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Spring 1998
Ph.D. Dissertation, 126 pages

DISSERTATION

GENETIC DETERMINANTS OF SINDBIS VIRUS ORAL INFECTIVITY IN *AEDES AEGYPTI* MOSQUITOES

Submitted by
Robert Craig Seabaugh
Department of Microbiology

In partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Spring 1998

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January 22, 1998

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ROBERT CRAIG SEABAUGH ENTITLED "GENETIC DETERMINANTS OF SINDBIS VIRUS ORAL INFECTIVITY IN *AEDES AEGYPTI* MOSQUITOES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Co-Advisor

Adviser

Department Head

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DEDICATION

To the memory of my Dad, whose quiet example taught me the importance of hard work and persistence, and that anything is possible...

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

Introduction

Mosquitoes transmit pathogens that cause well over 250 million new cases of malaria, filariasis, and viral disease each year (James, 1992). Malaria alone kills between 1.5 and 2.7 million people annually (Butler, 1997). Mosquito-borne diseases, once thought to be under control in many areas of the world, have resurged and emerging mosquito-borne diseases are being reported at an increasing rate. In 1994, the Centers for Disease Control (CDC) reported that of eight emergent infectious diseases, three (dengue, yellow fever, and Rift Valley fever) were caused by arthropod-borne (arbo-) viruses (CDC, 1994). Today, nearly half the world's population lives at risk of contracting dengue fever/dengue hemorrhagic fever (DEN/DHF), and hundreds of thousands of cases result each year (Gubler and Clark, 1995). In the American tropics, the risk of major outbreaks of arboviral disease is at its highest point in over 50 years due to the reinfestation of urban centers with *Aedes (Ae.) aegypti,* the mosquito vector of both urban yellow fever and DEN/DHF (Gubler, 1996).

The factors contributing to the increased threat of mosquito-borne disease are complex, involving changes in the social and economic structure of human populations around the globe, concomitant with altered regional ecologies. These changes are routinely accompanied by marked increases in the number and distribution of mosquitoes and other arthropod vectors of disease (Epstein, 1995; Gubler, 1996; Krause, 1994; Monath, 1994; Murphy and Nathanson, 1994). Clearly, a renewed emphasis on vector control is needed.

Past efforts to control vector-borne diseases have relied largely on strategies to eliminate or reduce the vector populations through habitat control measures. The

elimination of mosquito breeding sites and large-scale spraying of chemical pesticides or biological agents, such as the *Bacillus thuringensis* toxin, are examples of the methods employed (Crampton, 1994). However, these methods are costly and unsustainable, and some vector populations have developed resistance to the agents used (Butler, 1997; Goldman *et al.* 1986; Raymond *et al.* 1993). The mass release of sterile male mosquitoes into natural populations to reduce the population size has also yielded disappointing results (Crampton *et al.* 1990).

Molecular Approaches to Disease Vector Control

Advances in recombinant DNA technology have brought renewed hope to the field of vector control. The potential now exists to alter vector-pathogen interactions at the molecular level, allowing a new strategy for control to evolve. Using a variety of DNA delivery vehicles and methods of transformation, scientists are attempting to develop the means to produce transgenic mosquitoes (Besansky and Collins, 1992; Carlson *et al.* 1995; Crampton *et al.* 1990). This technology will permit the introduction into the mosquito genome of exogenous DNA sequences selected for the capacity to abrogate the mosquito's ability to harbor or transmit disease agents. If the transforming construct confers a selective advantage to the mosquito, the introduced "vector-incompetence" traits would be likely to spread through a native mosquito population, reducing the net capacity for pathogen transmission to humans. The genetic sequences employed to carry out this strategy could come from the mosquito or pathogen genomes or from knowledge of the molecular determinants of vector-pathogen interactions.

The transformation of *Anopheles (An.) gambiae, Ae.aegypti, and Ae. triseriatis* mosquitoes by microinjection of embryos with *Drosophila* P element transposons has been accomplished (McGrane *et al.* 1988; Miller *et al.* 1987; Morris *et al.* 1989), but is a relatively inefficient route to germline transformation (Besansky and Collins, 1992). Recently, the stable transformation of *Ae. aegypti* using Hermes element (Jasinskiene *et al.* 1997) and Mariner (Coates *et al.* 1997) transposons, and the transformation of *An. gambiae* using retroviral vectors (Matsubara *et al.* 1996) have been accomplished. Nevertheless, these are laborious procedures and a great deal of work remains before these methods are available for use in vector control strategies.

Investigations into the molecular biology of mosquito-pathogen interactions have identified a number of mosquito genes that could be used in a transforming construct to reduce mosquito vector competence. Examples from the *An. gambiae* genome include sequences encoding serine proteases (Collins *et al.* 1986, 1991) or a prophenolperoxidase enzyme (Paskewitz and Christensen, 1996), both of which are involved in the mosquito response to infection by malarial or filarial parasites. From the *Ae. aegypti* genome, sequences from the f^{m} locus, responsible for filarial susceptibility (MacDonald, 1962a,b), or a malate dehydrogenase gene, linked to yellow fever virus susceptibility (Lorenz *et al.* 1984) might be used. Depending on the gene in question and its function in the mosquito, inducible overexpression or ablation of the gene product(s) by a transforming construct could effectively block pathogen development or transmission.

Additionally, several classes of inducible mosquito immune peptides (e.g., diptericins, defensins, cecropins, lysozymes) (Cociancich *et al.* 1994; Hoffman and Hetru, 1992; Paskewitz and Christensen, 1996) that protect the mosquito against bacterial

infection (Crampton *et al.* 1990) might be exploited for molecular control measures. Lowenberger *et al.* (1996) have demonstrated that immune induction in *Ae. aegypti* is effective in limiting mosquito infection by the filarial nematode *Brugia malayi*. Other studies (Chalk *et al.* 1995; Gwadz *et al.* 1989) have reported that synthetic cecropins can kill *Plasmodium* and filarial nematodes *in vivo*. Further investigation into the effectors of the mosquito immune response will likely provide additional genetic sequences with the potential for use in transforming constructs.

Alternatively, an approach termed "intracellular immunization" (Baltimore, 1988) might be used to abrogate vector competence. The mosquito could be transformed with selected sequences from a potential pathogen under the control of appropriately inducible promoters. Overexpression of these sequences in the mosquito could interfere with subsequent infection by the targeted pathogen or one that possesses similar genetic sequences (Carlson *et al.* 1995). The utility of this approach has been demonstrated in transgenic plants engineered to be refractory to infection by a variety of plant viruses. Plant biologists have termed this "parasite-derived resistance" (Sanford and Johnston, 1985). Intracellular immunization has also been achieved through the expression of "antisense" oligonucleotide sequences. In 1978, replication of Rouse sarcoma virus was inhibited by an oligonucleotide complementary to the viral RNA (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). Many animal viruses have since been inhibited *in vitro* by this means. Depending on the expression system used and the targeted virus, antisense inhibition of viral gene expression has been observed to operate at several levels -- from inhibiting splicing of viral transcripts in the nucleus to blocking transcription of viral mRNAs in the cytoplasm of infected cells (Li and Rice, 1993). The

overexpression of pathogen-derived genetic sequences, expression of antisense oligonucleotides, or antiviral ribozymes (Sarver *et al.* 1990) are all potentially viable means of abrogating vector competence in the mosquito (Carlson *et al.* 1995).

When the two elements of this molecular approach to mosquito vector control (i.e., an effective transforming construct and exogenous genetic sequences with proven effectiveness) are available, the means to carry out a strategy of systematic infectious disease control based on transgenics will be in place. If successful, this strategy could reduce the number of vector-competent mosquitoes within a population, thereby interrupting the cycle of transmission and reducing the incidence of human cases of such mosquito-borne diseases as malaria, yellow fever, or dengue.

Evaluation of Genetic Constructs for Disease Vector Control

It is possible to evaluate the effectiveness of exogenous genetic sequences in reducing mosquito vector competence in the absence of germline transformation. Sindbis virus (SIN) expression systems have been successfully employed to transiently transform mosquito cells both *in vitro* and *in vivo* (Carlson *et al.* 1995; Higgs *et al.* 1993, 1995; Kamrud *et al.* 1995; Olson *et al.* 1992, 1994; Rayms-Keller *et al.* 1995). Because SIN can establish a persistent infection in many of the tissues of a wide variety of mosquito species, a gene of interest can be efficiently expressed in the mosquito for an extended period and the biological effects of its expression measured. SIN expression vectors do not transform the germline of infected mosquitoes, and so are limited to use as evaluative tools. Recently, the potential of this system was dramatically demonstrated when SIN expression systems were used to knock out 1) LaCrosse virus (*Bunyaviridae*) infection in

Ae. triseriatus mosquitoes (Powers *et al.* 1995) and 2) transmission of Dengue-2 virus (*Flaviviridae*) by *Ae. aegypti* mosquitoes (Olson *et al.* 1996). In both, the SIN system was used to express antisense RNAs targeted against the viruses.

It is significant that intrathoracic inoculation was used in these studies to introduce SIN constructs into the mosquitoes, and that expression of the viral antisense sequences in the mosquito salivary glands produced the observed effects. In both studies, virus-neutralizing effects were restricted to cells infected with the SIN construct and expression was not observed in the mosquito midgut epithelium.

Midgut expression of exogenous genetic sequences is an essential requirement of future efforts to abrogate mosquito vector competence. Arboviruses (and most other mosquito-borne pathogens) multiply in the mosquito before they can be transmitted (Chamberlain and Sudia, 1961). Following an infectious blood meal, the midgut is the first mosquito organ encountered by a pathogen. It is also the location of the encapsulation response of some mosquito species to a variety of infecting parasites, including the agents of malaria. For these reasons, it would be beneficial to be able to express interfering constructs in midgut cells, presenting a more formidable barrier to the initial infection and preventing the pathogen from early rounds of replication or dissemination to other mosquito tissues.

The Mosquito Midgut

The mosquito midgut (or mesenteron) consists of a single layer of columnar epithelial cells surrounded by a porous basal lamina on the ablumenal or hemocoel side (Figure 1.1). The entire organ is surrounded by a meshwork-like arrangement of

contractile muscle fibers, is extensively innervated, and permeated by respiratory tracheoles. The structure of the epithelium is relatively simple with a microvillar membrane on the lumenal margin, a continuous lateral junction between cells, and a highly involuted basolateral plasma membrane on the ablumenal margin (Hardy, 1988).

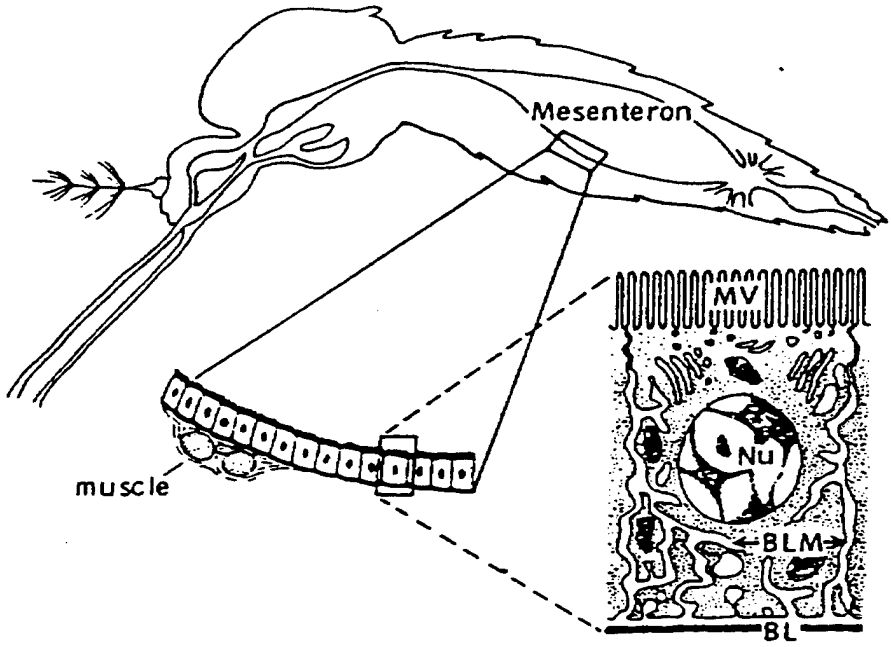

Figure 1.1. The Mosquito Midgut. Anatomy of the mosquito mesenteron (midgut), midgut epithelium, and midgut epithelial cell. MV=microvilli, Nu=nucleus, BLM=basolateral membrane, and BL=basal lamina. (Houk *et al.* 1985, with permission).

The infectious cycles and tissue tropisms of several arboviruses in their mosquito vectors have been reported (Beaty and Bishop, 1988; Beaty and Thompson, 1976; Larsen and Ashley, 1971; Scott *et al.* 1984; Weaver, 1986; Whitfield *et al.* 1973; Woodring *et al.* 1996). Bowers *et al.* (1995) recently described the temporal and spatial progression of SIN infection following the intrathoracic inoculation of *Ae. albopictus* mosquitoes with the virus. The infection began with an acute phase (48 to 72 hours post-infection)

coincident with detection of SIN antigen in fat bodies, hemolymph, gut visceral muscles, salivary glands, thoracic muscle, and neural tissues. Approximately 72 hours post-infection, a persistent infection phase occurred, accompanied by clearance of viral antigen from many of the infected tissues. Subsequently, viral antigen was restricted to the fat body, hindgut visceral muscles, and tracheoblasts from which the persistent infection was purportedly supported for the life of the mosquito. Though the gut-associated musculature and tracheoles were infected at an early stage in the infection, viral antigen was never detected in the epithelial cell layer that lines the midgut lumen.

Patterns of arboviral infection following oral introduction present quite a different picture. In the case of eastern equine encephalitis virus (EEE) in *Culiseta (Cs.) melanura* mosquitoes, Scott *et al.* (1984) reported that virus was first detected in the posterior midgut epithelium, from which it quickly disseminated (by 3 days post-infection) via the hemocoel to produce a systemic infection of most mosquito tissues. Weaver (1986) detailed the oral infection of *Culex (Cx.) taeniopus* with Venezuelan equine encephalomyelitis virus (VEE) by electron microscopy. In this study, large numbers of virions were observed on both sides of the basal lamina and budding from the basal membranes of midgut epithelial cells by 3 to 4 hours post-infection. This suggested that viral replication had occurred in the epithelial cells prior to release into the hemocoel. By 2 days post-infection dissemination of virus into abdominal nerve ganglia and the hindgut was detectible. Dissemination to other organs, including the salivary glands and brain, occurred between days 2 and 4 of extrinsic incubation.

Initiation of virus infection in the mosquito midgut requires that the female ingest a sufficient concentration of virus to overcome a "threshold of infection" (Hardy, 1988).

Jackson *et al.* (1993) determined the oral infectious dose for the SIN prototype, AR339, in *Ae. aegypti* mosquitoes to be $10^{5.3}$ tissue culture infectious dose fifty-percent endpoint ($TCID_{50}$) per mosquito or approximately 10^8 $TCID_{50}$/ml. The reason for minimum infectious dose requirements in mosquitoes is not known and varies among mosquito species for a given virus.

Taken together, these data support the long-held view that dissemination of arboviruses in competent mosquito vectors following *per os* infection involves an initial, unidirectional, productive infection of the mosquito midgut epithelium. This requirement is restrictive to non-compatible or low titer viruses and presents a "midgut infection barrier" (Hardy, 1988; Woodring *et al.* 1996) that arboviruses must overcome in order to disseminate to other mosquito organs (particularly the salivary glands) and continue their natural cycle. The molecular nature of this barrier has not yet been elucidated and may involve specific mosquito-pathogen interactions at the lumenal epithelial cell membrane or within the viral replicative machinery in the infected cells.

Mosquito inoculation has proven to be an ineffective means of introducing SIN constructs into the midgut epithelium, and past attempts at oral introduction of these expression systems into this cell layer were unsuccessful, yielding little expression of exogenous sequences (S. Higgs, personal communication). Diminished oral infectivity of SIN constructs, relative to the prototype virus, is a common observation (see Chapter 2: Results). The data presented in this dissertation suggest that genetic differences among closely related SIN viruses and viral expression systems play a role in the differences seen in oral infectivity in mosquitoes. Thus, barriers to normally efficient *per os* infection result from incompatablilties between the mosquito and virus introduced by viral passage,

selection, or genetic manipulation. Genetic engineering of SIN expression systems to increase oral infectivity and midgut tissue tropism should therefore be possible.

Sindbis Virus Characteristics

Host Range and Distribution

SIN is the prototype virus and one of the most widely studied members of the genus Alphavirus in the Family *Togaviridae* (Strauss and Strauss, 1994). All alphaviruses are arthropod-borne with most having mosquitoes as their exclusive vector (Calisher and Karabatsos, 1988). SIN was originally isolated from *Cx. univittatus* mosquitoes in the Sindbis health district near Cairo, Egypt in 1952 (Taylor *et al.* 1955). It has subsequently been isolated from several species of *Culex*, *Culiseta, Aedes*, *Anopheles*, and *Mansonia* mosquitoes (Chamberlain, 1980; Karabatsos, 1985). SIN has a very broad vertebrate host range as well. Isolates have been obtained from many species of birds and mammals (Karabatsos, 1985).

SIN has the most widespread geographic distribution among the alphaviruses. Various strains of SIN have been isolated from Europe, Africa, Asia (including India and the Phillipines), and Australia. The closely related SIN-like viruses, Whataroa and Aura, were isolated in New Zealand and South America, respectively (Strauss and Strauss, 1994). Like most arboviruses, SIN is maintained in natural cycles involving a mosquito vector and one or more vertebrate hosts. Most alphaviruses are thought to have an avian reservoir in nature, with migratory birds as the primary vertebrate component of the cycle (Calisher and Karabatsos, 1988). The local distribution of these viruses is closely tied to

that of their mosquito vector species, while their worldwide dispersion probably reflects the mobility of their avian hosts.

The Virion

The SIN virion consists of a single-stranded RNA genome, associated with a proteinaceous icosahedral capsid, surrounded by a lipid bilayer envelope in which glycoprotein "spikes" are embedded (Strauss and Strauss, 1994).

The virion nucleocapsid is approximately 35 nm in diameter with a sedimentation coefficient of 140S (Coombs and Brown, 1987). It is composed of 240 copies of a single virus-encoded capsid protein, arranged in an icosahedral lattice with T=4 symmetry (Coombs and Brown, 1987; Paredes *et al.* 1993). The virus envelope is derived from the plasma membrane of the host cell and is approximately 5 nm thick (Strauss and Strauss, 1994). There are 80 glycoprotein spikes extending from the virion envelope. Each spike consists of three heterodimers of the two virus-encoded glycoproteins (Rice and Strauss, 1982). The enveloped virion, including the glycoproteins, has a diameter of approximately 69 nm (Strauss and Strauss, 1994).

The Infection Cycle

SIN initiates infection of a susceptible cell by binding to the cell membrane. This binding is thought to result from specific interactions between the viral glycoprotein spikes and cellular membrane-associated receptor molecules. Studies addressing alphavirus receptor interactions have recently been reviewed (Strauss and Strauss, 1994) and suggest the following: "...that alphaviruses use protein receptors; that different alphaviruses may use the same receptor or different receptors; that more than one receptor can be used by one virus, leading to cases in which the major receptors used by one virus to enter

different cells are different; that the nature of the receptors used determines in part the virulence of the virus; and that one or a few amino acid changes in the envelope glycoproteins can lead to utilization of different sets of receptors." Wang *et al.* (1992) determined that SIN bound hamster cells (BHK-21) via a high affinity laminin receptor molecule associated with the cell plasma membrane. A monoclonal antibody (MAb) specific to this protein blocked SIN binding by up to 80%. The MAb was also found to efficiently block SIN binding to mouse, monkey, and human cells; however, it only partially blocked binding to chicken or mosquito cells. Ludwig *et al.* (1996) reported that VEE bound a membrane-associated, lamanin-binding protein from mosquito (C6/36) cells. They found that MAbs directed against either the cellular protein or the VEE E2 glycoprotein interfered with virus attachment. Also, infection inhibition experiments using a battery of anti-C6/36 MAbs demonstrated cross-reactivity with a number of other membrane associated polypeptides, suggesting that the receptor domain is common to multiple, antigenically related membrane proteins. The results from these studies of two different alphaviruses suggest that the broad host range of the virus is achieved in part by utilizing more than one protein receptor.

The major cell receptor binding activity of SIN has been localized to residues 170-220 of the E2 glycoprotein (Strauss and Strauss, 1994). Several studies have identified dramatic changes in alphavirus infectivity and/or virulence due to single amino acid changes in this domain (Kerr *et al.* 1993; Tucker and Griffen, 1991; Woodward *et al.* 1991). However, the E2 domain is not solely responsible for receptor interactions. Changes in other residues in E2 as well as in E1 result in altered viral tropism. Even in the

absence of a functional E2, alphaviruses are capable of binding to cells and penetrating their plasma membranes (Omar and Koblet, 1988).

After binding to the membrane receptor, the virus enters the cell by receptor-mediated endocytosis. The resulting virus-containing coated vesicles mature into phagolysosomes in the cytoplasm and their low interior pH causes the viral nucleocapsids to be released into the cytoplasm by permitting the viral and vesicle membranes to fuse. Fusion of the viral membrane with the vesicle is facilitated by a postulated fusion domain residing in a highly conserved sequence in the E1 glycoprotein (Strauss and Strauss, 1994).

After their release into the cytoplasm, the nucleocapsids are uncoated making the viral RNA accessible to ribosomes for initiation of translation. Several reports suggest that the binding of nucleocapsids to ribosomes triggers the uncoating process (Singh and Helinius, 1992; Wengler and Wengler, 1984) and a putative ribosome binding site within the capsid protein has been identified (Wengler *et al.* 1992). Once viral replication and expression of viral proteins has begun, genomic RNA and capsid protein subunits self-assemble into icosahedral nucleocapsids in the cytoplasm, which are transported to the plasma membrane. The viral envelope proteins are glycosylated as they are processed through the endoplasmic reticulum (ER)/Golgi apparatus of the cell and are subsequently transported to the plasma membrane. The virion envelope is obtained upon budding of the nucleocapsid through the cell plasma membrane at locations where viral glycoproteins have accumulated (Strauss and Strauss, 1994).

SIN infection of vertebrate cells is acute and causes the destruction of the cells. However, SIN establishes a persistent, non-cytolytic infection in invertebrate cells. In

adult mosquitoes, SIN infection produces no apparent pathology and in many cases the infection persists for the life of the mosquito (Bowers *et al.* 1995; Hardy, 1988; Rayms-Keller *et al.* 1995).

The Genome and Replication

The SIN genome is an 11,703 nucleotide (nt) non-segmented single strand of plus-sense RNA with a 7-methylguanosine cap at the 5' end and a 3' poly-adenosine (poly-A) tail (Figure 1.2). The genome consists of two distinct open reading frames (ORFs) and three non-coding regions (NCRs). Generally speaking, the 5' two-thirds of the genome encodes the nonstructural (replicase) proteins (nsP1-4); while the 3' one-third encodes the structural proteins that form the capsid (C) and envelope spikes (E1 and E2). The NCRs

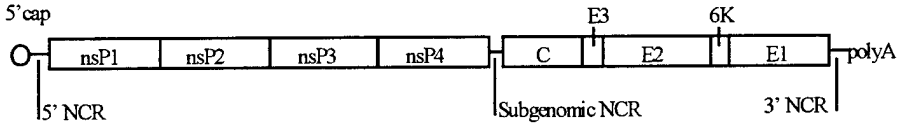

Figure 1.2. The SIN Genome.

are located at the extreme ends of the genome and between the two ORFs (Strauss and Strauss, 1994).

The nonstructural ORF is translated directly from the genome. In SIN, there is a "leaky" opal termination codon (UGA) between the nsP3 and nsP4 genes, resulting in the translation of two polyprotein species, P123 and P1234 (de Groot *et al.* 1990; Li and Rice, 1993; Shirako and Strauss, 1994). Both of these polyproteins are autocatalytically

cleaved to produce the four individual nsP proteins as well as a number of cleavage intermediates (de Groot *et al.* 1990) (Figure 1.3).

The SIN genome is replicated via a full length minus-sense RNA intermediate

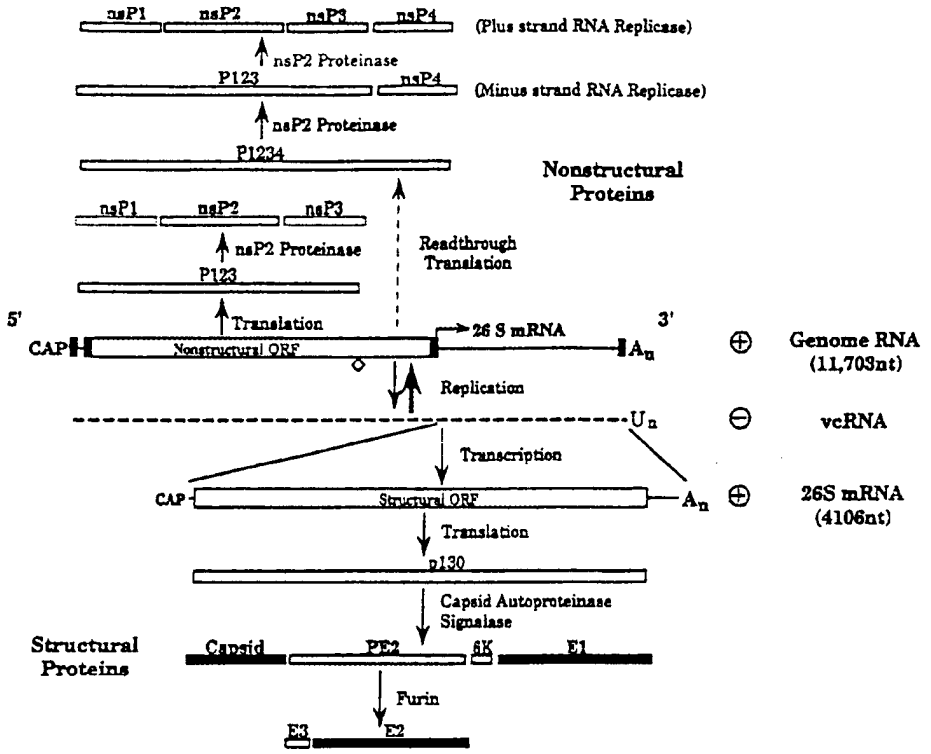

Figure 1.3. SIN Genome Replication and Expression. The genomic RNA is illustrated schematically in the center, with its translated ORF shown as an open box. Small black boxes are conserved sequence elements. The open diamond denotes the leaky opal termination codon. The nonsructural polyproteins and their processed products are shown above. The 26S subgenomic mRNA is expanded below to show the structural ORF and its translated products. Polypeptides present in the virion are shaded (Strauss and Strauss, 1994, with permission).

called the virus complementary (vc)RNA. Production of vcRNA is driven by a replicase complex, consisting of a combination of the P123 and nsP4 proteins in association with host cell factors (Shirako and Strauss, 1994). The vcRNA serves as a template both for genome replication and production of a subgenomic (26S) plus-sense RNA that is colinear

with the structural ORF. Again, a virus-encoded replicase complex, consisting of the four nsP proteins plus host cell factors, is required for transcription of the 26S RNA (Shirako and Strauss, 1994). The 26S RNA is immediately capped and poly-adenylated following its synthesis (Figure 1.3) making it available for translation (Strauss and Strauss, 1994).

