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Characterization of ecdysone-inducible early gene E74 in the mosquito (Aedes aegypti)

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Guoqiang Sun

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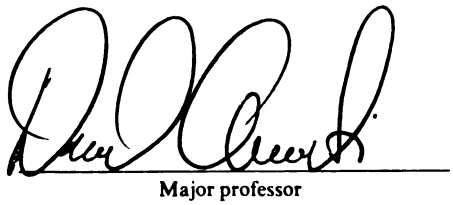

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CHARACTERIZATION OF ECDYSONE-INDUCIBLE EARLY GENE E74 IN THE MOSQUITO *AEDES AEGYPTI*

By

Guoqiang Sun

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ABSTRACT

CHARACTERIZATION OF ECDYSONE-INDUCIBLE EARLY GENE E74 IN MOSQUITO *AEDES AEGYPTI*

By

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In the anautogenous mosquito, *Aedes aegypti*, the reproduction depends on the acquisition of a blood meal. Consequently, mosquitoes are vectors of numerous devastating human diseases. Blood feeding triggers a hormonal cascade mediated by the steroid hormone 20-hydroxyecdysone (20E). This cascade activates expression of yolk protein precursor (*YPP*) genes in the female mosquito fat body, an insect metabolic tissue. This process is called vitellogenesis. The mediator genes of the ecdysteroid regulatory hierarchy are conserved between vitellogenesis in mosquitoes and metamorphosis in *Drosophila*. *E74*, an ecdysone-inducible early gene, is a key mediator gene in the hierarchy. In this dissertation, I present the cloning of the mosquito *E74* (*AaE74*) gene, which encodes two isoforms AaE74A and AaE74B. They have a common C-terminal Ets DNA-binding domain and unique N-termini. In the fat body, the transcript of AaE74B is induced by a blood meal, exhibiting a profile corresponding to those of *YYP* genes. In contrast, the transcript of AaE74A is activated at the termination stage of vitellogenesis, suggesting that AaE74B is an activator for *YYP* genes while AaE74A is a repressor during vitellogenesis in mosquitoes. Both AaE74 isoforms are ecdysone-inducible early gene products. Functional analyses have showed that AaE74B can activate the reporter

gene expression driven by the promoter from major yolk protein gene *vitellogenin* (*Vg*) in *Drosophila* Schneider 2 cells. In contrast, AaE74A exhibits a repression activity. Significantly, several putative E74 binding sites in the *Vg* promoter were demonstrated to be required for these activities, as deletion of these binding sites completely abolishes regulation by E74. These findings are congruent with their proposed roles in vitellogenesis. Remarkably, as assessed by transfection assays, AaE74B acts synergistically on *Vg* promoter with ecdysone receptor complex, and the action requires the identified E74 binding sites and ecdysone response elements in *Vg* promoter. Moreover, gel mobility shift assays have shown that both AaE74B and ecdysone receptor complex co-exist during vitellogenic period. Taken together, these results reveal a unique class of Ets-domain proteins with distinct functions in gene regulation in the mosquito fat body. Significantly, these studies provide evidence supporting a novel mechanism in insects by which target gene expression may be amplified by the cross talk between an Ets-domain protein and a nuclear receptor.

Dedicated to my lovely wife Qin Yu, and my respectful parents Songlin Son and Hongzhen Wang

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LIST OF ABBRVIEATIONS

20E: 20-hydrocxyecdysone

Aa: *Aedes aegypti*

AD: activation domain

AF-1: active activation function 1

AF-2: active activation function 2

AR: androgen receptor

ARC: activator-recruited cofactor

Bp: base pair

CA: corpora allata

CAT: chloramphenicol acetyltransferase

CBP: CREB binding protein

CC: corpora cardiaca

CDC: Center for Disease Control and Prevention

cDNA: complementary DNA

Chx: cycloheximide

DBD: DNA-binding domain

Dm: *Drosophila melanogaster*

DNA: Deoxyribonucleic acid

DR: direct repeats

DRIP: vitamin D receptor-interacting proteins

EcR: ecdysone receptor

EcREs: ecdysone response elements

ERG: ets-related gene

Elf-1: E74 like factor 1

ER: estrogen receptor

ESET: ERG (ets-related gene)-associated protein with a SET (suppressor of variegation, enhancer of zest and trithorax) domain

FOG-1: friend of GATA-1

GMSA: gel mobility shift assay

GR: glucocorticoid receptor

hid: head involution defective

HAT: histone acetyltransferases

HDAC: histone deacetylase

HNF3/fkh: hepatocyte nuclear factor3/forhead transcription factor

HREs: hormone response elements

HSF: Heat-shock factor

JHIII: juvenile hormone III

Kb: kilobase pair

LBD: ligand binding domain

Luc: luciferase

MR: mineralocorticoid receptor

NcoA: nuclear receptor coactivator

NcoR: nuclear receptor corepressor

NMR: nuclear magnetic resonse

NR: nuclear receptor

OEH: ovarian ecdysteriodogenic hormone

PAGE: polyacrylmide gel electrophoresis

PBP: PPAR-binding protein

PPAR: peroxisome-proliferator-activated receptor

PR: progesterone receptor

PBM: post blood meal

PCR: polymerase chain reaction

RAR: retinoid acid receptor

RBC: red blood cell

RER: rough endoplasmic reticulum

RNA: ribonucleic acid

rpt: reaper

RT: reverse transcription

RXR: retinoid X receptor

S2: *Drosophila* Schneider cell line 2

SDS: sodium dodecyl sulfate

SET: suppressor of variegation, enhancer of zest and trithorax

SMRT: silencing mediator of retinoid and thyroid hormone receptor

SMRTER: SMRT-related ecdysteroid receptor interacting factor

SRC: steroid receptor coactivator

TR: thyroid hormone receptor

TRAP: TR-associated proteins

USP: *Ultraspiracle*

VCP: vitellogenic carboxypeptidase

VCB: vitellogenic cathepsin-B

Vg: vitellogenin

VDR: vitamin D receptor

VgR: vitellogenin receptor

WHO: World Health Organization

wHTH: winged helix-turn-helix

YYP: yolk protein precursor

Chapter I Literature Overview

MEDICAL SIGNIFICANCE OF MOSQUITOES

The females of most mosquito species need to take blood in order to obtain protein resources for egg development. This blood feeding behavior allows mosquitoes to transmit numerous pathogens to animals and humans such as protozoan (Malaria), metazoan (Lymphatic filariasis) and viral pathogen (Dengue, Yellow fever, West Nile virus and Encephalitis). Mosquito-borne diseases are the most vicious diseases in the modern world, in both under-developing and developed countries.

Of these diseases, malaria is the most deadly disease in the world, which is endemic in the majority of the poorest countries the world. Based on the data from World Health Organization (WHO), about 500 million people are affected annually by malaria alone, leading to more than one million deaths per year–114 deaths from malaria every hour, mainly among young children in sub-Saharan Africa. In many malaria endemic countries, national malaria control programs that were established during 1960's and 1970's, when elimination of malaria was considered to be an achievable goal, have failed. Thus for many years, there has been little change in mortality and mortality from malaria, especially in Africa. Recently, the malaria is probably increasing in sub-Saharan Africa, due to insecticide resistance, environmental changes, climate changes, population migration and population increase. Moreover, because of the elimination of malaria from most developed countries, the pharmaceutical industry had lost interest in the development of anti-malaria drugs and governments had reduced the support for research on malaria.

The direct economic cost of malaria that results from treatment and from absence of work are tremendous, but the overall economic impact of malaria is likely to be more

substantial than suggested by estimates of direct cost alone, when are considers additional factors such as impact on tourism, trade and foreign investment.

Plasmodium falciparum and Plasmodium vivax are the main parasites and *Anopheles gambiae* is the major parasite transmission vector of malaria in Africa. Malaria parasites have a complex life cycle in a vertebrate host (often a human, monkey or rodent) and an insect vector (the female *Anopheles* mosquito). The bite of an infected mosquito initiates infection. During this process, the *Plasmodium* sporozoites in the salivary gland of the mosquito are injected into the tissue layers of the skin. The sporozoites then enter the circulatory system and are transported to infect hepatocytes in the liver. Within the hepatocyte, the parasite divides and matures to produce thousands of merozoites, which are released into the blood stream to infect red blood cells (RBCs). Merozoites within the RBCs replicate to produce new merozoites, which escape when the cell bursts. This cycle is continued, leading to the rapid increase of infected RBCs in the host and results in malaria-associated symptoms. Some of the parasites within the infected RBCs also undergo sexual differentiation, forming gametocytes, which are taken up by the mosquito during a blood meal to continue the life cycle (Beier, 1998; Kappe *et al.*, 2003).

Dengue is another mosquito-borne disease, which is found in tropical and sub-tropical regions around the world. The global prevalence of dengue has grown dramatically in recent decades (WHO report, 2002). Approximately 2,500 million people, two fifths of the world's population, are now at the risk of Dengue. The spread of dengue is attributed to increased spread of the dengue virus and their mosquito vectors, the most predominant of which is the urban species *Aedes aegypti.* Dengue fever is a

severe, flu-like illness, but seldom causes death. However, the other form-Dengue hemorrhagic fever, can be fatal, and is characterized by high fever, enlargement of liver, and circulatory failure.

West Nile virus is a human, equine, and avian neuropathogen. The virus is indigenous to Africa, Asia, Europe, and Australia, and has recently caused large epidemics in Romania, Russia, and Israel. The unexpectedly rapid spread of the West Nile virus within the North America further demonstrates the historical public health issue of mosquito-borne disease. As of the end of year 2002, the WHO and Center for Diseases Control and Prevention (CDC) have reported 3587 human cases of the West Nile Virus in the United States, with 211 deaths occurring in the 39 states and the district of Columbia (WHO disease Outbreak Report 14 November 2002). West Nile Virus is a member of the family *Flaviviridae* in the genus *Flavivirus*. Antigentically, it is a member of the Japanese encephalitis virus serocomplexes that include St. Louis encephalitis, Japanese encephalitis, and Murray Valley and Kunjin encephalitis viruses (Coia *et al.*, 1988; Nedry and Mahon, 2003). Each member of this serocomplexes can be transmitted by mosquito and cause fever, which is potentially fatal in human. West Nile Virus is primarily a bird virus that is spread by bird-feeding mosquitoes. As a consequence of the recent outbreak of West Nile Virus, it has already killed at least 100,000 crows, blue jays, and other birds, many of which are endangered species (Komar *et al.*, 2001).

In general, mosquitoes don't transmit West Nile Virus from person to person. Human illness from the West Nile Virus is rare, even in the areas where the West Nile Virus has reported. While some mosquito species are most likely to accelerate the virus life cycle in bird, others bite both birds and human and serve as "bridge species". The

complex dynamics of birds, mosquitoes and human also cause erratic outbreak patterns. The severity of the recent explosion was speculated to be caused by a mutant, more virulent form of the virus (Campbell *et al.*, 2002; Meek, 2002).

GENETICALLY MODIFIED MOSQUITOES

Though intense efforts have been made to improve the treatment of mosquito-transmitted diseases and to control insect vector numbers through environmental management, the overall impact on the incidence of insect-borne diseases has been limited. In principle, these diseases could be controlled by eliminating the mosquito, but in practice this approach has serious limitations. Another important class of weapons in the fight against malaria is the drugs that kill the parasite in the human. The effectiveness of this approach has been limited by problems of drug resistance, cost and drug side effects. Effective vaccines have proven to be much more difficult to develop than anticipated due to the capricious antigen of the parasite surface (Ghosh *et al.*, 2002).

The advent of molecular tools to manipulate insects, such as *Drosophila melanogaster*, presents researchers with the possibility to apply these techniques to vectors of insect-borne diseases, such as malaria. With these techniques, researchers can generate mosquitoes that block development and transmission of the malaria parasite. Furthermore, these transgenic mosquitoes could cross with wild-type mosquitoes in natural environments, dispersing the antiparasitic genes in the population (Atkinson and Michel, 2002; Ghosh *et al.*, 2002).

To begin such an effort, emphasis was first placed on developing successful genetic transformation of the insect vector, using strong promoters to drive expression of

exogenous genes in abundant quantities and in a tissue-specific manner. Although the requirement of a blood meal for reproduction renders the mosquito as a human pathogen vector, it provides an avenue for developing novel strategies to combat the diseases. One excellent example is the application of the *vitellogenin* promoter from *Aedes aegypti*, which is a sex-, stage-, and tissue- specific strong promoter induced by a blood meal to drive the expression of anti-pathogen gene products such as defensin (Kokoza *et al.*, 2000).

VITELLOGENESIS IN THE MOSQUITO *AEDES AEGYPTI*

In the anautogeneous mosquito *Aedes aegypti*, vitellogenesis initiates only after an uptake of a blood meal from a vertebrate host. Vitellogenesis involves the coordination of several processes. Firstly, massive syntheses of major yolk protein precursors (YPPs) such as vitellogenin (Vg) (Dhadialla and Raikhel, 1990), vitellogenic carboxypeptidase (VCP) (Cho *et al.*, 1991), and a 44kDa cathepsin B-like protease (VCB) (Cho *et al.*, 1999) and lipophorin (Sun *et al.*, 2000), by the fat body, a major metabolic organ of insect, analogous to vertebrate liver and adipose tissue combined; secondly, secretion of the YPPs into the hemolymph, the body fluid functionally similar to the vertebrate blood, finally the specific accumulation of these proteins by the developing oocytes (Raikhel and Lea 1983, 1986; Raikhel and Dhadialla, 1992). In *A. aegypti*, vitellogenesis depends on the interaction between neuropeptides and other signal molecules. The requirement of a blood meal, called anautogeny, results in a highly regulated cyclicity of egg production that is tightly coupled with food ingestion. The first cycle proceeds through two developmental periods, each of which is strictly under

control of two major insect hormones: juvenile hormone III (JHIII) and 20-hydroxylecdysone (20E) (molecular structures shown in figure 1) (reviewed in Raikhel *et al.*, 2002). The previtellogenic period begins at eclosion of the adult female and is divided into a preparatory stage and the state of arrest. The previtellogenic period is believed to be under control of JHIII (Shapiro *et al.*, 1986; Raikhel and Lea, 1990, 1991). The vitellogenic period starts with uptake of a blood meal and proceeds for 48 to 72 hours, depending on the availability of next blood meal, and is divided into a synthetic stage and a termination stage. The ecdysteroid hormone 20E has been demonstrated to control this period (figure 2) (Hagedorn, 1985, 1989; Raikhel and Lea, 1991; reviewed in Raikhel 2002).

The previtellogenic period

The previtellogenic period is a preparatory stage in which vitellogenic tissues, fat body and ovary, become competent for the requirements of intense physiological demands of vitellogenesis. The fat body is distributed throughout the head, thorax, and abdomen, however, most of the fat body is located in abdomen, where it forms sheets or lobes attached to the epidermis and surrounding midgut (Raikhel and Snigirevskaya, 1998). Previtellogenic development is critical for the mosquito fat body to acquire competence for Vg synthesis and responsiveness to 20E. When the adult emerges from the pupal stage, the corpora allata (CA) begins to secret JHIII, which signals the fat body to prepare for vitellogenesis (Shapiro and Hagedorn, 1982). During the preparatory stage, fat body trophocytes (the major fat body cell type) response to JHIII by two subcelluar

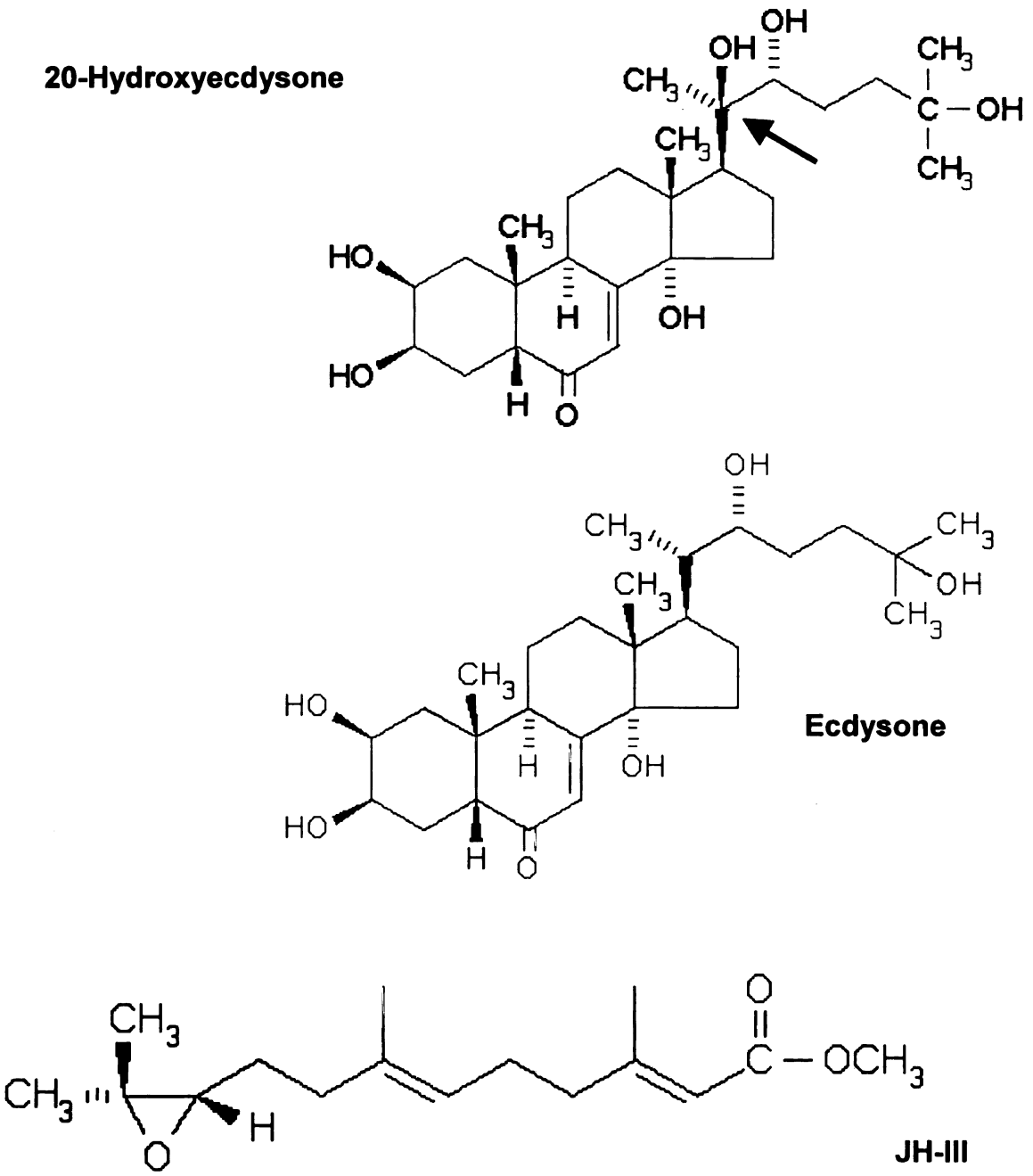

Figure 1. Chemical structures of ecdysone, its active form 20-Hydroxyecdysone and juvenile hormone III (JHIII). The arrow indicates the hydroxyl group at position 20 of the ecdysone steroid branch.

events: 1) increasing ploidy level from 2n to 4n, or even 8n by the third day postemergence, leading to an increase in RNA synthesis and accumulation of nonadenylated RNA (Dittermann *et al.*, 1989); 2) proliferating ribosome and total RNA increase by about 50% over first 3 days postemergence (Snigirevskaya *et al.*, 1997).

Previtellogenic period is also required for ovaries to become competent for uptake of YPPs from the hemolymph via the receptor-mediated endocytosis during vitellogenic period (Raikhel and Dhadialla, 1992). The machinery for this process such as clatherin-coated vesicles and coated pits is developed during this period (Kokoza *et al.*, 1997). The mosquito vitellogenin receptor (VgR), which is crucial for the receptor-mediated endocytosis, is also expressed at this stage (Sappington *et al.*, 1995, 1996; Sappington and Raikhel, 1998).

After acquirement of competence for 20E responsiveness, the female then enters a state of developmental arrest that persists until a blood meal. This provides a mechanism to synchronize the massive synthesis of YPPs in a sex-, stage- and fat body-specific manner triggered by ecdysteriod hormone.

In *A. aegypti*, *βFTZ-F1*, an orphan nuclear receptor (Laudet *et al.*, 1992; Ohno and Petkovich, 1993), was implicated as a competence factor (Li *et al.*, 2000a). The βFTZ-F1 mRNA is highly transcribed in mosquito fat bodies during previtellogenic period when 20E titer is low (Li *et al.*, 2000a). However, βFTZ-F1 protein is expressed only after 2-3 days postemergence in female mosquito, which coincides with onset of competence for 20E response. The JHIII titers increase gradually after eclosion, and reach a peak at 2-3 days postemergence. Over the next 5 days in female mosquitoes if not given a blood meal, the JHIII titers then slowly decline (Shapiro *et al.*, 1986).

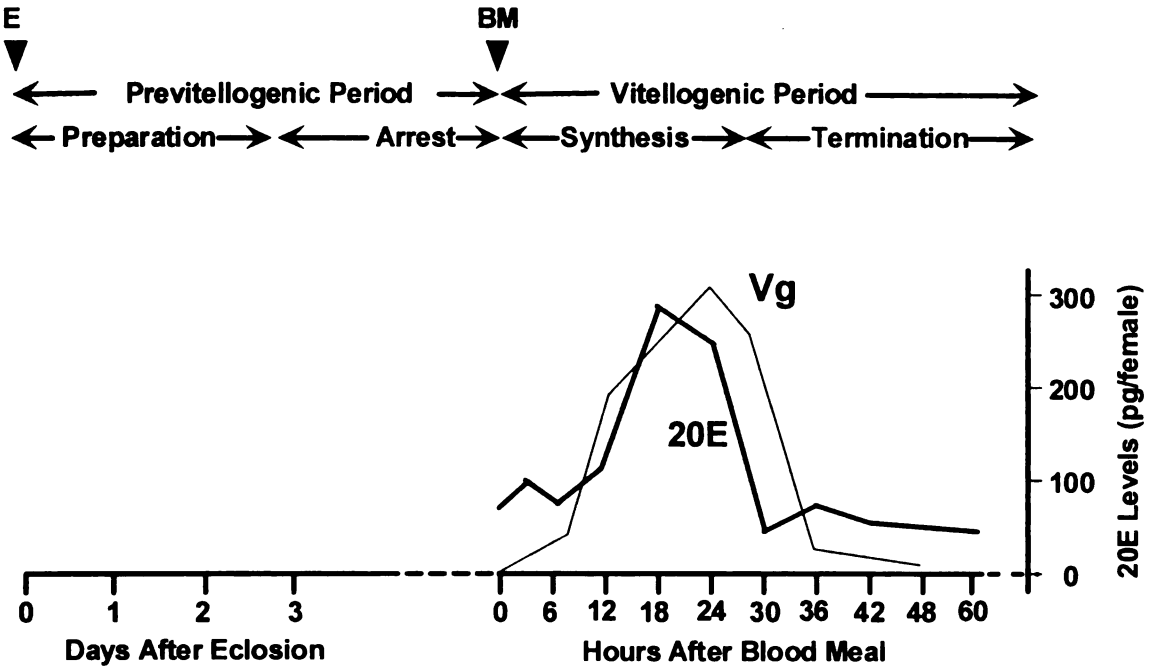

Figure 2. Summary of the vitellogenic cycle. In the mosquito, *Aedes aegypti*, vitellogenesis in the fat body can be divided into four phases: previtellogenic preparation, state of arrest, vitellogenic synthetic stage, and vitellogenic termination stage (Raikhel *et al.*, 1992). Transcription profile of *Vg* gene was determined by Northern and RT/PCR analyses (Redrawn from Raikhel 1992. 20E titers are from Hagedorn *et al.*, 1975).

The correlation of the translation of *βFTZ-F1* and JHIII titers brings up an intriguing question of whether the *βFTZ-F1* is regulated by JHIII at the translational level. Further study of the βFTZ-F1 protein profiles upon manipulation of the female mosquito fat bodies *in vitro* by JHIII will unveil their relationships as well as the mechanism of modulation of competence in mosquitoes.

State of arrest is an important adaptation for anautogeny of mosquito, which prevents the premature activation of *YYP* genes in previtellogenic competent females prior to a blood feeding. It has been demonstrated that the 20E signaling pathway is inhibited during the state of arrest (Li *et al.*, 2000a). In *A. aegypti*, the functional ecdysone receptor complex consists of ecdysone receptor (AaEcR) (Cho *et al.*, 1995) and its obligatory heterodimer partner *Ultraspiracle* (AaUSP) (Kapitskaya *et al.*, 1996). The complex is capable of activating *Vg* gene expression by recognizing the specific response elements in the *Vg* promoter (Martin *et al.*, 2001). The AaEcR and AaUSP proteins are abundant in the nuclei of the previtellogenic female fat body at the state of arrest (Zhu *et al.*, 2000). However, the AaEcR/AaUSP heterodimer is barely detectable in the fat body nucleus during this stage. Study has shown that AaUSP exists as a heterodimer with mosquito HR38 (AaHR38), which is a repressor that can compete with AaEcR for the common partner AaUSP under low 20E titer. However, the elevated 20E titer, triggered by a blood meal, allows AaEcR to efficiently displace AaHR38 and form a functional heterodimer with AaUSP and activate target gene expression (Zhu *et al.*, 2000). Significantly, *Vg* gene has been demonstrated to be the direct target of AaEcR/AaUSP complex (Martin *et al.* 2001).

Another repression mechanism of *Vg* gene expression is achieved probably by the mosquito GATA transcription factor, called AaGATAr. AaGATAr appears to repress *Vg* gene expression by occupying GATA binding sites in the 5' regulatory region of the *Vg* gene during previtellogenic period as well as postvitellogenic period (Martin *et al.*, 2000).

The vitellogenic period

During the previtellogenic period, fat body is remodeled into an efficient protein factory and becomes competent to response to 20E. When the mosquito takes a blood meal, JHIII titers drop rapidly, which is consistent with the proposed role in the competence establishment, but JHIII appears not to control YPPs syntheses (Dittermann *et al.*, 1989). The initiation of YPPs syntheses is triggered by another key insect hormone, 20E. Our current understating of the 20E signaling pathway is that the initiation of 20E signaling pathway requires coordination of signals from several tissues. Upon a blood meal taken by female mosquitoes, the peptide hormone, ovarian ecdysteroidogenic hormone (OEH) is released from corpora cardiaca (CC) when CC senses a combination of several signals: neural stimuli from a distended midgut and the increased levels of amino acids in the hemolymph (Lu and Hagedorn, 1986; Sappington and Raikhel, 1999). In response to OEH, ovaries produce ecdysone, which is hydroxylated to 20E in the fat bodies (Sappington and Raikhel, 1999; Dauphin-Villemant *et al.*, 1998). It was thought that 20E was the only active form of ecdysteroids stimulating yolk protein production. Recent results indicate that ecdysone, which has been considered to be a precursor of 20E, can function as ligand for the mosquito ecdysone receptor (Wang *et al.*, 2000). In

A. aegypti, the level of 20E is not detectable during previtellogenic period, and the level is low during the first 8 hours post blood meal (PBM), with only a small peak at 4 hours PBM. Thereafter, the 20E level increases dramatically, reaching maximum at 16-20h PBM, and then decline to previtellogenic levels at 36-42 h PBM (figure 2) (Raikhel and Dhadialla, 1992).

During the active synthesis stage, YPPs are produced by the fat bodies and accumulate in ovaries (Raikhel and Lea, 1986). Vitellin, the crystallized form of Vg, is the major source of nutrients in an insect egg (Raikhel and Lea, 1991). In most insects, Vg is a large oligomeric glycophospholipoprotein consisting of two or more subunits (Dhadialla and Raikhel, 1990). Both VCP and VCB are proenzymes (Cho *et al.*, 1991; Cho *et al.*, 1999), which are activated at the onset of embryogenesis, to digest vitellin. Lipophorin functions as a reusable lipid shuttle to transport lipid from the hemolymph to the developing eggs (Sun *et al.*, 2000).

All four yolk protein precursors share similar kinetics during vitellogenesis in mosquito. Their synthesis peaks at around 24h PBM, then drops sharply and terminates at 36-42 h PBM (Dhadialla and Raikhel, 1990; Cho *et al.*, 1991; Cho *et al.*, 1999; Sun *et al.*, 2000).

The rate of YPPs syntheses is closely correlated with titers of 20E. This observation led to the initial hypothesis that YPPs production is under control of 20E. However, 20E does not seem to directly activate these genes, instead it is thought to act through a newly synthesized mediator because application of a protein synthesis inhibitor to the fat bodies treated with 20E completely blocked the *Vg* gene activation (Deitsch *et*

al., 1995). Though many studies have focus on the mechanism of 20E regulation of the *Vg* gene, the exact nature of the 20E action in fat bodies is poorly understood.

Termination of vitellogenesis

Termination of vitellogenesis involves cessation of Vg synthesis by trophocytes, and 20E titers decline sharply to background levels, associated with the termination of yolk protein accumulation by oocytes. At subcellular level, lysosomes play two important roles during Vg production: 1) interruption of Vg secretion by degradation of the Vg containing secretary granules, 2) destruction of biosynthetic machinery, rough endoplasmic reticulum (RER) and Golgi complexes and subsequently remodeling of trophocytes (Raikhel, 1986). Therefore, it seems that there are two distinct mechanisms involved in regulating the cessation of Vg synthesis and secretion: first is repression of *Vg* expression due to decrease in 20E level, and second is the increase of lysosome activity for interrupting the Vg secretion and degrading organelles.

