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DISSERTATION

RNA-MEDIATED RESISTANCE TO DENGUE VIRUSES 1-4

Submitted by

Zachary N. Adelman

Department of Microbiology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2000

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ZACHARY N. ADELMAN ENTITLED "RNA-MEDIATED INTERFERENCE TO DENGUE VIRUSES 1-4" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Jonathan Carlson

Carol D Blair
Co-Advisor

Advisor

Department Head

ABSTRACT OF DISSERTATION

RNA-MEDIATED RESISTANCE TO DENGUE VIRUSES 1-4

Dengue (DEN) viruses (serotypes1-4) are transmitted to humans by the mosquito *Ae. aegypti*. DEN virus infection results in morbidity/mortality in 50-100 million people worldwide every year. Efforts to control dengue-caused disease through vaccination or by eliminating the mosquito vector have not been successful. The studies described in this dissertation attempted to generate a state of resistance to DEN viruses in mosquito cells. Resistance strategies were based on the transient or stable expression of DEN-derived RNA.

For transient assays, cDNA sequences derived from the genomes of DEN viruses 1-4 were expressed in cultured mosquito cells and *Ae. aegypti* mosquitoes using a double subgenomic Sindbis virus expression system. dsSIN-infected cells and mosquitoes were challenged with the homologous DEN virus serotype, and the level of virus resistance was determined. Recombinant dsSIN viruses designed to express RNA derived from the C, prM, and NS5 genes were the most effective in establishing virus resistance in mosquito cells. Resistance was established against each of the four DEN virus serotypes. Simultaneous resistance to two DEN virus serotypes was also established in mosquito cells, by inserting DEN genome sequences derived from two DEN viruses into a single recombinant dsSIN virus. cDNA inserts >220 bp were more effective at establishing resistance to DEN viruses than inserts of 105-190 bp. Interference to DEN viruses using recombinant dsSIN viruses was independent of the amount of effector RNA, and was likely due to the presence of dsRNA in the form of a SIN replicative intermediate.

For stable assays, transformed mosquito cell lines were developed to express sense, antisense, or dsRNA derived from the DEN-2 prM gene from a constituitive insect virus promoter. Forty-four percent of cell lines designed to express dsRNA were resistant to DEN-2 virus, while <15% of cell lines designed to express sense or antisense RNA alone were resistant to DEN-2 virus. Resistance to DEN-2 virus was not dependent on high levels of transgene mRNA, supporting the hypothesis that a post-transcriptional gene silencing mechanism exists in mosquitoes.

These results suggest that permanently transformed, virus-resistant mosquitoes can reasonably be developed with the introduction of a single transgene.

Zachary N. Adelman
Microbiology Department
Colorado State University
Fort Collins, CO 80523
Fall 2000

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DEDICATION

This work is dedicated to my mother, Patricia Aronson; my stepfather, Robert Aronson; my sisters, Libby and Becky; and my grandmother, Mary. They are my family, and everything I achieve, including this dissertation, stems from their wonderful influence.

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Chapter 1

Literature Review

Dengue viruses and diseases.

Dengue (DEN) viruses are members of the family Flaviviridae, genus flavivirus (Westaway *et al.*, 1985a). The flavivirus genus consists of approximately 70 members, which segregate into three main groups: tick-borne, mosquito-borne, and non-vector borne (Kuno *et al.*, 1998). DEN viruses form a distinct antigenic complex within the mosquito-borne group (Calisher *et al.*, 1989). Within the DEN virus antigenic complex are four distinct dengue virus serotypes (Calisher *et al.*, 1989), designated DEN 1-4. Other significant antigenic complexes within the mosquito-borne group include the Japanese encephalitis complex (ex. Japanese encephalitis, West Nile encephalitis, and Kunjin viruses), and yellow fever virus, which forms its own group.

All four DEN virus serotypes are capable of causing disease in humans. Disease ranges from subclinical to severe. The mild form of disease is called dengue fever (DF). Typically, DF manifests after an incubation period of 3-8 days, symptoms persist (and recur) for 5-7 days, followed by complete recovery of the affected individual (Monath, 1994). Symptoms of DF usually include fever, myalgia, arthralgia, headache, rash, and a generalized malaise.

Infection with any of the DEN virus serotypes can also result in a more serious illness, known as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). In addition to all of the symptoms of DF, DHF/DSS causes capillary leakage, severe fever and generalized hemorrhagic manifestations (Gubler, 1988; Gubler, 1998a). Unmanaged, the case fatality rate can be as high as 20-30%, primarily due to circulatory failure

(Gubler, 1988). However, with hospitalization and fluid replacement, this can be reduced to under 1% (Gubler, 1998a).

Evidence has been presented for two main hypotheses as to why some patients manifest DHF/DSS. Infection with virulent strains of virus may result in DHF/DSS. This hypothesis is supported by the observation that DHF did not occur in the Americas until an Asian strain of DEN-2 virus was introduced into Cuba in 1981 (Rico-Hesse, 1990; Rico-Hesse *et al.*, 1997). Major epidemics of DHF/DSS have occurred in the Americas ever since. While Asian strains of DEN-2 seem to be more virulent in the Americas than native strains, no single genotype or amino acid sequence has been successfully correlated with DHF (Igarashi, 1997; Leitmeyer *et al.*, 1999; Mangada and Igarashi, 1998).

The theory of antibody-dependent enhancement of infection as a cause of DHF/DSS has been summarized by Halstead (1988). He theorized that neutralizing antibodies that developed after a primary DEN virus infection, although protective against reinfection by the same serotype, aid in the entry of the other three DEN virus serotypes into monocytes. Indeed, studies in Thailand have shown that the risk of developing DHF after infection is up to 50 times greater in individuals with neutralizing antibody to a heterologous DEN virus serotype (Sangkawibha *et al.*, 1984; Thein *et al.*, 1997). This theory, however, does not explain cases of DHF that occur during a primary infection. Likely, a combination of virus strain, immune status, host genetics and a number of environmental factors influence whether a DEN virus infection presents as DF or DHF (Gubler, 1998a).

DEN viruses are endemic throughout the tropical regions of the world, with approximately 2.5 billion people at risk for DF and DHF/DSS (Gubler, 1998a; Igarashi, 1997; Monath, 1994). The emergence of DF and DHF/DSS as pandemic entities is thought to be due to factors such as population growth and expansion, increased urbanization, increased mosquito breeding sites, and increased travel between DEN endemic regions (Gubler, 1988). Even though with proper medical management DF and DHF/DSS are rarely fatal, these diseases represent an enormous economic burden to the countries in which they are endemic. This impact is felt in terms of missed work due to illness as well as the extensive cost of hospitalization and treatment. Meltzer *et al* (1998) assessed the economic impact of DEN disease in Puerto Rico to be over 650 disability-adjusted life years per million people, which puts DEN disease on the same scale as diseases such as tuberculosis and malaria. Similar estimates of the cost of DEN diseases have been made by Igarashi (1997) and Gubler (1998).

Three different transmission cycles have been described for DEN viruses; a forest cycle between canopy-dwelling mosquitoes (*Aedes* spp.) and primates; a rural cycle between sylvan mosquitoes (*Aedes* spp.) and humans; and an urban cycle between domesticated mosquitoes (*Aedes aegypti*) and humans (Gubler, 1988). The forest and rural transmission cycles may play a role in maintenance of DEN viruses between epidemics, but are not directly responsible for disease transmission. It is the urban transmission cycle that is largely responsible for epidemic and endemic DF and DHF/DSS throughout the tropical regions of the world. *Ae. aegypti* is thought to have originated in northern Africa, evolving a close relationship with human civilization after becoming isolated from forest-dwelling mosquitoes as the Sahara dried out (Tabachnick, 1991). *Ae. aegypti* has followed humankind around the world ever since, preferring to lay its eggs in small containers in and about human dwellings (Gubler, 1988). This close association with humans is what gives *Ae. aegypti* mosquitoes such a high vectorial capacity for transmitting disease agents such as the DEN viruses.

Vectorial capacity refers to the ability of a vector population to transmit disease agents. Vectorial capacity is influenced by the population density and the oral susceptibility of the vector to the virus, as well as the length of time between ingestion of a viremic blood meal and the ability to transmit the virus (extrinsic incubation period). Other factors influencing vectorial capacity include the vector feeding frequency, host preference, and life expectancy (Black and Moore, 1996). Female *Ae. aegypti* mosquitoes require a bloodmeal to support each gonotrophic cycle. While female *Ae. aegypti* raised in laboratory settings are regularly fed sugar in addition to blood, *Ae. aegypti* found in nature appear to feed solely on blood (Edman *et al.*, 1992). The preference to feed only on blood has been shown to increase the survivorship of wild *Ae. aegypti* mosquitoes in both Thailand and Puerto Rico (Costero *et al.*, 1998; Scott *et al.*, 1997). *Ae. aegypti* live in close association with humans, and prefer to feed on human blood, even if other sources are more abundant and readily available (Scott *et al.*, 1993a). *Ae. aegypti* mosquitoes are also easily interrupted while feeding, and frequently feed from multiple hosts before reaching repletion (Scott *et al.*, 1993b; Xue *et al.*, 1995). Finally, field caught *Ae. aegypti* from Thailand or Puerto Rico experienced high mortality after 2-3 days of starvation, indicating that these mosquitoes require feeding on human blood at least every third day to survive (Costero *et al.*, 1999; Scott *et al.*, 1997). In summary, *Ae. aegypti* mosquitoes are efficient vectors of DEN viruses because they live and breed in and around human dwellings, and feed almost daily with a diet consisting solely of human blood. This is augmented by the fact that most cities throughout the tropical regions of the world are experiencing major population growth, with poor waste and water management facilities (Gubler, 1988). Thus there is virtually no end to both the number of breeding sites and susceptible human hosts.

There are currently no reliable, safe and effective vaccines available for DEN viruses. Vaccine development has been slowed by the problem of antibody-dependent enhancement. Because neutralizing antibodies to one DEN virus serotype may predispose an individual to a more severe disease (DHF/DSS), any vaccination strategy must offer protection against all four DEN virus serotypes. Control of the mosquito population by eradicating breeding sites and larval habitats is currently the only effective way to reduce the vectorial capacity of *Ae. aegypti* populations (Gubler, 1998b; Lifson, 1996).

DEN virus molecular biology

DEN viruses have a (+) polarity, single-stranded RNA genome. DEN virus genomes are approximately 11kb in length and encode 3 structural proteins followed by 7 non-structural proteins, in the order: C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5. DEN virus genomes have a m^7GpppG cap at the 5' end, but are not polyadenylated. Instead, DEN viruses, like all flaviviruses, have a conserved stem-loop RNA secondary structure at the 3' end, which is thought to serve in the initiation of (-) strand synthesis (Wengler, 1981; Wengler and Castle, 1986).

The three structural proteins of DEN viruses are the capsid (C), premembrane (prM) and envelope (E). The C protein is cytoplasmic, and is presumed to have RNA binding activity. Both the prM and E proteins are translated directly into the ER lumen, where they remain membrane associated. The prM and E proteins form heterodimers once inside the ER, and the pr segment is cleaved at the time of virus release from the cell (Murray *et al.*, 1993; Wang *et al.*, 1999). The pr segment prevents E protein-mediated acid-dependent cell fusion from occurring before virus maturation for both DEN-2 virus and members of the tick-borne encephalitis virus complex (Heinz *et al.*, 1994; Murray *et al.*, 1993). Interestingly, the cleavage of prM to M in mature virions produced in mosquito cells has been observed to be incomplete as compared with virions produced in vertebrate cell cultures (Murray *et al.*, 1993). This may reflect a difference in the replication strategy of DEN viruses between humans and mosquitoes. The E protein is the major viral surface protein, and is responsible for cell binding and entry (Wang *et al.*, 1999).

All seven non-structural proteins are cytoplasmic, although the NS1 protein has cell surface as well as secreted forms (Chambers *et al.*, 1990). The precise role of the NS1 protein has not been clearly defined; however, several features are evident. NS1 proteins from both DEN-2 virus and Kunjin virus localize with virus-specific dsRNA (Mackenzie *et al.*, 1996; Westaway *et al.*, 1997). Temperature-sensitive mutations in the yellow fever virus NS1 protein prevent the accumulation of viral RNAs (Muylaert *et al.*, 1997). Furthermore, RNA accumulation is restored if functional NS1 protein is supplied *in trans* (Lindenbach and Rice, 1997). These data suggest that at least one function of the NS1 protein is involvement in viral RNA replication. NS1 may also play an indirect role in the pathogenesis of secondary DEN virus infections. Falconar (1997) has shown that anti-NS1 antibodies are capable of cross-reacting with human fibrinogen and thrombocytes, which is consistent with a higher risk of DHF upon secondary infection.

The NS2a, NS2b, NS4a, and NS4b proteins all contain hydrophobic, membrane anchoring domains. These small, membrane bound proteins are thought to provide the structural scaffolding required for DEN virus replication complex formation (Chambers *et al.*, 1990). The NS3 protein has several well-defined functions, including viral protease, NTPase and helicase (Cui *et al.*, 1998; Falgout *et al.*, 1991). The amino terminus of the NS3 protein, along with the NS2b protein, is responsible for all virus-mediated cleavage of the DEN polyprotein (Chambers *et al.*, 1990; Falgout *et al.*, 1991). In addition, RNA-binding to the 3' stem loop structure has been demonstrated for both Japanese encephalitis virus NS3 and DEN-1 NS3 proteins, suggesting a role in (-) strand synthesis (Chen *et al.*, 1997; Cui *et al.*, 1998).

The NS5 protein, at 110 kilodaltons, is the largest of the proteins encoded by DEN viruses. Based on its size and sequence homology to other viral polymerases, the NS5 protein is thought to be the viral RNA-dependent RNA polymerase (RdRP). The NS5 protein contains several motifs common to RdRPs, including the gly-asp-asp motif (GDD), which is common to both RNA and DNA polymerases (Argos, 1988). Since its initial designation as the viral RdRP, *in vitro* polymerase activity of the NS5 protein has been demonstrated when complexed with the NS3 protein (Bartholomeusz and Wright, 1993; Raviprakash *et al.*, 1998), confirming NS5 as the viral RdRP.

DEN viruses infect and replicate in both vertebrate and invertebrate cell cultures, reaching maximum titers in 3-7 days. Infection of vertebrate cells is usually cytolytic, while infection of invertebrate cells is accompanied by little to no cytopathic effects and a state of persistence (Igarashi, 1978). DEN (+) strand RNA serves directly as a mRNA upon uncoating of the virus. When sufficient accumulation of DEN non-structural proteins occurs, replication complexes begin to form on the perinuclear endoplasmic reticulum. NS3 and NS5 (and possibly NS1) bind to the 3' stem loop structure of the (+) strand DEN genome and initiate (-) strand synthesis, producing the double-stranded replicative form (RF, 20 S) (Cleaves *et al.*, 1981; Wengler, 1981). DEN virus (-) strand RNA serves as a template for (+) strand synthesis. It has been estimated that roughly 5 DEN (+) strands are concurrently synthesized from a single (-) strand, with an elongation time of about 15 minutes per strand (Cleaves *et al.*, 1981). This is the replicative intermediate (RI, 28 S) and has been shown to contain both single stranded and double-stranded regions (Cleaves *et al.*, 1981). DEN virus replication favors the production of (+) strands over (-) strands by a ratio of at least 10:1, although (-) strand synthesis occurs throughout the infection, unlike alphaviruses (Cleaves *et al.*, 1981). As more and more replication complexes form along the perinuclear ER, distinctive smooth membranous structures (SMS) appear throughout the infected cell (Leary and Blair, 1980). These SMS are a hallmark of flavivirus infection in both vertebrate and invertebrate cells (reviewed in Hase *et al* 1989). DEN (+) strand RNAs are then encapsidated, acquire both E and prM surface proteins during envelopment, and accumulate in cytoplasmic vacuoles (Hase *et al.*, 1989). Mature virions are then secreted, at which point prM is cleaved into M (Murray *et al.*, 1993; Wang *et al.*, 1999).

Alphaviruses.

The alphavirus genus belongs to the family Togaviridae (Westaway *et al.*, 1985b). Alphaviruses are arthropod-borne viruses, transmitted primarily between mosquito and avian species. Alphaviruses are divided into several antigenic complexes, including the Semliki Forest (SF) virus complex, the Venezuelan equine encephalitis/ eastern equine encephalitis (VEE/EEE) virus complex and the Sindbis (SIN) virus complex (Calisher and Karabatsos, 1988; Strauss and Strauss, 1994).

SIN virus has been designated as the prototypic member of the alphavirus genus (AR339 strain) (Westaway *et al.*, 1985b). SIN virus nucleocapsids have icosohedral symmetry (T=4), a diameter of 40 nm, and are enveloped (Strauss and Strauss, 1994). The genome of SIN virus is single-stranded, (+) sense RNA, approximately 11.7 kilobases in length and is both capped at the 5' end and polyadenylated at the 3' end. SIN virus RNA encodes 4 nonstructural proteins followed by 4 structural proteins: 5'-nsP1-nsP2-nsP3-nsP4-C-PE2-6K-E1-3' (Strauss *et al.*, 1984; Strauss and Strauss, 1994).

SIN virus replication occurs solely in the cytoplasm of infected cells, beginning with the SIN genomic RNA acting directly as a mRNA upon viral uncoating. Translation of the SIN mRNA results in a polyprotein consisting of nsP123 or nsP1234. nsP4 is immediately cleaved from the polyprotein, and both nsP123 and nsP4 are required for the initiation of (-) strand synthesis (Sawicki and Sawicki, 1998). Following (-) strand synthesis, replication complexes consisting of all four nsPs with full length (-) strand template (Barton *et al.*, 1991) synthesize either full length (+) strand genomic RNA (49 S) or a (+) strand subgenomic RNA (26 S) corresponding to only the structural genes. As SIN-specific proteins accumulate, nsP123 is cleaved to nsP1, nsP2 and nsP3 by the nsP2 portion of the polyprotein (Hardy and Strauss, 1989). When nsP2 becomes abundant, cleavage of the nsP123 polyprotein is complete. This results in the shut-off of viral (-) strand synthesis (3 h or 6 h after infection at 37°C or 30°C, respectively) (Strauss and Strauss, 1994). Replication complexes synthesizing 49 S or 26 S mRNAs are extremely stable (Sawicki and Sawicki, 1998), and will synthesize (+) strand RNA throughout infection. SIN virus replication complexes favor the production of 26 S RNA over 49 S, which leads to the production of large amounts of structural proteins necessary for virus packaging. This preference for 26 S RNA synthesis seems to be mediated by the nsP2 protein (Suopanki *et al.*, 1998).

SIN virus replicates similarly in both vertebrate and invertebrate cell cultures. In addition, SIN virus replication is dependent on host-derived factors in both vertebrate and invertebrate cells (Baric *et al.*, 1983; Condreay *et al.*, 1988; Erwin and Brown, 1983). The primary difference between SIN virus replication in vertebrate and invertebrate cells is the outcome. Mammalian cells infected with SIN virus experience strong cytopathic effects (CPE) within 18 h of infection, followed by the induction of apoptosis (Karpf and Brown, 1998; Levine *et al.*, 1993). Insect cells vary widely in the amount of CPE observed during a SIN virus infection (from severe to none), but a common feature is that surviving cells maintain persistent, low levels of virus production indefinitely (Brown and Condreay, 1986). Also, insect cell death that does occur is not due to apoptosis (Karpf and Brown, 1998).

The ability of SIN virus (and other alphaviruses) to form persistent infections in mosquito cells has been the subject of much research. Persistent SIN virus infections are characterized by low levels of virus production, progeny that form small plaques, and low numbers of cells (<2%) displaying SIN antigen (Brown and Condreay, 1986; Riedel and

Brown, 1977; Stollar and Shenk, 1973). Reidel and Brown (1977) have shown that cells may be "cured" of a persistent infection by culturing in the presence of SIN-neutralizing anti-serum, indicating that the state of persistence is maintained by the continuous reinfection of cells by the low-levels of virus present in the culture medium. This reinfection process can not be accelerated by providing high-titer virus (Stollar and Shenk, 1973), indicating that the state of persistence may also be conditioned by the cells as a means of controlling virus infection. Evidence for this was provided by Reidel and Brown (1979) and Newton and Dalgarno (1983), who described a soluble factor isolated from the medium of SIN or SF virus persistently infected cells that was able to condition persistence in cultures newly infected with SIN or SF viruses. This antiviral activity was found to be a small (3200 D) hydrophobic peptide (Luo and Brown, 1993) capable of inducing the synthesis of at least one cellular protein in addition to its own production (Luo and Brown, 1994). The antiviral peptide was only produced in certain mosquito cell lines (U4.4 but not C7-10), and only conditioned persistence to the homologous virus.

More recently, Karpf and Brown (1997) have shown that persistently infected mosquito cells cannot be productively re-infected by heterologous viruses (SF, Ross River viruses) within the alphavirus genus, or by the homologous virus. This heterologous interference among alphaviruses has been attributed to something quite different from an antiviral peptide. The hypothesis put forth to explain this observation is that cells in a persistently infected culture still contain nsP2 protein, which is able to cleave the nsP123 polyprotein of any super-infecting alphavirus before it is able to initiate (-) strand synthesis (Karpf *et al.*, 1997; Sawicki and Sawicki, 1998; Strauss and Strauss, 1994). Thus, replication of super-infecting viruses cannot occur. The ability of the SIN nsP2 protein to cleave the nsP123 polyproteins of related alphaviruses such as SF virus and Ross River virus remains to be demonstrated.

SIN expression systems

The first SIN cDNA infectious clone (Rice *et al.*, 1987) was produced just three years after the complete sequence was published (Strauss *et al.*, 1984). The ability to replicate to high RNA and protein levels in a short time (< 24 h) combined with the ability to infect a wide range of cell types (both vertebrate and invertebrate), made SIN virus seem like an ideal expression vector. Xiong *et al* (1989) described the construction of a cDNA in which the SIN structural genes had been replaced by the chloramphenicol acetyltransferase (CAT) gene. RNA transcribed *in vitro* from this cDNA and introduced into vertebrate cells resulted in RNA replication and expression of the CAT gene from the SIN virus subgenomic promoter. This SIN "replicon" was able to produce 10^8 CAT polypeptides per cell, the same magnitude as normal production of the SIN structural proteins (Xiong *et al.*, 1989). Bredenbeek *et al* (1993) followed this by describing various defective helper RNAs that could supply the SIN structural proteins *in trans*, leading to the production of infectious virus particles. Researchers at the AIDL have utilized infectious SIN replicon virus particles to study the morphogenesis of LaCrosse (LAC) virus structural proteins in mosquitoes and mosquito cells (Kamrud *et al.*, 1998), as well as the localization of YF and DEN-2 virus replicase (NS5) protein in vertebrate and invertebrate cells (Rayner, 1998).

While infectious SIN replicon virus particles have been used for the expression of large genes (4-5 kb), another SIN expression system has been designed specifically for the expression of smaller genes (≤1kb). Hahn *et al* (1992) described the construction of a cDNA containing the full SIN genome with a duplicated subgenomic promoter inserted immediately following the structural protein genes. RNA transcribed *in vitro* was shown to be capable of replication and production of infectious virus progeny after introduction into susceptible cells. This double-subgenomic SIN (dsSIN) virus was used initially to express epitopes derived from influenza virus HA protein in murine cells that could be recognized (and lysed) by cytotoxic T-cells (Hahn *et al.*, 1992), but has since found extensive use expressing genes in insect cells. Olson *et al* (1994) showed that the dsSIN virus capable of expressing the CAT gene in murine cells could also express high levels of CAT protein in mosquito cells and *Ae. triseriatus* mosquitoes. CAT protein expressed from a dsSIN virus was detected in mosquitoes up to 20 days after the dsSIN virus was delivered, demonstrating that dsSIN viruses are capable of providing long-term expression of heterologous genes in mosquitoes. This was followed by a study of dsSIN virus production and RNA levels in *Ae. aegypti* and *Culex pipiens*, as well as *Ae. triseriatus* mosquitoes (Rayms-Keller *et al.*, 1995). These studies showed that dsSIN viruses could productively infect most organ systems including the head and salivary glands, but were restricted in midgut epithelial cells and ovaries. The structural proteins included in the dsSIN virus expression system developed by Hahn *et al* (1992) were derived from a neuro-adapted strain (Griffin and Johnson, 1977; Lustig *et al.*, 1988) of the original AR339 SIN virus. When the structural proteins of this TE-based dsSIN virus were replaced with those of a mosquito-adapted Malaysian SIN virus (MRE16), the resulting MRE-based dsSIN virus readily infected midgut epithelial cells when delivered *per os* or by inoculation (Olson *et al.*, 2000; Seabaugh *et al.*, 1998). However, the MRE-based dsSIN viruses were found to replicate to lower titers than the original TE-based dsSIN virus in mosquitoes (Olson *et al.*, 2000).

Direct model of RNA-mediated interference

In the mid-80's reports appeared describing antisense-RNA inhibition of the translation of maternal mRNA transcripts in *Xenopus laevis* oocytes (Izant and Weintraub, 1984; Melton, 1985). Injected RNA of the opposite polarity (anti-sense) of a cellular mRNA was able to form Watson-Crick base-pairs with its target mRNA, and the resulting dsRNA could not be translated. At the same time, Sanford and Johnston (1985) proposed the concept of pathogen-derived resistance. They theorized that expression of RNA that was antisense relative to the RNA produced by an infecting pathogen (such as a virus) could inhibit the replication of the pathogen. Observations of antisense RNA action derive primarily from studies of bacterial gene regulation, where antisense RNAs serve as regulators of translation (reviewed in Simons 1988). Work on the *lac* operon has shown that to be effective in preventing the translation of *lac* proteins, antisense RNAs must be in excess (100-150:1) over the *lac*ZYA mRNA, and the antisense RNA must be capable of covering the ribosomal entry site (Pestka, 1992). Other researchers have found that RNase III, which non-specifically degrades dsRNA in prokaryotes, is essential for antisense regulation of plasmid R1 (Blomberg *et al.*, 1990). Melton (1985), in working with *X. laevis*, also found the necessity of an excess (50:1) of antisense RNA and that the 5' end of the target mRNA must be covered to prevent translation of β-globin mRNA. Many successful reports of using antisense RNA to prevent virus replication followed, including interference with RNA (HIV) and DNA (polyoma virus, human cytomegalovirus) viruses (Chatterjee *et al.*, 1992; Dal Monte *et al.*, 1996; Ottavio *et al.*, 1992). These reports echoed the mechanistic idea of a direct model based on (1) large amounts of antisense RNA compared to its complement, (2) antisense-sense hybridization, and (3) either translational blocking or immediate degradation of RNA:RNA hybrids. However, reports of antisense RNAs not performing as expected accumulated as well, and reviews of antisense RNA technology throughout the 90's usually included observations that did not fit the direct model of antisense RNA interference (Branch, 1996; Denhardt, 1992; Nellen and Lichtenstein, 1993; Sczakiel, 1997).

Indirect model of RNA-mediated interference

Research in plant systems produced the most dramatic examples of observations that did not fit the direct model of antisense interference. Attempts to increase the production of pigmentation in petunia plants by introducing extra gene copies resulted in a *decrease* in the amount of pigment in some of the plants (van der Krol *et al.*, 1990). Attempts to express a protein derived from tobacco mosaic virus in plants to study its function resulted in a number of plants being resistant to infection by the parent tobacco mosaic virus (Golembowski *et al.*, 1990). Other researchers found that expression of *sense*, as well as antisense transcripts could provide virus resistance in potato plants (Kawchuk *et al.*, 1991). There are no doubt countless other examples of these phenomena, and the phrase co-suppression began to refer to gene silencing induced by sense RNA, to differentiate it from antisense RNA effects. The common observation in these cases was that a virus-resistant or gene-silenced phenotype was often accompanied by low to non-detectable steady-state levels of transgene mRNA. Lindbo *et al.* (1993)

found that even though plants contained low-levels of transgene mRNA, the transgene was transcriptionally active, indicating that silencing was post-transcriptional. These researchers went on to propose that when mRNA production from an endogenous gene or transgene exceeded a certain threshold level, that gene product would be subject to post-transcriptional gene silencing. Importantly, this silencing effect would then apply to any viral or host RNA similar in sequence to the silenced RNA.

Post-transcriptional gene silencing (PTGS) has been shown to target viruses (English *et al.*, 1996; Golembowski *et al.*, 1990; Kawchuk *et al.*, 1991; Lindbo *et al.*, 1993; Pinto *et al.*, 1999) as well as transposable elements (Jensen *et al.*, 1999). This suggests that PTGS may have evolved to recognize and control these elements. This is supported by the observation that plants that "recover" from a virus infection can become resistant to the same virus through a PTGS mechanism (Lindbo *et al.*, 1993; Ratcliff *et al.*, 1997; Ratcliff *et al.*, 1999). Stronger evidence for PTGS being a defense mechanism, however, comes from the characterization of genes encoded by plant viruses whose protein products are capable of suppressing PTGS (Anandalakshmi *et al.*, 1998; Lucy *et al.*, 2000). In addition, plant viruses normally unable to replicate efficiently in some plants become much more robust in the presence of these PTGS inhibitors (Beclin *et al.*, 1998; Pruss *et al.*, 1997). Presumably these viruses are normally controlled by a PTGS mechanism, and when that mechanism is suppressed, viral replication proceeds uninhibited. Even more intriguing is the observation that viral proteins capable of inhibiting PTGS in some plants are critical to the ability of other plants to recognize and resist virus infection, by another unknown mechanism (Li *et al.*, 1999), indicating the "move-countermove" game between plants and viruses is extremely complex. It seems likely that examples of PTGS directed against transgenes or endogenous genes are a by-product of activating this well-established defense mechanism (Smyth, 1999).

Although descriptions of PTGS in plants have become common, until recently the precise trigger for this mechanism remained unknown. The idea of a threshold RNA level did not seem to be consistent, as RNA expression levels did not always correlate with the silencing phenotype. Also, transgenic plants exhibiting the PTGS phenotype were typically in the minority (2-30%) (Angell and Baulcombe, 1997; Grant, 1999) of the total transformed plants, and not all genes introduced resulted in silenced plants. The next step in the puzzle was provided by Fire *et al* (1998), who observed strong PTGS-like silencing of endogenous genes in *Caenorhabdites elegans* after the injection of dsRNA, which was termed RNA interference (RNAi). Injected dsRNA did not alter the targeted genes at the DNA level, and was not effective if directed against intronic sequence, indicating that the processed mRNA is the target for silencing. Purified antisense or sense RNA alone could not induce the silencing response, although unpurified RNA of either polarity could induce silencing to some extent. This activity was shown to be due to minute amounts of dsRNA present in crude *in vitro* transcription reactions (Fire *et al.*, 1998). This work (Fire, 1999; Montgomery and Fire, 1998; Montgomery *et al.*, 1998) served to bridge the gap between the growing body of plant research on PTGS and the scattered observations of RNA-mediated interference in other organisms that could not be explained by the direct model of antisense interference.

The next piece of the dsRNA puzzle was provided by Hamilton and Baulcombe (1999). These researchers observed that small RNA species (~25 nt) of both sense and antisense polarities were present only in cells displaying gene silencing (transgene silencing, endogenous gene silencing, virus-induced gene silencing, or recovery from virus infection) (Hamilton and Baulcombe, 1999). This confirmed that all forms of gene silencing observed in plants seemed to operate through a common mechanism. These findings were extended by the observations that dsRNA-induced silencing can occur in lysates of *Drosophila* cells or embryos (Tuschl *et al.*, 1999; Zamore *et al.*, 2000) and that the dsRNA is processed directly into 21-23 nucleotide lengths (Zamore *et al.*, 2000). These small RNAs then comprised part of a nuclease-complex, which mediated the specific degradation of single stranded RNA (mRNA) of either polarity into 21-23 nt lengths (Hammond *et al.*, 2000; Zamore *et al.*, 2000).