A single structural polyprotein (P130) is translated from the 26S RNA and is postranslationally cleaved to produce the C, E2, and E1 proteins, as well as two small polypeptides, E3 and 6K (Strauss and Strauss, 1994) (Figure 1.3).

Sindbis Virus Expression Systems

As cloned, double stranded cDNA, the genome of many RNA viruses can be altered by the insertion or deletion of genetic sequences, or by site-specific mutation (nucleotide substitution). With the addition of a transcriptional promoter (e.g., the bacteriophage SP6 promoter) upstream of the coding sequence, the modified cDNA can be transcribed back into RNA *in vitro* using a DNA-dependent RNA polymerase. The modified viral RNA can be transfected into permissive eukaryotic cells where the viral genome is replicated and the encoded proteins are expressed. If the viral genome is intact or if missing elements are provided *in trans* by a co-infecting virus, progeny virions are assembled and released from the cells. RNA virus-based expression systems have been developed to take advantage of the capacity of the genome to accept an additional sequence, encoding a heterologous protein, while retaining the ability to replicate at near normal levels.

Three types of RNA expression systems have been derived from infectious SIN genomes; all exploit the high level of gene expression obtained from the viral subgenomic promoter.

The first expression system to be developed was one in which a heterologous gene could be inserted downstream of the subgenomic promoter of a SIN defective interfering (DI) RNA. If introduced into a cell alone, the RNA would not replicate nor would its genetic cargo be expressed, but if complemented *in trans* by coinfection with wild type SIN, expression of the engineered construct via the subgenomic promoter proceeded, and the DI RNA could be (rarely) co-packaged in SIN virions. This was demonstrated in chicken embryo fibroblasts where co-infection with a SIN DI RNA containing the bacterial chloramphenicol acetyltransferase (CAT) gene and a SIN helper virus (Figure 1.4A) resulted in CAT expression in the cells (Levis *et al.* 1987).

In the second type of expression system (Figure 1.4B), a heterologous gene replaces the structural protein genes of the virus (Xiong *et al.* 1989). The vector is self-replicating (a replicon) but requires complementation to be packaged and released from cells as virion particles. DI RNAs modified to become "defective helper" RNAs provide the structural proteins for replicons, which can then be packaged under conditions in which the helper itself is not packaged (Bredenbeek *et al.* 1993). Such particles are infectious but self-limiting. They produce nsPs as well as genomic and subgenomic RNAs, but in the absence of structural proteins, new particles will not be formed. Some of the defective helpers retain the region of the genome that includes the packaging signal. These defective RNAs can be copackaged with the replicon, creating a virus with a bipartite genome (Geigenmuller-Gnirke *et al.* 1991). When replicons are packaged with

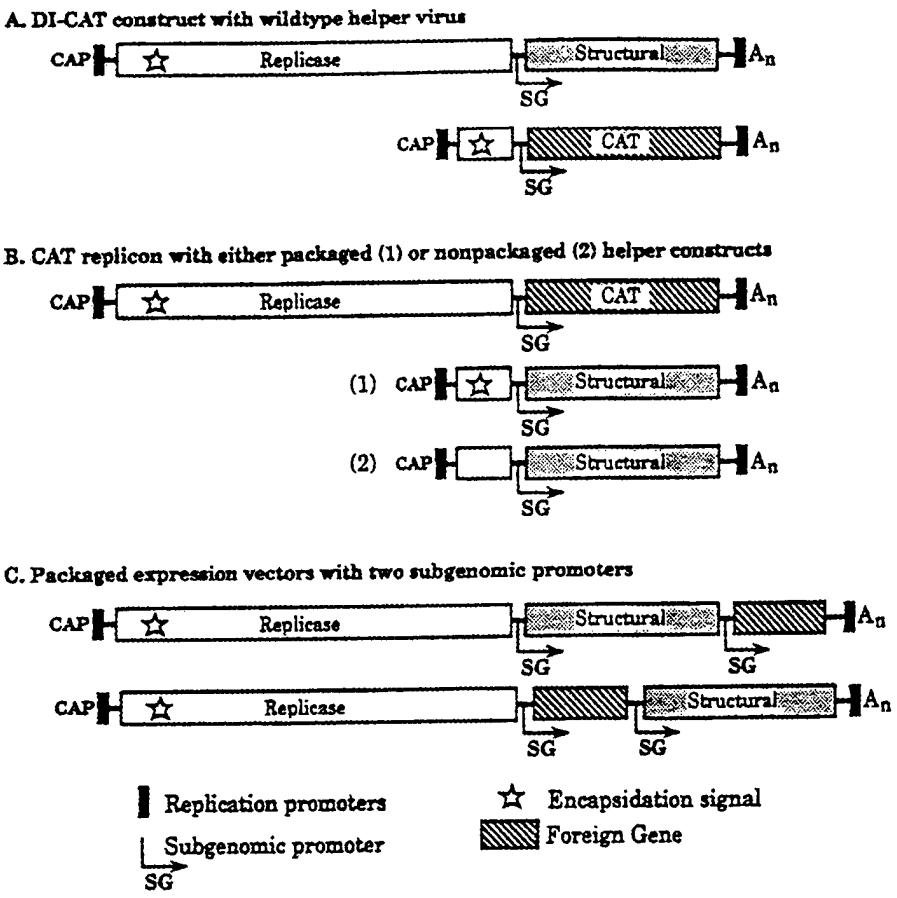

Figure 1.4. SIN Expression Systems. (A) The heterologous gene is inserted into a packaged DI RNA under the control of a SIN subgenomic promoter. Cells are infected with wild-type helper virus and transfected with the DI RNA. The yield from such an infection is a mixture of wild-type virus and packaged DI RNAs. (B) The foreign gene replaces the genes for the structural proteins in a full- length SIN RNA construct. Transfection of this RNA leads to replication of the genome and transient expression of the foreign gene upon transcription of the subgenomic RNA. The replicon can be packaged by cotransfection with a DI RNA construct that expresses the structural genes. DI RNA will also be packaged if it contains the packaging signal. (C) The foreign gene can be inserted into a full-length nondefective construct under the control of a second subgenomic promoter. Because of size limitations on packaging, such constructs are unstable if the foreign gene is much larger than 2 kb. (Strauss and Strauss, 1994, with permission).

this latter type of defective helper, they can be amplified through multiple passages (Bredenbeek *et al.* 1993).

The third type of expression system includes the complete SIN genome (Figure 1.4C) with the addition of a second subgenomic RNA promoter. One subgenomic

promoter controls the synthesis of the subgenomic mRNA that codes for the viral structural proteins, and the other controls the synthesis of a second subgenomic RNA that encodes a heterologous protein (Hahn *et al.* 1992; Raju and Huang, 1991). This double subgenomic SIN (dsSIN) vector is self-replicating and produces infectious virus particles. Rice *et al.* (1987) initially developed infectious dsSIN vectors from a heat-resistant, small plaque (HRsp) SIN isolate (Strauss *et al.* 1984) to facilitate studies of SIN genetics. One of these vectors was later modified by the replacement of the majority of the structural protein sequences with those from a mouse neurovirulent strain of SIN (Lustig *et al.* 1988). The resulting construct, pTE/3'2J, was used by Hahn *et al.* (1992) to study viral antigen processing in mammalian cells and is the primary expression system used in the studies presented here.

Summary and Goals

In an era of astounding achievement in the prevention and treatment of human illness, efforts to reduce the incidence of many arthropod-borne diseases are lagging behind. Conventional approaches to prevention have been relatively unsuccessful for a variety of reasons. The same plasticity of arthropod-borne pathogens that allows them to cycle between vertebrate and invertebrate hosts makes them difficult to immunize against. Attempts at large-scale vector control through the use of pesticides is both economically unfeasible and ecologically unsound.

As the human population expands people will continue to move into new habitats, where they will intersect previously unencountered pathogen maintenance cycles and become infected with "emerging" disease agents, some of them delivered by the bite of a

mosquito. Although the elimination of mosquito populations may be an unattainable goal, the reduction of their capacity to transmit disease through genetic modification might achieve the same net result. The tools are being developed for such a molecular approach to disease vector control.

The research described here was aimed at improving current SIN expression systems by increasing their oral infectivity and midgut tissue tropism in *Ae. aegypti* mosquitoes. SIN expression systems provide a powerful alternative to germline transformation of mosquito vectors. Their capacity for exogenous gene expression in the mosquito offers great opportunity to study the interactions between pathogen and vector at the molecular level. My goal has been to extend the usefulness of these constructs for further studies of the interactions that occur in the mosquito midgut epithelial cell layer, where first contact between vector and pathogen is made. The research described in this dissertation lays the groundwork for these future experiments.

The main hypotheses of this study were that 1) the envelope glycoproteins of the dsSIN expression system, TE/3'2J, were not optimal for *Ae. aegypti per os* infectivity, and 2) that infectivity could be improved by genetic manipulation of the genes encoding these proteins. Two approaches were used to address the hypotheses. The first approach centered on selection for variants of a TE/3'2J virus with increased mosquito infectivity by serial passage in adult *Ae. aegypti* mosquitoes. After selection, regions of the viral genome encoding the envelope glycoproteins were examined for changes from the original virus to determine if increased oral infectivity correlated to glycoprotein gene changes. The second approach involved the construction of a chimeric SIN in which the structural protein-encoding genes of a Malaysian SIN isolate (MRE16) were cloned downstream of

the subgenomic promoter of the SIN replicon (SINrep5). MRE16 is very infectious for *Ae. aegypti* when administered in an artificial blood meal. The chimeric virus was tested for altered phenotype in *Ae. aegypti* mosquitoes to assess the role of MRE16 structural proteins in mosquito infectivity and tropism.

CHAPTER 2

INCREASED ORAL INFECTIVITY OF A DOUBLE SUBGENOMIC SINDBIS VIRUS EXPRESSION VECTOR FOLLOWING SERIAL PASSAGE IN *AEDES AEGYPTI* MOSQUITOES

Introduction

The selection for phenotypic variants of a virus strain by serial *in vivo* passage is not new; many examples appear in the literature. Perhaps most relevant to this study was the selection of a strain of SIN that was highly lethal for mice by six alternating intracranial passages of AR339 virus in suckling and weanling mice (Griffen and Johnson, 1977). The resultant neuroadapted strain of SIN (NSV) was found to be genetically stable and could be discriminated from other SIN strains using anti-SIN E1 and E2 monoclonal antibodies (Stanley *et al.* 1985), suggesting that changes in the envelope glycoproteins were associated with the changes in virulence. Molecular studies to identify the determinants of neurovirulence (Lustig *et al.* 1988) revealed that the NSV E2 and E1 glycoproteins differed from those of the wild-type virus by four and two amino acid substitutions, respectively.

RNA viruses are particularly amenable to this type of selection. Their encoded RNA-dependent RNA polymerases misincorporate nucleotides at approximately 10^{-3} to 10^{-5} substitutions per nucleotide site per round of replication, which is about 300 times the error rate of DNA viruses (Domingo and Holland, 1994). Consequently, mutant viral RNA genomes are continuously being generated, and will arise in any RNA virus-infected cell. This continuous generation of variants underlies the "quasispecies" (extreme genetic heterogeneity) structure of RNA virus populations (Domingo *et al.* 1978, 1985; Holland *et al.* 1982; Steinhauer and Holland, 1987). Therefore, if an RNA virus replicates in an environment different from that where its parental genome replicated, the probability of a newly arising variant becoming dominant will increase and the average or "master" genome sequence will shift to one that is better adapted to the new environment. It would

seem logical that arboviruses would exhibit considerable evolutionary potential because of their need to bridge the phylogenetic gap between arthropods and vertebrates. Paradoxically, recent studies suggested that alphaviruses and flaviviruses exhibit approximately ten-fold lower rates of evolutionary change than many nonarthropod-borne RNA viruses (Weaver *et al.* 1992). Studies with LaCrosse virus (*Bunyaviridae*) similarly demonstrated genetic stability during laboratory transmission cycles (Baldridge *et al.* 1989). In reviewing these data, Scott *et al.* (1994) observed that arboviruses probably have mutation frequencies similar to those of faster evolving nonarthropod-borne RNA viruses, but that their evolution may be constrained by transmission cycles requiring alternating infection of vertebrate and invertebrate hosts.

In the studies reported here artificial selection by serial oral passage through *Ae. aegypti* mosquitoes was chosen as the primary approach to increasing the *per os* infectivity and midgut tissue tropism of the dsSIN expression vector, TE/3'2J/CAT. This method of selection avoided the constraints on virus evolution proposed for arboviruses by using only invertebrate hosts and host cells. It was reasoned that variants of the virus population that possessed enhanced midgut infectivity and that disseminated from the midgut to other mosquito organs would quickly dominate the population if the appropriate selective pressures were applied. To select for the desired SIN phenotype, mosquitoes were orally challenged with blood meals containing high concentrations of virus to overcome any infectivity barriers associated with titer rather than phenotype. Mosquitoes were then held for a 14 day extrinsic incubation to ensure maximum opportunity for dissemination to occur. Virus was isolated for subsequent rounds of selection from the heads of infected mosquitoes, enhancing the selection of variants with the capacity to

disseminate. Virus was amplified in mosquito (C6/36) cells to avoid vertebrate-specific mutations. The dsSIN virus, TE/3'2J/CAT, was chosen for these studies based on the rationale that the reporter gene would permit quantitative assays of CAT enzymatic activity in mosquito organs following selection. Extracted viruses were compared to unpassaged virus to measure their relative capacities to infect the mosquito midgut and disseminate to other organs. Genetic analyses of the viral glycoprotein-encoding sequences following four serial passages were performed.

Materials and Methods

Cells and Medium

BHK-21 (hamster kidney) and Vero (monkey kidney) cells 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. C6/36 (*Ae. albopictus*) cells (Singh, 1967) were grown in L-15 medium containing 5% FBS plus antibiotics at 28°C.

Viruses and Virus Constructs

The wild-type SIN, AR339, was obtained from the CDC, Division of Vector-borne Infectious Diseases Laboratory (Fort Collins, CO) and had been passed extensively in BHK-21 and Vero cells. The derivation of the dsSIN expression vector, TE/3'2J, has been described (Hahn *et al.* 1992; Raju and Huang, 1991; Rice *et al.* 1987) and was discussed in Chapter 1 (Sindbis Virus Expression Systems). The addition of the CAT gene to the construct was described by Hahn *et al.* (1992). Both the TE/3'2J and TE/3'2J/CAT virus stocks had been passed once in BHK-21 cells (electroporation) and

once in C6/36 cells. The virus designated 4P-TE/3'2J/CAT was passed once in BHK-21 cells (electroporation), then a total of four times by blood meal infection of *Ae. aegypti* mosquitoes with intervening amplification in C6/36 cells (see *Serial Oral Passage of TE/3'2J/CAT in Ae. aegypti Mosquitoes,* below).

Additionally, TE/3'2JΔ2SGP virus, which had the second internal promoter deleted, was generated. The pTE/3'2JΔ2SGP plasmid was produced by excision of the ApaI (11386) to XbaI (11550) DNA segment from the pTE/3'2J infectious clone. Following a sequential digest of the plasmid with ApaI and XbaI restriction endonucleases (Gibco-BRL, Gaithersburg, MD), 5 μg of the digested plasmid was incubated with 10 units T4 DNA polymerase (Gibco-BRL) at 11°C for 15 minutes to generate blunt ends according to standard protocols (Sambrook *et al.* 1989), then inactivated at 65°C for 15 minutes. After agarose gel isolation, the plasmid was recircularized using 1 unit T4 DNA ligase (Gibco-BRL) in a reaction containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 5% polyethylene glycol-8000 and incubated at 14°C overnight. Epicurian Coli® SURE® cells (Stratagene, LaJolla, CA) were transformed with the religated plasmid using ampicillin selection. pTE/3'2JΔ2SGP was extracted from the bacteria by alkaline lysis followed by anion exchange resin purification plasmid (Qiagen Inc., Chatsworth, CA). The plasmid was eluted in sterile, double-distilled water and quantified spectrophotometrically. Infectious virus was produced as described below (*in vitro Transcription* and *Electroporation*) yielding a titer of 9.2 $\log_{10}$ $TCID_{50}$/ml.

in vitro Transcription

To produce TE/3'2J, TE/3'2J/CAT, and TE/3'2JΔ2SGP viruses for these studies, each plasmid template was linearized with XhoI to allow run-off transcription from the

bacteriophage SP6 promoter. The 50 µl transcription reactions contained 40 mM Tris-HCl (pH 7.6); 6 mM $MgCl_2$; 4 mM DTT; 2 mM spermidine; 40 units rNAsin® ribonuclease inhibitor (Promega Corp., Madison, WI); 1 mM each ATP, CTP, UTP, and GTP (Promega); 1.2 mM capping analog [m^7G(5')ppp(5')G (Pharmacia, Pleasant Hill, CA)]; 100 µg BSA; 2 µg linearized DNA template; and 20 units SP6 polymerase (Ambion Inc., Austin, TX). The reactions were incubated for 1 hour at 39°C and transcribed genomic RNAs were electroporated immediately into cells.

Electroporation

Transfection of BHK-21 cells was accomplished using a BTX Electro Cell Manipulator 600 apparatus (BTX Inc., San Diego, CA). BHK-21 cells grown to 70-80% confluence were trypsinized, washed twice with cold phosphate buffered saline (PBS) (pH 7.4), and resuspended in PBS to a concentration of 10^7 cells/ml. For each electroporation, 0.4 ml of cells were transferred to a 2-mm-gap cuvette (BTX) and 10 µl viral RNA was added. The suspension was pulsed twice in succession at room temperature with 450 V, 100 µF, and 720 Ohms. Immediately after electroporation, 0.6 ml L-15 (5% FBS) was added to the suspension, and the entire volume was transferred to a 25-cm^2 flask containing 4 ml L-15 (5% FBS). The flask was incubated at 37°C until >70% of the cells exhibited cytopathic effects (CPE) (approximately 24 - 48 hours). Supernatant was removed from the cells, cell debris pelleted, and virus-containing medium stored at -70°C.

Virus Titrations

Titers of virus-containing samples were determined by endpoint dilution assays. BHK-21 cells in 96-well plates were infected with serial 10-fold dilutions of sample. Endpoints were determined by observation of virus-induced CPE, relative to sample

dilution, 2-4 days post-infection. All titrations were performed in triplicate. Virus titers were determined by the Karber method (Karber, 1931) and expressed as $\log_{10}$ $TCID_{50}$ /ml.

Comparative in vitro Virus Growth Rates

Rates of virus production (one-step growth curves) for TE/3'2J, TE/3'2J/CAT, and 4P-TE/3'2J/CAT were compared in BHK-21, C6/36, and Vero cell lines. Confluent monolayers of each cell type in 25-cm^2 flasks were infected at a multiplicity of infection (MOI) of 5.0 with each virus. Virus was absorbed to the cells for 1 hour under constant agitation at room temperature. Supernatants were removed for virus titration and replaced with 2.0 ml L-15 (2% FBS) at 2 hour intervals (t = 3, 5, 7, 9, 11, 13 hours) and stored at -70°C. Virus titers were determined for each sample as described above (*Virus Titration*). The results represent the combined data from two separate experiments for each virus in each cell type.

Mosquito Colonies

Ae. aegypti (RexD) mosquitoes originating from Rexville, Puerto Rico and *Ae. albopictus* mosquitoes obtained from the Vector Biology Laboratory, Notre Dame University were reared at 28°C, 80% relative humidity, and photoperiod of 16L:8D. *Ae. triseriatus* and *Cx. pipiens pipiens* mosquitoes originating from larval collections made in LaCrosse, Wisconsin were reared at 25°C, 80% relative humidity, and 16L:8D.

Mosquito Infection

For blood meal infections, frozen virus stocks were first amplified by infection of C6/36 cells at an MOI of 0.01. To accomplish this, medium was removed from confluent monolayers of cells in 25-cm^2 flasks, and the appropriate dilution of virus stock in a 1.0 ml volume, was added. The cells were incubated at room temperature under agitation for 1

hour. Medium was then removed and replaced with 2.0 ml of L-15 (5% FBS). The infected cells were incubated at 28°C for 48 hours, at which time the virus-containing supernatant was removed, a 1.0 ml aliquot was stored at -70°C for later virus titration, and the remaining 1.0 ml mixed with 2.0 ml of defibrinated sheep blood (Colorado Serum Co., Boulder, CO). The blood-virus mixture was warmed to 37°C and pipetted into the chamber of a water-jacketed (37°C) glass membrane feeder apparatus (Rutledge *et al.* 1964) fitted with a mouse skin membrane. The membrane feeder was secured to a carton, covered with nylon mesh, containing the mosquitoes. Adult female mosquitoes (5-7 days post-eclosion) were allowed to feed for 1-2 hours. Blood meal samples were collected pre- and post-feeding for virus titration. Following the blood meal, mosquitoes were cold anesthetized and only fully engorged individuals retained. The bloodfed mosquitoes were housed at species-specific insectary conditions 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, 1976).

Serial Oral Passage of TE/3'2J/CAT in Ae. aegypti Mosquitoes

TE/3'2J/CAT virus was passed four times by blood meal infection of *Ae. aegypti* mosquitoes with intervening virus amplification in C6/36 cells. For each passage, *in vitro* amplified virus was fed to approximately 150 adult female mosquitoes. After a 14 day extrinsic incubation, surviving mosquitoes were cold anesthetized, and their heads were removed and pooled for isolation of disseminated virus. Approximately 70-90 heads were macerated in a 1.7 ml tube containing 0.7 ml cold L-15 (5% FBS) using a sterile plastic

pestle. The suspension was then filtered through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI) to remove debris and any contaminating bacteria or fungi. The filtrate was stored at -70°C until the next passage. Ten to twenty additional mosquito heads were analyzed by indirect immunofluorescence assay (IFA) for the presence of SIN antigen (Olson *et al.* 1994).

IFA Analysis of Mosquito Tissues

IFA was used to detect SIN antigen in mosquito head squashes and dissected organs (Gould *et al.* 1985; Olson *et al.* 1994). Organs dissected from infected or control mosquitoes were placed on poly-D-lysine-coated slides in a drop of either L-15 (5% FBS) (midguts) or Elmer's glue solution (salivary glands) (Beaty and Thompson, 1976) and allowed to dry. Mosquito organs or head tissues were fixed to the slides by immersion in cold acetone for 1 hour. Tissues were incubated with mouse anti-SIN E1 MAb 30.11a (Chanas *et al.* 1982) (diluted 1:200 in PBS) at 37°C for 40 minutes. The tissues were then washed with PBS and incubated with biotinylated sheep anti-mouse antibody (Amersham Corp., Arlington Heights, IL) (1:200) at 37°C for 40 minutes. The tissues were washed again, and incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Amersham) (1:200) at 37°C for 10 minutes. After three final washes (2 x PBS, 1 x ddH_20), a drop of glycerol/PBS (9:1) containing 2.5% 1.4. diazobicyclo (2,2,2) octane (DABCO) was applied to each mosquito tissue sample, and a glass coverslip was mounted on the slide. SIN E1-specific fluorescence was detected using an Olympus BH2 epifluorescence microscope.

Comparative Virus Dissemination Rates

Adult female *Ae. aegypti* mosquitoes ingested blood meals containing either AR339, TE/3'2J, TE/3'2J/CAT, 4P-TE/3'2J/CAT, or TE/3'2JΔ2SGP viruses. The oral infectivities of each were measured by the ability of the viruses to infect and disseminate from the mosquito midgut. Similarly, *Ae. albopictus*, *Ae. triseriatus*, and *Cx. pipiens pipiens* mosquitoes were infected *per os* with either TE/3'2J/CAT or 4P-TE/3'2J/CAT. Blood meal infections were performed as described above (*Mosquito Infection*), and dissemination rates were determined as the percentage of infected mosquitoes displaying SIN antigen in head tissues by IFA (see *IFA Analysis of Mosquito Tissues*) following a 14 day extrinsic incubation.

Kinetics of Virus Dissemination

Adult female *Ae. aegypti* mosquitoes were infected with unpassaged TE/3'2J/CAT or 4P-TE/3'2J/CAT viruses by infectious blood meal and intrathoracic inoculation as described above (*Mosquito Infection*). Blood meal and inoculum virus titers were 9.0 and 9.5 $\log10_{10}$ $TCID_{50}$/ml, respectively. Control mosquitoes were either fed sheep blood without virus or inoculated with L-15 (2% FBS). On days 1, 3, 6, 9, 12, 15, and 21 post-infection, heads were removed and midguts and salivary glands were dissected from a number of the bloodfed mosquitoes. Similarly, inoculated mosquito dissections were performed on days 1, 3, 6, and 12 post-infection. Mosquito tissues were prepared and analyzed by IFA for SIN antigen as described above (*IFA Analysis of Mosquito Tissues*). Each tissue sample examined was scored for the degree of fluorescence observed using a relative scale from zero (0) to five (5); scores were averaged for each time point.