HORMONE REGULATION OF VITELLOGENESIS

The gene encoding Vg, a major YPP in most oviparous animals, is expressed in a sex-, tissue- and stage-specific manner. The YPPs not only share the common manufactory tissue, but also appear to share common regulatory mechanism by which the expression of YYP genes are under the control of 20E. There are several lines of evidences that support this hypothesis: 1) The expression patterns of YPPs are correlated with 20E titration profile during vitellogenesis (Raikhel, 1992), 2) In the *in vitro* fat body culture system, application of physiological dose of 20E (10^{-6} M) to fat body culture

media is sufficient to activate the *Vg* and *VCP* gene expression (Deitsch *et al.*, 1995), which represents an ideal system for mimicking the scenario *in vivo*; 3) Administration of protein inhibitor to the fat body culture media together with 20E completely blocks the *Vg* and *VCP* gene expression (Deitsch *et al.*, 1995). The unique features of *YYP* gene expression triggered by ecdysone cascade has led to study of the structures and functions of the regulatory region of these genes.

Insight of ecdysone mechanism is largely from the analyses of ecdysone effects on polytene chromosome puffing pattern in the salivary gland of *Drosophila*. Based on the these studies, Ashburner *et al.*, (1974) proposed an ecdysone hierarchy model by which ecdysone first activates the early ecdysone-responsive genes, leading to activation of late ecdysone responsive genes. Further studies have confirmed and extended this model. Ecdysone exerts its control via interaction with the intracellular receptor, a heterodimer consisting of two protein subunits, ecdysone receptor (EcR, NR1H1) and the retinoid X receptor homolog, *ultraspiracle* (USP, NR2B4); both subunits are members of nuclear receptor superfamily (Yao *et al.*, 1992, 1993). The ligand-activated EcR/USP heterodimer acts directly upon early genes such as the *BR-C*, *E74* and *E75* (DiBello *et al.*, 1991; Burtis *et al.*, 1990; Thummel *et al.*, 1990; Segraves and Hogness, 1990). The products of these early ecdysone-responsive genes subsequently activate the late genes while repressing their own activity. Detailed molecular and genetic analyses have validated this model, not only for the ecdysone regulated changes in gene expression in salivary glands and in metamorphosis, but also for other larval tissues that must be eliminated during the process of metamorphosis (Burtis *et al.*, 1990; Segraves and

Hogness, 1990; Thummel *et al.*, 1990; DiBello *et al.*, 1991; Karim and Thummel, 1992; Urness and Thummel 1995; Crossgrove *et al.*, 1996; Fletcher *et al.*, 1995).

The 20E mechanism in vitellogenesis in mosquito has been proposed to use pathway similar to those used in *Drosophila* metamorphosis (Li *et al.*, 2000a). Two isoforms of mosquito *EcR*, AaEcRA and AaEcRB are present in previtellogenic and vitellogenic fat bodies and ovaries (Cho *et al.*, 1995; Wang *et al.*, 2003). Two different transcripts of *USP* gene, presumably derived from differential splicing (Wang, unpublished data) have also been cloned and characterized in these tissues (Kapitskaya *et al.*, 1996; Wang *et al.*, 2000). The mosquito EcR/USP is a functional heterodimer complex, which can bind to various ecdysone response elements to regulate target gene expression (Wang *et al.*, 1998).

Previous studies by the *Drosophila* and *Aedes* transformation, as well as DNA-binding assays, have identified a 5' regulatory region of the *Aedes vitellogenin* gene (*Vg*), which is responsible for the stage-, and fat body-specific activation of the gene via a blood meal-triggered 20E cascade (Kokoza *et al.*, 2001). These analyses revealed at least three modules in the 2.1kb upstream portion of *Vg* gene. The proximal region, adjacent to the basal transcriptional start site, contains binding sites for several transcription factors: EcR/USP, GATA (GATA transcription factors) and HNF3/fkh (hepatocyte nuclear factor3/forkhead transcription factor). This region is required for the precise tissue-, and stage-specific expression, although this element alone has the ability to drive only low levels of transcription. The median region contains several putative ecdysone-inducible early gene binding sites. It is responsible for a stage-specific hormonal enhancement of *Vg* expression. Finally, the distal region is characterized by multiple sites for GATA

factor and is required for extremely high expression levels characteristic of *Vg* gene expression.

***DROSOPHILA* E74**

The life cycle of *Drosophila* includes embryonic, larval, pupal, and adult developmental stages. The steroid hormone 20E functions as an important signal during the life cycle of the animal and triggers dramatic changes in gene expression, cell physiology, and tissue organization. Increased levels of 20E are observed in all four developmental stages and the high titers of the hormone at the end of the third larval instar initiates puparium formation followed by metamorphosis during the pupal stage (Riddiford, 1993). Metamorphosis results in a complete transformation in form and function, from a crawling larva to a highly motile, reproductively active adult fly. Remarkably, the divergent developmental pathways are manifested simultaneously in an apparent response to a single steroid hormone 20E following ecdysone hierarchy model described earlier. One of the early genes activated in response to ecdysone action is the *E74* gene (Burtis *et al.*, 1990). E74 belongs to Ets transcription factor family. Different ecdysone-inducible *E74* promoters are used to generate transcripts for E74A and E74B, which are proteins having distinct N-terminal regions and sharing C-terminal Ets domain. Both proteins are expressed in many tissues throughout larval development and have been shown to bind to several late puff loci on salivary gland polytene chromosomes (Thummel *et al.*, 1990; Urness and Thummel, 1995). *74E* gene mutations have been characterized that are specific to the E74A or E74B products (Fletcher *et al.*, 1995). Loss-of-function studies demonstrated that E74A is a regulator of the late puff gene expression

and its proper function is required for the metamorphosis of both larval and adult tissues. Parallel genetic studies have identified an earlier requirement for E74B in the formation of a normal puparium (Kozlova and Thummel, 2002). The mutation of E74A or E74B cause defects in pupariation and pupation, and either mutation results in lethality during metamorphosis. Consistent with proposed role as an early gene, at the molecular level, the transcription of most ecdysone primary-response genes is unaffected by these mutations, whereas the E74 is required for appropriate regulation of many secondary-response genes. Significantly, Fletcher *et al* (1995) have demonstrated that L71-6, one of the secondary-response genes encoding a family of small secreted polypeptides that resemble defensins and venom in the newly formed prepupal salivary gland, is directly regulated by E74A, based on the several lines of evidence. These data provide a direct molecular link between a steroid-induced early gene and a secondary-response target promoter. Intriguingly, two E74 isoforms regulate the timing of the secondary-response gene expression depending on the 20E titers. However, they may serve distinct functions. It has been demonstrated that E74B functions as a potent repressor of several late gene expression while E74A contributes positively to the expression of the late genes (Fletcher *et al.*, 1997). For example, mutagenesis and ectopic expression analyses have shown that another later target gene *L71-1* is potentially repressed by E74B and can be induced prematurely by E74A. Given the presence of an identical Ets DNA binding domain in the two proteins, the E74 proteins most likely interact with different accessory factors to carry out their diverse functions.

More recently, the E74A function was found to be a regulator of apoptosis during metamorphosis, which is crucial for the elimination of obsolete tissues. A good example

is the degeneration of the larval salivary glands during metamorphosis. This occurs about 15 h after puparium formation and just after the prepupal pulse of ecdysone. A recent study demonstrated a direct link between an ecdysone triggered gene cascade and programmed cell death of the salivary gland cells (Jiang *et al.*, 2000). It is known that the activation of apoptosis is controlled by the balance of cell death activators and inhibitors in the cell. *Drosophila* activator genes include *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, while the fly apoptosis inhibitor genes include *diap1* and *diap2* (reviewed in Jiang *et al.*, 2000). Just prior to the selective histolysis of the salivary glands, first the *diap2* inhibitor and then the *rpr* and *hid* activator genes are expressed, suggesting their precise transcriptional control is important to establishing a correct balance for programming death in these cells (Jiang *et al.*, 1997). The recent report demonstrates that the Ets protein E74A plays a vital role in activating the *hid* gene in salivary gland cells, while other ecdysone induced transcription factor genes such as β*FTZ-F1*, *E75*, and *BR-C* function in either the repression of *diap2* or the activation of *rpr* and *hid*. This fine-tuning of the balance between the anti- and pro-apoptotic activities culminates in the prerequisite death of the larval salivary gland cells (Jiang *et al.*, 2000).

Ets DOMAIN TRANSCRIPTION FACTOR FAMILY

Ets genes are noted for wide distribution among metazoans. Multiple *Ets* genes have been characterized in species ranging from sponges, nematodes, insects to humans. Functional Ets protein binding sequences have been identified in promoter/enhancer regions of viral and cellular genes. The binding activities control gene expression and are critical for proper regulation of cellular proliferation, differentiation, development,

hematopoiesis, immune response, apoptosis, insect metamorphosis, and vitellogenesis (reviewed in Sharrocks *et al.*, 1997; Graves and Peterson 1997; Yordy *et al.*, 2000; Li *et al.*, 2000b; Sharrocks, 2001; Sementchenko *et al.*, 2000). Deregulation of Ets domain proteins by either inappropriate expression or expression as fusion with other proteins is believed to be critical in the generation of particular types of cancer (Dittmer and Nordheim, 1998).

All Ets family members contain DNA binding domains consisting of approximately 85 amino acids, designated as the Ets domain. Based on the phylogenetic analysis of the Ets domain, different Ets proteins are classified into subgroups. The Ets domain is both a structural and a functional domain, as the Ets domain can be produced as a stable protein fragment and is sufficient for DNA binding (Graves and Peterson 1997). The three dimensional structure of Ets domain by NMR (nuclear magnetic resonance) in both the presence and absence of DNA has revealed that Ets domain is composed of three α helices (H) and four β sheets (S) arrayed in the order: H1-S1-S2-H2-H3-S3-S4. The main protein-DNA contacts are from residues that are located in the third α-helix (Donaldson *et al.*, 1994; Liang *et al.*, 1994). Several highly conserved amino acids form the hydrophobic core of the Ets domain and play a role in the proper folding of the domain (Donaldson *et al.*, 1996). In addition to directing protein-DNA interactions, the Ets domain is also a target for protein-protein interactions that are mediated either intramolecularly or *in trans* by co-regulatory proteins (Li *et al.*, 2000b). The Ets transcription factors can be grouped into a larger structure class of DNA-binding proteins, termed the "winged helix-turn-helix" (wHTH) protein. The wHTH motif is found in a wide variety of DNA-binding proteins, including the HNF/*forkhead* family of

transcription factor (Clark *et al.*, 1993), Heat-shock factor HSF (Cicero *et al.*, 2001) and topoisomerase II (Berger *et al.*, 1996). The wHTH proteins utilize distinct oligomerization states for recognizing DNA. For instance, most Ets and HNF/*forkhead* proteins recognize DNA as a monomer while HSF recognizes DNA as a trimer.

The vast majority of Ets proteins bind to the specific purine-rich DNA sequences with a core motif 5'-GGA-3' that is found in promoters and enhancers. Such sequences are found in DNA sequences purified by *in vitro* binding to Ets proteins. However, low affinity binding sites with deviation from the consensus may play an important role *in vivo* for regulation of the expression of an inducible gene.

There is remarkable similarity among subgroups of the Ets proteins in the preferred nucleotides that flank the core GGA. Elf-1 (E74 like factor 1), E74 and Fli-1 prefer for adenine at a position immediately right of the GGA core, while Ets-1, Elk-1, PU.1 and ER81 select a thymidine or an adenine at this position (Graves and Peterson, 1997). In contrast, the nucleotides to the left of the core GGA are more tolerable to the variations. These nucleotides are believed for determining sequence specificity of individual Ets proteins.

Many Ets domain transcription factors are subject to autoregulation, whereby their DNA-binding activity is usually masked until an appropriate trigger takes place (Garvie et al., 2002). Two types of triggers that activate DNA binding are phosphorylation and interactions with a co-regulatory transcription factor. Conversely, phosphorylation and binding to the co-regulatory partner have also been shown to promote the dissociation of Ets domain proteins from DNA. For example, interactions between the *Drosophila* Ets domain protein Yan and its interaction partner, Mae, result in

Yan dissociation from DNA, but the interaction occurs between the Pointed domains of each protein (Baker *et al.*, 2001). The Pointed domain was originally identified in the *Pointed* gene in *Drosophila* and shed by subgroup of some Ets subfamilies.

Interaction with co-regulatory partner proteins seems to be one of the unifying themes among Ets domain transcription factors (Li *et al.*, 2000b). This allows combinatorial control of gene expression and enhances the specific action of Ets domain proteins. Moreover, the Ets proteins act synergistically with a variety of other transcription factors to regulate many cellular and viral promoters and enhancers, whereby synergy in this context describes the phenomenon in which the combined transcriptional activity of multiple factors is greater than additive. The importance of combinatorial control has been well documented in Ets family of transcription factors. For example, the Ets protein Fli-1 interacts *in vivo* with a protein complex containing transcription factor GATA-1 and its cofactor FOG-1 (friend of GATA-1), which is essential for the normal development of erythroid cells and megakaryocytes (Wang *et al.*, 2002). Another Ets transcription factor Ets-1 can cooperate with a variety of nuclear receptors, which conferred a ligand-independent activation to vitamin D receptor (VDR), estrogen receptor (ER) and peroxisome-proliferator-activated receptor α (PPARα). This direct physical interaction may induce a conformational change in the nuclear receptor, which creates an active interaction surface with coactivators (Tolon *et al.*, 2000).

CBP/p300 (CREB binding protein) is an adapter protein, bridging many specific transcriptional factors with components of the basal transcriptional machinery such as TFIID (Janknecht and Hunter, 1996). Both Ets1 and Ets2 recruit CBP/p300 to transcriptionally activate the human stromelysin promoter (Jayaraman *et al.*, 1999).

Moreover, Ets-1 and CBP/p300 form a stable complex in a DNA independent manner and this complex possesses histone acetyltransferases (HAT) activity (Yang *et al.*, 1998). Since Ets proteins interact with many transcription factors, the precise biological outcome depends upon on its partner. Interaction with chromatin remodeling factors provides a mechanism by which the partner of Ets can serve as a co-repressor, leading to transcriptional silencing. One good example is that an ERG (ets-related gene)-associated protein with a SET (suppressor of variegation, enhancer of zest and trithorax) domain (ESET) was found to have activity of histone H3-specific methyltransferase. The ESET histone methyltransferase can form a large, multi-protein complex(es) with mSin3A/B co-repressors and histone deacetylase 1 (HDAC1) and HDAC2 that participate in multiple pathways of transcriptional repression (Yang *et al.*, 2003). Another example is the interaction between corepressors CtBP and Ets protein NET that provides a bridge between NET and HDAC1 (Criqui-Filipe *et al.*, 1999), leading to transcriptional repression. The Ets protein TEL also has been demonstrated to be able to recruit co-repressors such as SMRT (silencing mediator of retinoid and thyroid hormone receptor) and mSin3A, a histone deacetyltransferase, resulting in transcriptional repression (Chakrabarti and Nucifora, 1999).

Interaction between Ets and other transcription factors results in either activation or repression of specific target genes. The factors that determine whether an individual Ets factor functions as an activator or a repressor include composition of DNA sequence, presence of tissue specific factors, alternative splicing and the combinatorial control by multiple transcription factors. For instance, PU.1 also functions as a repressor by interacting with GATA-1 (Rekhtman *et al.*, 1999). Many Ets domain transcription

factors are known to represent nuclear targets of signaling pathways. In particular, the MAPK pathways have been linked with a diverse series of regulatory events that involve Ets domain proteins (reviewed in Wasylyk *et al.*, 1998).

NUCLEAR RECEPTORS

Nuclear receptors (NRs) are one of the most abundant classes of transcriptional regulators in metazoans, in which they functions in diverse processes including reproduction, differentiation, development, metabolism, metamorphosis and homeostasis. Many NRs serve as ligand-activated transcription factors to mediate these processes. NRs are phylogenetically related receptors for small hydrophobic molecules such as steroids hormones (estrogens, glucocorticoids (GR), progesterone (PR), mineralocorticoids (MR), androgens (AR), vitamin D, ecdysone (EcR), etc.), retinoic acid (RAR) and 9-cis retinoic acid (RXR), thyroid hormone (TR) and fatty acids etc. (Mangelsdorf *et al.*, 1995; Riberio *et al.*, 1995; Escriva *et al* 2000; Olefsky, 2001). Also part of the family are the orphan receptors, which possess the similar structure but for which no ligands have been found. NRs share a common structural organization with a central, well-conserved DNA-binding domain (DBD, also termed C domain), a variable N-terminal region (A/B domain), a less variable hinge (D domain) and a carboxyl-terminus moderately conserved ligand-binding domain (LBD, E domain) (figure 3).

Some but not all NRs contain a carboxyl-terminal region F of unknown function. Two autonomous transactivation functions, a constitutively active activation function 1 (AF-1) originating in region A/B and a ligand-dependent AF-2 arising in the LBD, are responsible for the transcriptional activity of NRs.

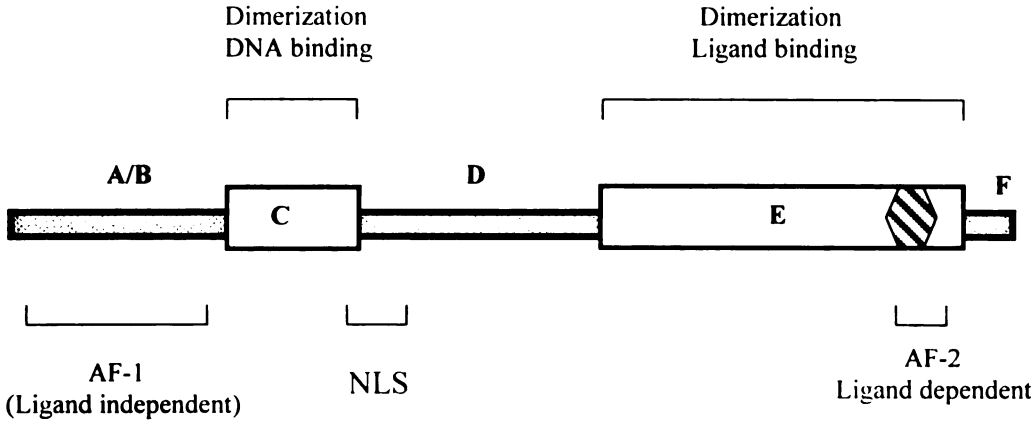

Figure 3. Structural and functional organization of nuclear receptors. Nuclear receptors consist of six domains (A–F) based on regions of conserved sequence and function. The DNA-binding domain (DBD; region C) is the most highly conserved domain and encodes two zinc finger modules. The ligand-binding domain (LBD; region E) is less conserved and mediates ligand binding and dimerization. The ligand-dependent transactivation function is termed AF-2. Within the AF-2, the integrity of a conserved amphipathic α-helix termed AF-2 activation domain (AD) has been shown to be required for ligand-dependent transactivation. The N-terminal A–B region contains a cell- and promoter-specific transactivation function termed AF-1. Region D is considered as a hinge domain. Region F is not present in all receptors and its function is poorly understood.

The DBD contains approximately 70 amino acids that fold into two highly conserved zinc-fingers motifs. Each zinc finger is coordinated in a tetrahedral geometry by the sulfurs from the conserved cysteins (Khorasanizadeh and Rastinejad, 2001; Chasse and Rastinejad, 2001). The DBD region has dual functions: dimerization and binding to specific DNA sequence called hormone response elements (HREs). Whereas the DBDs form the dimerization contacts in a DNA-dependent manner, the dimerization of LBDs, in some cases, can be DNA-independent.

Most NRs function as homo- or hetero- dimers, controlling the target gene expression by binding specifically to HRE (Khorasanizadeh and Rastinejad, 2001). The dimerization patterns of the receptor DBDs are reflected in their HRE configurations (Mangelsdorf et al., 1995). Steroid receptors, excluding ER, bind to response elements containing symmetric (palindromic) repeats of hexameric half-site sequence 5'-AGAACA-3'. ER is slightly different; its half-sites contain the sequence 5'-AGGTCA-3', although ER also binds symmetric repeats. In contrast, RXR homo- and heterodimers bind to direct repeats (DR) of the 5'-AGGTCA-3'sequence. Direct repeats with one to five base-pairs of spacing (DR1–DR5) are binding sites for various RXR heterodimers. Different NRs have different spacing preference. For example, a DR separated by five nucleotides (DR5) will be most often recognized by an RXR-RAR heterodimer, whereas a DR4 will be recognized by an RXR-TR heterodimer. Interestingly, some orphan receptors such as FTZ-F1 can bind to DNA as monomers through a single half-site.

Many NRs are transcriptional silencers in the absence of ligand as a result of interaction with co-repressors (Horwitz et al., 1996; Xu *et al.*, 1999). Three-dimensional structure analyses of NRs have shown that the LBD region is structured as a three-layered

α-helical antiparallel sandwich of 12 helices forming a hydrophobic pocket. Upon binding, the ligand makes different contacts with amino-acid residues, promoting a conformational change that closes the "lid" (helix 12, H12) on the pocket. In this conformation, the activation domain within the H12 (AF-2-AD) is able to interact with co-activators and promotes transcription of target genes (Moras and Gronemeyert, 1998; Bourguet *et al.*, 2000). The conformational change of the LBD upon ligand binding is therefore necessary for the transactivation function of some NRs. It is believed that co-repressors and co-activators modulate transcription at least in part by modifying the status of histone acetylation in chromatin (Xu *et al.*, 1999; Lemon and Freedman, 1999; Weatherman *et al.*, 1999; Moras and Gronemeyer, 1999).

Initial contact between activated NRs and coactivators is believed to be mediated in large part by an amphipathic helix conserved on the surface of most coactivators, the LXXLL motif (Heery *et al.*, 1997, 2001). Crystallographic evidence has shown that a ligand-dependent shift in the position of several critical helices in AF-2 of the receptor ligand binding domain, notably helix 12, creates a thermodynamically secure environment for the coactivators containing the LXXLL motif (Westin *et al.*, 1998; Darimont *et al.*, 1998; McInerney *et al.*, 1998).

COACTIVATORS AND COREPRESSORS OF NUCLEAR RECEPTORS

A number of nuclear receptors, including retinoid acid and thyroid hormone receptors, appear to bind to their target genes in the absence of ligand and actively repress transcription. NcoR (nuclear receptor corepressor) and its homolog SMRT seem to the major proteins associated with these nuclear receptors in the unliganded state (Horlein *et*

al, 1995; Wagner *et al.*, 1998; Cohen *et al.*, 1998; Chen, 2000). Both of these proteins contain a transferable repression domain. Protein–protein interaction studies revealed the association of NcoR/SMRT with mSin3, histone deacetylase (HADCs) (Heinzel *et al.* 1997; Nagy *et al.*, 1997; Xu *et al.*, 1999). Furthermore, HDAC activity is also found to be a critical component of the complex, which is required for repression by unliganded nuclear receptors (Heinzel *et al.* 1997; Nagy *et al.*, 1997). Nucleosome structures are thought to be essential in maintaining a repressive transcriptional environment at the promoter through electrostatic contacts between positively charged lysine side chains in histone and negatively charged DNA phosphate groups. Therefore, the hypoacetylated state of the histone generally is thought to repress the gene expression, while the hyperacetylated state indicates an active environment for gene expression. Recently, one corepressor SMRTER (SMRT-related ecdysteroid receptor interacting factor) was characterized in *Drosophila* to be able to directly interact with EcR. Similar to its counterpart in mammalian SMRT, SMRTER is recruited in the absence of ligand, which mediates repression by interacting with Sin3A, a repressor known to form a complex with the histone deacetylase Rpd3/HDAC (Tai *et al.*, 1999). Several *Drosophila* coactivators have been cloned and characterized: CBP and Taiman (Akimaru *et al.*, 1997; Bai *et al.*, 2000). However, no connection between CBP and nuclear receptor have been established so far. Interestingly, Taiman was demonstrated to directly interact with EcR in a ligand-dependent manner (Bai *et al.*, 2000).

Intriguingly, several studies have demonstrated that steroid hormone receptors, such as the estrogen receptor, recruit NcoR and SMRT in the presence of antagonist and that this recruitment is essential for full antagonist activity (Huang *et al.*, 2002). Upon

binding of hormone, these corepressors dissociate away from the DNA-bound receptor, and recruit a nuclear receptor coactivator (NCoA) complex. Prominent among these coactivators is the SRC (steroid receptor coactivator) family, which consists of SRC-1, TIF2/GRIP1, and RAC3/ACTR/pCIP/AIB-1 (Leo and Chen, 2000). These cofactors interact with nuclear receptors in a ligand-dependent manner and enhance transcriptional activation by the receptor via histone acetylation/methylation and recruitment of additional cofactors such as CBP/p300 (Hermanson *et al.*, 2002).

CBP/p300 functions as a versatile co-integrator (Goldman *et al.*, 1997; Shibata et al., 1997). The interaction with nuclear receptors could be ligand-dependent and relies on the AF-2 domain (Leo C, Chen, 2000; Ratajczak *et al.*, 2001). It also interacts with the Ets family proteins (Yamamoto *et al.*, 1999, 2002; Hong; *et al.*, 2002). *In vitro* biochemical analyses revealed the p160 family that interacts with liganded RAR and ER (Wong *et al.*, 2002; Sathya *et al.*, 2002; Cheskis *et al.*, 2003). In addition, p160 factors can also interact with CBP/p300. Structure and function analyses of p160 factors revealed the signature motif, LXXLL, are required for the interaction with nuclear receptors and CBP/p300 (Chen *et al.*, 2000; Demarest *et al.*, 2002). Intriguingly, different LXXLL motifs are required for PPARγ function in response to different ligands, suggesting distinct configuration of assembled complex. LXXLL motif contacts a "charged clamp" that is formed by AF-2 domain of nuclear receptors (Notle *et al.*, 1998).

In summary, ligand binding results in the dismissal of HDAC-containing corepressor complex and subsequently leads to recruitment of HAT-containing complex. This change of local chromatin remodeling activity underlies ligand-induced transcriptional activation.

One of the primary functions of the activated receptors is believed to facilitate access of the basal transcription machinery to the promoter to initiate transcription. Chromatin plays an important role in regulating the basal activity of many promoters. Chromatin remodeling factors are defined to be able to overcome the chromatin thermodynamics and are thought to be recruited at an early point in the model of receptor action. Perhaps the best characterized of these factors is the adenosine 5'-triphosphatase (ATPase)-containing multiprotein SWI/SNF complex containing proteins initially identified in yeast (Carlson and Laurent, 1994). ATPase-defective mutants of this complex can disrupt the transcriptional function of certain nuclear receptors in cell culture (Wade *et al.*, 1997; Wade and Wolffe, 1999). Chromatin remodeling factors are thought to mainly disrupt large chromatin domains noncovalently.

Local regulation of nucleosome structure are mediated in part by members of a family of proteins that were originally identified as nuclear receptor coactivators and the SRC/p160 family (Leo and Chen, 2000). SRC family members and other histone acetyltransferases such as CBP/ p300 are thought to disrupt interactions responsible for maintaining the promoter region in a repressive state by catalyzing the acetylation of histone lysines (Hermanson *et al.*, 2002). Of the SRC family members, SRC-1 and ACTR/SRC-3 have been shown to possess intrinsic acetyltransferase activity (Chen *et al.*, 1997; Spencer *et al.*, 1997).

A third prominent group of coactivators is represented by TR-associated proteins (TRAP)/vitamin D receptor-interacting proteins (DRIP) (Fondell *et al.*, 1996; Rachez *et al.*, 1998; Fondell, 2002). Studies have shown TRAP/DRIP to be capable of enhancing the function of several nuclear receptors beside TR and VDR (Shao *et al.*, 2000; Ge *et al.*,

2002; Mueller *et al.*, 2002), suggesting its general role in nuclear receptor-mediated signaling. In summary, *in vitro* molecular approaches have constructed a sequential model of coactivator action that initiates recruitment of chromatin modeling factors by nuclear receptor, followed by factors containing histone acetyltransferase activity such as TRAP/DRIP-like complexes, which ultimately contact basal transcription factors (McKenna and O'Malley, 2002).

Coactivators appear to participate in regulation of a wide variety of promoters by integrating nuclear receptor functions and a broad spectrum of cellular signals. One major question is how coactivators determine the specific spatiotemporal fates for given genes or gene sets. Posttranslational modifications such as phosphorylation, methylation or acetylation may provide information to solve the specify issue. Phosphorylation has been historically implicated in nuclear receptor function (Weigel, 1996). PPAR-binding protein (PBP) is an important coactivator for PPARγ and PBP is an integral component of a multiprotein TRAP/ DRIP/activator-recruited cofactor (ARC) complex required for transcriptional activity. It has been demonstrated that PBP phosphorylation by Raf/MEK/MAPK cascade exerts a positive effect on PBP coactivator function (Misra *et al.*, 2002).

Taken together, the transcription factor-specific, coactivator-specific, and promoter-customized sequences of posttranslational modification may contribute to the amplitude, timing and duration of transcription of individual genes.

