DISSERTATION

DEVELOPMENT OF *AEDES AEGYPTI* DENSOVIRUS AS A VECTOR FOR RNA INTERFERENCE

Submitted by
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Graduate Degree Program in Cell and Molecular Biology

In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
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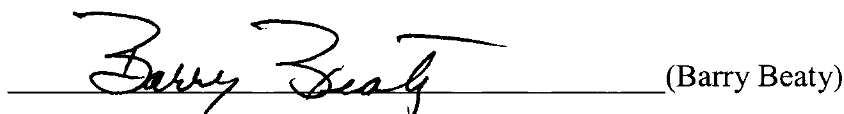
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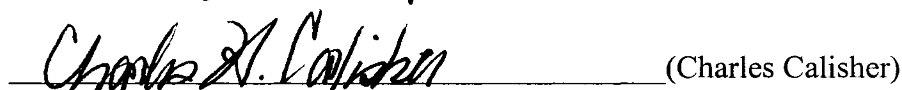
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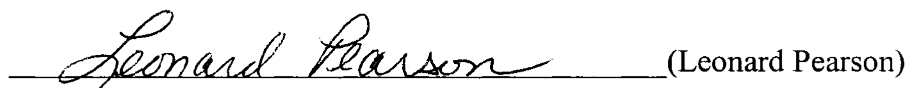
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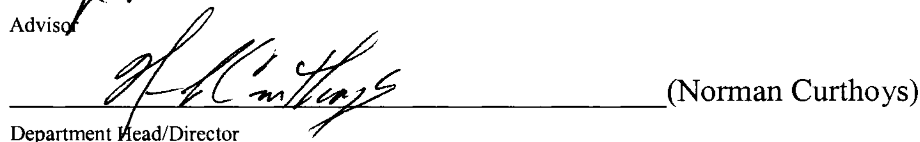
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ABSTRACT OF DISSERTATION

DEVELOPMENT OF *AEDES AEGYPTI* DENSOVIRUS AS A VECTOR FOR RNA INTERFERENCE

Aedes aegypti mosquitoes are the principal vector species for diseases of significant world health importance including yellow fever and dengue. *Aedes aegypti* densovirus (*Ae*DNV) is a parvovirus that infects and kills *Aedes aegypti* mosquitoes. *Ae*DNV has demonstrated potential as a biological control agent and as a vector for the expression of foreign genes in *Aedes aegypti* mosquitoes. The hypothesis for these studies was that *Ae*DNV could be developed as a vector for RNA interference (RNAi) in mosquitoes.

cDNA sequence analysis was used to determine that polyadenylation of *Ae*DNV transcripts occurs in the right-end UTR of the virus genome 13 nucleotides downstream of a canonical AATAAA polyadenylation hexamer. Mutational analyses of the *Ae*DNV right-end UTR were used to determine the sequence elements required for efficient gene expression from viral promoters. Efficient gene expression requires the presence of a 21 nucleotide upstream sequence element (USE) that is predicted to form a stem-loop secondary structure in the RNA transcript. Deletion analysis also identified non-essential sequences downstream of the poly(A) site suitable for replacement with an RNAi expression cassette.

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Polymerase III (Pol III) promoters capable of mediating RNAi in mosquito cells were cloned from the *Anopheles gambiae* and *Aedes aegypti* genomes. The Pol III promoters were tested for the ability to express short-hairpin RNAs (shRNA) targeted to firefly luciferase and to induce RNAi-mediated knockdown of a co-transfected luciferase reporter gene vector in AG-55 *Anopheles gambiae* and ATC-10 *Aedes aegypti* cells. Promoters capable of silencing expression of the co-transfected luciferase plasmid by up to 95% in AG-55 cells and up to 75% in ATC-10 cells were identified. RNase protection experiments allowed detection of the 19 nt luciferase short-interfering RNA (siRNA) in transfected cells.

The infectious clone of *Ae*DNV (pUCA) was modified to include a Pol III promoter-based RNAi expression cassette in the right-end UTR of the virus genome. The modified genomes were packaged into *Ae*DNV virions that were capable of infecting and killing *Aedes aegypti* mosquitoes. Sequence analysis of viral DNA harvested from adult mosquitoes infected as larvae indicated that the inserted RNAi cassettes are stable throughout the duration of the mosquitoes' life span.

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Most of all, I'd like to thank my family and friends for their support and encouragement.

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DEDICATION

I dedicate this dissertation to my parents Barry and Linda Konet. Words cannot express the gratitude I have for the investments you have always made in my continuing education.

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Chapter 1: Literature Review

Introduction

Significance of Mosquito-Borne Diseases

Mosquito-borne diseases cause significant morbidity and mortality in humans and animals worldwide. Adult female mosquitoes feed on animal blood and are potent vectors for the transmission of pathogens. The geographical distribution of a particular mosquito-borne disease parallels the distribution of the mosquito vector or vectors. Mosquito-borne pathogens include parasites, such as plasmodium and filarial worms, and arboviruses, including West Nile virus, yellow fever, the dengue viruses, and more than 100 other viruses, including members of the families *Bunyaviridae*, *Togaviridae*, *Rhabdoviridae*, *Flaviviridae*, and *Reoviridae* (Nasci and Miller, 1996).

Plasmodium ssp. parasites, which can cause malaria, are introduced to human hosts by the bite of an infected *Anopheles* ssp. mosquito. Malaria causes more than 1 million deaths annually, mostly in African children, although the disease is also prevalent in South America and areas of Asia (Guerra *et al.*, 2006). An estimated 350 to 500 million people are infected with malaria each year, and approximately 40% of the world's population is at risk for this disease. The economic impact of malaria in Africa is extremely severe and is believed to exceed $12 billion annually (Greenwood *et al.*, 2005).

Arboviruses are animal viruses transmitted by hematophagous arthropod vectors (Reeves, 2001). *Aedes aegypti* mosquitoes are the principal vector species for yellow

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fever virus and the dengue viruses. More than 2.5 billion people are at risk for dengue infection, with 50 million infections occurring annually and over 100 countries involved (Gubler, 2002b; Calisher, 2005). At least 500,000 people are hospitalized annually for dengue hemorrhagic fever, a more severe form of the disease, with fatality rates exceeding 20% in the absence of appropriate treatment. There are four serotypes of dengue virus and no cross-protection occurs between them. A recent increase in hyperendemicity (co-circulation of multiple dengue serotypes) has correlated with increased frequencies of dengue hemorrhagic fever and the serious dengue shock syndrome that may follow, presumably brought about by immune enhancement (Halstead, 2003). Development of an effective vaccine for dengue has been difficult because a vaccine must protect against all four serotypes. Chimeric flavivirus vaccines have shown some promise (Lai and Monath, 2003; Johnson *et al.*, 2004). Although an effective vaccine for yellow fever virus (YFV) exists, 200,000 cases of yellow fever, resulting in 30,000 deaths are estimated to occur annually (Anonymous, 2001). The rapid spread of West Nile virus, a flavivirus transmitted primarily by *Culex* mosquitoes, across the United States has demonstrated even the developed world's lack of preparedness for combating mosquito-borne disease (Lanciotti and Roehrig, 1999).

Many factors have contributed to the emergence and resurgence of mosquito-borne diseases. The lack of effective vaccines for malaria and dengue, pesticide resistance in mosquitoes, extreme poverty in many disease-endemic countries coupled with the erosion of public health infrastructures and lack of vector control programs have lead to the current, bleak situation (Beaty, 2005). New strategies for the control of mosquito-borne diseases are badly needed.

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<u>Control of Mosquito-Borne Diseases</u>

Mosquito-Pathogen Interactions

The life cycle of a mosquito-borne pathogen is intimately tied to the life cycle of the mosquito responsible for transmission. Until recently, the events occurring within the vector between pathogen infection and transmission to a new vertebrate host were largely unknown. This view of the "vector as a black box" is certainly outdated (Black *et al.*, 2002). In addition to pathogen transmission, mosquitoes provide a suitable environment for pathogen development, propagation, evolution and survival outside of a vertebrate host. The life cycle of malarial parasites provides an excellent example of mosquito-pathogen interactions. Male and female *Plasmodium* gametocytes are ingested when a female *Anopheles* mosquito takes a bloodmeal and develop into ookinetes in the mosquito's midgut. Ookinetes invade the midgut wall where they develop into oocycsts, which rupture and release thousands of sporozoites that invade the mosquito salivary gland allowing for transmission of the parasite to a new human host. The mosquito is absolutely required for malaria transmission because these stages of the parasite's life cycle do not occur in human hosts. Similarly, mosquito midgut epithelial cells provide an ideal environment for arbovirus replication. Generally, arbovirus infection is not believed to cause detrimental effects within the vector, although pathogenic lesions and reduced fitness have been documented in mosquitoes infected with eastern equine encephalitis virus and Sindbis virus (Scott and Lorenz, 1998; Bowers *et al.*, 2003). Arboviruses can be transmitted to vertebrate hosts directly, mechanically or biologically. Direct transmission of arboviruses to vertebrates via intranasal or venereal routes is well documented (Kuno, 2001). For example, vertebrates exposed to high concentrations of

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Rift Valley fever virus can become infected via aerosol, and insectivorous animals such as bats can become infected with YFV by eating infected prey (Kuno, 2001; Arishi, *et al.*, 2006). Mechanical transmission occurs via contaminated mouthparts, requires a high titer, and does not involve replication of the pathogen within the vector (Hoch *et al.*, 1985). Biological transmission involves amplification of the pathogen within the vector and requires an intimate relationship between pathogen, vector and vertebrate host.

Many factors influence the ability of a given pathogen to be transmitted biologically. Vector-specific factors such as host preference and host seeking contain genetic, behavioral and ecological components. For example, mosquito attraction and host seeking have been shown to have a molecular genetic basis (Hallem *et al.*, 2004). Vectors also enhance transmission of many pathogens by injecting a number of immunomodulatory substances with saliva (Rossignol *et al.*, 1985; Titus *et al.*, 1998). While vector-enhanced transmission was first demonstrated in sandflies that transmit leishmania, mosquito saliva has been shown to enhance vesicular stomatits virus infection and dengue virus replication in vertebrate hosts (Limesand *et al.*, 2000; Zhu *et al.*, 2002). Biologically transmitted pathogens must also undergo an extrinsic incubation period; the time from ingestion of an infectious blood meal to when the vector is capable of transmitting the pathogen to a new vertebrate host.

Effective pathogen transmission involves many complicated interactions between the pathogen and the mosquito following ingestion of an infected bloodmeal. Pathogens must escape from the peritrophic matrix, establish an infection in mosquito midgut epithelial cells (Jacobs-Lorena and Oo, 1996). Pathogens must then escape from the basal lamina of the midgut and establish secondary infections in other organs and tissues.

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Finally, the pathogen must infect salivary gland cells and escape into the salivary gland lumen for transmission to a new vertebrate host. Barriers to transmission can be encountered at any stage of the pathogen's dissemination within the mosquito (Woodring *et al.*, 1996). A midgut infection barrier refers to blockage in the earliest stages of infection such as receptor binding and virus replication. Inability of a virus to pass through the basal lamina of the midgut or produce a disseminated infection in other tissues is referred to as a midgut escape barrier. Blockage at the level of salivary gland infection or escape can also provide a barrier to transmission.

Vector competence refers to the intrinsic ability of a vector to become infected with a pathogen and transmit the pathogen to a new host. Vectorial capacity refers to the comprehensive ability of an organism to act as an efficient disease vector and includes factors such as survival, host preference, extrinsic incubation time, abundance and vector competence. Vector competence varies enormously between species and within populations of the same species. For example, *Ae. aegypti* populations from throughout the world exhibit natural variations in vector competence for flaviviruses (Gubler *et al.*, 1979). Urban *Aedes aegypti aegypti* populations are susceptible to flavivirus infection while sylvatic *Aedes aegypti formosus* are resistant to flavivirus infection (Tabachnick *et al.*, 1985). Differences in susceptibility to dengue-2 infection observed between different populations of *Ae. aegypti* mosquitoes have been shown to be due to the presence or absence of a midgut infection and/or midgut escape barrier (Bosio *et al.*, 1998; Bennett *et al.*, 2002).

The ability to artificially select susceptible and refractory mosquito strains in the laboratory provided evidence that vector competence has a genetic basis (Miller and

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Mitchell, 1991). Recent advances in molecular biology techniques have allowed researchers to more closely examine the genetic basis of vector competence. The construction of a linkage map for *Ae. aegypti* has allowed investigators to identify quantitative trait loci (QTL) that condition vector competence for dengue-2 virus (Antolin *et al.*, 1996). Crossbreeding experiments between refractory and susceptible strains of *Ae. aegypti* identified genomic regions that influence the susceptibility of *Ae. aegypti* midgut epithelial cells to infection with dengue-2 (Bosio *et al.*, 2000). The identification of QTL affecting the susceptibility of *Ae. aegypti* midgut epithelial cells to infection with dengue-2 virus and the discovery of QTL that determine whether mosquitoes with a dengue-2-infected gut are permissive to dissemination of the virus to other tissues have provided evidence for the genetic basis of midgut escape and midgut infection barriers in refractory strains of *Ae. aegypti* (Gomez-Machorro *et al.*, 2004; Bennett *et al.*, 2005a). Future experiments will be aided by the recent creation of a strain of *Aedes aegypti* possessing a definitive midgut escape barrier (Bennett *et al.*, 2005b).

Early trypsin has emerged as a candidate gene that conditions flavivirus susceptibility in *Ae. aegypti* mosquitoes. Although the early events of flavivirus infection of mosquito midgut epithelial cells are poorly understood, arboviruses belonging to the *Bunyaviridae* family require proteolytic processing of virion surface proteins for efficient infection of midgut cells (Ludwig *et al.*, 1989). In *Aedes* ssp. mosquitoes, a pool of early trypsin mRNA waits in the midgut until a bloodmeal containing free amino acids induces translation (Noriega *et al.*, 2001). Expression of early trypsin activates at least 12 other trypsins which fully digest the bloodmeal. Inhibition of trypsins in the midgut of *Ae. aegypti* mosquitoes significantly reduces the rate of dengue-2 infection and dissemination

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(Molina-Cruz *et al.*, 2005). Although early trypsin appears to be important for bloodmeal digestion and dengue virus dissemination, the first attempts to link early trypsin to QTL conditioning *Ae. aegypti* vector competence have proven unsuccessful (Gorrochotegui-Escalante *et al.*, 2005). Future experiments will continue to investigate the genetic determinants of vector competence.

Traditional Mosquito Control

Control of mosquito-borne pathogens requires effective control of the vector species. Traditional mosquito control methods focused on reducing the size of the vector populations or reducing the amount of contact between humans and vectors. Well-funded mosquito control campaigns between 1940 and 1960 based on traditional methods were highly effective. Malaria was largely eradicated from temperate regions by the mid-1950s by controlling *Anopheles* mosquitoes with widespread treatment of household interiors with DDT (Giglioli, 1956). The establishment of the *Aedes aegypti* eradication initiative by the Pan American Health Organization in 1947 was effective in controlling yellow fever, but the program's success led to its cessation, which resulted in resurgence of the vector (Gratz, 1999; Gubler, 2002a). Resurgence of *Ae. aegypti* in the Americas has led to the resurgence of yellow fever and dengue hyperendemicity (Beaty, 2000; Gubler, 2002b).

Chemical pesticides have been effective at reducing the size of mosquito populations in several instances. For example, treatment of household interiors with DDT greatly reduces the survival rate of *An. gambiae* and *Ae. aegypti* mosquitoes which prefer to rest and feed indoors. However, increasing pesticide resistance in vectors and

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widespread public fear of detrimental environmental effects due to pesticide usage are currently enormous problems. DDT was highly effective as a mosquito control agent, but toxicity in non-target species led to this compound being banned, even for small-scale indoor use (Roberts *et al.*, 1997; Attaran *et al.*, 2000). Mosquito habitat reduction has also been an effective method of vector control. Populations of *Aedes aegypti* mosquitoes have been urbanized over time and prefer to breed in man-made containers prone to collecting standing water such as tires, cans, and drainage ditches (Smolinski *et al.*, 2003). Removal of potential mosquito breeding sites not only reduces the vector population size, but also decreases the potential for human-vector contact (Mitchell, 1996). The use of chemicals such as DEET as mosquito repellents is another common method of reducing human-vector contact (Roberts and Reigart, 2004). Pyrethroid-treated bed nets are an example of a combination approach as they employ both an insecticide and a physical barrier aimed at reducing human-vector contact (Curtis *et al.*, 1990).

The shortcomings of traditional mosquito control methods highlight the need for novel approaches. The widespread public compliance required for effective mosquito control via habitat reduction is often difficult to achieve. Many mosquito species are becoming increasingly resistant to the chemical pesticides commonly used to control them (Campos and Andrade, 2003; Lima *et al.*, 2003), including DDT (Hemingway *et al.*, 2002; Sharma, 2003). New pesticides and new approaches for mosquito control are badly needed.

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Biological Control

Biological control of mosquito-borne diseases involves the use of an organism, or a component of an organism, such as a toxin, to regulate mosquito population size and/or disease transmission. The ideal biological control agent would possess a narrow host range and exert minimal effects on the surrounding environment. Animals, fungi, parasites, bacteria and viruses have all been explored as potential mosquito control agents. The killifish *Rivulus marmoratus* feeds on *Aedes taeniorhynchus* larvae and *Culex quinquefasciatus* egg rafts (Taylor *et al.*, 1992), but some evidence exists that mosquitoes deliberately avoid oviposition sites with large amounts of these fish (Ritchie and Laidlaw-Bell, 1994). The tree-hole-breeding mosquito *Toxorhynchites brevipalpis* feeds on larvae of other mosquito species including *Ae. aegypti,* but has limited efficacy for control of urban container-breeding mosquitoes. Some measure of success has been achieved using *Toxorhynchites amboinensis* to reduce *Ae. aegypti* densities in New Orleans, Louisiana (Focks *et al.*, 1983; Focks *et al.*, 1985). Fungi such as *Lagenidium*, *Coelomomyces* and *Culicinomyces* have been documented for their ability to infect and kill mosquitoes (for review see Scholte *et al.*, 2005). Elimination of dengue from communities in central Vietnam has been accomplished by reducing *Ae. aegypti* densities using predacious copepods of the genus *Mesocyclops* (Vu *et al.*, 2005).

Larvacidal bacteria such as *Bacillus thuringiensis israelensis* (BTI) have been explored as potential mosquito control agents, but concerns including high cost, limited efficacy and infection of off-target species have limited the utility of BTI (Davidson and Becker, 1996). However, recent efforts to develop recombinant strains of BTI with improved efficacy have shown promise (Federici *et al.*, 2003). Viruses belonging to the

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family *Baculoviridae* possess a very narrow host range and have been used as biopesticides, but problems with large-scale production and low pathogenicity have limited the usage of these viruses as mosquito control agents (for review see Szewczyk *et al.*, 2006). The newly discovered *Culex nigripalpus* nucleopolyhedrovirus has been shown to be highly pathogenic for *Culex* ssp. mosquitoes and is currently being investigated as a mosquito control agent (Andreadis *et al.*, 2003). Mosquito densoviruses have also demonstrated utility as mosquito control agents and are discussed in detail in the following sections.

Mosquito Densoviruses as Biological Control Agents

Mosquito densoviruses (MDV) (family *Parvoviridae*) have emerged as potential mosquito control agents. *Aedes aegypti* densovirus (*Ae*DNV) is the best characterized MDV in terms of basic biology. *Ae*DNV was originally isolated from a laboratory colony of *Ae. aegypti* mosquitoes in Ukraine and shown to infect mosquitoes of the genera *Aedes, Culex*, and *Culiseta* (Buchatsky, 1989). Mosquito larvae become infected during the aquatic instar phases of their development when they come into contact with virus in the water. Symptoms of infected larvae include loss of mobility, distortion of body shape and loss of pigmentation. Some larvae die from the infection, but many pupate and emerge as infected adults. Infected adult females vertically transmit the densovirus to their progeny. Vertical transmission allows for the virus to be spread to different breeding sites and infect new mosquito populations.

The MDVs characterized thus far exhibit dramatic differences in pathogenicity. An early histological study was the first to document the pathogenic effects of *Ae*DNV in

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infected *Ae. aegypti* larvae (Kuznetsova and Zelenko, 1975). A second MDV, *Aedes albopictus* parvovirus (*Aa*PV) (now designated *Aedes albopictus* densovirus (*Aal*DNV)) was isolated from C6/36 *Ae. albopictus* cells and was shown to be pathogenic for *Ae. aegypti* larvae (Jousset *et al.*, 1993; Boublik *et al.*, 1994). Several preliminary investigations attempted to quantify pathogenicity and mortality rates of MDVs, but differences in experimental design and uncertainty in the viral doses have prevented direct comparison between studies. Ninety percent larval mortality was reported following *per os* infection of first-instar *Ae. aegypti* larvae with *Aal*DNV (Barreau *et al.*, 1996). Infection of *Ae. albopictus* larvae with *Aedes* Thailand densovirus (*A*ThDNV) resulted in 82% larval mortality (Kittayapong *et al.*, 1999). Considerably less mortality (10%) was observed in *Aedes aegypti* larvae infected with *Hemagogus equinus* densovirus (*He*DNV) (O'Neill *et al.*, 1995). A recent preliminary study has reported low-level infection of *Anopheles minimus S.L.* larvae with *A*ThDNV resulting in minimal mortality (Rwegoshora and Kittayapong, 2004). Development of a real-time polymerase chain reaction assay (PCR) for quantification of MDV genomes and a standardized mosquito infection protocol facilitated direct comparison of infection and pathogenicity of *Ae*DNV, *He*DNV and *Aedes* Peruvian densovirus (*APe*DNV) in *Ae. aegypti* mosquitoes (Ledermann *et al.*, 2004). Although all three viruses replicated to similarly high titers, 75% larval mortality was achieved with *Ae*DNV compared to only 34% with *He*DNV and 28% with *A*PeDNV. *Ae*DNV-exposed larvae also exhibit delayed development compared to unexposed larvae and larvae exposed to the other two viruses. Ledermann *et al.* noted that *Ae*DNV was isolated from a laboratory colony of mosquitoes whereas *He*DNV and *A*PeDNV were isolated from insect cells and hypothesized that the

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decreased virulence observed with *He*DNV and *A*PeDNV could be linked to long-term adaptation of these viruses to cell culture. Strikingly, most MDVs, including *Ae*DNV, typically establish a persistent infection with little or no cytopathic effects (CPE) in C6/36 cells, whereas *He*DNV causes significant CPE consistent with induction of apoptosis in the same cell line (Paterson *et al.*, 2005).

*Ae*DNV possesses many attributes of the ideal biological control agent including a narrow host range, negligible effects on the surrounding environment, and the potential for rapid spread and persistence in mosquito populations. *Ae*DNV was patented as a mosquito control agent in the former Soviet Union under the name Viroden and tested extensively for environmental safety and toxicity (Buchatsky, 1989). Several species of mammals, birds, fish, worms, crustaceans, and insects were tested for susceptibility to *Ae*DNV infection. The inability of Viroden to infect any animals other than mosquitoes belonging to the genera *Aedes*, *Culex*, and *Culiseta* confirmed the narrow host range of *Ae*DNV. *Ae*DNV is also relatively resistant to extremes in temperature and pH, making it very environmentally stable.

Large-scale production of *Ae*DNV is a major obstacle to the successful implementation of the virus as a biological control agent. Purification from infected cell culture or mosquito larvae are the two traditional methods of *Ae*DNV production. Both of these methods have drawbacks. Cell-culture based production systems are easy to standardize and offer a simple purification scheme based on centrifugation, but the overall cost is quite high. Production of *Ae*DNV by C6/36 cells adapted to suspension culture in serum-free protein-free media has reduced preparation costs by eliminating the need for serum (Suchman and Carlson, 2004). Viroden was produced by harvesting

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*Ae*DNV from infected mosquito larvae. Although this technique is relatively inexpensive, the purification process is somewhat complicated and mosquito larvae are susceptible to other pathogens, which introduces the risk of contamination. New *Ae*DNV purification strategies are being explored. Recent studies have examined the binding affinity of *Ae*DNV for various ion exchange membranes (Specht *et al.*, 2004; Han *et al.*, 2005). Simple, large-scale purification of *Ae*DNV may be achieved by passing large amounts of unpurified virus preparations through a membrane that binds *Ae*DNV virions. Washing the membrane with appropriate solvents will remove extraneous cellular debris, and purified *Ae*DNV can be eluted.

Genetic Control

Genetic strategies for the control of mosquito-borne diseases have received an increasing amount of attention, as traditional methods have failed to reduce the burden imposed by mosquito-borne pathogens. The availability of sequence data including the *Ae. aegypti* genome project and the publication of the full genome sequence of *An. gambiae* has been a windfall for research aimed at determining the genetic basis of disease transmission (Holt *et al.*, 2002; Severson *et al.*, 2004). Genetic control strategies include sterile insect technique and mosquito transgenesis.

Sterile insect technique involves the sterilization of a large number of males by exposure to low doses of radiation followed by release of the sterilized males into an infested area. Decimation of entire populations of insects can be achieved if sterile males greatly outnumber fertile, wild males. Sterile insect technique was largely instrumental in the eradication of tsetse flies from Zanzibar (Vreysen *et al.*, 2000) and in the

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eradication of screwworm parasites in the Americas (Wyss, 2000). Although sterile insect technique has been effective at controlling certain species of insects, it has not been a successful strategy for mosquito control (Rai, 1996).

Genetic modification of mosquitoes rendering them incapable of pathogen transmission is a concept that has gained a considerable amount of support. Significant progress has been made in the laboratory towards the production of a genetically modified mosquito that is resistant to pathogen infection (Beaty, 2000). Transgenic, disease resistant mosquitoes would express a foreign effector gene that interferes with pathogen transmission. Effective control of a pathogen using mosquito transgenesis involves the identification of an effector gene, whose expression renders the vector incapable of transmitting the pathogen, a system for mosquito transformation, and a method for driving the gene into the wild population.

Transposable element systems are now widely used for transformation of mosquitoes. Inverted repeat sequences at each end of the transposon allow it to move from one DNA location to another, aided by a transposase. Transformation of flies with the P element from *Drosophila* prompted unsuccessful attempts at P element-mediated transformation of mosquitoes. In these studies, transformation of mosquitoes occurred by recombinantion rather than transposition. Failures with the P-element led to a search for transposons capable of transforming mosquitoes (Miller *et al.*, 1987; McGrane *et al.*, 1988). Successful transformation of *Ae. aegypti* has subsequently been achieved using the *Mariner*, *Hermes* and *PiggyBac* elements (Jasinskiene *et al.*, 1998; Coates *et al.*, 1998). Transformation of *Anopheles stephensi* has been achieved using the *Minos* element (Catteruccia *et al.*, 2000). *Anopheles gambiae*, *Culex pipiens* and several other

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mosquito species are now routinely transformed in the laboratory. Generating transgenic mosquitoes is a very difficult process. Transformation of mosquitoes with transposable elements involves the injection of mosquito eggs with DNA containing the transposon and gene of interest. This process is highly inefficient and extremely labor-intensive with success rates on the order of one successful transformant per 1000 injected eggs (For review see Handler, 2000). Therefore, genes of interest must be extensively characterized before committing to the creation of a new transgenic mosquito line.

Virus expression systems and RNA interference (RNAi) have emerged as tools for effector gene characterization in mosquitoes. The double-subgenomic Sindbis virus expression system (dsSIN) is a powerful tool for the characterization of gene function in mosquitoes (Shiao *et al.*, 2001) and has been used to express sequences or genes of interest in vectors (Olson, 2000; Pierro *et al.*, 2003). dsSIN has been used to engineer resistance to YFV and LaCross virus in vectors (Powers *et al.*, 1996; Higgs *et al.*, 1998) and has also been used to silence dengue virus gene expression in mosquitoes (Olson *et al.*, 1996; Adelman *et al.*, 2001). dsSIN-induced gene silencing occurs via the RNAi pathway, which is reviewed in the following sections (Sanchez-Vargas *et al.*, 2004; Blair *et al.*, 2006). Unfortunately, the utility of recombinant Sindbis viruses in nature is limited by the ability of the virus to infect many off-target species, including vertebrates.

Several promising effector molecules have been identified. One promising effector gene, the SM1 peptide, binds to mosquito midgut and salivary gland cells (Ghoush *et al.*, 2001). Expression of SM1 in transgenic *An. stephensi* inhibits transmission of *Plasmodium berghei* (Ito *et al.*, 2002). Inverted-repeat RNA (irRNA) bearing sequence homology to arboviral genomes has also emerged as an effector gene.

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Resistance to flavivirus infection has been observed in mosquitoes expressing irRNA (Sanchez-Vargas *et al.*, 2004; Franz *et al.*, 2006). Effective control of mosquito-borne disease by insect transgenesis will require the identification and characterization of new effector genes.

Replacement of wild mosquito populations with genetically modified mosquitoes incapable of pathogen transmission requires a method for driving the effector gene into the wild population. A gene drive system capable of rapidly introducing a foreign gene into wild populations is desirable as studies have indicated that elimination of dengue or malaria using a population replacement strategy would require incidences of the effector gene at levels approaching total fixation (Focks *at al.*, 2000; Boete and Koella, 2002). Infectious or infectious-like agents, such as transposons, viruses and symbionts, are attractive candidates as gene-drive systems because they circumvent Mendelian patterns of inheritance (James, 2005). Transposable element systems, including *Hermes, Minos,* and *Mariner*-based systems, are being explored as gene-drive mechanisms (O'Brochta *et al.*, 2003). Mosquito parvoviruses belonging to the *Densovirinae* subfamily have been used as gene transfer vehicles (Carlson *et al.*, 2006) and are discussed in detail in the following section. Endosymbiotic *Wolbachia* ssp. have also been suggested as a gene-drive system due to the phenomenon of cytoplasmic incompatibility and the ability of these symbionts to spread rapidly through mosquito populations. Male mosquitoes infected with *Wolbachia* possess modified sperm that is incapable of fertilizing the eggs of uninfected females, resulting in selection for mosquitoes infected with the endosymbionts. Expression of effector genes by *Wolbachia* ssp. could become a practical gene-drive mechanism (For review see Sinkins, 2004).

