (5' P-acggcattgaaacaatgcatcgcc 3') and reverse (5' P-gtacgtgacgttctttccagaaggggc 3') primers. DNA sequences containing the deleted portions of E2 were then cloned into the AgeI and AvrII unique restriction sites of the full-length MRE16ic cDNA.

Results

Nucleotide and Deduced Amino Acid Sequence Analysis

The nucleotide and deduced amino acid sequences of the 5′ NCR and nonstructural genes of MRE16 virus were compared to those of the HRSP AR399 SIN strain, and the results are summarized in Table 4.1. The 5′ NCR and nonstructural genes of MRE16 virus, excluding the 5′ cap, were 7,582 nucleotides in length. Using previously published sequence data, the complete MRE16 viral genome was determined to be 11,693 nucleotides, excluding the 5′ cap and 3′ poly(A) tail (Seabaugh et al., 1998). The MRE16 viral genome is 10 nucleotides shorter than the genome of the prototype SIN strain AR339.

The ClustalV algorithm was used to generate the alignments. The percentage of divergence between the nonstructural genes of AR339 HRSP and MRE16 virus was calculated to be 27.9 % at the nucleotide level and 13.8 % at the amino acid level. There were 2,132 nucleotide differences identified in nonstructural coding regions; 66.4 % of these differences did not alter the deduced amino acids. Of the 351 amino acid differences, 34.8 % were conservative. However, this analysis includes the C-terminal domain of nsP3. The sequence and length of the C-terminal two fifths portion of nsP3 are poorly conserved among alphaviruses (Strauss et al., 1988). A comparison of the nsP3 nonconserved domain of MRE16 virus with the same region of AR339 HRSP virus revealed the insertion of 78 nucleotides encoding 26 amino acids in one area of the C-terminal domain of MRE16 virus, and the deletion of 93 nucleotides encoding 31 amino acids in a second area of the C-terminal domain of MRE16 virus. The MRE16 nsP3 RNA was 15 nucleotides shorter than the HRSP nsP3 RNA, the net difference between the insertions and deletions present in the C-terminal domain. If the nonconserved C-terminal domain of nsP3 is excluded from the sequence analysis, then the total nucleotide and amino acid sequence divergence of the remaining nonstructural genome region becomes 24.9% and 9.2% respectively. Of the 1,710 nucleotide differences in coding regions remaining after excluding the nonconserved C-terminal domain of nsP3 from the sequence analysis, 77.6 % are silent and 45% of the 211 remaining amino acid differences are conservative. The 5′ NCR of the MRE16 viral genome was one nucleotide shorter than that of AR339 HRSP virus. An alignment of the 5′ NCRs of the two viruses revealed a deletion of 3 nucleotides and an insertion of 2 nucleotides in this region of the MRE16 viral genome. A 14.8 % divergence rate between the 5' NCR of these viruses was

calculated from the alignment. A similar comparison of the nucleotide and deduced amino acid sequences of the 26S subgenomic RNA, as well as the nucleotide sequences of the 3' NCR, of the MRE16 and AR339 HRSP viruses has been previously done (Seabaugh, 1998).

TABLE 4.1

Genomic Sequence Differences between AR339 HRSP and MRE16 Virus

Regions	Nucleotide differences[a]			Amino acid differences[a]		
	Total	%	% total 3rd nucleotide[b]	Total	%	% total conservative[c]
5′ NCR	9	14.8	–	–	–	–
nsP1	347	21.4	70.0	51	9.4	41.2
nsP2	615	25.4	78.5	83	10.3	57.8
nsP3						
Conserved[d]	254	26.1	79.1	31	9.5	48.4
Nonconserved[e]	422	54.7	46.5	140	54.5	19.3
nsP4	494	27.0	71.3	46	7.5	24.0
Total	2141	27.9	69.2	351	13.8	34.8

% = percent differences between AR339 HRSP and MRE16 viruses in the indicated genome region.
[a] Total number of differences include insertions and deletions.
[b] Percent of total nucleotide differences occurring at the third codon position in translated regions.
[c] Percent of total amino acid differences considered conservative: R = K, S = T, D = E, Q = N, V = L = I = M, A = G = V, Y = F.
[d] Conserved region of nsP3 nucleotides 4101 to 5075 (aa1 to aa325, AR339 HRSP numbering) or 4100 to 5074 (aa1 to aa325, MRE16 numbering)
[e]Nonconserved region of nsP3 nucleotides 5076 to 5768 (aa326 to aa556, AR339 HRSP numbering) or 5075 to 5752 (aa326 to 551, MRE16 numbering).

MRE16 Infectious cDNA Clone

A full genomic-length cDNA clone of the Malaysian SIN virus, MRE16, was constructed. At least two independent subclones were sequenced for each amplified region of the full-length MRE16 viral genome. The sequence of each subclone was compared to the viral sequence previously determined from overlapping uncloned RT-PCR amplicon fragments (5′ NCR, nsP1-nsP4) or previously published sequence (26S junction region, structural polyprotein, and 3′ NCR) (Seabaugh et al., 1998). Subclones containing errors resulting in a difference in the deduced amino acid sequence were not used in the final assembly of the full-length cDNA clone. Silent nucleotide substitutions

were retained as molecular markers to distinguish the clone derived virus from the parental virus and are summarized in Table 4.2. The cDNA subclone-derived sequences differed from the previously published sequence data at several nucleotide positions, presumably as a result of passage history. Differences were not considered to have arisen through cloning error if they were present in the sequence of at least two independent subclones. Nucleotide differences between the full-length MRE16 cDNA clone and the previously published sequence data are summarized in Table 4.2.

TABLE 4.2

Summary of nucleotide and amino acid sequence differences between the MRE16 full-length infectious cDNA clone and its parental MRE16 virus or previously published sequence data for MRE16 virus[1]

Genome nucleotide position	Nucleotide		Amino acid		Amino acid position
	MRE16 i.c.	Parental virus or published data	MRE16 i.c.	Parental virus or published data	
1279*	G	A	K	K	nsP1-407
8422	G	A	E	E	Capsid-264
9476	C	G	Q	E	E2-286
10011	C	U	P	L	6K-41
10021	G	A	V	V	6K-44
10858	A	G	A	A	E1-268
10892	C	G	L	V	E1-280
10930*	T	G	D	D	E1-292

* Error incorporated during cloning
[1](Seabaugh et al., 1998)

Identification of an MRE16 Deletion Mutant

An MRE16 mutant was identified and found to contain a deletion in the E2 region of the virus genome, including a portion of the proposed cell-receptor binding domain. During characterization of the MRE16 parental virus (AP61-6, C6/36-5) a small plaque phenotype was identified in the quasispecies population. Growth curves for MRE16 (AP61-6, C6/36-1) and MRE16ic viruses indicated that both replicated with nearly equal efficiency in both BHK-21 and C6/36 cells (Figures 4.2 and 4.3).

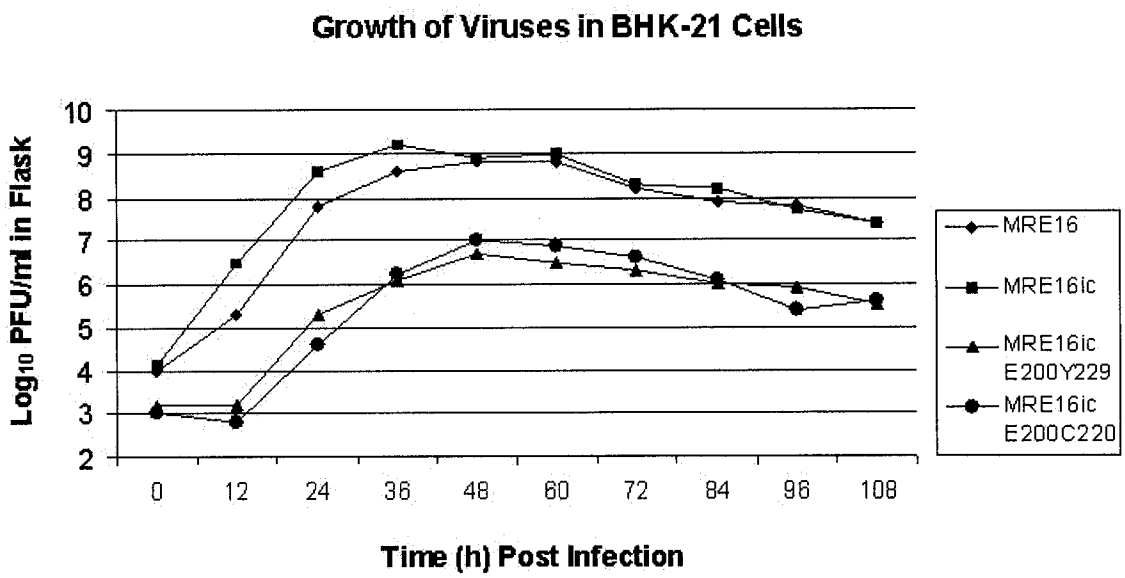

Figure 4.2. Comparative growth rates of MRE16, MRE16ic, MRE16ic ΔE200-Y229, and MRE16ic ΔE200-C220 viruses in BHK-21 cells. Viruses were grown in 25-cm^2 tissue culture flasks of BHK-21 cells. Multiplicity of infection was approximately 0.01 PFU/cell.

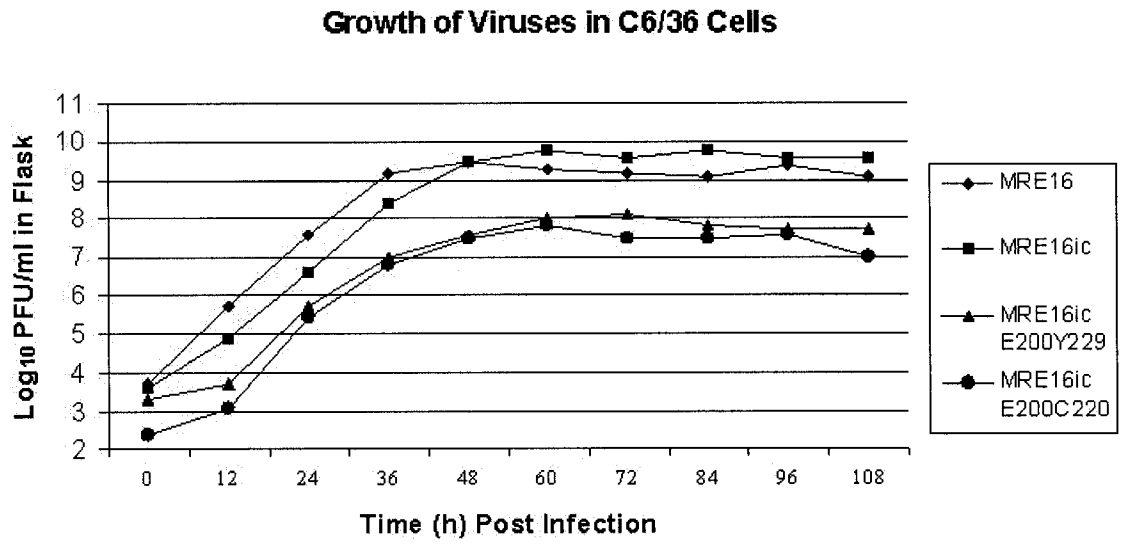

Figure 4.3. Comparative growth rates of MRE16, MRE16ic, MRE16ic ΔE200-Y229, and MRE16ic ΔE200-C220 viruses in C6/36 cells. Viruses were grown in 25-cm^2 tissue culture flasks of C6/36 cells. Multiplicity of infection was approximately 0.01 PFU/cell.

In BHK-21 cells, maximum titers of 8.8 - 9.2 $\log_{10}$ PFU/ml were reached at 36-48 hours post-infection. In C6/36 cells, titers of 9.5 - 9.8 $\log_{10}$ PFU/ml were achieved at 48–60 hours post-infection. However, growth of MRE16 in C6/36 cells resulted in a viral

population of predominantly small plaque phenotype by 36-48 hours post infection (Figure 4.4). Medium harvested at earlier time points contained virus that produced predominantly a large plaque phenotype. The large plaque size was consistent with that observed over the duration of growth in BHK-21 cells (Figure 4.4). The large plaque phenotype was found to predominate for the MRE16ic virus at each time point taken from either BHK-21 or C6/36 cells (Figure 4.4).

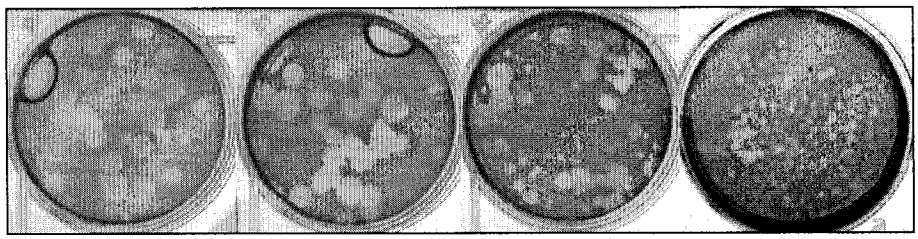

Figure 4.4. Plaque phenotypes for MRE16, MRE16ic, and MRE16ic ΔE200-Y229 viruses. Medium was harvested from infected BHK-21 or C6/36 cells during virus growth curve experiments (described previously in *Virus Growth Curves*) and plaqued on Vero cell monolayers as described previously in Chapter 3 (*Plaque Titrations*).

A spMRE16 virus seed stock obtained from a single round of plaque purification and passaged once in C6/36 cells reproduced the small plaque phenotype (data not shown). Sequencing of the spMRE16 structural genes revealed a deletion encompassing amino acid residues E-200 – Y-229 of E2 (Figure 4.5) as well as the two point mutations, S-60 → R in E2 and S-321 → L in E1.

Location of Deletions in spMRE16, MRE16ic ΔE200-Y229, and MRE16ic ΔE200-C220 Viruses

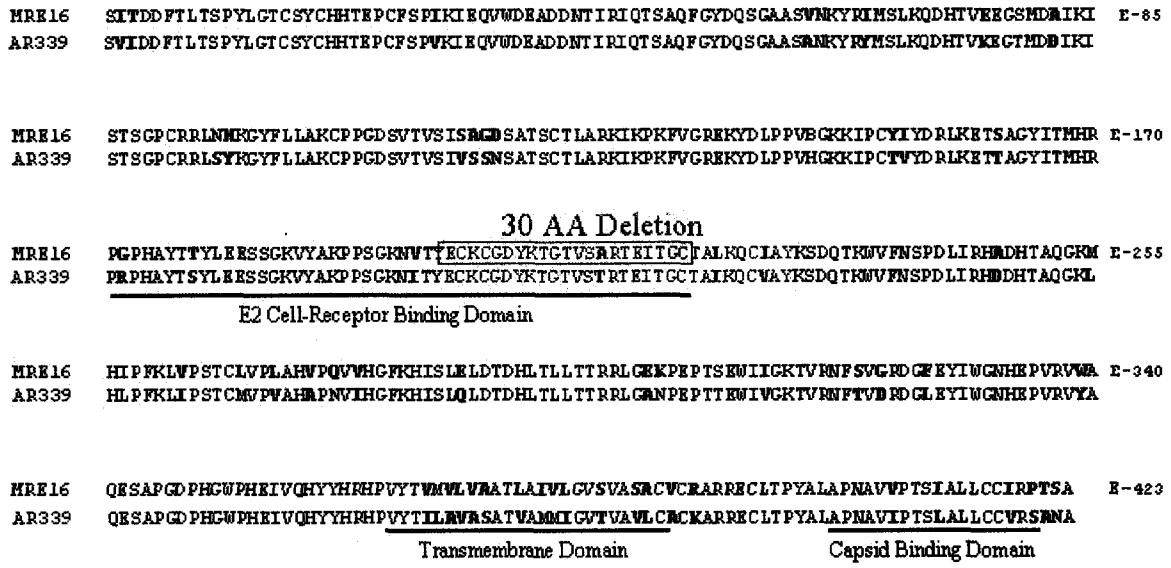

Figure 4.5. Deletion identified in spMRE16 and deletions engineered into MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 viruses. The locations of the deletion present in the E2 glycoprotein of spMRE16 (red letters) and deletions present in MRE16ic ΔE200-Y229 (red letters) or MRE16ic ΔE200-C220 (red letters within the blue box). Deduced a.a. residues from prototype AR339 virus sequence also shown.