Cloning and Sequencing of Viral cDNA Genome Segments

Total RNA was extracted from C6/36 cells infected at an MOI of 0.01 with TE/3'2J, TE/3'2J/CAT, or 4P-TE/3'2J/CAT viruses. A modified guanadinium isothiocyanate protocol using silica-based spin columns (RNeasy Total RNA System, Qiagen) was employed to purify total RNA from infected cells. The purified RNAs were then quantified spectrophotometrically. Double-stranded viral cDNA was synthesized by RT/PCR amplification of the purified RNA. First-strand priming of viral genome segments including the E2 glycoprotein-encoding region was accomplished using 100 pmoles reverse primer E2.R1 (5'GCAGCAGCGCATTAGAACG3'), 1μg RNA, and 200 units Superscript™ II reverse transcriptase (Gibco-BRL) in a reaction mix containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP mix, and 39 units rNAsin® ribonuclease inhibitor (Promega). The reaction was performed at 42°C for 1 hour and stopped by incubation at 70°C for 15 minutes. For subsequent PCR amplification, primers E2.R1 and E2.F1 (5'GGAAGGGACAGAAGAGTGG3') were used in 50 μl reactions consisting of 2 μl of first strand product, 100 pmoles forward and reverse primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100. The PCR profile consisted of denaturation at 95°C for 5 minutes followed by 25 cycles of amplification with 1.5 units Taq polymerase (Promega); 94°C for 1 minute, 56°C for 30 seconds, 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. Viral E1 glycoprotein-encoding genome segments were amplified similarly using primers E1.F1 (5'GCTCCTGCTGCCTGCCTTTTT3') and E1.R1 (5'TCTCAATGCCA-GTAGGACAGG3'). Genome segments encompassing the second internal promoter,

CAT gene, and 3'NCR were amplified using primers PolyA-R (oligo dT_{20}-GA) and E1.F4 (5'TCCCGAACGCTGCCTTTATCA3').

The E2 cDNA produced from 4P-TE/3'2J/CAT virus-infected cells was cloned into the TA vector pCR®II; E1 and 3'NCR cDNAs were cloned into the pCR®2.1 plasmid (Invitrogen, Carlsbad, CA). Insert-containing plasmids were purified from INVαF' *E. coli* (Invitrogen) by alkaline lysis followed by anion exchange resin isolation of plasmid DNA (Qiagen). Plasmid DNA was eluted in sterile, double distilled water and quantified spectrophotometrically.

Three separate clones of the E2-containing region (p4PE2.01 - .03) were sequenced bi-directionally using the *fmol*™ DNA Sequencing System (Promega), a modification of the dideoxy chain termination method (Sanger *et al.* 1977). Primers used for sequencing (Table 2.1) were first end-labeled with 12 μCi/μl [γ-^{32}P]-ATP in a reaction containing 10 pmoles primer, 10 pmoles γ-^{32}P-labeled ATP, 5 units T4 polynucleotide kinase, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, and 100 μM spermidine. For each set of sequencing reactions, four 16 μl reactions were prepared containing 2 μl of the appropriate d/ddNTP mix (either dideoxy-GTP, ATP, CTP, or TTP plus the remaining three nucleotides), 40 fmoles purified plasmid template DNA, 1.5 pmoles end-labeled primer, 50 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, and 5 units sequencing grade Taq polymerase (Stratagene). The reactions were preheated to 95°C for 2 minutes and subjected to 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 70°C for 1 minute. After completion of the thermocycling program, 3 μl of a stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each reaction. The four samples were heated to 90°C for 2 minutes prior to separation

on a 5% acrylamide sequencing gel. Sequence was determined by visualization of the gel following exposure to Fuji RX x-ray film at -70°C for 2 - 24 hours.

Table 2.1. Primers Used in Sequencing 4P-TE/3'2J/CAT PE2, 6K/E1, and 3'-Terminal Genome Segments[1]

Clones	Primer	Sequence (5'-3')	Location (5' end)
p4PE2.01-.03	E2.F1	GGAAGGGACAGAAGAGTGG	8420
	E2.F2	GCCTACGATACCCTGCTCA	8571
	E2.F3	CCATAGCGGAGCAGCAAGC	8792
	E2.F4	TGGCCCGCAAGATAAAACC	9007
	E2.R4	TTGGAGGCTGATGTGTTTA	9488
	E2.R3	CTGTACTATTTCGTGTGGC	9701
	E2.R2	GACCTAACGCAGCACAAGA	9889
	E2.R1	GCAGCAGCGCATTAGAACG	10007
p4PE1.01-.03	E1.F1	GCTCCTGCTGCCTGCCTTTTT	10006
	E1.F2	TCCCCTCCCCAAAAATCAAAT	10228
	E1.F3	TCATCGCCAGCACAGACATTA	10702
	E1.F4	TCCCGAACGCTGCCTTTATCA	10909
	E1.R4	CGACGTACGCCTCACTCATCT	10389
	E1.R3	ATGGGCGGCCTGAGTTGTTTT	10815
	E1.R2	GGTCATTTTTGTGCGGGGTGC	11250
	E1.R1	TCTCAATGCCAGTAGGACAGG	*[2]
p3'NCR	E1.F4	TCCCGAACGCTGCCTTTATCA	10909
	Poly-AR	TTTTTTTTTTTTTTTTTTTGA	*[3]

[1]The ends of some cloned sequences were obtained using plasmid-specific M13, SP6, or T7 promoter-complementary primers. [2]The E1.R1 primer location is within the internal promoter region of TE/3'2J; there is no corresponding location in the prototype SIN sequence. [3]The Poly-AR primer location is within the oligo-dT region included at the 3' end of the TE/3'2J genomic sequence.

Three clones of the E1-containing genome region (p4PE1.01 - .03) of 4P-TE/3'2J/CAT were sequenced using an ABI 377 DNA Sequencer (Perkin-Elmer Inc., Foster City, CA) and Taq Fluorescence Sequencing (FS) polymerase (Perkin-Elmer). The ends of each cloned cDNA segment were sequenced using plasmid-specific primers complementary to SP6, T7, or M13 promoters. Subsequent sequencing was performed in both directions using primers designed from previously determined E1 sequence. One

clone of the 3'NCR was also sequenced by this method. SeqAid™ II, version 3.6 computer software was used to align contiguous DNA sequences.

Results

Serial Oral Passage of TE/3'2J/CAT in Ae. aegypti Mosquitoes

The infectious dsSIN virus, TE/3'2J/CAT, was produced by run-off transcription of its parent clone and transfection of BHK-21 cells with the viral genomic RNA. Virus was recovered from the supernatant of the infected cells (9.5 $\log_{10}$ $TCID_{50}$/ml) and stored at -70°C until used. This virus stock was passed four times by *per os* infection of adult female *Ae. aegypti* mosquitoes with alternating amplification in mosquito (C6/36) cells. Virus that had disseminated to the head tissues of infected mosquitoes 14 days post-blood meal infection was recovered and used in each subsequent selective passage. The viral titers of blood meals used in each passage and the dissemination rates measured by head squash/IFA analysis at the end of each of the four extrinsic incubations were determined (Table 2.2). The virus recovered following the fourth passage (4P-TE/3'2J/CAT) caused disseminated infections in *Ae. aegypti* at over eight times the rate of the unpassaged TE/3'2J/CAT virus (62.5% vs. 7.5%; $p < 0.01$).

Table 2.2 Dissemination Rates After Each Passage of TE/3'2J/CAT Virus in *Ae. aegypti* Mosquitoes

Passage Number	Virus	Blood Meal Titer (Log_{10} $TCID_{50}$/ml)	Dissemination Rate[1]	Virus Recovered
1	stock TE/3'2J/CAT	8.0	nd[2]	1P TE/3'2J/CAT
2	1P TE/3'2J/CAT	8.5	20.0% (2/10)	2P TE/3'2J/CAT
3	2P TE/3'2J/CAT	9.5	26.7% (4/15)	3P TE/3'2J/CAT
4	3P TE/3'2J/CAT	9.0	45.0% (9/20)	4P TE/3'2J/CAT
-	4P TE/3'2J/CAT	9.0	62.5% (20/32)	-

[1]Dissemination rate is the percentage of infected mosquitoes with disseminated SIN infection (e.g., positive for SIN antigen by IFA of head squashes). [2]Dissemination rate was not determined (nd) for unpassaged virus in this experiment; however, subsequent dissemination experiments with unpassaged TE/3'2J/CAT virus in *Ae. aegypti* mosquitoes yielded a rate of 7.5% (see Table 2.3).

Comparative in vitro Virus Growth Rates

One-step growth curve experiments were performed to examine the differences in virus replication between TE/3'2J, TE/3'2J/CAT, and 4P-TE/3'2J/CAT viruses in different host cell types (Figure 2.1). In the mosquito cell line (C6/36), the passaged virus replicated to titers approximately 10 to 100 times those of TE/3'2J/CAT and TE/3'2J over the course of the experiment. By 13 hours post-infection, the titer of 4P-TE/3'2J/CAT was nearly 6.0 $\log_{10}$ $TCID_{50}$/ml. In BHK-21 cells the rates of virus production for 4P-TE/3'2J/CAT and TE/3'2J were very similar, reaching peaks of ~7.0 $\log_{10}$ $TCID_{50}$/ml at 11 hours post infection. Unpassaged TE/3'2J/CAT replicated less efficiently; titers were consistently 10 to 100 times lower throughout the infection. In Vero cells, the replicative kinetics of the three viruses were virtually indistinguishable until 9 hours post-infection. 4P-TE/3'2J/CAT virus titers then declined and were nearly 100-fold lower then TE/3'2J and TE/3'2J/CAT by 13 hours post-infection.

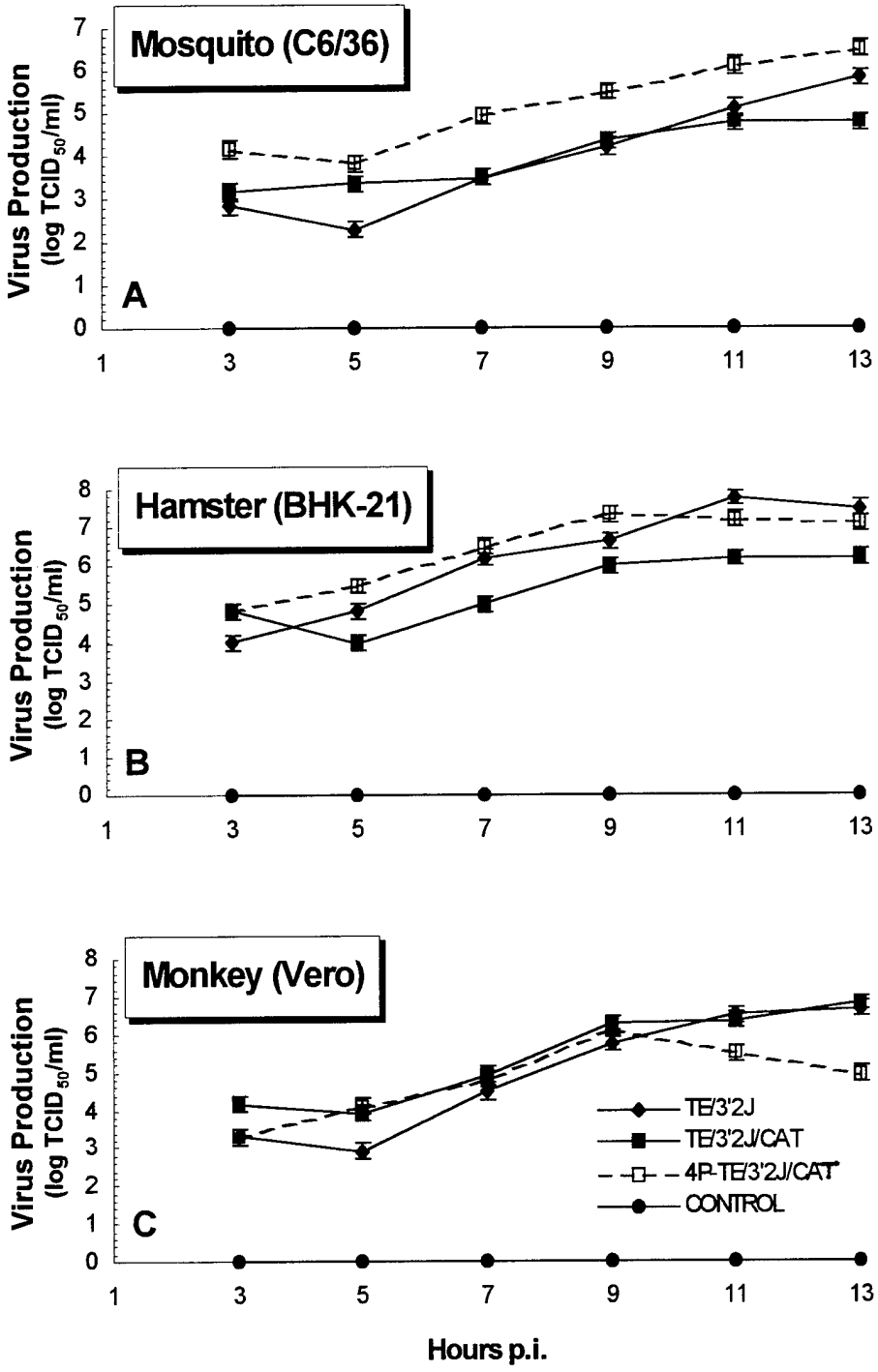

Figure 2.1. Comparative *in vitro* Growth Rates of SIN Virus Constructs. One -step growth curves in A) C6/36, B) BHK-21, and C) Vero cell lines infected at an MOI of 5.0. The results are from the combined data of two experiments. Error bars represent standard deviation.

Comparative Virus Dissemination Rates

Dissemination rate is a measure of productive infection. It is directly related to the capacity of the virus to infect the mosquito midgut epithelial cell layer and replicate prior to disseminating to other mosquito organs. A number of experiments were performed to compare the dissemination rate in *Ae. aegypti* mosquitoes of 4P-TE/3'2J/CAT virus to those of the prototype SIN (AR339), TE/3'2J, and unpassaged TE/3'2J/CAT viruses following blood meal infection (Table 2.3). Six separate experiments using 4P-TE/3'2J/CAT virus, with blood meal titers between 8.5 and 9.5 $\log_{10}$ $TCID_{50}$/ml, yielded dissemination rates between 48.2% and 93.3%. There was little statistical correlation ($r = -0.411$) between virus titer and dissemination rate in these experiments. Thus, the data was pooled yielding an overall dissemination rate of 63.9% (101 of 158 mosquitoes examined). Four experiments were performed to measure the dissemination rate of unpassaged TE/3'2J/CAT virus. Although there were two fewer experiments, the net number of mosquitoes examined was nearly the same (160) as with the passaged virus. Unpassaged TE/3'2J/CAT disseminated at a rate of 7.5% according to the pooled data ($r = 0.317$).

The dissemination rate of TE/3'2J was determined to be 7.2% in one experiment. This was strikingly similar to the dissemination rate of TE/3'2J/CAT, suggesting that the presence of the additional nucleotide sequence of the CAT gene (694 nt) was not a significant factor in infectivity or dissemination. Finally, the dissemination rate for the prototype SIN was 39.5% (Table 2.3). However, this rate was derived from two experiments with approximately 50-fold differences in blood meal titer, making it difficult to disregard the effect of virus titer on dissemination rate. The 33.3% AR339 dissemination rate resulting from the 8.5 $\log_{10}$ $TCID_{50}$/ml blood meal would seem to be

more relevant, as this titer was more similar to those of the other viruses. Chi square analyses revealed that significant differences in dissemination rates ($p<0.01$) existed between each pair of viruses except when comparing TE/3'2J to TE/3'2J/CAT ($p=0.94$).

Table 2.3. Comparative SIN Virus Dissemination Rates in *Ae. aegypti* Mosquitoes

Virus	Blood Meal Titer (Log_{10} $TCID_{50}$/ml)	Head Squash/IFA Results		
		# Positive	Total	% Positive
AR339	6.8	7	14	50.0%
AR339	8.5	8	24	33.3%
	pooled	15	38	**39.5%**
TE/3'2J	8.1	6	83	**7.2%**
TE/3'2J/CAT	9.0	0	24	0.0%
TE/3'2J/CAT	9.0	5	24	20.8%
TE/3'2J/CAT	8.8	3	28	10.7%
TE/3'2J/CAT	7.1	4	84	4.8%
	pooled	12	160	**7.5%**
4P-TE/3'2J/CAT	9.0	20	32	62.5%
4P-TE/3'2J/CAT	8.5	28	30	93.3%
4P-TE/3'2J/CAT	9.0	18	31	58.1%
4P-TE/3'2J/CAT	9.0	13	27	48.2%
4P-TE/3'2J/CAT	9.5	15	25	60.0%
4P-TE/3'2J/CAT	8.5	7	13	53.9%
	pooled	101	158	**63.9%**

To test whether the dissemination rates observed in *Ae. aegypti* mosquitoes were the result of a general phenomenon or a more host-specific interaction, blood meal infections of three other mosquito species were performed. Dissemination rates of unpassaged and passaged TE/3'2J/CAT viruses in *Ae. aegypti* (from Table 2.3), *Ae. albopictus*, *Ae. triseriatus*, and *Cx. pipiens* mosquitoes were determined (Table 2.4). Although these data are limited in the numbers of mosquitoes examined, they suggest a host component to viral dissemination rate. Chi square analyses showed that dissemination rate differences were significant ($p<0.01$) when comparing TE/3'2J/CAT in

Ae. aegypti, Ae. triseriatus, or Cx. pipiens to 4P-TE/3'2J/CAT in either *Ae. aegypti* or *Ae. albopictus* mosquitoes, or when comparing 4P-TE/3'2J/CAT in *Ae. aegypti* or *Ae. albopictus* to its own dissemination rates in *Ae. triseriatus* or *Cx. pipiens*. *Ae. aegypti* and *Ae. albopictus* are more closely related to each other (subgenus *Stegomyia*) than either is to the other two mosquito species tested (*Ae. triseriatus* is subgenus *Protomacleaya*). The similar high dissemination rates of 4P-TE/3'2J/CAT in *Ae. aegypti* and *Ae. albopictus* when compared to those in either *Ae. triseriatus* or *Cx. pipiens* suggest that serial passage selected for viral components involved in vector-specific interactions.

Table 2.4. Comparative SIN Virus Dissemination Rates in Selected Mosquito Species

Virus	*Ae. aegypti*	*Ae. albopictus*	*Ae. triseriatus*	*Cx. pipiens*
TE/3'2J/CAT	7.5% (12/160)	30.0% (3/10)	6.7% (1/15)	7.1% (2/28)
4P-TE/3'2J/CAT	63.9% (101/158)	72.2% (13/18)	4.2% (1/24)	3.6% (1/28)

Kinetics of Virus Dissemination

Ae. aegypti mosquito organs (midguts, salivary glands, and neural tissues) were examined by SIN-specific IFA analysis over a time course of 21 days following blood meal infection with either passaged or unpassaged TE/3'2J/CAT viruses (Figures 2.2 and 2.4). Organs from mosquitoes intrathoracically inoculated with the two viruses were similarly tested over a 12 day time course (Figure 2.3). Specific immunofluorescence in each of the organs examined was scored on a scale of 0 to 5 and the scores averaged at each time point. Differences in dissemination dynamics between bloodfed TE/3'2J/CAT and 4P-TE/3'2J/CAT are illustrated in Figure 2.2. The unpassaged virus was detected in mosquito midguts at levels marginally above background fluorescence from 3 to 6 and 12

to 15 days post-infection. Dissemination from the midgut to the head (neural tissues) was undetectable and only fluorescence at background level was observed in the salivary glands early (days 3 to 9) in the infection. In contrast, 4P-TE/3'2J/CAT antigen was detectable at significant levels in the midguts from 3 to 21 days post-infection, reaching peak levels at day 15. Dissemination to both the heads and salivary glands was evident by day 9 and continued to day 21. However, fluorescence levels had declined in all three tissues between 15 and 21 days post-infection. Organs were not dissected on day 18 for either of the mosquito groups.

Dissemination profiles for the two viruses following intrathoracic inoculation are illustrated in Figure 2.3. All of the examined mosquito tissues were heavily infected by day 3, regardless of the virus used. By day 12 the levels of fluorescence had declined slightly from those observed at 3-6 days post-infection. Dissections were not accomplished on day 9 or after day 12 for either group. There appeared to be very little difference in the tropisms of the two viruses following inoculation, suggesting that the effects of selective passage on 4P-TE/3'2J/CAT were specific to the capacity of the virus to escape the midgut after oral infection.

It is noteworthy that the fluorescence observed in the midguts of inoculated mosquitoes was qualitatively different from that seen following oral infection. In inoculated mosquitoes SIN antigen was restricted to the muscle fibers and respiratory tracheoles surrounding the midguts and was never detected in the underlying epithelial cell layer. In contrast, in the orally infected mosquitoes, SIN antigen was detectable only in the epithelial cell layer early in the infection with the passaged virus and appeared to

disseminate outward to involve the surrounding muscle, tracheoles, and nerves as the infection progressed. This can be clearly seen in Figure 2.4 (B and D).

Sequencing of 4P-TE/3'2J/CAT Envelope Glycoprotein Genes

To test the hypothesis that selection for midgut infectivity and dissemination following oral infection would result in alterations in the viral envelope glycoproteins, nucleotide sequences of three cDNA clones of the 4P-TE/3'2J/CAT PE2-encoding regions and three clones of the 6K/E1-encoding regions were determined. Primers used for sequencing and their positions in the genome are listed in Table 2.1 (SIN numbering) (Strauss *et al.* 1984). Nucleotide substitutions were detected, relative to unpassaged virus, in each of the clones (Figure 2.5). These data are compiled in Table 2.5 with derived amino acid substitutions indicated. It is apparent that selection by serial passage resulted in a very limited set of nucleotide substitutions with correspondingly few amino acid changes in the envelope glycoproteins. If changes occurring in only one of the three clones at a given position are disregarded, only five nucleotide substitutions occurred; one in the E2 gene and four in E1 (Table 2.5). The E2 cytosine to adenine change at position 8931 appeared in two of the clones, producing a conservative leucine to isoleucine amino acid substitution. A thymidine to adenine change in all three clones at position 10469 in E1 produced no amino acid substitution, as was the case with an adenine to guanine change at position 10946 in two clones. At position 10482, guanine was substituted for adenine in all three clones, changing the encoded threonine to alanine. At position 11340, all three clones had guanine substituted for adenine to encode a valine instead of methionine.

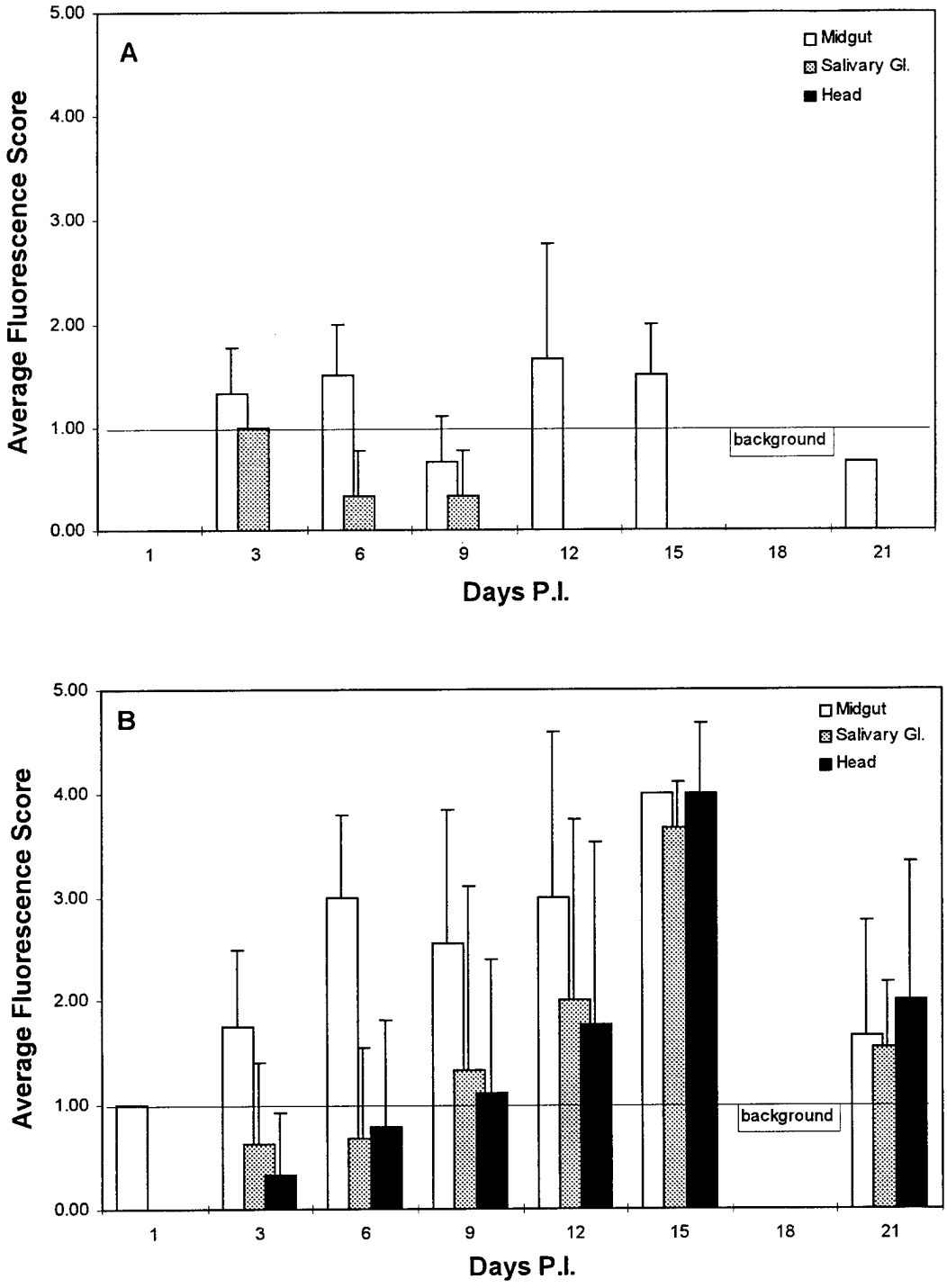

Figure 2.2. Comparative Virus Dissemination Profiles in Bloodfed *Ae. aegypti* Mosquitoes. Relative levels (scored on a scale from 0 to 5) of SIN E1-specific fluorescence were averaged for each time point in each of the tissues. Only scores above background levels of non-specific fluorescence were considered significant. A) Unpassaged TE/3'2J/CAT; B) 4P-TE/3'2J/CAT. Error bars indicate standard error, where applicable.