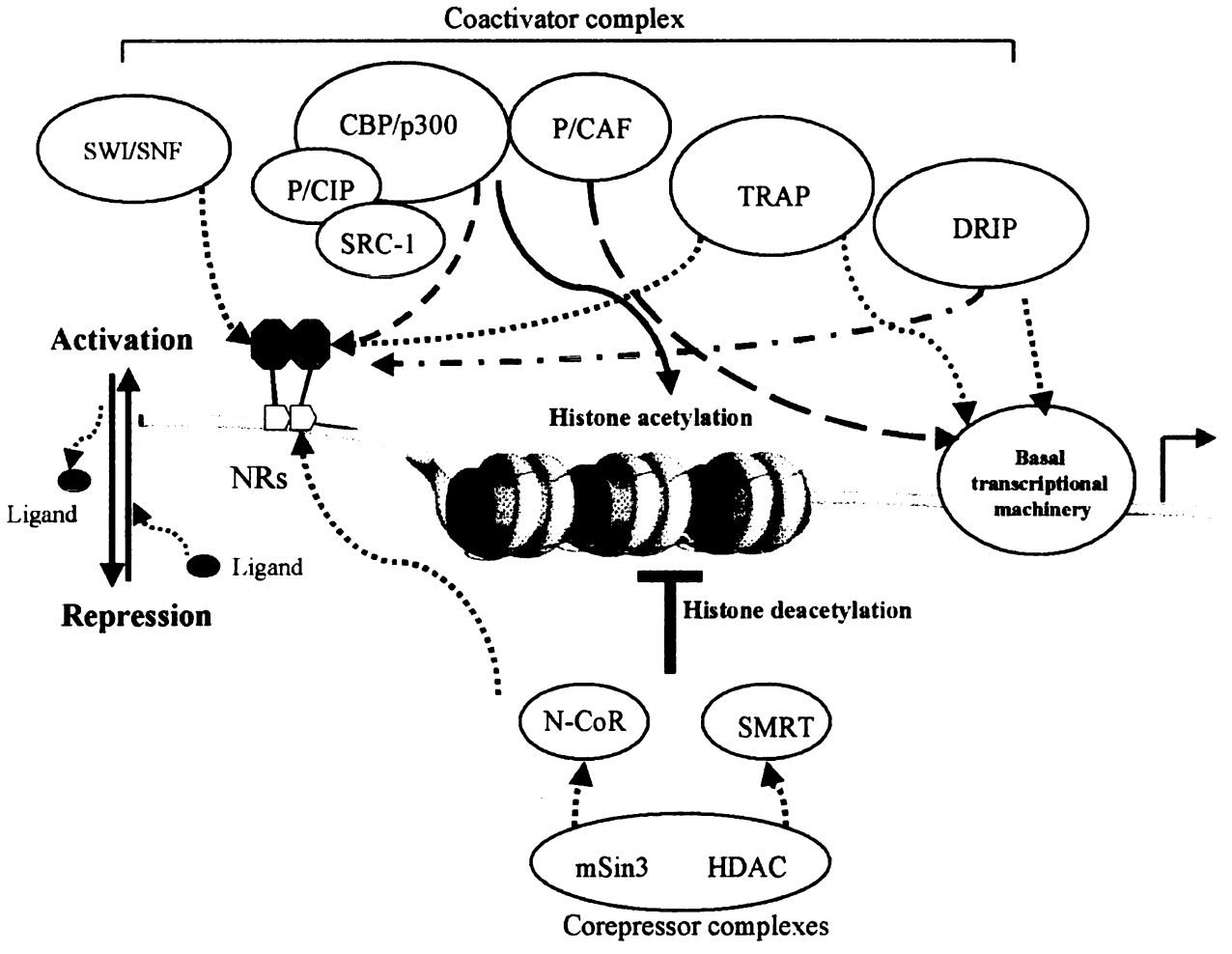
Coactivator complex
SWI/SNF
CBP/p300
P/CAF
P/CIP
SRC-1
TRAP
DRIP
Activation
Histone acetylation
NRs
Ligand
Ligand
Repression
Basal transcriptional machinery
Histone deacetylation
N-CoR
SMRT
mSin3
HDAC
Corepressor complexes

Figure 4. Regulation of nuclear receptor functions by multiple coactivators and corepressors. Proteins implicated in hormonal regulations of gene transcriptional are shown. Placement of each factor is arbitrary. In the absence of ligand, the mSin3/HDAC corepressor complexes, which harbor deacetylase activities, are linked to nuclear receptor via N-CoR or SMRT. While in presence of ligands, nuclear receptors induce the recruitment of coactivators, leading to activation of gene transcription in several sequential steps. First, chromatin remodeling is catalyzed by SWI/SNF or CBP/p/CAF complexes, followed by TRAP or DRIP complex activation of transcription via interaction with basal transcriptional machinery. The CBP/p300/p/CAF, TRAP and DRIP may provide a link between nuclear receptors and basal transcriptional machinery. For abbreviations of the complexes refer to the text. (Modified from Xu *et al.*, 1999).

Selective ligand/receptor/coactivators interactions represent an efficient system through which the pleiotropic effects of nuclear receptor ligands might be mediated, and are likely to be further determined by tissue-specific patterns of posttranslational modification of coactivators (Takeyama *et al.*, 1999; Bramlett *et al.*, 2001; Kraichely *et al.*, 2000).

The sequential model of regulation of nuclear receptor functions by multiple coactivators and corepressors, and is summarized in figure 4. In presence of ligands or absence of ligands, coactivators with HATs or corepressors with HDACs, respectively, are recruited by nuclear receptor to activate and repress gene transcription (Xu *et al.*, 1999).

RATIONAL FOR CURRENT RESEARCH

In anautogenous mosquitoes such as *A. aegypti*, a unique feature of the genes encoding YPPs is the requirement of a blood meal for their activation (Raikhel, 1992). Following blood meal activation, the major yolk protein *Vg* gene is transcribed at a very high level. The stringent control of the expression of *Vg* gene in mosquitoes by blood meal provides an outstanding model for elucidating hormonal and tissue-specific regulation in the context of complex physiological events surrounding reproduction. Furthermore, the *Vg* gene is a major regulatory target of the blood meal-activated cascade in the mosquito. Therefore, detailed analysis of the mechanisms governing the expression of this gene is essential.

It has been shown that physiological doses of 20E (10^{-6}M) activate *Vg* and *VCP* expression in an *in vitro* fat body culture system (Deitsch *et al.*, 1995), while application

of the protein inhibitor, cycloheximide (Chx), completely blocks expression. This suggests that activation of *YYP* is indirectly regulated by 20E (Deitsch *et al.*, 1995), presumably, through a mediator as suggested in the ecdysone cascade model in metamorphosis in *Drosophila* (Ashburner *et al.*, 1974; Thummel, 1996). Furthermore, the appearance of Vg mRNA in fat body shortly after a blood meal suggests that the control of Vg synthesis is at the stage of transcription rather than of translation.

My research interest is to elucidate the ecdysone regulation mechanism governing vitellogenesis in mosquitoes. This research will provide a knowledge base for future development of effective methods to control the pathogens transmitted by mosquitoes. Substantial progress has been made in our understanding of the molecular mechanism in this area. Notably, the key machinery of 20E hierarchy has been demonstrated to be conservatively utilized between vitellogenesis in mosquitoes and metamorphosis in *Drosophila*. First, functional ecdysone receptor is a heterodimer of ecdysone receptor (EcR) and its obligatory partner *Ultraspiracle* (USP) (Yao *et al.*, 1992). At least two isoform EcR and two USP isoforms have been cloned and characterized in *A. aegypti* (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996). It has been demonstrated that EcR/USP bound to 20E with high affinity and efficiently activated reporter gene expression in transient transfection assay (Wang *et al.*, 2000; Wang *et al.*, unpublished data). Several other key components such as an early ecdysone-inducible gene, *AaE75* (Pierceall *et al.*, 1999), early late ecdysone-inducible gene *AaHR3* (Kaptiskaya *et al.*, 2000) and competence factor, *βFTZ-F1*, were cloned and characterized in *A. aegypti* (Li *et al.*, 2000a). However, the exact nature of the mechanism of how *Vg* gene expression is controlled by 20E remains obscure.

E74 is a key mediator in the ecdysone hierarchy model, and *Vg* is a potential target gene in the mosquito fat bodies because the putative E74 response elements were identified in the 5' regulatory region of Vg. My research was designed to clone mosquito E74, identify its properties *in vitro* and explore its functions regarding the regulation mechanism of *Vg* gene expression in mosquito during vitellogenesis by *in vivo* studies.

Chapter II Materials and Methods

Animals

Mosquitoes were raised as described by Hays and Raikhel (1990). Larvae were fed a standard diet (Lea, 1964), and adults were fed on 10% sucrose continuously by wick. Adult females 3-5 days after eclosion were allowed to feed on anesthetized white rats to initiate vitellogenesis. All dissections were performed in *Aedes* physiological saline at room temperature (Hagedorn *et al.*, 1977).

Materials

Restriction Enzyme, superscript reverse transcriptase, primers used in PCR, and Taq polymerase were supplied by Gibco BRL. *Renilla* Luciferase (0.12μM) and the dilution buffer (0.01M KH_2PO_4, 0.5MNaCl, 0.001M EDTA, 0.1mg/ml BSA pH 7.5) were from Chemicon. *Firefly* Luciferase (Quantilium Recombinant luciferase) and its dilution buffer Passive Lysis Buffer were obtained from Promega. Perkin-Elmer Cetus provided the reagents for PCR, and Promega for *in vitro* transcription and translation (TNT®) assay. New England Nuclear supplied [^{32}P]α-dATP (3000Ci/mmol0 and [^{35}S] α-dATP (1000-1500Ci/mmol); ICN Radiochemicals supplied [^{35}S] Methionine (1200Ci/mmol). All other reagents used were of analytical grade from Sigma Chemical Co. and Baker Co.

Cloning and sequencing of cDNAs

The *A. aegypti* lambda Dash II genomic library, a kind gift of Dr. A. A. James of the Department of Molecular Biology and Biochemistry of the University of California at Irvine, was prepared from the Rockefeller strain of *Aedes aegypti*. The library was

custom made by Stratagene Cloning Systems (La Jolla, CA). The library was screened using a digoxigenin-dUTP labeled probe which corresponds to the Ets domain of a *Drosophila* E74 cDNA clone. In the preliminary screening, we obtained three genomic clones. They were digested with EcoRI and the EcoRI fragments were subcloned in the pBluescript SK vector. Restriction enzyme digestion and partial sequencing indicated that one of the clones contained a region almost identical to the Ets domain of the *DmE74* gene. This DNA fragment, with size of about 600 bp, was then utilized as a probe to screen a λZAP II cDNA library generated from the fat bodies of 6-48 h PBM vitellogenic female mosquitoes (Cho and Raikhel, 1992). Eight positive clones were identified. Based on the restriction endonuclease digestion mapping by PuvII and partial DNA sequencing these cDNA clones were divided into two groups. The two longest clones from each group, 4.2 kb and 5.1 kb long, were sequenced from both strands at Yale Keck Sequencing Center. However, both clones failed to support the *in vitro* coupled transcription-translation (TNT) reaction, suggesting the N-terminal open reading frames were not complete. Additional screening of the same library was performed using two specific probes at the 5' end of each cDNA clone, and two longer cDNA clones for each group were obtained. The group 1-specific cDNA clone of was 5.47 kb long, while the group 2-specific one was 5.13 kb long. Both cDNA clones supported TNT reaction radioactively labeled by [^{35}S] Methionine.

DNA and protein sequences were analyzed by GCG (Genetics Computer Group, Inc., University of Wisconsin) or EDITBASE, MAPDRAW, and MEGALIGH software (DNA Star) from Lasergene™ with minor manual changes of output.

Mosquito and *Drosophila* E74 alignment was performed using "Multiple Sequence Alignments" program from Baylor College of Medicine of HGSC at the website: http://dot.imgen.bcm.tmc.edu/multi-align/multi-align.html. The output shading format was conducted after alignment using "BOXSHADE 3.21" program at the website: http://www.ch.embnet.org/software/BOX_form.html.

Northern analysis

Total RNA was extracted from previtellogenic and vitellogenic stages of mosquito fat bodies or ovaries using the Guanidine Isothiocyanate method (Bose and Raikhel, 1988), or TRIZOL Reagent (Gibco BRL). Northern analyses were performed as described by Cho and Raikhel (1992). Probes corresponding to AaE74 isoform-specific and common regions were employed. The *YPP* gene, *VCP*, and the housekeeping gene, *actin*, were used as controls.

Plasmids constructs

The following plasmids were used as reporter constructs for transfection assays in S2 cells. The series deletion of Vg 5' region luciferase reporter construct (Vg^{2100}-Luc), Vg^{1071}-Luc, Vg^{618}-Luc Vg^{348}-Luc, pAcAaEcR and pAcAaUSPb were described in Wang *et al.* (2000). Two deletion constructs $Vg^{1071}\Delta$Cluster-Luc and $Vg^{1071}\Delta$C10-Luc were generated according to the instruction manual for the QuickChange site-directed mutagenesis system (Stratagene). Briefly, two synthetic oligonucleotide primers (125ng each), containing the desired mutation and 50nmol of Vg^{1071}-Luc plasmid were subjected to PCR amplification with *PfuTurbo* DNA polymerase. Two sets of primer sequences for

the mutagenesis are: PVgΔC10: 5'$_{-809}$tgaaggcttagagccg$_{-793}$ - $_{-755}$atgggttatacttcattgagg$_{-734}$3' and PVgΔCluster: 5'$_{-972}$tcatgagctgtccgc*aa$_{-955}$ - $_{-909}$ ccgc*aataagaatcgtga$_{-891}$3' (two asterisks indicate the mutations changed from G to C and the numbers represent the position relative to transcription start codon of *Vg* promoter). Each PCR reaction was performed using one primer set consisting of two primers with opposite sequence orientations (one sequence orientation is indicated above). The reaction was initiated with a denaturation at 95°C for 5 min, followed by 18 cycles of denaturation at 95°C for 50 sec, annealing at 58°C for 50 sec, and elongation at 72°C for 12 min. The PCR products were then treated with *Dpn*I (Stratagene) to remove the methylated template DNA, followed by gel purification, and transformation of the DNA into XL10-Gold Ultracompetent *E. coli*. Likewise, the double deletion construct Vg^{1071}ΔCluster/ΔC10-Luc (Vg^{1071}ΔDB-Luc) was created using Vg^{1071}ΔC10-Luc as template and PVgΔCluster as the primer set. The EcRE deletion Vg^{1071}ΔEcRE-Luc was created by insertion of a PCR fragment from the *Vg* promoter (–1071 to –618) into a vector $Vg^{\Delta RE1,2}$-Luc described previously (Martin *et al.*, 2001).

The pAS346(E74-CAT) reporter construct was a generous gift from Dr. Sharrocks (Ling, *et al.* 1997), which contains four Ets DNA binding sites of pBLCAT5. The vector comprises of the minimal tk promoter linked to a *CAT* reporter gene.

The following plasmids were constructed for use as expression vectors of the two AaE74 isoforms in the transfection assays. Two expression plasmids: pcDNA3.1AaE74B and pcDNA3.1AaE74A, containing the full-length AaE74 coding region were constructed by PCR amplification. The primers shown below contain additional terminal adaptor sequences of KpnI-Hind III restriction sites (in italic) and the flanking regions of

the translation start site were modified to include the Kozak sequence ACCACC (underlined) immediately before start site to enhance expression of the proteins. The primer sequences are:

AaE74B forward primer:

5'-gatc*aagctt*accaccATGCTGCAGCACATGTCACC-3'

AaE74A forward primer:

5'-gatc*aagctt*accaccATGCCCTTTATCGATGAGGAC-3'

AaE74 common reverse primer:

5'-gatc*tctaga*ACAGAAGTAGACCGCAATGG-3'.

The PCR products were then introduced in frame into pcDNA3.1/V5-His B (Invitrogen), which contains the CMV and T7 promoters as well as the V5 epitope and polyhistidine tag. Similarly, two expression plasmids were generated by PCR: pAcAaE74B and pAcAaE74A. Both constructs share the forward and reverse primers shown below containing the Xba I overhanging ends (in italic). The two primers sequences are: the common forward primer: 5'-GC*tctaga*AATTAACCCTCACTACTAAAGGGAAC-3', the common reverse primer: 5'-GAGATCGATTGCTCGGCCACA*tctaga*GC-3'. The forward primer also contains partial cDNA in pBluescript vector sequence. The PCR products were amplified with ProofStart™ DNA polymerase (Qiagen) and were digested and inserted into the Xba I site of pAc5.1/V5-His A (Invitrogen), which contains the constitutively expressed *Drosophila Actin 5C* promoter.

The following two plasmids were used for expression of two E74 isoform-specific regions in *E. coli*. Two AaE74 isoform expression constructs, pQEAaE74BIso (encoding

AaE74B amino acids from 1 to 311) and pQEAaE74AIso (encoding AaE74A amino acids from 1 to 262) were obtained by PCR amplification using primers covering each isoform-specific region with flanking sequences containing KpnI-Hind III overhanging ends (in uppercase). The primer sequences are:

AaE74B isoform primer: 5'-agatcGGTACC tgctgcagcacatgtcac-3'

AaE74A isoform primer: 5'-agatcGGTACC aggacctgctatggtgc-3'

AaE74A common reverse primer: 5'-agatcAAGCTTg *tta*tacagaagtagacc-3' (*tta* was introduced as a stop codon). The fragments were gel purified, digested and inserted into the IPTG-inducible expression vector pQE32 (Qiagen).

All the plasmids were confirmed, if possible, by partial sequencing or by SDS-PAGE using S^{35}-Met labeled TNT products if contain T7 or T3 promoter.

Antibodies

AaE74B and AaE74A polyclonal antisera were raised using standard protocols by Alpha Diagnostic International against synthetic peptides corresponding to the AaE74B isoform-specific amino acid sequence: (C) $_{170aa}$RYQPHHGGEPDEDEYDRER and the AaE74A isoform-specific amino acid sequence: $_{90aa}$NDLHLDEETSGQIFLQSC. These peptide region were selected based upon the analyses of antigenicity, hydrophilicity, and accessibility of the proteins. The antibodies were further affinity-purified using Antigen-Sepharose column supplied by the manufactory. The antibodies were tested by Western Blotting analyses using bacteria-expressed isoform specific proteins and TNT® expressed full-length proteins of AaE74B and AaE74A.

Bacterial expression of the AaE74A and AaE74B isoform-specific region

The expression vectors: pQEAaE74BIso and pQEAaE74AIso were transformed into *E. coli* strain M15. The cells were cultured overnight and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 30°C with vigorously shaking. The cells were lysed with B-PER protein Extraction Regents (Pierce). The proteins containing two isoform-specific E74 fragments were purified according to the manufacturer's instructions for QIAexpress system (Qiagen). The expressed proteins were analyzed and confirmed using SDS-PAGE.

Western blotting analysis

SDS-PAGE and western blot analysis was conducted as described previously (Zhu *et al.*, 2000). Protein from different sources were resolved on 7.5% SDS-PAGE, followed by electroblotting to polyvinylidene difuoride membranes (Amersham). The membrane was probed with AaE74 isoform specific antibodies. Immune complexes were visualized by the addition of SuperSignal® West Dura Extended Duration Substrate (Pierce) and autoradiography.

***In Vitro* Transcription-Translation**

In vitro transcription-translation was performed using TNT® rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine as described before (Zhu *et al.*, 2000). The AaE74B and AaE74A cDNAs encoding the full-length open reading frame were inserted into downstream of the T7 promoter of pCDNA3.1 (Invitrogen) and were transcribed under the control of this promoter.

In vitro fat body culture

The abdominal walls with adhering fat body (hereafter referred to as fat body) from 3-5 days old previtellogenic females were dissected as previous described (Li *et al.*, 2000a). The dissected fat bodies were treated with 20E (10^{-6}M, dissolved in 0.1% Ethanol) and /or cycloheximide (10^{-5}M) (Chx) as reported (Deitsch *et al.* 1995) for 4-16 hr and three repetitions of 9 fat bodies were collected at every 4 hr. As a control, fat bodies were incubated with 0.1% Ethanol in the culture medium.

RT-PCR/Southern analyses

RT-PCR/Southern analyses were performed as reported (Pierceall *et al.*, 1999). Total RNA was extracted as described in Northern analyses. The prepared RNA aliquots of 0.6 equivalent fat bodies were reversibly transcribed by Superscript II reverse transcriptase using random hexamers in a reaction volume of 10 ul. After the reaction the volume of each sample was brought to 24 ul with H_2O. In the analyses of development profiles, a 0.025 fat body equivalent cDNA was used as a PCR template for each time point. PCR reactions were carried out as reported (Pierceall *et al.*, 1999). The E74 PCR primers used for developmental profile and *in vitro* fat body culture were designed as one common reverse primer and two isoform-specific primers. This arrangement of primers offers the advantage of serving as a control for each other when they both were used in one PCR reaction. The sequences of each primer are listed below:

Name of primer	sequence 5'-3'
AaE74B forward	ACCGCCGACCGCAATACGAT

AaE74A Forward	AAAGCCGCCCGACATCGTCA
AaE74 common reverse	AGCTGTCGACATCCGAAACAC
VCP forward	AGCGCCCATTCTTGTTTGG
VCP reverse	CAGCTCATACAGGTATTCTCC
Actin forward	AAGGCCAACCGTGAGAAGATGAC
Actin reverse	GCTCGTTGCCAATGGTGATGAC
AaE75A forward	TAGTGCAATCAACGTATACCAATC
AaE75A reverse	CAAGGGCGATACTGGTTTCTG

Cell culture and transient transfection assay in Schneider 2 cell line

Drosophila Schneider 2 cell line (S2) (Invitrogen) was maintained at 26 to 28°C in *Drosophila* Serum-Free Medium (SFM) and supplemented with 18mM L-Glutamine. Transfection was conducted with CellFECTIN (Invitrogen) with an optimal DNA-lipid ratio of 1:4 (wt/wt), following the manufacturer's manual. Typically, 50 ng reporter pRL-CMV-Luc, 50 ng or 150 ng each transcription factor, and 1 μl or 3 μl CellFECTIN were mixed in a 24-well plate with a total volume of 250 ul of SFM and incubated at room temperature for 20 min. The expression vector pAc5.1/V5/HisA or pCDNA3.1 V5-HisB was used as carrier DNA so that each well received equal amount of total DNA. The transfection cocktail was then overlaid to attached *Drosophila* S-2 cells for 6 to 12 hr at 27°C. The transfection mixtures were removed and replaced with fresh SFM in absence of or in presence of the hormone treatment at a concentration of 10^{-6} M, and allowed to incubate for 48-72 hours. The activities of two luciferases, *Firefly* luciferase and *Renilla* luciferase in cell lysates, were subjected to dual luciferase analysis using Dual-Glo

Luciferase Assay System (Promega). Basically, the transfected cells were washed gently with a sufficient volume of phosphate buffered saline (PBS) and cell lysates were prepared using 100ul of Passive Lysis Buffer (PLB) for each well. 20µl of the cell lysate was subjected to dual luciferase assays by injecting 50 µl Luciferase Assay Reagent II (LARII) for *Firefly* luciferase and Stop & Glo Reagent 50 µl for *Renilla* luciferase. Measurement of each luciferase activity was conducted by programming the luminometer (Bio-Rad) to perform a two-second pre-measurement delay, followed by a ten-second measurement period for each reporter assay with a two-second interval between the two measurements. The substrates used for *Firefly* luciferase and *Renilla* luciferase equaled 50ul for each injection. The expression vectors pAc5.1/V5/HisA or pCDNA3.1 V5-HisB without transcription factor insert were used as negative controls.

The determination of expression efficiency of each expression vectors in S2 cells were performed in 6-well culture plate, cultured in the same way as that in the 24-well plate culture except that all the components increase proportionally. Nuclear extracts from the cells were prepared according to the protocol as described by Miura *et al.* (1999). AaE74A and AaE74B gel mobility shift assays (GMSA) were performed as described below. EcR and USP GMSA was conducted as described previously (Wang *et al.*, 1998).

Gel mobility shift assays (GMSAs)

Nuclear extracts from *A. aegypti* pre- and vitellogenic fat bodies were prepared according to the protocol for isolation of liganded EcR-USP receptor by the high salt extraction method as described by Miura *et al.* (1999). Transcription factors were

synthesized *in vitro* using a coupled transcription-translation (TNT®) kit from Promega. Reactions were performed in a 10 µl volume containing 2–3 µl of nuclear extracts equivalent to 10-15 fat bodies or 1µl of the appropriate TNT expressed protein samples, 25mM Herpes (pH 7.9) 50mM KCl 1mmDTT, 0.1% NP-40, 10% (v/v) Glycerol, 0.5 µg poly(dI-dC)·poly(dI-dC), 0.5 µg single-stranded DNA (5'-TGATCAAGCTTGTTATACAGAAGTAGACC- 3'). After 20-min incubation at room temperature, 1µl of [^{32}P] labeled DNA-probe with radioactivity of 20-60 kcmp was added, and the incubation was continued for another 20 min at the same temperature. Free and protein-bound DNA were separated on 5% nondenaturing polyacrylamide gels (pre-run for 15-20 min) at room temperature and a constant voltage of 100V to 150V in 0.5×TBE. Where indicated, competitor oligonucleotides or specific antisera were included in the DNA-binding reactions. The gel was then dried and subject to phosphoimager analyses or autoradiography exposure with an intensifying screen at -70°C.

DNA probes for GMSA were made by annealing together complementary oligonucleotides, and end-labeled by back-filling with Klenow fragment of DNA polymerase I by using [α-^{32}P]CTP (NEN). Oligonucleotides (only sense strands are shown and thereafter) containing overhanging CTAG used to generate the E74 consensus response element probes are as follows:

Consensus E74 probe: 5'-CTAGGATCAACCAGGAAGTGTTCGATT-3'.

Ten putative E74 response elements on *Vg* promoter are shown below. The locations of the fragments containing the E74 binding site relative to the transcriptional

start site of *Vg* are indicated in the brackets. The extra GATC are added to the 5' end of each sequence for future end labeling.

VGE74RE1(-1304 to -1274bp):

5'-*GATC*ATTTTGACAATCGTC*GGAA*AGGTCTTTCTG -3'

VGE74RE2(-1196 to –1166bp):

5'-*GATC*TATTAC*GGAA*AGCTTCTTATTCCGGACACT -3'

VGE74RE3(-974 to –944bp):

5'-*GATC*TTTCATGAGCTGTCC*GGAA*TTTGAATCAAA -3'

VGE74RE4(-950 to -920bp):

5'-*GATC*TTGAATCAAAGTGTCC*GGAA*TATGGGGCAA -3'

VGE74RE5(-924 to -894bp):

5'-*GATC*AAGTAA*GGAA*GCGTCCGGAATAAGAATCGT -3'

VGE74RE6(-804 to -774bp):

5'-*GATC*GCTTAGAGCCGA*GGAA*TCATACAGATTGAT -3'

VGE74RE7(-694 to -664bp):

5'-*GATC*CATGGCAACATT*TTCC*GAAACCTGGAGATA -3'

VGE74RE8(-389 to -359bp):

5'-*GATC*AATTGAATAATCT*GGAA*TCCATTGCAAGCT -3'

VGE74RE 9(-1543 to -1514bp):

5'-*GATC*CGCCGCCTGGA*TTCC*GCATATCTATTACCA -3'

VGE74RE10(-773 to -743bp):

5'-*GATC*TTATTTTATATGC*TTCC*TGATGGGTTATAC -3'

* All ten fragments contain the core E74 binding motif GGAA or TTCC as underlined. For competition reactions, unlabelled oligonucleotides, were added in 25- or 100-fold surplus depending on the nature of the oligonucleotides.

Chapter III Cloning and Characterization of Mosquito E74

*Most results have originally been published in Molecular and Cellular Endocrinology

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ABSTRACT

In the anautogenous mosquito, *Aedes aegypti*, vitellogenesis is under the strict control of 20-hydroxyecdysone (20E), which is produced via a blood meal-activated hormonal cascade. Several genes of the ecdysteroid-regulatory hierarchy are conserved between vitellogenesis in mosquitoes and metamorphosis in *Drosophila*. We report characterization of two isoforms of the mosquito early E74 gene (AaE74), which have a common C-terminal Ets DNA–binding domain and unique N-termini. They exhibited a high level of identity to *Drosophila* E74 isoforms A and B and showed structural features typical for Ets transcription factors. Both mosquito E74 isoforms bound to an E74 consensus motif C/AGGAA. In the fat body and ovary, the transcript of AaE74 isoform homologous to *Drosophila* E74B was induced by a blood meal exhibiting its highest level coinciding with the peak of vitellogenesis. In contrast, the transcript of AaE74 isoform homologous to *Drosophila* E74A was activated at the termination of vitellogenesis. These findings suggest that AaE74A and AaE74B isoforms play different roles in regulation of vitellogenesis in mosquitoes.

INTRODUCTION

The major insect steroid hormone, 20-hydroxyecdysone (20E), plays a crucial role in controlling key developmental events, including embryogenesis, larval development, metamorphosis, and in some insects, reproduction (Hagedorn, 1990; Riddiford, 1993; Dhadialla and Raikhel, 1994). In Dipteran insects, such as mosquitoes and flies, this hormone regulates vitellogenesis, which is an essential process in insect reproduction. Vitellogenesis involves massive synthesis of major yolk protein precursors (YPP) by the insect metabolic tissue, the fat body, secretion into the hemolymph and accumulation by developing oocytes. In the anautogenous mosquito, *Aedes aegypti*, vitellogenesis is initiated only after blood intake. A blood meal triggers a hormonal cascade, and consequently activates YPP genes in the fat body (Hagedorn, 1989; Raikhel, 1992; Dhadialla and Raikhel, 1994; Deitsch *et al.*, 1995). As a consequence of the blood meal requirement to activate vitellogenesis, mosquitoes transmit numerous pathogens of human diseases. Thus, it is essential to elucidate the molecular mechanism underlying the regulation of vitellogenesis.

