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DISSERTATION

# DESIGNING RETROVIRAL VECTORS FOR MOSQUITO TRANSFORMATION

Submitted by

Michael L. Bennett

Microbiology

In partial fulfillment of the requirements

For the Degree of Doctorate of Science

Colorado State University

Fort Collins, Colorado

Spring 2000

COLORADO STATE UNIVERSITY

SIGNATURE PAGE:

We hereby recommend that the dissertation prepared under our supervision by Michael L. Bennett entitled "Designing Retroviral Vectors for Mosquito Transformation" be accepted as fulfilling in part the requirements for the degree of Doctor of Philosophy.

January 12th, 2000

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ABSTRACT OF DISSERTATION:

## Designing Retroviral Vectors for Mosquito Transformation

The retroviral life cycle involves integration of its genome into that of the host cell, a fact that has been exploited in the development of retroviral vectors and their successful use in mammalian transgenesis. The generation of retroviral vectors pseudotyped with the vesicular stomatitis virus envelope glycoprotein (VSV G) has expanded the host range of this technology to non-mammalian systems, including arthropod species. The focus of this work was to test the hypothesis that VSV G-pseudotyped retroviral vectors could be used to transduce mosquito cells with foreign DNA sequences as an approach to generating a transgenic mosquito.

VSV G-pseudotyped retroviral vectors carrying the *Photinus pyralis* (firefly) luciferase gene were constructed and tested for their ability to mediate foreign gene expression in mosquito cells *in vitro* and *in vivo*. Two *Aedes* (C6/36 and AP-61) and one *Anopheles* (MOS-55) cell lines were able to be transduced, as indicated by luciferase activities up to 58-fold above background. A total 54 out of 1213 *Aedes triseriatus* mosquito eggs injected with high-titered virus survived to adulthood. PCR identified retroviral vector integration in 3 mosquitoes, but eggs collected from these failed to hatch and transgenic lines were unable to be established. Successful transformation of mosquito cells *in vitro* demonstrates that VSV-G pseudotyped retroviral vectors can

stably introduce foreign genes, but before this technique can be used to generate a transgenic mosquito the methods to deliver and the design of the retroviral vector need to be optimized.

The ability to generate high-level gene expression is an important aspect in the optimization of a retroviral vector for use in mosquito transformation. Although a number of candidate promoters are available, the efficiencies of these transcription initiating sequences have not been rigorously compared in mosquito cell lines. To address this issue, the constitutive expression rates of a series of ten promoter/enhancer elements were tested by transiently expressing luciferase in two *Aedes* mosquito cell lines. Both cell lines produced similar results; the baculovirus IE-1 promoter plus hr5 enhancer from the *Autographa californica* nuclear polyhedrosis virus produced the highest amount of expression, followed in descending order of expression by the *Aedes* densonucleosis virus p7 and p61 promoters transactivated with the NS1 protein, the *Drosophilia melanogaster* heat shock 70 protein (hsp70) promoter, the p7 and p61 promoters without the NS1 protein, and the *D. melanogaster* metallothionein (Mtn) promoter. No expression could be detected from the cytomegalovirus (CMV) immediate early promoter or the long terminal repeat (LTR) promoters from Moloney murine sarcoma virus (MoMSV) and Moloney murine leukemia virus (MoMLV).

The ability to detect gene expression via a reporter gene is another important aspect in the optimization of a retroviral vector for use in mosquito transformation. To address this issue, several fusion-genes were constructed from the green fluorescent protein (GFP) from *Aequorea victoria*, firefly luciferase (Luc) from *Photinus pyralis*, and hygromycin B phosphotransferase ($Hyg^R$) from *Escherichia coli*to combine and take

advantage of their distinct properties. The chimeric reporters maintained the functions of the original proteins, although the activity of the Luc gene in each fusion was lowered. These reporter fusion genes will be particularly advantageous in retroviral vectors; the expression of multiple reporters from one promoter conserves the limited coding capacity within the vector and decreases the likelihood of rearrangements stemming from additional promoters in between the LTRs.

Antisense-mediated intracellular immunity as a long term control strategy in arthropod-borne disease vectors will be dependent upon the ability of cellular and viral RNA transcripts to interact, a concept which has not been established in mosquito cells. To address this, a dsSIN virus vector expressing the 5' 595 bp of the firefly luciferase gene from the second subgenomic promoter was constructed and used to infect transformed *Ae. albopictus* cell lines constitutively expressing luciferase activity. The resulting 92-97% decrease in luciferase activity compared to cells infected with dsSIN vectors without the antisense luciferase sequence clearly demonstrates that cellular and viral RNA transcripts can hybridize and result in inhibition of protein synthesis in mosquito cells.

The demonstration that VSV G-pseudotyped retroviral vectors can infect and mediate stable expression in mosquito cell lines derived from several different mosquito species suggests that this system has the potential to be successfully used in the generation of transgenic mosquitoes, while the failure to actually produce a transgenic mosquito exposes obstacles that must still be overcome. The analysis of promoter function, the construction of multi-reporter fusion proteins, and the finding that RNA transcripts expressed from a cell's nucleus and an infecting virus can associate and

produce interference in mosquito cells address some of the key points important in the design of any mosquito transformation technique.

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Spring 2000

DEDICATION:

For my wife Ann, who believed in me in times when I did not believe in myself...

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# LITERATURE REVIEW

## Introduction

In the years following World War II, it was widely believed that humans were winning the centuries-long war against mosquito-borne infectious disease. The advent of powerful pesticides such as DDT led to organized campaigns to eradicate mosquito vector species that transmit the pathogens causing these diseases from infected to susceptible humans. By the 1950s and 1960s, these programs had dramatically reduced the population of mosquito vector species and almost eliminated the incidence of many mosquito-borne diseases from Central and South America, with similar successes in Asia. It became possible to imagine a world in which infectious vector-borne pathogens would no longer prey upon humanity.

This optimism, however, was premature. The initial success of vector control programs fostered an attitude of complacency towards arthropod-borne disease threats. The vector control eradication program was officially discontinued in the United States in 1970. With limited financial and human resources available, these programs gradually eroded elsewhere and the mosquito vector species began to reinfest areas from which they had been eliminated. The geographic distribution of many mosquito vector species is larger today than before the eradication programs and the incidence of mosquito-borne disease is greater than ever (Igarashi, 1997; Gubler, 1996; Robertson *et al.*, 1996; Gubler and Trent, 1993). There are over 500 million new cases of vector-borne disease per year (Table 1-1); another 2.5 billion people live in endemic areas and are at risk for infection. Needless to say, health experts no longer believe that the threat of arthropod-borne diseases is receding in the United States or elsewhere.

**Table 1-1. Estimated number of new cases of vector-borne disease per year.**

| Vector-Borne Disease | Estimated new cases per year |
|---|---|
| Malaria[a] | 300-500 million |
| Dengue and dengue hemorrhagic fever[a] | 40-60 million |
| Filariasis (elephantiasis)[a] | 20 million |
| Japanese encephalitis[a] | 50,000 to 100,000 |
| Ross river fever[a] | 20,000 to 30,000 |
| Yellow fever[a] | 15,000 to 20,000 |
| Venezuelan equine encephalitis[a] | 10,000 to 20,000 |
| California serogroup encephalitis[b] | 1,000 to 7,500 |
| St. Louis encephalitis[b] | 500 to 7,500 |
| Western equine encephalitis[b] | 500 to 1,000 |
| Eastern equine encephalitis[b] | 25 to 100 |

[a]World Health Organization
[b]Calisher, 1994

Prospects for reversing the recent trend of expanding geographic expansion of vector species and increased incidence of arthropod-borne disease using traditional methods is not promising. Several factors have contributed to the recent ineffectiveness of these measures. First, effective mosquito control is virtually nonexistent in most endemic countries, a result of the decay in control program infrastructure and the loss of trained personnel (Gubler and Clark, 1995). Second, major global demographic changes have occurred, the most important of which have been uncontrolled urbanization and concurrent population growth (Gubler, 1997; Knudsen and Slooff, 1992). These changes have resulted in substandard housing and inadequate water, sewer, and waste management systems, all of which increase vector population densities and facilitate the transmission of arthropod-borne disease. Third, once effective chemical and biological

control agents are now essentially useless due to the emergence of resistance in the vector populations (Chevillon *et al.*, 1997; Gullemaud *et al.*, 1997; Pasteur and Raymond, 1996; Roberts and Andre, 1994; Raymond *et al.*, 1991; Goldman *et al.*, 1986). These factors have combined to create a serious international public health threat from mosquito-borne diseases.

The dim prospect of controlling vector-borne disease by conventional means has prompted research towards the development of novel control strategies. Recent advances in molecular biology may provide the means necessary to control disease transmission by invertebrate vectors.

**Intracellular interference**

Most of the novel control strategies being developed for the control of arthropod-borne viruses (arboviruses) focus on disrupting pathogen transmission within the mosquito itself by some form of intracellular immunization. Intracellular immunization is defined as the protection of cells against infection by genetically engineering resistance into these cells (Baltimore, 1988). This means altering a permissive cell to make it refractory to infection by or unable to support replication of a particular virus (Powers *et al.*, 1994). Several applications of this idea are being investigated in vector-borne disease research (reviewed in Carlson *et al.*, 1995; Carlson, 1996). Genetic manipulation of the mosquito has the potential to render it incompetent for virus transmission. Unfortunately, relatively little is known about the molecular biology of the vector species, the viruses, and the determinants of infection, maintenance, and transmission. This lack of basic knowledge has hampered the identification and manipulation of arthropod genes involved in vector competence. Another approach to establishing intracellular immunization

centers on the phenomenon of pathogen-derived resistance (PDR; Sanford and Johnston, 1985; Coleman *et al.*, 1985; Grumet *et al.*, 1987). This idea is based on observations that certain viruses can confer resistance to homologous virus infection and replication, suggesting that the initial infection has set up a refractory state within the cell. While the exact molecular mechanism remains unclear, what is certain is that expression of a pathogen's own genomic sequences can cause interference. PDR-mediated interference has been demonstrated in animal and plant systems by the expression of viral coat, nucleocapsid, envelope, regulatory, or polymerase proteins or virus-specific non-coding sense and antisense RNA molecules (Reviewed in Baulcombe, 1994; de Haan, 1998; Wilson, 1993). In these studies transgenic organisms were used to express the antiviral effector molecules. Unfortunately, the production of transgenic mosquitoes is too inefficient to apply this technique to arthropod-borne disease studies; alternative techniques have had to be used to explore the antiviral potential of effector molecules in mosquitoes. A particularly effective technique has been the use Sindbis derived viral expression vectors to express possible antiviral sequences. Recombinant Sindbis viruses were constructed to express a region of the pathogen's genome and allowed to establish a persistent infection in mosquito cells. Expression of the pathogen-derived sequences resulted in resistance to infection with the wild-type pathogen, both in cells *in vitro* and *in vivo*. PDR in mosquito cells has been demonstrated with LaCrosse (Powers *et al.*, 1996; Powers *et al.*, 1994), dengue-2 (Gaines *et al.*, 1996; Olson *et al.*, 1996), and yellow fever viruses (Higgs *et al.*, 1998).

The successful generation of mosquitoes resistant to arbovirus infection has established that PDR can prevent the transmission of arboviral pathogens. While these

virus expression systems have been invaluable in demonstrating PDR in mosquito cells, they are not viable options for long term expression of an antiviral in mosquito populations outside the laboratory setting and do not offer the possibility for vertical transfer of the resistant phenotype. Stable, heritable expression necessitates insertion and expression from the DNA of the cell.

Transformation systems for mosquitoes would allow the introduction and testing of foreign DNA sequences in vector species. The successful development of such a system has the potential to greatly impact a wide range research, from basic vector biology to disease control strategies. The development of efficient transformation systems for mosquito vector species is a very active area of research.

**Mosquito transgenesis**

The lack of an effective and reliable method for germ-line transformation has been a major barrier to genetically engineering mosquitoes. Fifteen years ago, the successful development of *P* elements as a germ-line transformation system (Spradling and Rubin, 1982; Rubin and Spradling, 1982) provided a new generation of tools for the genetic manipulation and engineering of *Drosophila*. These tools revolutionized *Drosophila* research, spawning such powerful techniques as directed gene modification, analysis of *in vitro* mutagenized genes, gene cloning by transposon tagging, and enhancer trapping (Engels, 1995; Kaiser *et al.*, 1995). These accomplishments stimulated experiments in mosquito vector species, but *P* elements were found to be unsuitable for the transformation of non-*Drosophila* species due to the narrow host range of these elements (Rio, 1990; O'Brochta *et al.*, 1991; Handler *et al.*, 1993; Roche and Rio, 1998). Although these experiments did produce rare transgenic animals (Miller *et al.*, 1987;

McGrane *et al.*, 1988; Morris *et al.*, 1989), the insertions were found to be the result of random recombinational events instead of transposase-directed transpositional events. The low rate of transformation and instability of the foreign gene precludes this technique from being useful in mosquito transgenesis.

Despite these failures, the demonstration of the unprecedented capacity to investigate the molecular biology of a species by germ-line transformation emphasized the need for developing such a tool for mosquito transgenesis. Recently, two transformation vectors based on *P* related transposable elements have been shown to produce stable integration of foreign DNA into the genome of *Aedes aegypti* mosquitoes (Coates *et al.*, 1998; Jasinskiene *et al.*, 1998). The *mariner* and *Hermes* elements utilized in these studies originated in *D. mauritiana* (Haymer and Marsh, 1986) and *Musca domestica* (Warren *et al.*, 1994), respectively, and do not show the genus-specific restrictions that plagued *P* element based transformation. Whether these prove to be as significant in mosquito transgenesis as *P* was in *Drosophila* transgenesis has yet to be determined.

**Retroviruses**

Another possible transformation strategy is based on retroviruses. Retroviruses have long played a major role in the creation of transgenic mammals (Maclean, 1994) because their natural life cycle provides a mechanism to insert DNA into the genome of target cells.

The *Retroviridae* is a large family of viruses that derives its name from the Latin *retro* meaning "backward," in reference to the fact that these viruses convert their RNA genome into DNA. These viruses primarily infect vertebrates, although they can also be

found in invertebrate species (Teysset *et al.*, 1998; Poulet, 1994). Interactions between virus and host can range from completely benign to generally fatal. Due to the association of retroviruses with cancer, encephalopathies, and AIDS, the literature on these viruses far exceeds that on any other virus family.

Despite the variety of host species and interactions with the host cell, all retroviruses are quite similar in virion structure, genome organization, and mode of replication. The virion is invariably enveloped, ~100 nm in diameter, and contains two copies of a positive sense, single-stranded RNA genome. Retroviruses are the only positive sense RNA viruses that do not use their genome as an mRNA to direct translation immediately after infection. Instead, the RNA is used as a template for the synthesis of a double-stranded DNA copy of the virus genome that is then stably integrated into the host cell DNA. No viral transcription is required to initiate this process; all the proteins necessary to achieve integration are carried within the virion (Brown, 1997).

Like all viruses, retroviral infection is initiated by attachment to a cell-surface receptor molecule. This step is mediated by the specific interaction of the cellular receptor with the viral *env* gene products that are on the surface of the viral envelope. The products of the *env* gene form a protein structure consisting of a multimer of surface (SU) and transmembrane (TM) protein subunits (Kamps *et al.*, 1991) in which the former contains the receptor-binding function (Morgan *et al.*, 1993; Ott and Rein, 1992) and the latter spans the membrane and causes fusion with the target cell membrane (Gallaher, 1987; Perez *et al.*, 1987; Freed *et al.*, 1990). After attachment and fusion of virus envelope and cellular membrane, the virion core is released into the cytoplasm and the

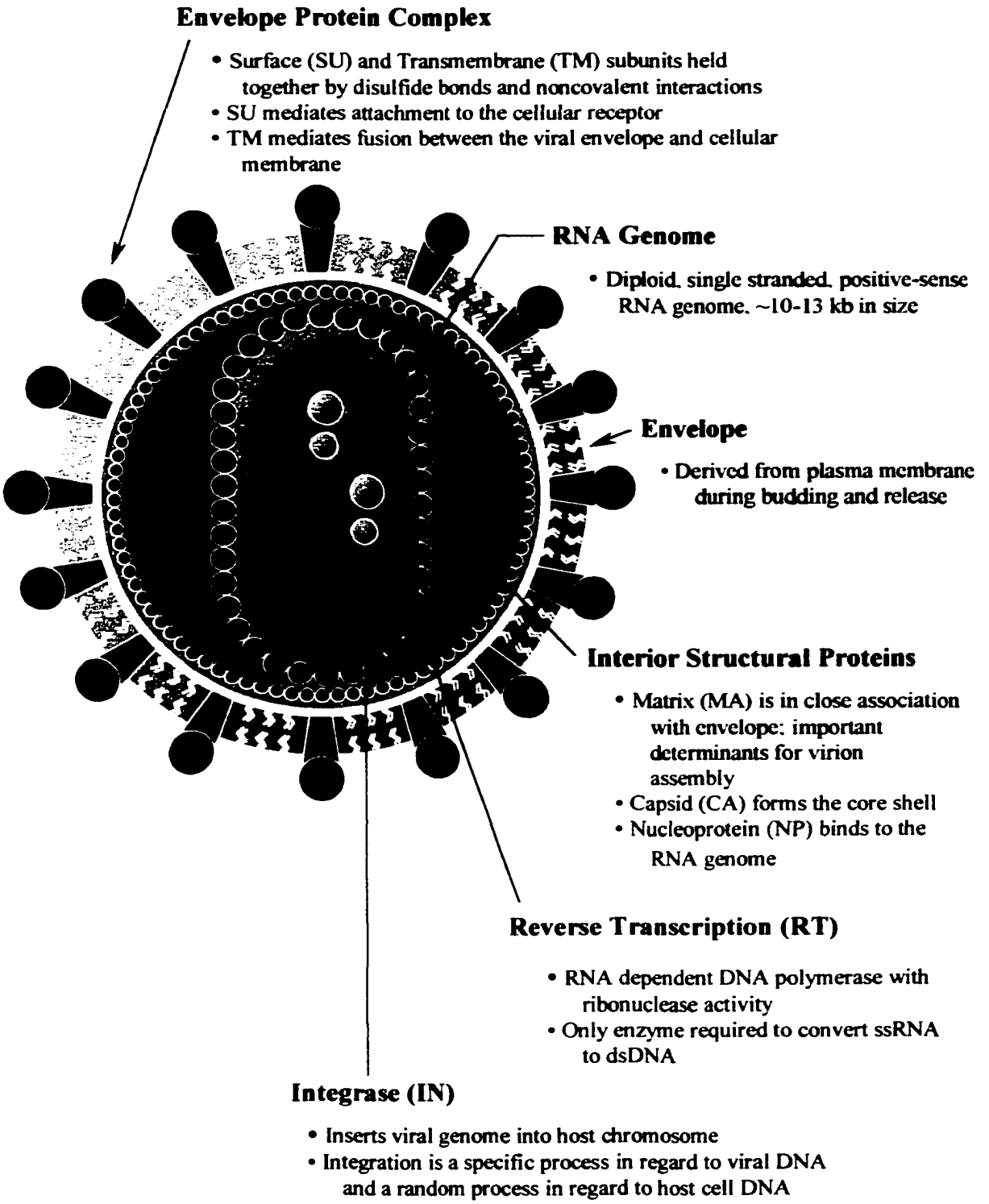



**Figure 1-1. Structure of the retroviral virion.**

RNA genome is copied into DNA. The only enzymatic protein needed to carry out this operation is reverse transcriptase. The first step is the synthesis of a minus-strand of DNA using the positive sense, single-stranded RNA as a template, which is destroyed in the process. The plus-strand of DNA is then produced using the minus-strand as template. Transport of the newly formed double-stranded DNA genome to the nucleus occurs as a complex with viral proteins from the infecting virion. Although the mechanism for entry into the nucleus varies among the different retroviruses, the majority require active mitosis and the accompanying breakdown of the nuclear membrane to achieve integration (Roe *et al.*, 1993). With access to the chromosomal DNA, the viral DNA/protein complex can proceed with integration utilizing the viral integrase enzyme. No cellular factors are required for this process (Bowerman *et al.*, 1989; Farnet and Haseltine, 1990). The viral DNA is inserted at random sites in the cellular DNA in a highly specific manner in which the organization of the viral genome is maintained. Once integration has occurred the provirus is treated as any other region of the chromosome; viral mRNA and proteins are produced using the host cell transcription and translation machinery, forming new virions that are released by budding.

Integration is the process most unique to retrovirus replication. No other animal virus has a regular mechanism for stably associating itself with the host DNA. The key part integration plays in the life cycle distinguishes retroviral integration from the sort of integration that is an occasional aberration of some DNA virus infections (Coffin, 1996). Once integrated, the provirus can be considered to be perfectly stable. There is no specific mechanism by which a provirus can be excised, moved from one location to another, or replicated independently of the chromosome in which it resides.

Researchers quickly realized they might be able to manipulate retroviruses and exploit their life cycle as a tool for inserting foreign genes into cellular DNA.

**Retroviral vectors**

Although considerable variation exists, there is a common framework of organization apparent within all retroviral genomes. The genome can be divided into coding and *cis*-acting regions. All viruses have the genes encoding virion proteins in a common order: *gag-pol-env*. This entire sequence is translated, with no internal noncoding regions present between the genes. The *cis*-acting regions are responsible for directing key steps in the life cycle of the virus, including packaging of the retroviral genome into budding virions, reverse transcription, integration, and expression of viral RNA from the integrated provirus. For the most part, the two functions are separated in the genome. The *cis*-acting regions are located in the terminal regions while the protein coding regions comprise the internal regions (Figure 1-2). This dichotomy is of central importance to the development of retroviruses as vectors.

The separation of the two functions allows for their physical separation. Retroviral genomes in which the coding sequences have been deleted are still capable of producing infectious virions when the *gag-pol-env* gene products are provided *in trans*. Cells can be engineered to constitutively express all the necessary retroviral proteins. Cell lines that synthesize the viral *gag*, *pro*, *pol*, and *env* gene products are called packaging cell lines (Mann *et al.*, 1983; Danos and Mulligan, 1988; Miller and Rosman, 1998). The introduction of a viral genome that carries all the *cis*-acting regions required to sustain the steps involved in the retrovirus life cycle will result in the production of infectious virion particles. This genome must include the Ψ signal for proper packaging,

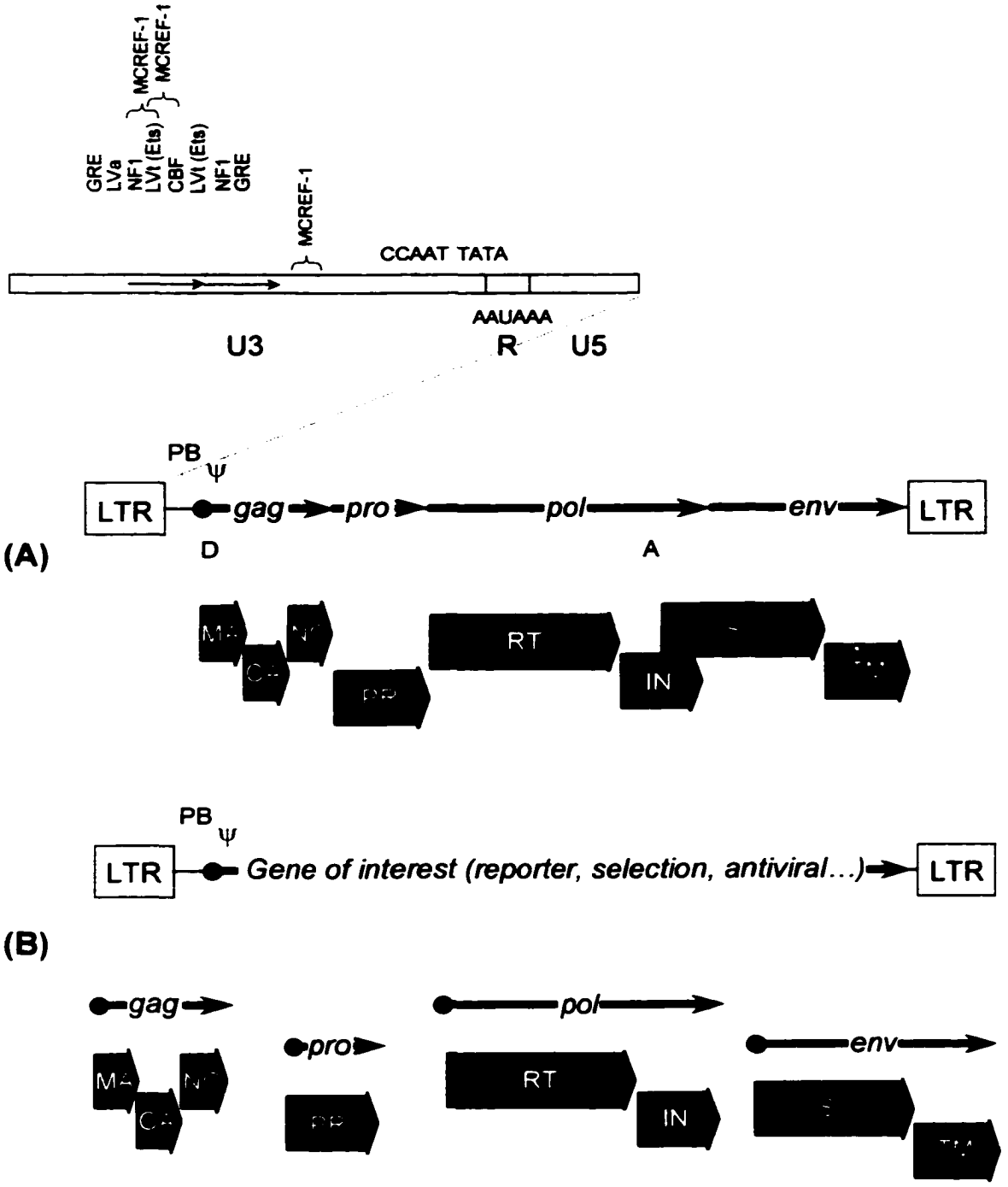



**Figure 1-2. Organization of the retroviral genome.** The Moloney murine leukemia retroviral genome as a provirus (A). The separation of coding and cis-acting regions (B) allows the formation of retroviral vectors. The LTR is divided into U3, R, and U5 regions with transcription signals and enhancers noted (Sun *et al.*, 1993; Manley *et al.*, 1993). PB=primer binding site; ψ=packaging signal; D=splice donor site; A=splice acceptor site.

the PB site for binding the tRNA primer and initiating reverse transcription, and the LTRs for completing the synthesis of the viral DNA genome and integration into the target DNA. The rest of the RNA, up to ~10,000 nt, can encode for virtually any gene of interest. The particles released from packaging cell lines are infectious but defective. Since the virus lacks the *gag*, *pro*, *pol*, and *env* genes, infected cells cannot produce progeny virus. Retroviral vector particles can undergo only the one round of infection, resulting in the integration of the gene of interest into the host cell genome.

