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NUTRITIONAL REGULATION OF *VITELLOGENIN* GENE TRANSCRIPTION IN THE YELLOW FEVER MOSQUITO *AEDES AEGYTPI*

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Major Professor's Signature

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NUTRITIONAL REGULATION OF *VITELLOGENIN* GENE TRANSCRIPTION IN THE YELLOW FEVER MOSQUITO *AEDES AEGYTPI*

By

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ABSTRACT

MECHANISMS OF NUTRITIONAL REGULATION OF *VITELLOGENIN* GENE TRANSCRIPTION IN THE YELLOW FEVER MOSQUITO *AEDES AEGYTPI*

By

Geoffrey Michael Attardo

The defining characteristic of anautogenous mosquitoes is the requirement of a blood meal to initiate reproduction. During vitellogenesis, a key event in reproduction, *yolk protein precursor* (*YPP*) gene expression is activated to a very high level. In the period prior to blood feeding (previtellogenesis), anautogenic mosquitoes maintain a state of reproductive arrest during which the *YPP* genes are repressed. Throughout vitellogenesis, 20-hydroxyecdysone (20E) is the major activating factor of *YPP* transcription. However, previtellogenic mosquitoes are not responsive to exogenous 20E treatment. In the anautogenous species *Aedes aegypti*, the regulatory region of the major *YPP* gene *vitellogenin* (*vg*) contains multiple GATA binding sites. Previous work suggested that a GATA factor (AaGATAr) acts as a repressor preventing activation of this gene prior to a blood meal. RNAi knockdown of the *AaGATAr* gene results in increased expression of the major *YPP* gene, *vg*, and its increased responsiveness to 20E stimulation. This research presents *in vivo* data confirming the role of AaGATAr as a repressor of the *vg* gene during the previtellogenic state of arrest.

Exogenous treatment with 20E is not capable of activation of vitellogenesis, suggesting that another signal is required for hormonal activation

of the *vg* gene. Previous data suggested that nutritional signals are important for egg development. It is shown here that amino acids (AAs) directly signal to the fat body, resulting in an increase in transcription of the *vg* gene. Furthermore, 20E activation of *vg* is dependant upon AA signaling. Based upon withdrawal studies a number of individual AAs are essential for this process. The AA signal is transmitted by the nutritionally regulated TOR kinase signal transduction pathway as was determined through inhibition of this system by the specific inhibitor of the TOR kinase, rapamycin.

Further analysis of AA signaling in vitellogenesis was performed through the cloning and characterization of the *Ae. aegypti* homologue of the cationic AA transporter *slimfast* (*slif*), which is associated with fat body nutritional signaling in *Drosophila melanogaster*. *Aaslif* is expressed in the fat body and appears to be negatively regulated by amino acids. RNAi knockdown of *Aaslif* results in inhibition of AA stimulation of *vg*, but does not affect 20E stimulation of *vg* in the presence of AAs.

These studies together demonstrate that AaGATAr inhibits *YPP* gene expression prior to a blood meal, and that after blood feeding AAs directly signal the fat body to derepress *YPP* genes and initiate vitellogenesis.

DEDICATION

To Douglas

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AA

Aas

adh

Ae.

Ae.

Ae.

Ae.

An.

AS

ATF

BM

CAT

C. pi

C. ta

C/EB

cDNA

CHC

CHO

CRE

D. me

KEY TO SYMBOLS OR ABBREVIATIONS

20E	20-hydroxyecdysone
AA	amino acid
AARE	amino acid response element
Aaslif	*Aedes aegypti* slimfast
adh	alcohol dehydrogenase gene
Ae. aegypti	*Aedes aegypti*
Ae. albopictus	*Aedes albopictus*
Ae. atropalpus	*Aedes atropalpus*
Ae. taeniorhynchus	*Aedes taeniorhynchus*
An. gambiae	*Anopheles gambiae*
AS	asparagine synthetase
ATF	activating transcriptic[illegible]actor
BM	blood meal
CAT	cationic amino acid transporter
C. pipiens	*Culex pipiens*
C. tarsalis	*Culex tarsalis*
C/EBP	CAAT box enhancer binding protein
cDNA	complementary DNA
CHC	clatherin heavy chain gene
CHOP	C/EBP homologus protein
CREB	cyclic AMP response element binding protein
D. melanogaster	*Drosophila melanogaster*

DBD	DNA binding domain
DNA	deoxyribonucleic acid
EcR	ecdysone receptor
EcRE	Ecdysone response element
EMSA	electrophoretic mobility shift assay
fbp1	fat body protein 1
hnf3/fkh	hepatocyte nuclear factor 3/forkhead
igf1	insulin like growth factor 1
IRES	internal ribosomal entry sequence
JH	juvenile hormone III
Lp	lipophorin
mRNA	messenger RNA
NCR	nitrogen catabolite repression
NSRE	nutrient sensitive response element
OEH	ovarian ecdysiotropic hormone
PBM	post blood meal
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
sgs	salivary gland secretion gene
slif	slimfast
TOR	target of rapamycin
tRNA	transfer RNA

USP	ultraspiracle
VCB	vitellogenic cathepsin B
VCP	vitellogenic carboxypeptidase
vg	vitellogenin
YPP	yolk protein precursor

CHAPTER 1: LITERATURE REVIEW

Introduction

Mosquito borne diseases are listed among the diseases having the greatest impact upon human health worldwide. Diseases such as dengue fever and malaria affect huge numbers of people. 2.5 billion people are at risk for dengue infection, and Malaria infects between 300-500 million people and kills roughly 1 million people (most of whom are young children) per year worldwide (CDC, 2003; WHO, 2003). These diseases were thought to have been brought under control during the 50's and 60's by reduction of mosquito populations through large-scale vector control programs. A resurgence of these diseases began during the late 70's and 80's and continues today due to a complex array of factors such as pesticide resistance, lack of effective vaccines, and drug resistance by the parasites in the case of malaria (Krogstad, 1996; Gubler, 1998). In locations such as sub-Saharan Africa, this situation in combination with other devastating diseases like AIDS and tuberculosis as well as economic, sociological, political, and environmental problems creates a crisis of vast proportions (Collins and Paskewitz, 1995; Butler et al., 1997; Corbett et al., 2002).

Another mosquito borne disease, West Nile virus, has recently emerged in the United States. West Nile virus emerged on the east coast of the U.S. in 1999. Within four years the virus spread across the entire country (CDC, 2004). While this disease does not have the impact that malaria or dengue have upon world health, it demonstrates how under current conditions new mosquito borne diseases can emerge and rapidly spread across a large geographic area. The

hypothetical consequences of a more virulent mosquito borne disease spreading as quickly are unnerving. The reproductive biology of the mosquito vectors of these diseases is an important target for the development of novel disease control strategies. A detailed understanding of how this process functions on a molecular level may reveal new ways to disrupt the process of disease transmission as well as yield new molecular tools and uncover the basic biology which will pave the way for further research.

Mosquitoes are effective disease vectors because the adult female mosquitoes obtain the nutrients needed for egg development from the blood of vertebrates (haematophagy). The need for blood brings mosquitoes in close contact with multiple host organisms making them an efficient vehicle by which parasites can be spread from host to host. Over millions of years, parasites have evolved to take advantage of this unique niche that facilitates their transmission from host to host. Mosquitoes are also successful as disease vectors due to their large reproductive capacity (>100 eggs per reproductive cycle), which results in large numbers of these vectors being generated in a short period of time (Clements, 1992). The tight link between blood feeding and reproduction in mosquitoes efficiently drives the spread of parasites from one host to another by mosquitoes.

The Physiology of Anautogeny

Many species of blood feeding insects have evolved to regulate their reproductive cycle around the acquisition of a blood meal. The requirement for blood to initiate reproduction is termed anautogeny. Consequentially, many of

these insects are potent disease vectors (Table 1). Mosquitoes are the most significant vectors on the list in terms of the number of diseases that they transmit and the number of people that those diseases affect.

Table 1. Anautogenous Arthropods and the Diseases They Vector

Common Name	Latin Name	Disease	Pathogen
Mosquito	Aedes aegypti	Yellow fever virus	Flaviviridae
Mosquito	Aedes spp.	Dengue fever virus	Flaviviridae
Mosquito	Anopheles spp.	Malaria	*Plasmodium falciprum*
Mosquito	Various species	Filarial elephantiasis	*Brugia malayi, Wucheria bancrofti*
Mosquito	Various species	West Nile virus	Flaviviridae
Mosquito	Culiseta melanura, Coquillettidia pertubans, Aedes vexans	Eastern equine encephalitis virus	Flaviviridae
Tsetse fly	Glossina sp.	African sleeping sickness	*Trypanosoma brucei*
Black fly	Simulium sp.	River blindness	*Onchocerca volvulus*
Sand fly	Phlebotomus sp.	Leishmaniasis	*Leishmania spp.*
Kissing bug	Rhodnius prolixus	Chagas' disease	*Trypanosoma cruzi*
Cat flea	Xenopsylla cheopis	Plague	*Yersinia pestis*
Body louse	Pediculus humanus	Epidemic typhus	*Rickettsia typhi*
Deer tick	Ixodes sp.	Lyme disease	*Borrelia burgdorferi*
Dog tick	Dermacentor sp.	Rocky mountain spotted fever	*Rickettsia rickettsii*

Different species of mosquitoes have developed variations on the requirement of blood for oogenesis. These variations range from mosquitoes that do not require blood at all, to mosquitoes that must have a blood meal to

begin oogenesis. Mosquitoes that do not require blood for reproduction are termed autogenous and can be either obligately or facultatively autogenous. Mosquitoes that do require blood are considered anautogenous; this condition can also be obligatory or facultative depending upon the species and conditions. The terms autogeny and anautogeny were first defined in 1929 (Roubaud, 1929). Since then many species of mosquitoes have been characterized as to the type of reproductive strategy they undertake (Vinogradova, 1965; Rioux et al., 1975).

Mosquitoes show a range of phenotypes in their autogenic or anautogenic tendencies. Strains of the pitcher plant mosquito *Wyeomyia smithii* in the northern region of North America (>40° N) do not blood feed at all (O'Meara and Lounibos, 1981). Some species of mosquitoes begin their adult life autogenously and switch to anautogeny after their first reproductive cycle. Many strains of *Aedes atropalpus*, only blood feed after having laid their first batch of eggs (O'Meara and Craig, Jr., 1969). Other facultatively autogenous mosquitoes will feed immediately if given the opportunity, but can develop a first batch of eggs without feeding if blood is not available, as is the case with the black marsh mosquito *Aedes taeniorhynchus*. There is a cost to autogenous egg development in these mosquitoes as egg numbers are significantly reduced in non-blood fed mosquitoes when compared to blood fed mosquitoes (O'Meara and Evans, 1973; O'Meara and Edman, 1975). Some facultatively autogenous mosquitoes require specific environmental and nutritional conditions during larval development to allow them to develop eggs autogenously (O'Meara, 1979).

Finally, obligately anautogenous mosquitoes under no circumstances develop eggs without a blood meal. Some species that for the most part are strictly anautogenous, have strains which are autogenous. An example of this is found in *Ae. aegypti*, which is usually obligately anautogenous. Autogenous "feral" strains of *Ae. aegypti* have been identified in Africa that live away from their normal human hosts in the jungle. These mosquitoes appear to have developed autogenous reproduction in response to a reduction in host density (Trpis, 1977).

Typically, the most effective disease vectors are obligatorily anautogenous for example: *Ae. aegypti*, the vector of dengue fever and *Anopheles gambiae*, the primary vector of malaria. The focus of this thesis was to identify and characterize some of the molecular mechanisms that specifically *Ae. aegypti* and more generally other anautogenous mosquitoes have developed to control their tightly regulated reproductive system.

To understand how expression of autogenous and anautogenous reproductive modes in mosquitoes are regulated, a number of studies have been conducted comparing the differences between these two reproductive types. Environmental factors are an important determinant of the expression of autogeny or anautogeny in some facultatively autogenous mosquito species. A study done on different populations of *Ae. taeniorhynchus* in Florida shows that the expression of autogenous egg development is very different from site to site. These differences are a result of a genetic factors, but it is hypothesized that host

abundance at the individual sites has shaped these predispositions in different populations (O'Meara and Evans, 1973).

Photoperiod and temperature are important regulators of autogenic expression as well. In the mosquito *Culex tarsalis*, longer photoperiods result in a large increase in the expression of autogeny while shorter photoperiods prompt anautogeny (Harwood, 1966). Temperature also plays an important role in the expression of autogeny in these mosquitoes. At temperatures below 21°C expression of autogeny is severely reduced (Brust, 1991). The sensitivity to photoperiod and temperature by these mosquitoes is most likely an adaptation that promotes survival and reproductive success during the fall and winter months when conditions are harsh and resources are scarce.

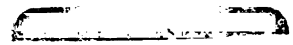
Another environmental factor shown to affect expression of autogeny is poor larval nutrition and stress. *C. tarsalis* larvae that were fed on a reduced diet and or exposed to crowding during development, developed into adults expressing much lower levels of autogenic egg development (Reisen et al., 1986). Finally, the mating status of the female mosquitoes has also been shown to have an effect on the ability to develop eggs without a blood meal. In *Ae. taeniorhynchus*, autogenous egg maturation will not begin until the female has mated with a male (O'Meara and Evans, 1976). It is hypothesized that a substance from the male accessory gland included with the sperm is responsible for this stimulus.

In addition to environmental stimuli, genetics are a critical determinant of the reproductive capabilities of a mosquito. Data from extensive crossbreeding

experiments between autogenous and anautogenous strains of *Culex pipiens* suggests that in these mosquitoes there are most likely multiple genes responsible for conferring the autogenous phenotype. Of the three chromosomes, these genes are linked to chromosome one (the sex chromosome) and chromosome three, creating a partial sex linkage. Expression of autogeny appears to be gene dosage dependant (Spielman, 1957).

In *Ae. atropalpus*, which has both autogenous and anautogenous strains, a similar genetic analysis was performed. The genetics of autogeny in this mosquito appear to be significantly different, as the trait of autogeny appears to be conferred by a single dominant autosomal gene (O'Meara and Craig, Jr., 1969). Further research demonstrated that while the gene conferring autogeny is dominant in this species, there are other modifier/enhancer genes that optimize the level of fecundity in mosquitoes carrying the autogeny gene. When these genes are removed through crosses with anautogenous strains, efficiency of autogenic egg development decreases (O'Meara, 1972). The genes responsible for conferring or supporting autogenic or anautogenic status have not yet been identified.

There are many factors regulating the expression of autogeny and anautogeny in mosquitoes. What are the physiological differences and changes that are happening in the mosquito as a result of these genetic and environmental stimuli and how do they result in one or the other form of reproductive strategy? Comparative physiological analyses between autogenous



and anautogenous mosquitoes show significant differences between mosquitoes expressing these two reproductive types.

Nutritional analysis of autogenous and anautogenous strains of *C. tarsalis* shows the autogenous mosquitoes having greater amounts of total lipids, total carbohydrates and total proteins. Interestingly, male mosquitoes from the autogenous strain were also found to have higher levels of nutrients than males from the anautogenous strain (Su and Mulla, 1997a). A similar analysis was performed in autogenous and anautogenous strains of *Aedes albopictus*. This analysis also found that the autogenous strains contained significantly higher energy reserves in the form of metabolizable protein and lipid stores (Chambers and Klowden, 1994).

A more specific nutritional analysis determined the levels of free AAs in the hemolymph of these autogenous and anautogenous strains immediately after eclosion. Total free AAs levels were significantly higher in the hemolymph of autogenous strains. Specifically, arginine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, threonine and valine showed the greatest difference in concentration between the two strains (Su and Mulla, 1997b).

Life cycle analysis of autogenic and anautogenic *C. tarsalis* shows that the autogenic strains take a day longer to complete larval development, but are able to lay eggs one to two days earlier than their anautogenous counterparts. However, the first batch of eggs laid by the autogenous mosquitoes contains significantly fewer eggs than those laid by the anautogenous strains (Reisen and Milby, 1987).

A disparity between nutritional reserves appears to be a common theme that arises upon comparison of autogenous with anautogenous mosquitoes. It appears that the environmental and genetic factors that facilitate autogeny to take place in some mosquitoes result in an accumulation of nutrients in the larval stage, which is then carried into the adult stage. This abundance of nutrients somehow triggers the activation of egg development in these mosquitoes. This information is important because it highlights the fact that anautogenous mosquitoes maintain relatively low levels of systemic nutrients and this most likely prevents egg development. Furthermore, it indicates that nutritional cues are playing a critical role in this system in mosquitoes. The nutritional deprivation in anautogenous mosquitoes is lifted when these mosquitoes feed on nutrient rich blood, which consequentially marks the beginning of egg development. To better understand the role of nutrition in egg development, we will examine what is known about reproduction in *Ae. aegypti*. The reproductive physiology of *Ae. aegypti* is well studied and is the system examined in this thesis.

Mosquito Vitellogenesis

Blood feeding initiates the process of vitellogenesis, a key physiological event in mosquito reproduction. Vitellogenesis is the utilization of nutrients from a blood meal for the large-scale synthesis and secretion of yolk protein precursors (YPPs) in a tissue called the fat body. The fat body is analogous to vertebrate liver and fat tissues. Fat body cells are specifically responsible for the synthesis of YPPs in mosquitoes (Hagedorn and Judson, 1972; Hagedorn et al., 1973). The main YPPs genes activated during vitellogenesis are *vitellogenin*

(*vg*), *vitellogenic carboxypeptidase* (*vcp*), *vitellogenic cathepsin B* (*vcb*) and *lipophorin* (*lp*). Out of these genes *vg* is the most highly expressed. Vitellogenesis occurs exclusively in the fat body and is divided into three main stages: previtellogenesis, vitellogenesis and post vitellogenesis (Raikhel et al., 2002).

The mosquito fat body is a functionally diverse tissue. Its functions range from storage and metabolism to protein synthesis during the mosquito's life cycle. These changes in function are regulated in a hormonally dependent manner (Raikhel, 1987a). Upon eclosion from pupae to adult, the fat body undergoes hormonally regulated changes that allow it to become responsive to signals that induce vitellogenesis. A transient peak in juvenile hormone III (JH) occurring immediately after eclosion is associated with these changes (Flanagan and Hagedorn, 1977; Raikhel and Lea, 1990; Hagedorn, 1994). These changes make the fat body competent to respond to the steroid hormone 20-hydroxyecdysone (20E) and to synthesize the massive amounts of protein required for egg maturation (Raikhel and Lea, 1983; Dittman et al., 1989). The orphan nuclear receptor AaFTZ-F1 has recently been shown to be essential for attainment of competence by the fat body to respond to 20E. Furthermore, the translation of AaFTZ-F1 is regulated by the presence of JH (Li et al., 2000; Zhu et al., 2003a). Once remodeling of the fat body has been completed and hormonal competence has been attained, the fat body enters a reproductive state of arrest during which it becomes dormant and *YPP* gene expression is repressed.

The state of arrest is relieved upon blood feeding by the female mosquito and vitellogenesis begins. Activation of vitellogenesis causes the fat body to undergo further structural changes that facilitate protein synthesis (Snigirevskaya et al., 1997). Blood feeding also stimulates an increase in JH esterase activity and inhibition of JH production causing a rapid decrease in the basal hemolymph JH titer (Readio et al., 1998). Another response to blood feeding is the secretion of a neurosecretory hormone releasing factor from the ovaries (Lea, 1967). This factor in turn causes the release of ovarian ecdysteroidogenic hormone (OEH) from the brain, which in turn stimulates the production of the steroid hormone, ecdysone, by the ovaries (Hagedorn et al., 1975). Ecdysone travels to the fat body and is hydroxylated to 20E. 20E titers increase and peak at 24 hours post blood meal (PBM), and then rapidly decline back to basal levels by 36 hours (Hagedorn, 1985; Hagedorn, 1989).

Molting, metamorphosis and reproduction are regulated in part by 20E in *D. melanogaster* and other insects (Hagedorn, 1989; Segraves, 1994; Thummel, 1996). 20E is the primary stimulus that up regulates *YPP* gene expression. *YPP* gene expression and protein synthesis positively correlate with 20E titers. When 20E titers decline, YPP synthesis declines as well (Hagedorn, 1985; Raikhel, 1992; Deitsch et al., 1995). After activation by 20E *YPP* genes are transcribed and translated specifically in the fat body. The YPP proteins are then processed in the fat body and secreted into the hemolymph where they travel to the ovaries and are endocytosed by the developing oocytes. At around 36 hours PBM the

fat body converts back to a nutrient storage and metabolism function until the next vitellogenic cycle is initiated (Figure 1)(Raikhel, 1992).