The synthesis of YPPs reaches dramatic levels with the maximum at 24 h post blood meal (PBM), and then rapidly terminates at 30-32 h PBM (Raikhel, 1992). The 20E titers in the female mosquito are tightly correlated with the expression of genes encoding YPPs and the synthesis of these proteins by the mosquito female fat body. The 20E titers exhibit a minor peak at 3-4 h PBM, and then rise drastically, reaching the major peak at 18-20 h PBM, which is followed by a drop to a basal level at 36 h PBM (Hagedorn *et al.*, 1975) (figure 1).

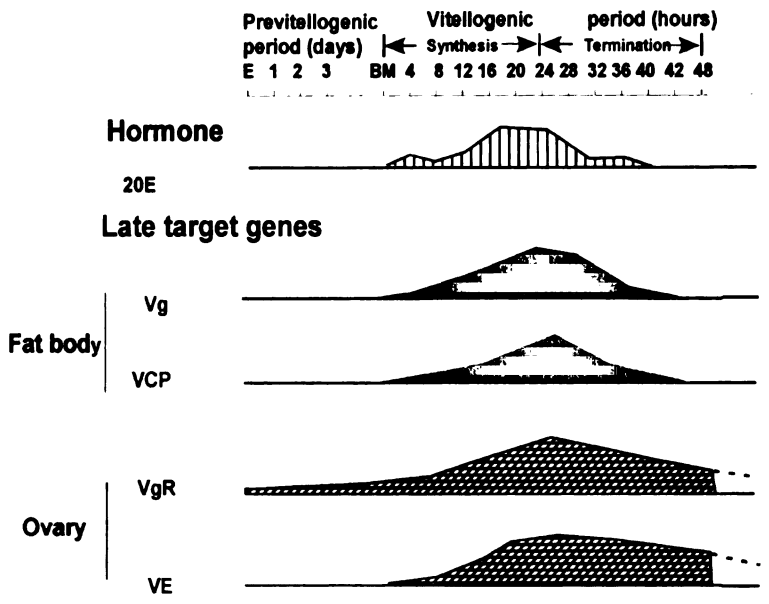


Figure 1. The profiles of 20-hydroxyecdysone (20E). (Hagedorn *et al.*, 1975) and transcripts of late genes in the fat body and ovary during the first vitellogenic cycle of the mosquito *Aedes aegypti*. Profiles of transcripts are based on data from Cho and Raikhel, 1992; Lin *et al.*, 1993; Sappington *et al.*, 1995; Pierceall *et al.*, 1999; Wang *et al.*, 2000. BM, blood meal; E, eclosion; 20E, 20-hydroxyecdysone; VCP, vitellogenic carboxypeptidase; VE, vitelline envelope; Vg, vitellogenin; VgR, vitellogenin receptor.

The 20E regulation of the expression of three major YPP genes: vitellogenin (*Vg*) (Dhadialla and Raikhel, 1990; Cho and Raikhel, 1992), vitellogenic carboxypeptidase (*VCP*) (Cho, *et al.*, 1991), and vitellogenic cathepsin B (*VCB*) (Cho, *et al.*, 1999), provides an ideal model for studying the molecular mechanism governing the hormonal control of genes involved in vitellogenesis. It has been shown that a physiological dose of 20E (10^{-6} M) activates *Vg* and *VCP* expression in the *in vitro* fat body culture system, while application of cycloheximide (Chx), a protein synthesis inhibitor, completely blocks their expression (Deitsch *et al.*, 1995). This suggests that activation of *YPP* genes is indirectly regulated by 20E, presumably through the 20E-regulated hierarchy involving early genes (Ashburner, 1972; Burtis *et al.*, 1990). On the other hand, recent structure-function analyses of the major YPP gene, *Vg*, has revealed a more complex regulation which involves both the direct action of 20E/ecdysteroid receptor, as well as the indirect action of early genes (Martin *et al.*, 2001; Kokoza *et al.*, 2001). The functional binding sites for two early gene products, E74 and E75, have been identified in the 5'-regulatory region of this gene (Kokoza *et al.*, 2001). Previously, we have characterized expression of *AaE75* (Pierceall *et al.*, 1999). In this study, we cloned and characterized another mosquito homologue of the *Drosophila* early ecdysone-inducible gene, *AaE74*.

Drosophila melanogaster E74 (*DmE74*) belongs to the Ets transcriptional factor superfamily (Sharrocks *et al.*, review 1997), and encodes two protein isoforms A and B, which share a common C-terminal Ets DNA–binding domain, yet have a unique N-terminal sequence (Burtis *et al.*, 1990). Mutations in *DmE74* are lethal during pupal development. Genetic studies have demonstrated that mutant E74 completely inactivates inducibility of the late gene, *L71*, in response to an ecdysone pulse in metamorphosis

(Fletcher *et al.*, 1995) and DmE74 protein directly regulates late gene *L71-6* transcription (Urness and Thummel, 1995). *E74* is also an essential component for the maximum expression level of the head involution defective (hid) gene, which controls the programming cell death during metamorphosis (Jiang *et al.*, 2000). Furthermore, *in situ* hybridizations of salivary gland polytene chromosomes have demonstrated that E74 proteins bound to both early and late ecdysone inducible puffs (Urness and Thummel, 1990). *E74* is necessary for the appropriate regulation of stage- and tissue-specific transcription of many ecdysone secondary-response genes (Fletcher *et al.*, 1995).

We report here that the mosquito *AaE74* gene also encodes two isoforms, AaE74A and AaE74B. The pattern of their expression clearly shows that both isoforms of the early E74 gene are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, *Aedes aegypti*. Furthermore, their differentiated expression suggests that the AaE74 isoforms may play different roles in regulation of mosquito vitellogenesis.

RESULTS

Cloning and sequence analysis of *Aedes aegypti* E74 homologue

The *A. aegypti* lambda Dash II genomic library was screened using the DNA from *Drosophila* E74 Ets domain as a probe. Three genomic clones were obtained. Sequencing of these genomic clones revealed that one of the clones, the 600-bp in length, contains a region identical to the Ets domain of the *DmE74* gene. This 600-bp fragment was then utilized as a probe to screen the mosquito vitellogenic fat body cDNA library. Several positive clones were cloned and characterized. Based on partial sequencing and restriction endonuclease analysis, the cDNA clones were divided into two groups. The

two longest cDNA clones from each group, 5.47-kb and 5.13-kb long, were entirely sequenced on both strands.

Sequence analysis was performed on the 5.47-kb long cDNA clone from the first group revealed two start codons at 107 and 156 bp, and a stop codon UAA at 2441 bp. Comparison of the start codon context to the Kozak sequence (Kozak, 1984) showed that the first start codon fitted two out of three, while the second fitted none. The longest ORF (788 AA) had a predicted molecular mass of 83.5kDa.

The 5.13-kb long cDNA clone from the second group had five ATG initiation codons that shared a stop codon UAA at 2970 bp. Four initiation codons were located close to each other at 289 bp, 292 bp, 295 bp and 307 bp and one distant initiation codon was at 484 bp. An attempt to search for in-frame stop codons in the upstream of the putative start codon revealed an in-frame stop codon at 19 bp. This suggested that the coding region was complete in this cDNA clone. Comparison of the sequence immediately upstream of the start codons with the optimal Kozak consensus sequence indicated that the longest open reading frame (ORF) starting from 289 bp, and the other, starting from 484 bp, showed a better fit with two matching positions out of three. The longest ORF (827AA) had a predicted molecular mass of 90.5 kDa.

Both cDNA clones contained poly (A) tracts, which were preceded by classical polyadenylation sites at an appropriate distance. A hydrophathy graphic analysis of the predicted amino acid sequences of showed that both protein products were predominantly hydrophilic (data not shown). Alignment analysis revealed that both deduced proteins were different in their N-terminal regions of 311 aa and 262 aa. However, they were 100% identical in the rest of the protein sequence, indicating that their respective cDNA

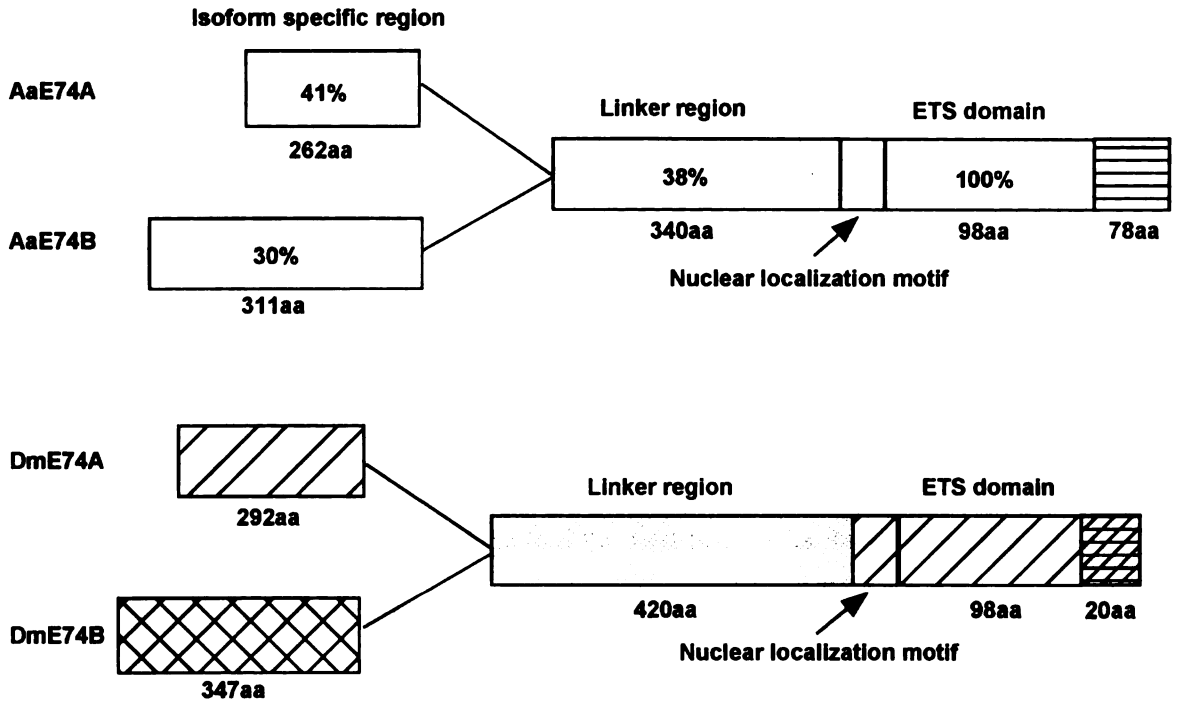


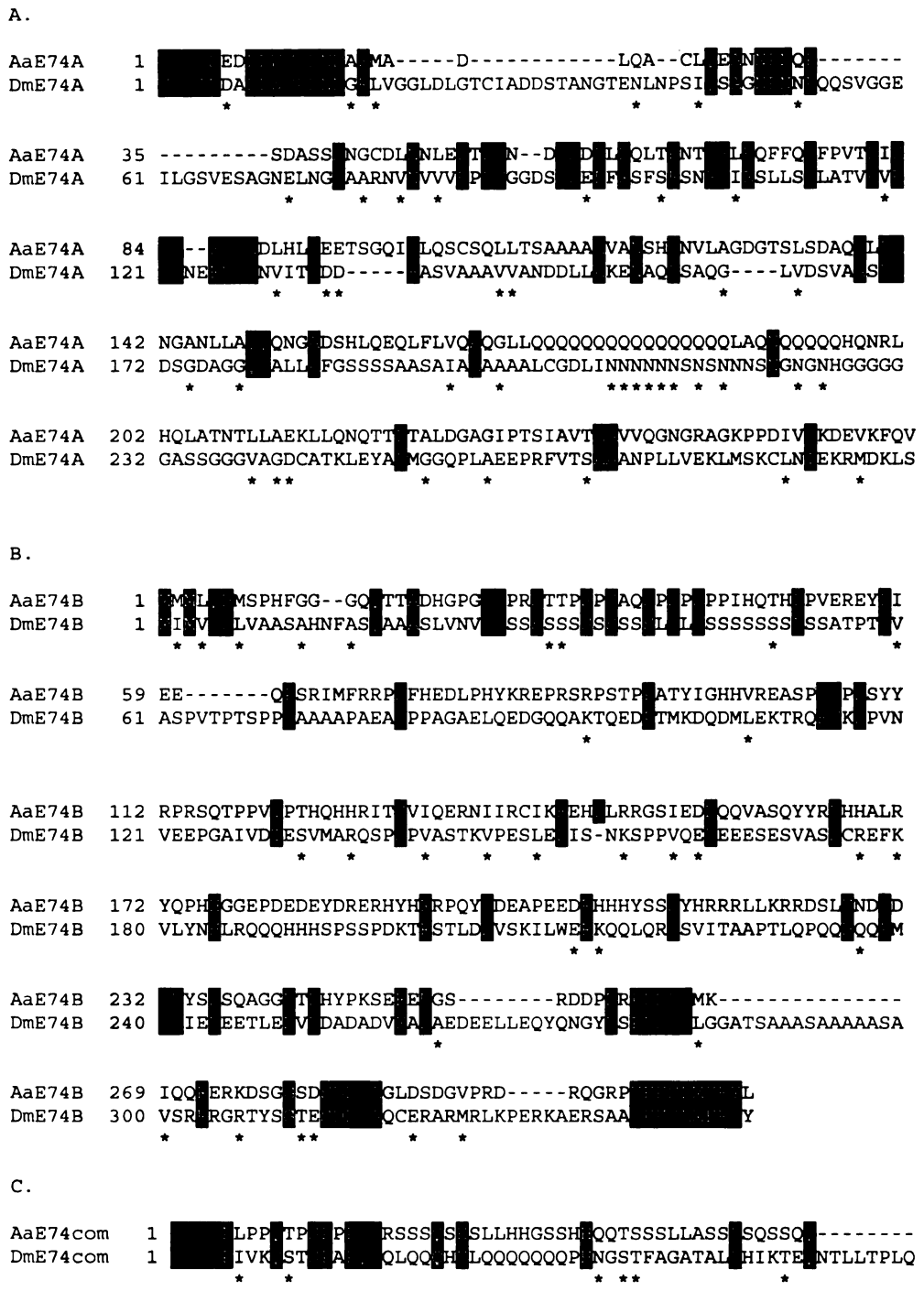
Figure 2. Schematic illustration of the structural comparison of mosquito and *Drosophila* E74 isoforms. The amino acid number is indicated below each domain. The percentage number shown in the mosquito E74 two isoform boxes are the similarity between mosquito E74 isoform to the *Drosophila* counterpart. Similarity was calculated manually based on the results of multiple alignment using "Multiple Sequence Alignments" and "BOXSHADE 3.21" programs indicated in figure 3.

clones encoded isoforms of the same protein (figure 2). The deduced amino acid sequences of these proteins were related to those of the members of the Ets family of transcription factors with the highest homology to *Drosophila* E74.

Alignment of the isoform-specific region of the first protein product demonstrated that it had 38% similarity and 22% identity to *Drosophila* E74A in this region (figure 3A). They were almost 100% identical in their first 31 amino acids. However, the alignment with DmE74B revealed only 16% similarity and 9% identity in the isoform-specific region. Therefore, we designated the first protein as *Aedes aegypti* E74A isoform (AaE74A). In addition to a relatively high level of homology, the isoform-specific regions of AaE74A and DmE74A were similar in length (figure 2).

The isoform-specific region of the second mosquito protein was close in its length to that of DmE74B. It displayed 30% similarity and 18% identity to DmE74B (figure 3B), while showed only 24% similarity and 16% identity to DmE74A. The last 50 amino acids in the isoform-specific regions of the second mosquito protein and DmE74B showed significantly higher identity (50%), which allowed us to assign the former as B isoform (AaE74B).

The 85 aa-long Ets domain located at the carboxyl terminus had 100% similarity between AaE74 and DmE74 (figure 2). In addition, there were clusters of homology in the linker region of these factors (figure 3C). There was also an identical adjacent 8-amino acid stretch, known as a nuclear localization signal motif (KR/KXR/K) (not shown).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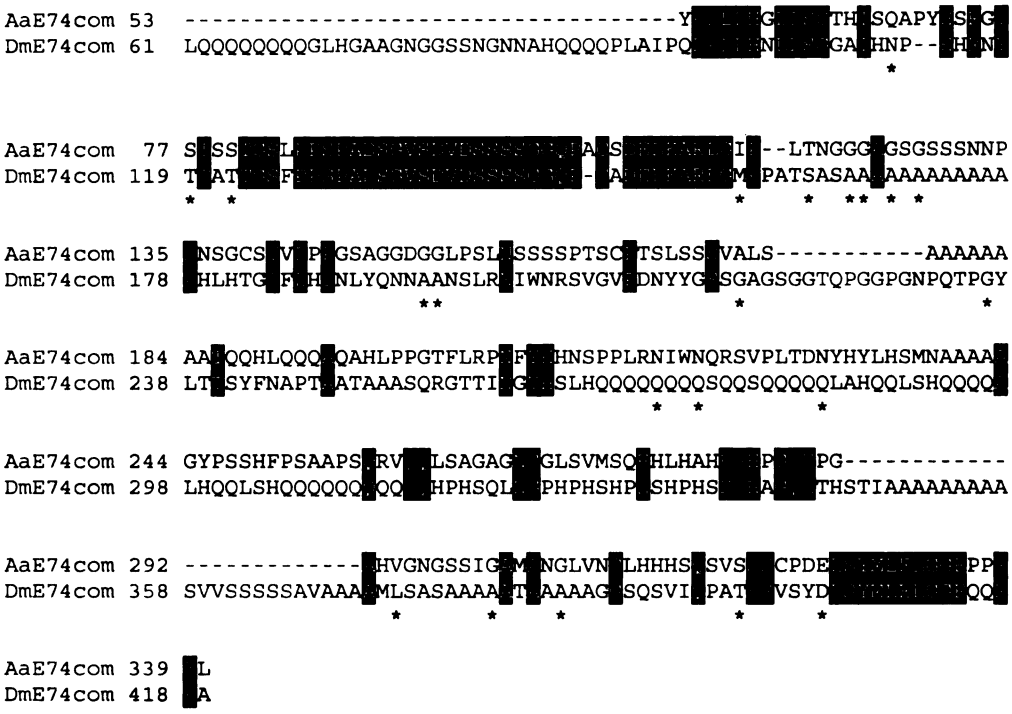
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AaE74com 292
DmE74com 358
AaE74com 339
DmE74com 418

Figure 3. Alignment of mosquito E74 two isoforms with *Drosophila* E74 isoforms. A. Alignment of mosquito E74A isoform specific region with *Drosophila* E74A isoform specific region. B. Alignment of mosquito E74B isoform specific region with *Drosophila* E74B isoform specific region. C. Alignment of mosquito E74 linker region with *Drosophila* E74 linker region. The alignment of four amino acid sequence from isoform specific regions of AaE74A (gene bank access number, AF435023), AaE74B (gene bank access number, AF435022) DmE74A (gene bank access number, A34692) and Dm74B (gene bank access number, B34692) were performed using "Multiple Sequence Alignments" program from Baylor College of Medicine of HGSC at the website: http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html. The output shading format was conducted after alignment using "BOXSHADE 3.21" program at the website: http://www.ch.embnet.org/software/BOX_form.html. Identical amino acids are shown in black, amino acids, which are similar, are indicated by "*" below the sequence.

The predicted conceptual translation of both AaE74 isoforms was generally supported by an *in vitro* transcription and translation experiment labeled by [^{35}S] Met and SDS-PAGE assay. Both *in vitro* translated Aa74A and AaE74B proteins had a higher than predicted molecular mass of about 98 kDa likely due to posttranslational modifications (figure 4A).

DNA binding properties of E74 isoforms

To determine the DNA binding activities of AaE74A and AaE74B isoforms, we performed an GMSA using the consensus E74 binding site sequence, which was originally identified by affinity chromatography screening (Urness and Thummel. 1990). *In vitro* translated full-length AaE74A and AaE74B proteins were used in GMSA. The oligonucleotide, containing the E74 consensus-binding site, formed high molecular weight complexes with *in vitro* expressed AaE74 proteins, but not with the control reticulocyte lysate. The complexes were competitively abolished by the cold consensus E74 binding site oligonucleotides. The GMSA experiment demonstrated that both AaE74 isoform proteins translated *in vitro* specifically bound to the consensus sequence (figure 4B).

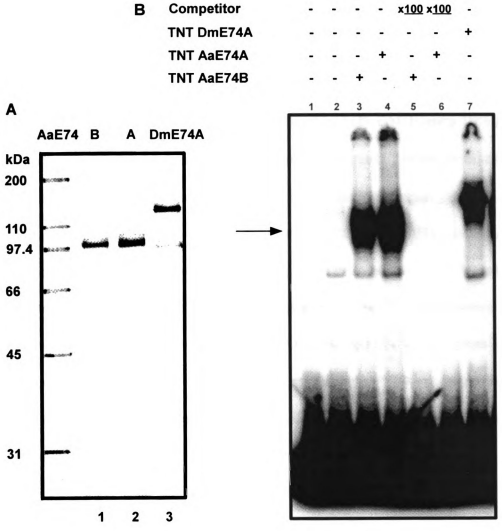

B
Competitor - - - - x100 x100 -
TNT DmE74A - - - - - - +
TNT AaE74A - - - + - + -
TNT AaE74B - - + - + - -
A
AaE74 B A DmE74A
kDa
200
110
97.4
66
45
31
1 2 3
1 2 3 4 5 6 7

Figure 4. *In vitro* translational analysis (A) and gel mobility shift assay (B) of AaE74 isoforms. A. The cDNA clones encoding AaE74A, AaE74B, and DmE74A were translated by a coupled *in vitro* transcription-translation system and labeled by [^{35}S]-Methionine. Translation products were resolved by SDS-PAGE using 10% gels followed by autoradiography. Molecular mass standards in decreasing order are myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase (Bio-Rad). B. AaE74 isoforms bind *in vitro* to the *Drosophila* E74 consensus DNA binding sequence. GMSA was performed with *in vitro*-translated AaE74 isoforms, and the *Drosophila* E74 consensus DNA binding sequence as ^{32}P-labeled probe. An arrow indicates the specific retardation complex. One hundred fold molar excess of unlabeled E74 consensus DNA binding sequence was used as a specific competitor. *In vitro*-expressed *Drosophila* E74 cDNA was used as a positive control. Lane 1, probe only, lane 2 containing cell lysate as mock.

Northern analysis of AaE74 isoforms

In order to examine the sizes of AaE74 isoform transcripts and the time of their expression, we first performed the Northern blot analysis using vitellogenic tissues of previtellogenic and vitellogenic mosquito females. Total RNA derived from mosquito fat bodies was subjected to Northern analysis using probes corresponding to isoform-specific regions of AaE74 or their common region. Northern blot analysis showed that both AaE74 isoforms were expressed in fat bodies of vitellogenic (24-30 h PBM), but not in previtellogenic, mosquitoes (figure 5). This analysis revealed two transcripts: 9-kb which hybridized to the AaE74B isoform-specific probe, as well as to a common region probe (figure 5, lanes 2 and 6) and a band of 5.5 kb which hybridized to the AaE74A isoform-specific probe, along with a common region probe (figure 5, lanes 4 and 6). Likewise, Northern blot analysis detected E74 transcripts only in vitellogenic ovaries and not in previtellogenic ones. Utilization of isoform-specific probes revealed that in the vitellogenic ovary AaE74A and AaE74B transcripts had the same sizes as those in the vitellogenic fat body (data not shown).

Temporal profile of AaE74 isoforms in female mosquito fat body and ovary during vitellogenesis

To determine the time course of E74 expression in further detail, we performed the RT-PCR. For comparison, an equivalent mosquito unit amount of tissue was used for each time point. No expression of either AaE74 isoform was detected in previtellogenic fat bodies and ovaries. However, in both the fat body and ovary, AaE74 isoforms were

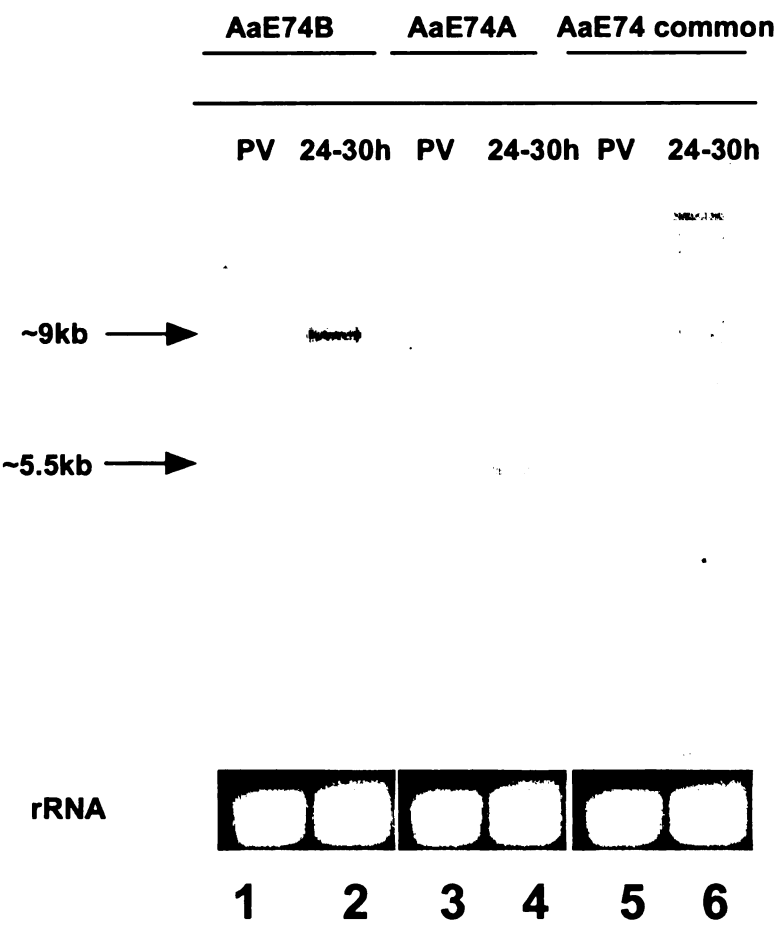


Figure 5. Northern blot analysis of AaE74 transcripts. Total RNA was isolated from previtellogenic (3-5 days after eclosion) and vitellogenic (24-30 h PBM) mosquito fat bodies. Five-mosquito-equivalent RNA was loaded on each lane. Isoform-specific probes corresponding to the AaE74A and AaE74B isoform regions and a probe containing common region were used for hybridization. Ribosomal RNA was included as a loading control.

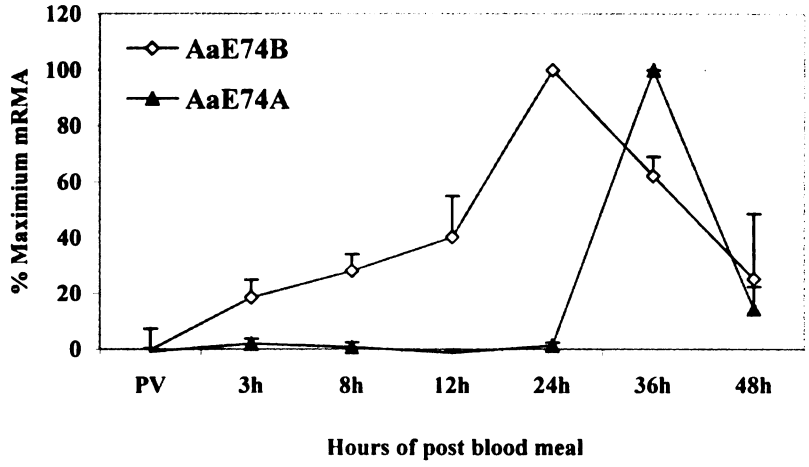
detected at the onset of vitellogenesis after female mosquitoes fed on blood. Remarkably, the two isoforms show distinct profiles in both vitellogenic tissues.

The RT-PCR analyses showed that in the fat body the AaE74B transcript was induced by a blood meal and exhibited moderate rise during the early vitellogenic stage, followed by a sharp elevation at 12 -24 h PBM. After 24 h PBM, AaE74B expression declined rapidly to a low level at the end of vitellogenesis (figure 6A). The expression pattern of AaE74B demonstrated by RT-PCR was also supported by Northern blot analysis (data not shown).

In contrast, the AaE74A transcript did not exhibit any significant increase until 24 h PBM when it displayed a dramatic elevation with a peak at 36 h PBM. Then AaE74A expression levels dropped rapidly to a low level at 48 h PBM (figure 6A).

