

## ABSTRACT

Title of Dissertation: THE INNATE IMMUNE RESPONSE OF  
*DROSOPHILA MELANOGASTER* AGAINST  
THE BIRNAVIRUS DROSOPHILA X VIRUS.

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The immune responses of *Drosophila melanogaster* to bacterial and fungal infections has been extensively studied. Here we expand from these two groups of pathogens and examined the immune response of *Drosophila* against a viral pathogen, specifically the birnavirus Drosophila X virus (DXV). We initially developed a screening system utilizing the anoxia sensitivity which is induced by DXV infection. This system allowed us to examine the effect of various mutations, in previously identified innate immune pathways as well as other possible antiviral pathways, on resistance to viral infection. Using this initial screening method we identified both the Toll pathway and RNAi pathway as possible antiviral responses in *Drosophila*. Furthermore, we found that increased susceptibility to viral infection by alteration of either of these pathways was generally associated with increased viral load in infected flies. Additionally, we developed cDNA clones of the entire DXV genome to use as the basis for examination of the effects of RNAi on DXV infection and for the development of a reverse genetics system. Using these clones, we show

that DXV is sensitive to RNAi. Although RNAi does not result in clearing of virus infection, it inhibits viral replication. Our results indicate that both the Toll and RNAi pathways are playing roles in *Drosophila*'s immune response to DXV infections.

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AGAINST THE BIRNAVIRUS DROSOPHILA X VIRUS.

By

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# Table of Contents

Acknowledgements.....	ii
Table of Contents .....	iv
List of Tables .....	vi
List of Figures .....	vii
Chapter 1 - Introduction and Literature Review.....	1
1.1    General Introduction .....	1
1.2 <i>Drosophila melanogaster</i> Immunity.....	3
1.2.1    Barrier Epithelia.....	4
1.2.2    Pathogen Recognition .....	5
1.2.3    Cellular Responses.....	7
1.2.3.1    Blood Cell Activation .....	8
1.2.3.2    Melanization .....	10
1.2.4    Humoral Responses .....	11
1.2.4.1    Toll Pathway .....	12
1.2.4.2    IMD Pathway .....	17
1.2.4.3    JAK-STAT Signaling.....	19
1.2.4.4    JNK Pathway .....	20
1.2.5    Antiviral Immune Responses .....	20
1.3    RNAi .....	22
1.3.1    Biological Functions of RNAi .....	24
1.3.2    Initiation Phase.....	25
1.3.3    Execution Phase .....	29
1.4    Drosophila X Virus.....	31
1.4.1    Discovery of Drosophila X Virus .....	31
1.4.2    Nomenclature and Classification .....	32
1.4.3    Epidemiology of Drosophila X Virus Infection.....	33
1.4.4    Viral Replication.....	34
1.4.5    Virus Release .....	36
1.4.6    Persistent Infection.....	36
1.4.7    Virion .....	38
1.4.8    DXV Genome Structure and Organization .....	38
1.4.9    Viral Proteins .....	41
1.4.10    Reverse Genetics System.....	46
1.5    Current Research Status .....	48
Chapter 2 - The Toll Pathway is Important for an Antiviral Response in <i>Drosophila</i> .....	51
ABSTRACT.....	51
INTRODUCTION .....	52
MATERIALS AND METHODS.....	56
RESULTS .....	60
Chapter 3 - RNAi is an Antiviral Immune Response Against a dsRNA Virus in <i>Drosophila melanogaster</i> .....	74

ABSTRACT .....	74
INTRODUCTION .....	75
MATERIALS AND METHODS.....	81
RESULTS .....	85
Chapter 4 - Conclusions and Future Studies.....	95
Appendix A: Development of a Drosophila X Virus Reverse Genetics System.....	112
INTRODUCTION .....	112
MATERIALS AND METHODS.....	118
RESULTS .....	133
DISCUSSION .....	136
References.....	139

## List of Tables

2.1	Quantitative RT-PCR Primers.....	57
2.2	<i>Drosophila</i> Lines Used in Genetic Screen.....	69

## List of Figures

1.1	Toll and IMD <i>Drosophila melanogaster</i> immune pathways.....	13
1.2	RNAi Pathway.....	26
1.3	Electron micrograph of Drosophila X Virus particles.....	39
1.4	Drosophila X Virus genome organization.....	40
1.5	Reverse Genetics system for recovering recombinant Birnaviruses from clone derived transcripts.....	47
2.1	DXV pathology in <i>Drosophila</i> wild type adults.....	61
2.2	Western blot of purified DXV using antibody generated against purified DXV.....	64
2.3	AMP expression levels in DXV-infected flies measured by quantitative RT-PCR.....	66
2.4	Survival of flies constitutively expressing a single AMP in an immunodeficient background.....	68
2.5	Survival of mutants with decreased susceptibility to DXV infection.....	71
2.6	Increase of susceptibility of <i>Toll</i> and <i>Dif</i> mutant fly lines.....	72
3.1	Survival of RNAi pathway mutants infected with DXV.....	86
3.2	Targeted RNAi of the DXV Genome.....	91
3.3	RNAi of the RNAi pathway in S2 cell culture.....	93
4.1	Model for <i>Drosophila</i> antiviral response.....	101
A.1	Protein sequence of the DXV 27kDA NS ORF.....	114
A.2	Location of NS ORFs in Birnaviruses.....	115
A.3	Protein sequence alignment of NS sequences of DXV and IPNV-Sp.....	117
A.4	Protocol for generation of a cDNA clone of the DXV genome.....	124
A.5	Protocol for generation of a $\Delta$ NS mutant cDNA of DXV genome segment A.....	126
A.6	Plasmid map of cDNA constructs of DXV (pCR2.1-DXV-A, pCR2.1-DXV-B, pCR2.1-DXV $\Delta$ NS).....	129

# Chapter 1

## Introduction and Literature Review

### 1.1 General Introduction

For years, *Drosophila melanogaster* has been used as a model system because of its ease of use, easily manipulated genetics, and extensive homology to various human systems that exist (Medzhitov and Janeway Jr, 2000). This homology has led to the development, study, and use of *Drosophila* as a model system for the study of the innate immune system. In addition, the lack of an adaptive immune system in flies allows for focused study of innate immune responses without the influence of the adaptive immune system.

To understand what responses can be feasibly implemented by an innate immune system one needs to consider that this system is non-variable in nature and does not undergo the extensive genetic shuffling and splicing characteristic of the mammalian adaptive immune system. Instead, the innate immune system makes use of molecular patterns shared by large groups of pathogens which are distinct from self-antigens. Examples of molecular structures which pathogens present and could be used as recognition sites for the innate immune system include select lipopolysaccharides (gram-negative bacteria), certain teichoic acids (gram-negative and gram-positive bacteria), the unmethylated CpG motif (characteristic of bacterial DNA), mannans (a conserved component of yeast cell walls), and double stranded RNA (a structural signature of RNA viruses) (Akira *et al.*, 2001; Aderem and Ulevitch, 2000). These pathogen-associated molecular patterns (PAMPs) provide the

means by which the innate immune system can recognize a diverse number of pathogens while having a relatively limited repertoire of receptor molecules.

The innate immune response in *Drosophila* has been categorized into three distinct response types based upon the pathogen eliciting the response. These broad classifications are the bacterial, fungal, and viral responses. Both bacterial and fungal immune responses have been characterized extensively in *Drosophila* because of the discovery of the role of the Toll and Imd pathways in the innate immune response by Lemaitre and coworkers (Lemaitre *et al.*, 1996). Until recently, however, most viral work in *Drosophila* focused more on the actual insect viruses themselves and not the host's response to viral infection. One reason behind this is the lack of an experimental system which can be used for rapid screening of mutant *Drosophila* lines to identify genes which play a role in antiviral resistance. Additionally, the drastic variation between viruses provides a very limited number of patterns which could be used effectively as PAMPs for identification purposes by the innate immune system. The discovery of Drosophila X virus in 1978, however, provided a virus whose pathology could be developed into a rapidly screenable means to detect alterations in the *Drosophila* antiviral immune response. This feature resulted in development of DXV infection as a model for the study of the *Drosophila* antiviral immune response – and led to our initial work into the discovery of aspects of the *Drosophila* antiviral immune response.

## **1.2 *Drosophila melanogaster* Immunity**

The immune system is key for survival for all multicellular organisms. Without it, something as small as a virus or microorganism can invade and kill even the largest organism in a short period of time. It is no different in something as small as a fruit fly, where the immune system is necessary for resistance to all forms of pathogens – be it bacteria, fungi, or virus.

The initial line of the flies' defense against pathogens involve barriers against their entry, primarily in the form of epithelial cells. These cells present a physical barrier that pathogens must succeed in crossing prior to any infection taking hold. A second line of defense, also exterior, involves the use of antimicrobial peptides on the surface of these external barriers to kill off invaders which are present prior to their entry into the organism.

Once inside the flies' body, however, pathogens still face a staunch response from the immune system. Unique chemical structures present on the pathogen are used by the body to recognize the infection and activate cellular and humoral immune responses to kill and eliminate the invaders. Hemocytes, the flies' blood cells, are the effectors of the cellular response and act via phagocytosis, encapsulation, and melanization. While phagocytosis and encapsulation act to partition off a pathogen from the rest of the organism, and then kill it once this is done, melanization is the direct act of poisoning pathogenic organisms using toxic metabolic byproducts, such as reactive oxygen radicals.

Despite the extensive response from the antimicrobial peptides on the barrier epithelia and the internal cellular response, a third mechanism also is activated by pathogens once they breach the barrier protection. Activated similarly to the cellular response, by unique chemical structures present on pathogens, the humoral response involves the expression of antimicrobial peptides specific for an invading pathogen. These antimicrobial peptides not only act directly against microorganisms, punching holes in their membranes and disrupting ion gradients, but also by signaling to other parts of the immune response, specifically the cellular responses, to activate and aid in pathogen destruction.

These responses, however, are not as simple and clear cut as presented. Large amounts of research and study over many years have provided detailed information on how these responses function and how they have diverged from a common ancestral system once common to both insects and mammal ancestral species.

### 1.2.1 Barrier Epithelia

Similar to antimicrobial peptide expression on surface epithelia of mammals, *Drosophila* barrier epithelia that is in contact with the environment (i.e. the respiratory tract, oral region, digestive tract, and the reproductive tracts) are able to induce and produce antimicrobial peptides (AMPs) when activated by the presence of pathogens (Onfelt Tingvall *et al.*, 2001; Tzou *et al.*, 2000; Huttner and Bevins, 1999; Ferrandon *et al.*, 1998; Ganz and Lehrer, 1998). All AMPs which are produced in this fashion, however, are under the control of the IMD pathway – even those

regulated by the Toll pathway systemically. Both of these pathways are detailed below (Sections 1.2.4.1 and 1.2.4.2). The reason for this method of induction control is not yet fully understood.

### 1.2.2 Pathogen Recognition

Because of the generally invariable nature of the recognition aspect of the *Drosophila* innate immune system, which lacks the ability to generate novel pathogen detecting motifs via genetic reshuffling as is done by the classic acquired immune system, alternate methods of pathogen identification are used. Pathogen associated molecular patterns (PAMPs) are highly conserved molecular motifs found across multiple species and genera of microbes and these patterns can be used for the recognition of a diverse set of microbes by a limited number of pattern recognition receptors. Examples of PAMPs are the specific molecules which activate the *Drosophila* innate immune pathway in response to bacteria and fungi. Bacterial pathogens are known to activate an innate immune response by the binding of peptidoglycans to peptidoglycan receptor proteins (PGRPs) (Michel *et al.*, 2001), and lipopolysaccharides and lipoteichoic acid to gram-negative binding proteins (GNBPs) (Gobert *et al.*, 2003; Kim *et al.*, 2000). Fungal pathogens, similarly, activate an immune response by the binding of β-glucan to GNBPs (Brown and Gordon, 2005).

The *Drosophila* genome encodes 13 PGRP genes, some of which have splicing variants (Werner *et al.*, 2003; Liu *et al.*, 2001; Werner *et al.*, 2000). All PGRPs, both in mammals and *Drosophila*, share a 160 amino acid peptidoglycan

recognition domain. Additionally, mammals and *Drosophila* both possess secreted and transmembrane PGRPs (Kang *et al.*, 1998). Thus far, only secreted forms have been linked to recognition of gram-positive bacteria and only transmembrane forms to recognition of gram-negative bacteria (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002b; Michel *et al.*, 2001).

Gram-negative bacterial recognition in *Drosophila* is achieved by the PGRP-LC transmembrane peptidoglycan receptor. This receptor has two primary splice variants that share the transmembrane and signaling (cytoplasmic) domains (Werner *et al.*, 2003; Choe *et al.*, 2002). Gram-positive pathogens are recognized by the secreted PGRP-SA peptidoglycan receptor and GNBP1. PGRP-SA is composed of one peptidoglycan recognition domain that binds Gram-positive peptidoglycan (Michel *et al.*, 2001; Werner *et al.*, 2000). GNBP1, a GNBP/ $\beta$ -(1,3)-glucan recognition protein, exists as both secreted and glycosylphosphatidylinositol (GPI)-linked form in the cytoplasmic membrane. However, only the secreted form of GNBP1 is required for response to Gram-positive bacterial infection (Gobert *et al.*, 2003; Kim *et al.*, 2000). Additionally, the  $\beta$ -1,3-D-glucans present in fungi can induce antimicrobial gene expression in *Drosophila* (blood) cell lines via a  $\beta$ -1,3-D-glucan binding protein (Kim *et al.*, 2000; Samakovlis *et al.*, 1992).

### 1.2.3 Cellular Responses

In addition to the barrier mechanisms used to block infection, *Drosophila* also has an extensive set of cellular response mechanisms to respond to infection. These cellular responses are mediated by the *Drosophila* blood cells, or hemocytes. Three types of hemocytes take part in the *Drosophila* cellular response, each with a specific activity. Plasmatocytes, which account for approximately 90% of the total hemocyte population, are macrophage like cells and perform the majority of the cellular responses' phagocytic activity (Lanot *et al.*, 2001; Rizki and Rizki, 1984b). Phagocytosis is rapid and efficient, as is observed by the uptake of bacteria injected into a fly within minutes after injection (Elrod-Erickson *et al.*, 2000; Rizki and Rizki, 1984b). Crystal cells, the second type of hemocyte, produce the various compounds and enzymes required for melanization reactions (Lackie, 1988). Lamellocytes, the third type of hemocyte, are flattened cells which differentiate in response to select immune challenges and mediate encapsulation of larger infectious agents, such as parasites. (Lanot *et al.*, 2001; Elrod-Erickson *et al.*, 2000; Rizki and Rizki, 1984b) Similar to the inactivation of the humoral responses, inactivation or removal of hemocytes results in a severe compromising of the flies' immune response.

In addition to the above functions, hemocytes also mediate wound closure and clotting (Galko and Krasnow, 2004; Scherfer *et al.*, 2004; Ramet *et al.*, 2002a; Lackie, 1988). Also, they are required during certain types of infections for signaling

to the fat body to activate the humoral immune responses, similar to the cross signaling between humoral and cellular responses in vertebrates (Foley and O'Farrell, 2003; Bassett *et al.*, 2000).

### **1.2.3.1 Blood Cell Activation**

Blood cells are activated by multiple mechanisms which vary based upon the infectious agent being detected and what response is being initiated. One method of activation, which can initiate both phagocytosis and encapsulation/melanization, is via the same mechanisms which activate the humoral AMP pathways. Research by Ramet and co-workers, for example, shows that the PGRP-LC receptor takes part in activation and recognition for a phagocytic response to gram-negative pathogens (Ramet *et al.*, 2002b). Additionally, Toll or JAK-STAT signaling hyperactivity can result in blood cell proliferation and appears to link these signaling pathways to the cellular immune response as well (Qiu *et al.*, 1998). These data suggest a link between the humoral response activation pathways, specifically the activation of the humoral response via PAMP receptors, and initiation of various cellular phagocytosis responses.

In addition to signaling of this type, cellular immune responses in the absence of PAMPs also occur in *Drosophila*. Alternate mechanisms that indicate damage to normal cells, like defective basement membranes or the presence of endogenous DNA in the hemolymph, can also activate a cellular response. Transplantation experiments involving species and cross-species fat bodies find that *Drosophila* are

able to recognize cross-species fat bodies and initiates an encapsulation reaction against it. However, if same-species fat bodies are transplanted and have damage to the basement membrane, they are encapsulated as well (Rizki and Rizki, 1980). A similar encapsulation response is used by *Drosophila* larvae to recognize parasitic wasp eggs, which present no identified PAMPs because of their insect origin (Lanot *et al.*, 2001; Russo *et al.*, 1996). These both suggest that abnormal basement membrane patterns may be able to trigger immune responses in lieu of PAMPs.

Endogenous DNA has also been found to initiate immune responses in studies of apoptosis. Mutants which lack the function of the fly proapoptotic homologs of the Ced-3 caspase and Ced-4/Apaf-1 develop melanotic capsules (Rodriguez *et al.*, 1999; Song *et al.*, 1997). It is hypothesized that mutants in Ced-3, which is known to fragment DNA, trigger an immune response because of the release of unfragmented DNA from cells undergoing improper apoptosis. This activation results in an autoimmune reaction in these Ced-3 mutants (Song *et al.*, 1997). Additionally, mutant flies which lack two other DNases used during apoptotic degradation of cellular DNA show constitutively active expression of an antimicrobial peptide, specifically Diptericin (Mukae *et al.*, 2002). Both of these observations suggest that endogenous DNA stimulates the innate immune system in *Drosophila*, similar to endogenous DNA activation of the mammalian immune system (Napirei *et al.*, 2000). This form of immune system activation would theoretically allow for an immune response to occur even in the absence of a detectable PAMP being presented during an infection.

### **1.2.3.2 Melanization**

In addition to blood cell activation and the ensuing endocytosis of microbial pathogens, a melanization response is also elicited in response to pathogens in *Drosophila*. The melanization response is a rapid and localized immune defense mechanism triggered by wounding, invasion of foreign bodies, or selected PAMPs. The response functions via production of toxic metabolic byproducts, including multiple reactive oxygen species, which aid in killing of the infectious agents as well as sterilization the wound site. Without this response *Drosophila* are unable to respond effectively to infection, encapsulation is ineffective, and wound closure is compromised due to clotting defects as well as inability to limit introduction of infectious agents through the wound (Ramat *et al.*, 2002a; Braun *et al.*, 1998).

Melanization is mediated by the prophenoloxidase (PPO) pathway, a serine protease cascade which activates phenoxoxidase, the catalyst for melanin production, from its zymogen form (Ashida, 1998; Soderhall and Cerenius, 1998; Aspan *et al.*, 1995; Yoshida *et al.*, 1986). While not completely elucidated, the activation mechanism of the PPO pathway relies on the inactivation of Serpin27A (Spn27A) the inhibitor of the PPO-activating enzyme (PPAE) (De Gregorio *et al.*, 2002b; Ligoxygakis *et al.*, 2002c). This inactivation is believed to occur either via induction of a Spn27A inhibitor or increased production of PPAE, both of which would result in an increase in uninhibited PPAE and PPO pathway activation. This activation can be triggered by various PAMPs and data suggests that localized Spätzle-Toll

signaling may be responsible for induction of the inhibitor of Spn27A, resulting in a depletion of active Spn27A (Ligoxygakis *et al.*, 2002c; Takehana *et al.*, 2002; Yoshida *et al.*, 1986). This is supported by the co-localization of melanization and Toll based responses during septic infections (De Gregorio *et al.*, 2002b; Ligoxygakis *et al.*, 2002c).

#### 1.2.4 Humoral Responses

The best characterized humoral response of *Drosophila* consists of the rapid expression of a diverse array of antimicrobial peptides in the fat body. These antimicrobial peptides are clustered into general pathogen specific action groups (anti-Gram-Positive, anti-Gram-Negative, and anti-fungal), although some are effective against multiple pathogen types. AMP expression patterns vary based upon the specific pathogen being responded to. The AMPs are an essential component of the *Drosophila* immune response, as flies with mutations that take out all AMP expression have severely compromised immune responses. Also, similar to the antimicrobial peptides found in mammals, *Drosophila* antimicrobial peptides are also expressed on the surface epithelia which contact the environment (Onfelt Tingvall *et al.*, 2001; Tzou *et al.*, 2000; Ferrandon *et al.*, 1998). While the exact effector mechanism of many AMPs is unknown, some have been demonstrated to function via disruption of bacterial membranes (Meister *et al.*, 2000; Bulet *et al.*, 1999).

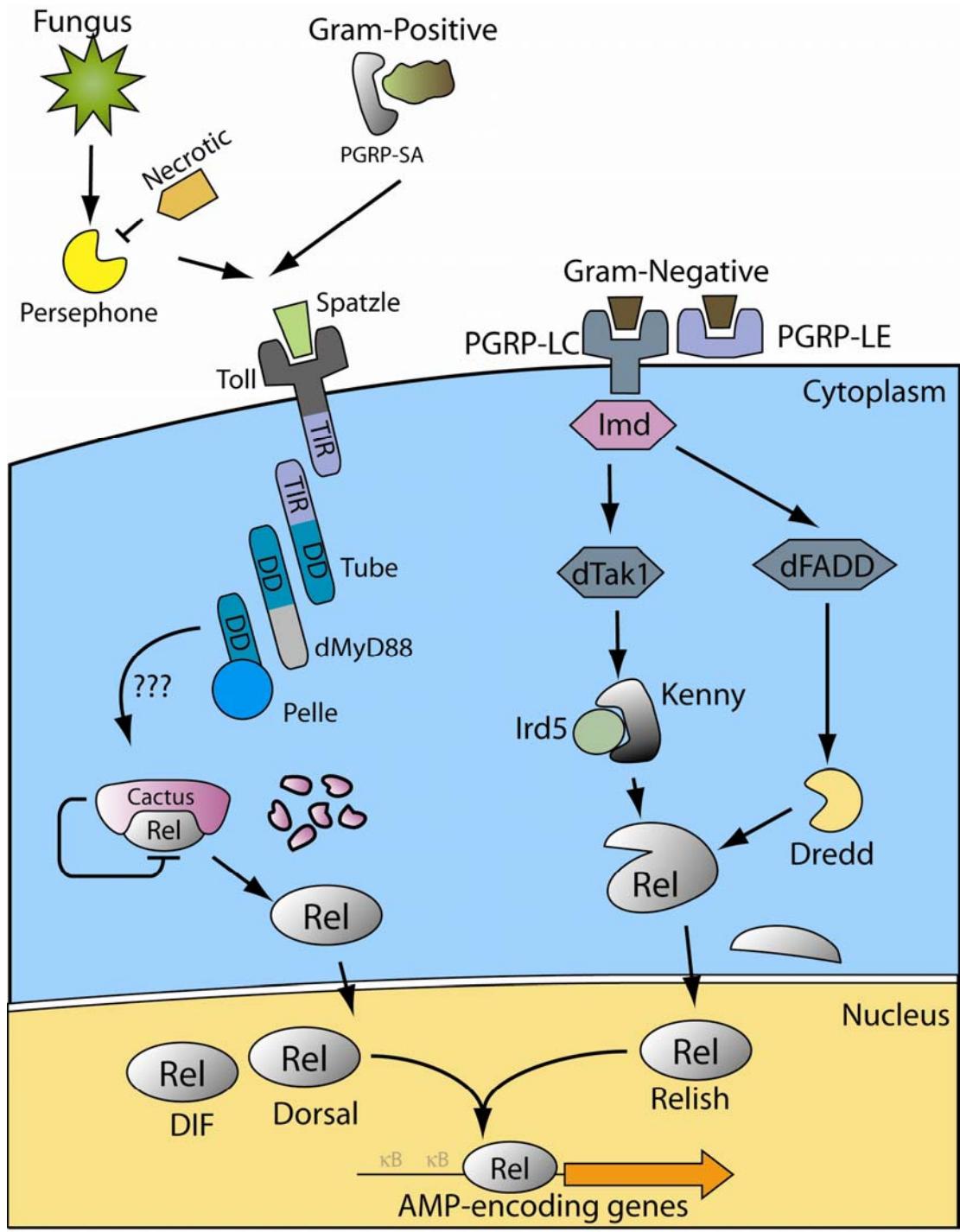
Study of these AMPs has led to the discovery and examination of the signaling pathways which regulate their expression. Two pathways, the Toll and IMD pathways, are the strongest linked to AMP expression in response to microbial infection at this time (Fig. 1.1). The Toll pathway is activated by Gram-positive and fungal pathogens, and the IMD pathway by Gram-negative pathogens. In addition to these two pathways, the JAK-STAT and JNK signaling pathways have been identified in recent years as inducing expression of immune specific genes during infection and playing a vital role in *Drosophila* immune responses.

#### **1.2.4.1 Toll Pathway**

The Toll pathway is activated in response to fungal and Gram-positive infections. Mutants that lack the ability to activate the Toll pathway are highly susceptible to these types of infection. Toll pathway mutants are, however, able to induce certain AMPs normally and can resist Gram-negative bacterial infections (De Gregorio *et al.*, 2002a; Tauszig-Delamasure *et al.*, 2002; Rutschmann *et al.*, 2000a; Meng *et al.*, 1999; Lemaitre *et al.*, 1996).

This pathway is activated in response to Gram-positive bacterial infections by recognition of Gram-positive peptidoglycan, by the secreted PGRP-SA protein (Michel *et al.*, 2001). Fungal activation of the Toll pathway is not as well understood, but some general information does exist. The serine protease Persephone has been found to take part in response against fungi, as mutants in the protein are

**Figure 1.1: Toll and IMD *Drosophila melanogaster* immune pathways.** The current model of Toll and IMD dependent induction of immune genes in fungal and Gram-positive bacterial infections (left-side) and gram-negative bacterial infections (right-side), respectively. In the Toll response, circulating pattern recognition proteins (PGRPs, GNBP<sub>s</sub>) cause proteolytic cleavage of the polypeptide Spaetzle, which then activates the Toll, which through a proteolytic cascade causes the degradation of cactus and nuclear translocation of the Rel transcription factor DIF. In the IMD response, membrane bound receptors (PGRPs) recognize gram-negative bacterial PAMPs and initiate another cascade resulting in the release and nuclear translocation of the Relish transcription factor. Once translocated to the nucleus, these transcription factors induce the expression of AMP encoding genes in a pathogen specific manner.



susceptible to fungal infection. Gram-positive bacteria can activate the Toll pathway without the presence of Persephone however, which suggests that Persephone represents a distinct signaling mechanism for fungal recognition (Ligoxygakis *et al.*, 2002b). It is unknown how fungal infection activates Persephone, but it is believed to be tied to the serine protease inhibitor Necrotic. Necrotic prevents constitutive activation of the Toll-dependent immune responses in the absence of infection, and may directly inhibit Persephone (Robertson *et al.*, 2003; Ligoxygakis *et al.*, 2002b). It is not known if Persephone is able to directly activate Spätzle or induces a secondary proteolytic cascade which results in Spätzle activation.

Both recognition events trigger a serine protease cascade which results in the proteolytic activation of the ligand Spätzle. Once activated, Spätzle binds to the transmembrane receptor Toll and likely causes the dimerization of Toll receptor molecules (Weber *et al.*, 2003). This dimerized receptor then induces the recruitment of a complex containing the adapter protein dMyd88 (a homolog of the human Myd88 protein), IRAK-like kinase Pelle, and the adaptor protein Tube (Sun *et al.*, 2002). Each of these three proteins contain a death domain (DD) (Sun *et al.*, 2002). Toll and dMyd88 interact through their Toll/Il-1 Receptor (TIR) domains, while Tube interacts with dMyd88 and Pelle through its death domain (Sun *et al.*, 2002). Pelle and dMyd88 do not directly interact (Sun *et al.*, 2002). The formation of this complex activates the Pelle kinase which then causes the hydrolysis of the I<sup>-</sup>B-like Cactus, an inhibitor of the NF-κB/Rel transcription factor family (Braun *et al.*, 1997). Of the three Rel proteins in the *Drosophila* genome, only two function in the Toll pathway, Dorsal-related immunity factor (DIF) and Dorsal. Relish, the third of these

Rel proteins is part of the IMD pathway, which is described below (Hedengren *et al.*, 1999). The hydrolysis of Cactus releases the two Toll pathway NF- B like transcription factors, allowing them to translocated to the nucleus (Weber *et al.*, 2003; Imler and Hoffmann, 2002; Tauszig-Delamasure *et al.*, 2002; Levashina *et al.*, 1999; Nicolas *et al.*, 1998; Wu and Anderson, 1998; Belvin and Anderson, 1996; Lemaitre *et al.*, 1996; Ip *et al.*, 1993; Schneider *et al.*, 1991).