Construction of Deletion Mutants MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220

The spMRE16 virus was assayed for oral infectivity in *Ae. aegypti* mosquitoes. IFA analysis of *Ae. aegypti* mosquito head tissues revealed that only 17% (8 of 47) of mosquitoes ingesting a blood meal containing spMRE16 virus had developed disseminated infections by 14 days post-infection (Table 4.3). Acting on the hypothesis that the deletion in E2 was solely responsible for the observed reduction in oral infectivity, experiments were performed to determine if the whole deletion or just the deleted portion of the proposed E2 cell-receptor binding domain was necessary to produce the observed phenotype of the spMRE16ic virus in mosquitoes. Two separate deletions were introduced into the full-length MRE16 cDNA clone, one encompassing residues E-200 to Y-229, and the second encompassing E-200 to C-220 of E2 (figure

4.5). This was done by PCR-based site-directed mutagenesis using a commercially available kit.

Growth of MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 Viruses in BHK-21 and C636 Cells and Plaque Sizes in Vero Cells

The growth and plaque sizes of virus generated from the MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 cDNA clones were characterized. Virus derived from the MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 infectious clones produced plaques of similar size in Vero cells (data not shown). MRE16ic ΔE200-Y229 plaques are shown in Figure 4.4 and were similar to plaque sizes observed for spMRE16 virus (data not shown). Growth curves for MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 viruses indicated that both replicated with nearly equal efficiency in BHK-21 and C6/36 cells (Figure 4.2 and 4.3). In BHK-21 cells, maximum titers of 6.7 – 7.0 $\log_{10}$ PFU/ml were reached at 48 hours post-infection. In C6/36 cells, titers of 7.8 – 8.1 $\log_{10}$ PFU/ml were achieved at 60-72 hours post-infection. The growth curve assays indicated that the replication kinetics of the MRE16ic deletion mutants in both cell types were altered nearly equally by each of the two deletions. The deletions had a greater effect on replication in vertebrate cells (BHK-21), reducing titers by approximately 100 fold at each time point, than in mosquito cells (C6/36) in which titers typically underwent only a 10 fold reduction at each time point. Interestingly the titer obtained for the spMRE16 virus seed stock obtained from a single round of plaque purification and passaged once in C6/36 cells for approximately 60 hours was 1.0 X 10^9 PFU/ml. Therefore, it is possible that in addition to the deletion encompassing amino acid residues E-200 – Y-229 of E2, one or both of the two point mutations, S-60 → R in E2 and S-321 → L in E1, identified

in the structural gene region of spMRE16 virus are required in order to achieve titers of this magnitude. This represents a possible area of future research.

Immunofluorescent Analysis of Mosquito Midgut Tissues

Mosquito midguts were assayed for the presence of MRE16, MRE16ic, MRE16ic ΔE200-Y229 or MRE16ic ΔE200-C220 viruses. *Ae. aegypti* mosquitoes ingested a blood meal containing approximately 4.0 X 10^9 PFU/ml of MRE16 virus, 2.5 X 10^9 PFU/ml of MRE16ic virus, 4.0 X 10^8 PFU/ml of MRE16ic ΔE200-Y229 virus, or 1.3 X 10^8 PFU/ml of MRE16ic ΔE200-C220 virus, respectively. *Cx. tritaeniorhynchus* mosquitoes ingested a blood meal containing approximately 4.0 X 10^9 PFU/ml of MRE16 virus. *Ae. aegypti* midguts were dissected and analyzed by IFA at 2-3, 4-5 and 8-9 days post-infection. At least 29 midguts from each group were examined for infection by detection of SIN virus-specific antigen. Nine *Cx. tritaeniorhynchus* midguts were dissected and analyzed by IFA for SIN virus-specific antigen at 2-3 days post-infection. Several midguts were dissected from mock-infected *Ae. aegypti* or *Cx. tritaeniorhynchus* mosquitoes and analyzed by the same IFA protocol. SIN virus-specific antigen was detected in all of the midguts examined from *Ae. aegypti* infected with MRE16ic or the MRE16 parental virus, and the distribution was similar at both 2-3 and 4-5 days post-infection. SIN virus-specific antigen was detected primarily in the epithelial cell layer in the majority of the midguts examined at 2-3 days post-infection in *Ae. aegypti.* Widespread distribution of SIN virus-specific antigen was observed in approximately 41.4% (12 of 29) of midguts infected with MRE16 and 32.3% (10 of 31) midguts infected with MRE16ic virus at 2-3 days (Figure 4.6 A and B). These results were not significantly different (P = 0.593, Fisher exact test). Only a few midguts displayed antigen in the overlying musculature and

respiratory tracheoles at the 2-3 day time point. At 4-5 days post-infection, evidence of infection was again seen in all of the midguts examined. However, the proportion of midguts displaying antigen throughout the epithelial cell layer lining the mesenteron had increased to 73.3% (22 of 30) for MRE16 virus and 65.5% (19 of 29) for MRE16ic virus. These results also were not significantly different (P = 0.580, Fisher exact test). SIN virus-specific antigen was also detected in the overlying musculature and respiratory tracheoles of a greater proportion of the infected midguts at 4-5 days in *Ae. aegypti*. In 8 of 9 *Cx. tritaeniorhynchus* orally infected with MRE16ic virus, SIN virus-specific antigen was distributed throughout the midgut musculature by 2-3 days post-infection, suggesting that virus had already escaped midgut epithelial cells and disseminated to other tissues (Figure 4.6 C). In one of the midguts SIN virus-specific antigen could not be detected.

Midguts were examined from *Ae. aegypti* mosquitoes ingesting the deletion mutants in order to determine if the deletion in E2 had any effect on the ability of the MRE16ic virus to initiate an infection of midgut epithelial cells. SIN virus-specific antigen could be detected in only 31.3% (10 of 32) of midguts from mosquitoes ingesting MRE16ic ΔE200-Y229 virus, and 35.5% (11 of 31) from those ingesting MRE16ic ΔE200-C220, 2-3 days after ingestion of the blood meal. The results were not significantly different (P = 0.793, Fisher exact test). These results indicated that these viruses were often unable to productively infect the midgut. In those midguts that did become infected, only 1 or 2 small foci of antigen per midgut were observed (Fig 4.6 D). Only 33.3% (10 of 30) and 23.3% (7 of 30) midguts taken 8-9 days after ingesting MRE16ic ΔE200-Y229 virus or MRE16ic ΔE200-C220 virus were infected, respectively.

Results were again not significantly different ($P = 0.568$, Fisher exact test). However, larger foci of antigen were observed, indicating that initial infection had spread to other cells of the midgut (Fig. 4.6 E). Immunofluorescence was not observed in any of the mock-infected midguts (data not shown). The oral infection trials of AaRexD mosquitoes with MRE16ic virus and the two deletion mutant viruses showed that both deletions significantly reduced the ability of MRE16ic virus to infect the midguts of these mosquitoes. Two to three days after ingesting an infectious blood meal approximately 100% (31 of 31) of *Ae. aegypti* mosquitoes orally exposed to MRE16ic virus developed a midgut infection compared with only 31.3% (10 of 32) ($P < 0.001$, Fisher exact test) and 35.5% (11 of 31) ($P < 0.001$, Fisher exact test) of mosquitoes orally exposed to MRE16ic ΔE200-Y229 virus and MRE16ic ΔE200-C220 virus, respectively. Furthermore, the number of mosquito midguts infected with either deletion mutant did not increase significantly by the 8-9 day time point.

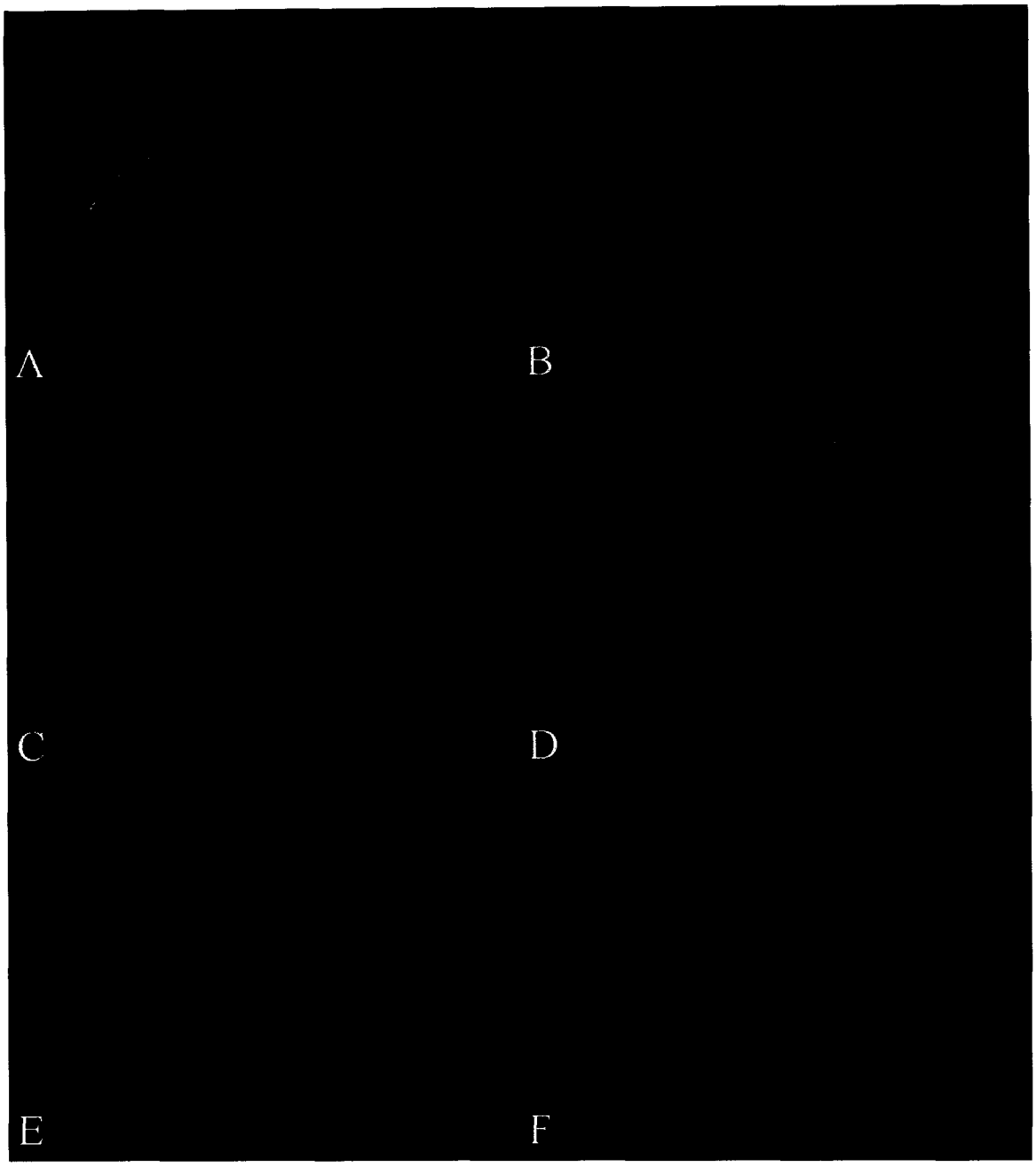

Figure 4.6. Intact mosquito midguts assayed for the presence of MRE16, MRE16ic, MRE16ic ΔE200-Y229 or MRE16ic ΔE200-C220 virus at various times post-infection. Composite images of *Ae. aegypti* mosquito midguts infected with either MRE16 virus (A) or MRE16ic virus (B) at 2-3 days post-infection and demonstrating widespread distributions of SIN E1 antigen. Composite image of a *Cx. tritaeniorhynchus* mosquito midgut infected with MRE16ic virus (C) at 2-3 days post-infection and demonstrating a disseminated infection. Composite images of *Ae. aegypti* mosquito midguts infected with either MRE16ic ΔE200-Y229 virus (D) or MRE16ic ΔE200-C220 virus (E) at 8-9 days post-infection, and MRE16ic ΔE200-Y229 virus (F) at 2-3 days post-infection. A focus of infection can be seen in the blue square. (SIN E1 antigen shown in green). Olympus F-IX70 fluorescent microscope; original magnification 125X.

Immunofluorescent Analysis of Mosquito Head Tissues

The rate of MRE16ic virus dissemination from the midgut of *Ae. aegypti* mosquitoes following *per os* infection was assayed and compared to that of MRE16. Dissemination of MRE16ic virus from the midgut of infected AaRexD mosquitoes was very similar to that observed for the parental MRE16 virus (Fig. 4.7). For each virus 100% of mosquitoes assayed were positive for a disseminated infection by 10 –12 days post-infection. Disseminated virus could be detected as soon as 2 days post-infection in the heads of mosquitoes given a blood meal containing MRE16 or MRE16ic virus. In each, viral E1 antigen was detected in greater than 90% of the heads of infected mosquitoes by 8 days post-infection. Ninety five percent of *Cx. tritaeniorhynchus* mosquitoes had E1 antigen in their heads 14 days after oral infection with MRE16 parental virus.

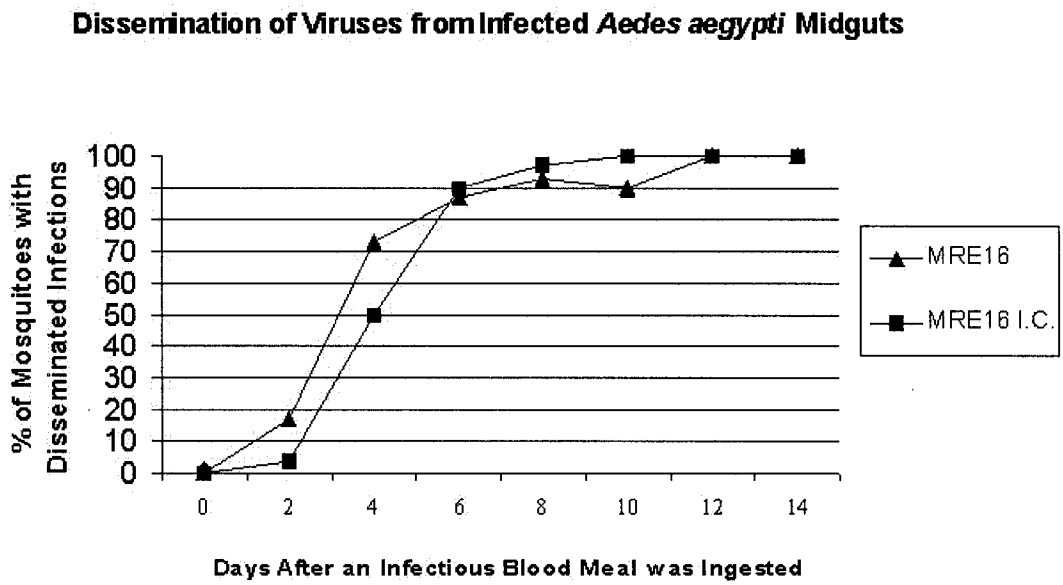

Figure 4.7. Comparative disseminated infection rates of MRE16 and MRE16ic viruses in *Ae. aegypti* mosquitoes. The percentage of mosquitoes orally exposed to either MRE16 or MRE16ic viruses with disseminated infections (e.g. positive for SIN-specific antigen by IFA of head tissues) at timed intervals (n ≥ 30).

