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REGULATORY REGIONS OF THE *ULTRASPIRACLE* GENE ISOFORMS FROM THE MOSQUITO *AEDES AEGYPTI*: ISOLATION AND CHARACTERIZATION

presented by

Renyuan Wang

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Department of Entomology

Major Professor's Signature

6/17/03

Date

REGULATORY REGIONS OF THE *ULTRASPIRACLE* GENE ISOFORMS FROM THE MOSQUITO *AEDES AEGYPTI*: ISOLATION AND CHARACTERIZATION

By

Renyuan Wang

A THESIS

Submitted to
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In partial fulfillment of t he requirements
for the degree of

MASTER OF SCIENCE

Department of Entomolo gy

2003

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ABSTRACT

REGULATORY REGIONS OF THE *ULTRASPIRACLE* GENE ISOFORMS FROM THE MOSQUITO *AEDES AEGYPTI*: ISOLATION AND CHARACTERIZATION

By

Renyuan Wang

The functional receptor for insect steroid hormone, ecdysteroid, is a heterodimer consisting of two nuclear receptors, ecdysone receptor (EcR) and a homologue of retinoid X receptor, Ultraspiracle (USP). There are two isoforms of USP, USP-A and USP-B in the mosquito *Aedes aegypti* and these two isoforms are alternatively spliced from one gene, but derived from different promoters. In mosquito fat body, the transcripts of USP-A are present during the previtellogenic period and the postvitellogenic period. However, the transcripts of USP-B are induced by the blood meal during the vitellogenic period. The transcriptional initiation sites of USP-A and USP-B were determined in orders to analyze the promoter regions of these two isoforms. With the *Aedes aegypti* genomic DNA library screening, the DNA fragments containing the regulatory regions of USP-A and USP-B have been isolated and sequenced. To identify the transcriptional regulation of USP-A and USP-B, the putative transcriptional factor binding sites were predicted by TESS and TRANSFAC programs. These putative transcription factor binding sites were then tested and confirmed by *in vitro* binding assays. Moreover, based on the transcription profiles and the potential binding sites, the hypotheses of the hormone regulation of USP-A and USP-B have been proposed.

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DEDICATION

I wish to dedicate this thesis to my parents, Yijun Wang and Ruoqin Zhu, who support me all the time.

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Chapter 3

Table 3.1 The s
USP-

LIST OF TABLE

Chapter 3

20E: 20-hydroc

Aa: *Aedes aegy*

AD: activation

AF-1: active act

AF-2: active act

ARC: activator-

Bp: base pair

CA: corpora alla

CAT: chloramp

CC: corpora car

CDC: Center fo

cDNA: compler

Chx: cyclohexi

DBD: DNA-bin

LIST OF ABBREVIATIONS

20E: 20-hydrocxyecdysone

Aa: *Aedes aegypti*

AD: activation domain

AF-1: active activation function 1

AF-2: active activation function 2

ARC: activator-recruited cofactor

Bp: base pair

CA: corpora allata

CAT: chloramphenicol acetyltransferase

CC: corpora cardiaca

CDC: Center for Disease Control and Prevention

cDNA: complementary DNA

Chx: cycloheximide

DBD: DNA-binding domain

Dm: *Drosophi*

DNA: Deoxyri

DR: direct rep

DRIP: vitamin

EcR: ecdyson

EcREs: ecdys

ER: estrogen

EMSA: mobil

GR: glucocor

hid: head inv

HAT: histone

HDAC: histo

HNF3/fkh: h

HREs: hormo

HSF: Heat-sh

JHIII: juvenil

Dm: *Drosophila melanogaster*

DNA: Deoxyribonucleic acid

DR: direct repeats

DRIP: vitamin D receptor-interacting proteins

EcR: ecdysone receptor

EcREs: ecdysone response elements

ER: estrogen receptor

EMSA: 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 p

LBD: ligand b

MR: mineralo

NcoA: nuclea

NcoR: nuclea

NMR: nuclea

NR: nuclear r

OEH: ovarian

PAGE: polya

PBM: post bl

PCR: polyme

RAR: retinoi

RBC: red blo

RER: rough e

RNA: ribonu

rpr: reaper

Kb: kilobase pair

LBD: ligand binding domain

MR: mineralocorticoid receptor

NcoA: nuclear receptor coactivator

NcoR: nuclear receptor corepressor

NMR: nuclear magnetic response

NR: nuclear receptor

OEH: ovarian ecdysteriodogenic hormone

PAGE: polyacrylmide gel electrophoresis

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: revers

RXR: reti

SDS: sodi

SET: supp

SRC: ster

TR: thyro

TRAP: T

USP: *Ult*

VCP: vit

VCB: vit

Vg: vitel

VDR: vi

VgR: vit

WHO: W

YYP: yo

RT: reverse transcription

RXR: retinoid X receptor

SDS: sodium dodecyl sulfate

SET: suppressor of variegation, enhancer of zeste and trithorax

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

YYP: yolk protein precursor

Chapter 1 – Literature Review

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Medical Importance of Mosquitoes

Insects have great importance to human because they are closely related to almost all kinds of activities. Insects appeared about four hundred million years ago. So far more than two million species of insects have been described, which make up 75% of all living species. New insect species are continuously being discovered and named. Insects live on every continent and in all kinds of terrestrial habitats. Therefore, they have become the major component of the earth's biodiversity, which contributes greatly to harmonious biosphere (Borror et al., 1989). Some insect species are beneficial in that they can be used as food, industrial resources or biological control agents. However, there are also a large number of harmful insects that cause serious losses in agriculture or a severely impact livestock and humans by disease transmission. Therefore, the knowledge of entomology from basic to applied science will create new methods of insect pest management and will contribute to sustainable agriculture, protection of the environment and the maintenance of biodiversity.

Taxonomically insects can be divided into 25 orders. The yellow fever mosquito, *Aedes aegypti*, with which I have been working, belongs to the order of *Diptera* and the family of *Culicidae*. This mosquito species has been used as one of the model insects for arthropod vector research in many labs due to its medical importance in transmitting human diseases and its synchronized egg development during adult stage in females. A great amount of information has been accumulated thus far for its physiology, biochemistry, and specifically for the regulatory mechanisms controlling vitellogenesis has made this species an ideal model system.

Mosquitoes serve as a vector for many harmful human diseases (Thomas et al.,

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1998). Malaria and the Dengue fever take a heavy toll on the human population in the world, especially in Africa and Asian. *Plasmodium falciparum* malaria is considered as one of the three most important human infectious diseases (tuberculosis, malaria and HIV/AIDS) in the world today. It is estimated that 3-5 million deaths every year are caused by this disease according to a WHO report (1998). In the recent years, the problem of malaria has become more serious because of the development and rapid spread of resistance in *P. falciparum* to the commonly used antimalarial drug, chloroquine. *Aedes aegypti* mosquitoes principally transmit dengue fever. Dengue virus causes a nonspecific febrile illness. Dengue threatens more than 2.5 billion people, with an annual incidence in the tens of millions and about 24,000 deaths per year (WHO Report, 1996). No effective vaccine candidates are available for preventing dengue cases and transmission. Last year the outbreak of West Nile virus in thirty-nine states gave us an example of how dangerous mosquito borne viruses can be and how quickly they can spread. The CDC has recently setup a surveillance system to monitor the spread of this virus in the US (CDC Report, 2002).

Therefore, there is an urgent need to explore every possible way to develop new control methods against these mosquito disease vectors. *Aedes aegypti* is a floodwater mosquito. The ability to store eggs and to synchronize hatching makes it much easier to rear and manipulate this mosquito in the laboratory than other disease vectors, such as *Anopheles gambiae* the malaria mosquito. The blood meal triggers a cascade of physiological events and the tight regulation of this system makes it an excellent model to study the genetic regulation.

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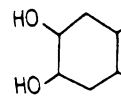
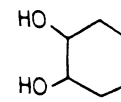
Hormonal Regulation of Vitellogenesis

Insect Hormones

It is now well known that the development and reproduction of insects are under the control of hormones. In general, there are three classes of hormones regulating these physiological events, namely, the neuropeptide hormone secreted by the brain, the terpenoid Juvenile hormones (JH) produced by corpora allata and the steroid hormone, ecdysteroids, produced by the epithelial glands (Figure 1.1). The pattern of hormonal control is probably similar in the larval stage of all the insect species investigated. When a larva reaches a threshold period (for example, a given weight) a neurohormone called prothoracicotropic hormone (PTTH) from brain neurosecretory cells is released into the hemolymph. This hormone stimulates the prothoracic glands to produce ecdysone (or 3-dehydoecdysone), which is converted into 20-hydroxyecdysone by the fat body. 20-hydroxyecdysone stimulates the molting process. The pattern of molting (larval-larval molt or larval-pupal molt) is determined by both juvenile hormones and ecdysteroids. When a high titer of juvenile hormones is present a larval-larval molt takes place.





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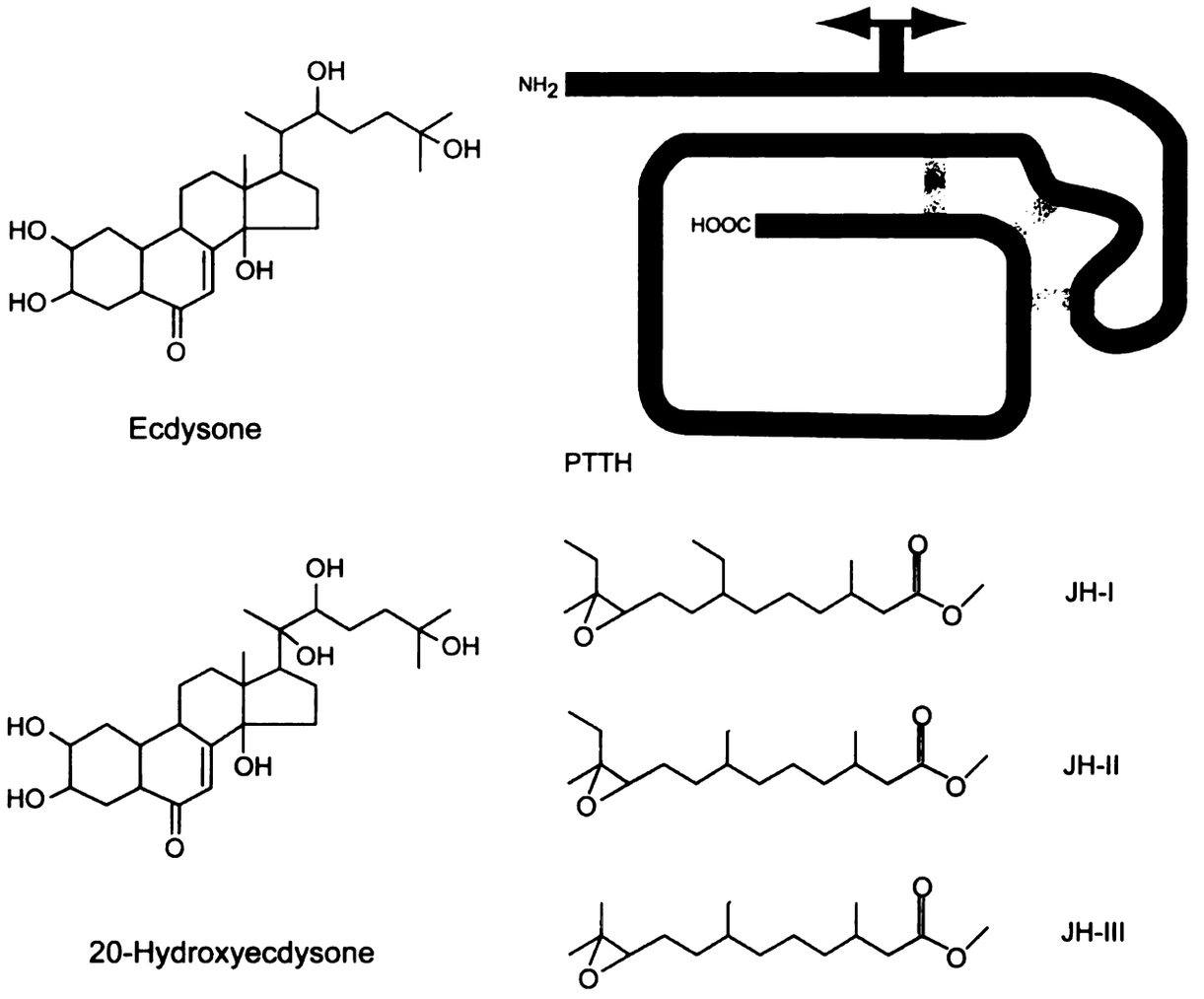

Figure 1.1 Structures of PTTH, JH-I, JH-II, JH-III, a-ecdysone, and 20-hydroxyecdysone.

Ecdysteroids in female mosquito

Growth and reproduction in insects are under the control of hormones. Ecdysteroids that were first described as molting hormones were subsequently found to play a role in reproduction as well. (Rees et al., 1989; Gäde et al., 1997). In the larval stage the prothoracic glands are the source of ecdysteroids. Since the glands no longer

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exist in the adult stage many people did not believe in the presence of ecdysteroids in adult insects. In 1975, Hagedorn's group first proved the ovary as the source of ecdysone in the mosquito *Aedes aegypti* using an *in vitro* organ culture method (Hagedorn et al., 1975). Since then, these hormones were also found to be present in the adult stage of other insects such as locusts, cockroaches, crickets, flies, bugs as well as termites (for a review see Hagedorn, 1985). It is now well established that the ovary is capable of synthesizing ecdysteroids during late pupal and adult stages. The ovarian follicle cells have shown to be determined as the precise site of ecdysteroid biosythesis in the adult locusts (Goltzene et al., 1978; Hoffmann et al., 1992). Recently, a putative early component of the ecdysteroid biosynthetic pathway has been functionally characterized in *Drosophila*. This gene, designated *dare* (defective in the avoidance of repellents), encodes a close homolog of the vertebrate adrenodoxin reductase (Freeman et al., 1999). The *dare* gene is greatly enriched in the ovary. The genetic studies and the expression patterns of dare, BRC, E74 and E75 during oogenesis in *Drosophila* suggest that ecdysteroids act in an autocrine or paracrine manner in the ovary (Kozlova and Thummel, 2000).

The physiological significance of the ovarian ecdysteroids varies in different insect species. For example, 2-deoxyecdysone is a predominant component in the ovary of *Locusta migratoria* and *Schistocerca gregraria* and it is mainly present in a conjugated form (Hoffmann et al., 1980). Since the biological activity of 2-deoxyecdysone is much lower than 20-hydroxyecdysone and also since the conjugated form of the ecdysteroid is considered as an inactive form, the ecdysteroids present in the ovary of locusts is proposed to have no role in the oogenesis of locusts. They might be later used in

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embryogenesis (Lagueux et al., 1977). In the cockroach *Nauphoeta cinerea*, the oocytes at a stage shortly before ovulation contain large amounts of free 20-hydroxyecdysone and a small quantity of the conjugated ecdysteroids. These free and conjugated ecdysteroids disappear at ovulation. Therefore, a function of ecdysteroids in chorion formation was suggested (Zhu et al., 1983). However, the mosquito *Aedes aegypti* represents a unique model in which the role of ovarian ecdysteroids in vitellogenesis has been clearly demonstrated (Hagedorn, 1985; Raikhel, 1992; Deitsch et al., 1995; Wang et al., 2000). The evidence as follows shows a role for ecdysteroids in vitellogenin synthesis in *Aedes aegypti* as was summarized by (Hagedorn, 1983).

1. A burst of ecdysteroid secretion occurs some 10-36 hours postblood meal (PBM), which coincides with the period of vitellogenin synthesis.
2. Ovaries taken from females 18 hours PBM synthesized ecdysone when incubated *in vitro* and ecdysone was found in the hemolymph. After the ovaries were removed from blood-fed females the rate of vitellogenin synthesis declined.
3. The fat body synthesizes vitellogenin when incubated with 20E. The dose that causes a half-maximal response (10^{-7}M) is similar to the amounts found in the hemolymph.
4. The kinetics of vitellogenin synthesis was very similar after a blood meal, after injection of 20E, and after incubation of fat body with hormone *in vitro*. In each case the peak of synthesis occurred 25 to 30 hours after stimulation, followed by a decline to low levels by 40 to 50 hours.
5. A factor isolated from an extract of heads, which is probably homologous to the egg development neurosecretory hormone (EDNH), stimulates ecdysone production by

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However, the role of ecdysteroids in inducing vitellogenin synthesis in this model was questioned because non-physiological doses of 20E were necessary to stimulate vitellogenesis *in vivo*. Hagedorn (1983) suggested that rapid elimination of injected 20E in the previtellogenic mosquito accounted for the insensitivity. If it is true, this speculation only provides a partial explanation, and other factor(s) may be involved in the regulatory hierarchy of ecdysteroids in mosquito vitellogenesis.

