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CHARACTERIZATION OF THE MOSQUITO ECDYSTEROID RECEPTOR

by

Sheng-Fu Wang

A DISSERTATION

submitted to

Michigan State University

In partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

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1999

ABSTRACT

CHARACTERIZATION OF THE MOSQUITO ECDYSTEROID RECEPTOR

BY

SHENG-FU WANG

The functional receptor for insect steroid hormone, ecdysteroid, is a heterodimer consisting of two nuclear receptors, ecdysteroid receptor (EcR) and a homologue of retinoid X receptor, *Ultraspiracle* (USP). I investigated properties of the mosquito *Aedes aegypti* ecdysteroid receptor (AaEcR-USP) with respect to its binding activity to DNA, transactivation and effect of ligand on these activities. AaEcR-USP binds DNA elements arranged either as inverted or direct repeats with the consensus half-site AGGTCA. Direct DNA binding and competition assays as well as estimation of equilibrium dissociation constants (K_d) indicated that a 1-bp spacer is optimal among inverted repeats (IR-1) and a 4-bp spacer optimal among direct repeats (DR-4) for binding to the mosquito EcR-USP complex. Co-transfection assays utilizing mammalian CV-1 cells demonstrated that EcR-USP is capable of transactivating reporter constructs containing either IR-1 or DR-4. The levels of transactivation are correlated with respective binding affinities of the response elements.

Two USP isoforms with different N-termini, USPa and USPb, display distinct tissue- and stage-specific patterns of expression during mosquito vitellogenesis. I showed that EcR-USPb binds DNA with twice the affinity than that of the EcR-USPa; accordingly EcR-USPb transactivates a reporter gene twice more efficiently than EcR-USPa in CV-1 cells. However, the roles of these two heterodimers in regulating mosquito genes remain to be elucidated.

Although ecdysone is commonly considered to be an inactive precursor of 20-hydroxyecdysone(20E), the steroid biochemistry in insects is not fully understood. Recent cloning of genes encoding EcR and USP proteins from different insect species has provided an opportunity to elucidate the molecular mechanisms underlying the physiological function of various ecdysteroids. As a step toward characterization of the ligand species specificity, I analyzed activity of various ecdysteroids using gel mobility shift assays and transfection assays in *Schneider-2* (S2) cells. As expected, Ecdysone did not activate the *Drosophila melanogaster* EcR-USP (DmEcR-USP). In contrast, this steroid functions as an active ligand on AaEcR-USP, significantly enhancing DNA binding and transactivating a reporter gene in *Drosophila* S2 cells. The mosquito receptor displays higher basal level of DNA binding activity in the absence of ligand than the fruit-fly receptor. Subunit swapping experiments indicated that the EcR protein, not the USP protein, is responsible for ligand specificity. Using domain swapping techniques, I made a series of *Aedes* and *Drosophila* EcR chimeric constructs. The ligand-specific region was mapped near the C-terminal of the ligand binding domain, termed as the I-box defining dimerization specificity of nuclear receptors. This region is located at the loop connecting Helices 9 and 10 and the N-terminal of Helix 10, as determined by comparison with available crystal structures obtained from other homologous nuclear receptors. Site-directed mutagenesis revealed that *Tyr611* in *Drosophila* EcR, whose corresponding residue in *Aedes* EcR is *Phe529*, is most critical for ligand specificity and basal level of DNA binding activity. These results demonstrate that Ecdysone could function as a *bona fide* hormone ligand in a species-specific manner.

To my wife, Xiaohong Hu

To my son, Kevin V. Wang

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LIST OF ABBREVIATIONS

22A 20-hydroxyecdysone -22-acetate

20E 20-hydroxyecdysone

2DE 2-deoxy-20-hydroxyecdysone

Aa *Aedes aegypti*

Aam tick *Amblyomma americanum*

β-Gal β-galactosidase

Bm silkworm *Bombyx mori*

bp base pair

CAT chloramphenicol acetyltransferase

Cc Mediterranean fruit fly *Ceratitis capitata*

cDNA complementary DNA

Ce *Caenorhabditis elegans*

Cf spruce budworm *Choristoneura fumiferana*

Cp Atlantic sand fiddler crab *Celuca pugilator*

Ct midge *Chironomus tentans*

DBD DNA binding domain

Dm *Drosophila melanogaster*

DMEM Dulbecco's modified Eagle's medium

DNA deoxyribonucleic acid

DR direct repeat

Ecd ecdysone

EcR ecdysteroid receptor

EcRE ecdysteroid response element

EDTA ethylenedinitrilo tetraacetic acid

Eip ecdysone inducible protein

EMSA electrophoretic gel mobility shift assay

ER estrogen receptor

GR Glucocorticoid receptor

HRE hormone response element

Hs *homo sapiens*

Hsp heat shock protein 27

Hv tobaco budworm *Heliothis virescens*

IR inverted repeat

JH juvenile hormone

Kb kilobase pair

Kd equilibrium dissociation constant

LBD ligand binding domain

Lc sheep blowfly *Lucilia cuprina*

Lm migratory locust *Locusta migratoria*

Luc luciferase

Met methoprene

MMTV mouse mammary tumor virus

Ms tobacco hornworm *Manduca sexta*

MR mineralacorticoid receptor

mRNA message RNA

MurA muristerone A

PAGE polyacrylamide gel electrophoresis

PBM post-blood meal

PCR polymerase chain reaction

Per perfect

PolB polypodine-B

PonA ponasterone-A

RAR retinoic acid receptor

RNA ribonucleic acid

RT reverse transcription

RXR retinoid X receptor

S2 *Drosophila* Schneider cell line 2

SDS sodium dodecyl sulphate

Sm *Schistosoma mansoni*

Tm yellow mealworm *Tenebrio moliter*

TR thyroid hormone receptor

USP *Ultraspiracle*

VCP vitellogenic carboxypeptidase

Vg vitellogenin

VDR vitamin D_3 receptor

INTRODUCTION

Mosquitoes transmit a variety of infectious diseases, namely malaria, yellow fever, dengue fever, dengue hemorrhagic fever, chikungunya, filariasis-bancroftian, Japanese encephalitis and Venezuelan equine encephalitis, among which malaria is the most deadly disease. Due to drug resistance of the parasites and insecticide resistance of the mosquito vector population, vector borne diseases are becoming more serious, and resurgences are occurring in areas once thought to be under control (Gratz, 1999). To make the situation even worse, recent ecological changes have fostered vector proliferation. Currently, World Health Organization estimates that the risk of malaria infection exists to at least some degree 100 countries and territories which account for over 40% of the world population. About 300-500 million people are infected and 1.5-2.7 million people are killed by malaria each year. Long term control strategies for mosquito-borne diseases depend upon the development of a vaccine against the parasite and genetic manipulation of the mosquitoes to confer resistance to the parasite. Recently, large effort has been invested on vaccine development, but a successful vaccine is not likely to be available in the foreseeable future due to the complexity of the parasite's life cycle.

Our research group, headed by Dr. Alexander S. Raikhel, has been focusing on investigating the molecular mechanism underlying mosquito vitellogenesis, the production of yolk proteins in the fat body and accumulation of yolk proteins in the developing oocyte. Mosquito vitellogenesis is initiated by a blood meal, through which the susceptible female acquires a pathogen from an infected host. The pathogen can be injected into a healthy individual when the female seeks a second blood meal. Understanding the mechanism of

vitellogenesis may lead to alternative mosquito control strategies. First, each procedure in the yolk protein production process can be targeted for insecticide development. Reciprocally, vitellogenesis can be utilized to deliver anti-parasite genes to render mosquitoes resistant to the parasites.

Upon blood ingestion, the female mosquito ovary is stimulated to produce the steroid hormone ecdysteroid, which stimulates the fat body to express yolk protein precursors, namely vitellogenin (Vg) and vitellogenic carboxy peptidase (VCP) (Raikhel, 1992). Accordingly, blood meal triggered yolk protein production is mediated by the ecdysteroid, which exerts function through the intracellular receptor, ecdysteoroid receptor. The functional ecdysteroid receptor is a heterodimer consisting of two protein subunits: ecdysteroid receptor (EcR) and ultraspiracle (USP), homologue of the vertebrate retinoid X receptor (RXR) (Yao et al., 1992; 1993; Thomas et al., 1993). This dissertation intends to characterize the functional properties of the mosquito *Aedes aegypti* ecdysteroid receptor, its DNA binding activity, transactivation efficiency and ligand specificity. I will first examine the candidacy of a variety of oligonucleatides as ecdysteroid response elements, then proceed to the characterization of USP isoforms, and conclude with the differential response of the mosquito and *Drosophila melanogaster* ecdysteroid receptors to the steroid ecdysone.

CHAPTER 1. MOLECULAR CHARACTERISTICS OF ECDYSTEROID RECEPTORS

Ecdysteroid regulated gene expression cascade in the fruit-fly and mosquito

Upon ecdysteroid injection, two series of chromosome puffs are induced from the salivary gland of the midge *Chironomus tentans.* The first series is not affected by the protein synthesis inhibitor cycloheximide whereas the later puff is prevented by the inhibitor (Clever and Karlson 1960). These results prompted Karlson to first propose that the steroid hormones act by way of activation of gene expression to stimulate mRNA synthesis (Karlson, 1963). Ashburner and his colleagues observed that ecdysteroid exerts similar effects on the chromosome puffing pattern of the salivary gland from the fruit fly *Drosophila melanogaster*. Moreover, Ashburner noticed that the early puffs require continued exposure to ecdysteroid to remain expanded and regress after a certain period of time. Based on these observation, Ashburner formulated a model to describe steroid regulation of gene expression. The steroid hormone, associated with a receptor, binds to DNA elements to stimulate the transcription of a small set of early genes and suppress the transcription of later genes. The early gene products activate the transcription of later genes and repress the transcription of early genes (Ashburner *et al.*, 1974).

A variety of steroid hormones, such as glucocorticoids, mineralcorticoids, estrogen, androgen and progesterone, have been identified in vertebrates to regulate a myriad of pathways ranging from development and morphogenesis to reproduction, behavior and homeostasis (Tsai and O'Malley 1994; Mangelsdorf et al., 1995). Although the first steroid hormone was isolated from insects, the first steroid hormone receptor cloned was the

vertebrate human glucocorticoid receptor (GR) (Hollenberg et al., 1985). In vertebrates, a large body of information has been accumulated about the mode of action of steroid hormones as well as their interaction with receptors and target genes. Along with receptors for thyroids, retinoids, vitamin D, prostaglandins and oxysterols, steroid hormone receptors form a family of lignad-regulated transcription factors known as the nuclear receptor superfamily (Tsai and O'Malley, 1994; Mangelsdorf and Evans, 1995). Recently, a growing number of homologous transcription factors with unknown ligand, called orphan receptors, have been added into this family (Mangelsdorf and Evans 1995). These receptors usually form homodimers or heterodimers to bind cognate response elements consisting of two hexameric half sites arranged as inverted, direct or everted repeats.

The developmental role of ecdysteroid has been most extensively studied in the *D. menaloganster* during metamorphosis (Riddiford, 1993). A peak of ecdysteroid combined with juvenile hormone (JH) induces the larval-pupae molt. Then both hormones drop to low level. Another surge of ecdysteroid without JH prompts the pupae molt into an adult. Recombinant DNA technology has allowed dissection of the Ashburner model at the molecular level. Three genes, namely E74, E75 and BR-C have been cloned from the early puffs 74EF, 75B and 2B5 respectively, each of which contains several isoforms. The ecdysteroid regulated gene expression cascade is elucidated using a combination of techniques including northern and western blotting, mutant analysis and ectopic expression of exogenous genes. In the early third-instar, low level of ecdysteroid allows transcription of EcR, E74B and BR-C. High level of ecdysteroid in late third instar stimulates a set of early genes including E75A, E74A and BR-C. These early genes activate expression of larval

specific late genes. A surge of ecdysteroid in pupae induces another series of early and late genes (Thummel, 1997).

Although ecdysteroid was originally identified as a molting hormone, it was later discovered that this steroid hormone plays profound roles in embryogenesis, nervous system development as well as reproduction (Lanot et al., 1989; Hagedorn, 1989). The mosquito *Aedes aegypti* provides a perfect model to study the functionality of ecdysteroid in reproduction as ecdysteroid induced vitellogenesis in female mosquito is totally controlled by a blood meal (Raikhel, 1992). More importantly, understanding the mechanism of mosquito vitellogenesis can lead to designing efficient strategies for combating this deadly disease vector. After adult emergence, the female mosquito enters the previtellogenenic stage accompanied by high level of JH which coordinates the fat body and ovary to gain commitment for large scale yolk protein production. A blood meal triggers an endocrine release of ecdysteroid, which stimulates the fat body to produce yolk protein precursors. These proteins are released into the hemolymph to be deposited into the ovary for later embryogenesis. Expression of the yolk protein genes, specifically vitellogenin (Vg) and vitellegenic carboxy peptidase (VCP), is induced by ecdysteorid but repressed by cycloheximide, suggesting a ecdysteroid regulated gene expression cascade similar to that in fly metamorphosis is employed by the mosquito fat body. In support of this hypothesis, transcription of the mosquito E75 homologue is activated by ecdysteroid and not repressed by cycloheximide; this activation resembles the fly early gene E75 (Pierceall et al., in press).

Understanding the functional properties of ecdysteroid receptor is indispensable in our attempt in elucidating the ecdysteroid regulated gene expression cascade. In this chapter, I am going to review the structural features of EcR and USP proteins cloned in various

arthropodes. In the following chapters, I will present a detailed analysis of the mosquito EcR-USP DNA binding properties and ligand specificity.

Structural properties of the EcR Protein, DBD and LBD

The first EcR gene was isolated from a *Drosophila melanogaster* genomic library by screening with an orphan nuclear receptor E75 probe (Koelle et al., 1991). PCR amplification with primers derived from DmEcR cDNA has permitted the isolation of EcR cDNAs from 12 other arthropod species including four more diptera species, the midge *Chironomus tentans* (CtEcR, Inhof et al., 1993), the yellow fever mosquito *Aedes aegypti* (AaEcR, Cho et al., 1995), the sheep blowfly *Lucilia cuprina* (LcEcR, Hannan and Hill, 1997) and the Mediterranean fruit fly *Ceratitis capitata* (CcEcR, Mintzas, A., Accession: 3393034). In addition, EcR cDNAs have been cloned from four lepdoptera species, the silkworm *Bombyx mori* (BmEcR, Swevers et al., 1995; Kamimura et al., 1996), the tobacco hornworm *Manduca sexta* (MsEcR, Fujiwara et al., 1995) the tobacco budworm *Heliothis virescens* (HvEcR, Martinez, Accession: 2440080) and the spruce budworm *Choristoneura fumiferana* (CfEcR, Kothapalli et al., 1995); one orthoptera species, the migratory locust *Locusta migratoria* (LmEcR, Saleh et al., 1998); one coleoptera species, the yellow mealworm *Tenebrio moliter* (TmEcR, Mouillet et al., 1997); one crustacean species, the Atlantic sand fiddler crab *Celuca pugilator* (CpEcR, Chung et al., 1998) and one ixodidae species, the tick *Amblyomma americanum* (AamEcR, Guo et al., 1997). Protein sequences deduced from these cDNA sequences display five characteristic nuclear receptor domains A/B (54-300aa), C (66aa), D (74-109aa), E (219-224aa) and F (3-227aa) (Fig. 1). The 66aa domain C is also called DNA-binding-doamin (DBD) as it harbors two C_2C_2 zinc modules