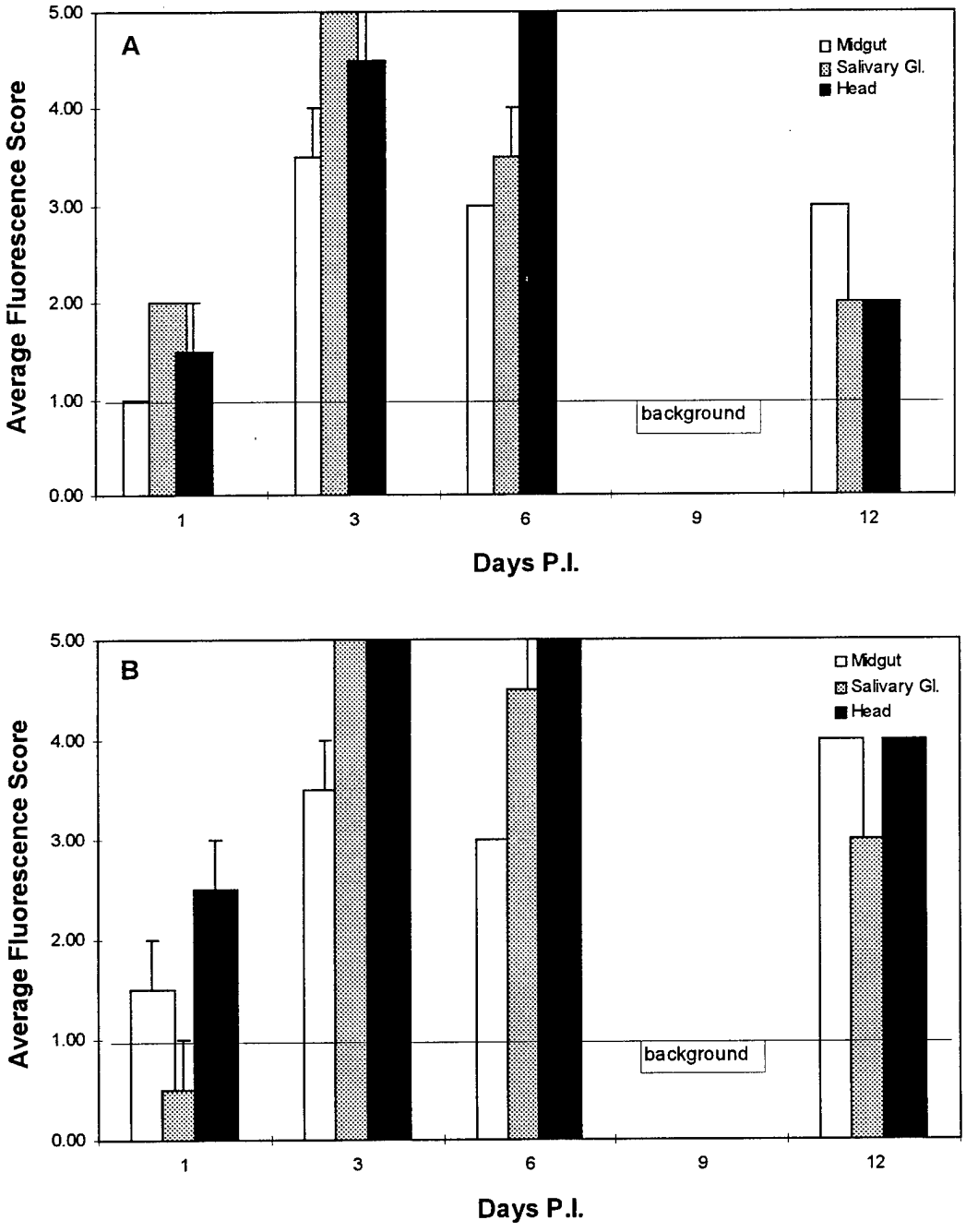

Figure 2.3. Comparative Virus Dissemination Profiles in Inoculated *Ae. aegypti* Mosquitoes. Averaged relative levels of SIN E1-specific fluorescence in dissected mosquito tissues following intrathoracic inoculation. A) Unpassaged TE/3'2J/CAT; B) 4P-TE/3'2J/CAT. Error bars indicate standard error, where applicable.

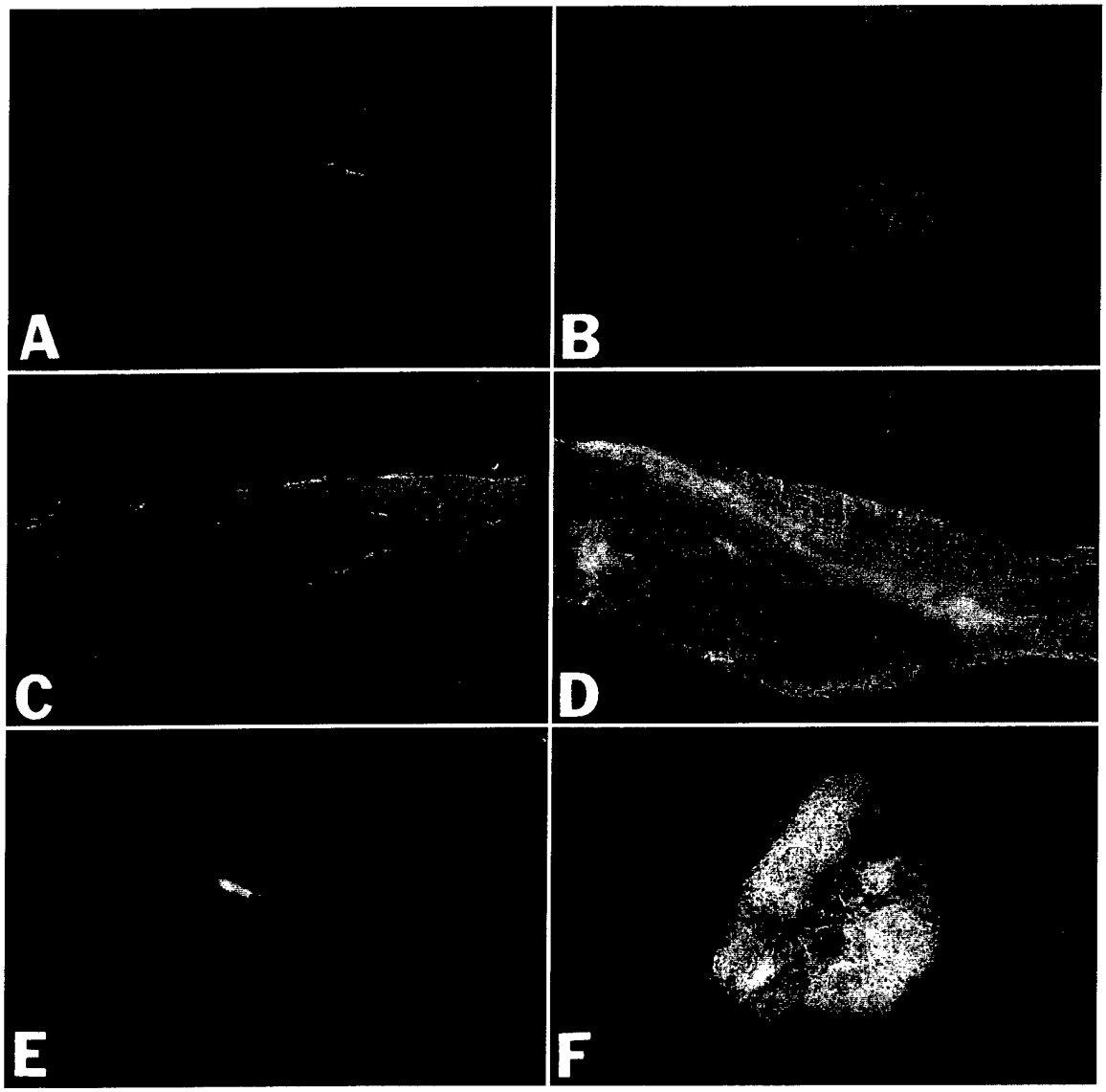

Figure 2.4. Comparative IFA Analysis of *Ae. aegypti* Organs Following Oral Infection with TE/3'2J/CAT and 4P-TE/3'2J/CAT Viruses. Representative organs of mosquitoes infected by blood meal with unpassaged (A,C,E) and 4th passage (B,D,F) TE/3'2J/CAT are shown. Mosquito midguts at 3 days post-infection (A,B) exhibiting focal epithelial cell fluorescence; and at 12 days post-infection (C,D) exhibiting incomplete dissemination in the epithelial cell layer with unpassaged virus but disseminated infection with the passaged 4P-TE/3'2J/CAT. Salivary glands from the same mosquitoes as C and D, respectively, 12 days post-infection (E,F) show no infection with unpassaged virus while antigen from the passaged virus can be seen in both lateral lobes. Magnification: (A-B) 50X; (C-F) 25X.

Figure 2.5. Nucleotide Sequence Comparison of 4P-TE/3'2J/CAT PE2 and 6K/E1 cDNA Clones to Unpassaged TE/3'2J/CAT Sequences. The underlined sequence is that of the unpassaged dsSIN (differences from HRsp are in lower case). The sequences of each of three clones of the passaged virus PE2 and 6K/E1 genes are below the line. Asterisks denote unchanged nucleotides; substitutions are in bold print (an N indicates uncertainty). Numbering in the right margin is from the SIN HRsp sequence (Strauss *et al.* 1984).

```
          >E3 (8439)
TE/3'2J TCC GCA GCA CCA CTG GTC ACG GCA ATG TGT TTG CTC GGA AAT GTG AGC TTC  8489
E2.01   *** *** *N* *** *** *** *** *N* *** *** *** N*N *** *** *N* *** ***
E2.02   *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
E2.03   *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

CCA TGC GAC CGC CCG CCC ACA TGC TAT ACC CGC GAA CCT TCC AGA GCC CTC GAC ATC  8546
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** **N *** *** *** *** *** *** *** *** *** *** ***

CTT GAA GAG AAC GTG AAC CAT GAG GCC TAC GAT ACC CTG CTC AAT GCC ATA TTG CGG  8603
*** *** *** *** *** *** *** *** *** *** *** **A *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** A** *** ***
                                  >E2 (8631)
TGC GGA TCG TCT GGC AGA AGC AAA AGA AGC GTC AcT GAC GAC TTT ACC CTG ACC AGC  8660
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *N* *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

CCC TAC TTG GGC ACA TGC TCG TAC TGC CAC CAT ACT GaA CCG TGC TTC AGC CCT GTT  8717
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

AAG ATC GAG CAG GTC TGG GAC GAA GCG GAC GAT AAC ACC ATA CGC ATA CAG ACT TCC  8774
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

GCC CAG TTT GGA TAC GAC CAt AGC GGA GCA GCA AGC GCA AAC AAG TAC CGC TAC ATG  8831
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

TCG CTT AAG CAG GAT CAC ACC GTT AAA GAA GGC ACC ATG GAT GAC ATC AAG ATT AGC  8888
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

ACC TCA GGA CCG TGT AGA AGG CTT AGC TAC AAA GGA TAC TTT CTC CTC GCA AAA TGC  8945
*T* *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** A** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** A** *** *** *** *** ***

CCT CCA GGG GAC AGC GTA ACG GTT AGC ATA GTG AGT AGC AAC TCA GCA ACG TCA TGT  9002
*** A** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

ACA CTG GCC CGC AAG ATA AAA CCA AAA TTC GTG GGA CGG GAA AAA TAT GAT CTA CCT  9059
*** *** *N* *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***

CCC GTT CAC GGT AAA AAA ATT CCT TGC ACA GTG TAC GAC CGT CTG AAA GAA ACA ACT  9116
*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
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Figure 2.5 (continued).

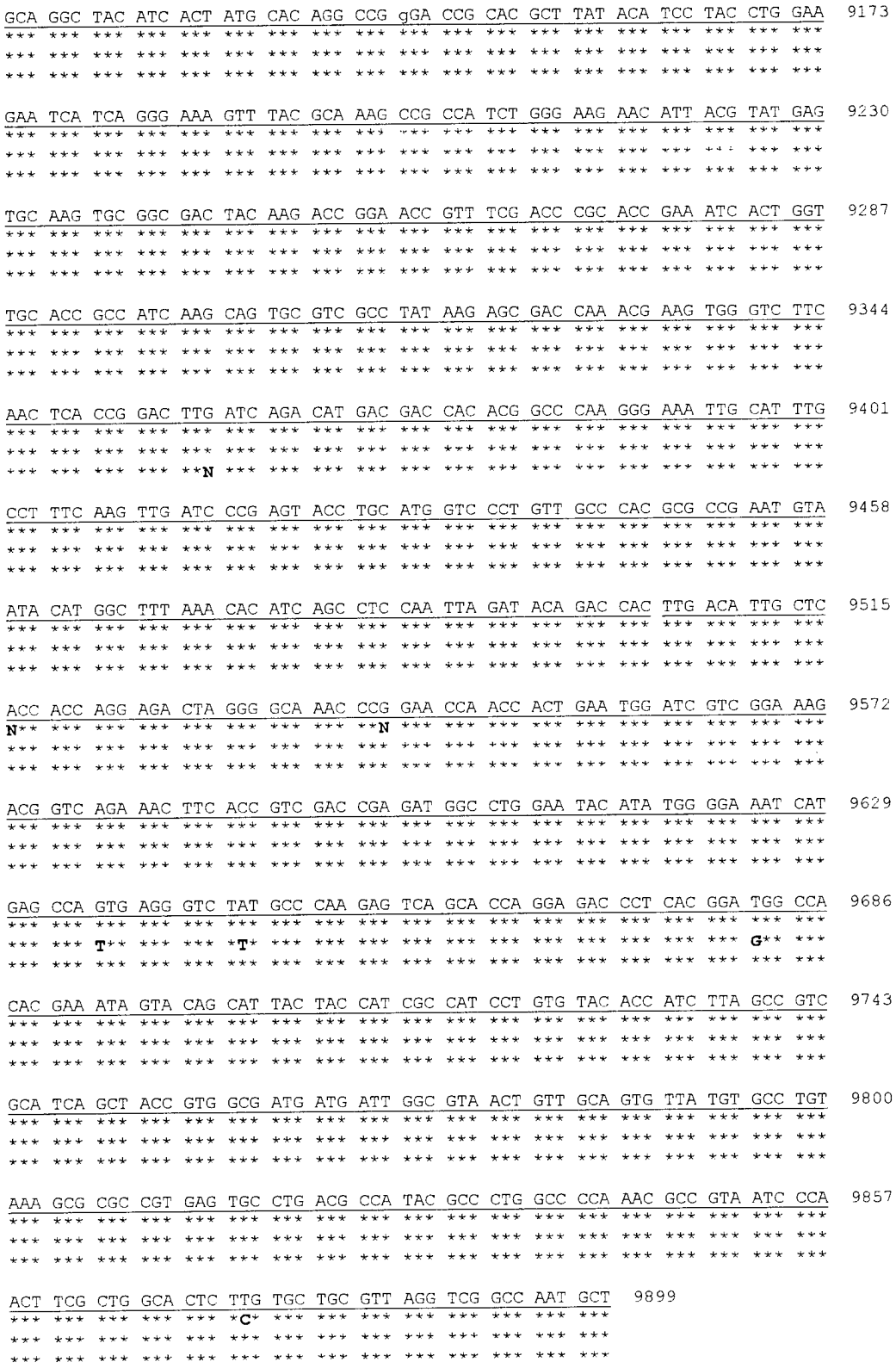

Figure 2.5 (continued).

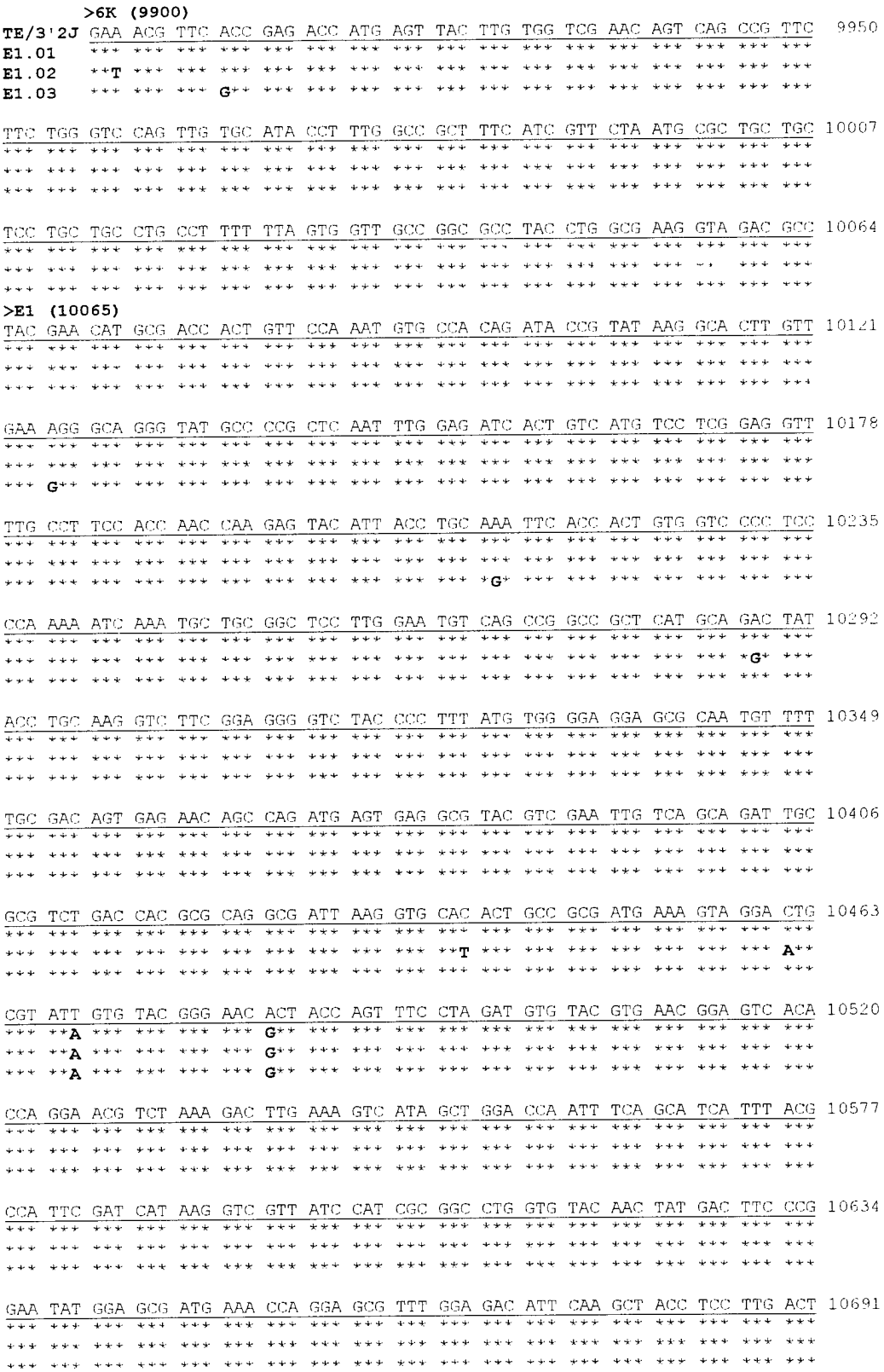

Figure 2.5 (continued).

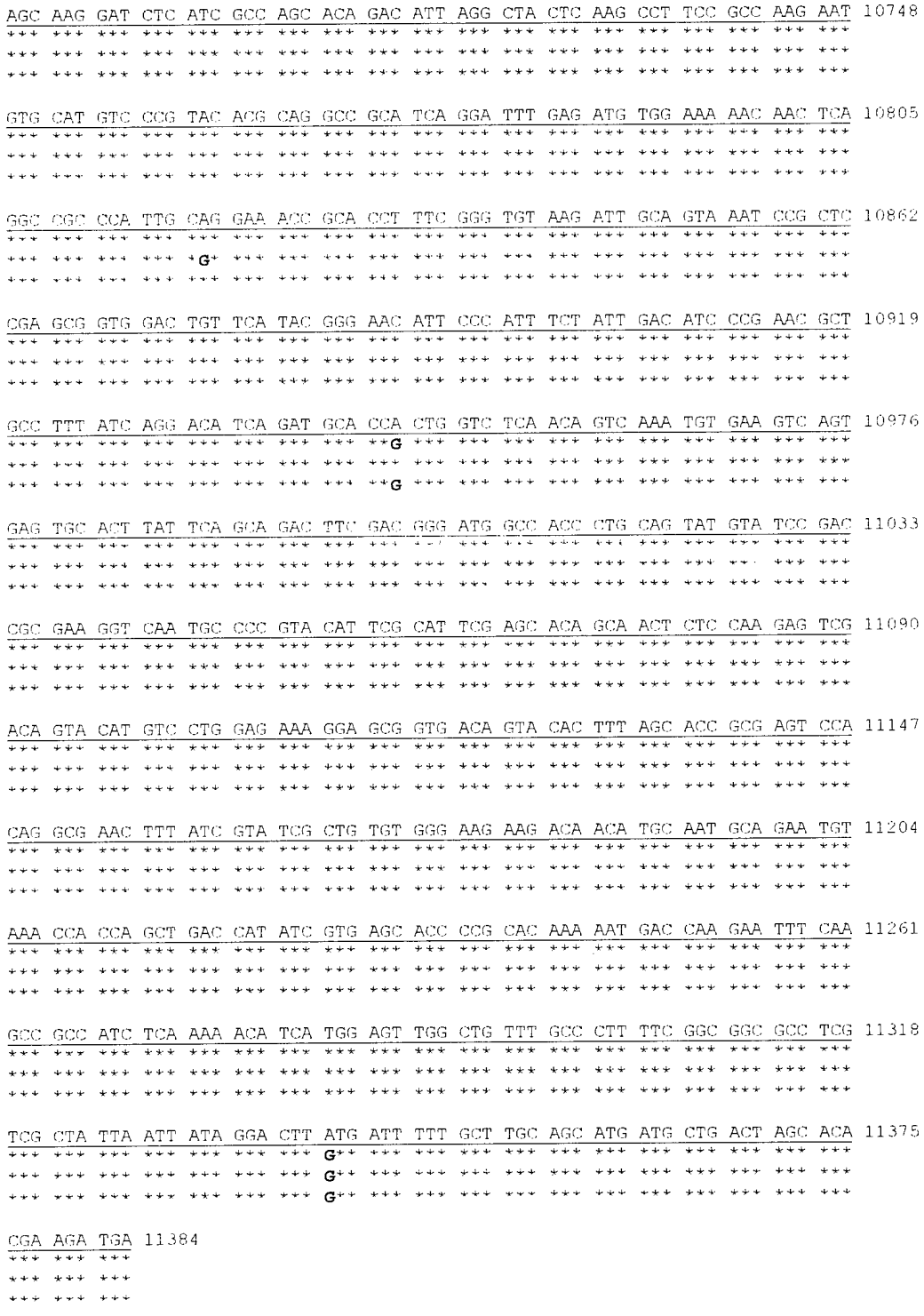

Table 2.5. Nucleotide and Derived Amino Acid Substitutions in 4P-TE/3'2J/CAT cDNA Clones[1]

		Nucleotide				Amino Acid[3]			
			Clone				Clone		
Gene	Position[2]	TE/3'2J	.01	.02	.03	TE/3'2J	.01	.02	.03
E3	8585	G	A			Leu	*		
	8598	T			A	Leu			Met
E2	8890	C	T			Thr	Ile		
	8931	C		A	A	Leu		Ile	Ile
	8949	C	A			Pro	Thr		
	9636	G		T		Val		Leu	
	9646	A		T		Tyr		Phe	
	9681	T		G		Trp		Gly	
	9874	T	C			Leu	Ser		
6K	9902	A		T		Glu		Asn	
	9909	A			G	Thr			Ala
E1	10125	A			G	Arg			Gly
	10213	A			G	Leu			Arg
	10288	A		G		Asp		Gly	
	10439	C		T		His		*	
	10461	C		A		Leu		Met	
	10469	T	A	A	A	Ile	*	*	*
	10482	A	G	G	G	Thr	Ala	Ala	Ala
	10819	A		G		Gln		Arg	
	10946	A	G		G	Pro	*		*
	11340	A	G	G	G	Met	Val	Val	Val

[1]Shaded rows indicate identical nucleotide substitutions were found in at least 2 of 3 clones at that position. [2]Nucleotide position reference is from Strauss and Strauss (1984) for HRsp SIN. [3]Unchanged amino acids are denoted by an asterisk.

Sequencing of 4P-TE/3'2J/CAT 3' Genome Terminus

In preparation for cloning and sequencing the 4P-TE/3'2J/CAT 3'NCR, RNA extracted from virus-infected C6/36 cells was amplified by RT/PCR using 3'NCR-flanking primers (Table 2.1). The resulting cDNA products were separated on an agarose gel for isolation prior to cloning (Figure 2.6). Interestingly, the cDNA band produced from passaged 4P-TE/3'2J/CAT RNA appeared to be smaller than that from the unpassaged virus and only slightly larger than TE/3'2J cDNA. These data indicated that a large (~500 nucleotide) deletion of the 4P-TE/3'2J/CAT genome had occurred within the amplified region during selective passage.

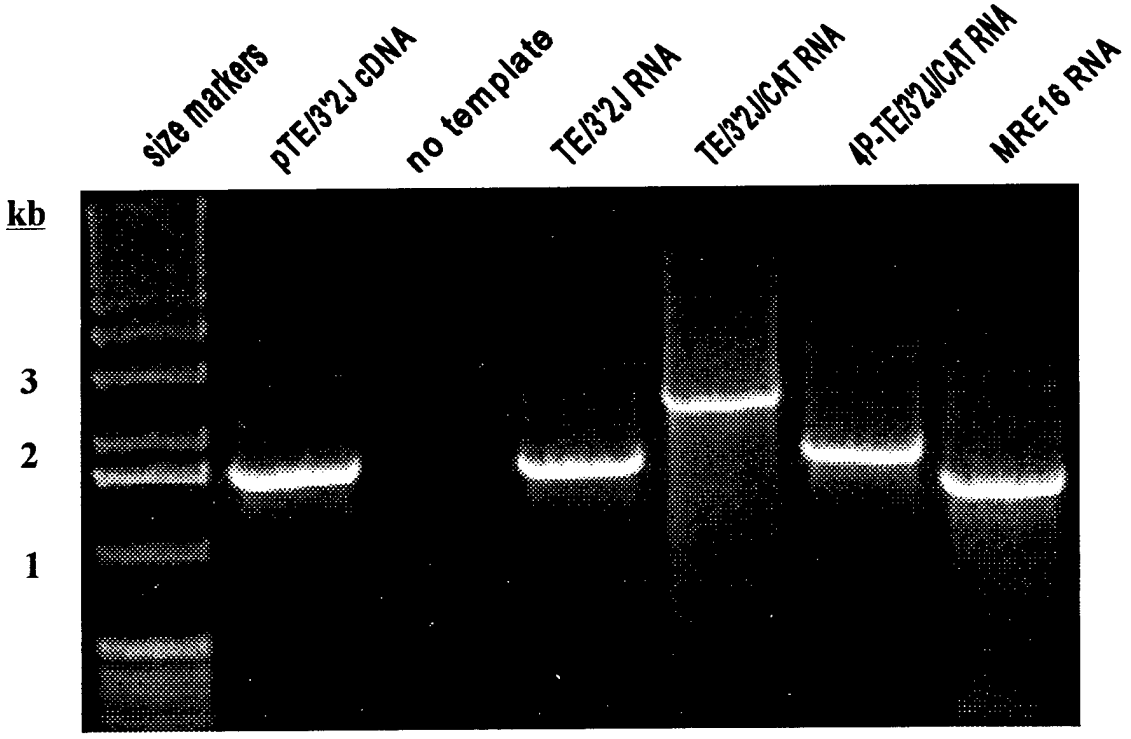

Figure 2.6. Comparative RT/PCR Analysis of Viral 3'-Genome Termini. 1% agarose/ethidium bromide gel of cDNAs produced by RT/PCR using primers flanking the viral 2nd subgenomic promoter and 3'NCR. The pTE/3'2J cDNA band in lane 2 depicts the expected band size for a dsSIN without insert; lane 3 was a negative RT/PCR control; lanes 4-7 contained the RT/PCR products of viral RNAs isolated from C6/36 cells infected with the indicated virus. The cDNA size difference between TE/3'2J (lane 4) and TE/3'2J/CAT (lane 5) was as expected. The cDNA from passaged 4P-TE/3'2J/CAT (lane 6) appeared to be about 500 bp smaller than expected, suggesting that a deletion had occurred in the amplified region during mosquito passage. MRE16 cDNA (lane 7) was slightly smaller than that of TE/3'2J due to its lack of a 2ndSGP and MCS.

The RT/PCR amplified 4P-TE/3'2J/CAT cDNA was cloned and sequenced both to identify the deleted genome segment and to determine if nucleotide substitutions in the 3'NCR had resulted from selective passage. The sequence data confirmed that a 600 nucleotide deletion had occurred: 127 bases of the second subgenomic promoter (2nd SGP) and the adjacent 473 bases of the CAT gene. Additionally, sequence analysis of all but the 3'-terminal 55 nucleotides of the viral 3'NCR revealed no changes from unpassaged TE/3'2J/CAT (Figure 2.7).