Dissemination of the MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 virus mutants were also assayed by IFA analysis of *Ae. aegypti* head tissues at 9 and 14 days. The number of mosquitoes that had disseminated infections at 9 days was not significantly different (P = 1.000, Fisher exact test). Only 3.3 % (1 of 30) of the mosquitoes ingesting MRE16ic ΔE200-Y229 virus and none (0 of 30) of those ingesting MRE16ic ΔE200-C220 virus exhibited a disseminated infection after 9 days. However, by 14 days, 20% (10 of 50) of *Ae. aegypti* ingesting MRE16ic ΔE200-Y229 virus were positive for a disseminated infection. Only 2% (1 of 50) of those ingesting MRE16ic ΔE200-C220 virus were positive at this time point, a significant difference (P = 0.008, Fisher exact test). The number of disseminated MRE16ic ΔE200-Y229 virus infections after 14 days was not significantly greater than the number observed in *Ae. aegypti* ingesting spMRE16 after 14 days (17% or 8 of 47) (P = 0.797, Fisher exact test). However, these results, 10 of 50 for MRE16ic ΔE200-Y229 virus, were in remarkable contrast to the number of disseminated infections observed in the mosquitoes ingesting MRE16ic virus after 14 days (100% or 60 of 60) ($P < 0.001$, Fisher exact test). One possible explanation for the low level of virus dissemination for MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 virus was that less virus was ingested by the mosquitoes; the blood meal titers of these viruses were not as high as the titer of MRE16 and MRE16ic viruses. To test for a possible titer-specific effect, the number of *Ae. aegypti* positive for a disseminated infection at 14 days after ingestion of serially diluted MRE16ic virus was determined. Table 4.3 summarizes the dissemination results obtained for all MRE16 viruses in *Ae. aegypti* and *Cx. tritaeniorhynchus* mosquitoes at 14 days post-infection. *Ae. aegypti* mosquitoes fed on a blood meal containing 1.6 x 10^7 PFU/ml of MRE16ic virus

still showed significantly higher virus dissemination than did the MRE16ic ΔE200-Y229 deletion mutant (4.0 x 10^8 PFU/ml in blood meal) ($P < 0.001$, Fisher exact test). These results clearly rule out a virus titer-specific effect.

These results indicate that the deletions introduced into MRE16ic are by themselves capable of dramatically reducing the ability of the virus to initiate an infection of the midgut epithelial cells of AaRexD mosquitoes. It is the inability of these viruses to efficiently infect the midguts of these mosquitoes that is responsible for the dramatic reductions observed in the number of mosquitoes developing disseminated infections following oral exposure. Furthermore, only the deleted portion of the proposed E2 cell-receptor binding domain was necessary to produce the observed reduction in midgut infectivity. In fact the present characterizations of the MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 viruses reveal that they are phenotypically identical except with respect to the number of mosquitoes developing disseminated infections 14 days after oral infection. This is an interesting and counterintuitive result. Possibly the smaller deletion may result in a change in the conformation of E2 as present in the larger deletion. Thus the conformation of E2 may be critical for dissemination from the midgut.

TABLE 4.3

Mosquitoes displaying virus dissemination 14 days after ingestion of infectious blood meal

Mosquito	SIN virus	Blood meal titer (PFU/ml)	Head tissue/immunofluorescence results		
			Positive	Total	%Positive
C. tritaeniorhynchus	MRE16	4.0 X 10^9	57	60	95
Ae. aegypti	MRE16	4.0 X 10^9	60	60	100
Ae. aegypti	MRE16ic	2.5 X 10^9	60	60	100
Ae. aegypti	spMRE16	1.0 X 10^9	8	47	17
Ae. aegypti	MRE16ic ΔE200-Y229	4.0 X 10^8	10	50	20
Ae. aegypti	MRE16ic ΔE200-C220	1.3 X 10^8	1	50	2
Ae. aegypti	MRE16ic (10^{-1})	2.3 X 10^8	56	60	93
Ae. aegypti	MRE16ic (10^{-2})	1.6 X 10^7	44	60	73

Virus replication in Aedes aegypti mosquitoes

In order to determine if the observed reduction in midgut infectivity by deletion mutant viruses was specific to midgut epithelial cells, the replication of the viruses MRE16ic ΔE200-Y229, MRE16ic ΔE200-C220, and MRE16ic was compared and contrasted following intrathoracic inoculation in *Ae. aegypti* mosquitoes. Mosquitoes were triturated at timed intervals, and virus titers were determined by plaque assay in Vero cells (Figure 4.8). All of the viruses replicated to between $10^{6.1}$ and $10^{7.7}$ PFU/ml by 48 hours post-infection. The replication kinetics of the two deletion mutants was similar, but the presence of the deletions altered the replication of the two mutants in comparison to MRE16ic virus (Figure 4.8). MRE16ic virus reached a maximum average titer of 7.8 $\log_{10}$ PFU/ml at 48 hours post-infection, followed by a more than ten-fold reduction in titer to 6.4. $\log_{10}$ PFU/ml at 96 hours. MRE16ic ΔE200-Y229 virus and MRE16ic ΔE200-C220 virus reached maximum titers of only 6.1 – 6.5 $\log_{10}$ PFU/ml at 48 hours post-infection, more than 10-fold lower titers than MRE16ic at the same time point. MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 virus titers were also lower than the MRE16ic virus titer at the 24 hour time point. However, the titers of all of these viruses were very similar at the 96 hour time point. This could be explained if fewer cells were initially infected with the deletion mutants than with MRE16ic virus. Therefore, replication of the deletion mutants would begin more slowly but eventually catch up. The current study has shown that the deletion mutants poorly infect midgut epithelial cells, and perhaps these cells account for the difference in the number of cells initially infected. Alternatively, the replication kinetics of all the viruses through 48 hours looks very similar to the kinetics observed during replication of the same viruses in C6/36 cells. Perhaps one or both of the

two point mutations, S-60 → R in E2 and S-321 → L in E1, identified in the structural gene region of spMRE16 virus might make the replication of the deletion mutants more like that of MRE16ic virus.

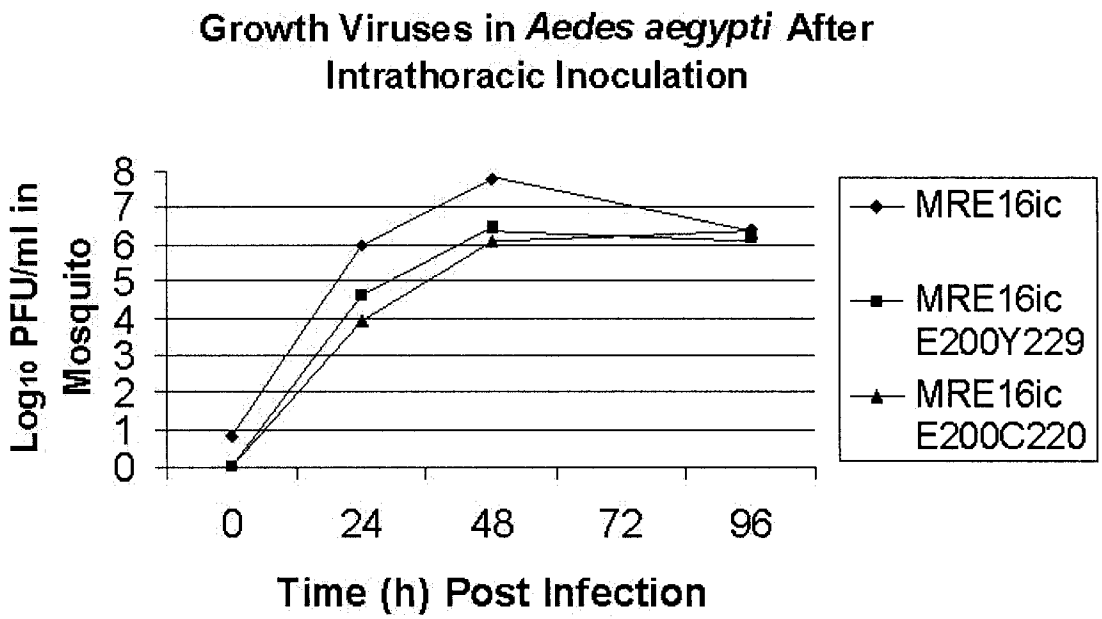

Figure 4.8. Comparative replication kinetics of MRE16ic, MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 viruses in *Ae. aegypti* mosquitoes. Mosquitoes were inoculated intrathoracically with between 10-15 PFU. Ten mosquitoes were taken at each time point for each virus. Individual virus titers obtained from triturated mosquitoes were averaged for each time point. A time zero was included to confirm the initial inoculating dose. The average inoculating dose, determined at the mosquito zero time point, for MRE16ic virus was 7 PFU. Virus was not recovered from mosquitoes inoculated with the deletion mutants at time zero. Virus was recovered from all of the mosquitoes that were triturated at all subsequent time points.

Discussion

On the basis of RNA-RNA hybridizations and RNase T1 oligonucleotide mapping, SIN viruses can be segregated into two genetically distinct types, Paleoarctic/Ethiopian and Oriental/Australian (Olson and Trent, 1985; Rentier-Delrue and Young, 1980). Thus far, limited nucleotide sequence analysis confirms these earlier genetic groupings (Sammels et al., 1999; Shirako et al., 1991). A comparison of the

sequence of the 5′ NCR and nonstructural genes (nsP1-nsP4) of MRE16 virus with those of the HRSP AR339 SIN strain showed significant genetic divergence between the two viruses, confirming the results of earlier sequence comparisons of the 26S RNA sequences of both viruses (Seabaugh et al., 1998). Nucleotide sequence analyses (Sammels et al., 1999; Seabaugh et al., 1998) have shown a close genetic relationship between MRE16 virus and other members of the Oriental/Australian genotype. An infectious cDNA clone for the SIN MRE16 virus was constructed. This is currently the only full-length clone for a member of the Oriental/Australian geographic type of SIN virus. Characterization of MRE16ic virus indicates that virus generated from the clone is identical to the parental MRE16 virus in both plaque size in Vero cells and replication rate in BHK-21 and C6/36 cells. The ability of infectious clone-derived virus (MRE16ic) to infect and disseminate within the *Ae. aegypti* mosquito was phenotypically identical to the parental virus.

The ability of mosquitoes to act as competent vectors of arboviruses such as SIN is influenced by many factors (Hardy, 1988; Hardy et al., 1983). Of particular importance are factors influencing differences in tissue susceptibility, transmission rates, and extrinsic incubation periods (Hardy, 1988). Viral determinants of midgut infection and escape influence all three. The mosquito midgut is the first organ system encountered after ingestion of an infectious blood meal. Infection and escape from the arthropod midgut is critical for maintenance of the transmission cycle, and as such its importance as a determinant of vector competence is well established (Hardy, 1988).

Numerous hypotheses have been proposed to explain the nature of the MI barrier, including diversion of the blood meal into the diverticula, inactivation of virus by

enzymes in the gut lumen, occlusion of virus by the peritrophic matrix, an absence or reduction of receptor sites, and abortive replication in the cells of the midgut (Hardy, 1988). However, it has been established by using several different experimental approaches that the resistance of *Cx. pipiens* to infection by WEE virus is associated with a failure of the virus to bind to the midgut microvillar membrane (Arcus, Houk, and Hardy, 1983; Houk et al., 1986). Furthermore, competitive binding experiments with midgut brush border fragments indicated that the binding of WEE virus was specific in the case of *Cx. tarsalis*, which is susceptible to infection with WEE virus, but non-specific in the case of *Cx. pipiens,* which is refractory to infection with WEE virus (Hardy, 1988). The results of these experiments suggest that the midgut brush border fragments of *Cx. pipiens* lack specific receptors for WEE virus.

Molecular approaches to the study of alphavirus-mosquito interactions have increased our understanding of virus entry, replication, and morphogenesis as well as cytopathic response and persistent infection in the mosquito. Recombinant DNA technology has in many cases aided in these studies. The majority of these studies have made use of cultured mosquito cells as a proxy for the mosquito. Horizontal transmission of virus involves a high degree of specificity between vector and virus, and is conditioned by multiple events under the influence of numerous variables. The intricacies of this relationship have directed the evolution of these viruses. In order to obtain a more detailed understanding of alphavirus infection of the invertebrate vector, molecular genetic approaches must be applied *in vivo*. Until now, these types of studies have been constrained by the limitations of available infectious clones. This chapter described the

use of the MRE16 full-length infectious clone in a study of pathogen-vector interactions *in vivo*.

A single deletion spanning a portion of the E2 cell-receptor binding domain of MRE16 virus was demonstrated to dramatically alter the ability of the virus to productively infect a mosquito vector. The replication of MRE16ic ΔE200-Y229 virus in C6/36 cells was altered by the presence of the deletion. This virus achieved maximum titers greater than 10-fold lower than those of the MRE16ic virus in C6/36 cells. However, the MRE16ic ΔE200-Y229 virus still replicated efficiently, reaching a maximum titer in excess of 10^8 PFU/ml in C6/36 cells at 72 hours post-infection. Interestingly, growth of viruses derived from clones MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 were more attenuated in BHK-21 than C6/36 cells, achieving maximum titers approximately 100-fold lower than MRE16ic virus in BHK-21 cells. Virus derived from MRE16ic ΔE200-Y229 replicated efficiently in *Ae. aegypti* mosquitoes following intrathoracic inoculation. Although replication of MRE16ic ΔE200-Y229 virus was less efficient than that of MRE16ic virus following intrathoracic inoculation, the mutant virus attained titers nearly identical to those of MRE16ic virus by 96 hours post-infection in mosquitoes. In contrast, when mosquitoes were challenged by the peroral route, the low midgut infectivity and dissemination of MRE16ic ΔE200-Y229 were in marked contrast to the high infectivity and dissemination observed for MRE16ic virus. However, some of the AaRexD mosquitoes did become infected with both of the deletion mutants after ingestion of an infectious blood meal. This may indicate that alphaviruses are able to infect midgut epithelial cells by an alternative mechanism that is less efficient. Although a number of studies have shown that the envelope glycoprotein

E2 is important for binding to cells, E1 alone also appears capable of binding and mediating penetration of cells. Omar and Koblet (1988) prepared SF virions lacking E2 and found the resulting virus to be infectious. This showed that virions with E1 alone can still induce binding and penetration. However, the removal of E2 from the virions resulted in a 90% loss in infectivity. This may have been due to the detergent treatment used to remove E2 reducing the stability of the virion. Alternatively, binding and penetration of cells using only the E1 envelope glycoprotein may be a less efficient mechanism for gaining entry into the cell than the mechanism used when a functional E2 is present. Sundin et al. (1987) have reported results suggesting that the fusion function of the G1 envelope glycoprotein of LAC virus is important for efficient infection of mosquito midgut epithelial cells. A monoclonal antibody-resistant (MAR) variant virus, V22, was identified that demonstrated a great reduction in fusion efficiency and mouse neuroinvasiveness. In addition, the midgut infectivity of V22 virus was dramatically reduced. A variant of V22 virus, V22M3, was identified that had reverted at the specific epitope following serial oral passage in mosquitoes. The reversion at this single epitope restored the fusion function of the virus, as well as the midgut infectivity of the virus. The fusion activity of alphaviruses is postulated to reside solely in the E1 envelope glycoprotein (Garoff et al., 1980; Kondor-Koch, Burke, and Garoff, 1983; Kondor-Koch et al., 1982; Rice and Strauss, 1981). Therefore, a functional E1 envelope glycoprotein may be sufficient for infection of some midgut cells.

