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dissertation entitled

CHARACTERIZATION OF NUCLEAR RECEPTORS
OF THE ECDYSONE GENE REGULATORY HIERARCHY
DURING MOSQUITO VITELLOGENESIS

presented by

Chao Li

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Genetics

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Major professor

6-24-2001
Date

CHARACTERIZATION OF NUCLEAR RECEPTORS OF THE ECDYSONE GENE REGULATORY HIERARCHY DURING MOSQUITO VITELLOGENESIS

By

Chao Li

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

2001

ABSTRACT

CHARACTERIZATION OF NUCLEAR RECEPTORS
OF THE ECDYSONE GENE REGULATORY HIERARCHY
DURING MOSQUITO VITELLOGENESIS

By

Chao Li

The insect steroid hormone, 20-hydroxyecdysone (20E), plays a critical role in regulating widely diverse physiological functions of development, metamorphosis and reproduction. The mode of 20E action is well understood in *Drosophila* development, where this steroid hormone regulates genes via a complex hierarchy containing several transcription factors belonging to the nuclear receptor superfamily. In contrast, our knowledge of 20E action in insect reproduction has been limited. In mosquitoes, a blood meal is required for egg development and as a consequence, they are vectors of numerous human and domestic animal diseases. The process of production of yolk protein precursors or vitellogenesis is central to egg development and is regulated by 20E. The goal of my thesis has been characterization of nuclear receptors of the 20E gene regulatory hierarchy involved in the mosquito vitellogenesis. The functional receptor for 20E, which is at the top of the hierarchy, is a heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and *Ultraspiracle* (USP). There are two isoforms for both EcR and USP in mosquitoes, which are differentially expressed in a stage- and tissue-specific manner during vitellogenesis. *In vitro* fat body culture experiments suggest that the ligand, 20E itself, differentially regulates expression of *AaEcR* and *AaUSP* isoforms. In *Drosophila*, the action of the EcR/USP heterodimer is mediated by the product of an early gene *E75*, which is an orphan nuclear receptor. Analysis of the *E75* gene has

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shown that the 20E-regulated hierarchy is used reiteratively during mosquito vitellogenesis. In the fat body, *AaE75* mRNA is not present during the previtellogenic stage, while *AaE75* is highly expressed during the vitellogenic stage. In *in vitro* fat body culture, *AaE75* is rapidly induced by 20E and this induction is not blocked by cycloheximide. Dose response experiments have revealed that *AaE75* is ten fold more sensitive to induction by 20E than the yolk protein gene, *Vg*. Furthermore, AaE75 is able to directly activate the *Vg* promoter in cell transfection assays, suggesting its role as an early gene. The mosquito becomes competent for a vitellogenic response to 20E after previtellogenic development. The nuclear orphan receptor AaFTZ-F1 serves as a competence factor for the stage-specific response to 20E. *AaFTZ-F1* transcript is expressed highly in the fat body during pre- and post-vitellogenic periods when 20E titers are low, but it disappears in mid-vitellogenesis when 20E titers are high. In *in vitro* fat body culture, *AaFTZ-F1* expression is inhibited by 20E and super-activated by its withdrawal. Following *in vitro AaFTZ-F1* super-activation, a secondary 20E challenge results in super-induction of the early *AaE75* gene and the late target *VCP* gene, demonstrating involvement of *AaFTZ-F1* in developing additional 20E responsiveness. Moreover, the onset of 20E-response competence in the mosquito fat body is correlated with the appearance of the functional AaFTZ-F1 protein at the end of previtellogenic development. Finally, I have identified another mosquito nuclear orphan receptor AHR78 that is expressed at a high level only in the ovary during the vitellogenic period. AHR78 inhibits the DNA binding and transactivation activities of AaEcR/AaUSP on the ecdysone response element of the *Vg* promoter.

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ACKNOWLEDGMENTS

I sincerely appreciate my major professor, Dr. Alexander Raikhel, for his guidance and continued support throughout my graduate studies. His thoughtful, patient and disciplined mentoring provided me invaluable guidance, while allowing sufficient academic freedom and thus my interest and understanding of biomedical research were enhanced. Second, I would like to thank my guidance committee, Drs. Donald Jump, David Arnosti, and Ke Dong, for their help and suggestions during my five-year studies. Third, I wish to thank the friends I made in the Raikhel lab and the Genetics Program for their friendship and advice. Especially, I greatly appreciated Dr. Ken Miura for his detailed teaching of molecular biology techniques at the beginning of my studies, Dr. Sheng-fu Wang for his helpful discussions during my experiment difficulties, and Mr. Alan Hays for culturing mosquitoes and other technical helps. I also thank people who have been doing excellent collaborative work with me including Drs. Marianna Kapitskaya, S-F. Wang, Jinsong Zhu, K. Miura, Avraham Biran, and Kook-Ho Cho. In addition, I would like to thank Drs. William Pierceall and William Segraves at Yale University for cloning the *AaE75* gene and editing my manuscripts. I am also very grateful to Michel Trail for editing a manuscript, and S-F. Wang and Geoffrey Attardo for editing my dissertation. Finally, I would like to thank my parents and brothers for their love, support and encouragement from the beginning to the end of my graduate studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

20E: 20-

Aa: Ac

AR: anc

β-Gal: β-

Bm: silk

Bp: base

CAT: ch

cDNA: c

Dm: *Dro*

DNA: De

DR: direct

EcR: ecdy

EMSA: el

EcRE: ecd

Elp: ecdy

ER: estrog

GR: gluco

GST: gluta

HRE: horm

Hsp: heat s

IR: inverte

JH: juvenil

LIST OF ABBREVIATIONS

20E: 20-hydroxyecdysone

Aa: *Aedes aegypti*

AR: androgen receptor

β-Gal: β-galactosidase

Bm: silkworm *Bombyx mori*

Bp: base pair

CAT: chloramphenicol aceyltransferase

cDNA: complementary DNA

Dm: *Drosophila melanogaster*

DNA: Deoxyrbonucleic acid

DR: direct repeat

EcR: ecdysteroid receptor

EMSA: electrophoretic mobility shift assay

EcRE: ecdysteroid response element

Eip: ecdysone inducible protein

ER: estrogen receptor

GR: glucocorticoid receptor

GST: glutathione S-transferase

HRE: hormone response element

Hsp: heat shock protein 27

IR: inverted repeat

JH: juvenile hormone

Kb: kilobase pair

Kd: equilibrium dissociation constant

LBD: ligand binding domain

Luc: luciferase

MR: mineralocorticoid receptor

mRNA: messenger RNA

MurA: muristerone A

NR: nuclear hormone receptor

PAGE: polyacrylamide gel electrophoresis

PR: progesterone receptor

PBM: post blood meal

PCR: polymerase chain reaction

RAR: retinoid acid receptor

RNA: ribonucleic acid

RT: reverse transcription

RXR: retinoid X receptor

S2: *Drosophila* Schneider cell line 2

SDS: sodium dodecyl sulfate

Tm: yellow mealworm *Tenebrio moliter*

TR: thyroid hormone receptor

USP: Ultraspiracle

VCP: vitellogenic carboxypeptidase

VCB: vitellogenic cathepsin-B

Vg: vitellogenin

VDR: vitamin D receptor

VgR: vitellogenin receptor

YPP: yolk protein precursor

Chapter 1

Literature Review

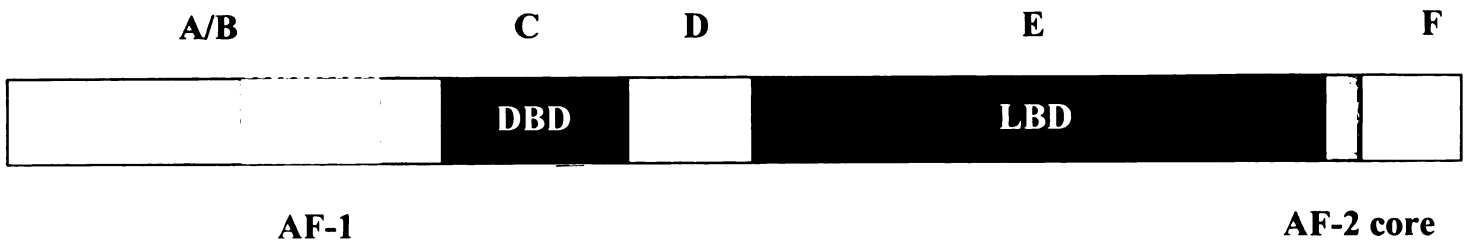
MOLECULAR MECHANISM OF NUCLEAR RECEPTOR ACTION

General structures and functions of nuclear receptors

Steroid/Thyroid hormones play critical roles in a wide array of biological processes such as development, reproduction and metabolism. Classical nuclear hormone receptors are ligand-activated transcription factors that activate gene expression upon binding to their respective ligands. The ligands for nuclear receptors are small lipophilic molecules such as steroids, retinoids, vitamin D, and thyroid hormone. The receptors for these ligands are: the sex steroid receptors including estrogen (ER), progesterone (PR), and androgen (AR) receptors; the adrenal steroid receptors including glucocorticoid (GR) and mineralocorticoid (MR) receptors; and others including thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR) and 9-cis retinoic acid (RXR), and ecdysone (EcR) receptors. In addition, a large number of so-called nuclear orphan receptors have been identified. While these receptors have similar structures to nuclear hormone receptors, 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).

One striking structural characteristic of the nuclear receptor superfamily is that most members contain five to six evolutionarily conserved functional domains, which can be divided into five regions starting from the N-terminal: A/B, C, D, E and F (Fig. 1). The A/B domain is most variable among family members and contains the ligand-independent transactivation function (AF1). The C domain is also named the DNA binding domain (DBD). The D domain acts as a hinge region between the C and E domains. It contains a nuclear localization signal, as well as corepressor binding sites on

Fig.1. Functional domains of nuclear receptors. The members of the nuclear receptor superfamily are composed of several functional domains, shown in the schematic with the N-terminus at left. A/B and F domains vary in size and primary sequence among the superfamily. The DNA binding domain (C) has the highest sequence conservation in the superfamily. The hinge region (D) contains some sequences important for nuclear localization and corepressor binding in some nuclear receptors. The ligand binding domain (E) has additional functions of heat shock protein (hsp) association, dimerization, coactivator-interaction and transactivation. AF-1 in the A/B domain refers to the ligand-independent transactivation function. AF-2 refers to the ligand-dependent activation function within the LBD. The AF-2 core is also known as Helix 12.



Functions:

Hormone-binding

Hsp association

Dimerization

DNA-binding

Co-repressor interaction

Co-activator interaction

Transactivation

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nuclear hormone receptors. The E domain is characterized as the ligand-binding domain (LBD), which binds to ligands and possesses activation functions (AF2). The F domain is at the very C-terminal and varies in size and primary sequence among the superfamily (Tsai and O'Malley, 1994).

A central and common feature of nuclear receptors is their DBDs. The DBD region has dual functions: hormone response element binding and dimerization. The sequences in the DBD exhibit remarkable conservation among the nuclear receptor superfamily, and this has proven useful for the cloning of many other nuclear receptors. Typically, the DNA-binding region consists of 66 highly conserved amino acid residues, which are referred to as the "core" region of the DBD in the nuclear receptors. The core DBD contains two zinc-nucleated subdomains. Each zinc is coordinated in a tetrahedral geometry by the sulfurs from conserved cysteines. The hinge region immediately following the C-terminal of the DBD can also contribute significantly to the DNA binding and target specificity of the receptor (Rastinejad, F., 1998; Luisi *et al.*, 1991).

Most receptors function as homo- or heterodimers, binding selectively to gene-regulatory sequences called hormone response elements (HREs). There are two distinct regions that maintain the dimerization function. First, certain receptors can dimerize via their LBDs in the absence of DNA. Second, more functionally relevant dimerization interfaces are formed through a separate interface between the receptors' DBDs only in the presence of an appropriate HRE (Perlmann *et al.*, 1993; Zechel *et al.*, 1994; Hard *et al.*, 1990).

Nuclear receptors often bind to bipartite DNA targets as a dimer. Despite the large number of nuclear receptors, only two types of consensus hexameric half-sites appear to

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be used as targets. The two most common half-site arrangements are the symmetric repeat and the direct repeat with different inter-half-site spacers. The steroid receptors for glucocorticoids, mineralocorticoids, androgens, and progesterone, bind to 5'-AGAACA-3' half sites; the estrogen receptor (ER), RXR, TR, VDR, RAR, PPAR, COUP-TF, and NGFI-B preferentially bind to consensus 5'-AGGTCA-3' half sites (Klock *et al.*, 1987; Umenoso *et al.*, 1991). Three critical residues in the DBDs, called the P-box, predispose receptors to preferentially bind the GR versus the ER elements. Receptors recognizing the AGAACA half sites contain a GSXXV in their P-box, while those that recognize AGGTCA half-sites typically contain EGXXG residues at the corresponding positions. Thus, the P-box is critical for half-site discrimination (Mader *et al.*, 1989).

The LBD of nuclear receptors is a large domain that sometimes encompasses almost the entire C terminus of these proteins. The LBD has been credited with having the additional functions of hormone-dependent dimerization and transactivation. Nuclear localization and heat shock protein association functions also require the LBD of some receptors. The binding of hormone results in receptor transformation and the initiation of a series of events by which receptors activate the expression of target genes (Kumar *et al.*, 1987; Dobson *et al.*, 1989; Allan *et al.*, 1992).

Interaction with a molecular chaperone-containing heterocomplex (MCH) is a pre-requisite for activation of steroid receptors. This interaction includes the molecular chaperones (Hsp90 and Hsc70), molecular chaperone interacting proteins (Hop, Hip, and p23), and peptidyl- prolyl isomerases (FKBP51, FKBP52, and Cyp40). In the case of PR and GR, the MCH is required for maturation to the aporeceptor state (DeFranco *et al.*,

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1998). Association of hsp90 is required for steroid binding to GR both *in vitro* and *in vivo* (Bresnick *et al.*, 1989; Picard *et al.*, 1990), and the binding of hsp90 to GR causes a conformational change in the GR LBD (Stancato *et al.*, 1996).

Roles of cofactors in gene activation and silencing by nuclear receptors

Upon hormone binding, receptors bind to DNA and activate target gene expression. Early studies have demonstrated that PR, ER and GR activate transcription by enhancing the formation of stable preinitiation complexes at their target promoters by either direct interaction with components of the transcription machinery, or through an intermediate factor such as a coactivator (Klein-Hitpass *et al.*, 1990; Tsai *et al.*, 1990; Elliston *et al.*, 1990). In the past few years, numerous nuclear receptor coactivators have been identified. They interact directly with the activation domain of a nuclear receptor in an agonist-dependent manner, and also interact with other factors and components of the basal transcription machinery but do not enhance the basal transcription activity on their own (Mckenna *et al.*, 1999; Robyr *et al.*, 2000; and Glass and Rosenfeld 2000).

The first cloned nuclear receptor coactivator, steroid receptor coactivator-1 (SRC-1), represents one of the well-documented coactivators for nuclear receptors (Onate *et al.*, 1995). Many other subsequently identified coactivators with structures and functions similar to SRC-1 comprise the SRC coactivator family. The SRC family can be divided into three subfamilies: SRC-1/NCoA-1, SRC-2/GRIP1/TIF2/NcoA-2, and SRC-3/p/CIP/RAC3/ACTR/AIB-1/TRAM-1. SRC coactivators share an ability to stimulate ligand-dependent transactivation by a rather large number of nuclear receptors in transient overexpression experiments. These coactivators also exhibit structure

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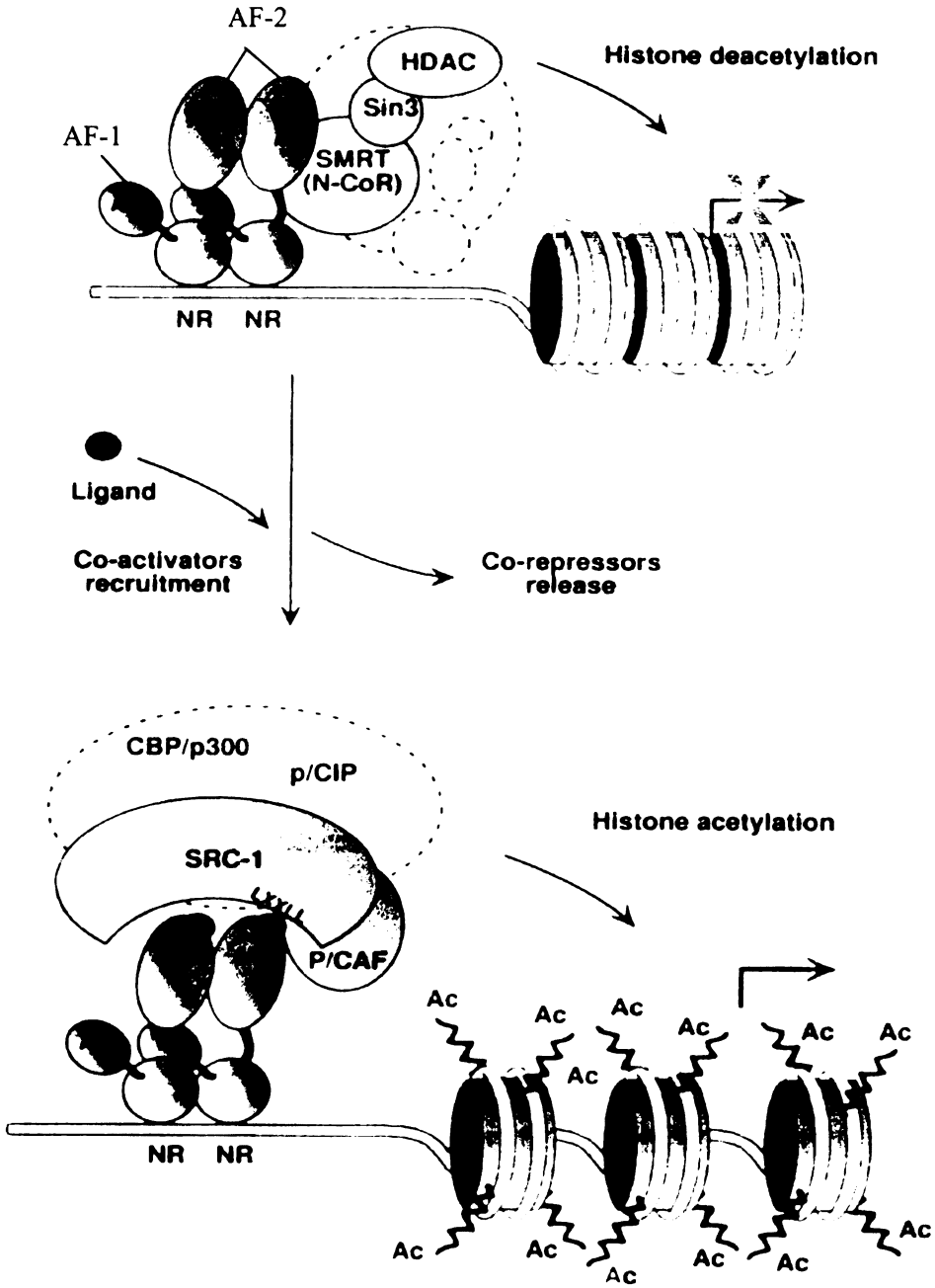
similarities. A distinct feature is the presence of multiple LXXLL signature motifs or NR boxes, which comprise of determinants for direct interactions with the nuclear receptor LBDs (Mckenna *et al.*, 1999).

Several LBD crystal structures have established that upon ligand binding, the α helices in the LBD, including the AF2 core (helix 12), undergo a major reorientation in the context of the overall LBD structure. This reorientation forms part of a "charged clamp" that accommodates SRC coactivators within a hydrophobic cleft of the LBD. This interaction appears to occur through direct contacts with the LXXLL motif. Furthermore, SRC coactivators interact with cointegrators CBP/p300 and together they can coactivate synergistically, while CBP/p300 itself interacts directly with nuclear receptors. SRC and CBP/p300 coactivators both possess intrinsic histone acetyl transferase (HAT) activity and therefore can act in concert to remodel chromatin. In addition, p/CIP and P/CAF coactivators are recruited to nuclear receptors in response to hormone binding as part of a growing, HAT-containing, chromatin-remodeling complex (Xu *et al.*, 1999; Freedman 1999).

Moreover, nuclear receptors can repress transcription under various circumstances. Repression occurs mostly in the absence of a ligand or when an antagonist is bound to the receptor. It has been established that the unliganded TR is able to bind DNA acting as a repressor. In the past several years, three active repressors have been identified: SMRT (silencing mediator for retinoid and thyroid hormone receptor) (Chen and Evans, 1995), N-CoR (nuclear receptor corepressor) (Kurokawa *et al.*, 1995; Horlein *et al.*, 1995) and SUN-CoR (small ubiquitous nuclear corepressor) (Zamir *et al* 1997). These corepressors are able to interact with the unliganded TR and RAR

Fig. 2. Activation and repression of a gene by nuclear receptors through coactivators or corepressors. The nuclear hormone receptor (NR) is associated with a corepressor (N-CoR, SMRT), which in turn recruits a histone deacetylase (HDAC) through its interaction with Sin3. Deacetylation of histone tails leads to transcriptional repression. Addition of the ligand disrupts this repression complex in favor of the association of a coactivator complex (SRC-1, P/CAF, p300/CBP, pCIP, and others). These proteins possess a histone acetyltransferase activity that allows chromatin decompaction through histone modifications. The interaction between the nuclear receptor AF-2 domain and the coactivation complex occurs through the LXXLL motif in many coactivators. The coactivator and corepressor complexes are represented with dashed lines since their exact composition *in vivo* is not determined. Modified from Robyr et al. (2000).


AF-2
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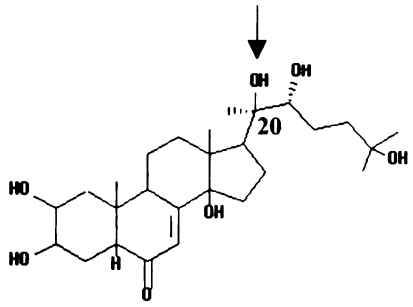
associated with RXR on DNA. The C terminus of N-CoR interacts with the hinge domain and a portion of the LBD in TR and RAR. The silencing activity is abolished upon ligand binding by the release of corepressors from the receptor. Further studies have demonstrated that SMRT and N-CoR mediate transcription repression through recruitment of a histone deacetylase complex. The N terminus repression domain (SD-1) of SMRT interacts with Sin3A, which in turn associates with a histone deacetylase HDAC-1 through one of its two silencing domains. Thus, chromatin modification through deacetylation plays an active role in nuclear receptor mediated gene silencing. In addition, TR silencing is mediated by its direct interaction with the general transcription factor TFIIB. This interaction is interrupted by thyroid hormone binding (Robyr *et al.*, 2000).

As a general model (Fig. 2), association of coactivators or corepressors with a nuclear hormone receptor is a ligand-dependent process. Coactivators and corepressors recruit HATs and HDACs, respectively, that finally result in gene activation or repression (Robyr *et al.*, 2000).

NUCLEAR RECEPTORS IN THE INSECT ECDYSONE GENE REGULATORY HIERARCHY

In insects, two classes of lipophilic hormones control many events in metamorphosis, development and reproduction. One class is the steroid hormones known as ecdysteroids. The active form of ecdysteroid appears to be 20-hydroxyecdysone (20E). The other class is the sesquiterpenoids also known as juvenile hormones (Fig. 3) (Riddiford, 1985; Bownes, 1986; Dhadialla and Raikhel, 1994). During the last decade,



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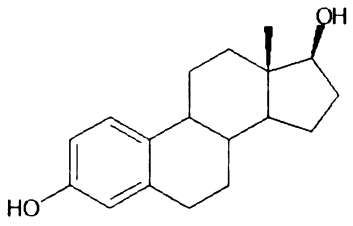

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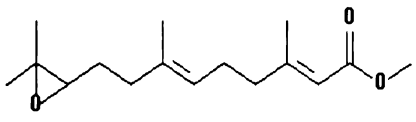

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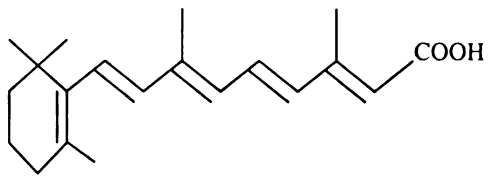

Retinoic acid

Fig. 3. Structures of major insect hormones and their vertebrate analogs. 20-hydroxy-ecdysone (20E) and juvenile hormone III (JH III) are insect hormones; Estradiol and 9-cis retinoic acid (retinoic acid) are two vertebrate hormones. The arrow on 20E indicates the difference between ecdysone and its active form (20E).

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the molecular mechanism of 20E action has been studied in detail (Thummel, 1995; Raikhel *et al.*, 1999; Kozlova and Thummel, 2000; Riddiford *et al.*, 2001), while the molecular basis underlying juvenile hormone action remains obscure (Riddiford, 1996).

The functional receptor for 20-hydroxyecdysone is the heterodimer of EcR (NR1H1) and Ultraspiracle (USP; NR 2B4) (Koelle *et al.*, 1991; Yao *et al.*, 1992, 1993). Both EcR and USP have multiple isoforms that differ only in their N-terminal A/B domains in many insects. In *Drosophila*, there exists one EcR gene, which encodes three EcR isoforms: EcR-A, -B1 and-B2. EcR-A and EcR-B result from utilization of alternative promoters while EcR-B1 and EcR-B2 are products of alternative splicing under the same promoter (Koelle *et al.*, 1991; Talbot *et al.*, 1993). The EcR isoforms identified in other insects are named similarly to the *Drosophila* EcR isoforms.

In *Drosophila*, only one isoform of USP encoded by a single gene is known (Oro *et al.*, 1990; Henrich *et al.*, 1990; Shea *et al.*, 1990). Yet two different molecular weight proteins are found, possible due to the use of an alternative translational start site (Henrich *et al.*, 1994). By contrast, two USP isoforms have been found in three other insects including, *Aedes aegypti* (Kapitskaya *et al.*, 1996), *Manduca* (Jindra *et al.*, 1997), and *Chironomus tentans* (Vogtli *et al.*, 1999). It is unclear thus far whether these isoforms result from alternative splicing of a single gene, or from different genes. The A/B domains of *Aedes* USP-A, *Chironomus* USP-1, and *Manduca* USP-1 exhibit the highest similarity, particularly at the N-terminus, to that of the single USPs of *Drosophila*, the silkworm *Bombyx mori* (Tzertzinis *et al.*, 1994), and spruce budworm *Choristoneura fumiferana* (Perera *et al.*, 1999).

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Both EcR and USP are required for 20E binding and binding to ecdysone responsive elements (EcRE). The first EcRE was identified in the promoter of the heat shock protein-27 gene in *Drosophila* (Riddihough and Pelman; 1987). It is an imperfect palindrome with only a 1-bp spacer (hsp27). Several other EcREs have also been identified in the regulatory regions of four *Drosophila* genes: Eip28/29 (Cherbas *et al.* 1991), Fbp-1 (Antoniewski *et al.*, 1994), Sgs-4 (Lehmann and Korge, 1995), and Lsp-2 (Antoniewski *et al.*, 1995), each of which contains an imperfect inverted repeat with a 1-bp spacer (IR-1). These findings suggest that natural *Drosophila* EcREs are predominantly IR-1s. Nonetheless, artificial direct repeats (DR) with different spacers appear to be functional as well (Horner *et al.*, 1995). In addition, a direct repeat EcRE with a 12-nucleotide spacer exists in the *Drosophila 3C/ng* (D'Avino *et al.*, 1995).

In invertebrates, perhaps the most surprising discovery in the nuclear receptor superfamily is the large number of genes that are regulated by ecdysone. Most of these genes are nuclear orphan receptors, which are either induced or repressed by this hormone at the level of transcription. In *Drosophila*, the discovery of most of these genes arose from efforts to understand how ecdysone directs early stages of metamorphosis. Early studies from polytene chromosome puffing patterns in larval salivary glands have revealed that ecdysone signaling is transduced and amplified via a two-step regulatory hierarchy (Ashburner *et al.*, 1974), which is widely cited as the Ashburner model. At the ends of larval and prepupal development, pulses of ecdysone directly induce a small number of "early genes", at least three of which encode transcription factors: E74 (Burtis *et al.*, 1990; Thummel *et al.*, 1990), and two nuclear receptors: E75 (Segraves and Hogness, 1990) and BR-C (DiBello *et al.*, 1991). These

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early genes both repress their own expression and induce more than 100 "late genes" as secondarily responsive genes. Interestingly, the early genes are expressed widely, while many late genes are expressed in a tissue-specific manner such as the Sgs-4 glue and L71 genes (Thummel, 1996).

E75 is a complex ecdysone–inducible early gene that encodes three isoforms, E75A, E75B and E75C as a result of alternative splicing (Feigl *et al.*, 1989; Segraves and Hogness, 1990). All three E75 isoforms are orphan receptors. E75A and E75C have distinct A/B domains joined to the identical DBD and LBD. In contrast, E75C lacks the first zinc finger of the DBD, suggesting that it is incapable of binding DNA. Consistent with the Ashburner model, several EcR/USP binding sites have been found in the E75 promoter (Talbot, 1993), and E75A protein is found to be bound to both early and late gene loci (Hill *et al.*, 1993). Moreover, recent studies have revealed that E75 is also required for development of egg chambers. This function was determined in E75 germline mutants in which nurse cells degenerate (Buszczak *et al.*, 1999).

There are some nuclear orphan receptors showing delayed early responses to ecdysone. HR3 is one of those, and therefore is called an "early-late" gene in the ecdysone gene regulatory hierarchy (Koelle *et al.*, 1992). *Drosophila* HR3 (DHR3) comprises a complex locus, encoding at least three different-sized classes of mRNAs. The *Drosophila* "early-late" genes are induced by ecdysone, but differ from the early genes in that they require ecdysone-induced protein for maximal levels of gene expression (Stone and Thummel, 1993; Horner *et al.*, 1995). Functional studies have revealed that DHR3 represses the ecdysone induction of early genes and induces βFTZ-

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F1, an orphan nuclear receptor that is essential for the appropriate response to the subsequent prepupal pulse of ecdysone (White *et al.*, 1997).

The orphan receptor *Drosophila* βFTZ-F1 has been identified as providing a temporal link between late larval and prepupal ecdysone responses (Woodard *et al.*, 1994). *Drosophila* βFTZ-F1 is expressed during the brief interval of low ecdysone titer in midprepupae. Consistent with its expression profile, 20E represses βFTZ-F1 transcript in *in vitro* cultured salivary glands. Ectopic expression of βFTZ-F1 in flies enhances ecdysone induction of the early genes; in βFTZ mutants the ecdysone-triggered genetic hierarchy is severely attenuated. Thus, βFTZ-F1 serves as a competence factor for stage-specific transcription responses to ecdysone, and its expression in the midprepupae appears to reset the system to allow reinduction of the early and late genetic responses to ecdysone (Woodard *et al.*, 1994; Broadus *et al.*, 1999).

In addition, it has been established that some insect nuclear orphan receptors appear to be involved in negative regulation of ecdysone responses. Three orphan receptors with this function have been identified, DHR38 (Sutherland *et al.*, 1995), Svp (Zelhof *et al.*, 1995a), DHR78 (Fisk and Thummel, 1995, 1998; Zelhof *et al.*, 1995b). The formation of inactive heterodimers between DHR38 and USP, SVP and EcR, as well as the DHR78 homodimer as a competitor, inhibit DNA binding and activation of EcR/USP on the ecdysone responsive elements, providing different levels at which ecdysone responses can be negatively controlled (Sutherland *et al.*, 1995; Zelhof *et al.*, 1995a; Fisk and Thummel, 1995, 1998; Zelhof *et al.*, 1995b).

Furthermore, recent studies on the molecular mechanism of EcR/USP action have demonstrated that cofactors are involved in activation or repression of ecdysone gene

regulation. Two *Drosophila* co-repressors have been characterized to interact with unliganded EcR/USP: Alien (Dressel *et al.*, 1999) and SMRTER (silencing mediator for RXR and TR-related ecdysteroid receptor interacting factor) (Tsai *et al.*, 1999). These proteins can interact directly with EcR, but not USP, in the absence of ligand. SMRTER has a similar function but different structure relative to the vertebrate corepressors SMRT and N-CoR. Antibody staining of salivary gland polytene chromosomes has displayed that SMRTER co-localizes with EcR/USP, suggesting these proteins function together to repress target gene expression. Like vertebrate SMRT and N-CoR, *Drosophila* SMRTER mediates repression by interaction with Sin3A, a repressor to form a complex with HDAC (Tsai *et al.*, 1999). *Drosophila* Alien is another co-repressor for EcR/USP, belonging to a novel class of co-repressors that are unrelated to SMRT and N-CoR. Alien proteins are highly conserved in evolution, showing 90% identity between human and fly. Vertebrate Alien interacts with TR only in the absence of hormone; *Drosophila* Alien shows similar activity, mediating repression through the Sin3A pathway (Dressel *et al.*, 1999).

It was believed that there was no SRC family coactivator in *Drosophila* and yeast (Freedman, 1999); however, a recent discovery has demonstrated that *Drosophila* TAI is an EcR/USP coactivator, the homolog of AIB1 (SRC3) (Bai *et al.*, 2000). Tai protein has the conserved sequence and overall domain structure of a SRC family member, containing bHLH, PAS and LXXLL motifs. TAI also shows a functional similarity with SRC proteins. TAI directly interacts with EcR in a ligand dependent manner, and co-localizes with EcR/USP in egg chambers and salivary gland polytene chromosomes. Furthermore, TAI augments transcriptional activation by EcR/USP in cultured cells (Bai

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et al., 2000). These findings provide the first evidence that nuclear receptor coactivators exist in both vertebrates and invertebrate flies.

Based on findings to date, most insect nuclear receptors have vertebrate homologs, exhibiting high levels of evolutionary conservation between vertebrates and invertebrates in the nuclear receptor superfamily. These conservations include similarities of protein structures and general molecular mechanisms of nuclear receptors and their cofactors (Table 1).

Table 1. Conservation of nuclear receptors and cofactors between insects and vertebrates

Insect Nuclear Receptor	**Vertebrate Homolog**	**% DBD/LBD Identity**
EcR	BAR (FXR)	81/34
USP	RXR	86/49
E75	Rev-Erb	79/35
HR3	RORα	75/20
FTZ-F1	SF1	88/33
HR78	TR2	74/26
HR38	NGFI-B	88/54
SVP	COUP-TF	94/93
HNF4	HNF4	90/67
Insect Co-activator	**Vertebrate Homolog**	**Domain Similarity**
TAI	AIB1	bHLH, PAS, LXXLL
Insect Co-repressor	**Vertebrate Homolog**	**% Sequence identity**
SMRTER	SMRT/N-CoR	High in SNOR, SANT, ITS, LSD, and GSI motifs
Alien	Alien	90

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NUCLEAR RECEPTORS IN MOSQUITO VITELLOGENESIS

Mosquitoes serve as vectors for many devastating human diseases (Collins and Paskewitz, 1995; Butler *et al.*, 1997; Beier, 1998) due to their ability to transmit pathogens during blood feeding, which is required for their egg development. In the anautogenous mosquitoes, vitellogenesis and egg maturation are initiated only after a female mosquito ingests vertebrate blood. A blood meal triggers a hormonal cascade resulting in production of 20E, the terminal signal, which activates yolk protein precursor (*YPP*) genes in the fat body (Hagedorn, 1983; 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994; Deitsch *et al.*, 1995a; 1995b).

In the anautogenous mosquito *Aedes aegypti*, which is being studied as a model vector, vitellogenesis proceeds through two periods (Fig. 4). The approximately 3-day-long previtellogenic development of both the ovary and fat body proceeds under the control of juvenile hormone (JH) III (Gwartz and Spielman, 1973; Flannagan and Hagedorn, 1977). JH titers are high during the previtellogenic period and drop immediately following the onset of vitellogenesis, which is initiated when the female mosquito takes a blood meal (Shapiro *et al.*, 1986). The fat body then produces YPP, which are internalized by the developing oocytes (Raikhel, 1992). In addition to vitellogenin (Vg), the major YPP, the mosquito fat body synthesizes three other YPPs, vitellogenic carboxypeptidase (VCP), vitellogenic cathepsin B (VCB) (Cho *et al.*, 1991; 1999), and lipophorin (Sun *et al.*, 2000). YPP synthesis reaches its maximal levels at 24 hr post-blood meal (PBM) and then proceeds until 30-32 hr PBM, when it is rapidly terminated (Raikhel, 1992).

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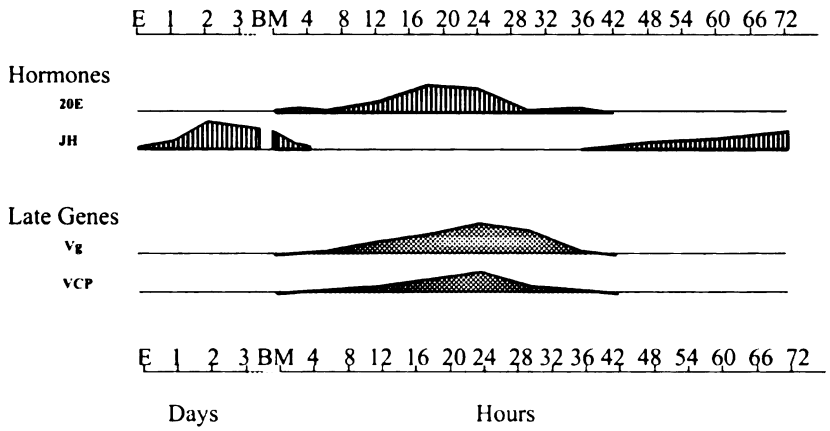

Fig. 4. Summary of events during the first cycle of vitellogenesis in the anautogenous mosquito, *Aedes aegypti*. The previtellogenic period begins at eclosion (E) of the adult female. During first ~2-3 days of post-eclosion life, the fat body becomes competent for subsequent vitellogenesis. The female then enters a state-of-arrest; yolk protein precursors, vitellogenin (Vg) and vitellogenic carboxypeptidase (VCP) are not synthesized during this previtellogenic period. Only when the female takes a blood meal (BM), is vitellogenesis initiated. Hormones: hormonal titers of juvenile hormone (JH) and ecdysteroids (20E) in *A. aegypti* females (Hagedorn *et al.*, 1975; Shapiro and Hagedorn, 1982). Transcript profiles of yolk protein precursors (Late Genes) in the mosquito fat body were determined by Northern blot and RT-PCR/Southern blot. Vg, vitellogenin (Cho and Raikhel, 1992); VCP, vitellogenic carboxypeptidase (Cho *et al.*, 1991). Modified from Li *et al.*, 2000.

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Ecdysteroid titers in the female mosquito hemolymph positively correlate with the rate of YPP synthesis in the fat body (Fig. 4). While the 20E titers are only slightly elevated at 4 hr PBM, they begin to rise sharply at 6-8 hr PBM, and reach their maximum levels at 18-24 hr PBM (Hagedorn *et al.*, 1975). Consistent with the initially proposed role of 20E in activating vitellogenesis in anautogenous mosquitoes (Hagedorn *et al.*, 1973; Hagedorn and Fallon, 1973), experiments using an *in vitro* fat body culture have shown that physiological doses of 20E (10^{-7} - 10^{-6} M) activate two *YPP* genes, *Vg* and *VCP* (Deitsch *et al.*, 1995a; 1995b). Utilization of the protein synthesis inhibitor, cycloheximide (Chx), has demonstrated that the activation of *YPP* genes in the mosquito fat body requires protein synthesis (Deitsch *et al.*, 1995a). Thus, it is likely that a regulatory cascade similar to that seen in *Drosophila* development mediates the action of 20E in the mosquito fat body.

The functional ecdysteroid receptor is a heterodimer of the ecdysone receptor (EcR) and a retinoid X receptor (RXR) homolog, Ultraspiracle (USP) (Yao *et al.*, 1992; 1993). In the mosquito *Aedes aegypti*, one isoform of ecdysone receptor (*AaEcR*) and two *USP* isoforms, *AaUSP-A* and *AaUSP-B* have been cloned (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996). The mosquito EcR/USP heterodimer has been shown to bind to various EcREs to modulate ecdysone regulation of target genes (Wang *et al.*, 1998). Two classes of EcREs, inverted repeats (IR) and directed repeats (DR), with different spacers are able to be bound by AaEcR/AaUSP. The IR-1 (IR with a one nucleotide spacer) and DR-4 (DR with a four nucleotide spacer) display optimal binding affinities in each class of EcRE. Changing the half sites of the *Drosophila* natural hsp27 EcRE from imperfect repeats to perfect repeats AGGTCA increases the DNA binding affinity of

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AaEcR/AaUSP 10 fold. The spacer-length requirement for DRs is less stringent relative to IRs. These findings suggest that although EcREs identified in some *Drosophila* gene promoters are predominantly IRs, some other native EcREs with different configurations may exist in other insects (Wang *et al.*, 1998). Indeed, most recent studies on the mosquito Vg gene promoter have revealed that an imperfect DR-1 EcRE (Vg EcRE) is located at the 348 bp upstream of the Vg promoter, and AaEcR/AaUSP is able to bind to the Vg EcRE. Moreover, deletion of the Vg EcRE from the Vg promoter-reporter results in loss of activation by AaEcR/AaUSP in cultured cells (Martin *et al.*, 2001).

AaUSP-A and AaUSP-B are the first two USP isoforms identified in insects (Kapitskaya *et al.*, 1996). AaUSP-A and AaUSP-B are identical proteins except at the N-terminal regions of the A/B domains: the first 31 aa in AaUSP-A different from the first 6 aa in AaUSP-B. Both mosquito USP isoforms can form heterodimers with AaEcR and bind to EcREs. In the fat body, AaUSP-A is expressed highly at the previtellogenic stage and post-vitellogenic stage while AaUSP-B is expressed at relative high levels in the vitellogenic stage (Kapitskaya *et al.*, 1996). These data suggest that the two isoforms may play different roles during mosquito vitellogenesis.

Previtellogenic, preparatory development is required for mosquito fat bodies to attain both the responsiveness to 20E and competence for massive yolk protein synthesis and secretion. Following this stage, mosquitoes enter a previtellogenic developmental arrest, namely the state-of-arrest, which prevents the activation of YPP genes in the previtellogenic female fat body prior to blood feeding (Raikhel and Lea, 1990). Recent studies have demonstrated that AHR38, a mosquito homolog of DHR38 and vertebrate NGFI-B, plays a crucial role in the state of arrest (Zhu *et al.*, 2000). AHR38 is able to

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disrupt binding of EcR/USP to EcREs and inhibit transactivation of a reporter gene by EcR/USP in cultured cells. GST pulldown and co-immunoprecipitation experiments have revealed that AaUSP, the obligatory heterodimer partner for AaEcR, forms a heterodimer with AHR38 at the state-of-arrest, preventing the formation of a functional EcR/USP complex (Zhu *et al.*, 2000). Consistent with these findings, binding of the EcR/USP complex to EcREs in the fat body nuclear extracts is barely detectable by EMSAs at the state-of-arrest; in contrast, a large amount of binding is visible in the 1hr PBM nuclear extracts (Miura *et al.*, 1999).

Another nuclear orphan receptor AaSVP, a homolog of *Drosophila* SVP and vertebrate COUP-TF, has been cloned recently in our lab. AaSVP is highly conserved with its homologs in term of amino acid sequence. AaSVP interacts with AaUSP in the post vitellogenic period, suggesting its involvement in terminating vitellogenic responses to ecdysone in mosquito fat bodies (Miura, Zhu, and Raikhel, unpublished data).

Finally, it is known that the mosquito Vg gene is activated in a tissue specific manner. The nuclear orphan receptor HNF-4, a member of the Hepatocyte Nuclear Factor 4 (HNF-4) family, is possibly implicated in controlling tissue specificity. Three HNF-4 isoforms are expressed in mosquito fat bodies with different developmental profiles during vitellogenesis, suggesting distinct functions for each isoform at different stages of vitellogenesis (Kapitskaya *et al.*, 1998).

RATIONALE FOR CURRENT STUDIES

One of the main research interests in Dr. Raikhel's lab is to understand the molecular mechanism underlying mosquito vitellogenesis. In his lab, several yolk

protein precursor genes have been cloned, which are expressed in a stage-, tissue- and sex-specific manner. These genes are activated in female fat bodies by the hormone 20E, which is triggered by a blood meal. This system of stringently controlled gene regulation provides an ideal model to study ecdysone gene regulation during insect reproduction. In contrast to detailed studies on the ecdysone regulatory cascade in *Drosophila* development and metamorphosis, however, the ecdysone gene regulatory hierarchy in insect reproduction was poorly understood.

In mosquitoes, one isoform of EcR and two isoforms of USP have been cloned in our lab, but some primary questions still need to be addressed. Three EcR isoforms have been identified in *Drosophila*; similarly, two or three isoforms have been found in many other insects. It has been demonstrated that the different EcR isoforms are expressed in different tissues and mediate functionally different ecdysone responses. Therefore, in mosquitoes, one of the key questions was whether any other EcR isoforms exist, and if they do, whether they are involved in vitellogenesis. Secondly, two USP isoforms have been identified in mosquitoes and they are expressed differentially during vitellogenesis. AaUSP-A mRNAs in fat bodies are at high levels during previtellogenic and postvitellogenic periods, but at low levels during the vitellogenic period. In contrast, AaUSP-B is expressed at high levels during the vitellogenic stage. Correlated with AaUSP mRNA levels, the two hormones, JHIII and 20E, display opposite titer dynamics during pre- and vitellogenic stages. JHIII titers are high during the previtellogenic stage and decline immediately following the onset of vitellogenesis. 20E remains at low levels during the previtellogenic stage and rises sharply after a blood meal. A recent study suggests that USP may be a candidate receptor for JH (Jones and Sharp, 1997).

Therefore, other more critical questions addressed in the first part of my research were how the mosquito USP gene(s) are regulated during vitellogenesis and whether AaUSP-A and AaUSP-B isoform expressions are under the control of JHIII or 20E.

The ecdysone gene regulatory hierarchy has been well documented in *Drosophila* metamorphosis. This regulatory cascade contains many transcription factors, most of which are nuclear orphan receptors. In mosquitoes, utilization of the protein synthesis inhibitor, cycloheximide (Chx), has demonstrated that the activation of *YPP* genes in the mosquito fat body requires protein synthesis (Deitsch *et al.*, 1995a). Thus, YPP genes serve as the ecdysone regulated late target genes. It is likely that a regulatory cascade similar to that seen in *Drosophila* development mediates the action of 20E in the mosquito fat body. Among the components of the ecdysone gene regulatory hierarchy in *Drosophila*, the early gene E75 and the early-late gene HR3 are two key ones. Therefore, in the second and third parts of my studies, mosquito homologs of E75 and HR3 were cloned and characterized as a beginning to elucidating the ecdysone regulation cascade in mosquito vitellogenesis.

A preparatory developmental previtellogenic period is required for the mosquito fat body to attain competence for ecdysone responsiveness (Sappington and Raikhel, 1999). Flannagan and Hagedorn (1977) have demonstrated that the acquisition of competence for the ecdysone response in the mosquito fat body is under the control of juvenile hormone III. However, the molecular mechanism underlying the change in sensitivity to ecdysone in the mosquito fat body remains obscure. In the fourth part of my research, I addressed the question of whether competence for the ecdysone response is controlled at the transcriptional level or translational level. Furthermore, the mosquito

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homolog of βFTZ-F1, a competence factor in the *Drosophila* ecdysone regulatory network, was cloned. I then designed experiments to address the question of what role mosquito FTZ-F1 plays during vitellogenesis, and whether the molecular mechanisms controlling the stage-specific ecdysone responses are conserved during *Drosophila* metamorphosis and mosquito vitellogenesis.

Finally, in the fifth part of my research, I cloned the mosquito homolog of *Drosophila HR78*, which is involved in negative regulation of ecdysone responses. I designed experiments and answered the questions of whether mosquito HR78 represses the DNA binding and transactivation functions of AaEcR/AaUSP, and what role it plays during vitellogenesis.

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Chapter 2*

Characterization of Two Isoforms of the Mosquito Ecdysone Receptor (EcR) and Ultraspiracle (USP)

*Most results have originally been published in Dev.Biol.

Ref: Wang, S.-F., Li, C.[#], Zhu, J., Miura, K., Miksicek, R. J., Raikhel, A. S. (2000). *Dev. Biol.* 218, 99-113.

[#] Underlined two authors contributed equally to this work.

ABSTRACT

Ultraspiracle (USP), the insect homologue of the vertebrate retinoid X receptor (RXR) is an obligatory dimerization partner for the ecdysteroid receptor (EcR). Two USP isoforms, USP-A and USP-B, with distinct N-termini occur in the mosquito, *Aedes aegypti*. In the fat body and ovary, USP-A mRNA is highly expressed during the pre- and late-vitellogenic stages, corresponding to a period of low ecdysteroid titer, while USP-B mRNA exhibits its highest levels during the vitellogenic period, correlating with a high ecdysteroid titer. Remarkably, 20-hydroxyecdysone (20E) has opposite effects on USP isoform transcripts in *in vitro* fat body culture. This steroid hormone up-regulates USP-B transcription and its presence is required to sustain a high level of USP-B expression. In contrast, 20E inhibits activation of USP-A transcription. Although EcR/USP-A recognizes the same ecdysteroid responsive elements, EcR/USP-B binds them with an affinity two-fold higher than that of EcR/USP-A. Likewise, EcR/USP-B transactivates a reporter gene in CV-1 cells two-fold more strongly than EcR/USP-A. These results suggest that USP-B functions as a major heterodimerization partner for EcR during the vitellogenic response to 20E in the mosquito.

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INTRODUCTION

In insects, as in higher metazoans, steroid-regulated gene expression is mediated by a family of intracellular receptors, which belong to the superfamily of nuclear receptors. These receptors have a highly conserved structure, including a DNA-binding domain (DBD) and a multifunctional ligand-binding domain (LBD). In addition to conferring ligand binding specificity, the LBD is responsible for dimerization, transactivation, co-activator/co-repressor interactions, and in the case of vertebrate steroid receptors, heat shock protein binding (O'Malley and Conneely, 1992; Mangelsdorf *et al.*, 1995; Onate *et al.*, 1995). Nuclear receptors are often encoded by several genes and each gene may produce several subtypes called isoforms due to utilization of alternative promoters, splicing or polyadenylation signals (Keightley, 1998). The major differences between isoforms appear to be centered in the N-terminal A/B domain. This least conserved region of nuclear receptors contains the activation function AF-1, which has been demonstrated to provide cell- and promoter-specific transactivation properties (Kastner *et al.*, 1990; Kuiper *et al.*, 1996; Paech *et al.*, 1997).

The major insect steroid hormones, ecdysteroids, are involved in regulating development, molting, metamorphosis and reproduction (Riddiford, 1993b; Raikhel, 1992). DNA binding, ligand binding and transfection assays indicate that the functional ecdysteroid receptor complex consists of two subunits, the ecdysteroid receptor itself (EcR) and the retinoid X receptor homologue Ultraspiracle (USP), both of which are members of the nuclear receptor superfamily (Koelle *et al.*, 1991; Yao *et al.*, 1992; 1993; Thomas *et al.*, 1993). The role of USP as a partner in the functional ecdysteroid receptor was further substantiated by mutational analysis. Fly *usp* mutants show multiple defects

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in ecdysteroid-regulated gene expression at the larval-prepupal transition, indicating that USP is required for ecdysteroid receptor activity *in vivo* (Hall and Thummel, 1998).

The pleiotropic effects of ecdysteroids are reflected by the existence of multiple EcR isoforms. EcR isoforms were first identified in *Drosophila* (Talbot *et al.*, 1993) and then in other insects including *Bombyx mori* (Swever *et al.*, 1995; Kamimura *et al.*, 1996; 1997) and *Manduca sexta* (Fujiwara *et al.*, 1995; Jindra *et al.*, 1996a). The three *Drosophila* EcR isoforms (EcR-A, EcR-B1 and EcR-B2) differ in the N-terminal A/B domain, as a result of utilization of different transcription start sites and alternative splicing. Distinct functions of DmEcR isoforms were first evidenced by their different tissue- and stage-specific expression profiles (Talbot *et al.*, 1993). In the *Drosophila* nervous system, EcR-A and EcR-B1 isoforms have clearly distinct functions (Truman *et al.*, 1994). A high level of EcR-A expression in the ventral CNS correlates with rapid degeneration of neurons after adult emergence (Robinow *et al.*, 1993). Moreover, *Drosophila* EcR mutations confirm that functional differences exist among receptor isoforms (Bender *et al.*, 1997). In particular, the EcR-B1 mutants can not survive through metamorphosis, and EcR-B1 and EcR-B2 mutants fail to prune back larval-specific dendrites to initiate larval neuron remodeling (Schubiger *et al.*, 1998).

Unlike *Drosophila* USP for which a single form of mRNA has been identified (Shea *et al.*, 1990; Henrich *et al.*, 1990, Oro *et al.*, 1990), two USP isoforms are reported in the mosquito *Aedes aegypti* (USP-A and USP-B, Kapitskaya *et al.*, 1996) and in the moth, *M. sexta* (USP-1 and USP-2, Jindra *et al.*, 1997). In both insects, USP isoforms exhibit distinct N-terminal A/B domains similar to isoform variation in the EcR isoforms. In *A. aegypti*, the N-terminal 31 aa in USP-A are different from the first 6 aa in USP-B.