Similar to the numerous Toll-like receptors in humans, *Drosophila* has been found to have at least nine Toll receptors. Of these nine, however, only two have thus far been significantly implicated in immune responses via genetic screens for immune system alterations. This is due, in part, to the lack of mutant fly strains available for some of the Toll receptors. In addition to Toll (dToll1), mentioned above, the Toll receptor 18Wheeler (dToll2) has been implicated in antibacterial action but there is some debate as to its role (Ligoxygakis *et al.*, 2002a; Williams *et al.*, 1997). dToll5 has been found to activate Drosomycin in S2 cells in examinations with a chimeric constitutively active form of the protein (Luo *et al.*, 2001; Tauszig *et al.*, 2000). The wild type form of dToll9 was also found to constitutively activate Drosomycin in S2 cells, even in the absence of Toll pathway activation (Ooi *et al.*, 2002). Morphogenetic disruption has also been observed in other Toll receptors not yet linked to immune function, suggesting that the *Drosophila* Toll receptor family consists of two sub-families with varying functions (Kimbrell and Beutler, 2001).

#### **1.2.4.2 IMD Pathway**

Original studies and studies of mammalian immunity suggested that lipid polysaccharide (LPS) was the PAMP responsible for recognition of Gram-negative bacteria in *Drosophila*. This was because LPS was a component of the bacteria's outer membrane, unlike peptidoglycan which is placed in the inner cell wall of Gram-negative bacteria where it was protected from possible receptor binding. It was found, however, that LPS does not induce the *Drosophila* humoral response *in vivo* (Kaneko *et al.*, 2005; Leulier *et al.*, 2003; Schleifer and Kandler, 1972). Work by Leulier and co-workers also found that the *Drosophila* humoral response is able to discriminate between gram negative and gram positive peptidoglycan – which vary only by one amino acid residue in their peptide region (Leulier *et al.*, 2003). This discovery clarified the PAMP responsible for activation of the IMD pathway.

The IMD pathway, activated by recognition of Gram-negative bacteria by the PGRP-LC transmembrane receptor protein, induces AMP production in the fat body. Additionally, the IMD pathway bear resemblance to the mammalian TNF- $\alpha$  pathway (Hultmark, 2003; Hoffmann and Reichhart, 2002; Khush *et al.*, 2001). *Drosophila* possesses 2 splice variants of this receptor protein, both of which share transmembrane and cytoplasmic domains, but have varying extracellular recognition domains (Werner *et al.*, 2003; Choe *et al.*, 2002). It should be noted that overexpression of PGRP-LE, a secreted molecule, is also able to induce the IMD pathway, suggesting that there are multiple receptors which activate the IMD pathway (Takehana *et al.*, 2002). PGRP-LE loss of function mutants have shown that the

molecule has non-cell autonomous effects on activation of the IMD pathway antibacterial peptide genes and acts synergistically with PGRP-LC (Takehana *et al.*, 2004).

Once PGRP-LC is activated, signaling occurs by via interaction with the Imd protein, which is similar to the mammalian receptor-interacting protein RIP (Choe *et al.*, 2005; Georgel *et al.*, 2001). This Imd/PGRP-LC interaction is hypothesized to induce a complex which contains the *Drosophila* Fas-associated DD (dFADD) and death-related ced-3/NEDD2-like protein (DREDD). This complex has a caspase activity which activates *Drosophila* transforming growth factor- $\beta$  (TGF-  $\beta$ ) – activated protein kinase 1 (dTak1), a mitogen-activated protein kinase kinase kinase (MAPKKK). dTak1 then activates via phosphorylation an Ik $\kappa$  kinase (IKK) complex containing Ird5, an Ikk- $\alpha$  homolog, and Kenny, an Ikk- $\beta$  homolog. (Lu *et al.*, 2001; Rutschmann *et al.*, 2000b; Silverman *et al.*, 2000b). This activated IKK complex then phosphorylates Relish, the NF-  $\kappa$ B which is further processed by proteins of a second branch of the IMD pathway. This second pathway involves DREDD which has been shown to associate with Relish (Stoven *et al.*, 2003). However, no evidence exists that shows the direct proteolytic activity of DREDD on Relish. Because DREDD is homologous to caspase 8, an activating caspase and not an effector protease, DREDD is believed to recruit and activate a downstream caspase instead of directly cleaving Relish. This recruited caspase then cleaves Relish into its active form, either directly or through other intermediaries.

#### **1.2.4.3 JAK-STAT Signaling**

As research continues into the immune system of *Drosophila melanogaster*, new immune functions of identified pathways are constantly being uncovered. One of these pathways, which also has a homologous pathway in mammals, is the JAK-STAT signaling pathway. JAK-STAT (JAK: Janus Kinase, STAT: signal transducers and activators of transcription) signaling is used by *Drosophila* to induce gene expression in the fat body in response to infection. Blood cells also activate JAK-STAT signaling during infection by producing Upd3, a cytokine-like protein, which signals through the Domeless receptor and the JAK-STAT pathway to activate transcription of *totA* (Agaisse *et al.*, 2003). Among the induced genes are *totA*, a stress induced gene of unknown function, and *Tep1*, a thiolester-containing protein that possibly acts as an opsonin (Agaisse *et al.*, 2003; Boutros *et al.*, 2002; Lagueux *et al.*, 2000). JAK-STAT signaling has also been implicated in activation of blood cells and as a possibly mechanism for viral resistance in *Drosophila* (Dostert *et al.*, 2005; Harrison *et al.*, 1995). It has been observed that JAK-STAT target gene expression during an immune response is generally delayed and transient in comparison to the Toll and IMD responses, although the reason why this is so is unclear (Boutros *et al.*, 2002).

#### **1.2.4.4 JNK Pathway**

As with the JAK-STAT pathway, the Jun N-terminal kinase (JNK) pathway has been linked to *Drosophila* innate immunity. Studies suggest that the JNK pathway may play a role in induction of *Drosophila* immune related genes. Genetic studies of homozygous adults are difficult, however, because of the early developmental event disruption, and resulting embryonic lethality, of null JNK pathway mutants (Noselli and Agnes, 1999). The JNK pathway is known, however, to control the rapid upregulation of cytoskeletal genes in response to infection in S2 cells (Boutros *et al.*, 2002). Additionally, a hypomorph of DFos, one of the transcription effectors of the JNK pathway, has been found to inhibit wound healing (Ramet *et al.*, 2002a). These data suggest the use of the pathway in *Drosophila* immunity.

#### **1.2.5 Antiviral Immune Responses**

Not much is known about the viral immune response of *Drosophila*, particularly when compared to the amount of research accomplished thus far into the *Drosophila* antifungal and antibacterial responses. The majority of work thus far has centered on two viruses, *Drosophila X virus* and *Drosophila C virus* (DCV). As DXV is a focus of this work and will be covered in greater detail in the next section?, only studies in DCV are reviewed here.

DCV is a non-enveloped single-stranded RNA virus which belongs to the picornavirus family, which also includes polio and foot-and-mouth disease virus. DCV infection causes death in adult flies within 4-10 days of injection, dependent upon the dose of virus administered (Dostert *et al.*, 2005; Cherry and Perrimon, 2004). DCV is also able to infect and cause the death of *Drosophila* cell culture cells (Cherry and Perrimon, 2004). DCV has also been shown to rely on endocytosis for cell entry and then host ribosomal machinery, utilizing internal ribosomal entry sites (IRESs) for translation and replication (Cherry *et al.*, 2005). It should be noted that DCV has not been shown to be a natural pathogen of *Drosophila* and a natural transmission route is still unclear (Roxstrom-Lindquist *et al.*, 2004).

Recent work using an RNAi-based microarray screening system to knock out various *Drosophila* genes and examine the effects upon DCV infection of *Drosophila* cells has found that approximately 140 genes are upregulated during DCV infection (Dostert *et al.*, 2005). Of these, only one third overlap the 230 genes which are upregulated during bacterial and fungal infections (De Gregorio *et al.*, 2001). Additionally, AMP genes are not induced by the virus.

Examination into these genes induced by DCV infection found that a few of these genes contained STAT binding motifs in their promoter regions indicating that the JAK-STAT pathway might be important in an antiviral response in *Drosophila* (Dostert *et al.*, 2005). The mammalian JAK-STAT pathway mediates cytokine signaling and T cell activation and is important for antiviral responses (Dupuis *et al.*, 2003; Karst *et al.*, 2003). Dostert et al. found that STAT binding activity from fly extracts was induced by DCV infection. Additionally, the STAT binding sites were

vital for virus-induced transgene activity. Also, over expression of a dominant negative version of the Domeless receptor and the negative regulator PIAS led to the modulation of a virus induced STAT-regulated target gene (*vir-1*). Lastly, the Hopscotch mutant fly line, a JAK loss-of-function mutation, displayed a decrease in *vir-1* expression and increased susceptibility to DCV infection. The role of *vir-1*, the STAT target gene, however, remains unclear. The protein is expressed mainly in non-infected tissues and has no antiviral activity, suggesting that the protein is either signaling or may only be induced because it contains a STAT response element. Even if *vir-1* is not directly active, however, the results do suggest that the JAK-STAT pathway is being activated during DCV infection and playing a role in antiviral response.

### 1.3 RNAi

RNA inhibition, known to be important in antiviral responses in plants, has begun to be examined in detail as a possible antiviral mechanism in various animal models. RNA inhibition, also known as Post-Transcription Gene Silencing, RNA silencing, RNA interference, and multiple other names depending on the species in question, was initially discovered in plants during experiments with transgenic petunias (Jorgensen, 1990). It has taken over a decade from that initial discovery to elucidate the mechanism behind this silencing phenomenon, where dsRNA is processed into enzyme complexes where it directs mRNA degradation or transcriptional inhibition (Fire, 1999). During this time, massive research has been

performed on the pathway and it has developed into one of the most complex and multifunctional pathways discovered – playing a role in aspects of development, gene regulation, immune defense, and others (Kim, 2005; Kuttenkeuler and Boutros, 2004; Montgomery, 2004).

One major aspect of RNAi being examined in depth are antiviral immune responses mediated by the RNAi pathway. These responses vary in understanding from the well understood role for RNAi in the genomic and mRNA degradation of dsRNA viruses to the unknown effects, if any, on dsDNA viruses. Even with extensive research and development, the interferon response present in mammalian model systems limits the ability to examine the specific RNAi response against a virus infection *in vivo*. Specifically, introduction of large (>30bp) dsRNA fragments into mammals results in abrupt and lethal induction of the interferon response, drastically limiting the ability to use unmodified mammalian systems for RNAi studies. Additionally, no system had yet been developed which relied on the organism's endogenous RNAi response to elicit an antiviral RNAi response, relying primarily on cell culture systems instead.

Because of lack of development and exploration into an animal model system, as well as the lack of information on antiviral responses of *Drosophila*, we expanded upon our initial *Drosophila* antiviral response research to include responses elicited via the RNAi pathway. Through use of systems developed in our initial examinations of the *Drosophila* immune systems response against viruses, we examined various mutants in the RNAi pathway to determine effects on viral susceptibility and viral replication *in vivo*. This research led to the discovery of an *in vivo* system in which

antiviral RNAi is occurring and provides the first model system of an antiviral RNAi response *in vivo* that does not rely on the introduction of foreign nucleic acids, only the virus itself.

### **1.3.1 Biological Functions of RNAi**

While RNAi was initially thought to simply be a method of silencing genes, it accomplishes other biological functions as well. In both *C. elegans* and *Drosophila*, RNAi pathway homologs have been implicated in the control of mobile genetic elements which possess an RNA intermediate phase. In *Drosophila*, for example, the loss of the RNA helicase SpindleE/Homeless causes a loss of gene silencing and an increased mobility of retrotransposons in the germline (Aravin *et al.*, 2001).

RNAi has also been found to play a pivotal role in viral immunity, particularly in plants. Alteration in certain genes in the RNAi pathway causes dramatically increased sensitivity to viral infection. Also, multiple viruses – both plant and animal – are found to encode inhibitors of RNAi, suggesting the need to overcome such a response for effective viral reproduction (Reed *et al.*, 2003; Li *et al.*, 2002; Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998).

RNAi has also been extensively linked to organism development in multiple species, ranging from plants (*Arabidopsis*) to insects (*Drosophila*) to mammals (mice). A third class of small RNAs has also been defined which includes RNAs that control developmental events – short temporary RNAs (stRNAs). These RNAs are processed into small RNAs in a similar manner as mi- and si- RNAs.

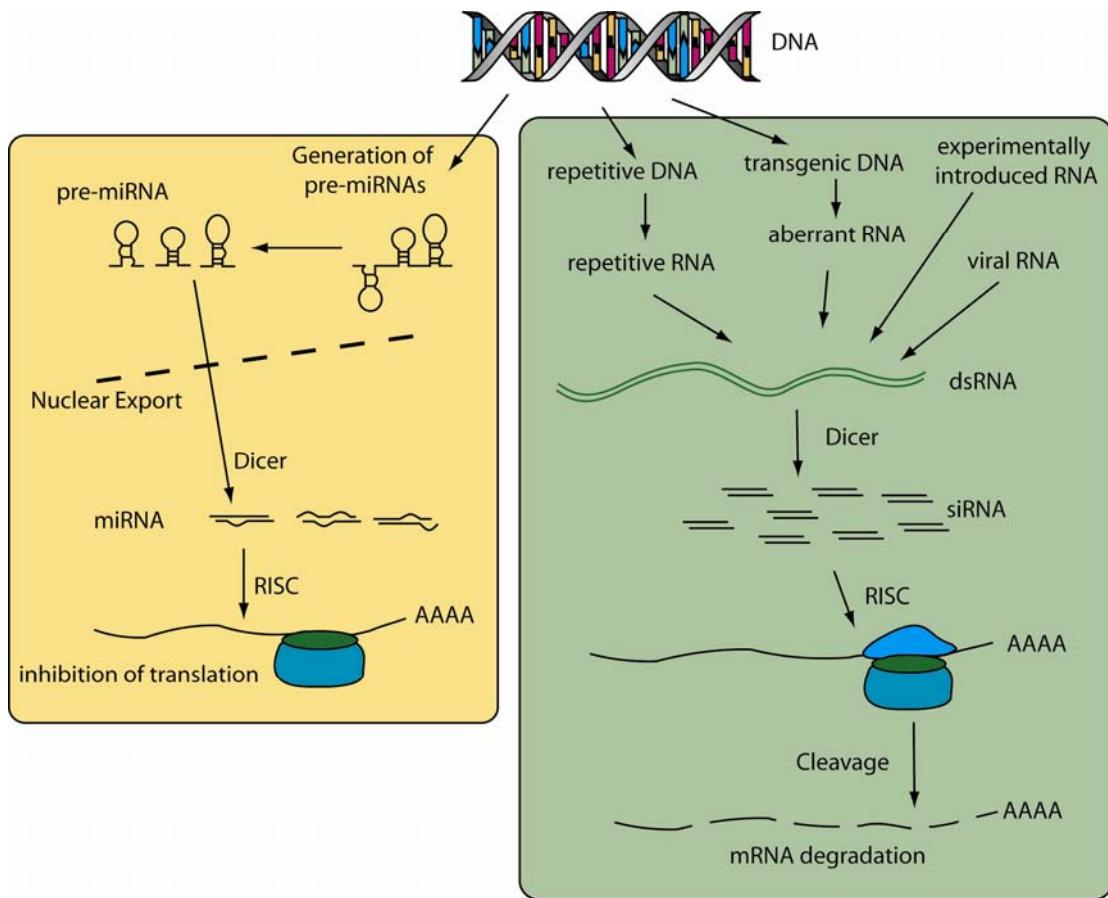
Heterochromatic formation, and therefore mitosis and meiosis, also appear linked to RNAi functions. In fission yeast, loss of RNAi function seems to cause disruption in heterochromatic formation regulation. Because certain meiotic genes are controlled via RNA-mediated heterochromatic formation, loss of RNAi genes causes the deregulation of these genes and the subsequent deregulation of meiosis (Schramke and Allshire, 2003).

The mechanism by which these RNAi responses occur has been extensively researched recently, and a general mechanism showing how RNAi occurs in two distinct phases has emerged. During the first phase of RNAi, the dsRNA source material, which provides the targeting mechanism of the later execution phase of RNAi pathway action, is processed into 21-23bp dsRNA fragments. These fragments are then used in the second phase of RNAi, which entails the execution of the silencing mechanism specific for the type of RNAi which is occurring. Both phases are detailed more specifically below.

### **1.3.2 Initiation Phase**

The initiation phase of RNAi involves the cleavage of dsRNAs into the 21-23bp dsRNA fragments used in RNAi execution (Hammond *et al.*, 2000; Zamore *et al.*, 2000; Tuschl *et al.*, 1999) (Fig. 1.2). The source dsRNA for RNAi can arise from endogenous or exogenous sources. Endogenous sources of dsRNA include short hairpin RNAs produced by the genome, aberrantly expressed transgenes, and

**Figure 1.2: RNAi pathway.** RNAi occurs by two similar paths, different in their source of dsRNA and their effector mechanism. In miRNA based RNAi (yellow, left side of figure), dsRNA is generated via transcription from an organisms genome and are processed into pre-miRNAs within the nucleus. pre-miRNA fragments are then exported to the cytoplasm and processed into miRNAs similarly to siRNAs but via an alternate Dicer enzyme (Dicer1). miRNAs contain a small number of mismatched based pairs. miRNA then incorporates into a RISC complex and effects the miRNA targeted mRNA. Because of the mismatches the mRNA has translation inhibited, but is not degraded. In siRNA based RNAi (green, right side), dsRNA derive from multiple sources but not for specific endogenous gene regulation. This long dsRNA is found in the cytoplasm and processed into siRNAs by the Dicer2 RNase. siRNAs are completely complementary to their targeting gene and when incorporated into a RISC cause the degradation of the targeted mRNA because of this.



transposons. Exogenous sources of dsRNA include natural sources, such as RNA viruses, and experimentally produced sources, both precleaved dsRNAs (21-23bp) and longer dsRNAs (>23bp).

During the initiation phase, Dicer, a conserved member of the RNase III gene family, cleaves the source dsRNA into 21-23bp. The Dicer protein contains a C-terminal dsRNA binding domain, an N-terminal RNA helicase domain, and two RNase III-like domains. The enzymatic activity of Dicer generates 21-23nt long dsRNAs with 3' overhanging ends (Blaszczyk *et al.*, 2001). The length is believed to be a byproduct of the presence of the two RNase III domains in the protein, one of which is believed to be inactive and acting as a spacer domain. This is supported by the fact that single domain bacterial RNase III enzymes cleave dsRNA into 11nt fragments (Blaszczyk *et al.*, 2001). Dicer homologs have thus far been identified in select yeasts, plants, *C. elegans*, *Drosophila*, mice, and humans (Bernstein *et al.*, 2001). However, no Dicer homolog (or any other RNAi pathway homolog) have been found in *S. cerevisiae*, *Archae*, and *Eubacteria* (Aravind *et al.*, 2000), suggesting development of the RNAi system after evolutionary divergences occurred separating these groups and the aforementioned groups of organisms.

### 1.3.3 Execution Phase

Once generated, siRNAs are incorporated into the RNA induced silencing complex (RISC) which is responsible for the subsequent action of the RNAi pathway (Nykanen *et al.*, 2001; Hammond *et al.*, 2000). During incorporation, the double-stranded siRNA is first unwound, and the antisense strand is incorporated into the RISC complex. (Schwarz *et al.*, 2002). This incorporated strand then acts as the targeting mechanism for the complex and directs its action to complementary mRNA. Additionally, microRNAs (miRNAs) can also associate with the RISC complex. These small RNAs are primarily present as a single-stranded RNA hairpin structure and regulate translation rather than induce mRNA degradation. These RNAs are believed to be encoded by mono- and polycistronic messages, transcribed by RNA polymerase II and III, and processed within the nucleus into pre-miRNAs. Once processed, the 60-70nt long pre-miRNAs form stem-loop structures (hairpins) which contain various bulges and loops because of mismatches present in their sequence (Pasquinelli, 2002). These pre-miRNAs are exported to the cytoplasm and processed into mature miRNAs which are of similar size to siRNAs (20-25 nt). This processing occurs via a similar mechanism to siRNAs, but evidence suggests that an alternate Dicer protein mediates this cleavage. Unlike siRNAs, miRNAs are primarily complementary to the 3' UTR sequences of target mRNAs and depending upon sequence identity can induce degradation (as that induced by siRNAs) or simply block translation (via an unknown mechanism).

Much of the RISC complex itself has not yet been identified and the only members identified so far are Argonaute and the related Argonaute protein family members Fragile X mental retardation protein (FMRP), the Vasa intronic gene (VIG), and Tudor-SN. The Argonaute protein family, also called PAZ-Piwi-Domain (PPD) proteins, are characterized by the presence of a PAZ domain and a C-terminal PIWI domain (Cerutti *et al.*, 2000). While the functional activities of these domains are unknown, it is believed that they may serve in protein to protein interactions. Members of the Argonaute family have been found to play a role in RNAi in multiple species, including *Drosophila*, *C. elegans*, *Arabidopsis*, and *Neurospora crassa*. It should be noted that Argonaute proteins are also involved in other cellular functions and do not function solely as members of the RNAi pathway and RISC complex.

The other three identified members of the RISC complex are suspected to play a role in the RNA-binding and nuclease functions of the RISC complex. It is known that FMRP and VIG complex with Tudor-SN and have RNA-binding properties (Caudy *et al.*, 2003). Additionally, Tudor-SN is related to micrococcal nucleases although this activity has not been shown to be the functional activity of the RISC. Recent studies also show a secondary nuclease activities of the RISC which are not believed to play a role in its RNAi function whatsoever (Schwarz *et al.*, 2004).

## 1.4 Drosophila X Virus

### 1.4.1 Discovery of Drosophila X Virus

Drosophila X virus (DXV), a viral pathogen of *Drosophila melanogaster*, was discovered during observations by Teninges and co-workers involving serial passages of Sigma virus in *Drosophila*. The rhabdovirus sigma causes acute anoxia sensitivity in *Drosophila* which results in the flies' death after 15 minutes of exposure to an anoxic environment, whereas uninfected flies can survive extended periods of anoxia (L'Heritier, 1958). During passages of the virus in adult flies, a group of control flies displayed anoxia sensitivity similar to that observed in flies infected with Sigma virus. Using negative contrast microscopy, the group found that the control flies displaying these symptoms contained no rhabdovirus, but instead a large volume of unidentified icosahedral particles. Further investigations revealed the presence of similar particles in various *Drosophila* cell lines. Because of the initial lack of information on the virus, and any relationships it might have with a virus family, Teninges and co-workers named the virus Drosophila X virus (Teninges *et al.*, 1979b).

The original source of DXV is unknown, but it has been suggested that DXV could have been introduced via calf serum during cell culture procedures and subsequent injections of culture derived material into flies. This is supported by the fact that no isolations of DXV have yet been obtained from *Drosophila* obtained in the field. However, while experiments passaging bovine serum through *Drosophila* resulted in the recovery of some non-replicating virus particles morphologically and

serologically identical to DXV, replication of DXV in any examined vertebrate system (calf kidney cells, chick embryo fibroblasts, human oral squamous cell carcinoma cells, HeLa, baby hamster kidney cells, Vero cells, suckling mouse brain cells) has not yet been observed. This suggests the virus is not derived from a bovine origin or if it is, it has evolved drastically to propagate in an invertebrate host, so much so that it can no longer replicate in a vertebrate system.

#### **1.4.2 Nomenclature and Classification**

Drosophila X virus (DXV) is a member of the family *Birnaviridae* and the prototype of the *Entomobirnavirus* genus. *Entomo* arises from Greek *entomon* meaning “insect” and signifies host range. *Bi* comes from the Latin prefix *bi*, meaning “two”, and signifies the bisegmented nature of the viral genome as well as the presence of dsRNA. *Rna* is from *ribo nucleic acid* which indicates the nature of the viral genome. The *Birnaviridae* family contains two other genera, genus *Avibirnavirus* (type species: Infectious bursal disease virus, IBDV), and genus *Aquabirnavirus* (type species: Infectious pancreatic necrosis virus, IPNV, also the prototype virus for the *Birnaviridae* family) (Dobos and Roberts, 1983). DXV is currently the only member of the *Entomobirnavirus* genus and only one serotype has been classified to date.

### **1.4.3 Epidemiology of *Drosophila* X Virus Infection**

DXV infection causes mortality in infected flies at a rate linearly related to the log of the DXV dose concentration (Teninges *et al.*, 1979b). Infection also causes sensitivity to oxygen deprivation, which is not normally observed in *Drosophila melanogaster*. This sensitivity appears 4 to 12 days prior to viral induced death of infected flies. The variation in symptom onset may be the result of variation between viral strains which have not as of yet been identified and typed, or variations in the immune responses between infected individuals. The oxygen deprivation sensitivity, or anoxia sensitivity, was initially detected by Teninges and co-workers via CO<sub>2</sub> tests, but was later found to be specific to oxygen debt (anoxia) and not CO<sub>2</sub>-sensitivity since pure nitrogen tests caused similar mortality results (Teninges *et al.*, 1979b).

Initial studies of DXV show that contact transmission of the virus is possible. Additionally, flies were found which were infected with virus but remained symptomless. After contact transmission, symptoms of infection – specifically anoxia sensitivity – are not observed until 20 days post transmission. At this point, the infection induced death of flies begins to occur on a similar time line as that of natural death in flies (Teninges *et al.*, 1979b).

During infection, virions have been observed spreading throughout the infected organism. Studies with ultrathin and paraffin sections show that at approximately the time of anoxia sensitivity onset, the gut, trachea, and muscle sheath cells are primarily affected, with other tissues showing low to undetectable levels of DXV antigen (Zambon *et al.*, 2005b; Teninges *et al.*, 1979b). This early invasion of

trachea cells may be what induces the anoxia sensitivity as a primary symptom of infection. As infection progresses, however, no tissue specificity was maintained as the virus propagated throughout the entire infected organism (Zambon *et al.*, 2005a). The highest intracellular concentrations of virus are often observed at the cell periphery just under the plasma membrane (Teninges *et al.*, 1979b).

#### 1.4.4 Viral Replication

The replication cycle of birnaviruses is not well understood, due primarily to their failure to block host cellular transcription and translation. This makes it difficult to label newly synthesized RNA and proteins. What is currently known has been elucidated by research into Infectious Pancreatic Necrosis Virus (IPNV) the prototype virus of the *Birnavirus* family. The birnavirus replication process consists of a number of discrete steps. First, the virus attaches to and enters the host cell. After entry, the virus initiates transcription and replication. Finally, virus particles are assembled and mature viruses are released from the host cells via lysis (Marsh and Helenius, 1989).

Little is known about the initial entry steps of birnaviruses. In IPNV both specific and non-specific binding between viral particles and host cells have been observed. Specific binding, however, is the only method by which a productive infection has been shown to occur (Kuznar *et al.*, 1995). No host receptors for birnaviruses have yet been discovered, in any species. The closest protein which has been found thus far is an unidentified 250-kDa protein which a marine birnavirus can

bind to and may serve as a common receptor for entry of *Aquabirnaviruses* to bind and enter various fish cell lines (Imajoh *et al.*, 2003). Entry itself seems to occur by receptor mediated endocytosis, similar to that of other non-enveloped viruses (Granzow *et al.*, 1997; Kuznar *et al.*, 1995).

All birnavirus replication appears to takes place in the cytoplasm and no viral components enter the nucleus. The time course of a single cycle of replication varies upon viral serotype and host, and has not yet been discerned for DXV. The extent of processing of the virion prior to transcription initiation is currently unknown, but *in vitro* data provides some hints. Data shows that birnavirus particles become transcriptionally active without any proteolytic pretreatment or degradation of the virion *in vitro* (Spies *et al.*, 1987; Cohen, 1975). This suggests that unlike reovirus, which also contains a dsRdRp, the birnavirus virion is not uncoated and instead functions as a replicative core without proteolytic or degredadative alteration (Skehel and Joklik, 1969). Viral particles initiate transcription and replication with minimal to no processing after entry into the cell, but the full extent of processing prior to transcription initiation is unknown.