To further define the genetic basis of the effect of the deletion, the deletion in MRE16ic ΔE200-Y229 was truncated to encompass only the ΔE200-C220 portion within the cell-receptor binding domain of the E2 envelope glycoprotein of the virus.

Characterization of virus derived from clone MRE16ic ΔE200-C220 indicated that it was identical to MRE16ic ΔE200-Y229 virus in both plaque size in Vero cells (data not shown) and replication rate in BHK-21 and C6/36 cells. The ability of the infectious clone-derived virus to infect the *Ae. aegypti* mosquito midgut was also phenotypically very similar to that of MRE16ic ΔE200-Y229 virus. However, dissemination of MRE16ic ΔE200-C220 virus from the midgut of *Ae. aegypti* mosquitoes at 14 days after peroral challenge was about 10-fold lower than that observed for MRE16ic ΔE200-Y229 virus (Table 4.3). This may indicate that the conformation of E2 is critical for dissemination from the midgut. Midgut leakage is a poorly understood phenomenon whose validity has been questioned in the past (Hardy et al., 1983). However, it is difficult to rule out the phenomenon as a possible explanation for the discrepancy, in large part because so little is known about it. Although most descriptions of midgut leakage indicate that it occurs shortly after ingestion of the blood meal, dissemination of both deletion viruses appeared to have occurred between 9 and 14 days post challenge (Boorman, 1960; Hardy et al., 1983; Miles, Pillai, and Maguire, 1973).

Residues 170-220 (SIN AR339 numbering) of E2 are postulated to be particularly important for cell binding, and appear to constitute a cell-receptor binding domain in the glycoprotein. The domain has been shown to be important for virus neutralization, and a domain of similar function and location has been identified in the E2 of VEE, and RR viruses. This domain may be well conserved among the alphaviruses (Smith et al., 1995; Strauss and Strauss, 1994). For SIN virus, two overlapping antigenic sites, A and B, have been defined within this domain (Stec et al., 1986; Strauss et al., 1991; Strauss and Strauss, 1994). Based on the changes seen in antibody-resistant variants, it seems likely

that all epitopes in antigenic sites A and B are contained within this domain (Strauss and Strauss, 1994). The deletions encompassed in MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220 viruses included at least portions of both A and B.

Mechanisms by which antibodies neutralize virus infectivity can vary. It has been suggested that SIN virus-specific antibodies that bind to E2 A and E2 B block attachment to cells (Wang et al., 1991). Additional studies utilizing anti-idiotypic antibodies have also illustrated the importance of this region to cell binding. Wang et al. (1991) demonstrated that anti-idiotypic antibodies generated from neutralizing antibodies recognizing epitopes within this domain (Strauss et al., 1991; Wang and Strauss, 1991) blocked the binding of SIN virus to chicken cells. The results suggested that the anti-idiotypic antibody was binding to a cellular receptor for the virus. Ubol and Griffin (1991) have reported that an anti-idiotypic antibody, also generated from a neutralizing antibody recognizing an epitope within this domain (Smith et al., 1995), was able to block virus binding to murine N18 neuroblastoma cells. Residue changes in this domain have been shown to affect the infection potential of alphaviruses for different cell types. Amino acid changes within the domain have been found to affect the sensitivity of chicken cells to infection with RR virus (Kerr, Weir, and Dalgarno, 1993; Strauss and Strauss, 1994). The results with RR virus were remarkable in that the parental virus infected only 2% of the cells, and only three amino acid changes in the envelope glycoproteins (one within the E2 cell-receptor binding domain) resulted in a virus capable of infecting all of the cells (Strauss and Strauss, 1994). Additionally, a single change at residue E2-219 of RR virus is thought to have adapted the virus to humans in one epidemic in Australia (Burness et al., 1988). Flynn et al. (1990) found that upon binding

of SIN to a cell, transitional epitopes were exposed. These conformational alterations were mimicked after exposure of virus to heat, and transitional epitopes neutralized by monoclonal antibodies (MAbs) (Strauss and Strauss, 1994). Selection for resistance to neutralization by these MAbs gave rise to viruses with amino acid residue changes at E2 position 200 or 202 (anti-E2 MAbs) or at E1 position 300 (anti-E1 MAbs). It is intriguing that the deletion mutant viruses generated in the present study, MRE16ic ΔE200-Y229 and MRE16ic ΔE200-C220, were lacking the transitional epitopes at E2 position 200 and 202. Most of the latter information was obtained using vertebrate host models, and it remains unclear what role this E2 domain plays in infection of the arthropod vector. A study by Woodward et al. (1991) demonstrated that the infectivity and dissemination of VEE virus in *Ae. aegypti* mosquitoes was altered as the result of a single amino acid change within this domain. This suggested that the domain was also important to productive alphavirus infection of arthropod vectors.

The current study indicates that residues within the cell-receptor binding domain of the E2 envelope glycoprotein of the MRE16 SIN virus, 200-220 (SIN AR339 numbering), are of particular importance to the midgut infectivity of the virus in AaRexD mosquitoes. The deletions introduced into the E2 envelope glycoprotein of MRE16ic, generating MRE16ic ΔE200-Y229 virus and MRE16ic ΔE200-C220 virus, may have impaired the ability of these viruses to interact with a cellular receptor on the mosquito midgut epithelium. If so, these results provide additional support for the existence of a specific receptor-ligand interaction by which alphaviruses gain entry to mosquito midgut epithelial cells. Nevertheless, the present study has likely identified an important molecular viral determinant of midgut infection. Elucidation of a specific mechanism will

require additional studies. Such studies could provide critical insights into the molecular nature of MI barriers and lead to a better understanding of vector specificity.

Arbovirus mutants have been previously isolated in the laboratory, including some with a small plaque phenotype, which have been shown to be non-transmissible by mosquitoes following peroral infection. These arbovirus mutants have included small plaque mutants of Middelberg (Pattyn and De Vleesschauwer, 1968), VEE (Gaidamovich et al., 1971), and SIN (Peleg, 1975) viruses. Such mutants would probably not arise in a natural transmission cycle, because of their inability to be efficiently transmitted. Nonetheless, arbovirus mutants identified in the laboratory can be important tools for elucidating the mechanisms associated with arboviral barriers to infection, dissemination, and transmission if they are well characterized. Infectious cDNA clones based on SIN viruses provide powerful tools for the characterization of these mutants. The present study demonstrated this by using the MRE16 infectious cDNA clone to identify a region of the alphavirus genome that appears to be important to midgut infection of *Ae. aegypti.* The availability of the MRE16 full-length infectious clone should also facilitate additional in *vivo* studies of pathogen-vector interactions, particularly early events at the initial site of replication, the epithelial cells of the mesenteron.

CHAPTER 5

SUMMARY

Vector-borne diseases have proven to be among the most intractable infectious diseases for both humans and livestock. Most medically important arboviruses are transmitted to vertebrate hosts by species of *Aedes* and *Culex* mosquitoes (Karabatsos, 1985; Mackenzie et al., 1994). However, little is known about the early events of arbovirus infection of these mosquito vectors. SIN viruses have many advantages for the study of early virus-vector interactions. The work described in this dissertation has developed and used the potential of a SIN model system to study arboviral-vector interactions in *Ae. aegypti* mosquitoes.

Expression systems have been developed previously from infectious clones of the prototype SIN AR339 strain and its derivatives (Hahn et al., 1992; Rice, 1992; Rice et al., 1987; Xiong et al., 1989). These expression systems are powerful tools for the expression and characterization of both endogenous and exogenous genes in the mosquito vector. However, these tools become ineffective in *Ae. aegypti* mosquitoes if administered through an infectious blood meal, requiring that the mosquitoes be intrathoracically inoculated with the viruses. Seabaugh et al. (1998) revealed that determinants of the high midgut infectivity and dissemination of the Malaysian SIN MRE16 virus in *Ae. aegypti* mosquitoes cosegregated with the structural gene sequences of the virus. This raised the possibility that the oral infectivity of currently used dsSIN expression systems might be improved. A chimeric SIN expression system designated MRE/3'2J/GFP was constructed by inserting the second subgenomic promoter and GFP gene of the double promoter vector TE/3'2J/GFP into the pMRE1001 cDNA infectious clone. Virus derived from MRE/3'2J/GFP was shown to effectively and efficiently transduce *Ae. aegypti* midgut epithelial cells with the GFP gene following infection *per os*. The availability of an

orally infectious SIN expression system makes available to researchers a powerful tool for the expression of exogenous genes in the midgut of *Ae. aegypti* mosquitoes. MRE/3'2J should facilitate additional studies of pathogen-vector interactions at an organ of great importance to vector competence.

Barriers to infection and dissemination of an arbovirus within the mosquito host are particularly important aspects of vector competence (Woodring, Higgs, and Beaty, 1996). The mechanisms responsible for these barriers have yet to be elucidated. The marked contrast of the midgut infection- and dissemination-potential of the MRE16 and AR339 strains of SIN virus in *Ae. aegypti* mosquitoes represent an intriguing opportunity to explore viral determinants of infectivity and transmissibility in a medically important arthropod vector. The midgut infection, dissemination, and transmission potential of MRE16 virus and virus derived from a cDNA clone, TR339, engineered to reflect the sequence of AR339 virus at the time of its isolation, were compared in *Ae. aegypti.* The results of this study suggested that the expression of a classic ME barrier is primarily responsible for the significantly lower dissemination and transmission potential of the TR339 virus. A second barrier is probably present in the salivary glands but appears to affect the transmission potential of both viruses nearly equally. The MRE16 and TR339 viruses now represent a well characterized model system for further studies of the viral genetic determinants of vector infectivity, particularly those affecting the expression of a ME barrier in a mosquito vector. In addition, a baseline was provided for the temporal and spatial infection/expression patterns in *Ae. aegypti* of the MRE16 and TR339 viruses. Expression systems have been developed from both of these viruses and this knowledge will certainly aid those using them.

The molecular determinants of vector-pathogen interactions are poorly understood. Arboviruses may gain entry to susceptible host cells through specific interactions with receptors on these cells. However, there is little *in vivo* evidence that this occurs in the midgut of the mosquito vector. Molecular approaches employing recombinant DNA technology to the study of alphaviruses have increased our understanding of virus entry, replication, and morphogenesis as well as cytopathic response and persistent infection in the mosquito. The full genomic sequence of MRE16 was determined and a full-length MRE16 cDNA infectious clone, designated MRE16ic, was constructed. The ability of MRE16ic to infect and disseminate from the midgut of *Ae. aegypti* mosquitoes was phenotypically identical to the parental virus. Thus the availability of MRE16ic provides opportunities for the *in vivo* application of molecular genetic approaches in order to obtain a more detailed understanding of alphavirus infection of the invertebrate vector. The power of this tool for this purpose was demonstrated when a small plaque phenotype MRE16 mutant was found to be present in the MRE16 virus quasispecies. The MRE16 virus small plaque mutant was found to contain a deletion in the E2 region of the virus genome, including a portion encoding the proposed cell-receptor binding domain. To test the hypothesis that the deletion in E2 was solely responsible for an observed reduction in oral infectivity, two separate deletions were introduced into the full-length MRE16 cDNA clone, one encompassing residues E-200 to Y-229, and the second encompassing E-200 to C-220 of E2. Both deletions significantly reduced the ability of MRE16ic virus to infect the midguts of AaRexD mosquitoes in oral infection trials. These results indicated that the deletions introduced into MRE16ic were by themselves capable of dramatically reducing the midgut

infectivity of the virus, and the inability of the deletion mutants to efficiently infect the midgut was responsible for the dramatic reductions observed in the number of mosquitoes developing disseminated infections after oral exposure. Furthermore, only the deleted portion of the proposed E2 cell-receptor binding domain (E-200 to C-220) was necessary to produce the observed reduction in midgut infectivity. These results provide indirect evidence for the hypothesis that alphaviruses gain entry to mosquito midgut epithelial cells through a specific receptor-ligand interaction. It is likely that the E2 envelope glycoprotein from amino acid residues E-200 to C-220 of MRE16 virus contains important molecular viral determinants of midgut infection. However, elucidation of a specific mechanism will require additional studies. Such studies might increase our understanding of the mechanisms of vector specificity and could suggest novel strategies for the control of arthropod-borne viruses.

In combination the results reported here have clearly illustrated the power of a SIN model and an infectious clone based approach to the study of arboviral-vector interactions. In addition the work has laid a solid foundation for additional studies encompassing several areas of the virus-vector relationship.

REFERENCES

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

Altman, R. M. (1963). The behavior of Murray Valley encephalitis virus in *Culex tritaeniorhynchus* Giles and *Culex pipiens quinquefasciatus* Say. *Am J Trop Med Hyg* **12,** 425.

Angell, S. M., and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *Embo J* **16,** 3675.

Anthony, R. P., and Brown, D. T. (1991). Protein-protein interactions in an alphavirus membrane. *J Virol* **65,** 1187.

Arcus, Y. M., Houk, E. J., and Hardy, J. L. (1983). Comparative *in vitro* binding of an arbovirus to midgut microvillar membranes from susceptible and refractory *Culex* mosquitoes. *Fed. Proc.* **42,** 2141.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds. (1994). Current Protocols in Molecular Biology. Current Protocols. Edited by K. Janssen. 2 vols.

Barillas-Mury, C. V., Noriega, F. G., and Wells, M. A. (1995). Early trypsin activity is part of the signal transduction system that activates transcription of the late trypsin gene in the midgut of the mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol* **25,** 241.

Barnett, H. C. (1956). The transmission of western equine encephalitis virus by the mosquito *Culex tarsalis* Coq. *Am J Trop Med Hyg* **5,** 86.

Baulcombe, D. (1999). Viruses and gene silencing in plants. *Arch Virol Suppl* **15,** 189.

Beaty, B. J., Holterman, M., Tabachnick, W., Shope, R. E., Rozhon, E. J., and Bishop, D. H. (1981). Molecular basis of bunyavirus transmission by mosquitoes: role of the middle-sized RNA segment. *Science* **211,** 1433.