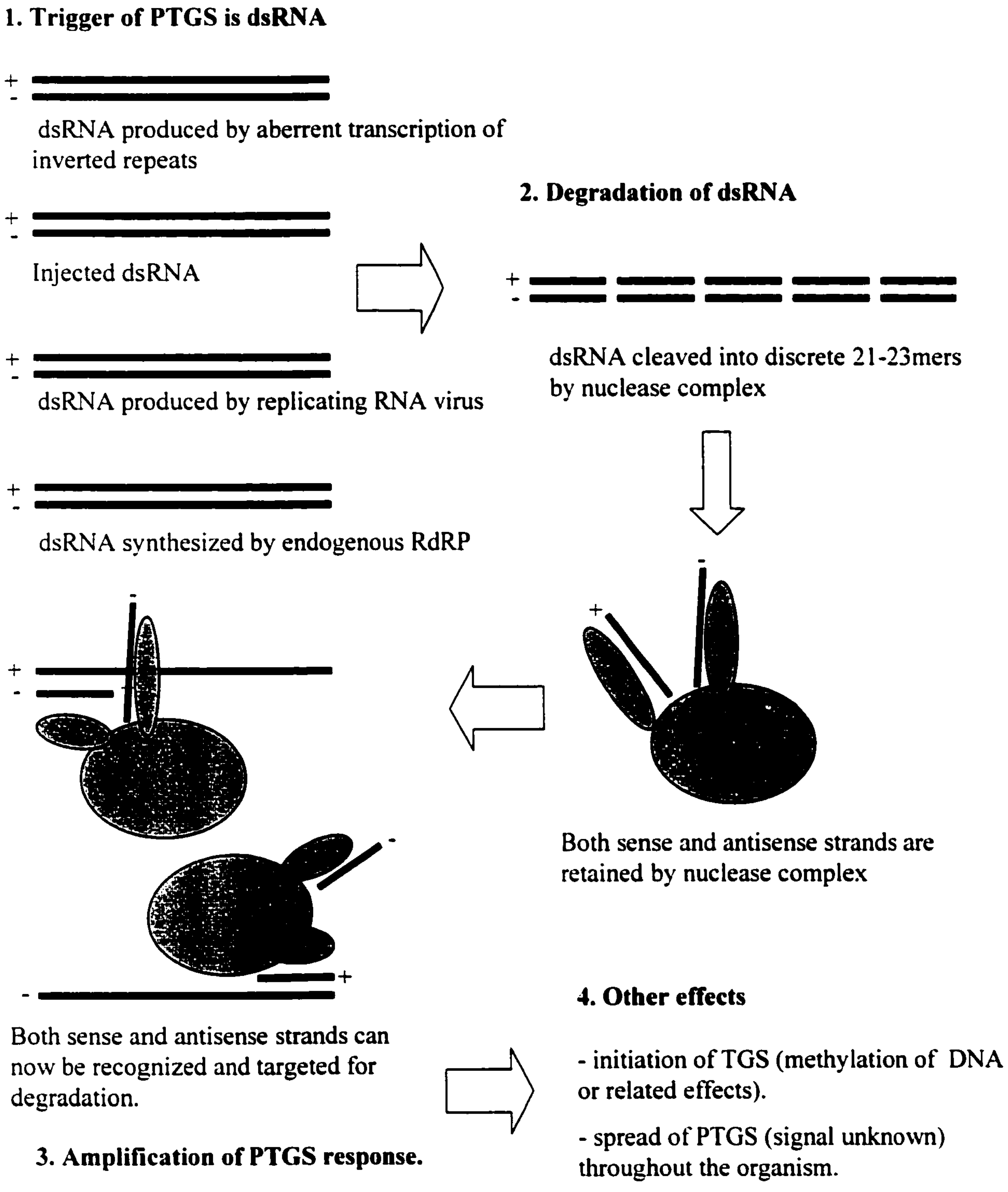
Introduction of dsRNA by direct (injection) or indirect methods (virus expression system, transgene) has since been shown to result in gene silencing phenomena in many organisms including *Drosophila* (Kennerdell and Carthew, 2000; Misquitta and Paterson, 1999), *H. virescens* (tobacco bud worm) (Hajos *et al.*, 1999), *Paramecium* (Ruiz *et al.*, 1998a), and *Trypanosoma brucei* (Ngo *et al.*, 1998). In addition, a higher proportion of PTGS phenotypes have been obtained in plants after transformation with transgenes designed to express dsRNA at a much higher frequency than with expression of sense or antisense RNA alone (Chuang and Meyerowitz, 2000; Smith *et al.*, 2000; Waterhouse *et al.*, 1998).

Lindbo *et al* (1993) first introduced the hypothesis that an endogenous plant-encoded RdRP may be responsible for amplifying a targeted RNA by synthesizing small complementary RNA strands. Since then RdRPs have been isolated from tomato plants (Schiebel *et al.*, 1998) and identified in *Arabidopsis* plants (Dalmay *et al.*, 2000b), *C. elegans* (Smardon *et al.*, 2000), and *Neurospora crassa* (Cogoni and Macino, 1999). In both *C. elegans* and *N. crassa* mutant RdRPs result in defective RNA interference (Cogoni and Macino, 1999; Smardon *et al.*, 2000). The amplification of both RNA strands by an induced RdRP could explain how extremely low levels of dsRNA could trigger the elimination of abundant messages. Alternatively, an induced RdRP could produce complementary RNA from a transcript recognized to be aberrent, creating dsRNA and triggering the silencing response. Work on the *Arabidopsis* RdRP has shown that mutants defective for this protein are unable to silence transgenes, consistent with the findings in both *N. crassa* and *C. elegans* (Dalmay *et al.*, 2000b). However, endogenous phytoene desaturase activity was silenced in *Arabidopsis* RdRP mutant plants infected with a tobacco rattle virus (TRV) carrying a portion of the phytoene desaturase gene (Dalmay *et al.*, 2000b). TRV produces a dsRNA replicative intermediate as part of its replication cycle. This suggests that the plant RdRP functions to synthesize complementary RNAs leading to the formation of the dsRNA trigger, but if dsRNA is already present the activity of the RdRP is redundant (Dalmay *et al.*, 2000b). A number of genetic loci have been found to encode proteins essential to the initiation and/or the maintenance of the gene silencing phenotype in plants (Dalmay *et al.*, 2000b; Mourrain *et al.*, 2000), *C. elegans* (Grishok *et al.*, 2000), *N. crassa* and others (Bosher and Labouesse, 2000). The precise function of these proteins in the establishment/ maintenance of gene silencing remains to be established, but will no doubt shed light on what has been an uncertain mechanism for a very long time.

One of the most interesting observations related to PTGS is its ability to spread throughout the organism (reviewed in Fagard and Vaucheret 2000). Researchers studying virus-induced gene silencing (where a virus expression system with a dsRNA replicative intermediate is used to express a host gene, resulting in the silencing of the host gene) observed that silencing occurred in a uniform fashion, even though virus replication occurred in a variegated manner (Ruiz *et al.*, 1998b). Other researchers took this a step further, and showed that scions of silenced plants were capable of causing PTGS when grafted onto naïve plants (Palauqui *et al.*, 1997; Palauqui and Vaucheret, 1998). Spreading of PTGS has also been observed in *C. elegans*, where the local injection of dsRNA caused silencing not only throughout the injected worm, but also in its progeny (Fire *et al.*, 1998). The precise nature of the PTGS signal, however, remains unknown.

A summary of the full mechanism of PTGS as it is understood presently, is presented in Fig 1.1.

dsRNA in the cytoplasm of vertebrate cells is associated with the presence of a replicating virus, and is a potent inducer of interferon α/β (Kumar and Carmichael, 1998). This leads to a local Th1 response and the targeted destruction of cells containing the dsRNA. Nuclear dsRNA may also play a role in the regulation of genes in vertebrate cells (Kumar and Carmichael, 1998). These dsRNA molecules do not induce interferon, but instead are recognized by adenosine deaminases, which convert adenosine residues to inosines (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). Adenosine deaminases are also capable of unwinding dsRNA duplexes, and the modified strands may then interact with nuclear DNA to modulate gene expression (Kumar and Carmichael, 1998). The precise relationship between PTGS in plants and invertebrates and dsRNA-mediated gene regulation in higher organisms with more complex immune systems remains to be determined.



1. Trigger of PTGS is dsRNA

dsRNA produced by aberrent transcription of inverted repeats

Injected dsRNA

dsRNA produced by replicating RNA virus

dsRNA synthesized by endogenous RdRP

2. Degradation of dsRNA

dsRNA cleaved into discrete 21-23mers by nuclease complex

Both sense and antisense strands are retained by nuclease complex

Both sense and antisense strands can now be recognized and targeted for degradation.

3. Amplification of PTGS response.

4. Other effects

- initiation of TGS (methylation of DNA or related effects).

- spread of PTGS (signal unknown) throughout the organism.

Fig 1.1. Model of Post-transcriptional gene silencing (PTGS)/ RNA interference.

PTGS may or may not be accompanied by transcriptional gene silencing (TGS). TGS and PTGS seem to be affected by transgene copy number and organization. Assaad *et al* (1993) described in detail the phenomenon of repeat-induced gene silencing (RIGS) in plants. Plants were transformed with antibiotic resistance genes, and progeny were scored as resistant or sensitive, and the results correlated with the structure of the transgene(s). Single copy transgene plants were largely resistant, indicating normal production of the antibiotic resistance gene product. Plants with highly repeated transgenes were largely antibiotic sensitive, and contained low levels of transgene mRNA. Repeated, silenced transgenes were consistently found to be methylated, while non-silenced trangenes remained unmethylated (Assaad *et al.*, 1993). Other plant researchers have observed that the silencing effect was associated with higher transgene copy number (Assaad *et al.*, 1993; Stam *et al.*, 1997; Stam *et al.*, 2000; van der Krol *et al.*, 1990) as well as methylation of the transgene (reviewed in Vaucheret *et al* 1998). It has been well documented in both vertebrates and *Drosophila* that chromosome structure and accessibility such as DNA methylation, histone deacetylation or ectopic pairing of repeated elements (Danam *et al.*, 1999; Garrick *et al.*, 1998; Henikoff, 1998; Razin, 1998) affect the transcription/silencing of genes (Boivin and Dura, 1998; Razin, 1998). It has been theorized that ectopic pairing of repeated elements leads to the production of aberrent RNAs (Assaad *et al.*, 1993; Wassenegger and Pelissier, 1998), which are then recognized and used as templates by the host RdRP, leading to the dsRNA trigger of gene silencing. Alternatively, aberrent RNAs may be transcribed from both strands, directly forming dsRNA. Thus ectopic pairing may be more important for providing the trigger for PTGS, while forms of TGS such as DNA methylation and histone deacetylation may be more important for the long-term maintenance of gene silencing.

Potential of PTGS/RNAi as a control measure for DEN viruses

With descriptions of PTGS being made in such a wide-ranging group of organisms, it seems likely that a PTGS/RNAi mechanism should exist in *Ae. aegypti* mosquitoes as well. This idea is supported by observations of PTGS in *Drosophila* (Kennerdell and Carthew, 2000; Misquitta and Paterson, 1999), an organism closely related to *Ae. aegypti*. If this is indeed the case, the expression of dsRNA derived from the genome of DEN viruses should then activate PTGS, which would target the degradation of a homologous DEN virus genome as well. This represents a powerful control strategy as it takes advantage of an endogenous cellular control mechanism, and does not involve the expression of foreign gene products. Also, virus escape from PTGS would be expected to be much more difficult than escape from a protein-mediated interference strategy, as >15-20 % nucleotide dissimilarity across the targeted region would be necessary to escape PTGS (Gaines *et al.*, 1996; Powers *et al.*, 1996). Several plant viruses have been shown to encode inhibitors of PTGS (Anandalakshmi *et al.*, 1998; Lucy *et al.*, 2000). DEN viruses may also encode such a protein, the function of which may be vital to establishing a lifelong infection in the mosquito. Identifying such an activity might provide further insights into controlling DEN virus replication in mosquitoes.

Germline transformation of plants and fruit flies has become routine, transformation of the mosquito *Ae. aegypti* has only recently been achieved. P-elements are used to transform *Drosophila*, however the P-element transposase has been shown to be non-functional in *Ae. aegypti* embryos (reviewed in Carlson *et al* 1995). The need for a transposable element-based transformation system in mosquitoes led to the isolation and characterization of other small, class II transposable elements such as *Hermes*, *mariner*, and *Minos* from insects (Atkinson *et al.*, 1993; Franz and Savakis, 1991; Medhora *et al.*, 1991), as well as *piggyBac*, isolated from an insect virus (Fraser *et al.*, 1996). Class II elements are small (typically ~3kb), and contain short inverted repeat sequences (<50 bp) flanking the coding region of the transpose which can act *in cis* or *in trans* via DNA-excision and re-insertion. Successful transformation events in *Ae. aegypti* have now been reported using the transposable elements *Hermes* (Jasinskiene *et al.*, 1998; Pinkerton *et al.*, 2000) and *mariner* (Coates *et al.*, 1998). Transformation with *mariner* was shown to be transposase-mediated, and not the result of illegitimate recombination. Also, the activities of salivary gland-specific and constitutive promoters have been described in *Ae. aegypti* (Coates *et al.*, 1999; Pinkerton *et al.*, 2000). The incorporation of these promoters in transposable elements to drive the expression of dsRNA (derived from DEN viruses) could then result in transformed mosquitoes, completely refractory to DEN infection in a tissue or temporally-specific manner. Thus the use of transposable elements such as *Hermes* or *mariner* to transform *Ae. aegypti* mosquitoes will not only accelerate studies of the genetics of this important disease vector, but will allow the stable expression of genes or sequences (dsRNA) shown to have a strong anti-viral activity. Because these elements can re-mobilize, it may be possible to use such elements to spread a virus-resistant phenotype throughout a natural mosquito population (Ribeiro and Kidwell, 1994).

The studies described in this dissertation aimed to identify genes capable of mediating RNA interference against each of the four DEN virus serotypes, using both transient and stable RNA expression systems. Candidate genes could then be used in future studies designed to generate virus-resistant transgenic mosquitoes. Additionally we aimed to determine whether resistance to DEN viruses in mosquitoes was more consistent with the direct or indirect models of RNA-mediated interference, as described above.

Chapter 2

Examination of the mechanism of RNA-mediated resistance to DEN-2 virus in mosquito cells, direct vs indirect models.

INTRODUCTION

RNA-mediated resistance to DEN-2 virus (Jamaica 1409) has been achieved in mosquito cells by using dsSIN viruses to express sense or antisense sequences derived from the pre-membrane and capsid genes of DEN-2 (Gaines *et al.*. 1996). Successful RNA-mediated interference against yellow fever (YF) virus also has been observed in dsSIN infected mosquito cells that express RNA encoding the GDD motif of the YF NS5 protein (Higgs *et al.*. 1998; Rayner, 1998). The following studies were designed to determine whether these observations of RNA-mediated interference to flaviviruses were more consistent with the direct model of antisense interference or the indirect model of gene silencing.

In the direct model. effector RNA molecules (in either sense or antisense orientation) would interact with the complementary DEN-2 genome RNA (either the minus or plus strand) directly and block translation or replication of the viral RNA (Nellen and Lichtenstein. 1993; Sczakiel. 1997). Effector RNA molecules must share enough sequence homology to allow complementary RNA sequences to form dsRNA duplexes. and these duplexes would be degraded by an RNase III (which degrades dsRNA)-like cellular housekeeping protein (reviewed in Simons 1988). Virus-resistant phenotypes in the direct model rely only on delivery of a sufficient amount of effector RNA sequence to the cells and the ability of the effector RNA to bind a target RNA sequence.

The indirect model involves a sequence-specific cellular response to a dsRNA trigger (Fire. 1999: Montgomery and Fire, 1998). The amount of effector RNA sequence introduced is less important than its double-stranded nature. Cellular proteins then cleave the dsRNA into discrete lengths (21-25 nt) (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Zamore *et al.*, 2000). These small RNA molecules of both sense and antisense polarities are then retained by an RNA nuclease complex, and used to identify and degrade any complementary RNAs that may be present within the cell (Hammond *et al.*, 2000).

A double subgenomic Sindbis virus expression system (dsSIN) was used to transiently express DEN-derived RNA sequences in mosquito cells. The dsSIN virus expression system was developed for the expression of heterologous genes or sequences <2 kilobases in length (Hahn *et al.*, 1992). dsSIN viruses replicate in the cytoplasm of mosquito cells, and produce high levels (10^4-10^5 copies per cell) of mRNA transcripts (Powers *et al.*, 1996). dsSIN viruses have been used for the expression of foreign proteins such as green fluorescent protein (GFP) and chloramphenicol acetyltransferase (CAT); mosquito proteins such as defensins A and C; viral proteins such as DEN-2 prM and LAC N; and for the expression of non-translatable RNA sequences (Olson *et al.*, 1994; Olson *et al.*, 2000), (Olson, unpublished results),(Gaines *et al.*, 1996; Higgs *et al.*, 1998; Johnson *et al.*, 1999; Olson *et al.*, 1996; Powers *et al.*, 1996; Powers *et al.*, 1994).

RNA virus-expression vectors have been used in plants to generate resistance to unrelated viruses, by expressing a portion of the target RNA virus genome (Pang *et al.*, 1997; Ratcliff *et al.*, 1999). This phenomenon has been called RNA-mediated cross protection (Ratcliff *et al.*, 1999), and has been shown to operate through the PTGS pathway (Hamilton and Baulcombe, 1999). RNA virus-expression systems have also been used to silence plant viruses (Ruiz *et al.*, 1998b). Similarly, the expression of luciferase RNA sequences from a dsSIN virus resulted in a decrease in the amount of luciferase activity in the salivary glands of mosquitoes transformed to stably express luciferase (Johnson *et al.*, 1999).

The direct model of RNA-mediated interference was the hypothesis underlying the design of previous challenge experiments with DEN viruses. These challenges were all performed when the amount of DEN RNA produced from a recombinant dsSIN virus was highest, thus providing the maximum concentration of effector RNA molecules. Peak levels of dsSIN RNA in C6/36 cells occur between 24 and 48 h post infection (Gaines *et al.*, 1996; Powers *et al.*, 1996). Forty eight hours after infection is also the peak in expression of CAT protein from a recombinant TE/3'2J (dsSIN) virus (Olson *et al.*, 1994).

The indirect model predicts that if the dsSIN virus induced a gene silencing response, resistance to DEN viruses would not require maintenance of high levels of dsSIN produced antisense or sense RNA. In this study cells were challenged at later time points after dsSIN virus infection to determine whether mosquito cells were resistant or became susceptible to DEN-2 virus after dsSIN viruses expressing DEN RNA sequences had shifted from productive to persistent infections (Riedel and Brown, 1977).

Another aspect of the mechanism of RNA-mediated interference that was examined in this study was the time after DEN-2 virus challenge when interference occurred. The indirect model predicts that interference would be immediate in cells exposed to DEN RNA sequences (dsRNA) prior to challenge. While in the direct model this is true for expressed antisense RNA, which could bind to DEN genome immediately after uncoating, sense RNA could not effect interference until negative strand synthesis begins. To test this we took advantage of an observation known as superinfection exclusion. Superinfection exclusion is a process whereby infection of a cell by a virus provides the cell with resistance to infection by related viruses. Superinfection exclusion, also referred to as homologous interference and heterologous interference (between closely related viruses) was reported as early as 1968 (Zebovitz and Brown, 1968). Homologous and heterologous interference have been observed in many virus families including flaviviruses (Dittmar *et al.*, 1982; Venugopal and Gould, 1992), alphaviruses (Karpf *et al.*, 1997; Stollar and Shenk, 1973) and bunyaviruses (Sundin and Beaty, 1988). Approximately 8 h after infection with one of the four serotypes of DEN virus, C6/36 cells begin to become resistant to superinfection with a heterotypic DEN virus (Dittmar *et al.*, 1982). These studies were driven by the hypothesis that expression of DEN RNA sequences in cells prior to infection with DEN-2 virus would prevent DEN-2 virus from establishing superinfection exclusion of heterologous DEN viruses.

MATERIALS AND METHODS

Cell lines. BHK-21 and C6/36 (*Aedes albopictus*) mosquito cells were grown in Liebowitz-15 (L-15) medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. C6/36 cells were maintained in L-15 medium containing 2% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Viruses. DEN-2 (Jamaica 1409) C6-5 and DEN-4 (H-241) p28C6-8 viruses were obtained from the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Ft. Collins, CO (Deubel *et al.*, 1988; Hammon *et al.*, 1960; Sabin, 1952). pXX indicates the number of passages in an unknown host, C6-X indicates the number of passages in C6/36 cells.

RT-PCR. Viral RNA was extracted from virus particles in the culture medium of infected C6/36 cells using the QiaAmp Viral RNA isolation kit. (Qiagen Inc, Chatsworth, CA) RNA was reverse transcribed using 75 units of Superscript II (Gibco BRL, Rockville, MD) and amplified using Taq polymerase (Promega, Madison, WI) in one reaction. (42^{0}C for 1 hr; 92^{0}C, 30 sec; 52^{0}C, 1 min, 72^{0}C, 2 min; 26 cycles; 72^{0}C, 7 min.). RT-PCR buffer contained 50 pmol of each primer (D2prM290 F 5' ACCACACGTAACGGAGAACCA 3'; R 5' TCCCACATGTGGAACGAGTGC 3') in 1X Taq buffer (Promega, Madison, WI), 250 pmol dNTPs and 2.5 mM $MgCl_2$.

Construction of dsSIN plasmids. The cloning strategy for inserting DEN-2 prM genome sequences into the dsSIN cDNA pTE/3'2J is shown in Fig 2.1. D2prM290 RT-PCR product was inserted directly into the EcoR V site of pBluescript (Stratagene, La Jolla, CA), which had been modified by incubation with 40mM dTTP, 5mM $MgCl_2$ and 15 units of Taq polymerase (Promega, Madison, WI), yielding pB2prM290. pB2prM290 was digested with Xba I/ Nhe I and the resulting D2prM290 fragment was inserted into the Xba I site of pTE/3'2J, producing pD2prM290As and pD2prM290S. Orientation of DEN-2 prM sequence with respect to the second subgenomic promoter was confirmed with PCR and restriction enzyme digests.

dsSIN virus production. dsSIN virus was produced as has been described (Gaines *et al.*, 1996; Hahn *et al.*, 1992). RNA was transcribed *in vitro* from the SP6 promoter of linearized dsSIN plasmids in the presence of 1 X transcription buffer, 1 mM cap analog, 50 U Rnasin, 40 U SP6 RNA polymerase (Ambion, Austin TX), 1mM rNTPs, 5 mM dithiothreitol and 1.0% bovine serum albumin (Promega, Madison, WI). *In vitro* transcribed RNA was electroporated into BHK-21 cells using a BTX electro cell

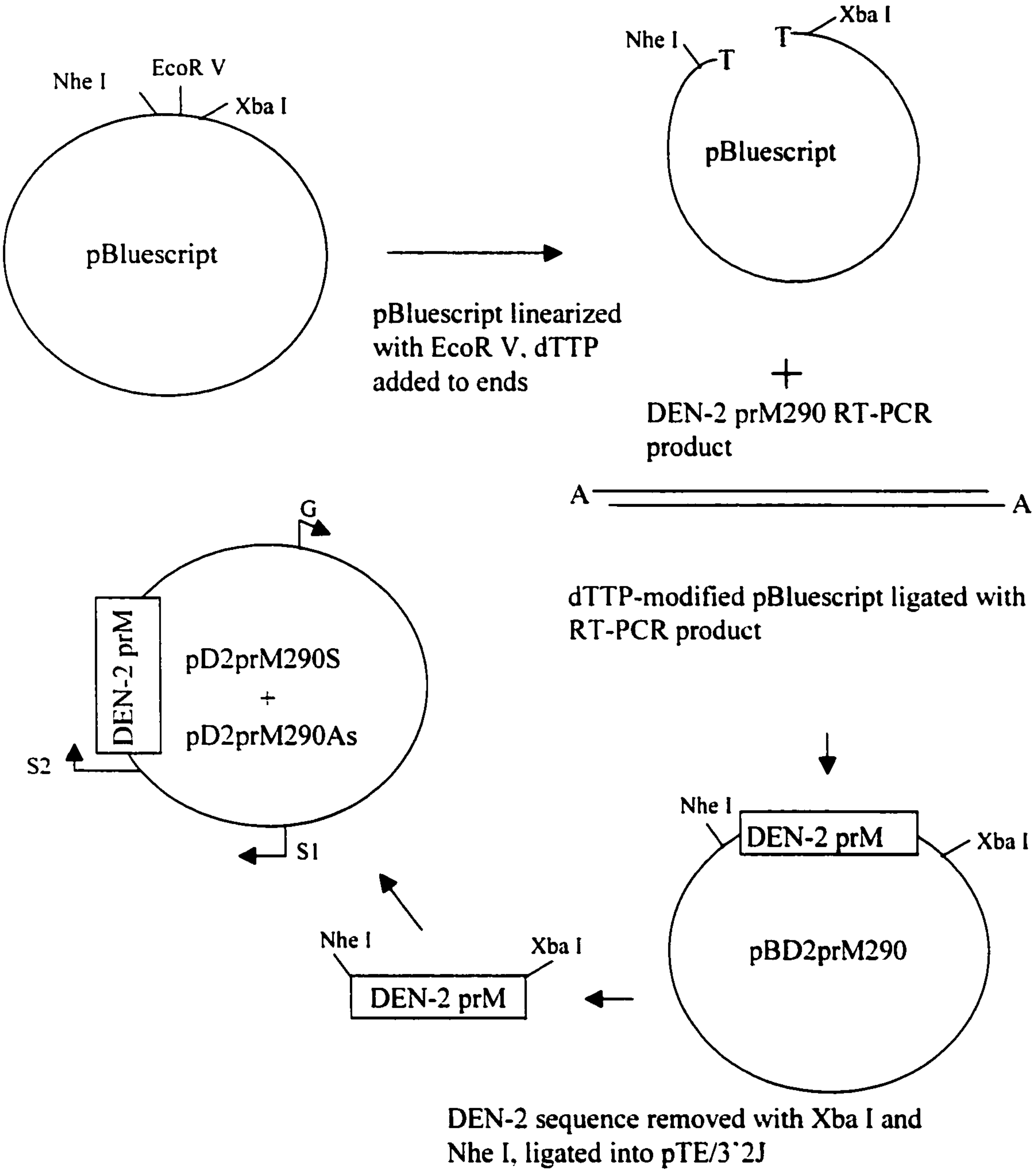


Fig 2.1. dsSIN cloning strategy. Cloning strategy used to create dsSIN plasmids with DEN-2 prM290 sequence in sense or antisense orientation downstream of the second subgenomic promoter (S2) of pTE/3'2J.

manipulator 600™ at 450 V, 100 µF, and 720 Ohms. Cells were pulsed twice, with a pulse length of 0.8-1.0 msec per pulse. Electroporated cells were seeded into 25cm^2 flasks in 5 ml of growth medium. Twenty-four hours later culture medium was collected, cell debris was removed by centrifugation, and virus was stored at –70° C. An aliquot of virus was titrated in an end-point assay using BHK-21 cells. Titers were expressed as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/ ml (Karber, 1931). Production of recombinant dsSIN virus RNA was analyzed by northern blot hybridization two days after infection of C6/36 cells.

Cell culture interference assay. C6/36 cells were seeded on 18mm glass coverslips in 12-well plates or 25cm^2 flasks and grown at 28°C. When the cells were approximately 80% confluent, they were infected at a multiplicity of infection (MOI) of 20–50 with either a control dsSIN virus (TE/3'2J) or a recombinant dsSIN virus containing a DEN sequence inserted in its genome. Virus was diluted into L-15 so that the total infection volume was 300 µl per well for a twelve well plate or 1 ml per 25cm^2 flask. Virus was allowed to adsorb to the cells for 30 min at ambient temperature with constant, gentle rocking. Infection medium was then removed and the cells were allowed to recover in L-15 growth medium (4 h to overnight). The virus-exposed cells were then transferred to maintenance medium to prevent overgrowth during the incubation period. At 48 h post infection a sample coverslip for each virus used was removed and assayed by immunofluorescence for the presence of SIN E1 antigen. If SIN E1 antigen was detected in all cells, the remaining monolayers and uninfected control cells were challenged with a

DEN virus at an MOI of 0.1. The protocol for infecting with challenge DEN viruses was the same as described for dsSIN virus infections.

Immunofluorescence assay. Immunofluorescence assays (IFA) were performed using monoclonal antibodies (Mab) 30.11a, which binds to SIN E1 antigen; 8613 or 4G2, which bind to DEN envelope antigen, as primary antibodies for detection of viral antigen in C6/36 cells (Clegg *et al.*, 1983; Gentry *et al.*, 1982; Gould *et al.*, 1985). Mab 1-H10-6 was used for DEN-4 specific staining (Henchal *et al.*, 1982). Acetone-fixed cells on glass coverslips in twelve-well plates were incubated in 400 µl (per well) of a 1:200 dilution of primary antibody in phosphate buffered saline (PBS) for 40 min at 37°C in a humid chamber. Coverslips were washed 3 X with 3-4 ml (per well) of PBS and then incubated with 400 µl of a 1:200 dilution of biotinylated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ), and a 1:200 dilution of 5.0% Evans blue counterstain for 40 min at 37°C in a humid chamber. Coverslips were washed 3 X with 3-4 ml of PBS, and incubated for 10 min in 400 µl of a 1:200 dilution of streptavidin-fluorescein (Amersham Pharmacia Biotech, Piscataway, NJ) at 37°C in a humid chamber. Coverslips were washed 3 X with 3-4 ml of PBS and mounted on glass slides with 3:1 glycerol:PBS. Immunostained cells were visualized with an Olympus BH-2 fluorescent microscope.

Estimate of the percentage of DEN infected cells. Six days following DEN virus infection cells in 12 well plates were fixed by decanting the medium, adding acetone: PBS (3:1), and incubating at -20°C for 5 min. The cells were washed once with PBS and examined by IFA to determine the proportion of cells containing DEN virus envelope antigen. Typically three fields of view from each coverslip were photographed using an

Olympus DP10 digital camera. All digital files were modified in Adobe Photoshop by adjusting the brightness by +20 and the contrast by +75. Cells containing DEN virus antigen were counted in Adobe Photoshop and expressed as a ratio to the total number of cells counted. All experiments were performed at least twice, each experiment analyzing duplicate coverslips each time, and the percentage of infected cells was expressed as the average of all trials.

RNA analysis. Total cellular RNA was extracted from infected or uninfected C6/36 cells in 25cm^2 flasks according to the manufacturer's specifications using the Qiashredder and RNeasy mini kits (Qiagen Inc., Chatsworth, CA). For northern blot analysis, 5 µg of total RNA was electrophoresed in a 1% agarose/ 1X MOPS (0.023M MOPS, 8.3 mM NaOAc, 0.2 mM EDTA)/ 2% formaldehyde gel and blotted to a positively charged nylon membrane (Brightstar from Ambion, Inc., Austin, TX). Northern blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored at -20°C until hybridization. For slot blot analysis, 5 µg total RNA was denatured in 150 µl of 6X SSC, 7% formaldehyde at 65°C for 10 min and loaded onto a nylon membrane (Brightstar from Ambion, Inc., Austin, TX) using a Minifold II slot blot apparatus (Schleicher +Shuell, Keane, NH). Wells were washed with 100 µl of 6X SSC, 7% formaldehyde. RNA slot blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored at -20°C until hybridization.

Hybridization and detection of RNA blots. An oligonucleotide (5' GCTGGTCGGATCATTGGGGCG 3') complementary to the 3' end of the SIN RNA non-coding region was used to detect dsSIN RNA. PCR products corresponding to portions of the DEN-2 E gene (nt 1642 – nt 1912) and DEN-4 C gene (nt 104 – nt 344) were used to detect DEN-2 and DEN-4, respectively. Probes were labeled with a psoralen-biotin conjugate according to the manufacturer's specifications (Ambion, Austin, TX). Labeled probes were added to hybridization buffer (7% SDS, 0.47 M Na_2HPO_4, 1.7 mM H_3PO_4) and hybridized overnight at 42°C for primer probe and 65°C for PCR product probes. Blots were washed twice with 2X SSC, 0.1%SDS at 20 min each, followed by 2 washes in 0.2X SSC, 0.1% SDS for 30 min each. Blots were then immediately subjected to Ambion's Brightstar Non-Isotopic Detection Kit and exposed to Kodak ML light film.

RESULTS

Characterization of recombinant dsSIN viruses. The titers of dsSIN viruses produced in BHK-21 cells ranged from 7.5-8.8 $\log_{10}TCID_{50}$/ml. Only dsSIN viruses that gave the required MOI of 10-50 were used in DEN challenge experiments. Northern blot analysis showed that C6/36 cells infected with recombinant dsSIN viruses D2prM290S and D2prM290As contained virus-specific, genomic RNA and two subgenomic RNAs. The size of each subgenomic RNA was larger than the TE/3'2J subgenomic RNA by the size of the insert sequence (Fig 2.2a). Subgenomic mRNAs from recombinant dsSIN viruses D2prM290As and D2prM290S were also shown to be of the expected strand polarity (Fig 2.2b).