The fact that IPNV's RdRp is transcriptionally active without any proteolytic treatment of the viral particles demonstrates that precursor molecules are able to reach the transcriptional complex through the shell of the capsid and initiate transcription (Becht, 1994).

#### **1.4.5 Virus Release**

There has been little research done in *avi-* and *aqua- birnaviruses* to determine the causes of apoptosis and lysis of infected cells – and even less in DXV. While evidence exists that IPNV can induce apoptosis in select cell types of fish, most evidence comes from *in vitro* cell culture studies. In DXV, only two pieces of research lend themselves to clarify this event. First, we find that apoptotic cell death inhibitor protein p35 has little to no effect on viral infection (Zambon, unpublished). Second, Contamine and co-workers observed that DXV is able to reach such high concentration in infected cells that it forms crystals (Teninges *et al.*, 1979b). This suggests that the cells are lysing solely due to sheer volume of DXV present within. No additional data exists on this in relation to DXV at this time.

#### **1.4.6 Persistent Infection**

Persistent infections are characterized as those in which the virus is not cleared but remains in specific cells of infected individuals. Once induced, a persistent infection may involve periods of both silent and productive infection without associated host cell death or host cell damage. It was noted during the initial studies of DXV that the virus was able to induce a persistent infection in cell culture (Teninges *et al.*, 1979b). A similar effect may be happening in infected adult flies, as some infected individuals not exposed to anoxia can survive for periods of time similar to the normal lifespan of an adult *Drosophila*.

Once infected with virus, *Drosophila* cell cultures are found to exhibit classic signs of viral infection – cytopathic effects of cell rupturing, distension of the cell membrane, and slowing of cell division. After a period of time, however, these effects subside and the cell culture will grow back up to normal cell density and display no further adverse effects aside from slightly slowed cell growth and replication. These cells continue to present DXV antigen, however, suggesting that the virus is still present within the culture.

Persistent infection of S2 cells can be detected via Western Blot, Quantitative PCR, Immunostaining, and Electron Microscopy, but is not observable under normal light microscopy conditions. Additional infection with DXV after a culture is in a state of persistent infection induces no increase in viral titers and no cytopathic effects. Because of this, it is important that extreme care is taken when handling DXV in relation to cell culture because of the possibility of unknown persistent infections occurring and not being detected until it is too late. There is no known way to completely clear a DXV infection once a culture is infected.

Similar persistent infections are observed in *Drosophila* cells when infected with Sigma Virus (Ohanessian, 1971), Vesicular Stomatitis Virus (Wyers *et al.*, 1980), or Sindbis virus (Bras-Herreng, 1975). Also, Togaviruses (Igarashi *et al.*, 1977; Esparza and Sanchez, 1975; Davey and Dalgarno, 1974; Singh, 1972) and rhabdoviruses (Artsob and Spence, 1974) can induce the same persistent infection type in mosquito cells. The diversity of these virus groups, and the similarity of their infection results, suggests that there is a viral replication control or immune process

common to these Dipteran cells which is causing the occurrence of this persistent infection as opposed to persistence of the high levels of virus replication observed early during infection and the death of all cells within a culture (Teninges *et al.*, 1979b).

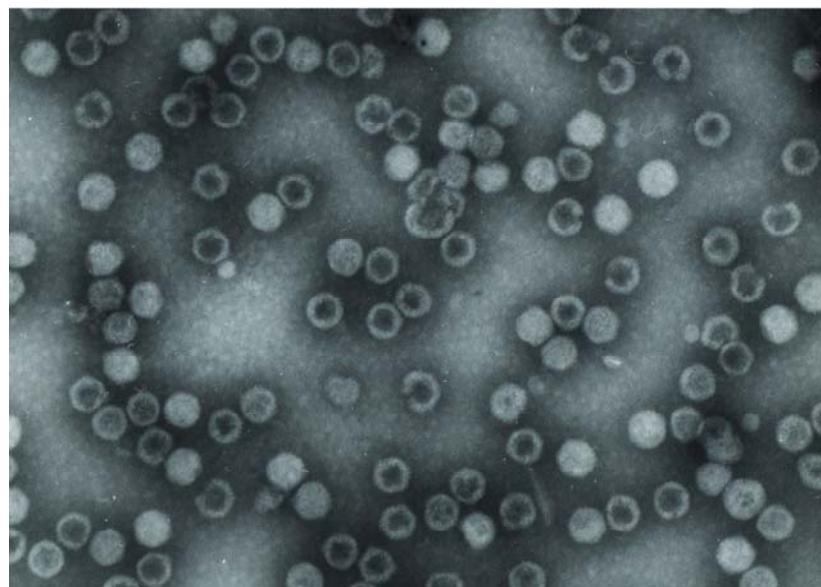
#### **1.4.7 Virion**

The virion of DXV is a non-enveloped, single shelled icosahedron, approximately 59nm in diameter (Fig 1.3). The capsid consists of 92 capsomers and a theorized icosahedral pattern of T=9. The molecular weight of the virion is  $81.2 \times 10^6$  daltons.

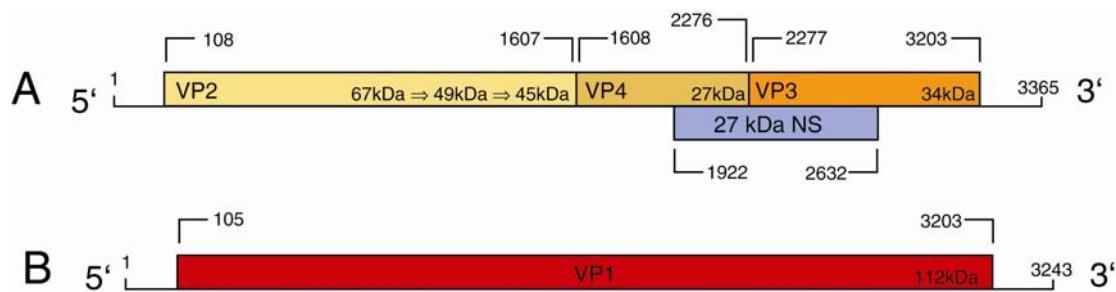
#### **1.4.8 DXV Genome Structure and Organization**

The dsRNA genome of DXV is composed of two segments, segment A and segment B (Fig. 1.4). Segment A is the larger of the two segments (3365 bp) and encodes two overlapping open reading frames (ORFs). The larger ORF encodes a 128 kDa precursor protein which is cleaved during translation by the viral protease VP4 (nonstructural, 27 kDa) into the major capsid proteins preVP2 (outer capsid, 67 kDa) and VP3 (inner capsid, 34 kDa). preVP2 is further cleaved into pVP2 (49 kDa) and then VP2 (45 kDa) during virus maturation. It is believed that cellular proteases are responsible for this cleavage and not VP4, based on data from other birnaviruses. The second ORF is in the -1 reading frame relative to the main ORF of segment A

Figure 1.3: **Electron micrograph of Drosophila X Virus particles.** The capsids are approximately 59nm in diameter. Hollow particles are defective capsids which are empty and lacking genomic material. Light colored full capsids are complete virions. Micrograph was taken by Hamp Edwards.



**Figure 1.4: Drosophila X Virus Genome Organization.** The DXV genome is made up of two double-stranded RNA segments. Segment A is 3.3kb and encodes a polyprotein of 128 kDa which is cleaved during translation into outer capsid protein (preVP2), inner capsid protein (VP3), and protease (VP4). This segment also encodes a secondary putative 27kDa ORF. Viral protein preVP2 is further cleaved after translational cleavage into pVP2 and then to its final 45kDa form VP2. Segment B is 3.2kb and encodes the 110kDa RNA-dependent RNA polymerase.



and potentially encodes a 27kDa NS protein. This protein has not yet been detected during viral infection. Genome segment B is 3203bp in length and encodes VP1 (112kDa), an RNA-dependent RNA polymerase (RdRp). This protein is found as a free polypeptide and is also covalently linked to the 5' ends of genome segments in birnaviruses, and believed to be equally distributed in DXV.

#### 1.4.9 Viral Proteins

VP1 is the viral encoded 112-kDa RNA-dependent RNA polymerase (RdRp). VP1 has a low copy number and is found in very low quantities in the virion itself – in the order of 4% of total protein present in the virion. It has not been fully determined whether or not birnavirus RdRps function as guanylyl transferases. This is because birnavirus VP1 can be guanylated *in vitro*, but the transfer of the GMP to an acceptor or reversibility of the reaction has not been demonstrated. This suggests that VP1 is not a guanylyl transferase or capping enzyme (Dobos, 1995b).

The birnavirus RdRps share several structural motifs (I, II, III and IV) that are common to RNA-dependent RNA polymerases (RdRp) of positive-strand RNA (ssRNA+) viruses which are conserved among viral RdRps (von Einem *et al.*, 2004; Gorbatenya and Koonin, 1988). In addition to these conserved motifs, however, are multiple features that are unique to the *Birnavirus* family. The birnavirus RdRp proteins are found in both free and genome-linked forms. The genome linked form is linked by a serine-5' GMP phosphodiester bond to the 5' end of both genome segments. The RdRp is able to self prime from this configuration using the hydroxyl

groups of amino acid residues present within the enzymes core (Calvert *et al.*, 1991). Also, the birnavirus RdRps lack the Gly-Asp-Asp (GDD) sequence of the VI motif, which (with a third conserved aspartate in motif IV) is believed to represent the catalytic site of the RdRp enzyme family by acting to coordinate the position of two metal ions, water, the free 3'hydroxyl of the nascent strand and the incoming ribonucleoside triphosphate during replication. While Infectious Bursal Disease virus (IBDV), a birnavirus which infects chickens, contains an LDD motif in place of this GDD, which may perform the catalytic activity noted, IPNV and DXV completely lack the GDD motif from the motif IV of their RdRps. IPNV does, however, contain an LDD motif downstream which may function as a replacement catalytic site (Shwed *et al.*, 2002; Duncan *et al.*, 1991). No examination for this motif in DXV has yet been performed. Also, the current mechanism of *in vitro* mRNA synthesis in birnaviruses is unknown, with data arguing both for and against a semi conservative strand displacement mechanism (Vakharia, 2001; Dobos, 1995b).

Viral proteins preVP2, VP4 and VP3 are all generated from the rapid cleavage, possibly cotranslationally, of the polyprotein produced by the main ORF of genome segment A. After initial cleavage from the polyprotein, preVP2 is further processed to pVP2, and finally to VP2 via cleavage at the protein's carboxy end (Azad *et al.*, 1987). These two cleavages of VP2, as well as one additional which has not yet been fully elucidated, result in four peptides which remain associated with the virion and may play a role in cell membrane destabilization during viral entry (Da Costa *et al.*, 2002). While it has been shown in IPNV that host proteases perform these cleavages, no data exists yet for other birnaviruses regarding these cleavages

and it is unknown if the viral protease, VP4, takes part. The final form of this protein, VP2, is the major outer capsid protein of birnaviruses and composes approximately 60% of a virion's total protein. (Dobos, 1995a). Data suggests that the final form of VP2 is glycosylated in the cytoplasm. (Espinoza and Kuznar, 2002; Hjalmarsson *et al.*, 1999).

VP2 is also the protein which has been found to elicit neutralizing antibodies and carries serotype-specific and group-specific antigenic determinants in animal models for IPNV and IBDV. This makes this protein especially vital for the development of anti-birnavirus vaccines, diagnostics, and serotyping of viral isolates.

VP3 is the inner capsid structural protein of birnaviruses and composes 34% of the total virion mass (Dobos, 1995a). VP3 has been shown to be an exclusively internal protein, with a possible exposure of select epitopes on the surface of the virion. Recent crystal structure data suggests that this exposure does not occur, however, and also suggests that VP3 plays a role in viral coat assembly (Coulibaly *et al.*, 2005). VP3 has been shown to bind to dsRNA and form a threadlike ribonucleoprotein complex. Also, VP3 interacts with the C-terminal segment of the VP2 precursor pVP2 and VP1 (Hjalmarsson *et al.*, 1999; Lombardo *et al.*, 1999). The domain of VP3 responsible for these interactions has been narrowed down to the basic carboxyl-terminal domain (Maraver *et al.*, 2003; Tacken *et al.*, 2000). These interactions allow VP3 to control polymerase and genome incorporation into the nascent particle. VP3 may also aid in VP2 maturation since pVP2's C-terminus is placed near its own 5-fold axes, where the presence of the C-terminus of the protein may sterically inhibit 5 fold contacts. Maturation would therefore only take place

upon interactions with VP3 which repositions the C-terminus of pVP2 and facilitates further pVP2 processing (Coulibaly *et al.*, 2005). The existence of these interactions, and their detection in multiple birnaviruses, suggests that VP3 is a key organizer of birnavirus structure, assembly, and replication.

VP4, a virus encoded protease, comprises the central region of the genome segment A polyprotein. It has been shown that VP4 forms a non-canonical RNA viral Lon protease, even though it does not contain an ATPase domain (Birghan *et al.*, 2000). Two residues in the birnavirus protease are conserved across the Lon/VP4 protease family and critical for its cleavage activity. These two residues form a Ser-Lys catalytic dyad responsible for cleavage functions (Birghan *et al.*, 2000). VP4 is only known to cleave at the pVP2-VP4 and VP4-VP3 junctions, both of which contain a (Ser/Thr)-X-Ala↓(Ser/Ala)-Gly motif. This target sequence has similarities to the cleavage sites of bacterial leader peptidases and herpes virus proteases (Birghan *et al.*, 2000).

In addition to the 128kDa polyprotein ORF, birnavirus genome segment A also encodes smaller reading frames which vary dependent upon both the virus and serotype examined. In IPNV, a 17-kDa nonstructural protein (VP5) is encoded and is detected in IPNV-infected cells (Magyar and Dobos, 1994). Reverse genetics has shown that IPNV VP5 is not required for replication in cell culture and that VP5-deficient mutant viruses have replication kinetics similar to that of wild type virus (Weber *et al.*, 2001). The function of this VP5 is still unknown, and it could possibly have a role *in vivo* which was not observed in *in vitro* experimentation. IBDV also encodes a 17kDa NS protein, also named VP5, from a small alternate open reading

frame, which has been detected in infected cells but not in the virion (Mundt *et al.*, 1995). This gene, while similar in sequence to the IPNV VP5, is not required for viral replication but is important for viral pathogenesis (Yao *et al.*, 1998). Again using reverse genetics, NS-deficient IBDV was found to delay replication kinetics and post-transfection titer levels which are a log fold lower than those of wild type virus. Additionally, transient expression of IBDV VP5 alone in chicken embryo fibroblast cells can induce apoptosis (Yao and Vakharia, 2001). It is believed that the IBDV VP5 putative transmembrane domain and its induction of cell lysis may play an important role in the release of IBDV from infected cells during viral replication.

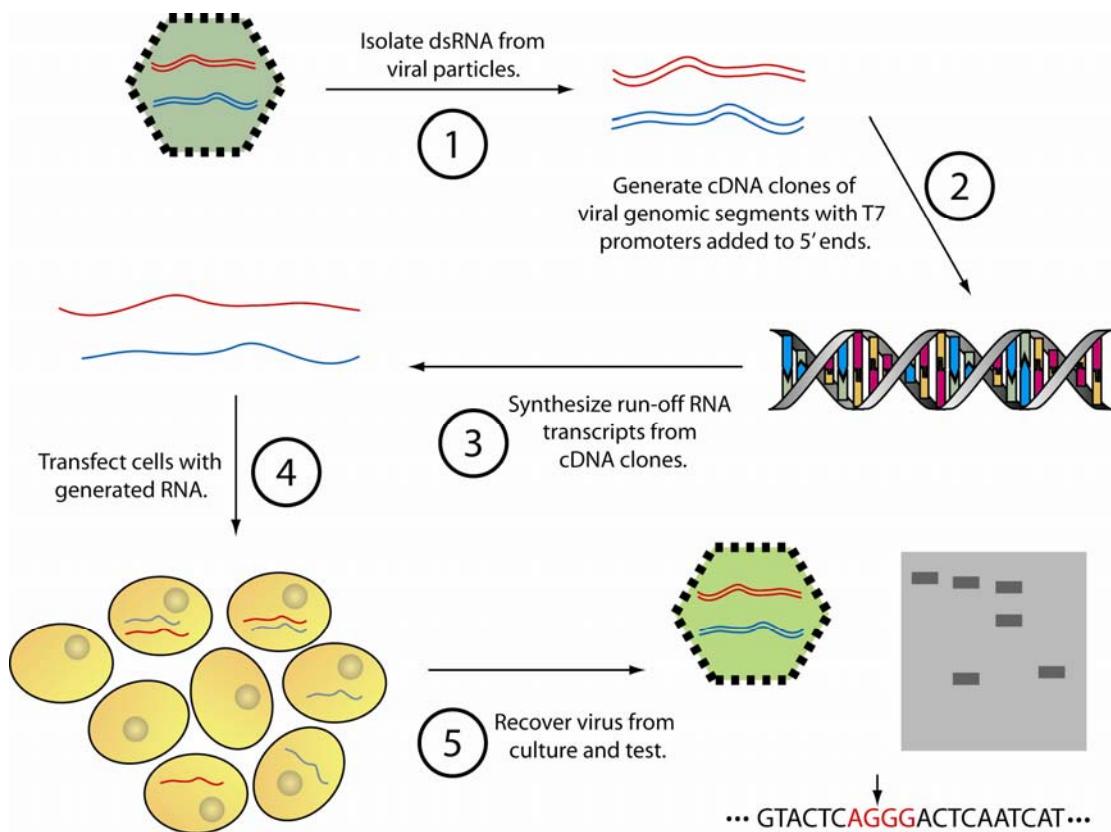
In addition to these expressed and detected proteins created by small ORFs, there is an additional ORF present further downstream in the genome, spanning the VP4-VP3 sections of the segment A polyprotein coding region and in the -1 reading frame relative to the polyprotein ORF. This protein is present in the DXV genome (27kDa) as well as IPNV strain Sp (25kDa). The ORF is of interest because the protein which it may encode is both arginine rich and contains a bipartite nuclear targeting sequence (Chung *et al.*, 1996; Shivappa *et al.*, 2004). However, the protein has not yet been detected and all attempts at reverse genetics examinations of its function in both DXV and IPNV proved unsuccessful.

#### **1.4.10 Reverse Genetics System**

One aspect of any research involving a virus that is always important is the ability to study various aspects of the virus itself via mutant viral strains generated by reverse genetics. These mutant strains allow for the elimination of select proteins, domains, and open reading frames from the virus, permitting a more complete understanding of how the virus itself works. This knowledge allows for a more complete understanding of how various other aspects of infection, from replication to immune responses resistance, are occurring. The first reverse genetics system for a birnavirus was developed by Mundt and Vakharia (1996) (Fig. 1.5). This system was developed for IBDV and has been successfully applied in IPNV also (Yao and Vakharia, 1998). The system functions through the transfection of synthetic positive-sense sRNA transcripts of both viral segments into appropriate host cells where they are transcribed and generate recombinant virus. It was found that no proteins, dsRNA or negative-sense ssRNA were necessary for virus recovery.

Because of the extensive homology between IPNV, IBDV, and DXV, this same system should theoretically function in generating recombinant DXV virus also. This system would allow for functional studies of viral proteins, virulence factors, alterations in immune responses, etc. These results will assist in determining what factors present in DXV elicit an antiviral immune response in *Drosophila*. Our work on the development of a reverse genetics system for DXV as well as to other aspects of the viral genome are detailed in Appendix A.

**Figure 1.5: Reverse genetics system for recovering recombinant Birnaviruses from clone derived transcripts.** dsRNA is isolated from Birnavirus particles (1) and then used to generate cDNA clones of viral genomic segments with T7 promoter sequences added to the 5' end of each segment (2). During this cDNA creation step alterations are made to the genome to insert marker restriction sites and mutations. These cDNA clones are then used to synthesize run-off RNA transcripts using a T7 transcription system (3) and transfected into host cell culture (4). Recombinant virus is then recovered from the culture and examined for marker sites and genomic sequence via restriction digestion, electrophoresis, and sequencing (5).



## 1.5 Current Research Status

As is apparent from the volume of information already provided, research into the immune responses of *Drosophila* is extensive. Details on the PAMPs required for pathogen recognition and the signaling pathways important in an immune response have been identified, and the activating and effector mechanisms of both the humoral and cellular responses have been elucidated. However, this research has primarily focused on responses against microbial, and not viral, pathogens. Because of this, we pursued research into how insects utilize their known repertoire of immune responses to combat a viral infection.

We began our examinations by developing a model system which could be used to screen for varying degrees of sensitivity to viral infection. Prior to our development of this model system using *Drosophila* X virus infection in *Drosophila*, no consistent viral infection model existed in *Drosophila*. When infected with DXV, flies develop an acute anoxia sensitivity infection pathology which allows for the screening of flies to detect variations in susceptibility to viral infection. Subsequent to developing this system, we screened a multitude of adult flies with mutations in various immune pathways. This screen uncovered mutants with significantly greater susceptibility to DXV infection and identified specific immune pathways that were important for antiviral immunity. We examined these flies to determine what effects these mutations of various pathways would have on the flies' viral titer levels. Determination of these titer levels would allow us to examine if viral replication was altered and causing the increased sensitivity to infection in these identified mutants.

We also explored the effects of previously identified *Drosophila* antimicrobial innate immune responses, specifically the antimicrobial peptides (AMPs) produced during microbial infection, on both viral infection and viral titer levels. From these examinations, we have been able to identify a novel role for the Toll pathway in *Drosophila* antiviral defense.

In addition to examining the previously identified *Drosophila* immune pathways, we also examined RNAi as a possible mechanism for antiviral defense in animals. While extensive research had previously been performed on antiviral applications of RNAi, all these studies relied upon cell culture or application of exogenous dsRNA to elicit a response against the virus. Using the methods we developed to examine the progression of viral infection, we were able to identify RNAi mutants which had dramatically increased susceptibility to viral infection. This was something never before shown with mutant adult animals?. Additionally, we found that RNAi compromised flies infected with DXV had higher titers of DXV than DXV infected wild type flies, further supporting the importance of RNAi in an antiviral response in *Drosophila*. These two studies show, for the first time, that endogenous RNAi is playing an antiviral role in adult *Drosophila*.

In summary, the main goals of this research focus on understanding the *Drosophila melanogaster* immune response against a viral pathogen, specifically DXV; identification of the genes and pathways that are utilized in this immune

response; and investigation of the potential of RNAi as an immune response in *Drosophila melanogaster* *in vivo*. All three of these goals have been successfully accomplished and this work has defined a previously unidentified antiviral response in *Drosophila*.

## **Chapter 2**

### **The Toll Pathway is Important for an Antiviral Response in *Drosophila***

#### **ABSTRACT**

The innate immune response of *Drosophila melanogaster* is governed by a complex set of signaling pathways that trigger antimicrobial peptide (AMP) production, phagocytosis, melanization and encapsulation. While immune responses against both bacteria and fungi have been demonstrated previously in *Drosophila*, identification of an antiviral response has yet to be found. To investigate what responses *Drosophila* mounts against a viral infection, we developed an *in vivo* *Drosophila* X virus (DXV) based screening system which identifies altered sensitivity to viral infection using DXV's anoxia induced death pathology. Using this system to screen flies with mutations in genes with known or suggested immune activity, we identified the Toll pathway as a vital part of the *Drosophila* antiviral response. Inactivation of this pathway instigated a rapid onset of anoxia induced death in infected flies and increases in viral titers compared to those in wild type (WT) flies. While constitutive activation of the pathway resulted in similar rapid onset of anoxia sensitivity, it also resulted in decreased viral titer. Additionally, AMP genes were induced in response to viral infection similar to levels observed during *E. coli* infection. However, enhanced expression of single AMPs did not alter resistance to

viral infection or viral titer levels, suggesting the main antiviral response is cellular rather than humoral. Our results show that the Toll pathway is required for efficient inhibition of DXV replication in *Drosophila*. Additionally, our results demonstrate the validity of using a genetic approach to identify genes and pathways utilized in viral innate immune responses in *Drosophila*.

## INTRODUCTION

The innate immune system plays an important role in immune responses against multiple pathogens in various species. In mammalian systems, it provides the first line of defense against pathogens prior to activation of acquired immune responses. In insects, the entire immune system is innate and has been shown to respond to bacteria, fungi, parasites, and, as our results show, viruses. Because of the striking homology between the *Drosophila* and mammalian innate immune systems, one example being the Toll pathway, *Drosophila* has become the model system of choice for the study of innate immune responses.

Because of the non-variable nature of innate immune responses, activation primarily occurs by recognition of distinct pathogen associated molecular patterns (PAMPs) which are shared by multiple pathogens (Medzhitov and Janeway, 1997). In mammalian systems, multiple Toll-like receptors (TLRs) have been found which activate the immune response via detection of a range of PAMPs including: lipopolysaccharide (TLR4), lipoproteins (TLR2), dsRNA (TLR3), flagellin (TLR5), CpG DNA (TLR9), and various antiviral compounds (TLR7) (Akira *et al.*, 2001;

Aderem and Ulevitch, 2000). In addition to these PAMPs, TLRs can also be activated by recognition of ‘self’ patterns normally present inside of cells such as heat shock proteins and uric acid (Akira, 2003; Shi *et al.*, 2003). When activated, these TLRs are involved in the expression of inflammatory cytokines and co-stimulatory molecules which activate the adaptive immune system (Akira *et al.*, 2001). In contrast, of the ten TLRs identified in *D. melanogaster*, only one has been definitively identified as playing a role in innate immunity. Additionally, unlike the limited PAMP sets recognized by each mammalian TLR, this one *Drosophila* Toll is able to respond to bacterial, fungal, and viral infections (De Gregorio *et al.*, 2001). *Drosophila* mounts an immune response against these pathogens through the use of both humoral and cellular responses. The identified humoral response in *Drosophila* consists primarily of the Toll pathway and the IMD pathway, which regulate antimicrobial peptide (AMP) expression in the fat body, a flattened tissue in the fly abdomen that is functionally equivalent to the mammalian liver (Rizki and Rizki, 1984a; Rizki, 1978).

The Toll pathway is activated by Gram-positive bacterial and fungal infections via binding of PAMPs to peptidoglycan receptor proteins (PGRPs) (-SA, -SD) and Gram-negative binding proteins (Bischoff *et al.*, 2004; Hoffmann, 2003; Akira *et al.*, 2001; Medzhitov and Janeway, 1997). This binding initiates a serine protease cascade which cleaves Spätzle, the ligand of the Toll transmembrane receptor protein (Hoffmann, 2003; Weber *et al.*, 2003). Once this cleaved form of Spätzle is bound, Toll signaling directs the phosphorylation and degradation of Cactus, an I $\kappa$ B-like protein which inhibits the NF- $\kappa$ B like transcription factors Dorsal

and Dif (Lemaitre *et al.*, 1996). Destruction of Cactus allows translocation of these transcription factors to the nucleus causing a rapid increase in expression of multiple AMPs (Weber *et al.*, 2003; Lemaitre *et al.*, 1996; Ip *et al.*, 1993). The Toll pathway also plays an important role in both maternal effect embryonic patterning and larval hematopoiesis (Qiu *et al.*, 1998; Morisalo and Anderson, 1995). This hematopoietic developmental effect may be significant for immune responses as hemocytes mediate nearly all cellular immune responses, including phagocytosis, melanization, encapsulation and also signal the fat body to initiate AMP production during infection.

