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DISSERTATION

EVALUATION OF EXPRESSION SYSTEMS AND ANTIVIRAL GENES TO INHIBIT LACROSSE VIRUS REPLICATION IN MOSQUITO CELLS

Submitted by
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In partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Colorado State University
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TANYA ALLEN MIURA ENTITLED "EVALUATION OF EXPRESSION SYSTEMS AND ANTIVIRAL GENES TO INHIBIT LACROSSE VIRUS REPLICATION IN MOSQUITO CELLS" BE ACCEPTED AS FULFILLING, IN PART, REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

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ABSTRACT OF DISSERTATION

"EVALUATION OF EXPRESSION SYSTEMS AND ANTIVIRAL GENES TO INHIBIT LACROSSE VIRUS REPLICATION IN MOSQUITO CELLS"

Novel control strategies are required to halt the resurgence of arthropod-borne diseases. One such control strategy involves the genetic modification of mosquitoes to reduce vector competence. Mosquito transgenesis is required to test this strategy. While stably transformed mosquitoes have been generated, transgenesis remains a difficult and laborious procedure. Until mosquito transgenesis is a routine procedure, genetic cassettes with potential for altering pathogen transmission in mosquitoes need to be tested in cell culture. In the studies presented here, several strategies for intracellular immunization for LaCrosse (LAC) virus were evaluated. Antisense strategies are effective at interfering with LAC replication when expressed from a Sindbis (SIN) expression system. While SIN virus-based expression systems are invaluable in the evaluation of antiviral strategies, they are not practical for long-term expression in mosquito populations. Evaluation of DNA-based expression of antiviral constructs is necessary before transgenic mosquitoes can be designed with reduced vector competence.

Plasmids using *Drosophila* metallothionein (Mtn) and heat shock protein 70 (hsp70) promoters to express LAC antisense RNA were used to

generate stably transformed cell lines, which were analyzed for plasmid integration, synthesis and cellular localization of antisense transcripts, and interference potential. Although LAC-specific RNAs are effective at interfering with virus replication when expressed from a SIN virus expression system, they are not effective when expressed from DNA constructs using *Drosophila* Mtn or hsp70 promoters. Cell lines transformed with the Mtn and hsp70-based plasmids showed little potential for interfering with LAC virus. Transcript numbers in these cell lines were much lower than is likely needed for antisense interference. Also, expression of the antisense transcript was only seen in ~30% of cells by fluorescence *in situ* hybridization (FISH). LAC replication in the remaining cells could account for the virus production detected in challenged cell lines.

A DNA-based SIN expression system using a baculovirus IE1 promoter to drive expression of a replicon RNA capable of expressing anti-LAC S segment RNA was also evaluated for interference potential. Higher transcript production was evident in the replicon-expressing cell lines as compared to those using the Mtn promoter. These cell lines were capable of interfering with LAC replication as evidenced by lower titers of LAC virus from transformed vs. non-transformed cells. Interference was dependent upon the dose of LAC virus used for challenge. However, all transformed cell lines tested inhibited LAC replication when challenged at a dose similar to that a mosquito would receive from an infectious blood meal in nature. Interference

from these cell lines may be mediated by the dsRNA molecules produced in the SIN replication cycle, rather than by classical antisense effects.

A protein-based strategy for intracellular immunization was also tested for interference with LAC virus. Human MxA protein was expressed in mosquito cell culture and evaluated for interference potential against LAC virus. MxA localization in mosquito cells was similar to its localization in mammalian cell lines. Expression of MxA in mosquito cells interfered with LAC replication, as seen by immunofluorescence (IFA) analysis. However, MxA expression did not interfere with replication of a recombinant SIN virus. MxA-expressing transgenic mosquitoes may be able to inhibit LAC virus replication, resulting in decreased transmission.

An alternate DNA-based expression strategy involves the use of *Aedes* densonucleosis (AeDNV) transducing viruses to infect mosquito larvae. A low packaging efficiency and genetic recombination resulting in the production of wild-type infectious virus are obstacles in the current system for producing densovirus transducing particles. A double subgenomic SIN virus (TE/3'2J/VP) was engineered that expresses the structural proteins (VPs) of AeDNV from the second subgenomic promoter. Expression of AeDNV VPs from TE/3'2J/VP was confirmed by northern analysis of RNA from infected C6/36 (*Aedes albopictus*) cells, and by indirect IFA in infected C6/36 cells and BHK-21 cells. TE/3'2J/VP was used to infect C6/36 cells expressing p7NS1-GFP, a plasmid expressing the nonstructural genes of AeDNV and green fluorescent protein (GFP) as a reporter gene. This infection resulted in

the production of AeDNV-GFP transducing virus, which is infectious to C6/36 cells and *Aedes aegypti* larvae, as determined by GFP expression. The TE/3'2J/VP packaging system produced comparable titers of transducing virus to the standard two-plasmid method. Employing an RNA virus expression system to supply AeDNV structural proteins eliminated the possibility of recombination resulting in wild-type infectious virus in transducing densovirus stocks. We are exploring ways to use the TE/3'2J/VP packaging system to improve the efficiency of producing AeDNV transducing viruses.

The knowledge gained from these studies will aid in the design of antiviral gene expression in mosquitoes. Antiviral gene expression can be accomplished by mosquito transgenesis or by infection with an AeDNV transducing virus. Specific antiviral genes and DNA-based expression systems will be necessary to evaluate genetic control strategies for arbovirus transmission in caged mosquito populations.

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Far away, there in the sunshine,
are my highest aspirations.
I may not reach them,
but I can look up and see their beauty,
believe in them,
and try to follow where they lead.

-Louisa May Alcott

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

Introduction

Mosquito-borne pathogens are a serious threat to the health and economic status of many developing countries. Vector control programs that involved the elimination of mosquito breeding sites and the use of chemical insecticides are now non-existent in many areas of the world. Insecticide resistance in mosquito populations (Brogdon & McAllister, 1998), increased human population in tropical areas, and a lack of vaccines have all contributed to the increased incidence of vector-borne diseases (Gubler, 1998). Reemergence of vector-borne diseases was noted in the 1970s, and has increased significantly during the past two decades. Dengue viruses, for example, have reemerged in tropical regions of the Americas, where *Aedes aegypti* eradication efforts had once eliminated the viruses. Today, greater than 2.5 billion people are at risk for dengue disease (Gubler, 1998). Novel control strategies aimed at eliminating virus transmission from mosquitoes to humans are needed to regain the ground lost in the past twenty years. New control strategies based on the genetic manipulation of mosquito vectors are currently being examined.

Genetic manipulation of invertebrates is critical in order to study the molecular biology of organisms of medical and agricultural importance, as well as for developing control strategies for vectors of human and veterinary pathogens. The genetic control hypothesis indicates that an insect's ability to vector a pathogen (vector competence) may

be altered by genetic modifications (Besansky & Collins, 1992; Carlson *et al.*, 1995; Carlson *et al.*, 1996; Collins & James, 1996; Crampton *et al.*, 1990). This hypothesis needs to be tested by genetically engineering mosquitoes and evaluating their potential to transmit pathogens.

The ability to efficiently engineer transgenic mosquitoes is critical for testing the genetic control hypothesis. The P element transposon, which allowed stable transformation of *Drosophila*, led to intense study of its molecular biology (Carlson *et al.*, 1995). Unfortunately, the P element is not capable of transposition in mosquitoes; the few successful gene integrations with P element constructs resulted from illegitimate recombination events (McGrane *et al.*, 1988; Miller *et al.*, 1987; Morris *et al.*, 1989). Transgenic mosquitoes have recently been engineered using transposable elements *Hermes*, from the housefly *Musca domestica*, and *Mariner*, from *Drosophila mauritiana* (Coates *et al.*, 1998; Coates *et al.*, 1999; Jasinskiene *et al.*, 1998; Pinkerton *et al.*, 2000; Sarkar *et al.*, 1997). This technology should aid in the genetic manipulation of mosquitoes, but, at the present time, mosquito transgenesis remains difficult.

Expressing genes of interest in cell culture is an efficient way to analyze their antiviral potential until mosquito transgenesis becomes an efficient laboratory procedure (Fallon, 1996). Stable transformation of mosquito cells in culture has been well documented. Early systems used expression of the *Ae. albopictus* dihydrofolate reductase (dhfr) gene from the *D. melanogaster* heat shock protein 70 promoter (hsp70) to render previously susceptible cells resistant to methotrexate (Fallon, 1996). The *D. melanogaster* hsp70 promoter was used in another study to express a hygromycin resistance gene in mosquito cells. It was found that the plasmid often existed in the

genome in high copy numbers, as large chromosomal arrays (Monroe *et al.*, 1992). A *D. melanogaster* metallothionein promoter (Mtn) has also been used for heavy metal-induced expression of reporter genes in mosquito cells (Hegadus *et al.*, 1998; Klimowski *et al.*, 1996; Kovach *et al.*, 1992; Wu & Fallon, 1997). Inducible expression of a heterologous sequence is desirable when controlled expression is necessary, in particular when a gene product is toxic to the cell or is required at a specific developmental stage.

Various antiviral gene constructs for interference with LaCrosse virus (LAC; family Bunyaviridae) have been evaluated for eventual expression in transgenic mosquitoes. The relationship between LAC and its vector, *Ae. triseriatus*, has been well characterized, thus interference strategies worked out for LAC may be a good model for other arbovirus:vector interactions. Genetic control strategies developed for LAC virus may be applied to other medically important members of the Bunyaviridae family, such as Rift Valley fever virus. LAC virus should provide a good model system for testing the genetic control hypothesis. Transovarial transmission of LAC virus will insure that most of the mosquitoes in a caged population are infected prior to introduction of transgenic mosquitoes carrying the effector genes. LAC will be transmitted to new members of the population, removing the need for artificial introduction of the virus to the mosquitoes. This will permit the study of the movement and efficacy of antiviral genes in a simulated mosquito natural infection cycle.

Bunyaviridae

The family Bunyaviridae consists of more than 300 viruses in five genera, *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*, most of which are arthropod-borne (Karabatsos, 1985). The viruses classified in the family Bunyaviridae are spherical, enveloped viruses with external glycoproteins. The virion contains three single-stranded genomic RNA molecules, designated small (S), medium (M), and large (L), which are complexed to hundreds of copies of nucleocapsid protein (N) and a polymerase protein. The genomic RNA segments have conserved termini capable of folding into a circular secondary structure (Obijeski *et al.*, 1976). The dsRNA panhandle structure formed by the pairing of segment termini may provide a recognition structure for the N protein to allow encapsidation to occur (Kolakofsky & Hacker, 1991). Virions consist of the S, M, and L ribonucleoprotein complexes, surrounded by a lipid envelope with embedded viral glycoproteins. Members of the family Bunyaviridae replicate in the cytoplasm of host cells and acquire their envelopes by budding through membranes of the Golgi apparatus (Andersson *et al.*, 1997; Bishop *et al.*, 1980; Elliott, 1990).

Closely related viruses within a serogroup are capable of undergoing segment reassortment during dual infection of a single cell (Elliott, 1990). Segment reassortment has been demonstrated in cell culture (Bishop, 1979) and in mosquitoes (Beaty *et al.*, 1981). Reassortant viruses from dual LAC and snowshoe hare (SSH) infections of *Ae. triseriatus* mosquitoes were transmitted transovarially and through blood-feeding

(Chandler *et al.*, 1990). Genetic reassortment between bunyaviruses provides an efficient mechanism of viral evolution.

LAC Medical Importance

LAC virus is a member of the California encephalitis serogroup of the genus *Bunyavirus*: it is endemic in many parts of the Midwest and eastern United States. LAC is the leading cause of pediatric encephalitis in the United States (Calisher, 1994). LAC virus was first isolated from a fatal case of encephalitis in LaCrosse, Wisconsin (Thompson *et al.*, 1965). Infection in children under 15 years of age can result in encephalitis, and symptoms range from stiff neck, lethargy, and seizures, to epilepsy, which occurs in 10% of encephalitis cases, and persistent paresis, which occurs in 2% of cases (Gonzalez-Scarano & Nathanson, 1996). Most adult infections are subclinical or may manifest as a flu-like illness.

LAC Life-Cycle

Vertebrate hosts of LAC virus include squirrels (*Sciurus carolinensis*) and chipmunks (*Tamius striatus*) (Pantuwatana *et al.*, 1972). No transmission occurs from a human infection because there is insufficient viremia to infect a mosquito during blood-feeding (Johnson, 1982). The woodland mosquito, *Ae. triseriatus*, is the primary vector of LAC and infection of the mosquito host is persistent and asymptomatic (Beaty & Bishop, 1988). *Ae. triseriatus* transmits LAC orally (Pantuwatana *et al.*, 1972).

transovarially (Watts *et al.*, 1973), and venereally (Thompson & Beaty, 1978). Transovarial transmission allows the virus to overwinter in diapaused mosquito eggs (Beaty & Thompson, 1975; Watts *et al.*, 1974). Transmission occurs after a mosquito ingests an infectious blood meal, the virus infects and replicates in midgut epithelial cells, and the virus is transported to salivary glands and ovaries through the hemolymph. The virus must pass through inherent barriers for a disseminated infection to occur.

The introduction of *Ae. albopictus* into LAC-endemic regions has potential for increasing the threat of LAC infections. *Ae. albopictus* has been shown to be an efficient vector for LAC in the laboratory (Cully *et al.*, 1991; Cully *et al.*, 1992; Francy *et al.*, 1990), and could potentially be a good vector in nature because of its close association to humans and aggressive biting behaviors (Francy *et al.*, 1990). *Ae. albopictus* have been collected in LAC-endemic areas, near chipmunks with antibodies to LAC virus (Cully *et al.*, 1991; Kitron *et al.*, 1998).

LAC Molecular Biology

The coding assignments for the three RNA segments of LAC have been determined (Beaty & Bishop, 1988). The L segment of LAC virus is 6980 nucleotides in length (Roberts *et al.*, 1995). It encodes the RNA-dependent RNA polymerase, which is associated with virion ribonucleocapsids and is required for all viral RNA synthesis. The M segment of LAC virus is 4526 nucleotides in length (Grady *et al.*, 1987). It encodes a polyprotein which is co-translationaly cleaved to form the two envelope glycoproteins, G1 and G2, and a non-structural protein designated NS_M (Matsuoka *et al.*, 1991). G1 is

thought to be responsible for attachment to vertebrate cells, while G2 is responsible for attachment to mosquito midgut cells (Ludwig *et al.*, 1989; Ludwig *et al.*, 1991). The protease-rich environment of the mosquito midgut is proposed to cleave G1, leaving G2 available for attachment to host cells. G1 has been shown to be critical for infection of non-midgut cells of mosquitoes and also for infection of vertebrate cells and cells in culture (Hacker & Hardy, 1997). G1 is the major neutralizing antigen of LAC virus (Pekosz *et al.*, 1995). The role of NS_M has not been clearly demonstrated, but it has been proposed to aid in viral protein transport (Elliott, 1990). The S segment of LAC virus is 981 nucleotides in length (Cabradilla *et al.*, 1983). It encodes the nucleocapsid protein (N) and a non-structural protein, designated NS_S, in overlapping reading frames which are translated from alternative initiation sites (Cabradilla *et al.*, 1983; Elliott, 1990). The initiation codon for the N protein is used preferentially by host ribosomes, thus there is more abundant translation of the N protein versus the NS_s protein (Elliott, 1990). Hundreds of copies of N protein (approximately 1 N protein per every 6 nucleotides) are associated with individual genome segments, forming viral ribonucleoprotein complexes (Obijeski *et al.*, 1976). The function of the NS_S protein is unknown.

The three RNA segments are produced in different abundance, likely a control mechanism based on the changing requirements for each gene product through the virus life-cycle (Rossier *et al.*, 1988). Transcription from the genomic RNA template involves a cap-scavanging mechanism. The viral polymerase, which is attached to the 3' end of the genomic RNA segment, contains an endonuclease function. It binds host mRNAs with methylated caps, and cleaves 10-18 nucleotides from the host mRNA molecule. The scavenged host RNA serves as a primer for viral transcription. The resulting viral mRNA

retains the cap and a portion of the host mRNA; therefore the ends of the RNA are no longer complementary and circularization, which would potentially impede translation, will not occur. The viral mRNAs are not poly-adenylated; however there is potential for a stem-loop structure near the 3'end of the mRNA, which may serve to enhance stability (Kolakofsky & Hacker, 1991; Patterson *et al.*, 1984). LAC virus requires a pool of host mRNA molecules for cap-scavanging and priming transcription (Rossier *et al.*, 1986). Transcription of viral mRNA is terminated 60-100 bases upstream of the end of the genomic RNA template, thus mRNA molecules are shorter than their genomic RNA counterparts (Kolakofsky & Hacker, 1991).

N, NS_S, and polymerase proteins are translated by free ribosomes in the cytoplasm of infected cells, and are not post-translationally modified (Schmaljohn, 1996). G1, G2, and NS_M proteins are translated by membrane-bound ribosomes from the M segment mRNA (Matsuoka *et al.*, 1991). G1 and G2 are processed through the ER and Golgi apparatus. Glycosylation of G1 and G2 must occur for LAC virus to be infectious (Schmaljohn, 1996).

LAC virus replication occurs after sufficient transcription and translation of viral proteins has taken place. The viral polymerase must switch from mRNA transcription to synthesis of virion complementary RNA (vcRNA), which serves as the template for genomic RNA synthesis. The mechanism for this switch has not been determined. Unlike the viral mRNA molecules, vcRNA is synthesized without the cap-scavenging mechanism, and is a full-length copy of genomic RNA (Schmaljohn, 1996). Upon synthesis of genomic RNA from vcRNA template, nucleocapsid proteins associate with the genomic RNAs, forming helical viral ribonucleocapsids (Schmaljohn, 1996). The

glycoproteins, G1 and G2, accumulate in Golgi membranes, where assembly takes place. The ribonucleoprotein complexes interact with the membrane-associated glycoproteins and virions bud through Golgi membranes, acquiring their envelope and glycoproteins. Mature virus is transported from the Golgi to the cell surface and released from vesicles of the exocytic pathway (Matsuoka *et al.*, 1991).

Intracellular Immunization

The antiviral strategies being designed for the control of arbovirus transmission by mosquitoes involve intracellular immunization. Intracellular immunization is defined as the genetic engineering of cells, to render them resistant to infection by an intracellular pathogen (Baltimore, 1988). Viruses are excellent targets of intracellular immunization strategies because they are obligate intracellular pathogens. Viral proteins, including coat and replicase proteins, and RNAs, including sense and antisense, have been used in intracellular immunization strategies (Inokuchi & Hirashima, 1987; Prins & Goldbach, 1996; Sanford & Johnston, 1985).

Coat protein-mediated resistance to tobacco mosaic virus (TMV) in transgenic plants is a classic example of intracellular immunization against a viral pathogen (Powell-Abel *et al.*, 1986; Register & Beachy, 1988; Reimann-Philipp & Beachy, 1993). Transgenic plants that express the TMV coat protein gene are no longer susceptible to infection with TMV. The mechanism of resistance may occur at an early step in infection, such as virus uncoating. Evidence for this mechanism is that resistance can be overcome by inoculating plants with naked RNA. There is, however, also evidence for

interference at a later step in infection. When a high concentration of TMV RNA is inoculated into transgenic plants, virus accumulates in leaves at the site of inoculation. Virus replication occurs; however transport of the virus, leading to a systemic infection, is delayed (Reimann-Philipp & Beachy, 1993).

Intracellular immunization against tomato spotted wilt virus (TSWV; genus *Tospovirus*, family Bunyaviridae) in tobacco plants has been established by expression of S segment-specific RNA sequences in transgenic plants. Antisense, sense, and untranslatable sense RNAs have been shown to be equally effective in this system and exhibit a gene-dosage effect (de Haan *et al.*, 1992; Prins & Goldbach, 1996; Prins *et al.*, 1996). Strong sequence homology is required for interference with related tospoviruses, and for cross-protection with various strains of TSWV. Effector RNAs may be interfering directly with their complementary viral RNAs or indirectly by competing for viral or host replication factors (de Haan *et al.*, 1992).

Gene Silencing

Nucleic acid-based intracellular immunization strategies can be designed based on knowledge of the triggers and mechanisms of gene silencing in nature. Gene silencing was discovered fortuitously in transgenic plants that had lost expression of their transgene. Most cases involve post-transcriptional gene silencing, in which the gene is transcribed, but increased degradation of the transcripts results in silenced expression. Host genes with homology to the transgene are also silenced, a phenomenon termed co-supression (Grant, 1999). Virus induced gene silencing (VIGS) occurs when a plant is

infected with a virus that contains sequence homologous to a host gene, resulting in silencing of the targeted host gene and eventual inhibition of the virus (Baulcombe, 1999).

Gene silencing is likely a natural mechanism plants use to inhibit genomic insertion of transposable elements and infection by viruses. Inverted repeats present in transposable elements and dsRNA produced during replication of an RNA virus have both been shown to trigger gene silencing. A plant may mistake a transgene for a transposable element or virus and respond by silencing the transgene (Ratcliff *et al.*, 1997). Gene silencing is also induced by repetitive DNA, especially inverted repeats. Methylation of repeated sequences may trigger gene silencing. Transcription through inverted repeats could result in an RNA with double-stranded regions, which could also induce sequence-specific gene silencing (Selker, 1999). Gene silencing in *Drosophila* involves position effect variegation, in which a transgene inserts near a region of condensed heterochromatin, resulting in little or no expression of the transgene. The compact structure of heterochromatin hinders access of transcriptional activators to promoter and enhancer sequences that are required for transgene expression (Boivin & Dura, 1998).

Transcriptional gene silencing (TGS) is heritable gene silencing, often involving hypermethylation of the promoter sequence within the chromosome (Grant, 1999). TGS has been seen when a transgene integrates in or near a hypermethylated region of a chromosome, similar to position effect variegation in transgenic *Drosophila*. However, other examples of TGS occur in which the target gene or promoter is methylated without any methylation in the neighboring sequence (Vaucheret *et al.*, 1998).

Post-transcriptional gene silencing (PTGS) involves homology-specific degradation of mRNA. The diversity of organisms in which PTGS has been seen indicates it may be a common mechanism for the regulation of gene expression. DNA methylation may also have a role in PTGS, as coding sequences of silenced genes are sometimes methylated. There are three models proposed for PTGS (Voinnet *et al.*, 1998). The threshold model involves a response to overexpression, in which genes with too high an expression level are silenced (Lindbo *et al.*, 1993). The aberrant RNA model predicts that gene silencing is activated by the presence of aberrant RNAs which are an indication of virus infection or ectopically altered chromosomal DNA (Grant, 1999). The third model proposes that ectopic interactions with the DNA encoding a specific gene result in its silencing (English *et al.*, 1996; Wassenegger & Pelissier, 1998). A 25 nucleotide antisense RNA that is complementary to the mRNA of a silenced gene has been isolated in many cases of PTGS (Hamilton & Baulcombe, 1999). Small antisense RNA molecules may be the key to signaling gene silencing, because they are large enough to be sequence specific, but could still be transported throughout the plant. This is significant because homologous gene silencing occurs throughout a plant after initial silencing at a localized site (Baulcombe, 1999).

dsRNA has been proposed to be a mediator of gene silencing in a variety of organisms including plants (Waterhouse *et al.*, 1998), nematodes (Fire *et al.*, 1998; Montgomery *et al.*, 1998; Timmons & Fire, 1998), trypanosomes (Ngo *et al.*, 1998), and arthropods (Kennerdell & Carthew, 1998). dsRNA has been shown to be more effective at interfering with gene expression than either sense or antisense RNAs expressed alone (Montgomery & Fire, 1998; Sharp, 1999). Interference by dsRNA is sequence specific, it

is able to spread from the initial site of introduction, and the target molecule is RNA (Montgomery & Fire, 1998). The mechanism of interference has not been characterized, but is suspected to act post-transcriptionally (Fire *et al.*, 1998). Organisms that respond to dsRNA interference may have a viral surveillance mechanism that detects dsRNA (a common signal of viral infection) and results in specific down-regulation of the target sequence by interference with mRNA processing, transport, or by increased degradation of the target mRNA (Montgomery & Fire, 1998).

Antisense RNA

Another model for nucleic acid-based interference strategies involves expression of antisense RNA complementary to a target mRNA, which can cause interference with expression of the target gene. Naturally-occurring antisense systems have been proposed to function in the regulation of gene expression (Dolnick, 1997) and include the regulation of *E. coli* IS10 transposase, the OmpF gene, and replication of the Col E1 plasmid (Kim & Wold, 1985). Izant and Weintraub (1984) first demonstrated the potential of using artificial antisense strategies to block gene expression as a tool to study gene function. They microinjected cells with antisense RNA to the thymidine kinase gene, thereby knocking out its function. Antisense oligonucleotides are now being used in many organisms to study gene function (Persidis, 1999). Artificial antisense strategies have been designed to interfere with viral gene expression, including LAC virus (Powers *et al.*, 1996; Powers *et al.*, 1994) flaviviruses (Higgs *et al.*, 1998; Olson *et al.*, 1996; Raviprakash *et al.*, 1995), measles virus (Bell *et al.*, 1997), hepatitis B virus (Putlitz *et*

al., 1998), vesicular stomatitis virus (Takacs & Banerjee, 1997), and retroviruses (Veres *et al.*, 1996; Von Ruden & Gilboa, 1989). The first antisense drug was approved by the FDA in 1998 for the treatment of human cytomegalovirus, while several others are being evaluated in clinical trials (Persidis, 1999).

Antisense interference strategies are based on the theory that a high enough concentration of antisense effector molecules in the appropriate cellular location will lead to hybridization to its target mRNA, resulting in a block in synthesis of the targeted gene product (Branch, 1996; Kim & Wold, 1985). It appears that an excess of antisense effector over target mRNA is required for interference to occur (Kim & Wold, 1985; Leiter *et al.*, 1989; Putlitz, *et al.*, 1998; Wang & Dolnick, 1993). The binding of antisense RNA to its target mRNA has been demonstrated (Bunch & Goldstein, 1989; Kim & Wold, 1985; Krystal *et al.*, 1990; Melton, 1985; Wang & Dolnick, 1993), and may prevent expression of the targeted gene product by various mechanisms. Some antisense effects include: arrest of translation, selected degradation of target RNA, premature transcription termination upon binding nascent mRNA, and irreversible modifications of the target mRNA (Branch, 1996; Helene & Toulme, 1990; Nellen & Lichtenstein, 1993; Putlitz *et al.*, 1998). A type I interferon response may be elicited by the dsRNA resulting from antisense binding to target mRNA in mammalian cells. The interferon response may be responsible for the inhibition of viral replication seen in these systems. Inherent factors that undermine antisense interference strategies include the secondary structure of target RNA, association of target RNA with proteins, and the intracellular localization of the target RNA (Branch, 1996). Viruses that replicate quickly

may also overwhelm a stable concentration of antisense molecules and escape interference (Leiter *et al.*, 1989).

Sindbis Virus Expression Systems

Recombinant Sindbis (SIN) viruses are effective at expressing antisense RNAs for viral interference (Gaines *et al.*, 1996; Higgs *et al.*, 1998; Olson *et al.*, 1996; Powers *et al.*, 1996; Powers *et al.*, 1994). SIN virus (family Togaviridae, genus *alphavirus*) is an enveloped, icosahedral virus with a single-stranded, positive-sense RNA genome. The genome of SIN is organized with the nonstructural genes at the 5' end, and the structural genes are expressed from a subgenomic promoter in the 3' portion of the genome. SIN has been developed as a gene expression tool because it has a broad host range, it expresses heterologous genes very efficiently, and it is relatively easy to engineer (Schlesinger, 1993; Xiong *et al.*, 1989). The SIN replicon expression system consists of a self-replicating RNA, which lacks the SIN structural genes. Heterologous sequences are cloned downstream of the subgenomic promoter and are expressed from the replicon RNA. Helper plasmids are used to supply structural proteins when packaging of recombinant virus is desired (Bredenbeek *et al.*, 1993). The double subgenomic SIN (dsSIN) expression plasmid, pTE/3'2J, was constructed with a second subgenomic promoter engineered downstream of the structural genes (Hahn *et al.*, 1992). Genes of interest are cloned downstream of the second subgenomic promoter in pTE/3'2J in order to engineer recombinant viruses that are capable of producing packaged, infectious virus.