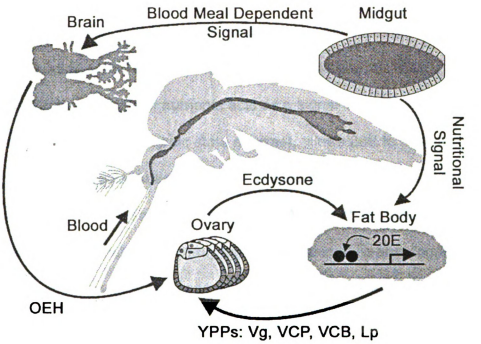


Figure 1. Schematic diagram of blood meal activation of vitellogenesis

20E is a main regulator of vitellogenesis (Spielman et al., 1971), but there are results that imply that 20E by itself is not sufficient to activate vitellogenesis. Observation of YPP secretion indicates that these genes are expressed before hemolymph levels of 20E begin to increase (Hagedorn et al., 1975). Other experimental results show that injection of physiological levels of 20E into previtellogenic mosquitoes is not sufficient to induce YPP synthesis and consequent egg development. Only very large (non-physiological) doses of 20E are able to activate low levels of YPP expression (Borovsky D. and Van Handel, 1979; Fuchs and Kang, 1981; Lea, 1982b). However, 20E injections into autogenous mosquitoes successfully activate YPP synthesis (Fuchs and Kang,

1981). These data suggest that a second control mechanism maintains the state of arrest in anautogenous mosquitoes. In contrast to these results, *in vitro* cultured fat bodies from 3-5 day previtellogenic mosquitoes treated with physiological levels of 20E successfully initiate vitellogenesis. The media in which the fat bodies are cultured in contains AAs (Raikhel et al., 1997). This suggests that the availability of nutrients acts as a signal to the fat body, which then deactivates the previtellogenic state of arrest, allows 20E to activate *YPP* genes and activates vitellogenesis. Further support for this hypothesis comes from experiments in which solutions containing AAs and physiological levels of 20E have been infused into the hemolymph of previtellogenic anautogenous mosquitoes; these mosquitoes successfully undergo egg development (Uchida K, 1998). These data together with the physiological analyses of autogenic and anautogenic mosquitoes further supports the idea of a nutritional requirement for vitellogenesis to begin in mosquitoes.

The Molecular Biology of Vitellogenesis

A more detailed understanding of how this system is regulated on a molecular level has been obtained through the cloning and characterization of the *vg* gene, which codes for the major YPP (Romans et al., 1995). Analysis of the regulatory region contained within the 2.1 kb 5' of the transcription start site for the *vg* gene yielded significant insights into the mechanisms regulating transcription of this gene in response to blood meal related stimuli. The *vg* 5' regulatory region has been divided into three main units (Figure 2) (Kokoza et al., 2001).

The most proximal region is responsible for fat body specificity and is required for 20E stimulation. This region contains functional binding sites for the

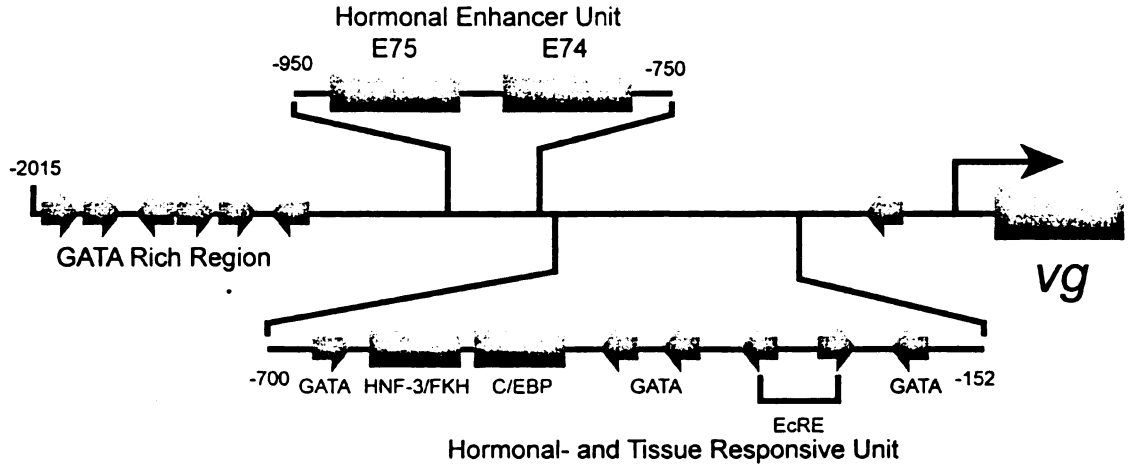


Figure 2. Schematic diagram of the regulatory region for the vitellogenin gene. EcRE – ecdysone response element; GATA – binding site for GATA family of transcription factors; C/EBP – binding site for C/EBP family of transcription factors; HNF-3/FKH – binding site for HNF3/FKH family of transcription factors; e75 – binding site for the e75 transcription factor; e74 – binding site for the e74 transcription factor; *vg* – open reading frame for the vitellogenin gene

EcR/USP, GATA, C/EBP, and HNF3/fkh transcription factors. 20E acts upon *vg* through the nuclear hormone receptor heterodimer EcR (Ecdysone receptor)/USP (retinoid X receptor homolog). Upon binding 20E, EcR/USP binds a EcR/USP response element (EcRE) and activates transcription (Yao et al., 1993; Riddiford et al., 2000). 20E stimulation works directly on *vg* through EcR/USP binding to the EcRE (Martin et al., 2001b; Zhu et al., 2003b). It also works indirectly through a hierarchy of 20E activated intermediate genes called early genes that code for transcription factors which bind in another region of the promoter (Sun et al., 2002; Sun et al., 2004). Comparison of the proximal regulatory region of *vg* and the regulatory regions of other fat body-specific

genes from *D. melanogaster* demonstrates the presence of similar binding sites and organization. Binding sites for factors from the HNF3, C/EBP, and GATA families of transcription factors are very common and appear to play a critical role in both activation and repression of gene expression in a tissue-specific manner. It is also common for these factors to be functioning in close proximity to hormonally regulated factors such as EcR/USP. These types of structural and mechanistic similarities can be seen in the regulatory regions of the *sgs, fbp-1* and *adh* genes from *D. melanogaster* (Abel et al., 1993; Roth et al., 1999; Brodu et al., 2001).

The second regulatory region of the *vg* gene appears to be a hormonal response enhancer, which has binding sites for the 20E regulated early gene factors E74 and E75 (Segraves and Hogness, 1990; Fletcher and Thummel, 1995). This region is responsible for enhancement of *vg* expression in a hormonally dependent manner. E74 has been shown to play a large part in the activation of *vg* and appears to work synergistically with the EcR/USP receptor (Sun et al., 2004).

Finally, the third region contains seven GATA binding sites. Deletion of this region in transgenic *Ae. aegypti* and *D. melanogaster* carrying *vg* promoter driven reporters drastically reduces the level of reporter expression when compared to the full-length *vg* promoter (Kokoza et al., 2001). GATA factors are important transcriptional regulators that are known to have both negative and positive effects upon transcription. One of the primary foci of this thesis is upon the role of an *Ae. aegypti* GATA factor (AaGATAr) and its role in the

maintenance of the previtellogenic state of arrest. GATA factors will be discussed in greater detail in the next section.

The mechanism by which the previtellogenic state of arrest is maintained and *YPP* genes are repressed has been a question that has been partially answered. A complex system regulates the formation of an active 20E receptor. During the state of arrest, the EcR/USP heterodimer is prevented from forming by an orphan nuclear receptor called AHR38. AHR38 forms a heterodimer with USP, thereby preventing EcR from heterodimerizing with USP. When a blood meal is taken and 20E titers increase, USP switches heterodimeric partners and joins with EcR to form the active 20E receptor. As vitellogenesis is terminating USP changes partners again and heterodimerizes with a transcription factor called AaSvp to shut down 20E signaling (Zhu et al., 2000; Zhu et al., 2003b). This elegant mechanism explains how premature activation of the EcR/USP system is repressed; however, it does not address the requirement of nutrients for activation and 20E function. Experimental evidence and previous work in the literature suggest that GATA factors may play a role in this regulatory system.

GATA Factors and Gene Expression

GATA factors are important for high levels of *vg* expression and most likely important in regulating the tissue specificity and possibly nutritional regulation of *vg* expression. GATA factors are a ubiquitous family of transcription factors found eukaryotic organisms. They typically consist of a conserved DNA binding domain (DBD) while the rest of the protein is unconserved. The non-

conserved regions share little or no sequence similarity and their functions are poorly defined (Lowry and Atchley, 2000).

A GATA factor DNA binding domain can contain either one or two highly conserved zinc finger motifs. The zinc fingers contain the consensus sequence of $CX_2CX_{17-18}CX_2C$ followed by a basic region containing a nuclear localization signal. The DNA binding domain recognizes the nucleotide motif (A/T)GATA(A/G) (Orkin, 1992). In two zinc finger GATA factors the C-terminal finger is responsible for DNA binding while the N-terminal finger defines binding specificity (Shim et al., 1995) and is involved in protein-protein interactions with cofactors (Haenlin et al., 1997; Fox et al., 1999; Ozawa et al., 2001). The C-terminal finger has also been shown to interact with a variety of cofactors, competitors and adjacent DNA binding transcription factors (Durocher et al., 1997; Blobel et al., 1998; Molkentin et al., 2000; Zhang et al., 2000; Morin et al., 2000; Gajewski et al., 2001).

In mammalian GATA factors, regions outside of the DNA binding domain have been shown to have activation properties (Morrisey et al., 1997). The diversity of protein-protein interactions that GATA factors are capable of causes their function to differ based upon the cellular and promoter context in which they are acting. In metazoans the complexity of these interactions is thought to act as a code that allows tissue specific gene expression even though a particular GATA factor is expressed throughout multiple tissues (Molkentin, 2000).

In *D. melanogaster* a number of fat body specific events are regulated by a GATA factor called DmGATAb or Serpent. The DmGATAb (serpent) factor has

been well characterized (Abel et al., 1993). It was recently discovered that the *DmGATAb* gene encodes two isoforms, the first with a single zinc finger domain and the second with two. These isoforms are thought to have differential regulatory functions due to different associations with cofactors as a result of the difference in the DNA binding domain (Waltzer et al., 2002).

DmGATAb is an essential factor for fat body formation during embryonic development in *D. melanogaster* (Sam et al., 1996; Hayes et al., 2001). After embryonic development, DmGATAb continues to regulate the expression of a number of genes in the fat body. One example, the *fbp1* gene, is expressed in the larval fat body and responds to stimulation by 20E. The promoter for this gene contains an EcRE, a binding site for a short-range repressor called Aef-1 and three GATA response elements (Brodu et al., 1999). The GATA elements seem to serve two roles; they are required for tissue specificity and for the promoter to be responsive to 20E. DmGATAb in this context acts to antagonize the Aef-1 repressor and allows activation by EcR/USP in response to 20E (Brodu et al., 2001).

GATA motifs are also required for fat body and blood cell specific activation of immune genes in response to immune challenge. The promoters for immune genes in *D. melanogaster* contain GATA elements adjacent to κB-like motifs. This combination of binding sites can be found in insect and in mammalian innate immune genes where they act synergistically to activate expression of these genes in a tissue specific manner (Kadalayil et al., 1997; Petersen et al., 1999).

In fungi, GATA factors regulate transcription in response to environmental stimuli. In *Saccharomyces cerevisiae* nitrogen catabolic gene expression and genes responsible for autophagy are regulated by multiple GATA factors in response to environmental nitrogen (Hardwick et al., 1999). One GATA factor, Gln3, acts as an activator. When it is phosphorylated, this factor is retained in the cytoplasm by the Ure2 protein (Cardenas et al., 1999). The phosphorylation state of this GATA factor is maintained by the TOR (target of rapamycin) kinase, which actively phosphorylates Gln3 and at the same time represses a phosphatase (Sit4), also by phosphorylation.

The TOR pathway is a nutrient sensitive signal transduction pathway, which is conserved from yeast to mammals (Rohde et al., 2001). The details of TOR regulation will be discussed in more detail in the next section. Limited quantities of environmental nitrogen sources results in the dephosphorylation of Gln3 by activated Sit4 allowing it to separate from Ure2, translocate to the nucleus and activate nitrogen catabolic genes (Figure 3) (Beck and Hall, 1999; Bertram et al., 2000). A GATA repressor called Dal80, which is upregulated by Gat1 and Gln3, further controls this system by competing for binding sites with the activators to down regulate the system (Cunningham et al., 2000).

GATA factors are involved in the regulation of the differentiation of preadipocyte cells to mature adipocytes in response to growth factors and nutrients in vertebrates (Tong et al., 2000). Treatment of preadipocytes with rapamycin (the TOR kinase inhibitor) inhibits them from differentiating into mature adipocytes (Bell et al., 2000). This data suggests that nutrient regulation

of GATA factor activity may work by a conserved mechanism from yeast up to man. Furthermore, as adipose tissue in mammals is analogous to fat body tissue in insects, the tissue specificity of this mechanism may be conserved as well.

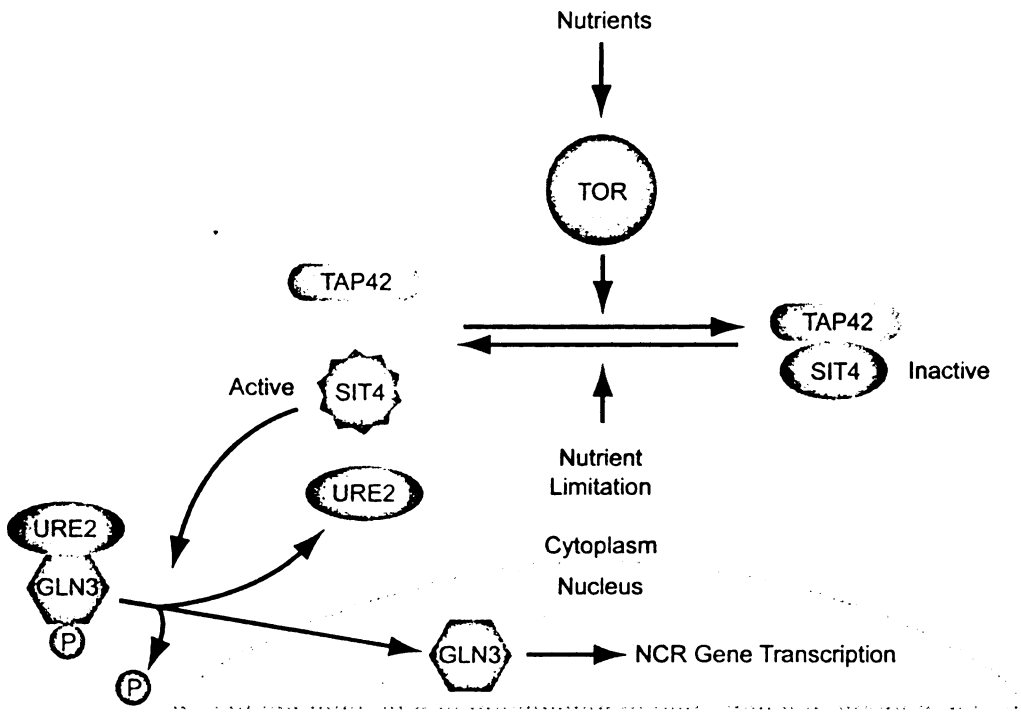


Figure 3. Schematic diagram of TOR regulation of GATA factors in *S. cerevisiae* adapted from Beck and Hall 1999

The number of GATA binding motifs found in the *Ae. aegypti vg* 5'-regulatory region and the negative effect on transcription of this gene when they are eliminated indicates that GATA factors are an important component to the regulation of this gene. Regulation of transcription by GATA factors in other systems suggests that this could be a mechanism by which nutritional signaling is taking place in the fat body. A search for GATA factors in the *Ae. aegypti* fat body has revealed a two-zinc finger GATA factor that is only found in the fat body (AaGATAr). Temporal expression analysis by northern blot shows that this factor is expressed in the previtellogenic and in the early and late vitellogenic period.

Electrophoretic mobility shift assay (EMSA) has shown that this factor binds the WGATAR consensus and specifically to GATA elements found in the *vg* regulatory region. Transient transfection analysis of the transcriptional regulatory activity of this factor in *D. melanogaster* S2 cells has shown that it has repression activity and can specifically inhibit 20E mediated activation in reporter constructs containing the proximal region of the *vg* regulatory region. EMSAs performed with fat body nuclear extracts from previtellogenic mosquitoes reveal a GATA binding activity with a mobility similar to that of recombinant AaGATAr (Martin et al., 2001a). These findings indicate that AaGATAr may be acting as a repressor of vitellogenesis during the previtellogenic state of arrest. Chapter two describes investigations of the role of AaGATAr in the maintenance of the previtellogenic state of arrest.

Amino Acid Regulation of Gene Expression

Nutrients appear to be a key stimulus in alleviating the vitellogenic state of arrest in anautogenous mosquitoes. However, the question of how they are doing this remains standing. Higher AA titers in the hemolymph of autogenous mosquitoes suggests AAs could act as a signal activate vitellogenesis (Su and Mulla, 1997b; Uchida et al., 1998). AAs would make an ideal signaling mechanism as hemolymph titers of AAs increase dramatically after a blood meal (Uchida et al., 1990; Uchida et al., 2003)

A more detailed examination of the role of AAs in stimulating egg development in mosquitoes shows that when the anautogenous mosquito *C.*

pipiens is infused with a balanced solution of AAs, it is capable of initiating egg development. This experiment was also performed with seven other species of anautogenous mosquitoes. Five of the seven underwent successful egg development (Uchida et al., 2001). Furthermore, specific AAs are essential for egg development as when they are omitted from the infusion mixture, egg development fails to occur. These essential AAs are leucine, isoleucine, lysine, phenylalanine, threonine, tryptophan, valine, cysteine, arginine and asparagine (Uchida K, 1998). This study agrees with previous work in which adult *Ae. aegypti* were fed artificial blood meals from which individual AAs were omitted and egg development was recorded. With the exception of asparagine the same AAs were found to be essential for egg development (Dimond et al., 1956). AAs essential for egg development are also essential for larval growth in *Ae. aegypti* and in general for growth in mammals (Figure 4) (Rose, 1938b; Singh and Brown, 1957).

These experiments clearly establish the link between AAs and reproduction in mosquitoes. They also reveal parallels between the AAs essential for egg development and those required for immature development in insects as well as in mammals. It is possible that the same conserved system regulating growth is also regulating reproduction in mosquitoes. A well-characterized nutritional signaling system that fits with the data presented above is the TOR kinase nutritional signaling pathway.

Amino Acids	*Aedes* Egg Development	*Culex* Egg Development	*Aedes* Larval Growth	Mammalian Growth
Leucine	XX	XX	XX	XX
Tryptophan	XX	XX	XX	XX
Methionine	X		XX	XX
Valine	XX	XX	XX	XX
Histidine	X	X	XX	XX
Lysine	XX	XX	XX	XX
Phenylalanine	XX	XX	XX	XX
Arginine	XX	XX	XX	XX
Asparagine		XX		
Threonine	XX	XX	XX	XX
Cysteine	X	XX	X	
Glycine				
Isoleucine	XX	XX	XX	XX
Tyrosine				
Aspartic Acid				
Serine			X	
Proline			X	
Glutamine				
Alanine				
Glutamic Acid	X			

Figure 4. Amino acids essential for growth and egg development. XX – represents absolutely essential amino acids, **X** – represents semi-essential amino acids. Mammalian growth data from (Rose, 1938b). Aedes larval growth data from (Singh and Brown, 1957). *Culex* egg development data from (Uchida K et al., 1992). Aedes egg development data from (Dimond et al., 1956)

The TOR kinase (Target of Rapamycin) is a serine/threonine kinase that is ubiquitously expressed in eukaryotes (Raught et al., 2001). It has been well characterized in its role as a nutrient sensor in multiple systems. *TOR* functions in concert with the insulin-signaling pathway. Crosstalk occurs between these pathways through TOR interactions with a small GTPase called RHEB (Saucedo et al., 2003) which functions downstream of the TSC proteins and the AKT and PI3 kinases (Hafen, 2004). The primary effect this pathway mediates is the

control of cellular growth via regulation of transcription and translation in response to environmental nutrients, specifically AAs (Figure 5).