Vitellogenesis in the mosquitoes *Aedes aegypti*

Vitellogenins are the precursors of the yolk protein, which are synthesized by the fat body of the female mosquito after taking a blood meal. The importance of this system has made it an active area of research. Significant progress has been specifically made in the molecular mechanism of vitellogenesis in the mosquito *Aedes aegypti* (Raikhel et al., 2002).

The mode of hormone action regulating vitellogenesis may be different depending on the species. So far as we know in the most insect species, juvenile hormones play a major role in stimulating vitellogenin synthesis (Koeppe et al., 1985). In *Diptera*, ecdysteroids are reported to induce vitellogenin synthesis and uptake. Since the mechanism of the hormonal regulation of vitellogenin synthesis and uptake has been largely elucidated in *Aedes aegypti* in the latest decades, I will summarize that the data obtained from this model insect and from other insects are also referred for a comparison to compare regulatory systems (Figure 1.2).

Vitellogenesis of *Aedes aegypti* can be divided into three phases: previtellogensis,

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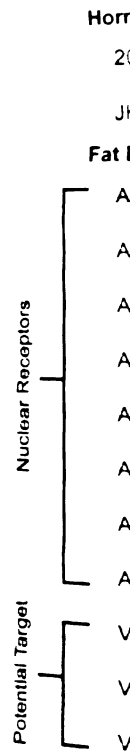

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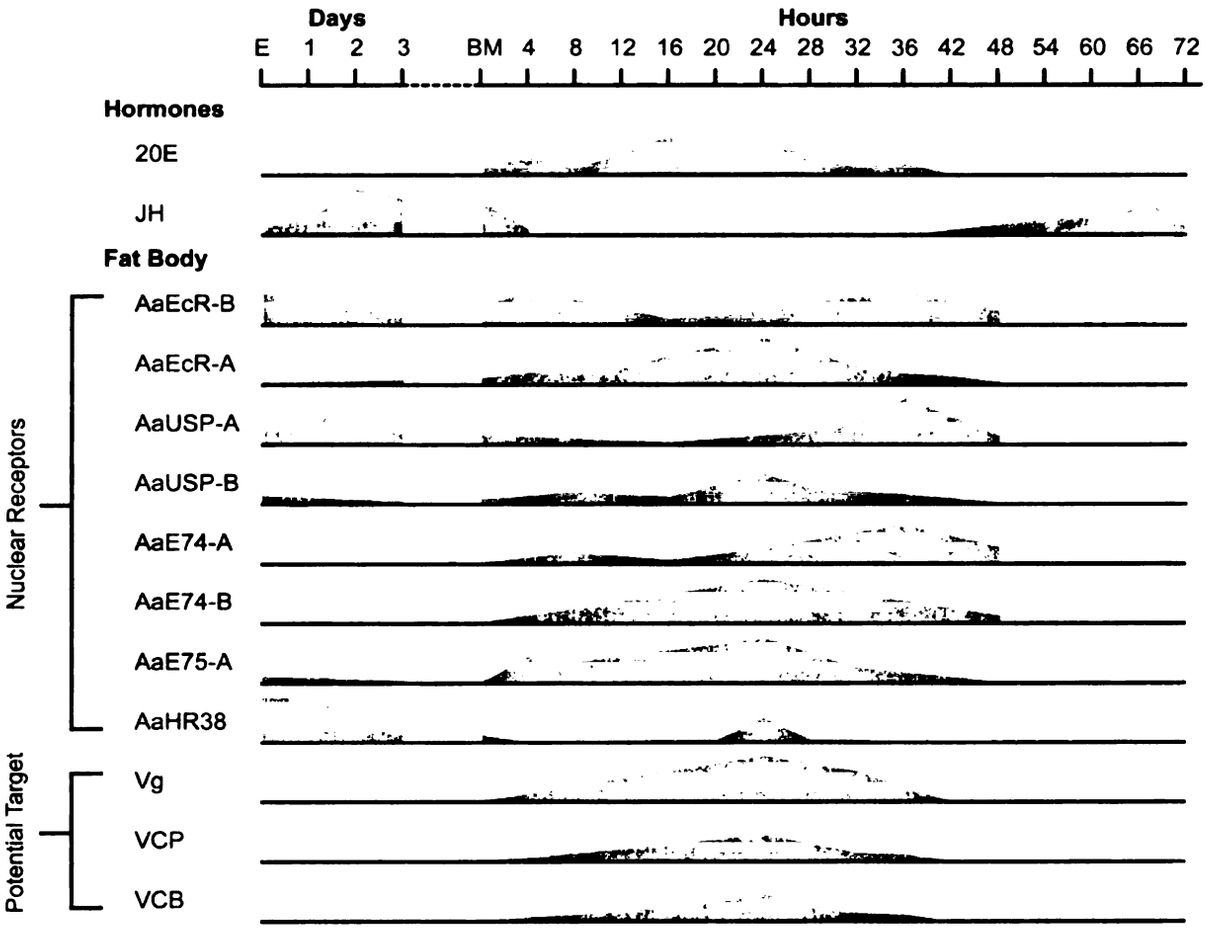

Figure 1.2 Profiles of major genes mRNA level of the ecdysteroid regulatory. (From Raikhel et al., 2002)

The previtellogenic period

During the previtellogenic phase, the fat body becomes capable of intense synthesis of yolk protein precursors (YPP). This is thought to be a preparatory phase under the control of juvenile hormone III (JH III) because the exposure of fat bodies and ovaries to JH III is necessary for the competence to respond to ecdysteroids and to the uptake of vitellogenin respectively. This phase is followed by a state of arrest which is maintained until a blood meal is taken.

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The vitellogenic period

The second stage is the vitellogenic phase during which a blood meal stimulates the secretion of OEH (ovarian ecdysteroidogenic hormone) by the brain. For invertebrates, the only steroidogenic gonadotropin identified to date is the ovary ecdysteroidogenic hormone (OEH I) from the yellow fever mosquito, *Aedes aegypti* (Brown *et al.*, 1998). Ingestion of a blood meal by females of this species leads to the release of OEH I from neurosecretory cells, and OEH I stimulates ovaries to secrete ecdysteroids, which modulate secretion of yolk proteins by the fat body (Clements, 1992). Native OEH I was isolated from female heads and partially sequenced. The head-specific cDNA has been cloned that encodes a prepropeptide, which can be processed into a bioactive peptide (Brown *et al.*, 1998). Recombinant OEH I was shown to have the same bioactivity as the native peptide, as it stimulates yolk deposition *in vivo* when injected into blood-fed, decapitated *Aedes aegypti*, and ecdysteroidogenesis when incubated *in vitro* with ovaries from sugar-fed females (Matsumoto *et al.*, 1989; Brown *et al.*, 1998). Numerous studies have clearly established that the ecdysteroid control of vitellogenesis is a central event in the blood meal-activated regulatory cascade leading to successful egg maturation (reviewed in Dhadialla and Raikhel, 1994; Raikhel et al., 1999). The hemolymph titers of ecdysteroids in female mosquitoes are correlated with the rate of Vg synthesis in the fat body. The ecdysteroid titers are only slightly elevated at 4 hours PBM; however they rise sharply at 6-8 hours PBM, and reach their maximum level at 18-20 hours PBM. Besides the major yolk protein (Vg), a second yolk protein called vitellogenic carboxypeptidase has also been identified in *Aedes aegypti* (Hays and Raikhel, 1990; Cho et al., 1991). The control of this protein is in to that of *Vg*. Yolk

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protein precursors (YPPs) are accumulated and stored in the yolk bodies of the oocytes. (Hagedorn, 1985, 1989; Raikhel, 1992; Dhadialla and Raikhel, 1994; Raikhel et al., 1999).

The post-vitellogenesis

The third stage is the postvitellogenic phase. The vitellogenic phase ends abruptly between 27 and 30 h PBM. During this time, the synthesis of YPPs is halted and YPP uptake by the oocytes is initiated. A proliferation of lysosomes from the fat body cells degrades the synthetic organ responsible for Vg production. The trophocytes are remolded in preparation of the next gonotrophic cycle. Since JH titers rise again 36 hours PBM, which correlates with the development of the secondary follicles until the resting stage, it is likely that JH is involved in this process. In addition, an oostatic hormone produced by mature primary follicles prevents the secondary follicles from breaking the state of arrest.

Synthesis and uptake of vitellogenins

Insect eggs contain a large amount material that is taken up from the maternal hemolymph. These materials include proteins, lipids and carbohydrates, all of which are used for embryogenesis. Among these materials, vitellogenins (Vgs), the major yolk protein precursors (YPPs), are large proteins that makes up 80-90% egg yolk (Kunkel and Nordin, 1985). Since Vgs are synthesized and accumulated in large amounts during vitellogenesis, they have attracted an attention from many scientists and have become a good model for the researches of hormonally stimulated gene activity, post translational

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processing, and mechanisms of sequestration by oocytes and proteolysis in developing eggs. In most insects, the native molecular masses of Vgs are in a range of 210-652 kDa, with subunit molecular weights of 150-200 kDa for large subunits and 40-65 kDa for small subunits, respectively (Izumi et al., 1994). Vgs isolated from various insects consist of polypeptides numbering from one in *Apis mellifera* to four or more in *Tenebrio molitor*, *Leucophaea maderae*, *Periplaneta americana*, *Locusta migritaoria* and *Rhodnius prolixus* (Harmish and White, 1982; Kanost et al., 1990). In the mosquito *Aedes aegypti*, the Vg has a molecular weight of about 260kDa with two large and several small subunits. The large subunits have a molecular weight of 200 kDa and the small subunits 65 kDa. Both subunits are phosphorylated and contain a high mannose oligosaccharide (Raikhel and Bose, 1988). Meanwhile, together with Vg, the vitellogenic fat body of the mosquito female's produces two other YPPs which are pro-enzymes deposited in developing oocytes and are activated during embryogenesis: 53-Kda vitellogenic carboxypeptidase (VCP), and a 44-Kda cathepsin B-like protease (VCB). Moreover, the regulation of these YP precursors appears to be similar: they are synthesized exclusively by the fat body in response to a blood meal and are maximally expressed at 24 h PBM (Hays and Raikhel, 1990; Cho et al., 1991). Recently, it has been shown that lipophorin (LP), the insect lipid transport molecule, also plays the role of a YPP in *Aedes aegypti* (Sun et al., 2000).

Studies on Vgs of several insect species showed that several vitellogenin apoproteins originate from a single large polypeptide. Most of the insect's Vgs are cleaved once in the fat body to produce two subunits (Sappington and Raikhel, 1998). In the mosquito, *Aedes aegypti* a fat-body-specific convertase has been cloned and the coexpression experiments have shown that this enzyme cleaves mosquito pro-Vg at the correct site (Chen et al.,

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1996). It is known that Vgs secreted by the fat body are taken up by insect oocytes via receptor-mediated endocytosis, a mechanism used by eukaryotic cells to internalize macromolecules (Raikhel and Dhadialla, 1992). After Vgs are sequestered by oocytes, these proteins are called vitellins (Vns). They were first discovered by Roth and Porter in 1964. The yolk accumulation in the oocytes of *Aedes aegypti* correlated with the increase in the number of coated vesicles (now known as clathrin-coated pits). Later Roth et al. (1976) further demonstrated that the internalization of Vgs from hemolymph into oocytes was mediated through these coated pits. These discoveries have become the milestone for our understanding of the process of the receptor-mediated endocytosis of macromolecules in eukaryotic cells. The coated pits are the receptor-ligand complexes clustered in specialized domains (Geuze et al. 1983, 1987; Bu and Schwartz 1994). The Vg internalization pathway has been thoroughly studied in mosquito oocytes through ultrastructural observation (Raikhel 1984a, b, 1987; Raikhel and Lea 1985). Insect Vg receptors (VgR) are large membrane-bound proteins (180-214 kDa). Most recent progress in the study of insect VgR has been made in the mosquito *Aedes aegypti* (Sappington and Raikhel, 1998). The entire coding region of *AaVgR* mRNA transcript was sequenced (Sappington et al., 1996). The immunocytochemical studies have clearly visualized the steps in Vg/VgR internalization, dissociation, sorting, and recycling of the receptor to the plasma membrane (Snigirevskaya et al. 1997).

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Hormone Regulation of Vitellogene Expression

Hormone Regulation cascade in *Drosophila*

Extensive characterization of the response of the cultured salivary glands from *Drosophila* to exogenous ecdysteroids has revealed three classes of puff sites related gene expression (Ashburner, et al., 1974). Based on their observations, Ashburner et al. (1974) proposed the hypothesis of the mechanism of gene regulation by the hormone.

According to this hypothesis, 20E first activates a small set of early genes, which then induce the expression of a large number of target late genes. Only when a sufficient concentration of the protein(s) encoded by the early gene been synthesized the ecdysone response element (EcR) can be displaced from the late gene site and transcription of that gene(s) can occur. Further studies in *Drosophila* support this hypothesis. For example, at the end of third larval instar in *Drosophila*, a high titer peak of ecdysteroids triggers puparium formation and the onset of metamorphosis. Several pulses of ecdysteroids during metamorphosis are responsible for further differentiation of the adult structures. It has been shown that 20E, bound to its receptor, directly induces a small number of primary-response early genes, including the *Broad-complex* (*BRC*), *E74* and *E75*. These early genes encode transcription factors that coordinate the induction of large sets of secondary-response late genes, leading to the appropriate stage and tissue-specific biological responses (Thummel et al., 1996; Segraves, 1998; Henrich, 1999; Figure 1.3)

Ecdysteroids are likely to play a critical role in *Drosophila* oogenesis and fertility. E75 mRNA can be up-regulated by exogenous 20E in cultured ovaries (Buszczak and Segraves, 1998). In addition, EcR and USP are present in both nurse and follicle cells at

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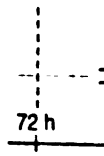

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relatively constant levels throughout oogenesis (Buszczak and Segraves, 1998). These results clearly show that the ecdysteroid-triggered regulatory hierarchy is activated by the hormone during *Drosophila* oogenesis.

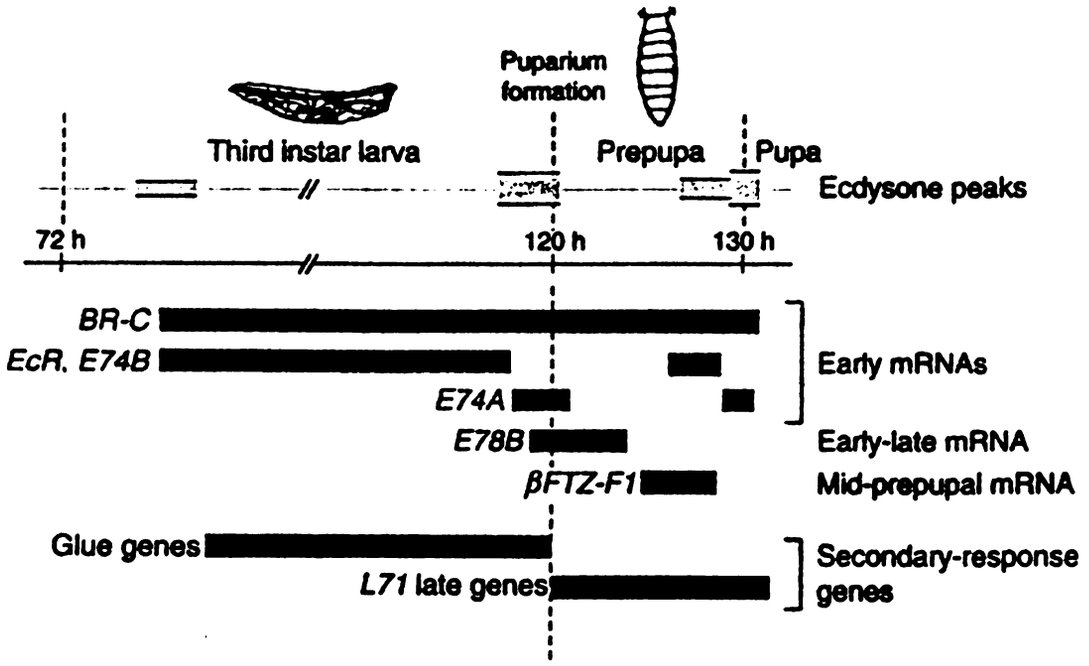

Figure 1.3 Early genes and later gene expression during *Drosophila* metamorphosis. (From Thummel et al., 1996).