Antisense Interference with LAC Virus

Powers *et al.* (Powers *et al.*, 1996; Powers *et al.*, 1994) have proven the effectiveness of RNA-mediated interference strategies against LAC virus using the dsSIN expression system. They expressed antisense, sense, and untranslatable sense RNAs of the M and S segments of LAC in dsSIN viruses. S segment RNAs were efficient at interfering with LAC virus replication as determined by reduction in LAC titers in cells co-infected with the recombinant dsSIN virus and wild-type LAC virus (Powers *et al.*, 1994). Mosquitoes infected with interfering dsSIN viruses were also resistant to LAC replication as determined by lack of viral dissemination to various mosquito tissues, and reduced viral titers in heads of infected mosquitoes (Powers *et al.*, 1996). These studies have shown that interference is mediated by RNA, not protein, based on interference when using antisense and untranslatable sense sequences.

Interference with heterologous viruses was also examined. Snowshoe hare (SSH) and Tahyna (TAH) viruses were inhibited by the dsSIN virus expressing anti-LAC S segment RNA. These viruses are most closely related to LAC, while other, more distantly related viruses, were not inhibited (Powers *et al.*, 1996). Co-infection of a mosquito with more than one bunyavirus in nature is possible because of overlapping geography and mosquito hosts, transovarial transmission, interrupted feeding leading to multiple subsequent blood meals, and life-long infection of the mosquito host (Beaty *et al.*, 1983). Superinfection exclusion prevents infection of a mosquito by a related virus by day three post-infection with the first virus. Exclusion only occurs when a mosquito is

infected with a closely related virus, such as members of the same serogroup (Beaty & Bishop, 1988; Beaty, *et al.*, 1983). Interference seen among closely related viruses by antisense RNA expression may operate in a similar homology-dependent manner as superinfection exclusion.

Interference to LAC virus using the dsSIN expression system was only seen when expressing RNAs from the S segment, in agreement with TSWV interference in tobacco plants. However, by blocking production of the N protein, expression of other viral proteins, such as G1, was inhibited. The S segment of LAC is the RNA most commonly seen in defective interfering particles, and its protein product, N, is the predominant viral protein in mosquitoes resistant to superinfection (Powers *et al.*, 1994). N protein accumulation is also an important signal for the virus to switch from mRNA transcription to genome replication (Kolakofsky *et al.*, 1987; Kolakofsky & Hacker, 1991).

Mx Proteins

Expression of the human MxA gene in mosquitoes represents a potential interference strategy against LAC virus using protein instead of nucleic acid. The Mx gene is one of over 50 interferon-induced genes involved in the antiviral immune response. The first Mx protein to be discovered was mouse Mx1, which was found to be responsible for influenza virus resistance in certain strains of mice (Lindenmann, 1962). Influenza susceptible mice, including most lab strains, have a structurally altered Mx1 gene (Arnheiter *et al.*, 1995). The human MxA protein was later found by cross-reactivity with a mouse antibody to Mx1 (Haller *et al.*, 1998). Mx proteins are effective

at interfering with virus production intracellularly; however, Mx1 is localized to the nucleus and MxA is localized to the cytoplasm. Thus mouse Mx1 and human MxA likely have different antiviral mechanisms (Arnheiter *et al.*, 1995). Mx proteins have been found in a wide range of vertebrates, including mammals, birds, and fish (Arnheiter *et al.*, 1995). Mx proteins interfere with a wide range of viruses having few shared characteristics: human MxA has been shown to inhibit certain viruses from the families Orthomyxoviridae, Rhabdoviridae, Bunyaviridae, Paramyxoviridae, and Togaviridae (Arnheiter *et al.*, 1995; Haller *et al.*, 1998). Human MxA inhibits replication of LAC virus in cell culture and in transgenic mice (Frese *et al.*, 1996; Hefti *et al.*, 1999).

AeDNV Background

Recombinant parvoviruses are a proven DNA-based expression system that may be a useful alternative to transgenic mosquitoes. *Aedes* densonucleosis virus (AeDNV; family Parvoviridae) is a mosquito-specific parvovirus that infects species of the genera *Aedes*, *Culex*, and *Culiseta* (Buchatsky, 1989). AeDNV infects a wide range of tissues of larvae and adult mosquitoes. The life-cycle of the virus is not well studied, but likely involves horizontal transmission of the virus by contaminated water and ingestion of infected dead larvae in larval rearing sites. Vertical transmission may play a role in spread of the virus to other rearing sites (Barreau *et al.*, 1997).

The AeDNV genome is approximately 4kb of single-stranded DNA, with terminal palindromes capable of folding into a T-shaped secondary structure critical for genome replication and packaging (Afanasiev *et al.*, 1994). The genome can be divided

into two coding regions. The left portion of the genome contains two open reading frames (ORFs) encoding nonstructural proteins NS1 and NS2, which are expressed from the p7 promoter in overlapping reading frames. NS1 is essential in replication and packaging, and also plays a role in the regulation of gene expression by transactivating viral promoters. The function of NS2 is unknown. The right portion of the genome contains one ORF encoding two structural virion proteins, VP1 and VP2, which are expressed from the p61 promoter (Afanasiev *et al.*, 1991).

Several members of the Parvoviridae family have been used as transducing viruses for the expression of heterologous genes (Bout, 1996; Corsini *et al.*, 1996; Hermonat & Muzyczka, 1984; Maxwell *et al.*, 1993; Muzyczka, 1992). The genomes of AeDNV and *Junonia coenia* DNV have been engineered to express heterologous genes in insect cells (Afanasiev *et al.*, 1994; Giraud *et al.*, 1992). AeDNV transducing virus may represent a future strategy for expression of heterologous genes in mosquito populations in nature. AeDNV may also be useful as a mosquito biocontrol agent.

Summary

Until mosquito transgenesis is a routine procedure, genetic cassettes with potential for altering pathogen transmission in mosquitoes need to be tested in cell culture. In the studies presented here, several strategies for intracellular immunization against LAC virus were evaluated. Antisense RNA from the S segment of LAC was analyzed for its ability to interfere with virus replication when expressed from DNA-based expression systems. Plasmids using *Drosophila* Mtn and hsp70 promoters were

analyzed for interference potential, antisense expression level, and cellular location of antisense transcripts. A SIN replicon expressing anti-LAC S segment RNA was expressed from a baculovirus IE1 promoter to evaluate interference potential. Human MxA protein was expressed in mosquito cell culture and was evaluated for interference potential against LAC virus. For evaluation of alternate DNA-based expression systems, a recombinant dsSIN virus expressing the structural genes of AeDNV was used to package recombinant transducing particles. The evaluation of a variety of arbovirus interference strategies including RNA- and protein-based effectors expressed from DNA-based systems will provide useful information for the design of arbovirus resistant mosquitoes.

CHAPTER 2: ANTISENSE INTERFERENCE WITH LACROSSE VIRUS USING CONSTRUCTS WITH *DROSOPHILA* PROMOTERS

Introduction

These studies were designed to evaluate the potential of antisense RNA for LAC virus interference when expressed from plasmids using *Drosophila* promoter elements. Expression of LAC virus-specific antisense RNA from the dsSIN expression system has been shown to be effective at interfering with LAC virus replication in mosquito cells and in live mosquitoes (See Chapter 1) (Powers *et al.*, 1996; Powers *et al.*, 1994). DNA-based constructs expressing these antisense sequences are needed for the evaluation of genetic constructs with potential for viral interference in a transgenic mosquito.

A DNA-based construct requires an effective promoter with or without enhancer sequences, and a termination sequence. Ongoing research is aimed at identifying mosquito promoters that may be effective for tissue-, developmental stage-, and sex-specific heterologous gene expression. For example, the *Ae. aegypti* apyrase promoter is only active in the medial and distal lateral lobes of the adult female mosquito (Coates *et al.*, 1999). Another approach is to use heterologous promoters from other dipterans or from viruses that infect mosquitoes. For example, promoters from *Drosophila* and baculoviruses have been used for gene expression in insect cells (Carbonell *et al.*, 1985; Hegadus *et al.*, 1998; Kovach *et al.*, 1992; Monroe *et al.*, 1992; Zhao & Eggleston, 1999).

A promoter that drives metallothionein gene expression in *D. melanogaster* was evaluated for expression of antiviral constructs in mosquito cells. Metallothioneins are ubiquitous proteins, whose expression is up-regulated by exposure to heavy metals. Two metallothionein genes, *mtn* and *mto*, have been discovered in *D. melanogaster*. The genetic sequence for the coding and regulatory elements of the *D. melanogaster* metallothionein genes has been determined (Lastowski-Perry *et al.*, 1985; Moroni *et al.*, 1986; Otto *et al.*, 1987). The metallothionein promoter (Mtn) consists of four conserved regulatory sequence repeats (metal response elements). Transcription from Mtn has been shown to increase in response to heavy metal exposure in *Drosophila* and mosquito cells (Kovach *et al.*, 1992; Otto *et al.*, 1987). Controlled regulation of transcription from Mtn has been useful in expressing genes in insect cells, particularly when the gene product is harmful or inhibitory to the cells in which it is expressed (Johansen *et al.*, 1989).

A second *Drosophila* promoter, which drives expression of the 70 kilodalton heat shock protein (hsp70) in response to increased temperatures, was also evaluated. Heat shock proteins are produced as a cellular response to heat and other stresses, allowing an organism to survive under adverse conditions. The hsp70 promoter is activated upon temperature increase and other situations that result in cellular stress. The mammalian hsp70 protein is proposed to function in the renaturing of proteins denatured because of increased temperature. Hsp70 renatures the disrupted proteins, allowing normal cell function to be sustained under increased temperatures, which leads to thermotolerance (Solomon *et al.*, 1991). Hsp70 has also been linked to regulation of transcription and translation in response to heat shock in *Drosophila* (Solomon *et al.*, 1991).

Previous studies used the Mtn (Powers, 1995) or hsp70 (Monroe, 1990) promoters to express LAC S segment-specific RNA for interference in mosquito cells. In the study involving the Mtn-based constructs, cells expressing the S segment in sense orientation were not resistant to LAC replication. Cells expressing the S segment in antisense orientation were not evaluated for interference because of low antisense transcript abundance (Powers, 1995). In the study involving the hsp70-based constructs, one cell line expressing the S segment in antisense orientation showed interference with LAC replication. Interference in this study was described as suboptimal, because a titer of 6-7 $\log_{10}TCID_{50}$/mL of LAC virus was still produced in the interfering cell line, while control cells produced 9-10 $\log_{10}TCID_{50}$/mL of virus (Monroe, 1990). The studies described here were done to repeat the previous work, including testing more cell lines for interference potential. One possible explanation for the lack of interference in the previous studies using these constructs is that the effector RNA molecules are not being transported to the cytoplasm where they would be required for LAC virus interference to occur. The cellular localization of LAC-specific RNA was evaluated to address this concern.

Genetic constructs using Mtn and hsp70 promoters from *D. melanogaster* were used to evaluate the interference potential of an antisense sequence from a DNA-based expression system. Constructs were used to generate stably transformed cell lines, which were analyzed for plasmid integration, concentration and cellular localization of antisense transcripts, and interference potential.

Materials and Methods

Plasmids. Plasmids pMtN and pMtAntiN contain the full-length S segment of LAC in sense and antisense orientation, respectively, under the control of the *Drosophila* Mtn promoter (Figure 2.1). The construction of pMtN and pMtAntiN is described in detail elsewhere (Powers, 1995). Plasmids phsN and phsAntiN contain the full-length S segment of LAC in sense and antisense orientation, respectively, under the control of the *Drosophila* hsp70 promoter (Figure 2.2). The construction of phsN and phsAntiN is described in detail elsewhere (Monroe, 1990). All of the plasmids also contain a hygromycin resistance gene cassette to allow for selection of stable transformants.

Cell culture and transformation. *Ae. albopictus* C6/36 cells (ATCC CRL-1660) were maintained at 28°C in Leibowitz's medium (L-15; Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS), 100U/ml penicillin and 0.1mg/ml streptomycin.

C6/36 cell transformation was accomplished using lipofectin reagent (Life Technologies, Gaithersburg, MD). Cells were seeded into 25cm^2 flasks and grown to 70-80% confluence. Fifty-two uL lipofectin were diluted in 100uL of phosphate-buffered saline (PBS) and incubated at room temperature for 15min. Cells were rinsed once with PBS and once with L-15 without FBS or antibiotics for 15min. Twenty-six ug of DNA were added to the diluted lipofectin and incubated at room temperature for 15min. The liposome/DNA complex was then added to the rinsed cells in a total volume of 2.5mL serum-free L-15.

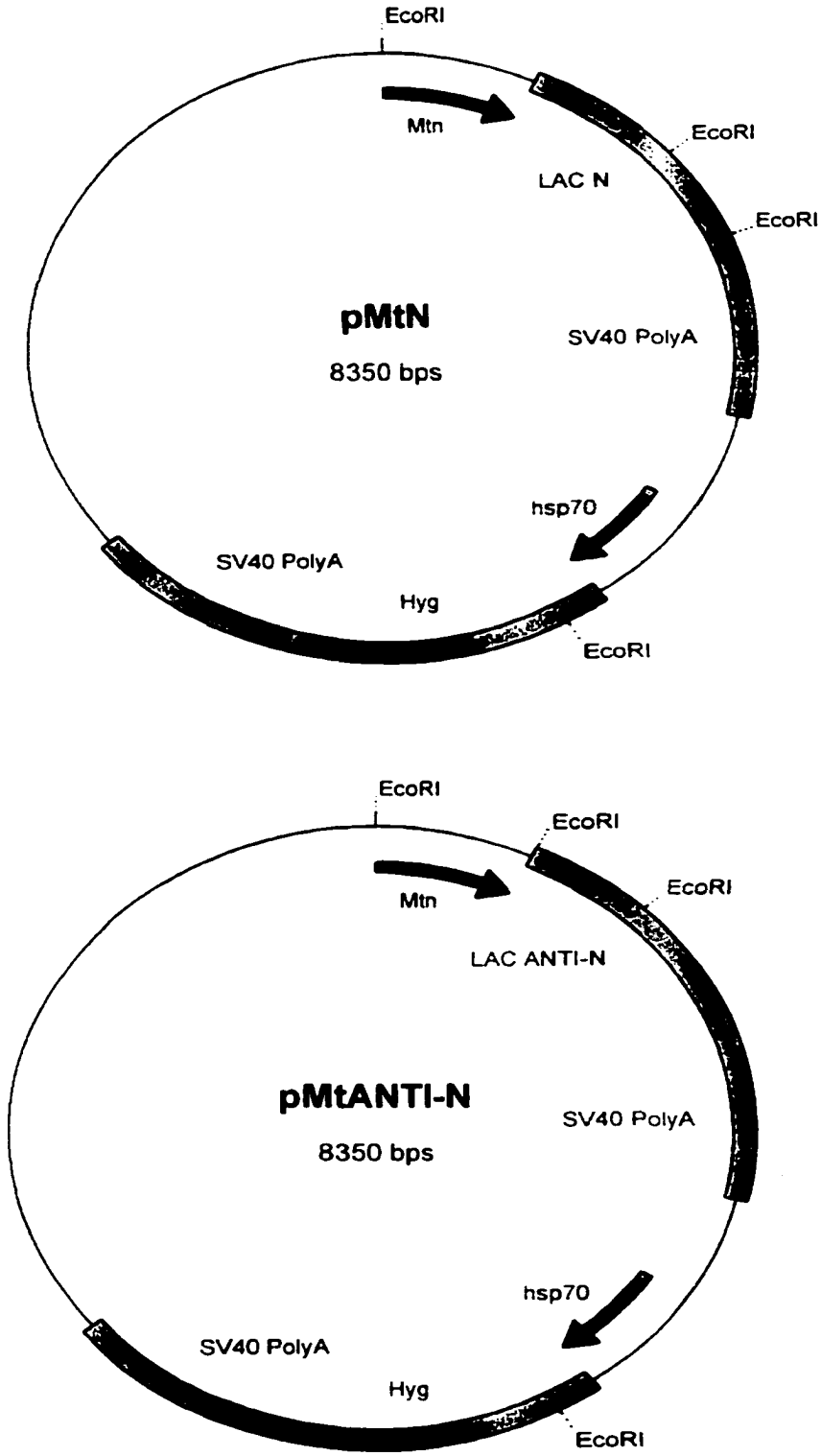


Figure 2.1. Plasmids pMtN and pMtAntiN contain the full-length S segment of LAC in sense and antisense orientation, respectively, under the control of the *D. melanogaster* Mtn promoter.

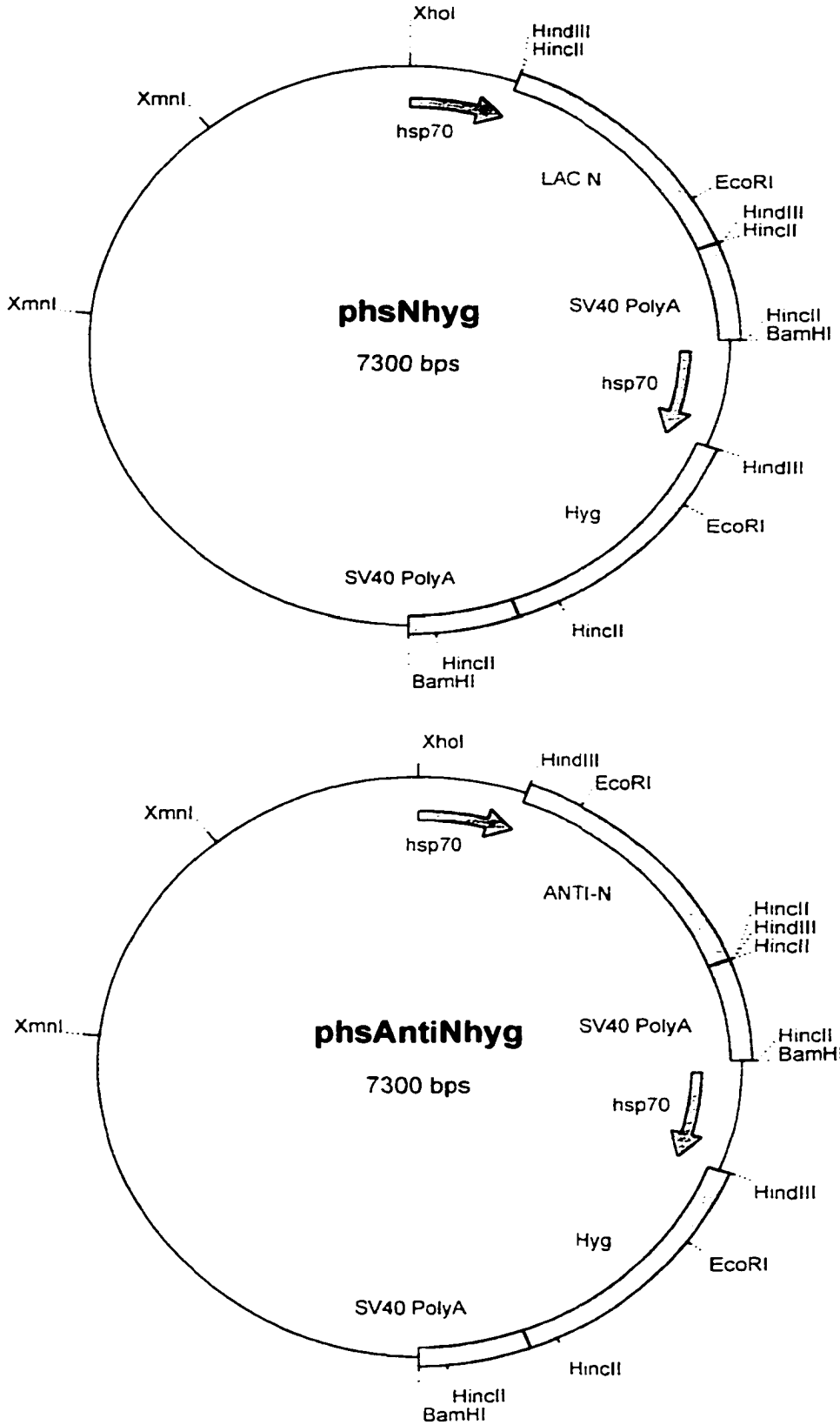


Figure 2.2. Plasmids phsN and phsAntiN contain the full-length S segment of LAC in sense and antisense orientation, respectively, under the control of the *Drosophila* hsp70 promoter.

Cells were incubated with the liposome/DNA complex for 15h at 28°C, at which time the medium was replaced with 5mL L-15 containing serum and antibiotics. The cells were placed under hygromycin selection after a 48h recovery period by adding 300units/mL hygromycin B (Calbiochem, La Jolla, CA) to the maintenance medium. Hygromycin-resistant colonies were isolated and expanded into transformed cell lines, using conditioned medium. Conditioned medium was prepared by collecting medium from actively growing C6/36 cell cultures, filtering the medium, and diluting it 1:2 in fresh L-15 containing 10% FBS, antibiotics, and L-glutamine.

RNA extraction. Total cellular RNA was extracted either by using a one-step guanidinium thiocyanate method (Chomczynski & Sacchi, 1987) or RNAwiz reagent (Ambion, Inc., Austin, TX) according to the manufacturer's specifications. Contaminating DNA was destroyed by treatment with RQ1 DNase (Promega Corp., Madison, WI) for 1h at 37°C followed by phenol/ chloroform extraction and isopropanol precipitation.

Northern blot analysis. Northern blot analysis was performed by separating 10ug of total cellular RNA by electrophoresis through a formaldehyde-agarose gel. The RNA was blotted onto a positively charged nylon membrane (Boehringer-Mannheim, Mannheim, Germany), and hybridized with a ^{32}P-labeled PCR product generated from an S segment cDNA template. Radioactive labeling was done using the Prime-It II kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications. The specific activity of the labeled probe was determined by scintillation counter measurement to be ~2.2 x 10^9 dpm/ug. Hybridizations were performed overnight at 60°C in hybridization

buffer containing 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 1M NaCl, 200ug/mL sheared, denatured DNA, and 20ng/mL probe. Membranes were washed twice in 2X standard saline citrate (SSC; 0.15M NaCl, 15mM sodium citrate, pH 7.0), 0.1% SDS for 10 min at room temperature, followed by 2 washes in 0.2X SSC, 0.1% SDS at 60°C for 30min. Membranes were exposed to x-ray film for 1-12 days.

RT-PCR analysis. RT-PCR analysis was performed using Superscript II reverse transcriptase (Life Technologies) and Taq DNA polymerase (Promega) according to the manufacturer's specifications. Primers specific for the LAC N coding sequence were used (Forward 5'-ATCAAGAGTGTGATGT-3'; Reverse 5'-GGAAGCCTGATGC CAAATTTC-3'). PCR was performed with an annealing temperature of 50°C for 30 cycles. Reaction products were separated by electrophoresis through a 1% agarose gel containing 0.5ug/mL ethidium bromide.

Slot blot analysis. Serially diluted, *in vitro* transcribed S segment RNA and 30ug total cellular RNA were used for slot blot analysis of RNA transcript levels. Samples were heat-denatured at 95°C for 20min in 7% formaldehyde, 6X SSC, and vacuum blotted onto a positively charged nylon membrane (BrightStar™, Ambion, Inc.) using a minifold II slot blot apparatus (Schleicher & Schuell, Keene, NH). The membrane was hybridized with an N-specific PCR product, and an identical blot was hybridized with an actin-specific PCR product to insure accurate measurement of RNA samples and loading of slot blot apparatus. Probes were labeled with psoralen-biotin according to the manufacturer's specifications (Ambion, Inc.). Membranes were incubated with UltraHyb buffer (Ambion, Inc.) for 1h at 42°C prior to hybridization. Hybridizations were done in UltraHyb buffer (Ambion, Inc.) containing 10ng/mL biotinylated probe overnight at

42°C. Membranes were washed in 2X SSC, 0.1% SDS twice at room temperature, followed by two washes in 0.2X SSC, 0.1% SDS at 50°C for 30min. Membrane-bound probe was detected using the BrightStar BioDetect kit according to the manufacturer's specifications (Ambion, Inc.), and exposed to x-ray film for 15-45 min. X-ray films were photographed and digitally analyzed for band density using GelExpert software (NucleoTech Corp., San Mateo, CA). Standardized band densities were used to estimate the number of transcripts per cell in transformed cell lines.

Genomic DNA extraction. Total cellular DNA was extracted from confluent cell cultures grown in 75cm^2 flasks. Cells were lysed in 10mL lysis buffer (100mM Tris, 10mM EDTA, 0.5% sarcosyl, 0.1mg/mL proteinase K) for 4h at 60°C. DNA was precipitated by incubating cell lysates with 10mL isopropanol at -20°C overnight. The DNA was pelleted at 16,000 x g for 15min, and resuspended in TE (10mM Tris-HCl, 1mM EDTA, pH 8). RNase A was added at 0.05mg/mL and was incubated for 1h at 37°C. The DNA was then treated with proteinase K, phenol/chloroform extracted, and ethanol precipitated. DNA was resuspended in TE and subsequently used for Southern blot analysis.

Southern blot analysis. Southern blot hybridization was performed by electrophoresis of 10ug of genomic DNA or control plasmid DNA digested with appropriate restriction enzymes on an 0.8% agarose gel. The gel was treated with 0.25M HCl for 30min, followed by 3M NaCl, 0.5N NaOH for 30min, and finally 10X SSC for 30min, prior to blotting on a positively charged nylon membrane (Brightstar^{TM}, Ambion, Inc.). Plasmid DNA or an S segment PCR product were used as probes for hybridization. Plasmid DNA was digested with EcoRI prior to labeling with a psoralen-biotin conjugate (Ambion,

Inc.) according to the manufacturer's specifications. Hybridizations were performed in 50% formamide, 1% SDS, 1M NaCl, 10% dextran sulfate, and 100ug/mL herring sperm DNA, and 20ng/mL biotinylated probe at 42°C overnight, following a 3h pre-hybridization incubation. Membranes were washed twice in 2X SSC for 5min, twice in 2X SSC, 1% SDS for 30min at 65°C, and twice in 0.1X SSC for 30min at room temperature. Biotinylated probe hybridization was detected using the BrightStar BioDetect kit (Ambion, Inc.) according to the manufacturer's specifications. Blots were exposed to x-ray film for 15min to 2h. X-ray film was digitized and analyzed by Un-Scan-It software (Silk Scientific Inc., Orem, UT) to estimate plasmid copy number.

Challenge with wild-type LAC virus. A $CuSO_4$ sensitivity experiment was performed to determine at what concentration healthy cells will be affected. Untransformed C6/36 cells were incubated with concentrations of $CuSO_4$ ranging from 0.1-1mM. Growth was inhibited in cells exposed to 0.8-1mM $CuSO_4$, but no cell death was seen at any concentration tested. Because previous studies showed that 0.2mM was the optimal concentrationof $CuSO_4$ for induction of the Mtn promoter in C6/36 cells (Kovach *et al.*, 1992), this concentration was used for these experiments. Cells transformed with the Mtn constructs were induced with 0.2mM $CuSO_4$ for 48h prior to challenge, and were maintained on medium with $CuSO_4$ throughout the infection. Cells transformed with the hsp70 constructs were incubated at 42°C for 1h prior to challenge, and were subsequently heat shocked for 1h every 24h during infection. Transformed cell lines were challenged with LAC virus at a multiplicity of infection (moi) of 0.01. Virus adsorption was allowed for 1h at room temperature on a shaker. Cells were then rinsed twice in PBS and fresh L-

15 containing 5% FBS and antibiotics was added. Infected cells were incubated at 28°C and supernatant was collected and stored at -70°C for titration analysis. Samples were titrated on BHK-21 cells using CPE endpoint dilutions to calculate viral titers as $\log_{10}TCID_{50}/mL$.