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Moreover, their 5' untranslated regions (UTRs) are different, suggesting that these isoforms are most likely derived from the same gene via utilization of alternative promoters (Kapitskaya, *et al.*, 1996).

In sharp contrast to the large body of information defining the roles of EcR isoforms, little is known about the properties of USP isoforms. The lack of USP isoforms in *Drosophila* makes it difficult to perform USP isoform-specific mutant analysis. However, identification of two forms of USP from *M. sexta* has permitted the study of USP isoform profiles during development (Jindra, 1997; Asahina *et al.*, 1997; Hiruma *et al.*, 1999; Lan *et al.*, 1999). The level of USP-2 mRNA correlates with the ecdysteroid titer whereas USP-1 transcription is complementary to that of USP-2. However, it is the EcR/USP-1 complex, not the complex containing EcR/USP-2, that activates the MHR-3 promoter although MHR-3 mRNA appears following a rise of ecdysteroid levels (Lan *et al.*, 1999). Likewise, the two *Aedes* USP isoforms enabled us to investigate the roles of distinct USP isoforms in the adult insect during reproduction. Indeed, mosquito vitellogenesis has provided a perfect model to study ecdysteroid-regulated gene expression during reproduction, as the endocrine release of ecdysteroids is tightly controlled by a blood meal (Fig. 1-A). The DNA binding properties of the *Aedes* EcR/USP-B complex have been studied in detail: it binds to various ecdysteroid responsive elements (EcRE) with the AGGTCA consensus half site arranged either as direct repeats or inverted repeats. A 1-bp spacer is optimal for inverted repeats, while a 4-bp spacer is optimal for direct repeats. DNA binding activity is correlated with transactivation as demonstrated by transfection assays in CV-1 cells (Wang *et al.*, 1998). Only the heterodimeric complex containing the mosquito EcR and one of the mosquito

USP isoforms (either USP-A or USP-B) represents a functional receptor capable of binding the EcRE or the ecdysteroid ligand (Kapitskaya *et al.*, 1996).

Here, we report the developmental profiles of the mosquito USP isoforms, their differential responses to 20-hydroxyecdysone (20E) as well as functional differences with respect to their DNA binding and transactivation abilities. Taken together, these findings suggest that USP isoforms clearly perform distinct functions during mosquito vitellogenesis. Furthermore, these data suggest that it is USP-B that serves as a major partner for EcR during the mosquito vitellogenic response to ecdysteroid.

MATERIALS AND METHODS

Animals

Mosquitoes, *A. aegypti*, were reared as described by Hays and Raikhel (1990). Vitellogenesis was initiated in 3-5 day old adult female mosquitoes by blood feeding them on rats.

RNA Isolation and RT-PCR/Southern Blot Analysis

Fat bodies and ovaries were dissected from female mosquitoes at different time points ranging from 0- to 5-day after eclosion at the previtellogenic stage, or from vitellogenic females ranging from 1 to 60 hours post-blood-meal (PBM). The temporal profiles of transcript abundance for the mosquito EcR, USP-A and USP-B in the mosquito fat body and ovary were examined using RT-PCR followed by Southern blotting with specific radioactive probes. Total RNA was prepared from fat body throughout the first vitellogenic cycle, using the guanidine isothiocyanate method as

described previously (Bose and Raikhel, 1988) with the modification that all isopropanol precipitation steps were done without low-temperature incubation to avoid co-precipitation of glycogen and salts. Alternatively, total RNA was extracted with TRIZOL Reagent (Gibco-BRL). RNA yields were determined spectrophotometrically, absorbency ratios A_{260}/A_{280} and A_{260}/A_{230} of RNA preparations were consistently above 1.7 and 2.0, respectively. Five microgram aliquots of each RNA preparation were reverse-transcribed by the SuperscriptII reverse transcriptase (Gibco-BRL) using random hexamer primers (Promega) in a reaction volume of 20 μl. The reverse transcription product was diluted to 40 μl with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at -20°C as a cDNA pool until use. For developmental profile analyses, 0.025 fat body-equivalents of cDNA pools were used as PCR templates in the case of mosquito fat body tissues whereas an equal amount of total RNA was used for reverse transcription in the case of ovarian tissues. A 522bp USP-A specific fragment was amplified with the primer pair: forward, 5'-TCATATCGTTCCGGAGATGTGG-3' and reverse, 5'-CCAATCCTGCCAGAGGTAGTG-3'. A 400bp USP-B specific fragment was amplified with the primer pair, forward, 5'-CTTCTCACAAGAGGTGCTGAGG-3'and reverse, 5'-TGGTATCCAACTGGAACTTGCG-3'. A 314bp AaEcR specific fragment was amplified with the primer pair, forward, 5'-GAGGAAGATCAACATGACGTGC-3' and reverse, 5'-ACCGTGAGGGAGAACATCTGC-3'. A 463-bp VCP (vitellogenic carboxypeptidase, Cho *et al*, 1991) specific fragment was amplified with the primer pair, forward, 5'-AGCGCCCATTCTTGTTTGG-3' and reverse, 5'-CAGCTCATACAGGTATTCTCC-3'.

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Thermal cycling conditions were as follows: the reaction was incubated at 94°C for 3 min followed by 17 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec. As a reference to the developmental changes of the fat body, the same cDNA was subjected to 10 or 15 cycles of RT-PCR followed by Southern blotting with a primer pair specific to the mosquito VCP. After PCR amplification, 10 μl each out of the total 25μl reaction was fractionated on a 2% agarose gel, and transferred onto Hybond N+ membrane (Amersham) under alkaline conditions. Radioactive cDNA probes were prepared from 15 ng each of the PCR fragments generated by the primer pairs of EcR, USP-A, USP-B, or VCP with the corresponding plasmids as templates. These amplified fragments were labeled by a random-primers DNA labeling system (Gibco BRL) to incorporate [α-^{32}P]-dATP (NEN). The membrane was hybridized with the probe at 65°C in a solution of 5 x SSC, 5 x Denhardt' solution, 0.5%SDS, 25mM NaH_2PO_4, 25mM Na_2HPO_4, 200 μg/ml of denatured salmon sperm DNA (Sigma) following prehybridization. The membrane was washed twice at 65°C in 2 x SSC, 0.1%SDS for 15 min, twice in 0.1 x SSC, 0.1%SDS for 15 min, then autoradiographed. RT-PCR/Southern blotting of the total RNA preparations without the reverse transcriptase did not result in any appreciable signals, indicating that contamination of RNA preparations with genomic DNA fragments was virtually negligible (not shown). The radioactive blots were quantified with a phosphorimager (Molecular Dynamics).

Examination of the EcR isoforms was performed in the same method as that in the USP isoforms. When conducting this experiment, AaEcR is named as AaEcR-B, and the new isoform is named AaEcR-A. The primers for AaEcR-A are: Forward, 5'-TGACGGCCATTCCGGCTTC; Reverse, GAGACGAGTAGCCATTGGAC.

In vitro Fat Body Culture

To examine the transcriptional inducibility of mosquito EcR, USP-A and USP-B by ecdysteroid, the abdominal walls with adhering fat body (hereafter referred to as the fat body) were dissected from 3~5-day-old previtellogenic females and incubated in an organ culture system as previously described (Deitsch *et al.*, 1995a; Raikhel *et al.*, 1997). The fat bodies were incubated either with culture media in the absence of ecdysteroid or in the presence of 10^{-6} M 20E (unless otherwise indicated). At every 4-hour interval after incubation, 9 fat bodies of each time point were collected and immediately stored in liquid nitrogen until RNA isolation. For the hormone withdrawal experiments, fat bodies were washed 3 times with culture media after incubating with 10^{-6} M 20E for 4 hours and then further incubated in hormone-free media. Total RNA extraction and RT-PCR/Southern blot analyses were performed as described above.

To confirm that the transcriptional response was due to the fat body and not the epidermis of the abdominal wall, the epidermis-free fat bodies were obtained by gently vortexing the dissected abdominal walls. The epidermis-free fat body was incubated in culture medium supplemented with 10^{-6} M 20E for 3-hr. The fat body adhering to the abdominal wall was incubated under the same conditions. Similar amounts of fat body tissue from both preparations were examined for the presence of two 20E-induced genes, an early gene *E75* and the late gene VCP (Deitsch *et al.*, 1995a; Pierceall *et al.*, 1999). The levels of induction of both genes were similar in fat bodies of both preparations. However, the epidermis-free fat body could be kept alive for only a short incubation time (data not shown).

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To test the effect of the protein synthesis inhibitor cycloheximide (Chx) on transcription, dissected 3-5 day old female mosquito fat bodies were pretreated for 1-hr with culture media containing 1mM Chx, then further incubated with the same concentration of Chx either without hormone or with 10^{-6} M 20E. Chx/hormone withdrawal experiments were conducted as for the hormone withdrawal experiment described above except that 1mM Chx was added to all culture media.

Antibodies and Western Blot Analysis

The monoclonal antibody AB11 against DmUSP (Christianson et al., 1992) was a generous gift from Dr. F.C. Kafatos (EMBL, Heidelberg, Germany). Nuclear proteins were extracted from the fat bodies of female mosquitoes as previously described (Miura et al., 1999). Protein extracts corresponding to 50 mosquito equivalents were resolved on 7.5% SDS-polyacrylamide gels, followed by electro-blotting to polyvinylidene difluoride membranes (Amersham). These membranes were probed with the anti-DmUSP monoclonal antibody (1:200 dilution) and then incubated with a secondary anti-mouse IgG antibodies (1:5,000 dilution) conjugated to horseradish peroxidase (Cappel). Immune complexes were visualized by addition of SuperSignal Ultra Chemiluminescent Substrate (Pierce) and autoradiography.

***In vitro Protein Synthesis and Electrophoretic Mobility Shift** Assay (EMSA)*

Nuclear receptors were synthesized by the coupled *in vitro* transcription-translation (TNT) system (Promega). The *EcoRI* fragment of mosquito USP-A cDNA in pBluescript was subcloned into the *EcoRI* site of pGEM7Z (Promega). The same vector

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was used to subclone DmEcR and DmUSP cDNA fragments (Wang, *et al.*, 1998) whereas mosquito EcR and USP-B cDNAs were cloned into pGEM3Z (Kapitskaya *et al.*, 1996). All these expression vectors utilized the SP6 promoter to synthesize proteins, which were first confirmed by ^{35}S-Methionine labeling and SDS-PAGE following the manufacturer's instruction. EMSA and measurement of equilibrium dissociation constants were conducted as described before (Wang *et al.*, 1998). In brief, four ecdysteroid response elements (EcREs):

IR^{hsp}-1 (agagacaagGGTTCAaTGCACTtgtccaat),

IR^{per}-1 (agcttcaaAGGTCAaTGACCTtgtccatcg),

DR-4 (aagcgaaAGGTCAaggaAGGTCAggaaaat)

and DR12 (aagcgaaAGGTCAagaggccaaagaAGGTCAggaaaat) were end-labeled with γ-^{32}P-ATP (DuPont NEN) with T4 polynucleotide kinase (Gibco BRL). Unless indicated, 50 fmol of labeled EcRE was incubated with 1μl each of the indicated *in vitro* synthesized nuclear receptor proteins. Binding reactions were resolved using a 6% native polyacrylamide gel, which was then vacuum dried at 80^{0}C and exposed to X-ray film. To measure the equilibrium dissociation constants, bound and free EcRE probes were quantified with a phosphorimager, permitting the construction of saturation curves and Scatchard analysis (Wang *et al.*, 1998).

Construction of Reporter and Expression Plasmids and Cell Transactivation Assay

Transactivation assays were conducted with the green African monkey kidney cell line CV-1 (ATCC). The *EcoRI* fragment of USP-A was subcloned into the mammalian

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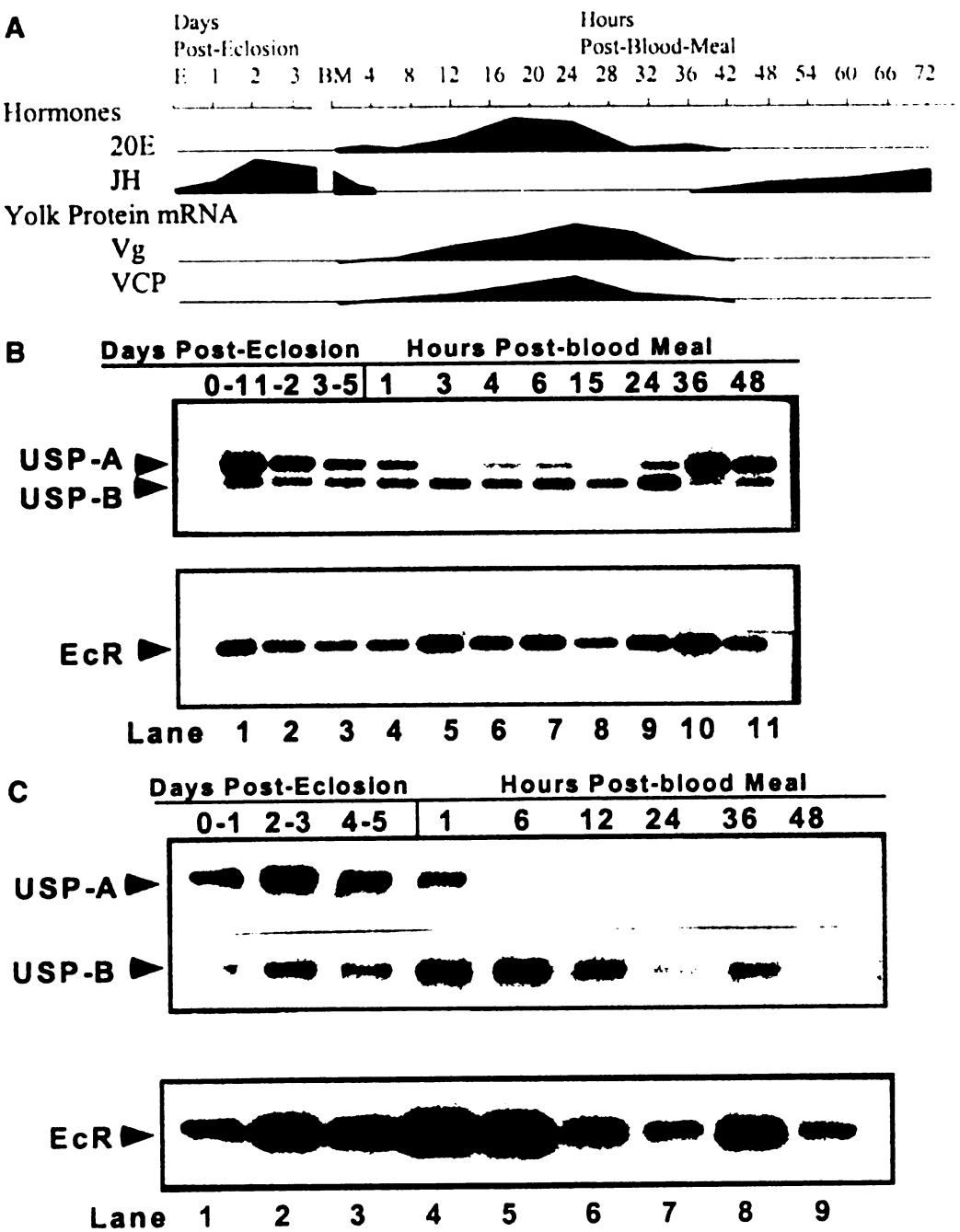
expression vector pCDNA3.1/Zeo (+), which was also used to express mosquito EcR and USP-B proteins (Wang *et al.*, 1998). CMV-LacZ was used as a co-reporter to normalize the reporter gene activities of the two reporter plasmids ΔMTV-5xIR^{hsp}-1-CAT and Δ MTV-3xDR4-CAT. Transfection assays were performed as described previously (Wang *et al.*, 1998). In brief, CV-1 cells were maintained in DMEM with 10% calf serum. Six-well plates were seeded at a density of $2x10^5$ cells/well the day before transfection. Following the manufacturer's instruction, cells were transfected for 5 hours with LipofectAMINE™ (Gibco BRL) and then added fresh medium together with either ethanol vehicle or 1μM Muristerone A. After a hormone treatment of 36 or 48 hours, cells were harvested for CAT (chloramphenicol acetyl transferase) activity and β-galactosidase activity. CAT activity was normalized with β-galactosidase activity. CAT activity was expressed as the percent of substrate converted to a product, and 1unit of CAT activity was defined as 1% of the chloramphenicol substrate converted to its acetylated forms.

RESULTS

Tissue- and Stage-Specific mRNA Expression of the Mosquito USP Isoforms

It has been previously reported that in vitellogenic tissues, the fat body and ovary, there are several transcripts corresponding to two *Aedes aegypti* USP isoforms (USP-A and USP-B) (Kapitskaya *et al.*, 1996). Here, more detailed analysis was undertaken for the developmental changes in transcripts of the two USP isoforms in the fat body and ovary during vitellogenesis. RT-PCR was performed utilizing USP isoform-specific primers followed by Southern-Blot hybridization with isoform-specific probes (Fig. 1).

Fig.1. Expression profiles of USP and EcR mRNA during vitellogenesis. (A) Hormone and Yolk protein mRNA profiles. The whole body ecdysteroid titer at various time points (hours) in post vitellogenic female mosquito is converted to 20E equivalent (Hagedorn *et al.*, 1975). JH (juvenile hormone) titer is from Shapiro *et al.*, 1986. The mRNA expression profiles of yolk protein Vg (Vitellogenin) and VCP (Vitellogenic carboxypeptidase) are from Deitsch *et al.*, 1995b. The titers are measured at various days' post-eclosion in previtellogenic mosquito and various hours' post-blood meal in vitellogenic mosquitoes. Other abbreviations: E, eclosion; BM: blood meal. **(B)** USP and EcR mRNA profiles in the fat body. Total RNA was isolated from fat body dissected from previtellogenic female mosquitoes at 0-1d, 1-2d, or 3-5d after eclosion (columns 1-3), or from vitellogenic mosquitoes 1h, 3h, 4h, 6h, 15h, 24h, 36h or 48h PBM (columns 4-11). **(C).** USP and EcR profiles in the ovary. Total RNA was isolated from ovaries dissected from previtellogenic female mosquitoes, 0-1d, 2-3d or 4-5d (columns 1-3) or vitellogenic mosquitoes 1h, 6h, 12h, 24h, 36h, or 48h PBM (column 4-9). RNA samples from each time point was subjected to reverse transcription and PCR amplification using either one pair of primers specific to AaEcR or two pairs of primers specific to USP-A and USP-B, respectively. Reverse transcribed products from equal number of fat bodies (fat body profile) or equal amount 5μg total RNA (ovary profile) were used for PCR amplification. The PCR products were resolved by agarose gel electrophoresis, followed by Southern blotting and autoradiography.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Expression of mosquito EcR was monitored in the same tissues as well. To minimize the experimental variability between PCR reactions, pooled RNA samples from fat body or ovary tissues were used in addition to low cycle numbers (17 cycles) for amplification. For control purposes, Northern blot analysis performed in parallel on samples from the same fat body or ovary tissues revealed identical mRNA profiles as RT-PCR/Southern blot analysis (data not shown).

In the fat body, USP-A mRNA was highly expressed at 0-1 day after emergence (Fig. 1B, lane 1), however its levels gradually decreased over the next 5 days of the previtellogenic period (Fig 1B, lanes 2 and 3). The level of USP-A mRNA declined even more dramatically immediately after a blood meal; it dropped at 3-hr PBM (Fig. 1B, lane 5) and remained low during the entire active vitellogenic period until 24-hr PBM (Fig 1B, lanes 6-9). The level of USP-A mRNA increased again in the fat body at the termination of the vitellogenic period, 36-hr PBM (Fig. 1B, lane 10). The expression pattern of USP-B in the fat body was significantly different from that of USP-A. The level of the USP-B transcript was relatively constant throughout the pre- and early vitellogenic periods, but exhibited an obvious enhancement by 24- hr PBM (Fig 1B, lane 9). However, this transcript declined at termination of vitellogenesis at 36-hr PBM (Fig. 1B, lane 10).

The USP-A mRNA was abundant in ovaries of newly emerged female mosquitoes (Fig 1C, lane 1). Its level increased during the second day of previtellogenic development (Fig. 1C, lane 2), but decreased slightly in 4-5 day old previtellogenic females (Fig.1C, lane 3). As in the fat body, a blood meal resulted in a dramatic drop in the USP-A mRNA level (Fig. 1C, lane 4) and the decline persisted throughout vitellogenesis (Fig. 1C, lanes 5-7). At 36-hr PBM, a small increase of USP-A mRNA was observed (Fig. 1C,

lane 8). Ovaries of newly emerged female mosquitoes also contained a detectable level of USP-B mRNA (Fig. 1C, lane 1) which was enhanced in 2- to 3-day old ovaries (Fig 1C, lanes 2-3). A blood meal triggered a considerable increase in the USP-B mRNA within one hour, and the level of USP-B transcript remained high during the first half of the vitellogenic period (Fig.1C, lanes 4-5). Thus, our results clearly demonstrated that in the adult mosquito female, the expression patterns of the two USP isoforms during the first vitellogenic cycle were dramatically different in both the fat body and ovaries.

Levels of the EcR transcript were monitored in the same tissues and times as those of the USP isoforms. The levels of EcR transcript were relatively constant in the female fat body, showing elevations at the beginning of the pre-vitellogenic phase (Fig. 1B, lane 1), early vitellogenesis (Fig. 1B, lane 5) and post-vitellogenesis (Fig. 1B, lane 10). In the ovary, the EcR transcript level also exhibited three increases at approximately similar times as in the fat body (Fig. 1C).

In Vitro Effect of 20-hydroxyecdysone on USP-A and USP-B Transcripts

The dramatic differences in the expression patterns of mosquito USP isoform transcripts observed in vitellogenic tissues suggested that mosquito USP isoforms were differentially regulated during vitellogenesis. I began studying the control of their expression by examining the effect of 20E on these USP transcripts. These experiments were conducted using an *in vitro* fat body culture system, which has been well-established and is more suitable for these studies than small mosquito ovaries (Raikhel *et al.*, 1997). In addition, unlike ovaries containing several cell types, the fat body is a relatively homogeneous tissue consisting of predominantly one cell type, trophocytes

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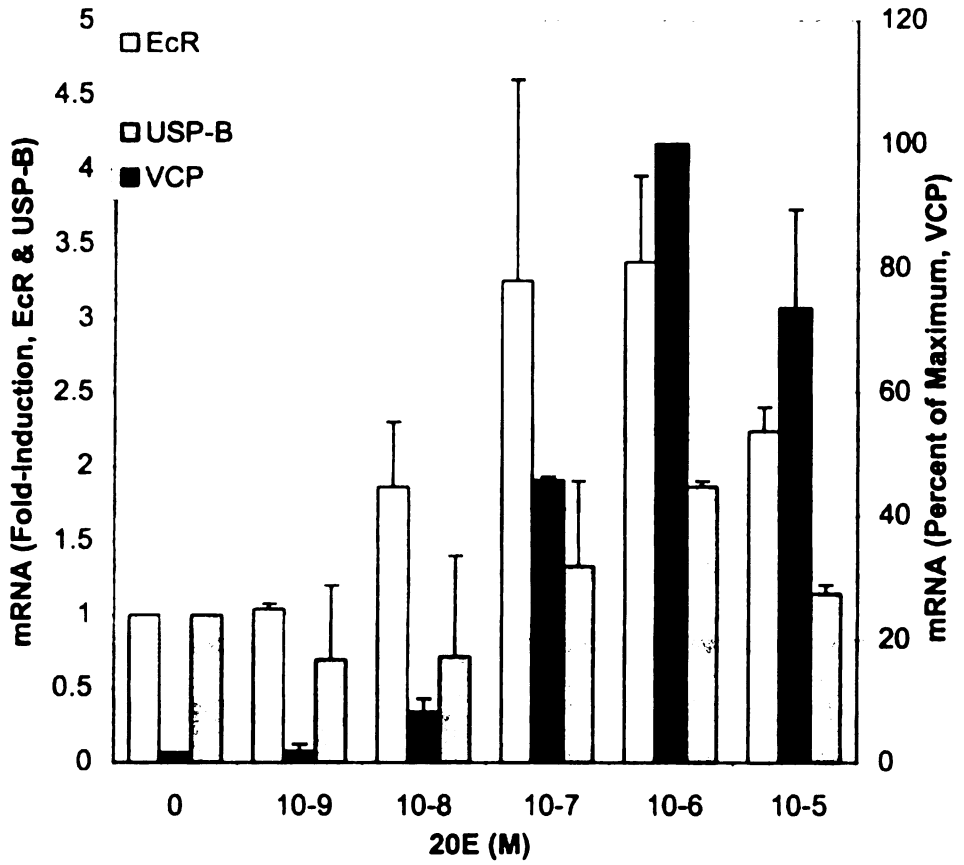

Fig. 2. Dose response of USP-B, EcR and VCP to 20E in *in vitro* fat body organ culture. Fat bodies dissected from nine previtellogenic female mosquitoes were cultured for four hours *in vitro* either in the absence of hormone or in the presence of increasing concentrations of 20E ranging from 10^{-9}M-10^{-5}M. Total RNA was isolated from these fat bodies and subjected to RT-PCR with specific primers to EcR, USP-B and VCP, respectively. The PCR products were resolved by the agarose gel electrophoresis, followed by Southern blotting and autoradiography, and were quantified by the phosphorimaging analysis. The levels of EcR and USP-B mRNA from fat bodies cultured without hormone were defined as 1 unit. The maximum level of VCP mRNA from fat bodies incubated with 10^{-6}M 20E was defined as 100, because VCP mRNA level was below detection in the previtellogenic fat bodies before the hormone treatment.

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(Raikhel, 1992), making interpretation of results easier. Abdominal walls with adhering fat bodies, isolated from previtellogenic female mosquitoes (3-5 days after eclosion) were incubated in culture medium either in the absence of hormone or in the presence of increasing amounts of 20E ranging from 10^{-9} M-10^{-5} M (Fig. 2). After a 4-hour incubation, these fat bodies were subjected to RNA isolation, RT-PCR and Southern blotting analyses. USP-B and EcR were activated by 20E in a dose-dependent manner. The mRNA levels of both EcR and USP-B were maximal at 10^{-6}M 20E (Fig. 2). In contrast, USP-A mRNA was not induced by any concentration of 20E (data not shown). As a positive control, I monitored the 20E dose response of vitellogenic carboxypeptidase (VCP) mRNA, one of the late target genes in the mosquito fat body (Cho *et al.*, 1991; Deitsch *et al.*, 1995a,b). This ecdysteroid-responsive gene exhibited a peak induction level at 10^{-6} M 20E (Fig. 2).

Next, I investigated the time course of 20E effects on the expression of USP isoforms. In this experiment, dissected previtellogenic fat bodies were incubated in culture media in the presence or absence of 10^{-6}M 20E (Fig. 3, A-D). At 4-hour intervals after incubation, USP transcripts were analyzed as described above. The effect of 20E on expression of the two USP isoforms in cultured previtellogenic fat bodies was opposite: continuous presence of 20E in the culture medium failed to induce the expression of USP-A (Fig. 3A, open squires), while it activated USP-B expression (Fig. 3B, open squares). USP-B transcript levels steadily increased with 5-, 7- and 10-fold enhancement at 8-, 12- and 16-hr of incubation, respectively (Fig. 3B, open squares). When fat bodies were incubated for four hours in the presence of 20E and then transferred into a hormone-free medium, the level of the USP-A transcript rose dramatically (Fig. 3A, open

triangles). In contrast, the USP-B transcript declined to its background level after an initial rise at 4-hr (Fig. 3B, open triangles).

The effect of 20E on the expression of AaEcR in *in vitro* cultured fat bodies was different from that of both USP isoforms. Incubation in hormone-free medium for 16-hr resulted in a steady increase from the high basal level of the EcR transcript present in the previtellogenic fat body (Fig. 3C, open circles). Continuous incubation of the fat body in 20E-containing culture medium first led to a rapid increase of the AaEcR transcript level by 4-hr incubation and then a considerably slower rise until 12-hr after incubation when the EcR level reached a plateau (Fig. 3C, open squares). Interestingly, withdrawal of the hormone after 4-hr of incubation caused the EcR transcript level to rise to a two-fold higher level (Fig. 3C, open triangles).

As a positive control, I monitored the effect of 20E on VCP. Previtellogenic fat bodies incubated in hormone-free media did not show any discernible level of VCP expression (Fig. 3D). This is consistent with results reported before (Deitsch *et al.*, 1995a). Continuous incubation in the presence of 20E resulted in the robust activation of VCP gene expression: the level of VCP mRNA doubled every 4 hours during the 12-hour incubation period and then continued to increase to the end of incubation (Fig. 3D, open squares). Hormone withdrawal after a 4-hr induction period reversed VCP gene activation, and the VCP mRNA levels returned to an undetectable level by 16-hr of incubation (Fig. 3D, open triangles).

Fig. 3. Effect of 20-hydroxyecdysone and cycloheximide on the transcription of USP-A (A and E, respectively), USP-B (B and F), EcR (C and G) and VCP (D and H) in the previtellogenic fat bodies. Previtellogenic fat bodies dissected from female mosquitoes (3-5 day after eclosion) were incubated in culture media only (open circles), with a 4-hour pulse treatment of 10^{-6}M 20E (open triangles), with continuous 20E treatment (open squares), with 1mM Chx (closed circles), with 1mM Chx and a 4-hour pulse 20E treatment (closed triangles) or with 1mM Chx and continuous 20E treatment (closed squares) for 16 hours. At every 4-hour interval, a group of 9 fat bodies were collected and subjected to RNA isolation, RT-PCR amplification and Southern blot analysis. The intensity of bands from Southern blots was quantified by phosphorimaging. For EcR, USP-A and USP-B, the level of amplified transcript detected from previtellogenic fat bodies prior to any treatment was defined as 1 unit. For the VCP mRNA, the level of amplified transcript detected from previtellogenic fat body treated only with 20E for 16 hours was defined as 100 units. For comparison, the mRNA profiles of fat bodies treated with 20E continuously were plotted in both, left and right, panels (dotted curve with open squares).

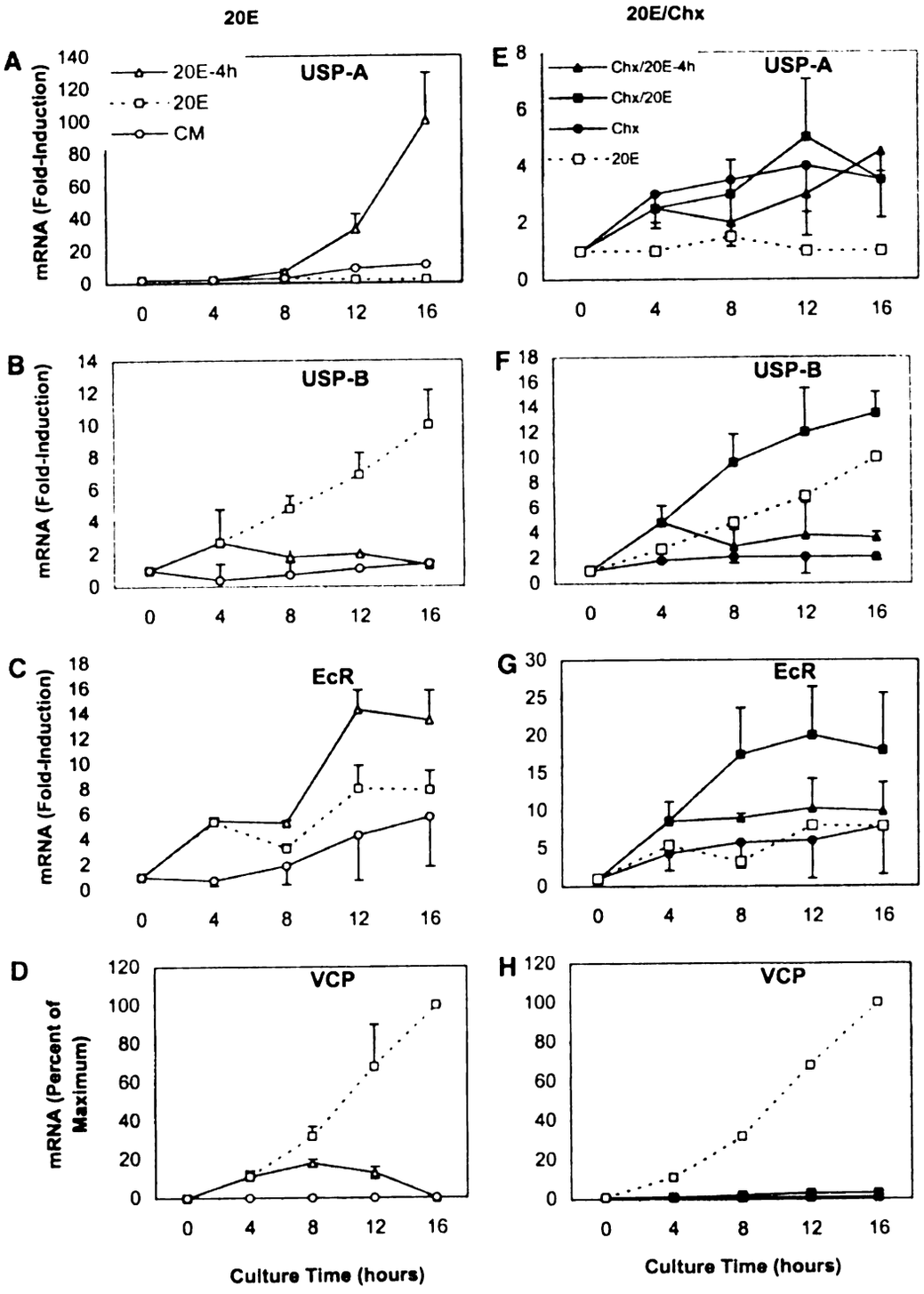
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Fig. 3. Effect of 20-hydroxyecdysone and cycloheximide on the transcription of USP-A (A and E, respectively), USP-B (B and F), EcR (C and G) and VCP (D and H) in the previtellogenic fat bodies. Previtellogenic fat bodies dissected from female mosquitoes (3-5 day after eclosion) were incubated in culture media only (open circles), with a 4-hour pulse treatment of 10^{-6}M 20E (open triangles), with continuous 20E treatment (open squares), with 1mM Chx (closed circles), with 1mM Chx and a 4-hour pulse 20E treatment (closed triangles) or with 1mM Chx and continuous 20E treatment (closed squares) for 16 hours. At every 4-hour interval, a group of 9 fat bodies were collected and subjected to RNA isolation, RT-PCR amplification and Southern blot analysis. The intensity of bands from Southern blots was quantified by phosphorimaging. For EcR, USP-A and USP-B, the level of amplified transcript detected from previtellogenic fat bodies prior to any treatment was defined as 1 unit. For the VCP mRNA, the level of amplified transcript detected from previtellogenic fat body treated only with 20E for 16 hours was defined as 100 units. For comparison, the mRNA profiles of fat bodies treated with 20E continuously were plotted in both, left and right, panels (dotted curve with open squares).

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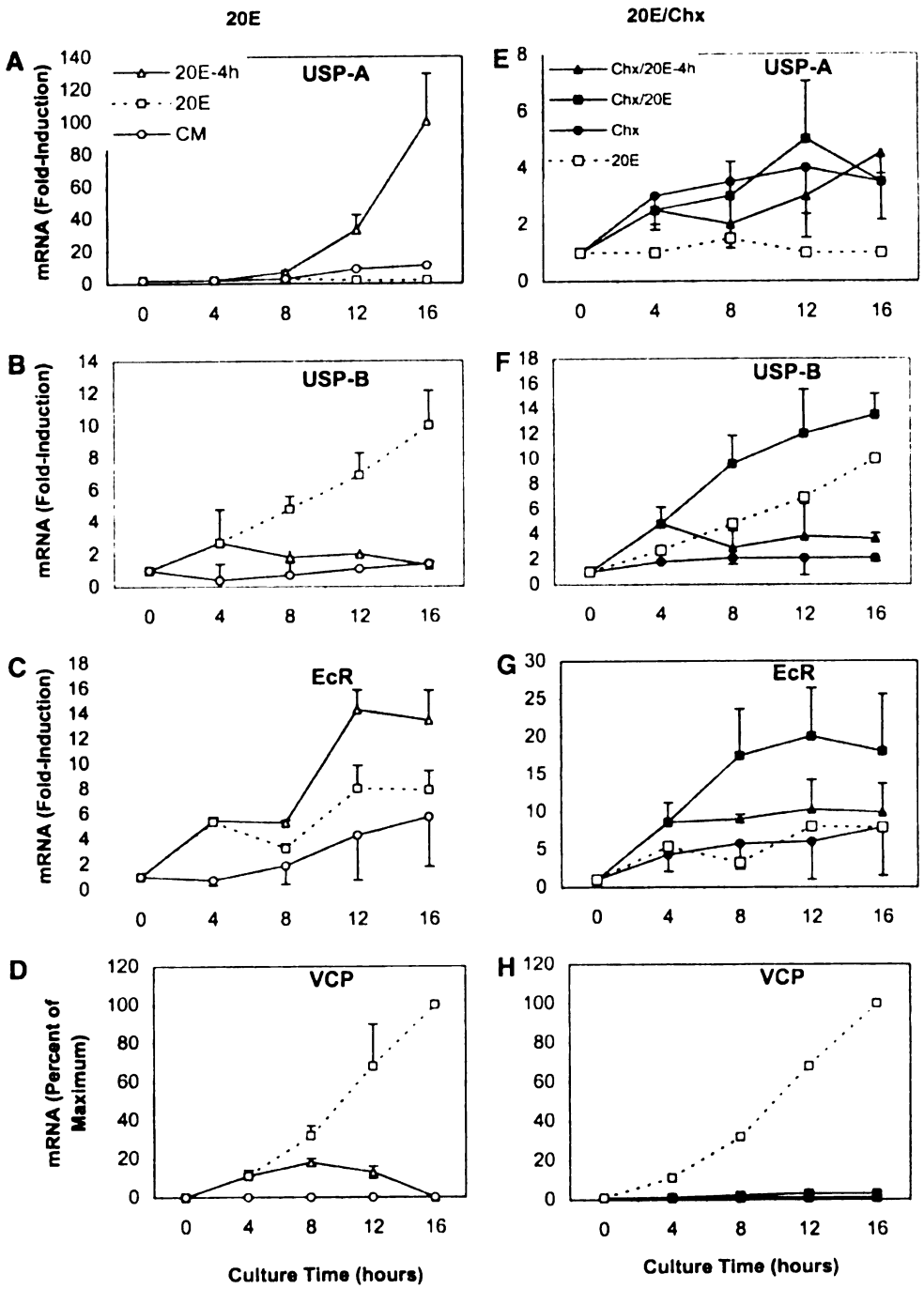

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Effect of Cycloheximide on USP Transcription

To assess whether mosquito USP isoforms are under direct or indirect control of 20E, I utilized a protein synthesis inhibitor cycloheximide (Chx) in the *in vitro* fat body culture experiments (Fig. 3, E-H). Previtellogenic Fat bodies were pretreated with culture media containing 1mM Chx, followed by further incubation with or without 10^{-6}M 20E. Fat bodies cultured continuously in the presence of 1mM Chx exhibited elevated USP-A transcription (near 4-fold induction, Fig 3E, closed circles), suggesting that Chx may repress the expression of inhibitors blocking USP-A expression. A similar profile was also observed when the fat bodies were incubated in media containing both 20E and Chx (Fig. 3E, closed squares). Importantly, when the 20E-withdrawal experiment was performed in the presence of Chx, no super-induction was observed in contrast to samples treated for only 4-hr with 20E alone (Fig. 3E, closed triangles).

The effect of Chx on the 20E induction of USP-B was different from that of USP-A. Overall, the presence of Chx in the medium led to slightly higher levels of the USP-B transcript than in similar treatments without Chx (Fig. 3B and 3F). In the Chx-containing medium, activation by 20E led to super-induction of USP-B mRNA (Fig. 3F), while its withdrawal resulted in considerably reduced, but still elevated levels of USP-B transcript (Fig. 3F, closed triangles).

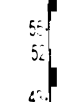
Fat bodies treated with Chx displayed enhanced levels of EcR transcripts, higher than in those incubated with a culture medium only (Fig.3C and 3G). In the presence of Chx, the effect of 20E on the AaEcR transcript in cultured fat bodies was very similar to that observed for USP-B (Fig. 3F). The level of VCP transcription was also monitored in

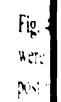
these fat bodies as a control. Addition of Chx to the culture medium resulted in complete inhibition of VCP mRNA irrespective of the presence or absence of 20E (Fig. 3H).

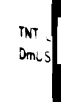
Protein Profiles of USP Isoforms in the Fat Body

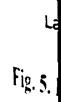
To determined USP protein profiles in the fat body during the vitellogenic cycle, nuclear extracts were isolated from fat bodies of female mosquitoes at various previtellogenic and vitellogenic stages. Nuclear proteins were then resolved by SDS-PAGE and the USP proteins were detected using anti-DmUSP monoclonal antibody in a Western blot assay. The 55-kDa USP-A and 52-kDa USP-B proteins produced from their respective cDNAs using an *in vitro* TNT transcription-translation system were used as positive controls (Fig. 4, lanes 1 and 2). Typically, the USP antibody recognized two major bands in the fat body nuclear extracts: the upper band which displayed a mobility similar to that of the TNT-produced USP-A, and the lower band which co-migrated with the TNT-produced USP-B (Fig. 4, lanes 3-10). These upper and lower bands were considered to be the *in vivo* counterparts of USP-A and USP-B, respectively.

There were no detectable USP-A and USP-B proteins in newly-eclosed, 0-0.5 day old female mosquitoes (Fig. 4, lane 3). Only a polypeptide of a considerably higher mobility (43 KDa), as well as a minor band, was recognized by the USP antibody. The identity of these lower molecular weight species and their relationship to mature USP-A and USP-B are currently unknown. The USP-A protein was not detected in the fat body of previtellogenic female mosquitoes until they were 3-5 days old, when a trace of USP-A was visible. However, this protein dramatically increased during the early stage of vitellogenesis, 3-6 hr PBM. Yet, by the peak of vitellogenic activity, 18-24 hr PBM, the









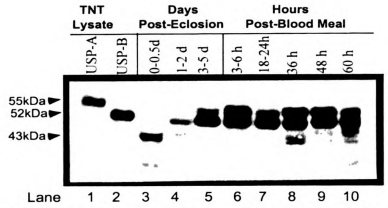


Fig. 4. Developmental profiles of USP proteins revealed by Western blot. Fat body nuclear extracts were isolated from female mosquitoes at previtellogenic stages 0-0.5 day, 1-2 day, 3-5 day (lanes 3-5), or post blood meal stages PBM 3-6h, 18-24h, 36h, 48h, 60h (lanes 6-10). Protein extracts, equivalent to 50 fat bodies per lane, were loaded in each lane for SDS-PAGE and Western blot analysis with the anti-DmUSP monoclonal antibody. In parallel, *in vitro* TNT expressed USP-A and USP-B proteins (lanes 1 and 2) were analyzed as positive controls. Arrowheads indicate the sizes of major bands.

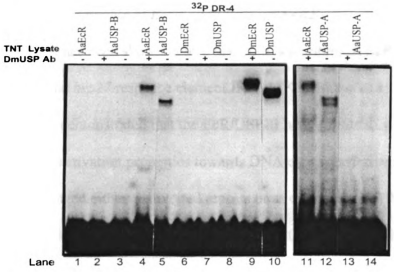


Fig. 5. EcR/USP binding to a direct repeat DR-4. ^{32}P labeled DR-4 EcRE was incubated with *in vitro* synthesized AaEcR (lane 1), USP-B (lanes 2 and 3), AaEcR and USP-B (lanes 4 and 5), DmEcR (lane 6), DmUSP (lanes 7 and 8), DmEcR and DmUSP (lanes 9 and 10), USP-A (lanes 13 and 14) or AaEcR and USP-A (lanes 11 and 12) and subjected to EMSA. Anti-DmUSP antibody was used for supershift assays to verify binding specificity (lanes 4, 9 and 11).

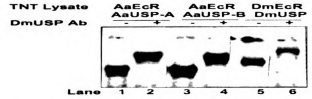


Fig. 6. EcR/USP binding to DR-12. ^{32}P-labeled DR12 was incubated with *in vitro* synthesized AaEcR and USP-A (Lanes 1-2), AaEcR and USP-B (lanes 3-4) or DmEcR and DmUSP (Lane 5-6) and subjected to EMSA. Supershift assays were conducted with anti-DmUSP antibody (Lanes 2, 4 and 6).

level of nuclear USP-A protein in the fat body drastically decreased (Fig. 4, lane 7). The level of USP-A increased again at the late vitellogenic stage, 36-48 hr PBM, before declining again at 60-hr PBM.

In contrast to USP-A, the USP-B protein appeared in the fat body of previtellogenic, 1-2 day old mosquitoes and reached a high level in 3-5 day old females. Moreover, the level of USP-B protein remained without any significant changes throughout the entire vitellogenic and postvitellogenic periods (Fig. 4).

DNA Binding Properties of the EcR/USP-A Heterodimer: the EcR/USP-A complex is Capable of Binding to Various EcREs

It has been shown before that both heterodimers, EcR/USP-A and EcR/USP-B, bind to the *hsp27* response element IR^{hsp}-1 (Kapitskaya *et al.*, 1996). More recently, it has been demonstrated that the EcR/USP-B heterodimer displays promiscuous binding and transactivation properties towards DNA elements derived from the AGGTCA half-site, arranged either as inverted repeats or as direct repeats (Wang *et al.*, 1998). Here, it was investigated whether the EcR/USP-A heterodimer exhibited DNA binding and transactivation properties similar to those of the EcR/USP-B heterodimer.

First, EMSA was conducted with the inverted repeats containing the perfect consensus sequence AGGTCA separated by a single nucleotide spacer (IR^{per}-1). These experiments showed that the EcR/USP-A heterodimer bound efficiently to IR^{per}-1 (data *not* shown).

Next, a direct repeat of the AGGTCA half-site with a four-nucleotide spacer (DR-4 element) was tested. This sequence has been shown to have an optimal spacer among

direct repeats for mosquito EcR/USP-B and *Drosophila* EcR/USP (Wang *et al.*, 1998; Antoniewski *et al.*, 1996; Vogtli *et al.*, 1998). TNT-expressed AaEcR, USP-A, USP-B, DmEcR or DmUSP proteins alone did not exhibit any binding to DR-4 (Fig. 5, lanes 1-3, 6-8, 13 and 14). A combination of mosquito AaEcR and USP-B lysates yielded efficient binding, whose specificity was confirmed by supershifts using the anti-DmUSP monoclonal antibody (Fig. 5, lanes 4 and 5). Similar binding activity was detected for the DmEcR/DmUSP heterodimer (Fig. 5, lanes 9 and 10). Likewise, the AaEcR/USP-A heterodimer displayed similar specific binding to the DR-4 element as confirmed by a supershift assay using anti-DmUSP monoclonal antibody (Fig 5, lanes 11 and 12).

Then it was investigated whether AaEcR/USP-A could bind to a direct repeat of the AGGTCA half-site with a 12-nucleotide spacer (DR-12), which displays robust binding activity with the AaEcR/USP-B and DmEcR/DmUSP heterodimers (D'Avino *et al.*, 1995; Wang *et al.*, 1998). EMSA revealed that the AaEcR/USP-A bound the DR-12 element efficiently, and the specificity of binding was demonstrated by a supershift with anti-DmUSP antibody (Fig 6).

Taken together, these experiments indicate that the AaEcR/USP-A complex, like its AaEcR/USP-B complex counterpart, is capable of binding to various EcREs including inverted and direct repeats as well as widely spaced repeats such as DR-12.

Estimation of Equilibrium Dissociation Constant (K_d): AaEcR/USP-B Bound on EcRE Twice as Strongly as AaEcR/USP-A

The similar binding properties of the AaEcR/USP-A and AaEcR/USP-B heterodimers prompted us to investigate whether they might possess differential binding

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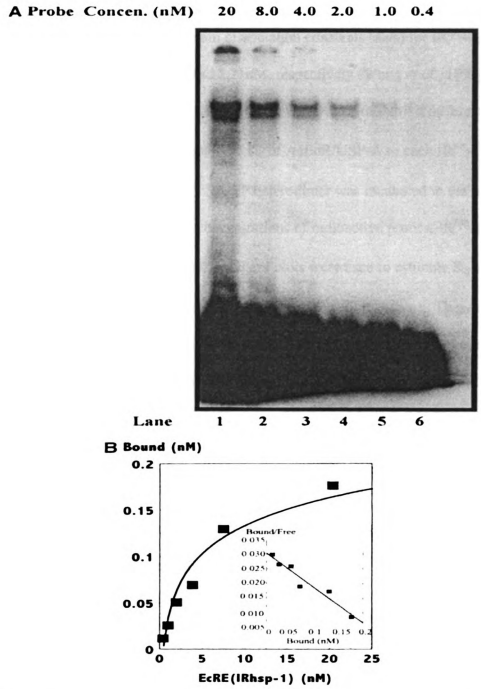

Fig.7. Direct measurement of equilibrium dissociation constants (*Kd*). Increasing amounts of ^{32}P labeled IR^{hsp}-1 EcRE probe ranging from 0.4nM-20nM was incubated with *in vitro* synthesized AaEcR and USP-A proteins and subjected to EMSA. Bound and free probes were quantified by phosphorimager analysis. Saturation curve (B) and Scatchard plot (inset in panel B) were prepared to calculate the *Kd*. This experiment was repeated three times and the mean value taken as the *Kd*.

affinities for EcREs. The AaEcR/USP-B heterodimer binds various EcREs with different binding affinities. The equilibrium dissociation constants (*Kd*s) for IR^{hsp}-1, IR^{per}-1 and DR-4 are 3.73 nM, 0.326 nM and 2.21nM, respectively (Wang *et al.*, 1998). To evaluate whether combinations of EcR with different USP isoforms exhibit different binding affinities for these DNA elements, the K_d of AaEcR/USP-A to each IR^{per}-1, IR^{hsp}-1and DR-4 was estimated. The AaEcR/USP heterodimer was incubated in the presence of $5x10^{-7}$ M 20E, with increasing concentrations of radioactive probes, IR^{hsp}-1 or DR-4. Saturation binding analyses and Scatchard plots were used to estimate K_d values for the heterodimeric pairs bound to IR^{hsp}-1 (Fig. 7) or DR-4 (not shown). These analyses indicated that like the AaEcR/USP-B heterodimer, the AaEcR/USP-A bound to various EcREs with different affinities. The differences in K_d values for these EcREs showed that the binding affinity of EcR/USP-A to a DR-4 was about 2-fold higher than to an IR^{hsp}-1 (Table 1), yet ten times weaker than to the IR^{per}-1 (not shown). More importantly, these results demonstrated the AaEcR/USP-A complex bound to each EcRE with a 2-fold lower affinity than the corresponding AaEcR/USP-B complex, suggesting that USP-B is a more potent heterodimerization partner for EcR (Table 1).

USP-B is a More Potent EcR Partner than USP-A for Transactivation via EcREs in CV-1 Cells

Transactivation properties of two mosquito heterodimers were next compared using the CV-1 cell line. This mammalian cell line has no endogenous EcR and contains very low endogenous level of RXR. It has been used to study transactivation of EcR/USP from several arthropod species including *Drosophila* and mosquito (Thomas

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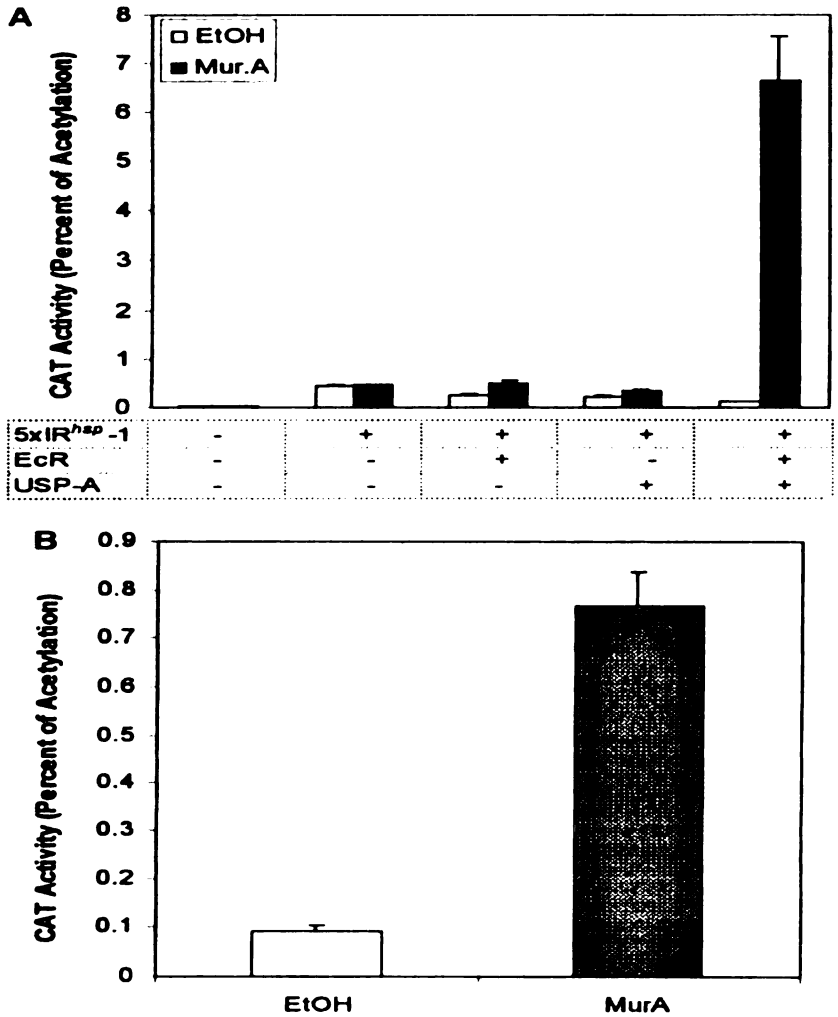

Fig.8. EcR/USP-A transactivated a reporter gene with either IR-1 or DR-4 EcREs in CV-1 cells. A. The ΔMTV-5xIR^{hsp}-1-CAT reporter gene (1.2μg) and 0.4μg CMV-LacZ were transfected into CV-1 cells with 0.4μg of either AaEcR or USP-A plasmid or 0.4μg each of the two expression vectors. The expression vector pCDNA3.1/zeo (+) was used as carrier DNA to equalize the total amount of plasmid so that each well received 2.4μg DNA. Cells receiving no exogenous DNA were used as a mock control. **B.** The ΔMTV-3xDR-4-CAT reporter gene (1.2μg) and 0.4μg CMV-LacZ were transfected into CV-1 cells with 0.4μg of AaEcR and USP-A expression vectors. After transfection cells were incubated with either vehicle (ethanol) or 1μM MurA at 37^{0}C for 36 hours and harvested for CAT activity and β-galactosidase activity, and CAT activity was normalized with β-galactosidase activity. The experiments were repeated more than three times.

et al., 1993; Yao *et al.*, 1992; 1993; Wang *et al.*, 1998). It has been shown previously that transfection of AaEcR/USP-B into CV-1 cells rendered them ecdysteroid responsive. Furthermore, it has been shown that the levels of reporter transactivation by AaEcR/USP-B correlated with the DNA binding affinities of this heterodimer when three distinct three functional EcREs were compared (Wang *et al.*, 1998).

