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DISSERTATION

**INHIBITION OF GENE EXPRESSION IN MOSQUITOES MEDIATED BY
RNA DELIVERED BY THE DOUBLE SUBGENOMIC SINDBIS VIRUS
EXPRESSION SYSTEMS**

**Submitted by
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**In partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Summer 2000**

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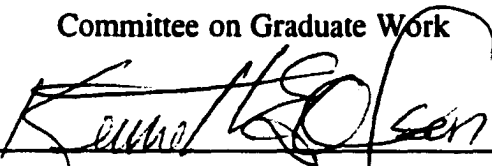
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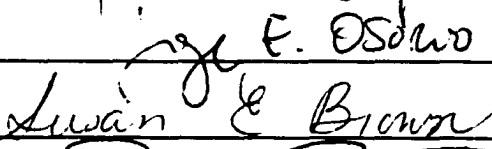
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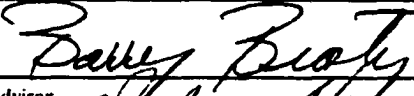
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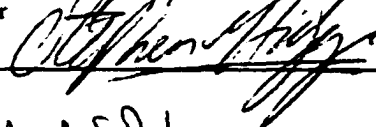
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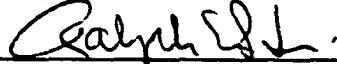
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ABSTRACT OF DISSERTATION

INHIBITION OF GENE EXPRESSION IN MOSQUITOES MEDIATED BY RNA DELIVERED BY THE DOUBLE SUBGENOMIC SINDBIS VIRUS EXPRESSION SYSTEMS

The double subgenomic Sindbis (dsSIN) virus expression system was used to deliver RNA transcripts complementary to endogenous mosquito mRNA to inhibit gene expression *in vivo*. dsSIN has been used to stably express genes in *Aedes aegypti* salivary glands and to inhibit flavivirus replication by expression of RNA transcripts complementary to the flavivirus genome. The dsSIN viruses TE/3'2J and MRE/3'2J were assessed for their ability to inhibit the expression of endogenous mosquito genes expressed exclusively in the salivary glands.

Experiments were conducted to inhibit expression of a reporter gene in a germline transformed *Ae. aegypti* line (APY-LUC43). These mosquitoes constitutively express luciferase from the mosquito apyrase promoter. APY-LUC43 mosquitoes were intrathoracically inoculated with TE/3'2J/ α -luc virus, which transcribed RNA complementary to the 5' end of the luciferase mRNA. Luciferase activity was monitored over time in mosquitoes infected with either TE/3'2J/ α -luc or control dsSIN viruses expressing unrelated antisense RNAs. Mosquitoes infected with TE/3'2J/ α -luc virus

exhibited 90% reduction in luciferase activity compared to uninfected and control dsSIN-infected mosquitoes at 5 and 9 days postinoculation.

Apyrase is a salivary gland protein with anti-platelet aggregating properties. TE/3'2J/ α -apyrase virus was engineered to contain 375 bases from the 5' coding region of the *Ae. aegypti* apyrase gene, inserted in antisense orientation. TE/3'2J/ α -apyrase was intrathoracically inoculated into female adult mosquitoes, and apyrase activity and protein production was assessed. TE/3'2J/ α -apyrase virus effectively infected salivary gland tissue; however, no diminishment of apyrase production was observed.

An orally infective dsSIN chimeric virus was engineered that contained the structural genes of MRE16 and the nonstructural genes and second subgenomic promoter of TE/3'2J/ α -apyrase. MRE/3'2J/ α -apyrase was tested for its ability to infect larvae *per os* and inhibit expression of apyrase in adults. Infection rates of up to 42% were obtained, but inhibition of apyrase expression was not observed.

These studies support the overall hypothesis of this dissertation, that expression of an endogenous mosquito gene can be specifically inhibited by RNA delivered by the SIN virus expression system. The dsSIN antisense RNA expression system provides an important tool for studying gene expression *in vivo*, and may lead to defining genetic determinants of mosquito vector competence.

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Chapter 1

Literature Review

INTRODUCTION

Arthropod-borne pathogens are important causes of mortality and morbidity worldwide, particularly in the tropical developing nations. Since the breakdown of mosquito eradication programs in the early 1970s, the abundance and geographical ranges of many mosquito vectors of disease are increasing; concomitantly, the incidence of mosquito-borne diseases is rising among animal and human populations worldwide. The causes for these increases are multifactorial, but include inadequate mosquito control programs, the development of insecticide resistance in mosquito populations, drug resistance in parasites, lack of vaccines, environmental changes, rapid urbanization in developing countries, increased international trade, and the advent of rapid international transportation (Division of Disease Prevention and Control, 1997; Gratz, 1999; Robertson *et al.*, 1996).

The anthropophilic mosquito *Aedes aegypti* is the major vector of dengue viruses, which cause dengue fever (DF) and dengue hemorrhagic fever (DHF) (Gubler, 1998). *Aedes aegypti* has infested sylvatic and urban environments throughout the tropics and subtropics; similarly, the four DEN serotypes are now found pantropically. Consequently, two and a half billion people, nearly half the world's population, live in areas where they are at risk for being infected with dengue viruses. There are an estimated 100 million cases of DF and several hundred thousand cases of DHF annually (Gubler, 1998).

In addition, there are 200 million malaria cases a year (Goddard, 2000). *Anopheles gambiae* is the primary mosquito vector for the plasmodium parasites that cause malaria. Except for Europe and the United States, where malaria has been eradicated, the geographic distribution is similar to that of one hundred years ago, throughout the tropics

and subtropics (Goddard, 2000). Dengue fever and malaria are the two arthropod-borne diseases that impact human health and morbidity the most. Despite intense research, development of efficacious vaccines against these two diseases have not been forthcoming.

Because of difficulties associated with conventional control measures, some new initiatives have focused on genetically reducing or eliminating a mosquito's ability to transmit a pathogen. However, more needs to be known about the biology of the mosquito and about the determinants of pathogen-mosquito interactions that lead to successful transmission. Such information is a prerequisite to determining which mosquito genes might be good targets for inhibition or overexpression, in which tissue introduced genes should be expressed, and what endogenous promoters should be used to express the genes. In particular, the function of genes expressed during blood feeding and during pathogen transmission in vector saliva need to be determined (Billingsley, 1994). These include genes expressed in the midgut that control blood feeding and those that are determinants of midgut infection and dissemination. Gene products produced in the salivary glands that facilitate blood feeding have also been shown to enhance pathogen transmission (Limesand *et al.*, 2000; Beerntsen *et al.*, 2000; Edwards *et al.*, 1998; Osorio *et al.*, 1996).

It is critical that new tools and approaches be developed to characterize gene function rapidly in arthropods (Carlson *et al.*, 1995; Crampton, 1994; Curtis, 1994; Handler & O'Brochta, 1991). In these studies, a double-subgenomic Sindbis virus expression system was used to inhibit gene expression *in vivo* by antisense RNA. Inhibition of gene expression in female adult salivary glands, specifically apyrase, was targeted. Inhibition

of gene expression *in vivo* using virus transducing systems provides a method for studying protein function in the mosquito. Such studies will increase our knowledge of mosquito molecular biology and further our understanding of the dynamic interactions between the pathogen and the mosquito vector.

Maintenance and Transmission of Pathogens in Mosquitoes. Many pathogens enter the mosquito in the blood meal. For transmission to occur, the pathogen must replicate and cycle through the mosquito to the salivary glands in order to be transmitted to a susceptible host (Woodring *et al.*, 1996). Although the life cycles of the various pathogens are distinct, they all are ingested in the blood meal and exposed to the midgut environment (Beerntsen *et al.*, 2000). In addition, they must infect and traverse the midgut, pass into the hemocoel, and be transported to the salivary glands. Entrance into each mosquito tissue presents a potential barrier to transmission, and in the case of viruses, is determined by a receptor-ligand interaction (Beerntsen *et al.*, 2000). Vector competence is hypothesized to be genetically determined, although the specific genes have not yet been identified (Beerntsen *et al.*, 2000; Bosio *et al.*, 1998; Calisher, 1994).

In the hematophagous mosquitoes, only female adults blood feed. The blood meal provides the protein nutrients for the developing embryos. Proteolytic enzymes activated by blood feeding digest the blood meal and the resultant amino acids are adsorbed by the midgut. Early trypsin transcription begins soon after adult eclosion and is abundant as mRNA before blood feeding (Noriega *et al.*, 1996a; Noriega *et al.*, 1996b; Noriega *et al.*, 1997). Blood feeding induces translation of early trypsin. Early trypsin is also a transcriptional activator of late trypsin, which is produced 6-8 hr after blood feeding (Noriega *et al.*, 1994). Ingested pathogens are also exposed to other midgut proteases,

which may aid or have a deleterious effect on the pathogen (Beerntsen *et al.*, 2000). The peritrophic matrix, which is formed within hours of blood ingestion separates the midgut epithelium from the blood meal. The peritrophic matrix keeps protease inhibitors within the lumen, provides a solid support around the blood meal, and functions as a semipermeable filter for digestive enzymes and blood protein (Jacobs-Lorena & Oo, 1996). It also may serve as a barrier to pathogen infection of midgut epithelial cells, although viruses and filarial worms generally enter epithelial cells before peritrophic matrix formation. *Plasmodium* sporozoites reproduce in the blood meal and generally can pass through the peritrophic matrix (Beerntsen *et al.*, 2000; Edwards & Jacobs-Lorena, 2000; Jacobs-Lorena & Oo, 1996). The midgut infection barrier is hypothesized to have a genetic basis, with intraspecific genetic variability determining vector competence. However, the full spectrum of genes that determine susceptibility to a pathogen has yet to be determined (Bosio *et al.*, 1998; Gorrochotegui-Escalante *et al.*, 2000).

Pathogens that successfully pass beyond the midgut enter the hemocoel and are transported in the hemolymph to the salivary glands. Hemocytes are part of the innate immune system of the mosquito (Hoffmann *et al.*, 1999). Antimicrobial molecules are also part of the immune system. These include defensins and cercropins (Paskewitz & Christensen, 1996). Defensins are antibacterial peptides produced in the fat body and transported to the hemolymph (Chalk *et al.*, 1995; Chalk *et al.*, 1994; Lowenberger *et al.*, 1995). Predominantly antibacterial peptides produced in response to bacterial infection, defensins have been shown to prevent or reduce infection by filarial worms and plasmodium parasites (Dimopoulos *et al.*, 1997; Dimopoulos *et al.*, 1998). Parasites may also be melanized by hemocytes. The effectiveness of melanization varies by species of

both the parasite and mosquito. Parasite density is also a factor (Beerntsen *et al.*, 2000). Parasites are physiologically costly to mosquitoes and melanotic encapsulation can be reproductively costly to the mosquito. Both melanotic encapsulation and egg tanning require tyrosine, resulting in a competition for this limited resource (Beerntsen *et al.*, 2000).

Entry into salivary gland tissue, the last barrier to transmission, is also hypothesized to be receptor-ligand mediated (Beerntsen *et al.*, 2000; Brown & Condreay, 1986). Salivary gland tissue consists of a single layer of epithelial cells surrounded by a basal lamina. Saliva proteins are synthesized in the epithelial cells then secreted into the connecting acinar spaces, where they are stored until the mosquito blood feeds. Saliva then passes into the salivary gland duct and is secreted (James, 1994; Ribeiro *et al.*, 1984; Ribeiro *et al.*, 1990). Pathogens infecting the salivary glands are secreted with saliva into the vertebrate host. Mosquito saliva contains antihemastatic factors that facilitate blood feeding (Stark & James, 1996) and immunomodulatory factors that enhance pathogen transmission (Edwards *et al.*, 1998; Limesand *et al.*, 2000; Osorio *et al.*, 1996). The studies described in this dissertation have focused on the salivary gland protein apyrase.

The Salivary Gland Protein Apyrase. Female *Aedes aegypti* salivary glands produce and secrete up to 20 different proteins into the host during blood feeding, approximately nine of which are of known function (James, 1994). Some facilitate hematophagy by initiating vasodilation and anti-inflammatory responses, or by inhibiting platelet aggregation in the vertebrate host (Titus & Ribeiro, 1990; Wikel *et al.*, 1996). Salivary glands are also the site of replication for viral pathogens, which are transmitted to the host via the saliva during blood feeding. For certain pathogens, mosquito saliva has been

shown to potentiate the infection process. Infections with arthropod-borne viruses (Bunyaviridae, Flaviviridae, Rhabdoviridae) and protozoa (*Leishmania*) are enhanced in experimental animals when the pathogen is administered through arthropod vector saliva (Edwards *et al.*, 1998; Lima, 1996; Limesand *et al.*, 2000; Osorio *et al.*, 1996; Titus & Ribeiro, 1990). Saliva proteins are hypothesized to give the pathogen a "head start" through their antihemostatic and immunomodulatory activities in the host, but the particular saliva proteins responsible have not been identified (Beerntsen *et al.*, 2000). Determining the effect specific salivary gland proteins have on pathogen transmission will help identify factors involved in vector competence.

Apyrase is a salivary gland protein that was chosen as a candidate for gene expression inhibition because its function is known. Apyrase is an antiplatelet aggregation protein secreted by many hematophagous arthropods. The genomic DNA and cDNA have been sequenced (Champagne *et al.*, 1995; Smartt *et al.*, 1995), a functional enzymatic assay has been developed (Ribeiro *et al.*, 1984; Ribeiro *et al.*, 1990), and a sensitive antibody to detect protein in immunoassays is available (Champagne *et al.*, 1995).

There are five Dipteran families that contain hematophagous genera. Hematophagy evolved at the family level, although in mosquitoes hematophagy evolved twice at the subfamily level (Champagne *et al.*, 1995; James, 1994). Hematophagy is considered to be a result of convergent evolution. The strategies used by arthropods to effect hemostasis vary tremendously except for apyrase, which suggests that it has a monophyletic origin (Champagne *et al.*, 1995; James, 1994; Ribeiro *et al.*, 1984).

The apyrase gene apparently existed before the divergence of the Diptera, and it possibly has a monophyletic origin from bacteria. Apyrases and 5'-nucleotidases belong

to a large family of enzymes that have the capacity to hydrolyze phosphates from a number of substrates (Champagne *et al.*, 1995). They are found in plants, where they may have a role as anti-phagostimulants and may facilitate phosphate transport (Roberts *et al.*, 1999; Thomas *et al.*, 1999). They are found widely in vertebrates, where they function to remove phosphates from charged nucleotides so that they can be transported across the cell membrane. They also function in vertebrate tissue and hematophagous arthropods as platelet aggregation inhibitors (Cheeseman, 1998; Mans *et al.*, 1999; Ribeiro *et al.*, 1984; Valenzuela *et al.*, 1998).

The apyrase gene of *Ae. aegypti* has been sequenced and compared with the amino acid sequences of other 5'-nucleotidases. Seven conserved regions were found with homology to other enzymes with 5'-nucleotidase activity in human and rat placental liver tissue. The mosquito apyrase lacks a hydrophobic carboxy-terminus. This facilitates apyrase secretion from salivary gland tissue in the mosquito, whereas in the other organisms apyrase remains membrane bound (Champagne *et al.*, 1995; Smartt *et al.*, 1995).

The ancestral form of apyrase is represented by a bacterial enzyme that has 5'-nucleotidase, UDP sugar hydrolase, and apyrase domains. Duplications of this broad-spectrum ancestral gene, was followed by divergent selection to produce apyrases (specialized to hydrolyze the pyrophosphate bond of nucleoside pyrophosphates), 5'-nucleotidases (specialized for the ester bond), and UDP sugar hydrolases. Domain loss or gain resulted in substrate specificity and cellular localization of apyrase. Two principal hypotheses have been promulgated for the evolution of apyrases. A gene for a single enzyme such as the 5'-nucleotidase may be the common progenitor for salivary apyrases

in the various arthropod taxa; alternatively, genes encoding enzymes with different cellular functions may have been selected to fill the new roles necessitated by the evolution to hematophagy (Champagne *et al.*, 1995). Analysis of the Hemiptera apyrase gene has shown that it belongs to a different enzyme family (Arca *et al.*, 1999; Law *et al.*, 1992), providing support for the alternate hypothesis of Champagne and colleagues.

A biological assay for apyrase has been developed, which measures the amount of orthophosphate produced from the removal of phosphate from ADP or ATP (Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1984). The assay was used to determine apyrase activities of whole salivary gland lysates in various organisms (Table 1.1).

Table 1.1 Apyrase activity

Apyrase activity	Units/mg salivary protein*
<i>Rhodnius prolixus</i>	5
<i>Aedes aegypti</i>	32
<i>Lutzomyia longipalpis</i>	40
<i>Phlebotomus papatasi</i>	55-70
<i>P. argentipes</i>	134-149
Pig epithelium	0.075
Potato tuber	1.3

* 1 unit of activity = amount of enzyme that releases 1 μ mole of orthophosphate/min at 30°C
(James, 1994; Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1984).

Studies have been conducted to determine if there is a relationship between the amount of apyrase produced and host preference, and if apyrase abundance affected probing time. Apyrase activity was compared among related *Anopheles* species (Cupp *et al.*, 1994). The ancestral species, which had the lowest apyrase activity, was represented by *An. quadrammulus*. *An. quadriannulatus* was also the most zoophilic species included. *An. merus* and *An. melas* are important vectors in sublittoral habitats but are primarily zoophilic. Anthropophilic species included *An. gambiae sensu stricto*, which

feeds strictly on humans; *An. arabiensis*, which has a lower human biting index when bovines are present; and *An. albimanus*, an important New World vector. There were significant variations in apyrase concentration between closely related mosquito species. In general, however, apyrase levels correlated with the mosquito species level of anthropophilicity. The authors concluded that the human host is important for evolution of apyrase levels (Cupp *et al.*, 1994). However, other mammals, including, cows and pigs, have greater concentrations of platelets than humans (human 250,000/ μ l, cow 350,000/ μ l, pig 445,000/ μ l) (Altman & Dittmer, 1964).

Apyrase activity was compared in another study between three species of *Anopheles*, one of which is not hematophagous (Ribeiro *et al.*, 1985). The most anthropophilic species in the study, *Anopheles freeborni*, produced the most apyrase, whereas the zoophilic *An. stephensi* produced about a third as much. Apyrase activity of the autogenous *An. salbaii* was significantly lower than that of the two hematophagous species (Table 1.2). There was an inverse relationship between the amount of stored apyrase in *Anopheles* spp. and probing time. The probing time of *An. stephensi* was not significantly less than that of *An. sp. nr. salbaii*, which does not need to blood feed to complete its life cycle (Ribeiro *et al.*, 1985). This may be because *An. stephensi* feed primarily on cattle, which shake off mosquitoes less readily than humans; therefore, there is no pressure on the mosquito to feed quickly.

Table 1.2 Apyrase activity in *Anopheles* spp.

Species	Apyrase activity*
<i>Anopheles freeborni</i>	20.7 +2.4
<i>An. stephensi</i>	7.8 + 0.7
<i>An. sp. nr. salbaii</i>	3.0 + 0.4

*1 unit of activity = amount of enzyme that releases
1 μ mole of orthophosphate/min at 30°C
(James, 1994; Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1984).

In the *Ae. aegypti* and *Plasmodium gallinacium* model system, probing time was compared to level of apyrase activity in infected and uninfected mosquitoes (Rossignol *et al.*, 1984). Mosquitoes that had the salivary glands removed probed for a significantly longer time than infected mosquitoes. Infected mosquitoes produced less apyrase, and probed longer than uninfected mosquitoes. Interestingly, the total volume of saliva in infected mosquitoes was the same as in uninfected mosquitoes.

Probing time of *Plasmodium*-infected and uninfected field-collected *Anopheles* spp. was also compared (Wekesa *et al.*, 1992). Infected mosquitoes fed for a longer period of time, with greater number of probes, than uninfected mosquitoes. In addition, a greater proportion of infected mosquitoes (65%) than uninfected mosquitoes (27%) blood fed. Of those that probed there was no difference in the proportion that imbibed blood, nor was there a difference in the proportion that had a full or partial blood meal between infected and uninfected mosquitoes. Blood-feeding behavior was independent of parasite density in individual mosquitoes. As a control, probing time between nulliparous and parous uninfected females was measured to ensure that older (infected) and younger (uninfected) mosquitoes were not being compared. There were no differences in these two control groups. Ribeiro and colleagues also found that apyrase production and secretion, as well as probing time, are altered in infected mosquitoes (Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1984). These results contradict those of other studies, which found no differences in probing time between uninfected and *P. berghei* infected *An. stephensi* (Cupp *et al.*, 1994). However, modification in probing time in sporozoite-infected mosquitoes is more likely in vector species having relatively higher levels of apyrase such as *An. gambiae*

and *An. arabiensis* than in those species with lower apyrase concentrations such as *An. stephensi* (Cupp *et al.*, 1994).

A factor to consider when analyzing these data is that transmission of *P. falciparum* by *An. stephensi* apparently occurs by ejection of sporozoites in a cluster in the first droplets of saliva, so longer probing time would not increase the probability of transmission (Rossignol *et al.*, 1985). Reduced apyrase and longer probing time may lessen the chance of transmission, as parasites are re-ingested by the feeding mosquito, and may reduce the fitness of the vector, which is at greater risk of being killed while blood feeding for an extended period of time. On the other hand, because malaria leads to thrombocytopenia, the vector may benefit from feeding on an infected host, even though it in turn is infected. The reduced production of antihemostatic factors in the saliva due to infection by the parasite are possibly overcome by the reduced hemostasis capabilities of the infected host (Rossignol *et al.*, 1985). However, it is not clear if this situation is significant in terms of pathogen transmission or vector competence.

Apyrase is clearly an antihemostatic factor activated during blood feeding, but whether or not apyrase is an important component of blood-feeding success is still to be determined. In addition, the role of apyrase in pathogen transmission has yet to be deciphered. Development of techniques to characterize gene expression and protein function *in vivo* are needed. A method of transiently inhibiting apyrase expression in mosquitoes is the subject of these studies.

Posttranscriptional RNA-mediated Inhibition of Gene Expression in Mosquitoes.

Introduction. Protein function can be determined by inhibiting gene expression and observing the biological consequence. Gene function in *Drosophila melanogaster* is often

analyzed by transposon-mediated insertional inactivation of the appropriate gene in a transgenic organism (Spradling *et al.*, 1995). Germline transformation of the *Ae. aegypti* mosquito genome has been described (Coates *et al.*, 1999; Jasinskiene *et al.*, 1998; Kokoza *et al.*, 2000). However, it remains a laborious and time-consuming procedure, and is not yet practical for studying characterization, mutagenesis, and expression of genes in mosquitoes. Transient transformation systems that express a gene of interest, or inhibit expression of a targeted endogenous gene, can provide a means of studying protein function *in vivo*. Posttranscriptional inhibition of gene expression by antisense RNA, double-stranded RNA, and gene silencing of transgenes is summarized in the following section.

Antisense RNA. Control of gene expression by production of transcripts in antisense orientation to an mRNA is a gene expression regulatory mechanism first identified in prokaryotes to control translation (Coleman *et al.*, 1984; Green *et al.*, 1986). The therapeutic potential of antisense inhibition of gene expression was described in 1985 (Izant & Weintraub, 1985). In insects, inhibition using antisense transcripts was first demonstrated in *Drosophila*. *Drosophila* cells stably transformed with a heat-inducible antisense hsp26 gene produced significantly less heat shock protein after heat induction than untransformed cells (Green *et al.*, 1986). Posttranscriptional regulation of gene expression *in vivo* was hypothesized to occur due to the Watson-Crick base pairing between the mRNA and complementary RNA at the ribosomal binding site (Coleman *et al.*, 1984; Green *et al.*, 1986; Mizuno *et al.*, 1984; Pestka, 1992). Initially, effector RNAs were designed complementary to the 5' end of a gene site (Coleman *et al.*, 1984; Green *et al.*, 1986; Mizuno *et al.*, 1984; Pestka, 1992). Complementary transcripts

binding downstream of the start site resulted in less efficiency of inhibition; once the ribosome was bound to the mRNA it was capable of stripping the effector RNA from the mRNA during translation (Pestka, 1992). Antisense transgenes were designed to down-regulate endogenous gene expression by hybridization and dsRNA degradation. This depended, it was hypothesized, on comparable amounts of antisense transcripts to the endogenous mRNA. It was calculated stoichiometrically that antisense RNA should outnumber mRNA by a factor of 150:1, as the level of inhibition was dependent on overabundance of antisense transcripts, with each mRNA molecule bound by an antisense molecule (Pestka, 1992). However, experimental evidence did not support this model, as low levels of antisense transcription showed equal gene silencing, and about the same inhibition as sense expression (Fire, 1999).

Double-stranded RNA. Later studies showed that inhibition could be achieved with antisense transcripts in much smaller abundance than target mRNA (Denhardt, 1992), and a few dsRNA transcripts could eliminate a much larger pool of endogenous mRNA (Fire, 1999; Fire *et al.*, 1998; Montgomery & Fire, 1998; Montgomery *et al.*, 1998). Down regulation of endogenous gene expression by dsRNA was shown in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998). The *unc-22* gene expresses a myofilament protein, resulting in a "twitching" phenotype. Antisense, sense, or dsRNA transcripts homologous to the *unc-22* gene were injected into *C. elegans* and then inhibition of gene expression was assessed, as measured by the *unc-22* gene mutant phenotype. dsRNA-inoculated transcripts inhibited *unc-22* gene expression significantly more than either antisense or sense transcripts alone (Montgomery & Fire, 1998; Montgomery *et al.*, 1998; Tabara *et al.*, 1998). In another experiment, transgenic plants expressing

either sense or antisense sequences to the potato virus Y were both susceptible to the virus, but crosses of the two were resistant (Waterhouse *et al.*, 1998). dsRNA injected into *Drosophila* embryos inhibited endogenous expression significantly more than antisense RNA (Hammond *et al.*, 2000).

dsRNA is a nucleic acid conformation not associated with normal cellular gene expression. Cells survive by unimpeded expression of their own genes, so that an RNA trigger for gene silencing must be recognized as distinct from normal cellular RNA. dsRNA is not found in normal cell gene expression and is part of the replication cycle of RNA viruses; therefore, it is an excellent inducer of cellular defense mechanisms. This gene regulation system must be finely tuned so that one virus particle is detected, the response is great enough to prevent virus replication, and, conversely, that the antiviral state does not continue out of control. It must also be specific, so that it does not interfere with or destroy normal cellular metabolism. Therefore, degradation of the dsRNA must be sequence specific and transient.

dsRNA is part of the replication cycle of RNA viruses as a replicative intermediate (Kumar & Carmichael, 1998). Subsequent degradation of the dsRNA by cellular RNases prevents viral replication in the cell and is a widespread mechanism of defense against viruses. Modulation of normal gene expression in response to physiological or developmental cues by dsRNA is widespread in primitive eukaryotes, Eubacteria, and Archaeobacter, vertebrates, and plants (Kumar & Carmichael, 1998; Merzendorfer *et al.*, 1997). It is probably an ancient eukaryotic function as trypanosomes to mammals recognize and degrade dsRNA (Bosher & Labouesse, 2000; Ngo *et al.*, 1998). In

mammals, as little as one dsRNA particle can induce the antiviral interferon α and β pathways (Green *et al.*, 1986; Kumar & Carmichael, 1998).

Inhibition of gene expression by dsRNA is posttranscriptional, as suppressed genes are actively transcribed (Montgomery *et al.*, 1998; Wassenegger & Pelissier, 1998). Transcription of the target locus is unaffected, but the half-life of target RNAs is decreased dramatically (Fire, 1999). Introduced antisense or dsRNA transcripts containing sequences homologous to the exon region of a gene will inhibit the gene's expression, whereas transcripts complementary to introns do not (Fire *et al.*, 1998; Sharp, 1999). Inhibition is specific to the target sequence and heterologous related viruses or introduced transcripts escape inhibition. Studies in *E. coli* and *C. elegans*, which express genes in operons, revealed that introduced dsRNA containing the sequence of an upstream part of the operon does not inhibit gene expression in the downstream transcripts (Montgomery *et al.*, 1998).