Interestingly, the AaE75A transcript, another 20E-inducible early gene, assayed in the same fat bodies, exhibited two peaks, the first at 3 hr PBM and second at 24 h PBM. The *VCP* gene, one of the *YPP* genes, used as a control, showed a typical profile, peaking at 24 h PBM (figure 6B). Both Northern and RT-PCR analyses demonstrated that the previtellogenic ovary was devoid of AaE74B mRNA (not shown). Upon a blood meal, the AaE74B transcript exhibited a barely detectable increase between 3 and 8 h PBM, after which it dramatically increased to its major peak at 24-36 h PBM. Thereafter, the expression dropped to a low level at 48 h PBM (figure 7A). Overall, the AaE74B expression pattern was similar to that of the AaE75A. The vitellogenin receptor mRNA, an ovarian germ-line cell specific gene (*VgR*) used as a control, showed its typical profile, being present in the previtellogenic and early vitellogenic ovaries. Its levels increased dramatically after 8-12 h PBM reaching the maximum at 24 h PMB (figure 7A).

A.



B.

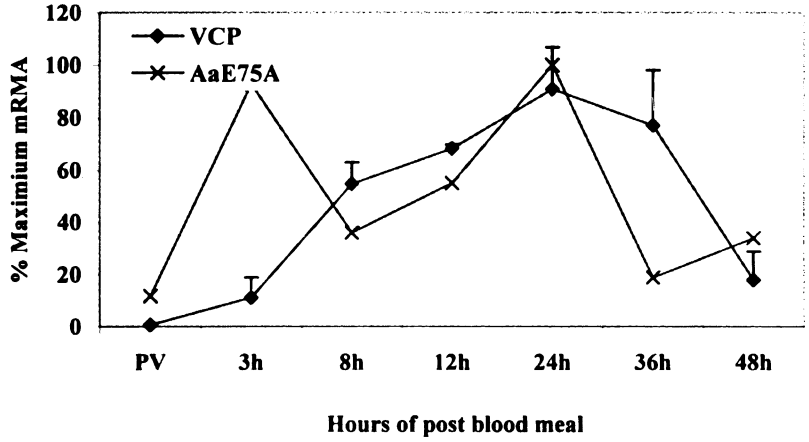


Figure 6. Developmental profiles of AaE74 in the fat body. The RT-PCR products were subjected to the Southern blot analysis and quantification by a phosphorimager. One mosquito equivalent of reverse-transcribed cDNA was applied as a template for PCR. The results are means of independent triplicate samples ± SD. An early gene *AaE75A* and the late gene *VCP* were used as controls.

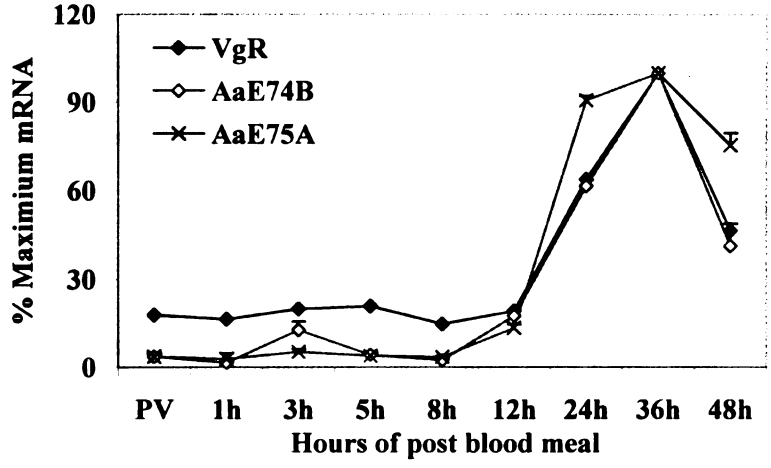
Similar to the fat body, the AaE74A transcript displayed a different profile in the ovary as well. Its levels remained at a background level in both previtellogenic and vitellogenic stages (1-24 h PBM), but exhibited a drastic increase thereafter, reaching the peak at 48 h PBM. This AaE74B expression pattern was similar to that of the vitelline envelope, an ovarian follicle cell specific gene (figure 7B).

DISCUSSION

We have demonstrated previously that several crucial genes of the ecdysteroid-regulatory hierarchy are conserved between vitellogenesis in mosquitoes and metamorphosis in *Drosophila* (Pierceall *et al.*, 1999; Raikhel *et al.*, 1999; Kapitskaya *et al.*, 2000; Li *et al.*, 2000a; Wang *et al.*, 2000). Like in *Drosophila* (Yao *et al.*, 1992), the functional mosquito ecdysteroid receptor is a heterodimer of the ecdysone receptor (EcR) and its obligatory partner *Ultraspiracle* (USP), with two EcR isoforms and two USP isoforms cloned and characterized in *A. aegypti* (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996; Wang *et al.*, 1998; Wang *et al.*, 2000; Wang *et al.*, unpublished data).

Several other key components such as an early ecdysone-inducible gene, *AaE75* (Pierceall *et al.*, 1999), an early late ecdysone-inducible gene *AaHR3* (Kapitskaya *et al.*, 2000) and a competence factor, *βFTZ-F1*, were also cloned and characterized in *A. aegypti* (Li *et al.*, 2000a). Here, we present further evidence that the key machinery is utilized reiteratively in these 20E-controlled developmental processes. Two isoforms of the *Drosophila* transcription factor E74 homologue, which share a common C-terminal Ets DNA-binding domain, yet have a unique N-terminal sequence, were identified and characterized in the mosquito *A. aegypti*. They exhibit a high level of identity to DmE74 isoforms A and B and

A.



B.

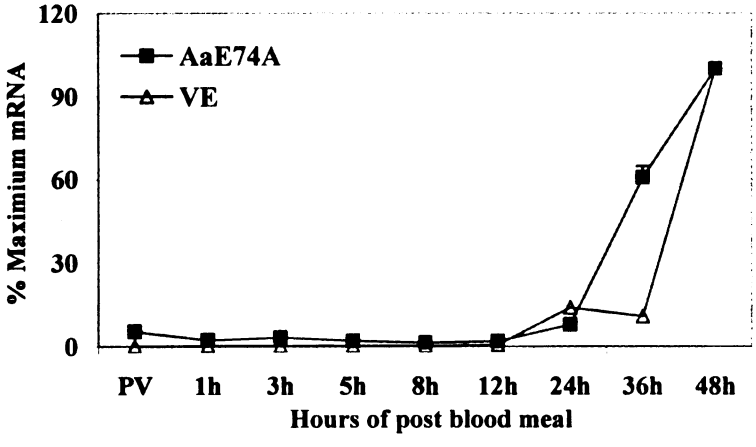


Figure 7. Developmental profiles of AaE74 in ovary. The RT-PCR was subject to the Southern blot assay and was quantified by a phosphorimager. One mosquito equivalent of reverse-transcribed cDNA was used as a template for PCR. The results are means of independent triplicate samples ± SD. The early gene *AaE75A*, the germ cell specific gene (*VgR*) and the follicular cell specific gene, *vitelline envelope* (*VE*) were included as three positive controls.

show structural features typical for members of the Ets transcriptional factor superfamily (Burtis *et al.*, 1990: Sharrocks *et al.*, 1997). Corresponding isoforms of both insects are identical in the Ets DNA binding domain and at a region immediately upstream from the Ets domain containing the nuclear localization signal motif. Furthermore, both mosquito E74 isoforms bind to a purine-rich *Drosophila* E74 binding site with the consensus core motif C/AGGAA (Urness and Thummel, 1990).

In the mosquito fat body, both AaE74 isoforms were not detectable at the previtellogenic stage. AaE74B mRNA showed moderate gradual enhancement at 3-8 h PMB, but exhibited dramatic elevation at 12-24 h PBM when 20E titers reached the major peak, then declined to a basal level at the termination of vitellogenesis, parallel to the dropping of 20E during that period. The overall AaE74B transcript profile correlated with those of yolk protein precursors genes, *Vg*, *VCP*, *VCB* and *Lp* (Cho *et al.*, 1991; Cho and Raikhel, 1992; Cho *et al.*, 1999; Sun *et al.*, 2000), suggesting its possible role in governing expression of these 20E-regulated genes. Previously, we have demonstrated that the AaE75A transcript, another 20E-inducible early gene, also appears after a blood meal and its profile correlates with transcript profiles of the *YPP* genes, exhibiting a major peak at 24 h PBM (Pierceall, *et al.*, 1999). However, in contrast to E74B, the E75A transcript has another early peak at 3 h PBM that corresponds to the first small peak of ecdysteroids that occurs at this time (Hagedorn *et al.*, 1975). Thus, although the expression of both early 20E-inducible genes *AaE74B* and *AaE75*, appear to be temporally linked to the initiation and maintenance of vitellogenesis in the mosquito fat body and ovary, their respective roles are likely different.

In both the fat body and ovary, the expression profiles of AaE74A differ dramatically from those of AaE74B. The AaE74A transcript does not show any significant increase during the most of the vitellogenic period but displayed a remarkable peak at the postvitellogenic period. Although protein profiles for two E74 isoforms are not available, the expression patterns of their transcripts imply that they may serve entirely different or even opposite functions in regulation of some late genes in mosquito vitellogenesis.

In the fruit fly, *Drosophila melanogaster*, there are two pulses of the steroid hormone ecdysone directing the major postembryonic developmental transitions, including molting and metamorphosis. The first small pulse activates the DmE74B transcription, while the second larger pulse induces the DmE74A expression (Karim and Thummel, 1991). These differential expression patterns of the two DmE74 isoforms presumably serve as a steroid-trigger switch to regulate late gene expression (Fletcher *et al.* 1997).

The functional binding sites for both these early gene products, E74 and E75, have been identified in the 5'-regulatory region of *Vg*, the major *YPP* gene (Kokoza *et al.*, 2001). A functional E74 binding site is located between -0.7 kb and -1.0 kb of the 5'-regulatory region relative to the *Vg* transcription start site (Kokoza *et al.*, 2001; Sun *et al.*, unpublished observation). Interestingly, the E75 binding site is adjacent to the E74 site (Kokoza *et al*, 2001). Transgenic analyses using *Drosophila* and mosquitoes have demonstrated that this region of the 5'-regulatory region of *Vg*, containing E74 and E75 binding sites, is required for a significant increase in the expression level of the reporter gene (Kokoza *et al.*, 2001). The two early genes, *AaE74* and *AaE75*, may act

cooperatively to regulate Vg expression *in vivo*. Alternatively, the two early genes may play different roles in regulating Vg expression as indicated in their control of salivary gland cell death during *Drosophila* metamorphosis. The genetic studies have demonstrated that DmE74A is required for maximum levels of *hid* (head involution defective) induction, while DmE75A and DmE75B are sufficient to repress a death inhibitor-diap2 (Jiang *et al.*, 2000). Future studies should differentiate the precise roles of each of these early gene products in regulation of the *Vg* gene expression and other genes involved in the mosquito vitellogenesis.

In summary, we report the cloning and characterization of two isoforms of the mosquito homologue of the 20E-inducible early gene *E74*. Both AaE74A and AaE74B isoforms are capable of binding to the E74 consensus sequence, however they exhibit distinct expression profiles in the fat body and ovary. Future analysis of the roles of *AaE74* is to better understand the mechanism by which AaE74 isoforms regulate late gene expression in vitellogenic tissues of anautogenous mosquitoes.

ACKNOWLEDGMENTS

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Chapter IV Functional Characterization of Mosquito E74

ABSTRACT

In the anautogenous mosquito, *Aedes aegypti*, successful reproduction depends on acquisition of a blood meal. Consequently, mosquitoes are vectors of numerous devastating human diseases. Blood feeding triggers hormonal cascade mediated by steroid hormone 20-hydroxyecdysone (20E), which activates yolk protein precursor (*YPP*) genes in the female mosquito fat body, an insect metabolic tissue. This process is named vitellogenesis. The mediator genes of the ecdysteroid regulatory hierarchy are conserved between vitellogenesis in mosquitoes and metamorphosis in *Drosophila*. *E74*, an ecdysone-inducible early gene, is a key mediator gene in the hierarchy. Previously, I cloned the mosquito *E74* (*AaE74*) gene, which encodes two isoforms AaE74A and AaE74B. They have a common C-terminal Ets DNA-binding domain and unique N-termini. In the fat body, the transcript of AaE74B is induced by a blood meal, exhibiting a profile corresponding to those of *YYP* genes. In contrast, the transcript of AaE74A is activated at the termination stage of vitellogenesis, suggesting that AaE74B is an activator for *YYP* genes while AaE74A is a repressor during vitellogenesis in mosquitoes. Both AaE74 isoforms are ecdysone-inducible early gene products. Functional analyses have showed that AaE74B can activate reporter gene expression driven by the promoter from major yolk protein *vitellogenin* (*Vg*) in *Drosophila* Schneider 2 cells. In contrast, AaE74A exhibits a repression activity. Significantly, several putative E74 binding sites in the *Vg* promoter were demonstrated to be required for these activities, as deletion of these binding sites completely abolishes the activities. These findings are in congruent with their proposed roles in vitellogenesis. Remarkably, as assessed by transfection assays, AaE74B acts synergistically on *Vg* promoter with ecdysone receptor complex,

and the action requires the identified E74 binding sites and ecdysone response elements in *Vg* promoter. Moreover, gel mobility shift assays have shown that both AaE74B and ecdysone receptor complex co-exist during vitellogenic period. Taken together, these results reveal a unique class of Ets-domain proteins with distinct functions in gene regulation in the mosquito fat body. Significantly, these studies provide evidence supporting a novel mechanism by which a target gene expression may be amplified by the cross talk between an Ets protein and a nuclear receptor.

INTRODUCTION

Successful reproduction in the anautogenous mosquito *Aedes aegypti* requires the ingestion of a blood meal from a vertebrate host. Vitellogenesis is the key process in egg maturation, which involves massive production of yolk protein precursors (YPPs) by the fat body, an insect metabolic tissue analogous to vertebrate liver (Raikhel and Lea 1990; Dittmann *et al.*, 1989; Raikhel et al., 2002). Vitellogenesis is initiated only after an anautogenous female mosquito takes blood. The unique properties of the requirement of a blood meal from a vertebrate host explain the success of the mosquito as efficient vector human pathogens. Elucidation of the mechanism, by which this process is regulated, could lead to the development of new strategies to combat this devastating human disease vector. The blood meal triggers the expression of a distinct set of transcription factors leading to a specific pattern of gene expressions tightly controlled by the insect hormone steroid 20-hydroxyecdysone (20E) (Hagedorn, 1990; Riddiford, 1993; Dhadialla and Raikhel, 1994).

The events of vitellogenesis are best understood in the yellow-fever mosquito *A. aegypti*. In this mosquito, the first cycle of vitellogenesis in the fat body can be divided into four phases: previtellogenic preparation, state of arrest, vitellogenic synthetic stage, and vitellogenic termination stage (figure 1)(Raikhel *et al.*, 1992). A newly emerged female requires about 3 days to become competent to undergo the intense physiological needs for vitellogenesis. During this phase, the vitellogenic tissue (the fat body) acquires responsiveness to 20E and becomes competent for yolk protein precursor synthesis and secretion. After 3 days of previtellogenic development, the fat body and ovary enter a state of arrest that persists until a blood meal is taken, which triggers the 20E cascade.

20E titers are only slightly elevated at 4 h post blood meal (PBM), they begin to rise sharply at 6-8 h PBM, and they reach their maximum level at 18-24 h PBM. During the active synthetic stage, major YPP, *vitellogenin* (*Vg*) (Dhadialla and Raikhel 1990; Cho and Raikhel 1992), *vitellogenic carboxypeptidase* (*VCP*) (Cho *et al.*, 1991), and *vitellogenic cathepsin B* (*VCB*) (Cho *et al.*, 1999) are produced by the fat body and accumulated by developing oocytes. The massive YPP syntheses peak at around 24 h PBM, and drop sharply and terminate by 36-42 h PBM. Finally, the fat body undergoes remodeling, waiting for the availability of the next blood meal to proceed with the next cycle of vitellogenesis (Hagedorn *et al.*, 1975) (YYP profiles are shown in figure 1).

The insight to the molecular mechanism of 20E action has been obtained from the studies of molting and metamorphosis in *Drosophila melanogaster*. 20E achieves the regulatory effect on gene expression through its receptor that is a heterodimer of two members of the nuclear receptor family, the ecdysone receptor (EcR) and a retinoid X receptor (RXR) homologue, *Ultraspiracle* (USP). The EcR/USP complex recognizes sequence-specific DNA sequences, named ecdysone response elements (EcREs). After binding to 20E, the EcR/USP complex directly induces several early response genes: *E74*, *E75*, and the *Broad-Complex(BR-C)*. As a result, products of these early genes activate late target genes, and meanwhile repress their own expression via a feedback inhibition mechanism (Thummel, 1996).

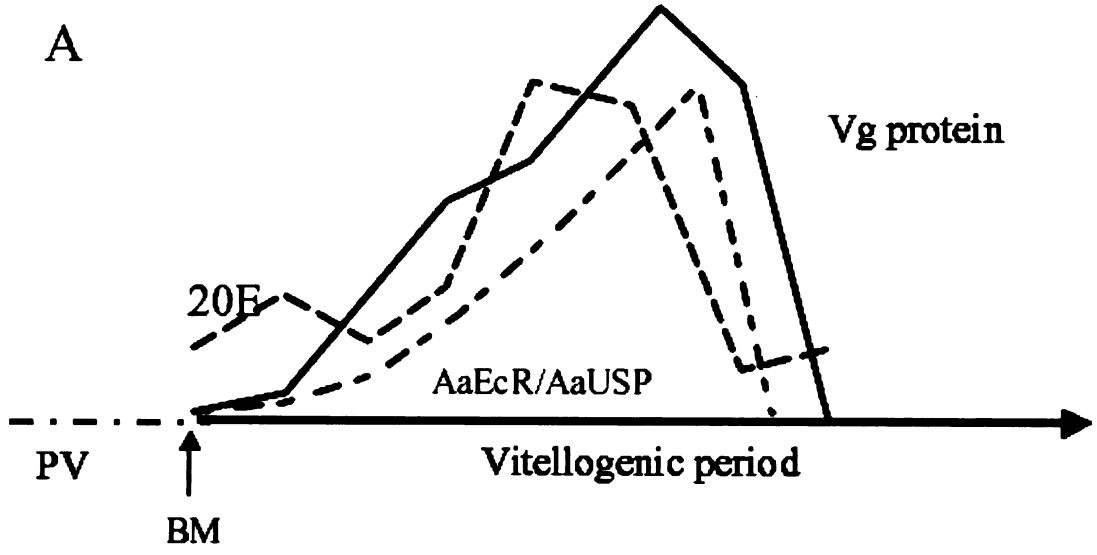
This ecdysteroid regulatory hierarchy appears to be conserved during vitellogenesis in the mosquito *A. aegypti* (Li *et al.*, 2000a). Two EcR isoforms (AaEcR-A and AaEcR-B) and two USP isoforms (AaUSP-A and AaUSP-B) are expressed during

Days
E 1 2 3 BM 4 8 12 16 20 24 28 32 36 42 48 54 60 66 72
Hours
Hormones
20E
JH
Fat Body
AaEcR-B
AaUSP-B
AaE74-A
AaE74-B
Vg
VCP
VCB
Transcription Factors
Potential Target

Figure 1. Summary of events during the first cycle of vitellogenesis in the anautogenous mosquito *Aedes aegypti*. The previtellogenic period begins to eclosion (E) of the adult female. During the 2-3 days post emergence, the mosquitoes become competent for vitellogenesis. The mosquito then enters a state of arrest. Yolk protein precursors, vitellogenin (Vg), vitellogenic carboxypeptidase (VCP) and vitellogenic capthsin B (VCB) are not synthesized until a blood meal is taken (BM). Hormones: hormone titers of juvenile hormone (JH) and 20-hydroxylecdysone (20E) in *A. aegypti* females (Hagedorn *et al.*, 1975; Shapiro an Hagedorn 1982). Transcription profiles of late genes (*Vg*, *VCP* and *VCB*) and transcription factors (AaEcR-B, AaUSP-B, AaE74A and AaE74B) were determined by Northern or RT/PCR analyses: *Vg* (Cho and Raikhel, 1992); *VCP* (Cho *et al.*, 1991); *VCB* (Cho *et al.*, 1999); AaEcR-B (Cho *et al.*, 1995; Wang *et al.*, 2003), AaUSP-B (Kapitskaya *et al.*, 1996), AaE74A and AaE74B (Sun *et al.*, 2002). Modified from Raikhel *et al.*, (2002), with permission.

pre- and vitellogenic periods in the fat body and ovaries (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996; Wang et a., 2000; Wang *et al.*, 2003) (the profiles of major isoforms AaEcR-B and AaUSP-B are shown in figure 1. The dominant mosquito EcR/USP heterodimer AaEcR-B/AaUSP-B will be referred to as AaEcR/AaUSP thereafter). AaEcR/AaUSP binds promiscuously to EcREs to modulate ecdysone regulation of target genes (Wang *et al.*, 1998). Significantly, it has been demonstrated that AaEcR/AaUSP complex from fat body nuclear extracts can bind to the EcRE present in the *Vg* promoter during the synthetic stage in vitellogenesis. This binding occurs in a pattern correlated with 20E titers and *Vg* gene expression (figure 2A) (Martin *et al.*, 2001).

Intensive studies have been performed to determine the mechanism of the transcriptional regulation of the major yolk protein gene *Vg*, however, the mechanism by which such a dramatic induction occurs *in vivo* after a blood meal is still unclear. The early gene *AaE74* was postulated to be responsible for further enhancement of the expression of *Vg* after its initial activation. There are several lines of evidence supporting the hypothesis. First, Vg protein synthesis is activated by a blood meal-initiated hormonal cascade. The activation can be mimicked in an *in vitro* fat body culture system. It has been demonstrated that a physiological dose of 20E can induce *Vg* gene expression in the competent fat bodies. This induction can be completely blocked by the administration of a protein synthesis inhibitor. This implies that activation of *Vg* is indirectly regulated by 20E (Deitsch *et al.*, 1995). Based on the ecdysone hierarchy model (Ashburner *et al.*, 1974; Thummel, 1996), *AaE74*, an ecdysone-inducible early gene, is an ideal candidate to directly regulate the expression of the potential late target gene, *Vg*. Second, AaE74B mRNA expression is activated at the onset of vitellogenesis.


A
Vg protein
20E
AaEcR/AaUSP
PV
Vitellogenic period
BM

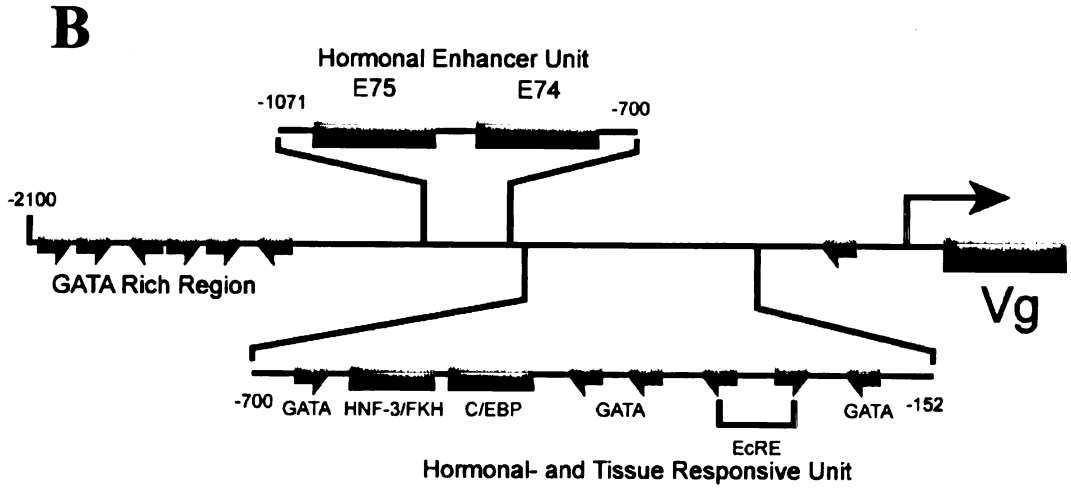

B
Hormonal Enhancer Unit
E75
E74
-1071
-700
-2100
GATA Rich Region
Vg
-700
GATA
HNF-3/FKH
C/EBP
GATA
GATA
-152
EcRE
Hormonal- and Tissue Responsive Unit

Figure 2. A) Developmental profile of the native AaEcR/AaUSP ecdysone receptor in *A. aegypti* fat body nuclear extracts during vitellogenesis (Martin *et al.*, 2001). Fat body nuclear extracts were prepared throughout previtellogenesis (PV) and at different hours during the vitellogenic period and subjected to GMSA with ^{32}P-labelled VgEcRE. Corresponding profiles of the 20E titer (Hagedorn *et al.*, 1975) and the expression of the *Vg* gene in the fat body (Cho and Raikhel, 1992). BM: blood meal. B) Schematic illustration of the regulatory region of the *Vg* gene. Numbers refer to the nucleotide positions relative to the transcription start site. C/EBP—response element of C/EBP transcription factor; EcRE—ecdysteroid response element; E74 and E75—response elements for respective early gene products of the ecdysone hierarchy; GATA—response element for GATA transcription factor; HNF3/fkh—response element for HNF3/forkhead factor; Vg—coding region of the *Vg* gene (from Kokoza *et al.*, 2001; Raikhel *et al.*, 2002).

The timing of AaE74B expression is a prerequisite for its ability to activate of the *Vg* gene expression. Finally, putative binding sites for E74 have been found in the 5'-regulatory region of the *Vg* gene.

E74 belongs to the family of Ets domain transcription factors. The Ets family shares a highly conserved DNA binding domain, the Ets domain, and binds to core DNA motif 5'-GGAA/T-3'. Ets proteins have been shown to be critical determinants of metazoan development and play crucial roles in transcriptional regulation of genes involved in tissue development, cellular differentiation, cell cycle control, and cellular proliferation as well as insect development (reviewed in Sharrocks *et al.*, 1997; Graves and Peterson 1997; Yordy *et al.*, 2000; Li *et al.*, 2000b; Sharrocks, 2000; Hsu and Schulz, 2000). Combinatorial control is a characteristic property of Ets family members, involving interaction between Ets and other key transcriptional factors such as GATA factors and nuclear receptors (reviewed in Li *et al.*, 2000b). Direct protein–protein physical interactions regulates DNA binding, subcellular localization, target gene selection and transcriptional activity of Ets proteins.

Previous studies utilizing *Drosophila* and *Aedes* transformation, as well as DNA-binding assays, have identified the 5' regulatory region of *Vg* gene, which is responsible for its sex-, stage- and tissue- specific expression (Kokoza *et al.*, 2001). The analyses revealed three modules in the 2.1kb upstream portion of the *Vg* gene. The distal region is characterized by multiple binding sites for GATA factors. This region is required for the extremely high expression levels characteristic to *Vg* gene expression. The median region contains several putative early gene binding sites. This region is essential for a stage-specific hormonal enhancement of *Vg* gene expression. Finally, the proximal

region, adjacent to the basal transcriptional start site, harbors binding sites for multiple transcription factors: EcR/USP, GATA factors (GATA transcription factors and HNF3/fkh (hepatocyte nuclear factor3/forkhead transcription factor). This region is required for the precise stage- and tissue-specific expression of the *Vg* gene, although the level driven by this region is low (figure 2 B) (Kokoza *et al.*, 2001; Raikhel *et al.*, 2002). Considering the modest level of 20E-dependent activation, it is speculated that the AaEcR/AaUSP acts cooperatively with other transcription factors to achieve the dramatically high level of *Vg* gene expression.

Recently, my colleagues and I isolated the mosquito *E74* gene (Sun *et al.*, 2002), which encodes two isoforms, which were named AaE74A and AaE74B (AaE74A and AaE74B profiles are shown in figure 1). The two isoforms have a common C-terminal Ets DNA-binding domain and unique N-termini, suggesting that these two isoforms might be derived through the differential usage of promoters. AaE74A and AaE74B exhibited a high degree of identity to *Drosophila* E74 isoforms A and B, respectively. Both isoforms can bind to an oligonucleotide harboring an E74 consensus motif C/AGGAA. In the fat body and ovary, AaE74B is induced by a blood meal, showing an expression pattern positively correlating with the levels of 20E and the Vg transcript during the vitellogenic cycle. In contrast, AaE74A is activated at the termination stage of vitellogenesis, implying AaE74A and AaE74B isoforms play distinct roles in regulation of *YYP* gene expression during vitellogenesis in mosquitoes.

In this study, I demonstrate that *AaE74* is an ecdysone-inducible early gene. The AaE74 isoforms displayed opposite effects on the *Vg* promoter, in congruence with their proposed roles in vitellogenesis. The functional importance of the E74 binding sites in

the *Vg* promoter has been revealed through mutagenesis studies in transfection assays. Notably, as evaluated by transfection assay, AaE74B acts synergistically on the *Vg* promoter with ecdysone receptor complex, which previously was demonstrated to directly target *Vg* gene during vitellogenesis (Martin *et al.*, 2001). Significantly, gel mobility shift assays have shown that AaE74B and the ecdysone receptor complex co-exist during vitellogenic synthesis stage, permitting their cooperative interaction on the *Vg* promoter *in vivo*. Collectively, these results demonstrate the existence of two AaE74 isoforms with distinct functions in regulation of the fat body gene expression. Significantly, this study supports a novel mechanism by which the high level of expression of a complex gene can be achieved by cross talk between two different transcription factor families.