```
          >2nd SGP
TE/3'2J:  CGGGCCCAGGTAGACAATATTACACCTGTCCTACTGGCATTGAGAACTTTTGCCCAGAGCAAAAGAGCATTCCAA
p3'NCR:   ************************************----------------------------------

GCCATCAGAGGGGAAATAAAGCATCTCTACGGTGGTCCTAAATAGTCAGCATAGTACATTTCATCTGACTAATACTACAACACC
---------------------------------DELETION-----------------------------------------
                                          >3'NCR
ACCACCT[CAT(694nt)]CTAGACCATGGATCCTAGACGCTACGCCCCAATGATCCGACCAGCAAAACTCGATGTACTTCCGA
---------------**************************************************************

GGAACTGATGTGCATAATGCATCAGGCTGGTACATTAGATCCCCGCTTACCGCGGGCAATATAGCAACACTAAAAACTCGATGT
**********************************************************************************

ACTTCCGAGGAAGCGCAGTGCATAATGCTGCGCAGTGTTGCCACATAACCACTATATTAACCATTTATCTAGCGGACGCCAAAA
**********************************************************************************

ACTCAATGTATTTCTGAGGAAGCGTGGTGCATAATGCCACGCAGCGTCTGCATAACTTTTATTATTTCTTTTATTAATCAACAA
*********************************************

AATTTTGTTTTTAACATTTCA-polyA
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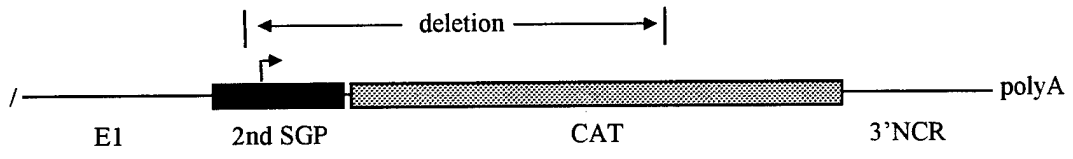

Figure 2.7. Nucleotide Sequence of 4P-TE/3'2J/CAT 3' Genome Terminus. Unpassaged TE/3'2J/CAT sequence is listed above the solid line in the upper figure. Asterisks denote nucleotide identity of p3'NCR; dashes indicate missing sequence. The lower diagram illustrates the deletion from 4P-TE/3'2J/CAT.

Dissemination of 4P-TE/3'2J/CAT Compared to TE/3'2JΔ2SGP

The data suggested that the loss of the 2nd SGP during selective passage could have contributed to the improved *in vitro* and *in vivo* growth and dissemination characteristics of 4P-TE/3'2J/CAT virus. To test this possibility, a control virus (TE/3'2JΔ2SGP) was engineered from pTE/3'2J by deletion of the 2nd SGP, and the deletion was confirmed by RT/PCR analysis of RNA isolated from the C6/36 cells used for virus amplification prior to blood feeding (Figure 2.8). *Ae. aegypti* mosquitoes were infected by blood meal with TE/3'2J, TE/3'2JΔ2SGP, and 4P-TE/3'2J/CAT viruses, and dissemination rates were determined. The head squash/IFA data following 14 days extrinsic incubation suggested that the loss of the 2nd SGP from the 4P-TE/3'2J/CAT virus genome was not entirely responsible for the altered virus phenotype (Table 2.6). Eight out of 42 mosquitoes (19.1%) infected with TE/3'2JΔ2SGP developed disseminated infections. Only 2 of 42 (4.8%) mosquitoes infected with TE/3'2J and 11 of 32 (34.4%) infected with 4P-TE/3'2J/CAT developed disseminated infections. The latter two dissemination rates did not differ significantly from those obtained in previous studies with these two viruses (Table 2.3). The dissemination rate of TE/3'2JΔ2SGP virus did not differ significantly from those observed with either TE/3'2J or 4P-TE/3'2J/CAT viruses. However, dissemination rates of the latter two viruses did differ significantly ($p < 0.01$). Thus, the 2nd SGP deletion from the 4P-

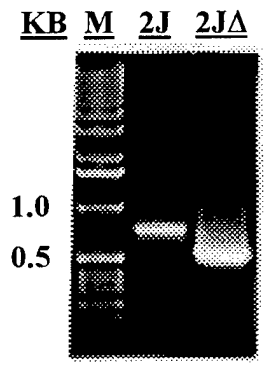

Figure 2.8. Confirmation of TE/3'2JΔ2SGP Virus Deletion. Agarose gel migration of RT/PCR cDNAs from TE/3'2J (2J) and TE/3'2JΔ2SGP (2JΔ) produced with primers flanking the 2ndSGP demonstrate reduced size of 2JΔ. Size markers (M).

TE/3'2J/CAT genome was probably not solely responsible for the infectious phenotype of the virus. At best, the data is suggestive of a partial effect on dissemination rate.

Table 2.6. Dissemination Rate of 4P-TE/3'2J/CAT vs. TE/3'2JΔ2SGP Virus in *Ae. aegypti* Mosquitoes

Virus	Blood Meal Titer (Log_{10} $TCID_{50}$/ml)	Head Squash/IFA Results		
		# Positive	Total	% Positive
TE/3'2J	7.8	2	42	4.8%
TE/3'2JΔ2SGP	8.2	8	42	19.1%
4P-TE/3'2J/CAT	7.8	11	32	34.4%

Discussion

The dsSIN expression vector, TE/3'2J, was originally engineered from the pTE12 full-length infectious clone of SIN, a construct in which the wild type (HRsp) envelope glycoprotein genes had been replaced by those of a strain selected for neurovirulence in mice (Lustig *et al.* 1988). The TE/3'2J virus, whether carrying the bacterial CAT gene downstream of its 2nd SGP (TE/3'2J/CAT) or not, was poorly suited for *per os* infection of *Ae. aegypti* mosquitoes. It produced disseminated infection only about 7.5% of the time compared to a nearly 40% rate for AR339 (Table 2.3). This relatively low level of infectivity was observed in four species of mosquitoes tested (Table 2.4). IFA analyses of orally infected *Ae. aegypti* showed that the virus rarely established a stable infection in the midgut epithelium and almost never escaped the midgut to infect other mosquito organs (Figures 2.2 and 2.4).

Selection for increased midgut infectivity and dissemination was successfully accomplished by *per os* serial passage of TE/3'2J/CAT in *Ae. aegypti* mosquitoes. Pooled data from six experiments testing the virus recovered after four serial passages in

mosquitoes (4P-TE/3'2J/CAT) demonstrated an 8-fold increase in dissemination rate over the unpassaged virus. Also, analysis of the kinetics of *Ae. aegypti* infection with 4P-TE/3'2J/CAT by IFA staining of dissected mosquito organs over a 21 day time course showed that the passaged virus exhibited an enhanced capacity to sustain an infection of the midgut epithelium, infect adjacent cells in the midgut, and disseminate to other mosquito organs (Figures 2.2 and 2.4). Interestingly, the altered viral phenotype resulting from selection was limited by host species (Table 2.4), suggesting that the selected changes in the virion were involved in host-specific interactions that take place in the mosquito midgut. Additionally, 4P-TE/3'2J/CAT replicated more efficiently in mosquito (C6/36) cells than unpassaged virus (with or without CAT) (Figure 2.1). The results in mammalian cells (BHK-21 or Vero) differed, again suggesting a host-specific component to the viral phenotype selected by serial passage.

Three general mechanisms for enhanced, host-specific infectivity can be envisioned: 1) Alterations in the structure of viral envelope glycoproteins that increase the specificity of interactions with receptors located in the epithelial cell membrane; 2) changes in the viral non-structural (replicase) proteins that improve interactions with host replication factors; or 3) changes in the noncoding regions of the viral genome (e.g., promoters) that alter interactions with the viral replicase complex acting in association with host cell factors. The experiments reported here only examined the first possibility in detail; some inferences can be made regarding the third.

Nucleotide sequence analysis of three separately cloned PE2 and 6K/E1 glycoprotein gene segments from 4P-TE/3'2J/CAT revealed five consensus changes resulting from selective passage (Figure 2.5 and Table 2.5); no nucleotide changes were

detected in the 3'NCR. Only three of the nucleotide changes would produce amino acid substitutions in the final protein products. The leucine to isoleucine substitution in the E2 glycoprotein is not likely to bring about a change in virus tropism; the conservative nature of the substitution and its location outside the receptor-binding domain of E2 (Strauss and Strauss, 1994) argue against such an effect. However, the two substitutions in the E1 glycoprotein may play more significant roles in virus tropism, E2-E1 dimerization, or virus assembly. The threonine to alanine substitution lies in the protein's ectodomain, but not within any currently recognized functional motif. However, the nonconservative nature of this change, from hydrophilic to hydrophobic, could have a major impact on protein folding, indirectly contributing to enhanced function. The methionine to valine substitution appears in the putative membrane spanning region of the E1 glycoprotein, and both amino acids are hydrophobic; little change in function would be expected from this amino acid substitution. Experiments using site-directed mutagenesis of pTE/3'2J will be necessary to fully explore the roles played by these amino acid substitutions in enhancing dsSIN midgut infectivity.

The 2nd SGP/CAT deletion in 4P-TE/3'2J/CAT did not appear to have a major impact on dissemination in *Ae. aegypti* (Table 2.6). One might assume that a virus containing one less promoter than normal would be more efficient at recruiting host transcription or translation co-factors, especially if their concentrations in the cell were limiting. The data suggest that the infectious characteristics of 4P-TE/3'2J/CAT virus in *Ae. aegypti* are only partly the result of the 2nd SGP deletion. Thus, other genomic changes resulting from selective passage are likely to be responsible for increased virus infectivity and dissemination following *per os* infection.

This study did not explore selected alterations in the non-structural proteins of 4P-TE/3'2J/CAT or in noncoding regions other than the 3'NCR. A single amino acid change in the nsP2 protein of SIN is sufficient to alter the viral phenotype from cytopathic to persistent in BHK cells (Dryga *et al.* 1997). Synergistic effects between SIN 5'NCR, nsP1, and E2 genetic changes resulting in increased neuroinvasiveness in weanling mice have also been reported (Dubuisson *et al.* 1997). These two examples point out that further examination of the 4P-TE/3'2J/CAT genome is necessary for a full understanding of the determinants of its oral infectivity in *Ae. aegypti* mosquitoes.

CHAPTER 3

CHARACTERIZATION OF THE MRE16 SINDBIS VIRUS STRAIN

Introduction

Studies were conducted to evaluate possible genetic determinants of SIN oral infectivity and midgut tropism in mosquitoes. As noted previously, TE/3'2J virus, engineered from the Egyptian AR339 and NSV strains (Lustig *et al.* 1988; Rice *et al.* 1987), is restricted in its ability to productively infect *Ae. aegypti* mosquitoes when administered orally. In contrast, a SIN strain (MRE16), isolated from mosquitoes in Malaysia, was found to be highly infectious in *Ae. aegypti* mosquitoes (Seabaugh *et al.* 1998). Studies were conducted to quantify the infectivity of MRE16 in several mosquito species and elucidate the genetic determinants of the infectious phenotype. MRE16's structural protein-encoding genes were cloned and sequenced to evaluate the differences from AR339 and to provide the sequence information necessary to test the hypothesis that viral structural proteins determine mosquito oral infectivity (see Chapter 4). The sequence data also provided the means to determine the phylogenetic relationship of MRE16 to other geographic isolates of SIN.

Materials and Methods

Virus and Cells

The SIN MRE16 strain was originally isolated from a pool of *Cx. tritaeniorhynchus* mosquitoes collected in Malaysia in the late 1960s (Pudney *et al.* 1979). The virus was isolated in *Ae. psuedoscutellaris* (AP61) cells (Varma *et al.* 1974) and passaged exclusively in either AP61 or C6/36 cells. Cells were maintained in L-15 medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml

streptomycin at 28°C. MRE16 titrations were performed as described in Chapter 2 (*Virus Titrations*, page 28).

MRE16 in vitro Replication

One-step growth curves for MRE16 in C6/36, BHK-21, and Vero cell lines were determined as described in Chapter 2 (*Comparative in vitro Virus Growth Rates*, page 29) and compared to those of the TE/3'2J virus. The results represent the combined data from two separate experiments in each cell type.

MRE16 Dissemination Rates

MRE16 dissemination rates following blood meal infection of *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus*, and *Cx. pipiens* mosquitoes were measured by head squash and SIN-specific IFA as described in Chapter 2 (*Comparative Virus Dissemination Rates*, page 32).

MRE16 Dissemination Kinetics

Adult female *Ae. aegypti* mosquitoes were infected with MRE16 virus by infectious blood meal as described in Chapter 2 (*Mosquito Infection*, page 29). The blood meal titer was 8.0 log $TCID_{50}$/ml. On days 1, 3, 6, and 9 post-infection mosquito heads, midguts, and salivary glands were dissected, affixed to glass slides, and analyzed by IFA for SIN-specific E1 antigen using the anti-SIN MAb 30.11a as described in Chapter 2 (*IFA Analysis of Mosquito Tissues*, page 31). Each tissue sample was scored for the degree of fluorescence observed (0 to 5 scale) and scores averaged for each time point (see *Kinetics of Virus Dissemination Rates*, page 32).

cDNA Cloning

To produce viral RNA for subsequent investigations, C6/36 cell monolayers (previously tested for SIN contamination by IFA) in 25-cm^2 flasks were infected with MRE16 at an MOI of 0.01 and incubated at 28°C for 48-72 hours. Total RNA was extracted from the cells and genome sequences encompassing all of the MRE16 structural protein-coding regions and 3'NCR were converted to cDNA by RT/PCR and cloned as described in Chapter 2 (*Cloning and Sequencing of Viral cDNA Genome Segments*, page 33). Primers used for amplification of the genome segments are listed in Table 3.2. The cDNA products were cloned into the TA vector, pCR®2.1 (Invitrogen). Insert-containing plasmids were purified as previously described (see *Cloning and Sequencing of Viral cDNA Genome Segments*, page 33).

DNA Sequencing

Four overlapping segments of MRE16 cDNA were cloned and sequenced (Figure 3.4) using an ABI 377 DNA Sequencer (Perkin-Elmer) with Taq FS polymerase (Perkin-Elmer) as previously described (page 33). The ends of each cloned cDNA segment were sequenced using plasmid-specific primers complementary to SP6, T7, or M13 promoters. Subsequent sequencing reactions were performed in both directions using primers designed from previously determined MRE16 sequences.

Computer Analyses

DNA sequence analyses were aided by the use of SeqAid™ II, version 3.6, to align contiguous stretches of sequence data (Figure 3.5). Alignments of sequences from different viruses were accomplished using Clustal W™, version 1.6 software. Clustal W uses a progressive multiple alignment algorithm that employs the neighbor-joining method

and full dynamic programming for distance calculations (Thompson *et al.* 1994). For protein alignments (Figure 3.6), gap penalties were employed based on the BLOSUM30 series amino acid weight matrix. For phylogenetic analyses of nucleotide sequences (Figure 3.7), Clustal W produced unrooted trees with branch lengths proportional to estimated genetic divergence between virus strains. The output files from these calculations were analyzed with TreeView™ (Win16), version 1.3, for visualization of the resultant phylogenetic trees.

Results

MRE16 in vitro Replication

Replication of MRE16 and TE/3'2J viruses was compared in mosquito (C6/36), hamster (BHK-21), and monkey (Vero) cell lines, respectively (Figure 3.1). In mosquito cells, MRE16 titered to between 2.3 and 3.3 $\log_{10}$ $TCID_{50}$/ml at each sample period over the 13 hour time course. TE3/2J virus titer ranged from 2.8 $\log_{10}$ $TCID_{50}$/ml at 3 hours to 5.8 $\log_{10}$ $TCID_{50}$/ml at 13 hours post-infection. In Vero cells, the MRE16 titer declined from an initial 5.2 $\log_{10}$ $TCID_{50}$/ml at 3 hours to 3.8 $\log_{10}$ $TCID_{50}$/ml at 13 hours post-infection. In the same interval, TE/3'2J virus titer increased from 3.3 $\log_{10}$ $TCID_{50}$/ml to 6.6 $\log_{10}$ $TCID_{50}$/ml. In the BHK-21 cell line, MRE16 titered at 4.0 $\log_{10}$ $TCID_{50}$/ml initially and reached maximum titer of 5.0 $\log_{10}$ $TCID_{50}$/ml by 11 hours post-infection. In contrast, TE/3'2J titer increased from 4.0 $\log_{10}$ $TCID_{50}$/ml to 7.8 $\log_{10}$ $TCID_{50}$/ml over the same period.

Thus, both viruses replicated to highest titer in BHK-21 cells and lowest titer in C6/36 cells. However, there was a 1000-fold difference between MRE16 and TE/3'2J

virus titers at peak time points in each of the three cell types examined. These data suggest that a fundamental difference in replicative efficiency exists between the two viruses. Not surprisingly, TE/3'2J and MRE16 viruses used for mosquito blood meals [collected after 48 hours in C6/36 cells (MOI=0.01)] routinely yielded titers that differed by 2 to 3 $\log_{10}$ $TCID_{50}$/ml (data not shown).

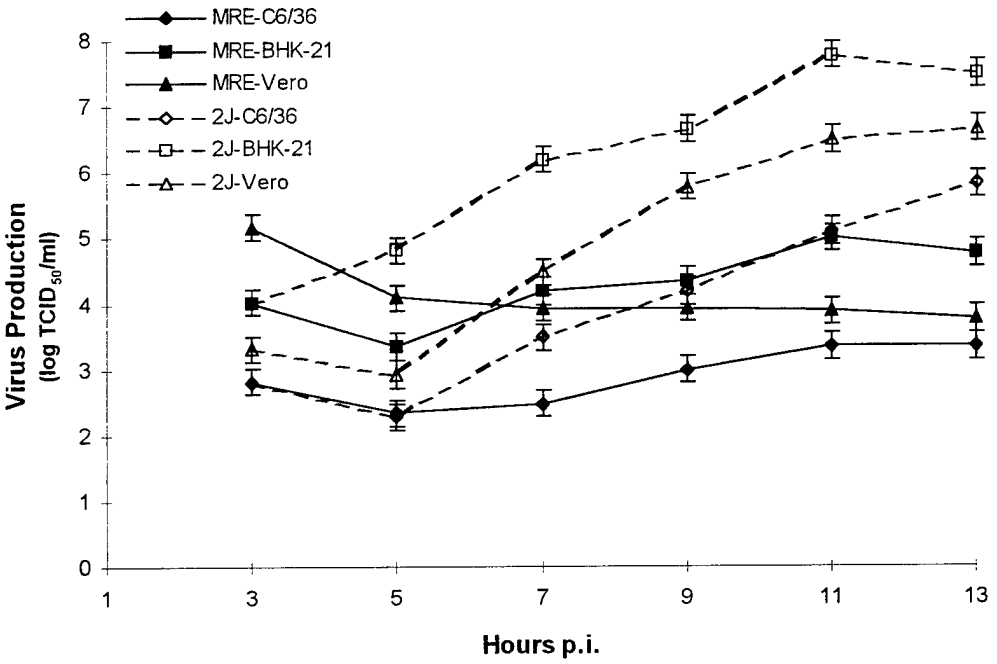

Figure 3.1. Comparative *in vitro* Growth Rates of MRE16 and TE/3'2J Viruses. One-step growth curves in C6/36, BHK-21, and Vero cell lines infected at an MOI of 5.0. The results represent combined data of two experiments. Error bars represent standard deviation.

MRE16 Dissemination Rates

The oral infection and dissemination rates of MRE16 in *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus*, and *Cx. pipiens* mosquitoes were determined by head squash/IFA following blood meal challenge of each mosquito species. Pooled results from four experiments in *Ae. aegypti* were compared to the results of single experiments with

three other mosquito species (Table 3.1). The data for TE/3'2J/CAT (from Tables 2.3 and 2.4) are included for comparison.

MRE16 produced disseminated infections in nearly 96% of the *Ae. aegypti* mosquitoes tested. Similarly, in *Ae. albopictus*, MRE16 infected and disseminated from the midguts of 100% of the challenged mosquitoes. In *Ae. triseriatus*, MRE16 infected and disseminated at the significantly lower rate of 34.78% ($p<0.05$). Finally, in *Cx. pipiens*, MRE16 did not produce any disseminated infections in the 13 mosquitoes tested. In contrast, AR339, TE/3'2J, and TE/3'2J/CAT virus dissemination rates in *Ae. aegypti* were 39.5%, 7.2%, and 7.5%, respectively (Table 2.3, page 40), all significantly lower than that of MRE16 ($p<0.01$). In *Ae. albopictus* and *Ae. triseriatus* mosquitoes TE/3'2J/CAT virus disseminated only 30% and 6.7% of the time, respectively, significantly less than MRE16 ($p<0.01$). Only dissemination rates in *Cx. pipiens* were similar between the two viruses (Table 3.1). It is especially noteworthy that these infection rates were obtained with MRE16 blood meal titers approximately 100-fold lower than any of the other viruses tested. TE/3'2J or TE/3'2J/CAT infections with virus diluted to MRE16-equivalent titer (~7.0 $\log_{10}$ $TCID_{50}$/ml) routinely yielded no detectable midgut infection or dissemination from the mosquito midgut to other tissues (data not shown).

Table 3.1. MRE16 Dissemination Rates in Selected Mosquito Species

Virus	Titer	*Ae. Aegypti*	*Ae. albopictus*	*Ae. triseriatus*	*Cx. pipiens*
TE/3'2J/CAT	9.0	7.5% (12/160)	30.0% (3/10)	6.7% (1/15)	7.1% (2/28)
MRE16	7.0	95.8% (113/118)	100% (8/8)	34.8% (8/23)	0% (0/13)

These data clearly demonstrate that MRE16 is more infectious orally in *Ae. aegypti* and *Ae. albopictus* mosquitoes than TE/3'2J (with or without CAT). The data

also support the concept that host-specific interactions play a role in mosquito infectivity (discussed in Chapter 2).