Fluorescent *in situ* hybridization (FISH). C6/36 cells grown on glass coverslips were fixed in 4% formaldehyde, 5% acetic acid (in PBS) for 20min. Fixed cells were stored in 70% ethanol at 4°C until used. Cells were rehydrated in PBS, permeabilized in 0.1% pepsin in 0.01M HCl, post-fixed in 4% formaldehyde, and dehydrated in a series of ethanol washes. Cells were hybridized in FISH buffer (60% deionized formamide, 2X SSC, 50mM sodium phosphate, 10% dextran sulfate) containing 250ng/uL yeast tRNA (Life Technologies), 250ng/uL herring sperm DNA (Promega), and 5ng/uL fluorescein-labeled probe, overnight at 42°C in a humidified box. RNA probes were labeled with fluorescein using a T7 polymerase run-off transcription reaction, as recommended by the manufacturer (NEN Life Sciences, Boston, MA). Cells were rinsed twice in 60% formamide/2X SSC for 15min, followed by 2X SSC for 3min. Tyramide signal amplification was performed after hybridization according to the manufacturer's specifications (NEN Life Sciences). Briefly, an anti-fluorescein antibody conjugated to horseradish peroxidase (HRP) was allowed to bind the fluorescein-labeled probe, followed by reaction of fluorescein tyramide with the HRP to deposit numerous fluorescein molecules at the site of probe binding. The coverslips were mounted on glass slides with a glycerol solution containing diazobicyclo(2.2.2) octane (DABCO; Sigma) and viewed by fluorescence microscopy.

Results

Derivation of transfomed cell lines. Stably transformed cell lines expressing LAC RNA were engineered to test our hypothesis that LAC-specific RNAs expressed from DNA-based constructs interfere with viral replication when they are present in the same cellular compartment as LAC virus. C6/36 cells were transformed with plasmids pMtN, pMtAntiN, phsN, and phsAntiN (Figures 2.1 and 2.2) using Lipofectin reagent (Life Technologies). Hygromycin resistant colonies were isolated and expanded into stably transformed cell lines. Pooled hygromycin resistant cells were also perpetuated to determine if interference could be detected in a cell line which was not clonaly derived, which would be more representative of a population of cells in a transformed mosquito. A total of 78 cell lines were derived from the C6/36 cell transformations. Northern blot analysis was used to choose cell lines expressing LAC-specific RNA, which were screened for LAC interference. An interference experiment was undertaken to screen for transformed cell lines that interfered with virus replication. Cells were induced either by addition of heavy metals or by heat shock, and were challenged with LAC virus at an moi of 0.01. Five days post-infection, the cell supernatants were collected and titrated on BHK-21 cells. Cell lines that produced at least 1.7 $\log_{10}TCID_{50}$/mL (Beaty *et al.*, 1989) lower titer of LAC virus than the C6/36 controls were chosen for further interference studies (Table 2.1). None of the cell lines transformed with the heat shock plasmids interfered with LAC virus replication.

Table 2.1. Initial Screening for Interference Potential

Cell Line	Titer[r] at day 5 (SD)	Cell Line	Titer[r] at day 5 (SD)
C6/36	5.2 (0.7)	HsAN 2.8	5.7 (0.9)
HsN 1.7	5.6 (0.2)	HsAN 2.11	5.8 (0.4)
HsN 1.5	4.7 (0.7)	MtN 1.8*	3.9 (0.2)
HsN 1.2	5.4 (0.2)	MtN 1.13*	3.7 (0.8)
HsN 2.4	5.7 (0.4)	MtN NC*	**3.4** (0.5)
HsN 1.6	6.3 (0.2)	MtN 1.1*	**3.5** (0.3)
HsN 1.1	5.5 (0.3)	MtN 1.12*	**3.2** (0.9)
HsN 1.1*	5.9 (0.2)	MtAN 1.17*	3.8 (0.7)
HsN NC	4.7 (0.4)	MtAN 1.6*	**2.5** (0.4)
HsN NC*	5.6 (0.2)	MtAN 1.10*	3.9 (0.8)
HsAN 2.7	5.6 (0.2)	MtAN 1.13*	4.6 (0.2)
HsAN 2.7*	5.8 (0.6)	MtAN 1.14*	5.0 (0.3)
HsAN 2.1	4.5 (0)	MtAN NC*	3.7 (1.3)

*Asterisks indicate induced cell lines.

[r]Bold type indicates titers at least 1.7 $\log_{10}TCID_{50}$/mL lower than C6/36 control.

Cell lines exhibiting potential to interfere with LAC virus were used in further genetic evaluation and interference time course experiments. For a comparison, cell lines expressing LAC RNA which did not interfere with viral replication were also evaluated. Because none of the hsp70-based plasmids showed potential for interference, they were excluded from the following genetic analysis.

Detection of LAC S segment-specific transcripts from transformed cell lines. Total cellular RNA from cell lines transformed with pMtN or pMtAntiN was analyzed by northern hybridization with a probe specific to the LAC S segment RNA. LAC-specific bands were detected at approximately 1.3 kb, which is the expected size of the S segment RNA and SV40 poly A sequence (Figure 2.3). The RNA samples used in the northern analysis were not equal in concentration as judged from viewing ethidum bromide-

stained rRNA bands under ultraviolet light (data not shown), therefore no quantitative comparison can be made from this analysis.

RT-PCR was performed on cell line RNAs that had been treated with DNase, to confirm that the band seen by northern blot was specific for the LAC S segment. A 750bp PCR product was detected in all reactions using RNA from the transformed cell lines (Figure 2.4). No product was detected in PCR reactions without prior cDNA synthesis, indicating there was no LAC-specific DNA in the RNA samples (data not shown). A second, slightly smaller band was seen in the RT-PCR reactions. This band was from a nonspecific reaction, because it is also present in the untransformed C6/36 cell lane (Figure 2.4).

Total cellular RNA from transformed cell lines was analyzed for LAC-specific transcript numbers by slot blot and digital analysis (Figure 2.5, Table 2.2). Transcript numbers ranged from 38-360 transcripts per cell. Some cell lines did not respond to induction with heavy metals with an increase in LAC-specific transcripts. Cell line MtAN 1.6 showed the strongest response to induction, increasing by 100 transcripts per cell upon heavy metal exposure (Figure 2.5, Table 2.2).

Analysis of plasmid insertion by Southern blot hybridization. Genomic DNA from transformed cell lines was analyzed by Southern blot, using restriction enzymes EcoRI and NcoI (Figure 2.6). Digestion of pMtN and pMtAntiN with EcoRI gives distinctive band patterns for each plasmid, while NcoI linearizes both plasmids. The expected sizes of DNA fragments from digestion with EcoRI are indicated to the left of the films, and those expected with NcoI are indicated to the right of the films (Figure

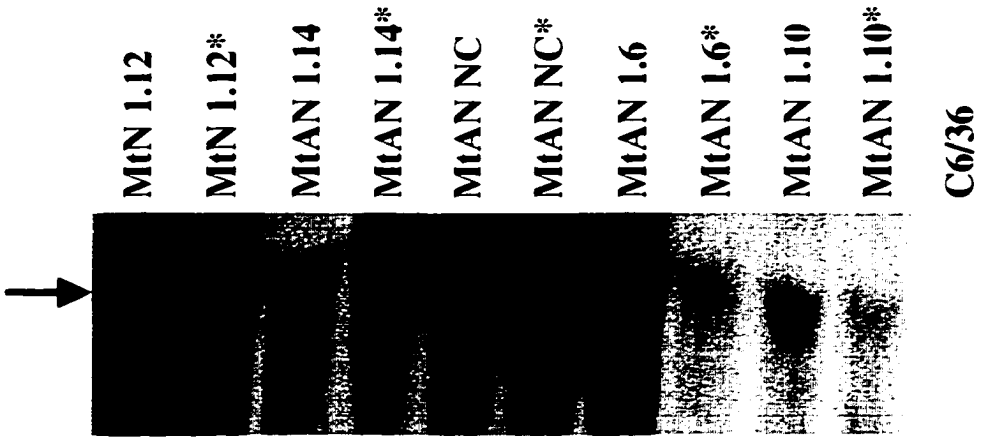


Figure 2.3. Northern blot analysis of representative MtN and MtAN cell line RNAs. Arrow indicates position of 1.3kb bands. Asterisks indicate $CuSO_4$-induced samples.

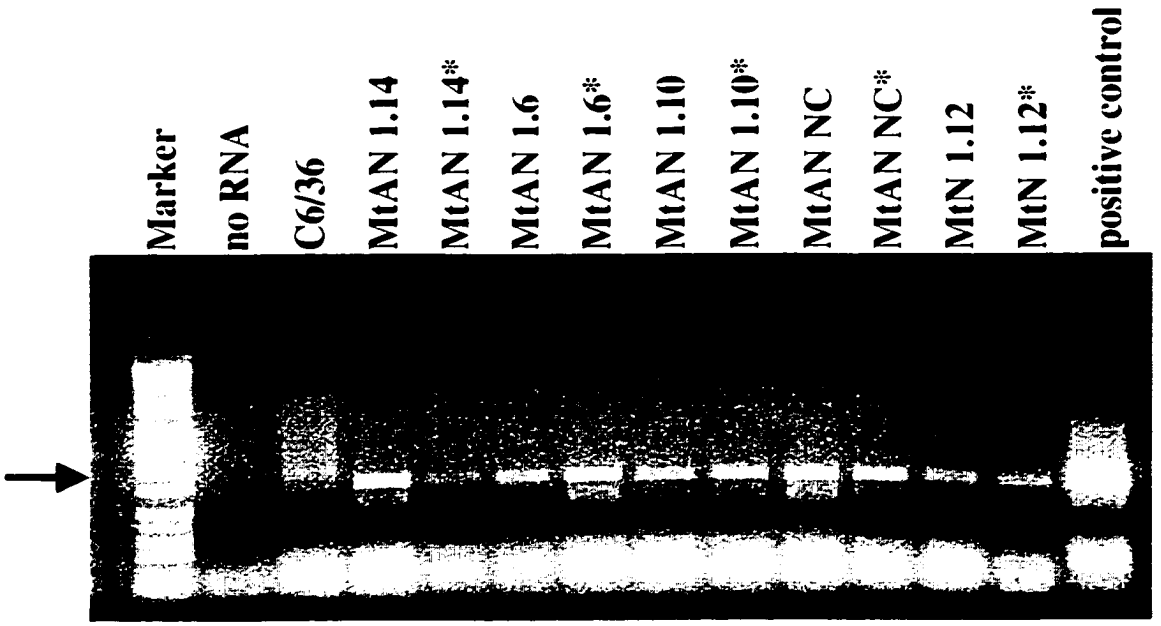


Figure 2.4. RT-PCR of representative MtN and MtAN cell line RNAs. Arrow indicates position of 750bp LAC N gene-specific bands. Asterisks indicate $CuSO_4$-induced samples.

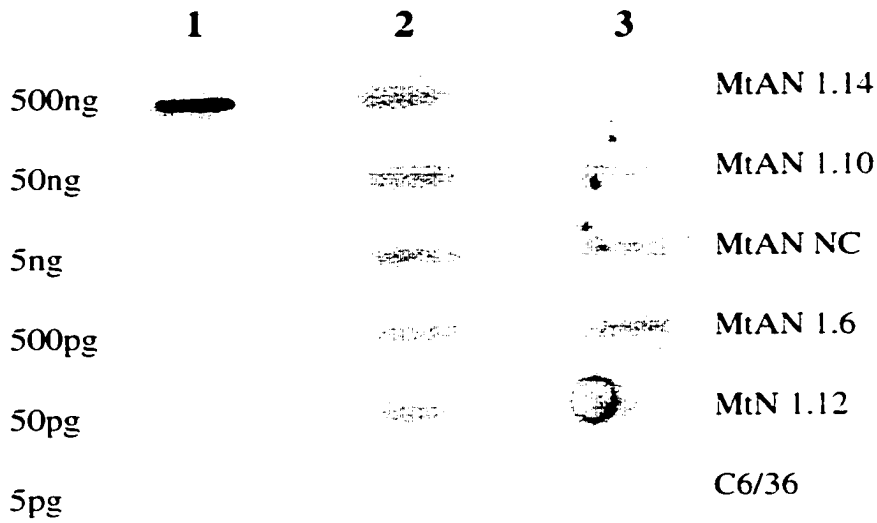


Figure 2.5. Slot blot of RNA from MtN and MtAN cell lines. Lane 1: S RNA standards in concentrations listed at left. Lanes 2 and 3: RNAs from MtAN and MtN samples listed at right. Lane 2: uninduced. Lane 3: induced with 200mM $CuSO_4$.

Table 2.2. N-specific transcript numbers

Cell Line	Transcripts/Cell
MtAN 1.14	3.5×10^2
MtAN 1.14*	3.8×10^1
MtAN 1.10	3.6×10^2
MtAN 1.10*	8.5×10^1
MtAN NC	2.7×10^2
MtAN NC*	2.1×10^2
MtAN 1.6	2.3×10^2
MtAN 1.6*	3.2×10^2
MtN 1.12	1.5×10^2
MtN 1.12*	1.8×10^2

"Slot blot in Figure 2.5 was evaluated by GelExpert software (NucleoTech Corp.) to determine transcript number/cell. Asterisks indicate $CuSO_4$-induced samples.

2.6). Restriction digests of genomic DNA from transformed cell lines were compared to parallel digests of plasmid DNA. Uncut plasmid and genomic DNA and digested DNA from C6/36 cells were also analyzed. Two distinct bands were seen when MtAN 1.6 was digested with NcoI. Because NcoI will cut the plasmid once, this indicates that one copy of the plasmid was integrated into the genome (Figure 2.6 B). The laddering of an indeterminant number of bands in the MtN 1.12 sample digested with NcoI indicates multiple copies were integrated in the genome (Figure 2.6 A). The multiple bands representing the 6kb fragment in the EcoRI digest confirms this observation. The x-ray films were analyzed digitally to estimate copy number. The number of plasmid copies inserted in all cell lines analyzed was estimated to be between 1-10 copies per genome. Plasmid DNA could not be detected in any cell lines by ethidium bromide-staining as seen with high copy number plasmid insertions (Monroe, 1990). Cell line MtN 1.12 was not expressing N protein, as determined by immunofluorescence assay (IFA) (data not shown).

Determination of cell line potential to inhibit LAC virus production. Three interference time course experiments were undertaken to determine the specific potentials of the chosen cell lines to interfere with LAC virus production (Figure 2.7, Table 2.3). These experiments had varied results. Some of the transformed cell lines showed lower production of LAC virus than C6/36 controls (Figure 2.7); however this interference was not consistent throughout the experiments (Table 2.3). The data from Table 2.3 was compiled from four separate experiments. When these data were combined, no trend of interference was seen in any of the cell lines.

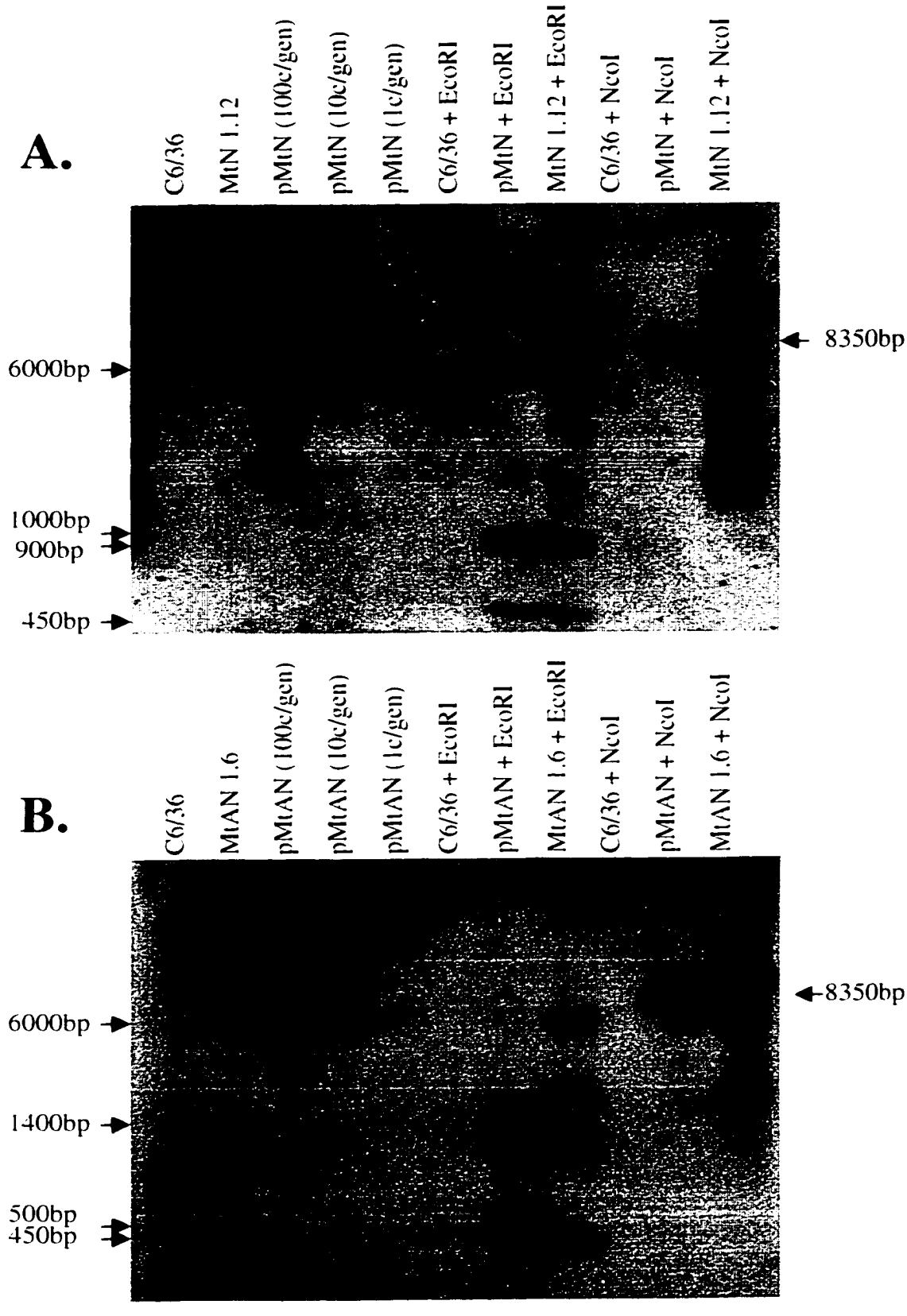


Figure 2.6. Southern blot analysis of cell lines (A) MtN 1.12 and (B) MtAN 1.6. Samples are described above the lanes, see text for details. C/gen=copies per genome. Sizes of plasmid fragments digested with EcoRI are at left, and with NcoI are at right of films.

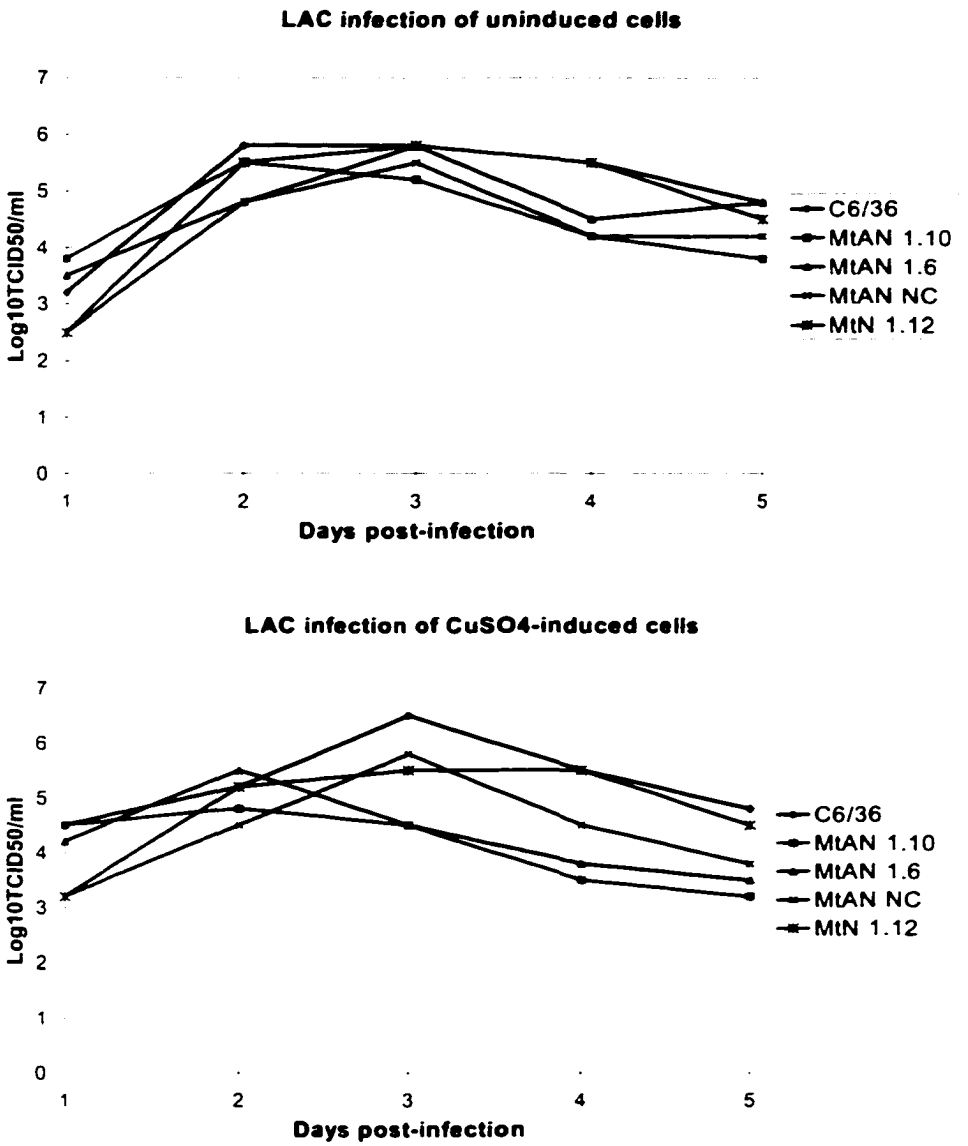


Figure 2.7. Interference time course experiment comparing uninduced (top graph) and $CuSO_4$-induced (bottom graph) cell lines transformed with pMtN or pMtAN. Cells were challenged with LAC virus at an moi of 0.01. Cell supernatents were collected and titrated each day for five days. Titers are expressed as $\log_{10}TCID_{50}$/mL.

Table 2.3. Titers of LAC virus escaping interference in pMtAntiN and pMtN transformed cell lines.

	LAC titer (SD)[r]				
Cell Line	24h	48h	72h	96h	120h
C6/36	4.4 (1.2)	7.0 (1.1)	6.9 (1.4)	6.3 (0.7)	5.7 (0.9)
C6/36 *	4.6 (0.2)	5.5 (0.3)	7.0 (1.1)	6.0 (0.7)	5.7 (1.3)
MtAN 1.10	4.7 (1.2)	5.5 (0)	6.2 (1.4)	5.0 (1.1)	4.5 (1.0)
MtAN 1.10 *	4.9 (0.5)	4.7 (0.2)	6.0 (2.1)	5.2 (2.3)	4.4 (1.6)
MtAN 1.6	4.6 (1.0)	**5.3** (1.4)	**5.2** (2.4)	5.7 (1.2)	4.7 (1.2)
MtAN 1.6 *	3.6 (0.5)	4.7 (1.1)	5.7 (1.2)	6.3 (2.2)	4.2 (0.7)
MtAN NC	3.2 (0.9)	**5.0** (0.3)	6.5 (1.4)	5.7 (2.1)	4.7 (0.7)
MtAN NC *	3.4 (0.2)	4.0 (0.7)	5.7 (0.2)	5.7 (1.6)	4.7 (1.2)
MtN 1.12	4.3 (1.9)	6.4 (0.9)	6.3 (2.0)	5.2 (2.5)	4.9 (1.7)
MtN 1.12 *	4.3 (1.4)	4.6 (1.3)	6.1 (0.5)	6.4 (0.8)	5.1 (0.5)

*Asterisks indicate induced cell lines.

[r]Bold type indicates titers at least 1.7 $\log_{10}TCID_{50}$/mL lower than C6/36 control.

Localization of antisense transcripts. FISH was used to determine the localization of antisense transcripts in transformed cell lines, in comparison to localization of LAC virus RNA in infected C6/36 cells (Figure 2.8). In transformed cell lines, antisense S segment transcripts were localized in the cytoplasm of cells, in several very distinct foci. Antisense transcripts were detected in a fraction of cells, and induction by heavy metals resulted in an increase in antisense expression as visualized by increased fluorescence. In C6/36 cells infected with LAC virus, genomic RNA and mRNA species were also detected in very distinct foci in the cytoplasm. There was only one or two very strong foci of fluorescence seen in LAC-infected cells, while most of the transformed cells had several smaller foci of fluorescence.

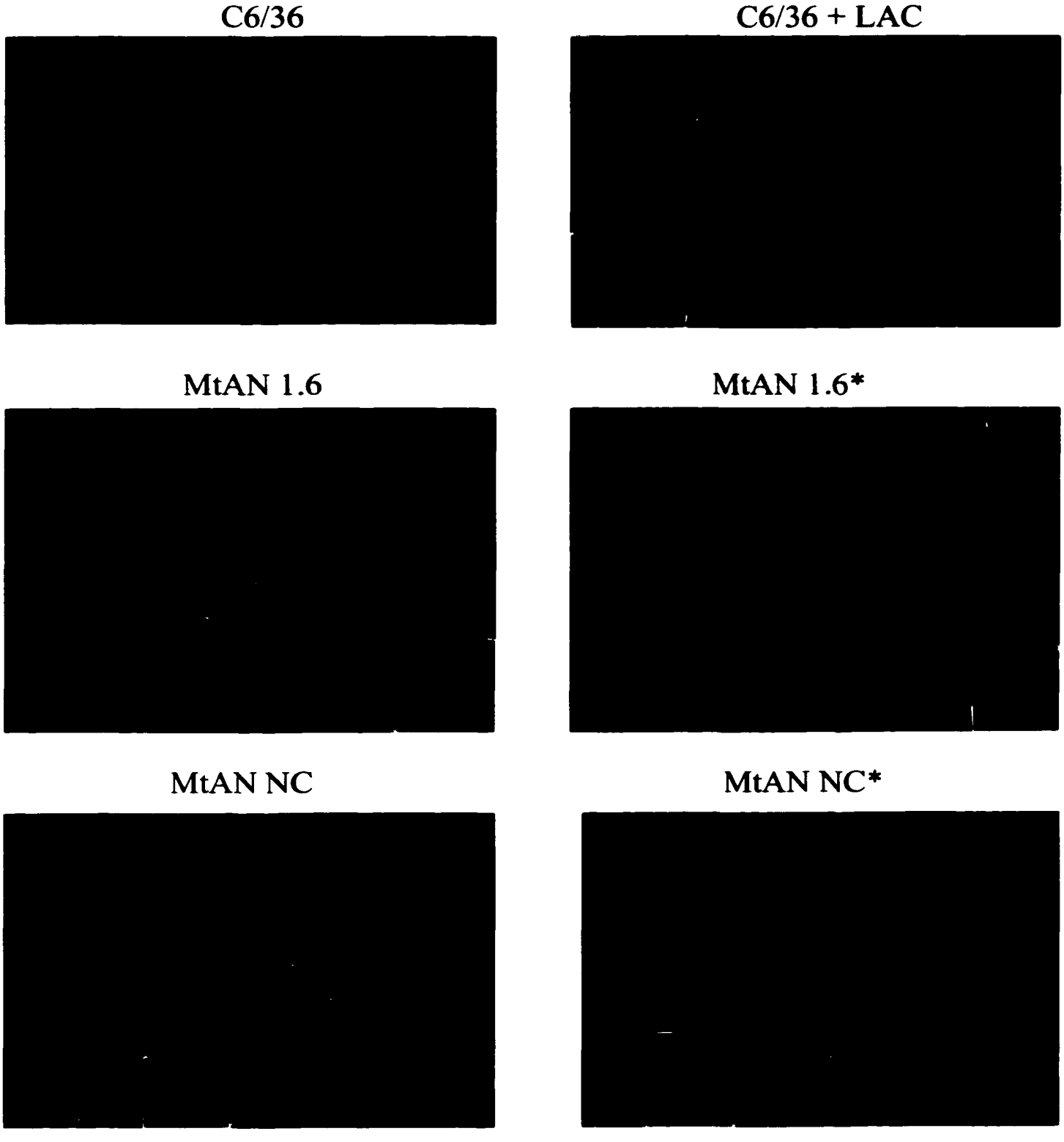


Figure 2.8. FISH analysis of antisense transcripts in pMTAntiN-transformed cell lines. Cell line NC was generated from a pool of hygromycin resistant cells. Asterisks indicate $CuSO_4$-induced samples.