Retroviral vectors have proven to be powerful tools because of the efficiency, tidiness, and permanence with which they insert genes into cells. Most recombinant retroviral vectors and packaging lines produced so far have been based on murine leukemia viruses (MLV; Miller, 1992). These vectors have played major roles in the creation of transgenic animals (Botteri *et al.*, 1986; Rossant, 1989; Gordon, 1993; Shuldiner, 1996). The success of this system has been noticed by researchers attempting to develop techniques for the transgenesis of mosquito vector species. Unfortunately, MLV does not infect insect cells and therefore MLV vectors are not suitable for insect transformation.

**Vesicular stomatitis virus glycoprotein-pseudotyped retroviral vectors**

Retrovirus infection is primarily determined by the interaction between the envelope protein complex on the lipid envelope of the retroviral particle and specific receptors on the host cell surface (Coffin, 1996). Recent advances in retroviral vector design provide the possibility of adapting these systems to be used as transformation tools in mosquitoes by altering viral tropisms.

It has long been known that when two viruses infect the same cell, the proteins coded by one viral genome can sometimes package the genome of the other. This process, called phenotypic mixing, is not surprising when the viruses are closely related. However, phenotypic mixing can also occur between viruses belonging to completely different taxonomic groups. In 1972, this phenomenon was demonstrated in cells infected with retrovirus and vesicular stomatitis virus (VSV; Zavada, 1972), a member of the *Rhabdoviridae* family. VSV is a lytic virus that replicates in the cytoplasm, has a single negative-stranded RNA genome of ~11,000 nt, a bullet-shaped virion surrounded by a membrane, and the ability to shut off host transcription and translation (Wagner and Rose, 1996). This is very different from retroviruses that have a diploid, positive-stranded RNA genome of 7-13,000 nt, replicate via a DNA intermediate that must integrate into the host genome to be efficiently transcribed, and in most cases do not kill the cells they infect (Weiss, 1982). About the only similarity is that both viruses release progeny virions by budding from the plasma membrane.

Unlike retroviruses that typically have a limited host range (Albritton *et al.*, 1989), VSV infects many cell types from vertebrate and invertebrate animals. This very broad host range is mediated by the viral encoded VSV G-glycoprotein which binds phosphatidylinositol, phosphatidylserine, and $G_{M3}$ ganglioside phospholipid moieties that are universal components in the cell membranes of a wide variety of species and cell types (Mastromarino *et al.*, 1987; Schlegel *et al.*, 1983; Conti *et al.*, 1991; Akkina *et al.*, 1996). When VSV and MMLV infect the same cell, the resulting progeny are a mixture of parental types and pseudotypes, the latter formed by phenotypic mixing. These pseudotypes include VSV virions with retroviral env proteins, retroviral virions with

VSV G proteins, and virions with combinations of envelope proteins derived from both viruses. The mechanism responsible for this is unknown. VSV G protein and *env* gene products have no amino acid sequence similarity (Rose and Gallione, 1981), suggesting that the incorporation of the receptor protein may not be occurring as a function of specific recruitment but rather simply because the proteins are not attached to anything else (Dong *et al.*, 1992; Perez *et al.*, 1987). VSV seems particularly prone to phenotypic mixing; this phenomenon has been demonstrated not only with numerous members of the family *Retroviridae* (Weiss *et al.*, 1977; Witte and Baltimore, 1977; Altstein *et al.*, 1976) but also with members of the *Togaviridae*, *Bunyaviridae* (Dragunova *et al.*, 1986), *Chordopoxvirinae* (Lukashevich and Zavada, 1982), *Arenaviridae* (Sengupta and Rawls, 1979; Bruns and Lehmann-Grube, 1984), *Herpesviridae* (Gonczol *et al.*, 1980), *Flaviviridae* (Dragunova and Gresikova, 1986), *Paramyxovirinae* (Metsikko and Garoff, 1989), and *Orthomyxoviridae* (Zavada and Rosenbergova, 1972). The important result pertaining to retroviral vector production is the generation of virions that have the expanded host range of VSV.

The VSV G was found to be the only VSV-coded protein required to confer the expanded host range. This information led to the construction of retroviral packaging cell lines expressing VSV G and the successful generation of VSV G-pseudotyped retroviral vectors (Emi *et al.*, 1991). Titers were comparable to those of non-pseudotyped retroviruses at $10^2$-$10^3$ infectious units/ml. The next step was to increase virus titers and this was accomplished by the transformation of a highly transfectable cell line with a *gag-pol* coding construct. Cells expressing the highest levels of the gag and pol proteins produced the highest virus titer (Burns *et al.*, 1993). This line was then transfected with a

plasmid construct carrying the gene/sequences of interest between LTRs to make the desired retroviral vector. Stably transformed cells were then selected using an antibiotic resistance marker (included in plasmid). The *vsv-g* gene was expressed from a transiently transfected plasmid. Pseudotyped retroviral vector particles were collected within 96 hours before the cells die from VSV-G glycoprotein induced membrane fusion and syncytium formation (Yee *et al.*, 1994). Titers $>10^6$ infectious units/ml can be generated in this fashion. These titers were further increased by concentrating the virus from culture fluids. Unlike the fragile retroviral envelope complex that is held together by relatively weak disulfide bonds and noncovalent interactions, the single polypeptide of VSV-G glycoprotein is extremely resistant to the shear stresses of ultracentrifugation. Titers $>10^9$ infectious units/ml were generated after two rounds of ultracentrifugation without significant loss in infectivity (Burns *et al.*, 1993). These titers are six to seven orders of magnitude higher than previous reports using MMLV derived vectors.

VSV G-pseudotyped vectors were initially used to successfully deliver genes into hamster cells that are refractory to retrovirus infection. This result was not altogether unexpected since expression of the appropriate receptor gene in these cells confers MLV-susceptibility (Hopkins, 1993). More striking was the ability of the vectors to deliver genes into non-mammalian cell lines derived from fish, frogs, and insects (Burns *et al.*, 1996; Burns *et al.*, 1993).

**Summary**

With the demonstration that the natural cycle of an arbovirus can be interfered with in its invertebrate vector, a renewed emphasis has been placed on the development

of efficient stable gene transfer techniques in arthropod vectors. The retroviral life cycle involves integration of its genome into that of the host cell, a fact that has been exploited in the development of retroviral vectors and their successful use in mammalian transgenesis. The generation of VSV G-pseudotyped retroviral vectors has expanded the host range of this technology to include non-mammalian systems. In this study, the main hypothesis to be tested was whether VSV G-pseudotyped retroviral vectors could be used in mosquito transgenesis. Additionally, aspects of promoter and reporter gene design were addressed, and the ability of cellular and viral RNA transcripts to produce interference was investigated, as these are critical components of engineering pathogen-derived resistance in mosquito vectors.

## - Chapter 2 -

# TRANSFORMATION OF MOSQUITO CELLS USING A PSEUDOTYPED RETROVIRAL VECTOR.

## INTRODUCTION

Currently available techniques to introduce and express heterologous DNA sequences in mosquito cells in culture, including transfection and infection with Sindbis virus vectors, do not permit heritable, stable gene expression in whole organisms. This lack of an efficient transformation system in arthropod-borne disease vectors is a major obstacle to the advancement of novel disease control strategies. The production of transgenic mosquitoes promises huge potential benefits in the study of vector biology and vector competence and has generated great interest in the development of efficient stable transformation techniques.

While retroviruses have long played a major role in the creation of transgenic mammals, they have not been used in insect transformation because of limitations in the range of host cells that can be infected. Recent advances in retroviral vector design have generated retroviral vectors in which the envelope protein has been replaced with that of another virus. The resulting retroviral pseudotypes adopt the host cell range of the virus donating the envelope protein. Vesicular stomatitis virus, which is transmitted by insect vectors, is able to infect a wide variety of mammalian and non-mammalian cell types. Pseudotyping retroviral vectors with the VSV envelope glycoprotein produces particles that can infect a variety of non-mammalian cells *in vitro* and *in vivo* including fish (Lin *et al.*, 1994), newt (Burns *et al.*, 1994), and frog cells (Burns *et al.*, 1996). The host range of VSV also includes mosquito cells (Schloemer and Wagner, 1975) and raises the possibility that VSV G-pseudotyped retroviral vectors could be used in the generation of transgenic mosquitoes.

This chapter details efforts to utilize VSV G-pseudotyped retroviral vectors to mediate foreign gene expression in mosquito cells *in vitro* and *in vivo* as a first step towards developing this as a transformation system in mosquitoes.

## MATERIALS & METHODS

### *Cell lines*

All mammalian cell lines were maintained in Dulbecco's modified Eagle medium (DMEM)-high glucose supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) and grown at 37°C in 90% air/10% $CO_2$. PA317 cells (*Mus musculus*; ATCC Number: CRL-9078) are derived from NIH/3T3 cells (ATCC Number: CCL-163) and stably express the Moloney murine leukemia virus *gag*, *pol*, and *env* gene products, whereas 293GP cells (*Homo sapiens*; Burns *et al.*, 1993) are derived from 293 cells (ATCC Number: CRL-1573) and stably express only the *gag* and *pol* gene products. Madin-Darby canine kidney (MDCK) cells (*Canis familiaris*; ATCC Number: CCL-34) were used to determine viral titer.

Mosquito cell lines C6/36 (*Aedes albopictus*; ATCC Number: CCL-126), AP-61 (*Ae. pseudoscutellaris*; Varma and Pudney, 1969), and MOS-55 (*Anopholes gambie*; Marhoul and Pudney, 1972) were maintained in Liebovitz (L-15) medium supplemented with either 5% (C6/36) or 10% (AP-61 and MOS-55) FBS, 2 mM L-glutamine, penicillin (100 U /ml), and streptomycin (100 μg /ml) and grown at 28°C.

***Vector construction***

The plasmid pLNhsp70lucL was constructed by digesting pLhsp70hygL (Matsubara *et al.*, 1996) with *Bgl*II, filling in the ends using the large fragment of DNA polymerase I, and then digesting with *Hin*dIII to remove the β-galactosidase cDNA (Z). The cDNA for the *Photinus pyralis* (firefly) luciferase gene (*luc*) was inserted as the *Hin*dIII-*Sac*I digestion product of pcLuc to form pLhsp70lucL. This plasmid was digested with *Hpa*I and *Xho*I and ligated to the *Eco*RI-digested, Klenow-repaired, *Xho*I-digested fragment containing the cDNA for neomycin phosphotransferase ($neo^R$) derived from pLNpolacZL (Adam *et al.*, 1991).

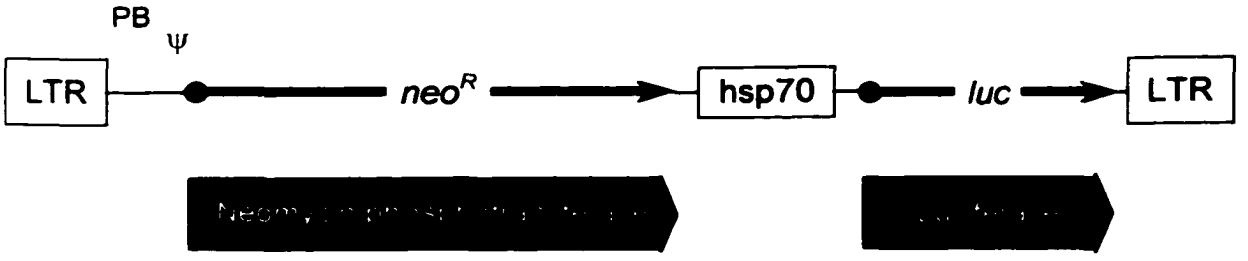



**Figure 2-1. Genetic organization of LNhsp70LucL.**

***Packaging cell lines and pseudotyped virus production***

The 293GP/LNhsp70lucL packaging cell line was constructed according to Yee *et al.* (Yee *et al.*, 1994). Briefly, PA317 cells were transfected with pLNhsp70lucL DNA. Amphotropic retroviral vector particles were collected from the culture medium and used to infect 293GP cells. G418 (400 μg/ml) was used to select for stably transformed 293GP cells; resistant colonies were isolated and subcultured. Luciferase activity for

each clone was assayed according to the instructions for the Enhanced-Luciferase Assay kit (Analytical Luminescence Laboratory, Ann Arbor, MI). Cells were seeded into 12-well tissue culture plates, allowed to reach confluence, and then harvested in 250 µl of 1×Cell Lysis buffer. The lysate was centrifuged at 13,000×g for 2 minutes to remove debris, and 10 µl was assayed for luciferase activity according to the manufacturer's instructions using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA) set for a 2 second delay and 10 second integrated measurement. The 293GP/LNhsp70lucL clone expressing the highest amount of luciferase activity was used to produce pseudotyped virus vector. These cells were transfected with the pHCMV-VSV-G plasmid, which expressed the vesicular stomatitis virus G envelope protein from the cytomegalovirus immediate early promoter (Matsubara *et al.*, 1996), by calcium phosphate co-precipitation. After 8 hours of transfection, the calcium/DNA precipitate was replaced with fresh culture medium without G418. Medium with virus was collected once a day between 24 and 96 hours post-transfection, filtered (0.45 µm pore), and stored at -70°C.

***Concentration of virus***

The frozen culture medium harvested from pHCMV-G transfected 293/LNhsp70lucL cells was thawed in a water bath at 37°C, and ultracentrifugation was done using a Beckman (Fullerton, CA) Model L3-50 centrifuge in an SW41 rotor at 50,000 × *g* at 4°C for 90 minutes. The pellet was resuspended overnight in 0.5-1% of its original volume in TNE (50 mM Tris-HCl, pH 7.8/130 mM NaCl/1 mM EDTA) or 0.1% Hank's balanced salt solution. A second cycle of ultracentrifugation was performed. Virus titer was determined by infecting MDCK cells and selecting for G418 resistant colonies as described above.

***Infection of mosquito cell lines***

$1 \times 10^5$ VSV G-pseudotyped retroviral vector colony forming units (cfu) were suspended in 1.6 ml of L-15/10% FBS plus polycation and used to infect various mosquito cell lines grown to ~70% confluence in 12-well tissue culture plates at 28°C. The retroviral vector containing medium was replaced after 4 hours with normal growth medium, and the cells were incubated for 24 hours at 28°C. Luciferase activity was determined as described above.

***Microinjection of mosquito eggs***

*Aedes triseriatus* (originally collected as larvae in LaCrosse, WI) mosquito eggs were obtained from long established, breeding colonies maintained at 25°C with 70-80% relative humidity on a 16 hour light, 8 hour dark photoperiod. Eggs were collected on oak water soaked oviposition substrates (OPs) four days after female mosquitoes were bloodfed on mice. The eggs were lightly desiccated by air drying for ~20 minutes and immediately used for microinjection experiments. Injections were performed with glass capillaries (Drummond, 50 µl) drawn to a fine tip with a Narshige PB7 needle puller and held in an Aus Jena micromanipulator with the volume controlled by an Eppendorf Microinjector 5242 using nitrogen gas. 0.5-1.0 nl of concentrated, high-titer virus (~$5 \times 10^8$ cfu/ml) with 4 µg/ml of polybrene (Sigma) was injected into the posterior of the egg (Figure 2-2). Eggs were allowed to embryonate for 14 days at 25°C with 70-80% relative humidity on a 16L:8D photoperiod. Hatching was induced by submerging eggs in hatching solution (1:100 dilution of Difco (Detroit, MI) brain heart infusion in

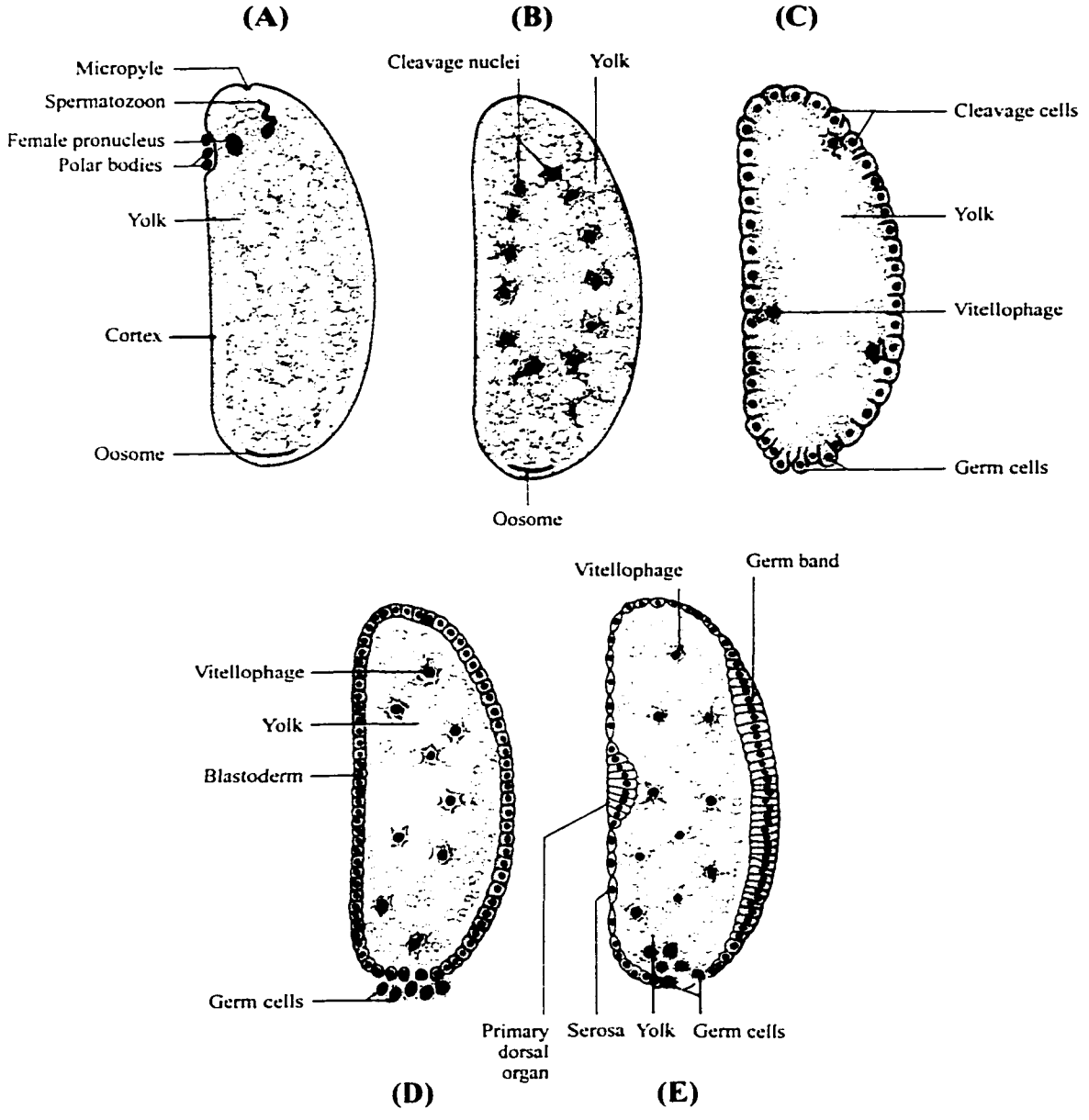



**Figure 2-2. Development of the mosquito embryo.** The spermatozoon and female pronucleus (A) fuse to form a fusion nucleus that functions as an individual cell and proliferates mitotically to form cleavage nuclei (B). These migrate to the periphery of the egg, develop cell membranes, and form the blastoderm (C and D). Some of the cleavage cells pass through a specialized region of the egg called the oosome and are signaled to differentiate into germ cells (C, D, and E). These cells eventually give rise to the gametes or reproductive cells in the late larval, pupal, and adult stages. Microinjection was targeted to the oosome to preferentially infect cells giving rise to the reproductive tissues. Modified from Johannsen and Butt, 1941.

deoxygenated, double-distilled water) for ~45 minutes. Larvae were collected and reared to adulthood.

***Analysis of mosquitoes microinjected as eggs***

Adult mosquitoes microinjected as eggs were mated and females bloodfed on mice. Eggs were collected on OPs and stored in plastic bags at 25°C and a photoperiod of 16L:8D. The parental mosquitoes were ground on dry ice with a disposable pestle (Kontes, Vineland, NJ), suspended in 150 µl of luciferase lysis buffer containing 1% Triton-X100 (Analytical Luminescence Laboratories, Ann Arbor, MI), and incubated at 4°C for 15 minutes. Nuclei and cell debris were pelleted by centrifugation at 13,000×g for 2 minutes at room temperature. 96 µl of supernatant was transferred to a clean tube, combined with 4 µl of 25× protease inhibitor cocktail (50 µg/ml aprotinin, 250 µM leupeptin, 25 µM pepstatin, 25 mM benzamidine, in 25 mM EDTA), and assayed for luciferase activity according to the manufacturer's instructions (Analytical Luminescence Laboratories, Sparks, MD). The pellet was resuspended in 500 µl of a digestion buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, 10 mM Tris pH 7.4) containing 10 µl proteinase K (10 mg/ml) and incubated overnight at 37°C. The aqueous phase was extracted with phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol. The DNA was precipitated with isopropanol and resuspended in 100 µl sterile water. 1 µl of extracted DNA was used for PCR analysis in a reaction mixture containing 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 200 µM each dNTP, and 1 U AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA). PCR was performed using primers specific to the muscle actin gene (upstream primer: 5'-CCCAGAGCAAGAGAGGTA-3', downstream primer: 5'-GCGTATCCTTCGTAGATGGG-3') at a 55°C annealing

temperature were used to confirm the recovery of amplifiable DNA. A nested set PCR was used to detect integrated retrovirus sequences (first-round LTR PCR: upstream primer: 5'- AGGACCTGAAATGACCCTGT -3', downstream primer: 5'- ACGGGTAGTCAATCACTCAG-3'; nested LTR PCR: upstream primer: 5'- ACCAATCAGTTCGCTTCTCG –3', downstream primer: 5'- AAGGAACAGCGAGACCACAA -3') with an annealing temperature of 60°C. 1μl of the first-round PCR product was used for the nested PCR.

## RESULTS

### *Infection of mosquito cell lines*

Initially experiments focused on determining whether mosquito cells could be infected with VSV G-pseudotyped retroviral vectors. Two *Aedes* (C6/36 and AP-61) and one *Anopheles* (MOS-55) mosquito cell lines were exposed to VSV G-pseudotyped LNhsp70lucL vector particles in the presence of 4 μg/ml of the polycation polybrene. All three mosquito cell lines were able to be infected, as demonstrated by an increase in luciferase activity above background (Figure 2-3). MOS-55 and AP-61 cells produced increases of 55- and 58-fold, respectively, while C6/36 cells produced only a 4-fold increase above background. While the increase in C6/36 cells was small in comparison to the other mosquito cell lines examined, it was nonetheless statistically significant ($P < 0.05$).

To optimize infection in mosquito cells, the effect of different concentrations of three polycations were examined for their ability to increase infection. Polybrene

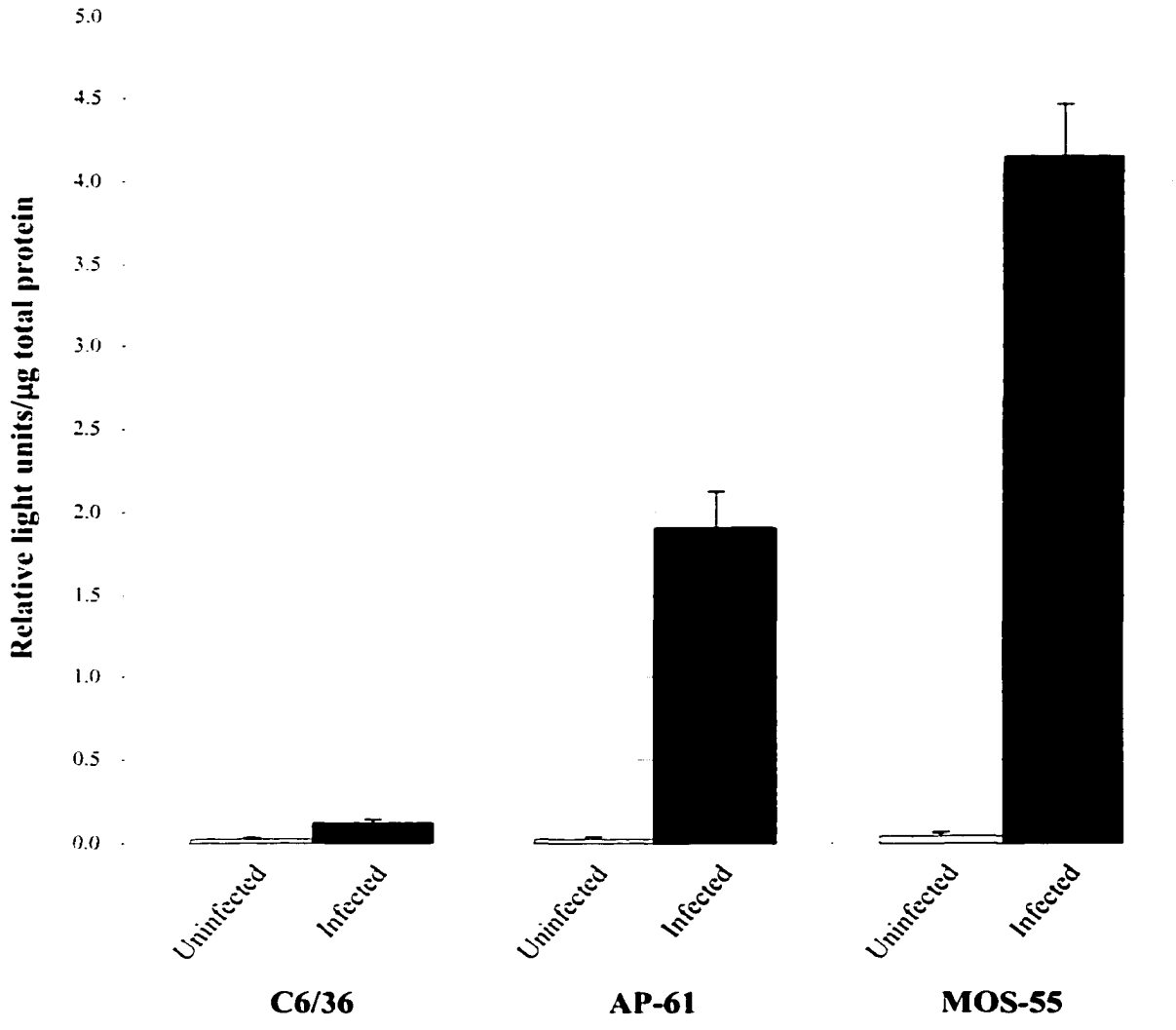



**Figure 2-3. Infection of mosquito cell lines with VSV G-pseudotyped LNhsp70Luc retroviral vector.** Cells were infected in duplicate and the bars represent the standard error.