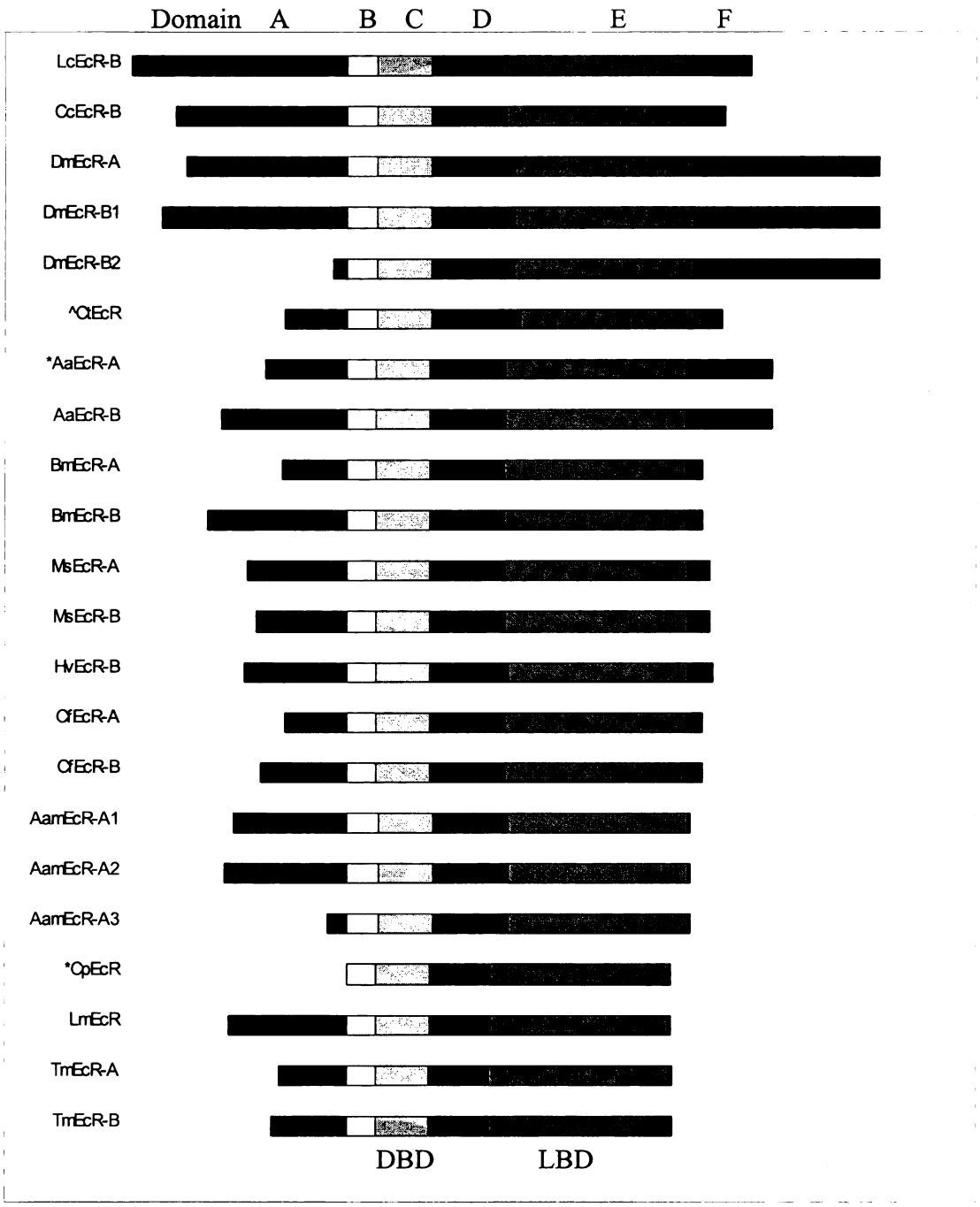


Fig.1. Schematic diagram of EcR proteins. EcR proteins contain 6 characteristic domains. Domain A is usually isoform specific. Domain B is designated as a short stretch of aa proceeding the domain C, which is the DNA binding domain. Domain D is the hinge region. Domain E is the ligand binding domain. Domain F is the C-terminal tail with unknown function. N-termini of AaEcR-A and CpEcR, indicated by an asterisk, are not complete.

```
                                                                DBD
                                                                ---->
HvEcR GREELSPASS VNGCST.... DGEARRQKKG PAPRQQEELC LVCGDRASGY
MsEcR GREELSPASS INGCST.... DGEPRRQKKG PAPRQQEELC LVCGDRASGY
CfEcR GREELSPASS INGCST.... DGEARRQKKG PAPRQQEELC LVCGDRASGY
BmEcR GREELSPASS INGCSA.... DADARRQKKG PAPRQQEELC LVCGDRASGY
LcEcR GRDDLSPSSS LNGFSTSDAS DVKK..IKKG PAPRLQEELC LVCGDRASGY
CcEcR GRDDLSPSSS LNGYSANDSC DVKK..IKKG PAPRLQEELC LVCGDRASGY
DmEcR GRDDLSPSSS LNGYSANESC DAKK..SKKG PAPRVQEELC LVCGDRASGY
AaEcR GREDLSPSSS LNGYT..DGS DAKK..QKKG PTPRQQEELC LVCGDRESGY
CtEcR ~~PNSKLDDG NMSVHMGDGL DGKKSSSKKG PVPRQQEELC LVCGDRASGY
LmEcR GREDLSPLS. ..SLNGYSAD SC.DAKKKKG AAPRQQEELC LVCGDRASGY
TmEcR GREDLSP.S. ..SLNGYSAD SC.DSKKKKG PTPRQQEELC LVCGDRASGY
CpEcR ~RDNMSPPS. ..SLSNFGAD SYGDLKKKKG PIPRQQEEMC LVCGDRASGY
AamEcR ~KEEMSPSSG GGGLNGYFVD SFGDPKKKKG PAPRQQEELC LVCGDRASGY

      DR-Box P-Box                    D-Box
HvEcR HYNALTCEGC KGFFRRSVTK NAVYICKFGH ACEMDIYMRR KCQECRLKKC
MsEcR HYNALTCEGC KGFFRRSVTK NAVYICKFGH ACEMDMYMRR KCQECRLKKC
CfEcR HYNALTCEGC KGFFRRSVTK NAVYICKFGH ACEMDMYMRR KCQECRLKKC
BmEcR HYNALTCEGC KGFFRRSVTK NAVYICKFGH ACEMDMYMRR KCQECRLKKC
LcEcR HYNALTCEGC KGFFRRSVTK NAVYCCKFGH ACEMDMYMRR KCQECRLKKC
CcEcR HYNALTCEGC KGFFRRSVTK NAVYCCKFGR SCEMDMYMRR KCQECRMKKC
DmEcR HYNALTCEGC KGFFRRSVTK SAVYCCKFGR ACEMDMYMRR KCQECRLKKC
AaEcR HYNALTCEGC KGFFRRSVTK NAVYCCKFGH ACEMDMYMRR KCQECRLKKC
CtEcR HYNALTCEGC KGFFRRSVTK NAVYCCKFGH ECEMDMYMRR KCQECRLKKC
LmEcR HYNALTCEGC KGFFRRSITK NAVYQCKYGN NCEIDMYMRR KCQECRLKKC
TmEcR HYNALTCEGC KGFFRRSITK NAVYQCKYGN NCEIDMYMRR KCQECRLKKC
CpEcR HYNALTCEGC KGFFRRSITK NAVYQCKYGN NCEMDMYMRR KCQECRLKKC
AamEcR HYNALTCEGC KGFFRRSITK NAVYQCKYGN NCDIDMYMRR KCQECRLKKC
              Helix                                  Helix

       DBD  T-Box         A-Box
       <----
HvEcR LAVGMRPECV VPENQCAMKR KEKKAQREKD KLPVSTTTVD DHMPPIMQCD
MsEcR LAVGMRPECV VPESTCKNKR REKEAQREKD KLPVSTTTVD DHMPAIMQCD
CfEcR LAVGMRPECV VPETQCAMKR KEKKAQKEKD KLPVSTTTVD DHMPPIMQCE
BmEcR LAVGMRPECV IQEPS.KNKD RQRQKKDKGI LLPVSTTTVE DHMPPIMQCD
LcEcR LAVGMRPECV VPENQCAMKR REKKAQKEKD KI.......Q TS.....VCA
CcEcR LAVGMRPECV VPENQCAMKR REKKAQKEKE KQ.......T TG.NSPIICK
DmEcR LAVGMRPECV VPENQCAMKR REKKAQKEKD KM.......T TS.PSSQHGG
AaEcR LAVGMRPECV VPENQCAIKR KEKKAQKEKD KV.......Q TN........
CtEcR LAVGMRPECV VPENQCAIKR KEKKAQKEKD KVPGIVGSNT SS.SSLLNQS
LmEcR LTVGMRPECV VPEYQCAVKR KEKKAQKDKD KPNSTTNGSP EVMMLKDIDA
TmEcR LSVGMRPECV VPEVQCAVKR KEKKAQKEKD KPNSTTNGSP DVIKIE..PE
CpEcR LNVGMRPECV VPESQCQVKR EQKKARDKDK TYPSLGSPIA EDKAAPISPV
AamEcR LSVGMRPECV VPEYQCAIKR ESKKHQ.KD  RPNSTTRESP SALMAPSSVG

HvEcR PPPPEAARIL ECVQHEVVPR FLN.......  ......EKLM EQNRLKNVPP
MsEcR PPPPEAARI. ....HEVVPR FLT.......  ......EKLM EQNRLKNVTP
CfEcR PPPPEAARI. ....HEVVPR FLS.......  ......DKLL ETNRQKNIPQ
BmEcR PPPPEAARI. ....HEVVPR YLS.......  ......EKLM EQNRQKNIPP
LcEcR T......... E.IKKEILD. LMTCEPPSHP TCPLLPEDIL AKCQARNIPP
CcEcR T......... ESIKNEILE. LMNCEPPSHP TCPLLPDDIV AKCKASNIPP
DmEcR NGSLASGGGQ DFVKKEILD. LMTCEPPQHA TIPLLPDEIL AKCQARNIPS
AaEcR ...ATVSTTN STYRSEILPI LMKCDPPPHQ AIPLLPEKLL QENRLRNIPL
```

```
 CtEcR LNNGSLKNLE ISYREELLEQ LMKCDPPPHP MQQLLPEKLL MENRAKGTPQ
 LmEcR KVEPER.... .......... ........PL SNGIKPVSPE QE........
 TmEcR LSDSEK.... .......... ........TL TNGRNRISPE QE........
 CpEcR SKDMSA.... .......... ........AP RLNVKPLTRE QE........
AamEcR GVSPTSQPMG GGGSSLGSSN HEEDKKPVVL SPGVKPLSSS QE........
```

```
                          LBD                    AF2a
 HvEcR LTANQKSLIA RLVWYQEGYE QPSEEDLKRV TQS....DED DEDSDMPFRQ
 MsEcR LSANQKSLIA RLVWYQEGYE QPSEEDLKRV TQTWQLEEEE EEETDMPFRQ
 CfEcR LTANQQFLIA RLIWYQDGYE QPSDEDLKRI TQTWQQADDE NEESDTPFRQ
 BmEcR LSANQKSLIA RLVWYQEGYE QPSDEDLKRV TQTWQ.SDEE DEESDLPFRQ
 LcEcR LSYNQLAVIY KLIWYQDGYE QPSEEDLKRI M.S..SPDEN ESQHDASFRH
 CcEcR LTRNQLAVIY KLIWYQDGYE QPSEEDLKRI M.S..TPDEN ESPNDISFRH
 DmEcR LTYNQLAVIY KLIWYQDGYE QPSEEDLRRI M.S..QPDEN ESQTDVSFRH
 AaEcR LTANQMAVIY KLIWYQDGYE QPSEEDLKRI MIG..SPNEE EDQHDVHFRH
 CtEcR LTANQVAVIY KLIWYQDGYE QPSEEDLKRI TTE..LEEEE DQEHEANFRY
 LmEcR ....E..LIH RLVYFQNEYE SPSEEDLRRV TS..QPT.EG EDQSDVRFRH
 TmEcR ....ELILIH RLVYFQNEYE HPSEEDVKRI IN..QPI.DG EDQCEIRFRH
 CpEcR ....E..LIN TLVYYQEEFE QPTEADVKKI RF..N.F.DG EDTSDMRFRH
AamEcR ....D..LIN KLVYYQQEFE SPSEEDMKKT TP..FPLGDS EEDNQRRFQH
                              Helix 1
                        Signature Sequence
 HvEcR ITEMTILTVQ LIVEFAKGLP GFAKISQSDQ ITLLK ACSSE VMMLRVARRY
 MsEcR ITEMTILTVQ LIVEFAKGLP GFSKISQSDQ ITLLK ASSSE VMMLRVARRY
 CfEcR ITEMTILTVQ LIVEFAKGLP GFAKISQPDQ ITLLK ACSSE VMMLRVAR Y
 BmEcR ITEMTILTVQ LIVEFAKGLP GFSKISQSDQ ITLLK ASSSE VMMLRVARRY
 LcEcR ITEITILTVQ LIVEFAKGLP AFTKIPQEDQ ITLLK ACSSE VMMLRMARRY
 CcEcR ITEITILTVQ LIVEFAKGLP AFTKIPQEDQ ITLLK ACSSE VMMLRMARRY
 DmEcR ITEITILTVQ LIVEFAKGLP AFTKIPQEDQ ITLLK ACSSE VMMLRMARRY
 AaEcR ITEITILTVQ LIVEFAKGLP AFTKIPQEDQ ITLLK ACSSE VMMLRMARRY
 CtEcR ITEVTILTVQ LIVEFAKGLP AFIKIPQEDQ ITLLK ACSSE VMMLRMARRY
 LmEcR ITEITILTVQ LIVEFAKRLP GFDKLLREDQ IALLK ACSSE VMMFRMARRY
 TmEcR TTEITILTVQ LIVEFAKRLP GFDKLLQEDQ IALLK ACSSE VMMFRMARRY
 CpEcR ITEMTILTVQ LIVEFSKQLP GFATLQREDQ ITLLK ACSSE VMMLRAARRY
AamEcR ITEITILTVQ LIVEFSKRVP GFDTLAREDQ ITLLK ACSSE VMMLRGARKY
          Helix 3                 Helix 4     Helix 5
```

```
 HvEcR DAATDSVLFA NNQAYT RDNY RKAGMAYVIE DLLHFCRCMY SMMMDNVHYA
 MsEcR DAATDSVLFA NNQAYT RDNY RKAGMSYVIE DLLHFCRCMY SMSMDNVHYA
 CfEcR DAASDSVLFA NNQAYT RDNY RKAGMAYVIE DLLHFCRCMY SMALDNIHYA
 BmEcR DAASDSVLFA NNKAYT RDNY RQGGMAYVIE DLLHFCRCMF AMGMDNVHFA
 LcEcR DHNSDSIFFA NNRSYT RDSY KMAGMADNIE DLLHFCRQMY SMKVDNVEYA
 CcEcR DHNSDSIFFA NNRSYT RDAY KMAGVADNIE DLLHFCRQMY SMKVDNVEYA
 DmEcR DHSSDSIFFA NNRSYT RDSY KMAGMADNIE DLLHFCRQMF SMKVDNVEYA
 AaEcR DAATDSILFA NNRSYT RDSY RMAGMADTIE DLLHFCRQMF SLTVDNVEYA
 CtEcR DHDSDSILFA NNTAYT KQTY QLAGMEETID DLLHFCRQMY ALSIDNVEYA
 LmEcR DVNSDSILFA NNQPYT KDSY NLAGMGETIE DMLRFCRQMY AMKVDNAEYA
 TmEcR DVQSDSILFV NNQPYP RDSY NLAGMGETIE DLLHFCRTMY SMKVDNAEYA
 CpEcR DAKTDSIVFG NNYPYT QASY ALAGLGESAE ILFRFCRSLC KMKVDNAEYA
AamEcR DVKTDSIVFA NNQPYT RDNY RSASVGDSAD ALFRFCRKMC QLRVDNAEYA
          S1      S2  Helix 6     Helix 7
```

```
                                        I-Box
HvEcR LLTAIVIFSD RPGLEQPLLV EEIQRYYLNT LRVYILNQNS ASPRGAVIFG
MsEcR LLTAIVIFSD RPGLEQPLLV EEIQRYYLKT LRVYILNQHS ASPRCAVLFG
CfEcR LLTAVVIFSD RPGLEQPQLV EEIQRYYLNT LRIYILNQLS GSARSSVIYG
BmEcR LLTAIVIFSD RPGLEQPSLV EEIQRYYLNT LRIYIINQNS ASSRCAVIYG
LcEcR LLTAIVIFSD RPGLEEAELV EAIQSYYIDT LRIYILNRHC GDPMSLVFFA
CcEcR LLTAIVIFSD RPGLEKAQLV EEIQSYYIDT LRVYIINRHC GDSMSLVFFA
DmEcR LLTAIVIFSD RPGLEKAQLV EAIQSYYIDT LRIYILNRHC GDSMSLVFYA
AaEcR LLTAIVIFSD RPGLEQAELV EHIQSYYIDT LRIYILNRHA GDPKCSVIFA
CtEcR LLTAIVIFSD RPGLEKAEMV DIIQSYYTET LKVYIVNRHG GESRCSVQFA
LmEcR LLTAIVIFSE RPSLVEGWKV EKIQEIYLEA LKAYVDNRR. .RPKSGTIFA
TmEcR LLTAIVIFSE RPSLIEGWKV EKIQEIYLEA LRAYVDNRR. .SPSRGTIFA
CpEcR LLAAIAIFSE RPNLKELKKV EKLQEIYLEA LKSYVENRR. .LPRSNMVFA
AamEcR LLTAIVIFSE RPSLVDPHKV ERIQEYYIET LRMYSENHR. .PPGKN.YFA
      Helix 8                Helix 9

                                                  AF2-AD core
b-eHV EILGILTEIR TLGMQ NSNMC ISLKLKNRKL PPFLEEIWD
MsEcR KILGVLTELR TLGTQ NSNMC ISLKLKNRKL PPFLEEIWD
CfEcR KILSILSELR TLGMQ NSNMC ISLKLKNRKL PPFLEEIWD
BmEcR RILSVLTELR TLGTQ NSNMC ISLKLKNRKL PPFLEEIWD
LcEcR KLLSILTELR TLGNQ NAEMC FSLKLKNRKL PKFLEEIWD
CcEcR KLLSILTELR TLGNQ NAEMC FSLKLKNRKL PKFLEEIWD
DmEcR KLLSILTELR TLGNQ NAEMC FSLKLKNRKL PKFLEEIWD
AaEcR KLLSILTELR TLGNQ NSEMC FSLKLKNRKL PRFLEEIWD
CtEcR KLLGILTELR TMGNK NSEMC FSLKLRNRKL PRFLEEVWD
LmEcR KLLSVLTELR TLGNQ NSEMC FSLKLKNKKL PPFLAEIWD
TmEcR KLLSVLTELR TLGNQ NSEMC ISLKLKNKKL PPFLDEIWD
CpEcR KLLNILTELR TLGNI NSEMC FSLTLKNKRL PPFLAEIWD
AamEcR RLLSILTELR TLGNM NAEMC FSLKVQNKKL PPFLAEIWD
       Helix 10        Helix 11         Helix 12
```

Fig. 2 Alignment of EcR domains B-E. Sequence alignment was conducted with GCG pileup. By comparison with available crystal structure of homologous nuclear receptors, typical secondary structure α-helix and β-sheet are underlined, and known functional sub-domains are in bold. Boundaries of DBD and LBD are indicted by arrows.

(Fig.2) conferring sequence specific DNA binding activity. This domain is most highly conserved. The DBDs from lepidopteran BmEcR, MsEcR and CfEcR are 100% identical. Even the most divergent DBDs from the fly DmEcR and the tick AamEcR display 86.6% identity.

Domain swapping technique has allowed the identification of several critical regions in the DBD defining DNA binding specificity. The P-box, originally identified by ER and GR swap experiment, determines half site sequence selection for response elements (Green and Chambon 1987). The D-box, originally identified by the swap experiment by Umesono and Evans (1989), is responsible for detecting half site spacing in a response element. The DR box is required for heterodimerization of TR and RAR with RXR to bind direct repeats (Perlmann et al., 1993). The T-box and A-box are responsible for high affinity binding of NGFI-B monomers (Wilson et al., 1992). Crystal structure indicated that the A-box in thyroid hormone receptor (TR) contacts DNA phosphates and the minor groove to stabilize protein-DNA interaction (Rastinejad et al., 1995).

All the Arthropod EcR proteins contain an identical P-box EGckG, which suggests that they have a similar half site sequence preference. The DR-box is also identical among these EcR proteins, yet the D-box has some variation. The D-box in Lepidopteran HvEcR, MsEcR, CfEcR and BmEcR and the Dipteran LcEcR share a D-box of KFGHA. The coleopteran TmEcR, orthopteran LmEcR, tick AamEcR and crab CpEcR share another D-box KYGNNC whereas the diptran AaEcR, DmEcR and CcEcR display variable D-boxes. These observations suggest EcR proteins may have different spacing preferences. In contrast to the D-box, the T- and A-Boxes from diptera are almost identical whereas more variability is observed for those in the lepidoptera (Fig.2).

The LBDs of EcR proteins are less conserved. than the DBD. LBDs from the silkworm BmEcR and the crab CpEcR are the most diverse, with only 54.4% identity. However, dipteran LBDs exhibit the high level of identity. LBDs from DmEcR, LcEcR and CcEcR are more than 94% identical. Crystal structures of retinoid X receptor (RXR, Bourguet et al., 1995), retinoic acid receptor (RAR, Renaud et al., 1995), thyroid hormone receptor (TR, Wagner et al., 1995), estrogen receptor (ER, Brzozowski, et al., 1997; Tanenbaum et al., 1998) and progesterone receptor (PR, Williams and Sigler, 1998) indicate that the LBD have the same overall tertiary structure of an anti-parallel α-helical sandwich consisting of 11-12 α-helices, five of which, helices 3, 4, 5, 8 and 9, compose the LBD core. LBDs from the 13 EcR proteins can be aligned with 11 helices displaying the same length except Helix 1 and the bridge connecting helices 9 and 10. The lepidopteran MsEcR, CfEcR and BmEcR are 2-4 aa longer than other receptors in the helix 1 region. A transactivation domain AF2-a has been identified in GR and ER (Milhon et al., 1994; Pierrat et al., 1994) in Helix 1. If a trasactivation domain can be identified at this region of EcRs, the variation of Helix 1 could account for differential transactivation activity. Helix 12 contains a weak autonomous activation domain, termed AF2-AD, which is required for transactivation and ligand dependent interaction of LBD with cofactors including co-activators and co-repressors (Reviewed by Simons, 1998). The dipteran and lepidopteran receptors contain identical AF2-AD, PFLEEI motif, suggesting that they may interact with homologous cofactors. Helix 12 in LmEcR, TmEcR, CpEcR and AamEcR has one aa different from that in dipteran and lepidopteran insects. It would be interesting to see whether this substitution could lead to interaction with alternative cofactors. A signature sequence spanning Helices 3 and 4 is thought to be a major stabilizing component of the folded LBD, and these two helices

constitute putative Hsp90 binding site for vertebrate steroid receptors (Simons, 1998). The signature sequence is exclusive to species within a specific Order. Helices 9 and 10 constitute the I-Box (Identity box, Perlmann et al., 1996), which is critical in the formation of RXR-RAR and RXR-TR heterodimers and COUP-TF homodimers. The I-box in LmEcR, TmEcR, CpEcR and AamEcR is two amino acids shorter than that in the other receptors. This suggests these receptors may display different dimerization capacity. The I-boxes in AaEcR and DmEcR are highly conserved, yet they display dramatically different heterodimerization capacities (Chapter 5).

These analyses indicate that the EcR proteins from various species are highly related. However, each receptor protein has its distinct features, suggesting that each one may have its special functionality. It is noteworthy that the LBD is more diverse than the DBD, implying that each receptor may possess its unique ligand specificity.

To assess the similarity of these EcR proteins, a phylogenetic analysis was performed using sequences from domains B-E. Domain A was not included for this analysis since it varies among isoforms. Domain F was also excluded because of the difference of its length among species (Fig. 1). The phylogenetic analysis indicated EcR proteins from diptera and lepidoptera clustered together, respectively. Surprisingly, the insect yellow mealworm TmEcR and locust LmEcR proteins are more related to the tick AamEcR and crab CpEcR than to other insect EcR proteins. Such a relation suggests the EcR protein may have evolved before the splitting of tracheata, crustacea and chelicerata (Fig. 3).

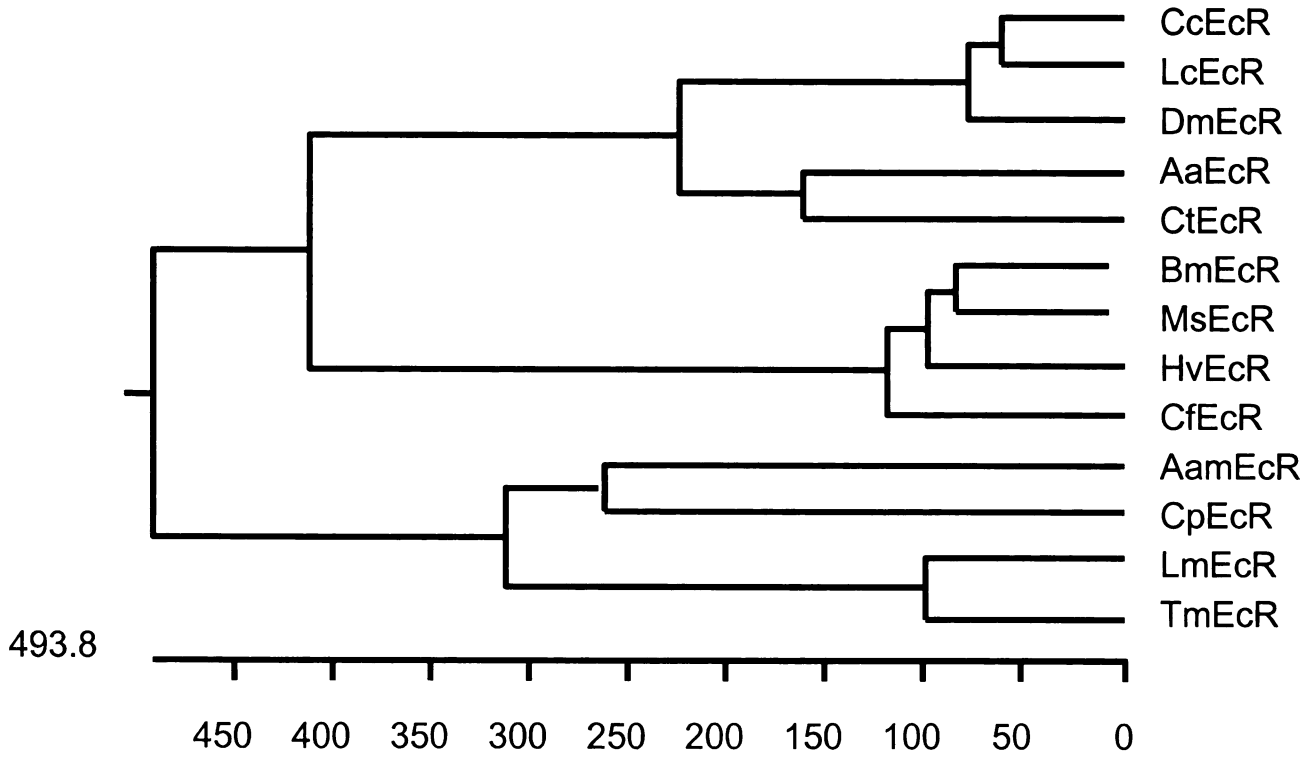
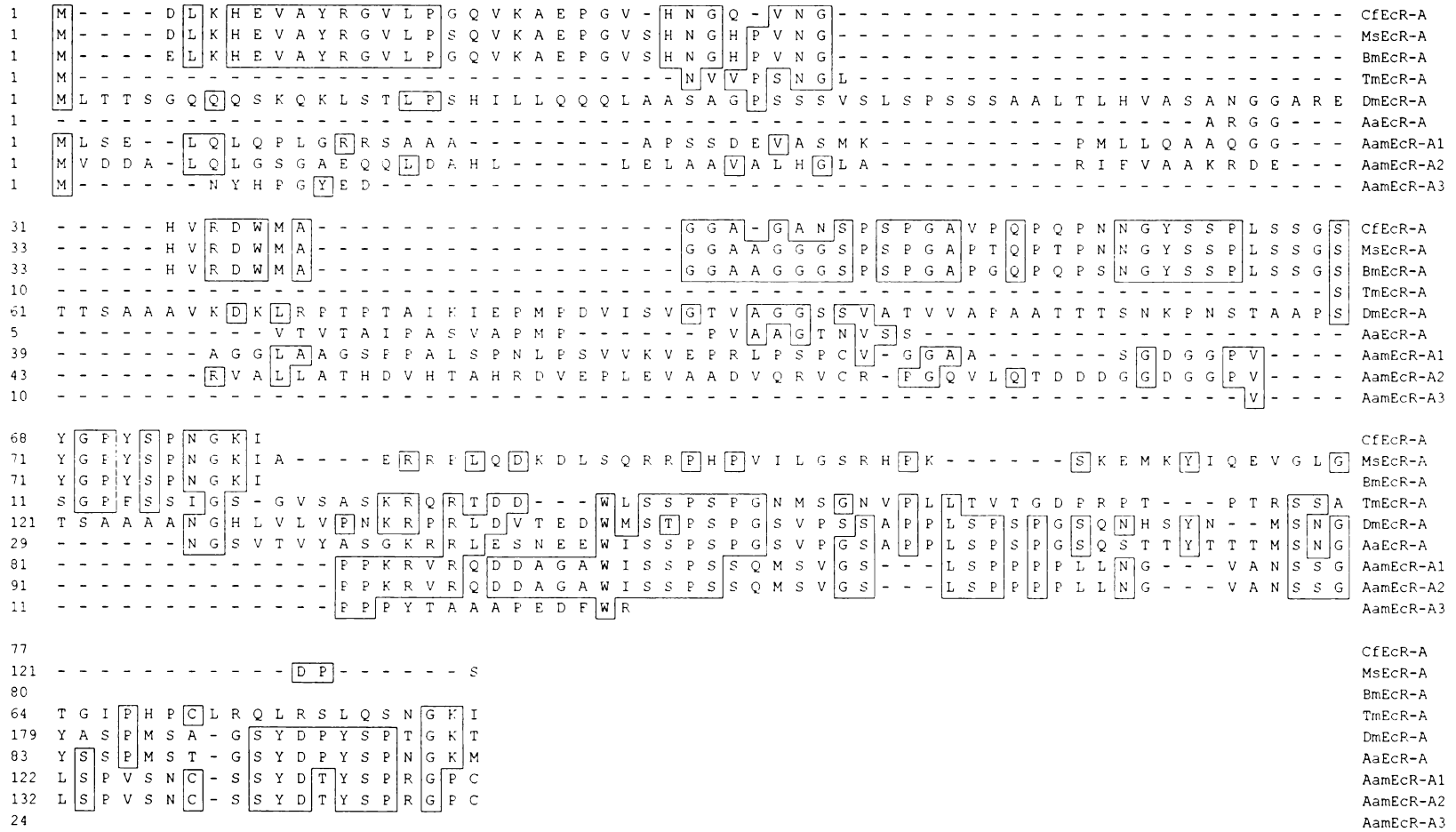


Fig.3 Phylogenetic tree of EcR domains B-E. Phylogenetic tree was constructed with DNA Star using J. Hein method with PAM250 residue weight table.

EcR isoforms.