Beaty, B. J., Miller, B. R., Shope, R. E., Rozhon, E. J., and Bishop, D. H. (1982). Molecular basis of bunyavirus per os infection of mosquitoes: role of the middle-sized RNA segment. *Proc Natl Acad Sci U S A* **79,** 1295.

Beaty, B. J., and Thompson, W. H. (1978). Tropisms of La Crosse virus in *Aedes triseriatus* (Diptera: Culicidae) following infective blood meals. *J Med Entomol* **14,** 499.

Beerntsen, B. T., James, A. A., and Christensen, B. M. (2000). Genetics of mosquito vector competence. *Microbiol Mol Biol Rev* **64,** 115.

Bergold, G. H., Suarez, O. M., and Munz, K. (1968). Multiplication in and transmission by *Aedes aegypti* of vesicular stomatitis virus. *J Invertebr Pathol* **11,** 406.

Black, W. C. t., Bennett, K. E., Gorrochotegui-Escalante, N., Barillas-Mury, C. V., Fernandez-Salas, I., de Lourdes Munoz, M., Farfan-Ale, J. A., Olson, K. E., and Beaty, B. J. (2002). Flavivirus susceptibility in *Aedes aegypti. Arch Med Res* **33,** 379.

Blair, C. D., Adelman, Z. N., and Olson, K. E. (2000). Molecular strategies for interrupting arthropod-borne virus transmission by mosquitoes. *Clin Microbiol Rev* **13,** 651.

Boorman, J. (1960). Observations on the amount of virus present in haemolymph of *Aedes aegypti* infected with Uganda S, yellow fever and Semliki Forest Viruses. *Trans. R. Soc. Trop. Med. Hyg.* **54,** 362.

Bosio, C. F., Beaty, B. J., and Black, W. C. t. (1998). Quantitative genetics of vector competence for dengue-2 virus in *Aedes aegypti. Am J Trop Med Hyg* **59,** 965.

Bosio, C. F., Fulton, R. E., Salasek, M. L., Beaty, B. J., and Black, W. C. (2000). Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti*. *Genetics* **156,** 687.

Bowers, D. F., Abell, B. A., and Brown, D. T. (1995). Replication and tissue tropism of the alphavirus Sindbis in the mosquito *Aedes albopictus*. *Virology* **212,** 1.

Brault, A. C., Powers, A. M., and Weaver, S. C. (2002). Vector infection determinants of Venezuelan equine encephalitis virus reside within the E2 envelope glycoprotein. *J Virol* **76,** 6387.

Bredenbeek, P. J., Frolov, I., Rice, C. M., and Schlesinger, S. (1993). Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J Virol* **67,** 6439.

Brown, D. T., and Condreay, L. D. (1986). Replication of alphaviruses in mosquito cells. *In* "The Togaviridae and Flaviviridae" (S. Schlesinger, and M. J. Schlesinger, Eds.), p. 171. Plenum Press, New York.

Burge, B. W., and Pfefferkorn, E. R. (1966). Isolation and characterization of conditional-lethal mutants of Sindbis virus. *Virology* **30,** 204.

Burness, A. T., Pardoe, I., Faragher, S. G., Vrati, S., and Dalgarno, L. (1988). Genetic stability of Ross River virus during epidemic spread in nonimmune humans. *Virology* **167,** 639.

Butler, D., Maurice, J., and O'Brien, C. (1997). Time to put malaria control on the global agenda. *Nature* **386,** 535.

Calisher, C. H., and Karabatsos, N. (1988). Arbovirus serogroups: definition and geographic distribution. *In* "The arboviruses: epidemiology and ecology" (T. P. Monath, Ed.), Vol. 1, p. 19. CRC Press, Boca Raton, FL.

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

Carlson, J., Olson, K., Higgs, S., and Beaty, B. (1995). Molecular genetic manipulation of mosquito vectors. *Annu Rev Entomol* **40,** 359.

Castronovo, V., Claysmith, A. P., Barker, K. T., Cioce, V., Krutzsch, H. C., and Sobel, M. E. (1991). Biosynthesis of the 67 kDa high affinity laminin receptor. *Biochem Biophys Res Commun* **177,** 177.

Castronovo, V., Taraboletti, G., and Sobel, M. E. (1991). Functional domains of the 67-kDa laminin receptor precursor. *J Biol Chem* **266,** 20440.

Chamberlain, R. W. (1980). Epidemiology of arthropod-borne togaviruses: the role of arthropods as hosts and vectors and of vertebrate hosts in natural transmission cycles. *In* "The togaviruses: biology, structure, replication" (R. W. Schlesinger, Ed.), p. 175. Academic Press, Inc, New York.

Chamberlain, R. W., Kissling, R. E., and Sikes, R. K. (1954). Studies on the North American arthropod-borne encephalitides. VII. Estimation of the amount of eastern equine encephalitis virus inoculated by infective *Aedes aegypti. Am J Hyg* **60,** 286.

Chamberlain, R. W., and Sudia, W. D. (1961). Mechanisms of transmission of viruses by mosquitoes. *Ann. Rev. Entomol.* **6,** 371.

Chanas, A. C., Gould, E. A., Clegg, J. C., and Varma, M. G. (1982). Monoclonal antibodies to Sindbis virus glycoprotein E1 can neutralize, enhance infectivity, and independently inhibit haemagglutination or haemolysis. *J Gen Virol* **58,** 37.

Chandler, L. J., Blair, C. D., and Beaty, B. J. (1998). La Crosse virus infection of *Aedes triseriatus* (Diptera: Culicidae) ovaries before dissemination of virus from the midgut. *J Med Entomol* **35,** 567.

Cheng, L. L., Bartholomay, L. C., Olson, K. E., Lowenberger, C., Higgs, S., Beaty, B. J., and Christensen, B. M. (2001). Characterization of an endogenous gene expressed in *Aedes aegypti* using an orally infectious recombinant Sindbis virus. 1:10 ed. Journal of Insect Sciences.

Chernesky, M. A. (1968). Transmission of California encephalitis virus by mosquitoes. *Can J Microbiol* **14,** 19.

Coates, C. J., Jasinskiene, N., Miyashiro, L., and James, A. A. (1998). Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci U S A* **95,** 3748.

Collins, W. E., Harrison, A. J., and Jumper, J. R. (1965). Infection and transmission thresholds of eastern encephalitis virus to *Aedes aegypti* as determined by a membrane feeding technique. *Mosq. News.* **25,** 293.

Coombs, K., and Brown, D. T. (1987). Topological organization of Sindbis virus capsid protein in isolated nucleocapsids. *Virus Res* **7,** 131.

Davis, N. L., Fuller, F. J., Dougherty, W. G., Olmsted, R. A., and Johnston, R. E. (1986). A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. *Proc Natl Acad Sci U S A* **83,** 6771.

Davis, N. L., Pence, D. F., Meyer, W. J., Schmaljohn, A. L., and Johnston, R. E. (1987). Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. *Virology* **161,** 101.

Davis, N. L., Powell, N., Greenwald, G. F., Willis, L. V., Johnson, B. J., Smith, J. F., and Johnston, R. E. (1991). Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. *Virology* **183,** 20.

Davis, N. L., Willis, L. V., Smith, J. F., and Johnston, R. E. (1989). In vitro synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. *Virology* **171,** 189.

de Lara Capurro, M., Coleman, J., Beerntsen, B. T., Myles, K. M., Olson, K. E., Rocha, E., Krettli, A. U., and James, A. A. (2000). Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium gallinaceum*-infected *Aedes aegypti*. *Am J Trop Med Hyg* **62,** 427.

Doherty, R. L., Carley, J. G., Filippich, C., Kay, B. H., Gorman, B. M., and Rajapaksa, N. (1977). Isolation of Sindbis (alphavirus) and Leanyer viruses from mosquitoes collected in the Northern Territory of Australia, 1974. *Aust J Exp Biol Med Sci* **55,** 485.

Doherty, R. L., Carley, J. G., Kay, B. H., Filippich, C., Marks, E. N., and Frazier, C. L. (1979). Isolation of virus strains from mosquitoes collected in Queensland, 1972-1976. *Aust J Exp Biol Med Sci* **57,** 509.

Doi, R. (1970). Studies on the mode of development of Japanese encephalitis virus in some groups of mosquitoes by the fluorescent antibody technique. *Jpn J Exp Med* **40,** 101.

Doi, R., Shirasaki, A., and Sasa, M. (1967). The mode of development of Japanese encephalitis virus in the mosquito *Culex tritaeniorhynchus* summorosus as observed by the fluorescent antibody technique. *Jpn J Exp Med* **37,** 227.

Dougherty, W. G., Lindbo, J. A., Smith, H. A., Parks, T. D., Swaney, S., and Proebsting, W. M. (1994). RNA-mediated virus resistance in transgenic plants: exploitation of a cellular pathway possibly involved in RNA degradation. *Mol Plant Microbe Interact* **7,** 544.

Dubuisson, J., Lustig, S., Ruggli, N., Akov, Y., and Rice, C. M. (1997). Genetic determinants of Sindbis virus neuroinvasiveness. *J Virol* **71,** 2636.

Dubuisson, J., and Rice, C. M. (1993). Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J Virol* **67,** 3363.

Duda, E., and Berencsi, K. (1980). Sindbis virus receptor protein of BHK cells. *Acta Virol* **24,** 149.

Felix, C. R., Betschart, B., Billingsley, P. F., and Freyvogal, T. A. (1991). Post-feeding induction of trypsin in the midguts of *Aedes aegypti* (Diptera: Culicidae) is separable into two cellular phases. *Insect Biochem* **21,** 197.

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

Flynn, D. C., Meyer, W. J., Mackenzie, J. M., Jr., and Johnston, R. E. (1990). A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. *J Virol* **64,** 3643.

Freier, J. E., and Beier, J. C. (1984). Oral and transovarial transmission of La Crosse virus by *Aedes atropalpus*. *Am J Trop Med Hyg* **33,** 708.

Gahmberg, C. G., Utermann, G., and Simons, K. (1972). The membrane proteins of Semliki Forest virus have a hydrophobic part attached to the viral membrane. *FEBS Lett* **28,** 179.

Gaidamovich, S. Y., Khutoretskaya, N. V., Lvova, A. I., and Sveshnikova, N. A. (1973). Immunofluorescent staining study of the salivary glands of mosquitoes infected with group A arboviruses. *Intervirology* **1,** 193.

Gaidamovich, S. Y., Tsilinsky, Y. Y., Lvova, A. I., and Khutoretskaya, N. V. (1971). *Aedes aegypti* mosquitoes as an experimental model for studies on the ecology and genetics of Venezuelan equine encephalomyelitis virus. *Acta Virol* **15,** 301.

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

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

Garoff, H., Frischauf, A. M., Simons, K., Lehrach, H., and Delius, H. (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature* **288,** 236.

Garoff, H., and Simons, K. (1974). Location of the spike glycoproteins in the Semliki Forest virus membrane. *Proc Natl Acad Sci U S A* **71,** 3988.

Geigenmuller-Gnirke, U., Weiss, B., Wright, R., and Schlesinger, S. (1991). Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome. *Proc Natl Acad Sci U S A* **88,** 3253.

Gould, E. A., and Clegg, J. C. S. (1985). Growth, Titration and Purification of Togaviruses. *In* "Virology: a practical approach." (B. W. J. Mahy, Ed.), p. 43. IRL Press, Oxford, U.K.

Gratz, N. G. (1999). Emerging and resurging vector-borne diseases. *Annu Rev Entomol* **44,** 51.

Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F. (1958). Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am J Trop Med Hyg* **7,** 365.

Griffin, D. E., and Johnson, R. T. (1977). Role of the immune response in recovery from Sindbis virus encephalitis in mice. *J Immunol* **118,** 1070.

Grimstad, P. R. (1988). California Group virus disease. *In* "The arboviruses: epidemiology and ecology" (T. P. Monath, Ed.), Vol. 2, p. 99. CRC, Boca Raton, FL.

Grimstad, P. R., Paulson, S. L., and Craig, G. B., Jr. (1985). Vector competence of *Aedes hendersoni* (Diptera: Culicidae) for La Crosse virus and evidence of a salivary-gland escape barrier. *J Med Entomol* **22,** 447.

Grosso, L. E., Park, P. W., and Mecham, R. P. (1991). Characterization of a putative clone for the 67-kilodalton elastin/laminin receptor suggests that it encodes a cytoplasmic protein rather than a cell surface receptor. *Biochemistry* **30,** 3346.

Gubler, D. J. (1998). Resurgent vector-borne diseases as a global health problem. *Emerg Infect Dis* **4,** 442.

Gubler, D. J. (2001). Human arbovirus infections worldwide. *Ann N Y Acad Sci* **951,** 13.

Gubler, D. J., and Rosen, L. (1976a). A simple technique for demonstrating transmission of dengue virus by mosquitoes without the use of vertebrate hosts. *Am J Trop Med Hyg* **25,** 146.

Gubler, D. J., and Rosen, L. (1976b). Variation among geographic strains of *Aedes albopictus* in susceptibility to infection with dengue viruses. *Am J Trop Med Hyg* **25,** 318.

Gwadz, R. W., Kaslow, D., Lee, J. Y., Maloy, W. L., Zasloff, M., and Miller, L. H. (1989). Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infect Immun* **57,** 2628.

Hahn, C. S., Hahn, Y. S., Braciale, T. J., and Rice, C. M. (1992). Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. *Proc Natl Acad Sci U S A* **89,** 2679.

Hardy, J. L. (1988). Susceptibility and resistance of vector mosquitoes. *In* "The arboviruses: ecology and epidemiology" (T. P. Monath, Ed.), Vol. 1, p. 87. CRC Press, Boca Raton, FL.

Hardy, J. L., Houk, E. J., Kramer, L. D., and Reeves, W. C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annu Rev Entomol* **28,** 229.

Harrison, S. C. (1986). Alphavirus structure. *In* "The Togaviridae and Flaviviridae" (S. Schlesinger, and M. J. Schlesinger, Eds.), p. 21. Plenum Publishing Corp., New York.

Harrison, S. C., David, A., Jumblatt, J., and Darnell, J. E. (1971). Lipid and protein organization in Sindbis virus. *J Mol Biol* **60,** 533.

Harrison, S. C., Strong, R. K., Schlesinger, S., and Schlesinger, M. J. (1992). Crystallization of Sindbis virus and its nucleocapsid. *J Mol Biol* **226,** 277.

Hayles, L. B., McLintock, J., and Saunders, J. R. (1972). Laboratory studies on the transmission of western equine encephalitis virus by Saskatchewan mosquitoes. *I. Culex tarsalis*. *Can J Comp Med* **36,** 83.

Helenius, A., Morein, B., Fries, E., Simons, K., Robinson, P., Schirrmacher, V., Terhorst, C., and Strominger, J. L. (1978). Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. *Proc Natl Acad Sci U S A* **75,** 3846.

Higgs, S., and Beaty, B. J. (1996). Rearing and Containment of Mosquito Vectors. *In* "The Biology of Disease Vectors" (B. J. Beaty, and W. C. Marquardt, Eds.), p. 595. University Press of Colorado, Niwot, CO.

Higgs, S., Olson, K. E., Klimowski, L., Powers, A. M., Carlson, J. O., Possee, R. D., and Beaty, B. J. (1995). Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Sindbis virus vector. *Insect Mol Biol* **4,** 97.