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Mosquito-borne diseases are a serious worldwide public health issue. Failure of traditional control methods to reduce the burden imposed by mosquito-borne diseases has prompted research aimed at developing new approaches. Although considerable progress has been made towards the goal of developing pathogen-resistant mosquitoes, novel strategies for testing candidate genes and transducing mosquitoes are needed.

*Ae*DNV Molecular Biology

Parvovirus Overview

Parvoviruses are small, non-enveloped DNA viruses, 18-26 nm in diameter, with icosahedral symmetry and terminal genomic sequences capable of secondary structure formation (Muzyczka and Berns, 2001). Parvovirus virions contain a single molecule of single-stranded DNA 4-6 kb in size. The family *Parvoviridae* contains two subfamilies: the *Parvovirinae*, which infect vertebrates, and the *Densovirinae*, which infect invertebrates. The subfamily *Parvovirinae* contains three genera: *Parvovirus, Erythrovirus,* and *Dependovirus*. The *Parvovirus* genus includes mammalian parvoviruses, such as the prototype minute virus of mice (MVM) and the canine and feline parvoviruses. The genomic organization of MVM is typical of most parvoviruses (Fig. 1.1). The MVM genome encodes all viral proteins on the same strand and contains two open reading frames (ORF) that cover the left and right halves of the genome. The left half of the genome encodes two non-structural (NS) proteins, NS1 and NS2, and the right half encodes two structural proteins, VP1 and VP2. Mutational analyses have shown that NS1 is required for virus replication and genome packaging (Rhode, 1982). VP1 and VP2 comprise the virus capsid along with a third structural protein generated in

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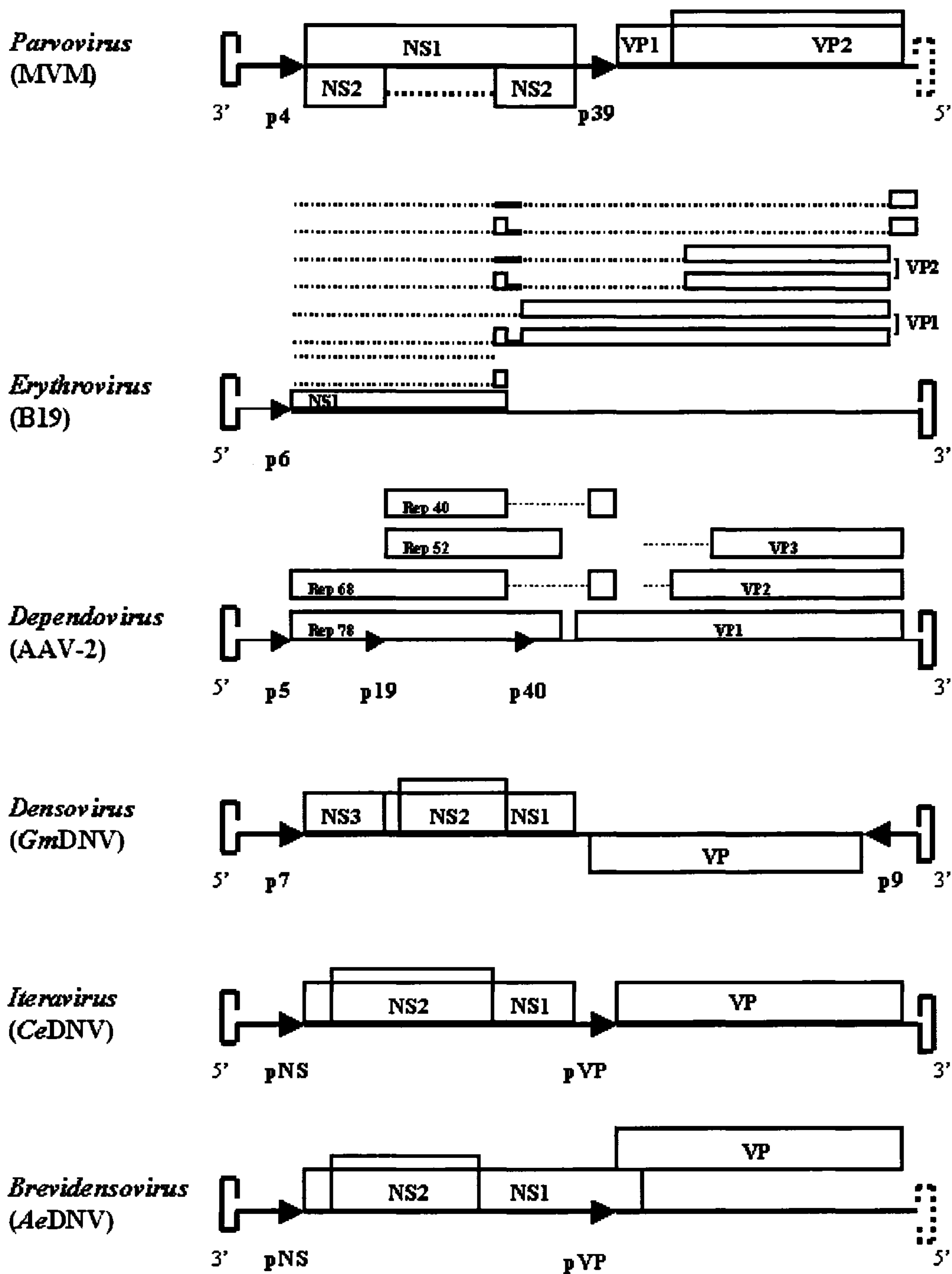

Fig. 1.1. *Parvoviridae* genome organization. Open reading frames are indicated by boxes, arrows represent promoters and their direction of transcription. Splicing is indicated by dotted lines. (Reproduced with modification from T. Ward, Ph.D. dissertation.)

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the intact capsid by proteolytic cleavage of VP2 (Tattersall *et al.*, 1977). MVM capsids predominately package the negative strand. Transcription initiates from two promoters located immediately upstream of the left and right ORFs and terminates at a single site in the right-end untranslated region (UTR) of the MVM genome. Alternative splicing of the two transcripts generates mRNAs coding for NS1, NS2, VP1, and VP2. Members of the *Erythrovirus* genus, including the human B19 parvovirus, express all of their transcripts from a single promoter located in the left end of the virus genome (Fig. 1.1). The *Dependovirus* genus includes the mammalian adeno-associated viruses (AAV) which require a helper virus, such as an adenovirus or herpesvirus, to establish a fully productive infection (Atchison *et al.*, 1965; Buller *et al.*, 1981). AAV integrates into the host genome in the absence of a helper virus resulting in a latent infection. A productive infection can be initiated upon rescue of the integrated AAV genome by a helper virus (Cheung *et al.*, 1980). Dependoviruses package positive and negative strands in equimolar ratios and possess genomes containing three promoters (Fig. 1.1). Most members of the *Parvovirinae* have two promoters, the non-structural gene promoter at the left end of the virus genome and a structural gene promoter in the middle of the genome.

The invertebrate parvoviruses are classified in the *Densovirinae* subfamily, the etiology of which stems from the densely stained, hypertrophied appearance of infected nuclei. The *Densovirinae* subfamily is further subdivided based on genome organization into the genera *Densovirus*, *Iteravirus*, and *Brevidensovirus* (for review see Bergoin and Tijssen, 2000). Ambisense genome organization, a characteristic shared by members of the *Densovirus* genus, is a unique feature among members of the *Parvoviridae* (Fig. 1.1).

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NS and VP genes are located on strands of opposite polarity. The two strands are encapsidated separately in an equimolar ratio. Lepidopteran densoviruses, including *Galleria mellonella* densovirus (*Gm*DNV) and *Junonia coenia* densovirus (*Jc*DNV), are the best studied members of the *Densovirus* genus. *Iteravirus* genomes possess identical terminal sequences and are organized in a monosense fashion (Fig. 1.1). Representative members of the *Iteravirus* genus include *Bombyx mori* densovirus and *Casphalia extranea* densovirus. Finally, members of the *Brevidensovirus* genus have small (4 kb), monosense genomes that are flanked by non-identical terminal sequences (Fig. 1.1). Brevidensoviruses preferentially package genomes of negative polarity. Most members of this genus infect mosquitoes, although a brevidensovirus of shrimp has been isolated (Fediere, 2000). At least 8 mosquito brevidensoviruses have been described. Phylogenetic analysis based on genome sequence data suggests two clades: one for new world mosquito brevidensoviruses and another for old world brevidensoviruses. The old world mosquito densoviruses (MDV) include *Aedes aegypti* DNV (*Ae*DNV), *Aedes* Thailand DNV (*A*ThDNV), and C6/36 DNV, a Chinese isolate. *Aedes* Peruvian DNV (*APe*DNV), *Hemagogus equinus* DNV (*He*DNV), and *Aedes albopictus* DNV (*Aal*DNV) belong to the new world clade.

AeDNV Molecular Biology and Gene Expression

Until very recently, little was known about the molecular biology of MDVs. The development of infectious clones for *Ae*DNV (pUCA) and *A*PeDNV (pUCP) has facilitated experiments investigating MDV molecular biology and gene expression by providing a reverse genetic system (Afanasiev *et al.*, 1991; Afanasiev *et al.*, 1994). The

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molecular biology of *Ae*DNV has received the most attention. *Ae*DNV is a negative-sense DNA virus with a small, linear genome, 4 kb in size. The nonstructural genes (NS1 and NS2) and the viral protein genes (VP1 and VP2) are expressed from the left and right halves of the same strand by two promoters, pNS and pVP, respectively (Afanasiev, 1990; Afanasiev, 1991). The NS1 proteins of many parvoviruses are necessary for genome replication and packaging and for transactivation of transcription from parvovirus promoters (Vanacker and Rommelaere, 1995; Muzyczka and Berns, 2001; Ward *et al.*, 2001a). Consistent with other parvoviruses, *Ae*DNV genome replication and transactivation by NS1 requires the presence of the viral ends (Hanson and Rhode, 1991; Ward *et al.*, 2001a). The open reading frame for NS2 lies entirely within the coding sequence for NS1, but is frameshifted by one nucleotide. The function of NS2 is currently unknown. VP1 and VP2 are encoded by the same ORF. Production of VP2 is believed to result from initiation at a different translation codon or from proteolytic cleavage of VP1 (Afanasiev *et al.*, 1991; Kimmick *et al.*, 1998). *Ae*DNV expresses two polyadenylated transcripts, one each from pNS and pVP, that terminate in the right-end untranslated region (UTR) of the virus genome (Ward, *et al.*, 2001a).

Identification of sequence elements comprising promoters or regulatory regions has been accomplished by insertion of reporter genes, such as β-galactosidase, in the ORF of NS1, NS2, or VP followed by reverse genetic analysis. mRNA expressed from pNS contains two AUG translation initiation codons. The AUG closest to the 5' end of the message corresponds to the NS1 ORF while the NS2 ORF begins with a downstream AUG. The NS1 and NS2 ORFs are expressed at roughly equal efficiencies, indicating that initiation at either AUG occurs at similar frequencies (Kimmick *et al.*, 1998). This

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differs from the classic scanning model of translation initiation where the first AUG from the 5' end of the mRNA is the most efficiently used (Kozak, 1980). A more optimal sequence context for the NS2 AUG compared to the NS1 AUG may explain why both translation initiation codons are efficiently used. Kimmick *et al.* also demonstrated that sequences downstream of the pNS TATA-box, including a predicted RNA secondary structure, are required for expression of both ORFs in C6/36 cells. Deletion of sequences forming the predicted secondary structure resulted in ablation of protein expression but did not significantly affect RNA levels, indicating that regulation of gene expression by sequence elements downstream of the pNS TATA-box occurs at the level of translation (Kimmick *et al.*, 1998). Primer extension analysis was used to map the transcriptional start site of the VP gene to a CAGT arthropod initiator motif occurring upstream of the TATAA sequence previously thought to define the pVP promoter (Ward *et al.*, 2001a). Deletion analysis confirmed that the CAGT sequence, and neither of the surrounding TATA-boxes, is absolutely required for efficient gene expression from the *Ae*DNV pVP promoter. The CAGT motif has been described as a required promoter element in many arthropod transcription units (Smale *et al.*, 1990; Blissard *et al.*, 1992; Cherbas and Cherbas, 1993; Pullen and Freisen, 1995).

Production of empty *Ae*DNV virus like particles has been accomplished using a methylotrophic yeast-based protein expression system. The *Ae*DNV VP gene was optimized for yeast codon usage and inserted into the *Pichia pastoris* genome downstream of the alcohol oxidase gene promoter. Induction with methanol results in the production of *Ae*DNV VPs which self-assemble into virus-like particles (R. Specht,

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unpublished observations). *Ae*DNV virus-like particles are being used in experiments aimed at determining the host-cell receptor involved in virus binding.

Densoviruses as Gene Delivery Vehicles

Parvoviruses are being developed as delivery vehicles for the introduction of foreign genes to animal hosts. Many studies have demonstrated the utility of AAV-2 for gene therapy in humans (for review see Grieger and Samulski, 2005). The availability of infectious clones for many parvoviruses has facilitated experiments with recombinant viral genomes. Replacement of wild-type non-structural or structural gene coding sequences with exogenous DNA creates a recombinant AAV-2 genome that can be encapsidated into recombinant AAV-2 virions if the missing wild-type factor is provided *in trans*. Expression of the foreign gene can be accomplished by infecting cells or organisms with parvoviruses containing recombinant genomes. Two members of the *Densovirinae*, *Jc*DNV (genus *Densovirus*) and *Ae*DNV (genus *Brevidensovirus*), have been developed as systems for the expression of foreign genes in insect hosts.

*Jc*DNV was the first densovirus to be tested as an expression system. Insertion of the *E. coli lac*Z gene as a fusion gene in the major ORF of the *Jc*DNV genome resulted in β-galactosidase (β-gal) expression upon transfection of the recombinant *Jc*DNV infectious clone into permissive cells (Giraud *et al.*, 1992). Encapsidation of the recombinant *Jc*DNV genomes was accomplished by co-transfection with the wild-type *Jc*DNV infectious clone to supply the missing factors. Expression of chloramphenicol acetyltransferase (CAT), green florescent protein (GFP), and firefly luciferase from the *Jc*DNV genome has been accomplished by insertion of the reporter gene in-frame with

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either VP or NS (Bergoin *et al.*, 1998). Stable GFP expression in insect cells resulting from integration of transfected *Jc*DNV-derived vectors into the host genome has been recently reported (Bossin *et al.*, 2003).

*Ae*DNV has been developed as a vector for the expression of foreign genes in mosquitoes and in cell culture. Development of an infectious clone of *Ae*DNV, pUCA, allowed for the creation of β-gal expression vectors containing *lacZ* sequences in the NS or VP ORFs (Afanasiev *et al.*, 1994). Subsequent experiments have shown that GFP, β-gal, β-glucuronidase (GUS), and antibiotic resistance genes can all be expressed from both viral promoters at reasonable efficiencies (Kimmick *et al.*, 1998; Afanasiev *et al.*, 1999; Ward *et al.*, 2001a). Foreign genes expressed from pNS must be expressed as NS1 or NS2 fusion proteins because expression of genes as non-fusion proteins using their native initiation codons has proven unsuccessful (Kimmick *et al.*, 1998). NS1 functionality does not seem to be impaired in NS1-fusion protein constructs, as long as the fusion is to the C-terminus. Similar results have been obtained with foreign genes expressed from the pVP promoter. However, foreign genes expressed from pVP can be expressed as VP-fusion proteins or as nonfused proteins initiating at their native AUG codons (Ward *et al.*, 2001a). VP-fusion proteins accumulate in the nucleus due to the presence of a nuclear localization signal in the VP1 N-terminus (Afanasiev *et al.*, 1994; Afanasiev *et al.*, 1999). This observation suggests that VP1 functionality is not impaired in VP1-fusion constructs. Interestingly, the highest level of GFP expression occurs when GFP is fused to the C-terminus of NS1 (Afanasiev *et al.*, 1999; Allen-Miura *et al.*, 1999).

Successful foreign gene expression has prompted the development of *Ae*DNV as a transducing virus (for review see Afanasiev and Carlson, 2000). Transduction involves

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the delivery of foreign genes by infectious recombinant *Ae*DNV virions. *Ae*DNV transducing genomes contain a foreign gene expressed from one of the viral promoters, the terminal sequences necessary for packaging and replication, and a polyadenylation signal sequence for transcription termination. Infectious transducing virions are generated by co-transfection of mosquito cells with a plasmid containing the transducing genome and a plasmid supplying the missing factor (either NS or VP). Parvovirus genome packaging efficiency decreases significantly for genomes larger than the wild type with 120% of normal being the upper limit (Dong, *et al.*, 1996; Hermonat *et al.*, 1997; Kimmick *et al.*, 1998). Therefore, the transducing capacity of *Ae*DNV is limited to approximately 4 kb. Packaging of *Ae*DNV-GFP transducing genomes has also been accomplished by expression of *Ae*DNV structural proteins from a Sindbis virus expression system (Allen-Miura *et al.*, 1999). Transducing virions carrying an NS1-GFP genome are capable of infecting *Ae. aegypti* larvae with detectable GFP expression in the midgut, hindgut, malphigian tubules and anal papillae (Afanasiev *et al.*, 1999; Ward *et al.*, 2001b). Anal papillae regulate ion concentration in the hemolymph of mosquito larvae and are usually the first organ system infected by *Ae*DNV. Infected anal papillae occasionally have been observed to melanize and fall off, terminating the infection, thereby providing evidence that anal papillae are the primary route of *Ae*DNV infection (Ward *et al.*, 2001b). Transovarial transduction of *Ae. aegypti* mosquitoes has been reported for *Ae*DNV-GFP (T. Ward, Ph.D. Dissertation). Ward and colleagues have also demonstrated that *Ae*DNV transducing virions are capable of infecting the anal papillae of *An. gambiae* larvae although disseminated infection does not occur. Although considerable effort has been expended to develop *Ae*DNV as a transducing virus, less-

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pathogenic MDVs, such as *A*PeDNV, may be more effective as gene-delivery vehicles because of reduced mortality in transduced mosquito populations.

The studies described above have demonstrated the utility of *Ae*DNV as a vector for the expression of foreign genes in mosquitoes and as a potential biological mosquito control agent. Reporter gene experiments have identified sequence elements required for efficient expression of foreign genes from each of the *Ae*DNV promoters. Because the coding capacity of the *Ae*DNV genome is limited, identification of sequence elements present in the *Ae*DNV right-end UTR required for polyadenylation of viral transcripts would be beneficial. Analysis of the right-end UTR may also identify non-essential sequences suitable for replacement with exogenous DNA, such as an RNAi-expression cassette.

RNA Interference and Mosquito-Borne Disease

RNA Silencing Historical Overview

It is now known that RNA gene silencing phenomena such as post-transcriptional gene silencing (PTGS) or co-suppression in plants, quelling in fungi, and RNA interference (RNAi) in animals all share a common mechanism that reflects an ancient origin in a common ancestor of animals, plants and fungi (Mello and Conte, 2004). The groundbreaking 1998 discovery by Fire and colleagues that double-stranded RNA (dsRNA) functions as the trigger for induction of these seemingly disparate responses provided the foundation for subsequent experiments that would uncover the mechanism governing RNA silencing and propel the field of RNAi to the very forefront of molecular biology. Since the discovery of the trigger, RNA silencing has become a widely used

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tool for the study of gene function, and has been shown to be an important player in diverse biological processes including heterochromatin remodeling and regulation of gene expression by microRNAs. RNA silencing is also an important defense against viruses and transposable elements (Keene *et al.*, 2004). Much has been learned about the mechanism and utility of RNA silencing, but many questions remain unanswered.

Wingard's 1928 paper on tobacco ringspot virus is perhaps the first report documenting a phenomenon that would later be proven to result from RNA silencing (Wingard, 1928). Wingard reported that tobacco plants infected with the virus showed symptoms of disease only on the lower leaves, while the upper leaves remained healthy and resistant to secondary infection. Many decades later, researchers have shown that the resistance to tobacco ringspot virus exhibited in the upper leaves is due to silencing of the viral RNA by PTGS (Ratcliff *et al.*, 1997; Covey *et al.*, 1997). Two papers published in April of 1990 document what would come to be known as co-suppression in plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). The researchers were attempting to produce petunias with brighter colored petals by giving the plants extra copies of the gene coding for the desired pigmentation. Surprisingly, introducing extra copies of the gene resulted in white flowers that were devoid of any pigmentation. The investigators were able to link this phenotype with a 50-fold reduction in mRNA levels for the pigmentation gene. Shortly after this silencing phenomenon was described in plants, researchers made similar observations in fungi and called it "quelling" (Romano and Macino, 1992). It was also demonstrated that virus resistance in plants could be accomplished by intracellular expression of a small segment of viral RNA (van Kammen, 1997). This was termed virus-induced gene silencing (VIGS). PTGS, co-suppression, VIGS and quelling all

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shared the common thread of seemingly inexplicable gene silencing due to the introduction of homologous RNA. Concurrent work in the model organism *Caenorhabditis elegans* would provide an explanation.

Discovery of the RNA Silencing Trigger

In the 1990s, several groups of researchers were utilizing a technique called antisense RNA-mediated gene silencing to study gene function in the nematode *C. elegans*. Antisense RNA-mediated gene silencing involves the introduction of single-stranded RNA (ssRNA) complementary to the messenger RNA (mRNA) being silenced. Base-pairing of the antisense RNA with the target mRNA blocks the process of translation and silences expression of the gene encoded by the target mRNA (Izant *et al.*, 1984; Nellen *et al.*, 1993).

Guo and Kemphues were the first to observe that gene silencing could be induced in *C. elegans* by the introduction of either antisense or sense RNA. Guo and Kemphues were attempting to block expression of the *C. elegans par-1* gene using an antisense RNA approach, yet they observed silencing of the target gene with both sense and antisense RNA preparations (Guo and Kemphues, 1995). Two initial possibilities for this apparent lack of strand specificity existed. The first postulated that the introduced ssRNA was inducing a silencing response independent of the polarity of the ssRNA. The second possibility suggested that the silencing observed with the control sense RNA preparation was actually due to a small amount of contaminating antisense RNA being present in the sense RNA preparation. This type of contamination is common in the *in vitro* transcription reactions that are used to generate antisense RNA. Since the observed

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silencing appeared to differ from conventional antisense RNA, Rocheleau and colleagues created a new term for this phenomenon called RNA-mediated interference (RNAi) (Rocheleau *et al.*, 1997).

In 1998, Fire and colleagues realized that contamination of the RNA preparations used in *C. elegans* gene silencing with a small amount of RNA of opposite polarity may result in the formation of double stranded RNA (dsRNA). Fire and colleagues postulated that this dsRNA might be the trigger for RNAi (Fire *et al.*, 1998). To test if dsRNA could induce gene silencing, Fire and colleagues purified sense and antisense ssRNA preparations and compared the ability of these preparations to silence the target gene against a dsRNA preparation. Sense and antisense ssRNA preparations exhibited only marginal interference activity and required large doses of RNA to produce an observable effect. In contrast, the dsRNA preparation resulted in 100-fold greater silencing of the target gene when compared to the ssRNA preparations. Fire's initial study also showed that the silencing effect could spread between tissues and be transmitted in the germ line of *C. elegans* injected with dsRNA. Fire's finding that dsRNA was the trigger for induction of the RNAi response is of such significance that it was recognized with a Nobel award this year. Put simply by Mello and Conte:

> "the observation by Fire *et al.* that dsRNA is a potent trigger for RNAi in the nematode *C. elegans* was important because it suggested a simple approach for efficient induction of gene silencing in *C. elegans* and other organisms, and accelerated the discovery of a unifying mechanism that underlies a host of cellular and developmental pathways (Mello and Conte, 2004)."

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Shortly after Fire's discovery of the trigger, dsRNA was shown to induce silencing in *Drosophila melanogaster* and promote mRNA degradation in *Trypanasoma brucei* suggesting that the RNAi response was not exclusive to nematodes (Kennerdell and Carthew, 1998; Ngo *et al.*, 1998).

RNAi Mechanism

Discovery of the trigger allowed for the development of methods that resulted in the identification of genes required for RNAi in *C. elegans*. Fire and Mello's research group found that RNAi could be induced in *C. elegans* by feeding the animals dsRNA or soaking them in dsRNA (Timmons and Fire, 1998; Tabara *et al.*, 1998). By selecting *C. elegans* mutants exhibiting resistance to RNAi, genes required for the RNAi response in *C. elegans* were identified (Tabara *et al.*, 1999). In particular, the rde-1 locus was implicated as being important for RNAi and was shown to be a member of a gene family that is highly conserved among eukaryotes. Identification of rde-1 and other genes required for RNAi in *C. elegans* allowed for comparison of these genes to those required for RNAi in other species including plants, fungi and *Drosophila* (Schmidt *et al.*, 1999; Fagard *et al.*, 2000; Catalanotto *et al.*, 2000; Arvin *et al.*, 2001). Some of these genes were found to encode cellular nucleases necessary for RNAi-mediated gene silencing in *Drosophila* and *C. elegans* (Hammond *et al.*, 2000; Parrish and Fire, 2001). Concurrent research demonstrated the presence of small RNAs in organisms undergoing RNAi (Hamilton and Baulcome, 1999; Zamore *et al.*, 2000). These small RNAs are now known as short-interfering RNAs (siRNAs).

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A link between the genetics and biochemistry of RNAi came in 2001 when dsRNAs were shown to be processed into small RNAs by the cellular nuclease Dicer (Bernstein *et al.*, 2001). Dicer is a member of the RNase III family of nucleases. Two Dicer genes, Dicer-1 and Dicer-2, have been identified in *Drosophila.* Dicer-1 is responsible for processing dsRNAs that are miRNA precursors, while Dicer-2 preferentially processes longer dsRNAs (Lee *et al.*, 2004). The products of Dicer are 19-23 nt duplexes of RNA possessing a 3' overhang (Elbashir *et al.*, 2001).

The siRNAs produced by Dicer are loaded onto the RNA-induced silencing complex (RISC), which was initially described in *Drosophila* (Hammond *et al.*, 2000). The exact makeup of proteins comprising RISC is still unknown and several different forms of this silencing complex have been reported. Components of RISC include members of the Argonaute (Ago) protein family (Hammond *et al.*, 2001). It has been suggested that the Ago protein binds RISC to the siRNA, but further investigation is needed. Dicer products are double stranded, but functional RISC contains only single-stranded RNA. It is postulated that some component of RISC possesses an RNA helicase activity that is responsible for unwinding the siRNA duplexes. In support of this hypothesis is the fact that RISC assembly is ATP-dependent (Nykanen *et al.*, 2001; Pham *et al.*, 2004). Studies investigating RISC assembly in *Drosophila* have implicated the RNA helicase Armitage as being important for siRNA duplex unwinding (Tomari *et al.*, 2004). RISC is guided by the bound siRNA to mediate sequence-specific degradation of a target mRNA possessing exact or near-exact sequence complementarity (Martinez *et al.*, 2002; Martinez and Tuschl, 2004). Target mRNAs are cleaved near the middle of the

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complimentary region (Elbashir *et al.,* 2001). RISC is capable of cleaving multiple target mRNAs in the presence of ATP (Hutvagner and Zamore 2002).

Amplification of the RNAi effect has been observed in nematodes and plants. Amplification of the silencing effect requires an RNA-dependent RNA polymerase (RdRP) (Wassenegger and Pelissier, 1998; Sijen *et al.,* 2001). RdRPs are absent in vertebrates and *Drosophila* (Schwarz *et al.*, 2002; Roignant *et al.*, 2003; Stein *et al.*, 2003). The absence of RdRPs makes amplification of the RNAi response unlikely in mosquitoes (Hoa *et al.,* 2003).

RNAi as a tool for control of mosquito-borne disease

The RNAi pathway appears to be highly conserved among eukaryotes, and has recently been demonstrated in mosquitoes. RNAi-based approaches could form the basis of novel strategies for the control of mosquito-borne diseases. Discovery of the RNAi response in mosquitoes began with investigations of antisense RNA and followed a path similar to the research with *C. elegans*. Researchers were exploring the utility of antisense RNA technology as a method to produce mosquitoes resistant to arbovirus infection. The double-subgenomic Sindbis virus expression system was being used to deliver antisense RNA targeted against the dengue virus type 2 (DEN-2) structural genes to C6/36 *Aedes albopictus* cells (for review of Sindbis expression systems see Olson, 2000). Expression of antisense RNA complimentary to the full-length DEN-2 premembrane (prM) mRNA produced cells that were resistant to challenge with DEN-2, but not DEN-3 (Gaines *et al.*, 1996). The same approach was also shown to produce DEN-2 resistance in adult female *Ae. aegypti* mosquitoes (Olson *et al.*, 1996). However,

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expression of DEN-2 prM sense RNA also made mosquitoes resistant to challenge with DEN-2. This observation bears a striking similarity to the observation made one year earlier by Guo and Kemphues who were using antisense RNA for the study of gene function in *C. elegans* (Guo and Kemphues, 1995). The ability of RNA of either polarity to make cells resistant to infection by an RNA virus suggested that this was not a conventional antisense RNA phenomenon. Subsequent findings in *C. elegans* and *D. melanogaster* indicated that the RNAi pathway was responsible for the observed resistance to virus infection.