Cells must communicate with other cells to induce resistance in surrounding tissues prior to viral invasion (Fire, 1999; Kennerdell & Carthew, 1998; Merzendorfer *et al.*, 1997). Gene-specific agents that stimulate or mediate the suppression effects are amplifiable or part of a catalytic process. For example, *C. elegans* was inoculated in the body cavity with 10^7 dsRNA *unc-22* particles. Even if each dsRNA particle entered a cell, this is far fewer than the number of cells affected in the organism (Fire *et al.*, 1998; Sharp, 1999). Cells outside of inoculation site exhibited the *unc-22* mutant phenotype. In addition, inhibition was carried over to the F1 generation (Fire *et al.*, 1998; Sharp, 1999). Interference has been shown to be inherited, possibly by an extragenic factor such as short sequences of RNA (Catalanotto *et al.*, 2000; Grishok *et al.*, 2000; Hamilton &

Baulcombe, 1999; Hammond *et al.*, 2000). In mammals, the antiviral state activated by interferon α and β is communicated to surrounding cells to protect them from virus infection, and in plants virus resistance is spread through the phloem to other cells (Baltimore, 1988; Montgomery & Fire, 1998).

The mechanism of inhibition using transiently expressed antisense RNA or dsRNA have not yet been elucidated and may involve more than one mechanism. One hypothesized mechanism is degradation of dsRNA by cellular RNases by the cell's antiviral defense, with the dsRNA acting as the target and initiator of inhibition (Fire, 1999). This may involve conversion of adenine residues in dsRNA to inosine by cellular adenosine deaminases. The product RNA is then positionally modified and bound by proteins so that the two strands dissociate (Patel *et al.*, 1999). This modified strand interacts with cytoplasmic mRNA, and forms at least a short double strand, which signals its destruction by RNases (Sharp, 1999). dsRNA containing sequences of the tubulin gene electroporated into *Trypanosoma brucei* resulted in transient knockout of endogenous tubulin gene expression (Ngo *et al.*, 1998). Apparently, upon entering the cell cytoplasm, the dsRNA is unwound by helicases, and the antisense RNA strand forms a duplex with the target mRNA (Ngo *et al.*, 1998). This picture seems to conflict with the observation that antisense transcripts alone do not inhibit expression as well as dsRNA (Fire, 1999). However, dsRNA is more stable than single-stranded RNA, and so might be effective for longer in the cytoplasm than single antisense strands.

Cosuppression. Plant geneticists introduced multiple copies of an endogenous gene, with a more active promoter than an endogenous promoter, in hopes of getting higher endogenous gene expression. Instead of higher expression, gene expression was

significantly reduced, silenced, or the mRNA transcripts were in much lower abundance than expected (Bruening, 1998). Multiple copies of the transgene, or very active promoters producing abundant transcripts, were more likely to induce gene silencing than single copy transgenes or low-level transcription. In transgenic organisms both the endogenous and introduced copies of the gene were silenced, thus the term cosuppression (Jorgensen, 1995). Similarly, RNA complementary to an endogenous mRNA is also recognized as potentially harmful RNA, resulting in blockage of expression of the endogenous gene (Fire, 1999). Gene silencing of transgenes and endogenous genes occurs frequently in plants (Bruening, 1998), in primitive eukaryotes [*Dictyostelium* (Montgomery & Fire, 1998), *Paramecium* (Ruiz *et al.*, 1998a)], as well as *Drosophila* (Kennerdell & Carthew, 1998).

RNA-mediated resistance to RNA viruses in plants has been shown using transgenes that transcribe an RNA sequence homologous to a virus coat protein gene (Prins & Goldbach, 1996). Initially, it was thought that overproduction of the viral coat protein interfered with uncoating of the virus in the transgenic plants. However, interference did not rely on a protein product, but on the amount of transgenic mRNA transcribed; more transcripts were associated with interference and few copies resulted in no interference (Prins & Goldbach, 1996).

Both virus-induced and transgene gene silencing may work by the same mechanism (Ruiz *et al.*, 1998b). Methylations of the transgene may result in transcription of aberrant or truncated mRNAs in the nucleus (Grant, 1999; Kasschau & Carrington, 1998; Wassenegger & Pelissier, 1998). Transgenes may have altered termination sequences or 3'-end processing (Litiere *et al.*, 1999). In plants an RNA-dependent RNA polymerase

present in the cytoplasm transcribes RNA complementary to the transgene mRNA, as well as the endogenous mRNA or viral RNA (Hamilton & Baulcombe, 1999). A gene in *C. elegans* has been identified with sequence similarity to tomato and *Neurospora crassa* RNA-dependent RNA polymerases, which are implicated in gene silencing (Smardon *et al.*, 2000). Hybridization between the complementary transcript and infecting viral RNA or endogenous mRNAs may trigger cell-mediated dsRNA degradation (Baulcomb, 1999; Ratcliff *et al.*, 1999; Ruiz *et al.*, 1998b). This could explain why this mechanism is so versatile in targeting different introduced sequences, and yet so strictly sequence specific. It also could explain why transgenic antisense and sense sequences are equally effective (Montgomery & Fire, 1998; Prins & Goldbach, 1996). If an endogenous gene can be silenced posttranscriptionally by an antisense transgene, the silencing mechanism must operate on both strands (Prins & Goldbach, 1996). Indeed, cosuppression and gene silencing by dsRNA may work by the same mechanism (Bass, 2000; Boshier & Labouesse, 2000; Catalanotto *et al.*, 2000; Ketting & Pasterk, 2000). However, there is no direct evidence that silencing involves formation of dsRNA (Henikoff, 1998; Roche & Rio, 1998).

Short RNA sequences, 21-23 bases long, have been identified in gene-silenced cells. They may function as signal and specificity determinants for gene silencing. Whether they are by-products of gene silencing or effector molecules has not been determined (Bass, 2000; Hamilton & Baulcombe, 1999; Hammond *et al.*, 2000).

RNA interference using virus vectors. Virus vectors have been used to deliver interfering RNA transcripts both to inhibit endogenous gene expression and to prevent replication of viruses. Plant RNA viruses have been used to inhibit expression of green

fluorescent protein transiently expressed in the nucleus of host plants (Ratcliff *et al.*, 1999). Examples of gene silencing in insects include antisense RNA introduced by the baculovirus vector carrying antisense sequences to juvenile hormone esterase of tobacco budworm (Hajos *et al.*, 1999). Although the majority of the infected budworms died from the virus infection, 25% showed aberrant morphology associated with JHE gene expression inhibition.

The double subgenomic Sindbis (dsSIN) viruses, based on the mosquito-borne virus Sindbis (SIN; *Alphavirus*; *Togaviridae*), allows long-term, stable, cytoplasmic expression of genes of interest in mosquitoes (Carlson *et al.*, 1995; Higgs *et al.*, 1993; Olson *et al.*, 1998; Olson *et al.*, 1994; Rayms-Keller *et al.*, 1995). dsSIN virus has been used to deliver RNA transcripts, both in sense and antisense orientation to the genomes of bunyavirus and flaviviruses, in cultured mosquito cells and in mosquitoes, preventing replication of these viruses (Higgs *et al.*, 1998; Olson *et al.*, 1996; Powers *et al.*, 1996; Powers *et al.*, 1994; Smardon *et al.*, 2000). The dsSIN viruses TE/3'2J and MRE/3'2J were used in the studies described in this dissertation to deliver RNA sequences complementary to endogenous mosquito mRNA in an effort to inhibit gene expression *in vivo*.

Sindbis (SIN) Virus Expression.

Sindbis molecular biology. SIN viruses are positive-stranded, enveloped, mosquito-borne RNA viruses of the genus *Alphavirus*, family *Togaviridae*, which cycle primarily between *Culex* mosquito species and birds (Strauss & Strauss, 1994). Alphaviruses are able to infect a wide variety of cells within an individual organism and across species. SIN virus infects and is able to replicate in mosquitoes, amphibians, birds and mammals,

including humans, as well as a variety of vertebrate and invertebrate cell cultures (Strauss & Strauss, 1994). The virus likely uses more than one cell surface molecule for attachment and entry (Klimstra *et al.*, 1998). The 5' and 3' nontranslated regions (NTR) in SIN are *cis*-acting regulatory regions. It is hypothesized that host proteins bind to these regions to promote replication. Mutations in the 5' or 3' NTR changes the host cell binding efficiency (Kuhn *et al.*, 1992). These regions are highly conserved, reflecting the optimum consensus sequence for replication in the three host cell types. Whereas infection of vertebrate cells by SIN virus triggers apoptosis and eventual wide-spread cell death, in mosquito cells SIN establishes a persistent infection with very little cytopathic effect (Jupp & Phillips, 1998; Karpf & Brown, 1998).

The 11.7-kb, single-stranded, plus-sense RNA genome is associated with the virus capsid, which consists of multiple copies of a single capsid protein species (Strauss & Strauss, 1994). The envelope consists of two envelope proteins, E1 and E2, arranged in a stable heterodimer embedded in a host-derived lipid bilayer. Three heterodimers associate to form the virus surface spike. The E2 glycoprotein is hypothesized to be involved in cell receptor binding (Klimstra *et al.*, 1998; Woodward *et al.*, 1991). During infection the E1 glycoprotein mediates fusion with the cell membrane (London *et al.*, 1992), and the E1-E2 heterodimers dissociate to form E1 monomers and E2 homotrimers. Upon entry into the cell cytoplasm, SIN nonstructural proteins are directly translated from the 5' end of the genome as a polyprotein from which four nonstructural proteins, ns1-ns4, are posttranslationally cleaved. A full-length minus strand of the genome is produced during replication, which serves as the template for positive strand transcription and also for transcription of the 26S RNA, which encodes viral structural proteins. The

26S RNA is transcribed from an internal initiation site, the subgenomic promoter, on the negative strand and is capped at the 5' end and polyadenylated at the 3' end (Strauss & Strauss, 1994; Xiong *et al.*, 1989). Structural proteins are translated as a polyprotein from the 26S mRNA, and processed cotranslationally and posttranslationally to produce the envelope proteins, the capsid protein, and the two small polypeptides E3 and 6K. Nontranslated nucleotides at the 5' and 3' ends and between the nonstructural and structural regions of the genome are cis-acting signal sequences for replication of the RNA and cellular protein binding sites (Kuhn *et al.*, 1990; Strauss & Strauss, 1994).

Double-subgenomic Sindbis infectious clones. An infectious clone of the SIN virus consensus sequence AR339 was constructed (Xiong *et al.*, 1989), and from that the double subgenomic SIN (dsSIN) virus TE/3'2J was developed (Hahn *et al.*, 1992). A second SIN subgenomic RNA promoter was inserted 3' to the structural genes of the virus in the 3' noncoding region (NCR), followed by the multiple cloning site, and 3' NCR (Fig 1.1). Heterologous mRNA gene sequences ligated into the multiple cloning site of TE/3'2J are transcribed in infected cells from the second subgenomic promoter (Hahn *et al.*, 1992; Higgs *et al.*, 1997; Olson *et al.*, 1998; Olson, 2000). Thus, in the double subgenomic Sindbis (dsSIN) virus three mRNA species are transcribed in infected cells: (1) the full-length genome; (2) RNA transcribed from the first subgenomic promoter, which also transcribes the heterologous sequences at the second subgenomic site; and (3) RNA transcribed from the second subgenomic promoter. All three of the RNA transcripts include the gene of interest (Hahn *et al.*, 1992). Heterologous sequences can include a gene of interest that produces a protein (Carlson *et al.*, 1995; Hahn *et al.*, 1992; Higgs *et al.*, 1993; Olson *et al.*, 1994; Xiong *et al.*, 1989), a truncated sequence,

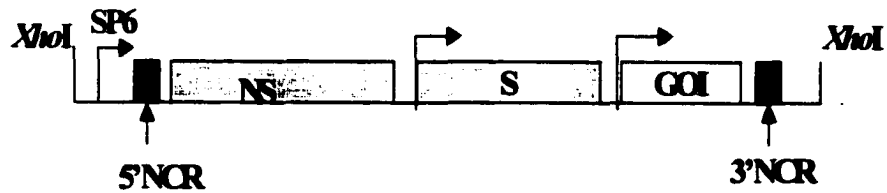


Figure 1.1 The dsSIN virus expression system. NS, nonstructural proteins; S, structural proteins; NCR, noncoding region.

or a nontranslatable effector RNA sequence (Olson *et al.*, 1996; Powers *et al.*, 1996; Powers *et al.*, 1994).

The dsSIN virus expression system is an effective and efficient system for gene delivery, because it infects a variety of cells, both vertebrate and invertebrate. Also, SIN virus gene expression occurs exclusively in the cytoplasm, which eliminates the need for processing and translocation signal sequences that complicate DNA gene delivery systems. SIN replicates rapidly and to a high titer, so that abundant RNA sequences or heterologous protein products can be produced (Powers *et al.*, 1996; Xiong *et al.*, 1989).

The dsSIN viruses TE/3'2J and MRE/3'2J readily infect *Ae. aegypti* and *Ae. triseriatus* mosquitoes. Effector RNAs complementary to specific gene sequences within the genomes of yellow fever (YF) and dengue-2 (DEN-2) viruses (Flaviviridae) and LaCrosse virus (Bunyaviridae) have been identified that inhibit the replication of YF and DEN-2 viruses in *Ae. aegypti*, and LaCrosse virus in *Ae. triseriatus* (Higgs *et al.*, 1998; Olson *et al.*, 1996; Powers *et al.*, 1996; Powers *et al.*, 1994).

TE/3'2J. The dsSIN virus TE/3'2J was developed from the consensus cDNA SIN virus AR339 (Hahn *et al.*, 1992). Long-term cell culture of SIN strain AR339 in vertebrate cells and mice has resulted in adaptive mutations in the virus (Hahn *et al.*,

1992). The virus readily infects mosquito cells *in vitro*, and mosquito cells *in vivo* when it is intrathoracically inoculated, but it is not orally infective and does not infect midgut tissue by the intrathoracic inoculation route (Olson *et al.*, 1996; Rayms-Keller *et al.*, 1995; Seabaugh *et al.*, 1998). Virus attachment and penetration studies using strains of Sindbis differing by a single amino acid change in the structural glycoproteins shows that adaptive mutations are often not relevant *in vivo* but can occur in one passage in cell culture (Klimstra *et al.*, 1998).

MRE16. In contrast, the Malaysian SIN virus strain MRE16 infects midgut cells upon oral infection, and disseminates throughout the mosquito. Structural protein genes of MRE16 are similar in sequence to the Sindbis strains circulating in nature (Seabaugh *et al.*, 1998).

Comparison of TE/3'2J and MRE16 nucleotide sequences. The mutations that contribute to the attenuation of a virus have been determined by nucleotide sequence comparisons between virulent viruses and their progeny vaccine strains (Kuhn *et al.*, 1992; Miller *et al.*, 1982; Woodward *et al.*, 1991; Yoksan *et al.*, 1986). Mutations causing amino acid substitutions in the structural proteins of the virus have been shown to modulate virus entry into particular host cell types and to determine tissue tropisms (Kuhn *et al.*, 1992). As noted previously, the SIN virus MRE16 readily infects the midgut *per os*, but TE/3'2J does not (Hahn *et al.*, 1992). MRE16 structural gene sequences are more similar to the SIN strains in nature, whereas TE/3'2J is based on laboratory passaged viruses. To determine the nucleotide sequences that condition this virus phenotype, the nucleotide sequence of TE/3'2J was compared to MRE16 (Seabaugh *et al.*, 1998). Determinants for midgut infectivity are located in the structural proteins of the

viruses. Nucleotide differences between the two strains were noted in the N-terminal of the capsid protein, the E3 protein and the membrane-spanning domain of the E2 glycoprotein (Seabaugh *et al.*, 1998). Mutations in these sequences yielded differences in virus production depending on the host cell used in the assay and in virus structural proteins (Kuhn *et al.*, 1990; Kuhn *et al.*, 1992). E2 is the least conserved of the two glycoproteins (London *et al.*, 1992). One amino acid change in E2, hypothesized to mediate virus attachment, can alter cellular tropism or cell membrane binding efficiency (Klimstra *et al.*, 1998; Kuhn *et al.*, 1992; Strauss & Strauss, 1994; Tucker & Griffin, 1991; Woodward *et al.*, 1991; Yao *et al.*, 1996; Brown & Condreay, 1986). An amino acid change of isoleucine to phenylalanine in the E2 glycoprotein of Venezuelan equine encephalitis reduced infectivity in C6/36 cells 1000-fold, possibly by changing the E2 conformation (Woodward *et al.*, 1991). Wild-type Sindbis virus (AR339) causes encephalitis in mice, yet one amino acid change in E2 at amino acid 172 position from glycine to arginine reduced neurovirulence and neural cell binding affinity. Nonneural cell binding was not affected and replication capacity was not altered (Tucker & Griffin, 1991).

MRE/3'2J chimeric virus. Chimeric viruses have been constructed to study determinants of virus entry and tissue tropism (London *et al.*, 1992). Sindbis-Rift Valley fever chimeric virus has been used to study the interactions between the structural and nonstructural regions of the Alphavirus genome (Kuhn *et al.*, 1990; Kuhn *et al.*, 1991; Kuhn *et al.*, 1992; Yao *et al.*, 1996). A chimeric virus was constructed containing the structural genes of MRE16 and the nonstructural genes and 3' noncoding region of TE/3'2J. The chimeric virus, designated MRE1001, had growth characteristics and tissue

tropism of MRE16. Importantly, it infected mosquito midgut tissue when administered in a blood meal (Seabaugh *et al.*, 1998). From MRE1001, the dsSIN expression system MRE/3'2J was constructed; it contains the structural genes of MRE16 and the nonstructural genes, second subgenomic promoter, and 3' noncoding region of TE/3'2J (Olson *et al.*, 2000). This dsSIN expression system was used to introduce antisense RNA genes *per os* to mosquito larvae. The dsSIN systems provide powerful new tools to rapidly characterize genes *in vivo*.

CONCLUSIONS

The incidence of diseases caused by mosquito-borne pathogens is increasing. Attempts to eradicate diseases through vector control or vaccination have not been successful. Indeed, the distribution of *Ae. aegypti*, the vector of DEN and YF virus has increased. Currently, nearly half the world population lives in areas where they are at risk for being infected with an mosquito-borne virus, and vaccines are lacking for many important diseases. In addition, mosquitoes are increasingly becoming resistant to insecticides. Clearly, innovative approaches to control mosquito populations are needed. In addition, methods for reducing vector competence are essential. However, factors that condition vector competence, such as genetic determinants and environmental conditions, are still largely unknown. Much less is known about the mosquito than the medically unimportant *Drosophila*. As mosquito transgenesis is extremely time consuming and labor intensive, transient methods of determining gene function are needed. Inhibition of gene expression and observing the biological effect has been used to characterize genes in many organisms, including *Drosophila*. The SIN virus transducing system expressing antisense sequences has been used successfully to inhibit viral replication *in vitro* and *in vivo*.

The overall hypothesis for this research is that endogenous gene expression can be posttranscriptionally inhibited by association of RNA complementary to specific endogenous mRNA in the mosquito cell cytoplasm. Such gene expression studies will help identify factors involved in pathogen transmission and potential target sites for gene alteration. Expression of an endogenous mosquito gene can be specifically inhibited through antisense RNA delivered by the dsSIN virus expression system. In this dissertation, the dsSIN expression system was used to deliver RNA complementary to mosquito mRNA to *Ae. aegypti* salivary glands. TE/3'2J and MRE/3'2J viruses were used as gene expression systems. Both TE/3'2J and MRE/3'2J viruses were evaluated for their ability to infect salivary gland tissue and to inhibit target gene expression.

Chapter 2

Inoculation of the Double-Subgenomic Sindbis Virus Vector

TE/3'2J/ α -apyrase to Inhibit Apyrase Expression in *Aedes aegypti*

INTRODUCTION

TE/3'2J virus readily infects *Aedes aegypti* salivary gland tissue when intrathoracically inoculated into adults (Higgs *et al.*, 1993; Olson *et al.*, 1998). Heterologous genes inserted into the multiple cloning site by the second subgenomic promoter are efficiently expressed in mosquito cells and mosquitoes (Higgs *et al.*, 1995; Olson *et al.*, 1994). RNA sequences complementary to yellow fever and dengue viruses genomic sequences when inserted in TE/3'2J have inhibited the replication of these viruses in *Ae. aegypti* (Higgs *et al.*, 1998; Olson *et al.*, 1996). A similar experimental design was used to try to inhibit expression of and to characterize the endogenous mosquito gene apyrase.

Apyrase is produced in the distal lateral and medial lobes of the salivary glands of female adult mosquitoes upon eclosion (James, 1994; Ribeiro *et al.*, 1984; Ribeiro *et al.*, 1990; Smartt *et al.*, 1995). Expression is under the control of a constitutive promoter, and apyrase protein is abundant throughout the lifetime of the adult mosquito. Apyrase functions as a platelet aggregating inhibitor by cleaving phosphate from ADP and ATP. ADP and ATP released from damaged cells are powerful signals for platelet aggregation to effect hemostasis. Apyrase is an antihemostatic salivary gland protein common to many hematophagous arthropods (Champagne *et al.*, 1995). Therefore, characterization of apyrase by inhibition of expression is relevant to a large group of medically important insects.

The hypothesis for this study was that apyrase gene expression could be inhibited by infection with TE/3'2J/ α -apy virus, which contains RNA complementary to the *Ae. aegypti* apyrase mRNA. A sequence containing 375 bases of the coding region of the 1.7 kb *Ae. aegypti* apyrase cDNA (Champagne *et al.*, 1995) was inserted in antisense

orientation into pTE/3'2J at the multiple cloning site in the second subgenomic promoter to produce TE/3'2J/ α -apy virus (A. Powers and K. Olson, unpublished). TE/3'2J/ α -apy virus was inoculated into *Ae. aegypti* adults and larvae, and apyrase protein production in adult salivary gland tissue was analyzed for inhibition.

MATERIALS AND METHODS

Construction of TE/3'2J/ α -apy Virus. The construction of pTE/3'2J has been described (Hahn *et al.*, 1992; Higgs *et al.*, 1997). cDNA was obtained of the 1.7 kb *Ae. aegypti* apyrase gene (Champagne *et al.*, 1995); 375 bp from the 5' end of the apyrase cDNA was inserted at the *Xba*I site of pTE/3'2J (Fig. 2.1) in antisense orientation. The insert sequence, size, and orientation was confirmed by nucleotide sequencing of pTE/3'2J/ α -apy (data not shown).

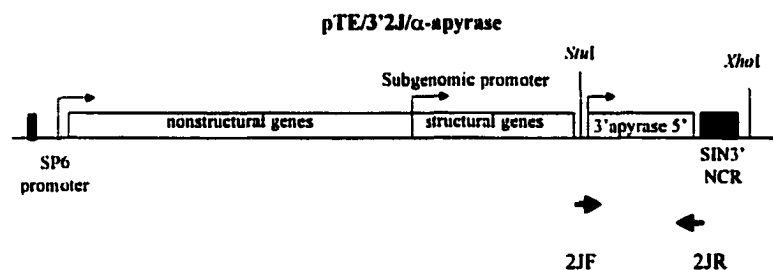


Figure 2.1 dsSIN virus expression system pTE/3'2J/ α -apy. The first 375 bases from the coding region of the apyrase gene were inserted in 3'-to-5' orientation into pTE/3'2J in the multiple cloning site after the second subgenomic promoter. Nucleotide sequence and insert orientation were confirmed by sequence analysis of the PCR product amplified using primers 2JF and 2JR (Table 2.1).

Generation of dsSIN Viruses. Recombinant virus was produced from pTE/3'2J/ α -apy by linearization with *Xho*I, transcription from the SP6 promoter, and electroporation (450 V, 100 μ F, 720 ohms, 0.7-0.9 msec) into baby hamster kidney 21 (BHK-21) cells (Powers *et al.*, 1994). Tissue culture 50% infective dose (TCID₅₀) of the virus, measured

as log₁₀ per ml, was determined by titration in triplicate in BHK-21 cells. A TCID₅₀ of 9.2-9.5 log₁₀/ml was used for inoculations.

Mosquito Rearing. Rexville strain from Puerto Rico (RexD) *Ae. aegypti* mosquitoes were maintained during the course of the experiments at 28°C and 80% humidity, with 12-hr light/dark cycles, and sugar and water provided *ad libitum*. In experiments requiring blood-fed mosquitoes, the preparation of blood and presentation of the blood meal was performed as previously described (Higgs & Beaty, 1996).

Intrathoracic Inoculation of Mosquitoes.

Adults. Adult female mosquitoes were cold anesthetized and intrathoracically injected using a Drummond 50- μ l microcapillary needle that had been prepared with a needle puller (Narishige Co., Toyko, Japan). Approximately 1 μ l of dsSIN virus ($\cong 10^5$ infectious particles) in Leibovitz (L-15) medium containing 10% FBS and 1% penicillin/streptomycin was injected into each mosquito (Rosen & Gubler, 1974).

Larvae. Fourth instar larvae were pipetted onto a wet paper towel. Larvae were injected intrathoracically with 1 μ l of dsSIN virus using a dissecting microscope. After inoculation, larvae were removed from the paper towel with a paintbrush and placed into a hatching pan.

Indirect Immunofluorescence Assay (IFA). Salivary glands from each treatment group were dissected in water, mounted on acid-washed slides in a droplet of diluted (1:1000) Elmer's glue, dried, and fixed in acetone for 10 min at -20°C (Higgs *et al.*, 1997). Infection of the salivary glands with dsSIN virus was confirmed by IFA using a primary antibody that recognizes Sindbis E1 antigen (Chanas *et al.*, 1982), biotinylated sheep anti-mouse secondary antibody (Amersham)/1% Evan's blue in PBS, and streptavidin-

fluorescein tertiary antibody (Amersham), all diluted 1:200 in PBS. Salivary glands were visualized using an Olympus BH-2 epifluorescence microscope.

Apyrase Activity Assay. Apyrase activity was determined using a published protocol (Ribeiro *et al.*, 1984) and an inorganic phosphorus commercial kit (Sigma). Salivary glands were dissected in 0.15 M NaCl and transferred to 100 μ l of 10 mM TrisCl (pH 7.5). Salivary glands were homogenized in an Aquasonics waterbath (VWR Scientific) for 2 min at 50°C (power level 5), then centrifuged at 8000 g for 1 min. Five microliters of salivary gland homogenate were added to 95 μ l of reaction medium (100 mM TrisHCl buffer pH 9.0, 200 mM NaCl, 10 mM CaCl₂, and 20 mM ADP sodium salt) and incubated at 37°C for 30 min. The reaction was stopped by adding 25 μ l of ammonium molybdate in 2.5N sulfuric acid (Sigma). Three microliters of F&S reducing reagent (Sigma) was added. then the plate was placed in the dark for 20 min. The amount of inorganic phosphate released was measured using a 450 Plate Reader (BioRad, Richmond, CA) with a 665-nm filter. Apyrase activity was calculated as the total amount of monophosphate released per pair of salivary glands.

Polymerase Chain Reaction. PCR amplification was conducted in a 50- μ l reaction containing 50 ng of template, 50 pmoles forward and reverse primer, 0.2 mM dNTPs, 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-Cl (pH 9.0), and 0.1% Triton X-100. The PCR amplification cycle consisted of 95°C for 5 min, 80°C for 2 min, during which time the Taq polymerase was added, followed by 25 amplification cycles: 93°C for 1 min, 56°C for 30 sec, 70°C for 2 min, with a final extension at 72°C for 2 min. Primers used in PCR reactions in these experiments are listed in **Table 2.1**.