Higgs, S., Oray, C. T., Myles, K., Olson, K. E., and Beaty, B. J. (1999). Infecting larval arthropods with a chimeric, double subgenomic Sindbis virus vector to express genes of interest. *Biotechniques* **27,** 908.

Higgs, S., Powers, A. M., and Olson, K. E. (1993). Alphavirus expression systems: applications to mosquito vector studies. *Parasitology Today* **9,** 444.

Higgs, S., Rayner, J. O., Olson, K. E., Davis, B. S., Beaty, B. J., and Blair, C. D. (1998). Engineered resistance in *Aedes aegypti* to a West African and a South American strain of yellow fever virus. *Am J Trop Med Hyg* **58,** 663.

Higgs, S., Traul, D., Davis, B. S., Kamrud, K. I., Wilcox, C. L., and Beaty, B. J. (1996). Green fluorescent protein expressed in living mosquitoes without the requirement of transformation. *Biotechniques* **21,** 660.

Houk, E. J., Chiles, R. E., and Hardy, J. L. (1980). Unique midgut lamina in the mosquito, *Aedes dorsalis* (Meigan) (Insecta:Diptera). *Int. J. Insect Morphol. Embryol.* **9,** 161.

Houk, E. J., Hardy, J. L., and Chiles, R. E. (1981). Permeability of the midgut basal lamina in the mosquito, *Culex tarsalis* Coquillett (Insecta, Diptera). *Acta Trop* **38,** 163.

Houk, E. J., Kramer, L. D., Hardy, J. L., and Chiles, R. E. (1985). Western equine encephalomyelitis virus: in vivo infection and morphogenesis in mosquito mesenteronal epithelial cells. *Virus Res* **2,** 123.

Houk, E. J., Kramer, L. D., Hardy, J. L., and Presser, S. B. (1986). An interspecific mosquito model for the mesenteronal infection barrier to western equine encephalomyelitis virus (*Culex tarsalis* and *Culex pipiens*). *Am J Trop Med Hyg* **35,** 632.

Howard, J. J., and Wallis, R. C. (1974). Infection and transmission of eastern equine encephalomyelitis virus with colonized *Culiseta melanura* (Coquillett). *Am J Trop Med Hyg* **23,** 522.

Huang, M. J., and Summers, J. (1991). Infection initiated by the RNA pregenome of a DNA virus. *J Virol* **65,** 5435.

Hurlbut, H., and Thomas, J. (1960). The experimental host range of the arthropod-borne animal viruses in arthropods. *Virology* **12,** 391.

Hurlbut, H. S. (1956). West Nile virus infection in arthropods. *Am J Trop Med Hyg* **5,** 76.

Hurlbut, H. S. (1966). Mosquito salivation and virus transmission. *Am J Trop Med Hyg* **15,** 989.

Igarashi, A. (1978). Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *J Gen Virol* **40,** 531.

Jackson, A. C., Bowen, J. C., and Downe, A. E. (1993). Experimental infection of *Aedes aegypti* (Diptera: Culicidae) by the oral route with Sindbis virus. *J Med Entomol* **30,** 332.

Jasinskiene, N., Coates, C. J., Benedict, M. Q., Cornel, A. J., Rafferty, C. S., James, A. A., and Collins, F. H. (1998). Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. *Proc Natl Acad Sci U S A* **95,** 3743.

Johnson, B. W., Olson, K. E., Allen-Miura, T., Rayms-Keller, A., Carlson, J. O., Coates, C. J., Jasinskiene, N., James, A. A., Beaty, B. J., and Higgs, S. (1999). Inhibition of luciferase expression in transgenic *Aedes aegypti* mosquitoes by Sindbis virus expression of antisense luciferase RNA. *Proc Natl Acad Sci U S A* **96,** 13399.

Jorgensen, R. (1990). Altered gene expression in plants due to trans interactions between homologous genes. *Trends Biotechnol* **8,** 340.

Jupp, P. G. (1985). Culex theileri and Sindbis virus; salivary glands infection in relation to transmission. *J Am Mosq Control Assoc* **1,** 374.

Jupp, P. G., and I., P. J. (1998). An electron microscopical study of Rift Valley fever and Sindbis viral infection in mosquito salivary glands (Diptera:Culicidae). *African Entomology* **6,** 75.

Jupp, P. G., and McIntosh, B. M. (1970a). Quantitative experiments on the vector capability of *Culex* (*Culex*) *pipiens* fatigans Wiedemann with West Nile and Sindbis viruses. *J Med Entomol* **7,** 353.

Jupp, P. G., and McIntosh, B. M. (1970b). Quantitative experiments on the vector capability of *Culex* (*Culex*) *univittatus* Theobald with West Nile and Sindbis viruses. *J Med Entomol* **7,** 371.

Jupp, P. G., McIntosh, B. M., and Dickinson, D. B. (1972). Quantitative experiments on the vector capability of *Culex* (*Culex*) *theileri* Theobald with West Nile and Sindbis viruses. *J Med Entomol* **9,** 393.

Kamrud, K. I., Olson, K. E., Higgs, S., Powers, A. M., Carlson, J. O., and Beaty, B. J. (1997). Detection of expressed chloramphenicol acetyltransferase in the saliva of *Culex pipiens* mosquitoes. *Insect Biochem Mol Biol* **27,** 423.

Kamrud, K. I., Powers, A. M., Higgs, S., Olson, K. E., Blair, C. D., Carlson, J. O., and Beaty, B. J. (1995). The expression of chloramphenicol acetyltransferase in mosquitoes and mosquito cells using a packaged Sindbis replicon system. *Exp Parasitol* **81,** 394.

Karabatsos, N., Ed. (1985). International Catalogue of Arboviruses, including certain other viruses of vertebrates. 3rd ed. San Antonio, TX: American Society of Tropical Medicine and Hygiene.

Karber, G. (1931). Bietrag zur kollkktiven behandlung pharmakologischer reiheversuche. *Arch Exp Pathol Pharmakol* **162,** 480.

Kerr, P. J., Weir, R. C., and Dalgarno, L. (1993). Ross River virus variants selected during passage in chick embryo fibroblasts: serological, genetic, and biological changes. *Virology* **193,** 446.

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

Kinney, R. M., Chang, G. J., Tsuchiya, K. R., Sneider, J. M., Roehrig, J. T., Woodward, T. M., and Trent, D. W. (1993). Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. *J Virol* **67,** 1269.

Klimstra, W. B., Ryman, K. D., and Johnston, R. E. (1998). Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J Virol* **72,** 7357.

Kondor-Koch, C., Burke, B., and Garoff, H. (1983). Expression of Semliki Forest virus proteins from cloned complementary DNA. I. The fusion activity of the spike glycoprotein. *J Cell Biol* **97,** 644.

Kondor-Koch, C., Riedel, H., Soderberg, K., and Garoff, H. (1982). Expression of the structural proteins of Semliki Forest virus from cloned cDNA microinjected into the nucleus of baby hamster kidney cells. *Proc Natl Acad Sci U S A* **79,** 4525.

Kramer, L. D., Hardy, J. L., Presser, S. B., and Houk, E. J. (1981). Dissemination barriers for western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low viral doses. *Am J Trop Med Hyg* **30,** 190.

Kuberski, T. (1979). Fluorescent antibody studies on the development of dengue-2 virus in *Aedes albopictus* (Diptera: Culicidae). *J Med Entomol* **16,** 343.

Kuhn, R. J., Griffin, D. E., Owen, K. E., Niesters, H. G., and Strauss, J. H. (1996). Chimeric Sindbis-Ross River viruses to study interactions between alphavirus nonstructural and structural regions. *J Virol* **70,** 7900.

Kuhn, R. J., Griffin, D. E., Zhang, H., Niesters, H. G., and Strauss, J. H. (1992). Attenuation of Sindbis virus neurovirulence by using defined mutations in nontranslated regions of the genome RNA. *J Virol* **66,** 7121.

Kuhn, R. J., Niesters, H. G., Hong, Z., and Strauss, J. H. (1991). Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterization of defined chimeras with Sindbis virus. *Virology* **182,** 430.

Lamotte, L. C. (1960). Japanese B encephalitis virus in the organs of infected mosquitoes. *Am J Hyg* **72,** 73.

Lane, D., and Harlow, E., Eds. (1990). Antibodies: A Laboratory Manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor laboratory Press.

Lanzen, H. G., and Wright, K. A. (1971). The salivary glands of *Aedes aegypti* (L.): an electron microscopic study. *Can J Zool* **49,** 1343.

Lee, C. H., and Schloemer, R. H. (1981a). Identification of the antiviral factor in culture medium of mosquito cells persistently infected with Banzi virus. *Virology* **110,** 445.

Lee, C. H., and Schloemer, R. H. (1981b). Mosquito cells infected with Banzi virus secrete an antiviral activity which is of viral origin. *Virology* **110,** 402.

Levine, B., Jiang, H. H., Kleeman, L., and Yang, G. (1996). Effect of E2 envelope glycoprotein cytoplasmic domain mutations on Sindbis virus pathogenesis. *J Virol* **70,** 1255.

Levis, R., Huang, H., and Schlesinger, S. (1987). Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells. *Proc Natl Acad Sci U S A* **84,** 4811.

Liljestrom, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991). *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J Virol* **65,** 4107.

Liu, I. K., and Zee, Y. C. (1976). The pathogenesis of vesicular stomatitis virus, serotype Indiana, in *Aedes aegypti* mosquitoes. I. Intrathoracic injection. *Am J Trop Med Hyg* **25,** 177.

Lobo, N. F., Hua-Van, A., Li, X., Nolen, B. M., and Fraser, M. J., Jr. (2002). Germ line transformation of the yellow fever mosquito, *Aedes aegypti*, mediated by transpositional insertion of a piggyBac vector. *Insect Mol Biol* **11,** 133.

Lowenberger, C. A., Ferdig, M. T., Bulet, P., Khalili, S., Hoffmann, J. A., and Christensen, B. M. (1996). *Aedes aegypti*: induced antibacterial proteins reduce the establishment and development of *Brugia malayi*. *Exp Parasitol* **83,** 191.

Lowenberger, C. A., Kamal, S., Chiles, J., Paskewitz, S., Bulet, P., Hoffmann, J. A., and Christensen, B. M. (1999). Mosquito-*Plasmodium* interactions in response to immune activation of the vector. *Exp Parasitol* **91,** 59.

Ludwig, G. V., Christensen, B. M., Yuill, T. M., and Schultz, K. T. (1989). Enzyme processing of La Crosse virus glycoprotein G1: a bunyavirus-vector infection model. *Virology* **171,** 108.

Ludwig, G. V., Kondig, J. P., and Smith, J. F. (1996). A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J Virol* **70,** 5592.

Lustig, S., Jackson, A. C., Hahn, C. S., Griffin, D. E., Strauss, E. G., and Strauss, J. H. (1988). Molecular basis of Sindbis virus neurovirulence in mice. *J Virol* **62,** 2329.

Maassen, J. A., and Terhorst, C. (1981). Identification of a cell-surface protein involved in the binding site of Sindbis virus on human lymphoblastic cell lines using a heterobifunctional cross-linker. *Eur J Biochem* **115,** 153.

Mackenzie, J. S., Lindsay, M. D., Coelen, R. J., Broom, A. K., Hall, R. A., and Smith, D. W. (1994). Arboviruses causing human disease in the Australian zoogeographic region. *Arch Virol* **136,** 447.

Malherbe, H., and Strickland-Cholmley, M. (1963). Sindbis virus infection in man: report of a case with recovery of virus from skin lesions. *S Afr Med J* **37,** 547.

Mastromarino, P., Conti, C., Petruzziello, R., Lapadula, R., and Orsi, N. (1991). Effect of polyions on the early events of Sindbis virus infection of Vero cells. *Arch Virol* **121,** 19.

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

McLean, D. M. (1955). Multiplication of viruses in mosquitoes following feeding and injection into the body cavity. *Aust J Exp Biol Med* **33,** 53.

McLintock, J. (1978). Mosquito-virus relationships of American encephalitides. *Annu Rev Entomol* **23,** 17.

Mellink, J. J. (1982). Estimation of the amount of Venezuelan equine encephalomyelitis virus transmitted by a single infected *Aedes aegypti* (Diptera: Culicidae). *J Med Entomol* **19,** 275.

Mendoza, Q. P., Stanley, J., and Griffin, D. E. (1988). Monoclonal antibodies to the E1 and E2 glycoproteins of Sindbis virus: definition of epitopes and efficiency of protection from fatal encephalitis. *J Gen Virol* **69,** 3015.

Miles, J. A., Pillai, J. S., and Maguire, T. (1973). Multiplication of Whataroa virus in mosquitoes. *J Med Entomol* **10,** 176.

Miller, B. R., Beaty, B. J., and Lorenz, L. H. (1982). Variation of La Crosse virus filial infection rates in geographic strains of *Aedes triseriatus* (Diptera: Culicidae). *J Med Entomol* **19,** 213.

Miller, B. R., DeFoliart, G. R., and Yuill, T. M. (1977). Vertical transmission of La Crosse virus (California encephalitis group): transovarial and filial infection rates in *Aedes triseriatus* (Diptera: Culicidae). *J Med Entomol* **14,** 437.

Miller, B. R., DeFoliart, G. R., and Yuill, T. M. (1979). *Aedes triseriatus* and La Crosse virus: lack of infection in eggs of the first ovarian cycle following oral infection of females. *Am J Trop Med Hyg* **28,** 897.

Miller, B. R., and Mitchell, C. J. (1986). Passage of yellow fever virus: its effect on infection and transmission rates in *Aedes aegypti. Am J Trop Med Hyg* **35,** 1302.

Miller, B. R., and Mitchell, C. J. (1991). Genetic selection of a flavivirus-refractory strain of the yellow fever mosquito *Aedes aegypti. Am J Trop Med Hyg* **45,** 399.

Monath, T. P. (2001). Yellow fever: an update. *Lancet Infect Dis* **1,** 11.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T. A., and Vaucheret, H. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101,** 533.

Murphy, F. A. (1975). Cellular resistance to arbovirus infection. *Ann N Y Acad Sci* **266,** 197.

Murphy, F. A., Whitfield, S. G., Sudia, W. D., and Chamberlain, R. W. (1975). Interactions of vector with vertebrate pathogenic viruses. *In* "Invertebrate Immunity" (K. Maramorosch, and R. E. Shope, Eds.), p. 25. Academic Press, New York.

Napoli, C. A., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthetase gene in Petunia results in reversable cosuppression of homologous genes *in trans. Plant Cell* **2,** 279.

Newton, S. E., Short, N. J., and Dalgarno, L. (1981). Bunyamwera virus replication in cultured *Aedes albopictus* (mosquito) cells: establishment of a persistent viral infection. *J Virol* **38,** 1015.

Niklasson, B. (1988). Sindbis and Sindbis-like viruses. *In* "The arboviruses: epidemiology and ecology" (T. P. Monath, Ed.), p. 167. CRC Press, Inc, Boca Raton, Fla.

Noriega, F. G., Pennington, J. E., Barillas-Mury, C., Wang, X. Y., and Wells, M. A. (1996a). *Aedes aegypti* midgut early trypsin is post-transcriptionally regulated by blood feeding. *Insect Mol Biol* **5,** 25.

Noriega, F. G., Wang, X.-Y., Pennington, J. E., Barillas-Mury, C. V., and Wells, M. A. (1996b). Early trypsin, a female-specific midgut protease in *Aedes aegypti*: isolation, aminoterminal sequence determination, and cloning and sequencing of the gene. *Insect Biochem Mol Biol* **26,** 119.