Adelman and colleagues used an antisense RNA approach to silence dengue viral gene expression in *Ae. aegypti* mosquitoes and were the first to suggest that a pathway similar to the RNAi response exists in mosquitoes (Adelman *et al.*, 2001). Similarly, Caplen and colleagues demonstrated that RNAi could be used to inhibit replication and expression of Semliki forest virus (Caplen *et al.*, 2002). Hoa and colleagues characterized RNAi in an *An. gambiae* cell line and showed that Dicer-2 and members of the Ago family of proteins are required for RNAi in the malaria mosquito (Hoa *et al.*, 2003). Additionally, RNAi has been shown to act as a natural antiviral response to O'nyong-nyong virus (Keene *et al.*, 2004). Antisense RNA and RNAi-based gene silencing approaches have been used in the study of mosquito gene function (Johnson *et al.*, 1999; Attardo *et al.*, 2003; Tamang *et al.*, 2004; Boisson *et al.*, 2006; Riehle *et al.*, 2006). RNAi is also being explored as a tool for the disruption of arthropod-borne disease transmission (Adelman *et al.*, 2002; Brown *et al.*, 2003; Meister *et al.*, 2005).

Several studies have investigated the utility of antisense RNA and RNAi as tools for control of the dengue viruses. Transfection of C6/36 cells with DEN-1 specific

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dsRNA has been shown to inhibit DEN-1 replication (Caplen *et al.*, 2002). More recent work has focused on expression of anti-dengue inverted-repeat RNAs (irRNAs) in transformed mosquitoes and mosquito cells. irRNAs are recognized as dsRNA by the cellular RNAi machinery. Expression of irRNA transcribed from a plasmid construct targeting the DEN-2 prM gene has been shown to produce DEN-2 resistance in transfected C6/36 cells (Adelman *et al.*, 2002). This study also confirmed the presence of DEN-2 specific 21-25 nt siRNAs indicating that the observed resistance is a result of RNAi. Sanchez-Vargas and colleagues have shown that DEN-2 replication in C6/36 cells can be inhibited by transfection of cells with synthetic 21 bp siRNAs targeted to the DEN-2 prM gene (Sanchez-Vargas *et al.*, 2004). This study also successfully used the recombinant Sindbis virus expression system to silence the endogenous early trypsin gene in *Ae. aegypti* mosquitoes.

The identification of promoters for dsRNA expression in mosquitoes and the availability of transposable element systems for mosquito transgenesis have resulted in the creation of genetically modified, dengue-resistant mosquitoes. Promoters have been described that are transcriptionally active throughout the entire mosquito as well as promoters that function only in specific tissues (for review see Olson *et al.*, 2002). For example, the midgut-specific carboxypeptidase promoter is capable of expressing dsRNA in adult mosquito midguts following induction of the promoter upon ingestion of a blood meal (Moreiera *et al.*, 2000). The Mariner *Mos*I transposable element system (for review see Torti *et al.*, 1997) has been used to create genetically modified *Ae. aegypti* capable of expressing DEN-2 specific dsRNA transcribed from the carboxypeptidase promoter (Sanchez-Vargas *et al.*, 2004). Transgenic mosquitoes expressing dsRNA targeted to the

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DEN-2 prM gene are inhibited in their ability to support dengue virus replication (Franz *et al.*, 2006). The production of transgenic mosquitoes exhibiting resistance to pathogen infection is a significant step towards the goal of controlling mosquito-borne disease by replacement of competent wild vectors with incompetent transgenic vectors.

Pol III Promoter-Based RNAi Cassettes

The use of RNA polymerase III promoters (Pol III) for expression of shRNA was initially developed in mammalian systems where dsRNA longer than 30 bp is recognized by the RNA-dependent protein kinase inducing the interferon response (Paddison and Hannon, 2002). shRNA expression cassettes circumvent the problematic interferon response and are well characterized in mammalian systems (for review see Wadhwa *et al.*, 2004; Sandy *et al.*, 2005). No response analogous to the interferon pathway exists in insects. Pol III promoters for expression of dsRNA have not received much scrutiny in mosquitoes or other invertebrates, although they have emerged as powerful tools for mammalian RNAi (Dykxhoorn *et al.*, 2003). For this approach, a Pol III promoter is used to transcribe an inverted-repeat sequence to produce short-hairpin RNA (shRNA) that contains a double-stranded stem of 19-25 bp with sequences complementary to the target gene, and a 5-10 base loop sequence connecting them (Fig. 1.2). Pol III transcription is terminated by including a poly(T) stretch at least 6 nt long after the target sequence hairpin. Dicer cleaves the loop sequence, RISC unwinds the dsRNA forming siRNAs, and mediates degradation of mRNAs containing the target sequence (Meister and Tuschl, 2004). These expression cassettes are usually under 200 nt in size, making

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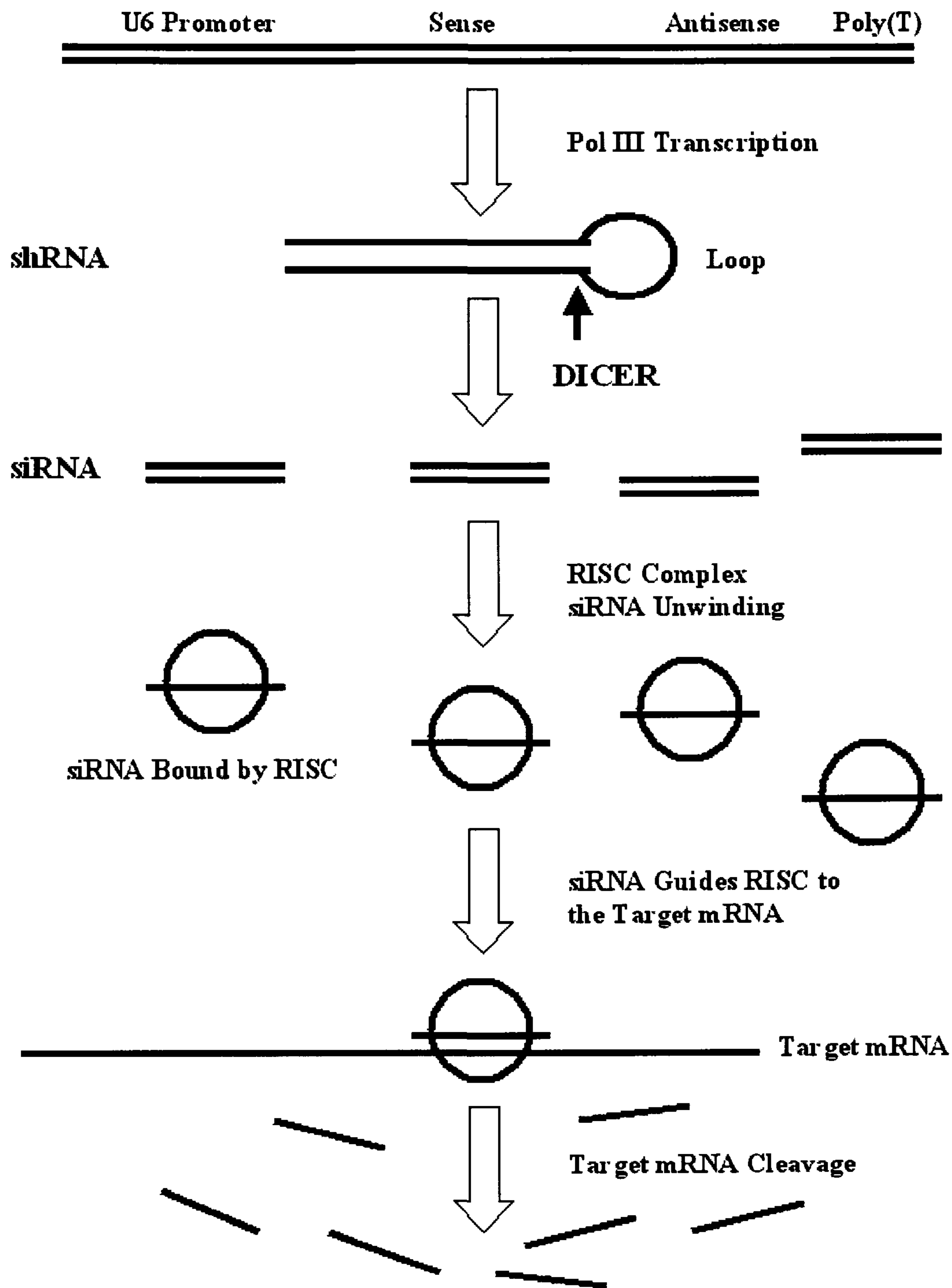

Fig. 1.2. Induction of RNAi by shRNAs expressed from Pol III promoters. Pol III transcription generates shRNAs that are cleaved by Dicer into 19-25 nt siRNAs. RISC is guided by the bound siRNA to mediate sequence-specific degradation of a target mRNA (Reproduced with modification from Genscript.com.)

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them attractive options for DNA-based RNAi induction in expression vectors with limited coding capacities.

We hypothesize that *Ae*DNV can be developed as a vector for RNAi in mosquitoes. Studies have already demonstrated *Ae*DNV's utility as a transducing agent, but the limited coding capacity of the viral genome precludes the production of fully functional recombinant *Ae*DNV virions because either NS or VP must be substituted for and the missing factor supplied *in trans*. However, a Pol III RNAi cassette is small enough to be added to the *Ae*DNV genome without necessitating the removal of NS or VP coding sequences. Insertion of an RNAi cassette in a non-coding region of the *Ae*DNV genome could allow for the delivery of shRNA by *Ae*DNV virions capable of replication and packaging in the absence of a helper virus. The right-end UTR of the *Ae*DNV genome is the most obvious candidate region for the insertion of exogenous DNA comprising an RNAi expression cassette, but the role of sequences contained in this region with respect to virus viability has not been ascertained. Specifically, the right-end UTR contains sequences directing polyadenylation of viral transcripts that must be determined and maintained in any *Ae*DNV genomes modified to include an RNAi expression cassette in the *Ae*DNV right-end UTR.

Polyadenylation

Messenger RNA 3'-End Processing Overview

Most eukaryotic pre-mRNAs undergo several co- and post-transcriptional modifications necessary for the formation of mature mRNAs. These modifications

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include addition of the 5' cap structure, splicing, and processing of the pre-mRNA 3'-end involving transcription termination and polyadenylation. Most eukaryotic pre-mRNAs, with the exception of histone pre-mRNAs, acquire a poly(A) tract at their 3' end (for review of histone mRNA processing see Gilmartin, 2005). Proper processing of the pre-mRNA 3'-end resulting in addition of the poly(A) tail is a crucial step in gene expression and has been linked to processes such as translation, mRNA transport, splicing, transcription termination, and mRNA stability. The eukaryotic polyadenylation process is remarkably complex and requires the interaction of at least six cellular factors, several of which are multi-subunit protein complexes. The mammalian, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* polyadenylation processes are the best studied. Research has indicated that the polyadenylation process in *S. cerevisiae* and *A. thaliana* does not differ from that of mammals as much as was previously assumed. Therefore, this section focuses on polyadenylation within the animal kingdom (for review of polyadenylation in yeast see Edmonds, 2002; for review of polyadenylation in plants see Quesada *et al*., 2006).

The following sections review the discoveries that formed the basis for our current understanding of the polyadenylation process. Due to the complexity of this process, some context is required before an historical review of the key discoveries is possible. A current model of the polyadenylation process in animals is illustrated in Figure 1.3. The polyadenylation process begins with the recognition of a hexanucleotide sequence motif on the nascent transcript by cleavage and polyadenylation specificity factor (CPSF). The most common hexanucleotide motif is AAUAAA. A GU-rich

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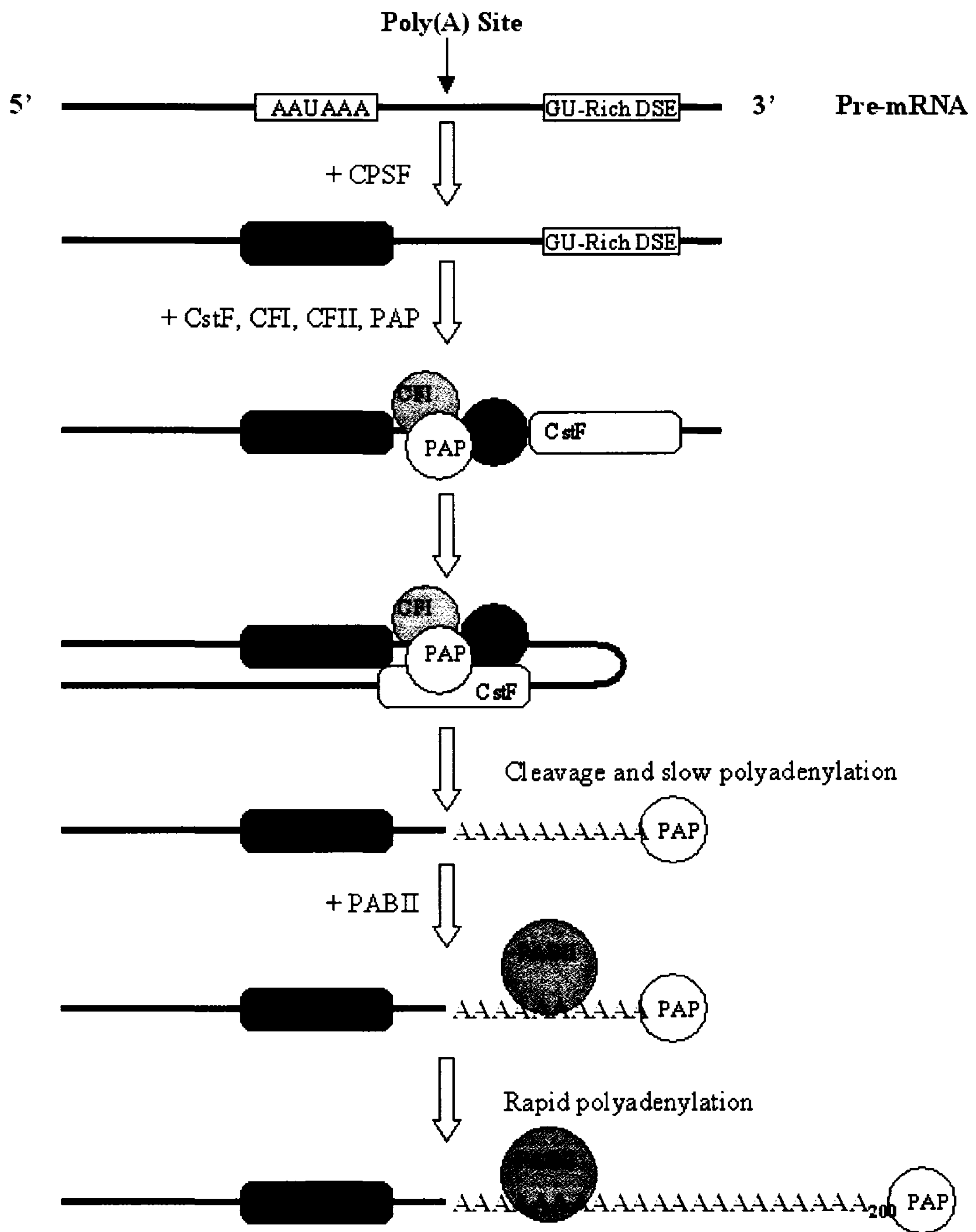

Fig. 1.3. The polyadenylation process in animals. CPSF is recruited to the nascent transcript by the AAUAAA hexamer. CstF binds the pre-mRNA at the GU-rich DSE and recruits CFI, CFII, and PAP to the transcript. Initial polyadenylation following cleavage is slow, but is accelerated by the addition of PABIII. (Reproduced with modification from http://sites.huji.ac.il/malaria/maps/mRNAcleavage.html.)

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sequence element downstream of the AAUAAA hexamer recruits cleavage stimulation factor (CstF) to the pre-mRNA. Binding of CstF and CPSF to the pre-mRNA is cooperative and commits the transcript to undergo cleavage and polyadenylation. Cleavage factors I and II are also required for pre-mRNA cleavage, but the role(s) of these factors are not well described. Addition of the poly(A) tail requires poly(A) binding protein (PAB) and poly(A) polymerase (PAP) to catalyze addition of adenosine residues. Therefore, polyadenylation is a three-step process. First, the cellular machinery must recognize a poly(A) signal on the pre-mRNA. Second, the cellular machinery cleaves the pre-mRNA at a location referred to as the poly(A) site. Third, up to 200 adenosine residues are added to the cleaved pre-mRNA, forming the poly(A) tail.

Early investigations followed one of two routes: identification of sequence motifs required for polyadenylation or identification of cellular factors required for polyadenylation. It took over a decade of investigation before the relationships between the sequence elements and the cellular polyadenylation machinery began to be described. Since that time, considerable progress has been made in determining the precise mechanism by which eukaryotic pre-mRNAs are cleaved and polyadenylated by cellular factors. This section attempts to deliver an historical account of the discoveries leading up to our present understanding of the polyadenylation process. Polyadenylation sequence elements, the cellular polyadenylation machinery, and specific examples of polyadenylation in insects and parvoviruses are discussed.

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Early Polyadenylation Experiments

Edmonds and Abrams published the first paper in the field of polyadenylation in 1960. They identified an enzyme that was capable of polymerizing adenylate units from ATP and called it poly(A) polymerase (PAP) (Edmonds and Abrams, 1960). Interest in PAP was sparked in 1971 by the publication of three studies that demonstrated the presence of poly(A) tails at the 3'-ends of mature mRNAs (Darnell *et al.*, 1971; Edmonds *et al.*, 1971; Lee *et al.*, 1971). A possible role for the poly(A) tail in mRNA stability was suggested in 1975 (Marbaix *et al.*, 1975).

The first discovery of a signal involved in the polyadenylation process came in 1976 (Proudfoor and Brownlee, 1976). Proudfoot and Brownlee's landmark paper reported that an AAUAAA hexanucleotide motif occurred approximately 20 nt upstream of the poly(A) tail in the mRNAs surveyed. Very few nucleic acid sequences were available in 1976. In fact, Proudfoot and Brownlee's study was restricted to the six purified mRNA molecules available at the time. Identification of the AAUAAA poly(A) signal formed the basis for subsequent experiments describing sequence elements involved in polyadenylation.

Much of the early work investigating poly(A) signal sequences was performed with either the major late transcript of adenovirus type 2 (Ad2) or the simian virus 40 (SV40) late mRNA. Transcription of the SV40 late mRNA was shown to extend past the site of polyadenylation suggesting that generation of eukaryotic mRNA 3'-ends is not accomplished by simple transcription termination (Ford and Hsu, 1978). Studies examining the utilization of four different poly(A) sites in the adenovirus major late mRNA suggested that polyadenylation proceeded by an unknown mechanism directed by

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one of several AAUAAA motifs (Nevins and Darnell, 1978). Transcription past the site of poly(A) addition was also confirmed for the Ad2 major late mRNA (Nevins *et al.*, 1980). Studies demonstrating that the mouse β-globin mRNA was processed by cleavage of an extended transcript indicated that the continuation of transcription past the poly(A) site was a phenomenon not exclusive to viral systems (Hofer and Darnell, 1981).

Research throughout the 1980s demonstrated the importance of AAUAAA and other signal sequences for efficient polyadenylation. Deletion of small regions near the SV40 late mRNA poly(A) site revealed that AAUAAA was required for virus viability (Fitzgerald and Shenk, 1981). Mutation of the SV40 late AAUAAA resulted in decreased polyadenylation (Wickens and Stephenson, 1984). Concurrent work with the α-globin mRNA demonstrated that AAUAAA was also important for pre-mRNA cleavage when mutation of the AAUAAA motif generated transcripts that extended beyond the normal cleavage site (Higgs *et al.*, 1983). Sequences downstream of the AAUAAA motif were shown to be important for polyadenylation and cleavage of several pre-mRNAs including the adenovirus E2A (McDevitt *et al.*, 1984), SV40 early (Sadofsky and Alwine, 1984) and β-globin genes (Gil and Proudfoot, 1984). The required downstream sequence elements (DSE) were shown to be GU-rich (McDevitt *et al.*, 1986).

Discovery of the sequence motifs and cellular machinery required for polyadenylation were greatly accelerated by the development of HeLa cell nuclear extracts capable of reproducing the polyadenylation reaction *in vitro* (Manley, 1983; Moore and Sharp, 1984; Moore and Sharp, 1985). These nuclear extracts were also instrumental in the discovery of the spliceosome complex (Grabowski *et al.*, 1985).

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Work with the nuclear extracts demonstrated that a complex is formed on the SV40 late pre-mRNA and that complex formation and pre-mRNA cleavage requires the AAUAAA signal (Conway and Wickens, 1987). Further mutational analysis of AAUAAA solidified this hexanucleotide's role in the efficiency and accuracy of pre-mRNA cleavage and polyadenylation (Sheets *et al.*, 1990). The early studies with nuclear extracts suggested that cleavage and polyadenylation of pre-mRNAs involves formation of a complex and that every base of the AAUAAA motif is important, although a variant AUUAAA motif is tolerated (Wilusz *et al.*, 1989).

Identification and characterization of the cellular components forming the complexes involved in the polyadenylation and cleavage of pre-mRNAs paralleled the discovery of sequence elements directing complex formation, polyadenylation and cleavage. Further discussion of sequence elements comprising the polyadenylation signal, including AAUAAA and DSEs, would be incomplete without a discussion of the cellular complexes and proteins that are now known to interact with these sequence elements.

Discovery of the Polyadenylation Machinery

As mentioned previously, nuclear extracts capable of reproducing cleavage and polyadenylation suggested that mRNA 3'-end formation involved the participation of cellular complexes. Fractionation of nuclear extracts revealed that specific pre-cleavage and post-cleavage complexes were involved in SV40 late mRNA 3'-end processing (Zarkower and Wickens, 1987). Takagaki and colleagues demonstrated that cleavage and polyadenylation could be uncoupled, implicating the involvement of two distinct cellular

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complexes (Takagaki *et al.*, 1988). Further fractionation of HeLa cell nuclear extracts was used to describe the makeup of the complexes involved in pre-mRNA cleavage and polyadenylation. Fractionation yielded one fraction possessing only cleavage activity and another fraction capable of sequence-specific cleavage and polyadenylation (Christofori and Keller, 1988). Even further fractionation produced two factors required only for cleavage (Takagaki *et al.*, 1989). A fraction with polymerase activity was also identified (McDevitt *et al.*, 1988).

The identification of complexes involved in 3'-end processing was greatly aided by studies using UV-crosslinking to identify complexes bound to the 3'-end of pre-mRNAs. UV-crosslinking showed that protein binding to the SV40 early, SV40 late and adenovirus L3 AAUAAA signal could be disrupted by mutagenesis of this hexanucleotide motif (Wilusz and Shenk, 1988). Crosslinking experiments also began to implicate downstream sequences (Gilmartin and Nevins, 1991).

The results of the fractionation and crosslinking experiments culminated in the first models of the polyadenylation process (Fig. 1.3) (Gilmartin and Nevins, 1989; Weiss *et al.*, 1991). The poly(A) signal, containing the AAUAAA hexamer and a GU-rich DSE, is recognized by cleavage and polyadenylation specificity factor (CPSF). Binding of cleavage stimulation factor (CstF) to the DSE enhances the binding of CPSF. Cleavage of the pre-mRNA occurs if the additional cleavage factors I and II (CFI and CFII) and poly(A) polymerase are present. This model was quickly refined to include poly(A) binding protein (PABII) as a required component (Wahle *et al.*, 1993).

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Components of the Polyadenylation Machinery

Cleavage and Polyadenylation Specificity Factor (CPSF)

Cleavage and polyadenylation specificity factor (CPSF) recognizes AAUAAA and is required for cleavage and poly(A) addition (Keller *et al.*, 1991). Initial binding of CPSF to the pre-mRNA is weak. Cooperative interaction of CPSF with CstF bound to the DSE strengthens CPSF binding to the AAUAAA (MacDonald *et al.*, 1994; Weiss *et al.*, 1991; Wilusz *et al.*, 1990). CPSF was shown to have a stimulatory effect on poly(A) polymerase (PAP) (Wahle *et al.*, 1991). CPSF is also required for splicing of single-intron pre-mRNAs *in vivo* (Li *et al.*, 2001).

The CPSF complex contains at least four protein subunits designated CPSF-160, CPSF-100, CPSF-73 and CPSF-30 according to their molecular weights (Bienroth *et al.*, 1991; Murthy and Manley 1992). Although the requirement for CPSF in both cleavage and polyadenylation is well established, the role of each CPSF subunit in performing these tasks is still being ascertained. Murthy and Manley showed that the CPSF-160 subunit binds the AAUAAA motif on pre-mRNAs (Murthy and Manley, 1995). The stimulatory effect of CPSF on PAP has been attributed to the human Fip1 subunit of CPSF (Kaufmann *et al.*, 2004). There are conflicting reports as to which CPSF subunit is responsible for cleavage of the pre-mRNA. It was generally accepted that CPSF-30 was the subunit required for pre-mRNA cleavage due to its homology to the *Drosophila* clipper protein (CLP) which possesses an endonuclease activity (Bai and Tolias, 1998; Zarudnaya *et al.*, 2003). However, more recent UV-crosslinking and mutagenesis experiments have identified CPSF-73 as the subunit responsible for pre-mRNA cleavage (Ryan *et al.*, 2004).

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Cleavage Stimulation Factor (CstF)

Early experiments demonstrated that CstF is required for cleavage, but not poly(A) addition (Gilmartin and Nevins, 1989; Takagaki *et al.*, 1989). CstF was the first cleavage factor isolated (Wilusz *et al.*, 1990). CstF contains at least 3 subunits, designated CstF-50, CstF-64 and CstF-77, that have been expressed using a recombinant baculovirus system (Takagaki and Manley, 1994). CstF binds the pre-mRNA at the GU-rich DSE and interacts cooperatively with CPSF (MacDonald *et al.*, 1994; Takagaki *et al.*, 1997). The CstF subunits of *A. thaliana* have recently been cloned and shown to be functionally similar to the CstF subunits of yeast and mammals (Yao *et al.*, 2002). CstF has also been implicated as a regulator of the cell cycle (Martincic *et al.*, 1998).

CstF-64 is the best characterized subunit of CstF. Binding of CstF-64 to pre-mRNAs is influenced by heterogeneous nuclear ribonucleoprotein (hnRNP) F (Veraldi *et al.*, 2001). Structural data have shown that CstF-64 is the subunit that binds GU-rich DSEs (Perez-Canadillas *et al.*, 2003). Recognition of GU-rich elements by RNA binding motifs within CstF-64 has recently been described (Deka *et al.*, 2005; Salisbury *et al.*, 2006). Perhaps most strikingly, different forms of CstF-64 have been found in different tissues. For example, a variant form of CstF-64, designated τCstF-64, has been found in mouse male germ cells. Interestingly, mouse male germ cell mRNAs lack AAUAAA but are efficiently polyadenylated (Wallace *et al.*, 1999).

Poly(A) Polymerase (PAP)

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PAP catalyzes addition of the poly(A) tail and is also involved in cleavage. The first purified preparations of PAP showed a lack of specificity for RNA substrates (Christofori and Keller, 1991). Addition of CPSF conferred specificity to purified bovine PAP preparations (Raabe *et al.*, 1991; Wahle, 1991a). Multiple forms of PAP have been described in human cells (Thurreson *et al.*, 1994). PAP has also been shown to contain multiple functional domains, including a putative nuclear localization signal (Raabe *et al.*, 1994). Availability of the crystal structures of bovine and yeast PAP has allowed for the identification of putative RNA binding sites and demonstrated that the PAP C-terminal domain is required for processivity (Gershon, 2000; Martin *et al.*, 2000). Nuclear proteins inhibiting PAP activity have been characterized, indicating that gene expression can be controlled at the level of polyadenylation inhibition (Ko *et al.*, 2002). Characterization of protein factors controlling poly(A) tail synthesis and maturation is ongoing (Mangus *et al.*, 2004).

Poly(A) Binding Protein II

Poly(A) binding protein II (PABII) binds the nascent poly(A) tail and stimulates poly(A) tail elongation (Wahle *et al.*, 1993). Wahle and colleagues identified a novel protein that acts as a specificity factor for polymerization of the poly(A) tail (Wahle, 1991b). This protein was shown to bind and stimulate extension of the poly(A) tail, even in the absence of AAUAAA, provided that a short poly(A) tail already existed on the mRNA (Wahle *et al.*, 1993). This protein was designated poly(A) binding protein II. PABII is localized to the nucleus and PABI is localized to the cytoplasm (Kraus *et al.*, 1994). Efficient elongation of the poly(A) tail requires the presence of PAP, CPSF, and

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PABII, suggesting that these three factors remain in contact with each other during poly(A) elongation. Regulation of poly(A) tail length by PABII has been identified as a mode of translational control regulating *Drosophila* development (Benoit *et al.*, 2005).

Cleavage Factors I and II (CFI and CFII)

Cleavage factor I was first described by fractionation experiments in the late 1980s (Takagaki *et al.*, 1989). CFI and CFII are required for cleavage, but not polyadenylation (Ruegsegger *et al.*, 1998). Ruegsegger and colleagues demonstrated that CFI stimulates the cleavage reaction and assembly of the cleavage complex. Sequence motifs within a subunit of CFI mediating RNA binding, protein-protein interactions and subcellular localization have been identified (Dettwiler *et al.*, 2004). The function of CFII is still not clear, but it is known to bridge CFI and CPSF and displays homology to yeast cleavage factors (de Vries *et al.*, 2000).