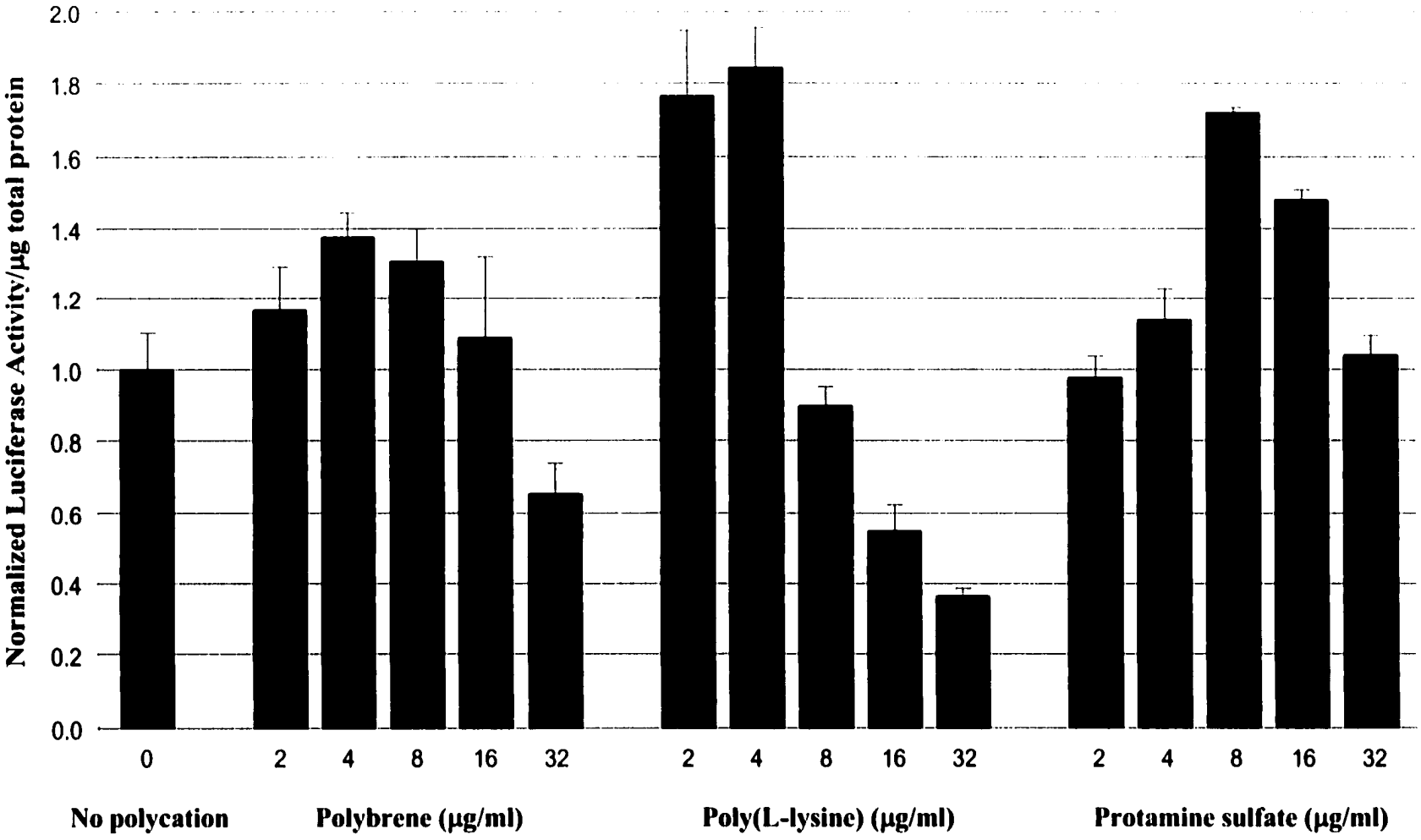



**Figure 2-4. Effect of varying concentrations of polycations polybrene, poly(L-lysine), and protamine sulfate on VSV G-pseudotyped retroviral vector infection in AP-61 cells.** Luciferase measurements were normalized to the activity from infection in the absence of polycation. Each column represents cells infected in triplicate and the bars represent percent standard error.

(Sigma,), poly(L-lysine)(Sigma), and protamine sulfate (Sigma) were tested at concentrations ranging from 0 to 32 μg/ml. The greatest luminescence was observed from AP-61 cells infected with pseudotyped LNhsp70LucL in the presence of 4 μg/ml of poly(L-lysine) (Figure 2-4).

***Microinjection of mosquito eggs***

A total of 1213 mosquito eggs were microinjected with high titered, pseudotyped LNhsp70LucL virus (Table 2-1), of which 54 survived to emerge as adults (4.4%). After mating, bloodfeeding, and collecting eggs from individual females, the mosquitoes were analyzed for infection by the retroviral vector. Nested set PCR identified 3 mosquitoes (5.4% of the survivors, 0.25% of the microinjected eggs) positive for integration of LNhsp70LucL; no mosquitoes were found to produce luciferase activity above background. Of the 3 nested set PCR positive mosquitoes, 1 was male and 2 were female. The females laid a total of 20 eggs. Unfortunately, multiple attempts to hatch these eggs did not result in the emergence of any larvae.

## DISCUSSION

VSV G-pseudotyped retroviral vectors were shown to be capable of infecting mosquito cells and expressing a foreign gene product. Infection *in vitro* showed that *Aedes* and *Anopheles* mosquito cell lines produced luciferase activity after incubation with VSV G-pseudotyped LNhsp70LucL virus. In mammalian transduction, retroviral infection efficiencies can be increased by the addition of polycations to the cell culture medium that act by overcoming the repulsive charges between viral envelope and cell membrane. The effect of three polycations (polybrene, poly(L-lysine), and protamine

**Table 2-1. Microinjection of *Aedes triseriatus* eggs with high-titered (~5 × $10^8$ cfu/ml) VSV G-pseudotyped LNhsp70LucL retroviral vector.**

| Date | Eggs | Hatching | | PCR analysis | | Luciferase assay | |
|---|---|---|---|---|---|---|---|
| | microinjected | # hatched | % survival | # positive | % positive | # positive | % positive |
| 09/06/95 | 52 | 2 | 3.8% | 0 | 0.0% | 0 | 0.0% |
| 09/08/95 | 37 | 1 | 2.7% | 0 | 0.0% | 0 | 0.0% |
| 09/14/95 | 57 | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |
| 09/29/95 | 110 | 2 | 1.8% | 0 | 0.0% | 0 | 0.0% |
| 10/03/95 | 13 | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |
| 10/26/95 | 115 | 10 | 8.7% | 0 | 0.0% | 0 | 0.0% |
| 11/01/95 | 123 | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |
| 11/02/95 | 155 | 5 | 3.2% | 0 | 0.0% | 0 | 0.0% |
| 11/07/95 | 109 | 7 | 6.4% | 0 | 0.0% | 0 | 0.0% |
| 11/21/95 | 46 | 2 | 4.3% | 0 | 0.0% | 0 | 0.0% |
| 11/24/95 | 88 | 9 | 10.2% | 0 | 0.0% | 0 | 0.0% |
| 12/04/95 | 54 | 4 | 7.4% | 3 | 75% | 0 | 0.0% |
| 12/05/95 | 120 | 4 | 3.3% | 0 | 0.0% | 0 | 0.0% |
| 01/16/96 | 70 | 3 | 4.3% | 0 | 0.0% | 0 | 0.0% |
| 01/23/96 | 64 | 7 | 10.9% | 0 | 0.0% | 0 | 0.0% |
| **Totals** | **1213** | **56** | **4.4%** | **3** | **5.4%** | **0** | **0.0%** |

sulfate) on infection efficiency were examined in the *Ae. pseudoscutellaris* AP-61 mosquito cell line. The presence of polycation in the culture medium did improve infection efficiency, but no treatment resulted in a >1.85-fold increase over infection in the absence of polycation (Figure 2-4). A similarly small increase of 2.2-fold was observed in *An. gambiae* MOS-55 cells (J.C. Burns, personal communication). This small benefit brings into question whether polycations should be added to virus when injected into mosquito eggs. The higher concentrations of polycation were seen to be toxic to mosquito cells *in vitro*. VSV G-pseudotyped retroviral vectors have been used in mammalian *in vivo* applications in the absence of polycation without loss of infection efficiency (Douar *et al.*, 1996). Evidence for VSV G-pseudotyped retroviral vector infection *in vivo* in this study is less persuasive. The survival rate for microinjected eggs was 4.4%. Three of the 56 survivors were identified by nested set PCR to possess retroviral DNA. While this is a respectable 5.4% of the survivors, no expression of the transgene was detected in any of these animals. The three $PCR^-$ mosquitoes came from eggs injected on the same day and assayed by nested set PCR in the same group. Without the confirmation of protein expression, it is possible that the PCR reaction became contaminated and produced a false positive signal. Microinjection of LNhsp70LucL into *Drosophila melanogaster* eggs resulted in 36% of the animals positive for virus infection and expression (Jordan *et al.*, 1998). Because of reservations about the accuracy of the nested PCR and the absence of transgene expression, it is believed that VSV G-pseudotyped retroviral vector transduction *in vivo* did not occur.

The infection of mosquito cells *in vitro* by VSV G-pseudotyped retroviral vectors has been further examined in the *Anopheles gambiae* MOS-55 cell line (Matsubara *et al.*,

1996). The strong increase of reporter gene activity above background, similar to the results seen in this study, confirmed the ability of these vectors to infect mosquito cells. A hemi-nested, "one-sided" PCR reaction (Sarkar *et al.*, 1993) was used to demonstrate integration of the retroviral provirus. Sequencing of the insertion junction between retroviral DNA and the host cell chromosome revealed a 2-bp deletion from the 3'-end of the LTR, a feature of retroviral integration in mammalian genomes. Integration was also found to occur at variable sites in the mosquito genome. These findings demonstrate that retroviral infection, reverse transcription, and integration can occur in mosquito cells and share many characteristics with mammalian host cell infection. Recently, the *Drosophila melanogaster* gypsy retrotransposon has been found to be an infectious insect retrovirus (Kim *et al.*, 1994) and has homology to mammalian retroviruses (Alberola *et al.*, 1997). These studies strengthen the idea that retroviruses can be utilized to stably transform mosquitoes.

VSV G-pseudotyped retroviral vectors have recently been shown to infect mosquito cells *in vivo* when injected into late-instar larvae (Jordan *et al.*, 1998). PCR analysis and luciferase assays were used to demonstrate the presence of retroviral DNA and foreign gene expression (see Appendix, page 126). This successful transformation of mosquito cells *in vivo* is in contrast to results with microinjection of *Ae. triseriatus* embryos. These differences may be due to the delivery of the retroviral vector. The size of the mosquito larvae allowed a volume of ~1 µl of high-titer VSV G-pseudotyped viral vector (corresponding to ~1 × $10^6$ cfu) to be microinjected into an environment surrounded by dividing cells of the growing organism. The size of the *Aedes triseriatus* egg allowed a volume of only ~1 nl to be microinjected (corresponding to ~1 × $10^3$ cfu).

In addition, the early environment of the embryo may not be ideal for retroviral infection. The initial hours of egg development are characterized by the division of cleavage nuclei, which are basically cells without cell membranes (Romoser and Stoffolano, 1994). Because infection is initiated by binding of the envelope protein (in this case VSV G) to cell surface receptors (in this case phospholipid moieties), facilitating penetration and uncoating of the virion, the retroviral vector may not be able to efficiently begin the infection process until formation of the cell membranes (~8 hours post-oviposition in *Aedes aegypti*; Raminani and Cupp, 1978). However, Jordan *et al.* were able to transform *D. melanogaster* by microinjecting eggs in a procedure similar to that used in this work. Embryogenesis in these insects is also characterized by cleavage nuclei division, but the time until cell membrane formation is much less (~2 hour post-oviposition; Okada, 1998). This difference may be significant as retroviruses lose infectivity rapidly over time (Miller *et al.*, 1993). Additionally, the procedure for microinjection of *Drosophila* eggs has been optimized from years of working with *P* elements transformation systems.

The successful transformation of mosquito cells *in vitro* and *in vivo* (larvae) and *D. melanogaster in vivo* (eggs) demonstrate that VSV G-pseudotyped retroviral vectors can stably introduce foreign genes in insect cells. These are encouraging results that support the possibility that VSV G-pseudotyped retroviral vectors can be developed as an efficient technique for the generation of transgenic mosquitoes. Optimization of methods to deliver vector stocks to the gametes or early embryos should enable germline transformation of mosquitoes.

## - Chapter 3 -

# COMPARISON OF RELATIVE PROMOTER EFFICIENCIES IN TRANSIENTLY TRANSFECTED *AEDES* MOSQUITO CELL LINES.

## INTRODUCTION

Applying the principle of intracellular immunization to disrupt pathogen transmission at the molecular level within the mosquito is the goal of the many of the novel disease control strategies being developed. Many years of research in plant-pathogen systems has provided extensive background information on PDR with which to design antiviral strategies in mosquito cells. Effective strategies have included both protein- and RNA-based resistance (Kavanagh and Spillane, 1995; Lomonossoff, 1995; Truve *et al.*, 1994; Gadani *et al.*, 1990). Initially, no correlation could be made between expression levels of the transgene and observed resistance levels. But follow-up experiments found that resistant plants have high nuclear expression levels, a lower steady state level of transgene RNA in the cytoplasm, and more methylation at the transgene DNA sequence (Prins and Goldbach, 1996). A proposed mechanism to explain these relationships involves a post-transcriptional RNA degradation pathway that is induced by the detection of aberrantly high levels of an RNA transcript. The lack of an efficient transformation system has precluded the production of transgenic mosquitoes and therefore it is unknown whether such a system exists in mosquito cells. It seems clear that the efficacy of controlling vector-borne diseases using intracellular immunization strategies to disrupt viral replication and transmission in mosquito cells is predicated on the high level expression of antipathogen effector molecules. This may have been a key feature in the effective interference seen against LaCrosse (Powers *et al.*, 1996; Powers *et al.*, 1994), dengue (Gaines *et al.*, 1996; Olson *et al.*, 1996), and yellow fever (Higgs *et al.*, 1998) viruses using the Sindbis virus expression system. Obtaining similar results from a DNA-based expression system will require the identification of

effective promoters that can transcribe and express high levels of the transgene in the vector (Bennett *et al.*, 2000).

Considerable efforts have been made to discover and analyze endogenous and heterologous promoters that might successfully be used to initiate high levels of transcription in mosquito cells (Benedict *et al.*, 1993; Salazar *et al.*, 1994; Ibrahim *et al.*, 1996; Smartt *et al.*, 1995; Pfeifer *et al.*, 1997; Kovach *et al.*, 1992; Shotkoski *et al.*, 1996; Zhao and Eggleston, 1999). A number of candidate promoters are now available, but the efficiencies of these transcription initiating sequences have not been rigorously compared in mosquito cell lines. To address this issue a series of plasmids were constructed containing candidate promoters/enhancers regulating the expression of the *Photinus pyralis* (firefly) luciferase gene and their constitutive levels of gene expression compared. Constitutive promoter expression was examined because the techniques to induce promoters are often difficult to maintain and the mosquitoes don't survive long under such harsh conditions. The promoter/enhancer elements examined in this study were the *Drosophila melanogaster* heat shock 70 (hsp70) promoter (Ingolia *et al.*, 1980), the cytomegalovirus enhancer (CMV) promoter (Foecking and Hofstetter, 1986), the *D. melanogaster* metallothionein (Mtn) promoter (Otto *et al.*, 1987), the baculovirus immediate early promoter with the hr5 enhancer element ($^{hr5}$IE1) from *Autographa californa* nuclear polyhedrosis virus (Nissen and Friesen, 1989), the *Aedes* densonucleosis virus p7 and p61 promoters with and without the NS1 protein (Afanasiev *et al.*, 1994; Boublik *et al.*, 1994), and the Moloney murine sarcoma virus (MMSV) LTR (Reddy *et al.*, 1980) and Moloney murine leukemia virus (MMLV) LTR promoters (Van Beveren *et al.*, 1980).

Firefly luciferase was employed as the reporter for these promoter efficiency studies because of its sensitivity, quantitativity, and linearity over a wide range of enzyme concentrations (Wood, 1995). The 61 kDa monomeric protein achieves photon emission through the enzymatic oxidation of luciferin in a reaction that requires ATP, $Mg^{2+}$ and $O_2$ (Figure 3-1). Firefly luciferase does not require post-translational modification for enzymatic activity (Wood *et al.*, 1984; de Wet *et al.*, 1985) and therefore functions as a genetic reporter immediately upon translation. Firefly luciferase is recognized as one of the most sensitive, rapid, safe, and easy reporter gene assay systems available (Sherf and Wood, 1995).

Luciferase assays, as well as all reporter gene assays, are subject to large errors due to differences in transfection efficiency, cell density, cell health, and lysis efficiency. These errors can undermine accuracy and compromise the significance of findings (Hollon and Yoshimura, 1989). Experimental variability can be accounted for by measuring two individual reporter enzymes in a single sample. Cells are co-transfected with an experimental construct and a control construct that encode for distinct reporter genes. In measurements of gene expression, relative changes in the expression of the experimental reporter gene correlate to changes in the activity of the promoter while the internal control produces background expression (transcribed from a constitutive promoter) based on the overall transfection efficiency. Normalizing expression from the experimental reporter to expression from the internal control has been shown to reduce standard errors and minimize the influence of experimental variability (Behre *et al.*, 1999; Mitsuda *et al.*, 1997; Swoap, 1998). The *Renilla reniformis* (sea pansy) luciferase gene was used as the internal control reporter in these experiments. *Renilla* luciferase is a

36 kDa monomeric protein that is composed of 3% carbohydrate when purified from its natural source (Matthews *et al.*, 1977); it does not require post-translational modification for activity. The reaction catalyzed by *Renilla* luciferase utilizes coelenterazine and $O_2$ (Figure 3-1). Firefly and *Renilla* luciferases share the property of light emission but are of distinct evolutionary origins. The differences in enzyme structure and substrate requirements make it possible to selectively discriminate between their respective bioluminescent reactions.

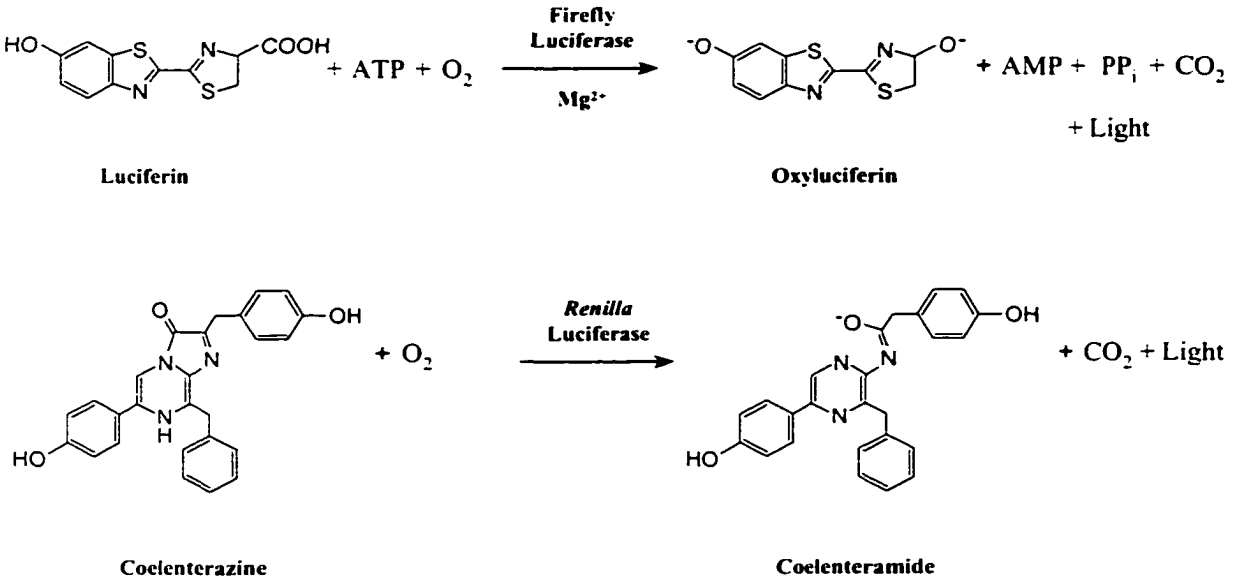



**Figure 3-1. The different bioluminescent reactions catalyzed by firefly and *Renilla* luciferases.**

The dual-luciferase assay system was utilized to compare promoter efficiency in mosquito cells. The activity of the promoters was tested by transiently transfecting the recombinant plasmids into two mosquito cell lines, C6/36 (*Aedes albopictus*) and AP-61 (*Ae. pseudoscutellaris*). Changing only the promoter eliminated any effect that differences in the surrounding DNA sequence might have on reporter gene expression.

Any observed differences in luciferase activity could therefore be attributed to the efficiency of the promoter.

In this chapter, promoter/enhancer elements were compared for their ability to constitutively express luciferase with the aim that the most efficient promoter/enhancer would be a good candidate for transcribing antipathogen effector molecules in an integrated, DNA-expression systems.

## MATERIALS & METHODS

### *Cell Lines*

*Aedes albopictus* C6/36 cells (ATCC Number: CCL-126) and *Ae. pseudoscutellaris* AP-61 cells (Varma and Pudney, 1969) were maintained at 28°C in L-15 medium supplemented with 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, and 5% or 10% heat-inactivated fetal bovine serum (FBS), respectively.

### *Plasmid Construction*

*Experimental Reporter Constructs*

pBS[*Null-Luc*]: The *Ph. pyralis* (firefly) *luc+NF* luciferase gene was used in these studies because of its ability to form N-terminal luciferase fusion proteins. The pSP-*luc+NF* plasmid (Promega, Madison, WI) was digested with *Kpn*I and *Xba*I to release the *luc+NF* gene. This fragment was ligated into pGL3-Basic (Promega), which had previously been digested with the same two enzymes. The *luc+NF*-poly(A) sequence from this plasmid was removed using *KpnI* and *Bam*HI and ligated into pBluescriptII (SK+) (Stratagene, La Jolla, CA) to create the vector pBS[*Null-Luc*].

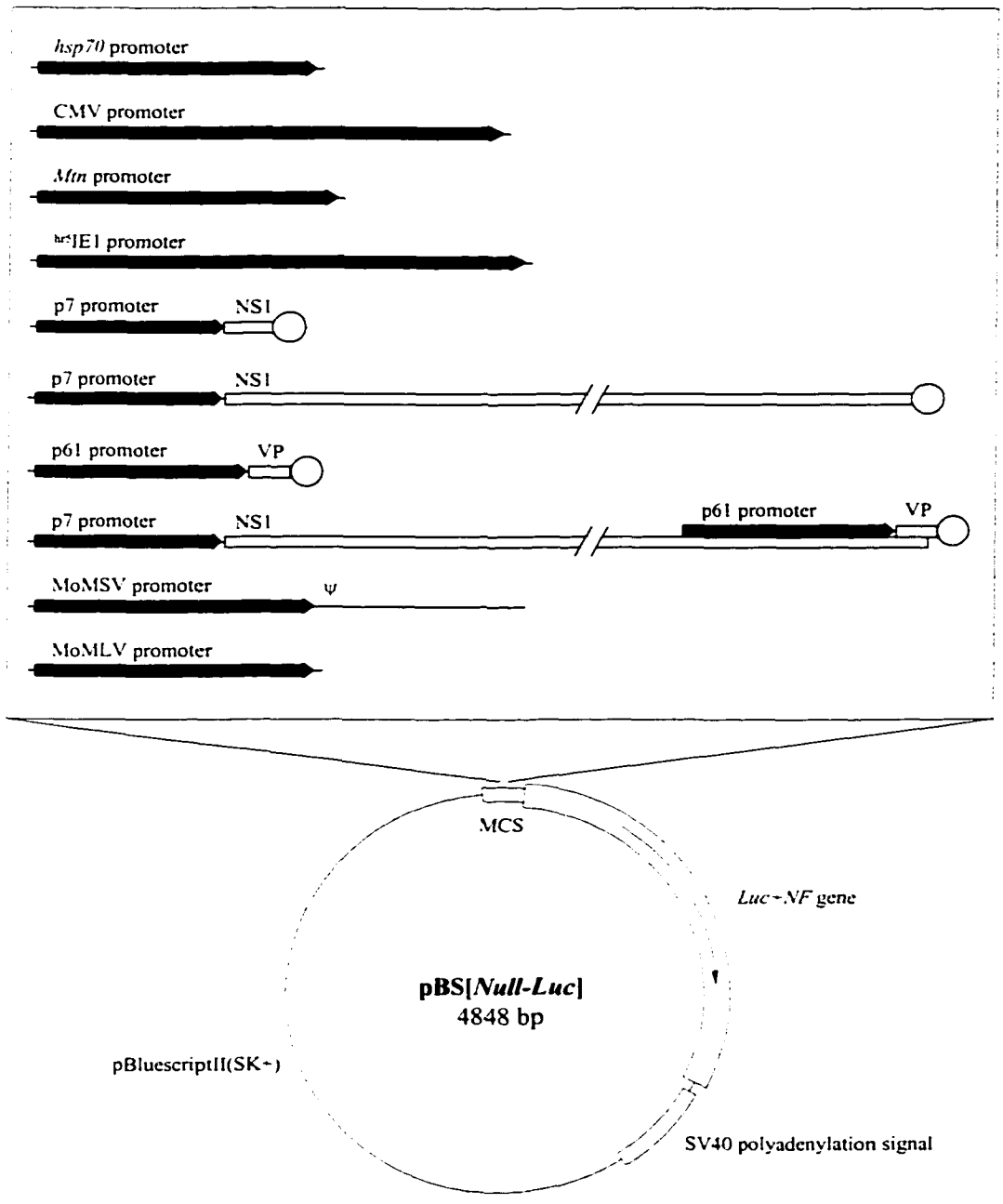



**Figure 3-2. Construction of experimental promoter/enhancer plasmids.** The promoter elements were inserted into the multiple cloning site of pBS[*Null-Luc*] upstream of the *luc+NF* gene. VP = *Aedes* densonucleosis virus virion protein; NS1 = *Aedes* densonucleosis virus nonstructural-1 protein; ψ = retroviral packaging signal; circle indicates fusion site with the *luc+NF* gene.

pBS[*hsp70-Luc*]: The *luc+NF*-poly(A) sequence from the pBS[*Null-Luc*] plasmid was removed using *Kpn*I and *Bam*HI, and inserted into the pUChshyg plasmid (Monroe *et al.*, 1992) downstream of the *D. melanogaster* hsp70 promoter. The hsp70-*luc+NF*-poly(A) sequence was removed using the restriction enzyme *Bsp*106I and ligated into the *Bsp*106I site in pBluescriptII (SK+).

pBS[*CMV-Luc*]: The cytomegalovirus enhancer promoter (CMV) was removed from the pcDNA3 plasmid (Invitrogen, Carlsbad, CA) vector using *Bgl*II and *Hin*dIII and inserted upstream of the luciferase gene in pBS[*Null-Luc*] using the same two enzyme sites.

pBS[*Mtn-Luc*]: The *D. melanogaster* metallothionein promoter (Mtn) was removed from the pMt-2 plasmid (Kovach *et al.*, 1992) using *Pvu*II and *Eco*RV and inserted into the *Eco*RV site in pBluescriptII (SK+). The *Mtn* promoter was excised from this intermediate construct using *Bam*HI and *Hin*dIII and ligated into same sites in pBS[*Null-Luc*] upstream of the luciferase gene.

pBS[$^{hr5}$*IE1-Luc*]: The AcMNPV immediate early promoter with the hr5 enhancer element ($^{hr5}$IE1) was removed from the pIE1-3 plasmid (Novagen, Madison, WI) using *Sma*I and inserted into the *Sma*I site in pBluescriptII (SK+) to form the intermediate plasmid pBS2(SK+)[$^{hr5}$IE1]. The $^{hr5}$IE1 region was removed from this construct using *Xba*I and inserted into the *Nhe*I site upstream of the luciferase gene in pBS[*Null-Luc*].

pBS[*p7-Luc*]: The *luc+NF* gene was removed from the pSP-*luc+NF* plasmid using *Bst*EII and *Xho*I and ligated downstream of the *Aedes* densonucleosis virus p7 promoter in the pUC-A plasmid (Afanasiev *et al.*, 1994). This inserted the luciferase gene in frame with the NS1 gene 100 bp downstream of the start ATG. The *p7-luc+NF* sequence was then removed using *Kpn*I and *Xba*I and ligated upstream of the luciferase gene into the

same enzyme sites in pBluescriptII (SK+). The SV40 poly(A) signal was added downstream of the luciferase gene by digesting pGL3-Basic with *Xba*I and *Bam*HI and ligating the 313 bp fragment into the same sites present in the intermediate pBluescriptII (SK+)-*p7-luc+NF* plasmid.

pBS[*p7-Luc+NS1*]: A 55 bp region of pBS[*p61-Luc+NS1*] was removed by digestion with *Sna*BI and *Avr*II. The 5' *Avr*II overhang was removed by Mung Bean nuclease treatment and the resulting blunt-ended plasmid was ligated to itself to form an NS1-luciferase fusion in which the entire NS1 gene except the stop codon was intact.

pBS[*p61-Luc*]: The *Aedes* densonucleosis virus p61 promoter was removed from pBS [*p61-Luc+NS1*] using *Nco*I and ligated into the *Nco*I site in pBS [*Null-Luc*]. This created a VP-luciferase fusion protein with the first 100 bp of the VP gene.

pBS[*p61-Luc+NS1*]: Plasmid pUC-A was digested with *Kpn*I to release a 2.7 kb fragment carrying the *Aedes* densonucleosis virus NS1 gene transcribed from the p7 promoter and the first 100 bp of the VP gene transcribed from the p61 promoter. This fragment was ligated into the *Kpn*I site in pBS[*Null-Luc*].

pBS[*LTR(MoMSV)-Luc*]: The Moloney murine sarcoma virus (MoMSV) LTR was amplified from the 5' LTR of the pLNCX plasmid (Clontech, Palo Alto, CA) using *TaqPlus Precision* polymerase (Stratagene) and primers engineered with *Xba*I sites. The resulting 1517 bp product included the LTR and the ψ retroviral packaging signal and was ligated into the pCR2.1-TOPO vector using the TOPO TA-cloning kit (Invitrogen). The LTR(MoMSV) + ψ sequence was removed from this intermediate plasmid by *Xba*I digestion and ligated into the *Nhe*I in pBS[*Null-Luc*].

pBS[*LTR(MoMLV)-Luc*]: The Moloney murine leukemia virus (MoMLV) LTR was amplified from the 3' LTR of the pLNCX plasmid using *TaqPlus Precision* polymerase and primers engineered with *Avr*II sites. The resulting 602 bp product was ligated into the pCR2.1-TOPO vector using the TOPO TA-cloning. The LTR(MoMLV) sequence was removed by *Avr*II digestion and ligated into the *Avr*II in pBS[*Null-Luc*].