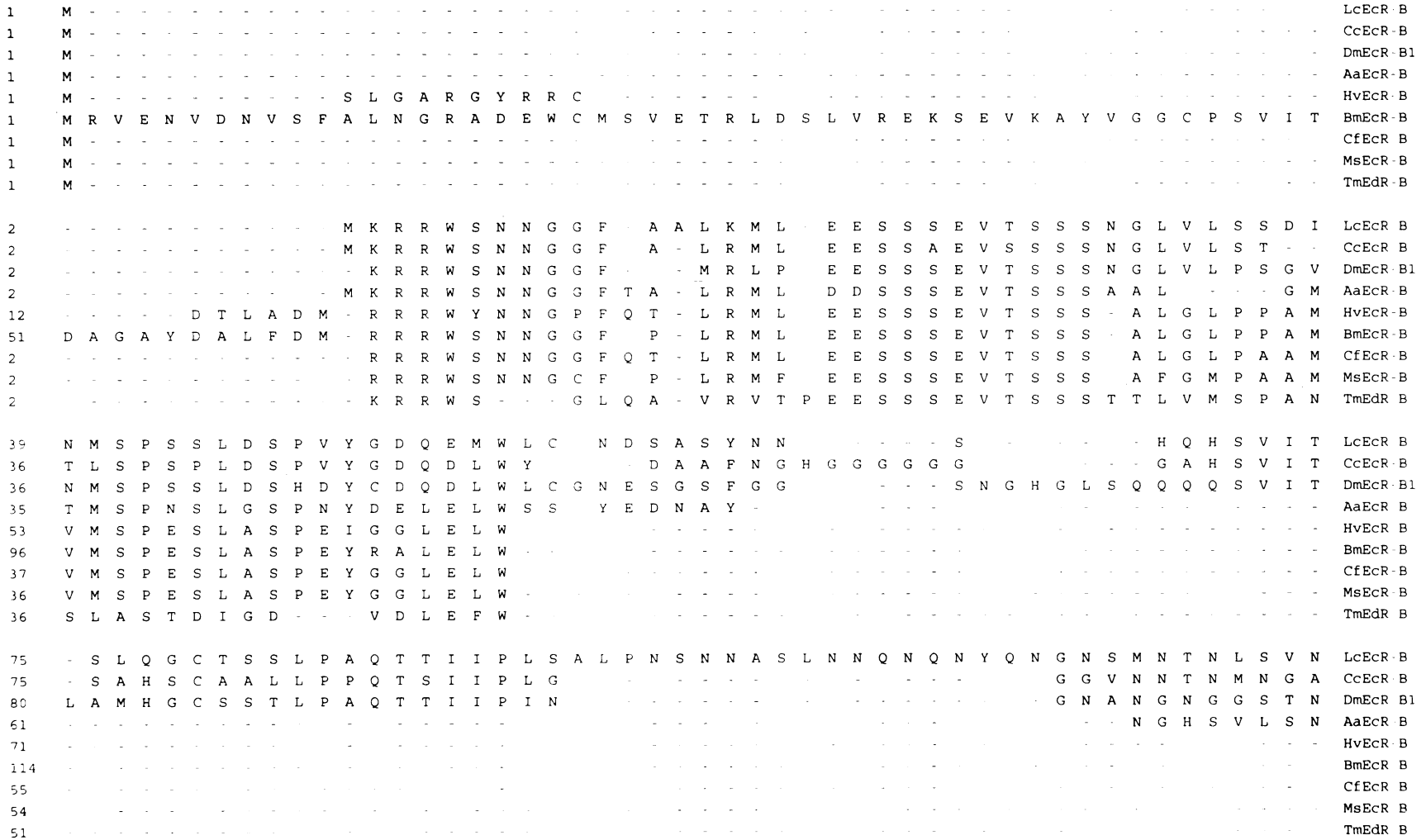
Three EcR isoforms were first identified from the *D. melanogaster*. DmEcR-A uses a 5' promoter, DmEcR-B1 and B2 are derived from alternative splicing utilizing the 3' downstream promoter (Talbot et al., 1993). These isoforms differ only in their N-termini (designated domain A). The isoform specific region starts from 37 aa (designated domain B) proceeding the DBD first Zn module (Fig. 1). Other arthropod EcR isoforms display similar structural features, yet the length of domain B is species specific. The EcR-A homologues have been isolated from *Aedes* (Wang and Raikhel, unpublished results), *Choristeneura* (Perera et al., Accession: 3659899), *Manduca* (Jindra et al., 1996), *Bombyx* (Kamimura et al., 1997) *Tenebrio* (Mouillet et al., 1997) and *Amblyomma* (Guo et al., 1997). The EcR-B1 homologue has been cloned from *Aedes* (Cho et al., 1995), *Ceratitis*(Verras et al., Accession: 3393034), *Lucilia*(Hannan et al., 1997), *Bombyx*(Swevers et al., 1995, Kimimura et al., 1996), *Manduca*(Fujiwara et al., 1995), *Heliothis*(Martinez,A, Accession: CAA70212), *Choristoneura* (Kothapalli et al., 1995) and *Tenebrio* (Mouillet et al., 1997). The DmEcR-B2 has only 17aa at its domain A. The N-terminus of the dipteran midge CtEcR is more similar to that in the orthopteran LmEcR (25.2% identity) than to any other receptors. Both CtEcR and LmEcR are more similar to DmEcR-B2 than to isoform B1 or isoform A, suggesting the B2 isoform could be the most primitive one (Fig. 6).

The lepidopteran CfEcR, MsEcR and BmEcR isoform specific region share around 90% identity. Likewise the dipteran AaEcR-A and DmEcR-A are highly identical, albeit the AaEcR-A N-terminus is incomplete. The N-terminus of TmEcR-A is more diverse from lepidopteran and dipteran receptors with only 18.7% identity to that in MsEcR-A. Three



Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Fig. 4. Alignment of EcR-A isoform specific region domain A. Alignment was conducted with DNA Star using Clustal method with PAM250 residue weight table. Residues conserved among species are boxed.


LcEcR B
CcEcR B
DmEcR B1
AaEcR B
HvEcR B
BmEcR B
CfEcR B
MsEcR B
TmEdR B

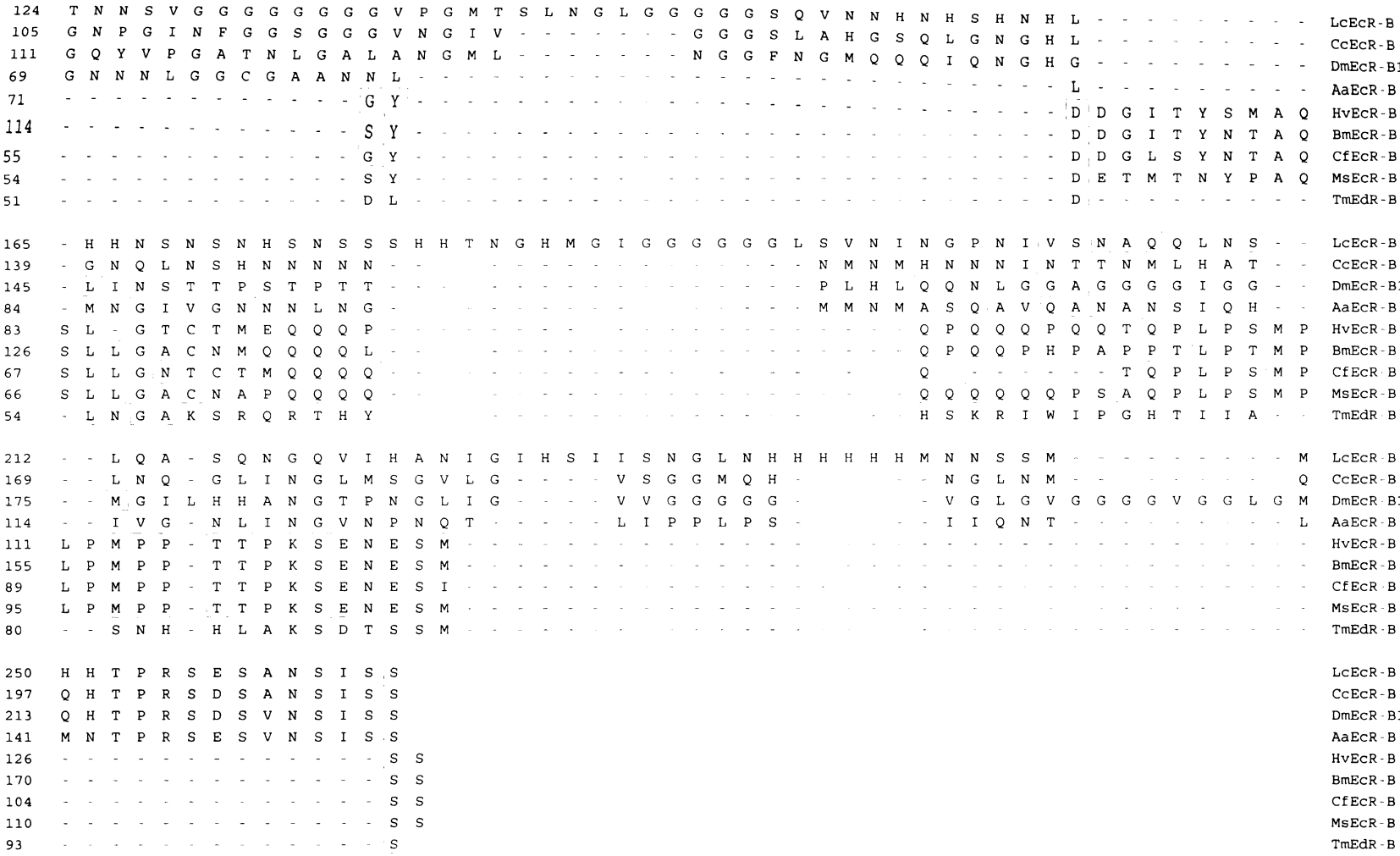


Fig. 5. Alignment of EcR-B1 isoform specific region domain A. Alignment was conducted with DNA Star using Clustal method with PAM250 residue weight table. Residues conserved among species are boxed.

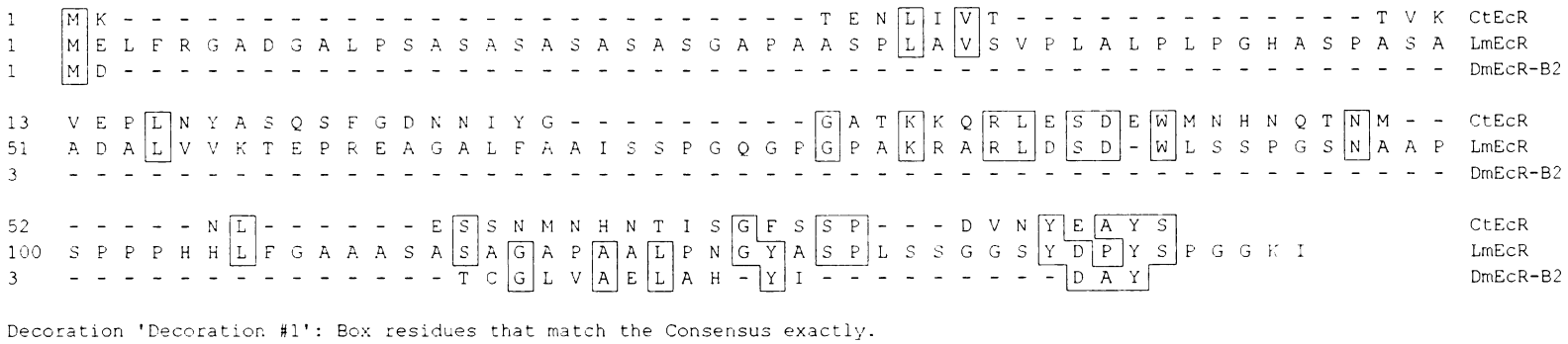


Fig. 6. **Alignment of DmEcR-B2, LmEcR and CtEcR isoform specific region domain A.** Alignment was conducted with DNA Star using Clustal method with PAM250 residue weight table. Residues conserved among species are boxed.

EcR-A isoforms have been cloned from the tick, but their N-terminal similarity with those in insect EcR-As is very low (Fig. 4).

Most groups first cloned the EcR-B1 isoform in their experiments, which implies that EcR-B1 is the most abundant transcript. Lepidopteran CfEcR, HvEcR, BmEcR and MsEcR B1 specific regions share around 80% identity. The dipteran LcEcR, CcEcR, DmEcR and AaEcR B1 specific region are less conserved with an identity ranging from 35,5%-51.2%. The orthopteran TmEcR isoform B1 specific region, the most diverse region among the known EcR-B1, displays 31.5%-39.6% identity with domain A in EcR-B1 of other species (Fig. 5).

The role of EcR isoforms is best characterized in *Drosophila* by genetic analysis. EcR-B1 predominates during proliferative or repressive responses (Truman et al., 1994). EcR-B mutants can not survive through metamorphasis and they fail to prune back larval-specific dendrite to initiate larval neuron remodeling (Bender *et al.*, 1997; Schubiger *et al.*, 1998). EcR-A predominates during maturational responses (Truman *et al.*, 1994). High level EcR-A expression in ventral CNS correlates with their rapid degeneration after adult emergence (Robinow *et al.*, 1993). The molecular mechanism of these isoform specificity is not well understood. Domain mapping technique indicates that the first 53 aa of DmEcR-B1 possesses an autonomous transactivation domain in yeast and mammalian cells. However, in these cells the N-terminal of DmEcR-A and DmEcR-B2 fail to activate an reporter gene (Lezzi's group, unpublished results).

Characteristics of the EcR heterodimerization parter, *Ultraspiracle*.

EcR cDNA has only been discovered in Arthropoda, yet the heterodimerization partner USP is more widely distributed in metazoa. USP homologues have been discovered

in species ranging from Cnidaria to human. In arthropoda, USP cDNA has been reported for eight species, The dipteran *Drosophila menalogaster* (DmUSP, Henrich et al., 1990; Shea et al., 1990; Oro, et al, 1990), *Aedes aegypti* (AaUSP, Kapitskaya et al., 1996), and *Chironomus tentans* (CtUSP, Vogtli et al., Accession: 2895868), lepidopteran *Bombyx mori* (BmUSP, Tzertzinis et al., 1994) and *Manduca sexta* (MsUSP, Jindra et al., 1997), the crustacea, *Celluca pugilator* (CpRXR, Chung et al., 1998) and the ixodida *Amblyomma americanum* (AamRXR, Guo et al.1998,). In lower metazoa, USP homologues have been cloned from nematoda *Caenorhabditis elegans* (CeUSP, Wilson et al., 1994) and Trematoda *Schistosoma mansoni* (SmUSP, Freebern et al., 1999) (Fig. 7). In addition, Laudet's group cloned the partial USP cDNA from Cnidaria, *Anemonia Sulcata* and Mollusca *Biomphalaria glabrata* (Escriva et al., 1997).

The N-terminal A/B domains of invertebrate USPs fail to show significant similarity except within the order of lepidopteran (Fig. 8). In contrast, the 66aa DBD is impressively conserved, even the most divergent nemotode CeUSP and the blood fluke SmUSP display 50% identity in their DBDs. Among arthropods, the DBD identity is in about 90%, while BmUSP and MsUSP DBDs are 98.5% identical.

The P-box in USPs, cEGcKg, which is conserved in all species except for a one amino acid substitution in CeUSP (Fig. 8), is identical to that in EcR proteins, which implies that both subunits in the EcR-USP dimer have the same half site sequence preference. In accordance with the diversity of D-boxes in EcR proteins, the D-boxes in USPs have higher variability than their P-box. Even within the Lepidoptera, the D-box in CfUSP, REERN, is one amino acid different from those in MsUSP and BmUSP, REDRN. The conservation of USP proteins extends from the DBD core into the T-box and A-box except in CeUSP.

