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DISSERTATION

MOLECULAR BIOLOGY AND PATHOGENESIS OF *AEDES AEGYPTI* DENSOVIRUS

Submitted by

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In partial fulfillment of the requirement

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2001

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TODD W. WARD ENTITLED "MOLECULAR BIOLOGY AND PATHOGENESIS OF *AEDES AEGYPTI* DENSOVIRUS" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MOLECULAR BIOLOGY AND PATHOGENESIS OF *AEDES AEGYPTI* DENSOVIRUS

Aedes aegypti densovirus (AeDNV) is a parvovirus that efficiently infects and kills *Aedes aegypti* mosquitoes. AeDNV has potential for use as a biological control agent, or a transducing vector for the introduction of genes of interest into *Aedes aegypti* mosquitoes.

AeDNV has two promoters shown to be functional by reporter gene analysis. Expression from the structural protein gene promoter (pVP) and nonstructural protein gene promoter (p7) was analyzed by deletion and mutation of constructs containing the *lac*Z reporter gene fused to the VP and NS1 open reading frames. Primer extension was used to map the transcriptional start site of the structural transcript to a consensus initiator sequence (CAGT) 60 nucleotides upstream from the map unit 61 TATAA sequence previously thought to define the promoter. Mutagenesis and deletion analysis showed that both the TATAA sequence at map unit 59.5 and the initiator sequence are required for efficient expression of LacZ-VP fusions from pVP and mutation of the map unit 61 TATAA sequence had little effect. Mutation of the p7 TATAA sequence abolished gene expression, however, in contrast to the pVP promoter, mutation of the consensus initiator sequence only reduced expression of NS1-lacZ fusion proteins by 60%. Temporal expression analysis of the NS1, NS2, and VP fusion proteins showed that all viral proteins are expressed simultaneously. VP protein is expressed to a higher level than the NS proteins and NS2 is expressed to much higher levels than NS1.

A mixture of wild-type AeDNV and transducing virus carrying the GFP gene were used to infect *Aedes aegypti* larvae. The pathogenesis of AeDNV

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infection was followed by the observation of GFP expression by fluorescence microscopy. The anal papillae were the primary route of infection, with dissemination to the fat bodies taking approximately two days after infection. Older larvae were less susceptible to infection and more likely to survive into adult stages still expressing GFP. Fifty-six percent of infected larvae lost infected anal papillae after they were observed to shrink or occasionally melanize, suggesting an anti-viral immune response. Adult *Aedes aegypti* were infected with a mixture of wild-type AeDNV and transducing virus by intrathoracic injection. Ninety-five percent of mosquitoes showed GFP expression 72 hours after infection. Injection of mosquitoes 24 hours after the ingestion of a blood meal provided optimal infection rates in progeny larvae. Efficiency of vertical transmission of virus paralleled the endocytic activity of developing follicles within the ovaries. GFP expression was detectable in 50% of progeny, however PCR detected the presence of wild type AeDNV in 100% of young larvae. However, GFP expression was found to be transient, usually disappearing by 4 days post hatching. PCR detected AeDNV or GFP DNA in only 16% of surviving adults. These results suggest that intrathoracic injection of AeDNV derived vectors may provide an efficient means of introducing genes of interest into mosquitoes.

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Dedication

For my parents Bill and Suzy Ward. You taught me that success was always achieved if I gave my best, even if it wasn't very good. Your love, encouragement, and support always gave me the guts to try.

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Chapter 1: Literature Review

Introduction

Impacts of mosquito-borne disease.

Mosquitoes are potent vectors of many pathogens that cause disease in humans and animals. Mosquitoes have aptly been described as flying syringes as they are extremely efficient at transmitting pathogens from one host's blood to another. Mosquito-borne diseases are distributed around the world in association with the distribution of the mosquitoes that transmit them. Pathogens transmitted include arboviruses, such as dengue and yellow fever, and parasites, such as plasmodium and filarial worms.

Malaria is caused by *Plasmodium ssp.* and is transmitted by many mosquitoes of the *Anopheles* genus, most effectively by *Anopheles gambiae*. Malaria is one of the most costly of mosquito-borne diseases affecting over 200 million people world wide each year. 1-2 million people are estimated to die each year from malaria with over 1 million deaths occurring in Africa alone (Anonymous, 2001). In addition to the high mortality of diseases such as malaria, mosquito-borne diseases have a major impact on the quality of life and economic well being of countries in endemic areas. Malarial endemic countries are estimated to have 20% less economic development in the past 15 years than equivalent malaria-free countries (Anonymous, 2000c).

Arboviruses are viruses that replicate within and are transmitted by arthropods to animal hosts. Of the over 500 arboviruses catalogued, approximately 100 are recognized for their ability to infect humans (Nasci and

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Miller, 1996). The *Aedes aegypti* mosquito is a potent vector of viruses that cause yellow fever and dengue fever. An effective vaccine for yellow fever has been developed and has helped to reduce incidence of this disease. However, no such vaccine exists for dengue and over 2-billion people are at risk for dengue fever with approximately 50-million cases reported each year in tropical regions around the world (Anonymous, 2000a,b). Temperate regions, including North America, are endemic to many arboviruses such as LaCrosse and St. Louis encephalitis viruses and dengue has encroached upon the southern borders of Texas (Rawlings *et al.*, 1995).

Relief from the effects of these diseases requires successful control of the disease within the host, or control of the mosquito vector. Traditionally, mosquito control strategies concentrated upon the reduction of mosquito populations by use of chemical pesticides or through the reduction of breeding habitat by the drainage of wetlands and removal or sealing of water containers. Mosquitoes are able to efficiently maintain pathogen transmission; consequently, control programs often require a 90% or more reduction in mosquito populations to be effective (Focks *et al.*, 2000). Unfortunately, mosquito control measures have suffered from emerging resistance in mosquitoes to pesticides, and lack of long-term commitment from governments and human populations to maintain control efforts. Any one of the approaches to mosquito-borne disease control discussed below may have some success on its own, but the levels of success needed to break transmission cycles will likely require integrated control programs that include many various techniques (Mitchell, 1996).

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Control of Mosquito-borne disease

Mosquito pathogen interactions.

Mosquito-borne pathogens are intimately linked to the vector that transmits them. Mosquito-borne pathogens are generally unable to be transmitted between humans. Thus, the relationship between the mosquito and pathogen, and between the mosquito and its host, is critical to the propagation of disease. This is where the flying syringe analogy becomes oversimplified. The mosquito not only transmits pathogens, but plays a central role in the propagation, development, evolution, and survival of the pathogen outside the human host. Vector competence is a measure of a mosquito's intrinsic ability to support the development and propagation of a pathogen and to transmit the pathogen to a new host. This depends upon the genetics of the mosquito and varies from species to species as well as within species.

It is common, especially in the case of arboviruses, for the mosquito to appear to suffer no deleterious effects for carrying a heavy pathogen load. Viruses that cause serious, life threatening disease in humans have no apparent effect on the mosquito. After a relatively small number of pathogens are ingested during a blood meal the pathogen must get from the midgut lumen to the mosquito's salivary glands to be subsequently transmitted to a new host. There are many stages at which a mosquito may present a barrier to the invading pathogen. Within the midgut the pathogen must be able to pass through the peritrophic matrix and infect the epithelial cells lining the midgut (midgut infection barrier) (Jacobs-Lorena and Oo, 1996; Woodring *et al.*, 1996). Once within the cells of the midgut the pathogen must pass to the basal surface and penetrate into the hemolymph (midgut escape barrier) (Woodring *et al.*, 1996). Finally, the pathogen must invade the salivary glands

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(salivary gland infection barrier) where it may be transmitted during the next blood meal. At each stage of the infectious process the pathogen must have traits that allow it to overcome these barriers. In the case of arboviruses the mosquito serves as a source of amplification for the pathogen. Small numbers of arboviruses are ingested. However, after replication and dissemination from the midgut the virus infects and replicates further in the salivary glands and the mosquito is then able to transmit virus to a new host. This propagation is necessary for many pathogens to create a high enough dose to infect the next host.

Mosquitoes also provide a suitable environment for the development of many parasites. Malarial parasites (*Plasmodium ssp.*) utilize the mosquito as a cyclo-propagative host. The parasite develops into an ookinete within the midgut, penetrates through the midgut epithelium and forms an oocyst which gives rise to 1000's of infective sporozoites which migrate to the salivary glands. These developmental stages are absolutely dependent upon the mosquito as they do not occur within the vertebrate host (Gwadz and Collins, 1996). Alternatively, filarial parasites use a mosquito only for development of microfilaria to the infective larval stage that then can reinfect a host. There is no increase in pathogen numbers, therefore, this is considered a cyclo-developmental relationship (Woodring *et al.*, 1996).

In addition to being a developmental and propagative participant, mosquitoes also serve as reservoirs protecting pathogens and maintaining the transmission cycle when no hosts are available, or when feeding is not possible due to climatic changes. In sub-Saharan Africa mosquitoes, and the pathogens they carry, must survive the dry season. In temperate regions they must survive cold seasons. A short photo period, or other factors may induce the mosquito to enter diapause, a dormant, non-developing period (Nasci and

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Miller, 1996). Mosquitoes may diapause in the form of an egg, larvae, or adult. During this period the pathogen must also maintain itself without damaging the mosquito. When conditions permit diapause is broken and the pathogen is ready to be transmitted, usually through the first blood meal.

Genetic variation of arboviruses also takes place mainly within the mosquito and not within the human host (Beaty, 1996). Surprisingly, during diapause viruses are not completely dormant. Supporting this theory is the observation that bunyavirus cap scavenging continues during diapause (Dobie *et al.*, 1997).

Vectorial capacity is a measure of the effectiveness of a mosquito population, at a given time and place, to transmit pathogens to hosts and propagate disease. Apart from a mosquito's vector competence, additional factors such as population density of both host and vector, and associations between these populations play a large role in a mosquito's ability to effectively propagate diseases (Black and Moore, 1996). Disease propagation requires a constant supply of pathogen that can cycle between human hosts to mosquito vectors. A period of time in which a population of mosquitoes is unable to bite infected hosts, or infected mosquitoes are unable to find susceptible hosts may cause a break in the transmission cycle. Ecological considerations such as host seeking behavior, preferred breeding habitat, presence of a susceptible population of hosts, fecundity, and voltinism all play essential roles in bringing mosquitoes and humans together and thereby in propagating disease (Nasci and Miller, 1996). Obviously, as mosquito populations rise, so do the interactions between mosquitoes and their hosts. Anthropophilic mosquitoes such as *Anopheles gambiae* feed almost exclusively on humans, and prefer to rest indoors. Likewise *Aedes aegypti* mosquitoes breed in artificial containers like cisterns and tires, and also prefer to rest

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indoors. These ecological factors increase the vectorial capacity of the mosquito. However, it is possible to have an extremely competent vector, one that supports pathogen propagation and has the ability to transmit an infectious dose of pathogens, but, due to the separation of the mosquitoes from an appropriate host, low mosquito populations, or lack of a susceptible population, is unable to transmit the disease to a suitable host. Understanding factors affecting vector competence and vectorial capacity allow for directed attempts at controlling vector-borne diseases.

Control of mosquito vectors.

The complex interactions between the mosquito, pathogen, and vertebrate host have many aspects that could be perturbed to break the transmission cycle. Control measures traditionally focus on reducing the vectorial capacity of mosquito populations to transmit disease. Vectorial capacity can be reduced by limiting the interaction between human hosts and mosquitoes. This can be achieved by reducing mosquito population size or by reducing mosquito-human contact. The reduction of a vector's competence, its ability to support the propagation or development of pathogens, is a relatively new area of research and requires manipulation of the mosquito itself.

Control measures have been developed and modified as more knowledge has been gained about the ecology of mosquitoes. Traditionally, control of mosquito-borne disease has focused upon the reduction of mosquito populations. One of the most effective methods of achieving this is the reduction of mosquito breeding habitat. *Aedes aegypti* mosquitoes breed well in artificial containers including old tires, cisterns, and soda cans, that fill with rain water (Tun-Lin *et al.*, 2000). This brings the mosquito into close association with populations of humans, who inadvertently provide the

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containers for the mosquitoes (Mitchell, 1996). Control of yellow fever and dengue fever has been accomplished by the removal of *Aedes aegypti* breeding containers near human domiciles, which reduces vector-human interactions. Through forceful compliance to control measures, including the destruction of breeding containers and drainage of wetlands, General Gorgas was able to eradicate yellow fever from Cuban cities and was instrumental in the control of yellow fever and malaria during the construction of the Panama canal (Cashman, 1998). Reduction of breeding habitat is still a useful strategy. However, apart from the control of water impoundments and reduction of artificial containers, large scale drainage of wetlands has been abandoned in many countries due its effects on the environment. Additionally, the removal of artificial containers requires high compliance rates and long-term commitment of the population and governments involved to remain effective.

Chemical pesticides, such as DDT, have been widely used and have also proven very successful. However, unforeseen effects on non-target organisms, and the surprisingly quick emergence of DDT-resistant mosquito populations have all but ended the use of DDT (Ferrari, 1996). Generally, chemical pesticides are applied to large areas by aerial spraying. This inundation of mosquito habitat requires the application of large quantities of pesticides and does not specifically target mosquitoes. Problems are encountered when low doses reach mosquitoes. This is not only ineffective, but can help foster resistance (Ferrari, 1996). Mosquitoes such as *Aedes aegypti* and *Anopheles gambiae* prefer to rest inside homes. Thus sprayed pesticides are not effective unless sprayed within each home. Breeding containers, such as tires, also protect mosquito larvae from these types of applications (Perish, 2000). Newly developed ultra-low volume aerosols produce droplets small enough to penetrate houses and kill mosquitoes hiding

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within homes (Perish, 2000). Though this technology is promising, concentrations delivered within homes must be evaluated carefully to ensure efficacy. Due to the toxicity, resistance, and environmental problems of traditional chemical pesticides, research has been conducted on safer pesticides or alternate methods of delivery. Safer pesticides have been produced such as insect hormone analogs, (i.e. methoprene), but many suffer from low stability or require a long time to kill the mosquito (for review see Palchick, 1996). In malaria endemic areas the use of pyrethroid impregnated bed nets has been successful and only kills those mosquitoes that try to feed upon humans. Unfortunately, the use of these bed nets is limited by the ability of people to afford and maintain a tear free net and to use them properly (Rashed *et al.*, 2000).

Biological control

An alternative to the chemical control of mosquitoes is biological control. Biological control of mosquitoes encompasses the use of mosquito predators, pathogens, or toxins produced by organisms. The benefits of biological control include low environmental impact, high specificity, and safety, but biocontrol strategies typically suffer from high cost of implementation. The mosquito guppy *Gambusia affinis* effectively controls mosquito populations when introduced into water sources containing mosquito larvae. By devouring hundreds of mosquito larvae daily these fish effectively reduce mosquito population size. Effective control of rice paddy mosquitoes by edible fish has been shown to be attainable and has the added benefit of increasing rice yield and providing fish for food (Wu, 1991). This is an example of an ideal control method, as it is effective and encourages participation in the control program by giving the participant obvious and

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tangible benefits. These predacious fish obviously require a large body of water to be effective and are useless against container breeding mosquitoes such as *Aedes aegypti.* Additionally the larval habitat must be compatible with that needed by the fish.

Parasites, bacteria, fungi, and viruses have been discovered that have potential use as control agents and have little or no harmful effect on the environment. These organisms have the potential for large scale spraying and can also be applied to the control of container breeders. To date the only widely used biocontrol agent of this type for mosquitoes has been the larvacidal bacterium *Bacillus thuringiensis israelensis* (BTI) (for review see Davidson and Becker 1996). BTI has many advantages, including a relatively simple and cheap means of production, and its target specificity for the digestive tracts of insects. BTI produces endospores that are collected, packaged and distributed into mosquito breeding habitat. Once activated by the alkaline digestive tract of an insect larva, the spores germinate releasing preformed toxins that kills the larva. Despite its success, BTI has a relatively wide spectrum of action, killing many insect larvae other than the target species, and resistance in wild populations has been observed (Ferre, 1991). Fungi such as *Lagenidium giganteum* and *Culicinomyces clavisporus* also have been developed that infect and kill mosquito larvae (for review see Davidson and Becker, 1996). However, *Culicinomyces clavisporus* must be ingested by larvae and its effectiveness is therefore related to the abundance of alternative food in the environment. In fact, introduction of fungal spores has been shown to increase mosquito survival rates and size as a result of killing weaker larvae which reduces competition for food (Wilson *et al.*, 1990). Copepods such as *Mesocyclops ssp.* can kill mosquito larvae and are attractive as they are normally found within larval habitats, can be grown in the

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laboratory, and are easily reintroduced to control target mosquito populations (Nam *et al.*, 2000).

Densoviruses as a control agent

Viral control of agricultural pests has been accomplished by the use of baculoviruses. Baculoviruses are large DNA viruses that are pathogenic for Lepidoptera insects. Improvements in killing efficiency have been made by the introduction of toxic genes into the viral genome. Until recently no, baculoviruses have been found that productively infect mosquitoes. However, a newly characterized baculovirus may have potential for the control of *Culex nigripalpus* and *Culex quinquefasciatus* (Becnel *et al.*, 2001; Moser *et al.*, 2001). Many viruses have been evaluated for their potential for mosquito control. These include mosquito iridescent viruses, nuclear polyhedrosis viruses and cytoplasmic polyhedrosis viruses. These viruses have been all but abandoned due to low mortality of affected populations, and difficulty of achieving infection in the laboratory. Despite these drawbacks viruses cannot be excluded as potential control agents.

Densoviruses of mosquitoes have not been given much scrutiny but may have better potential for the control of vector mosquito species than previously characterized viruses. *Aedes aegypti* densovirus (AeDNV) infects and kills mosquitoes of the genera *Aedes*, *Culex* and *Culiseta*, with the most dramatic pathology within *Aedes aegypti* mosquitoes. It was originally found as a contaminant in a laboratory mosquito colony in Kiev, Ukraine (Buchatsky *et al.*, 1987). Viroden, an AeDNV-derived biocontrol agent licensed in the former Soviet Union, has been shown to control mosquito populations in field trials (Buchatsky *et al.*, 1987). AeDNV is maintained in nature by horizontal as well as vertical transmission (Buchatsky *et al.*, 1989). *Aedes albopictus*

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parvovirus (AaPV), a closely related parvovirus, also causes pathology in *Aedes aegypti* mosquitoes, and can transmit vertically (Barreau *et al.*, 1996, 1997). Other densoviruses have been isolated from insect cell cultures (O'Neill *et al.*, 1995) and some have been shown to persist in laboratory colonies for up to 7 generations (Kittayapong *et al.*, 1999). The high mortality associated with infection is encouraging, and densoviruses appear to be widespread in nature in various mosquito species (Kittayapong *et al.*, 1999).

Statements that the ability of a virus to be vertically transmitted implies lack of pathogenicity are over simplified (Woodring and Davidson, 1996). As with all infectious agents, the route of infection and dose can effect the outcome of disease. Detailed examination of AeDNV and other mosquito densoviruses has indicated that young larvae are most susceptible to infection and suffer greater mortality rate than infections in older larvae (Buchatsky *et al.*, 1989; Barreau *et al.*, 1996). Surviving mosquitoes allow transmission to the next generation (Buchatsky *et al.*, 1989; Barreau *et al.*, 1996). Densoviral infection can be detected by behavioral changes, visible whitish coloration, electron microscopy, staining of affected nuclei, or by PCR detection of viral DNA (Buchatsky *et al.*, 1989; Barreau *et al.*, *1996*, O'Neill *et al.*, 1995). Infection of young larvae *Aedes albopictus* densovirus yields 100% mortality prior to adulthood (Barreau *et al.*, 1996). Inoculation of mosquito breeding sites with densoviruses has been shown to provide temporary reduction of larval numbers (Buchatsky *et al.*, 1987).

Unlike other mosquito viruses, densoviruses are extremely resistant to harsh environmental conditions and show high infection rates in laboratory colonies (Buchatsky *et al.*, 1989; Barreau *et al.*, 1996; Kittayapong *et al.*, 1999). Parvoviruses resist pH from 3-9, temperatures of 60°C, and are relatively resistant to UV light. This makes it possible to produce virus preparations, like

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Viroden, that are similar in stability to BTI preparations (Buchatsky *et al.*, 1987). An obvious hurdle to the development of any virus control agent is the high cost of production. Densovirus production in cell culture is low. Densovirus purification from infected larvae (Viroden) provides a cheaper and much more effective means for virus production (Buchatsky *et al.*, 1987; B. Duda personal communication). As discussed below, AeDNV has potential for the control of mosquitoes in addition to its pathogenicity; the AeDNV genome can be manipulated to express genes of interest in vectors.

Genetic control and transformation of mosquitoes.

Genetic control of mosquitoes is another potential means to reduce vector capacity. However, unlike chemical or biological control, genetic control can also be utilized to reduce vector competence by altering a mosquitoes ability to transmit disease. Genetic control of vector capacity maintains the goal of mosquito population control but by very different means. Sterile insect techniques have been developed that introduce massive quantities of sterile male insects into a natural insect population. Females mated to a sterile male do not produce viable offspring. Although successful in the control of some insects, this technique has not been effective in combating large mosquito populations (Rai, 1996). This may be due in part to a lack of knowledge of the mating behavior of the target mosquitoes. Competition with non-sterile males is problematic as some mosquitoes may mate with more than one male. Another problem is that the number of sterile males may be insufficient to compete with the enormous natural population (Rai, 1996).

The reduction of a vector's competence, its ability to support the propagation or development of pathogens, is a relatively new area of research

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and requires manipulation of the mosquito itself. An alternative type of genetic control is the modification of the mosquito at the genetic level. This involves the introduction of a foreign gene into mosquitoes. Presently, this is the only type of control that has the capability to reduce vector competence. Transgenic mosquitoes would express a foreign gene that, for example, could block arbovirus replication within salivary glands, or create a formidable midgut escape barrier. In theory, this would not yield a reduction in the mosquito's fitness, and it actually may provide an advantage by relieving damage done by larger parasites such as *Plasmodium ssp.*

Successful transformation of mosquitoes has been achieved using transposable elements (TE). TEs are flanked by inverted repetitive sequences that are able to transpose from one location in DNA to another. Original experiments in mosquitoes used the P-element of *Drosophila* . Successful integration was achieved in *Anopheles gambiae* (Miller *et al.*, 1987) and *Aedes triseriatus* (McGrane *et al.*, 1988) both using selection with G418. However, it was determined that the P-element was not actually involved in transposition. Subsequently, *Hermes* transposon mediated transformation was achieved with *Anopheles gambiae* cell lines (Zhoa and Eggleston, 1998). Transformed *Aedes aegypti* mosquitoes have been produced that express the *Drosophila melanogaster* cinnabar gene and fire-fly luciferace protein (Jasinskiene *et al.*, 1998; Coates *et al.*, 1999), both using the *Hermes* transposition systems. *Hermes* has also been used to transform *Culex quinquefasciatus* (M. Allen, unpublished observations). Additionally, the *Mariner* transposon has also been used to transform *Aedes aegypti* mosquitoes (Coates *et al.*, 1998). Transformation of *Anopheles stephensi* has been achieved with the *Minos* transposon (Catteruccia *et al.*, 2000).

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Attempts to introduce transgenic, refractory mosquitoes into the wild would benefit from a transposons ability to drive itself into a population through transpositions. P-element inundation of *Drosophila* laboratory populations have been shown to require an introduction of 5% of the existing population (Carareto *et al.*, 1997). However, computer modeling suggests that the introduction of a TE into a mosquito population would require a less than 1% introduction of transformed mosquitoes into an established population (Ribeiro and Kidwell, 1994).

Though potentially useful as a control method in itself, cytoplasmic incompatibility due to the presence of *Wolbachia ssp.* bacterial endosymbionts could be used to help drive transgenes into mosquito populations by selecting against non-transformed/*Wolbachia*-free mosquitoes. *Wolbachia*-free mosquitoes would not produce young when mated with *Wolbachia ssp.* infected, transgenic mosquitoes. This is due to the death of incompatible sperm within the egg cytoplasm (Rai, 1996). This drive mechanism would help to reduce natural non-transformed populations, and propagate the newly introduced strain.

Unfortunately, the current methods for developing a single transformed mosquito line requires the injection of DNA, carrying transposons and genes of interest, into mosquito eggs. Because of its low efficiency this procedure requires a large investment in man-hours, expensive equipment, and a certain knack for the procedure (For review see Handler, 2000). Because of this, genes of interest must be tested for efficacy prior to committing to the production of a transformed mosquito line. Transient expression systems have been developed for the evaluation of potentially useful genes. Sindbis virus, an Alphavirus of the family Togavirus, has been developed into a successful transducing system for the testing of

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genetic methods of disease transmission control (For review see Olsen, 2000). To perturb the vector competence of mosquitoes, antisense RNA, RNA interference, single chain antibodies, or ribozymes, all of which may interfere with pathogen replication or transmission, have been investigated using the Sindbis system. The gene of interest is cloned into a plasmid containing a DNA copy of the viral genome, downstream from a viral promoter. RNA is then generated, transfected into cell culture and recombinant virus is produced. This recombinant virus is then introduced into mosquitoes or cell culture, where it infects most cells and tissues. The gene of interest is expressed in the affected cells and tissues which allows study of the recombinant gene's effects. This system is very effective and has been successful in demonstrating RNA mediated knock out of luciferase gene expression within transformed *Aedes aegypti* mosquitoes (Johnson *et al.*, 1999). The Sindbis system has also been used to induced resistance to dengue, yellow fever, and LaCrosse viruses in mosquitoes (Olsen *et al.*, 1996; Powers *et al.*, 1996). The main drawback of the Sindbis system is that the virus is also known to infect vertebrates. This complicates experimental procedures and limits Sindbis to laboratory studies as it could never be released into a natural population. The importance of controlling mosquito-borne diseases has prompted research to discover additional tools for the testing of genes of interest within living mosquitoes and more efficient methods of transducing mosquitoes.

AeDNV Molecular Biology

Properties of Parvoviruses.

Parvoviruses are small (20-30 nm) icosohedral, nonenveloped, ssDNA viruses that have characteristic terminal genomic sequences that are able to

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form secondary structures. The family Parvoviridae is divided into two subfamilies, Parvovirinae, which include the mammalian parvoviruses, and Densovirinae, which include the parvoviruses of arthropods. The subfamily Parvovirinae includes three genera, *Erythrovirus*, *Parvovirus*, and *Dependovirus*. B19 virus represents the *Erythrovirus* genus and is the only autonomous parvovirus known to cause disease in humans. The *Erythrovirus* genus has a genome organization unique to parvovirus with all transcripts originating from a single promoter at the left end (Fig. 1.1). The *Parvovirus* genus includes mammalian parvoviruses, such as the canine and feline parvoviruses, and rodent parvoviruses, such as minute virus of mice (MVM) and H1 of hamsters. These viruses have two promoters, unique terminal sequences, and with the exception of LUIII parvovirus, package predominately the negative strand (Fig. 1.1.) Finally, the *Dependovirus* genus encompasses the adeno-associated viruses (AAVs) of humans and other mammals. This genus is unique in that members require coinfection with a helper virus, such as adenovirus or herpes simplex virus, to complete their life cycle. Additionally, in the absence of a helper virus, AAVs are able to integrate specifically into the host genome and wait for a helper virus in a latent state. *Dependoviruses* have identical terminal inverted repeat (TIR) sequences at either end of the virus genome and thus have a slightly different replication strategy than other parvoviruses. Furthermore, AAV has 3 promoters and packages both positive and negative genomic DNA equally. Members of the *Parvovirinae* subfamily encode all viral proteins on the same strand and, with the exception of erythroviruses, have two promoters, the nonstructural protein gene promoter at the left end and the structural protein gene promoter near the middle of the genome (Fig. 1.1).

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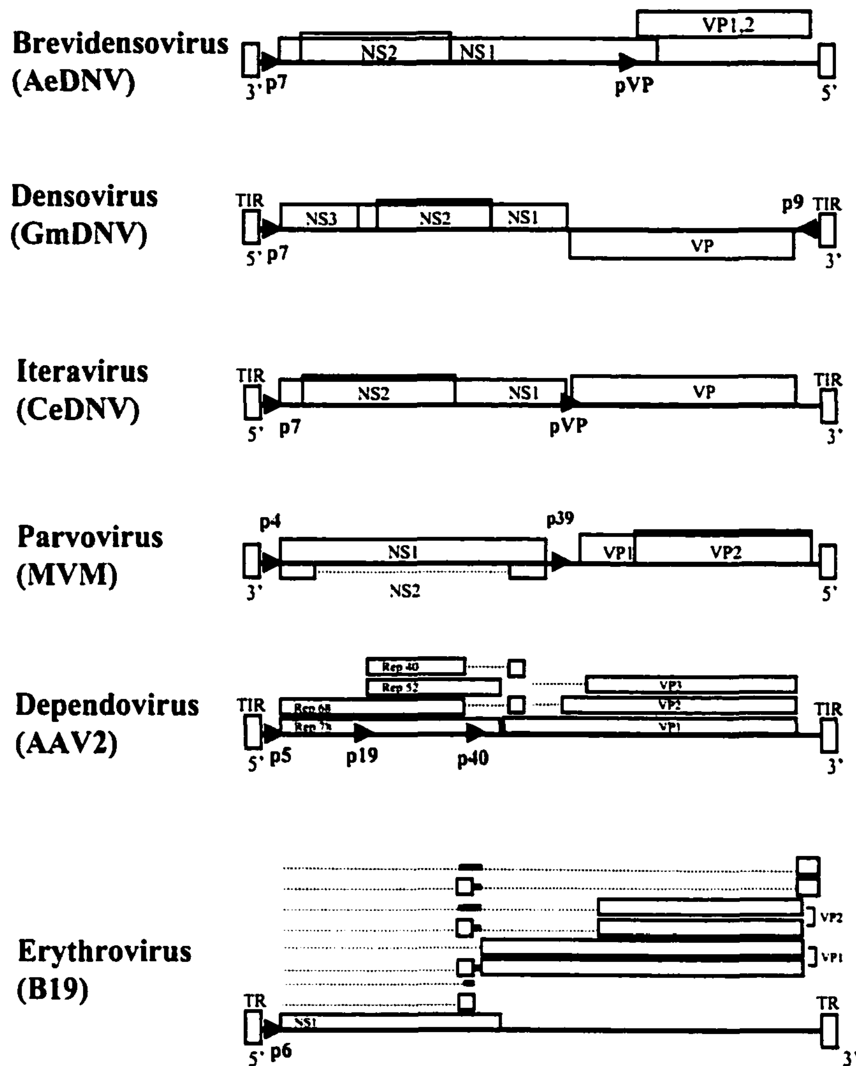

Fig. 1.1. Genome organization of the *Parvoviridae*. Boxes represent open reading frames, dotted lines indicate splicing, and filled arrows indicate promoter location and their direction of transcription.