The transactivation potential of the AaEcR/USP-A heterodimer was assessed with the ΔMTV-5xIR^{hsp}-1-CAT reporter plasmid, which contains five tandem repeats of IR^{hsp}-1. Transfection of CV-1 cells with the reporter alone resulted in a very low basal level of CAT activity (Fig. 8A). Co-transfection of the reporter with USP-A alone did not confer MurA responsiveness. Weak response to MurA was detected in cells transfected with the reporter gene and AaEcR expression vector, suggesting that AaEcR can heterodimerize with endogenous RXR to activate this reporter gene. However, strong activation of the reporter gene by 1μM MurA (50-fold) was noted only in cells co-transfected with AaEcR and USP-A expression vectors (Fig. 8A). This indicated that the AaEcR/USP-A heterodimer formed a functional hormone-dependent transactivator in CV-1 cells.

Next, to test whether the AaEcR/USP-A heterodimer could also activate a reporter gene containing the DR-4 as an EcRE, the ΔMTV-3xDR-4-CAT reporter plasmid containing three tandem repeats of DR-4 was transfected into CV-1 cells. Co-transfection of this reporter construct with either the AaEcR or the USP-A alone did not render CV-1 cells ecdysteroid-responsive (data not shown). However, co-transfecting the reporter construct with both AaEcR and USP-A expression vectors rendered CV-1 cells highly responsive to the ecdysteroid MurA giving 6-fold induction (Fig. 8B).

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Table 1. The equilibrium dissociation constants (K_d) of different DNA sequences binding to AaEcR/USP-A and AaEcR/USP-B.

EcRE	IR^{hsp}-1(nM)*	DR-4 (nM)*
AaEcR/USP-A	7.36±0.81	4.06±0.05
AaEcR/USP-B	3.73±0.85	2.21±0.36

To measure the K_d values, *in vitro* synthesized AaEcR and USP-A were incubated with increasing concentrations of ^{32}P labeled IR^{hsp}-1 or DR-4 as described in Fig. 6. EMSA and quantification for each element were performed a minimum of three times and the mean values were taken as the K_d. For comparison, K_d values for EcR/USP-B to the same elements are also listed (Wang *et al.*, 1998. with permission).
* Mean of three determinations ± SE

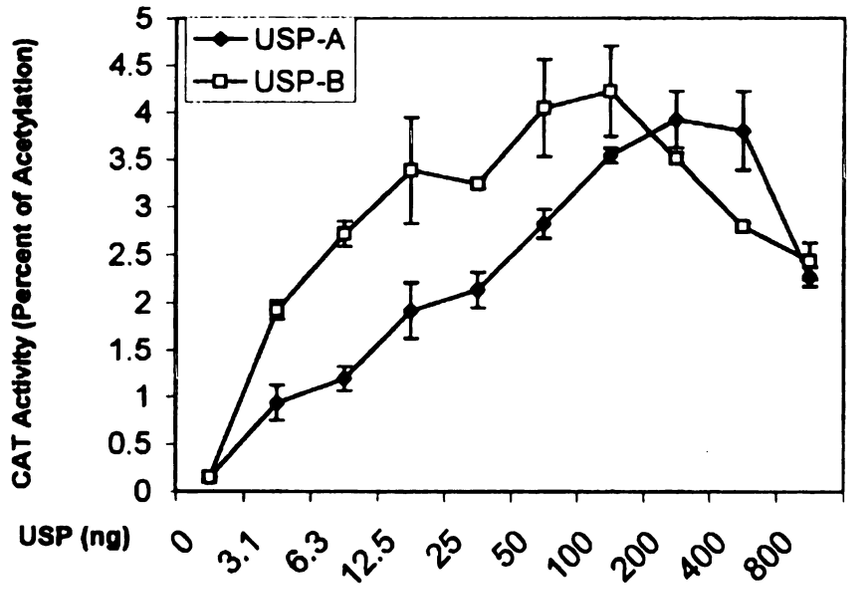


Fig. 9. AaEcR/USP-B mediated more efficient transactivation than EcR/USP-A. Reporter plasmid (0.6μg), CMV-LacZ (0.2μg) and AaEcR plasmid (0.2μg) were transfected into CV-1 cells with increasing amounts of either USP-A or USP-B expression vector ranging from 3.1ng-800ng. The expression vector pCDNA3.1/zeo (+) was used as carrier DNA to equalize the total amount of plasmid so that each well received 2.0μg DNA. Cells receiving no exogenous DNA were used as a mock control. After transfection, cells were incubated with 1μM MurA at 37°C for 48 hours and harvested for CAT activity and β-galactosidase activity. CAT activity was normalized to β-galactosidase activity. The experiments were repeated three times.

The transactivation potentials of the two heterodimers were then compared. In this experiment (Fig. 9), a fixed amount of reporter plasmid (ΔMTV-5xIR^{hsp}1-CAT, 600ng) and an AaEcR expression vector (200ng) were co-transfected into CV-1 cells along with increasing amounts of either USP-A or USP-B expression vectors, ranging from 3 ng to 800 ng. Cells transfected with a reporter plasmid and the AaEcR expression vector alone displayed less than 0.5 units of CAT activity (not shown). Co-transfecting these cells with the USP-A plasmid increased the Mur A-dependant CAT activity in proportion to the amount of USP plasmid added. The activation reached saturation with 200 ng of USP-A plasmid (Fig. 9). Co-transfecting the cells with USP-B instead of USP-A resulted in higher Mur A-dependant activation of the reporter at lower levels of input plasmid. Notably, the USP-B mediated transactivation reached its peak with about half the amount of input plasmid compared to that of USP-A (100ng versus 200 ng). In a separate experiment, transactivation potentials of the two heterodimers were compared using the ΔMTV-3xDR-4-CAT reporter plasmid. Co-transfection of this reporter with the AaEcR and AaUSP-A expression plasmids consistently led to a lower level of reporter induction than with the AaEcR and AaUSP-B plasmids (data not shown). Taken together, these results further suggested that USP-B is a more potent partner than USP-A for EcR mediated transactivation in CV-1 cells.

DISCUSSION

In this work, the expression profiles of USP isoforms were characterized during the first vitellogenic cycle in the female mosquito *A. aegypti*. The transcriptional responses of USP isoforms to 20E were investigated, and the DNA-binding and

transactivation properties of the EcR/USP-A and EcR/USP-B heterodimers were compared by employing EMSA and transfection assays. Importantly, here two mosquito USP isoforms, USP-A and USP-B, displayed distinct stage-specific expression in two vitellogenic tissues, the fat body and ovary. USP-A mRNA was highly expressed in previtellogenic and late vitellogenic fat bodies and ovaries, coinciding with a period of low ecdysteroid titer, whereas USP-B mRNA was enriched at the vitellogenic stage when the ecdysteroid titer in the female mosquito is high. Significantly, USP-B, but not USP-A mRNA levels, correlated with the level of EcR in both tissues.

The mRNA profiles of the USP isoforms strongly suggest that ecdysteroids be involved in differential regulation of their respective promoters. Indeed, when I utilized the fat body *in vitro* organ culture to investigate the effect of 20E on the transcription of USP isoforms, these experiments unequivocally established ecdysteroid engagement in controlling the transcription of both USP-A and USP-B. Remarkably, the effect of 20E on USP-A expression was opposite to that of USP-B expression. Continuous exposure to 20E abrogated the transcription of USP-A. However, a 4-hour pulse treatment of fat bodies with 20E followed by continued incubation in hormone-free medium resulted in a drastic augmentation of USP-A transcription. Interestingly, these USP-A mRNA responses to 20E *in vitro* are similar to those of AaFTZ-F1. According to our observations, AaFTZ-F1 is also suppressed by 20E, but is super-activated under conditions of 20E withdrawal (Li C., Kapitskaya, M.Z. and Raikhel, A.S., unpublished observation). FTZ-F1, an insect homologue of the steroidogenic factor 1 (SF-1), has been implicated in regulating a stage-specific response to ecdysteroid (Woodard *et al.*, 1994). This similarity in the effect of 20E suggests that, as in the case of FTZ-F1, the

nuclear regulatory receptor, HR3 (Hormone Receptor 3) may be involved in mediating the 20E effect on USP-A. HR3 is involved in mediating 20E responsiveness in *Drosophila* (Lam *et al.*, 1997; White *et al.*, 1997). Moreover, the HR3 homologue has been identified in the mosquito and has been found to respond positively to 20E in an *in vitro* fat body culture system (Kapitskaya, M.Z., Li, C. and Raikhel, A.S., unpublished results). The observation that Chx completely inhibited USP-A super-induction following 20E withdrawal further substantiated the involvement of HR3 or other labile factors in activation of USP-A expression.

In contrast to USP-A, the USP-B transcript was highly elevated by the continuous presence of 20E in an *in vitro* fat body assay. Furthermore, continuous exposure of the fat body to 20E was required for maximal activation of USP-B transcription. In the presence of Chx, 20E super-induced USP-B transcription, suggesting that it represents an early gene of the ecdysteroid gene regulatory hierarchy. One critical criterion to differentiate early and late genes is the effect of a protein synthesis inhibitor such as Chx on their response to hormone (Ashburner *et al.*, 1974; Huet *et al.*, 1995). Vg and VCP genes, which are transcriptionally induced by ecdysteroid but inhibited by Chx, are typical late genes in the mosquito (Deitsch *et al.*, 1995a). In contrast, addition of cycloheximide into the culture media super-induces AaE75, a mosquito early gene (Pierceall *et al.*, 1999). Of note, the effects of 20E on EcR in the mosquito fat body, as well as the EcR mRNA response to Chx were generally similar to those of USP-B. However, as in the case of EcR expression, a more complex pattern of response to 20E compared to that of USP-B was observed (Fig. 3). This may reflect the presence of more than one isoform of EcR as has been shown for *M. sexta* (Jindra *et al.*, 1996a). In

contrast to USP, only a single EcR isoform corresponding to *Drosophila* EcR-B1 has been identified so far in the mosquito (Cho *et al.*, 1995). However, our recent studies suggest that there is at least one additional EcR isoform present in the mosquito during vitellogenesis (Wang, S.F., Li, C., Zhu, J.S. and Raikhel, A.S. unpublished data).

Interestingly, USP isoforms have been found to be differentially expressed during the *Manduca* epidermis during larval molts (Jindra *et al.*, 1997). When the ecdysteroid titer is high, the *Manduca* USP1 transcript (the counterpart of USP-A) disappears and the levels of the USP2 transcript (the counterpart of USP-B) are elevated, resembling the USP isoform expression profiles in the adult female mosquito during vitellogenesis. Moreover, *in vitro* exposure of the day-2 fourth instar larval epidermis to 20E led to the decrease of *Manduca* USP-1 mRNA, whereas the effect of 20E on USP-2 mRNA was opposite causing its levels to rise (Hiruma *et al*, 1999). A differential effect of 20E has also been reported for *Manduca* EcR isoforms (Jindra *et al.*, 1996b). In vertebrates, ligands can cause different or even opposite responses of various receptor isoforms (Syvala *et al.*, 1996; Paech *et al.*, 1997; Keightley, 1998).

In the fat body of newly eclosed female mosquitoes, transcripts of both USP isoforms and EcR are present with the USP-A transcript being particularly abundant. However, our immunoblot analysis showed that the corresponding proteins were not detectable at this stage in the fat body. A high mobility 43-kDa protein, recognized by the USP monoclonal antibody, was observed in the newly eclosed female fat body. During the extraction of the fat body nuclear proteins, a cocktail of proteinase inhibitors was added and all samples were processed under identical conditions. Therefore, it is unlikely that the 43-kDa protein is a degradation product of USP-A or USP-B. Our previous

Northern blot analyses have demonstrated the existence of four USP transcripts of different sizes in both the fat body and ovary (Kapitskaya *et al.*, 1996). Utilization of isoform-specific probes derived from the 5'- and 3'- untranslated regions of USP-A and USP-B revealed that the largest 3-kb transcript corresponds to USP-A, while two smaller transcripts of 2.5 and 2.2 kb correspond to USP-B (Kapitskaya *et al.*, 1996). The identity of the smallest 1.8-kb transcript remains unknown. It is possible that a newly identified 43-kDa USP-related polypeptide is a product of the 1.8-kb transcript. This suggestion, however, requires further study.

Traces of the USP-B protein appeared in 1-2 day old mosquitoes and reached high levels in 3-5 day-old mosquitoes. By contrast, only a low level of the USP-A protein was detectable in 3-5 day-old mosquitoes. EMSA utilizing nuclear extracts from different stages of previtellogenic fat bodies also showed the appearance of functional AaEcR/USP heterodimers only in fat bodies of previtellogenic females 3-5 days after eclosion (Zhu, J.S. and Raikhel, A.S., unpublished data). Thus, since the USP-B protein is the most abundant USP isoform in the fat body of 3-5 day old previtellogenic females, an increase in the USP-B protein along with EcR protein, which is also appeared at this stage, may promote competence for the vitellogenic response to a blood meal (Zhu, J.S., Wang, S.F., Li, C. and Raikhel, A.S., unpublished data).

During the previtellogenic phase which covers the period from adult emergence to 3-5 days after eclosion, the female mosquito becomes competent for vitellogenic events (reviewed by Hagedorn, 1989; Raikhel, 1992). The previtellogenic phase is accompanied by a rise in the juvenile hormone (JH) titer, which reaches its peak two days after eclosion and then declines (Fig. 1-A; Shapiro *et al.*, 1986). Acquisition of competency to

vitellogenic events and responsiveness to ecdysteroid have been shown to be controlled by JH (reviewed in Raikhel, 1992; Dhadialla and Raikhel, 1994). In the ovary, the development of the receptor-mediated complex responsible for yolk protein uptake is controlled by JH (Raikhel and Lea, 1985). In the fat body, JH-dependent events include an increase in ploidy and proliferation of ribosomes (Dittmann *et al.*, 1989; Raikhel and Lea, 1990). However, the molecular mechanism of JH action in the mosquito is unknown. In view of our observations on the delayed appearance of USP protein during previtellogenic development, it is possible that JH acts at the translational level regulating coordinated production of a set of transcription factors required for rapid activation of target genes in response to blood-meal induced hormones. Verification of this hypothesis requires further study.

The level of the USP-A protein increased dramatically in the fat body in response to a blood meal. However, at the active phase of vitellogenesis, 18-24 hr PBM, the USP-A protein dropped to quite a low level, consistent with the decrease in USP-A mRNA level, which was also absent at this time. In contrast to the fluctuation of USP-A mRNA and protein levels during the vitellogenic stage, USP-B mRNA and protein levels are expressed evenly high throughout this stage, suggesting once again that USP-B may be the major partner for EcR function during vitellogenesis.

In order to provide additional insights into the possible roles of USP isoforms in the mosquito, we have compared the DNA-binding and transactivation properties of the EcR/USP-A heterodimer with those of the EcR/USP-B heterodimer which we have characterized previously (Wang *et al.*, 1998). Although the extent of our present analysis has not been as detailed as for USP-B (Wang *et al.*, 1998), it appears that USP-A can

effectively dimerize with EcR, and the resulting heterodimer exhibits a wide specificity of EcRE recognition similar to that of the AaEcR/USP-B heterodimer. The EcR/USP-A heterodimer bound inverted repeats (IR^{hsp}-1 and IR^{per}-1), direct repeats (DR-4) and widely spaced EcREs such as DR-12. However, by measuring binding affinities of both heterodimers we have clearly shown that the EcR/USP-B consistently displayed higher binding affinity than EcR/USP-A to either direct or inverted repeats. Moreover, although EcR/USP-A mediates ligand-induced transactivation in CV-1 cells from either IR^{hsp}-1 or DR-4 EcREs, once again the EcR/USP-B heterodimer appears to be the more effective transactivator. The functional difference between USP-A and USP-B in mosquito cells could be more profound than observed in CV-1 cells. The *Manduca* EcR heterodimerized with either USP-1 or USP-2 bind IR^{hsp}-1 and another imperfect palindrome (EcRE1) from the MHR3 promoter. Yet only EcR·USP1, but not EcR/USP-2, efficiently transactivated the MHR3 promoter in *Manduca* GV-1 cells (Lan *et al.*, 1999).
Thus, these findings have demonstrated that throughout the vitellogenic cycle, the mosquito USP isoforms are differentially regulated at both the transcriptional and translational levels. They exhibit strikingly different responses to 20E in fat body cultured *in vitro*. Our functional studies of the DNA binding and transactivation properties indicate that, although both isoforms form functional heterodimers with EcR capable of recognizing a wide range of EcREs, USP-B forms a more efficient functional ecdysteroid receptor than USP-A. Taken together, our findings strongly suggest that USP-B is the major heterodimeric partner of EcR responsible for mediating the ecdysteroid response during vitellogenesis in the mosquito. Elucidation of the possible role of USP-A in the adult female mosquito requires further investigation.

Sequence comparison of the AaEcR with *Drosophila* EcR isoforms revealed that the AaEcR shows high similarity with *Drosophila* EcR-B. Since many insect EcR isoforms have been identified, it was likely to exist more than one EcR isoform in mosquitoes. Indeed, most recently, the second AaEcR isoform has been identified in mosquitoes by means of PCR amplification using degenerated primers based on highly conserved regions in the EcR-A A/B domain between *Drosophila* and other insects (Fig. 10A; Wang, S-F, Li, C. and Raikhel, A., unpublished). Subsequently, the PCR generated DNA fragment was used to screen the mosquito vitellogenic cDNA library. However, extensive library screening failed to identify a cDNA clone in this library (Li, C. and Raikhel, A., unpublished). When I performed PCR using a primer located on the PCR-generated AaEcR-A fragment and a second primer on the 3'UTR of the AaEcR common region, I amplified the entire nucleotide sequence of AaEcR-A from the vitellogenic-staged fat body cDNA. This result further has confirmed existence of AaEcR-A in mosquito fat bodies (now shown).

Next, I used AaEcR isoform-specific primers to examine the expression levels of AaEcR-A in both fat bodies and ovaries (Fig. 10B). In fat bodies, AaEcR-A is barely detectable during the previtellogenic stage; whereas, its levels increase after a blood meal with a peak level at 24hr PBM followed by a rapid decline to the previtellogenic level at 48hr PBM. In ovaries, AaEcR-A is expressed at relatively high levels throughout the previtellogenic and vitellogenic stages with elevated levels after a blood meal (Fig. 10B). Thus, the AaEcR-A expression levels appear to correlate with 20E levels during vitellogenesis.

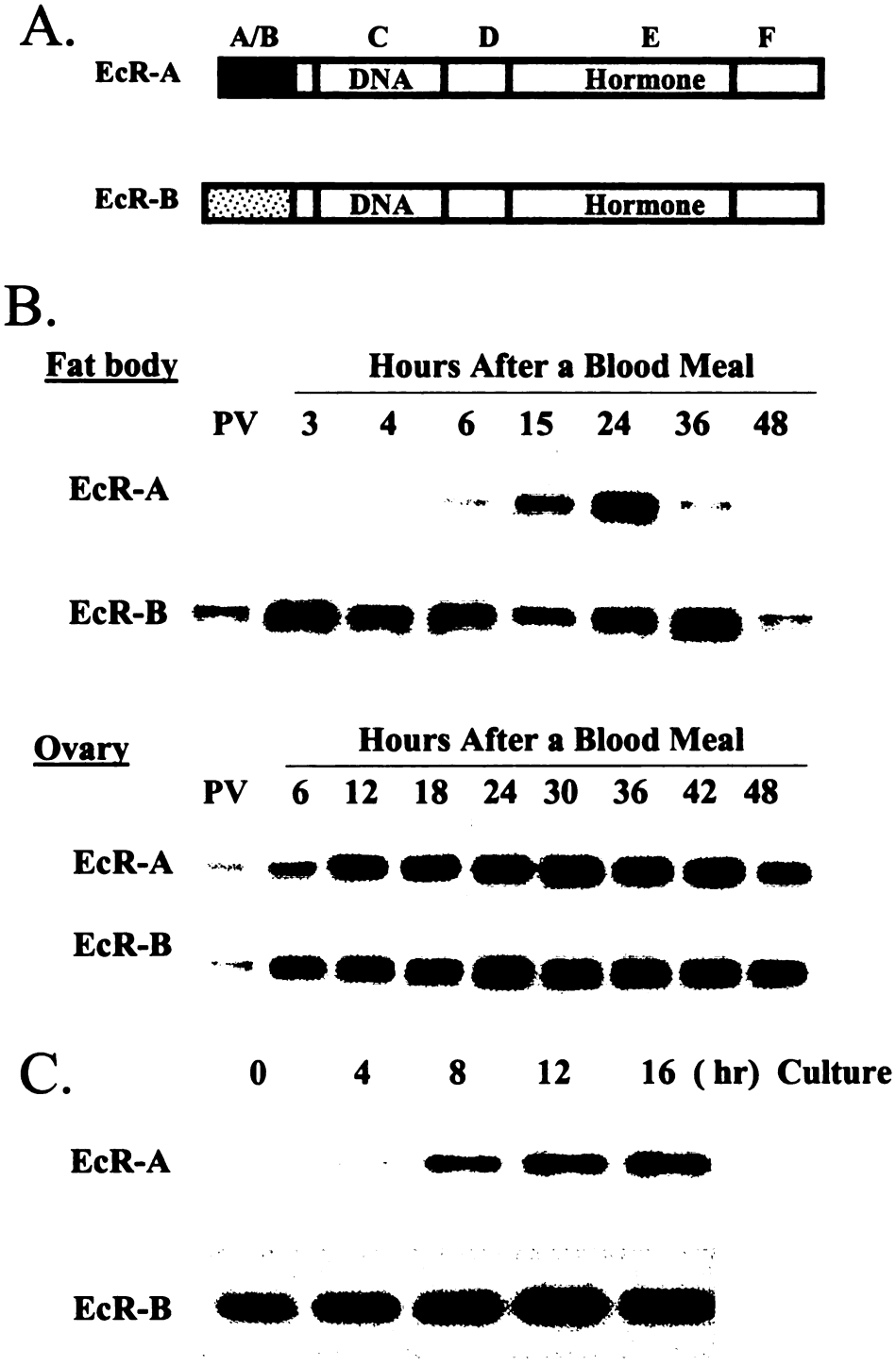


Fig.10. Characteristics of the AaEcR-A isoform. (A). Schematic domain structures of the mosquito EcR isoforms. (B). Expression profiles of AaEcR-A in fat bodies and ovaries by RT-PCR/Southern analysis. (C). Induction of AaEcR-A mRNA in the fat body culture.

The correlation of the expression pattern of AaEcR-A in the fat body with 20E levels suggests that AaEcR-A expression may be under the control of 20E. To test this hypothesis, previtellogenic fat bodies were incubated with 20E for 16hr, and the mRNA levels of AaEcR-A were monitored during this period of time. Indeed, I detected that the AaEcR-A mRNA levels rose gradually during the 16hr culture with a maximal level at 16hr, indicating that AaEcR-A is more inducible by 20E than the AaEcR-B (Fig. 10C).

In the fat body, the substantial low level of AaEcR-A expression during the previtellogenic stage, and relatively high level expression only within a limited time frame during the vitellogenic stage, suggest that AaEcR-A may not be a major receptor for the ecdysone response during vitellogenesis. Elucidation of the possible role of AaEcR-A in mosquito vitellogenesis requires further investigation.

ACKNOWLEDGMENTS

I thank Sheng-fu Wang for DNA binding and cell transfection assays and initiation of the AaEcR isoform project, Jinsong Zhu for Western blot analysis and Ken Miura for some RT-PCR experiments. I also thank Alan R. Hays' assistance in mosquito rearing and Dr. F. C. Kafatos for providing us with the *Drosophila* USP monoclonal antibody. This work was supported by NIH grant AI-36959.

Chapter 3*

Characterization of the Mosquito Early Gene *E75*

*Most results have originally been published in Mol. Cell. Endocrinol.

Ref: Pierceall, W. E., Li, C.#, Biran, A., Miura, K., Raikhel, A. S., and Segraves, W. A. (1999). *Mol. Cell Endocrinol.* 150, 73-89.

# Underlined two authors contributed equally to this work.

ABSTRACT

The steroid hormone ecdysone# controls genetic regulatory hierarchies underlying insect molting, metamorphosis and, in some insects, reproduction. Cytogenetic and molecular analysis of ecdysone response in *Drosophila* larval salivary glands has revealed regulatory hierarchies including early genes which encode transcription factors controlling late ecdysone response. In order to determine whether similar hierarchies control reproductive ecdysone response, ecdysone-regulated gene expression in vitellogenic mosquito ovaries and fat bodies has been investigated. Here, the homologue of the *Drosophila E75* early ecdysone inducible gene has been identified in the yellow fever mosquito *Aedes aegypti*, and show that, as in *Drosophila*, the mosquito homologue, *AaE75*, consists of three overlapping transcription units with three mRNA isoforms, AaE75A, AaE75B, and AaE75C, originating as a result of alternative splicing. All three *AaE75* isoforms are induced at the onset of vitellogenesis by a blood meal-activated hormonal cascade, and highly expressed in the mosquito ovary and fat body, suggesting their involvement in the regulation of oogenesis and vitellogenesis, respectively. *In vitro* fat body culture experiments demonstrate that *AaE75* isoforms are induced by 20-hydroxyecdysone, an active ecdysteroid in the mosquito, independently of protein synthesis. Furthermore, AaE75 is able to activate the *Vg* promoter in cell transfection assays, suggesting its role as an early vitellogenic gene. These findings suggest that related ecdysone-triggered regulatory hierarchies may be used reiteratively during developmental and reproductive ecdysone response.

Following the recommendation of Cherbas *et al.* (1984), ecdysone is used as the generic term for hormones with the appropriate biological activities. The active hormone in the *Drosophila* salivary gland hierarchies and mosquito vitellogenesis and oogenesis has been shown to be 20-hydroxyecdysone.

INTRODUCTION

The insect steroid hormone ecdysone controls developmental processes including larval molting, metamorphosis and, in some insects, reproduction. In *Drosophila*, gene regulation by ecdysone is best understood in the late larval and prepupal salivary gland, where hierarchies of gene expression are reflected in the activities of polytene chromosome puffs. Extensive characterization of puffing patterns has revealed that ecdysone acts first to activate a small number of early ecdysone inducible genes, and that their products control subsequent progression of the genetic response to ecdysone (Ashburner *et al.*, 1974). Molecular characterization of this hierarchy has led to the identification of the ecdysone receptor as a heterodimer of the EcR (Ecdysone Receptor) and USP (Ultraspiracle) proteins, and has shown that the early genes are direct targets of the ecdysone-receptor complex (Cherbas *et al.*, 1991; Koelle *et al.*, 1991; Yao *et al.*, 1992; Yao *et al.*, 1993). Consistent with their proposed regulatory role, the early genes, *BR-C, E74* and *E75*, each encode transcription factors which have been implicated in the regulation of early and late gene expression (Burtis *et al.*, 1990; Segraves and Hogness, 1990; Cherbas *et al.*, 1991; Guay and Guild, 1991; Koelle *et al.*, 1991; DiBello *et al.*, 1992; Hill *et al.*, 1992; Yao *et al.*, 1992, 1993; Fletcher and Thummel, 1995). The expression of these genes in other tissues and at other stages of development is consistent with the idea that these genes are also involved in controlling spatially and temporally parallel regulatory hierarchies mediating ecdysone response in other tissues and stages (Segraves, 1988; Thummel *et al.*, 1990; Huet *et al.*, 1993). Among likely ecdysone-regulated processes in *Drosophila*, two of the least understood are the progression of oogenesis and vitellogenesis. The ecdysone-inducibility of yolk proteins (Bownes, 1986)

and the female sterility of mutations affecting ecdysone synthesis and ecdysone response are suggestive of a role for ecdysone in female reproduction, but little is known of the specific role of ecdysone in these processes (Garen *et al.*, 1977; Oro *et al.*, 1992).

In contrast, the role of ecdysone in female reproduction is well established in anautogenous mosquitoes, especially the yellow fever mosquito *Aedes aegypti*. In this mosquito, initiation of egg maturation and vitellogenesis requires a blood meal, which stimulates neuroendocrine production of the ecdysiotropic neuropeptide EDNH (egg development neurosecretory hormone) or OEH (ovarian ecdysteroidogenic hormone) (Lea, 1967, 1972; Matsumoto *et al.*, 1989). This hormone stimulates ovarian ecdysone production, which in turn controls the induction and progression of vitellogenesis and egg development. In *A. aegypti*, ecdysone levels are low during the first 8-10 hr post blood meal (PBM), with only a small peak at 4 hr. Thereafter, the ecdysone level increases dramatically to a maximum at 16-20 hr PBM, then rapidly declines to previtellogenic levels in preparation for the termination of vitellogenesis at 30-32h PBM (Hagedorn, 1983, 1985; Hays and Raikhel, 1990; Raikhel, 1992). Aspects of the oogenic and vitellogenic responses can be induced by exogenous 20-hydroxyecdysone administration (Hagedorn, 1983, 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994), but the action of 20-hydroxyecdysone (20E) on the mosquito yolk protein genes, vitellogenin (*Vg*) and vitellogenic carboxypeptidase (*VCP*) requires protein synthesis (Deitsch *et al.*, 1995a). This indicates that, as in the *Drosophila* salivary gland, ecdysone response occurs via a cascade of regulatory events. Other functions associated with ecdysone during mosquito egg maturation include the formation of vitelline envelope (Raikhel and Lea, 1991; Lin *et al.*, 1993) and the separation of the secondary follicle in the ovary (Beckemeyer and Lea,

1980). Thus, the mosquito provides an ideal opportunity to investigate hierarchical control of ecdysone-induced gene expression during reproduction. Moreover, the critical role of anautogenous mosquitoes as disease vectors amplifies the importance of understanding this process, a potential target in the development of pest control strategies.

Consistent with the proposed role of ecdysone in controlling oogenesis and vitellogenesis, *EcR* and *USP* transcripts can be detected in pre- and post-vitellogenic ovaries and fat bodies. Three size classes of transcripts for *AaEcR*, the *A. aegypti* homologue of *Drosophila EcR*, are expressed in pre- and post-vitellogenic ovaries and fat bodies (Cho *et al.*, 1995). The other critical component, *USP*, is also expressed in these tissues with one predominating in the previtellogenic stage and the other in the vitellogenic stage (Kapitskaya *et al.*, 1996).

Here, the *A. aegypti* homologue of the *Drosophila E75* gene has been identified and characterized, a putative representative of the next level in the ecdysone response hierarchy. As one of the classical early genes, *E75* has been proposed to play a role in controlling ecdysone response in the late third instar salivary gland. *E75* transcripts are induced during all developmental ecdysone pulses, and genetic analysis suggests that *E75* is required for all critical developmental ecdysone responses, during embryogenesis, larval development and metamorphosis (W.A. Segraves, P. Jenik, C. Fichtenberg and C. Hughes, unpublished). It is shown here that *A. aegypti E75* is expressed in ovary and fat body following a blood meal, and that *AaE75* transcripts are ecdysone-inducible in isolated fat body *in vitro*. These findings suggest that *AaE75* plays an important role in mediating ovarian and fat body ecdysone response, and that ecdysone-triggered

regulatory hierarchies such as those implicated in the initiation of metamorphosis may be reiteratively utilized in the control of reproductive ecdysone response.

MATERIALS AND METHODS

Degenerate PCR/Inverse PCR amplifications

Several conserved regions with relatively low codon degeneracy, within the DNA and hormone binding domains of *Manduca sexta* (Segraves and Woldin, 1993) and *D. melanogaster E75* were used to design degenerate oligonucleotides for PCR amplification of mosquito *E75* sequences (Figs. 1 and 2). Oligonucleotides used for amplification of the 421 bp hormone binding domain fragment were E2F, GNCTNATHTGYATGTTYGAY, annealing to common region nucleotides 560-579 and E4R, CCACATYTGYTGNCKNAG, annealing to common region nucleotides 981-964 (Fig.1). Each PCR amplification reaction contained ~100 ng of genomic DNA, 100 ng of each primer, 250 mM each dNTP, 2 mM MgCl2, 33.5 mM Tris pH 8.8, 16.6 mM ammonium sulfate, 10 mM b-mercaptoethanol, and 1.0 unit of Taq polymerase (Amersham) in 32 µl final reaction volume, and was conducted for 35 cycles of 94°C for 1 min, 46°C for 1 min, 72°C for 1.5 min. If no product was observed after the initial amplification, 1 µl of the initial reaction was diluted into fresh reaction mix and re-amplified for 20 to 35 cycles. Where possible, re-amplifications were performed using nested primer sets. For PCR amplifications using non-degenerate primers, annealing temperatures were increased to 50-52°C and the extension cycle was lengthened to 3 min. For mapping of genomic clones using PCR amplification, 10 ng of template DNA was used in each amplification reaction.

Inverse PCR templates were prepared by digestion with the appropriate restriction enzyme, followed by ethanol precipitation in the presence of ammonium acetate, dilution to a volume of 1 ml and self-ligation overnight at 18°C. The template DNA was then re-precipitated and 250 ng was used in each PCR reaction. Oligonucleotides used for the specific amplification of exon A0 sequences were GTCGAATTCDATRKNAGYTC, annealing to exons A0 and A1 from nucleotide 8 of exon A1 to nucleotide 588 of exon A0, and IN3, GTGTGTGCGGTGATAAAGC, annealing to nucleotides 28-46 of exon A1.

cDNA and genomic libraries

An *A. aegypti* cDNA library prepared from poly (A+) selected mRNA from fat body 0-48 hr post blood meal (PBM) (Cho *et al.*, 1991) and an *A. aegypti* genomic library constructed from Sau3A-digested DNA cloned into the λFix II vector (Deitsch and Raikhel, 1993) have been described previously. For size-selected genomic DNA libraries, fragments of the desired size were excised from low melting point agarose gels, digested with b-agarase (NEB), phenol/chloroform extracted, ethanol precipitated and ligated into *EcoRI*-digested phage arms of the replacement vector l607 (Davis *et al.*, 1980). Phage were packaged with Gigapak II XL packaging extract (Stratagene) and propagated on *E. coli* C600 hflA to select for recombinant phage, which are cI-.

DNA sequencing

Vitellogenic fat body cDNA clones in λZAPII were propagated in Bluescript KS- following *in vivo* excision of the recombinant Bluescript phagemid in the presence of a

helper filamentous phage (Stratagene). Sequence analysis of cDNA clones, genomic clones and PCR products was performed by thermal cycle sequencing using a modified Taq polymerase and chain terminating dideoxynucleotides (Perkin-Elmer). All sequence data obtained from PCR-derived templates was confirmed by comparison to unamplified genomic or cDNA sequence. Some preliminary and confirmatory sequence analysis was generated on the IBI automated sequencing system at the Keck Biotechnology Center (Yale University School of Medicine). Sequence data was assembled and analyzed using Geneworks sequence analysis software.

Animals

Mosquitoes (*Aedes aegypti*) were reared as described (Hays and Raikhel, 1990). Vitellogenesis was initiated in adult females 3-5 days after eclosion by allowing them to feed on rats.

Northern blot analyses

Total RNA was prepared from ovaries or fat bodies dissected from female mosquitoes 3-5 days old (previtellogenic), or at 6 hr intervals post blood meal (PBM), using the guanidine isothiocyanate method (Cho *et al.*, 1991). For analysis of transcription events early after the onset of vitellogenesis, ovaries and fat bodies were also dissected 1, 3, and 4 hr PBM. Northern blot analyses were performed with the same amounts of mosquito equivalents of total RNA (6-48 hr PBM ovaries) loaded on 1% formaldehyde-agarose gels. Isoform-specific probes (designated A625 for AaE75A, B660 for AaE75B and C350 or C796 for AaE75C) were derived from isoform-specific

exons. A625 contains all of the indicated exon A0 sequences. B660 extends from the *EcoRI* site (nt 337) to the PstI site (nt 1007) of exon B1. C796 was obtained by amplification of exon C0 sequences using oligonucleotides CF1 and CR1 (Fig. 1). C350 is derived from an AaE75C cDNA clone, and extends from nucleotide 504 of exon C0 to the *EcoRI* site formed at the junction of exons C0 and A1 (Fig. 1). The probe common to all isoforms extends from an *EcoRI* site (nt 862) to the end of the indicated exon 4/5 sequences in the AaE75B cDNA 2-3A6 (Fig. 1). All probes were used for hybridization under high stringency conditions. The following cDNA fragments were used as probes to control for stage- and tissue-specificity: mosquito vitellogenic carboxypeptidase (VCP), the fat body-specific vitellogenic gene expressed exclusively during vitellogenesis (Cho *et al.*, 1991); the vitellogenin receptor (VgR), an ovary-specific gene expressed in the germ-line cells, i.e., the oocyte and nurse cells (Sappington *et al.*, 1996); the vitelline envelope gene 15a-1, an ovary-specific gene expressed in the follicular epithelium (Lin *et al.*, 1993). In addition, a cDNA probe for *Aedes aegypti* cytoplasmic actin (Deitsch *et al.*, 1995a,b) served as a control for a constitutively expressed gene.

Developmental RT-PCR analyses

The temporal changing profiles of the transcript abundance of the three *E75* isoforms were examined by using RT-PCR followed by Southern blotting with specific radioactive probes. Total RNA was prepared from fat body or ovaries throughout the first vitellogenic cycle as described above (Cho *et al.*, 1991) with some modification in which all isopropanol precipitation steps were done without low-temperature incubation to avoid the co-precipitation of glycogen and salts. RNA preparations were determined

spectrophotometrically. A_{260}/A_{280} and A_{260}/A_{230} of RNA preparations were always above 1.7 and 2.0, respectively and quite consistent irrespective of developmental stages of the mosquitoes. Five microgram each of the RNA preparation was reverse-transcribed by SuperscriptII reverse transcriptase (Gibco-BRL) and random hexamer (Promega) in a reaction volume of 20 μl, diluted to 40 μl with TE buffer (10 mM Tris-Cl, pH 8.0, containing 1 mM EDTA) and stored at -20°C as a cDNA pool until use. In the case of mosquito tissues, 0.025 fat body- or ovary-equivalent cDNA pools ranging from 0.3 μl to 1.4 μl were used as PCR templates because of drastic changes in size of the tissue during vitellogenesis. PCR-amplification of three isoform-specific DNA fragments was done either simultaneously in a single tube with 25 μl of reaction mixture containing three isoform-specific sense primers (75A-F, 75B-F, and 75C-F) and one common antisense primer (75com-R), or separately using only one set of primers. For the details of the primers, see below.

Thermal cycling conditions were as follows: the reaction was incubated at 94°C for 3 min; this was followed by 18 (for fat body samples) or 16 (for ovarian samples) cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. As a reference of the development in the fat body and ovary, the same cDNA was subjected to the 10 cycles of RT-PCR followed by Southern blotting with a primer pair specific to the mosquito VCP (Cho *et al.*, 1991), and vitellogenin receptor (Sappington *et al.*, 1996), respectively. After PCR, 10 μl each of the reaction was fractionated 2.5 % agarose gel, transferred onto Hybond N+ membrane (Amersham) under alkaline conditions. Radioactive cDNA probe was prepared from 15 ng each of three PCR fragments generated by the primer pairs of 75A-F/75com-R (generating 517 bp fragment), 75B-F/75com-R (394 bp) and 75C-

F/75com-R (280 bp), respectively, by random-priming labeling with [α^{32}P]-dATP (NEN), and the membrane hybridized with the probe at 65°C in a solution of 5 x SSPE, 5 x Denhardt' solution, 200 μg/ml of denatured salmon sperm DNA (Sigma) following prehybridization. The membrane was washed twice at 65°C in 2 x SSPE for 15 min, once in 1 x SSPE for 15 min and finally twice in 0.1 x SSPE for 15 min, all containing 0.1% SDS, then autoradiographed. PCR/Southern blotting of the total RNA preparations without reverse transcription did not result in any appreciable signal on autoradiogram, indicating that the contamination of genomic DNA fragments into the total RNA fraction was virtually negligible (not shown). The radioactive blots were quantified with a phosphorimager (Molecular Dynamics). Primers used for the developmental RT-PCR:

75A-F, TAGTGCAATCAACGTATACCAATC;

75B-F, CGTGGAAGAAGACCACGATCG;

75C-F, AACATAACCACGTGACCTCAATG;

75com-R, CAAGGGCGATACTGAATTTTCTG.

In vitro fat body culture

To examine the ecdysone inducibility of the mosquito *AaE75* homologue, the abdominal walls with adhering fat body (hereafter referred to as the fat body) were dissected from 3 to 5-day-old previtellogenic females and incubated in an organ culture system for 2 to 4 hr with increasing doses of 20E as previously described (Deitsch *et al.*, 1995a,b; Raikhel *et al.*, 1997). Cycloheximide (Chx) (Calbiochem) was dissolved in water immediately before use (Deitsch *et al.*, 1995). Total RNA was extracted as indicated above (Cho *et al.*, 1991). As a control, RNA was extracted from fat bodies

incubated without the hormone or Chx. Northern blot or RT-PCR analyses were utilized as described above. Hybridization using a probe for the ecdysone-inducible VCP gene provided a positive control, while a cDNA probe for mosquito actin served as a control for a constitutively expressed gene (Deitsch *et al.*, 1995a,b).

Cell culture and transient transfection assay

Cell culture and transient transfection assay were performed basically as previously described (Wang *et al.*, 2000b). The pAc5-AaEcR and pAc5-AaUSPb constructs contained the full length AaEcR and AaUSPb (Wang *et al.*, 2000b). The Vg-promoter-luc is the 2.1kb Vg promoter driving a luciferase reporter gene in the pGL3-basic vector. The full-length of AaE75A was subcloned into the pAc5 *NotI* and *ApaI* sites for transfection.

RESULTS

Cloning of an E75 homologue from Aedes aegypti cDNA and genomic libraries

To identify the *A. aegypti* homologue of the *Drosophila E75* early ecdysone inducible gene, a PCR-based strategy was utilized to take advantage of scattered highly conserved regions in the DNA-binding domain and the E domain of the *D. melanogaster* and *Manduca sexta* E75 proteins; the latter domain has strong similarity to the hormone binding domain of receptors in the nuclear hormone receptor superfamily (Segraves and Hogness, 1990; Segraves and Woldin, 1993). Several combinations of degenerate primers within the hormone-binding domain amplified genomic DNA fragments of the predicted size (see Materials and Methods). The largest of these, a 421 bp fragment, was cloned and sequenced. It encodes a pollypeptide with ~90% amino acid identity to the

corresponding region of *Drosophila* E75. Thus the gene from which these sequences had been amplified was tentatively named *A. aegypti E75* or *AaE75*.

Using the internal sequence of the 421-bp fragment, a second set of oligonucleotide primers was designed, directed away from one another, for use in inverse PCR reactions. DNA prepared from a fat body cDNA library was cleaved with *EcoRI*, recircularized and subjected to inverse PCR, yielding a 2.2 kb fragment. Direct sequence analysis indicated that this fragment contained sequences extending 5' into the D region which links the DNA and hormone binding domains and 3' through the carboxyl terminal domain to the 3' untranslated region. Further PCR analysis of the cDNA library, using degenerate primers for the DNA binding domain and primers derived from the sequence of the 2.2 kb fragment, indicated that the fat body cDNA library contained cDNAs extending at least into the DNA binding domain.

To obtain vitellogenic fat body cDNA clones, the 2.2-kb inverse PCR fragment was used as a probe to screen approximately 2 x 10^6 recombinants. The cDNA clone extending farthest in the 5' direction, 2-3A6, was sequenced in its entirety. Analysis of this sequence indicated that this was a full length *Aedes aegypti E75B* cDNA (Fig. 1, exon B1 and common region).

A second type of cDNA clone contained a region closely resembling the *E75* A1 exon, which encodes the first finger of the DNA binding domain, preceded by sequences closely resembling the E75C-specific sequences of *Drosophila*. However, this clone contained only 350 bp of E75C-specific sequence and appeared to be truncated at its 5' end. Three sequential rounds of inverse PCR "walking" gave rise to an additional 2 kb of AaE75C-specific genomic sequence. PCR of vitellogenic fat body cDNA using

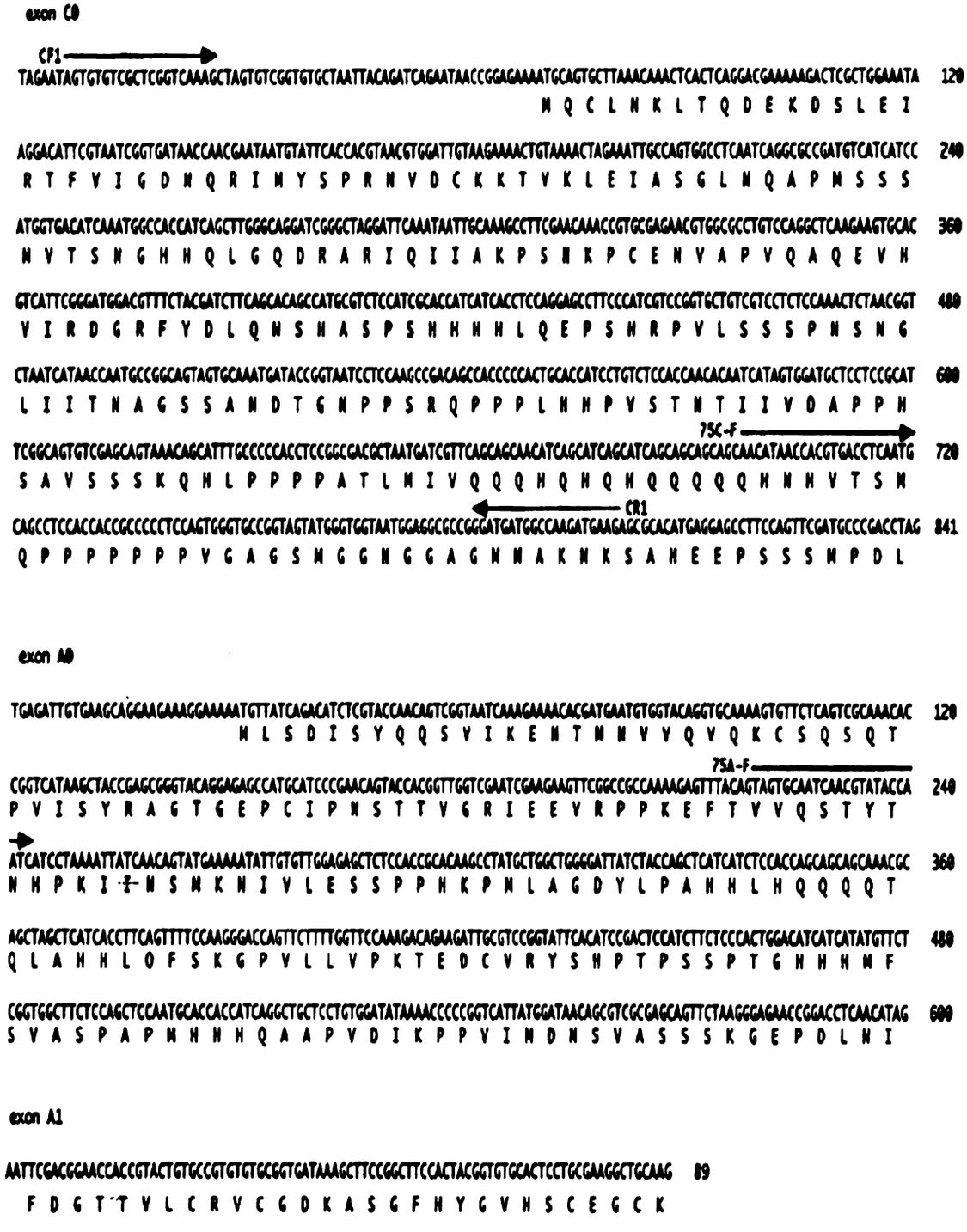
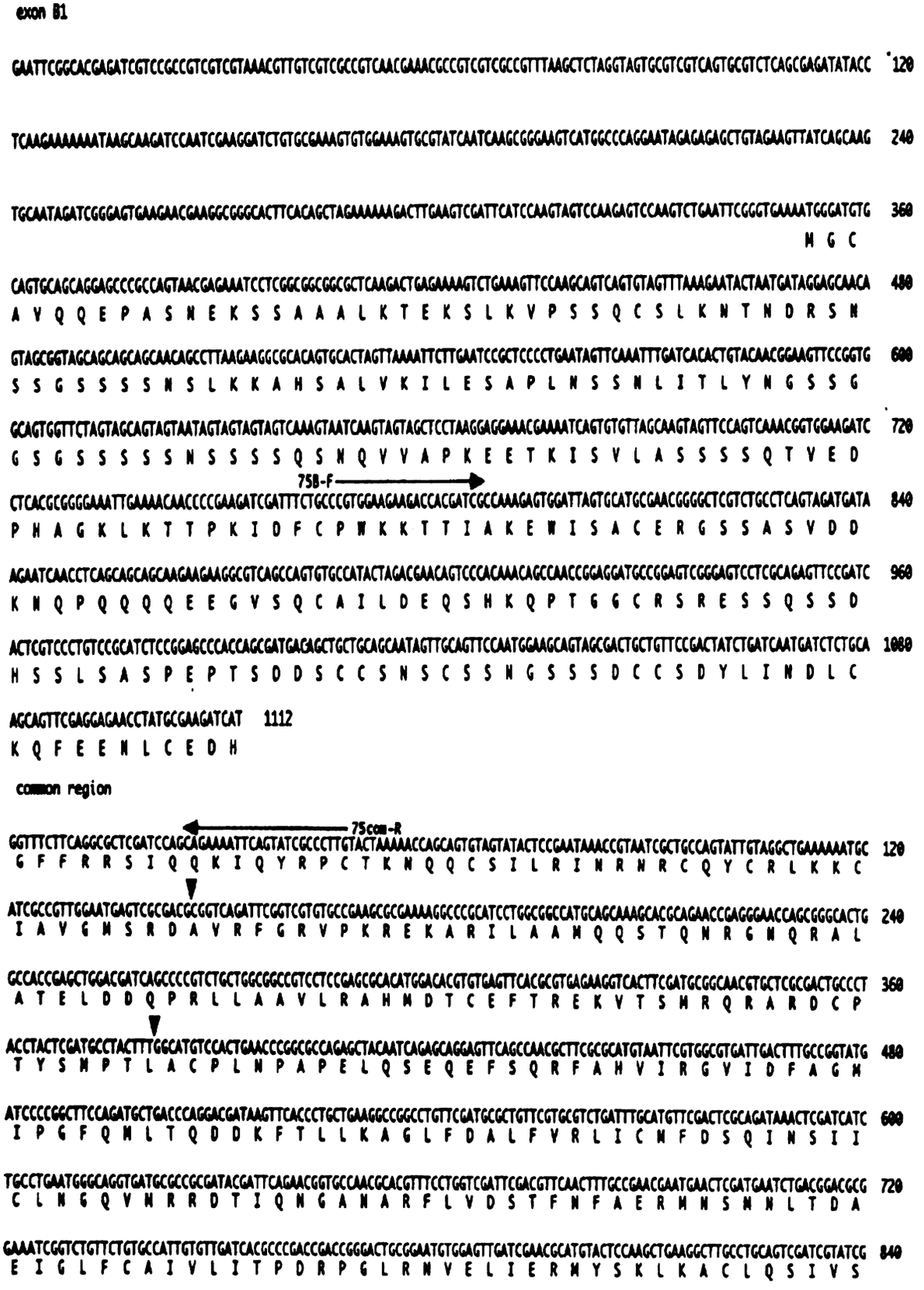


Fig. 1. Nucleotide and amino acid sequences of AaE75. Sequences for the transcription unit-specific exons, C0, A0, A1 and B1, and for the common region, shared by all three AaE75 transcription units, are shown. Oligonucleotides used for RT-PCR analysis and other probe generation are indicated above the nucleotide sequence. Introns separating common region exons 2, 3 and 4/5 are indicated by vertical arrows.


exon B1
GAATTCGGCACGAGATCGTCCGCCGTCGTCGTAAACGTTGTCGTCGCCGTCAACGAAACGCCGTCGTCGCCGTTTAAGCTCTAGGTAGTGCGTCGTCAGTGCGTCTCAGCGAGATATACC 120
TCAAGAAAAAAATAAGCAAGATCCAATCGAAGGATCTGTGCGAAAGTGTGGAAAGTGCGTATCAATCAAGCGGGAAGTCATGGCCCAGGAATAGAGAGAGCTGTAGAAGTTATCAGCAAG 240
TGCAATAGATCGGGAGTGAAGAACGAAGGCGGGCACTTCACAGCTAGAAAAAAGACTTGAAGTCGATTCATCCAAGTAGTCCAAGAGTCCAAGTCTGAATTCGGGTGAAAATGGGATGTG 360
M G C
CAGTGCAGCAGGAGCCCGCCAGTAACGAGAAATCCTCGGCGGCGGCGCTCAAGACTGAGAAAAGTCTGAAAGTTCCAAGCAGTCAGTGTAGTTTAAAGAATACTAATGATAGGAGCAACA 480
A V Q Q E P A S N E K S S A A A L K T E K S L K V P S S Q C S L K N T N D R S N
GTAGCGGTAGCAGCAGCAGCAACAGCCTTAAGAAGGCGCACAGTGCACTAGTTAAAATTCTTGAATCCGCTCCCCTGAATAGTTCAAATTTGATCACACTGTACAACGGAAGTTCCGGTG 600
S S G S S S S S N S L K K A H S A L V K I L E S A P L N S S N L I T L Y N G S S G
GCAGTGGTTCTAGTAGCAGTAGTAATAGTAGTAGTAGTCAAAGTAATCAAGTAGTAGCTCCTAAGGAGGAAACGAAAATCAGTGTGTTAGCAAGTAGTTCCAGTCAAACGGTGGAAGATC 720
G S G S S S S S N S S S S Q S N Q V V A P K E E T K I S V L A S S S S Q T V E D
75B-F
CTCACGCGGGGAAATTGAAAACAACCCCGAAGATCGATTTCTGCCCGTGGAAGAAGACCACGATCGCCAAAGAGTGGATTAGTGCATGCGAACGGGGCTCGTCTGCCTCAGTAGATGATA 840
P H A G K L K T T P K I D F C P W K K T T I A K E W I S A C E R G S S A S V D D
AGAATCAACCTCAGCAGCAGCAAGAAGAAGGCGTCAGCCAGTGTGCCATACTAGACGAACAGTCCCACAAACAGCCAACCGGAGGATGCCGGAGTCGGGAGTCCTCGCAGAGTTCCGATC 960
K N Q P Q Q Q Q E E G V S Q C A I L D E Q S H K Q P T G G C R S R E S S Q S S D
ACTCGTCCCTGTCCGCATCTCCGGAGCCCACCAGCGATGACAGCTGCTGCAGCAATAGTTGCAGTTCCAATGGAAGCAGTAGCGACTGCTGTTCCGACTATCTGATCAATGATCTCTGCA 1080
H S S L S A S P E P T S D D S C C S N S C S S N G S S S D C C S D Y L I N D L C
AGCAGTTCGAGGAGAACCTATGCGAAGATCAT 1112
K Q F E E N L C E D H
common region
75com-R
GGTTTCTTCAGGCGCTCGATCCAGCAGAAAATTCAGTATCGCCCTTGTACTAAAAACCAGCAGTGTAGTATACTCCGAATAAACCGTAATCGCTGCCAGTATTGTAGGCTGAAAAAATGC 120
G F F R R S I Q Q K I Q Y R P C T K N Q Q C S I L R I N R N R C Q Y C R L K K C
ATCGCCGTTGGAATGAGTCGCGACGCGGTCAGATTCGGTCGTGTGCCGAAGCGCGAAAAGGCCCGCATCCTGGCGGCCATGCAGCAAAGCACGCAGAACCGAGGGAACCAGCGGGCACTG 240
I A V G M S R D A V R F G R V P K R E K A R I L A A M Q Q S T Q N R G N Q R A L
GCCACCGAGCTGGACGATCAGCCCCGTCTGCTGGCGGCCGTCCTCCGAGCGCACATGGACACGTGTGAGTTCACGCGTGAGAAGGTCACTTCGATGCGGCAACGTGCTCGCGACTGCCCT 360
A T E L D D Q P R L L A A V L R A H M D T C E F T R E K V T S M R Q R A R D C P
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T Y S M P T L A C P L N P A P E L Q S E Q E F S Q R F A H V I R G V I D F A G M
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I P G F Q M L T Q D D K F T L L K A G L F D A L F V R L I C M F D S Q I N S I I
TGCCTGAATGGGCAGGTGATGCGCCGCGATACGATTCAGAACGGTGCCAACGCACGTTTCCTGGTCGATTCGACGTTCAACTTTGCCGAACGAATGAACTCGATGAATCTGACGGACGCG 720
C L N G Q V M R R D T I Q N G A N A R F L V D S T F N F A E R M N S M N L T D A
GAAATCGGTCTGTTCTGTGCCATTGTGTTGATCACGCCCGACCGACCGGGACTGCGGAATGTGGAGTTGATCGAACGCATGTACTCCAAGCTGAAGGCTTGCCTGCAGTCGATCGTATCG 840
E I G L F C A I V L I T P D R P G L R N V E L I E R M Y S K L K A C L Q S I V S

Fig. 1 (cont'd).