Discussion

Mosquito cells transformed with constructs expressing LAC S segment RNA were evaluated for the potential to inhibit LAC virus replication. Although S segment-specific RNAs are effective at interfering with LAC virus when expressed from a SIN virus expression system (Chapter 1) (Powers *et al.*, 1996; Powers *et al.*, 1994), they are not effective when expressed from DNA constructs using *Drosophila* Mtn or hsp70 promoters. The FISH analysis indicates that LAC effector RNA is present in the cytoplasm of cells transformed with the Mtn constructs (Figure 2.8). From this analysis, it is not clear if the effector RNA is in the same cytoplasmic compartment as the target RNA. Proper trafficking of effector molecules is essential for effective intracellular immunization strategies.

It has been shown that a 50-100 fold excess of antisense effector RNA over target RNA is required for antisense inhibition of gene expression to occur (Izant & Weintraub, 1984). The concentration of antisense RNA in pMtAN-transformed cell lines might be sufficient if LAC replication is inhibited at an early time point post-infection. However if allowed to replicate, LAC RNA synthesis may quickly overwhelm the concentration of antisense RNA. If DNA expression of antisense RNA is going to work, expression must be at a comparable level to the dsSIN expression system, or interference would not be expected. Transcript numbers in our pMtAntiN-transformed cell lines (38-360 transcripts/cell) are much lower than those in dsSIN-infected cells (9,900-90,000 antisense transcripts/cell at 96h post-infection, (Powers, 1995). A stronger DNA

promoter, such as the actin 5C or baculovirus IE1 promoter (Zhao & Eggleston, 1999), may be needed to optimize this interference system.

Stably transformed cell lines were maintained that expressed LAC S segment RNA from the nucleus. This RNA was transported to the cytoplasm of transformed cells, as seen using FISH analysis. The presence of anti-LAC S segment RNA in the cytoplasm of mosquito cells should interfere with LAC virus replication; however, the expression is limited to approximately 30% of cells in line MtAN 1.6. If active virus replication takes place in the remaining 70% of cells, interference may not be detected by sampling the population of cells as a whole. A procedure for detecting viral interference on a single cell basis is required to fully evaluate viral interference in this system. Interference may be occurring in the cells that are actively expressing the antisense construct; however, interference from a small proportion of cells may not be detected by titrating virus from cell supernatants.

The inconsistent interference when expressing anti-LAC sequences from *Drosophila* promoters may be addressed by expressing antisense sequences in live mosquitoes using mosquito-specific promoters such as apyrase or maltase promoters, which are active in the salivary glands of adult mosquitoes. Expression of antisense RNA in the salivary glands may specifically inhibit LAC virus replication in an epidemiologically relevant organ, resulting in a block in transmission through blood-feeding. It is not known if interference in a cell culture system will directly predict interference in live mosquitoes.

CHAPTER 3: INTERFERENCE STRATEGIES WITH LACROSSE VIRUS USING SINDBIS VIRUS REPLICON-BASED CONSTRUCTS

Introduction

In order to boost antisense transcript production over the *Drosophila*-based expression plasmids, a DNA-based SIN replicon expression system was designed for antisense RNA expression in mosquito cells. The use of a DNA-based SIN replicon capable of cytoplasmic self-amplification provides a two-tiered expression system (Figure 3.1). Primary transcription from a eukaryotic promoter in the nucleus results in a replicon RNA that is transported to the cytoplasm, where SIN non-structural proteins are translated. The SIN replicon then undergoes self-amplification and expression of heterologous genes cloned in place of the SIN structural genes. There is no virus packaging because the structural genes have been deleted. These studies test the prediction that expression of LAC-specific antisense RNA from a DNA-based SIN replicon will boost effector RNA transcription, resulting in greater interference potential against LAC virus.

DNA-based replicon expression strategies have been tested in various mammalian cell lines and *in vivo* in mice. These studies compared expression of a luciferase reporter gene from a DNA-based replicon and a standard plasmid that does not express a SIN replicon. The replicon-based constructs have increased expression of heterologous sequences when compared to corresponding plasmids without the replicon sequence (Driver *et al.*, 1995; Dubensky *et al.*, 1996; Herweijer *et al.*, 1995).

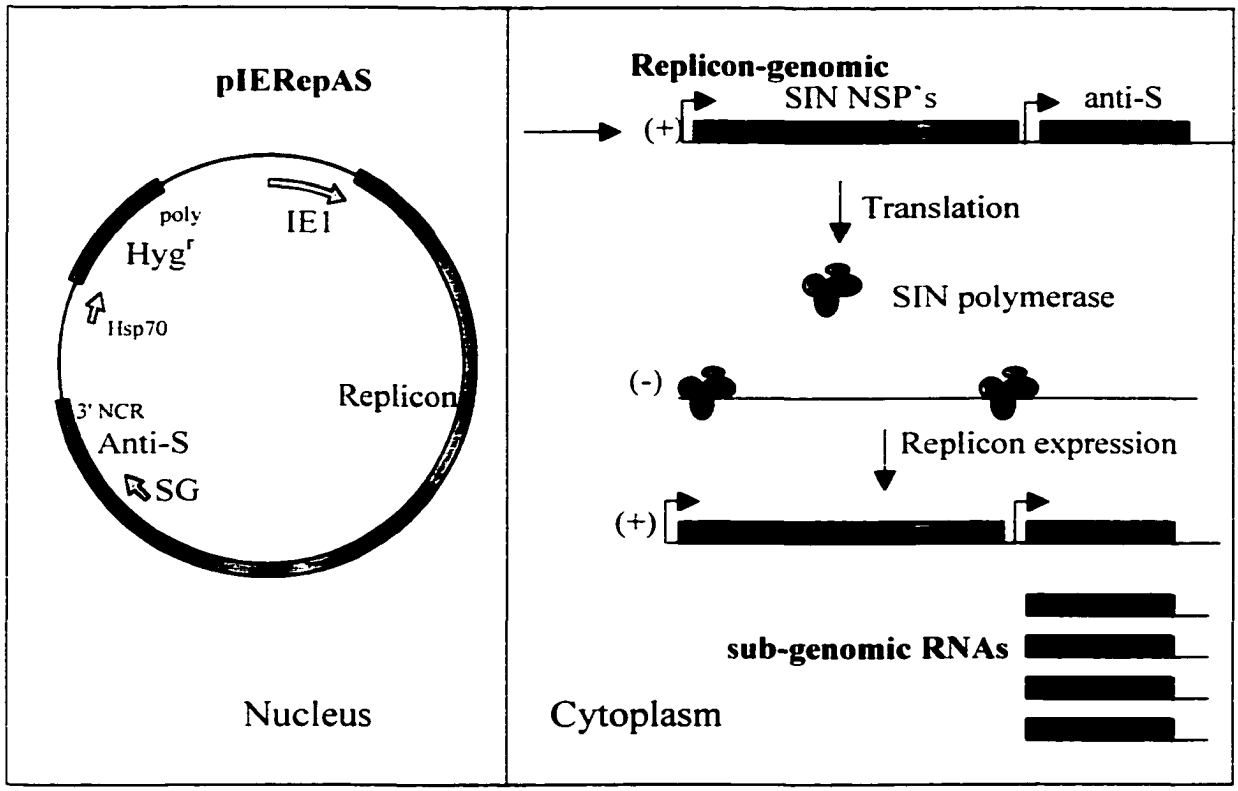


Figure 3.1. Expression of antisense RNA to LAC S segment from a DNA-based Sindbis replicon. pIErepAS. SG=subgenomic promoter; NCR=non-coding region: NSP=non-structural protein.

The IE1 promoter from the baculovirus of *Autographa californica* was used for expression of a SIN replicon in insect cells. The baculovirus IE1 promoter is constitutively active in mosquito cells in culture (Zhao & Eggleston, 1999). The immediate early genes of baculoviruses are expressed in the absence of viral protein synthesis, their gene products are required for the expression of later classes of viral genes (Guarino & Dong, 1991). The IE1 gene product is important in the regulation of viral gene expression. It is involved in transactivation of late gene promoters, inhibition

of IE0 gene expression, and autoregulation of the IE1 promoter through the hr5 enhancer (Kovacs *et al.*, 1992; Kovacs *et al.*, 1991).

This is the first example in which a DNA-based SIN replicon is expressed from a eukaryotic promoter in insect cells. Expression of LAC S segment antisense RNA from a DNA-based SIN replicon results in interference with LAC virus replication in a dose-dependent manner.

Materials and Methods

Plasmid construction. pIERepAS (Figure 3.1) was constructed using standard cloning procedures (Sambrook *et al.*, 1989). A hygromycin resistance gene cassette was excised from the plasmid pUChshyg (Monroe *et al.*, 1992) with XmnI and BamHI and ligated into the EcoRV/BamHI site of pBluescript KS (Stratagene), creating pBlu/hshyg. pBlu/hshyg was then linearized with NotI and SacI and cloned into the NotI/SacI site of pSINRep5 (Bredenbeek *et al.*, 1993), creating pRep/hshyg. The IE1 promoter from pIE1-3 (Novagen Inc., Madison, WI) was amplified by PCR and the product was cloned into a TA cloning vector, pCR2.1 TOPO, (Invitrogen, Carlsbad, CA) using primers with modified SacI ends. IE1 was subsequently excised from pCR2.1 TOPO with SacI and cloned into the SacI site of pRep/hshyg, creating pIERep. The LAC anti-S sequence was inserted downstream of the replicon subgenomic promoter by first cloning an S segment PCR product into the pCR2.1 TOPO vector (Invitrogen), and then excising with XbaI at sites that had been engineered into the primers used for PCR amplification. The XbaI fragment was then cloned into the XbaI site of pIERep creating pIERepAS. Clones were

screened by PCR analysis and restriction enzyme digests to insure the S segment was inserted into the replicon sequence in the antisense orientation.

***In vitro* transcription and electroporation.** Plasmid pIERepAS was linearized with XhoI at a site at the 3' end of the replicon sequence. The DNA template was treated with proteinase K, phenol/chloroform extracted, and ethanol precipitated. *In vitro* transcription was done with SP6 polymerase (Promega Corp.), using the manufacturer's suggested reaction conditions. RNA from the *in vitro* transcription reaction was electroporated into BHK-21 cells using previously described conditions (Powers *et al.*, 1994).

C6/36 cell line transformation. C6/36 cells were transformed with pIERepAS as described in Chapter 2. Hygromycin resistant colonies were isolated and expanded into stably transformed cell lines.

Northern hybridization. Total cellular RNA was extracted using RNAwiz reagent (Ambion, Inc.) according to the manufacturer's instructions. RNA was DNase-treated and extracted with phenol/chloroform, followed by precipitation in ethanol. Ten ug of RNA were separated by electrophoresis on a formaldehyde agarose gel, and blotted onto a positively charged nylon membrane (BrightStar™, Ambion, Inc.) by capillary transfer. Membranes were hybridized with a biotinylated oligonucleotide (5'-GCTGGTCGGA TCATTGGGGCG-3': Life Technologies) specific for the replicon 3' non-coding region (NCR) or with an S segment specific PCR product labeled using psoralen-biotin according to the manufacturer's recommendations (Ambion, Inc.). Hybridization was performed in 0.25M Na_2HPO_4, pH 7.2, 10% SDS, 0.1mM EDTA, and 10ng/mL biotinylated probe at 50°C overnight. Membranes were rinsed in 2X SSC, 0.1% SDS at

room temperature, followed by two rinses in 0.2X SSC, 0.1% SDS at 60°C for 30min. Hybridization of biotinylated probe was detected using Ambion's BioDetect kit according to the manufacturer's instructions. Membranes were exposed to x-ray film for 10min to 1h at room temperature.

Slot blot analysis. Ten ug of total cellular RNA from IERepAS transformants, C6/36 control cells, and MtAN 1.6 cells (see Chapter 2) were heat-denatured at 95°C for 20min in 7% formaldehyde, 6X SSC, and vacuum blotted onto a positively charged nylon membrane (BrightStar™, Ambion, Inc.) using a minifold II slot blot apparatus (Schleicher & Schuell, Keene, NH). Serial dilutions of *in vitro* transcribed S segment RNA ranging in concentration from 500ng to 5pg were also blotted on the membrane. An S segment-specific PCR product probe was labeled with psoralen-biotin according to the manufacturer's specifications (Ambion, Inc.). The membrane was hybridized using the conditions described for northern hybridization above. A duplicate blot was hybridized under the same conditions with a biotinylated histone-specific oligonucleotide. Binding of biotinylated probe to the membranes was detected using Ambion's BrightStar BioDetect kit according to the manufacturer's protocol. Membranes were exposed to x-ray film which was photographed and analyzed by GelExpert Software (NucleoTech Corp.). Band densities of RNA concentration standards were used to calculate S segment-specific transcript numbers per cell.

Virus challenge and titration. pIERepAS-transformed and non-transformed C6/36 cells were infected with LAC virus at an moi of 0.001 or 0.05. Virus was adsorbed to cells for 1h with gentle rocking. Cells were rinsed three times with PBS after the 1h

adsorption, and placed on medium containing 2% FBS and antibiotics, with or without hygromycin B. Cell culture supernatants were collected every day for 5 days and were titrated on BHK-21 cells. Serial dilution endpoints were used to calculate titers as $\log_{10}TCID_{50}$/mL.

Results

Detection of functional replicon produced by pIERepAS in BHK-21 cells. Three different clones (#3, #4, and #28) of plasmid pIERepAS were tested for the ability to produce functional replicon RNA in BHK-21 cells. RNA was transcribed *in vitro* from the plasmids and electroporated into BHK-21 cells. Twenty-four hours post-electroporation, total RNA was collected from the cells and was analyzed by northern blot hybridization with a probe specific for the 3' NCR of the replicon (Figure 3.2). One clone (Figure 3.2, clone #28) had genomic (9kb) and subgenomic (1.4kb) bands similar to RNA from a control plasmid, pIERep/GFP (Figure 3.2, GFP), which has been shown to express GFP in BHK-21 and C6/36 cells (data not shown). Ribosomal RNA bands of approximately 2kb and 4.5kb were present in all samples. No SIN-specific bands were detected in non-electroporated BHK-21 cells (Figure 3.2, BHK-21).

Generation of stably transformed cell lines and RNA analysis. The pIERepAS construct that was shown to make functional replicon RNA by northern blot analysis was introduced into C6/36 cells using lipofectin reagent. Hygromycin-resistant cell lines were propagated and tested for replicon RNA production by northern blot analysis. Total

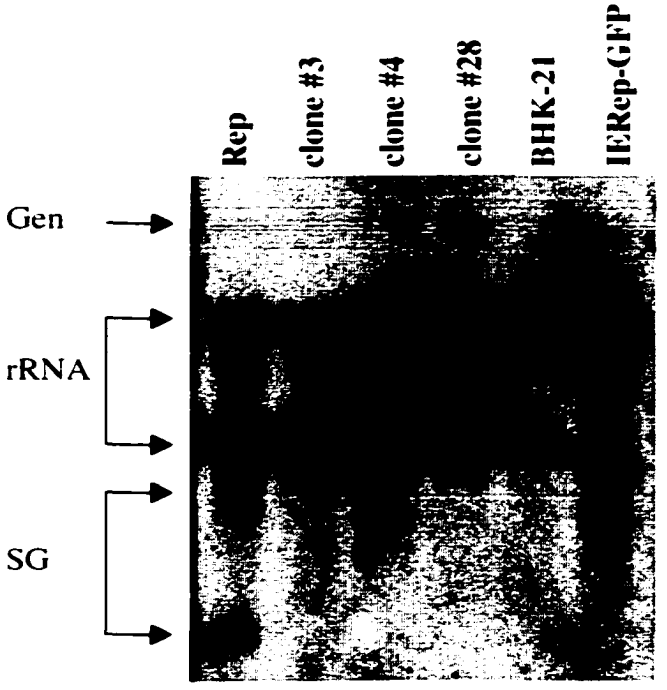


Figure 3.2. Northern blot analysis of RNA from BHK-21 cells electroporated with RNA transcribed *in vitro* from replicon plasmids. Rep=empty replicon; clones 3, 4, and 28 are separate clones of pIERepAS; BHK-21=non-electroporated control; GFP=pIERepGFP control; Gen=rep genomic RNA; SG=subgenomic RNA.

cellular RNA was blotted and hybridized with a biotinylated probe specific for the LAC S segment RNA (Figure 3.3). Genomic (9kb) and subgenomic (1.4kb) bands were seen in most of the pIERepAS-transformed cell lines. These bands were not seen in non-transformed C6/36 cells. A similar northern blot membrane was hybridized with a SIN 3' NCR-specific probe. This probe also bound to the genomic and subgenomic bands in the IERepAS-transformed cell lines, and did not bind to non-transformed C6/36 cell RNA (data not shown). Transformed cell lines that expressed varying levels of subgenomic RNA, as determined by northern blot analysis, were used for quantification of LAC-specific RNA and challenge experiments.

Quantification of LAC-specific RNA. LAC-specific RNA produced in IERepAS-transformed cell lines was quantified by slot blot analysis. Ten ug of total cellular RNA and *in vitro* transcribed S RNAs of known concentration were blotted onto a nylon membrane and hybridized with a biotinylated S segment-specific probe (Figure 3.4, A). A duplicate blot was hybridized with a histone sequence-specific probe to insure accurate loading of the slot blot apparatus (Figure 3.4, B). Films exposed to the slot blot membranes were photographed and analyzed by GelExpert Software (NucleoTech Corp.). Band intensities were used to estimate the number of S-specific transcripts per cell (Table 3.1). Similar quantities of total cellular RNA were blotted as determined by analysis of band intensities from the membrane hybridized with the histone-specific probe (Figure 3.4, B, and data not shown). Transcript quantification using the membrane hybridized with the S-specific probe (Figure 3.4, A) indicated that transcript numbers in IERepAS-transformed cell lines ranged from 5,300 to 10,000 transcripts/cell (Table 3.1). The transcript quantification analysis indicated that there were less than 940 copies of S-

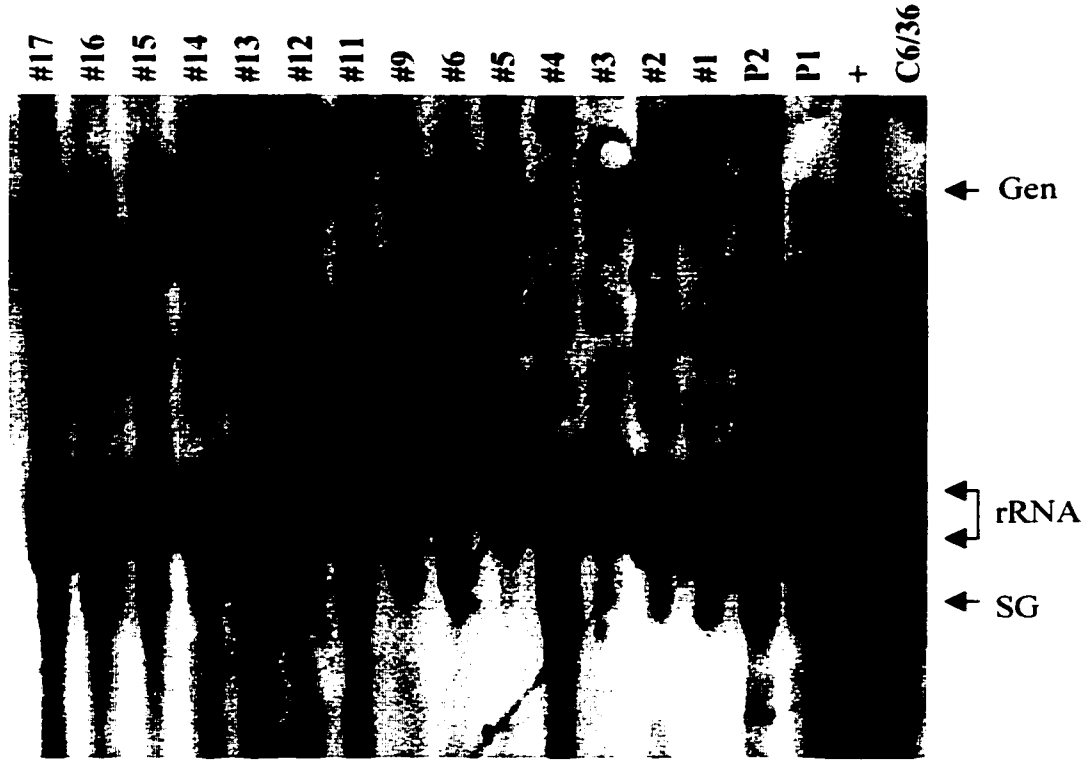


Figure 3.3. Northern blot analysis of total cellular RNA from IERepAS stable transformants. Membrane was probed with an S segment-specific DNA probe. Clonal cell lines listed as numbers above lanes, P1 and P2 are pooled lines. Gen=replicon genomic; SG=replicon subgenomic. Positive control RNA was *in vitro* transcribed S segment RNA. C6/36=non-transformed cells.

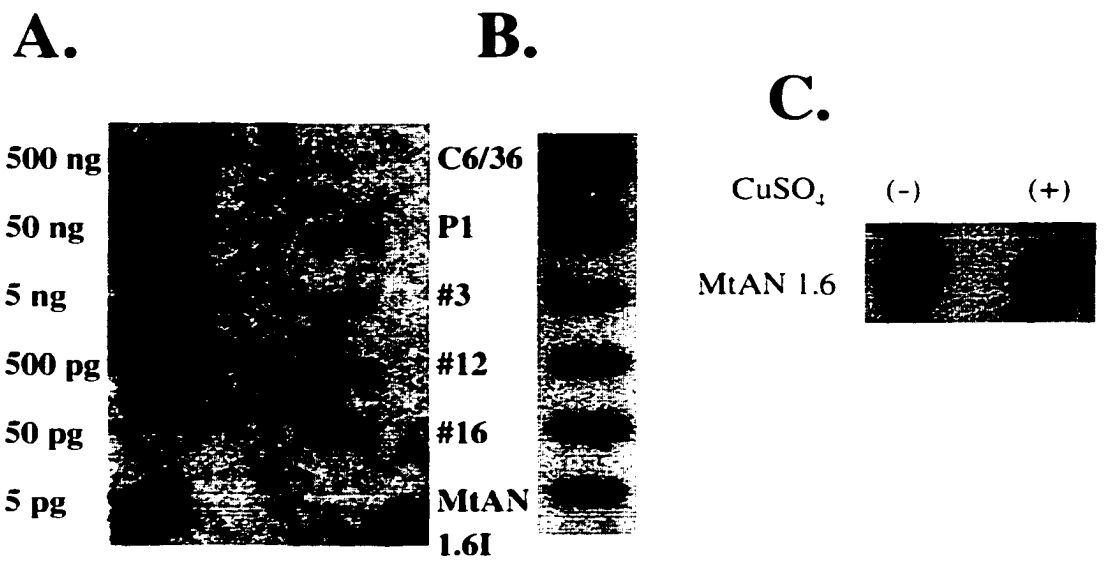


Figure 3.4. Slot blot analysis of total cellular RNA from IERepAS stable transformants. A.) Lane 1, *in vitro* transcribed S segment antisense RNAs of known concentrations. Lane 2, 10ug total cellularRNA from lines listed to right. I=induced with $CuSO_4$. Membrane was probed with S-specific probe. B.) 10ug total cellular RNA from lines listed to left. Membrane was probed with histone-specific probe. C.) 30ug total cellular RNA from uninduced and induced MtAN 1.6 cell line, probed with S segment-specific probe.

Table 3.1. Quantification of Anti-S transcripts in IERepAS stable transformants.

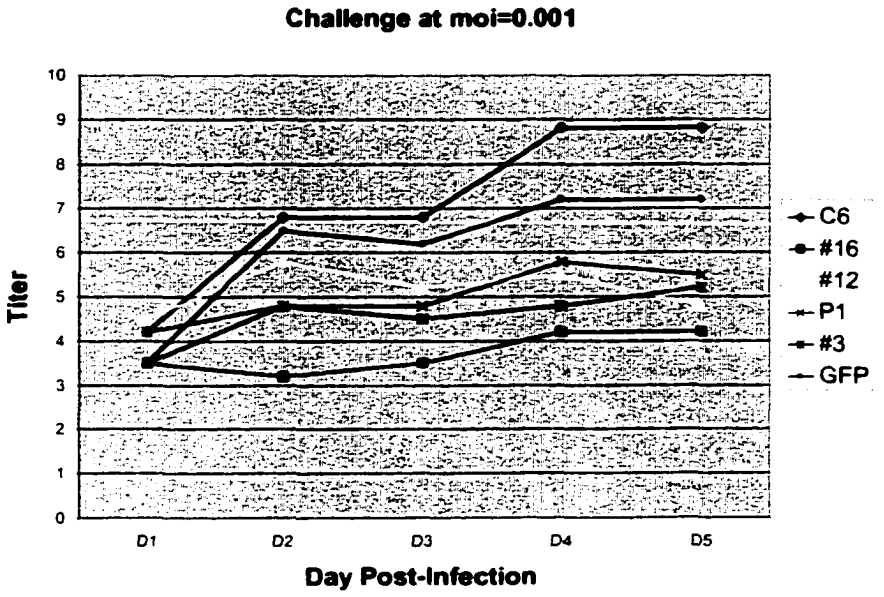
Cell line	Transcripts/Cell
P1	6800
#3	5300
#12	10000
#16	8600
MtAN1.6I	<940

*Calculations based on band intensity given by GeleExpert (Nucleotech) software.

specific transcripts per cell in the MtAN 1.6 cells that were induced with $CuSO_4$. (The lowest concentration detected by this assay was 940 copies per cell.) This is in agreement with an earlier analysis of this cell line that indicated it produces 320 transcripts per cell upon induction with $CuSO_4$ (See Chapter 2). An S segment-specific band was not seen in the lane with MtAN 1.6 induced RNA, when 10ug of RNA were loaded (Figure 3.4, A), although the histone blot indicated the correct amount of RNA was loaded (Figure 3.4, B). In another slot blot in which 30ug of total RNA were loaded, S-specific bands were seen in MtAN 1.6 induced and uninduced lanes (Figure 3.4, C).