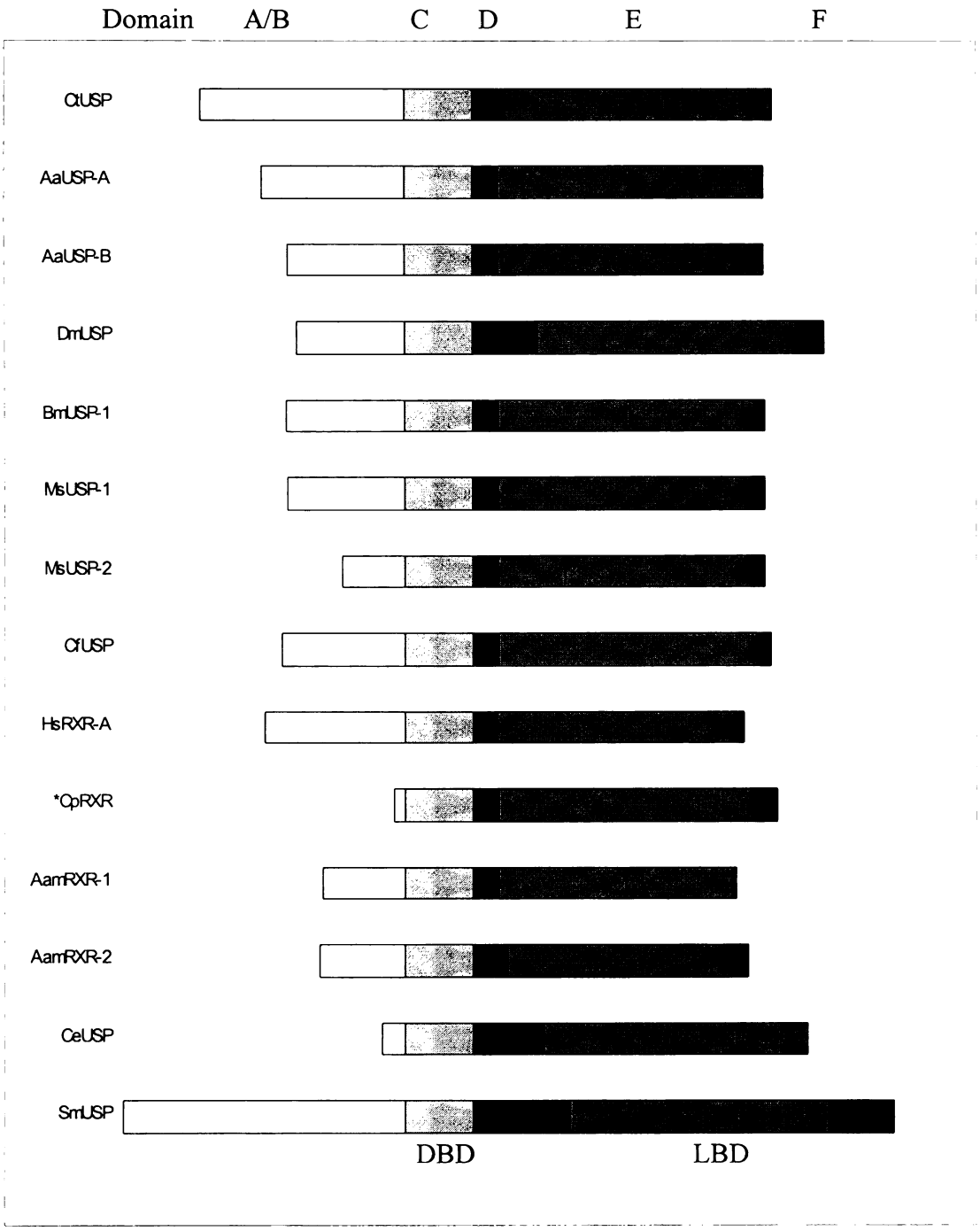


Fig.7. Schematic diagram of USP proteins. USP proteins contain 5 characteristic domains. Part of domain A/B, the N-terminal, is usually isoform specific. Domain C is the DNA binding domain. Domain D is the hinge region. Domain E is the ligand binding domain. Domain F is the C-terminal tail with unknown function. N-termini of CpEcR, indicated by an asterisk, is not complete.

```
  BmUSP T.....T.PN VE..LDIQWL NIESGFMSPM SPPEMKPDT. .AMLDGFRDD
  MsUSP  T.....T.PS VE..LDIQWL NIEPGFMSPM SPPEMKPDT. .AMLDGLRDD
  CfUSP  MQSLNHI.PT VDCSLDMQWL NLEPGFMSPM SPPEMKPDT. .AMLDGLRDD
  CpRXR ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
AamRXR1 SNGVSSS.LP PQPSLPAERL RRALGSITVG RPAAQQRSS. .G...DTATA
 HsRXRa SPMGPHS.MS VPTTPTLGFS TGSPQLSSPM NPVSSSEDI. .K...PPLGL
 AaUSP  NCGPASP.GA FNQQVAAALQ QQQQNVNSLN SQQSGGGGG. .A...GGGTP
  CtUSP VTFNQIK.LQ SPSPSNASSS STLSGPLTTT PPATNANNI. .LGMGNGNCG
  DmUSP SFSPKAE.SP VPFMQAMSMV HVLPGSNSAS SNNNSAGDAQ MAQAPNSAGG
  SmUSP SNETSTSYLP QVTKVETNSL TVSQSPILLF VDPNKTKPES RECFPTQNPS
  CeUSP ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~MR

                                    DBD                  P-Box
  BmUSP STPPPPFKNY PPNHPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
  MsUSP STPPPAFKNY PPNHPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
  CfUSP ATSPPNFKNY PPNHPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
  CpRXR ~~~~~~~~~~ ~~~HPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
AamRXR1 AAATSH.HAL STKPPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
 HsRXRa NGVLKV.PAH PSGNMASFTK HICAICGDRS SGKHYGVYSC EGCKGFFKRT
 AaUSP  TTPTNMSQQY PPNHPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
  CtUSP NTANGKQSQY PPNHPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
  DmUSP SAAAAVQQQY PPNHPLSGSK HLCSICGDRA SGKHYGVYSC EGCKGFFKRT
  SmUSP ELASQSSSAT SVN..TTNLN PICVICGDKA SGKHYGVISC EGCKGFFKRT
  CeUSP LMSKDSSQSL VANHKAT... LFCAVCGDTA LGKHYGVTAC NGCKGFFRRS
                                                        Helix
                  D-Box                        DBD     T-Box
  BmUSP VRKDLTYACR EDKNCIIDKR QRNRCQYCRY QKCLACGMKR EAVQEERQR.
  MsUSP VRKDLTYACR EDRNCIIDKR QRNRCQYCRY QKCLACGMKR EAVQEERQR.
  CfUSP VRKDLSYACR EERNCIIDKR QRNRCQYCRY QKCLACGMKR EAVQEERQR.
  CpRXR VRKDLTYACR EERSCTIDKR QRNRCQYCRY QKCLTMGMKR EAVQEERQR.
AamRXR1 VRKDLSYACR EERTCIIDKR QRNRCQYCRY QKCLACGMKR EAVQEERQR.
 HsRXRa VRKDLTYTCR DNKDCLIDKR QRNRCQYCRY QKCLAMGMKR EAVQEERQR.
  AaUSP VRKDLSYACR EDKNCTIDKR QRNRCQYCRY QKCLACGMKR EAVQEERQR.
  CtUSP VRKDLSYACR EERNCVIDKK QRNRCQYCRY QKCLNCGMKR EAVQEERQRG
  DmUSP VRKDLTYACR ENRNCIIDKR QRNRCQYCRY QKCLTCGMKR EAVQEERQRG
  SmUSP VRKQLVYVCR ESGQCPVDRR KRTRCQHCRF EQCLAKGMKK EAVQEERHRQ
  CeUSP IWKNRTYACR YQGKCGVAKE QRNACRSCRL KECIKVGMNP RAVQGDIDTA
                                        Helix

                                    A-Box
  BmUSP ..........  ..........  ......AARR TE..DAHPS.  ......SSVQ
  MsUSP ..........  ..........  ......AARG TE..DAHPS.  ......SSVQ
  CfUSP ..........  ..........  ......NARG AE..DAHPS.  ......SSVQ
  CpRXR ..........  ..........  ......TKGD KG..DGDTE.  ......SSCG
AamRXR1 ..........  ..........  ......TKDR AD..SEVES.  ......TSGG
 HsRXRa ..........  ..........  ......GKDR NE..NEVES.  ......TS.S
 AaUSP  ..........  ..........  ......SSKF SI..KSEEI.  ......NSTS
  CtUSP ..........  ..........  ......GKSQ KG..DDMSI.  ......SSTQ
  DmUSP ..........  ..........  ......ARNA AG..RLSASG GGSSGPGSVG
  SmUSP PSSNPVPLIS KPPKSEKKGP GRRSTFGNKS AE..SIVTDQ PPNINQXSTP
  CeUSP STSSASPNLV SVEMHPYTKS DFREVECQTE VSTISSHPTS PSTLPFVSSG
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        LBD
BmUSP ------>...E LSIERLLE.. LEALVADSAE EL........ .QIL...RV.
MsUSP .........E LSIERLLE.. IESLVADPPE EF........ .QFL...RV.
CfUSP VS......DE LSIERLTE.. MESLVADPSE EF........ .QFL...RV.
CpRXR AI......SD MPIASIRE.. AELSVDPIDE QPL....... DQGV...RL.
AamRXR1 AP......PE MPLERILE.. AELRVE.... .......... ..........
HsRXRa AN......ED MPVERILE.. AELAVEPKTE TYV....... EANM...GL.
AaUSP  SV......RD VTIERIHE.. AEQLSEQKSG DNA....... IPYL...RV.
CtUSP SLVNNGPGRD ITVERLME.. ADQMSEARCG DKS....... IQYL...RVA
DmUSP GSSSQGGGGG GGVSGGMG.. SGNGSDDFMT NSV....... SRDFSIERII
SmUSP NISITPTTTD CVQPNQVK.. SXSSTTCIQS NNVLLSDXTD LPNLTLRCLL
CeUSP SSICSVSSSC SSVSTVYSNC LAMLPPNPSL PHAFYNPHLI CNRTKLTPTG
                          Helix 1           Helix 2

BmUSP GPESGVPAKY RA........ .......... ..PVSSLCQI GNKQIAALIV
MsUSP GPESGVPAKY RA........ .......... ..PVSSLCQI GNKQIAALVV
CfUSP GPDSNVPPRY RA........ .......... ..PVSSLCQI GNKQIAALVV
CpRXR QVPLAPPDSE KCSFTLPFHP VSEVSCANPL QDVVSNICQA ADRHLVQLVE
AamRXR1 .......... ........SQ TGTLSESAQQ QDPVSSICQA ADRQLHQLVQ
HsRXRa N......... .......... ......PSSP NDPVTNICQA ADKQLFTLVE
AaUSP  GSNSMIPPEY KGA....... .......... ...VSHLCQM VNKQIYQLID
CtUSP ASNTMIPPEY RA........ .......... ..PVSAICAM VNKQVFQHMD
DmUSP EAEQRAETQC GDRALTFLRV GPYSTVQPDY KGAVSALCQV VNKQLFQMVE
SmUSP SAELSMDPKL AVSERGEAIY EDIPGDDDTG LHPLTIICQS IEQQLPRIVN
CeUSP ERIATLPEVL QDFRRIFVLF TDVLSILPEF SRLDESDRMV LAKSRFSFFY
                                                  Helix 3

BmUSP W......... ..ARDIPHFG Q..LEID..D QILLIKG SWN ELLLFAIAWR
MsUSP W......... ..ARDIPHFG Q..LELE..D QILLIKN SWN ELLLFAIAWR
CfUSP W......... ..ARDIPHFG Q..LELD..D QVVLIKA SWN ELLLFAIAWR
CpRXR W......... ..AKHIPHFT D..LPIE..D QVVLLKA GWN ELLIASFSHR
AamRXR1 W......... ..AKHIPHFE E..LPLE..D RMVLLKA GWN ELLIAAFSHR
HsRXRa W......... ..AKRIPHFS E..LPLD..D QVILLRA GWN ELLIASFSHR
AaUSP  F......... ..ARRVPHFI N..LPRD..D QVMLLRC GWN EMLIAAVAWR
CtUSP F......... ..CRRLPHFT K..LPLN..D QMYLLKQ SLN ELLILNIAYM
DmUSP Y......... ..ARMMPHFA Q..VPLD..D QVILLKA AWI ELLIANVAWC
SmUSP W......... ..ARQLPVFS SVYLSFD..D QFCLIKA AWP ELVLISSAYH
CeUSP WWLTCCWTAK VGCPGVCYAN GAYHPSDKRQ QAFPDVK GVT ELSVETVSKP
                                      Helix 4      Helix 5

BmUSP SMEFLNDERE NVD....... ....SRNTAP .PQLICLMPG MTLHRNSALQ
MsUSP SMEYLTDERE NVD....... ....SRSTAP .PQLMCLMPG MTLHRNSALQ
CfUSP SMEYLEDERE NGDG...... ....TRSTTQ .PQLMCLMPG MTLHRNSAQQ
CpRXR SMGV...... EDG....... .......... ....IVLATG LVIHRSSAHQ
AamRXR1 SVDV...... RDG....... .......... ....IVLATG LVVQRHSAHG
HsRXRa SIAV...... KDG....... .......... ....ILLATG LHVHRNSAHS
AaUSP SMEYIETERS SDGS...... ....RITVRQ .PQLMCLGPN FTLHRNSAQQ
CtUSP SIQYVEPDRR NADG...... ....SLERRQ ISQQMCLSRN YTLGRNMAVQ
DmUSP SIVSLDDGGA GGGGGGLGHD GSFERRSPGL QPQQLFLNQS FSYHRNSAIK
SmUSP STVIRDG... .......... .......... ....LLLSIG RHLGREVAKS
CeUSP LANINITDAE MLVGSVFAIF YEYPLPPKVS YASTHILNEA RDLYTQCMIT
                                                     Helix 6
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  BmUSP AGVGQIFDRV LSELSLKMRS LRMDQAECVA LKAIILLNPD VKGLKNKQEV
  MsUSP AGVGQIFDRV LSELSLKMRT LRMDQAEYVA LKAIILLNPD VKGLKNKPEV
  CfUSP AGVGAIFDRV LSELSLKMRT LRMDQAEYVA LKAIVLLNPD VKGLKNRQEV
  CpRXR AGVGAIFDRV LSELVAKMKE MKIDKTELGC LRSIVLFNPD AKGLNCVNDV
AamRXR1 AGVGAIFDRV LTELVAKMRE MKMDRTELGC LLAVVLFNPE AKGLRTCPSG
 HsRXRa AGVGAIFDRV LTELVSKMRD MQMDKTELGC LRAIVLFNPD SKGLSNPAEV
  AaUSP AGVDTLFDRI LCELGIKMKR LDVTRAELGV LKAIILFNPD IRGLKCQKEI
  CtUSP AGVVQIFDRI LSELSVKMKR LDLDATELCL LKSIVVFNPD VRTLDDRKSI
  DmUSP AGVSAIFDRI LSELSVKMKR LNLDRRELSC LKAIILYNPD IRGIKSRAEI
  SmUSP HGLGPLVDRI LHELVARFRD LSLQRTELAL LRAIILFNPD ANGLSSRHRV
  CeUSP VSP...YSQL EAKSASRLAE ISLIFSSITN LKYLTSDNIE LSDVLHVMEV
              Helix 7              Helix 8

  BmUSP DVLREKMFLC LDEYCRRSRG GEEGRFAALL LRLPALRSIS LKSFEHLYLF
  MsUSP VVLREKMFSC LDEYVRRSRC AEEGRFAALL LRLPALRSIS LKCFEHLYFF
  CfUSP DVLREKMFSC LDDYCRRSRS NEEGRFASLL LRLPALRSIS LKSFEHLYFF
  CpRXR EILREKVYAA LEEYTRTTYP DEPGRFAKLL LRLPALRSIG LKCLEYLFLF
AamRXR1 GPEGESV.SA LEEHCRQQYP DQPGRFAKLL LRLPALRSIG LKCLEHLFFF
 HsRXRa EALREKVYAS LEAYCKHKYP EQPGRFAKLL LRLPALRSIG LKCLEHLFFF
  AaUSP DGMREKIYAC LDEHCKQQHP SEDGRFAQLL LRLPALRSIS LKCLDHLNFI
  CtUSP DLLRSRIYAS LDEYCRQKHP NEDGRFAQLL LRLPALRSIS LKCLDHLFYF
  DmUSP EMCREKVYAC LDEHCRLEHP GDDGRFAQLL LRLPALRSIS LKCQDHLFLF
  SmUSP EAVREQLYSA LHSYCTTNQP QDTSRFTKLL LRLPPLRSIA SKCLEHLVFV
  CeUSP DQL...ITEA FDVHRPME~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
              Helix 9                 Helix 10             Helix

                        LBD
  BmUSP HLVAEGSVSS YIRDALCNHA PPIDTNIM~~ ~~~~~~~~~~ ~~~~~~~~~~
  MsUSP HLVADTSIAS YIHDALRNHA PSIDTSIL~~ ~~~~~~~~~~ ~~~~~~~~~~
  CfUSP HLVAEGSISG YIREALRNHA PPIDVNAMM~ ~~~~~~~~~~ ~~~~~~~~~~
  CpRXR KLIGDTPLDS YLMKMLVDNP NTSVTPPTS~ ~~~~~~~~~~ ~~~~~~~~~~
AamRXR1 KLIGDTPIDN FLLSMLEAPS DP~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsRXRa KLIGDTPIDT FLMEMLEAPH QMT~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  AaUSP RLLSDKHLDS FIVEMLDMPI ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  CtUSP QLIDDKNVEN SVIEEFHKLN ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  DmUSP RITSDRPLEE LFLEQLEAPP PPGLAMKLE~ ~~~~~~~~~~ ~~~~~~~~~~
  SmUSP KLAAEDPTSC RLINLVEHGV WPIQEKSFEL ATLPSDSAST DSVPSQITMV
  CeUSP ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
         11   Helix 12
```

Fig. 8. **Alignment of USP protein domains C-E.** Sequence alignment was conducted with GCG pileup. By comparison with available crystal structure of homologous nuclear receptors, typical secondary structure α-helix and β-sheet are underlined, and known functional sub-domains are in bold. Boundaries of DBD and LBD are indicted by arrows. The *Homo sapiens* RXRα (HsRXRa) is also included for alignment as its crystal structure is available.

Most of the USP proteins have a short hinge region (24-35aa long, however, the hinge regions in DmUSP (63aa), CeUSP and SmUSP are much longer than those in other species(Fig. 7). LBDs in arthropoda USP (35.3-88.2% identical) are less conserved than those in EcR proteins (54.4%-94.6% identical). The highest conservation lies within Lepidopterans, with 88.2% identity for the LBDs in BmUSP and MsUSP. Yet LBDs in the lepidopteran and dipteran USPs are only 35.3% identical. Interesting, phylogenetic analysis indicated that the arthropod tick and crab USP homologues are more similar to vertebrate RXR protein sequences than to other arthropod USP proteins (Fig. 9), hence they were originally named AamRXR and CpRXR, respectively, instead of USPs. Moreover, tick RXR is encoded by two different genes, unlike insect USPs, which are encoded by a single gene based on available data. The LBD of the nematode CeUSP is only 10% identical to that in other species, yet the LBD in the blood fluke SmUSP is as high as 40.5% identical to that in human RXRα. The LBD core, helices 3, 4, 5, and 8 can be aligned nicely for all these USP homologues except in CeUSP, suggesting they form similar antiparallel alpha-helical sandwich structure analogous to that of the human RXRα (Fig. 8).

USP isoforms

Three genes encoding RXRα, RXRβ and RXRγ have been identified in several vertebrate species, including human, mouse, rat, chicken and frog. Two additional subtypes, RXRδ and RXRε have been cloned from zebra fish (Johns et al., 1995). Each gene may encode several isoforms due to utilization of alternative promoter and/or splicing variation. Among insects, only one form of USP cDNA has been identified from *Drosophila, Bombyx, Chironomus, Choristoneura*. In contrast, two USP cDNA isoforms have been cloned from *Aedes* (AaUSPa

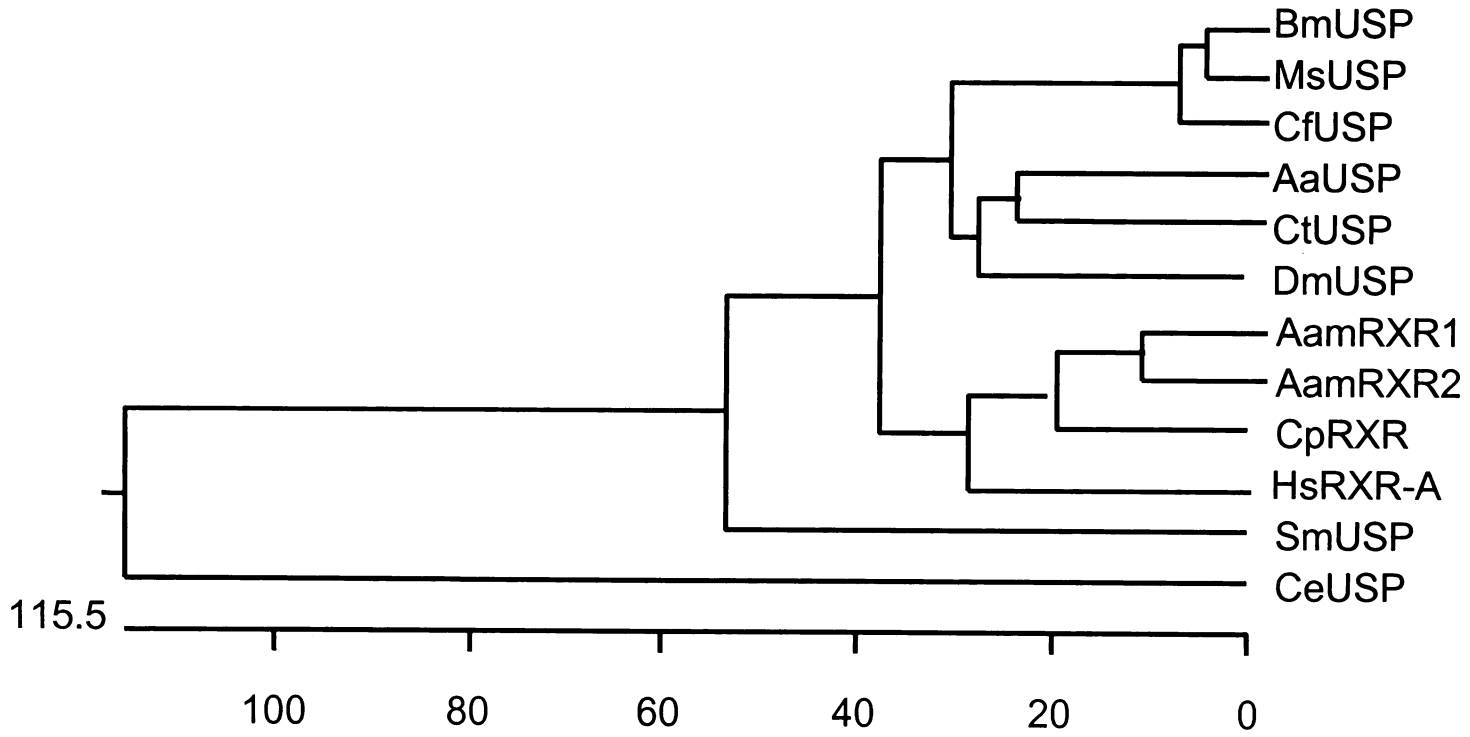


Fig. 9. **Phylogenetic tree of USP domains C-E.** Phylogenetic tree was constructed with DNA Star usnig Clustal method with PAM250 residue weight table.

(A)

```
1  M - - - L K K E K P M L S V A A I I Q - - - - - - - A Q G R W D R T L P - - - - - - - L A G L A        AaUSP-A
1  M S S V A K K D K R T M S V T A L I N R A W P L T P A P H Q Q Q S M P S S Q P S N F L Q P L A T P S    MsUSP-1

31                                                                                                      AaUSP-A
51 T T P S V E L D I Q W L                                                                              MsUSP-1

Decoration 'Decoration #1': Box residues that match the Consensus exactly.
```

(B)

```
1 M D P S D R                                                                                           AaUSP-B
1 M E P S R E S G L                                                                                     MsUSP-2

Decoration 'Decoration #1': Box residues that match the Consensus exactly.
```

Fig.10. Alignment of USP isoform specific region from *Aedes* and *Manduca* proteins. AaUSPa is more related with MsUSP1 (A) and AaUSPb is more related with MsUSP2 (B). Identical residues are boxed

and AaUSPb, Kapitskaya et al., 1996) and *Manduca* (MsUSP-1 and MsUSP-2, Jindra et al., 1997). Like the EcR isoforms, these USP isoforms differ only in their N-termini, suggesting they are derived from the same gene.

In lepidoptera, N-termini from CfUSP and BmUSP are similar to MsUSP-1 (Fig.8). However, N-termini from the dipteran CtUSP and DmUSP do not show much identity with either AaUSPa or AaUSPb. MsUSP-1 isoform specific region is longer than MsUSP-2 and is more similar to AaUSPa, allowing alignment with AaUSPa. Likewise, MsUSP-2 N-terminus can be aligned with that of AaUSPb (Fig.10).

The failure to identify USP isoforms from *Drosophila* has made it difficult to define their distinct roles by genetic analysis. Interestingly, AaUSPb and MsUSP-2 mRNA are expressed with high titer of ecdysteroid (Jindra et al., 1997; Chapter 3). My analysis indicated that the AaEcR heterodimerized with different mosquito USP proteins display distinct DNA binding and transactivation activities (Chaper 3).

Ecdysteroid responsive elements (EcREs)

The canonical steroid receptor binding site is a DNA element that binds one or two receptor molecules, either as monomers, homo- and/or heterodimers (Tsai and O'Malley 1994). The binding sites can be arranged as single half-sites, inverted, direct or everted repeats. Although DNA binding by EcR-USP is sequence specific, a surprising variety of sequences have been identified as ecdysteroid response elements in *Drosophila* (See Table 1). The EcREs identified in the promoters of *hsp27* (Riddihough and Pelham, 1987), *Eip28/29* (Cherbas et al., 1991), *Fbp-1* (Antoniewski et al., 1994), *Sgs-4* (Lehman and Korge 1995)

and *Lsp-2* (Antoniewski et al., 1995) contain degenerate palindromic repeats with a 1-bp

Table 1. EcRE identified in *Drosophila* target genes

Target Gene	HRE sequence	HRE location	sequence analyzed	Technique	reference
hsp27	GGTTCAaTG CACT	-547/-535		Cell transfection/ Gel Shift	Riddihough and Pelham, 1987
Eip28/ 29 dist	AGGACTtTG ACCCcaaTG AACT	+4433/+4 469	-2kb/+7kb (9kb)	Cell transfection/ affinity purify	Cherbas et al., 1991
Sgs-4	AGTTCGaGG CACC	-308/-296	2.5kb/+5.2 kb (8.7kb)	Gel shift and P element transformation	Korge et al., 1990; Lehmann and Korge, 1994
Fbp-1	GGTTGAaTG AATT	-91/-105		P element transformation and gell shift	Antoniewski et al., 1994
LSP-2	CGGCCAaTG ACCG	-63/-75	-3100/+16 (3kb)	Cell tansfection and gel shift	Antoniewski et al., 1995
3C/ng	AGGTCAagA GGCCAaaga AGGTCA	coding region	3.5kb	affinity purify and gel shift	D'Avino et al., 1995

spacer (IR-1). This observation led to the conclusion that EcREs are characteristically IR-1s. However, Horner et al (1995) have demonstrated that direct repeats with a 3-5 base- pair spacer also bind DmEcR-DmUSP. It was shown recently that the DmEcR- DmUSP heterodimer can activate DR-0 through DR-5 reporter genes (Antoniewski et al., 1996), suggesting direct repeats also function as EcREs. In fact, Eip28/29 dist*, one of the Eip28/29 EcREs, contains an imperfect palindrome (IR-1) and an imperfect direct repeat (DR-3). *In vitro* binding studies have shown that the DR-3 also interacts with DmEcR-DmUSP (Zelhof et al., 1995). Indeed, the EcRE identified from the 3C/ng element is a direct repeat with a 12-bp spacer (DR-12, D'Avino et al., 1995). Cell transfection, gel shift assays and/or specific DNA-protein interaction assays were utilized to identify these elements. In addition, P-element mediated fly transformation, which is actually gaining more popularity, was also used to identify EcREs (See Table 1). Information obtained by this technique is more convincing since it reflects real *in vivo* physiological situation. However, the variety of EcR-

USP binding sequences has prevented the derivation of EcRE that can account for all these sites.

The DNA binding characteristics of mosquito EcR-USP are similar to those of their *Drosophila* homologues. Gel mobility shift assays demonstrated that AaEcR-AaUSP binds hsp-27, Eip28/29 dist*, DR-1 - DR-5 and DR-11-DR-13 elements (Wang et al, 1998, Chapter 2). Transactivation assays in CV-1 cells showed that ecdysteroid responsiveness requires both AaEcR and AaUSP. Despite the recent identification of EcR-USP binding sites in more *Drosophila* promoters, an EcRE has not been reported from other insects.

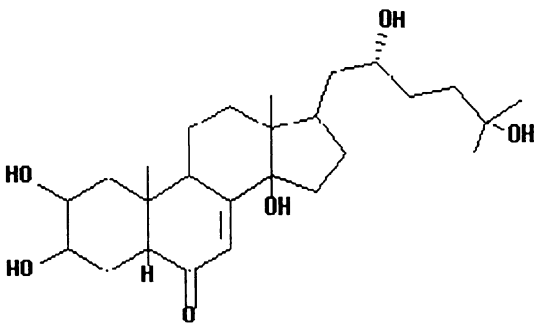
Bioassays and ligand specificity

The first isolated steroid hormone is ecdysone, which was originally purified as a molting hormone from the silkworm *Bombyx mori* pupae monitored by a bioassay *Calliphora* test, in which the hormone is examined by its activity to stimulate sclerotization of the ligated mature larvae of the blowfly *Calliphora erythrocephala* (Butenandt and Karlson, 1954). Following this breakthrough, numerous compounds structurally related to ecdysone have been identified from various animals and plants to form a large family of ecdysteroids (Reviewed by Rees, 1989; Lafont and Horn 1989) (See Fig. 11 for some of their structures).