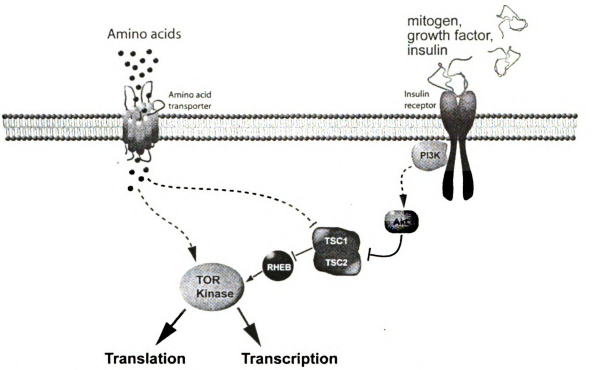


Figure 5. Schematic diagram of the TOR and Insulin signaling pathways

In *S. cerevisiae* AA and nitrogen source deprivation results in transcriptional activation of nitrogen catabolite repression genes via the TOR pathway (Cooper, 2002). In *D. melanogaster* AAs act directly upon the fat body via the TOR pathway to regulate the growth of the whole organism by the secretion of an insulin binding protein originating from the fat body (Colombani et al., 2003). AAs and TOR are also involved in regulation of blastocyst trophoectoderm differentiation and embryonic implantation in mammals (Martin and Sutherland, 2001).

The exact mechanism of how TOR senses the presence of AAs is not known. However, some of the AAs that stimulate TOR activity are known.

Branched chain AAs and leucine in particular have been identified as activators of TOR in mammalian adipocyte tissue. Stimulation with leucine results in increased protein synthesis, hyperplastic growth and tissue morphogenesis in rat adipocytes (Lynch et al., 2003). In addition, limitation of the cationic AA arginine inhibits global *D. melanogaster* growth, which is regulated by TOR (Colombani et al., 2003).

The mechanisms by which TOR transduces its response to AA stimulation are better understood. TOR is involved in the regulation of both transcription and translation. TOR regulates translation through the maintenance of the phosphorylation state of two translational regulatory proteins. TOR phosphorylates and activates the p70 S6 kinase in response to nutritional stimuli (Chung et al., 1992; Brown et al., 1995; Fox et al., 1998). The S6 kinase phosphorylates the ribosomal protein S6 that specifically facilitates the translation of mRNAs containing a 5'-polypyrimidine tract. These types of mRNAs typically encode ribosomal proteins, translation elongation factors, and growth control proteins (Jefferies et al., 1997). TOR also regulates the phosphorylation state of the PHAS-I/4E-BP1 factor. This factor in a dephosphorylated state binds to and inhibits the eIF-4E translational initiation factor which functions in cap recognition and recruitment of ribosomes to the mRNA. Phosphorylation of 4E-BP1 inactivates it and derepresses eIF-4E allowing translation to proceed (Brunn et al., 1997).

TOR has also been well documented in its ability to regulate transcription. As discussed in the section on GATA factors, TOR regulates the nuclear

localization of a GATA type transcription factor named Gln3 via maintenance of its phosphorylation state in yeast (Beck and Hall, 1999; Bertram et al., 2000). In mammals the *CHOP* (C/EBP homologus protein) and *AS* (Asparagine synthetase) genes are both negatively regulated in the presence of AAs. Specifically, both are upregulated during leucine starvation. Control of these genes has been localized to *cis* elements found in the 5' regulatory region. The *CHOP* gene contains an element called the AARE (AA response element) while the *AS* gene contains two elements called NSRE –1 and –2 (Nutrient sensitive response element). Transfer of the AARE element alone to a basal promoter sequence confers AA responsiveness to it, while both NSRE's are necessary to confer nutrient sensitivity. The AARE and NSRE-1 elements both show homology to the binding sites for the C/EBP and ATF/CREB families of transcription factors (Bruhat and Fafournoux, 2001). *CHOP* expression was linked to TOR in a study showing that insulin like growth factor 1 (IGF 1) is required for *CHOP* activation and that treatment with the TOR inhibitor rapamycin as well as inhibitors of the insulin pathway prevented activation (Entingh et al., 2001).

The nutritional differences between autogenous and anautogenous mosquitoes, the requirement for specific AAs for egg development and the involvement of GATA factors in vitellogenic regulation point towards the possibility that vitellogenic gene expression is being activated by an AA based signaling pathway. Chapter 3 will focus on the role of AAs in vitellogenic gene regulation.

Cationic Amino Acid Transport and Signaling

The mechanism by which cells sense the presence of AAs remains unknown. However, AA transporters are suspected of being direct initiators of nutritional signaling as well as being indirectly involved in regulating the flow of AAs in and out of the cell. AA transporters function primarily by two mechanisms. Transport is either driven by electrical and chemical gradients or by antiport mechanisms that use AAs at high intracellular levels to drive the transport of extracellular AAs (Broer, 2002).

There are a number of proposed mechanisms by which AA transporters may act to signal the presence of AAs. One possibility is that the transporter acts as a receptor at the top of a signal transduction pathway that directly regulates the activity of downstream proteins (Figure 6a). The second possibility is that AAs flowing into and other solutes flowing out of the cell results in physiological changes in membrane polarity, cellular volume, pH and salt concentrations, which are then detected by the cell (Figure 6b). Another possibility is that an intracellular receptor is detecting increased concentration of AAs, charged tRNAs, or AA metabolites (Fig 6c). The fourth possibility is if AAs are sensed by an extracellular receptor, nearby transporters affecting local extracellular AA concentrations could regulate these receptors by their level of transport activity (Figure 6d) (Hyde et al., 2003).

Recently a cationic AA transporter called Slimfast (Slif) was shown to be involved in regulation of body size in *D. melanogaster* as part of a nutritional signaling system in the fat body. This system is TOR regulated and controls

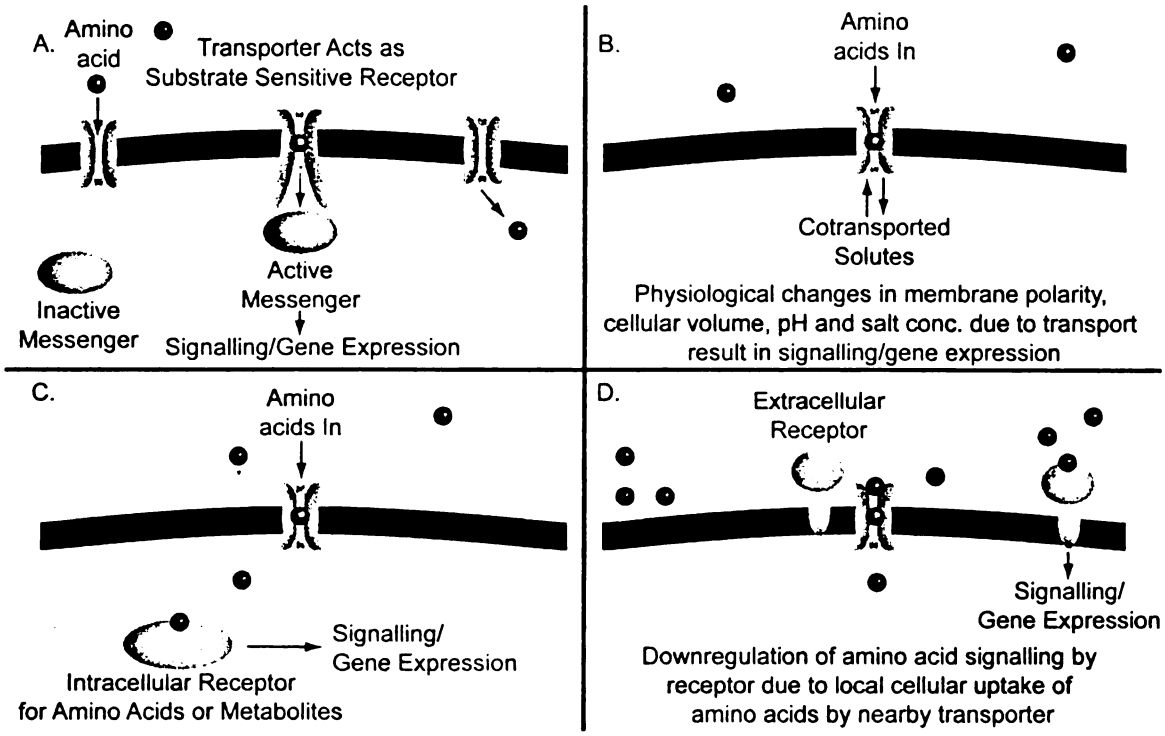


Figure 6. Schematic diagram of potential amino acid transporter signaling mechanisms adapted from Hyde et al., 2003

whole body growth of the fly. Knockout of this transporter results in sensitivity to arginine withdrawal, reduced body size and in some cases interrupted larval growth and development (Colombani et al., 2003). Slif is homologus to the mammalian CAT family of AA transporters, which are considered system y^+ type transporters. System y^+ transporters are specific for cationic AAs (Deves and Boyd, 1998). Structural predictions of CAT proteins suggest that they contain either 12 or 14 transmembrane segments depending upon the modeling software used. However, immunostaining against the 3rd and 4th extracellular loops suggests that the 14 transmembrane domain model is correct (Closs, 1996).

While the mechanism of how Slif regulates the TOR pathway is unknown, it is interesting that CAT type transporters themselves are regulated by AAs. In mammals mRNA of the *cat-1* gene is stabilized in the absence of AAs resulting in

mRNA accumulation (Hyatt et al., 1997). The stabilization of *cat-1* mRNA is due to the presence of *cis*-acting RNA sequences in the 3'-UTR of the transcript which are acted upon by unknown *trans* factors (Aulak et al., 1999). Translation of this transcript also increases during AA starvation. This is interesting as the *TOR* pathway under those conditions down regulates most cap-dependant translation. Translation is possible due to the presence of an internal ribosomal entry sequence (IRES) in the 5'-UTR of this transcript. IRES dependant translation is increased in response to AA starvation conditions (Fernandez et al., 2001).

The regulation of growth in *D. melanogaster* by Slif may be part of a conserved nutritional pathway in insects. Chapter four of this thesis will focus on the cloning and characterization of the *Ae. aegypti* homologue of the *slif* gene and a test of the hypothesis that the *Ae. aegypti* homologue of Slif plays a role in regulation of vitellogenesis.

Rationale for Current Studies

A great deal of research has been done on the reproductive physiology and molecular biology of reproduction in *Ae. aegypti*. However, a number of issues remain to be resolved. The mechanisms that maintain the previtellogenic state of arrest have been partially elucidated. Prevention of premature formation of the active 20E receptor in the absence of hormone is accomplished by alternative dimerization of the USP receptor. However, a GATA factor, AaGATAr, is expressed during the previtellogenic period and has been shown to have repression activity that inhibits hormonal stimulation *in vitro*. One of the

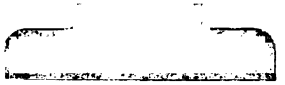
main goals of this research is to determine if AaGATAr is repressing YPP gene expression *in vivo* during the previtellogenic state of arrest. This goal is covered in chapter 2 where we use RNAi to knockdown this GATA factor and determine the effects upon *vg* gene expression.

Another unsolved issue that remains is the identity of the signal that releases the transcriptional repression from YPP genes after a blood meal. Based upon background data, a nutritional signal is the likely candidate to be responsible for vitellogenic derepression. However, it is not known what tissue this signal is working on or how the signal is transmitted. In chapter three, we address whether AA signaling and transcriptional regulation of vitellogenesis are connected. Using a fat body tissue culture system we test if AAs directly signal to the fat body, what specific AAs are responsible for this signaling and how this signal is transduced to the *YPP* genes.

Finally, based upon the evidence from *D. melanogaster*, the cationic AA transporter Slif appears to be an component of fat body based nutritional signaling. In chapter four, we clone and characterize the *Ae. aegypti* homologue of the *slif* gene to determine if this transporter is playing a role in the nutritional regulation of vitellogenesis. We then use RNAi to knock down this transporter and look at the effects it has upon YPP gene regulation.

These three lines of analysis contribute significantly to what is currently known about how vitellogenesis and more generally how reproduction in anautogenous mosquitoes is regulated. The idea of a nutritional signal regulating vitellogenesis is not new, but it has never been investigated in detail at

the molecular level. These studies are the first to analyze the connection between blood meal acquired nutrients and regulation of gene expression in *Ae. aegypti*.



Chapter 2: RNA Interference-mediated Knockdown of a GATA Factor Reveals a Link to Anautogeny in the Mosquito Aedes aegypti*

*Results have originally been published in Proc. Natl. Acad. Sci.

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Abstract

Blood feeding tightly regulates the reproductive cycle in anautogenous mosquitoes. Vitellogenesis (the synthesis of yolk protein precursors) is a key event in the mosquito reproductive cycle and is activated in response to a blood meal. Prior to blood feeding, *Ae. aegypti* is in a state of reproductive arrest during which the yolk protein precursor genes (*YPP*s) are repressed. The regulatory region of the major *YPP* gene *vitellogenin* (*vg*) has multiple GATA binding sites required for the high expression level of this gene. However, a GATA factor (AaGATAr) likely acts as a repressor preventing activation of this gene prior to a blood meal. Here we report *in vivo* data confirming the role of AaGATAr as a repressor of the *vg* gene at the state of previtellogenic arrest. Using an RNAi mediated gene knockdown technique in conjunction with the Sindbis viral expression system we show that disruption of *AaGATAr* results in an increased basal level of expression of the *vg* gene and an elevated response to the steroid hormone 20-hydroxyecdysone in mosquitoes in a state of arrest. These experiments have revealed a component in the molecular mechanism by which anautogeny is maintained in *Ae. aegypti*.

Introduction

The fundamental basis of mosquitoes' success as disease vectors is the requirement for vertebrate blood as a nutrient source for egg maturation. In anautogenous mosquitoes, the reproductive system is held in a state of arrest during which *YPP* genes are repressed and ovarian development halts until the mosquito acquires a blood meal (BM). A result of this strategy is that it provides an evolutionary drive for increased vector/host interactions and as a consequence increased disease transmission. The molecular mechanisms of how anautogenous mosquitoes maintain the state of arrest are poorly understood.

Autogenous mosquitoes are capable of egg development soon after eclosion without a blood meal. The physiological choice of autogeny versus anautogeny can be either obligate or facult e depending upon the species and strain of mosquito. Studies have shown that the expression of autogeny or anautogeny can be regulated by multiple factors. In *C. tarsalis* autogeny is a dominant polyfactorial inheritable trait (Eberle and Reisen, 1986). Environmental factors such as photoperiod and temperature can also influence the reproductive path taken by this mosquito (Brust, 1991). Other factors such as diet and larval population density affect the proportion of autogenic *C. tarsalis* in a population (Reisen et al., 1986). Comparative studies of the physiological differences between autogenic and anautogenic mosquitoes of different strains of the same species and different species show that differences become evident during the larval stage. A general trait appears to be that autogenous mosquitoes emerge

with more carbohydrates, lipids and protein and in some cases take longer to develop in the larval stage (Klowden and Chambers, 1992; Chambers and Klowden, 1994; Su and Mulla, 1997a).

Characterization of the larval storage proteins, hexamerins, has shown that the autogenous mosquito *Ae. atropalpus* produces three different hexamerins one of which is female specific. In the anautogenous *Ae. aegypti* two hexamerins are produced, but a third sex specifically expressed hexamerin is not present (Gordadze et al., 1999; Zakharkin et al., 2001). AA titers in the hemolymph also appear to be higher in autogenous mosquitoes (Su and Mulla, 1997b). The physiological characterizations of the phenomena of autogeny/anautogeny have revealed fundamental differences between the two systems in different mosquito species on a nutritional level, but do not address how these physiological differences regulate vitellogenic transcriptional control.

The central event in mosquito reproduction is vitellogenesis. Vitellogenesis involves synthesis and secretion of yolk protein precursors (YPPs) on a massive scale by the fat body (a tissue analogous to the vertebrate liver). This is followed by YPP accumulation by developing oocytes (Raikhel and Dhadialla, 1992; Raikhel et al., 2002). *YPP* gene transcription is regulated by hormonal and possibly nutritional cues. These cues are associated with the onset of blood feeding and acquisition of a BM in anautogenous mosquitoes. Levels of the endogenous steroid hormone, 20-hydroxyecdysone (20E), increase over the 24 hours following a blood meal (PBM) and stimulate *YPP* gene transcription. 20E works directly through its heterodimeric receptor, which consists of two nuclear

receptors. These receptors are the ecdysone receptor protein (EcR) and the Ultraspiracle protein (USP). The EcR/USP heterodimer can directly and indirectly up regulate the *vg* gene (Kokoza et al., 2001; Raikhel et al., 2002).

A newly emerged female *Ae. aegypti* requires three days of post eclosion development to reach reproductive competence. During this period she does not blood feed and her reproductive organs undergo previtellogenic development (Dittman F. et al., 1989; Raikhel and Lea, 1990). Once the mosquito has become competent to undergo vitellogenesis, it enters a state of arrest and *YPP* genes are maintained under transcriptional repression. Hormonal activation of *YPP* genes is repressed during the previtellogenic period by the sequestration of the USP protein via a nuclear receptor called Ahr38 which prevents the formation of the active receptor (Zhu et al., 2000). *In vitro* 20E can disrupt the AaHR38-USP complex and activate the EcR-USP heterodimer formation (Zhu et al., 2000), however, large non-physiological doses of 20E are required to activate low levels of YPP production and enable egg development *in vivo* (Lea, 1982a). These data suggest that an additional system of repression exists at the state of arrest in anautogenous mosquitoes.

In the search for such a repressor in *Ae. aegypti* we have cloned a two-zinc finger GATA factor gene (AaGATAr) with an ability to repress transcription and specifically to inhibit 20E-mediated activation of the vitellogenin (*vg*) gene in vitro (Martin et al., 2001a). Electrophoretic mobility shift assay (EMSA) has shown that the AaGATAr protein binds specifically to GATA elements located in the *vg* gene, the major *YPP* gene. Furthermore, fat body nuclear extracts from

previtellogenic mosquitoes contain an abundant GATA binding moiety with mobility similar to that of *in vitro* synthesized AaGATAr protein (Martin et al., 2001a). These results have led us to suggest that in the anautogenous mosquito *Ae. aegypti*, the AaGATAr protein is functioning as a transcriptional repressor of the *vg* gene at the state of arrest.

In this report we used an RNAi based technique to confirm the potential role of the *AaGATAr* gene *in vivo*. A fold-back anti-*AaGATAr* RNAi construct was expressed in five to seven day old previtellogenic mosquitoes using the Sindbis viral expression system. Expression of *AaGATAr* specific RNA duplexes caused a significant increase in the level of *vg* gene expression, indicating that repression of this gene was alleviated. Moreover, *vg* responsiveness to exogenous treatment by physiological doses of 20E, was highly elevated in *AaGATAr* RNAi knockdowns. These experiments provide direct experimental proof of the role of the AaGATAr protein as a repressor of the vitellogenin gene during the state of arrest and have revealed a component of the molecular mechanism by which anautogeny is regulated in *Ae. aegypti*.

Materials and Methods

Animals. Mosquitoes for the *AaGATAr* mRNA expression profile experiment were reared, fed and dissected as described in (Deitsch et al., 1995).

Construction of the *AaGATAr* RNAi Construct. The RNAi fold back construct was comprised of two fragments from the *AaGATAr* gene open reading frame in

the sense and antisense conformation respectively. These were separated with an 80 bp intron from the *Ae. aegypti clathrin heavy chain* gene (*CHC*) (Kokoza and Raikhel, 1997). The construct was cloned into the pBluescript SK (+) (Stratagene, LaJolla, CA) vector. All fragments were amplified using PFU polymerase (Promega, Madison, WI). The *CHC* Intron was amplified with the following primers using genomic DNA: *CHC* Intron Forward: 5'CGGGATCCCGGGTATGTATGACAGGGAAAAC (contains *Bam*HI site); *CHC* Intron Reverse: 5'CTGCAGTTGTTTCCTAGTAGATGAGATGTC (contains *Pst*I site).