Sequence Elements Comprising the Polyadenylation Signal

AAUAAA

AAUAAA recruits CPSF to the pre-mRNA (Keller *et al.*, 1991). Early studies suggested that the AAUAAA hexamer was nearly ubiquitous in the 3'-ends of pre-mRNAs, with the exception of a commonly found variant motif AUUAAA (for review see Zhao *et al.*, 1999). Incidences of AAUAAA and AUUAAA polyadenylation signals in the 3' ends of cDNAs used in earlier experiments were reportedly in the range of 80% to 100% (Proudfoot and Brownlee, 1976; Wickens and Stephenson, 1984; Sheets *et al.*,

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1990; Salamov and Solovyev, 1997). However, the sample sizes of the 3'-ends surveyed in these studies were very small with a range of only 6 to 250 cDNAs. More recent analyses of much larger sequence databases have indicated that the incidence of the AAUAAA hexamer is much lower than previously assumed. A survey of 6,210 mouse expressed sequence tags (ESTs) reported AAUAAA at only 26.1% and AUUAAA at 7.9% (Graber *et al.*, 1999). In the same study, a survey of 4,427 human ESTs reported AAUAAA at 53.2% and AUUAAA at 11.7%. Another survey using a database containing 5,647 human ESTs reported AAUAAA at 58.2% and AUUAAA at 14.9% (Beaudoing *et al.*, 2000). Another study compared the incidence of AAUAAA and AWUAAA (where W stands for A, U, C, or G) motifs between 10 different species (Salisbury *et al.*, 2006). The incidence of the AAUAAA hexamer was notably lower for invertebrates (51.0% to 61.8%), compared to vertebrates (69.9% to 77.9%). Nearly 40% of the 3'-ends surveyed from *D. melanogaster* and *An. gambiae* lacked AAUAAA and nearly 30% lacked AWUAAA (Salisbury *et al.*, 2006).

The disparities involving the incidence of pre-mRNA AAUAAA-like motifs between the early and more recent studies are the subject of an excellent review written by MacDonald and Redondo (MacDonald and Redondo, 2002). Tissue-specific variants of the polyadenylation machinery such as τCstF-64 have been postulated to play a role in alternative polyadenylation of pre-mRNAs (Dass *et al.*, 2001).

Downstream Sequence Elements (DSE)

Deletion experiments with the adenovirus E2A and SV40 pre-mRNAs implicated somewhat interchangeable GU-rich sequence elements downstream of the AAUAAA

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hexamer and cleavage site as being important for the polyadenylation process (McDevitt *et al.*, 1986). McDevitt's original study suggested the existence of two separate elements, a U-rich element and a GU-rich element, comprising the DSE. Although many experiments have been performed to characterize DSEs, our current understanding of the sequence composition of DSEs is still relatively poor. DSEs are most often described as GU-rich, diffuse and poorly conserved.

Some progress has been made in characterizing the sequence and functional significance of DSEs in the polyadenylation process. DSEs characterized by single or multiple stretches of up to 5 consecutive U resides, often interrupted by a single G, were found in approximately 70% of mammalian pre-mRNAs (MacDonald *et al.*, 1994; Takagaki and Manley, 1997). GU-rich DSEs were generally found 12 to 40 nt downstream of the pre-mRNA cleavage site. The AAUAAA hexamer and the DSE form the core polyadenylation signal and specify the site of pre-mRNA cleavage and polyadenylation (Chen *et al.*, 1995). Chen and colleagues also reported that a C-A residue is preferred at the cleavage site, but is not essential.

The GU-rich DSE serves as a binding site for the 64 kDa subunit of CstF (MacDonald *et al.*, 1994). AAUAAA recruits CPSF to bind the hexamer via its 160 kDa subunit (Keller *et al.*, 1991; Murthy and Manley, 1995). CPSF-160 has been shown to interact with PAP and CstF-77 indicating a cooperative relationship for the binding of CPSF and CstF to the core polyadenylation signal (Murthy and Manley, 1995). Binding of CPSF and CstF near the cleavage site recruits other factors involved in polyadenylation and cleavage and commits the pre-mRNA to undergo cleavage

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(MacDonald *et al.*, 1994; Gilmartin and Nevins, 1991; Weiss *et al.*, 1991; Wilusz *et al.*, 1990).

A consensus sequence for GU-rich DSEs has been difficult to determine. Additional auxiliary sequences downstream of the core DSE have been implicated in stimulating 3'-end processing by enhancing the binding of CstF to the DSE (Chen *et al.*, 1998). Chen and colleagues proposed that these auxiliary DSEs (AUX DSE) may promote 3'-end processing by forming a stable secondary structure that focuses binding of CstF to the core DSE. Considerable effort has been expended to characterize the sequence content of DSEs, but a consensus has not emerged (for review see Zarudnaya *et al.*, 2003). The most exhaustive study of DSE content to date involved the characterization of DSEs from 10 species performed by Salisbury and colleagues in 2006. They concluded that most DSEs have two parts: a proximal, poorly conserved GU-rich element and a distal, highly conserved U-rich element (Salisbury *et al.*, 2006). Salisbury's study also defined the DSE-recognition motif of CstF-64.

Upstream Sequence Elements

Auxiliary elements located upstream of the AAUAAA hexamer have also been described, but investigations of the sequence content and regulatory role(s) of upstream elements have not been as thorough as investigations involving downstream elements. Several early studies demonstrated the importance of upstream sequence elements (USE) for efficient 3'-end processing in systems including SV40 late, ground squirrel hepatitis virus and adenovirus type 2 (DeZazzo and Imperiale, 1989; Carswell and Alwine, 1989; Russnak, 1991). Sequences upstream of many retroviral polyadenylation signals are

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required for efficient cleavage and polyadenylation (Valsamakis *et al.*, 1991; Valsamakis *et al.*, 1992). Sequence replacement experiments demonstrated that the USE from HIV-1 can substitute for either the SV40 late USE or the ground squirrel hepatitis virus USE (Russnak and Ganem, 1990; Valsamakis, *et al.*, 1991).

A few examples of interaction between an USE and components of the cleavage and polyadenylation machinery have been demonstrated. CPSF binding is promoted by the presence of sequences upstream of the adenovirus major late hexamer (Gilmartin *et al.*, 1996). The HIV-1 USE forms an RNA secondary structure that increases pre-mRNA processing efficiency by enhancing CPSF binding (Gilmartin *et al.*, 1995 and Gravely *et al.*, 1996). The USE of the SV40-late polyadenylation signal is required for efficient pre-mRNA processing and has been shown to interact with the U1 small nuclear ribonucleoprotein particle (snRNP) (Lutz and Alwine, 1994). The C2 complement gene USE has been shown to enhance the binding affinity of CstF and also interacts with the polypyrimidine tract-binding protein (PTB), a well-known regulator of splicing (Moreira *et al.*, 1998). Pre-mRNA cleavage is enhanced in the presence of PTB presenting a possible link between polyadenylation and splicing.

Poly(A) Sequence Motifs and RNA Secondary Structure

RNA secondary structure created by base-pairing interactions between nucleotides in the vicinity of the poly(A) signal plays a significant role in the efficiency of pre-mRNA 3'-end processing. Current evidence suggests that the AAUAAA hexamer is presented as part of a double-stranded structure, often in the form of a stem-loop (Zarudnaya *et al.*, 2003). The first report that local secondary structure near poly(A)

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signals influences cleavage and polyadenylation determined that the HTLV-1 poly(A) signal required formation of a stem-loop structure that brings the hexamer and DSE into close proximity with each other (Bar-Shira *et al.*, 1991). Stem-loop structure is also important for polyadenylation at the murine IgM poly(A) site (Phillips *et al.*, 1999). The ability of the HIV-1 AAUAAA signal to bind polyadenylation factors is controlled by local RNA structure (Klasens *et al.*, 1999). Moreover, Flaherty and colleagues have hypothesized that interactions between the polyadenylation machinery bound at the pre-mRNA 3' end with the capping machinery bound at the 5' end creates mRNAs that exist as closed loops (Flaherty *et al.*, 1997).

The role of AUX DSEs in the formation of local secondary structures important for the polyadenylation process has been investigated. Chen and colleagues proposed that AUX DSEs may form secondary structures required for efficient pre-mRNA 3'-end processing by promoting binding of CstF to U-rich elements (Chen *et al.*, 1998). Determination of the SV40 late poly(A) signal secondary structure indicates that efficient pre-mRNA cleavage requires formation of a stem-loop containing the U-rich DSE and the AAUAAA hexamer (Hans and Alwine, 2000). Hans and Alwine suggest that the secondary structure of the SV40 late poly(A) signal may promote interaction between CstF and the DSE and also propose a catalytic role for this structure in pre-mRNA cleavage.

Research by Zarudnaya and colleagues has shown that G-quadruplexes found in many AUX DSEs form secondary structures that maintain the poly(A) signal in an accessible conformation and may serve as binding sites for proteins influencing polyadenylation efficiency including hnRNP (Zarudnaya *et al.*, 2003). Members of the

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hnRNP H family of ribonucleoproteins are regulators of pre-mRNA splicing (Chou *et al.*, 1999) and have been shown to bind RNA sequences resembling G-rich AUX DSEs (Caputi and Zahler, 2001). Stimulation of polyadenylation following hnRNP binding to the G-rich AUX DSE may be the result of secondary structure formation that promotes assembly of polyadenylation and cleavage complexes (Arhin *et al.*, 2002).

Coupling of Polyadenylation to Other Cellular Processes

Components of the polyadenylation machinery have been implicated in various other cellular processes. The connection between mRNA 3'-end processing and transcription has received much attention in recent years (for review see Proudfoot, 2004). Interaction between the 3' and 5' ends of pre-mRNAs has been shown to involve the association of polyadenylation factors with the 5' cap-binding complex (Flaherty *et al.*, 1997). Deletion analysis of the large subunit of mouse RNA polymerase II (RNA Pol II) revealed that CPSF and CstF bind the C-terminal domain (CTD) of the polymerase (McCracken *et al.*, 1997; Dantonel *et al.*, 1997). Destruction of the RNA Pol II CTD greatly reduces polyadenylation efficiency. Efficient polyadenylation requires contact between the RNA Pol II CTD and CstF-50 (Fong and Bentley, 2001). This interaction is mediated by heptad repeats occurring in the RNA Pol II CTD (Rosonina and Blencowe, 2004).

The polyadenylation process is also coupled to pre-mRNA splicing. Experiments using synthetic pre-mRNA substrates consisting of an SV40 late poly(A) signal and an adenovirus splicing cassette demonstrated that polyadenylation signals are necessary for efficient splicing (Cooke *et al.*, 1999). As mentioned in the section describing upstream

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sequence elements, the splicing regulator polypyrimidine tract-binding protein has been UV-crosslinked to the poly(A) signal of the C2 complement pre-mRNA (Moreiera *et al.*, 1998). CPSF has also been shown to be important for efficient pre-mRNA splicing *in vivo* (Li *et al.*, 2001).

Polyadenylation in Insect Systems

The machinery and sequence motifs required for efficient polyadenylation of insect pre-mRNAs are nearly identical to those required for 3'-end processing of mammalian pre-mRNAs. Although the frequency of the AAUAAA hexamer is lower in *D. melanogaster* and *An. gambiae* pre-mRNAs when compared to the frequency of the hexamer in vertebrate pre-mRNAs (Salisbury *et al.*, 2006), many components of the *Drosophila* cleavage and polyadenylation complexes have been shown to be similar to their mammalian counterparts. Zarudnaya and colleagues have stated that it is likely that the DSEs of poly(A) signals are identical in insects and mammals (Zarudnaya *et al.*, 2003).

Several homologues of the mammalian cleavage and polyadenylation machinery have been described in *Drosophila*. The suppressor of forked (Su(f)) gene product is the *Drosophila* homologue of mammalian CstF-77 (Takagaki *et al.*, 1994). Reduction in pre-mRNA 3'-end processing efficiency has been reported for mutants of Su(f) (O'Hare, 1995) and the role of Su(f) in regulation of poly(A) site choice has recently been confirmed (Juge *et al.*, 2000). Human CstF-77 and drosophilid Su(f) perform the same function in pre-mRNA 3'-end formation in their respective organisms (Benoit *et al.*, 2002). CstF-64 is also highly conserved between humans and *Drosophila* (Takagaki and

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Manley, 1997). Cloning and characterization of CstF-64 from *Drosophila* demonstrated that the Drosophilid homologues of CstF-64 and CstF-77 interact with each other in a fashion that is identical to their interaction in humans (Hatton *et al.*, 2000).

Drosophilid homologues of mammalian CPSF subunits and PAB2 have also been identified. Murthy and Manley's hypothesis that the interaction of CPSF with CstF may be impaired in Su(f) mutants was confirmed when the cloned drosophilid homologue of CPSF-160 was shown to interact with CstF-77/Su(f) (Murthy and Manley, 1995; Salinas *et al.*, 1998). The *Drosophila* "clipper" (CLP) protein has been identified as a CPSF-30 homologue (Bai and Tolias, 1998). Cloned *Drosophila* PAB2 has been shown to be functionally analogous to bovine PAB2 and has been implicated in *Drosophila* development (Benoit *et al.*, 1999). Cloning and characterization of components of the *Drosophila* polyadenylation and cleavage complexes has indicated that regulation of the polyadenylation process in insects involves machinery analogous to mammalian polyadenylation factors.

Parvovirus Polyadenylation

Very few studies have examined polyadenylation and pre-mRNA 3'-end processing within the family *Parvoviridae*. Most reports investigating parvovirus polyadenylation have merely identified potential AAUAAA polyadenylation signals without demonstrating that this particular signal actually directs polyadenylation of viral transcripts. The handful of studies investigating the polyadenylation process within the family *Parvoviridae* have indicated that polyadenylation and cleavage of viral pre-mRNAs is directed by polyadenylation signal sequences similar to those described for

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mammals and *Drosophila*. Polyadenylation and cleavage of parvovirus pre-mRNAs is accomplished by the host cell's polyadenylation machinery. Most components of the polyadenylation machinery are highly conserved in structure and function between mammals and insects.

Several studies have investigated polyadenylation within the vertebrate parvoviruses. The polyadenylation process of the human parvovirus B19 is the most extensively characterized. The initial transcription map for B19 indicated the probable utilization of variant polyadenylation signals in the middle of the virus genome (Ozawa *et al.*, 1987). More recent analysis has characterized the hexamers involved in B19 polyadenylation and defined required downstream and upstream sequence elements (Yoto *et al.*, 2006). Yoto and colleagues have determined that utilization of the nonconsensus AUUAAA motif requires upstream and downstream sequence elements and is also influenced by an adjacent AAUAAC hexamer. The nonconsensus hexamer affords expression of the downstream virus capsid gene by allowing some transcription to continue past the poly(A) site in the middle of the genome. A second poly(A) site approximately 260 nt downstream of the main poly(A) site was shown to be used 10% of the time. U-rich sequences characteristic of previously described DSEs were found downstream of both B19 poly(A) sites. Mutagenesis experiments also identified a 22 nt stretch upstream of the main AUUAAA hexamer required for efficient polyadenylation. Interestingly, the second poly(A) site utilized only 10% of the time occurs immediately downstream of a consensus AAUAAA hexamer. The authors note that it is intriguing as to why the poly(A) site downstream of the more consensus hexamer is not favored.

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Polyadenylation studies have been conducted in several other vertebrate parvoviruses. Polyadenylation of Minute virus of mice (MVM) mRNAs has been shown to occur predominantly at a single site downstream of the final AATAAA in the virus genome (Clemens and Pintel, 1987). Polyadenylation of adeno-associated virus type 5 transcripts has been shown to occur at a site lying within the intronic region in the center of the genome (Qiu *et al.*, 2002). A polyadenylation signal in the middle of the simian parvovirus genome directs polyadenylation of both spliced and unspliced mRNAs (Liu *et al.*, 2004). The transcription profile of Aleutian mink disease parvovirus was recently reevaluated, but sequence motifs governing polyadenylation were not discussed (Qiu *et al.*, 2005a). Efficient polyadenylation of goose parvovirus mRNAs requires a DSE and additional upstream sequences that were not completely defined (Qiu *et al.*, 2005b).

Most examinations of the polyadenylation process within the invertebrate parvoviruses have been confined to the genus *Densovirus*. Potential polyadenylation signals have been identified in *Periplaneta fuliginosa* densovirus (*Pf*DNV), which is postulated to regulate expression of its structural protein genes by alternative polyadenylation and splicing (Yamagishi *et al.*, 1999). More detailed cDNA sequence analysis has revealed that the actual site of polyadenylation for *Galleria mellonella* densovirus (*Gm*DNV) and in *Mythimna loreyi* densovirus (*Ml*DNV) pre-mRNAs occurs immediately downstream of an AAUAAA hexamer (Tijssen *et al.*, 2003; Fediere *et al.*, 2004). Mutational analysis of putative polyadenylation signals and deletion analysis of potential regulatory noncoding regions have not been performed on these densoviruses.

*Ae*DNV expresses two mRNAs that undergo polyadenylation at a site somewhere in the right-end UTR of the virus genome. It is likely that polyadenylation occurs

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immediately downstream of the first AATAAA hexamer following the VP gene stop codon. However, determination of the poly(A) site and identification of sequence elements present in the *Ae*DNV right-end UTR that are required for efficient viral gene expression are necessary before experiments aimed at replacing non-coding sequences with exogenous DNA can be undertaken.

Summary

New strategies for controlling mosquito-borne diseases are badly needed. *Ae*DNV infects mosquitoes that act as vectors for diseases of significant world health importance and has shown great promise as a biological control agent and as a tool for mosquito transduction. The hypothesis of the studies contained herein is that *Ae*DNV can be developed as a vehicle for the delivery of shRNAs to *Ae. aegypti* mosquitoes with the eventual goal of disrupting arbovirus transmission via RNAi and that the right-end UTR of the *Ae*DNV genome is a suitable site for the insertion of a Pol III shRNA expression cassette. Towards this end, experiments describing sequence elements present in the *Ae*DNV right-end UTR required for polyadenylation of viral transcripts and robust gene expression are presented in Chapter 2. The data presented in Chapter 2 also identified non-essential sequences in the *Ae*DNV right-end UTR suitable for replacement with exogenous DNA. Studies resulting in the cloning and characterization of mosquito Pol III promoters capable of mediating RNA interference in *Ae. aegypti* and *An. gambiae* cells are presented in Chapter 3. Chapter 4 reports that *Ae*DNV particles containing genomes that have been modified to include a putative RNAi expression cassette in the right-end UTR are viable and capable of infecting *Ae. aegypti* mosquitoes.

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Chapter 2: Polyadenylation and gene expression in *Aedes aegypti* densovirus

This chapter presents data that in part have been submitted as a manuscript to the Journal of Virology:

Konet, D. S., Carter, D., Afanasiev, B. N., Robinson, E., and Carlson, J. O. (submitted). Polyadenylation and gene expression in *Aedes aegypti* densovirus. J. Virol.

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Introduction

Aedes aegypti mosquitoes are the major vector species for flaviviruses such as yellow fever virus and the dengue viruses. Dengue infects 50 to 100 million people every year and an estimated 2.5 billion people are at risk for the disease (Anonymous, 2002). The lack of effective vaccines, pesticide resistance in mosquitoes, extreme poverty in many disease endemic countries coupled with the erosion of public health infrastructures and lack of vector control programs, have lead to the resurgence of mosquito-borne pathogens including dengue (Beaty, 2005). The failure of traditional methods to reduce the global burden imposed by mosquito-borne diseases has highlighted the need for alternative approaches.

Mosquito densonucleosis viruses, such as *Aedes aegypti* densovirus (*Ae*DNV), have emerged as candidates for use in integrated vector-borne disease control programs (for review see Carlson *et al.*, 2006). *Ae*DNV belongs to the family *Parvoviridae*, subfamily *Densovirinae*, whose members infect only arthropods (Afanasiev and Carlson, 2000; Bergoin and Tijssen, 2000). The *Densovirinae* subfamily is further subdivided into the genera *Densovirus, Iteravirus,* and *Brevidensovirus. Ae*DNV belongs to the *Brevidensovirus* genus. *Ae*DNV possesses many attributes of the ideal biological control agent including a narrow host range, negligible effects on the surrounding environment, and the potential for rapid spread and persistence in mosquito populations. *Ae*DNV has also been developed as a vehicle for the delivery and expression of foreign genes in *Ae. aegypti* mosquitoes.

Effective development of *Ae*DNV as a biological control agent requires knowledge of the virus' molecular biology. *Ae*DNV is a negative-sense DNA virus with

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a small, linear genome 4 kb in size (Afanasiev *et al.*, 1991). The nonstructural genes (NS1 and NS2) and the viral protein genes (VP1 and VP2) are expressed from the left and right halves of the same strand by two promoters, pNS and pVP, respectively (Afanasiev *et al.*, 1990; Afanasiev *et al.*, 1991).

It has been shown previously that *Ae*DNV expresses two transcripts that terminate somewhere in the right-end untranslated region (UTR) of the virus genome (Kimmick *et al.*, 1998; Ward *et al.*, 2001a), but the sequences regulating transcription termination and polyadenylation of viral transcripts have not been identified. The polyadenylation process usually begins with the recognition of a hexanucleotide sequence motif on the nascent transcript by the host cell's polyadenylation machinery (for review of the polyadenylation process see Edmonds, 2002). The most common hexanucleotide motif is AAUAAA. GU-rich sequence elements downstream of the AAUAAA hexamer (DSE) may recruit additional cellular factors to the transcript. DSEs vary tremendously in sequence composition and are absent from many pre-mRNAs (Chen *et al.*, 1998). Auxiliary elements located upstream of the AAUAAA hexamer have also been described, but investigations of the sequence content and regulatory role(s) of upstream elements (USE) have not been as thorough as investigations involving downstream elements (Moreira *et al.*, 1998). RNA secondary structure created by base-pairing interactions between nucleotides in the vicinity of the poly(A) signal may also play a significant role in the efficiency of pre-mRNA 3'-end processing. Current evidence suggests that the AAUAAA hexamer is presented as part of a double-stranded structure, often in the form of a stem-loop (Zarudnaya *et al.*, 2003).

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Several studies have investigated polyadenylation within the vertebrate parvoviruses. The initial transcription map for B19 indicated the probable utilization of variant polyadenylation signals in the middle of the virus genome (Ozawa *et al.*, 1987). More recent analysis has characterized the hexamers involved in B19 polyadenylation and determined that utilization of a nonconsensus AUUAAA motif requires upstream and downstream sequence elements and is also influenced by an adjacent AAUAAC hexamer (Yoto *et al.*, 2006). Polyadenylation of minute virus of mice (MVM) mRNAs occurs at a single site downstream of the final AATAAA in the virus genome (Clemens and Pintel, 1987). Polyadenylation of adeno-associated virus type 5 transcripts has been shown to occur at a site lying within the intronic region in the center of the genome (Qiu *et al.*, 2002). A polyadenylation signal in the middle of the simian parvovirus genome directs polyadenylation of both spliced and unspliced mRNAs (Liu *et al.*, 2004). The transcription profile of Aleutian mink disease parvovirus was recently reevaluated, but sequence motifs governing polyadenylation were not discussed (Qiu *et al.*, 2005a). Efficient polyadenylation of goose parvovirus mRNAs requires a DSE and additional upstream sequences that were not completely defined (Qiu *et al.*, 2005b).

Most examinations of the polyadenylation process within the invertebrate parvoviruses have been confined to the genus *Densovirus*. Members of this genus have their genomes organized in an unusual ambisense fashion. Potential polyadenylation signals have been identified in *Periplaneta fuliginosa* densovirus (*Pf*DNV), which is postulated to regulate expression of its structural protein genes by alternative polyadenylation and splicing (Yamagishi *et al.*, 1999). More detailed cDNA sequence analysis has revealed that the actual site of polyadenylation for *Galleria mellonella*

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densovirus (*Gm*DNV) and in *Mythimna loreyi* densovirus (*Ml*DNV) pre-mRNAs occurs immediately downstream of an AAUAAA hexamer (Tijssen *et al.*, 2003; Fediere *et al.*, 2004). Mutational analysis of putative polyadenylation signals and deletion analysis of potential regulatory noncoding regions have not been performed on these densoviruses.

This study examined polyadenylation in the *Brevidensovirus* genus. The sequences between the VP gene and the right-end of the *Ae*DNV genome were examined for their possible roles in polyadenylation and regulation of viral gene expression. The site of polyadenylation for *Ae*DNV transcripts was determined. Site-directed mutagenesis was used to alter sequences in the right-end UTR of *Ae*DNV to determine the effects on gene expression and virus growth.

Materials and Methods

Plasmid Composition

pUCA is the infectious clone of *Ae*DNV (Afanasiev *et al.*, 1994). p61NcoRE contains a β-gal reporter gene expressed from the pVP promoter of *Ae*DNV. The reporter gene is followed by the right end terminal sequences of *Ae*DNV (Ward *et al.*, 2001a). pBSLuc contains the *Drosophila* Hsp70 promoter driving expression of the pGL-3 firefly luciferase gene (Promega, Madison, WI), (M. Bennett, Ph.D. Dissertation). pEGFP-IEI contains the baculovirus IEI promoter driving expression of enhanced green fluorescent protein (C. Wilusz, personal communication).

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Mutagenesis

For mutagenesis of p61NcoRE, two primers flanking the region of interest (*Ae*DNV structural gene region nucleotides 3635 to 3837) were synthesized (Invitrogen, Carlsbad, CA). Primer DelFlank Fwd binds to *lac*Z sequences upstream of the region of interest. Primer DelFlank Rev binds to pUC sequences downstream of the region of interest (Table 2.1). Mutations were created using two complimentary primers introducing a diagnostic restriction enzyme recognition site at the area being mutagenized. Two PCR reactions were run, one with DelFlank Fwd and the reverse mutagenic primer, and a second with DelFlank Rev and the forward mutagenic primer (Table 2.1). The PCR reagents were obtained from the Qiagen Taq Polymerase PCR Core Kit (Qiagen, Valencia, CA). Each PCR reaction mix contained 5.0 μl 10X PCR buffer (contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$ (pH 8.7)), 1.0 μl 10 mM dNTPs, 1.0 μl of a 5 μM solution of each primer, 40.7 μl H_2O, 0.3 μl Taq (5 units/μl) and 1 μl of p61NcoRE template DNA (10 ng/ul). The PCR reactions were cycled at 95°C for one minute, 47°C for one minute, and 72°C for one minute for a total of thirty cycles. The completed reactions were run on a 1.5% agarose gel and the desired fragments were extracted using the Qiagen Gel Extraction Kit. 1.0 μl of each purified PCR product were mixed together with 10 μl 10X PCR Buffer and denatured for 10 minutes at 95°C, then annealed for 10 minutes at 53°C. Two μl of each flanking primer, 2 μl of 10 mM dNTPs, 81.5 μl of H_2O, and 0.5 μl of Taq polymerase (5 units/μl) were added. The mixture was heated at 74°C for 4 minutes, then cycled 30 times at 95°C for one minute, 47°C for one minute and 72°C for one minute. The fragment containing the desired mutation was gel extracted as described above. The same internal primers were used for mutagenesis of

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Name	Sequence 5'-3'
Del Flank Fwd	ATCAGCCGCTACAGTCAACA
Del Flank Rev	TTATGCTTCCGGCTCGTATG
pUCA Mut Fwd	CAAACACTACTATTCAATGCAAACAACGACAGA
pUCA MutRev	CGAAGGGAGAAAGGCGGACAGGTA
AATAAA Mut Fwd	CATATATAATCCCTAGGGCATTCAAAAAAC
AATAAA Mut Rev	GTTTTTTGAATGCCCTAGGGATTATATATG
ATTATA Mut Fwd	CAAAACTTAAATGCTAGCTTCAACTTGTATCAAC
ATTATA Mut Rev	GTTGATACAAGTTGAAGCTAGCATTTAGTTTTG
Del #1 Fwd	CAAAACTTAAATATTATAAATAAAGCATTCAAAAAAC
Del #1 Rev	GTTTTTTGAATGCTTTATTTATAATATTTAAGTTTTG
Del #2 Fwd	TATATAATCAATAAAGTTCCACCTTAAAACATAA
Del #2 Rev	TTATGTTTTAAGGTGGAACTTTATTGATTATATA
Del #3 Fwd	TATATATCACAATATATTCCACCTCAG
Del #3 Rev	CTGAGGTGGAATATATTGTGATATATA
Del #4 Fwd	CCACCTCCGGGATCCCCG
Del #4 Rev	CGGGGATCCCGGAGGTGG
Del #1A Fwd	CTTAAATATTATATGTATCAACTATAAC
Del #1A Rev	GTTATAGTTGATACATATAATATTTAAG
Del #1B Fwd	ATTATATTCAACTACTATAACACATA
Del #1B Rev	TATGTGTTATAGTAGTTGAATATAAT
Del #1C Fwd	TTCAACTTGTATCACACATATATAATC
Del #1C Rev	GATTATATATGTGTGATACAAGTTGAA
Del #1D Fwd	TATCAACTATAATATAATCAATAA
Del #1D Rev	TTATTGATTATATTATAGTTGATA
Del #1E Fwd	CTATAACACATAAATAAAGCATTC
Del #1E Rev	GAATGCTTTATTTATGTGTTATAG
Del #1F Fwd	CTTAAATATTATACACATATATAATC
Del #1F Rev	GATTATATATGTGTATAATATTTAAG
Del #1G Fwd	TATCAACTATAAAATAAAGCATTC
Del #1G Rev	GAATGCTTTATTTTATAGTTGATA
β-gal qPCR Left	CTCACGCGTGGCAGCATCAGG
β-gal qPCR Right	AGCGGTCAAAACAGGCGGCAGTAA
EGFP-3	GACGTAAACGGCCACAAGTT
EGFP-4	AAGTCGTGCTGCTTCATGTG

Table 2.1. Oligonucleotides used for site-directed mutagenesis and RT-qPCR.