Ogunbi, O. (1968). Ukauwa virus proliferation in mosquitoes. *Can J Microbiol* **14,** 125.

Oldstone, M. B., Tishon, A., Dutko, F. J., Kennedy, S. I., Holland, J. J., and Lampert, P. W. (1980). Does the major histocompatibility complex serve as a specific receptor for Semliki Forest virus? *J Virol* **34,** 256.

Olson, K., and Trent, D. W. (1985). Genetic and antigenic variations among geographical isolates of Sindbis virus. *J Gen Virol* **66,** 797.

Olson, K. E., Beaty, B., and Higgs, S. (1998). Sindbis virus expression systems for the manipulation of insect vectors. *In "The Insect Viruses"* (L. K. Miller, and L. A. Ball, Eds.), p. 371. Plenum Press, New York.

Olson, K. E., Carlson, J. O., and Beaty, B. J. (1992). Expression of the chloramphenicol acetyltransferase gene in *Aedes albopictus* (C6/36) cells using a non-infectious Sindbis virus expression vector. *Insect Mol Biol* **1,** 49.

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

Olson, K. E., Higgs, S., Hahn, C. S., Rice, C. M., Carlson, J. O., and Beaty, B. J. (1994). The expression of chloramphenicol acetyltransferase in *Aedes albopictus* (C6/36) cells and *Aedes triseriatus* mosquitoes using a double subgenomic recombinant Sindbis virus. *Insect Biochem Mol Biol* **24,** 39.

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

Omar, A., and Koblet, H. (1988). Semliki Forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. *Virology* **166,** 17.

Orr, C. W. M., Hudson, A., and West, A. S. (1961). The salivary glands of *Aedes aegypti.* Histological-histochemical studies. *Can J Zool* **39,** 265.

Pal-Bhadra, M., Bhadra, U., and Birchler, J. A. (1997). Cosuppression in *Drosophila*: gene silencing of Alcohol dehydrogenase by white-Adh transgenes is Polycomb dependent. *Cell* **90,** 479.

Paredes, A. M., Brown, D. T., Rothnagel, R., Chiu, W., Schoepp, R. J., Johnston, R. E., and Prasad, B. V. (1993). Three-dimensional structure of a membrane-containing virus. *Proc Natl Acad Sci U S A* **90,** 9095.

Paredes, A. M., Simon, M. N., and Brown, D. T. (1992). The mass of the Sindbis virus nucleocapsid suggests it has T = 4 icosahedral symmetry. *Virology* **187,** 329.

Patrican, L. A., DeFoliart, G. R., and Yuill, T. M. (1985). Oral infection and transmission of La Crosse virus by an enzootic strain of *Aedes triseriatus* feeding on chipmunks with a range of viremia levels. *Am J Trop Med Hyg* **34,** 992.

Pattyn, S. R., and De Vleesschauwer, L. (1968). The multiplication of Middleburg s and l plaque viruses in *Aedes aegypti* mosquitoes. *Acta Virol* **12,** 347.

Peers, R. R. (1972). Bunyamwera virus replication in mosquitoes. *Can J Microbiol* **18,** 741.

Peleg, J. (1975). In vivo behavior of a Sindbis virus mutant isolated from persistently infected *Aedes aegypti* cell cultures. *Ann N Y Acad Sci* **266,** 204.

Pence, D. F., Davis, N. L., and Johnston, R. E. (1990). Antigenic and genetic characterization of Sindbis virus monoclonal antibody escape mutants which define a pathogenesis domain on glycoprotein E2. *Virology* **175,** 41.

Pfefferkorn, E. R., and Hunter, H. S. (1963). Purification and partial chemical analysis of Sindbis virus. *Virology* **20,** 433.

Pierce, J. S., Strauss, E. G., and Strauss, J. H. (1974). Effect of ionic strength on the binding of Sindbis virus to chick cells. *J Virol* **13,** 1030.

Polo, J. M., Davis, N. L., Rice, C. M., Huang, H. V., and Johnston, R. E. (1988). Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. *J Virol* **62,** 2124.

Polo, J. M., and Johnston, R. E. (1991). Mutational analysis of a virulence locus in the E2 glycoprotein gene of Sindbis virus. *J Virol* **65,** 6358.

Porterfield, J. S. (1980). Antigenic characteristics and classification of togaviridae. *In* "The togaviruses: biology, structure, replication" (R. W. Schlesinger, Ed.), p. 13. Academic Press, Inc., New York.

Powers, A. M., Kamrud, K. I., Olson, K. E., Higgs, S., Carlson, J. O., and Beaty, B. J. (1996). Molecularly engineered resistance to California serogroup virus replication in mosquito cells and mosquitoes. *Proc Natl Acad Sci U S A* **93,** 4187.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111,** 229.

Pudney, M., Leake, C. J., and Varma, M. G. R. (1979). *In* "Arctic and Tropical Arboviruses", p. 245. Academic Press, New York.

Raju, R., and Huang, H. V. (1991). Analysis of Sindbis virus promoter recognition *in vivo*, using novel vectors with two subgenomic mRNA promoters. *J Virol* **65,** 2501.

Rayms-Keller, A., Powers, A. M., Higgs, S., Olson, K. E., Kamrud, K. I., Carlson, J. O., and Beaty, B. J. (1995). Replication and expression of a recombinant Sindbis virus in mosquitoes. *Insect Mol Biol* **4,** 245.

Rentier-Delrue, F., and Young, N. A. (1980). Genomic divergence among Sindbis virus strains. *Virology* **106,** 59.

Rice, C. M. (1992). Examples of expression systems based on animal RNA viruses: alphaviruses and influenza virus. *Curr Opin Biotechnol* **3,** 523.

Rice, C. M. (1996). Flaviviridae: the viruses and their replication. 3rd ed. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), p. 961. Lippincott-Raven, Philadelphia.

Rice, C. M., Bell, J. R., Hunkapiller, M. W., Strauss, E. G., and Strauss, J. H. (1982). Isolation and characterization of the hydrophobic COOH-terminal domains of the Sindbis virion glycoproteins. *J Mol Biol* **154,** 355.

Rice, C. M., Levis, R., Strauss, J. H., and Huang, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J Virol* **61,** 3809.

Rice, C. M., and Strauss, J. H. (1981). Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc Natl Acad Sci U S A* **78,** 2062.

Rice, C. M., and Strauss, J. H. (1982). Association of Sindbis virion glycoproteins and their precursors. *J Mol Biol* **154,** 325.

Riedel, B., and Brown, D. T. (1979). Novel antiviral activity found in the media of Sindbis virus-persistently infected mosquito (*Aedes albopictus*) cell cultures. *J Virol* **29,** 51.

Romano, N., and Macino, G. (1992). Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol Microbiol* **6,** 3343.

Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10,** 937.

Rumenapf, T., Strauss, E. G., and Strauss, J. H. (1995). Aura virus is a New World representative of Sindbis-like viruses. *Virology* **208,** 621.

Russell, D. L., Dalrymple, J. M., and Johnston, R. E. (1989). Sindbis virus mutations which coordinately affect glycoprotein processing, penetration, and virulence in mice. *J Virol* **63,** 1619.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." 2 ed. 3 vols. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sammels, L. M., Lindsay, M. D., Poidinger, M., Coelen, R. J., and Mackenzie, J. S. (1999). Geographic distribution and evolution of Sindbis virus in Australia. *J Gen Virol* **80,** 739.

Scherer, W. F., Cupp, E. W., Dziem, G. M., Breener, R. J., and Ordonez, J. V. (1982). Mesenteronal infection threshold of an epizootic strain of Venezuelan encephalitis virus in *Culex (Melanoconion) taeniopus* mosquitoes and its implication to the apparent disappearance of this virus strain from an enzootic habitat in Guatemala. *Am J Trop Med Hyg* **31,** 1030.

Scherer, W. F., Cupp, E. W., Lok, J. B., Brenner, R. J., and Ordonez, J. V. (1981). Intestinal threshold of an enzootic strain of Venezuelan encephalitis virus in *Culex (Melanoconion) taeniopus* mosquitoes and its implications to vector competency and vertebrate amplifying hosts. *Am J Trop Med Hyg* **30,** 862.

Schiefer, B. A., and Smith, J. R. (1974). Comparative susceptibility of eight mosquito species to Sindbis virus. *Am J Trop Med Hyg* **23,** 131.

Schlesinger, M., and Schlesinger, S. (1986). Formation and assembly of alphavirus glycoproteins. *In* "The Togaviridae and Flaviviridae" (S. Schlesinger, and M. J. Schlesinger, Eds.), p. 121. Plenum Publishing Corp., New York.

Schoepp, R. J., and Johnston, R. E. (1993a). Directed mutagenesis of a Sindbis virus pathogenesis site. *Virology* **193,** 149.

Schoepp, R. J., and Johnston, R. E. (1993b). Sindbis virus pathogenesis: phenotypic reversion of an attenuated strain to virulence by second-site intragenic suppressor mutations. *J Gen Virol* **74,** 1691.

Schols, D., De Clercq, E., Balzarini, J., Baba, M., Witvrouw, M., Hosoya, M., Andrei, G., Snoeck, R., Neyts, J., Pauwels, R., Nagy, M., Gyorgyi-Edelenyi, J., Machovich, R., Horvath, I., Low, M., and Gorog, S. (1990). Sulphated polymers are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, vesicular stomatitis virus, respiratory syncytial virus, and toga-, arena-, and retroviruses. *Antivir. Chem. Chemother.* **1,** 233.

Scott, T. W., Hildreth, S. W., and Beaty, B. J. (1984). The distribution and development of eastern equine encephalitis virus in its enzootic mosquito vector, *Culiseta melanura. Am J Trop Med Hyg* **33,** 300.

Scott, T. W., Lorenz, L. H., and Weaver, S. C. (1990). Susceptibility of *Aedes albopictus* to infection with eastern equine encephalomyelitis virus. *J Am Mosq Control Assoc* **6,** 274.

Seabaugh, R. C. (1998). Dissertation. Colorado State University, Fort Collins, CO.

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

Severini, C., Romi, R., Marinucci, M., and Raymond, M. (1993). Mechanisms of insecticide resistance in field populations of *Culex pipiens* from Italy. *J Am Mosq Control Assoc* **9,** 164.

Shiao, S. H., Higgs, S., Adelman, Z., Christensen, B. M., Liu, S. H., and Chen, C. C. (2001). Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito *Armigeres subalbatus*. *Insect Mol Biol* **10,** 315.

Shirako, Y., Niklasson, B., Dalrymple, J. M., Strauss, E. G., and Strauss, J. H. (1991). Structure of the Ockelbo virus genome and its relationship to other Sindbis viruses. *Virology* **182,** 753.

Sibley, C. H., Hyde, J. E., Sims, P. F., Plowe, C. V., Kublin, J. G., Mberu, E. K., Cowman, A. F., Winstanley, P. A., Watkins, W. M., and Nzila, A. M. (2001). Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? *Trends Parasitol* **17,** 582.

Simpson, D. A., Davis, N. L., Lin, S. C., Russell, D., and Johnston, R. E. (1996). Complete nucleotide sequence and full-length cDNA clone of S.A.AR86 a South African alphavirus related to Sindbis. *Virology* **222,** 464.

Singh, I., and Helenius, A. (1992). Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. *J Virol* **66,** 7049.

Smith, A. L., and Tignor, G. H. (1980). Host cell receptors for two strains of Sindbis virus. *Arch Virol* **66,** 11.

Smith, T. J., Cheng, R. H., Olson, N. H., Peterson, P., Chase, E., Kuhn, R. J., and Baker, T. S. (1995). Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. *Proc Natl Acad Sci U S A* **92,** 10648.

Sriurairatna, S., and Bhamarapravati, N. (1977). Replication of dengue-2 virus in *Aedes albopictus* mosquitoes. An electron microscopic study. *Am J Trop Med Hyg* **26,** 1199.

Stec, D. S., Waddell, A., Schmaljohn, C. S., Cole, G. A., and Schmaljohn, A. L. (1986). Antibody-selected variation and reversion in Sindbis virus neutralization epitopes. *J Virol* **57,** 715.

Stollar, V. (1980). Togaviruses in cultured arthropod cells. *In* "The Togaviruses: Biology, Structure, Replication" (R. W. Schlesinger, Ed.), p. 583. Academic Press, New York.

Strauss, E. G., Levinson, R., Rice, C. M., Dalrymple, J., and Strauss, J. H. (1988). Nonstructural proteins nsP3 and nsP4 of Ross River and O'Nyong-nyong viruses: sequence and comparison with those of other alphaviruses. *Virology* **164,** 265.

Strauss, E. G., Rice, C. M., and Strauss, J. H. (1984). Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology* **133,** 92.

Strauss, E. G., Schmaljohn, A. L., Stec, D. S., and Strauss, J. H. (1990). Mapping of Sindbis virus neutralization epitopes. *In* "New aspects of positive-strand RNA viruses." (M. A. Brinton, and F. X. Heinz, Eds.), p. 305. American Society for Microbiology, Washington, D. C.

Strauss, E. G., Stec, D. S., Schmaljohn, A. L., and Strauss, J. H. (1991). Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. *J Virol* **65,** 4654.

Strauss, J. H., Rumenapf, R. C., Weir, R. J., Kuhn, R. J., Wang, K. S., and Strauss, E. G. (1994). Cellular receptors for alphaviruses. *In* "Cellular receptors for animal viruses" (E. Wimmer, Ed.), p. 141. Cold Spring Harbor Laboratory Press, Plainview, N. Y.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58,** 491.

Stubbs, M. J., Miller, A., Sizer, P. J., Stephenson, J. R., and Crooks, A. J. (1991). X-ray solution scattering of Sindbis virus. Changes in conformation induced at low pH. *J Mol Biol* **221,** 39.

Sundin, D. R., Beaty, B. J., Nathanson, N., and Gonzalez-Scarano, F. (1987). A G1 glycoprotein epitope of La Crosse virus: a determinant of infection of *Aedes triseriatus*. *Science* **235,** 591.

Symington, J., and Schlesinger, M. J. (1978). Characterization of a Sinbis virus variant with altered host range. *Arch Virol* **58,** 127.

Takahashi, M. (1982). Differential transmission efficiency for Japanese encephalitis virus among colonized strains of *Culex tritaeniorhynchus*. *Japanese Journal of Sanitary Zoology* **33,** 325.

Takahashi, M., and Suzuki, K. (1979). Japanese encephalitis virus in mosquito salivary glands. *Am J Trop Med Hyg* **28,** 122.

Taylor, R. M., Hurlbut, H. S., Work, T. H., Kingsbury, J. R., and Frothingham, T. E. (1955). Sindbis virus: A newly recognized arthropod-transmitted virus. *American Journal of Tropical Medicine and Hygiene* **4,** 844.

Thomas, L. A. (1963). Distribution of the virus of western equine encephalomyelitis in the mosquito vector, *Culex tarsalis*. *Am J Hyg* **78,** 150.

Tooker, P., and Kennedy, S. I. (1981). Semliki Forest virus multiplication in clones of *Aedes albopictus* cells. *J Virol* **37,** 589.

Tucker, P. C., and Griffin, D. E. (1991). Mechanism of altered Sindbis virus neurovirulence associated with a single-amino-acid change in the E2 Glycoprotein. *J Virol* **65,** 1551.