RESULTS

AaE74 is an ecdysone-inducible early gene

Although the mosquito E74 proteins share significant degree of homology with *Drosophila* E74 in the Ets domain and linker region, it is possible that what we cloned could be another Ets domain protein and not an ecdysone-inducible early gene. To investigate the responsiveness of *E74* to 20E stimulation, we first took advantage of the *in vitro* fat body culture system, which previously has been demonstrated to possess the ability to induce *Vg* gene expression by 20E (Deitsch *et al.*, 1995), mimicking *in vivo* blood meal initiated vitellogenesis.

Fat bodies from previtellogenic female mosquitoes (3-5 days after eclosion), which are competent for 20E response, were incubated in the culture media either in absence of hormone or in presence of increasing concentration of 20E, ranging from 10^{-9}M to 10^{-5}M. After a 4-hour incubation, these fat bodies were collected. The RNA was extracted from the cultured fat bodies and was subjected to quantitative RT-PCR analyses. Both AaE74 isoforms were inducible by 10^{-7}M to 10^{-5}M 20E (figure 3), similar to the potential target gene *VCP*, but not to the structural gene *actin,* which did not exhibit any 20E inducibility. As controls, both AaE74 isoforms showed almost no discernable expression when treated by culture media or ethanol, the 20E solvent. The mRNA level of AaE74B was maxim at approximately 10^{-7}M 20E and had a 50% maximal response at ~10^{-8}M. In contrast, AaE74A reached its peak at about 10^{-6}M 20E and half of maximal induction is ~10^{-7}M.

Next, I examined the time course of the 20E effect on the expression of the AaE74 isoforms in the same culture system. Previtellogenic fat bodies from 3-5 days after eclosion were dissected and incubated under different treatments at 27°C for from 0 to 16 hours, to mimic natural conditions. Every four-hour incubation interval, fat bodies were collected and analyzed as described above. As seen in figure 4A and 4B, both AaE74 isoforms transcripts steadily increase during the time course when treated with 20E (indicated in solid diamond curve). The induction profile is also correlated with that of VCP as shown in figure 4C. Based on the ecdysone hierarchy model, to be qualified for an early gene, it must meet the two criteria: 1) it must be ecdysone-inducible; 2) it should eliminate self-repression and show super-induction. Therefore, I designed a protein synthesis inhibition experiment, in which the fat bodies were treated with 20E and

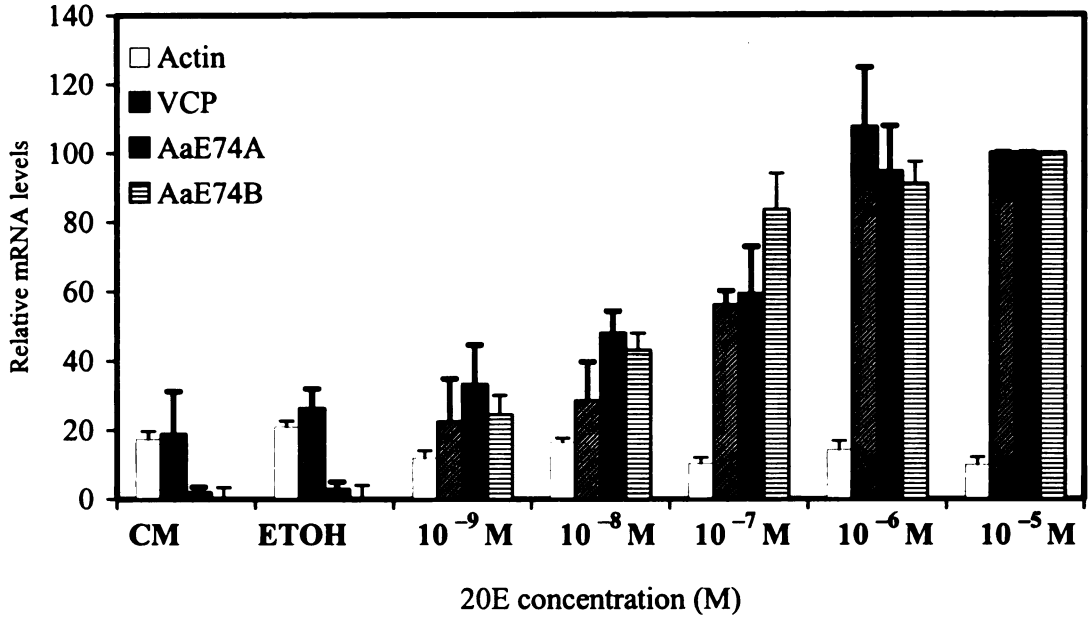
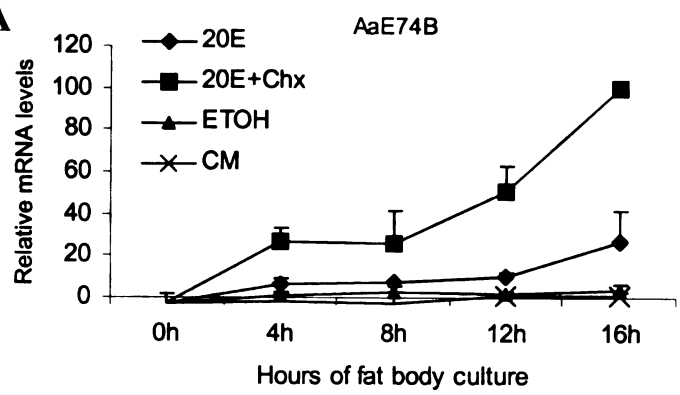
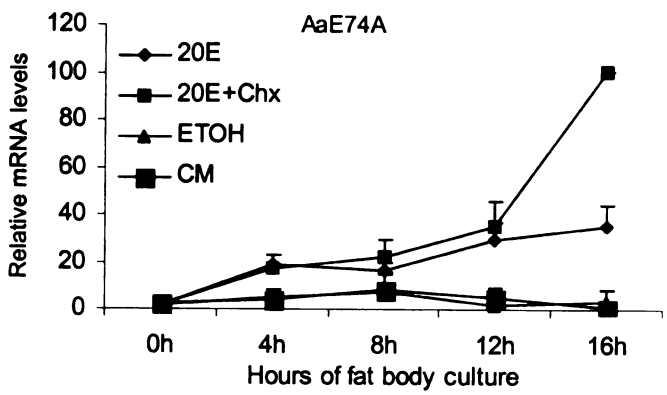


Figure 3. AaE74 dose response to 20-hydroxyecdysone (20E) in the *in vitro* fat body culture. The transcriptional responses of two AaE74 isoforms to 20E were monitored in an *in vitro* fat body culture system as described in Materials and Methods. Total RNA was extracted from previtellogenic mosquitoes 3-5 days after eclosion, which were subjected to several treatments as indicated in the graph. RT-PCR and Southern hybridization were conducted using isoform-specific probes for AaE74A and AaE74B. VCP and Actin were used as positive controls. For AaE74B AaE74A and VCP, data sets were normalized against the amount at 10^{-5}M of 20E. For Actin, data were normalized against the amount at 10^{-5}M of 20E of VCP. Quantitative analysis was performed using phosphoroimager. The results represent the means of three independent experiments ±S.D.

A


AaE74B
20E
20E+Chx
ETOH
CM
Relative mRNA levels
120
100
80
60
40
20
0
0h
4h
8h
12h
16h
Hours of fat body culture

B


AaE74A
20E
20E+Chx
ETOH
CM
Relative mRNA levels
120
100
80
60
40
20
0
0h
4h
8h
12h
16h
Hours of fat body culture

C

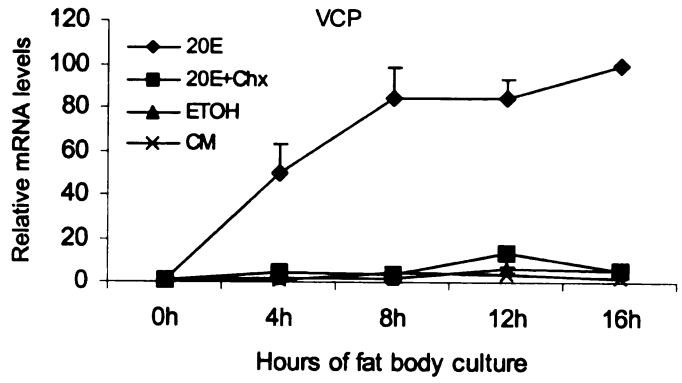

VCP
20E
20E+Chx
ETOH
CM
Relative mRNA levels
120
100
80
60
40
20
0
0h
4h
8h
12h
16h
Hours of fat body culture

Figure 4. Time course of AaE74 induction in the in *vitro* fat body culture. Fat bodies from 3-5 days post eclosion previtellogenic mosquitoes were sterilized and dissected. They were then incubated under various conditions: culture media only (CM, cross curve), ETOH (0.1%ethanol,)(solid triangle curve), 20-hydroxyecdysone (20E) (10^{-6} M, solid diamond curve) and 20E together with 10^{-4} M cyclohexamide (Chx) (solid square curve). A) AaE74B, B) AaE74A, and C) VCP. The results represent the means of independent triplicate ±SD. Total RNA was extracted from cultured fat bodies, RT-PCR/Southern analyses were performed as descried in the Materials and Methods with isoform-specific probes for AaE74A and AaE74B. VCP serves as a positive control.

cyclohexamide (Chx), a protein synthesis inhibitor. As shown in figure 4, both AaE74A and AaE74B were superinduced under this treatment, while VCP expression was suppressed (shown in solid square curve in figure 4 A, B, and C), vividly demonstrating that AaE74 isoforms behave as early gene products. As controls, AaE74A, AaE74B and VCP showed almost no detectable expression in the treatment with either culture media (figure 4, cross curve) or in the media containing ethanol (figure 4, solid triangle curve curve).

Production and characterization of the AaE74 isoform-specific antibody

Two antibodies against the AaE74 isoform-specific region were raised and purified as described in Materials and Methods. To test the two antibodies, Western blotting analysis was carried out with a series dilution of antibodies using either *in vitro* expressed full-length AaE74 proteins or *E. coil* expressed isoform specific regions. As shown in figure 5, AaE74B antibody could specifically recognize the AaE74B proteins from both sources, while the AaE74A could only recognize the bacterial expressed AaE74A protein. The failure of AaE74A to detect the full version of the protein could be due to the fact that the epitope for the antibody is buried inside the protein body and not accessible to the antibody.

Further assessment of the AaE74B antibody was carried out by gel retardation assay using an oligonucleotide harboring consensus E74 binding site as a probe and TnT expressed proteins as protein sources. As shown in figure 6, AaE74B forms a retardation complex with the probe (lane 2), which can only be effectively competed away by

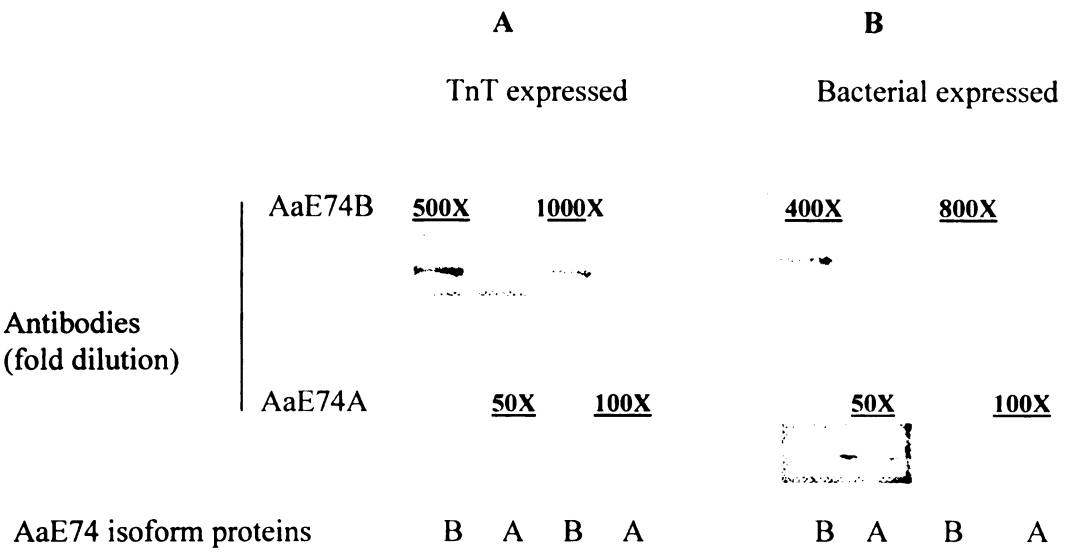


Figure 5. Examination of two antibodies against AaE74 isoforms by Western blotting analysis. TnT expressed full length AaE74A and AaE74B (panel A) and bacterial expressed His tagged AaE74A and AaE74B (panel B) were subjected to Western blotting analysis using peptide-generated antibodies against AaE74A and AaE74B with serial dilutions (the fold dilutions are indicated above each lane).

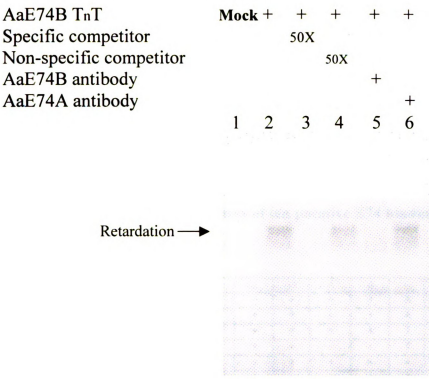


Figure 6. Gel mobility sift assay of AaE74B. GMSA was performed using *in vitro*-transcribed and -translated AaE74B and ^{32}P-labeled oligonucleotide containing consensus E74 binding site as a probe. The AaE74B-specific complex is indicated as an arrow (lane 2). Fifty-fold molar excesses of cold probe (lane 3) were included as specific competitors. The same excess amount of cold double-stranded non-specific oligonucleotide was included in lane 4. The DNA-AaE74B complex was abolished by the AaE74B antibody (lane 5), but not by AaE74A antibody (lane 6).

specific DNA competitor (lane 3) but not by the non-specific DNA competitor (lane 4). The identity of retardation complex was revealed by addition of AaE74B antibody. The complex was abolished by the AaE74B antibody (lane 5), but not by AaE74A antibody (lane 6), which was prepared in parallel, suggesting the AaE74B antibody disrupts the AaE74B DNA binding ability. Examination of AaE74A antibody failed to abolish or supershift the AaE74A-DNA retardation complex (data not shown).

Table 1. Alignment of the sequences of ten putative E74 binding sites to the consensus E74 sequence.

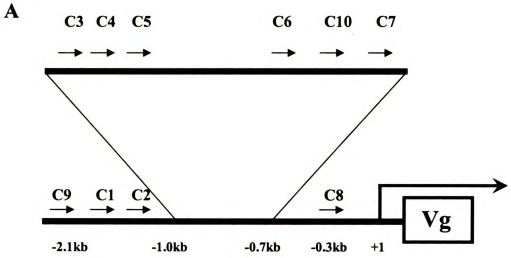
Consensus SeQ	A	A	T/C	C	C/a	G	G	A	A	G	T	fit Number
C1	T	C	G	T	+	+	+	+	+	A	G	5
C2	+	T	+	A	+	+	+	+	+	A	G	7
C3	T	G	+	+	+	+	+	+	+	T	+	8
C4	T	G	+	+	+	+	+	+	+	T	A	7
C5	+	G	+	A	+	+	+	+	+	+	C	8
C6	G	C	+	G	+	+	+	+	+	T	C	6
C7	G	T	+	T	+	+	+	+	+	A	A	6
C8	+	+	+	+	T	+	+	+	+	T	C	8
C9	T	+	+	G	+	+	+	+	+	T	C	7
C10	C	+	+	+	+	+	+	+	+	+	C	9

The symbol "+" indicates match. The consensus sequence is shown in the first row, if the letter case is shown in capital/capital, it indicates that the two nucleotides are equally dominant, if the letter case shown in capital/lowercase, it indicates that the capital case is dominant.

Two AaE74 isoforms bind *in vitro* to the putative E74 response elements located in the median region of the *Vg* promoter

Previously, transgenic assays showed that the proximal regulatory region of *Vg* gene could drive reporter gene expression in a tissue- and stage- specific manner, albeit at very low levels (Kokoza *et al.*, 2001), while the median region is required for hormonal

enhancement of *Vg* expression. A detailed inspection of the nucleotide sequence in the entire 2.1kb Vg regulatory region against E74 consensus sequence: AAT/cCC/aGGAAGT identified by Urness *et al.* (1990) revealed the presence of ten potential response elements, named C1- C10 (illustrated in table 1). The sequences of these response elements are described in Materials and Methods. Their relative locations in the *Vg* promoter are illustrated in figure 7A. Comparing these sequences to the consensus E74 binding sequence (summarized in table 1), C10 displays the highest match 9/11. The decreasing order of match to consensus sequence for the ten binding sites are: C10 (9/11)> C3, C5, C8 (8/11)>C2, C4, C9 (7/11)>C6, C7 (6/11)>C1 (5/11), where the fractions in brackets represent the numbers of nucleotides matching the 11 consensus shown in figure 7B, the C10 and C5, which are located in the median region of *Vg* regulatory region, displayed the strongest competitive ability. C2, C3 and C4 constituted a secondary strongest competitive group, the rest of sites belong to the weakest competitive group. Both isoforms appear to have the same preferences for these putative binding sites (figure 7B and 7C). Therefore, the order of binding ability based on competition assay is: C10, C5 >C2, C3, C4, C9 > C1, C6, C7, C8. In general, the order of binding ability is consistent with nucleotide match order except for C8, which showed weakest binding, although it has a reasonable match of 8/11. Examination of the nucleotide sequence reveals that a position (C/a) immediately before GGAA is important for AaE74 binding, because only C8 violates consensus rule at this position. This is in agreement with previous statistical analysis of consensus sequence by random oligonucleotide selection, which allows no tolerance at this position (Urness *et al.*, 1990). Interestingly, C3, C4, and C5 form a cluster about 50bp apart, designated the E74 cluster


A
C3 C4 C5
C6 C10 C7
C9 C1 C2
C8
Vg
-2.1kb
-1.0kb
-0.7kb
-0.3kb
+1

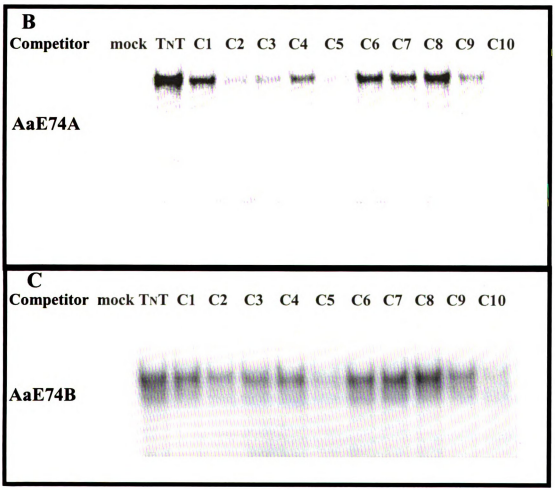

B
Competitor mock TNT C1 C2 C3 C4 C5 C6 C7 C8 C9 C10
AaE74A
C
Competitor mock TNT C1 C2 C3 C4 C5 C6 C7 C8 C9 C10
AaE74B

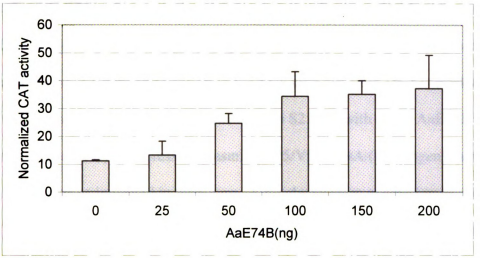
Figure 7. Competition assay in GMSA of the putative E74 binding sites in the *Vg* 5' - regulatory region. (A) Schematic depiction of the location of the ten putative E74 binding sites in the *Vg* 5'-regulatory region. Numbers refer to the nucleotides positions relative to the *Vg* transcription start site. (B and C) Competition assay in GMSA of ten putative E74 binding sites on *Vg* 5' - regulatory region. GMSA was performed using *in vitro*-transcribed and -translated AaE74A (panel B) and AaE74B (panel C), and ^{32}P-labeled oligonucleotide containing consensus E74 binding site as a probe. Fifty-fold molar excesses of the native oligonucleotide harboring each putative E74 binding site for *Vg* promoter was included as a specific competitor.

(from –963bp to –912bp). It is worth noting that both E74 cluster and C10 are located in the median region of *Vg* promoter (figure 7 A).

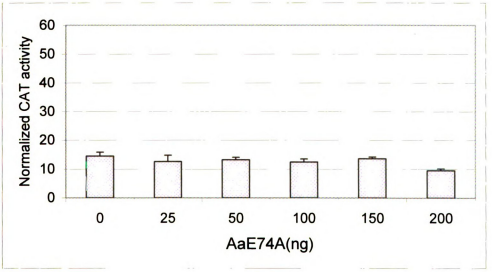
Two AaE74 isoforms exhibit distinct transactivation activity in a *Drosophila* Schneider-2 cell line.

Previous studies have revealed two distinct profiles for AaE74A and AaE74B during vitellogenesis in mosquito, implying they might play divergent roles in the mosquito vitellogenesis, although both AaE74 isoforms displayed similar affinity to the oligonucleotide containing consensus E74 binding site (Sun *et al.*, 2002). To investigate whether AaE74A and AaE74B possess different transactivation activities, transient transfection assays were conducted in the *Drosophila* Schneider-2 cell line (S2). This cell line was transfected with a chimeric reporter gene pAS346(E74-CAT), which contains four copies of consensus E74 binding sites fused upstream to thymidine kinase minimal promoter (tk) driving CAT gene expression. This S2 transfection system has been successfully utilized to identify AaEcR/AaUSP mediated transactivation activity through their corresponding response elements (Wang *et al.*, 1998, Wang *et al.*, 2000, Martin *et al.*, 2001). CAT activities showed as relative activity were normalized against LacZ, which serves as an internal and transfection efficiency control. As becomes clear in figure 8, different scenarios exist. AaE74B can stimulate reporter gene expression up to 3-fold of induction in a dose-dependent manner (figure 8A), as the extent of activation increased with the amount of the exogenous AaE74B added. While, under similar conditions,AaE74A displayed no induction (figure 8B). This difference in activity is

A


Normalized CAT activity
60
50
40
30
20
10
0
0
25
50
100
150
200
AaE74B(ng)

B


Normalized CAT activity
60
50
40
30
20
10
0
0
25
50
100
150
200
AaE74A(ng)

C

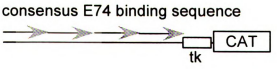

consensus E74 binding sequence
CAT
tk

Figure 8. Two AaE74 isoforms displayed distinct transactivation activities in transfection assays. Oligonucleotides containing four copies of consensus E74 sites were placed upstream of the tkCAT gene (pAS346 (E74-CAT), a kind gift from Dr. Sharrocks. The reporter plasmid was co-transfected into *Drosophila* S2 cells with either AaE74B (panel A) or AaE74A (panel B) in expression plasmid pAc5/V5.1/HisA (Invitrogen), with increasing amounts as indicated in the figure. A β-galactosidase expression vector pAc5.1/V5-His/LacZ (Invitrogen) (0.1 μg) was cotransfected as an internal control, and CAT activity was normalized to β-galactosidase activity. Each data point represents an average of three experiments. The error bars indicate S.D.

consistent with the proposed roles based on their expression profiles during vitellogenesis. Therefore, it is conceivable that the two isoforms may serve different functions on their native promoters. One of the best candidate target genes is *Vg*, which possesses three attractive features: dramatic induction after a blood meal, 20E-inducibility and expression in a precise spatiotemporal fashion.

AaE74B, not AaE74A, can transactivate *Vg* expression

GMSA has revealed two E74 binding portions E74 cluster and C10 in the median region of *Vg* promoter. To determine whether the *Vg* gene is a direct target for AaE74, I utilized the transfection strategy. I started with the Vg^{1071}-Luc reporter construct, which possesses the median region of *Vg* promoter. As shown in figure 9, AaE74B conveyed close to a 3-fold induction of luciferase activity in a dose-dependent manner. In sharp contrast, the reporter was repressed up to 2 fold by the AaE74A protein in a dose-dependent manner. This differs from the previous transfection results using the pAS346 (E74-CAT) reporter construct. Once again, these findings support the roles of the two AaE74 isoforms: AaE74B as a potential activator and AaE74A as a potential repressor of the *Vg* gene.

Characterization of E74 response elements in *Vg* promoter

In order to map the functional E74 binding sites more precisely in *Vg* promoter, I co-transfected the S2 cells with AaE74 and various lengths of the 5' *Vg* regulatory region ligated to the luciferase reporter gene. I utilized four reporter constructs previously used for characterization of EcR response elements (EcRE) on *Vg* promoter: Vg^{2100}-Luc,

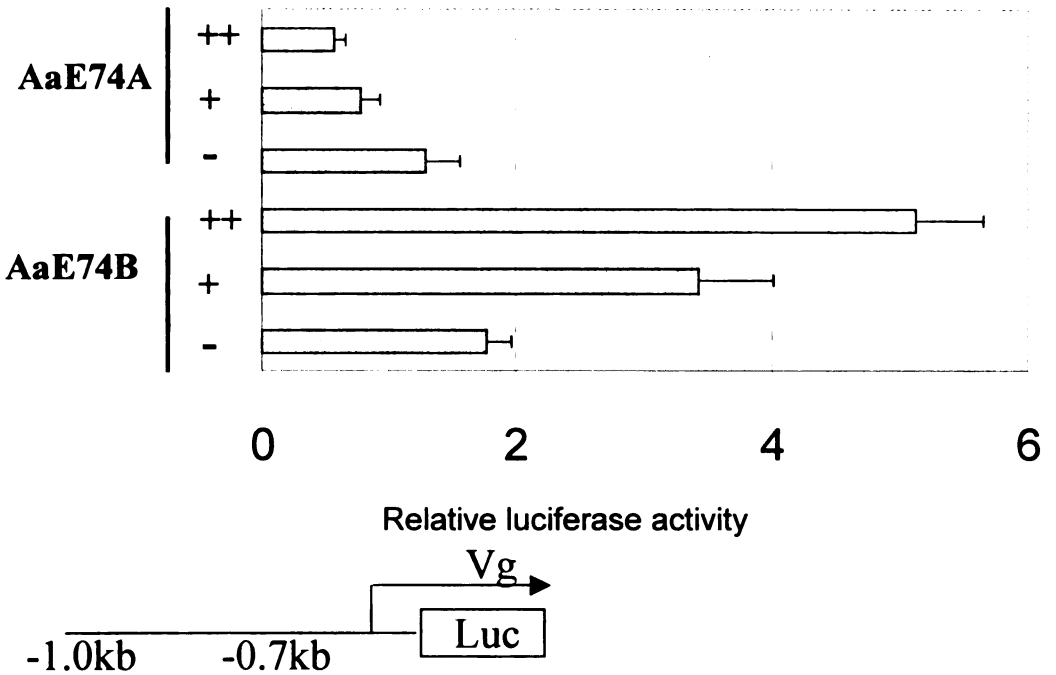


Figure 9. AaE74B, not AaE74A, can transactivate *Vg* 5'- regulatory region. S2 cells were transfected with 100 ng of LacZ co-reporter and 100 ng Vg^{1071}-Luc (containing *Vg* genomic sequences from -2100 to +115 bp). Transfections without AaE74 expression vector are indicated as "-", and those with increasing amounts of AaE74B and AaE74A are indicated as "+" (50 ng) and "++"(100 ng). After transfection, cells were incubated for 2 days at room temperature and harvested for luciferase activity assay. Data represent ratios of *Firefly* luciferase to LacZ activity (Relative luciferase activity) and values are means of duplicate or triplicate independent transfection experiments, with error bars representing the S. D. of the mean.

Vg^{1071}-Luc, Vg^{618}-Luc and Vg^{348}-Luc (Martin *et al.* 2001), each of which contains successive deletion of the 5' *Vg* regulatory region as shown in the diagram in figure 10. The 2.1kb 5' regulatory region of Vg-luciferase reporter gene includes the entire regulatory region from -2100 bp to +115 bp, hence named Vg^{2100}-Luc. The other three constructs were named accordingly. Vg^{1071}-Luc retains median region and proximal region of *Vg* promoter, and Vg^{618}-Luc contains only proximal region. The shortest reporter construct Vg^{348}-Luc includes only partial proximal region but contains the essential ecdysone response elements (EcRE) identified previously (Martin *et al.*, 2001). All constructs include the transcription start site (+1) and TATA box (-30) characterized previously (Romans *et al.*, 1995). In the co-transfection with AaE74B (figure 10), Vg^{2100}-Luc showed modest induction with about 1.5 fold luciferase activity induction, significantly lower than that of the induction of luciferase activity on Vg^{1071}-Luc. This observation suggests that the region between -1071bp and -2100bp in *Vg* promoter may contain response elements that can interact with transcriptional repressors in the S2 cells. In contrast, further deletion construct Vg^{618}-Luc and Vg^{348}-Luc abolished the induction activity, clearly indicating that the region from –618bp to –1071bp of the *Vg* promoter is critical for AaE74B transactivation activity. As a control, the promoterless vehicle (pGL3 basic) alone displayed basal level of luciferase activity.