*Internal Control Construct*

pRL-hsp70: The hsp70 promoter was removed from the pUChshyg plasmid using the enzymes *Xmn*I and *Hin*dIII. The resulting fragment was then inserted upstream of the *R. reniformis* (sea pansy) luciferase gene in pRL-Null (Promega) which had been previously digested with *Xho*I and *Hin*dIII. An initial ligation allowed the *Hin*dIII overhangs to come together and was followed by a fill-in reaction using the large fragment of DNA polymerase I to generate blunt ends that were joined by a second ligation reaction.

Unless otherwise stated, all reagents were purchased from Life Technologies (Gaithersburg, PA). All primers used in cloning and screening methods were purchased from Genosys, Woodlands, TX.

In preparation for transfection experiments, each completed plasmid was transformed into SURE cells (Life Technologies) and plasmid DNA was isolated on a Maxiprep column (Qiagen, Hilden, Germany). DNA was ethanol precipitated and resuspended in 10 mM Tris (pH 8.5). DNA concentration was determined on a DU-640 Spectrophotometer (Beckman). All DNAs were then diluted to 250 μg/ml in 10 mM Tris (pH 8.5) prior to being used for transfection except the pRL-hsp70 internal control plasmid, which was diluted to 100 μg/ml.

### *Determination of Firefly and Renilla Luciferase Expression*

C6/36 or AP-61 cells were grown to confluence in a T-75 tissue culture flask. The cells were suspended in phosphate-buffered saline (PBS) and cell density determined using a hemocytometer. C6/36 cells ($8.0 \times 10^4$) or AP-61 cells ($4.0 \times 10^4$) were seeded into each well of a 12-well plate with L-15/5% or 10% FBS medium, respectively, and allowed to adhere for 12 hours. For transfection, the growth medium was replaced with 400 µl L-15 medium without antibiotics or serum. A total of 4.08 µg of DNA was transfected into each well (Table 3-1) using Lipofectin reagent (Life Technologies). Because these experiments were investigating relative promoter strengths, the transient transfection was designed to deliver the same molar concentration of experimental promoter construct DNA molecules; ~$3.83 \times 10^{11}$ molecules based on the number of DNA plasmid molecules in 2.0 µg of the negative control plasmid pBS[*Null-Luc*], the smallest sized plasmid used in this work. pBluescriptII (SK+) was used to increase the amount of DNA to 4.0 µg per well and 0.08 µg of pRL-hsp70 was added as the internal

**Table 3-1. DNA amounts transiently transfected into C6/36 and AP-61 cells.**

| Experimental Promoter Plasmid | Plasmid Size (bp) | Exp. plasmid (µg/well) | Mock plasmid (µg/well) | pRL-hsp70 (µg/well) |
|---|---|---|---|---|
| pBS[*Null-Luc*] | 4848 | 2.00 | 2.00 | 0.08 |
| pBS[*hsp70-Luc*] | 5337 | 2.20 | 1.80 | 0.08 |
| pBS[*CMV-Luc*] | 5745 | 2.37 | 1.63 | 0.08 |
| pBS[*Mtn-Luc*] | 5446 | 2.25 | 1.75 | 0.08 |
| pBS[$^{hr5}$*IE1-Luc*] | 5958 | 2.45 | 1.55 | 0.08 |
| pBS[*p7-Luc*] | 5427 | 2.24 | 1.76 | 0.08 |
| pBS[*p7-Luc+NS1*] | 7480 | 3.09 | 0.91 | 0.08 |
| pBS[*p61-Luc*] | 5489 | 2.27 | 1.73 | 0.08 |
| pBS[*p61-Luc+NS1*] | 7539 | 3.12 | 0.88 | 0.08 |
| pBS[*LTR(MoMSV)-Luc*] | 6365 | 2.46 | 1.54 | 0.08 |
| pBS[*LTR(MoMLV)-Luc*] | 5450 | 2.25 | 1.75 | 0.08 |

control. After 8.0 hours, the DNA/Lipofectin/medium complex was replaced with 2.0 ml growth medium. After ~28 hours, the cells were assayed according to the instructions for the Dual-Luciferase Assay kit (Promega). This assay measures and distinguishes firefly and *Renilla* luciferase activity. Cells were harvested in 250 µl of 1× Passive Cell Lysis buffer, centrifuged to remove debris, and 10 µl of each lysate assayed using a TD 20/20 Luminometer (Turner Designs) set for a 2 second delay and 10 second integrated measurement.

***Normalization and Comparison of Luciferase Expression***

Each firefly luciferase measurement ($LA_F$) was normalized to the internal control *Renilla* luciferase measurement ($LA_R$) from the same sample to generate normalized luciferase activity ($NLA_F$).

Normalized Luciferase Activity,

$$NLA_F = (LA_F/LA_R)$$

To account for variations between experiments, these values were then normalized to the luciferase activity produced by the pBS[*hsp70-Luc*] plasmid in the same experiment. Direct comparison of these normalized data was found to yield inaccurate results because of the differences in expression from the internal control when co-transfected with different experimental constructs (see results, Figure 3-3). To correct for this, the normalized data were multiplied by the average of the *Renilla* luciferase activities from cells transfected with the same experimental promoter construct in the same experiment.

This eliminated the sample-to-sample variation yet maintained the magnitude of the original firefly luciferase values.

Comparable Luciferase Data,

$$CLA_F = [(LA_F/LA_F) \times \text{Ave } LA_{R\ (\text{same promoter, same experiment})}]$$

Again, variations between experiments were taken into account by normalizing to the luciferase activity produced by the pBS[*hsp70-Luc*] plasmid in the same experiment. This data transformation allowed for the accurate comparison of firefly luciferase expression ($CLA_F$) from cells transfected with different experimental promoter constructs in three separate experiments despite the differences in *Renilla* luciferase activities.

## RESULTS

### *Confirming the need to normalize to a co-transfected internal control*

Initial experiments to compare promoter efficiency resulted in large standard errors. This difficulty led to an assessment of the effectiveness of co-transfecting an internal control to increase accuracy. pBS[*hsp70-Luc*] and pRL-hsp70 were transiently transfected at various ratios and firefly and *Renilla* luciferase activities assayed after 28

**Table 3-2. Comparison of methods to reduce standard error**

| Ratio of pBS[*hsp70-Luc*] to pRL-hsp70 | $LA_F$ | Percent standard error from: $LA_F$ normalized to total protein | $LA_F$ normalized to *Renilla* luciferase |
|---|---|---|---|
| 100:1 | 10.15 | 8.12 | 3.03 |
| 50:1 | 19.47 | 20.67 | 9.74 |
| 25:1 | 9.72 | 7.94 | 2.14 |
| 10:1 | 16.96 | 15.13 | 4.98 |

hrs. Standard errors were determined for $LA_F$ when it was not normalized, normalized to total protein concentration, or normalized to $LA_R$ (Table 3-2). Normalizing the data to expression from the co-transfected internal control reduced standard errors between 2.0- and 8.4-fold, with an average reduction of 4.3-fold, whereas normalizing to total protein resulted in only a minimal reduction. This finding definitively shows that using a co-transfected internal control is the most effective method for reducing the standard error in transient transfections.

***Effects of the two promoters on firefly luciferase expression***

To determine whether the presence of two promoters would influence expression of the firefly luciferase gene (Farr and Roman, 1992), C6/36 and AP-61 cells were transiently transfected with each of the cloned promoter/luciferase plasmids with and without the internal control. A heteroscedastic two-tailed T-test (unequal variance) was used to determine that transient transfection with and without the internal control revealed no statistically significant difference ($P < 0.05$) in firefly luciferase activity (Table 3-3). P values ranged from 0.321 to 0.993 with a mean of 0.721, well above $\alpha$ of 0.05 and indicating no interference when the various experimental promoter constructs were co-transfected with pRL-hsp70. Thus, using the hsp70 promoter to express the *Renilla* luciferase gene in a DNA ratio of 1:25 with the experimental plasmid constructs did not alter the observed activity from any of the promoters examined in this study.

***Effects of the two promoters on* Renilla *luciferase expression***

Cells transfected with experimental and internal control plasmids were also assayed for expression of *Renilla* luciferase activity. Using the criteria described above, the mean *Renilla* luciferase activities were determined not to be the same and differed

**Table 3-3. The presence of the internal control does not influence firefly luciferase activity.** Neither C6/36 or AP-61 cells show significant differences in $LA_F$ measurements when transfected with (w/ IC) or without (w/out IC) the internal control plasmid.

C6/36 Cells

| Promoter Construct | pBS[*Null-Luc*] | | pBS[*hsp70-Luc*] | | pBS[*CMV-Luc*] | | pBS[*Mtn-Luc*] | | pBS[*IE1-Luc*] | | pBS[*p7-Luc*] | | pBS[*p7+NS1-Luc*] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC |
| Average $LA_F$ (n=6) | 0.230 | 0.251 | 975.8 | 847.5 | 0.115 | 0.104 | 10.01 | 9.47 | 3091.5 | 2860.8 | 916.4 | 773.3 | 1994.5 | 1806.2 |
| Standard Error (%) | 30.74 | 15.40 | 15.55 | 18.97 | 15.70 | 19.08 | 22.15 | 18.07 | 8.63 | 15.42 | 14.58 | 21.02 | 7.05 | 18.68 |
| T-Test P value | 0.796 | | 0.574 | | 0.673 | | 0.851 | | 0.666 | | 0.513 | | 0.623 | |

| Promoter Construct | pBS[*p61-Luc*] | | pBS[*p61+NS1-Luc*] | | pBS[*LTR(MoMSV)-Luc*] | | pBS[*LTR(MoMLV)-Luc*] | |
|---|---|---|---|---|---|---|---|---|
| | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC |
| Average $LA_F$ (n=6) | 336.4 | 335.9 | 1134.2 | 1253.5 | 0.168 | 0.157 | 0.450 | 0.410 |
| Standard Error (%) | 10.47 | 13.83 | 6.60 | 6.89 | 12.24 | 20.06 | 18.84 | 9.54 |
| T-Test P value | 0.993 | | 0.321 | | 0.767 | | 0.677 | |

AP-61 Cells

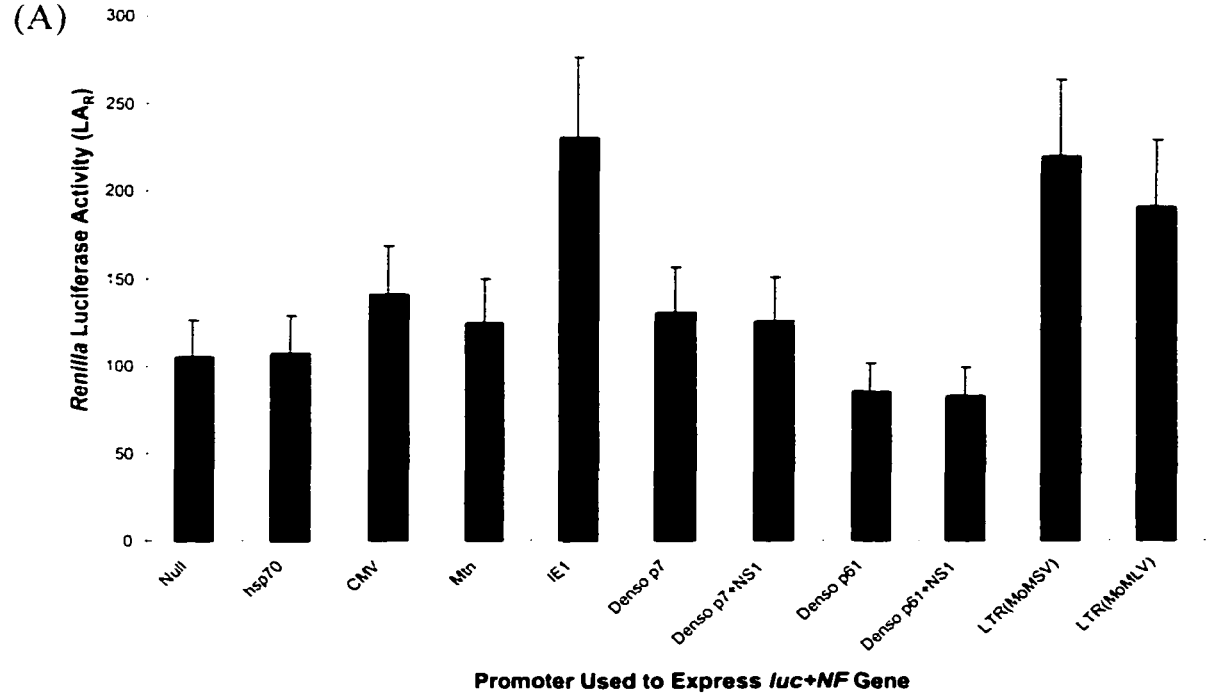
| Promoter Construct | pBS[*Null-Luc*] | | pBS[*hsp70-Luc*] | | pBS[*CMV-Luc*] | | pBS[*Mtn-Luc*] | | pBS[*IE1-Luc*] | | pBS[*p7-Luc*] | | pBS[*p7+NS1-Luc*] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC |
| Average $LA_F$ (n=6) | 0.821 | 0.588 | 2960.0 | 2742.7 | 0.435 | 0.411 | 133.39 | 145.04 | 25296 | 26337 | 1997.7 | 1928.7 | 4270.2 | 4321.8 |
| Standard Error (%) | 32.87 | 26.17 | 6.92 | 11.20 | 8.34 | 12.70 | 30.53 | 14.87 | 7.83 | 9.91 | 6.47 | 16.46 | 19.81 | 17.22 |
| T-Test P value | 0.475 | | 0.571 | | 0.717 | | 0.807 | | 0.758 | | 0.846 | | 0.964 | |

| Promoter Construct | pBS[*p61-Luc*] | | pBS[*p61+NS1-Luc*] | | pBS[*LTR(MoMSV)-Luc*] | | pBS[*LTR(MoMLV)-Luc*] | |
|---|---|---|---|---|---|---|---|---|
| | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC | w/out IC | w/ IC |
| Average $LA_F$ (n=6) | 1538.2 | 1412.0 | 2809.5 | 2709.5 | 0.597 | 0.623 | 0.649 | 0.829 |
| Standard Error (%) | 12.55 | 19.70 | 12.49 | 29.00 | 13.50 | 27.88 | 18.55 | 28.41 |
| T-Test P value | 0.718 | | 0.911 | | 0.895 | | 0.516 | |

depending on which experimental promoter construct was co-transfected with the pRL-hsp70 plasmid (Figure 3-3). The magnitude of this effect was dependent on the promoter driving the expression of the *luc+NF* gene and both C6/36 and AP-61 cells showed these differences despite each sample being transfected with the same amount of internal control. *Renilla* luciferase activity showed standard errors ranging from 2-35% with an average of 15.7% in cells co-transfected with the same experimental promoter plasmid, compared to cells transfected with different experimental promoter plasmids that could show >7-fold variation. *Renilla* luciferase activity could not be correlated with high firefly luciferase activity. C6/36 and AP-61 cells generated similar but not identical patterns of *Renilla* luciferase expression. The most noticeable differences were increased *Renilla* luciferase activity in cells co-transfected with pBS[$^{hr5}$*IE1-Luc*] (both lines), the mammalian LTR constructs (C6/36), pBS[*CMV-Luc*] (AP-61), and pBS[*Mtn-Luc*] (AP-61) and lowered activity in cells co-transfected with pBS[*p61-Luc*] and pBS[*p61-Luc+NS1*] (both lines). The precise reasons for these differences is not known but may be related to the availability of transcription factors or other perturbations of the cells. The finding that *Renilla* expression but not *luc+NF* expression was altered by co-transfection of the internal control is probably a result of the ratios of DNA transfected. Firefly luciferase expression is likely being affected to a degree by the presence of the second promoter, but the variations seen in the unnormalized transient transfections would conceal any small difference.

***Determination of promoter efficiencies in C6/36 and AP-61 cells.***

Firefly luciferase activity from the experimental promoter construct was initially .



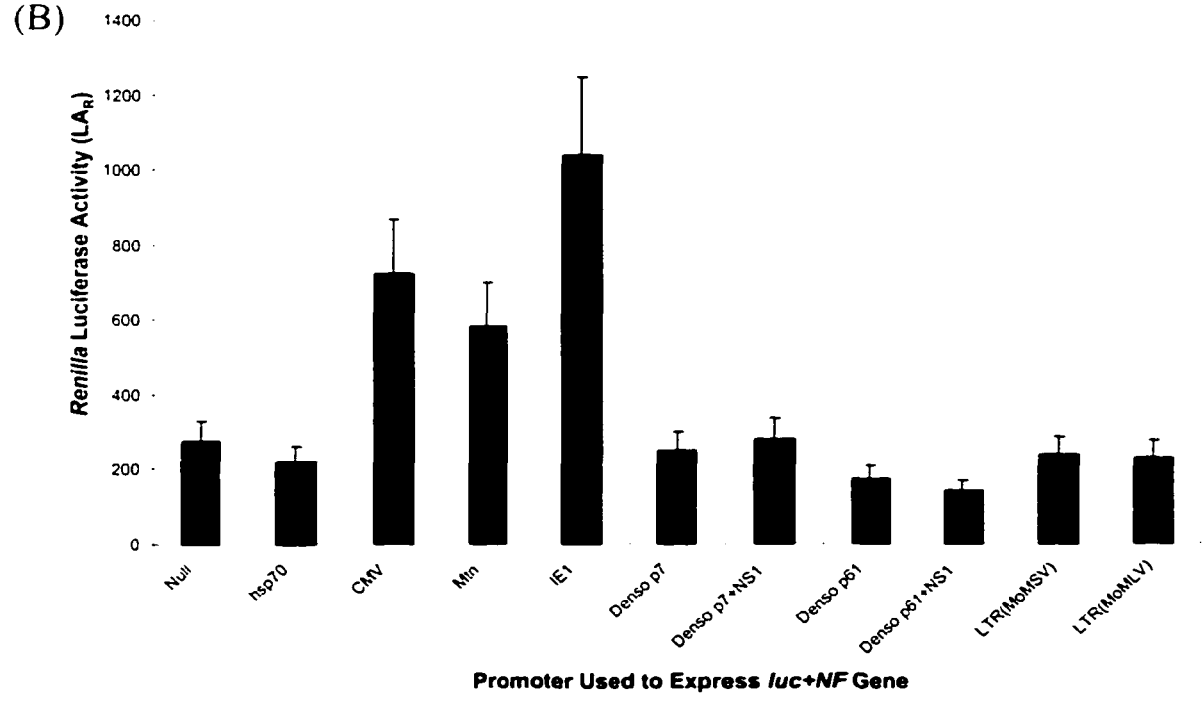




**Figure 3-3. *Renilla* luciferase expression in C6/36 (A) and AP-61 (B) cells co-transfected with the experimental reporter constructs and the pRL-hsp70 internal control plasmid.** Each column represents the average of six transfected cell samples and error bars equal percent standard error.

normalized to the *Renilla* luciferase activity from the pRL-hsp70 plasmid (Figure 3-4). Direct comparison of these normalized data was determined to be inaccurate because of the large observed differences in *Renilla* luciferase activity observed when co-transfected with the various experimental promoter constructs. The rationale behind this conclusion is that an internal control should yield values dependent upon a single variable, in this case the level of transfection. The consistent finding that the *Renilla* luciferase measurement varied corresponding to the co-transfected plasmid indicated the internal control was flawed and necessitated an alternate approach to analyze the data.

The technique used to eliminate sample-to-sample variation despite the observed variations in *Renilla* luciferase activity from the internal control is described in the materials and methods (page 45). Standard errors before normalizing to *Renilla* luciferase activity ranged from 2.8-37.7% with an average of 16.9%. *Renilla* normalized expression data from each experimental promoter construct yielded much more consistent results within the same experiment, with standard errors ranging from 0.7-12.1% with an average of 3.9%. This increased accuracy (measured as a decrease in the standard error) ranged from a low of 1.2-fold to a high of 24.8-fold with an average of 6.0-fold. In no case did standard error increase as a result of normalization. This increased accuracy produced consistent results when each of the 10 different promoter-luciferase experimental promoter plasmids were transiently transfected in separate experiments (Figure 3-5). The data from the three separate experiments generated standard errors ranging from 1.09-14.78% with an average of 7.97% from all the constructs that produced firefly luciferase activity well above background. The Mtn promoter produced a

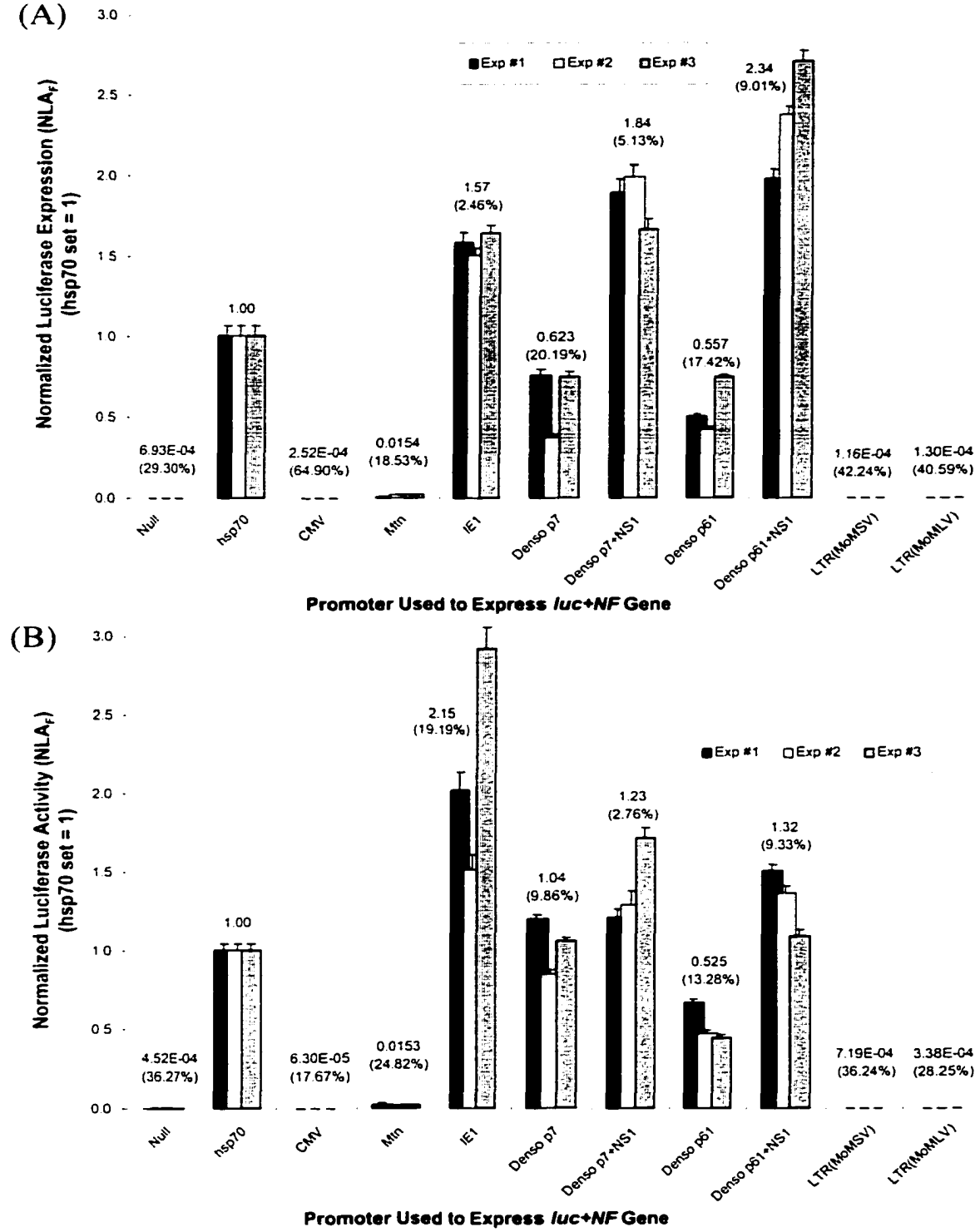



**Figure 3-4. Normalized firefly luciferase activity ($NLA_F$) in C6/36 (A) and AP-61 (B) cells co-transfected with experimental reporter constructs and the internal control pRL-hsp70 plasmid.** Each bar represents the average $NLA_F$ in transfection experiments with n=6 for experiment #1 and n=3 for experiments #2 and #3; error bars indicate standard error for each set. The numbers above the columns indicate the average from the three independent experiments with the standard error expressed as a percentage of the average (indicated in brackets).