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the same region in pUCA, but with different flanking primers and pUCA as the template DNA. Primer pUCAMut Fwd binds to VP sequences upstream of the region of interest. Primer pUCAMut Rev binds to pUC sequences downstream of the region of interest (Table 2.1).

Cloning

Gel-purified PCR products containing the desired mutation were subcloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) and mutations were verified by sequencing. Transferring the mutation from the pCR4-TOPO subclone to p61NcoRE was accomplished by digesting each construct with BseRI and XhoI (New England Biolabs, Beverly, MA). Transferring the mutation from the subclone to pUCA was accomplished by digesting each construct with NsiI and XhoI (New England Biolabs, Beverly, MA). The restriction digests were run on a 1.5% agarose gel and the desired fragments were isolated. Ligations were performed overnight at 17°C with T4 DNA Ligase (MBI Fermentas, Hanover, MD). Ligated DNA was ethanol precipitated, resuspended in water, and electroporated into *Escherichia coli* DH5-α cells. Electroporated cells were plated on LB-ampicillin plates and incubated at 37°C overnight. Colonies were picked and analyzed by restriction digests of minipreps. Positive colonies were inoculated into 500 ml LB-amp media, grown overnight, and purified using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA).

Additional deletion mutants of p61NcoRE and pUCA were created by restriction digest. Mutagenesis of the consensus AATAAA motif creates the unique AvrII restriction enzyme recognition site, CCTAGG (construct ΔAATAAA). Mutagenesis of

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the ATTATA motif creates the unique NheI restriction enzyme recognition site, GCTAGC (construct ΔATTATA). A 154 bp deletion was created in both constructs by performing a partial digest of the consensus mutant constructs with AvrII and BamHI. The digested parent plasmid was treated with T4 DNA polymerase to fill in the ends, then ligated. This deletion removes all of the right end noncoding sequence between the mutagenized consensus motif and *Ae*DNV's terminal hairpin (construct ΔUTR).

Cells and Transfections

Aedes albopictus C6/36 cells were grown in L-15 medium with 10% fetal bovine serum at 27°C in 6-well plates. The cells were transfected using the Effectene transfection reagent (Qiagen, Valencia, CA) when they were approximately 50% confluent as judged by visual inspection in the microscope at low magnification. Transfections were performed according to the manufacturer's instructions for transfection of wells in a 6-well plate. A fixed amount (40 ng) of luciferase reporter gene plasmid (pBSLuc) was included in each transfection as a control for cell lysis and transfection efficiency.

Protein Expression Assays

At 48 hours post-transfection, cells were rinsed twice with sterile PBS, lysed, and harvested using the Galacto-Light kit (Tropix, Bedford, MA). β-gal expression was quantified by incubating 20 μl of a 1:10 dilution of the cell lysates with Galacton reagent (Tropix, Bedford, MA) and measuring the light produced with a TD-20e luminometer (Turner Designs, Sunnyvale, CA). This procedure is described in detail elsewhere

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(Afanasiev *et al.*, 1999; Kimmick *et al.*, 1998). Luciferase levels, from pBSLuc, were determined using the Luciferase Assay System (Promega, Madison, WI) and a TD-20e luminometer. Arbitrary light units from the β-gal assays were normalized to the average light units of luciferase in order to control for transfection and cell lysis efficiency. Lysates from mock-transfected C6/36 cells were included as negative controls. All assays were performed in triplicate.

RNA Harvest, RT-PCR, and cDNA Sequence Analysis

cDNAs were generated from cells transfected with pUCA in order to determine the site of polyadenylation. Cells were transfected as described previously and harvested at 48 hours post-transfection. Total RNA was harvested using the RNEasy kit (Qiagen, Valencia, CA) and a reverse transcription reaction (RT) was performed. All RT reagents were obtained from the Thermoscript RT-PCR System kit (Invitrogen, Carlsbad, CA). Five µl of RNA was added to 2 µl 10 mM dNTPs, 4 µl DEPC H_2O, and 1 µl of a 5 µM solution containing a degenerate oligo(dT) primer (5'-T_{29}V-3'). The degenerate base anchors the poly(T) primer directly to the polyadenylation site. The above mixture was incubated for 5 minutes at 65°C, then placed on ice. Four µl of cDNA synthesis buffer (contains 250 mM Tris acetate (pH 8.4), 275 mM potassium acetate and 40 mM magnesium acetate), 1 µl of 0.1 mM DTT, 1 µl RNase-Out (40 units/µl), 1 µl DEPC H_2O, and 1 µl Thermoscript RT (15 units/µl) were added to the reaction. The RT reaction mix was incubated for one hour at 55°C, followed by 5 minutes at 85°C to terminate the reaction. 1 µl of RNase-H was added to the completed RT reaction and incubated for 20 minutes at 37°C. 2 µl of the resulting cDNA were added to a PCR

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mixture containing the template cDNA, 5 µl 10X PCR buffer, 1 µl 10 mM dNTPs, 1 µl of pUCAMut Fwd primer, 1 µl of degenerate oligo(T) primer, 39.7 µl H_2O, and 0.3 µl Taq polymerase (5 units/µl). The PCR reaction was incubated for 1 minute at 95°C, 1 minute at 45°C, and 1 minute at 72°C for 30 cycles. The resulting PCR fragments were gel extracted and cloned into the pCR4-TOPO vector as described above. Twelve clones per construct were sequenced using an automated sequencer at Colorado State University.

_Ae_DNV Quantitative Real-Time PCR Analysis

Virus yield was quantitated using a real-time polymerase chain reaction (qPCR) assay (Ledermann *et al.*, 2004). A set of primers and a TaqMan probe were designed within a region of the *Ae*DNV NS1 gene (Table 2.1). The Brilliant Quantitative PCR Core Reagent kit provided the polymerase, dNTPs, $MgCl_2$, and other proprietary components (Stratagene, La Jolla, CA). Three hundred nM of each primer and 225 nM of probe were found to be optimal for each 50 µl reaction. The PCR reactions were placed in individual wells of a 96-well optical reaction plate, covered with optical caps and cycled with an ABI Prism model 7700 sequence detection system (ABI, Foster City, CA). The following thermocycler program was used: (1) 50°C for 2 minutes, (2) 95°C for 10 minutes, (3) 95°C for 15 seconds, (4) 60°C for 1 minute, and (5) 40 repetitions (step 3).

The pANS1-GFP plasmid (Afanasiev *et al.*, 1999) was used as a standard to calculate viral genome numbers. UV spectrophotometry (Model 500 Beckman, Fullerton, CA) was used to determine the concentration of the plasmid. The pANS1-GFP plasmid has a calculated weight of 7.1×10^{-12} µg/plasmid. The standard was serially

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diluted to obtain plasmid concentrations of 1 X 10^7-1 X 10^1 genome equivalents/µl (geq/µl). A "working set" of standards was discarded after a maximum of three freeze/thaw cycles.

Quantification of β-gal mRNA Levels Using Quantitative Reverse-Transcription Real-Time PCR

A quantitative reverse-transcription real-time PCR (RT-qPCR) assay was used to measure the amount of β-gal mRNA produced by mutant and wild-type versions of p61NcoRE. C6/36 cells were grown in individual wells of a 6-well plate and were transfected with 400 ng of the appropriate DNA using the Qiagen Effectene transfection kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Forty ng of the GFP reporter gene plasmid, pEGFP-IEI, was included in each transfection mixture to control for transfection and cell lysis efficiency.

mRNA was harvested from cells at 48 hours post-transfection using a Trizol-based RNA extraction protocol (Invitrogen, Carlsbad, CA). Cells were scraped into the growth media, transferred to a 1.7 ml eppendorf tube, and pelleted by centrifugation at 5,000 rpm for 2 minutes. The growth medium was decanted and the cells were suspended in 500 µl of Trizol by vortexing. The cells were incubated in Trizol for 5 minutes at room temperature. Following incubation, 100 µl of chloroform were added and each sample was centrifuged at 12,000 rpm for 10 minutes at 4°C. The aqueous phase was transferred to a new 1.7 ml eppendorf tube, 250 µl of isopropanol were added and the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The resulting pellet was washed with 70% ethanol and dried for 5 minutes in a Savant model SVC-

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100H Speedvac concentrator (Savant, Farmingdale, NY). The pellet was resuspended in 160 μl of RNase-free water. DNase digestion was accomplished by adding 20 μl of DNase I buffer (contains 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 1 mM $CaCl_2$) and 20 μl of RNase-free DNase I enzyme (MBI Fermentas, Hanover, MD). The samples were incubated for 20 minutes at 37°C and extracted using 200 μl of phenol-chloroform. The aqueous phase was transferred to a new 1.7 ml eppendorf tube. RNA was precipitated by adding 66 μl of 10 M ammonium acetate and 500 μl of 100% ethanol, followed by centrifugation at 12,000 rpm for 10 minutes at room temperature. The resulting pellet was washed with 70% ethanol, dried for 5 minutes in a Speedvac concentrator and resuspended in 20 μl of water. RNA concentration was determined by UV-spectrophotometry using an Eppendorf Biophotometer (Eppendorf, Westbury, NY). Five hundred ng of total RNA per sample were reverse-transcribed using the Superscript III RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol for random hexamer primed RT reactions. cDNAs generated by the RT reaction were used immediately for real-time PCR.

β-gal mRNA levels were determined using a SYBR Green-based real-time PCR strategy. Primers β-gal qPCR Left and β-gal qPCR Right were designed to amplify a 237 bp region near the 3'-end of the β-gal gene (Table 2.1). Primers EGFP-3 and EGFP-4 amplify the control GFP reporter gene (Table 2.1). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each 25 μl reaction contained 12.5 μl of Supermix (contains 100 mM KCl, 40 mM Tris-HCL (pH 8.4), 0.4 mM of each dNTP, iTaq DNA polymerase (50 units/ml), 6 mM $MgCl_2$, 20 nm flouresein, and a proprietary amount of SYBR Green 1 and stabilizers), 1 μl of upstream primer (2.5 μM),

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1 μl of downstream primer (2.5 μM), 8.5 μl of water, and 2 μl of template cDNA generated by the RT reaction. The PCR reactions were placed in individual wells of a 96-well optical reaction plate, covered with optical caps and cycled with an MJ Research DNA Engine Opticon 2 Continuous Fluorescence Detector (Bio-Rad, Hercules, CA). The following thermocycler program was used: (1) 50°C for 10 minutes, (2) 95°C for 5 minutes, (3) 95°C for 10 seconds, (4) 60°C for 30 seconds, (5) 85°C for 2 seconds, (6) plate read, (7) 40 repetitions (step 3), (8) 95°C for one minute, (9) 55°C for 1 minute. A plate read temperature of 85°C was found to be optimal by melt curve analysis.

The p61NcoRE wild-type plasmid was used as a standard to calculate β-gal mRNA transcript numbers. UV spectrophotometry (Eppendorf, Westbury, NY) was used to determine the concentration of the plasmid. The p61NcoRE plasmid has a calculated weight of 7.34×10^{-12} μg/plasmid. The standard was serially diluted to obtain plasmid concentrations of 1×10^{7}-1×10^{2} plasmids/μl. mRNA levels were calculated using MJ Opticon Monitor analysis software version 3.1 (Bio-Rad, Hercules, CA). β-gal mRNA levels were normalized to GFP mRNA levels to control for transfection and cell lysis efficiency. Lysates from mock-transfected C6/36 cells were included as negative controls. All assays were performed in triplicate.

RNA Secondary Structure Predictions

The effect of each deletion or mutation on local RNA secondary structure was determined using the mfold program for RNA folding version 3.2 by Zuker and Turner (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi).

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Results

cDNA analysis of *Ae*DNV polyadenylation

To identify the site of polyadenylation, RT-PCR, using an *Ae*DNV-specific forward primer in the VP gene and a degenerate oligo(T) primer, was performed on mRNA harvested from cells transfected with pUCA. The resulting fragment was cloned into a Topo-TA cloning vector and 12 clones were sequenced to determine the site of polyadenylation. These cDNA sequence analyses revealed that polyadenylation of *Ae*DNV transcripts occurs at nucleotide (nt) 3698 in the *Ae*DNV genome (Fig. 2.1a). This site is 13 nt downstream of the canonical AATAAA polyadenylation signal. No heterogeneity in the point of polyadenylation was observed in the twelve clones that were sequenced. Site-directed mutagenesis was used to change the AATAAA polyadenylation signal to CCTAGG. This mutation shifts the site of polyadenylation 34 bases upstream to nt 3664, indicating that the canonical AATAAA motif acts as the polyadenylation signal for wild-type *Ae*DNV (Fig. 2.1a). The location of the new polyadenylation site suggests that the ATTATA sequence, occurring at nt 3640, may direct polyadenylation of *Ae*DNV transcripts when the wild-type motif is unavailable. The ATTATA sequence located at nt 3640 occurs in the same position as the conserved AATAAA polyadenylation signal in closely related brevidensoviruses, including *Aedes Peruvian* densovirus (*A*PeDNV) and *Aedes albopictus* densovirus (*AAl*DNV) (Genbank accession numbers: AY310877 and X74945) (Boublik *et al.*, 1994). Mutagenesis of the ATTATA sequence alone has no effect on the site of polyadenylation.

Previous experiments with *Ae*DNV have utilized a construct, pUCAinv, for transactivation of viral promoters (Afanasiev *et al.*, 1994; Ward *et al.*, 2001a). In this

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a)

3675

*Ae*DNV: CTATAACACATATATAATC**AATAAA**GCATTCAAAAAACATATAAG

*Ae*DNV cDNA: CTATAACACATATATAATC**AATAAA**GCATTCAAAAAAC**AAAAAA**$_n$

*Ae*DNV ΔAATAAA cDNA: CTAT**AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**$_n$

*Ae*DNV ΔATTATA cDNA: CTATAACACATATATAATC**AATAAA**GCATTCAAAAAAC**AAAAAA**$_n$

b)

2750

*Ae*DNV: ATG**AATAAA**GGCAACCACACGGTATATCATGTAGTAAAGCAGCAAAAATAC

*Ae*DNV cDNA: ATG**AATAAA**GGCAACCACACGGTATATCATGTAGTAAAGCAG**AAAAAAAAA**$_n$

Fig. 2.1. Polyadenylation sites in the *Ae*DNV genome. (a) Effects of mutagenesis on the site of *Ae*DNV transcript polyadenylation. (b) Polyadenylation at a site following the NS1 gene of the *Ae*DNV genome. The canonical AATAAA polyadenylation motif is shown in bold.

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construct, the sequences containing the wild-type polyadenylation signal described here were inverted, and therefore unavailable for transcription termination. However, the level of transactivation was similar to the level observed when pUCA was used as a helper. This implies that NS1 protein can be expressed efficiently when other sequences are used for polyadenylation and termination of the transcript. To determine the alternate site of polyadenylation, RT-PCR using a virus-specific forward primer located near the end of the coding sequences for NS1 along with the degenerate oligo(T) primer was performed on mRNA harvested from transfected cells. Cloning and sequencing the products obtained from the RT-PCR identified a second polyadenylation site located at nt 2767, downstream of an AATAAA motif observed downstream of the NS1 gene (Fig. 2.1b).

Mutational analysis of the *Ae*DNV right-end UTR

To determine the sequences critical for gene expression and likely involved in the transcription termination process, a construct, p61NcoRE, containing the *lac*Z reporter gene fused to the VP reading frame at nt 2674 was used (Afanasiev *et al.*, 1994; Ward *et al.*, 2001a). The *lacZ* gene is followed by the right-end UTR of *Ae*DNV. Mutations or deletions were introduced into viral sequences in the right-end UTR as shown in Figure 2.2. These mutant constructs were co-transfected into C6/36 cells along with the pBSLuc luciferase control plasmid and then assayed for reporter gene expression. β-gal expression was normalized to the luciferase expression and compared to the expression of unmodified p61NcoRE arbitrarily set at 100%. The mutagenesis results are summarized in Figure 2.3. Changing the canonical AATAAA polyadenylation signal at nt 3680 to

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```
                              3650                              3675
AeDNV:       ACTTAAATATTATATTCAACTTGTATCAACTATAACACATATATAATCAATAAAGCATTCA
Deletion #1: ACTTAAATATTATA-----------------------------------------AATAAAGCATTCA
Deletion #2: ACTTAAATATTATATTCAACTTGTATCAACTATAACACATATATAATCAATAAAG-------
Deletion #3: ACTTAAATATTATATTCAACTTGTATCAACTATAACACATATATAATCAATAAAGCATTCA
Deletion #4: ACTTAAATATTATATTCAACTTGTATCAACTATAACACATATATAATCAATAAAGCATTCA

                       3700                         3725                      3750
AeDNV:       AAAAACATATAAGTCAAATTAATATATATCACAATAAAAATCCACCTTAAAACATAAGCT
Deletion #1: AAAAACATATAAGTCAAATTAATATATATCACAATAAAAATCCACCTTAAAACATAAGCT
Deletion #2: -------------------------------------------- TCCACCTTAAAACATAAGCT
Deletion #3: AAAAACATATAAGTCAAATTAATATATATCACAATAAAAA-------------------------
Deletion #4: AAAAACATATAAGTCAAATTAATATATATCACAATAAAAATCCACCTTAAAACATAAGCT

                                  3775                          3800
AeDNV:       TAATTTCCACCTCCGTATTCCACCTCAGAATATTGGCTTAAAATCCACCTCCAATGATACA
Deletion #1: TAATTTCCACCTCCGTATTCCACCTCAGAATATTGGCTTAAAATCCACCTCCAATGATACA
Deletion #2: TAATTTCCACCTCCGTATTCCACCTCAGAATATTGGCTTAAAATCCACCTCCAATGATACA
Deletion #3: ---------------- TATTCCACCTCAGAATATTGGCTTAAAATCCACCTCCAATGATACA
Deletion #4: TAATTTCCACCTCC-----------------------------------------------------