Table 2.1 Primers used in PCR reactions

Primer name	Nucleotide sequence
2JF	5'-GTCAGCATAGTACATTTTCATC-3'
2JR	5'-GCTGGTCGGATCATTGGGGCG-3'
EndApyF	5'-AGCCGCATGATCCAAAGGACA-3'
EndApyR	5'-TTCCGAAAATTGCCTGCTTGAAT-3'

Polyacrylamide Gel Electrophoresis (PAGE). Salivary glands were dissected in 0.15 M NaCl then transferred to a tube containing 12 μ l loading buffer (50 mM Tris-Cl pH 7.5, 1% SDS, 2 mM phenyl methyl sulphuryl fluoride, 1 mM EDTA, 10% glycerol, 5% mercaptoethanol, 0.2% bromophenol blue). Homogenization consisted of three cycles of freeze-thawing, and boiling for 2 min. One complete pair of salivary glands was loaded per lane, and proteins were separated on a 10% SDS-PAGE 1-mm gel (Novex, Hercules, CA) by electrophoresis in a BioRad II gel box at 150 mV for 1 hr.

Silver Staining of Salivary Gland Proteins. Proteins in the PAGE gel were fixed by washing for 1 hr in ice-cold 10% acetic acid, followed by three 5-min washes in distilled water. The PAGE gel was incubated on a shaker for 30 min in silver-staining solution consisting of 5.8 mM silver nitrate and 0.056% formaldehyde in distilled water, followed by a brief wash in distilled water. The silver stain was developed in a chilled (4°C) 2.8 mM sodium carbonate, 0.056% formaldehyde, 2.8 mM sodium thiosulfate solution. Development was stopped by a rinse in cold (4°C) 7% acetic acid.

Western Blot Assay. Proteins were transferred from the PAGE gel to a nitrocellulose membrane (Trans-blot, BioRad) in a BioRad transfer apparatus (transfer buffer: 25 mM Tris, 192 mM glycine, 10% methanol; pH 8.3) at 200 mA for 3 hr. Proteins were detected using the ECL-Western blotting kit according to manufacturer's instructions (Amersham), except that PBS-0.05% NP-40 was used for blocking and all washes. A

polyclonal rabbit anti-apyrase antibody raised to recombinant apyrase peptide, diluted 1:20,000, detected the 68-kd apyrase protein (Champagne *et al.*, 1995). As an internal control, polyclonal rabbit anti-D7 antibody, diluted 1:2000, detected the 37-kd D7 protein (James, 1994). Amersham anti-rabbit HRP secondary antibody, diluted 1:1000, was used as secondary antibody. The membrane was exposed to Kodak X-Omat AR (VWR Scientific) X-ray film for 2 sec. Total salivary gland protein was detected by rinsing the membrane twice in PBS-0.05% NP-40 then incubating with 50-ml colloidal gold (Bio-Rad) for 1 hr.

Virus Assays of Infected Mosquitoes. Mosquito heads and abdomens in L-15 diluent were triturated with a pestle, and the suspension was passed through a 0.2- μ m pore-size filter, as previously described (Higgs *et al.*, 1997). Virus titers were determined by end point dilution assay in triplicate in Vero cells (Karber, 1931).

RNA Collection and Isolation. Total cellular RNA was isolated from infected C6/36 cells using the one-step guanidinium thiocyanate extraction method (Chomczynski & Sacchi, 1987). Salivary glands were dissected in PBS (pH 7.2) and collected in a tube containing 50 μ l of ice-cold PBS. Five hundred microliters of Trizol (GibcoBRL) was added to the tube, the salivary glands were immediately homogenized with a pestle, then frozen at -70°C until RNA isolation. Mosquito salivary gland RNA was isolated using the manufacturer's directions (Trizol, GibcoBRL). Briefly, tubes were thawed, then incubated at room temperature for 5 min. Chloroform (0.2 ml chloroform/1 ml Trizol) was added to the tube, and after thorough mixing by vigorous shaking, the tube was incubated for 5 min at room temperature. Centrifugation at 11,000g for 15 min at 4°C separated the RNA, DNA, and protein into the top aqueous layer, the interphase, and the

bottom pink layer, respectively. The RNA in the top aqueous layer was removed into a clean tube. The remaining DNA/protein mixture was stored at -70°C . RNA was precipitated by addition of isopropanol (0.5 ml/1 ml Trizol), incubation at room temperature for 10 min, then centrifugation for 10 min at 12,000g. The RNA was washed once with 500 μl 70% ethanol in RNase-free water, then pelleted by centrifugation at 4°C , 47,500g for 5 min. RNA was resuspended in RNase-free water warmed to 60°C and stored at -70°C until use.

RNA Slot-Blot Assay. Five micrograms of total RNA in 150 μl of 6X SSC, 7% formaldehyde was pipetted onto a BrightStar-Plus membrane (Ambion) in a Minifold II (Schleicher and Schuell, Keene, NH) slot-blot apparatus connected to a vacuum. A PCR product specific for apyrase mRNA downstream of the apyrase insert in TE/3'2J/ α -apy was labeled with a psoralen-biotin conjugate according to manufacture's instructions (BrightStar, Ambion). The probe was hybridized to the membrane in Church's hybridization buffer (1mM EDTA, 0.5 M NaPO_4 , 7% SDS) in a 60°C rotating water bath for 18 hr. Following hybridization, the membrane was washed twice for 10 min in 2X SSC, 0.1% SDS, followed by two high-stringency washes for 30 min at 60°C in 0.2X SSC, 0.1% SDS.

RNA was visualized using the BrightStar BioDetect detection kit according to manufacturer's instructions (Ambion). The membrane was exposed to Kodak X-Omat AR (VWR Scientific, West Chester, PA) X-ray film for 5 min.

RESULTS

Characterization of TE/3'2J/ α -apy. pTE/3'2J/ α -apy was characterized by PCR amplification and sequencing of the apyrase insert and the multiple cloning site of

pTE/3'2J. Primers 2JF and 2JR amplified the insert and 95 bases of the surrounding pTE/3'2J plasmid (Fig 2.1). Both strands were sequenced from the PCR product from the 2JF and 2JR positions (University of California, Davis) and the nucleotide sequence was aligned with the GeneBank apyrase sequence (Champagne *et al.*, 1995) using the DNASTar (DNA Star Inc., Madison, WI) program. The insert contained the ATG start codon, and 375 bases of the coding sequence in 3'-to-5' orientation in the pTE/3'2J plasmid (data not shown).

Profile of Apyrase Expression in Salivary Glands of Mosquitoes Infected with TE/3'2J/ α -apy Virus. Female adult mosquitoes that had eclosed within 24 hr were cold anesthetized and intrathoracically injected with TE/3'2J or TE/3'2J/ α -apy virus. Controls were cold anesthetized only. At 48 hr postinoculation, immediately following a blood meal (5 days), and 48 hr after blood feeding (7 days), salivary glands were dissected and transferred to Eppendorf tubes containing 10 mM Tris-Cl (pH 7.5), and then assayed for apyrase activity (Table 2.2). Mosquito carcasses were titrated to confirm virus infection.

Table 2.2 Apyrase activity in salivary glands before and after blood feeding

	Mean virus titer (TCID ₅₀)	N	Mean apyrase activity* (\pm SD)
<i>Before blood meal</i>			
Uninoculated control		6	202 (54)
TE/3'2J inoculated	4.7	6	171 (81)
TE/3'2J/ α -apy inoculated	4.1	5	235 (62)
<i>After blood meal</i>			
Uninoculated control		8	186 (81)
TE/3'2J inoculated	4.2	5	115 (74)
TE/3'2J/ α -apy inoculated	3.6	6	231 (53)
<i>48 hr post blood meal</i>			
Uninoculated control		5	218 (62)
TE/3'2J inoculated	3.8	3	183 (55)
TE/3'2J/ α -apy inoculated	3.5	4	229 (80)

*Apyrase acitivity was measured as the amount of monophosphate produced per salivary gland homogenate.

Apyrase activity in the TE/3'2J/ α -apy infected mosquitoes, uninoculated controls, and TE/3'2J-inoculated controls did not differ significantly ($p=0.5$) throughout the time course of the experiment. After blood feeding, apyrase activity decreased by 10-30% in all groups, then increased to pre-blood-fed levels within 48 hr, as measured by apyrase activity assay (Table 2.2). This included the mosquitoes infected with TE/3'2J/ α -apy virus.

Salivary Gland Protein Profile. Total salivary gland protein was separated on an SDS-PAGE gel and silver stained (Fig 2.2). Apyrase appeared as a band at 66 kd. Although the band intensity varied between samples, a quantitative measurement could not be made. Therefore, a Western blot was done using a primary antibody specific for apyrase (Fig. 2.3). At all time points, including immediately after blood feeding and 24 hr later, the amount of apyrase protein per pair of salivary glands was equivalent in both uninoculated control and TE/3'2J/ α -apy virus infected mosquitoes.

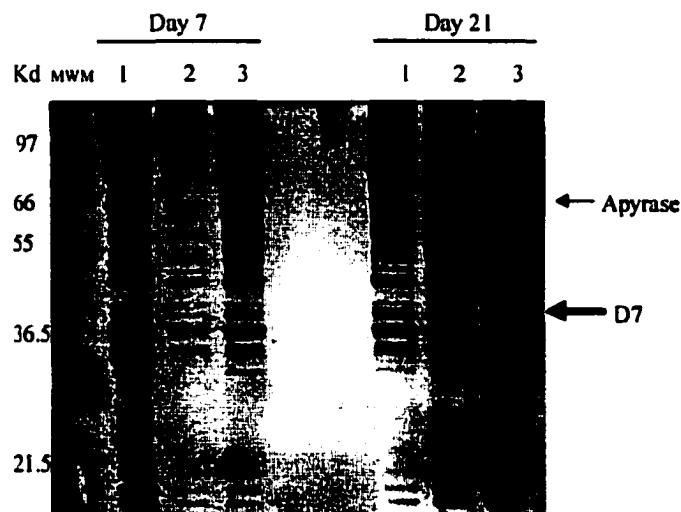


Figure 2.2 Salivary gland protein profile. Salivary glands were dissected and proteins were separated on an SDS-PAGE gel. Proteins were visualized by silver stain. Apyrase is seen at 66 kd (*thin arrow*), D7 protein at 37 kd (*heavy arrow*). Each lane represents one pair of salivary glands. Lane 1, uninoculated control; lane 2, TE/3'2J control inoculated; TE/3'2J/ α -apy inoculated. MWM, molecular weight marker.

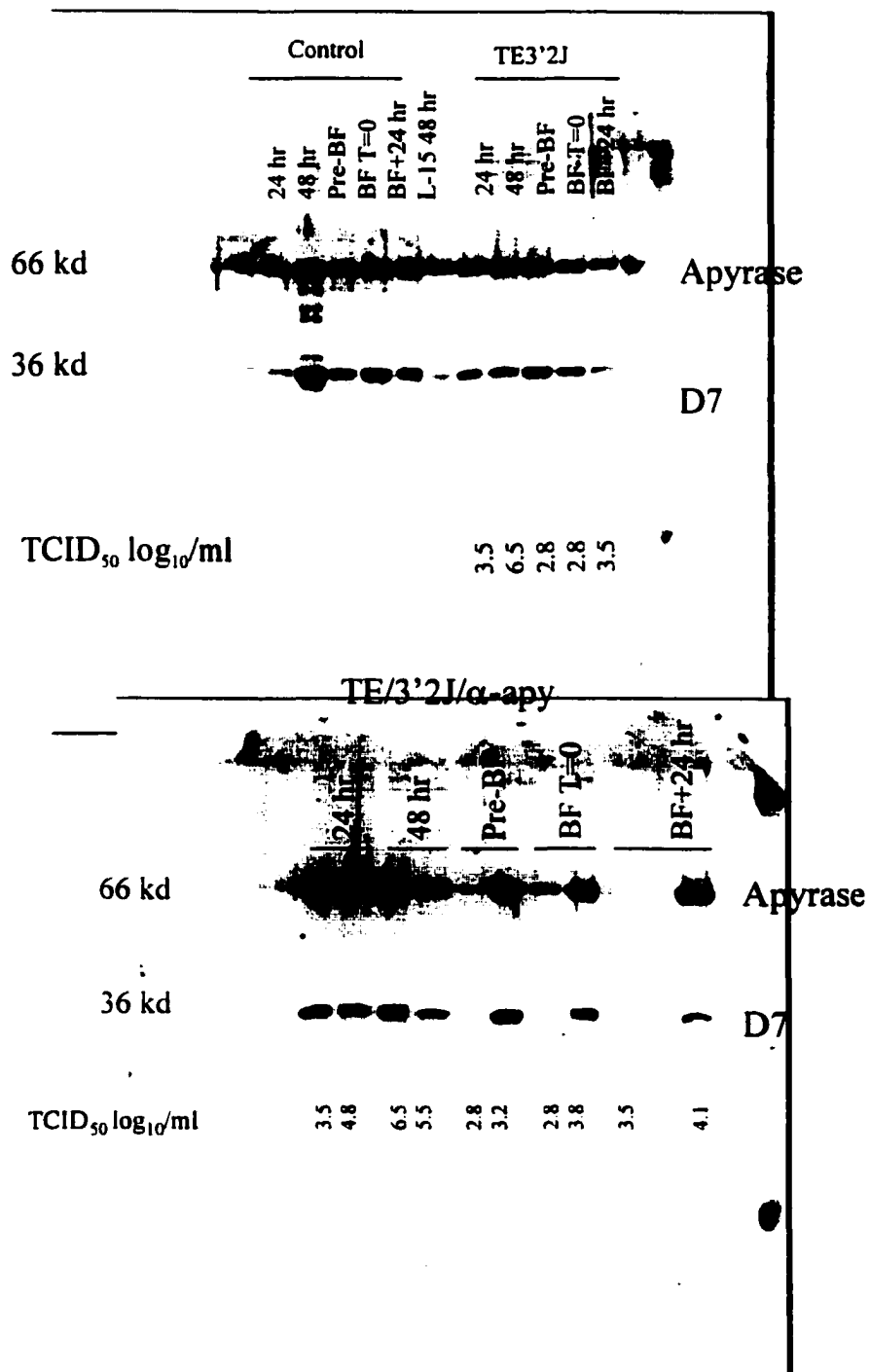


Figure 2.3 Immunoassay detection of apyrase and D7 in salivary glands of uninoculated, TE/3'2J control, and TE/3'2J/α-apy infected mosquitoes. Salivary glands were dissected and proteins separated on an SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with primary antibodies to apyrase and D7. D7 served as a control salivary gland protein. Abbreviation: BF, blood fed.

In another experiment, fourth instar larvae were inoculated with either TE/3'2J/ α -apy, TE/3'2J, or L-15 medium. Apyrase assay was measured in adult females, both by apyrase activity assay and Western blot assay. No difference was seen in apyrase activity or production between control inoculated and TE/3'2J/ α -apy infected mosquitoes (data not shown). However, mortality of inoculated larvae was great: 30-50% in L-15, 90-92% in TE/3'2J, and 63-93% in TE/3'2J/ α -apy over two experiments.

IFA of Salivary Glands. SIN antigen was detected by IFA in salivary glands of all mosquitoes inoculated with TE/3'2J/ α -apy or TE/3'2J (**Fig 2.4**) Approximately 20% of infected salivary glands visualized by IFA were incompletely infected. In these salivary glands the medial lobe was the area most frequently not infected by SIN.

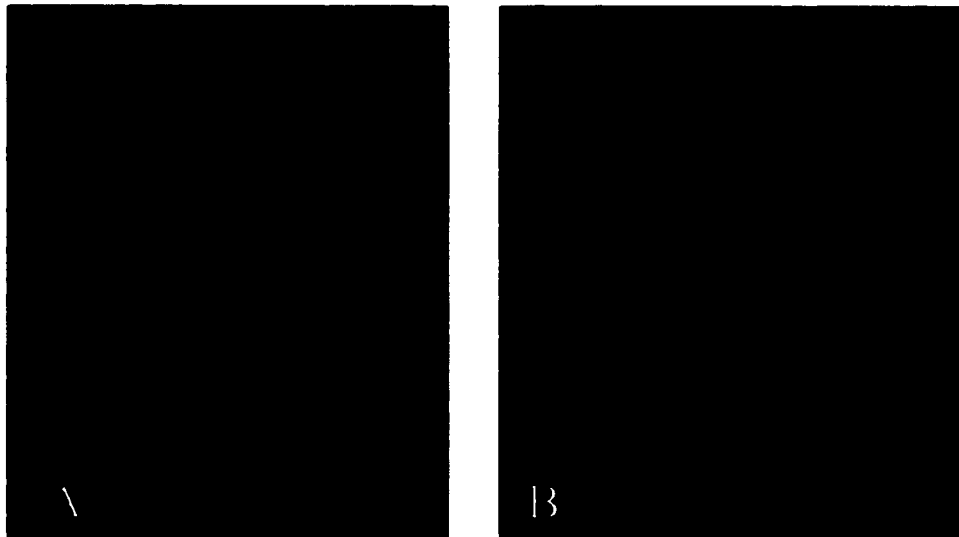


Figure 2.4 SIN infection of salivary glands. IFA using antibody 30.11a, which recognizes SIN E1 envelope protein. (A) TE/3'2J/ α -apy infected salivary glands 48 hr postinoculation. (B) Salivary glands infected with TE/3'2J dissected immediately following a blood meal. Original magnification 200X.

Analysis of Endogenous Apyrase RNA. To determine if degradation of apyrase RNA could be detected in mosquitoes infected with TE/3'2J/ α -apy virus, RNA extracted from pooled salivary gland homogenates was assayed by slot blot. An RT-PCR psoralen-biotin labelled probe was used that was specific for apyrase mRNA downstream of the apyrase insert in TE/3'2J/ α -apy (EndApyF and EndApyR, Table 2.1) (Champagne *et al.*, 1995). However, no difference in apyrase total RNA between TE/3'2J/ α -apy inoculated mosquitoes and uninoculated controls was seen (Fig. 2.5).

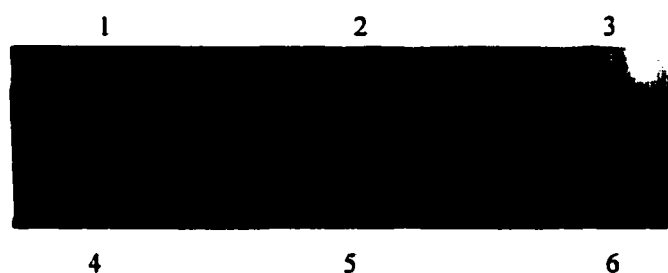


Figure 2.5 Slot blot analysis of apyrase RNA from uninfected, TE/3'2J, and TE/3'2J/ α -apy infected mosquitoes. 1, C6/36 negative control; 2, uninfected 48 hr posteclosion; 3, uninfected immediately after bloodfeeding (5 days); 4, uninfected; 5, TE/3'2J infected; 6, TE/3'2J/ α -apy infected. Samples 4-6 were all non-blood-fed, 5-day-old mosquitoes. Five micrograms of pooled salivary gland RNA was loaded per lane. A biotin-labeled PCR product specific for apyrase downstream of the insert detected endogenous apyrase RNA.

DISCUSSION

Jose Ribeiro and colleagues originally characterized apyrase in the salivary glands of *Ae. aegypti* (Ribeiro *et al.*, 1984). The apyrase enzyme activity assay measured the amount of orthophosphate cleaved from ATP or ADP by apyrase in salivary gland extracts. By this assay, apyrase was not detectable until 24-48 hr posteclosion. Apyrase activity reached peak activity at 2-3 days posteclosion, decreased by 45% after a blood meal, then

increased to preblood meal levels by 24 hr post blood feeding (Marinotti *et al.*, 1990). In contrast, using a specific antibody (Champagne *et al.*, 1995), apyrase was detectable upon eclosion, and no decrease in apyrase protein was seen after blood feeding (Smartt *et al.*, 1995). Possibly apyrase is produced and stored initially as a zymogen, which is activated upon blood feeding. This would explain the discrepancy in the amount of apyrase measured by these two assays. However, no data in the literature suggests that this is the case.

The experimental design in this dissertation was based on these previous studies. It was hypothesized that if the apyrase antisense RNA could be expressed in the salivary glands directly after eclosion, the abundant RNA transcripts produced by the dsSIN vector TE/3'2J/ α -apy potentially could inhibit apyrase protein synthesis. Therefore, adult mosquitoes were intrathoracically inoculated as soon after eclosion as possible, at the latest at 24 hr.

In addition, it was reasoned that even if apyrase was abundant in the salivary glands at 48 hr, by the time the TE/3'2J/ α -apy virus established a productive infection, blood feeding might deplete the stored apyrase. After blood feeding, apyrase production would increase to preblood feeding levels in uninfected controls. Inhibition of apyrase production might be observed in the TE/3'2J/ α -apy infected mosquitoes at this time point (Marinotti *et al.*, 1990). However, in all studies described in this dissertation, no decrease in apyrase protein was observed at any time point in uninfected controls. Similarly, apyrase protein was also abundant in the TE/3'2J/ α -apy virus-infected mosquitoes.

Larvae were inoculated with TE/3'2J/ α -apy virus in another experiment in order to infect the mosquitoes prior to development of salivary glands, and hence before

transcription of apyrase mRNA. Larvae inoculated in this experiment developed normally until pupal stage, when approximately 90% died during eclosion. SIN infection of salivary glands in surviving infected mosquitoes was incomplete compared to adult inoculated mosquitoes, as determined by IFA. Correspondingly, in mosquitoes inoculated as larvae, apyrase activity was not decreased in adult salivary glands.

Apyrase is constitutively expressed in adult female mosquitoes. Apyrase is abundant upon adult eclosion and throughout the lifetime of the mosquito, including immediately following a blood meal (**Fig. 2.3**). Saliva is stored in acinar spaces in the salivary glands until the mosquito probes for a blood meal, at which time the saliva passes into the salivary gland ducts and is secreted into the host. Apyrase may be a very stable protein that can be stored for a long time. This would seem to be likely because the mosquito saliva must be available to feed immediately upon encountering a host, as there would be no time to synthesize, then secrete the necessary proteins. If salivary proteins were not immediately available, then the time required for protein synthesis might result in missed feeding opportunities

Antisense inhibition of gene expression is hypothesized to occur posttranscriptionally before protein translation. Duplex formation between the antisense RNA and mRNA signals dsRNA degradation by cellular RNases. To determine if stored apyrase protein was masking mRNA degradation in mosquitoes infected with TE/3'2J/ α -apy, apyrase RNA levels were compared between uninfected and TE/3'2J/ α -apy-infected salivary glands by slot blot (**Fig. 2.5**). Again, no difference in apyrase RNA was observed between uninfected and infected mosquitoes.

Another possible explanation for the lack of inhibition of apyrase gene expression in TE/3'2J/ α -apy virus infected salivary glands is that the viral RNA is not localized in the same cellular compartments as endogenous apyrase mRNA. The model for antisense inhibition of gene expression assumes that the antisense RNA and mRNA hybridize to form dsRNA, which are then degraded by cellular RNases (Fire, 1999). In previous studies using the dsSIN vector to deliver antisense transcripts to block replication of arboviruses, both the target and effector molecules were RNA viruses, which probably were processed and replicated in the same cellular sites (Higgs *et al.*, 1998; Olson *et al.*, 1996).

Thus attempts to knock out apyrase expression by antisense inhibition were unsuccessful. This could be due to many factors including use of the virus vector TE/3'2J that is ill adapted for *in vivo* mosquito studies. In addition, the selection of apyrase as a target molecule may be ill suited for antisense suppression, because the protein is too stable, too abundantly expressed, and differences in expression difficult to measure. In subsequent chapters some of these factors are addressed.

In Chapter 3, an endogenous gene model system was designed using a transgenic mosquito. APY-LUC43 transgenic mosquitoes are stably transformed with a luciferase gene controlled from a cloned *Ae. aegypti* apyrase promoter. Hence, luciferase is expressed in the same salivary gland tissue and at the same developmental stage in these transgenic mosquitoes as apyrase is expressed in wild-type *Ae. aegypti*. Additionally, luciferase activity can be readily assessed, with slight differences in expression easily measurable in a luminometer. Results of these experiments are reported in the next chapter.

Chapter 3

Inhibition of Luciferase Expression in Transgenic *Aedes aegypti* by Intrathoracic Inoculation of the dsSIN Expression Vector TE/3'2J/ α -luc

INTRODUCTION

The dsSIN virus TE/3'2J can be engineered to express homologous antisense RNA and thereby inhibit replication of yellow fever (Higgs *et al.*, 1998), dengue (Olson *et al.*, 1996), and LaCrosse (Powers *et al.*, 1996; Rayms-Keller *et al.*, 1995) viruses in mosquito cells and mosquitoes. The mechanism of interference may occur through base pairing of the virus RNA and the complimentary sequence expressed by TE/3'2J, with subsequent degradation of the double-stranded RNA by cellular ribonucleases (Coleman *et al.*, 1984; Green *et al.*, 1986; Izant & Weintraub, 1985; Olson *et al.*, 1996). Alternatively, the dsRNA replicative intermediate structure of the virus vector may trigger inhibition (Ratcliff *et al.*, 1999). Both the SIN virus and the infecting virus are RNA viruses; they replicate entirely in the cytoplasm, and probably in the same cellular compartments. Thus, to inhibit expression of an endogenous gene, the SIN virus may need to be in the same cellular compartment as the mRNA coming from the nucleus.

The ability of antisense RNA delivered by the dsSIN virus vector to specifically inhibit expression of an endogenous gene was tested in a transgenic line of *Ae. aegypti* (Jasinskiene *et al.*, 1998). This line was developed using a binary *Hermes* transposable element system derived from *Musca domestica* (Jasinskiene *et al.*, 1998; Sarkar *et al.*, 1997; Warren *et al.*, 1994) to transform a white-eye mutant (*kh*^v) from the Rockefeller strain of *Ae. aegypti* (Bhalla, 1968). The *kh*^v mutant has a defect in the gene for *kynurenine hydroxylase*, an eye pigment enzyme. The *Hermes* donor plasmid contained a phenotypic marker gene, *cinnabar*, derived from *Drosophila melanogaster* (Cornel *et al.*, 1997), which complements the *kynurenine hydroxylase* gene, and a quantifiable reporter gene, *luciferase (luc)*, regulated by the *Ae. aegypti* *Apyrase (Apy)* promoter

(Coates *et al.*, 1999). In the transgenic mosquitoes, luciferase is expressed in the same developmental- and tissue-specific manner as the endogenous *Apy* gene in wild-type *Ae. aegypti* (Coates *et al.*, 1999; Smartt *et al.*, 1995), in the distal-lateral and medial lobes of the salivary glands of female adult mosquitoes. dsSIN viruses infect the salivary glands of *Aedes* spp. when intrathoracically injected into adults (Olson *et al.*, 1994; Rayms-Keller *et al.*, 1995). To test the hypothesis that the dsSIN could be exploited to inhibit gene expression of luciferase in these mosquitoes, a dsSIN virus, designated TE/3'2J/anti-luc, was engineered to express a 595-base RNA sequence complementary to the 5'-end of the luciferase mRNA (Johnson *et al.*, 1999). The results from these experiments demonstrate the sequence-specific inhibition of luciferase protein production in salivary glands of transgenic mosquito through expression of antisense RNA.

MATERIALS AND METHODS

Construction of TE/3'2J/ α -luc and Control dsSIN Viruses. The construction of pTE/3'2J (Hahn *et al.*, 1992; Higgs *et al.*, 1997) and pTE/3'2J/ α -luc (Johnson *et al.*, 1999) has been described. pTE/3'2J/ α -luc contained 595 bp from the 5' end of the luciferase gene in the pGEM-luc plasmid inserted in the *Xba*I site of pTE/3'2J in antisense orientation (Fig. 3.1). The antisense orientation of the luciferase insert in pTE/3'2J was confirmed by restriction endonuclease digestion and polymerase chain reaction.