MRE16 Dissemination Kinetics and Tissue Tropism in Ae. aegypti Mosquitoes

Following blood meal infection with MRE16, mosquito organs (midguts, salivary glands, and neural tissues) were assayed by SIN-specific IFA over a 9 day time course. SIN-specific fluorescence was detected in mosquito midguts above background levels one day after ingestion of infectious blood meals (Figure 3.2). By 3 days post-infection SIN

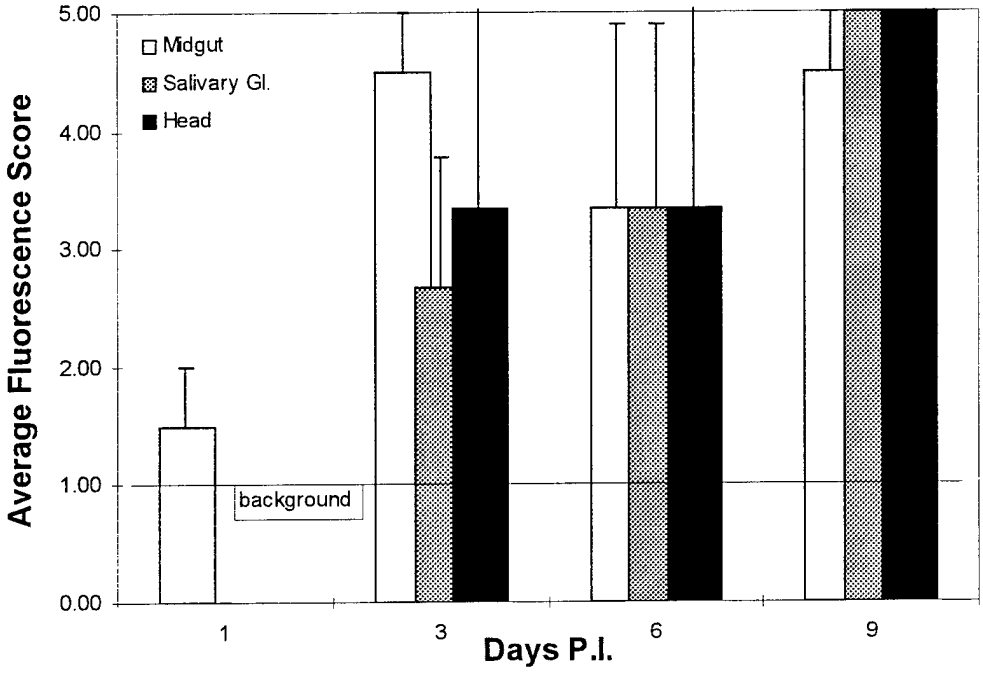

Figure 3.2. MRE16 Dissemination Profile in Bloodfed *Ae. aegypti* Mosquitoes. Averaged relative levels of SIN E1-specific fluorescence in dissected mosquito tissues following oral infection with MRE16 virus. Error bars indicate standard error.

antigen was detectable at relatively high levels in both salivary glands and neural tissues. By 9 days post-infection most of the midguts, salivary glands, and neural tissues examined displayed nearly maximum fluorescence levels suggesting that barriers to midgut infection

and/or dissemination were virtually nonexistent. This is in contrast to the low levels of SIN antigen detected in these mosquitoes following oral infection with TE/3'2J/CAT virus (Figure 2.2, page 44).

MRE16-infected mosquito midguts dissected on days 3 and 9 post-infection showed characteristic, although apparently accelerated, patterns of infection. At 3 days post-infection much of the epithelial cell layer lining the midgut and some of the respiratory tracheoles appeared to be infected (Figure 3.3A). This is in contrast to the relatively small foci of epithelial infection seen with TE/3'2J/CAT virus (Figure 2.4A, page 46) at the same time point. By day 9, the MRE16 infection involved much of the midgut epithelium and had disseminated to overlying muscles, nerves, and tracheoles (Figure 3.3B). Salivary glands dissected from MRE16-infected mosquitoes 9 days post-infection displayed SIN antigen in all three lobes (Figure 3.3C). TE/3'2J/CAT virus never disseminated to the salivary glands (Figures 2.2 and 2.4E), and 4P-TE/3'2J/CAT only infected the distal lateral lobes (Figure 2.4F). These results illustrate the robustness of MRE16 invasiveness in *Ae. aegypti* mosquitoes following *per os* infection.

Nucleotide Sequencing Strategy

To provide flexibility in future genetic manipulations of SIN expression vectors, overlapping regions of the MRE16 genome corresponding to its 26S RNA were cloned and sequenced. The locations of cDNA clones, relative to a generic SIN 26S RNA map, are illustrated in Figure 3.4. The primers used for amplification of each cDNA are listed in Table 3.2. Initially, primers were designed for RT/PCR amplification of the E1 gene from conserved flanking regions of the SIN sequence (Strauss *et al.* 1984). Primers 5 and 6

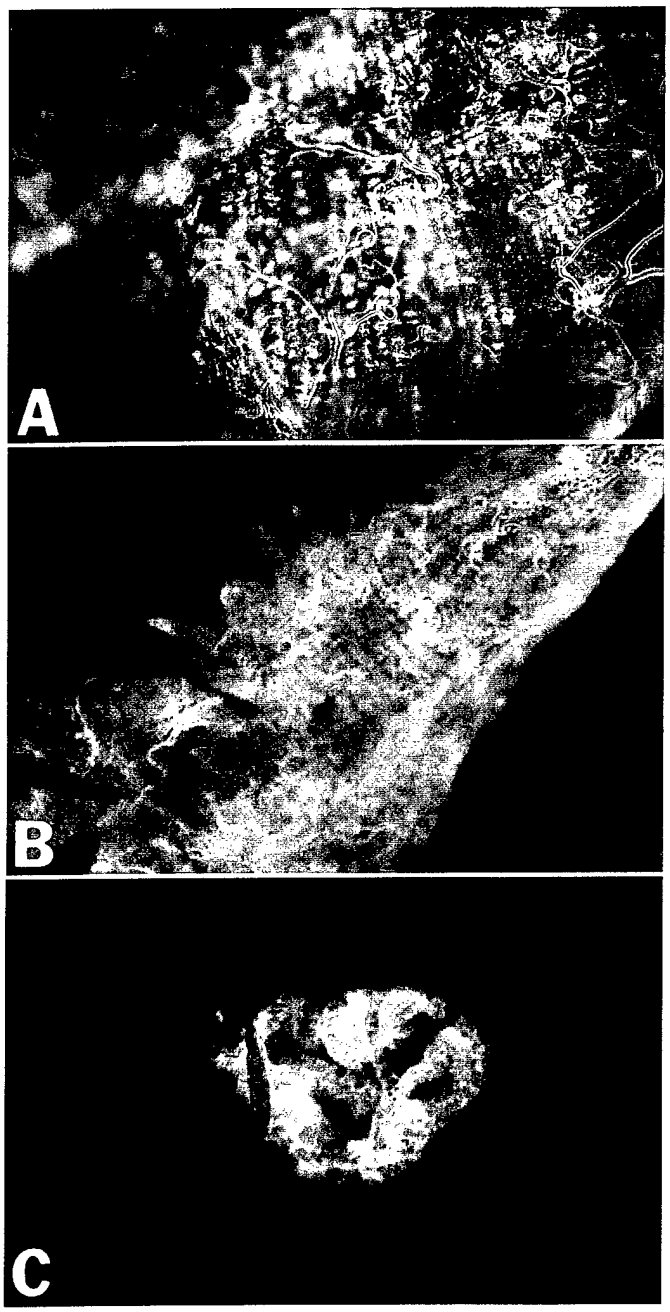

Figure 3.3. IFA Analysis of *Ae. aegypti* Organs Following Oral Infection with MRE16 Virus. Representative organs from mosquitoes infected by blood meal are shown. A) mosquito midgut at 3 days post-infection; B) midgut at 9 days post-infection; C) salivary glands from the same mosquito as B 9 days post-infection. Magnification: (A) 50X; (B-C) 25X.

corresponded to nucleotides 10007-10037 and 11405-11425 (SIN numbering), respectively. After the resulting cDNA was cloned (pME1) and sequenced, primers were designed to amplify and clone PE2 and the 3'NCR (pME2 and p3'NCR). Primer 3 was designed from SIN sequence corresponding to nucleotides 8420-8438, and primer 4 from MRE16 sequence at position 2650-2668 (Figure 3.5 numbering). Primer 7 corresponded to MRE16 nucleotides 3671-3691, while primer 8 was an oligo dT primer that included the two 3'-terminal nucleotides of the SIN sequence. The capsid-encoding region (pMCAP) was amplified with primer 1, corresponding to nucleotides 7577-7597 (SIN numbering), and primer 2, designed from MRE16 position 1937-1957.

Each of the four cDNA clones were sequenced bi-directionally with the exception of the 3'-most 62 nucleotides of p3'NCR, where sequence data were obtained in only the forward direction. Approximately 300-600 nucleotides of sequence data were determined at a time. Primers for succeeding reactions were designed from previously determined sequence with the aid of Oligo™ version 4.0 computer software, and chosen to provide a minimum of 50 nucleotides of overlap. Where cDNA clones overlapped, four separate sets of sequence data were available for comparison. A single nucleotide (position 988 within the E3 coding region) remained unresolved due to a difference in the overlapping sequences of the pMCAP and pME2 clones. For pMCAP, the base resolved to an adenosine (AAU) resulting in an encoded asparagine residue, while the pME2 sequence revealed a guanosine (GAU) resulting in an aspartic acid at this position.

Table 3.2. Primers Used to Amplify MRE16 Genome Segments for Cloning

Primer	Sequence (5'-3')	Position[1]	Source
1	GCATCTCTACGGTGGTCCTAA	21	conserved SIN
2	TTGGCTCTGGTTTCTCTCCTA	1937	pME2
3	GGAAGGGACAGAAGAGTGG	828	conserved SIN
4	GCGTTCCACAACACTTCAC	2668	pME1
5	GCTCCTGCTGCCTGCCTTTTT	2414	conserved SIN
6	GTACATCGAGTTTTGCTGGTC	3833	conserved SIN
7	CAGCCGTCTCCCAAACATCAT	3671	pME1
8	TTTTTTTTTTTTTTTTTTTTGA	4111	conserved SIN + poly(A)

[1]Primer 5'-end locations are listed with reference to Figure 3.5.

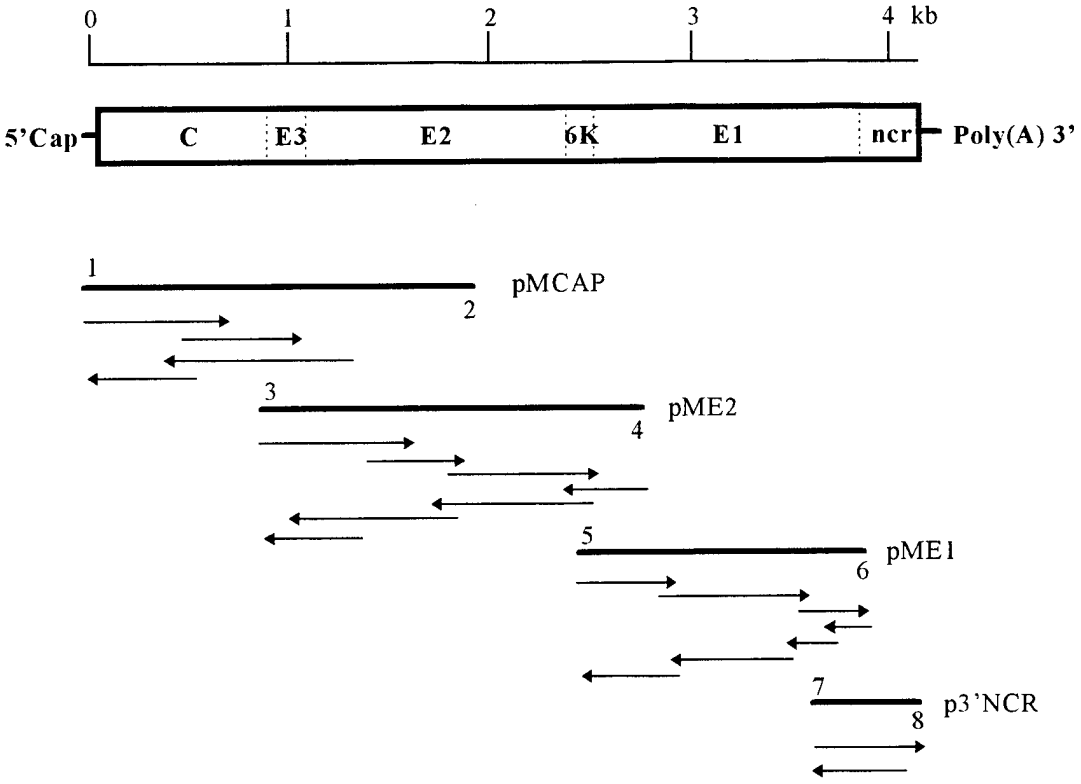

Figure 3.4. Schematic of Sequencing Strategy for MRE16 26S RNA. The organization of the 26S RNA is depicted with a scale bar showing kilobase intervals; C - capsid, E2 and E1 - envelope glycoproteins, E3 and 6K - unpackaged polypeptides, ncr - 3' noncoding region. Thick bars represent the four overlapping cDNA clones of segments of the viral 26S RNA; pMCAP, pME2, pME1, and p3'NCR. Numbers at either end of each bar denote the primers (listed in Table 3.2) used for RT/PCR amplification of each clone. Arrows represent the direction and length of sequences obtained from each of the clones.

Nucleotide and Deduced Amino Acid Sequences

The nucleotide and deduced amino acid sequences of the MRE16 26S RNA and structural polyprotein are presented in Figure 3.5. An open reading frame, beginning with an AUG codon at the 5' end of the capsid gene and ending with an opal termination codon at the 3' end of the E1 glycoprotein gene, was maintained throughout the coding sequence of the RNA. The total number of nucleotides was found to be 4,111, numbered from the 5' end of the 26S RNA to the 3' end of the genome, excluding the poly(A) tail. The lengths of both coding and noncoding regions of the 26S RNAs of AR339 (HRsp) (Strauss *et al.* 1984), Aura virus (Rumenapf *et al.* 1995), EEE (Chang and Trent, 1987), VEE (Kinney *et al.* 1986), and SFV (Garoff *et al.* 1980a,b) are compared in Table 3.3. The AR339 and the MRE16 26S RNA were of the same size in all regions except the nonconserved 5' end of the capsid-encoding segment, where MRE16 contained two codon insertions.

Table 3.3. Comparison of Alphavirus 26S RNAs[1,2]

Virus	26S leader	Structural Genes					3'NCR	Total
		C	E3	E2	6K	E1		
AR339 (HRsp)	48	792	192	1269	165	1320	319	4105
MRE16	48	798	192	1269	165	1320	319	4111
Aura	53	801	183	1272	162	1317	462	4250
EEE	66	780	189	1260	171	1326	358	4150
VEE	38	825	177	1269	165	1329	118	3921
SFV	41	801	198	1266	180	1317	261	4064

[1]Size, in nucleotides, of each of the indicated RNA regions. [2]Adapted from Strauss and Strauss (1994).

The sequence differences between AR339 and MRE16 are shown in Figure 3.5 and 3.6 and summarized in Table 3.4. There were 994 nucleotide differences and 171

amino acid differences between the two viruses for divergence rates of 24.2% and 13.8%, respectively. Of the 925 nucleotide differences in coding regions, 73.8% occurred in the third nucleotide position of the codon and 76.2% were silent mutations. Of the amino acid differences, 48.0% could be considered conservative.

At the nucleotide level, the noncoding MRE16 26S RNA leader sequence was more different from AR339 (43.8%) than the average 24.2% divergence, while the 3'NCR was more conserved (only 16.6% divergence). In the coding regions, nucleotide divergence rates averaged 25.8%. Only the E3-encoding region exhibited a greater degree of divergence (37.0%) than average.

Table 3.4. Differences Between AR339 and MRE16 Virus Sequences

Region	Nucleotides			Amino Acids		
	total	%	% total 3rd nt.[1]	total	%	% total conservative[2]
26S leader	21	43.8	-	-	-	-
Capsid[3]	179	22.4	74.9	32	12.2	46.9
E3	71	37.0	57.7	19	29.7	21.1
E2	317	25.0	75.1	62	14.7	56.5
6K	33	20.0	66.7	8	14.5	50.0
E1	323	24.5	77.1	50	11.4	48.0
3'NCR[4]	53	16.6	-	-	-	-
Total	994	24.2	73.8	171	13.8	48.0

[1]Nucleotide differences occurring at codon third positions in translatable regions. [2]Amino acid differences considered conservative: R=K, S=T, D=E, Q=N, V=L=I=M, A=G=V, Y=F. [3]Insertions in the nonconserved 5' end of the capsid-encoding sequence of MRE16 are not included in calculations. [4]Gaps introduced in the 3'NCR of either virus to align sequences are included in calculations.

At the amino acid level, concentrations of divergence occurred in the N-terminal one-third of the capsid protein, the E3 protein, and in the membrane-spanning domain of the E2 glycoprotein (Figure 3.6). In contrast, the C-terminal two-thirds of the capsid

protein was highly conserved; its sequence had diverged only 7% from that of AR339, and 8 of the 12 altered amino acids were conservative substitutions. A putative ribosome binding domain (Wengler *et al.* 1992) in the capsid protein had been precisely conserved, as had the site of autocatalytic cleavage of capsid protein from the structural polyprotein molecule.

The five glycosylation sites within the PE2/6K/E1 polyprotein were functionally intact, and no additional NXT/S glycosylation signals had been introduced into the sequence. In the E3 polypeptide, 19 of the 64 amino acids differed from AR339 and only 4 of these were conservative substitutions. Sixty-two of 423 (14.7%) E2 amino acids were different from AR339; about 57% of these were conservative substitutions.

Three functional domains within the E2 sequence deserve further mention (Figure 3.6). First, residues 170-220 constitute a cell-receptor binding domain that is susceptible to neutralizing antibody (Strauss and Strauss, 1994). Within this region, MRE16 exhibited only four amino acid differences from AR339: R-172 to G, S-178 to T, I-197 to V, and T-213 to A. It should be noted that single amino acid changes in this domain have resulted in dramatic alterations in alphavirus virulence, tropism, and mosquito infectivity (Kerr *et al.* 1993; Tucker and Griffen, 1991; Woodward *et al.* 1991). Second, the transmembrane domain of the MRE16 E2 glycoprotein had significantly diverged from that of AR339; thirteen of 26 residues were different. Most of the substitutions were conservative, however, and the number of hydrophobic residues was unchanged. Third, the C-terminal 33 amino acids of E2 comprise the cytoplasmic domain of the glycoprotein, believed to interact with the nucleocapsid during assembly (Strauss and Strauss, 1994). Twelve of the 33 residues in this domain are invariant among alphaviruses, suggesting a

conserved structure that is important to the interaction. MRE16 conformed to this model; its sequence differed from AR339 at 7 locations in this domain, all outside of the conserved residues.

The 55 amino acid 6K protein of MRE16 differed by only 8 amino acids (14.5%) from AR339, half of which were conservative. The 6K protein has been found to be important for alphavirus assembly (Liljestrom *et al.* 1991) and is incorporated into virions in small amounts (Gaedigk-Nitschko and Schlesinger, 1990). Its exact role is unknown.

The E1 glycoprotein was the most conserved at the amino acid level of the proteins encoded by the MRE16 26S RNA. There were 50 amino acid differences from AR339 (11.4% divergence) and 48% of them were conservative substitutions. A putative fusion domain (Strauss and Strauss, 1994) within E1 was exactly conserved. Although the transmembrane domain of E1 has not been precisely mapped, a presumed 28 residue membrane-spanning segment of the MRE16 E1 protein (Figure 3.6) contained only 4 amino acid differences from AR339. All of these were conservative, hydrophobic substitutions.

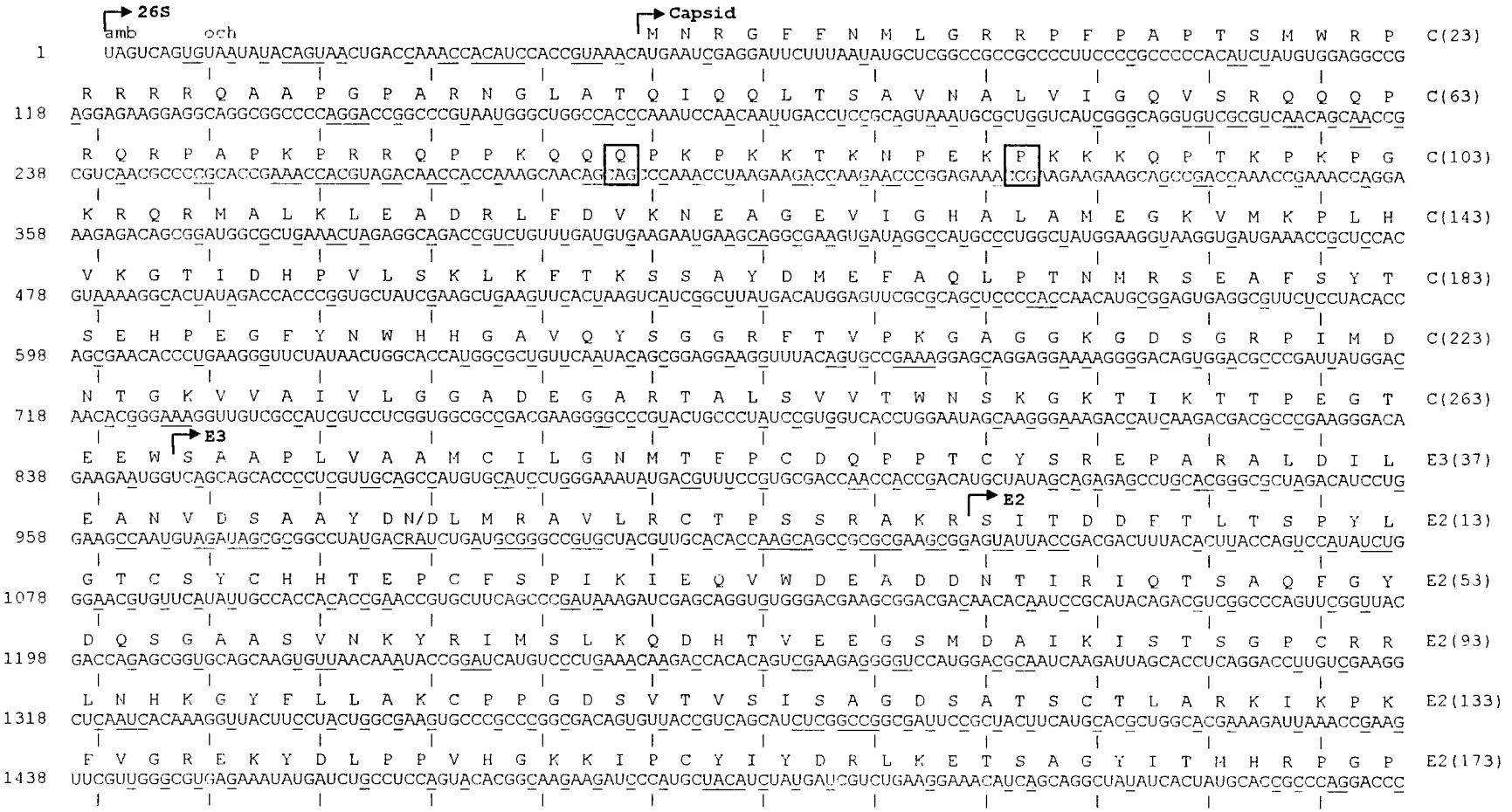

Figure 3.5. Nucleotide Sequence of the MRE16 26S RNA and Deduced Amino Acid Sequence of the Encoded Structural Polyprotein. Nucleotides are numbered (left margin) from the 5' end. Amino acids are indicated by single letter code and numbered (right margin) from the start of each final protein product. Nucleotides different from those in AR339 (HRsp) are underlined. Two triplet nucleotide insertions within the capsid coding region are boxed. An unresolved purine at position 988 is indicated by an R and the resultant two possible amino acids as N/D. Termination codons are labeled amb, och, or opa. The three 40 nt repeat elements in the 3' NCR are boxed.

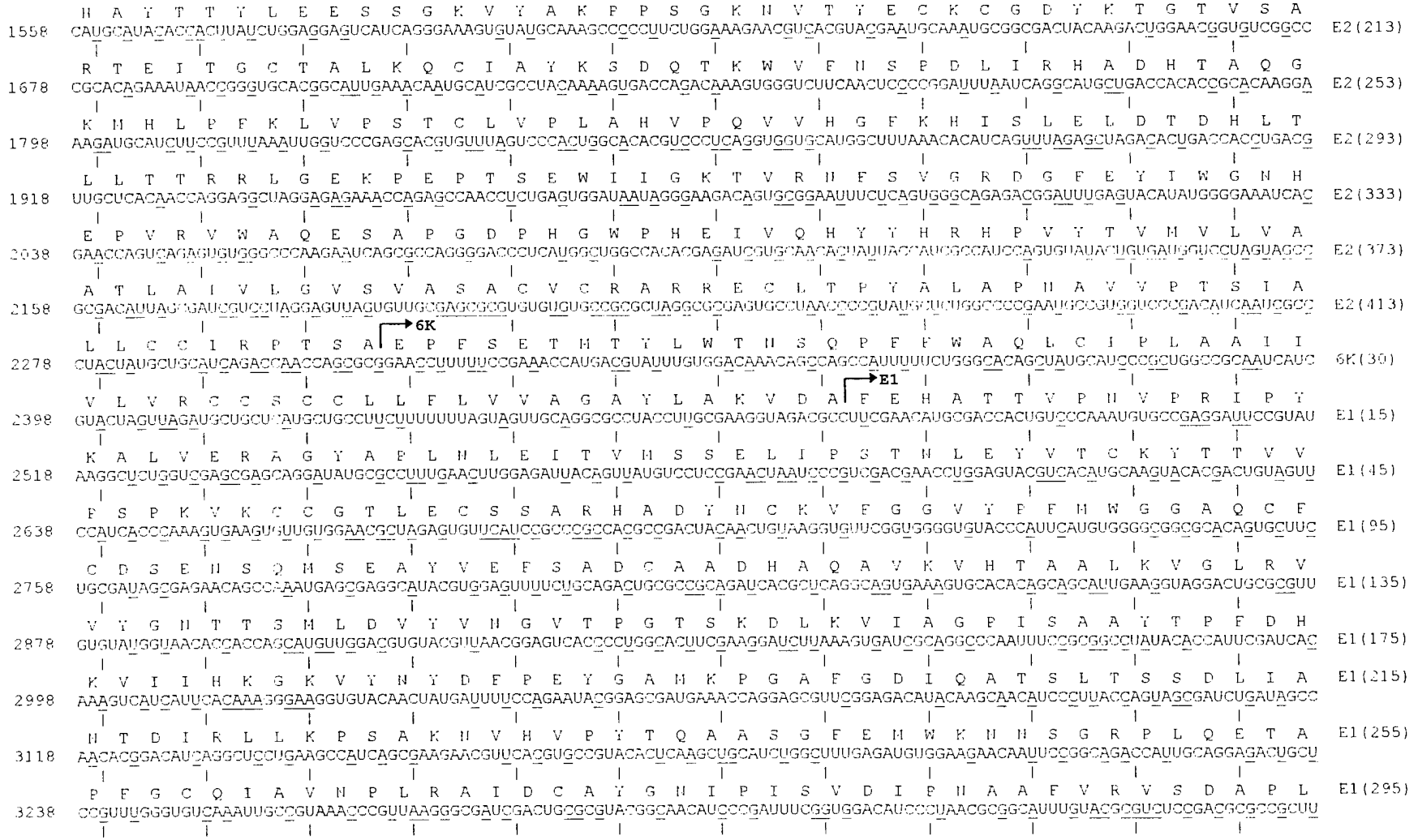

Figure 3.5. (Continued).