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 L  L  R  Q  H  M  W  N  A  E  E  D  L  A  K  S  P  S  S  N  T  W  S  C  D  G  N  N  V  E  D  V  A  K  S  P  M  S  S  V

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GCTCCCCTCAGGCGGACTCGTGCCGAATTC  2790
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Fig. 1 (cont'd).

oligonucleotides based on this sequence indicated that the cDNA library contained sequences extending nearly to the start of the AaE75C open reading frame, but screening of an additional 2 X 10^6 cDNAs did not reveal any full length E75C cDNAs. Therefore inverse PCR on *EcoRI*-digested recircularized templates was utilized to isolate the C-specific region from the rare or truncated cDNA or cDNAs responsible for this signal. At the 3' end of the isolated sequences, the presence of an *EcoRI* site derived from the exon C0-exon A1 splice (see Fig. 1) confirms that these sequences derive from spliced RNA and not from genomic DNA. At the 5' end, sequence analysis indicates that these sequences extend to within 30 bp of a putative initiator Met closely preceded by an in-frame stop codon (Fig. 1). RT-PCR analysis indicates, however, that the 5' end of AaE75C likely lies significantly upstream of this region (data not shown).

No AaE75A-specific cDNAs were identified in the screens described above. In order to isolate the AaE75A-specific coding region from the fat body library, primer sets were designed to amplify specifically AaE75A sequences. First, primers annealing within the A1 exon were used for an initial round of inverse PCR amplification, then a predicted exon A0-specific degenerate primer was used to re-amplify these products (see Materials and Methods). This yielded 183 bp of sequence closely matching predicted AaE75A-specific sequences. Primers derived from this sequence were used in a second inverse PCR reaction on recircularized *EcoRI*-digested fat body cDNA library material, giving rise to a fragment extending beyond the 5' end of the AaE75A-specific ORF (Fig. 1).

Defining the Aedes aegypti E75 genomic structure

To elucidate the genomic structure of the *AaE75* gene, the cDNA fragments described above were used as probes to identify genomic clones from size-selected and complete *A. aegypti* genomic libraries, yielding a collection of overlapping genomic clones within each region. Exons were localized by restriction mapping, hybridization and PCR using exon-specific and vector primers (Fig. 2). The intron between exons A0 and A1 is approximately 15.6 kb in length and the intron between the AaE75B-specific region and exon 2 is 150-200 bp in length.

The failure of clones A8A and B10 to cross-hybridize indicated that there are additional intron sequences separating these two clones. Genomic clones A8A and B10 were attempted to use to define the length of this intron through genomic walking. Three rounds of chromosomal walking from the A8A side yielded an additional 20+ kb of genomic DNA before the presence of repetitive sequences prevented further walking. Screening with a 5' terminal fragment of clone B10 yielded no additional clones, indicating a potential gap in the genomic library. While the complete length of the intron between the A1 and B1 exons thus cannot be determined, these findings suggest that it is in excess of 30 kb.

Within the common region, restriction mapping, hybridization, PCR and direct sequence analysis showed that there is no intron separating the hormone binding domain and the carboxyl terminal F domain, but that there is an intron of approximately 3.8 kb separating exon 3 from exon 4/5. To determine the length of the intron separating exon 2 from exon 3, a 5' terminal fragment of clone E7 was used to isolate a clone, L13, which also hybridized with the B10 clone. Further restriction mapping and Southern blot

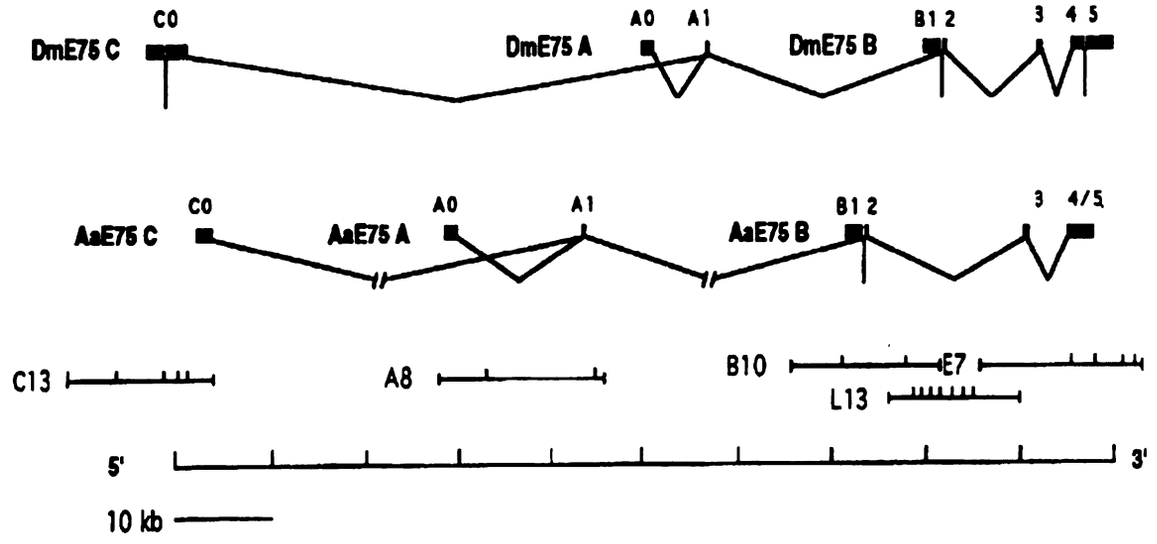


Fig. 2. Genomic organization of AaE75. The intron-exon structure and partial restriction map of genomic clones are shown. The DNA binding domain is encoded within exon A1 and exon 2. The region of the steroid receptor hormone binding domain is located within the first ~200 amino acids of exon 4/5. The intron-exon map of *D. melanogaster E75* (*DmE75*) is shown for comparison. *EcoRI* sites are indicated on the genomic clones.

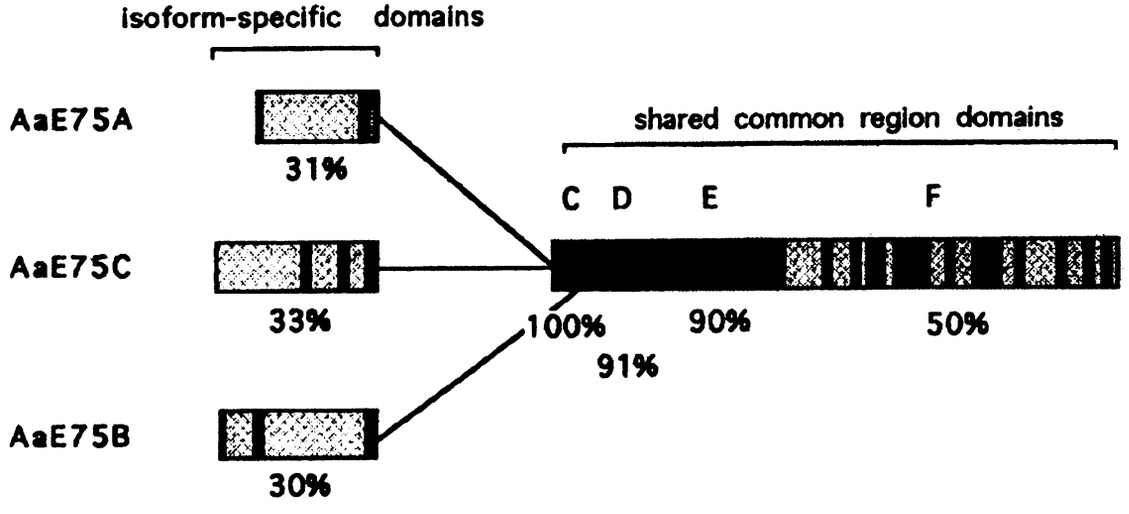


Fig. 3. Comparison of *Aedes aegypti* and *D. melanogaster* E75 amino acid sequences. Schematic representation of sequence identity within structural domains of the *A. aegypti* E75 A, E75B, and E75C-specific regions as well as the DNA binding domain (C), linking region (D), hormone binding domain (E), and repressor region (F). Overall percentage of amino acid identity between *A. aegypti* and *D. melanogaster* E75 domains are presented below each region. Sub-regions showing high levels of identity within less conserved domains are indicated as dark stippled bars.

analysis allowed alignment of these clones and showed that the intron separating exons 2 and 3 is approximately 17 kb in length (Fig. 2).

Evolutionary conservation of the dipteran E75 proteins

The predicted amino acid sequences of the AaE75A, AaE75B and AaE75C proteins are shown above the corresponding nucleic acid sequences (Fig. 1). Fig. 3 schematically depicts the comparison between these sequences and those of E75 A, B and C from *D. melanogaster*. The DNA binding, linking, and hormone binding regions display high levels of identity with *Drosophila* E75: 100%, 91% and 93%, respectively, at the amino acid level. The terminal domains are substantially more substantially divergent, but show a similar organization and contain several highly conserved subregions. More significantly, the amino terminal domains specific to the AaE75A, AaE75B and AaE75C isoforms are clearly conserved with each showing ~30% identity to the amino terminal domain of the corresponding *Drosophila* protein.

AaE75 transcript analysis

The transcript size of the *AaE75* gene was examined by the Northern blot analysis of vitellogenic tissues of female mosquitoes during the first cycle of vitellogenesis. Mosquito ovarian RNA 24-30 hours PBM was hybridized with isoform-specific probes (A625 for AaE75A, B660 for AaE75B and C350 or C796 for AaE75C; see Materials and Methods), revealing two transcripts per isoform (Fig. 4): 6.2 and 5, 6.9 and 5.6, and 6.8 and 5.4 kb, for isoforms A, B, and C, respectively. When a probe to the region common to all three *AaE75* isoforms was utilized, 5 bands were observed (Fig. 4). These findings

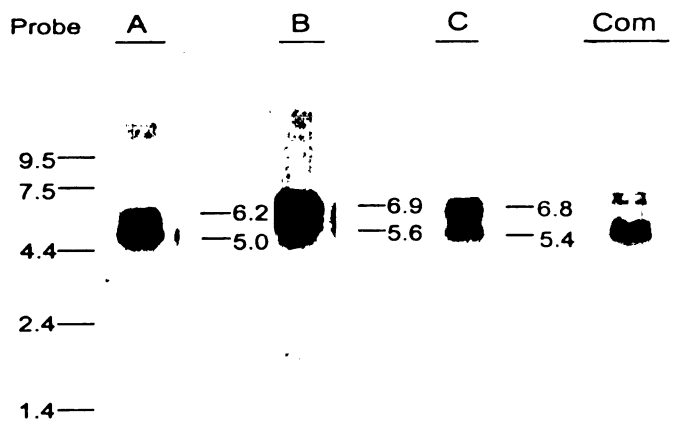


Fig. 4. *AaE75* transcripts in the mosquito ovary. Total RNA was extracted from ovaries dissected from vitellogenic mosquitoes 24-30 hr after a blood meal. Northern blots were hybridized with AaE75 isoform-specific probes. (Lane A, AaE75A-specific probe; Lane B, AaE75B-specific probe; Lane C, AaE75C-specific probe; Com, common region AaE75 probe).

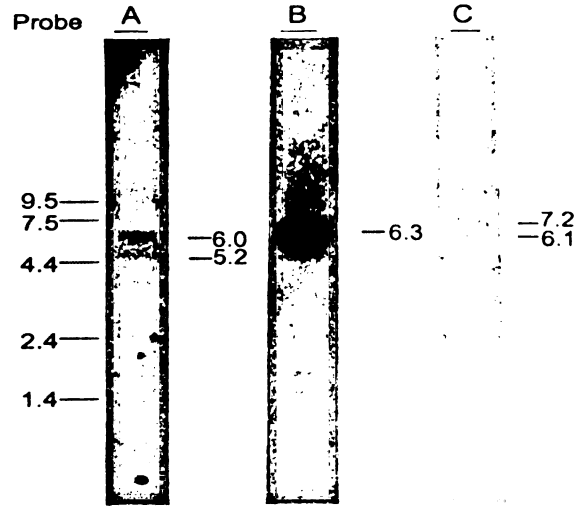


Fig. 5. AaE75 transcripts in the mosquito fat body. Total RNA was extracted from fat bodies dissected from vitellogenic mosquitoes 24-30 hr after a blood meal. Northern blots were hybridized with AaE75 isoform-specific probes. (Lane A, AaE75A-specific probe; Lane B, AaE75B-specific probe; Lane C, AaE75C-specific probe).

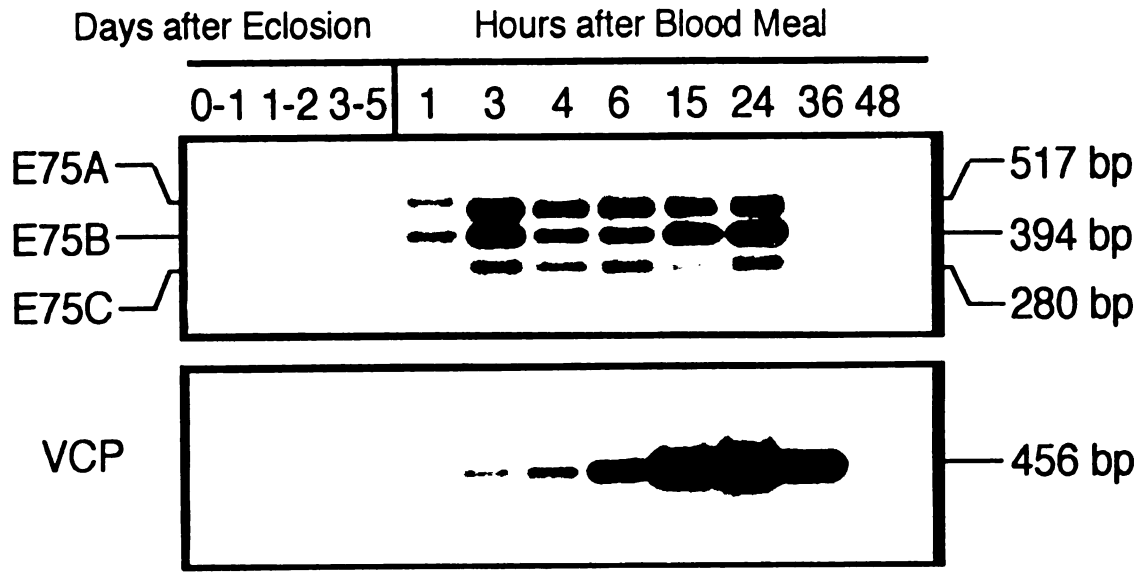
are consistent with the presence of several different 3' ends in the cDNA clones analyzed (data not shown), suggesting that these RNA size-classes arise from utilization of alternative polyadenylation signals, as in the case of *Drosophila E75* (Segraves and Hogness, 1990).

In the fat body, transcript size was also established using Northern blot hybridization of RNA from the vitellogenic fat bodies 24-30 hr PBM with isoform-specific *AaE75* probes. As in the ovary, two transcripts are clearly observed for the AaE75A and AaE75C isoforms. Similarly, it is likely that two transcripts existed for AaE75B (Fig. 5). Overall, the level of *E75* RNA in the fat body was considerably lower than in the ovary, making the Northern blot analysis a relatively undesirable alternative for further analysis in this tissue.

Time course of AaE75 expression in the fat body and ovary during vitellogenesis

To determine the temporal expression pattern of *AaE75* mRNA levels in the vitellogenic tissues, the fat body and ovary, RT-PCR analysis was primarily used. Complementary DNA was reverse transcribed from fat body total RNA and then amplified in PCR reactions using isoform-specific primers (see Fig. 1 and Materials and Methods). In both vitellogenic tissues, *AaE75* expression was induced at the onset of vitellogenesis by a blood meal-activated hormonal cascade, and *AaE75* isoforms were highly expressed in the mosquito ovary and fat body, suggesting an involvement in the regulation of oogenesis and vitellogenesis, respectively. In the fat body (Figs. 6a and 6b), *E75* transcripts exhibit a sharp rise immediately after the onset of vitellogenesis reaching

(a).



(b).

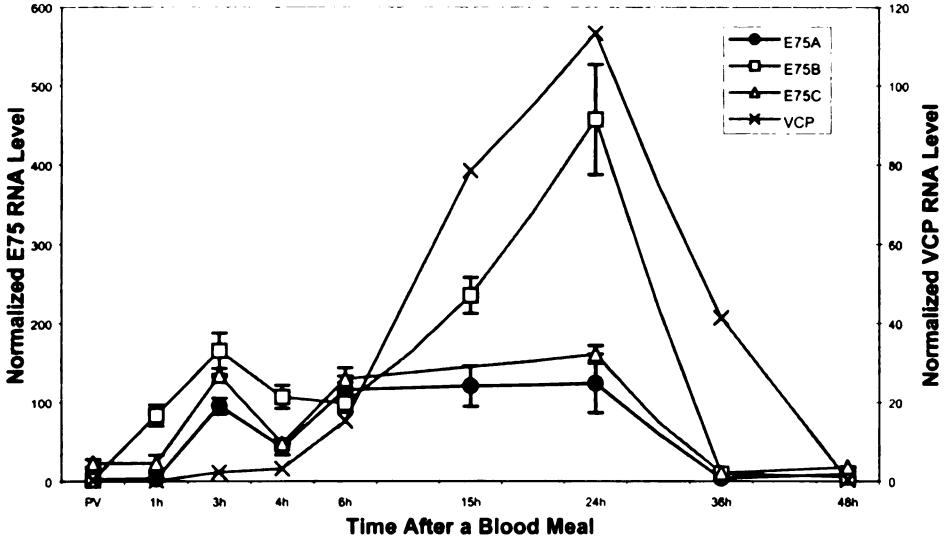


Fig. 6. Accumulation of AaE75 mRNA in the mosquito fat body. This experiment was performed by Southern blotted RT-PCR (see Materials and Methods). The results are expressed normalized units of the highest readings per fat body and represent means of three determinations ± S.E. Shown also are the results of a RT-PCR with VCP, which serves to illustrate the course of vitellogenesis in the fat body. A. a representative Southern blot of the RT-PCR; B. quantitative analysis of the data.

a peak at 3-4 hr PBM when ecdysone titer shows a first small peak (Hagedorn *et al.*, 1975). After the initial drop, levels of AaE75A and AaE75B transcripts gradually increased again reaching a second peak at the 24 hr PBM when ecdysone levels just have passed their major peak, at 18-20 hr PBM (Hagedorn *et al.*, 1975). The second gradual increase in *AaE75* transcripts closely correlated with the accumulation of RNA for YPPs in the fat body as demonstrated by the VCP transcript kinetics. The second peak of isoforms A and C transcripts was not as pronounced as that of isoform B. After the second peak, the amounts of *AaE75* transcripts decreased to very low levels at the end of vitellogenesis, 42 and 48 h PBM (Figs. 6a and 6b).

The temporal expression of the *AaE75* gene in the ovary was first examined by Northern blot analyses utilizing total ovarian RNA for time points spanning the first vitellogenic cycle from 0 to 48 hr PBM. For each time point, the same number of ovary-equivalents of RNA was loaded, and blots were probed with the three isoform-specific *AaE75* probes. To provide a reference with follicle development, two ovary-specific probes were utilized: vitellogenin receptor VgR cDNA, (Sappington *et al.*, 1996), which is specific to the germ-line cells (i.e., the oocyte and nurse cells); and the VE gene 15a-1 (Lin *et al.*, 1993), which is specifically expressed in the follicular epithelium surrounding the germ cells in the mosquito follicle. These Northern blots showed that transcripts for all three *AaE75* isoforms exhibited similar patterns of accumulation and disappearance in the mosquito ovary (Fig. 7). The *AaE75* transcripts accumulated slowly during the first 10-12 hr PBM; however AaE75A and Aa75B transcripts exhibited dramatic accumulation during the next 12 hr, reaching a peak by 24 hr PBM. The amount of these *AaE75* transcripts declined between 24 and 36 hr PBM, the time coinciding with the

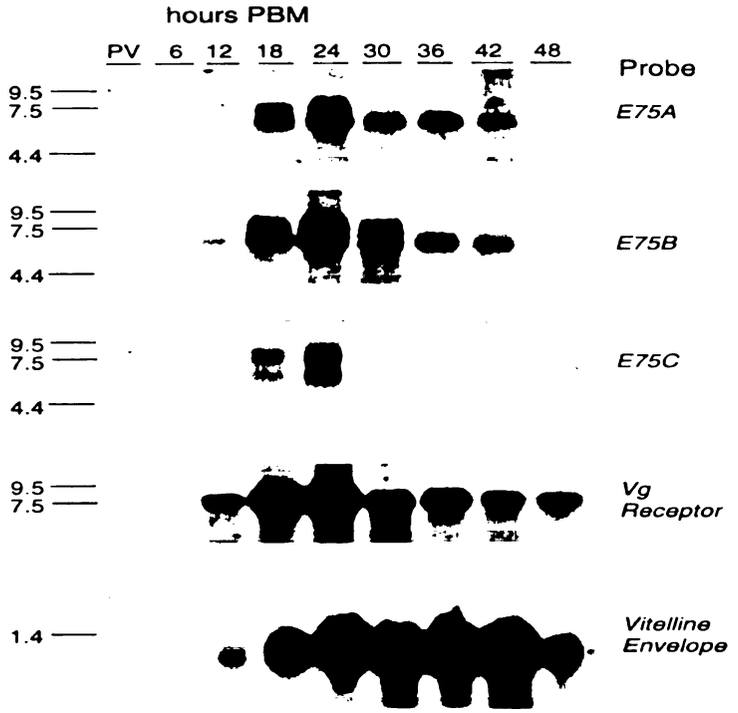


Fig. 7. Expression of AaE75 RNA isoforms in the mosquito ovary during the first vitellogenic cycle. Total RNA was extracted from the same amount of ovary equivalents dissected from previtellogenic mosquitoes 3-5 days after eclosion and from vitellogenic mosquitoes at the indicated hours after a blood meal. Northern blots were hybridized with AaE75 isoform-specific probes (see Materials and Methods), and with Vitellogenin Receptor (VgR) and Vitelline Envelope (VE) cDNA clones.

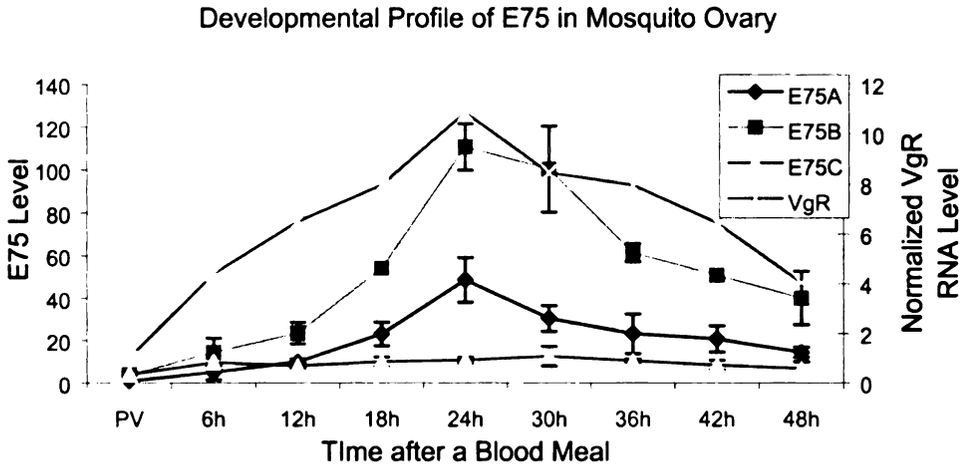


Fig. 8. Accumulation of AaE75 mRNA in the mosquito ovaries, quantified by phosphorimager analysis of Southern blotted RT-PCR products (see Materials and Methods). The results represent means of three determinations ± S.E. Shown also are the results of a RT-PCR with VgR, which serves to illustrate the course of ovarian development.

termination of vitellogenesis and cessation of yolk accumulation by the ovary, and was followed by a more gradual decline over the next 12 hr, reaching low levels by 48 hr PBM. The AaE75C showed only a modest increase with a small peak at 24 hr PBM (Fig. 7). The onset of high *AaE75* expression in the vitellogenic ovary closely paralleled that of the ecdysone-inducible VE genes. However, the VE gene transcripts remain in the ovary at peak levels well beyond the peak of *AaE75* expression (Fig.7). The overall expression profiles of *AaE75* transcripts resembled even more closely the vitellogenic profile of VgR expression (Sappington *et al.*, 1996), which increased to a peak at 24 hr PBM, and then declined during the latter portion of vitellogenesis (Fig. 7). In contrast to *AaE75*, however, VgR was also expressed at a low level in previtellogenic ovaries (not shown). The quantitative analysis of the temporal expression of *AaE75* mRNA levels in the vitellogenic ovary, performed using the RT-PCR analysis as described for the fat body, is in agreement with the Northern blot analysis (Fig. 8).

Response of AaE75 to ecdysone in vitro

The pattern of *AaE75* transcript kinetics in the mosquito ovary and fat body following a blood meal suggests that ecdysone produced in response to a blood meal is likely responsible for the induction of the *AaE75* gene. To determine whether *AaE75* induction following a blood meal is ecdysone-mediated, we have tested the *in vitro* effect of 20-hydroxyecdysone on the appearance of *AaE75* mRNA in isolated previtellogenic female fat body, under conditions previously demonstrated *in vitro* induction of the *VCP* gene (Deitsch *et al.*, 1995a) (Fig. 9). As it has been previously established (Deitsch *et al.*, 1995a), the levels of the *VCP* mRNA increased in response to increasing concentration of

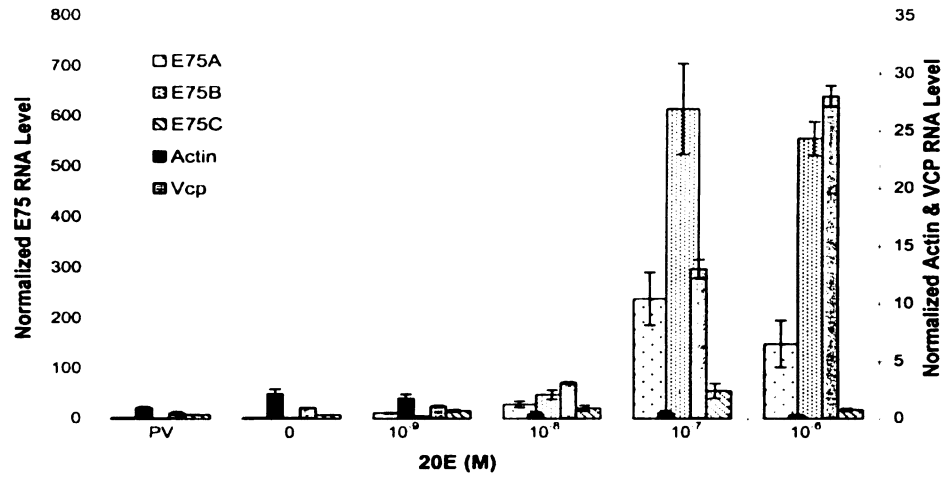


Fig. 9. AaE75 response to 20-hydroxyecdysone in *in vitro* fat body organ culture. The transcriptional response of the mosquito E75 homologue to 20-hydroxyecdysone was examined in an *in vitro* culture system of female previtellogenic fat body. Total RNA also was extracted from fat bodies dissected from previtellogenic (PV) mosquitoes to provide a control for the *in vitro* system. RT-PCR and Southern hybridization were performed with isoform-specific probes for AaE75A, AaE75B, or AaE75C (see Materials and Methods, and Fig. 6). RT-PCR for VCP provided a positive control, while Actin was used as control for loading.

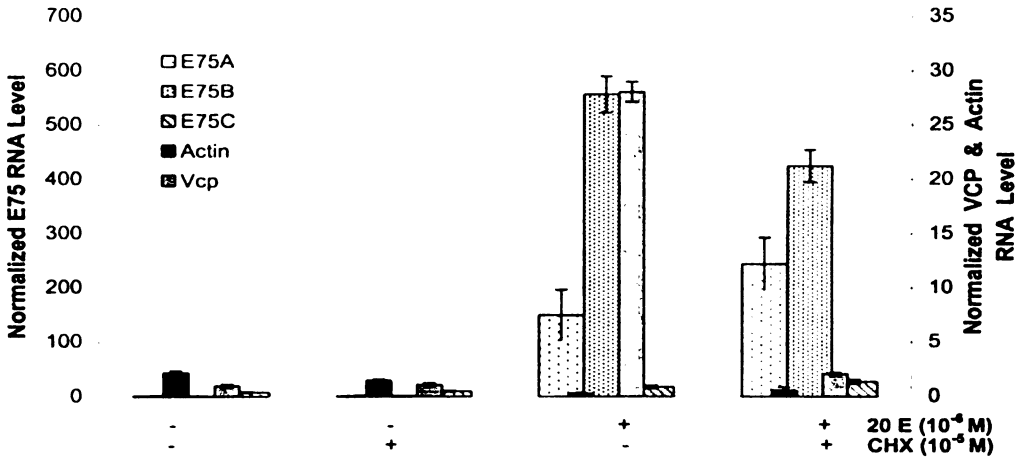


Fig. 10. Effect of cycloheximide (Chx) on the activation of the *AaE75* gene. The previtellogenic fat bodies were first pre-incubated in the culture medium for 1 hr in the absence or presence of 10^{-5} M Chx, and then continued incubating with the same dose of Chx but with the addition of 10^{-6} M 20E for 2 hr. RT-PCR and Southern hybridization were performed with isoform-specific probes for AaE75A, AaE75B, or AaE75C (see Materials and Methods, and Fig. 6).

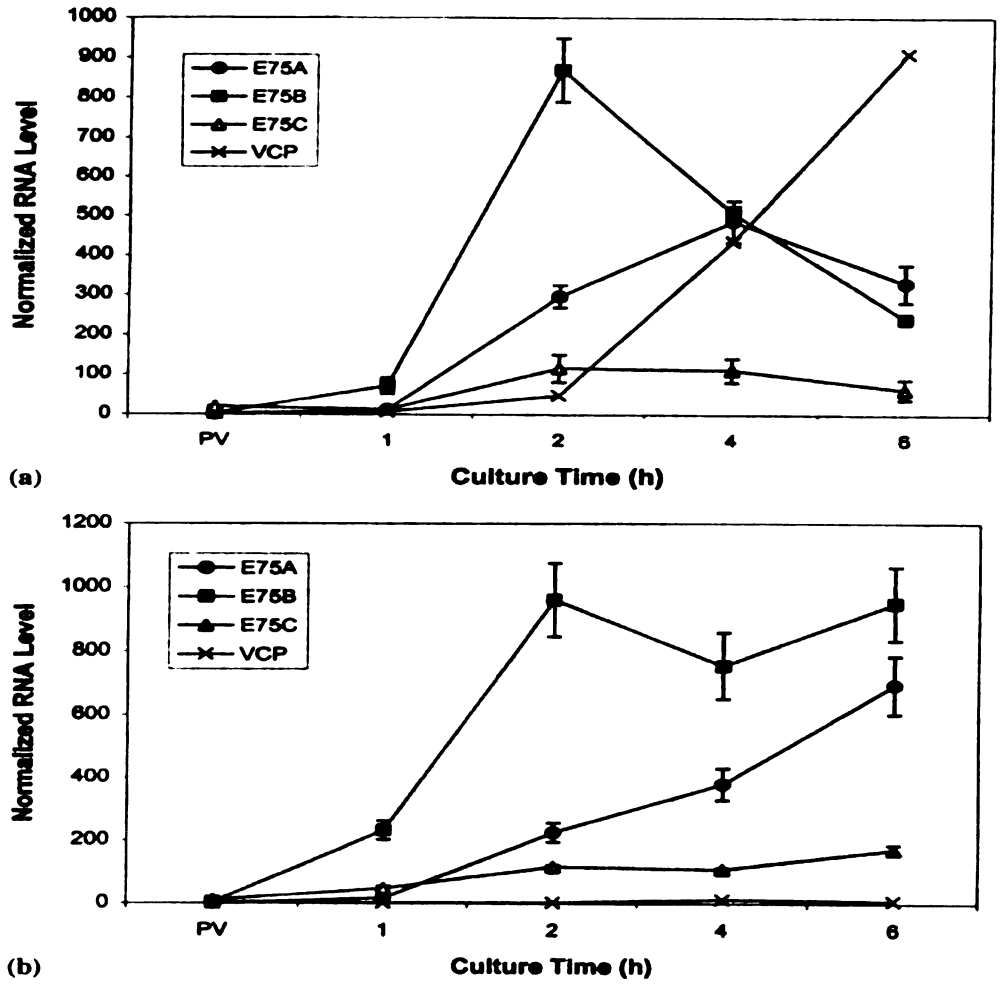


Fig. 11. Time course of *AaE75* induction in *in vitro* fat body organ culture. Total RNA was extracted from previtellogenic (PV) fat bodies or fat bodies incubated in the presence of 20-hydroxyecdysone for the indicated times. RT-PCR and southern blotting were performed with isoform-specific probes for AaE75A, AaE75B or AaE75C (see Section 2, Fig. 6). VCP was included as a positive control. The results represent the means of three independent experiments +SE: (a). RNA accumulation of E75A, E75B, E75C and VCP during exposure to 10^{-7} M 20-hydroxyecdysone; (b) RNA accumulation of E75A, E75B, E75C and VCP during exposure to 10^{-7} M 20-hydroxyecdysone and 10^{-5} M cycloheximide

20-hydroxyecdysone, reaching maximum at 10^{-6} to 10^{-5} M of the hormone in the medium (not shown). The levels of actin mRNA remained relatively constant. The mRNA levels of all three *AaE75* isoforms increased in a dose-dependent manner in response to 20-hydoxyecdysone. The levels of AaE75A and AaE75B were elevated significantly, while only small increase was observed for AaE75C mRNA. Importantly, the *AaE75* transcripts reached their maximal levels at the concentration of 20-hydroxyecdysone in the culture medium at least 10-fold lower (10^{-7} M) than that for the *VCP* and *Vg* genes (Fig. 9).

To determine the requirement of protein synthesis for activation of the *E75* early gene in the mosquito fat body by 20E, the previtellogenic fat bodies were incubated with cycloheximide (Chx), a protein synthesis inhibitor which reversibly represses protein synthesis in this tissue in a dose-dependent manner (Deitsch *et al.*, 1995a). The fat bodies were first pre-incubated in the culture medium for 1 hr in the absence or presence of 10^{-5} M Chx, and then continued incubating with the same dose of Chx but with the addition of 10^{-6} M 20E for 2 hr. Transcripts for all three *AaE75* isoforms appeared in response to 20E in the presence of Chx, in contrast to the *VCP* gene, which was inhibited by Chx (Fig 10). Incubation of the non-stimulated, previtellogenic fat bodies in the presence of Chx alone activated neither the *AaE75* gene nor *VCP* (not shown). The time course of response also differentiated between *AaE*75 and *VC*P. AaE75 transcripts are induced rapidly and reach a peak by 2–4 h before beginning to regress. In contrast, VCP transcripts continue to accumulate to 6 h and beyond in response to hormonal stimulation. When the accumulation of early gene protein products is blocked by cycloheximide, both

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the marked regression of early gene expression and the increase in VCP expression are blocked (Fig. 11).

Activation of the Vg promoter by E75 in S2 cells

On the Vg promoter, a number of potential transcription factor binding sites have been identified including E75 and EcR/USP (Martin *et al.*, 2001; Martin and Raikhel, unpublished). To examine whether E75 is capable of inducing activation of the Vg promoter, cell transfection assays were performed. The expression plasmid carrying AaE75A was transfected into *Drosophila* S2 cells along with the reporter plasmid containing the Vg-promoter-luciferase. Either in the presence or absence of 20E, E75A activated the reporter gene to more than two fold (Fig. 12, 100ng), and the activation increased in a dose-dependent manner in response to increasing amounts of E75A (Fig.12). Interestingly, higher levels of activation were detected without 20E than with 20E using this 2.1kb Vg promoter construct (Fig. 12, -20E and +20E).

Next, to investigate whether E75 affects the EcR/USP-induced transactivation of the target Vg gene, which has been reported previously (Martin *et al.*, 2001), the expression plasmids carrying EcR/USP and E75A were co-transfected into S2 cells along with the Vg-luciferase plasmid. In the presence of 20E, the reporter gene activities were significantly enhanced when both EcR/USP and E75A were present, compared with EcR/USP or E75A alone (Fig. 12). This synergistic activation was dependant on the amount of E75A, exhibiting stronger activation when higher amounts of E75A were present with constant amount of EcR/USP (11ng) (Fig. 12).

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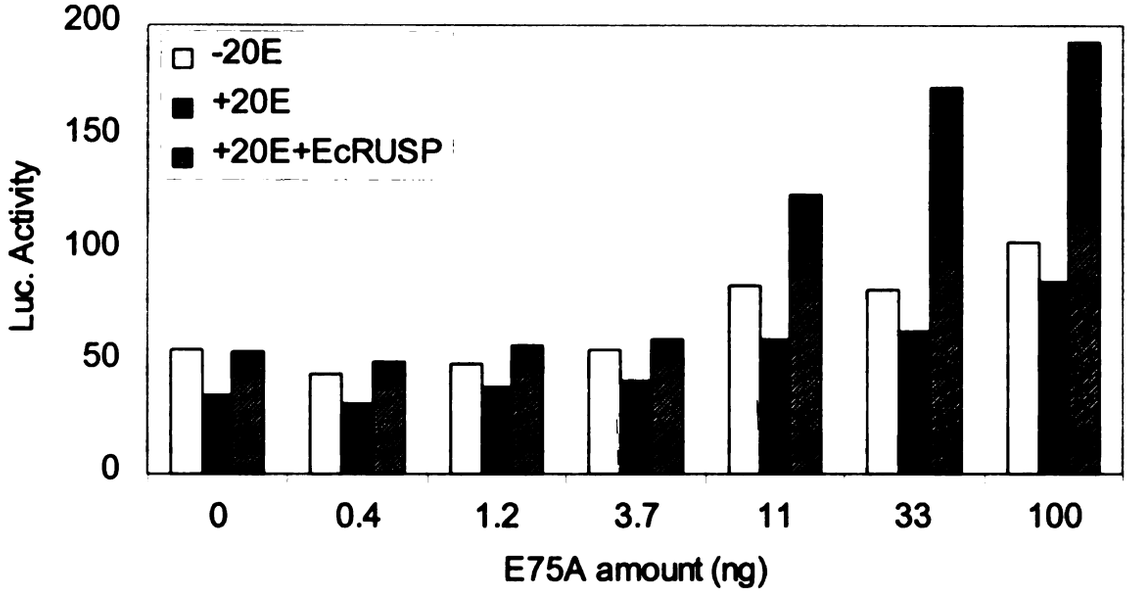

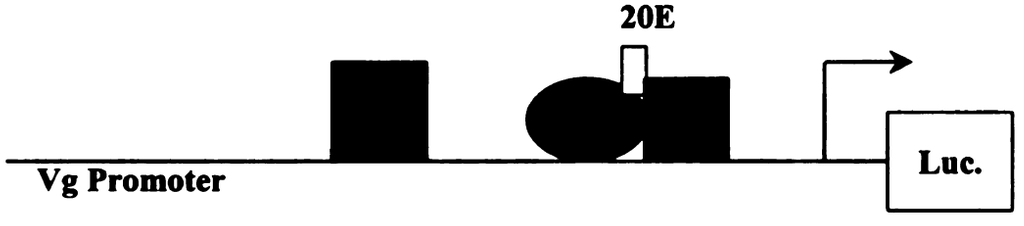

Fig.12. Action of E75 and EcR/USP on the Vg Promoter.
Increasing amounts of the expression plasmid for AaE75A were cotransfected with the 2.1kb-Vg promoter-luciferase construct and a constant amount of AaEcR/AaUSP plasmids (+EcRUSP) into *Drosophila* S2 cells in the presence (+20E) or absence of 20E (-20E). Reporter gene activities were measured after 1 day culture (see Materials and Methods).

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These results indicate that the early gene *E75* may be involved in the activation of the Vg gene and be part of the ecdysone gene regulatory hierarchy during mosquito vitellogenesis.

DISCUSSION

The yellow fever mosquito *Aedes aegypti* is an ideal system for investigating the molecular basis of ecdysone action in insect reproduction, because of the stringent control of egg maturation and vitellogenic events triggered by a blood meal (Raikhel, 1992). Using PCR-based strategies to isolate genomic and cDNA clones, the *Aedes aegypti* homologue of the *Drosophila* early ecdysone-inducible gene E75 has been identified. Its genomic organization, including the structure of the three overlapping transcription units AaE75A, AaE75B, and AaE75C, is highly conserved, with the exon-intron structure of all three transcription units closely resembling that seen in *Drosophila* (Fig.1). By contrast, in lepidoptera there is an altered structure of the E75B transcription unit, in which the E75B-specific region is fused to exon 2, and E75C transcripts have never been observed (Zhou *et al.*, 1998; Segraves and Woldin, 1993; Jindra *et al.*, 1994a). These differences are paralleled by a striking difference in the length of both the E75A and E75B transcript units. In *Drosophila* and mosquito, the E75A transcript units, are, e.g. 50 and >65kb, respectively, comparing to ~16 kb in Manduca (Zhou *et al.*, 1998)

A similar relationship is seen for the E75 proteins, in which the conservation of E75 sequences among species is consistent with their phylogenetic relatedness. For example, within the largest block of conserved sequence, the E region, a comparison of AaE75 to E75 from *Drosophila*, another dipteran, shows approximately half as much

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divergence (93% identity) as the comparison of either diperan E75 protein to E75 proteins of lepidopteran *Manduca Sexta, Galleria mellonella and Choristoneura fumiferana* (87% identity). The mosquito, *Drosophila* and lepidopteran E75 E regions each show approximately 36-37% identity within the E region to the Rev-erb/RVR family of mammalian E75 homologues (Miyajima *et al.*, 1989; Lazar *et al.*, 1990).

The comparison between AaE75 and *Drosophila* E75 also shows clear conservation of each of the three independent isoforms; the AaE75A, AaE75B and AaE75C-specific amino termini each show several clusters of conserved amino acids, resulting in overall identity of 31, 30 and 33% respectively. While evolutionary conservation of E75A- and E75B-specific sequences has been seen in *Manduca*, the conserved regions consisted of only a small number of amino acids within the much smaller amino terminal domains of the lepidopteran E75 proteins (Segraves and Woldin, 1993; Jindra *et al.*, 1994a; Palli *et al.*, 1997; Zhou *et al.*, 1998). In contrast, the AaE75A- and AaE75B-specific regions much more closely resemble those of the *Drosophila* proteins both in size and amino acid sequence. The AaE75C-specific region is also clearly conserved, and represents the first E75C isoform seen outside of *Drosophila*. These observations extend our knowledge of the evolutionary divergence of E75 isoforms and support the hypothesis that these represent evolutionarily conserved functionally independent forms of the E75 protein.

Each of the three *AaE75* transcription units is expressed in the fat body and ovary of vitellogenic female mosquitoes, and *in vitro* culture experiments indicate that *AaE75* is ecdysone-inducible in isolated fat body. These findings suggest that *E75*, which plays a critical role in the control of ecdysone response during *Drosophila* development, is also

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involved in the control of reproductive ecdysone response in the mosquito. The *AaE75* gene may be one of the key genes responsible for controlling downstream ecdysone signaling in the regulation of vitellogenic genes in the mosquito. Insofar, *E75* appears to be expressed and required for all ecdysone responses; it may thus be an integral part of an ecdysone response cascade used reiteratively in developmental and reproductive events governed by this hormone.

In the fat body (Fig. 6), *E75* transcripts exhibit a sharp rise immediately after the onset of vitellogenesis reaching a peak at 3 hr PBM when 20E titer shows a first small peak (Hagedorn *et al.*, 1975). In contrast, the YPP transcripts show only a small gradual increase during first 4 hr PBM. After the initial drop, levels of *AaE75* transcripts gradually increase again reaching second peak of their expression at the 24 hr PBM when 20E levels have passed their major peak, at 18 hr PBM (Hagedorn *et al.*, 1975). However, the second gradual increase in *AaE75* transcripts closely correlates with the accumulation of RNA for YPPs in the fat body, suggesting that *AaE75* may be involved in the indirect regulation of these genes by 20E (Fig. 6). Indeed, E75 binding sites have been found in the regulatory regions of mosquito *YPP* genes, *Vg* and *VCP* (D. Martin and A. Raikhel, unpublished observation).

While the transient early induction of *AaE75* may readily be explained in the context of *E75*'s role in *Drosophila* developmental ecdysone response hierarchies, the sustained later response during the period of peak vitellogenesis is less easily understood. Elucidating the conditions, which allow this sustained response of the *AaE75* gene will no doubt be critical to our understanding of the hormonal regulation of developmental events which require prolong expression of genes at a high level, such as vitellogenic

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genes in the mosquito fat body. Interestingly, AaE75B reinduction reaches a particularly high level. Recent studies of White *et al.* (1997), showing the role of E75B in attenuating ecdysone response, suggest that AaE75B may be acting here to help terminate some vitellogenic events in the mosquito fat body.

In vitro organ culture experiments have demonstrated that 20E induces transcripts corresponding to all three *AaE75* isoforms in the previtellogenic fat body. The 20E induction of the *AaE75* gene was independent of protein synthesis, thus demonstrating that *AaE75* acts as an early gene in ecdysone hierarchy, as previously seen for larval tissues in other insects (Segraves and Hogness, 1990; Jindra and Riddiford, 1996; Palli *et al.*, 1997; Zhou *et al.*, 1998). Unlike *Galleria* and *Manduca E75* (Jindra and Riddiford, 1996; Zhou *et al.*, 1998), in which E75A exhibited higher sensitivity to 20E than E75B, mosquito *E75* isoform transcripts show similar sensitivity to this hormone *in vitro*. Importantly, however, *AaE75* is ten-fold more sensitive to 20E induction than vitellogenic genes, *VCP* and *Vg* (Fig. 10). *AaE75* transcripts were induced by 20E at 10^{-7}M *vs.* 10^{-6}M required for *in vitro* induction of *VCP* and *Vg*, suggesting that *AaE75* may participate in activation of genes important for vitellogenic events preceding the high level of expression of *YPP* genes. This suggestion is agreement with the early time of the first *AaE75* expression peak in the fat body *in vivo*, when the transcripts for YPP genes are hardly detectable. Previously, a similar concentration-dependent pattern of activation of early and late chromosome puffs and genes by 20E has been observed in *Drosophila melanogaster* (Karim and Thummel, 1992; Russell and Ashburner, 1996), reflecting the requirement for a threshold level of early gene activation required for maximal induction of the late genes.

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As in the fat body, the highest level of *AaE75* expression in the ovary was reached by 24 hr PBM. More sensitive RT-PCR analysis indicates a much earlier detectable level of *AaE75*, as early as 1 hr PBM, in conjunction with the initial onset of ecdysone response. However, in contrast to the fat body, there was no evident early *AaE75* transcript peak in the ovary soon after the onset of vitellogenesis. In addition, AaE75A and AaE75B transcripts were present in the ovary at the high levels until 42 hr PBM, considerably longer than in the fat body. It is important to remember that unlike the fat body, which is relatively homogeneous tissue consisting predominantly of one cell type, the ovary contains both somatic and germ-line tissues which may have distinct *AaE75* requirements. Indeed, the kinetics of expression of the ecdysone-inducible genes, VgR the germ-line cells and VE in the follicular epithelium, are different. Thus, the expression patterns of *AaE75* transcripts in these ovarian tissues may differ as well.

The timing of *AaE75* expression within the ovary raises interesting questions about its position within the reproductive ecdysone response hierarchy. In *Drosophila* salivary glands, *E75* transcripts peak within approximately 2-4 hr, and then begin to decline as a result of negative autoregulation. One factor proposed to influence the timing of ecdysone response in *Drosophila* is the time required to transcribe the unusually long early genes, *E74, BR-C* and *E75* (Thummel, 1992). It also seems plausible that the full progression of vitellogenic ecdysone response may be most closely analogous to the series of ecdysone responses in the *Drosophila* third instar larva and prepupa, in which each ecdysone pulse results in the synthesis of factors required to set the stage for next (Hagedorn *et al.*, 1975), rather than to a single discrete ecdysone response.