Hormone Regulation of *Vitellogenin* (Vg) Gene

The role of ecdysteroids in vitellogenesis is best understood in *Aedes aegypti* (Figure 1.2). In *Aedes aegypti* mosquito, a blood meal activates the genes encoding YPPs (Raikhel, 1992). When fat bodies excised from previtellogenic female mosquitoes are incubated *in vitro* in the presence of physiological doses of 20E, the expression of the YPP genes are induced. As mentioned above, the mosquito EcR-USP complex has been shown to bind to various ecdysone response elements (EcREs) to modulate ecdysone

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regulation of target genes (Wang et al., 1998). Meanwhile, the *Aedes aegypti* homologue of the *Drosophila E75* gene a putative representative of the next level in the ecdysone response heirachy has been identified and characterized. AaE75 is expressed in ovary and fat body following a blood meal, and AaE75 transcripts, corresponding to three E75 isoforms, are ecdysone-inducible in isolated fat bodies cultured in vitro. E75 transcripts show two peaks, with a small peak coinciding with the first peak of 20E. The correlation between mid-vitellogenic expression of E75 and vitellogenin (Vg) genes suggests that the YPP genes are direct targets of E75 (Pierceall et al., 1999). Two isoforms of the homolog to the *Drosophila* transcription factor E74, which share a common C-terminal Ets DNA-binding domain, yet have unique N-terminal sequences, are present in the mosquito *Aedes aegypti*. The AaE74B transcript is induced by a blood meal-activated hormonal cascade in fat bodies and peaks at 24 hours PBM, the peak of vitellogenesis. In contrast, AaE74A is activated at the termination of vitellogenesis, exhibiting a peak at 36 hours PBM in the fat body and 48 hours PBM in ovary. These findings suggest that AaE74A and AaE74B isoforms play different roles in regulation of vitellogenesis in mosquitoes, as an activator and a repressor of YPP gene expression respectively (Sun et al., 2002). The *Vg* gene regulatory portion containing E74 binding sites are required for the high level of Vg expression (Kokoza et al., 2001). Recent results indicate that ecdysone can function as ligand for the mosquito EcR-USP heterodimer (Wang et al., 2000). Vg mRNA and protein are detected as soon as 1 hour after blood feeding, reaching their peak at 24 hours, declining at 30 hours and disappearing by 48 hours (Raikhel and Lea, 1983; Cho and Raikhel, 1992). The pattern is in parallel the profile of the ecdysteroid titer. Expression of YPP gene transcription factors is super-induced by 20E in the presence of

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the protein synthesis inhibitor, cycloheximide. In contrast, this inhibitor abrogates 20E-induced YPP gene transcription in the fat body (Deitsch et al., 1995; Li et al., 2000; Sun et al., 2000). Cloning and analysis of the 5' upstream regulatory region of the mosquito *Vg* gene has revealed the presence of putative binding sites for EcR-USP along with those to the early genes, *E74* and *E75*. Thus, the *Aedes aegypti Vg* gene is the target of direct and indirect regulation by 20E.

Transcriptional Factors

Any protein that regulates the transcriptional activity of a gene is called a transcription factor. There are several thousands of transcription factors in the eukaryotes. I will introduce some factors that have been studied in the regulation of *vitellogene*.

Nuclear receptors

Classical nuclear hormone receptors are ligand-activated transcription factors which active gene expression after binding to their respective ligands. The ligands for nuclear receptors are small lipophilic molecules such as steroids, retinoids, vitamin D and thyroid hormone. The receptor for these ligands include the estrogen receptor (ER), androgen receptor (AR), Vitamin D receptor, retinoid X receptor (RXR), and ecdysone receptor (EcR) (Mangelsdorf et al., 1995, Riberio et al., 1995, Escriva et al., 2000, Olefsky, 2001). Additionally, there are a large number of orphan nuclear receptors. These receptors share the similar structure, but their ligands are unknown. The nuclear receptor superfamily is comprised of both nuclear hormone receptors and orphan receptors (Tsai and O'Malley, 1994; Mangelsdorf et al., 1995).

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When hormones reach their target cells they must interface with the target cells through specific receptors. So far to our knowledge, there are three cellular mechanisms by which hormones act. For proteins and peptides their receptors are located in the cell membrane while for steroid hormones or thyroid hormones their receptors are in the nucleus. The membrane receptors transduce the signal by generating second messengers, while nuclear receptor-ligand complexes interact directly with the genome.

The superfamily share the same structure with which all members contain five conserved functional domains starting from the N-terminal: A/B, C, D, E, F (Tsai and O'Malley, 1994). The A/B domain (the most variable in all the domains) contains the ligand-independent transactivation function (AF1). The C domain is the DNA binding domain (DBD). The D domain is a hinge region between the C and E domains and it may act as a corepressor binding site. The E domain is the ligand binding domain (LBD), which binds to the ligand and possesses activation functions (AF2). The F domain is at the C-terminal and varies in size and primary sequence among the superfamily.

Ecdysone receptor (EcR)

EcR was first cloned from *Drosophila melanogaster* (Koelle et al., 1991). The ecdysone receptor belongs to the nuclear hormone receptor superfamily and is defined by a 66-68 amino acid sequence, the DNA binding domain, which contains two cysteine-cysteine zinc fingers, and is necessary for the recognition of, and binding to a hormone response element. The functional ecdysone receptor of *Drosophila* has been identified as a heterodimeric complex consisting of EcR and its retinoid-like partner, ultraspiracle (USP), and it is thought that the complex is stabilized by 20E (see Henrich et al., 1999

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In the mosquito *Aedes Aegypti* a body of evidence shows that 20E acts directly on the vitellogenin gene via its functional receptors, the heterodimer composed of ecdysone receptor (EcR) and the ultraspiracle (USP) protein (Kapitskaya et al., 1996; Wang et al., 2000). There are two kinds of AaEcR in *Aedes aegypti*, AaEcR-A and AaEcR-B and these proteins can be divided into five domains (Raikhel A.S. et al. 1998). AaEcR-B A/B domain is a 189 amino acid terminal region of the nuclear receptor and is implicated in transactivation. The C domain is a 66 amino acid region containing two C2-C2 zinc fingers involved in DNA binding. The D domain is a linker region of variable length including 92 amino acids. The E domain is a 222 amino acid region required for hormone binding.The F domain is a 106 amino acid region on the carboxyl terminal portion of the receptor. Compared with the *Drosophila* EcR, it has ~55-65% identity within the DNA binding domain and ~25-35% identity within hormone binding domain. AaEcR is similar to the EcR-B1 isoform of *Drosophila melanogaster*. There are three AaEcR transcripts of 4.2 kb, 6 kb and 11 kb in adult mosquitoes. 4.2kb mRNA is predominantly expressed in female mosquitoes during vitellogenesis. In both the fat body and ovaries of the female mosquito, the level of AaEcR mRNA is high during the previtellogenic period and after the onset of vitellogenesis (6 hours PBM).

Ultraspiracle (USP)

Ultraspiracle (USP) is the insect homolog mammalian of the retinoid X receptor (RXR). RXR is involved in the regulation of the hormonal signaling pathways through the formation of heterodimer complexes to bind to DNA. The first discovery of USP was

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in *Drosophila* (Yao et al., 1992, 1993). The USP gene has already been cloned in *Drosophila* (Yao et al., 1992, 1993), *Lucilia cuprina* (Hannan et al., 2001), *Choristoneura fumiferana* (Perera et al., 2003), *Aedes aegypti* (kapitskaya et al., 1996), *Manduca sexta* (Jindra et al., 1997) and *Bombyx mori* (Tzertzinis et al., 1994). The localization of USP on polytene chromosomes suggests that the protein can be colocalized to the site of ecdysteroid-responsive chromosome puffs (Buszczak et al., 1998). In insects, the USP and EcR to form a functional complex mediate the effects of 20E by activating and repressing expression of ecdysone responsive genes (Ghbeish et al., 2001; Schubiger et al., 2000).

USP Gene in *Aedes aegypti*

Two AaUSP isoforms were cloned by Dr. Kapitskaya (Kapitskaya et al., 1996). The 2.33-kb AaUSP-A cDNA has an open reading frame (ORF) of 484 amino acids encoding a polypeptide of 54kDa, and the 2.14kb AaUSP-B ORF of 459 amino acids encodes a 51.3kDa polypeptide. The difference between USP-A and USP-B is only at the N-terminal portion of the A/B domain. Specifically, in the N-terminal of USP-A there are 31amino acid while there are only 6 amino acid in N-terminal of USP-B. Their binding domain is the 92% and 97% identical to the domains of *Drosophila* (DmUSP) amd *Bombyx* (BmUSP), respectively. The ligand-binding domain is only 57% and 52% identical to those of DmUSP and BmUSP, respectively.

The similarity of USP-A and USP- B suggests that they are derived from the same gene with different promoters (Kapitskaya et al., 1996). But their responses to 20-hydroxyecdysone (20E) indicate that the isoforms of USP perform distinct functions during mosquito vitellogenesis.

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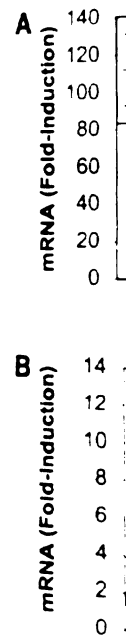
By using *in vitro* and *in vivo* tests we have found that USP-B is predominant in the fat body whereas the USP-A is in the ovary. At different vitellogenic stages the titer of the isoforms also show opposite profiles in fat body. Although USP-A and USP-B can form a functional receptor with EcR, the affinity of EcR-USP-B is two fold higher than EcR-USP-A. In *Aedes aegypti* USP-A mRNA is highly expressed during the pre- and late vitellogenic stages, corresponding to a period of low ecdysteroid titer, whereas USP-B mRNA exhibits its highest level during the vitellogenic period (Wang et al., 2000). It appears that 20E has an opposite effect on the two USP isoforms transcripts in *in vitro* fat body culture (Figure 1.4). 20E inhibits action of USP-A transcription, and on the other hand it upregulates USP-B. This result indicates that the USP-B functions as a major heterodimerization partner for EcR during the vitellogenic response to 20E in the mosquito.

E75

E75 belongs to the nuclear receptor superfamily similarly to EcR and USP. Three isoforms of E75 have been identified in the mosquito (Pierceall et al., 1999). Like other nuclear receptors, they have the A/B, C, D, E, F domains. The different isoforms share the same C, D, E, F domains but have different A/B domains. Further study of E75 regulation is forthcoming.



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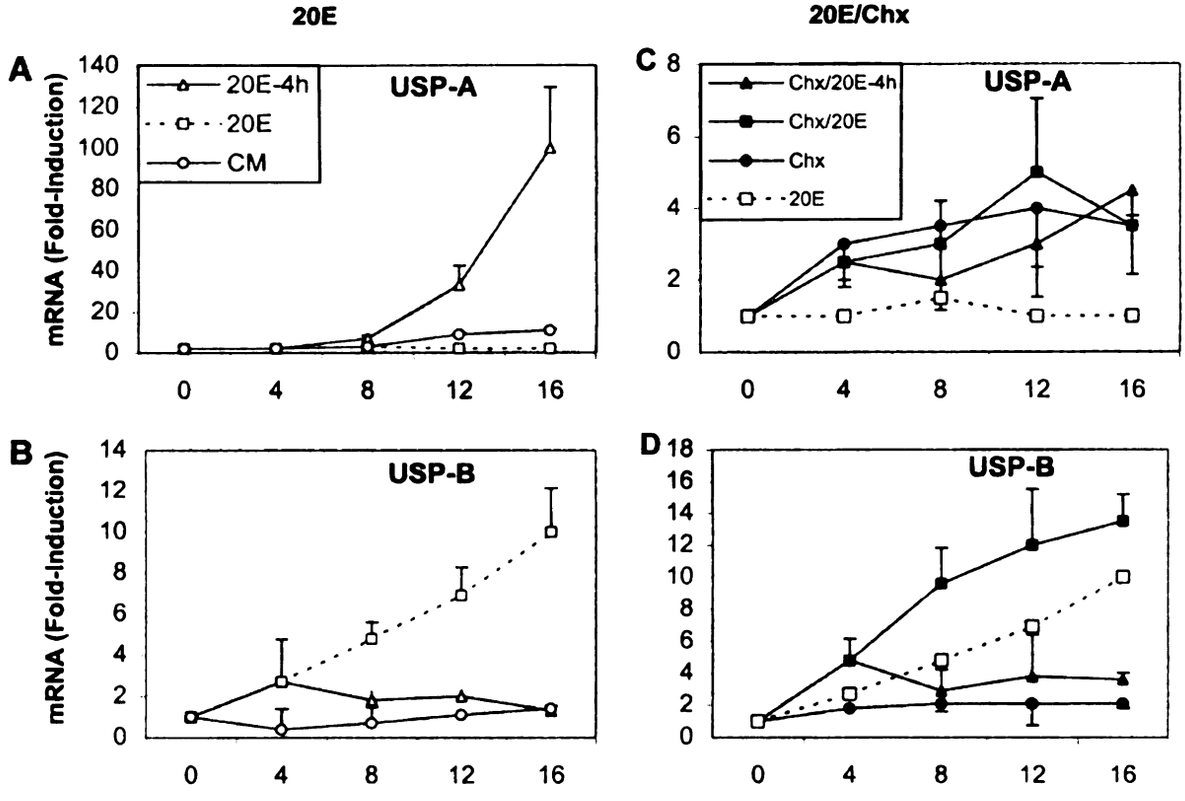

Figure 1.4 USP-A and USP-B expression profiles in vitro fat body culture with 20E and cyclohexomide treatment. (From Wang et al., 2000)

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Broad Complex family

The Broad Complex is another early ecdysone-response gene, which is defined by three complementory functions and encodes several distinct zinc-finger-containing isoforms (Mugat et al., 2000). They play a critical role in the *Drosophila* metamorphosis and different isoforms have the opposite functions. In *Aedes aegypti*, we have discovered 4 isoforms of the Broad complex namely, BRC Z1, BRC Z2, BRC Z3, and BRC Z4 (Chen et al., unpublished), however, the particular function of these broad complex genes has not been identified. Studies in *Drosophila* have given us ideas of how they may be functioning. (Mugat et al., 2000; Figure 1.5)

In the larval stage, BRC Z2 acts as a repressor to prevent the gene expression, however, at the beginning third instar the BRC Z3 is expressed and competes with Z2 to the binding site. Z3 does not appear to activate gene expression, and its job seems to be to compete away Z2 binding during the puparium formation, 20E is created and the EcR-USP complex is produced. Z1 and Z4 are then recruited to the promoter region to initiate the gene expression.

In *Drosophila* studies, the role of broad complex Z2 is a repressor, while Z1 and Z4 act as activators and the Z3 is as a competitor to compete the repressor Z2. Z3 itself is without any activation function.

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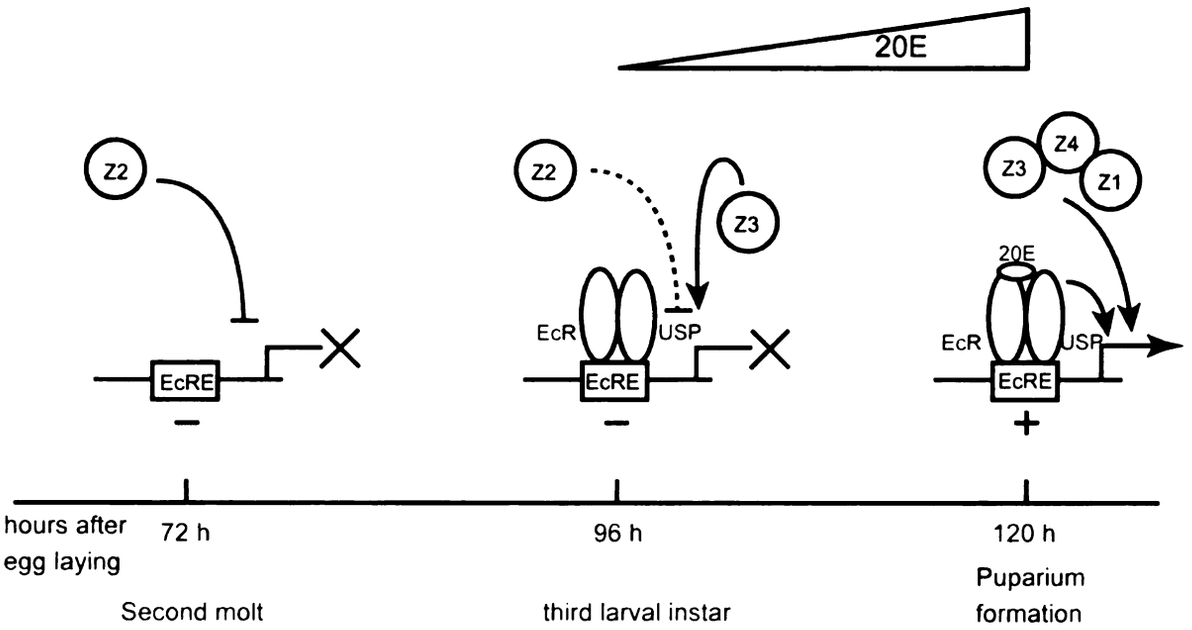

Figure 1.5 Putative model for the regulation of the broad complex in *Drosophila melanogaster* development. Broad complex isoforms control the Fbp1 enhancer. Z2 as a repressor suppresses the gene expression. However, with only Z3, but not EcR-USP the gene still can not be induced. In the later stage of third instar, the titer of 20E increases and Z1, Z3, Z4 and EcR-USP cooperatively up-regulate the gene expression. (From Mugat et al., 2000)

E74

E74 belongs to the Ets transcriptional factor superfamily (Sharrocks et al., 1997). This family has a conserved primary sequence of its DNA-binding domain. This domain is also similar on a structural level and binds to the specific GGAA motif. E74 and E75 are early genes in the mosquito. In *Aedes aegypti,* there are two isoforms of the E74 protein, E74A and E74B (Sun et al. 2002). E74A appears to function as a repressor and E74B appears as an activator and has the synergistic function with EcR-USP complex.