Next, to determine whether the median region is also required for AaE74A activity, I performed the co-transfection assays with AaE74A (figure 11), the Vg^{2100}-Luc showed slight repression, but much lower than that of Vg^{1071}-Luc. Further deletion construct Vg^{618}-Luc displayed no inhibitory effect, implying that the median region is not only required for AaE74B stimulation of *Vg* promoter, but also for AaE74A repression

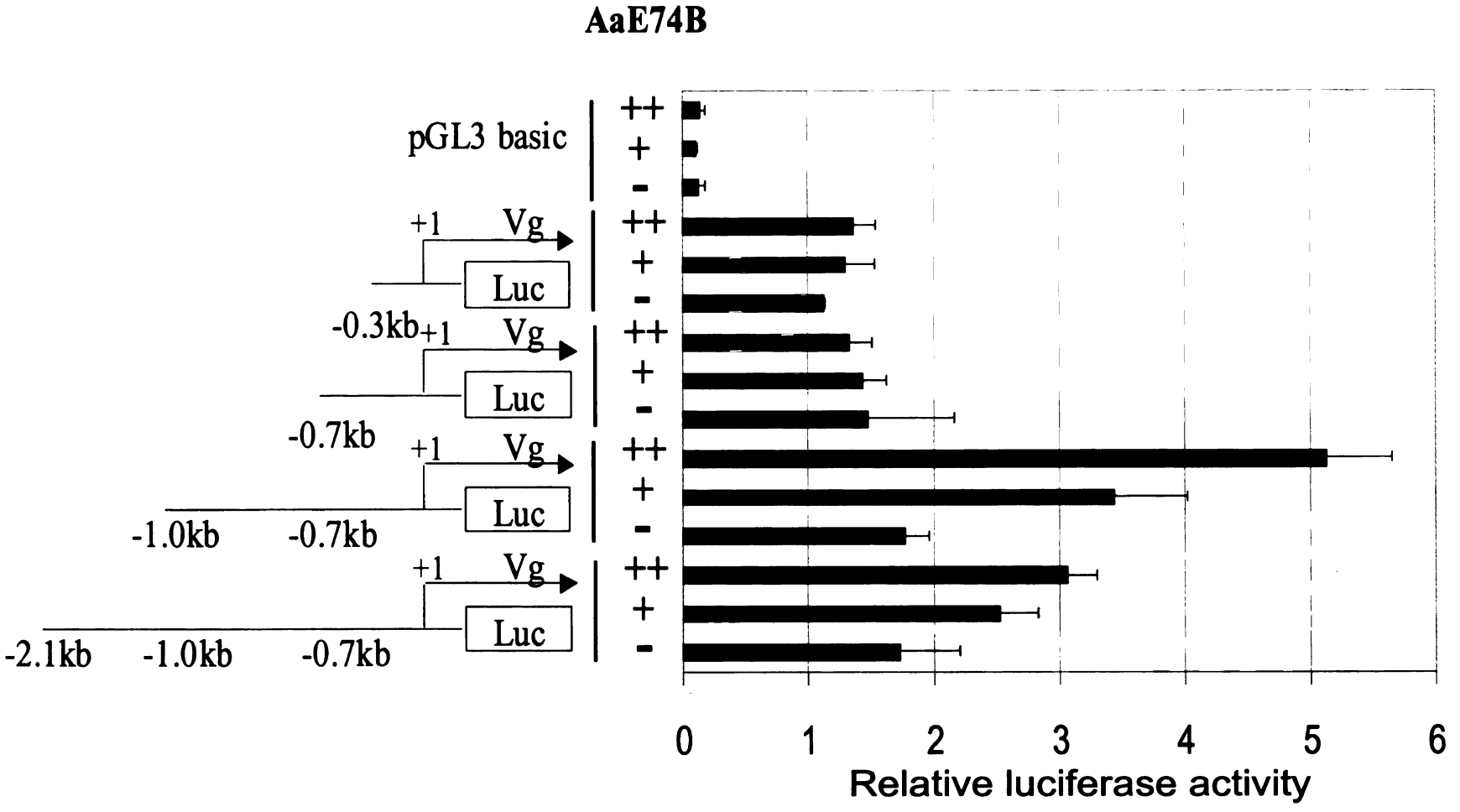

AaE74B
pGL3 basic
++
+
-
+1
Vg
Luc
-0.3kb
-0.7kb
-1.0kb
-2.1kb
0
1
2
3
4
5
6
Relative luciferase activity

Figure 10. Mapping AaE74B binding sites in *Vg* promoter by transfection assay. Activity of a series of 5'deletion mutations of the Vg ^{2100}-Luc were constructed and transfected into S2 cells. A schematic representation of each reporter construct is depicted in the left panel. S2 cells were transfected with 100 ng of LacZ co-reporter, 100 ng of reporter construct. The transfections without the AaE74B expression vector are indicated as "-", and those with increasing amounts of AaE74B are indicated as "+" (50 ng) and "++" (100 ng). Data represent ratios of *Firefly* luciferase to LacZ activity (Relative luciferase activity) and values are means of duplicate of triplicate independent transfection experiments, with error bars representing the S. D. of the mean.

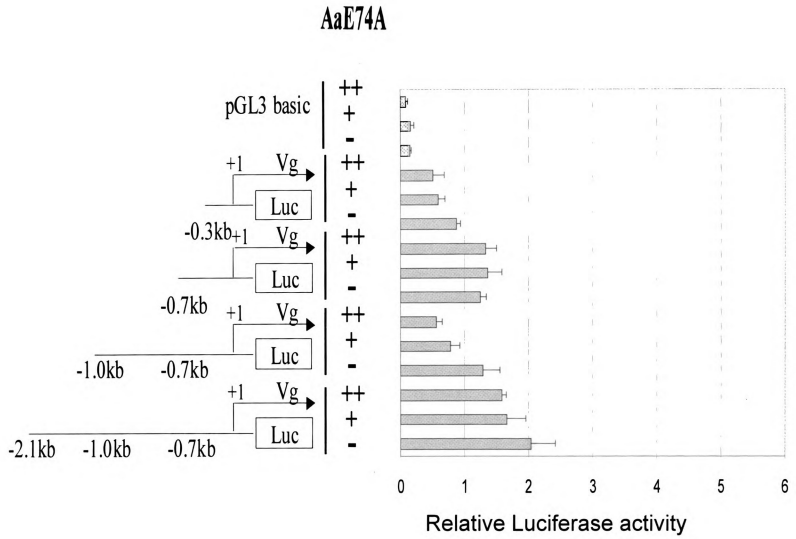

AaE74A
pGL3 basic
++
+
-
+1
Vg
Luc
-0.3kb
-0.7kb
-1.0kb
-2.1kb
0
1
2
3
4
5
6
Relative Luciferase activity

Figure 11. Mapping AaE74A binding sites in *Vg* promoter by transfection assay. Activity of a series of 5'deletion mutations of the Vg ^{2100}-Luc were constructed and transfected into S2 cells. A schematic representation of each reporter construct is depicted in the left panel. S2 cells were transfected with 100 ng of LacZ co-reporter, 100 ng of reporter construct. The transfections without the AaE74A expression vector are indicated as "-", and those with increasing amounts of AaE74A are indicated as "+" (50 ng) and "++" (100 ng). Data represent ratios of *Firefly* luciferase to LacZ activity (Relative luciferase activity) and values are means of duplicate of triplicate independent transfection experiments, with error bars representing the S. D. of the mean.

activity. These findings are congruent with the fact that AaE74A and AaE74B share the same DNA binding domain and they both possess a strong affinity to the putative binding portions (E74 cluster and C10) in the *Vg* promoter. As for the shortest construct Vg^{348}-Luc, slight repression activity was observed, the reason for such a repression is unclear. We found that, in absence of exogenous AaE74 protein, there is a low level of transactivation activity for the four reporter constructs, reflecting the contribution by endogenous Ets proteins in S2 cells.

To further investigate whether AaE74B-mediated activation and AaE74A-mediated repression associate with the putative E74 response elements in the median region of the *Vg* promoter, I made three deletion reporter constructs: $Vg^{1071}\Delta$cluster-Luc, in which E74 cluster was deleted from Vg^{1071}-Luc, $Vg^{1071}\Delta$C10-Luc, in which the singleE74 binding site C10 was deleted from Vg^{1071}-Luc, and $Vg^{1071}\Delta$DB-Luc, in which both E74 cluster and C10 were deleted from Vg^{1071}-Luc. As shown in figure 12, when transfected with AaE74B, both $Vg^{1071}\Delta$cluster-Luc and $Vg^{1071}\Delta$C10-Luc displayed significantly reduced transactivation activities in comparison to the intact construct Vg^{1071}-Luc. The double deletion construct $Vg^{1071}\Delta$DB-Luc showed a completely abrogated transactivation activity, clearly demonstrating that both putative E74 binding portions are required for AaE74B to achieve their effects in *Vg* promoter. Interestingly, in absence of AaE74B, the double deletion construct $Vg^{1071}\Delta$DB-Luc produced an even lower induction activity than the single deletion or intact reporter constructs did. This is probably because of the existence of endogenous *Drosophila* Ets proteins in S2 cells. The

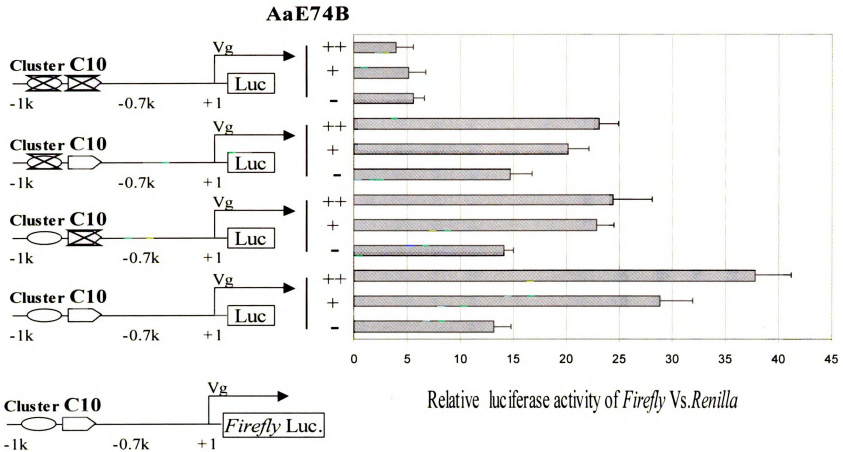

AaE74B
Cluster C10
Vg
Luc
-1k
-0.7k
+1
++
+
-
0
5
10
15
20
25
30
35
40
45
Relative luciferase activity of Firefly Vs.Renilla
Cluster C10
Vg
Firefly Luc.
-1k
-0.7k
+1

Figure 12. The E74 cluster and C10 are required for AaE74B transactivation activity in *Vg* promoter. Mutation of E74 cluster and C10 on the *Vg* promoter obliterates AaE74B induction activity. A schematic diagram of the deletion reporter constructs is depicted in the left panel. S2 cells were transfected with 100 ng pRLCMV, 100 ng expression vector pAc5-AaE74B and 00 ng different reporter plasmids. Data represent the ratios of *firefly* luciferase to *Renilla* luciferase activity (relative luciferase activity *Firefly* Vs. *Renilla*) and values are means of duplicate or triplicate independent transfection experiments, with error bars representing the S. D. of the mean.

two E74 binding portions might work redundantly or cooperatively to activate gene expression to a full extent, or simply because multiple binding sites offer more opportunities for the transcription factor to bind.

In contrast, co-transfection with AaE74A, both single deletion constructs Vg^{1071}Δcluster-Luc or Vg^{1071}ΔC10-Luc showed less repression, but the attenuation of repression was not significant. However, the double mutant construct Vg^{1071}ΔDB-Luc abolished any repression activity (figure 13). Taken together, these observations demonstrated that both the E74 cluster and C10 in the *Vg* promoter are required for AaE74A repression activity as well.

AaE74B protein from fat body nuclear extracts recognize the putative E74 binding sites in *Vg* promoter by gel mobility shift assay

To further establish the relevance of natural AaE74B to activation of the *Vg* gene, I attempted to examine whether the AaE74B from fat body nuclear extracts can bind to the consensus E74 DNA fragment by GMSA.

As shown in figure 14, pre-vitellogenic fat body nuclear extract contained no discernable protein-DNA complex (figure 14, lane 1). After incubation of the probe with nuclear extract from 6 h PBM, a strong retarded band was observed (figure 14, lane 2). The intensity of the AaE74B binding complex declined at 12 h PBM and 24 h PBM (figure 14, lane 3 and 4). The complex disappeared at 36 h and 48 h PBM (figure 14, lane 5 and 6), the termination period when *Vg* gene expression has completely ceased. The identity of the protein-DNA complex was revealed by application of specific antibody.

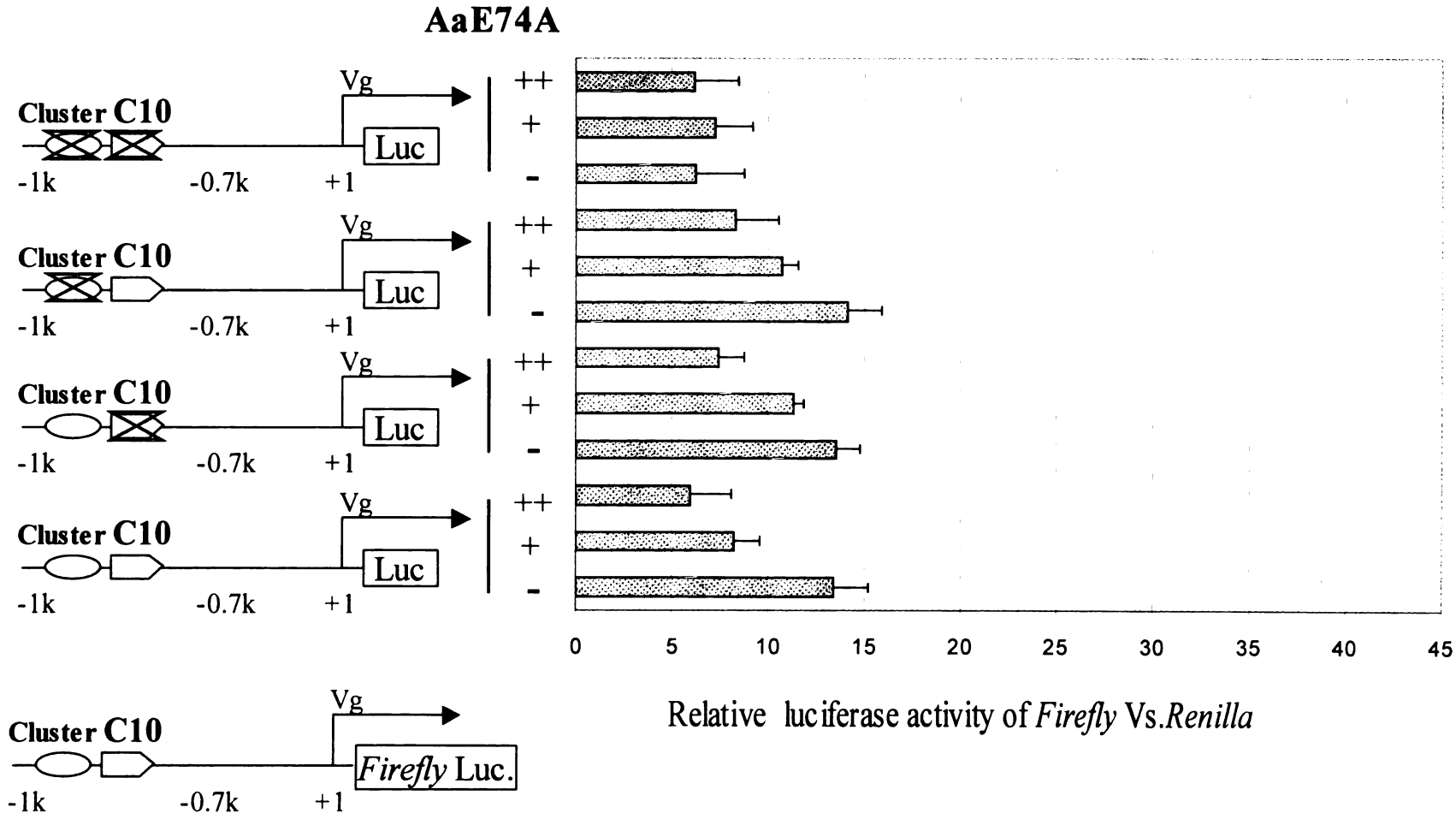

AaE74A
Vg
Cluster C10
Luc
-1k
-0.7k
+1
++
+
-
0
5
10
15
20
25
30
35
40
45
Relative luciferase activity of *Firefly* Vs.*Renilla*
Vg
Cluster C10
Firefly Luc.
-1k
-0.7k
+1

Figure 13. Mutations of E74 cluster and C10 in *Vg* promoter abolish the AaE74A repression activity. A schematic diagram of the deletion reporter constructs is depicted in the left panel. S2 cells were transfected with 100 ng pRLCMV, 100 ng expression vector pAc5-AaE74A and 100 ng different reporter plasmids. Data represent ratios of *Firefly* luciferase to *Renilla* luciferase activity (relative luciferase activity *Firefly* Vs *Renilla*) and values are means of duplicate or triplicate independent transfection experiments, with error bars representing the S. D. of the mean.

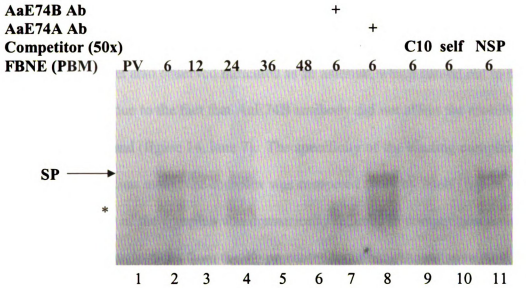


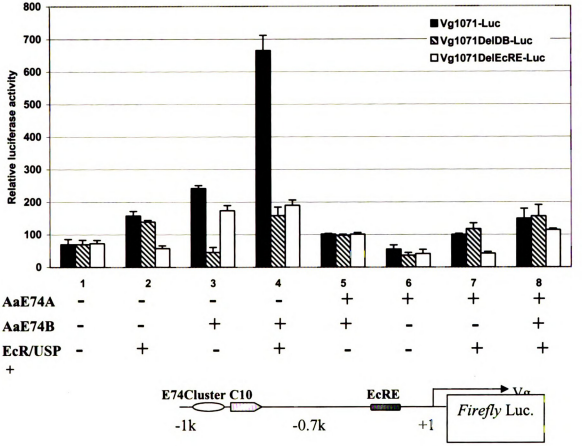
Figure 14. AaE74B binding profile in the fat body nuclei during vitellogenesis. Fat body nuclear extracts fat bodies from the *A. aegypti* previtellogenic period (PV) and at different hours during the vitellogenic period (lane 2-6) were prepared as described by Miura *et al.* (1999). Reactions were performed in a 10 µl volume containing 2–3 µl of nuclear extracts, 25mM Herpes (pH 7.9) 50mM KCl 1mmDTT, 0.1%NP-40, 10% (v/v) Glycerol, 0.5 µg poly(dI-dC)·poly(dI-dC), 0.5 µg single-stranded DNA (5'-TGATCAAGCTTG*TTA*TACAGAAGTAGACC -3'). ^{32}P-labeled oligonucleotide containing consensus E74 binding site was used as a probe. Fifty-fold molar excesses of the oligonucleotide containing each C10 (lane 9), cold probe (lane 10) and non-specific oligonucleotide (NSP) (lane 11) were included as competitors. The identity of the retardation band was determined by AaE74B antibody (lane 7) and AaE74A antibody (lane 8). SP: specific retarded DNA–protein complex. The asterisk indicates a non-specific band.

The complex could only be abolished by anti-AaE74B antibody (figure 14, lane 7), but not by the anti-AaE74A antibody, which was produced in parallel (figure 14, lane 8). One fast migration band was also observed indicated as an asterisk, which turned out to be non-specific binding, due to the fact that AaE74B antibody did not affect the mobility and intensity of this band (figure 14, lane 7). The specificity of the binding complex was confirmed by competition assay: the complex was competed away by itself (figure 14, lane 10). The intensity of the complex was dramatically reduced in competition assays with unlabelled oligonucleotides from the *Vg* promoter containing the putative AaE74 binding site C10 (figure 14, lane 9). Conversely, the non-specific oligonucleotide showed much weaker competition than C10.

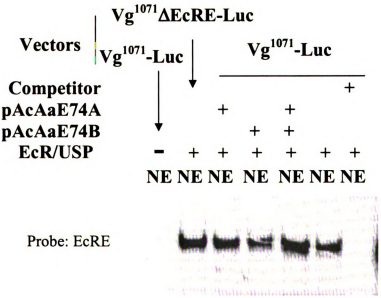
AaE74B acts synergistically with AaEcR/AaUSP in *Vg* promoter

On the 5'-regulatory region of the *Vg* promoter, a functional ecdysone receptor response element (VgEcRE) was previously identified to be necessary to elicit responsiveness to 20E (Martin *et al.*, 2001) (figure 2B). Moreover, the VgEcRE was manifested to directly bind the AaEcR/AaUSP complex from vitellogenic fat body nuclei. Therefore, AaE74B might work together with AaEcR/AaUSP on the *Vg* promoter. This possibility was explored by transient transfection assay. Vg^{1071}-Luc was transfected into S2 cells with expression vectors encoding AaE74B, AaEcR and AaUSP, and the expression efficiency of each expression vectors was determined in figure 15 B and C. Figure 15 A shows that, in agreement with previous report (Martin *et al.*, 2001), AaEcR/AaUSP alone could confer to the Vg^{1071}-Luc reporter construct an approximately 2.5 fold-induction in

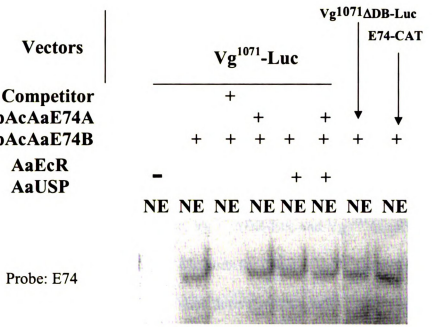
A


Vg1071-Luc
Vg1071DelDB-Luc
Vg1071DelEcRE-Luc
Relative luciferase activity
800
700
600
500
400
300
200
100
0
1 2 3 4 5 6 7 8
AaE74A - - - - + + + +
AaE74B - - + + + - - +
EcR/USP - + - + - - + +
E74Cluster C10
EcRE
Vg
Firefly Luc.
-1k
-0.7k
+1

B


Vg1071ΔEcRE-Luc
Vectors
Vg1071-Luc
Vg1071-Luc
Competitor
pAcAaE74A
pAcAaE74B
EcR/USP
NE NE NE NE NE NE NE
Probe: EcRE

C


Vg1071ΔDB-Luc
E74-CAT
Vectors
Vg1071-Luc
Competitor
pAcAaE74A
pAcAaE74B
AaEcR
AaUSP
NE NE NE NE NE NE NE NE
Probe: E74

D

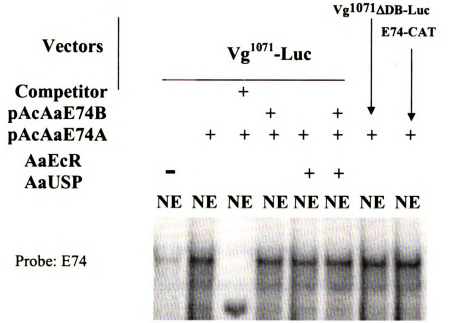

Vg1071ΔDB-Luc
E74-CAT
Vectors
Vg1071-Luc
Competitor
pAcAaE74B
pAcAaE74A
AaEcR
AaUSP
NE NE NE NE NE NE NE NE
Probe: E74

Figure 15 A. AaE74B acts synergistically with AaEcR/AaUSP and AaE74A antagonizes AaE74B in transactivation of *Vg* promoter. A) AaE74A, AaE74B, AaEcR and AaUSP expression vectors (100ng each, wherever the "-"sign is shown, an equivalent amount of empty pAc5.1/V5/His vector was supplemented to ensure the equal amount of total DNA introduced), in the combinations as indicated in the figure, were co-transfected with different reporter constructs: Vg^{1071}-Luc, Vg^{1071}ΔDB-Luc and Vg^{1071}ΔEcRE-Luc, into the *Drosophila* S2 cells (see Materials and Methods for detail). Vg^{1071}-Luc denotes Vg^{-1071}-Luc, Vg^{1071} ΔDB-Luc denotes a Vg^{-1071}-Luc derivative that lacks the E74 cluster and C10, and Vg^{1071}ΔEcRE-Luc denotes a Vg^{1071}-Luc derivative that lacks functional EcRE identified previously (Martin *et al.*, 2001). Data represent ratios of *Firefly* luciferase to *Renilla* luciferase activity (Relative luciferase activity) and values are shown from duplicate or triplicate independent transfection experiments. The bottom diagram is the schematic illustration of the luciferase reporter construct Vg^{1071}-Luc with the numbers indicating the relative position to the *Vg* transcriptional start site. B), C) and D The determination of expression efficiency of each expression vectors in S2 cells were performed in 6-well culture plate, cultured in the same way as that in the 24-well plate culture except that all the components increase proportionally. Nuclear extracts from the cells were prepared according to the protocol as described by Miura *et al.* (1999). GMSA were performed as described in Materials and Methods. NE: nuclear extract from the S2 cells. Fifty-fold molar excesses of cold probe were included as specific competitors. The ^{32}P-labeled oligonucleotide probes used in the GMSA are DR-1 in panel B (Wang *et al.*, 1998) and E74 consensus in panels C and D (used in figure 6)

response to 20E (figure 15 A, lane 2, solid bar). Remarkably, AaE74B, which by itself caused only up to 3 fold induction (figure 15 A, lane 3, solid bar), had a synergistic effect with AaEcR/AaUSP (figure 15 A, lane 4, solid bar). The synergistic effect was further enhanced upon incubation with increasing concentrations of AaE74B and AaEcR/AaUSP (data not shown).

Therefore, although AaE74B alone induced low transcriptional activation, it is capable of a functional interaction with AaEcR/AaUSP and potentiating *Vg* transcriptional enhancement.

Since AaE74B can binds *in vitro* to the E74 cluster and C10 located in median region *Vg* promoter, it is likely that the synergistic stimulation of *Vg* gene transcription by AaE74B is mediated via its binding to these binding sites. Meanwhile, the proximal region of the *Vg* promoter contains the sequences responsible for AaEcR/AaUSP responsiveness to 20E. To evaluate whether the AaE74 binding sites (E74 cluster and C10) and VgEcRE contribute to the synergistic activation, transfection assays were performed on the double deletion construct $Vg^{1071}\Delta$DB-Luc and VgEcRE mutation construct $Vg^{1071}\Delta$EcRE-Luc (see Materials and Methods). As shown in figure 15 A striped bars, AaEcR/AaUSP alone induced a 2-fold increase in the $Vg^{1071}\Delta$DB-Luc reporter construct (figure 15 A, lane 2, striped bar), indicating the AaEcR/AaUSP induction does not require the intact E74 binding sites. While the E74 cluster and C10 are essential for the AaE74B activation of the construct Vg^{1071}-Luc construct, because AaE74B alone did not elicit any induction in the $Vg^{1071}\Delta$DB-Luc reporter construct (figure 15 A, lane 3, striped bar), consistent with previous results (figure 10). In the presence of AaE74B, AaEcR/AaUSP displayed no further induction on $Vg^{1071}\Delta$DB-Luc

construct (figure 15 A, lane 4, striped bar), demonstrating that the C10 and E74 cluster in the median region of the *Vg* promoter are required for AaE74B synergistic action with AaEcR/AaUSP.

As for the Vg^{1071} ΔEcRE-Luc construct (open bars), the induction activity was almost retained when transfected with AaE74B alone, in comparison to the Vg^{1071}-Luc (figure 15 A, lane 3, open bar), but the promoter activity was abrogated when transfected with AaEcR/AaUSP alone (figure 15 A, lane 2, open bar). This is reminiscent to the response by the construct Vg^{348} ΔEcRE-Luc, in which the VgEcRE was removed from the shorter *Vg* promoter reporter construct Vg^{348}–Luc (Martin *et al.*, 2001). The Vg^{1071} ΔEcRE-Luc construct displayed no the synergistic activity when transfected together with AaE74B and AaEcR/AaUSP (figure 15 A, lane 4, open bar). It was evident from these studies, therefore, that the VgEcRE in the *Vg* promoter is also essential for the synergistic activation by AaE74B and AaEcR/AaUSP.

AaE74A antagonizes AaE74B in transactivation of *Vg* promoter

Since the AaE74A repressed promoter activity in the Vg^{1071}-Luc construct, I attempted to examine the influence of AaE74A on these deletion constructs when cotransfection with or without AaE74B and AaEcR/AaUSP, the expression efficiency of each expression vectors were determined in figure 15 B and D. Figure 15 A showed that when transfected with AaE74A alone, the deletion constructs Vg^{1071}ΔEcRE-Luc exhibited repressed activity (figure 15 A, lane 6, open bar). Similar repression activity was obtained with Vg^{1071}-Luc (figure 15 A, lane 6, solid bar), indicating that the VgEcRE is dispensable for AaE74A repression activity. In agreement with previous observations

(figure 13), the Vg^{1071}ΔDB-Luc showed reduced promoter activity (figure 15 A, lane 6, striped bar). Intriguingly, when AaE74A was cotransfected with AaE74B, Vg^{1071}-Luc and Vg^{1071}ΔEcRE-Luc showed decreased induction activity in comparison to that transfected with AaE74B alone (figure 15 A, lane 3, solid bar and open bar), demonstrating that AaE74A can antagonize the induction activity of AaE74B on *Vg* promoter.