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The subfamily *Densovirinae* encompasses autonomous parvoviruses that infect arthropods. This subfamily contains three genera, *Densovirus*, *Iteravirus*, and *Brevidensovirus*. The three genera are separated based upon their genome organization (Fig. 1.1). The genus *Densovirus* includes viruses that infect many insects of the order Lepidoptera with *Galleria mellonella* densonucleosis virus (GmDNV), and *Junonia coenia* densonucleosis virus (JcDNV) being the best studied (for review see Bergoin and Tijssen, 2000). All members of this genus encode their nonstructural (NS) proteins on the opposite strand to that of the structural (VP) proteins with promoters located in either of the identical TIR sequences at the ends of the viral genome (for review see Bergoin and Tijssen, 2000). Like AAV, members of the *Densovirus* genus encapsidate both strands equally. *Iteraviruses* include the economically important parvovirus of the silk worm *Bombyx mori* (BmDNV) and the *Casphalia extranea* densovirus (CeDNV). These viruses are most similar to the *densovirus* genus with identical TIRs. However, iteraviruses use the same DNA strand for production of both NS and VP proteins. Brevidensoviruses, like autonomous parvoviruses of mammals, have unique terminal sequences, and encode all proteins on the same DNA strand. Brevidensoviruses encapsulate predominately the negative DNA strand. Molecularly, AeDNV is the best characterized member of the Densovirus subfamily and is the prototype member of the *Brevidensovirus* genus. Another distinction of the brevidensoviruses is that the NS1 open reading frame (ORF) overlaps with the beginning of the VP ORF, a feature unique within the Parvoviridae (Afanasiev *et al.*, 1991). Within the genus *Brevidensovirus*, genome sequences indicate AeDNV is closely related to other members with 72.5%, 70% and 67.8% nucleic acid identity to the *Aedes albopictus* parvovirus (AaPV), a Peruvian isolate (ApeDNV), and *Haemogogous equinis* densovirus (HeDNV), respectively

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(Afanasiev *et al.*, 1994; Boublik *et al.*, 1994; B. Afanasiev personal communication; A. Paterson, personal communication). All four viruses are pathogenic to *Aedes aegypti* mosquitoes. The similarities of all known mosquito densoviruses suggests that information about the molecular biology and pathogenesis gained from studies on AeDNV presented herein should be applicable to all mosquito brevidensoviruses.

Host range

AeDNV was shown to be able to infect mosquitoes of the genera *Culex*, *Culisita* and *Aedes* (Buchatsky *et al.*, 1989), and morbidity resulted from lysis of infected cells. Interestingly, AeDNV does not cause cytopathology within *Aedes albopictus* C6/36 cell culture (Afanasiev *et al.*, 1994) and does not efficiently infect *Aedes triseriatus* mosquitoes. Broad host range with varying pathology has been observed for many parvoviruses. MVM replicates well within many cell cultures and is nonpathogenic in its natural host except in certain neonatal, inbred mice (Palmer and Tattersall, 2000). MVM and other rodent parvoviruses also replicate well within many human cell lines. The cellular receptor for autonomous parvoviruses has not been elucidated, but the broad host range of rodent parvoviruses, suggests that it is a relatively common cellular protein. The dependovirus AAV, has been found to utilize common heparin sulfate proteoglycans as receptors with various co-receptors contributing to tissue tropism *in vitro* (Carter, 2000).

Minor changes within the VP2 protein, in locations known to be at the capsid surface, drastically affect host range. Two subtypes of MVM; MVMp and MVMi, show reciprocal cell tropism for murine fibroblasts and T-lymphocytes respectively (Tattersall and Bratton, 1983). A two amino acid change in VP2, within the shared domain of VP1 and VP2, can confer the ability of each

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subtype to replicate within the opposite cell line (reviewed in Palmer and Tattersall, 2000). A similar situation has been observed in Aleutian mink disease parvovirus (ADV); the ADV-G strain is unable to replicate within mink but can replicate within Crandal feline kidney (CrFK) cells. A five amino acid difference separates this strain from the highly pathogenic ADV-Utah virus (Bloom *et al.*, 1998). A single amino acid change within VP2 conferred the ability of ADV-G to replicate and cause pathology within mink, though the disease is not identical to Aleutian disease (Fox, 1999). The most dramatic example of host range change is the emergence, and rapid world-wide spread, of canine parvovirus (CPV) in the late 1970's. CPV is a host range variant of the feline panleukopenia virus (FPV) and causes a highly fatal diarrheal disease in dogs, but is unable to infect felines. CPV is only 1% divergent from FPV at the nucleic acid level, and the host range determinant maps to five amino acid changes in VP2 (Parrish, 1999).

Although currently there is no crystal structure for a mosquito densovirus, GmDNV virions have been crystallized and the 3-D structure analyzed to a resolution of 3.7 angstroms (Simpson *et al.*, 1998). The external loop portions of VP4 were identified and are likely to be host range determinants as has been observed for mammalian parvoviruses.

It is tempting to speculate that with proper knowledge AeDNV could be manipulated to alter its host range. Densoviruses infecting many species of mosquitoes have been detected by PCR (Kittayapong *et al.*, 1999) and may yield valuable information if the determinants of tropism could be determined. It seems possible that like mammalian parvoviruses, small changes in the VP proteins of densoviruses could be engineered that allow infection of other important vectors of human disease, such as *Anopheles gambiae*.

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Interestingly, evidence indicates that CPV, FPV, ADV, and MVM tropism is not determined at the cell surface. These viruses have been shown to enter cells via endocytosis, but entry is not sufficient for infection (Linser *et al.*, 1979; Vihinen-Ranta *et al.*, 1998; Parker *et al.*, 2000). Mutations in VP2 of these viruses do not affect entry into cells. MVMi and MVMp productively infect murine fibroblasts and T-lymphocytes, respectively, but can compete for binding and uptake by both cell types (discussed above and reviewed in Palmer and Tattersall, 2000). This indicates that the tropism is based upon events that occur after virus entry, but prior to transcription (Spalholz and Tattersall, 1998; Ball-Goodrich and Tattersall, 1992). VP proteins have been shown to be necessary to direct viral DNA to the nucleus (Vihinen *et al.*, 2000). Antibodies against VP2 of MVM can block productive infection when injected into cells up to 8 hours after virus adsorption and entry (Vihinen *et al.*, 2000). In addition to the requirement for capsid proteins, productive infection of MVM also relies upon cellular factors that have yet to be identified (Spalholz and Tattersall, 1998). This suggests that release of viral DNA into the nucleus may take place by a similar mechanism to that of the human herpes viruses (HSV). HSV capsid proteins have been shown to specifically interact with nuclear pores and subsequent release of the viral DNA into the host nucleus requires the herpes HFES protein (for review see Roizman and Sears, 1996).

Replication

Once the viral genome is released into the nucleus of permissive cells, replication commences. Replication has been studied extensively in AAV and MVM (for review see Burns, 1996). Parvoviruses do not code for polymerases and thus rely upon the host's replication machinery. Replication of MVM is discussed here, because MVM has a genomic organization similar to AeDNV and

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it is assumed that their replication mechanisms are similar. The special secondary structures formed by the genomic terminal sequences (TS), at the 3' and 5' ends, mediate many aspects of replication. Initially the 3' TS hairpin provides a 3' hydroxy as a primer for DNA replication. DNA polymerase, possibly DNApol delta (Bashir *et al.*, 2000), synthesizes a new strand up to the 5' end of the genome formed by the 5' TS. The remaining nick is then ligated by cellular ligase (Fig. 1.2b). This initial conversion produces a double-stranded, monomer replicative form (RF) and can be detected *in vitro* and *in vivo*. Initiation and completion of conversion is solely dependent upon cellular factors as the virus does not carry any enzymes with it. Furthermore, transcription of viral genes cannot take place until after conversion of the genome into a double-stranded molecule.

Once conversion into a double stranded monomer is completed gene expression is possible and the genome is further converted into a dsDNA dimer RF. In MVM a binding motif proximal to p4 binds a host factor, E2F, that presumably activates NS1 expression (Delue, 1999). NS1 is produced and binds specifically to sequences within the TS. Binding sites within both the 3' and 5' hairpins have been found to be necessary for replication (Cotmore, 2000). NS1 initially binds to the 5' TS and introduces a nick from which replication proceeds (Fig. 1.2c). Formation of a dimer RF is accomplished via hairpin transfer (Fig 1.2e-g). The double stranded 5' hairpin sequences melt and the newly synthesized DNA strand forms a hairpin with itself to allow continuation of replication. Elongation to the other end of the viral genome creates a dimer that contains 5' TSs at either end and 3' TS in the middle of the molecule (Fig. 1.2g). This process can be repeated to create larger RFs. Resolution of the RF into ssDNA genomes requires the viral NS1 protein. To resolve the RF, NS1 nicks the DNA within the 5' TS and remains bound to the 5'

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Fig. 1.2. Replication model of MVM and most likely model for AeDNV. +,- indicate positive and negative DNA strands. Dotted lines indicate newly synthesised DNA. The 3' hairpin is shown in red and the 5' hairpin is green. Green circle and arrow indicate replication. a) Viral DNA as it enters the nucleus. b) Replication begins at the 3' end and is terminated at the 5' hairpin. The resulting nick is then ligated by cellular ligase. The viral NS1 protein is expressed. c) Viral NS1 binds the 5' hairpin and introduces a single stranded nick in the DNA. d) Replication commences and unfolds the 5' hairpin. e) Hairpin transfer. f) Replication continues to form a dimer replicative form. g-h) Dimer RF. NS1 binds to the 3' hairpin sequences. i) The 3' sequences can fold to form a holiday like structure. NS1 nicks the 3' sequences. j) Replication initiates from the 3' end of one strand and a recombination event resolves the secondary structure. k) Recombination results in two monomer RFs. l) Further replication from a second hairpin transfer (i) displaces a negative sense strand that can be packaged into a capsid. NS1 remains bound until final encapsidation.

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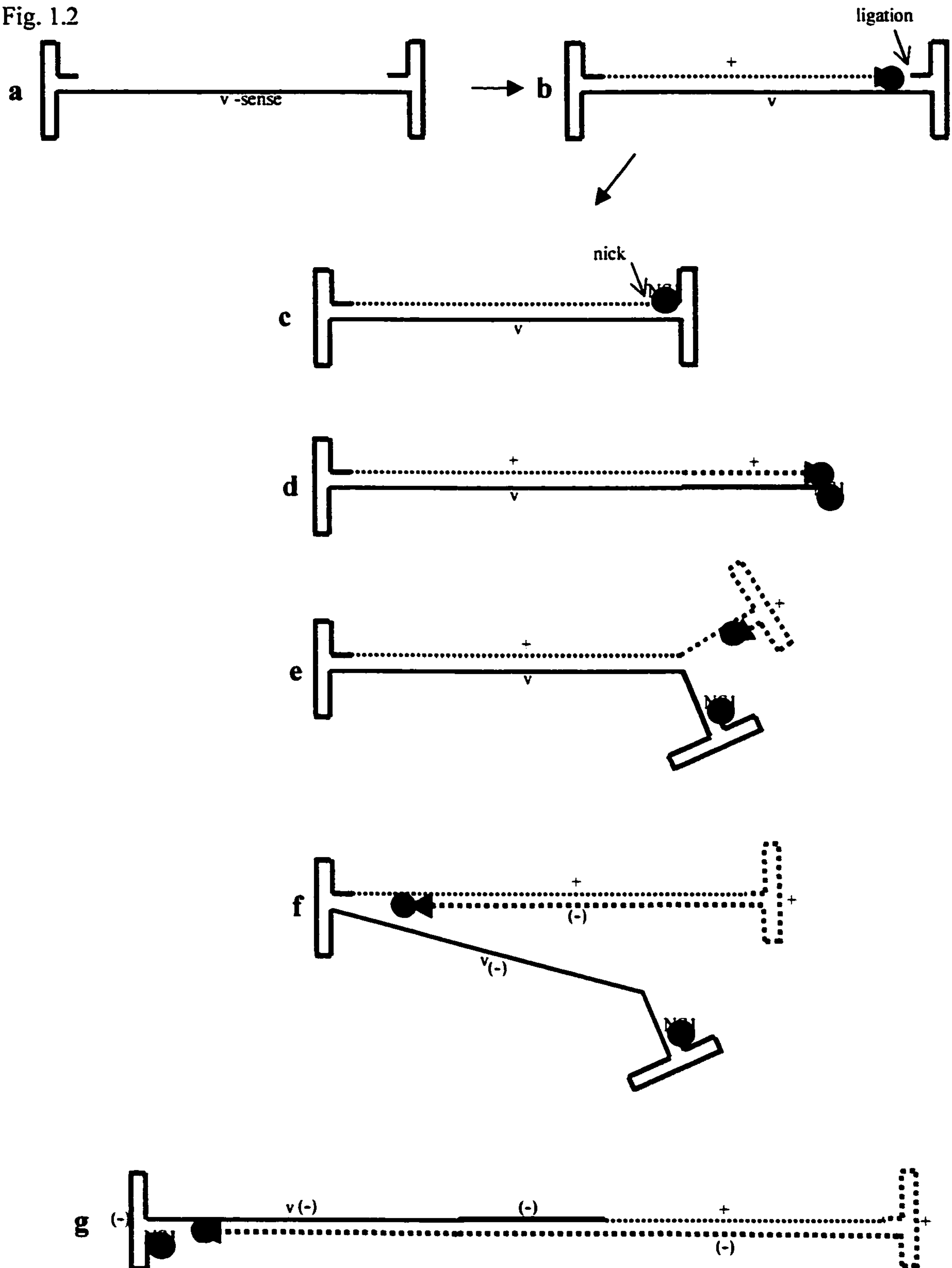

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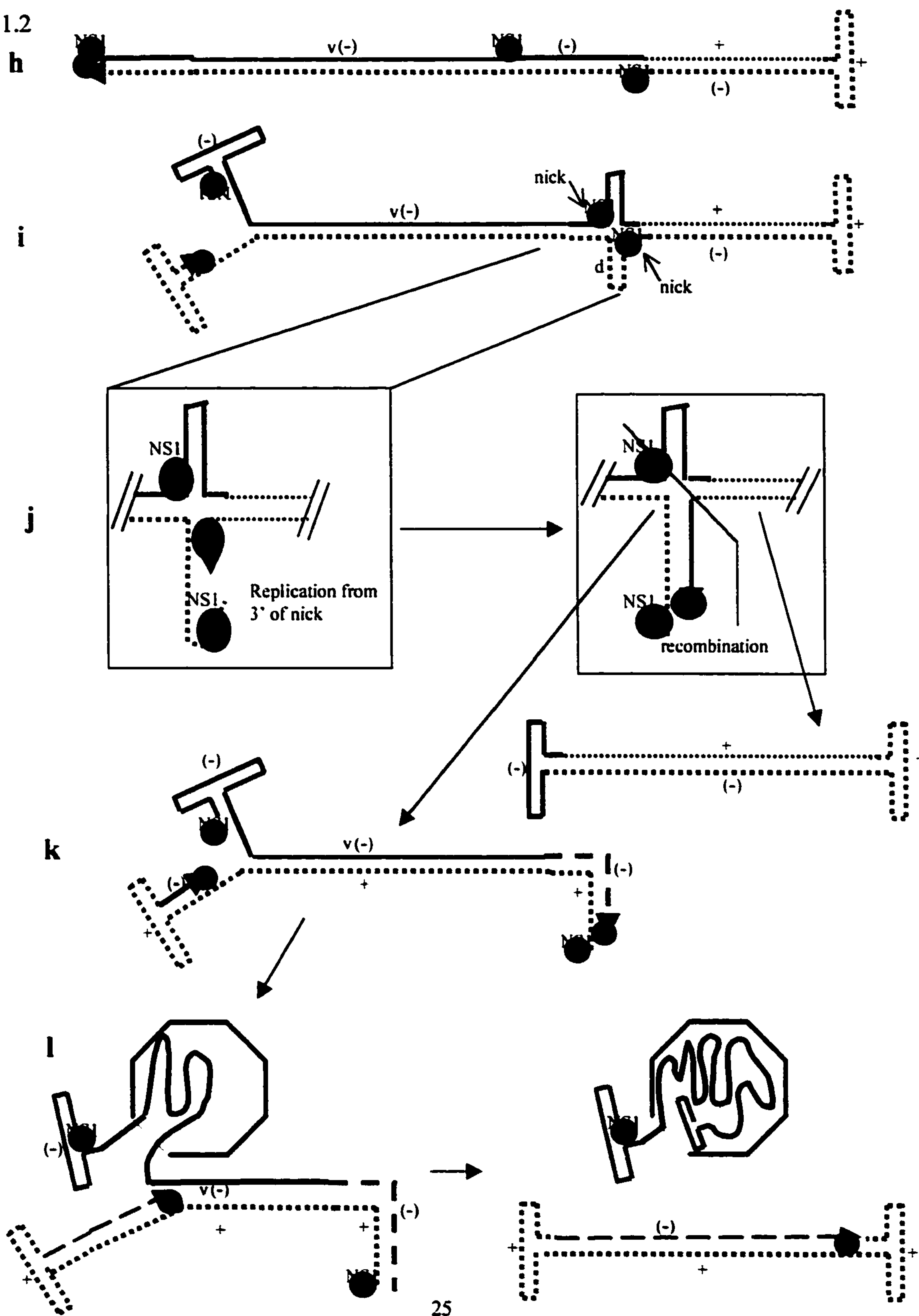

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end of the DNA strand (Fig. 1.2h,i) (for review see Berns, 1996). The new 3' end created can serve as a primer for DNA polymerases which then displace the ssDNA that is bound to NS1 (Fig. 1.2j). It is then theorized that a holiday type DNA structure is formed and a recombination event occurs (Fig. 1.2j). Resolution of this strand transfer produces a new monomer RF and a second RF that can be used for packaging (Fig. 1.2k). Continued replication from the hairpin transfer then displaces the (-)ssDNA, which is packaged into empty virions (Fig. 1.2l). The cycle is then repeated. The cellular factors required for the induction of NS1 synthesis, and polymerases for parvovirus replication are expressed during the S-phase of the cell cycle and help explain why parvovirus replication only occurs during this time (Delue, 1999). Interestingly in parvovirus H1, replication and gene expression are associated with unique nuclear structures, parvovirus-associated replication bodies, that contain associated cellular factors and NS1 can be detected by fluorescent microscopy (Cziepluch, 2000).

Gene Expression

Promoter structure, function, and gene expression for the mammalian parvoviruses has been investigated in detail but the densoviruses have received little attention. AeDNV's 4-kb genome can be divided roughly into two parts. Like members of the *Parvovirus* genus, the nonstructural protein genes are encoded on the 3' (left) portion and the structural protein genes on the 5' (right) third of the genome. Like most parvoviruses AeDNV has two promoters that separately express the nonstructural and structural protein genes.

In mammalian parvoviruses the late promoter (structural protein gene promoter) is quiescent in the absence of NS1 and the early nonstructural

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protein gene promoter is constitutive. Core promoter elements of the constitutive early (p4) promoter of MVM include a TATA box and SP1 binding site, with the full left end sequence being necessary for maximal expression (Lorson *et al.*, 1998). Late promoter (p38) elements also include a TATA box and SP1 binding motif (Lorson *et al.*, 1998); however, transcripts are undetectable in the absence of NS1 (Lorson and Pintel, 1997). AeDNV promoters contain TATA sequences and sequences matching consensus arthropod initiators (Cherbas and Cherbas, 1993), but lack SP1 sites as insects do not express an SP1 protein. Based on sequence analysis of AeDNV, promoters were tentatively located at map units 7 and 61 for the nonstructural and structural genes respectively (Afanasiev *et al.*, 1991). Though AeDNV seems to resemble autonomous parvoviruses of mammals, late gene expression from the structural protein gene promoter (pVP) was found to be constitutive in cell culture (Afanasiev *et al.*, 1994). Expression from the nonstructural protein gene promoter, p7, was found to be constitutive for both NS1- and NS2-ß-gal fusions (Afanasiev *et al.*, 1994; Kimmick *et al.*, 1998).

The NS1 protein of parvoviruses functions both in replication and in the transactivation of viral promoters. In all parvoviruses NS1 acts to increase transcription of both the structural gene promoter and its own promoter (for review see Berns, 1996; Hirt, 2000). In MVM the quiescent structural gene promoter (p38) is activated to approximately 2 times the maximal levels achieved by the early (p4) promoter when NS1 is present (Lorson and Pintel, 1997). Similarly, VP transcripts from AeDNV were shown to out number NS transcripts 2.2:1 (Kimmick, 1997; Ward *et al.*, 2001a). This gene expression strategy is necessary to ensure adequate production of structural proteins as approximately 60 copies are needed for each virion (Hirt, 2000). Genome replication also plays a role in gene expression as it adds templates for

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transcription. This increase in template number is likely to be vital to the production of sufficient viral capsid proteins (Delue *et al.*, 1999). The AeDNV early promoter, p7, is also transactivated by the NS1 protein, but the late promoter, pVP, is active without NS1 (Afanasiev *et al.*, 1994).

In MVM, NS1 binds specifically to DNA sequences ($[ACCA]_{2-3}$) upstream from viral promoters and to the terminal sequences during replication (for review see Hirt, 2000). Sequences upstream from the p38 promoter of MVM appear to contain all necessary elements for expression and NS1 responsiveness in the absence of other virus sequence (Lorson *et al.*, 1998). However, in H-1 parvovirus the upstream sequences are not enough and the genomic terminal sequences are necessary for full stimulation by NS1 (Hanson and Rhode III, 1991). Repression of the p38 promoter of MVM and other autonomous parvoviruses is not an effect of NS1, but is likely mediated by cellular factors, the effects of which are then alleviated by NS1 (Krauskopf and Aloni, 1994). The Rep protein of AAV (NS1 equivalent) is the only parvovirus protein to be implicated in the repression of viral promoters (for review see Linden and Berns, 2000), as lack of gene expression is beneficial to the virus when a helper virus is not present. In AeDNV the structural promoter constructs lacking the viral terminal sequences were insensitive to NS1 (T. Ward, unpublished observation).

Separating early and late gene expression by the induction of late promoters with products from early promoters is common throughout virus families. In the case of MVM and other mammalian parvoviruses the silent p38 promoter is activated by NS1, thus yielding a clear early to late transition in gene expression.

Once transcription begins, mammalian parvoviruses use extensive alternative splicing to yield large numbers of transcripts. B19 parvovirus

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produces 9 transcripts from a single promoter, MVM and AAV produce 3 and 6 transcripts from their two promoters respectively (Fig. 1.1). By contrast, Northern analysis indicates that AeDNV produces one transcript from each promoter. Both transcripts appear to utilize the same terminator near the 5' end of the viral genome (Kimmick, 1997). Recent evidence indicates that a parvovirus of cockroaches (*Periplaneta fuliginosa* densovirus [PfDNV]), a member of the genus *Densovirus*, utilizes alternative splicing to switch reading frames downstream from initiation codons (Yamagishi *et al.*, 1999). Anecdotal evidence suggests a similar situation for JcDNV (Bergoin and Tijssen, 2000). In AeDNV, and other parvoviruses, the various nonstructural coding regions overlap within a single sequence of DNA and would be expressed by the translation of different reading frames. Without evidence for splicing it is not obvious how these different proteins are expressed in AeDNV.

Translation of the various parvovirus proteins is typically accomplished by initiation at an AUG that is has been brought into the proper reading frame by splicing events. In AeDNV, the NS2 AUG codon is downstream from a putative secondary structure, the integrity of which is required for the efficient translation of both NS1 and NS2 with the NS2 protein being more dependent (Kimmick *et al.*, 1998). This secondary structure may provide a type of internal ribosomal entry site or may aid in the positioning of a scanning ribosome (Kimmick *et al.*, 1998). FPV structural gene transcripts contain a minicistron that begins at an AUG codon upstream from the structural protein AUG. This minicistron is dispensable for expression and its deletion increases structural protein expression (Clemens and Carlson, 1989). This suggests that a ribosome is scanning the transcript and passing the first AUG often enough to produce capsid proteins. Similarly the AUG of the NS1 protein of AeDNV is in

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poor context for initiation compared to the AUG of the NS2 protein (Kimmick, 1998). B19 parvovirus contains many AUG codons upstream of the structural protein AUG, deletion of which increases gene expression (Ozawa *et al.*, 1988) and suggests that upstream AUG codons are widely utilized by parvoviruses to regulate translation efficiency. The quantity of viral protein production may be involved in pathogenesis as weaker promoters are associated with long-term diseases, such as Aleutian disease of mink, and robust promoters are associated with rapidly fatal infections like CPV (Christensen *et al.*, 1993).

AeDNV as a potential genetic tool and genetic control agent.

AeDNV and many mammalian parvoviruses are being developed as gene transfer vectors (For review see Corsini *et al.*, 1996; Shaughnessy *et al.*, 2000) to deliver foreign genes into living organisms for gene therapy. The small DNA genome of parvoviruses is relatively easy to manipulate. The production of infectious plasmid clones has facilitated the study and modification of parvoviral genomes and allowed the insertion of foreign genes. The full genomic sequence of AeDNV is known (Afanasiev *et al.*, 1991) and an infectious clone was produced that allowed the insertion of reporter genes for the study of promoter functions (Afanasiev *et al.*, 1994).

Though AeDNV has not been developed to the extent of the Sindbis expression system, AeDNV has proven to be effective in the transduction of *Aedes aegypti* mosquitoes (Afanasiev *et al.*, 1999, 2000; Allen-Muira *et al.*, 1999). Cell cultures producing wild-type AeDNV as well as the recombinant virus carrying GFP have been used to evaluate AeDNV's transduction potential. Infection of larvae with virus particles carrying a recombinant genome with the green fluorescent protein (GFP) (s65t) gene fused to the viral NS1 protein revealed that a variety of tissues can be infected and efficiently express the

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GFP protein (Afanasiev *et al.*, 1999). Infected larvae showed expression initially within the anal papillae (Afanasiev *et al.*, 1999; Allen-Muira *et al.*, 1999) or rarely in the midgut (Afanasiev *et al.*, 1999). GFP expression could be detected in as little as 24 hours post infection, but the highest proportion of affected larvae was observed 48 hours post infection (Afanasiev *et al.*, 1999). This was superior to previously reported methods of detecting densoviral infection; infection was detected 3 to 4 days earlier than other means (discussed above). Additionally, assay for infection by GFP is harmless to larvae and provides the ability to identify individual cells and tissues infected.

The Dependovirus AAV has been shown to integrate specifically into the human chromosome 19 and has been intensively studied because of its potential as a gene delivery vector for humans (For review see Tal, 2000). Recently, the autonomous parvovirus MVM was shown to be able to integrate into episomes (Corsini *et al.*, 1997) suggesting that integration may be a widespread phenomenon, even in autonomous parvoviruses.

Parvoviruses lack genes required for viral DNA replication and can only productively infect cells during S-phase. This dependence on active cell division has led to the discovery that many parvoviruses efficiently infect and kill cancerous cells (For review see Shaughnessy *et al.*, 1996 and Corsini *et al.*, 1996). The addition of genes such as the herpes virus thymidine kinase can render infected cancerous cells susceptible to treatment by gancyclovir (DuPont *et al.*, 2000). Replacement of the structural genes of the H1 parvovirus of hamsters with genes encoding cytokines has been shown to enhance the tumor suppressive qualities of this virus (Haag *et al.*, 2000). For long term gene therapy AAV's ability to integrate may be beneficial. However, the use of autonomous parvoviruses in the treatment of cancer would benefit from a lack

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of integration, as this would avoid possible oncogenic side effects of integration.

If the genome of AeDNV could integrate into mosquito chromosomes this virus could provide an easy means for the production of transgenic mosquitoes. Integration can be detected within C6/36 cell cultures that have been transfected with a hygromycin expression construct flanked by the viral terminal sequences (Carlson *et al.*, 2000). Unfortunately, the integrated construct was found to have major rearrangements and it is not clear whether integration was due to illegitimate recombination or by NS1 mediated virus integration (Carlson *et al.*, 2000). Interestingly, the densovirus of *Junonia coenia* has been shown to integrate in somatic cells in *Junonia coenia* and in *Drosophila* (H. Bossin personal communication). However, even in the absence of integration, recombinant AeDNV viruses carrying genes limiting disease transmission could be used to test the genes efficacy. This is illustrated by the studies presented in chapters 4 and 5 where GFP transgene expression in infected larvae and adult *Aedes aegypti* is analyzed in detail. The difference between using AeDNV and the Sindbis system is that if successful, a recombinant AeDNV virus could be used to transiently affect natural populations of mosquitoes. To develop AeDNV into a useful tool for the evaluation of potential mosquito-borne disease control a detailed knowledge of AeDNV biology is needed to ensure effective manipulation of the AeDNV genome.

Much of what we know about densoviruses is derived from studies of mammalian parvoviruses. Many aspects of the molecular biology of these viruses is likely to be similar; however, detailed studies of AeDNV infection and molecular biology are needed to confirm these potential similarities and scrutinize differences. Detailed knowledge of AeDNV will increase our

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understanding of all mosquito densoviruses and is pivotal in the development of this virus as a transducing vector and biological control agent. Towards this end, the molecular biology of gene expression in AeDNV is investigated in chapters 2 and 3 by determining the temporal gene expression patterns and genomic sequence requirements for expression of the structural and nonstructural protein genes. Analysis of AeDNV pathogenesis, vertical transmission, and use as a gene transfer vector is presented in chapters 3 and 4.