The amplified fragment was purified, digested with *Bam*HI and *Pst*I and ligated into the pBluescript SK (+) multiple cloning site. GATA sense and antisense fragments were identical with the exception of restriction sites on the end of the primers that allowed for proper orientation and positioning of the fragments into the vector. The fragments were amplified using the following primers (engineered restriction sites are listed after the sequence): *AaGATAr* Sense Forward: 5'GCTCTAGAGCGTGTTGATATGGGACACCCA (*Xba*I site); *AaGATAr* Sense Reverse: 5'CGGGATCCCGCGAGGTACGTTGATCAACTG (*Bam*HI site); *AaGATAr* Antisense Forward: 5'GGAATTCCGTGTTGATATGGGACACCCA (*Eco*RI site and nested *Xba*I site); *AaGATAr* Antisense Reverse: 5'CTGCAGTTCGAGGTACGTTGATCAACTG (*Pst*I site).

The region used extends from bp 1399 bp to1699 bp in the *AaGATAr* cDNA. The sense fragment was digested with *Xba*I and *Bam*HI and ligated into the pBluescript SK (+) vector between the T3 promoter and the *CHC* intron. The

antisense fragment was digested with *Eco*RI and *Pst*I and inserted in the antisense orientation into the vector between the *CHC* intron and the T7 promoter. This 680 bp fold-back construct was digested with *Xba*I and cloned into the *Xba*I site of the pTE/3'2J SIN vector. The construct was transformed into DH5α competent *E.coli*, purified and viral RNA was produced as described previously (Higgs et al., 1997).

Production and Injection of Sindbis Recombinant *AaGATAr* RNAi Virus and 20-Hydroxyecdysone Treatments. Recombinant virus was produced and adult female mosquitoes, five to seven days old were inoculated with virus as described previously (Higgs et al., 1997). The Rex-D strain of *Ae. aegypti* mosquitoes used for injections originated from Rexville, Puerto Rico and were maintained as previously described (Miller and Mitchell, 1991; Higgs and Beaty, 1996; Wendell et al., 2000). At five days post-infection, mosquitoes were reaneasthetized and were randomly allocated to treatment or control groups. The treatment mosquitoes were inoculated with 0.5 µl of a solution containing ethanol (control) or 10^{-6} M 20 hydroxyecdysone (Sigma Chemicals, St. Louis, MO)). Following incubation for 6 hours at 28°C, 80% rh, mosquitoes were frozen at -80°C until processed.

Real Time PCR Analysis. Total RNA from mosquitoes or fat bodies was extracted by the Trizol method (Gibco BRL/Invitrogen, Carlsbad, CA). Three µg of total RNA was treated with amplification grade Dnase I (Gibco BRL/Invitrogen)

and two µg of DNase I treated RNA were used in cDNA synthesis reactions using the Omniscript Reverse Transcriptase kit (Quiagen). cDNA levels in the different samples were quantified by real time PCR using Taqman primers/probes for *vg* and *Actin* and SYBR green primers for *AaGATAr*. We used a real time PCR master mix, iQ Supermix (Biorad) for the Taqman reactions or the Quantitech SYBR green Mastermix (Quiagen) for the SYBR green reactions. Primers and probes are as follows (all probes use the Black Hole Quencher and were synthesized by Quiagen): *actin* Forward: 5'ATC ATT GCT CCA CCA GAA CG; *actin* Reverse: 5'AAG GTA GAT AGA GAA GCC AAG; Hex Labeled *actin* Probe: 5'ACT CCG TCT GGA TCG GTG GCT CC; *vg* Forward: 5'ATG CAC CGT CTG CCA TC; *vg* Reverse: 5'GTT CGT AGT TGG AAA GCT CG; Texas Red Labeled *vg* Probe: 5'AAG CCC CGC AAC CGT CCG TAC T; *AaGATAr* Forward: 5'GAG CTG CAC GAT TTG AAG GAC; *AaGATAr* Reverse: 5'CAC TGC TGT TGC CAT TGT TGT

Final reaction concentration for all primers and probes was 250 nM. Total reaction volumes were 25 µl and run in 96 well PCR plates (Biorad). All reactions were run in triplicate using two µl of cDNA per reaction. Reactions were run on an iCycler real time PCR machine (Biorad). Standard Curves used to quantify relative gene concentrations were made from 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest or from a dilution of a plasmid standard. The program used for amplifying the reactions was as follows. 1. Melting: 95°C 3 Min, 2. Melting: 95°C 10 sec, 3. Annealing 59°C 45 sec (Florescence recorded), 4. Repeat to step 2 for 50 cycles. Real time data was

collected by iCycler iQ Real Time Detection System Software V.3.0 for windows. Raw data was exported to Microsoft excel for analysis.

Northern Blot . Northern blots were performed using the Northernmax kit and following its protocols (Ambion). Each lane contains 7.5 µg of total RNA isolated as described above and was blotted to a N+ Hybond nylon membrane (Amersham Pharmacia, Buckinghamshire, England). Blots were probed with single stranded ^{32}P PCR labeled DNA probes against *vg*, *Actin*, and the RNAi construct. Probes were synthesized by asymmetrical PCR in the presence of alpha labeled ^{32}P dCTP to yield single stranded labeled probes. Signal was detected using a Personal FX phosphoimager (BioRad).

Protein Isolation and Western Blot. Mosquitoes were homogenized in cracking buffer (125 mM Tris (pH 6.8), 5% β -mercaptoethanol, 2% SDS, 4M Urea). Homogenate was boiled for 5 min and then centrifuged for 5 min at 28000 G's. Supernatant was removed and protein concentration was determined by Bradford assay (BioRad). Western blotting was performed as previously described (Hays and Raikhel, 1990). Blot was probed using a previously characterized monoclonal antibody against the *vg* apoprotein small subunit (Raikhel et al., 1985).

Results and Discussion

Expression profile of *AaGATAr*. We used real time PCR to obtain a detailed expression profile of the *AaGATAr* gene in the fat body of the adult mosquito. RNA was collected from previtellogenic mosquito fat bodies at 12-hour intervals beginning immediately after eclosion and extending to 288 hours post eclosion. A vitellogenic profile was also created using fat bodies dissected from mosquitoes at two or four hour time intervals during vitellogenesis for 48 hours. Equal amounts of total RNA were used to synthesize cDNA from each time point. Analysis of *AaGATAr* mRNA levels was determined using real time PCR primers to the region of *AaGATAr* used in the RNAi construct.

During previtellogenesis *AaGATAr* is expressed constitutively with a small decrease in abundance between 96 and 144 hours. Previous work has shown that *in vitro*, AaGATAr acts as a repressor (Martin et al., 2001a). The fact that there is a GATA binding activity present in previtellogenic fat body nuclear extracts and that *AaGATAr* is expressed constitutively in the previtellogenic fat body suggests that it is acting as a mechanism to maintain the transcriptional state of arrest. During the vitellogenic period there is an increase in *AaGATAr* expression early in vitellogenesis, and a large increase occurs during the time when vitellogenesis is being terminated. The peak at the beginning of vitellogenesis is significant, but is occurring early in vitellogenesis before *vg* levels have started to increase. *AaGATAr* levels recede back to baseline as levels of *vg* increase significantly. Repression activity of *AaGATAr* at this time is most likely overridden by the vitellogenic stimuli that activate the *vg* gene. Based

upon its large increase at the end of vitellogenesis AaGATAr may also be involved in the termination of vitellogenesis. Future assays of AaGATAr protein levels with specific antibodies are essential for understanding of its role throughout the vitellogenic cycle (Figure 1).

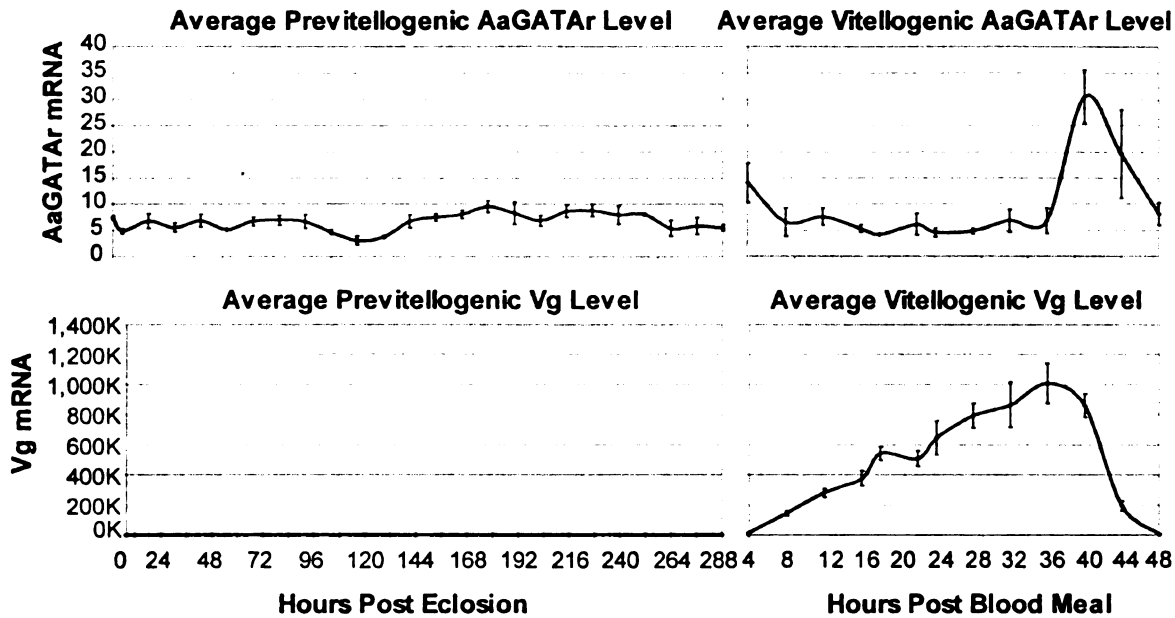

Figure 1. *AaGATAr* mRNA expression profile. Real-time PCR analysis was performed upon cDNA samples obtained from mosquito fat bodies dissected at 12-hour time intervals during the previtellogenic state and either two or four hour time intervals during the vitellogenic state. cDNA was synthesized from total RNA from groups of five fat bodies per time point. Reactions were performed in triplicate. Data represent means ± SE of triplicate samples.

RNAi-mediated knockdown of *AaGATAr* results in increased transcription of the *vg* gene. We have used a reverse genetic approach to disrupt the function of the AaGATAr factor *in vivo* through the utilization of RNAi. RNAi causes the disruption of specific mRNA in response to the presence of double stranded RNA homologous to the mRNA of interest (Hannon, 2002). To express double-stranded RNA homologous to the *AaGATAr* mRNA in *Ae. aegypti*, we used the SIN viral vector system. SIN is a positive strand RNA virus from the

Togaviridae family and the alphavirus genera. In insect cells, infection with this virus results in a noncytopathic, widespread, and persistent infection (Bredenbeek et al., 1993). The double subgenomic SIN is a proven system for the ectopic expression of genes and antisense transcripts in mosquitoes (Higgs et al., 1995; Kamrud et al., 1997; Kamrud et al., 1998; Higgs et al., 1998; Johnson et al., 1999; Shiao et al., 2001).

We used a fold-back construct of the double-stranded *AaGATAr* RNA inserted in front of the second subgenomic Sindbis promoter. This construct consists of a 300-bp fragment from a region of low conservation within the *AaGATAr* open reading frame. This fragment was placed in the sense conformation followed by the same fragment inserted in the antisense conformation. An intron from another *Ae. aegypti* gene (*Clathrin heavy chain* gene, *chc*) (Kokoza and Raikhel, 1997) was placed as a spacer between the sense and antisense fragments (Figure 2a). Introns have been shown to increase the effectiveness of these types of constructs, possibly due to the spliceosome facilitating the formation of the double-stranded RNAi structure (Smith et al., 2000). When expressed the homologous regions of the construct base pair and fold into a double stranded structure. During infection the virus was expected to expresses a full length genomic RNA (12.4 kb with RNAi insert) and two subgenomic RNAs (4.8 kb and ~1 kb with RNAi insert) (Figure 2b). As a negative control, mosquitoes were infected with dsSIN virus without the *AaGATAr* RNAi insert. Five to seven day old mosquitoes were injected with virus and collected at five days post infection for analysis. Sindbis virus infections by intrathoracic

injection typically peak at five to six days post infection, so mosquitoes were collected at that time point for analysis (Rayms-Keller et al., 1995).

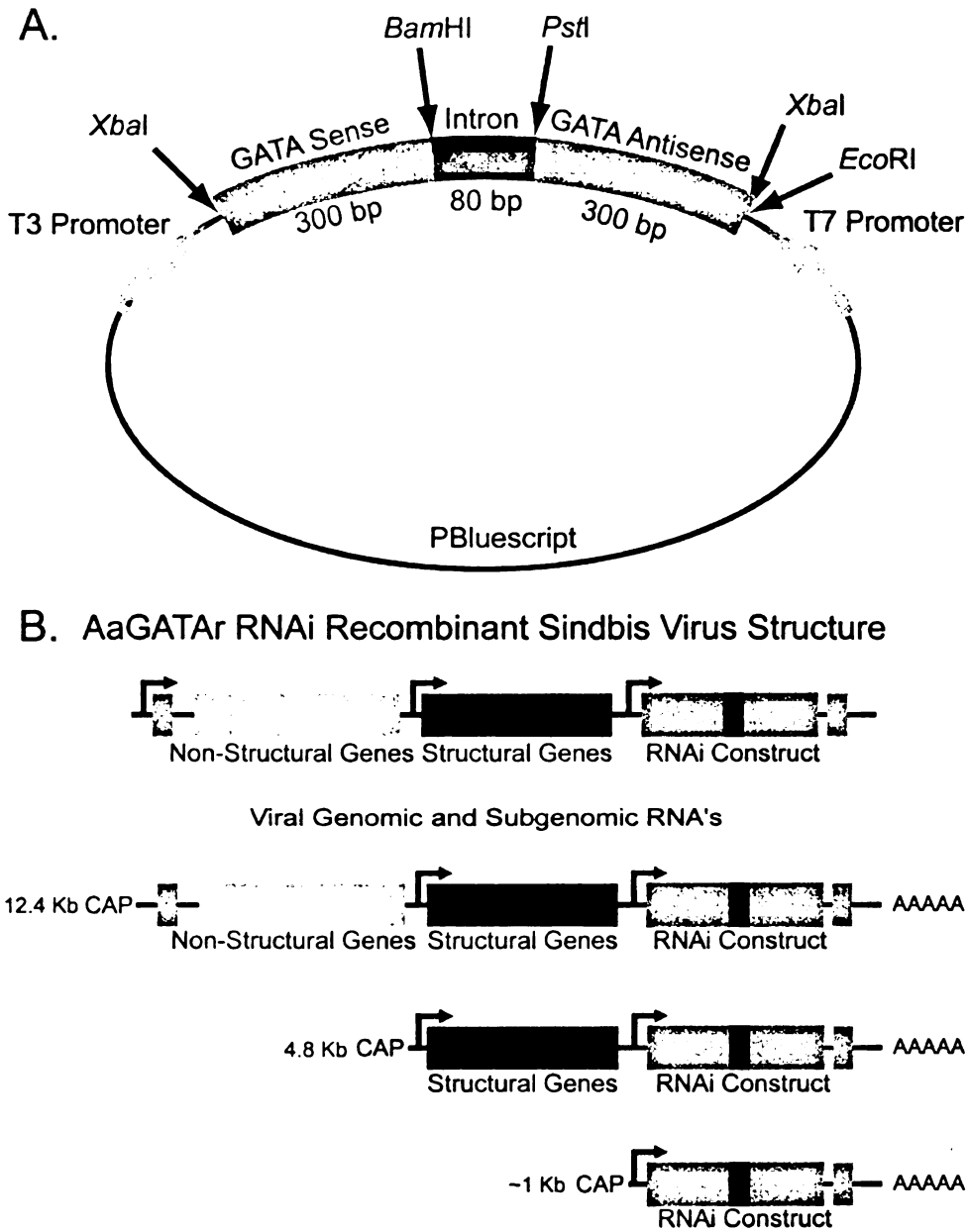

Figure 2. (A) Schematic drawing of *AaGATAr* RNAi construct. (B) Schematic representation of the *AaGATAr* RNAi Sindbis virus and subgenomic RNA's expressed during infection

To confirm that the virus was expressing the RNAi construct, a northern blot was performed using a probe to the *AaGATAr* fragment used in the RNAi

construct. In the *AaGATAr* RNAi treated mosquitoes specific bands can be seen at the expected sizes indicating the presence of the RNAi construct in the genomic, 1st subgenomic, and 2nd subgenomic viral RNA's. The 2nd subgenomic RNA is the major transcript and consists mainly of the RNAi construct. There is also a fourth band at around 300 bp, which most likely represents the double stranded form of the construct. The presence of this band indicates that the construct is being effectively expressed and is folding into the correct double stranded conformation (Figure 3).

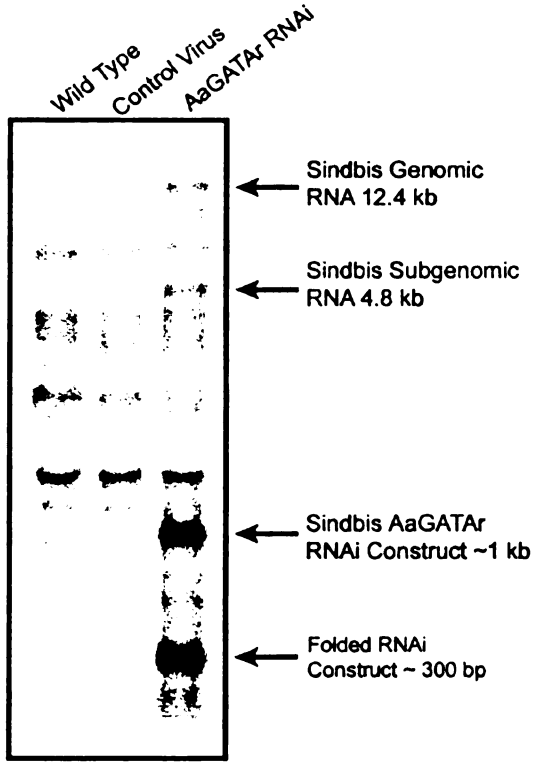

Figure 3. Expression of the RNAi construct subgenomic RNA by Sindbis virus in infected mosquitoes. Five to seven day old mosquitoes were infected with either control Sindbis virus (lacking insert) or Sindbis virus carrying the *AaGATAr* RNAi construct and allowed to incubate for five days at 28°C, 80% rh. Wild type mosquitoes are uninfected. 7.5 µg of total RNA from each sample group was blotted and analyzed by Northern blot using a probe against the *AaGATAr* fragment used in the RNAi construct

We hypothesized that a knockdown of *AaGATAr* in previtellogenic mosquitoes would result in an increase in the transcription level by a target of AaGATAr repression, the *vg* gene. To test this we performed a northern blot to check for levels of *vg* expression in the RNAi treated mosquitoes and real time PCR to confirm and quantify the differences between treatments. Our present analysis has revealed that the *vg* gene is expressed during the previtellogenic period at an extremely low level not detected in previous studies. This previtellogenic *vg* gene expression was not detected because the comparison was always made in the context of its vitellogenic expression, which is a million fold higher than the previtellogenic level (Cho and Raikhel, 1992; Deitsch et al., 1995). Experiments performed in this paper were done relative to the previtellogenic basal *vg* mRNA level permitting comparison of levels different only 5-20 fold. *vg* expression in the *AaGATAr* RNAi mosquitoes was significantly higher than in the wild type or control-infected mosquitoes (Fig 4a+b). We used western blot analysis to confirm increased expression of *vg* at the protein level. As was seen at the RNA level *vg* synthesis is increased in the knockdown mosquitoes over the controls (Fig 4c). These results support our hypothesis that AaGATAr is acting as a repressor of *vg* transcription.

To determine if the mRNA for *AaGATAr* was being degraded we used real time PCR to quantify its mRNA levels. While there is a significant decrease in the amount of *AaGATAr* mRNA in the RNAi treated mosquitoes over the controls, it was not dramatic. This could be due to incomplete penetrance by the knockdown

or incomplete activation of the RNAi system, resulting in cells escaping the effects of the RNAi (Fig 4d).