Two TE/3'2J viruses expressing antisense RNA sequences unrelated to luciferase mRNA were used as control dsSIN viruses. TE/3'2J/ α -D1GDD virus expressed a 240-base sequence complementary to the dengue-1 (DEN-1) virus nonstructural protein 5

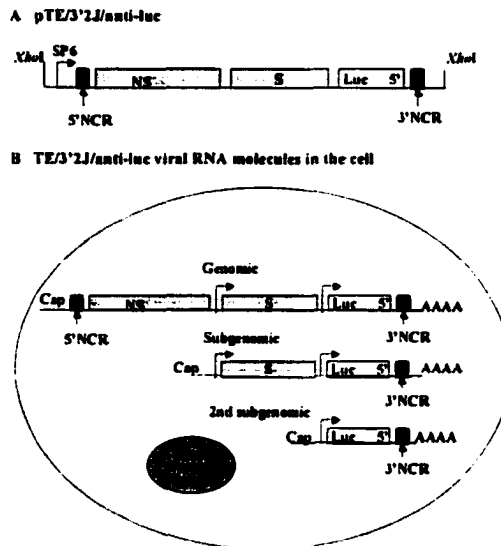


Figure 3.1 pTE/3'2J/ α -luc. (A) pTE/3'2J/ α -luc linearized by digestion with *Xho*I. A 595-base fragment from the 5' end of the *Luc* gene was ligated into the multiple cloning site behind the second subgenomic promoter in antisense orientation. (B) The three predicted species of viral RNA transcripts expressed in mosquito cells. Viral nonstructural proteins are translated directly from genomic RNA. Structural protein mRNA is transcribed from the negative-sense strand at the subgenomic promoter; the heterolous anti-sense luciferase segment is also transcribed. Antisense luciferase mRNA is also transcribed from the negative-sense strand at the second subgenomic promoter.

(NS5) at the GDD motif (position 9399-9641 of the DEN-1 virus genome); TE/3'2J/ α -prM contained a 290-base fragment complementary to the DEN-2 premembrane (prM) coding region (position 448-738 of the DEN-2 virus genome) (Z. Adelman, personal communication).

Generation of dsSIN Viruses. Recombinant virus was produced from pTE/3'2J/ α -luc by linearization with *Xho*I, transcription from the SP6 promoter, capping with RNA cap analog (1 mM), and electroporation (450 V, 100 μ F, 720 ohms, 0.7-0.9 msec) into baby hamster kidney 21 (BHK-21) cells (Powers *et al.*, 1994). Virus titer [tissue culture 50% infective dose (TCID₅₀)], measured as log₁₀ per ml, was determined by titration in triplicate in BHK-21 cells (Hsiung, 1994; Karber, 1931). For all three viruses, a TCID₅₀ of 8.2-8.5 log₁₀/ml was used.

Infection of *Aedes albopictus* C6/36 Cells. C6/36 cells were cultured in 25-cm² flasks containing Leibovitz's (L-15) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. When approximately 80% confluent, the cells were infected with control or TE/3'2J/α-luc virus at a multiplicity of infection (MOI) of 10. Virus was adsorbed to the cells for 1 hr, with constant agitation. Nonadsorbed virus was removed and the flask was washed twice with PBS. Medium was replaced and the cells were incubated for 48 hours at 27°C. Supernatant for end point dilution assays was collected at 0, 24, and 48 hr postinfection.

Northern Blot Analysis of dsSIN Transcripts. Total cellular RNA was isolated from infected C6/36 cells after 48 hr of infection using the one-step guanidinium thiocyanate extraction method (Chomczynski & Sacchi, 1987). Five micrograms of isolated RNA was analyzed by Northern blot assay as described in Chapter 2. The membrane was probed with a BrightStar psoralen-biotin (Ambion, Austin, TX) labeled PCR product specific for the luciferase insert in the TE/3'2J/α-luc virus in hybridization buffer in a 60°C rotating water bath for 18 hr. Following hybridization, the membrane (Ambion) was washed twice in 2X SSC, 0.1% SDS for 10 min, then washed twice for 30 min in 0.2X SSC, 0.1% SDS warmed to 60°C. RNA was visualized using the BrightStar BioDetect (Ambion) detection kit according to manufacturer's instructions. The membrane was exposed to Kodak X-Omat AR (VWR Scientific, West Chester, PA) X-ray film for 5 min.

Polymerase Chain Reaction. PCR reactions were carried out as described in Chapter 2. The primers are listed in **Table 3.1**. See **Fig. 2.1** for location of primers on pTE/3'2J.

Table 3.1 Primers used in PCR reactions

Primer name	Nucleotide sequence
2JF	5'-GTCAGCATAGTACATTTTCATC
2JR	5'-GCTGGTCGGATCATTGGGGCG
LucF	5'-ATGGAAGACGCCAAAAAC
LucR	5'-AAAACCGGGAGGTAGATG

Mosquito Rearing. The generation and characterization of the transgenic *Ae. aegypti* pH[*cn*]APY(1.6)LUC line #43 (APY-LUC43) has been described (Coates *et al.*, 1999). APY-LUC43 generation 8 (G8) mosquitoes were obtained as eggs; G11 and G12 adults were used in these experiments. Adult APY-LUC43 mosquitoes were maintained during the course of the experiments at 28°C and 80% humidity, with 12-hr light/dark cycles, and sugar and water provided *ad libitum*. In experiments requiring blood-fed mosquitoes, the preparation of blood and presentation of the blood meal was performed as previously described (Higgs & Beaty, 1996).

Intrathoracic Inoculation of Mosquitoes. Adult female mosquitoes were cold anesthetized and intrathoracically injected using a Drummond 50- μ l microcapillary needle that had been prepared with a needle puller (Narishige Co., Toyko, Japan). Approximately 1 μ l of dsSIN virus ($\cong 10^5$ infectious particles) in L-15 medium containing 10% FBS and 1% antibiotics was injected into each mosquito (Olson *et al.*, 1996; Rosen & Gubler, 1974).

Indirect Immunofluorescence Assay (IFA). Salivary glands from each treatment group were dissected in phosphate-buffered saline, mounted on acid-washed slides in a droplet of diluted (1:1000) Elmer's glue, dried, and fixed in acetone for 10 min at -20°C (Higgs *et al.*, 1997). Infection of the salivary glands with dsSIN virus was confirmed by IFA using a primary antibody that recognizes Sindbis E1 antigen (Chanas *et al.*, 1982), a

biotinylated sheep anti-mouse secondary antibody (Amersham) with 1% Evan's blue in PBS, and streptavidin-fluorescein tertiary antibody (Amersham), all diluted 1:200 in PBS, then visualized on an Olympus BH-2 epifluorescence microscope.

Luciferase Activity Assay. Thoraces or salivary glands were dissected in 0.15 M NaCl buffer and stored in 30 μ l lysis buffer (Promega) at -70°C . Thorax or salivary gland samples were thawed at room temperature, sonicated in an Aquasonics waterbath (model 75S, VWR Scientific) for 2 min at 50°C , power level 5, then centrifuged briefly at 13,000 rpm (Hermle Z233M, Wehningen, Germany). Luciferase assays were performed according to the manufacturer's directions (Promega). Briefly, 20 μ l of each sample was added to 100 μ l of luciferase assay reagent. Luciferase activity was measured as relative light units (RLU) using a Turner TD-20e luminometer (Promega), which was standardized to 10-fold dilutions of recombinant luciferase protein (Gibco) mixed with 1 mg/ml BSA; 1-3 RLU=1 μ g/ml luciferase protein.

Apyrase Activity Assay. Apyrase activity was determined using the protocol of Ribeiro (Ribeiro *et al.*, 1984) and an inorganic phosphorus commercial kit (Sigma). Salivary glands were dissected in 0.15 M NaCl and transferred to 100 μ l of 10 mM TrisCl (pH 7.5). Salivary glands were homogenized in an Aquasonics waterbath (VWR Scientific) for 2 min at 50°C , power level 5, then centrifuged at 13,000 rpm for 1 min. Five microliters of salivary gland homogenate was added to 95 μ l of reaction medium (100 mM Tris-Cl buffer pH 9.0, 200 mM NaCl, 10 mM CaCl_2 , and 20 mM ADP sodium salt) and incubated at 37°C for 30 min. The reaction was stopped by adding 25 μ l of ammonium molybdate in 2.5N sulfuric acid (Sigma). Three microliters of F&S reducing reagent (Sigma) was added, then the plate was placed in the dark for 20 min. The amount

of inorganic phosphate released was measured on a 450 Plate Reader (BioRad, Richmond, CA) with a 665 nm filter. Apyrase activity was calculated as the total amount of monophosphate released per microgram of salivary gland protein.

Western Blot Assay. Salivary glands were dissected in 0.15 M NaCl then transferred to a tube containing 12 μ l loading buffer (50 mM Tris-Cl pH 7.5, 1% SDS, 2 mM phenyl methyl sulphuryl fluoride, 1 mM EDTA, 10% glycerol, 5% mercaptoethanol, 0.2% bromophenol blue). Homogenization consisted of three cycles of freeze-thawing, and boiling for 2 min. One complete salivary gland was loaded per lane, and proteins were separated on a 10% SDS-PAGE 1-mm gel (Novex, Hercules, CA) run at 150 mV for 1 hr. Proteins were transferred to a nitrocellulose membrane (Trans-blot, BioRad) by electrophoresis using a BioRad transfer apparatus (transfer buffer: 25 mM Tris, 192 mM glycine, 10% methanol; pH 8.3) run at 200 mA for 3 hr. Proteins were detected using the ECL-Western blotting kit (Amersham) according to manufacturer's instructions, except that PBS-0.05% NP-40 was used for blocking and all washes. A polyclonal rabbit anti-apyrase antibody raised to recombinant apyrase peptide diluted 1:20,000 in PBS-0.05% NP-40 detected the 68-kd apyrase protein (Champagne *et al.*, 1995). Amersham anti-rabbit HRP secondary antibody, diluted 1:1000, was used as secondary antibody. The membrane was exposed to Kodak X-Omat AR (VWR Scientific) X-ray film for 2 sec. The membrane was stripped according to manufacturer's directions (Amersham) and reprobbed with a polyclonal rabbit anti-D7 antibody diluted 1:2000, which detected the 37-kd D7 protein (James, 1994). Total salivary gland protein was detected by rinsing the membrane twice in PBS-0.05% NP-40 then incubating with 50-ml colloidal gold (BioRad) for 1 hr.

Total Protein Concentration. Protein assays of salivary glands were performed in a 96-well plate using the Pierce-Coomassie Plus Protein assay reagent (Pierce, Rockford, Ill) according to manufacturer's instructions. Protein concentration was measured colorimetrically on a 450 Plate Reader with a 595-nm filter. Protein concentration was determined by comparison to bovine serum albumin standard concentrations.

Virus Assays of Infected Mosquitoes. Mosquito heads and abdomens were triturated in 1 ml L-15 diluent with a pestle then passed through a 2- μ m pore-size filter, as previously described (Higgs *et al.*, 1997). Virus titers were determined by end point dilution assay in triplicate in Vero cells (Karber, 1931).

Statistical Analysis. Because of the variation of luciferase protein expression among individual mosquitoes within a treatment group, the standard deviations were larger than the mean in some cases. To normalize the data points so that treatment groups could be compared, analysis of variance (ANOVA) and contrast statements of the sample values were calculated from \log_{10} of RLU values using the SAS 6.12 statistical program.

RESULTS

TE/3'2J/ α -luc Virus Characterization. Antisense orientation of the luciferase insert in pTE/3'2J was screened by PCR using primers derived from the 3' end of the luciferase insert (LucR) and flanking the multiple cloning site of pTE/3'2J (2JF, 2JR) (**Table 3.1 and Fig. 3.2**).

Northern blot analysis of infected C6/36 cells showed transcription of the three viral mRNA species of TE/3'2J/ α -luc virus (**Fig. 3.3**). Virus infection was confirmed by determining viral titers in mosquito carcasses. Mean titers ranged from 4.0 to 5.6 \log_{10} TCID₅₀ per mosquito.

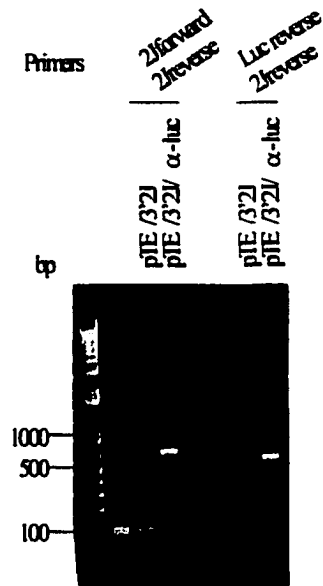


Figure 3.2 PCR screening of luciferase insert size and orientation in pTE/3'2J/ α -luc. Primers 2JF and 2JR amplify 95 bases upstream and downstream of multiple cloning site in pTE/3'2J and the 400-base luciferase insert + 95 bases in pTE/3'2J/ α -luc (*left*). The Luc reverse primer, specific for the 3'-end of the luciferase insert, with the 2JR primer resulted in a PCR product in the pTE/3'2J/ α -luc reaction only (*right*).

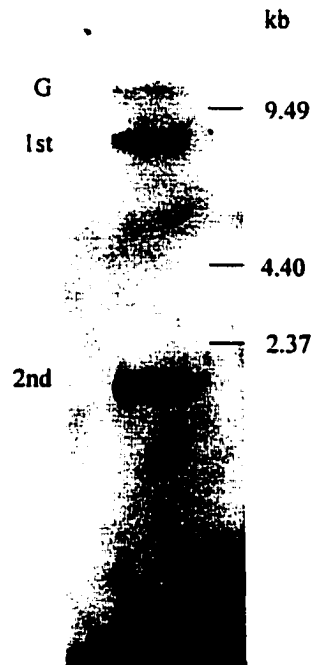


Figure 3.3 Northern blot analysis of TE/3'2J/ α -luc transcripts in infected C6/36 cells. A DNA probe complementary to the 595-base region of the *luc* gene hybridized to the luciferase insert of the genomic (G), first subgenomic (1st), and second subgenomic (2nd) transcripts of the virus.

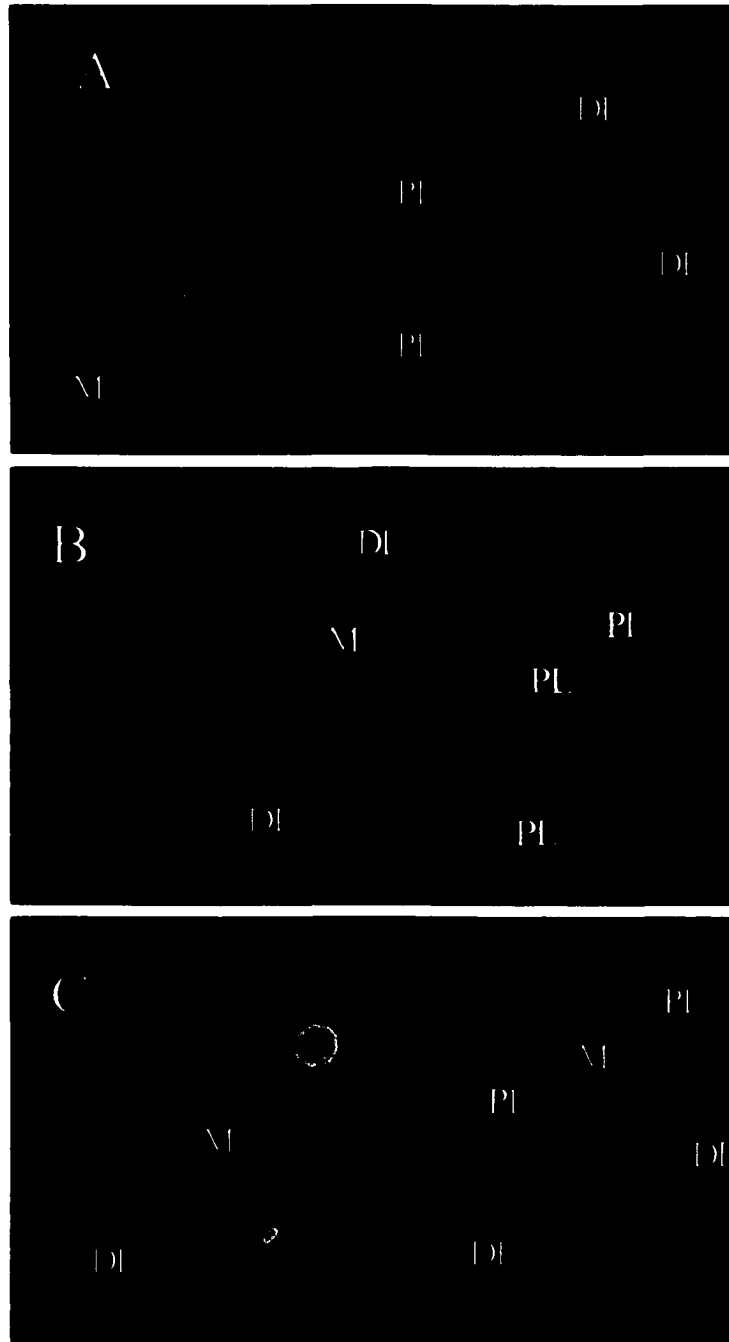


Figure 3.4 SIN virus infection in APY-LUC43 female mosquito salivary glands 10 days after intrathoracic inoculation. (A) L-15, (B) TE/3'2J/α-luc virus, or (C) TE/3'2J/α-D1GDD virus. Primary antibody 30.11A, which recognizes SIN E1 (envelope) protein, was used. DL, distal-lateral lobe; M, medial lobe; PL, proximal-lateral lobe. A and B, original magnification 200X; C, original magnification 100X.

SIN antigen was detected by IFA in all lobes of the salivary glands of mosquitoes infected with TE/3'2J/ α -luc and control viruses at 2 and 10 days postinoculation (**Fig. 3. 4B and C**). This demonstrated that dsSIN viruses infect the same salivary gland tissue that expresses luciferase in the transgenic mosquitoes, specifically the distal-lateral and medial lobes (Coates *et al.*, 1999).

Profile of Luciferase Expression in APY-LUC43 Mosquitoes Infected with TE/3'2J/ α -luc Virus. Four separate experiments were conducted. In the first experiment, female adult mosquitoes that had eclosed less than 24 hr earlier were cold anesthetized and intrathoracically injected with TE/3'2J/ α -luc virus. Controls were cold anesthetized only. Thoraces were collected and luciferase activity measured. Luciferase activity was significantly inhibited in the TE/3'2J/ α -luc virus-infected mosquitoes compared to uninfected controls throughout the 8 days of the experiment (**Fig. 3.5**). Uninoculated controls averaged 43-69 RLU; in contrast, the TE/3'2J/ α -luc treatment group ranged between 0.2-2.8 RLU. At 48 hr postinoculation and directly following the blood meal at 7 days, luciferase activity in TE/3'2J/ α -luc-infected mosquitoes was reduced 90% compared to controls. At 6 days (before blood feeding) and at 8 days (24 hr after the blood meal) postinoculation, luciferase activity was reduced 99% in mosquitoes infected with TE/3'2J/ α -luc virus. Luciferase activity was greater (range 42-110 RLU) in mosquitoes inoculated with TE/3'2J control virus than in uninfected controls over the 8 days of the experiment.

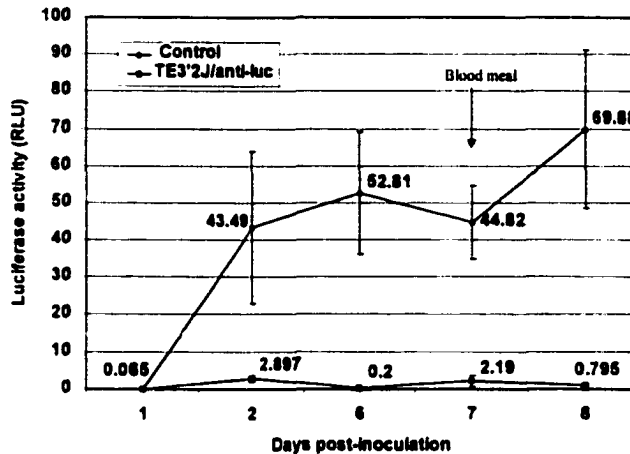


Figure 3.5 Luciferase activity in APY-LUC43 mosquitoes. Mean luciferase activity (\pm SE), measured as relative light units (RLU), in control uninoculated APY-LUC43 mosquitoes and mosquitoes intrathoracically injected with TE/3'2J/ α -luc virus within 24 hr of eclosion. Luciferase activity was measured at 1 day post-eclosion (control $n=2$), 2 days post-eclosion (control $n=3$, TE/3'2J/ α -luc $n=8$), before a blood meal at 6 days (control $n=3$, TE/3'2J/ α -luc $n=2$), immediately after the blood meal at 7 days (control $n=4$, TE/3'2J/ α -luc $n=4$), and 24 hr post-blood meal at 8 days (control $n=3$, TE/3'2J/ α -luc $n=5$). Baseline luciferase activity was established by RLU readings of *kh^w* (white eye) mosquitoes; *kh^w* = 0.043 RLU.

In the second experiment, larger sample sizes were used, and two additional control treatments were added. Mosquitoes were intrathoracically inoculated with L-15 medium to control for inoculation effects on luciferase expression. In addition, TE/3'2J/ α -D1GDD virus, carrying a nonhomologous antisense RNA insert, was included as a control virus to determine whether inhibition of luciferase activity by dsSIN virus was sequence specific. Mosquitoes were again inoculated within 24 hr of eclosion. Thoraces were collected at 5 and 9 days postinoculation, but mosquitoes were not blood fed.

Although all mosquitoes used in the experiment came from one hatch, the development time varied and eclosion occurred over 8 days. There was considerable variation in luciferase activity between the first mosquitoes to eclose and those that developed more slowly (Table 3.2). In general, mosquitoes that eclosed quickly produced

Table 3.2 Luciferase activity in transgenic (APY-LUC43) mosquitoes: exp. 2 and 3

	<i>n</i> (% survival)	Ave TCID ₅₀ (log ₁₀ /ml)	Mean RLU (SE)	<i>P</i> value*
<i>Experiment 2: 5 days postinoculation</i>				
Replicate 1				
Uninoculated control	7 (70)	NI	12.8 (3.5)	
L-15	ND	ND	ND	
TE/3'2J/α-D1GDD	7 (90)	4.7	8.3 (3.0)	
TE/3'2J/α-luc	7 (100)	4.1	3.4 (0.7)	<i>P</i> =0.001
Replicate 2				
Uninoculated control	10 (100)	NI	26.9 (6.2)	
L-15	10 (62)	NI	43.6 (10.1)	
TE/3'2J/α-D1GDD	6 (50)	3.7	17.7 (4.8)	
TE/3'2J/α-luc	9 (56)	3.2	6.87 (1.7)	<i>P</i> =0.0003
Replicate 3				
Uninoculated control	17 (100)	NI	22.4 (4.3)	
L-15	10 (65)	NI	53.2 (16.8)	
TE/3'2J/α-D1GDD	6 (55)	4.6	139.1 (82.7)	
TE/3'2J/α-luc	10 (55)	3.9	18.3 (9.0)	<i>P</i> =0.002
5 day total				
Uninoculated control	34 (93)	NI	21.8 (2.9)	
L-15	20 (64)	NI	48.4 (9.7)	
TE/3'2J/α-D1GDD	19 (61)	4.3	56.2 (27.5)	
TE/3'2J/α-luc	26 (65)	3.7	10.3 (3.7)	<i>P</i> =0.0001
<i>Experiment 2: 9-days postinoculation</i>				
Replicate 1				
Uninoculated control	10 (60)	NI	8.1 (2.4)	
L-15	9 (64)	NI	9.2 (3.7)	
TE/3'2J/α-D1GDD	8 (56)	4.1	13.5 (5.2)	
TE/3'2J/α-luc	12 (70)	2.7	1.7 (1.0)	<i>P</i> =0.0001
Replicate 2				
Uninoculated control	10 (59)	NI	40.1 (7.2)	
L-15	9 (76)	NI	41.8 (7.3)	
TE/3'2J/α-D1GDD	10 (70)	4.0	80.9 (20.0)	
TE/3'2J/α-luc	7 (35)	2.5	3.3 (0.8)	<i>P</i> =0.0001
9 day total				
Uninoculated control	20 (59)	NI	24.1(5.2)	
L-15	18 (71)	NI	25.5 (5.6)	
TE/3'2J/α-D1GDD	18 (64)	4.0	50.9 (13.8)	
TE/3'2J/α-luc	19 (51)	2.7	2.3 (0.7)	<i>P</i> =0.0001
<i>Experiment 3: 9 days postinoculation</i>				
Uninoculated control	10 (94)	NI	23.3 (5.7)	
L-15	10 (85)	NI	15.4 (3.4)	
TE/3'2J/α-D2prM	11 (80)	3.4	21.2 (2.4)	
TE/3'2J/α-luc	11 (50)	3.2	2.9 (1.0)	<i>P</i> =0.0001

*Luciferase activity in TE/3'2J/anti-luc-infected mosquitoes vs. uninoculated, L-15-inoculated, and TE/3'2J/anti-D1GDD virus-infected controls compared by contrast statement.

Abbreviations: ND, not done; NI, not infected; SE, standard error.

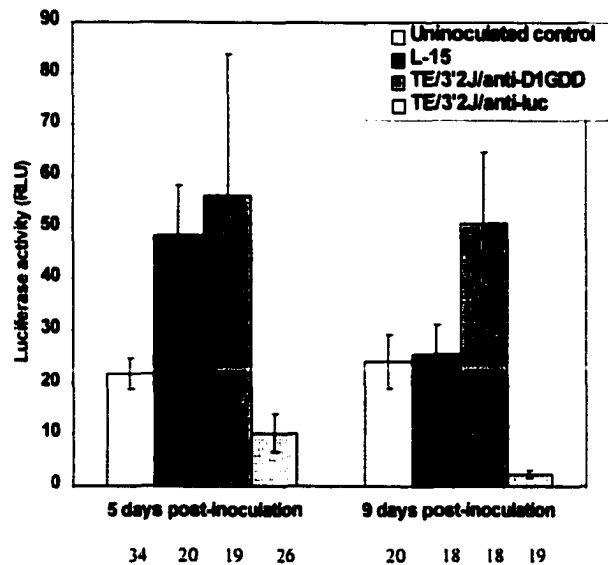


Figure 3.6 Luciferase activity in salivary glands of APY-LUC43 mosquitoes 5 and 9 days postinoculation. Mean luciferase activity (\pm SE) measured as relative light units (RLU), in APY-LUC43 mosquitoes 5 and 9 days after intrathoracic injection with L-15, TE/3'2J/ α -D1GDD virus, or TE/3'2J/ α -luc virus. Uninoculated control mosquitoes were cold anesthetized only.

less luciferase than those that eclosed 5-7 days later. In order to minimize the effect this variation might have, a series of replicates were conducted, with all treatments included in each replicate. Each day pupae were separated from the larvae. The next day when the group of pupae eclosed, the adult females were divided and received one of the treatments. Each day's eclosion group was considered a replicate, and included all treatments of the experiment. A total of seven eclosion groups, or replicates, were inoculated, but only replicates containing at least six surviving mosquitoes in each treatment group were included in analyses. This resulted in three replicates of the 5-day group and two replicates of the 9-day group (Table 3.2 and Fig 3.6).

Even with larger sample sizes (total: control $n=54$, L-15 $n=38$, TE/3'2J/ α -D1GDD $n=37$, TE3'2J/ α -luc $n=45$), individual luciferase activity varied greatly. The standard deviation was greater than the mean RLU of the treatment group in some cases. This was

especially true of the uninoculated controls, which did not receive any treatment that might affect luciferase activity. To normalize the distribution within the treatment groups, the RLU readings were converted to \log_{10} , which was used to calculate ANOVAs and make comparisons by contrast statements of the combined replicates (**Table 3.2**).