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Chapter 2: Identification of structural gene core promoter elements and requirements for transactivation

This chapter presents data that in part has been published in:

Ward T. W., Kimmick M. W., Afanasiev, B. N. , Carlson, J. O. (2001) Characterization of the structural gene promoter of *Aedes aegypti* densovirus. J. Virol. 75:1325-1331

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Introduction

Densoviruses are autonomous parvoviruses that infect arthropods. *Aedes aegypti* densonucleosis virus (AeDNV) is in the genus *Brevidensovirus* (Afanasiev *et al.*, 2000; Bergoin and Tijssen, 2000). Its 4-kb, negative-sense, single-stranded DNA genome can be divided into two parts, with the nonstructural protein genes at the left end and the structural protein gene at the right end (Afanasiev *et al.*, 1991). AeDNV has two proteins, NS1 and NS2 that are encoded within the same DNA sequence in two different open reading frames (ORFs). NS1 is required for viral replication and has been implicated in the transactivation of viral promoters (Afanasiev *et al.*, 1994, 1999). The structural proteins VP1 and VP2 are encoded within the same ORF (Afanasiev *et al.*, 1991). VP2 may be a proteolytic cleavage product of VP1 or the result of a different translation initiation codon.

Brevidensoviruses, like vertebrate parvoviruses, encode all of their proteins on the same strand (Afanasiev *et al.*, 1991; Boublik *et al.*, 1994). Based on the location of TATAA boxes and ORFs, promoters were previously predicted to be at map units 7 and 61 for the nonstructural and structural genes of AeDNV respectively (Afanasiev *et al.*, 1991, 1994). These regions are conserved between AeDNV, *Aedes albopictus* parvovirus (AaPV), and a new isolate from mosquito cells (Afanasiev *et al.*, 1991; Boublik *et al.*, 1994; B. N. Afanasiev, unpublished observations).

Extensive study of parvovirus promoter structure and transcriptional regulation has been mainly confined to the mammalian parvoviruses (Berns, 1996; Hirt, 2000). These viruses use alternative splicing to yield different

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coterminal transcripts from the same promoter (for a review see Berns, 1996 and Hirt, 2000), which increases the number of protein species produced. Core promoter elements, which have been found to include a TATAA element and an upstream SP1 binding site (Lorson *et al.*, 1996), are sensitive to the presence of the viral NS1 protein (Afanasiev *et al.*, 1994; Cotmore *et al.*, 1995; Kimmick *et al.*, 1998; Rhode III, and Richard, 1995; Vanacker and Rommelaere, 1995). However, the core promoter structure of densoviruses is not well defined. A parvovirus of cockroaches (*Periplaneta fuliginosa* DNV), of the genus *Densovirus*, is likely to utilize alternative splicing (Yamagishi *et al.*, 1999), but it is not clear whether members of the genus *Brevidensovirus* or *Iteravirus* do so as well. Some indirect evidence does suggest that AeDNV and members of the *Densovirus* genus can initiate translation at multiple AUG codons to produce multiple proteins from the same transcript (Bergoin and Tijssen, 2000; Kimmick *et al.*, 1998). Promoters of baculoviruses, another family of arthropod viruses, have been studied intensively. Early genes have been found to utilize TATAA sequences and an initiator sequence CAGT (Blissard *et al.*, 1992; Pullen and Freisen, 1995). Late genes are expressed using a viral polymerase that initiates transcription at a TAAG sequence (Blissard *et al.*, 1992; O'Reilly *et al.*, 1995; Pullen and Freisen, 1995). The CAGT motif of the early genes can function without an accompanying TATAA sequence (Blissard *et al.*, 1992; O'Reilly *et al.*, 1995; Pullen and Freisen, 1995). This CAGT sequence has been shown to be important for expression from many arthropod and mammalian promoters whether or not they contain a TATAA sequence (Blissard *et al.*, 1992; Cherbas and Cherbas, 1993; Smale and Baltimore, 1989). This CAGT motif is observed downstream of TATAA sequences of both putative promoter regions of AeDNV.

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This chapter presents analysis of expression from the structural protein gene promoter of AeDNV. Primer extension was used to map the transcription start site for the structural gene. Deletion analysis and site-directed mutagenesis were used to test the hypothesis that DNA sequences surrounding, and including, the p61 TATA sequence are involved in viral protein gene expression. Furthermore, the hypothesis that the viral terminal sequences are necessary for transactivation of the structural protein gene promoter by NS1 was tested using southern analysis and gene expression analysis of ß-gal expression constructs varying in their content of genomic termini.

Materials and Methods

Cloning and mutagenesis. All plasmid clones were grown in *Escherichia coli* DH5-alpha cells. **pUCA**, the infectious clone of the AeDNV genome, is described in detail elsewhere (Afanasiev *et al.*, 1994). **pUCAINV** is the transactivating construct used to supply NS1 without VPs (Afanasiev *et al.*, 1994). **nsp61gal** was derived from pUCA by inserting the *lacZ* gene in frame with the VPs at the *Sna*BI site at nucleotide 2674, as described elsewhere (Afanasiev *et al.*, 1994). **pVPNco** contains virus sequences from nucleotides 2043 to 2674 including the structural gene promoter driving expression of the VP-ß-galactosidase fusion protein. This was accomplished by deleting the left end of the virus from nsp61gal by ligating Klenow enzyme-filled *Sst*I and *Nco*I restriction digested nsp61gal DNA. This left 420 nucleotides of virus sequence upstream from the p61 TATA sequence. The right hand terminal sequences were deleted by digestion with *Hind*III followed by religation. **pVPFsp** is identical to pVPNco but with only 83 nucleotides (2381 to 2674) upstream of the p61 TATA sequence. **pVPMsc** is identical to pVPFsp but contains only 24 nucleotides (2440 to 2674) upstream of the p61 TATA to the *Msc*I site. This was

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created by digesting nsp61gal with *Msc*I and religating to create a p7-p61 fusion. The p7 sequence was deleted by digestion with *Msc*I and *Nar*I, filling in with Klenow polymerase, and ligation. **ΔFsp/Msc** is identical to pVPNco but with a deletion of nucleotides 2381 to 2440 including the Inr sequence. This was created by digestion with *Msc*I, and partial *Fsp*I digestion to remove the region of interest, followed by Klenow polymerase repair of the *Fsp*I cohesive end and ligation. **pATG.1** is a fusion of the *lacZ* gene to the first ATG in the structural gene transcript. It was made by partial *Bam*HI digestion of pVPNco followed by *Msc*I digest, mung bean nuclease treatment, gel isolation of the 5,500-bp fragment, and ligation. This removes the region between the VP-*lacZ* fusion and the first ATG.

pVPNcoRLE is pVPNco with the right and left ends (5' and 3' nucleotides 1 to 268 and 3736 to 3999 respectively) of the virus left intact, including the TIRs. It was made by digestion of nsp61gal with *Eco*NI and *Nco*I. These overhangs were filled in with Klenow enzyme and ligated. **pVPNcoRE** is pVPNco with the right end of the virus still intact. It was created by digestion of nsp61gal with *Nar*I and *Nco*I; these ends were filled in with Klenow enzyme and ligated. **pVPNcoLE** is pVPNco with the left end of the virus intact. It was created by digestion of pVPNcoRLE with *Hin*dIII and religation.

For PCR mutagenesis, primers flanking the region of interest (structural gene region nucleotides 2045 to 2674) were synthesized (Gibco BRL, Gaithersburg, Md.). Primer Kasfwd (CAGATGCGTAAGGAGAAAATACCGC) binds to pUC sequences upstream of the region of interest. Primer ßgalrev (GTTGTAAAACGACGGGATCC) binds to ß-galactosidase sequences 120 nucleotides downstream from the VP-*lacZ* fusion.

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To create mutations, two complementary primers were designed with a diagnostic restriction enzyme recognition site at the desired location. Two separate PCRs (one with Kasfwd and the mutation reverse primer and one with ß-galrev and the mutation forward primer) were performed to yield two fragments with the mutation at either end. These fragments were purified from 1% agarose gel using a GeneClean kit (Bio 101, La Jolla, Calif.). Purified fragments were mixed together and denatured at 95°C for 10 min and allowed to anneal by cooling to 45°C for 10 min. *Taq* polymerase and deoxynucleotide triphosphates were then added, and the mixture was incubated for 10 min, at 72°C. Finally, 10.5 pmole of the flanking primers was added, and the mixture was cycled 29 times at 95°C for 1 min, 45°C for 1 min, and 72°C for 2.5 min. To mutate the map unit 61 TATATAA sequence (designated p61) two complementary primers were made with a *Dra*I restriction site (underlined) at nucleotide 2470 (CACAAAAATTTAAAATCTAATAGCAGAAGAAG [point mutations in bold]). For mutation of the map unit 60 TATAA sequence complementary primers with an *Xho*I restriction sequence (GACAATATACCTCGAGTGCGCAAATAC) in the map unit 60 TATA sequence were used. For mutation of the transcription start site sequence, complementary primers with an *Eco*RV restriction sequence (CAAAATAAATTAGATATCCGTCCTCCAACTC) within the consensus start sequence were used. Fragments with these mutations were cut with *Nar*I and *Bam*HI and inserted into the pVPNcoΔgal subclone digested with the same enzymes. Successful insertions were then cut with *Bam*HI and the *lacZ* gene was added. The *lacZ* gene was obtained from nsp61gal digested with *Bam*HI (3072-bp fragment). All mutations were confirmed by sequencing using an automated sequencer at Colorado Sate University or the University of Colorado.

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Cells and transfections. For transfection, *A. albopictus* C6/36 cells were grown in L15 medium with 10% fetal bovine serum at 25°C in 6 well plates at a density of 1.6 x 10^6 cells/well (Kimmick *et al.*, 1998). Eighteen hours later, the cells were rinsed twice with phosphate buffered saline (PBS) and 150 µl of the transfection mixture was added. The transfection mixture was made by combining 5 µg of plasmid DNA in 50 µl of L15 (3 µg of ß-galactosidase expression construct, 1.5 µg of transactivating construct or pUC19, and 0.5 µg pBSLuc) with 100 µl of L15-20% Lipofectin reagent (Gibco BRL). Cells were then incubated at 28°C for 6 h. The transfection mixture was removed, the cells were rinsed twice with PBS, and fresh complete L15 medium was added.

Protein expression assays. At 48 h post-transfection, cells were rinsed twice with sterile PBS, lysed, and harvested using a Galactolight kit (Tropix, Bedford, Mass.). ß-Galactosidase expression was quantified by incubating 5 µl of a 1:10 dilution of the cell lysates with Galacton reagent (Tropix) and measuring the resulting reaction with a TD-20e luminometer (Turner Designs, Sunnyvale, Calif.) as described previously (Afanasiev *et al.*, 1999; Kimmick *et al.*, 1998). Luciferase levels were determined using a luciferase assay system (Promega, Madison, Wis.) and TD-20e luminometer. Arbitrary light units from the ß-galactosidase assays were normalized to the average light units of luciferase, which controls for transfection and lysis efficiency. Lysates from nontransfected C6/36 cells were included as a negative control.

Primer extension analysis. A 10.5-pmole aliquot of oligonucleotide was labeled with γ-[^{32}P]-ATP using T4 polynucleotide kinase as recommended by the manufacture (New England Biolabs, Beverly, Mass.). Oligonucleotide ßgalrev (CCTAGGGCAGCAAAATGTTG) binds to the 5' end of the *lacZ* gene just downstream from the *Bam*HI site. p61rev108 (GGTACTGCCTCTTGTTGCT) binds to

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the viral sequence 108 bp down stream from the p61 TATA at nucleotides 2583 to 2604. ßgalrev and p61rev108 were used for primer extension on total RNA from cells transfected with nsp61gal and pUCA, respectively. RNA harvested from nontransfected C6/36 cells was used as a negative control. C6/36 cells were transfected as above except that 75-cm^2 flasks seeded with 2.25 x 10^7 cells were incubated with 800 µl of transfection mixture (150 µl of Lipofectin, 30 µg of plasmid DNA in L15). Total RNA was collected from cells transfected as above by the guanidinium isothiocyanate method (Kimmick *et al.*, 1998; Kingston *et al.*, 1995) or by passage through an RNeasy spin column (Qiagen, Valencia, Calif.). RNA was aliquoted in 30 µg samples, treated with 100 U of DNase (Gibco BRL) for 30 min, precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate, and washed twice with 70% ethanol. The RNA was then resuspended in 12.5 µl of hybridization buffer (final concentration: 150 mM KCl, 10 mM Tris-HCl [pH 8.3], 1 mM EDTA); 0.8 pmol of labeled probe was added and allowed to anneal for 30 min at 65°C after being denatured for 5 min at 95°C. Primer extension buffer was added (final concentration, 50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol) with 10,000 U of Superscript AMVRT (Gibco BRL). The reaction mixture was incubated at 42°C for 50 min and the reaction was stopped by heating to 70°C for 10 min. Then 4 µl of gel loading/stop buffer was added (New England Biolabs), and the samples were denatured at 95°C for 10 min and separated on a 5% acrylamide-8 M urea sequencing gel at 1,500 V for 2.5 h. To determine the precise transcriptional start site, sequencing was performed on nsp61gal or pUCA as a template with the same oligonucleotides as used for primer extension, using a Circumvent sequencing kit (New England Biolabs). These sequencing ladders were denatured as above and loaded next to the primer extension products for visualization of the transcriptional start site. Gels were then transferred to

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Whatman filter paper and dried. Gels were visualized by autoradiography using Fuji medical X-ray film (Fuji Medical Systems, Stamford, Conn.) for 5 to 72 h at -70°C.

Replication analysis. C6/36 cells (7.5 x 10^6) were transfected with constructs containing the VP promoter and the viral ends; 48 h post-transfection, low-molecular-weight DNA was extracted by the Hirt method (1). The DNA was precipitated with 10 M ammonium acetate and ethanol, washed twice with 70% ethanol, and resuspended in 50 µl of water. Each sample was digested with *Dpn*I overnight at 37°C. The enzyme was heat killed; then the samples were loaded onto a 1% agarose gel and run for 6 h at 40 V. The DNA was transferred to a Genescreen Plus membrane (DuPont), which was prehybridized (50% formamide, 10% Dextran sulfate, 2x SSC, 10% SDS) for 1 h at 45°C. Probe was prepared by random prime labeling (Boehringer Mannheim, Indianapolis, Ind.) a 3,072-bp *lacZ* gene fragment obtained by digesting nsp61gal with *Bam*HI and purifying the 3,072 bp fragment by using agarose gel electrophoresis and a GeneClean kit (Tropix); 10^7 dpm of probe was hybridized to the membrane in 4.5 ml of hybridization buffer (50% formamide, 10% Dextran sulfate, 2x SSC, 10% SDS) for 12 h at 45°C. The membrane was then washed twice with 2x SSC for 10 min and visualized by autoradiography using Fuji medical X-ray film (Fuji Medical Systems) and intensifying screens (DuPont) for 5 to 72 h at -70°C.

Results

Structural gene transcript initiation site. Primer extension analysis was used to precisely map the initiation site for the structural gene transcript. Primers that bound either to viral sequences (p61rev108) or to the 5' end of the *lacZ* reporter gene (ßgalrev) 108 or 200 nucleotides downstream

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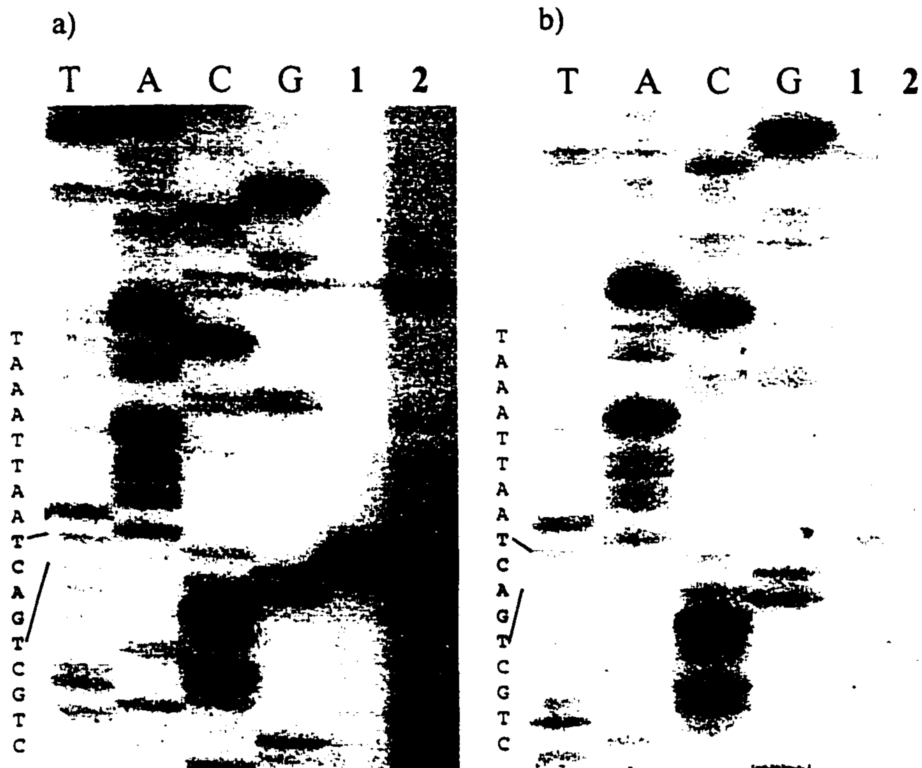

FIG. 2.1. Primer extension analysis of the structural gene transcript (sequence reads from top to bottom). a) Primer extension using virus specific primer p61rev108. Lanes T, A, G, C, and sequencing ladder produced using nsp61gal and primer p61rev108; lane 1; RNA from cells transfected with VP-*lacZ* fusion construct nsp61gal; lane 2; RNA from cells infected with AeDNV. (b) Primer extension using *lacZ* gene specific primer βgalrev. Lanes T, A, G, C, and sequencing ladder produced using nsp61gal and primer βgalrev; lane 1; RNA from cells transfected with VP-*lacZ* fusion construct nsp61gal; lane 2; RNA from nontransfected cells.

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from the map unit 61 TATAA sequence were used. *LacZ* fusions were included to confirm the identity of the transcription start site of reporter gene constructs, and the results were identical to those from the AeDNV infected cells. As shown in Fig. 2.1a, when the ^{32}P-labeled p61rev108 oligonucleotide was extended by reverse transcriptase, a band was observed that was approximately 200 nucleotides in length that corresponds to nucleotide 2402, which is 60 nucleotides upstream of the map unit 61 TATAA sequence. The putative start site is the first C within the sequence TCAGTC. Primer ßgalrev (Fig. 2.1b) also mapped the transcript initiation to the C in the CAGT site using nsp61gal transfected cellular RNA.

Mutational analysis of the structural gene promoter. To determine the sequences critical for expression of the structural gene, different constructs containing the *lacZ* reporter gene fused to the VP reading frame at nucleotide 2674 (Afanasiev *et al.*, 1994) were compared for the efficiency of ß-galactosidase expression. These constructs contained deletions of the viral sequences upstream of the VP gene. Plasmid constructs pVPMsc, pVPFsp, and pVPNco contain 24, 83, and 420 bp, respectively, upstream of the map unit 61 TATAA (Fig. 2.2). The level of expression from pVPNco was arbitrarily set to 100%. pVPFsp contains the initiation site defined by primer extension but lacks a TATAA sequence at map unit 60, 26 nucleotides upstream of the initiation site. This construct expressed at 30% of the level of pVPNco. pVPMsc lacks the initiation site and did not express above the background level. ΔFsp/Msc has a deletion of the 60 nucleotides between the *Fsp*I and *Msc*I sites (nucleotides 2381 to 2440) including the initiator (Inr) site; no ß-galactosidase expression was detected from this construct. This suggested that the region containing the Inr is critical for expression. Fusion of the *lacZ*

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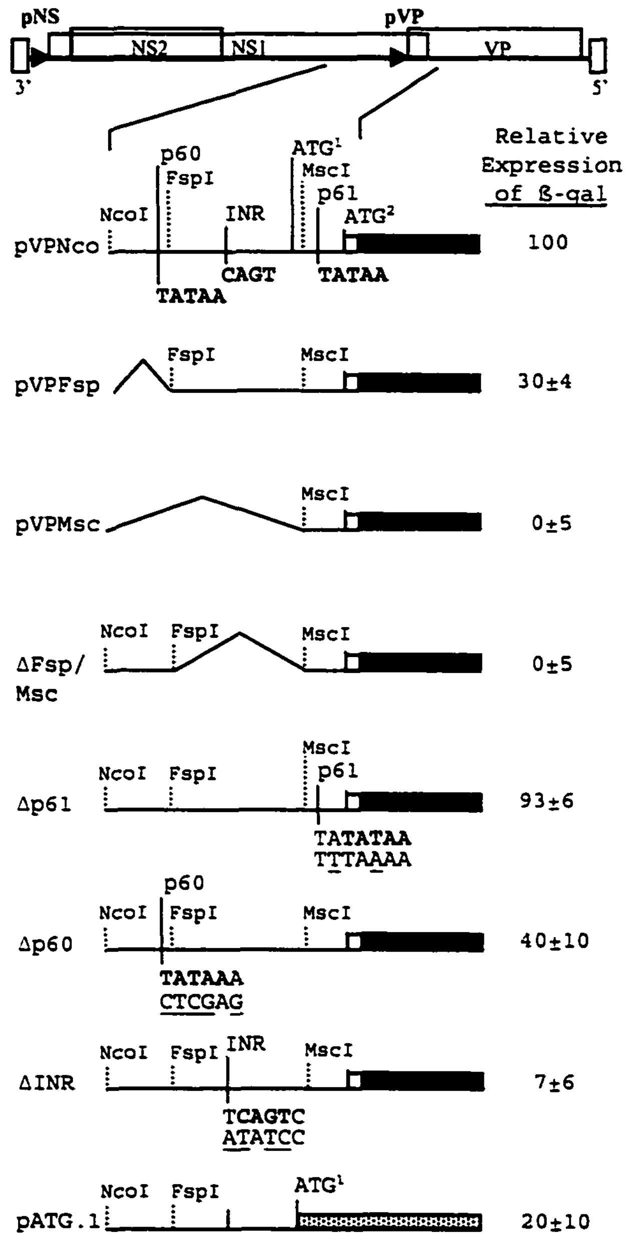

FIG. 2.2. (a) Effects of deletions and mutations on VP/ β -galactosidase fusion protein expression. The VP-*lacZ* fusion is indicated by black boxes with the relative upstream deletions or mutations indicated. The mutated sequences are shown below the wild-type viral sequence. Relative expression levels represent 3 experiments performed in duplicate and are normalized to the β-galactosidase expression of pVPNco. The dotted box (pATG.1) represents β-galactosidase fused to the first AUG of the transcript. AUG 1, the first AUG in the VP transcript, which is not in frame with the VP ORF; AUG 2, AUG for the VP ORF.

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```
  p60    FspI                            +1
CTATAA [ATGCGCA] AATACAAAATAAATTAATCAGTCGT