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While these factors may contribute to the relatively slow appearance of high levels of *AaE75* transcripts, the broad, sustained response suggests that *AaE75* may be acting as an early-late in this system, such that it is further induced rather than repressed during the late ecdysone response. Hierarchically, the early and early-late genes are equivalent (Woodard *et al.*, 1994); however, the early-late genes are expressed over a broader time frame than the transiently expressed early genes which are subject to autoregulatory repression. Comparison between different tissues in the *Drosophila* larva suggests that there can be substantial differences in the kinetics with which various early gene isoform levels rise and fall (Huet *et al.*, 1993; 1995); presumably, these reflect differences in the temporal requirements of these genes for the regulation of target genes within each tissue. Comparison of *AaE75* expression to the expression of other genes active during the peak of vitellogenesis suggests that it may be required for the regulation of ecdysone induced genes, such as vitelline envelope protein, for a sustained period during oogenesis. The specific *AaE75* isoforms which might be involved in this process remain to be determined; although AaE75C expression is low in the ovary as a whole relative to fat body, the ovary consists of both somatic and germ line tissues which may have different *AaE75* requirements.

Despite these intriguing differences, the present findings suggest that elements of 20E-triggered regulatory hierarchies are used reiteratively during development and reproduction. Indeed, *E75* is thus far expressed and required for all characterized 20E responses; it may be an integral part of all or nearly all 20E-responsive regulatory hierarchies. Parallel genetic studies in *Drosophila* support the suggestion that reproductive and developmental 20E responses are controlled by closely related primary

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responses. Of the three transcription factor-encoding classical early genes, *E75, E74* and *BR-C*, each is involved in the regulation of oogenesis as well as development (Buszczak *et al.*, 1999). Remarkably, *E75* and *E74* appear to be involved in progression through a mid-oogenesis checkpoint, which may be analogous to the previtellogenic ovarian arrest in mosquitoes, suggesting that the fundamental mechanisms underlying the control of reproductive 20E response may be highly conserved. By combining the complementary strengths of *A. aegypti* physiological studies and *Drosophila* genetics, it should be possible to dissect the mechanisms of through which 20E and *E75* control critical aspects of insect reproduction, and to understand the ways in which these mechanisms have evolved.

ACKNOWLEDGMENTS

I thank William Pierceall and William Segraves at Yale University for cDNA and genomic cloning of *AaE75*, Avraham Biran for Northern blot. I also thank Judy Cole and Brad Dunlap for assistance in the isolation and analysis of genomic clones, Dr. Tom Sappington for critical reading of the manuscript, and Dr. H. Hagedorn (University of Arizona) for the gift of a mosquito vitelline envelope gene probe. This work was supported by a grant from the National Institutes of Health (AI36959) to A.S.R. and W.A.S. W.E.P was supported in part by the Seessel Anonymous Fund.

Chapter 4*

Cloning and Characterization

of the Mosquito Early-late Gene *HR3*

*Originally published in Mol. Cell. Endocrinol.

Ref: Kapitskaya, M. Z., Li, C.#, Miura, K., Segraves, W., and Raikhel, A. S. (2000).

Mol. Cell. Endocrinol. 160, 25-37.

# Underlined two authors contributed equally to this work.

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ABSTRACT

The insect steroid hormone, 20-hydroxyecdysone (20E), is a key factor controlling critical developmental events of embryogenesis, larval molting, metamorphosis, and, in some insects, reproduction. Our lab is interested in understanding the molecular basis of the steroid hormone ecdysone action in insect egg development. The yellow fever mosquito, *Aedes aegypti*, in addition to being an important vector of human diseases, represents an outstanding model for studying molecular mechanisms underlying egg maturation due to stringently controlled, blood meal-activated reproductive events in this insect. To elucidate the genetic regulatory hierarchy controlling the reproductive ecdysone response, ecdysone-regulated gene expression in vitellogenic mosquito ovaries and fat bodies has been investigated. It has been previously demonstrated the conservation of a primary ecdysone-triggered regulatory hierarchy, implicated in development of immature stages of *Drosophila*, represented by the ecdysone receptor/Ultraspiracle complex and an early gene *E75* during the reproductive ecdysone response (Chapter 2, 3). The present study demonstrates that conservation of the factors involved in the ecdysone-responsive genetic hierarchy regulating female reproduction extends beyond the early genes. Here, it is a report on *AHR3, a* highly conserved homologue of the *Drosophila HR3* early-late ecdysone-inducible gene in the mosquito. *AHR3* is expressed in both vitellogenic tissues of the female mosquito, the fat body and the ovary. The expression of *AHR3* correlates with the ecdysteroid titer, reaching a peak at 24 hr after a blood meal. Moreover, *in vitro* fat body culture experiments demonstrate that the kinetics and dose response of *AHR3* to 20E, an active ecdysteroid in the mosquito, is similar to those of the late vitellogenic genes rather

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than the early *E75* gene. However, as shown for other early and early-late genes, the 20E activation of *AHR3* is not inhibited by the presence of cycloheximide, a protein synthesis inhibitor. Taken together, these findings strongly suggest *AHR3* involvement in regulating the vitellogenic response to ecdysone in the adult mosquito.

INTRODUCTION

The insect steroid hormone 20-hydroxyecdysone (20E) regulates multiple developmental processes including embryogenesis, larval molting, metamorphosis and, in some insects, reproduction (Bownes, 1986; Riddiford, 1993a,b; Thummel, 1995). Analysis of the ecdysone effect on polytene chromosome puffing patterns in the late larval and prepupal salivary gland of *Drosophila* suggested that the initial activation of a small number of early ecdysone-inducible genes leads to subsequent inducement of a large number of late target genes (Ashburner *et al.*, 1974). Elucidation of this genetic hierarchy at the molecular level has led to the identification of the ecdysone receptor as a heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and the homologue of the retinoid X receptor, Ultraspiracle (USP) (Koelle *et al.*, 1991; Yao *et al.*, 1992; 1993). Furthermore, these studies have shown that the action of the ecdysone-receptor complex is indeed mediated by early genes such as the *BR-C, E74* and *E75* genes encoding transcription factors involved in regulation of late gene expression (Burtis *et al.*, 1990; Segraves and Hogness, 1990; Thummel *et al.*, 1990; Guay and Guild, 1991; DiBello *et al.*, 1991; Fletcher and Thummel, 1995). The ecdysone-mediated genetic regulatory network is further refined by the presence of a set of genes which are involved in setting up the timing and stage-specificity of gene activation by this genetic hierarchy

(Thummel, 1997). The orphan nuclear receptor *DHR3*, an early-late gene with induction characteristics including properties of both early and late genes, is one of the key genes in the ecdysone-mediated genetic regulatory network. Recent studies have revealed that during insect metamorphosis DHR3 has a dual role in repressing the early genes while activating another orphan nuclear receptor, *βFTZ-F1*, which in turn provides competence for the stage-specific late prepupal response to 20E (Woodard *et al*, 1994; Lam *et al*, 1997; White *et al*, 1997; Broadus *et al*., 1999). *DHR3* homologues cloned from several Lepidopteran insects are highly conserved and their expression suggests *HR3* involvement in mediating ecdysone response during Lepidopteran development and metamorphosis (Palli *et al*., 1992; 1996; Jindra *et al*., 1994b). Recent analysis of *Drosophila DHR3* mutants has revealed that *DHR3* function is, in addition, required to complete embryogenesis (Carney *et al*., 1997), prepupal-pupal transition and differentiation of adult structures during *Drosophila* metamorphosis (Lam *et al*., 1999).

In contrast to the wealth of information concerning the molecular basis of the ecdysone-mediated genetic hierarchy during development and metamorphosis, comparatively little is known about the ecdysone-regulated aspects of insect female reproduction, oogenesis and vitellogenesis. In *Drosophila*, ecdysone involvement in female reproduction has been suggested by the female sterility of mutations affecting ecdysone synthesis and ecdysone response (Garen *et al*., 1977; Oro *et al*., 1992), by the ecdysone-inducibility of yolk protein genes (Bownes, 1986) and most recently by studies implicating the ecdysone response hierarchy in egg chamber maturation (Buszczak *et al*., 1999). The role of ecdysone in female reproduction is best documented in anautogenous mosquitoes, especially the yellow fever mosquito, *Aedes aegypti*. In anautogenous

mosquitoes, a blood meal is required for egg maturation. Ingestion of blood stimulates the brain to secrete the ecdysiotropic neuropeptide, egg development neurosecretory hormone or ovarian ecdysteroidogenic hormone (Lea, 1967; 1972; Matsumoto *et al.*, 1989; Brown *et al.*, 1998). These hormones activate the ovary to produce ecdysone, which controls the synchronous progression of egg development in the ovary and the synthesis of yolk protein precursors by the fat body. In *A. aegypti*, ecdysteroid levels are relatively low during the first 8 hr post blood meal (PBM), with only a small peak at 4 hr. Later, the ecdysone level rapidly increases to the maximum at 18-24 hr PBM, then abruptly declines to the previtellogenic level in preparation for the termination of vitellogenesis at 30-32 hr PBM (Hagedorn, 1983; 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994). Action of 20-hydroxyecdysone on the mosquito yolk protein genes, vitellogenin (*Vg*) and vitellogenic carboxypeptidase (*VCP*), requires protein synthesis, suggesting indirect control by the genetic regulatory cascade involving early genes (Deitsch *et al.*, 1995a,b). In the mosquito ovary, established functions associated with ecdysone include the formation of the vitelline envelope (Raikhel and Lea, 1991; Lin *et al.*, 1993) and the separation of the secondary follicle in the ovary (Beckemeyer and Lea, 1980). Thus, the mosquito provides an ideal system to investigate control of ecdysone-induced gene expression during female reproduction. Furthermore, the critical role of anautogenous mosquitoes as disease vectors increases the significance of elucidating the ecdysone-mediated gene regulatory hierarchy, a potential target in the development of novel strategies for vector control.

In full agreement with the suggested role of ecdysone in regulating female reproduction, ecdysone receptor transcripts can be detected in pre- and vitellogenic

ovaries and fat bodies (Cho *et al.*, 1995). Transcripts encoding two different USP isoforms are differentially expressed in these tissues (Kapitskaya *et al.*, 1996; Wang *et al.*, 2000a). The mosquito EcR/USP heterodimer has been shown to bind to various ecdysone response elements (EcREs) to modulate ecdysone regulation of target genes (Wang *et al.*, 1998). Recently, the *A. aegypti* homologue of the *Drosophila E75* gene, a putative representative of the next level in the ecdysone response hierarchy, has been identified and characterized. *A. aegypti E75*, *AaE75*, is expressed in ovary and fat body following a blood meal. *AaE75* transcripts, corresponding to three E75 isoforms, are ecdysone-inducible in isolated fat body cultured *in vitro*. These findings suggest that *AaE75* mediates ovary and fat body ecdysone responses, and that ecdysone-triggered regulatory hierarchies such as those implicated in the initiation of metamorphosis may be reiteratively utilized in the control of the reproductive ecdysone response (Pierceall *et al.*, 1999).

The present study demonstrates that conservation of the factors involved in the ecdysone genetic hierarchy regulating female reproduction extends beyond the early genes. It is shown here that a highly conserved homologue of *DHR3* is expressed in the vitellogenic tissues of the female mosquito, the fat body and the ovary. The expression of the mosquito homologue, which is named *AHR3*, correlates with the titer of 20E, peaking at 24 hr PBM. Furthermore, 20E activates the *AHR3* transcript in the previtellogenic fat body *in vitro;* this activation is not inhibited by cycloheximide, a protein inhibitor, suggesting that *AHR3* is acting as an early-late gene in this system. Taken together, these findings strongly suggest *AHR3* involvement in regulating the vitellogenic response to ecdysone in the adult mosquito.

MATERIALS AND METHODS

Animals

Mosquitoes, *A.aegypti*, were reared according to Hays and Raikhel (1990). Larvae were fed on a standard diet as described before (Lea, 1964). Vitellogenesis was initiated by allowing females 3-5 days after eclosion to feed on an anesthetized white rat.

Materials

The RNA ladder was purchased form Life Technologies, Inc.; Sequenase was from U. S. Biochemical Corp.; restriction enzymes were from Boehringer Mannheim. All primers used in PCR were ordered from Gibco-BRL. Superscript II reverse transcriptase and Taq DNA polymerase were from Gibco-BRL; random hexamer primers from Promega. Perkin-Elmer was the source of reagents for the polymerase chain reaction (PCR). MSI CO supplied nitrocellulose-blotting membranes. Radionucleotides for labeling of probes and DNA sequencing were from NEN life Science Products. All other reagents were of analytical grade from Sigma or Baker.

Cloning, sequencing, and 5'RACE of cDNA

The cDNA fragment of *AHR3* was first obtained by PCR, for which degenerate primers were designed based on the sequences of its homologues in other insect species. Amplification was achieved in a Perkin Elmer thermal cycler using the template cDNA reverse-transcribed from 20 μg of total RNA prepared from the fat bodies of vitellogenic female mosquitoes. The PCR-generated fragment was used as a probe to screen a

λZAPII cDNA library, which was prepared from the fat bodies of vitellogenic female mosquitoes 6-48 hr post-blood meal (PBM) as previously reported (Cho and Raikhel, 1992). Several positive cDNA clones were subsequently isolated and sequenced in W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Analyses of nucleotide and deduced amino acid sequences were performed using the software of GCG (Genetics Computer Group, University of Wisconsin, Madison).

5' RACE (Rapid Amplification of cDNA Ends) was performed with a 5' RACE kit from GIBCO/BRL using the manufacturer's protocol. The first strand of cDNA for the mosquito *HR3* gene was prepared with the gene-specific primer HR3-SP1: ATTTGCGCTAACATGCTATCG and total RNA from post blood meal 24 hr fat bodies. After the first round PCR, the gene-specific primer HR3-SP2: CAGCCATTTCAAGTTCACTACG for *HR3* was used for the second round nested PCR. The PCR fragments positively confirmed by Southern Blot hybridization were then subcloned into pGEM-T-easy vectors (Promega) and sequenced.

Northern blot hybridization

Total RNA was isolated from mosquitoes of different stages and tissues using the guanidine isothiocyanate method as described previously (Bose and Raikhel, 1988). Polyadenylated mRNA was isolated using Niomag oligo $(dT)_{20}$ magnetic beads and the manufacturer's protocols (PerSeptive Diagnostics, Inc.). For Northern blot analysis, total or poly(A)$^+$ RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels in MOPS buffer, blotted to a nitrocellulose membrane, and hybridized with ^{32}P-labed DNA

probes under high stringency conditions. Autoradiography was conducted at -80°C using intensifying screens.

RT-PCR/Southern blot analyses

The temporal profile of transcript abundance of *HR3* in the mosquito fat body was examined by RT-PCR followed by Southern blotting with the gene-specific radioactive probe. Total RNA was prepared from fat bodies throughout the first vitellogenic cycle, as described in Northern Blot above, with the modification that all isopropanol precipitation steps were done without low-temperature incubation to avoid co-precipitation of glycogen and salt. RNA concentration was determined spectrophotometrically; Absorbance ratios, A_{260}/A_{280} and A_{260}/A_{230}, in RNA preparations were always above 1.7 and 2.0, respectively, quite consistent irrespective of the developmental stages of mosquitoes. Five microgram aliquots of each RNA preparation were reverse-transcribed by SuperscriptII reverse transcriptase (Gibco-BRL) and random hexamer (Promega) in a reaction volume of 20 μl. After the reaction, the volume of each sample was brought to 40 μl with TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 8.0). cDNA samples were stored at -20°C until use. In the case of mosquito tissues, 0.025 fat body- or pupae-equivalent cDNA pools were used as PCR templates. See Fig. 1 for the details of the primer design.

Thermal cycling conditions were as follows: the samples were incubated at 94°C for 3 min, followed by 18 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. As a reference to the developmental change of the fat body, the samples of the same cDNAs were subjected to the 10 cycles of PCR with a primer pair specific to the

mosquito vitellogenic carboxypeptidase; the reaction was followed by Southern blotting hybridization using a *VCP* specific probe generated by PCR using DNA from a *VCP* cDNA clone as a template (Cho *et al.*, 1991). The primers for *VCP* were: forward, 5'-AGCGCCCATTCTTGTTTGG-3' and reverse, 5'-CAGCTCATACAGGTATTCTCC-3'. RT-PCR/Southern blotting of the total RNA preparations without reverse transcriptase did not result in any appreciable signal, indicating that contamination of RNA preparations with genomic DNA fragments was virtually negligible (not shown). The radioactive blots were quantified with a phosphorimager (Molecular Dynamics).

In vitro fat body culture

To examine the effect of ecdysone on expression of the mosquito *HR3* gene, the abdominal walls with adhering fat bodies (hereafter referred to as the fat body) were dissected from 3-5-day-old previtellogenic females and incubated in an organ culture system as previously described (Raikhel *et al.*, 1997). The fat bodies were incubated with 20E either for 16 hr, or after 4hr with 20E followed by washing with the hormone-free medium 3 times and 12hr continuing incubation in the hormone-free medium. At every 4 hr interval, a sample of 9 fat bodies was collected and stored in liquid nitrogen until use. Total RNA was extracted as described in RT-PCR above. As a control, RNA was extracted from fat bodies incubated in the hormone-free medium. RT-PCR/Southern blot analyses were utilized as described above. Hybridization using probes for the ecdysone-inducible *VCP* gene was provided as a positive control.

To test the effect of the protein synthesis inhibitor cycloheximide (Chx) on transcription, dissected 3-5 day old female mosquito fat bodies were pretreated with

culture media containing 1mM Chx for 1 hr then further incubated with the same concentration of Chx either without or with 10^6 M 20E. The Chx/hormone withdrawal experiment was conducted as described for the hormone withdrawal experiment above except that 1mM Chx was added to all culture media.

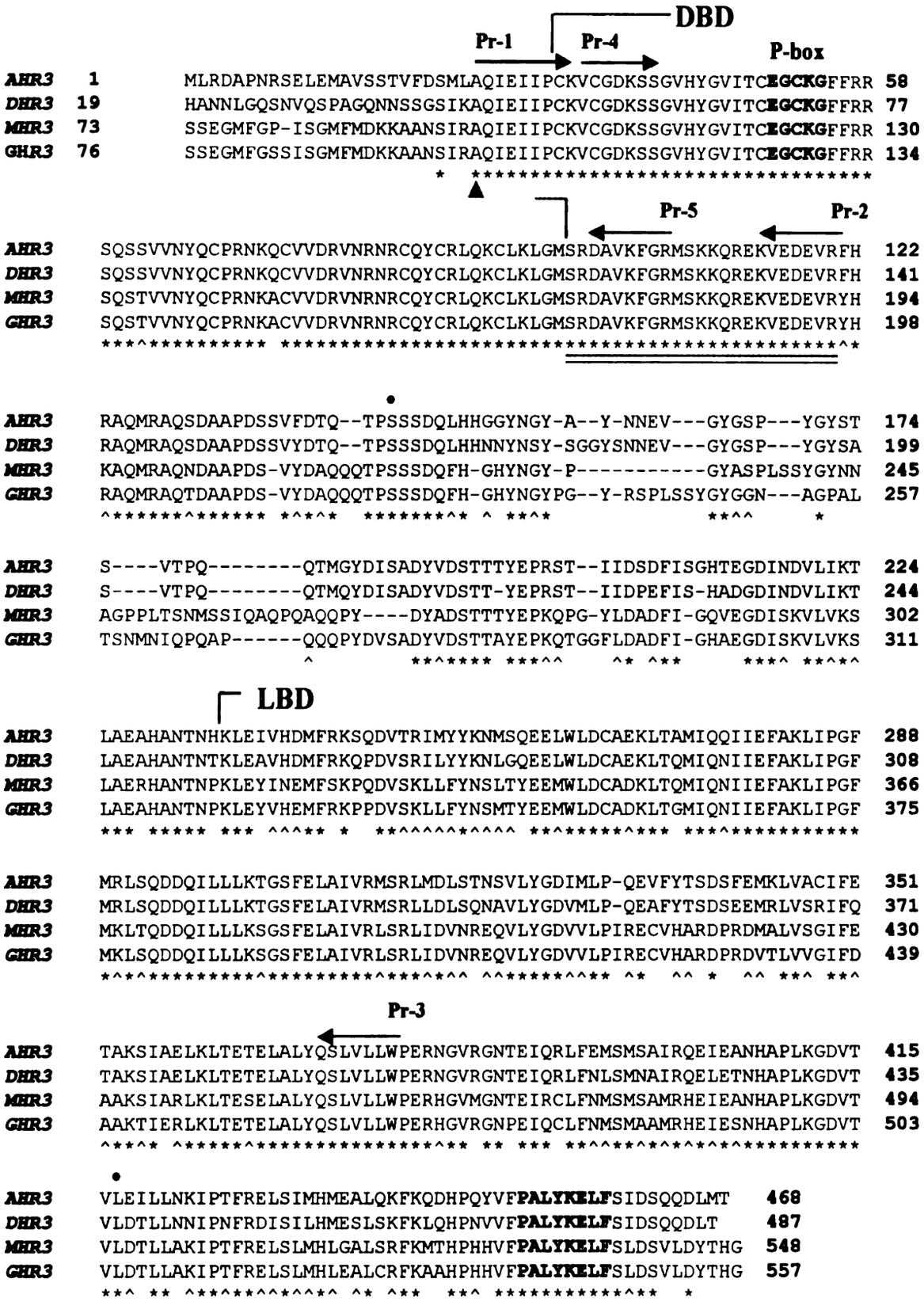
RESULTS

Isolation and characterization of a cDNA clone encoding the mosquito AHR3 nuclear receptor

To obtain a mosquito *HR3* clone, degenerate primers were designed, based on the amino acid comparison of its insect homologues. The sense primer Pr-1 conformed to the conserved sequence QIEIIPCK adjacent to the C (DNA-binding) domain; the antisense primer Pr-2 corresponded to the sequence EKVEDEVR from the conserved part of the D domain of these nuclear receptors (Fig. 1). After a mosquito PCR-fragment of the expected size, 285 bp, was sequenced and confirmed to encode a *DHR3*-related polypeptide, it was used as a probe to screen the mosquito fat body cDNA library (see Materials and Methods). The cDNA clone with the largest insert of 2.56 kb (designated as *AHR3* clone) was fully sequenced. This cDNA clone contained an open reading frame (ORF) of 1.42-kb and 3'-untranslated region (3'-UTR) of 1.14 kb. It did not have in-frame stop-codons at the 5'-end, indicating the ORF could potentially be truncated at the 5'-terminus; it also lacked a canonical site of polyadenylation and poly(A)-tail at the 3'-end.

In the isolated *AHR3* cDNA clone, there were 20 base pairs without any in-frame termination codon located upstream of potential translation initiation site ATG. In order

Fig.1. Alignment of the deduced amino acid sequence of the mosquito *AHR3* cDNA with its insect homologues: *DHR3* from *Drosophila* (Koelle *et al,* 1992), *MHR3* from *Manduca* (Palli *et al*, 1992), and *GHR3* from *Galleria* (Jindra *et al*, 1994b). Asterisks or hats indicate amino acids, which are identical or similar, respectively. The borders of DNA-binding domain (DBD) and putative ligand-binding domain (LBD) are marked with square braces. The P-box in DBD is in bold. The carboxy-terminal extension (CTE) of DBD, defined according to Giguere *et. al.* (1994), is double-underlined. The conserved 8-amino acid stretch at the C-termini of LBD is in bold. Only part of A/B-domains, adjacent to DBDs, are shown for DHR3, MHR3, and GHR3 (notice a high percentage of identical amino acids between lepidopteran MHR3 and GHR3). The vertical arrowhead marks a position from which a high similarity between different HR3 receptors starts. Horizontal double arrows indicate sequences corresponding to the degenerate sense primer (Pr-1) and antisense primer (Pr-2) used for *AHR3* cloning by PCR. An arrow shows the sequence-specific antisense PCR-primer Pr-3, and two black dots stand for two XhoI restriction sites of the *AHR3* cDNA, which were utilized to prepare a single-stranded DNA hybridization probe (see Fig. 3, 4). Two arrows indicate the sequence-specific sense (Pr-4) and antisense (Pr-5) primers used for RT-PCR analysis (see Fig. 5, 6, 7, 8). The sequence of the mosquito *AHR3* has been submitted to the GenBank (http://www.ncbi.nlm.nih.gov); its accession number is AF230281.


DBD
Pr-1
Pr-4
P-box
Pr-5
Pr-2
LBD
Pr-3
AHR3 1 MLRDAPNRSELEMAVSSTVFDSMLAQIEIIPCKVCGDKSSGVHYGVITCEGCKGFFRR 58
DHR3 19 HANNLGQSNVQSPAGQNNSSGSIKAQIEIIPCKVCGDKSSGVHYGVITCEGCKGFFRR 77
MHR3 73 SSEGMFGP-ISGMFMDKKAANSIRAQIEIIPCKVCGDKSSGVHYGVITCEGCKGFFRR 130
GHR3 76 SSEGMFGSSISGMFMDKKAANSIRAQIEIIPCKVCGDKSSGVHYGVITCEGCKGFFRR 134
AHR3 SQSSVVNYQCPRNKQCVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRFH 122
DHR3 SQSSVVNYQCPRNKQCVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRFH 141
MHR3 SQSTVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRYH 194
GHR3 SQSTVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRYH 198
AHR3 RAQMRAQSDAAPDSSVFDTQ--TPSSSDQLHHGGYNGY-A--Y-NNEV---GYGSP---YGYST 174
DHR3 RAQMRAQSDAAPDSSVYDTQ--TPSSSDQLHHNNYNSY-SGGYSNNEV---GYGSP---YGYSA 199
MHR3 KAQMRAQNDAAPDS-VYDAQQQTPSSSDQFH-GHYNGY-P----------GYASPLSSYGYNN 245
GHR3 RAQMRAQTDAAPDS-VYDAQQQTPSSSDQFH-GHYNGYPG--Y-RSPLSSYGYGGN---AGPAL 257
AHR3 S----VTPQ--------QTMGYDISADYVDSTTTYEPRST--IIDSDFISGHTEGDINDVLIKT 224
DHR3 S----VTPQ--------QTMQYDISADYVDSTT-YEPRST--IIDPEFIS-HADGDINDVLIKT 244
MHR3 AGPPLTSNMSSIQAQPQAQQPY----DYADSTTTYEPKQPG-YLDADFI-GQVEGDISKVLVKS 302
GHR3 TSNMNIQPQAP------QQQPYDVSADYVDSTTAYEPKQTGGFLDADFI-GHAEGDISKVLVKS 311
AHR3 LAEAHANTNHKLEIVHDMFRKSQDVTRIMYYKNMSQEELWLDCAEKLTAMIQQIIEFAKLIPGF 288
DHR3 LAEAHANTNTKLEAVHDMFRKQPDVSRILYYKNLGQEELWLDCAEKLTQMIQNIIEFAKLIPGF 308
MHR3 LAERHANTNPKLEYINEMFSKPQDVSKLLFYNSLTYEEMWLDCADKLTQMIQNIIEFAKLIPGF 366
GHR3 LAEAHANTNPKLEYVHEMFRKPPDVSKLLFYNSMTYEEMWLDCADKLTGMIQNIIEFAKLIPGF 375
AHR3 MRLSQDDQILLLKTGSFELAIVRMSRLMDLSTNSVLYGDIMLP-QEVFYTSDSFEMKLVACIFE 351
DHR3 MRLSQDDQILLLKTGSFELAIVRMSRLLDLSQNAVLYGDVMLP-QEAFYTSDSEEMRLVSRIFQ 371
MHR3 MKLTQDDQILLLKSGSFELAIVRLSRLIDVNREQVLYGDVVLPIRECVHARDPRDMALVSGIFE 430
GHR3 MKLSQDDQILLLKSGSFELAIVRLSRLIDVNREQVLYGDVVLPIRECVHARDPRDVTLVVGIFD 439
AHR3 TAKSIAELKLTETELALYQSLVLLWPERNGVRGNTEIQRLFEMSMSAIRQEIEANHAPLKGDVT 415
DHR3 TAKSIAELKLTETELALYQSLVLLWPERNGVRGNTEIQRLFNLSMNAIRQELETNHAPLKGDVT 435
MHR3 AAKSIARLKLTESELALYQSLVLLWPERHGVMGNTEIRCLFNMSMSAMRHEIEANHAPLKGDVT 494
GHR3 AAKTIERLKLTETELALYQSLVLLWPERHGVRGNPEIQCLFNMSMAAMRHEIESNHAPLKGDVT 503
AHR3 VLEILLNKIPTFRELSIMHMEALQKFKQDHPQYVFPALYKELFSIDSQQDLMT 468
DHR3 VLDTLLNNIPNFRDISILHMESLSKFKLQHPNVVFPALYKELFSIDSQQDLT 487
MHR3 VLDTLLAKIPTFRELSLMHLGALSRFKMTHPHHVFPALYKELFSLDSVLDYTHG 548
GHR3 VLDTLLAKIPTFRELSLMHLEALCRFKAAHPHHVFPALYKELFSLDSVLDYTHG 557

to identify the missing 5'-end of *AHR3* cDNA, 5'RACE PCR was carried out using total RNA from the fat body 24 hr post blood meal (PBM) as a template (see Materials and Methods). A 200-bp fragment was the major amplification product (data not shown). To verify the absence of genomic contamination, 5'RACE was performed under the same conditions except without superscript II reverse transcriptase. This control did not result in any amplification product, indicating that the 200-bp PCR fragment originated from mRNA. This fragment was subcloned into an pGEM-T-easy vector (Promega) and sequenced. It shared an expected 36-bp sequence overlapping with the 5'end of the *AHR3* cDNA clone. This observation confirmed that the 200-bp fragment was indeed a portion of the mosquito *HR3* cDNA. There were two in-frame termination codons, TAG and TGA, located at the 45 bp and 42 bp upstream of the initiation site ATG, suggesting that the 5' end of the *AHR3* ORF was complete.

Conceptual translation of the mosquito *HR3* clone showed that it exhibited a structural organization similar to that of other members in the HR3-subfamily of nuclear receptors (Figs.1 and 2). The most conserved regions were the DNA-binding domain (DBD) and so-called carboxy-terminal extension (CTE) of the DNA-binding domain (Figs.1 and 2). As illustrated in Fig.1 and Fig.2, the mosquito AHR3 had the P-box which shared 100%, 97% and 76% identical amino acids in the DBD, and 100%, 91% and 74% identical amino acids in CTE with *Drosophila* HR3, *Choristoneura* HR3 and human homologue RORα, respectively. This indicated that the AHR3 and other HR3 receptors could bind to similar target DNA-sites.

A putative LBD of AHR3 did not show high similarity with those of the nematode CeHR3 and human RORα counterparts, though it was still significantly conserved within

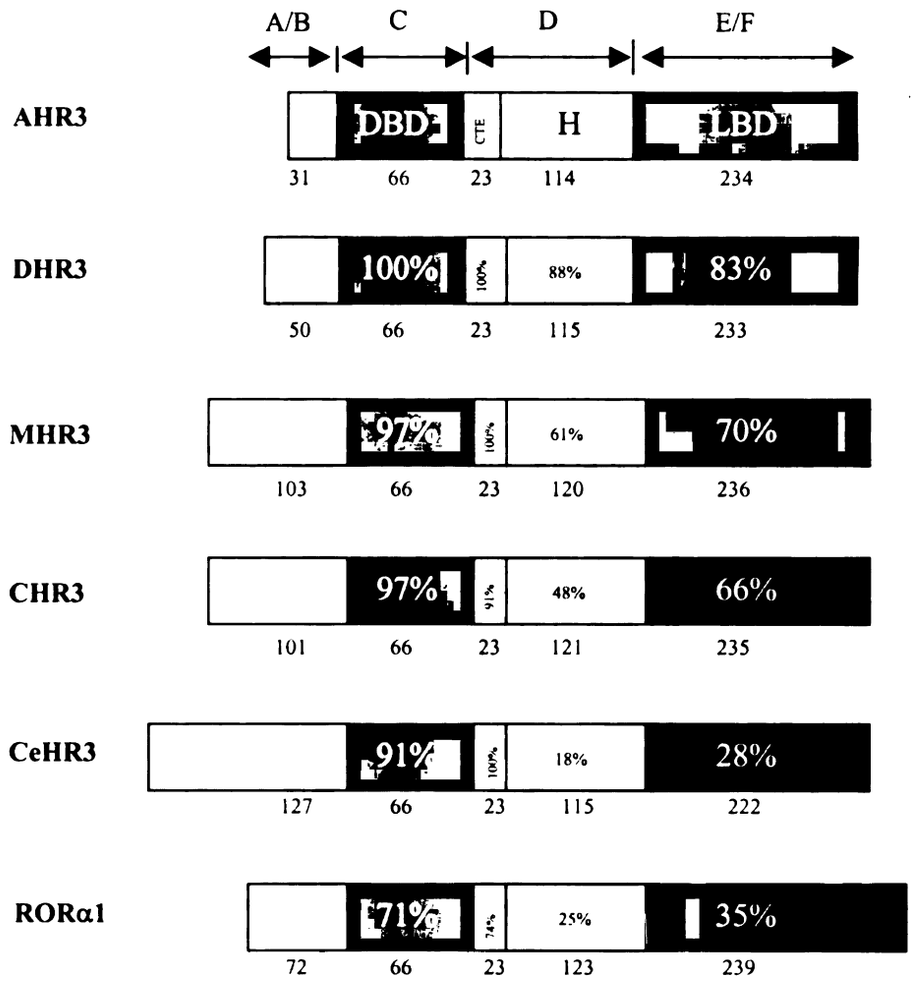


Fig. 2. Domain comparison of the mosquito HR3 with selected members of the ROR/HR3 subfamily of nuclear receptors. Shown are the HR3 nuclear receptors: AHR3 from *Aedes aegypti* (this study), DHR3 from *Drosophila* (Koelle *et al*, 1992), MHR3 from *Manduca* (Palli *et al*, 1992), CHR3 from *Choristoneura* (Palli *et al*, 1996), CeHR3 from nematode *Caenorhabditis elegans* (Kostrouch *et al*, 1995), and human RORα1 (Giguere *et al*, 1994). The letters above the mosquito HR3 protein denote functional domains. The D-domain is divided into the CTE (carboxy-terminal extension) and H (hinge) subdomains. Numbers below proteins indicate the number of amino acids in each subdomain. The percentages of identities between corresponding domains of AHR3 and other homologues are indicated within each domain; the ratios were calculated as a number of amino acids identical between comparative sequences divided by the total amino acid number of the shorter sequence.

insect LBDs (Fig. 2). Notably, there were 8 conserved amino acids in all members of the HR3 subfamily at the C-terminus of LBD (shown in bold in Fig. 1). They contained a stretch LYKELF similar to the AF-2 core motif (Beato *et al*, 1995), which strongly suggested the functional significance of this sequence. The high similarity between HR3s started in A/B-domains from the 7th amino acid preceding DBDs; however, the rest of the HR3 A/B domain was poorly conserved in all members of the insect HR3.

The AHR3 transcripts and the time course of AHR3 expression in the mosquito ovary and fat body during the first vitellogenic cycle

To determine the size of *AHR3* transcripts, Northern analysis was conducted for poly (A) RNA of the ovary and fat body isolated from females in the middle of the vitellogenic stage (24 hr PBM), when female mosquitoes were exposed to a high concentration of the steroid hormone 20E. Analysis of the ovarian RNA revealed the presence of a major 7.0 kb transcript, and two minor transcripts of 9.5 kb and 5.9 kb in size. In the vitellogenic fat body, the same three *AHR3* transcripts were observed. In contrast to the ovary, however, all three transcripts were expressed evenly in the fat body. Interestingly, in pupae only the major 7.0 kb and minor 9.5 kb transcripts were present (Fig. 3).

Northern blot analysis of *AHR3* RNA in the ovary from female mosquitoes taken at different stages of vitellogenesis showed a transcriptional pattern of *AHR3* with a prominent peak at 24-30 hr PBM, soon after a peak of 20E titer in the mosquito hemolymph (Fig. 4).

Due to the relatively low abundance of AHR3 mRNA, the RT-PCR analysis was used for elucidation of the *AHR3* mRNA kinetics in the female mosquito fat body during

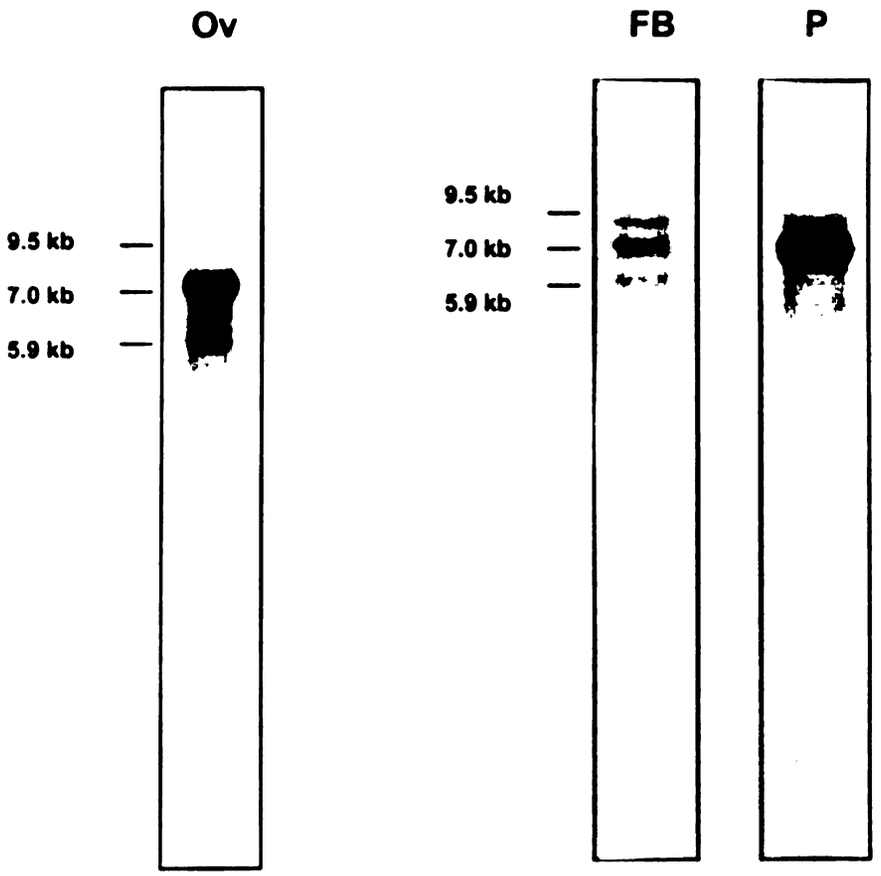


Fig. 3. *AHR3* transcripts in the mosquito *Aedes aegypti*. Northern blot analysis of poly(A) RNA extracted from ovaries (Ov) and fat bodies (FB) of female mosquitoes, and whole body pupae (P). A 0.82 kb XhoI-fragment of the *AHR3* cDNA (marked by two black dots in Fig. 1) was a template for a sequence-specific anti-sense primer Pr-3 (shown in Fig. 1) to prepare a hybridization probe by PCR. The resulting single-stranded DNA-probe of 0.68 kb corresponded to the C-terminal part of the hinge-subdomain and the N-terminal part of the LBD of the mosquito HR3.

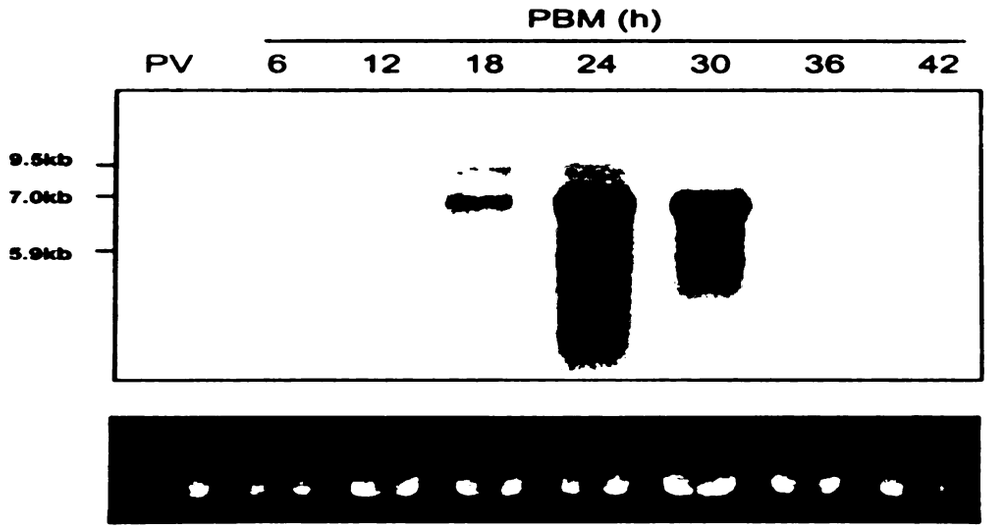


Fig. 4. Expression of *AHR3* in the mosquito ovary during the first vitellogenic cycle. Northern blot analysis of total RNA extracted from ovaries of female mosquitoes dissected before (PV) or after a blood meal (PBM) at the indicated times. Hybridization was performed as described in Fig. 3. The bottom lanes present ribosomal RNA in the same gel stained by ethidium bromide prior to blotting.

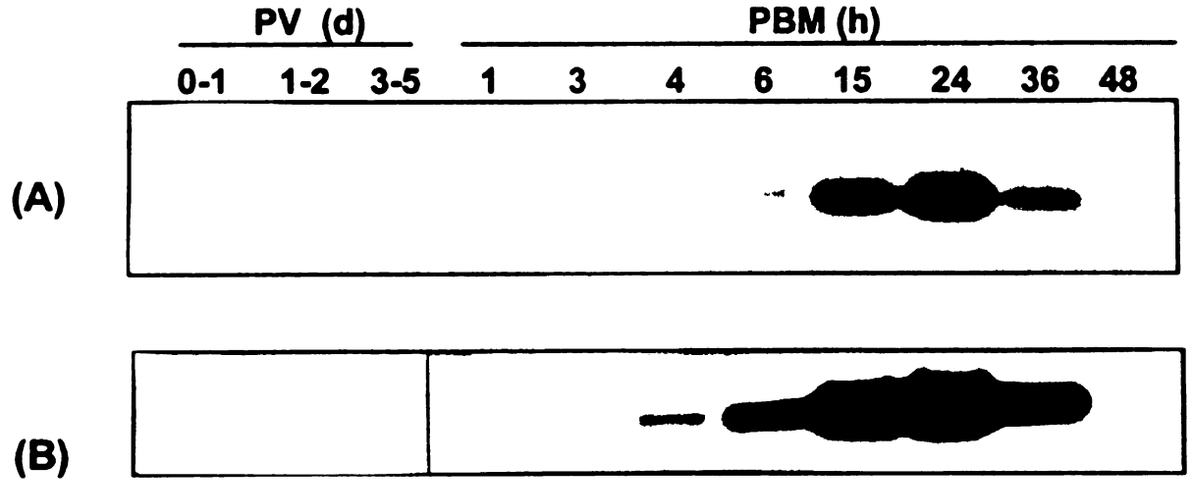


Fig. 5. Expression of *AHR3* in the mosquito fat body during the first vitellogenic cycle. Total RNA, extracted from mosquito fat bodies, was reverse transcribed and cDNA equivalents to 0.025 mosquito fat body were subjected to PCR, using primers specific to either *AHR3* or *VCP*. The resulting PCR-fragments were hybridized with ^{32}P-labeled gene-specific DNA-probes. (A) RT-PCR/Southern analysis of the *AHR3* expression. The 0.22 kb PCR-fragment was generated with the *AHR3*-specific primers Pr-4 and Pr-5 (shown in Fig. 1). (B) RT-PCR/Southern analysis of the *VCP* expression. *VCP* is a late gene regulated by 20E in the mosquito fat body (Deitsch *et al.*, 1995a).

the first vitellogenic cycle. *AHR3* mRNA was absent during the previtellogenic period, the transcript could be detected only after the onset of vitellogenesis triggered by a blood meal. A peak of *AHR3* expression was clearly seen at 24 hr PBM. Determination of the *VCP* mRNA levels in the same samples clearly showed that this expression of *AHR3* mRNA coincided with the peak expression of yolk protein genes (Fig. 5).

In vitro 20E induction of AHR3 mRNA in the female mosquito fat body

To determine whether the positive correlation between the *AHR3* expression and the peak of ecdysteroids observed *in vivo* reflected the involvement of 20E in regulation of expression of the *AHR3* gene, I investigated the patterns of *AHR3* transcript accumulation in *in vitro* cultured fat bodies in response to 20E. For these experiments, previtellogenic fat bodies of 3-5 days after eclosion were incubated in the culture medium in the presence or absence of the hormone. The RT-PCR analysis was used to monitor the transcript levels in the cultured tissues (see Materials and Methods). In the preliminary experiments it was found that the *AHR3* transcript, which was absent in the previtellogenic fat body, appeared after exposure of the tissue to 20E (not shown). In the next series of experiments, I determined the 20E concentration required for maximal activation of *AHR3* in the fat body by culturing this tissue with increasing concentrations of the hormone. Fat bodies from previtellogenic 3-5 day-old females were used to determine the background levels of transcripts for an early gene *E75*, a late gene *VCP*, a housekeeping gene *actin*, and the early-late gene under investigation, *AHR3*. Actin was expressed in detectable amounts in previtellogenic fat bodies. Culturing in the medium without the hormone did not result in any measurable increase in mRNA of all these genes (Fig. 6). The levels of actin mRNA remained relatively constant in the presence of

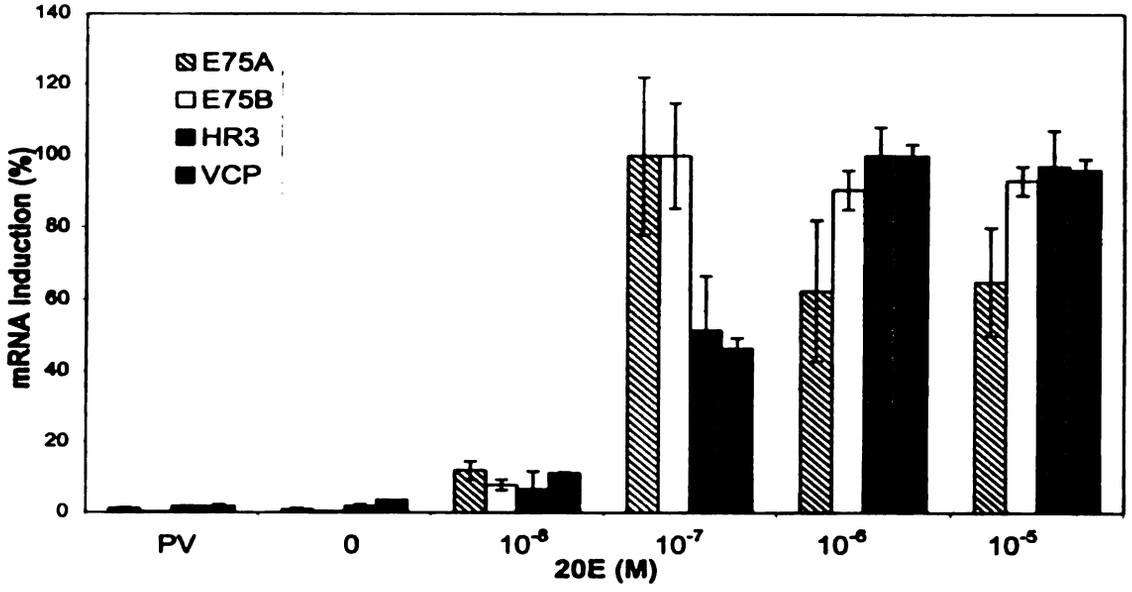


Fig. 6. *AHR3* response to 20-hydoxyecdysone (20E) in *in vitro* fat body organ culture. The transcriptional response of *AHR3* to 20E was examined in an *in vitro* culture system of female previtellogenic fat bodies (Deitsch *et al.*, 1995a; Raikhel *et al.*, 1997). Total RNA was extracted from untreated previtellogenic (PV) fat bodies, or from fat bodies incubated for 2 hr at the indicated 20E concentrations. RT-PCR/Southern blot hybridization was performed using probes specific for *AHR3* or each other gene (see Materials and Methods, and Fig. 1). *AaE75*A and *AaE75*B provided controls for an early gene, *VCP* for a late 20E-responsive gene. The results represent the average of three independent experiments ± S.D.

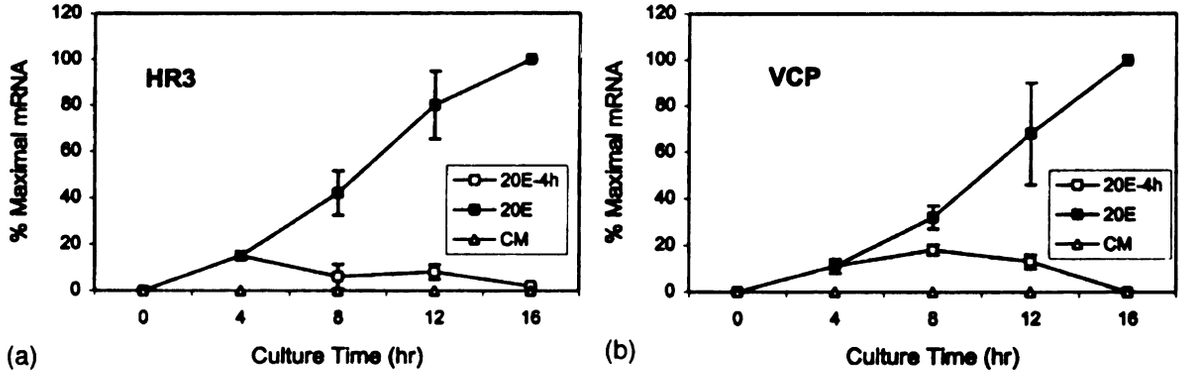


Fig.7. Time course of *AHR3* induction by 20E in *in vitro* fat body organ culture. Accumulation of *AHR3* mRNA (A) and *VCP* mRNA (B) during culturing in the hormone-free medium (CM), in the continuous presence of 10^{-6} M 20E (20E), or 4 hr exposure to 20E followed by culturing in the hormone-free medium (20E-4hr). RT-PCR/Southern blot hybridization was performed using probes specific for either *AHR3,* or *VCP* as a late 20E-responsive gene. The results represent the average of three independent experiments ± S.D.

every tested concentration of 20E (not shown). In contrast, *E75*, *HR3* and *VCP* genes responded to increasing concentrations of 20E in the culture medium by dramatic rise in their mRNA levels. As it has been previously established, the *VCP* mRNA reached its maximal levels at 10^{-6} M of 20E (Deitsch *et al.*, 1995a), while *E75A* and *E75B* mRNAs at 10^{-7} M of 20E (Pierceall *et al.*, 1999). The mRNA levels of *AHR3* increased in a dose-dependent manner in response to 20E. The *AHR3* transcript reached its maximal level at the 20E concentration of 10^{-6} M (Fig. 6).

Culturing the previtellogenic fat bodies in the hormone-free medium up to 16 hr did not induce *AHR3* and *VCP* mRNAs (Fig. 7). In contrast, *AHR3* mRNA appeared rapidly in the fat bodies cultured in the medium containing 10^{-6} M 20E. The levels of *AHR3* mRNA then gradually increased over the 16-hr culturing period and did not show any regression (Fig. 7A). The *VCP* mRNA induction profile was similar to that of *AHR3*, the levels of this transcript increased continuously throughout the entire 16 hr incubation with 10^{-6} M 20E (Fig. 7B). In the next experiment, the fat bodies were cultured in this hormone-containing medium for only 4 hrs, washed three times with the hormone-free medium and continued incubation in the hormone-free medium for 12 more hrs. Both *AHR3* and *VCP* mRNA levels increased after first the 4 hr of incubation, however, their levels decreased following withdrawal of the hormone from the medium. Interestingly, the *AHR3* mRNA level dropped more rapidly that that of *VCP* (Fig.7A and 7B).