Interestingly, when AaE74A was cotransfected with AaEcR/AaUSP, Vg^{1071}ΔDB-Luc showed marginal induction activity, in congruence with previous results of the requirement of C10 and E74 cluster in median region of Vg promoter for AaE74A repression activity. This further demonstrates that AaE74A also antagonizes the synergistic activation of *Vg* expression by AaE74B. Whereas the construct Vg^{1071}-Luc was slightly repressed in comparison to the results obtained with AaEcR/AaUSP alone (figure 15 A, lane 7, solid bar). This implies that AaE74A also antagonizes the AaEcR/AaUSP activation of the *Vg* promoter. As for Vg^{1071}ΔEcRE-Luc, the reporter gene expression was repressed at a similar level to that transfected with AaE74A alone (figure 15 A, lane 7, open bar), supporting the notion of AaE74A repression activity on the *Vg* promoter. Remarkably, when introducing AaE74A, AaE74B and AaEcR/AaUSP into S2 cells, Vg^{1071}-Luc exhibited no synergistic activity (figure 15 A, lane 8, solid bar), whereas Vg^{1071}ΔDB-Luc displayed comparable induction activity to that when cotransfection with AaE74B and AaEcR/AaUSP (figure 15 A, lane 4, stripped bar) and AaEcR/AaUSP alone (figure 15 A, lane 2, stripped bar), indicating the induction activity was solely a result of AaEcR/AaUSP. However, Vg^{1071}ΔEcRE-Luc displayed marginally weak induction activity (figure 15 A, lane 8, open bar), comparable to the cotransfection

of AaE74A and AaE74B (figure 15 A, lane 5, open bar), but less than that when cotransfected by AaE74B and AaEcR/AaUSP (figure 15 A, lane 4, open bar).

DISCUSSION

Previously, I cloned the mosquito *E74* gene, which encodes two isoforms AaE74A and AaE74B, probably from alternative splicing products, and demonstrated that both isoforms can bind to the oligonucleotide containing consensus E74 binding sites. The two AaE74 isoforms displayed distinct profiles during vitellogenesis in the mosquito, in which AaE74B was induced by a blood meal exhibiting its highest-level coinciding with the peak of vitellogenesis. In contrast, the transcript of AaE74A was activated during the termination stage of vitellogenesis. In this study, I presented evidence from the *in vitro* fat body culture, administrated with 20E and cyclohexamide, to demonstrate that the AaE74 isoforms are the early gene products (figure 4). Preliminary studies showed that the two AaE74 isoforms displayed different responses to 20E with AaE74B being more sensitive than AaE74A (figure 3). This scenario is reminiscent of the two *Drosophila* E74 isoforms, in which E74B can be activated at a much lower 20E concentration than that of E74A (Karim *et al.*, 1991). The temporal difference may reflect the difference in sensitivities of two E74 isoforms to 20E, representing an elegant ecdysone-induced E74 switch during metamorphosis in *Drosophila melanogaster* (Thummel, *et al.*, 1990). Likewise, the difference of the temporal profile and biological functions of the two AaE74 isoforms may reflect their difference in the sensitivity.

Utilizing a cell transfection strategy, as employed to identify the ecdysone receptor response elements in *Vg* gene (Martin *et al.*, 2001), I demonstrated that AaE74B activation and AaE74A repression activity were localized to the median region of the *Vg* promoter. Detailed mapping and gel retardation assay revealed two strong E74 binding site portions from the median region of the *Vg* promoter, C10, the strongest binding site, and E74 cluster, which consists of three strong E74 binding sites (figure 7). The functionality of the E74 binding site C10 was further supported in GMSA, using the AaE74B protein from fat body nuclear extracts by the specific competition of these response elements against the probe containing consensus E74 binding sites (figure 14).

The AaE74B DNA binding profile was obtained by GMSA (figure 14, lane 9). AaE74B binding begins immediately after blood meal, reaches its peak at 6 h PBM, gradually declines to 24 h PBM, and terminates at 36 h PBM. The discrepancy between the AaE74B binding profile and the mRNA expression profile may indicate that AaE74B DNA binding activity is controlled at post-transcriptional levels. This could be the result of multiple levels of regulation. The availability of the AaE74B protein could be controlled at the translational level in a similar manner to that of the mosquito *βFTZ-f1* gene (reviewed in Raikhel *et al.*, 2002). Another possibility is that AaE74B is being regulated at the level of translocation of the protein to the nucleus. A third possibility is that E74B is being post translationally modified in a way that it regulates its DNA binding activity. The final scenario resembles the case of the Ets protein Elk1, in which multiple residues in the C-domain of Elk1 are phosphorylated, leading to modified DNA binding and transactivation (Price *et al.*, 1995; Cruzalegui *et al.*, 1999). This AaE74B binding peak precedes those of hemolymph 20E levels and *Vg* gene expression during

vitellogenesis, supporting its role as an ecdysone-inducible early gene, and more importantly, reinforcing the notion that AaE74B, as a positive regulator, directly target *Vg* gene expression.

The levels of induction obtained in the cell transfection experiments (2 to 3-fold activation) correspond to the levels of induction observed when the native promoters were introduced into *Drosophila* S2 cells (Martin *et al*, 2001; Antoniewski *et al.*, 1996). Such a modest activation is far from the physiological situation in the mosquito where a more than 100-fold increase of the Vg mRNA accumulation has been observed (Cho *et al.*, 1991). Furthermore, the appearance of Vg mRNA in fat body shortly after a blood meal suggests that the control of Vg synthesis is at the stage of transcription rather than of translation. This dramatic level of *in vivo* activation of the mosquito *Vg* gene is likely a synergistic contribution of multiple hormonal-, tissue- and/or stage-specific factors. Indeed, the mosquito *Vg* 5'-region contains putative sites for several tissue-specific factors as forkhead, C/EBP and GATA, as well as sites for ecdysone response elements and several other early ecdysteroid-response factors, such as the Broad-Complex (BC) and E75 (Raikhel *et al.*, 2002; Li, unpublished data).

Our current understanding of the molecular mechanism of 20E action, called the ecdysone hierarchy model, is largely based on the puffing pattern studies from polytene chromosome in salivary gland and genetic analyses of the transcription factors involved in the 20E signaling pathway in *Drosophila.* According to this model, the hormone as a ligand binds to the ecdysone receptor complex, which directly induces a small set of ecdysone-inducible early genes. These genes encode transcription factors that can both repress their own expression and directly activate late genes. Whether the ecdysone

receptor complex is involved in the direct regulation of late genes or not was unclear. In the present work, using the activation of mosquito *Vg* promoter as a model, I demonstrated the existence of a novel mechanism of induction by synergistic interaction between an early gene, E74 and the ecdysone receptor complex.

The functional AaE74B binding profile overlaps with the AaEcR/AaUSP binding profile detected using the native VgEcRE from the *Vg* promoter (Mirua *et al.*, 1999; Martin *et al.*, 2001), providing an opportunity for the interaction between AaE74B and AaEcR/AaUSP complex. It has been shown that the functional AaEcR/AaUSP complex only forms after a blood meal, presumably through a derepression mechanism associated with 20E titer alteration. In previtellogenic period, the ecdysone receptor is repressed by the formation of an alternative heterodimer consisting of AaEcR and AHR38. The blood meal-triggered elevated 20E titers disrupt the repressed form of ecdysone receptor complex and allow the formation of AaEcR/AaUSP complex (Zhu *et al.*, 2000). Significantly, Zhu *et al* (2003) have demonstrated that the *Vg* promoter was occupied *in vivo* by both AaEcR and AaUSP in fat body during early vitellogenic period (3-12 h PBM) using the chromatin immunoprecipitation assay (ChIP). A functional ecdysone response element was identified in the proximal region of the *Vg* promoter and has been demonstrated to be required for the basal level of expression of the *Vg* gene (Kokoza *et al.*, 2001). The *Vg* expression is thought to be further enhanced by more distant enhancer elements (Kokoza *et al.*, 2001). Significantly, multiple functional AaE74 binding sites (the E74 cluster and C10) were identified in the median region in this study. These observations implicated that AaE74B may act cooperatively with AaEcR/AaUSP in the *Vg* promoter. Combinatorial control is a characteristic property of Ets family members,

involving interaction with other key transcription factors such as GATA and nuclear receptors. Such interactions coordinate many cellular processes, leading to either synergistic transactivation of the promoter (Tolon *et al.*, 2000) or reciprocally repression (Darby *et al.*, 1997).

Indeed, I demonstrated that AaE74B and AaEcR/AaUSP exerted a synergistic effect on the *Vg* promoter as assessed by cell transfection assays. A working model of synergistic action of AaE74B and AaEcR/AaUSP on *Vg* promoter during vitellogenesis in mosquito *A. aegypti* is summarized in figure 16. The synergistic action required the existence of several E74 binding sites in the median region and ecdysone response element in the proximal region of *Vg* promoter, as mutation of them obliterated the synergistic activity. The synergistic activation is reminiscent of the expression of Cyp24, an enzyme necessary for the metabolism of vitamin D, which is induced via two vitamin D response elements (VDREs) (Dwivedi *et al.*, 2000). An adjacent RAS-dependent Ets-binding site is required for maximal expression. Instead, a ternary complex between the vitamin D receptor, retinoid X receptor and Ets1 results in the synergistic activation of the Cyp24 promoter and requires RAS-dependent phosphorylation of Ets1 for maximal transactivation (Dwivedi *et al.*, 2000).

The cooperative activity may require the direct physical interaction as demonstrated in the mammalian *prolactin* promoter. Like the complex system of the *Vg* promoter, *prolactin* promoter consists of multiple Ets binding sites and hormone response elements. The association of Ets-1 with nuclear receptor confers to the nuclear receptors new properties: ligand-independent and AF2-independent activation via conformational change. Intriguingly, there is a direct interaction of the nuclear receptors with Ets-1,

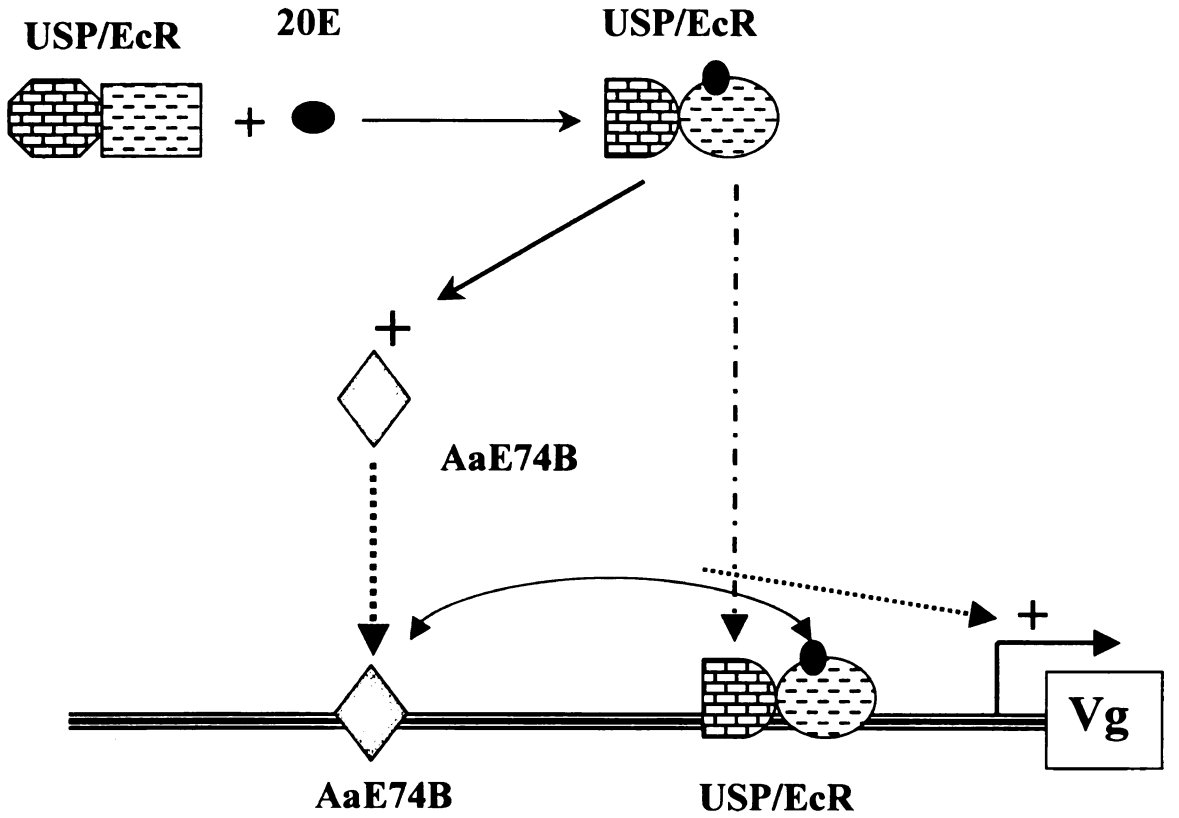


Figure 16. Working model of synergistic action of AaE74B and AaEcR/AaUSP on *Vg* promoter during vitellogenesis in mosquito *A. aegypti*. Direct and indirect activation of yolk protein precursor gene *Vg* is controlled by 20E signaling pathway in the mosquito fat body. After binding 20E, the AaEcR/AaUSP heterodimer undergoes conformational change, resulting in activation of the early gene *E74*. The product AaE74B acts synergistically with the AaEcR/AaUSP complex on the *Vg* promoter (modified from Raikhel *et al.*, 2002).

which requires the highly conserved DNA binding domains (DBD) of both Ets-1 and nuclear receptors (Tolon *et al.*, 2000). Therefore it is likely that the AaE74B and/or AaE74A interact with AaEcR or AaUSP via their corresponding DBD. Further studies using GMSA or coimmunoprecipitation (Co-IP) assay will confirm their direct physical interaction.

Our transfection experiments demonstrate that AaE74B, but not AaE74A, can positively regulate the Vg promoter. Since, like AaE74B, AaE74A can specifically recognize the E74 cluster and C10 in the Vg promoter, AaE74A may function as a dominant-negative regulator of Vg gene expression during the termination stage of vitellogenesis. We believe this because the presence of AaE74A abolished the synergistic activation activity of AaE47B and AaEcR/AaUSP on *Vg* promoter. However, the absence of binding activity of AaE74A during the termination stage of vitellogenesis is unexpected. It is possible that AaE74A DNA binding ability is inhibited but AaE74A is still able to exert a repressive function by interacting with other transcription factors such AaEcR/AaUSP or other cofactors. Further western blotting analyses and RNA interference experiments on AaE74A will help to clarify its role in vitellogenesis.

AaE74A and AaE74B have opposing effects upon the regulation of the *Vg* promoter. A similar scenario has been observed in the R7 retinal cells of *Drosophila*, where two Ets factors, Pointed and Yan, target the same genes in the same cell, but with opposing functions (Lai and Rubin, 1992; Brunner *et al.*, 1994; O'Neill *et al.*, 1994). The same transcriptional switch was also observed in *Drosophila* E74, by which two E74 isoforms also have been demonstrated to regulate distinct sets of late target genes

depending on the different titers of 20E available or displayed opposite effect on the same target gene (Fletcher *et al.*, 1995).

The distinct functions of AaE74A and AaE74B may be due to unique posttranslational modifications such as phosphorylation, differences in DNA binding, and differences in protein–protein interactions with transcription factors or co-activators/co-repressors interacting with other regulatory elements. The repression activity of AaE74A, which is transcribed only during the termination stage of vitellogenesis in the fat body, offers a termination mechanism of vitellogenesis, which is critical for the completion of the cyclicity of mosquito reproduction. Similar to the requirements of multiple factors to activate the *Vg* promoter, the termination of the *Vg* promoter may also involve several factors. Recently, Zhu *et al.* (2003) has demonstrated a novel mechanism involving competition of AaSVP with AaEcR for AaUSP, therefore preventing the formation of a functional AaEcR/AaUSP complex during the termination stage of vitellogenesis due to the alteration of 20E titers. Here I propose another mechanism by which functional AaEcR/AaUSP complex is repressesed via physical interaction with AaE74A, presumably through a mediator, resulting in the dissociation of AaEcR/AaUSP from the *Vg* promoter as the *Vg* promoter was demonstrated to be unoccupied by AaEcR/AaUSP *in vivo* (Zhu *et al.*, 2003).

Taken together, functional AaE74 binding sites were localized to the median region of the *Vg* promoter. These putative binding sites serve as a platform for AaE74B and AaE74A, which have opposite functions on the *Vg* promoter. This balance of positive and negative roles of the two AaE74 isoforms provides an elegant mechanism for controlling both the rise and subsequent rapid decrease in activity required for tightly

controlling *Vg* gene expression. Significantly, the data reveal the existence of a novel 20E working mechanism, by which ecdysone-inducible early gene product AaE74B activates synergistically, with ecdysone receptor complex, the complex regulatory program of the *Vg* gene expression during vitellogenesis in the fat body of the mosquito *A. aegypti*.

Chapter V Summary and Future Research Prospects

The hallmark of the vitellogenin (Vg) promoter in the mosquito *Aedes aegypti* is a sex-specific and precise spatiotemporal synchronized expression induced by the female mosquito ingestion of a blood meal, which offers an elegant model for studying ecdysteriod regulation of gene expression at the transcriptional level. Though many aspects of the hormone regulation are deduced based on the conservation between *Drosophila* metamorphosis and mosquito vitellogenesis, due to lacking of the robust genetic techniques and resources available in the model insect *Drosophila*, understanding the mechanisms governing the blood meal-triggered ecdysteriod cascade in mosquito *A. aegypti* at the molecular level is essentially poor.

In this dissertation, I have presented an intensive investigation of the molecular basis of ecdysone signaling pathway in the vitellogenesis of the mosquito, with emphasis on the ecdysone-inducible early genes, which is believed to directly regulate the late target gene expression. Cloning and characterization of the mosquito ecdysone-inducible early gene *E74* have demonstrated its key roles in the initiation and termination of vitellogenesis. Two AaE74 isoforms are proposed to serve opposite functions during vitellogenesis: AaE74B as an activator and AaE74A as a repressor. Remarkably, a novel mechanism involving AaE74B synergistic action with ecdysone receptor complex AaEcR-AaUSP on Vg promoter during vitellogenesis was proposed as an extension for the ecdysone hierarchy model. In the future research, I propose to continue in focusing on the investigation of the functions of the two AaE74 isoforms: what and how they play in regulation of Vg expression, and how AaE74 gene(s) expression is regulated in the following aspects:

Investigation of the possible direct interaction between AaE74 protein and AaEcR-AaUSP heterodimer

Protein–protein interactions regulate DNA binding, subcellular localization, target gene selection and transcriptional activity of Ets proteins. Combinatorial control is a characteristic property of Ets family members, involving interaction between Ets and other key transcriptional factors such as nuclear receptors and GATA factors (Li, *et al.*, 2000; Sharrocks, 2001). One example of co-dependence between Ets and other transcription factors is the synergistic binding and cooperative activation in prolactin promoter via Ets-1 and nuclear receptors: vitamin D receptor (VDR), estrogen receptor (ER) and peroxisome proliferator-activated receptorα (PPARα), via the DNA binding domains of both proteins (Tolon *et al.*, 2000), which is highly conserved within the two transcription factor family. However, the direct physical interaction may not extended to all nuclear receptors, for example, ETS-1 does not associated with the retinoid X receptor (RXR) (Tolon *et al.*, 2000). Unique combinations of protein–protein interactions are likely to direct different Ets factors to regulate the expression of specific target genes. It is this precise assembly of multiple transcription factors onto a chromatin template that enhances transcriptional specificity and defines activation or repression function.

In the previous chapters, AaE74B and AaEcR-AaUSP were demonstrated to synergistically stimulate the Vg promoter activity. In order to investigate whether the synergistic action is caused by direct physical protein-protein interaction, direct GST-pull down experiments will be designed using *in vitro* expressed proteins. Further evaluation of the interaction under the condition of DNA binding can be obtained from gel mobility shift assays (GMSA) using oligonucleotide sequence of a probe containing either E74

binding sites or nuclear receptor response elements. Incubation of AaE74B and AaEcR-AaUSP proteins will result in super-retardation of protein-DNA complex if there is direct interaction. Addition of the insect hormone 20E into the reaction will permit the determination of the involvement and requirement of the hormone.

Further identity of the complex can be assessed by application of corresponding antibodies. If the direct interaction is observed, detailed characterization of the domains of both proteins involved in the interaction can be achieved by the GST-pull down experiment using various deleted versions of each protein expressed *in vitro*. Finally, the importance of the domains, if they directly interact with each other, can be evaluated in the transfection assay using the protein containing mutated domains, in comparison to those using the intact proteins.

The ability of individual Ets factors to function as activators or repressors is also dependent upon promoter and cell context. Likewise, it is worth exploring the possibility of interaction between AaEcR-AaUSP and AaE74A, which was demonstrated to repress Vg promoter activity.

Study of the AaE74 function by gene disruption using RNAi.

Recently Fire and colleagues demonstrated that injection of double-stranded RNA (dsRNA) corresponding to the coding region of a particular gene would effectively and specifically interfere with the ability of a gene in *Caneohabdtis elegens* (Fire *et al.*, 1998). This method of RNA interference (RNAi) has provided a powerful new tool for functional genetic studies in a wide range of model organisms including trypanosomes, zebra fish, fruit flies and mice (Hunter, 1999). In *Drosophila*, dsRNA injection in

embryos is widely used for assessing gene function in early development. However, in adult flies RNA interference is routinely mediated by the expression of hairpin dsRNA in transgenic strains (St Johnston, 2002). Recently Blandin and his colleagues (2002) have reported successfully injecting dsRNA into the adults mosquito *Anopheles gambiae* and observed efficient and reproducible silencing of the mosquito antimicrobial peptide gene *Defensin*.

The same strategy will be adopted for study in the functions of AaE74 during vitellogenesis. The AaE74 RNAi disruption experiment will consist of three steps as below, mainly following the procedure as described (Blandin *et al.*, 2002). First, each AaE74 isoforms specific coding region will be subcloned in the plasmid LITMUS (New England Biolabs), which possesses two T7 promoters. Therefore, the sense and antisense RNAs can be synthesized and annealed in one step *in vitro* using Ambion T7 kit. A nano-injector (Nanoject, Drummond) will be used to introduce the dsRNAs into the thorax of CO_2-anesthetized mosquito females, and then allowed to recover for 4 days.

In comparison to the approach in which dsRNA are introduced into mosquito genome via germ-line transgenesis, this system of direct microinjection of dsRNA into the body of adult mosquito possesses two obvious merits: 1) it is rapid and direct; 2) timing of gene-knockout is maneuverable, which, otherwise, maybe detrimental or lethal to during the embryogenesis or later stage development in the case of E74 and adult mosquitoes survival rate will be extremely low.

After the dsRNA injection, I will focus on the studies of mutant effect on the major transcriptional regulators involved in the20E cascade and their effectors in vitellogenesis, which have been well documented in Dr. Raikhel's lab, such as EcR/USP,

competent factor βFTZ-F1, early gene AaE75, early later gene AaHR3 and the potential later genes yolk protein precursors. To investigate the null mutant effect, the *in vitro* fat body culture system and the Real time PCR method will be used to determine the expression. The abnormal phenotype of the mutant strains will also be carefully examined.

Further analyses of the functions of AaE74 can be performed in different genetic background. At least two transgenic lines have been generated in Dr. Raikhel's lab, which contain Vg entire -2.1 kb regulatory region and Vg-1071bp correspondent to the Vg ^{1071}-Luc (see Materials and Methods) fused to defensin gene (Kokoza *et al.*, 2000). The defensins gene can be used as a reporter gene, in parallel to the endogenous Vg gene. Although the positions in the chromosome where the transposons of the two transgenic mosquito strains integrated into have not been identified, introduction of AaE74 two isoforms via RNAi into these two mosquito strains will presumably produce some interesting results.

The major concern of the direct injection approach is the fat body or other tissue cell population affected by RNAi may be limited and, hence, the mutant effects of loss-of-function may not be so significant, while the gain-of-function will be relatively easier to identify.

One caveat/limitation to interpret the RNAi results is that one mutant effect could be resulted from both direct and indirect target genes. Expression of direct target genes is due to the interaction of AaE74 proteins with the regulatory elements present in the gene. In contrast, the regulation of indirect target genes may be controlled via one or multiple intermediate proteins that are controlled by the direct target genes.

Determination of the properties and dynamics of AaE74 protein binding of chromatin *in vivo* by Chromatin Immunoprecipitation (CHIP)

Functional EcR response elements were identified in the proximal region of the Vg promoter and has been demonstrated to be required for basal level expression of the *Vg* gene (Kokoza *et al.*, 2001; Martin *et al.*, 2001), which is thought to be further enhanced by more distant enhancer elements. Significantly, Zhu and colleagues (2003) have demonstrated that the Vg promoter was occupied *in vivo* by both AaEcR and AaUSP at early vitellogenic period (3-12 h PBM) as examined by chromatin immunoprecipitation assay (ChIP). This correlation of AaE74B and AaEcR-AaUSP expression and binding profiles provides an opportunity for examining the interaction between AaE74B and AaEcR-AaUSP complex *in vivo* . The determination of the occupancy of AaE74B on the Vg promoter *in vivo* simultaneously with AaEcR and AaUSP will provide compelling evidence for their relevant interaction. One strategy for identifying the specific protein that associates with a response element in the context of chromatin is to use CHIP assay (Shang *et al.*, 2000; Chen *et al.*, 1999). The principle strength of CHIP assay is that it is the only method currently available for detection of the interaction between a protein and a response element *in vivo*. If compelling data is obtained, the method can provide strong and direct evidence that a site is occupied by a specific protein *in vivo*. In addition to direct response element binding factors, the presence of proteins that are not bound directly to DNA and that depend on the protein for binding can also be determined.

In brief, an antibody specific to a target protein is used to immunoprecipitate formaldehyde cross-linked sonicated chromatin from cells treated with or without a reagent, or from the cells in different developmental stages. The resulting partitioned genomic DNA is then analyzed by quantitative PCR using primer spanning the response element for changes in the relative levels of DNA-associated target protein *in vivo* under different physiological conditions.

Specifically, AaE74 CHIP assay can be performed using AaE74B antibody. Subsequent PCR will be carried out using the primers covering the media region containing AaE74 binding sites identified previously in the Vg promoter. AaEcR and AaUSP occupancy will be also evaluated as controls. Either fat body tissue or fat body nuclear nuclei at onset of vitellogenesis female mosquito will be used as initial material for cross-linking.

Of primary concern is that the feasibility of using tissue from adult mosquitoes as a cell source needs evaluation and testing, in contrast to traditional method using cell line. There are three options that could circumvent this problem. First, the fat body cells can be separated by partial digestion with protease. Second the fat body nuclei, instead of whole cell, can be used for cross-linking. However, the physiological condition where the protein-DNA interaction is required may be perturbed. Third, physical separation of the individual cell by grinding the frozen fat body tissue can be tested. Of secondary concern is the quality of AaE74B antibody, despite the fact that it has been successfully applied in GMSA. CHIP is technically challenging, the attractive merit of the method will justify it as a new research direction.

Dissection of regulatory region of AaE74 gene(s)

The distinct expression patterns of AaE74 two isoforms during the vitellogenesis in mosquito and their opposite effects on Vg promoter suggest that the two isoforms may be subject to regulation of differential expression. In *Drosophila*, the E74 two isoforms were produced by alternative splicing and differential promoter utilization. Characterization of the AaE74 promoter will provide a platform for studying the regulation mode of its expression. Although no EcR response elements was found in *Drosophila* promoter (Thummel, 1989), it will be interesting to find the EcR response elements in its regulatory region, since AaE74 is thought to be directly activated by 20E.

In order to characterize the gene structure of AaE74, the initial step is to obtain the AaE74 genomic clone from mosquito genomic library and determine transcription start site by primer extension, and/or 5' RACE and/or RNase protection. The potential transcription factor binding sites will be examined in detail manually or with the help of on transcription factor finding softwares such as MatInspector and TFSEARCH. The synthetic oligonucleotides containing the presumptive candidate binding sites will be used in gel mobility shift assay with the proteins prepared from nuclear extracts or by *in vitro* transcription/translation of specific transcription factor. Competition using excess oligonucleotide or oligonucleotide containing mutation in the presumptive sites will be tested for specificity. The transcription factor responsible for the specific DNA-protein complex will be identified by antibody inhibition/supershift analyses. The functional importance of the specific sequence can be further analyzed using deletions of various region of the AaE74 promoter. The importance of particular candidate binding sites can be assessed by mutagenesis. Further assessment of the validity of these putative response

elements in regulation of AaE74 will require *in vivo* experiments such as the transgenic mosquito system, which is well established in Dr. Raikhel's lab (Kokoza *et al.*, 2000; Shin *et al.*, 2002).

Detailed AaE74 domain analysis

The two isoforms of AaE74 share the N terminal DNA binding domain, while differing in the C-termini. Since the two isoforms of AaE74 exhibited opposite function in Vg promoter by transient transfection assay, it is intriguing to identify the domains, which is responsible for such distinct activity. It is mostly likely that the difference of activity is contributed by the isoform-specific region of the AaE74. Therefore, the domain swapping experiments will be designed. A series of domains swapping constructs between AaE74B and AaE74A isoform specific coding region will be generated by PCR and subject to transfection analyses of the promoter activity of Vg ^{1071}-Luc reporter construct in S2 cells as previously described (see Materials and Methods).

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