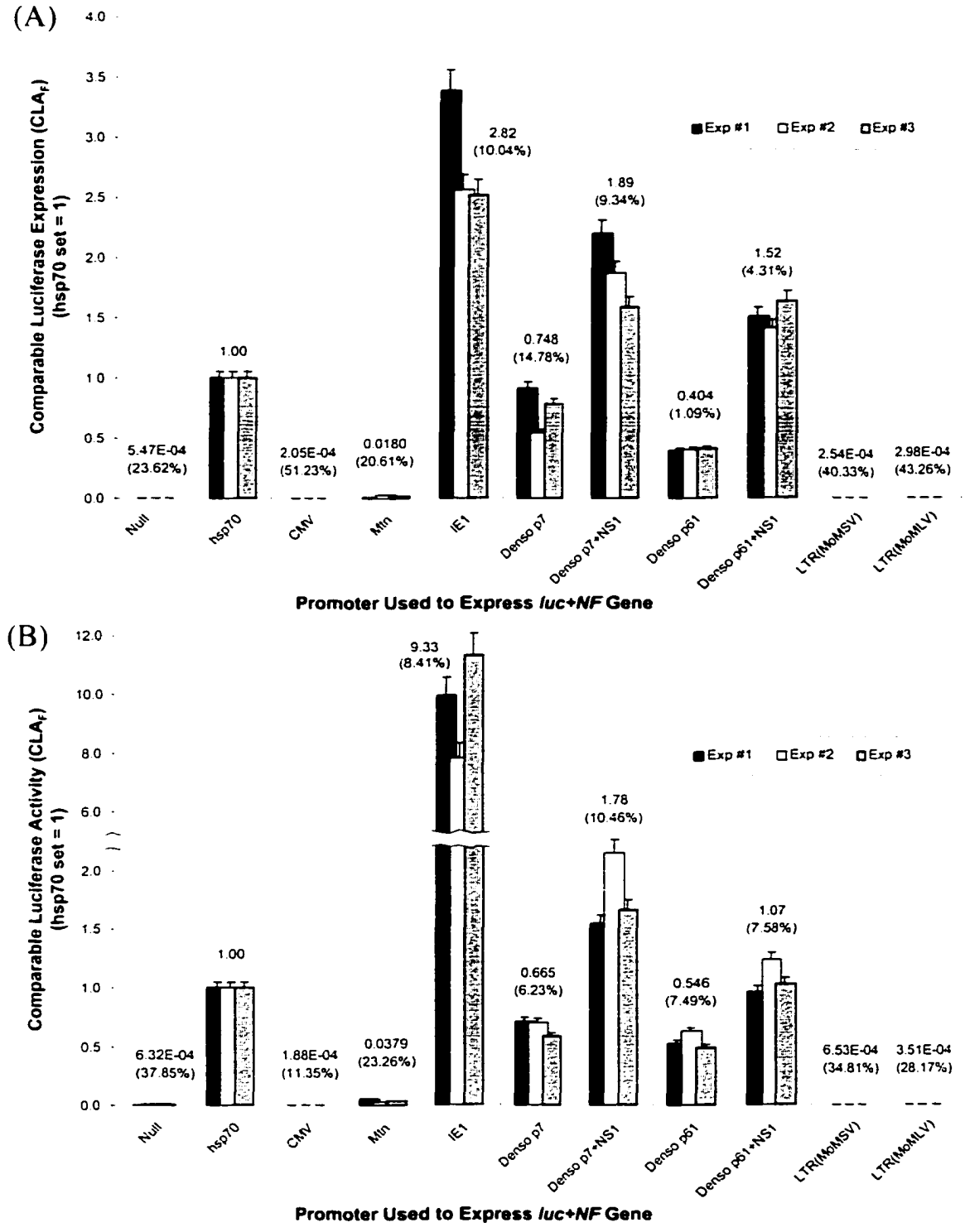



**Figure 3-5. Comparable, normalized firefly luciferase activity ($CLA_F$) in C6/36 (A) and AP-61 (B) cells co-transfected with experimental reporter constructs and the internal control pRL-hsp70 plasmid.** Each bar represents the average $CLA_F$ in transfection experiments with n=6 for experiment #1 and n=3 for experiments #2 and #3; error bars indicate standard error for each set. The numbers above the columns indicate the average from the three independent experiments with the standard error expressed as a percentage of the average (indicated in brackets).

relatively low level of firefly luciferase and had a high standard error of ~22%. Those promoters that did not show expression above background produced much higher standard errors, ranging from 11.4-51.2% with an average of 33.83%. The baculovirus $^{hr5}$IE1 promoter/enhancer produced the highest amount of firefly luciferase activity, followed in descending order by p7-Luc+NS1, p61-Luc+NS1, hsp70, p7, p61, and Mtn promoters. CMV, MMSV LTR, and MMLV LTR promoters showed no activity above pBS[*Null-Luc*] background in either C6/36 or AP-61 cell lines, despite their ability to strongly drive expression in the mammalian BHK-21 cell line (data not shown). The same general pattern of expression was observed in both *Aedes* cell lines tested (Figure 3-6).

## DISCUSSION

The relative constitutive expression rates of a series of promoter elements were tested by transiently expressing luciferase in two *Aedes* mosquito cell lines. Promoter efficiency was assessed using a plasmid encoding the *Ph. pyralis* (firefly) *luc+NF* luciferase gene downstream of a multiple cloning site into which promoter/enhancer regions could be inserted to drive expression of the reporter gene. By changing only the promoter, any effect that differences in the surrounding DNA sequence might have on reporter gene expression was eliminated. Observed differences in luciferase activity could therefore be attributed solely to the efficiency of the promoter.

C6/36 *Ae. albopictus* and AP-61 *Ae. pseudoscutellaris* cells had similar promoter expression profiles in these experiments. The *D. melanogaster* hsp70 promoter was used to compare relative promoter efficiency because it has been highly characterized (Amin

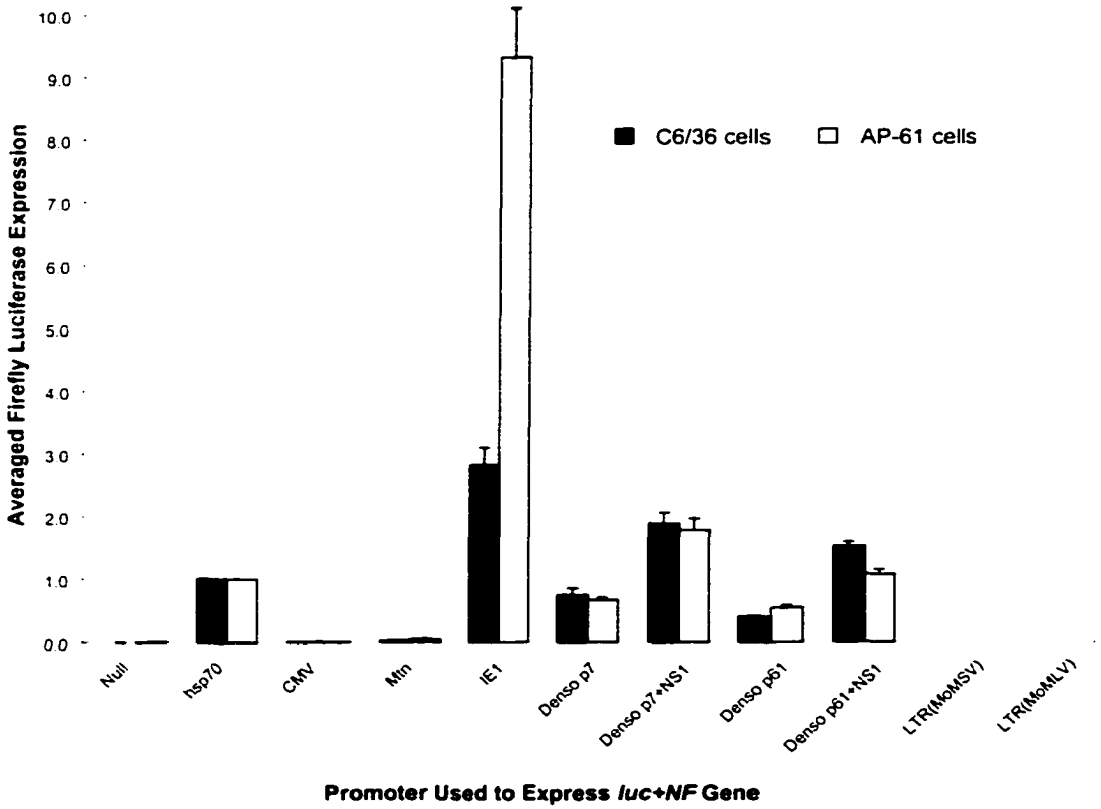



**Figure 3-6. Direct comparison of the firefly luciferase expression from the experimental reporter constructs in C6/36 and AP-61 cells.** Each bar represents the average of the three independent experiments in each cell line with error bars representing the standard errors expressed as a percentage of the average. Note: there are no error bars associated with expression from the hsp70 promoter because the data was normalized to hsp70 expression and was set equal to 1.

*et al.*, 1987), has been used previously in transient transfection experiments of mosquito cells (Durbin and Fallon, 1985; Gerenday *et al.*, 1999; Lycett and Crampton, 1993), and was known to express constitutively in both cell types used in this study. Of the promoters tested, the baculovirus IE-1 promoter plus hr5 enhancer from the *Autographa californica* nuclear polyhedrosis virus produced the highest amount of expression of the firefly luciferase reporter gene. The level of expression observed from the $^{hr5}$IE1 promoter/enhancer in C6/36 and AP-61cells was ~3- and ~10-fold higher, respectively, than from uninduced hsp70. The reason for the more than 3-fold difference in expression in the two cell lines is unclear, but not unprecedented. A similar disparity in promoter efficiency was observed from the *Bombyx mori* IE1 and *D. melanogaster* actin 5C promoters in two *Anopheles* cell lines (Zhao and Eggleston, 1999). None of the other promoters examined in this study demonstrated this magnitude of difference in the two *Aedes* mosquito cell lines. The *Aedes* densonucleosis virus p7 and p61 promoters produced expression above that of hsp70 only when transactivated by the NS1 protein. Transactivation of the p7 and p61 promoters by NS1 led to a 2.5-fold and 3.8-fold increase in expression, respectively, in C6/36 cells and a 2.7-fold and 2.0-fold increase, respectively, in AP-61 cells. Although significant, these increases are lower than expected as previous observations have shown that supplying the NS1 protein *in trans* can generate >18-fold induction from these promoters (data not shown). Constitutive expression of the Mtn promoter in the two *Aedes* cell lines was detected at relatively low levels. Even an induction of 10- to 20-fold (Klimowski *et al.*, 1996) with high levels of heavy metals would generate a lower level of expression compared to the viral promoters described above. The lack of expression from the CMV promoter coupled with similar

observations made in *Drosophila* Kc1 and SL2 cell lines and the *Spodoptera* Sf9 cell line (Pfeifer *et al.*, 1997) indicate a general inability of this promoter to function in insect cells, probably due to a lack of necessary transcription factors (Ghazal *et al.*, 1988). The identification of the *D. melanogaster* gypsy element as an infectious retrovirus with sequence and organizational similarity to mammalian retroviruses (Kim *et al.*, 1994; Alberola *et al.*, 1997) together with the successful infection of mosquito cells with retroviral vectors *in vitro* and *in vivo* (Matsubara *et al.*, 1996; Jordan *et al.*, 1998) made including the mammalian MoMSV- and MoMLV-LTR promoters in these comparison experiments important. Neither the MoMSV- nor the MoMLV-LTR produced detectable expression of the reporter gene in these experiments. This finding has implications toward the design of retroviral vectors to be used in attempted mosquito transgenesis, although expression from a transiently transfected plasmid may not truly portray expression from an integrated provirus.

This work contributes to a growing database of information about promoter activity in mosquito cells. The applicability of these findings to mosquito transgenesis will depend on whether expression from transient transfection will correlate to expression from elements integrated into the host cell genome. Caution should be used in assuming results from transient transfection experiments can be directly extrapolated to expression from elements integrated into the host genome. Expression from stably transformed cells can be dramatically affected by proximity to enhancer elements and other position effects (Heinrich *et al.*, 1995; Guy *et al.*, 1996). This may be the basis for the recent finding that the MoMSV- and MoMLV-LTR promoters mediate expression during retroviral infection of mosquito cells (J.C. Burns, personal communication). The applicability of

these results will also depend on whether the level of *in vivo* promoter efficiency will parallel the levels observed *in vitro*. The biology of immortalized cells can be substantially different from that of cells within an organism. Promoter activity can be specific for individual insect cell lines and vary even in lines derived from the same genus (Zhao and Eggleston, 1999). The most likely explanation involves promoter recognition mediated by differences in the transcriptional factors carried by cell lines derived from a range of cell types. Promoters identified to be highly efficient *in vitro* could be examined for activity *in vivo* using techniques to assay promoter function in developing mosquito embryos or primary tissue cultures (Morris *et al.*, 1995).

Despite these shortcomings, transient transfection studies are one of the best approaches currently available to rapidly evaluate and compare promoter efficiencies in mosquito cells. The precise comparison of expression from different promoter/enhancer regions requires controlling for potential variability in the transfection efficiency (Hollon and Yoshimura, 1989). By transfecting equal molar amounts of the experimental promoter plasmids and using an internal control to account for transfection variability, expression levels were able to be determined to within <5% standard errors (although there were instances of standard errors as high as 12%) within the same experiment. Standard errors were ~20% (and as high as 40%) when no internal control was used. This increased accuracy translated into consistent expression results when independent transfection experiments were compared. The only instances of high variability were from those promoters that did not produce luciferase activity above background. A disadvantage of using an internal control is that the interactions of the two promoters must be assessed (Farr and Roman, 1992). In addition, the ratios of each plasmid

construct in each cell line must be optimized to ensure experimental accuracy . It is most likely an interaction between the promoter of the experimental plasmid and the *hsp70* promoter of the internal control that resulted in the variability observed in the *Renilla* luciferase measurements.

Knowledge of constitutive rates of expression from promoter elements is essential for the design and optimization of novel strategies for the control of vector-borne diseases. These results and those of others (Zhao and Eggleston, 1999) describing the promoter efficiencies in transiently transfected mosquito cell lines have important implications for the eventual use of transformation technologies to engineer transgenic mosquitoes.

- Chapter 4 -

# CONSTRUCTION OF MULTI-REPORTER FUSION PROTEINS FOR USE IN VSV G-PSEUDOTYPED RETROVIRAL VECTORS

## INTRODUCTION

After the identification of efficient promoter regions that facilitate high-level expression in mosquito cells (Chapter 3), the next question to be addressed was "What should be expressed from these promoters?" The ultimate goal of this research is the stable expression of antipathogen effector molecules in mosquitoes and the generation of a virus resistant phenotype using VSV G-pseudotyped retroviral vectors to deliver the refractory sequences. These vectors have never before been used to generate transgenic mosquitoes; an initial success using this system would support the feasibility of this approach. Success or failure of VSV G-pseudotyped retroviral vector-mediated transgenesis will depend on refining both the design of the vectors and the execution of the technique. Unfortunately, the methods for directly measuring gene expression are time consuming and costly and therefore preclude the screening of a large numbers of individual mosquitoes. In addition, accurate quantification by these methods is limited. The expression of a reporter gene would permit the rapid, convenient, and cost effective analysis of large numbers of potentially transformed mosquitoes and allow for the potential of VSV G-pseudotyped retroviral vectors to mediate mosquito transgenesis to be assessed.

The central concept of a reporter gene is simple. It is a defined nucleotide sequence, which when introduced into a biological system yields a readily measurable phenotype upon expression (Wood, 1995). There are numerous reporter systems available, with more potential reporter systems identified each year. Each has its own set of advantages and disadvantages. Selecting the appropriate reporter system can be a critically important aspect of an experiment.

The properties that make reporters useful for different experiments can be quite distinct. In certain situations, the function of more than one reporter gene is desirable. The construction of fusion genes encoding chimeric proteins can combine the advantages and functions of different reporter systems. The versatility of multi-reporter proteins could enable diverse data to be collected from the same experiment. Experiments requiring long periods of development, such as those employing pseudotyped retroviral vector production (Yee *et al.*, 1994), would especially benefit by the use of multi-reporters. To address this need, several fusion-genes were constructed to combine the functions of the green fluorescent protein (GFP) from *Aequorea victoria* (Prasher *et al.*, 1992), firefly luciferase (Luc) from *Photinus pyralis* (de Wet *et al.*, 1985), and hygromycin B phosphotransferase ($Hyg^R$) from *Escherichia coli* (Gritz and Davies, 1983). These three reporter systems were chosen in order to take advantage of their distinct properties and because each has previously been expressed in mosquito cells.

GFP has been expressed in a variety of cell types (Chalfie *et al.*, 1994; Plautz *et al.*, 1996) and proven itself as a non-invasive indicator of expression in living cells. When illuminated by light of wavelength 395 nm, GFP yields a bright green fluorescence (wavelength of 509 nm) that does not require cofactors, substrates, or additional gene products (Chalfie *et al.*, 1994; Higgs *et al.*, 1996; Gerdes and Kaether, 1996; Kain *et al.*, 1995). Luc is especially useful as a quantitative reporter of transcription in animal (Geusz *et al.*, 1997; Castano *et al.*, 1996), plant (Ishitani *et al.*, 1998; Chen *et al.*, 1998; Walbot *et al.*, 1987), and insect (Plautz *et al.*, 1997; Rutter *et al.*, 1995; White *et al.*, 1995) systems at the single cell, tissue, and whole organism levels. Luciferase catalyzes the oxidation of the substrate molecule luciferin with an accompanying emission of light

(de Wet *et al.*, 1985). $Hyg^R$ is a selectable marker that specifically inactivates the aminocyclitol antibiotic hygromycin B (hyg) by aminoglycoside phosphorylation (Pardo *et al.*, 1985). Both prokaryotic and eukaryotic cells are sensitive to hyg-mediated inhibition of protein synthesis, but expression of the hygromycin B phosphotransferase protein induces protection and is commonly used in the selection of transformed cells (Blochlinger and Diggelmann, 1984; Cox *et al.*, 1996; Aubrecht *et al.*, 1997).

This chapter details the construction and characterization of multi-reporter fusion proteins for eventual use in VSV G-pseudotyped retroviral vectors. Because the production of different high titered vectors for different experiments is extremely time consuming, combining reporter functions would allow one vector to be used in a variety of research applications.

## MATERIALS & METHODS

### *Cell lines*

*Aedes albopictus* C6/36 cells (ATCC Number: CCL-126) were maintained at 28°C in L-15 medium supplemented with 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, and 5% heat-inactivated fetal bovine serum (FBS).

### *Plasmid Construction*

pMt2-Luc: This plasmid was previously constructed (Klimowski *et al.*, 1996). Briefly, the *Ph. pyralis* (firefly) luciferase gene was isolated from pGL2-Basic (Promega) and inserted into the pMt2 plasmid (Kovach *et al.*, 1992) downstream of the *Drosophila* metallothionein (Mtn) promoter.

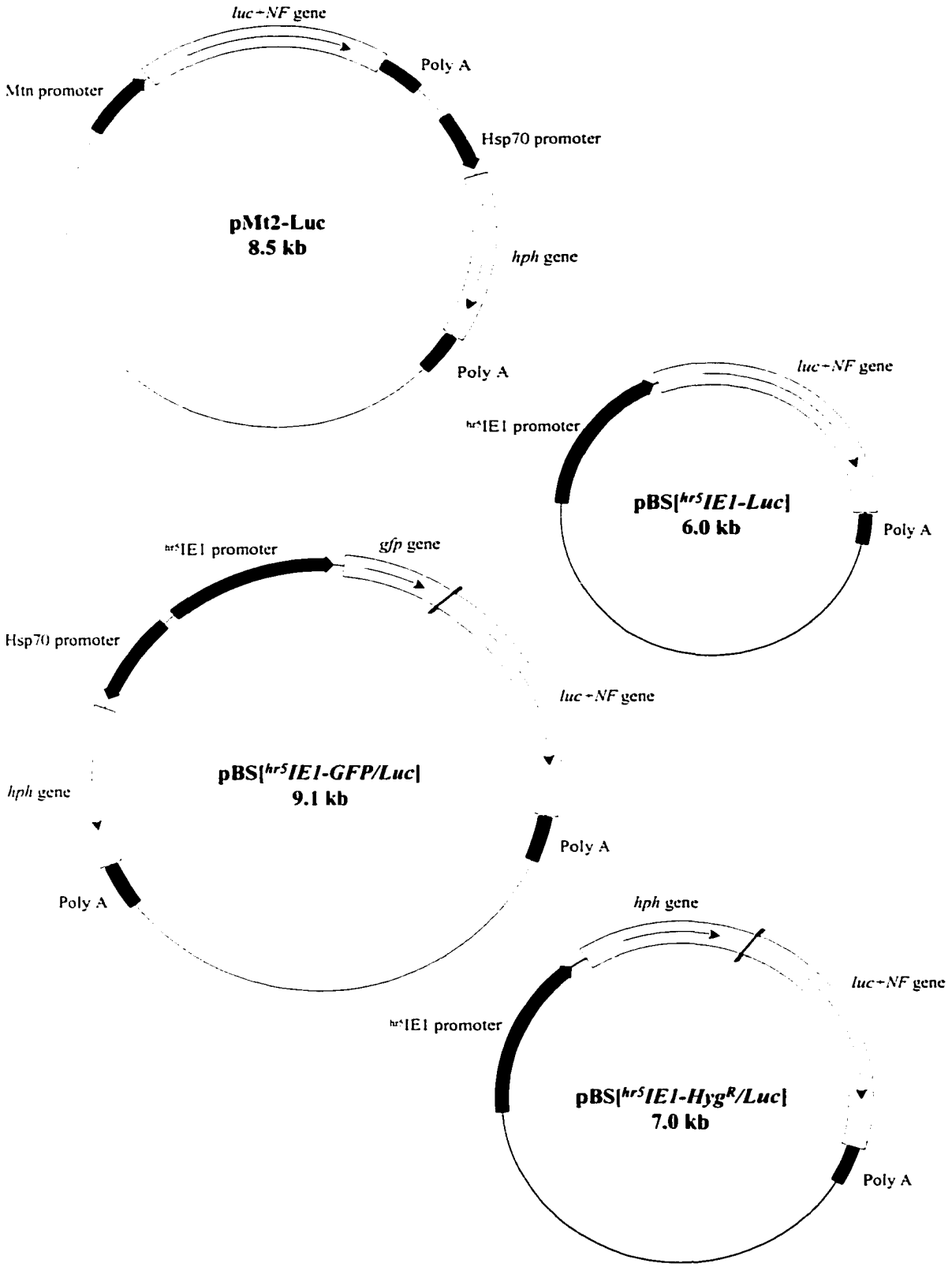



**Figure 4-1. Plasmids used to analyze multi-reporter fusion proteins.**

pBS[$^{hr5}$*IE1-Luc*]: The pSP-luc+NF plasmid (Promega) was digested with *Kpn*I and *Xba*I to release the firefly luciferase *luc+NF* gene. The *luc+NF* gene is a version of the firefly luciferase gene has been engineered to function in fusion proteins. The 1753 bp fragment was ligated into pGL3-Basic (Promega), which had previously been digested with *Kpn*I and *Xba*I. The luc+NF-poly(A) sequence from this plasmid was removed using *Kpn*I and *Bam*HI and ligated into pBluescriptII (SK+) (Stratagene), which had been previously digested with the same two enzymes to form pBS[*Null-Luc*]. The baculovirus IE1 promoter plus hr5 enhancer ($^{hr5}$IE1) was removed from the pIE1-3 vector (Novagen) using *Sma*I and ligated into pBluescript II (SK+) vector (Stratagene), which was previously digested with the same enzymes to form the intermediate plasmid pBS2(SK+)[$^{hr5}$IE1]. The $^{hr5}$IE1 sequence was removed from this construct by *Xba*I digestion and inserted into the *Nhe*I site of pBS[*Null-Luc*] to form pBS[$^{hr5}$*IE1-Luc*] (Bennett *et al.*, 2000).

pBS[$^{hr5}$*IE1-GFP/Luc*]: The pS65T-C1 plasmid (Clontech) was digested with *Nhe*I and *Bgl*II to release a 698 bp fragment encoding the green fluorescent protein (GFP) gene version S65T. This fragment was ligated into the same sites in pBS[*Null-Luc*]. The GFP/Luc fusion gene was removed from this plasmid using *Nhe*I and *Xba*I and inserted into pBS[$^{hr5}$*IE1-Luc*] previously digested with *Avr*II and *Xba*I. This plasmid was then digested with *Kpn*I and *Spe*I and a 2.5 kb *Kpn*I-*Xba*I fragment carrying the *hph* gene under the control of the *D. melanogaster* hsp70 promoter inserted. The fragment originated from pUChshyg and was inserted (via a pCR2.1 shuttle) upstream of the baculovirus promoter/enhancer region, oriented to transcribe in the opposite direction as GFP/Luc.

pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*]: The *hph* gene coding for hygromycin B phosphotransferase (Gritz and Davies, 1983) followed by an SV40 polyadenylation signal and expressed from the *D. melanogaster* heat shock 70 promoter was removed from the pUChshyg plasmid (Monroe *et al.*, 1992) using *Xho*I and *Bam*HI. This 2.7 kb fragment was and ligated into pBluescript II (SK+) previously digested with the same two enzymes. The hsp70-*hph* sequence was amplified using TaqPlus polymerase (Stratagene), a T7 primer (5'-GTAATACGACTCACTATAGGGC-3'), and a primer at the 3' end of the *hph* gene incorporating a *Bst*EII site (5'-TACGGTGACCTTTGCCCTCGGACGAGT GC-3'). Amplification was performed in a thermocycler (MJ Research Inc., Watertown, MA) for 15 cycles at an annealing temperature of 55°C. The 1.5 kb amplification product was TA cloned (Marchuk *et al.*, 1991)into the pCR2.1-TOPO vector using the TOPO-TA cloning kit (Invitrogen). The *hph* gene was removed from this plasmid by digesting with *Hin*dIII and *Bst*EII and ligated into pBS[$^{hr5}$*IE1-Luc*] digested with the same two enzymes to form the plasmid pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*].

pRL-hsp70: The hsp70 promoter was removed from the pUChshyg plasmid using the enzymes *Xmn*I and *Hin*dIII. The resulting fragment was then inserted upstream of the *R. reniformis* (sea pansy) luciferase gene in pRL-Null (Promega), which had been previously digested with *Xho*I and *Hin*dIII. An initial ligation allowed the *Hin*dIII overhangs adhere. This was followed by a fill-in reaction using the large fragment of DNA polymerase I to generate blunt ends that were subsequently joined by a second ligation reaction.