Sense and antisense RNA-mediated interference to DEN-2. Recombinant dsSIN viruses D2prM290S and D2prM290As were tested for their ability to interfere with homologous and heterologous challenge viruses in C6/36 cells. Prior infection with both recombinant dsSIN viruses substantially reduced the amount of DEN E antigen

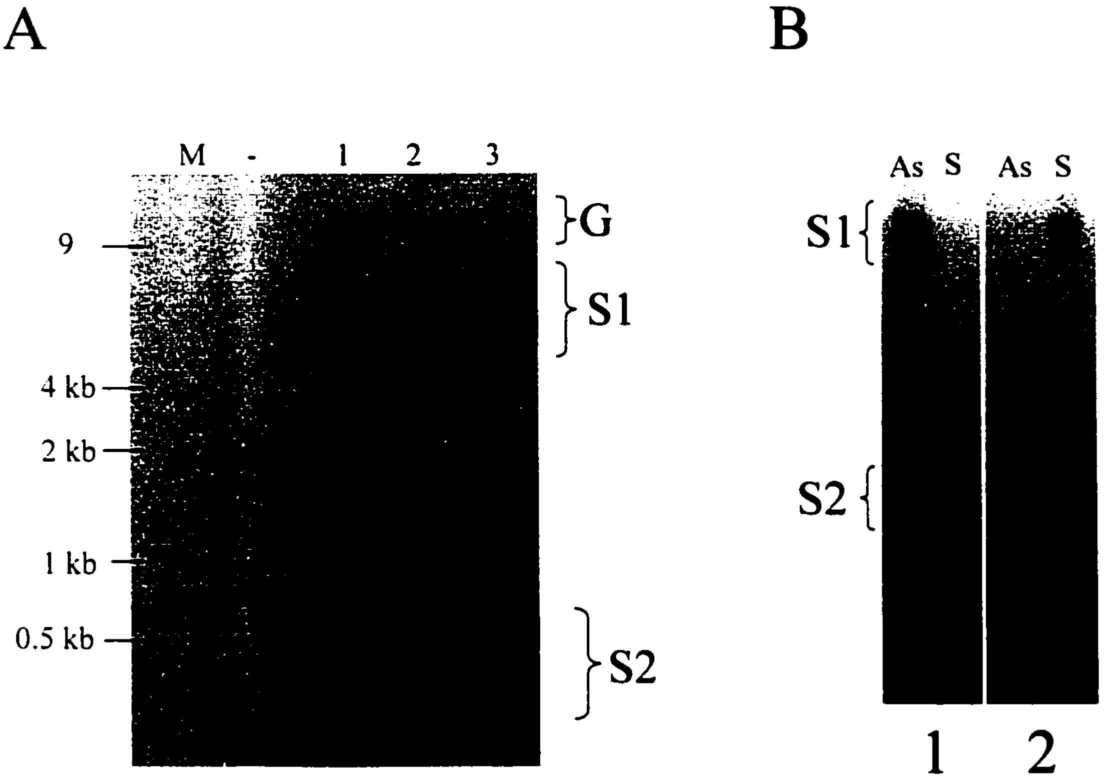


Fig 2.2. Analysis of dsSIN-expressed D2prM290 RNA. (A) Northern blot analysis of total cellular RNA extracted from uninfected C6/36 cells (-) or C6/36 cells 48 h after infection with recombinant dsSIN viruses (1) TE/3'2J, (2) D2prM290As, (3) D2prM290S. Blot was hybridized to a SIN specific probe; genomic (G), 1st subgenomic (S1) and 2nd subgenomic (S2) mRNAs are indicated. (B) Total RNA extracted from C6/36 cells infected with D2prM290As and D2prM290S was probed for (1) antisense DEN-2 prM or (2) sense DEN-2 prM sequence.

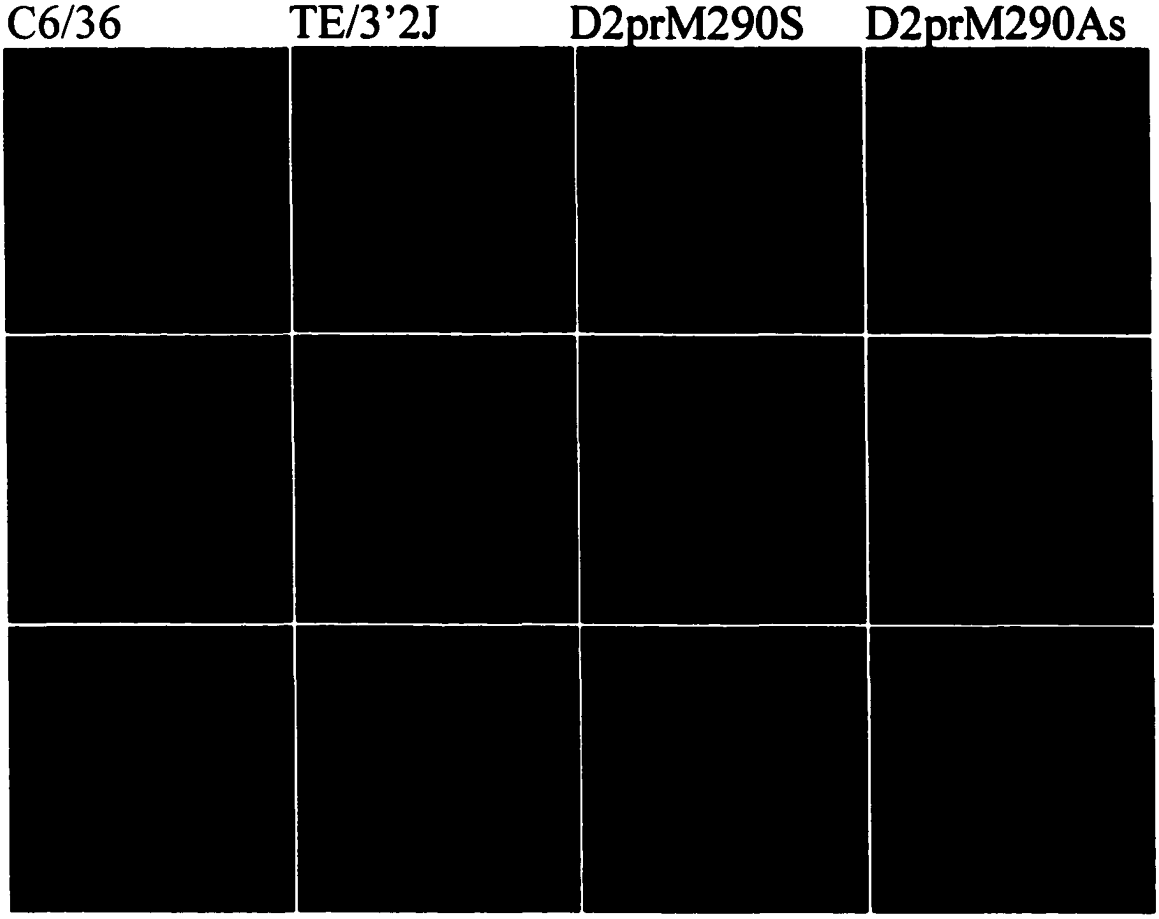
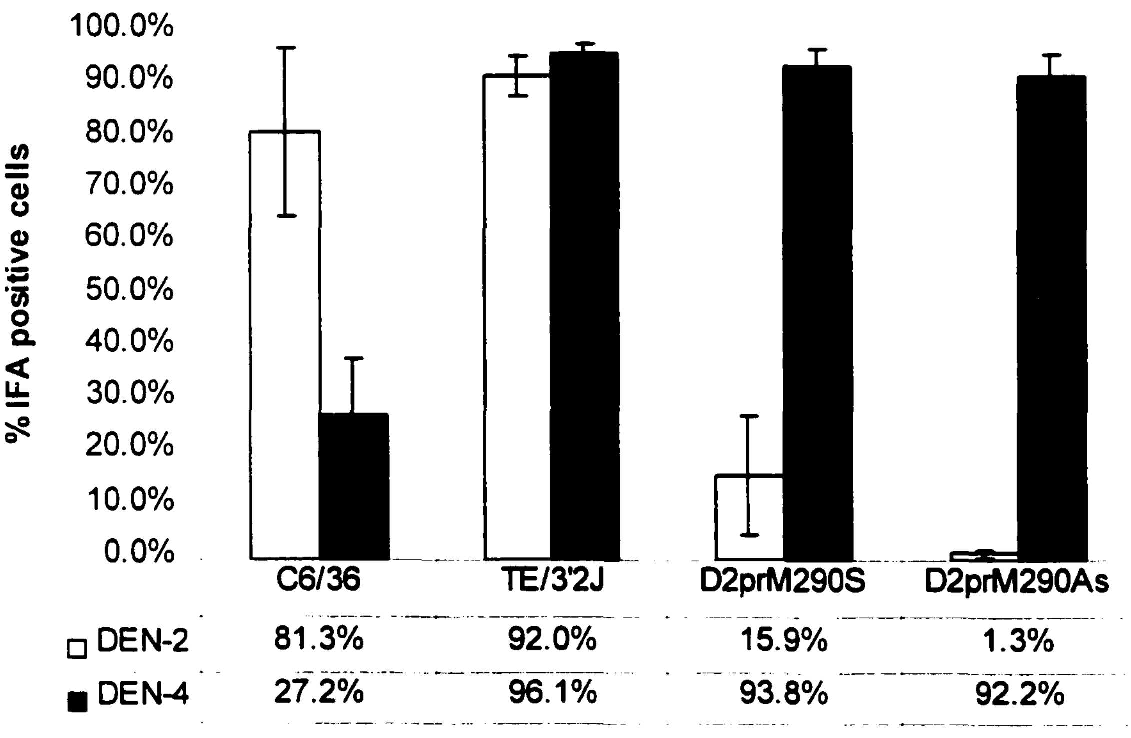


Fig 2.3. IFA of C6/36 cell culture interference assays. C6/36 cells infected with dsSIN viruses (indicated at top) were challenged with DEN-2 (middle row) or DEN-4 (bottom row) virus. Top row: cells stained with antibodies specific for SIN E1 at 48 h post SIN infection; Middle row: cells stained with antibodies specific for DEN-2 E antigen (3H5); Bottom row: cells stained with antibodies specific for DEN-4 E antigen (1-H10-6).

containing cells when challenged with the homologous DEN-2 virus, but not when challenged with the heterologous DEN-4 virus as determined by IFA (Fig 2.3). Counting cells showed that prior infection with the D2prM290As virus protected >98% of the cells from DEN-2 virus (Fig 2.4). The D2prM290S virus prevented the replication of DEN-2 in about 84% of the cells, while 80-90% of C6/36 or TE/3'2J-infected C6/36 cells supported the replication of DEN-2 virus. More than 90% of the cells pre-infected with dsSIN viruses TE/3'2J, D2prM290S or D2prM290As contained DEN-4 antigen, indicating that the D2prM290 sequence does not offer cross-protection to the heterologous DEN-4 virus.

The ability of recombinant dsSIN viruses D2prM290S and D2prM290As to inhibit DEN-2 and DEN-4 virus RNA accumulation was also examined (Fig 2.5 a+b). Both recombinant dsSIN viruses were able to inhibit DEN-2 virus RNA accumulation as compared to mock-infected C6/36 cells and TE/3'2J infected C6/36 cells, but D2prM290As was clearly more effective than D2prM290S. Neither D2prM290As nor D2prM290S affected the accumulation of DEN-4 virus RNA (Fig 2.5b). Duplicate blots probed for the mosquito actin mRNA confirmed equal loading of wells (not shown).

dsSIN RNA accumulation over time. dsSIN virus mRNA levels were also examined in the same samples as described in Fig 2.4a. Three northern blot analyses were performed using RNA extracted from cells on days 2-8 post dsSIN virus infection (TE/3'2J, D2prM290S, and D2prM290As viruses). There was no observed difference between the levels of genomic and subgenomic dsSIN mRNAs in TE/3'2J infected/DEN-2 challenged and D2prM290As infected/DEN-2 challenged cells (Fig 2.6a). dsSIN RNA levels in D2prM290S infected/DEN-2 challenged cells were similar initially, but seemed to



	C6/36	TE/3'2J	D2prM290S	D2prM290As
□ DEN-2	81.3%	92.0%	15.9%	1.3%
■ DEN-4	27.2%	96.1%	93.8%	92.2%

Fig 2.4. Percentage of C6/36 cells containing DEN-2 or DEN-4 antigen 6 days post challenge. C6/36 cells were pre-infected with recombinant dsSIN viruses (TE/3'2J, D2prM290S, D2prM290As) and challenged with DEN-2 or DEN-4 virus 48 h later.

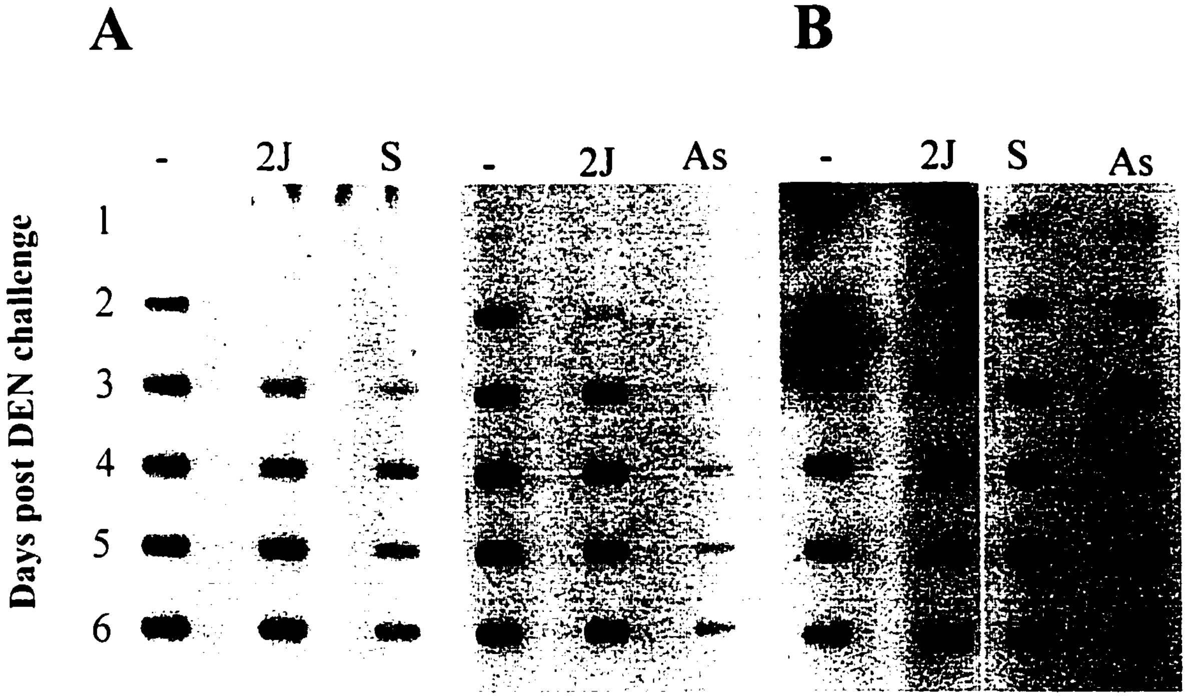


Fig 2.5. DEN-2 and DEN-4 RNA accumulation in C6/36 cells. Slot blot analysis comparing either DEN-2 (A) or DEN-4 (B) RNA accumulation in C6/36 cells that had been either mock-infected (-), or infected with the recombinant dsSIN viruses TE3'2J (2J), D2prM290As (As) or D2prM290S (S) 48 h prior to DEN challenge.

decline faster (by day 4 compared to day 5) than TE/3'2J or D2prM290As infected cells. However, for each virus, the amount of mRNA peaked at two days post dsSIN infection, and all viral mRNA species declined at least 100 fold by 8 days post infection (Fig 2.6b).

Is dsSIN mediated RNA interference dose-dependant? The observation that dsSIN mRNA declined precipitously by 8 days post infection allowed us to test if a high expression level of RNA effector molecules was required for sense or antisense D2prM290 RNA-mediated interference to DEN-2 virus. C6/36 cells were infected with D2prM290S or D2prM290As; TE/3'2J or mock-infected cells were used as controls. Cells were analyzed by IFA for SIN E1 antigen at 2, 5 and 8 days post infection (Fig 2.7). Fluorescence was most intense at 2 days post infection, and at five days was still seen throughout the cytoplasm in a majority of cells. By 8 days, however, less than half the cells were completely fluorescing and many displayed no SIN E1 antigen. This pattern was the same for all three dsSIN viruses (TE/3'2J, D2prM290S, D2prM290As). Cells were challenged with DEN-2 virus at each of these time points and analyzed 6 days after challenge for the presence of DEN E antigen (Fig 2.8). If cells were challenged forty-eight hours after D2prM290S or D2prM290As infection, resistance to DEN-2 virus was observed in infected cells. Interference was also observed if DEN-2 challenge was delayed until 5 days after D2prM290S or D2prM290As infection, as only 1-2% of the cells contained DEN-2 antigen. If DEN-2 challenge was delayed until 8 days after D2prM290S or D2prM290As infection, resistance to DEN-2 virus was still seen in approximately 80% of the cells. Cells infected with TE/3'2J were equally permissive for DEN-2 virus, regardless of the time of challenge.

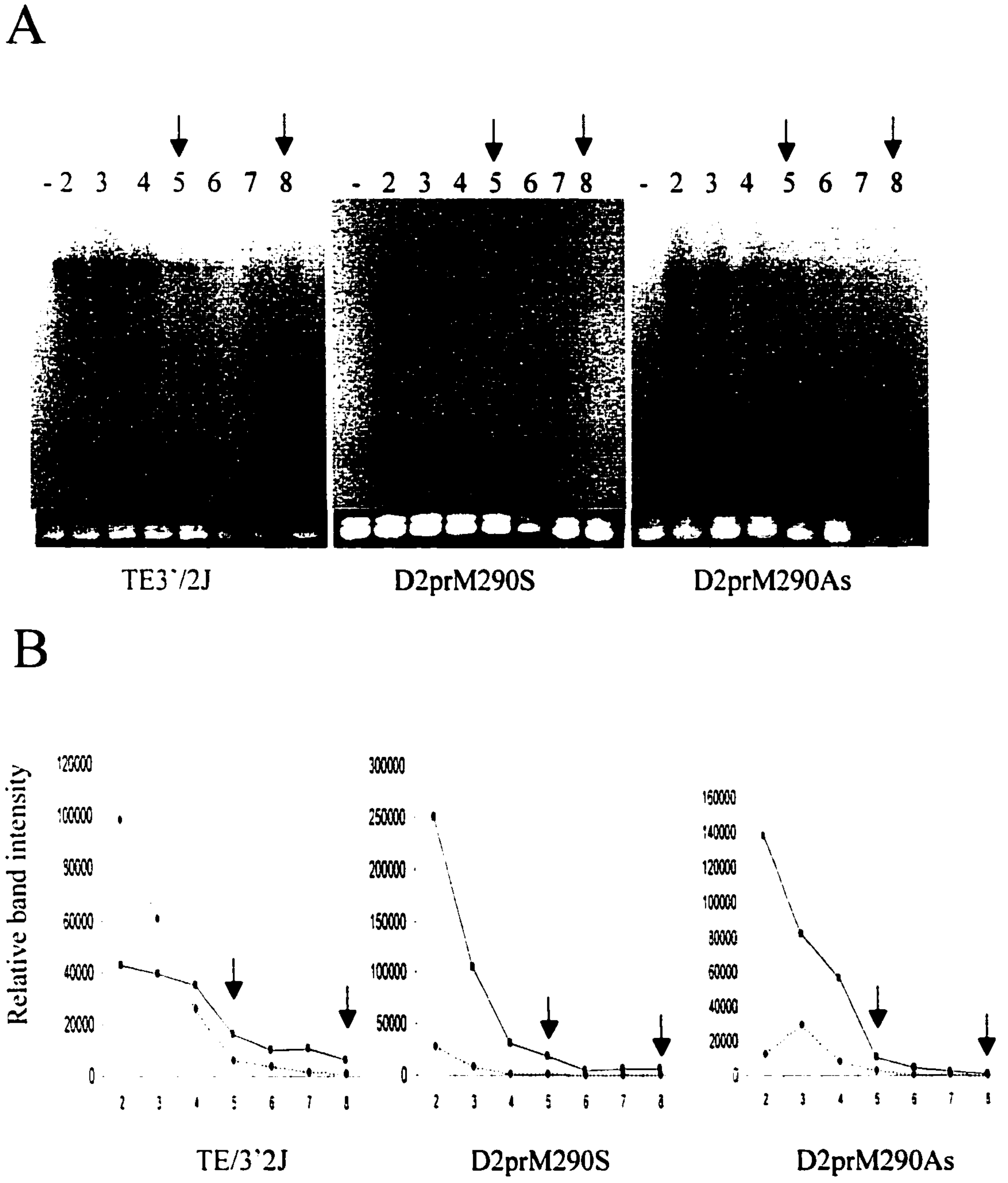


Fig 2.6. Decline in recombinant dsSIN virus mRNA over time. (A) Northern blot analysis of dsSIN transcripts 2-8 days after infection with TE/3'2J, D2prM290S or D2prM290As viruses (challenged with DEN-2 at day 2). Ethidium bromide stained ribosomal RNA shown underneath each blot to verify equal loading of lanes. (B) Graphic representation of the decline in dsSIN 1st subgenomic (dotted line) and 2nd subgenomic (solid line) mRNAs on days 2-8 post infection.

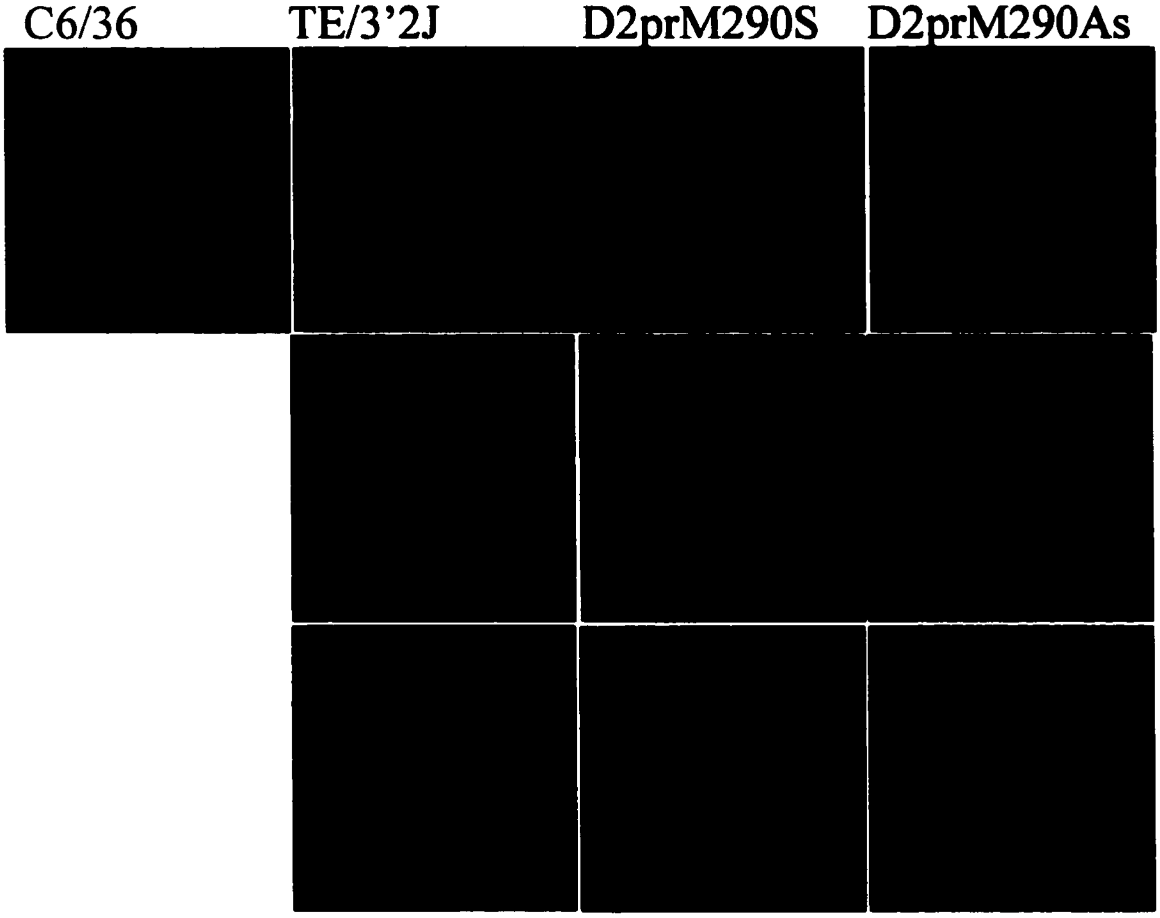
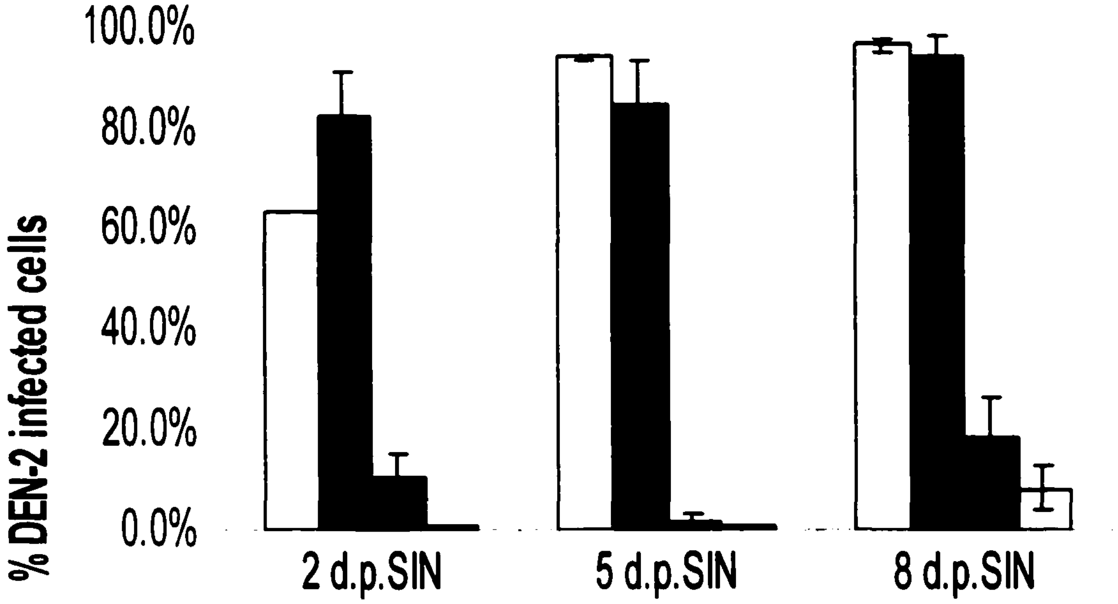


Fig 2.7. IFA of SIN E1 antigen at 2, 5, and 8 days post dsSIN virus infection. C6/36 cells were infected with recombinant dsSIN viruses TE/3'2J, D2prM290S or D2prM290As. Top row: 2 days post dsSIN virus infection. Middle row: 5 days post dsSIN virus infection. Bottom row: 8 days post dsSIN virus infection. Uninfected C6/36 cells are shown for comparison (top left)



	2 d.p.SIN	5 d.p.SIN	8 d.p.SIN
□ C6/36	63.2%	94.1%	96.5%
■ TE/3'2J	82.6%	84.7%	94.1%
■ D2prM290S	10.4%	2.0%	18.1%
□ D2prM290As	0.4%	0.8%	8.2%

Fig 2.8. RNA-mediated interference to DEN-2 virus at 2, 5 and 8 days after dsSIN infection. C6/36 cells were infected with TE/3'2J, D2prM290S or D2prM290As virus and then challenged with DEN-2 virus 2, 5, or 8 days later. Cells were assayed by IFA for the presence of DEN-2 E antigen 6 days after challenge.

Timing of dsSIN-expressed RNA-mediated interference. To determine the time after DEN-2 virus challenge when replication is inhibited in the presence of D2prM290 RNA, the phenomenon of heterologous interference was examined in the presence and absence of dsSIN-generated D2prM290 RNA. C6/36 cells were infected with D2prM290As or TE/3'2J virus. Duplicate cultures were challenged with DEN-2 48 h after recombinant dsSIN infection, and at specific time points were superinfected with DEN-4 virus. Cells were assayed for the presence of DEN-4 antigen 6 days after DEN-4 challenge. C6/36 cells alone, or cells initially infected with the TE/3'2J virus displayed resistance to DEN-4 eight hours after challenging with DEN-2: 20% and 50% of the cells contained DEN-4 antigen, respectively (Fig 2.9). The observed resistance increased by 20 h post DEN-2 virus challenge, with only 10% of the C6/36 cells and 20% of the TE/3'2J-infected cells containing DEN-4 antigen. However, at all time points, cells initially infected with D2prM290As virus and challenged with DEN-2 virus remained permissive to DEN-4 virus. This indicates that the D2prM290As virus established a state of resistance to DEN-2 virus in C6/36 cells, and silencing of the DEN-2 genome occurred within the first 8 h after DEN-2 virus challenge.

DISCUSSION

These studies confirm the finding that RNA-mediated resistance can be initiated in mosquito cells by expressing heterologous RNA with a recombinant dsSIN virus. RNA-mediated resistance described here was sequence specific, as reported previously (Gaines *et al.*, 1996; Powers *et al.*, 1996). Both the sense and the antisense prM290

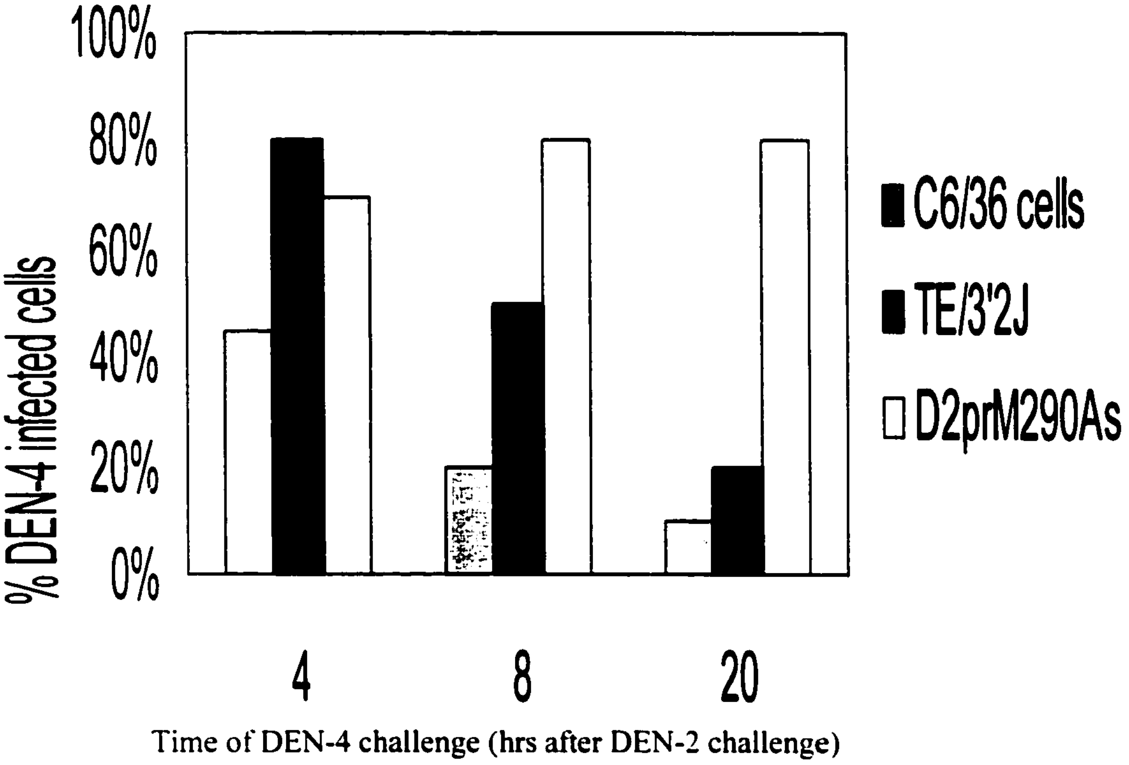


Fig 2.9. Elimination of DEN-2 mediated superinfection exclusion using dsSIN expressed D2prM290As sequence. C6/36 cells were infected with TE/3'2J, D2prM290As or no dsSIN virus. Two days later cells were challenged with DEN-2 virus, followed by a DEN-4 virus challenge 4, 8, or 20 h later. Cells were assayed for the presence of DEN-4 E antigen 6 days post DEN-4 challenge.

sequences were able to inhibit the accumulation of DEN-2 RNA genomes. This refutes the hypothesis that only the translation of DEN proteins (E antigen) was affected by the interfering RNA sequences, but not the process of RNA replication. The studies that followed shed further light on the mechanism of dsSIN produced RNA-mediated interference to DEN viruses.