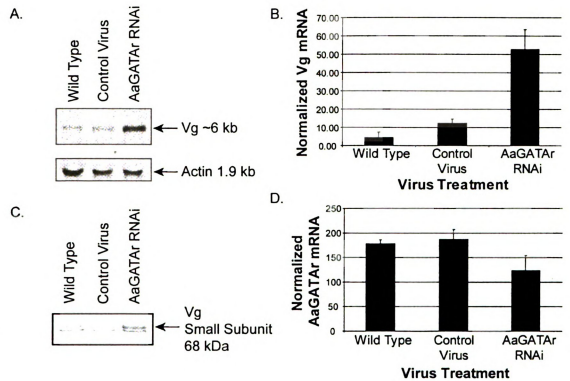

Figure 4. Vitellogenin expression is de-repressed in *AaGATAr* RNAi treated mosquitoes. Five to seven day old mosquitoes were infected with either control Sindbis virus (lacking insert) or Sindbis virus carrying the *AaGATAr* RNAi construct and allowed to incubate for five to six days at 28°C, 80% rh. Wild type mosquitoes are uninfected. (A) Northern blot analysis of *Vg* expression in *AaGATAr* RNAi mosquitoes. 7.5 µg of total RNA from each sample group was blotted and analyzed by Northern blot using a probe against vitellogenin. (B) Real-time PCR analysis of *Vg* expression in RNAi treated mosquitoes. cDNA was synthesized from total RNA from groups of five fat bodies per time point. Reactions were performed in triplicate. Data was normalized by real time PCR analysis of *Actin* levels in the cDNA samples. Data represent means ± SE of triplicate samples. (C) Western blot analysis of Vg expression in *AaGATAr* RNAi mosquitoes. Total protein was extracted from groups of five mosquitoes. Twenty µg of total protein was blotted and probed with a monoclonal antibody against the 68 Kd small subunit of *Vg*. (D) Real-time PCR analysis of *AaGATAr* expression in RNAi treated mosquitoes. Analysis was performed as in B. Data represent means ± SE of triplicate samples.

The *vg* gene is sensitized to the presence of 20-hydroxyecdysone in *AaGATAr* knockdown mosquitoes. The steroid hormone 20E is a key factor in the activation of *YPP* genes. In anautogenous mosquitoes, 20E is not the only factor required for stimulation of *YPP* genes. In previous *AaGATAr* transient transfection experiments, it was shown that in *in vitro* cell culture, the presence of AaGATAr not only represses the basal expression level of the *vg* gene expression, but it is also capable of repressing the ability of the *vg* gene to respond to hormonal stimulation by 20E (Martin et al., 2001a). To test if *AaGATAr* is inhibiting the response by the *vg* gene to 20E *in vivo*, we used *AaGATAr* RNAi treatments in combination with 20E injections. At five days post infection mosquitoes were injected with a physiological dose of 20E (10^{-6} M) or with .5 ul of ethanol (control) and allowed to incubate for six hours. We then measured levels of *vg* expression in the mosquitoes using real time PCR.

Treatment with 10^{-6} M 20E caused a 2-2.5 fold increase in *vg* mRNA expression in both control groups of mosquitoes over control mosquitoes not having been treated with 20E, indicating that there is some response by the *vg* gene to the presence of 20E. However, in the RNAi treated mosquitoes the level of *vg* expression was elevated 4-4.5 fold in response to the hormone as compared to ethanol. These results support the hypothesis that the AaGATAr factor serves as a repressor of the *vg* gene during the previtellogenic state of arrest overriding hormonal stimuli. Knockdown of the *AaGATAr* factor by the RNAi treatment not only results in increase of basal level of *vg* gene expression

but also allows the hormonal cascade to more effectively activate the gene (Figure 5).

The role of the *AaGATAr* factor in anautogeny. A considerable base of physiological and molecular knowledge has been developed regarding the hormonal regulation of vitellogenesis in *Ae. aegypti*. In the ecdysteroid regulatory cascade, the sequestration of the USP protein by Ahr38 prevents early formation of the functionally active ecdysone receptor. *In vitro*, this repression can be lifted by the presence of physiological levels of 20E (Zhu et al., 2000).

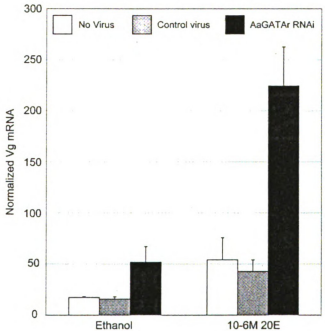

Figure 5. The *vg* gene is sensitized to the presence of 20E in *AaGATAr* knockdown mosquitoes. Five to seven day old mosquitoes were infected with either control Sindbis virus (lacking insert) or Sindbis virus carrying the *AaGATAr* RNAi construct and allowed to incubate for five days at 28°C, 80% rh. No virus treatments are uninfected wild type mosquitoes. After five days mosquitoes were injected with 0.5 µl of either ethanol (control) or 10^{-6} M 20E. *Vg* expression was quantified by Real-time PCR as described in figure 4b. Data represent means ± SE of triplicate samples.

However, *in vivo* treatment with 20E does not stimulate vitellogenesis. The mechanism mosquitoes have developed to maintain this repression system *in vivo* has been elusive. The *AaGATAr* gene was implicated as a repressor of *YPP* genes for multiple reasons. It was expressed in a fat body specific manner and its protein binds to GATA response elements present in the *vg* gene. It also represses the basal levels of expression from the *vg* promoter as well as 20E-mediated activation in transient transfection assays. AaGATAr contains a protein-protein interaction motif for a well-known co-repressor protein, dCtBP. GST pull-down assays show that AaGATAr indeed binds to the *D. melanogaster* CtBP (Martin et al., 2001a).

Our goal in this work was to gain *in vivo* evidence that the AaGATAr protein is acting as a mechanism to repress *YPP* gene expression in anautogenous mosquitoes. We achieved this goal using RNAi to knockdown AaGATAr function and demonstrated the phenotype by observing the effect of the knockdown on the expression of the major *YPP* gene *vg*. While the level of *vg* expression was not close to the level seen during vitellogenesis, this was expected as the gene was derepressed in the absence of additional stimulation that would be occurring during the natural vitellogenic cycle. In addition to higher basal expression the gene showed a much higher level of response to 20E in the knockdown mosquitoes than in the controls. This suggests that the repression mediated by the AaGATAr protein was alleviated, allowing other factors such as EcR/USP to function more effectively on the gene (Figure 6).

While we see increased *vg* expression in the knockdown treatments, mosquitoes dissected after RNAi treatment did not undergo egg development. This may be due to the fact that the levels of *vg* being expressed were very low relative to a vitellogenic mosquito. It is also likely that requirements for initiation

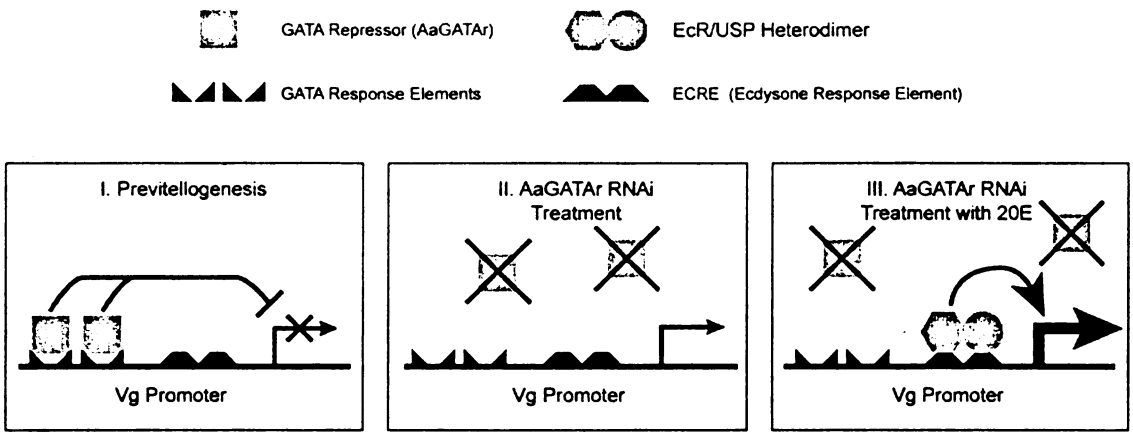

Figure 6. Schematic diagram of the effects of the AaGATAr RNAi knockdown on the *vg* promoter

of the complex regulatory processes in other tissues (such as the brain and the ovaries) necessary for the initiation of oogenesis are not sufficient with the knockdown of a single gene. It has been shown that initiation of *vg* uptake and protein synthesis in the *Ae. aegypti* ovary needs a brain factor, that is most likely is a peptide hormone because the requirement of cAMP for these processes (Koller and Raikhel, 1991; Sappington et al., 1997).

These results further supports the role of the AaGATAr factor in the maintenance of the repression of the vitellogenin gene during the previtellogenic state of arrest in *Ae. aegypti*. The exact signal that is causing the de-repression and activation of *vg* and how it is being mediated is still unknown. Further studies are necessary to gain more insight into this regulatory system. A detailed understanding of the mechanisms regulating expression of anautogeny will lead

to the future development of novel control strategies for prevention of pathogen transmission by mosquitoes.

Acknowledgments

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Chapter 3: TOR-mediated amino acid signaling in mosquito anautogeny *

*Some results have originally been published in Proc. Natl. Acad. Sci.

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Proc. Natl. Acad. Sci. In Press

# Underlined two authors contributed equally to this work

Abstract

A defining characteristic of anautogenous mosquitoes is their requirement for a blood meal to initiate reproduction. During vitellogenesis, a key event in mosquito reproduction, *yolk protein precursor* (*YPP*) gene expression is activated over a million fold from its basal state. The steroid hormone 20E regulates *YPP* gene transcription during vitellogenesis. However, exogenous treatment of mosquitoes with physiological levels of 20E does not result in activation of vitellogenesis, indicating that another regulatory mechanism is required for the hormonal system to function. In an in vitro fat body culture system, the presence of amino acids (AAs) caused an increase in basal transcription of the major *YPP* gene, *vitellogenin* (*vg*). Moreover, activation of *vg* transcription by 20E activity is dependant upon the presence of AAs to activate *vg* transcription. Withdrawal of individual AAs demonstrates that ten specific AAs are essential for the transcriptional response to 20E by the *vg* gene. We also demonstrate the involvement of the TOR signaling pathway in the AA response, by treatment of fat bodies with the TOR kinase inhibitor rapamycin. Rapamycin treatment results in blockage of the basal response to AAs by *vg* and a severe reduction in 20E induction, suggesting that the TOR kinase regulatory cascade transduces the AA signal. Treatment with the translational inhibitor cyclohexamide also inhibits the AA response by *vg*, suggesting that translational regulation by TOR may be playing a role in this system. Taken together, this work demonstrates that hemolymph AA levels play a key role in the onset of vitellogenesis in anautogenous mosquitoes.

Introduction

The mosquito reproductive cycle is a finely tuned system permitting mosquitoes to thrive in a variety of strenuous environmental conditions. The reproductive system of anautogenous mosquitoes has been adapted so that it is only active after a blood meal. In contrast, autogenous mosquitoes are capable of laying a clutch of eggs without a blood meal. Both of these strategies confer advantages under different environmental circumstances. The expression of autogeny or anautogeny can be obligatory or facultative, depending upon the species. There are also examples of both reproductive strategies occurring in different strains of the same species of mosquito. Multiple factors control the expression of either the autogenic or anautogenic phenotype. These factors are a mixture of genetic predisposition and environmental cues (Trpis, 1977; Eberle and Reisen, 1986; Brust, 1991; Sota and Mogi, 1995).

There are significant physiological differences between autogenic and anautogenic mosquitoes. Comparison of autogenous versus anautogenous *C. tarsalis* strains shows that autogenous strains require a day longer to complete the larval stage of development, but are capable of laying eggs one to two days earlier than their anautogenous counterparts. However, the number of eggs laid in the first batch by autogenous mosquitoes is significantly less than anautogenous mosquitoes (Reisen and Milby, 1987). Physiological analyses of both strains show that autogenous strains emerge with higher overall levels of stored nutrients and a heavier body weight (Su and Mulla, 1997a). A similar study showed that autogenous strains of *Ae. albopictus* also emerge with greater

amounts of metabolizable protein, lipids, and total available energy than anautogenous strains (Chambers and Klowden, 1994). Furthermore a study specifically focusing upon the levels of AAs present in the hemolymph of autogenous versus anautogenous *C. tarsalis* shows that autogenous mosquitoes emerge with significantly higher levels of total AAs in the hemolymph. The difference in AA content lasts to somewhere between seven and ten days post emergence (Su and Mulla, 1997b). Comparative analysis between the autogenous mosquito *Ae. atropalpus* and the anautogenous mosquito *Ae. aegypti* reveals differences in the expression of larval storage proteins called hexamerins. *Ae. atropalpus* produces three different hexamerins, one of which is found exclusively in female larvae and pupae. In contrast, *Ae. aegypti* only produces two hexamerins which are expressed in both males and females (albeit more highly in females) (Gordadze et al., 1999; Zakharkin et al., 2001).

These physiological studies illustrate clear differences between autogenous and anautogenous mosquitoes. How these differences impact reproductive processes at a molecular level has yet to be determined. The key difference between the two reproductive groups appears to be on a nutritional level. Studies concerning the effects of hemolymph AA levels upon egg development in mosquitoes have shown that a number of AAs are essential for oogenesis and that a steady infusion of a balanced mixture of AAs into the hemolymph can stimulate egg development in a variety of mosquito species. Furthermore, withdrawal of specific AAs from the infused solution resulted in a failure of egg development (Lea et al., 1956; Uchida K, 1998; Uchida et al.,

2001). A study of the dynamics of AA concentration in the hemolymph shows significant increases in total AA concentration in the hemolymph within eight hours post blood meal which lasts to three days post blood meal (Uchida et al., 1990). It is during this period that the process of vitellogenesis occurs

A key process in reproduction, vitellogenesis, is the synthesis and secretion of YPPs by the fat body (a tissue analogous to the vertebrate liver) (Raikhel and Dhadialla, 1992; Raikhel et al., 2002). In *Ae. aegypti* (the species used in this work), the mosquito requires a three-day period after emergence during which it undergoes physiological changes that prepares it for vitellogenesis. These changes allow the mosquito to become competent to respond to signaling by the steroid hormone 20-hydroxyecdysone (20E) and capable of the extremely high levels of protein synthesis required during vitellogenesis (Raikhel and Lea, 1983). Once the mosquito has achieved competence, its reproductive system enters a state of arrest (previtellogenesis) during which *YPP* gene transcription is repressed until stimulation by a blood meal (Martin et al., 2001a; Attardo et al., 2003). After blood meal, *YPP* genes are transcriptionally activated specifically in the fat body. The focus of our study is upon the transcriptional activation of the major *YPP* gene, *vitellogenin* (*vg*) in the anautogenous mosquito *Ae. aegypti*. *vg* expression peaks at around 24 hours and subsides between 36 and 48 hours after a blood meal. *vg* has been shown to be both directly and indirectly regulated by the steroid hormone 20E, the titers of which directly correlate with *vg* expression during vitellogenesis (Deitsch et al., 1995; Kokoza et al., 2001; Martin et al., 2001b). However,

hormonal regulation is not the complete story as exogenous treatment of competent mosquitoes with physiological levels of 20E do not result in activation of vitellogenesis (Lea, 1982a). However, 20E infusion in combination with AAs results in egg development in *C. pipiens* (Uchida et al., 1998). Based upon this background evidence we suspected that AAs act as a direct signal to the fat body to stimulate vitellogenesis.

AA's have been shown to play a signaling role in cellular sensing of nutrients via the TOR kinase signaling pathway. The TOR kinase (Target of Rapamycin) is a serine/threonine kinase that is ubiquitously expressed in eukaryotes (Raught et al., 2001). It has been well characterized in its role as a nutrient sensor in multiple systems. In *S. cerevisiae* AA and nitrogen source deprivation results in transcriptional activation of nitrogen catabolite repression genes via this pathway (Cooper, 2002). In *D. melanogaster* AAs act directly upon the fat body via the TOR pathway to regulate the growth of the whole organism by a process originating from the fat body (Colombani et al., 2003). In vertebrates, AAs (particularly leucine) are involved in regulation of adipocyte differentiation and metabolism via the TOR pathway (Lynch et al., 2000). AAs and TOR are also involved in blastocyst trophoectoderm differentiation and embryonic implantation in mammals (Martin and Sutherland, 2001).

In this work we show that this AA signaling mechanism has been utilized to regulate *YPP* gene expression in mosquitoes. Our experiments demonstrate that the presence of AAs increases the basal levels of *vg* expression and is essential for *vg*'s transcriptional response to 20E stimulation. Further

characterization of this response by withdrawal of individual AAs shows that there are specific AAs that are essential for this process. We also demonstrate the involvement of the TOR kinase in the transmission of this signal by blocking TOR signaling with rapamycin, a well characterized inhibitor of the TOR kinase (Heitman et al., 1991; Koltin et al., 1991). Treatment with cycloheximide, a translational inhibitor, reveals a similar effect to rapamycin treatment indicating that one of the ways TOR may be functioning to control this process is through translational regulation.

Materials and Methods:

Animals. Mosquitoes for fat body culture experiments were reared, fed and dissected as described (Deitsch et al., 1995).

Fat Body Culture. The fat body tissue culture system was described previously (Deitsch et al., 1995; Raikhel et al., 1997). Media in which AAs were removed individually or completely were supplemented with an equal molar amount of mannitol (Sigma-Aldritch Chemicals, St. Louis, MO), to compensate for changes in osmotic pressure. Fat bodies were incubated for three hours at 27°C before collection and processing. Rapamycin-treated fat bodies were given a one hour pretreatment in *Ae. aegypti* physiological saline (APS) with the appropriate concentration of rapamycin. After an hour, fat bodies were transferred to culture media also containing the appropriate concentration of rapamycin. Cycloheximide treatments were performed as described previously (Li et al., 2000).

Real Time PCR Analysis. Total RNA from fat bodies was extracted by the Trizol method (Gibco BRL/Invitrogen, Carlsbad, CA). Three µg of total RNA was treated with amplification grade DNase I (Gibco BRL/Invitrogen) and two µg of DNase I treated RNA were used in cDNA synthesis reactions using the Omniscript Reverse Transcriptase kit (Quiagen). cDNA levels in the different samples were quantified by real time PCR using Taqman primers/probes for *vg* and *actin* and SYBR green primers for *e74*. We used a real time PCR master mix, iQ Supermix (Biorad) for the Taqman reactions or the Quantitech SYBR green Mastermix (Qiagen) for the SYBR green reactions. Primers and probes are as follows (all probes use the Black Hole Quencher and were synthesized by Qiagen): *actin* Forward: 5'ATC ATT GCT CCA CCA GAA CG; *actin* Reverse: 5'AAG GTA GAT AGA GAA GCC AAG; Hex Labeled *actin* Probe: 5'ACT CCG TCT GGA TCG GTG GCT CC; *vg* Forward: 5'ATG CAC CGT CTG CCA TC; *vg* Reverse: 5'GTT CGT AGT TGG AAA GCT CG; Texas Red Labeled *vg* Probe: 5'AAG CCC CGC AAC CGT CCG TAC T; *e74B* Forward: 5'GAC CTC GTT CGC AAA CAC CTC; *e74B* Reverse: 5' AAG CCA CCT GTT GAT CGT CTT C. Final reaction concentration for all primers and probes was 250 nM. Total reaction volumes were 20 µl and run in 96 well PCR plates (Biorad). All reactions were run in duplicate using two µl of cDNA per reaction. Reactions were run on an iCycler real time PCR machine (Biorad). Standard curves used to quantify relative gene concentrations were made from 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest or from a dilution of a plasmid

standard. The program used for amplifying the reactions was as follows. 1. Melting: 95°C 3 Min, 2. Melting: 95°C 10 sec, 3. Annealing 59°C 45 sec (Florescence recorded), 4. Repeat to step 2 for 50 cycles. Real time data was collected by iCycler iQ Real Time Detection System Software V.3.0 for windows. Raw data was exported to Microsoft excel for analysis.