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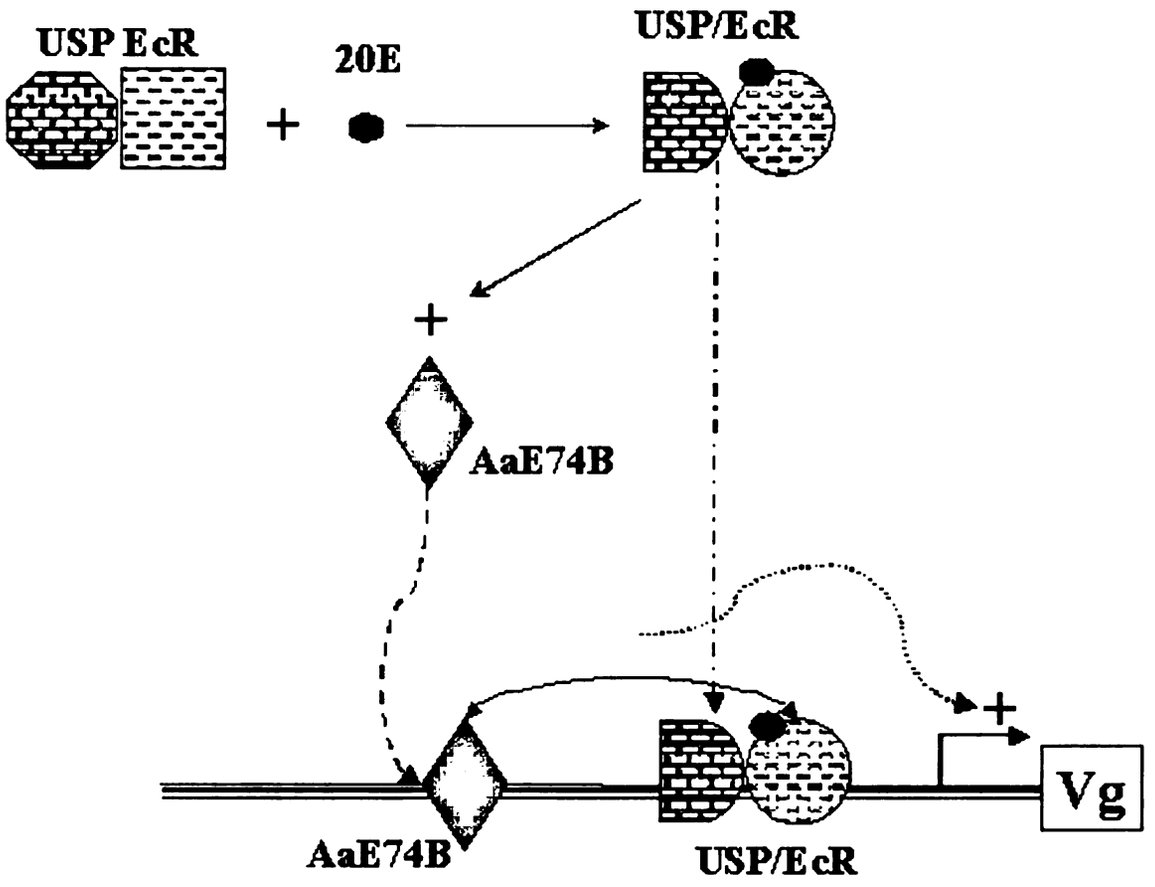

Figure 1.6 E74B functions synergistically with EcR-USP on Vg promoter during vitellogenesis in mosquito. (From Sun, G. Ph.D. thesis, MSU 2003)

GATA factors

The GATA family is the named as such because they bind to the (A/T)GATA(A/G) motif (Lowry et al., 2000). GATA factors contain one or two distinctive zinc-finger DNA binding domains. GATA factors play a critical role in the development. There are 6 GATA factors in vertebrates and variable numbers in invertebrates. In the mosquito, a GATA factor (AaGATAr) has been cloned and characterized (Martin et al., 2001). A hypothetical model of GATA regulation has been proposed. (Attardo et al., Unpublished work; Figure

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1.7) During the previtellogenic period, AaGATAr binds to the Vg promoter to repress the expression of the gene. However, during the vitellogenic period, an unidentified GATA factor appears to translocate from the cytoplasm into the nucleus. It then competes with the AaGATAr and binds to the *Vg* promoter. At the same time, 20E titer increase and the EcR-USP complex forms and initiates Vg expression. We believe the uncharacterized GATA factor acts as an activator increase Vg expression.

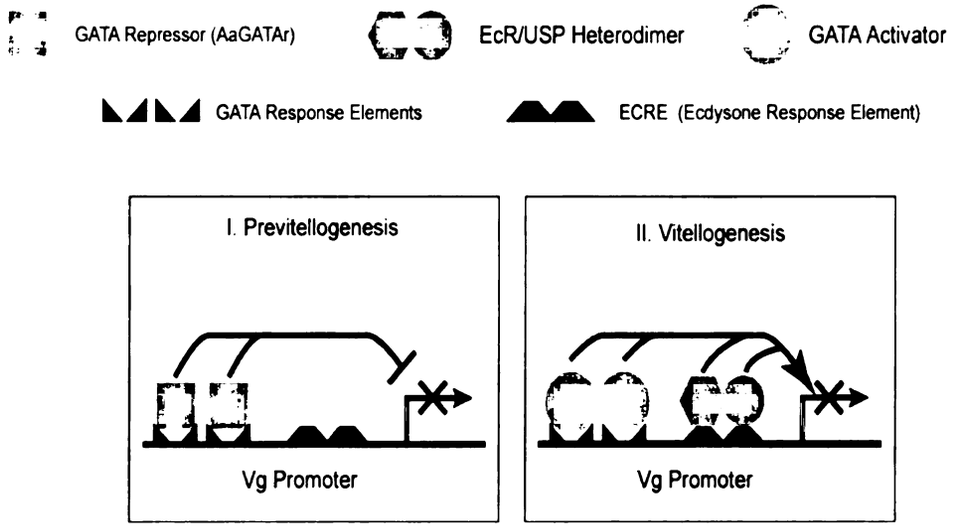

Figure 1.7 Hypothetical model of GATA factors regulation of the Vg promoter. During previtellogenic period, AaGATAr binds to the Vg promoter and represses gene expression. During vitellogeneic period, in conjunction with the EcR-USP/20E complex and other factors, a GATA activator competes with AaGATAr and induces the gene expression. (Attardo et al., PNAS submitted)

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Functional Analysis of USP

There are several functional studies of USP in insects. There is only one isoform of USP in *Drosophila,* and the mRNA of this USP has been detected all developmental times (Henrich et al., 1994). The function of USP in *Drosophila* is not only in the female reproduction, but also in the eye morphogenesis (Oro et al., 1992).

In *Manduca Sexta,* there are two isoforms of *USP* gene called, *USP-1* and *USP-2.* In the epidermis during the larval and pupal molts, the expression of these two isoforms switches from USP-1 to USP-2 due to the rising titer of ecdysteriod (Asahina et al., 1997). The transfection assays shows that USP-1 and USP-2 have different functions to regulate the delayed-early gene MHR3 in epidermis (Lan et al., 1999). The USP-1 and EcR-B1 (the predominant EcR isoform in the epidermis) form the functional heterodimer to up-regulate the gene. However, the other isoform USP-2 has the suppress function to regulate the gene, although all this two isoforms could form the EcR-USP heterodimer complex.

It is also believed that USP could be the nuclear receptor for juvenile hormones (Jones et al., 1997; 2001). The EMSA and fluorescence-based binding assays both showed that the USP has the ability to bind the JH III and change the conformation during the binding.

In the absence of USP, some early hormone responsive genes such as *DHR3* and *E75B* are unable to up-regulate in response to 20E, however, other genes that usually express later are expressed precociously such as FTZ-F1 and Broad complex Z1 (BRC-Z1). The unliganded EcR-USP complex suppresses the gene expression during the early phases of steroid driven development. USP independently acts as a repressor to control

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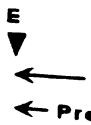

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some early gene expression in laval stages of insects (Ghbeish et al., 2001).

Current studies in *Aedes aegypti* have suggested a working model of modulation of the cyclicity of vitellogenic ecdysteroid-mediated signaling through alternative heterodimerization of USP. USP changes different patterns during the vitellogenesis (Zhu et al., 2000; Zhu et al., 2003; Figure 1.8). In previtellogenic period, AHR38 as the partner of USP prevents the formation of the EcR-USP complex by binding to USP and 20E dependent transactivation is blocked. After a blood meal, in the vitellogenic period, the EcR-USP complex forms and activates the early genes and the late genes such as Vg due to the increasing titer of 20E. However, in the termination stage, USP changes partners again from EcR to Seven-up (Svp), repressing USP-based hormone responses.

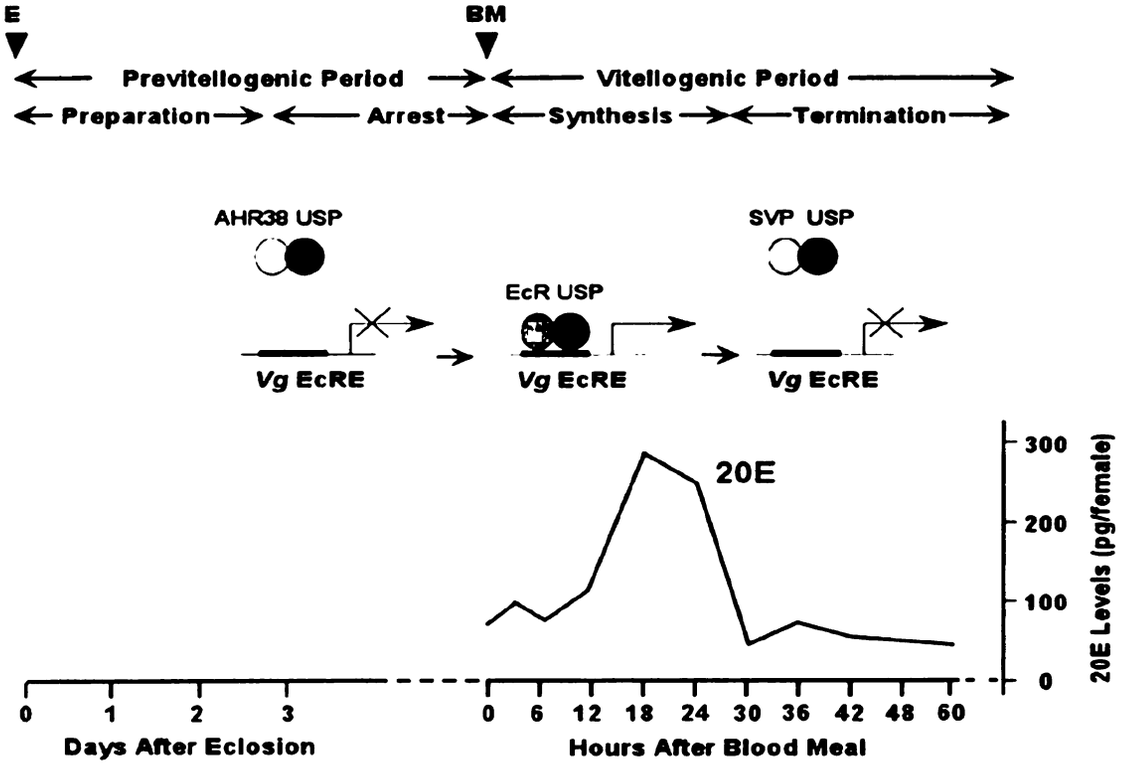

Figure 1.8 Model of the different heterodimers of USP during the vitellogenesis in mosquito. USP changes different partners during the whole vitellogenesis from AHR38-USP, to EcR-USP, to Svp-USP. (From Zhu et al., 2003)

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Significance of current research

The primary focus of Dr. Raikhel's research is to characterize the molecular regulatory hierarchy mediating vitellogenesis in the adult mosquito *Aedes aegypti.* There are several yolk protein precursor genes which have been studied. These genes are regulated in the fat body by the hormone 20E and are triggered by blood feeding. This hierarchy system is an ideal model on which to research ecdysone related genes during the insect reproduction.

In the mosquito, two isoforms of USP that are the partners in the heterodimeric EcR-USP complex have been cloned in our lab. Questions regarding these two isoforms still need to be addressed. There is only one isoform of USP found in the *Drosophila*, and the expression of this gene is constitutive. In *Manduca sexta,* two USP isoforms have different functions in regulating the delayed-early gene. Therefore, the key questions are why are two isoforms of AaUSP, which have different expression pattern in fat body, and what is the role of these two isoforms in the ecdysone regulation system. Another question is what factors control the differential expression of USP-A and USP-B during the vitellogenesis, and what the function is of these regulatory elements.

My research focuses on these questions and provides more information on the basic mechanism of transcription regulatory in the mosquito. Furthermore, the cloning and characterization of the regulatory regions of the two isoforms of USP will be of use in the construction of new transgenic mosquitoes for the purpose of controlling the disease transmission.

Chapter 2 – Materials and Methods

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The *Aedes aegypti* mosquitoes were reared as described by Hays and Raikhel (Hays et al., 1990). After 3 to 5 days of post-eclosion, vitellogenesis in the female mosquitoes was initiated by blood feeding on anesthetized rats. For dissection, mosquitoes were placed at 4° C. Fat bodies were isolated in Aedes physiological saline (APS) (150 mM NaCl, 4 mM KCl, 0.1mM $NaHCO_3$, 0.6 mM $MgCl_2$, 25 mM HEPES buffer, 1.7 mM $CaCl_2$, pH 7.0). Dissected previtellogenic and vitellogenic fat bodies were then quickly frozen in liquid nitrogen and stored at -80 ° C.

Oligonucleotides and Probes

Oligonucleotides were purchased from the Macromolecular Structure Facility of the Biochemistry Department at Michigan State University and GIBCO BRL. For DNA binding studies, a pair of sense and antisense olionucleotides were annealed, and resolved by 4% to 20% non-denaturing poly-acrylamide TBE gel (Bio-Rad) (Bio-Rad Laboratories, Inc.), and the appropriate bands of double-stranded oligonucleotides were isolated.

Nucleotide Sequence

Sequencing analysis was performed at the DNA sequence facility in Michigan State University and at University of California, Riverside.

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Single Strand DNA Labeling

Single strand DNA labeling was used for genomic screening. Only one single stand primers was added into each reaction mixture with α-^{32}P dCTP. The mixture was prepared as followed: 10X PCR buffer, Mg^{2+} 25 mM, 25ng denatured single strand DNA, anti-sense primer 10 μM, 0.5 mM dATP, dGTP, dTTP mixtures 1 μl, cold dCTP 1.65 μl, Taq polymerase (5U/μl) 0.5 μl, and the α-^{32}P dCTP(3000Ci/mM) 10 μl. PCR amplification was performed using the following conditions: initial denaturation at 94℃ for 2 minutes, denaturation at 94℃ for 30 seconds, annealing at 60℃ for 30 seconds, extension at 72℃ for 5 minutes for 35 cycles. The labeling probes were purified by using Bio-Rad (P-30) columns and the cpm value was counted.

Genomic DNA Extraction and Southern Blot Analysis

For mosquito genomic DNA isolation, an individual mosquito was ground in microcentrifuge tubes with extraction buffer (100mM Tris-HCl PH 8.0, 100mM EDTA PH 8.0, 100mM NaCl, 200ug/ml Proteinase K, 0.5% SDS, abd 1% Nonidet P-40). The extraction was incubated at 55℃ overnight. RNase A (5mg/ml) was then added, and incubated at room temperature for about 30 minutes. The treated product was extracted with phenol and chloroform, and ethanol precipitated. The pellet was washed with 70% ethanol and resuspended in TE buffer. The genomic DNA and then digested separately with restriction enzyme Bgl II, Xba I, and Sal I. The digestion products were separated on 1% aragose gel, and analyzed by Southern blot.

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RNA Extraction

The fat bodies of previtellogenic and 24 hours post-vitellogenic mosquitoes were homogenized in Triazol reagent (Gibco BRL/Invitrogen, Carlsbad, CA), and the fat bodies were homogenized by a mechanical homogenizer (South Jersey Precision Tool and Mold Inc., Vineland, NJ). Optional Triazol were followed in the first step of homogenization and third step of RNA precipitation to remove insoluble cuticle and to prevent contamination of proteoglycan and polysaccharide. Purified RNA was then resuspended in RNAse/DNAse free ddH_2O. RNA yield, quality, and purity were determined by spectrophotometric analysis using a Beckman DU 530 UV/Vis Spectrophotometer.