                               #1 MscI
CCTCCAACTCTCATTGAGCCAATACATA [TGGCCA] TAGT

                           p61
GTTTATAAAAAATTTCACAAAAATATATAATCTAATAGCA

GAAGAAGACAAAGCACACACAGTAAACGAGAAGGCAATAC

                                  VP orf
AAATCAACAACGAAGTGAAAGAAGAAGCAGAATCATG
```

Fig. 2.2b. Sequence of the structural protein gene promoter region (nucleotides 2372-2563).

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gene to the first ATG codon in the transcript, which is not in the VP reading frame (Fig. 2.2a, pATG.1), reduced expression by 80%.

To test their importance, the CAGT, map unit 60 TATAA, and map unit 61 TATAA sequences were modified via PCR based mutagenesis. Changing the map unit 60 TATAA from TATAA to CTCGA reduced expression by 60% (Fig. 2.2, Δp60) compared to the wild type construct pVPNco. Two nucleotide changes from T to A or A to T in the map unit 61 TATATAA sequence were made, yielding the sequence TTTAAAA. These changes were chosen because the region surrounding this sequence is very AT rich, with only 5 GC base pairs within 25 nucleotides. This mutation did not have a significant effect on expression of the ß-galactosidase fusion protein (93% of that of pVPNco). The most dramatic reduction in expression was seen when the sequence surrounding the transcriptional start site was changed. A four nucleotide change from TCAGTC to ATATCC (Fig. 2.2, ΔINR) reduced expression by 93% compared to pVPNco. This reduction is similar to the reduction observed with ΔFsp/Msc, in which the CAGT sequence and the surrounding 60 nucleotides are deleted.

Requirements for transactivation. It has been reported that both of the AeDNV promoters can be transactivated by NS1 (Afanasiev *et al.*, 1994, 1999; Kimmick *et al.*, 1998). The construct pVPNco was relatively insensitive to the presence of NS1 (provided by pUCAINV); it exhibited a 1.7-fold increase, compared to constructs studied previously that showed a 7-fold increase in gene expression (Afanasiev *et al.*, 1994; unpublished observations). Since NS1 is thought to interact with the terminal sequences of the viral genome, the 5'- and 3'-terminal sequences were added back to pVPNco. Constructs containing the right (5') end (pVPNcoRE), left (3') (pVPNcoLE), or both ends (pVPNcoRLE) (Fig. 2.3) were transfected into C6/36 cells and analyzed for expression of ß-galactosidase in the presence and absence of the transactivating construct

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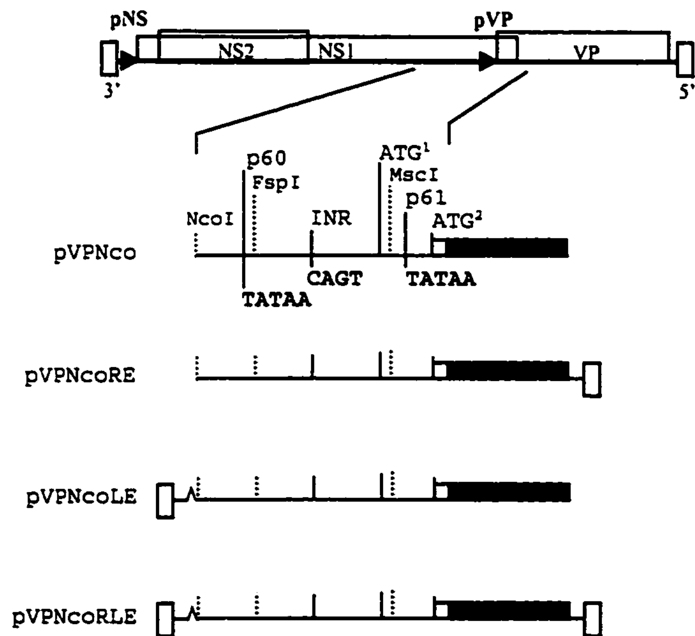

FIG. 2.3. VP-*lacZ* fusion constructs used for analysis of terminal sequence requirements for transactivation by NS1. The VP-*lacZ* fusion is indicated by black boxes.

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Table 2.1. Effect of NS1 on expression of pVP/ß-galactosidase from constructs containing viral terminal sequences.

Construct	% Relative expression levels (mean ± SD)		
	-NS1	+NS1	Fold Increase (mean ± SD)
pVPNco	100[b]	175 ± 22.8	1.7 ± 0.2
pVPNcoRE	140 ± 69.7	1650 ± 563	9.7 ± 3.4
pVPNcoLE	93.3 ± 13.8	965 ± 297	7.9 ± 0.2
pVPNcoRLE	102 ± 41	2750 ± 1790	37.6 ± 8.3
control[b]	0.01	0.01	

[a]Data are presented as relative light units and represent two to four experiments. The expression of ß-galactosidase from pVPNco was set to 100% to allow standardization between experiments.

[b]Lysates from nontransfected cells.

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pUCAINV. All four constructs had similar basal levels of gene expression without pUCAINV (Table 2.1). However, expression from the constructs containing viral terminal sequences was greatly enhanced by cotransfection with pUCAINV. pVPNcoRE and pVPNcoLE had increases of 9.7- and 7.9-fold, respectively, whereas pVPNcoRLE showed a 37-fold increase in ß-galactosidase expression (Table 2.1). These results demonstrate that viral termini are necessary for increased expression from the structural gene promoter when NS1 is present.

Since NS1 is involved in both the transactivation of parvovirus promoters and the replication of viral genomes (Hanson and Rhode III, 1991; Vanacker and Rommelaere, 1995), I sought to differentiate between increased gene expression due to transactivation and replication of the template. Low molecular weight DNA was extracted from cells transfected as above with the construct pVPNco, pVPNcoRE, pVPNcoLE, or pVPNcoRLE with and without pUCA. This DNA was then digested with *Dpn*I, which cleaves all Dam-methylated GATC sites in DNA of bacterial origin while leaving unmethylated viral replicative form DNA intact. These samples were analyzed by Southern blotting. The membrane was probed with a *lacZ* gene-specific probe to detect any replicated construct DNA. When both viral ends were present (pVPNcoRLE), a 4.6-kb, *Dpn*I-resistant band was observed in the presence of NS1, indicating replication of the construct (Fig. 2.4, lane 8).
Other constructs lacking both ends only show *Dpn*I digestion fragments. Thus, the increase in protein expression observed when both viral ends and NS1 are present is due to both template replication and transactivation by NS1 binding to the ends. This is consistent with previous observations with AeDNV and other parvoviruses (Afanasiev *et al.*, 1994; Hanson and Rhode III, 1991; Rhode III, and Richard, 1995).

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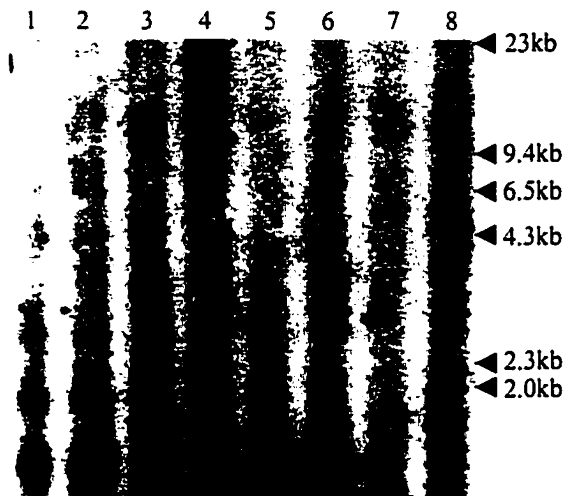

FIG. 2.4. Replication analysis of β -galactosidase expression constructs. Southern blot of Hirt extracts from C6/36 cells digested with *Dpn*I after transfection as follows: with lane 1, pVPNco: lane 2, pVPNco plus NS1; lane 3, pVPNcoRE; lane 4, pVPNcoRE plus NS1; lane 5, pVPNcoLE; lane 6, pVPNcoLE plus NS1; lane 7, pVPNcoRLE; lane 8, pVPNcoRLE plus NS1. The blot was probed with a ^{32}P-labeled *lacZ* gene-specific probe.

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Discussion

Previous studies have shown the existence of two functional promoters within the AeDNV genome, which were designated p7 and p61 according to the location of suspected TATAA boxes (Afanasiev *et al.*, 1991, 1994, 1999). Additionally, Northern blot analysis detected the presence of two RNA transcripts (Kimmick, 1997) and implies that RNAs expressed from the p7 promoter, bypasses the previously proposed polyadenylation site at the end of the NS1 gene (nucleotide 2730) (Afanasiev *et al.*, 1991), and uses the same polyadenylation site as the structural gene transcript (nucleotide 3679) (Kimmick, 1997; Ward *et al.*, 2000).

By using primer extension analysis, the transcriptional initiation site of the structural gene promoter was mapped to nucleotide 2402. It is located within a consensus Inr sequence TCAGTC, with the first C being +1. This Inr sequence fits the consensus Inr (TCA[G/T]T[T/C]) sequence of arthropods, including *Drosophila*, except the +1 is at the C and not A position. This motif is common in baculoviruses, arthropods, and mammalian systems (Cherbas and Cherbas, 1993; Pullen and Freisen, 1995; Smale and Baltimore, 1989). The Inr is 60 nucleotides upstream of the TATAA sequence previously assumed to define the VP promoter (p61) (Afanasiev *et al.*, 1991, 1994; Boublick *et al.*, 1994). Deletions which encompass the Inr region (Fig. 2.2a, ΔFsp/Msc and pVPMsc), but retain the putative p61 TATAA sequence, severely crippled gene expression. Mutation of the putative p61 TATAA sequence (Δp61) had an insignificant effect on gene expression (figure 2.2a). Thus, the p61 TATAA sequence does not seem to be involved in gene expression. A different TATAA sequence located upstream of the transcriptional start site, at nucleotide 2373, and the Inr were mutated by PCR mutagenesis to confirm their function. A four nucleotide change in the Inr sequence (ΔINR) resulted in a 93% reduction

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of gene expression (Fig. 2.2a). The TATAA sequence upstream of the Inr was found to be less important since constructs retained 30% (pVPFsp) to 40% (Δp60) of expression with this sequence deleted or mutated, respectively (Fig. 2.2a). These observations together place the dispensable TATA box of the structural gene promoter at nucleotide 2372 and demonstrate that the consensus Inr sequence, CAGT, is critical for efficient gene expression. This requirement of an Inr sequence for gene expression with a TATAA sequence only enhancing expression has been observed with a variety of *Drosophila* and mammalian genes, as well as baculovirus genes, containing the consensus sequence CAGT (Blissard *et al.*, 1992; Cherbas and Cherbas, 1993; O'Reilly *et al.*, 1995; Smale and Baltimore, 1989; Smale *et al.*, 1990). Baculovirus early genes contain this Inr sequence (O'Reilly *et al.*, 1995), which is known to interact with general transcription factors such as TFIID or TFII-I (Smale *et al.*, 1990; Roeder, 1991) and would also be required for expression of densovirus genes, which rely on the cellular transcription machinery. In contrast, baculovirus late genes utilize a baculovirus specific polymerase that recognizes the sequence TAAG and, with rare exceptions, lack a functional CAGT motif (O'Reilly *et al.*, 1995; Pullen and Freisen, 1995).

Interestingly, with transcription beginning at nucleotide 2402, there is a short ORF starting 125 nucleotides upstream of the putative initiation codon of the VP gene (Fig. 2.2b). If expressed, this ORF would produce an 80-amino acid protein corresponding to the carboxy terminus of the viral NS1 protein and could interfere with translation of the VP gene. A *lacZ* gene fusion to this upstream AUG (pATG.1) was expressed at a much lower level than VP fusions (Fig. 2.2a). The context of an AUG codon is important for the efficiency of translation initiation at that site, the optimal context being (A/G)CCAUGG (Kozak, 1986). The context surrounding the AUG of the small ORF at nucleotide

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2440 (CAUAUGG) has a 4-of-7 match to that of the optimal sequence (Kozak, 1986). However, the first AUG in the VP reading frame (AUCAUGG) is more optimal, having matches of 6-of-7 nucleotides. These observations may explain the reduced level of expression of the small ORF and the robust translation of the structural proteins from the downstream AUG (Kozak, 1999). The sequences surrounding these AUG codons, the Inr, and the TATA box are completely conserved between AeDNV, AaPV and a newly isolated mosquito DNV (Afanasiev *et al.*, 1991; Boublick *et al.*, 1994; unpublished observations), suggesting that they are important for regulation of gene expression. It is interesting that the feline panleukopenia parvovirus and B19 virus were shown to contain one and many AUG codons, respectively, upstream from the structural gene AUG (Clemens and Carlson, 1989; Ozawa *et al.*, 1988). Deletion of these upstream AUG triplets resulted in increased gene expression supporting the theory that a scanning ribosome was leaking past the first AUG to produce structural proteins. The presence of an upstream ORF may be another method of fine-tuning viral protein expression within infected cells, and may affect the pathogenesis of parvovirus diseases (Christensen *et al.*, 1993). It is interesting that expression of the NS2 protein of AeDNV would also require the ribosome to miss the NS1 start codon and scan further to translate this protein or perhaps to initiate via an internal ribosome entry site (Kimmick *et al.*, 1998). Detailed examination of translation initiation will be required to elucidate the true function, if any, of the small ORF.

AeDNV promoters are known to be affected by the viral NS1 protein (Afanasiev *et al.*, 1994, 1999). Expression from the base construct pVPNco, which lacks either of the viral ends, was found in this study to be relatively insensitive to stimulation by NS1 (1.7 fold, [Table 2.1]). This is in contrast to previous work which showed that the structural protein gene promoter can be

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transactivated by NS1 (Afanasiev *et al.*, 1994). However, the constructs used in the previous study contained the left end of the viral genome, which in many parvovirus systems is known to interact with NS1 (Cotmore *et al.*, 1995; Cotmore and Tattersall, 1988; Vanacker and Rommelaere, 1995). To determine the effect of the viral genome termini on transactivation of the structural protein gene promoter the virus terminal sequences were added back to the pVPNco construct. Adding either the right or the left end had a dramatic effect on expression in the presence of NS1, increasing expression by 9.7- or 7.9-fold with the right or left end respectively (Table 2.1). This is in contrast to what has been found with minute virus of mice and feline panleukopenia virus, where sequences proximal to the viral promoter are fully functional in transactivation by NS1 without the viral ends (Clemens and Carlson, 1989; Lorson *et al.*, 1996, 1997). It remains possible that viral sequences other than the termini affect transactivation, since sequences of the VP gene and between the viral left end and the *Nco*I site were not tested. NS1 of other parvoviruses have been shown to bind sequences in the viral terminal regions (Cotmore *et al.*, 1995; Cotmore and Tattersall, 1988; Hanson and Rhode III, 1991; Vanacker and Rommelaere, 1995). This may indicate the presence of an enhancer like sequence in the AeDNV viral ends similar to those observed under certain conditions for adeno-associated virus type 2 (Beaton *et al.*, 1989). The addition of both viral ends had a synergistic effect above that of either end alone with a 37-fold increase in expression in the presence of NS1 (Table 2.1). Similar observations were made with the p4 promoter of the LUIII parvovirus (Hanson and Rhode III, 1991). This is to be expected because the viral ends allow excision from the plasmid and subsequent replication of flanked sequences (Beaton *et al.*, 1989). Southern blot analysis (Fig. 2.4) confirms that replication of the viral DNA does indeed take place, provided that

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both viral ends and NS1 are present. Thus, the increase in template number was at least partially responsible for the greater increase in transactivated expression levels of pVPNcoRLE over constructs containing only one viral end. It is not clear whether both viral ends can produce a greater enhancer effect or if replication accounts for the entire increase in gene expression in the presence of NS1.

It is obvious from this study that although AeDNV has one of the smallest of DNA virus genomes, much can be learned from it that may apply to other mosquito densovirus promoters and add to a deeper understanding of gene expression and regulation in mosquitoes.

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Chapter 3. Characterization of the p7 promoter of AeDNV

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Introduction

Despite small genomes and relatively few open reading frames of the parvoviruses, detailed analysis has alluded to complex regulation of gene expression and interactions with host cells (for review see Vanacker and Rommelaere, 1995; Berns, 1996). Early viral genes are usually involved in the regulation of gene expression and replication, whereas the late genes typically encode structural proteins or are otherwise involved in maturation of virus particles. Parvoviruses of mammals differ in their regulation of expression from late promoters. The structural promoter (p39) of MVM is quiescent in the absence of NS1 and requires activation by the NS1 protein (Rhode III, 1985; Lorson *et al.*, 1996, Lorson and Pintel, 1997). Additionally, it has been shown that cellular factors are involved in the repression of the late promoter (Krauskopf and Aloni, 1994). Dependovirus expression patterns are further complicated by a latent-like state during the absence of a helper virus (for review see: Linden and Berns, 2000). The Rep protein of AAV (analogous to NS1 of autonomous parvoviruses) is detectable immunologically during the latent state, though transcription levels are below detectable limits (Mendelson *et al.*, 1986). Evidence supports that Rep is responsible for repression of viral promoters. However, in the permissive state Rep also acts as a transcriptional activator of virus promoters (Beaton *et al.*, 1989; Pereira *et al.*, 1997). In this case there is a definite distinction between late and early gene expression, which is dependent upon the NS1 or Rep protein. AeDNV does not have a clear cut transition between expression of nonstructural (early) and structural genes (late) as the pVP promoter efficiently produces VP

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protein in the absence of NS1. Although NS1 transactivates structural protein gene expression, it also increases expression of its own promoter (p7) (Afanasiev *et al.*, 1994; Kimmick *et al.*, 1998).

AeDNV, like other autonomous parvoviruses, has two viral promoters. The p7 and pVP promoters express the NS and VP proteins, respectively (Afanasiev *et al.*, 1994). The sequences of the promoter regions of AeDNV are not identical, although they share some canonical sequences such as the Inr. For pVP the Inr was found to be most important sequence for gene expression (chapter 2), with a TATAA sequence augmenting expression. The p7 TATATAA sequence is also upstream from a TCAGTC Inr-like sequence, although a link between this sequence and the transcription start site has not been established.

As described in Chapter 2, the transactivation of parvovirus promoters requires NS1 and sometimes requires the viral terminal sequences. It was determined that the pVP promoter could be transactivated 1.7-fold in the absence of viral ends, 8- to 10-fold with one end, and over 37-fold with both ends intact. It appears likely that the p7 promoter would also function in this way.

This chapter explores the core promoter elements and requirements of viral termini for transactivation of the p7 promoter and compares findings to that of pVP (Chapter 2). Site-directed mutagenesis was used to test the hypothesis that the p7 TATA, and consensus INR sequences are involved in NS protein gene expression similar to that found for pVP. Furthermore, the hypothesis that the viral terminal sequences are necessary for p7 promoter transactivation by NS1 was investigated using gene expression analysis of ß-gal expression constructs varying in their content of genomic termini. Finally, it was hypothesized that because AeDNV has a constitutivly active late

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promoter it would lack a temporal dichotomy of early and late gene expression observed in other parvoviruses.

Materials and Methods

ß-Galactosidase expression constructs. p7galNS1 has the *lacZ* gene in place of the VP gene, fused to the NS1 reading frame and additionally retains both the 3' and 5' TSs as described previously ([p7galNS1] Kimmick, 1998 and Kimmick *et al.*, 1998). **p7galNS1(RE)** is identical to p7galNS1 except that it has only the right TS. This construct retains 75 nt upstream of the p7 promoter and was made by transferring the *Xmn*I - *Eco*NI fragment from pUCAdE5d.Bpm (kindly provided by B.N. Afanasiev) into p7galNS1 digested with *Xmn*I and *Eco*NI. **p7galNS1(LE)** is identical to p7galNS1 except with only the left end remaining. The right (5') end was removed by digestion of p7galNS1 with *Hind*III followed by religation. **p7galNS1(NOE)** is p7galNS1 without either viral terminal sequence. It was made by deleting the right TS from p7galNS1(RE) by digestion with *Hind*III followed by religation. **Δp7galNS1** was created by transferring the *Xmn*I-*Dra*III fragment of Δp7,ΔΔ into p7galNS1. The p7galNS1 backbone was gel isolated after *Xmn*I digestion and a partial *Dra*III digest. This was then ligated to the Δp7,ΔΔ *Xmn*I-*Dra*III fragment containing the mutation. **p7ΔCAGTgalNS1** was created identically as described for Δp7galNS1 except that the *Xmn*I-*Dra*III fragment containing the mutation was transferred from ΔCAGT (provided by B. N. Afanasiev). **p7galNS2** is identical to p7galNS1(RLE) except that it has the *lacZ* gene fused, in place of the VP gene, to the NS2 reading frame ([p7galNS2] Kimmick, 1997; Kimmick *et al.*, 1998). pVPNcoRLE has *lacZ* fused to the structural protein gene and is described in Chapter 2 (Ward *et al.*, 2001a). pUCA is the infectious clone

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of AeDNV which expresses all viral proteins (Afanasiev *et al.*, 1994), as described in Chapter 2.

Protein expression assays. Transfection and detection of ß-gal and luciferase expression were performed as described in Chapter 2.

Temporal expression analysis. Plasmids expressing ß-gal-fusions to NS1, NS2, and VP (p7galNS1, p7galNS2, and pVPNcoRLE respectively) were mixed with pUCA and pBSLuc (2.5 µg, in a ratio of 2:2:1, respectively) and transfected into 12 well plates as described in Chapter 2. After transfection cells were harvested every 4 h for 48 h. All samples were then analyzed for ß-gal and luciferase activity.

Results and Discussion

Identification of core promoter elements. Mutation of the p7 TATATAA sequence to TGGATCC reduced gene expression by 99% when compared to wild type p7galNS1 (Δp7galNS1; Fig. 3.1, 3.2). Mutation of the Inr sequence downstream from the p7 TATA box from TCAGTC to ATATCC had less of an impact reducing expression to 40% that of p7galNS1 (p7ΔCAGTgalNS1; Fig 3.1, 3.2). This is in direct contrast to the situation found for pVP where the identical TATATAA sequence (previously known as p61 Afanasiev *et al.*, 1991, 1994) was not found to be involved with gene expression (Fig. 2.1). Instead, pVP has characteristics similar to that of a TATA-less promoter with the Inr required for efficient gene expression and a non-canonical TATA sequence at map unit 60 being slightly less important (Fig 2.1). Interestingly, the distance between the p7 promoter and the Inr sequence is 40 nt, 14 nt more than that found for the Inr of pVP. Whether this is relevant to the differences in expression of these promoters is not obvious at this time. It is interesting that the p7 CAGT sequence is 7 nt downstream from the presumed AUG of the NS1

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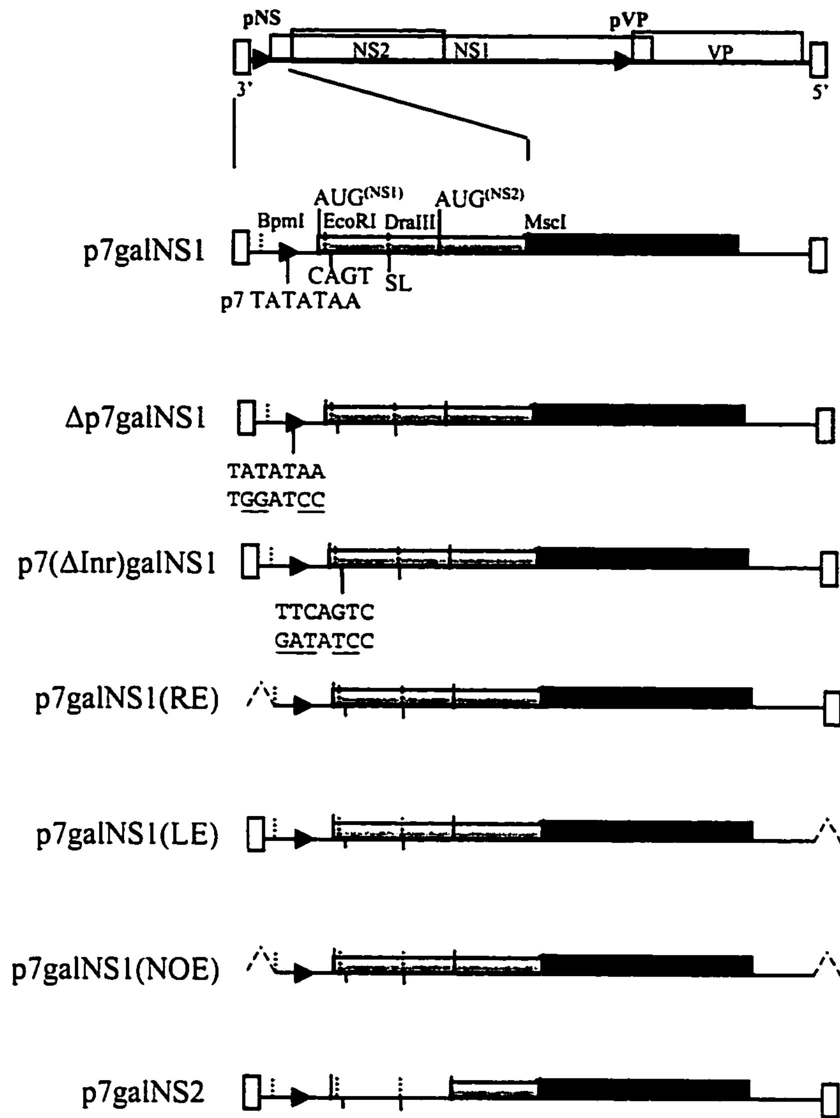

Fig. 3.1. Deletions and mutations of *lacZ* fusion constructs expressed from the p7 promoter. Black boxes indicate *lacZ* sequences fused to NS1 or NS2 sequences (gray boxes). The mutated sequences are shown below the wild-type viral sequence. SL = stem-loop structure.

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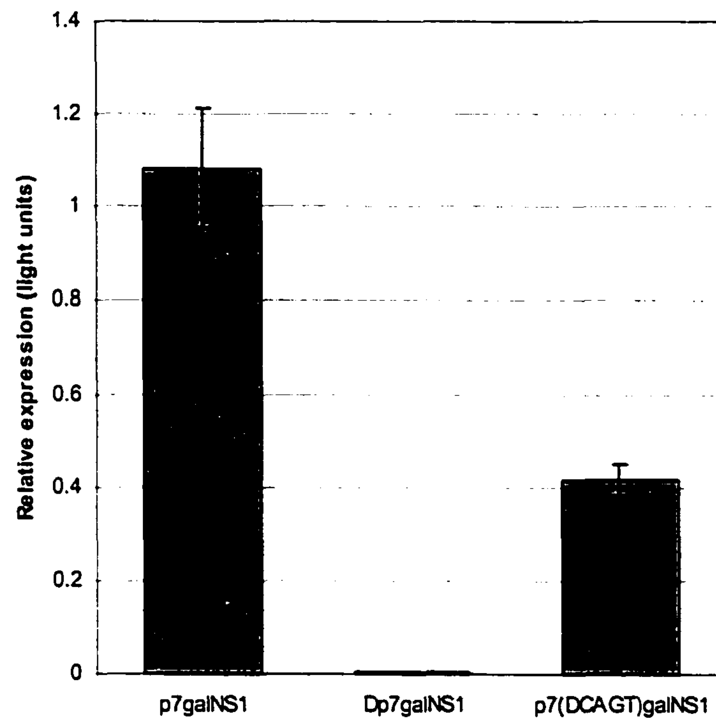

Fig. 3.2. β-galactosidase expression from NS1-*lacZ* fusion constructs. Dp7galNS1 has the p7 TATA sequence mutated, and p7(DCAGT)galNS1 has the Inr-like sequence mutated. Expression is relative to p7galNS1, ± standard deviation.