Luciferase production was significantly diminished by TE/3'2J/ α -luc virus infection compared to the three controls at both 5 ($P=0.0001$) and 9 ($P=0.0001$) days postinoculation (**Table 3.2 and Fig. 3.6**). At 5 days postinoculation, luciferase activity in the TE/3'2J/ α -luc virus-infected mosquitoes was half that of the uninoculated control mosquitoes (10.3 vs. 21.8 RLU). The overall luciferase activity in 81% of the TE/3'2J/ α -luc infected mosquitoes was less than 10 RLU. However, in the third replicate, the mean RLU was 18.3, resulting in a 5-day total mean of 10.3 RLU. At 9 days postinoculation, luciferase expression was decreased 90% in the mosquitoes infected with TE/3'2J/ α -luc virus compared to uninoculated controls.

Luciferase expression in the uninoculated controls remained constant at both time points, with the means ranging from 21.3 to 21.8 RLU. In contrast, luciferase expression in the L-15-inoculated control mosquitoes was almost twofold higher at 5 days (48.4 RLU) than at 9 days (25.5 RLU), when it decreased to uninoculated control levels. Production of luciferase in the TE/3'2J/ α -D1GDD virus-infected mosquitoes was twofold higher than uninoculated controls both at 5 and 9 days postinoculation.

In the third experiment, TE/3'2J/ α -D2prM virus was used to control for non-antisense effects. Mosquitoes were intrathoracically injected within 24 hr of eclosion, and thoraces were collected 9 days later. To confirm that the individual variation in luciferase activity was not due to incomplete collection of salivary glands, total protein of each thorax was

determined and compared with the luciferase RLU value of the thorax. There was no correlation between high or low luciferase activity and total protein concentration of the thorax (data not shown).

As in the previous experiment, infection by TE/3'2J/ α -luc virus inhibited luciferase production 90% compared to the three controls (Table 3.2). In contrast to the second experiment, in this experiment luciferase activity in L-15 controls was lower than in uninoculated controls (15.4 vs 23.3 RLU), although not significantly ($P=0.1$). Luciferase production in TE/3'2J/ α -D2prM virus-infected mosquitoes was nearly equal to the uninoculated controls (23.3 vs. 22.2 RLU).

In the fourth experiment luciferase activity was assayed in dissected salivary glands standardized to total salivary gland protein to eliminate the effect of variable salivary gland size or dissection errors. Female mosquitoes that had eclosed less than 24 hr earlier were cold anesthetized and intrathoracically injected with TE/3'2J/ α -luc or TE/3'2J/ α -D1GDD virus. Uninoculated controls were cold anesthetized only. Salivary glands were collected at 5 and 9 days postinoculation. The 9-day group was blood fed on day 6.

Luciferase activity in dissected salivary glands was measured and divided by the total protein of the salivary gland sample (average protein concentration per salivary gland: 2.2 μ g in uninfected, 2.6 μ g in TE/3'2J/ α -D1GDD virus, and 1.8 μ g in TE/3'2J/ α -luc virus-infected mosquitoes). Mean luciferase activity at 5 days postinoculation was 272 RLU/ μ g protein in uninoculated controls, 210 RLU/ μ g protein in mosquitoes infected with TE/3'2J/ α -D1GDD virus, compared to 18 RLU/ μ g protein in mosquitoes infected with TE/3'2J/ α -luc virus (Table 3.3). Mean luciferase activity at 9 days was 21, 14, and 3 RLU/ μ g protein for uninfected, TE/3'2J/ α -D1GDD virus-infected, and

Table 3.3 Luciferase activity in salivary glands of transgenic (APY-LUC43) mosquitoes: exp. 4

	N	Average TCID ₅₀ (log ₁₀ /ml)	Mean RLU/μg protein (SE)	% survival	P value*
<i>5 days postinoculation</i>					
Uninoculated	20	NI	272 (142)	78	
TE/3'2J/α-DIGDD	19	5.6	210 (125)	73	
TE/3'2J/α-luc	20	5.2	18 (9)	78	P=0.0001
<i>9 days postinoculation</i>					
Uninoculated	17	NI	21 (4)	83	
TE/3'2J/α-DIGDD	14	4.5	14 (4)	73	
TE/3'2J/α-luc	16	4.0	3 (1)	70	P=0.0001

*Luciferase activity (RLU/μg protein) in TE/3'2J/α-luc virus infected vs. uninfected and TE/3'2J/α-DIGDD virus infected mosquitoes as calculated by contrast statement.

Abbreviations: ND, not done; NI, not infected; SE, standard error.

TE/3'2J/α-luc virus-infected mosquitoes, respectively. Data were transformed to log₁₀ and analyzed for evidence of significant luciferase inhibition. Luciferase activity in TE/3'2J/α-luc infected mosquitoes was reduced >90% compared to uninfected controls at both 5 (0.7 vs. 2.0 log₁₀ RLU/μg protein; *P*=0.0001) and 9 days (-0.7 vs. 1.2 log₁₀ RLU/μg protein; *P*=0.0001) postinfection (**Fig. 3.7**). Mean luciferase activity in salivary glands of TE/3'2J/α-DIGDD virus-infected controls and uninfected controls did not differ significantly at 5 days (1.7 vs. 2.0 log₁₀ RLU/μg protein; *P*=0.17) or 9 days (0.9 vs. 1.2 log₁₀ RLU/μg protein; *P*=0.2) postinfection (**Fig. 3.7**).

Apyrase and Western Blot Assays. Mean salivary gland apyrase activity did not differ statistically at 5 or 9 days postinfection between uninfected salivary glands and those infected with TE/3'2J/α-luc virus (**Table 3.4**). Apyrase and D7 protein concentrations in TE/3'2J/α-luc virus-infected and uninfected salivary glands were shown to be comparable by Western blot assay (**Fig. 3.8A and B**). In addition, total salivary gland protein profiles and concentrations assayed by Western blot did not differ between TE/3'2J/α-luc virus-infected and uninfected salivary glands (**Fig. 3.8C**).

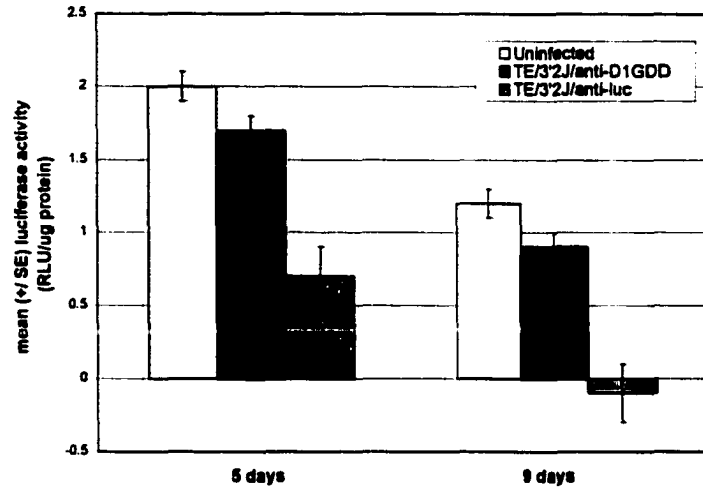


Figure 3.7 Luciferase activity in salivary glands of APY-LUC43 mosquitoes. Mean log₁₀ luciferase activity, measured as relative light units standardized to total salivary gland protein, in APY-LUC43 mosquitoes 5 and 9 days after intrathoracic injection with either TE/3'2J α -luc or control virus. Uninoculated control mosquitoes were cold anesthetized only.

Table 3.4 Apyrase activity* in salivary glands

	5 day		9 day	
	mean \pm SE	<i>N</i>	mean \pm SE	<i>N</i>
Uninfected	252 \pm 61	6	252 \pm 59	7
TE/3'2J/ α -luc	182 \pm 49	7	272 \pm 57	5

*Apyrase activity was measured as the amount of monophosphate produced per microgram of salivary gland protein (PO₄/μg protein). The 9-day mosquitoes were blood fed on day 6.

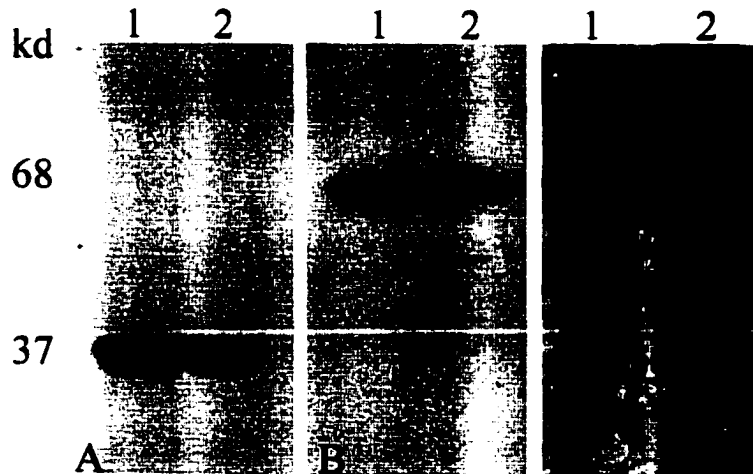


Figure 3.8 Western blot assay of salivary glands probed for apyrase and D7. (A) The 37-kd D7 protein, (B) the 68-kd apyrase protein, and (C) total protein stained from the nitrocellulose membrane. Lane 1: one pair of uninfected salivary glands; lane 2: one pair of salivary glands infected with TE/3'2J α -luc virus for 5 days.

DISCUSSION

The *luciferase* gene in transgenic APY-LUC43 mosquitoes is regulated by the *Ae. aegypti* *Apyrase* promoter, and thus is expressed in the same developmental- and tissue-specific manner as the endogenous *Apy* gene (Coates *et al.*, 1999). This provides a measurable model of endogenous gene expression. Apyrase and luciferase are both produced exclusively in the distal-lateral and medial lobes of the salivary glands of female adult mosquitoes. At 24 hr post-eclosion there is no detectable luciferase (Fig. 3.5); similarly, in wild-type *Ae. aegypti*, apyrase is not detectable immunologically until 48 hr after eclosion (Marinotti *et al.*, 1990). Luciferase activity decreases 16% directly after a blood meal, but 24 hr later increases 25% above pre-blood-meal levels (Fig. 3.5).

Apyrase in wild-type *Ae. aegypti* mosquitoes is not significantly diminished following a blood meal (Smartt *et al.*, 1995).

Initially, we wanted to see if luciferase production in APY-LUC43 mosquitoes was inhibited by TE/3'2J/ α -luc virus infection, and if so, the duration and magnitude of inhibition. In the first experiment, 90% of the luciferase expression was inhibited by TE/3'2J/ α -luc virus compared to uninfected controls by 6 days. Inhibition increased to 99% in blood-fed mosquitoes (Fig. 3.5). However, sample sizes in this initial experiment were small (n ranged from 2-8), and the variability in luciferase activity between individuals in the infected control group was large.

In the second experiment, a series of replicates was combined to increase the sample size. To control for possible effects of the inoculation on luciferase activity, one treatment group consisted of mosquitoes intrathoracically injected with L-15 medium. Luciferase expression in the L-15-inoculated mosquitoes was approximately twofold over uninoculated controls at 5 days postinoculation, then decreased to uninoculated control levels by 9 days. The transient increase in luciferase expression in mosquitoes injected with L-15 may be due to activation of the *Apy* promoter by the injection, or alternatively, the *Apy* promoter might be transactivated by factors produced in response to the injection, such as stress proteins, or by sugars or proteins contained in the L-15 medium.

Antisense RNA-mediated inhibition of gene expression in both plant and animal cells is dependent on a high level of sequence identity between the effector and target RNAs (Branch, 1996; Higgs *et al.*, 1998). However, suppression of gene expression by sense or heterologous related antisense RNA has been reported in both plant and animal cells (Branch, 1996). Because the endogenous mRNA and the sense or nonhomologous

transcript cannot base pair with each other, inhibition of target gene expression is not the result of RNA duplex formation in these cases. Therefore, to ensure that inhibition of luciferase activity by SIN virus was sequence specific, a nonhomologous antisense RNA control virus was included in subsequent experiments. Possible nonspecific inhibition of luciferase expression by SIN virus infection of the salivary glands could be detected by these control dsSIN viruses.

Infection of mosquitoes with control dsSIN viruses did not inhibit luciferase expression. Rather, luciferase expression in mosquitoes infected with TE/3'2J/ α -D1GDD virus increased twofold over uninoculated controls at 5 and 9 days postinfection in the second experiment; however, the difference occurred in two of the five replicates (**Table 3.2**). In the third experiment, mosquitoes infected with TE/3'2J/ -D2prM virus showed no increased - or decreased - luciferase expression over uninoculated controls (**Table 3.2**). In the fourth experiment, the level of luciferase expression in mosquitoes that were infected with control virus was slightly depressed compared to uninfected mosquitoes, although not significantly (**Table 3.3 and Fig. 3.7**). Therefore, infection by control dsSIN viruses did not significantly affect luciferase expression. Survival rates were lower in infected mosquitoes compared to uninoculated mosquitoes at 5 days in experiment 2 and 9 days in experiment 3 (**Table 3.3**). No deleterious effects of the inoculation or SIN infection was observed in previous experiments using wild-type *Ae. aegypti* (Jupp & Phillips, 1998). Reduced survival in these experiments may be due to the less robust mutant-transgenic line of mosquitoes.

The individual variability of luciferase expression seen in the first experiment continued to be a confounding factor in all experiments, despite larger sample sizes.

Luciferase expression in uninoculated controls varied by as much as 100-fold. For example, in the second experiment at 9 days postinoculation luciferase expression in uninoculated controls ranged from 2 to 90 RLU. We were unable to analyze the transcriptional activity of luciferase, because luciferase mRNA is not detectable by Northern blot analysis in the transgenic APY-LUC43 line (Coates *et al.*, 1999). The APY-LUC43 line was derived from one male and 10 females, and a wide range of luciferase expression in individuals has been noted since establishment of the line (C.J. Coates and A.A. James, unpublished data). Factors such as insertion sites and enhancers could result in individuals with *Apy* promoters of varying robustness, which could account for some of the variability in luciferase production among control mosquitoes. In natural populations, genetic variability is often a confounding factor in measuring a treatment effect. Environmental factors such as nutritional status and body size upon eclosion could also contribute to variability in *apyrase* promoter activity. Luciferase is expressed exclusively in the distal-lateral and medial lobes of the salivary glands; therefore it is essential that the TE/3'2J/ α -luc virus infect every cell in this tissue to completely inhibit luciferase expression. The dsSIN virus infects salivary gland tissue; however, it is possible that the lack of inhibition of luciferase expression seen in a few individuals infected with TE/3'2J/ α -luc virus (particularly replicate 3 in experiment 2) could be due to lack of infection of all salivary gland tissue by the virus. Infection of each mosquito used in luciferase assays was confirmed by end point dilution assay of the carcass, but it was not possible to determine the extent of salivary gland infection by IFA and measure luciferase activity on the same salivary gland sample, as whole salivary glands were needed for each assay.

Despite the individual variability of expression and the few cases of breakthrough expression in mosquitoes infected with TE/3'2J/ α -luc, luciferase expression in APY-LUC43 mosquitoes was significantly inhibited by TE/3'2J/anti-luc virus infection; 90% at 9 days postinfection in the second, third, and fourth experiments. In addition, inhibition of luciferase activity by TE/3'2J/ α -luc virus was specific; production of other salivary gland proteins was not affected. Apyrase enzymatic activity was not significantly different between uninfected mosquitoes and those infected with TE/3'2J/ α -luc virus (Table 3.4). Apyrase protein, as measured by Western blot assay, was abundant in both treatment groups (Fig. 3.8B). Similarly, D7, a female-specific salivary gland protein, was present in equivalent amounts in both uninfected and TE/3'2J/ α -luc infected mosquitoes (Fig. 3.8A). Total salivary gland protein profiles and concentrations were equivalent between uninfected and TE/3'2J/ α -luc virus-infected mosquitoes (Fig. 3.8C).

Thus intrathoracic injection of mosquitoes with TE/3'2J/ α -luc virus has proved to be an effective and specific method for inhibiting gene expression *in vivo*. This method of gene expression inhibition has potential in studying mosquito biology, particularly for inducible genes expressed exclusively in adults, such as late trypsin (Noriega *et al.*, 1994).

However, inoculation of the virus vector is an inefficient method of gene delivery, and TE/3'2J does not readily infect mosquitoes *per os*. A dsSIN vector, MRE/3'2J, has been developed that can orally infect mosquitoes (Olson *et al.*, 2000; Seabaugh *et al.*, 1998) and might provide a more efficient gene delivery system. In subsequent experiments, MRE/3'2J was tested for its potential to orally infect larvae. In addition, MRE/3'2J containing antisense RNA to apyrase inserted at the second subgenomic

promoter (MRE/3'2J/ α -apyrase) was evaluated for its ability to inhibit *apyrase* gene expression in *Ae. aegypti*. These experiments are described in the following chapter.

Chapter 4

Oral Infection of *Aedes aegypti* Larvae with MRE/3'2J/ α -apyrase Virus

INTRODUCTION

The double-subgenomic Sindbis (dsSIN) virus TE/3'2J is an efficient gene delivery system (Hahn *et al.* 1992) when intrathoracically injected into mosquitoes, but when orally administered in a blood meal, it infects mosquitoes poorly (Higgs *et al.*, 1997). In contrast, the Malaysian SIN virus strain MRE16 is highly infectious *per os* (Seabaugh *et al.*, 1998). The oral infectivity phenotype is determined by the SIN structural genes. A chimeric virus, designated MRE1001, containing MRE16 structural genes and TE/3'2J nonstructural genes, displayed the enhanced orally infectious phenotype (Seabaugh *et al.*, 1998). MRE/3'2J was developed from MRE1001, with the addition of the TE3'2J second subgenomic promoter and 3' noncoding region. This dsSIN virus vector efficiently transduces a heterologous gene sequence via the *per os* route (Olson *et al.*, 2000). MRE/3'2J/GFP, when delivered in a blood meal to female *Aedes aegypti*, was nearly 100% infectious, and expressed green fluorescence protein (GFP) in the midguts of infected mosquitoes. However, even though the virus caused a disseminated SIN infection in infected mosquitoes, GFP protein was not observed in organs other than the midgut (Olson *et al.*, 2000).

Ae. aegypti larvae fed on C6/36 cells infected with MRE/3'2J/GFP had a greater oral infection rate than larvae fed with TE/3'2J/GFP virus-infected C6/36 cells (Higgs *et al.*, 1999). This method holds promise for gene delivery early in mosquito development. Inhibition of expression of a gene normally expressed in the adult stage might be achieved by infecting larvae with MRE/3'2J carrying homologous antisense RNA. The effector antisense transcripts would then be expressed in the mosquito tissue as it develops, and be "in place" at the time of mosquito mRNA transcription. Thus there

would be sufficient antisense transcripts in cell cytoplasm to hybridize to the endogenous mRNA coming from the nucleus.

Previous attempts to knock out production of apyrase protein in salivary glands of mosquitoes intrathoracically inoculated with TE/3'2J/ α -apyrase virus were unsuccessful (see Chapter 2). Apyrase is produced in salivary glands soon after adult eclosion, and is abundant throughout the lifetime of the mosquito. TE/3'2J virus infects the salivary glands within 48 hr of inoculation. Thus, even if newly emerged adults are inoculated with the virus, by the time the virus productively infects the salivary glands, apyrase protein is abundant.

In the studies described in this chapter, a chimeric dsSIN virus expression system was constructed and used to orally infect *Ae. aegypti* larvae. The second subgenomic promoter, antisense apyrase insert, and 3' noncoding region from TE/3'2J/ α -apy were cloned into the chimeric MRE1001 virus, producing MRE/3'2J/ α -apy virus (Fig. 4.1). *Ae. aegypti* larvae were infected *per os* with MRE/3'2J/ α -apyrase virus, before the apyrase gene was expressed in adults. Apyrase gene expression was then assayed in female adults. The hypothesis of these experiments was that apyrase expression in adult mosquitoes could be inhibited through *per os* infection of larvae by MRE/3'2J/ α -apyrase. Theoretically, antisense apyrase RNA transcripts would be in salivary gland tissues before eclosion, and would hybridize to mosquito apyrase mRNA. Two mosquito egg hatching methods and three strategies of larvae infection, including addition of DTT and removal of FBS, were investigated in order to optimize virus infection rates.

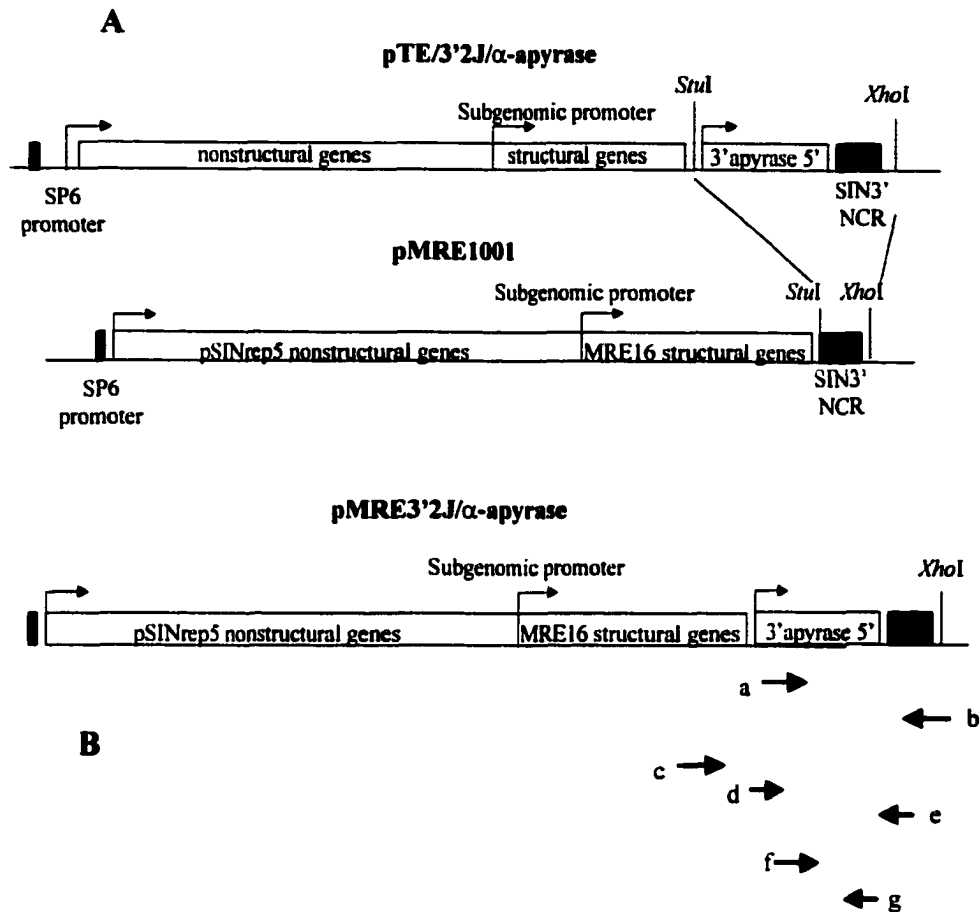


Figure 4.1 Construction of the MRE/3'2J/α-apy chimeric virus. (A) The PCR product amplified with TE/3'2JStuIF and TE/3'2JXhoIR primers was ligated into the pCR2.1 cloning plasmid (Invitrogen). pCR2.1/3'2J/α-apy was restriction digested with *StuI* and *XhoI*. The restriction digest product was ligated into pMRE1001 at the *StuI* and *XhoI* restriction sites to produce MRE/3'2J/α-apy. (B) Primer sites used in cloning and screening reactions: a, TE/3'2JStuIF; b, TE/3'2JXhoIR; c, MRE16E1F; d, 2JF; e, 2JR; f, ApyR; g, ApyF. See Table 4.1 for primer nucleotide sequences.

MATERIALS AND METHODS

Construction of pMRE/3'2J/α-apy Virus. The construction of pTE/3'2J (Hahn *et al.*, 1992) and insertion of a heterologous gene in the multiple cloning site has been described (Hahn *et al.*, 1992; Higgs *et al.*, 1993; Olson *et al.*, 1996; Olson *et al.*, 1994). pTE/3'2J/α-apyrase was produced by inserting apyrase cDNA from the 5' end (position

14-377) (Champagne *et al.*, 1995) into the dsSIN vector pTE/3'2J at the *Xba*I cloning site (A. Powers and K. Olson, data not shown). The development of pMRE 1001 has been described (Seabaugh *et al.*, 1998). The TE/3'2J second subgenomic promoter, antisense apyrase insert, and 3' noncoding region of TE/3'2J/ α -apy was PCR amplified from the pTE/3'2J/ α -apy template using primers TE/3'2J*Stu*I F, creating a *Stu*I restriction site, and TE/3'2*Xho*I R (Fig. 4.1 and Table 4.1). PCR amplification was conducted in a 50- μ l reaction containing 50 ng of pTE/3'2J/ α -apy template, 50 pmoles forward and reverse primer, 0.2 mM dNTPs, 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-Cl (pH 9.0), and 0.1% Triton X-100. The PCR amplification cycle consisted of 95°C for 5 min, 80°C for 2 min, during which time the Taq polymerase was added, followed by 25 amplification cycles: 93°C for 1 min, 56°C for 30 sec, 70°C for 2 min, with a final extension at 72°C for 2 min. Two microliters of the resultant 1.1-kb PCR product was inserted into the pCR2.1 cloning plasmid per manufacturer's instructions (Invitrogen). Plasmids containing the insert were screened by PCR and restriction enzyme digest analysis.

pMRE1001 and pCR2.1/3'2J/ α -apy were digested separately with *Stu*I and *Xho*I in a reaction medium containing 6 μ l 10X react 2 buffer (GibcoBRL), 6 μ l 10X bovine serum albumin (BSA), 36 U *Stu*I, 36 U *Xho*I, and 30 μ g cDNA. The linearized pMRE1001 was dephosphorylated by the addition of 2 U shrimp alkaline phosphatase (SAP) and 6 μ l 10X SAP buffer (Amersham). The pCR2.1/3'2J/ α -apy 1-kb digest product was gel isolated and purified (Qiaquick), then ligated into MRE1001 using DNA T4 ligase, incubated overnight at 14°C. SURE *E.coli* (Stratagene) cells were transformed with the ligation medium per manufacturer's instructions. Bacterial colonies were screened by PCR amplification with primers specific for the apyrase insert (Table 4.1). Bacterial colonies

testing positive by PCR were cultured in TB broth (Sambrook *et al.*, 1989) overnight at 37°C. Plasmid DNA was extracted as described (Sambrook *et al.*, 1989). Purified plasmids were further screened by restriction digest.

Table 4.1 Primers used in PCR Reactions

Primer name	Nucleotide sequence
TE/3'2J <i>Stu</i> I	5'-GAAGGCCTTCAGGTAGACAATATTACAC-3'
TE/3'2J <i>Xho</i> I	5'-CTTACCGCTGTTGAGATC-3'
MRE16E1F	5'-CCC GCAGGCTAACTTCATA-3'
2JF	5'-GTCAGCATAGTACATTTTCATC-3'
2JR	5'-GCTGGTCCGATCATTGGGGCG-3'
ApyF	5'-GGCTGGAAACCGGGTTACAG-3'
ApyR	5'-CCTTAGAAGATTGTACCACAG-3'

Construction of pMRE/3'2J/GFP Virus. The construction of pMRE/3'2J/GFP has been described (Olson *et al.*, 2000). Derived from *Aequorea victoria* jellyfish, the 1200-bp green fluorescent protein (GFP) gene encoded a red-shifted mutant, designated GFP-S65T (Clonetech, Palo Alto, CA).