The IMD pathway is activated in a similar fashion to the Toll pathway. It is believed that the IMD pathway is triggered via interaction between the Gram-negative bacteria PAMP diaminopimelic acid peptidoglycan and the PGRP-LC transmembrane receptor (Kaneko *et al.*, 2004; Hoffmann, 2003). Imd, a death domain adaptor protein with significant similarities to the mammalian receptor interaction protein (RIP), is then recruited by and binds to dFadd (Naitza *et al.*, 2002; Georgel *et al.*, 2001). dFadd interacts with the caspase Dredd which in turn is thought to associate with and cleaves phosphorylated Relish, a bipartite NF- $\kappa$ B type transcription factor (Stoven *et al.*, 2003; Naitza *et al.*, 2002; Hu and Yang, 2000). Relish is phosphorylated by the *Drosophila* IKK complex which is activated by the MAPKKK Tak1 in an Imd dependent manner (Lu *et al.*, 2001; Vidal *et al.*, 2001; Rutschmann *et al.*, 2000b; Silverman *et al.*, 2000a). The cleaved N-terminal domain of Relish then translocates to the nucleus where it regulates the transcription of various immune response related genes (Stoven *et al.*, 2003).

Inactivation of either these pathways results in increased susceptibility to select microorganisms. Inactivation of the Toll pathway, for example, eliminates induction of the antifungal peptide *Drosomycin* and increases susceptibility to fungal and Gram-positive infections. These flies are able to induce the antibacterial peptide Diptericin normally, however, and can resist Gram-negative bacterial infections (Tauszig-Delamasure *et al.*, 2002; Rutschmann *et al.*, 2000a; Meng *et al.*, 1999; Lemaitre *et al.*, 1996).

In addition to bacterial and fungal pathogens, multiple viruses that can infect *Drosophila* have been identified. *Drosophila C Virus*, for instance, has been studied and its pathogenesis examined in depth (Cherry and Perrimon, 2004). Another virus, *Drosophila X virus* (DXV), is a member of the *Birnavirus* family and has an icosahedral nucleocapsid and bisegmented dsRNA genome. Despite extensive research into DXV's genome, many of its pathological effects in *Drosophila* have yet to be thoroughly defined (Chung *et al.*, 1996; Teninges *et al.*, 1979a). Infection was shown to induce anoxia sensitivity and eventual death, but the specific cause was unknown (Teninges *et al.*, 1979a).

In our studies, we developed several assays to identify mutant lines with altered sensitivity to DXV infection. Additionally, we find that the Toll pathway is a essential component of viral resistance in flies. *Dif<sup>d</sup>* mutants, which do not have a functional Toll pathway, develop higher DXV titers and succumb to death by anoxia more rapidly then wild type (WT) flies. *Tl<sup>10b</sup>*, a Toll gain-of-function mutant, succumbs to similar early onset death but has a reduced DXV titer. These results provide the first example of an identified *Drosophila* innate immune related pathway

playing a role in viral susceptibility. Our results suggest that the Toll pathway is able to reduce replication of DXV, and possibly other viral pathogens. Further characterization of this pathway in relation to viral resistance should yield insight into this branch of the innate immune response in *Drosophila*.

## MATERIALS AND METHODS

### Fly Rearing.

All flies used were 3-5 day old adults and reared at 22°C on standard yeast/agar media. OregonR flies were used as WT. Fly lines were obtained from D. Ferrandon, B. Lemaitre, K. Anderson, and the Bloomington *Drosophila* Stock Center.

### Reverse Transcription and Quantitative Real-Time PCR.

RNA was isolated from adult flies by homogenizing flies in STAT-60 buffer according to the manufacturer's protocol (Isotex Diagnostics). Quantitatitive RT-PCR was then performed using a PE Biosystems 5700 GeneAmp Sequence detection system and Invitrogen Lux Primers. Specific LUX primers were designed against DXV strand B and used to quantify relative viral titer. LUX primer sets were also designed to measure AMP gene expression. *Ribosomal protein 49* was used as a control in all experiments. All primers are listed in Table 2.1.

**Table 2.1:** Quantitative RT-PCR Primers

DXV		
Left Primer	GGAGTTGAAGCCACGGTTG	
Right Primer	GACGATCTGCCAGTTGGCTCATCG[FAM]C	
<i>AttacinA</i>		
Left Primer	CACCAGATCCTAACATCGTGGCCCTGG[FAM]G	
Right Primer	ACGCGAATGGGTCTGTTGT	
<i>Cecropin A1</i>		
Left Primer	TTTCGTCGCTCTCATTCTGG	
Right Primer	GACAATCCCACCCAGCTTCCGATTG[FAM]C	
<i>Defensin</i>		
Left Primer	CCACATGCGACCTACTCTCCA	
Right Primer	GACAAGAACGCAGACGGCCTTG[FAM]C	
<i>Diptericin</i>		
Left Primer	TTTGCAGTCCAGGGTCACCA	
Right Primer	CACGAGCCTCCATTCAAGTCCAATCTCG[FAM]G	
<i>Drososcin</i>		
Left Primer	GTGAGGCGCGAGGCAGT	
Right Primer	CACCTGGATGGCAGCTTGAGTCAGG[FAM]G	
<i>Drosomycin</i>		
Left Primer	ATCCTGAAGTGCTGGTGCAGAGGA[FAM]G	
Right Primer	ACGTTCATGCTAATTGCTCATGG	
<i>Metchnikowain</i>		
Left Primer	CAGTGCTGGCAGAGCCTCAT	
Right Primer	CAACCATAAATTGGACCCGGTCTGG[FAM]TG	
<i>rp49</i>		
Left Primer	CACGATAGCATACAGGCCAAGATCG[FAM]G	
Right Primer	GCCATTGTGCGACAGCTAG	

### **Histology.**

Flies 5 days post eclosion were injected with DXV and kept at 25°C. Flies were fixed in a FAAG solution [80% ethanol, 4% formaldehyde, 5% acetic acid, 1% glutaraldehyde (EM Grade, 25%)], dehydrated, and embedded in paraffin. Staining of DXV was performed by application of primary Ab consisting of anti-DXV antiserum to sections at 1:1000 dilution and left overnight at 4°C. Sections were washed in 1% PBSB and incubated in a 1:2000 dilution of peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 2 hrs at 25°C. After washing in PBS, sections were stained via DAB peroxidase based indirect detection protocol as per (Harlow and Lane, 1999). TUNEL staining was performed with the Tdt-FragEL DNA Fragmentation Detection Kit from Calbiochem as per the manual. Alternate sections of the fly stained with TUNEL were also immunostained against DXV.

### **Stress Experiments.**

Flies were exposed to heat (42°C), cold (4°C), or desiccation for 15 min. at 3, 5, 7, and 10 days post-infection. Assessment of survival was done 2 hrs post stress treatment.

### **Infection Method.**

For viral infection, flies were injected using a Drummond Nanoject or WPI PicoPump with approximately 30nL of a  $10^5$  fold dilution of purified DXV. Purified DXV was generated from an initial stock provided by Peter Dobos. Bacterial infection was done with 30nL of an overnight culture of *E. coli* washed once in and resuspended in PBS. Control injections were done with double-distilled H<sub>2</sub>O.

### **Anoxia Sensitivity Survival Assay.**

Anoxia sensitivity survival assaying was performed via 15 mins of CO<sub>2</sub> exposure in a sealed chamber. Flies were then assayed for survival 2 hrs post-treatment. For genetic screening, flies were assayed at 3, 7, and 10 d.p.i. For WT survival curves, flies were assayed every 24 hrs p.i. Sample size was no fewer than 100 flies per line tested. Flies were transferred to new vials every 3 days.

### **Western Blots.**

Purified DXV was used to produce rabbit antisera (Duncroft Inc., Lovettsville, VA). Antisera cleaned with acetone powder made from S2 cells and OregonR adult *Drosophila* and utilized at a 1:1000 fold dilution in Western blots. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) was utilized as a secondary antibody at 1:1000 fold dilution. Western blotting was performed as per standard protocol, utilizing ECL Western Blotting Detection Reagents (Amersham Biosciences).

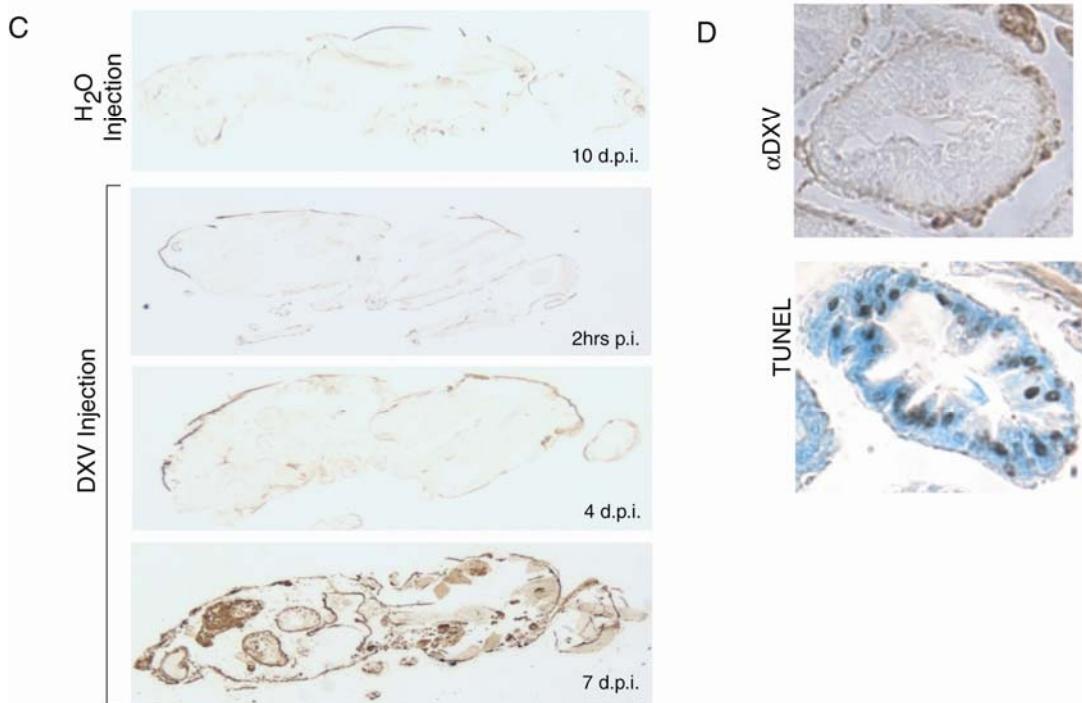
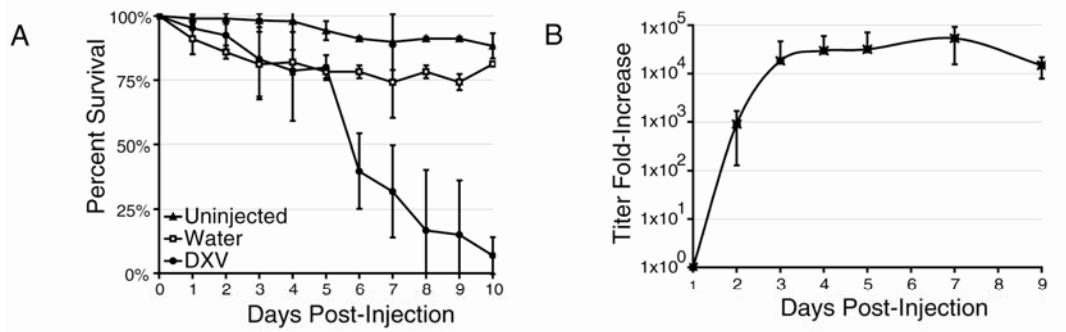
## RESULTS

### **DXV Infection in Wild Type *Drosophila*.**

WT *Drosophila* infected with DXV die 20-25 days post-infection (d.p.i.). It was previously noted that anoxia stress results in death at approximately 7 d.p.i. We found that this anoxia phenotype could be used as a reproducible assay for rapid identification of mutations that influence the viral immune response. We initially generated survival curves for WT *Drosophila* mock injected, injected with distilled H<sub>2</sub>O, and injected with DXV (Fig. 2.1A). Flies injected with DXV displayed a dramatic increase in anoxia sensitivity induced death 6 d.p.i. in comparison to the control groups. This increase in death was found to be anoxia specific and not the result of a general stress response as DXV-infected *Drosophila* displayed no alteration in survival in response to heat, cold, or dehydration stresses over the course of 10 days (data not shown). The specific and reproducible nature of this assay allowed for its use in identifying mutant *Drosophila* lines with altered sensitivity to viral infection.

To determine if the antiviral response was inhibiting viral replication, reducing pathogenic effects, or both, we examined a time course of viral titer in WT flies. Because of technical limitations, we were unable to utilize plaque assays with DXV and S2 cells for viral titer measurements. As an alternate method, quantitative

**Figure 2.1: DXV Pathology in *Drosophila melanogaster* wild type adults.** (a) The anoxia sensitivity phenotype correlates with injection of DXV into WT flies, with a dramatic drop in survival at 6 d.p.i. Over 200 flies were utilized for each survival curve. Error bars show 1 standard deviation. (b) Viral titer as measured by quantitative RT-PCR, shows a rapid increase in DXV. Each point represents a reaction from a pooled sample of 10 flies. A relative viral titer measurement of 1 represents DXV titer level at 1 d.p.i. Error bars show 1 standard deviation. (c) Immunostaining of DXV in sagittal sections of WT whole flies after injection with water or DXV. The head is oriented to the right. Immunostaining of H<sub>2</sub>O injection control shows no significant background. DXV infected samples show a steady spread of virus through entire WT organism over time. (d) DXV immunostaining and TUNEL staining of sagittal sections of a 7 d.p.i *Drosophila* showing cell death in the tissue where DXV antigens are detected. No TUNEL staining was observed in H<sub>2</sub>O injected flies. Sections are ~25um apart in the same organism.



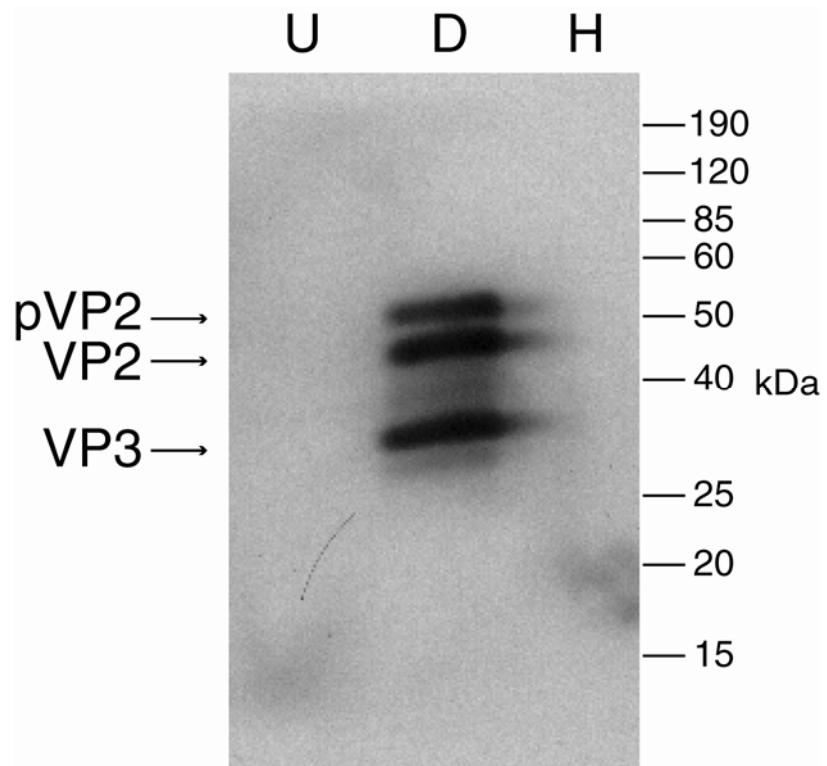
RT-PCR against a 62-bp region of DXV strand B was used for determination of relative titer increase. In WT *Drosophila* infected with DXV, viral titer was found to increase logarithmically before slowing to a near linear rate of increase (Fig. 2.1B). The timing of the increase in titer correlates with the increase in anoxia induced death in infected WT flies.

To examine tissue specificity of a DXV infection, we examined paraffin tissue sections of DXV infected WT *Drosophila* with an anti-DXV polyclonal antibody which recognizes DXV's capsid proteins (Fig. 2.2). We observed initial punctate staining at 4 d.p.i. followed by rapid viral spread throughout the organism by 7 d.p.i. (Fig. 2.1C). As with viral titer levels, we found that increased viral dispersal correlates with the earlier onset of anoxia induced death. We also find that cell death observed via TUNEL assay occurs in the same location where DXV staining is observed (Fig. 2.1D). It appears that some selectivity may occur during the initial stages of DXV infection but by the time that anoxia induced death occurs, the virus has pervaded multiple tissues and it is not clear if infection of a specific tissue is the cause of this increased anoxia induced death.

### **Antimicrobial Peptides' Effects on Viral Resistance.**

Previous work has shown that AMP gene expression is increased in response to bacterial and fungal infections in *Drosophila*. This expression is dependent on activation of the Toll and/or IMD pathways, so AMP expression has been utilized as a means to determine if these pathways are activated during infection. To determine

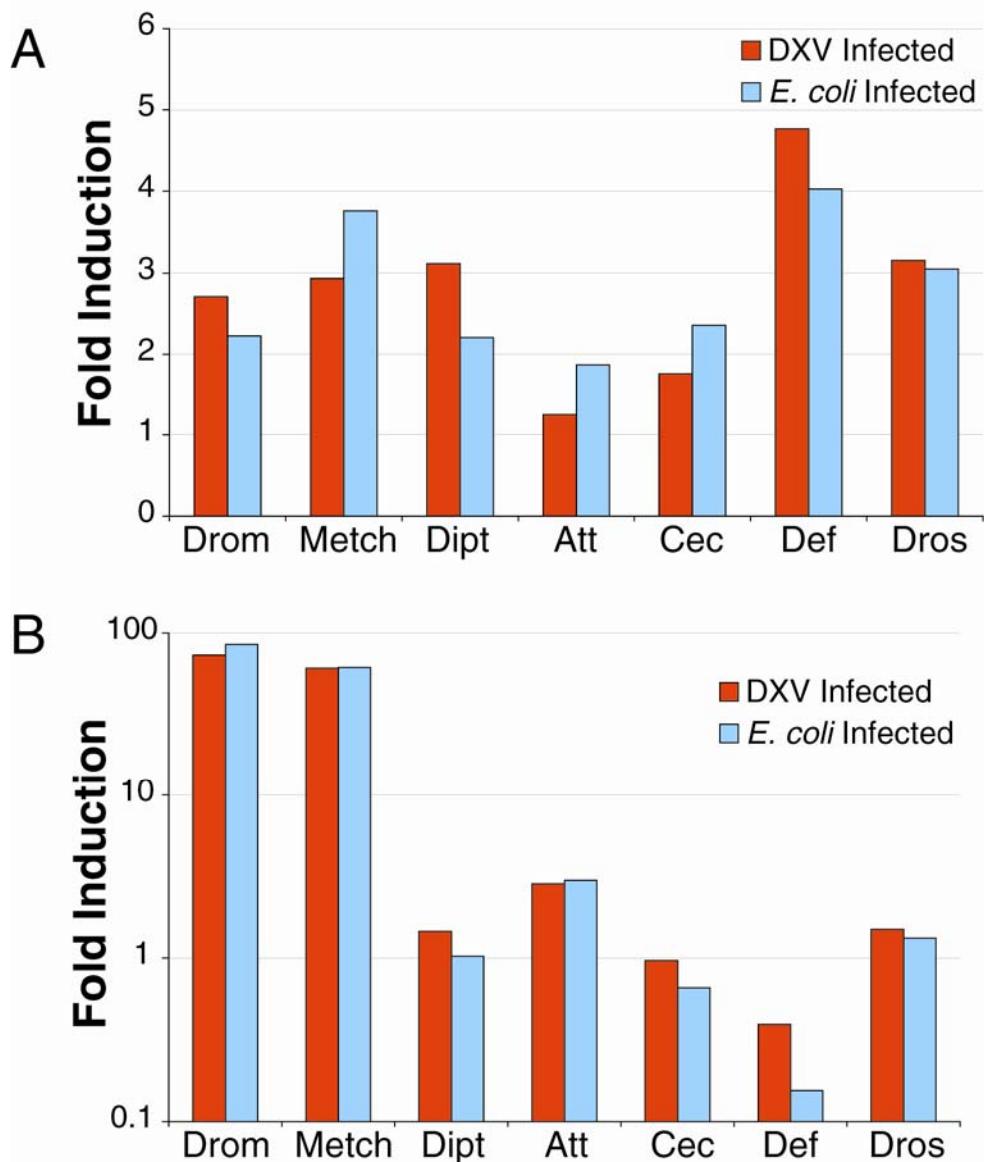
**Figure 2.2: Western blot of purified DXV using antibody generated against purified DXV.** U: Homogenized uninfected adult *Drosophila*. D: Purified DXV. H: ddH<sub>2</sub>O. Proteins pVP2, VP2, and VP3 are readily detectable in DXV infected S2 cell samples. Detection of these proteins shows minimal background in uninfected cell culture and is usable for detection of DXV in infected adult flies and infected S2 cell culture.



whether DXV infection induces expression of known *Drosophila* AMPs, we examined the expression levels of seven *Drosophila* AMPs via RT-PCR at 2 and 24 hrs post-viral infection. We utilized *E. coli* infection, a known activator of the Toll and IMD pathways, as a control to compare the levels of AMP gene expression in viral infection to those found in a Gram<sup>-</sup> bacterial infection. Wounding controls were used for baseline levels of AMP expression. We found that both DXV and *E. coli* infection induce similar expression levels of the Toll and IMD pathway target genes at both time points (Fig. 2.3A & B). This suggests that the Toll and IMD signaling pathways are both activated in response to viral infection.

Because of this up regulation of AMP expression during viral infection, we wanted to determine if AMPs were acting as effectors in an antiviral response. To do this, we examined the effect of constitutive expression of various singly expressed AMPs in an immunodeficient background. Flies of this genotype were generated through the use of the UAS-Gal4 system in an IMD and Toll pathway deficient background (*imd; spz*) (Tzou *et al.*, 2002b; Brand and Perrimon, 1993). Two fly lines deficient in both the Toll and IMD pathways, one containing the daughterless Gal4 driver (*imd; daGal4 spz*) and one containing a UAS-promoted AMP (*UAS-AMP imd; UAS-AMP spz*), were crossed to generate flies in which the UAS-promoted AMP is driven by the daughterless Gal4 driver. The daughterless Gal4 driver expresses GAL4 constitutively in all tissues. Attacin, Cecropin, Defensin, Diptericin, Drosomycin, Drosocin, and Metchnikowin UAS promoted fly lines were all tested (Tzou *et al.*, 2002a) . Offspring were infected with DXV and assayed for anoxia survival at 3, 7, and 10 d.p.i. Of the 7 AMPs analyzed in triplicate, less than 10%

**Figure 2.3: AMP expression levels in DXV infected flies measured by quantitative RT-PCR.** (a) 2 hrs post DXV injection (b) 24 hrs post DXV injection. No significant difference between the DXV and *E. coli* infected groups exists, with even the greatest difference being under a one-fold alteration. Data is representative of three experiments.

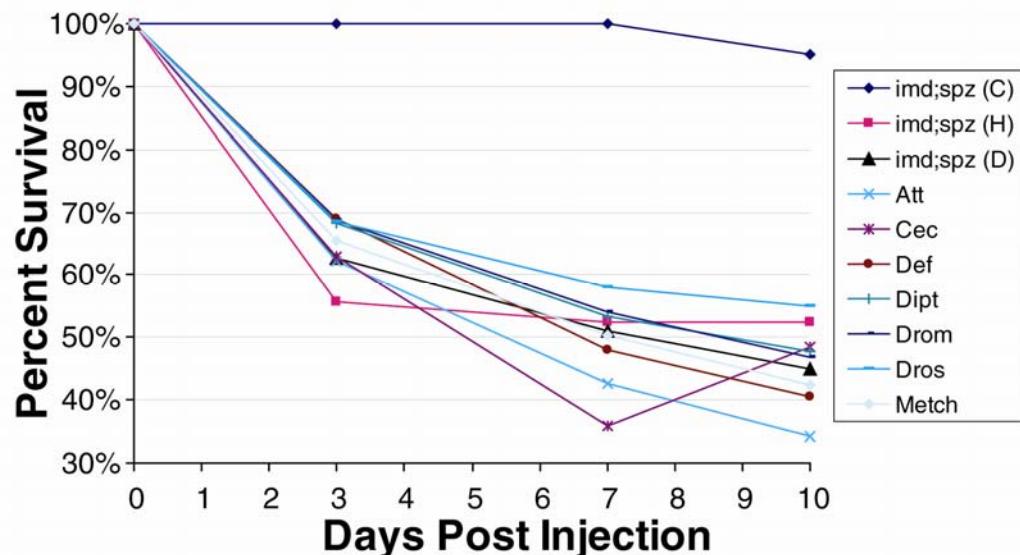


difference ( $\leq 10\%$ ) from the DXV effect on the *imd;spz* parental lines was observed (Fig. 2.4). Additionally, viral titer measurements in these flies were similar to those observed in WT flies. These findings indicate that expression of any of these AMPs alone is not sufficient to confer viral resistance in *Drosophila*.

### **Genetic Screen for Antiviral Immune Response Genes.**

In order to identify genes important for an antiviral response in *Drosophila*, we utilized anoxia induced death to screen a collection of mutant *Drosophila* lines. All screened lines were known to be or predicted to be important in immune responses against bacteria or fungi (Table 2.2). Due to various genetic backgrounds and lack of parental stocks for many of these lines, intragroup comparisons were performed to quantify the severity of viral susceptibility of the screened lines. Lines which were outside of one standard deviation from the average survival at two or more time points were selected as having significantly altered sensitivity to viral infection. Five lines were found to have increased resistance to viral infection (Fig 2.5). Only the *Dif*<sup>d</sup> and *Tl*<sup>10b</sup> mutant lines were found to be more susceptible to DXV infection. It is important to note that *rel*<sup>E20</sup> flies, a null mutant for the NF- $\kappa$ B in the IMD pathway, displayed no significant alteration in its resistance to viral pathogenic effects and had viral titers similar to those observed in WT flies (Fig. 2.6). These results suggest that while both the Toll and IMD pathways are activated by viral infection, only the activity of the Toll pathway imparts specific resistance against DXV.

**Figure 2.4: Survival of flies constitutively expressing a single AMP in an immunodeficient background.** Survival for UAS/Gal4 driven single AMP expression flies (Att (Attacin), Cec (Cecropin), Def (Defensin), Dipt (Diptericin), Drom (Drosomycin), Dros (Drosocin), & Metch (Metchnikowin)) and *imd*;spz flies uninjected (*imd*;spz (C)), injected with H<sub>2</sub>O (*imd*;spz (H)), and injected with DXV (*imd*;spz (D)). There is no significant difference in survival between AMP expressing lines and the *imd*;spz (D) line. A minimum of 150 flies of each line were tested.