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protein. It is tempting to speculate about the possibility of two transcripts being produced from the p7 promoter. Because the p7 TATA sequence is required for expression it is reasonable to assume transcription would begin approximately 30 nt downstream. This transcript would contain both NS1 and NS2 AUGs. However, if the CAGT sequence downstream from p7 (which shares 6 identical nt to that of pVPs Inr) also acts to initiate transcription, only the NS2 AUG would be included, precluding NS1 from being translated. It is also possible that the consensus Inr sequence of p7 is not involved in transcription, but mutation of this sequence somehow destabilizes the 5' end of the transcript. Analysis of the same mutations effects on protein expression from the NS2 reading frame may further our understanding of nonstructural gene expression in AeDNV. The effects of these sequences on differential gene expression is not obvious. Baculoviruses have TATA-less early promoters, but then use a viral polymerase for late gene expression. The requirement of a TATA box in the case of p7 indicates that the TATA binding protein/RNApolII is responsible for accurate initiation of transcription with involvement of TFIIA, whereas the pVP promoter is more likely to depend upon the interaction of TFII(I) with the Inr sequence.

Transactivation of the p7 promoter. The NS1-ß-gal fusion construct p7galNS1 contains both viral terminal sequences (Fig 3.1) and can be transactivated, producing a 53-fold increase in gene expression when NS1 is supplied *in trans* (Table 3.1). As observed for the structural gene promoter, this increase in gene expression relies upon the presence of the viral ends which include the terminal sequences. The presence of NS1 increased protein expression from constructs that contained the right (p7galNS1(RE); Fig. 3.1) or left end (p7galNS1(LE); Fig. 3.1) by 25.1- or 34.4-fold, respectively (Table 3.1). p7galNS1(NOE), which does not contain either viral terminal sequences, was

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Table 3.1. Effect of NS1 on expression of ß-galactosidase from p7 in constructs containing viral terminal sequences.

Construct	Relative expression levels ± S D		
	-NS1	+NS1	Fold Increase
p7NOE	100[b]	393 ± 148	3.5 ± 1.3
p7RE	85 ± 29	2120 ± 963	25.1 ± 7.2
p7LE	92 ± 22	4580 ± 2060	34.4 ± 8.8
p7RLE	68 ± 28	3810 ± 1690	53.2 ± 37.6
control	0.01	0.01	

[a]Data is presented as relative light units and represents 2-4 experiments. [b]The expression of ß-galactosidase from p7galNS1(NOE) was set to 100% to allow standardization between experiments.

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much less responsive to the presence of NS1 with only a 3.5-fold increase in gene expression (Table 3.1). It is apparent that the p7 promoter responds to the presence of NS1 in a nearly identical manner as pVP. Both promoters function regardless of terminal sequence presence and are maximally stimulated by the presence of both ends and NS1. In the case of pVP it was shown that template replication is likely to contribute to this phenomenon (Chapter 2). It is likely that the greater transactivation of the p7 promoter in the presence of both ends would also be due, at least in part, to template replication. It is interesting that the viral promoters appear to be stimulated, in the presence of NS1, to a greater extent by the presence of the genomic end which is closest to the promoter in the wild type genome. The p7 promoter responds better to the left end (34-fold) than to the right end (25-fold) and vice versa for the pVP promoter (7.9-fold for the left TS and 9.7-fold for the right; Table 2.1). Actual distance between the promoter and the TS does not correlate with transactivation levels. The left TS in the construct pVPNcoLE is 590 nt upstream from the pVP TATAA whereas the right TS is over 1600 nt downstream. In the case of the p7 promoter constructs the closer, left, TS (approximately 180 nt upstream from the p7 TATA sequence) yields more transactivation with NS1 than the right end (approximately 3800 nt downstream from p7), indicating that there may be an effect of distance; however, this was not addressed directly. NS1 is known to bind to the TSs of many parvoviruses (discussed in Chapter 1 and 2); however, it is unclear whether sequences proximal to AeDNV promoters are also necessary for transactivation as is the case for MVM (Lorson *et al.*, 1998). It remains to be determined whether NS1 binding to viral DNA is required for transactivation of AeDNV promoters. MVM NS1 protein has been shown to bind sequences proximal to the promoter as well as the viral termini, however, evidence

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indicates that the TS is not necessary for transactivation (Lorson *et al.*, 1996, 1998). Other autonomous parvoviruses also show requirements for viral termini to achieve transactivation (Hanson and Rhode III, 1991).

Temporal gene expression. In transient expression assays NS1- and NS2-ß-gal fusion proteins have similar levels of expression (Afanasiev *et al.*, 1994; Kimmick *et al.*, 1998). VP-ß-gal-fusion constructs have a slightly higher level of basal expression and transactivate to a higher level as well (Afanasiev *et al.*, 1994). This is expected due to the high quantity of capsid proteins needed per virion. Analysis of temporal expression of NS1, NS2 and VP ß-gal fusions was achieved by transfecting NS1-, NS2-, and VP-*lacZ* fusion constructs with pUCA as helper to provide all viral proteins. Samples were collected for analysis every four hours post transfection. ß-gal expression analysis indicated that all three proteins are produced simultaneously (Fig. 3.3). VP production rises at a rate twice that of the NS proteins within the first 8 hours. It is also apparent that there is an association with the presence of the NS1 protein and higher levels of all viral proteins. This is not surprising since it is known that NS1 transactivates both viral promoters (Afanasiev *et al.*, 1994). Surprisingly, the NS2 protein fusion was expressed to twice the levels of the NS1 protein fusions. No function has yet to be ascribed to AeDNV NS2 though it may be required for efficient virus production (B. Afanasiev personal communication). Previous studies of NS1 and NS2 fusion protein expression did not show a significant difference in their levels of expression (Afanasiev *et al.*, 1994; Kimmick *et al.*, 1998). However, in these experiments gene expression was analyzed 48-72 hours post transfection. In this experiment all viral protein expression is observed to drop after 40 hours post transfection. In accordance with previous work, the NS2 protein levels drop sharply and are similar to that of NS1 by 48 hours (Fig. 3.3). If only one transcript is produced

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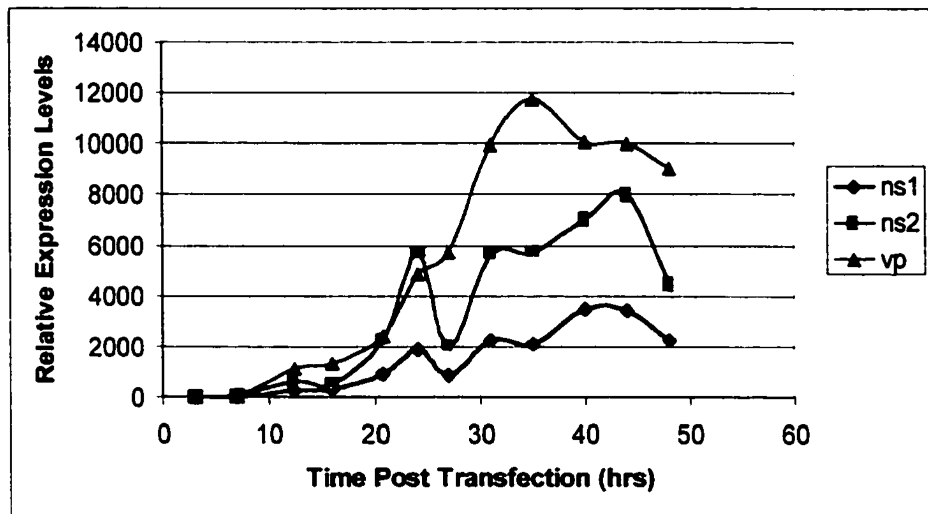

Fig. 3.3. Temporal β-galactosidase expression from cells transfected with *lacZ* fusion constructs. Cells were cotransfected with pUCA and: (ns1) NS1-*lacZ* fusion construct p7galNS1, (ns2) NS2-*lacZ* fusion construct p7galNS2, or (vp) VP-*lacZ* fusion construct pVPNcoRLE. All samples were normalized to luciferace (internal control) expression.

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by the p7 promoter (Kimmick, 1997; Ward *et al.*, 2001a) it is not obvious how NS1 protein expression could fall at a slower rate than that of NS2. This observation suggests another level of regulation that could repress NS2 expression without having as strong effect on NS1. Regulation could take place and the level of translation. This is supported by observations that deletion of a putative secondary structure within the transcript, downstream of the NS2 AUG, represses NS1 more than NS2 (Kimmick *et al.*, 1998). Alternatively, it is possible that two transcripts are made from the p7 promoter, one positioned by the TATAA box and another by the Inr. In this hypothetical model the long transcript would include both the NS1 and NS2 initiation codons, but the shorter one, beginning at the Inr, would lack the NS1 AUG. This could provide transcripts that would produce more NS2 than NS1, then by shutting down short transcript synthesis could reduce NS2 levels to that of NS1. Regulation of initiation site preference could be achieved by modulating TFIIA and TFII-I interactions with the upstream promoter region and Inr, respectively, as these molecules direct the entry of the RNApolII holoenzyme and thus affect initiation site selection (Roeder, 1991).

Conclusions

It is apparent that the pVP and p7 promoters utilize different DNA sequences for efficient gene expression with the p7 promoter relying less upon the Inr sequence than that of pVP. Efficient transactivation of both promoters requires the viral terminal sequences and both promoters respond in a similar way to the presence of NS1. The p7 transcript is likely initiated as determined by the TATA sequence as opposed to using the consensus Inr which is approximately 14 nt further downstream than would be expected. Primer extension has been unable to elucidate the true transcriptional start site,

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though it is likely to be 14 nt upstream of the Inr so as to include the AUG of the NS1 ORF. Analysis of NS2-ß-gal fusions with mutations in the TATA and Inr may help to clarify how the NS1 and NS2 proteins are differentially expressed. The transient, high levels of NS2 expression, compared to NS1, suggests a complex regulatory mechanism which may be more intricate than the scanning ribosome model suggested in previous work (Kimmick *et al.*, 1998). Northern analysis is not sensitive enough to differentiate between two transcripts produced by differential initiation sites (Kimmick, 1997; Ward *et al.*, 2001a) and primer extension analysis has not yielded reliable data. Primer extension showed transcription initiation at 11, 23, and 37 nt downstream from the p7 TATA box in three different experiments (T. Ward, observations). These putative transcripts would include both NS1 and NS2 AUG's except in the case of initiation at the Inr (37 nt downstream of the p7 TATA). Repeatable 5' mapping of these transcripts is required to clarify this question. Additionally NS2-*lacZ* fusion constructs with mutations within the TATA and Inr sequences could determine if NS2 expression is regulated in a different manner than NS1.

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Chapter 4. AeDNV Transducing Densovirus Pathogenesis and Expression in *Aedes aegypti* Mosquitoes

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

Ward T. W., M. S. Jenkins, B. N. Afanasiev, B. A. Duda, E. Suchman, M. Edwards, M. Jacobs-Lorena, B. J. Beaty, J. O. Carlson. (submitted) *Aedes aegypti* transducing densovirus pathogenesis and expression in *Aedes aegypti* and *Anopheles gambiae* larvae. Ins. Mol. Biol.

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Introduction

Mosquitoes are vectors of many important human and animal diseases. Control of these diseases either has focused upon preventing infections in the human or animal host or controlling the vector. Unfortunately, attempts to chemically control mosquito populations have led to widespread resistance within mosquito populations (Ferrari, 1996). Biological control agents include pathogens such as nematodes, fungi, bacteria, viruses, predators such as the mosquito fish, or toxins produced by microorganisms (Woodring and Davidson, 1996). The use of biological control agents targeting vectors of human pathogens has been slow in development. However, the bacteria *Bacillus thuringiensis* and *B. sphaericus* have been used successfully to control mosquito larvae (Federici 1995; Davidson and Becker, 1996; Woodring and Davidson, 1996). Some viruses, such as the baculoviruses, have been used successfully to control agricultural arthropod pests (Hunter-Fujita, *et al.*, 1998; Moscardi, *et al.*, 1999).

Densoviruses are autonomous parvoviruses that infect a variety of insects and crustaceans (Fédière, 2000). Mosquito densoviruses have been isolated from natural mosquito populations (Kittayapong *et al.*, 1999), but are more commonly found as contaminants in mosquito cell cultures or laboratory colonies (Buchatsky, 1989; O'Neill *et al.*, 1995; Jousset *et al.*, 1993). *Aedes aegypti* mosquitoes, which transmit dengue and yellow fever viruses, are highly susceptible to infection by the *Aedes aegypti* densovirus (AeDNV) (Buchatsky, 1989). This virus also infects other mosquitoes of the genera *Aedes*, *Culex* and *Culiseta*, and thus has the potential for use as a biological control agent

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(Buchatsky *et al.*, 1987). AeDNV, like all densoviruses, has potent larvacidal activity and additionally can persist in populations of *Aedes aegypti* by vertical transmission (Buchatsky, 1989).

An infectious clone of AeDNV (pUCA) has been developed that, when transfected into C6/36 *Aedes albopictus* cells, produces wild type virus (Afanasiev *et al.*, 1994). Reporter genes have been inserted into the infectious clone, and have facilitated the study of viral promoter activity, regulation, and sequence requirements for gene expression (Afanasiev *et al.* 1994, 1999; Kimmick *et al.*, 1999, Afanasiev and Carlson, 2000; Ward *et al.*, 2001a).

AeDNV transducing vectors have been designed to transduce genes of interest into live mosquitoes. This could potentially provide a genetic means of controlling mosquito borne diseases (Carlson *et al.*, 2000). A recombinant transducing virus expressing the green fluorescent protein (GFP) gene of *Aequorea victoria*, but lacking the viral VP proteins required for packaging, has been developed and used to detect viral infection of cultured mosquito cells and *Aedes aegypti* larvae (Afanasiev *et al.*, 1999; Allen-Muira *et al.*, 1999; Carlson *et al.*, 2000). This recombinant genome can be replicated and packaged into virions in the presence of helper virus making transducing particles, which can then be used to infect larvae and to study the tissue distribution of the virus (Afanasiev *et al.*, 1999; Allen-Muira *et al.*, 1999). Using transducing particles the portal of larval infection has been revealed to be the anal papillae rather than the alimentary tract (Afanasiev *et al.* 1999; Allen-Muira *et al.*, 1999). Additionally, when wild type virus is also present, GFP expression is observed to spread from the anal papillae to other larval tissues (Afanasiev *et al.*, 1999). This is because transducing genomes can only be packaged and spread to new cells when the viral structural genes are supplied *in trans*.

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This study explores the pathogenesis of AeDNV transducing particles in *Aedes aegypti* larvae by infecting them with a mixture of transducing particles carrying the GFP gene and wild type virus. Previous studies followed populations of infected larvae (Afanasiev *et al.*, 1999), however, because detection of GFP by fluorescence microscopy is harmless to larvae, we were able to determine the details of pathogenesis of transducing particles in 255 individual mosquito larvae by separating infected larvae and observing GFP expression daily. Pathogenesis data was accumulated into a database designed to analyze questions pertaining to GFP transduction and pathogenesis and is presented in Appendix 2. This database yielded all of the pathogenesis and gene expression data presented here, and can be utilized to answer specific questions, pertaining to GFP expression patterns or pathogenesis, as they arise. In previous studies of mosquito densoviruses, larvae were exposed to the virus by placing them into flasks containing infected cells (Barreau *et al.*, 1994; Afanasiev *et al.*, 1999) or by feeding infected dead larvae to healthy larvae (Barreau *et al.*, 1996). When exposed to infected cell culture only 10% of mosquito larvae became infected (Afanasiev *et al.*, 1999). These methods of infection are likely to be sub-optimal as it was determined that AeDNV rarely enters via the alimentary tract (Afanasiev *et al.*, 1999; Allen-Muira *et al.*, 1999). We sought to improve infection and transduction efficiency through concentration of virus particles and manipulation of the larval environment by altering the salt concentration.

Methods and Materials

Transducing particle production. pUCA, the infectious clone of AeDNV, expresses the nonstructural proteins and the structural proteins of the virus, and is described in detail elsewhere (Afanasiev *et al.*, 1994). The plasmid

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pANS1-GFP is identical to the infectious clone pUCA except that the viral structural protein gene is deleted and replaced with a fusion of the green fluorescent protein of *Aequorea victoria* to the c-terminus of the viral NS1 protein (Afanasiev *et al.*, 1999). Transducing particles were produced as described previously (Afanasiev *et al.*, 1999). pUCA and pANS1-GFP (10 ug each) were transfected into 2.25 x 10^7 C6/36 *Aedes albopictus* cells using Lipofectin (Life Technologies Gaithersburg, MD) This allows for the packaging of both wild type and transducing genomes. 48 hours post transfection transducing particles were harvested by scraping the cells and centrifuging them at 32,000 rpm in a Beckman SW41ti rotor for 2 hours. The resulting pellet was then resuspended in 1 ml water. Three treatments were assessed for their ability to release the virus from cells. The resuspended pellet was divided into three equal aliquots, one was sonicated, one subjected to 3 freeze thaw cycles, and the last was resuspended only. Sonication was performed using a Branson Sonifier 450 at an output of 5, 50% duty, for 30 seconds on ice. Three freeze/thaw cycles were performed: The suspension was frozen at -70°C for 24 hours, then thawed to 37°C. Two further cycles of freezing at -70°C for 20 minutes and thawing at 37°C were performed. All treatments yielded similar infection rates in cell culture and *Aedes aegypti* larvae (data not shown). All further transducing particle mixtures used in this study were treated by sonication, because this provided a more homogenous suspension.

The effect of salt concentration on larval susceptibility to infection. *Aedes aegypti*, strain REX D, larvae were hatched in deoxygenated water for 15-20 minutes. Larvae were separated into groups of 100 and placed into 1.7 ml tubes with 100 µl water. 775 µl of L-15 (Life Technologies) medium was added to a final concentration of 1x (physiological concentration), 0.66x,

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0.33x, and 0x for 0.15 M. 0.10 M, 0.05 M, and 0.0 M NaCl equivalent, respectively. To each group of larvae 125 µl transducing particles was then added for a final volume of 1 ml. Controls for each NaCl equivalent concentration were also prepared, as above, except using 50 larvae per tube without transducing particles. Additionally, 12.5 µl of L-15 was added to each control tube to account for the amount of salt present in the transducing particle preparation. All larvae were then incubated for 24 hours at 27°C with cycles of 12 hours of light and 12 hours of darkness as described previously (Allen-Miura *et al.*, 1999). After infection, the larvae were transferred to separate 50 ml conical tubes containing 30 ml of ddH_2O water and 0.01 g of flake, fish food. At 48 and 96 hours post infection, infected groups of larvae were screened for expression of GFP by fluorescence microscopy, and both the infected and control groups were monitored for mortality. The GFP positive larvae were removed to avoid recounting them on subsequent days.

Pathogenesis in individual larvae. *Aedes aegypti* eggs were hatched for 5 consecutive days. Each day 100 newly hatched larvae were placed into 25 ml deionized water and fed with 25 mg flake fish food. On the fifth day the freshly hatched and 1-4 day old larvae were transferred into 100 µl water, and 900 µl of transducing particles (sonicated) were added to each. Larvae were incubated with the transducing particles for 20 hours at 27°C and then transferred to 50 ml deionized water containing 25 mg flake fish food. Larvae were screened for GFP expression by fluorescent microscopy every 24 hours. GFP positive larvae were separated into individual test tubes with 5 ml water containing 0.01g larvae food and were examined daily to determine GFP expression patterns.

Anopheles gambiae mosquitoes were infected as third or fourth instar larvae in 500 µl of a 1:4 dilution of virus stock in water for three hours.

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(Martin Edwards personal communication) The volume was then increased to 20 ml and the larvae were incubated overnight. Larvae were screened daily for GFP expression by fluorescent microscopy. Fluorescence microscopy images were obtained using a BioRad MRC600 confocal microscope.

Results and Discussion

Effect of salt concentration on infection. Mosquito anal papillae are known to be involved in the control of ion balance between the mosquitoes hemolymph and its surrounding environment (Wigglesworth, 1938; Edwards and Harrison, 1983). As this was found to be the initial site of infection, we sought to determine whether stressing the anal papillae by adjusting the salt concentration in the environment would affect larval susceptibility to infection. Freshly hatched larvae were challenged with transducing particles in the presence of four different NaCl equivalent concentrations ranging from 0.0 M to 0.15 M. Infection was scored by visual observation of GFP expression within living larvae (Table 4.1). NaCl concentrations above 0.05 M drastically inhibited infection. On day 5 (96 hours pi), only 5.3% and 5.7% of larvae were infected for the 0.10 and 0.15 M NaCl groups, respectively. However, 42% and 22.7% of larvae were infected in the 0.0 and 0.05 M NaCl groups, respectively. Mortality was highest in the infected 0.05 and 0.0 M NaCl groups (79.7% and 76.7% respectively). Ninety-six hours post infection, the mortality rates for each control group averaged 14% with 0.15 M salt having a higher mortality of 36%. Optimal infection rates at low ionic strengths suggest that this virus has adapted to the *Aedes aegypti* larval environment, which is typically containers holding rain water. This also indicates that infection of larvae by placing them into flasks of infected cell culture (Barreau *et al.*, 1994,

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Table 4.1. Effect of salt concentration upon viral infection rates[1].

[NaCl]	% infected larvae at 48 hours pi	% infected larvae at 96 hours pi	mortality %	Control mortality
0.0M	40.7±18	42.0±17.4	76.7±19.3	10.7±11.1
0.05M	22.3±11.4	22.7±11.9	79.7±18.0	13.3±3.1
0.10M	3.3±3.5	5.3±4.2	45.7±26.8	9.3±7.6
0.15M	2.7±1.5	5.7±2.1	25.0±28.6	36.0±24.6

[1] Mean of three experiments ± standard deviation.

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Afanasiev *et al.*, 1999) would substantially limit infection rates as the cell culture medium typically contains 0.15 M equivalent in salts.