***Transient Transfection in C6/36 cells***

In preparation for transient transfection experiments, each completed plasmid was transformed into DH10B cells (Life Technologies) and plasmid DNA was isolated on a Maxiprep column (Qiagen). DNA was ethanol precipitated and resuspended in 10 mM Tris (pH 8.5). DNA concentration was determined on a DU-640 Spectrophotometer (Beckman). All DNAs were then diluted to 250 μg/ml in 10 mM Tris (pH 8.5) prior to being used for transfection except the pRL-hsp70 internal control plasmid, which was diluted to 50 μg/ml. C6/36 cells were grown to confluence in a T-75 tissue culture flask. The cells were suspended in phosphate-buffered saline (PBS) and cell density determined using a hemocytometer. $8.0 \times 10^4$ cells were seeded into each well of a 12-well plate with the appropriate growth medium and allowed to adhere for 24 hours. For transfection, the growth medium was replaced with 400 μl L-15 medium without antibiotics or serum and a total of 4.08 μg of DNA was transfected into each well using Lipofectin reagent (Life Technologies). The transient transfection was designed to deliver the same molar concentration of plasmid DNA molecules as described in Chapter 3. pBluescriptII (SK+) was used to increase the amount of DNA to 4.00 μg per well and 0.08 μg of the *R. reniformis* luciferase encoding pRL-hsp70 was added as an internal control. After 8.0 hours of transfection, the DNA/Lipofectin/ medium complex was replaced with 2.0 ml growth medium. After ~28 hours the cells were assayed for GFP or luciferase activity. GFP activity was detected using an Olympus BH-2 epifluorescence microscope; UV illumination at a wavelength of 395 nm produced fluorescence at a wavelength of 509 nm. Firefly and *Renilla* luciferase activities were determined according to the instructions for the Dual-Luciferase Assay kit (Promega). This assay

measures and distinguishes firefly and *Renilla* luciferase activity. Cells were harvested in 250 µl of 1 × Passive Cell Lysis buffer, centrifuged to remove debris, and 10 µl of each lysate assayed using a TD 20/20 Luminometer (Turner Designs) set for a 2 second delay and 10 second integrated measurement. Firefly luciferase measurement ($LA_F$) was normalized to the internal control *Renilla* luciferase ($LA_R$) from the same sample to remove sample-to-sample experimental variation.

***Stable Transfection in C6/36 cells***

C6/36 cells were allowed to grow to ~70% confluence in a T-25 tissue culture flask. The growth medium was replaced with 2.6 ml of L-15 medium without antibiotics or serum and transfected as described above for 16 hours with 26 µg of pMt2-Luc, pBS[$^{hr5}$*IE1-GFP/Luc*], or pBS[$^{hr5}$*IE1-Hyg$^R$/Luc*]. 48 hours after removing the DNA/Lipofectin/medium complex, cells were selected for hygromycin resistance in L-15/10% FBS medium containing 300 U/ml hygromycin B (Calibiochem, La Jolla, CA). After 6 days, cell lines were isolated using 100 µl pipette tips and propagated for analysis. Early expansion was aided by the used of a 1:1 ratio of fresh to conditioned L-15/10% FBS medium.

***Protein half-life determination***

Stably transformed cell lines derived from transfections with the pMt-2, pBS[$^{hr5}$*IE1-GFP/Luc*], or pBS[$^{hr5}$*IE1-Hyg$^R$/Luc*] plasmids were seeded into 12-well plates and allowed to grow to ~90% confluence. The L-15/10% FBS + 300 U hygromycin B/ml selection medium was replaced with L-15/10% FBS + 4 µg/ml actinomycin D medium (Condreay *et al.*, 1988). Cells were harvested from 1 to 72 hours after addition of the actinomycin D and assayed for firefly luciferase activity as described

above. Protein concentration was assayed using Coomassie Plus reagent (Pierce, Rockford, IL) (5 µl of cell lysate in 150 µl reagent) and the absorbance measured at 600 nm using a microplate reader. $LA_F$/mg protein data was used to calculate an exponential trendline describing the decay rate of the luciferase activity in the form:

$$LA_F=LA_F(t_0)e^{-\lambda(t)}$$

With the determination of λ, protein half-life ($t_{1/2}$) was solved as:

$$t_{1/2}=(\ln 2)/\lambda$$

## RESULTS

### *Transient luciferase activity of multi-reporter fusion genes*

Luciferase activity from the two multi-reporter fusion gene products was readily detected in C6/36 mosquito cells transiently transfected with pBS[$^{hr5}$*IE1-GFP/Luc*] or pBS[$^{hr5}$*IE1-Hyg$^R$/Luc*], although the presence of the N-terminal GFP or Hyg$^R$ polypeptide chain affected the activity of the luciferase molecule in these fusions (Table 4-1). In

**Table 4-1. Luciferase activity from Luc, GFP/Luc, and Hyg$^R$/Luc proteins expressed in transiently transfected cells.**

| | Plasmid | | |
|---|---|---|---|
| | pBS[$^{hr5}$*IE1-Luc*] | pBS[$^{hr5}$*IE1-GFP/Luc*] | pBS[$^{hr5}$*IE1-Hyg$^R$/Luc*] |
| RLA (n=6) | 11.64 | 3.27 | 0.51 |
| SE | 4.46% | 2.03% | 3.90% |
| Relative Activity | 100% | 28.1% | 4.4% |

Relative Light Units. RLA = $LA_F/LA_R$; SE is the standard error

transient transfections, firefly luciferase activity (normalized to *Renilla* luciferase activity) from pBS[$^{hr5}$*IE1-Luc*] was 11.64 RLU (with a standard error of 4.46%) compared to 3.27 RLU (2.03%) and 0.51 RLU (3.90%) from pBS[$^{hr5}$*IE1-GFP/Luc*] and pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*], respectively. These are statistically significant decreases in luciferase activity, with heteroscedastic two-tailed T-tests yielding P values of 0.000012 between pBS[$^{hr5}$*IE1-Luc*] and pBS[$^{hr5}$*IE1-GFP/Luc*] and 0.000004 between pBS[$^{hr5}$*IE1-Luc*] and pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*].

***Transient GFP activity***

C6/36 cells transiently transfected with pBS[$^{hr5}$*IE1-GFP/Luc*] demonstrated bright green fluorescence when exposed to UV light comparable to cells transfected with a pBS[$^{hr5}$*IE1-GFP*] construct (data not shown). Non-transfected or pBS[$^{hr5}$*IE1-Luc*] transfected C6/36 cells showed no green fluorescence.

***Hygromycin B phosphotransferase activity***

The activity of the *hph* gene product in the Hyg$^{R}$/Luc fusion protein was determined by the formation of stably transformed cell lines derived from C6/36 cells. pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*] transfection followed by selection in medium containing 300 U/ml hygromycin B resulted in the isolation of 17 clonal, hygromycin resistant cell lines. No clones were isolated from non-transfected or cells transfected with pBS[$^{hr5}$*IE1-Luc*]. Luciferase activity from pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*] transformed cells ranged from 1.7 × background to 62,024 × background with a median level of 8358 × background (Table 4-2). Three of these (clones H/L-2A12, -1D4, and -1A7, representing low, medium, and high level luciferase expressing lines, respectively) were exposed to increasing concentrations of hygromycin B. $TCLD_{50}$ was determined as the concentration of

**Table 4-2. Luciferase activity from cell lines stably transformed with plasmids pMt2-Luc, pBS[$^{hr5}$*IE1-GFP/Luc*], and pBS[$^{hr5}$*IE1-Hyg$^{R}$-Luc*].**

| Cell Line | | LAP/mg total protein | × Background | |
|---|---|---|---|---|
| Untransformed | | | | |
| C6/36 | | 154.4 | 1 | |
| Transformed with pMt2-Luc | | | | |
| Clone | MTL25 | 6004500 | 38885 | |
| | MTL26 | 8350 | 54.1 | |
| | MTL27 | 10300 | 66.7 | |
| | MTL28 | 30500 | 197.5 | |
| | MTL29 | 9772000 | 63283 | |
| | MTL33 | 121 | 0.8 | |
| | MTL34 | 566700 | 3670 | |
| | MTL35 | 2120 | 13.7 | |
| | MTL51 | 1145000 | 7415 | Median expression above background = 1055 |
| | MTL54 | 162900 | 1055 | |
| | MTL55 | 15310 | 99.1 | |
| | MTL57 | 18270 | 118.3 | |
| | MTL59 | 2023700 | 13105 | |
| | MTL61 | 1041000 | 6741.5 | |
| | MTL67 | 50400 | 326.4 | |
| | MTL77 | 3434900 | 22244 | |
| | MTL79 | 2436700 | 15780 | |
| Transformed with pBS[$^{hr5}$*IE1-GFP/Luc*] | | | | |
| Clone | 5A1 | 202529 | 1311.6 | |
| | 5A2 | 175545 | 1136.8 | |
| | 5A4 | 20175 | 130.7 | |
| | 5A5 | 82084 | 531.6 | |
| | 5A6 | 245954 | 1592.8 | |
| | 5B10 | 4654 | 30.1 | |
| | 5B11 | 13261 | 85.9 | Median expression above background = 531.6 |
| | 5B2 | 27276 | 176.6 | |
| | 5B3 | 30288 | 196.1 | |
| | 5B4 | 85330 | 552.6 | |
| | 5B5 | 165483 | 1071.7 | |
| | 5B6 | 31175 | 201.9 | |
| | 5B7 | 393340 | 2547.3 | |
| Transformed with pBS[$^{hr5}$*IE1-Hyg$^{R}$/Luc*] | | | | |
| Clone | 1A5 | 989126 | 6406 | |
| | 1A7 | 9577640 | 62024 | |
| | 1A11 | 1290622 | 8358 | |
| | 1B2 | 3284677 | 21271 | |
| | 1B8 | 2765705 | 17911 | |
| | 1C5 | 3287625 | 21291 | |
| | 1D4 | 1111279 | 7197 | |
| | 1D6 | 3312392 | 21451 | |
| | 1D8 | 3389693 | 21951 | Median expression above background = 8358 |
| | 1D10 | 6890413 | 44622 | |
| | 1D12 | 7180235 | 46499 | |
| | 2A1 | 482654 | 3126 | |
| | 2A2 | 259.9 | 1.7 | |
| | 2A3 | 20759 | 134.4 | |
| | 2A5 | 36298 | 235.1 | |
| | 2A12 | 3352 | 21.7 | |
| | 2B5 | 12701 | 82.3 | |

hygromycin B at which >50% of the cells died within 96 hours of exposure. Greater levels of luciferase activity corresponded to a greater $TCLD_{50}$. A $TCLD_{50}$ of $2 \times 10^3$ U/ml was determined for clone H/L-2A12, $3 \times 10^4$ U/ml for clone H/L-1D4, and $1 \times 10^5$ U/ml for clone H/L-1A7.

***Generation of stably transformed cell lines***

Transfection of C6/36 cells with pMt2-Luc or pBS[$^{hr5}$*IE1-GFP/Luc*] resulted in 17 and 13 stable, hygromycin-resistant cell lines, respectively. Cell lysates from each cell line were analyzed for firefly luciferase activity (Table 4-2). Luciferase activity from pMt2-Luc transformed cell lines ranged from undetectable to 63,283 × background with a median level of 1055 × background; luciferase activity from pBS[$^{hr5}$*IE1-GFP/Luc*] transformed cell lines ranged from 30.1 to 2,547 × background with a median level of 531.6 × background. Despite the easily detected luciferase activity, no green fluorescence was observed from any cell line stably transformed with pBS[$^{hr5}$*IE1-GFP/Luc*].

***Luciferase, GFP/Luc, and Hyg$^R$/Luc half-life determination in C6/36 cells***

Actinomycin D treatment caused an initial increase in luciferase activity in clone MTL25 cells (stably transformed with pMt2-Luc), which was seen to peak after ~5 hours. Due to this increase, only $LA_F$/mg protein data points from 7 to 72 hours post-actinomycin D addition were used in the calculation of t½ (Figure 4-2b). Firefly luciferase t½ was calculated to be 15.61 hours and 15.47 hours in two separate experiments. This corresponds extremely well with a t½ of 15.47 hours determined previously by Mtn promoter induction (Figure 4-2a). This method for half-life determination was then applied to the determination of the half-lives of the multi-reporter

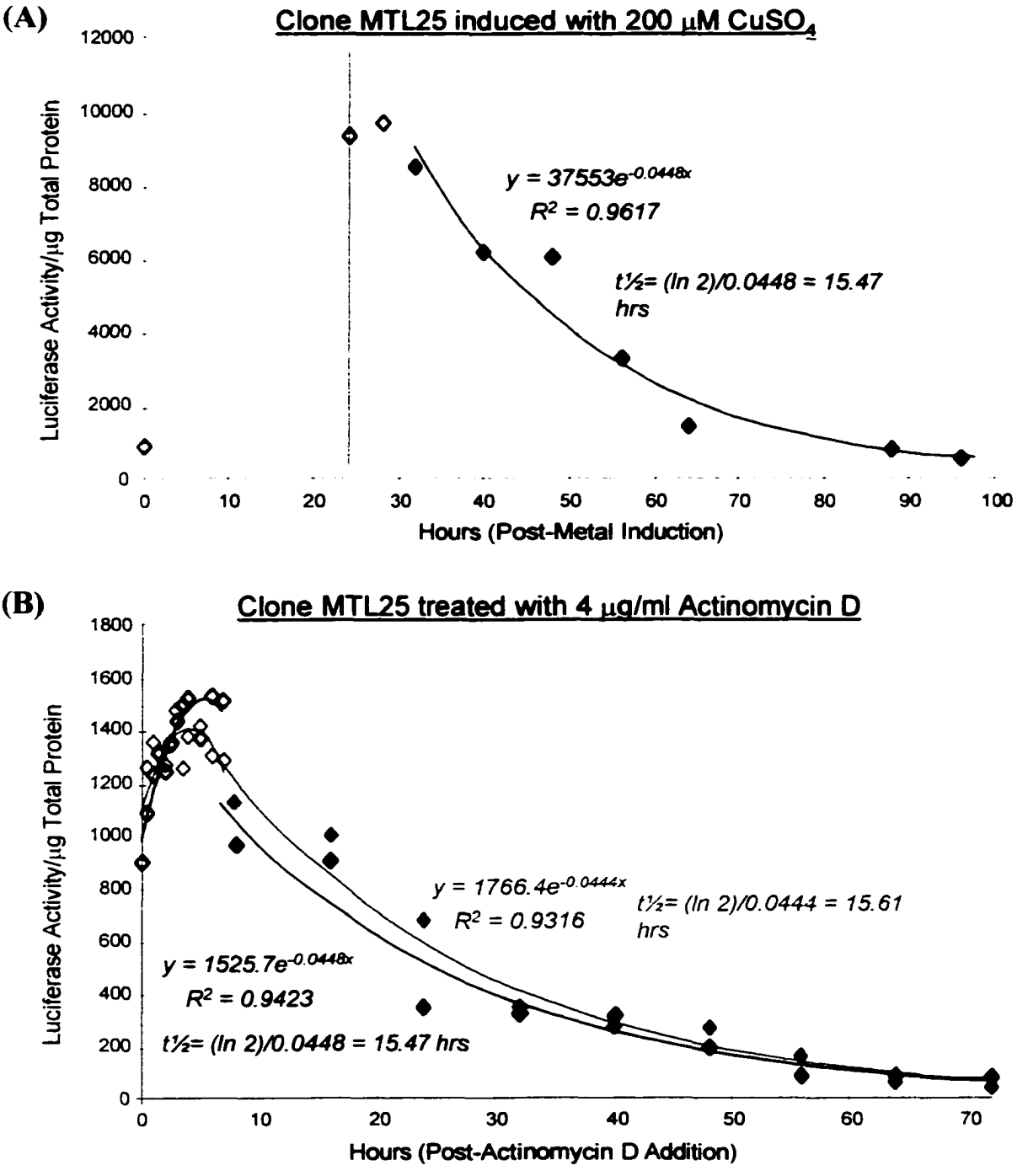



**Figure 4-2. Determination of luciferase half-life in C6/36 cells by metal induction (A) or actinomycin D treatment (B).** Closed diamonds indicate data points used in the calculation of t½; open diamonds were not.

fusion proteins. $LA_F$/mg protein did not decrease until 7 hours after the addition of actinomycin D. Therefore only data from 7 to 72 hours post-actinomycin D addition was used in the calculation of protein half-life. GFP/Luc t½ was determined to be 29.75 and 30.40 hours from clones G/L-5A1 and G/L-5B7, respectively; $Hyg^R$/Luc t½ was determined to be 19.80 and 19.69 hours from clones H/L-1A7 and H/L-1D4, respectively.

## DISCUSSION

Chimeric proteins combining the strengths of fluorescent, bioluminescent, and antibiotic resistance were engineered to take advantage of the properties that make the *gfp*, *luc*, and *hph* reporter genes useful for particular experimental applications. The GFP/Luc and $Hyg^R$/Luc constructed here preserved the functions of the original proteins. These multi-reporter fusion proteins will be beneficial in experiments involving VSV G-pseudotyped retroviral vectors because of their increased versatility, eliminating the need to design new vectors for separate experiments.

The observed activity of a bioreporter is a function of protein expression and stability. To accurately compare reporter activity, both issues must be addressed. In these studies, the first aspect was addressed by comparing transient transfections in which the same promoter was used to direct expression. The second aspect was addressed by the determination of protein half-life using stably transformed cell lines. The two multi-reporter proteins were found to retain the GFP, luciferase, and hygromycin B phosphotransferase functions, although the quantitative activity of the fused luciferase in GFP/Luc and $Hyg^R$/Luc produced only 16.9% and 3.7%, respectively, the luciferase activity of non-fused Luc (Table 4-3). This lowered function is probably the result of

**Table 4-3. Luciferase activity from Luc, GFP/Luc, and Hyg$^R$/Luc proteins expressed in transiently transfected cells, including affect of protein half-life.**

| | Plasmid: | | |
|---|---|---|---|
| | pBS[$^{hr5}$*IE1-Luc*] | pBS[$^{hr5}$*IE1-GFP/Luc*] | pBS[$^{hr5}$*IE1-Hyg$^R$/Luc*] |
| RLA (n=6) | 11.64 | 3.27 | 0.51 |
| SE | 4.46% | 2.03% | 3.90% |
| t½ factor | 1.000 | 1.663 | 1.190 |
| Relative Activity | 100% | 16.9% | 3.7% |

Relative Activity = (RLA(plasmid) × t½ factor)/RLA(pBS[$^{hr5}$*IE1-Luc*])

steric interference as the proximity of the GFP and Hyg$^R$ peptides could result in either altered protein folding or decreased accessibility to substrate molecules. The insertion of a spacer may restore enzymatic efficiency to its original level (Day *et al.*, 1998). GFP and Hyg$^R$ activities from the fusion proteins were not altered, but the assays utilized to determine a discrepancy are not nearly as sensitive as the luciferase assay (Wood, 1995).

It is interesting to note that although transient transfections of the GFP/Luc construct demonstrated that both reporters functioned in this fusion protein, cell lines stably transformed with this construct failed to fluoresce despite producing detectable luciferase activity. GFP has been suggested to be moderately toxic and unstable in long term expression systems (Hanazono *et al.*, 1997). However GFP has been used to produce stable cell lines and transgenic animals (Kandel *et al.*, 1997; Bagley *et al.*, 1998; Ramiro *et al.*, 1998; van den Pol and Ghosh, 1998). In this study, stable GFP/Luc transformed cell lines produced only 6.36% the luciferase activity of stable Hyg$^R$/Luc transformed cell lines. Adjusting for enzymatic efficiency reveals that GFP/Luc expressing cells produce ~1% the number of transgene encoded protein molecules as Hyg$^R$/Luc expressing cells. This difference of two orders of magnitude does suggest a disadvantage for cells expressing the GFP/Luc fusion gene and might also explain why

these cells were not observed to fluoresce. There simply may not have been enough of the less sensitive GFP reporter present to produce a detectable signal.

A feature that distinguishes firefly luciferase from other reporter proteins is its relatively short half-life. In mammalian cells, t½ is typically ~3 hours (Thompson *et al.*, 1991). This value can be dramatically affected by the temperature at which cells are incubated (White *et al.*, 1996). The t½ determination of ~15.5 hours from cells grown at 28°C confirms this theory. Fusing proteins typically produces a product with an altered half-life. Both GFP/Luc and $Hyg^R$/Luc fusion proteins demonstrated this, as t½ increased 93% and 27%, respectively. In contrast, a similar fusion protein with N-terminal addition of GFP to luciferase was not seen to have an altered t½ in mammalian cells incubated at 37°C (Day *et al.*, 1998). The lower temperature used to grow mosquito cell cultures may have helped identify differences in t½, as small, but significant changes could be more noticeable at 16 hours than at 3 hours. The short half-life of luciferase has allowed it to be used as a monitor of rapid change in protein expression (Wood, 1995). The stability of GFP (Chalfie, 1995), chloramphenicol acetyl transferase (CAT; Thompson *et al.*, 1991), and β-galactosidase (β-gal; Langridge, 1969) have hampered their use in some transcriptional analyses. Once synthesized, these proteins produce signal over a long period of time. Fusing proteins may be an effective strategy for altering the half-life of other reporters and increasing their versatility.

The main reason to design and utilize a multi-reporter fusion protein is the increased versatility. Multi-reporters could be applied to virtually any situation that a single reporter could. The GFP/Luc and $Hyg^R$/Luc proteins described here combine functions that are very effective as cellular markers useful in mosquito transgenesis. The

different systems increase the chances for identifying possible transgenic animals. These reporter fusion genes will be particularly advantageous in retroviral vectors; the expression of multiple reporters from one promoter conserves the limited coding capacity within the vector and decreases the likelihood of rearrangements stemming from additional promoters in between the LTRs.

Assessing the ability of new techniques to generate stable transformation will require the screening of large numbers of individual mosquitoes. The most effective reporters identifying transgenic animals are those that induce an easily detectable phenotype. Eye color is a commonly used phenotypic marker in *Drosophila* and several of these systems are promising transformation markers for mosquito species (Rong and Golic, 1998; Coates *et al.*, 1997; Besansky *et al.*, 1995). The disadvantage of these markers is that they must be expressed within specific tissues and they offer no simple means to measure transcriptional activity. Constructing a fusion incorporating one of these genes with luciferase, for example, would allow large numbers of possibly transformed mosquitoes to be examined by eye color and the expression level quantified by luciferase assay. In addition, transgenic animals expressing the transgene in tissues other than the eye could be identified. Such a multi-reporter protein would be especially beneficial to optimize both the transfection efficiency and transcriptional efficiency of a transformation technique.

# - Chapter 5 -

# INTERFERENCE BETWEEN RNA TRANSCRIPTS EXPRESSED FROM A CELL'S NUCLEUS AND AN INFECTING VIRUS

## INTRODUCTION

As natural gene delivery vehicles, viruses have been exploited to introduce and express genes into cells *in vitro* and *in vivo*. Adenoviruses (Berkner, 1988; Chengalvala *et al.*, 1991; Ragot *et al.*, 1997), alphaviruses (Liljestrom, 1994; Xiong *et al.*, 1989), herpesviruses (Fink and Glorioso, 1997; Efstathiou and Minson, 1995), parvoviruses (Carter, 1992; Snoeck *et al.*, 1997), papovaviruses (Menck *et al.*, 1989), poxviruses (Carroll and Moss, 1997; Moss, 1993), and retroviruses (Maclean, 1994) have been utilized as expression systems in mammalian cells, while alphaviruses (Olson *et al.*, 1998), parvoviruses (Afanasiev *et al.*, 1994), and retroviruses (Chapter 2; Matsubara *et al.*, 1996) have been utilized in insect cells. These viral vectors have contributed greatly to the current knowledge of fundamental cellular processes in a wide range of biological systems.

Members of the *Alphavirus* genus have received considerable attention for use as virus-based expression vectors in both vertebrate and insect cells (Xiong *et al.*, 1989; Hahn *et al.*, 1992; Bredenbeek *et al.*, 1993; Raju and Huang, 1991; Levis *et al.*, 1987; Dubensky *et al.*, 1996; Huang, 1996). Properties that make alphavirus vectors desirable as expression systems include high-level expression, a broad host range, and infection of nondividing cells (Strauss and Strauss, 1994). In addition, replication occurs entirely within the cytoplasm of the infected cell as an RNA molecule, eliminating possible problems associated with mRNA splicing or transport from the nucleus. The development of full-length cDNA clones has allowed the easy manipulation of these vectors for use as research tools. Alphaviruses have proven themselves efficient vectors for short-term, high-level expression of foreign genes and sequences.

In nature, the alphavirus life cycle alternates between vertebrates and hematophagous arthropods, usually mosquitoes (Calisher, 1994). Infection of vertebrates can result in fever, rash, encephalitis, or death; in contrast, infection of mosquitoes has generally been assumed to have no overall pathological consequence (Brown and Condreay, 1986). However, specific mosquito tissues do show pathology following alphavirus infection (Weaver *et al.*, 1992; Weaver *et al.*, 1988). This trend has also been noted in cell culture systems. Nearly all vertebrate cell lines infected with alphaviruses do not survive the acute infection (Brown, 1984), while most mosquito cell lines establish a long-term persistent infection (Karpf *et al.*, 1997). It is interesting to note that acute infection can result in cytopathic effects (CPE) in some mosquito cell lines (Karpf and Brown, 1998; Miller and Brown, 1992; de Wet *et al.*, 1985) and this has been attributed to higher levels of virus replication (Tooker and Kennedy, 1981). The general absence of virus-induced cytopathology has allowed alphaviruses to be used as viral vectors to express foreign genes and sequences in mosquito cells.