Two model systems have been proposed to explain RNA-mediated interference. In the direct model. DEN-2 prM RNA sequence expressed from dsSIN viruses would act directly as an effector molecule. forming a stable RNA:RNA duplex with the target RNA: other studies in vertebrate cells suggest that such duplexes are likely to be involved in antisense interference (Sczakiel. 1997). dsSIN-transcribed antisense DEN-2 RNA would bind to the full-length. (+) strand genomic RNA of the challenge DEN-2 virus. while sense DEN-2 prM RNA expressed from a dsSIN virus would bind to the (-) strand template synthesized during replication of the challenge DEN-2 virus. Once these duplexes were formed. they would effectively prevent the translation or replication of the target RNA. The one crucial aspect of this hypothesis is that an excess of effector RNA to target RNA is necessary to bind up the target sequence (Pestka, 1992).

The alternative explanation for RNA-mediated interference is an indirect model, where expression of DEN-2 RNA by the dsSIN virus activates a cellular mechanism capable of recognizing and silencing the DEN-2 prM sequences. This silencing would then apply to the challenge DEN-2 virus RNA as well. resulting in interference. Such an indirect mechanism has been described in plants as RNA-mediated cross-protection (Ratcliff *et al.*. 1999). and is part of a more generalized mechanism known as post-transcriptional gene silencing (Baulcombe. 1999: Bruening, 1998: Grant, 1999). Models of gene silencing propose that the key inducer is dsRNA (Fire, 1999; Waterhouse *et al.*, 1998), and that even trace amounts of dsRNA can act as a trigger (Fire *et al.*, 1998). The dsSIN virus expression system used in these studies produces both dsRNA (as a replicative intermediate) and vast quantities of mRNA that could potentially act directly as effector molecules.

We attempted to distinguish between the direct and indirect models of interference by challenging cells at later time points in the dsSIN replicative cycle. Infection of C6/36 cells with SIN virus is characterized by an acute phase of high levels of virus production, followed by a state of persistence that continues indefinitely (Brown and Condreay, 1986). We repeated the finding that by 6-8 days after SIN virus infection less than half of C6/36 cells contain SIN virus-specific antigen (Riedel and Brown, 1977). Our results confirm that mRNA transcripts derived from dsSIN viruses decrease very rapidly after a peak at 2 days after infection.

Two major explanations have been put forth previously for SIN virus persistence in mosquito cells. One is cell-mediated, while the other is virus-mediated. The cell-mediated hypothesis is based on the observation that persistently infected cells produce and release a soluble peptide that conditions the state of persistence in neighboring cells. Several groups have described a dialyzable, heat-sensitive, proteinase K-sensitive factor that conditions persistence in mosquito cells for either SIN virus or Semliki Forest virus (Newton and Dalgarno, 1983; Riedel and Brown, 1979). This "antiviral activity" was virus specific, and has been shown to induce the translation of at least one cellular protein (Luo and Brown, 1994).

The virus-mediated hypothesis for the establishment of persistence is based on the observation that SIN virus regulates its own replication (Sawicki and Sawicki, 1998). Viral negative strand RNA is only produced in the presence of the P123 polyprotein, and once the nsP2 protease reaches sufficient levels, the polyprotein is cleaved, effectively ending negative strand synthesis. Negative strand shut-off occurs within six hours of SIN virus infection, thus infection is self-limiting after that point. This mechanism has been proposed to explain heterologous interference between alphaviruses as well as homologous interference (leading to persistence) between SIN viruses (Karpf *et al.*, 1997) and has also been observed in vertebrate cells (Strauss and Strauss, 1994). It is likely that dsRNA in the form of SIN replicative intermediates would only be accessible to proteins involved in establishing PTGS during this period of negative strand synthesis. After that the replication complexes (containing dsRNA) that produce positive strand RNAs are found in membrane-bound vesicles that are extremely stable (Sawicki and Sawicki, 1998). It has been observed that plant viruses can induce PTGS (Ruiz *et al.*, 1998b), and this replication strategy used by SIN virus may represent an adaptation of SIN viruses to escape PTGS effects in mosquitoes.

By delaying the DEN-2 virus challenges until SIN virus had shifted from the productive to persistent stage of replication, it was hypothesized that the indirect model of RNA-mediated interference could be tested. The results presented here show that even at 8 days after dsSIN infection the majority of cells remained resistant to the challenge DEN virus, indicating that the large excess of dsSIN-produced mRNAs are not required for interference to DEN-2 virus. These results favor the indirect model of post-transcriptional gene silencing as the mechanism of the DEN RNA-mediated interference observed against DEN-2 virus.

DEN-mediated superinfection exclusion is a process that may be quite distinct from RNA-mediated interference, which is based on close homology between the dsRNA trigger and the single stranded RNA target. While the process by which different DEN virus serotypes exclude heterologous superinfection is not understood, the fact that it occurs allowed further insight into the timing of RNA-mediated interference. Expression of an RNA sequence derived from DEN-2 virus inhibited the replication of challenge DEN-2 virus and thus ablated the ability of that DEN-2 virus to exclude DEN-4. In the original description of heterologous interference between DEN viruses (Dittmar *et al.*, 1982), it was found that this phenomenon was dependent upon protein synthesis and began to manifest within the first 8 h of DEN replication. The data presented here suggest that replication of the challenge DEN-2 virus was inhibited within the first eight hours after uncoating.

It is also important to note that although the direct model is not supported by the data presented here, it is possible that dsSIN-produced DEN-2 RNA sequences *are* acting directly as effector molecules, and that the resulting dsRNA initiates a post-transcriptional gene-silencing response. In this scenario antisense RNA effector molecules might be more likely to bind to a plus-strand DEN genome than sense RNA effector molecules to a minus-strand, as the plus-sense strand is about 10-fold more abundant then the minus-strand during DEN replication (Cleaves *et al.*, 1981). Future experiments to determine whether SIN virus itself induces a PTGS response would further clarify the results described here.

Chapter 3

RNA-mediated interference to multiple DEN virus serotypes by DEN RNA sequences expressed from a single dsSIN virus

INTRODUCTION

The goal of these studies was to document RNA-mediated interference to the remaining three DEN virus serotypes using the dsSIN virus expression system, and to determine if resistance could be generated to multiple DEN serotypes using a single, hybrid RNA sequence.

DEN viruses -1, -2, -3, and -4 share only 60-80% nucleotide sequence identity across the entire genome. Since PTGS is homology-dependent, RNA-mediated interference strategies effective against DEN-2 may not be effective against the other DEN virus serotypes. To determine whether DEN-1, DEN-3 and DEN-4 viruses are susceptible to RNA-mediated interference, RNA sequences derived from regions previously established to be capable of initiating RNA-mediated interference (capsid, prM, GDD motif encoding region of NS5; (Gaines *et al.*, 1996; Higgs *et al.*, 1998; Rayner, 1998) were tested for interference potential against DEN viruses 1, 3, and 4.

Although all sequences derived from the flavivirus genome (C, prM, NS5) tested so far have been capable of initiating RNA-mediated resistance, other work has shown that only sequences derived from the LAC virus S segment were able to provide resistance to LAC virus (Powers *et al.*, 1996). RNA sequences derived from the M segment did not initiate resistance to LAC virus (Powers *et al.*, 1996). To determine whether the sequences derived from the DEN virus genome also could vary in their ability to initiate RNA-mediated interference, sequences from two additional regions of the DEN genome we tested for interference potential. These sequences are derived from the 5' ends of the NS3 and NS5 genes. These genes were targeted because they had the highest amount of sequence conservation between serotypes (Fu *et al.*, 1992). It was reasoned that because of this greater sequence conservation, they would be better targets of RNA-mediated resistance, as they were less tolerant of sequence variation. Fig 3.1 shows the locations on the parent genome of all DEN sequences tested in this study.

Finally, recombinant dsSIN viruses were constructed to express in mosquito cells hybrid RNA sequences from multiple serotypes of DEN viruses as a single mRNA molecule. The length of each RNA segment forming the hybrid was 200-300 nt per segment. This size limit was necessary because of the packaging restrictions of the dsSIN system. Before assembly of recombinant dsSIN constructs containing hybrid sequences derived from multiple DEN viruses, a study was undertaken to determine the minimum length required for a DEN RNA sequence to initiate RNA-mediated interference. Here the interference potentials of DEN RNA sequences 105 nt to 240 nt in length are described.

Previously, RNA-mediated interference was demonstrated in C6/36 cells against DEN-2 virus by expressing 567nt of sense or antisense RNA encompassing the prM gene from a recombinant dsSIN virus (Gaines *et al.*, 1996). Similar resistance was observed to yellow fever virus by expressing 774nt of RNA derived from the prM gene or 1,009nt of antisense RNA complementary to the region of the NS5 gene encoding the GDD motif (Higgs *et al.*, 1998). Pang *et al* (1997) have determined the minimum effective length of homologous sequence necessary to generate tospovirus-resistant plants to be between 236-387 bp when expressed alone. More recently, Jan *et al* (2000a) observed this lower limit to be 110 bp when the homologous sequence was transcribed as a hybrid RNA with a non-target sequence such as the GFP gene. As these examples have been shown to be

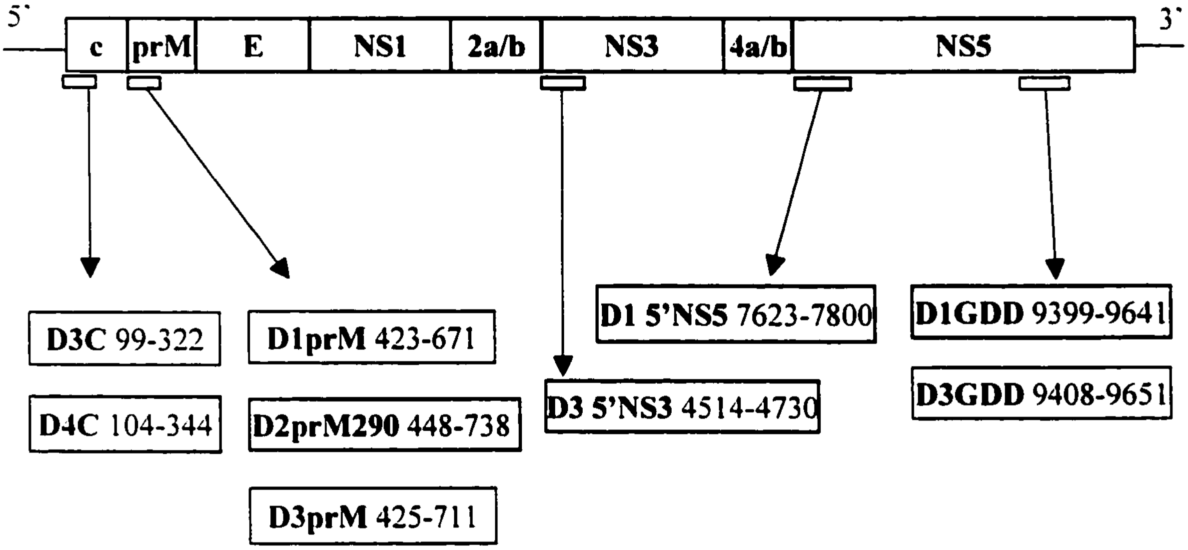


Fig 3.1 Map of the flavivirus genome. The locations of DEN sequences used in this study are indicated. The location of the D2prM290 sequence described in chapter 2 is also shown for comparison. Boxes show the names used in the text to refer to each sequence, followed by the nucleotide position of each sequence on the respective genome it was derived from.

part of the post-transcriptional gene silencing mechanism in plants, we would expect that similar limitations on the length of homologous virus sequence in mosquito cells would apply (Ratcliff *et al.*, 1999).

Materials and Methods

DEN viruses, Cells and Antibodies. DEN-1 (Hawaii) p28C6-7 and DEN-3 (H-87) p28C6-7 viruses were obtained from the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Ft. Collins, CO (Hammon *et al.*, 1960; Sabin, 1952). pXX indicates the number of passages in an unknown host, C6-X indicates the number of passages in C6/36 cells. DEN-2 and DEN-4 viruses used were the same as described in chapter 2. BHK-21 and C6/36 cells were grown and maintained in Leibowitz-15 medium containing 100U/ml penicillin, 100 μg/ml streptomycin and 10% FBS or 2% FBS, respectively. Mab's 4G2 and 30.11a were used to detect DEN E antigen and SIN E1 antigen in immunofluorescence assays as described in chapter 2.

RT-PCR. Viral RNA was extracted from virus particles in the culture medium of infected C6/36 cells using the QiaAmp Viral RNA isolation kit. (Qiagen Inc, Chatsworth, CA) RNA was reverse transcribed using 75 units of Superscript II (Gibco BRL, Rockville, MD) and amplified using Taq polymerase (Promega, Madison, WI) in one reaction. (42^0C for 1 hr; then 92^0C, 30 sec, 52^0C, 1 min; 72^0C, 2 min; 26 cycles; 72^0C, 7 min.). RT-PCR buffer contained 50 pmol of each primer (Table 3.1) in 1X Taq buffer (Promega, Madison, WI), 250 pmol dNTPs and 2.5 mM $MgCl_2$.

Table 3.1. Primers used to amplify specific regions of DEN-1, DEN-3 and DEN-4 virus genomes.

DEN genome region	Forward primer (5'-3')	Reverse primer (3'-5')	Position on genome (nt)
D1prM	TTCCATTTGACTACACGA	TACCTTGTACAAGGGTTTGA	423 - 671
D1GDD	ACCTCTAGAAGCCCAGCTAATAAGAC	ATGGTGTTACCCTTGGGAGATCTT	9399 - 9641
D1-5'NS5	CGGGAGCCAAGGGGAAACAG	CGTGAGCAAGGTGTACACCCT	7623 - 7800
D3C	ACAACCAACGGAAAAAGAC	TGGAAATTCTTCAGCCCC	99 - 322
D3prM	GCAACACTTGCTTTCCACTTA	CGTATCTGCGCTATTCTGTAG	425 - 711
D3-5'NS3	CCGGCGTCCTATGGGACCTA	TTGGTTTGACCCGATCGCAC	4514 - 4730
D3GDD	ACCTCTAGAAGCCCAGCTAATAAGAC	ATGGTGTTACCCTTGGGAGATCTT	9408 - 9651
D4C	AACCAACGAAAAAAGGTCGTT	TTCCGGTAGTTCTATGACTAA	104 - 344

[underlined sequence denotes bases added to generate Xba I restriction sites]

Construction of dsSIN plasmids with a single DEN sequence. RT-PCR products were subcloned into the pTE/3'2J plasmid in one of two ways (see Fig 3.2). (1) D1GDD, D3GDD RT-PCR products were inserted into pCR 2.1 (Invitrogen, Carlsbad, CA), yielding plasmids pCRD1GDD and pCRD3GDD. DEN sequences were isolated as Xba I fragments and inserted into the Xba I site of pTE/3'2J, yielding pD1GDDAs and pD3GDDAs. (2) D1 5'NS5, D1prM, D3 5'NS3, D3prM, D3C and D4C RT-PCR products were inserted in pCR 2.1 (Invitrogen, Carlsbad, CA), producing plasmids pCRD1NS5, pCRD1prM, pCRD3prM, pCRD3C, pCRD3NS3, and pCRD4C. DEN sequences from serotypes 1, 3 and 4 were isolated as EcoR I fragments and inserted into the EcoR I site of pBluescript (Stratagene, La Jolla, CA), previously modified to contain an Nhe I site in place of the Hind III site. This produced plasmids pBD1NS5, pBD1prM, pBD3prM, pBD3NS3, pBD3C and pBD4C. All DEN sequences in pBluescript were isolated as Xba I and Nhe I fragments and inserted into the Xba I site of pTE/3'2J, producing pD1 5'NS5As, pD1prMAs, pD3prMAs, pD3CAs, pD3 5'NS3As, pD4CAs, and pD4CS. Orientation of all DEN sequences with respect to the second subgenomic promoter was confirmed with PCR and restriction enzyme digests.

Construction of dsSIN plasmids with truncated DEN sequences. Recombinant dsSIN plasmids containing inserts of DEN sequence of 104 - 180nt were constructed. The cDNA of the C gene of DEN-4 contains an Nhe I site; consequently, digesting pBD4C with Xba I and Nhe I to completion produced two fragments (134 bp and 106 bp). These two fragments were individually ligated into the Xba I site of pTE/3'2J to create pD4C 5'134As and pD4C 3'106As. dsSIN plasmids containing sequences of various lengths

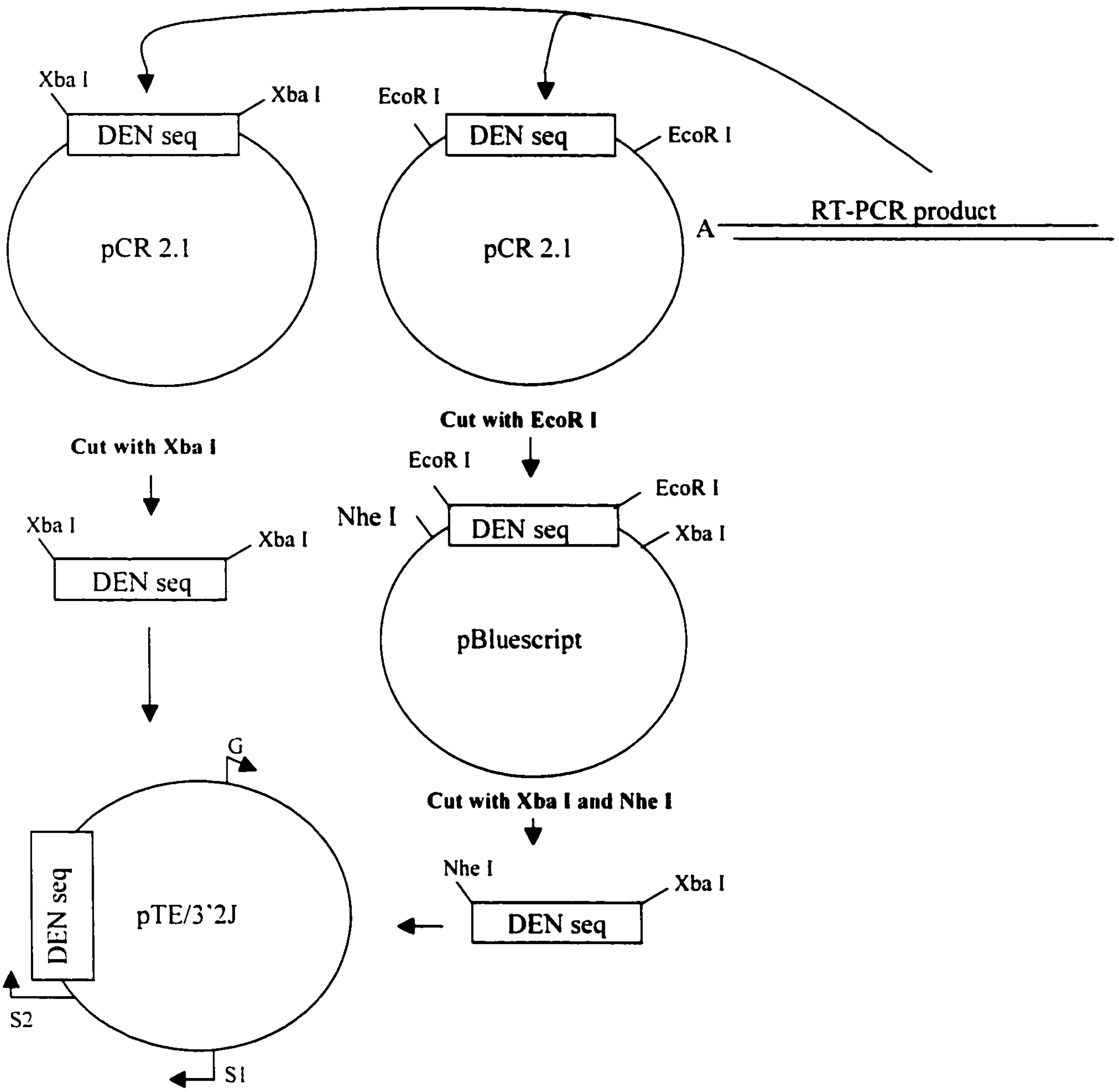


Fig 3.2. Cloning strategies. Two different cloning strategies used to create dsSIN plasmids with DEN sequences downstream of the second subgenomic promoter (S2) of pTE/3'2J.

from the DEN-1 NS5 gene (GDD motif region) were constructed as follows: RT-PCR was performed using (i) the Fsh forward primer (5' ACCTAGTTCTCGACTGGCTGGAA 3') with the D1GDDR primer (5' ACCTCTAGAAGCCCAGCTAATAAGAC); (ii) the Rsh reverse primer (5' TCTAGAAGGCTGTTGCGAACCTGT 3') with the D1GDD F primer (5' TTCTAGAGGGTTCCCATTGTGGTA 3'), and (iii) both the Fsh and Rsh primers together. All primers were engineered to contain Xba I restriction sites. RT-PCR product was cloned into pCR 2.1 (Invitrogen, CA), yielding pCRD1GDDFsh, pCRD1GDDRsh and pCRD1GDDFshRsh. DEN sequence was excised with Xba I and inserted into the Xba I site of pTE/3'2J. Orientation of inserts was confirmed with PCR. dsSIN viruses generated from these plasmids were named (i) D1GDDFshAs, (ii) D1GDDRshAs, and (iii) D1GDDFshRshAs.

Construction of dsSIN plasmids with two DEN sequences. pCRD4C was digested with EcoR I to release the D4C fragment, which was then inserted into the EcoR I site of pBD2prM290, yielding pBD2prM290-D4C. Orientation of the D4C sequence was determined to be the same as that of the D2prM290 sequence by PCR and restriction enzyme digest. D2prM290-D4C sequence was isolated as an Xba I/ Nhe I fragment and ligated into the Xba I site of pTE/3'2J, producing plasmids pD2prM290S-D4CS and pD4CAs-D2prM290As.

pCRD1GDD was digested with Xba I, and the D1GDD sequence was ligated into the Spe I site of pBD4C, producing pBD4C-D1GDD. D4C-D1GDD sequence was isolated as an Xba I/ Nhe I fragment and ligated into the Xba I site of pTE/3'2J, yielding pD1GDDAs-D4CAs.

pBD3C was digested with Xba I and Nhe I to release D3C sequence, which was then inserted into the Spe I site of pBD4C, yielding pBD4C-D3C. This insert was then isolated as an Xba I/Nhe I fragment and ligated into the Xba I site of pTE/3'2J, yielding pD4CAs-D3CAs.

pCRD3GDD was digested with EcoR I to release D3GDD sequence, which was then ligated into the EcoR I site of pBluescript (Stratagene, La Jolla, CA) to create pBD3GDD. The D1GDD sequence was then isolated from pCRD1GDD as an Xba I fragment and ligated into the Spe I site of pBD3GDD, yielding pBD1GDD-D3GDD. This plasmid was partially digested with Xba I, and the D1GDD-D3GDD fragment was isolated and cloned into the Xba I site of pTE/3'2J, yielding pD1GDDAs-D3GDDAs.

pCRD1prM was digested with Xba I, and the resulting fragment was ligated into the Spe I site of pBD3prM, yielding pBD1prM-D3prM. After digestion with Xba I and Nhe I, the D1GDD-D3GDD fragment was inserted into the Xba I site of pTE/3'2J, yielding pD1GDDAs-D3GDDAs.

dsSIN virus production. dsSIN virus was produced as has been described (Gaines *et al.*, 1996; Hahn *et al.*, 1992). RNA was transcribed *in vitro* from the SP6 promoter of linearized dsSIN plasmids in the presence of 1 X transcription buffer, 1 mM cap analog, 50 U Rnasin, 40 U SP6 polymerase (Ambion, Austin TX), 1mM rNTPs, 5 mM dithiothreitol and 500 ng bovine serum albumin (Promega, Madison, WI). *In vitro* transcribed RNA was electroporated into BHK-21 cells using a BTX electro cell manipulator 600™ at 450 V, 100 μF, and 720Ω. Electroporated cells were seeded into 25cm^2 flasks in 5 ml of growth medium. Twenty-four hours later culture medium was collected, cell debris was removed by centrifugation, and virus was stored at –70° C. An aliquot of virus was titrated in an end-point assay using BHK-21 cells. Titers were expressed as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/ ml (Karber, 1931). Production of recombinant dsSIN virus RNA was analyzed by northern blot hybridization two days after infection of C6/36 cells.

Cell culture interference assay. C6/36 cells were seeded on 18mm glass coverslips in 12-well plates or $25cm^2$ flasks and grown at 28°C. When the cells were approximately 80% confluent, they were infected at a multiplicity of infection (MOI) of 20–50 with either a control dsSIN virus (TE/3'2J) or a recombinant dsSIN virus containing a DEN sequence inserted in its genome. Virus was diluted into L-15 so that the total infection volume was 300 µl per well for a twelve well plate or 1 ml per $25cm^2$ flask. Virus was allowed to adsorb to the cells for 30 min at ambient temperature with constant, gentle rocking. Infection medium was then removed and the cells were allowed to recover in L-15 growth medium (4 h to overnight). The virus-exposed cells were then transferred to maintenance medium to prevent overgrowth during the incubation period. At 48 h post infection a sample coverslip for each virus used was removed and assayed by immunofluorescence for the presence of SIN E1 antigen. If SIN E1 antigen was detected in all cells, the remaining monolayers and uninfected control cells were challenged with a DEN virus at an MOI of 0.1. The protocol for infecting with challenge DEN viruses was the same as described for dsSIN virus infections.

At six days post infection with a given DEN virus serotype challenged cells were fixed in acetone, immunostained, visualized, and the percentage of DEN antigen containing cells estimated, as described in chapter 2.

RNA analysis. Total cellular RNA was extracted from 25cm^2 flasks containing infected or uninfected C6/36 cells according to the manufacturer's specifications using the Qiashredder and RNeasy mini kits (Qiagen Inc., Chatsworth, CA). 5 μg of total RNA was electrophoresed in a 1% agarose/ 1X MOPS (.023M MOPS, 8.3 M NaOAc, 0.2 mM EDTA)/ 2% formaldehyde gel and blotted to a positively charged nylon membrane (Brightstar from Ambion, Inc., Austin, TX). Blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored until hybridization. A biotinylated oligonucleotide (5' GCTGGTCGGATCATTGGGGCG 3') complementary to the 3' end of the SIN non-coding region was used to detect dsSIN RNA. Probe was added to hybridization buffer (7% SDS, 0.47 M Na_2HPO_4, 1.7 mM H_3PO_4) and hybridized overnight at 42°C. Blots were washed twice with 2X SSC, 0.1%SDS at 20 min each, followed by 2 washes in 0.2X SSC, 0.1% SDS for 30 min each. Blots were then immediately subjected to Ambion's Brightstar Non-Isotopic Detection Kit and exposed to Kodak ML light film.

RNA quantification. Exposed films were analyzed using an AGFA Duoscan T1200™ scanner. Relative band intensities were quantified using the program UN-SCAN-IT gel™ version 3.1 (Silk Scientific Corporation).

RESULTS

Characterization of recombinant dsSIN viruses containing sequences from DEN-1, DEN-3, and DEN-4. The titers of dsSIN viruses produced in BHK-21 cells ranged from 7.5-8.8 $\log_{10}TCID_{50}$/ml. Only dsSIN viruses that gave the required MOI of 10-50 were used in DEN challenge experiments. Northern blot analysis showed that C6/36 cells infected with recombinant dsSIN viruses containing sequences derived from DEN viruses 1, 3 and 4 contained virus-specific genomic RNA, and two subgenomic mRNAs of the expected size (Fig 3.3).

RNA interference to DEN-1, DEN-3 and DEN-4 viruses. Eight recombinant dsSIN viruses were constructed with 219-290nt inserts complementary to portions of the capsid (C), premembrane (prM), NS3, and NS5 genes of DEN-1, -3, or -4. Recombinant dsSIN viruses expressing DEN sequences that conferred >99% homologous interference in C6/36 cells were developed for all four DEN virus serotypes (Table 3.2). As in the case of DEN-2, the expression of DEN-1 or -3 antisense prM sequence conferred >99% resistance to the homologous DEN virus. The antisense C and NS5 (GDD motif encoding region) RNA sequences were also completely protective against DEN-4 and DEN-1, respectively. Expression of the antisense C, NS3 and NS5 (GDD motif region) based sequences provided interference with DEN-3 replication in 85-95% of C6/36 cells. Interestingly, the D1-5'NS5 virus did not provide any interference to DEN-1 in C6/36 cells.

Characterization of recombinant dsSIN viruses containing truncated DEN sequences. The D4C and D1GDD sequences were selected to examine the minimum length of DEN RNA sequence necessary to initiate RNA-mediated interference in mosquito cells. These sequences are derived from the 5' and 3' ends of the coding regions of the DEN-4 and DEN-1 virus genomes, respectively. The 240 nt D4C sequence was divided into two segments of 134 nt and 106 nt (Fig 3.4a). The D1GDD sequence was truncated by removing either the first 82 nt, the final 56 nt, or both the first 82 nt and the final 56 nt (Fig 3.4b). Recombinant dsSIN viruses were generated with each of these

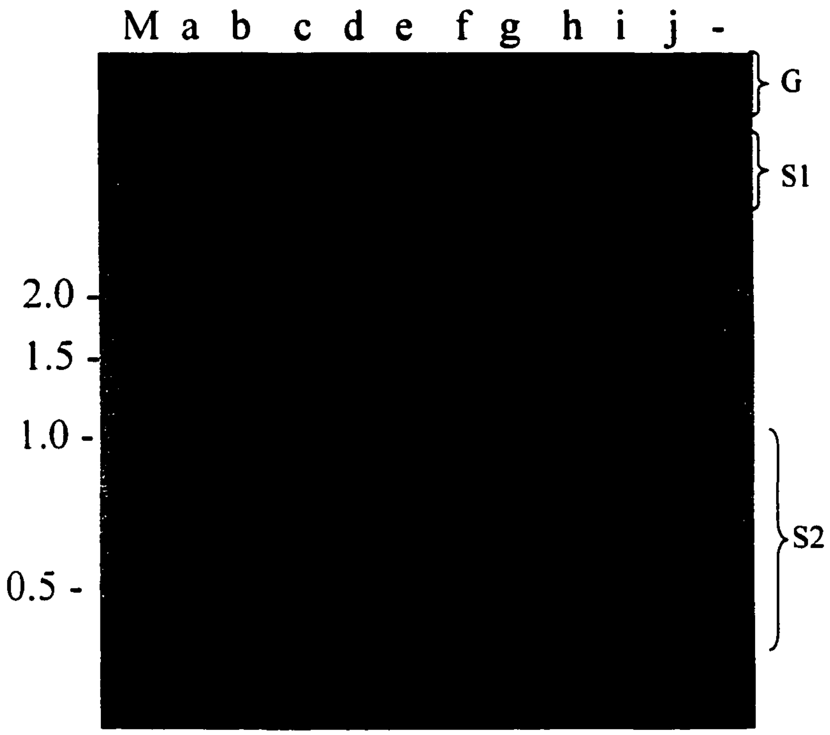


Fig 3.3. Northern blot analysis of recombinant dsSIN virus transcripts present in C6/36 cells 48 h post infection. Blot was probed with a SIN-specific oligonucleotide. (M) molecular weight marker (kb); (a) TE/3'2J; (b) D1 5'NS5As: (c) D1GDDAs; (d) D1prMAs; (e) D2prM290As; (f) D3prMAs; (g) D3 5'NS3As; (h) D3GDDAs; (i) D3CAs; (j) D4CAs; (-) C6/36 cells. G= genomic RNA, S1= first subgenomic mRNA, S2= second subgenomic mRNA.

Table 3.2. Interference with DEN-1, DEN-3 and DEN-4 virus replication#

Recombinant dsSIN virus	Insert length (nt)	Percentage of cells containing DEN E antigen
-	-	50-100%*
TE3'/2J	-	80-100%*
D1prMAs	248	<1%
D1GDDAs	243	<1%
D1 5'NS5As	177	90-100%
D3prMAs	286	<1%
D3 5'NS3As	216	~15%
D3GDDAs	243	~5%
D3CAs	223	~5%
D4CAs	240	<1%

*represents range of results from challenge with DEN-1, -3, or -4.

#Cell culture interference assays were done in C6/36 cells using recombinant dsSIN viruses that expressed RNA sequences derived from DEN-1, 3, and -4 viruses. Cells were challenged with the homologous DEN virus serotype.