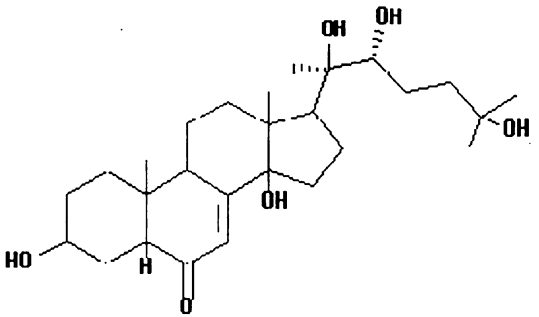
The *Calliphora* sclerotization test was originally used to isolate ecdysone (Butenandt and Karlson, 1954). To improve yield, sensitivity and efficiency, several other organisms including *insects Musca domestica, Sarcophaga peregrina, Samia cynthia, Chilo suppressalis* and the horseshoe crab *Limulus polyphemus* were subsequently used for bioassays (Reviewed by Smith, 1985). The activity order of seven ecdysteroids detected by

bioassay with *S. peregrina* is ponasterone A>cyasterone>20-hydroxyecdysone> ponasterone B>ponasterone C>ecdysone>inokosterone. However, a distinct activity order is obtained by bioassay with another insect *S. cynthia*: cyasterone>ponasterone B=ponasterone A>ponasterone C=ecdysone>20-hydroxyecdysone=inokosterone, suggesting ecdysteroids have differential effect on insects (Reviewed by Dinan, 1989). However, these bioassays can not distinguish between the effect of a test compound and subsequent metabolites. Accordingly, assays involving less metabolism like organ culture and cell culture have been developed. Imaginal disks and salivary gland from some dipteran and lepidopteran insects, namely, *D. melanogaster* and *S. bullata*, respond to ecdysteroids. In these *in vitro* organ culture assays, 20E can elicit developmental responses at physiological concentration 10^{-7}M, whereas ecdysone can only exert response at supra-physiological concentration, 10^{-5}M (reviewed by Smith, 1985). This observation has led to the hypothesis that ecdysone is synthesized as a prohormone and converted to the active form, 20-hydroxyecdysone. In support of this hypothesis, binding assays utilizing *Drosophila* cell extracts with ^{3}H labeled ponasterone A and competition assays with other ecdysteroid has yielded estimated Kd values 1nM, 0.1μM and 2μM for ponasterone A, ecdysone and 20-hydroxyecdysone, respectively (Reviewed by Dinan, 1989).

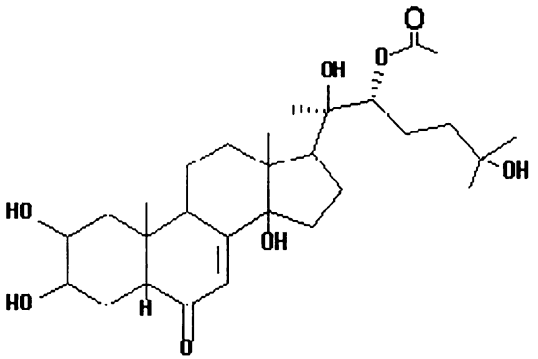
Although the prohormone hypothesis could be valid for some species including *D. melanogaster*, other species may have different pathways. In the *M. sexta* prothoracic gland, which is the site for ecdysteroid synthesis, 3-dehydroxyecdysone is the major product. In crustacea, ecdysone, 3-dehydroxyecdysone and 25-deoxyecdysone in various combination have been detected from the ecdysteroid synthesis site, Y-organ. (reviewed by Rees, 1995). In the adult female mosquito *A aegypti*, both 20E and ecdysone are detected from whole



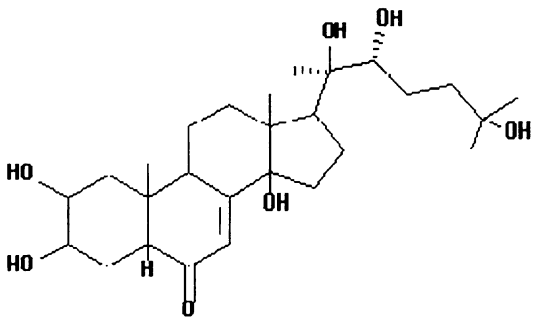
Ecdysone



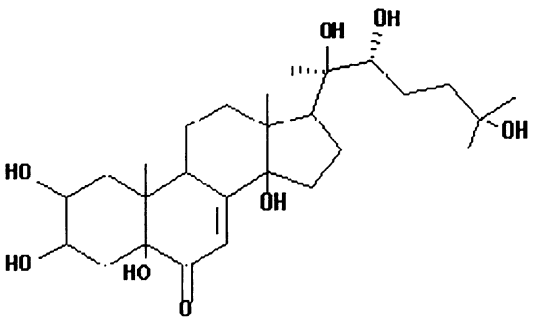
2-deoxy-20-hydroxyecdysone



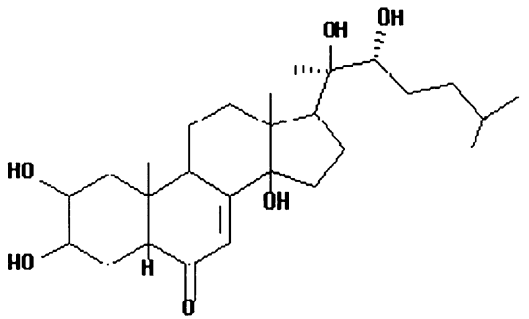
20-hydroxyecdysone-22-acetate



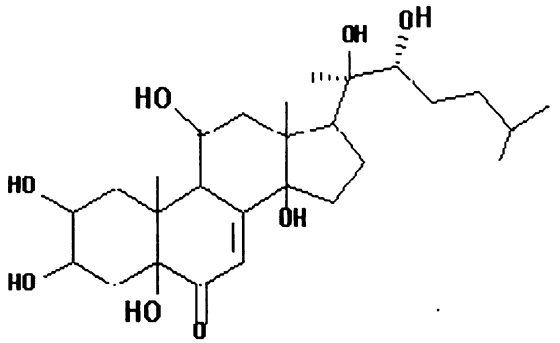
20-hydroxyecdysone



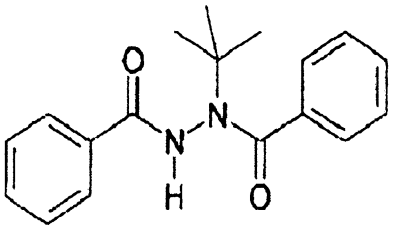
polypodine-B



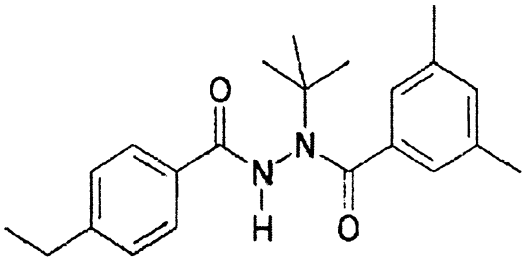
ponasterone-A



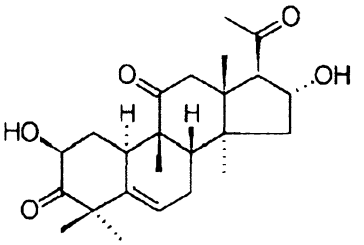
muristerone-A



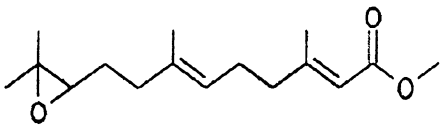
RH-5849



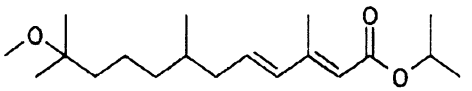
RH-5992, tebufenozide



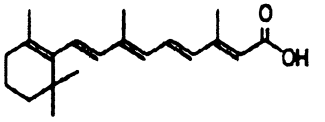
hexanorcucurbitacin D



juvenile hormone III



methoprene



retinoic acid

Fig. 11 Chemical structure of ecdysteroid, juvenile hormone and their analogues. Seven ecdysteroids, ecdysone, 2-deoxy-20-hydroxyecdysone, 20-hydroxyecdysone-22-acetate, 20-hydroxyecdysone, polypodine-B, ponasterone-A and muristerone-A are used for ligand specificity studies in chapter 4. RH-5849 and RH5992 are ecdysteroid agonists and hexanorcucurbitacin is an antagonist. Methoprene is an agonist of juvenile hormone. The structure of retinoid acid is also listed to show its similarity with JH.

body extract, but only ecdysone is detected in the ovary (Hagedorn, 1989), suggesting ecdysone in the ovary may function as a ligand.

The *Drosophila* embryonic cell lines, B_{II} and Kc cells, have been utilized to test the ecdysteroid activity. Upon hormone treatment, the normally small round Kc cells became flattened and spindle-shaped, developing axon like structure. They aggregate, undergo arrest of cell cycle and eventually die (Cherbas et al, 1980). Ecdysteroids prompt B_{II} cells to form phagocytotic clumps with commitment increase in cell size and reduction in cell density. The Kc cell assay has been utilized to identify the ecdysteroid agonist RH5849 (Wing, 1988) and the B_{II} cell assay was used to identify the ecdysteroid antagonist cucurbitacin (Dinan et al., 1997).

All these assays indirectly analyze the interaction between an ecdysteroid and the receptor. Cloning of cDNAs encoding EcR and USP proteins from different animals has brought investigation of ecdysteroid specificity to a new era, which permits the investigation of the direct interaction of ligand and receptor in a species specific manner.

Rationale for current studies

Steroid hormone receptors bind response elements to regulate target gene expression. Vertebrate steroid hormone receptors form homodimers to bind response elements arranged as inverted repeats with a 3-bp spacer. In contrast, the insect steroid receptor is a heterodimer. Several EcREs have been identified from *Drosophila* ecdysteroid responsive genes. Based on the sequence for hsp27 and Eip EcREs, Cherbas *et al.* (1991) proposed RG(G/T)TCANTGA(C/A)CY as the consensus EcRE. Likewise, Antoniewski *et al.* (1993) analyzed the EcREs from hsp27 and Fbp-1 and proposed

PuG(G/T)T(C/G)ANTG(C/A)(C/A)(C/t)Py as the consensus EcRE. These consensus sequences are imperfect inverted repeats with a 1-bp spacer. In addition, the *Drosophila* EcR-USP can also bind direct repeats. However, no EcRE has been reported from other insect species including mosquito. Furthermore, as discussed above, it is not clear whether a single consensus EcRE can be devised. I speculated that the mosquito EcR-USP possessed DNA binding activity similar to that of *Drosophila* EcR-USP as homologous receptor proteins from these two species are highly conserved. The P-box in the nuclear receptor determines half site selection. ER type with a P-box EGckA selects half site AGGTCA, while GR type with a P-box GSckV selects half site AGAACA (Tsai and O'Malley, 1994). The P-box in EcR and USP proteins, EGckG, is identical to that in TR, VDR, RAR, PPAR and RXR, similar to that in ER, EGckA, suggesting AGGTCA as the consensus half-site. Before I joined Dr. Raikhel's lab, cDNAs encoding the mosquito EcR-B1, USPa and USPb had been cloned. My research project was involved characterization of the functional properties of the mosquito ecdysteorid receptor. The first part of my research was designed to define the features required for a functional EcRE for the mosquito ecdysteroid receptor. I addressed the following questions: 1) Can the mosquito EcR-USP bind *Drosophila* EcREs? 2) Is AGGTCA the consensus half site for an EcRE? 3) Can EcR-USP bind inverted repeats besides IR-1? 4) Can EcR-USP bind direct repeats? 5) What is the optimal spacer among direct repeats? 6) Is DNA binding activity correlated with transactivation efficiency?

Nuclear receptors usually contain several isoforms. Our research group was first to cloned two cDNA ultraspiracle isoforms, which differ primarily in their 5' untranslated regions and N-terminal A/B domain. These isoforms are likely involved with regulation of tissue and stage specific gene expression. RT-PCR analysis indicated that these two isoforms

exhibit distinct transcription profiles. The transcription of USPa correlated with the peak of juvenile hormone in the previtellogenic female mosquito. More intriguingly, Jones and Sharp (1997) recently reported that the *Drosophila* USP functioned as a receptor for juvenile hormone. Accordingly, EcR-USP transactivation can either be agonized or antagonized by juvenile. I speculated that these two USP isoforms could mediate differential DNA binding activity when heterodimerized with EcR. Therefore, in the second part of my research project, I addressed: 1) Do EcR-USPa and EcR-USPb display different DNA binding activity? 2) Can the USP isoforms mediate differential transactivation? 3)Can the juvenile hormone agonist methoprene affect EcR-USP transactivation?

The third part of my research involved the ligand specificity of EcR-USP. Although numerous ecdysteroids have been identified from animals and plants, little is known about the active form of ecdysteroid. In the mosquito *Aedes aegypti,* only ecdysone is detected by radioimmunoassay in the ovary, however, 20-hydroxyecdysone is the predominant one in the whole body. This observation has led Hagedorn et al (1975) to conclude that the ovary produces the ecdysone prohormone and it is converted to the active form 20E in the fat body. However, I speculated that ecdysone could also be an active hormone as some mosquito ovarian genes are ecdysteroid responsive. I designed experiment to answer the following questions: 1) Can ecdysone activate the mosquito EcR-USP? 2) Do ecdysteroid exert differential effects on mosquito and fruit-fly receptors? 3) What determines ligand specificity?

Under the guidance of Dr. Raikhel, I have focused my research to these specific aims and have provided clear answers to these questions.

CHAPTER 2. DNA BINDING AND TRANSACTIVATION CHARACTERISTICS OF THE MOSQUITO ECDYSTEROID RECEPTOR-*ULTRASPIRACLE* COMPLEX

ABSTRACT

The steroid hormone ecdysteroid is a key regulatory factor, controlling blood-meal triggered egg maturation in mosquitoes. To elucidate the ecdysteroid hierarchy governing this event, our research group cloned and characterized the ecdysteroid receptor (AaEcR) and the nuclear receptor *Ultraspiracle* (AaUSP), a retinoid X receptor homologue, from the mosquito, *Aedes aegypti*. I demonstrated that these mosquito nuclear receptors form a functional complex when binding the *Drosophila* heat shock protein 27-ecdysteroid response element (IR^{hsp}-1). Because natural ecdysteroid response elements (EcREs) from mosquito genes are not yet known, I analyzed the DNA binding properties of the AaEcR-AaUSP heterodimer with respect to the effects of nucleotide sequence, orientation and spacing between half sites in natural *Drosophila* and synthetic EcREs. Using an electrophoretic gel mobility shift assay (EMSA), I showed that AaEcR-AaUSP exhibits a broad binding specificity, forming complexes with inverted (IR) and direct (DR) repeats of the nuclear receptor response element half site consensus sequence AGGTCA separated by spacers of variable length. A single nucleotide spacer was optimal for both imperfect (IR^{hsp}-1) and perfect (IR^{per}-1) inverted repeats; adding or removing one base pair in an IR^{hsp}-1' spacer practically abolished binding. However, changing the half site to the consensus sequence AGGTCA (IR^{per}-1) increased binding of AaEcR-AaUSP ten fold over IR^{hsp}-1, and at the same time, reduced the stringency of the spacer length requirement, with IR^{per}-0 to IR^{per}-5 showing

detectable binding. Spacer length was less important in DRs of AGGTCA (DR-0 to DR-5): although 4-bp was optimal, DR-3 and DR-5 bound AaEcR-AaUSP almost as efficiently as DR-4. Furthermore, AaEcR-AaUSP also bound DRs separated by 11-13 nucleotide spacers. Competition experiments and direct estimation of binding affinity (*Kd*) indicated that, given identical consensus half sites and an optimal spacer, the AaEcR-AaUSP heterodimer bound an IR with higher affinity than a DR. Co-transfection assays utilizing CV-1 cells demonstrated that the mosquito EcR-USP heterodimer is capable of transactivating reporter constructs containing either IR-1 or DR-4. The levels of transactivation are correlated with the respective binding affinities of the response elements (IR^{per}-1 > DR-4 > IR^{hsp}-1). Taken together, these analyses predict broad variability in the EcREs of mosquito ecdysteroid-responsive genes.

INTRODUCTION

Nuclear hormone receptors activate or repress transcription through direct association with specific sequences known as hormone response elements (HREs), in the regulatory regions of responsive genes (Evans, 1988; Beato, 1989; Tsai and O'Malley, 1994; Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995). Known HREs contain characteristic 6-base pair core sequences, found either singly or as half sites within inverted, direct, or everted repeats. The specificity of an HRE is derived from four important characters: the nucleotide sequence of each half site, the spacing between half sites, half site orientation, and composition of the flanking regions (Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995; Cooney and Tsai, 1994). Steroid hormone receptors, including the estrogen (ER), progesterone (PR), glucocorticoid (GR), and mineralocorticoid (MR)

receptors, were originally thought to bind exclusively as homodimers to inverted repeats (IR) with the consensus half site AGGTCA or AGAACA separated by three nucleotides (IR-3) (Tsai and O'Malley, 1994; Cooney and Tsai, 1994). However, glucocorticoid and estrogen receptors have recently been shown to form complexes on direct repeats with variable spacing between half sites (Aumais *et al.*, 1996).

The vitamin D receptor (VDR) and non-steroid nuclear receptors, including thyroid hormone (TR) and retinoic acid (RAR) receptors, bind to cognate response elements as heterodimers with a shared partner, the retinoid X receptor (Mangelsfdorf and Evans, 1995). Response elements for these receptors are composed of direct repeats with consensus half sites, AGGTCA, spaced by 3, 4, or 5 nucleotides (DR-3, DR-4, and DR-5) (Umesono *et al.*, 1991). The subsequent demonstration that a DR-1 serves as an RXR and peroxisome proliferator activating receptor response element, and that a DR-2 serves as a second RAR response element has expanded the model to the so-called 1- to 5- rule (Mangelsdorf *et al.*, 1994). More recently, widely spaced, directly repeated AGGTCA elements have been shown to act as promiscuous enhancers for different classes of nuclear receptors; for example, a DR-15 reporter gene can be activated by RAR/RXR, VDR/RXR, and ER (Kato et al., 1995). These heterodimers can also regulate target gene expression by binding to response elements consisting of inverted or everted repeats. For example, an inverted repeat without spacing (IR-0) has been reported to function as a response element for TR, RAR, and VDR, while everted repeats with a 6-bp spacer or a 12-bp spacer serve as response elements for TR and VDR respectively (Umesono *et al.*, 1988; Baniahmad *et al.*, 1990; Carlberg *et al.*, 1993).

The insect steroid hormone, ecdysteroid, regulates essential processes in development, molting, metamorphosis, and reproduction (Riddiford, 1985; Bownes et al., 1986; Hagedorn, 1989; Segraves, 1994; Thummel, 1996). The functional ecdysteroid receptor in *Drosophila* is a heterodimer of the ecdysteroid receptor (EcR) protein (Koelle et al., 1991) and a RXR homologue, *Ultraspiracle* (USP) (Thomas et al., 1993; Yao et al., 1992; 1993). The first ecdysteroid response element (EcRE) was identified in the promoter of the *Drosophila* heat shock protein-27 gene. It is an imperfect palindrome with only a 1-bp spacer (IR^{hsp}-1), rather than the 3-bp spacer typical of vertebrate steroid HREs (Riddihough et al., 1987). Several EcREs have been identified in the regulatory regions of four more *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 containing imperfect inverted repeats with a 1-bp spacer (IR-1). These findings, together with DNA binding and *in vitro* transactivation studies, suggested that natural *Drosophila* EcREs are predominantly IR-1s. However, it was later found that *Drosophila* EcR-USP (DmEcR-DmUSP) can bind synthetic DRs of A/GGGTCA with spacers of 3-5 nucleotides, and can activate reporter gene constructs containing these direct repeats in *Drosophila* Schneider-2 cells (Horner et al., 1995; Antoniewski et al., 1996). Finally, the EcRE of the *Drosophila nested gene (ng)* is a direct repeat of AGGTCA with a 12-nucleotide spacer (D'Avino et al., 1995).

The maintenance and dispersal of mosquito-borne disease depends upon successful reproduction of the mosquito, and 20E plays a crucial role in regulation of vitellogenesis and oogenesis (Bownes 1986; Hagedorn, 1989; Dhadialla and Raikhel, 1994; Raikhel et al., 1999). The processes of egg maturation and disease transmission are intimately associated

through the mutual requirement for blood. Therefore, elucidation of the role of ecdysteroid receptor in mosquito reproduction is of significant biological and epidemiological importance. Although several target genes for the 20E-mediated regulatory cascade have been identified (32-34), native EcREs in mosquitoes are still unknown. In the mosquito *Aedes aegypti,* cDNAs of one ecdysteroid receptor (AaEcR) (Cho et al., 1995) and two USP isoforms (Kapitskaya et al., 1996) have been cloned. Compared to vertebrate nuclear receptors, insect EcR and USP homologues show unexpectedly high levels of sequence diversity (Kapitskaya et al., 1996, Cherbas and Cherbas, 1996). It is, therefore, difficult to predict the DNA binding specificity of the mosquito EcR-USP heterodimer. While DNA binding domain determinants of half site sequence specificity have been identified (Umesono et al., 1989, Mader et al., 1993; Danielsen et al., 1989), determinants of half site spacing and orientation as well as flanking sequence preferences are less well understood. In order to address these questions, I have analyzed the DNA binding properties of the AaEcR-AaUSP heterodimer. I used electrophoretic gel mobility shift assays (EMSA) with synthetic oligonucleotides and *in vitro* synthesized AaEcR and AaUSP to investigate the effects of the sequence, orientation, and spacing of half sites on the DNA binding properties of the mosquito EcR-USP heterodimer. Finally, I have used CV-1 cells in order to correlate the DNA binding properties of AaEcR-AaUSP with their ability to transactivate the reporter gene constructs containing EcREs.

MATERIAL AND METHODS

In vitro synthesis of nuclear receptor proteins - The nuclear receptor proteins were synthesized by coupled in vitro transcription-translation using the TNT system (Promega). AaEcR and AaUSPb cDNAs containing full open reading frames (ORFs) were subcloned into pGEM3Z (Promega) as previously described (Kapitskaya et al., 1996). For comparison, the 2.1-kb EcoRI fragment from pZ7-1-DmUSP (Henrich et al., 1990) and the 3.3-kb BamHI fragment from pACT-DmEcR-B1 (Koelle et al., 1991) bearing the entire ORFs of *Drosophila* USP and EcR, respectively, were subcloned into pGEM7Z (+) (Promega). The in vitro transcription/translation reactions programmed by the circular plasmid DNAs utilized the SP6 promoter. To confirm the synthesis of proteins with expected sizes, control TNT reactions were performed in the presence of [^{35} S]-methionine, and the resulting reactions analyzed by SDS-PAGE and autoradiography.

Oligonucleotides and probes - Oligonucleotides were purchased either from the Macromolecular Structure Facility of the Biochemistry Department at Michigan State University or GIBCO BRL. For DNA binding studies, a pair of sense and antisense oligonucleotides was annealed, resolved by 15% or 20% non-denaturing poly-acrylamide gel electrophoresis (PAGE), and the appropriate bands of double-stranded oligonucleotides were electro-eluted. Ten picomoles of double-stranded oligonucleotide were end-labeled with T4 DNA kinase (GIBCO BRL) and 50 μCi of [γ^{32} P]-ATP (DuPont NEN), and the unincorporated radioactivity removed through a Sephadex G-25 (Pharmacia) spin-column.

Electrophoretic gel mobility shift assay (EMSA) - One microliter of each TNT reaction was used alone or in combination as a protein source for EMSA. Proteins were incubated for 30 min at room temperature in 20 μl of the electrophoretic mobility shift buffer, containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 4% (v/v) glycerol, 0.05 mg/ml of poly (dI-dC)-poly (dI-dC), 0.3 mg/ml of a single-stranded DNA (5'-CTAACAAAGTTCGCCTGGACTAGAACGGCC-3'), 0.5 μM 20E and, for competition experiments, the indicated amounts of unlabeled competitor oligonucleotides. This was followed by the addition of 0.05 picomole of [^{32} P]-probe and incubating for another 30 min. The reaction mixture was resolved using a 6% non-denaturing PAGE at a constant voltage of 150 V for 90 min at room temperature. The gel was dried, and the distribution of radioactivity visualized either by autoradiography or by phosphor imaging for quantitative analysis using ImageQuant™ software (Molecular Dynamics).

Equilibrium dissociation constant (K_d) estimation - K_d values of AaEcR-AaUSP binding to potential EcREs were estimated according to Fawell et al. (1990) using the EMSA procedure described above. Protein samples were first incubated in the electrophoretic mobility shift buffer containing 0.5 μM 20E for 30 min, then with several different concentrations of labeled double-stranded oligonucleotides for another 30 min. Bound and free probes were separated by non-denaturing PAGE and quantified by the phosphor-imager. Saturation curves and Scatchard plots (Scatchard, 1969) were calculated for at least three independent experiments, and the mean value taken as the K_d.