Sindbis virus is the prototype member of the alphaviruses, which are enveloped viruses containing a single-stranded, positive-sense RNA genome (Strauss and Strauss, 1994). The 11,703 nt RNA genome of Sindbis virus is infectious upon introduction into the cytoplasm of susceptible cells. During viral replication, the Sindbis virus genomic 49S RNA serves as the template for synthesis of a complementary negative strand by the virus-encoded replicase complex (Figure 5-1). The negative strand in turn serves as the template for additional genomic RNA and for an abundant internally initiated 26S subgenomic RNA. The nonstructural proteins are translated from the 5' two-thirds of the genomic RNA, while the structural proteins are translated from the subgenomic 26S

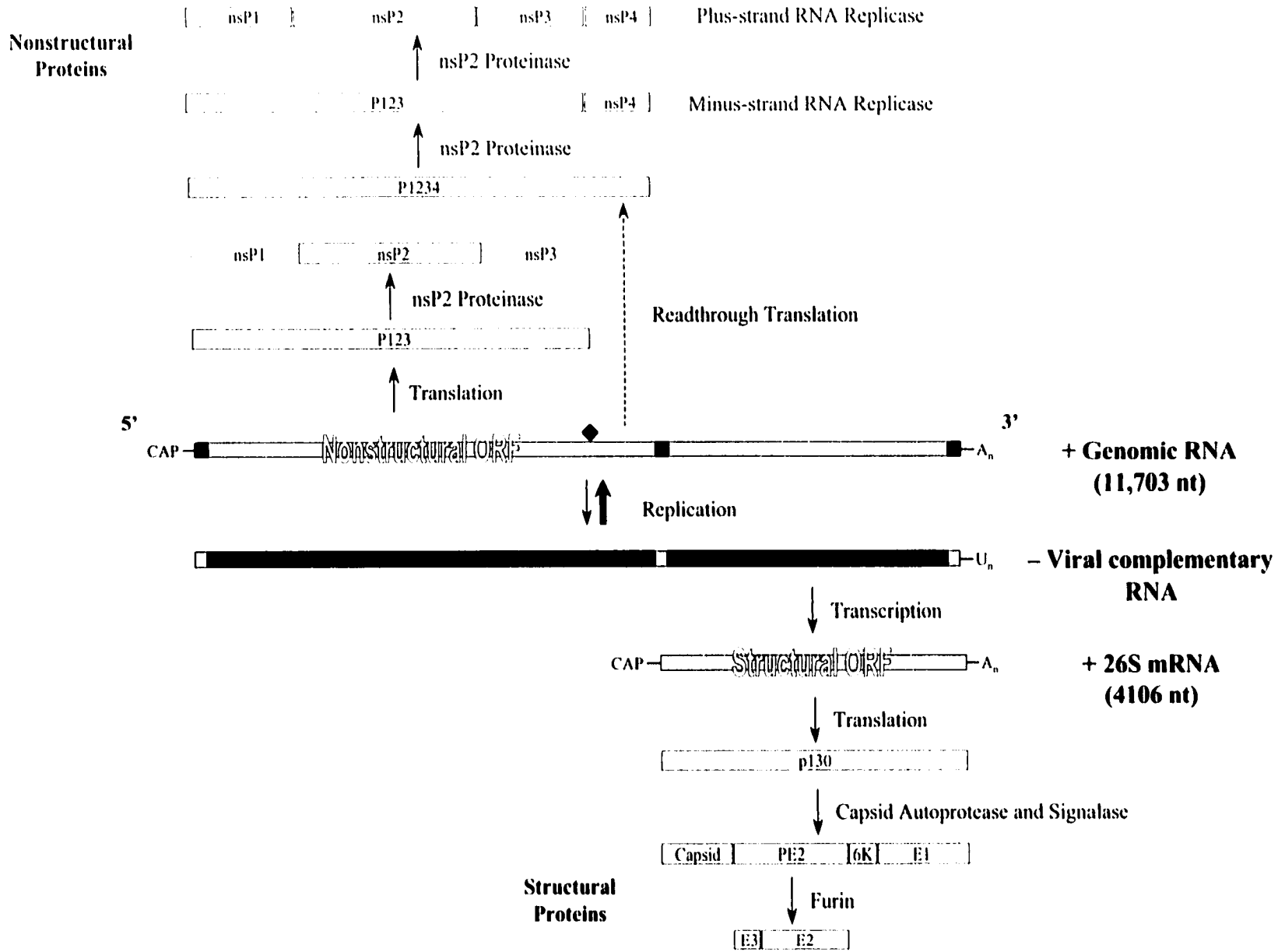



**Figure 5-1. Genomic organization of SIN virus.** The 49S genomic RNA is initially utilized as an mRNA to translate viral plus- and minus-strand RNA replicases. The small block boxes on the genomic RNA are conserved sequence elements and the black diamond denotes the leaky opal termination codon. Termination at the opal codon produces P123, while readthrough produces P1234. The structural proteins are translated from the 26S subgenomic mRNA genomic RNA. Capsid, E2, and E1 are found in the virion.

RNA that represents the 3' one-third of the genome. The nonstructural and structural proteins are each expressed as polypeptides and post-translationally processed into the individual proteins (Strauss and Strauss, 1994). Expression from Sindbis virus vectors, as well as other alphavirus vectors, is based on the same strategy wild-type virus uses to express its structural proteins. Heterologous sequences or genes of interest are inserted after an internal initiation site and abundantly transcribed as subgenomic RNA, which in turn serves as the transcriptional template for protein synthesis. Sindbis defective interfering and replicon expression vectors are missing part or all of the nonstructural and structural genes, respectively, and therefore require a helper virus to supply the missing components (Olson *et al.*, 1998). These systems produce replication incompetent vectors unable to generate new particles. Another approach utilizes a duplicated subgenomic promoter within the viral genome and results in the production of two subgenomic RNA molecules. This double-subgenomic Sindbis vector (dsSIN) system produces infectious, replication competent vectors capable of spreading from cell to cell.

Recombinant dsSIN viruses have proven to be especially valuable as expression vectors in mosquito cells. Examples include expression of chloramphenicol acetyltransferase (CAT) leading to the high levels of protein production, over $10^5$ polypeptides molecules per cell (Olson *et al.*, 1994), expression of the green fluorescent protein (GFP) allowing infected mosquito tissues to be visualized *in vivo* (Higgs *et al.*, 1996), and expression of an insect-specific neurotoxin that mediated killing in infected mosquitoes (Higgs *et al.*, 1995). In addition to protein production, dsSIN vectors have been employed in strategies to combat arbovirus transmission. Expression of antisense RNA molecules resulted in the establishment of resistance to LaCrosse (Powers *et al.*,

1996; Powers *et al.*, 1994), dengue (Gaines *et al.*, 1996; Olson *et al.*, 1996), and yellow fever viruses (Higgs *et al.*, 1998).

While Sindbis-derived vectors have proven their utility for transient expression in mosquitoes, these systems offer no possibility of establishing long-term, heritable expression. To accomplish this requires germ-line transformation and expression from the DNA of the mosquito. The success of control strategies designed to express antipathogen effector RNA molecules from DNA will require the interaction of viral and cellular RNA transcripts. Potential problems associated with mRNA splicing, transport from the nucleus, and compartmentalization, precluded by the use of Sindbis-derived vectors, may jeopardize the establishment of intracellular immunity (Scherczinger *et al.*, 1992; Denhardt, 1992).

This chapter investigated the ability of cellular and viral RNA transcripts to interact in mosquito cells using a dsSIN vector engineered to produce a 595 bp RNA transcript complementary to the 5' portion of the firefly luciferase gene to infect mosquito cell lines stably expressing luciferase or luciferase fusion proteins (Chapter 4).

## MATERIALS AND METHODS

### *Cell Lines*

*Aedes albopictus* C6/36 cells (ATCC Number: CCL-126) and *Mesocricetus auratus* BHK-21 cells (ATCC Number: CCL-10) were maintained in L-15 medium supplemented with 2 mM L-glutamine, 100 U penicillin/ml, 0.1 mg streptomycin/ml, and 5% or 10% heat-inactivated fetal bovine serum (FBS), respectively. Clones MTL25, G/L-5B7, and H/L-1A7 were established by transfecting plasmids pMt2-Luc, pBS[$^{hr5}$IE1-

GFP/Luc], and pBS[$^{hr5}$IE1-Hyg$^{R}$/Luc], respectively, into C6/36 cells using Lipofectin reagent (Life Technologies) and selected for stable transformation in L-15/10% FBS medium containing 300 U/ml of hygromycin B (Calibiochem).

***dsSIN Plasmid Construction***

The pTE/3'2J plasmid contains a full-length Sindbis viral cDNA with a second internal subgenomic promoter positioned downstream of the structural genes (Hahn *et al.*, 1992). pGEM-luc plasmid (Promega) was digested with *Bam*HI and *Eco*RI to release a 595 bp fragment containing the 5' end of the *Photinus pyralis* (firefly) luciferase gene and inserted into pBluescriptII(SK+) (Stratagene) using the same two enzymes. An *Nhe*I site was created by digesting with *Hin*dIII, filling in the 5' overhang, and ligating the plasmid to itself. The luciferase fragment was then removed using *Nhe*I and *Xba*I and cloned into the *Xba*I site downstream of the second internal subgenomic promoter of pTE/3'2J (orientation was confirmed by restriction enzyme analysis) to form pTE/3'2J-αLuc (Figure 5-2).

***dsSIN Virus Production***

Recombinant Sindbis virus was produced as previously described (Rice *et al.*, 1987; Powers *et al.*, 1994). Briefly, dsSIN DNA plasmids were linearized with *Xho*I and utilized as a template for *in vitro* RNA synthesis using SP6 polymerase. The resulting RNA was electroporated (BTX Inc., San Diego, CA) into BHK-21 cells at 500 V, 100 μF, and 720 Ω for a duration of approximately 0.8 ms. Cells from each electroporation were immediately seeded into 25-cm$^2$ cell culture flasks with 5 ml of L-15/10% FBS medium and incubated at 37°C for 24-36 hours. dsSIN viruses were harvested from the medium and titrated in BHK-21 cells by using an end point assay (Smith, 1969).

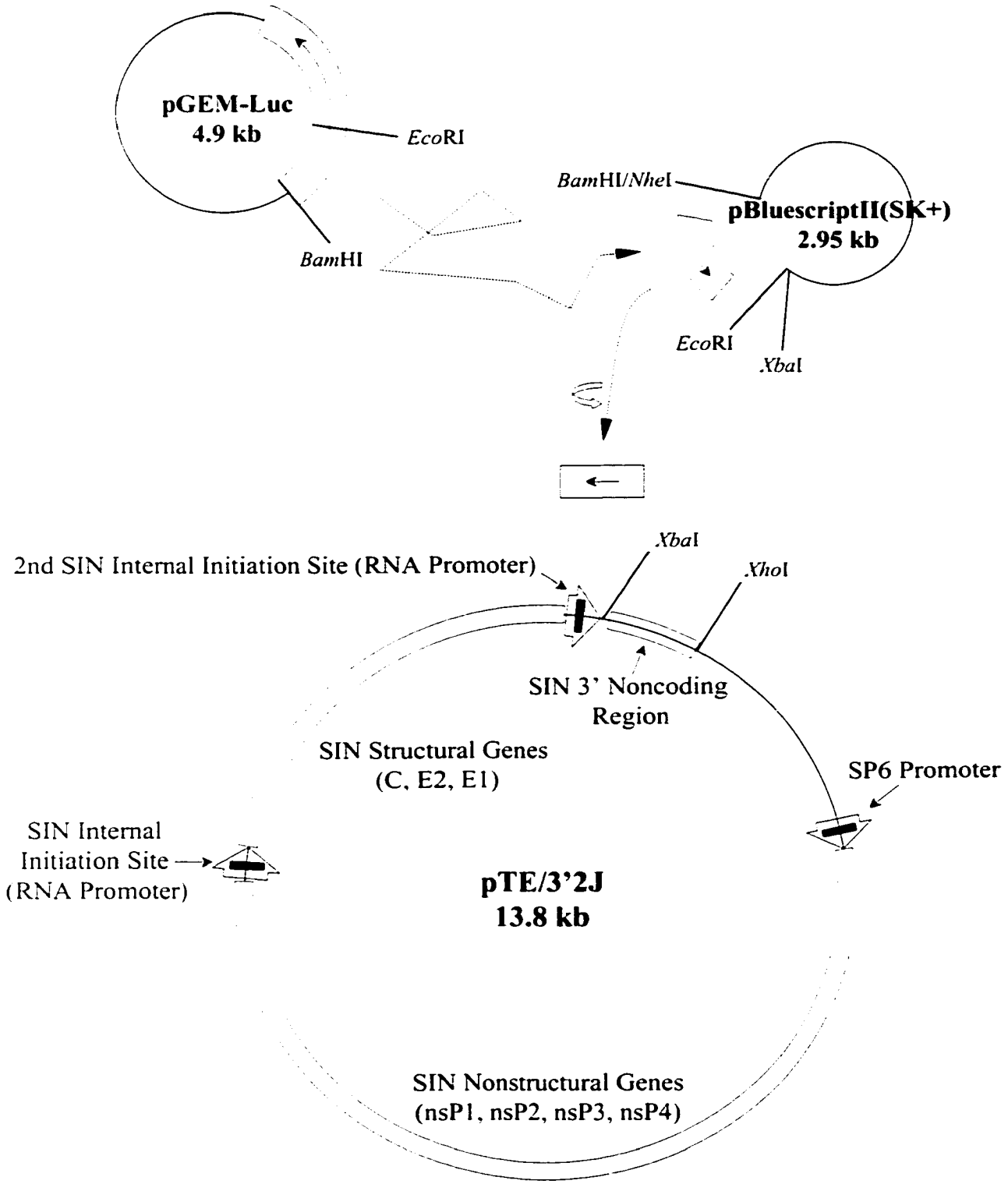



**Figure 5-2. Construction of pTE/3'2J-αLuc.**

***Virus Infection and RNA Analysis***

C6/36, MTL25, H/L-1A7, or G/L-5B7 cells were seeded into 25-$cm^2$ cell culture flasks and allowed to grow to ~95% confluence in L-15/5% FBS medium. Medium was removed and cells infected with $3.8 \times 10^6$ TE/3'2J or TE/3'2J-αLuc virus particles (MOI of 2) suspended in 2.0 ml of L-15 medium. Infection was allowed proceed for 1 hour at room temperature. 4 ml of L-15/2% FBS were added to each flask to raise the volume and the cells were incubated at 28°C. Total RNA was harvested four days post-infection using RNAWiz (Ambion, Austin, TX) and quantified on a Beckman DU-640 (Beckman). 10 μg of RNA from each sample was fractionated on a formaldehyde denaturing gel, passively transferred to a nylon membrane (BrightStar; Ambion), and analyzed by northern hybridization (Sambrook *et al.*, 1989). An oligonucleotide probe (5'-gctggtcggatcattggggcg-3') complementary to the dsSIN 3' non-coding region was non-radioactively labeled with Psoralen-Biotin (Ambion) and used to detect the various viral RNA species resulting from dsSIN infection. Two RNA probes were used to distinguish between sense and anti-sense luciferase messages. The plasmid pBS[*Null-Luc*] (Chapter 3) was linearized at a *Bst*BI site in the region of luciferase cloned into pTE/3'2J-αLuc and utilized as template for *in vitro* RNA synthesis (MAXIscript, Ambion) from a bacteriophage T7 promoter (anti-sense probe) or T3 promoter (sense probe). The RNA probes were quantified and non-radioactively labeled with Psoralen-Biotin. Hybridization of the DNA probe was performed at 42°C and the RNA probes at 65°C for 16 hrs in modified Church's buffer (50 mM $Na_2HPO_4$, 2 mM $H_3PO_4$, and 7% sodium dodecyl sulfate; Church and Gilbert, 1984). The bound probe was visualized using a

streptavidin-alkaline phosphatase conjugate and the chemiluminescent reagent CDP-Star (BrightStar BioDetect, Ambion) and exposed to X-ray film for 1 to 5 minutes.

***Virus Infection and Luciferase Interference***

MTL25, H/L-1A7, or G/L-5B7 cells were seeded into 24-well plates and allowed to grow to ~95% confluence in L-15/5% FBS medium. Medium was removed and cells infected with $7.6 \times 10^6$ TE/3'2J, TE/3'2J-αDen (expressing an ~550 bp RNA from the second subgenomic promoter complementary to the dengue-2 prM gene; generous gift of Z. Adelman), or TE/3'2J-αLuc virus particles (MOI of 10) suspended in 250 µl of L-15 medium. Infection was allowed to proceed for 1 hour at room temperature. 2 ml of L-15/2% FBS were added to each well and the cells were then incubated at 28°C. Infection was confirmed after two days by immunofluorescence assay (IFA) of cells fixed on glass coverslips in -20°C acetone for 5 minutes. Viral proteins were detected using the primary monoclonal antibody 30.11 (anti-SIN E1;Chanas *et al.*, 1982) and a secondary biotinylated sheep anti-mouse antibody (Amersham, Arlington Heights, IL). Fluorescence produced by bound fluorescein-streptavidin (Amersham) was viewed with an Olympus BH-2 epifluorescence microscope. After confirming ~100% infection, cells were harvested in triplicate every two days for 10 days and assayed for firefly luciferase activity following the instructions for the Enhanced Luciferase Assay kit (Promega) in 250 µl of 1× Passive Cell Lysis buffer. Lysates were centrifuged to remove debris, and 10 µl was assayed for firefly luciferase activity using a TD 20/20 Luminometer (Turner Designs) set for a 2 second delay and 10 second integrated measurement. Each sample was assayed for total protein using the Coomassie Plus reagent (Pierce) and absorbance measured at wavelength (λ) of 600 nm.

## RESULTS

### *Virus Infection and RNA Analysis*

RNA was extracted 4 days post-infection from uninfected cells and cells infected with TE/3'2J or TE/3'2J-αLuc viruses; 80 to 188 μg of total RNA was isolated from each 25-$cm^2$ flask. Northern analysis of these RNAs with an oligonucleotide probe specific for the 3' noncoding region of Sindbis virus showed three intracellular mRNA species from cells infected with dsSIN vectors (Figure 5-3). In cells infected with TE/3'2J, the genomic mRNA was detected at approximately 12 kb, the first subgenomic RNA was detected at 4.5 kb, and the second subgenomic RNA was detected at 0.7 kb. The RNA species were approximately 0.6 kb larger in cells infected with TE/3'2J-αLuc, corresponding to the size of the inserted luciferase sequence.

The DNA probe was stripped from the membrane and the RNAs hybridized with RNA probes specific for sense and anti-sense luciferase messages (Figure 5-4). The sense probe detected a single RNA in uninfected H/L-1A7, MTL25, and G/L-5B7 cells corresponding to the size of the luciferase mRNA (3.4, 2.3, and 3.0, respectively), while no signal was seen from uninfected C6/36 cells. These mRNAs were also seen in cells infected with TE/3'2J or TE/3'2J-αLuc viruses. The anti-sense luciferase RNA probe did not produce any signal in uninfected or TE/3'2J infected cells, but did produce bands in TE/3'2J-αLuc infected cells that matched those produced using the SIN specific DNA probe.

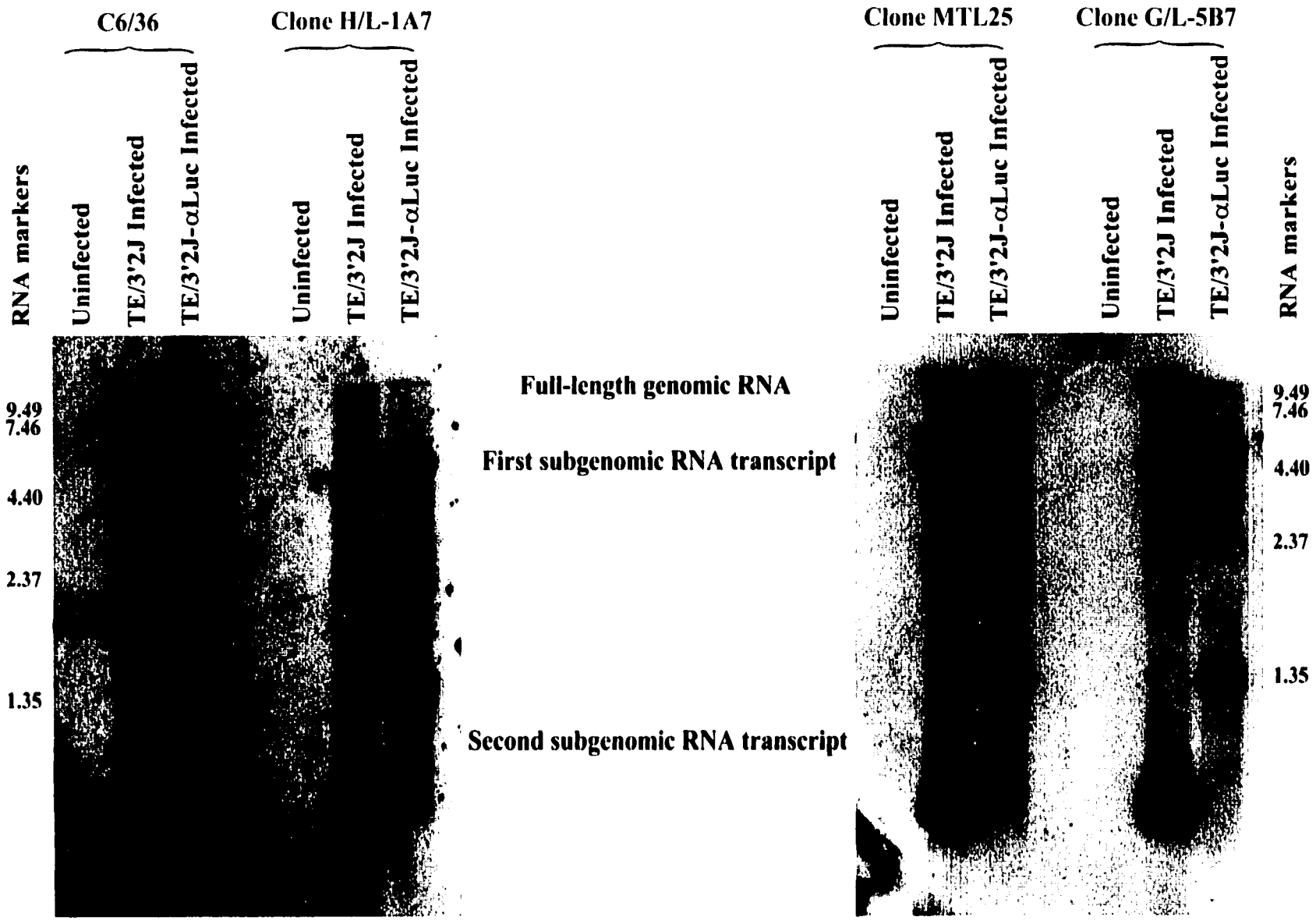



**Figure 5-3. Northern blot analysis using a Sindbis specific oligonucleotide probe.**

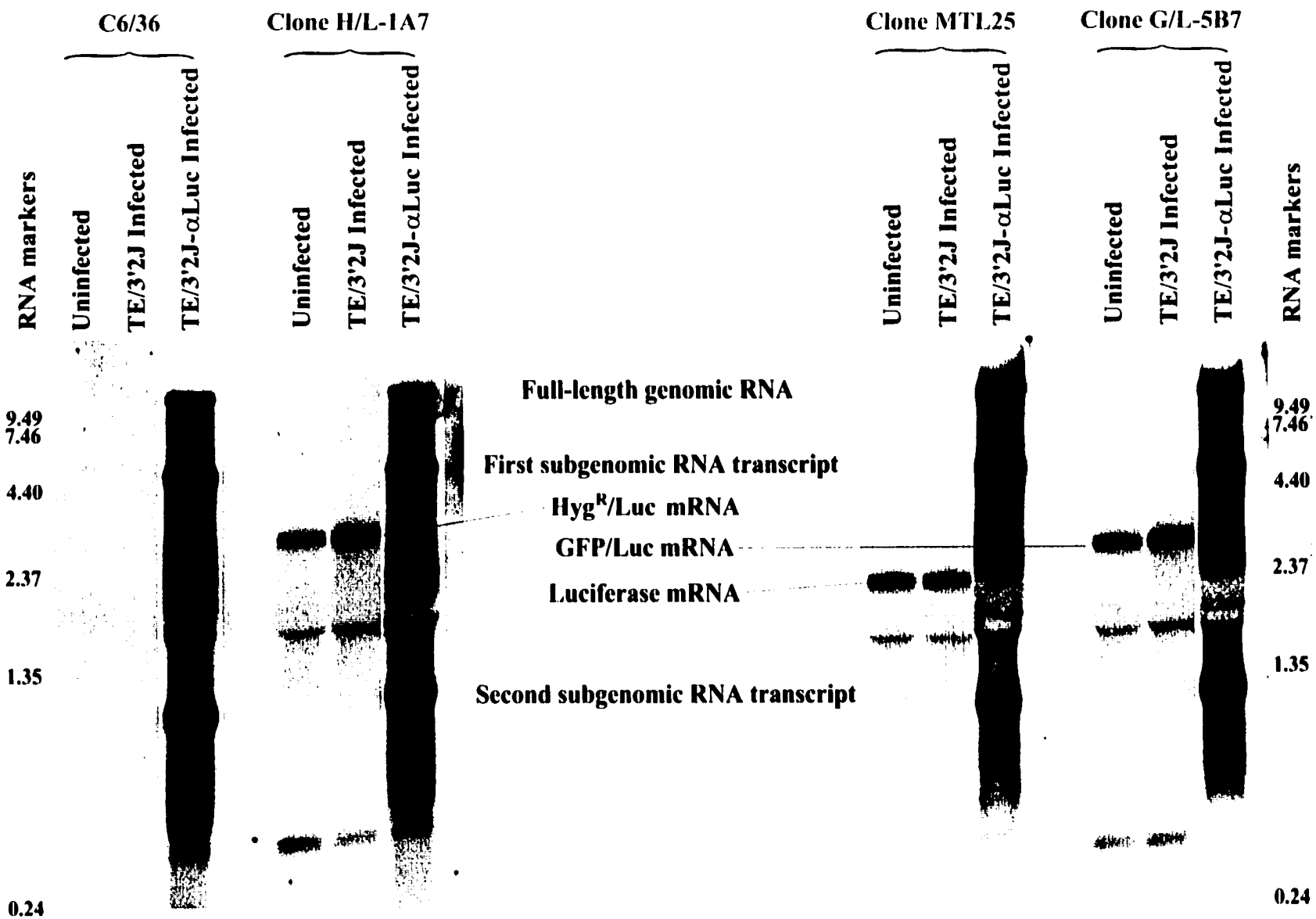



**Figure 5-4. Northern blot analysis using luciferase sense and antisense specific RNA probes.**

### *Virus Infection and Luciferase Interference*

MTL25, G/L-5B7, and H/L-1A7 cells were uninfected or infected (MOI of 10) with TE/3'2J, TE/3'2J-αDen, or TE/3'2J-αLuc virus. IFA was used to ensure that approximately ~100% of the dsSIN infected cells were positive for virus antigen 2 days after infection. Firefly luciferase activity was assayed every 2 days for 10 days post-infection and normalized to total protein within each sample. Luciferase activity from uninfected, TE/3'2J and TE/3'2J-αDen infected cells was relatively equivalent and consistent over time. In contrast, the luciferase activity from TE/3'2J-αLuc infected cells was significantly less. TE/3'2J-αLuc infected MTL25, G/L-5B7, and H/L-1A7 cells produced only 3.07, 3.19, and 8.17% of the activity, respectively, on day 10 compared to cells infected with the other dsSIN viruses (Figure 5-5). The rate at which the luciferase activity decayed corresponded to the half-life ($t_{1/2}$) of the luciferase protein (Chapter 4). Decreases in activity paralleled predicted values based on the $t_{1/2}$ of the luciferase and luciferase fusion proteins, although in all cases the observed value was slightly higher than the calculated value. For example, luciferase activity from TE/3'2J-αLuc infected MTL25 cells dropped 85.7% from 48- to 96-hours post-infection. This is very close to the 88.3% drop that would be expected given the $t_{1/2}$ of 15.5 hours. G/L-5B7 and H/L-1A7 demonstrated reduced luciferase activity in accordance with the half-life of the respective luciferase fusion protein each line produced. Overall, expression of the antisense luciferase fragment from the second subgenomic internal promoter in the dsSIN vector resulted in a 96, 97, and 92% decrease in firefly luciferase activity in MTL25, G/L-5B7, and H/L-1A7 cells, respectively. Although decreased, luciferase levels in the