To determine the requirement of protein synthesis for activation and maintenance by 20E of the *HR3* early-late gene in the mosquito fat body, the previtellogenic fat bodies were incubated with cycloheximide (Chx), a protein synthesis inhibitor which reversibly represses protein synthesis in this tissue in a dose-dependent manner (Deitsch *et al.*,

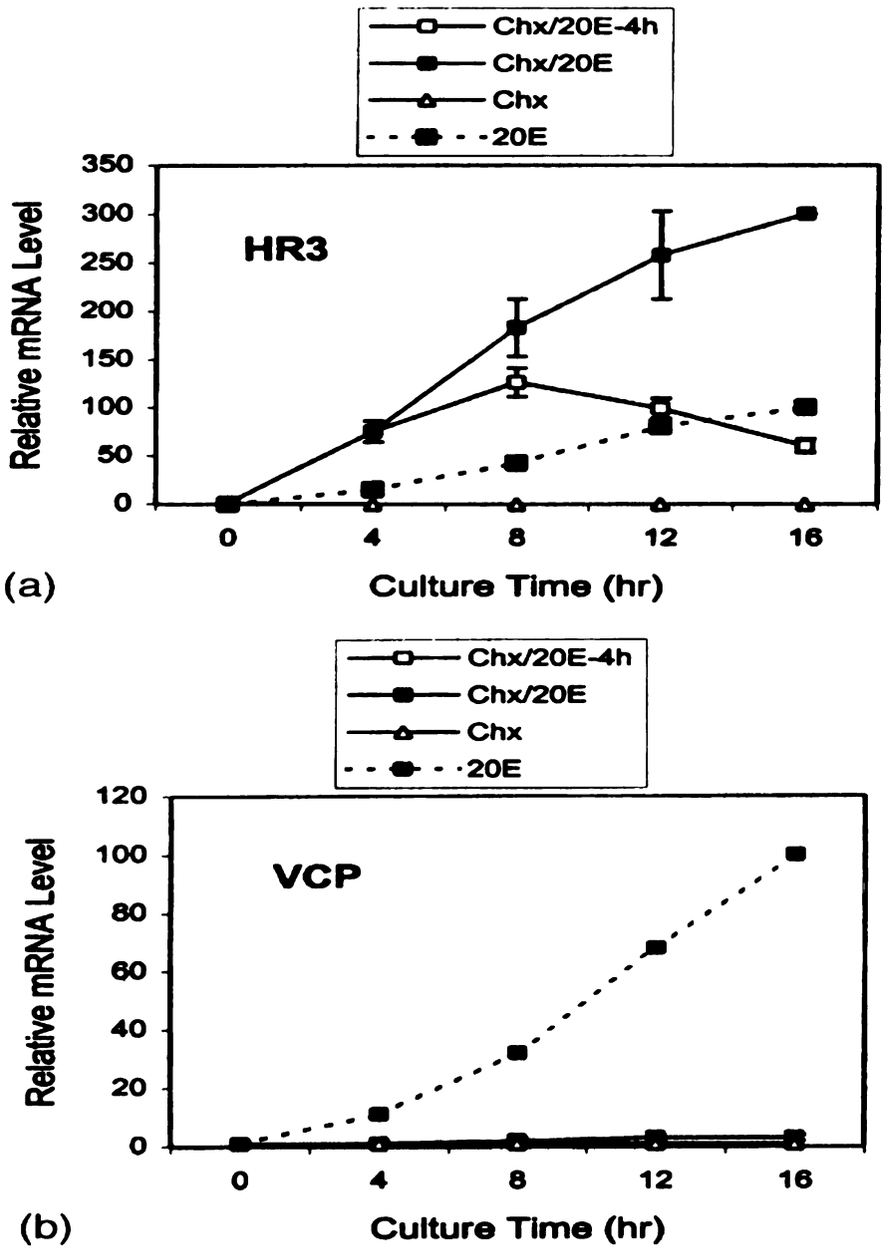


Fig. 8. Effect of cycloheximide on the *AHR3* induction by 20E in *in vitro* fat body organ culture. Accumulation of *AHR3* mRNA (A) and *VCP* mRNA (B) during culturing with 10^{-3} M Chx in the hormone-free medium (Chx), in the continuous presence of 10^{-6} M 20E (Chx/20E), or in the 4 hr exposure to 20E followed by culturing in the hormone-free medium (Chx/20E-4hr). Dashed lines in (A) and (B), marked (20E), are controls from the previous experiment (Fig. 7). RT-PCR/Southern blot hybridization was performed using probes specific for either *AHR3,* or *VCP* as a late 20E-responsive gene. The results represent the average of three independent experiments ± S.D.

1995a,b). The fat bodies were first pre-incubated in the culture medium for 1 hr in the presence of 10^{-3} M Chx, and then continued incubation with the same dose of Chx but with the addition of 10^{-6} M 20E for various times (Fig. 8). Incubation of the previtellogenic fat bodies in the presence of Chx in the hormone-free medium induced neither the *AHR3* gene nor *VCP*. Addition of 10^{-6} M 20E to the Chx-containing medium activated the *AHR3* gene in contrast to the *VCP* gene, which was inhibited by Chx. Furthermore, the *AHR3* gene was induced to a higher level by Chx/20E than by 20E alone. Likewise, withdrawal of 20E from the Chx-containing medium after the first 4 hrs of incubation, caused a slower decline in the *AHR3* mRNA level in fat bodies (Fig. 8A) than withdrawal of 20E from the Chx-free medium (Fig. 7A).

DISCUSSION

In this work, cloning and characterization of the mosquito nuclear receptor *HR3* (*AHR3*) is reported. Analysis of the *AHR3* deduced amino acid sequence showed that it was highly similar to its insect counterparts. The most conserved were the DNA-binding domain (DBD) and so-called carboxy-terminal extension (CTE) of the DNA-binding domain (Figs.1 and 2). Giguere *et al.* (1994, 1995) have performed a detailed analysis of RORα nuclear receptor and have shown that this human HR3 courterpart binds DNA as a monomer, recognizing an asymmetric response element (RORE) composed of two distinct half-sites, a 6 bp AT-rich sequence at the 5'-end and a PuGGTCA core half-site motif at the 3'-end (Giguere *et al*, 1994). The specificity of RORα binding to the RORE is provided by amino acids from the P-box and the CTE: the P-box recognizes the core half-site motif, and a group of amino acids from the CTE recognizes the AT-rich half-site (Giguere *et al*, 1995). Subsequently, the presence of similar DNA-sites has been shown

for insect HR3s from *Drosophila* (Lam *et al*, 1997; Horner *et al*, 1995) and *Choristoneura* (Palli *et al*, 1996). The mosquito AHR3 had a highly conserved P-box and CTE, suggesting that it likely recognized a similar target DNA-site as a monomer.

The ROR/HR3 transcription factors are classified as orphan nuclear receptors due to the lack of any known ligand (Mangelsdorf *et al*, 1995). The lack of ligand-dependent transactivation could lead to the relaxation in conservation of the orphan receptors' LBDs, which in nuclear receptors are generally responsible for dimerization and ligand-binding functions. This may be one reason why putative LBD of AHR3 did not show a high similarity with those of nematode CeHR3 and human RORα counterparts (Giguere *et al*, 1994; Kostrouch *et al*, 1995), though it was still significantly conserved among HR3 receptors (Fig. 2).

The high similarity between HR3s started in A/B-domains from the 7th amino acid preceding DBDs. Interestingly, an intron-exon junction (marked by arrowhead in Fig.1) has been determined at this position after the structure of *DHR3* and human *RORα* genes were characterized (Koelle *et al*, 1992; Giguere *et al*, 1994). The rest of the mosquito A/B domain was poorly conserved, as is consistent with the generally low conservation of highly variable nuclear receptor A/B domains. However, in the case of the ROR/HR3 subfamily, analysis of the A/B-domains merits special attention. In fact, cloning of three human RORα isoforms, which differ only in their A/B-domains, has revealed that the distinct A/B-domains modulate the DNA-binding specificity of each RORα isoform (Giguere *et al*, 1994; McBroom *et al* 1995). So far no similar *HR3* isoform variation has been demonstrated in insects. In this context, it is interesting to note that in lepidopteran HR3 receptors from *Manduca* (Palli *et al*, 1992), *Galleria*

(Jindra *et al*, 1994b), and *Choristoneura* (Palli *et al*, 1996), isolated from larval stages of these insects, the A/B-domains are not only similar in size but also possess a high percentage of identical amino acids (Palli *et al*, 1996). The mosquito *HR3* is the first *HR3* isolated from an adult insect. It had a distinct A/B-domain and might represent a distinct adult-specific *HR3* isoform.

Although, *HR3* was one of the first insect nuclear receptors to be cloned (Palli *et al*., 1992; Koelle *et al*., 1992), understanding of its role in regulating the ecdysone-mediated genetic network has been achieved only recently (Lam *et al*, 1997; White *et al*, 1997). These studies have demonstrated that the orphan nuclear receptor *DHR3* is one of the key genes in the ecdysone-mediated genetic regulatory network. During *Drosophila* metamorphosis, DHR3 has a dual role in repressing the early genes while activating another orphan nuclear receptor, *βFTZ-F1*, which has been implicated in providing competence for stage-specific responses to ecdysone (Lam *et al*, 1997; White *et al*, 1997). DHR3 homologues cloned from several Lepidopteran insects are highly conserved, and their expressions suggest HR3 involvement in mediating ecdysone response during Lepidopteran development and metamorphosis (Palli *et al*., 1992; 1996; Jindra *et al*., 1994b). Moreover, recent analysis of *Drosophila DHR3* mutants revealed that *DHR3* function is required to complete embryogenesis (Carney *et al*., 1997), prepupal-pupal transition and differentiation of adult structures during *Drosophila* metamorphosis (Lam *et al*., 1999). In this study, the novel evidence provided strongly suggests that the involvement of *HR3* in the ecdysone gene regulatory hierarchy during female reproduction.

In insects, transcription of the *HR3* gene is under the regulation of 20E (Horner *et al*, 1995; Palli *et al*, 1996; Jindra *et al*, 1994b; Palli *et al*, 1992). Expression analysis of the mosquito *HR3* concurs with the studies in other insects, which have previously shown the hallmarks of early-late gene expression, direct positive regulation of the *HR3* expression by 20E followed by a sustained expression during the late ecdysteroid response. The *in vivo* developmental profiles of *AHR3* in both fat body and ovary showed that the peak of the *AHR3* transcript coincided with a high titer of 20E in hemolymph. In *in vitro* cultured fat bodies, in contrast to the early gene *AaE75*, which showed rapid peak activation by 20E at 2~4 hr of culturing (Pierceall *et al.*, 1999), the *AHR3* induction slowly and continuously increase in its mRNA level up to 16 hr of culturing. This pattern of *AHR3* activation by 20E was consistent with observations on the successive puffing expression of the corresponding loci 75B (encoding *E75*) and 46F (encoding *DHR3*) in the polytene chromosomes (Ashburner, 1972; Walker and Ashburner, 1981), and with findings in cultured *Drosophila* organs (Horner *et al.*, 1995) and salivary glands (Huet *et al.*, 1995). Likewise, in the cultured silk glands of *Galleria mellonella,* 20E induction of *GHR3* mRNA has been observed 2 hr after the peak of *E75A* mRNA (Jindra and Riddiford, 1996). Our *in vitro* study suggested that delayed induction of the early-late *HR3* gene was due to the relatively low sensitivity of its maximal response to 20E, which was more similar to late genes rather than to early genes. It seems likely that the high level of 20E needed for maximal response is required to trigger the expression of factors that contribute to the sustained induction, presumably through elements in addition to the EcREs responsible for early expression. However,

like other early-late genes, *AHR3* is also directly regulated by 20E; in contrast to the late *VCP* gene, *AHR* can be activated by 20E in the absence of new protein synthesis.

Previously, I have described on the expression and function of the ecdysone receptor genes *AaEcR* and *AaUSP* and the induction of the early ecdysone-inducible gene *AaE75* in conjunction with the initiation of vitellogenic ecdysteroid response. Here, this study demonstrates that conservation of ecdysone responsive genes, originally elucidated in development of immature stages of *Drosophila*, extends to the next level in this hierarchy, to the early-late genes involved in the transition from the early to the late ecdysone response. The expression pattern of the early-late gene *AHR3* suggests that it may play a central role in mediating this transition during the vitellogenic response to ecdysone in the adult mosquito.

ACKNOWLEDGMENTS

I thank Marianna Kapitskaya for partial cloning of *AHR3* and Northern analysis, Ken Miura for RT-PCR analysis, and William Segraves for critical reading. This work was supported by a grant from the National Institutes of Health (AI36959).

Chapter 5*

Cloning and Characterization of the Mosquito *FTZ-F1* Gene

*Modified from the original publication in Dev. Biol.

Ref: Li, C., Kapitskaya, M. Z., Zhu, J., Miura, K., Segraves, W., Raikhel, A. S. (2000). *Dev. Biol.* 224, 96-110

ABSTRACT

In the mosquito *Aedes aegypti*, the adult female becomes competent for a vitellogenic response to ecdysone after previtellogenic development. Here, βFTZ-F1, the nuclear receptor implicated as a competence factor for stage-specific responses to ecdysone during *Drosophila* metamorphosis, serves a similar function during mosquito vitellogenesis. *AaFTZ-F1* is expressed highly in the mosquito fat body during pre- and post-vitellogenic periods when ecdysteroid titers are low. The mosquito *AaFTZ-F1* transcript nearly disappears in mid-vitellogenesis when ecdysteroid titers are high. An expression peak of *HR3*, a nuclear receptor implicated in the activation of *βFTZ-F1* in *Drosophila*, precedes each rise in mosquito *FTZ-F1* expression. In *in vitro* fat body culture, *AaFTZ-F1* expression is inhibited by 20-hydroxyecdysone (20E) and super-activated by its withdrawal. Following *in vitro AaFTZ-F1* super-activation, a secondary 20E challenge results in super-induction of the early *AaE75* gene and the late target *VCP* gene. Electrophoretic mobility shift assays show that the onset of ecdysone-response competence in the mosquito fat body is correlated with the appearance of the functional AaFTZ-F1 protein at the end of the previtellogenic development. These findings suggest that a conserved molecular mechanism for controlling stage specificity is reiteratively used during metamorphic and reproductive responses to ecdysone.

INTRODUCTION

Competence to respond to specific developmental signals allows the spatially and temporally restricted utilization of global signals within a multicellular organism. A signal molecule, which is spread throughout an organism via the circulatory system, elicits a specific response only from competent cells and tissues, leaving other parts of an organism unaffected. Recent studies have given rise to considerable insight into the molecular mechanisms underlying competence. One of the general means for accomplishing competence is the time- and cell-specific expression of a receptor or receptor cofactor needed to render a cell responsive to a corresponding hormonal signal (Shi *et al.*, 1996; Puigserver *et al.*, 1998). Regulation of competence can also be achieved through chromatin remodeling via involvement of linker histones, which repress transcription (Steinbach *et al.*, 1997; Lee and Archer, 1998). Finally, the stage-specificity of a response to a hormonal signal can be determined by a specialized competence factor. *Drosophila* metamorphosis provides one of the best-studied examples of how competence affects the specificity of response to a hormonal signal. In this organism, the βFTZ-F1 orphan nuclear receptor functions as a competence factor which directs the developmental transition from larval to pupal-specific gene expression in response to the insect steroid hormone ecdysone (Woodard *et al.*, 1994; Broadus *et al.*, 1999).

Mosquitoes serve as vectors for many harmful human diseases (Collins and Paskewitz, 1995; Butler *et al.*, 1997; Beier, 1998) because they require blood feeding for their egg development. In the so-called anautogenous mosquitoes, vitellogenesis and egg maturation are initiated only after a female mosquito ingests vertebrate blood. A blood

meal triggers a hormonal cascade resulting in production of 20-hydroxyecdysone (20E), the terminal signal, which activates yolk protein precursor (*YPP*) genes in the fat body (Hagedorn, 1983; 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994; Deitsch *et al.*, 1995a; 1995b).

In the anautogenous mosquito *Aedes aegypti*, which is being used as a model vector, vitellogenesis proceeds through two periods (Fig. 1). The approximately 3-day-long previtellogenic development of both the ovary and fat body proceeds under the control of juvenile hormone (JH) III (Gwartz and Spielman, 1973; Flannagan and Hagedorn, 1977). The JH titers are high during the previtellogenic period and drop immediately following the onset of vitellogenesis, which is initiated when the female mosquito takes a blood meal (Shapiro *et al.*, 1986). The fat body then produces yolk protein precursors (YPP), which are internalized by the developing oocytes (Raikhel, 1992). In addition to vitellogenin (Vg), the major YPP, the mosquito fat body synthesizes two other YPPs, vitellogenic carboxypeptidase (VCP) and vitellogenic cathepsin B (VCB) (Cho *et al.*, 1991; 1999). YPP synthesis reaches its maximal levels at 24 hr post-blood meal (PBM) and then proceeds until 30-32 hr PBM, when it is rapidly terminated (Raikhel, 1992).

The titers of ecdysteroid in the mosquito female hemolymph are correlated with the rate of YPP synthesis in the fat body (Fig. 1). While the 20E titers are only slightly elevated at 4 hr PBM, they begin to rise sharply at 6-8 hr PBM, and reach their maximum levels at 18-24 hr PBM (Hagedorn *et al.*, 1975). Consistent with the initially proposed role of 20E in activating vitellogenesis in anautogenous mosquitoes (Hagedorn *et al.*, 1973; Hagedorn and Fallon, 1973), experiments using an *in vitro* fat body culture have

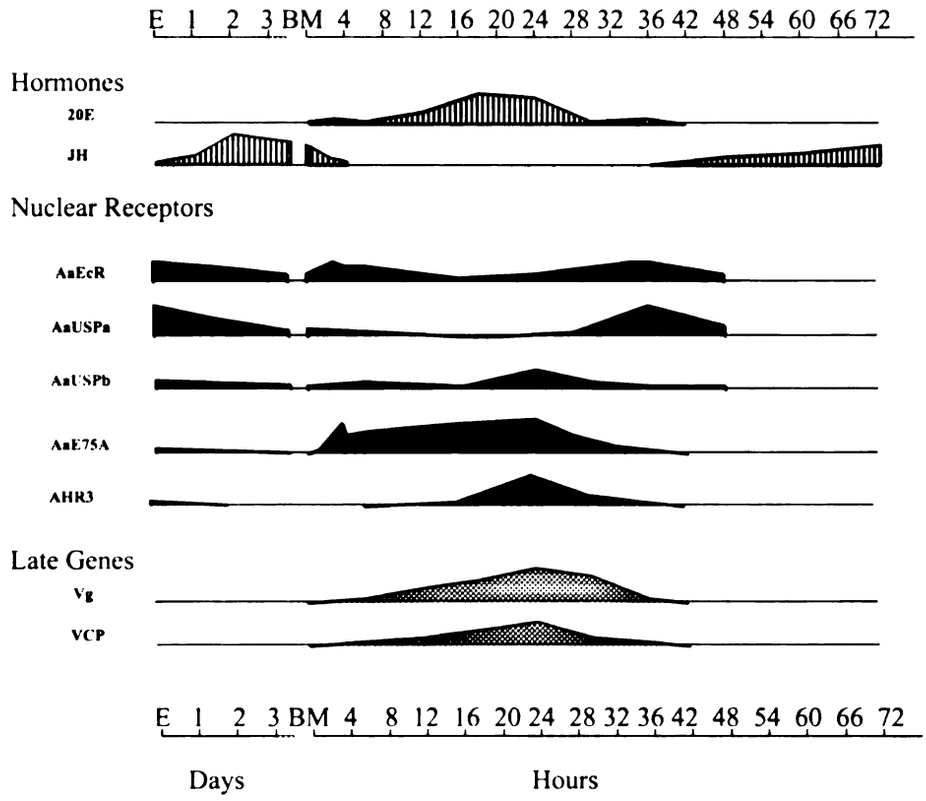


Fig. 1. Summary of events during the first cycle of vitellogenesis in the anautogenous mosquito, *Aedes aegypti*. The previtellogenic period begins at eclosion (E) of the adult female. During first ~2-3 days of post-eclosion life, the fat body becomes competent for subsequent vitellogenesis. The female then enters a state-of-arrest; yolk protein precursors, vitellogenin (Vg) and vitellogenic carboxypeptidase (VCP) are not synthesized during this previtellogenic period. Only when the female takes a blood meal (BM), vitellogenesis is initiated. Hormones: hormonal titers of juvenile hormone (JH) and ecdysteroids (20E) in *A. aegypti* females (Hagedorn *et al.*, 1975; Shapiro and Hagedorn, 1982). Transcript profiles of nuclear receptors (Nuclear Receptors) in the mosquito fat body were determined by RT-PCR/Southern blot; Transcript profiles of yolk protein precursors (Late Genes) in the mosquito fat body were determined by Northern blot and RT-PCR/Southern blot. AaEcR, ecdysone receptor; AaUSPa, Ultraspiracle isoform A; AaUSPb, Ultraspiracle isoform B (Wang *et al.*, 2000); AaE75A, early gene *AaE75* isoform A (Pierceall *et al.*, 1999); AHR3, early-late gene *AHR3* (Kapitskaya *et al.*, 2000); Vg, vitellogenin (Cho and Raikhel, 1992); VCP, vitellogenic carboxypeptidase (Cho *et al.*, 1991).

shown that physiological doses of 20E (10^{-7} - 10^{-6} M) activate two *YPP* genes, *Vg* and *VCP* (Deitsch *et al.*, 1995a; 1995b). Utilization of the protein synthesis inhibitor, cycloheximide (Chx), has demonstrated that the activation of *YPP* genes in the mosquito fat body requires protein synthesis (Deitsch *et al.*, 1995a). Thus, it is likely that a regulatory cascade similar to that seen in *Drosophila* development mediates the action of 20E in the mosquito fat body.

The functional ecdysteroid receptor is a heterodimer of the ecdysone receptor (EcR) and a retinoid X receptor (RXR) homolog, Ultraspiracle (USP) (Yao *et al.*, 1992; 1993). In the mosquito *Aedes aegypti*, one isoform of ecdysone receptor (*AaEcR*) and two *USP* isoforms, *AaUSP-A* and *AaUSP-B* have been cloned (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996). The mosquito EcR/USP heterodimer has been shown to bind to various ecdysone response elements (EcREs) to modulate ecdysone regulation of target genes (Wang *et al.*, 1998). A mosquito homolog of the *Drosophila* early gene *E75* is involved in mediating the ecdysteroid response during vitellogenesis (Pierceall *et al.*, 1999). Furthermore, a highly conserved homolog of the DHR3 orphan nuclear receptor previously implicated in activation of *Drosophila βFTZ-F1* is expressed in vitellogenic tissues, the fat body and ovary (Kapitskaya *et al.*, 2000).

A preparatory, developmental, previtellogenic period is required for the mosquito fat body to attain competence for ecdysone responsiveness (Sappington and Raikhel, 1999). Flannagan and Hagedorn (1977) have demonstrated that the acquisition of competence for ecdysone response in the mosquito fat body is under the control of juvenile hormone III. However, the molecular mechanisms underlying the acquisition of sensitivity to ecdysone in the mosquito fat body remain obscure.

Here this chapter reports that βFTZ-F1, the orphan nuclear factor implicated as a competence factor for stage-specific responses to ecdysone during *Drosophila* metamorphosis, likely serves a similar function during mosquito vitellogenesis. A homolog of *Drosophila βFTZ-F1* is expressed relatively highly in the mosquito fat body during pre- and post-vitellogenic periods when ecdysteroid titers are low. In *in vitro* fat body culture, *AaFTZ-F1* expression is inhibited by 20E and super-activated by its withdrawal. Moreover, after the *in vitro* super-activation of *AaFTZ-F1* expression in the previtellogenic mosquito fat body, a secondary 20E challenge results in super-induction of the early *AaE75* gene and the late target *VCP* gene. Although *AaFTZ-F1* mRNA is abundant even in newly emerged mosquitoes, electrophoretic mobility shift assays show that it is the appearance of functional AaFTZ-F1 protein that correlates with the onset of ecdysone-response competence in the mosquito fat body. Our findings suggest that the regulation and function of FTZ-F1 during mosquito reproduction closely resemble those shown at the onset of *Drosophila* metamorphosis, and that FTZ-F1 may therefore be part of a conserved and broadly utilized molecular mechanism controlling the stage specificity of ecdysone responses.

MATERIALS AND METHODS

Animals

Mosquitoes, *Aedes aegypti*, were reared according to the method of Hays and Raikhel (1990). Larvae were fed on a standard diet as previously described (Lea, 1964). Vitellogenesis was initiated by allowing females, 3-5 days after eclosion, to feed on an anesthetized white rat.

Materials

The RNA ladder was purchased from Gibco-BRL; Sequenase, from U. S. Biochemical Corp.; restriction enzymes, from Boehringer Mannheim. All primers used in PCR were ordered from Gibco-BRL. Superscript II reverse transcriptase and Taq DNA polymerase were from Gibco-BRL; random hexamer primers, from Promega. Perkin-Elmer was the source of reagents for the polymerase chain reaction (PCR). MSI CO and Amersham Pharmacia Biotech supplied nitrocellulose and Hybond-N^+ blotting membranes, respectively. Radionucleotides for labeling probes and DNA sequencing were from NEN Life Science Products. All other reagents were of an analytical grade from Sigma or Baker.

cDNA cloning

Degenerate primers based on the sequences of *FTZ-F1* insect homologs were used for PCR to obtain a mosquito homolog cDNA fragment. Amplification was carried out in a Perkin Elmer thermal cycler using template cDNA produced by reverse transcription from total RNA prepared from the fat bodies of vitellogenic female mosquitoes. The PCR-generated fragment was used as a probe to screen a λZAPII cDNA library prepared from the fat bodies of vitellogenic female mosquitoes during 6-48 hr post-blood meal (PBM) as previously reported (Cho and Raikhel, 1992). Several positive cDNA clones were subsequently isolated and sequenced at W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University. Analyses of nucleotide and deduced amino acid

sequences were performed using the software of GCG (Genetics Computer Group, University of Wisconsin, Madison).

Northern blot analysis

Total RNA was isolated by using the guanidine isothiocyanate method as described previously (Bose and Raikhel, 1988), or using TRIZOL Reagent (Gibcol-BRL). Polyadenylated mRNA was isolated using Niomag oligo $(dT)_{20}$ magnetic beads and the manufacturer's protocols (PerSeptive Diagnostics, Inc.). For Northern blot analysis, total or poly(A) RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels in MOPS buffer, blotted to nitrocellulose membranes, and hybridized with ^{32}P-labeled DNA probes (Random Primers DNA Labeling Systems, Gibco-BRL) under high stringency conditions. Autoradiography was conducted at -80°C with intensifying screens.

RT-PCR/Southern blot

RT-PCR/Southern analyses were performed basically as previously described (Pierceall *et al.*, 1999). Total RNA was prepared from fat bodies throughout the first vitellogenic cycle, as described in the Northern blot procedure above, with the modification that all isopropanol precipitation steps were done without low-temperature incubation, to avoid co-precipitation of glycogen and salt. RNA concentration was determined spectrophotometrically; absorbance ratios, A_{260}/A_{280} and A_{260}/A_{230}, in RNA preparations were always above 1.7 and 2.0, respectively. Five microgram aliquots of each RNA preparation were reverse-transcribed by Superscript II reverse transcriptase

(Gibco-BRL) and random hexamer (Promega) in a reaction volume of 20 μl. After this reaction, the volume of each sample was brought to 40 μl with TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 8.0). cDNA samples were stored at -20°C until use. In the analyses of developmental profiles, 0.025 fat body- or pupa-equivalent cDNA pool was used as a PCR template for each point. Thermal cycling conditions were as follows: 94°C for 3 min, followed by 18 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. As a reference to the developmental change of the fat body, the samples of the same cDNAs were subjected to the 13 cycles of PCR with a primer pair specific to the mosquito vitellogenic carboxypeptidase (*VCP*). The reaction was followed by Southern blot hybridization using a *VCP* specific probe, which was generated by PCR using cloned *VCP* cDNA as a template (Cho *et al.*, 1991). The PCR primers for each gene examined in this paper were listed in Table 1.

Table 1

Primers	Forward	Reverse
AaE75A	TAGTGCAATCAACGTATACCAATC	GGCACACGACCGAATCTGAC
AaE75B	CGTGGAAGAAGACCACGATCG	GGCACACGACCGAATCTGAC
AHR3	GTGTGCGGCGACAAGTCGTC	TCGACCGAATTTGACCGCGTC
AaFTZ-F1	TGAAGGTGGACGACCAGATGAA	TGTTCTGCAGCTCGTTGAAGTG
VCP	AGCGCCCATTCTTGTTTGG	CAGCTCATACAGGTATTCTCC
Vg	TACTTGAAGACAAGATGCTAGCG	CGTTCTTGTAACTGTAGCCTTG

RT-PCR/Southern blotting of total RNA preparations without reverse transcriptase did not result in any appreciable signal, indicating that contamination of RNA preparations with genomic DNA fragments was virtually negligible (not shown). As a quantitative control for the RT-PCR, each cDNA was diluted to 1/2, 1/4, 1/8, and 1/16, and PCR amplifications of the cDNA dilutions were monitored by different PCR cycles ranging from 13 to 22 cycles followed by Southern blot quantification. The analysis of normalized results showed that RT-PCR amplifications were linear (not shown). To further test whether results of the RT-PCR/Southern analyses correlate with those from the Northern blot analysis, both of these techniques were used in parallel to measure the *VCP* mRNA levels in fat bodies during the vitellogenic cycle as well as over the course of *in vitro* treatments with 20-hydroxyecdysone. *VCP* mRNA levels were analyzed by either Northern blot or RT-PCR/Southern blot analyses. In both cases, the results were quantified using a phosphorimager (Molecular Dynamics). The Northern blot and RT-PCR/Southern blot analyses yielded very similar results (data not shown).

In vitro organ culture

The abdominal walls with adhering fat bodies (hereafter referred to as the fat body) were dissected from 0~0.5-day or 3~5-day-old previtellogenic females and incubated in an organ culture system as previously described (Raikhel *et al.*, 1997). For the stage-specific responses to 20E, the dissected fat bodies were exposed to 10^{-6} M 20E for 6 hr; for all other cultures, the fat bodies were incubated with 20E either for 16 hr, or for 4 hr with 20E followed by washing three times with hormone-free medium and an additional 12 hr incubation in hormone-free medium. At every 4 hr interval, a sample of

nine fat bodies was collected and stored in liquid nitrogen until use. As a control, the fat bodies were incubated in hormone-free medium. RT-PCR/Southern blot analyses were utilized as described above. Hybridization using probes for the ecdysone-inducible *VCP* gene was provided as a positive control.

To test the effect of the protein synthesis inhibitor cycloheximide (Chx) on the expression of each gene, dissected fat bodies from 3-5 day old female mosquitoes were pre-treated with culture medium containing 10^{-4} M Chx for 1 hr, then further incubated with the same concentration of Chx either with or without 10^{-6} M 20E. The Chx/hormone withdrawal experiment was conducted as described previously in the hormone withdrawal experiment above except that 10^{-4} M Chx was added to all culture media.

The experiments of secondary responses to 20E or Chx/20E in cultured fat bodies were an extension of the 20E-withdrawal experiments described above. After the initial 4 hr exposure to 20E followed by 12 hr culturing without 20E, the fat bodies were exposed again to 20E, or pre-treated with Chx for 1 hr and then exposed to 20E/Chx for an additional 4 or 16 hr. In addition to these two experiments, as the third experiment, after pre-treatment with Chx for 1 hr and the initial 4 hr exposure to 20E/Chx followed by 12 hr culturing with Chx only, the fat bodies were exposed again to 20E/Chx for an additional 4 or 16 hr. RT-PCR/Southern blot were utilized to analyze the responses of each gene under investigation.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were carried out as described previously (Wang *et al.*, 1998). The nucleotide sequence of the F1RE used in EMSA is 5'GCAGCACCGTCTCAAGGTCGCCGAGTAGGAGAA3' (Ohno *et al.*, 1994). The F1RE oligo was purified before EMSA. The *in vitro* synthesized AaFTZ-F1 protein was made by the TNT system (Promega). Fat body nuclear extracts (FBNE) were prepared as previously described (Miura *et al.*, 1999). The DmFTZ-F1 antibody was a generous gift from Dr. Carl Thummel (University of Utah).

RESULTS

Ecdysone-responsive vitellogenic competence in the mosquito fat body

Previously, Flanagan and Hagedorn (1977) have shown that the fat body of newly emerged female mosquitoes is not responsive to 20E *in vitro* with respect to vitellogenin protein synthesis. However, responsiveness to this hormone can be detected in the fat body as early as 12 hr post-eclosion. Furthermore, the levels of the 20E-activated Vg synthesis in the fat body *in vitro* reach its maximum in 36-48-hr-old mosquitoes. Here, I utilized the fat body from mosquitoes of different post-eclosion ages and monitored the appearance of mRNAs for two *YPP* genes, *Vg* and *VCP*, in response to 20E *in vitro*. These experiments confirmed that the fat body of a newly emerged mosquito was not competent to respond to hormonal stimulation and that the *YPP* genes were not activated (Fig. 2). In contrast, both *Vg* and *VCP* genes were expressed in the fat body of 3-5 day old females treated with 10^{-6} M 20E *in vitro* (Fig. 2). Moreover, the RT-PCR analysis of both *Vg* and *VCP* mRNAs showed that the ability of the *YPP* genes to respond to

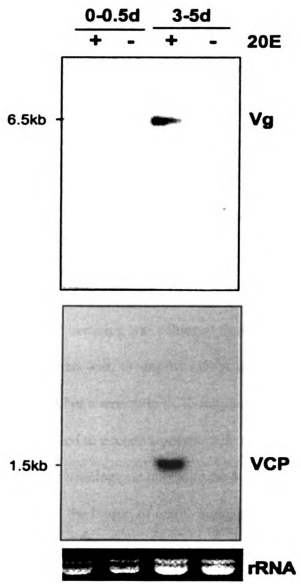


Fig. 2. Northern blot analysis of stage-specific induction of the vitellogenin (Vg) and vitellogenic carboxypeptidase (VCP) transcripts by the hormone 20E. Nine previtellogenic fat bodies of different stages were cultured in the presence (+) or absence (-) of 10^{-6} M 20E for 6 hr. Total RNA from 3 fat bodies (~10 μg) was loaded in each lane. Hybridization was performed with the ^{32}P- labeled *Vg* or *VCP* probe (see Materials and Methods). Ribosomal RNA (rRNA) stained with ethidium bromide is shown as a loading control. 0-0.5d: fat bodies from newly eclosed previtellogenic females, 0-12 hr post-eclosion; 3-5d: fat bodies from previtellogenic females, 3-5 day post-eclosion.

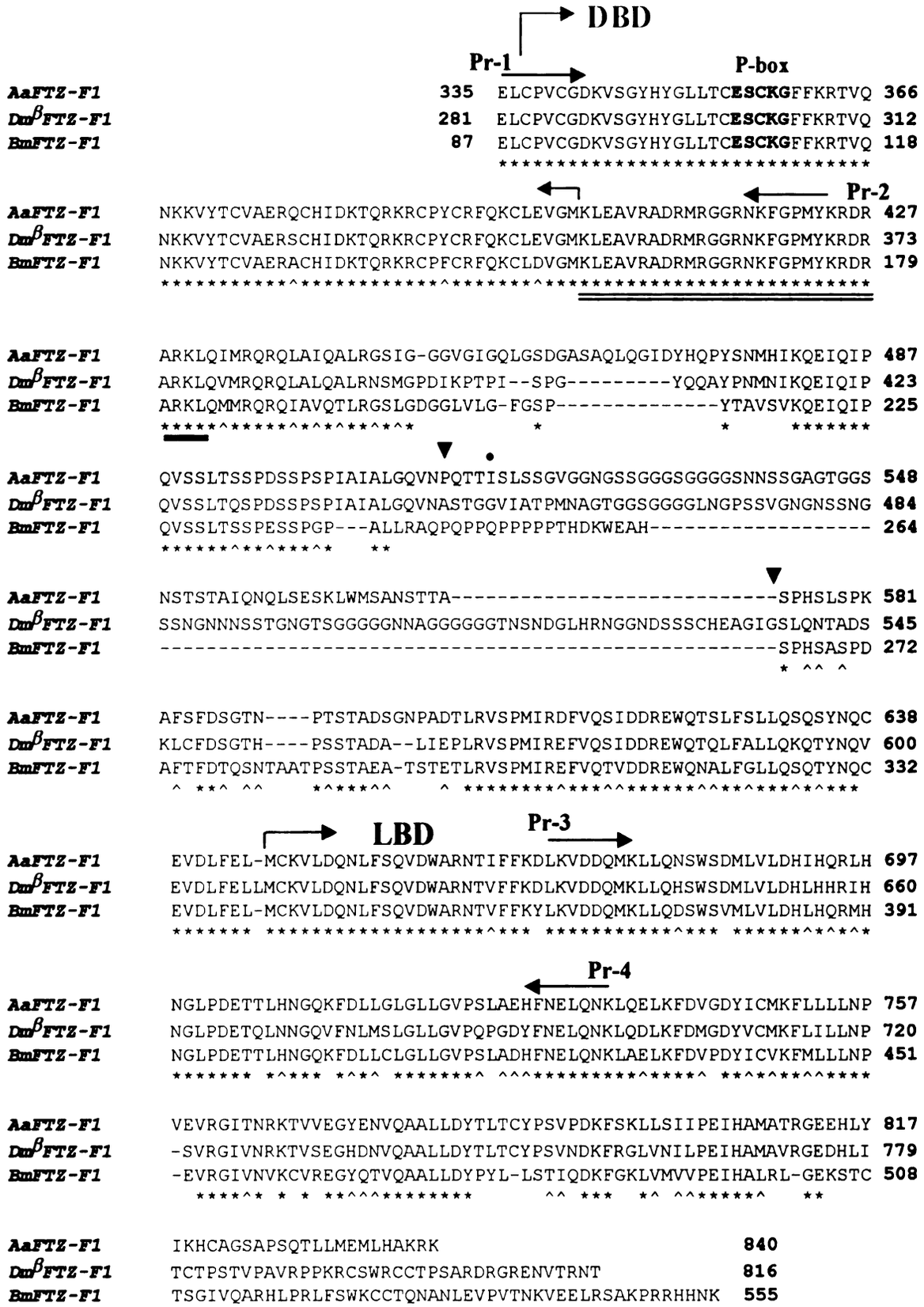
hormonal activation increased gradually over the previtellogenic period from 1 to 3 day post-eclosion (not shown). These data indicated that the ecdysone-responsive vitellogenic competence in the mosquito fat body was under transcriptional control.

Isolation and characterization of a cDNA clone encoding the mosquito AaFTZ-F1 nuclear receptor

To investigate whether the mosquito homolog of the competence factor βFTZ-F1 could be involved in the control of competence in the mosquito fat body, a mosquito homolog of *Drosophila* βFTZ-F1 was cloned. The initial isolation of a cDNA fragment encoding the mosquito *FTZ-F1* homolog was achieved through a PCR-based approach. A pair of degenerate PCR-primers used to amplify cDNA of the mosquito *FTZ-F1* homolog is shown in Fig. 3. After a mosquito PCR-fragment of the appropriate size, 266 bp, was sequenced and confirmed to encode a polypeptide of the expected sequence, it was used as a probe to screen a vitellogenic mosquito fat body cDNA library. Several positive clones were identified, the largest of which contained an insert of 4.6 kb. The first methionine residue at the N-terminal region of the ORF (811 aa) was located at 1.4 kb downstream of this cDNA clone. Before the first Met, there were several in-frame stop codons in the 5'UTR, suggesting that the first Met was the translation start site. There was a poly (A) tail at the 3'UTR end of this clone.

The conceptual translation of AaFTZ-F1 amino acid sequence revealed distinctly high similarity with its insect counterparts of *Drosophila* and *Bombyx* (Lavorgna *et al.*, 1991; 1993; Sun *et al.*, 1994), particularly in the DBD and the adjacent stretch of 29 amino acids known as the FTZ-F1 box (Fig. 3). According to recent data, members of

Fig. 3. Alignment of the deduced amino acid sequence of the mosquito *AaFTZ-F1* cDNA with its insect homologs: DmβFTZ-F1 from *Drosophila* (Lavorgna *et al.*, 1993) and BmFTZ-F1 from *Bombyx* (Sun *et al.*, 1994). Asterisks or carets indicate amino acids which are identical or similar, respectively. The borders of DNA-binding domain (DBD) and putative ligand-binding domain (LBD) are marked with square braces. The P-box in the DBD is in bold. The FTZ-F1-box is double-underlined. Two vertical arrowheads flank the non-conserved insertions in the hinge-regions of insect FTZ-F1s. Horizontal arrows indicate sequences corresponding to the degenerate sense primer (Pr-1) and antisense primer (Pr-2) used for *AaFTZ-F1* cDNA cloning. A black dot marks an EcoRV restriction site. Two other arrows represent the regions of the sequence-specific sense (Pr-3) and antisense (Pr-4) primers utilized in RT-PCR analysis of *AaFTZ-F1*. The Pr-4 was also used to generate a single-stranded DNA-probe for Northern blot (see Fig. 4A). Most part of A/B domains in FTZ-F1s are not shown due to very low similarity. The complete sequence of mosquito *AaFTZ-F1* has been submitted to the GenBank (http://www.ncbi.nlm.nih.gov); its accession number is AF274870.


DBD
Pr-1
P-box
AaFTZ-F1 335 ELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQ 366
DmβFTZ-F1 281 ELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQ 312
BmFTZ-F1 87 ELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQ 118
Pr-2
AaFTZ-F1 NKKVYTCVAERQCHIDKTQRKRCPYCRFQKCLEVGMKLEAVRADRMRGGRNKFGPMYKRDR 427
DmβFTZ-F1 NKKVYTCVAERSCHIDKTQRKRCPYCRFQKCLEVGMKLEAVRADRMRGGRNKFGPMYKRDR 373
BmFTZ-F1 NKKVYTCVAERACHIDKTQRKRCPFCRFQKCLDVGMKLEAVRADRMRGGRNKFGPMYKRDR 179
AaFTZ-F1 ARKLQIMRQRQLAIQALRGSIG-GGVGIGQLGSDGASAQLQGIDYHQPYSNMHIKQEIQIP 487
DmβFTZ-F1 ARKLQVMRQRQLALQALRNSMGPDIKPTPI--SPG---------YQQAYPNMNIKQEIQIP 423
BmFTZ-F1 ARKLQMMRQRQIAVQTLRGSLGDGGLVLG-FGSP----------YTAVSVKQEIQIP 225
AaFTZ-F1 QVSSLTSSPDSSPSPIAIALGQVNPQTTISLSSGVGGNGSSGGGSGGGGSNNSSGAGTGGS 548
DmβFTZ-F1 QVSSLTQSPDSSPSPIAIALGQVNASTGGVIATPMNAGTGGSGGGGLNGPSSVGNGNSSNG 484
BmFTZ-F1 QVSSLTSSPESSPGP---ALLRAQPQPPQPPPPPTHDKWEAH---------- 264
AaFTZ-F1 NSTSTAIQNQLSESKLWMSANSTTA----------SPHSLSPK 581
DmβFTZ-F1 SSNGNNNSSTGNGTSGGGGGNNAGGGGGGTNSNDGLHRNGGNDSSSCHEAGIGSLQNTADS 545
BmFTZ-F1 ----------SPHSASPD 272
AaFTZ-F1 AFSFDSGTN----PTSTADSGNPADTLRVSPMIRDFVQSIDDREWQTSLFSLLQSQSYNQC 638
DmβFTZ-F1 KLCFDSGTH----PSSTADA--LIEPLRVSPMIREFVQSIDDREWQTQLFALLQKQTYNQV 600
BmFTZ-F1 AFTFDTQSNTAATPSSTAEA-TSTETLRVSPMIREFVQTVDDREWQNALFGLLQSQTYNQC 332
LBD
Pr-3
AaFTZ-F1 EVDLFEL-MCKVLDQNLFSQVDWARNTIFFKDLKVDDQMKLLQNSWSDMLVLDHIHQRLH 697
DmβFTZ-F1 EVDLFELLMCKVLDQNLFSQVDWARNTVFFKDLKVDDQMKLLQHSWSDMLVLDHLHHRIH 660
BmFTZ-F1 EVDLFEL-MCKVLDQNLFSQVDWARNTVFFKYLKVDDQMKLLQDSWSVMLVLDHLHQRMH 391
Pr-4
AaFTZ-F1 NGLPDETTLHNGQKFDLLGLGLLGVPSLAEHFNELQNKLQELKFDVGDYICMKFLLLLNP 757
DmβFTZ-F1 NGLPDETQLNNGQVFNLMSLGLLGVPQPGDYFNELQNKLQDLKFDMGDYVCMKFLILLNP 720
BmFTZ-F1 NGLPDETTLHNGQKFDLLCLGLLGVPSLADHFNELQNKLAELKFDVPDYICVKFMLLLNP 451
AaFTZ-F1 VEVRGITNRKTVVEGYENVQAALLDYTLTCYPSVPDKFSKLLSIIPEIHAMATRGEEHLY 817
DmβFTZ-F1 -SVRGIVNRKTVSEGHDNVQAALLDYTLTCYPSVNDKFRGLVNILPEIHAMAVRGEDHLI 779
BmFTZ-F1 -EVRGIVNVKCVREGYQTVQAALLDYPYL-LSTIQDKFGKLVMVVPEIHALRL-GEKSTC 508
AaFTZ-F1 IKHCAGSAPSQTLLMEMLHAKRK 840
DmβFTZ-F1 TCTPSTVPAVRPPKRCSWRCCTPSARDRGRENVTRNT 816
BmFTZ-F1 TSGIVQARHLPRLFSWKCCTQNANLEVPVTNKVEELRSAKPRRHHNK 555

the FTZ-F1 subfamily bind as a monomer to a 5'-PyCAAGGPyCPu-3' DNA response element. The P-box from the DBD recognizes the core motif 5'-AGGPyCPu-3', and the FTZ-F1-box recognizes the first three base pairs 5'-PyCA-3' of the target DNA-element (Ueda and Hirose, 1991; Wilson *et al.*, 1993; Ueda *et al.*, 1992). The mosquito AaFTZ-F1 had a P-box with conserved CESCKG amino acids, which were characteristic for members of the FTZ-F1 subfamily. The DBD and FTZ-F1 box of AaFTZ-F1 were highly conserved relative to the corresponding sequences in insect homologs, and also shared a high similarity with those in vertebrate FTZ-F1 homologs, exhibiting 83% identity with mouse LRH-1 (Tugwood *et al.*, 1991) and 85% identity with bovine Ad4BP (Honda *et al.*, 1993).

In the hinge subdomain, which connects the FTZ-F1 box and the putative LBD, significant conservation was retained only among insect FTZ-F1s. The conserved sequences were interrupted by a non-conserved spacer sequence (marked with vertical arrowheads in Fig. 3) in each insect receptor. The A/B-domains of the insect FTZ-F1 receptors were quite divergent (not shown).

Comparison of the mosquito LBD with the LBDs of insect and non-insect FTZ-F1 homologs showed that there was limited identity in LBDs between mosquito FTZ-F1 and its vertebrate counterparts, exhibiting only 46% identity with mouse LRH-1 and 42% with bovine Ad4BP. However, a high level of identity was retained in the LBDs of insect FTZ-F1s, showing 70% with DmFTZ-F1 and 66% with BmFTZ-F1.

Temporal expression of AaFTZ-F1 in the mosquito fat body during the first vitellogenic cycle

To determine the pattern of *AaFTZ-F1* transcripts in the female fat body, Northern blot was performed for analysis of poly(A) RNA isolated from 100 fat bodies at each time point. Three critical time points during mosquito vitellogenesis were chosen: the early previtellogenic stage at 1 day post emergence (Fig. 4A, PV), the late vitellogenic stage at 36 hr PBM (Fig. 4A, PBM 36h) when the 20E titers are low, and the middle vitellogenic stage at 18 hr PBM (Fig.4A, PBM 18h) when 20E is at the peak level. The 10 kb and 5.2 kb *AaFTZ-F1* mRNAs were detected in the fat body of 1d PV and 36h PBM mosquitoes; in contrast, the fat bodies at 18 hr PBM contained no detectable *AaFTZ-F1* message (Fig. 4A, PBM 18h).

In order to examine the *AaFTZ-F1* developmental profile in this vitellogenic tissue in more details, a more sensitive RT-PCR analysis was utilized. First, RT-PCR was conducted by two pairs of primers located in the 5'UTR or between the hinge region and LBD, and it appeared that the expression patterns of AaFTZ-F1 during vitellogenesis were the same (not shown). In the later experiments, the primer pair in the hinge region and LBD was chosen. As demonstrated in Fig. 4B, the level of the *AaFTZ-F1* transcript was high in the fat bodies of newly eclosed females, and its level declined slightly over the course of previtellogenic development. After the onset of vitellogenesis, triggered by a blood meal, the *AaFTZ-F1* transcript level dropped dramatically during the first 4 hr PBM and then stayed at background levels until 24 hr PBM. Then, it rose again, reaching the previtellogenic levels at 36 hr PBM, and stayed at a high level for the next 12 hr of post-vitellogenesis. Previously, it has been demonstrated that the *AHR3* gene is

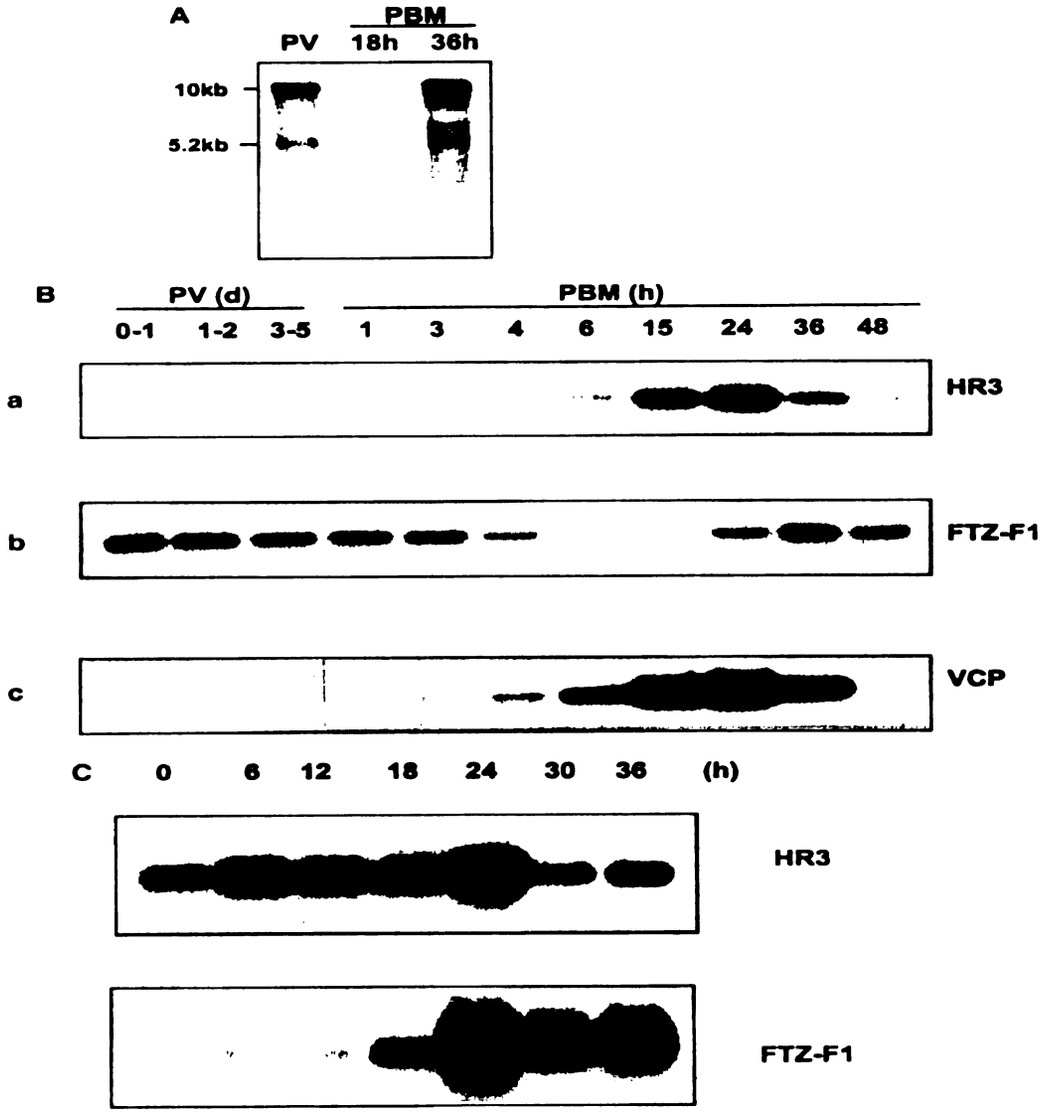


Fig. 4. *AaFTZ-F1* expression profiles in the female mosquito fat body and pupae. (A) Northern blot analysis of *AaFTZ-F1* in fat bodies. Poly(A) RNA was extracted from fat bodies of 100 female mosquitoes at each point, PV: previtellogenic stage 0-1 day post-eclosion; PBM 18h or 36h: post blood meal 18 or 36 hours. The region between EcoRV site and Pr-4 (Fig.3) of AaFTZ-F1 was used as a probe for hybridization. **(B)** Temporal expression of *AaFTZ-F1* in the fat body during the first vitellogenic cycle by RT-PCR/Southern analyses. **A.** The early-late gene *AHR3* expression profile. The 0.22 kb PCR product was generated with the *AHR3*-specific primers and hybridized with a ^{32}P-labeled *AHR3* specific DNA probe (see Methods and Materials). **B.** *AaFTZ-F1* expression profile. The 0.2 kb PCR-fragment was generated with the *AaFTZ-F1*-specific primers Pr-3 and Pr-4 and hybridized with a ^{32}P *AaFTZ-F1* specific probe (shown in Fig. 3). **C.** Expression profile of the mosquito vitellogenic carboxypeptidase (*VCP*) (Cho *et al.*, 1991). *VCP* is shown as an independent control for RT-PCR/Southern analyses of an ecdysone-inducible late gene in the mosquito fat body. **(C)** Temporal expression of *AaFTZ-F1* during pupa development analyzed by RT-PCR/Southern analyses. Expression profiles of *AHR3* (HR3) and *AaFTZ-F1* (FTZ-F1) are shown. Each lane contained the same mosquito pupa equivalents of total RNA subjected to RT-PCR/Southern analyses.

expressed in a narrow window correlated with the high levels of ecdysteroid at 18-24 hr PBM in the mosquito fat body (Kapitskaya *et al.*, 2000). The post-vitellogenic rise in *AaFTZ-F1* mRNA levels occurred immediately after the *AHR3* mRNA peak and in correlation with a drop in the ecdysteroid titer (Fig. 4B).