Antibodies - The antiserum raised against AaEcR (anti-AaEcR) was prepared as follows. The HincII-EcoRI fragment of the AaEcR cDNA clone was subcloned into pMAL-c2 (New England Biolabs), and expressed in Escherichia coli TB1 strain. The AaEcR protein, which was fused to maltose binding protein, was concentrated by amylose resin according to the manufacturer's instructions. The fusion protein was further purified by SDS-PAGE followed by electroelution. A New Zealand white rabbit was immunized by subcutaneous injection with 100 μg of the purified fusion protein emulsified in TiterMax (CytRx) adjuvant. Blood was collected at two-week intervals, and the titer of specific antibodies estimated by Western blotting of the TNT reaction programmed by pGEM3Z-AaEcR.

The monoclonal antibody against DmUSP (anti-DmUSP), described in Khoury Christianson *et al.* (1992), was a gift from Dr. F. C. Kafatos (European Molecular Biological Laboratories, Heidelberg, Germany).

Reporter and expression plasmids and cell transfection assays - The BamHI-EcoRI fragment of AaEcR cDNA (35), and the EcoRI fragments of AaUSPb cDNA (36) were subcloned into the corresponding sites of pCDNA3.1/zeo (Invitrogen). Translatability of these constructs was checked by *in vitro* TNT coupled transcription/translation (Promega), which was followed by EMSA to verify binding properties of the expressed receptors. The reporter plasmid ΔMTV-5 x IR^{hsp}-1-CAT (chloramphenicol acetyltransferase), containing five copies of IR^{hsp} -1 was used in initial transfection assays (Yao et al., 1992). To make other reporter plasmids, oligonucleotides IR^{hsp}-1(agcttcaaGGGTTCaTGCACTtgtccatcg), DR-4 (agcttcaagTGACCTcctgTGACCTtgtccatcg), and IR^{per}-1

(agcttcaagAGGTCAaTGACCTtgtccatcg) were ligated into the HindIII site of ΔMTV-CAT (Hollenberg et al., 1988). Constructs harboring a single copy of either IR^{hsp}-1, IR^{per}-1, or DR-4 were used for a comparative study of transactivation by EcR-USP. Three copies of DR-4 were placed before the CAT gene to make the reporter construct ΔMTV-3xDR4-CAT. All reporter constructs were confirmed by sequencing. The expression plasmid CMV-β-Gal was a kind gift from Dr. L. Karl Olson (Department of Physiology, Michigan State University).

The green African monkey kidney CV-1 cell line (American Tissue Culture Collection, Bethesda, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. 2 x 10^5 cells were seeded in 6-well plates for 18-24 hours before transfection. Transfection was performed using Lipofectamine (Gibco BRL) according to the manufacturer's instruction. In brief, 0.4 μg each of AaEcR, AaUSP and CMV-β-Gal (β-galactosidase) expression plasmid, and 1.2 μg of the reporter plasmid were mixed with lipofectamine and transfected in OPTI-MEM (Gibco-BRL) for 3-5 hours. The transfection mixture was removed, and the cells further incubated in OPTI-MEM supplemented by 5% charcoal-stripped calf serum for 36-48 hours in the presence of 100% ethanol vehicle or Muristerone A (Sigma). Each well received 2.4 μg of total DNA. pCDNA3.1/Zeo (+) was used as a carrier for equalizing the amount of DNA allocated to each well. CAT assays were performed as described by Herbomel et al.(1984) for two hours, and β-Gal assays 60-90 min. CAT activity was normalized with β-Gal activity.

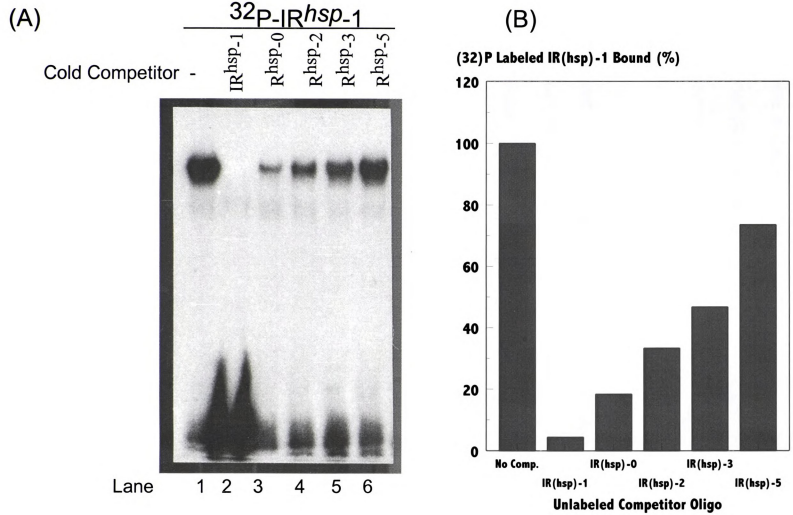
RESULTS

Binding of the AaEcR-AaUSP heterodimer to inverted repeats: the effect of spacer length and half site nucleotide sequence - Using EMSA with *in vitro* TNT-expressed mosquito EcR and USP isoforms, I previously demonstrated that the AaEcR-AaUSP complex bound a 30-base-pair oligonucleotide corresponding to the *Drosophila* hsp27 EcRE (Kapitskaya et al., 1996). This element was designated as IR^{hsp}-1 (an imperfect inverted repeat with a 1-bp spacer). In this study, I report in detail the DNA binding characteristics of mosquito EcR heterodimerized with the mosquito USPb isoform (designated hereafter as USP). My analyses indicated, however, that the binding properties of the AaEcR-AaUSPa heterodimer are generally similar to those of AaEcR-AaUSPb (not shown).

First, I confirmed binding of the AaEcR-AaUSP heterodimer to IR^{hsp}-1 by utilizing anti-AaEcR and anti-DmUSP antibodies: when EMSA were performed in the presence of either of the antibodies, AaEcR-AaUSP/IR^{hsp}-1 complexes were supershifted (not shown). Next, I investigated the role of IR spacer nucleotide length in the binding of the AaEcR-AaUSP heterodimer. In the first series of experiments, I tested the ability of IR^{hsp}s with various spacer-lengths to compete against IR^{hsp}-1 binding to the AaEcR-AaUSP complex (Fig. 1). A fifty-fold molar excess of the appropriate cold IR was added to each EMSA reaction with radiolabeled IR^{hsp}-1, and the intensity of the resulting bands measured. Self-competition with cold IR^{hsp}-1 led to a 96% reduction in binding intensity (Fig. 1A and 1B). IR^{hsp}-0 also was revealed to be an efficient competitor, displacing 82% of the bound IR^{hsp}-1 probe. However, the ability of IR^{hsp}s to compete with IR^{hsp}-1 progressively declined as the number of spacer nucleotides increased from 2 to 5. In a second series of experiments, the direct binding of

Fig. 1. Effect of spacer length in the imperfect inverted repeats (IR^{hsp}s) on binding with AaEcR-AaUSP. (A). EMSAs of *in vitro*-synthesized AaEcR-AaUSP incubated with 0.05 pmole of ^{32}P-labeled IR^{hsp}-1 in the absence (lane 1) or presence of a 50-fold molar excess of unlabeled IR^{hsp}-1 (lane 2) or its variants with varying length of spacer nucleotides between the two half-sites (IR^{hsp}-0, IR^{hsp}-2, IR^{hsp}-3, IR^{hsp}-5, lanes 3-6, see below for each sequence). The position of the AaEcR-AaUSP/^{32}P-IR^{hsp}-1 complex is indicated by an arrow head and the free probe by an asterisk. (B) The radioactivity associated with the protein/DNA complexes in (A) was quantified by phosphor-imaging as described in Materials and Methods. Each bar represents percentage binding relative to the control reaction without unlabeled competitors appearing in lane 1 of panel A. Oligonucleotides used in this experiment (only one strand of each probe is shown):

IR^{hsp} -0: agagacaagGGTTCATGCACTtgtccaa
IR^{hsp} -1: agagacaagGGTTCAaTGCACTtgtccaat
IR^{hsp} -2: agagacaagGGTTCAatTGCACTtgtccaa
IR^{hsp} -3: agagacaagGGTTCAaatTGCACTtgtccaa
IR^{hsp} -5: agagacaagGGTTCAaataaTGCACTtgtccaa


(A)
32P-IRhsp-1
Cold Competitor -
IRhsp-1
Rhsp-0
Rhsp-2
Rhsp-3
Rhsp-5
Lane 1 2 3 4 5 6
(B)
(32)P Labeled IR(hsp)-1 Bound (%)
120
100
80
60
40
20
0
No Comp.
IR(hsp)-1
IR(hsp)-0
IR(hsp)-2
IR(hsp)-3
IR(hsp)-5
Unlabeled Competitor Oligo

IR^{hsp}-0 -5 to AaEcR-AaUSP was tested by EMSA: apart from IR^{hsp}-1, only IR^{hsp}-0 exhibited a detectable retarded band (not shown).

Next, I tested AaEcR-AaUSP binding to a perfect inverted repeat consisting of AGGTCA half sites with a 1-bp spacer (IR^{per}-1). The EMSA revealed a strong retarded band when both TNT-generated AaEcR and AaUSP proteins were included in the reaction (Fig. 2A, lane 1). Binding of AaEcR-AaUSP to IR^{per}-1 was confirmed by supershift assays incorporating anti-AaEcR and anti-DmUSP (Fig. 2A, lanes 2 and 3). Interestingly, a faster migrating weak band, which was supershifted by anti-DmUSP, was detected when AaUSP alone was tested with IR^{per}-1 (not shown).

Perfect inverted repeats with nucleotide spaces from 0 to 5 (IR^{per}-0 –5) had much higher affinity to AaEcR-AaUSP than the imperfect repeats motifs (IR^{hsp}-0 –5): the retarded bands were clearly recognizable in all reactions (Fig. 2B). The retarded bands with IR^{per}-0 and IR^{per}-1 were considerably more intense than those formed by IR^{per}-2 to IR^{per}–5. Together with the results of IR^{hsp}s, this indicates preferential binding of AaEcR-AaUSP to IR motifs with 1 and 0 nucleotide spacers (Fig. 2B). This preference was also confirmed by competition EMSA (Fig. 3). AaEcR-AaUSP were incubated with a fixed amount of radiolabeled IR^{per}-1 and competed by increasing amounts of unlabeled IR^{per}s of various spacers. In the self-competition control, binding was almost completely competed away by inclusion of a 25-fold molar excess of cold IR^{per}-1. IR^{per}-0 also competed well, with a 25-fold molar excess competing away 93% of IR^{per}-1 binding to AaEcR-AaUSP. Twenty five-fold molar excess of IR^{per}-2, IR^{per}-3, and IR^{per}-5 displaced only 56%, 11%, and 32% of binding, respectively. IR^{per}-5 consistently showed stronger competition than IR^{per}-3.

Fig. 2. AaEcR-AaUSP bound to the perfect inverted repeats, IR^{per} sequences. (A) EMSAs were done with AaEcR-AaUSP and an IR^{per}-1 probe. The specifically retarded AaEcR-AaUSP/IR^{per}-1 complex is indicated by an arrow head (lane 1). The complex was supershifted by anti-AaEcR (lane 2, an arrow with open head) and anti-DmUSP (lane 3, an arrow with solid head), and could be competed away by a 50-fold molar excess of the unlabeled IR^{per}-1 (lane 4). The position of unbound IR^{per}-1 is indicated by an asterisk. (B) Direct binding EMSA assay. EMSAs were carried out with AaEcR-AaUSP and 0.05 pmole each of radiolabeled IR^{per}s with 0 to 5 spacer nucleotides. Position of the shifted AaEcR-AaUSP/DNA complex is indicated by an arrow head and of the free probe by an asterisk. Oligonucleotide probes used:

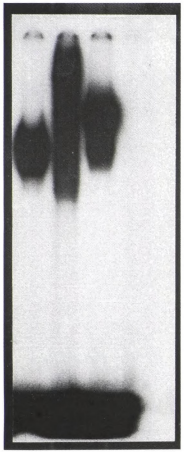
IR^{per} -0: agagacaagAGGTCATGACCTtgtccaa
IR^{per} -1: agagacaagAGGTCAaTGACCTtgtccaat
IR^{per} -2: agagacaagAGGTCAatTGACCTtgtccaa
IR^{per} -3: agagacaagAGGTCAaatTGACCTtgtccaa
IR^{per} -5: agagacaagAGGTCAaataaTGACCTtgtccaa

(A)

AaEcR Ab +

DmUSP Ab +

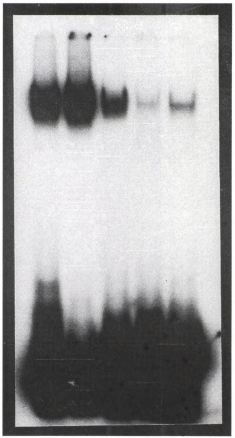
Cold Competitor +



Lane: 1 2 3 4

(B)

Hot probe: IR^{per-} 0 1 2 3 5



Lane: 1 2 3 4 5

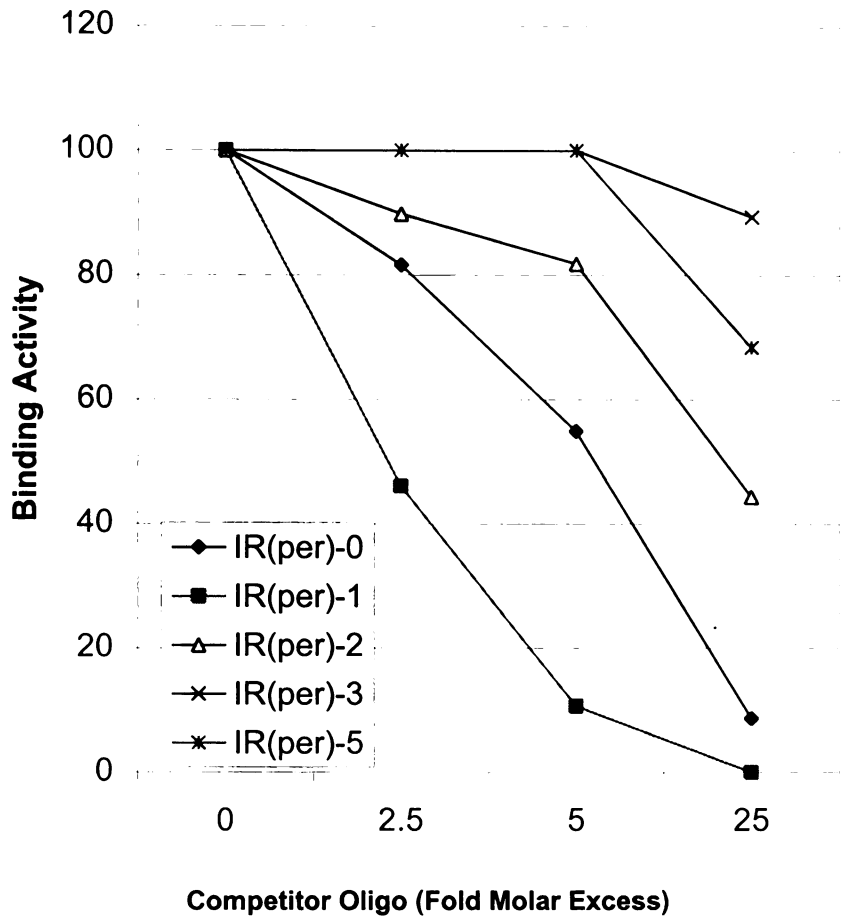


Fig. 3. Effect of spacer length of the perfect inverted repeats (IR^{per}s) on binding with AaEcR-AaUSP. AaEcR-AaUSP was incubated with 0.05 pmole of ^{32}P-labeled IR^{per}-1 in the absence of unlabeled competitor or in the presence of different molar excess of unlabeled competitor oligo IR^{per}0-5 (see Fig. 2 for sequences). Reactions were subjected to EMSA, and the radioactivity in the specific protein/DNA complexes counted by phosphor-imaging. The radioactivity associated with the DNA/protein complex observed without competition was taken as the control and defined as 100%.

Efficiency of competition indicates that the DNA-binding affinity of AaEcR-AaUSP towards IR^{per}s follows the order: IR-1 > IR-0 > IR-2 > IR-5 > IR-3.

Binding of the AaEcR-AaUSP heterodimer to direct repeats of AGGTCA - I also measured the binding of mosquito EcR-USP to a set of synthetic elements containing direct repeats with spacers ranging from 0-5. The mosquito EcR-USP complex effectively bound direct repeats (DRs) of AGGTCA containing a four-bp spacer (DR-4) (Fig. 4). The composition of the AaEcR-AaUSP complex was verified by supershift experiments using either anti-AaEcR or anti-DmUSP. The latter supershifted the AaEcR-AaUSP/DR-4 complex as efficiently as it did the control complex DmEcR-DmUSP/DR-4 (not shown).

I investigated the possible effect of DR-4 flanking regions on AaEcR-AaUSP binding by testing three DR-4 response elements: DR-4/3C with flanking regions from *Drosophila ng* elements (D'Avino et al., 1995), DR-4/HS with flanking regions from *Drosophila* hsp27 EcRE (Riddihough et al., 1987), and DR-4/VT with flanking regions from the thyroid response element (Glass et al., 1988). Radiolabeled IR^{hsp}-1 was displaced from AaEcR-AaUSP equally efficiently by 50-fold molar excess of cold DR-4s containing any of the three flanking regions (Fig 4, lane 1- 5). Labeled DR-4s with different flanking regions strongly bound AaEcR-AaUSP, forming specific retardation bands of similar size and intensity (Fig. 4, lanes 6 - 11). Thus, the flanking DNA sequences did not much affect specific binding of the heterodimer. Also of note is that incubation of AaEcR-AaUSP with DR-4/3C resulted in an additional band of higher mobility than the specific heterodimer band (Fig. 4, lane 6). This high mobility band was competed by an excess of the cold specific probe (Fig 4, lane 7),

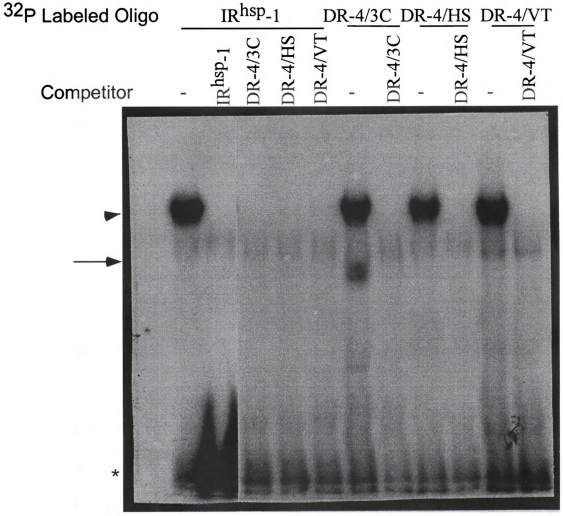


Fig. 4. AaEcR-AaUSP binding to a perfect direct repeat of AGGTCA with a 4-bp spacer (DR-4). Three types of DR-4s (see sequence below) were used in competition assays against radiolabeled IRhsp-1 (lanes 1-5), and in direct binding assays (lanes 6-11). For competition assays, EMSAs were performed with AaEcR-AaUSP and 0.05 pmole of 32P-labeled IRhsp-1 in the absence (lane 1) or presence of a 50-fold molar excess of unlabeled IRhsp-1 (lane 2) or of DR-4s with different flanking regions (DR-4/3C in lane 3; DR-4/HS in lane 4; and DR-4/VT in lane 5). Direct binding of these DR-4s with AaEcR-AaUSP was examined by EMSAs using radiolabeled DR-4/3C (lanes 6), DR-4/HS (lanes 8) and DR-4/VT (lanes 10), with self-competition assays by 50-fold molar excess of corresponding unlabeled nucleotides (lanes 7, 9 and 11). The position of the AaEcR-AaUSP/DNA complexes is indicated by an arrow head. An arrow points to the position of the complex formed by AaUSP monomer with DR-4/3C in lane 6 (for details, see text). An asterisk indicates the position of free probe. Oligonucleotides used in this experiment: DR-4/3C: aagcgaaAGGTCAaggaAGGTCAggaaaat
DR-4/HS: ttggacaAGGTCAcaggAGGTCActtgtct
DR-4/VT: tagcttcAGGTCAcaggAGGTCAgagag

and was specifically supershifted with anti-DmUSP, but not anti-AaEcR antibodies (not shown), suggesting that it might represent the binding of AaUSP alone.

The role of the spacer nucleotides in DRs was also investigated. In EMSA competition experiments, the AaEcR-AaUSP complex was incubated with labeled DR-4 in the absence or presence of 5- or 25- fold molar excess of cold DRs containing nucleotide spacers of various lengths (DR-0 to DR-5). Twenty five-fold molar excess of cold DR-4 was sufficient to displace the bound probe almost completely (Fig. 5). DR-3 and DR-5 were almost as efficient as DR-4 itself. DR-1 and DR-2 seemed to be slightly less efficient, but still displaced around 90% of the labeled probe. DR-0 was the weakest competitor, with only 2/3 of the bound probe displaced by a 25-fold molar excess of this response element (Fig. 5). Indeed, in direct EMSA binding experiments, DR-0 was the only DR sequence, which did not exhibit detectable binding to AaEcR-AaUSP (not shown). Thus, spacer length in DRs appeared to be less critical for binding to the mosquito EcR-USP complexes than in IRs. Nevertheless, efficiency of competition (Fig. 5) indicates that the DNA-binding affinity of AaEcR-AaUSP towards DRs follows the order: DR-4 > DR-3 > DR-5 > DR-2 > DR-1 > DR-0.