                      3825
AeDNV:       GTTAGGAAGCTAATATTAGTCCGGGATCCCCG
Deletion #1: GTTAGGAAGCTAATATTAGTCCGGGATCCCCG
Deletion #2: GTTAGGAAGCTAATATTAGTCCGGGATCCCCG
Deletion #3: GTTAGGAAGCTAATATTAGTCCGGGATCCCCG
Deletion #4: --------------------------- GGATCCCCG
```

Fig. 2.2. The right-end UTR of *Ae*DNV. The nucleotides removed by each deletion are shown by the dashed lines. The TAA stop codon for the VP gene and the AATAAA polyadenylation motif are shown in bold. The variant ATTATA motif and the GGATCC BamHI recognition site marking the beginning of *Ae*DNV's terminal hairpin are underlined.

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Construct	β-gal	qPCR
Wild-Type	100%	100%
AATAAA Mut	56±3	63±8
ATTATA Mut	15±1	60±9
Double Mut	34±4	15±2
ΔUTR	0±0.3	0±0.5
Del #1	0±0.2	0±0.3
Del #2	84±9	36±7
Del #3	130±19	230±38
Del #4	98±5	2±0.3

Fig. 2.3. Effects of mutations and deletions on β-galactosidase expression (β-gal) and virus production (qPCR).

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CCTAGG reduces β-gal expression by 44% (Fig. 2.3 AATAAA Mut). Changing the ATTATA sequence at nt 3640 to GCTAGC reduces expression by 85% (Fig. 2.3 ATTATA Mut). A double mutant construct was also assayed, yet showed no further reduction in reporter gene expression (Fig. 2.3 Double Mut).

To identify potential auxiliary downstream sequence elements (DSE), four deletions were constructed in the right end UTR (Fig. 2.2). Deletion of the 34 nucleotides between the ATTATA sequence and the canonical AATAAA polyadenylation motif (deletion #1) results in no detectable β-gal expression (Fig. 2.3). Deletion #2 removes the 45 nucleotides of sequence following the AATAAA signal. This construct deletes the C-A sequence identified as the site of polyadenylation for *Ae*DNV, and expresses β-gal at 84% of the wild-type. cDNA sequence analysis of the mRNA showed that polyadenylation was shifted to a C-A sequence located 15 nt downstream of the AATAAA signal in this deletion construct (data not shown). Deletions #3 and #4 have little or no detrimental effect on β-galactosidase production, expressing at 130% and 94% of wild-type AeDNV respectively (Fig. 2.3).

To determine the effects of these mutations on virus growth, the same mutations and deletions were made in the infectious clone of *Ae*DNV, pUCA. Quantitative real-time PCR (qPCR) was performed on *Ae*DNV virions harvested from the supernatants of cells transfected with different versions of pUCA to determine the effect of each mutation or deletion on virus growth. In general, the effect of each mutation or deletion on virus growth, as assayed by qPCR, agrees with the trend observed in the β-gal expression experiments (Fig. 2.3). Mutagenesis of either the consensus AATAAA motif or the ATTATA sequence results in approximately a 40% reduction in virus growth when

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compared to pUCA. Mutagenesis of both motifs causes an 85% reduction in virus growth (Fig. 2.3). Surprisingly, a similar reduction was not observed in β-gal constructs containing the same double mutation. Increased virus growth was observed for the pUCA deletion #3 construct, confirming the β-gal expression data. Interestingly, removal of the 69 nucleotides of sequence upstream of *Ae*DNV's terminal hairpin (deletion #4) reduces virus growth by 98%, but has no significant effect on the production of β-gal. Since this construct is capable of producing protein, the crippling effect on virus production is likely due to disruption of the virus replication process.

Characterization of an *Ae*DNV Upstream Sequence Element

The ablation of β-gal expression and virus growth observed when the 34 nt upstream of the AATAAA hexamer are removed (deletion #1) suggests that a required upstream sequence element (USE) exists in this region of the *Ae*DNV right-end UTR (Figures 2.2 and 2.3). To determine sequences comprising the USE, additional, smaller deletions were created within the deletion #1 region of p61NcoRE (Figure 2.4a). The resulting constructs (deletion #1a-deletion #1g) were assayed for β-gal expression (Figure 2.4b). Removal of any 6-7 nt stretch within the deletion #1 region does not completely abolish reporter gene expression (deletions #1a-1e). However, deletion of 21 nt comprising the 5'-end of deletion #1 does result in ablation of β-gal expression (deletion #1f). The 21 nt removed in deletion #1f appear to comprise the minimal essential region of the USE.

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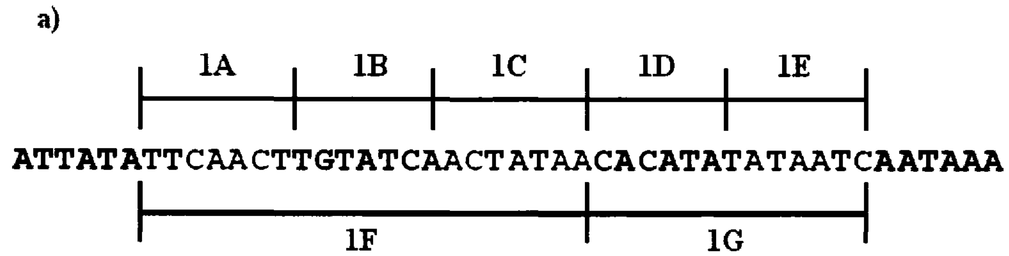
a)



b)

Construct	% β-Gal Expression	% mRNA Expression
AATAAA Mut	56 ± 3	89 ± 5
ATTATA Mut	15 ± 1	101 ± 9
Double Mut	34 ± 4	66 ± 7
ΔUTR	0 ± 0.3	28 ± 9
Deletion 1	0 ± 0.2	0.2 ± 0.2
Deletion 1A	114 ± 3	102 ± 15
Deletion 1B	111 ± 4	92 ± 9
Deletion 1C	124 ± 7	145 ± 26
Deletion 1D	62 ± 7	97 ± 7
Deletion 1E	83 ± 6	222 ± 18
Deletion 1F	0 ± 0.3	0.3 ± 0.2
Deletion 1G	55 ± 5	130 ± 19
Deletion 2	84 ± 9	38 ± 13

Fig. 2.4. Deletion analysis of the *Ae*DNV right-end UTR. a) Sequences deleted in each construct. b) The effect of each deletion on β-gal expression and mRNA expression.

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mRNA Quantification and RNA Secondary Structure Predictions

The relative amounts of β-gal mRNA expressed by mutants of p61NcoRE were determined using RT-qPCR. The results of this experiment are shown in Figure 2.4b. Mutagenesis of the hexanucleotide motifs did not have a significant effect on mRNA levels. Removal of UTR sequences from the mutagenized AATAAA hexamer to sequences comprising the terminal hairpin (ΔUTR) results in ablation of β-gal expression, although mRNA expression at 28% of wild-type was observed. These observations suggest that the ΔUTR mutant is most likely deficient at the level of translation. The near-total reduction in both β-gal and mRNA expression observed with the deletion #1 and deletion #1f constructs indicates that the sequences removed by these deletions are necessary for expression of transcripts from the pVP promoter of *Ae*DNV.

The effect of each mutation or deletion on local RNA secondary structure within the *Ae*DNV right-end UTR was predicted using mfold (Fig. 2.5). Pre-cleavage (Fig. 2.5a) and post-cleavage (2.5b) structures were determined for wild-type *Ae*DNV. Post-cleavage complexes contained sequences from the UAA VP stop codon to the C-A residue identified as the site of polyadenylation. Pre-cleavage complexes contained 34 nt of additional downstream sequences. Deletion analysis demonstrated that sequences downstream of the AATAAA hexamer were not essential for gene expression (Figure 2.3, deletion #2). Therefore, secondary structure predictions for mutated *Ae*DNV right-end UTRs were restricted to post-cleavage complexes. The RNA secondary structure predictions assume that cleavage and polyadenylation occurs at the wild-type poly(A) site (Fig. 2.1a) with the exception of the consensus mutant (ΔAATAAA).

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Fig. 2.5. *Ae*DNV right-end UTR RNA secondary structure predictions. Black lines indicate the locations of the AAUAAA and AUUAUA hexamers. Black lines are also used to indicate mutagenized hexamers. a) Wild-type pre-cleavage structure. The poly(A) site is indicated by an arrow. b) Wild-type post-cleavage structure. The sequences removed by each deletion are shown. c) ΔAATAAA post-cleavage structure. d) ΔATTATA post-cleavage structure. e) Double mutant post-cleavage structure. f) Deletion #1 post-cleavage structure. g) Deletion #1a post-cleavage structure. h) Deletion #1b post-cleavage structure. i) Deletion #1c post-cleavage structure. j) Deletion #1d post-cleavage structure. k) Deletion #1e post-cleavage structure. l) Deletion #1f post-cleavage structure. m) Deletion #1g post-cleavage structure.

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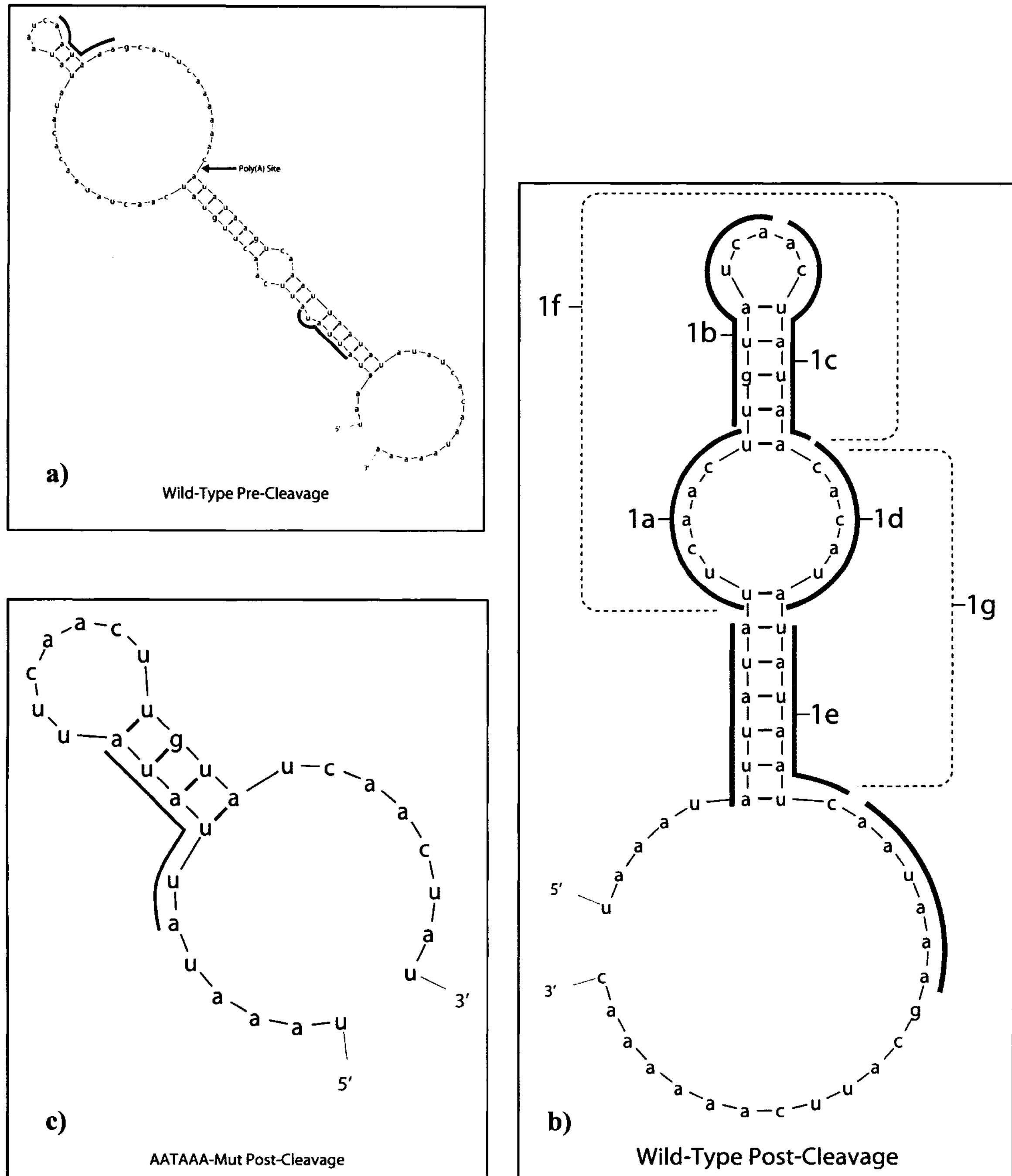

Fig. 2.5

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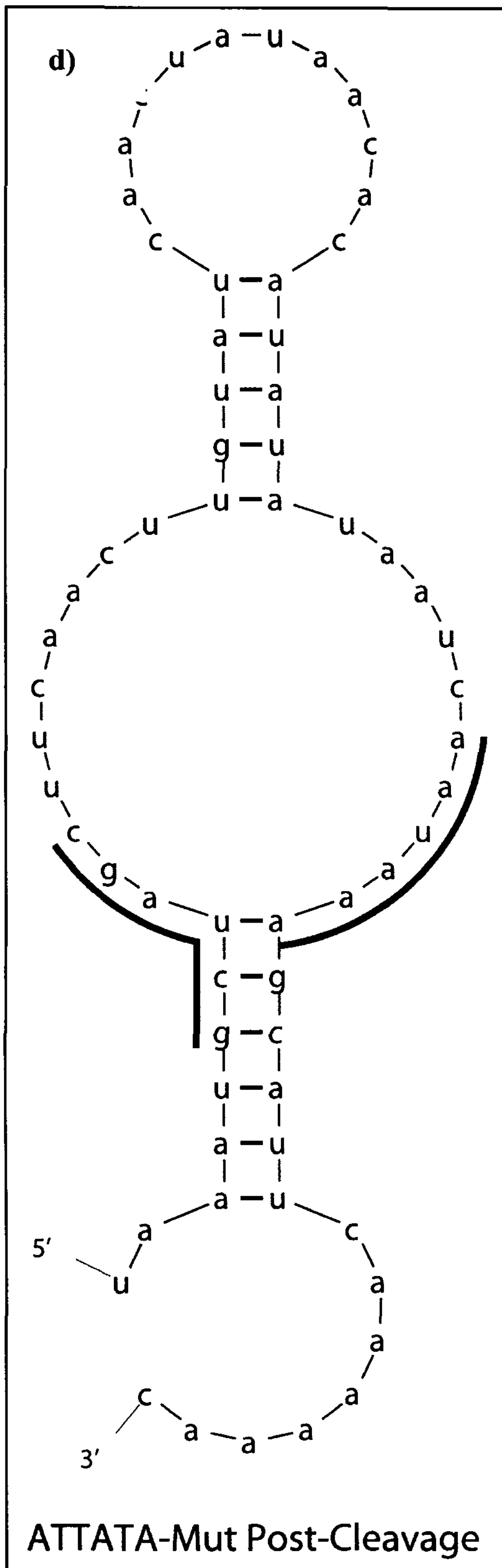

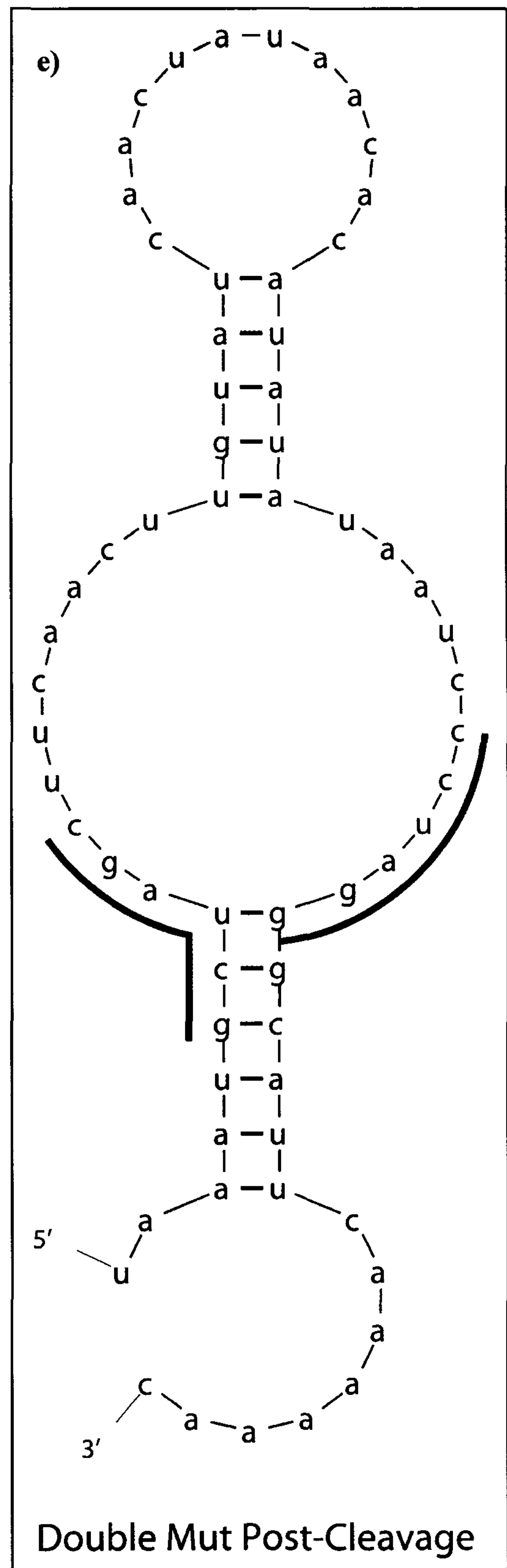

Fig. 2.5

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f)

Deletion 1 Post-Cleavage

g)

Deletion 1A Post-Cleavage

h)

Deletion 1B Post-Cleavage

i)

Deletion 1C Post-Cleavage

Fig. 2.5

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j)

Deletion 1D Post-Cleavage

k)

Deletion 1E Post-Cleavage

l)

Deletion 1F Post-Cleavage

m)

Deletion 1G Post-Cleavage

Fig. 2.5

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The predicted pre-cleavage (Fig. 2.5a) and post-cleavage (Fig. 2.5b) RNA secondary structures for wild-type *Ae*DNV suggest the presence of stem-loop structures in the right-end UTR of the viral genome. The AAUAAA hexamer is presented as part of the stem-loop in the pre-cleavage structure (Fig. 2.5a) and immediately following the stem-loop in the post-cleavage structure (Fig 2.5b). The AUUAUA motif forms the base of the stem in both structures. The sequences removed in the deletion #1 construct form the stem-loop. The sequences removed by each deletion are shown in Fig. 2.5b. The predicted effect of each mutation or deletion on local RNA secondary structure is shown in figures 2.5c-2.5m. Deletion #1 destroys the majority of the stem-loop and is deficient in β-gal mRNA expression (Fig. 2.5f). Deletions 1a through 1g all disrupt the conformation of the stem-loop structure, yet constructs containing these deletions are all capable of expressing mRNA with the exception of deletion 1f (Fig. 2.4b). The observation that constructs forming structures such as deletion 1b (Fig. 2.5h) or deletion 1g (Fig. 2.5m) are capable of mRNA expression while the similar structure formed by deletion 1f (Fig. 2.5l) is not suggests that the ablation of mRNA expression observed with deletion #1 and deletion 1f is most likely not attributable solely to changes in local RNA secondary structure.

Discussion

According to the previous northern blot data, two RNA transcripts are expressed from the *Ae*DNV genome (Ward *et al.*, 2001a). These data imply that transcripts expressed from pNS, as well as transcripts expressed from pVP, terminate in the right-end UTR of the *Ae*DNV genome. We used cDNA sequence analysis to demonstrate that

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polyadenylation of *Ae*DNV transcripts occurs in the right-end UTR, 13 nucleotides downstream of a canonical AATAAA polyadenylation signal. The hypothesis that the AATAAA hexamer is responsible for directing polyadenylation of wild-type *Ae*DNV transcripts was confirmed by mutagenesis of this motif that caused the site of polyadenylation to be shifted 34 bases upstream. This 34 base shift places the polyadenylation site in close proximity to the ATTATA sequence which is located at the position where closely related brevidensoviruses including *Aal*DNV, *A*PeDNV and *He*DNV contain the canonical AATAAA polyadenylation motif (Boublik *et al.*, 1994). This may indicate that the ATTATA sequence directs polyadenylation of *Ae*DNV transcripts when the canonical motif is unavailable. The ATTATA hexamer of *Ae*DNV may also influence pre-mRNA processing in a manner similar to the AAUAAC hexamer of B19 (Yoto *et al.*, 2006).

This type of transcriptional profile in which all transcripts are polyadenylated at an AATAAA signal in the right-end of the virus genome is common among members of the family *Parvoviridae* (Muzyczka and Berns, 2001), with the exception of B19 (Ozawa *et al.*, 1987), simian parvovirus (SPV) (Liu *et al.*, 2004) and members of the *Densovirus* genus (Bergoin and Tijssen, 2000). Previous experiments with pUCAinv, a construct with an inversion of the right-end UTR of *Ae*DNV, indicated that polyadenylation of transcripts expressed from pNS must be occurring somewhere else (Afanasiev *et al.*, 1994; Ward *et al.*, 2001a). We demonstrated that polyadenylation of NS transcripts can occur following the NS1 gene at nt 2767 downstream of an AATAAA motif located at nt 2734. A transcript terminating at this site would contain only NS genes. However, a transcript of expected size was not observed on the northern blot indicating that

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polyadenylation at this location is relatively rare during normal infection (Ward *et al.*, 2001a).

Site-directed mutagenesis was used to explore other sequences in the right-end UTR of the *Ae*DNV genome that may be important for transcription termination and robust expression of viral genes. These are the first studies to report the effects of mutagenesis on the polyadenylation process within the virus subfamily *Densovirinae*. We mutagenized potential polyadenylation signals and created a series of deletions in the right-end UTR of *Ae*DNV to identify sequence elements required for viral gene expression. Mutagenesis of the consensus AATAAA polyadenylation motif causes a significant decrease in expression of proteins from the pVP promoter as well as a decrease in virus production compared to wild-type *Ae*DNV (Fig. 2.3). The effects on gene expression were quantitated in the β-gal assay system and the effect on virus growth was demonstrated by qPCR analysis of mutant viruses produced by modified infectious clones of *Ae*DNV. Surprisingly, mutagenesis of the ATTATA sequence results in a similar reduction in virus growth when compared to mutagenesis of the consensus motif as determined by qPCR (Fig. 2.3). Mutagenesis of either of these two motifs did not significantly alter the amount of mRNA produced by constructs containing these mutations (Figure 2.4b).

Constructs in which both the consensus AATAAA polyadenylation motif and the ATTATA sequence have been mutagenized show a significant reduction in their ability to express viral proteins. Real-time PCR analysis has shown that mutagenesis of both motifs causes a nearly ten-fold decrease in virus growth (Fig. 2.3). However, a significant amount of mRNA expression continues in the absence of these motifs (Figure

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2.4b). These observations strongly suggested the existence of additional sequence elements, such as a GU-rich DSE, in the noncoding region following the VP gene. GU-rich DSEs required for efficient polyadenylation have been described in numerous mammalian systems, as well as in the insect virus family *Baculoviridae* (Van Oers *et al.*, 1999; Edmonds, 2002; Jin and Guarino, 2000). The right-end UTR of *Ae*DNV lacks any significant stretches of G/U residues, although it is very U-rich. However, the sequence composition of previously described DSEs remains poorly described (Chen and Wilusz, 1998; Xie *et al.*, 2005).

Although the right-end UTR of *Ae*DNV contains no sequences of significant similarity compared to previously described DSEs, several of these deletions have notable affects. A near-total reduction in mRNA levels, protein expression and virus viability was observed in the case of deletion #1 that removed the 34 intervening nucleotides between the two candidate polyadenylation hexamers. This observation indicates that critical sequences are contained in these 34 nucleotides that would need to be maintained in any gene delivery applications involving insertion of exogenous DNA in the right-end UTR of *Ae*DNV. Deletion #2 removes 46 nucleotides including the C-A sequence identified as the site of transcript cleavage and polyadenylation. This deletion reduces virus production and mRNA expression by approximately 65%. cDNA sequence analysis confirmed that polyadenylation of *Ae*DNV deletion #2 transcripts occurs at the next C-A residue of suitable proximity when the site of wild-type polyadenylation is unavailable (data not shown). Deletion #4 removes the 69 nucleotides immediately upstream of the sequences responsible for creating *Ae*DNV's terminal hairpin. β-gal constructs containing this deletion continue to express protein at a level comparable to

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the wild-type (Fig. 2.3). However, the lack of viral yield suggests that these sequences are necessary for virus production and are likely involved in the replication process (Brunstein and Astell, 1997). Deletion #3 removes 35 nucleotides in the right-end UTR of *Ae*DNV (Fig. 2.2). Constructs containing deletion #3 exhibit increased levels of protein expression as well as increased *Ae*DNV yield (Fig. 2.3). These mutational analyses identified non-essential sequences downstream of the *Ae*DNV poly(A) site that may be suitable for replacement with exogenous DNA.

Mutational analysis of the *Ae*DNV right-end UTR demonstrated that sequences upstream of the AATAAA hexamer are required for efficient mRNA expression (Fig. 2.4b). Required upstream sequence elements (USE) have been described in several systems, but have not received much scrutiny. The ablation of mRNA expression caused by the removal of these sequences (deletion #1) suggests that a required USE exists in this 34 nt region of the *Ae*DNV right-end UTR. Constructs containing smaller deletions in this area (deletions 1a-1g) were used to narrow the location of the USE to the 21 nt removed in deletion #1f (Fig. 2.4a). Surprisingly, none of the smaller deletions contained within deletion #1f (deletions 1a, 1b, and 1c) ablated mRNA expression by themselves (Fig. 2.4b). The sequences removed in deletion #1 are predicted to form an RNA stem-loop structure (figure 2.5b). Several studies have described RNA secondary structures occurring near poly(A) signals that are important for efficient pre-mRNA 3'-end processing (Zarudnaya *et al.*, 2003). For example, the ability of the HIV-1 AAUAAA signal to bind polyadenylation factors is controlled by local RNA structure (Klasens *et al.*, 1999). Disruption of the predicted stem-loop structure present in the *Ae*DNV right-end UTR may negatively affect mRNA levels by presenting the AAUAAA hexamer in an

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unfavorable context or by decreasing the stability of the pre-mRNA. Deletion of sequences forming the stem-loop has effects on the predicted RNA structure in all cases, but most are still capable of forming secondary structures (Fig. 2.5f-2.5m). The observation that only the construct containing the predicted structure for deletion #1f (Fig. 2.5l) is incapable of mRNA expression while other constructs containing superficially similar structures remain viable suggests the influence of factors other than predicted formation of secondary structures. RNA secondary structure prediction is inexact at best and it is possible that the structures presented in this study may not accurately represent the conformation of these structures in nature. We hypothesize that the 21 nt USE removed by deletion #1f contains both primary and secondary RNA structure elements that influence the binding efficiency of cellular polyadenylation factors and/or pre-mRNA stability.

These studies have identified the sequences that control polyadenylation of *Ae*DNV gene transcripts and determined additional sequences in the right-end UTR that are required for expression of mRNA from *Ae*DNV promoters. Sequences forming a predicted RNA stem-loop that may comprise a USE were identified. These experiments also identified a region of the *Ae*DNV right-end UTR that may be suitable for replacement with exogenous DNA. These findings contribute to a deeper understanding of polyadenylation and gene regulation within the *Brevidensovirus* genus and may be of use in the development of these viruses as more effective disease control or transducing agents.

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Chapter 3: Short-hairpin RNA expressed from polymerase III promoters mediates RNA interference in mosquito cells

This chapter presents data that in part have been submitted as a manuscript and accepted for publication in Insect Molecular Biology:

Konet, D. S., Anderson, J., Piper, J., Akkina, R., Suchman, E., and Carlson, J. O. (in press). Short-hairpin RNA expressed from polymerase III promoters mediates RNA interference in mosquito cells. Insect. Mol. Biol.

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Introduction

RNA interference (RNAi) is a sequence-specific gene-silencing pathway that holds great promise for studies of gene function and perhaps gene therapy (Fire *et al.*, 1998; Hannon, 2002; Anderson and Akkina, 2005). Double stranded RNA (dsRNA), a potent inducer of the RNAi response, is processed by Dicer, a member of the ribonuclease III family of nucleases, into single stranded, 19-25 nt short-interfering RNAs (siRNAs) that associate with the RNA-induced silencing complex (RISC) and target the protein complex to degrade messenger RNAs (mRNAs) that have exact sequence complementarity (Caplen *et al.*, 2001; Meister and Tuschl, 2004). The RNAi pathway appears to be highly conserved among eukaryotes, and has recently been demonstrated in mosquitoes (Adelman *et al.*, 2001; Caplen *et al.*, 2002; Hoa *et al.*, 2003; Keene *et al.*, 2004). RNAi-based gene silencing approaches have been used in the study of mosquito gene function (Johnson *et al.*, 1999; Attardo *et al.*, 2003; Tamang *et al.*, 2004; Boisson *et al.*, 2006; Riehle *et al.*, 2006). RNAi is also being explored as a tool for disruption of arthropod-borne disease transmission (Adelman, *et al.*, 2002; Brown *et al.*, 2003; Meister, *et al.*, 2005; Franz *et al.*, 2006).

Both siRNA and dsRNA have been used as triggers for induction of the RNAi response. siRNA or dsRNA complementary to the gene to be silenced can be introduced to cells by transfection or to organisms by injection. A common alternative to direct synthesis and transfection of siRNAs is DNA cassette-based expression of short-hairpin RNAs (shRNAs) (Dykxhoorn *et al.*, 2003). For this approach, an RNA polymerase III (Pol III) promoter is used to transcribe an inverted-repeat sequence to produce short-hairpin RNA (shRNA) that contains a double-stranded stem of 19-25 bp with sequences

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complementary to the target gene, and a 5-10 base loop sequence connecting them. Pol III transcription is terminated by including a poly(T) stretch at least 6 nt long after the target sequence hairpin. Dicer cleaves the loop sequence, RISC unwinds the dsRNA forming siRNAs, and mediates degradation of mRNAs containing the target sequence (Meister and Tuschl, 2004).

RNA polymerase III is responsible for transcription of short structural or catalytic RNAs including 5S rRNA, tRNA, H1 and the U6snRNA that forms a component of the spliceosome (Bogenhagen *et al.*, 1980; Galli *at al.*, 1981; Baer *et al.*, 1989; Das *et al.*, 1988). Most Pol III promoters contain intragenic control elements that make them of little use for expression of shRNAs. However, some Pol III promoters, including those responsible for expression of U6snRNA, contain all of their control elements in the external, 5' flanking region of the transcribed gene. These promoters are comprised of a TATA-like element located approximately 30 bp upstream of the transcription initiation site and a proximal sequence element (PSE) located upstream of the TATA box (Kunkel and Pederson, 1988). Some also have a distal sequence element (DSE) further upstream from the PSE that enhances transcription from the core promoter (Kunkel and Danzeiser, 1992). These promoters have been widely used in mammalian systems for expression of shRNA (Castanotto *et al.*, 2002; Gou *et al.*, 2003). Recently, a bovine U6snRNA promoter was characterized for expression of shRNA targeted to EGFP or GAPDH (Lambeth *et al.*, 2005), and a *Drosophila melanogaster* U6 promoter has been used to express shRNA in the *D. melanogaster* Schneider 2 cell line (Wakiyama *et al.*, 2005). The *D. melanogaster* U6 promoter used for shRNA expression has been extensively

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characterized and the critical TATA-like element and the PSE have been identified (Jensen *et al.*, 1998; McNamara-Schroeder *et al.*, 2001).

We report here the cloning of four putative U6snRNA promoters, two each from the *Ae. aegypti* and *An. gambiae* genomes. We assayed each promoter's ability to express an shRNA targeted to firefly luciferase and to mediate knockdown of a co-transfected luciferase reporter gene in ATC-10 *Ae. aegypti* and AG-55 *An. gambiae* cells. Additionally, we were able to detect expression of the 19 nt siRNA transcript. Pol III promoters for expression of shRNA in mosquitoes should be useful in studies of mosquito gene function and could form the basis of novel control strategies for mosquito-borne diseases involving RNAi-based gene silencing (Caplen *et al.*, 2002; Travanty *et al.*, 2004; Bian *et al.*, 2005; Franz *et al.*, 2006).

Materials and Methods

Mosquito U6snRNA Promoter Cloning

The *D. melanogaster* U6snRNA sequence (Genbank Accession no. NR002083) was used to perform a BLAST search of the *An. gambiae* and *Ae. aegypti* genomes (Fig. 3.1a). Sequences upstream of predicted mosquito U6snRNA genes were aligned with sequence analysis software (DNASTAR, Madison, WI). PCR primers were designed to amplify the putative promoter sequences (Table 3.1). PCR was performed using reagents from the Qiagen Taq Polymerase PCR Core Kit (Qiagen, Valencia, CA). Each 50 µl PCR reaction contained 5.0 µl 10X PCR buffer (contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$ (pH 8.7)), 1.0 µl 10 mM dNTPs, 1.0 µl of a 10 µM solution of each primer, 41.6 µl H_2O, 0.3 µl Taq (5 units/µl) and 0.1 µg of genomic mosquito DNA. The PCR

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a)

```
                      (-65) PSE                        (-30) TATA-Like Element
ACTACTTTTA[illegible]AGACTCAGTTCGTATATATA-GACCTAT            Drosophila
AATATCCTTTATGCATCGCTAGAGCAAGGATTGAAAGCGCAAAGTATATATACAACCTTT  AnGam-1
TTCTAACGTTATCCATCGCTAGAAGTGAAACGAGCGTGCGTAGGTATATATATGAAATGG  AnGam-2
TGATACGTCCATCCATGGCTAGAACCGCGTTCGCTGTAGAAGACTATATAAG-AGCAGAG  Aedes-1
TACGCAGTCTGTGCATCGCTAGAATCATATTTACGGAAGAGTATTATATA--TACCCGAT  Aedes-2
TTAACTCCTAG-------------AGATGCATGAAAAAAAAAAAAAAAAAACAC------  Aedes-3
-AAACTCCTAGTTGCATGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACACACACAC  Aedes-4
TGAATATGTTATTTGGCGTTTTTATTGTTTCAGAAGGGGTTATTTACATATTATGTAACT  Aedes-5
AAATATTTTCGCCCATCGCTAGAACTTTTACCGTTTCCATTGAGTATATAACTAAGATGA  Aedes-6
AAAATCTCCCAGCGCACCCTGCGAT-GCTCCGAAAACTGTCACCAATCGGTAAGATTCAT  Aedes-7

                         U6snRNA
TT--TCAATTTAACGT--CGTTCTTGCTTCGGC  Drosophila
TT--TCCCCTCGTCCT--TGTCCTTGCTTCGGC  AnGam-1
A---GTTGCTCTCTGCT-TGTCCTAGCTTCGGC  AnGam-2
GC--AAGAGTAGTGAAA-TGTCTTTGCTTCGGC  Aedes-1
GC--GTTGCTCTTCGAT-TGTCCTAGCTTCGGC  Aedes-2
-----------------GTCCTAGCTTCGGC    Aedes-3
ACACACACACACACACAGTCCTAGCTTCGGC    Aedes-4
TTA-ATAAAAAT-------GTTCTAGCTTCGGC  Aedes-5
AT--GAGGCTAATTGA--TGTCTTTGCTTCGGC  Aedes-6
TCG-CCAAAGGTACGC---GTCCTTGCTTCGGC  Aedes-7
```

b)

Forward Primer

Mosquito U6 Promoter

Reverse Primer

PCR Product

PacI	Mosquito U6 Promoter	Luciferase Sense	Loop	Luciferase Anti-Sense	Terminator	AgeI

Fig. 3.1. U6snRNA promoter alignments and shRNA cassette construction. a) Alignment of U6snRNA gene sequences produced by BLAST searches of the *Aedes aegypti* and *Anopheles gambiae* genomes using the *Drosophila melanogaster* U6snRNA gene sequence as a query. The TATA-like elements and the first 14 bases of the U6snRNA sequence are shown. The putative PSEs, including the full *Drosophila* PSE, are also shown. The *Drosophila* PSE is shown in a darker shade. Ten base tick marks are also provided. b) Schematic representation of RNAi cassette design by single-step PCR amplification. The forward primer contains an overhang that introduces a PacI restriction enzyme recognition sequence to facilitate cloning in future experiments. The reverse primer contains the stem-loop luciferase target sequence and a poly(T) tract to serve as a terminator of Pol III transcription. The reverse primer also introduces an AgeI restriction enzyme recognition sequence to facilitate cloning in future experiments involving other vectors.

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c)

```
-----------------+------------------+------------------+------------------+------------------+-
--------------------------------------------------   Full Hairpin
-----------------------------------------------TTA   Aedes-1
--------------------------------------------------   Aedes-2
--------------------------------------------------   AnGam-1
TTAATTAATTTGTATGCGTGCGCTTGAAGGGTTGATCGGAACCTTACAAC   AnGam-2 Long
--------------------------------------------------   AnGam-2 Short
          Pac I

-----------------+------------------+------------------+------------------+------------------+-
--------------------------------------------------   Full Hairpin
ATTAAGAATGAAATCGCCCATCG-AGTTGATACGTCCATCCATCGCTAGA   Aedes-1
------------TTAATTAAGTACAGTTACGCAGTCTGTGCACTGCTAGA   Aedes-2
--------TTAATTAATCCTTCTTGAATATC--CTTTATGCATCGCTAGA   AnGam-1
AGTTGTAGCTATACGGCTGCGTGTGGCTTCTAACGTTATCCATCGCTAGA   AnGam-2 Long
------------TTAATTAAGTGTGGCTTCTAACGTTATCCATCGCTAGA   AnGam-2 Short
                                      (-65) PSE

-----------------+------------------+------------------+------------------+------------------+-
--------------------------------------------------   Full Hairpin
A-CCGCGTTCGCTGTAGAAGACTATATAAGAGCAGAGGCAAGAGTAGTGA   Aedes-1
A-TCAGATTTACGGAAGAGTATTATATATACCCAATGC-GTTGCTCATCG   Aedes-2
GCAAGGATTGAAAGCGCA-AAGTATATATACAACCT----TTTTTCCCCT   AnGam-1
A-GTGAAACGAATGTGCGTAGGTATATATATGAAATGGAGTTGCTCTCTG   AnGam-2 Long
A-GTGAAACGAATGTGCGTAGGTATATATATGAAATGGAGTTGCTCTCTG   AnGam-2 Short
                (-30) TATA-Like Element

-----------------+------------------+------------------+------------------+------------------+-
---CTTACGCTGAGTACTTCGACCTCAGCTCGAAGTACTCAGCGTAAG     Full Hairpin
AATCTTACGCTGAGTACTTCGACCTCAGCTCGAAGTACTCAGCGTAAGTT   Aedes-1
TTTCTTACGCTGAGTACTTCGACCTCAGCTCGAAGTACTCAGCGTAAGTT   Aedes-2
CGTCTTACGCTGAGTACTTCGACCTCAGCTCGAAGTACTCAGCGTAAGTT   AnGam-1
CTTCTTACGCTGAGTACTTCGACCTCAGCTCGAAGTACTCAGCGTAAGTT   AnGam-2 Long
CTTCTTACGCTGAGTACTTCGACCTCAGCTCGAAGTACTCAGCGTAAGTT   AnGam-2 Short