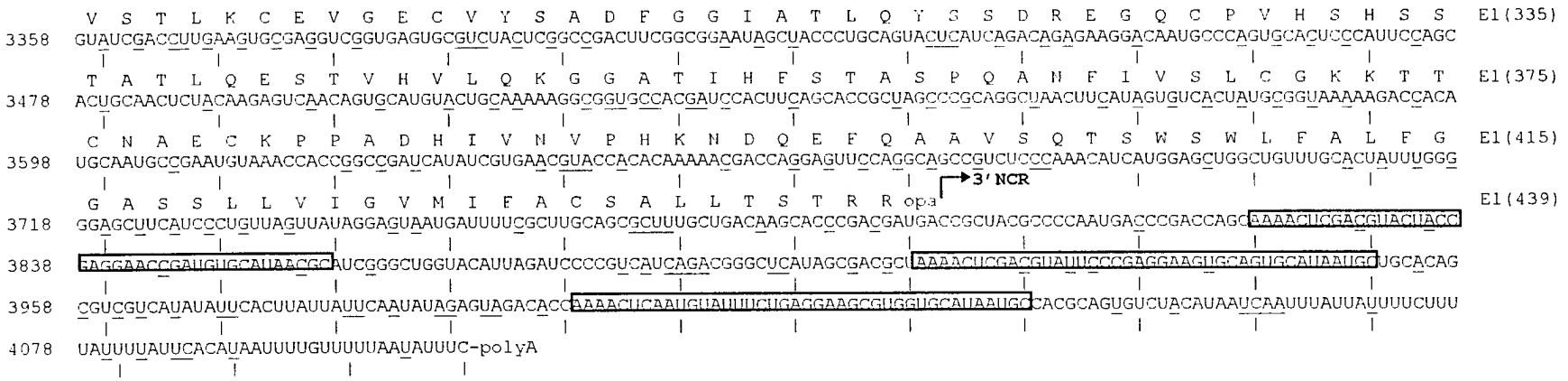

Figure 3.5. (Continued).

Figure 3.6. Comparison of Deduced Structural Polyprotein Sequences of MRE16 and AR339 SIN Strains. The amino-termini of each final protein product are labeled accordingly. Amino acid differences between the two viruses are highlighted in bold print. Asterisks denote glycosylation sites. Important functional domains are indicated by overlying bars and are labeled.

```
        E1>                                                                                                  E1 fusion
MRE16 A FEHATTVPNVPRIPYKALVERAGYAPLNLEITVMSSELIPSTNLEYVTCKYTTVVPSPKVKCCGTLECSSARHADYNCKVFGGVYPFM (E1)88
AR339 A YEHATTVPNVPQIPYKALVERAGYAPLNLEITVMSSEVLPSTNQEYITCKFTTVVPSPKIKCCGSLECQPAAHADYTCKVFGGVYPFM
       domain                                                *
MRE16 WGGAQCFCDSENSQMSEAYVEFSADCAADHAQAVKVHTAALKVGLRVVYGNTTSMLDVYVNGVTPGTSKDLKVIAGPISAAYTPFDHKVI (E1)178
AR339 WGGAQCFCDSENSQMSEAYVELSADCASDHAQAIKVHTAAMKVGLRIVYGNTTSFLDVYVNGVTPGTSKDLKVIAGPISASFTPFDHKVV
                                                                 *
MRE16 IHKGKVYNYDFPEYGAMKPGAFGDIQATSLTSSDLIANTDIRLLKPSAKNVHVPYTQAASGFEMWKNNSGRPLQETAPFGCQIAVNPLRA (E1)268
AR339 IHRGLVYNYDFPEYGAMKPGAFGDIQATSLTSKDLIASTDIRLLKPSAKNVHVPYTQASSGFEMWKNNSGRPLQETAPFGCKIAVNPLRA

MRE16 IDCAYGNIPISVDIPNAAFVRVSDAPLVSTLKCEVGECVYSADFGGIATLQYSSDREGQCPVHSHSSTATLQESTVHVLQKGGATIHFST (E1)358
AR339 VDCSYGNIPISIDIPNAAFIRTSDAPLVSTVKCEVSECTYSADFGGMATLQYVSDREGQCPVHSHSSTATLQESTVHVLEKGAVTVHFST
                                                          E1 transmembrane domain(?)
MRE16 ASPQANFIVSLCGKKTTCNAECKPPADHIVNVPHKNDQEFQAAVSQTSWSWLFALFGGASSLLVIGVMIFACSALLTSTRR          (E1)439
AR339 ASPQANFIVSLCGKKTTCNAECKPPADHIVSTPHKNDQEFQAAISKTSWSWLFALFGGASSLLIIGLMIFACSMMLTSTRR
```

Figure 3.6. (Continued).

Relationship of MRE16 to Other Sindbis Virus Isolates

The 3' NCR sequence data obtained from MRE16 was added to a phylogenetic analysis of SIN strains performed by Shirako *et al.* (1991) to illustrate the relationship of MRE16 to several other geographic isolates of SIN. The nucleotide sequences of the 3'NCRs of four African-European isolates (including AR339) and three Asian-Australian isolates (including MRE16) were aligned. The resulting distance matrix was used to construct an unrooted radial phylogenetic tree (Figure 3.7). The result confirmed the geographic divergence of the two SIN subgroups and revealed that the Malaysian isolate, MRE16, is more closely related to Australian and Indian strains than to the African-European strains of SIN.

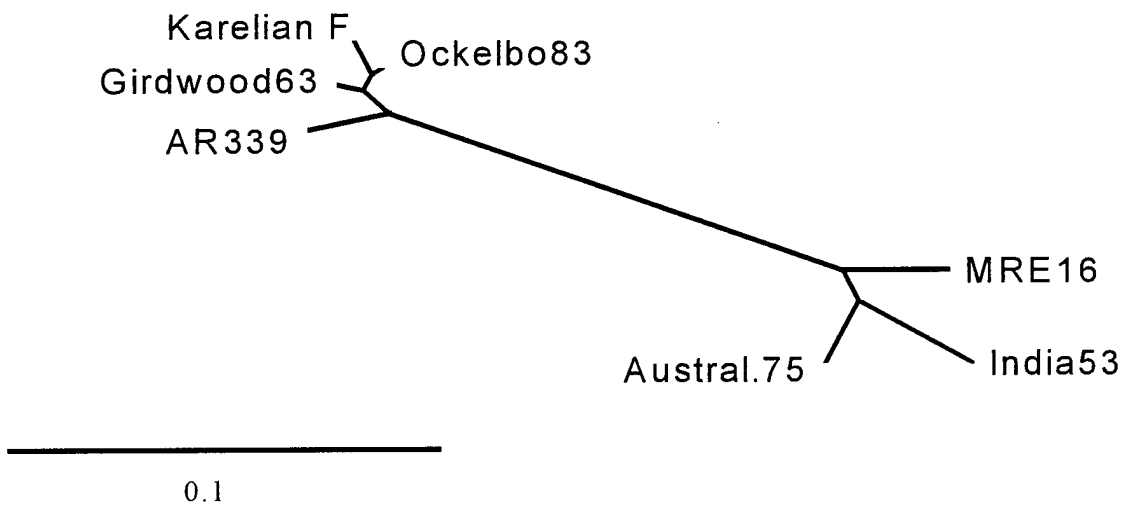

Figure 3.7. Radial Phylogenetic Tree of Selected SIN Strains. Branch lengths are proportional to estimated divergence rates based on nucleotide sequences of the 3'NCRs. Gaps introduced to align sequences were counted as mismatches. The scale bar represents approximately 10% divergence. The sequences of Ockelbo 83M107, Karelian fever, South African Girdwood, Indian A1036, and Australian MRM18520 strains are from Shirako *et al* (1991). The AR339 (HRsp) sequence is from Strauss *et al* (1984). The MRE16 sequence is from Figure 3.5.

Discussion

This work was undertaken as a prelude to re-engineering SIN-based expression systems for increased *per os* infectivity and midgut tissue tropism in selected mosquito species. The demonstrated genetic and phenotypic (e.g., oral infectivity) differences between MRE16 and the SIN prototype virus (from which SIN expression systems were derived) provide a starting point for identifying the viral determinants of mosquito-specificity and tissue tropism.

MRE16 administered in an artificial blood meal infected >95% of challenged *Ae. aegypti* and *Ae. albopictus* mosquitoes. In contrast, AR339 infected fewer than 40% and unpassaged SIN-based expression viruses infected fewer than 10% of challenged mosquitoes. IFA analyses of infected mosquito midguts demonstrated distinct differences in the patterns of infection of these viruses in the epithelial cell layer and in their rates of dissemination to other mosquito tissues. In preparation for testing the hypothesis that these variant phenotypes were determined by the viral structural proteins (e.g., envelope glycoproteins), the genetic differences between MRE16 and AR339 were determined. These sequence data provided the information needed for the construction of a chimeric SIN virus that possesses 26S RNA elements derived from MRE16 (see Chapter 4).

Based on nucleotide sequence data corresponding to its subgenomic (26S) RNA, MRE16 differs from the Egyptian SIN prototype, AR339, by approximately 24%. Comparisons of functionally significant regions of the genomes of these two viruses provided clues to their differences in mosquito infectivity.

The 26S noncoding regions function in viral replication, probably in association with host cellular proteins. Mutations in these sequences have been shown to result in

differences in virus production depending on the type of host cell used (Durbin *et al.* 1991; Kuhn *et al.* 1990, 1992). Though the sequence data reported here did not include the active domain of the 26S subgenomic promoter, a high degree of divergence was found in the noncoding leader sequence upstream of the structural polyprotein open reading frame. It is possible that this region plays a role in the recruitment of cellular factors to the promoter, which may differ depending on the species of the host.

In contrast, the 3'NCR was found to be relatively more conserved. Within this region, three 40 nucleotide repeat elements (Figure 3.5) exhibit an even greater degree of conservation among SIN strains (Shirako *et al.* 1991). The data suggest that this conserved region of the genome interacts with host proteins in a highly specific manner. Minor differences in sequence could potentially modulate virus replication in different host species or in various tissues within a given host organism.

The N-terminal domain of the capsid protein is not conserved among alphaviruses and is thought to protrude into the interior of the nucleocapsid, where it interacts electrostatically with the viral RNA (Strauss and Strauss, 1994). The amino acid substitutions in this domain of MRE16 would not appear to result in any change in this function. Coupled with the high degree of conservation in the C-terminal two-thirds of the protein, there do not appear to be any significant differences in the capsid proteins of the two viruses that would result in altered host specificity.

A high degree of nonconservative amino acid divergence was found in the E3 polypeptide. E3 functions as the N-terminus of PE2 (pre-E2 glycoprotein) as it is processed through an infected cell's ER/Golgi apparatus, dimerizes with E1, and is transported to the plasma membrane (Strauss and Strauss, 1994). The functional

significance of E3, other than providing the signal sequence for translocation of the PE2/6K/E1 polyprotein into the ER, is unknown. However, studies with SFV have shown that E3 cleavage from PE2 occurs at different stages of glycoprotein processing in vertebrate cells than in mosquito cells (de Curtis and Simons, 1988; Naim and Koblet, 1990; Scharer *et al.* 1993). Such differences may be important in the stability of glycoprotein dimers or in the membrane localization of virus budding from polarized cells of different host species. Cleavage of E3 from PE2 is thought to be catalyzed by a furin-like host-specific serine proteinase (Steiner *et al.* 1992; Strauss and Strauss, 1994). The furin cleavage signal (RXK/RR) at the C-terminus of E3 has been functionally maintained in MRE16. It would be very interesting to examine species-specific differences in glycoprotein processing to clarify the role of E3 in viral replication.

Differences in the glycoproteins are potentially very significant with regard to host specificity, especially amino acid changes in the ectodomains where specific cellular interactions occur. A single nucleotide change in the SIN E2, resulting in an R-172 to G amino acid substitution, causes increased neurovirulence in weanling mice (Tucker and Griffen, 1991). A single amino acid change in an E2 epitope of the TC-83 strain of VEE (I-207 to F) causes decreased oral infectivity in *Ae. aegypti* mosquitoes (Woodward *et al.* 1991). In these studies MRE16 was found to have four amino acid substitutions in the E2 cell-receptor binding domain relative to the prototype SIN. All were conservative substitutions, but the potential role of these changes, individually or together, could be significant with regard to infectivity and tropism in the mosquito. The significance of changes in E1 glycoprotein residues is more difficult to predict. Although no critical alterations in currently recognized functional domains were detected, the role of E1 in

viral infectivity and tropism is not well understood. Any of the 26 nonconservative amino acid differences detected could be important with regard to protein folding, dimerization with E2, or direct interaction with host cell receptors.

Alphaviruses belonging to the SIN group have an Old World distribution, including Africa, Europe, Asia, and Australia. Aura virus, isolated in South America, is the sole New World representative of the SIN-like viruses (Rumenapf *et al.* 1995). Divergent evolution between European-African and Indian-Far Eastern-Australian isolates of SIN has been demonstrated by nucleic acid homology studies (Rentier-Delrue and Young, 1980) and divergence between Paleoarctic-Ethiopian and Oriental-Australian SIN strains has been demonstrated by RNase T1 fingerprinting of genomic RNA and tryptic peptide mapping of structural proteins (Olson and Trent, 1985). Most recently, nucleotide sequence analyses of the 3'-genomic termini of several SIN strains revealed that the European-African and Asian-Australian subgroups differed by 17% (Shirako *et al.* 1991). The data presented here place MRE16 in the Asian-Australian branch of the SIN evolutionary tree (Figure 3.7) and demonstrate 24% nucleotide sequence divergence from AR339. These results confirm the validity of a geographic basis for evolutionary divergence among the SIN strains. However, the relationship between geographic divergence among SIN strains and their infectivity in *Ae. aegypti* remains to be determined.

CHAPTER 4

A CHIMERIC SINDBIS VIRUS POSSESSING MRE16 STRUCTURAL PROTEINS EXHIBITS MRE16-LIKE ORAL INFECTIVITY IN *AEDES AEGYPTI* MOSQUITOES

Introduction

Chimeric alphaviruses have been produced from infectious clones of SIN and Ross River virus (RRV) by reciprocal exchanges of structural and nonstructural genes or noncoding genome regions (Kuhn *et al.* 1991, 1996; Lopez *et al.* 1994; Yao *et al.* 1996). These studies demonstrated the usefulness of engineered chimeric viruses in characterizing alphavirus gene function and showed that viable virus could be produced from hybrid genomes of distantly related alphaviruses.

The dsSIN virus, TE/3'2J, was previously shown to have poor oral infectivity in *Ae. aegypti* mosquitoes (Chapter 2). In contrast, the MRE16 strain of SIN exhibited a highly infectious phenotype in these mosquitoes when administered orally (Chapter 3). It was hypothesized that SIN oral infectivity for mosquitoes is determined by one or more of the viral structural genes. A chimeric infectious clone was engineered by combining the nonstructural genes of pTE/3'2J (the pSINrep5 replicon; see Figure 1.4B) and the structural genes of MRE16. The resultant infectious clone was used to produce a SINrep5/MRE16 chimeric virus (MRE1001). It was reasoned that if SIN structural genes were solely responsible for oral infectivity, the chimeric virus would exhibit the MRE16 infectious phenotype. Alternatively, if differences in oral infectivity resulted from genetic variation in the nonstructural genes or was a polygenic trait resulting from differences in both the structural and nonstructural genes or in noncoding sequences, the chimera would show phenotypic differences from MRE16 in both tropism for mosquito midguts and dissemination rate. The infectivity of the chimeric virus following oral administration to *Ae. aegypti* mosquitoes was evaluated relative to both the TE/3'2J and MRE16 parent viruses. The results from these experiments suggested that oral infectivity for *Ae. aegypti*

is encoded within the structural proteins (probably the envelope glycoproteins) of the virus.

Materials and Methods

Assembly of Cloned MRE16 Structural (C/E2/E1) Genes

The pMCAP, pME2, and pME1 plasmids (pCR®2.1, Invitrogen) containing MRE16 capsid, PE2, and 6K/E1 gene segments, respectively (Figure 3.4), were assembled as a single plasmid designated pMCAP/E2/E1.

Approximately 2 μg of purified pMCAP and pME2 were digested separately with 10 U XmnI restriction endonuclease (Stratagene) in a reaction mix containing 25 mM Tris-HCl (pH 7.7), 10 mM $MgCl_2$, 1 mM DTT, and 30 μg/ml BSA at 37°C for 1 hour, then 65°C for 20 minutes to inactivate the enzymes. The resultant 3.1 kb pMCAP and 3.4 kb pME2 DNA fragments (Figure 4.1A) were isolated by 1% agarose gel electrophoresis and purified using a GENECLEAN II® kit (BIO 101, Inc., La Jolla, CA) per manufacturer's instructions. The pMCAP fragment was dephosphorylated by treatment with 3 U shrimp alkaline phosphatase (SAP) (Amersham LIFE SCIENCE, Inc., Arlington Heights, IL) in a reaction mix containing 20 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ at 37°C for 1 hour. The pMCAP and pME2 fragments were then joined by incubation at 14°C for 16 hours in the presence of 10 U T4 DNA ligase (Gibco-BRL), 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 5% (w/v) polyethylene glycol-8000. The ligation mix was used to transform INVαF' *E. coli* (Invitrogen) as previously described (see *Cloning and Sequencing of Viral Genome Segments*, page 33). Plasmids purified from individual bacterial colonies were screened both by restriction enzyme digest

analysis and PCR using standard protocols (Sambrook *et al.* 1989). A plasmid containing MRE16 capsid through E2 gene sequences was purified and quantified as previously described (see *Cloning and Sequencing of Viral Genome Segments*, page 33), and designated pMCAP/E2.

pMCAP/E2 and pME1 plasmids (Figure 4.1B) were digested separately with 20 U SalI and BamHI restriction endonucleases (Gibco-BRL) in a reaction mix containing 150 mM KOAc, 37.5 mM Tris-Acetate (pH 7.6), 15 mM MgOAc, 0.75 mM β-mercapto-ethanol, and 15 μg/ml BSA at 37°C for 1 hour, then 65°C for 20 minutes to inactivate the enzymes. The resultant pMCAP/E2 restriction fragments were purified using a WIZARD® DNA Clean Up kit (Promega) per manufacturer's instructions and dephosphorylated by SAP treatment, as described above. The 1.2 kb pME1 restriction fragment was gel isolated and purified using a GENECLEAN II® kit (see above). The pMCAP/E2 and pME1 fragments were joined by treatment with T4 DNA Ligase (Gibco-BRL), and the ligation reaction was used to transform INVαF' *E. coli* (Invitrogen), as described above. Plasmids purified from individual bacterial colonies were screened both by restriction enzyme digest analysis and PCR using standard protocols (Sambrook *et al.* 1989). A plasmid containing MRE16 capsid through E1 gene sequences was purified and quantified as described above, and designated pMCAP/E2/E1.

Construction of a Chimeric Infectious Clone and MRE1001 Virus Production

The MRE16 capsid through E1 genome segment was amplified by PCR from a pMCAP/E2/E1 template using primers MRE-FX (5'-GCTCTAGAGCATGAATCGA-GGATTCTTTAA-3') and MRE-RX (5'-GCTCTAGAGCTCATCGTCGGGTGCTTG-TCA-3') (containing 5' XbaI tails) and a high fidelity polymerase (Figure 4.2A). PCR

amplification was performed in a 50 µl reaction containing approximately 50 ng plasmid template DNA, 100 pmoles forward and reverse primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 2 U rTth DNA polymerase XL (Perkin Elmer). The PCR profile consisted of denaturation at 95°C for 5 minutes followed by 25 cycles of amplification; 94°C for 1 minute, 55°C for 3 minutes, 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. The resultant 3.8 kb cDNA product was isolated by separation on a 1% agarose gel and purified using a GENECLEAN II® kit (BIO 101) per manufacturer's instructions. The PCR product was first TA cloned into the pCR® 2.1 vector (Invitrogen) as previously described (see *Cloning and Sequencing of Viral Genome Segments*, page 33), then excised using XbaI and recloned by sticky-end ligation into the pZErO™-1 vector (Invitrogen) to enhance clonal selection by Zeocin™ (Invitrogen) antibiotic resistance. The ligation reaction was used to transform TOP10 *E. coli* (Invitrogen). Plasmids purified from individual bacterial colonies were screened both by restriction enzyme digest analysis and PCR using standard protocols (Sambrook *et al.* 1989). The resultant plasmid was designated pZ-MCAP/E2/E1-X.

pZ-MCAP/E2/E1-X and pSINrep5 were digested separately with XbaI (Figure 4.2B). The linearized pSINrep5 was dephosphorylated by SAP treatment (see above) and the 3.8 kb pZ-MCAP/E2/E1-X digest product (containing MRE16 capsid through E1 sequences) was gel isolated and purified using a GENECLEAN® II kit (BIO 101). The two cDNA fragments were joined by ligation at 14°C overnight. SURE® *E.coli* (Stratagene) were transformed with the ligation reaction mix. Plasmids purified from

individual bacterial colonies were screened by restriction digest analysis (Sambrook *et al.* 1989).

Chimeric MRE1001 viral RNA was transcribed *in vitro* from three of the plasmid isolates and used to transfect BHK-21 cells (see *in vitro Transcription*, page 27, and *Electroporation*, page 28). BHK-21 supernatants were collected and titrated when CPE reached approximately 80% (see *Virus Titrations*, page 28) yielding 6.5 $\log_{10}$ $TCID_{50}$/ml of virus 36 hours post-transfection. The resultant MRE1001 virus was replication and packaging competent.

Comparative Virus Dissemination Rates and IFA Analysis of Infected Mosquito Tissues

TE/3'2J, MRE16, and MRE1001 viruses were amplified by infection of C6/36 cells at 0.01 MOI. After incubation at 28°C for 48 hours 1.0 ml of each virus-containing supernatant was mixed with 2.0 ml of defibrinated sheep blood (Colorado Serum Co.) and fed to approximately 100 adult female *Ae. aegypti* mosquitoes (see *Mosquito Infection*, page 29). Virus titrations were performed on each infectious blood meal (see *Virus Titrations*, page 28). At 10 days post-infection midguts were dissected from 3 to 5 mosquitoes in each group, fixed, and analyzed by IFA to detect the presence of SIN antigen using MAb 30.11a (see *IFA Analysis of Mosquito Tissues*, page 31). The oral infectivity of each virus was measured by IFA analysis of mosquito neural tissues 14 days post-infection using MAb 30.11a (see *IFA Analysis of Mosquito Tissues*, page 31, and *Comparative Virus Dissemination Rates*, page 32).

Results

Production of Chimeric MRE1001 Virus

The procedures used to construct a plasmid (pCR® 2.1 background) containing the assembled MRE16 structural genes are illustrated in Figure 4.1. The plasmid clones used as templates for nucleotide sequence analyses of MRE16 capsid, PE2, and 6K/E1 genes (Chapter 3) possessed overlapping ends. Restriction sites were identified within the overlaps between pMCAP and pME2 (XmnI), and pME2 and pME1 (SalI) that allowed the sequences to be joined without loss of genetic information. Digestion of pMCAP and pME2 with XmnI produced two restriction fragments from each that could be separated by agarose gel electrophoresis. Ligation of the 3.1 kb pMCAP fragment to the 3.4 kb pME2 fragment reconstituted the pCR® 2.1 vector and joined contiguous MRE16 capsid and PE2 gene sequences. A similar approach was used to add MRE16 6K/E1 gene sequences to the pMCAP/E2 construct. Co-digestion of pMCAP/E2 and pME1 with SalI and BamHI produced two restriction fragments of each. Ligation of the 1.4 kb pME1 fragment to the major pMCAP/E2 fragment reconstituted the vector and joined contiguous MRE16 6K/E1 to capsid/PE2 gene sequences. The resultant plasmid, pMCAP/E2/E1, contained the uninterrupted structural genes of MRE16 as well as portions of the 5' and 3' NCRs. Confirmation of the correct insert size was achieved by restriction digest analysis, and E1 gene inclusion was confirmed by PCR using E1-flanking primers (data not shown).

The procedures used to insert MRE16 structural genes into pSINrep5 are illustrated in Figure 4.2. The SINrep5 replicon contains SIN nonstructural gene

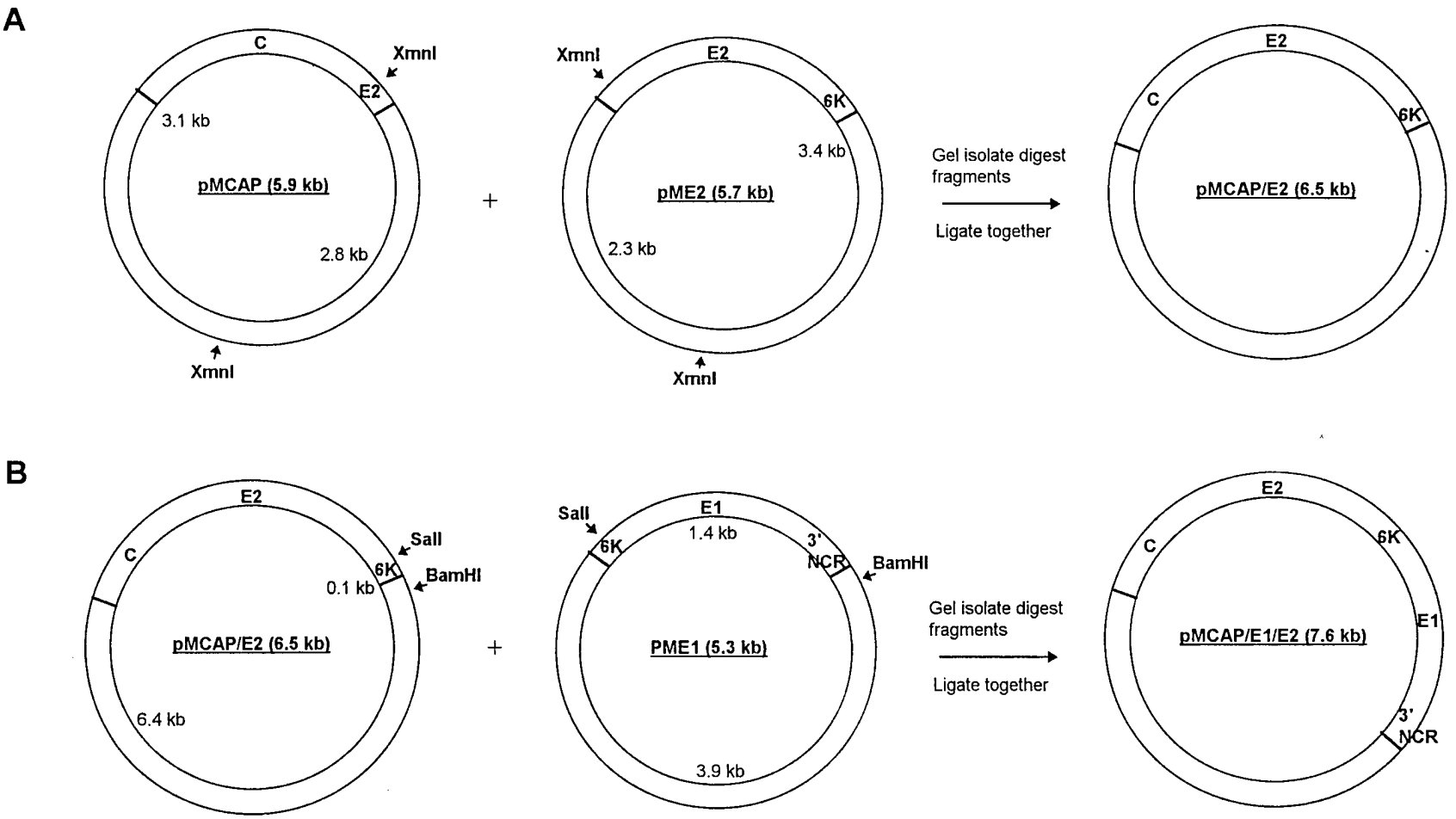

Figure 4.1. Assembly of Cloned MRE16 Structural Genes. A) Plasmids containing MRE16 capsid (pMCAP) and PE2 (pME2) gene sequences were cleaved with XmnI and fragments religated to produce a pMCAP/E2 clone. B) pMCAP/E2 and a plasmid containing MRE16 6K/E1 gene sequences (pME1) were cleaved with SalI and BamHI and fragments religated to produce pMCAP/E2/E1, containing all MRE16 structural genes. All illustrated plasmids were constructed in the pCR®2.1 (Invitrogen) TA cloning vector.