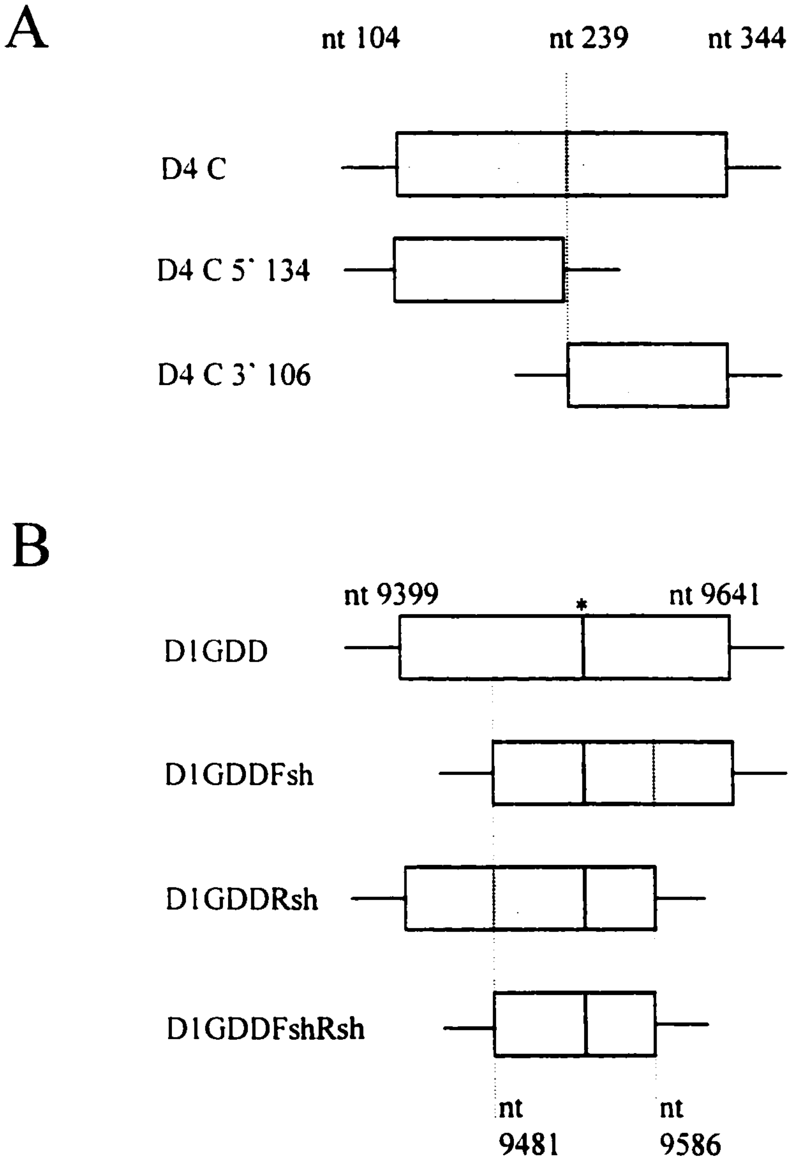


Fig 3.4. Cartoon of truncated DEN sequences inserted downstream of the second subgenomic promoter of the pTE/3'2J plasmid. (A) sequences derived from the DEN-4 capsid gene. (B) sequences derived from the DEN-1 NS5 gene. * indicates the location of the GDD motif encoding region.

sequences downstream from the second subgenomic promoter, and total cellular RNA was examined 48 h after infection to verify production of the genomic and subgenomic RNA (Fig 3.5 a & b). All recombinant dsSIN viruses produced SIN transcripts of the expected size.

Determination of minimum size of interfering DEN RNAs. Recombinant dsSIN viruses containing truncated DEN sequences were tested in cell culture interference assays. The D1GDDFshAs and D1GDDRshAs, containing 160 and 187 nt of DEN-1 RNA sequence, respectively, retained the same interference potential to DEN-1 as the original D1GDDAs (240nt) sequence (Table 3.3). The D1GDDFshRshAs virus, while it was not quite as effective as its larger counterparts, was still able to prevent DEN-1 replication in 85% of the cells.

Reducing the length of the D4C RNA sequence to 134nt or 106nt resulted in a significant decrease in interference to DEN-4 (Table 3.4). Over 50% of the cells infected with either the D4C 5'134As virus or the D4C 3'106As virus remained permissive for the challenge DEN-4 virus.

Characterization of recombinant dsSIN viruses containing sequences from two DEN serotypes. Six recombinant dsSIN viruses were constructed by combining sequences from two different DEN virus serotypes, previously shown to confer >99% interference to the homologous DEN virus when expressed in C6/36 cells individually. Northern blot analysis showed that at 48 h post infection, C6/36 cells contained virus-specific genomic RNA, and two subgenomic mRNAs of the expected size for all recombinant dsSIN viruses (Fig 3.6).

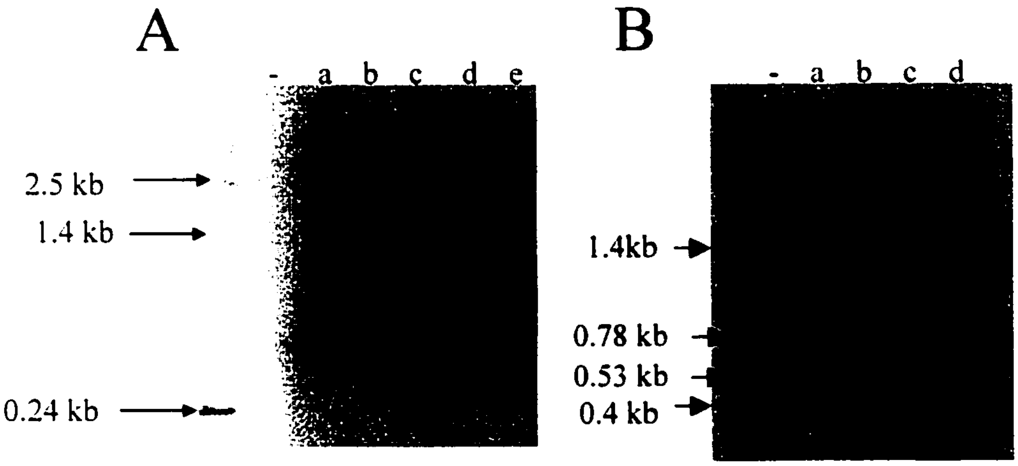


Fig 3.5. Northern blot analysis of recombinant dsSIN viruses expressing truncated DEN genome inserts at 48 h post infection. Blot was probed with a SIN-specific oligonucleotide (A) Uninfected cells (-), TE/3'2J (a), D1GDDAs (b), D1GDDFshAs (c), D1GDDRshAs (d), D1GDDFshRshAs (e). (B) Uninfected C6/36 cells (-), TE/3'2J (a), D4CAs (b), D4C 5'134As (c), D4C 3'106As (d).

Table 3.3. Cell culture interference assays of recombinant dsSIN viruses expressing DEN-1 RNA sequences of 105-243nt.

Recombinant dsSIN virus	Insert length (nt)	Percentage of cells containing DEN-1 E antigen
-	-	80%
TE/3'2J	-	90%
D1GDDAs	243	<1%
D1GDDFshAs	160	<1%
D1GDDRshAs	187	2%
D1GDDFshRsh	105	15%

Table 3.4. Cell culture interference assays of recombinant dsSIN viruses expressing DEN-4 RNA sequences of 106-240nt.

Recombinant dsSIN virus	Insert length (nt)	Percentage of cells containing DEN-4 E antigen
-	-	90%
TE/3'2J	-	90%
D4CAs	240	<1%
D4C 5'134	134	50%
D4C 3'106	106	75%

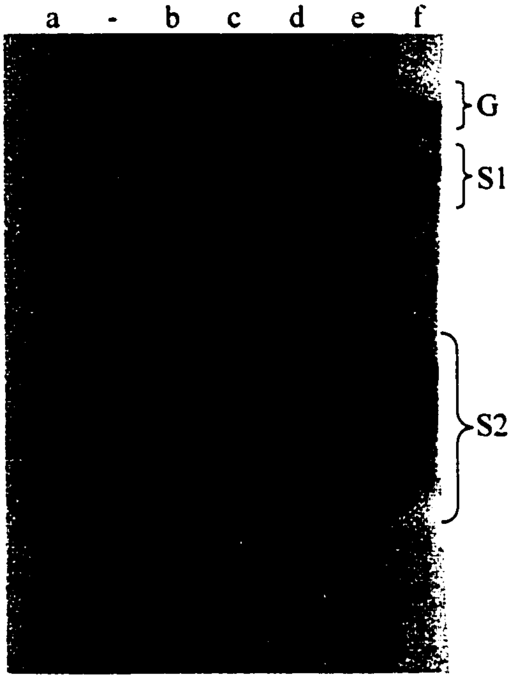


Fig 3.6. Northern blot analysis of dsSIN produced transcripts containing inserts from two different DEN virus serotypes in C6/36 cells 48 h post infection. Blot was probed with a SIN-specific oligonucleotide (a) TE/3'2J. (-) C6/36, (b) D4CAs-D3CAs, (c) D4CAs-D2prM290As, (d) D4CAs-D1GDDAs, (e) D1prMAs-D3prMAs, (f) D1GDDAs-D3GDDAs.

Interference by recombinant dsSIN viruses expressing two DEN RNA sequences. Recombinant dsSIN viruses containing sequences derived from two DEN virus serotypes were tested for interference to each homologous DEN virus in cell culture interference assays. All recombinant viruses conferred significant interference to one or both DEN serotypes, however in no case was the interference against both DEN serotypes >99% (Table 3.5). D4CAs was able to confer >99% interference to DEN-4 when combined with the D2prM290As sequence, but not when combined with D1GDDAs or D3CAs. D2prM290As and D3CAs both showed decreased ability to confer interference when combined with D4CAs, while D1GDDAs continued to confer >99% interference when combined with D4CAs. Interestingly, D4CAs-D2prM290As showed the same interference pattern as D2prM290S-D4CS, indicating that the polarity of the expressed DEN sequence may not be significant.

DISCUSSION

The dsSIN expression system provides a convenient, transient method of expressing RNA sequences to determine their effectiveness in inhibiting the replication of DEN viruses. Sequences derived from five different regions of the genome of DEN viruses were tested for dsSIN-expressed DEN RNA interference. Sequences derived from the prM and GDD regions were tested for interference potential because sense or antisense RNA sequences of >500nt derived from these regions had successfully conferred interference in other studies (Gaines *et al.*, 1996; Higgs *et al.*, 1998; Olson *et al.*, 1996). Sequences derived from the 5' regions of the C, NS3 and NS5 genes were tested for RNA-mediated interference to observe whether any DEN sequence expressed

Table 3.5. Cell culture interference assays of recombinant dsSIN viruses expressing RNA from two DEN virus serotypes.

C6/36 cells infected with recombinant dsSIN virus:	Tandem insert length (nt)	% DEN positive cells by IFA			
		DEN-1	DEN-2	DEN-3	DEN-4
-	-	50-100	50-100	50-100	50-100
TE/3'2J	-	80-100	80-100	80-100	80-100
D1GDDAs-D3GDDAs	243-243	2	-	10	-
D1prMAs-D3prMAs	248-286	3	-	3	-
D4CAs-D2prM290As	240-290	-	33	-	<1
D2prM290S-D4CS	290-240	-	33	-	<1
D4CAs-D1GDDAs	240-243	<1	-	-	20
D3CAs-D4CAs	223-240	-	-	10	10

could exhibit equal protection. All DEN RNAs derived from the prM gene were able to confer >99% resistance to the homologous DEN virus in C6/36 cells. DEN RNAs derived from the C, NS3, and NS5 genes (GDD motif region) conferred between 85-99% interference. It is unknown whether these differences truly represent significant differences in the abilities of these sequences to interfere with the homologous DEN viruses, or whether they are the result of variation in the replication of the various dsSIN viruses, as they are all distinct. Finally, DEN RNA derived from the 5' end of the NS5 gene conferred no resistance in C6/36 cells to the homologous DEN-1 challenge virus. This may be due to the fact that the DEN-1 5' NS5 sequence was only 177 nt in length, roughly 40 - 110 nt shorter than the rest of the sequences tested.

The minimum effective length of DEN RNA sequence expressed from a dsSIN virus that conferred resistance to the homologous DEN virus was examined. Six recombinant dsSIN viruses were tested that expressed DEN sequences less than 200nt long (D1GDDFshAs, D1GDDRshAs, D1GDDFshRshAs, D4C 5'134As, D4C 3'106 As, D1 5'NS5As), only three of which were able to confer substantial resistance to the challenge DEN virus. This is in contrast to 7/7 sequences longer than 216nt (D1prMAs, D1GDDAs, D3prMAs, D3CAs, D3GDDAs, D3 5'NS3As, D4CAs) providing substantial resistance to the homologous challenge virus. This is similar to observations in transgenic plants where RNA-mediated interference is not observed consistently with expression of RNA of lengths below 240 nt (Pang *et al.*, 1997). Jan *et al* (2000a) have also shown that when linked to a non-target sequence, expression of 110 nt of viral sequence seems to be the lower limit required to generate virus-resistant plants. Other researchers found that transfection of dsRNA sequences of 100 bp can induce a post-transcriptional gene silencing response in *Trypanosoma brucei* (Ngo *et al.*, 1998). They, noted, however, that the silencing phenotype was stronger when dsRNA of 450 nt were used. These similarities reinforced the hypothesis that DEN sequences expressed from the dsSIN system activate a post-transcriptional gene-silencing mechanism in the mosquito cell, which is then capable of silencing the challenge DEN virus.

Recombinant dsSIN viruses designed to express sequences from the NS5 (GDD region) or the prM region of both DEN-1 and –3 were the most successful in interfering with the replication of both homologous challenge viruses in mosquito cells. For the D1prMAs-D3prMAs, only 3% of the cells contained either DEN-1 or DEN-3 antigen 6 days after challenge. The D4C and D2prM290 sequences were expressed together in either the sense or antisense orientation. Surprisingly, both dsSIN viruses displayed identical resistance patterns, with both completely resisting DEN-4, but allowing DEN-2 replication in 33% of the cells. This reinforces the idea that the polarity of the RNA sequence is not important in the establishment of RNA-mediated resistance with dsSIN viruses. The two other combinations tested the D4C sequence, which had provided the best interference when combined with D2prM290, with either the D1GDD or the D3C sequences. In these cases, however, ability to interfere with DEN-4 was diminished. Ability to maintain <99% interference did not seem to correlate with whether a particular DEN RNA sequence was expressed at the 5' or 3' end of a pair.

The results from these studies show quite clearly that RNA-mediated resistance to all four DEN serotypes is achievable in cultured *Ae. albopictus* cells, and that resistance can be conferred to multiple DEN serotypes simultaneously using the dsSIN virus expression system. The next set of studies were designed to determine if RNA-mediated interference to multiple DEN virus serotypes can be established in the natural mosquito vector of DEN viruses 1-4, *Ae. aegypti.*

Chapter 4

RNA-mediated resistance to DEN viruses 1, 2, 3, and 4 in *Ae. aegypti* mosquitoes.

INTRODUCTION

In chapters 2 and 3, sequences derived from the genomes of DEN serotypes 1-4 were described, that when expressed in cultured mosquito cells from a dsSIN virus expression system conferred high levels of resistance to the homologous DEN virus. Past studies have shown a strong correlation between the ability of an RNA sequence to interfere with DEN-2 replication in mosquito cells and in whole mosquitoes (Gaines *et al.*, 1996; Olson *et al.*, 1996). Therefore it should be possible to protect *Ae. aeypti* mosquitoes from infection by each of the four DEN virus serotypes by expressing homologous, intracellular DEN RNA sequences.

The TE/3'2J virus expression system has been used for the expression of both proteins (Olson *et al.*, 2000) and non-translatable RNA sequences in *Ae. aegypti* mosquitoes (Higgs *et al.*, 1998; Johnson *et al.*, 1999; Olson *et al.*, 2000). The virus replicates very quickly in almost all mosquito tissues, including the salivary glands. Notably, the TE/3'2J virus poorly infects midgut epithelial cells (Olson *et al.*, 2000).

DEN viruses vary greatly in their abilities to cause a disseminated infection in *Ae. aegypti* mosquitoes. Genetic variation and environmental factors likely dictate whether an infectious bloodmeal will result in a disseminated DEN infection (Bosio *et al.*, 1998; Gubler *et al.*, 1979). We have also observed that the TE/3'2J virus does not readily infect and disseminate in *Ae. aegypti* following ingestion of an infected blood meal (Olson *et al.*, 2000). With these observations in mind, recombinant dsSIN viruses and DEN viruses were simultaneously intrathoracically inoculated into the *Ae. aegypti* mosquito. This allows both viruses to bypass any midgut infection and escape barriers and insures that 100% of mosquitoes receive both the dsSIN virus and the challenge DEN virus. The fact that SIN virus replicates faster in mosquito cells than do DEN viruses insures that any resistance phenotypes will be initiated in most cells of the mosquito prior to DEN virus infection.

MATERIALS AND METHODS

Viruses and Mosquitoes. *Aedes aegypti* mosquitoes (Rexville D strain, Puerto Rico) were reared in an insectary at 28°C and 80% humidity, with a photoperiod of 18h light and 6h darkness. Recombinant dsSIN viruses and DEN viruses used were the same as described in chapters 2 and 3, materials and methods.

Mosquito interference assay. Adult female mosquitoes were inoculated intrathoracically with 0.5 µl containing approximately 10^5 $TCID_{50}$ of recombinant dsSIN virus and 3 X 10^3 PFU of DEN-1, -2, -3 or -4 virus. Injected mosquitoes were held for 14 days at 28°C and 80% humidity and fed only sugar and water. Mosquito heads were removed and fixed to acid-washed glass slides (1M HCl for 4 h, rinsed with tap water followed by distilled water), in cold, 100% acetone for 5 min at –20°C and allowed to dry. The slides were stored at –20°C until IFA was performed. Slides were analyzed by IFA using the primary antibody 4G2 as described in chapter 2, materials and methods. Mosquito heads were scored as either positive (some or all tissue contained antigen) or negative (no antigen present) (Fig4.1).

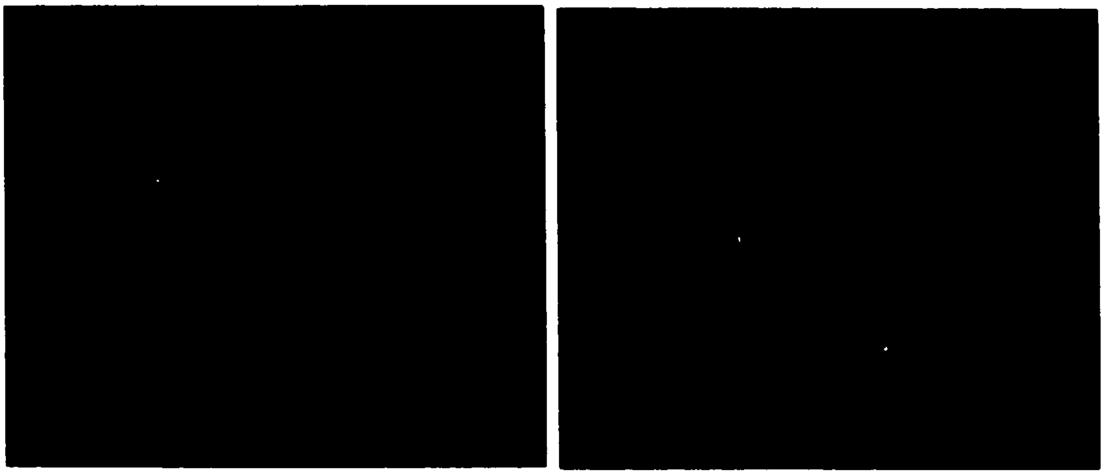


Fig 4.1. ***Ae. aegypti*** **head tissue either negative (-) or positive (+) for DEN E antigen at 14 days post inoculation.**

RESULTS

dsSIN-mediated interference: single DEN genome inserts. Recombinant dsSIN viruses that conferred the highest level of interference in cell culture were tested in *Ae. aegypti* mosquito interference assays. In the case of DEN-1, -2, and -3, expression of the prM sequence in either sense or antisense orientation conferred complete interference, as all mosquitoes were negative for DEN E antigen (Table 4.1). The D4CAs and D1GDDAs viruses also completely protected mosquitoes against DEN-4 and -1, respectively. The D4CS virus protected all but 1 of 38 mosquitoes, or 97% of the total mosquitoes tested from DEN-4. However, coinjection of recombinant dsSIN virus D3GDDAs with DEN-3 did not confer resistance to DEN-3 in mosquitoes: 94% of mosquitoes were positive for DEN-3 antigen, and this represents the sum of multiple experiments.

dsSIN-mediated interference: truncated DEN genome inserts. The recombinant dsSIN viruses described in chapter 3 expressing truncated antisense sequences from DEN-1 NS5 (GDD motif region) and DEN-4 (C region) were also analyzed in mosquito interference assays. The D1GDDFshAs (160 nt) virus protected all mosquitoes injected, while the D1GDDRshAs (187 nt) virus protected 89% of injected mosquitoes (Table 4.2). Interference in mosquitoes was reduced to 74% when the D1GDDFshRshAs (105 nt) dsSIN virus was coinjected with DEN-1. When mosquitoes were coinjected with DEN-1 and TE/3'2J or with DEN-1 alone, 84% and 80% of the mosquitoes were positive for DEN antigen at 14 days post inoculation. In contrast to results presented in chapter 3, where approximately 50% of cells expressing the D4C 5'134As sequence supported the replication of DEN-4, the D4C 5'134As virus protected 99% of mosquitoes from DEN-4 replication (Table 4.3). Only 1 mosquito out of 103 showed DEN antigen after 14 days,

Table 4.1. Interference to DEN virus serotypes 1-4.*

Recombinant dsSIN virus name	Length DEN sequence (nt)	Number of DEN positive *Ae. aegypti* mosquitoes (percent positive)
D1prMAs	248	0/60 (0)
D1GDDAs	243	0/46 (0)
D2prM290As	291	0/44 (0)
D2prM290S	291	0/46 (0)
D3prMAs	286	0/60 (0)
D3GDDAs	243	73/78 (94)
D4CAs	240	0/95 (0)
D4CS	240	1/38 (3)

*Recombinant dsSIN viruses expressing sense or antisense DEN RNA were coinjected into *Aedes aegypti* mosquitoes along with the homologous DEN virus serotype. IFA of mosquito head tissue at 14 days post injection were scored as positive or negative for DEN viral antigen.

Table 4.2. Mosquito interference assays of recombinant dsSIN viruses expressing DEN RNA sequences of varying length derived from the D1NS5 gene (GDD-motif region).

Recombinant dsSIN virus name	Length DEN sequence (nt)	Number of DEN-1 positive *Ae. aegypti* mosquitoes (percent positive)
none	-	16/20 (80)
TE/3'2J	none	38/45 (84)
D1GDDAs	243	0/46 (0)
D1GDDFshAs	160	0/44 (0)
D1GDDRshAs	187	4/45 (9)
D1GDDFshRshAs	105	12/47 (26)

Table 4.3 Mosquito interference assays of recombinant dsSIN viruses expressing DEN RNA sequences of varying length derived from the D4C gene.

Recombinant dsSIN virus name	Length DEN sequence (nt)	Number of DEN-4 positive *Ae. aegypti* mosquitoes (percent positive)
none	-	50/50 (100)
TE/3'2J	none	78/78 (100)
D4CAs	240	0/95 (0)
D4C 5'134As	134	1/103 (1)
D4C 3'106As	106	48/68 (71)

and this represents the sum of several experiments. However, expression of the D4C 3'106As sequence did not provide significant interference in mosquitoes, as 71% of heads contained DEN-4 antigen. All mosquitoes injected with TE/3'2J virus and DEN-4 virus or DEN-4 virus alone supported the replication of DEN-4, as evidenced by detecting DEN-4 E antigen in all head tissues.

dsSIN-mediated interference: multiple DEN genome inserts. The recombinant dsSIN viruses D1GDDAs-D3GDDAs, D1prMAs-D3prMAs, D2prM290S-D4CS, and D4CAs-D2prM290As, each of which expressed RNA sequences from two different DEN virus serotypes, were tested in mosquito interference assays. The D1GDDAs-D3GDDAs virus protected approximately 90% of mosquitoes against infection by each of the homologous DEN viruses (Table 4.4). This contrasts with the results presented in Table 4.1, which showed that when expressed alone, D3GDDAs virus was not protective in mosquitoes. The D1prMAs-D3prMAs virus was capable of protecting 97% of mosquitoes from infection by DEN-3 and 99% of mosquitoes from DEN-1 infection. This recombinant dsSIN virus did not confer any resistance to heterologous DEN-4 virus, as 24/25 co-inoculated mosquitoes contained DEN-4 antigen. The D2prM290S-D4CS and D4CAs-D2prM290As conferred similar interference when coinjected with DEN-2 or DEN-4 viruses. IFA showed that 4% and 13% of mosquitoes supported the replication of DEN-2 virus, while roughly 6% and 4% of mosquitoes supported DEN-4 virus, respectively. This contrasted with control mosquitoes injected with DEN-2 virus or DEN-4 virus, or coinjected with TE/3'2J /DEN-2 or TE/3'2J /DEN–4, 100% of which were positive for either DEN-2 or DEN-4 viruses. Neither of these recombinant dsSIN viruses conferred significant interference when coinjected with DEN-1 virus.

Table 4.4 *Ae. aegypti* coinjected with a DEN virus and a recombinant dsSIN virus expressing sense or antisense sequences derived from more than one DEN serotype.

Recombinant dsSIN virus name	Tandem insert length (nt)	Number of DEN positive *Ae. aegypti* mosquitoes (percent positive)							
		DEN-1		DEN-2		DEN-3		DEN-4	
none	-	64/73	(88)	26/26	(100)	74/75	(99)	32/32	(100)
TE/3'2J	none	108/115	(94)	21/21	(100)	108/108	(100)	16/16	(100)
D2prM290S-D4CS	290-240	12/25	(48)	2/52	(3.8)	-	-	4/70	(5.7)
D4CAs-D2prM290As	240-290	16/29	(55)	3/23	(13)	-	-	2/49	(4.1)
D1prMAs-D3prMAs	248-286	1/96	(1)	-	-	2/66	(3)	24/25	(96)
D1GDDAs-D3GDDAs	243-243	7/79	(8.9)	-	-	5/64	(7.8)	-	-

DISCUSSION

The goal of these studies was to determine if RNA-mediated interference to DEN virus serotypes 1-4 in cultured mosquito cells was predictive of whether or not RNA-interference occurred in whole mosquitoes. This study clearly shows that all four DEN serotypes can be prevented from replicating in *Ae. aegypti* mosquitoes when co-inoculated with a recombinant dsSIN virus designed to express a homologous DEN sequence. This is not entirely unexpected, as recombinant dsSIN viruses have been used to prevent the replication of DEN-2 virus, yellow fever virus and LaCrosse virus in mosquitoes (Higgs *et al.*, 1998; Olson *et al.*, 1996; Powers *et al.*, 1996). Both sense and antisense prM sequences of 290 nt were capable of conferring resistance to DEN-2 virus at the same magnitude as a 567 nt sequence derived from the entire prM gene of DEN-2 virus in previous interference studies (Olson *et al.*, 1996). In that study, virus accumulation in mosquito head tissue correlated with the mosquitoes' ability to transmit DEN-2 virus, and, conversely, a lack of virus accumulation in head tissue correlated with the inability to transmit virus through its saliva. The expression of DEN RNA sequences from recombinant dsSIN viruses described in this study that prevent virus dissemination in tissues such as the head also would be expected to ablate transmission of the virus, although this has yet to be tested.

Secondly, we have shown that sequences derived from two DEN serotypes can confer resistance to both homologous DEN viruses when the dsSIN virus genome contains hybrid DEN RNAs. This chimeric approach has been successful in preventing the replication of tomato spotted wilt virus and turnip mosaic virus in transgenic tobacco plants (Jan *et al.*, 2000b). The interference we observed did not seem to be related to the polarity of the inserts, as both D2prM290S-D4CS and D4CAs-D2prM290As conferred similar amounts of resistance. This is consistent with the indirect model of post-transcriptional gene silencing described in chapters 2 and 3. We also observed that interference was sequence specific, as none of the dsSIN viruses tested in mosquitoes conferred resistance to heterologous DEN viruses. This was previously found to be the case by Powers *et al.* (1996), who found that homologous RNA sequences could interfere with LaCrosse virus and closely related bunyaviruses, but not with more distantly related California serogroup bunyaviruses. Gaines *et al* (1996) found that sequences derived from DEN-2 virus were protective against DEN-2, but not DEN-3 or DEN-4. Sequence specificity has also been shown to be important in gene silencing in plants (English *et al.*, 1996; Jones *et al.*, 1998; Wassenegger and Pelissier, 1998).

Comparison of the results in this chapter with those described in chapters 2 and 3 reveals several differences between the ability of expressed RNA sequences to interfere with homologous DEN viruses in cultured cells and mosquitoes. The D3GDDAs sequence, while protective on its own in cell culture, had no real effect in mosquitoes. Mosquitoes injected with D3GDDAs virus were not tested for the presence of D3GDD specific RNA or dsSIN virus specific RNA. However, D3GDDAs virus from the same stock used to inject mosquitoes was re-tested in cell culture interference assays against DEN-3. The results were consistent with the finding that in cell culture the D3GDDAs sequence confers resistance to DEN-3. Mosquitoes were then re-inoculated with D3GDDAs virus and DEN-3, and it was again found that the D3GDDAs sequence did not confer resistance to DEN-3. When, however, the D3GDDAs sequence was combined with the D1GDDAs sequence in a single recombinant dsSIN virus, resistance to DEN-3 was restored. Pang *et al* (1997) showed that when viral RNA sequences unable to confer resistance on their own are linked to a non-target sequence such as the gene encoding the GFP, then both the GFP and the challenge virus were subject to post-transcriptional gene-silencing. If the D1GDD RNA sequence were responsible for activating post-transcriptional gene silencing, the D3GDD RNA sequence may have been silenced as well. Why the D3GDD sequence was unable to activate such a mechanism in mosquitoes when it appeared able to do so in cultured mosquito cells is not known. Also, the 5' 134 nt of the capsid gene of DEN-4, when expressed in mosquito cells was not protective, but in mosquitoes complete interference could be established. Here we saw the opposite effect, and again, the reason for the inconsistency is not known. C6/36 cells were cloned more than twenty years ago (Igarashi, 1978) from a mosquito cell line first established more than thirty years ago (Singh, 1967), so some variation in the activation of post-transcriptional gene silencing between C6/36 cells and *Ae. aegypti* mosquitoes is not too surprising. Also, C6/36 cells were derived from *Ae. albopictus* mosquitoes, not *Ae. aegypti*, and additional variation could be expected between them. It is important to note, however, that while important, these are nevertheless exceptions to the rule. The majority of DEN sequences tested for interference potential showed similar or identical effects in both cultured mosquito cells and whole mosquitoes.

The dsSIN virus expression system has allowed the examination of the interference potentials of various transiently-expressed, DEN-derived RNA sequences in mosquitoes, without the need for transformation of the mosquito genome. However, the development of a more genetically stable method of studying RNA-mediated resistance to DEN viruses is necessary to generate PTGS in mosquitoes and mosquito cells. The next two chapters describe studies using DNA-based constructs designed to express DEN RNA sequences in mosquito cells, with the goal of testing different strategies that will be the most effective in generating virus-resistant transgenic mosquitoes.

Chapter 5

RNA-mediated resistance to DEN-2 virus generated in transformed non-clonal mosquito cell lines

INTRODUCTION

Permanently transformed, non-clonal cell lines were established to determine if RNA-mediated interference to DEN-2 virus could be generated by expression of transcripts from the nucleus of mosquito cells. Virus-resistant transgenic plants have been developed by transformation with DNA-based constructs designed to express RNA derived from plant RNA viruses (English *et al.*, 1996; Jan *et al.*, 2000b; Pinto *et al.*, 1999; Prins and Goldbach, 1996; Waterhouse *et al.*, 1998). Other researchers, however, have been unable to generate virus-resistant mosquito cells by transformation with DNA constructs shown to express LAC-derived RNA sequences (Allen-Miura, 2000; Monroe, 1990; Powers, 1995). In order to evaluate RNA-mediated resistance in stably transformed mosquito cells, a DNA-based system was developed to transcribe a self-replicating SIN RNA or replicon from the nucleus of mosquito cells. Plant geneticists have shown that DNA-based virus replicons (or amplicons) can initiate a strong and consistent post-transcriptional gene silencing response in plants (Angell and Baulcombe, 1999; Dalmay *et al.*, 2000a). Also, at the time these studies were conducted the direct model of antisense interference had not been ruled out as a possible mechanism for RNA-mediated interference, and it was thought that the self-amplification of the SIN replicon would be necessary for any DNA-based interference strategies.