Results

To determine if AAs have an effect on *YPP* gene transcription, we cultured fat body tissue from mature mosquitoes and used real time PCR to quantify expression of the *vg* gene in the presence or absence of AAs. The fat body culture system used was established previously and is well documented (Deitsch et al., 1995; Raikhel et al., 1997). The tissue culture media contains salts, buffer, glucose and AAs.

Amino acids are essential for activation of the *vg* gene. Fat bodies exposed to AAs show an eight-fold increase in the basal level of *vg* expression in comparison with fat bodies without exposure to AAs (Fig 1a). When the same experiment is performed in the presence of 20E the difference in the response between the AA- and AA+ treatments becomes striking, with a 66-fold increase in *vg* expression in the AA+ treatment over the AA- treatment (Fig 1b). To determine if lack of AAs causes global transcriptional inhibition, we looked at levels of the 20E responsive early gene *e74* in treatments with and without AAs. *e74* is responsive to 20E stimulation in AA- treated fat bodies and actually shows

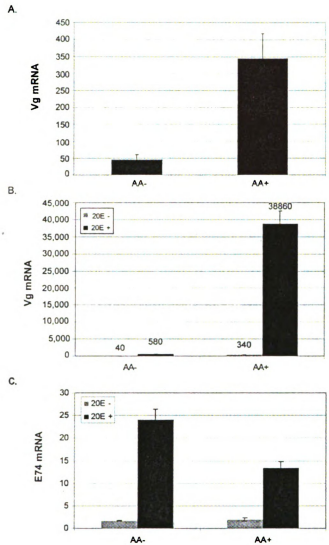


Figure 1. AA stimulation is required for *vg* gene activation. A. Fat body exposure to AAs causes an increase in basal *vg* transcription. Fat bodies from 3-5 day old mosquitoes were incubated in culture with or without AAs for three hours at 27°C and then collected. Total RNA was isolated from groups of six fat bodies. cDNA was synthesized from equal amounts of DNase I treated total RNA. Real-time PCR reactions were performed in duplicate. Data was normalized by real time PCR analysis of *actin* levels in the cDNA samples. Data represent means ± SE of triplicate samples. **B.** AAs are required for *vg* to respond to 20E stimulation. Fat bodies were cultured with and without amino acids and with and without 20E (10^{-6} M) treatment respectively and then processed as above. Data represent means ± SE of triplicate samples. **C.** Absence of amino acids does not inhibit transcriptional activation of the 20E responsive early gene *e74*. cDNA samples were from B. and were analyzed as above using *e74* specific primers. Data represent means ± SE of triplicate samples.

a higher 20E response in the absence of AAs (Fig 1c). This clearly demonstrates that in the absence of AAs the tissues capability to respond transcriptionally to hormonal stimulation has not been compromised. Taken together these experiments demonstrate that AAs act as a direct signaling mechanism upon the fat body and that this signal is affecting specific targets such as the *vg* gene.

Specific amino acids are essential for *vg* activation. We performed experiments using 20 different culture media formulations where each of the individual AAs were removed to determine which were essential for hormonal activation of the gene. The individual withdrawal media were osmotically balanced with mannitol in the same manner as with the total AA withdrawal media. We identified 10 AAs which, when withdrawn, result in a dramatic reduction (more than 90% decrease) in the response by *vg* to 20E induction relative to complete media. Based upon the severity of the effect caused by their withdrawal these AAs have been labeled as essential to vitellogenesis. The essential AAs are listed here in order of the potency of the effect their withdrawal has upon the response to 20E: leucine > tryptophan > methionine > valine > histidine > lysine > phenylalanine > arginine > asparagine > threonine. Three AAs, cysteine, glycine and isoleucine resulted in a significant decrease in the response by *vg* to 20E (50-80% decrease). Withdrawal of tyrosine, aspartic acid, serine, proline, glutamine, alanine and glutamic acid resulted in a low to non-statistically significant reduction in response by *vg* (30-0% decrease)(Figure 2).

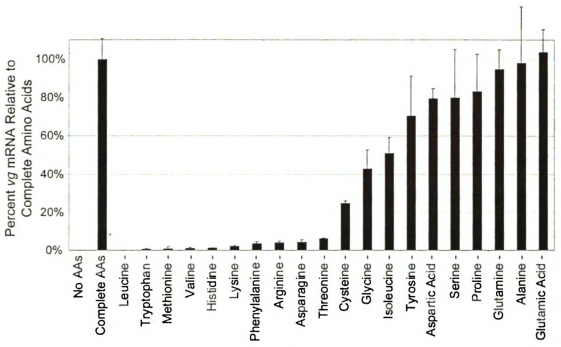


Figure 2. Specific AAs are essential for 20E responsiveness by *vg*. Fat bodies were cultured in media lacking individual AAs with 20E (10^{-6} M). Culture protocols and sample analysis were the same as in figure 1. Responses to 20E stimulation in the withdrawal medias are presented as the mean percentage relative to the response observed in media containing complete amino acids ± SE of triplicate samples.

Based upon the properties of the AAs identified as essential, we recognized some patterns occurring in this group. All cationic AAs and two of the three-branched chain AAs were found to be essential. The last branched chain AA, isoleucine, also caused a significant decrease in *vg* expression, but the result was not as dramatic as that seen with leucine and valine. Both cationic and branched chain AAs have been implicated in nutritional signaling via the TOR kinase pathway, and leucine in particular has been shown in a number of studies to be especially important (Lynch et al., 2000; Kimball and Jefferson, 2001; Colombani et al., 2003; Jacinto and Hall, 2003).

The TOR signaling pathway transmits the amino acid signal. Based upon the fact that this system appears to be driven by AAs, we next decided to test whether the nutritionally regulated TOR kinase pathway is involved in mediating the AA signal. We used a TOR specific inhibitor called rapamycin. Rapamycin is a macrolide and functions by forming a complex with a 12 kD protein called FKBP12. This complex then binds specifically to the TOR kinase and inhibits its kinase activity (Sigal and Dumont, 1992; Cardenas et al., 1995). Fat bodies in culture were treated with rapamycin to observe its effect upon AA stimulation of *vg*.

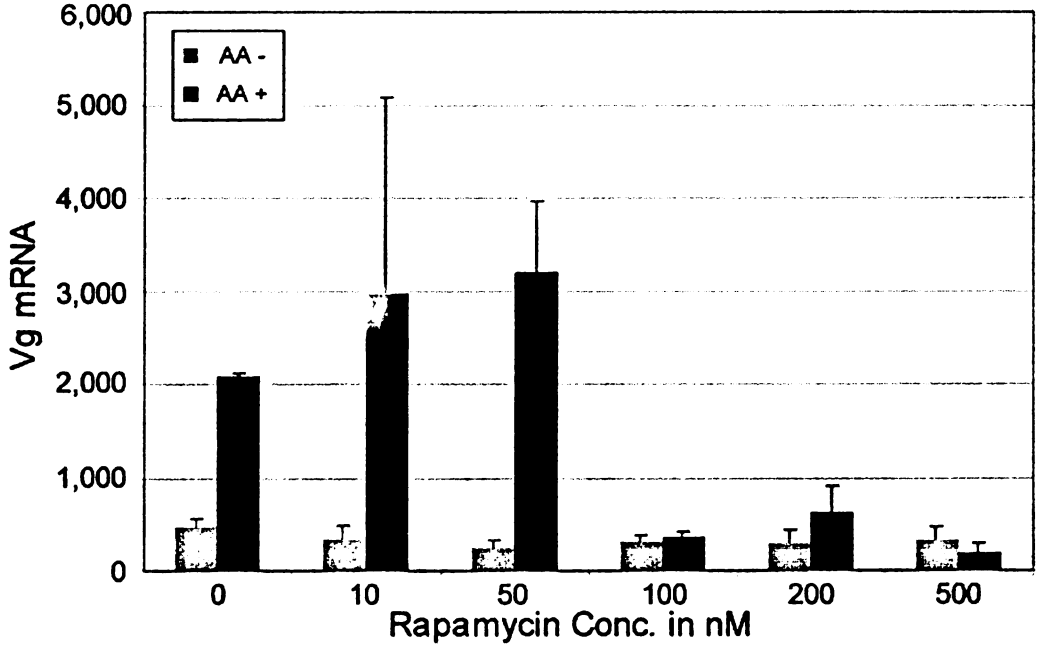


Figure 3: Rapamycin inhibits the stimulatory response by *vg* to the presence of amino acids. Fat bodies were pretreated for 1 hour in APS with varying concentrations of rapamycin. Fat bodies were then transferred to media with or without AAs and the appropriate concentration of rapamycin, incubated for three hours and collected for analysis. Sample analysis was as in figure 1. Data represent means ± SE of triplicate samples.

After preincubation in physiological saline for 1 hour with various concentrations of rapamycin ranging from 10 to 500 nM, the fat bodies were transferred to culture media containing the appropriate levels of rapamycin with or without AAs. We found that at 100 nM of rapamycin the basal response by *vg* to the presence of AAs was completely inhibited to the levels seen in the AA-control (Figure 3).

This data confirms the hypothesis that TOR is mediating this signal. To determine the effect of rapamycin on 20E stimulation of the promoter in the presence of AAs, we treated fat bodies in the presence of AAs and 20E with and without 100 nM rapamycin. Rapamycin treatment resulted in a 71% reduction in the response by *vg* to 20E stimulation (Figure 4a). To rule out the possibility that the rapamycin effects observed are being caused by general toxic effects on the fat body tissue, we again examined the response of the early gene *e74* to 20E stimulation in the presence of rapamycin. The *e74* gene remains capable of responding to 20E stimulation in the presence of rapamycin and was not significantly different from the control, demonstrating that while rapamycin specifically blocks the TOR based signal to *vg* it does not inhibit 20E responsiveness in general (Figure 4b).

Global inhibition of translation blocks amino acid signaling. TOR regulates cellular responses to nutrients through both transcriptional and translational regulation mechanisms (Rohde et al., 2001). To determine if translational regulation is a mechanism by which TOR might be regulating *vg* expression, we used the translational inhibitor cycloheximide.

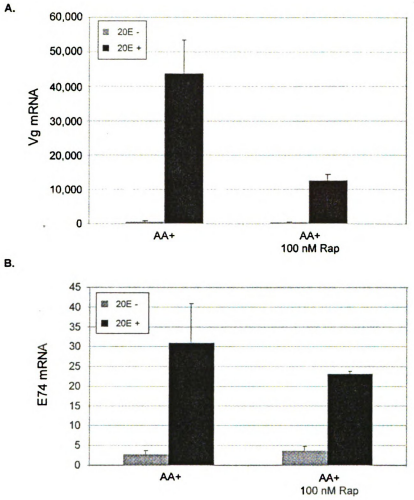


Figure 4. Rapamycin inhibits 20E induction of *vg*. A. Rapamycin inhibition of 20E induction of *vg*. Fat bodies were pretreated with 100 nM rapamycin for 1 hour and transferred to culture media with AAs, 100 nM rapamycin and with or without 10^{-6} M 20E for 3 hours. Sample analysis was as in figure 1. Data represent means ± SE of triplicate samples. B. Rapamycin treatment does not inhibit induction of the 20E responsive gene *e74*. cDNA samples were from A. and were analyzed as above using *e74* specific primers. Data represent means ± SE of triplicate samples.

Fat bodies were treated with and without AAs in the presence and absence of cycloheximide. Cycloheximide treatment completely inhibited the response by the *vg* gene to AA stimulation (Figure 5). This result illustrates that translation is required for AA based transcriptional activation of *vg* and that activation of the TOR pathway is required for the translation of factors critical to *vg* activation.

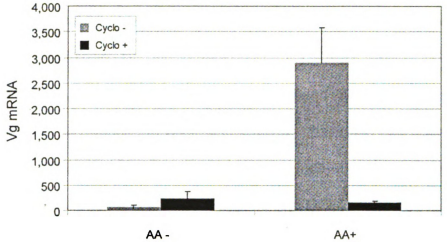


Figure 5. Cycloheximide inhibits AA stimulation of *vg*. Fat bodies were cultured for three hours in media with or without amino acids and with and without 10^{-4} M cycloheximide. Sample analysis was as in figure 1. Data represent means ± SE of triplicate samples.

Discussion

This work demonstrates that in *Ae. aegypti,* vitellogenic gene expression is dependent upon AA signaling and that a number of specific AAs are essential for this process. The AAs found to be essential for vitellogenesis completely overlap with the ten AAs essential for growth in mammals with the exception of isoleucine which was on the borderline for being essential for vitellogenesis (Rose, 1938a). One AA found to be essential for vitellogenesis, but not for mammalian growth was asparagine. Comparison of AAs essential for *Ae. aegypti vg* gene expression and those essential for complete egg development in *Ae. aegypti* and *C. pipiens pallens* also reveals a high degree of conservation of the AAs essential for these processes. The only exception again was asparagine which was found to be essential for vitellogenesis in this assay but not for egg development when omitted from an artificial blood meal in *Aedes* (Dimond et al.,

1956). Studies examining the requirements of amino acids for larval growth show that the same AAs essential for vitellogenesis are also essential for larval growth (Singh and Brown, 1957) (Figure 6).

Both cationic and branched chain AAs have been implicated in nutritional signaling via the TOR kinase pathway, and leucine in particular has been shown in a number of studies to be especially important (Lynch et al., 2000; Kimball and Jefferson, 2001; Colombani et al., 2003; Jacinto and Hall, 2003). The fact that leucine is the most critical AA for vitellogenesis strongly supports that TOR is regulating this system.

A number of AAs were identified as non-essential. It is possible that these AAs are playing other roles in the mosquito. Hemolymph concentrations of these AAs may be high before a blood meal making them poor vitellogenic signaling molecules. Interestingly, a recent study showed that *Ae. aegypti* may use proline for nitrogen storage before and after blood meal. Proline as well as alanine and glutamic acid are at a high concentration relative to the other free AAs in the hemolymph of competent pre-blood fed mosquitoes (Goldstrohm et al., 2003). This finding is in agreement with the fact that these AAs also appear to be non-essential for vitellogenic activation.

The mechanism by which TOR senses the concentration of hemolymph AAs is still unclear, but the downstream mechanisms of how TOR transduces this signal are better understood. TOR is involved in the regulation of both transcription and translation. TOR regulates translation through the maintenance of the phosphorylation state of two translational regulatory proteins. TOR

Amino Acids	*Aedes vg* Gene Expression	*Aedes* Egg Development	*Culex* Egg Development	*Aedes* Larval Growth	Mammalian Growth
Leucine	XX	XX	XX	XX	XX
Tryptophan	XX	XX	XX	XX	XX
Methionine	XX	X		XX	XX
Valine	XX	XX	XX	XX	XX
Histidine	XX	X	X	XX	XX
Lysine	XX	XX	XX	XX	XX
Phenylalanine	XX	XX	XX	XX	XX
Arginine	XX	XX	XX	XX	XX
Asparagine	XX		XX		
Threonine	XX	XX	XX	XX	XX
Cysteine	X	X	XX	X	
Glycine	X				
Isoleucine	X	XX	XX	XX	XX
Tyrosine					
Aspartic Acid					
Serine				X	
Proline				X	
Glutamine					
Alanine					
Glutamic Acid		X			

Figure 6. Comparison of amino acids essential for vitellogenic gene expression, growth and egg development. XX – represents absolutely essential amino acids, **X** – represents semi-essential amino acids. Mammalian growth data from (Rose, 1938b). Aedes larval growth data from (Singh and Brown, 1957). *Culex* egg development data from (Uchida K et al., 1992). Aedes egg development data from (Dimond et al., 1956)

phosphorylates and activates the p70 S6 kinase in response to nutritional stimulus (Chung et al., 1992; Brown et al., 1995; Fox et al., 1998). Activated S6 kinase phosphorylates the ribosomal protein S6 that specifically facilitates the translation of mRNA's containing a 5'-polypyrimidine tract. These types of

mRNAs typically encode ribosomal proteins, translation elongation factors, and growth control proteins (Jefferies et al., 1997). TOR also regulates the phosphorylation state of the PHAS-I/4E-BP1 factor. This factor in a dephosphorylated state binds to and inhibits the eIF-4E translational initiation factor which functions in CAP recognition and recruitment of ribosomes to the mRNA. Phosphorylation of 4E-BP1 inactivates it and derepresses eIF-4E allowing translation to proceed (Brunn et al., 1997). The experiment showing cyclohexamide blockage of the AA signal suggests that translational regulation is an important mechanism for this pathway. It is possible that in the absence of AAs, TOR is inactive. This inactivity may prevent translation in the fat body and prevent the synthesis of essential factors or cofactors required for transcriptional activation of *vg*.

TOR has also been well documented in its ability to regulate transcription. In yeast, TOR regulates the nuclear localization of a GATA type transcription factor named Gln3 via its phosphorylation state. Lack of nutrients causes the dephosphorylation of Gln3 and its cytoplasmic binding partner Ure2. This results in the translocation of Gln3 into the nucleus. Upon translocation to the nucleus, Gln3 displaces a GATA repressor Dal80 which results in the activation of a set of genes known as the nitrogen catabolite repressed genes (Cardenas et al., 1999; Beck and Hall, 1999; Oliveira et al., 2003). We have suggestive evidence that TOR may directly regulate transcriptional activation of *vg* by a similar mechanism. Analysis of the *vg* regulatory region has revealed the significance of GATA transcription factors in this system. We showed previously that the *vg*

gene is directly repressed by a GATA type transcription factor, named AaGATAr, during the previtellogenic state of arrest (Martin et al., 2001a; Attardo et al., 2003). We also have EMSA evidence that there is a second cytoplasmic GATA factor that translocates into the nucleus within an hour of blood feeding (Data not shown). This data creates striking parallels between the two systems.

The cycloheximide results in combination with GATA factor history suggest that TOR could be working by both transcriptional and translational control mechanisms to regulate *vg* expression in response to blood meal generated AA signals.

We show here that AAs play a critical role in the vitellogenic regulation of the major *YPP* gene *vg*. As female mosquitoes use blood as a primary nutritional source, detection of AA's coming from the digestion of a blood meal is an ideal signal to trigger initiation of vitellogenesis. While it has been shown in other systems that the TOR pathway regulates growth and metabolism, this is the first demonstration that the TOR pathway has been adapted to regulate reproduction.

The use of the TOR pathway to regulate reproduction demonstrates the adaptation of a major signaling pathway to facilitate the unique reproductive requirements of mosquitoes. In some mosquito species capable of both autogenous and anautogenous egg development, this adaptation may allow flexibility in choosing the reproductive path taken in response to its global nutritional status. As observed in previous studies, autogenous mosquitoes typically emerge from the pupal stage with higher levels of nutrients, specifically higher hemolymph titers of AAs than anautogenous mosquitoes (Chambers and

Klowden, 1994; Su and Mulla, 1997b). It is possible that the higher hemolymph AA titers in autogenous mosquitoes are above the threshold required for activation of the TOR signaling system and allows the mosquito to initiate reproduction.

Further study of this system is required on multiple levels. The exact mechanism as to how AAs are detected by TOR remains unknown. Furthermore, is TOR regulating GATA factor activity, do AAs regulate other processes via similar pathways in other organs during vitellogenesis in the mosquito and what role do these processes play in reproduction? Deeper analysis of this system will provide insight into the molecular mechanics of mosquito reproduction and that of other blood feeding insects.

Chapter 4: Cloning and Characterization of the *Aedes aegypti slimfast* Gene *

Abstract

In *Aedes aegypti* AA signaling is essential for the initiation of vitellogenesis. The nutrient-regulated TOR kinase signal transduction pathway conducts the AA signal. This signal results in basal activation of the *vg* gene and most likely other *yolk protein precursor* (*YPP*) genes. Furthermore, AA signaling is required for activation of *vg* by the steroid hormone 20-hydroxyecdysone (20E). While TOR is the confirmed mediator of this signal, it is not known what proteins lie upstream in this pathway. AA transporters have been suspected as being an integral part of AA signaling. A cationic AA transporter called Slimfast (Slif) was recently shown to be required for TOR based nutritional regulation of organismal growth in *Drosophila melanogaster*. To determine if Slif is also involved in nutritional regulation of vitellogenesis in mosquitoes the *Ae. aegypti* homologue of *slif* was cloned. *slif* is well conserved between *D. melanogaster*, *Anopheles gambiae* and *Ae. aegypti* and is related to the cationic AA transporter genes (*cat* genes) in mammals. Genomic searches also revealed the presence of other *cat*-like genes in the *D. melanogaster* and *An. gambiae* genomes. *Aaslif* mRNA is present in the fat body during previtellogenesis and decreases at the peak of vitellogenesis. *Aaslif* levels also decrease in fat body culture upon exposure to amino acids and TOR knockdown, suggesting expression of *Aaslif* is negatively nutritionally regulated. Functional analysis of *Aaslif* by RNAi shows that it is involved in the AA signaling pathway that regulates vitellogenesis. Knockdown of *Aaslif* reduces the basal AA activation of *vg*. However, this

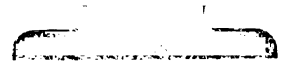
knockdown is not capable of inhibiting hormonal stimulation with 20E, suggesting that there may be redundancy in the system compensating for *Aaslifs* function.