Interference with LAC virus is dose-dependent. IERepAS stable transformants and C6/36 control cells were challenged with LAC virus at an moi of 0.001 or 0.05. C6/36 cells that had been transformed with a plasmid, pIERepGFP, expressing GFP in place of the anti-S sequence, were also challenged to insure interference is not a result of replicon expression. Cell supernatants were collected from infected cells each day for 5 days and were titrated on BHK-21 cells to calculate viral titers as $\log_{10}TCID_{50}$/mL. pIERep-GFP-transformed cells produced similar titers of LAC virus as compared to C6/36 control cells. All anti-S expressing cell lines tested inhibited LAC replication when challenged at an moi of 0.001 (Figure 3.5, A). Titers from IERepAS cell lines were approximately 3-5 logs lower than from C6/36 cells at days 4 and 5 post-infection. The same cell lines were permissive for LAC replication when challenged with 50 times more virus, at an moi of 0.05 (Figure 3.5, B). No difference in titers was seen between IERepAS and C6/36 control cells.

A.



B.

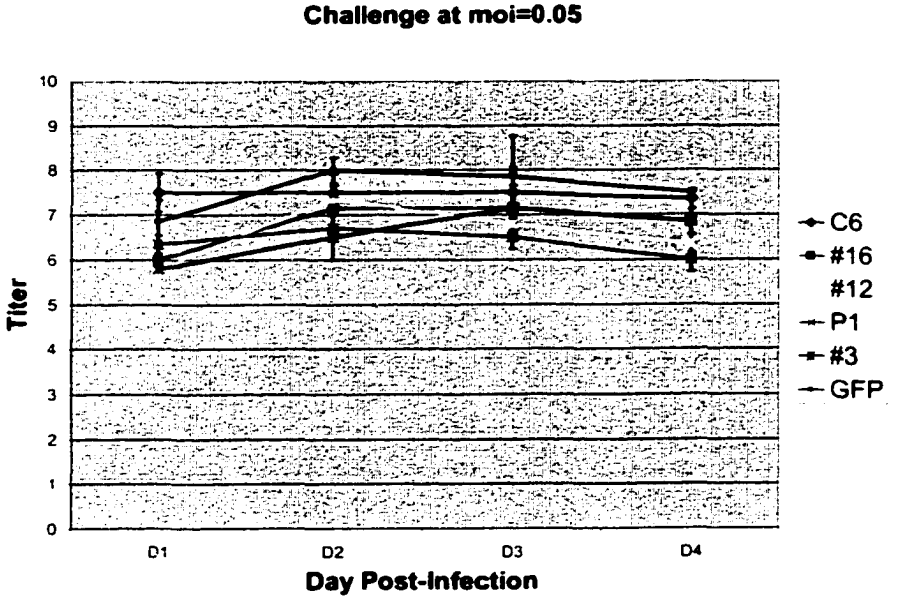


Figure 3.5. Challenge of IERepAS stable transformants with LAC virus. A.) Challenge at moi of 0.001. B.) Challenge at moi of 0.05. Titers are represented as $\log_{10}TCID_{50}$/mL. C6=untransformed C6/36 cells; GFP= C6/36 cells transformed with pIERepGFP; #16, #12, and #3 are clonally derived IERepAS transformants; P1 is a pool of IERepAS transformants.

A dose-response experiment was performed to confirm the findings of the initial challenge experiments. IERepAS cell line #16 and C6/36 control cells were challenged with LAC at various doses (moi=0.001-0.05) to determine at what dose the virus overcomes antisense inhibition (Figure 3.6). Three replicates from each cell line were challenged at each dose and supernatants were titrated on day 4 post-infection. Interference was only seen when IERepAS #16 cells were challenged at an moi of 0.001. The next lowest moi tested was 0.005; there was no interference at this dose or at the higher doses.

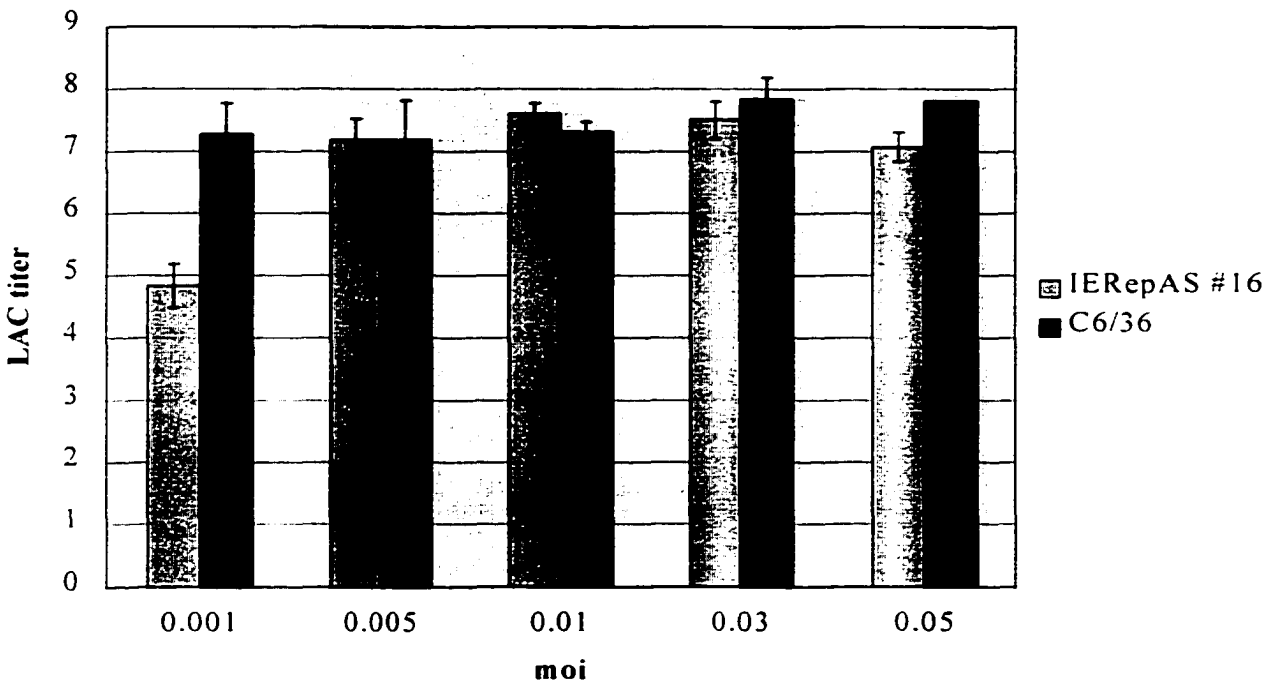


Figure 3.6. Challenge of C6/36 and IERepAS #16 cell lines with LAC virus at various doses. Cell supernatants were titrated on day 4 post-infection. Titers represent mean $\log_{10}TCID_{50}/mL \pm SD$ of three replicates.

Conclusions

A DNA-based SIN replicon capable of expression and RNA self-amplification in mosquito (C6/36) cells was engineered. Expression of LAC S segment-specific antisense molecules was seen in IERepAS-transformed cell lines by northern analysis of total cellular RNA. Quantification of S-specific transcripts by slot blot analysis indicates much higher expression of antisense molecules (5,000 to 10,000 transcripts per cell, Table 3.1) as compared to cells transformed with a plasmid using the *Drosophila* Mtn promoter (180-360 transcripts per cell, see Chapter 2). The concentration of antisense transcripts in pIERepAS-transformed cells is approaching that seen when expressed from the dsSIN expression system (9,900-90,000 antisense/cell at 96h post-infection) (Powers, 1995). A DNA-based SIN replicon expression system allows for increased transcript production over traditional plasmid systems because of self-amplification of the replicon RNA in the cytoplasm of transformed cells.

IERepAS-transformed cells inhibited LAC virus replication when challenged at an moi of 0.001. A mosquito taking an infectious blood meal from a small mammal in nature will ingest virus at a maximum moi of 0.001. Therefore, resistance at this low moi is relevant to what is observed in nature. It is not necessary to obtain resistance at a higher moi (0.05), because this exceeds the dose of virus a mosquito would ingest in nature. It is not known what proportion of cells are expressing the replicon antisense construct in our transformed cell lines. As discussed in Chapter 2, if a portion of the cells in a population are not expressing the antisense effector RNA, LAC replication in these cells could overwelm the interference strategy.

There may be several explanations for the increased inhibition of LAC virus replication in pIERepAS-transformed cells as compared to pMtAN-transformed cells. The pIERepAS-transformed cell lines generate much more effector RNA than the pMtAntiN-transformed cell lines. Anti-S transcript production was 100 times higher in pIERepAS-transformed cell lines. This high concentration of antisense RNA may prevent the virus from replicating early in infection, thus the virus is not able to overcome inhibition through rapid replication.

An alternative explanation is the presence of dsRNA in the replicon-expressing cell lines. It has been shown in a number of systems that dsRNA is more efficient at interfering with gene expression than sense or antisense RNA alone (Fire, *et al.*, 1998; Kennerdell & Carthew, 1998; Montgomery & Fire, 1998; Ngo, *et al.*, 1998; Waterhouse, *et al.*, 1998). dsRNA has also been proposed to signal PTGS, leading to sequence-specific degradation of mRNA (Montgomery & Fire, 1998). The existence of dsRNA intermediates with homology to LAC RNA, which are inherent in the replication of SIN viruses, may be responsible for interfering with LAC virus replication. This would explain why sense and antisense LAC sequences expressed from a SIN virus expression system are both effective at interfering with LAC virus. This also would explain the lack of consistent interference when the same sequences are expressed from a standard DNA plasmid. A dsRNA molecule with LAC sequence specificity may be equally efficient at inhibiting LAC virus replication. A plasmid designed to make both strands capable of folding into a dsRNA molecule could be designed to test the hypothesis that dsRNA is the mediator of LAC inhibition in mosquito cells expressing SIN-based antisense constructs, rather than classical antisense effects.

It is not known if a live mosquito would tolerate the genetic burden of a DNA-based SIN replicon expression plasmid. Mosquitoes tolerate persistent infections with SIN and recombinant SIN viruses; however, not necessarily during development. A mosquito promoter that is only active in the adult mosquito might be useful in delaying replicon expression until development is complete. Presumably, once expression of the replicon RNA from the nucleus occurs, the replicon genomic RNA can persist in the cytoplasm and undergo self-amplification, mimicking a SIN persistent infection, without the production of packaged virus. Because of the cytoplasmic production of effector RNAs, this system might actually be more stable in adult mosquitoes than antisense RNA expression from a traditional nuclear plasmid system. Another problem with using a DNA-based replicon for expression in transgenic mosquitoes is the potential difficulty with mobilization of a large genetic construct by a transposable element. However, in studies using the P element in *D. melanogaster*, insert size did not affect mobilization potential (Meister & Grigliatti, 1993).

CHAPTER 4: INTERFERENCE WITH LACROSSE VIRUS BY EXPRESSION OF HUMAN MxA PROTEIN IN MOSQUITO CELLS

Introduction

A protein-mediated interference strategy involving expression of the human MxA protein was evaluated to complement RNA-mediated interference strategies. The human MxA gene is induced by alpha and beta interferons, often in response to viral infection. Mx proteins belong to the dynamin superfamily of large GTPases found in yeast, plants, and animals (Haller *et al.*, 1998). The N terminal portion of the protein contains the highly conserved GTP-binding domain involved in enzyme activation. The C terminal domain contains a leucine zipper motif, which is often involved in protein-protein interactions. Dynamins are involved in endocytosis and vesicle transport, and tend to assemble in helical arrays around tubular structures. Human MxA has been shown to form helical aggregates in solution (Haller *et al.*, 1998). MxA also interacts with cellular proteins, including cytoskeletal components. The pattern of MxA localization seen by IFA is indicative of association with intracellular vesicles. The high abundance of MxA protein in interferon-induced cells suggests a stoichiometric interaction with cellular proteins, rather than a true enzymatic function (Horisberger, 1992).

The antiviral activity of Mx proteins has been demonstrated for a wide range of viruses with very different replication strategies. Interferons are produced in response to viral infection and act in a paracrine fashion, setting up an antiviral state in surrounding

cells. Binding of alpha and beta interferons to specific receptors activates the JAK/STAT signaling pathway which results in the activation of over 50 genes, including Mx (Stark *et al.*, 1998).

Human MxA protein has been shown to inhibit LAC virus in cell culture and in MxA-transgenic mice (Frese *et al.*, 1996; Hefti *et al.*, 1999). Interferon alpha/beta receptor knock-out mice are unable to respond to type I interferons, do not have intact Mx genes, and are highly susceptible to viral infections despite an otherwise intact immune system. When these knock-out mice transgenically express the human MxA gene, they become resistant to previously lethal virus infections (Hefti *et al.*, 1999). These mice allow for the evaluation of MxA-induced virus resistance *in vivo*, without the involvement of other interferon-induced gene products. The observation that MxA is able to interfere with LAC and other viruses in the absence of the normal interferon response pathway is central to the hypothesis that MxA is capable of interfering with LAC virus replication in mosquito cells.

These studies were designed to test the prediction that expression of the human MxA gene in mosquito cells will result in inhibition of LAC virus replication. It was shown that mosquito cells in culture are capable of expressing the human MxA gene from a baculovirus IE1 promoter. MxA expression in mosquito cells renders them resistant to LAC virus, but not to a recombinant dsSIN virus, as determined by IFA analysis.

Materials and Methods

Plasmid construction. Plasmid pIE1-MxA (Figure 4.1) contains the human MxA gene downstream from a baculovirus immediate early promoter. It was constructed by PCR-amplification of the ~2kb MxA sequence from pHMGMxA (obtained from Dr. Richard Bowen via Dr. Peter Staeheli; Pavlovic *et al.*, 1990) using primers (5'-GGATCCGGAA GATGGTTGTTTCCG-3' and 5'-GGATCCGGACAGAGTGTGGTTAACC-3') containing BamHI sites flanking the primer sequence. The PCR product was cloned into a TA cloning vector (pCR2.1 TOPO; Invitrogen) and was subsequently excised with BamHI. The BamHI fragment was cloned into the BamHI site of pIE1-3 (Novagen), creating pIE1-MxA.

Cells and viruses. *Ae. albopictus* C6/36 cells were grown in L-15 medium supplemented with 10% FBS and antibiotics. Wild-type LAC virus (tissue culture passage 10) was grown on BHK-21 cells for 48h to generate virus stocks for challenge experiments. The stock virus had a titer of 7.2 $\log_{10}TCID_{50}$/mL. The construction of recombinant SIN virus TE/3'2J/VP is described in Chapter 5. Stock TE/3'2J/VP virus had a titer of 6.5 $\log_{10}TCID_{50}$/mL.

MxA expression and challenge experiments. C6/36 cells growing on glass coverslips were transformed with pIE1-MxA using Effectene reagent (QIAGEN Inc., Valencia, CA) according to the manufacturer's recommendations. Twenty-four hours after the transformation reaction began, cells were challenged with LAC virus or TE/3'2J/VP virus at an moi of 0.01. Virus adsorption was done on a shaker for 1h, after which cells were rinsed twice in PBS and L-15 containing 5% FBS and antibiotics was added. Twenty-

four and 48h after infection, cells were fixed to the coverslips and analyzed by IFA as described below. MxA-positive and MxA-negative cells were counted in random, non-overlapping microscope fields and were analyzed for LAC or VP-specific antigen. Cell numbers were statistically analyzed in a two by two table with chi square analysis using SAS statistical software (SAS Institute, Inc., Cary, NC).

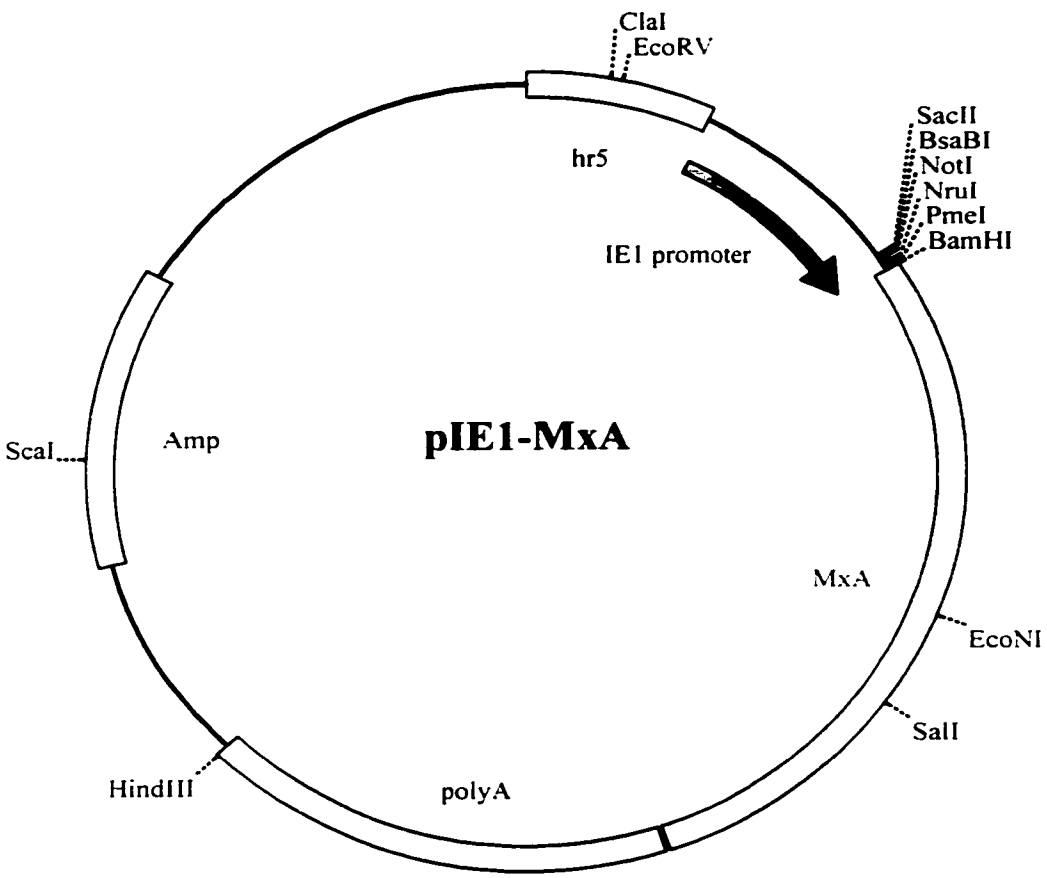


Figure 4.1. Plasmid pIE1-MxA contains the human MxA gene expressed from a baculovirus IE1 promoter. hr5 is a *cis*-acting enhancer. Unique restriction enzyme sites are shown.

Indirect IFA. Cells were rinsed with PBS, fixed in 4% paraformaldehyde for 8min, rinsed twice with PBS, permeabilized with 0.05% TRITON X-100 (Sigma) for 8min, and rinsed again with PBS for 20min with gentle aggitation. Fixed cells were incubated with primary antibody for 2h at 37°C, followed by incubation with secondary antibody for 40min at 37°C. An MxA-specific monoclonal antibody, 2C12 (Staeheli & Haller, 1985), was used as a primary antibody with a fluorescein (FITC)-conjugated anti-mouse secondary antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). LAC antigen was detected using rabbit polyclonal serum and a tetramethylrhodamine (TRITC)-conjugated anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories). VP antigen was detected using a rabbit antibody that binds AeDNV virions and a TRITC-conjugated secondary antibody (Kirkegaard & Perry Laboratories). Cells were viewed using an epifluorescence Olympus BH-2 microscope with a dual FITC/TRITC cube filter (Chroma Technology Corp., Brattleboro, VT).

Results

Expression of human MxA from a baculovirus promoter in mosquito cell culture. The *Ae. albopictus* cell line C6/36 was transformed with the plasmid pIE1-MxA (Figure 4.1), which has the human MxA gene cloned downstream of a baculovirus IE1 promoter. Forty eight hours after pIE1-MxA was introduced into cells, they were fixed and analyzed for MxA protein expression by IFA (Figure 4.2). MxA-specific fluorescence

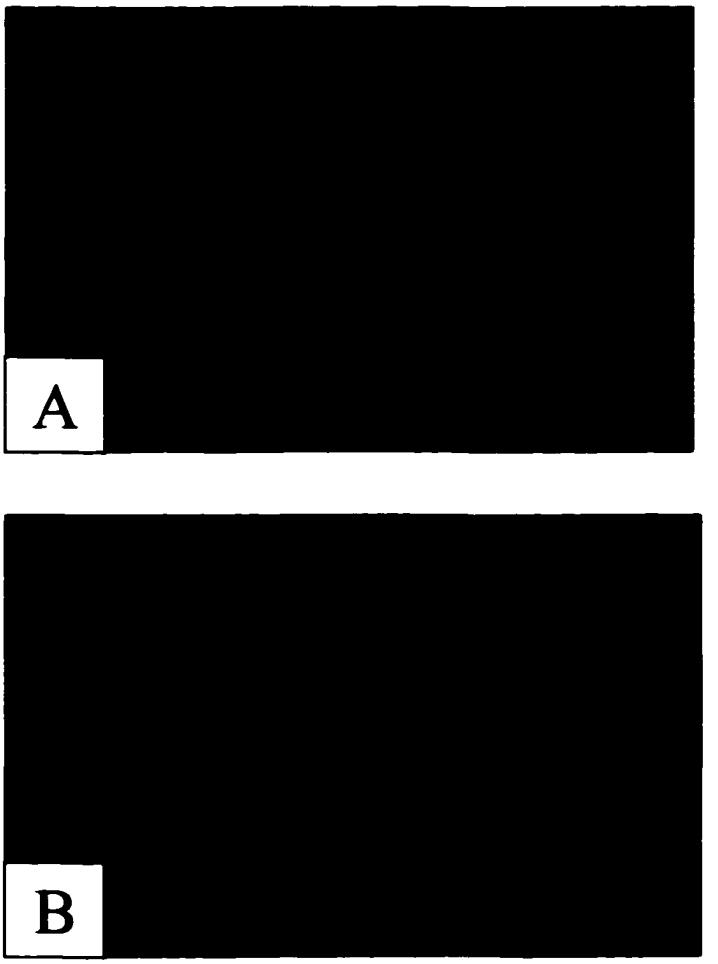


Figure 4.2. Expression of human MxA protein in mosquito cell line C6/36. pIE1-MxA-transfected cells were analyzed by IFA. A.) untransfected C6/36 cells (200X); B.) C6/36 cells transfected with pIE1-MxA (1000X).

was seen in the cytoplasm of positive cells (Figure 4.2, B) in a punctate pattern similar to that seen in vertebrate cell lines expressing the human MxA protein. The pattern of MxA localization seen by IFA is indicative of association with intracellular vesicles. No MxA-specific fluorescence was seen in non-transformed C6/36 cells (Figure 4.2, A).

Expression of human MxA in mosquito cells interferes with LAC virus replication. C6/36 cells expressing pIE1-MxA were challenged with LAC virus at an moi of 0.01. At 24 and 48h post-infection, cells were fixed and analyzed by IFA to detect Mx and LAC antigen (Figure 4.3). pIE1-MxA-transformation efficiencies of approximately 25% were seen using Effectene reagent (QIAGEN). LAC antigen was not evident in the majority of MxA-expressing cells (Figure 4.3, B and D); however, dual fluorescence was detected in a small number of cells (Figure 4.3, C). These cells appear to have less MxA-specific fluorescence than cells that have no detectable LAC antigen. Cells with lower levels of MxA expression may be more susceptible to LAC infection than high expressing cells. Numbers of MxA positive and MxA negative cells with or without LAC antigen were compared and analyzed statistically (Table 4.1). The number of LAC-infected MxA+ cells was significantly lower than infected MxA- cells at both time points ($p<0.0001$). LAC virus infection rates from three replicate experiments were compared at 24h and 48h post-infection (Figure 4.4). The mean infection rate in MxA negative cells was 67.6% and the mean infection rate in MxA positive cells was 7.4%, at 24h post-infection. These rates were 81.4% and 6.8%, respectively, at 48h post-infection.

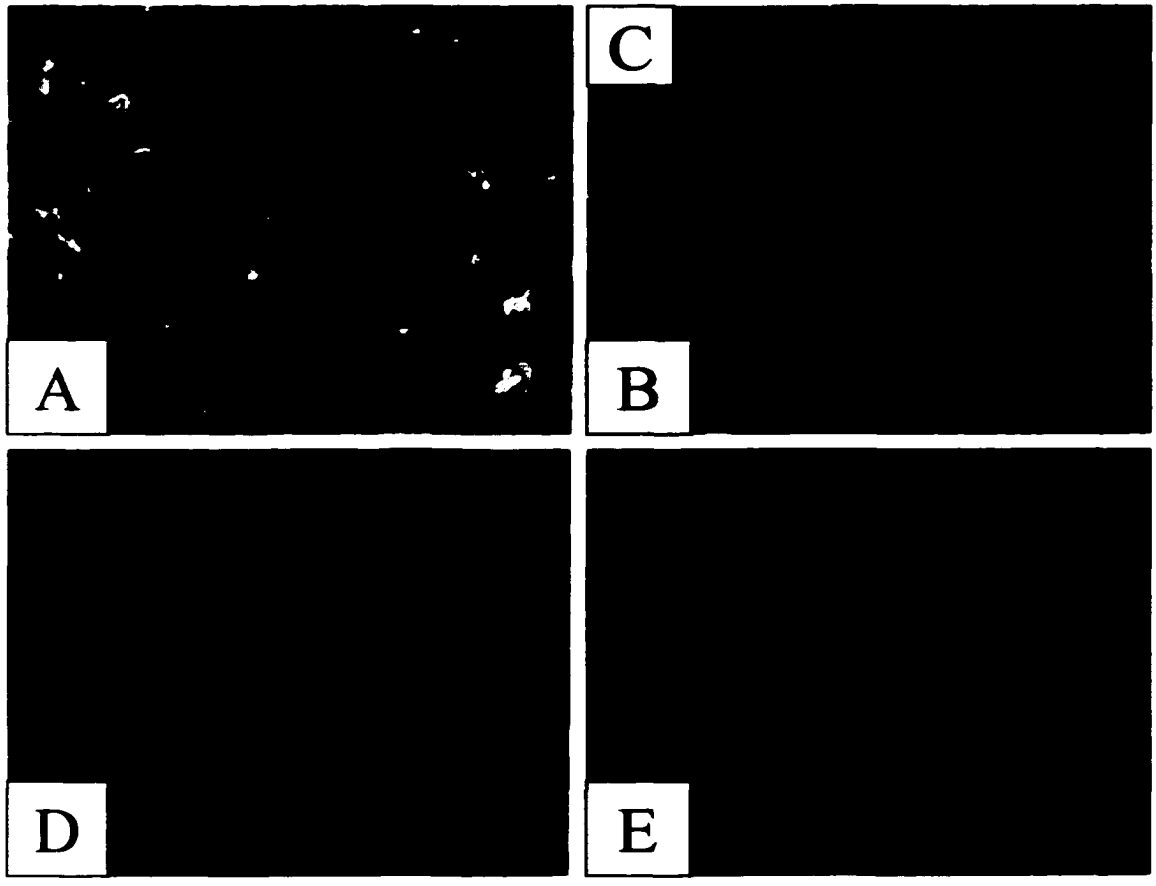


Figure 4.3. Analysis of viral infection in MxA-expressing C6/36 cells. Cells that were transformed with pIE1-MxA and challenged with LAC or TE/3'2J/VP virus were analyzed by IFA. A.) MxA-/LAC- at 24h, 200X; B.) MxA+/LAC+ at 24h, 200X; C.)MxA+/LAC+ at 24h, 1000X; D.) MxA+/LAC+ at 48h, 400X; E.) MxA+/TE3'2J/VP+ at 24h, 1000X

Table 4.1. Comparison of LAC-infected cell numbers in MxA positive and MxA negative cell populations.