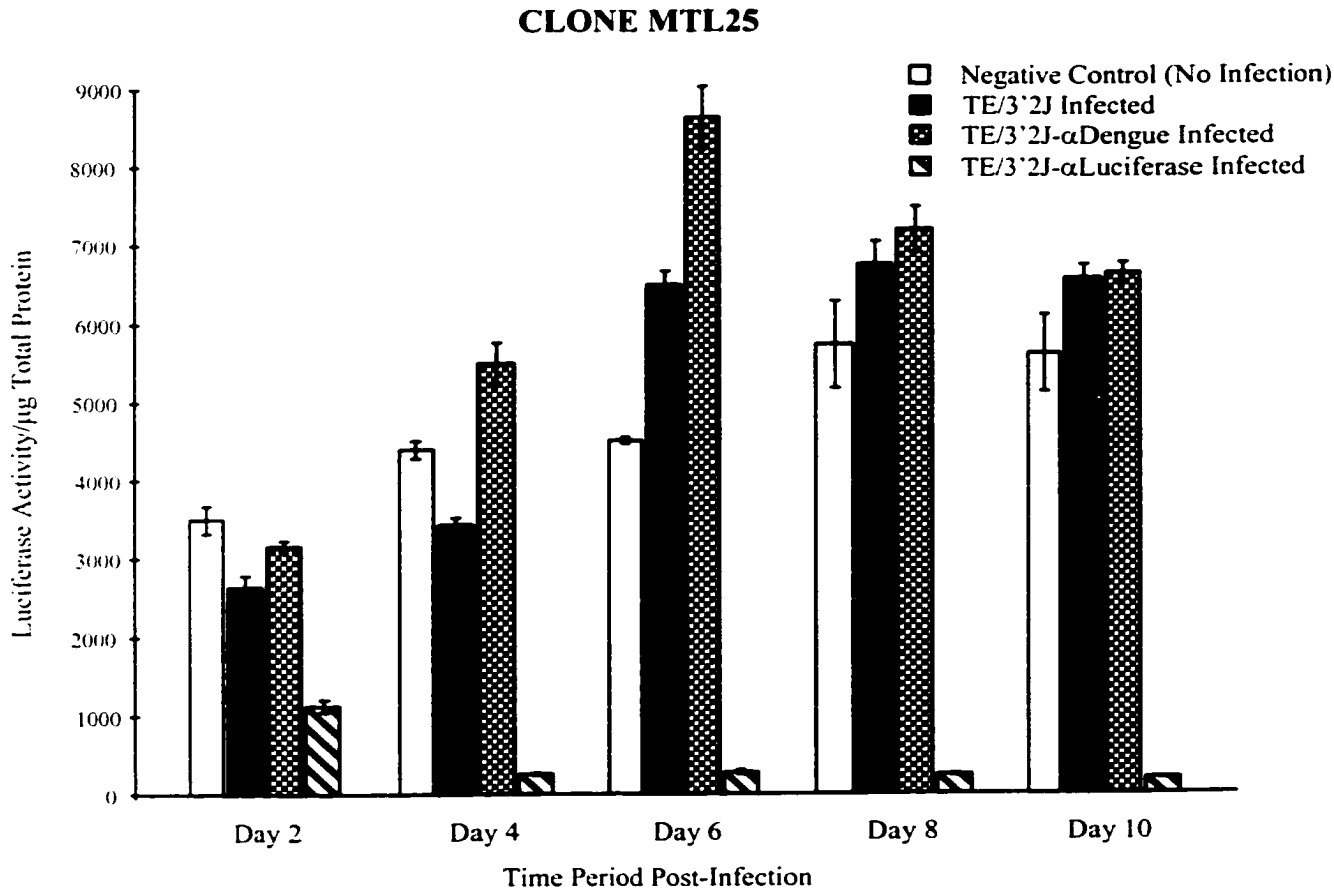
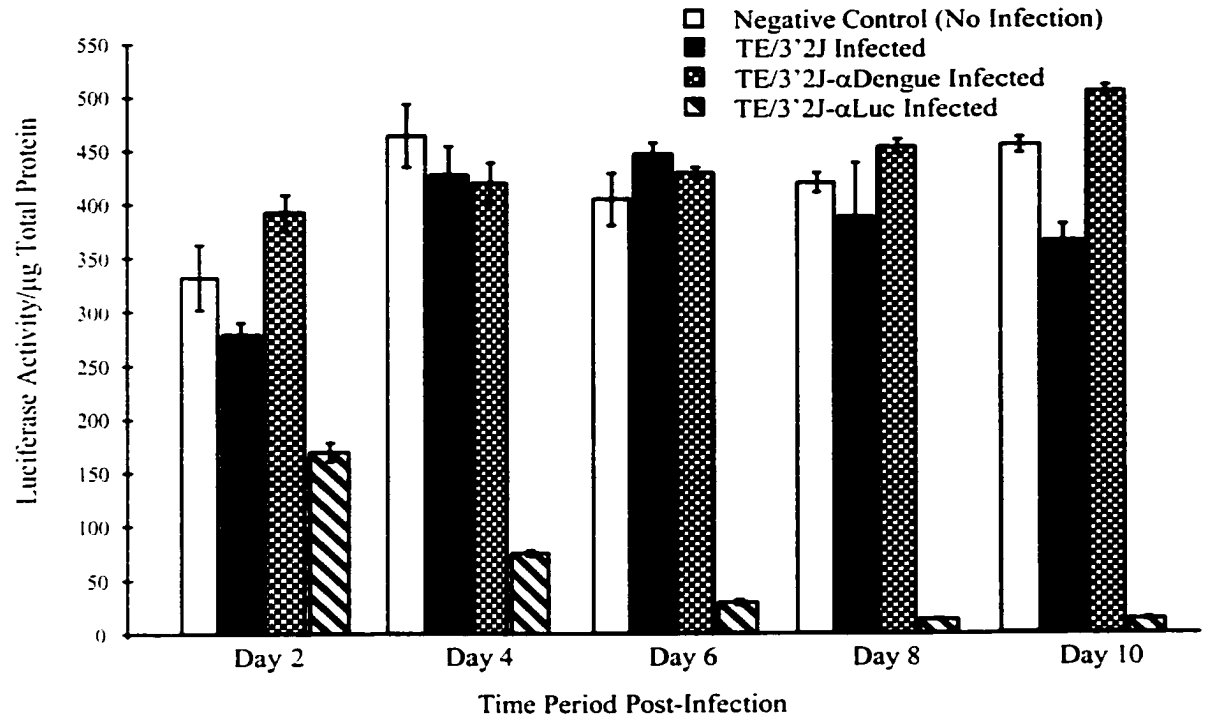



**Figure 5-5. Luciferase activity in cells infected with TE/3'2J, TE/3'2J-αDengue, or TE/3'2J-αLuc.** Each column represents the average of three wells and bars indicate standard error.



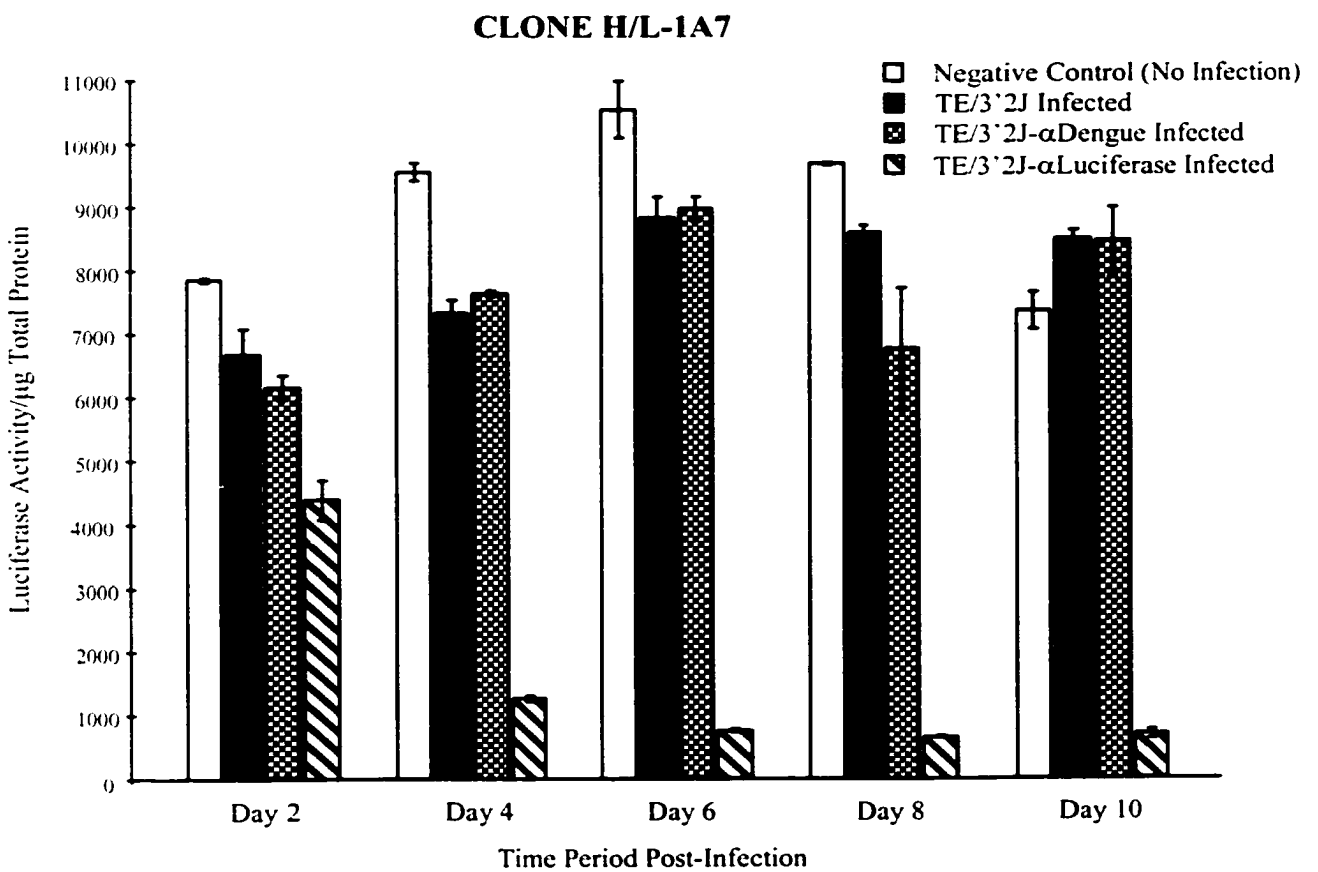




**Figure 5-5 (Continued)**

TE/3'2J-αLuc infected cells remained significantly above the background level of the parental C6/36 cell line.

## DISCUSSION

Antisense-mediated intracellular immunity as a long term control strategy in arthropod-borne disease vectors will be dependent upon the ability of cellular and viral RNA transcripts to interact. To address this, a dsSIN virus vector expressing the 5' 595 bp of the firefly luciferase gene from the second subgenomic promoter was constructed and used to infect transformed *Ae. albopictus* cell lines constitutively expressing luciferase activity. The resulting 92-97% decrease in luciferase activity compared to cells infected with dsSIN vectors without the antisense luciferase sequence clearly demonstrates that cellular and viral RNA transcripts can hybridize and result in inhibition of protein synthesis in mosquito cells. This corresponds well with a recent publication in which transgenic *Ae. aegypti* expressing luciferase were infected with the same TE/3'2J-αLuc and a 90% reduction in luciferase activity was observed (Johnson *et al.*, 1999).

The mechanisms of antisense RNA mediated interference can be observed at the level of mRNA splicing, transport from the nucleus, and/or translation (Denhardt, 1992). Because the RNA genome of dsSIN vectors replicates in the cytoplasm of infected cells, interference is mediated exclusively at the level of translation. The mechanism of this interference is hypothesized to result from the expressed antisense binding to the target transcript and preventing ribosome attachment and/or elongation and facilitating degradation of the RNA duplex. The overall decrease in luciferase activity was similar in all three cell lines examined in this study, suggesting that the mechanism of interference

is the same. The reduction in luciferase activity in TE/3'2J-αLuc infected MTL25 cells could be attributed to blocking ribosome attachment (Nelson *et al.*, 1993; Melton, 1985; Helene and Toulme, 1990) since binding of the antisense occurs at the 5' end of the gene, but would not be the case in G/L-5B7 and H/L-1A7 cells since binding is >700 and >1100 bp, respectively, downstream of the translational start site. The other mechanisms of interference are not mutually exclusive and both have been attributed to effective antisense RNA applications (Colman, 1990; van der Krol *et al.*, 1988; Kumar and Carmichael, 1998).

Antisense RNA inhibition of protein synthesis is notoriously unpredictable. While >200-fold reductions have been reported (Knecht and Loomis, 1987), most reductions are more modest (Pestka, 1992). In this study, expression of the antisense luciferase transcript did not completely ablate protein synthesis. Luciferase activity in TE/3'2J-αLuc infected cells ranged from 100 to 3200-fold higher than background levels in untransformed C6/36 cells (Figure 5-5). Once reached, these levels of luciferase activity were maintained until the experiment was terminated. There are two possibilities to explain this residual luciferase activity. First, newly synthesized mRNA could be associating with ribosomes before antisense transcripts can hybridize. Second, the binding of the antisense transcript to the target transcript is in a state of equilibrium and when the two molecules dissociate, ribosomes positioned on the mRNA finish translating the polypeptide chain. The consistent degree of interference among the three cell lines suggests which scenario is likely occurring. New mRNA emerging from the nucleus can be thought of as being in a race to bind either ribosomes or antisense transcripts; the overall number of target mRNA would not affect this race. The overall number of target

mRNA will, however, influence the equilibrium. Changing the number of luciferase mRNA molecules would shift the proportion bound to the antisense transcript. This would be observed as a difference in the percent knockdown in luciferase activity. The AcMNPV $^{hr5}$IE1 promoter directs the expression of 200- to 500-fold more RNA transcripts than the *Drosophila* Mtn promoter (Chapter 3), yet the percent knockdown of luciferase activity from cells expressing luciferase from the two promoters was similar. This suggests that the residual luciferase activity seen in TE/3'2J-αLuc infected cells was the result of translation before the antisense transcript was able to hybridize and interfere with protein synthesis.

While this work clearly establishes the ability of cellular and viral RNA transcripts to associate and inhibit protein synthesis in mosquito cells, using antisense strategies to interfere with vector-borne disease transmission will be more complex. Successful intracellular immunization will not be measured by the percent knockdown of expression of the target gene, but what influence this percent knockdown has on the replication of the pathogen. If there is considerably more mRNA and protein in the cell than is required, then even a substantial reduction in the expression of the targeted gene may not result in interference. If, on the other hand, a small change in the amount of the gene product can affect pathogen replication, then antisense-mediated interference will be successful. Therefore, identifying areas of the pathogen genome that are especially susceptible to antisense-mediated interference is extremely important and will increase the likelihood of successfully interfering with pathogen replication.

Antisense RNA has the potential to be a powerful tool in strategies designed to control vector-borne disease by disrupting pathogen gene expression and replication in

mosquitoes. Many examples of antisense-mediated intracellular immunization exist in plant and animal systems, but without a technique for the stable insertion of DNA into the mosquito genome, analogous experiments have not been performed in mosquitoes. The finding that cellular and viral RNA molecules can interact confirms that expression from a DNA-based systems will be able to target infecting pathogens. Applying this information to arthropod-borne disease control strategies will aid in the production of long-term intracellular immunity and the possibility of pathogen resistant mosquitoes.

# - Chapter 6 -

# SUMMARY AND DISCUSSION

## SUMMARY

The effectiveness of traditional methods used to control vector mosquitoes and the diseases they transmit has declined in recent years. With the prospect of long-term control discouraging the use of traditional methods, researchers are turning to advances in molecular biology and genetics to open up new avenues for controlling these diseases. Novel strategies have emerged with the goal of disrupting pathogen transmission at the molecular level within mosquitoes. Preliminary work has shown that the natural cycle of an arbovirus can be interfered with in its invertebrate vector. These findings have spurred a renewed emphasis on the development of efficient, stable gene transfer techniques in arthropod vectors. This dissertation describes efforts to utilize VSV G-pseudotyped retroviral vectors in mosquito transgenesis and also examines aspects critical to their successful use.

Transgenesis has the potential to reveal a wealth of information on the fundamental molecular biology of mosquito vector species and vector-pathogen interactions. The demonstration that VSV G-pseudotyped retroviral vectors can infect and mediate stable gene expression in mosquito cell lines derived from several different mosquito species suggests that this system has the potential to be successfully used in the generation of transgenic mosquitoes. The failure to actually produce a transgenic mosquito exposes obstacles that must still be overcome. Retroviral vector design, delivery by microinjection, and detection of the integrated provirus are all elements that need refinement before this system can be exploited as a transgenesis technique in mosquitoes. The analysis of promoter function and construction of multi-reporter fusion proteins address some of these points and will help in the construction of vectors with an

optimal chance to produce a transgenic mosquito. This work, plus the finding that RNA transcripts expressed from a cell's nucleus and an infecting virus can associate and produce interference in mosquito cells (Powers *et al.*, 1996; Powers *et al.*, 1994; Gaines *et al.*, 1996; Olson *et al.*, 1996; Higgs *et al.*, 1998), are important to any method predicated upon expression of antipathogen effector molecules from stably inserted DNA.

## DISCUSSION

VSV G-pseudotyped retroviral vectors are only one of a number of systems with the potential for heritable interference with arthropod-borne disease transmission, including transposable elements (O'Brochta and Atkinson, 1996), bacterial symbionts (Beard *et al.*, 1998), double-strand RNA-induced gene silencing (Montgomery and Fire, 1998; Montgomery *et al.*, 1998; Kennerdell and Carthew, 1998; Sharp, 1999), small fragment homologous replacement (Goncz *et al.*, 1998), and chimeraplasty (Kren *et al.*, 1997; Bandyopadhyay *et al.*, 1999; Zhu *et al.*, 1999). Recently, the generation of transgenic mosquito lines using the transposable elements *Hermes* and *mariner* has invigorated the field of vector-borne disease research. Given such success, the question arises "Should retroviral vectors continue to be pursued as tools for the stable transformation of mosquitoes?" Each system has advantages and disadvantages. Undoubtedly, transposable elements are technically easier to employ than retroviral vectors because they are delivered as DNA. Once constructed, the plasmid DNA can be utilized directly. Retroviral vectors require the additional step of virus production; the generation of high titer virus entails the stable transformation of the packaging cell line.

Microinjection delivers ~1 nl per egg, which translates to ~1000 infectious units (IU) from a $10^9$/ml stock of VSV G-pseudotyped retroviral vector. Injecting the same volume of a 1 μg/μl stock of DNA supplies ~$1.5 \times 10^8$ plasmid molecules carrying transposable element. This provides >$3.0 \times 10^5$-fold more opportunities for integration into the target germ cell population. The successful use of *P*-elements, as well as *hobo* (Blackman *et al.*, 1989), *Hermes* (O'Brochta *et al.*, 1996), *mariner* (Garza *et al.*, 1991; Lidholm *et al.*, 1993), *Minos* (Loukeris *et al.*, 1995), and *piggyBac* (Handler and Harrell, 1999),to stably insert DNA into *Drosophila melanogaster* has demonstrated the utility of transposable elements in transgenesis as well as identified potential problems. *D. melanogaster* strains lacking endogenous transposable elements were critical in the development of *P*-elements as a gene-transfer system because constitutive expression of the transposase can result in the inhibition of integration and compromise transgene stability. The presence of endogenous transposable elements may prove problematic in mosquito transgenesis because of cross-mobilization of the transposition vector. Cross-mobilization occurs when transposases interact between elements. This can occur if the same or related transposons are present, and has even been observed between different transposon families (O'Brochta and Atkinson, 1996). In addition, a stably inserted transposon vector in a host devoid of endogenous elements can still undergo movement and duplication if transposase is subsequently added. Retroviral vectors, on the other hand, have no specific mechanisms for excision, movement, or duplication once they have integrated into the host chromosome. For these reasons, transposable element-mediated transformation may be the most suitable technique for use in a laboratory setting and retroviral vector-mediated transformation for use in a field setting. In the laboratory,

specialized mosquito strains devoid of endogenous transposable elements can be used as recipients of the transgene to control for problems associated with transposase activity. In a field environment, the target population will be relatively heterogeneic and can be expected to possess numerous endogenous transposable elements (Robertson and Lampe, 1995). The stability of a retroviral provirus would prevent loss of the transgene and thus the desired phenotype. Clearly, transgenesis using transposable elements will be a powerful tool in vector-borne disease research, but the realization that there will be limitations requires that alternate systems be developed. VSV G-pseudotyped retroviral vectors also possess potential as tools for the stable transformation of mosquito vectors.

Advances in the design and production of pseudotyped retroviral vectors may increase the prospects of using these systems in mosquito transgenesis. New packaging cell lines have been constructed that are stably transformed with the *vsv g* gene. Membrane fusion and syncytium formation do not kill these cells because VSV G expression is tightly controlled by a tetracycline-regulated promoter derived from the *Escherichia coli* Tn10 transposon (Gossen and Bujard, 1992). After the stable insertion of the retroviral genome and release of transcriptional control, high numbers of VSV G-pseudotyped particles are produced as 100% of the cells make infectious virus. Lentiviral vectors are another development that could find application in mosquito transgenesis. Delivery of the retrovirus vector into the egg by microinjection was designed to target the growing embryo when all the cells would be dividing and susceptible to infection. Lentiviruses have the unique ability among retroviruses to integrate into the genome of nondividing cells, and vectors derived from a lentiviral genome share this feature (Naldini *et al.*, 1996). These vectors have been pseudotyped with the VSV G envelope

and concentrated by ultracentrifugation to titers >1 × $10^9$ IU/ml (Kafri *et al.*, 1999). Integration into terminally differentiated cells raises the possibility of infecting the reproductive organs after they have developed. Considerably more volume can be injected into an adult mosquito versus an egg, so more virus particles can be delivered. VSV G-pseudotyped lentiviral vectors would offer another approach to mosquito transgenesis.

Mosquito transgenesis will be a significant aid in the fight against vector-borne disease. Despite this promise, the mission of controlling vector-borne disease transmission using pathogen-resistant mosquitoes will be problematic. While the scientific barriers appear to be falling, the release of a genetically engineered pest insect will undoubtedly encounter political barriers. Overcoming these concerns will be as challenging as the laboratory work itself. Continued research now will ensure that the information necessary to control vector-borne disease will be in place once these issues are resolved.

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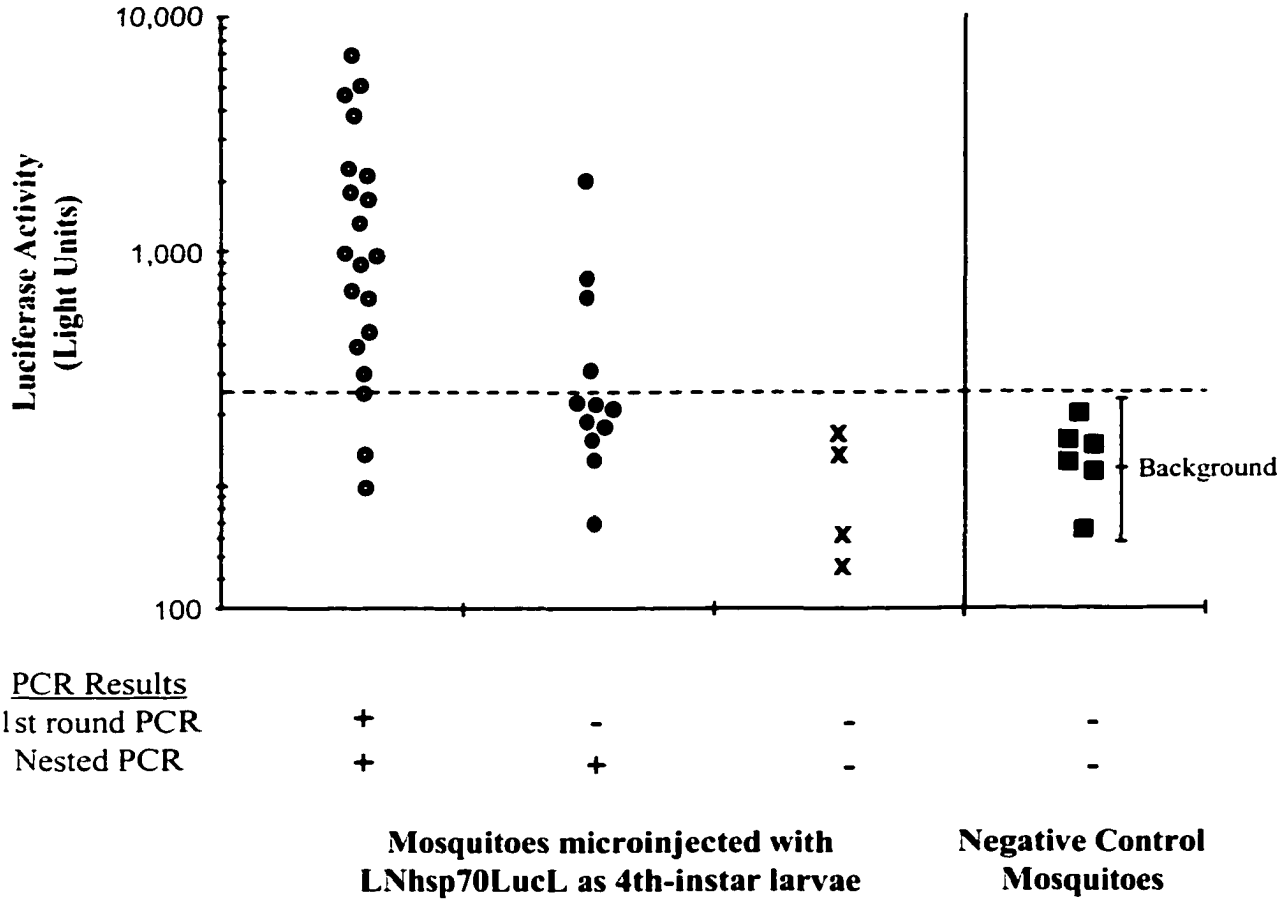
## APPENDIX




Key: ○, first-round PCR$^+$ and nested PCR$^-$ microinjected mosquitoes
●, first-round PCR$^-$ and nested PCR$^+$ microinjected mosquitoes
x, first-round PCR$^-$ and nested PCR$^-$ microinjected mosquitoes
■, uninjected negative control mosquitoes

**Luciferase expression in adult *Anopheles gambiae* mosquitoes.**
(modified from Jordan *et al.*, 1998)