The high level of the *AaFTZ-F1* transcript in the fat body of the early previtellogenic females suggested that it might be activated prior to emergence by a falling ecdysteroid titer in late pupae. To test this hypothesis, RT-PCR was performed for analysis of the levels of *AaFTZ-F1* and *AHR3* transcripts during development of female mosquito pupae. Developing female pupae were collected at 6 hr intervals, from 0 hr (newly-pupated) to the final collection of late pupae at 36 hr post-pupation (adult mosquitoes normally emerge within the next 6 hr). RT-PCR analysis was then performed using RNA collected at different intervals of pupal development. *AHR3* transcript levels increased during early and mid-pupal stages, reaching its peak in 24 hr old pupae. By 30 hr, the *AHR3* transcript level decreased dramatically and remained low in late pupae. The *AaFTZ-F1* transcript level exhibited a sharp increase in 18-24 hr old pupae and remained at the elevated levels until the end of pupal development (Fig. 4C).

Dual effects of 20E on AaFTZ-F1 mRNA in cultured mosquito fat bodies

To determine whether the negative correlation between *AaFTZ-F1* expression and the levels of ecdysteroids observed *in vivo* reflected the involvement of 20E in regulation of the *AaFTZ-F1* gene, I investigated the patterns of *AaFTZ-F1* transcript accumulation in *in vitro* cultured fat bodies. Previtellogenic fat bodies, 3-5 days after eclosion, were incubated for up to 16 hr in culture medium either in the presence or absence of 10^{-6} M

20E. RT-PCR analysis was used to monitor the transcript levels of *AaFTZ-F1* in the cultured organs (see Material and Methods). Fat bodies from previtellogenic 3-5 day-old females were used to determine the background levels of transcripts for each ecdysone-regulated gene including two isoforms of an early gene *E75* (*AaE75A* and *AaE75B*), an early-late gene *AHR3*, and a late gene *VCP*. Culturing the previtellogenic fat bodies in hormone-free medium up to 16 hr did not induce *AaE75*, *AHR3*, or *VCP* mRNAs (Fig. 5). The *AaFTZ-F1* mRNA level, which was relatively high in the previtellogenic fat body, was not effected by the 16 hr incubation in hormone-free medium (Fig. 5, FTZ-F1). In contrast to *AaE75*, *AHR3*, and *VCP* genes, which were induced by the presence of 20E in culture medium, the *AaFTZ-F1* mRNA dropped to an undetectable level after a 12 hr incubation with 10^{-6}M 20E (Fig. 5, 20E).

In the next experiment, fat bodies were cultured in hormone-containing medium for only 4 hr, then washed three times in hormone-free medium and incubated in hormone-free medium for an additional 12 hr. Both *AaE75A* and *AaE75B* levels dramatically increased after a 4 hr incubation in 20E-containing medium, and then dropped sharply following the withdrawal of the hormone from medium (Fig. 5, E75A, E75B). *AHR3* and *VCP* mRNA levels, which rose moderately during 4 hr incubation with 20E, also declined (Fig. 5, HR3, VCP). In contrast, the *AaFTZ-F1* mRNA exhibited a considerable elevation of its level following the withdrawal of 20E from the incubating medium (Fig. 5, FTZ-F1).

To determine the requirement of protein synthesis for 20E-dependent activation or repression of the *AaFTZ-F1* gene in the mosquito fat body, the previtellogenic fat bodies were incubated with cycloheximide (Chx), a protein synthesis inhibitor which reversibly

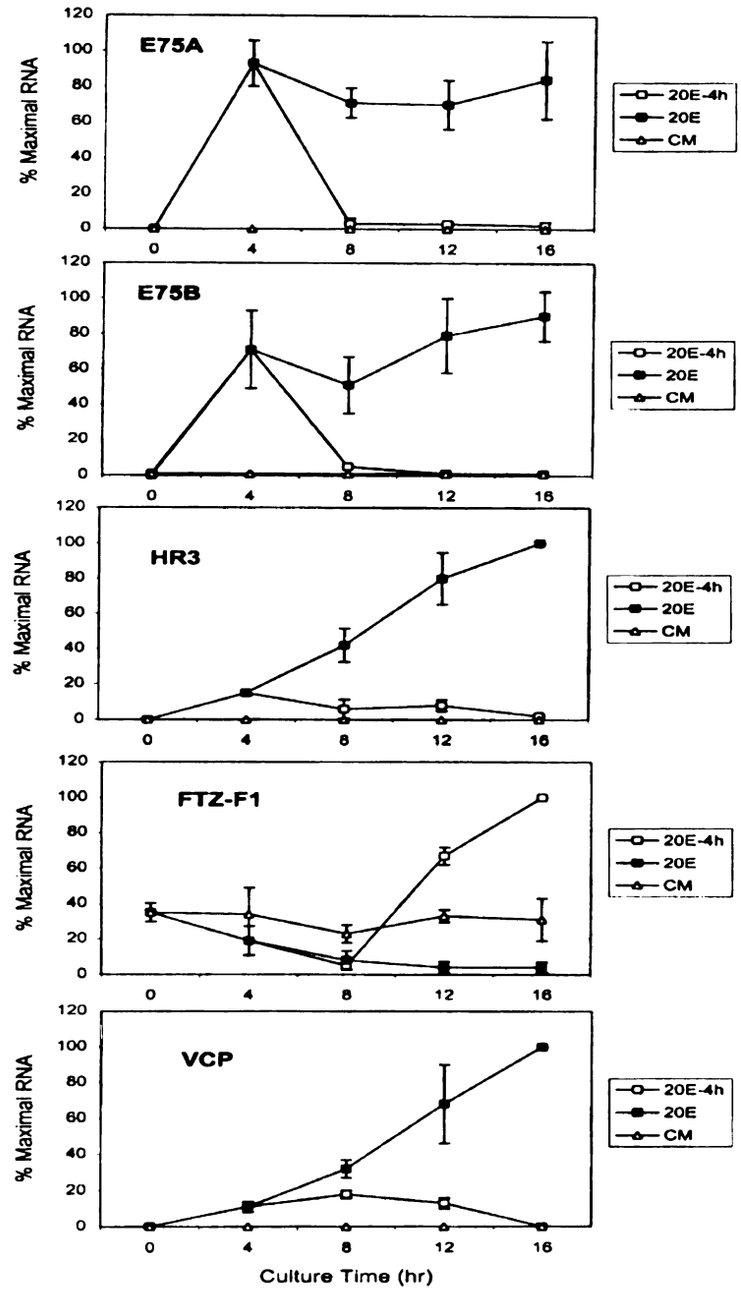


Fig. 5. *In vitro* repression of *AaFTZ-F1* expression by the hormone 20E, and super-induction of *AaFTZ-F1* by withdrawal of 20E in fat body organ culture. RT-PCR/Southern analyses were performed for each ecdysone-regulated gene in mosquitoes as follow: early gene *AaE75A* (E75A) and *AaE75B* (E75B) isoforms (Pierceall *et al.*, 1999); early-late gene *AHR3* (HR3) (Kapitskaya *et al.*, 2000); *AaFTZ-F1* (this paper); late gene *VCP* (VCP) (Deitsch *et al.*, 1995a; 1995b). For the *in vitro* fat body culture and RT-PCR/Southern analyses, see Methods and Materials. Closed squares represent a continuous treatment with 20E (20E); open squares, a 4hr treatment with 20E followed by an additional culture in 20E-free medium (20E-4h); open triangles, a control medium without 20E (CM). Each point represents an average of three independent experiments ± S.D.

represses YPP protein synthesis in this tissue in a dose-dependent manner (Deitsch *et al.*, 1995a; 1995b). The fat bodies were first pre-incubated in hormone-free culture medium for 1 hr in the presence of 10^{-4}M Chx, and then incubated with the same dose of Chx but with the addition of 10^{-6}M 20E for various times (Fig. 6). While incubation of the previtellogenic fat bodies in the presence of Chx in the hormone-free medium did not induce *AaE75*, *AHR3*, and *VCP* mRNAs, the *AaFTZ-F1* transcript levels were considerably elevated (Fig. 6, Chx). Addition of 10^{-6} M 20E to Chx-containing medium activated *AaE75* and *AHR3* genes to the levels much higher than those attained with 20E alone; the levels of *AaFTZ-F1* mRNA were reduced compared with those in hormone-free, Chx-containing medium (Fig. 6, Chx/20E). Withdrawal of 20E from Chx-containing medium after 4-hr incubation caused a slower decline in the *AaE75A*, *AaE75B* and *AHR3* mRNA levels than 20E withdrawal from Chx-free medium (Fig. 5, 20E-4h; Fig. 6, Chx/20E-4h). Interestingly, the *AaFTZ-F1* gene was super-induced under the same conditions (Fig. 6, FTZ-F1). The *VCP* gene was not responsive to any stimulation in the presence of Chx (Fig. 6, VCP).

In vitro enhancement of ecdysone-responsive vitellogenic competence following over-expression of AaFTZ-F1

Withdrawal of 20E from the fat body culture after an initial 4 hr incubation in the presence of 20E resulted in a considerable elevation of *AaFTZ-F1* mRNA (Fig. 5, FTZ-F1, 20E-4h). If AaFTZ-F1 is a competence factor for 20E sensitivity, one might expect that these conditions should lead to enhanced responsiveness of the fat body to 20E. To test this hypothesis, I re-introduced the fat body, after treatment for 16 hr as in Fig. 5

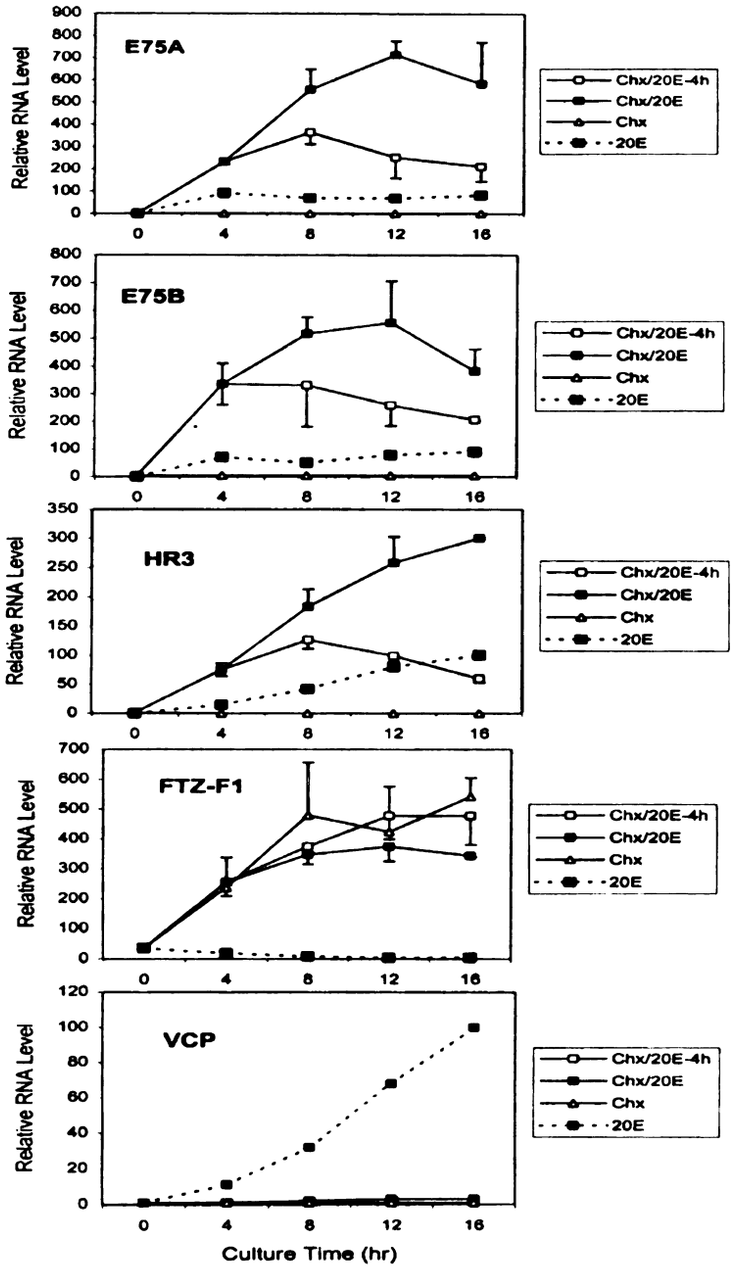


Fig. 6. Effects of cycloheximide (Chx) on the 20E regulation of *AaFTZ-F1* in *in vitro* fat body organ culture. The mRNA levels of each *AaE75A* (E75A), *AaE75B* (E75B), *AHR3* (HR3), *AaFTZ-F1* (FTZ-F1) or *VCP* (VCP) gene were examined during culturing with 10^{-4} M Chx in hormone-free medium (Chx), in the continuous presence of 10^{-4} M Chx and 10^{-6} M 20E (Chx/20E), or in the 4 hr exposure to 10^{-4} M Chx and 10^{-6} M 20E followed by culturing with 10^{-4} M Chx in hormone-free medium (Chx/20E-4h). Dashed lines (20E) are from the previous experiment (Fig. 5, 20E), and all other data are relative to these values. RT-PCR/Southern blot hybridization were performed using a probe specific for each 20E-responsive gene. Each point represents an average of three independent experiments ± S.D.

(20E-4h) with the elevated level of *AaFTZ-F1* mRNA, to a 20E-containing medium and examined its capacity for a secondary 20E-activation of the early *E75*, early-late *HR3*, and late *VCP* genes (Fig. 7). In the first experiment (Fig. 7A), the tissue was incubated in the presence of 10^{-6} M 20E for 4 hr or for 16 hr. The 4-hr secondary incubation resulted in super-induction of the early *AaE75* gene, with the level of *AaE75A* mRNA more than three-fold higher than that seen in the 4-hr primary 20E-incubation (Fig. 7A). The *HR3* and *VCP* transcript levels were also increased compared to the primary incubation. However, their levels increased even further after a 16-hr secondary incubation. In particular, this secondary incubation resulted in a 2-fold induction of the late *VCP* gene compared to that in the primary 20E incubation (Fig. 7A). Thus, this test suggests that the fat body with the experimentally increased level of *AaFTZ-F1* mRNA was competent to activate the early, early-late, and late 20E-responsive genes to a much higher level.

Next, I modified the second part of the experiment. As in the first experiment, the previtellogenic female fat bodies were first incubated in 20E-containing medium for 4 hr, followed by 12 hr incubation in the hormone-free medium. However, the secondary 20E incubations were performed in the presence of 10^{-4} M Chx (Fig. 7B). Under these conditions, *AaE75A* showed even higher induction after 4-hr incubation (8-fold). Furthermore, *AaE75A* transcript continued accumulation reaching the 16-fold level of increase after 16-hr incubation. Likewise, the early-late *HR3* gene transcript showed continuing accumulation. In contrast, the secondary activation of the late *VCP* gene was inhibited (Fig.7B).

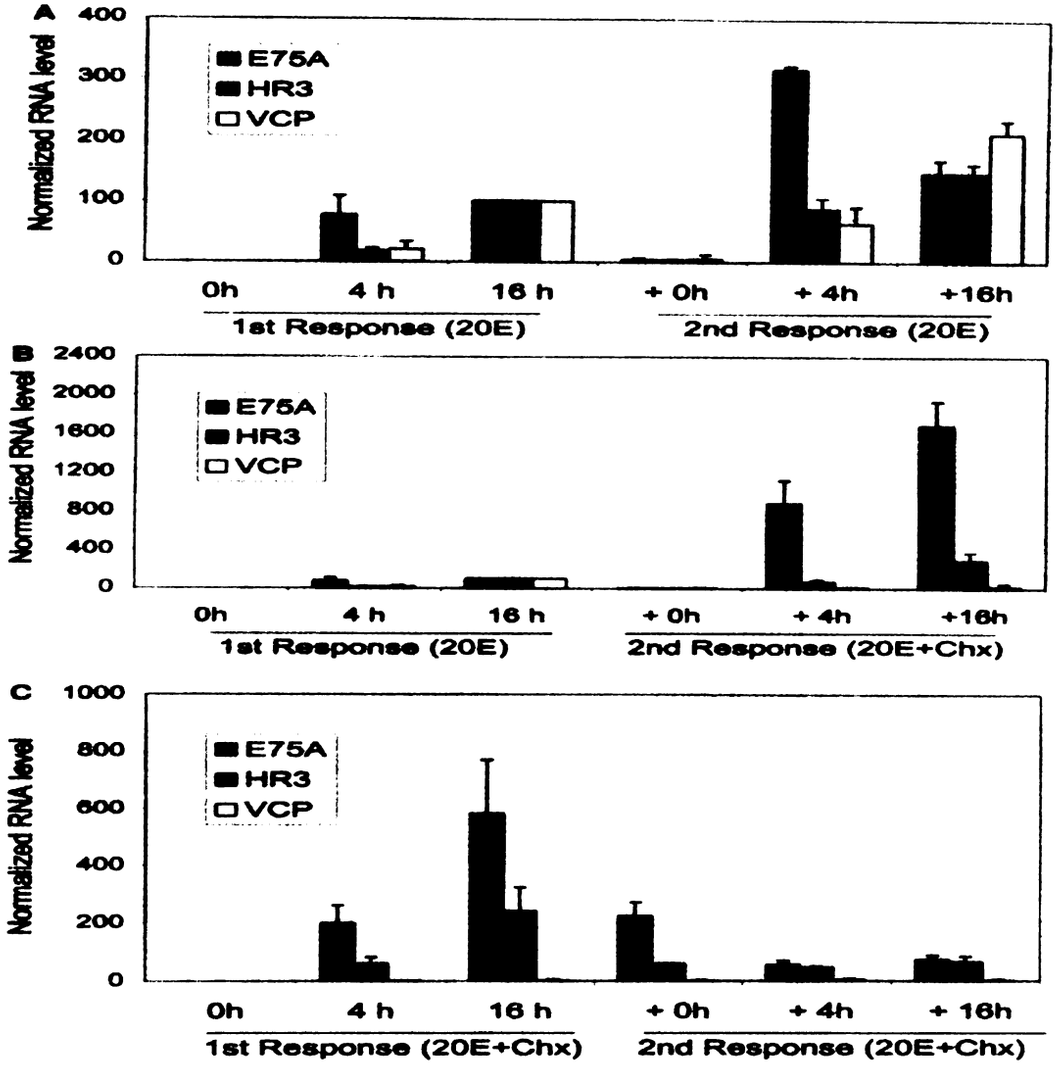


Fig. 7. Secondary responses to 20E of the ecdysone-inducible genes in *in vitro* fat body culture. The primary response experiments were performed as previously described (Fig. 5, 20E, or Fig. 6, Chx/20E) and presented for comparison with the secondary responses. The secondary response experiments were performed following the 20E withdrawal experiment: the previtellogenic competent fat body was first incubated with 10^{-6} M 20E for 4 hr and then in hormone-free medium for 12 hr in the absence or presence of 10^{-4} M Chx (Fig. 5, 20E-4h, or Fig. 6, Chx/20E-4h, respectively). The secondary responses were evaluated in such a fat body after additional incubation with 20E for 4 hr or 16 hr in the absence (20E) or presence of Chx (20E + Chx). RT-PCR/Southern analyses were conducted for the cultured fat bodies. **(A)** Super-induction of ecdysone-inducible genes (E75A, HR3 and VCP) in the secondary responses to 20E. Both the primary and secondary response experiments were performed in the presence of 10^{-6} 20E. **(B)** Effects of Chx on the secondary responses of 20E-responsive genes. The primary response experiment was performed as in Panel A. The 20E withdrawal prior to the secondary incubation was performed in the presence of 10^{-6} M 20E only (as in Fig. 5, 20E-4h), while the secondary response experiment was in the presence of 10^{-4} M Chx and 10^{-6} M 20E. **(C)** Abolishment of the secondary responses of 20E-inducible genes by Chx present in the initial 20E withdrawal incubation. In the primary responses, *E75* and *HR3* genes were super-induced by 20E in the presence of 10^{-4} M Chx. However, after the 20E-withdrawal experiment performed in the presence of the same concentration of Chx, as in Fig. 6, Chx/20E-4h, the secondary induction of *E75* and *HR3* genes was inhibited. For each 20E-inducible gene, data were normalized based on the 16-hr 20E induction in the primary response, which was referred to as 100. Each point represents an average of three independent experiments $\pm$ S.D.

In the final experiment, I conducted both the initial (Fig. 6, FTZ-F1, Chx/20E-4h) and secondary incubations in the presence of 10^{-4} M Chx. Under these conditions, activation of all 20E-responsive genes during the secondary response was abolished (Fig. 7C). This suggests that the AaFTZ-F1 protein synthesis resulted from the initial incubation without Chx was required for the secondary activation of the 20E-responsive genes.

The onset of ecdysone-response competence was correlated with the appearance of functional AaFTZ-F1 protein

The fat body of a newly emerged female mosquito was not competent to respond to 20E, and the competence was acquired by this tissue as a result of the previtellogenic development (Fig. 2). However, the *AaFTZ-F1* gene was highly expressed in this tissue throughout the previtellogenic development (Fig. 4B, FTZ-F1). To resolve this apparent contradiction, investigation was conducted on whether the AaFTZ-F1 protein was present at the first day after eclosion, or the appearance of the functional AaFTZ-F1 protein could correlate with the acquisition of competence for 20E-vitellogenic response by this tissue. To test this hypothesis, EMSA was performed for nuclear extracts from previtellogenic fat bodies and the FTZ-F1 recognition element (F1RE) from in the upstream regulatory region of *Drosophila fushi tarazu* (*ftz*) gene. This 9-bp binding sequence for FTZ-F1 (5'-YCAAGGYCR-3') was identified by an *in vitro* DNase I footprinting assay (Ohno *et al.*, 1994).

First, it was evaluated whether the *in vitro* translated AaFTZ-F1 protein was able to bind to the F1RE (Fig. 8A, lane 2). The specificity of binding was confirmed by

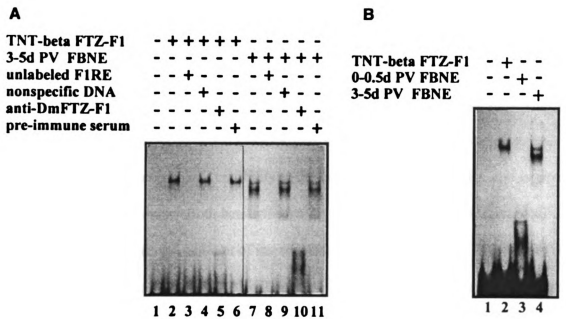


Fig. 8. Identification of the functional AaFTZ-F1 protein in fat body nuclear extracts (FBNE) of previtellogenic female mosquitoes. **(A)** EMSA analysis of the AaFTZ-F1 protein translated *in vitro* (TNT-beta FTZ-F1) or in the FBNE from mosquitoes, 3-5 days after eclosion. The protein samples were incubated with the ^{32}P-labeled FTZ-F1 response element, F1RE. The specificity of the interaction between the F1RE and AaFTZ-F1 was examined by the addition of 50-fold molar excess of the unlabeled F1RE (lanes 3 and 8) or a double-stranded nonspecific DNA (lanes 4 and 9). In addition, anti-DmFTZ-F1 antibodies (lanes 5 and 10) and pre-immune serum (lanes 6 and 11) were added to the reactions. **(B)** EMSA analysis of the AaFTZ-F1 protein in FBNE at different stages of the previtellogenic mosquitoes: lane 1, negative control; lane 2, TNT-AaFTZ-F1; lane 3, FBNE from newly eclosed previtellogenic females, 0-12 hr post-eclosion; lane 4, FBNE from previtellogenic females, 3-5 days post-eclosion.

competition with an excess amount of the specific unlabeled F1RE (Fig. 8A, lane 3) and by co-incubation with anti-DmFTZ-F1 antibodies (Fig. 8A, lane 5). Both of these treatments eliminated the binding complex. The EMSA analyses were further carried out by incubating the fat body nuclear extracts (FBNE) of the 3-5-day old female mosquitoes with ^{32}P-labeled F1RE. Two retarded DNA-protein complexes were observed (Fig. 8A, lane 7), one of which was similar in mobility to the complex formed by F1RE and *in vitro* translated AaFTZ-F1 protein. Both bands formed by the nuclear extract were removed by competition with an excess amount of the specific unlabeled F1RE (Fig. 8A, lane 8) or by co-incubation with anti-DmFTZ-F1 antibodies (Fig. 8A, lane 10), indicating that these complexes were the specific binding of AaFTZ-F1 to F1RE.

Next, the DNA binding activity of AaFTZ-F1 in the FBNE of the female mosquitoes at 0-0.5 day after eclosion was tested. No AaFTZ-F1-containing complex was detected in the FBNE of the newly-eclosed females (Fig. 8B, lane 3). In contrast, the AaFTZ-F1 binding was clearly seen in the 3-5 day-old FBNE used as a positive control in the same experiment (Fig. 8B, lane 4). Testing of the FBNE from mosquitoes of intermediate previtellogenic ages, 1 and 2 day-old, was inconclusive due to variability in development and limitation in the sensitivity of the assays. Interestingly, when the FBNE from the vitellogenic mosquitoes were used in EMSA, the results showed that the intensity of the AaFTZ-F1-containing complexes decreased sharply after a blood meal and disappeared completely by the peak of vitellogenesis. The AaFTZ-F1-containing complexes appeared again in the FBNE from the post-vitellogenic mosquitoes (data not shown). Thus, these analyses confirmed that the acquisition of competence for 20E-

vitellogenic response by the mosquito fat body correlated with the appearance of the functional AaFTZ-F1 protein.

DISCUSSION

The *Drosophila FTZ-F1* gene, located within the 75CD mid-prepupal salivary gland polytene chromosome puff, includes two overlapping transcription units encoding αFTZ-F1 and βFTZ-F1, two orphan nuclear receptor isoforms differing only in the N-terminal part of their A/B domains (Ueda *et al.*, 1990; Lavorgna *et al.*, 1991; 1993). βFTZ-F1 has been shown to play a central role in the acquisition of competence for the late prepupal ecdysone response at the onset of *Drosophila* metamorphosis (Woodard *et al.*, 1994; Broadus *et al.*, 1999). This nuclear receptor is repressed by ecdysone and expressed during the low hormone titer interval in mid-prepupae (Lavorgna *et al.*, 1993; Woodard *et al.*, 1994). Immunocytochemical staining of polytene chromosomes with anti-βFTZ-F1 antibodies shows that βFTZ-F1 protein is bound to several sites corresponding to ecdysone-regulated genes, including the early genes, *E75* and *E74*, suggesting its direct involvement in regulation of these genes (Lavorgna *et al.*, 1993). Indeed, ectopic over-expression of βFTZ-F1 significantly increases the ecdysone induction of the early genes *BR-C*, *E74A* and *E75A* (Woodard *et al.*, 1994). Finally, a recent study has shown that the ecdysone-triggered hierarchy, which directs the stage-specific metamorphic transition, is attenuated in βFTZ-F1 mutants (Broadus *et al.*, 1999). These observations support the initial suggestion made from studying ecdysone responses of puffs of *Drosophila* salivary gland polytene chromosomes, which has showed that competence is acquired during the mid-prepupal period for re-induction of the *BR-C*,

E74A and *E75A* early puffs (Richards, 1976a; 1976b; 1976c), and clearly define βFTZ-F1 as a key competence factor for stage-specific ecdysone responses in *Drosophila* metamorphosis.

Ecdysone plays a critical role in controlling oogenesis and vitellogenesis in flies and mosquitoes (Bownes, 1986; Hagedorn, 1985; Raikhel, 1992; Buszczak *et al.*, 1999; Raikhel *et al.*, 1999); however, limited information has been available concerning the molecular basis of these ecdysone-mediated events. In particular, an important question to be answered is how the same hormone, which regulates larval molts and pupal metamorphosis, activates entirely different sets of target genes in the reproductive tissues of an adult insect. In the case of mosquito, it is shown here that AaFTZ-F1 likely serves as a competence factor for the stage-specific responses to ecdysone in the fat body during vitellogenesis. A homolog of *Drosophila βFTZ-F1* is expressed at relatively high levels in the mosquito fat body during pre- and post-vitellogenic periods when ecdysteroid titers are low. However, the *AaFTZ-F1* transcript nearly disappears during the vitellogenic period with high ecdysteroid levels.

In *Drosophila,* another orphan nuclear receptor (DHR3) is implicated in regulation of *βFTZ-F1* gene transcription. During *Drosophila* metamorphosis, DHR3 has dual roles in both repressing the early genes and activating *βFTZ-F1* transcription (Lam *et al.*, 1997; White *et al.*, 1997). The mosquito *HR3* homolog, *AHR3*, has been cloned (Kapitskaya *et al.*, 2000), and its expression analysis concurs with the studies on *Drosophila* metamorphosis, suggesting its role in *AaFTZ-F1* gene activation. The *in vivo* developmental profiles of *AHR3* in both the fat body and ovary have shown that the peak of the *AHR3* transcript coincides with a high ecdysteroid titer in the hemolymph

(Kapitskaya *et al.*, 2000). In this study, the developmental profile of *AHR3* in pupae was also examined. Remarkably, the peak expression of *AHR3* preceded expression of the *AaFTZ-F1* gene in pupae and in the adult vitellogenic tissues, the fat body (this study) and ovary (Kapitskaya, M., Li, C., and Raikhel, A., unpublished observation). The sequential expression of *AHR3* and *AaFTZ-F1* in the late pupa-early adult suggests that these factors are involved in setting up stage-specific responses to ecdysone in the previtellogenic fat body for the first vitellogenic cycle. Likewise, expression of *AHR3* and *AaFTZ-F1* during the late vitellogenic period is likely preparing the fat body for a second vitellogenic cycle.

Experiments with *in vitro* organ culture strongly supported the hypothesis that AaFTZ-F1 expression is involved in the acquisition of competence for ecdysone response in the adult mosquito fat body. In the previtellogenic fat body, *AaFTZ-F1* expression was inhibited by 20E and strongly activated by its withdrawal. Similarly, in the silkworm *Bombyx mori*, *BmFTZ-F1* is inducible by a decline in the ecdysteroid titer (Sun *et al.*, 1994). Addition of Chx into the medium in withdrawal experiments also led to super-induction of the mosquito *FTZ-F1*, suggesting that *AaFTZ-F1* expression is repressed by one or more unstable repressors. Importantly, after *in vitro* over-expression of *AaFTZ-F1* in the previtellogenic mosquito fat body, a secondary 20E challenge resulted in super-induction of the early *AaE75* gene and the late *VCP* gene. These experiments are consistent with *in vitro* organ culture experiments in *Drosophila* which have shown that *βFTZ-F1* is repressed by ecdysone, and that *in vivo* ectopic expression of *βFTZ-F1* in late-third instar larvae enhances early gene induction (Woodard *et al.*, 1994). The acquisition of competence for ecdysone responsiveness by the mosquito fat body occurs

over the first two days of the previtellogenic development (Flanagan and Hagedorn, 1977; this study). Although the newly emerged mosquitoes were not competent for ecdysone response, *AaFTZ-F1* mRNA was abundant in their fat bodies. The explanation for this apparent contradiction was revealed by EMSA analysis of AaFTZ-F1 DNA binding activity, which showed that the appearance of functional AaFTZ-F1 protein, rather than its mRNA, was correlated with the ecdysone-response competence in the mosquito fat body. The previtellogenic development of the mosquito fat body, which involves an increase in ploidy (Dittmann *et al.*, 1989) and ribosome proliferation (Raikhel and Lea, 1991), is controlled by JH III. Flanagan and Hagedorn (1977) have shown that the fat body responsiveness to ecdysone is also under JH regulation. Further studies are required to elucidate whether JH is involved in the post-transcriptional control of AaFTZ-F1 activity.

In conclusion, our findings indicate that a conserved molecular mechanism for the stage specificity may be used during insect metamorphic and reproductive responses to ecdysone. However, the unique feature of mosquito vitellogenesis is the apparent involvement of post-transcriptional control of AaFTZ-F1 in the acquisition of the ecdysone-response competence. Elucidation of the molecular mechanism underlying this unique characteristic of mosquito vitellogenesis represents an exciting and challenging task for future research.

ACKNOWLEDGMENTS

I thank Marianna Kapitskaya for partial cloning of AaFTZ-F1, Jinsong Zhu for EMSA analysis and Ken Miura for RT/PCR analysis. I also thank William Segraves for

critical reading, Alan Hays for excellent technical assistance, Michel Trail for editing the manuscript, and Carl Thummel for his generous gift of *Drosophila* FTZ-F1 antibodies. This grant was supported by NIH grant AI-36959.

Chapter 6

Cloning and Characterization of the Mosquito *HR78* Gene

ABSTRACT

The ecdysone gene regulatory hierarchy is mediated by a number of transcription factors belonging to the nuclear receptor superfamily. Here, *AHR78* has been identified, which is the mosquito homolog of *Drosophila* hormone receptor 78 (DHR78) and its vertebrate counterpart, testicular receptor 2 (TR2). A full-length cDNA clone was obtained from a mosquito vitellogenic fat body cDNA library. The complete ORF of AHR78, consisting of 630 amino acids, exhibits the highest homology to DHR78 and TR2. The AHR78 DBD displays 95% identity (98% similarity) with the DHR78 DBD and 79% identity (89% similarity) with the TR2 DBD. During the previtellogenic period, *AHR78* is expressed at extremely low levels in the fat body and previtellogenic ovary, however, the AHR78 transcript is considerably elevated in the ovary during the vitellogenic period, reaching its peak level at 24 hr post blood meal, which correlates with the expression peak of the vitellogenin gene (Vg), a target of 20E in the fat body. In *in vitro* culture, *AHR78* mRNA is induced by 20E in the ovary but not in the fat body. Furthermore, AHR78 protein is capable of inhibiting the DNA binding and transactivation activities of AaEcR/AaUSP on various ecdysone response elements (EcRE), including *Drosophila* hsp27- and Eip28/29-EcREs, and the *Aedes* Vg-EcRE. These results suggest that AHR78 may be involved in inhibition of the Vg gene expression in the ovary during vitellogenesis.

INTRODUCTION

The major insect steroid hormone, 20E, is involved in regulating multiple developmental processes including embryogenesis, larval molting, metamorphosis and reproduction (Bownes, 1986; Raikhel, 1992; Riddiford, 1993a,b; Thummel, 1995). The functional ecdysteroid receptor is a heterodimer of the ecdysone receptor (EcR) and a retinoid X receptor (RXR) homolog, Ultraspiracle (USP) (Koelle *et al.*, 1991; Yao *et al.*, 1992; 1993). EcR and USP play the key roles in the ecdysone gene regulatory hierarchies during *Drosophila* metamorphosis (Bender *et al.*, 1997; Hall and Thummel 1998). In addition, the formation of inactive heterodimers between DHR38 and USP, SVP and EcR, as well as the DHR78 homodimer as a competitor, inhibit DNA binding and activation by EcR/USP on the ecdysone responsive elements, providing different levels at which ecdysone responses can be negatively controlled (Sutherland *et al.*, 1995; Zelhof *et al.*, 1995a; Fisk and Thummel, 1995, 1998; Zelhof *et al.*, 1995b).

In contrast to the large body of information concerning the molecular basis of the ecdysone-mediated gene regulation during development and metamorphosis, comparatively little is known about the ecdysone-regulated aspects of insect female reproduction, especially the negative regulation of ecdysone responses. The mosquito is an ideal model animal to study ecdysone-mediated gene regulation during reproduction. This is due to the fact that mosquito reproduction is triggered by blood feeding, which is a requirement for egg development. In addition, mosquitoes act as vectors for many devastating human diseases, which increases the significance of current research (Collins and Paskewitz, 1995; Butler *et al.*, 1997; Beier, 1998). In the anautogenous mosquito *Aedes aegypti*, the process of vitellogenesis is the cornerstone of egg maturation.

Vitellogenin is initiated only after a female mosquito ingests vertebrate blood. A blood meal triggers a hormonal cascade which results in production of 20E, the terminal signal that activates yolk protein precursor (*YPP*) genes in the fat body (Hagedorn, 1983; 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994; Deitsch *et al.*, 1995a; 1995b). Vitellogenin (Vg) is one of the major YPPs. In addition, the mosquito fat body synthesizes several other YPPs, vitellogenic carboxypeptidase (VCP), vitellogenic cathepsin B (VCB) and lipophorin (Cho *et al.*, 1991, 1999; Sun *et al.*, 2000). YPP synthesis reaches maximal levels at 24 hr post-blood meal (PBM) and then proceeds until 30-32 hr PBM, when it is rapidly terminated (Raikhel, 1992). YPP genes are activated in the fat body and their protein products are translocated through the hemolymph and finally internalized into the developing oocytes (Raikhel, 1992).

The titers of ecdysteroid in the female mosquito hemolymph positively correlate with the rate of YPP synthesis in the fat body. While 20E titers are only slightly elevated at 4 hr PBM, they begin to rise sharply at 6-8 hr PBM, and reach their maximum levels at 18-24 hr PBM (Hagedorn *et al.*, 1975). Consistent with the initially proposed role of 20E in activating vitellogenesis in anautogenous mosquitoes (Hagedorn *et al.*, 1973; Hagedorn and Fallon, 1973), experiments using an *in vitro* fat body culture have shown that physiological doses of 20E (10^{-7} - 10^{-6} M) activate two *YPP* genes, *Vg* and *VCP* (Deitsch *et al.*, 1995a; 1995b). Utilization of the protein synthesis inhibitor, cycloheximide (Chx), has demonstrated that the activation of *YPP* genes in the mosquito fat body requires protein synthesis (Deitsch *et al.*, 1995a). Thus, it is likely that a regulatory cascade similar to that seen in *Drosophila* development mediates the action of 20E in the mosquito fat body.

In *A. aegypti*, two isoforms of both the ecdysone receptor (*AaEcR-A* and *AaEcR-B*) and Ultraspiracle (*AaUSP-A* and *AaUSP-B*) have been identified. Also AaEcR-B and AaUSP-B function as major heterodimerization partners during vitellogenesis (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996; Wang *et al.*, 2000a; Wang, Li, and Raikhel, unpublished data). Mosquito *EcR* and *USP* are expressed in pre- and vitellogenic fat bodies and ovaries (Cho *et al.*, 1995; Wang *et al.*, 2000a). The AaEcR/AaUSP heterodimer has been shown to bind to various ecdysone response elements (EcREs) to modulate ecdysone regulation of target genes (Wang *et al.*, 1998). Most recently, we have demonstrated that the *Vg* 5'- regulatory region contains a functional EcRE that is necessary to confer its responsiveness to 20E. AaEcR/AaUSP binds directly to the Vg EcRE, and activates the Vg promoter in the presence of 20E in *Drosophila* S2 cells (Martin *et al.*, 2001).

Furthermore, studies in the previous chapters have demonstrated that conservation of the ecdysone-responsive gene regulatory hierarchy extends beyond *EcR* and *USP*. A mosquito homolog of the *Drosophila* early gene *E75* is involved in mediating the ecdysteroid responses during vitellogenesis (Pierceall *et al.*, 1999). Mosquito AaFTZ-F1 serves as a competence factor for the vitellogenic response to ecdysone, which is implicated in activation of AaE75 and YPP genes (Li *et al.*, 2000). The orphan nuclear receptor AHR3, a mosquito homolog of DHR3, has also been cloned, and is thought to be involved in the activation of *AaFTZ-F1* during vitellogenesis (Kapitskaya *et al.*, 2000; Li *et al.*, 2000). In addition, AHR38, the mosquito homolog of DHR38 and vertebrate NGFI-B, inhibits the ecdysone response in the mosquito fat body during the previtellogenic stage by strongly interacting with the AaUSP protein and preventing the formation of a functional ecdysteroid receptor (Zhu *et al.*, 2000). Thus,

despite the functional diversity of genes targeted by ecdysone-mediated gene regulation, some molecular mechanisms of the ecdysone gene regulatory hierarchy appear to be conserved between mosquito vitellogenesis and *Drosophila* metamorphosis.

In this study, I report the cloning of mosquito HR78, a homolog of *Drosophila* DHR78 (Zelhof *et al.*, 1995a; Fisk and Thummel, 1995) and vertebrate TR2 (Chang and Kokontis, 1988). DHR78 is an orphan receptor, which can inhibit ecdysone signaling and is required for ecdysone-regulated gene expression in mid-third instar larvae in *Drosophila* (Fisk and Thummel, 1995, 1998; Zelhof *et al.*, 1995b). While the mosquito AHR78 DBD is highly conserved relative to the DHR78 DBD, the LBDs in this subfamily show less similarity relative to the other nuclear receptor subfamilies, suggesting that the AHR78 subfamily has evolved rapidly, particular in the E domain. AHR78 is expressed at extremely low levels in the fat body. In the ovary, AHR78 mRNA levels are relatively low during the previtellogenic stage; however, its levels increase dramatically during the vitellogenic stage with a peak level at 24 hr post blood meal. Moreover, AHR78 inhibits binding by EcR/USP to natural EcREs and inhibits the transactivation of EcR/USP in cell transfection assays. Most significantly, AHR78 inhibits DNA binding and transactivation of EcR/USP on the Vg promoter, suggesting that AHR78 may be involved in inhibition of *Vg* gene activation in the ovary during vitellogenesis.

MATERIALS AND METHODS

Animals

Mosquitoes, *Aedes aegypti*, were reared according to the method of Hays and Raikhel (1990). Larvae were fed on a standard diet as previously described (Lea, 1964). Vitellogenesis was initiated by allowing females, 3-5 days after eclosion, to feed on an anesthetized white rat.

cDNA cloning

Degenerate primers based on the specific-conserved sequences in DBDs between *DHR78* and *mTR2* were designed for PCR to obtain a mosquito homolog HR78 cDNA fragment. Two forward degenerate primers, AHR78-F1 (GCN WSI GGI MGI CAY TAY GII G) and AHR78-F2 (TGY GAR GGI TGY AAR GGN TTY TT), and one reversed primer, AHR78-R (TGR CAI CKR TTI CKR TGR TGY TT) were utilized for nested PCR. Amplification was carried out in a Perkin Elmer thermal cycler using a template of cDNA produced by reverse transcription from total RNA prepared from the fat bodies of the vitellogenic female mosquitoes. The PCR-generated fragment was used as a probe to screen a λZAPII cDNA library prepared from the fat bodies of vitellogenic female mosquitoes during 6-48 hr post-blood meal (PBM) as previously reported (Cho and Raikhel, 1992). Several positive cDNA clones were subsequently isolated and the one with longest cDNA insert was sequenced at W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University. Analyses of nucleotide and deduced amino acid sequences were performed using the software of GCG (Genetics Computer Group, University of Wisconsin, Madison).

RT-PCR/Southern blot

RT-PCR/Southern analyses were performed as previously described (Li *et al.*, 2000). Total RNA was prepared from fat bodies throughout the first vitellogenic cycle by TRIZOL Reagent (Gibco BRL). After reverse-transcription by Superscript II reverse transcriptase (Gibco BRL), 0.025 fat body equivalent of the cDNA pool was used as a PCR template for each point for the analyses of developmental profiles. Thermal cycling conditions were as follows: 94°C for 3 min, followed by 18 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The PCR primers for AHR78 are AHR78-F33 (GCT TCC CAA CTA CCT GAG TGC G) and AHR78-R73 (GCC AGC TGG CCG CTA CTC T), both of which are located in the ligand binding domain of AHR78. As a reference to the developmental change of the fat body and the ovary, the samples of the same cDNAs were subjected to the 13 cycles of PCR with each primer pair specific to the mosquito vitellogenic carboxypeptidase (*VCP*) or vitellogenin receptor (*VgR*). Southern blot hybridization was performed under high stringency conditions using each gene-specific probe, which was generated by PCR using each cloned cDNA as a template.

In vitro fat body and ovary culture

Mosquito fat body culture experiments were performed as previously described (Li *et al.*, 2000). The ovary culture experiments were performed according to the method by Sappington *et al.* (1997). After culturing, RT-PCR/Southern blot were utilized to analyze the mRNA levels.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were carried out as described previously (Wang *et al.*, 2000a). Nuclear receptor proteins were synthesized *in vitro* using a coupled transcription-translation (TNT) kit from Promega. AaEcR and AaUSPb proteins were expressed with the SP6 promoter from the vectors pGEM3Z-AaEcR and pGEM7Z-AaUSPb, respectively. AHR78 protein was expressed from pBluescriptSK-AHR78 with the T3 promoter. The double strands of EcREs are: Vg-EcRE, agctgtgTGACCTagcgggAGGCCAaTGGTCGagt; Hsp27, agagacaagGGTTCAaTGCACTtgtccaat; Eip28/29, ttaaAGGATCtTGACCCcaaT GAACTtcat. Five pmols of each EcRE was end-labeled with γ-^{32}P ATP (Dupont NEN) by T4 polynucleotide kinase (Gibco BRL). In each binding reaction, typically 1μl TNT protein and 100 fmol labeled EcRE were incubated in total 20μl of binding buffer containing 20mM HEPES (pH7.5), 2mM DTT, 100mM KCl, 7.5% Glycerol, 0.1% NP-40, 2μg of poly (dI-dC)• poly (dI-dC) (Pharmacia Biotech). In some experiments, the binding buffer also contained 2 μg of non-specific single stranded DNA to eliminate non-specific binding (Wang *et al.*, 1998). The binding reaction was conducted for 30 min at room temperature.

Cell culture and transient transfection assay

Cell culture and transient transfection assay were performed as previously described (Wang *et al.*, 2000b). The *Drosophila* S2 cell line (Invitrogen) was maintained at 22-24 °C in Schneider *Drosophila* medium supplemented with 10% heat-

inactivated fetal bovine serum, penicillin (100U/ml), and streptomycin (100μg/ml) (Gibco BRL). Transfection was conducted with Cellfectin (Gibco BRL). Typically, 100ng of the luciferase reporter plasmid and 12.5 ng of the reporter pAc5-LacZ plasmid as well as a total 180ng of all receptors and pAc5 plasmids, were mixed in a 48-well plate with 2 μg of Cellfectin in a total volume of 20μl, and then incubated at room temperature for 30min. The transfection cocktail was overlaid with 200 μl of S2 cells (2×10^6 cells/ml) in *Drosophila* serum-free medium. Transfection was terminated 12 hr later by adding 200μl 20% fetal bovine serum media containing either 2×10^{-6} M 20E or an equal amount of vehicle ethanol. After treatment with the hormone 20E for 27hr, cells were collected and lysed by 100 μl 1×reporter lysis buffer (Promega) with one cycle of freezing and thawing. Reporter gene assays were conducted as described by the Promega firefly luciferase reporter and β-galactosidase systems. A luminometer (Turner Designs model TD20e) was used to detect luciferase activity with 10 sec pre-delay and 30 sec integration time. Luciferase activities were normalized by β-galactosidase activities in each experiment. The reporter plasmids ΔMTV-Eip28/29-luc and ΔMTV-hsp27-luc were kind gifts from M. Mckeown (Salk Institute, San Diego, CA). The ΔMTV-Eip28/29-luc construct contains four copies of Eip28/29 EcRE and the ΔMTV-hsp27-luc has five copies of hsp27 EcRE. The pAc5-AaEcR and pAc5-AaUSPb constructs contained the full length AaEcR and AaUSPb (Wang *et al.*, 2000b). The pAc5-AHR78 plasmid was constructed by digesting pBluesript SK-AHR78, which was directly from the mosquito λZapII cDNA library, with *XbaI* and *ApaI* and subcloning the ORF into the pAc5 *XbaI/ApaI* sites. The Vg-luc plasmid was the Vg promoter (-348 to +115bp) subcloned in the pGL3-basic vector (Promega).

RESULTS

Cloning and characterization of AHR78 cDNA

Three degenerate primers were designed (see Materials and Methods), based on specific-conserved regions of the DNA binding domain (DBD) in the DHR78/TR2 family. After the first round of PCR amplification (using the fat body cDNA as a template) by the primer pair AHR78-F1 and AHR78-R, and purification of the amplified DNA fragment, the second round of nested PCR amplification was conducted using the primers AHR78-F2 and AHR78-R. The final PCR amplified DNA fragment (~110 bp) was then subcloned into a pGEM-T vector and sequenced. The sequencing data indicated that this DNA fragment highly resembled the DHR78/TR2 family, and the deduced amino acid sequence was more similar to that of DHR78 than that of any other nuclear receptors in the GeneBank. Subsequently, this DNA fragment was used as a probe to screen the mosquito fat body cDNA library that was generated from fat bodies of 6-48hr PBM mosquitoes by the poly (T) method (Cho and Raikhel, 1992).

Several positive clones were identified by screening of the cDNA library. The clone with the largest insert was completely sequenced on both strands. This clone of a 2.8 kb insert contained an open reading frame (ORF) of 630 amino acids. There were 3 stop codons before the first putative start codon (ATG), and polyadenylation signaling (AATAAA) after the ORF, suggesting the ORF is complete (the GeneBank accession number is.....).