Introduction

Mosquitoes are responsible for the transmission of diseases that have a large negative impact upon human life. The primary reason for this is that mosquitoes have evolved to use mammalian blood as a source of nutrition to drive reproduction. In many species of mosquitoes, a blood meal must be taken before they can undergo reproduction. Mosquitoes that utilize this reproductive strategy are called anautogenous and maintain their reproductive system in a state of arrest in the absence of blood (Roubaud, 1929).

A key process in the reproductive system is vitellogenesis. Vitellogenesis is the synthesis and secretion of yolk protein precursors (YPPs) by the fat body, a tissue analogous to the vertebrate liver (Raikhel, 1987b). Transcription of *YPP* genes is repressed during the state of arrest. It was demonstrated previously that the key trigger that releases the reproductive system from the state of arrest and activates vitellogenesis are amino acids (AAs). AAs are released from the midgut as the blood meal is being digested. AAs in the hemolymph directly stimulate the fat body. Stimulation of the fat body results in a basal increase in the transcription of the *vg* gene (the major *YPP* gene) and prepares it for stimulation by 20E. Furthermore, we showed that the AA signal is transduced by the TOR kinase nutritional pathway (Hansen et al., 2004).

A similar system has been identified in *D. melanogaster*. In the *D. melanogaster* fat body, TOR mediated AA signaling regulates the growth rate of the whole organism. This system was identified during a search for growth phenotypes coming from a transposon mediated mutagenesis study. The



disrupted gene encoded a cationic AA transporter called *slimfast (slif).* Disruption of *slif* resulted in global inhibition of growth as well as a sensitivity to arginine starvation. The phenotypic effects of *slif* knockdown resemble those of rapamycin treatment, the inhibitor of the TOR pathway (Colombani et al., 2003). The mechanism of how AAs are detected by the TOR pathway remains unknown. However, AA transporters, if not the direct sensor for AAs, are thought to be at least indirectly involved in sensing AAs.

There are four proposed general mechanisms by which AA transport might regulate nutritional signaling. One possibility is that the transporter acts as a receptor at the top of a signal transduction pathway. In this type of mechanism the receptor detects AA influx through a change in conformation that would be transduced to a signaling protein such as a G-protein. The second possibility is that AAs flowing into and other solutes flowing out of the cell result in physiological changes in membrane polarity, cellular volume, pH and salt concentrations, which are then detected by the cell. A third possibility is that an intracellular receptor is detecting increased concentration of AAs, charged tRNAs, or AA metabolites. Fourth and finally, if AAs are sensed by an extracellular receptor, nearby transporters affecting local extracellular AA concentrations could regulate these receptors by their level of transport activity (Hyde et al., 2003).

Slif is most closely related to the CAT or cationic AA transporter family of proteins in mammals. CAT proteins are defined as transporters exhibiting affinities and translocation rates for cationic AAs, that are higher than or

equivalent to those for other types of AAs. There are different cationic AA transport systems and the CAT proteins specifically are members of the y^+ system of cationic transporters. The y^+ system is specific for the basic AAs arginine and lysine (Deves and Boyd, 1998).

Previous results show that withdrawal of either arginine or lysine from the fat body severely inhibits vitellogenic activation even when the other 19 AAs are present. Most likely, these two AAs, as well as the other AAs found to be essential, are working through a specific detection system linked to the TOR pathway. To determine if Slif is involved in this detection system, we cloned the *Ae. aegypti* homologue of *slif* (*Aaslif*). We show here that *Aaslif* is expressed in the fat body and is differentially regulated in response to AAs and nutritional stimulation. RNAi knockdown of this gene results in an inhibition in the response to AA stimulation by the *vg* gene. However, the knockdown of *Aaslif* is not able to inhibit 20E activation in the presence of AAs, indicating that there may be redundancy in the sensory system.

Materials and Methods

Molecular biology techniques & cloning. Standard procedures were used for recombinant DNA manipulations (Ausubel et al., 1991). Homologues to *D. melanogaster slif* were identified in the *An. gambiae* genome project database and aligned using ClustalW (http://clustalw.genome.ad.jp/). The phylogenic tree of *slif* homologues was also created using ClustalW using the following sequences obtained by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/):

Aedes-slif (Accession #: AY654299); Anopheles-slif (Accession #: XP_314535.1); Drosophila-slif (Accession #: NP_649428.1); Drosophila-1 (Accession #: NP_649019.1); Anopheles-1 (Accession #: XP_314536.1); Homo-CAT2A (Accession #: NP_003037.1); Mus-CAT1 (Accession #: NP_031539.1); Rattus-CAT1 (Accession #: NP_037243.1); Mus-CAT2 (Accession #: NP_031540.1); Homo-CAT3 (Accession #: Q8WY07); Rattus-CAT2A (Accession #: NP_072141.1); Xenopus-1 (Accession #: AAH63720.1); Homo-CAT1 (Accession #: NP_003036.1); Mus-CAT3 (Accession #: NP_031541.1); Rattus-CAT3 (Accession #: NP_058913.1); Xenopus-2 (Accession #: AAH42222.1); Drosophila-2 (Accession #: NP_996088.1); Anopheles-2 (Accession #: XP_314539.1); Drosophila-3 (Accession #: AAR88535.1); CaenorhabditisE-1 (Accession #: NP_493662.1); CaenorhabditisB-1 (Accession #: CAE62808.1); Danio-CAT4 (Accession #: NP_919408.1); Homo-CAT4 (Accession #: NP_004164.1); Oryza-1 (Accession #: NP_921650.1.

Highly conserved regions were chosen as a template for primers to amplify a partial cDNA of the *Ae. aegypti* homologue of *slif* from a fat body cDNA library. The 5'- and 3'-ends of the cDNAs were amplified by rapid amplification of cDNA ends (RACE) PCR using the Smart cDNA RACE Amplification Kit (BD Clontech, Palo Alto, CA). All PCR products were cloned in pCRII-TOPO vector (Invitrogen, Carlsbad, CA). Analysis of primary predicted protein structure was performed at EXPASY (http://us.expasy.org/). Transmembrane domain prediction was created using the TMHMM software at (http://bioweb.uwlax.edu/Default.htm).

Animals. Mosquitoes for fat body culture experiments were reared, fed and dissected as described (Deitsch et al., 1995).

Fat Body Culture. The fat body tissue culture system was described previously (Deitsch et al., 1995; Raikhel et al., 1997). Media from which AAs were removed was supplemented with an equal molar amount of mannitol (Sigma-Aldrich Chemicals, St. Louis, MO), to compensate for changes in osmotic pressure. Fat bodies were incubated for three hours at 27°C before collection and processing.

Real Time PCR Analysis. Total RNA from mosquitoes or fat bodies was extracted by the Trizol method (Gibco BRL/Invitrogen, Carlsbad, CA). Three µg of total RNA was treated with amplification grade DNase I (Gibco BRL/Invitrogen) and two µg of DNase I-treated RNA were used in cDNA synthesis reactions using the Omniscript Reverse Transcriptase kit (Quiagen). cDNA levels in the different samples were quantified by real time PCR using Taqman primers/probes for *vg* , *slimfast* and *actin*. A real time PCR master mix, iQ Supermix (Biorad), was used for the Taqman reactions. Primers and probes are as follows (all probes use the Black Hole Quencher and were synthesized by Qiagen): *actin* forward: 5'ATC ATT GCT CCA CCA GAA CG; *actin* reverse: 5'AAG GTA GAT AGA GAA GCC AAG; Hex labeled *actin* probe: 5'ACT CCG TCT GGA TCG GTG GCT CC; *vg* forward: 5'ATG CAC CGT CTG CCA TC; *vg* reverse: 5'GTT CGT AGT TGG AAA GCT CG; Texas red labeled *vg* probe:

5'AAG CCC CGC AAC CGT CCG TAC T; *slimfast* Forward: 5'CTG GTT GGC TTC GTG AT; *slimfast* Reverse: 5'CTC TAG TTG ACT TTC CGA C; FAM labeled *slimfast* probe: 5'CAT TCG ACA TTC GGT TCT TGG CTC CG. Final reaction concentration for all primers and probes was 250 nM. Total reaction volumes were 20 µl and run in 96 well PCR plates (Biorad). All reactions were run in duplicate using two µl of cDNA per reaction. Reactions were run on an iCycler real time PCR machine (Biorad). Standard curves used to quantify relative gene concentrations were made from 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest or from a dilution of a plasmid standard. The program used for amplifying the reactions was as follows. 1. Melting: 95°C 3 Min, 2. Melting: 95°C 10 sec, 3. Annealing 59°C 45 sec (Florescence recorded), 4. Repeat to step 2 for 50 cycles. Real time data was collected by iCycler iQ Real Time Detection System Software V.3.0 for windows. Raw data was exported to Microsoft Excel for analysis.

RNA interference. Generation of double-stranded RNAs (dsRNA) was accomplished by cloning *slif* and *TOR* template cDNAs into the pLitmus 28i vector. dsRNA was produced by *in vitro* transcription with T7-RNA polymerase using the Hiscribe RNAi Transcription Kit (New England Biolabs, Beverly, MA). Approximately 0.5 to 1 µg of dsRNA in 0.3 to 0.5 µl of H2O was injected into the thorax of CO_2 anesthetized three to five day old female mosquitoes. The mosquitoes were allowed to recover for three days before further processing.

Results and Discussion

Cloning and characterization of *slimfast*. The *Ae. aegypti slif* (*Aaslif*) cDNA (Accession #: AY654299) consists of 2256 basepairs and codes for a protein consisting of 428 AAs with a predicted molecular wieght of 64 kD. *Aaslif* is 68% identical and 79% similar to a predicted *An. gambiae* homologue (Accession #: XM_314535). Relative to *D. melanogaster slif*, *Aaslif* is 52% identical and 66% similar. Protein folding prediction algorithms predict that *Aaslif* contains a total of 14 transmembrane helices (Figure 1). Blast analysis of *Aaslif* indicates that the most closely related mammalian homologues are the CAT (cationic AA transporter) proteins. Phylogenic analysis of the *D. melanogaster, An. gambiae and Ae. aegypti slif* homologues (Figure 2) places them in a group that lies outside the well-characterized mammalian CAT 1, 2 and 3 proteins, indicating that variants of the CAT proteins probably arose in mammals from a precursor cationic transporter after divergence from insects. There also appear to be additional uncharacterized cationic AA transporters in both the *D. melanogaster* and *An. gambiae* genomes indicating the likelihood that there are more cationic transporters present in *Ae. aegypti*.

***Aaslif* Expression.** *Aaslif* is expressed in the fat body during the previtellogenic time period. Within the 12 hours after emergence, *Aaslif* mRNA levels are high and then drop to a basal level after 12-24 hours (Figure 3). After a blood meal *Aaslif* mRNA levels rise slightly at six hours and then drop to their lowest point by

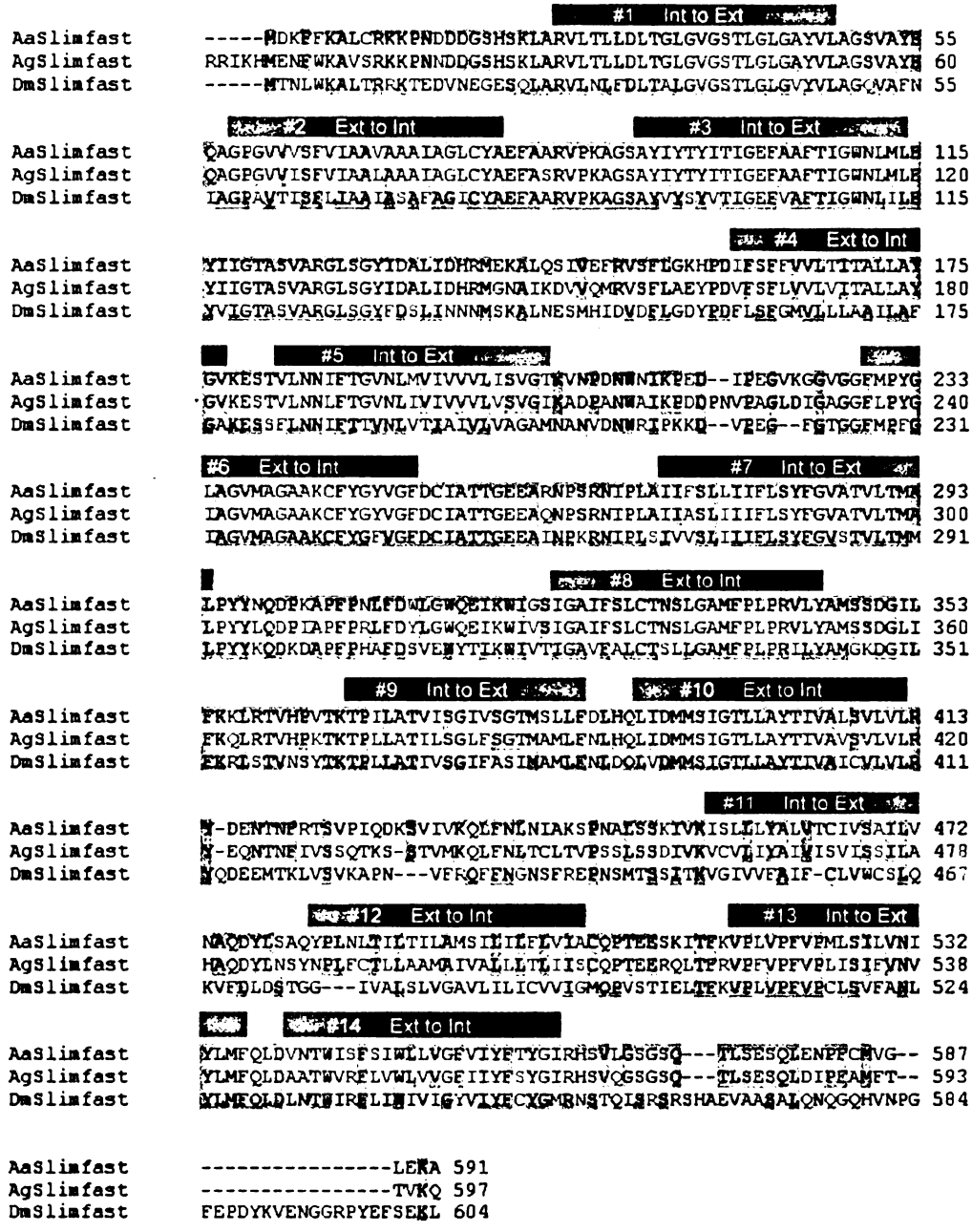


Figure 1. ClustalW alignment of the *Ae. aegypti*, *An. gambiae* and *D. melanogaster* slimfast protein sequences. AaSlimfast – Ae. aegypti slimfast (Accession #: AY654299); AgSlimfast – Ag. Gambiae slimfast (Accession #: XM_314535); DmSlimfast – D. melanogaster slimfast (Accession #: NP_649428.1). Bars above the alignment represent transmembrane domains. Labels on the bars represent the orientation of the domain. Int to Ext – Intracellular to Extracellular; Ext to Int – Extracellular to Intracellular

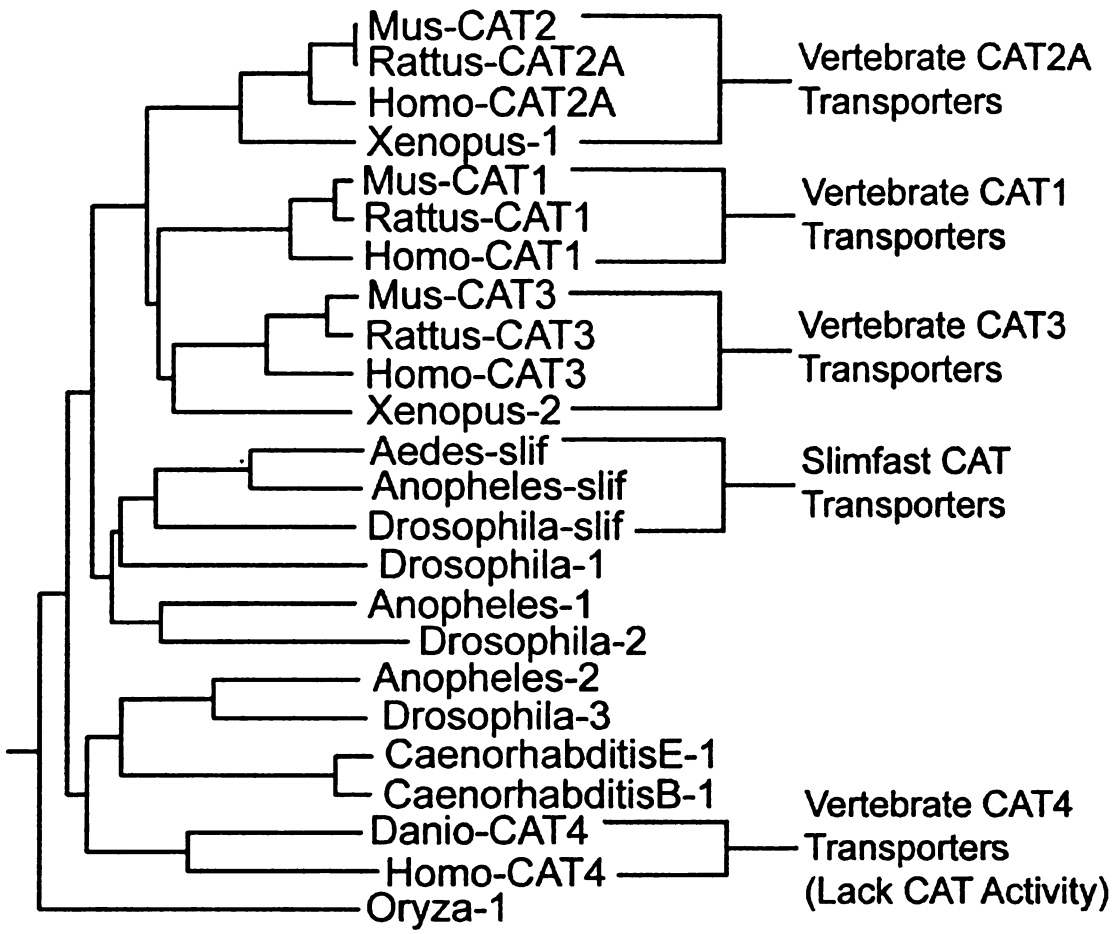


Figure 2. Phylogenic tree of *Ae. aegypti slimfast* and homologues. Mus: *Mus muscolus*; Rattus: *Rattus norvegicus*; Homo: *Homo sapiens*; Xenopus: *Xenopus laevis*; Aedes: *Aedes aegypti*; Anopheles: *Anopheles gambiae*; Drosophila: *Drosophila melanogaster*; CaenorhabditisE: *Caenorhabditis elegans*; CaenorhabditisB: *Caenorhabditis briggsae*; Danio: *Danio rerio*; Oryza: *Oryza sativa*. (See materials and methods for gene accession #'s)

30 hours. This drop coincides with the peak of vitellogenesis that occurs at around 30 hours post blood meal (PBM). As vitellogenesis is terminating (between 36 and 72 hours) *Aaslif* mRNA levels rise significantly although not to the level seen during the initial peak at 0 to 12 hours post emergence.

The high levels seen soon after emergence might be due to the structural remodeling occurring in the fat body during which it prepares itself to become

receptive to vitellogenic stimuli. During this time it is likely that the fat body is synthesizing the transporter to permit detection and transport of AAs released from a future blood meal. The lower mRNA levels present after this period may be used to maintain a steady state level of active transporter in the fat body. The peak in mRNA seen at termination is most likely the fat body preparing itself again for the next vitellogenic cycle by replenishing transporter protein levels.