**Table 2.2: *Drosophila* Lines Used in Genetic Screen**

Stock Number / Reference	Genotype	From
78	os <sup>0</sup>	Bloomington
79	os <sup>s</sup>	Bloomington
511	gro <sup>1</sup>	Bloomington
4719	f <sup>5</sup> l(1)17Aa <sup>9130</sup> os <sup>1</sup>	Bloomington
12406	w <sup>1118</sup> ; P{GT1}CG9339 <sup>BG00162</sup>	Bloomington
12407	w <sup>1118</sup> P{GT1}CG1532 <sup>BG00171</sup>	Bloomington
12409	w <sup>1118</sup> P{GT1}BG00218	Bloomington
12457	w <sup>1118</sup> ; P{GT1}Rab14 <sup>BG01134</sup>	Bloomington
12459	w <sup>1118</sup> ; P{GT1}Aef1 <sup>BG01171</sup>	Bloomington
12482	w <sup>1118</sup> P{GT1}Tis11 <sup>BG00309</sup>	Bloomington
12539	w <sup>1118</sup> ; P{GT1}drongo <sup>BG01320</sup>	Bloomington
12542	w <sup>1118</sup> ; P{GT1}BG01483	Bloomington
12562	w <sup>1118</sup> P{GT1}l(1)G0289 <sup>BG00227</sup>	Bloomington
12566	w <sup>1118</sup> P{GT1}CG2471 <sup>BG01767</sup>	Bloomington
12572	w <sup>1118</sup> P{GT1}BG01439	Bloomington
12598	w <sup>1118</sup> ; P{GT1}BG01672	Bloomington
12617	w <sup>1118</sup> ; P{GT1}BG02150	Bloomington
12631	w <sup>1118</sup> ; P{GT1}CG33100 <sup>BG01713</sup>	Bloomington
12637	w <sup>1118</sup> ; P{GT1}BG01176	Bloomington
12647	w <sup>1118</sup> ; P{GT1}CG31705 <sup>BG01777</sup>	Bloomington
12652	w <sup>1118</sup> ; P{GT1}BG02028	Bloomington
12669	w <sup>1118</sup> ; P{GT1}CG13512 <sup>BG02301</sup>	Bloomington
12670	w <sup>1118</sup> P{GT1}Pat1 <sup>BG02303a</sup> P{GT1}CG3960 <sup>BG02303b</sup>	Bloomington
12676	w <sup>1118</sup> P{GT1}CG32675 <sup>BG02353</sup>	Bloomington
12683	w <sup>1118</sup> ; P{GT1}Hsp22 <sup>BG02409</sup>	Bloomington
12712	w <sup>1118</sup> P{GT1}pod1 <sup>BG02604</sup>	Bloomington
12727	w <sup>1118</sup> P{GT1}Adar <sup>BG02235</sup>	Bloomington
12730	P{GT1}BG01736 w <sup>1118</sup>	Bloomington
12756	w <sup>1118</sup> ; P{GT1}BG01884	Bloomington
12767	w <sup>1118</sup> ; P{GT1}CG30015 <sup>BG02336</sup>	Bloomington
12785	w <sup>1118</sup> ; P{GT1}BG01560	Bloomington
12804	w <sup>1118</sup> ; P{GT1}BG02675	Bloomington
12807	w <sup>1118</sup> ; P{GT1}BG02256	Bloomington
12830	w <sup>1118</sup> ; P{GT1}BG02852	Bloomington
12871	w <sup>1118</sup> ; P{GT1}BG02772	Bloomington
12917	y <sup>1</sup> w <sup>67c23</sup> ; P{SUPor-P}CG6145 <sup>KG02707</sup>	Bloomington
13128	w <sup>1118</sup> P{GT1}BG02770	Bloomington
13208	y <sup>1</sup> P{SUPor-P}Ptp4E <sup>KG02328</sup>	Bloomington

**Table 3.1: *Drosophila* Lines Used in Genetic Screen (continued)**

Stock Number / Reference	Genotype	From
13232	$y^1 w^{67c23}; P\{SUPor-P\}KG02786$	Bloomington
13293	$y^1 w^{67c23}; P\{SUPor-P\}KG00562$	Bloomington
13765	$y^1 w^{67c23}; P\{SUPor-P\}Mes2^{KG02901} ry$	Bloomington
13767	$y^1 w^{67c23}; P\{SUPor-P\}KG03017$	Bloomington
13973	$y^1 w^{67c23}; P\{SUPor-P\}CG1600^{KG03987}$	Bloomington
14553	$y^1 w^{67c23}; P\{SUPor-P\}KG07726$	Bloomington
14555	$y^1; P\{SUPor-P\}CG30460^{KG07759}; ry^{506}$	Bloomington
14629	$y^1 w^{67c23}; P\{SUPor-P\}KG07439$	Bloomington
14816	$y^1 w^{67c23}; P\{EPgy2\}EY00205$	Bloomington
14842	$y^1 w^{67c23}; P\{EPgy2\}EY00412$	Bloomington
14908	$y^1; P\{SUPor-P\}KG05948/CyO; ry^{506}$	Bloomington
a	$Dif^1 cn bw$	Ferrandon, D.
b	$stat92E^F$ (grown @ 29°C)	Dearolf, C.
c	$rel^{E20}$	Hultmark, D.
d	$Toll^{10b}$	Anderson, K.
e	$Tum^L$	Dearolf, C.
	$Df(3R)ro^{XB3}/Tl^{9QRX}$	Anderson, K.
	$pll^{21}/pll^{16}$	Anderson, K.
	$snk^{073}/snk^{229}$	Anderson, K.
	$spz^{rm7}/spz^{197}$	Anderson, K.
	$Df(3R)XM3/tub^{R5.6}$	Anderson, K.

a. Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D. (2000) Imm. 12, 569-580.

b. Baksa, K., Parke, T., Dobens, L.L., & Dearolf, C.R. (2002) Dev. Bio. 243, 166-175.

c. Hedengren M, Asling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, Hultmark D. (1999) Mol. Cell. 4, 827-837.

d. Schneider,D.S., Hudson, K.L., Lin, T.Y., & Anderson, K.V. (1991) Genes & Dev. 5, 797-807.

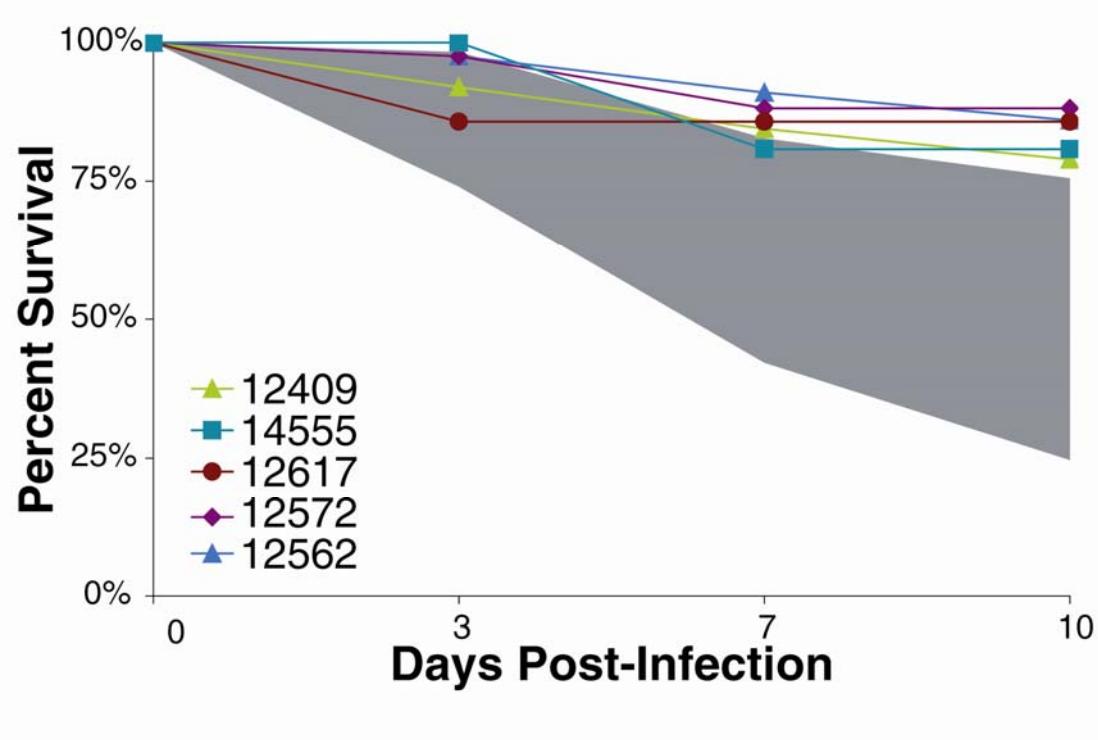
e. Luo, H., Hanratty, W.P., & Dearolf, C.R. (1995) EMBO J. 14, 1412-1420.

Red Lettering: More susceptible than average to viral infection.

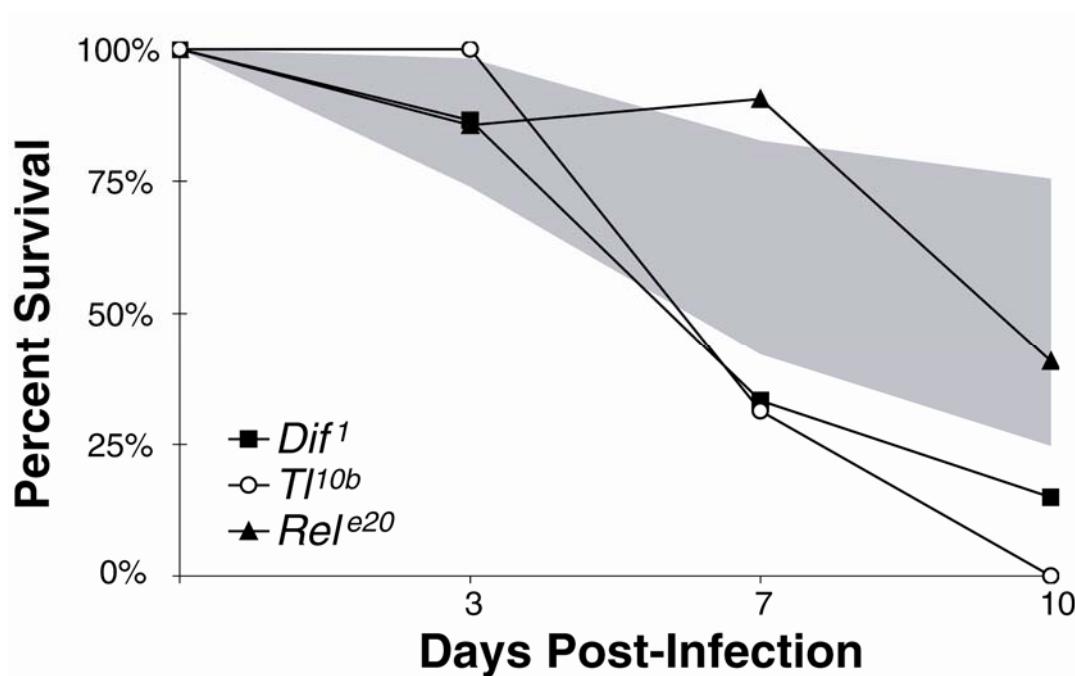
Green Lettering: Less susceptible than average to viral infection.

**Figure 2.5: Infection survival curves of mutant flies with decreased DXV**

**infection susceptibility.** We examined lines in IMD, Toll, and Stat pathways as well genes found to be upregulated by infection in microarray screens. Of the screened lines (Table 2.2), only these 5 lines display significant increased resistance to viral infection, defined as higher survival than the screened pools average survival range plus or minus one standard deviation (gray region) at two time points.



**Figure 2.6: Increased susceptibility of *Toll* and *Dif* mutant fly lines to DXV infection.** *Dif*<sup>l</sup> lacks the ability to activate the Toll pathway. *Tl*<sup>l<sup>0b</sup></sup> is a constitutively active mutant of the Toll pathway. *rel*<sup>E20</sup> is a mutant in the IMD pathway. All measurements normalized to H<sub>2</sub>O injected samples of the same mutant *Drosophila* line. Only *Dif*<sup>l</sup> and *Tl*<sup>l<sup>0b</sup></sup> lines have significant alteration in survival, defined as 2 (or more) time points being outside of one standard deviation of the screened lines average survival (gray region). *rel*<sup>E20</sup> displays average survival. The 7 day time point increase in *rel*<sup>E20</sup> survival is due to base line effects from the wounding controls.



### **Toll pathway mutants are more sensitive to viral infection.**

To confirm that the Toll pathway was involved in an antiviral response, we examined *Dif*<sup>d</sup>, a loss of function mutant in an NF-κB-like transcription factor in the Toll pathway. As noted above, we find that the mutant line succumbs to anoxia induced death approximately 48 hrs earlier than WT *Drosophila* (Fig. 2.6). In addition, viral titer levels were found to be approximately 40 times higher than that observed in WT flies at 3 d.p.i. This increased viral titer correlates to a 5 d.p.i. titer in WT *Drosophila*, the time point at which the main spike in anoxia induced death is observed. This suggests that Dif plays a role in an antiviral response that both inhibits viral replication and limits the pathogenic effects of infection.

To determine if constitutive activation of the Toll pathway would confer increased resistance to DXV infection, we examined the *Tl*<sup>10b</sup> gain of function mutant fly line. Contrary to expectations, the *Tl*<sup>10b</sup> line also demonstrated early onset of anoxia sensitivity similar to that of the *Dif*<sup>d</sup> line (Fig. 2.6). In addition, we find that these flies have viral load approximately ½ that of WT flies at the same time point. This decreased viral titer suggests that the constitutive activation of the Toll pathway is able to retard viral replication but is not able to affect the overall outcome of DXV infection. This suggests that DXV titer may be partially independent of the pathogenic effects of infection.

## Chapter 3

# RNAi is an Antiviral Immune Response Against a dsRNA Virus in *Drosophila melanogaster*

### ABSTRACT

*Drosophila melanogaster* has a robust and efficient innate immune system, which reacts to infections ranging from bacteria to fungi and, as discovered recently, viruses as well. The known *Drosophila* immune responses rely on humoral and cellular activities, similar to those found in the innate immune system of other animals. Recently, RNAi or ‘RNA silencing’, has arisen as a possible means by which *Drosophila* can react to pathogens, transposons, and retroviral elements, in a fashion similar to that of a traditional adaptive immune system. RNAi is a highly conserved regulation and defense mechanism, which suppresses gene expression via targeted RNA degradation directed by either exogenous dsRNA (cleaved into siRNAs) or endogenous miRNAs. In plants, RNAi has been found to act as an antiviral immune response system. Here we show that RNAi silencing is an antiviral response used by *Drosophila* to combat infection by Drosophila X virus, a birnavirus, as well. Additionally, we identify multiple core RNAi pathway genes, including *piwi*, *vasa* *intronic gene (vig)*, *aubergine (aub)*, *armitage (armi)*, *Rm62*, *r2d2*, and *Argonaute 2 (AGO2)* as having vital roles in this response in whole organisms. Our findings establish *Drosophila* as an ideal model for the study of antiviral RNAi responses in animals.

## INTRODUCTION

*Drosophila melanogaster* is an excellent model for the study of the innate immune system because of its genetic malleability, its lack of an adaptive immune system, and the conservation of these signaling pathways with those of higher organisms. To date, innate immune responses against bacteria, fungi, and viruses have all been identified in flies. These responses are known to occur via the Toll and IMD immune signaling pathways (reviewed in (Brennan and Anderson, 2004)).

*Drosophila* also possesses a robust RNA interference (RNAi) system homologous to the Post-Transcriptional Gene Silencing (PTGS)/RNAi systems found in plants and other animals.

The PTGS/RNAi system was initially described in plants over a decade ago during studies of transgenic petunias (van der Krol *et al.*, 1990). Shortly after its initial descriptions, it was found that PTGS was able to induce an immune response against certain viral pathogens in plants, which possess only an innate immune system and are unable to generate traditional adaptive immune molecules (e.g. antibodies). It was found that transgenic plants, which express portions of viral genomic RNA, were specifically resistant to that same virus (Lindbo *et al.*, 1993). Also, the levels of the proteins associated with these viral genome portions were greatly reduced. Viruses were found which carried copies of host genes within their genomes that could cause a reciprocal effect against the host – possibly reducing the associated host gene expression and causing increased susceptibility to viral infection (Kumagai *et al.*, 1995). It was not until later that work in *C. elegans* would identify

dsRNA as the initiating factor in these silencing responses and the process would be named RNA inhibition (RNAi) (Fire, 1999). Further research over the years since this discovery has uncovered the molecular workings of this complex pathway (further reviewed in (Lecellier and Voinnet, 2004)).

The RNAi pathway occurs in two main steps, the initiation and the execution steps. To initiate RNAi, dsRNA can be introduced by either endogenous or exogenous sources. Endogenous sources include short hairpin RNAs produced by the genome, aberrantly expressed transgenes, and transposons. Exogenous sources of dsRNA include naturally occurring dsRNA, such as that originating from RNA viruses, and dsRNA experimentally produced and introduced (Hannon, 2002). During the initiation phases of RNAi, dsRNAs greater than 23bp are processed into 21-23bp dsRNA fragments with 3' overhanging ends by either Dicer1 or Dicer2, depending upon whether the originating dsRNAs are endogenous, and involved in gene regulation, or non-self dsRNAs, respectively (Lee *et al.*, 2004; Blaszczyk *et al.*, 2001). The Dicer proteins are members of the RNase III gene family and contain an N-terminal RNA helicase domain, two RNase III-like domains, and a C-terminal dsRNA-binding domain. The cleaved length of the produced dsRNAs is caused by the dimerized RNase III domains in the protein, one of which is inactive and acts simply as a spacer domain. (Blaszczyk *et al.*, 2001). To date, homologs of Dicer have been identified in yeast, plants, worms, flies, mice, and humans (Bernstein *et al.*, 2001). These small RNAs are then utilized as templates in the subsequent RNAi execution steps (Hammond *et al.*, 2000; Zamore *et al.*, 2000; Tuschl *et al.*, 1999).

The small RNA fragments are incorporated into the RNA induced silencing complex (RISC) which is responsible for the execution phase of the RNAi pathway (Blaszczyk *et al.*, 2001; Nykanen *et al.*, 2001). The small dsRNAs are unwound, and the anti-sense strand is then incorporated into the RISC complex with the aid of the protein R2D2 (Liu *et al.*, 2003; Schwarz *et al.*, 2002). This incorporated strand acts as the targeting mechanism for the RISC and directs its action to complementary mRNA. RNAi is also used for gene regulation through the use of endogenously encoded RNA stem loops. These stem-loop structures are processed into micro-RNAs (miRNAs), which can also associate with the RISC complex. miRNA control of gene regulation is required for the control of normal cell processes (reviewed in (Pasquinelli *et al.*, 2005)) and this may be one of the factors contributing to the lethality of select mutations in the RNAi pathway. Studies of miRNA mediated gene regulation led to the discovery that variations in the complementarity of the template small RNA determine the final method of inactivation of the target message. Complete matching of the entire template RNA results in degradation of the targeted mRNA, while mismatching at a small number of base pairs (2-3bp) leads instead to inhibition of translation via a currently unknown mechanism. This difference in final activity could play a vital role in antiviral immunity since failure to degrade the viral genome would allow its persistence and use in viral replication.

The only components of the RISC complex that have been identified so far are Argonaute2 and the related Argonaute protein family members Fragile X mental retardation protein (FMRP), the Vasa intronic gene (VIG), and Tudor-SN. The Argonaute protein family is also called PAZ-Piwi-Domain (PPD) proteins and are

characterized by the presence of a PAZ domain and a C-terminal PIWI domain (Cerutti *et al.*, 2000). These domains are believed to function in protein-protein interactions. Members of the Argonaute family have been found to play a role in RNAi in multiple species, including *Neurospora crassa*, *Drosophila*, *C. elegans*, and *Arabidopsis* (Catalanotto *et al.*, 2002; Bernstein *et al.*, 2001; Parrish and Fire, 2001; Fagard *et al.*, 2000). It should be noted that Argonaute family proteins are also involved in other cellular functions, that are distinct from siRNA based RNAi, such as developmental control and stem cell maintenance (Carmell *et al.*, 2002).

The three Argonaute related proteins in the RISC complex are suspected to play a role in RNA-binding and nuclease functions. Both FMRP and the VIG have RNA-binding properties and complex with Tudor-SN (Caudy *et al.*, 2002). Additionally, Tudor-SN is related to micrococcal nucleases. However, the nuclease activity of Tudor-SN has not been identified as the functional nuclease activity of the RISC, but may instead be part of the secondary nuclease activities of the RISC, uncovered in recent research (Schwarz *et al.*, 2004).

The *Drosophila* genes *armitage* and *aubergine* are also essential for RNAi, but have not yet been linked to a specific function in the process. Armitage is necessary for silencing of select mRNAs involved in oogenesis, and *armitage* mutants display defects in RISC assembly (Cook *et al.*, 2004; Tomari *et al.*, 2004). Aubergine, in addition to being a member of the Argonaute gene family, has been linked to RNAi in the oocyte and co-localizes with known RNAi pathway components (Findley *et al.*, 2003; Kennerdell *et al.*, 2002). Rm62, an ortholog of human p68, unwinds short but not long segments of dsRNA in an ATP-dependent

fashion, and has been shown to interact with dFMR1, the *Drosophila* homolog of the human FMRP1. Rm62 has been found to be necessary for RNAi function (Huang and Liu, 2002; Ishizuka *et al.*, 2002). Piwi, also an Argonaute gene family member, has been shown to control male germ-line stem cell division and has been linked to PTGS (Pal-Bhadra *et al.*, 2002).

The initial identification of the RNAi phenomenon and its antiviral role in plants has resulted in considerable interest into the use of RNAi as an antiviral strategy in animal systems. In mosquitoes, for example, expression of Dengue virus genome fragments in cells or whole mosquitoes was able to inhibit later Dengue virus infection and replication (Adelman *et al.*, 2001; Gaines *et al.*, 1996; Olson *et al.*, 1996). Another study found that infection of *Drosophila* cells with Flock House virus (FHV) resulted in accumulation of viral genome specific siRNAs which could promote RNAi based degradation of viral RNA (Li *et al.*, 2002). Additionally, FHV was found to encode an RNAi inhibitor protein (B2), which can protect the virus from host RNAi responses and promote its survival (Li *et al.*, 2002). The fact that the virus produces this protein suggests that RNAi is imposing selection pressure upon the virus and that the RNAi inhibitor is needed for efficient replication. This advantage would explain how the cost of producing the protein and retaining it within the viral genome is overcome. Work in mammalian cell culture is also uncovering a possible role for RNAi in a mammalian antiviral immune response. The fact that some viruses are susceptible to RNAi when it is induced in cell culture (e.g. poliovirus, human papillomavirus, HIV) suggests that RNAi can interfere with viral genome expression in certain types of mammalian infections (Gitlin *et al.*, 2002; Jacque *et al.*, 2002;

Jiang and Milner, 2002). Whereas other viruses, such as FHV, hepatitis delta virus, and influenza virus are resistant to RNAi, suggesting that these viruses evolved a means to surmount an RNAi response (Li *et al.*, 2004; Chang and Taylor, 2003; Li *et al.*, 2002).

While these and other experiments using RNAi specifically induced against the virus provide some support that RNAi acts as a semi-adaptive innate immune defense in animals, they also raise the question of whether or not the host endogenous RNAi response can provide protection in whole organisms similar to that observed when antiviral RNAi is induced in cell culture or whole animals.

To examine a possible role for RNAi as an antiviral immune response, we use a model system that was developed using *Drosophila* X virus (DXV), a bisegmented dsRNA birnavirus (Zambon *et al.*, 2005a). *Drosophila* infected with this virus develop acute anoxia sensitivity and die within two weeks of symptom onset. The anoxia sensitivity to DXV infection phenotype can be used as a means to identify mutants that are more susceptible to virus. Previously, from screening known immunity mutants, it was found that the Toll pathway, but not the IMD pathway, plays a key antiviral role in *Drosophila*. By screening *Drosophila* lines having mutations in key components of the RNAi pathway, we determined that the RNAi pathway is also an essential part of the antiviral response in *Drosophila*.

## MATERIALS AND METHODS

### Viral Infection of Flies

All flies used were 3-5 day old adults and reared at 22°C on standard yeast/agar media. OregonR flies were used as WT. Flies were injected using a Drummond Nanoject or WPI PicoPump with approximately 30nL of a 10<sup>5</sup> fold dilution of purified DXV. This same concentration was used for the pilot screen (Zambon *et al.*, 2005b) and for all subsequent viral infections. Purified DXV was generated from an initial stock provided by Peter Dobos (University of Guelph, Guelph, Canada). Control injections were done with double-distilled H<sub>2</sub>O.

### Anoxia Sensitivity Survival Assay

Assaying was performed via 15 mins of CO<sub>2</sub> exposure at 3, 7, and 10 d.p.i. in a sealed chamber. Following anoxia, flies were then assayed for survival. Flies were transferred to new vials every 3 days. All flies were base lined to water injected *Drosophila* of the same line to control for wound effects. Fly lines were selected as significantly altered if their survival at 2 of the 3 time points examined fell outside 1 standard deviation of the average survival curve. The average wild type survival and range was developed in our previous pilot screen (Zambon *et al.*, 2005b) from 50 examined mutant fly lines and the OregonR wild type fly line. The sample size for each RNAi mutant tested was no fewer than 100 flies per line with all mutants rigorously re-tested to confirm their phenotype.

## Generation of dsRNA

For the anti-DXV VP1 dsRNA generation, a cDNA clone of DXV genome segment B was created using two separate reverse transcription reactions utilizing the following primers: 5'-GAG CTC TAA TAC GAC TCA CTA TAG GAA AAT AAT TGG CGG CCG ATA AGG-3'; 5'-GGT CTG AGA TAG GTA AGC CAC-3'; 5'- GTG AGT TAC GTC CCA GCC CAG CAT GTC CAT C-3' 5'-CTG CAG GGA GCC GCC CAA TTT ACA TTT GG-3'. The reactions generated two 1.6kb fragments with overlapping regions containing an *MfeI* restriction site. Both segments were then restriction digested with *MfeI* and ligated to form a full length cDNA clone, which was subcloned into pCR2.1-TOPO using the Invitrogen TOPO-TA Kit as described by the manufacturer. A dsRNA generating DNA fragment was then created from this DXV cDNA clone using primers flanking an 470bp central region of VP1, the viral RdRp, which had 5' flanking T7 promoters: Forward: 5'- TAATACGACTCACTATAGCCAGTCAAGGGTGGAT-3' Reverse: 5'- TAATACGACTCACTATAGAGGCCGCATCTCCTAGA-3'). This fragment was also cloned into pCR2.1-TOPO as above.

To generate an anti-AGO2 dsRNA, *Drosophila* RNA was isolated from S2 cells using STAT-60 buffer according to the manufacturer's protocol (Isotex Diagnostics), then reverse transcription and PCR against a 1kb fragment of the respective gene was performed. Primers used contained 5' flanking T7 promoters, and are as follows: Forward: 5'-TGT AAT ACG ACT CAC TAT AGG GAC AAT CGT TCG CTT TGC GT-3', Reverse: 5'-TGT AAT ACG ACT CAC TAT AGG

GAT GAC GAA CGC AGC CTA GA-3'. Fragments were then subcloned into pCR2.1-TOPO using the Invitrogen TOPO-TA Kit as described by the manufacturer.

The pCR2.1 vectors containing the dsRNA generation templates were then amplified by PCR, using primers noted above to generate specific short linear DNA templates to generate ssRNA with the help of the Ambion MegaScript T7 Kit, as per manufacturer's instructions. The resulting ssRNA was ethanol precipitated and resuspended in DEPC-treated H<sub>2</sub>O. The ssRNA was then heated to 65°C for 30 minutes, cooled (1°C/min) to 4°C, and stored at -20°C. The dsRNA was then fractionated by gel electrophoresis and visualized to confirm uniformity and size.

### **Reverse Transcription and Quantitative Real-Time PCR**

RNA was isolated from adult flies by homogenizing flies in STAT-60 buffer according to the manufacturer's protocol (Isotex Diagnostics). Quantitative RT-PCR was then performed using a PE Biosystems 5700 GeneAmp Sequence detection system and Invitrogen Lux Primers. Specific LUX primers were designed against DXV strand B and used to quantify relative viral titer. *Ribosomal protein 49 (rp49)* was used as a control in all experiments. The primers used are listed in Table 2.1.

### **RNAi in S2 Cells**

S2 cells were grown in *Drosophila* SFM medium (Invitrogen). Cells were plated in six-well plates at 1x10<sup>6</sup> cells in 2ml SFM. 20µg of appropriate dsRNA was immediately added and plates were then incubated at room temperature for 60 minutes. 2mL of SFM with Penicillin (50 units/ml) and Streptomycin (50µg/ml) was

then added to each well and plates were incubated at RT for 72 hours to allow for protein turnover before DXV infection. Transfection of dsRNA was not found to have an improved effect on RNAi and was therefore not done. Cells were then infected with DXV at an M.O.I. of ~1. Samples were taken every 24 hours. At the time of infection, and every 3 days subsequent, 15 $\mu$ g of anti-RdRp dsRNA was added to each experimental well to ensure that DXV replication was still occurring under RNAi conditions, and not limited by degradation of the RNAi inducing dsRNA.