Pathogenesis and portal of entry of AeDNV. Zero to four day old larvae were infected with transducing particles to determine the affect of age on infection and pathogenesis. Each larva expressing GFP was separated into an individual test tube to follow the course of infection. The primary infection site, or portal of entry, was the anal papillae. Forty-nine percent (126/255) of larvae first expressed GFP in the anal papillae. Eight percent (21/255) of larvae first expressed GFP within a bristle cell, and a single cell at the base of an anal papilla first expressed GFP in 7% (18/255) of larvae (Table 4.2). Other tissue locations, such as the fat bodies, muscles, or neurons, accounted for only 2% (5/255) of the primary infection sites and no infection of the midgut was observed. However, in many cases larvae showed primary infection in more than one tissue (Table 4.2). In these cases the anal papillae were involved with 67% (172/255) of primary infections, the bristle cells in 25% (63/255), and a single cell at the base of an anal papillae in 20% (50/255). This predominance of infection of the anal papillae confirms previous results using these transducing particles (Afanasiev *et al.*, 1999). Experiments using pure transducing virus, that is not contaminated with wild type virus and thus unable to spread from cell to cell, also indicated the papillae were the portal of infection, not the alimentary tract (Allen-Muira *et al.*, 1999).

The age of the larvae did not influence the portal of entry (Figure 4.1). In 0, 1, 3, and 4 day old larvae the anal papillae was the primary infection site. However, the two day old larvae had a higher rate of primary infection within bristle cells, and the four day old larvae that had an increased frequency of "other" locations being the primary infection site. As expected, older larvae were less susceptible to infection. Susceptibility peaked with 92% of the 2 day-

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Table 4.2. Location and frequency of primary infection sites in 255 infected larvae.

Primary Infection Site	Site alone	Site included with others
Anal papillae	126	172
Bristle Cell	21	63
Base of Anal papillae	18	50
Other[1]	5	114

[1] All locations other than the anal papillae, bristle cells, or base of anal papillae. The midgut was not observed.

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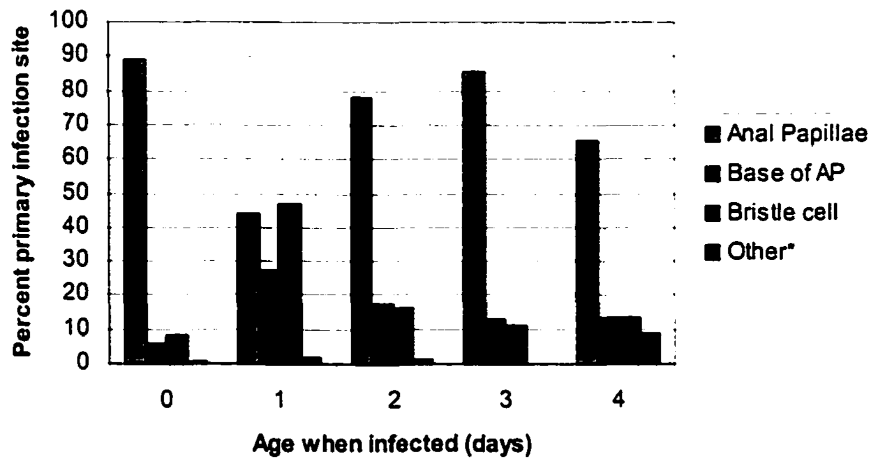

Figure 4.1. Effect of age on primary infection site. AP = Anal papilla. *Locations other than the three listed, the alimentary tract was not observed to be a primary infection site.

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old larvae becoming infected and decreased to 22% for the four day old larvae (Figure 4.2). The overall infection rate in the experiment was 51% (255/500), which is higher than that obtained by incubating larvae with infected cell culture (Afanasiev *et al.*, 1999). Greater susceptibility of two-day-old larvae was observed in other infection experiments as well (Fig. 4.2b) and may be explained by the timing of molts; molting could occur during the 24 hour infection period providing a thinner cuticle for the virus to penetrate.

In addition to being less susceptible to infection, the older larvae were also more likely to survive infection through pupation and into adulthood (Figure 4.3). None of the larvae infected at day zero survived to pupation, whereas larvae infected at three and four days had a 25% survival rate to pupation and 14% survival into adulthood. Death typically occurred during pupation or eclosion, which is consistent with earlier studies of wild type AeDNV infection (Buchatsky, 1989) and other mosquito densoviruses (Barreau *et al.*, 1996).

Dissemination from anal papillae and tissue tropisms. Separation of larvae that were infected, at 0-5 days of age, into individual tubes permitted monitoring of virus pathogenesis in individual mosquitoes on a daily basis based upon GFP expression. The typical course of pathogenesis of AeDNV in an individual larva is shown in Figure 4.4. The primary route of infection was through the anal papillae. Dissemination from infected anal papillae to cells of the fat bodies occurred in 103/172 larvae after an average of 2 days. This was generally followed by infection of the muscle fibers and other tissues. In most cases dissemination led to death (72/103), taking an average of 2 days after dissemination. However, older larvae (22/103, 3-4 days old) tended to survive at least one day longer. In previous infection studies, tissues of the fat body exhibited the first signs of histological changes at about

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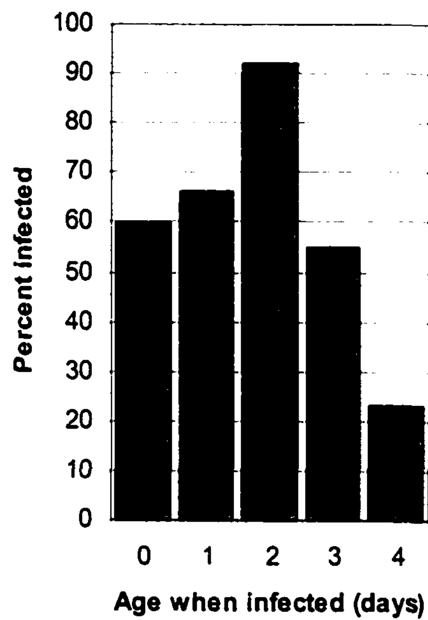

Figure 4.2. Effect of age on larval susceptibility to AeDNV transducing particles. Bars indicate infection rates within the 500 infected larvae discussed.

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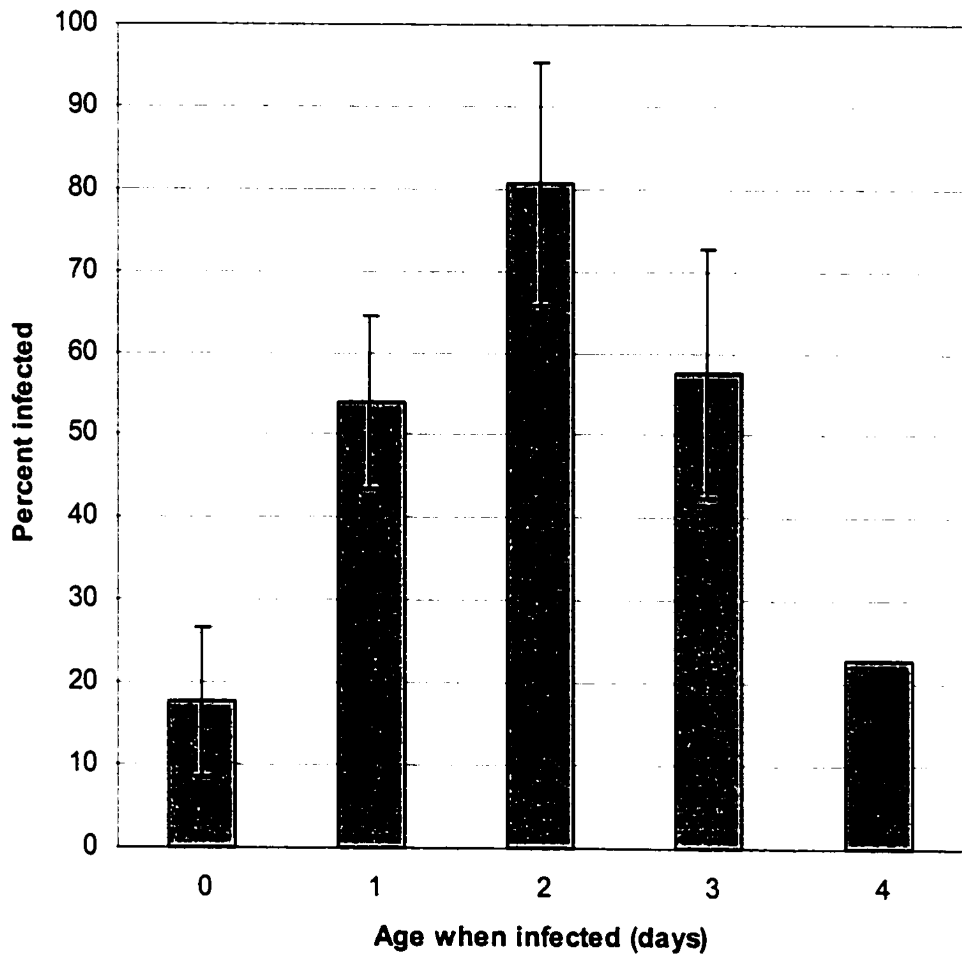

Figure 4.2b. B) Infection by age in 3 experiments including 255 case histories. The line indicates the average of 3 experiments ± SD.

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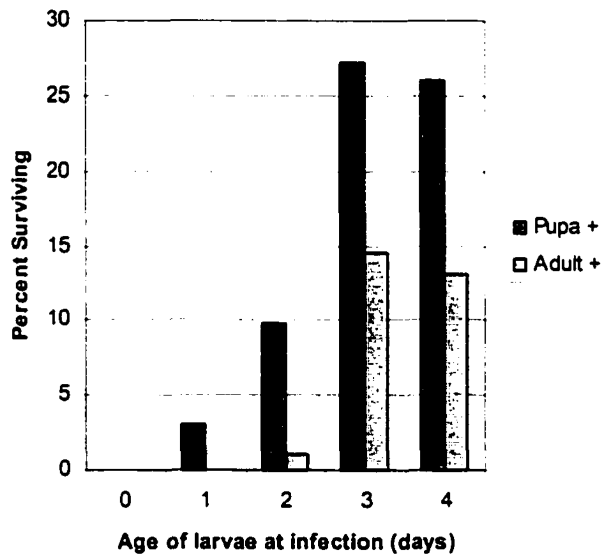

Figure 4.3. Effect of larval age when infected upon survival through pupation and into adulthood. Bars indicate the percent surviving and still expressing GFP.

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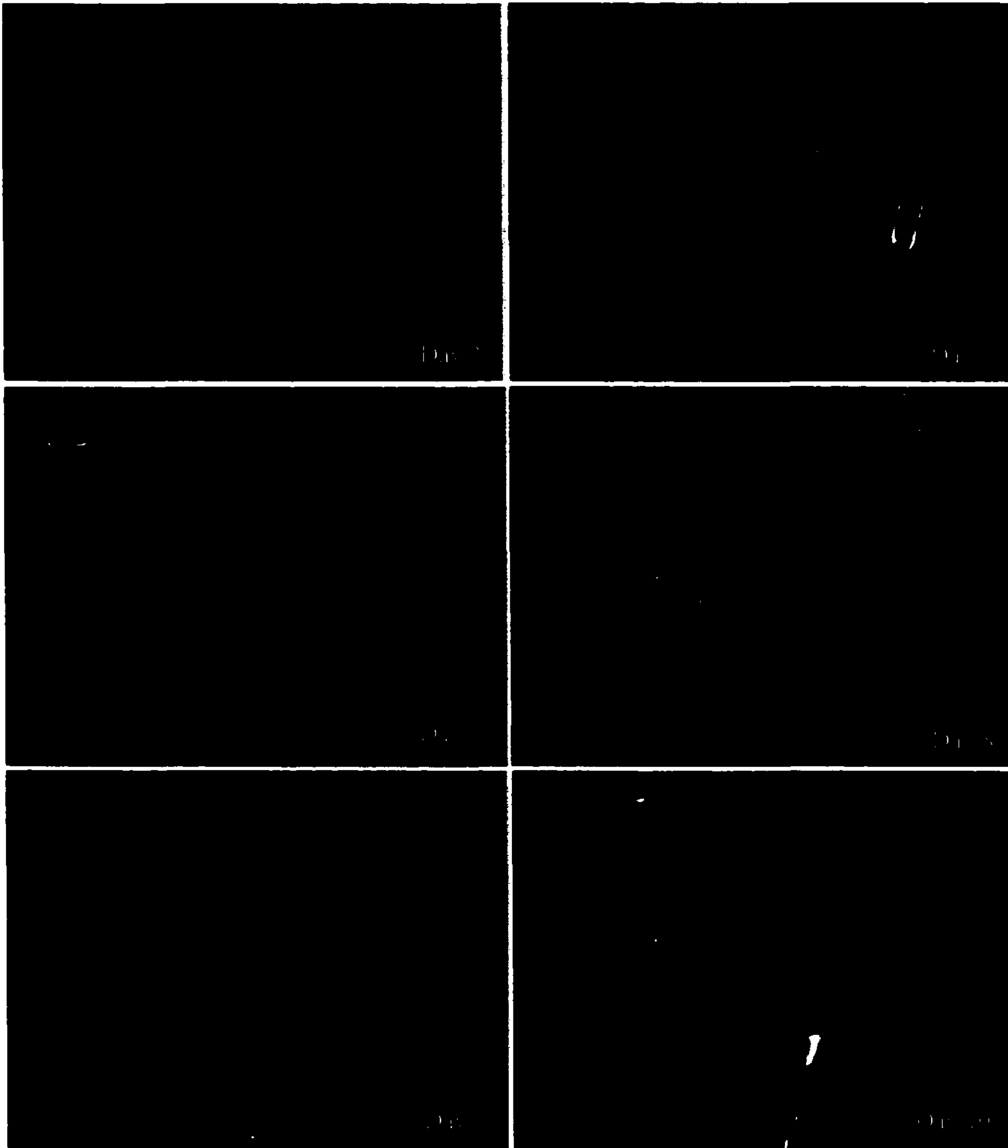

Figure 4.4a. Typical course of infection in an individual mosquito larvae from 2 to 10 days post infection. GFP expression was first observed within the anal papillae. By day 4 dissemination to the fat body has occurred, and at 8 and 10 days post infection many tissues including muscle fibers are infected.

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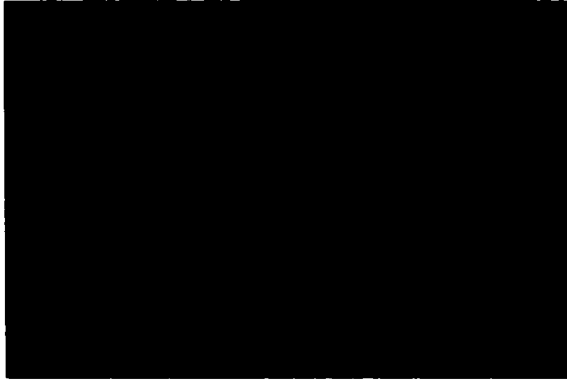
B)



C)

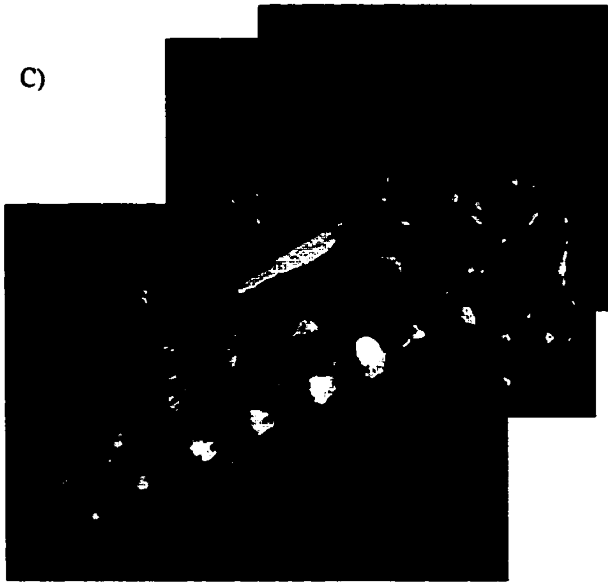


Fig. 4.4b,c. Persistance of GFP expression into pupal and adult stages. B) Disseminated GFP expression within a pupa. C) Disseminated infection within an adult.

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4 days post infection (Barreau *et al.*, 1996; Buchatsky *et al.*, 1989); however, the infection likely began in other tissues as GFP expression in the present study was observed within 24 hours. Other characteristics of infection including reduced response to stimuli, whitish coloration, or body distortion were also not apparent until at least 4 days post infection (Buchatsky 1989, Barreau *et al.*, 1996). These were likely to be larvae with disseminated viral infections. The larvae in the current study, with virus restricted to the anal papillae, did not show these pathological symptoms. Disseminated infection, in the absence of involvement of an anal papilla, only occurred in 5 of 255 infected larvae where the infection appeared to begin within a bristle cell.

Surprisingly, *Anopheles gambiae* larvae (3rd and 4th instar) were susceptible to infection, though at a lower rate than observed with *Aedes aegypti* (Martin Edwards personal communication, Ward *et al.*, 2001b). Only the tip of the *Anopheles gambiae* anal papillae expressed GFP, whereas in *Aedes aegypti* larvae, the entire papillae expressed GFP (Figure 4.4a, 4.5a). The reason for this is not obvious as the anal papilla of mosquitoes is a syncytium of cells (Edwards and Harrison, 1983), which should allow for the spread of the replicating transducing genome from nuclei to nuclei through the connected cytoplasm. This may indicate anatomical differences between the anal papillae of these mosquitoes that may present a barrier to infection, or a reduced capacity of the host to support viral genome replication. Tropism of mammalian parvoviruses has been shown mapped to determinants on the capsid surface and affects the viruses ability to replicate and express genes, but does not usually affect its ability to gain entry into cells. This suggests that AeDNV is able to gain entry into *Anopheles gambiae* cells, but they are unable to fully support viral replication and gene expression (Ward *et al.*, 2001b). It is

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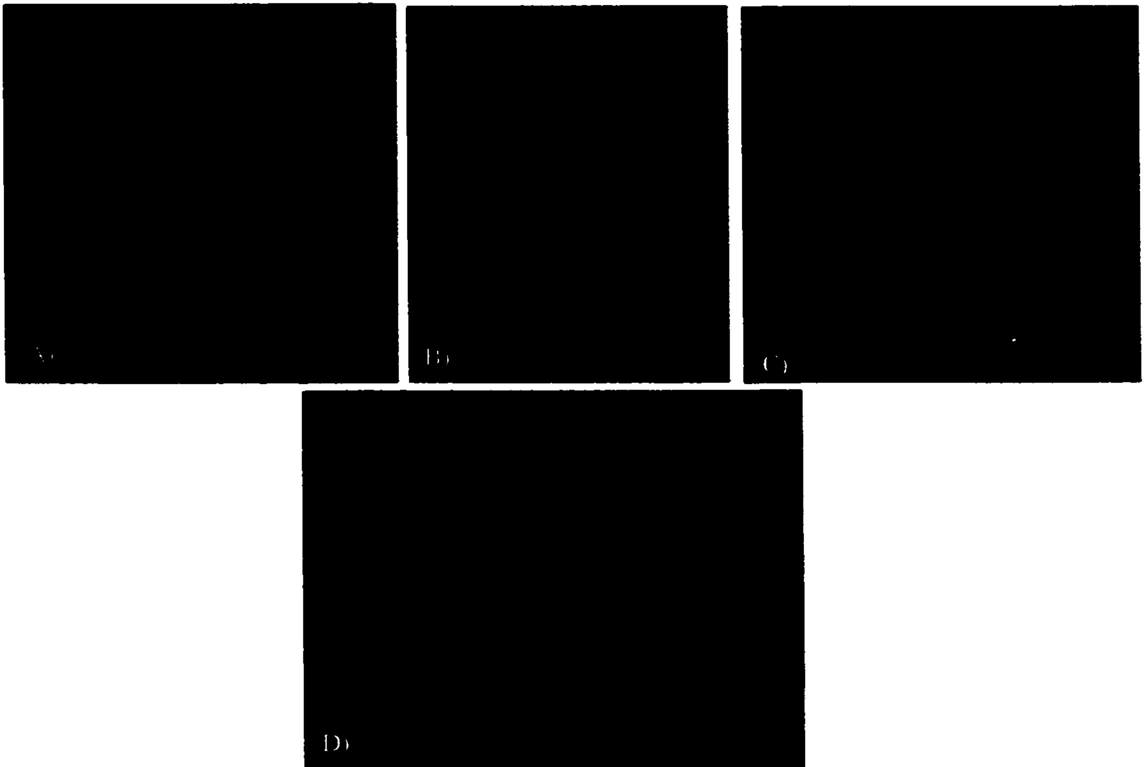

Figure 4.5. Anal papillae shrinkage and loss. A) Infected anal papilla with distinct nuclei. B) Shrinking papilla, the nuclei are no longer distinct. C) Melanization of an infected anal papilla. D) Infected larva with all four papillae missing.

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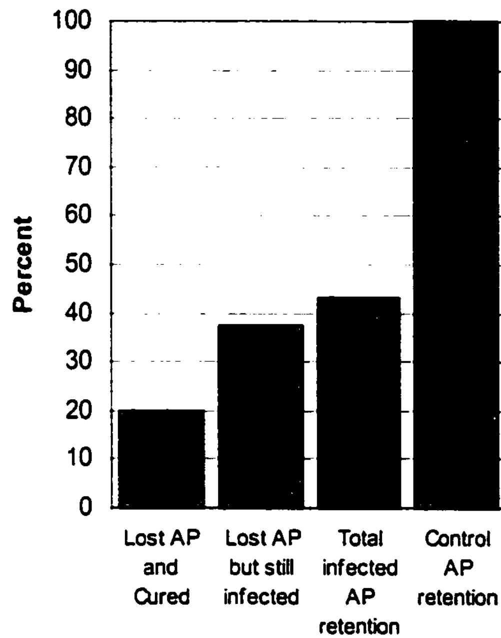

Figure 4.6. Loss of infected anal papillae. AP = anal papilla.

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tempting to speculate that host range determinants could be explored and modified to allow better infection in *Anopheles gambiae* and other mosquitoes.

Loss of anal papillae. Larvae with positive anal papillae were observed closely to follow the course of infection. Amazingly, many infected anal papillae were lost. This occurred in 56% (144/255) of infected mosquitoes. In addition a pattern of shrinkage and loss was observed in 47/144 larvae. The anal papillae begin to shrink, the nuclei became indistinct and then the papilla was lost (Fig. 4.5). In one case (1/144) a darkening of the papilla occurred (Fig. 4.5c), which is indicative of melanization. This may be the first evidence of an as yet uncharacterized immune response against the virus. This process of shrinking and loss was very rapid with 59% occurring within 24 hours. This rapid loss may explain why shrinkage and melanization was not observed in all cases anal of papillae loss. 35% (51/144) of larvae that lost anal papillae were subsequently cured of the infection since there were no further signs of GFP expression and normal development occurred (Fig. 4.6). The other 65% (93/144) of larvae retained additional positive papillae or progressed to a disseminated infection prior to the loss of the papillae. Uninfected control larvae (screened after one week) retained all four anal papillae suggesting that shrinkage and loss was a response to virus infection. Melanization is typical of immune responses against larger parasites such as *Plasmodium ssp.* or *Dirofilaria ssp.* (Paskewitz and Christensen, 1996) that cannot readily be phagocytosed and would not be expected in the case of a viral infection.

Conclusions

This study exploited an AeDNV transducing vector to investigate viral pathogenesis and transduction of genes of interest into *Aedes aegypti* larvae. The use of GFP allowed monitoring of infection and gene expression in living

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mosquito larvae, pupae and adults without harming the organism. Observation of 255 infected larvae, daily, yielded a database of information that allowed the characterization of many aspects of AeDNV pathogenesis in mosquito larvae. Infection was observed after as little as 24 hours with GFP compared to the 4-5 days required for non-reporter gene based methods (Buchatsky 1989; Barreau *et al.*, 1996). When transducing genes of interest, there is now a clear picture of where these genes will be expressed, how long it will take, and the frequency at which it should occur. AeDNV transducing systems should be useful in the study of the effects of other genes of interest. GFP was shown to be expressed in a high percentage of larvae, in multiple tissues, and can persist in larvae through to adulthood. The ability of GFP expression to persist into adulthood raises the question of whether it may also persist into future generations by vertical transmission. The virus can persist in such a manner (Buchatsky, 1987, 1989) and if the gene of interest does also, this system could allow for their introduction into a mosquito population. Unfortunately, *Anopheles gambiae* larvae were found to be refractory to viral dissemination. The loss of infected anal papillae in *Aedes aegypti* larvae is suggestive of an anti-viral immune response and may be the first report of such a phenomenon in mosquitoes. The importance of this observation is unknown; however, it may have implications in our ability to control arboviral diseases.

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Chapter 5. Transovarial Transduction of *Aedes aegypti*

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Introduction

Transduction of mosquitoes with genes of interest is an important method of testing candidate genes for the control of mosquitoes or mosquito-borne diseases. Due to the labor and cost of current transformation techniques for mosquitoes, genes must be extensively tested for their efficacy prior to committing to the production of a transformed mosquito line. Transient gene expression for testing genes of interest has successfully been accomplished *in vitro* and *in vivo* using the recombinant Sindbis virus system (reviewed in Olsen, 2000). Although it has been very effective this system is limited to BSL2 laboratory studies since this virus also infects mammals and birds. AeDNV efficiently transduces reporter genes into cell culture and live *Aedes aegypti* larvae (Afanasiev *et al.*, 1999, 2000; Carlson *et. al.*, 2000; Ward *et al.*, 2001b). Additionally, AeDNV is not an arbovirus as it only infects mosquitoes of the genera *Culex*, *Culiseta*, and *Aedes* and not vertebrates (Buchatsky *et al.* 1987, 1989). These properties give AeDNV the potential for use in biocontrol as well as for the introduction of genes of interest into a natural mosquito population (Buchatsky *et al.*, 1987; Carlson *et. al.*, 2000). It has been observed that densoviruses can persist in laboratory colonies for many generations (Barreau *et al.* 1996, Buchatsky *et al.* 1989; Kittayapong *et al.*, 1999) and is likely to persist in nature by horizontal as well as vertical transmission. It was therefore hypothesized that AeDNV transducing viruses would also be able to persist into subsequent generations, though they require the presence of a helper to allow packaging.

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After a blood meal is ingested the development of embryonic follicles proceeds. The major storage protein vitellogenin is produced and transferred into the developing follicles by receptor mediated endocytosis (Snigirevskaya *et al.*, 1997; Sappington *et al.*, 1995). Parvoviruses are known to gain entry into cells via endocytosis and are found associated with clathrin coated pits (Linser *et al.*, 1979; Vihinen-Ranta *et al.*, 1998; Parker *et al.*, 2000). It was hypothesized that the efficiency of infection of developing follicles would be greatest during vitellogenin endocytosis. In *Aedes aegypti* the rate of vitellogenin endocytosis peaks at approximately 30 hours post blood meal and declines sharply as follicle development comes to completion. Eggs are then laid at about 48 hours post blood meal. As the developing follicle has a very high endocytic rate it was hypothesized whether virus could be transferred into developing follicles in association with vitellogenin (Sappington *et al.*, 1995). This study explores the ability of *Aedes aegypti* mosquitoes, infected as larvae or adults, to transmit transducing and viral genomes to subsequent generations.

Materials and Methods

Infection of *Aedes aegypti* larvae was carried out as described in Chapter 4. Pools of 100 larvae at 48 hours of age were incubated with transducing particle-wild type-virus for 24 hours as described in Chapter 4. These larvae were allowed to pupate and were then screened for expression of GFP. GFP positive pupae were isolated and allowed to eclose within a one pint ice cream cup supplied with water and sugar. After eclosion the mosquitoes were dissected and observed by fluorescence microscopy to analyze GFP expression.

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Intrathoracic injection of adult *Aedes aegypti* mosquitoes. Adult mosquitoes were chilled at 4°C for 5 min. and transferred to a glass petri dish on ice. Mosquitoes were injected in the thorax, just under the wing, with 1µl of traducing particle preparation using a pulled capillary syringe. The mosquitoes were then transferred to an cage and incubated at 27°C, 80% humidity, with 12 h light and 12 h dark cycles.

Optimization of injection time. Mated adult female *Aedes aegypti* mosquitoes were injected at the following time points relative to the ingestion of a blood meal: 48 hours prior to, 4, 6, 8, 10, 14, 18, 24, 30, and 36 hours after ingestion of a blood meal. The 4 to 36 hour groups were fed upon a mouse, whereas the 48 hour prior to blood meal group was fed via an artificial membrane feeder at 37°C with 1 ml sheep blood for 1 hour with a parafilm membrane rubbed on human skin . Oviposition cups were placed in each mosquito cage at 48 hours after blood meal ingestion. Egg liners were collected 72-96 hours post blood meal, dried at room temperature for 2 hours, sealed in a petri dish and incubated at 27°C, 80% humidity, with 12 h light and 12 h dark cycles for 48 hours. Larvae were hatched by placing the eggs in distilled water and incubated as above. Larvae were observed by fluorescence microscopy every 24 hours to determine GFP expression patterns.

PCR detection of viral and transducing DNA. Mosquitoes were collected from each of the following groups: Larva infected as larvae, adults infected as larvae, larvae from adults injected with transducing particles (F_1) and adults subsequently raised from these larvae. Adult and larval mosquito were prepared for PCR by grinding once with a pipette tip in a total of 30 µl of STE. The mosquito was then sonicated in a sonicating water bath for 15 or 20 cycles for larvae or adults, respectively, using a Branson Sonifier at 100% output and 20% duty cycle. 10 units of Proteinase K was added and the mixture

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was incubated at 37°C for 1 hour followed by 95°C for 15 min. 5 µl of the treated sample was then added to 20 µl PCR reaction mix (final concentration: 1.5 mM MgCl, 20 mM Tris-HCl[pH 8.0], 50 mM KCl, 0.2 mM dNTPs, 0.5 mM each vp-up, vp-down, GFP-up, GFP-down primers, and 2.5 units Taq). The mixture was then cycled 35 times at 95°C for 30 sec, 55°C for 1 min, 72°C for 1 min. The resulting PCR was then analyzed on a 2% agarose gel. The primers vp-up (AGACAACGCTTGCTAAC) and vp-down (GTTGTTGTATATACTCCTTC) amplify only wild type AeDNV and yield a band of 340 nt in length. The primers GFP-up (CGCCACCATGGGTAAAGG) and GFP-down (TCGGGCCATGGCACTCTTG) amplify GFP sequences and yield a 280 nt product.

Preparation of transducing particles is described in chapter 3. A mixture of wild type AeDNV and AeDNV transducing particles carrying the GFP gene were produced as described previously (Afanasiev *et al.* 2000; Chapter 3). Briefly, C6/36 *Aedes albopictus* cell culture was transfected with pUCA (AeDNV infectious clone, Afanasiev *et al.*, 1994) and pANS1-GFP (transducing genome with GFP in place the VP gene). Virus particles were harvested 48 hours post transfection, concentrated by ultracentrifugation and suspended in 1 ml water by sonication.

Results and Discussion

Infection of larvae. A small proportion of larvae infected with transducing particles survived into adulthood (Chapter 4). However, adult mosquitoes that demonstrated infection as larvae were found to suffer high morbidity. Dissection of the mosquitoes that were GFP positive as pupae revealed GFP expression in tissues of the fat bodies, neurons, ovaries, and testes (Fig 5.1), indicating that this virus would likely be transmitted to subsequent generations. However, GFP positive pupae were unlikely to

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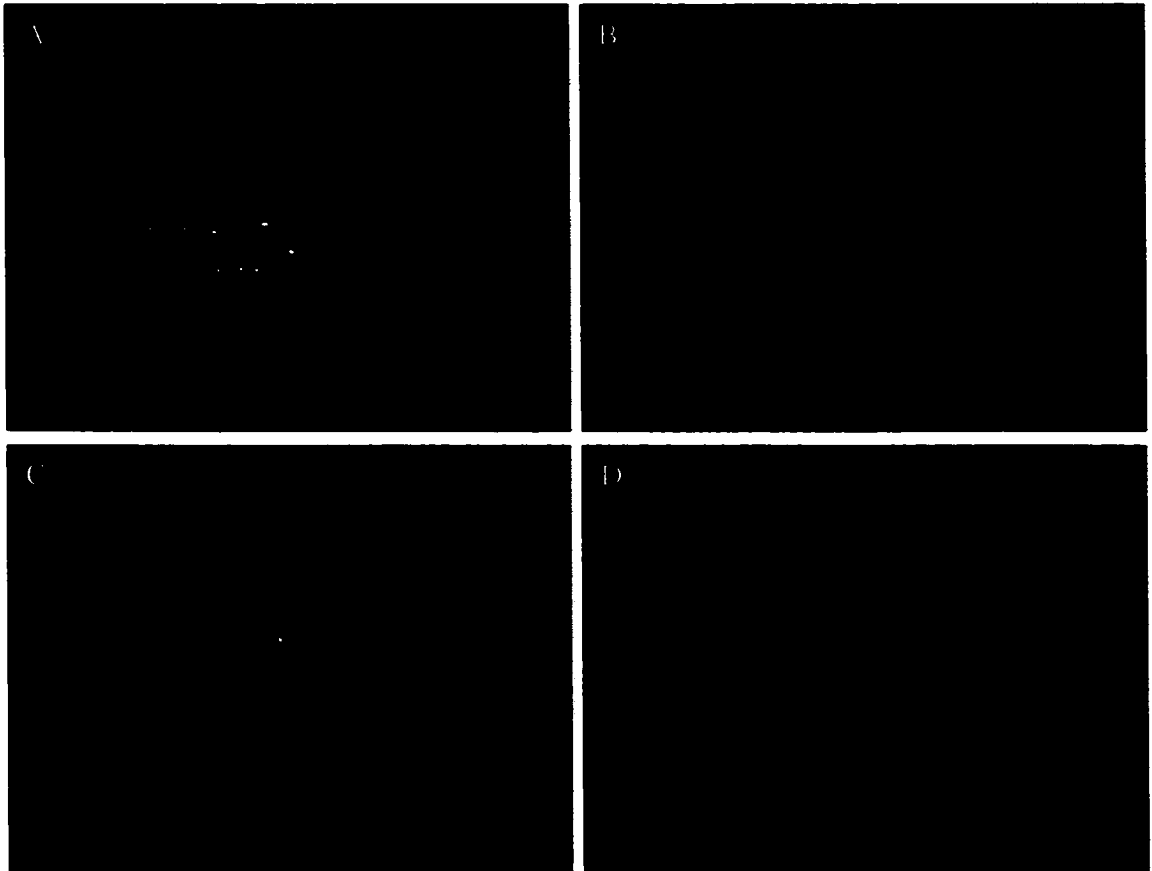

Figure 5.1. GFP expression in tissues of adult mosquitoes infected as larvae with transducing particles and wild-type AeDNV. A) Neurons within the midgut. B) Many cells within the fat bodies. C) Testes. D) Ovaries.

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survive eclosion (98% mortality) and the surviving adults were unresponsive to stimuli, unable to fly, and thus were unable to mate. This observation suggests that vertical transmission may be very infrequent, however, the dose of virus used in these experiments is likely to be vastly greater than that encountered in nature. Larvae infected as 3rd or 4th instar had a higher likelihood of survival (Chapter 4), but were unable to fly or mate. The vertical transmission of AeDNV and similar viruses has been well characterized (Buchatsky *et al.* 1989; Barreau *et al.* 1997; Kittayapong *et al.*, 1999). It is likely that the high viral load needed to allow for visible GFP expression is detrimental to any surviving adult mosquitoes.

Infection of adult mosquitoes by intrathoracic injection. Injection of virus into adult mosquitoes caused mortality in 57% of injected females 24 hours post injection; mortality in mock injected mosquitoes was 30%. 48 hours post injection with transducing particles 84% of surviving mosquitoes successfully oviposited. 95% of females injected with transducing particles were GFP positive, when dissected 72 hours post injection and observed by fluorescence microscopy. 98% of those infected showed GFP expression within cells of the ovarian sheath and ovarial sheath (Fig. 5.2a), and rarely within nerve cells of the midgut (Fig. 5.2b).

Despite lack of apparent infection in follicular endothelial cells, eggs hatched from injected females yielded a large percentage of GFP positive larvae indicating uptake of the virus by the developing follicles. The optimal time of injection was found to be at 24 h post blood meal; these progeny exhibited a 48% filial infection rate as assayed by GFP (Fig. 5.3). The average for time points prior to 24 h was 18% and the infection rates declined sharply after 24 hours to zero when the mosquitoes were injected at 36 hours post blood meal (Fig 5.3). Filial infection rates by time of injection closely resemble the

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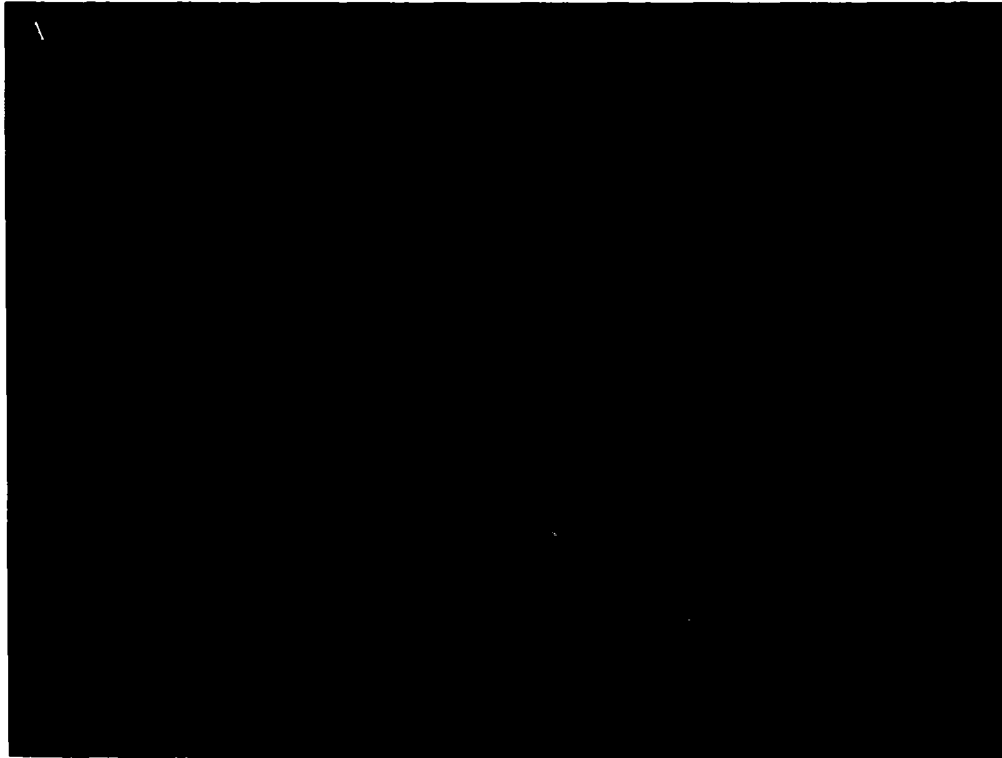

Fig. 5.2. GFP expression in adult female *Aedes aegypti* 72 hours after injection with transducing particles and wild-type AeDNV. A) Cells on the surface of the ovaries. B) Neurons of within the midgut.

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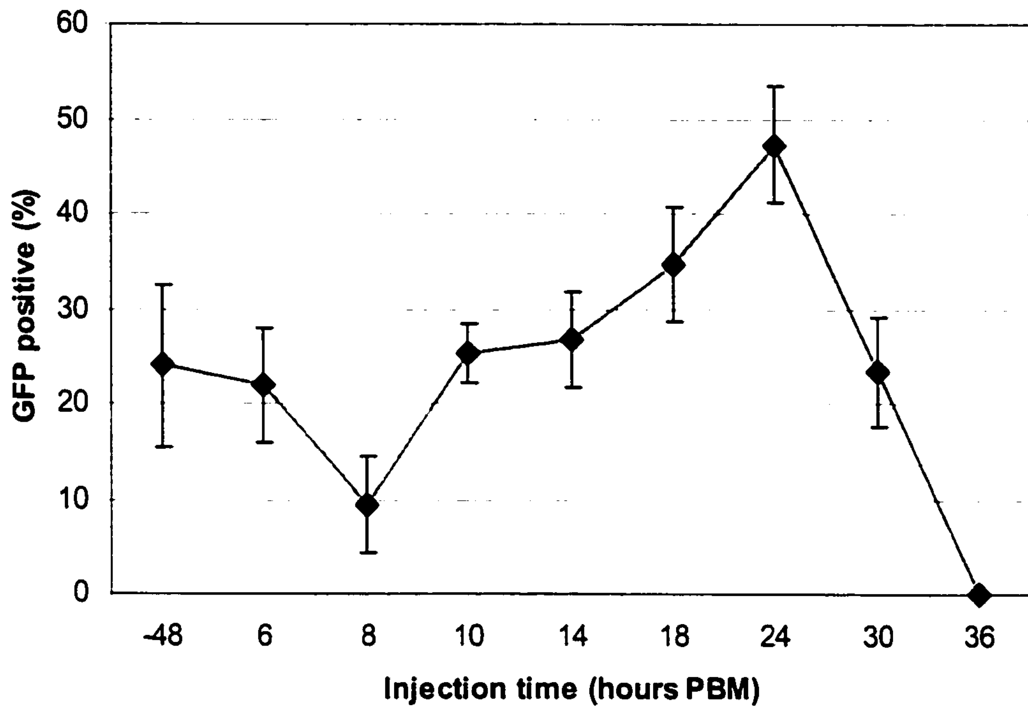

Figure 5.3. Optimization of injection time by GPF expression in F_1 progeny. The progeny from the injected females were screened for GFP expression. PBM = post blood meal.

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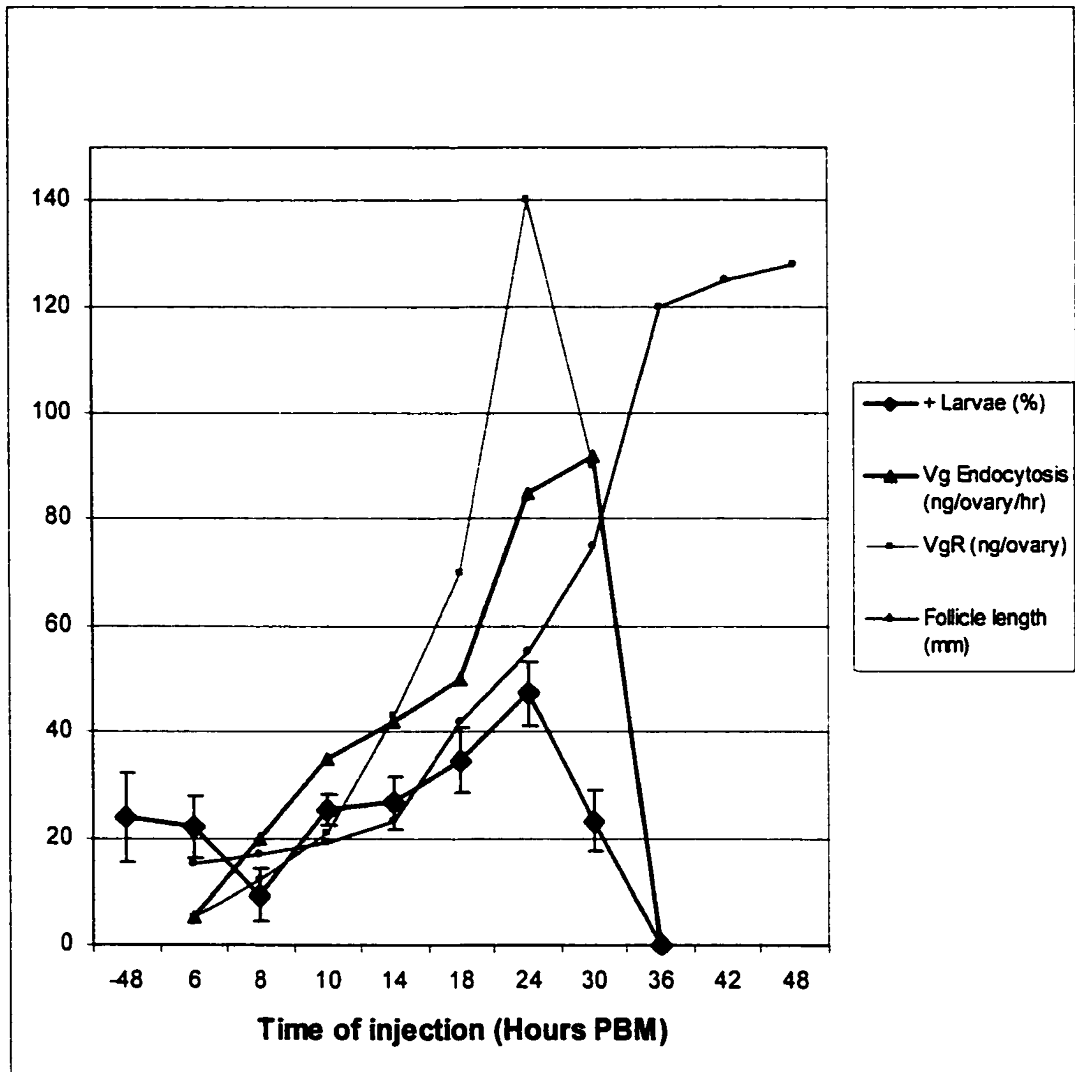

Figure 5.4 Comparison of filial infection rates with various physiological aspects of follicle delvelopment. Vg = Vitellogenin. VgR = Vitellogenin receptor. Vg endocytosis, VgR concentration, and follicle length adapted from Sappington *et al*., 1995.

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kinetics of vitellogenin endocytosis that peaks at 30 hour post blood meal (Sappington *et al.*, 1995) (Fig. 5.4). It is possible that vertical transmission requires that the virus infects the tissues of the ovaries and is subsequently transmitted to the follicles. Alternatively, it is possible that the transducing virus is endocytosed along with vitellogenin, either nonspecifically by being present in high concentrations within the mosquito hemolymph, or specifically with the virus binding to receptors on follicle epithelial cells. In either case the high endocytic rate at 24 to 30 hours post blood meal appears to correlate with infection of offspring (Fig 5.4).

Mosquitoes injected 48 hours prior to blood feeding were also infected and transmitted transducing virus to their progeny. Infection of adult females by intrathoracic injections occurred in 100% (13/13) of mosquitoes injected (GFP expression observed before ([11/13] or after dissection [2/13]). Filial infection rates were 26% for this group indicating that infection prior to egg development can also yield vertical transmission.