I was also interested in determining whether the mosquito EcR-USP complex might be capable of recognizing more widely spaced direct repeats. It has been reported that the *Drosophila* EcR-USP complex recognizes a DR-12 sequence found within the *Drosophila ng* gene (the original element was called DR-11 because 7-bp were taken as the consensus half site) (D'Avino et al., 1995). The mosquito EcR-USP was capable of binding to the *ng* EcRE (Figure 6), however, my analysis of this element identified more closely-spaced cryptic direct

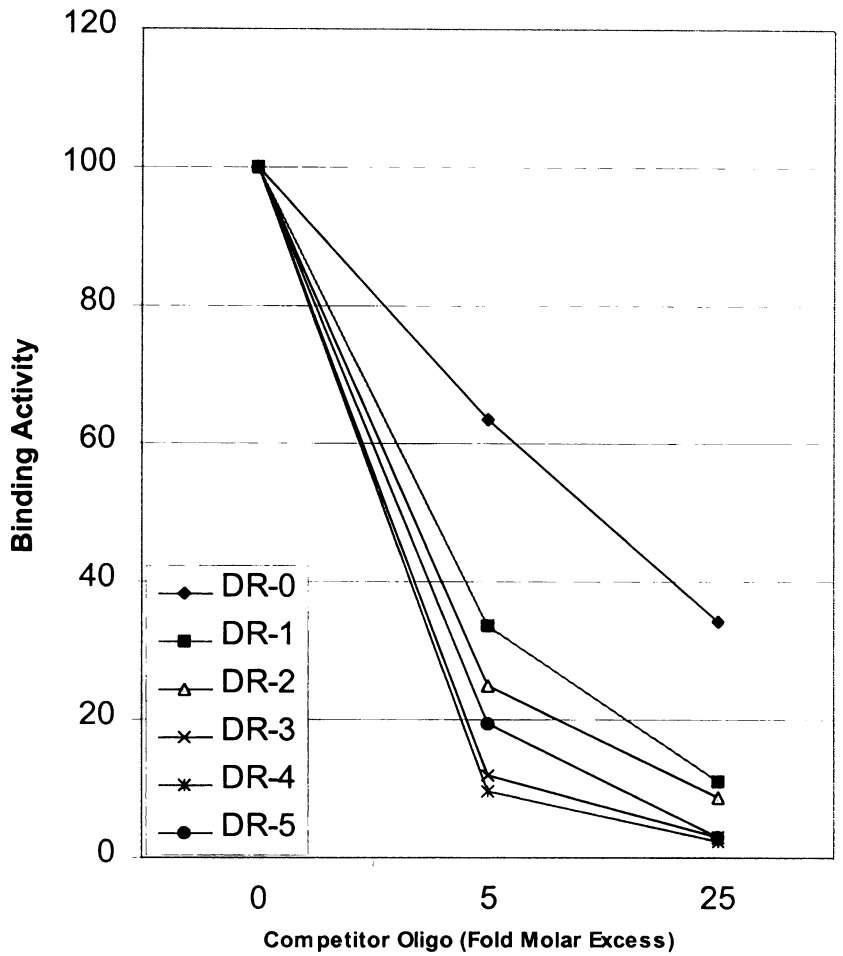


Fig. 5. Effect of spacer length of the perfect direct repeats on binding with AaEcR-AaUSP. AaEcR-AaUSP was incubated with 0.05 pmole of ^{32}P-labeled DR-4/3C in the absence of unlabeled competitor or in the presence of different molar excess of unlabeled competitor oligo DR0-5. Reactions were subjected to EMSA, and the radioactivity in the specific protein/DNA complexes counted by phosphor-imaging. The radioactivity associated with DNA/protein complex observed without competition was taken as the control and defined as 100% Data are reported as a percentage of the control. Oligonucleotides used in this experiment:

DR-0: aagcgaaAGGTCAAGGTCAaggaaaat
DR-1: aagcgaaAGGTCAgAGGTCAaggaaaat
DR-2: aagcgaaAGGTCAggAGGTCAaggaaaat
DR-3: aagcgaaAGGTCAaggAGGTCAaggaaaat
DR-4: aagcgaaAGGTCAaggaAGGTCAaggaaaat
DR-5: aagcgaaAGGTCAagagaAGGTCAaggaaaat

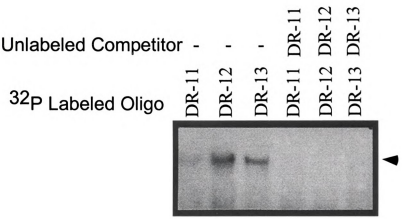


Fig.6. AaEcR-AaUSP binding to a direct repeat of AGGTCA with 11-, 12-, and 13-bp spacers (DR-11, DR-12, and DR-13, respectively). EMSAs were done with AaEcR-AaUSP and radiolabeled DR-11 (lanes 1), DR-12 (lanes 2) and DR-13 (lanes 3). Lanes 4-6, self-competition with a 50-fold molar excess of respective cold nucleotides. The position of the shifted AaEcR-AaUSP/DNA complex is indicated by an arrow head. Oligonucleotides used in this experiment:
DR-11: aagcgaaAGGTCAagaggcaaagaAGGTCAggaaaat
DR-12: aagcgaaAGGTCAagaggccaaagaAGGTCAggaaaat
DR-13: aagcgaaAGGTCAagaggcgcaaagaAGGTCAggaaaat

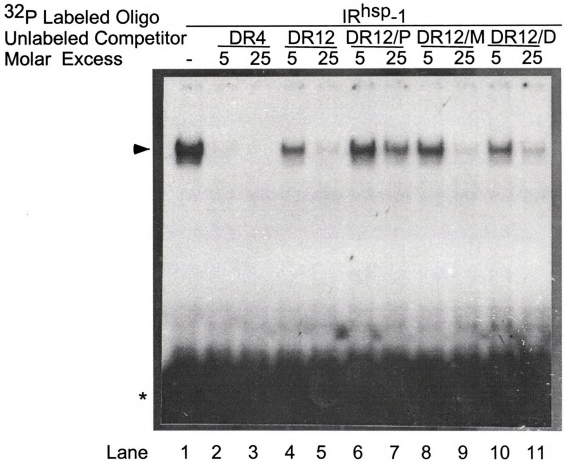


Fig. 7. Binding properties of DR-12 and its mutant sequences. EMSAs were performed with AaEcR-AaUSP and radiolabeled IRhsp-1 in the absence (lane 1) or presence of a 5- or 25-fold molar excess of cold DR-4 (lanes 2 and 3), DR-12 (lanes 4 and 5), DR-12/P (lanes 6 and 7), DR-12/M (lanes 8 and 9), and DR-12/D (lanes 10 and 11). The reactions were subjected to EMSA and autoradiographed. The AaEcR-AaUSP/DNA complexes are indicated by an arrow head and the free probe by an asterisk. Oligonucleotides used in this experiment:

DR-4/HS: ttggacaAGGTCAcaggAGGTCActtgtct
DR-12: aagcgaaAGGTCAagAGGCCAaagaAGGTCAggaaaat
DR-12/P: aagcgaaAGacatagAGGCCAaagaAGGTCAggaaaat
DR-12/M: aagcgaaAGGTCAagAGacataagaAGGTCAggaaaat
DR-12/D: aagcgaaAGGTCAagAGGCCAaagaAGacatggaaaat

repeats within the *ng* EcRE. Within the *ng* EcRE, there are consensus half sites at either end and an imperfect half site (AGGCCA) in the middle, such that it could form a DR-2 or DR-4 in combination with one of the terminal elements. While binding of DmEcR-DmUSP was abolished when both terminal consensus half sites in the DR-12 were mutated simultaneously (D'Avino et al., 1995), this does not rule out the possibility that the DR-2 or DR-4 might be the active element or might be a functionally significant part of a compound element.

To investigate the nature of AaEcR-AaUSP binding to the *ng* EcRE, I performed mutational analyses of all three half sites in this sequence, in which a mutation was introduced independently into the 5'-proximal (DR-12/P), middle (DR-12/M), or 3'-distal (DR-12/D) half sites. First, I conducted a competition assay with ^{32}P-labeled IR^{hsp}-1 and 5- or 25-fold molar excess of cold DR-12, DR-12/P, DR-12/M, or DR-12/D (Fig.7). Twenty five-fold molar excess of cold DR-4, used as a positive control, removed 98% of radioactive probe binding (Fig. 7, lane 3), while 25-fold molar excess of the *ng* EcRE eliminated about 92% (Fig. 7, Lane 5). Mutating the proximal half-site (DR-12/P) cripples the DR-12, leaving only the imperfect DR-4 intact. DR-12/P was much a weaker competitor (Fig. 7, lanes 6 and 7). In contrast, DR-12/M and DR-12/D, in which only the imperfect DR-2 was preserved, retained most of the binding ability of the original *ng* EcRE, with DR-12/M binding more strongly than DR-12/D (Fig. 7, lanes 8-11). The overall order of relative affinity of tested elements was DR-4 > *ng* EcRE = DR12/M > DR12/D >> DR12/P. Importantly, DR-12/M exhibited the same level of competition as the original DR-12, suggesting that the latter indeed serves as a response element with a 12-nucleotide spacer.

In order to determine whether other widely spaced direct repeats might also function as EcREs, I tested binding to DR-11 and DR-13 elements (Fig. 6). These studies show that the EcRE/USP has considerable flexibility in its spacing requirements. While binding to DR-12 was strongest of the three, specific binding was also clearly demonstrated for the DR-11 and DR-13 elements.

In addition, I tested binding of the AaEcR-AaUSP complex to Eip28/29, a composite element from the promoter of the *Drosophila Eip28/29* gene, containing an imperfect IR-1 and an imperfect DR-3 (Cherbas et al., 1991). EMSA analyses showed that AaEcR-AaUSP bound this composite motif as a heterodimer (data not shown).

Binding affinity of the AaEcR-AaUSP heterodimer: *effect of sequence and orientation of half sites in the response element* - I compared the effect of half site orientation and sequence on the DNA binding affinity of the AaEcR-AaUSP heterodimer. For these analyses, I utilized only IR^{hsp}-1, IR^{per}-1 and DR-4, because these elements exhibited maximal binding in the respective categories. First, I performed competitive EMSA, in which ^{32}P-labeled IR^{hsp}-1 was competed with 2.5-, 5.0-, and 10.0-fold molar excess of cold IR^{hsp}-1, IR^{per}-1, DR-4, or Eip-28/29 (Fig. 8). The results suggest that the binding affinity of IR^{per}-1 to AaEcR-AaUSP was stronger than that of DR-4 or IR^{hsp}-1, which varied insignificantly from each other. The Eip28/29 appeared to have the weakest binding affinity to AaEcR-AaUSP. Finally, to resolve quantitatively the differences in DNA binding affinity, I calculated the equilibrium dissociation constants (*Kd*) for IR^{hsp}-1, IR^{per}-1 and DR-4 binding to AaEcR-AaUSP. The EcR-USP heterodimer was incubated with increasing concentrations of radiolabeled probes (IR^{hsp}-1, IR^{per}-1, or DR-4) in the presence of $5x10^{-7}$ M

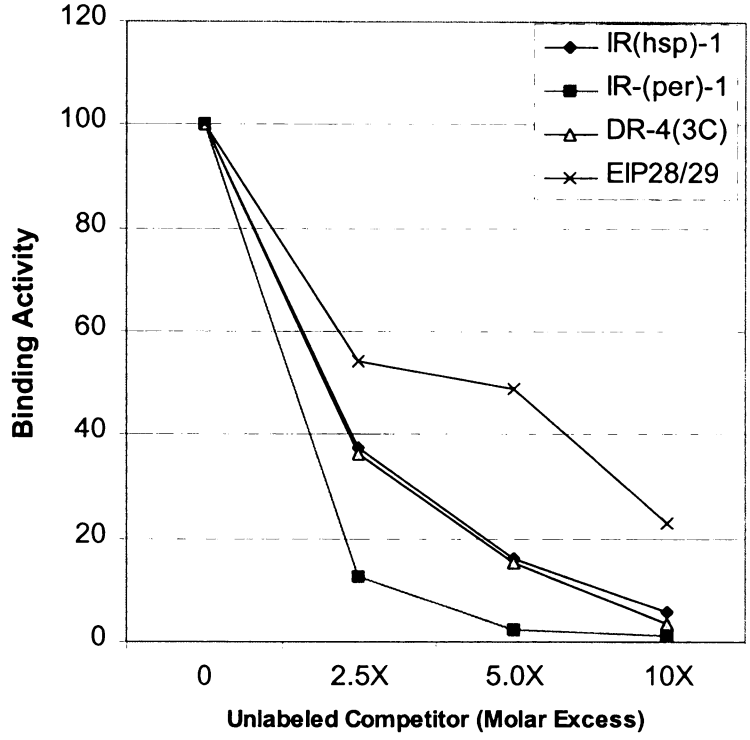
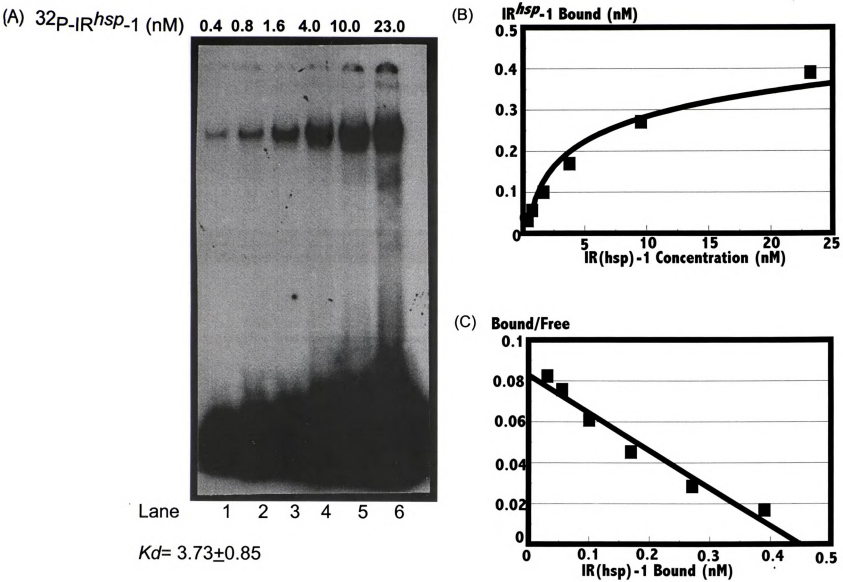


Fig. 8. Comparison of binding affinity to AaEcR-AaUSP among IR^{hsp}-1, Eip28/29, IR^{per}-1 and DR-4 by competition assay. AaEcR-AaUSP was incubated with 0.05 pmole of ^{32}P-labeled IR^{per}-1 in the absence of unlabeled competitor or in the presence of different molar excess of unlabeled competitor IR^{hsp}-1, Eip28/29, IR^{per}-1 or DR-4/3C (for sequences, see Figs. 1, 2 and 4). Reactions were subjected to EMSA, and the radioactivity in the specific protein/DNA complexes counted by phosphor-imaging. The radioactivity associated with protein/DNA complex observed without competition was taken as the control and defined as 100%. Data are reported as a percentage of the control.

Fig. 9. Binding affinity (Kd) of IR^{hsp}-1 to the AaEcR-AaUSP complex. (A) EMSAs of AaEcR-AaUSP and radiolabeled IR^{hsp}-1 through the indicated range of DNA probe concentrations. Radioactivity associated with free oligonucleotides and with protein/oligonucleotide complexes was determined separately, permitting the construction of a saturation curve (B) and a Scatchard Plot (C). EMSA and quntification were repeated at least three times. The shifted DNA/protein complex is indicated by an arrow and the free probe by an asterisk.


(A) 32P-IRhsp-1 (nM) 0.4 0.8 1.6 4.0 10.0 23.0
Lane 1 2 3 4 5 6
Kd= 3.73±0.85
(B) IRhsp-1 Bound (nM)
0.5
0.4
0.3
0.2
0.1
0
5 10 15 20 25
IR(hsp)-1 Concentration (nM)
(C) Bound/Free
0.1
0.08
0.06
0.04
0.02
0
0 0.1 0.2 0.3 0.4 0.5
IR(hsp)-1 Bound (nM)

20E, the optimal concentration for EcR-USP DNA binding (S. Wang and A. Raikhel, unpublished observation). Saturation binding analyses and Scatchard analyses were used to estimate *Kd* values for the binding of AaEcR-AaUSP to IR^{hsp}-1 (Fig. 9), IR^{per}-1, and DR-4 (not shown). The differences in *Kd* values for IR^{hsp}-1, IR^{per}-1, and DR-4, which are in agreement with the results of the competition analyses (Fig. 8), indicate that AaEcR-AaUSP binds IR^{per}-1 with an 8-fold higher affinity than to DR-4, and with 10-fold higher affinity than to IR^{hsp}-1 (Table 1).

Transactivation of AaEcR-AaUSP: DNA binding affinity corresponds to transactivation activity - Transactivation of AaEcR-AaUSP was studied using the CV-1 cell line. This mammalian cell line has no endogenous EcR and contains very low endogenous levels of RXR. It has been used to study transactivation of DmEcR-DmUSP (20-22). The transactivation ability of the AaEcR-AaUSP 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 plasmid alone resulted in a very low basal level of CAT activity (Fig.10A). Co-transfection of the reporter plasmid with either AaEcR or AaUSP expression vector alone did not confer ecdysteroid responsiveness. However, strong induction (40 fold) of CAT activity was observed when the reporter plasmid was co-transfected with both AaEcR and AaUSP expression vectors and incubated with 1 mM MurA, demonstrating that the AaEcR-AaUSP heterodimer activated reporter gene expression in a ligand-dependent manner.

Next, I tested whether DR-4 could function as an EcRE in CV-1 cells. I constructed a reporter plasmid (ΔMTV-3xDR-4-CAT) containing three copies of DR4. Co-

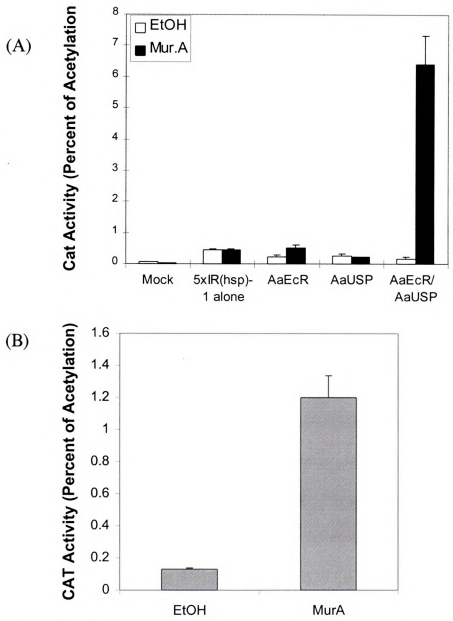


Fig.10. AaEcR-AaUSP renders CV-1 cells ecdysteroid responsive. (A) 0.4 μg of CMV-β-Gal and 1.2 μg reporter plasmid ΔMTV-5xIR^{hsp}1-CAT were transiently transfected into CV-1 cells (column 2), with 0.4 μg of AaEcR (column 3), 0.4 μg AaUSP (column 4) or 0.4 μg each of AaEcR and AaUSP (column 5) expression vectors. (B). 0.4 μg of CMV-β-Gal and 1.2 μg reporter plasmid ΔMTV-3xDR4-CAT were transiently transfected into CV-1 cells with 0.4 μg each of AaEcR and AaUSP expression vectors. After transfection, cells were incubated in the presence of vehicle ethanol or 1 μM MurA for 36 hours and harvested for CAT assay. CAT activity was normalized by β-Gal activity. Transfection was performed in triplicate and the normalized CAT activity averaged (mean±SE).

transfection of this reporter construct with either the AaEcR or the AaUSP expression vector did not render CV-1 cells ecdysteroid responsive (data not shown). However, co-transfecting the reporter construct with both AaEcR and AaUSP expression vectors rendered CV-1 cells highly responsive to ecdysteroid with 9-fold induction (Fig. 10-B).

Finally, I elucidated whether the level of transactivation by AaEcR-AaUSP depends on the sequence and orientation of the half-sites in the EcRE, and whether it is correlated with DNA binding affinity. I constructed ΔMTV-CAT reporter plasmids containing a single copy of IR^{hsp}-1, DR-4 or IR^{per}-1. Co-transfection of these reporter plasmids with either AaEcR or AaUSP expression vector did not render CV-1 cells ecdysteroid responsive similar to results with the ΔMTV- 5xIR^{hsp}-1-CAT reporter plasmid (data not shown). Co-transfection of both AaEcR and AaUSP with each of these reporter plasmids resulted in an increase in CAT activity in the presence of 1mM MurA (Fig. 11). The level of transactivation of the reporter plasmid containing only one copy of the EcRE is considerably lower than that with five copies or three copies (Figs. 10). The differences in levels of activation between tested EcREs were of lower magnitude than the differences between their binding affinities. However, the strength of binding directly corresponds to the level of transactivation for each class of EcRE, with IR^{per}-1 > DR-4 > IR^{hsp}-1 (Table 1). A nonparametric *STP* test (Sokal and Rohlf, 1987) indicated that the differences in transactivation between the different elements are statistically significant (α=0.01).

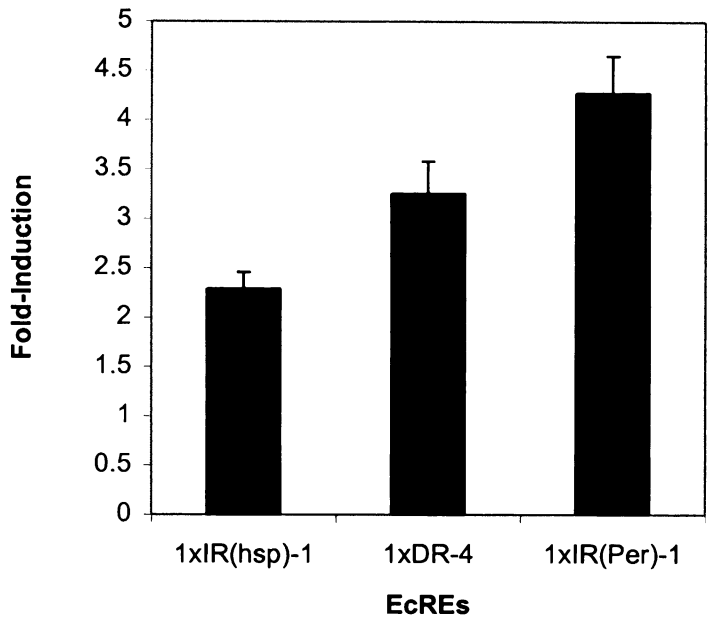


Fig.11. Comparison of MurA transcriptional induction conferred by IR^{hsp}-1, DR-4 and IR^{per}-1 elements on ΔMTV-CAT reporter constructs. 0.4 μg each of CMV-β-Gal, AaEcR and AaUSP expression vectors were transiently co-transfected with 1.2 μg of the ΔMTV-CAT reporter plasmid harboring one copy of IR^{hsp}-1 (column 1), DR-4 (column 2), and IR^{per}-1 (column 3). After transfection, cells were incubated in the presence of vehicle ethanol or 1 μM MurA for 36 or 48 hours and harvested for CAT assays. Transfection was performed in two independent experiments in triplicate, and the CAT activity, normalized by β-Gal activity, was expressed as fold-induction (mean±SE). The differences in transactivation between the different elements are statistically significant ($P<0.01$).

Table 1

The equilibrium dissociation constants (K_d) of different DNA sequences binding to AaEcR-AaUSP, and the corresponding level of reporter gene transactivation in CV-1 cells (means±SE).

EcRE	IR^{hsp}-1	IR^{per}-1	DR-4
Kd (in nM):	3.73±0.85	0.326±0.026	2.21±0.36
Fold Induction	2.29±0.17	4.27±0.38	3.26±0.38

Binding affinities (Kds) of IR^{hsp}-1, IR^{per}-1 and DR-4 to the AaEcR-AaUSP complex were measured by EMSA. *In vitro* translated AaEcR-AaUSP was incubated with increasing amounts of radiolabeled elements (0.4 nM-20 nM) and resolved by electrophoresis. Radioactivity associated with free oligonucleotides and with protein/oligonucleotide complexes was quantitated by phosphor-image analysis, permitting the construction of a saturation curve and a Scatchard Plot (Fig. 9). EMSAs and quantifications were repeated at least three times and the mean taken as the Kd. For transfection assays, 0.4 μg each of CMV-β-Gal, AaEcR and AaUSP expression vectors were transiently co-transfected into CV-1 cells with 1.2 μg of ΔMTV-CAT reporter plasmid harboring one copy of IR^{hsp}-1, DR-4 and IR^{per}-1 (Fig. 11). After transfection, cells were incubated in the presence of vehicle 100% ethanol or 1μM MurA for 36 or 48 hours and harvested for CAT and β-Gal assays. Fold induction was calculated from normalized CAT activity from two triplicate experiments. Standard errors (SE) were calculated by Microsoft Excel™. IR^{hsp}-1: agagacaagGGTTCAaTGCACTtgtccaat IR^{per}-1: agagacaagAGGTCAaTGACCTtgtccaat DR-4: aagcgaaAGGTCAaggaAGGTCAggaaaat

DISCUSSION

In this paper, I provide further evidence that the AaEcR-AaUSP heterodimer is the functional mosquito ecdysteroid receptor, and that it is capable of binding various DNA motifs oriented either as inverted or direct repeats. Data presented here parallels previous observations from several insect species that heterodimerization of EcR and USP is required for efficient binding of both the ligand and the response elements, as well as for gene transactivation (Thomas et al., 1992; Yao et al., 1992; 1993, Kapitskaya et al., 1996; Swever et al., 1996; Elke et al., 1997). Analyses utilizing EMSA and anti-EcR and anti-USP antibodies clearly demonstrated that the AaEcR-AaUSP heterodimer exhibits specific binding to the various sequences of naturally-occurring *Drosophila* EcREs as well as to the synthetic response elements tested in this study. This and a previous study (Kapitskaya et al., 1996) demonstrate that the AaEcR-AaUSP heterodimer is capable of binding to various DNA motifs with the consensus half-site sequence AGGTCA oriented as inverted repeats. Indeed, EcREs found in native *Drosophila* genes are predominantly inverted imperfect palindromes; the binding affinities of Sgs-4, Lsp-2, Fbp-D, and Eip28/29 EcREs are weaker than the hsp27 EcRE (IR^{hsp}-1), which is the most efficient natural EcRE identified for *Drosophila* to date (Riddihough et al., 1987; Cherbas et al., 1991; Lehmann and Korge, 1995; Antoniewski et al., 1995; Antoniewski et al., 1993). I obtained similar results when testing mosquito ecdysteroid receptor binding to the Eip28/29 and hsp27 EcREs (Fig. 8). Here, I have shown that the perfect palindrome IR^{per}-1 binds 10 times more efficiently than IR^{hsp}-1.