-----------------+-----
               Full Hairpin
TTTTTTACCGGT   Aedes-1
TTTTTTACCGGT   Aedes-2
TTTTTTACCGGT   AnGam-1
TTTTTTACCGGT   AnGam-2 Long
TTTTTTACCGGT   AnGam-2 Short
Terminator  Age I
```

Fig. 3.1 (cont.). c) Alignment of *Anopheles gambiae* and *Aedes aegypti* U6snRNA promoters driving expression of the luciferase target hairpin. The conserved TATA-like element, PSE, terminator sequence and restriction enzyme recognition sites are shown. The full luciferase target hairpin sequence is shown for reference. Ten base tick marks are also provided.

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Name	Sequence 5'-3'
AnGam-1 U6 Fwd	CCTTTGCCGGCTCTTTCTTCTCTT
AnGam-1 U6 Rev	AGGGGCCATGCTAATCTTCTCTGT
AnGam-2 U6 Fwd	ACAAGGCCAAAAATCCCACTCGTC
AnGam-2 U6 Rev	AGGGGCCATGCTAATCTTCTCTGTATC
Aedes-1 U6 Fwd	CCGTCGGCTGGCTGGATT
Aedes-1 U6 Rev	TCTATGTTTGGGTTTGATGGTTGC
Aedes-2 U6 Fwd	AGCCAAGTCAATAACAGGTCCAGT
Aedes-2 U6 Rev	AGGGGCCATGCTAATCTTCTCT
AnGam-1 RNAi Fwd	GCGTGCGTTACGGCAGGATCAAAACCTTATAACAGT CACAC
AnGam-1 RNAi Rev	ACTACCGGTAAAAAAAACTTACGCTGAGTACTTCGA GCTGAGGTCGAAGTACTCAGCGTAAGACGAGGGGA AAAAAGGTTGTATATATAC
AnGam-2 RNAi Fwd Long	GTCTTAATTAATTTGTATGCGTGCGCTTGAAGGGTTG ATC
AnGam-2 RNAi Fwd Short	GTCTTAATTAAGTGTGGCTTCTAACGTTATCCATCGC TAG
AnGam-2 RNAi Rev	ACTACCGGTAAAAAAAACTTACGCTGAGTACTTCGA GCTGAGGTCGAAGTACTCAGCGTAAGAAGCAGAGA GCAACTCCATTTCATATATATAC
Aedes-1 RNAi Fwd	GACTTAATTAAGAATGAATCGCCCATCGAGTTGATA CGTC
Aedes-1 RNAi Rev	ACTACCGGTAAAAAAAACTTACGCTGAGTACTTCGA GCTGAGGTCGAAGTACTCAGCGTAAGATTTCACTAC TCTTGCCTCTGCTCTTATATAG
Aedes-2 RNAi Fwd	GTCTTAATTAAGTACAGTTACGCAGTCTGTGCACTGC TAG
Aedes-2 RNAi Rev	ACTACCGGTAAAAAAAACTTACGCTGAGTACTTCGA GCTGAGGTCGAAGTACTCAGCGTAAGAAACGATGAG CAACGCATTGGGTATATATAATAC
Luciferase RNA Probe	CUUACGCUGAGUACUUCGAGGACAGAGGG

Table 3.1. Oligonucleotides used for promoter cloning, shRNA expression cassette construction and siRNA detection .

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reactions were cycled at 95°C for one minute, 47°C for one minute, and 72°C for one minute for a total of thirty cycles. The reaction products were run on a 1.5% agarose gel and the desired fragments were extracted using the Qiagen Gel Extraction kit. The purified PCR product containing the desired promoter sequences was cloned into the PCR4-topo plasmid vector using the Topo-TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). All clones were verified by sequencing using an automated sequencer at Macromolecular Resources or the Arthropod-Borne and Infectious Diseases Laboratory at Colorado State University.

shRNA Expression Cassette Construction

The anti-luciferase shRNA expression vectors were created by a single-step PCR-based method (Fig. 3.1b), (Castanotto *et al.*, 2002). The mosquito U6snRNA clones described above were used as template for the PCR reaction. The forward primers contained a Pac I restriction enzyme recognition site immediately upstream of 20 bases of the desired promoter sequence. The reverse primers contained 20 nt of sequence complimentary to the last 20 bases of the promoter, the 19 nt sense luciferase target sequence, the 7 bp loop sequence, the 19 nt antisense luciferase target sequence, a poly(T) tract to terminate Pol III transcription and an Age I restriction enzyme recognition site (Figure 3.1b). Production of PCR products containing full-length hairpin sequences was accomplished by using a proofreading polymerase contained in the Advantage 2 PCR kit (Clontech, Mountain View, CA). Each 50 µl PCR reaction contained 5 µl of 10x Advantage PCR buffer (400 mM Tricine-KOH, 150 mM KOAc, 35 mM $Mg(OAc)_2$, 37.5 µg/ml BSA, 0.05% Tween 20, and 0.05% Nonidet-P40), 0.3 µl of

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topo-cloned promoter template DNA (1 μg/μl), 1 μl of 10 mM dNTP mix, 1 μl each of forward and reverse primers (10 μM), 40.7 μl of water and 1 μl of 50x Advantage 2 Polymerase Mix (contains 50% glycerol, 15 mM Tris-HCl, 75 mM KCl, 0.05 mM EDTA and a proprietary mixture of Titanium Taq DNA Polymerase, a small amount of proofreading polymerase, and BD TaqStart Antibody (1.1 μg/μl)). The PCR reactions were incubated for 3 minutes at 95°C followed by 30 cycles of 95°C for 15 seconds and 68°C for 3 minutes. The resulting PCR product contained all components of the anti-luciferase shRNA cassette (Fig. 3.1c). The PCR product was cloned into the PCR4-topo plasmid backbone as described above. All oligonucleotides used in this study are shown in Table 3.1.

Cells and Transfections

AG-55 *An. gambiae* and ATC-10 *Ae. aegypti* cells were grown at 27°C in L-15 media with 20% fetal bovine serum and 1% penicillin-streptomycin. Confluent cell flasks were split by scraping with a rubber policeman and resuspending 5 times with a glass 5ml pipet to reduce cell clumping. ATC-10 cell flasks must be split at 1:3 or lower. AG-55 cells could be split at 1:10. The cell suspensions were seeded into individual wells of a 12-well Costar plate and incubated at 27°C. The cells were transfected using the Effectene transfection reagent (Qiagen, Valencia, CA) when they were approximately 50% confluent as judged by visual inspection in the microscope at low magnification. Transfections were performed according to the manufacturer's instructions for transfection of wells in a 12-well plate.

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pBSLuc contains the *D. melanogaster* Hsp70 promoter driving expression of the luciferase target gene. A fixed amount (20 ng) of a β-galactosidase (β-gal) reporter gene plasmid (pVPNcoRE) was included in each transfection as a control for cell lysis and transfection efficiency. pVPNcoRE contains the *Ae. aegypti* densovirus structural gene promoter driving expression of *LacZ*/β-gal (Ward *et al.*, 2001.). The total DNA amount of 420 ng was kept constant for the transfection experiments while the ratio of interfering construct to luciferase construct was varied. For example; the 3:1 experiment contained 300 ng of the interfering construct, 100 ng of the luciferase construct, and 20 ng of the β-gal construct.

Reporter Gene Expression Assays

Co-transfected cell populations were harvested at appropriate time points according to the instructions provided with the Galacto-Light assay kit (Applied Biosystems, Foster City, CA). β-gal expression was quantified by incubating an aliquot of the cell lysates with Galacton reagent (Tropix, Bedford, MA) and measuring the light produced with a TD-20e luminometer (Turner Designs, Sunnyvale, CA). This procedure is described in detail elsewhere (Afanasiev *et al.*, 1999; Kimmick *et al.*, 1998). Luciferase levels, from pBSLuc, were determined using the luciferase assay system (Promega, Madison, WI) and a TD-20e luminometer. Arbitrary light units from the luciferase assays were normalized to the average light units of β-gal in order to control for transfection and cell lysis efficiency. Luciferase assays were performed in triplicate and β-gal assays were performed in duplicate.

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The mean, standard deviation, and 95% confidence intervals (95% CI) were calculated for the luminescence data for luciferase and β-gal. The background of luciferase and β-gal activity observed in the mock-transfected cell lysates were subtracted out. Finally, the luciferase expression was divided by the β-gal expression to yield a ratio. The ratio observed with a co-transfection of pBSLuc, an AnGam-1 promoter-based construct lacking the luciferase target hairpin, and pVPNcoRE was used as a control and set to 100% expression. Uncertainty generated by each mathematical function was propagated through the calculations to maintain a 95% confidence interval for all data and used to generate the error bars used in Figures 3.2 and 3.3.

siRNA Detection

Detection of anti-luciferase siRNAs was accomplished by performing an RNase protection assay on transfected AG-55 cell extracts that were harvested using the *mir*Vana miRNA isolation kit (Ambion, Austin, TX). 1 μg of the AnGam-2 RNAi expression vector was transfected into a T-75 tissue culture flask containing 50% confluent AG-55 cells using the Effectene transfection kit (Qiagen, Valencia, CA). An untransfected flask was used as a negative control. The cells were harvested, lysed and enriched for small RNAs according to the manufacturer's instructions. The 29 nt RNA oligonucleotide, "Luc RNA Probe", containing the 19 nt luciferase target sequence (5'-CTTACGCTGAGTACTTCGA-3') followed by 10 non-luciferase nucleotides was 5' end-labeled with [gamma-^{32}P] ATP using the *mir*Vana Probe and Marker Kit (Ambion, Austin, TX). The radiolabeled probe was hybridized overnight at room temperature to cell extracts using the *mir*Vana miRNA detection kit (Ambion, Austin, TX). The RNA fragments were run on a denaturing 15% TBE-urea gel (Bio-Rad, Hercules, CA).

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Hybridization was detected by autoradiography. The gel appearing in Fig. 3.4 is a composite of two time exposures of the same gel.

Results and Discussion

Characterization of *Aedes aegypti* and *Anopheles gambiae* U6 Promoters and shRNA Expression Vector Construction

The *D. melanogaster* U6snRNA gene sequence (Genbank Accession no. NR002083) was used in a BLAST search to identify putative U6snRNA genes in the *Ae. aegypti* and *An. gambiae* genomes. Several copies of the U6snRNA gene sharing 97% to 99% homology to the *D. melanogaster* sequence were found in the mosquito genomes, seven in *Ae. aegypti* and two in *An. gambiae* (Fig. 3.1a). Four promoters (AnGam-1, AnGam-2, Aedes-1 and Aedes-2) contained a TATA-like element at approximately (-30) and a highly conserved upstream sequence element at approximately (-65) similar to proximal sequence elements characteristic of previously described Pol III promoters (Jensen *et al.*, 1998). These four promoters were used in this study and those lacking the two sequence motifs were not further characterized. No other conserved sequence elements were found within 300 nt upstream of the genes. The TATA-like sequence (5'-TATATA-3') is highly conserved in sequence and proximity to the predicted transcription start site between *D. melanogaster*, *An. gambiae* and *Ae. aegypti.* The upstream (-65) putative PSE sequence (5'-CATCGCTAGA-3') is highly conserved between *Ae. aegypti* and *An. gambiae*, but exhibits no significant sequence homology to the *D. melanogaster* PSE (Fig. 3.1a).

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Using each putative mosquito U6snRNA promoter sequence as a template, a PCR-based cloning strategy was used to generate shRNA expression vectors targeting firefly luciferase (Fig. 3.1b) (Castonotto *et al.*, 2002). All final cDNA shRNA expression cassettes contained an *An. gambiae* or *Ae. aegypti* promoter, a luciferase target sense sequence, a loop sequence, a luciferase target antisense sequence and an 8 nt poly(T) tract to terminate Pol III transcription (Fig. 3.1c). The 19 nt luciferase target sequence suggested by Genscript, Inc. has been shown to silence luciferase expression by approximately 80% (Lewis *et al.*, 2002).

Activity of Mosquito Promoters Measured By Firefly Luciferase Knockdown

Each shRNA expression vector (interfering construct) was assayed for its ability to silence the expression of a co-transfected luciferase reporter gene vector in AG-55 *An. gambiae* and ATC-10 *Ae. aegypti* cells. The AnGam-1 promoter containing the native U6snRNA gene sequence in place of the luciferase target hairpin was used as a control because it expresses a transcript that does not interfere with luciferase expression. Figure 3.2a shows the knockdown of luciferase in *An. gambiae* AG-55 cells at 24-hour intervals after transfection with each interfering construct. The interfering constructs exhibited a wide variation in their ability to silence luciferase. This is similar to a recent report that demonstrated varying transcriptional efficiencies of different human U6snRNA genes (Domitrovich and Kunkel, 2003). Both of the *Ae. aegypti* promoter-based constructs performed poorly in AG-55 cells, silencing expression of the luciferase vector by at most 38%. Both of the *An. gambiae* promoter-based constructs exhibited increased silencing in AG-55 cells compared to the *Ae. aegypti*-based constructs. The AnGam-1 construct

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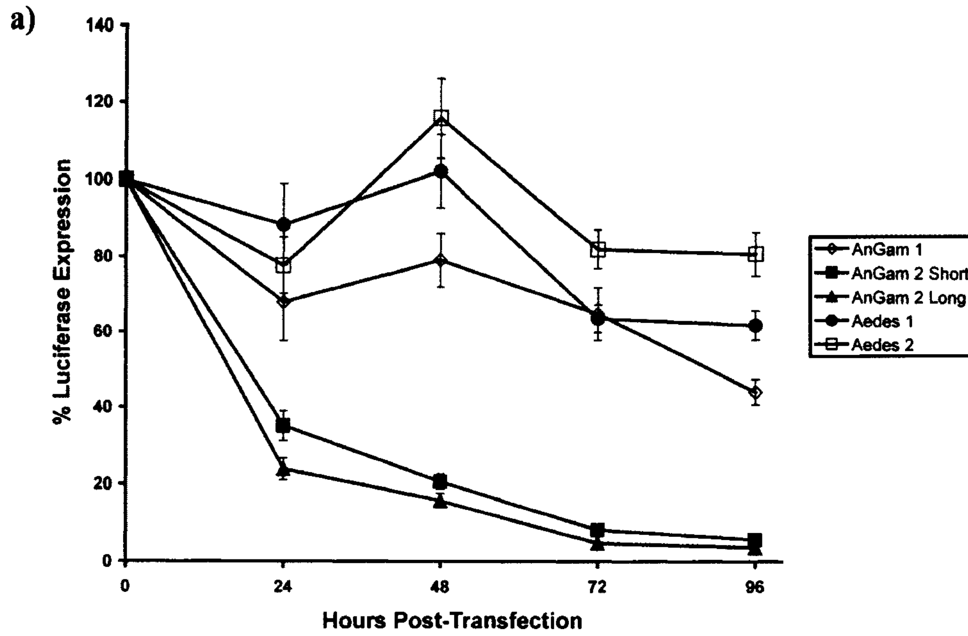

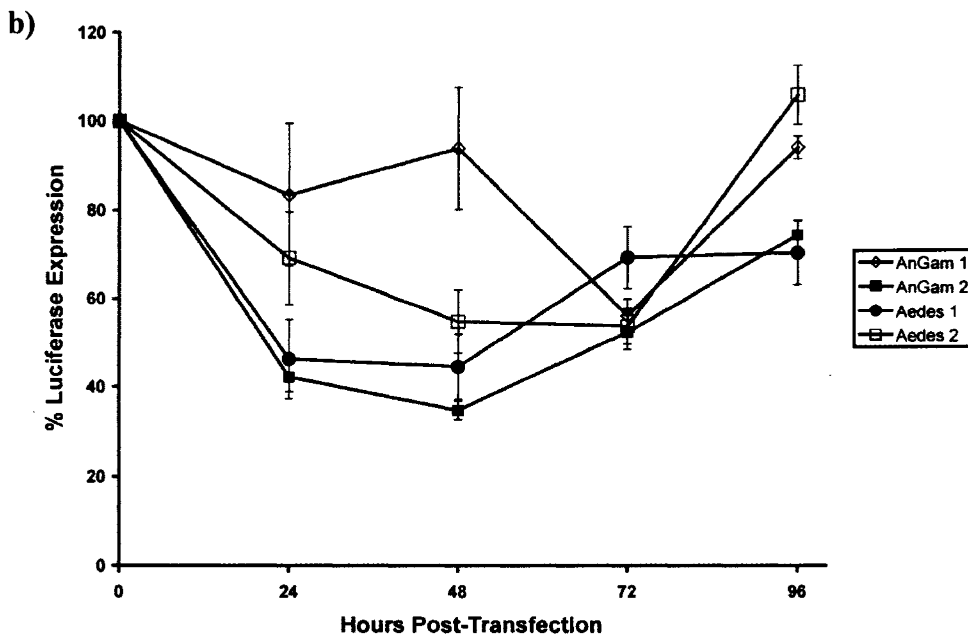

Fig. 3.2. shRNA inhibits luciferase expression in mosquito cells. a) Luciferase reporter gene expression observed in *Anopheles gambiae* AG-55 cells at 24 hour intervals. Each transfection mixture contained 200 ng of interfering construct and 200 ng of luciferase target construct. The error bars represent 95% confidence intervals for reporter gene luminescence data collected at each time point. b) Luciferase reporter gene expression observed in *Aedes aegypti* ATC-10 cells at 24 hour intervals. Each transfection mixture contained 200 ng of interfering construct and 200 ng of luciferase target construct. The error bars represent 95% confidence intervals for reporter gene luminescence data collected at each time point.

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was able to silence expression of luciferase by approximately 55% at 96 hours post-transfection. The AnGam-2 interfering construct exhibited rapid and sustained silencing of luciferase expression. Luciferase silencing observed with this construct exceeds 90% at 72 and 96 hours post-transfection. A version of the AnGam-2 interfering construct containing 60 bp of additional sequences upstream of the PSE (AnGam-2 Long) did not demonstrate a marked increase in the construct's ability to mediate knockdown indicating that the core promoter consisting of the TATA-like element and the PSE is sufficient for robust expression of shRNA demonstrated by the 95% knockdown (Fig. 3.2a). This observation suggests that additional distal sequence elements, such as those described in mammalian U6snRNA gene promoters, are not required for efficient shRNA-mediated gene silencing in mosquito systems (Lambeth *et al.*, 2005). Additionally, distal sequence elements are not required for efficient transcription of the *D. melanogaster* U6snRNA gene (Jensen *et al.*, 1998; McNamara-Schroeder *et al.*, 2001). Consequently, we used the shorter version of the AnGam-2 interfering construct (AnGam-2 Short) in the rest of the experiments described in this report.

Figure 3.2b shows the knockdown of luciferase in *Ae. aegypti* ATC-10 cells at 24-hour intervals after transfection for each interfering construct. The best silencing was seen with the Aedes-1 and AnGam-2 constructs at 48 hours post-transfection. Unlike AG-55 cells, knockdown peaks at 48 hours post-transfection, then wanes over the subsequent time points (Fig. 3.2b). In an attempt to further optimize silencing in ATC-10 cells, the ratio of Aedes-1 or AnGam-2 interfering construct to luciferase target construct was varied from 30:1 to 1:30 while the total amount of plasmid DNA used in each transfection was kept constant at 420 ng. The results of this experiment are shown in

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Figure 3.3. Even at high ratios of interfering to target construct, only about 75% silencing was achieved with both the Aedes-1 and AnGam-2 interfering constructs. Lower ratios of interfering to target construct caused a linear reduction in silencing efficiency for both constructs, although luciferase inhibition of at least 30% was still observed with the AnGam-2 construct at the lowest 1:30 ratio. The differing characteristics of the RNAi effect observed in *An. gambiae* versus *Ae. aegypti* cells could be due to a number of factors. The growth characteristics of the two cell lines are considerably different with ATC-10 cells exhibiting a markedly slower growth rate when compared to AG-55 cells. The luciferase levels are lower and peak earlier in ATC-10 cells even though the cells did not reach confluence until 96 hours post-transfection. We hypothesize that factors such as growth rate, confluence, transfection efficiency and perhaps differences in luciferase half-life, mRNA stability, or variations in transcription factor availability for ATC-10 cells versus AG-55 cells could all have an effect on reporter gene expression and the efficiency of RNAi-mediated gene silencing.

siRNA Detection

To confirm that the observed luciferase knockdown was due to RNAi, an siRNA detection experiment was performed using an RNase protection assay. AG-55 cells were transfected with the AnGam-2 interfering construct that exhibited the highest level of luciferase silencing. Total RNA was harvested and enriched for small RNAs. This was hybridized to a 5'-^{32}P radiolabeled probe (29 nt) containing 19 nt of luciferase target sequence at the 5' end and 10 nt of non-luciferase sequence at the 3' end. Because RNase treatment degrades only single-stranded RNA, hybridization of the radiolabeled

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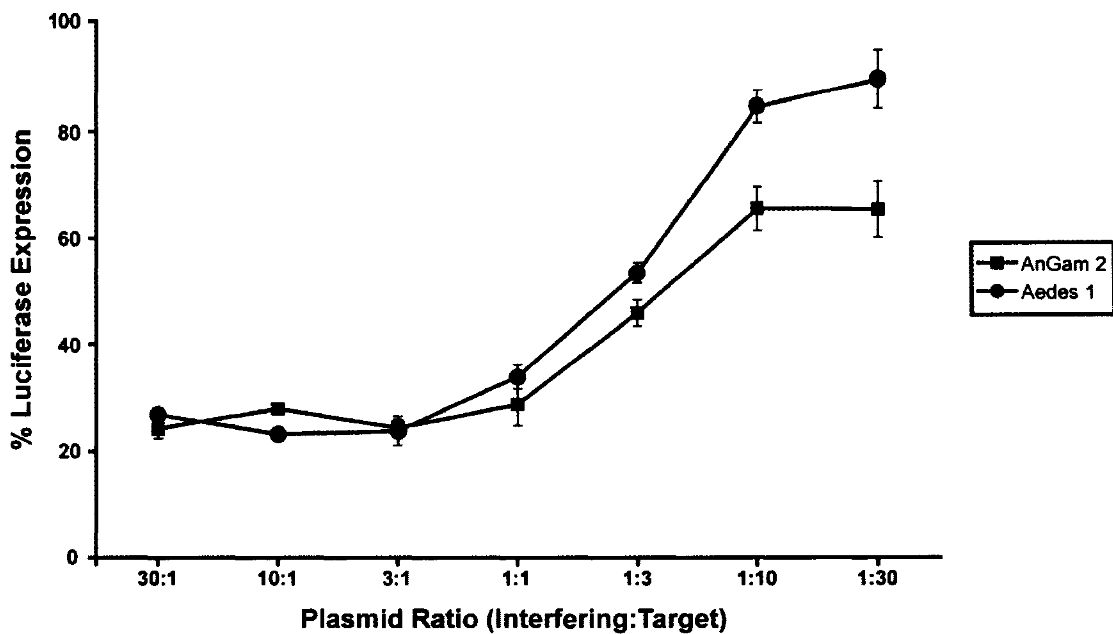

Fig. 3.3. Variation of RNAi with ratio of interfering to target construct in ATC-10 cells. Luciferase reporter gene expression in *Aedes aegypti* ATC-10 cells at 48 hours post-transfection when the ratio of interfering to target construct was varied from 30:1 to 1:30. The error bars represent 95% confidence intervals for reporter gene luminescence data.

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probe to the 19 nt siRNA protects the luciferase target sequence in the probe from digestion. A positive result is indicated by the presence of a 19 nt band representing the portion of radiolabeled probe that was protected from RNase digestion due to its hybridization with the siRNA transcript (Anderson and Akkina, 2005; Franz *et al.*, 2006). The results of this experiment are shown in Fig. 3.4. Lane 5 contains the undigested, radiolabeled probe with the 10 bp overhang. Lane 4 contains RNA harvested after transfection with the AnGam-2 interfering construct that was hybridized to the labeled probe and subjected to RNase digestion. The 10 bp shift observed between lanes 4 and 5 indicates that the probe hybridized with the 19 nt anti-luciferase siRNA and was subsequently protected from the RNase digestion. The band shown in lane 4 indicates that the AnGam-2 promoter is capable of expressing shRNA that is converted to siRNA, the hallmark of the RNAi response.

Conclusions

Although induction of RNAi by transfection or injection of siRNA or dsRNA is well established in mosquito systems, a DNA based system for induction of RNAi allows new strategies such as delivery by a virus or by transgenesis. We used the *D. melanogaster* U6snRNA gene sequence to locate putative Pol III U6snRNA gene promoters in the *An. gambiae* and *Ae. aegypti* genomes. Two putative promoters from each mosquito species were cloned and assayed for their ability to express shRNA targeted to firefly luciferase in ATC-10 *Ae. aegypti* and AG-55 *An. gambiae* mosquito cells. A promoter consisting of only a TATA-like element and a putative PSE was identified that silences expression of a co-transfected luciferase reporter gene vector by

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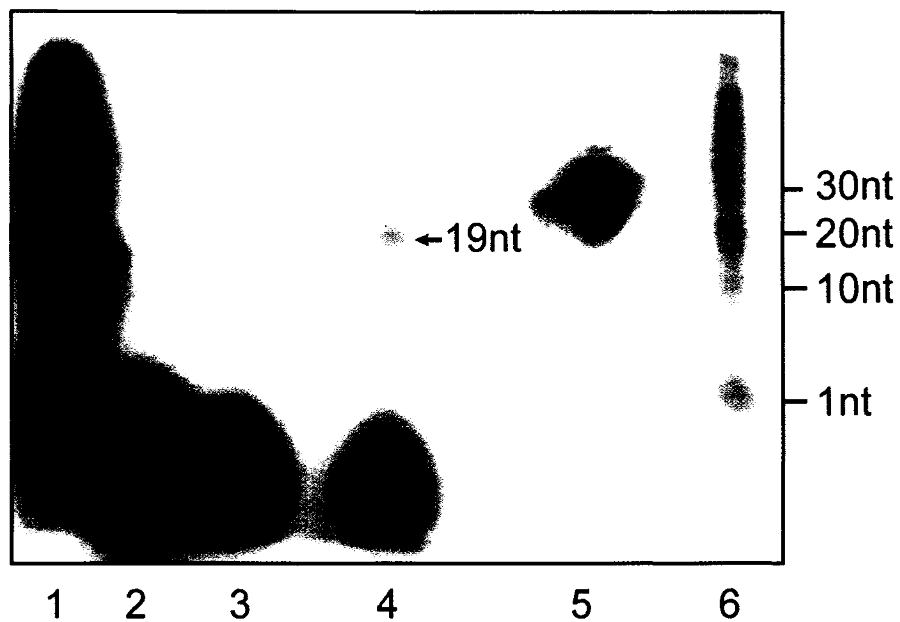

Figure 3.4. Detection of siRNA by RNase protection. Lane 1=RNA ladder; lane 2=Luc RNA Probe + RNase; lane 3=negative control + RNase; lane 4=AnGam-2 + RNase; lane 5=Luc RNA Probe - RNase; lane 6=RNA ladder. The figure is a composite of two exposure times. Lanes 1-4 were exposed for 72 hours to allow visualization of the protected probe fragment in lane 4. Lanes 5 and 6 were exposed for 24 hours to avoid over-darkening of the undigested probe and RNA ladder markers.

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up to 95% in *An. gambiae* cells and up to 75% in *Ae. aegypti* cells. We also demonstrated the ability of this promoter to express shRNA by performing a radiolabeled RNase protection assay that detected the siRNA transcript. The promoters characterized in this study should be useful for many applications in mosquito systems including functional genomic experiments, and development of RNAi-based strategies aimed at disruption of arbovirus or malaria parasite transmission.

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Chapter 4: Development of *Aedes aegypti* densovirus as an siRNA delivery vehicle

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Introduction

Mosquito-borne diseases cause significant morbidity and mortality in humans and animals worldwide. *Aedes aegypti* mosquitoes are the principal vector species for yellow fever virus and the dengue viruses. Approximately 2.5 billion people are at risk for dengue infection, with 50 million infections occurring annually (Anonymous, 2002). The lack of effective vaccines, pesticide resistance in mosquitoes, extreme poverty in many disease endemic countries coupled with the erosion of public health infrastructures and lack of vector control programs, have lead to the resurgence of mosquito-borne pathogens including dengue (Beaty, 2005). The failure of traditional methods to reduce the global burden imposed by mosquito-borne diseases has highlighted the need for alternative approaches.

RNAi has emerged as a potential tool for the control of mosquito-borne pathogens including arboviruses. Methods based on RNAi-mediated silencing of viral genes or endogenous mosquito genes involved in vector competence could form the basis of novel control strategies (for review see Blair *et al.*, 2006). Numerous studies have demonstrated the efficacy of RNAi-based approaches for the disruption of arbovirus transmission, but an ideal system for the delivery of RNAi to mosquitoes in a natural setting is still lacking. *Ae*DNV is an excellent candidate as a vector for RNAi in mosquitoes because of its narrow host range, environmental stability and potential to spread rapidly through mosquito populations (for review see Carlson *et al.*, 2006). Studies demonstrating the utility of *Ae*DNV as an agent for mosquito transduction suggest that induction of RNAi in mosquitoes by infection with *Ae*DNV-derived vectors is entirely plausible.

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This chapter presents data suggesting that *Ae*DNV can be developed as a vector for RNA interference (RNAi) in mosquitoes. Although the coding capacity of *Ae*DNV is severely limited, a Pol III-based RNAi expression cassette is small enough to be added to the *Ae*DNV genome without necessitating the removal of NS or VP coding sequences (Fig. 4.1). Previous research has identified non-essential sequences in the right-end UTR of the *Ae*DNV genome suitable for replacement with exogenous DNA comprising an RNAi expression cassette (Chapter 2). Promoters capable of expressing shRNA and inducing the RNAi response in mosquito cells have also been described (Chapter 3). The studies contained in this chapter demonstrate that 1) an RNAi expression cassette can be inserted in the right-end UTR of the *Ae*DNV genome, 2) *Ae*DNV virions containing these modified genomes are capable of infecting *Ae. aegypti* mosquitoes, and 3) the RNAi expression cassettes are stable for the duration of the mosquito's lifespan.

Materials and Methods

Mosquito Strains and Insectary Conditions

The mortality assay was performed at the Colorado State University arthropod-borne infectious diseases laboratory (AIDL) using Rexville-D *Aedes aegypti* mosquitoes (Ballinger-Crabtree *et al.*, 1992). Insectary conditions were maintained at 28°C and 80% relative humidity. Temperature and humidity were recorded and adjusted on a daily basis. Humidity was controlled by a floor unit evaporative humidifier with an internal humidistat and temperature was controlled by the facility's HVAC system.

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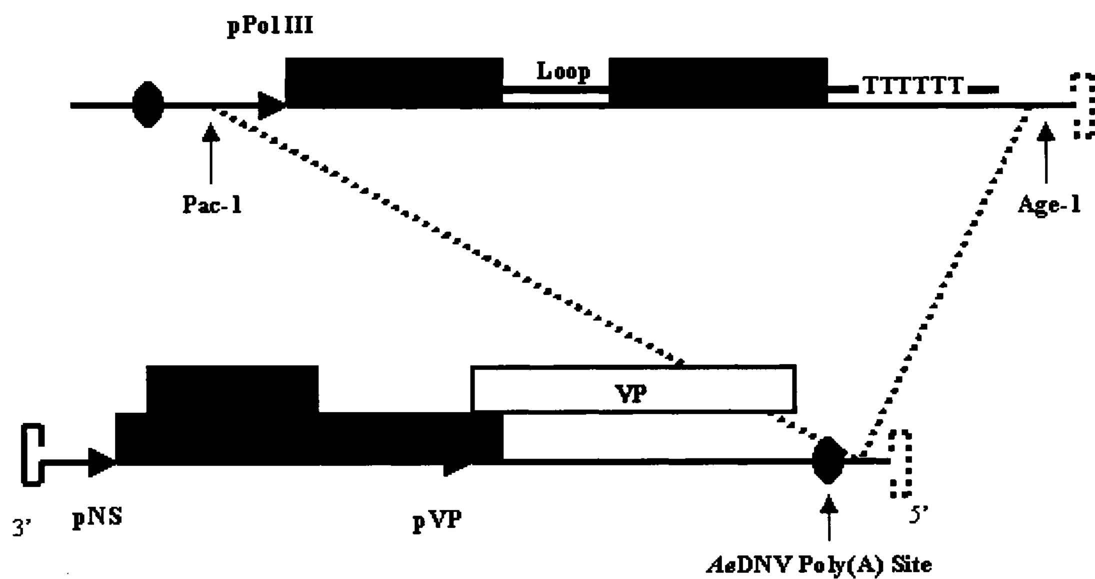

Fig 4.1. Insertion of an RNAi expression cassette in the right-end UTR of the *Ae*DNV genome. A Pol III promoter drives expression of sense and antisense target sequences. Pol III transcription is terminated by including a poly(T) stretch. The *Ae*DNV poly(A) site is shown in red. The Pac-1 and Age-1 restriction enzyme recognition sites used for cloning are also shown.