	24h		48h	
LAC:	-	+	-	+
MxA-	207	433	56	245
MxA+	358	30	221	16

Expression of MxA does not interfere with replication of a recombinant Sindbis virus. A recombinant dsSIN virus, TE3'2J/VP, expressing the virion proteins (VP) from *Aedes* densovirus (see Chapter 5) was used to challenge pIE1-MxA-transformed C6/36 cells. At 24h post-infection, cells were analyzed by indirect IFA to detect MxA and VP specific antigens (Figure 4.3, E). VP antigen was detected in a majority of cells expressing MxA. In cells expressing both MxA and VP antigens, MxA-specific fluorescence was seen in the cytoplasm, while VP-specific fluorescence was seen in the nucleus. No significant difference in the number of cells with or without VP antigen in MxA positive and MxA negative cell populations was found by chi square analysis (Table 4.2; $p=0.5723$). There was also no significant difference between infection rates in MxA positive and MxA negative cell populations (Figure 4.4).

Table 4.2. Comparison of TE/3'2J/VP-infected cell numbers in MxA positive and MxA negative cell populations

	24h	
TE'3'2J/VP:	-	+
MxA -	54	258
MxA +	35	146

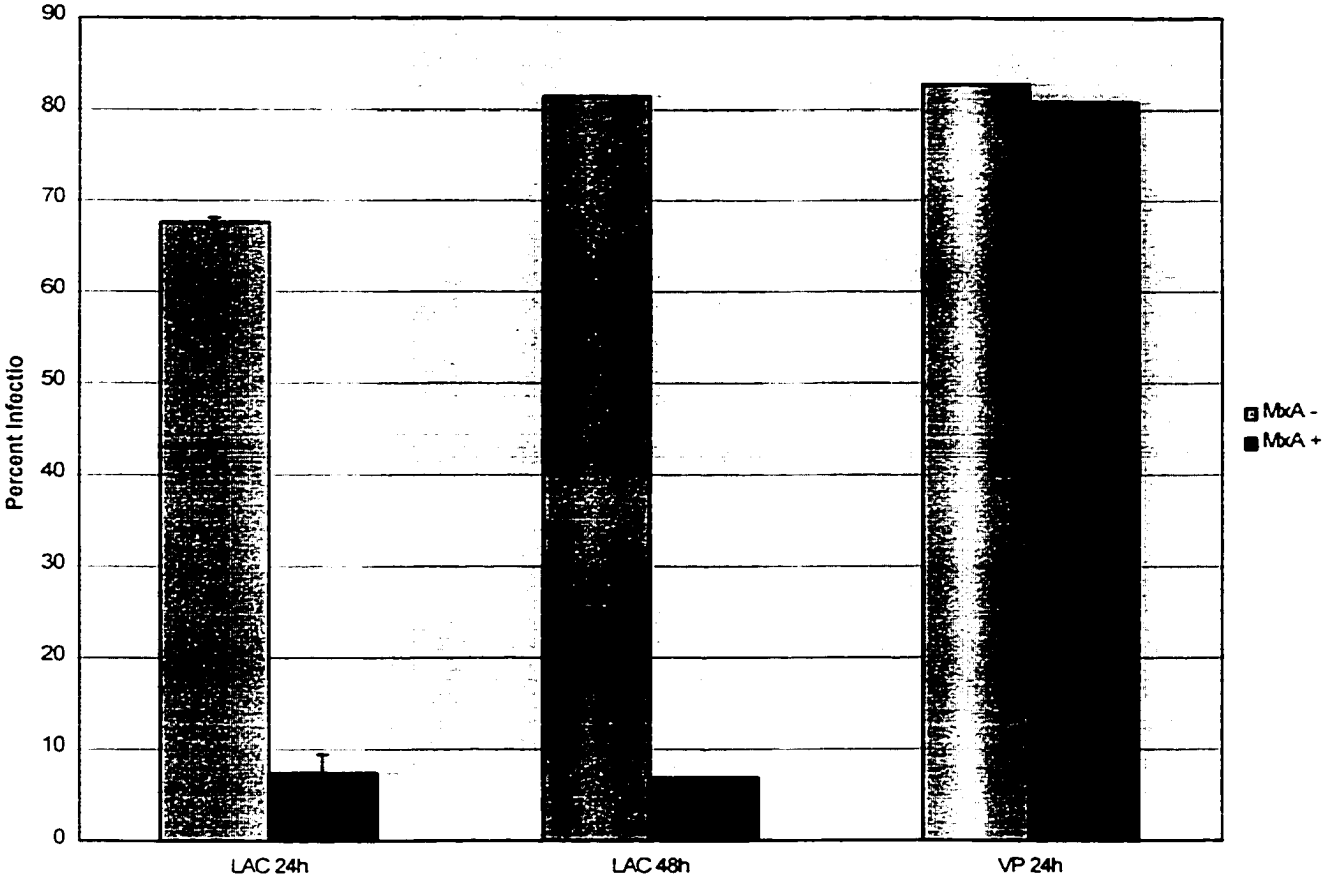


Figure 4.4. Comparison of LAC and TE/3'2J/VP (VP) infection rates in MxA- and MxA+ cell populations at 24h and 48h post-infection. LAC infection rates were statistically different by chi square analysis ($p<0.0001$). TE/3'2J/VP infection rates were not statistically different by chi square analysis.

Discussion

These data support the findings that MxA protein inhibits LAC virus, independent of other components of the interferon alpha/beta pathways. Hefti *et al.* (Hefti *et al.*, 1999) found that interferon alpha/beta receptor knockout mice that transgenically express human MxA protein were protected from a lethal challenge with LAC virus. Thus, mosquito cells, which do not have the interferon alpha/beta response pathway would also be expected to inhibit LAC virus replication upon expression of the human MxA protein. There was a significant reduction in LAC virus replication in mosquito cells expressing MxA (Table 4.1 and Figure 4.4).

Most of the viruses susceptible to MxA inhibition are negative sense RNA viruses (Haller *et al.*, 1998); however, a positive sense RNA virus, Semliki Forest virus, is inhibited by MxA (Landis *et al.*, 1998). Because SIN and Semliki Forest virus are genetically related, it was predicted that MxA would inhibit replication of a SIN virus. Interference was not observed, however, when MxA expressing cells were challenged with a recombinant dsSIN virus (Figures 4.3, 4.4, and Table 4.2). Different susceptibilities to MxA have been seen with similar viruses and even with the same virus in different cell types. For example, MxA inhibits measles virus in human cells, but not in mouse cells. The spectrum of antiviral activity of MxA needs to be determined in mosquitoes. Because MxA does not inhibit a recombinant SIN virus, a replicon expressing MxA could be designed to quickly test for interference to other arboviruses. The advantage of using a SIN replicon in place of our transient expression system is that a replicon would allow for MxA expression in a majority of cells in a population.

Challenged cells could then be analyzed by a virus titration protocol instead of IFA analysis and counting of positive and negative cells under the microscope.

The mechanism of MxA-specific interference with LAC virus replication is currently unknown, but there is strong evidence that MxA interferes with transcription and replication of LAC RNA (Frese *et al.*, 1996). LAC S segment RNA, including genomic, virion complementary, and mRNA, and N protein are detected in MxA-expressing cells in the absence of virus production. However, M and L segment RNA and their respective proteins are not detected in the same cells. Similar results have been seen in Mx1-expressing mouse cells infected with influenza virus (Pavlovic *et al.*, 1992). This indicates that while the RNA polymerase is able to initiate replication, it is blocked before full-length RNA is made; however there is some completion of S segment RNA because it is the shortest segment (Frese *et al.*, 1996). This is in agreement with the interference with influenza by mouse Mx1, in which inhibition of transcription is correlated to genome segment length (Krug *et al.*, 1985; Pavlovic *et al.*, 1992).

An alternative hypothesis for the mechanism of interference with LAC virus is that MxA is binding to LAC ribonucleoprotein complexes, thereby preventing their transport. LAC virions must bud through Golgi membranes to acquire envelope before becoming infectious. MxA has been shown to bind Thogoto virus, thereby preventing its transport into the nucleus (Kochs & Haller, 1999).

It is unknown whether or not LAC-specific RNAs can be detected in mosquito cells expressing the MxA protein. Because hyperimmune serum was used to detect LAC antigens by IFA, it is not clear if antigen detected in MxA positive cells is a result of active virus replication or if it is N protein-specific, as seen with LAC infection of MxA-

expressing mammalian cells (Frese *et al.*, 1996). Determining the specific LAC RNAs and proteins present in MxA-expressing mosquito cells might provide a clue to the mechanism of interference by MxA in this system.

Mosquitoes are important in the transmission of many viruses that affect humans, including LAC virus. Transgenic mosquitoes expressing MxA may potentially be valuable in blocking LAC virus transmission to humans. Other arboviruses also need to be tested in our mosquito cell culture system for sensitivity to MxA interference. The transmission of important pathogens could potentially be interrupted in MxA-expressing mosquito vectors. Expression of MxA in epidemiologically significant organs of the mosquito would be important for interference with arbovirus transmission. In the case of LAC virus, expression of MxA in the mosquito midgut cells might prevent spread of virus infection to other tissues involved in transmission. Induction of MxA expression in the midgut upon blood-feeding might be an effective way to block LAC virus replication from an important source of infection. The salivary glands and ovaries would be important targets for blocking LAC virus transmission by blood-feeding and by transovarial transmission, respectively.

CHAPTER 5: PACKAGING OF AeDNV-GFP TRANSDUCING VIRUS BY EXPRESSION OF DENSOVIRUS STRUCTURAL PROTEINS FROM A SINDBIS VIRUS EXPRESSION SYSTEM

Introduction

Aedes densonucleosis virus (AeDNV) is being investigated as an alternate means for mosquito transduction and may be useful in expressing antiviral or anti-mosquito constructs in live mosqutioes. AeDNV also has potential as a biocontrol agent for mosquito control. Genetic manipulation of invertebrates is important for studying organisms of medical and agricultural importance, as well as for developing control strategies for vectors of human and veterinary pathogens (Buchatsky, 1989; Carlson *et al.*, 1995; Carlson *et al.*, 1996; Corsini *et al.*, 1996; Tal & Attathom, 1993). Several members of the Parvoviridae family have been used as transducing viruses for the expression of heterologous genes (Bout, 1996; Corsini *et al.*, 1996; Hermonat and Muzyczka, 1984; Maxwell *et al.*, 1993; Muzyczka, 1992). The genomes of AeDNV and *Junonia coenia* DNV have been engineered to express heterologous genes in insect cells (Afanasiev *et al.*, 1994; Giraud *et al.*, 1992). The use of AeDNV as a mosquito control agent is currently being examined. For example, expression of a scorpion toxin gene from an AeDNV transducing virus may increase the toxicity of the virus for mosquitoes.

Genetic recombination resulting in the production of wild-type infectious virus is an obstacle in the current system for producing densovirus transducing particles. Generation of AeDNV transducing virus requires co-expression of a plasmid containing a transducing genome carrying the gene of interest, and a helper plasmid supplying virus

structural proteins in *trans* (Afanasiev *et al.*, 1994; Afanasiev *et al.*, 1999). This method allows for production of AeDNV transducing virus that expresses a gene of interest, but there is a high level of wild-type virus production due to recombination between the transducing genome and helper plasmids (Afanasiev *et al.*, 1999).

In this study, a dsSIN virus-based packaging system was designed to bypass the problem of wild-type virus contamination of AeDNV transducing virus stocks. A dsSIN virus, TE/3'2J/VP, was engineered to express AeDNV structural proteins (VPs) downstream of the second subgenomic promoter. Expression of AeDNV VPs allows packaging of a transducing genome expressing green fluorescent protein (GFP) (Figure 5.1). AeDNV-GFP transducing viruses readily infect C6/36 cells and *Aedes aegypti* larvae, as determined by GFP expression. This system allows for production of AeDNV transducing particles without genetic recombination resulting in wild-type infectious virus, and it is comparable in efficiency to the two-plasmid method of generating recombinant AeDNV viruses.

Materials and Methods

Cell culture and mosquitoes. *Ae. albopictus* C6/36 cells (ATCC CRL-1660) were grown at 28°C in Leibowitz-15 (L-15) medium (GIBCO, Gaithersburg, MD) supplemented with 10% FBS, 100U/ml penicillin, and 0.1mg/ml streptomycin. BHK-21 cells (ATCC CCl-10) were grown at 37°C in L-15 plus 10% FBS and antibiotics.

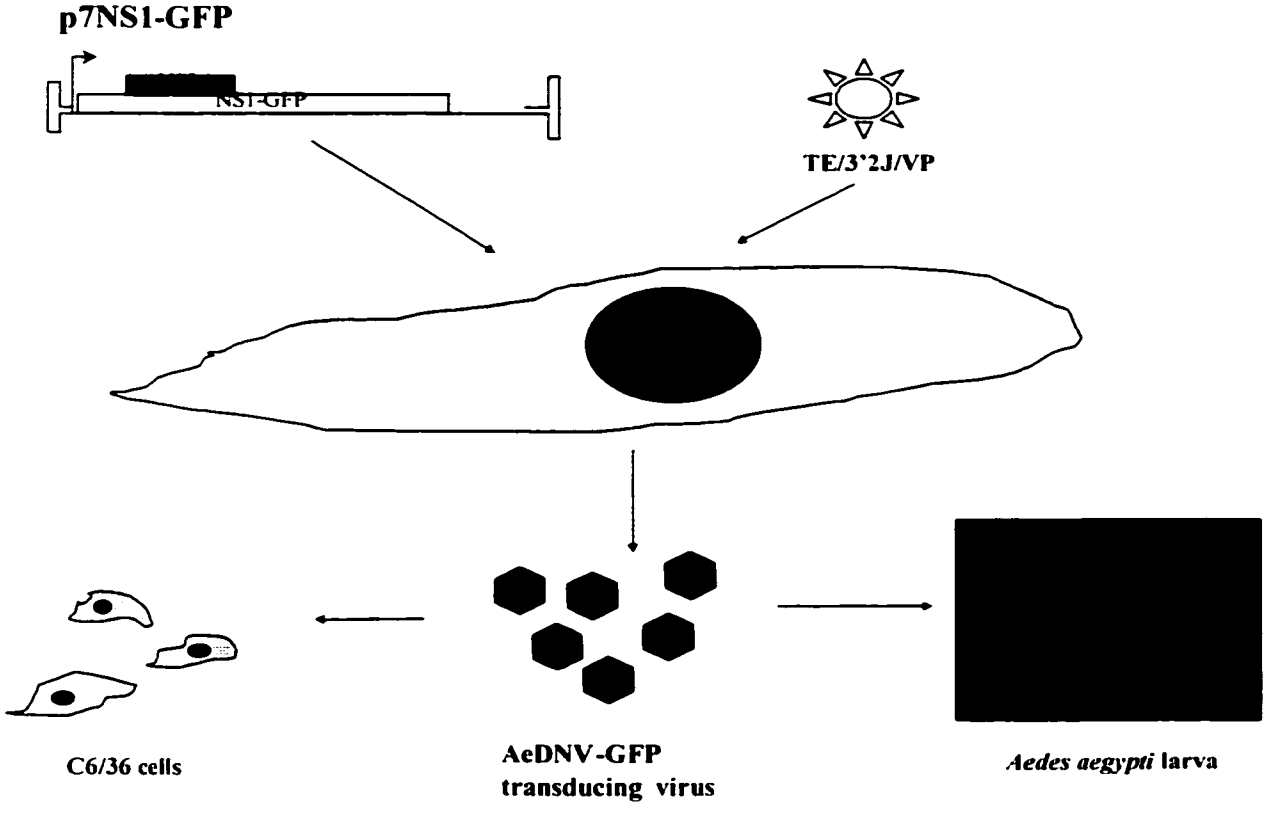


Figure 5.1. Protocol for producing AeDNV-GFP virus using the TE/3'2J/VP packaging system. C6/36 cells are transformed with the transducing genome plasmid, p7NS1-GFP, using Lipofectin Reagent (Life Technologies). The cells are then infected with the TE/3'2J/VP virus. AeDNV-GFP transducing virus is collected by ultracentrifugation and sonication, see Materials and Methods, and is then applied to C6/36 cells or *Ae. aegypti* larvae.

Ae. aegypti mosquitoes were maintained at 27°C and 80% humidity with a photoperiod of 12h light/12h dark.

Plasmid construction. Plasmids used in this study are depicted in Figure 5.2. All plasmids were constructed using standard cloning techniques (Sambrook *et al.*, 1989). p7NS1-GFP (Figure 5.2B) expresses an NS1-GFP fusion protein from the p7 promoter. The construction of p7NS1-GFP is described in detail elsewhere (Afanasiev *et al.*, 1999). pUCA (Figure 5.2C) is the infectious clone of AeDNV; its construction is described elsewhere (Afanasiev *et al.*, 1994).

Plasmid pTE/3'2J/VP (Figure 5.2A) is the dsSIN template for producing TE/3'2J/VP recombinant virus. It was constructed by PCR-amplification of the AeDNV VP gene sequence using primers with engineered XbaI sites flanking the VP sequence (forward primer: 5'-GCTCTAGAGCATGGCAGACAGCACTTCA-3'; reverse primer: 5'-GCTCTAGAGCTTAAGTTTTGATTTCATAC-3'). The PCR product was inserted into a TA cloning plasmid, pCR 2.1 (Invitrogen). The VP sequence was subsequently excised with XbaI and cloned into the XbaI site of pTE/3'2J, downstream of the second subgenomic promoter.

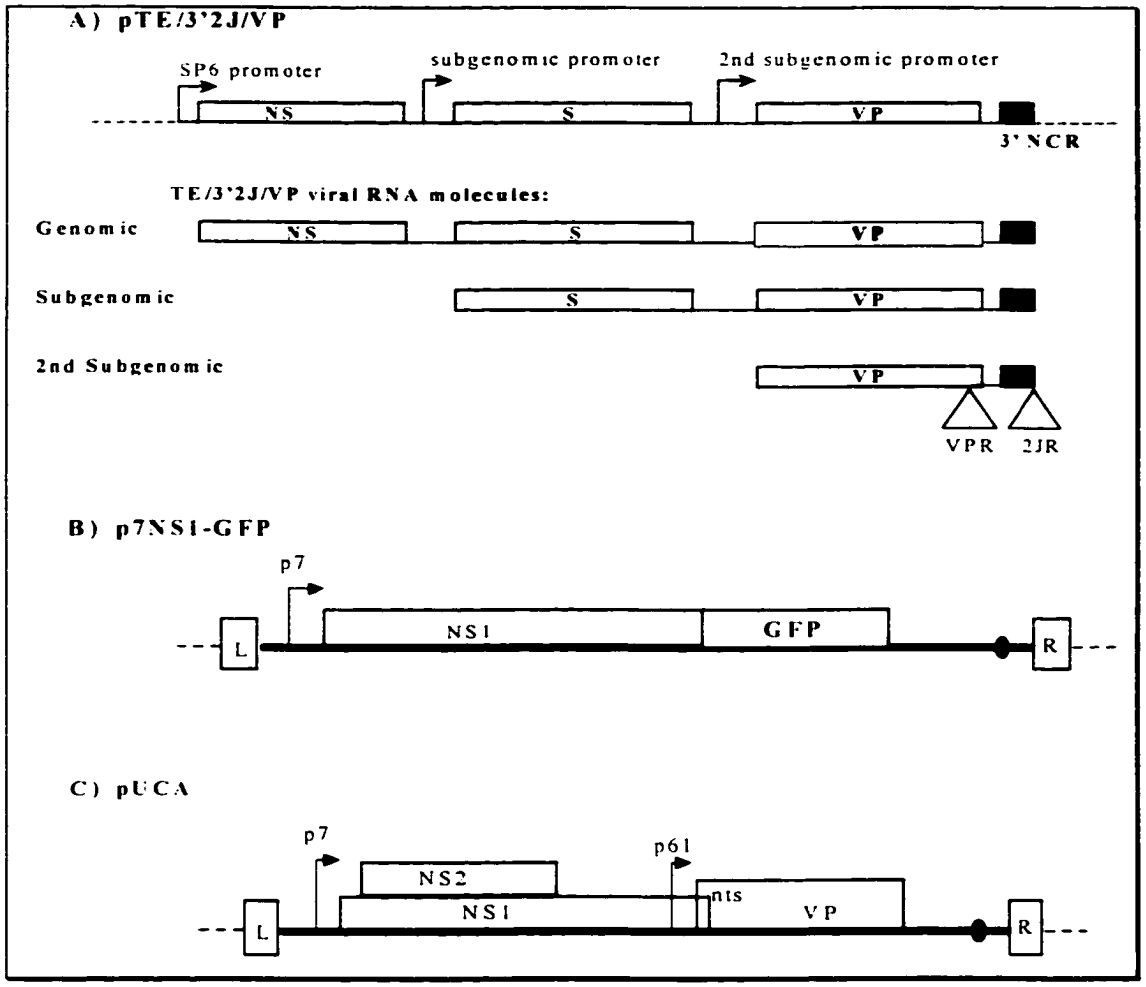


Figure 5.2. Plasmids used in this study. A) pTE/3'2J/VP, recombinant dsSIN virus template; NS, nonstructural genes; S, structural genes; VP, AeDNV structural protein genes; 3' NCR, non-coding region; TE/3'2J/VP viral RNA molecules present upon infection are indicated; Location of probes used in northern blot analysis are indicated: VPR, VP-specific probe; 2JR, NCR-specific probe. B) p7NS1-GFP, AeDNV-GFP transducing virus genome. C) pUCA, infectious clone of AeDNV; L, left hairpin sequence; R, right hairpin sequence; nts, nuclear targeting sequence. Blackened ovals represent polyadenylation signals. Promoters are depicted as arrows. SIN non-coding region is designated by a hatched box. Foreign sequences are within shaded rectangles. Dashed lines indicate plasmid sequences.

Production of recombinant Sindbis virus, TE/3'2J/VP. pTE/3'2J/VP was used as a template for SP6-catalyzed in vitro transcription as described elsewhere (Rice *et al.*, 1987). The resulting RNA was electroporated into BHK-21 cells, as previously described (Powers *et al.*, 1994). Virus stocks were titrated on BHK-21 cells, using serial dilution endpoints to calculate titers as $\log_{10}TCID_{50}$ per mL (Higgs *et al.*, 1993).

Northern blot analysis. C6/36 cells were infected with TE/3'2J/VP or TE/3'2J control virus at an moi of 1. The cells were harvested after 48h and RNA was extracted by a single-step guanidinium thiocyanate protocol (Chomczynski & Sacchi, 1987). RNA was fractionated on a formaldehyde-agarose gel and blotted onto a positively charged nylon membrane (Boehringer-Mannheim). The membrane was hybridized with an oligonucleotide, 2JR (5'-GCTGGTCGGATCATTGGGGCG), which is complementary to the SIN 3' NCR (Figure 5.2A). 2JR was labeled with [γ-^{32}P]ATP using polynucleotide kinase (Promega Corp.) according to the manufacturer's specifications. Hybridization conditions were 42°C, in 10% dextran sulfate, 1% SDS, 1M NaCl, and 200μg/ml sheared, denatured DNA, for 4h. The membrane was washed in 2X SSC/0.1% SDS twice at room temperature, followed by two 30min washes in 0.2X SSC/0.1% SDS at 50°C. The membrane was exposed to Fuji x-ray film for 2h.

The 2JR probe was removed from the membrane using an alkaline stripping solution (200mM NaOH, 0.1% SDS) for 20min at 37°C. The membrane was exposed to film overnight to ensure all of the probe had been removed. It was then re-hybridized with a ^{32}P-labeled oligonucleotide specific for the VP sequence (VP reverse primer described above), using hybridization conditions described above.

Indirect IFA. C6/36 cells were seeded onto glass coverslips in a 12-well plate and infected with TE/3'2J/VP at an moi of 2. Coverslips were rinsed in PBS and fixed in cold acetone 48h post-infection. Mouse anti-SIN E1 antibody was applied for 40min at 37°C to detect SIN infection. Immunofluorescent labeling was done using a biotin-streptavidin immunofluorescence technique (Gould *et al.*, 1985; Olson *et al.*, 1996). Coverslips were mounted on slides and examined using an Olympus BH-2 microscope. Cells were examined for VP expression by a similar protocol using rabbit anti-VP antibody. The same procedure was followed for BHK-21 cells, except coverslips were fixed at 12h post-infection.

Larval tissues were fixed on glass slides with cold acetone. An antibody to the SIN E1 glycoprotein was used to assay for SIN infection as described above for assay of cell culture, and a streptavidin-Texas red conjugate was used for detection.

Production of AeDNV-GFP transducing virus. C6/36 cells in a 75cm^2 flask at 70% confluence were transformed with p7NS1-GFP with or without pUCA helper plasmid using Lipofectin reagent (Life Technologies). A Lipofectin-DNA complex was made by incubating 30μg of plasmid DNA with 150μl of Lipofectin reagent in a total volume of 500μl of serum-free L-15 medium for 15min at room temperature. Cells were rinsed twice with 5ml of sterile PBS. The PBS rinse was removed and replaced with the Lipofectin-DNA complex in a total volume of 5ml. Following incubation for 8h at 28°C, the Lipofectin-DNA complex was replaced with L-15 medium containing 10% FBS and antibiotics. Cells were examined using an epifluorescent inverted microscope (Olympus) to detect GFP expression.

Cells were infected 20h post-transformation with TE/3'2J/VP virus at an moi of 10. Cells and transducing particles were pelleted 48h post-infection by centrifugation at 32,000rpm for 2h. Cells were lysed by sonication to release transducing virus which was applied to C6/36 cells that were 80% confluent. In experiment 2 (Table 5.1) the cell lysate was clarified by centrifugation at 1,500rpm for 5min prior to inoculation of C6/36 cells. C6/36 cells were incubated for 72h with transducing particles, and GFP expression was observed by fluorescent microscopy. Titer of AeDNV-GFP transducing virus was determined in C6/36 cells by counting transduced cells in randomly chosen microscopic fields.

Infection of *Ae. aegypti* larvae with AeDNV transducing particles was achieved by hatching larvae in flasks containing the whole cell lysate from transducing particle producer cells(Barreau *et al.*, 1994). The lysate was treated with anti-SIN neutralizing antibody for 2h at 4°C (Olson *et al.*, 1996) prior to larval infection. Larvae were transferred to pans with fresh water and were fed liver powder 48h after hatching. Larvae were then examined for GFP expression by fluorescent microscopy.