Generation of MRE/3'2J/ α -apy Virus. Recombinant virus was produced from pMRE/3'2J/ α -apy by linearization with *Xho*I, transcription from the SP6 promoter, and electroporation (450 Volts, 100 μ F, 720 Ohms) into BHK-21 cells (Powers *et al.*, 1994). Virus titer [tissue culture 50% infective dose (TCID₅₀), measured as log₁₀ per ml] was determined by titration in triplicate in baby hamster kidney 21 (BHK-21) cells.

Oral Infection of *Aedes aegypti* Larvae with MRE/3'2J/ α -apy Virus.

First instar larvae infection. C6/36 cells (80% confluent) were infected with MRE/3'2J/ α -apy at a multiplicity of infection (MOI) of 10. Virus was adsorbed to the cells for 1 hr, with constant agitation. Afterwards, 4 ml (25-cm² flasks) or 10 ml (75-cm²

flasks) Leibovitz's (L-15) medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin was added to the flask.

Protease cleavage by trypsin of the SIN virus PE2-E1 heterodimer allows the virus to infect midgut epithelial cells (Strauss & Strauss, 1994). In addition, FBS is a trypsin inhibitor (Sambrook *et al.*, 1989). Alternative infection studies were carried out without fetal bovine serum in the medium in an attempt to increase the infection rate. In this procedure the virus supernatant was removed after the initial 1-hr adsorption step. The cells were washed twice with PBS followed by addition of 10 ml L-15 medium with 100 U/ml penicillin and 100 µg/ml streptomycin. One hundred microliters of supernatant were collected directly after infection (T=0) and at 24, 48, and 72 hr postinfection. Virus was amplified for 48 hr in C6/36 cells cultured in 25-cm² or 75-cm² flasks, or until GFP expression was observed in 100% of the cells infected with MRE/3'2J/GFP.

Larvae or eggs were added to the flasks when 100% of the cells expressed GFP in the MRE/3'2J/GFP-infected cells. This was determined empirically as 48 hr postinfection in flasks containing FBS in the medium, and 72 hr postinfection in the flasks without FBS in the medium (Fig. 4.4). Two hatching methods were used. In the first method, *Ae. aegypti* (Rockefeller strain) eggs were hatched in a 15-ml centrifuge tube with nitrogen gas in tap water for 20 min. Infected C6/36 cells were scraped from the bottom of the flask, and approximately 100 larvae in 1 ml of water were added to the cell-supernatant suspension in the flask. After 3 days larvae were transferred to hatching pans. Pupae were transferred to cartons, and upon eclosing, were provided with water and sugar *ad libitum*.

In the second hatching method the egg liner was soaked in 60% ethanol for 20 min to surface sterilize the eggs, then rinsed twice in PBS, and dried on filter paper. Infected

C6/36 cells were scraped from the bottom of the flask. The egg liner was placed directly into the flask, or eggs were brushed from the liner onto filter paper, counted and then brushed into the flask. Larvae generally consumed all of the C6/36 cells within 24 hr. They were maintained in the flask for a further 24 hr, then transferred to hatching pans; tap water and food were added to the hatching pans.

Third instar larvae infection. Larvae infected at third instar were hatched in tap water, and maintained in the hatching pans until development to third instar. Larvae were collected in 1 ml tap water, and added to the flask of infected C6/36 cells. At approximately 48 hr, developing pupae were transferred to cartons.

Diothiothreitol (DTT) is a reducing agent that has been shown to allow greater sized particles to pass across the larval peritrophic matrix into midgut epithelial cells in mosquitoes (Edwards & Jacobs-Lorena, 2000). An experiment was carried out to determine if the infection rate might be improved by the addition of DTT to the C6/36 supernatant. When the third instar larvae were introduced to the flasks, 10 mM of DTT was added to the supernatant. Larvae and pupae were allowed to develop as above.

Intrathoracic Inoculation of Mosquitoes. Adult female mosquitoes were cold anesthetized and intrathoracically injected using a Drummond 50- μ l microcapillary needle prepared with a needle puller (Narishige Co., Toyko, Japan). Approximately 1 μ l of dsSIN virus ($\cong 10^4$ infectious particles) in L-15 medium containing 10% FBS and 1% antibiotics was injected into each mosquito (Olson *et al.*, 1996; Rosen & Gubler, 1974).

Indirect Immunofluorescent Assay. For infection screening, one hind leg or head was pulled from each adult mosquito and mounted and pressed onto an acid-washed slide with a glass coverslip. Salivary glands were dissected in PBS (pH 7.2) and mounted on a

slide. Mosquito tissue was fixed to the slide in acetone at -20°C for 10 min. SIN virus infection of tissue was assayed by IFA with primary antibody anti-SIN E1 monoclonal antibody 30.11A, which recognizes the E1 protein of SIN virus (Chanas *et al.*, 1982). The antibody was diluted 1:200 in PBS, dropped onto the slide, and incubated at 37°C for 40 min. Slides were washed twice with PBS, and incubated for 40 min at 37°C with biotinylated sheep anti-mouse secondary antibody (Amersham)/1% Evan's blue in PBS diluted 1:200 in PBS. After two washes in PBS, the leg tissue was incubated 10 min at 37°C with streptavidin-fluorescence (Amersham) diluted 1:200 in PBS. The slides were subsequently washed three times in PBS and twice in distilled water. A drop of DABCO (2.5 g DABCO, 90 ml glycerol, 10 ml PBS, pH 8.6) was applied to each tissue sample, then the slide was overlaid with a glass coverslip. SIN antigen was visualized on an Olympus BH-2 epifluorescence microscope with FITC filters.

Paraformaldehyde Fixation. Mosquito midguts or salivary glands were dissected in PBS on an acid-washed slide. Midguts were immersed in 4% paraformaldehyde; salivary glands were transferred to a drop of 4% paraformaldehyde on the slide. Tissues were fixed for 20 min. The midguts were rinsed twice in PBS for 10 min. The paraformaldehyde solution was pipetted from the salivary glands. A drop of PBS was added to the salivary glands to rinse.

Virus Titer. Mosquito carcasses in 1 ml L-15 diluent were triturated with a pestle then passed through a 2- μ m pore-size filter, as previously described (Higgs *et al.*, 1997). Virus titers were determined by end point dilution assay in triplicate in BHK-21 cells (Karber, 1931) after incubation at 37°C for 5 days. Cells were stained with 100 μ l of titration cell

stain (0.1% amido black, 25% isopropanol, 10% acetic acid, 64.9% distilled water) for 30 min, then rinsed with water.

RNA Collection and Isolation. To isolate RNA from C6/36 cells, 4 ml of RNAwiz (Ambion) was added to the 25-mm² flasks containing C6/36 cells. Cells were scraped from the bottom of the flask and pipetted into centrifuge tubes. RNA was extracted per manufacturer's instructions.

Salivary glands were dissected in PBS (pH 7.2) and collected in a tube containing 50 µl of ice-cold PBS. Five hundred microliters of Trizol (GibcoBRL) was added to the tube, the salivary glands were immediately homogenized with a pestle, then frozen at -70°C until RNA isolation. Mosquito salivary gland RNA was isolated using the manufacturer's directions. Briefly, tubes were thawed, then incubated at room temperature for 5 min. Chloroform (0.2 ml chloroform/1 ml Trizol) was added to the tube, thoroughly mixed by vigorous shaking, then the tube was incubated for 5 min at room temperature. Centrifugation at 11,000g for 15 min at 4°C separated the RNA, DNA, and protein into the top aqueous layer, the interphase, and the bottom pink layer, respectively. The RNA in the top layer was removed into a clean tube. The remaining DNA/protein mixture was stored at -70°C. RNA was precipitated by addition of isopropanol (0.5 ml/1 ml Trizol), incubation at room temperature for 10 min, then centrifugation for 10 min at 12,000g. The RNA was washed once with 500 µl 70% ethanol in RNase-free water, then pelleted by centrifugation at 4°C, 7,500g for 5 min. RNA was resuspended in RNase-free water warmed to 60°C and stored at -70°C until use.

Reverse Transcription. cDNA was reverse transcribed from mosquito RNA using Superscript II RNase H reverse transcriptase in a reaction containing 15 pmol of primer, 3 ug total RNA, and RNase-free water to 12 µl total volume. The mixture was heated to 70°C for 10 min, then quickly chilled on ice to remove RNA secondary structure. After brief centrifugation, 7 µl of a reaction mixture containing 5X first strand buffer (50mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂ final concentration), 10 mM DTT, and 0.5 mM dNTP mix was added to each tube. The mixture was incubated at 42°C for 2 min, after which 1µl of Superscript II was added, then incubated for 1 hr at 42°C, and 15 min at 70°C. Three microliters of the RT reaction was used in PCR reactions.

2JR, a reverse primer located downstream of the insert in the 3' noncoding region of the virus (**Table 4.1**) was used to reverse transcribe the virus RNA. Full-length genomic, first subgenomic, and second subgenomic transcripts all containing the apyrase or GFP insert, would be detectable by this primer. Two regions of the virus sequence were amplified by PCR. Primers 2JF and 2JR amplified the regions on both sides and outside of the insert. The second primer set consisted of a forward primer located in the E1 region of MRE16 (primer MRE16E1F), with 2JR the reverse primer, located 45 bases downstream of the insert (**Fig. 4.1 and Table 4.1**).

Northern Blot Analysis. Ten micrograms of total RNA extracted from C6/36 cells or salivary glands was fractionated in a 1.25% agarose-formaldehyde gel [1.25 g agarose, 10 ml 10X MOPS, 5.1 ml formaldehyde (37%), 84 ml RNase-free water] run at 180 V in 1X MOPS/0.66 M formaldehyde running buffer. RNA was transferred to a BrightStar-Plus (Ambion) positively charged nylon membrane overnight in 20X sodium chloride-sodium citrate (SSC) solution (175.3 g NaCl, 88.2 g citric acid in 1 L total vol H₂O, pH 7.0).

The RNA was hybridized first with a BrightStar psoralen-biotin (Ambion) labeled PCR product specific for the apyrase insert. Hybridization was at 60°C in a rotating water bath for 18 hr. Following hybridization, the membrane was washed twice for 10 min in 2X SSC, 0.1% SDS, followed by two high-stringency washes for 30 min at 60°C in 0.2X SSC, 0.1% SDS. RNA was visualized using the BrightStar BioDetect (Ambion) detection kit according to manufacturer's instructions. The membrane was exposed to Kodak X-Omat AR (VWR Scientific, West Chester, PA) X-ray film for 5 min.

The membrane was dehybridized by rinsing four times in 160 ml of boiling RNase-free water for 10 min, incubating in 5% SDS in the rotating water bath at 65°C for 40 min, then rinsing three times in RNase-free water (A. Rayms-Keller and R. Mackie, personal communication). The membrane was rehybridized with biotinylated (GibcoBRL) labeled 2JR primer (**Table 4.1**) in the rotating water bath at 42°C, overnight. Detection was the same as above.

Apyrase Activity Assay. Apyrase activity was determined using the protocol of Ribeiro (Ribeiro *et al.*, 1984) and an inorganic phosphorus commercial kit (Sigma). Salivary glands were dissected in 0.15 M NaCl and transferred to 100 µl of 10 mM Tris-Cl (pH 7.5). Salivary glands were homogenized in an Aquasonics waterbath (VWR Scientific) for 2 min at 50°C (power level 5), then centrifuged at 13,000 rpm for 1 min. Ten microliters of salivary gland homogenate was added to 90 µl of reaction medium (100 mM Tris-Cl buffer pH 9.0, 200 mM NaCl, 10 mM CaCl₂, and 20 mM ADP sodium salt) in a 96-well plate. Samples were incubated at 37°C for 30 min. The reaction was stopped by adding 25 µl of ammonium molybdate in 2.5N sulfuric acid (Sigma). Three microliters of F&S reducing reagent (Sigma) was added, then the plate was placed in the

dark for 20 min. The amount of inorganic phosphate released was measured on a 450 Plate Reader (BioRad, Richmond, CA) with a 665-nm filter. Apyrase activity was calculated as the total amount of monophosphate released per microgram of salivary gland protein.

Western Blot Assay. Salivary glands were dissected in 0.15 M NaCl then transferred to a tube containing 12 μ l loading buffer (50 mM TrisCl pH 7.5, 1% SDS, 2 mM phenyl methyl sulphuryl fluoride, 1Mm EDTA, 10% glycerol, 5% mercaptoethanol, 0.2% bromophenol blue). Homogenization consisted of three cycles of freeze-thawing, and boiling for 2 min. One complete pair of salivary gland was loaded per lane, and proteins were separated on a 10% SDS-PAGE 1-mm gel (Novex, Hercules, CA) run at 150 mV for 1 hr. Proteins were transferred to a nitrocellulose membrane (Trans-blot, BioRad) by electrophoresis using a BioRad transfer apparatus (transfer buffer: 25 mM Tris, 192 mM glycine, 10% methanol; pH 8.3) set at 200 mA for 3 hr. Proteins were detected using the ECL-Western blotting kit (Amersham) according to manufacturer's instructions, except that PBS-0.05% NP-40 was used for blocking and all washes. A polyclonal rabbit anti-apyrase antibody raised to recombinant apyrase peptide diluted 1:20,000 in PBS-0.05% NP-40 detected the 68-kd apyrase protein (Champagne *et al.*, 1995). A polyclonal rabbit anti-D7 antibody diluted 1:2000 detected the 37-kd D7 protein (James, 1994). Amersham anti-rabbit HRP secondary antibody, diluted 1:1000, was used as secondary antibody. The membrane was exposed to Kodak X-Omat AR (VWR Scientific) X-ray film for 30 seconds. Band density was calculated using the Un-SCAN-IT (Silk Scientific Co., Orem, UT) Program. Total salivary gland protein was detected by rinsing the membrane twice in PBS-0.05% NP-40 then incubating with 50-ml colloidal gold (Bio-Rad) for 1 hr.

RESULTS

MRE/3'2J/ α -apy Virus Characterization. pMRE/3'2J/ α -apy was screened by PCR and restriction endonuclease digestion to confirm presence, size, and orientation of the apyrase insert (**Fig. 4.2**). A PCR reaction was carried out using a forward oligonucleotide primer specific for the MRE16 E1 gene (MRE16E1F primer, **Table 4.1**) and a reverse oligonucleotide primer from the 3'-noncoding region of pTE/3'2J (2JR primer, **Table 4.1**). Northern blot assay of RNA from infected C6/36 cells confirmed transcription of the three viral mRNA species of MRE/3'2J/ α -apy and MRE/3'2J/GFP (**Fig. 4.3**).

Oral Infection of *Aedes aegypti* Larvae. Virus was amplified in C6/36 cells prior to *per os* infection of larvae. Supernatant was collected at 24-hr time points and virus titer calculated in order to establish an optimum time for introduction of larvae (**Fig. 4.4**). MRE/3'2J/GFP was used as the control heterologous virus. Expression of GFP in the C6/36 cells could be observed in the flask using the microscope. Generally, 100% of cells expressed GFP by 48 hours, at which time the larvae were added to the flask (**Fig. 4.5**). Infection by MRE/3'2J/ α -apy of C6/36 cells was also visualized by IFA staining using the Sindbis E1 protein primary antibody (data not shown). Virus titer, GFP expression, and IFA all confirmed 48 to 72 hr as the optimal time for larvae introduction.

Infection of larvae hatched in nitrogen gas. In the first series of experiments, eggs were hatched in tap water using nitrogen gas. After 20 min, the newly hatched larvae were transferred to the flasks containing infected C6/36 cells. The larvae generally consumed all the cells by 24 hr. Larvae were maintained in the flasks for a further 24-48 hr, because the supernatant was shown to contain virus, which could potentially infect the larvae (**Fig. 4.4**). GFP was expressed in the anal pupillae in most larvae infected with

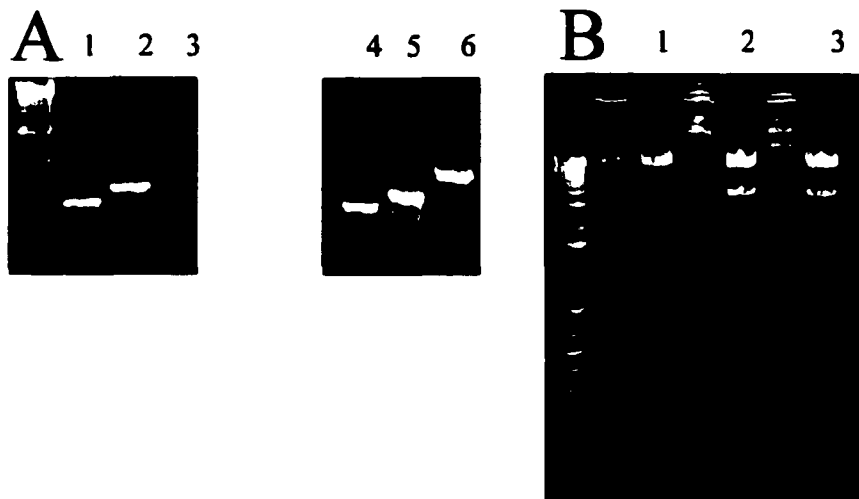


Figure 4.2 PCR and restriction digest screening for insert presence and orientation in pMRE/3'2J/α-apy. (A) PCR screening of pTE/3'2J/α-apy (lanes 1-3) and pMRE/3'2J/α-apy (lanes 4-6). Lanes 1 and 4 PCR amplification with ApyF and ApyR primers. Lanes 2 and 5 PCR amplification with 2JF and 2JR primers. Lanes 3 and 6 PCR amplification with MRE16E1F and ApyF primers, confirming the insert presence and orientation in pMRE/3'2J/α-apy. (B) pTE/3'2J/α-apy (lane 1), pMRE1001 (lane 2), and pMRE/3'2J/α-apy (lane 3) were restriction digested with *Xba*I. pTE/3'2J/α-apy has one *Xba*I restriction site, which linearizes the plasmid. pMRE1001 and pMRE/3'2J/α-apy have two *Xba*I restriction sites, resulting in three products.

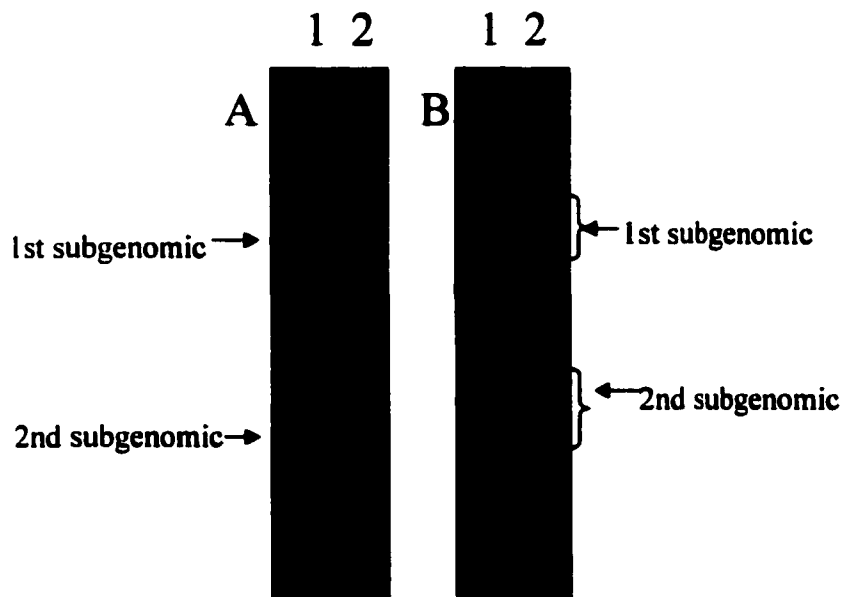


Figure 4.3 Northern blot assay of MRE/3'2J/α-apy and MRE/3'2J/GFP. RNA was isolated from C6/36 cells infected with MRE/3'2J/α-apy (lanes 1) and MRE/3'2J/GFP (lanes 2) and probed with (A) a biotin-labeled PCR product of the apyrase insert or (B) biotin-labeled 2JR primer.

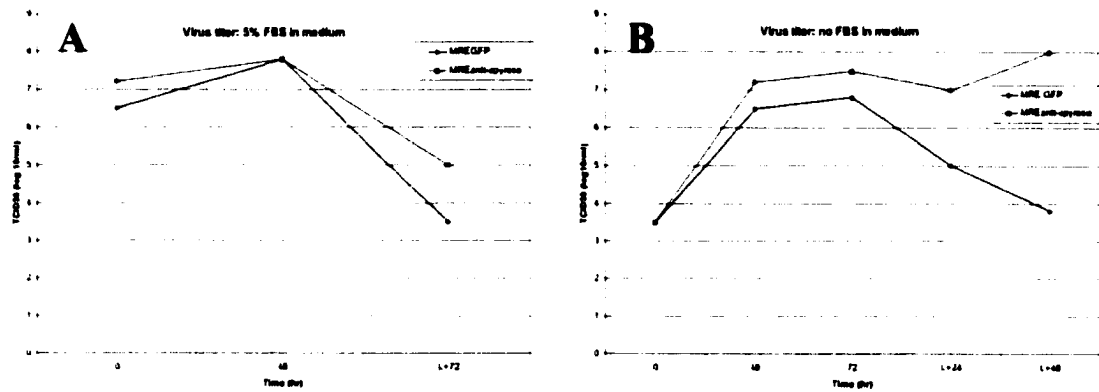


Figure 4.4 Replication of MRE/3'2J/α-apy and MRE/3'2J/GFP in C6/36 cells. C6/36 cells were infected at an moi of 10. After 1 hr, L-15 medium (A) with or (B) without 5% FBS was added to the flasks. Supernatant was collected at 24 hr intervals. Larvae were added to flasks at (A) 48 or (B) 72 hr postinfection.

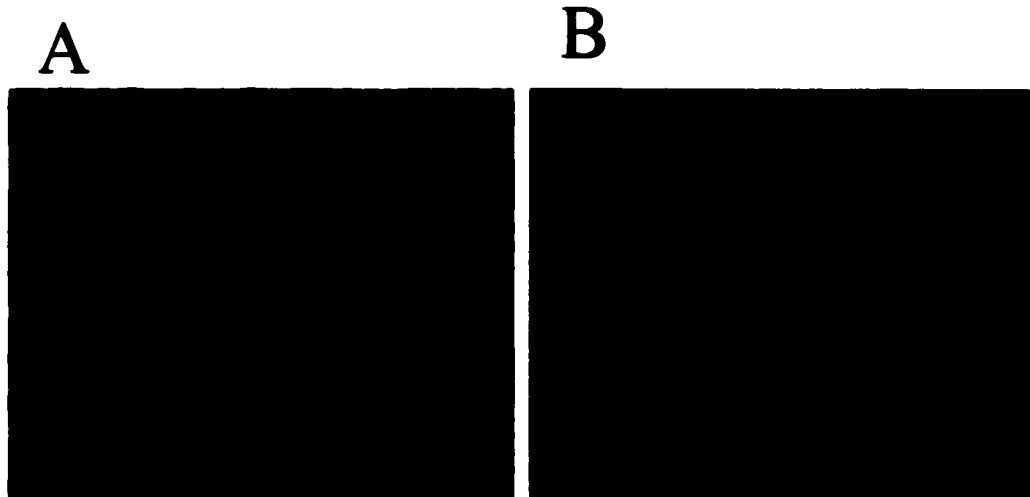


Figure 4.5 GFP expression in C6/36 cells infected with MRE/3'2J/GFP. (A) C6/36 48 hr postinfection in 25 cm² flasks. (B) Uninfected C6/36 cells.

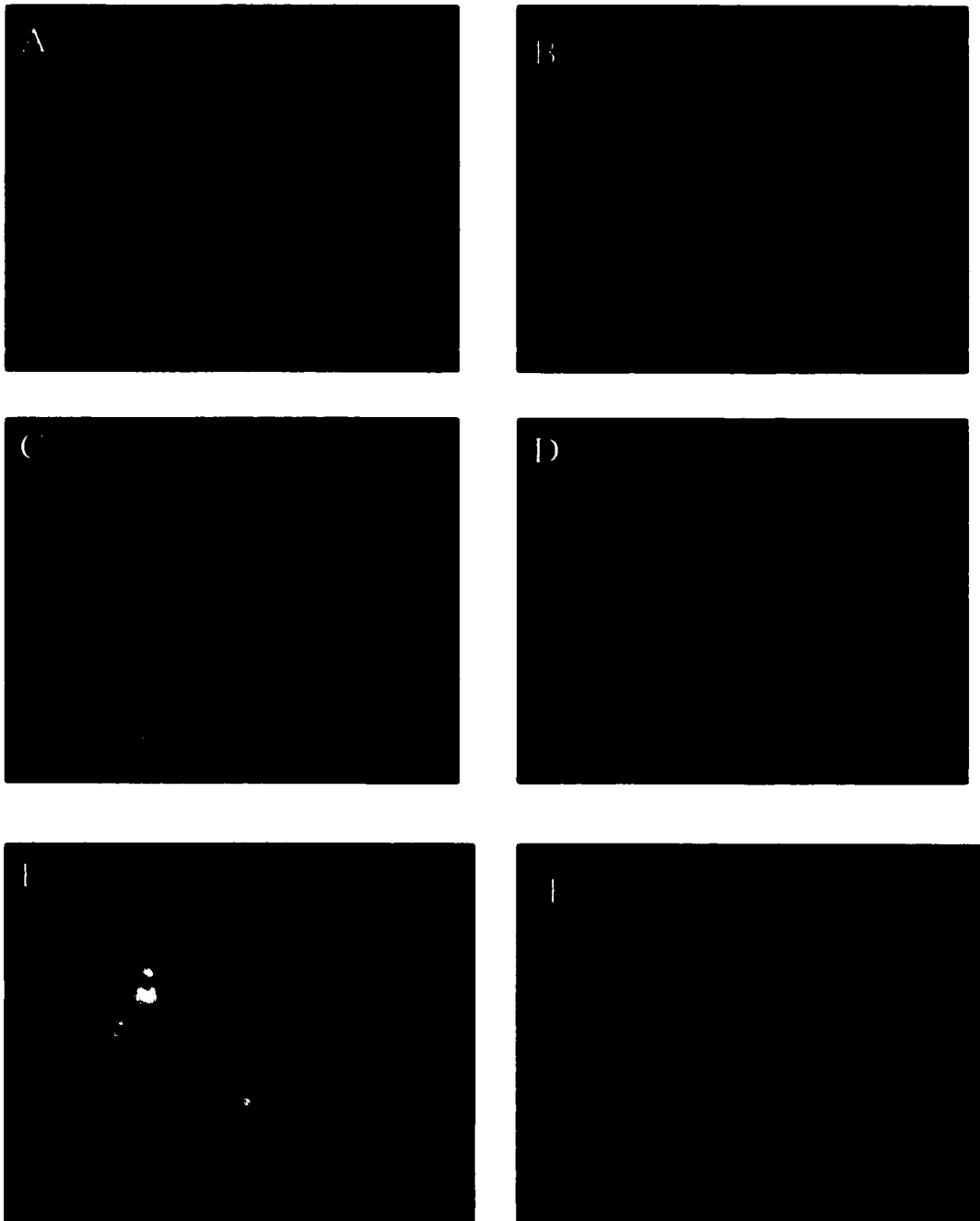


Figure 4.6 GFP expression in larvae 48 hr posthatching in supernatant of MRE/3'2J/GFP infected C6/36 cells. (A) Larvae photographed in flask. One of six shown shows GFP expression. (B) Close up of head. (C) Larva in flask infected with MRE/3'2J/GFP vs (D) larva in uninfected flask. (E and F) Anal papillae of C and D, respectively.