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Plasmid Composition and Cloning

pUCA is the infectious clone of *Ae*DNV (Afanasiev *et al.*, 1994). Construction of RNAi expression cassettes containing different mosquito U6snRNA promoters driving expression of firefly luciferase target sequences is described in detail in Chapter 3. Three different PCR4-topo-based plasmids, each containing a different Pol III promoter driving expression of luciferase target sequences, were used to create three different versions of pUCA-RNAi. The AnGam-2-long, Aedes-1 and Aedes-2 topo constructs were used to create *Ae*DNV-RNAi-AnGam, *Ae*DNV-RNAi-Aedes-1, and *Ae*DNV-RNAi-Aedes-2, respectively. pUCA was mutagenized at nt 3711 to introduce a unique PacI restriction enzyme recognition site and at nt 3765 to introduce a unique AgeI restriction enzyme recognition site. Mutagenesis was accomplished using pUCA-MCS primers according to the PCR strategy described in Chapter 2. Each PCR4-topo-based plasmid contained a unique PacI site upstream of the RNAi expression cassette and a unique AgeI site downstream of the cassette to facilitate cloning. Transferring each RNAi expression cassette from PCR4-topo to pUCA was accomplished by digesting each construct with PacI and AgeI (New England Biolabs, Beverly, MA). The restriction digests were run on a 1.5% agarose gel and the desired fragments were isolated using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Ligations were performed overnight at 17°C with T4 DNA Ligase (MBI Fermentas, Hanover, MD). Ligated DNA was ethanol precipitated, resuspended in water, and electroporated into *Escherichia coli* DH5-α cells. Electroporated cells were plated on LB-ampicillin plates and incubated at 37°C overnight. Colonies were picked and analyzed by restriction digests of minipreps.

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Positive colonies were inoculated into 500 ml LB-amp media, grown overnight, and purified using the Qiagen Plasmid Midi kit (Qiagen, Valencia, CA).

Virus Production and Quantification

*Ae*DNV was produced by transfecting wild-type or modified pUCA into C6/36 *Ae. albopictus* cells grown in T-75 flasks using L-15 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Transfection was accomplished using the Qiagen Effectene transfection kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Transfected cells were incubated at 28°C for 48 hours. After the incubation period, cells were lysed by placing the flasks at -80°C for 20 minutes followed by 10 minutes in a room temperature water bath. The freeze-thaw was repeated for a total of three cycles. Whole lysate was collected and centrifuged for 15 minutes at 3,750 rpm using a Beckman G56R centrifuge (Beckman-Coultier, Fullerton, CA). Supernatents were transferred to a 50 ml polystyrene ultra-centrifuge tube (Beckman-Coultier, Fullerton, CA) and centrifuged at 28,000 rpm for 2.5 hours using an SW28Ti rotor. The virus pellets were resuspended in 900 μl of double-deionized water. The virus suspension was subjected to digestion with DNase by adding 100 μl of DNase-1 buffer (contains 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 1 mM $CaCl_2$) and 20 μl of RNase-free DNase-1 (MBI Fermentas, Hanover, MD) and incubating at 37°C for 30 minutes. *Ae*DNV was quantified using the real-time PCR assay described in Chapter 2 (Ledermann *et al.*, 2004).

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Mosquito Infection Protocol

Mosquito eggs were hatched in tap water. Approximately 1-3 hours after hatching, 125 larvae per group were counted into 500 μl of water in a 1.5 ml eppendorf tube. The larvae were transferred to a 15 ml conical tube and given 1 X 10^{10} genome equivalents/ml of the appropriate version of *Ae*DNV in a final volume of 2 ml. The mosquitoes were fed and placed at 28°C for 24 hours. After 24 hours, 25 larvae were placed into each of four 100 ml plastic cups (Dixie, Atlanta, GA) and given food. The final volume in each cup was 80 ml. Controls received no virus, but were treated equally. The cups were placed in aluminum trays and incubated in the insectary at 80°F and 80% relative humidity. The cups were monitored and given appropriate amounts of food until pupation. After pupation, the pupae were transferred into new cups and placed into a sealed cage consisting of a one-gallon ice cream carton with a screened lid. Water and raisins were placed on the screen. Adults were allowed to emerge in the new cages. The experiment was ended 28 days after exposure.

Calculation of Larval and Total Mortality

The number of larvae and live or dead pupae in each cup were counted on a daily basis until the end of the experiment. Based on the initial count of larvae, which was taken on day one after exposure to the virus, the larval and pupal mortalities were calculated for each cup. The adult mortality was calculated by counting the number of dead adults on day 28 and dividing by the initial larval count. Total mortality was

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calculated by adding the larval, pupal, and adult mortalities. Sixteen cups were counted for each version of the virus and for the control. The mean, standard deviation, and 95% confidence intervals (95% CI) were calculated for each group and used to generate the error bars shown in Fig. 4.2.

DNA Extractions, Conventional PCR and Sequencing

Individual adult mosquitoes harvested at 28 days post-infection were ground with a pestle into a 1.7 ml eppendorf tube containing 100 μl of sterile PBS. The pestle was rinsed into the tube used for grinding with an additional 100 μl of PBS. Isolation of DNA from mosquitoes was accomplished by using the Qiagen DNeasy kit according to the manufacturer's protocol B for isolation of DNA from insects (Qiagen, Valencia, CA).

Primers designed to amplify the VP region of *Ae*DNV (Table 4.1) were used to screen DNA samples extracted from adult mosquitoes for *Ae*DNV infection. The PCR reagents were obtained from the Qiagen Taq Polymerase PCR Core kit (Qiagen, Valencia, CA). Each PCR reaction mix contained 5.0 μl 10X PCR buffer (contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$ (pH 8.7)), 1.0 μl 10 mM dNTPs, 1.0 μl of a 5 μM solution of each primer, 40.7 μl H_2O, 0.3 μl Taq (5 units/μl) and 1 μl of extracted DNA. One μl of pUCA (10 ng/μl) was added to the positive control reaction and 1 μl of distilled, deionized water was added to the negative control reaction. The PCR reactions were cycled at 95°C for one minute, 52°C for one minute, and 72°C for one minute for a total of thirty cycles. The completed reactions were run on a 1.5% agarose gel and stained with ethidium bromide. The presence of a 355 bp band was considered a positive result (Fig. 4.3).

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Name	**Sequence 5'-3'**
Real-time NS1 Fwd	CATACTACACATTCGTCCTCCACAA
Real-time NS1 Rev	CTTGGTGATTCTGGTTCTTGACTCTT
Real-time NS1 Probe	FAM-CCAGGGCCAAGCAAGCGCC-TAMRA
*Ae*DNV VP Fwd	AGACAACGCTTGCTAAC
*Ae*DNV VP Rev	GTTGTTGTATATACTCCTTC
*Ae*DNV Seq Fwd	ATGGCAGAATTAACAGAAC
*Ae*DNV Seq Rev	AGCTTCCTAACTGTATCAT
pUCA-MCS Fwd	CAAATTAATTAATATCACAATATCCTCAGCTAGTATAC CGGTATTCCACCTCAG
pUCA-MCS Rev	CTGAGGTGGAATACCGGTATACTAGCTGAGGATATTG TGATATTAATTAATTTG
pUCAMut Fwd	CAAACACTACTATTCAATGCAAACAACGACAGA
pUCAMut Rev	CGAAGGGAGAAAGGCGGACAGGTA

Table 4.1. Oligonucleotides used for *Ae*DNV quantification and mutagenesis.

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Primers flanking the RNAi expression cassette insertion site in the right-end UTR of *Ae*DNV were used to amplify DNA samples from mosquitoes positive for *Ae*DNV infection. PCR was performed according to the above protocol using the *Ae*DNVSeq primer set (Table 4.1). The completed reactions were run on a 1.5% agarose gel, stained with ethidium bromide, and the desired fragments were extracted using the Qiagen Gel Extraction kit (Qiagen, Valencia, CA). All PCR fragments were sequenced using an automated sequencer at Colorado State University's Macromolecular Resources facility.

Results

Mosquito Mortality Assay

To determine whether insertion of an RNAi expression cassette in the right-end UTR of the *Ae*DNV genome alters the effect of the virus on mosquitoes, wild-type *Ae*DNV and *Ae*DNV-RNAi produced from modified infectious clones were used to infect *Ae. aegypti* larvae as described in the mosquito infection protocol. Control larvae received no virus. The larval and total mortality rates for mosquitoes exposed to different versions of *Ae*DNV were calculated and are reported in Fig. 4.2.

Viruses containing genomes with modified right-end UTRs exhibited variations in their ability to cause mortality in *Ae. aegypti* mosquitoes. Viruses containing *Ae. aegypti*-based Pol III cassettes killed mosquitoes at rates comparable to those attained with wild-type *Ae*DNV. Significantly lower mortality rates were observed when viruses containing an *An. gambiae*-based cassette were used. Exposure of *Ae. aegypti* larvae to unmodified *Ae*DNV resulted in 68% larval mortality and 79% total mortality. Comparable mortality

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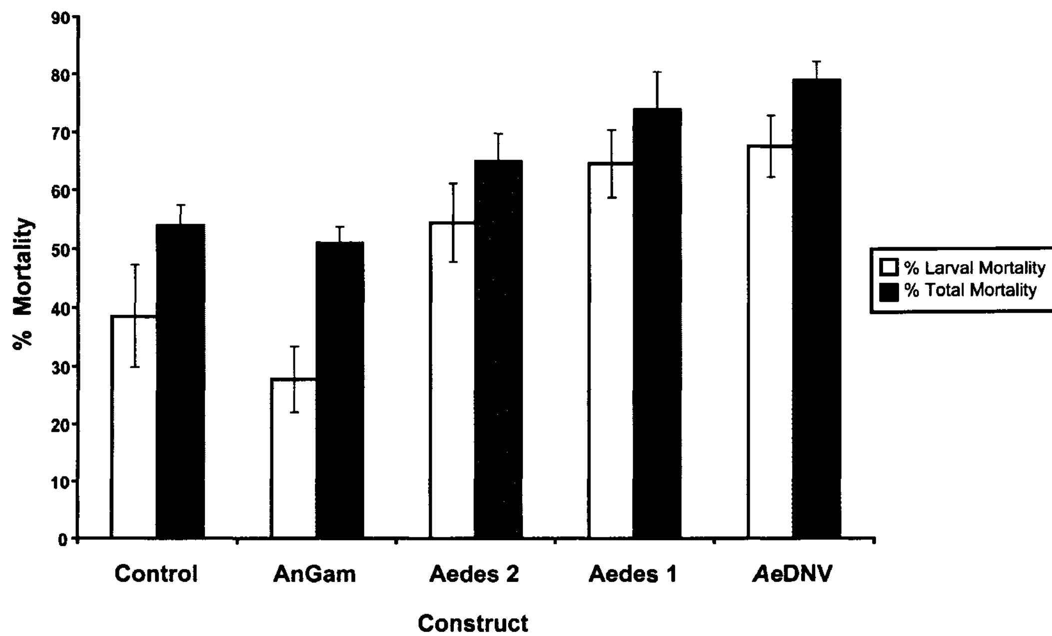

Fig. 4.2. *Aedes aegypti* mortality assay. Mortality achieved with wild-type and modified versions of *Ae*DNV. Error bars represent 95% confidence intervals.

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(65% larval and 74% total) was achieved with *Ae*DNV-RNAi-Aedes-1. Exposure of larvae to *Ae*DNV-RNAi-Aedes-2 resulted in 55% larval mortality and 65% total mortality. Larval mortality of only 28% was observed following exposure of larvae to *Ae*DNV-RNAi-AnGam. The larval mortality achieved with *Ae*DNV-RNAi-AnGam is lower than the larval mortality observed within the unexposed control larvae (39%), although this difference is not statistically significant. The 51% total mortality achieved with *Ae*DNV-RNAi-AnGam was comparable to the total mortality of 54% observed in the unexposed control group.

Infection of *Ae. aegypti* with *Ae*DNVs containing modified right-end UTRs

PCR was used to determine if viruses modified to include an RNAi expression cassette in the right-end UTR of the *Ae*DNV genome are capable of infecting *Ae. aegypti* mosquitoes. *Ae. aegypti* larvae were exposed to wild-type or modified *Ae*DNV and reared to adults. DNA samples harvested from surviving adult mosquitoes at 28 days post-exposure were amplified by PCR using primers specific for the *Ae*DNV VP gene. Completed reactions were run on a 1.5% agarose gel. The results of this experiment are shown in Fig. 4.3. The presence of a 355 bp band in lanes 6-8 suggests that *Ae*DNV genomes containing RNAi expression cassettes in the right-end UTR package into virions capable of initiating a productive infection in *Ae. aegypti* mosquitoes. A positive result was achieved with all three versions of *Ae*DNV-RNAi.

Confirmation of RNAi Expression Cassette Stability by Sequencing

DNA sequencing was used to confirm the stability of RNAi expression cassettes inserted in the right-end UTR of the *Ae*DNV genome. DNA samples extracted from

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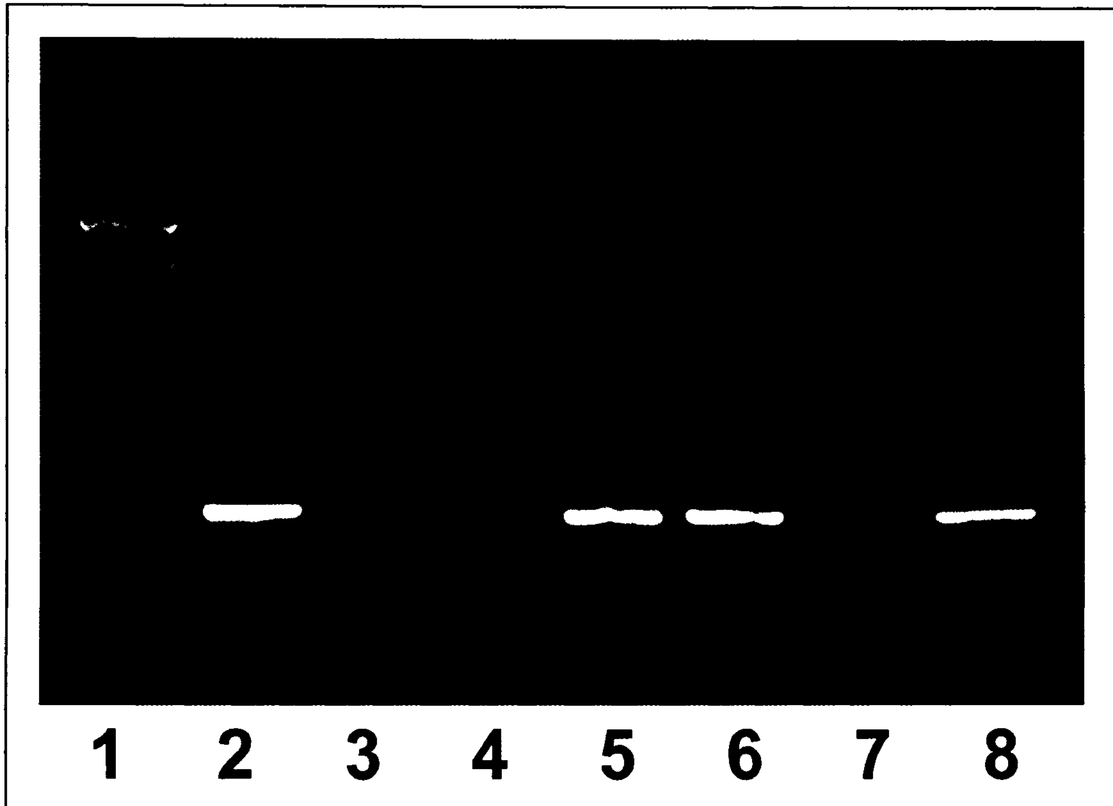

Fig. 4.3. Detection of *Ae*DNV by PCR. Adult *Aedes aegypti* mosquitoes surviving larval infection with wild-type or modified versions of *Ae*DNV were screened for *Ae*DNV infection using PCR. Control mosquitoes received no virus. Lane 1=DNA ladder; lane 2=positive control; lane 3=negative control; lane 4=uninfected control; lane 5=wild-type *Ae*DNV; lane 6=*Ae*DNV-RNAi-AnGam; lane 7=*Ae*DNV-RNAi-Aedes-1; lane 8=*Ae*DNV-RNAi-Aedes-2.

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adult *Ae. aegypti* mosquitoes positive for *Ae*DNV infection were amplified by PCR using primers flanking the inserted RNAi cassette. Sequence analysis confirmed that exogenous DNA comprising an RNAi cassette was still present in *Ae*DNV-RNAi genomes harvested from adult mosquitoes at 28 days post-exposure (data not shown). No mutations in the inserted cassette were observed with any version of *Ae*DNV-RNAi. The results of these experiments indicate that RNAi expression cassettes can be inserted in the *Ae*DNV right-end UTR and that genomes containing an RNAi expression cassette can be packaged into *Ae*DNV virions capable of infecting mosquito larvae. Sequence analysis of *Ae*DNV-RNAi genomes harvested from infected adult *Ae. aegypti* also suggests that the RNAi expression cassettes remain stable throughout the mosquito's development.

Discussion

The right-end UTR of the *Ae*DNV genome appears to be capable of tolerating the insertion of exogenous DNA comprising a Pol III-based RNAi expression cassette. *Ae*DNV genomes modified to include an RNAi expression cassette in the right-end UTR were packaged into *Ae*DNV-RNAi virions capable of infecting *Ae. aegypti* larvae. The *Ae*DNV-RNAi genomes could be recovered from adult mosquitoes using PCR. Figure 4.3 demonstrates that mosquitoes surviving larval infection with *Ae*DNV-RNAi remain infected following metamorphoses to pupae and adults. Persistence of *Ae*DNV-RNAi throughout the mosquito's life cycle is necessary for spread of the virus to new breeding sites by infected females transmitting the virus vertically. Sequence analyses confirmed the integrity of the inserted RNAi cassettes, indicating that potential pitfalls such as

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reversion of the virus back to the wild-type or alteration of the inserted sequences by recombination or spontaneous mutation should not pose problems.

The modified versions of *Ae*DNV were capable of killing *Ae. aegypti* larvae at rates comparable to the wild-type virus with the exception of *Ae*DNV-RNAi-AnGam (Fig. 4.2). The decreased killing efficiency observed with this version of *Ae*DNV may be attributable to the presence of a highly active Pol III promoter in the virus genome or decreased virus viability caused by genome packaging constraints. The insertion of a highly active exogenous Pol III promoter in the *Ae*DNV right-end UTR may reduce *Ae*DNV viability by interfering with viral processes such as transcription or DNA replication. Constructs based on the AnGam-2 and Aedes-1 U6snRNA promoters exhibited the highest amount of target gene silencing in ATC-10 *Ae. aegypti* cells, suggesting that these two promoters are the most transcriptionally active (Chapter 3). Binding of transcription factors to a highly active Pol III promoter inserted in the *Ae*DNV right-end UTR could interfere with DNA replication causing a reduction in virus viability. Data presented in Chapter 3 demonstrated that comparable target gene silencing, and presumably comparable levels of transcription, were achieved using Aedes-1 or AnGam-2-based promoter constructs. This observation indicates that the reduction in larval mortality observed with *Ae*DNV-RNAi-AnGam compared to *Ae*DNV-RNAi-Aedes-1 is most likely not due to the presence of a highly active Pol III promoter in the *Ae*DNV right-end UTR.

The reduction in larval mortality observed with *Ae*DNV-RNAi-AnGam may be attributable to reduced virus viability caused by a reduction in viral genome packaging efficiency. The wild-type *Ae*DNV genome contains 3978 nt. *Ae*DNV-RNAi genomes

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are slightly larger than wild-type *Ae*DNV (Table 4.2). Although parvovirus genomes as large as 120% of wild-type have been packaged successfully, the packaging efficiencies for genomes larger than the wild-type are often greatly reduced (Dong *et al.*, 1996; Hermonat *et al.*, 1997; Kimmick *et al.*, 1998). The longer version of the AnGam-2 promoter was used for the creation of *Ae*DNV-RNAi-AnGam. The additional 49 nt contained in the *Ae*DNV-RNAi-AnGam genome compared to *Ae*DNV-RNAi-Aedes-1 may decrease virus viability by hindering genome packaging. Reduction in virus viability due to increased genome size may explain the reduction in *Ae. aegypti* larval mortality observed with *Ae*DNV-RNAi-AnGam (Fig. 4.2). While this reduction in killing efficiency is significant, an *Ae*DNV vector exhibiting decreased mosquito lethality may be a more desirable gene delivery vehicle. Mosquitoes infected with a less lethal *Ae*DNV-derived gene vector would have a higher survival rate resulting in a greater probability for spread of the virus to other mosquito populations.

The data presented in this chapter suggest that *Ae*DNV can be developed as a vector for RNAi in mosquitoes. The infectious clone of *Ae*DNV (pUCA) was modified and used to produce three versions of *Ae*DNV-RNAi. *Ae*DNV virions containing genomes modified to include an RNAi cassette in the right-end UTR remain viable and are capable of infecting *Ae. aegypti* larvae. The cloning strategy used to modify pUCA will facilitate the creation of new versions of *Ae*DNV-RNAi containing hairpin sequences targeting genes necessary for arbovirus transmission. Further development of *Ae*DNV as a vector for RNAi in mosquitoes may form the basis of novel strategies for the control of mosquito-borne diseases.

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Construct	**Total Genome Size (nt)**	**% of Wild-Type *Ae*DNV**
*Ae*DNV	3978	N/A
*Ae*DNV-RNAi-AnGam	4131	103.8%
*Ae*DNV-RNAi-Aedes-1	4082	102.6%
*Ae*DNV-RNAi-Aedes-2	4067	102.2%

Table 4.2. Modified *Ae*DNV genome sizes. The size of *Ae*DNV genomes containing modified right-end UTRs are compared to unmodified *Ae*DNV.

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Chapter 5: Summary and Perspectives

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Summary

Mosquito-borne diseases are a global problem. The failure of traditional methods to effectively control mosquito-borne diseases has demonstrated the need for alternative control strategies. Mosquito densonucleosis viruses, including *Ae*DNV, have emerged as candidates for use in integrated vector-borne disease control programs. *Ae*DNV kills *Ae. aegypti* mosquitoes and can also be used as a transducing vector for the expression of foreign genes in mosquitoes. RNAi-based approaches for the disruption of arbovirus transmission have demonstrated considerable promise, but an environmentally safe vector for the delivery of RNAi to mosquitoes in nature is lacking. *Ae*DNV is an excellent candidate as a vector for RNAi in mosquitoes because of its environmental stability, narrow host range, and potential to spread through mosquito populations. The studies presented here were undertaken to determine if the right-end UTR of the *Ae*DNV genome is a suitable site for the insertion of an RNAi expression cassette.

Sequence elements necessary for polyadenylation of *Ae*DNV transcripts were investigated in Chapter 2. cDNA sequence analysis was used to determine that polyadenylation of *Ae*DNV transcripts occurs in the right-end UTR of the virus genome 13 nt downstream of a canonical AATAAA polyadenylation hexamer. Mutation and deletion analyses of the *Ae*DNV right-end UTR were used to determine the sequence elements required for efficient gene expression from viral promoters. The effect of each mutation or deletion on protein expression was assayed using a reporter gene system consisting of a β-gal gene expressed from the pVP promoter and followed by the *Ae*DNV right-end UTR. Relative levels of β-gal mRNA expression for each mutant were determined by performing RT-qPCR on RNA harvested from C6/36 cells transfected

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with the reporter gene constructs. The same mutations were also made in pUCA, the infectious clone of *Ae*DNV. The effect of each mutation or deletion on virus growth was assayed using quantitative real-time PCR analysis of *Ae*DNV virions harvested from the supernatants of cells transfected with different versions of pUCA. Efficient mRNA expression requires the presence of a 21 nt USE that is predicted to form a stem-loop RNA secondary structure. Deletion analysis also identified non-essential sequences downstream of the poly(A) site suitable for replacement with exogenous DNA.

Cloning and characterization of Pol III promoters capable of mediating RNAi in *Ae. aegypti* and *An. gambiae* cells was described in Chapter 3. It was hypothesized that a Pol III-based RNAi expression cassette would be small enough to be added to the right-end UTR of the *Ae*DNV genome without necessitating the removal of NS or VP coding sequences. Pol III promoters expressing shRNA are widely used for induction of the RNAi response in mammalian systems, but analogous promoters capable of inducing RNAi in mosquitoes had not been described. Putative U6snRNA Pol III promoters were cloned from the *An. gambiae* and *Ae. aegypti* genomes. The Pol III promoters were tested for the ability to express shRNAs targeted to firefly luciferase and to mediate RNAi knockdown of a co-transfected luciferase reporter gene vector in AG-55 *An. gambiae* and ATC-10 *Ae. aegypti* cells. Promoters capable of silencing expression of the co-transfected luciferase plasmid by up to 95% in AG-55 cells and up to 75% in ATC-10 cells were identified. RNase protection experiments allowed detection of the 19 nt luciferase siRNA in transfected cells. These findings indicated that mosquito U6snRNA gene promoters could be used for production of shRNA capable of inducing the RNAi response in mosquito cells.

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The experiments described in Chapter 4 investigated the viability and pathogenicity of *Ae*DNV virions that were modified to include an RNAi expression cassette in the right-end UTR of the virus genome. An RNAi cassette consisting of luciferase target sequences driven by one of the Pol III promoters characterized in Chapter 3 was inserted in the right-end UTR of the *Ae*DNV genome. Three different versions of pUCA-RNAi were created and used to produce *Ae*DNV virions containing the modified genomes.

Viruses harvested from C6/36 cells transfected with the modified infectious clones were used to infect *Ae. aegypti* larvae. The ability of the modified viruses to cause mortality in *Ae. aegypti* mosquitoes was compared to the mortality achieved with unmodified *Ae*DNV. Viruses containing *Ae. aegypti*-based Pol III cassettes killed mosquitoes at rates comparable to those attained with wild-type *Ae*DNV. Viruses containing the larger *An. gambiae*-based cassette exhibited decreased killing efficiency, although this may be due to genome packaging constraints. Infection of mosquitoes with the modified versions of *Ae*DNV was confirmed using PCR. Detection of *Ae*DNV in adult mosquitoes surviving larval infection indicated that *Ae*DNV genomes modified to include an RNAi expression cassette in the right-end UTR were capable of packaging into infectious virions. Sequence analysis of viral DNA harvested from infected adult mosquitoes confirmed that the inserted cassettes remain stable throughout the mosquito's development.

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Perspectives

Mosquito-borne diseases are a scourge upon humanity. The failure of traditional methods to reduce the global burden imposed by mosquito-borne diseases has demonstrated the need for novel approaches. *Ae*DNV has demonstrated utility as a biological control agent and as an agent for mosquito transduction. Realization of *Ae*DNV's full potential will require additional investigations of the virus's molecular biology and pathogenesis.

These studies have demonstrated that *Ae*DNV has potential for use as a vector for RNAi in mosquitoes. The characterization of Pol III promoters for the expression of shRNA capable of mediating RNAi in mosquito cells provides a new tool for RNAi-mediated gene silencing applications in mosquito systems. The conclusion that RNAi cassettes based on these Pol III promoters can be inserted in the right-end UTR of the *Ae*DNV genome provides the foundation for further development of *Ae*DNV as a vector for RNAi. RNAi mediated silencing of genes involved in the transmission of mosquito-borne pathogens may be achieved using modified versions of *Ae*DNV. Induction of the RNAi response using *Ae*DNV as a vehicle for the delivery of shRNA to mosquitoes may form the basis of novel strategies for the control of mosquito-borne diseases.

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