### **Western Blotting**

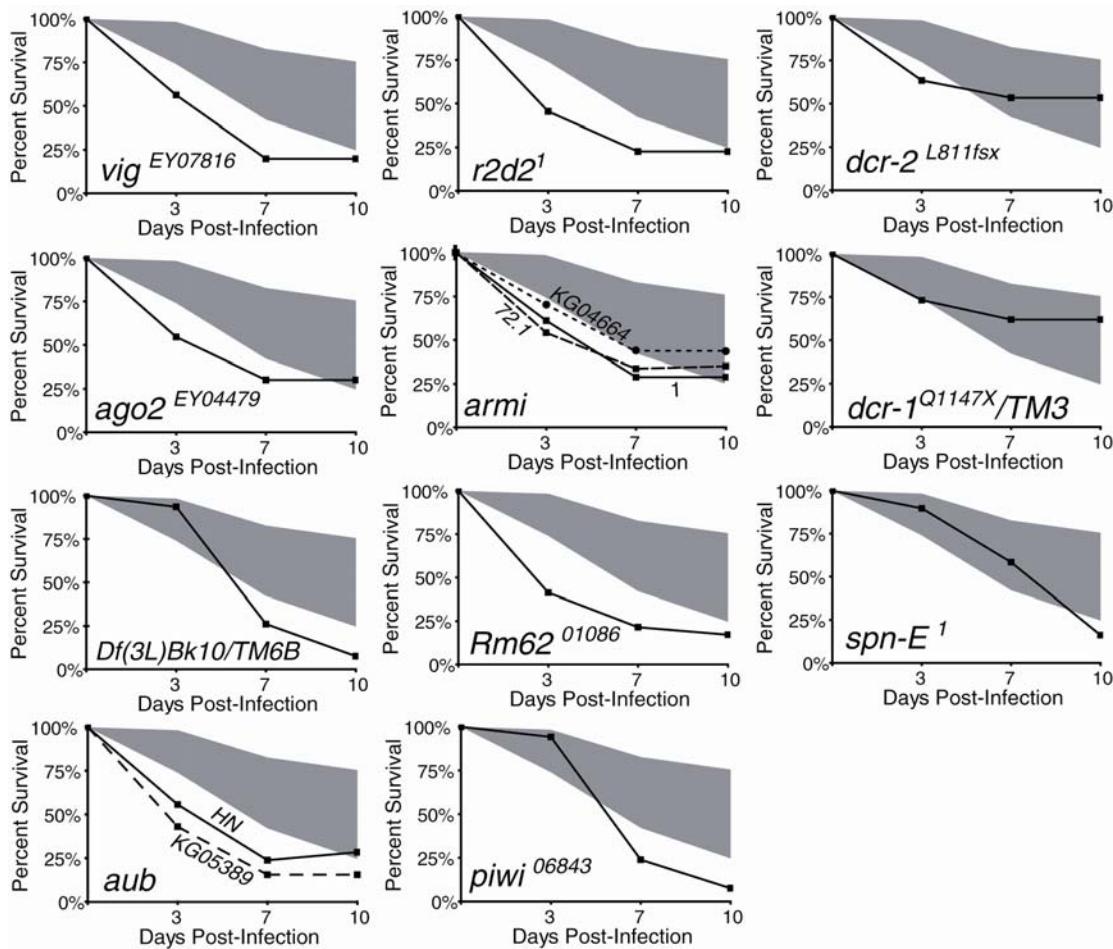
Purified DXV was used to produce rabbit antisera (Duncroft Inc., Lovettsville, VA). Antiserum was adsorbed with acetone-treated powder prepared from S2 cells and OregonR adult *Drosophila*, and utilized at 1:1000 dilution in Western blots. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) was utilized as a secondary antibody at 1:1000 dilution. Western blotting was performed as per standard protocol, utilizing ECL Western Blotting Detection Reagents (Amersham Biosciences).

## RESULTS

To determine whether RNAi might play an antiviral role in *Drosophila*, we examined all available adult viable *Drosophila* lines affecting known or predicted RNAi pathway genes. To do this, we screened 14 fly lines with different mutations in the RNAi pathway at 3, 7, and 10 days post infection (d.p.i.) for alterations in anoxia sensitivity induced death caused by infection with DXV (Zambon *et al.*, 2005b). Of the RNAi pathway mutants, all but three lines were found to be highly susceptible to viral infection. The criteria for susceptibility was defined from our previous pilot screen as having survival rates outside one standard deviation of the screen average at two of the three time points. The fact that 11 out of the 14 mutants are more susceptible to infection is striking (Fig. 3.1), and suggests that the RNAi pathway is a key antiviral defense.

Vig and AGO2 which encode essential proteins in the RISC complex (Hammond *et al.*, 2001), were both identified as having increased sensitivity to viral infection. We found that *vig*<sup>EY07816</sup> mutants had a dramatic increase in viral sensitivity at all time points, with approximately 80% of the flies dying by 7 d.p.i. *AGO2*<sup>EY04479</sup> mutants displayed similar increases in anoxia sensitivity during DXV infection compared to the average. Of note, flies heterozygous for the deficiency, *Df(3L)Bk10*, which deletes the genomic region encompassing *AGO2*, also resulted in an increase in sensitivity. As a heterozygote, flies with this deficiency would be predicted to express half the amount of AGO2 as wild type. Consistent with this, the increase was

**Figure 3.1: Altered DXV infection survival of RNAi pathway mutant *Drosophila melanogaster*.** Mutant Drosophila line survival (black line) compared to the average infection response (+/- 1 Standard Deviation). Significant alteration in survival was defined from our previous screen as 2 (or more) time points being outside of one standard deviation of the average survival range (gray region). Shown, from top to bottom, left to right, are: *vig*<sup>EY07816</sup>, *AGO2*<sup>EY04479</sup>, *Df(3L)Bk10/TM6B*, *aub* (*aub*<sup>HN</sup> - solid line, *aub*<sup>KG05389</sup> - dashed line), *r2d2*<sup>1</sup>, *armi* (*armi*<sup>1</sup>-solid line w/ squares; *armi*<sup>72.1</sup> – dashed line w/ squares; *armi*<sup>KG04664</sup> – dashed line with circles), *Rm62*<sup>01086</sup>, *piwi*<sup>06843</sup>, *dcr-2*<sup>L811fsx</sup>, *dcr-1*<sup>Q1147X/TM3</sup>, and *spn-E*<sup>1</sup>. The mutant lines in the first two columns are significantly more sensitive to viral infection.



shifted to the later time points (7 and 10 d.p.i.), as compared to the earlier onset observed in the homozygous AGO2 mutant line. This suggests that the host antiviral response is severely sensitive to the amount of AGO2 present and that it may be a limiting component for RISC formation.

In addition to the RISC associated genes identified above, lines with mutations for *aubergine*, *r2d2*, *armitage*, and *Rm62* were also found to have increased susceptibility to viral infection. Mutants in *aubergine* (*aub*<sup>HN</sup> and *aub*<sup>KG05389</sup>), an Argonaute1 homolog that co-localizes with RNAi pathway components (Tomari *et al.*, 2004), displayed dramatic increases in viral susceptibility following infection. Similarly, a mutation in the *r2d2* protein (*r2d2*<sup>I</sup>), which chaperones miRNA/siRNA incorporation into the RISC complex (Liu *et al.*, 2003), also resulted in a similar sensitivity. Two of three examined alleles of *armitage* (*armi*<sup>I</sup> and *armi*<sup>72.I</sup>), which plays a role in RISC assembly (Tomari *et al.*, 2004), had similar increases in viral infection sensitivity. The third *armi* allele (*armi*<sup>KG04664</sup>) showed a response within the wild type range. This allele is due to a P-element insertion approximately 65bp upstream of the *armi* start site. This *armi*<sup>KG04664</sup> allele has not been well-characterized, and our results indicate that it is likely a weak mutation as compared to the other two *armitage* alleles. Mutants in *Rm62* (*Rm62*<sup>01086</sup>), a *Drosophila* ortholog of human p68 which unwinds short dsRNAs in an ATP-dependent manner (Huang and Liu, 2002), also displayed a dramatic increase in viral infection sensitivity. Lastly, *piwi* (*piwi*<sup>i06843</sup>) mutants were also identified as having increased sensitivity to viral infection, resulting in significantly higher mortality at 7 and 10 days post-infection (d.p.i.). The specific role of *piwi* in *Drosophila* RNAi is

not yet fully understood, but *piwi* has been shown to be important in the RNAi pathway (Pal-Bhadra *et al.*, 2002). *piwi* was originally identified for its role in germ line stem cell division (Pal-Bhadra *et al.*, 2002; Cox *et al.*, 2000); how this process might relate to its function in RNAi is currently not understood.

It was interesting to find that the elimination of *Dicer2* (*dcr-2*<sup>L811fx</sup>) or a heterozygous mutant of *Dicer1* (*dcr-1*<sup>Q1147X</sup>/*TM3*) caused no significant increases in susceptibility to viral infection as compared to the wild type range. The *Dicer2* and *Dicer1* genes are essential for the generation of the 21-23bp fragment siRNAs and miRNAs, respectively (Lee *et al.*, 2004). They have been shown to have some overlap in function (Lee *et al.*, 2004). Our results support this, because single mutations affecting either of these viral RNAi pathway RNases fails to cause a deleterious effect on viral immunity.

The mutation in *spindle-E* (*spn-E*<sup>l</sup>), which is involved in intracellular mRNA localization and has been associated with RNAi (Kennerdell *et al.*, 2002; Gonzalez-Reyes *et al.*, 1997), did cause an increase in sensitivity at 10 d.p.i. However, the difference from wild type was not severe enough by our criteria to be considered significant.

These results suggest that, individually, *spn-E*, *dcr-1*, and *dcr-2*, are not as important for an antiviral RNAi response, and that redundancy exists in the RNAi pathway at these select steps. In contrast, the dose effect of the heterozygous *AGO2* deficiency suggests that the activity level of the RISC complex is critical and its decrease results in a dramatically reduced ability to use RNAi against the DXV genome. This is further supported by previous research showing that the most

effective way to reduce RNAi in tissue culture is by knock down of Ago2 (Li *et al.*, 2002). Since RNAi, in general, is less than 100% effective at eliminating a gene transcript, this suggests that AGO2 protein levels are vitally important for RNAi function.

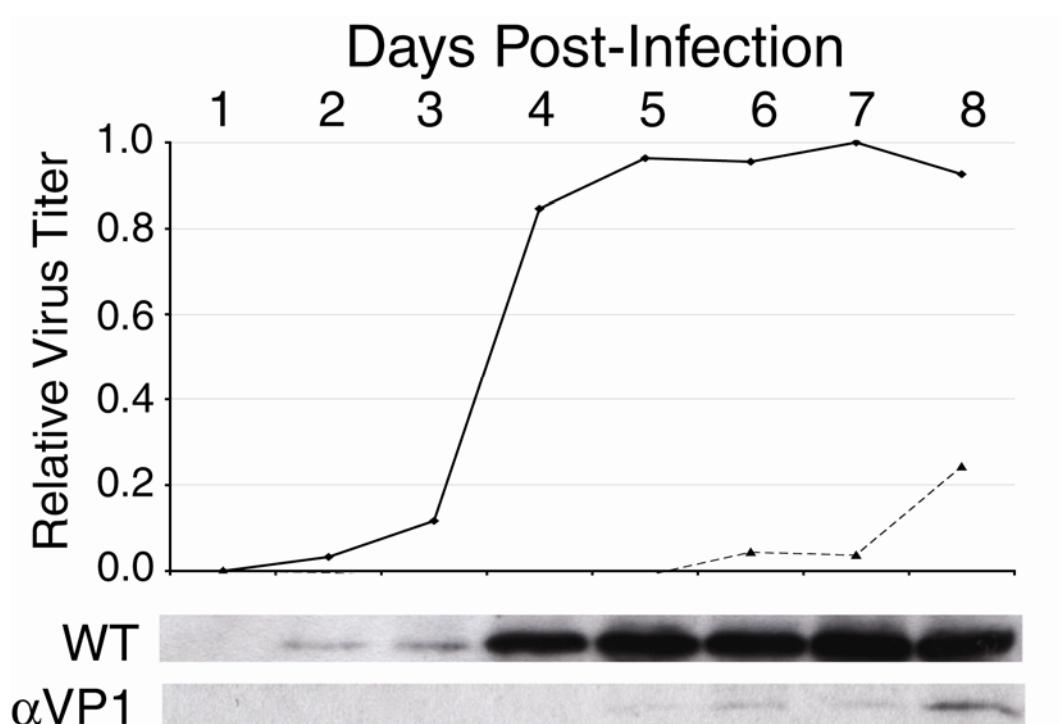
The susceptibility of multiple mutants in the RNAi pathway indicates that this pathway is playing a key role in resistance against DXV infection in *Drosophila*. Also, the results suggest that Piwi may play a larger role in antiviral directed RNAi than previously known.

We next wanted to determine if the increased viral sensitivity in our mutant lines correlated with higher viral titer in these flies. To do this, we used RT-PCR against a 62-bp region of DXV segment B to examine viral titer levels in the mutants relative to wild type *Drosophila* infected with DXV. We previously found increased viral titer at earlier time points correlates well with an earlier onset of anoxia induced death in other mutant lines with increased viral susceptibility (Zambon *et al.*, 2005a). At 3 d.p.i., we found that all the RNAi lines with increased sensitivity to viral infection also had increased levels of viral titer as compared to that found in wild type flies infected with DXV. The increased viral levels ranged from 13 to 40 times that of wild type levels at the same time point (data not shown). These increased viral titers are similar to titer levels found 24 hours later in DXV-infected wild type flies. This suggests that the RNAi pathway is essential for effective inhibition of viral replication in wild type animals.

One reason for the use of Drosophila X virus in our studies is it would theoretically be completely susceptible to RNAi because of the dsRNA nature of its genome and replicative intermediates. During DXV replication, plus-sense ssRNA is extruded from the viral capsid into the cytoplasm. This RNA is then translated and eventually used in viral genome generation. These genome copies, once packaged inside the viral capsid, are believed to be protected from RNAi. Because of this, and our findings that flies impaired in RNAi are more susceptible to infection, we next wanted to determine if inducing RNAi could render cells resistant to DXV infection. To accomplish this, we generated a dsRNA fragment from the coding region (bps 337-791) of the DXV RNA-dependent RNA polymerase (RdRp) VP1 gene, which is essential for viral replication, and knocked down the gene in *Drosophila* S2 cells. Viral protein levels during infection were then examined in media samples taken every 24 hours post infection by Western Blot using a polyclonal antibody against DXV (Fig. 3.2). In untreated DXV infected S2 cells, DXV protein was detectable at 2 d.p.i. and reached a plateau by 4 d.p.i., similar to the plateau observed when examining viral titer via Q-PCR methods in cell culture. Over the course of 8 days, however, DXV protein in RNAi treated DXV-infected S2 cells remained largely undetectable, only appearing faintly at 7 d.p.i.

These results demonstrate, for the first time, that a birnavirus is susceptible to replication inhibition by genome directed RNAi. Additionally, the appearance of viral protein at days 7 and 8 suggests that RNAi is not 100% efficient under DXV infection conditions and/or the viral capsid is protecting the genome from degradation. Protection provided by the virus capsid structure would allow for

**Figure 3.2: Targeted RNAi of the DXV Genome.** Western blots and densitometry measurements of DXV proteins in media samples of infected cell culture untreated (WT, solid line with diamonds) and treated with dsRNA against the DXV RdRp (VP1, dashed line with triangles). VP3, the DXV inner capsid protein, was visualized using Western Blotting and densitometrically quantified using EagleSight software v3.2 (Stratagene) for data analysis. A value of 1 is equal to the density of untreated S2 cells at 7 d.p.i. Westerns were done using polyclonal antibody against DXV, as per (Zambon *et al.*, 2005). The experiment shown is representative of 3 replicates.

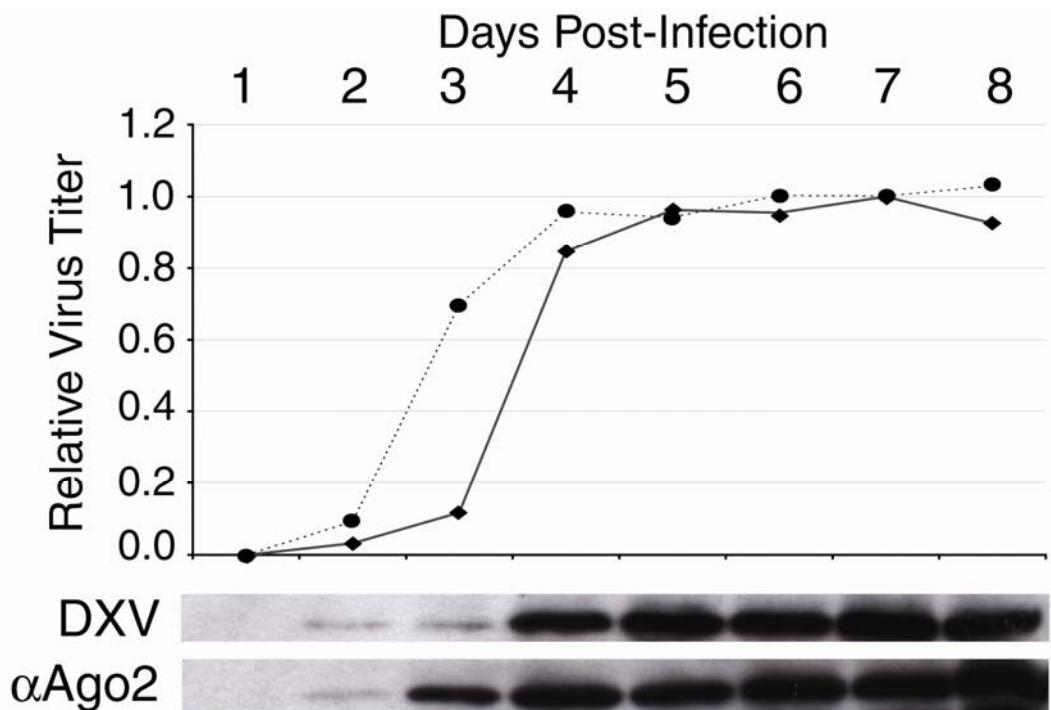


continued production of ssRNA even under highly induced and specific RNAi conditions, essentially overcoming the RNAi reaction by mass generation of plus-sense RNAs which cannot all be degraded prior to the generation of additional viruses. It is important to note that because our system uses 300-500bp dsRNA instead of the 21-23bp siRNAs used in mammalian RNAi systems, it is highly unlikely that viral mutations and selection are the cause of the eventual virus evasion of RNAi (Gitlin *et al.*, 2005).

Once we had determined that RNAi based inhibition was able to limit DXV replication, we wanted to determine if specific RNAi knockdown of the RNAi pathway would result in increased viral replication. To do this, we utilized the S2 cell system so that we could look at cell autonomous effects and confirm our *in vivo* results. It was previously noted that the RNAi pathway can be effectively knocked down by using RNAi to target Ago2, one of the genes identified in our screen (Li *et al.*, 2002). The fact that loss of Ago2 is more effective at inhibiting the RNAi pathway than loss of either of the Dicer RNases suggests little to no redundancy of Ago2 compared to other RNAi components. Additionally, Ago2's role as part of the RISC may be causing its reduction to result in a bottleneck for all RNAi activity.

This allowed for an examination of both the effects of Ago2's knockdown specifically as well as the likely knockdown of the RNAi pathway as a whole. We found that DXV protein levels in the Ago2/RNAi knockdown cell culture reproducibly increased to detectable levels 24 hours prior to the detection in untreated DXV-infected S2 cell cultures (Fig. 3.3). To insure that our results were gene specific and not a nonspecific dsRNA induced response, we performed the above

**Figure 3.3: RNAi of the RNAi pathway in S2 cell culture.** Western blots and densitometry measurements of DXV proteins in media samples of infected cell culture untreated (WT, solid line with diamonds) or treated with dsRNA against AGO2 ( $\alpha$ -AGO2, dotted line with circles). VP3, the DXV inner capsid protein, visualized using Western Blotting and densitometrically quantified using EagleSight software v3.2 (Stratagene) for data analysis. A value of 1 is equal to the density of untreated S2 cells at 7 d.p.i. Westerns were done using polyclonal antibody against DXV, as per (Zambon *et al.*, 2005). The experiment shown is representative of 3 replicates.



experiments using dsRNA against the *LacZ* gene and found that while RNAi could suppress LacZ expression, it had no effect on DXV protein levels over the course of a DXV infection (data not shown). This suggests that the RNAi response must be gene specific and that DXV likely does not encode a general RNAi inhibitor.

In summation, our results demonstrate that the RNAi pathway is being utilized in an antiviral capacity during a DXV infection in *Drosophila*, causing a reduction in both DXV replication and protein production. Additionally, the results in the cell culture knock-down of the RNAi pathway correlate well with the increased viral titers observed in the whole organism. Our studies demonstrate that in cell culture, as well as in animals, the endogenous RNAi pathway is limiting DXV replication during infection, and this is critical for increased host survival.

## Chapter 4

### Conclusions and Future Studies

The innate immune system of *Drosophila melanogaster* is both robust and varied in its responses. While little information previously existed as to if and how *Drosophila* responded to viral infection, our work demonstrates that *Drosophila* possess robust and vital antiviral responses. These responses, based in both traditional immune pathways, such as the Toll pathway, and in traditionally non-immune response pathways, such as the RNAi pathway, provide clues into previously unknown aspects of the innate immune response. Together with development of the DXV infection model system and generation of cDNA clones of the virus' genome, we establish a new model for study of antiviral innate immunity.

Our initial research goal was to develop a model system for the genetic study of the antiviral innate immune response using the genetically tractable *D. melanogaster*. We find that injection of DXV, a dsRNA birnavirus, establishes an infection in *Drosophila* which causes anoxia sensitivity and death. Our examinations of this phenotype defined the timepoint and virus dosage levels required for reproducible use for a genetic screen. We found that over the course of infection there is a correlation between viral titer increase and sensitivity to anoxia.

## Using immunohistology and TUNEL staining in DXV infected WT

*Drosophila*, we observed the viral progression and associated cellular death. It has been proposed that selective tissue infiltration by DXV may be the cause of the anoxia phenotype observed in infected flies (Teninges *et al.*, 1979a). While previous examinations indicate some tissue selectivity for the trachea and fat body that may be occurring early in infection, virus proliferation occurs so rapidly that it is not clear if this specificity is the underlying cause of anoxia sensitivity (Teninges *et al.*, 1979b).

Taken together, we believe these results qualify DXV infection as a viable screening method for several reasons. First, the anoxia based screening is consistent, easily performed, and rapid. Second, the time period between initial infection and anoxia sensitivity onset allows for assessment of immune system compromise severity. Third, the ability to accurately measure viral titer levels *in vivo* allow for determination of whether viral replication in mutants correlates with the pathogenic effects. Lastly, the ability to immunostain sections of animals for viral spread allows for localized tissue effects to be identified in tested mutant lines.

Using this system we have examined the innate immune response in *Drosophila* to determine what pathways play a necessary role in an antiviral response. One aspect of *Drosophila* immunity, the humoral immune response, has been extensively studied as a model for innate immune signaling in mammals (Khush *et al.*, 2001). Current models involve the parallel IMD and Toll pathways. Both pathways result in production of large quantities of secreted AMPs via the activation of NF- $\kappa$ B-related transcription factors as well as signaling for the activation of cellular responses (De Gregorio *et al.*, 2002a; De Gregorio *et al.*, 2001; Irving *et al.*,

2001). While evidence exists that some AMPs may play an antiviral role in mammals, most of the characterized *Drosophila* AMPs work by membrane disruption, and it was not known if they affected viruses (Meister *et al.*, 2000; Bulet *et al.*, 1999). Our examinations of AMP expression in DXV infected *Drosophila* showed an increase in expression of AMPs comparable to that found during an *E. coli* infection of WT flies. It has been previously shown that transgenic expression of single AMPs can rescue survival in immune compromised *Drosophila* (Tzou *et al.*, 2002a). To examine if this was true for viral resistance, anoxia DXV infection experiments were performed on *Drosophila* lines which constitutively expressed a single AMP in all tissues. We found that contrary to what has been found during fungal and bacterial infection, constitutive expression of a single AMP provides no resistance to viral infection or decrease in viral titer. These results concur with known AMP action mechanisms of membrane disruption since viruses lack the cellular membrane structure necessary for these AMPs to be effective. Additionally, viral capsid diversity provides no known conserved target for AMPs to work on across a broad viral range. The increase in AMP expression does show, however, that the IMD and Toll pathways are both activated by DXV infection. This suggests that if AMPs play a role in an antiviral response, they are most likely playing an indirect role.

The lack of direct action of AMPs during viral infection suggests that *Drosophila* mount a preemptive strategy against secondary infections when infected with a viral pathogen. This secondary infection could be caused by the damage resulting from a viral infection - be it damage to the normal barrier tissues that protect from these microbial pathogens or damage to immune tissues, such as the fat body, as

viral infection progresses. By expressing AMPs, even when not directly effective against the primary viral pathogens, secondary infections may be prevented from taking hold. This would lower the stress the flies system is subject to and decrease mortality among virus infected flies. This hypothesis could be tested by examining AMP expression profiles during infection of *Drosophila* with other viruses. It should be possible to determine if virus infection induced AMP expression is simply to prevent secondary infections, as discussed above, or if there may be another reason - which varied AMP expression profiles would suggest. This hypothesis could also be tested examining if flies are more or less susceptible to bacterial infection following virus induced upregulation of AMP.

It should be noted that the possibility also exists that AMPs may be playing a role in the destruction of virally infected cells of the fly. While this is contrary to our results which indicate that the expression of single AMPs fails to be protective during aseptic viral infection, it may be something worth examining further to ensure that AMPs are not acting combinatorially some fashion to elicit an antiviral response. Application of various AMPs to virus-infected S2 cell culture would be an efficient, and easy, means to examine this. What would you assay to see this?

In addition to examining the role of AMPs in a viral infection, we also screened a collection of fly lines mutant in genes predicted or identified as active in the *Drosophila* immune responses against bacteria or fungi (Wu, 2001). *Dif*<sup>1</sup> and *Tl*<sup>10b</sup> mutant fly lines were both identified as being significantly more sensitive to viral infection than other lines examined. Additionally, the *rel*<sup>E20</sup> mutants had no

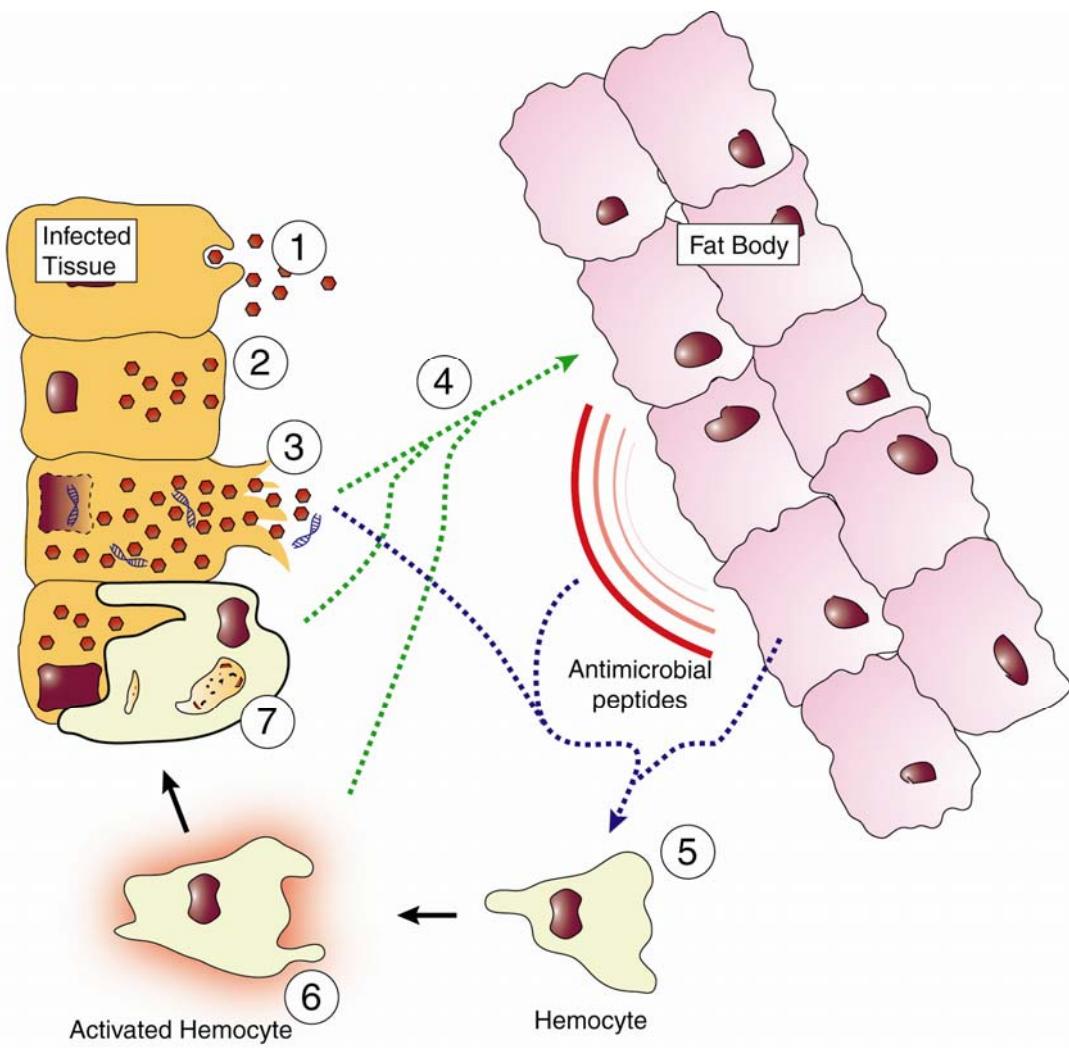
effect on sensitivity to viral infection or viral titer levels. These results indicate that although both the IMD and Toll pathways are activated during viral infection, only the Toll pathway plays a role in viral resistance.