The Sindbis replicon, pSINrep5, was designed for the expression of large genes or sequences, up to 5 kb in length (Bredenbeek *et al.*, 1993; Xiong *et al.*, 1989). RNA transcribed *in vitro* from the pSINrep5 plasmid can be delivered into either mammalian or insect cells by electroporation (Kamrud *et al.*, 1998). Following transfection by electroporation of the SIN replicon RNA, replication of the SIN virus (+) and (-) strands occurs normally, but due to the deletion of the SIN structural genes viral RNA is not packaged and no progengy virus is produced (Xiong *et al.*, 1989). Packaged SIN replicon virus particles can be generated if a defective helper RNA, encoding the SIN structural proteins, is co-electroporated with the SIN replicon RNA (Bredenbeek *et al.*, 1993; Kamrud *et al.*, 1998; Kamrud *et al.*, 1995; Olson, 2000; Rayner, 1998).

The expression of SIN replicon with inserted DEN-derived sequences from DNA plasmids was controlled by the baculovirus immediate-early 1 (IE1) promoter. Transcription from this promoter in mosquito cells is constituitive, so no prior treatment or induction steps were needed to initiate transcription of recombinant SIN replicon mRNAs or cytoplasmic amplification of DEN-derived mRNAs.

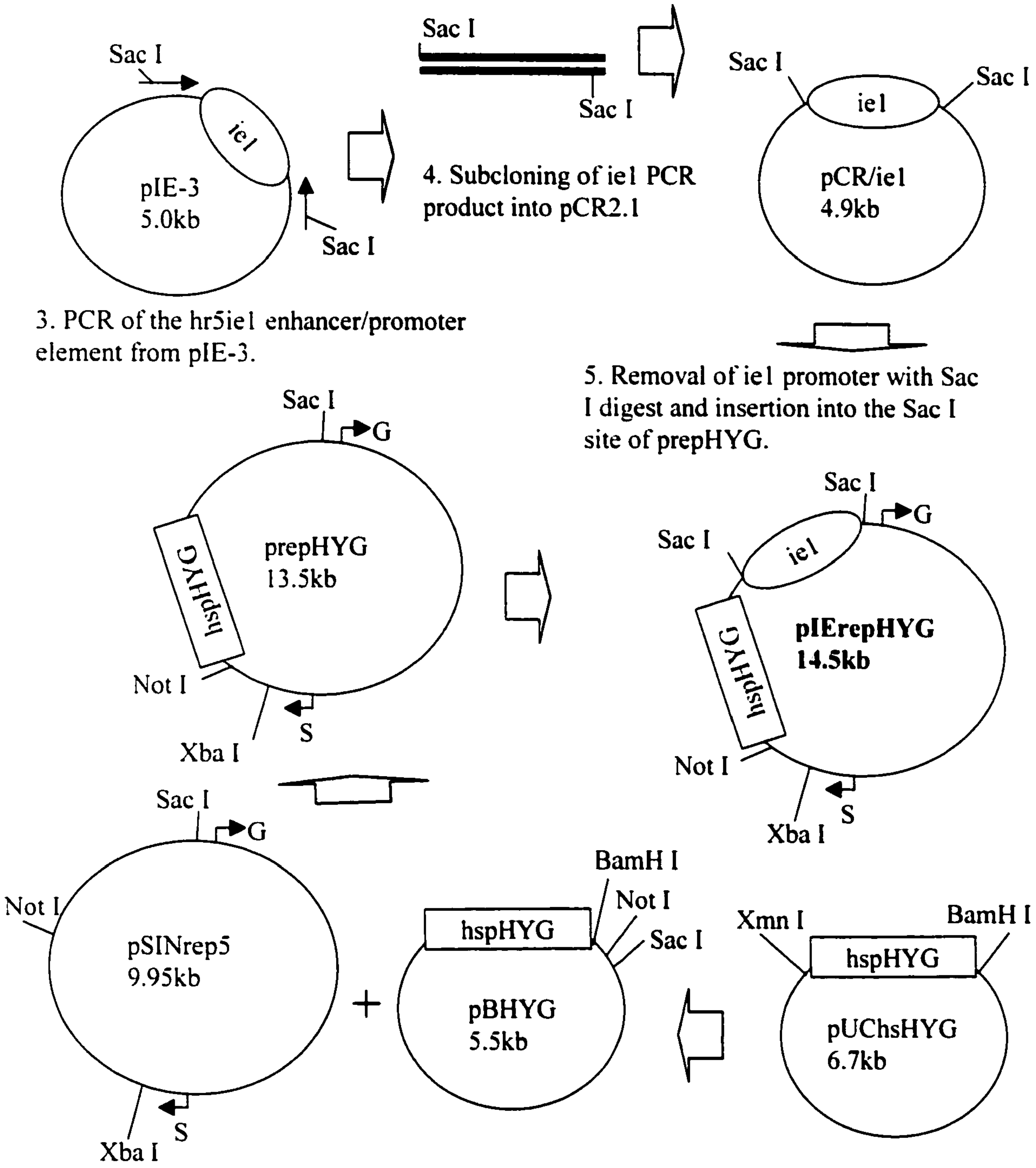
Transformed mosquito cells in this study were selected based on resistance to the antibiotic hygromycin B. Plasmids containing the hygromycin-resistance gene (HYG) have been used previously for the selection of transformed mosquito cells (Allen-Miura, 2000; Kovach, 1995; Monroe *et al.*, 1992; Powers, 1995). In these studies, and in the present study, expression of the HYG gene was driven by the *Drosphila* heat shock promoter, which has been shown to act constitutively in mosquito cells (Allen-Miura, 2000; Monroe *et al.*, 1992; Powers, 1995).

Cells transformed with the cDNA of the SIN replicon were not clonally selected for several reasons. First, non-clonal lines can be established after several weeks, while clonal selection requires 2-3 months. It was important to minimize the time of selection because it was unknown how long expression of the complete SIN replicon would be maintained, as it has an open reading frame of almost 10 kb. Also, it was thought that because the SIN replicon is self-replicating, cells transcribing the SIN replicon would contain similar amounts of the expressed DEN RNA sequences, irrespective of any positional effects associated with transcription of the nuclear-SIN DNA plasmid. All cells expressing the SIN replicon would then also be exposed to double-stranded RNA (as a SIN replicative intermediate). Thus a non-clonal SIN replicon-based cell line would behave exactly as a clonal line with regard to the initiation of RNA-mediated resistance.

MATERIALS AND METHODS

Construction of DNA-based SIN replicon plasmids. The cloning strategy used for constructing pIErepHYG is shown in Fig 5.1. pSINrep5 (Xiong *et al.*, 1989) was modified by excising the majority of the bacterial plasmid sequence by digesting with the restriction enzymes Not I and Sac I. This sequence was replaced by a linearized pBHYG Not I/ SacI fragment, creating pRepHYG. pBHYG had been created by inserting the BamH I/ Xmn I fragment of pUChsHYG containing the *Drosophila* heat shock promoter followed by the hygromycin resistance gene into the BamH I/ EcoR V site of pBluescript (Stratagene, La Jolla, CA). The construction of pUChsHYG has been described (Monroe, 1990). The hr5IE1 enhancer/promoter region of the pIE-3 plasmid (Novagen, Madison, WI) was subcloned into pCR 2.1 TOPO (Invitrogen, Carlsbad, CA) following amplification with primers modified to contain Sac I sites, creating pCR/IE1. The hr5IE1 enhancer/promoter sequence was then isolated as a Sac I fragment and ligated into the Sac I site of pRepHYG, creating pIErepHYG.

pIErepGFPHYG was created by inserting the Xba I/ Not I fragment from pRepGFP (Higgs *et al.*, 1996) containing the GFP gene into the Xba I/ Not I site of pIErepHYG.



3. PCR of the hr5ie1 enhancer/promoter element from pIE-3.

4. Subcloning of ie1 PCR product into pCR2.1

5. Removal of ie1 promoter with Sac I digest and insertion into the Sac I site of prepHYG.

2. Ligation of pBHYG, pSINrep5 after Not I/Sac I digest.

1. Insertion of hspHYG into pBluescript

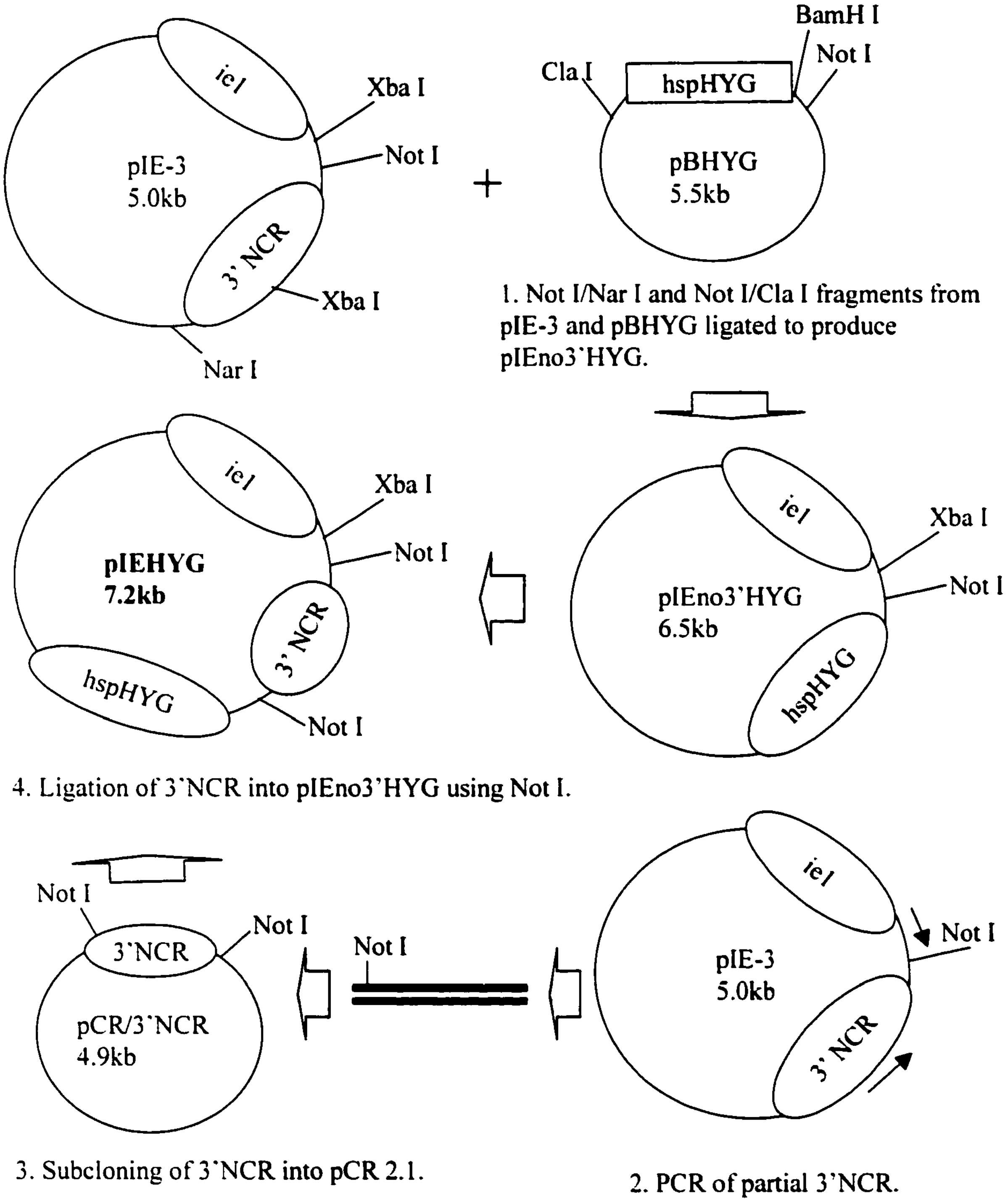
Fig 5.1. Cloning strategy of DNA-based SIN replicon, pIErepHYG (shown in bold, see text for details). G = genomic promoter. S = subgenomic promoter.

pIErep2prM290AsHYG was constructed by ligating the Xba I/Nhe I fragment from pBD2prM290 containing the D2prM290 sequence into pSINrep5, yielding pRep2prM290As. The D2prM290 sequence was then isolated as an Xba I/ Not I fragment and ligated into the Xba I/ Not I site of pIErepHYG.

The construction of pIErepAnti-SHYG has been described (Allen-Miura, 2000).

Construction of pIE-3 –based plasmids. The pIE-3 plasmid (Novagen, Madison, WI) was modified to contain the hygromycin resistance gene before the insertion of sense or antisense dengue sequences (Fig 5.2). The 3'NCR of the pIE-3 plasmid was removed by digestion with Not I/ Nar I, and was replaced by hspHYG, which was isolated from pBHYG as a Not I/ Cla I fragment, creating pIEno3'HYG. A portion of the 3' NCR (derived from the SV40 3' NCR) from pIE-3 was subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA) following amplification, was excised with Not I, and was inserted into the Not I site of pIEno3'HYG, creating pIEHYG. Dengue 2 sequences were then excised as Xba I/ Nhe I fragments from pBD2prM290 (Chapter 2, materials and methods), and inserted into the Xba I site of pIEHYG, creating plasmids pIE2prMAsHYG, pIE2prMSHYG. Orientation of D2prM290 sequences was confirmed by sequencing.

Cells and Viruses. Growth medium (L-15 with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) and maintenance medium (L-15 with 2% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) were used to culture C6/36 cells and BHK-21 cells, as described in chapter 2. Hygromycin B-resistant cell lines were grown on L-15 with 10%FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and 300 U hygromycin B/ml. The DEN-2 (Jamaica 1409) virus was used as described in chapter 2.



1. Not I/Nar I and Not I/Cla I fragments from pIE-3 and pBHYG ligated to produce pIEno3'HYG.

4. Ligation of 3'NCR into pIEno3'HYG using Not I.

3. Subcloning of 3'NCR into pCR 2.1.

2. PCR of partial 3'NCR.

Fig 5.2 Cloning strategy for plasmid pIEHYG (shown in bold, see text for details.

***In vitro* transcript production.** pIErep2prM290AsHYG and pIErepGFPHYG were linearized with Not I, proteinase K treated, phenol/chloroform extracted, and ethanol precipitated. RNA was transcribed *in vitro* from the SP6 promoter of linearized replicon cDNA in the presence of 1 X transcription buffer, 1 mM cap analog, 50 U RNasin, 40 U SP6 polymerase (Ambion, Austin TX), 1mM rNTPs, 5 mM dithiothreitol and 500 ng bovine serum albumin (Promega, Madison, WI). *In vitro* transcribed RNA was electroporated into BHK-21 cells using a BTX electro cell manipulator 600™ at 450 V, 100 μF, and 720 Ohms. Electroporated cells were seeded into 25cm^2 flasks in 5 ml of growth medium.

Establishment of transformed cell lines. Plasmids pIErep2prM290AsHYG, pIErepGFPHYG, pIErepAnti-SHYG and pIED2prM290AsHYG were used to establish transformed, non-clonal mosquito cell lines. Lipofectin (GibcoBRL, Rockland, MD) was mixed with L-15 medium, in a ratio of 1:2, and incubated 45 min at ambient temperature. Ten micrograms of plasmid DNA was mixed with 30-50 μl of L-15 and combined with 150 μl of the lipofectin mixture for 15 min. C6/36 cells in 25cm^2 flasks that had reached 70-80% confluency were washed 2X with 2-3 ml of PBS, followed by the addition of 2 ml of L-15 and finally the L-15/Lipofectin/DNA mixture. Cells were incubated in the L-15/Lipofectin/DNA mixture overnight (~16 h) at which point the mixture was replaced by growth medium. After 24 h this medium was replaced with growth medium containing 300 U hygromycin B/ ml. Fresh medium was applied every 2-3 days, and hygromycin B-resistant cell lines developed after 3-5 weeks of selection.

RNA analysis. Total cellular RNA was extracted from 25cm^2 flasks containing infected or uninfected C6/36 cells or BHK-21 cells according to the manufacturer's specifications using the Qiashredder and RNeasy mini kits (Qiagen Inc., Chatsworth, CA). For northern blot analysis, 5 µg of total RNA was electrophoresed in a 1% agarose/MOPS/formaldehyde gel and transferred to a positively charged nylon membrane (Brightstar from Ambion, Inc., Austin, TX). Blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored until hybridization.

For slot blot analysis, 5 µg total RNA was denatured in 150 µl of 6X SSC, 7% formaldehyde at 65°C for 10 min and vacuum blotted onto a positively charged nylon membrane (Brightstar from Ambion, Inc., Austin, TX) using a Minifold II slot blot apparatus (Schleicher +Shuell, Keane, NH). Wells were washed with 100 µl of 6X SSC, 7% formaldehyde. Slot blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored until hybridization.

An oligonucleotide (5' GCTGGTCGGATCATTGGGGCG 3') complementary to the 3' end of the SIN RNA non-coding region was used to detect dsSIN RNA. A PCR product consisting of both D2prM290 and SIN replicon sequence was used as a hybridization probe to detect SIN and D2prM290 RNA transcripts simultaneously. A PCR product corresponding to a portion of the DEN-2 E gene (nt 1642 – nt 1912) was used to detect DEN-2 RNA. PCR products to be used as probes were labeled with a psoralen-biotin conjugate according to the manufacturer's specifications (Ambion, Austin, TX). Labeled probes were added to hybridization buffer (7.0% SDS, 0.47 M Na_2HPO_4, 1.7 mM H_3PO_4) and hybridized overnight at 42°C for primer probe and 65°C for PCR product probes. Blots were washed twice with 2X SSC, 0.1% SDS for 20 min each, followed by 2 washes in 0.2X SSC, 0.1% SDS for 30 min each. Blots were then immediately subjected to Ambion's Brightstar Non-Isotopic Detection Kit and exposed to Kodak ML light film.

RT-PCR. RNA extracted from cells was reverse transcribed using 75 units of Superscript II (Gibco BRL, Rockville, MD) and the cDNA was amplified using Taq polymerase (Promega, Madison, WI) in one reaction. (42^{0}C for 1 hr; 92^{0}C, 30 sec; 52^{0}C, 1 min; 72^{0}C, 2 min; 26 cycles; 72^{0}C, 7 min.). RT-PCR buffer contained 50 pmol of primer (D2prM290 F 5' ACCACACGTAACGGAGAACCA 3'; R 5' TCCCACATGTGGAACGAGTGC 3') in 1X Taq buffer (Promega, Madison, WI), 250 pmol dNTPs and 2.5 mM MgCl.

Challenge of transformed cell lines. C6/36 cells or non-clonal transformed cell lines were seeded on 18mm glass coverslips in 12-well plates or 25cm^2 flasks and grown at 28^{0}C. When the cells were approximately 80-90% confluent, they were challenged with DEN-2 virus at an m.o.i of 0.1 in a total volume of 300 μl for 18mm coverslips or 1 ml for 25cm^2 flasks. Virus was allowed to adsorb for 30 min at ambient temperature with gentle rocking. Infection medium was then removed and cells were allowed to recover in growth medium (non-transformed C6/36 cells) or growth medium containing 300U HYG/ml (transformed cells) for 4 hr to overnight. Cells were transferred to maintenance medium (non-transformed C6/36 cells) or maintenance medium with 300U HYG/ml (transformed cells) to prevent overgrowth during the incubation period.

Estimate of DEN infected cells. At six days post challenge cells were fixed in 3:1 acetone:PBS for 5 min at –20^{0}C and stored in PBS until IFA was performed. IFA was performed using primary Mab 4G2, as described in chapter 2. Three fields of view from each coverslip were photographed using an Olympus DP10 digital camera. All digital files were modified in Adobe Photoshop by adjusting the brightness by +20 and the contrast by +75. Cells containing DEN virus antigen were counted in Adobe Photoshop and expressed as a ratio to the total number of cells counted. Experiments were performed with duplicate coverslips, and percent DEN-2 infected cells was expressed as the average of all trials.

RESULTS

Detection of SIN transcripts in BHK-21 cells. *In vitro* transcription was performed using pIErep2prM290AsHYG and pIErepGFPHYG as templates to verify that the SIN replicon reading frames were intact prior to transfection of cDNA. RNA was electroporated into BHK-21 cells, which were harvested 16-18 h later. Analysis of RNA from these cells showed that genomic and subgenomic mRNAs were produced in transfected cells after electroporation of RNA transcribed from both the pIErep2prM290AsHYG and pIErepGFPHYG plasmids (Fig 5.3). The difference in transcription of subgenomic mRNA between pIEreGFPHYG and pIErepD2prM290AsHYG may be due to different electroporation efficiency or to differences in the integrity of the electroporated RNAs. Regardless, subgenomic mRNAs were detected, indicating that there were no disruptions in the reading frames of these plasmids during the multiple subcloning steps. Non-specific bands most likely representing ribosomal RNAs were observed in all lanes, including mock-transfected BHK-21 cell RNA. Transfection of pIErepGFP RNA also resulted in the expression of GFP fluorescence in >80% of BHK-21 cells (not shown).

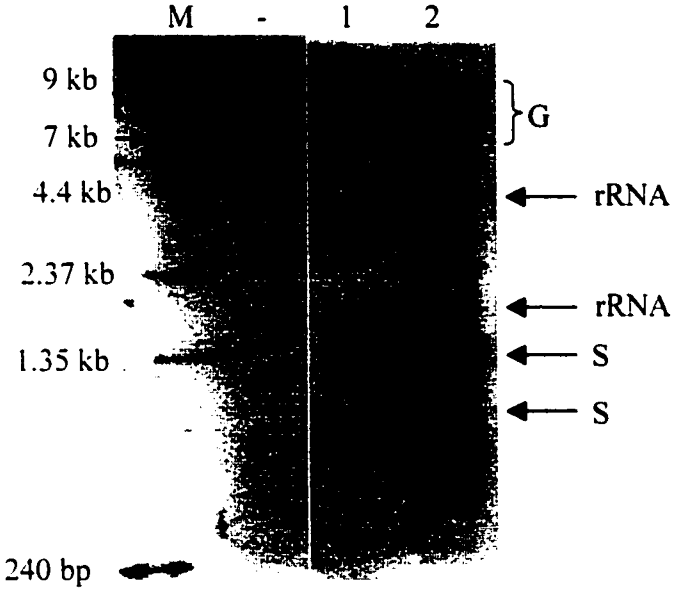


Fig 5.3. Detection of SIN mRNA transcripts in BHK-21 cells. RNA was isolated from BHK-21 cells 16 h after electroporation of *in vitro* transcribed RNA from SIN replicon cDNAs. Blot was hybridized to an oligonucleotide complementary to the 3' end of the SIN non-coding region, as described in the materials and methods section. (1) pIErep2prM290As, (2) pIErepGFP. (M) Marker, (-) mock-transfected BHK-21 cells. (G) indicates position of genomic mRNA transcript, (S) indicates subgenomic mRNA transcript.

Detection of mRNA transcripts in transformed cells. Northern blot analysis was conducted in order to determine if the transformed, non-clonal cell lines were expressing detectable levels of D2prM290As mRNA. Fig 5.4a shows results of hybridization to RNA isolated from transformed, hygromycin resistant cell lines (passage <5) IErepD2prM290As, IErepGFP, and IED2prM290As compared to that of non-transformed C6/36 cells and TE/3'2J-infected C6/36 cells. Genomic and subgenomic mRNA species were detected for the TE/3'2J infected cells, as expected. A band corresponding to the expected size of the subgenomic mRNA was also detected in IErepD2prM290As cell RNA. Genomic-length mRNA was not detected in these cells. No hybridization was detected to RNA isolated from IErepGFP, IED2prM290As or C6/36 cells. To determine if mRNA transcription was occurring in IED2prM290As cells, but below the level of detection by northern blot hybridization, RT-PCR was performed using primers specific for the D2prM290 sequence (Fig 5.4b). Consistent with the northern analysis, D2prM290 sequence was detected in the IErepD2prM290As cells (arrow). No product was seen in the absence of reverse-transcriptase. D2prM290 sequence was not detected in IED2prM290As, IErepGFP or C6/36 cells. IErepGFP cells were examined for the presence of GFP under an inverted UV light microscope before and after hygromycin selection. GFP-specific fluorescence was observed in both cases, but at extremely low levels (<0.001% of cells, data not shown).

DEN-2 RNA accumulation in SIN replicon-cDNA transformed cells. Transformed, hygromycin-resistant IErepD2prM290As cells (passage <10) were challenged with DEN-2 and RNA was collected from cells at 3 and 6 days post challenge. As controls,

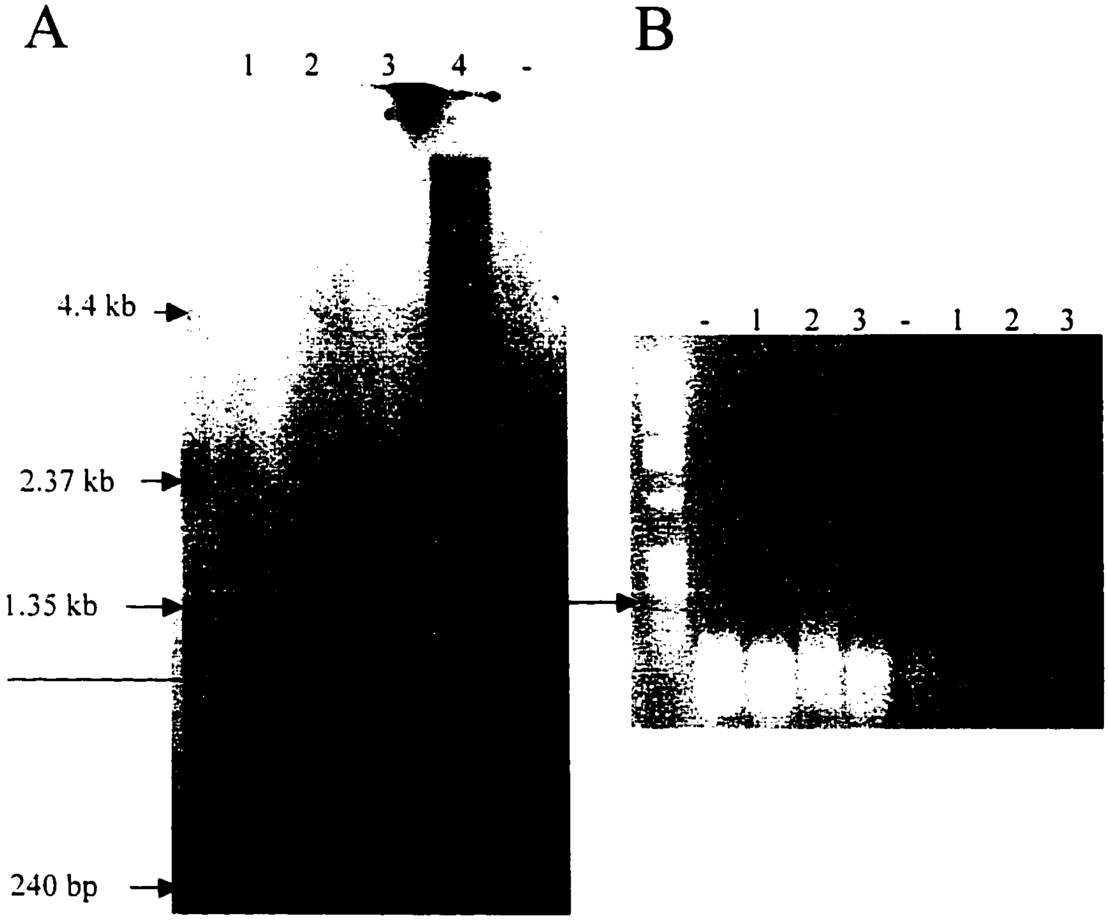


Fig 5.4. Detection of D2prM290 RNA in transformed mosquito cells. (**A**) Northern blot of RNA extracted from transformed cell lines. Blot was hybridized to a probe containing both SIN replicon and D2prM290 sequence. as described in the materials and methods. (1) IErepGFP, (2) IErepD2prM290As, (3) IED2prM290As, (4) TE/3'2J, (-) C6/36 cells. Expected size of subgenomic mRNA in IErepD2prM cells is ~750 bp (indicated by arrow). (**B**) RT-PCR of RNA from transformed cells (-) C6/36, (1) IED2prM290As, (2) IErepGFP, (3) IErepD2prM290As using primers (D2prM290 F + R) specific for the inserted DEN-2 sequence. RT = in the presence of reverse transcriptase. No RT= no reverse transcriptase was added. Arrow indicates band of the expected size (290 nt of the prM).

IErepGFP, IErepAnti-S (cells transformed with a replicon cDNA designed to express RNA derived from LAC virus) and non-transformed C6/36 cells were also challenged. Fig 5.5a shows that DEN-2 RNA accumulates in C6/36, IErepGFP and IErepAnti-S cells by 6 days post challenge, although RNA accumulation seemed to be reduced in IErepAnti-S cells. In contrast, by 6 days post infection RNA did not accumulate in IErepD2prM290As cells. Longer exposures revealed that DEN-2 RNA is weakly detectable in IErep2prM290 cells at 6 days post challenge, but background becomes substantial (not shown). Fig 5.5b shows a duplicate RNA blot probed for a cellular actin mRNA, verifying equal loading of all wells.

Percentage of DEN-2 infected cells after challenge in transformed cell lines. The percentage of IErepD2prM290As cells (passage 10-20) containing DEN-2 E antigen was estimated at six days post challenge. IErepGFP, IErepAnti-S, IED2prM290As and non-transformed C6/36 cells were also challenged with DEN-2 virus as controls. Non-transformed C6/36 cells and IErepGFP cells fully supported the replication of DEN-2 virus, as evidenced by the observation that >80% of cells contained DEN-2 E antigen (Fig 5.6). Cells transformed with pIErepAnti-SHYG, a plasmid that conditions RNA-mediated resistance to LAC virus (Allen-Miura, 2000), were also permissive for DEN-2 virus. Non-clonal cells derived from transfection with pIE2prM290As, a plasmid designed to express only antisense DEN-2 prM RNA (but not dsRNA) permitted DEN-2 virus replication in >40% of cells. In contrast, only 5% of cells transformed with the pIErep2prM290AsHYG plasmid contained DEN-2 antigen at six days post infection.

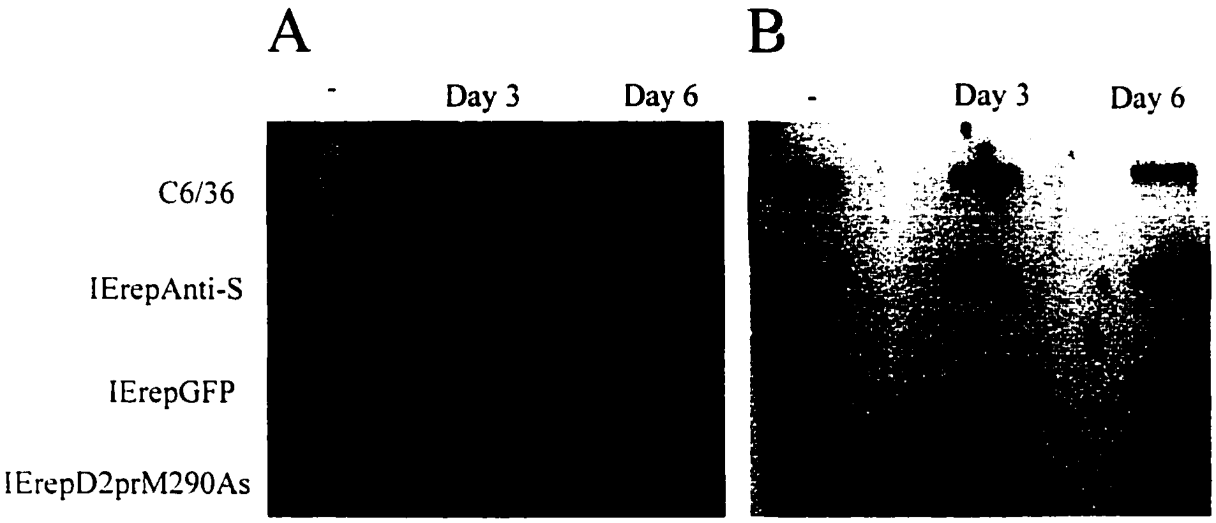


Fig 5.5. Challenge of SIN replicon cDNA transformed non-clonal cell lines with DEN-2. RNA was isolated from cells harvested at 3 or 6 days post challenge. Duplicate blots were probed either for (A) DEN-2 RNA or (B) B-actin mRNA.