Tucker, P. C., Strauss, E. G., Kuhn, R. J., Strauss, J. H., and Griffin, D. E. (1993). Viral determinants of age-dependent virulence of Sindbis virus for mice. *J Virol* **67,** 4605.

Turell, M. J. (1988). Horizontal and vertical transmission of viruses by insect and tick vectors. *In* "The Arboviruses: ecology and epidemiology" (T. P. Monath, Ed.), Vol. 1, p. 127. CRC Press, Boca Raton, FL.

Turell, M. J., Gargan, T. P., 2nd, and Bailey, C. L. (1984). Replication and dissemination of Rift Valley fever virus in *Culex pipiens*. *Am J Trop Med Hyg* **33,** 176.

Ubol, S., and Griffin, D. E. (1991). Identification of a putative alphavirus receptor on mouse neural cells. *J Virol* **65,** 6913.

van der Krol, A. R., Mur, L. A., de Lange, P., Mol, J. N., and Stuitje, A. R. (1990). Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. *Plant Mol Biol* **14,** 457.

Varma, M. G., Pudney, M., and Leake, C. J. (1974). Cell lines from larvae of *Aedes (Stegomyia) malayensis Colless* and *Aedes (S) pseudoscutellaris (Theobald)* and their infection with some arboviruses. *Trans R Soc Trop Med Hyg* **68,** 374.

Voinnet, O., Lederer, C., and Baulcombe, D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana. Cell* **103,** 157.

Wahlberg, J. M., Bron, R., Wilschut, J., and Garoff, H. (1992). Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. *J Virol* **66,** 7309.

Wang, K. S., Kuhn, R. J., Strauss, E. G., Ou, S., and Strauss, J. H. (1992). High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J Virol* **66,** 4992.

Wang, K. S., Schmaljohn, A. L., Kuhn, R. J., and Strauss, J. H. (1991). Antiidiotypic antibodies as probes for the Sindbis virus receptor. *Virology* **181,** 694.

Wang, K. S., and Strauss, J. H. (1991). Use of a lambda gt11 expression library to localize a neutralizing antibody-binding site in glycoprotein E2 of Sindbis virus. *J Virol* **65,** 7037.

Weaver, S. C. (1986). Electron microscopic analysis of infection patterns for Venezuelan equine encephalomyelitis virus in the vector mosquito, *Culex (Melanoconion) taeniopus. Am J Trop Med Hyg* **35,** 624.

Weaver, S. C., Lorenz, L. H., and Scott, T. W. (1992). Pathologic changes in the midgut of *Culex tarsalis* following infection with Western equine encephalomyelitis virus. *Am J Trop Med Hyg* **47,** 691.

Weaver, S. C., Lorenz, L. H., and Scott, T. W. (1993). Distribution of western equine encephalomyelitis virus in the alimentary tract of *Culex tarsalis* (Diptera: Culicidae) following natural and artificial blood meals. *J Med Entomol* **30,** 391.

Weaver, S. C., Scherer, W. F., Cupp, E. W., and Castello, D. A. (1984). Barriers to dissemination of Venezuelan encephalitis viruses in the Middle American enzootic vector mosquito, *Culex (Melanoconion) taeniopus. Am J Trop Med Hyg* **33,** 953.

Weaver, S. C., Scott, T. W., and Lorenz, L. H. (1990). Patterns of eastern equine encephalomyelitis virus infection in *Culiseta melanura* (Diptera: Culicidae). *J Med Entomol* **27,** 878.

Weaver, S. C., Scott, T. W., Lorenz, L. H., Lerdthusnee, K., and Romoser, W. S. (1988). Togavirus-associated pathologic changes in the midgut of a natural mosquito vector. *J Virol* **62,** 2083.

Weaver, S. C., Scott, T. W., Lorenz, L. H., and Repik, P. M. (1991). Detection of eastern equine encephalomyelitis virus deposition in *Culiseta melanura* following ingestion of radiolabeled virus in blood meals. *Am J Trop Med Hyg* **44,** 250.

Wengler, G. (1984). Identification of a transfer of viral core protein to cellular ribosomes during the early stages of alphavirus infection. *Virology* **134,** 435.

Wengler, G., and Wurkner, D. (1992). Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. *Virology* **191,** 880.

Whitfield, S. G., Murphy, F. A., and Sudia, W. D. (1971). Eastern equine encephalomyelitis virus: an electron microscopic study of *Aedes triseriatus* (Say) salivary gland infection. *Virology* **43,** 110.

Whitfield, S. G., Murphy, F. A., and Sudia, W. D. (1973). St. Louis encephalitis virus: an ultrastructural study of infection in a mosquito vector. *Virology* **56,** 70.

WHO (1996). The World Health Report 1996: fighting disease, fostering development. Geneva: World Health Organization.

WHO (1997). "Dengue haemorrhagic fever: diagnosis,treatment, prevention and control." 2nd ed. ed. World Health Organization, Geneva, Switzerland.

WHO (1999). Report on Infectious Diseases: Removing Obstacles to Healthy Development. World Health Organization.

Woodring, J. I., Higgs, S., and Beaty, B. J. (1996). Natural cycles of vector-borne pathogens. *In* "The Biology of Disease Vectors" (B. J. Beaty, and W. C. Marquardt, Eds.), p. 51. Universtiy of Colorado Press, Niwot, CO.

Woodward, T. M., Miller, B. R., Beaty, B. J., Trent, D. W., and Roehrig, J. T. (1991). A single amino acid change in the E2 glycoprotein of Venezuelan equine encephalitis virus affects replication and dissemination in *Aedes aegypti* mosquitoes. *J Gen Virol* **72,** 2431.

Wright, K. A. (1969). The anatomy of salivary glands of *Anopheles stephensi Liston. Can J Zool* **47,** 579.

Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M., and Huang, H. V. (1989). Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* **243,** 1188.

Yao, J. S., Strauss, E. G., and Strauss, J. H. (1996). Interactions between PE2, E1, and 6K required for assembly of alphaviruses studied with chimeric viruses. *J Virol* **70,** 7910.

APPENDIX A

ADDITIONAL TABLES

TABLE A.1

Temporal distribution of SIN virus-specific antigen in posterior midguts and other tissues of the alimentary canal from TR339 infected *Ae. aegypti*

Days Post Infection	Score (posterior midgut)	Number of positives according to tissue					
		Trachea	Musculature (circular or longitudinal)	Anterior Midgut or Foregut	Anterior Intestine or Hindgut	Pyloric Ampulla	Malpighian Tubules
2	Light: 30/30 (100%)	1	2		1	1	
	Moderate: 0						
	Heavy: 0						
	Total: 30/30 (100%)	**1/30 (3.3%)**	**2/30 (6.7%)**	**0/30 (0.0%)**	**1/30 (3.3%)**	**1/30 (3.3%)**	**0/30 (0.0%)**
4	Light: 26/31 (83.9%)	2	2	2	1		
	Moderate: 3/31 (9.7%)						
	Heavy: 2/31 (6.5%)	1					
	Total: 31/31 (100%)	**3/31 (9.7%)**	**2/31(6.5%)**	**2/31 (6.5%)**	**1/31 (3.2%)**	**0/31 (0.0%)**	**0/31 (0.0%)**
6	Light: 26/30 (86.7%)	4	5	4	5	3	2
	Moderate: 3/30 (10%)						
	Heavy: 1/30 (3.3%)						
	Total: 30/30 (100%)	**4/30 (13.3%)**	**5/30 (16.7%)**	**4/30 (13.3%)**	**5/30 (16.7%)**	**3/30 (10.0%)**	**2/30 (6.7%)**
8	Light: 21/31 (67.7%)	4	4	3	1	1	3
	Moderate: 6/31 (19.4%)	2	3	3	2	2	2
	Heavy: 4/31 (12.9%)	2	3	2	2	2	1
	Total: 31/31 (100%)	**8/31 (25.8%)**	**10/31 (32.3%)**	**8/31 (25.8%)**	**5/30[a] (16.7%)**	**5/30[a] (16.7%)**	**6/31 (19.4%)**
10	Light: 21/31 (67.7%)	5	5	5	5	5	5
	Moderate: 10/31 (32.3%)	2	3	2	2	2	2
	Heavy: 0/31 (0.0%)						
	Total: 31/31 (100%)	**7/31 (22.6%)**	**8/31 (25.8%)**	**7/31 (22.6%)**	**7/31 (22.6%)**	**7/31 (22.6%)**	**7/31 (22.6%)**
12	Light: 19/33 (57.6%)	2	3	2	3	1	2
	Moderate: 9/33 (27.3%)	5	5	5	5	5	5
	Heavy: 4/33 (12.1%)	3	3	3	2	2	2
	Total: 32/33[b] (97.0%)	**10/33 (30.3%)**	**11/33 (33.3%)**	**10/33 (30.3%)**	**10/30[a] (33.3%)**	**8/30[a] (26.7%)**	**9/33 (27.3%)**
14	Light: 18/30 (60.0 %)	7	8	8	5	5	5
	Moderate: 8/30 (26.7%)	3	4	4	3	3	3
	Heavy: 4/30 (13.3%)	1	2	1	2	1	2
	Total: 30/30 (100%)	**11/30 (36.7%)**	**14/30 (46.7%)**	**13/30 (43.3%)**	**10/28[a] (35.7%)**	**9/28[a] (32%)**	**10/30 (33.3%)**

[a] In some of the dissected midguts the pyloric ampulla and hindgut were missing.
[b] One midgut was not infected.

TABLE A.2
Temporal distribution of SIN virus-specific antigen in posterior midguts and other tissues of the alimentary canal from MRE16 infected *Ae. aegypti*

Days Post Infection	Score (posterior midgut)	Number of positives according to tissue					
		Trachea	Musculature (circular or longitudinal)	Anterior Midgut or Foregut	Anterior Intestine or Hindgut	Pyloric Ampulla	Malpighian Tubules
2	Light: 21/31 (67.7%)	3	4	3	2	1	1
	Moderate: 8/30 (26.7%)		1	1			
	Heavy: 2/30 (6.7%)			1			
	Total: 31/31 (100%)	**3/31 (9.7%)**	**5/31 (16.1%)**	**5/31 (16.1%)**	**2/31 (6.5%)**	**1/31 (3.2%)**	**1/31 (3.2%)**
4	Light: 7/30 (23.3%)	3	3	2	2	2	
	Moderate: 10/30 (33.3%)	7	8	6	6	7	5
	Heavy: 13/30 (43.3%)	5	6	6	5	5	5
	Total: 30/30 (100%)	**15/30 (50.0%)**	**17/30 (56.7%)**	**14/30 (46.7%)**	**13/29[a] (44.8%)**	**14/29[a] (48.3%)**	**10/30 (33.3%)**
6	Light: 7/31 (22.6%)	7	7	7	5	5	7
	Moderate: 12/31 (38.7%)	9	9	9	7	8	10
	Heavy: 12/31 (38.7%)	6	12	10	10	10	12
	Total: 31/31 (100%)	**22/31 (71.0%)**	**28/31 (90.3%)**	**26/31 (83.9%)**	**22/26[a] (84.6%)**	**23/26[a] (88.5%)**	**29/31 (93.6%)**
8	Light: 5/32 (15.6%)	5	5	5	5	5	4
	Moderate: 18/32 (56.3%)	16	18	18	17	16	16
	Heavy: 9/32 (28.1%)	9	9	9	9	9	8
	Total: 32/32 (100%)	**30/32 (93.8%)**	**32/32 (100%)**	**32/32 (100%)**	**31/32 (96.9%)**	**30/32 (93.8%)**	**28/32 (87.5%)**
10	Light: 8/35 (22.9%)	8	8	8	8	8	8
	Moderate: 13/35 (37.1%)	13	13	13	12	12	13
	Heavy: 14/35 (40.0%)	14	14	14	14	14	14
	Total: 35/35 (100%)	**35/35 (100%)**	**35/35 (100%)**	**35/35 (100%)**	**34/34[a] (100%)**	**34/34[a] (100%)**	**35/35 (100%)**
12	Light: 18/33 (54.6%)	18	18	18	17	17	18
	Moderate: 12/33 (36.4%)	12	12	12	11	11	12
	Heavy: 3/33 (9.1%)	3	3	3	1	1	3
	Total: 33/33 (100%)	**33/33 (100%)**	**33/33 (100%)**	**33/33 (100%)**	**29/29[a] (100%)**	**29/29[a] (100%)**	**33/33 (100%)**
14	Light: 15/30 (50.0%)	15	15	15	14	14	15
	Moderate: 12/30 (40.0%)	12	12	12	12	12	11
	Heavy: 3/30 (10.0%)	3	3	3	2	2	3
	Total: 30/30 (100%)	**30/30 (100%)**	**30/30 (100%)**	**30/30 (100%)**	**28/28[a] (100%)**	**28/28[a] (100%)**	**29/30 (96.7%)**

[a] In some of the dissected midguts the pyloric ampulla and hindgut were missing.

TABLE A.3

SIN virus titers obtained from triturated whole *Ae. aegypti* mosquitoes used in transmission attempts 12 days after ingesting an infectious blood meal

MRE16		TR339	
Cage #	Titer (PFU/ml)	Cage #	Titer (PFU/ml)
1	2.5×10^7	1	5.3×10^6
	6.0×10^5		3.3×10^6
	8.0×10^5		1.2×10^5
	1.1×10^6		2.0×10^6
	4.0×10^6		2.5×10^6
2	2.7×10^5	2	8.7×10^5
	7.3×10^5		2.7×10^5
	4.0×10^5		7.3×10^5
			7.3×10^6
3	6.7×10^5		
	4.7×10^5	3	1.1×10^6
	4.0×10^5		1.5×10^6
	3.3×10^5		1.7×10^5
	5.3×10^5		9.3×10^5
	3.3×10^5		
		4	2.5×10^4
			2.7×10^3
			1.1×10^6

Titers were determined from single mosquitoes triturated in 1 ml of diluent.
Titers were determined by plaque assay in Vero cells.

TABLE A.4

SIN virus titers obtained from the homogenized brains of newborn mice used in transmission attempts

MRE16		TR339	
Cage #	Titer (PFU/ml)	Cage #	Titer (PFU/ml)
1	8.7×10^6	1	1.1×10^7
	$\geq 1.0 \times 10^7$		Uninfected
	1.8×10^5		Uninfected
	Uninfected		Uninfected
	$\geq 1.0 \times 10^7$		Uninfected
2	Uninfected	2	1.0×10^5
	3.3×10^6		Uninfected
	3.3×10^6		7.3×10^5
			Uninfected
3	1.1×10^7		
	1.0×10^6	3	2.1×10^6
	2.4×10^5		Uninfected
	2.7×10^6		7.3×10^5
	$\geq 1.0 \times 10^7$		Uninfected
	$\geq 1.0 \times 10^6$		
		4	1.3×10^6
			Uninfected
			Uninfected

SIN virus titer obtained from the brain of a newborn mouse injected (IP) with MRE16 virus = 6.0×10^6 PFU/ml.
SIN virus titer obtained from the brain of a newborn mouse injected (IP) with TR339 virus = 8.7×10^6 PFU/ml.
Titers were determined from supernatant corresponding approximately to a 20% mouse brain preparation (20% wt:vol dil).
Titers were determined by plaque assay in Vero cells.