MRE/3'2J/GFP (Fig 4.6). Larvae were then transferred to hatching pans and allowed to develop. At 5 days post-eclosion, legs were pulled from female adults, and screened for SIN virus infection by IFA (Table 4.2). Mosquitoes were maintained in 96-well plates until screening was complete. Salivary glands were dissected within 24 hr of IFA screening. SIN infection was confirmed in mosquitoes positive by IFA by titration of mosquito carcasses. The SIN infection rate varied from 6.5% in MRE/3'2J/GFP infected mosquitoes to 30% in MRE/3'2J/ α -apy infected mosquitoes. SIN infection of salivary glands in mosquitoes positive for infection was detected by IFA. All salivary glands were shown to be positive for SIN infection in orally infected mosquitoes. However, infection of salivary gland tissue was incomplete (Fig. 4.7A). Salivary glands from mosquitoes intrathoracically inoculated with virus were 100% infected (Fig. 4.7B).

Table 4.2 *Per os* infection rates in adult mosquitoes: nitrogen hatched

	% infected	Mean titer (TCID ₅₀ log ₁₀ /ml)
Uninfected control	0	
MRE/3'2J/GFP	6.5 (15/230)	4.7
MRE/3'2J/ α -apy	30 (23/76)	4.8



B

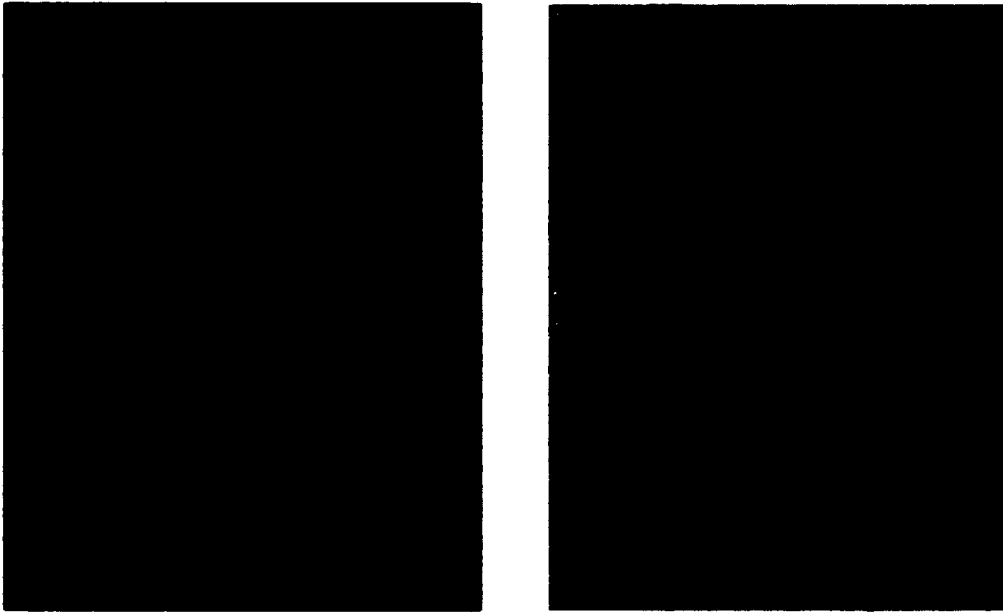


Figure 4.7 SIN virus infection in salivary glands of mosquitoes. (A) orally infected as larvae and (B) inoculated as adults. A primary antibody that recognizes the E1 envelope protein of SIN was used in IFA. Original magnification 200X.

Infection of larvae hatched directly in flask. In another experiment, egg liners were surface sterilized with 60% ethanol. Eggs were brushed off the egg liner onto a piece of filter paper, counted, brushed into the flask, and hatched directly in the L-15 medium. The infection rate of the flask-hatched mosquitoes was higher than the nitrogen-hatched mosquitoes for both MRE/3'2J/GFP (21% vs. 3%) and MRE/3'2J/ α -apy (32% vs. 8.5%) viruses (**Table 4.3**). Eggs were hatched directly in flasks in subsequent experiments.

Table 4.3 Infection rates of mosquitoes hatched in nitrogen or in flask

	% infected
Uninfected	0
MRE/3'2J/GFP flask	21 (34/162)
MRE/3'2J/GFP N ₂	3 (3/111)
MRE/3'2J/ α -apyrase flask	32 (48/147)
MRE/3'2J/ α -apyrase N ₂	8.5 (10/117)

Comparison of infection rates with and without FBS in medium. In another series of experiments eggs were hatched in flasks containing infected C6/36 cells with supernatant consisting of L-15 medium with or without 5% FBS. Larvae were maintained in the flasks for an additional 24-48 hr, then transferred to hatching pans and fed as usual. Adult head tissue of both males and females was screened for SIN virus infection by IFA. The resulting SIN infection rates are presented in **Table 4.4**. The highest infection rate observed was 31%, in MRE/3'2J/GFP infected mosquitoes hatched in supernatant without FBS. The lowest infection rate was observed in the mosquitoes hatched in flasks containing 5% FBS (8-16%). Because of possible overcrowding in the flasks with medium without FBS (N=213 in MRE/3'2J/ α -apy infected flasks), the experiment was repeated with fewer larvae. However, the infection rate did not increase in the MRE/3'2J/ α -apy infected mosquitoes (15%).

Table 4.4 Comparison of *per os* infection methods in larvae

Virus	Treatment	N	% infected	% survival*	Ave TCID ₅₀ (log ₁₀ /ml)
1st instar infection					
Control	+FBS	30	0	100	
MRE/3'2J/GFP	+FBS	41	8	41	4.7
MRE/3'2J/α-apy	+FBS	39	16	39	4.8
Control	-FBS	50	0	50	
MRE/3'2J/GFP	-FBS	113	31	113	4.7
MRE/3'2J/α-apy	-FBS	173	13	173	5.0
3rd instar infection					
Control	DTT+FBS	37	0	37	
MRE/3'2J/GFP	DTT+FBS	41	42	41	4.8
MRE/3'2J/α-apy	DTT+FBS	35	25	35	5.2
MRE/3'2J/GFP	+FBS	10	40	10	4.7
MRE/3'2J/GFP	-FBS	19	5	19	4.6

*Survival rates were calculated as adults/pupae.

Oral infection of third instar larvae. Third instar larvae were added to a flask containing L-15 medium consisting of 5% FBS 10 mM DTT, 5% FBS, or no FBS. MRE/3'2J/GFP infected mosquitoes in 5% FBS + DTT (42%) and 5% FBS (40%) accounted for the highest infection rates in this experiment (**Table 4.4**).

SIN Infection of Salivary Glands. IFA of salivary glands dissected from orally infected or inoculated female mosquitoes revealed that all salivary glands from infected mosquitoes contained SIN virus antigen (**Fig 4.7**). Complete infection of all the lobes of the salivary glands was observed in mosquitoes intrathoracically inoculated with virus; however, incomplete infection was seen in orally infected mosquitoes.

GFP Expression in Salivary Glands of MRE/3'2J/GFP Infected Mosquitoes.

Salivary glands were dissected from mosquitoes infected with MRE/3'2J/GFP virus and fixed in 4% paraformaldehyde. GFP was detected in 5 midguts and 2 salivary glands dissected from 10 infected mosquitoes hatched in the flask (**Fig 4.8**). GFP was expressed in all salivary glands from mosquitoes inoculated with MRE/3'2J/GFP. However, GFP

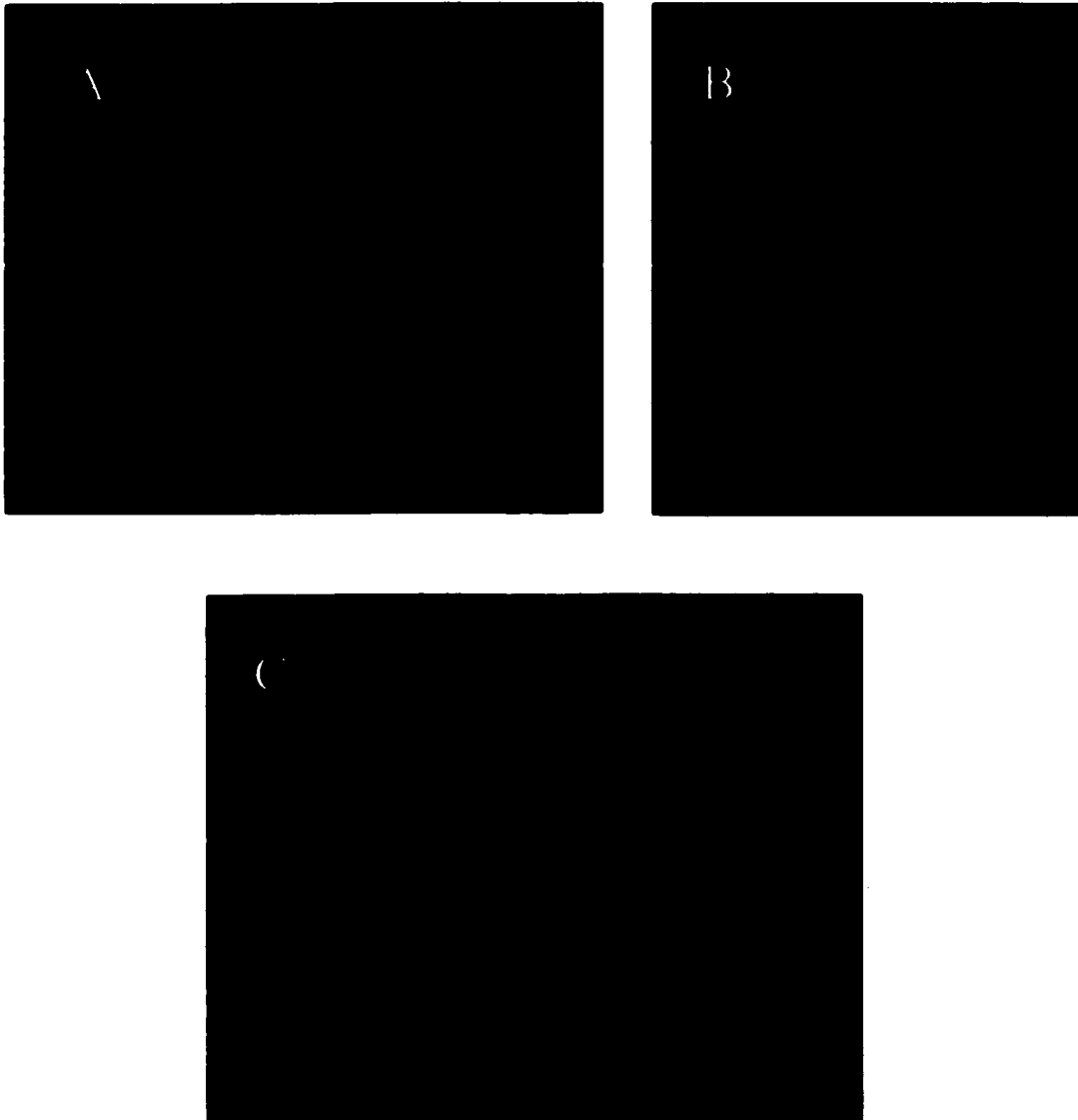


Figure 4.8 GFP expression in (A) midguts and (B) salivary glands of *Ae. aegypti* mosquitoes that had been orally infected with MRE/3'2J/GFP as larvae. (A) GFP expression was seen throughout the midguts of 5 of 10 dissected mosquitoes. (B) GFP expression was limited to one medial lobe in the salivary glands; 2 out of 10 salivary glands dissected showed GFP expression. (C) GFP expression in salivary glands of intrathoracically inoculated adults. GFP was shown in all salivary glands. Original magnification (A and C) 200X; (B) 400X.

was not expressed throughout the salivary gland tissue in any of the inoculated mosquitoes; GFP was expressed in the medial lobes, but not the lateral lobes of the salivary glands (Fig 4.8).

RT-PCR Screening for Insert in Salivary Glands of Infected Mosquitoes. To determine if the dsSIN virus infecting the salivary glands contained the apyrase or GFP insert, RT-PCR was done on RNA isolated from 20 pooled salivary glands dissected from mosquitoes infected as larvae, and shown to be positive by IFA of leg tissue. Two PCR products were amplified from the salivary glands of MRE/3'2J/ α -apy infected mosquitoes. The 95-bp band corresponded to the surrounding TE/3'2J multiple cloning site without the apyrase insert. The second band of approximately 500-bp was the size of the surrounding TE/3'2J vector with the 375-bp apyrase insert. One PCR product of 95 bp was amplified from salivary glands of mosquitoes infected with MRE/3'2J/GFP (Fig. 4.9). Thus, the GFP insert was deleted from the virus.

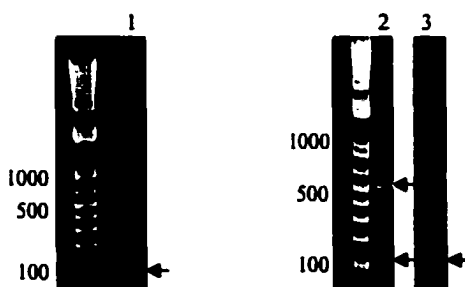


Figure 4.9 RT-PCR from pooled salivary glands of adult mosquitoes infected *per os* as larvae with MRE/3'2J/ α -apy (lane 2) or MRE/3'2J/GFP (lane 3). cDNA was reverse transcribed from RNA with the 2JR primer. Using primers 2JF and 2JR a 95-bp sequence of the multiple cloning site and the heterologous sequence was amplified. Lane 1: PCR amplification of pMRE/3'2J showing the 95-bp PCR product without an insert. Lane 2: RT-PCR from pooled salivary glands of mosquitoes infected with MRE/3'2J/ α -apy showing both the 95-bp and 500-bp PCR product. Lane 3: RT-PCR from pooled salivary glands of mosquitoes infected with MRE/3'2J/GFP with one 95-bp PCR product.

Analysis of Apyrase Enzymatic Activity and Protein Production. Salivary glands were dissected from infected mosquitoes and assayed for apyrase activity. Although apyrase activity was reduced in MRE/3'2J/ α -apy infected mosquitoes compared to uninfected controls ($p=0.01$), apyrase activity in mosquitoes orally infected with the control virus, MRE/3'2J/GFP, also was reduced compared to uninfected controls ($p=0.0006$) (Table 4.5). However, apyrase activity in inoculated mosquitoes was inhibited only in MRE/3'2J/ α -apy infected salivary glands compared to the two control groups ($P=0.0006$).

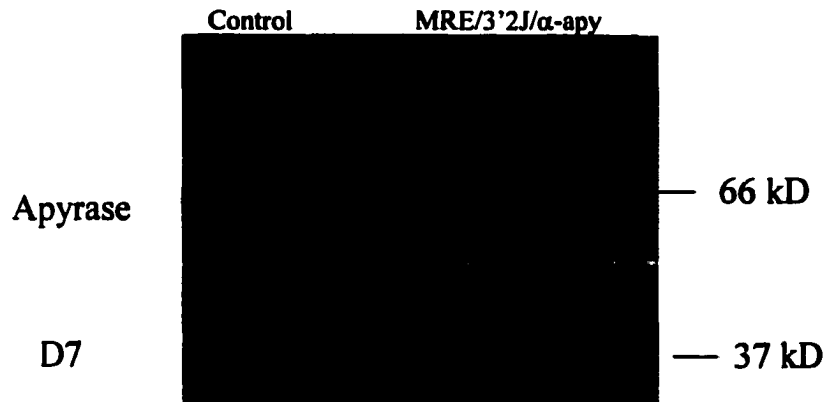
Table 4.5 Apyrase activity in adult mosquitoes orally infected as larvae or inoculated as adults

	N	Mean apyrase activity* (\pm SD)
Uninfected control	15	203 (109)
Orally infected MRE/3'2J/GFP	12	113 (93)
Orally infected MRE/3'2J/ α -apy	13	95 (98)
Uninoculated control	3	102 (21)
Inoculated MRE/3'2J/GFP	3	101 (16)
Inoculated MRE/3'2J/ α -apy	3	47 (8)

*Apyrase activity was measured as the amount of monophosphate produced per microgram of salivary gland protein ($\text{PO}_4/\mu\text{g}$ protein).

A western blot assay further characterized apyrase protein production (Fig. 4.10). There was no reduction of apyrase protein in orally infected mosquitoes compared to uninfected controls. Diminished apyrase concentration was observed visually in mosquitoes intrathoracically inoculated with MRE/3'2J/ α -apy virus compared to uninfected controls. However, by densitometric analysis (pixel density compared between D7 and apyrase bands, expressed as a proportion), apyrase was reduced in both MRE/3'2J

A



B

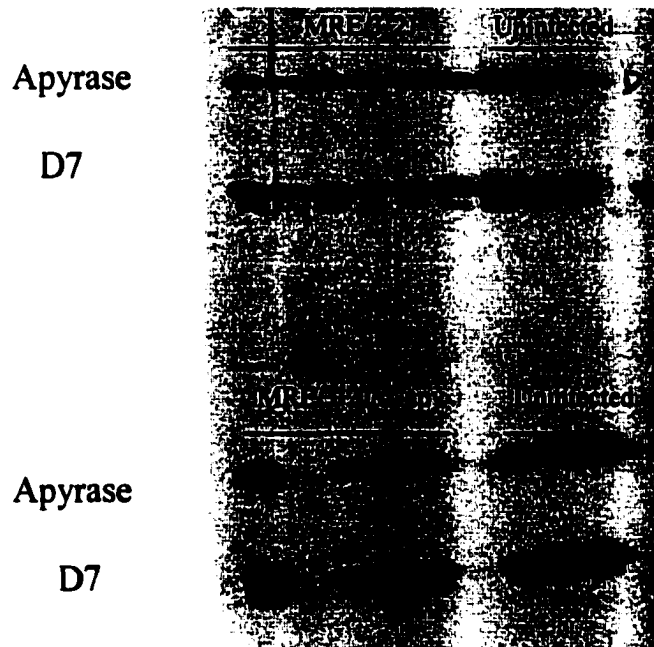


Figure 4.10 Western blot analysis of apyrase protein production in salivary glands of mosquitoes. (A) Orally infected as larvae (uninfected $n=2$, MRE/3'2J/ α -apy infected $n=4$) and (B) intrathoracically inoculated with MRE/3'2J/ α -apy ($n=4$) or MRE/3'2J control virus ($n=6$) (uninfected control $n=6$). (A) There was no difference in amounts of apyrase between uninfected controls and mosquitoes that had been orally infected with MRE/3'2J/ α -apy at larval stage. (B) Apyrase production was reduced in both mosquitoes intrathoracically inoculated with MRE/3'2J/ α -apy and MRE/3'2J control viruses compared to uninoculated mosquitoes, but the difference was not significant ($p=0.4$).

infected control (mean 0.75, n=6) and MRE/3'2J/ α -apy infected (mean 0.79, n=4) salivary glands compared to uninfected controls (mean 0.94, n=6), although not significantly ($p=0.4$) (Fig. 4.10B).

DISCUSSION

In the initial experiments, mosquito eggs were hatched in a tube with nitrogen gas. The larvae were counted and added to the flask containing infected C6/36 cells within 20 min of hatching. This infection procedure, based on previous studies (Higgs et al. 1999), was repeated in three experiments. Infection rates ranged from 6%-30%. In an attempt to attain a consistently greater infection rate, other methods were investigated. Formation of the larval peritrophic matrix may have prevented midgut infection by MRE/3'2J/ α -apy and MRE/3'2J/GFP viruses (Edwards & Jacobs-Lorena, 2000; Higgs *et al.*, 1999). Therefore, mosquito eggs were added directly to the flask, and the larvae hatched in the supernatant. They were exposed to the virus in the supernatant and in the infected C6/36 cells, which they immediately began eating upon hatching. The infection rate of the flask-hatched mosquitoes was slightly higher than the nitrogen-hatched larvae (Table 4.3). Therefore, in subsequent experiments eggs were hatched directly in the flask.

Protease cleavage of the SIN virus PE2-E1 heterodimer by trypsin is a necessary processing step which enables the virus to infect the midgut epithelial cells (Strauss & Strauss, 1994). Fetal bovine serum is a trypsin inhibitor (Sambrook *et al.*, 1989). Virus is harvested in BHK-21 supernatant containing 5-10% fetal bovine serum (see Materials and Methods; Generation of MRE/3'2J/ α -apy Virus) and C6/36 cells are maintained in L-15 medium containing 5-10% FBS. To determine if the FBS had an effect on virus

infection of larvae, a series of infection studies were carried out with all FBS removed from the cell medium before the mosquito eggs were added to the flasks. For MRE/3'2J/ α -apy infected mosquitoes, the infection rates were comparable for mosquitoes infected as first instar larvae: 16% vs 13% for +FBS and -FBS, respectively (**Table 4.4**). Infection rates for mosquitoes infected as first instar larvae with MRE/3'2J/GFP ranged from 30% in -FBS to 8% in +FBS treatments. However, in mosquitoes infected as third instar larvae, only 5% of those with the FBS removed were infected, compared to 40% of those infected in flasks containing FBS (**Table 4.4**).

Infection of third instar larvae yielded the highest infection rates (**Table 4.4**). The highest infection rate in mosquitoes hatched in flasks containing 5% FBS was 32%, whereas the highest infection rate for mosquitoes introduced to the flask at third instar was 42% (**Table 4.3**). Therefore, the larval peritrophic matrix probably does not inhibit passage of virus through the larval midgut.

Addition of DTT to the MRE/3'2J/GFP infected cells resulted in an infection rate of 42%. However, 40% infection was attained without DTT, in supernatant containing FBS (**Table 4.4**). Therefore, it was concluded that addition of DTT does not significantly increase SIN virus infection.

Curiously, the survival rate to adults was similar in larvae infected at third instar to those hatched in the flask, which were infected at first instar. Intuitively, the mortality rate of larvae infected upon hatching would be greater than those infected at third instar. The more instar stages that a mosquito passes through while infected would seem to increase the probability for mortality due to the virus. However, in both groups most of the mortality was seen between the pupal stage and ecdysis (**Table 4.4**).

Apyrase activity as measured by enzymatic assay was reduced in both the MRE/3'2J/GFP control and MRE/3'2J/ α -apy orally infected mosquitoes (Table 4.5). In mosquitoes inoculated with MRE/3'2J/ α -apy virus, apyrase activity was significantly lower compared to uninoculated and MRE/3'2J/GFP-inoculated control mosquitoes. However, enzymatic activity may have been affected by virus infection or the MRE/3'2J/GFP construct.

Expression of the *GFP* gene in mosquito cells may have unexpected or confounding effects and may be deleterious to the metabolism of the cell (K. Olson, personal communication). In these experiments, expression of GFP in the salivary glands may have interfered with general salivary gland protein production, including apyrase. The nonspecific inhibitory effects of MRE/3'2J/GFP may have masked the specific inhibition of apyrase by MRE/3'2J/ α -apy. More experiments need to be done to determine the effect of GFP expression on mosquito cell processes.

To determine if the reduction of apyrase activity corresponded to a reduction of apyrase protein production, an immunoassay was done using MRE/3'2J as a control virus. Apyrase was abundant in all salivary glands from orally infected mosquitoes (Fig. 4.10). In inoculated mosquitoes, apyrase was reduced in both MRE/3'2J/ α -apy and MRE/3'2J control groups compared to uninfected controls, although not significantly ($P=0.4$) (Fig. 4.10). Thus, infection by MRE/3'2J virus itself has an inhibitory effect on apyrase production, although the difference was not significant.

Apyrase protein is synthesized early after eclosion, and probably is stored as a stable protein in the salivary glands (James, 1994; Ribeiro *et al.*, 1990; Smartt *et al.*, 1995). Analysis of apyrase protein by the apyrase activity assay and by immunoassay may not

detect inhibition of apyrase mRNA by the antisense RNA transcripts. No apyrase mRNA was detected by Northern blot assay of polyA selected apyrase mRNA from salivary gland RNA using biotinylated probes. Further analysis of apyrase mRNA with a more sensitive detection technique may reveal degradation of apyrase mRNA in mosquitoes infected with MRE/3'2J/ α -apy virus.

The MRE/3'2J virus is an efficient *per os* gene delivery system when introduced in a blood meal to adult mosquitoes. Nearly 100% of midguts can be infected by this method (Olson, 2000). However, infecting larvae *per os* is less efficient; 42% was the highest infection rate achieved by this method. Virus infection in larvae may cause mortality in one of the five developmental stages to adult. The highest infection rate in these experiments was 42%, although other researchers have reported higher infection rates (L.Beaty, personal communication).

The MRE/3'2J chimeric virus is unstable after many replicative cycles in the mosquito (Olson *et al.*, 2000). Infection of the developing larvae increases the possibility of the heterologous gene being deleted. RT-PCR analysis of salivary glands in MRE/3'2J/ α -apy infected salivary glands showed two bands, one without the apyrase insert and one with the insert. The GFP insert was absent in mosquitoes infected with MRE/3'2J/GFP, as measured by RT-PCR (Fig. 4.9). In addition, GFP was detected in only 2 of 10 salivary glands positive for SIN infection (Fig. 4.8), whereas SIN infection was detected in all infected salivary glands by IFA (Fig. 4.9). These findings were consistent with previous *in vivo* experiments using this expression vector (Olson *et al.*, 2000). Development of a dsSIN expression system based on the MRE16 infectious clone will hopefully eliminate the instability of this expression vector.

Per os infection of larvae with the MRE/3'2J virus expression vector has potential for transducing heterologous genes in larval, pupal, and adult tissue. However, infection of larvae with SIN virus results in higher mortality than normal throughout development, especially during adult eclosion. In addition, for those viruses without a phenotypic marker, such as MRE/3'2J/ α -apy, the IFA method of screening for infected individuals is not efficient. A leg must be pulled for IFA screening. At the same time the mosquito must be maintained alive until screened. Those mosquitoes positive for infection are then dissected and analyzed for gene expression, in this case, apyrase activity. Nonetheless, with optimization of infection and screening methods, this method of gene delivery will become a valuable tool for use in transient gene transducing studies.

Chapter 5

Summary

The dsSIN expression vector TE/3'2J has been used to express heterologous genes in *Aedes aegypti*. In addition, replication of flaviviruses in mosquitoes has been inhibited by infection of the mosquito with dsSIN virus carrying sequences homologous to the flavivirus genome in antisense orientation. The hypothesis that endogenous mosquito gene expression could be specifically inhibited by complementary RNA carried by the dsSIN virus was based on these successful *in vivo* expression and inhibition studies.

Inhibition of luciferase expression in the transgenic mosquito by infection with the dsSIN expression vector TE/3'2J/ α -luc supports the hypothesis of this dissertation. Endogenous gene expression can be posttranscriptionally inhibited by association with RNA complementary to specific endogenous mRNA in mosquito cytoplasm. TE/3'2J/ α -luc virus readily infected the salivary glands tissue, where luciferase was expressed. Luciferase activity decreased 90% in TE/3'2J/ α -luc infected mosquitoes compared to control mosquitoes. Inhibition was sequence specific, as infection by control viruses carrying nonhomologous sequences did not result in diminished luciferase expression. In addition, TE/3'2J/ α -luc infection of the salivary glands did not affect the production of other salivary gland proteins.

In this model endogenous gene system, luciferase was expressed at very low levels; luciferase mRNA was not detectable (Coates *et al.*, 1999). However, luminometric analysis of luciferase activity is very sensitive and accurate, so that even small differences in luciferase production by the transgenic mosquitoes could be measured.

There are many variables working in an *in vivo* system that are not encountered when testing a system in tissue culture. Efforts to inhibit apyrase expression in adult mosquitoes by inoculation with the dsSIN virus TE/3'2J/ α -apy were hampered by

problems inherent in an *in vivo* system. First, it is imperative that the effector RNA reach the target tissue at the developmental stage that the gene is expressed. Apyrase is produced soon after adult eclosion, and is either continuously translated, or alternatively, after a burst of production, apyrase may be stored in the salivary glands as a stable protein until needed. TE/3'2J/ α -apy inoculated into adults could be introduced too late to effect inhibition, or the abundant stable protein could mask the inhibitory effect of antisense transcripts on mRNA.