The conceptual translation of AHR78 amino acid sequence revealed distinctly high similarity with its insect counterparts of *Drosophila* and *Tenebrio molitor* (Zelhof

```
          1                                                  50
 AHR78    ~~~MEPHNFD IKPNLQQMKL EPGTQQFGSS SLDDKGPSLQ HLSSLNSMSI
TmHR78    ~~~~~~~~~~ ~~~~~~~MDI E...HSIKSE ILSDK...IR NNSNNLCLTV
 DHR78    MDGVKVETFI KSEENRAMPL IGGGSASGGT PLPGGGVGMG AGAS.ATLSV

          51 ──▶ DBD                                         100
 AHR78    ELCLVCGDRA SGRHYGAISC EGCKGFFKRS IRKQLGYQCR GSMNCEVTKH
TmHR78    ELCVVCGDRA SGRHYGAISC EGCKGFFKRS IRKQLGYQCR GSKNCEVTKH
 DHR78    ELCLVCGDRA SGRHYGAISC EGCKGFFKRS IRKQLGYQCR GAMNCEVTKH

          101                 ◀──                           150
 AHR78    HRNRCQYCRL QKCLACGMRS DSVQHERKPI IDKKDG.... ......SGGP
TmHR78    HRNRCQYCRL QKCLACGMRS DSVQHERKPI IDKKDY.... ......SN..
 DHR78    HRNRCQFCRL QKCLASGMRS DSVQHERKPI VDRKEGIIAA AGSSSTSGGG

          151                                               200
 AHR78    NPNSKYNPHR .N........ ...KEYHNEQ KSNTTVTPSY LNLFQGFNLA
TmHR78    NIGNNYNSNS VN........ ...KIF..IR KDLSTDSPGL LP..APFNPC
 DHR78    NGSSTYLSGK SGYQQGRGKG HSVKAESAPR LQCTARQQRA FNLNAEYIPM

          201                                               250
 AHR78    ELAVANLSKR A..SKSPPAP SHLTSSSTTT SSNPFSSLHS FKSQDDRQPT
TmHR78    DLGLQFLNKR IGNSGSSDLP YHLSPSQASI .......... ...EDD...V
 DHR78    GLNFAELTQT LMFATQQQQQ QQQQHQQSGS YSPDIPKADP EDDEDDSMDN

          251                                               300
 AHR78    PAD.SIAALM QLGPTAAAAV AMSGLGPPPQ QPPPSLEPPE LPSKPTPPSS
TmHR78    SMD.S..... .......... TNTGAG.... .........E L.........S
 DHR78    SSTLCLQLLA NSASNNNSQH LNFNAGEVPT ALPTTSTMGL IQSSLDMRVI

          301                                               350
 AHR78    H.SLPMETTD SNSMEKNLIC NSLEFIQNLE HELNNSVNNN YGIKSELN..
TmHR78    D.ALIM.... ..ARDKQLIS KALDTMARVQ C.LNGT.... ..DLSSLT..
 DHR78    HKGLQILQPI QNQLERN.GN LSVKPECDSE AEDSGTEDAV DAELEHMELD

          351                                               400
 AHR78    ....NGRDDD RDDDCLNYDY GSLELSDNCI SFDIQVPNVL PNYLSAHYVC
TmHR78    ....AA.... .DEKC..YEY EGPILQEQHI SFNLQIPGPV PPYLNIHYIC
 DHR78    FECGGNRSGG SDFAINEAVF EQDLLTDVQC AFHVQPPTLV HSYLNIHYVC
                                                        ──▶ LBD
          401                                               450
 AHR78    ETGSRLLFAT VHWMKKNHLF SMLSDSFQSE LMRQTWPELF MIGLAQSSGQ
TmHR78    ESGSRLLFLS IHWTRNIPAF QYLTTETQIT LLRGCWAELF TLGLAQCSQT
 DHR78    ETGSRIIFLT IHTLRKVPVF EQLEAHTQVK LLRGVWPALM AIALAQCQGQ

          451                                               500
 AHR78    LAFNTVMLAL IQYMKNLILN KKYGSDVINY LTKYILLIQE FVSDVQKLNL
TmHR78    LSLSTILSAL ISHLHTLIAQ DKMSATKVKQ VSDHIVKLQD YANTMNRLNV
 DHR78    LSVPTIIGQF IQSTRQLADI DKIEPLKISK MANLTRTLHD FVQELQSLDV

          501                                               550
 AHR78    TDQEFAYMRL LCIFNPDNIL QDNVKNQHLA KIQDMVLSSF RDYYKQKHSR
TmHR78    DEHEYAYLKA ITLFSADQ.. PDILLRKHVE KLQEKSFQAL KTY.......
 DHR78    TDMEFGLLRL ILLFNP.... ...TLFQHRK E......RSL RGYVRRVQLY
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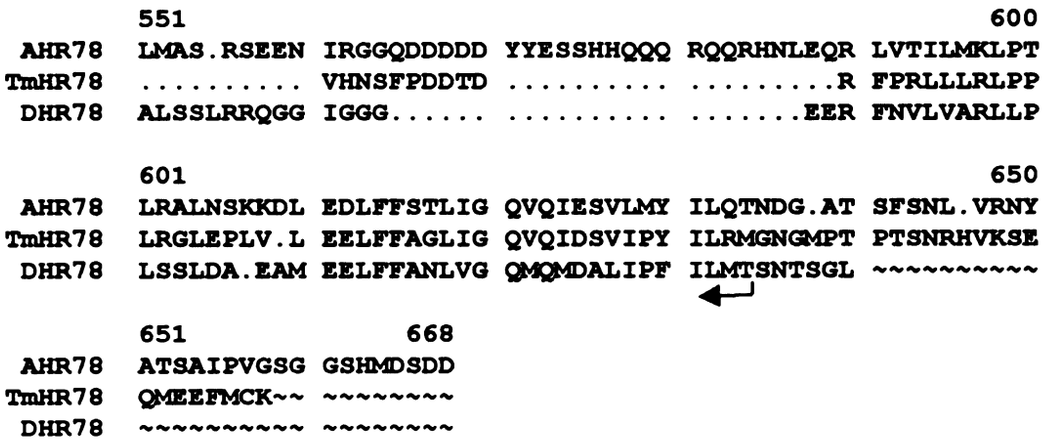

Fig. 1. Alignment of the deduced amino acid sequence of the mosquito AHR78 cDNA with its insect homologs: DHR78 from *Drosophila* (Fisk and Thummel, 1995; Zelhof, *et al.*, 1995b) and TmHR78 from *Tenebrio molitor* (Mouillet *et al.*, 1999). The borders of DNA-binding domain (DBD) and putative ligand-binding domain (LBD) are marked with square braces. The complete nucleotide sequence of *AHR78* has been submitted to the GenBank (http://www.ncbi.nlm.nih.gov); its accession number is .....

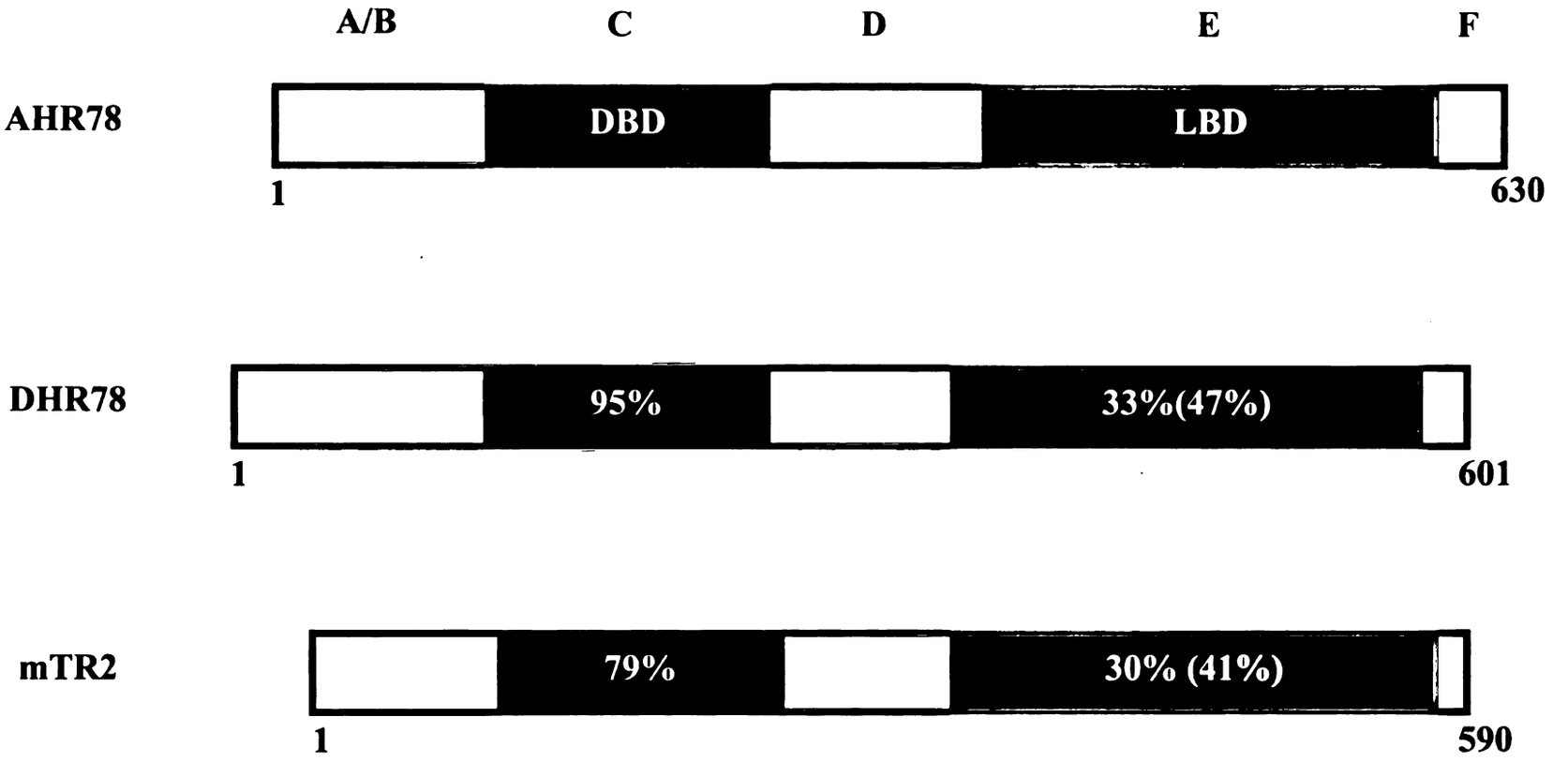

Fig. 2. Domain comparison of the mosquito AHR78 with selected members of the HR78/TR2 subfamily nuclear receptors. Shown are the HR78 nuclear receptors: AHR78 from *Aedes aegypti* (this study), DHR78 from *Drosophila* (Fisk and Thummel, 1995; Zelhof, *et al.*, 1995b), and mouse TR2 (Chang and Kokontis, 1988). The letters above the mosquito HR78 protein denote functional domains. Numbers below proteins indicate the number of amino acids in each receptor. The percentages of identities (or similarities) between corresponding domains of AHR78 and other homologues are indicated within each domain; the ratios were calculated as a number of amino acids identical between comparative sequences divided by the total amino acid number of the shorter sequence.

et al., 1995a; Fisk and Thummel, 1995; Mouillet *et al.*, 1999) (Fig. 1). AHR78 contains the classic nuclear receptor functional domains: A/B domain, DNA binding domain (DBD) or C domain, hinge region or D domain, ligand binding domain (LBD) or E domain, and F domain. Comparison of AHR78 with *Drosophila* DHR78 and its vertebrate counterpart, human testicular receptor 2 (TR2) showed that these proteins were of similar length in regards to their amino acid sequences: AHR78, 630aa; DHR78 601aa; mTR2, 590aa. Moreover, the AHR78 DBD is highly conserved in relation to the DBDs in *DHR78* and TR2, exhibiting 95% identity (98% similarity) and 79% identity (89% similarity), respectively. The LBD of AHR78 exhibits 33% identity (47% similarity) with DHR78 and 30% identity (41% similarity) with mTR2. The other domains of this family are relatively less conserved (Fig. 2).

AHR78 is highly expressed in the vitellogenic ovary, but not the fat body

To examine the AHR78 expression profiles, RT-PCR was utilized to analyze the mRNA levels during mosquito vitellogenesis. The two critical vitellogenic tissues, the fat body and ovary, were chosen for examination of AHR78 expression levels during the first vitellogenic cycle. In the fat body, traces of AHR78 were expressed at the relatively constant levels throughout the previtellogenic and vitellogenic stages. Vitellogenic carboxypeptidase (VCP), one of the major YPPs (Cho *et al*, 1991), was used as a positive expression control in the fat body. In the ovary, AHR78 mRNA levels were considerably higher than those in the fat body, with dramatic increases after a blood meal peaking at 18-24h PBM, followed by a gradual decline after 30hr PBM (Fig. 3). Interestingly, expression dynamics of AHR78 in the ovary correlates with 20E level changes during

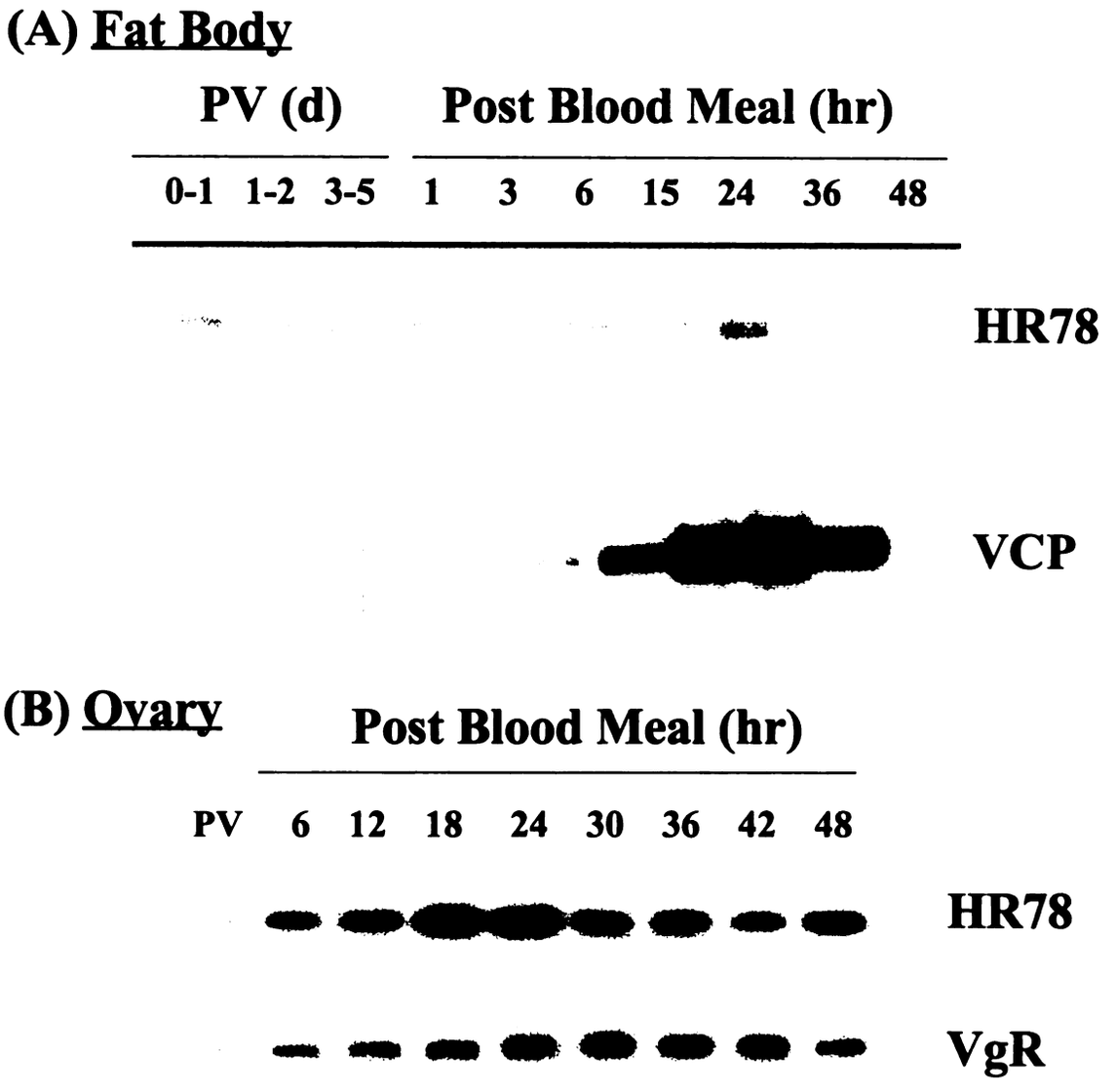

Fig. 3. *AHR78* expression profiles in the female mosquito fat body and ovary. (A) Temporal expression of *AHR78* in the fat body during the first vitellogenic cycle examined by RT-PCR/Southern analyses. The expression profiles of *AHR78* (upper panel) and vitellogenic carboxypeptidase (*VCP*) as a control (lower panel) were examined (see Materials and Methods). **(B)** Temporal expression of *AaHR78* in the ovary during the first vitellogenic cycle. The expression profiles of *AHR78* (upper panel) and vitellogenin receptor (*VgR*) as a control (lower panel) were examined (see Materials and Methods). Each lane contained the same mosquito equivalents of total RNA subjected to RT-PCR/Southern analyses. PV: previtellogenic stage.

this period as well as the accumulation of yolk protein precursors by the oocytes. As a positive control, the expression profile for vitellogenic receptor (VgR) (Sappington *et al* 1996) was provided for the ovary. In addition, AHR78 expression patterns and levels in both the fat body and ovary were monitored by using different pairs of PCR primers, which were located in either the 5'UTR or LBD. These results were the same, suggesting that the expression profiles of AHR78 detected by the RT-PCR method were specific to the AHR78 gene (not shown).

AHR78 is inducible by 20E in the ovary but not in the fat body

To investigate whether the high levels of AHR78 mRNA in the ovary during the vitellogenic stage are under the control of 20E, ovaries of previtellogenic 3-5d-old or 2h PBM were incubated with 20E for 6h followed by RT-PCR/Southern analysis. The AHR78 mRNA levels increased significantly after the 20E treatment in both previtellogenic and 2h PBM ovaries (Fig. 4B). For control, treatment of ovaries with ethanol, which is solvent for 20E, did not show any effect on AHR78 mRNA levels (Fig. 4B).

Next, fat bodies were tested in a parallel experiment. After previtellogenic 3-5d-old fat bodies were cultured with 10^{-6} M 20E for 4h and 8h, AHR78 did not exhibit detectable changes in mRNA levels (Fig. 4A). In the control, VCP mRNA was highly induced by 20E in the same experiment.

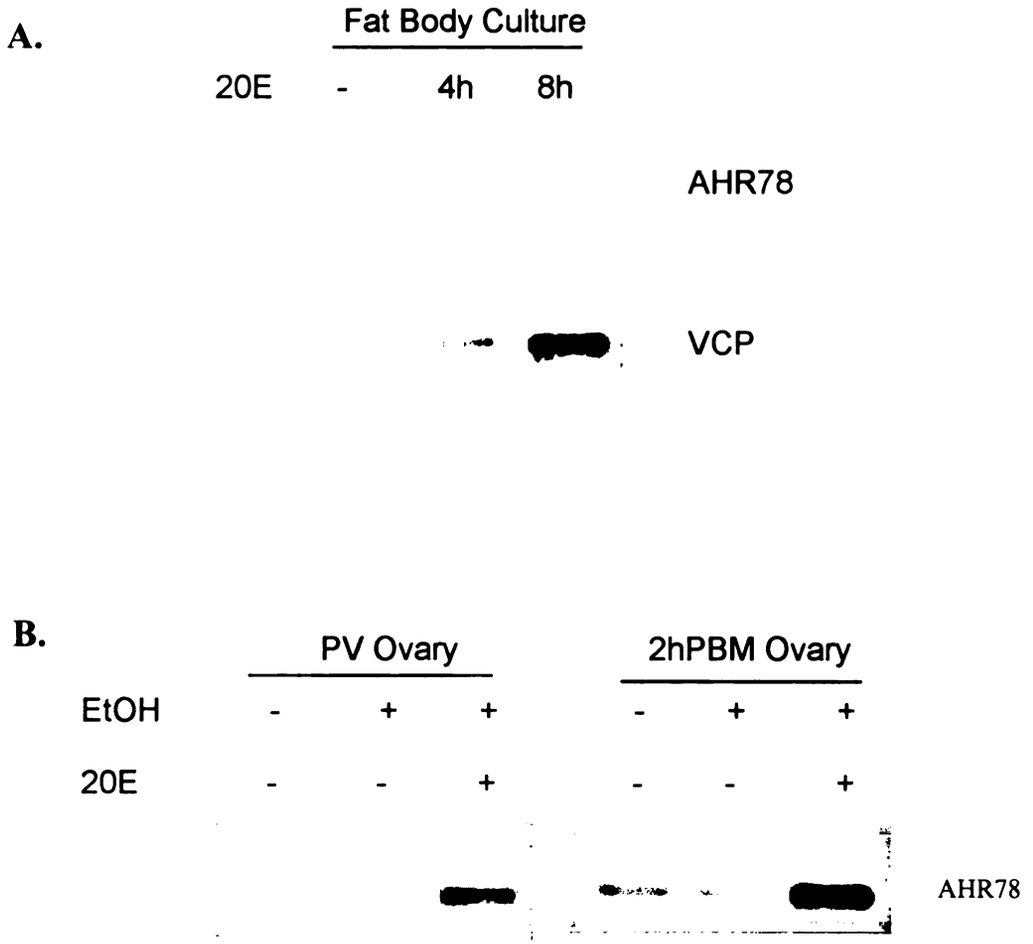

Fig. 4. Induction of AHR78 mRNA by 20E in the ovary but not the fat body.
A). The AHR78 gene was not responsive to 20E in the fat body. Previtellogenic 3-5d-old female fat bodies were incubated with 10^{-6} M 20E for 4 or 8 hr followed by RT-PCR/Southern analysis. The VCP gene was used as a positive control. **B).** Induction of AHR78 mRNA in the ovary. Previtellogenic 3-5d-old female ovaries (PV Ovary) or 2h-PBM ovaries were treated with 20E or 20E solvent ethanol (EtOH) for 6hr, and then analyzed by RT-PCR/Southern blot.

AHR78 inhibits the DNA binding of EcR/USP to EcREs

To test the effect of AHR78 on EcR/USP DNA binding, EMSA was performed using two well characterized EcREs, hsp27 and Eip28/29. In the absence of AHR78, AaEcR/AaUSPb bound to hsp27 (Fig.5A, lane 4, 7) as previously reported (Wang *et al.*, 1998). Addition of increasing amounts of AHR78 to the binding reactions dramatically decreased EcR/USP binding to EcRE (lane 8, 9). In the control, addition of another nuclear orphan receptor AaE75A did not have any effect on DNA binding of mosquito EcR/USP (Fig. 5A, lane 10, 11). Unexpectedly, AHR78 alone was not able to bind to hsp27 (Fig. 5A, lane 12).

When testing the effect of AHR78 on EcR/USP DNA binding by using the Eip28/29 EcRE, results similar to those in the hsp27 experiment were obtained: addition of AHR78 dramatically decreased the DNA binding of EcR/USP (Fig. 5B, lane 8, 9).

Furthermore, I examined whether AHR78 had a similar effect on EcR/USP binding to Vg-EcRE, an EcRE identified in the Vg promoter (Martin, *et al.*, 2001), and found that AHR78 inhibited the binding of EcR/USP to the Vg-EcRE (Fig. 5C, lane 2, 3). Moreover, I tested whether 20E had any effect on the AHR78 inhibiting action. As shown in Fig. 5C, Lane 7, in the presence of 20E, inhibition of AHR78 on the EcR/USP DNA-binding was similar to that seen in the absence of 20E (Fig. 5C, lane 3). As a control, addition of increasing amounts of another nuclear orphan receptor AaFTZ-F1 protein into the binding reactions did not show any detectable effect in the absence of 20E (Fig. 5C, lane 4, 5). Likewise, in the presence of 20E (Fig. 5C, lane 8), AaFTZ-F1 protein did not affect the DNA binding of EcR/USP.

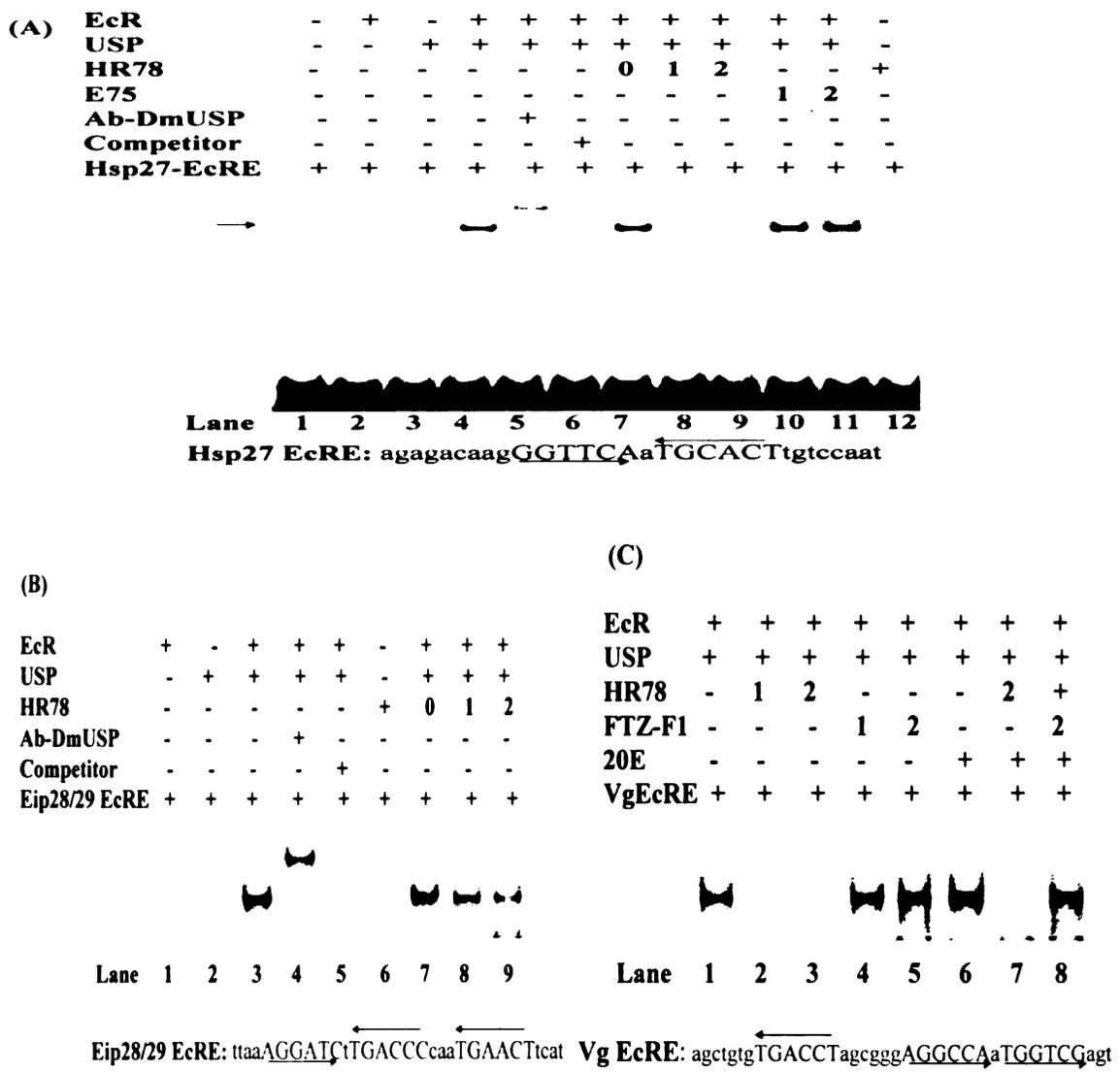

Fig. 5. Inhibition of EcR/USP binding to the natural EcREs by AHR78. *In vitro*-translated AaEcR and AaUSP proteins alone or along with *in vitro* translated AHR78 protein were incubated with ^{32}P-labeled different natural EcREs. The reaction mixtures were subjected to EMSA and autoradiography. **(A)** Inhibition of EcR/USP binding to the hsp27-EcRE by AHR78. In addition to 1 μl of each TNT-AaEcR and AaUSP (lane 4 and 7), 1 μl or 2 μl of TNT-AHR78 (lane 8 and 9) or AaE75A (lane 10 and 11) were added to the reactions. For controls, antibody against *Drosophila* USP supershifted the EcR/USP binding (lane 5); 50 times molar excess of cold hsp27 EcRE was added as competition (lane 6). All reactions were in the absence of 20E. The arrow indicates the specific binding. **(B)** Inhibition of EcR/USP binding to the Eip28/29-EcRE by AHR78. This was a similar experiment to that in the panel A except that Eip28/29-EcRE was used as ^{32}P-labeled or cold oligonucleotides. **(C)** Inhibition of EcR/USP binding to the mosquito Vg-EcRE by AHR78. This was a similar experiment to that in the panel A except that Vg-EcRE was used as ^{32}P-labeled oligonucleotides, and some experiments in the presence of 5×10^{-6}M 20E (lane 6, 7 and 8) as well as AaFTZ-F1 protein as controls (lane 4 and 5).

AHR78 inhibits 20E-stimulated transactivation by EcR/USP

To investigate whether AHR78 affects the EcR/USP-induced transactivation of target genes, a cell transfection assay was utilized. The reporter plasmid Eip-luc containing the *Drosophila* Eip28/29-EcRE, which has been previously shown to be activated by the mosquito EcR/USP (Wang *et al.*, 2000b), was transfected into *Drosophila* S2 cells alone or along with the expression plasmids carrying *AaEcR*, *AaUSP* or *AHR78* cDNAs. After transfection, cells were incubated either in the absence of hormone or in the presence of 20E at 10^{-6}M for 27 hr. When no *EcR/USP* plasmid was transfected, cells receiving Eip-luc alone exhibited a low level of 20E-stimulated reporter gene activities, which was presumably due to the presence of endogenous *Drosophila* EcR/USP in S2 cells (Fig. 6A, column 1). Transfection of both mosquito *EcR* and *USP* plasmids did not affect this baseline activity in the absence of 20E (Fig. 6A, column 2); however, addition of 20E resulted in 2.5 fold induction in reporter gene activities (Fig.6A, column 2). When increasing amounts of AHR78 plasmid was co-transfected with *EcR/USP* plasmids, the induction was reduced continuously with virtually no induction at 8-fold molar excess of the AHR78 plasmid (Fig. 6A, column 5). Interestingly, in the absence of 20E, AHR78 also reduced the basal reporter activities by up to 50% (Fig. 6A, column 5).

Similarly, using another hsp27-EcRE construct in the ΔMTV promoter driving a luciferase reporter, co-transfection of *AHR78* plasmids along with *EcR/USP* plasmids also reduced 20E-stimulated activation. The effect of AHR78 was less dramatic in the hsp27-luc construct than that in the Eip28/29-luc construct, presumably due to the high

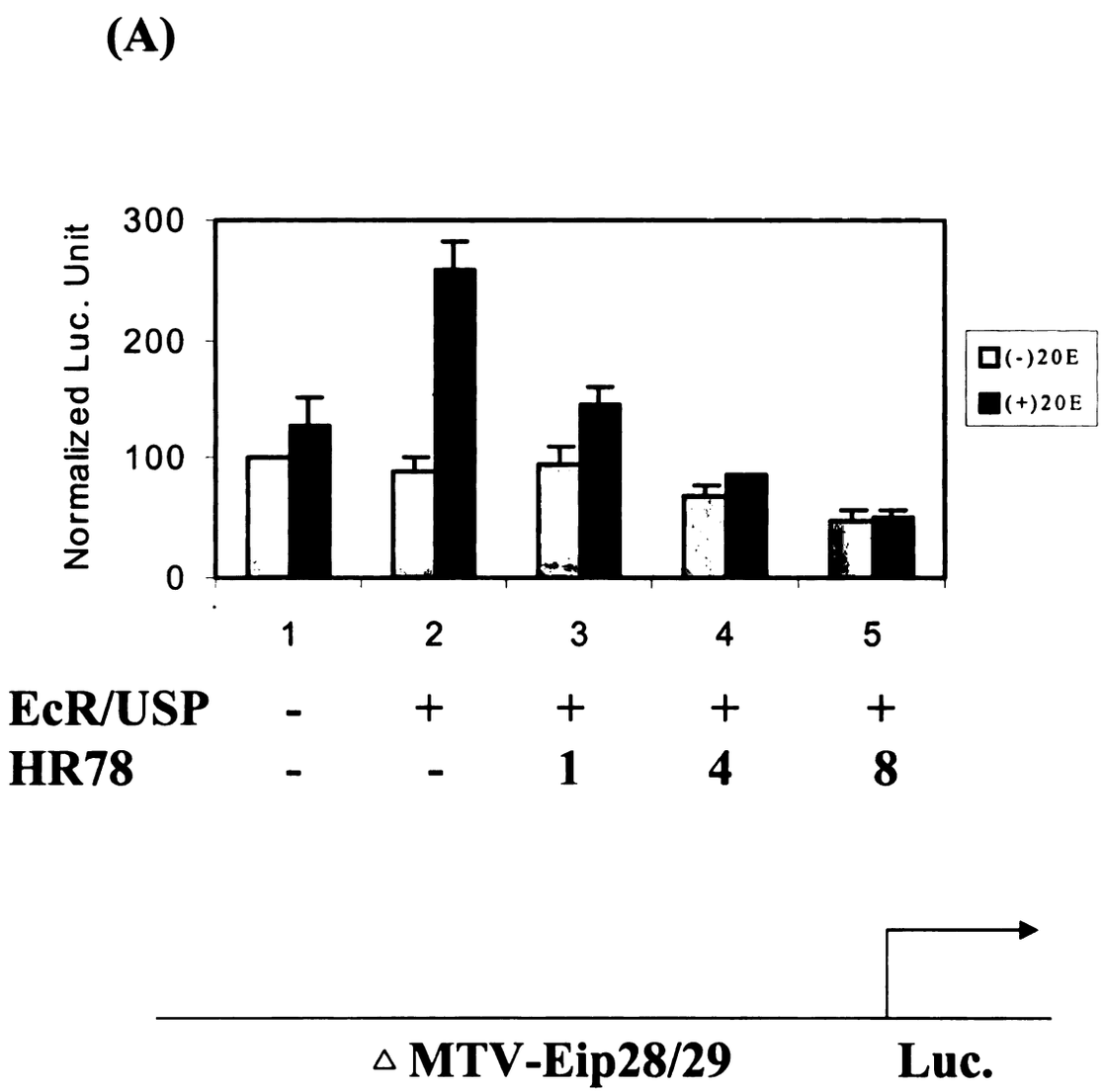

Fig 6. AHR78 inhibition of 20E-stimulated transactivation by AaEcR/AaUSP in S2 cells. **(A)** AHR78 inhibition of 20E-stimulated transactivation of the Eip-luc by AaEcR/AaUSP in S2 cells. The reporter plasmids (Eip-luc,) were transfected into *Drosophila* S2 cells with no expression plasmid (column 1), or with AaEcR/AaUSP expression plasmids (lane 2) and increasing molar excesses of AHR78 expression plasmids (lane 3, 4 and 5) (see Materials and Methods). After transfection, cells were incubated either in the absence or in the presence of hormone 20E at 10^{-6}M for 27 hr and harvested for β-galactosidase and luciferase activity assays. Luciferase activity was normalized with β-galactosidase activity.

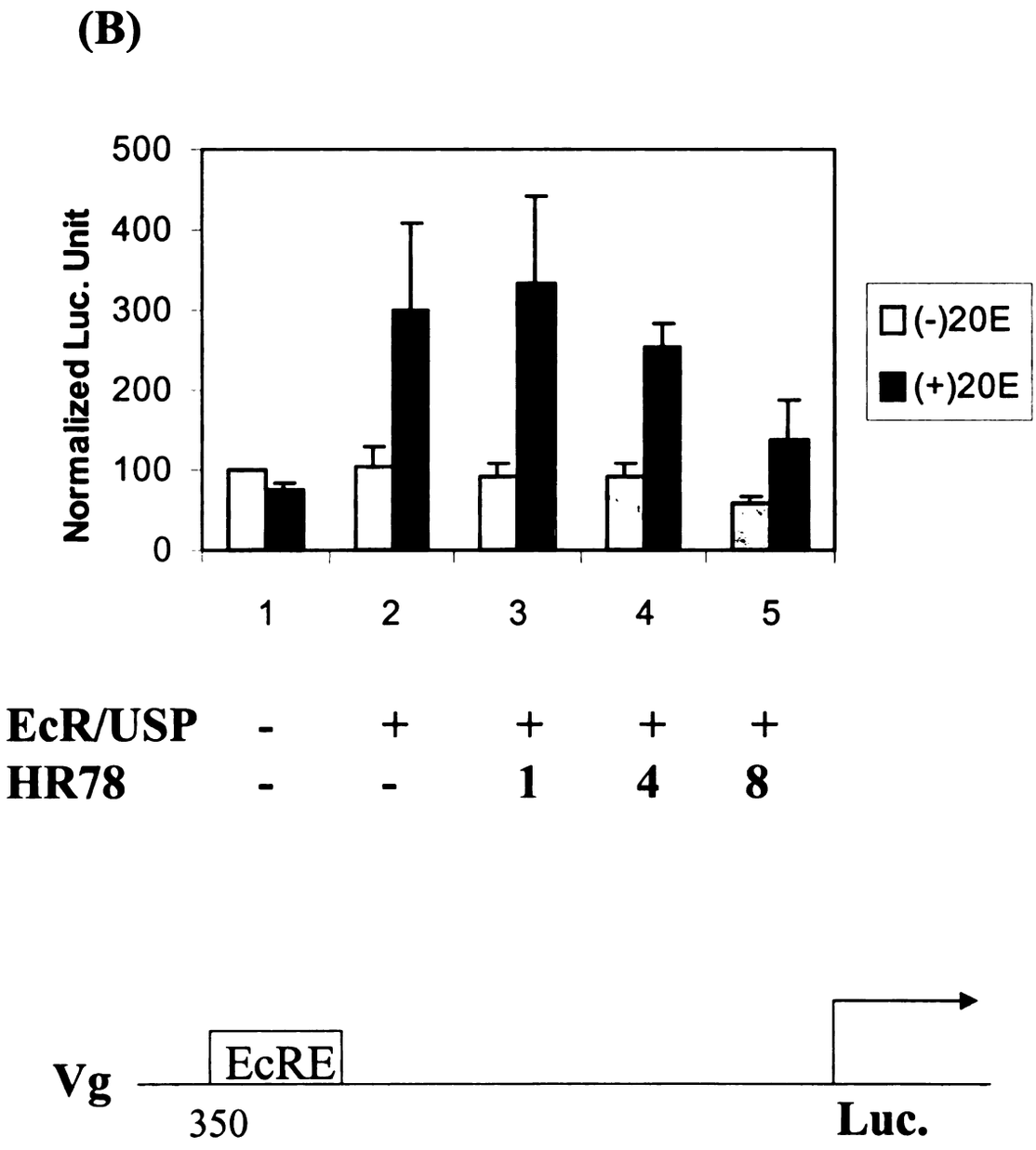

Fig. 6. (Cont'd). **B)** AHR78 inhibition of 20E-stimulated transactivation of the Vg-luc by AaEcR/AaUSP in S2 cells. This experiment was performed similarly to the panel A except that the Vg-luc reporter plasmid was used in this experiment. Each value represents the mean ±SD (n=3).

affinity of hsp27-EcRE with EcR/USP and, as a result of it, high background levels of reporter gene activities in this experiment (not shown).

In addition to examining the effect of AHR78 on the two *Drosophila* EcREs, I investigated the effect of AHR78 on the Vg-EcRE, which has been demonstrated to be responsive to EcR/USP (Martin *et al.*, 2001). The reporter activities, driven by a 348bp upstream region of the Vg promoter, increased by 3-fold in responsive to EcR/USP in the presence of 20E (Fig. 6B, columns 1 and 2.). The activation by 20E was not affected by a small amount of AHR78 (Fig. 6B, column 3); whereas it was decreased significantly upon co-transfection of increasing amounts of AHR78 (Fig. 6B, columns 4 and 5). Other promoter-reporter constructs consisting of either 2kb, 1kb or 600bp of the Vg promoter yielded less conclusive results (not shown). This is likely due to the complexity of the Vg promoter and interfering factors in the S2 cells, making the effect of AHR78 invisible. Taken together, these results suggest that AHR78 inhibits the EcR/USP transactivation on various EcREs, most importantly on the native Vg EcRE.

DISCUSSION

In this study, I report the cloning and characterization of AHR78, the mosquito homolog of HR78 in other insects and vertebrate TR2. A search of the gene data bank using the full AHR78 amino acid sequence revealed that AHR78 exhibits the highest similarity with the members of this HR78/TR2 subfamily, rather than other subfamilies of the nuclear receptor superfamily (not shown). AHR78 consists of 5 classical functional domains of the nuclear receptors (Fig. 2). While DBDs of this subfamily are highly conserved, LBDs are less similar relative to other members of the nuclear receptor

superfamily. Although the AHR78 LBD shows 33% identity with DHR78, this is the closest homology it shares among nuclear receptors. Similarly, *Tenebrio molitor* TmHR78 shows only 31% identity with DHR78 LBD (Mouillet *et al.*, 1999). These results suggest that members of the HR78/TR2 subfamily of nuclear receptors have evolved rapidly, particularly in this domain.

Analysis of expression profiles of *HR78*s during insect development reveals the similar temporal correlations with ecdysteroid levels. In the mosquito fat body, AHR78 exhibited only negligible expression levels throughout the previtellogenic and vitellogenic stages. In contrast, in the ovary, AHR78 levels rose dramatically after a blood meal with peak expression occurring at 18-24 hr PBM correlating positively with the ecdysteroid peak in the mosquito (Fig. 3). During *Drosophila* metamorphosis, DHR78 is expressed throughout development with peaks of expression in third instar larvae and prepupae that is correlated with known ecdysteroid pulses (Fisk and Thummel, 1995; Zelhof *et al.*, 1995b). Similar correlation between expression levels and ecdysteroid variations has been reported for TmHR78 (Mouillet *et al.*, 1999). The correlations between expression levels of HR78 and ecdysteroid levels further support that AHR78 is a member of the HR78 family, despite low conservation levels of the LBD amino acid sequences.

In *Drosophila,* it has been demonstrated by DHR78 mutants that DHR78 functions at the top of the ecdysteroid regulatory hierarchies. DHR78 mutations block ecdysteroid-regulated gene expression in mid-third instar larvae (Fisk and Thummel, 1998). In addition, DHR78 protein binds as a homodimer to a subset of EcR/USP binding sites *in vitro*; therefore, it has been proposed that DHR78 can function to regulate

ecdysone responses negatively (Thummel, 1995). However, in mosquitoes direct binding of AHR78 to EcREs was not observed. This was probably due to sensitivity of the experiment, or the evolutionary divergence among insect HR78, particularly in LBDs. One of the LBD functions in a classical nuclear receptor is to form a receptor homodimer. The less conserved LBDs in the HR78 subfamily may explain why AHR78 did not bind to EcREs directly. In mosquitoes, the direct interaction of AHR78 and EcR/USP was observed *in vitro* in the GST-pulldown assays (Li, C. and Raikhel, A, unpublished). This result suggested that AHR78 negatively regulated the ecdysone responses in a different way. The direct interaction of AHR78 and EcR/USP may prevent the AaEcR/AaUSP binding to EcREs. Although it has not been reported in other insects that HR78 proteins can interact directly with EcR or USP, DHR78 protein is bound to many ecdysteroid-regulated puff loci, suggesting that DHR78 may interact directly with EcR or USP (Fisk and Thummel, 1998). In addition, consistent with findings in *Drosophila* DHR78, AHR78 inhibits the 20E-induced activation of target genes in cell transfection assays (Fisk and Thummel, 1995; Zelhof *et al.*, 1995b; this paper).

One unique characteristic of AHR78 is that it is highly expressed in the ovary during the vitellogenic stage (Fig. 3B). YPP genes are silent in the ovary despite the presence of both EcR and USP (Wang *et al.*, 2000a). Taken together with the role of AHR78 in inhibition of the Vg promoter, it suggests that AHR78 may be responsible for the tissue-specific expression of the YPP genes, such as the inactivity of the Vg gene in the ovary. Further elucidation of the functions of AHR78 may provide new insights in our understanding the nature of ecdysone gene regulatory hierarchies in general as well as ecdysteroid regulation of mosquito vitellogenesis in particular.

ACKNOWLEDGMENTS

This work was supported by a grant (AI36959) from the National Institutes of Health. I thank Dr. Kook-Ho Cho for performing the ovary culture experiment, Mr. Alan Hays for his excellent assistance in maintaining the mosquito culture, Dr. M. Ken for contributive help at the initiation of this project, Dr. F. Kafatos for the generous gift of anti-DmUSP antibodies, Dr. M. McKeown for providing reporter constructs, and Dr. D. Martin for providing Vg-luc constructs.

Chapter 7

Summary and Future Research Perspectives

Summary and Future Research Perspectives

Ecdysone gene regulation is one of best systems for studying eukaryotic transcription regulation. In *Drosophila*, this system has been studied for several decades, and recent discoveries have made significant advances in the molecular mechanism of the ecdysone gene regulatory hierarchies during metamorphosis. In contrast, in other insects, though ecdysone is one of the key hormones and regulates many events including development, metamorphosis and reproduction, the molecular basis of the ecdysone gene regulation hierarchy was poorly understood. This is particularly so in ecdysone gene regulation of insect reproduction. This insufficiency has made the general fundamental molecular mechanism of ecdysone gene regulation in insects unclear.

In this dissertation, I have reported the extensive investigation of the molecular basis of ecdysone gene regulation in female mosquito reproduction. Characterizations of many elements of the ecdysone gene regulation cascade have demonstrated that the general molecular mechanism underlying ecdysone gene regulation appears to be conserved between mosquito vitellogenesis and fly metamorphosis. These suggest that despite the diversities of physiological functions of ecdysone in insects, the fundamental molecular features are considerably similar. In the future research, I propose to study the following aspects:

Regulatory regions of the AaUSP gene(s).

As described in Chapter 2, mosquito USP displays interesting characteristics: 1). The presence of two isoforms with distinct N-termini, 2). Differential expression and regulation by 20E of the two mosquito USP isoforms during vitellogenesis. 3). Direct

regulation of AaUSP-B by 20E and indirect regulation of AaUSP-A by 20E. The AaUSP-A expression profile negatively correlates with ecdysteroid titers while the AaUSP-B isoform expression shows positive correlation with ecdysteroid titers during mosquito vitellogenesis. *In vitro* organ culture experiments have suggested that the differential expression of mosquito USP isoforms is controlled by 20E. AaUSP-A expression is upregulated by the falling 20E titer while AaUSP-B is upregulated by the rising 20E titer. Moreover, the upregulation of AaUSP-B is enhanced in the presence of cycloheximide (Chx), a protein synthesis inhibitor; whereas the upregulation of AaUSP-A is abolished in the presence of Chx. These interesting differential regulation features lead us to ask the questions: what is the molecular basis of remarkable differential regulation of the two USP isoforms? How does the gene structure of *USP* facilitate this regulation? Does it result from alternative splicing of a single gene or from two separated genes? In insects, most known nuclear receptor isoforms are generated by alternative splicing and/or differential promoter utilization, though in vertebrates many nuclear receptor isoforms result from different genes. Examination of the mosquito *USP* gene will definitely provide more information about gene structure and regulation in general as well as those specifically involved in vitellogenesis.

The clear answers to the questions above will first rely on genomic cloning of the mosquito *USP* gene. Using the two *AaUSP* cDNA clones to screen the genomic library can be the method to obtain the genomic structure of AaUSP. Genomic Southern blot can also be utilized to perform hybridization with probes generated by different 5'UTR or N-termini of *AaUSP* cDNAs. In this way, it will be known whether the two AaUSP isoforms are derived from different genes or from different promoters of a single gene.

Moreover, further identification of the transcriptional units of *AaUSP* is necessary, which can be done by primer extension or RNase protection assays. The next step will be to clone the promoter regions of the two AaUSP isoforms and to functionally study the differential regulation of *AaUSP* by 20E.

Once the two promoters are identified, I propose to construct a promoter-reporter plasmid and co-transfect them with EcR/USP into the *Drosophila* S2 cell line. We will then be able to examine how the two *USP* promoters respond to the presence or absence of 20E. Furthermore, a series of deletions of the *AaUSP* promoters will be constructed to map the differential responsive regions on the different promoters. EMSAs can be performed to test whether there is a direct binding site of EcR/USP on the AaUSP-B promoter. Through this procedure, we will be able to obtain the answer concerning the ecdysone responsive regions of two AaUSP isoforms.

The early gene (E75) regulation in the ecdysone genetic regulatory hierarchy.

As described in Chapter 3, the mosquito homolog of *Drosophila E75* has been cloned, which is a crucial early gene involved in *Drosophila* metamorphosis. E75 belongs to the nuclear receptor superfamily and has a counterpart in vertebrates, Rev-erb. Previous data has demonstrated that *E75* is directly induced and responsive earlier than Vg in the mosquito fat body by the steroid hormone 20E, suggesting that *E75* is involved in the activation and maintenance of the mosquito vitellogenic response to ecdysone. Next, I propose to use the biochemical and genetic approaches to investigate how *E75* is regulated and how it controls its target genes during mosquito vitellogenesis.

Three cDNA isoforms and the genomic DNA of mosquito *E75* have been cloned. I propose to extend these studies by identifying the transcription start site of each transcriptional unit, and further to identify the promoter of mosquito *E75*.

As shown in Chapter 3, *E75* gene expression is induced by 20E. After identifying the *E75* promoter, we can scan the promoter sequence for putative EcR/USP binding sites using the consensus binding site of EcR/USP as a reference. These putative binding sites will be tested using *in vitro* DNA binding assays to identify the EcR/USP binding sites on the *E75* promoter. Furthermore, I propose to test whether these binding sites are functional *in vivo* by cell transfection assays, using reporter genes driven by the different *E75* promoter deletions, co-transfected with EcR/USP expression plasmids. Moreover, we can study the molecular basis of the auto-inhibition of the E75 gene. It has been demonstrated that the induction by 20E of *E75* is significantly increased in the presence of cycloheximide, a protein synthesis inhibitor, suggesting that the E75 protein likely represses its own gene promoter. I propose to utilize the same strategy used above to identify potential binding sites of E75 on its own gene promoter, and test the action of the E75 protein on its own promoter by cell transfection assays. Finally, we can characterize the interaction between E75 and EcR/USP on the *E75* promoter, and investigate how activation by EcR/USP and repression by E75 on the *E75* gene promoter lead to sustained expression of *E75*.

The induction of the *E75* gene positively correlates with that of vitellogenin gene both *in vitro* and *in vivo*. The putative E75 binding sites have been found on the Vg promoter (Martin and Raikhel, unpublished). Furthermore, it has been shown in cell transfection assays using the Vg promoter carrying a reporter gene that E75 up-regulates

the reporter expression (Chapter 3). Next, I propose to identify the functional binding sites of E75 on the Vg promoter, and then compare the differences between each E75 responsive element on the *E75* and *Vg* promoters. Additionally, we can test whether the different responsive elements result in different E75 activities. As shown in Chapter 3, EcR/USP and E75 synergistically activated the Vg promoter in *Drosophila* S2 cells. Next, I propose to characterize the potential different molecular mechanisms of the action of both EcR/USP and E75 on different promoters (Vg and *E75*). Furthermore, we can use *Drosophila* to further characterize mosquito *E75*, by crossing the *Drosophila E75* mutant or the ectopically inducible *E75 Drosophila* with the mosquito-Vg-promoter-transgenic *Drosophila,* both of which are available. In the future, if possible, we can make a transgenic mosquito to characterize the action of E75 and EcR/USP on the Vg promoter, so that we can obtain a much better understanding of the molecular mechanism of indirect ecdysone control of vitellogenic genes.

Molecular basis of competence and its regulation for vitellogenic responses to ecdysone.

Results from AaFTZ-F1 studies (Chapter 5) indicate that AaFTZ-F1 serves as a competence factor for controlling vitellogenic responses to ecdysone during the previtellogenic period. This is based on two lines of evidence: one is that in *in vitro* fat body culture, following the high level of AaFTZ-F1expression, induction of E75A and VCP by 20E increases significantly; the second lines of evidence is that binding of nuclear extracts from newly enclosed, incompetent female fat bodies to the FTZ-F1 responsive element is undetectable, while binding is clearly detected in nuclear extracts

from 3~5-day-old competent fat bodies. These observations lead us to believe that competence of mosquito vitellogenic responses to ecdysone correlates with the appearance of functional AaFTZ-F1 protein. Since AaFTZ-F1 mRNA is abundant throughout the entire previtellogenic stage, regulation of AaFTZ-F1 binding activities could be by either translational and/or post-translational control. Preliminary Western blot analysis with *Drosophila* FTZ-F1 antibodies indicated that AaFTZ-F1 protein was present during the entire previtellogenic stage. This result needs to be confirmed by Western blot using antibodies specific to AaFTZ-F1. If the possibility of translational control is ruled out, we can focus on examining post-translational control. A common post-translation modification is phosphorylation. We will first determine if this regulating system is being mediated by phosphorylation. Interestingly, steroidogenic factor 1 (SF-1), a vertebrate homolog of the insect FTZ-F1, has been demonstrated to be post-translationally regulated. Activation of SF-1 mediated transcription via interaction with nuclear receptor coactivators depends on the phosphorylation of a single serine residue in the AF-1 domain of SF-1 (Hammer *et al.*, 1999). Moreover, the silkworm BmFTZ-F1 activation function is mediated by the coactivator MBF1, which stabilizes the BmFTZ-F1-DNA complex (Li *et al.*, 1994). To test whether phosphorylation of AaFTZ-F1 contributes to vitellogenic competence, we can introduce protein kinases or their inhibitors into EMSA experiments; or alternatively, in organ culture experiments, we can enhance/inhibit the phosphorylation in the fat body by kinases or their inhibitors.

Furthermore, another interesting question is whether the acquisition of competence via AaFTZ-F1 is under hormonal control. Early studies have established that the acquisition of competence by the mosquito fat body for the vitellogenic response to

ecdysone is controlled by juvenile hormone (JH) (Flannagan and Hagedorn, 1977). Treatment of previtellogenic incompetent fat bodies with JH renders this tissue capable of synthesizing vitellogenin protein in response to 20E (Flannagan and Hagedorn, 1977; Ma *et al.*, 1988). Our preliminary experiments have revealed that after incubation of newly eclosed incompetent fat bodies with 10^{-6} M JHIII for 24 hr followed by addition of 10^{-6} M 20E, the induction of VCP mRNA was significantly increased, as shown by RT-PCR/Southern blot (Li C, and Raikhel, A., unpublished). Therefore, one interesting question is whether JH-controlled competence and AaFTZ-F1-involved competence are in the same pathway. To answer this question, we can perform EMSAs to examine the DNA binding activity of nuclear extracts from newly eclosed, 24hr JH III-treated female fat bodies. This experiment will provide direct evidence of whether the active DNA-binding AaFTZ-F1 protein results from JH action in the fat body.

In vertebrates, recent discoveries have established that two ligands, oxysterols and 25-Hydroxycholesterol, are capable of stimulating SF-1 dependant transcription. This suggests that SF-1 may be a nuclear hormone receptor rather than an orphan receptor (Bertherat, 1998). Therefore, a more interesting question for AaFTZ-F1 will be whether it is a JH receptor. A simple experiment can be performed by cell transfection assay, using a promoter-reporter construct containing FTZ-F1 responsive elements. Co-transfection of both this reporter construct and AaFTZ-F1 expression plasmids into *Drosophila* S2 cells (and/or CV-1 cells) in the presence or absence of JHIII or its analog methoprene should show if FTZ-F1 is responsive to JH or its analogs.

AHR78 function in vitellogenic ovaries

In Chapter 6, AHR78 studies have demonstrated that AHR78 is expressed at very low levels in the fat body and the previtellogenic ovary, but expressed at considerably high levels in the vitellogenic ovary. Functional analysis has established that AHR78 acts as a repressor, inhibiting DNA binding and transactivation of EcR/USP. These results suggest that AHR78 may be involved in inhibiting activation of *YPP* genes in the ovary. To further support this hypothesis, first, we should specify the localization of AHR78 expression within the ovary due to the fact that the mosquito ovary contains several different tissues including oocytes, nurse cells and follicle cells. The main challenge of this experiment lies in the relatively low levels of expression of AHR78 mRNA. An AHR78 anti-sense mRNA probe can be used in *in situ* hybridization experiments. Antibodies against the AHR78 can also be raised to examine the AHR78 protein levels in the ovary. Second, further EMSA analysis of AHR78 inhibition of EcR/USP binding to EcREs will be conducted by nuclear extracts from both fat bodies and ovaries. Previous studies in our lab have successfully detected specific binding to EcREs of EcR/USP from nuclear extracts in both fat bodies and ovaries (Miura *et al.*, 1999). In this proposed study, *in vitro* produced AHR78 protein will be added into the binding reactions to test whether it will abolish the native EcR/USP proteins binding to EcREs. Furthermore, preliminary GST pull-down experiments indicate that AHR78 interacts with EcR and USP. Next, co-immunoprecipitation experiments will be designed to examine whether the interactions of AHR78 with EcR/USP occur *in vivo*. These studies will provide more valuable information regarding the potential inhibitory role of AHR78 on EcR/USP action.

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