Rapid Amplification of cDNA Ends (RACE) Analysis

To determine the transcriptional start sites of the two USP isoforms, 5'-RACE experiment was performed using Invitrogen's RACE kit (Invitrogen Corporation). One hundred micrograms of female fat body were collected at 24 hours post blood meal. The 5' primer and 5' nest primer were provided by the kit. The 3' primers used were USP-A original primer: 5'-TTCACATTCACGCGTTGTTCAC-3', USP-A nest primer: 5'-GGGATATCCCACCATGATCAT-3', USP-B original primer: 5'-AACGTCGTCGTCTTCCGTCC-3', and USP-B nest primer: 5'-AGTCGTTTGACGTTCCTCCACG-3'.

The single band representing USP-A and USP-B was sequenced and checked by southern blot.

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RNAse Protection Assay

Total RNA from female mosquitoes 24 hours post-blood-meal was extracted using the Trizol. The concentration and purity of the RNA were determined by UV spectroscopy at 260 and 280 nm. USP-A and USP-B PCR products that were around 125 bp in length were cloned into PCR IV vector (Invitrogen) using the TA cloning Kit (Invitrogen). The resulting plasmids were sequenced by T7 and T3 primers to determine the specific promoter. Radiolabeled antisense RNA probes for USP-A and USP-B were transcribed with T7 RNA polymerase and T3 RNA polymerase respectively after linearization of cloned USP-A and USP-B PCR IV vectors. The RNase protection assay (Ambion Europe Ltd.) was performed on α-^{32}p-labeled UTP labeled USP-A and USP-B probes. Briefly, labeled transcripts were mixed with approximately 100 μg RNA, and were then suspended in hybridization buffer. The RNA mixture was heated for 10 minutes at 90℃ and hybridized for 36 hours at 42℃. For studies on RPA, 1.0 pg RNase A buffer was added. Digestions were performed at 37℃ for 30 minutes. The ribonuclease digestions were terminated by addition of RNase inactivation/Precipitation Solution (Ambion). The RNase-treated mixtures were incubated at -20℃ for 15 minutes for removing the supernatant. USP-A and USP-B were sequenced by using USP-A primer: 5' AACCGACCGACAAACCGAAG-3' and USP-B primer: 5' CAACAATCGAAACAAAACTTTTCC-3'. The samples were subjected to denatured polyacrylamide gel electrophoresis which were dried and exposed to Kodak X-OMAT X-ray films (Eastman Kodak Co., Rochester, NY, USA) for 6 to 72 hours for generation of autoradiograms.

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DNA Sequence Analysis

The promoter regions of USP-A and USP-B were obtained and compared to the sequence database using NCBI Blast and Chromas 2.0. The presence of transcriptional regulatory elements in USP-A and USP-B promoters were searched through the TRANSFAC database (Heinemeyer et al., 1998) and the TESS database.

Polymerase Chain Reaction (PCR) and Genomic Library Screening

For USP-A and USP-B, PCR primers were designed based on sequences that were obtained from RACE assays. The following PCR primers were used:

USP-A Forward: 5'-TCACCATACAGAAGCGAGGG-3'

USP-A Reverse: 5'-GGGATATCCCACCATGATCAT-3'

USP-B Forward: 5'-GATTGTTGATGTTGCAATTTCC-3'

USP-B Reverse: 5'-AGTCGTTTGACGTTCCTCCACG-3'

USP-A Sequence: 5'-CAACGACGATAACACCCCACAT-3'

USP-B Sequence: 5'-CAACAATCGAAACAAAACTTTTCC-3'

USP cDNA was prepared by Shengfu Wang (Michigan State University). The PCR condition for amplification of USP-A and USP-B screening probes was as followed: initial denaturation at 95℃ for 15 min, followed by 30 cycles of denaturation at 95℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds. A band of approximately 200 bp was PCR amplified. Isolated PCR products were labeled using Invitrogen Random

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Labeling Kit (Invitrogen life technologies) and purified by Bio-Rad purification columns. The labeled probes were then used to screen the *Aedes aegytpi* genomic library (prepared in Alexander Raikhel's library). The hybridized phage clones were extracted using QIAGEN Lambda Midi Kit (Qiagen Inc.). Isolated genomic DNA clones were sequenced by primers designed according to the sequence results from the RACE assays.

The genomic fragments were subcloned into pBluescript and sequenced.

Real-time Quantitative PCR

RNA was collected from previtellogenic mosquito fatbodies at 12-hour intervals immediately after eclosion to 96 hours post eclosion. A vitellogenic profile was also created using mosquito fatbodies from 0 hour to 96 hours post blood meal. The expression profiles were taken at 4 hour intervals. Equal amounts of total RNA were used to synthesize cDNA from each time point. Analyses of mRNA levels were determined using gene-specific real time PCR primers. All data obtained were normalized by the amount of actin mRNA level which is constitutively expressed. The known *vitellogene* expression profile was used as control (Raikhel et al., 1999).

Total RNA (3μg) was treated with DNAseI (Gibco BRL/Invitrogen) to remove genomic DNA contamination. DNAse I- treated RNA was then directly used for cDNA synthesis using the Omniscript Reverse Transcriptase kit (Qiagen). cDNA levels form fifty samples were quantified by real-time PCR. A real time PCR master mix was used for cDNA amplification. The following primers were used at 250mM.

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Actin Reverse: 5' GCACAGCTTCTCCTTAATGTCAC-3'

Vg Forward: 5' GCAGGAATGTGTCAAGCGTGAAG-3'

Vg Reverse: 5' ACGAGGACGAAGAATCGGAAGAG-3'

USP-A Forward: 5' CGAACCAAATCTACGCTGACG-3'

USP-A Reverse: 5' CACGGTTATTGATGGTAAAGTG-3'

USP-B Forward: 5' ACGTAAACAACAGGACCAGTAGG-3'

USP-B Reverse: 5' CCGAAATTCGCGACGGCGATC-3'

All real-time PCR reactions were duplicated with 2 μl of cDNA per reaction. All duplicated samples were then run on the iCycler real time PCR machine (Biorad). Standard curves used to quantify relative gene concentrations were made from 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest. All experiments were performed using the same serial dilution set for generating the standard curve. The program used for amplifying the reactions was in the following: 1. Cycle 1: melting 95℃ for 15 minutes; Cycle 2: melting 94℃ for 15 seconds, annealing 59℃ for 45 seconds (Florescence recorded), repeating 40 times; Cycle 3: melting 95℃ for 1 minute; Cycle 4, melting 95℃ for 10 seconds, repeat 70 times and for every repeat the temperature was decreased 1℃.

Real time data, collected with the iCycler iQ Real Time Detection System Software V.3.0 (Bio-rad), were analyzed.

In vitro Synthesis of Proteins

Proteins (E74, Broad complex Z1, Z2, Z4, EcR and USP) used for EMSAs were

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synthesized using a coupled *in vitro* transcription-translation TNT system (Promega). The E74 cDNA was cloned downstream of the T3 promoter in pBluescript (Sun et al., 2002), The Broad complexes Z1, Z2, and Z4 were cloned downstream of the T7 promoter in PCR II (Invitrogen). The ECR and USP cDNAs were cloned downstream of the T7 promoter in pBluescript. Each of cDNAs contained full length open reading frames (ORFs). Control TNT reactions were performed in the presence of [^{35}S]-methionine in order to confirm proteins with the expected sizes that were expressed. The resulting reactions were analyzed by SDS-PAGE and autoradiography. The TNT reactions were conducted at 30℃ for 1.5 hours, and stored at -80℃.

Electrophoretic Mobility Shift Assays

EMSAs were performed using TnT *in vitro* synthesized proteins as mentioned above. Each TnT reaction was used alone or in combination for EMSA. Proteins were incubated for 15 minutes at room temperature in electrophoretic mobility shift buffer (Promega). Binding of EcR and USP proteins to EcRE was assayed in the presence of 0.5μM 20E. The probes were labeled with [γ-^{32}p]dATP by T4 DNA kinase (Promega). For competition experiments, 25-fold to 100-fold excess of unlabeled competitor oligonucleotide DNA probes were used. Direct binding and competitive binding products were loaded on a 5% TBE nondenaturing polyacrylamide gel (Bio-Rad) and electrophoresed in 0.5X TBE buffer at 100V for 90 minutes at room temperature. After electrophoresis, the gels were dried and autoradiographed either by an intensifying screen at -80℃ or by phosphor imaging.

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The oligonucleotides used are listed below.

USP-A E74 C1: 5'-ATGTAGAATTTCCTGAGGAAATTGCCTACTT-3'

USP-A E74 C3: 5'-GCGAAAAGTTTTCCGGACTGAAGCGGGAAT-3'

USP-A BRC C-Z1/4: 5'-AATCTTTCGAAAAACAAAATAAAGTAC-3'

ECRE DR1: 5'-GATCCGTAGGGGTCAGAGGTCACTCGAGATC-3'

USP-A ECRE C5: 5'-TCACATGAGTTCAGCAGACGATGTGATCAAGGA-3'

USP-B BRC Z1-C1/4: 5'-TACTGGTCCTGTTGTTTATAGT-3'

USP-B BRC Z2 C1: 5'-CGCATTCTTGAGCTATTCCTTGCAGA-3'

USP-B BRC Z4-C1: 5'-TATTATTTTGAAATGCAAGCTGA-3'

USP-B BRC Z4-C2: 5'-GGAATCGCTAAAGAAATTTCTC-3'

USP-B ECRE C5: 5'-CTTGGGGTTCATTCACATATTTCATAACGC-3'

USP-B ECRE C6: 5'-GAAATTGACCATTTTTGACACCCACC-3'

Chapter 3 –Results

Introduction

As described previously in Chapter 1, the expression of the two USP isoforms is differentially regulated by 20E. The goal of this study was to identify and to characterize the regulatory regions of USP-A and USP-B. These two isoforms display great conservation in both tissue- and stage- specificity in mosquitoes. Thus, it is important to understand different mechanisms that regulate the USP-A and USP-B promoters. Additionally, the USP protein is a critical component of the ECR-USP heterodimer. Characterization of the promoters will enrich our knowledge about mechanisms of the 20E regulatory hierarchy in the mosquito fat body.

Some of the work described in this chapter was previously reported (Wang et al., 2000), and (Kapitskaya et al., 1999). The two *USP* isoforms from *Aedes aegypti* were cloned previously. The expression profiles of both isoforms were examined, and it was determined that USP-A and USP-B are differentially regulated in the presence or absence of 20E (Wang et al., 2000). In this report, I will describe my work on the cloning and characterization of the USP-A and USP-B regulatory regions.

The Copy Number of USP Gene in Mosquito Genome

To determine how many copies of the USP gene exist in the mosquito genome, genomic southern blot was performed by using newly emerged female mosquitoes. The southern blot was performed using genomic DNA that was isolated from two different

mosquitoes.

A specific probe of about 200 bp in length, in the common portion of cDNA from the two USP isoforms, was used in hybridization. In lane 1, the cDNA was used as positive control. Genomic DNA digested separately with either Bgl I, Sal I or Xba I only showed one hybridization band (Figure 3.1 lanes 2-4). Although the sizes of the bands were different, the signals indicated a single copy of the USP gene is present in the mosquito genome. To further verify this result, another mosquito from a different batch (group 2) was used to perform the same experiment (Figure 1, lanes 5-7). Though the DNA yield of the second group was less than that of the first, hybridization results confirmed the presence of a single copy of the USP gene in the mosquito genome.

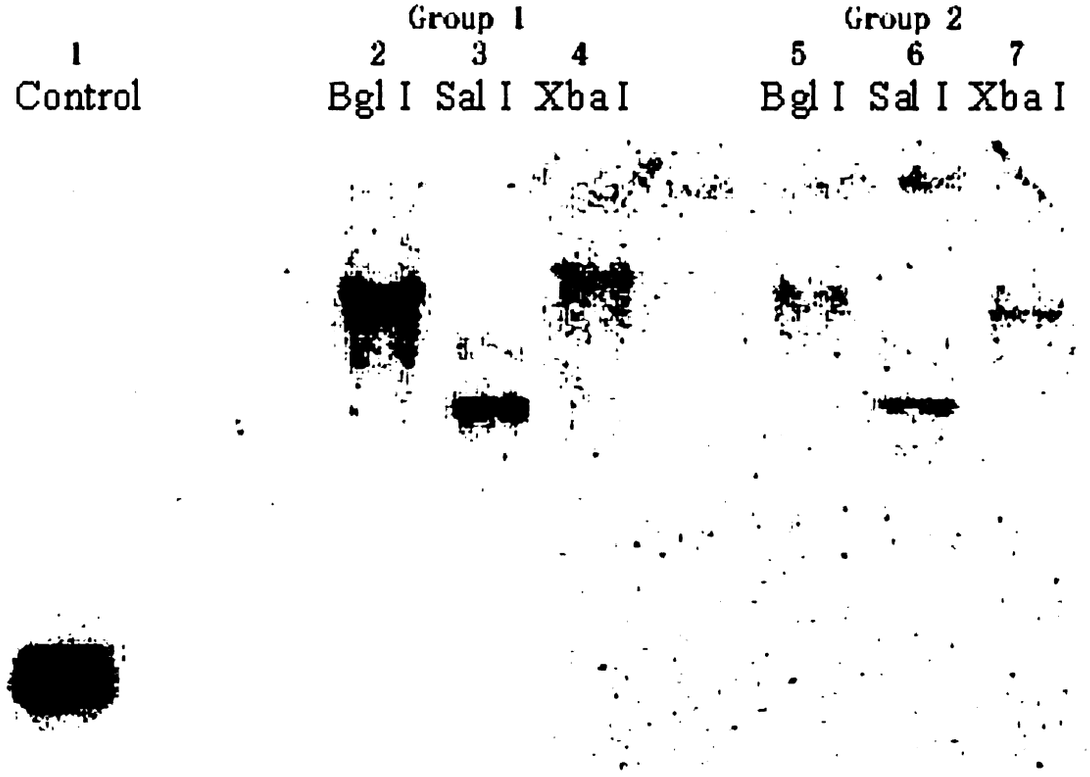
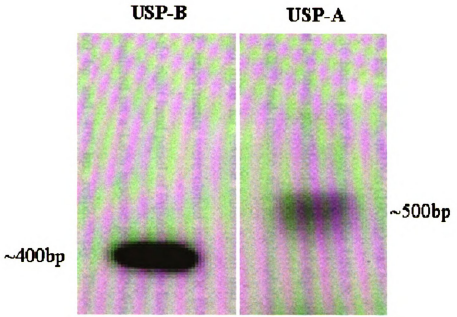


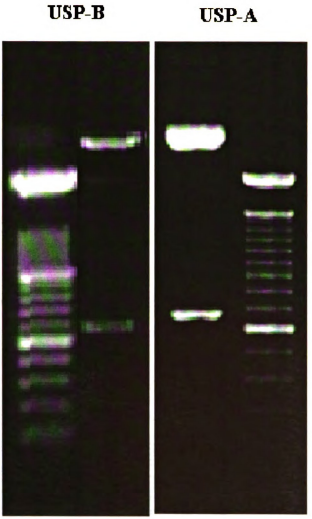
Figure 3.1 Genomic southern blot of the USP gene. Genomic DNA was extracted from a single mosquito. The DNA was separated into three parts and digested by different enzymes and then performed southern blot. Group 1 and 2 represent genomic DNA from 2 mosquitoes. The USP cDNA was used as positive control.

Figure 3.2 The RACE products cloning and verification. RACE products were cloned into the pBluescript vector and verified by the southern blot. The RACE products of USP-A and USP-B were cloned into pBluescript and digested by the EcoR I as shown in panel B. These gels were then used to perform a southern blot and probes were selected in the specific region according to the cDNA sequence from the USP-A and USP-B.

A.



B.



Identification of Transcriptional Start Site

The accurate mapping of the initiation site is vital to isolate the promoter regions of USP-A and USP-B. Generally, three are four methods to identify the transcriptional start site of a gene, namely the primer extension assay (PE) (Boorstein et al., 1989; Ghosh et al.,1978), the RNase protection assay (RPA) (Melton et al., 1984), the S1 nuclease assay (Berk and Sharp 1977), and the rapid amplification of cDNA ends (RACE) (Frohman et al., 1988). Due to the difficulty and importance of the initiatial site, for most genes, two or more methods should be used to avoid the mistakes in identifying the start site. The parallel results from the experiments can give us most accurate information about the transcriptional initial site for further study.