Our experiments also indicate that viral titer levels in the *Dif*<sup>d</sup> and *Tl*<sup>10b</sup> mutant fly lines diverge from levels observed in the WT flies. *Dif*<sup>d</sup> flies have increased viral titer early during infection compared to WT, which directly correlates to the observed increase in anoxia induced death in this line. *Tl*<sup>10b</sup> flies, however, have decreased viral titer compared to WT. Because of the lack of direct antiviral effects provided by single AMPs, we believe that these changes occur due to an alteration of the cellular response in Toll pathway mutants.

The Toll pathway plays a role in the proliferation of hemocytes, which are the effectors of the cellular immune response in *Drosophila* (Qiu *et al.*, 1998). Hemocytes play a critical part in the immune response in flies via phagocytosis and signaling to the fat body (Basset *et al.*, 2000). The importance of these blood cells in fighting infection is apparent when phagocytosis is blocked or in mutants that lack blood cells (Elrod-Erickson *et al.*, 2000; Braun *et al.*, 1998). Hemocytes also play a role in surveillance of healthy and damaged basement membranes and encapsulate and destroy aberrant tissue (Rizki and Rizki, 1984a). Previous observations show that cells undergoing normal apoptosis are recognized by hemocytes through Croquemort, a CD36-like receptor, and that hemocytes are able to act as a correctional mechanism when apoptosis goes wrong (Franc *et al.*, 1999). It has been suggested that cells which do not undergo proper apoptosis are recognized as aberrant. Additionally, it has been shown in Lepidoptera that hemocytes are able to recognize virally infected

cells (Trudeau *et al.*, 2001). We therefore theorize that virally infected cells, which may be displaying characteristics of damaged cells as well as undergoing abnormal apoptosis, are recognized as aberrant and targeted by the hemocytes for phagocytosis and destruction (Fig. 4.1). Cells which are not recognized by hemocytes as aberrant and rupture due to viral replication would also release internal cellular compounds, such as endogenous DNA, into the hemocoel of the fly. In both *Drosophila* and mammals, materials of this type have been shown to instigate immune responses. Undigested endogenous DNA in caspase and DNase mutant *Drosophila* lines has been previously noted to cause multiple immunostimulatory responses, among them melanotic encapsulation of self tissue and constitutive *Diptericin* expression (Mukae *et al.*, 2002; Napirei *et al.*, 2000; Rodriguez *et al.*, 1999; Song *et al.*, 1997). Uric acid, produced during the catabolism of purines, is present at high concentration in cytosol and has been shown to enhance CD8+ T cell responses in mammalian systems (Shi *et al.*, 2003). Our AMP profile data from 24 hrs p.i. shows that of the AMPs whose expression is altered in comparison to *E.coli* infection, *Diptericin* is shifted upwards compared to levels observed in the Gram-negative bacterial infection expression profile. *Diptericin* expression is used as a flag for activation of the IMD pathway, and this activation via undigested endogenous DNA may account for the pathway's activation during viral infection without a corresponding role in viral resistance. Additionally, this suggests that the IMD pathway plays little to no role in stimulating the cellular response during viral infection. These observations suggest

**Figure 4.1: Model for the *Drosophila* antiviral response.** 1. Virus enters system and infects cell. 2. Virus replication. 3. Lysis of infected cell, releasing internal cellular compounds and virus. 4. Released materials activate the IMD and Toll humoral immune pathways and cause local activation of the cellular response. 5. Global activation of hemocytes via Toll pathway signaling. 6. Activated hemocytes signal to the fat body enhancing Toll and IMD pathways activation. 7. Hemocytes recognize aberrant infected cells and engulf and eliminate these cells. Activated hemocytes then enforce stringent recognition of aberrant tissue and destroy infected cells more efficiently. Green arrows indicate signaling to activate the humoral response. Blue arrows indicate signaling to activate the cellular response.



that the decreased viral titer in  $Tl^{10b}$  flies is a result of their increased hemocyte number and increased cellular immune response. Increased sensitivity to anoxia is most likely due to an inability, even in this highly activated immune state, to prevent destruction of select tissues which cause the anoxia sensitivity phenotype.

Studies into *Drosophila* mutants with defects in various aspects of blood cell development, differentiation, and activation would allow for a more complete understanding of how each blood cell, and specific blood cell related activity, plays a role in antiviral immunity. Additionally, these studies would provide more details on our current theory of why constitutively active Toll pathway mutants are more highly sensitive to viral infection yet have lower viral titers than wild type flies.

In addition to mutant examination, a slightly less obvious, and much more difficult, study which would answer some of the questions regarding the role of blood cells during viral infection would be a direct examination of viral interaction with hemocytes. By bleeding out the hemocytes and culturing them in media as a primary cell culture where they are exposed to virus, it could be determined if hemocytes are being infected by DXV and how rapidly it is occurring. Hemocytes from constitutively active Toll mutants could then be examined to determine if changes in the hemocyte-virus interaction could be the cause of the increased sensitivity to viral infection. A determination of whether blood cell phagocytic activity or some other activity is causing the increased sensitivity to viral infection in constitutively active Toll pathway mutant fly lines could then be made.

Based upon the current data, we theorize that the required activation of the Toll pathway for antiviral immunity may be occurring via cellular debris released during cell rupture due to viral replication. Internal compounds may initiate a chain of events which results in Toll pathway activation, similar to the activation of the IMD pathway by endogenous DNA. This may be occurring in a fashion analogous to the activity of cytokines in mammals with hemocytes releasing a signal which activates the Toll pathway.

In addition to our studies into the innate immune system of *Drosophila*, we also examined a collection of RNAi pathway mutant *Drosophila* lines. *AGO2*, *vig*, *aub*, *armi*, *r2d2*, *piwi*, and *Rm62* mutant fly lines were all identified as having increased sensitivity to viral infection when examined by our anoxia screening method. Additionally, all identified lines displayed increased viral titer levels at 3 d.p.i. Our previous research into the antiviral function of the Toll pathway shows a similar increase in viral titers at early time points in select mutants, suggesting that this RNAi response is being utilized alongside a Toll based response to restrict viral replication. These results indicate that the RNAi pathway is essential for an antiviral immune response *in vivo*.

Additionally, we determined that RNAi against the RdRp of DXV is highly efficient at restricting viral replication in cells. This suggests that an RNAi response mounted against DXV is being used by *Drosophila* to restrict virus propagation. However, our results indicate that virus replication is only being restricted by RNAi, not completely halted, as the virus is not being fully cleared. This is apparent by the detection of DXV proteins 7 d.p.i. in cell culture despite continuous addition of

microgram quantities of dsRNA to induce RNAi against the DXV RdRp. This strongly suggests that RNAi, as we propose, is only used to slow virus replication. This slowing of replication would allow for other immune responses, such as the Toll related responses we previously detailed, to be mounted against the virus and begin clearing the infection before it reaches a terminal point.

Four main pieces of evidence are generally cited as support for RNAi as an antiviral mechanism in plants, the system in which RNAi as an antiviral response was first established. Our work provides the final piece of evidence needed to fulfill these criteria for animal systems as well.

Primary among the evidence cited, and the most vital, is that mutations in the RNAi pathway should cause increased susceptibility to viral infection in whole organisms. While it has been demonstrated that RNAi knockdown of one specific part of the RNAi pathway, specifically AGO2, reduces an antiviral response in mosquitoes, our studies broaden that observation by examining flies mutant for multiple RNAi pathway components and determining that these mutations affect virus susceptibility *in vivo*. There are several limitations inherent to the RNAi knockdown of AGO2 as a means to examine the RNAi pathway. First, RNAi itself does not result in total elimination of a gene product, as would occur with a null mutant for an examined gene. The effectiveness of injected dsRNA-induced RNAi to reduce the given gene product *in vivo* is often dependent on both the natural turnover rate of the protein and the ability of the targeted or relevant tissue to take up the dsRNA. Secondly, the use of RNAi to knockdown the RNAi pathway can bias results since producing the desired phenotype (loss of the pathway) depends on activation or use of

the same pathway. Our studies examine how true mutants in RNAi genes are affected by viral infection, and hence, provide the final crucial evidence that demonstrates that RNAi is acting as an effector against viral infection *in vivo*.

Second, induced targeted RNAi is able to suppress viral replication. Many examples exist of such studies showing targeted RNAi is able to suppress viral replication in animals, including FHV (S2 cells), Taura Syndrome virus (*L. vannamei*), and hepatitis C (Huh7.5 cells) (Randall and Rice, 2004; Robalino *et al.*, 2004; Li *et al.*, 2002). Studies using the O'nyong-nyong virus, a dsRNA *Alphavirus*, in *Anopheles gambiae*, have also shown a similar outcome (Keene *et al.*, 2004). Their work shows that *in vivo* co-injection of virus and dsRNA complementary to the virus genome results in reduced viral titers and spread of virus infection through the infected organism, as compared to injection of virus alone. Our work with DXV shows, similarly, that induced targeted RNAi against the DXV virus genome is able to suppress DXV replication in *Drosophila* cells as well.

Third, many viruses have a means of evading total replication suppression by RNAi in the host during infection. This is demonstrated by the FHV's genome encoded RNAi inhibitor and by DXV's ability to eventually continue replication, despite strong genome specific RNAi pressure in S2 cells. Hence, similar evidence for virus evasion of RNAi exists in animal models.

Lastly, infection by viruses induces a strong RNAi response. As observed with FHV, infection by a virus induces a strong RNAi response in *Drosophila* cells, as can be seen by the increased amount of virus specific siRNA post infection (Li *et al.*, 2002). While definitive results have not yet been obtained that show siRNA

production during DXV infection in *Drosophila*, preliminary results (data not shown) have found small RNAs (~50bp) which are detected on Northern blots enriched for small (<200bp) RNAs by DXV genome specific probes. This presents one important future studies which needs to be done to demonstrate that RNAi is important for antiviral immunity in flies. Specifically, measurement of siRNAs specific for the DXV genome in infected flies and cell culture cells needs to be accomplished and optimized into a repeatable assay. Detection of these siRNAs would show definitively that the DXV genome is exposed at one portion of the viral replication cycle to Dicer digestion and that the flies generate siRNAs against DXV. While suppression of RNAi is still possible once this occurs, as is seen with Flock house virus and the use of its RNAi suppressor protein B2, our data showing that LacZ RNAi based inhibition during DXV infection occurs normally would strongly suggest that DXV's primary means of protection from RNAi is provided by the mature virions capsid. Detection of small RNAs, can be done using Northern blots with radiolabelled probes against the DXV genome. . Initial studies would be undertaken in cell culture to eliminate some variables present in whole organisms, and once optimized in cell culture the detection of siRNAs in a whole organism system should be substantially easier to accomplish.

Further studies examining possible RNAi inhibition mechanisms during DXV infection could be accomplished by examining the effects of ectopic expression of various DXV proteins on RNAi of a reporter gene in cell culture systems. By introducing virus proteins individually, instead of during an actual infection, it would be possible to observe and quantify what effects they have on both RNAi, as well as

other cellular processes. These studies have been initiated via the development of cDNA clones of the DXV genome which is detailed in Appendix A, but much work still needs to be done.

Taken as a whole, our studies into antiviral RNAi in *Drosophila* add greatly to the cited evidence to provide the same support for RNAi as an antiviral mechanism in animals and define another aspect of the *Drosophila* antiviral immune response. In addition, when examined with what we have shown in regards to the Toll pathway being required for an effective immune response against DXV in *Drosophila*, additional aspects of antiviral immunity in flies are beginning to become clear. Similarities in susceptibility to viral infection observed between mutant *Drosophila* deficient in the Toll pathway and the RNAi pathway suggest that both are required for an effective antiviral response against DXV infection. Our previous work suggests that the Toll antiviral effects occur systemically (Zambon *et al.*, 2005b). In *Drosophila*, unlike in plants and worms, RNAi effects are not transmitted to neighboring cells and occur only in cells in which dsRNA is initially present or taken up (Van Roessel *et al.*, 2002). Because of this, we believe that RNAi is acting on a cell autonomous level and not systemically, limiting viral replication on a cell to cell basis and slowing infection progression so that the Toll mediated response can act systemically against virus-infected cells and their surrounding tissue.

Overall, we show that *Drosophila X* virus replication is highly sensitive to RNAi based silencing, although the virus can eventually overcome the inhibition. Additionally, and more importantly, we demonstrate that *Drosophila* utilize RNAi as

an antiviral immune response against a dsRNA virus and shown that *Drosophila melanogaster* is an excellent model for the study of antiviral RNAi responses in both whole organisms and cell culture.

While our work in RNAi pathway and Toll pathways has opened the door on an entirely new use of the *Drosophila* as a model organism, additional studies which examine the two antiviral responses jointly still need to be performed.

One aspect of this is to continue forward genetic screens utilizing the DXV based anoxia sensitivity as an assay. Continuation of this screen will allow for the identification of completely novel antiviral pathways. Additionally, further testing of untested genes of identified antiviral pathways, may yield novel components of the Toll and RNAi pathways which have roles specific for antiviral responses.

Additionally, the anoxia sensitivity system can be used to examine interactions between these newly identified antiviral pathways. By examining flies mutant in both the Toll and RNAi pathways we will be able to determine if antiviral effects are combinatorial or synergistic in nature by comparing virus infection susceptibility in joint mutants to that of single pathway mutants. This research into the interactions between the various antiviral pathways should provide more clarity to how *Drosophila* reacts to a viral pathogen on an organismal level, and not solely how one pathway reacts independently of others.

In addition, comparative studies need to be done into other viral pathogens of *Drosophila*. Examination of the mutant lines which were resistant to DXV infection may shed more light on the anoxia sensitivity aspect of the DXV infection pathology. By determining the role of these genes and where they are

expressed (in a localized or global fashion), it may be possible to identify specific tissues and protein functions that are causing anoxia sensitivity to arise in DXV infected flies. Additional studies examining anoxia sensitivity effects symptomatic of other viral infections, such as that of Sigma virus, could be done to determine if similar tissues and pathways are affected to cause anoxia sensitivity. It is not known how these insect viruses induce anoxia sensitivity, so a comparisons of virus localization, receptor binding, and tissue specificity, to determine commonalities in pathology could be highly beneficial for further research involving multiple viruses.

An examination of how the *Drosophila* immune system responds to other viral pathogens, like DCV, would give insight as to which aspects of the *Drosophila* antiviral response are generalized for all viral pathogens or are virus specific. If antiviral responses in *Drosophila* are induced in a pathogen tailored fashion, this work becomes even more intriguing. The *Drosophila* model would then provide a chance to examine what aspects of the virus structure or infection pathology induce a tailored antiviral response. The identification of general antiviral responses extend beyond *Drosophila* immunology and would provide information/knowledge? of significant import to the medical field in regards to potential treatments for viral infections.

A similar comparative study between the birnaviruses DXV, IBDV, and IPNV could give insight into establishment of an infection and host immune responses in regards to the virus itself to determine the extent of similarity between various birnavirus infections. Because of the economic import of both IBDV and IPNV, comparisons of this type could be of great value to the poultry and aquaculture

industries, respectively. The ability to use the genetically malleable *Drosophila* system to shed light on various aspects of IBDV and IPNV infection may allow for more rapid development of vaccines for use in animal populations as well as possible treatments for infected animals because of the ease of use of the *Drosophila* system. Because of *Drosophila*'s rapid lifecycle and low maintenance cost, multiple experiments could be accomplished prior to movement into a fish or chicken system for finalization of various antiviral therapies.

In conclusion, our work has succeeded in revealing antiviral responses in *Drosophila melanogaster* for the first time. The existence of these responses opens up the *Drosophila* model system to what until now has been a relatively unexplored field of research. With the extensive innate immune system homology between *Drosophila melanogaster* and mammalian systems, discovery of these responses will allow examination of various aspects of the innate immune system which were either too difficult – or impossible – to examine when using mammalian models. The increased reproduction rate and short lifespan of *Drosophila*, as well as release from restrictions imposed upon studies in mammalian systems, allows for more rapid studies to be performed on many aspects of the innate immune system. Additionally, lack of a traditional mammalian adaptive immune system in *Drosophila* allows for unfettered examination of innate immune responses. The use of immune system research into *Drosophila* results from these studies will allow for a more comprehensive understanding of the mammalian innate immune system, and more targeted and effective study design for research on mammalian systems.

## **Appendix A: Development of a Drosophila X Virus Reverse Genetics System**

### **INTRODUCTION**

In the current post-genomic age, it is not only advantageous but necessary to have a developed molecular genetics toolbox to perform meaningful genetic studies of a system. At the outset of our research, the tools available for use with DXV were limited to small samples of purified virus and minute quantities of purified genomic RNA which degraded rather rapidly. Sequencing of the genome of DXV had been done by performing stepwise reverse transcription-coupled PCR followed by traditional sequencing methods, but these small fragments only provided a means to obtain the DXV genome sequence and were not sufficient for looking into the genome and proteome of DXV (Accession Numbers: DXV-A: NC\_004177; DXV-B: NC\_004169). Even more importantly, it provided no material for generating a mutant virus. It did however provide the initial information needed to develop the materials needed to make this possible.

DXV, a member of the genus *Entomobirnavirus* of the *Birnaviridae* family, has a bisegmented double-stranded RNA genome (Teninges *et al.*, 1979b). The smaller of the two segments encodes VP1, the viral RNA dependent DNA polymerase. The larger segment encodes a 128 kDa polyprotein in a single large ORF which is processed co-translationally into VP2, VP3 and VP4. VP2 and VP3

are the major capsid proteins of the virus, and VP4 is a non-structural processing protease. Additionally on segment A is a secondary ORF which encodes a 27kDa putative non-structural protein.

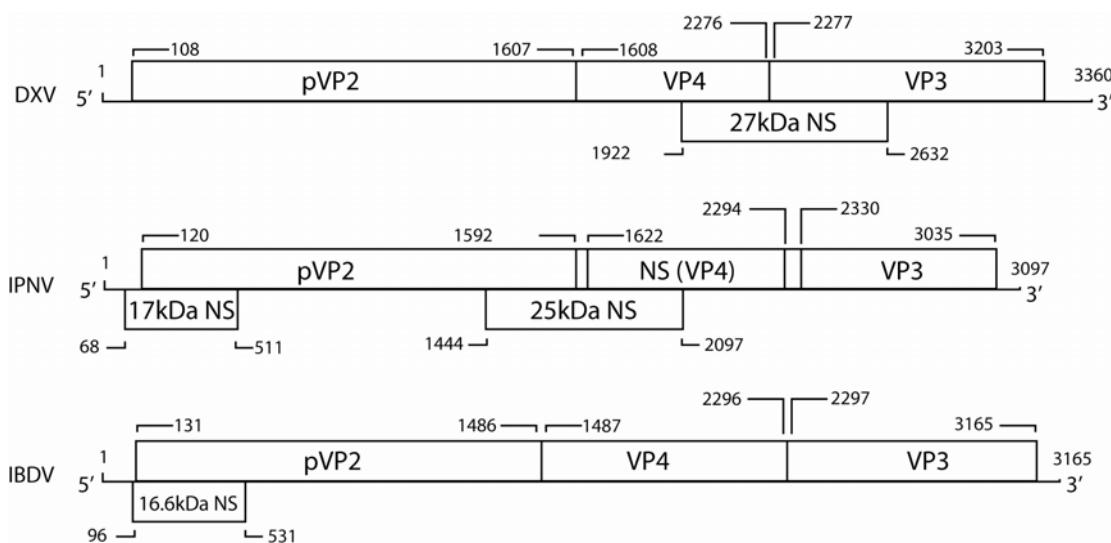
This putative protein, the focus of the work described here, is interesting because of a number of features it possesses. First, a very high proportion (27%) of its 237 amino acids are basic, 31 of which are arginines (Fig. A.1). This gives the hypothetical protein a pI value of 11 (Chung *et al.*, 1996). Additionally, the C-terminal end of the protein contains a bipartite nuclear targeting sequence. This sequence and the highly basic nature of the protein suggest that it has a nucleic acid binding function, although no evidence exists of this yet.

Sequencing data from other birnaviruses, specifically IPNV and IBDV, reveals that both of these viruses also possess small non-structural arginine-rich peptides in secondary reading frames on segment A. These peptides (IPNV-VP5 and IBDV 17-kDa NS), however, are placed at the 5' end of the segment, not overlapping the central region of the polyprotein between the VP4 and VP3 junction (Fig. A.2) (Chung *et al.*, 1996; Mundt *et al.*, 1995; Magyar and Dobos, 1994). They do however, have 23% overall residue homology with the DXV-NS protein (Chung *et al.*, 1996). The function of these small non-structural proteins during IPNV and IBDV infection is currently unclear, but evidence exists that suggests they play a role in apoptosis. Expression of the IDBV-NS protein from a plasmid expression vector induces apoptosis in chick embryo fibroblast (CEF) cells, and mutant IBDV viral

Figure A.1: **Protein sequence of the DXV-NS 27kDa putative ORF.** The NS proteins bipartite nuclear targeting sequence motif (residues 204 to 220) is defined by two basic residues (bold), a space of ten amino acids (italics), and at least three basic residues out of the next five (bold).

MLSIIRRKTRIVDITKQGNGNVPPACHLC S RRLTKEWSFG	1 - 40
EGTICSFHRIRCRVQCGRSFWHQPETPIDGLSWDSTTWEL	41 - 80
TRISSSKDPDISGQKDKRYGRRKEKNPKTDPPALDSRVRE	81 - 120
HEPIHEHEPIPGRVGPADTKQRCKANLRGDSGFVSIGRSN	121 - 160
HPKLSREDCHNTRVPPGTQGVRGGNVQLDKPRERPVSYQH	161 - 200
GS <b>KKR</b> SEHRNPVSR <b>S</b> <b>HRRKK</b> A KTRTKTSKL GKS RDIC	201 - 237

**Figure A.2: Placement of NS ORF's in various Birnaviruses.** Drosophila X Virus (DXV), Infectious Pancreatic Necrosis Virus (IPNV), and Infectious Bursal Disease Virus (IBDV) genome segment A's are diagrammed above showing the placement of the NS proteins which are present in ORFs other than the main ORF which expresses the segments main polyprotein product. Note that IPNV is diagrammed as the Sp serotype which possesses a 25kDa NS ORF unique to the serotype as well as the 17kDa NS ORF found in all IPNV serotypes.



strains lacking the protein have reduced apoptotic effect *in vivo* (Jungmann *et al.*, 2001; Yao and Vakharia, 2001). Limited evidence exists regarding the IPNV-NS protein, with data only demonstrating that the protein is not essential for replication or virulence in cell culture (Song, 2003).

It's unclear as to whether these NS proteins are analogous to DXV's 27kDa NS ORF because of their altered placement within the viral genome. There does exist, however, a strain of IPNV (Sp serotype) that contain a strikingly similar internal ORF in segment A that may encode another putative non-structural protein in addition to the one coded for by the secondary ORF at the 5' end of genome segment A. This 25kDa protein, similar to the small NS proteins mentioned earlier, is highly basic. It also contains a nuclear targeting signal similar to the bipartite nuclear targeting signal in the DXV-NS protein. These two proteins, DXV-NS and IPNV-Sp-NS, share a 12.7% identity and 44% similarity (Fig. A.3). Unfortunately, as with the DXV-NS protein, this IPNV NS protein has not yet been detected *in vivo*. Additionally, recombinant viruses with the ORF start site altered appeared to have no effect on virus replication in CHES cell culture trials (Song, 2003).

**Figure A.3: Alignment of the deduced amino acid sequence of the putative 25 kDa protein of IPNV-Sp and 27 kDa protein of Drosophila X virus (DXV).**

Potential bipartite nuclear targeting sequences in IPNV (residues 46-71 and 97-113) and DXV (residues 203-221) are italicized. Modified from Shivappa *et al.*, 2004.

The high degree of similarity between all of these proteins, when compared directly to each other, is striking. Also of great interest is that these two ORFs, which overlap the primary reading frame of the virus genome, are placed in drastically different positions yet still share high homology with one another. Even though the a between DXV-NS and the 25-kDa putative protein of IPNV-Sp is unknown, they imply a need for their presence. This is strongly supported by the studies showing the importance of IPNV-VP5 and IBDV 17-kDa in their respective viruses.

Because of the previous work on the homologous NS proteins in IPNV and IBDV and the strong possibility that DXV-NS could be a factor in DXV virulence and host interaction, we developed the basic components of a reverse genetics system for the DXV virus. During this development we also generated cDNA clones of the entire virus genome, a mutant cDNA clone of DXV genome segment A that does not possess the 27kDa NS ORF, and performed initial attempts at generation of transgenic viruses.

## MATERIALS AND METHODS

### Agarose Gel Visualization of DNA

Agarose gels of 1% were created with 1uL of 40mg/mL EtBr solution per 10mL of gel and run on an Owl EasyCast™ Mini Gel Electrophoresis System. Gels were run at room temperature at 100-200V and visualized under UV light. Photographs were taken using the Eagle Eye II Still Video System (Stratagene).

## **DNA Gel Extraction**

All DNA gel extraction was performed with the QIAGEN QIAquick Gel Extraction Kit using the manufacturers centrifuge based protocol. Unless noted, nucleic acids purified in this fashion were run in an agarose gel as per the Agarose Gel Visualization methods noted above.

## **DXV Nucleic Acid Extraction**

An aliquot of stock virus was taken and diluted into an equal volume of RNase free H<sub>2</sub>O. This solution was then treated with Proteinase-K treated for 90 minutes. 5M NaCl was then added to the solution to bring it to 0.15M with respect to NaCl. An equal volume of Phenol/Chloroform/Isoamyl Alcohol(25:24:1) mixture was then added. The solution was vortexed briefly, centrifuged at 10,000g+ for 5 minutes, and the aqueous layer was transferred to a new tube. A volume of Chloroform/Isoamyl (24:1) mixture equal to the original solution volume was then added, the solution vortexed, centrifuged at 10,000g+ for 5 minutes, and the aqueous layer was then transferred to a new tube. This step was then repeated once again. 100% Ethanol was then added to the solution (2.5x the volume of the original solution), incubated at -20°C for 30 minutes, then spun at 10,000g+ for 5 minutes.

All liquid was then aspirated off and the pellet was washed in 70% ethanol. The ethanol was then removed and the pellet was air dried at room temperature for 10 minutes. It was then resuspended in 20uL of DEPC H<sub>2</sub>O.