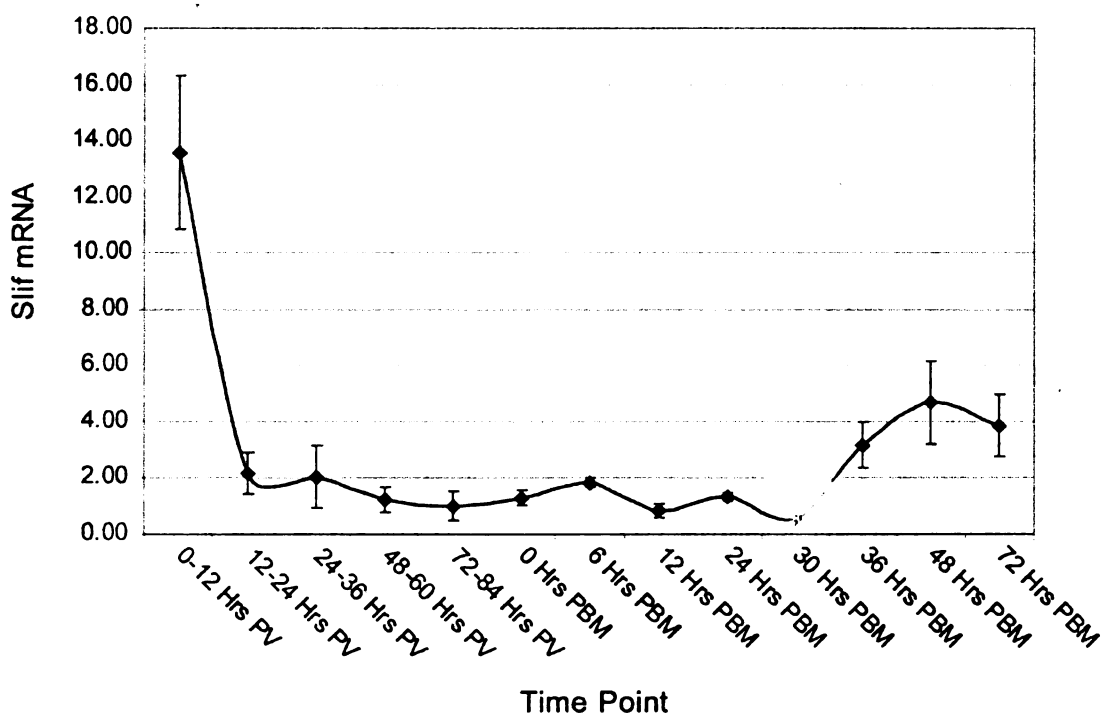


Figure 3. *Aaslif* fat body mRNA profile. Real-time PCR analysis was performed upon cDNA samples obtained from mosquito fat bodies dissected at 12 hour previtellogenic intervals and 6 to 12 hour intervals during the vitellogenic state. cDNA was synthesized from total RNA from three groups of six fat bodies per time point. Reactions were performed in triplicate. Data represent means ± SE of triplicate samples.

***Aaslif* RNAi and nutritional regulation of mRNA stability.** Double stranded RNA (dsRNA) was produced to a 774 nucleotide (nt) region of *Aaslif* spanning from nt 1192 to nt 1965. Three to five day old non-blood fed mosquitoes were injected with *Aaslif* dsRNA. As a control for comparison of *Aaslif* knockdown

effects on nutritional signaling, *AaTOR* was knocked down in parallel in another group of mosquitoes using the same protocol. After three days of recovery the mosquitoes were dissected and fat bodies were cultured in the presence and absence of AAs. Analysis of the level *Aaslif* mRNA present in the knockdown mosquitoes shows a significant decrease in the *Aaslif* dsRNA treated mosquitoes (Figure 4a). Interestingly, *Aaslif* levels in the control fat bodies decrease significantly upon the addition of AAs, suggesting that the presence of AAs negatively regulates *Aaslif* transcription. This would explain the decrease in *Aaslif* mRNA levels in the fat body after blood feeding during the time course.

This observation is not without precedent as this also occurs in the case of the mammalian *cat-1* gene. It was shown that *cat-1* transcription itself was not affected by the presence or absence of AAs, but that the *cat-1*mRNA stability is regulated. The mRNA is stable in the absence of AAs and unstable in their presence. This is due to the presence of *cis* acting RNA sequences in the 3'-UTR of the mRNA (Hyatt et al., 1997; Aulak et al., 1999). It is possible that a similar system is regulating *Aaslif* mRNA stability. TOR may be affecting this process as the mRNA levels of *Aaslif* actually rise higher in the TOR knockdown mosquitoes than the controls in the absence of AAs.

***Aaslif* knockdown affects amino acid stimulation of the *vg* gene.** Analysis of *vg* mRNA in the *Aaslif* knockdown mosquitoes indicated a significant decrease in the response by *vg* to AAs as compared to the *mal* control. The decrease in *vg* stimulation is not statistically different from that seen in the TOR knockdown,

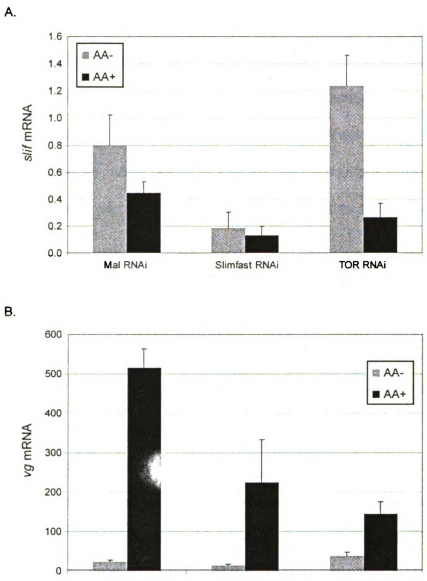


Figure 4. *Aaslif* knockdown inhibits amino acid stimulation of *vg*. A. Treatment with *Aaslif* dsRNA results in a significant reduction in *Aaslif* mRNA. Three to five day old mosquitoes were given injections of 0.6-1.0 µg of dsRNA coding for the non-coding region of a bacterial gene (Mal RNAi), the coding region of the *Aaslif* gene (Slimfast RNAi) or the coding region of the *AaTOR* gene. Mosquitoes were allowed to recover for three days. Fat bodies from these mosquitoes were then dissected and cultured in either the presence or absence of amino acids. Culture protocols and sample analysis were the same as in figure 1 of chapter 3. Gene expression was analyzed using *Aaslif* specific real time PCR primers. Data represent means ± SE of triplicate samples. **B.** Treatment with *Aaslif* dsRNA results in inhibition of AA stimulation of the *vg* gene. cDNA samples from A. were analyzed using *vg* specific real time PCR primers. Data represent means ± SE of triplicate samples.

although the *TOR* knockdown effect appears to be somewhat stronger than that seen with the *Aaslif* knockdown (Figure 4b). However, while a decrease in the activation of *vg* was seen with AA stimulation alone, *vg* activation was not affected when fat bodies were treated with AAs in combination with 20E in *Aaslif* knockdown mosquitoes, while the *TOR* knockdown mosquitoes showed a significant decrease in the response to 20E treatment (data not shown).

The role of amino acid transport in vitellogenesis. AA based regulation of vitellogenesis appears to be a complex system that most likely combines inputs from multiple sources and funnels them through the TOR kinase in order to regulate gene expression and translation. *Aaslif's* role as a cationic AA transporter affects the perceived input of arginine and lysine into this system as can be seen by the effect of its knockdown on AA stimulation of *vg*. In AA withdrawal experiments the withdrawal of either arginine or lysine results in severe attenuation of the response of fat bodies to hormonal stimulation. However, the ability of the fat body to respond to hormonal stimuli after RNAi knockdown of *Aaslif* suggests that either: a complete knockout (rather than knockdown) of this transporter is required to see an effect or there is enough redundancy in the AA transport system to cover the loss of one cationic AA transporter. The presence of other sequences homologus to cationic AA transporters in the *An. gambiae* and *D. melanogaster* genomes suggests that these genes could provide redundant function in the absence of Aaslif. Another possibility is that other cationic AA transport systems besides the y^+ system are

capable of transporting arginine and lysine if necessary. Further characterization of other cationic transporters is required for complete analysis of the role of cationic amino acid transport in the regulation of vitellogenesis.

Chapter 5: Summary and Future Research Directions

The work in this thesis covers important aspects of the molecular mechanisms regulating the process of vitellogenesis in the yellow fever mosquito *Ae. aegypti.* This work has shown that the *AaGATAr* gene is part of a mechanism maintaining the previtellogenic transcriptional state of arrest by repressing *YPP* genes; that AAs from a blood meal signal to the fat body to activate vitellogenesis through the TOR pathway; and that a cationic AA transporter acts as part of the AA signaling pathway (Figure 1). In this section I will give a detailed summary of the findings of these studies and propose future research to be done to further clarify how these mechanisms work to regulate vitellogenesis.

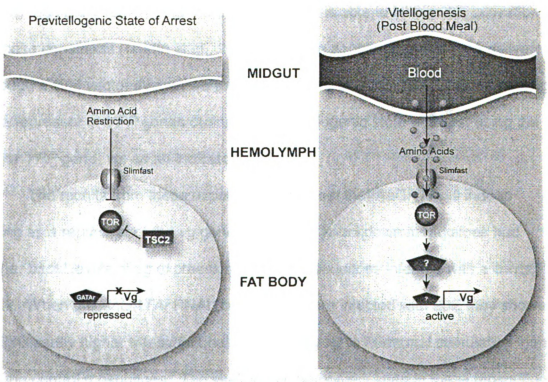


Figure 1. Schematic diagram of nutritional regulation of vitellogenesis.

GATA Factor Regulation of Vitellogenesis

In the first section of this thesis the role of a GATA type transcription factor in the regulation of the previtellogenic state of arrest was analyzed *in vivo*. The GATA factor AaGATAr, had been suspected of being a repressor of vitellogenesis as a result of *in vitro* studies. These studies showed a number of lines of evidence supporting this hypothesis. *AaGATAr* mRNA is present during the previtellogenic period. *In vitro* expressed AaGATAr protein binds to GATA response elements present in the *vg* promoter. Nuclear extracts from previtellogenic fat bodies contain a GATA binding activity. Cell culture experiments showed AaGATAr is able to repress expression constructs driven by the *vg* regulatory region, and more specifically it is able to inhibit 20E activation of these constructs (Martin et al., 2001a). Using double stranded RNA delivered through a viral vector system we tested the hypothesis that AaGATAr was acting as a repressor of *YPP* genes during the previtellogenic state of arrest using the major *YPP* gene, *vg*, as an indicator.

The results from these experiments suggest that AaGATAr is indeed acting as a repressor of the *vg* gene. *AaGATAr* knockdown mosquitoes had higher basal levels of *vg* expression than did mosquitoes infected with a control virus. When the *AaGATAr* RNAi mosquitoes were injected with 20E they showed a significantly higher transcriptional response by *vg* to hormonal stimulation than did the control mosquitoes. The vitellogenic responses seen in these mosquitoes were not equal to what is seen after a blood meal, but do represent a significant derepression of the *vg* gene. This indicates that in knocking out *AaGATAr* we

derepressed the gene resulting in an increased basal state, but the absence of stimulatory signals did not allow the gene to be completely activated as would be seen after a blood meal.

Questions still remain pertaining to how blood meal stimulation relieves the previtellogenic repression caused by AaGATAr. Based upon the demonstrated mechanisms present in the literature, nuclear translocation of another GATA factor might be the way this occurs. In yeast a GATA factor repressor called Dal80 inhibits the activation of nitrogen catabolite repression genes (NCR genes) in the presence of nutrients. Dal80 is replaced under nutrient starved conditions by another GATA factor called Gln3. The cellular localization of Gln3 is regulated by its phosphorylation state, which is controlled by the TOR kinase. In the presence of nutrients Gln3 is cytoplasmic and in the absence of nutrients Gln3 translocates into the nucleus to displace Dal80 and activate the NCR genes (Oliveira et al., 2003). GATA factor regulation by translocation has also been observed in another insect in the regulation of choriogenesis. In the silkworm *Bombyx mori* a GATA factor called BCFI has been identified which has three isoforms resulting from differential splicing of the same gene. In follicle cells one of these variants is cytoplasmic at the beginning of choriogenesis and translocates into the nucleus during late choriogenesis (Drevet et al., 1994; Skeiky et al., 1994; Drevet et al., 1995).

Interestingly, in *Ae. aegypti*, another GATA factor besides AaGATAr is suspected to be in the fat body. The cytoplasm of previtellogenic fat body nuclear extracts contains a GATA binding factor that has a different mobility from

the GATA factor present in the nucleus. After a blood meal the cytoplasmic GATA factor appears to translocate into the nucleus within an hour of blood feeding. Recently the cDNA for another GATA factor, an isoform of *AaGATAr*, was cloned and is suspected of playing a role as an activator of vitellogenesis (Park J.H. and Raikhel, A.S. Unpublished data). Further study of this isoform (*AaGATAa)* should be performed. To determine its tissue and stage specificity, an expression profile of this isoform should be examined. AaGATAa DNA binding capabilities should also be examined to see if it has the capacity to bind to response elements in the *vg* promoter. To determine its transcriptional activity, cell transfection experiments should be performed using expression constructs driven by the *vg* regulatory region. If possible, isoform-specific antibodies should be raised against AaGATAr and AaGATAa and immunocytochemistry studies should be performed to determine the cellular localization of these isoforms during the previtellogenic state of arrest and after blood feeding. To answer the question of its effect upon *YPP* transcription, an RNAi knockdown analysis specific to *AaGATAa* should be performed in vitellogenic mosquitoes to see if it affects *YPP* gene expression.

Amino acid regulation of vitellogenesis

Previous studies have shown high levels of nutrients and AAs specifically to be correlated with egg development in anautogeneous mosquitoes (Uchida K, 1998). The question remained however, how do high levels of AAs initiate vitellogenesis in the mosquito? The hypothesis that this thesis tests is that AAs work directly on the fat body to initiate vitellogenesis. This was tested through

the use of an *in vitro* tissue culture system that allowed the testing of the effects of AAs on gene expression in the fat body using *vg* again as the representative *YPP* gene. Treatment of the fat body with all 20 AAs resulted in a basal up regulation of *vg* expression. Furthermore, 20E treatment of fat bodies with and without AAs showed that hormonal stimulation is only functional in the presence of AAs.

Specific AAs are critical for this system to function. We identified 10 AAs which, when withdrawn result in a dramatic reduction (more than 90% decrease) in the response by *vg* to 20E induction relative to complete media. Based upon the severity of the effect caused by their withdrawal these AAs have been labeled essential to vitellogenesis. The essential AAs are listed here in order of the potency of the effect their withdrawal has upon the response to 20E: leucine > tryptophan > methionine > valine > histidine > lysine > phenylalanine > arginine > asparagine > threonine. Interestingly, the essential AAs contained all the basic AAs and two of the three branched chain AAs. Both of these groups have been associated with nutritional signaling by the TOR kinase pathway (Colombani et al., 2003; Lynch et al., 2003).

Finally the TOR kinase pathway was shown to be the effector of the AA signal as treatment with the TOR inhibitor rapamycin blocked basal stimulation and reduced 20E activation in the presence of AAs. Furthermore, treatment with the translational inhibitor cycloheximide also blocks AA signaling, suggesting that translational control of some factors is another possible mechanism regulating

vitellogenesis. This is significant as one of the cellular functions the TOR pathway controls is translation.

AAs act as the signal from the blood meal that initiates vitellogenesis. TOR then transduces this signal. What remains to be understood is what occurs downstream of the TOR kinase itself. TOR is known to control the regulation of transcription as well as translation (Rohde et al., 2001). The cycloheximide experiment indicates that translation is important for gene expression during vitellogenesis. This does not remove the possibility that TOR is also controlling transcription directly through interactions with transcription factors. TOR regulates the phosphorylation state and cellular localization of the yeast GATA factor Gln3. As GATA factors have been demonstrated to be important in the regulation of *vg* transcription, the posttranslational regulation of AaGATAr and AaGATAa by TOR should be considered as a possibility. As was proposed earlier, isoform specific antibodies and either an immunocytochemical assay for nuclear translocation of the GATA factors or western blot of nuclear and cytoplasmic proteins would be a good test for TOR regulation of AaGATAr and AaGATAa. Translocation events could be tested upon exposure of *in vitro* cultured fat bodies to AAs and rapamycin as well as fat bodies from mosquitoes before and after blood meal. These antibodies could also be used in chromatin immunoprecipitation assays to determine if exposure of fat bodies to AAs results in a switch in the GATA factor present at the *vg* regulatory region. Connecting AA signaling to GATA factor translocation would clearly link TOR to transcriptional regulation of YPP genes.

The role of Slimfast in Amino Acid Signaling

How exactly the TOR kinase senses AAs remains unknown. However, a cationic AA transporter Slif is suspected to be part of the mechanism of AA detection based upon recent work in the *D. melanogaster* fat body (Colombani et al., 2003). Whether this is due to an increased intracellular concentration of AAs and their metabolites or through a direct signaling mechanism that is inherently part of the transporters function is not understood. To test if this transporter is involved in nutritional regulation of vitellogenesis the *Ae. aegypti* homologue of *slif, Aaslif* was cloned and analyzed.

Aaslif has significant conservation between *D. melanogaster*, *An. gambiae*, and *Ae. aegypti*. A BLAST search using the *Aaslif* sequence reveals it is most closely related to the *cat* transporter genes in mammals, which exclusively transport arginine and lysine (Closs, 1996). It also revealed the presence of other cationic transporters in the *D. melanogaster* and *An. gambiae* genomes. *Aaslif* is expressed highly immediately after eclosion and after termination of vitellogenesis, at low levels in the fat body during the previtellogenic period and appears to be down regulated at the peak of vitellogenesis. In fat body culture the addition of amino acids causes a reduction in *Aaslif* mRNA, suggesting that it may be negatively nutritionally regulated. Interestingly, when *TOR* is knocked down, *Aaslif* expression goes up in the fat body. TOR knockdown would simulate a starved condition and adds further evidence to the possibility that *Aaslif* is nutritionally regulated. RNAi knockdown

of *Aaslif* itself inhibits the transcriptional response by *vg* to AA stimulation, but *Aaslif* knockdown does not inhibit 20E stimulation of *vg* in the presence of AAs.

As it appears that there are other cationic AA transporters present in the *An. gambiae* genome, there are most likely others present in the *Ae. aegypti* genome. To comprehensively analyze the role of cationic AA transporters in the regulation of vitellogenesis these other putative transporters should also be cloned and characterized in the same way as *Aaslif* was. The combinatorial effects of these transporters upon vitellogenesis could then be determined by attempting multiple gene knockdowns by injection of a mixture of dsRNA homologus to all the transporters and subsequent monitoring of *vg* transcription after exposure to AAs and to AAs and 20E.

A remaining question to answer is whether these transporters are sending the signal through the TOR pathway. While monitoring *vg* activity in response to AAs is a good indicator that TOR is functioning, it is not a direct analysis of TOR activity. A direct assay of TOR activity has been used in *D. melanogaster*. This assay monitors the phosphorylation state of threonine 389 in the P70 S6 kinase, using a monoclonal antibody and western blot to observe TOR kinase activity under different nutritional conditions (Stocker et al., 2003). The P70 S6 kinase is a highly conserved protein and would most likely be conserved enough between *D. melanogaster* and *Ae. aegypti* to allow the same antibody to be used for the same purpose in mosquitoes. Using this assay, tissue culture could be performed on fat body tissue from AA transporter knockdown mosquitoes to determine if in these mosquitoes, TOR kinase activity is being inhibited.

If a signal is being sent down the TOR signal transduction pathway by an AA transporter, these transporters may be directly interacting with other proteins at the plasma membrane. An experiment to identify putative transporter interacting proteins would be to express the intracellular domains of Aaslif as bait in a yeast two-hybrid system and to use previtellogenic and vitellogenic fat body cDNA libraries as prey. Based upon the types of proteins interacting with the transporter an educated guess could be formed as to if the transporter is directly signaling down a pathway, what pathway that might be and in what direction to take the research next.

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