First the rapid amplification cDNA ends (RACE) was used to isolate isoform-specific 5' cDNA region. For USP-A, mRNA (100ng) was isolated from the fat bodies of female mosquitoes at 36 hours post blood meal. For USP-B, mRNA (100ng) from fat bodies was obtained at 24 hours post blood meal. After digested by two enzymes, calf intestinal phosphatase (CIP) and tobacco acid pyrophosphatase (TAP), the RNA was linked to the RNA oligo linke for reverse transcription and PCR amplification. The products of the RACE experiments were cloned into PCR IV (Invitrogen) and were digested by EcoR I. In the right hand panel of Figure 3.2, the EcoR I digested DNA from positive clones was visualized on a 1.0% agarose gel. USP-A and USP-B products are indicated at about 500 bp and 400 bp, respectively. These DNA fragments were transferred to nylon membranes for southern blot analyses.

Panel A of Figure 3.2 shows that both RACE products hybridized to USP-A and

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USP-B probes corresponding to specific regions in the A/B domains. These clones were then sequenced to determine the transcription start sites.

Verification of USP-A and USP-B Transcription Start Sites

RNase protection assay (RPA) was then performed to verify the transcriptional initial sites of USP-A and USP-B. Based on RACE results, the DNA fragments for RPA were selected from -50 to +100 bp around the putative initiation sites in the genomic sequences of USP-A and USP-B. Using these as template, single strand complementary RNA was transcribed and labeled *in vitro* with α- ^{32}P UTP.

One hundred micrograms of fat body RNA was hybridized to USP-A and USP-B mRNA probes overnight. The resulting double stranded USP-A and USP-B RNAs formed were digested by RNase I and compared to the genomic sequences.

Results indicated in Figure 3.3 showed that the size of the protected products of USP-A (87 bp) and USP-B (56 bp) confirm the transcriptional initial sites as previously identified by RACE.

Cloning the Promoter Regions of the Mosquito USP Isoforms

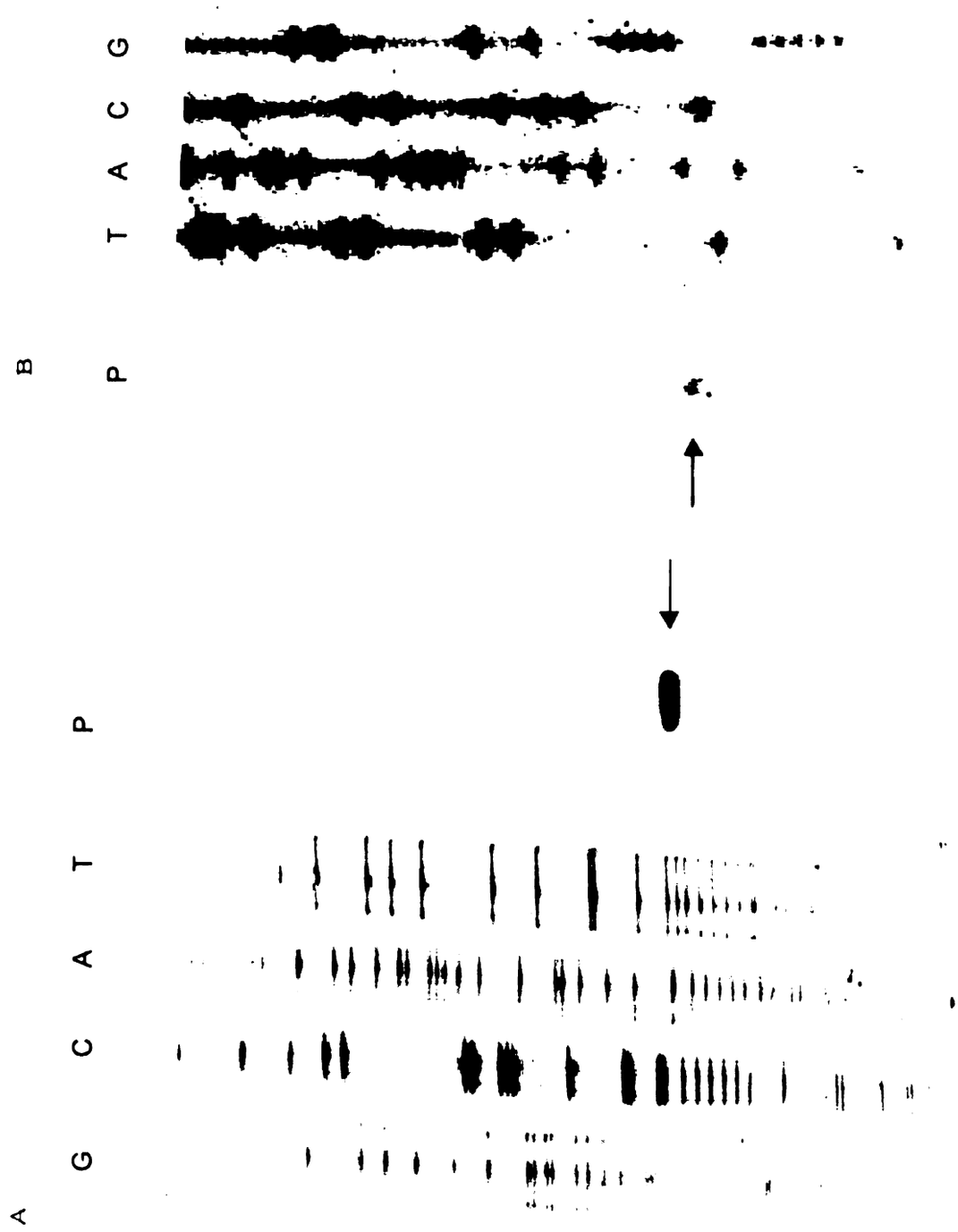
The promoter regions of USP-A and USP-B were obtained by screening a Lambda Fix II genomic library from *Aedes aegypti.* The probes were selected from the

cDNA sequences of USP-A and USP-B and labeled by random labeling kit with α-^{32}P dCTP. The probes were then hybridized to the genomic library and positive plagues were picked and extracted the DNA. These phagure DNA then cloned into the pBluescript vector and sequenced to compare them to the cDNA sequences. In the first two rounds of screening, it was found that both USP-A and USP-B 5' untranslated regions (UTR) spanned more than 20 kb in length, most of which were intron's sequences. The third round of primers for USP-A and USP-B were moved further upstream (+100 bp) for screening. The promoter regions of USP-A and USP-B were isolated. The lambda DNA was then extracted and sub-cloned into pBluescript. The sequenced promoter regions obtained for USP-A and USP-B were 1.6 kb and 1.5 kb in length, respectively (Figure 3.4). Both USP-A and USP-B promoter are TATAless, but they all contain the initiator element which consensus sequence is PyPyA+1NT/APyPy (Carey et al., 1999).

Figure 3.3 RNase protection assays of USP-A and USP-B.

A. The mRNA from 24 hours PBM fat body annealed to the complementary mRNA that was transcribed *in vitro* by the T7 polymerase. The synthesized mRNA is a fragment from 125 bp around the transcription initial site. The sequencing ladder (GCAT) was prepared from the USP-A genomic clone with the same primer as that used for RNase protection. P denotes the RNase protection products.

B. The mRNA from 24 hours PBM fat body annealed to the complementary mRNA that was transcribed *in vitro* by the T7 polymerase. The synthesized mRNA is a fragment from 125 bp around the transcription start site. The sequencing ladder (TACG) was prepared from the USP-B genomic clone with the same primer as that used for RNase protection. P denotes the RNase protection products.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CATAAGCTAATGATAATTAAGGTTTTTGCCAAAATTGCGTTTAGTTCA
TGCGCAAATTTGTCAACTTTTTGAAACAATGATTTCTATCATTTTCAC
TGACACTGCCGAAAATTCATGTCTCACATGAGTTCAGCAGACGATG
TGATCAAGGAAAATGCGGTTCCTACTAAAAATGACATCGCCGAAAA
CGATTCCGTTGTCAAATCTTTCATAAGTTTATTCAATTAGGCAACATT
AACTAATTACTATTACGAATGTAGAATTTCCTGAGGAAATTGCCTACT
TTTAGGCGTATTCCGCGGTTCAAGGTGTTTTTAATTTTGAAATAACTC
AAAAAGTAAATGACTTGTAAGAGTTTGGTCTTCATATTCGGCTTCAG
GGGCCCTGATTTTAGTAAGTAACGACATTTCCAATATTAGCGAACGT
TGTTTACTTGGGTGATCAATGTTACCCCATATCAGCTGAATCAAAAA
ATTACTTAAAACATTTATTTAAAAATGTTTAAATCTTTCGAAAAACAA
AATAAAGTACATAGTTAGGAGCGGTGGGTGCCAGTACTTGTTTTAAA
AATATGAAACTTGTAACATTTGTATTGTGAAATGAAAGATTTAAGAA
AAACTAAAAAAAAATGATCAATGTTACCCCGGATTACGGTACCTGACT
TGTTTTTTAATGCTTCCAGTGGGCGCTTTGCCCTTTGAAGGAAGCAC
CACACTAGACAACGGACTAGCATGCAACGCCGAGTGGCACAGTCG
AAATACATTTCTGGCGAAAAGTTTTCCGGACTGAAGCGGGAATCGA
ACCCACACCTTGACTTGATGCGGCTAAAATGCTTGGTAACACTAACC
GCACGGCCACGAGGCCCACAAATACTTATGCTATGGGAAAGGCGAC
ACATCAAGACAGGTCCTACGAATTTTGAAAATTTTCGTAGACATGCA
GCTAGTCATAGAAATAGATTGTCCAGCACATTTTTTCCCGGTAATAAC
GTTCTGAGGAGCACTGCGACAGGAGCCGTGTAGGAGCAGCGCTTG
AACCCTCTCCCCTTGAGGGGAGCTTCTTCCTTGCCGGTATGTTTTGA
TGCCATTGCGACGAGCTTGTTCGCTCCGCTCTCTCGCTAGTCGCTAT
CTCCGGTGGCGCGGAGTCCCTCTCGCGGTATTCTGCTGCGCTCTGCT
GCTGCGGTAGCTTCTATATTGTTTTTATTGTTTTCGTTCATAGCCGTG
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TCTGGTCTACCTTGTTCAAAGTCAACTCCCGTCCGTCGTCACAGTC
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GTCGGTTGGTCGATCCAATTTGGGCGCCAATTTTCATCTCTAGTCCC
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AACAGCTAGAAAAATAATATTTAATCGATAATACAGCGAAGCTGTA
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TTCATTCACATATTTCATAACGCCGAAATTGACCATTTTTGACACCCA
CCCACCCCCTCGTAACGCTTTTTGTATGAATATTCTAAAAACTTCGTA
TGAGCCGTAACATCGTGAGGACAACCGCCCACCCACCCCCTTCAGCG
TCATGAAATTTGTGAATAAGCCCTTGTCAGCAGCGAATCGGGCTATA
CTCCACTGCACTGTGACGTCACGCATCAATTTTGACAGGTACTGGTC
CTGTTGTTTACAGTTTGGACTTAGTACTTTTTTCGAATCATTCTTAATT
TTGTGAAAGTTTGCTGCGAGGAGAGGTCAAATCCACTGCAGATACCC
ACAGATCGTATTATTCTATCTTCCTACCGTGGAAAATCGTCACCATC
AGCATGCTGGTGATAGAAATGCGAAGATGAAAAAATGATGGAAAAA
ACGACTAAGTCCGAAACGTAAACAACAGGACCAGTAGGCTAGTCAT
ATGCAGCGCTCTATATCCAAAAATGTTGTATGCAAATTTTGTTCCGT
GTATGTATAACGGGCAGCCTCTTGAGCACCCCTGAGTACACTTTTC**A**
GCTTTTTCTGCTGCTGCTTGCTCGAAGTAAACTCGCGGATCGTCGTCG
CGAATTTCGGAAAAGTTTTGT

Figure 3.4 Sequences of the regulatory regions of the two USP isoforms. These sequences show the promoter regions of two USP isoforms. The USP-A (panel A) and the USP-B (panel B) promoter regions are shown. The bold red letters indicate the transcription start sites.

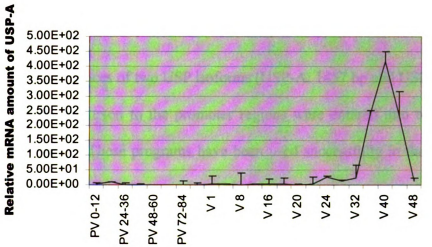
Expression Profile of USP Isoforms

To determine the kinetics of USP-A and USP-B expression, real time PCR was performed to examine the mRNA levels in the fat body of female adult mosquitoes.

In the previtellogenic period, USP-A transcripts showed a 2-fold increase above basal level (1 relative mRNA amount) within 12 to 24 hours after eclosion, and then they dropped to a basal level and remained there from 24 to 96 hours post emergence. However, after a blood meal, the mRNA level started to increase at 20 hours PBM. After 28 hours PBM, the level of USP-A mRNA rose dramatically. The amount of mRNA increased by up to 400-fold and peaked at 40 hours PBM. The peak then started to decrease to the basal level at 48 hours PBM, which is the termination stage of vitellogenesis.

USP-B displayed a different expression profile as compared to that of USP-A. During the previtellogenic period, the amount of mRNA remained at the basal level (1 relative mRNA amount). However, the USP-B mRNA level increased after blood meal and reached its highest point at 24 hours PBM. After that, the amount of transcript decreased sharply and reached the basal level at 40 hours PBM.

A.



B.

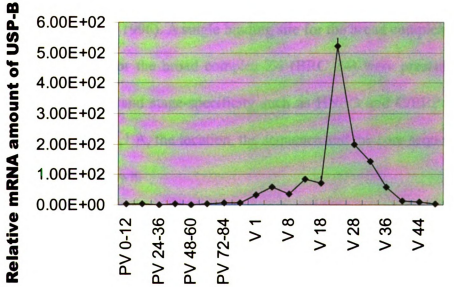


Figure 3.5 Profiles of relative mRNA amount of USP-A and USP-B. The relative mRNA amount of USP-A is shown in panel A, and that of USP-B is shown in panel B.

Analysis of USP Promoter Regions

The DNA sequences of two USP isoforms (USP-A: 1457 bp and USP-B: 1646 bp) spanning from the first exon to the promoter regions were entered into two programs, TESS and TRANFAC. These programs have been used successfully in the past for this purpose to search for putative transcriptional binding sites.

Two E74 binding sites with the consensus sequence 5'-GGAA-3' were identified in the USP-A promoter. A search for ecdysone response elements (ECREs) using to the consensus sequence (PuG(G/T)TCA) reveal a set of inverted repeats with 0 to 12 spacers inserted. (Antoniewski et al., 1996). A single binding site for the broad complex Z1 (BRC Z1) and two binding sites for the broad complex Z4 (BRC Z4) were present. Binding sites associated with tissue- and stage-specificity such as HNF/3 and C/EBP were also located. As shown in Table 3.1 A, the location, the sequence and the core sequence of the putative binding sites are shown.

Similar analysis was done with the USP-B promoter. A single Broad Complex Z1 and Z2, as well as three broad complex Z4 binding sites were located. Eight EcREs, C1 to C8, were found in the promoter region. The USP-B promoter, like that of USP-A also contained HNF/3 and C/EBP binding sites (Table 3.1b).

A.

Binding site	Sequence	Core sequence	Beginning site (bp)
E74 C1	ATGTAGAATTTCCTGAGGAAATTGCCTACTT	GGAA/TTCC	-1056
E74 C3	GCGAAAAGTTTTCCGGACTGAAGCGGGAAT	GGAA/TTCC	-547
Broad Complex CZ1/4	AATCTTTCGAAAAACAAAATAAAGTAC	ACAA/TAAA	-810
Ecdysone Response Element C5	TCACATGAGTTCAGCAGACGATGTGATCAAGGA	(A/G)G(G/T)TCA TGA(A/C)C(C/T)	-1188

B.

Binding site	Sequence	Core sequence	Beginning site (bp)
Broad Complex CZ1/4	TACTGGTCCTGTTGTTTATAGT	ACAA/GAAA	-1010
Broad Complex Z2	CGCATTCTTGAGCTATTCCTTGCAGA	CTAT	-624
Broad Complex Z4 C2	TATTATTTTGAAATGCAAGCTGA	TAAA	-1007
Broad Complex Z4 C3	GGAATCGCTAAAGAAATTTCTC	TAAA	-324
Ecdysone Response Element C5	CTTGGGGTTCATTCACATATTTCATAACGC	(A/G)G(G/T)TCA TGA(A/C)C(C/T)	-550
Ecdysone Response Element C6	GAAATTGACCATTTTTGACACCCACC	(A/G)G(G/T)TCA TGA(A/C)C(C/T)	-489

Table 3.1 The sequences, core sequences and location of the putative binding site in USP-A (Panel A) and (Panel B) promoter

Identification of Cis-Regulatory Elements of USP-A and USP-B Promoter Regions by EMSAs with *in vitro* Produced Transcription Factors

Electrophoretic mobility shift assays (EMSAs) were used to determine whether the putative protein binding sites were functional. Different proteins, such as E74A, the Broad Complex proteins (Z1, Z2 and Z4), ECR, and USP, cDNA fragments were sub-cloned into transcription vectors, and synthesized using a coupled transcription/translation (TnT) system.