### **Reverse Transcription Protocol**

For all reverse transcription reactions involving DXV genomic DNA, the same standard protocol was used, the only changes being the primers utilized. DMSO (1uL) and one of the two primers used in the RT-PCR (100 pM) reaction were added to the genomic RNA (5uL), and the solution was denatured at 97°C for 7 minutes. The mixture was then brought to 42°C and RT-Mix (3uL 10x PCR Buffer, 6uL 25mM MgCl<sub>2</sub>, 8uL dNTPs, 4.5uL DEPC-treated H<sub>2</sub>O, 1uL RNase inhibitor, 1uL Reverse Transcriptase (Invitrogen)) was then added to the solution. The solution was then incubated at 42°C for 60 minutes, heated to 95°C for 5 minutes to inactivate the Reverse Transcriptase, and then placed on ice. PCR-Mix (7uL 10x PCR buffer, 2uL 25mM MgCl<sub>2</sub>, 0.5uL TAQ polymerase, 100pM second primer, DEPC water to 70uL total volume when added to RT-PCR reaction) was then added and the entire mixture was subjected to PCR (95°C for 30s, 37°C for 30s, 72°C for 1.5min, Repeat 35x, 72°C for 10min, Hold 4°C).

## **Restriction Digestion Protocol**

All restriction digests were done as per manufacturer's protocol for the particular restriction enzyme being utilized. All digests were done at 37°C for 1 hour. Double digests were done in the buffer which provided the highest combined activity for both enzymes used.

## **Generation of Full Length cDNA clones of the DXV Genome**

To generate the full length genome of DXV RNA segments A & B, viral RNA was first isolated and then used as templates in an RT-PCR reaction (as above) with four separate reverse transcription reactions, one for overlapping regions from each end of the segment. This was done using the following primers, which also add a T7 site (underscore, green) and *SacI* (bold, red) site to the 5' ends of each genomic segment and a *PstI* site (italics, blue) to the 3' end of each genomic segment (Fig. A.4):

DXV-A Reactions:

Reaction 1 (DXV-A 5' End)

DXVA-F1: 5'-**GAG CTC** TAA TAC GAC TCA CTA TAG GAA AAT AAA TGG CGG CCG ACA AGG-3'

DXVA-R1: 5'- GTT CCC AAG TAG TGG AAT CCC AAG -3'

Reaction 2: (DXV-A 3' End)

DXVA-F2: 5'-CGC TTA CTC AGC TGA CTC C-3'

DXVA-R2: 5'-**CTG CAG** GGA ACC ACC ACG GAT TCG ATA CCG -3'

DXV-B:

Reaction 1: (DXV-B 5' End)

DXVB-F1: 5'-**GAG CTC** TAA TAC GAC TCA CTA TAG GAA AAT AAT TGG  
CGG CCG ATA AGG-3';

DXVB-R1 5'-GTG AGT TAC GTC CCA GCC CAG CAT GTC CAT C-3'

Reaction 2: (DXV-B 3' End)

DXVB-F2: 5'-GGT CTG AGA TAG GTA AGC CAC-3';

DXVB-R2: 5'-**CTG CAG** GGA GCC GCC CAA TTT ACA TTT GG-3'.

These reactions generated two 1.6kb fragments with overlapping regions containing a restriction site (*MfeI*) for each segment. All reactions were visualized on 1% TAE agarose gels using EtBr staining. Bands of the correct size were excised and DNA was extracted from the gel fragments using the Qiagen QIAquick Gel Extraction Kit (Qiagen Inc). Isolated DNA was then cloned into the pCR2.1 using the Invitrogen TOPO-TA Kit as described by the manufacturer. Plasmid DNA was obtained from transformed culture using the Promega Wizard Plus Mini- and Midi-

Prep kits as per manufacturer's instructions. Clones were sequenced inward in the 5' and 3' direction to ensure proper sequences were obtained.

Segment clones were then restriction digested with *MfeI* (37°C, 1hr) and each segment pair (DXVA-5' and DXVA-3'; DXVB-5' and DXVB-3') were ligated together at this site to form a full length cDNA clone of that segment. This full length cDNA was also ligated into the pUC19 vector, which had been previously digested with *SacI* and *PstI*. All clones were sequenced following ligation. The DXV-A clone was named pUC19-DXVA, and the DXV-B clone was named pUC19-DXVB.

This method is diagrammed in Figure A.4 for further clarification.

### **Generation of the DXV-ΔNS cDNA clone**

Using pUC19-DXVA as source DNA material, a site-directed mutational PCR was performed using three primers which encompass a region containing the 27kDa ORF's start site and is flanked by an *NcoI* restriction site near its 5' and an *MfeI* restriction site near its 3' end. This is further diagrammed in Figure A.5 for clarification. The first primer used is DXVA-F2, and the other two are as follows:

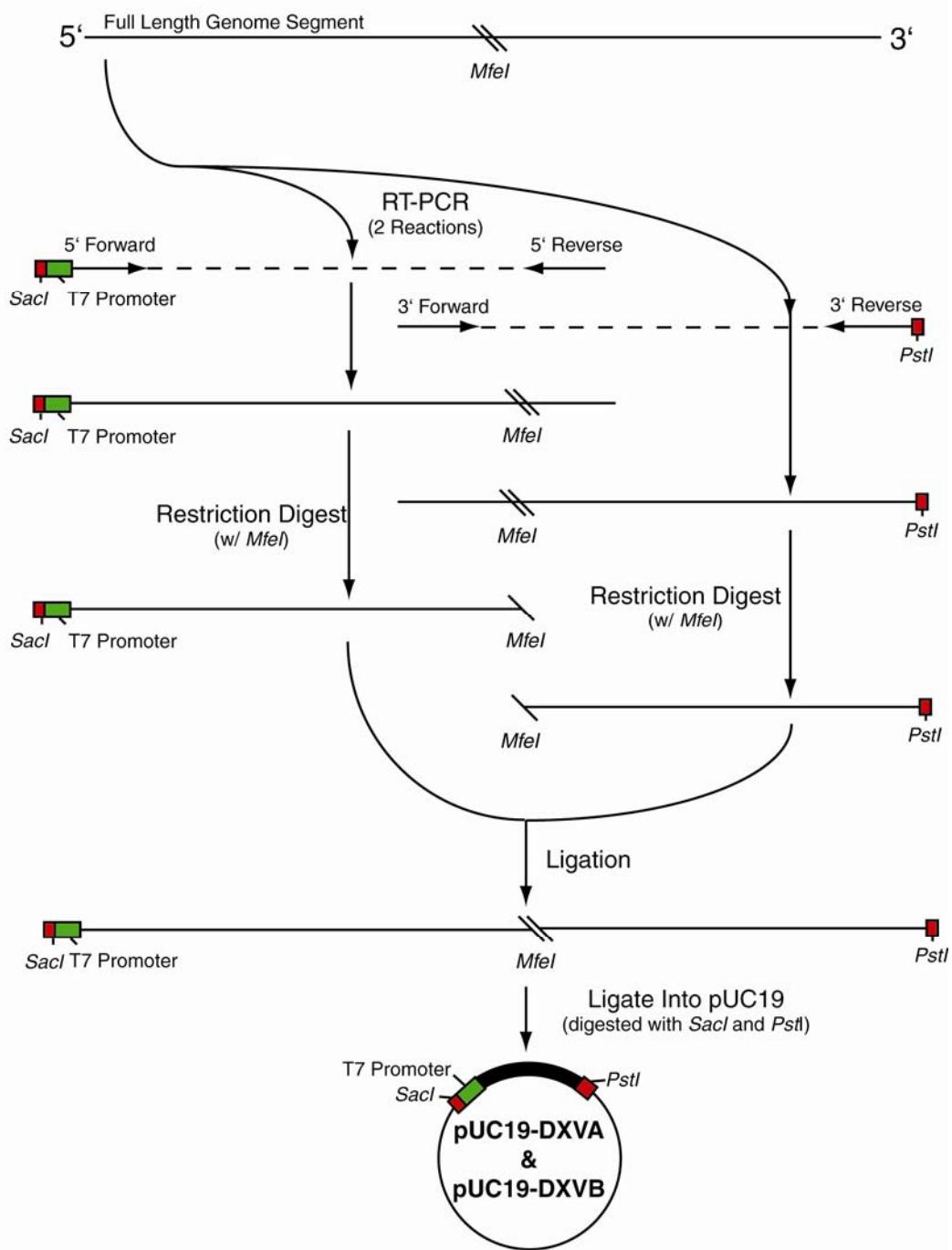
DXVA-R3: 5'-GTT CCC AAG TAG TGG AAT CCC AAG-3'

DXVA-Mut: 5'-CAT CCT CCT ACC GTG CTA TC-3'

The PCR product was visualized by gel electrophoresis and the appropriate sized band (195bp) was purified from the gel and cloned into pCR2.1-TOPO using the Invitrogen TOPO-TA Kit as described by the manufacturer and sequenced.

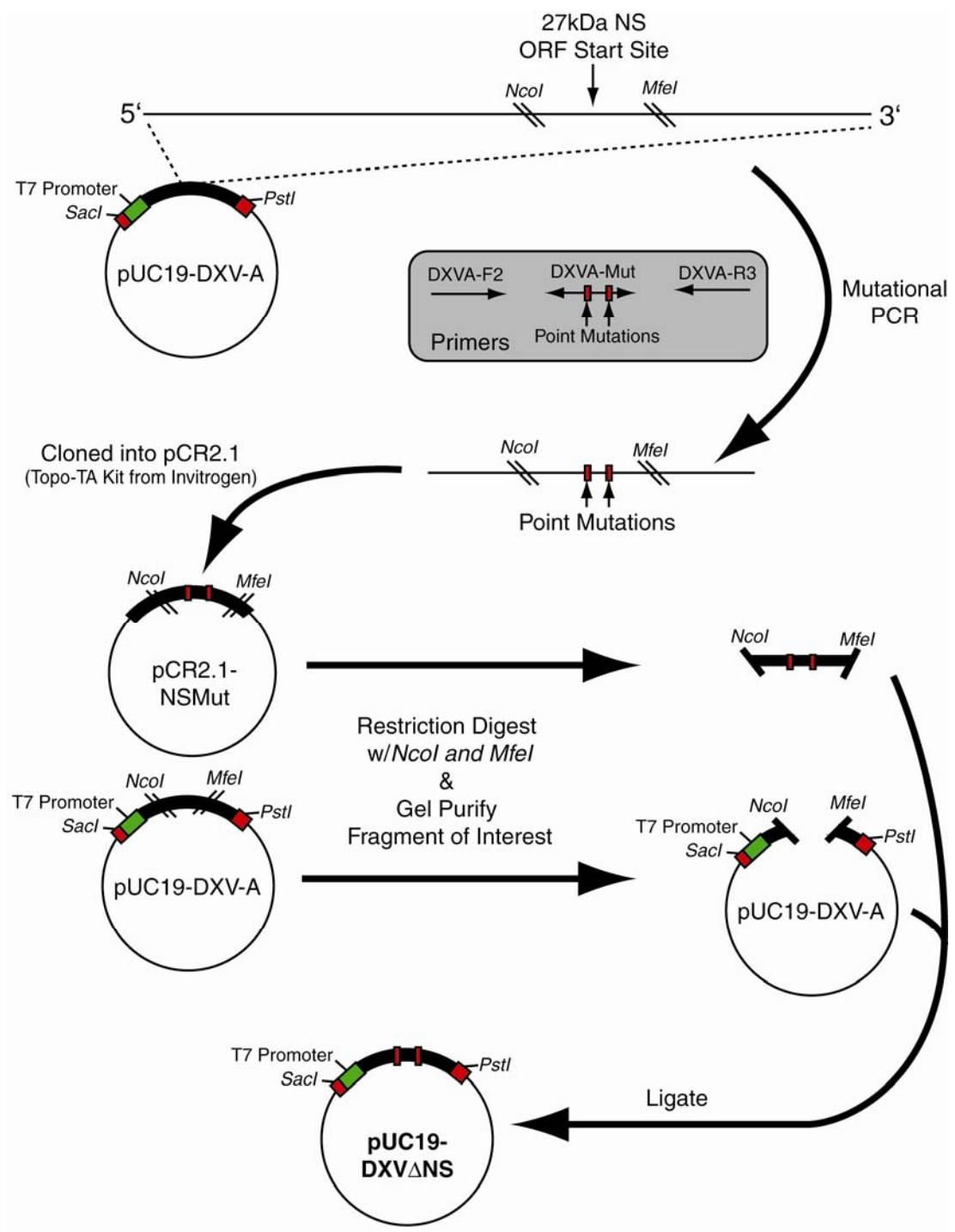
**Figure A.4: Generation of a cDNA clone of a DXV genome segment.** Viral RNA is first extracted from purified viral particles. This full length RNA is then used as a template in four RT-PCR reactions, two for each genome segment. Each segment is reverse transcribed using primers which generate two ~1.7kb cDNA fragments. In addition, the 5' segments 5' primer adds a *SacI* restriction site and T7 promoter site to the 5' end of the fragment. The 3' segments 3' end primer adds a *PstI* restriction site to the 3' end of the fragment. Each genome segment will thus have a 5' fragment and 3' fragment generated by this set of RT-PCRs. The fragments from each respective genome segment are then restriction digested using *MfeI* and then ligated together to form a full length cDNA of that fragment. The full length cDNA is then clones into pCR2.1 and transformed into *E. coli* for long term storage.

## Purified Viral RNA



**Figure A.5: Protocol for the generation of a mutant DXV cDNA genome segment**

**A.** Using the full length cDNA clone of DXV genome segment A (pCR2.1-DXVA) as template material, mutation PCR is performed using 3 primers, a 5' primer, 3' primer, and a mutational primer. These primers are all included with the template DNA during a PCR reaction and introduce the mutations noted in Fig. 5.6 (pCR2.1-DXV $\Delta$ NS). This mutated fragment is then cloned into pCR2.1 using the Invitrogen Topo-TA kit, plasmid is generated, and then restriction digested with *NcoI* and *MfeI*. The small mutated portion of the plasmid is then purified by gel purification. Concurrently, the pCR2.1-DXVA plasmid is similarly restriction digested and the larger portion of the plasmid is gel purified. These two gel purified DNA fragments are then ligated together and retransformed into *E. coli*. This ligated plasmid, pCR2.1-DXV $\Delta$ NS, now contains an additional restriction site (*ClaI*) and a mutated 27kDa NS ORF start codon.



Plasmid DNA was obtained from transformed culture using the Promega Wizard Plus Mini- and Midi- Prep kits as per manufacturer's instructions. A clone identified with the correct sequence (with mutations at both points, as noted in Fig. A.6) was named pCR2.1-NSMut. Separately, pCR2.1-NSmut and pUC19-DXVA were restriction digested with *NcoI* and *MfeI*, then fragments of appropriate size (195bp for pCR2.1-NSMut; ~3kb for pUC19-DXVA) were gel purified. These purified DNA fragments were then ligated together. The clones were then sequenced and, based upon confirmation of the sequence, a correct clone was found and named pUC19-DXV $\Delta$ NS.

### **Ethanol Precipitation**

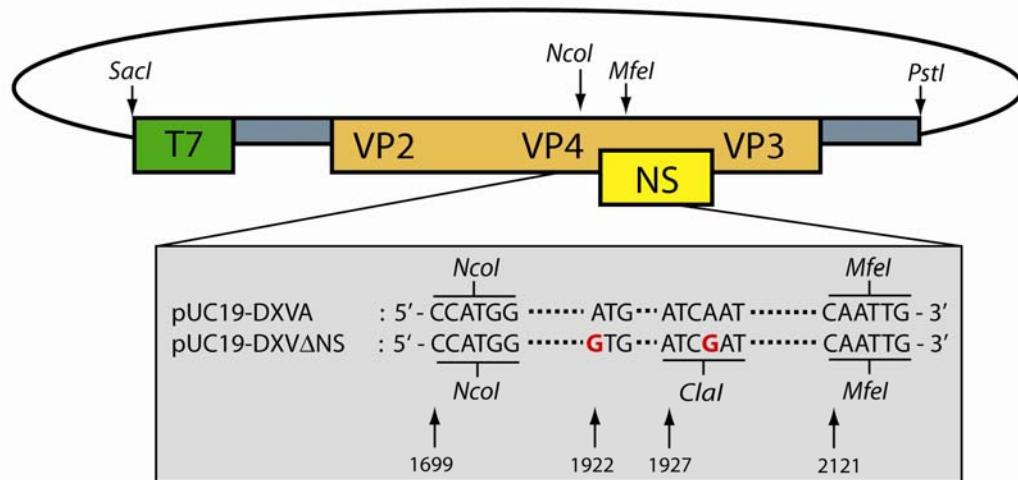
3M sodium acetate was added to the nucleic acid sample at a ratio of 1/10 the total volume and mixed. Three volumes of EtOH were then added to the sample and mixed. The solution was then precipitated overnight at -20°C and the nucleic acid recovered by centrifugation. The pellet was air dried and resuspended in DEPC-treated H<sub>2</sub>O and the concentration was quantified by spectrophotometry.

### **Phenol/Chloroform Extraction**

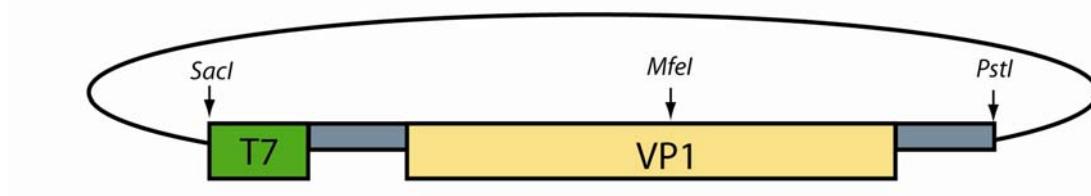
5M NaCl was added to the sample being extracted to bring the solution to 0.15M with respect to NaCl concentration. An equal volume of Phenol/Chloroform/Isoamyl Alcohol (25:24:1) mixture was then added. The solution was vortexed briefly, centrifuged at 10,000g+ for 5 minutes, and the aqueous layer was transferred to a new tube. A volume of Chloroform/Isoamyl Alcohol (24:1)

**Figure A.6: Plasmid maps of cDNA constructs of DXV (pCR2.1-DXVA, pCR2.1-DXVB, pCR2.1-DXV $\Delta$ NS).** A genomic map of segments A and B under the control of a T7 RNA polymerase promoter cloned into plasmid vector pCR2.1 is shown. Restriction enzymes and their restriction sites used for cloning are indicated. Plasmid pCR2.1-DXVA $\Delta$ NS is modified as shown in the inserted box; the resulting nucleotide changes are in red boldface.

### pUC19-DXVA



### pUC19-DXVB



mixture equal to the original solution volume was then added, the solution vortexed, centrifuged at 10,000g+ for 5 minutes, and the aqueous layer was then transferred to a new tube. This step was then repeated once again. 100% Ethanol was then added to the solution (2.5x the volume of the original solution), incubated at -20°C for 30 minutes, then spun at 10,000g+ for 5 minutes. All liquid was then aspirated off and the pellet was washed in 70% Ethanol. The ethanol was then removed and the pellet was air dried at room temperature for 10 minutes. The pellet was then resuspended in DEPC-H<sub>2</sub>O, in a volume dependent upon the original sample and the procedure it was being used in.

### **DXV Nucleic Acid Extraction**

An aliquot of stock virus was taken and diluted into an equal volume of RNase free H<sub>2</sub>O. This solution was then Proteinase-K treated for 90 minutes. The solution was then Phenol/Chloroform extracted as above. Once the pellet was dried, it was resuspended in 20uL DEPC-treated H<sub>2</sub>O.

### **Transcription and Transfection of Synthetic RNAs**

Plasmids pUC19-DXVA, pUC19-DXVB, and pUC19-DXVΔNS, were used in a PCR reaction with the 5' and 3' genomic strand end primers used in cDNA creation (pUC19-DXVA & pUC19-ΔNS: DXVA-F1 and DXVA-R2; pUC19-DXVB: DXVB-F1 and DXVB-F2), ethanol precipitated, and used as templates for *in vitro*

transcription with the Ambion MegaScript T7 Kit (used as per manufacturer's instructions). T7 transcriptions were incubated at 37°C for 2hrs. Synthetic RNA transcripts were purified by Phenol/Chloroform extraction and ethanol precipitation. The concentration was brought to 1 ug RNA per uL with DEPC-treated H<sub>2</sub>O. Once purified, transcripts were immediately used in transfections.

*Drosophila* S2 cells were transfected using the lipid-based reagent CellFectin in a protocol adapted from the published procedure (Invitrogen). Log phase cells of at least 95% viability were centrifuged at 1000 x g and the supernatant removed from the cell pellet by aspiration. Cells were resuspended in *Drosophila* Serum-Free Media (dSFM) (Invitrogen) without antibiotics and quantified using a hemacytometer and trypan blue stain to determine viable cell numbers.

Cells suspended in serum-free Schneider's media were added to 6-well plates at approximately 3 x 10<sup>6</sup> cells per well. The total volume per well was 3 mL. The S2 cells were placed in a sealed container to maintain humidity and prevent evaporation and incubated at 28°C for at least one hour to allow attachment.

For each transfection, 3µg of a DXV-A (DXV-A or DXV-NS) and 3µg of a DXV-B prepared synthetic RNA transcript were added to 250µL of dSFM (6µg of synthetic RNA transcript in total). In a separate tube, 8 µL of the CellFectin reagent was diluted in 250 µL serum-free media. The two solutions were mixed well separately, combined, and mixed well together. The solution was incubated at room temperature for 20 minutes. The transfection mixture was then diluted with a further 800 µL of serum-free media. Media was aspirated from the wells and the dilute transfection mixture overlaid on the cells.

The cells were incubated at 28°C for 4 hours and the transfection mixture aspirated from the wells. The cells were washed once with dSFM and then incubated at 28°C after the addition of 3mL of fresh dSFM.

Samples were collected every 24 hours and examined via Western Blotting to check for the presence of DXV proteins in cell culture.

### **Transcription and Translation Assay**

Plasmids pUC19-DXVA and pUC19-DXVB (2 $\mu$ g/each) were used in the TNT Reticulocyte Lysate System containing [ $^{35}$ S]-labeled methionine as per manufacturer (Promega) instructions. The reactions were carried out at 30°C for 60 minutes. Methionine-labeled products were then resolved by SDS-PAGE and detected by autoradiography.

## RESULTS

### Generation of cDNA clones of DXV Genome Segments A and B

Generation of cDNA clones of genome segments A and B were performed as noted in Materials and Methods (Fig 5.4). During generation, sequencing was performed of the first clone products created (~1.6kb each, 2 for each segment) to compare their sequence to the previously known DXV sequence. This sequencing was done in both the 5' and 3' direction using the M13 primer sites located within the pCR2.1-TOPO vector. Sequencing using these primers allowed for analysis of the sequence of each segment, leaving a small gap of less than 100bp within each central region which overlapped between clones. Each preliminary fragment was also visualized on agarose gels to ensure proper overall size.

After ligation and subcloning of the newly generated full length cDNA clone of each segment, the full length clones were sequenced in both the 5' and 3' directions as well. This was done using the M13 primer sites preplaced within the pUC19 vector. This sequencing allowed for confirmation that no alterations occurred within the end regions of the full segment as well as ensuring proper placement within the vector. As above, visualization was also done on agarose gels to ensure proper segment size.

Once all full length clones were generated, they were examined via a Transcription and Translation (TnT) assay, again noted in the materials and methods. This assay shows, crudely, the expression profile of the two genomic cDNA clones.

Due to time constraints, knowledge of the sequence, and technical issues surrounding the use of radioactive materials, this assay was never repeated in a better fashion to get cleaner results. This is discussed below in regards to the reverse transgenic system.

Together, the sequencing data most of all, along with further work using the constructs in other research, has fully validated these cDNA clones as full and correct clones of the DXV RNA genome and supports their use in various molecular methodologies where they are needed.

The final clones are diagrammed in Figure A.6.

### **Generation of NS cDNA clone of DXV Genome Segment A**

A DXV genome segment A with site directed mutations to remove the 27-kDa NS ORF start codon and introduce a restriction site, was generated using mutational PCR as noted in Materials and Methods (Fig. A.5). During generation of this cDNA clone all validation methods were replicated as those used during the generation of the DXV-A and DXV-B clones. In addition, the final generated clone was restriction digested using the mutationally generated *ClaI* site to ensure that the clone was in fact a mutant clone. The final ligated clone, pUC19-DXV $\Delta$ NS, was included in the aforementioned TnT assay. Additionally, sequencing data and agarose visualization gels show the validity of the mutated clone as a full length DXV-A clone with the two PCR generated point mutations.

The final clone is diagrammed in Figure A.6.

## **Generation of DXV via a reverse genetics system**

To generate a NS-DXV virus it was necessary to first develop a reverse genetics system for DXV in general. Reverse genetics systems have been developed for IBDV and IPNV, two other birnaviruses. We attempted to use the same methods with our generated cDNA clones of the DXV genome to generate transgenic virus.

In short, the generalized birnavirus reverse genetics system functions via the transfection of positive-sense viral mRNA into cell culture cells which are translated by the cells, generating recombinant virus (Fig. 1.5). This required a full length ss-mRNA to be introduced into the culture cells, in our case S2 cells. We first attempted this by transfection of the pUC19-DXVA and pUC19-DXVB based mRNA into S2 cell culture using the CellFectin Transfection reagent (as per Materials and Methods). Trials were performed using this system with multiple concentrations of mRNA, ranging from the standard 3ug quantities as recommended by the manufacturer to the entire quantity of a T7 reaction (>50ug). Additionally, trials were done with mRNA obtained directly from the T7 reaction, with no cleaning step prior to transfection. Lastly, attempts were made at transfection using chemical based methods instead of the CellFection reagent. Unfortunately, none of these attempts generated wild type virus. We believe this may be due to the rapid degradation of the mRNA within the cell culture media and we currently have no method to circumvent this issue. It is unclear what differences exist between the IBDV and IPNV systems and the DXV system which cause these problems to arise, although neither the IBDV nor IPNV

systems are extremely efficient. It has been noted, in passing, that these systems work relatively poorly, if at all, and this low success rate could be the cause of much of our problems.

## **DISCUSSION**

Our development of the basic molecular tools needed to perform research into DXV and its host interactions has been met with both success and failure. Marked among the successes is the development of the cDNA clones from the viral RNA genome and generation of a mutant NS cDNA clone of DXV genome segment A. Unfortunately, we were not successful in developing a functioning reverse genetics system. Extensive trials were performed to try to generate virus, specifically wild type, utilizing a reverse genetics system similar to that developed previously by Vakharia and co-workers for IBDV and IPNV. Unfortunately, these trials met with no success.

However, the successes that were obtained were quite substantial. The development of the cDNA clone of DXV has provided the basic materials needed to further work with DXV on a molecular level. Examples of this work is the ability to generate RNAi constructs targeting specific portions of the DXV genome as well as the ability to generate expression constructs for specific DXV proteins. Previously this would rely upon reverse transcription of a constantly shifting virus RNA genome, but we now have a stable genetic profile from which all future study can be performed. Additionally, the ability to use this cDNA to develop RNAi constructs

was very important. This ability has allowed control experiments to be done which let us examine virus pathology and *Drosophila* anti-viral immune responses in various conditions, such as the knockdown of Ago2 to examine the effects of a reduced RNAi response on viral infection (see Chapter 3 for more information). In addition to these successes and failures, other work has been done (Savage, 2004) which studied further the NS protein and relied heavily upon these cDNA clones to accomplish its goals.

While the initial reasons for examining the DXV-NS protein still exist, it is clear that further examination of the protein will require more work into the successful development of a reverse genetics system for DXV. Without this system, a true understanding of the protein's role in infection will not be known and, at best, can only be inferred by effects which transgenically expressed 27kDa protein has on cells. Unfortunately, this is unlikely to exactly match its expression during viral infection and hence would only provide a partial picture of what is occurring. One of the first steps in this research will be a proper TnT assay examination of the cDNA clones of DXV-A and DXV-B. With elimination of background it can be determined if the cDNAs are able to express the correct proteins alone. This will be a major piece of data for troubleshooting the DXV reverse genetics system and advancing the system to full functionality in the future.

Overall, these studies developed the basic molecular tools needed for research into DXV in multiple other areas, and provided us with the ability to look into places which we could not have before. The inability to develop a reverse genetics system is only a minor setback, and is likely due to the difficulty inherent to the reverse

genetics system. Even without the reverse genetics system, however, the majority of other work performed into DXV infection and immune responses to this infection would not have been possible without the development of the cDNA clones of the DXV genome.

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