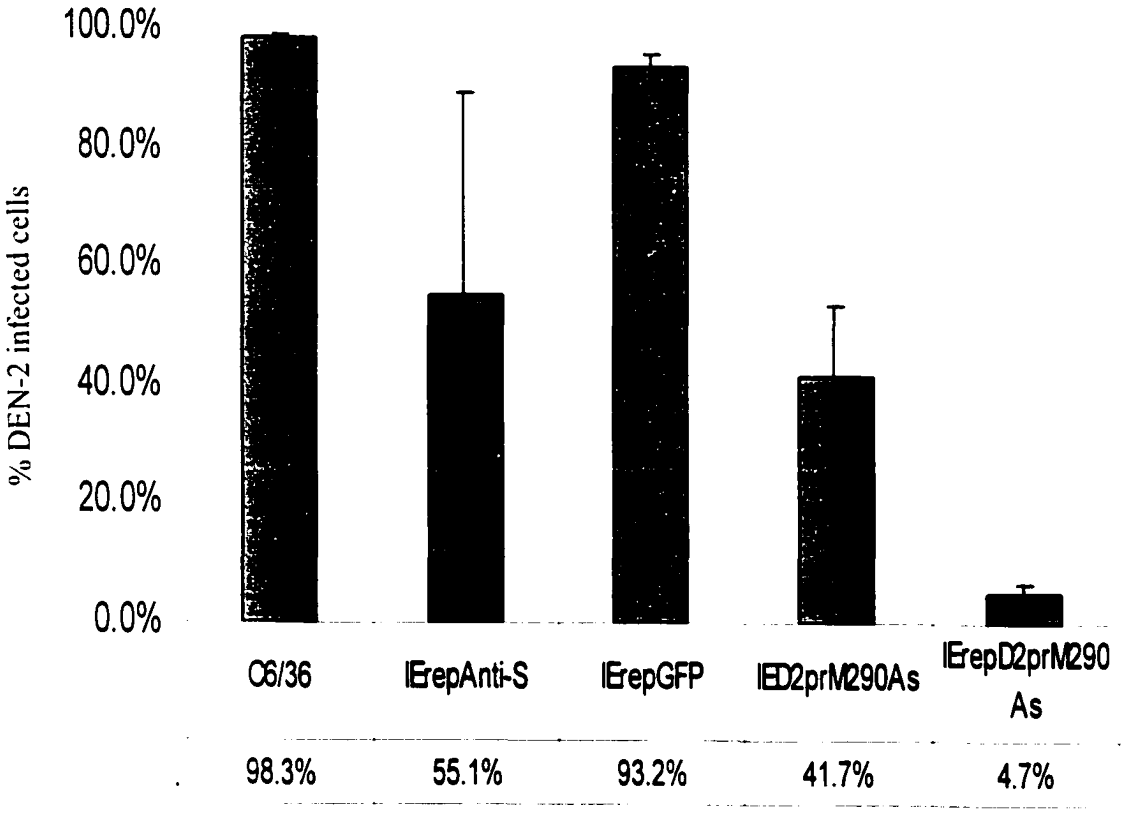


Fig 5.6. Percentage of DEN-2 infected cells after challenge in transformed cell lines. The percentage of C6/36, IErepAnti-S, IErepGFP, IED2prM290As and IErepD2prM290As cells expressing DEN-2 antigen six days after challenge with DEN-2 virus was determined by IFA.

DISCUSSION

Transformation by DNA encoding the SIN replicon under the control of the baculovirus IE1 promoter resulted in the expression and self-amplification of SIN replicon RNA. DNA-based alphavirus replicons have also been developed for expression in mammalian cells for the purpose of DNA vaccination (DiCiommo and Bremner, 1998; Dubensky *et al.*, 1996) and for the sensitive detection of alphaviruses (Olivo *et al.*, 1994). DNA-based alphavirus expression systems offer several advantages over the RNA-based SIN system described in chapters 2-4. The need to generate capped, *in vitro* transcribed RNA, which is both costly and sensitive to contamination/ degradation, is eliminated. Also the need to generate, titrate, and store SIN virus stocks is removed. The DNA-based approach also simplified challenge experiments, as challenge experiments involving infectious SIN viruses and DEN viruses require precise timing to avoid cell death due to overgrowth.

Expression of DEN-2 prM sequence (290 nt) in C6/36 cells by a SIN replicon transcribed from the nucleus resulted in >90% of cells being resistant to DEN-2 virus. Both DEN-2 specific RNAs and DEN-2 E antigen failed to accumulate in these cells as shown by RNA slot blot hybridization analysis and immunofluorescence assay. Other researchers have utilized DNA-based replicons (termed "amplicons") based on the positive-strand RNA genome of potato X virus (Angell and Baulcombe, 1999; Dalmay *et al.*, 2000a). These amplicons were capable of generating a post-transcriptional gene-silencing response in a much higher percentage of plants than traditional DNA plasmids encoding only a sense or antisense sequence (Angell and Baulcombe, 1999). This is consistent with the indirect model of dsRNA as the inducer of gene silencing, since potato virus X also has a dsRNA intermediate as part of its replication cycle (Riechmann *et al.*, 1992). The results presented here support the important role of the SIN replicon (dsRNA) in triggering the RNA-mediated response, as the antisense D2prM290 RNA sequence did not protect cells from DEN-2 virus when expressed directly from the IE1 promoter to the same extent as when transcribed by the SIN replicon.

Use of the DNA-based SIN replicon system also has its drawbacks. Mainly, the large size of SIN replicon cDNA makes it not well suited for the transformation of mosquitoes. Maintaining the integrity of such a large transcriptional unit while attempting to drive the SIN replicon into mosquito populations also seems unlikely. Also, transfection of the large (14.5kb) DNA plasmids into C6/36 cells via lipofectin was extremely inefficient. When the gene encoding the jellyfish green fluorescent protein (GFP) was placed downstream of the subgenomic promoter (in pIErepGFPHYG), GFP fluorescence was observed in a very small number of cells (10^{-4}-10^{-5}) 24-48 h after transfection. To overcome the low efficiency of transfection, transformed cells in this study were selected by resistance to the antibiotic hygromycin B. Attempts to improve transfection efficiency by electroporating DNA using various conditions were unsuccessful, and resulted in low efficiencies of GFP expression as well (not shown). Future attempts to improve the efficiency of transfection would certainly increase the utility of the DNA-based SIN replicon system, by eliminating the need for antibiotic selection. The DNA-based SIN replicon could then serve as an important tool in the process of developing new antiviral agents in mosquito cells, obviating the need to produce, titrate, and store dsSIN viruses.

Chapter 6

dsRNA-mediated interference to DEN-2 virus in stably transformed, clonal mosquito cell lines

INTRODUCTION

In this study mosquito cells were transformed with DNA plasmids designed to express sense, antisense or double-stranded RNA derived from the genome of DEN-2 virus. Double-stranded RNA, derived from the genome of DEN-2 virus and produced from the nucleus of mosquito cells, should trigger PTGS and result in resistance to DEN-2 virus challenge. Similar expression of sense or antisense RNA alone should not be capable of conferring resistance, as the dsRNA trigger would not be expected to be present.

Transformation of plants with transgenes designed to express sense or antisense RNA homologous to endogenous plant genes results in PTGS of both the transgene and the endogenous gene in a limited number of plants (2-30%) (Grant, 1999). With the discovery that PTGS is triggered by dsRNA (Fire *et al.*, 1998), many researchers have redesigned DNA constructs used to generate PTGS phenotypes. Waterhouse *et al* (1998) reported that 50% of plants transformed to express dsRNA derived from potato virus Y were resistant to virus challenge, whereas less than 15% of plants designed to express sense or antisense RNA alone were virus-resistant. *Drosophila* geneticists have reported PTGS of endogenous genes with the expression of dsRNA from a plasmid was similar to injecting dsRNA directly (Kennerdell and Carthew, 2000). This represents an improvement over PTGS initiated by injected dsRNA, which has been shown to be incapable of penetrating into later stages of development (Kennerdell and Carthew, 1998). PTGS induced by dsRNA in *Drosophila* can be therefore be controlled temporally if expression of the dsRNA is controlled at the DNA level by inducible promoters

(Fortier and Belote, 2000; Kennerdell and Carthew, 2000). The expression of dsRNA from stably introduced transgenes is likely to be more useful in generating virus-resistant mosquitoes than direct dsRNA injection, as PTGS can then be controlled and regulated by tissue-specific, inducible or constituitive promoters, and will be passed on to subsequent generations. The activities of midgut-specific, salivary gland-specific, as well as constituitive promoters have been described in *Ae. aegypti* mosquitoes (Coates *et al.*, 1999; Pinkerton *et al.*, 2000). The potential then exists to generate transformed *Ae. aegypti* mosquitoes capable of expressing dsRNA derived from one or more DEN viruses in specific tissues upon an induction signal, with the result being mosquitoes refractory to virus infection. Before such ground-breaking studies are undertaken we aimed to demonstrate that such a state of virus resistance could be generated in cultured mosquito cells.

Several attempts to generate stably transformed, virus-resistant mosquito cells have been made over the past ten years at the AIDL. However, all attempts were made with the direct model of antisense interference in mind. Following transfection, cells were clonally selected and the resulting lines were screened for mRNA expression. The lines expressing the highest amounts of antisense or sense RNA were the lines that were further characterized and challenged. Not surprisingly (in retrospect...) these lines did not offer significant, reproducible resistance to LAC virus challenge (Allen-Miura, 2000; Monroe, 1990; Powers, 1995). Cell lines with low-level or no expression of the sense or antisense RNA were always found, but these lines, which may have been subject to PTGS, were never challenged. Because PTGS may or may not be accompanied by TGS, the presence or absence of transgene mRNA alone is not predictive for whether or not a given cell line will be resistant to virus challenge. Therefore, in the studies described in this chapter, all transformed cell lines were screened directly for their resistance/ susceptibility to DEN-2 virus.

MATERIALS AND METHODS

Plasmids, Cells, Viruses. The construction of plasmids pIEHYG, pIED2prM290As and pIED2prM290S was described in chapter 5. An additional plasmid was designed to express dsRNA corresponding to the DEN-2 prM gene. pIEMnpD2prM290As was constructed by inserting the Mnp sequence (567 nt of the DEN-2 genome corresponding to the 3' end of the C gene, the entire prM-M gene and a 5' portion of the E gene; np= no protein) in sense orientation into the Xba I site of pIED2prMAs. Mnp was isolated from pBMnp as an Nhe I/ Xba I fragment, as described by Gaines *et al* (1996). The pIEHYG plasmid, as well as the expected mRNA transcripts for pIE2prM290S, pIED2prM290As, and pIEMnpD2prM290As are shown diagrammatically in Fig 6.1 (A&B). Growth medium (L-15 with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) and maintenance medium (L-15 with 2% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) were used to culture C6/36 cells, as described in chapter 2. Conditioned medium used during the cell selection process consisted of 50% C6/36 cell culture fluid (medium removed from confluent C6/36 cells and filtered through a 0.2 μm filter), 10%FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 37% L-15 and 300 U hygromycin B/ml. Hygromycin B-resistant cell lines were grown on L-15 with 10%FBS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2mM L-glutamine and 300 U

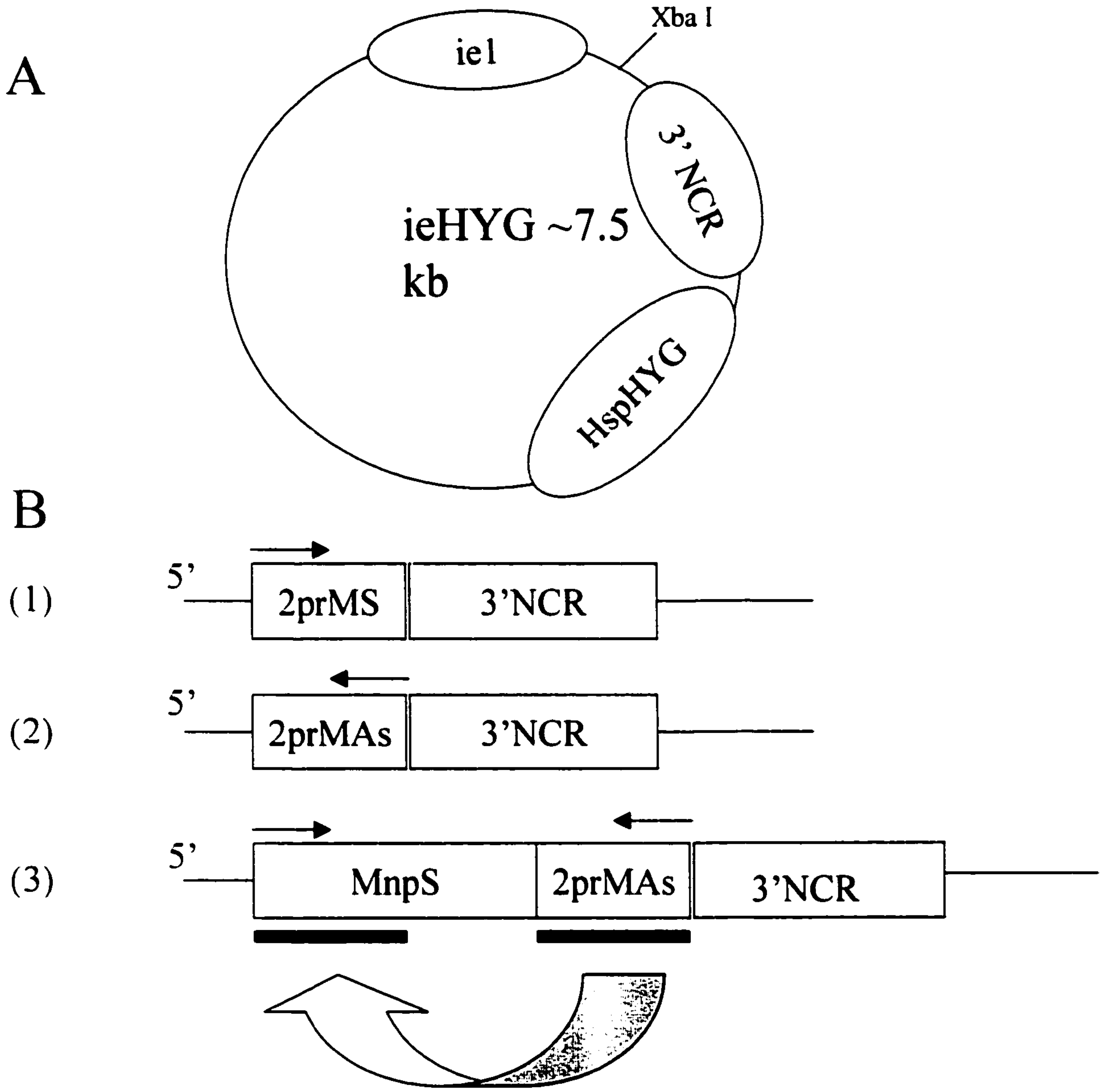


Fig 6.1. Map of pIEHYG plasmid and expected mRNA transcripts. (A) The pIEHYG plasmid was used for transformation and expression of DEN-2 sequences in C6/36 cells. DEN-2 prM sequence was cloned into the Xba I site in sense and/or antisense orientation. (B) Theoretical mRNA species produced by (1) sense, (2) antisense, or (3) foldback plasmids. Bars indicate complementary sequence.

hygromycin B/ml. DEN-2 (New Guinea C) virus was used interchangeably with the DEN-2 (Jamaica 1409) virus described in chapter 2 for challenges.

Establishment of clonal cell lines. Plasmids pIEHYG, pIED2prM290S, pIED2prM290As and pIEMnpD2prM290As were used to transform mosquito cell lines. Lipofectin (GibcoBRL, Rockville, MD) was mixed with L-15 medium, in a ratio of 1:2, and incubated 45 min at room temperature. Following incubation, 0.1-1.0 μg of plasmid DNA was mixed with 30-50 μl of L-15 and combined with 150 μl of the lipofectin mixture for 15 min. C6/36 cells in 25cm^2 flasks that had reached 50-70% confluency were washed 2X with 2-3 ml of PBS, followed by the addition of 2 ml of L-15 and finally the L-15/Lipofectin/DNA mixture. Cells were incubated in the L-15/Lipofectin/DNA mixture overnight (~16 h) at which point the mixture was replaced by growth medium. 24 h later this medium was replaced with growth medium containing 300 U Hygromycin/ml. Fresh medium was applied every 2-3 days, until the majority of cells had died. Remaining living cells were then placed on conditioned medium. Colonies were picked using extra-long gel-loading micropipet tips and inoculated into 25cm^2 flasks containing 5 ml of conditioned medium. Upon colony formation this process was repeated, with all clonal cell lines established being derived from at least two rounds of colony purification.

DEN-2 challenge of clonal cell lines. Clonal cell lines were seeded on 18mm glass coverslips in 12-well plates and grown at 28°C. When the cells were approximately 80-90% confluent, they were challenged with DEN-2 at an m.o.i of 0.01 in a total volume of 300 μl. Virus was allowed to adsorb for 30 min at ambient temperature with gentle rocking. Infection medium was then removed and cells were allowed to recover in growth medium containing 300U HYG/ml for 4 hr to overnight, at which point they were transferred to maintenance medium with 300U HYG/ml. At seven days post challenge cells were fixed in 3:1 acetone:PBS for 5 min at –20°C and stored in PBS until immunofluorescence assays (IFA) were performed. IFA was performed as described in chapter 2, using monoclonal antibody 4G2.

Determination of the percentage of DEN-2 infected cells. Three fields of view from each coverslip were photographed using an Olympus DP10 digital camera. All digital files were modified in Adobe Photoshop by adjusting the brightness by +20 and the contrast by +75. Cells containing DEN-2 virus antigen were counted in Adobe Photoshop and expressed as a ratio to the total number of cells counted. All experiments were performed with duplicate coverslips each time, and percent DEN-2 infected cells was expressed as the average of all trials.

DNA isolation. DNA was isolated from clonal cell lines according to the manufacturer's specifications using the DNeasy mini kit (Qiagen Inc., Chatsworth, CA), except DNA was eluted from the DNeasy spin column twice in a volume of 150 µl of AE elution buffer each time.

DNA slot blot analysis. DNA isolated from clonal cell lines was digested with the restriction enzyme EcoR I for 3-4 h at 37°C. Digested DNA was then precipitated at –20°C for 20 min with 1/10 volume of 3M sodium acetate, pH 5.5 and three volumes of 100% ethanol. Precipitated DNA was centrifuged for 20 min at 4°C, 17500 X g, washed with 70% ethanol, and centrifuged for 5 min at 4°C, 17500 X g. Pellets were allowed to air dry, and were resuspended in a small volume of sterile H_2O. DNA samples were denatured for 1 h at 60°C after the addition of 1/10 volume 3M NaOH. One volume of 6X SSC was added and samples were vacuum blotted onto a positively charged nylon membrane (Brightstar from Ambion, Austin, TX) using a Minifold II slot blot apparatus (Schleicher +Shuell, Keane, NH). DNA slot blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored at –20°C until hybridization.

Plasmid pIED2prM290S was used as a probe to detect plasmid DNA isolated from transformed cells. pIED2prM290S was digested with EcoR I and labeled with a psoralen-biotin conjugate according to the manufacturer's specifications (Ambion, Austin, TX). Labeled probe was denatured at 99°C for 10 min, plunged into ice (1-2 min) and added to hybridization buffer (7.0% SDS, 0.47 M Na_2HPO_4, 1.7 mM H_3PO_4). Hybridization was allowed to proceed overnight at 65°C. Following hybridization blots were washed twice with 2X SSC, 0.1% SDS at 20 min each, followed by 2 washes in 0.2X SSC, 0.1% SDS for 30 min each. Blots were then immediately subjected to Ambion's Brightstar Non-Isotopic Detection Kit and exposed to Kodak ML light film.

Determination of DNA plasmid copy number per cell. Exposed films were scanned using an AGFA Duoscan T1200™ scanner. Bands were quantified using the program UN-SCAN-IT gel™ version 3.1 (Silk Scientific Corporation). DNA plasmid copy number was then estimated by assuming the that the average weight of a nucleotide pair was 660 daltons/ bp, that there were 1.67 x 10^{-24} g/dalton; and that 1×10^{7} cells contained 50 μg of total high molecular weight DNA (Sambrook *et al.*, 1989). (ex. A 7500 bp plasmid x 660 d/bp = 5×10^{6} d; 5×10^{6} d x 1.67×10^{-24} g/d = 8.3×10^{-18} g; 2 μg of cell DNA= 4×10^{5} cells: 8.3×10^{-18} g x 4×10^{5} cells = 3.3×10^{-12} g or 3.3 pg corresponds to 1 plasmid copy per cell). Several exposures were scanned and quantified, and the results were averaged to give the approximate DNA plasmid copy number per cell.

Southern blot hybridization analysis. Genomic DNA was digested with the appropriate restriction enzyme for approximately 5 h at 37°C. Digested genomic DNA was then precipitated at –20°C for 20 min with 1/10 volume of 3M sodium acetate, pH 5.5 and three volumes of 100% ethanol. Precipitated DNA was centrifuged for 20 min at 4°C, 17500 X g, washed with 70% ethanol, and centrifuged for 5 min at 4°C, 17500 X g. Pellets were allowed to air dry, and were resuspended in a small volume of sterile H_2O. DNA was loaded onto a 0.7% agarose, 1X TAE gel and electrophoresed at 100 V for 2-3 h. DNA was depurinated by soaking gels for 10 min in 0.2N HCl; denatured for 45 min in 1.5M NaCl, 0.5M NaOH; and neutralized for 30 min and 10 min in 1.5M NaCl, 1.0M Tris, pH 7.4. Following neutralization DNA was transferred via capillary action to a positively charged nylon membrane (Brightstar™, from Ambion, Austin, TX). DNA blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored at –20°C until hybridization.

Probes were labeled by randomly-primed incorporation of [α-^{32}P]dCTP (3000 Ci/mmol) using the Megaprime DNA labeling system (Amersham-Pharmacia Biotech, Piscataway, NJ), and using the pIEMnp2prM290As plasmid as template. Specific activity of probes was 4.0x 10^8 dpm/ug. Probes were denatured by boiling for 10 min, plunged into ice (1-2 min) and added to hybridization buffer (7.0% SDS, 0.47 M Na_2HPO_4, 1.7 mM H_3PO_4). Hybridization was allowed to proceed overnight at 65°C. Following hybridization blots were washed twice with 2X SSC, 0.1% SDS at 20 min each, followed by 2 washes in 0.2X SSC, 0.1% SDS for 30 min each. Blots were then exposed at –70°C with an intensifying screen for 1-7 days to Kodak Biomax MR film.

RNA analysis. Total cellular RNA was extracted from clonal cell lines according to the manufacturer's specifications using the Qiashredder and Rneasy mini kits (Qiagen Inc., Chatsworth, CA). For northern blot analysis, 5 μg of total RNA was electrophoresed in a 1% agarose/MOPS/formaldehyde gel and blotted to a positively charged nylon membrane (Brightstar from Ambion, Inc., Austin, TX). Blots were UV crosslinked using the autocrosslink function on a Stratalinker 1800 (Stratagene, La Jolla, CA) and stored at –20°C until hybridization. RNA probes corresponding to the D2prM290 sense and antisense strands were transcribed *in vitro* by using T3 or T7 RNA polymerase and Xba I or Nhe I linearized pBD2prM290 as templates. Transcription reactions were performed using the MAXIscript™ kit (Ambion, Austin, TX) including 10 mCi/ ml of α-^{32}P labeled UTP with an activity of 3000 Ci/mmol. Specific activity of RNA probes was determined to be 1.0×10^{11} cpm/μg by TCA precipitation using salmon sperm DNA as a carrier, as recommended by the manufacturer (Ambion, Austin, TX). Blots were hybridized with 2.0×10^{8} cpm/ml of RNA probe in UltraHyb™ (Ambion, Austin, TX) hybridization buffer at 68°C for 12-16 h. Blots were washed twice with 2X SSC, 0.1%SDS for 20 min each, followed by 2 washes in 0.2X SSC, 0.1% SDS for 30 min each. Blots were then exposed at –70°C with an intensifying screen for 1-7 days to Kodak Biomax MR film.

Plaque assays. Plaque assays were performed using the method of Miller *et al* (1986). Vero cells were seeded in six well plates in M199 medium containing 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 250 μg/L fungizone, 10 μg/ml gentamycin, 0.23% sodium bicarbonate (growth medium). Vero cells were incubated at 37°C in 5% CO_2 until complete monolayer had formed. Serial dilutions (ten-fold) of cell culture medium from DEN-2 infected cells were made by adding 200 μl of infected cell medium to 1.8 ml of M199 medium containing 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 250 μg/L fungizone, 50 μg/ml gentamycin, 0.12% sodium bicarbonate. Diluted medium (100 μl) was added to each well (typically two wells per dilution) containing 0.5 ml of growth medium and incubated at 37°C for 1 hr, with rocking every 10 min. After incubation, 4 ml of primary overlay was added to each well. Primary overlay consisted of a 1:1 mixture of 2% Seaplaque agarose (FMC Bioproducts, Rockland, ME) and nutrient medium (2X Earle's balanced salt solution, 1.0% yeast extract, 5.0% lactalbumin hydrolysate, 0.46% sodium bicarbonate, 4.0% FBS, 50 μg/ml gentamcin, 1.25 μg/ml fungizone) which were incubated separately at 42°C until immediately before use. After agar had solidified plates were inverted and kept at 37°C and 5% CO_2 for six days, at which point 2 ml of secondary overlay was added to each well. Secondary overlay was identical to primary overlay, except it also contained 0.01% neutral red. Plates were then wrapped in aluminum foil to protect from light and kept inverted at 37°C and 5% CO_2. Plaques were counted daily until no new plaques were observed (10-11 days after infection), at which point virus titer was calculated and expressed as PFU/ ml.

RESULTS

Establishment/ Challenge of transformed cell lines. After 2-3 months of selection and colony purification, 4 groups of transformed cell lines were obtained: 10 IEHYG (H, null) cell lines containing no DEN sequence; 10 IED2prM290S (sense, S) cell lines designed to transcribe 290 nucleotides of the DEN-2 prM gene in sense orientation; 18 IED2prM290As (antisense, As) cell lines designed to transcribe 290 nucleotides of the

DEN-2 prM gene in antisense orientation and 18 IEMnp2prM290As (foldback, FB) cell lines designed to express dsRNA derived from the DEN-2 prM gene. All cell lines were challenged with DEN-2 virus within ten passages of establishment to determine whether they were susceptible or refractory to DEN-2 infection.

Ten null cell lines were challenged with DEN-2, and nine out of ten lines supported DEN-2 virus replication in almost 80% of cells (Table 6.1). Line H9.1 only supported DEN-2 virus replication in 20-50% of cells in the first trial, but subsequent trials at later passages showed this line to be capable of supporting high levels of DEN-2 virus replication.

Ten sense cell lines were challenged with DEN-2 virus (Table 6.2). Results of the first trial at cell passage 3-5, indicated several lines (S1.3, S8.1, S9.1, S9.3) were resistant to DEN-2 virus infection. However, subsequent DEN-2 virus challenges at later cell passages (8-24) showed that lines S1.3, S8.1 and S9.1 were capable of supporting DEN-2 virus replication in >30% of cells. However, one line, S9.3, remained resistant to DEN-2 virus over at least five challenges encompassing over 50 cell passages (over 3 months) (Table 6.2).

Seventeen antisense cell lines were challenged with DEN-2 virus at cell passages 3-5. Several lines (As4.2, As4.3, As6.1, As10.1, As13.1) appeared to be resistant to DEN-2 virus, as only 1-10% of cells supported DEN-2 virus replication (Table 6.3). Subsequent challenges showed that lines As4.3, As6.1, As10.1 and As13.1 all become permissive for DEN-2 virus by cell passage 7-9. One line, As4.2, only supported DEN-2 virus replication in 8.4% of cells by passage 9, but by passage 59 more than half of the cells supported DEN-2 virus replication. As4.2 cells at passage 3 were further colony

Table 6.1. Challenge of null cell lines with DEN-2 virus.#

Null (H) Line #	Trial 1	Trial 2	Trial 3
2.1	p4 80 -100%	p7 48.9%±4.4%	
2.2	p3 80 -100%		
3.1	p3 50 -80%		
3.2	p4 50 -80%		
4.1	p3 80 -100%		
5.1	p4 80 -100%		
6.1	p3 80 -100%		
6.2	p3 80 -100%		
9.1	p4 20 -50%	p24 50.6%±11.2%	p47 84.4%±6.2%
9.2	p4 50 -80%		

#Cell lines derived from tranfection with the pIEHYG plasmid were challenged with DEN-2 virus, and the percentage of cells containing DEN-2 E antigen 7 days after challenge was determined by IFA. Each column represents a separate challenge experiment.

Table 6.2. Challenge of sense cell lines with DEN-2 virus.#

Sense (S) Line #	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
1.1	p4 20-50%*				
1.2	p4 20-50%*				
1.3	**p3 1%**	p8 20-50%			
4.1	pX 80-100%				
8.1	p4 1-20%	p12 10-20%	p24 31.9%±1.8%		
9.1	p4 1-20%	p8 10-20%	p15 20.7%±4.8%	p17 35.6%±4.7%	
9.2	**p4 1-10%***				
9.3	**p3 <1%**	**p8 <1%**	**p12 0.5%±0.2%**	**p28 2.0%±1.3%**	**p51 4.1%±2.0**
10.1	p5 80-100%				
10.2	p4 80-100%				

* significant cell death occurred during the challenge period.

#Cell lines derived from tranfection with the pIED2prM290S plasmid were challenged with DEN-2 virus, and the percentage of cells containing DEN-2 E antigen 7 days after challenge was determined by IFA. Each column represents a separate challenge experiment.. Bold indicates >90% resistance to DEN-2 virus.

Table 6.3. Challenge of antisense cell lines with DEN-2 virus.

Antisense (As) Line #	Trial 1	Trial 2	Trial 3
3.1	p4 20-50%		
4.1	p3 20-50%		
4.2	**p3 ~1%**	**p9 8.4%±1.4%**	p19 14.6%±1.5%
		@p27 28.7%±5.0%	p59 53.6%± 7.3%
4.2c	**p5 1.2%±1.3%**	**p7 13.3%±5.4%**	**p10 0.8%+0.5%**
4.3	**p4 ~1%**	p9 40.2%±7.1%	
5.1	p4 80-100%		
6.1	**p3 ~1%***	p7 28.5%	
6.2	**p4 1-10%***		
6.3	p3 ~50%		
8.1	p4 20-50%		
9.1	p4 10-20%		
9.2	p4 20-50%		
9.3	p4 10-20%		
10.1	**p3 1-10%***	p9 10-20%	
12.1	p4 10-20%*		
13.1	p4 1-20%	pX 49.2%±8.1%	
13.2	p3 20-50%*		
14.2	p3 20-50%		

* significant cell death occurred during the challenge period.

@ indicates trials 4 and 5.

**Cell lines derived from transfection with the pIED2prM290As plasmid were challenged with DEN-2 virus, and the percentage of cells containing DEN-2 E antigen 7 days after challenge was determined by IFA. Each column represents a separate challenge experiment.. Bold indicates >90% resistance to DEN-2 virus.

purified, under the assumption that an initially small population of susceptible cells was becoming more predominant after months of passage. Cell line As4.2c was obtained from this third round of colony purification, and was challenged with DEN-2 virus at passages 5, 7, and 10 (Table 6.3). As4.2c cells were almost completely resistant to DEN-2 virus at passages 5 and 10, with only 1% of cells containing DEN-2 antigen.

Eighteen foldback cell lines were challenged with DEN-2 virus at cell passages 4-16. Six foldback lines (FB1.2, FB2.1, FB 7.1, FB8.1, FB9.1, FB9.2) were consistently resistant to DEN-2 virus challenge, with less than 2% of cells containing DEN-2 antigen in most trials (Table 6.4). Two additional lines (FB1.1, FB18.2) were permissive for DEN-2 virus replication in the first trial, but at later passages became more and more resistant, with less than 2% of cells containing DEN-2 antigen by passage 11. One line, FB17.1, was initially resistant to DEN-2 virus, but by passage 16 supported DEN-2 virus replication in >40% of cells.

DEN-2 virus production in resistant cell lines. Cell culture medium from cell lines shown to be resistant to DEN-2 virus by IFA was collected at 7 days post DEN-2 challenge. This medium was titrated in Vero cells by plaque assay to determine the amount of DEN-2 virus produced. Untransformed C6/36 cells and null cell line H9.1 were included as a positive controls. Table 6.5 shows that sense (S9.3), antisense (As4.2c) and foldback (FB2.1, FB9.1) cell lines all produced 100-1000 fold less DEN-2 virus than either the null cell line H9.1 or untransformed C6/36 cells.