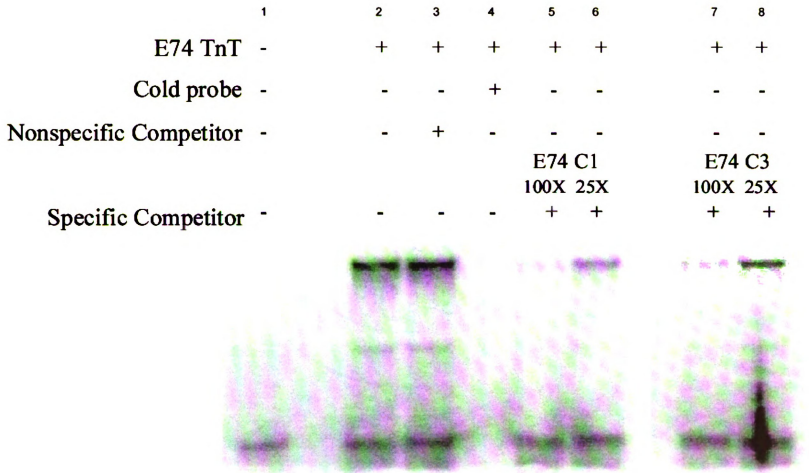
To investigate the putative binding sites on the USP-A promoter, two E74 (E74-C1 and E74-C3), one BRC Z1 (BRC-CZ1/4), one BRC Z4 (BRC-CZ1/4), and six EcRE (EcRE C1-C6) potential binding sites were tested by EMSAs. E74 TnT protein was first tested in both competition and direct binding experiments (Figure 3.5). In panels A and B of figure 3.5, the first four lanes indicate binding of the E74 TnT protein to the labeled E74 consensus probe (lanes 1-4). The labeled E74 consensus probe was used as a positive control for E74 binding. Competition assay showed that 100-fold excess of cold competitors E74-C1 and E74-C3 were able to compete away binding of E74 TnT protein to labeled E74 consensus probe (Figure 3.5 A lanes 5, 7). E74 direct binding assays showed in panel B of Figure 3.5 that labeled E74-C1 and E74-C3 probes was able to bind to the E74 TnT protein (Lanes 6 and 10). Furthermore, the E74-C1 and E74-C3 probes were competed away by the E74 consensus sequence (lanes 7 and 11) but not by the nonspecific sequence (lanes 8 and 12).

Figure 3.6 Competition and direct binding assay of putative E74 binding sites in USP-A promoter by EMSAs.

A. The putative binding sites on the USP-A promoter can efficiently compete against the *Drosophila* E74 binding site. The consensus DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. Twenty-five or one hundred fold molar excess of unlabeled E74 putative DNA was used as a specific competitor. *Drosophila* E74 consensus sequence was used as a positive control.

B. AaE74 protein was bound *in vitro* to the *Drosophila* E74 consensus DNA binding sequence and the putative E74 binding sequences on USP-A promoter. The DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. *Drosophila* E74 consensus sequence was used as a positive control.

A.


1 2 3 4 5 6 7 8
E74 TnT - + + + + + + +
Cold probe - - - + - - - -
Nonspecific Competitor - - + - - - - -
E74 C1 E74 C3
100X 25X 100X 25X
Specific Competitor - - - - + + + +

B.

	1	2	3	4	5	6	7	8	9	10	11	12
E74 TnT	-	+	+	+	-	+	+	+	-	+	+	+
Cold probe	-	-	-	+	-	-	+	-	-	-	+	-
Nonspecific Competitor	-	-	+	-	-	-	-	+	-	-	-	+
						E74 C1				E74 C3		
Specific probe	-	-	-	-	+	+	+	+	+	+	+	+

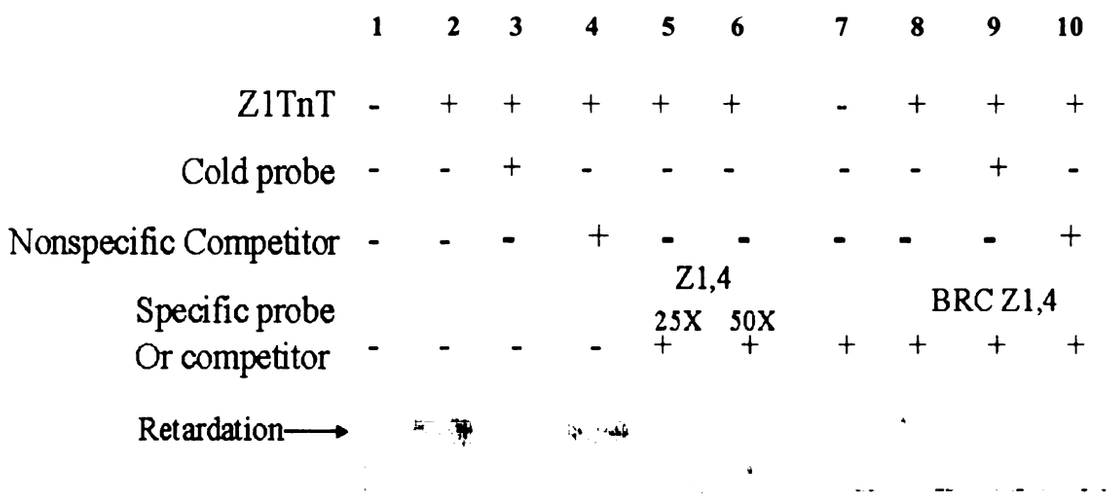


Figure 3.7 Competition and direct binding of the putative binding sequence on the USP-A promoter to the BRC Z1 protein. BRC Z1 protein was bound *in vitro* to the *Drosophila* BRC Z1 consensus DNA binding sequence and binding was competed away by the putative binding sites from the USP-A promoter. The consensus DNA and the potential binding sequences were ^{32}P labeled. An arrow indicates the specific retardation complex. Twenty-five or fifty fold molar excess of unlabeled BRC Z1 putative binding site DNA was used as a specific competitor. *Drosophila* BRC Z1 consensus sequence was used as a positive control.

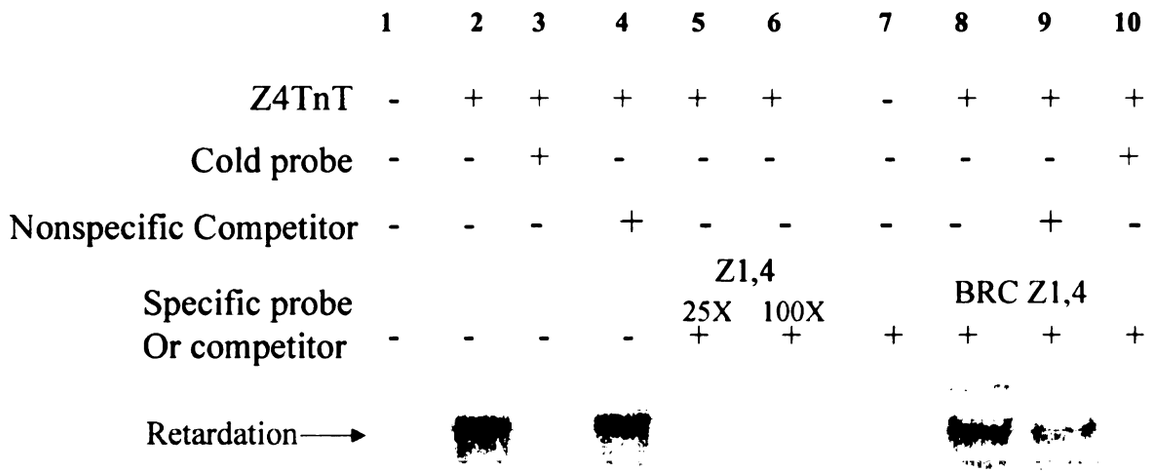


Figure 3.8 Competition and direct binding of the putative binding sequence from the USP-A promoter to the BRC Z4 protein. BRC Z4 protein was bound *in vitro* to the *Drosophila* BRC Z4 consensus sequence. Binding was competed away with the putative binding sites from the USP-A promoter. The consensus DNA and the potential binding sequence were labeled with ^{32}P labeled. An arrow indicates the specific retardation complex. Twenty-five and one hundred fold molar excess of unlabeled BRC Z4 putative DNA was used as a specific competitor. The *Drosophila* BRC Z4 consensus sequence was used as a positive control.

Figure 3.9 Competition and direct binding assay of putative EcREs in USP-A promoter by EMSAs.

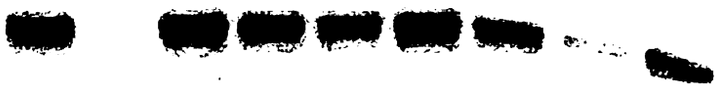
A. AaEcR and AaUSP proteins with 20E were bound *in vitro* to the *Drosophila* EcRE consensus DNA binding sequence Competition assays were performed using cold binding sites from the USP-A promoter. The consensus DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. One hundred fold molar excess of unlabeled putative EcRE DNA was used as a specific competitor. *Drosophila* EcRE consensus sequence was used as a positive control.

B. AaEcRE and AaUSP proteins with 20E were bound *in vitro* to the *Drosophila* EcRE consensus DNA binding sequence and the putative EcRE binding sequences from the USP-A promoter. The DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. *Drosophila* EcRE consensus sequence was used as a positive control.

A.

	1	2	3	4	5	6	7	8	9	10
ECR TnT	-	+	+	+	+	+	+	+	+	+
USP TnT	-	+	+	+	+	+	+	+	+	+
20E	-	+	+	+	+	+	+	+	+	+
Cold probe	-	-	+	-	-	-	-	-	-	-
Nonspecific Competitor	-	-	-	+	-	-	-	-	-	-
					C1	C2	C3	C4	C5	C6
Specific Competitor (100X)	-	-	-	-	+	+	+	+	+	+

Retardation⟶



B.

	1	2	3	4	5	6	7	8
ECR TnT	-	+	+	+	-	+	+	+
USP TnT	-	+	+	+	-	+	+	+
20E	-	+	+	+	-	+	+	+
Cold probe	-	-	+	-	-	-	+	-
Nonspecific Competitor	-	-	-	+	-	-	-	+
							ECRE C5	
Specific probe	-	-	-	+	+	+	+	+

Retardation⟶

The same competition and direct binding EMSAs performed for BRC Z1 were also performed for BRC Z4 using the binding sites CZ1/4 as shown in figure 3.6.

The results indicate that the putative binding site BRC CZ1/4 has the ability to bind the BRC Z4 TnT protein (lanes 8, 9 and 10) and this site can compete away the consensus *Drosophila* BRC Z4 sequence (lanes 5 and 6).

Since the software could not identify the potential EcRE binding sites, I searched the sequences manually and found 6 putative binding sites of EcRE, namely EcRE C1-6, and then analyzed them by EMSAs (Figure 3.8). The consensus sequence (DR1) of EcRE was used as a positive control in both competition and direct binding assay (Lanes 1-4 in panels A and B). Competition assay showed that only EcRE C5 and C6 had the ability to compete away the consensus sequence with 100 fold excess in concentration and the competition capability of EcRE C5 was higher than that of EcRE C6 (Lanes 9 and 10). The EcRE direct binding assay indicated that EcRE C5 was able to bind to the EcR-USP and 20E complex (Lane 6). In addition, the EcRE C5 probe was competed away by EcRE DR1 consensus sequence (Lane 7) but not by the nonspecific sequence (Lane 8) which indicate the specific binding.

The same assays were performed to examine the putative binding sites on the USP-B promoters. One BRC Z1 (BRC CZ1/4), one BRC-CZ2 (BRC Z2C1), three BRC Z4 (BRC CZ1/4, BRC C1Z4, and BRC C2Z4), and eight EcRE (EcRE C1-C8) potential binding sites were tested.

Figure 3.9 shows the competition and direct binding assays of the putative binding site BRC CZ1/4 to the BRC Z1 TnT protein. The results showed that the putative binding site has competitive and direct binding ability (lanes 5, 6 and 8).

The same assays were performed for the putative BRC Z2C1 binding site (Figure 3.10). It shows that the BRC Z2C1 site can compete away the consensus sequence and directly binding to the BRC Z2 TnT protein (lanes 5, 6 and 7).

Three putative BRC Z4 binding sites were tested (Figure 3.11). These three sites were capable of competing away the binding by the BRC Z4 TnT protein, as shown in the upper bands (panel A. lanes 6, 7, 8 and 9). Direct binding assays demonstrate that the three putative binding sites can specifically bind the BRC Z4 protein (lanes 1-9).

Finally, all eight potential EcRE sites on the USP-B promoter region were tested (Figure 3.12). In panel A, only EcRE C5 and EcRE C6 have the ability to compete away the consensus DR1 sequence (lanes 9 and 10). Further direct binding assays showed that the EcRE C5 has a much stronger specifically binding ability than the EcRE C6 (Panel B lanes 6-12).

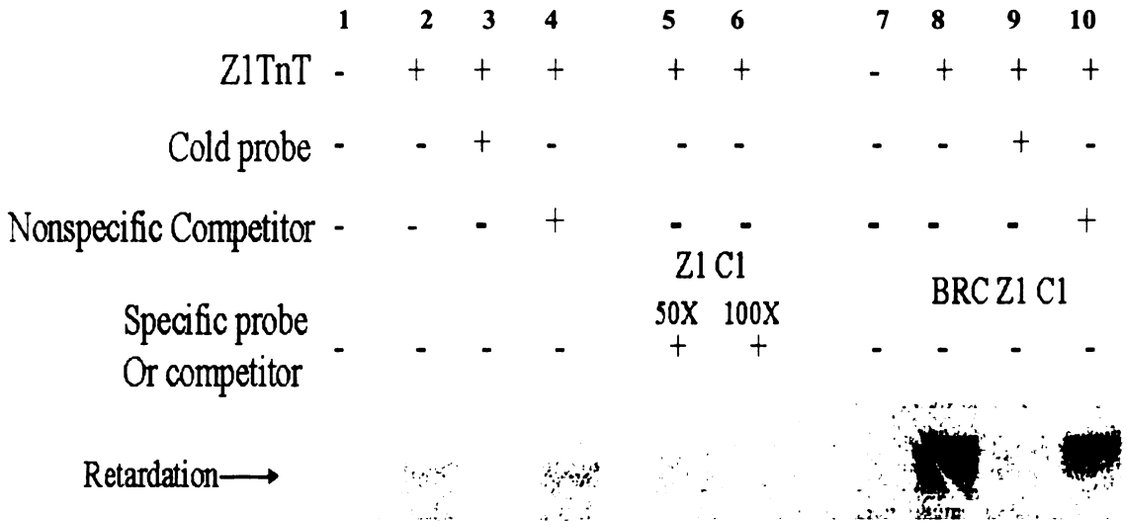


Figure 3.10 Competition and direct binding by the putative BRC Z1 site in the USP-B promoter to the BRC Z1 protein. AaBRC Z1 protein was bound *in vitro* to the *Drosophila* BRC Z1 consensus DNA binding sequence and competed against putative binding BRC Z1 sites on the USP-B promoter. The consensus DNA and the potential binding sequence were ^{32}P labeled. An arrow indicates the specific retardation complex. One hundred and fifty fold molar excess of unlabeled BRC Z1 putative DNA was used as a specific competitor. *Drosophila* BRC Z1 consensus sequence was used as a positive control.

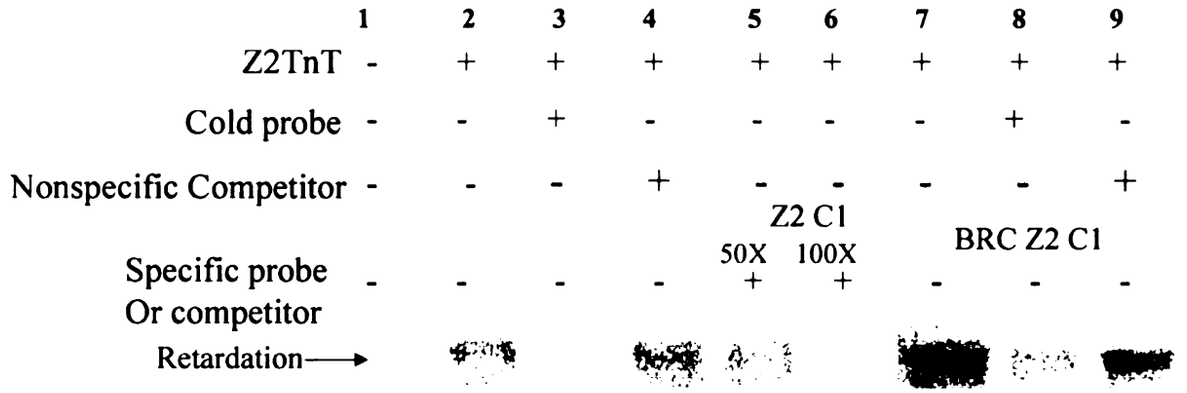


Figure 3.11 Competition and direct binding of the putative BRC Z2 binding sequence in USP-B promoter to BRC Z2 protein. The AaBRC Z2 protein was bound *in vitro* to the *Drosophila* BRC Z2 consensus DNA binding sequence and competed against the putative BRC Z2 binding sites on the USP-B promoter. The consensus DNA and the potential binding sequence were ^{32}P labeled. An arrow indicates the specific retardation complex. One hundred and fifty fold molar excess of unlabeled BRC Z2 putative DNA was used as a specific competitor. *Drosophila* BRC Z2 consensus sequence was used as a positive control.