Vertical transmission occurred in 83% of mosquitoes. This is much higher than the vertical transmission rate observed for A*Th*DNV (Kittayapong *et al.*, 1999), a closely related densovirus, though it is not possible to compare the dose of virus used between these experiments and mosquito strains. It is possible that intrathoracic injection of concentrated particles would to deliver a greater viral load into the mosquitoes than would be expected within a mosquito infected as a larvae and surviving into adulthood.

It is curious that some adult *Aedes aegypti* are able to cope with such a large dose of virus; 18% (45/247) survived 7 days post injection to blood feed a second time. Ingestion of a second blood meal appeared to induce mortality; only 40% (27/45) surviving the next 24 hours. Only 2 of the 27 remaining

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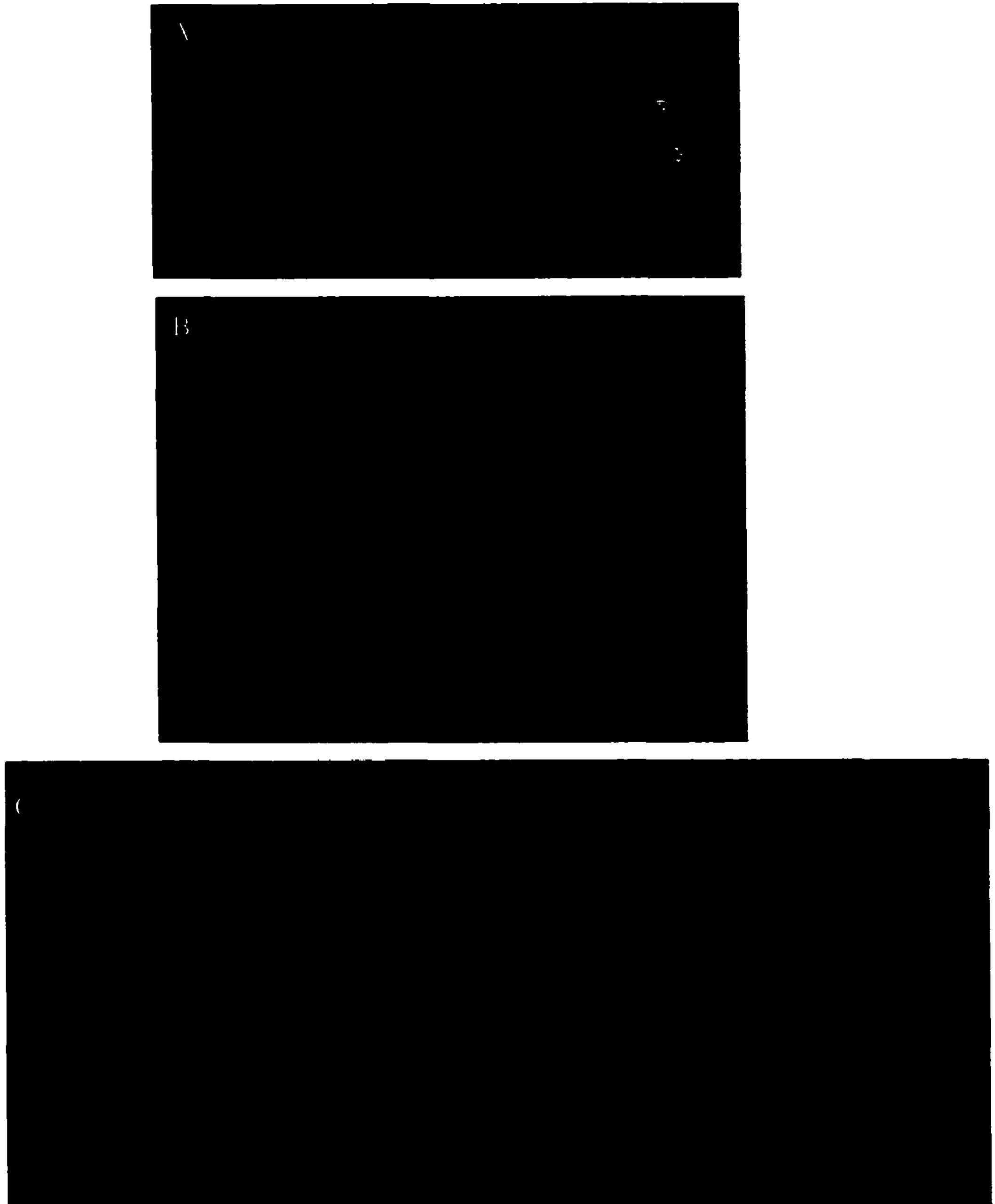

Figure 5.5. F_1 larvae expressing GFP. A) Negative control. B) Larvae with expression within the fat bodies. C) Larvae with expression within imaginal disks.

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mosquitoes successfully oviposited, and none of the resultant larvae showed GFP expression.

GFP expression characteristics. GFP expression was weaker than that observed in larvae directly infected by transducing particles (Chapter 4). PCR confirmed the presence of both wild-type AeDNV and GFP genomes in weakly GFP positive larvae. GFP expression was evident immediately upon hatching in infected larvae and appeared to be limited to the fat bodies (Fig. 5.5b) or imaginal disks (Fig. 5.5c). For unknown reasons GFP expression disappeared in affected larvae after approximately 4 days post hatching. In the absence of wild-type virus, the recombinant GFP genome would lack the ability to package itself and disseminate which may explain the loss of GFP expression. In support of this, GFP negative larvae still contained of wild type AeDNV DNA (100%), but lacked recombinant GFP DNA when analyzed by PCR (84%). Only 16% of adults screened retained AeDNV DNA and only 5% had GFP DNA (as shown by PCR), suggesting that the larvae are able to cure the viral infection.

Intrathoracic injection of pure transducing particle preparations may overcome some of the problems encountered and augment the success of this procedure by eliminating the toxic effects of wild-type AeDNV. These methods may provide a useful method of delivering genes of interest into germline cells as the embryo is infected prior to differentiation which takes place after eggs have been fertilized and oviposited.

Intrathoracic injection of naked DNA. The efficiency of transduction of progeny of injected adult females suggested that injection of naked DNA may also have potential to deliver transgenes to developing embryos. The rapid endocytosis at the follicle could provide a means of entry for the DNA. Successful introduction of transgenes by DNA injection has been

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accomplished in certain phytoseiids (Presnail and Hoy, 1994). This would be a preferable method of introducing genes of interest, because it would eliminate transducing particle preparation. Plasmid DNA was injected alone, or as a mixture of DNA and BSA or DNA and Lipofectin transfection reagent. DNA concentrations up to 1 µg/µl were not observed to cause harm to the mosquitoes. 100% of injected mosquitoes survived to 48 hours post injection (70% survival for mock injected). Unfortunately, GFP expression was not observed and plasmid DNA was undetectable in progeny larvae by PCR for all DNA mixtures tested. Though disappointing, this suggests that the virus capsid is necessary for passage of viral and transducing DNA into developing follicles. Furthermore, it is likely that specific binding of the virus to the surface of embryonic cells is required. Alternatively, that the capsid may simply protect the DNA from degradation within the mosquito hemoceol. Powerful DNases may be present within the hemolymph as have been found in the supernatants of C6/36 cells (A. Patterson, personal communication).

Conclusions

Adult *Aedes aegypti* that are infected as larvae with AeDNV and GFP transducing virus are generally unfit and unable to mate. This indicates that vertical transmission may be a rare event. However, in nature mosquito larvae are unlikely to encounter the high titers of virus used in these experiments. It is likely that with lower doses of virus a mosquito is more able to survive into adulthood and be fit enough to transmit virus to its progeny. Intrathoracic injection of AeDNV transducing virus illustrated that, under certain conditions, vertical transmission of AeDNV is extremely efficient. Additionally, vertical transmission of transducing virus is also efficient. It is not clear from these studies whether replication of the virus is necessary

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prior to transmission to progeny. It is unlikely that the 12-24 hours between injection and egg laying is enough time for a full replication cycle to take place. Supporting this is the observation that filial infection rates peak when injected 24 hours post blood meal. If replication in maternal tissues was required prior to vertical transmission it would be expected that injection at earlier times would yield higher transmission rates. This suggests that the injected virus directly infects the developing follicle. Further study is required to confirm this; however, if true, it implies that AeDNV transducing vectors could be used to deliver a variety of genes into germline cells of *Aedes aegypti*. Further usefulness of intrathoracic injection of transducing particles could be achieved by the use of pure transducing particle preparations produced by the Sindbis system (Allen-Muira *et al.*, 1999) and would not be complicated by the pathogenic properties of AeDNV.

It is curious that GFP expression disappeared from GFP positive larvae. This observation suggests that the larvae are able to suppress gene expression or clear viral infections. Supporting this is the observation that third and fourth instar F_1 larvae have no detectable AeDNV DNA when the same group of larvae showed 100% infection by PCR when more than 2 days old. Buchatsky also observed some mosquito larvae apparently clearing viral infection (Buchatsky *et al.* 1989). These observations and the ability of larvae to shed infected anal papillae all point an anti-viral immune response in *Aedes aegypti* mosquitoes.

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Chapter 6: Summary and Perspectives

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Summary

Mosquito-borne diseases are a global problem and current control strategies are failing to control mosquito populations and disease incidence. Development of new, effective control methods is necessary to reduce morbidity and mortality associated with these diseases and improve quality of life. Interest in AeDNV derives from its ability to kill *Aedes aegypti* mosquitoes. In addition, AeDNV transducing vectors expressing toxic genes may provide an improved biological control agent. Transducing vectors carrying other genes of interest hold promise as useful tools for the investigation of mosquito biology, and as a testing platform for genes that have potential for reducing disease transmission. To realize the true potential of AeDNV detailed knowledge of its molecular biology and biology is needed. Furthermore, knowledge of AeDNV's pathogenesis and biology is required to ensure its safe and efficacious use as a biocontrol agent. The studies presented herein were undertaken to expand our knowledge of AeDNV, to illustrate the complexity of this virus and to investigate its potential for future application.

Sequence requirements for efficient gene expression from the two promoters of AeDNV were investigated in Chapters 2 and 3. It was hypothesized that sequences upstream from the map unit 61 TATA sequence (previously known as p61) are required for efficient structural protein gene expression. This was investigated by gene expression analysis of deletion and mutation constructs expressing a VP-ß-gal fusion protein. The true structural protein gene promoter was found to be located at map unit 59.5 and was renamed pVP. Efficient expression from pVP relies upon an Inr sequence and

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an upstream TATA box for full expression. Deletion or mutation of either of these sequences reduces but does not abolish gene expression. This suggests that pVP has properties of both a TATA-less and TATA containing promoter. It appears that the Inr is able to direct transcription initiation in the absence of a TATA sequence. However, the TATA sequence is required for maximal expression. Primer extension analysis demonstrated that the structural protein gene transcriptional start site was within the Inr sequence of the pVP promoter, which is consistent with the function of an Inr.

Identification of functional sequences within pVP led to the hypothesis that the consensus Inr and TATA sequence of the p7 promoter would also be required for expression. Mutation of these sequences showed that, in contrast to pVP, the nonstructural promoter, p7, absolutely requires the TATA box sequence for expression. Mutation of the TATA reduces gene expression to undetectable levels. The integrity of the consensus Inr sequence is required for maximal expression but mutation of this sequence does not reduce expression to the extent of a similar mutation in the Inr of the pVP promoter indicating that the Inr sequence may not be directing transcription as is likely for pVP.

The viral NS1 protein is thought to transactivate both viral promoters. Transactivation of both promoters by the viral NS1 protein was hypothesized to require the presence of the viral terminal sequences. Addition of the viral terminal sequences to p7 and pVP constructs increased their expression in response to NS1. Maximal expression is achieved only in the context of both viral terminal sequences and NS1. Additionally, in the case of the pVP promoter, maximal expression is at least in part due to template amplification as shown by Southern analysis.

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Expression from the structural gene promoter was found in previous studies to be constitutive. Temporal regulation of gene expression was therefore hypothesized to lack the early to late shift in gene expression observed with other parvoviruses. Temporal analysis of expression of the NS1, NS2 and VP reading frames indicated that all viral proteins are expressed simultaneously. This is in contrast to mammalian parvoviruses, which have a transition from early (NS) expression to late (VP) expression. The VP protein, which is needed in many copies per genome for proper encapsulation, was expressed at higher levels than the nonstructural proteins. Surprisingly, the NS2-ß-gal fusion protein was expressed to levels twice those of NS1 early in infection. The role of NS2 is unknown at this time, but is likely to be important in the virus life cycle based upon its abundance.

The pathogenesis of AeDNV was examined in Chapter 4 with the use of a recombinant virus expressing GFP. It was hypothesized that transducing virus carrying the GFP gene could be used to investigate many aspects of AeDNV pathogenesis, and that AeDNV could persist into adult stages and subsequently be transmitted to progeny. Larvae were coinfected with the GFP virus and AeDNV, and GFP expression was used as a marker to follow the tissue distribution of virus gene expression (and thus replication) as it disseminated through infected larvae. Separation and observation of individual infected larvae yielded a detailed information about AeDNV infection and dissemination, and transgene expression. In support of previous work, the initial portal of entry was found to be the anal papillae. Dissemination from the anal papillae to tissues of the fat bodies required an average of 2 days, with death of the infected larvae usually following two days later. Tissues including muscles and neurons were found to become infected after the fat bodies. Survival of larvae was associated with the age when they became infected;

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older larvae were more likely to survive into pupal and adult stages. Additionally, larvae older than 2 days of age were less susceptible to infection.

Another interesting result of this study was the observation that infected larvae specifically lost infected anal papillae. This loss typically took less than 24 hours, however, in many cases a distinct pattern of anal papillae shrinkage was observed, but rarely included melanization. This is potentially the first direct observation of an anti-viral immune response in mosquitoes. Melanization is typical of responses to large pathogens such as *Plasmodium* ssp. and is also observed in response to mechanical damage. It is possible that the melanization of anal papillae is not induced by AeDNV, but is instead a non-specific response to the damage being caused by the virus.

The study of AeDNV pathogenesis revealed important information about the tissue distribution of transgene expression. Recombinant AeDNV viruses have potential for general use as a gene delivery system. All of the pathogenesis data also applies to the expression of foreign genes by this transduction system. Application of transduction for the study of other genes of interest will benefit from this study by having prior knowledge of the infection rates, expression patterns, and dissemination characteristics of AeDNV. Furthermore, the potential application of AeDNV as a biocontrol agent has been increased; infection rates of 92% were achieved in these studies which is up to 10 fold higher than that achieved previously (Afanasiev *et al.*, 1999). The factors contributing to this include the concentration of virus particles and the determination of the optimal age of larvae for infection (2 days post hatching). Further increases in the efficiency of virus production could come from better transfection procedures or reagents, or by producing virus in larvae. All would drop the potential cost of large scale virus production.

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AeDNV transducing particle's ability to infect *Anopheles gambiae* (Ward *et al.*, 2001b) illustrates how little we know about the host range determinants of this virus. Among mammalian parvoviruses 1-5 amino acid changes within the capsid proteins can completely alter the host range of the virus. It is evident that *Anopheles gambiae* are susceptible, but not permissive to infection by AeDNV. It is tempting to speculate that knowledge of the tropism determinants of AeDNV could allow for the production of a strain of AeDNV that can efficiently infect and kill this malaria vector. Unfortunately, *Anopheles gambiae* infections were not carried out under the optimal conditions (M. Edwards, personal communication) that were determined during the course of these experiments. The larvae were too old, and they were not exposed to the same high concentration of virus particles. Repetition of these experiments under optimal conditions is important, and should include investigation of whether or not *Anopheles gambiae* also are able to lose infected anal papillae.

The ability of AeDNV and GFP transducing virus to be transmitted vertically was demonstrated in Chapter 5. Mosquitoes surviving infection as larvae and GFP positive as adults were unfit and unable to mate. However, tissues of the testes and ovaries expressed GFP, suggesting that vertical transmission of AeDNV was possible. Adult mosquitoes infected by intrathoracic injection of virus also revealed GFP expression within tissues of the ovaries. Vertical transmission to F_1 progeny was observed by fluorescence microscopy and confirmed by PCR. GFP expression showed that the optimal time to inject mosquitoes was 24 hours post blood meal ingestion. These results correlated with the endocytic activity of the developing follicles within the ovaries. The most likely mechanism of transmission is via a direct infection of the developing follicle without replication within maternal tissues. PCR

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analysis indicated that vertical transmission can occur in 100% of progeny, though by the fourth instar, few larvae still retain viral DNA. This may be due to the mosquito clearing the virus infection, and combined with the observation of virus induced loss of anal papillae (Chapter 4) suggests an anti-viral immune response.

Despite the loss of GFP expression and the lack of persistence of viral genomes, delivery of foreign DNA by AeDNV particles by intrathoracic injection is likely to be useful for the transduction of germline cells in mosquitoes.

Perspectives

Mosquito-borne diseases are a burden upon humanity. Attempts to control these diseases have met with only marginal success and the development of new control agents and tools for the study of mosquito-pathogen interactions is needed. Transformation technologies have provided a new tool for the investigation of mosquito molecular biology. However, because of the cost and time involved, genes of interest must by analyzed carefully prior to the production of a transformed mosquito line. AeDNV has been proposed to have potential as a transducing vector, because it can be used to deliver foreign genes into live mosquitoes, and as a biocontrol agent, because it can kill *Aedes aegypti* mosquitoes. AeDNV is still in the preliminary stages of development as a transducing vector for the testing of genes of interest. Realization of AeDNV's potentials requires a detailed knowledge of AeDNV pathogenesis and molecular biology.

These studies have illustrated that AeDNV has the potential for use as a biological control agent and a transducing vector. Detailed knowledge of the molecular biology of virus gene expression and regulation increases our

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ability to construct useful transducing genomes. Knowledge of AeDNV pathogenesis and the characteristics of foreign gene expression gained in these studies can launch the introduction of genes that may affect the mosquito's ability to survive, or transmit disease.

Many new mosquito densoviruses have recently been identified. Any of these viruses may have a greater potential for biocontrol and transduction than AeDNV. However, the studies presented here add to a body of knowledge making AeDNV the best studied member of the densovirus subfamily and providing a model for gene expression and pathogenesis of all mosquito densoviruses.

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Appendix 1: Gene expression from the p7/p61 fusion construct ΔΔ

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Introduction

The ß-galactosidase (ß-gal) expression construct ΔΔ contains the p7 promoter fused upstream of the map unit 61 TATAA sequence previously thought to define the structural protein gene promoter (Fig. A1.1) (for discussion refer to chapter 2). Originally constructed by Joe Corsini, ΔΔ was intended to investigate potential enhancer sequences in the 3' end of the virus genome. ß-gal is fused to the structural protein gene as described in chapter 2. ΔΔ was found to express ß-gal-VP-fusion proteins to high levels (40% that of pVPNco, Fig 2.2) and could be transactivated by NS1 (unpublished observations). However, in light of findings presented in Chapter 2 it became an enigma as to how this gene was being expressed; the construct is missing the TATAA and Inr sequences found to be required for structural protein gene expression. This suggests that the p7 promoter is responsible for gene expression. Confounding this theory are 2 AUG codons (for NS1 and NS2) upstream and out of frame with the VP-ß-gal AUG (Fig. A1.1). If the transcript begins at the p7 promoter these upstream AUGs would likely interfere with the translation of the structural protein by binding passing ribosomes (Kozak, 1999). The following is a brief discussion of current data on ΔΔ and how it seems to contradict the rules of AeDNV gene expression as set forth in chapter 2 and 2b.

Materials and Methods

Cell culture, transfection, and reporter gene analysis was performed as described in Chapter 2.

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Constructs. Refer to Fig. A1.1. **ΔΔ**, **ΔNSΔBE**, and **p61ΔΔ,Δp7** were constructed by Joe Corsini. **ΔΔ** was produced by digestion of nsp61gal (Chapter 2, Afanasiev *et al.*, 1994) with *Msc*I, followed by religation. The 5' sequence was then removed by digestion with *Hind*III followed by religation (as per Chapter 2, pVPNcoLE). This places the p7 promoter 200 nt upstream from the map unit 61 TATAA sequence (290 nt upstream of the VP AUG). **ΔNSΔBE** was made by deleting 300 nt from the 3' end of ΔΔ. This deletes viral sequence up to the *Eco*RI restriction site 39 nt downstream from p7. **p61ΔΔ,Δp7** has the 3' 260-nucleotides deleted up to the *Eco*NI restriction that lies in the middle of the p7 TATAA sequence. **Δp7,ΔΔ** was created by PCR based mutagenesis as per Chapter 2 except the mutation primers were: Bammid fwd (CCACATGGTCCACCCCGGATCCAGGAGTACAAAAG [Mutation underlined]) and its complement, Bammid rev. The flanking primers were: upstream, CCTCTTCGCTATTACGCCAGCTGGCCGAAAG (380 nt upstream of p7) and downstream, GCTGTCTGCCATGATTCTGCTTCTTCTTTC (310 downstream of p7) that bound to viral sequences up or downstream of the region of interest. **Δp61,ΔΔ** was created by transferring the *Msc*I to *ClaI* fragment containing the p61 point mutation from Δp61 (Chapter 2) into ΔΔ digested with the same enzymes.

Results and Discussion

As presented in Chapter 2, the structural gene promoter consists of a powerful Inr sequence that is able to direct transcription in the absence of a TATAA box. The TATAA sequence at map unit 60 does contribute to gene expression; however, ΔΔ does not contain either of these sequences. The TATAA sequence at map unit 61 is present but was found to be dispensable for gene expression (Fig. 2.2a, Δp61). Additionally, it is of note that the p7 TATAA

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sequence was found to be required for gene expression (Chapter 2b) and is present in ΔΔ.

Deletions were made within the ΔΔ construct to determine the location of sequences that act on expression efficiency. Deletion of the p7 promoter and the left end of the virus (ΔNSΔBE) or deletion of the p7 region between *Eco*NI and *Msc*I (p61,ΔΔ,Δp7) (Fig. A.1.1) abolished gene expression. This suggests that the p7 promoter is responsible for expression from ΔΔ, though p61,ΔΔ,Δp7 also lacks the secondary structure found to be required for efficient translation of the NS proteins (Kimmick *et al.*, 1998), which may account for this constructs lack of expression. This supports results showing that the p61 TATAA sequence does not act as a promoter (Chapter 2). However, point mutation of the p7 TATAA sequence within ΔΔ (Fig. A1.1, p7,ΔΔ) did not completely destroy gene expression (22% that of ΔΔ) as would be expected. This same mutation completely eliminates ß-gal-NS1 protein expression in the construct Δp7galNS1 which expresses NS1-ß-gal-fusion protein from the p7 promoter (Chapter 2b and Fig. A1.1).