DNA plasmid copy number in transformed cell lines. In order to determine whether resistance or susceptibility to DEN-2 virus was related to the number of DNA plasmid copies/ cell, DNA from all cell lines was analyzed. DNA from null, sense and antisense

Table 6.4. Challenge of foldback cell lines with DEN-2 virus.[a]

Foldback (FB) Line #	Trial 1	Trial 2	Trial 3
1.1	p4 32% ± 14%	**p7 4.5% ± 2%**	**p11 1.2% ± 0.6%**
1.2	**p6 2.3% ± 1%**	**p10 1.8% ± 0.7%**	**p11 1.6% ± 1%**
2.1	**pX 0.3% ± 0.4%**	**p10 9.8% ± 4.2%**	**p13 0.8% ± 0.2**
	*** p14 2.0% ± 2.4%**		
3.2	p11 18.9% ± 9.5%	p14 27.4% ± 13.2%	
4.2	p6 58% ± 5.8%		
7.1	**p5 2.1% ± 1.6%**	**p11 1.3% ± 0.6%**	**p12 1.3% ± 0.6%**
8.1	**p10 2.1% ± 1.4%**	**p11 1.8% ± 0.8%**	
9.1	**p5 0.0% ± 0.0%**	**p11 0.9% ± 0.1%**	
9.2	**p6 0.1% ± 0.2%**		
11.1	p4 20.3% ± 5.3%		
11.2	p9 13.7% ± 4.7%	p10 11.2% ± 5.1%	
14.1	p5 19.4% ± 10.9%	p13 19.4% ± 9.9%	
14.2	p6 45.7% ± 14.3%		
15.1	p8 36.9% ± 6.5%		
16.1	p7 14.3% ± 7.6%		
17.1	**p5 1.4% ± 1.1%**	p15 13.7% ± 2.0%	p16 44.9% ± 19.3%
17.2	p5 74.3% ± 4.9%		
18.2	p7 28.4% ± 3.6%	**p10 2.5% ± 1.5%**	**p11 1.8% ± 1.3%**

* indicates 4th trial

[a] Cell lines derived from tranfection with the pIEMnpD2prM290As plasmid were challenged with DEN-2 virus, and the percentage of cells containing DEN-2 E antigen 7 days after challenge was determined by IFA. Each column represents a separate challenge experiment.. Bold indicates >90% resistance to DEN-2 virus.

Table 6.5. DEN-2 virus production in transformed cell lines challenged with DEN-2.[#]

Cell line	PFU/ml*
C6/36	1.7×10^5
H9.1	4.1×10^5
S9.3	5.1×10^3
As4.2c	2.3×10^2
FB2.1	2.1×10^3
FB9.1	1.2×10^3

* represents the average of 2-4 samples

[#] Amount of DEN-2 virus present in cell culture medium of null (H9.1), sense (S9.3), antisense (As4.2c), and foldback (FB2.1, FB9.1) transformed cell lines at 7 days post DEN-2 challenge, expressed as PFU/ml.

cell lines (passages 8-12) was analyzed on a single slot blot (Fig 6.2). This slot blot also contained known amounts (100ng-1pg) of plasmid DNA added to C6/36 genomic DNA, which was used to calculate the approximate DNA plasmid copy number/cell (Table 6.6). Plasmid copy number in null, sense and antisense cell lines ranged from 1-1500, with most lines shown to contain <50 plasmid copies. All null cell lines contained fewer than 10 copies of the IEHYG plasmid per cell. Cell lines As4.2 and As4.2c contained ~130 copies of the ieD2prM290As plasmid, the most out of all the lines derived from transfection with that plasmid. Four of the sense lines (S8.1, S9.1, S9.2, S9.3) contained over 500 copies of the ieD2prM290S plasmid. The remaining 6 sense lines contained fewer than 20 copies per cell. Plasmid DNA could not be detected in three antisense lines (As13.1, As13.2, As14.2) and two sense lines (S10.1, S10.2).

DNA from foldback cell lines was analyzed for plasmid copy number as well (Fig 6.3). Known amounts of pIED2prM290S DNA (1ng-2.5pg) were blotted to estimate the approximate DNA plasmid copy number in transformed cell lines. These estimates, along with the previously obtained DEN-2 challenge results, are shown in Table 6.7. Plasmid copy number in 17 foldback lines (FB8.1 was not analyzed) ranged from 1-250 copies per cell. Cell lines resistant to DEN-2 virus had copy numbers ranging from 1 to 65 copies per cell, while DEN-2 susceptible cell lines had plasmid copy numbers ranging from 2-250. No correlation between plasmid copy number and DEN-2 resistance/susceptibility was apparent in the foldback lines.

Data presented in Tables 6.1-6.7 is summarized in Table 6.8. Only 14 % and 7 % of cell lines transformed with a plasmid designed to express sense or antisense RNA derived from DEN-2 were found to be resistant to DEN-2 virus. Resistance to DEN-2

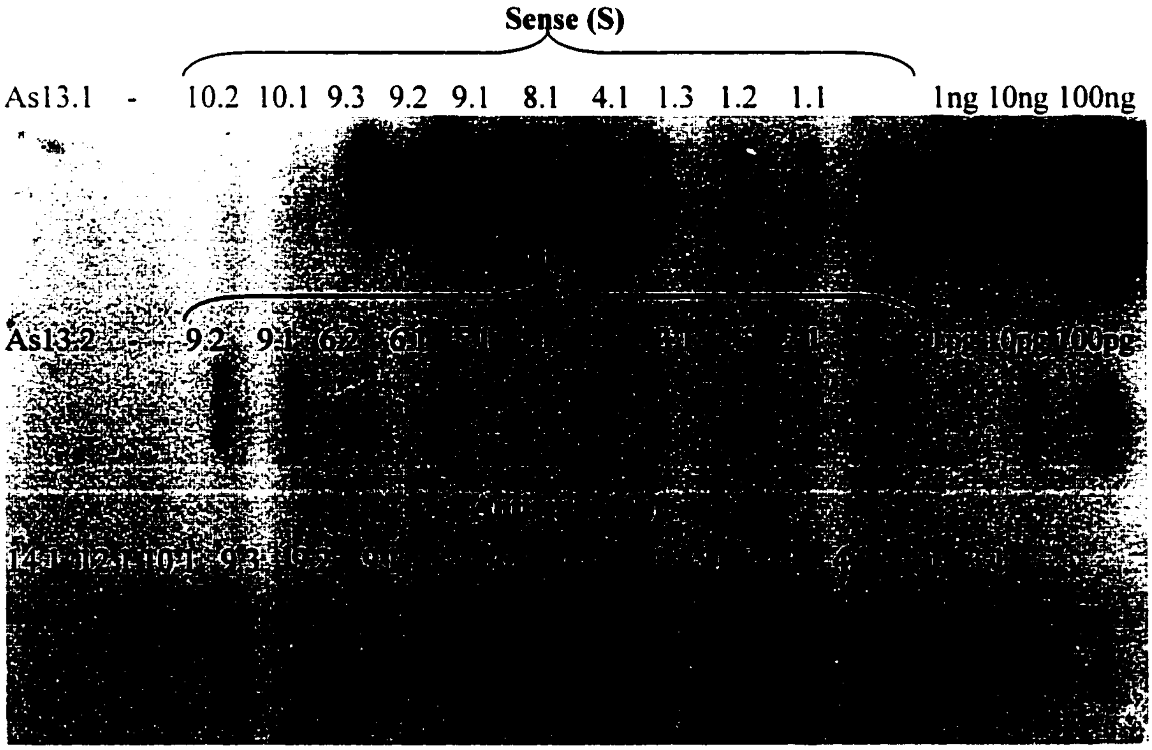


Fig 6.2. Slot blot analysis of null, sense and antisense cell line DNA. DNA isolated from IE2prM290S(sense), IEHYG (null), IE2prM290As (antisense) cell lines (passages 6-9), was digested with the restriction enzyme EcoRI. pIED2prM290S DNA (1pg-100ng) was included to calculate plasmid copy number per cell. (Film shown was one of several used to calculate the average plasmid copy number per cell).

Table 6.6 Approximate DNA plasmid copy number per cell for IED2prM290As (antisense), IEHYG (null) and IED2prM290S (sense) cell lines.*

Antisense (As)		Null (H)		Sense (S)	
Line #	Copy #	Line #	Copy #	Line #	Copy #
3.1	3-4	2.1	1	1.1	19
4.1	60	2.2	1	1.2	6
4.2	130	3.1	2	1.3	8
4.2c	**130**	3.2	2	4.1	5
4.3	88	4.1	1-2	8.1	1500
5.1	25	5.1	1-2	9.1	500
6.1	55	6.1	1-2	9.2	500
6.2	68	6.2	1-2	**9.3**	**500**
6.3	67	9.1	9	10.1	<1
8.1	18	9.2	8	10.2	<1
9.1	11				
9.2	2-3				
9.3	2-3				
10.1	50				
12.1	44				
13.1	<1				
13.2	<1				
14.2	<1				

*Cell lines that showed stable resistance to DEN-2 virus are indicated in bold.

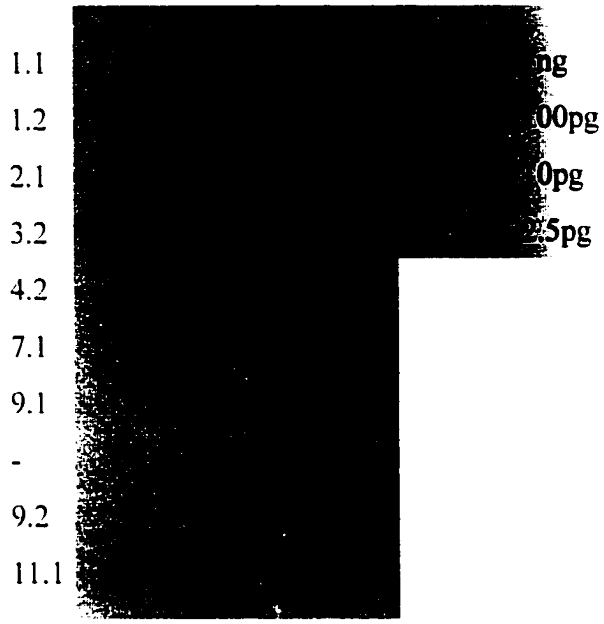


Fig 6.3. Slot blot analysis of foldback cell line DNA. DNA isolated from ieMnp2prM290As (foldback) cell lines was digested with the restriction enzyme EcoR I. pIED2prM290S DNA (2.5 pg-1ng) was included to calculate plasmid copy number per cell. (Film shown was one of several used to calculate the average plasmid copy number per cell).

Table 6.7. Comparison of plasmid DNA copy number with the ability to interfere with the replication of DEN-2 virus in foldback cell lines.

Foldback (FB) Line #	Trial 1	Trial 2	Trial 3	Approximate DNA Plasmid copy number
1.1	p4 32% ± 14%	**p7 4.5% ± 2%**	**p11 1.2% ± 0.6%**	**2-4**
1.2	**p6 2.3% ± 1%**	**p10 1.8% ± 0.7%**	**p11 1.6% ± 1%**	**5-6**
2.1	**pX 0.3% ± 0.4%** ***p14 2.0% ± 2.4%**	**p10 9.8% ± 4.2%**	**p13 0.8% ± 0.2**	**5-6**
3.2	p11 18.9% ± 9.5%	p14 27.4% ± 13.2%		250
4.2	p6 58% ± 5.8%			35
7.1	**p5 2.1% ± 1.6%**	**p11 1.3% ± 0.6%**	**p12 1.3% ± 0.6%**	**44**
8.1	**p10 2.1% ± 1.4%**	**p11 1.8% ± 0.8%**		**x**
9.1	**p5 0.0% ± 0.0%**	**p11 0.9% ± 0.1%**		**1**
9.2	**p6 0.1% ± 0.2%**			**1**
11.1	p4 20.3% ± 5.3%			23
11.2	p9 13.7% ± 4.7%	p10 11.2% ± 5.1%		100
14.1	p5 19.4% ± 10.9%	p13 19.4% ± 9.9%		97
14.2	p6 45.7% ± 14.3%			74
15.1	p8 36.9% ± 6.5%			83
16.1	p7 14.3% ± 7.6%			28
17.1	**p5 1.4% ± 1.1%**	p15 13.7% ± 2.0%	p16 44.9% ± 19.3%	2
17.2	p5 74.3% ± 4.9%			2
18.2	p7 28.4% ± 3.6%	**p10 2.5% ± 1.5%**	**p11 1.8% ± 1.3%**	**65**

* indicates 4th trial

virus for both sense and antisense cell lines was associated with high plasmid copy number (>100 copies per cell). In contrast, 44% of cell lines transformed with a plasmid designed to express dsRNA derived from DEN-2 were resistant to DEN-2 virus challenge. In these "foldback" cell lines, resistance to DEN-2 virus was not correlated with plasmid copy number, as resistant lines were isolated that ranged from 1 copy per cell to 65 plasmid copies per cell (Table 6.8).

Plasmid organization in resistant cell lines. Southern blot analysis was performed to determine the organization of plasmid DNA in several of the DEN-2 resistant cell lines. DNA from cell lines S9.3 and As4.2c was digested with EcoR I, an enzyme that recognizes multiple sites within the pIED2prM290S and pIED2prM290As plasmids (Fig 6.4a). Digestion of pIED2prM290S yielded three major bands of roughly 1, 2 and 3 kb (lane 3). Digestion of DNA from cell line S9.3 yielded the same three bands (lane 5), indicative of repeated plasmid DNA sequences, with few or no integration sites. Digestion of DNA from cell line As4.2c with EcoR I (lane 6) also yielded a pattern identical to digested plasmid DNA (not shown). DNA was also digested with restriction enzymes Xmn I, which has one recognition site in the plasmid DNA, and Kpn I, which does not have any recognition sites within the plasmid DNA (Fig 6.4 b & c). The Xmn I digest showed that the majority of both S9.3 and As4.2c plasmid sequences in transformed cells were unit length, but fainter bands were observed that may represent junction fragments with mosquito cell DNA. Digestion with Kpn I did not reveal any distinctive bands, only high molecular weight DNA that was likely undigested. To determine the methylation status of the pIED2prM290S and pIED2prM290As plasmids in cell lines S9.3 and As4.2c, DNA from these lines was digested with the restriction

Table 6.8. Proportion of cell lines resistant to DEN-2 virus.*

DNA plasmid used to transfect C6/36 cells		Number of cell lines generated that showed stable resistance to DEN-2 virus (% resistant)	Plasmid copy number per cell (CPC) in DEN-2 resistant cell lines
pIEHyg	(null)	0/6 (0)	-
pIED2prM290S	(sense)	1/7 (14)	high, >100 CPC
pIED2prM290As	(antisense)	1/14 (7)	high, >100 CPC
pIEMnpD2prM290As	(foldback)	7/16 (44)	low, 1-65 CPC

*A summary of data tables 6.1-6.7, showing the number of DEN-2 resistant cell lines generated after transfection with each DNA plasmid. Cell lines derived from the same primary colony and found to have to the same DNA plasmid copy number were considered duplicates, and were only counted once.

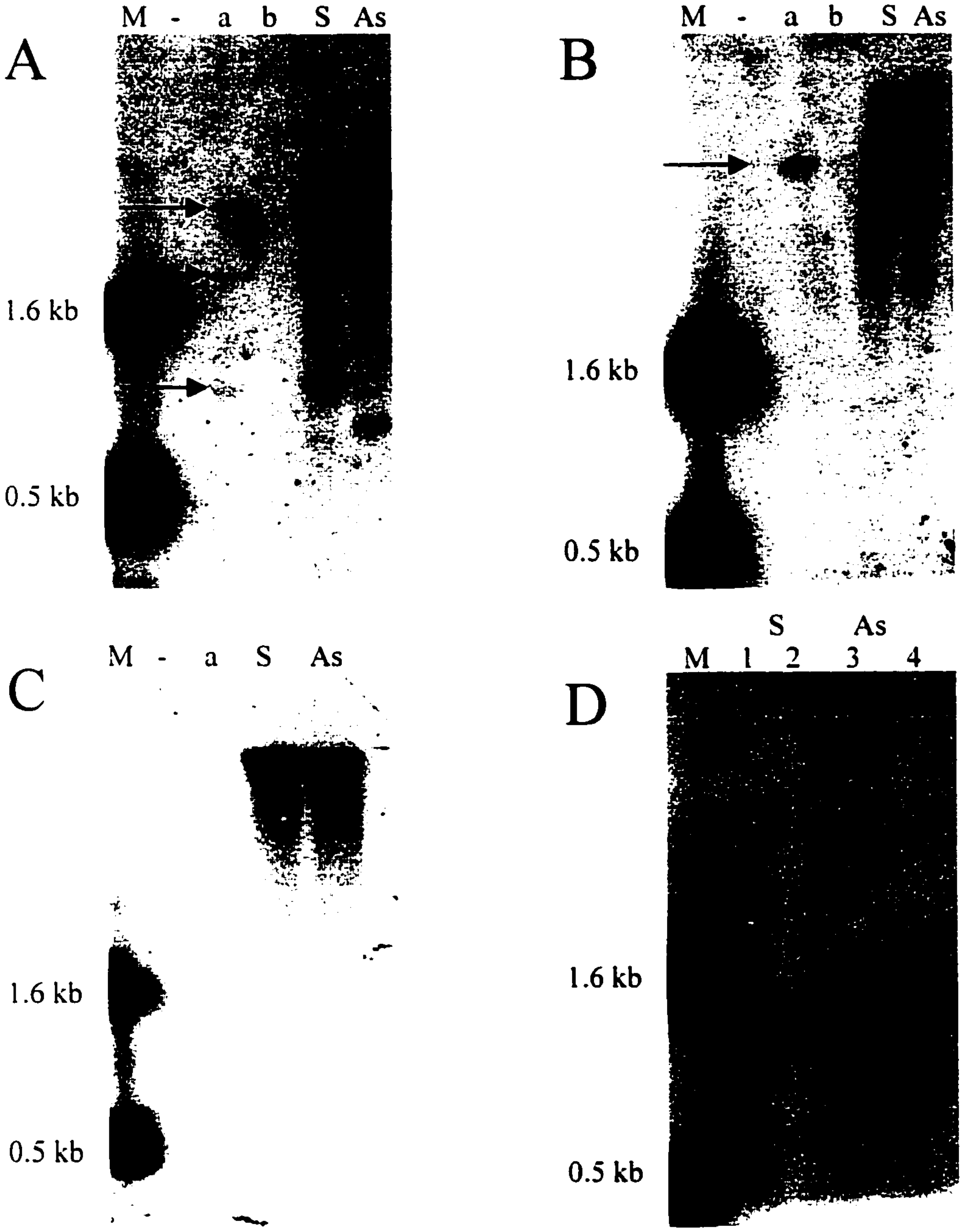


Fig 6.4. Southern blot analyses of DNA isolated from cell lines S9.3 (S) and As4.2c (As). DNA was digested with (**A**) EcoR I; (**B**) Xmn I; (**C**) Kpn I; and (**D**) Sau3A I (lanes 1&3) or Mbo I (lanes 2&4). M = molecular weight marker. (-) = untransformed C6/36 cell DNA. (a. b) = 100 pg and 10 pg of pIED2prM290S plasmid added to C6/36 genomic DNA prior to digestion. Arrows indicate expected bands for pIED2prM290S plasmid DNA.

enzymes Sau3A I or Mbo I. Both enzymes recognize the sequence GATC, but Sau3A I will not cleave GATm4C or GATm5C methylated sequences. Fig 6.4d shows that DNA from cell lines S9.3 and As4.2c gave the same digestion pattern for both Sau3A I and Mbo I, indicating that the plasmid DNA was not methylated in either of these cell lines.

mRNA transcript analysis in resistant cell lines. PTGS in transgenic plants is characterized by low steady-state levels of transgene mRNA. Cell lines resistant to DEN-2 virus were analyzed by northern blot hybridization for the presence of sense or antisense D2prM290As mRNA transcripts (Fig 6.5). No distinct mRNA species was detected in cell lines S9.3, As4.2c, FB2.1, FB7.1, or FB9.1 when probed for sense D2prM290 mRNA (Fig 6.5a). Similarly, no distinct mRNA species was detected when probed for the antisense D2prM290 sequence (Fig 6.5b). Cell lines S9.3, FB2.1 and FB7.1 contained a smear of hybridization signal, indicating transcripts of varying sizes were produced from the D2prM290 template in those lines. Prior treatment of RNA samples with DNase did not affect the hybridization signal, indicating that probe was binding to an RNA target, and not to contaminating plasmid DNA (not shown). No hybridization was seen with C6/36 cell RNA alone, or with C6/36 cell RNA plus *in vitro* transcribed RNA of the same polarity as the probe. Strong hybridization was seen with C6/36 RNA plus *in vitro* transcribed RNA of the opposite polarity as the sense probe, corresponding to 20 or 200 copies of mRNA per cell (Fig 6.5a, lanes 8 &9).

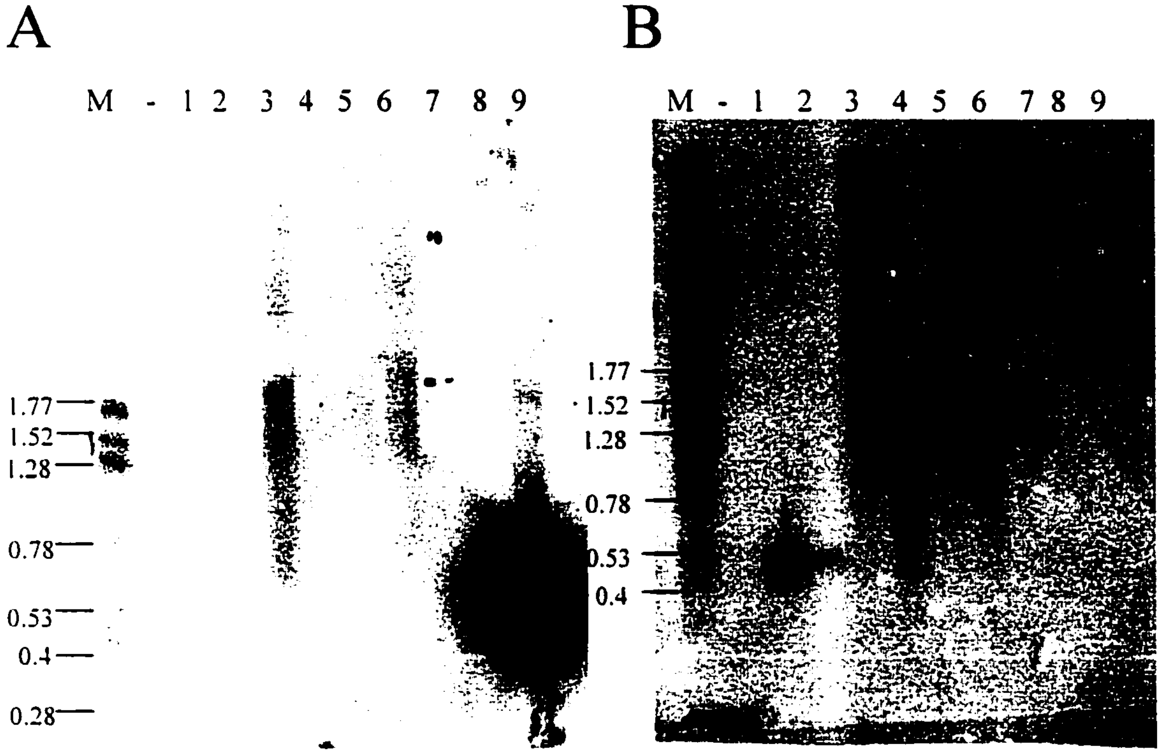


Fig 6.5. Northern blot analysis of mRNA transcripts produced in DEN-2 resistant transformed cell lines. Blots were probed for either sense (A) or antisense (B) D2prM290 sequence. RNA was isolated from (-) C6/36; (3) S9.3; (4) As4.2c; (5) FB2.1; (6) FB7.1; and (7) FB9.1 cells. Lanes 1& 2 contain C6/36 cell RNA with 100 or 10 pg of D2prM290 RNA transcribed *in vitro* with T3 polymerase (antisense strand). Lanes 8 & 9 contain C6/36 cell RNA with 100 or 10 pg of D2prM290 RNA transcribed *in vitro* with T7 polymerase (sense strand). M indicates molecular size marker (kb).

DISCUSSION

Stable resistance to DEN-2 virus has been generated in mosquito cells after transformation with DNA plasmids designed to express sense, antisense or dsRNA derived from the the prM gene of DEN-2. Several cell lines derived from transfection of sense or antisense constructs seemed to be resistant to DEN-2 virus at very early passages (p3-5), but not at later passages. This was most likely an artifact reflecting the metabolic state of the transformed cells. Although no metabolic labeling experiments were performed, it was observed that several passages (around 10) were often required before newly established transformed cells grew and divided consistently. Thus only cell lines that showed resistance to DEN-2 at 10 or more passages were considered truly resistant. This phenomenon was not observed in the foldback cell lines designed to express dsRNA, as these lines were initially assayed for resistance to DEN-2 at later passages (4-10).

Resistance to DEN-2 virus was associated with non-methylated, high DNA plasmid copy number for both the S9.3 and As4.2c cell lines. Although methylation of transgenes subject to PTGS is consistently seen in plants (reviewed in Wasseneger and Pelissier 1998), this is not the case in *Drosophila*, where sequences are rarely methylated (Razin, 1998). In lieu of DNA methylation, *Drosophila* has been shown to use DNA-binding proteins (products of the Polycomb group genes) to alter chromatin structure and effect transcriptional gene silencing (Boivin and Dura, 1998). Mutations in the *Drosophila* Polycomb group genes affect the ability to silence P elements (Roche and Rio, 1998) as well as transgenes (Pal-Bhadra *et al.*, 1997). It is possible that mosquitoes use a similar group of proteins to effect TGS, rather than DNA methylation.

High plasmid copy number has been correlated with gene silencing in *Drosophila* (Pal-Bhadra *et al.*, 1997) and in plants (Assaad *et al.*, 1993; van der Krol *et al.*, 1990). It was hypothesized by those researchers that ectopic pairing of repeated DNA sequences led to the transcription of aberrent RNAs (Assaad *et al.*, 1993). Aberrent transcription of both DNA strands would result in the formation of dsRNA, triggering PTGS. Recently, Wang and Waterhouse (2000) reported in a study of PTGS in rice that all silenced plants generated after transformation with sense or antisense constructs contained inverted repeats of the introduced DNA. Similarly, other researchers have shown that inverted repeats are found in transgenes of silenced plants, while single copies of the same genes remained active (Stam *et al.*, 1997; Stam *et al.*, 2000; Van Houdt *et al.*, 2000). It seems likely that the sense (S9.3) and antisense (As4.2c) cell lines described here, which contained hundreds of copies of the introduced plasmid DNA, contained inversions that led to the production of dsRNA, resulting in PTGS.

Consistent with the model of dsRNA as a trigger of PTGS, cells transformed with a DNA plasmid already containing an inverted repeat (pIEMnpD2prM290As) were resistant to DEN-2 virus at a much higher frequency than sense or antisense alone (44% compared to 14% and 7%). These results mirror those reported by Waterhouse *et al* (1998), who showed that 44-54% of plants were resistant to potato virus Y (PVY) after transformation with an inverted repeat DNA, while less than 15% of plants were resistant to PVY after transformation with sense or antisense DNA constructs. Other researchers have reported similar increases in the frequency of PTGS when inverted repeat DNA constructs were used in place of traditional sense or antisense DNA (Chuang and Meyerowitz, 2000; Fortier and Belote, 2000; Kennerdell and Carthew, 2000; Stam *et al.*, 1997; Stam *et al.*, 2000; Wang and Waterhouse, 2000). Taking this observation one step further, Smith *et al* (2000) have shown that when an intron was included between inverted repeats, subsequent processing of the intron yielded perfectly matched dsRNA, with no hairpin loop. When this DNA construct was introduced into plants the frequency of PTGS was virtually 100%, again echoing the ability of perfectly matched dsRNA to act as a potent trigger of PTGS.

The most significant finding described in this chapter was the observation that resistance to DEN-2 virus could be generated with as little as a single copy of the introduced plasmid DNA present in each cell. Transformation of *Ae. aegypti* with the transposable elements *Hermes* and *mariner* has recently been described (Coates *et al.*, 1998; Jasinskiene *et al.*, 1998; Pinkerton *et al.*, 2000). In all cases to date only a single copy of the introduced element was found to be stably integrated into the mosquito genome. Taken together, the opportunity to generate virus-resistant mosquitoes using transposable elements has never been more feasible. The results presented here, as well as by numerous other researchers over the past few years, indicates that inverted repeat sequences derived from the genomes of one (or more) DEN viruses and delivered into *Ae. aegypti* as part of a *Hermes* or *mariner* transposable element *will* result in mosquitoes permanently refractory to infection by DEN viruses via a PTGS mechanism. Both salivary gland-specific (Coates *et al.*, 1999) and constitutive promoters (Pinkerton *et al.*, 2000) have been described in *Ae. aegypti*. This provides the additional options of establishing PTGS solely in the salivary glands (an organ DEN viruses must replicate in successfully to be transmitted) or throughout the entire organism (or both). It is also possible to link expression of the dsRNA to the induction of mosquito genes such as late trypsin and vitellogenin, which are activated upon acquisition of a bloodmeal. These options for temporal or spatial expression of DEN-derived dsRNA may prove to be superior to constitutive-whole body expression of dsRNA if a negative side effect of artificially induced PTGS is observed in living mosquitoes. Also, activation of PTGS in a specific tissue may result in the spread of PTGS throughout the mosquito, as has been observed in plants (Palauqui *et al.*, 1997). Future work is now warranted to assess the feasibility of driving *Hermes* or *mariner* (or similar elements) bearing virus-resistance genes into naïve mosquito populations. If such a drive mechanism is possible, then PTGS may be the best long-term solution available to combat the ever-worsening global problem of dengue fever and dengue hemorrhagic fever.

Chapter 7

SUMMARY

Dengue fever and dengue hemorrhagic fever/ dengue shock syndrome represent the most important arthropod-borne viral diseases in the world. DF and DHF/DSS affect 50-100 million people every year, with 10-20 times as many at risk. Strategies to control the mosquito vector, *Ae. aegypti*, have not been successful, due to both the lack of funding and organization. Attempts to develop DEN vaccines have also not been successful. In light of these facts, control strategies based on driving virus-resistance genes into mosquito populations are now being explored.

Strategies based on driving virus-resistance genes into naïve mosquito populations depend on three main objectives: 1) the identification of genes capable of generating a strong, refractory phenotype to the target viruses; 2) the ability to generate stable transgenic mosquito lines; and 3) the ability of transgenes to mobilize and spread through naïve mosquito populations. The work presented in this dissertation dealt with objective (1); the identification of genes capable of generating virus-resistant mosquitoes, and the mechanism by which such interference to DEN viruses occurs.

Interference strategies described in this study were based on RNA-mediated resistance. Evidence was presented that supported the hypothesis that resistance to DEN viruses was due to the presence of dsRNA initiating a specific cellular response, and not simply to stoichiometric annealing of an effector to a target RNA. This supports the idea that a mechanism analogous to post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in *C. elegans* and *Drosophila* exists in mosquitoes. Several interesting questions come to light with the understanding that PTGS occurs in arthropod vectors. Primarily, how do arboviruses such as Sindbis and the DEN viruses deal with PTGS in mosquitoes? Do these viruses encode proteins that suppress or delay the PTGS response? Both Sindbis and the DEN viruses replicate in membranous structures in mosquito cells; is this an attempt to escape from PTGS? Resolving questions such as these about the virus-vector interactions of arboviruses will offer further insight into both controlling arboviral disease and advancing the capabilities of arbovirus expression systems.

Transient interference assays were performed in this study in both cultured mosquito cells and whole mosquitoes using a replication-competent Sindbis virus expression system. This allowed the rapid characterization of the interference potentials of numerous RNA sequences without the need for germline transformation. Sequences were identified that could effect interference against each of the four DEN virus serotypes. This indicated that all four DEN viruses can be targets of PTGS. This is encouraging, as it suggests that an RNA-based strategy targeting all four DEN serotypes will be effective in generating mosquitoes refractory to all four viruses. This was further shown by the success of hybrid RNAs containing sequences derived from two DEN virus serotypes at initiating PTGS against both homologous viruses. It seems likely then that hybrid RNAs derived from all four DEN serotypes would be capable of initiating PTGS as well. Unfortunately the packaging limitations of the Sindbis expression system did not allow direct testing of this hypothesis.

Interference assays were also performed in this study using stably transformed mosquito cells. These studies showed that the introduction of DNA plasmids encoding sense, antisense or dsRNA derived from DEN-2 virus were all capable of initiating PTGS, without the need for high levels of RNA expression. Resistant cell lines that were developed shared the characteristic of low to non-detectable levels of transgene mRNA.

This is significant, as it implies that there is almost no metabolic cost associated with the resistance phenotype. This contrasts with protein-mediated interference strategies, as well as antisense RNA strategies based on the direct model of RNA-mediated interference, both of which require continual production of either the transgene protein product or the transgene mRNA. Because the final goal of these studies is to drive virus-resistance genes into mosquito populations, it is important that there be no reproductive or metabolic disadvantage to individuals carrying the transgene. PTGS induced by dsRNA seems to be the best solution to this problem, as it takes advantage of what appears to be an ancient mechanism specifically designed to counteract invading RNAs.

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