Figure 3.12 Competition and direct binding assays of potential BRC Z4 binding sites in USP-B promoter by EMSAs.

A. AaBRC Z4 protein was bound *in vitro* to the *Drosophila* BRC Z4 consensus DNA binding sequence and competed against the putative binding sites from the USP-B promoter. The consensus DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. Fifty and one hundred fold molar excess of unlabeled BRC Z4 putative DNA was used as a specific competitor. *Drosophila* BRC Z4 consensus sequence was used as a positive control.

B. The AaBRC Z4 protein was bound *in vitro* to the *Drosophila* BRC Z4 consensus DNA binding sequence and the putative BRC Z4 binding sequences from the USP-B promoter. The DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. *Drosophila* BRC Z4 consensus sequence was used as a positive control.

A.

	1	2	3	4	5	6	7	8	9
Z4 TnT	+	+	+	+	+	+	+	+	+
Cold Probe	-	+	-	-	-	-	-	-	-
Non Specific Competitor	-	-	+	-	-	-	-	-	-
Specific Competitor	-	-	-	Z1,4 50X	Z1,4 100X	Z4-C1 50X	Z4-C1 100X	Z4-C2 50X	Z4-C2 100X

Retardation→

B.

	1	2	3	4	5	6	7	8	9
	Z4-C2			Z4-C1			Z1,4		
Z4 TnT	+	+	+	+	+	+	+	+	+
Non Specific Competitor	-	-	+	-	-	+	-	-	+
Specific Competitor	-	+	-	-	+	-	-	+	-

Retardation→

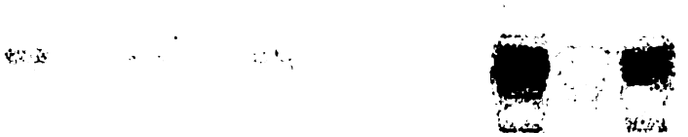


Figure 3.13 Competition and direct binding assay of putative EcREs in USP-B promoter by EMSA.

A. AaEcR and AaUSP proteins with 20E were bound *in vitro* to the *Drosophila* EcRE consensus DNA binding sequence and competed against the putative EcRE sites from the USP-B promoter. The consensus DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. One hundred fold molar excess of unlabeled putative EcRE DNA was used as a specific competitor. The *Drosophila* EcRE (DR1) consensus sequence was used as a positive control.

B. AaEcRE and AsUSP proteins with 20E were bound *in vitro* to the *Drosophila* EcRE consensus DNA binding sequence and the putative EcRE binding sequences from the USP-B promoter. The DNA was ^{32}P labeled. An arrow indicates the specific retardation complex. *Drosophila* EcRE consensus sequence was used as a positive control.

A.

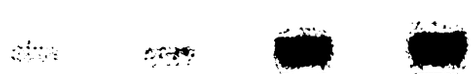
	1	2	3	4	5	6	7	8	9	10	11	12
ECR TnT	-	+	+	+	+	+	+	+	+	+	+	+
USP TnT	-	+	+	+	+	+	+	+	+	+	+	+
20E	-	+	+	+	+	+	+	+	+	+	+	+
Cold probe	-	-	+	-	-	-	-	-	-	-	-	-
Nonspecific Competitor	-	-	-	+	-	-	-	-	-	-	-	-
					C1	C2	C3	C4	C5	C6	C7	C8
Specific Competitor (100X)	-	-	-	-	+	+	+	+	+	+	+	+

Retardation ⟶

B.

	1	2	3	4	5	6	7	8	9	10	11	12
ECR TnT	-	+	+	+	-	+	+	+	-	+	+	+
USP TnT	-	+	+	+	-	+	+	+	-	+	+	+
20E	-	+	+	+	-	+	+	+	-	+	+	+
Cold probe	-	-	+	-	-	-	+	-	-	-	+	-
Nonspecific Competitor	-	-	-	+	-	-	-	+	-	-	-	+
						ECRE C5				ECRE C6		
Specific Competitor	-	-	-	-	+	+	+	+	+	+	+	+

Retardation ⟶



Chapter 4 – Summary and Conclusions

Introduction

In this study, I have determined the transcriptional start sites and cloned the promoter regions of USP-A and USP-B. The transcriptional profiles of these two isoforms were characterized during the pre-vitellogenic and vitellogenic period, and the profiles are consistent with previous observations (Wang et al., 2000). Sequence analyses of both USP-A and USP-B promoters reveal putative transcription factor binding sites, some of which have been confirmed by EMSAs. According to the putative binding sites and the profile study, I have proposed general hormone regulation models for USP-A and USP-B.

USP a Single Gene Copy

From the previous studies, USP-A and USP-B have been shown to have identical C, D, E and F domain, but the A/B domain is different. Genomic DNA digestion and Southern blot analyses (Figure 3.1) showed that although the genomic DNA was digested by three different enzymes, the different digestions only resulted in one hybridization product. These results demonstrated that USP-A and USP-B originate from the same gene, but they are derived from different promoters and alternative splicing.

Identification of the USP-A and USP-B Promoters

Rapid amplification of cDNA ends (RACE) and RNase protection assay (RPA) have been used to identify the transcriptional start sites. The results from the RACE experiments (Figure 3.2) were checked by southern blot, and the sequences have been compared with the results from the RPA (Figure 3.3). The results from these two assays

are in agreement as to the transcriptional start sites of USP-A and USP-B. Once identified, I cloned and sequenced the promoter regions of these two isoforms, which contain the conserved initiator element.

Expression of USP mRNA in Fat body

According to previous research (Wang et al., 2000), there are huge differences in the mRNA expression profiles between USP-A and USP-B during vitellogenesis. I used real-time PCR to quantify the USP-A and USP-B expression profiles due to the fact that it is more accurate than previous methods. I adopted this technique to detect the mRNA from the two USP isoforms in the fat body during the vitellogenesis.

Specific primers were chosen from the A/B domain, which is different between USP-A and USP-B. The results correctly demonstrated the mRNA level distinction between these two isoforms.

When the 20E is absent, prior to blood feeding, the mRNA of USP-A was expressed. The level of the transcripts stayed at a basal level for the next few days. However, during the vitellogenic period as the titer of 20E increases, it represses the expression of the USP-A. Furthermore, 40 hours PBM, which is the termination period, the 20E level decreases and the level of USP-A mRNA increases and appears to be a super induced during the 40 hours PBM to a peak level 400 fold higher than the basal level.

The USP-B profile shows that its mRNA level increases with the appearance of the 20E after a blood meal, it peaks at 24 hours post blood meal, and decreases after this point back to the basal level by 36 hours PBM.

In conclusion, the two isoforms of USP-A and USP-B have different expression profiles during the vitellogenesis. According to the relationship between transcripts of USP-A and USP-B and the 20E titer, when 20E is present, USP-A is repressed and the USP-B is induced, but when 20E is absent, the USP-B is repressed and the USP-A is induced. Specifically, when 20E is present then disappears, the mRNA level of USP-A will show a super-induction. These results are in agreement with previous observations (Wang et al., 2000).

Potential Binding Sites in the USP-A and USP-B Promoter Regions

The sequence results of the promoter regions of USP-A and USP-B were entered into two programs (TESS and TRANSFAC) to search for the potential transcription factor binding sites. According to the search results, I selected some of the binding sites which are related to our previous work and some regulatory proteins previously discovered in *Aedes aegypti* for further study.

Using electrophoretic mobility shift assays (EMSAs), I identified two E74 binding sites, one BRC Z4 binding sites, one BRC Z1 binding site, and one EcR-USP binding site in USP-A promoter. Similar analysis was also done on the USP-B promoter. There are three BRC Z4 binding sites, one BRC Z1 and Z2 binding site, and two EcR-USP binding sites (Figure 4.1). Other binding sites, such as GATA, HNF 3, and C/EBP are present in both promoter regions and will be tested in future research.

To further understand the function of these binding sites and to construct a hypothesis of hormone regulation of USP-A and USP-B, it will be necessary to compare

the USP promoters to other 20E regulated mosquito gene promoters. In our lab, the promoters of vitellogenin (Vg) (Kokoza et al., 2001) and vitellogenic carboxypeptidase (VCP) (Deitsch et al., unpublished) have been investigated. These genes also have the tissue-, stage- and sex-specific expression. Vg, VCP and USP all have the stage/tissue specific binding sites such as HNF3 and C/EBP. These binding proteins determine when and where Vg, VCP and USP are express. These four promoters all contain one or two ecdysone response elements where the EcR-USP heterodimer binds to when 20E is present. However, the function of the EcR and USP heterodimer may not have the same function to USP-A and USP-B expression as compared to the later genes Vg and VCP, which are induced by the heterodimer. In previous studies, Dr. Shengfu Wang proposed that USP-B is the partner of EcR-B to form the heterodimer. USP-B, as an early gene, appears to act by a positive feedback mechanism when the heterodimer is present. At the same time this heterodimer seems to repress the other isoform of USP, USP-A. Other regulatory proteins, E74 and Broad Complex which bind the USP-A promoter, have different functions on different genes. The E74 gene also has two isoforms, which appear to have different functions and could be acting positively or negatively on USP-A.

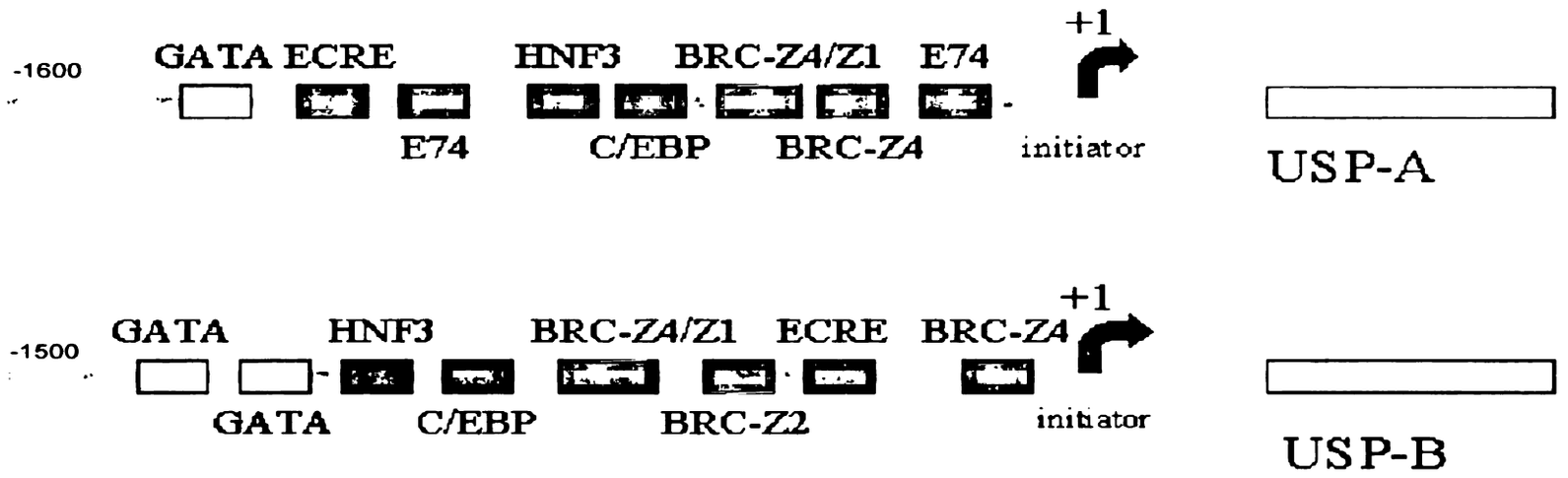


Figure 4.1 The potential binding sites in USP-A and USP-B promoter regions.

Hypothesis of the Hormone Regulation of USP-A and USP-B

According to the results from the EMSA experiments and the 20E treatment and withdrawal experiments, I have proposed preliminary models of the hormonal regulation of the two USP isoforms.

In previous research, USP-A was repressed when 20E was present, and after withdrawal of 20E it is super-induced. That suggests that the EcR-USP act as a repressor on the promoter of USP-A when the 20E is present. However, at the same time, some other early genes are induced by this EcR-USP heterodimer. When the titer of 20E decreases after 24 hours PBM, some early genes, such as E74A and Broad Complex, which have already been expressed, will be as activators to super-induce the expression of USP-A. During the previtellogenic period, there may be a very weak activator working on the promoter to induce a small peak of the USP-A transcript.

USP-B is induced by the EcR-USP complex, when 20E is present. Positive feed back can induce the expression of USP-B until the amount of 20E decreases and USP switchs to another partner, such as AHR38 or Svp (Zhu et al., 2003). After that, the complex disappears and stops the induction of the USP-B expression. The HNF3 and C/EBP constitute the tissue specific control of the expression and the other factors like GATA and broad complex could have positive or negative effect on the regulation. They maybe have synergistic function to regulate the expression of the USP-B.

Due to the fact that E74 (Sun et al., 2002) and Broad Complex family (Mugat et al., 2000) can act as repressors or activators in the gene regulation, the model only briefly touches on these proteins and their function in USP regulation.

Chapter 5- Future research prospects

Introduction

This project is one part of the characterization of the genetic regulatory hierarchy mediating ecdysteroid action in the adult mosquito. The aim of this part was to understand the molecular basis of the differential expression of the two USP isoforms. The USP-A and USP-B promoter regions have been cloned and characterized allowing further work as described below.

Characterization of Cis-Regulatory Elements of USP-A and USP-B by EMSA with *in vivo-produced* Transcription Factors

In the current work, all proteins which included E74, BRC Z1, Z2, Z4, EcR and USP used in binding assays were the *in vitro* expression proteins (TnT). However, to further verify the binding abilities of the sites in the promoters of USP-A and USP-B, fat body nuclear extracts need to be used to provide native proteins. Fat body nuclear extracts will be prepared at time points, 0, 4, 8 and 12 hours after emergence, and 0, 12, 20, 24, 30, 36, 42 and 48 hours during vitellogenic period. These extracts can be used for EMSAs with the binding sites that have been analyzed. Super-shift assays can also be used to verify that the protein(s) of interest are really bound to the regulatory elements. These *in vivo* results will provide us not only evidence for functional binding sites, but also more information about the titers of the proteins of interest in the nucleus.

The DNAase I protection or footprinting assays could also be useful in understanding the specific binding sites of certain transcription factors. These results will

pave the road for future studies on the regulation of the USP promoters.

Transfection Assays of USP-A and USP-B Promoter Regions

Currently the embryonic cell line (Aag2) of *Aedes aegypri* has been established by A. Fallon, University of Minnesota (Gao et al., 2000). This system provides an advantage in performing transfection assays to study USP-A and USP-B promoter regions in comparison to other insect cell lines.

The constructs of both USP-A and USP-B promoters can be sub-cloned into vectors such as the promoterless plasmid pGL3basic that contains a luciferase reporter. These constructs can be transfected into *Aag2* cell line. Transcription factors, such as E74 and broad complex, which have been characterized in this study by EMSAs may be tested in Aag2 cells. Furthermore, the transfection assays with the deletion of the binding sequences from the promoter will tell us more about the function of these binding sites. Results of these experiments will further help us to understand the activation and repression functions of these proteins in order to construct a working model of USP-A and USP-B regulation.

Transformation Analysis of USP-A and USP-B Promoter Regions

The ideal cell line to perform transfection assay is the mosquito fat body cell line. However, this system currently is not available. Since our lab has successfully

constructed several lines of transgenic mosquitoes, it may be feasible to use germ-line transformation to analyze the promoter regions of USP-A and USP-B.

The constructs of USP-A and USP-B promoters can be transformed into UGAL mosquito using the piggy-Bac transposable element. Using transformants, several puzzles could be answered. It is possible to use various portions of the promoter and the mutation constructs to test the expression in the transgenic mosquito. The fact that USP-A undergoes super induction after 20E withdrawal might make this promoter an effective driver for anti-pathogen genes in transgenic mosquitoes to reduce disease transmission.

Appendices

Appendix 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2003-02

Title of thesis or dissertation (or other research projects):

Regulatory regions of the *ultraspiracle* gene isoforms from the mosquito *Aedes aegypti* isolation and characterization.

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)

Renyuan Wang

Date 19 May 2003

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America.
Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults †	Adults °	Other	Museum where deposited
Aedes aegypti	Michigan, Ingham Co., East Lansing MSU Campus - lab culture 16 April 2003					10	10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Renyuan Wang

Date 19 May 2003

Voucher No. 2003-02

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator 21 May 2003

Date

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