With this in mind, the chimeric virus MRE/3'2J was constructed. MRE16 virus easily infects mosquitoes *per os*, whereas TE/3'2J virus infects mosquitoes poorly unless introduced by inoculation. MRE/3'2J/ α -apy consists of the structural proteins of MRE16, which determine the orally infective phenotype, in the TE/3'2J backbone. In order to infect mosquitoes before development of salivary glands and production of apyrase in adults, *Ae. aegypti* larvae were hatched in flasks containing MRE/3'2J/ α -apy-infected C6/36 cells. The larvae ate the C6/36 cells, thereby becoming infected with the MRE/3'2J/ α -apy virus. Apyrase activity was then assessed in infected adults.

Infection rates in these experiments ranged from 6-42%. Upon inoculation, MRE/3'2J/ α -apy infected salivary glands readily. However, the chimeric virus was shown to be unstable, as the gene of interest inserted in the second subgenomic promoter region was deleted after many rounds of replication in the mosquito. A significant decrease in apyrase protein production was not observed in these experiments. Further studies, including *per os* infection of adults with MRE/3'2J/ α -apy in a blood meal, could provide the efficiency and effective inhibition not seen in this endogenous gene expression study. Future target genes for knockout studies should include inducible

genes. One potential inducible gene is late trypsin. Late trypsin is induced by a burst of translation of early trypsin, which in turn is activated by blood feeding (Noriega *et al.*, 1996a; Noriega *et al.*, 1996b; Noriega *et al.*, 1994). Another factor to consider when looking at gene expression studies is having an accurate method for determining differences in gene expression. In the experiments conducted in this dissertation, apyrase appeared to be inhibited in some cases when measured by apyrase activity assay. However, upon immunoassay analysis of apyrase, no specific diminishment of apyrase was detected. This difference may be particular to apyrase, which may be stored as an inactive proenzyme in the salivary glands.

Other types of constructs with potential for inhibiting gene expression include sense transcripts, or dsRNA. Sense or dsRNA sequences homologous to endogenous mosquito genes may provide an effective means of achieving specific, efficient gene silencing.

Use of the dsSIN virus TE/3'2J to express heterologous genes in mosquitoes is a powerful tool in gene expression studies. Further development of the dsSIN expression system based on the MRE16 infectious clone has the potential to be an efficient, effective transducing system when optimized.

References

- Altman, P. L. & Dittmer, D. S. (1964). *Biology Data Book*, pp. 271. Washington, DC: Federation of American Societies for Experimental Biology.
- Arca, B., Lombardo, F., deLara Capurro, M., della Torre, A., Dimopoulos, G., James, A. & Coluzzi, M. (1999). Trapping cDNAs encoding secreted proteins from the salivary glands of the malaria vector *Anopheles gambiae*. *Proceedings of the National Academy of Sciences* 96, 1516-1521.
- Baltimore, D. (1988). Intracellular immunization. *Nature* 335, 395-396.
- Bass, B. L. (2000). Double-stranded RNA as a template for gene silencing. *Cell* 101, 235-238.
- Baulcomb, D. (1999). Viruses and gene silencing in plants. *Archives in Virology* 15, 189-201.
- Beerntsen, B. T., James, A. A. & Christensen, B. M. (2000). Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews* 64, 115-137.
- Bhalla, S. C. (1968). White eye, a new sex-linked mutant of *Aedes aegypti*. *Mosquito News* 28, 380-385.
- Billingsley, P. F. (1994). Molecular targets in the insect midgut. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 88, 136-140.
- Bosher, J. M. & Labouesse, M. (2000). RNA interference: genetic wand and genetic watchdog. *Nature Cell Biology* 2, E31-E36.
- Bosio, C., Beaty, B. J. & Black IV, W. (1998). Quantitative genetics of vector competence for dengue-2 virus in *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene* 59, 965-070.

- Branch, A. D. (1996). A hitchhiker's guide to antisense and nonantisense biochemical pathways. *Hepatology* 24, 1517-1529.
- Brown, D. T. & Condreay, L. D. (1986). Replication of alphaviruses in mosquito cells. In *The Togaviridae and Flaviridae*, pp. 171-207. Edited by S. Schlesinger & M. Schlesinger. New York: Academic Press.
- Bruening, G. (1998). Plant gene silencing regularized. *Proceedings of the National Academy of Sciences* 95, 13349-13351.
- Calisher, C. H. (1994). Medically important arboviruses of the United States and Canada. *Clinical Microbiology Reviews* 7, 89-116.
- Carlson, J., Olson, K., Higgs, S. & Beaty, B. J. (1995). Molecular genetic manipulation of mosquito vectors. *Annual Review of Entomology* 40, 359-388.
- Catalanotto, C., Azzalin, G., Macino, G. & Cogoni, C. (2000). Gene silencing in worms and fungi. *Nature* 404, 245.
- Chalk, R., Albuquerque, C. M., Ham, P. J. & Townson, H. (1995). Full sequence and characterization of two insect defensins: immune peptides from the mosquito *Aedes aegypti*. *Proceedings of the Royal Society London* 261, 217-221.
- Chalk, R., Townson, H., Desmond, N. H. & Ham, P. (1994). Purification of an insect defensin from the mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 24, 403-410.
- Champagne, D. E., Smartt, C. T., Ribeiro, J. M. & James, A. A. (1995). The salivary gland-specific apyrase of the mosquito *Aedes aegypti* is a member of the 5'-nucleotidase family. *Proceedings of the National Academy of Sciences* 92, 694-698.
- Chanas, A. C., Ellis, D. S., Stamford, S. & Gould, E. A. (1982). The interaction of monoclonal antibodies directed against envelope glycoprotein E1 of Sindbis virus with virus-infected cells. *Antiviral Research* 2, 191-201.
- Cheeseman, M. (1998). Characterization of apyrase activity from the salivary glands of the cat flea *Ctenocephalides felis*. *Insect Biochemistry and Molecular Biology* 28, 1025-1030.
- 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., 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.
- Coleman, J., Green, P. J. & Masayori, I. (1984). The use of RNAs complementary to specific mRNAs to regulate the expression of individual bacterial genes. *Cell* 37, 429-436.
- Cornel, A. J., Benedict, M. Q., Rafferty, C. S., Howells, A. J. & Collins, F. H. (1997). Transient expression of the *Drosophila melanogaster cinnabar* gene rescues eye color in the white eye (WE) strain of *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 27, 993-997.
- Crampton, J. M. (1994). Prospects for genetic manipulation of insect vectors. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 88, 141-143.
- Cupp, E., Cupp, M. S. & Ramberg, F. (1994). Salivary apyrase in African and New World vectors of *Plasmodium* species and its relationship to malaria transmission. *American Journal of Tropical Medicine and Hygiene* 50, 235-240.
- Curtis, C. (1994). The case for malaria control by genetic manipulation of its vectors. *Parasitology Today* 10, 371-374.
- Denhardt, D. T. (1992). Mechanism of action of antisense RNA. In *Annals of the New York Academy of Sciences*, pp. 70-76.
- Dimopoulos, G., Richman, A., Muller, H.-M. & Kafatos, F. C. (1997). Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences USA* 94, 11508-11513.
- Dimopoulos, G., Seeley, D., Wolf, A. & Kafatos, F. C. (1998). Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *The EMBO Journal* 17, 6115-6123.
- Division of Disease Prevention and Control, C. D. P. (1997). Re-emergence of dengue in the Americas. *Epidemiological Bulletin/PAHO* 18, 1-6.
- Edwards, J. F., Higgs, S. & Beaty, B. J. (1998). Mosquito feeding-induced enhancement of Cache Valley virus (Bunyaviridae) infection in mice. *Journal of Medical Entomology* 35, 261-265.
- Edwards, M. & Jacobs-Lorena, M. (2000). Permeability and disruption of the peritrophic matrix and caecal membrane from *Aedes aegypti* and *Anopheles gambiae* mosquito larvae. *Journal of Insect Physiology* in press.

- Fire, A. (1999). RNA-triggered gene silencing. *Trends in Genetics* 15, 359-363.
- Fire, A., Xu, S., Montgomery, M., Kostas, S., Driver, S. & Mello, C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.
- Goddard, J. (2000). *Infectious Diseases and Arthropods*, pp. 231. Totowa, NJ: Humana Press.
- Gorrochotegui-Escalante, N., Munoz, M. D. L., Fernandez-Salas, I., Beaty, B. J. & Black IV, W. C. (2000). Genetic isolation by distance among *Aedes aegypti* populations along the northeastern coast of Mexico. *American Journal of Tropical Medicine and Hygiene* 62, 200-209.
- Grant, S. R. (1999). Dissecting the mechanisms of posttranscriptional gene silencing: divide and conquer. *Cell* 96, 303-6.
- Gratz, N. (1999). Emerging and resurging vector-borne diseases. *Annual Review of Entomology* 44, 51-75.
- Green, P. J., Pines, O. & Masayori, I. (1986). The role of antisense RNA in gene regulation. *Annual Review of Biochemistry* 55, 569-597.
- Grishok, A., Tabara, H. & Mello, C. C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* 287, 2494-2497.
- Gubler, D. J. (1998). Dengue and dengue hemorrhagic fever. *Clinical Microbiology Reviews* 11, 480-496.
- Hahn, C. S., Hahn, Y. S., Braciale, T. J. & Rice, C. M. (1992). Infectious Sindbis virus transient expression vectors for studying antigen procession and presentation. *Proceedings of the National Academy of Sciences, USA* 89, 2679-2683.
- Hajos, J., Vermunt, A., Zuidema, D., Kulcsar, P., Varjas, L., de Kort, C., Zavodszky, P. & Vlak, J. (1999). Dissecting insect development: baculovirus-mediated gene silencing in insects. *Insect Molecular Biology* 8, 539-544.
- Hamilton, A. & Baulcombe, D. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950-952.
- Hammond, S. M., Bernstein, E., Beach, David & Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293-295.

- Handler, A. M. & O'Brochta, D. A. (1991). Prospects for gene transformation in insects. *Annual Review of Entomology* 36, 159-183.
- Henikoff, S. (1998). Conspiracy of silence among repeated transgenes. *BioEssays* 20, 532-535.
- Higgs, S. & Beaty, B. J. (1996). Rearing and containment of mosquito vectors. In *The Biology of Disease Vectors*, pp. 595-605. Edited by B. J. Beaty & W. C. Marquardt. Niwot, CO: University Press of Colorado.
- Higgs, S., Olson, K., Kamrud, K. I., Powers, A. M. & Beaty, B. J. (1997). Viral expression systems and viral infections in insects. In *Molecular Biology of Insect Disease Vectors: A Methods Manual*, pp. 459-483. Edited by J. Crampton, C. Beard & C. Louis. London: Chapman and Hall.
- Higgs, S., Olson, K. E., Klimowski, L., Powers, A., Carlson, J., Possee, R. & Beaty, B. (1995). Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Sindbis virus vector. *Insect Molecular Biology* 4, 97-103.
- Higgs, S., Oray, C. T., Myles, K., Olson, K. E. & Beaty, B. J. (1999). Infecting larval arthropods with a chimeric double subgenomic Sindbis virus vector to express genes of interest. *Biotechnics* 27, 908-911.
- 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.
- Hoffmann, J., Kafatos, F. C., Janeway, C. A. J. & Ezekowitz, R. (1999). Phylogenetic perspectives in innate immunity. *Science* 284, 1313-1318.
- Hsiung, G. (1994). Virus assay, neutralization test, and antiviral assay. In *Virus Isolation and identification*, pp. 46-49: Yale University.
- Izant, J. G. & Weintraub, H. (1985). Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. *Science* 229, 345-352.
- Jacobs-Lorena, M. & Oo, M. M. (1996). The peritrophic matrix of insects. In *The Biology of Disease Vectors*, pp. 318-332. Edited by B. J. Beaty & W. C. Marquardt. Niwot: University Press of Colorado.

- James, A. A. (1994). Molecular and biochemical analyses of the salivary glands of vector mosquitoes. *Bulletin Institut Pasteur* 92, 133-150.
- 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 Sciences, USA* 95, 3743-3747.
- Johnson, B. W., Olson, K. E., Allen-Miura, T., Rayms-Keller, A., Carlson, J. O., Coates, C. J., Jasinskiene, N., James, A. A., Beaty, B. J. & Higgs, S. (1999). Inhibition of luciferase expression in transgenic *Aedes aegypti* mosquitoes by Sindbis virus expression of antisense luciferase RNA. *Proceedings of the National Academy of Sciences* 96, 13399-13403.
- Jorgensen, R. (1995). Cosuppression, flower color patterns, and metastable gene expression studies. *Science* 268, 686-691.
- Jupp, P. & Phillips, J. (1998). An electron microscopical study of Rift Valley fever and Sindbis viral infection in mosquito salivary glands (Diptera: Culicidae). *African Entomology* 6, 75-81.
- Karber, G. (1931). Beitrag zur Kollektiven Behandlung Pharmakologischer Reihenversuche. *Archives of Experimental Pathology and Pharmacology* 162, 480-483.
- Karpf, A. R. & Brown, D. T. (1998). Comparison of Sindbis virus-induced pathology in mosquito and vertebrate cell cultures. *Virology* 240, 193-201.
- Kasschau, K. & Carrington, J. (1998). A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* 95, 461-470.
- Kennerdell, J. & Carthew, R. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 95, 1017-1026.
- Ketting, R. F. & Pasterk, R. H. (2000). A genetic link between co-suppression and RNA interference in *C. elegans*. *Nature* 404, 296-298.
- Klimstra, W. B., Ryman, K. D. & Johnston, R. E. (1998). Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *Journal of Virology* 72, 7357-7366.
- Kokoza, V., Ahmed, A., Cho, W., Jasinskiene, N., James, A. & Raikhel, A. (2000). Utilization of transgenic technology for modeling blood meal-activated systemic immunity in the mosquito vector, *Aedes aegypti*. *in press*.

- Kuhn, R., Hong, Z. & Strauss, J. (1990). Mutagenesis of the 3' nontranslated region of Sindbis virus RNA. *Journal of Virology* 64, 1465-1476.
- Kuhn, R., Niesters, H., Zhang, H. & Strauss, J. (1991). Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterization of defined chimeras with Sindbis virus. *Virology* 182, 430-441.
- Kuhn, R. J., Griffin, D. E., Zhang, H., Niesters, H. G. & Strauss, J. H. (1992). Attenuation of Sindbis virus neurovirulence by using defined mutations in nontranslated regions of the genome RNA. *Journal of Virology* 66, 7121-7127.
- Kumar, M. & Carmichael, G. G. (1998). Antisense RNA; function and fate of duplex RNA in cells of higher eukaryotes. *Microbiology and Molecular Biology Reviews* 62, 1415-1434.
- Law, J. H., Ribeiro, J. M. & Wells, M. A. (1992). Biochemical insights derived from insect diversity. *Annual Review of Biochemistry* 61, 87-111.
- Lima, H. C. (1996). Effects of sand fly vector saliva on development of cutaneous lesions and the immune response to *Leishmania braziliensis* in BALB/c mice. *Infection and Immunity* 64, 5442-5445.
- Limesand, K., Higgs, S., Pearson, L. & BJ, B. (2000). Potentiation of vesicular stomatitis New Jersey virus infection in mice by mosquito saliva. *Parasite Immunology* 22, in press.
- Litiere, K., van Eldik, G., Jacobs, J., Van Montagu, M. & Cornelissen, M. (1999). Posttranscriptional gene silencing of *gn1* in tobacco triggers accumulation of truncated *gn1*-derived RNA species. *RNA* 5, 1364-1373.
- London, S., Schmaljohn, A., Dalrymple, J. & Rice, C. (1992). Infectious enveloped RNA virus antigenic chimeras. *Proceedings of the National Academy of Sciences USA* 89, 207-211.
- Lowenberger, C., Bulet, P., Charlet, M., Hetru, C., Hodgeman, B., Christensen, B. M. & Hoffmann, J. A. (1995). Insect immunity: isolation of three novel inducible antibacterial defensins from the vector mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 25, 867-873.
- Mans, B., Gaspar, A., Louw, A. & Neitz, A. (1999). Apyrase activity and platelet aggregation inhibitors in the tick *Ornithodoros savignyi*. *Experimental and Applied Acarology* 22, 353-366.
- Marinotti, O., James, A. A. & Ribeiro, J. M. (1990). Diet and salivation in female *Aedes aegypti* mosquitoes. *Journal of Insect Physiology* 36, 545-548.

- Merzendorfer, H., Harvey, W. & Wiczorek, H. (1997). Sense and antisense RNA for the membrane associated 40 kDa subunit M40 of the insect v-ATPase. *FEBS Letters* 411, 239-244.
- Miller, B. M., Beaty, B. J., Eckels, K., Aitken, T. & Russel, P. (1982). Dengue-2 vaccine: oral infection, transmission, and reversion rates in mosquito, *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene* 31, 1232-1237.
- Mizuno, T., Chou, M.-Y. & Inouye, M. (1984). A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proceedings of the National Academy of Sciences, USA* 81, 1966-1970.
- Montgomery, M. & 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., SiQun, X. & Fire, A. (1998). RNA as a target of double-stranded RNA mediated genetic interference in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences USA* 95, 15502-15507.
- Ngo, H., Tschudi, C., Gull, D. & Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences USA* 95, 14687-14692.
- Noriega, F., Pennington, J., Barillas-Mury, C., Wang, X. & Wells, M. (1996a). *Aedes aegypti* midgut early trypsin is post-transcriptionally regulated by blood feeding. *Insect Molecular Biology* 5, 25-29.
- Noriega, F., Wang, X.-Y., Pennington, J. E., Barillas-Mury, C. V. & Wells, M. A. (1996b). Early trypsin, a female-specific midgut protease in *Aedes aegypti*: isolation, amino-terminal sequence determination, and cloning and sequencing of the gene. *Insect Biochemistry and Molecular Biology* 26, 119-126.
- Noriega, F. G., Barillas-Mury, C. & Wells, M. A. (1994). Dietary control of late trypsin gene transcription in *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 24, 627-631.
- Noriega, G., Shah, D. & Wells, M. (1997). Juvenile hormone controls early trypsin gene transcription in the midgut of *Aedes aegypti*. *Insect Molecular biology* 6, 63-66.
- Olson, K., Beaty, B. & Higgs, S. (1998). Sindbis virus expression systems for the manipulation of insect vectors. In *The Insect Viruses*, pp. 371-404. Edited by L. K. Miller & L. A. Ball. New York: Plenum Press.

- Olson, K. E. (2000). Sindbis virus expression systems in mosquitoes: background, methods, and applications. In *Insect Transgenesis: Methods and Applications*, pp. 159-188. Boca Raton, FL: CRC Press.
- 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.
- Olson, K. E., Higgs, S., Hahn, C., Rice, C. M., Carlson, J. O. & Beaty, B. J. (1994). The expression of chloramphenicol acetyltransferase in *Aedes albopictus* (C6/36) cells and *Aedes triseriatus* mosquitoes using a double subgenomic recombinant Sindbis virus. *Insect Biochemistry and Molecular Biology* 24, 39-48.
- Olson, K. E., Myles, K. M., Seabaugh, R. C., Higgs, S., Carlson, J. O. & Beaty, B. J. (2000). Development of a Sindbis virus expression system that efficiently expresses green fluorescent protein in midguts of *Aedes aegypti* following *per os* infection. *Insect Molecular Biology* 9, 57-65.
- Osorio, J. E., Godsey, M. S., Defoliart, G. R. & Yuill, T. M. (1996). La Crosse viremias in white-tailed deer and chipmunks exposed by injection or mosquito bite. *American Journal of Tropical Medicine and Hygiene* 54, 338-342.
- Paskewitz, S. M. & Christensen, B. M. (1996). Immune responses of vectors. In *The Biology of Disease Vectors*, pp. 371-392. Edited by B. Beaty & W. C. Marquardt. Niwot: University Press of Colorado.
- Patel, R., Vestal, D., Xu, Z., Bandyopadhyay, S., Guo, W., Erme, S., Williams, B. & Sen, G. (1999). DRBP76, a double-stranded RNA-binding nuclear protein, is phosphorylated by the interferon-induced protein kinase, PKR. *Journal of Biological Chemistry* 274, 20432-20437.
- Pestka, S. (1992). Antisense RNA. History and perspective. *Annals of the New York Academy of Sciences* 660, 251-62.
- 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 in Virology* 141, 2259-2276.

- Ratcliff, F. G., MacFarlane, S. A. & Baulcombe, D. C. (1999). Gene silencing without DNA: RNA-mediated cross-protein between viruses. *The Plant Cell* 11, 11207-1215.
- Rayms-Keller, A., Powers, A. M., Higgs, S., Olson, K. E., Kamrud, K. I., Carlson, J. O. & Beaty, B. J. (1995). Replication and expression of a recombinant Sindbis virus in mosquitoes. *Insect Molecular Biology* 4, 245-251.
- Ribeiro, J. M. (1987). Role of saliva in blood feeding by arthropods: diversity and redundancy. *Annual Review of Entomology* 32, 463-478.
- Ribeiro, J. M., Rossignol, P. & Spielman, A. (1985). Salivary gland apyrase determines probing time in anopheline mosquitoes. *Insect Physiology* 31, 689-692.
- Ribeiro, J. M., Sarkis, J. J., Rossignol, P. A. & Spielman, A. (1984). Salivary apyrase of *Aedes aegypti*: Characterization and secretory fate. *Comparative Biochemistry and Physiology* 79B, 81-86.
- Ribeiro, J. M., Vaughan, J. A. & Azad, A. F. (1990). Characterization of the salivary apyrase activity of three rodent flea species. *Comparative Biochemistry and Physiology* 95B, 215-218.
- Roberts, N., Brigham, J., Wu, B., JB, M., Volpin, H., Phillips, D. & Etzler, M. (1999). A Nod factor-binding lectin is a member of a distinct class of apyrases that may be unique to the legumes. *Molecular and General Genetics* 262, 261-267.
- Robertson, S. E., Hull, B. P., Tomori, O., Bele, O., LeDuc, J. W. & Esteves, K. (1996). Yellow fever: a decade of reemergence. *Journal of the American Medical Association* 276, 1157-1162.
- Roche, S. E. & Rio, D. C. (1998). *Trans*-silencing by P elements inserted in subtelomeric heterochromatin involves the *Drosophila* polycomb group gene, Enhancer of zeste. *Genetics* 149, 1839-1855.
- Rosen, L. & Gubler, D. (1974). The use of mosquitoes to detect and propagate dengue viruses. *American Journal of Tropical Medicine and Hygiene* 23, 1153-1160.
- Rossignol, P., Ribeiro, J. M., Jungery, M., Turell, M., Spielman, A. & Bailey, C. (1985). Enhanced mosquito blood-finding success on parasitemic hosts: evidence for vector-parasite mutualism. *Proceedings of the National Academy of Sciences* 82, 7725-7727.
- Rossignol, P., Ribeiro, J. M. & Spielman, A. (1984). Increased intradermal probing time in sporozoite-infected mosquitoes. *American Journal of Tropical Medicine and Hygiene* 33, 17020.

- Ruiz, F., Vayssie, L., Klotz, C., Sperling, L. & Madeddu, L. (1998a). Homology-dependent gene silencing in *Paramecium*. *Molecular Biology of the Cell* 9, 931-943.
- Ruiz, M. T., Voinnet, O. & Baulcombe, D. C. (1998b). Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* 10, 937-946.
- Sambrook, J., Fritsch, E. & Maniatis (1989). *Molecular Cloning: A Laboratory Manual*, second ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- 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.
- Seabaugh, R. C., Olson, K. E., Higgs, S., Carlson, J. O. & Beaty, B. J. (1998). Development of a chimeric Sindbis virus with enhanced *per os* infection of *Aedes aegypti*. *Virology* 243, 99-112.
- Sharp, P. (1999). RNAi and double-strand RNA. *Proceedings of the National Academy of Sciences USA* 13, 139-141.
- Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N. & Maine, E. M. (2000). EGO-1 is related to RNA-directed RNA polymerase and function in germ-line development and RNA interference in *C. elegans*. *Current Biology* 10, 169-178.
- Smartt, C. T., Kim, A. P., Grossman, G. L. & James, A. A. (1995). The *apyrase* gene of the vector mosquito, *Aedes aegypti*, is expressed specifically in the adult female salivary glands. *Experimental Parasitology* 81, 239-248.
- Spradling, A., Stern, D., Kiss, I., Roote, J., Laverly, T. & Rubin, G. (1995). Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proceedings of the National Academy of Sciences USA* 92, 10824-10830.
- Stark, K. & James, A. A. (1996). The salivary glands of disease vectors. In *The Biology of Disease Vectors*, pp. 333-348. Edited by B. J. Beaty & W. C. Marquardt. Niwot: University Press of Colorado.
- Strauss, J. & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiological Reviews* 58, 491-562.
- Tabara, H., Grishok, A. & Mello, C. C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282, 430-431.

- Thomas, C., Y, S., Naus, K., A, L. & Roux, S. (1999). Apyrase functions in plant phosphate nutrition and mobilizes phosphate from extracellular ATP. *Plant Physiology* 119, 543-552.
- Titus, R. G. & Ribeiro, J. M. (1990). The role of vector saliva in transmission of arthropod-borne disease. *Parasitology Today* 6, 157-160.
- Tucker, P. C. & Griffin, D. E. (1991). Mechanism of altered Sindbis virus neurovirulence associated with a single amino acid change in the E2 glycoprotein. *Journal of Virology* 65, 1551-1557.
- Valenzuela, J., Charlab, R., Galperin, M. & Ribeiro, J. (1998). Purification, cloning, and expression of an apyrase from the bed bug *Cimex lectularius*: a new type of nucleotide-binding enzyme. *Journal of Biological Chemistry* 273, 30583-30590.
- Warren, W. D., Atkinson, P. W. & O'Brochta, D. A. (1994). The *Hermes* transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the *hobo*, *Ac*, and *Tam3 (hAT)* element family. *Genetic Research of Camberra* 64, 87-97.
- Wassenegger, M. & Pelissier, T. (1998). A model for RNA-mediated gene silencing in higher plants. *Plant Molecular Biology* 37, 349-362.
- Waterhouse, P., Graham, M. & Wang, M. (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 USA* 95, 13595-13064.
- Wekesa, J. W., Copeland, R. S. & Mwangi, R. W. (1992). Effect of *Plasmodium falciparum* on blood feeding behaviour of naturally infected *Anopheles* mosquitoes in western Kenya. *American Journal of Tropical Medicine and Hygiene* 47, 484-488.
- Wikel, S. K., Ramachandra, R. N. & Bergman, D. K. (1996). Arthropod modulation of host immune responses. In *The Immunology of Host-Ectoparasitic Arthropod Relationships*, pp. 107-130. Edited by S. K. Wikel, et al. Wallingford: CAB International.
- Woodring, J. L., Higgs, S. & Beaty, B. J. (1996). Natural cycles of vector-borne pathogens. In *The Biology of Disease Vectors*, pp. 51-72. Edited by B. J. Beaty & W. C. Marquardt. Niwot: University Press of Colorado.
- Woodward, T. M., Miller, B. M., Beaty, B. J., Trent, D. W. & Roehrig, J. T. (1991). A single amino acid change in the E2 glycoprotein of Venezuelan equine encephalitis virus affects replication and dissemination in *Aedes aegypti* mosquitoes. *Journal of General Virology* 72, 2431-2435.

- Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M. & Huang, H. V. (1989). Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* 243, 1188-1191.
- Yao, J. S., Strauss, E. G. & Strauss, J. H. (1996). Interactions between PE2, E1, and 6K required for assembly of alphaviruses studied with chimeric viruses. *Journal of Virology* 72, 7910-7920.
- Yoksan, S., Bhamarapavati, N. & Halstead, S. (1986). Dengue virus vaccine development: study on biological markers of uncloned dengue 1-4 viruses serially passaged in primary kidney cells. In *Arbovirus research in Australia, Proceedings of the Fourth Symposium*, pp. 35-38. Edited by T. St. George, B. Kay & J. Blok. Brisbane, Australia.