Results

Expression of AeDNV structural proteins from TE/3'2J/VP. A recombinant dsSIN construct was designed with the AeDNV structural protein genes downstream from the second subgenomic promoter (pTE/3'2J/VP, Figure 5.2A). This construct was used in an *in vitro* transcription reaction to generate RNA that was electroporated into BHK-21 cells to produce TE/3'2J/VP transducing virus. C6/36 cells were infected with either

TE/3'2J/VP or control TE/3'2J virus lacking the VP sequence behind the second subgenomic promoter, and RNA transcription was analyzed by northern blot analysis (Figure 5.3). Total cellular RNA was hybridized with a ^{32}P-labeled oligonucleotide complementary to the SIN 3' NCR (Figure 5.3A), which will bind the three viral RNA species: full-length genomic, subgenomic, and second subgenomic (Figure 5.2A). The RNA transcripts from the TE/3'2J/VP virus are expected to be 1.1kb larger than the corresponding RNA's from the empty TE/3'2J virus, based on the size of the inserted VP sequence. Two transcripts were detected in C6/36 cells infected with TE/3'2J/VP (Figure 5.3A, lane 3). The 5.8kb (subgenomic) transcript was the predicted size of the SIN structural genes, VP sequence, and the 3' NCR. The 1.7kb (second subgenomic) transcript was the predicted size of the VP sequence plus the 3' NCR. Two transcripts were detected in C6/36 cells infected with TE/3'2J virus (Figure 5.3A, lane 2). The first subgenomic transcript was approximately 4.7kb, indicative of the SIN structural genes and the 3' NCR. The second subgenomic transcript from TE/3'2J was 0.6kb, which is the predicted size of the 3' NCR. Full-length genomic bands were not detected in either sample. The RNA was extracted late in infection; therefore, genomic RNA was not expected to be in high abundance. No virus-specific transcripts were detected in uninfected C6/36 cells (Figure 5.3A, lane 1).

The 3' NCR-specific probe was removed from the membrane, and the membrane was re-hybridized with a ^{32}P-labeled oligonucleotide (VPR; Figure 5.2A) specific for the VP sequence (Figure 5.3B). This probe bound to the 5.8kb (subgenomic) and the 1.7kb (second subgenomic) transcripts in C6/36 cells infected with TE/3'2J/VP (Figure 5.3B,

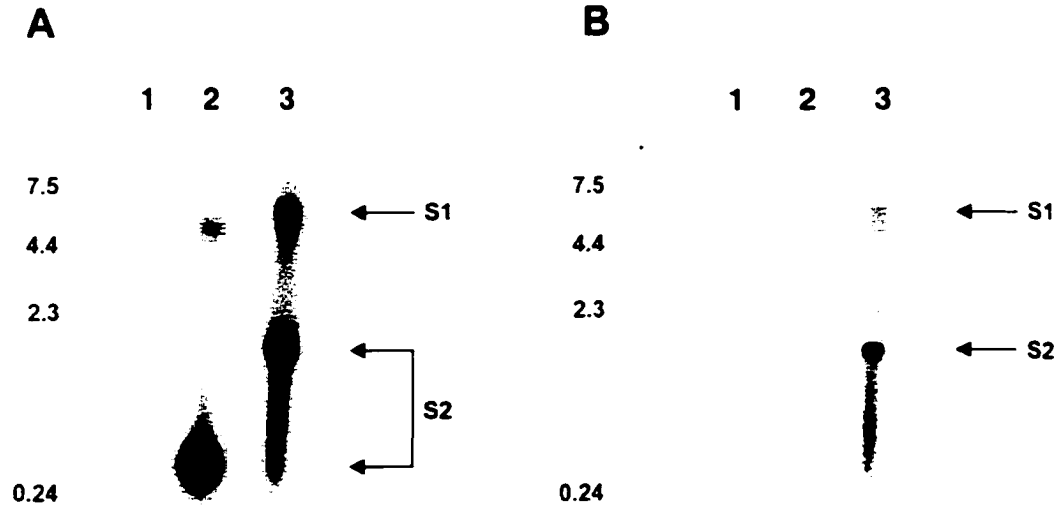


Figure 5.3. Northern analysis of VP RNA expression from TE/3'2J/VP-infected C6/36 cells. Total cellular RNA from (1) uninfected, (2) TE/3'2J-infected, or (3) TE/3'2J/VP-infected cells was blotted as described in Materials and Methods, and probed with a A.) 3'NCR-specific, or B.) VP-specific radiolabeled probe. Molecular weight markers in kilobases are at left. S1=subgenomic RNA; S2= second subgenomic RNA.

lane 3). No VP-specific bands were detected in C6/36 cells infected with control TE/3'2J virus or in uninfected cells (Figure 5.3B, lanes 1 and 2).

C6/36 cells and BHK-21 cells were infected with TE/3'2J/VP, and virus encoded protein expression was analyzed by indirect IFA. TE/3'2J/VP infection was confirmed using an antibody specific to the SIN E1 envelope glycoprotein (data not shown). A VP-specific antibody was used to detect AeDNV structural proteins in C6/36 and BHK-21 cells infected with TE/3'2J/VP (Figure 5.4). VPs were localized in the nucleus in C6/36 cells (Figure 5.4B). VPs were limited to the cytoplasm, however, in BHK-21 cells (Figure 5.4D). No fluorescence was detected in uninfected C6/36 cells (Figure 5.4A) or in uninfected BHK-21 cells (Figure 5.4C).

Transduction of C6/36 cells and *Aedes aegypti* mosquitoes with particles produced by the TE/3'2J/VP virus packaging system. The transducing genome p7NS1-GFP (Figure 5.2B) was used to assess the ability of TE/3'2J/VP virus to provide structural proteins for transducing virus packaging. p7NS1-GFP encodes an NS1-GFP fusion protein that retains the functions of the NS1 protein; thus NS1 is able to mediate excision of the transducing genome from p7NS1-GFP and subsequent replication in transformed cells (Afanasiev *et al.*, 1999).

AeDNV-GFP transducing virus was generated by transformation of C6/36 cells with p7NS1-GFP, followed by infection with the TE/3'2J/VP virus. AeDNV-GFP transducing particles were collected and applied to C6/36 cells, which were shown to express GFP (Figure 5.5A). AeDNV-GFP was also generated by co-expression of p7NS1-GFP and pUCA, an infectious clone of AeDNV. Titers of AeDNV-GFP virus generated by the two methods were not significantly different (Table 5.1). Lysate from

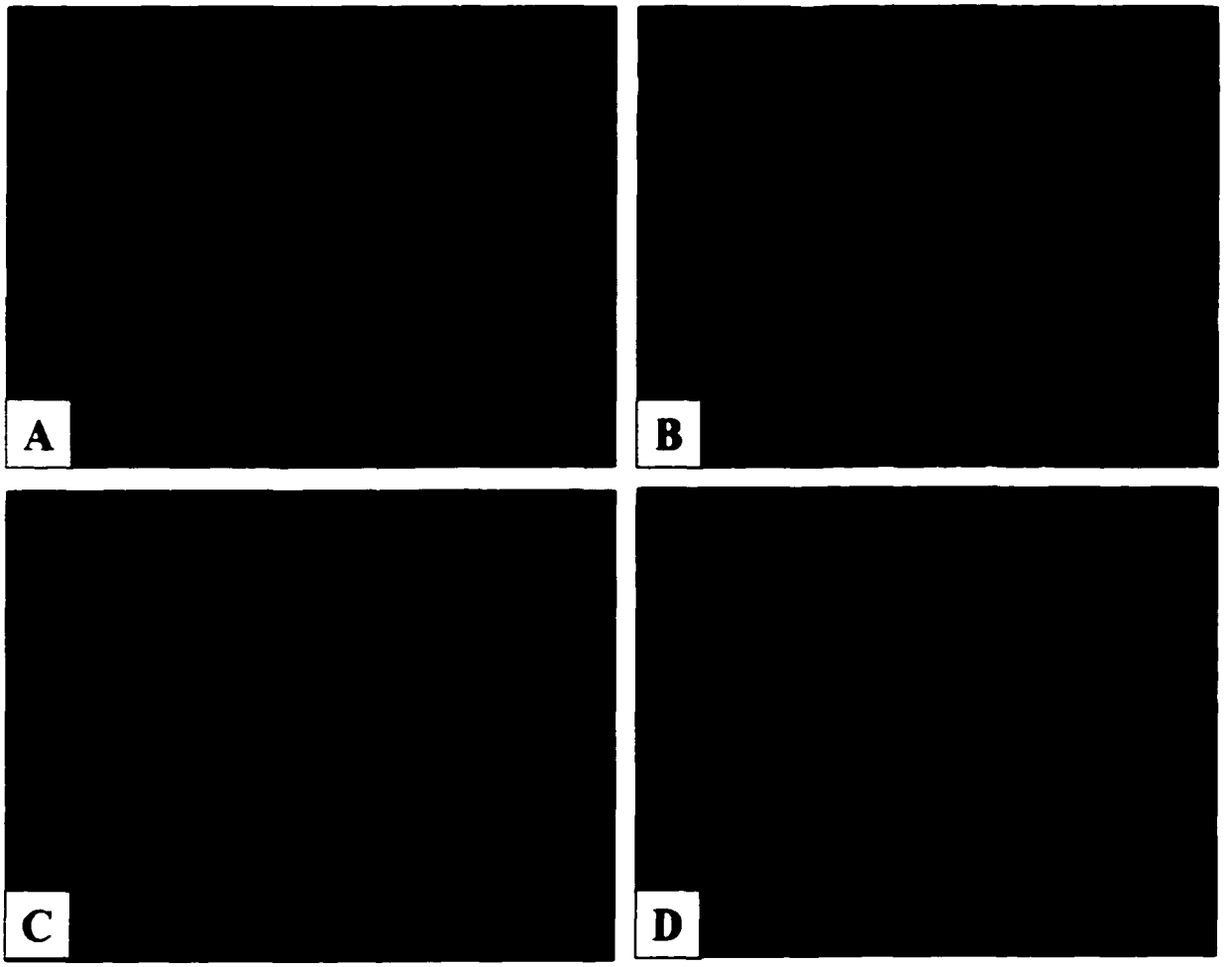


Figure 5.4. Detection of VPs expression by indirect IFA. Uninfected and TE/3'2J/VP-infected cells were assayed using a VPs-specific antibody. A.) uninfected C6/36 cells; B.) C6/36 cells + TE/3'2J/VP; C.) uninfected BHK-21 cells; D.) BHK-21 cells + TE/3'2J/VP.

producer cells was used to inoculate C6/36 cells in the first experiment. Cell debris from the producer cells was removed prior to inoculation of C6/36 cells in the second experiment. While transducing virus titers differed between the two experiments, little difference was seen in titers generated by the TE/3'2J/VP packaging method as compared to the two plasmid method. No transducing particles were detected when C6/36 cells were infected with TE/3'2J/VP virus prior to transformation with p7NS1-GFP.

TABLE 5.1

COMPARISON OF TITERS[a] OF AeDNV-GFP GENERATED BY TWO PLASMID SYSTEM AND TE/3'2J/VP PACKAGING SYSTEM

	Experiment: 1[b]	2[c]
p7NS1-GFP + pUCA	6.5×10^3	1.1×10^4
p7NS1-GFP + TE/3'2J/VP	7.1×10^3	1.1×10^4

[a]Titers are expressed as transducing particles per milliliter. AeDNV-GFP virus was generated by co-expression of the transducing genome, p7NS1-GFP, and helper plasmid pUCA, or by cellular expression of p7NS1-GFP followed by infection with TE/3'2J/VP virus, which supplies structural proteins for transducing genome packaging. AeDNV-GFP was applied to C6/36 cells at 80% confluence. Cells expressing GFP were counted to determine virus titer.
[b]AeDNV-GFP was collected by sonication of producer cells.
[c]AeDNV-GFP was collected by sonication of producer cells followed by pelleting of cell debris at 1,500 rpm for 5min.

Figure 5.5. Transduction of (A) C6/36 cells, and (B) *Ae. aegypti* larva with AeDNV-GFP transducing virus generated by the dsSIN packaging system. Larva at 100X magnification showing GFP expression in anal papillae.

Ae. aegypti larvae were also transduced by AeDNV-GFP virus packaged by structural proteins produced from TE/3'2J/VP. GFP expression was most frequently localized to the anal papillae of transduced larvae (Figure 5.5B), similar to infection with particles generated by the two-plasmid system (Afanasiev *et al.*, 1999). GFP expression was evident in the nuclei of the syncytial cells of the anal papillae. *Ae. aegypti* larvae that had been exposed to transducing virus were examined by indirect IFA for SIN infection using an antibody to the SIN E1 envelope glycoprotein. No larvae were found to be positive for SIN infection (data not shown).

Discussion

An AeDNV transducing virus packaging system based on a recombinant dsSIN virus was developed to remove wild-type virus contamination from transducing virus stocks. Infectious AeDNV-GFP transducing particles are produced when TE/3'2J/VP virus is applied to cells expressing p7NS1-GFP, as demonstrated by GFP expression in transduced C6/36 cells and *Ae. aegypti* larvae. The TE/3'2J/VP packaging system is currently being optimized to obtain higher concentrations of transducing virus.

Corsini *et al.* (Corsini *et al.*, 1996) used a SIN replicon to provide essential genes to package a LuIII (rodent parvovirus) transducing genome expressing luciferase. The NS1 protein was supplied by a SIN replicon, and the packagable LuIII-luciferase transducing genome and structural proteins were provided as separate plasmids. Transducing particles were produced using this system; however, the efficiency was much lower than the standard plasmid-based system. The use of an unpackaged SIN

replicon in their study may have contributed to the low efficiency of transduction. Because a packaged dsSIN expression system was used to supply parvovirus proteins in *trans*, the problem of co-electroporation of DNA and RNA constructs has been eliminated, and the resulting efficiency of transduction is comparable to the standard method of recombinant AeDNV production.

VPs expressed from TE/3'2J/VP are localized in the nucleus in invertebrate cells as demonstrated by indirect IFA. There is a putative nuclear targeting sequence in the amino terminus of the VP1 protein (Figure 5.2C). The lysine/arginine motif is similar to the nuclear localization signals of the SV40 large T antigen and of the polyhedrin of *Autographa californica* nuclear polyhedrosis virus (Afanasiev *et al.*, 1994). This sequence allows for transport of the VPs to the nucleus during productive infection with wild-type virus. Transport of reporter proteins to the nucleus is observed when a reporter gene is fused to the amino terminus of VP1. This has been shown using β-galactosidase (Afanasiev *et al.*, 1994) or GFP (Afanasiev *et al.*, 1999) as a reporter gene. The VPs remained in the cytoplasm when vertebrate cells were infected with TE/3'2J/VP virus. Thus the putative nuclear targeting sequence of AeDNV structural proteins is not recognized in BHK-21 cells. It is unknown whether this phenomenon is seen with other vertebrate cell lines. The lack of recognition of the putative nuclear targeting sequence in vertebrate cells was unexpected, because vertebrate parvoviruses have similar lysine/arginine motifs, which may be involved in nuclear localization (Tullis *et al.*, 1993).

Expression of VPs from a recombinant dsSIN virus eliminates the critical problem of recombination resulting in wild-type virus. The current method for generating recombinant AeDNV viruses involves co-expression of transducing genome

and helper plasmids. The transducing genome plasmid contains the gene of interest and the sequences required for replication and excision of the cloned sequence. The helper plasmid supplies proteins essential for virus packaging in *trans*. Recombination between transducing genome and helper plasmids occurs, producing wild-type virus (Afanasiev *et al.*, 1999; Brandenburger & Russell, 1996; Maxwell *et al.*, 1993).

The problem of wild-type virus contamination of transducing particle stocks is not unique to parvoviruses, and other DNA virus gene expression systems could benefit from this work. The possibility of recombination is eliminated by supplying essential genes from a recombinant RNA virus; therefore transducing virus stocks are without wild-type virus contamination. This is especially significant when considering the use of a virus expression system in gene therapy of humans (Brandenburger & Russell, 1996; Corsini, *et al.*, 1996).

The packaging system described here may also be useful as a tool to investigate the biology of AeDNV. It appears that the anal papillae are organs of primary AeDNV infection, because the occurrence of wild-type virus has been eliminated and TE/3'2J/VP virus is not present in transduced larvae. The possibility that anal papillae are infected by secondary spread of transducing virus is unlikely. Anal papillae are involved in ion balance regulation and have direct contact with the hemolymph (Wigglesworth, 1938). Thus infection of anal papillae may play an important role in viral spread and pathogenesis.

CHAPTER 6: SUMMARY AND DISCUSSION

The current resurgence of mosquito-borne diseases is a reminder of the failed attempts at controlling mosquito vectors and the diseases they transmit. Novel control strategies involving genetic manipulation of vector populations are being evaluated as alternative ways to control vector-borne diseases. Genetic modification of mosquitoes resulting in reduced vector competence may prevent transmission of mosquito-borne pathogens to humans.

The goal of this research is to design genetic constructs capable of interfering with LAC virus replication in mosquito cell culture. Knowledge gained from this work will be used in the design of strategies for interfering with virus transmission from mosquitoes to humans. The LAC virus/*Ae. triseriatus* model system is advantageous for these studies because transovarial transmission permits maintenance of the virus in a laboratory population of mosquitoes. Transovarial transmission of LAC virus will insure that most of the mosquitoes in a caged population are infected prior to introduction of transgenic mosquitoes carrying the effector genes. This will permit the study of the movement and efficacy of antiviral genes in a simulated natural population of infected mosquitoes.

Previous studies have demonstrated that a full length antisense RNA to the S segment of LAC is efficacious at interfering with viral replication when expressed from a dsSIN expression system. While SIN virus-based expression systems are invaluable in the evaluation of antiviral sequences, they are not practical for long-

term expression in mosquito populations. Evaluation of DNA-based expression of antiviral constructs is necessary before transgenic mosquitoes with reduced vector competence can be designed. To accomplish this, DNA constructs were evaluated in cell culture, and the packaging of AeDNV transducing viruses was evaluated for expression in live mosquitoes.

The first part of this project involved the expression of LAC S segment RNAs in sense and antisense orientations, using Mtn and hsp70 promoters from *Drosophila*. The Mtn promoter is inducible by the addition of heavy metals, but expression is also seen in uninduced cells. The hsp70 promoter is inducible by increasing the incubation temperature of *Drosophila* cells; however it is not inducible by heat shock in C6/36 cells (Monroe, 1990). An inducible promoter with very tight regulation would be useful in expressing heterologous sequences in a controllable manner. Unfortunately, neither of the promoters used in this study fit this need. Interference was seen in cell lines that expressed the antisense sequence from the Mtn promoter; however, it was not reproducible. Antisense RNA synthesis was only detected in approximately 30% of cells in the most inducible line. If interference occurred in the cells actively expressing the antisense RNA, and non-expressing cells permitted LAC replication, interference may not be detected when sampling the cell supernatant. A system to evaluate interference on a single cell basis is needed to examine possible interference in a non-homogeneous population of cells. FISH analysis detecting antisense effector and target molecules simultaneously, or a combined FISH/IFA system would be helpful in evaluating interference on a single cell basis.

The second part of this project involved designing a DNA-based SIN replicon expression system for expressing antisense RNA in mosquito cells. This system provides for increased RNA synthesis in the cytoplasm of expressing cells, as compared to standard plasmid expression systems. Theoretically, a DNA-based replicon could increase the amount of antisense RNA being synthesized and insure the effector RNA was present in the cytoplasm of transformed cells. An increase in RNA production was seen using the replicon construct as compared to our highest expressing metallothionein-based cell line. Interference was detected in cell lines transformed with the replicon construct, in a dose-dependent manner. When cells were challenged with a dose of virus comparable to what a mosquito would receive in nature, a 1,000-10,000 fold decrease in LAC virus replication was seen. If viral replication can be significantly inhibited in a mosquito vector, the dose it delivers to a human may be too low to result in infection.

It is not clear if interference in this system is due to classical antisense effects, or is a result of dsRNA, which is inherent in the replication strategy of SIN virus. It has been shown in a number of systems that dsRNA is more efficient at interfering with gene expression than sense or antisense RNA alone (Fire, *et al.*, 1998; Kennerdell & Carthew, 1998; Montgomery & Fire, 1998; Ngo, *et al.*, 1998; Waterhouse, *et al.*, 1998). dsRNA has also been proposed to signal PTGS, leading to sequence-specific degradation of mRNA (Montgomery & Fire, 1998). A dsRNA molecule with LAC sequence specificity may be equally efficient at inhibiting LAC virus replication. A plasmid designed to make both strands capable of folding into a dsRNA molecule could be designed to test the hypothesis that dsRNA is the mediator

of LAC inhibition in mosquito cells expressing SIN-based antisense constructs, rather than classical antisense effects.

A human antiviral peptide, MxA, was evaluated for interference with LAC virus when expressed in mosquito cells. MxA effectively interferes with LAC virus replication in mammalian systems. It has also proven to be effective when expressed in mice lacking the interferon α/β response pathway. Human MxA was expressed from a baculovirus IE1 promoter in mosquito cells. Significant interference was seen with LAC virus in MxA expressing cells (Figures 4.3, 4.4, and Table 4.1). Most of the viruses susceptible to MxA inhibition are negative sense RNA viruses (Haller *et al.*, 1998), however, a positive sense RNA virus, Semliki Forest virus, is inhibited by MxA (Landis *et al.*, 1998). Because SIN and Semliki Forest virus are genetically related, it was proposed that MxA might also inhibit replication of a recombinant SIN virus. However interference was not observed when MxA expressing cells were challenged with a recombinant dsSIN virus (Figures 4.3, 4.4, and Table 4.2). Because MxA does not appear to inhibit expression from a dsSIN expression system, a SIN replicon expressing MxA could be designed to quickly evaluate interference potential with other arboviruses. If other arboviruses are inhibited by MxA in mosquito cells, MxA would be a good candidate to express in transgenic mosquitoes for broad-spectrum arboviral interference.

Finally, a dsSIN virus was used to express structural proteins for packaging of recombinant AeDNV virus, which is an alternate DNA-based system for the expression of heterologous genes in mosquitoes. The plasmid-based method of making transducing densoviruses results in genetic recombination and production of

wild type infectious virus. A dsSIN virus was used to supply densovirus structural proteins in order to package recombinant densovirus without wild-type virus contamination. This system was evaluated using a GFP-expressing transducing virus, and was compared to the plasmid-based system. Comparable production of recombinant densovirus was seen with the two systems. Transducing densoviruses are useful in the study of infection and pathogenesis in live mosquitoes and in designing a virus-based larvicide for mosquito control. One of the obstacles in using AeDNV as a mosquito control agent is the low efficiency of generating infectious virus. The dsSIN system may be exploited to make large quantities of AeDNV structural proteins to increase production of transducing virus. Current studies investigating how virus particles are assembled will hopefully aid in the design of a more effective packaging system.

These results suggest that high levels of antisense RNA, dsRNA, and MxA expression from effective DNA-based constructs have potential for interfering with LAC virus replication in mosquito cell culture. Expression of these antiviral sequences in epidemiologically significant organs (midgut, ovaries, salivary glands) of mosquitoes may be effective at preventing LAC virus transmission to humans. These results can also be used to design genetic control strategies for other arboviruses. Genetic control strategies designed to interfere with transmission of arboviruses to humans may be the key to reversing the resurgence of mosquito-borne diseases.

REFERENCES

Afanasiev, B. N., Galyov, E. E., Buchatsky, L. P. & Kozlov, Y. V. (1991). Nucleotide sequence and genomic organization of *Aedes* densonucleosis virus. *Virology* **185**, 323-336.

Afanasiev, B. N., Kozlov, Y. V., Carson, J. O. & Beaty, B. J. (1994). Densovirus of *Aedes aegypti* as an expression vector in mosquito cells. *Experimental Parasitology* **79**, 322-339.

Afanasiev, B. N., Ward, T. W., Beaty, B. J. & Carlson, J. O. (1999). Transduction of *Aedes aegypti* mosquitoes with vectors derived from *Aedes* densovirus. *Virology* **257**, 62-72.

Andersson, A. M., Melin, L., Bean, A. & Pettersson, R. F. (1997). A retention signal necessary and sufficient for golgi localization maps to the cytoplasmic tail of a Bunyaviridae (Uukuniemi virus) membrane glycoprotein. *Journal of Virology* **71**, 4717-4727.

Arnheiter, H., Frese, M., Kambadur, R., Meier, E. & Haller, O. (1995). Mx transgenic mice-animal models of health. *Current Topics in Microbiology and Immunology* **206**, 119-147.

Baltimore, D. (1988). Intracellular immunization. *Nature* **335**, 395-396.

Barreau, C., Jousset, F.-X. & Bergoin, M. (1997). Venereal and vertical transmission of the *Aedes albopictus* parvovirus in *Aedes aegypti* mosquitoes. *American Journal of Tropical Medicine and Hygiene* **57**, 126-131.

Barreau, C., Jousset, F.-X. & Cornet, M. (1994). An efficient and easy method of infection of mosquito larvae from virus-contaminated cell cultures. *Journal of Virological Methods* **49**, 153-156.

Baulcombe, D. (1999). Viruses and gene silencing in plants. *Archives of Virology* **Supplementum 15**, 189-201.

Baulcombe, D. C. (1999). Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology* **2**, 109-113.

Beaty, B. J. & Bishop, D. H. L. (1988). Bunyavirus-vector interactions. *Virus Research* **10**, 289-302.

Beaty, B. J., Bishop, D. H. L., Gay, M. & Fuller, F. (1983). Interference between Bunyaviruses in *Aedes triseriatus* mosquitoes. *Virology* **127**, 83-90.

Beaty, B. J., Calisher, C. H. & Shope, R. E. (1989). Arboviruses. In *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, pp. 797-855. Edited by N. J. Schmidt & R. W. Emmons. Washington D.C.: American Public Health Association, Inc.

Beaty, B. J., Rozhon, E. J., Gensemer, P. & Bishop, D. H. L. (1981). Formation of reassortant bunyaviruses in dually-infected mosquitoes. *Virology* **111**, 662-665.

Beaty, B. J. & Thompson, W. H. (1975). Emergence of LaCrosse virus from endemic foci: Fluorescent antibody studies of overwintered *Aedes triseriatus*. *The American Journal of Tropical Medicine and Hygiene* **24**, 685-691.

Bell, A. F., Whitton, J. L. & Fujinami, R. S. (1997). Antisense-mediated resistance to measles virus infection in HeLa cells. *The Journal of Infectious Diseases* **176**, 258-261.

Besansky, N. J. & Collins, F. H. (1992). The mosquito genome: organization, evolution and manipulation. *Parasitology Today* **8**, 186-192.

Bishop, D. H. L. (1979). Genetic potential of bunyaviruses. *Current Topics in Microbiology and immunology* **86**, 1-33.

Bishop, D. H. L., Calisher, C. H., Casals, J., Chumakov, M. P., Gaidamovich, S. Y., Hannoun, C., Lvov, D. K., Marshall, I. D., Oker-Blom, N., Pettersson, R. F., Porterfield, J. S., Russell, P. K., Shope, R. E. & Westaway, E. G. (1980). Bunyaviridae. *Intervirology* **14**, 125-143.

Boivin, A. & Dura, J.-M. (1998). *In vivo* chromatin accessibility correlates with gene silencing in *Drosophila*. *Genetics* **150**, 1539-1549.

Bout, A. (1996). Prospects for human gene therapy. *European Journal of Drug Metabolism and Pharmacokinetics* **21**, 175-179.

Branch, A. D. (1996). A hitchhiker's guide to antisense and nonantisense biochemical pathways. *Hepatology* **24**, 1517-1529.

Brandenburger, A. & Russell, S. (1996). A novel packaging system for the generation of helper-free oncolytic MVM vector stocks. *Gene Therapy* **3**, 927-931.

Bredenbeek, P. J., Frolov, I., Rice, C. M. & Schlesinger, S. (1993). Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *Journal of Virology* **67**, 6439-6446.

Brogdon, W. G. & McAllister, J. C. (1998). Insecticide resistance and vector control. *Emerging Infectious Diseases* **4**, 605-613.

Buchatsky, L. P. (1989). Densonucleosis of bloodsucking mosquitoes. *Diseases of Aquatic Organisms* **6**, 145-150.

Bunch, T. A. & Goldstein, L. S. B. (1989). The conditional inhibition of gene expression in cultured *Drosophila* cells by antisense RNA. *Nucleic Acids Research* **17**, 9761-9781.

Cabradilla, C. D., Holloway, B. P. & Obijeski, J. F. (1983). Molecular cloning and sequencing of the LaCrosse virus S RNA. *Virology* **128**, 363-368.