Mutation of the p61 TATAA sequence within the VP-ß-gal-fusion construct pVPNco had no effect on gene expression (Chapter 2, Fig. 2.2), indicating that it is not the true promoter. This same mutation, when cloned into ΔΔ (Δp61,ΔΔ), appeared to enhance gene expression to 150% that of ΔΔ (Fig. A.1.1). It is possible that p61 TATAA sequence is competing for transcription factors (TATA binding protein) but is unable to successfully form the preinitiation complex. The destruction of this interaction would then increase the availability of transcription factors for the p7 promoter. In support of this, internal control expression constructs appeared to compete

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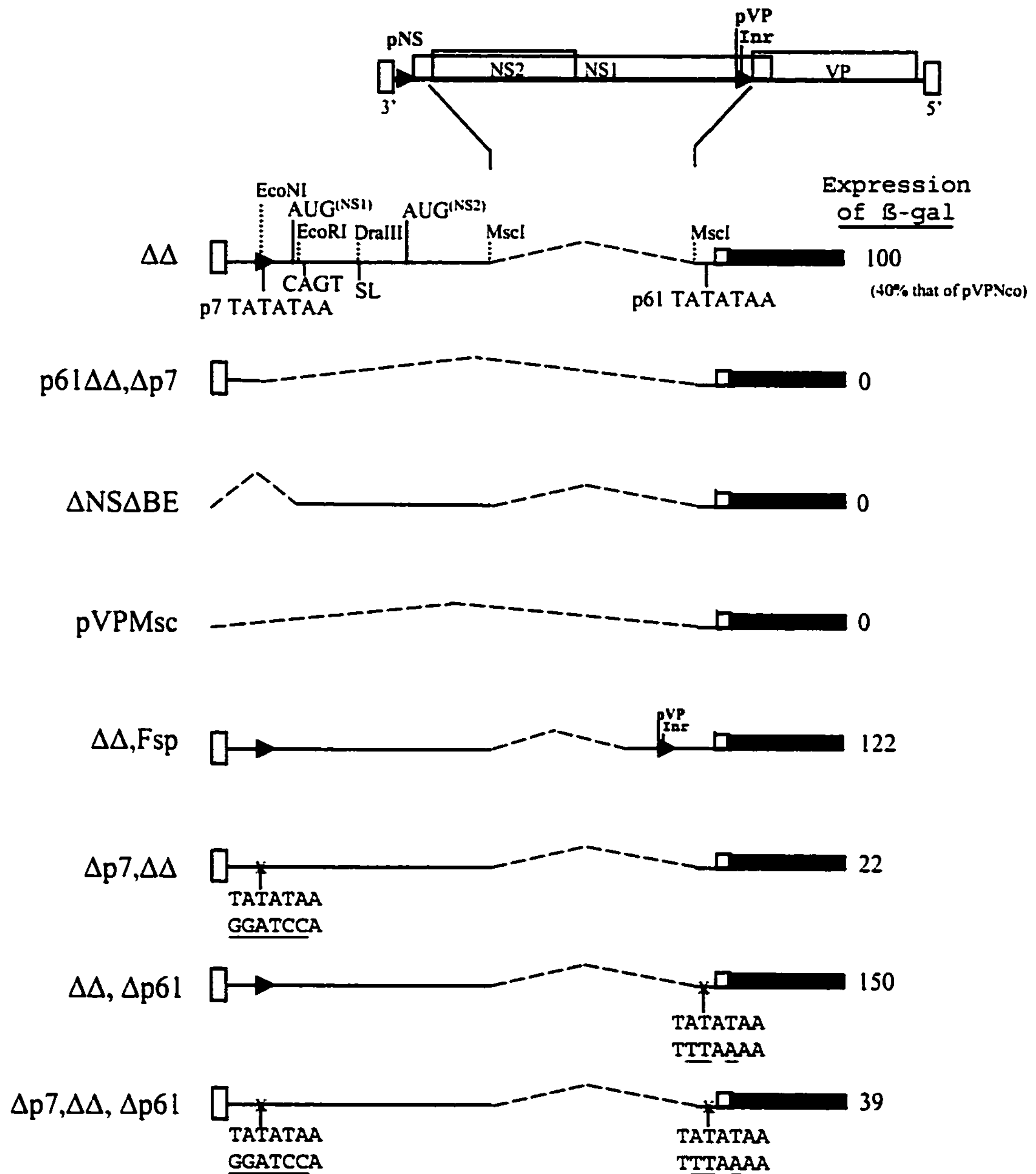

Fig A.1.1 ΔΔ and related deletion and mutation constructs with expression of β-galactosidase relative to ΔΔ. Black boxes indicate *lacZ* sequence fused to the viral VP gene. X = point mutations with mutated sequence shown below wild-type sequence.

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with ß-gal constructs for transcription factors as high ß-gal expression repressed control glucuronidase expression and vice versa (unpublished observations). Due to this phenomenon control constructs in these studies are supplied at low levels and are expressed from non-viral promoters (pBSLuc, chapter 2).

Deletion of both the p7 and p61 TATAA sequences (Δp7,ΔΔ,Δp61) failed to extinguish expression from this construct (40% that of ΔΔ, Fig. A1.1). Thus their are few possible explanations left. The TCAGTC sequence downstream from the p7 promoter is identical in sequence to the Inr of pVP, but is 10 nt further downstream from the accompanying p7 TATAA box. Because of the strength of the pVP Inr it would be reasonable to expect the p7 Inr sequence to be capable of transcription initiation. However, Δp7galNS1 and ΔNSΔBE do not express NS1-ß-gal-fusion proteins. Both contain the CAGT sequence but lack a functioning p7 TATAA. Thus, it appears that the CAGT sequence downstream from p7 does not function in the absence of other known promoter sequences as the Inr does for pVP (chapter 2).

One remaining possibility is that the p0.5 TATAA sequence, upstream of p7 (Afanasiev *et al.*, 1991), may have limited function in binding transcription factors and initiating transcription. Afanasiev and coworkers showed that ß-gal can be expressed from p0.5 though at very low levels (Afanasiev *et al.*, 1994). This explanation fits more of the data than any other. It explains how without the p7 TATAA, p61 TATAA, or either sequences intact, expression still occurs from Δp7,ΔΔ, Δp61,ΔΔ, and Δp7,ΔΔ,Δp61 respectively. This also explains the lack of expression from constructs when both p7 and p0.5 sequences are missing (ΔNSΔBE, pVPMsc). The construct p61,ΔΔ,Δp7 retains the p0.5 TATAA sequence but does not express. However, this could be due to the deletion of the secondary structure downstream of p7 found to be needed for efficient NS

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protein translation (Kimmick *et al.*, 1998). Finally, the only remaining quandary is the lack of expression from Δp7galNS1 which retains the p0.5 sequence. This comparison, however, may not be meaningful as this construct expresses a NS1-ß-gal-fusion protein instead of the VP-ß-gal-fusions of the ΔΔ constructs.

In conclusion, the best fit theory is that the ΔΔ constructs express proteins from the p7 promoter, however the p0.5 promoter is able to contribute measurable ß-gal expression even in the absence of p7. Obviously mutation of the p0.5 TATAA sequence or knowledge of the 5' end of the transcripts produced by these various constructs would lend to a deeper understanding of these phenomena. Unfortunately, primer extension was unable to identify the NS transcript's 5' end, though this procedure was successful in mapping the VP transcript.

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Appendix 2. Database of 255 individual, densoviral infection case histories in *Aedes aegypti* larvae as determined by GFP expression patterns.

Database layout.

This database uses Microsoft excel spread sheet software. Data is arranged in rows of individual larvae. The columns represent the identifying number of the larvae (Table A2.1), and distribution of GFP expression and observations for each day of the experiment. The larvae are grouped by age from freshly hatched "0-day" to "4-day" indicating age in days when infected with virus/transducing virus mixture (Chapter 4). Next to the data for each infection group are columns labeled with infection sites such as the anal papilla (Table A2.2) that are used for calculations and the answering of questions.

Instructions for answering questions using the database.

This database was designed to quickly and easily answer yes/no type questions about AeDNV pathogenesis. Simple questions like " what percentage of larvae had anal papillae as the primary infection site" can be answered quickly. More complex questions such as "How long does it take for a larvae to die after dissemination of the virus" can also be answered quickly.

Example 1. "The percentage of larvae with primary infection in the anal papillae." Step 1. Add a column after the raw data and label it "Primary in the

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AP" or something similar. Step 2. Scroll down the data and place a number 1 in the corresponding row of the "Primary in the AP" column if that larvae had primary infection in an anal papilla. Step. 3 At the bottom of the "Primary in the AP" column calculate the total number of 1's placed in the column using the summation tool[1]. Divide this by the total number of larvae in the sample and you have the percentage.

Example 2. More complex questions are answered in a similar way, with more steps. "Does infection of a bristle cell lead to a disseminated infection without involvement of the anal papillae?" Step 1. Score all larvae that had involvement of an anal papilla prior to dissemination in a column labeled "anal papillae involved". Step 2. Sort[2] all data based upon the "anal papillae involved" column information entered for step 1. This will separate all of the larvae marked with a #1 in the "anal papillae involved" column from those not marked. Step 3. Score remaining larvae for infection of bristle cells prior to disseminated infection. Total this column[1] and divide by the total number not marked in the "anal papillae involved" column. This gives the percentage of bristle cells that occur prior to infection without anal papillae involvement.

Example 3. "What is the average time to death after dissemination." Step 1. label a column "disseminated" and score larvae for the occurrence of a disseminated infection. Step 2. Sort[2] data by the "disseminated" column. Step 3. Label another column with "death in 24 hours", a second with "death in 48 hours", etc. then finally label one "survived". Score larvae based upon number of days between notation of dissemination and notation of death. Step 4. Total the numbers from each column[1], calculate the median time to death using the proper equation.

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[1]Select the box at the bottom of the column to be added. Click the "Σ" button and ensure that the entire column is highlighted. Press "return".

[2]Sort data by clicking the column number at the top of the spreadsheet. This will highlight the entire column. Goto the "Data" pull down menu and select "Sort". Ensure that the proper column has been entered and press "enter".

Table A2.1. Definition of notations.

Notation	Definition
#,#(ie 1,43)	1,43 = 1 day old when infected, mosquito #43 (n and 0 = 0 days old)
(#)AP+(m)	# number of positive anal papillae in all nuclei (m=weak GFP)
(#)ap	# number of single cell at the base of an anal papillae
bt or BT	bristle cell on thorax
bm or BM	bristle cell on mid-body
g ?	questionable midgut
all	disseminated infection (in fat bodies)
allm	minor dissemination
mall	major dissemination (more than fat bodies)
pup	pupal stage
Ad	Adult stage
-	negative for GFP
x	dead
mu	muscle cells
nec	following AP = shrinking anal papilla
(#)apl	# number of anal papillae remaining

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day PI	2	3	4	7
mosquito#				
0,1	1bm	1bm	1bm	x
0,2	mg	-	x	
1,1	AP+	AP+ 1ap 5bt 2bm	AP+ 1ap fb all	2AP+nc all
1,10	1bt	1bt 1ap allm	all	x
1,11	1ap	1ap 1bt	allm	x
1,12	2ap 3bt 1bm	1ap	all	AP+ all 2apl
1,13	AP+m	x		
1,14	AP+	AP+ 2mg	mg+	all 1aplnc
1,15	AP+m 1ap 1bt	AP+m 1ap 1bt	0ap+nc	x
1,16	AP+	allm 3apl	allm	pup all eye
1,17	2AP+m	x		
1,18	3AP+ 2bt	1ap 3apl	1bm	3AP+ all 3apl
1,19	2bt	x		
1,2	AP+	AP+	2apl	x
1,20	1bm	2bt	allm	x
1,21	1bt	1bm	allfb	x
1,22	AP+m	1bt allm	-	x
1,23	AP+m 2ap	AP+m 2ap	all	x
1,24	AP+ 2ap 3bt	4apl -	AP+m allm	3AP+ all
1,25	2ap 2bt	x		
1,26	1bt 1bm	2AP+m 1bt 1bm	AP+m allm	all 2apl
1,27	1bt hair	2bt 2ap	2bt 1ap	AP+m 3aplnc all
1,28	1ap 1bt	2ap allfb	all	pup all
1,29	AP+ 1ap 2bm	AP+ 1ap 2bm	1bt	x
1,3	1ap 2bt	2apl	mgallm	x
1,30	2AP+ 1bt 2bm	x		
1,31	AP+ 2ap 3bt 1bm	2bt 2ap 2apl	allg	allfb
1,32	1bt	allm	x	
1,33	1ap	1bt 1b,	allm	AP+ all
1,34	AP+	allm	x	
1,35	2ap 1bt	AP+nc	-	x
1,36	1bt	2ap 1bm	allm	2AP+ all 2apl
1,37	1ap	2AP+ 2bt 3bm	2AP+ all	x
1,38	1ap 1bt 1bm	1ap 1bt 1bm	allm	x
1,39	1ap	allm	allm	x
1,4	AP+ 1bt 1bm	mbt 2bm 3apl	2AP+	x
1,40	AP+ 1bt	3apl	2ap	2bm mu
1,41	2ap 3bt 1bm	allm	3AP+	all AP+m
1,42	AP+ 2bt	2apl allm	allm	x
1,43	1bt	2AP+	1ap	1bm 2apl
1,44	2AP+	allm 2apl	allm 2apl	1ap
1,45	2ap	allm	x	
1,46	AP+ 1ap 1bt	3apl-	3apl-	pup-
1,47	AP+m	AP+ allm	AP+m AP+	x
1,48	1bt 1bm	all	2AP+nc 2apl	x
1,49	1bt 1bm	1bm	AP+m 1bm mg	x
1,5	1bt 1bm	1bt 1ap 2apl	2AP+ all 3apl	x
1,50	2bt hair	all	mgall	all mu 0apl
1,51	1ap 1bt	x		

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1,52	1bt	-	-	all 3apl
1,53	1bm	1bm 1apl	1bm 1apl	all 1apl
1,54	2bt 1ap	x		
1,55	1ap	-	-	x
1,56	AP+ 2bt	2bt 3apl	2bt mg	x
1,57	AP+	AP+ allm	AP+ allm	all 1aplnc
1,58	AP+	2apl -	allm	allm 3bm
1,59		AP+ 1bt 1bm	AP+ 1bt 2bm	allm mu
1,6	2ap	2bm 2apl	AP+ all 2apl	AP+nc all 1apl
1,60		1ap 1bt 1bm	1ap allm 1bt 1bm	allfb 0apl
1,61		4bt 7mgm	allm	x
1,62		AP+ 1ap	AP+	2AP+ all
1,63	-	3bt 1bm	all	x
1,64			allm	3ap all
1,65			1bt	1apl-
1,66			1bt 1bm	x
1,7	2AP+	allm 3apl	3AP+ all	x
1,8	AP+m	allm	-	x
1,9	1bt	3apl	3apl	x
2,1	2AP+	2AP+	AP+m 4bt 4bm	4AP+ all
2,10	1ap	AP+m 2bt	2AP+ allmaj	x
2,11	AP+m 2bm 1bt	AP+m 4bm	AP+ all	all 0apl
2,12	2AP+	2AP+m	1bt 0apl	mufb 0apl
2,13	1ap	-	1ap	all
2,14	AP+m 2ap 3bt 1bm	AP+m 2AP 3bt 1bm	2ap AP+m 1bt 1bm	3AP+ all 3apl
2,15	AP+	AP+ 1bm	AP+m allm 2apl	pup all
2,16	2AP+	2AP+ 2bt	AP+nc 2ap allm 3apl	2AP+ all 2apl
2,17	2AP+	2AP+	2AP+1nc	2aplnc
2,18	AP+	2AP+	2AP+	allm 1apl
2,19	AP+m	AP+m	AP+	1apl -
2,2	2AP+	2AP+ 1bt	2AP+m allm	AP+m all 2apl
2,20	AP+m	2ap 4bt 1bm	-	x
2,21	1bt 1bm	2AP+1m 4bt 1bm	2AP+ fb 3bt 3apl	AP+ all 1apl
2,22	AP+	AP+	AP+ allm 3apl	3AP+nc hd all
2,23	3AP+m 3bt	3AP+m 3bt	2AP+1m 3apl	AP+ 1apl all
2,24	2AP+ 4bt	2AP+ 4bt 2bm	AP+nc	all 0apl
2,25	2ap 2bt	AP+m 1ap	AP+	x
2,26	2ap 4bt 2bm	AP+m 1ap 3bt 2bm	AP+m 1ap 3bt 2bm	x
2,27	AP+ 1ap 2bt	allm 2apl	allm 2apl	x
2,28	2AP+ 2bt 1bm	2AP+ 2bt 1bm	AP+nc	x
2,29	2ap 2bt	2ap 2bt	2ap 2bt	4AP+ all 3m
2,3	2AP+ 3bt	AP+m 1ap 3bt	AP+m all	AP+ all 1apl
2,30	AP+ 2bt	3ap 2bm	3ap 2bm allm	x
2,31	AP+m	AP+m	AP+	pup -
2,32	2ap 4bt	2ap 4bt 3apl	AP+ 2ap 4bt 3apl	AP+ 3aplnc all
2,33	AP+ ap	3apl 1bm	1bm 3apl	2AP+nc all
2,34	4AP+ 2bt 1bm	AP+m allm	x	
2,35	AP+ 1bt	AP+ 2ap 2bt 3apl	AP+ 2ap 2bt 3apl	x
2,36	2AP+m	3AP+	x	
2,37	3AP+	3AP+ 3bt	AP+nc 3apl 3bt	all 0apl
2,38	AP+	AP+ 1bt	AP+nc 1bt	allm 0apl

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2,39	3AP+ 1ap 1bt	AP+nc 2apl	AP+nc 2apl	all 0apl
2,4	3AP+	3AP+ 1bt	3AP+ all	all 0apl
2,40	AP+	AP+m 3apl	AP+m 2ap 3apl	pup allm
2,41	AP+ 1bt 1bm	AP+ 1bt 1bm 3apl	-	x
2,42	2AP+1m	AP+ 3apl 2bt 3bm	AP+ 3apl allm	all 0apl
2,43	AP+m	AP+ 3apl allm	AP+ 3apl allm	pup all
2,44	2AP+ 1bt 1bm	2AP+1m	AP+nc AP+m	all 0apl
2,45	AP+m 4bt 1bm	AP+m 4bt 3bm	AP+ allm	AP+ 2apl allg
2,46	2AP+1m	2AP+	2AP+	1bt 2bm
2,47	2AP+m	2AP+ 2bt	2AP+ 2bt	AP+ 1apl all
2,48	1ap 1bt 1bm	AP+ 2bt	AP+ allm	x
2,49	AP+ 3bt	AP+ alm	AP+ all	all 0apl
2,5	AP+m	AP+	AP+ 3apl	pup fb
2,50	AP+ 1bt	AP+nc 1bt	AP+ 3apl 2bt	allfb 3apl
2,51	2AP+m	2AP+m allm	2AP+m allm	AP+ 3aplnc all
2,52	AP+m 4bt 4bm	2AP+ 4bt 4bm	2AP+ 4bt 4bm	x
2,53	2AP+ 1bt 1bm	2AP+ 3bt 2bm	AP+nc 3bt 2bm	all 0apl
2,54	AP+ 1ap 4bt 1bm	AP+ 1ap 4bt 1bm	AP+ 1ap 4bt 1bm	2AP+ 4apl all
2,55	AP+ 1ap 4bt	AP+ 1ap 4bt	AP+ 1ap 4bt	2AP+1nc 2apl all
2,56	2AP+m 1ap 4bt 4bm	2AP+m 1ap 4bt 4bm	2AP+m 1ap 4bt 4bm	x
2,57	AP+m	AP+m	AP+	pup -
2,58	AP+m	AP+m	AP+nc	x
2,59	AP+	AP+m	AP+m	3apl gm?
2,6	2ap 4bt	2ap 4bt	2ap alm	all
2,60	3AP+ 2bt	3AP+ 2bt	2AP+ 3apl 2bt	all 0apl
2,61	1ap	-	4bt 3bm	all 3apl
2,62	3AP+2m	AP+m 1apl	AP+nc	x
2,63	AP+ 1ap 4bt	AP+m 3apl	2AP+1m 3apl	all 1apl
2,64	2AP+ 2ap 4bt 1bm	2AP+ 2ap 4bt 1bm	2AP+m 2ap allm	all 0apl
2,65	1ap 1bm	1ap 1bm	AP+m allm	x
2,66	2AP+ 1ap 4bt 5bm	2AP+ 1ap 4bt 5bm	x	
2,67	2AP+ 1bm	AP+ 1ap 2apl	1apl allm	all 0apl
2,68	AP+ 4bt AP+ 4bt 1bm		2AP+ 1ap allm	2AP+ 2apl all
2,69	AP+m 1ap 1bt	AP+m 1ap 3apl 1bt	AP+m 1ap 3apl 1bt allm	pup all
2,7	2AP+ ap 4bt 1bm	2AP+ ap 4bt 1bm	2AP+ 2bt 2bm	AP+ all 2apl
2,70	3AP+ 4bt	2AP+ 3apl	0apl	all 0apl
2,71	AP+ 1bt	AP+ 1bt	x	
2,72	AP+	x		
2,73	2ap 4bt bm	2ap 4bt bm	AP+	AP+ 3apl all
2,74	**2ap 2bt 2bm**	**allm**	**all**	**x**
2,75	AP+m 2ap 4bt 2bm	AP+m 2ap 4bt 2bm	allm 2ap	pup all
2,76	AP+m	3AP+1m	3AP+ all	x
2,77	AP+m	AP+m	AP+	x
2,78	AP+ 1bt 1bm	AP+ 1bt 1bm	AP+m	pup allm
2,79	1bm	1bm	all mu 3apl	AP+ all 1apl
2,8	AP+m	AP+	2AP+	all 0apl
2,80	3AP+ 4bt 3bm	3AP+ 4bt 3bm	1bm 2apl	4apl -nc
2,81	-	AP+m	AP+ allm	AP+nc 2apl
2,81	AP+ 1bm	2AP+1nc 3apl 4bt 3bm	all 1apl	pup -
2,82	AP+ 1bt	2AP+1nc 1bm	all 2apl	AP+ 1ap all 1apl
2,82	-	1bt	all	all 0apl

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2,83	-	AP+m	AP+ allm	all 0apl
2,83	AP+m 2ap 4bt	AP+ 1bt	1bt 3apl	x
2,84	-	AP+m	AP+	pup -
2,85	-	1ap 3bt 3m	2ap 3bt 3bm	4AP+m all
2,86		1ap 1bm 1bt	1ap mu 1bm 1bt	x
2,87		1ap 1bt	AP+m 1ap 1bt	3AP+1m allm fb
2,88			fb	allm 4apl1n
2,89			1ap	1ap 4bm 2bt
2,9	2AP+ 4bt 1bm	2AP+ 4bt 1bm	allfb 0apl	pup allm
3,1	AP+	AP+	AP+nec	1AP 3apl
3,10	AP+m	2AP+m 1bt	2AP+ allm	X
3,11	3AP+	3AP+ 1bt 1bm	2AP+ allm	allg AP+2nec 3apl
3,12	2AP+	AP+nec	AP+m	X
3,13	1AP AP+m	1AP AP+m 3bt	AP+2bt	AP+nec 3apl all
3,14	AP+m 1AP 3bt	AP+m 2AP 3bt	AP+m allm	all 1aplnec
3,15	AP+m 3ap 2bt 1bm	AP+ 2ap 2bt 1bm	2AP+m 1ap 2bt	AP+ all 2apl
3,16	AP+m	2AP+m	X	
3,17	AP+m	AP+ 1bm	AP+ g	AP+ allm 3apl
3,18	AP+m	3AP+m 1bm	3aP+ 1bm	3AP+ allm
3,19	1AP 1bt	AP+ 1ap 1bt 1bm	AP+ allm	all 0apl
3,2	AP+m	AP+m	AP+	1apl -
3,20	AP+m	AP+	1apl -	X
3,21	AP+m	AP+ 1bt 1bm	2AP+ allm	3AP+1nc 1mall
3,22	2AP+	1AP+ 4apl	2AP+	3apl-
3,23	AP+m 2ap	2AP+ 3bt	2AP+ allm	AP+nc all 3apl
3,24	AP+m	AP+ 1bm	AP+ allm	pup all
3,25	AP+	AP+	AP+	3apl -
3,26	AP+m	AP+m	AP+ 3apl	AP+ 1ap 2apl
3,27	AP+m	AP+m	AP+	X
3,28		AP+m	AP+ 1bm	AP+m
3,28			1ap 2bt	3bm 1bt 3apl
3,29		AP+m	AP+m 1ap 1bt	x
3,29a			AP+ 4bm	AP+ allm
3,3	AP+m	AP+	AP+	fb 3apl
3,30		AP+m	AP+ 1ap	1bt 3bm
3,30			1ap	x
3,31		2AP+ 1bt	2AP+ 2bt	AP+
3,31			2ap	1ap 4bt mu
3,32	-	2AP+m 2bm	2AP+ fb	pup fb
3,32a	-	AP+m	AP+ fb	1bm allm
3,33	-	AP+m	2AP+1m	1bt 1bm allm
[illegible]	-	AP+	AP+	AP+ all 3apl
3,35	-	AP+m	AP+ allm	1bt 1bm
3,36	-	1ap	AP+m 2ap 2bt	AP+ fb
3,37		AP+	x	
3,38		1bt	1ap 1bt	allm fb
3,39		AP+	3apl-	allm 0apl
3,4	AP+m	AP+m	AP+	AP+nec
3,40		AP+	2AP+	1ap allm 1apln
[illegible]	-	1ap 1bt	1ap 1bt 1bm	pup allm
3,42	-	AP+m	2AP+ fbm	pup all fb

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3,43	-	2bt	2bt	1bt 0apl
3,44	-	1ap 1bm	AP+ 1fb	1bm
3,45	-	AP+m 1ap 1bt	AP+ 1bt	pup all
[illegible]	-	AP+m	AP+ allfbm	pup allm
3,47		1ap 1bm	1ap 1bm	allfb
3,48		AP+m 1bt	AP+m1ap 1bt	AP+ 1ap allmu
3,49		AP+	AP+	allm 1bt 3apl
3,5	AP+m	AP+	1AP AP+nec	3apl -
3,50		AP+m	AP+m	1ap 3apl
3,6	AP+m	2AP+m	2AP+	all 1apl
3,7	AP+m	AP+m 2bt	1bt 1ap	AP+nec 3apl allm
3,8	AP+m	AP+m 2bt	AP+ 2bt 1bm	AP+nec 3apl allm
3,9	AP+m	AP+ 1bt	AP+ 1bm	AP+nec all 2apl
4,1	AP+	AP+	AP+	3apl FB
4,10		1ap	1ap	7bm mu
4,11		AP+m	AP+	AP+ allfb 3apl
4,12		AP+m	AP+	1bm 3apl
4,13		AP+m	AP+m	AP+m allm
4,14		1bt	1bt	allfb
4,14a		3bt 1bm	2bt 1bm	2ap allmfb
4,15		AP+	AP+	AP+n
4,16		1ap	1ap	2ap mucm
4,17		AP+m	AP+m	x
4,18			g?	x
4,19			g?	apn 2bt 2apl
4,2	AP+	AP+	AP+	3apl -
4,20			AP+m	AP+ 4bt
4,21			AP+m	AP+
4,22			1bt	1bt
4,23			1ap	1ap 4bt 1bm
4,3		AP+m	AP+	pup allm
4,5		AP+m 1ap	AP+	AP+ fb
4,6		AP+m	AP+	1ap allm
4,7		AP+m	AP+m	mum 3apl
4,8		AP+	AP+	allm 3apl
4,9		AP+m	AP+m	AP+ 1bm
n1	-	AP+	x	
n11		AP+m	AP+	2bm mg 2apl
n12		1ap 2bt	1ap 2bt	x
n13		AP+	AP+ 1bm	AP+m allfb 1apl
n14		1ap	2AP+1m	allm
n15		1bt	1bt 1ap	x
n17		AP+	AP+	3bm 1apl
n2	-	3bt 1ap	AP+	3bt 5bm allm
n21		AP+	1bm	3bm 2bt allm 4apl
n22			AP+m	AP+n 1bm
n23			1bt	x
n24			1ap 1bt	allm
n25			1bt 1bm	allfb
n26			AP+ 1ap	AP+n 2ap
n3	-	1bt	-	2bm mu

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n4	-	1ap 1bt 1bm	x	
n5		AP+	AP+	allm 4apl
n6		AP+m	AP+	x
n8		1ap	1ap	AP+m all 3apl

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8	day PI	9	10	11	12+	1st day infe
	mosquito#					
	0,1					
	0,2					
x	1,1					1
	1,10					
	1,11					2
AP+ all 2apl	1,12	x				2.2
	1,13					1
x	1,14					1
	1,15					1.1
pup -	1,16	x				1
	1,17					1
pup all	1,18	pup all	x			1
	1,19					1
	1,2					1
	1,20					
	1,21					
	1,22					1
	1,23					1.1
pupx	1,24					1.1
	1,25					2.2
all	1,26	all	allfb	x		
x	1,27					
pup all	1,28	x				2.2
	1,29					1.1
	1,3					2.2
	1,30					1.1
x	1,31					1.1
	1,32					
AP+ all 2apl	1,33	x				2
	1,34					1
	1,35					2.2
AP+m2nc 3apl all	1,36	all	x			
	1,37					2
	1,38					2.2
	1,39					2
	1,4					1.1
pup -	1,40	pup -	AK			1.1
x	1,41					2.2
	1,42					1.1
pup -	1,43	x				
1ap oapl	1,44	pup -	AK			1
	1,45					2
pup-	1,46	x				1.1
	1,47					1.1
	1,48					
	1,49					
	1,5					
x	1,50					1
	1,51					2.2

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allm 2apl	1,52	x			
x	1,53				
	1,54				2.2
	1,55				2
	1,56				1.1
x	1,57				1
allmfb	1,58	x			1
x	1,59				1.1
x	1,6				2
allm 0apl	1,60	x			2.2
	1,61				
x	1,62				1
	1,63				
x	1,64				
x	1,65				
	1,66				
	1,7				1
	1,8				1
	1,9				
AP+ all 1apl	2,1	AP+ all 1apl	X		1
	2,10				2
x	2,11				1.1
mufb 0pal	2,12	x			1
all 4apl	2,13	all	mall	allfb 3apl	2
2AP+ 2apl all	2,14	AP+ all 2apl	mallfb 1aplnc	allfbm 0apl	1.1
x	2,15				1
AP+ all 1apl	2,16	mallm 0apl	x		1
x	2,17				1
all 1apl	2,18	mall 1apl	mall hd	1mu oapl	1
pup -	2,19	pup -	mall 1apl	1aplg	1
AP+m all 2apl	2,2	AP+ all 2apl	AP+ allfbg 1apl	AP+ allfb 2apl	1
	2,20				1
AP+ all 1apl	2,21	AP+ mall 1apl	x		
2aplnc hd al	2,22	1aplnc allgm	x		1
AP+ allm	2,23	x			1
x	2,24				1.1
	2,25				2.2
	2,26				2.2
	2,27				1.1
	2,28				1.1
x	2,29				2.2
all 0apl	2,3	x			1.1
	2,30				1.1
AK	2,31				1
x	2,32				2.2
x	2,33				1.1
	2,34				1
	2,35				1.1
	2,36				1
x	2,37				1
allm 0apl	2,38	allm 0apl	x	x	1

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x	2,39					1
all 0apl	2,4	x				1
Ax	2,40					1
	2,41					1.1
x	2,42					1
x	2,43					1
x	2,44					1.1
2AP+ allg	2,45	2AP+ allg head	2AP+ allgm	2AP+1nc allg sph		1.1
x	2,46					1
AP+ all 1apl	2,47	x				1
	2,48					2.2
x	2,49					1.1
x	2,5					1
allgm 2apl	2,50	x				1.1
x	2,51					1
	2,52					1.1
x	2,53					1.1
x	2,54					1.1
x	2,55					1.1
	2,56					1.1
pup -	2,57	AK				1
	2,58					1
fb 3apl	2,59	x				1
all 3apl	2,6	mall 3apl	allgm sph 3apl	allgmfb 3apl		2.2
x	2,60					1
x	2,61					2
	2,62					1
all 1aplnc	2,63	mall 1apl	mgallm 0apl	x		1.1
x	2,64					1.1
	2,65					2.2
	2,66					1.1
x	2,67					1.1
x	2,68					1.1
x	2,69					1.1
2AP+ all 2apl	2,7	3AP+ mall 3apl	2apl+1nc allfbm	x		1.1
x	2,70					1
	2,71					1.1
	2,72					1
AP+ allg 2apl	2,73	AP+nc allgm 2apl	allgm 1apl	allgmfb		2.2
	2,74				Ad	2.2
Ax	2,75					1.1
	2,76					1
	2,77					1
pup all	2,78	Ax				1.1
x	2,79					
all 0apl	2,8	mall 0apl	mall 0apl	x		1
4apl -nc	2,80	4apl -nc	3apl -	pup -		1
pup -	2,81	pup mu	pup 1ap eye	Ad-K		1
pup -	2,81	AK				1.1
x	2,82					1.1
all	2,82	alll	allg	x		

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all	2,83	x				1
	2,83					1.1
pup -	2,84	pup-	x			1
3AP+ all 3apl	2,85	3AP+ all 3apl	mall 3apl+	x		2.2
	2,86					2.2
2AP+ all 3apl	2,87	2AP+ mall 2apl	2AP+n mallfb	x		2.2
all 3apln	2,88	gall 1AP+n	2AP+m allfbg	all 1apln		
1ap 3bm	2,89	allm 3apl	2ap allm	1ap 3fb allmg		2
x	2,9					1.1
3apl -	3,1	3apl -	fb 3apl	pup allm	Ad	1
	3,10					1
2AP+nec g	3,11	2AP+nec all	1apl+m	allfbm 1apl+		1
	3,12					1
AP+nec 3apl all	3,13	AP+nec 3apl all	allfbm	X		1
allm 0apl	3,14	allgm 0apl	allgmfb	allgmfb		1.1
AP+ allm 2apl	3,15	allm 2apl+	allm 2apl+	allm 2apl+1nec		1.1
	3,16					1
AP+ fb 3apl	3,17	AP+ allfb	allmfb 2apl	X		1
2AP+ all 2apl	3,18	2AP+ allg 3apl	allfbg 1apl	all 1apl		1
all 0apl	3,19	X				2.2
2bt 1apl	3,2	fb 1apl	fb 1g	1fb 1apl		1
	3,20					1
3AP+1nc allm 4apl	3,21	2AP+1nc mall 3apl	3AP+2nc allgfbm	3APL+nc fballm		1
2apl -	3,22	3apl -	3apl -	pup -	Ad	1
pup all	3,23	pup all	X			1.1
pup all	3,24	X				1
3apl -	3,25	3apl -	3apl -	3apl -		1
x	3,26					1
	3,27					1
pup 1ap	3,28	Ad+	Ad+	Ad+	Ad	1
g? 3apl	3,28	gut 3apl	AP+ 3fb 3apl	2APl+ all		2.2
	3,29					1
x	3,29a					1.1
3apl -	3,3	3apl -	3apl -	1musc 3apl		1
allm 0apl	3,30	allm 0apl	allfbm 0apl	mall		1
	3,30					2
pup-	3,31	x				1.1
g?	3,31	1ap 1bt g?	2ap	vmall		2
pup all	3,32	x				1.1
pup fb	3,32a	pup fb	Ax			1
pup -	3,33	pup allm	Ax			1
pup all	[illegible]	pup all	A+		Ad	1
1ap 3apl	3,35	3apl -	-	pup fb	Ad	1
pup all	3,36	pup all	x			2
	3,37					1
x	3,38					
3apl-	3,39	pup-	pup-	Adx		1
4apl1nec	3,4	3apl -	3apl -	3apl -		1
pup all	3,40	pup all	Adx			1
pup allm	[illegible]	A+			Ad	2.2
pup all	3,42	x				1

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-	3,43	1bt 2bm	x			
pup -	3,44	pup-	AK			2.2
pup all	3,45	x				1.1
pup allm	.·	A+			Ad	1
all 3apl1n	3,47	x				2.2
x	3,48					1.1
all 3apl	3,49	pup all	pup 4dots	Ad+	Ad	1
3apl -	3,5	3apl - 1AP	3apl 1AP	3apl -		1
2apl-	3,50	2apl-0	2apl-	x		1
all 1apl	3,6	allmg	1apl+ allfbm	pup all	pupX	1
AP+nec 3apl allm	3,7	2apl all	allgfb 2apl	allfb 2apl		1
AP+ all 2apl	3,8	allg 2apl	3apl AP+nec	allfb 2apl1+		1
2apl allg	3,9	allgm 0apl	X	X		1
allm	4,1	3apl fball	6fb 3apl	allm 3apl		1
pup allm	4,10	pup allm	Adx			2
AP+ allm 3apl	4,11	AP+ all 2apl	fbgall	1AP+L fb mall		1
3apl-	4,12	3apl-	3apl-	3apl-	pupX	1
4apl-	4,13	pup-	pup-	x		1
pup all	4,14	x				
allfb 3apl	4,14a	3ap 1bt fb 3apl	gfball	mgfball		
pup -	4,15	ad-?	Ad+			1
2ap 4apl	4,16	2ap 4apl	all pup	all pup	Ad	2
	4,17					1
	4,18					
1apn 2apl-	4,19	AP+	AP+ g	AP+		
2apl -	4,2	-	-	-		1
AP+	4,20	x				1
AP+	4,21	AP+ 3apl	AP+mn	1m1g 3apl		1
1bt 3apl	4,22	3apl-	3apl-	3apl-		
1ap	4,23	1ap	1g	-		2
pup -	4,3	Adk				1
all 3apl	4,5	gall 3apl	pup all	x	pupX	1.1
pup vmall	4,6	pup vmall	Ad+	Ad+		1
3apl-	4,7	g?	3apl-			1
3apl-	4,8	pup-	pup-	Adx		1
pup -	4,9	pup-	AdX			1
	n1					1
2apl-	n11	-	1g 2apl	x		1
	n12					2.2
allm 0apl	n13	allm 0apl	allmgfb 0apl	all 0apl		1
3apl-	n14	3apl-	3apl -	pup-	Ad	2
	n15					
1apl-	n17	-	1g 1apln	1apl-		1
bmall 3apl	n2	allm	allm	vmall		2.2
g?-	n21	4apl-	4apl-	pup-	Ad	1
AP+ fb 2apl1n	n22	x				1
	n23					
allm 3apl	n24	3apl-	2apl-	2apl-		2.2
x	n25					
AP+n	n26	2AP+n	mallfb 0apl	all 0apl		1.1
-' '3apl'	n3	x				

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	n4				2.2
1ap 3apl	n5	1ap 3apl	1fb 3apl	1bt 1m	1
	n6				1
AP+ all 3apl	n8	AP+ 3apl all	x		2

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