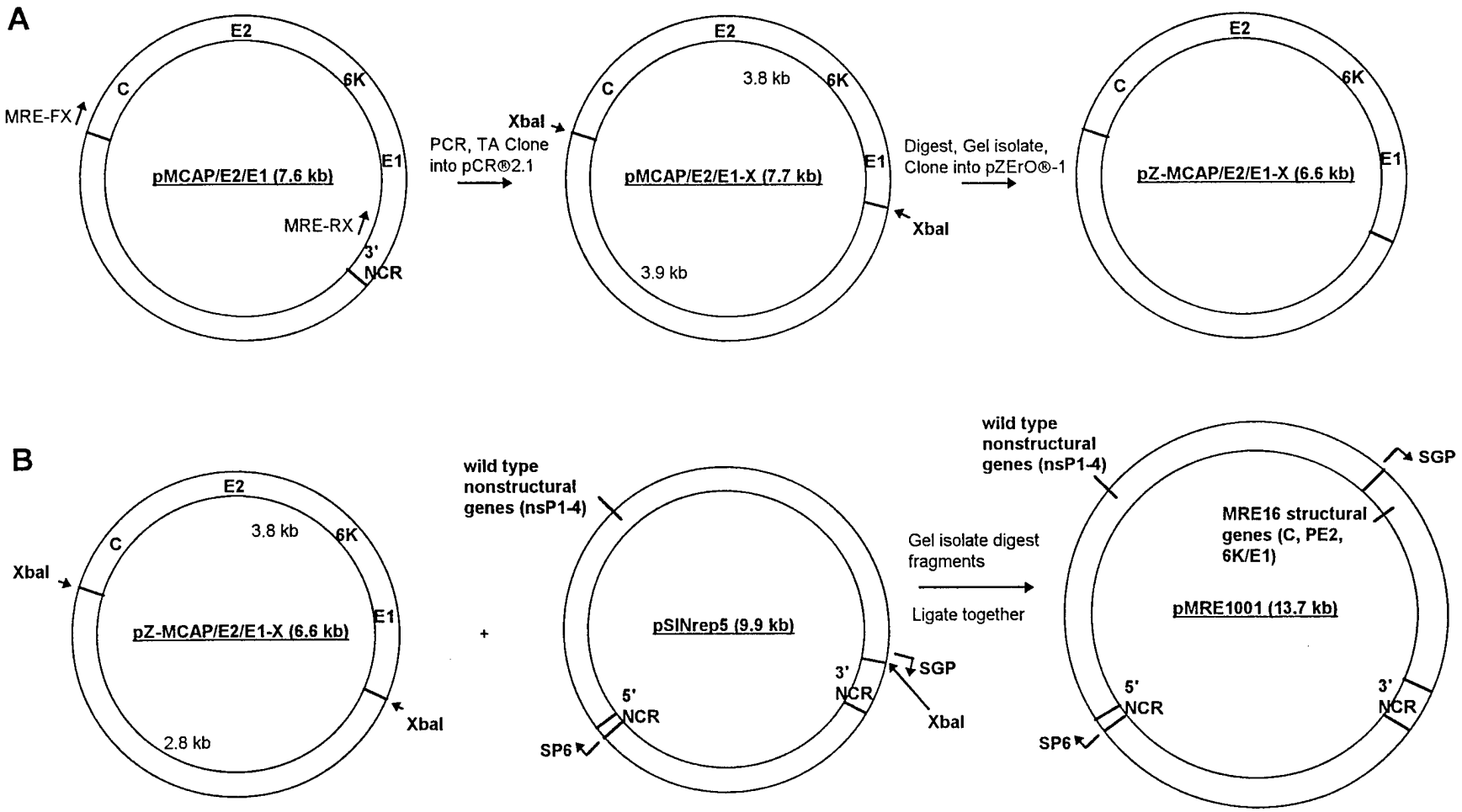

Figure 4.2. Construction of a Chimeric Infectious Clone. A) MRE16 structural genes were amplified by PCR using primers containing XbaI tails; the PCR product was cloned into pCR® 2.1, then excised by XbaI restriction and cloned into pZErO®-1 to produce pZ-MCAP/E2/E1-X. B) MRE16 structural genes were excised from pZ-MCAP/E2/E1-X and ligated into the pSINrep5 XbaI site located downstream of the subgenomic promoter (SGP) producing a chimeric infectious clone, pMRE1001. Viral RNA was produced by *in vitro* transcription from the bacterial SP6 promoter.

(replicase) sequences identical to those of TE/3'2J but lacks structural genes (Xiong *et al.* 1989) (see Figure 1.4). pSINrep5 has a multiple cloning site (MCS) located between the viral subgenomic promoter and 3' NCR. The MCS includes a unique XbaI restriction site. To produce a chimeric infectious clone XbaI 'tails' were first added to the MRE16 genes by PCR using primers designed to anneal at the termini of the MRE16 coding sequences and to contain XbaI restriction sites at their 5' ends. The PCR product was cloned into pCR®2.1, then recloned by XbaI "sticky-end" ligation into the pZErO®-1 vector (Invitrogen) to enhance antibiotic selection. MRE16 capsid through E1 sequences were excised from pZ-MCAP/E2/E1-X by XbaI digestion and ligated into the pSINrep5 XbaI cloning site. Confirmation of insert size and the correct (forward) orientation was attained by BglII/AatII digest analysis (Figure 4.3). Chimeric virus was produced from three of the resultant clones by transfection of BHK-21 cells with viral RNA transcribed from the plasmid templates. All three transfections yielded 6.5 $\log_{10}$ $TCID_{50}$/ml of infectious virus.

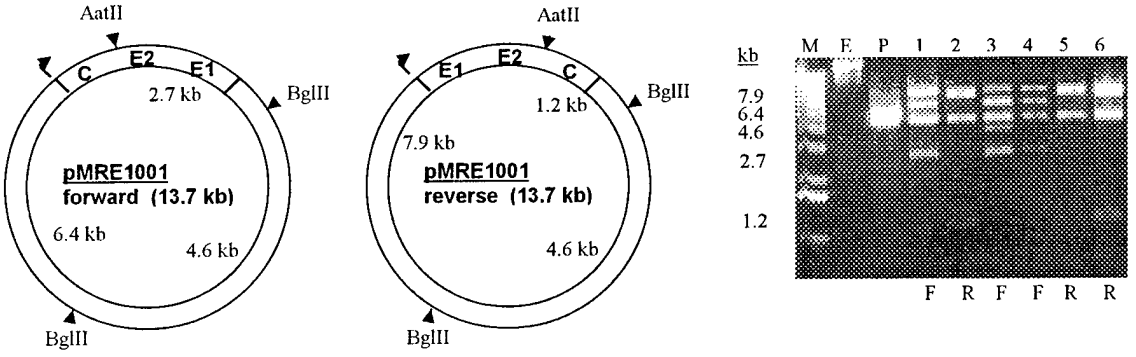

Figure 4.3. Confirmation of MRE16 Gene Insertion in pSINrep5. Insert size and orientation were determined by AatII/BglII restriction. The expected fragment sizes for either forward or reverse orientation are illustrated. Digest fragments from six individual clones (1-6) were separated by agarose gel electrophoresis. Size markers (M); no enzyme (E) and pSINrep5 (P) controls were included. Deduced insert orientations are indicated for each plasmid; F (forward), R (reverse).

Virus from the supernatant of one of the transfections was amplified in C6/36 cells, and cDNA was produced by RT/PCR of the E1/3'NCR junction region of the viral genome. The RT/PCR product was analyzed by MluI restriction digest to confirm that MRE16 E1 and SINrep5 3'NCR sequences were present in the virus (data not shown). Additionally, the nucleotide sequence of the RT/PCR product was determined (Figure 4.4). These data revealed that the MRE1001 genome contained MRE16 E1 glycoprotein sequences and SINrep5 MCS and 3' NCR sequences, confirming the chimeric nature of the viral genome.

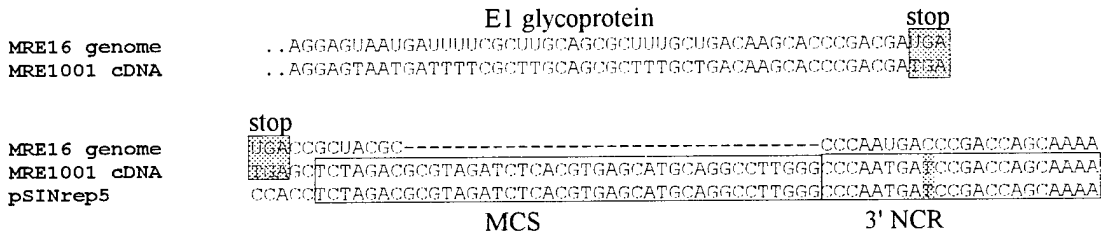

Figure 4.4. Alignment of MRE16, SINrep5, and Chimeric Virus Nucleotide Sequences. The nucleotide sequence of MRE1001 virus cDNA aligned with that of MRE16 E1 from position 3744 (see Figure 3.5) through the stop codon (upper illustration) Downstream of the stop codon the chimeric virus sequence aligned with the pSINrep5 MCS and 3'NCR but not with the MRE16 3'NCR (lower illustration).

Comparative Virus Dissemination Rates

TE/3'2J, MRE16, and MRE1001 viruses were amplified in C6/36 cells and administered to *Ae. aegypti* mosquitoes in infectious blood meals. At 10 days post-infection mosquito midguts were analyzed for the presence and distribution of SIN antigen (Figure 4.5). MRE16 and MRE1001 viruses showed similar infection patterns in midguts with extensive immunofluorescence detected in midgut epithelial cells (Figure 4.5A and C, respectively) and surrounding muscle and tracheole tissues of the anterior midgut (Figure 4.5B and D, respectively). Mosquitoes infected with TE/3'2J virus displayed SIN antigen

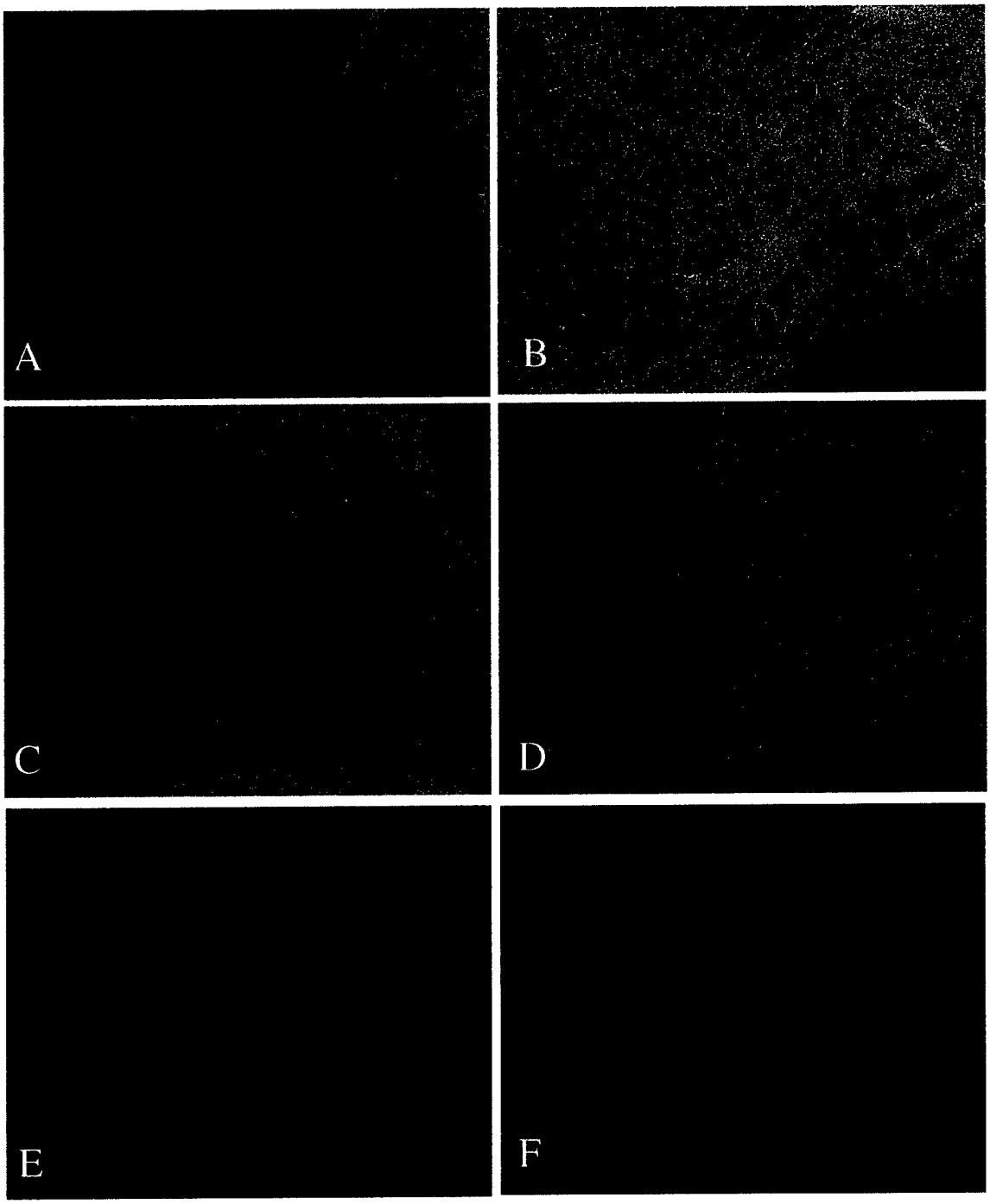

Figure 4.5. IFA Analysis of *Ae. aegypti* Midguts Following Oral Infection with TE/3'2J, MRE16, and MRE1001 Viruses. Midgut epithelial cells of mosquitoes infected with A) MRE16, C) MRE1001, and E) TE/3'2J viruses (magnification 40X). Muscle tissues surrounding anterior midgut showing infection with B) MRE16, D) MRE1001, and F) TE/3'2J. Magnifications were 40X (B and D) and 20X (F). SIN E1 was detected by IFA using monoclone 30.11a as the primary antibody.

only rarely in midgut epithelial cells and not at all in surrounding muscle, nerve, or respiratory (tracheole) tissues (Figure 4.5E and F, respectively).

At 14 days post-infection viral dissemination rates were measured by IFA analysis of mosquito neural tissues using a SIN-specific MAb (Table 4.1). Only 11.3% (9/80) of TE/3'2J-infected mosquitoes exhibited disseminated infections. In contrast, 96.3% (77/80) of MRE16-infected and 91.3% (73/80) of MRE1001-infected mosquitoes were positive for SIN E1 antigen in neural tissues. Dissemination of MRE16 and MRE1001 viruses did not differ statistically ($p=0.81$); however, both viruses more efficiently established disseminated infections than TE/3'2J virus ($p<0.01$). These data strongly suggest that oral infectivity for *Ae. aegypti* mosquitoes is a genotypic characteristic of particular SIN strains encoded by one or more of the structural genes of the virus. Also, the data demonstrate that the substitution of MRE16 structural protein-encoding genes into SIN expression systems is possible and could be employed to improve the oral infectivity of these systems in *Ae. aegypti* mosquitoes.

Table 4.1. Comparative Virus Dissemination Rates in *Ae. aegypti* Mosquitoes

Virus	Titer (log_{10} $TCID_{50}$/ml)	Head Squash/IFA Results		
		# Positive	Total	% Positive
TE/3'2J	10.0	9	80	11.3%
MRE16	7.5	77	80	96.3%
MRE1001	7.5	73	80	91.3%

Discussion

Rice *et al.* (1987) developed the first full-length infectious cDNA clones of SIN. One of the goals of their work was to provide the means to..."define precisely the sequence changes responsible for the phenotype of any SIN variant by the strategy of

exchanging segments of the prototype clone with cDNA of the variant and determining the phenotype of the resulting clones." At that time, impressive results had already been achieved using infectious clones of poliovirus (Kohara *et al.* 1985; Pincus and Wimmer, 1986).

Bredenbeek *et al.* (1993) introduced SINrep5, a refined version of the SIN replicon expression system, that lacked structural genes but could be complemented *in vitro* by several different defective helper (DH) viruses. The pSINrep5 cDNA contained the first 7,646 nucleotides of the prototype SIN genomic cDNA (Toto1101) (Rice *et al.* 1987), followed by a polylinker, the 3'-terminal 310 nucleotides of the SIN genome, plus 37 A residues. pSINrep5 was constructed to provide a variety of sites for the insertion of heterologous genes downstream of the efficient SIN subgenomic promoter. A co-infecting DH virus provided the structural genes necessary to produce infectious virus.

To prepare the chimeric MRE1001 virus, the structural genes of the MRE16 SIN strain were cloned into the XbaI restriction site within the polylinker of pSINrep5, reconstituting a full length infectious SIN clone. The clone retained all of the nucleotides of the prototype SIN except for the sequences encoding the structural polypeptide, which was entirely from MRE16. Thus, transfection of BHK-21 cells with viral RNA transcribed from the linearized cDNA template yielded chimeric virus possessing an MRE16 capsid and envelope glycoproteins, but prototype SIN promoters, non-coding sequences, and genes for viral replicase proteins.

The chimeric MRE1001 virus was shown to be as orally infectious as the MRE16 parent virus in *Ae. aegypti* mosquitoes. Over 90% of mosquitoes ingesting either virus in an infectious blood meal developed disseminated infections within a 14 day extrinsic

incubation period. In contrast, approximately 11% of mosquitoes ingesting TE/3'2J virus were similarly infected. Examination of the midguts of infected mosquitoes at 10 days post-blood meal by SIN-specific IFA revealed that both MRE16 and chimeric virus-infected midguts exhibited patterns of SIN antigen expression suggestive of virus dissemination through the midgut "barriers" (Hardy *et al.* 1983). In contrast, TE/3'2J-infected midguts exhibited only focal distribution of SIN antigen in the epithelial cell layer lining the lumen of the midgut. Thus, it can be concluded that very little TE/3'2J virus was able to penetrate the midgut infectivity barrier or escape from this cell layer, preventing dissemination from the epithelium to other mosquito tissues.

The double-subgenomic SIN expression system, TE/3'2J (Hahn *et al.* 1992; Raju and Huang, 1991), was previously shown to be relatively non-infectious when administered *per os* to *Ae. aegypti* mosquitoes (see Chapter 2). pTE/3'2J is a complete prototype SIN infectious clone that possesses an additional viral subgenomic promoter and polylinker sequence positioned downstream of the structural genes. In Chapter 2 it was demonstrated that loss of the second subgenomic promoter and polylinker (TE/3'2JΔ2SGP) did not by itself result in a significant difference in viral infectivity. Thus, the results described above could not be due simply to the increased size or complexity of the TE/3'2J genome over that of MRE16 or the chimeric virus. Nor is it likely that differences in virus replication in the midgut epithelium resulted in the phenotypic differences observed. The chimeric virus possessed the same promoters and nonstructural genes as TE/3'2J. Rather, the data suggest that differences in the structural proteins confer the different phenotypes seen and that the oral infectivity of MRE16 for *Ae. aegypti* mosquitoes is encoded in its structural genes.

Further studies are required to precisely map the sequences responsible for the observed phenotypes; however, the viral capsid protein-encoding gene is an unlikely candidate. Although nearly 47% of the deduced amino acids in the MRE16 capsid protein differ from those in the prototype virus (Chapter 3), most are clustered in the nonconserved N-terminal one-third of the protein, which is believed to bond via nonspecific electrostatic interactions with the viral RNA genome during packaging (Strauss and Strauss, 1994). In the conserved C-terminal two-thirds of the protein, MRE16 differs from the prototype by only 7%, and 8 of the 12 amino acid differences are conservative substitutions (Chapter 3). Therefore, it is most likely that oral infectivity for mosquitoes is dependent on viral envelope glycoprotein sequences, although differences between SIN variants due to relative packaging efficiencies resulting from capsid-RNA interactions cannot be ruled out.

Nevertheless, it is now possible to re-engineer existing SIN expression systems (e.g., TE/3'2J) for improved oral infectivity in mosquitoes. Substitution of MRE16 structural sequences for the prototype genes should result in enhanced infectivity, virus dissemination, and increased expression of heterologous genes in mosquito tissues. Experiments to evaluate the effectiveness of selected exogenous sequences in reducing disease vector competence can proceed with increased vigor, and possible solutions to the growing problem of mosquito-borne diseases may soon be at hand.

CHAPTER 5

SUMMARY

The data presented in this dissertation support the hypothesis that SIN infectivity for *Ae. aegypti* mosquitoes, following *per os* introduction, is in large part determined by the genetic sequences of the virus that encode its envelope glycoproteins. This may not be surprising to some, as it has been well established that variations in SIN infectivity, virulence, and/or pathogenicity in vertebrates can result from minor changes in viral glycoprotein sequences (Davis *et al.* 1986,1987; Griffin, 1986; Lustig *et al.* 1988; McKnight *et al.* 1996; Stanley *et al.* 1985; Tucker and Griffin, 1991; Ubol and Griffin, 1991). However, the effects of such changes in mosquitoes have not previously been studied in detail.

These are significant data when viewed in the context of developing efficient mechanisms for molecular control of disease vectors. Expression systems have been developed from infectious clones of SIN (Bredenbeek and Rice, 1992; Hahn *et al.* 1992; Rice *et al.* 1987; Xiong *et al.* 1989) and used to study the effectiveness of exogenous gene expression in reducing the vector competence of mosquitoes that transmit disease agents to humans (Higgs *et al.* 1993,1995; Olson *et al.* 1996; Powers *et al.* 1994,1996). However, the expression systems are ineffective if administered to mosquitoes in infectious blood meals, requiring that mosquitoes be individually inoculated with virus suspensions. Improving the oral infectivity of SIN-based expression systems would accelerate these studies. Additionally, inoculated viral expression systems do not efficiently infect the epithelial cell layer that lines the lumen of the mosquito midgut. This tissue layer plays a critical role in vector-pathogen interactions. Increasing the tropism of SIN expression systems to include this tissue layer would be a significant advance. The midgut is clearly a principal target organ for blocking disease transmission through the

selected expression (or interference with expression) of mosquito gene products associated with vector competence.

This dissertation describes two experimental approaches used to address the hypothesis. First, increased oral infectivity of a dsSIN expression virus, TE/3'2J/CAT, was selected for by serial passage in *Ae. aegypti* mosquitoes. Oral infectivity and midgut tropism increased significantly after four passages. Genetic analysis of the resultant virus revealed three consensus amino acid changes in the glycoprotein-encoding genes. However, a deletion of the second subgenomic promoter of the virus also occurred. Comparative infectivity analyses of the passaged virus with a promoter-deleted parent virus indicated that the deletion was not entirely responsible for the increase in infectivity. However, the possibility that additional genetic changes may have occurred as a result of selective passage could not be ruled out.

The second approach involved the construction of a chimeric SIN infectious clone possessing the structural protein-encoding genes of MRE16 (a Malaysian SIN strain) and the nonstructural genes and noncoding sequences of TE/3'2J. MRE16 was previously shown to be highly orally infectious in *Ae. aegypti*. A chimeric virus produced from the infectious clone exhibited essentially the same phenotype as the MRE16 parent, but was nearly nine-fold more infectious than TE/3'2J in *Ae. aegypti* and exhibited a pronounced capacity to infect and disseminate from the mosquito midgut. Nucleotide sequence analysis of the MRE16 26S RNA demonstrated only minor differences in the capsid protein-encoding genes of MRE16 and chimeric viruses relative to the prototype virus, but significant differences in several regions of the PE2 and E1glycoprotein-encoding regions.

Taken together, these studies strongly support the contention that the relative infectivity of SIN strains (or expression systems derived from them) in a given mosquito host species is largely determined by the genetic sequences encoding the viral glycoproteins. Though the precise genetic determinants of mosquito infectivity remain to be resolved, these studies provide the basis for engineering new SIN-based expression systems with enhanced capacity for oral infection of mosquitoes and exogenous gene expression in their midgut epithelia. Development of SIN expression systems that efficiently express exogenous gene sequences in epidemiologically significant target organs of medically important vectors (e.g., *Anopheles*, *Aedes*, *Culex*. species) will be an important addition to the armamentarium of vector biologists and vector-borne disease specialists.

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