Calisher, C. H. (1994). Medically important arboviruses of the United States and Canada. *Clinical Microbiology Reviews* **7**, 89-116.

Carbonell, L. F., Klowden, M. J. & Miller, L. K. (1985). Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells. *Journal of Virology* **56**, 153-160.

Carlson, J., Olson, K., Higgs, S. & Beaty, B. (1995). Molecular genetic manipulation of mosquito vectors. *Annual Reviews on Entomology* **40**, 359-388.

Carlson, J. O., Afanasiev, B. N., Higgs, S., Matsubara, T. & Burns, J. C. (1996). Viral transducing vectors for mosquitoes. In *Molecular biology of insect disease vectors: a methods manual*, pp. 444-458. Edited by J. M. Crampton, C. B. Beard & C. Louis. London: Chapman and Hall.

Chandler, L. J., Beaty, B. J., Baldridge, G. D., Bishop, D. H. L. & Hewlett, M. J. (1990). Heterologous reassortment of bunyaviruses in *Aedes triseriatus* mosquitoes and transovarial and oral transmission of newly evolved genotypes. *Journal of General Virology* **71**, 1045-1050.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156-159.

Coates, C. J., Jasinskiene, N., Miyashiro, L. & James, A. A. (1998). *Mariner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proceedings of the National Academy of Science* **95**, 3748-3751.

Coates, C. J., Jasinskiene, N., Pott, G. B. & James, A. A. (1999). Promoter-directed expression of recombinant fire-fly luciferase in the salivary glands of *Hermes*-transformed *Aedes aegypti*. *Gene* **226**, 317-325.

Collins, F. H. & James, A. A. (1996). Genetic modification of mosquitoes. *Science and Medicine* **3**, 52-61.

Corsini, J., Afanasiev, B., Maxwell, I. H. & Carlson, J. O. (1996). Autonomous parvovirus and densovirus gene vectors. *Advances in Virus Research* **47**, 303-351.

Corsini, J., Maxwell, I. H., Maxwell, F. & Carlson, J. O. (1996). Expression of parvovirus LuIII NS1 freom a Sindbis replicon for production of LuIII-luciferase transducing particles. *Virus Research* **46**, 95-104.

Crampton, J., Morris, A., Lycett, G., Warren, A. & Eggleston, P. (1990). Transgenic mosquitoes: A future vector control strategy? *Parasitology Today* **6**, 31-36.

Cully, J. F. J., Heard, P. B., Wesson, D. M. & Craig, G. B. J. (1991). Antibodies to LaCrosse virus in eastern chipmunks in Indiana near an *Aedes albopictus* population. *Journal of the American Mosquito Control Association* **7**, 651-653.

Cully, J. F. J., Streit, T. G. & Heard, P. B. (1992). Transmission of LaCrosse virus by four strains of *Aedes albopictus* to and from the eastern chipmunk (*Tamias striatus*). *Journal of the American Mosquito Control Association* **8**, 237-240.

de Haan, P., Gielen, J. J. L., Prins, M., Wijkamp, I. G., van Schepen, A., Peters, D., van Grinsven, M. Q. J. M. & Goldbach, R. (1992). Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. *BioTechnology* **10**, 1133-1137.

Dolnick, B. J. (1997). Naturally occurring antisense RNA. *Pharmacology and Therapeutics* **75**, 179-184.

Driver, D. A., Latham, E. M., Polo, J. M., Belli, B. A., Banks, T. A., Chada, S., Brumm, D., Chang, S. M. W., Mento, S. H., Jolly, D. J. & Dubensky Jr., T. W. (1995). Layered amplification of gene expression with a DNA gene delivery system. *Annals of the New York Academy of Sciences* **772**, 261-261.

Dubensky, T. W., Jr., Driver, D. A., Polo, J. M., Belli, B. A., Latham, E. M., Ibanez, C. E., Chada, S., Brumm, D., Banks, T. A., Mento, S. J., Jolly, D. J. & Chang, S. M. W. (1996). Sindbis virus DNA-based expression vectors: Utility for *in vitro* and *in vivo* gene transfer. *Journal of Virology* **70**, 508-519.

Elliott, R. M. (1990). Molecular biology of the Bunyaviridae. *Journal of General Virology* **71**, 501-522.

English, J. J., Mueller, E. & Baulcombe, D. C. (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *The Plant Cell* **8**, 179-188.

Fallon, A. M. (1996). Transgenic insect cells: mosquito cell mutants and the dihydrofolate reductase gene. *Cytotechnology* **20**, 23-31.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Francy, D. B., Karabatsos, N., Wesson, D. M., C.G. Moore, J., Lazuick, J. S., Niebylski, M. S., Tsai, T. F. & G.B. Craig, J. (1990). A new arbovirus from *Aedes albopictus*, an Asian mosquito established in the United States. *Science* **250**, 1738-1740.

Frese, M., Kochs, G., Feldmann, H., Hertkorn, C. & Haller, O. (1996). Inhibition of bunyaviruses, phleboviruses and hantaviruses by human MxA protein. *Journal of Virology* **70**, 915-923.

Gaines, P. J., Olson, K. E., Higgs, S., Powers, A. M., Beaty, B. J. & Blair, C. D. (1996). Pathogen-derived resistance to dengue type 2 virus in mosquito cells by expression of the prememrane coding region of the viral genome. *Journal of Virology* **70**, 2132-2137.

Giraud, C., Devauchelle, G. & Bergoin, M. (1992). The densovirus of *Junonia coenia* (Jc DNV) as an insect cell expression vector. *Virology* **186**, 207-218.

Gonzalez-Scarano, F. & Nathanson, N. (1996). Bunyaviridae. In *Fields Virology*, pp. 1473-1504. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott-Raven Publishers.

Gould, E. A., Buckley, A. & Cammack, N. (1985). Use of the biotin streptavidin interaction to improve flavivirus detection by immunofluorescence and ELISA tests. *Journal of Virological Methods* **11**, 41-48.

Grady, L. J., Sanders, M. L. & Campbell, W. P. (1987). The sequence of the M RNA of an isolate of LaCrosse virus. *Journal of General Virology* **68**, 3057-3071.

Grant, S. R. (1999). Dissecting the mechanisms of posttranscriptional gene silencing: Divide and conquer. *Cell* **96**, 303-306.

Guarino, L. a. & Dong, W. (1991). Expression of an enhancer-binding protein in insect cells transfected with the *Autographa californica* nuclear polyhedrosis virus IE1 gene. *Journal of Virology* **65**, 3676-3680.

Gubler, D. J. (1998). Resurgent vector-borne diseases as a global health problem. *Emerging Infectious Diseases* **4**, 442-450.

Hacker, J. K. & Hardy, J. L. (1997). Adsorptive endocytosis of California encephalitis virus into mosquito and mammalian cells: A role for G1. *Virology* **235**, 40-47.

Hahn, C. S., Hahn, Y. S., Braciale, T. J. & Rice, C. M. (1992). Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. *Proceedings of the National Academy of Sciences, USA* **89**, 2679-2683.

Haller, O., Frese, M. & Kochs, G. (1998). Mx proteins: mediators of innate resistance to RNA viruses. *Revue Scientifique et Technique de l'Office International des Epizooties* **17**, 220-230.

Hamilton, A. J. & Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952.

Hefti, H. P., Frese, M., Landis, H., Paolo, C. D., Aguzzi, A., Haller, O. & Pavlovic, J. (1999). Human MxA protein protects mice lacking a functional alpha/beta interferon system against LaCrosse virus and other lethal viral infections. *Journal of Virology* **73**, 6984-6991.

Hegadus, D. D., Pfeifer, T. A., Hendry, J., Theilmann, D. A. & Grigliatti, T. A. (1998). A series of broad host range shuttle vectors for constitutive and inducible expression of heterologous proteins in insect cell lines. *Gene* **207**, 241-249.

Helene, C. & Toulme, J.-J. (1990). Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochimica et Biophysica Acta* **1049**, 99-125.

Hermonat, P. L. & Muzyczka, N. (1984). Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammaliam tissue culture cells. *Proceedings of the National Academy of Sciences, USA* **81**, 6466-6470.

Herweijer, H., Latendresse, J. S., Williams, P., Zhang, G., Danko, I., Schlesinger, S. & Wolff, J. A. (1995). A plasmid-based self-amplifying Sindbis virus vector. *Human Gene Therapy* **6**, 1161-1167.

Higgs, S., Powers, A. M. & Olson, K. E. (1993). Alphavirus expression systems: applications to mosquito vector studies. *Parasitology Today* **9**, 444-452.

Higgs, S., Rayner, J. O., Olson, K. E., Davis, B. S., Beaty, B. J. & Blair, C. D. (1998). Engineered resistance in *Aedes aegypti* to a West African and a South American strain of yellow fever virus. *American Journal of Tropical Medicine and Hygiene* **58**, 663-670.

Horisberger, M. A. (1992). Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins. *Journal of Virology* **66**, 4705-4709.

Inokuchi, Y. & Hirashima, A. (1987). Interference with viral infection by defective RNA replicase. *Journal of Virology* **61**, 3946-3949.

Izant, J. G. & Weintraub, H. (1984). Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis. *Cell* **36**, 1007-1015.

Jasinskiene, N., Coates, C. J., Benedict, M. Q., Cornel, A. J., Rafferty, C. S., James, A. A. & Collins, F. H. (1998). Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. *Proceedings of the National Academy of Science* **95**, 3743-3747.

Johansen, H., van der Straten, A., Sweet, R., Otto, E., Maroni, G. & Rosenberg, M. (1989). Regulated expression at high copy number allows production of a growth-inhibitory oncogene product in *Drosophila* Schneider cells. *Genes and Development* **3**, 882-889.

Johnson, R. T. (1982). Chapter 5: Meningitis, Encephalitis, and Poliomyelitis. In *Viral Infections of the Nervous System*, pp. 87-128 (115-116). New York: Raven Press.

Karabatsos, N. (1985). International catalogue of arboviruses and certain other viruses of vertebrates. In *American Society of Tropical Medicine and Hygiene*. San Antonio, Texas.

Kennerdell, J. R. & Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* **95**, 1017-1026.

Kim, S. K. & Wold, B. J. (1985). Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA. *Cell* **42**, 129-138.

Kitron, U., Swanson, J., Crandell, M., Sullivan, P. J., Anderson, J., Carro, R., Haramis, L. D. & Grimstad, P. R. (1998). Introduction of *Aedes albopictus* into a LaCrosse virus-enzootic site in Illinois. *Emerging Infectious Diseases* **4**.

Klimowski, L., Rayms-Keller, A., Olson, K. E., Yang, R. S. H., Tessari, J., Carlson, J. & Beaty, B. J. (1996). Inducibility of a molecular bioreporter system by heavy metals. *Environmental Toxicology and Chemistry* **15**, 85-91.

Kochs, G. & Haller, O. (1999). Interferon-induced human MxA GTPase blocks import of Thogoto virus nucleocapsids. *Proceedings of the National Academy of Sciences, USA* **96**, 2082-2086.

Kolakofsky, D., Bellocq, C. & Raju, R. (1987). The translational requirement for LaCrosse virus S-mRNA synthesis. *Cold Spring Harbor Symposia on Quantitative Biology* **52**. 373-379.

Kolakofsky, D. & Hacker, D. (1991). Bunyavirus RNA synthesis: Genome transcription and replication. *Current Topics in Microbiology and Immunology* **169**. 143-158.

Kovach, M. J., Carlson, J. O. & Beaty, B. J. (1992). A *Drosophila* metallothionein promoter is inducible in mosquito cells. *Insect Molecular Biology* **1**. 37-43.

Kovacs, G. R., Choi, J., Guarino, L. A. & Summers, M. D. (1992). Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein. *Journal of Virology* **66**. 7429-7437.

Kovacs, G. R., Guarino, L. A. & Summers, M. D. (1991). Novel regulatroy properties of the IE1 and IE0 transactivators encoded by the *baculovirus Autographa californica* multicapsid nuclear polyhedrosis virus. *Journal of Virology* **65**. 5281-5288.

Krug, R. M., Shaw, M., Broni, B., Shapiro, G. & Haller, O. (1985). Inhibition of influenza viral mRNA synthesis in cells expressiong the interferon-induced Mx gene product. *Journal of Virology* **56**. 201-206.

Krystal, G. W., Armstrong, B. C. & Battey, J. F. (1990). N-myc mRNA forms an RNA-RNA duplex with endogenous antisense transcripts. *Molecular and Cellular Biology* **10**. 4180-4191.

Landis, H., Simon-Jodicke, A., Kloti, A., Paolo, C. D., Schnorr, J.-J., Schneider-Schaulies, S., Hefti, H. P. & Pavlovic, J. (1998). Human MxA protein confers resistance to Semliki Forest virus and inhibits the amplification of a Semliki Forest virus-based replicon in the absence of viral structural proteins. *Journal of Virology* **72**. 1516-1522.

Lastowski-Perry, D., Otto, E. & Maroni, G. (1985). Nucleotide sequence and expression of a *Drosophila* Metallothionein. *The Journal of Biological Chemistry* **260**. 1527-1530.

Leiter, J. M. E., Krystal, M. & Palese, P. (1989). Expression of antisense RNA fails to inhibit influenza virus replication. *Virus Research* **14**. 141-160.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. & Dougherty, W. G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *The Plant Cell* **5**. 1749-1759.

Lindenmann, J. (1962). Resistance of mice to mouse adapted influenza A virus. *Virology* **16**, 203-204.

Ludwig, G. V., Christensen, B. M., Yuill, T. M. & Schultz, K. T. (1989). Enzyme processing of LaCrosse virus glycoprotein G1: a bunyavirus-vector infection model. *Virology* **171**, 108-113.

Ludwig, G. V., Israel, B. A., Christensen, B. M., Yuill, T. M. & Schultz, K. T. (1991). Role of LaCrosse virus glycoproteins in attachment of virus to host cells. *Virology* **181**, 564-571.

Matsuoka, Y., Chen, S. Y. & Compans, R. W. (1991). Bunyavirus protein transport and assembly. *Current Topics in Microbiology and Immunology* **169**, 161-179.

Maxwell, I. H., Maxwell, F., Rhode, S. L., III, Corsini, J. & Carlson, J. O. (1993). Recombinant LuIII autonomous parvovirus as a transient transducing vector for human cells. *Human Gene Therapy* **4**, 441-450.

McGrane, V., Carlson, J. O., Miller, B. R. & Beaty, B. J. (1988). Microinjection of DNA into *Aedes triseriatus* ova and detection of integration. *American Journal of Tropical Medicine and Hygiene* **39**, 502-510.

Meister, S., and Grigliatti, T. (1993). Rapid spread of a P element/Adh gene construct through experimental populations of *Drosophila melanogaster*. *Genome* **36**, 1169-1175.

Melton, D. A. (1985). Injected anti-sense RNAs specifically block messenger RNA translation in vivo. *Proceedings of the National Academy of Sciences* **82**, 144-148.

Miller, L. H., Sakai, R. K., Romans, P., Gwadz, R. W., Kantoff, P. & Coon, H. G. (1987). Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. *Science* **237**, 779-781.

Monroe, T. J. (1990). Approaches to intracellular immunization: genetic manipulation of arboviral host cells, Ph.D. dissertation. Colorado State University.

Monroe, T. J., Muhlmann-Diaz, M. C., Kovach, M. J., Carlson, J. O., Bedford, J. S. & Beaty, B. J. (1992). Stable transformation of a mosquito cell line results in extraordinarily high copy numbers of the plasmid. *Proceedings of the National Academy of Sciences, USA* **89**, 5725-5729.

Montgomery, M. K. & Fire, A. (1998). Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. *Trends in Genetics* **14**, 255-258.

Montgomery, M. K., Xu, S. & Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proceedings of the National Academy of Science* **95**. 15502.

Moroni, G., Otto, E. & Lastowski-Perry, D. (1986). Molecular and cytogenetic characterization of a metallothionein gene of *Drosophila*. *Genetics* **112**. 493-504.

Morris, A. C., Eggleston, P. & Crampton, J. M. (1989). Genetic transformation of the mosquito *Aedes aegypti* by micro-injection of DNA. *Medical and Veterinary Entomology* **3**. 1-7.

Muzyczka, N. (1992). Use of adeno-associated virus as a general transduction vector for mammalian cells. *Current Topics in Microbiology and Immunology* **158**. 97-129.

Nellen, W. & Lichtenstein, C. (1993). What makes an mRNA anti-sense-itive? *Trends in Biochemical Science* **18**. 419-423.

Ngo, H., Tschudi, C., Gull, K. & Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proceedings of the National Academy of Science* **95**. 14687-14692.

Obijeski, J. F., Bishop, D. H. L., Palmer, E. L. & Murphy, F. A. (1976). Segmented genome and nucleocapsid of LaCrosse virus. *Journal of Virology* **20**. 664-675.

Olson, K. E., Higgs, S., Gaines, P. J., Powers, A. M., Davis, B. s., Kamrud, K. I., Carlson, J. O., Blair, C. D. & Beaty, B. J. (1996). Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. *Science* **272**. 884-886.

Otto, E., Allen, J. M., Young, J. E., Palmiter, R. D. & Maroni, G. (1987). A DNA segment controlling metal-regulated expression of the *Drosophila melanogaster* metallothionein gene Mtn. *Molecular and Cellular Biology* **7**. 1710-1715.

Pantuwatana, S., Thompson, W. H., Watts, D. M. & Hanson, R. P. (1972). Experimental infection of chipmunks and squirrels with LaCrosse and Trivittatus viruses and biological transmission of LaCrosse virus by *Aedes triseriatus*. *The American Journal of Tropical Medicine and Hygiene* **21**. 476-481.

Patterson, J. L., Holloway, B. & Kolakofsky, D. (1984). LaCrosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *Journal of Virology* **52**. 215-222.

Pavlovic, J., Haller, O. & Staeheli, P. (1992). Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. *Journal of Virology* **66**. 2564-2569.

Pavlovic, J., Zurcher, T., Haller, O. & Staeheli, P. (1990). Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. *Journal of Virology* **64**, 3370-3375.

Pekosz, A., Griot, C., Stillmock, K., Nathanson, N. & Gonzalez-Scarano, F. (1995). Protection from LaCrosse virus encephalitis with recombinant glycoproteins: Role of neutralizing anti-G1 antibodies. *Journal of Virology* **69**, 3475-3481.

Persidis, A. (1999). Antisense therapeutics. *Nature Biotechnology* **17**, 403-404.

Pinkerton, A. C., Michel, K., O'Brochta, D. A. & Atkinson, P. W. (2000). Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. *Insect Molecular Biology* **9**, 1-10.

Powell-Abel, P., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R. T. & Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.

Powers, A. M. (1995). Genetically engineered resistance to bunyavirus infection in mosquito cells. Ph.D. dissertation. Colorado State University.

Powers, A. M., Kamrud, K. I., Olson, K. E., Higgs, S., Carlson, J. O. & Beaty, B. J. (1996). Molecularly engineered resistance to California serogroup virus replication in mosquito cells and mosquitoes. *Proceedings of the National Academy of Sciences, USA* **93**, 4187-4191.

Powers, A. M., Olson, K. E., Higgs, S., Carlson, J. O. & Beaty, B. J. (1994). Intracellular immunization of mosquito cells to LaCrosse virus using a recombinant Sindbis virus vector. *Virus Research* **32**, 57-67.

Prins, M. & Goldbach, R. (1996). RNA-mediated virus resistance in transgenic plants. *Archives of Virology* **141**, 2259-2276.

Prins, M., Resende, R. d. O., Anker, C., Van Schepen, A., De Haan, P. & Goldbach, R. (1996). Engineered resistance to tomato spotted wilt virus is sequence specific. *Molecular Plant-Microbe Interaction* **9**, 416-418.

Putlitz, J. Z., Wieland, S., Blum, H. E. & Wands, J. R. (1998). Antisense RNA complementary to hepatitis B virus specifically inhibits viral replication. *Gastroenterology* **115**, 702-713.

Ratcliff, F., Harrison, B. D. & Baulcombe, D. C. (1997). A similarity between viral defense and gene silencing in plants. *Science* **276**, 1558-1560.

Raviprakash, K., Liu, K., Matteucci, M., Wagner, R., Riffenburgh, R. & Carl, M. (1995). Inhibition of dengue virus by novel, modified antisense oligonucleotides. *Journal of Virology* **69**, 69-74.

Register, J. C. & Beachy, R. N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology* **166**, 524-532.

Reimann-Philipp, U. & Beachy, R. N. (1993). The mechanism(s) of coat protein-mediated resistance against tobacco mosaic virus. *Seminars in Virology* **4**, 349-356.

Rice, C. M., Levis, R., Strauss, J. H. & Huang, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. *Journal of Virology* **61**, 3809-3819.

Roberts, A., Rossier, C., Kolakofsky, D., Nathanson, N. & Gonzalez-Scarano, F. (1995). Completion of the LaCrosse virus genome sequence and genetic comparisons of the L proteins of the Bunyaviridae. *Virology* **206**, 742-745.

Rossier, C., Patterson, J. & Kolakofsky, D. (1986). LaCrosse virus small genome mRNA is made in the cytoplasm. *Journal of Virology* **58**, 647-650.

Rossier, C., Raju, R. & Kolakofsky, D. (1988). LaCrosse virus gene expression in mammalian and mosquito cells. *Virology* **165**, 539-548.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*, Second edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanford, J. C. & Johnston, S. A. (1985). The concept of parasite-derived resistance-deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113**, 395-405.

Sarkar, A., Yardley, K., Atkinson, P. W., James, A. A. & O'Brochta, D. A. (1997). Transposition of the *Hermes* element in embryos of the vector mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* **27**, 359-363.

Schlesinger, S. (1993). Alphaviruses-vectors for the expression of heterologous genes. *Trends in Biotechnology* **11**, 18-22.

Schmaljohn, C. S. (1996). Bunyaviridae: The viruses and their replication. In *Fundamental Virology*, pp. 649-673. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott-Raven Publishers.

Selker, E. U. (1999). Gene silencing: Repeats that count. *Cell* **97**, 157-160.

Sharp, P. A. (1999). RNAi and double-strand RNA. *Genes and Development* **13.** 139-141.

Solomon, J. M., Rossi, J. M., Golic, K., McGarry, T. & Lindquist, S. (1991). Changes in hsp70 alter thermotolerance and heat-shock regulation in *Drosophila. The New Biologist* **3.** 1106-1120.

Staeheli, P. & Haller, O. (1985). Interferon-induced human protein with homology to protein Mx of influenza virus-resistant mice. *Molecular and Cellular Biology* **5.** 2150-2153.

Stark, G. R., Kerr, I. M., Williams, B. R. G., Silverman, R. H. & Schreiber, R. D. (1998). How cells respond to interferons. *Annual Reviews in Biochemistry* **67.** 227-264.

Takacs, A. M. & Banerjee, A. K. (1997). Inhibition of vesicular stomatitis virus in cells constitutively expressing an antisense RNA targeted against the virus RNA polymerase gene. *Journal of General Virology* **78.** 125-130.

Tal, J. & Attathom, T. (1993). Insecticidal potential of the insect parvovirus GmDNV. *Archives of Insect Biochemistry and Physiology* **22.** 345-356.

Thompson, W. H. & Beaty, B. J. (1978). Venereal transmission of LaCrosse virus from male to female *Aedes triseriatus. The American Journal of Tropical Medicine and Hygiene* **27.** 187-196.

Thompson, W. H., Kalfayan, B. & Anslow, R. O. (1965). Isolation of California encephalitis group virus from a fatal human illness. *American Journal of Epidemiology* **81.** 245-253.

Timmons, L. & Fire, A. (1998). Specific interference by ingested dsRNA. *nature* **395.** 854.

Tullis, G. E., Burger, L. R. & Pintel, D. J. (1993). The minor protein VP1 of the autonomous parvovirus minute virus of mice is dispensable for encapsidation of progeny single-stranded DNA but is required for infectivity. *Journal of Virology* **67.** 131-141.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Mourrain, P., Palauqui, J.-C. & Vernhettes, S. (1998). Transgene-induced gene silencing in plants. *The Plant Journal* **16.** 651-659.

Veres, G., Excaich, S., Baker, J., Barske, C., Kalfoglou, C., Ilves, H., Kaneshima, H. & Bohnlein, E. (1996). Intracellular expression of RNA transcripts complementary to the human immunodeficiency virus type 1 *gag* gene inhibits viral replication in human CD4+ lymphocytes. *Journal of Virology* **70.** 8792-8800.

Voinnet, O., Vain, P., Angell, S. & Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**, 177-187.

Von Ruden, T. & Gilboa, E. (1989). Inhibition of human T-cell leukemia virus type I replication in primary human T cells that express antisense RNA. *Journal of Virology* **63**, 677-682.

Wang, S. & Dolnick, B. J. (1993). Quantitative evaluation of intracellular sense:antisense RNA hybrid duplexes. *Nucleic Acids Research* **21**, 4383-4391.

Wassenegger, M. & Pelissier, T. (1998). A model for RNA-mediated gene silencing in higher plants. *Plant Molecular Biology* **37**, 349-362.

Waterhouse, P. M., Graham, M. W. & Wang, M.-B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences* **95**, 13959-13964.

Watts, D. M., Pantuwatana, S., DeFoliart, G. R., Yuill, T. M. & Thompson, W. H. (1973). Transovarial transmission of LaCrosse virus (California encephalitis group) in the mosquito, *Aedes triseriatus*. *Science* **182**, 1140-1141.

Watts, D. M., Thompson, W. H., Yuill, T. M., DeFoliart, G. R. & Hanson, R. P. (1974). Overwintering of LaCrosse virus in *Aedes triseriatus*. *The American Journal of Tropical Medicine and Hygiene* **23**, 694-700.

Wigglesworth, V. B. (1938). The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae. *Journal of Experimental Biology* **15**, 235-247.

Wu, C. C. M. & Fallon, A. M. (1997). Evaluation of a heterologous metallothionein gene promoter in transfected mosquito cells. *Comparative Biochemistry and Physiology* **116B**, 353-358.

Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M. & Huang, H. B. (1989). Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* **243**, 1188-1191.

Zhao, Y.-G. & Eggleston, P. (1999). Comparative analysis of promoters for transient gene expression in cultured mosquito cells. *Insect Molecular Biology* **8**, 31-38.

APPENDIX

List of Abbreviations

AeDNV	*Aedes* densonucleosis virus
BHK-21	baby hamster kidney cells (clone 21)
C6/36	*Aedes albopictus* cells (clone C6/36)
CPE	cytopathic effect
dsSIN	double subgenomic Sindbis expression system
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FITC	fluorescein conjugate
h	hour
HRP	horseradish peroxidase
hsp70	70 kilodalton heat shock protein (promoter)
hyg	hygromycin phosphotransferase gene
IE1	immediate early 1 promoter
IFA	immunofluorescence assay
IFN	interferon
kb	kilobases
L	LaCrosse large segment RNA

LAC	LaCrosse virus
M	LaCrosse middle segment RNA
mL	milliliter
moi	multiplicity of infection
Mtn	metallothionein promoter
N	LaCrosse nucleocapsid protein
NCR	non-coding region
nts	nuclear targeting signal
ORF	open reading frame
PBS	phosphate buffered saline
PTGS	post-transcriptional gene silencing
S	LaCrosse small segment RNA
SDS	sodium dodecyl sulfate
SIN	Sindbis virus
SSC	standard saline citrate
SSH	snowshoe hare virus
TAH	Tahyna virus
TCID	tissue culture infectious dose
TGS	transcriptional gene silencing
TMV	tobacco mosaic virus
TRITC	tetramethyl rhodamine conjugate
TSWV	tomato spotted wilt virus
ug	microgram

uL	microliter
VIGS	virus induced gene silencing
VP	AeDNV virion or structural protein