My results suggest that spacer length likewise plays an important role in both imperfect (IR^{hsp}) and perfect (IR^{per}) inverted repeats, with a single nucleotide spacer being optimal for

both. This finding is in agreement with conclusions drawn from studies performed with DmEcR-DmUSP (Riddihough et al., 1987; Cherbas et al., 1991; Antoniewski et al., 1993). Moreover, I also found that whereas adding or removing one base pair from a spacer in IR^{hsp}-1 practically abolishes binding, changing the half site to the consensus sequence AGGTCA (IR^{per}-1) reduces the stringency of the spacer length requirement, so that IR^{per}-0 to IR^{per}-5 exhibit detectable binding.

It has been demonstrated that *Drosophila* EcR-USP binds to direct repeats, and that a 4-bp spacer is optimal (Horner et al., 1995; Antoniewski et al., 1996). My observations on the mosquito EcR-USP heterodimer suggest that this aspect of EcR-USP DNA binding specificity also displays a high degree of functional conservation. Direct binding and competition assays demonstrate that the nucleotide spacer length is less important in direct repeats of AGGTCA (DR-0 to DR-5) than in IRs. Although 4-bp is an optimal spacer length in the direct repeats, DR-3 and DR-5 bind AaEcR-AaUSP almost as efficiently as DR-4. The order of binding affinities of AaEcR-AaUSP to DRs (DR-4 > DR-3 > DR-5 > DR-2 > DR-1 > DR-0) corresponds closely to that recently reported for DmEcR-DmUSP (DR-4 > DR-5 > DR-3 > DR-1 > DR-2 > DR-0) (Antoniewski et al., 1996).

Competition experiments and direct estimations of *Kd* indicate that binding affinity depends on the sequence of the half-site and is higher when the consensus is used for each half site. However, given the same consensus half site and an optimal spacer, the AaEcR-AaUSP heterodimer binds an inverted repeat with considerably more strength than a direct repeat. My results significantly extend DNA binding studies on insect EcR-USP heterodimers by providing accurate measurements of dissociation constants for DR-4, IR^{hsp}-1 and IR^{per}-1.

Kato et al. (1992) showed that in the chicken ovalbumin promoter region, there are multiple AGGTCA motifs arranged as direct repeats separated from each other by more than 100 bp. In spite of such large spacers, they can act synergistically as a complex estrogen response element (ERE), indicating that widely spaced half-sites can cooperate to generate an efficient ERE. Moreover, widely spaced direct repeats (10-200 bp) can function as cis-acting response elements for retinoic acid and vitamin D receptors (Kato et al., 1995). In contrast to the specificity observed with shortly-spaced DRs (DR-1 to DR-5), different receptors bind promiscuously to these widely spaced repeats to activate transcription in the presence of retinoic acid, vitamin D, or estrogen. My tests have shown that although the AaEcR-AaUSP heterodimer exhibits relatively strong binding to DR-12, it is considerably weaker than to DR-3 or DR-4. Furthermore, AaEcR-AaUSP binds to other direct repeats separated by more than 10 nucleotides (DR-11 and DR-13), but with considerably lower affinity than to DR-12. Presently, it is not known whether insect EcR-USP heterodimers are capable of utilizing widely spaced half sites (e.g., >100 bp) as response elements.

I observed that the *ng* EcRE, previously described as a DR-12, is a composite of three half-sites, suggesting the possibility that an internal 5'-proximal DR-2 and/or 3'-proximal DR-4 might contribute to the functionality of this element. Mutating the 5' half site in this element dramatically reduced its binding affinity, revealing its critical role in EcR-USP binding. In contrast, mutating the 3' half site decreased binding affinity only slightly, while mutating the middle half site did not have any obvious effect on the binding of the element. Thus, both competition and direct binding analyses of mutated *ng* EcRE suggest that in

addition to functioning as a true DR-12, the *ng* element may also have a functional imperfect DR-2 located at its 5' end (Fig. 7).

Transactivation assays in CV-1 cells confirmed the finding of the DNA binding assays, demonstrating that the AaEcR-AaUSP heterodimer is indeed the functional ecdysteroid receptor. Co-transfection of AaEcR and AaUSP expression vectors into CV-1 cells conferred 40-fold induction of the reporter plasmid ΔMTV-5xIR^{hsp}-1-CAT and 9-fold induction of ΔMTV-3xDR4-CAT in response to 1 mM MurA. I observed that the number of EcREs in a reporter construct is not directly proportional to the magnitude of reporter transactivation. A reporter plasmid containing a single copy of IR^{hsp}-1 was induced only 2.5-fold compared to a 40-fold induction of the reporter containing five copies of the same EcRE. Therefore, in order to compare transactivation activities of IR and DR elements, I utilized the reporter plasmids containing only one copy of either IR^{hsp}-1, DR-4 or IR^{per}-1. Importantly, all three response elements were able to mediate ecdysteroid responsiveness of the reporter in CV-1 cells. The transactivation efficiencies of tested response element followed the order IR^{per}-1 > DR-4 > IR^{hsp}-1. Thus, despite the fact that the differences were not as dramatic as those for binding affinities measured for the same elements, the two sets of data are in agreement with one another (Table 1). Using transfection in *Drosophila* Schneider-3 cells and endogenous receptor pools, Martinez et al. (1991) also showed that for DmEcR-DmUSP, IR^{per}-1 was transactivated about twice as well as IR^{hsp}-1 when placed before the TK promoter. Using four copies of EcREs ahead of the *hsp*70 promoter, Vogtli et al (1998) reported that IR^{per}-1 activated the reporter gene twice stronger than IR^{hsp}-1 and DR-4 in the presence of

endogenous DmEcR-DmUSP in Schneider-2 (S2) cells. These results agree with my observations for CV-1 cells.

DR-4, the optimal DR for binding to AaEcR-AaUSP (Fig. 5) and DmEcR-DmUSP (Horner et al., 1995; Antoniewski et al., 1996), so far has not been identified as a natural EcRE in ecdysteroid responsive genes in any organism. The functionality of DR-4 as EcRE is controversial in the literature. Antoniewski et al. (1996 showed that various DRs, including DR-4, could act as functional EcREs for *Drosophila* EcR-USP in (S2) cells. More recently, these results have been confirmed by Vogtli et al. (1998). However, DR-4 failed to render *Drosophila* Kc cells ecdysteroid responsive (Cherbas and Cherbas, 1996). Our data demonstrate that DR-4 also can act as a functional EcRE for AaEcR-AaUSP in mammalian CV-1 cells. Taken together, these observations suggest that direct repeats may serve as cell-specific EcREs. By placing two copies of either IR^{hsp}-1 or DR-4 upstream of the thymidine kinase (TK) promoter for transfection assays in S2 cells, Antoniewski et al. (1996) demonstrated that IR^{hsp}-1 was a more potent EcRE than DR-4 for transactivation by exogenous DmEcR-DmUSP. In contrast, the situation was the reverse in my experiments in CV-1 cells. These differences could be due to the number of EcREs in the reporter constructs, to the type of cells used for the transactivation experiments, or they could also reflect true differences in the transactivation properties of *Drosophila* and mosquito EcR-USP heterodimers.

Taken together, my findings suggest that both IR and DR response elements can act as functional EcREs in the activation of mosquito genes. My data predict wide variability among natural EcREs in mosquito genes. Moreover, because the level of gene

transactivation by ecdysteroid depends on an EcRE sequence, differences between the various EcREs may be utilized as one of the mechanisms regulating the levels of ecdysteroid responsiveness. Recently, support for this hypothesis has been provided by discovery of EcREs in two mosquito yolk protein precursor genes, vitellogenin (Vg) and vitellogenic carboxypeptidase (VCP). Regulatory regions of both *Vg* and VCP genes, expression of which is controlled by 20E (Dhadialla and Raikhel, 1990, Cho et al., 1991), contain imperfect DR-1 and DR-2 elements, respectively. Several lines of analysis strongly suggest that both Vg DR-1 and VCP DR-2 are functional EcREs. First, both of them specifically bind AaEcR-AaUSP in EMSA utilizing *in vitro* expressed receptors, as well as nuclear extracts from vitellogenic mosquito fat bodies. Second, a portion of the VCP promoter containing the DR-2 EcRE confers ecdysteroid responsiveness in CV-1 cells. Finally, a reporter gene containing the regulatory region of either the *Vg* or VCP gene with the respective EcRE was expressed at the correct stage when transformed into *Drosophila* (Martin, D., Wang, S.-F., Miura, K., Kokoza, V. and Raikhel, A., unpublished data). Thus, the present study provides a solid foundation to search for native EcREs in mosquito genes. It will aid in the analysis of the regulatory mechanisms governing gene expression during the blood-meal activated events of reproduction and pathogen transmission in this critically important insect vector for both humans and animal diseases.

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CHAPTER 3. CHARACTERIZATION OF MOSQUITO *ULTRASPIRACLE* ISOFORMS

ABSTRACT

Ultraspiracle (USP), the insect homolog of vertebrate retinoid X receptor (RXR), is an obligatory dimerization partner for ecdysteroid receptor (EcR). Two USP isoforms, USPa and USPb, with distinct N-termini occur in the mosquito, *Aedes aegypti*. I report here functional characterization and developmental profiles of the two USP isoforms during mosquito vitellogenesis. RT-PCR analysis of RNA isolated from fat body and ovary revealed that USPa mRNA was highly transcribed in the fat body and ovary during the pre-vitellogenic stage, corresponding to the high titer of juvenile hormone. This suggests that USPa may be regulated by juvenile hormone. In contrast, USPb mRNA correlated with the 20-hydroxyecdysone (20E) titer in the fat body while in the ovary UPSb mRNA peaks paralleled small 20E plateaus in early and late stages of vitellogenesis. These results implicate USPb as the major partner for mosquito EcR mediating 20E regulated gene expression during vitellogenesis. Electrophoresis mobility shift assays (EMSAs) revealed USPa heterodimerized with EcR to bind *Drosophila* hsp-27 ecdysteroid responsive element (EcRE), which was an inverted repeat with a 1-bp spacer (IR^{hsp}-1), and direct repeats with 4-bp (DR-4) or 12-bp (DR-12) spacers. Transactivation in CV-1 cells demonstrated USPa together with EcR was capable of rendering CV-1 cells ecdysteroid responsive via either IR^{hsp}-1 or DR-4 elements. Titration of USP expression vectors indicated EcR-USPb transactivated a reporter gene around two-fold higher than EcR-USPa at low receptor concentrations in accordance with

their DNA binding activities, in which EcR-USPb binds DNA with twice the affinity than that of EcR-USPa. These results demonstrate that USPb is a more efficient dimerization partner for EcR. Methoprene, the JH analog, exerted no significant agonistic or antagonistic effect on EcR-USP complexes.

INTRODUCTION

Steroids regulated gene expression is mediated by their intracellular receptors. In vertebrates, these receptors are usually encoded by several genes and each gene may encode several subtypes called isoforms due to utilization of alliterative promoters and/or polyadenylation signals. In insects, ecdysteroids are the major steroid hormones regulating development, molting, metamorphosis and reproduction (Riddiford, 1993; Raikhel, 1992). The functional ecdysteroid receptor complex consists of two subunits, ecdysteroid receptor (EcR) and *Ultraspiracle* (USP) proteins, both of which are members in the nuclear receptor superfamily (Yao et al., 1992; 1993; Thomas et al., 1993). Recently, cDNA encoding EcR and USP have been cloned from several insect species including the mosquito *Aedes aegypti* (Henrich and Brown, 1995). The pleiotropic effect of ecdysteroids is reflected by the existence of multiple receptor isoforms. EcR isoforms were first identified from *Drosophila* (Talbot *et al.*, 1993) and then from other species including *Bombyx mori* (Swever et al., 1995; Kamimura et al., 1996; 1997) and *Manduca sexta* (Fujiwara et al., 1995: Jindra et al., 1996). These EcR isoforms differ in the N-terminal A/B domain, suggesting they are derived from utilization of distinct promoters and/or alternative splicing. Likewise, two USP isoforms have been identified from *A. aegypti* (USPa and USPb, Kapitskaya et al., 1996) and *M. sexta* (USP1 and USP2, Jindra

et al., 1997). *Drosophila* and *Bombyx* contain USP transcripts of variable sizes (Shea et al., 1990; Henrich *et al.*, 1994; Tzertzinis *et al*, 1994) as revealed by northern blots, suggesting the existence of USP isoforms in these species. In addition, USP proteins with various sizes were detected by western blot in *Drosophila* (Henrich et al., 1994). USP isoforms exhibit distinct N-terminal A/B domain in accordance with those in the EcR isoforms. In mosquitoes, the N-terminal 31 aa in USPa are different from 6 aa in USPb. Moreover, their 5' and 3' untranslated regions (UTRs) are quite different, suggesting these isoforms are most likely derived from utilization of alternative promoters as well as polyadenylation signals (Kapitskaya, *et al.*, 1996).

In the anautogenous mosquito, *A. aegypti*, vitellogenesis is triggered by a blood meal, which stimulates the production yolk protein precursors by the fat body and the accumulation of these proteins by the growing oocytes (Raikhel, 1992). The *A. aegypti* vitellogenesis can be divided into two phases, previtellogenic and vitellogenic phases. The previtellogenic phase covers the period from adult emergence to 3-5 days after eclosion or until a blood meal is available. The vitellogenic phase is initiated by a blood meal and lasts 48-72 hours (reviewed by Hagedorn, 1989; Raikhel, 1992). These two phases are featured by distinct titers of two lipophilic hormones, juvenile hormone (JH) and ecdysteroids. The fat body and oocytes acquire competence to vitellogenesis in the previtellogenic phase accompanied by a rise of JH titer, reaching its peak at two days after eclosion and then declining (Shapiro et al., 1986). In the vitellogenic phase, the blood meal induces the ovary to produce ecdysteroids which stimulate the fat body to express yolk protein precursor, particularly vitellogenin and vitellogenic carboxy

peptidase (VCP). Ecdysteroid titer reaches its peak at 18- to 24-hour post-blood-meal (PMB18-24), the most active transcription phase of Vg and VCP genes.

The fluctuation of JH and ecdysteroid concentration is reminiscent of the hormone titer oscillation during insect metamorphosis, with a high titer of JH maintaining development at a certain larval stage whereas high titer of ecdysteroid provoke molting or metamorphosis (Riddiford, 1993). Although little is known about the mode of JH action, a rapid progress has been achieved in the past decade to unravel the mechanism governing ecdysteroid-regulated gene expression especially during the *Drosophila* development (Thummel, 1997).

Distinct functions of DmEcR isoforms were first evidenced by their different tissue and stage specific expression profiles (Talbot *et al.*, 1993). EcR-B1 predominates during proliferative or repressive responses (Truman et al., 1994). EcR-B mutants can not survive through metamorphasis and they fail to prune back larval-specific dendrite to initiate larval neuron remodeling (Bender *et al.*, 1997; Schubiger *et al.*, 1998). EcR-A predominates during maturational responses (Truman *et al.*, 1994). High level EcR-A expression in the ventral CNS correlates with their rapid degeneration after adult emergence (Robinow *et al.*, 1993).

In sharp contrast to the large body of information accumulated to define the roles of EcR isoforms, little is known about USP isoform specificity. The failure to clone USP isoforms from *Drosophila* has made it difficult to perform isoform specific mutant analysis in flies. Identification of two USP proteins from *M. sexta* has permitted the study of USP isoform profile during development (Jindra, 1997). Likewise, the two *Aedes* USP isoforms have enabled us to investigate different roles of distinct USP in the adult insect

during reproduction. Indeed, mosquito vitellogenesis has provided a perfect model to study the ecdysteroid regulated gene expression as the endocrine release of ecdysteroids is tightly controlled by a blood meal. The functionality of the *Aedes* EcR-USPb complex was illustrated in great detail as it binds to various ecdysteroid response elements with the AGGTCA consensus half site arranged either as direct repeats or inverted repeats. One base-pair is optimal for inverted repeats and four base-pairs are optimal for direct repeats. DNA binding activity is correlated with transactivation as demonstrated by transfection assays in CV-1 cells. (Wang *et al.*, 1998). Both mosquito USP isoforms (USPa and USPb) form functional heterodimeric complexes with the mosquito EcR, when binding to either the EcRE or the ligand (Kapitskaya et al., 1996). I describe here the USP mRNA developmental profile, DNA binding properties and transactivation functions of the AaEcR heterodimerized with USPa or USPb.

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 analysis.

Fat body and ovary were dissected from female mosquito at different time points ranging 0- to 5- day after eclosion or from vitellogenic females ranging from 1- to 48-

hours post-blood-meal (PBM). Total RNA was isolated from fat bodies and ovaries using guanidine isothiocyanate method as described previously (Cho et al., 1991). For reverse-transcription, 5μg total RNA was primered with random hexamers and then transcribed with SuperScript™ II RNaseH$^-$ Reverse Transcriptase (Gibco BRL) in 40μl total volume; and 1μl reverse transcription product was subjected to PCR amplification with Taq polymerase (Gibco BRL). Primers designed from the 5' region of USPa and USPb cDNAs or the LBD of AaEcR were used for PCR amplification. A 522bp USPa fragment was amplified with the primer pair: forward, 5'-TCATATCGTTCCGGAGATGTGG-3' and reverse, 5'-CCAATCCTGCCAGAGGTAGTG-3'. A 400bp USPb fragment was amplified with the primer pair, forward, 5'-CTTCTCACAAGAGGTGCTGAGG-3'and reverse, 5'-TGGTATCCAACTGGAACTTGCG-3'. A 314bp AaEcR fragment was amplified with the primer pair, forward, 5'-GAGGAAGATCAACATGACGTGC-3' and reverse, 5'-ACCGTGAGGGAGAACATCTGC-3'. PCR reactions were performed with an initial denaturation at 94^{0}C for 2 min followed by 18 cycle of danaturation for 30 sec at 94^{0}C, annealing for 30 Sec at 60^{0}C and elongation for 30 sec at 72^{0}C. One sixth volume of the PCR product was resolved in 2% agarose gel, transferred to nylon membrane for southern hybridization under high stringency condition. Hybridization probe was obtained by PCR amplification of plasmid containing corresponding cDNA; and the amplified fragment was gel purified, primered with random hexamer and elongated with Klenow (Giboco BRL) to incorporate ^{32}P-dATP (DuPont NEN).

In vitro Protein Synthesis and Electrophoresis Mobility Shift Assay (EMSA)

The nuclear receptors were synthesized by coupled *in vitro* transcription/translation (TNT) system (Promega). The EcoR1 fragment of USPa cDNA in pBluescript was cloned into the EcoR1 site of pGEM7Z (Promega). The same vector was used to clone DmEcR and DmUSP cDNA fragments (Wang, et al., 1998) whereas AaEcR and AaUSPb 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-Met 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), DR-4(aagcgaaAGGTCAagga AGGTCA ggaaaat) and DR12 (aagcgaaAGGTCAagaggccaaagaAGGTCAggaaaat) were labeled with $^{\gamma\text{-}32}$P-ATP (DuPont NEN) with T4 nucleotide kinase (Gibco BRL). Unless indicated, 50 fmol labeled EcRE was incubated with 1μl *in vitro* synthesized nuclear receptor protein. Bound and free probes were resolved by 6% native acrylamide gel, which was then vacuum dried and exposed to X-ray film. To measure the equilibrium dissociation constants, bound and free EcRE probes were quantified by phosphor-imaging, permitting the construction of saturation curve and Scatchard plot.

Transactivation assays

Transactivation assays were conducted with the green African monkey kidney cell line CV-1. The EcoRI fragment of USPa was cloned into the mammalian expression vector pCDNA3.1/Zeo(+), which was also used to express AaEcR and AaUSPb proteins (Wang

et al., 1998). CMV-LacZ was used as a coreporter to normalize the reporter gene activities of the two reporter plasmids ΔMTV-5xIR^{hsp}-1-CAT and ΔMTV-3xDR4-CAT. Transfection assays were done as described previously (Wang et al., 1998). In brief, CV-1 cells were maintained in DMEM with 10% calf serum. Six –well plates were seed with $2x10^5$ cell/well the day before tansfection. Following the manufacturer's instruction, I transfected cells for 5 hours with LipofectAMINE™ (Gibco BRL) and then added fresh medium together with vehicle ethanol or hormone. After 36- or 48-hour hormone treatment, cells were harvested for CAT activity and β-galactosidase activity. CAT activity was normalized with β-galactosidase activity. CAT activity was expressed as the percent of substrate converted to product, and 1unit of CAT activity was defined as 1% of the chlorophenecol substrate converted to product.

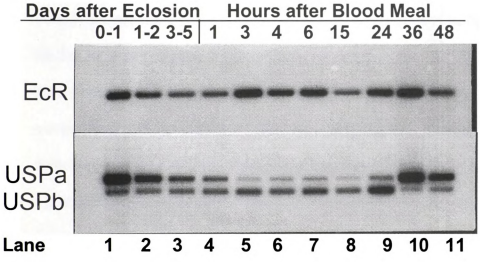
RESULTS

Tissue and Stage specific expression of AaUSP isoforms

I previously reported that the two *Aedes* USP isoforms are transcribed in the fat body and ovary (Kapitskaya et al., 1996). Using isoform specific probes, I performed reverse transcription followed by polymerase chain reaction (RT-PCR) and Southern-Blot to characterize the transcriptional profile of the two USP isoforms and EcR in the fat body and ovary during vitellogenesis.

To investigate USP isoform developmental profiles in the fat body, I isolated total RNA from various time points of previtellogenic and post blood meal female mosquito fat bodies. Equal amounts of total RNA were used for reverse transcription primered with random hexamer, followed by PCR amplification using isoform specific 5' primers.

(A) Fat Body



(B) Ovary

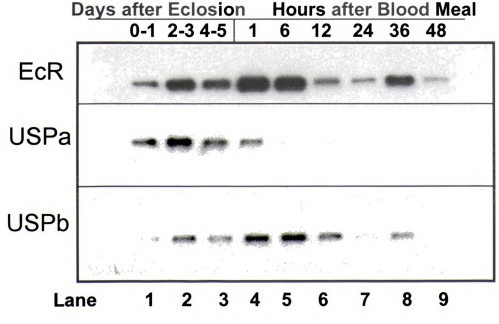


Fig.1. Expression profile of EcR and USP mRNA during vitellogenesis. (A) Fat body profile. Total RNA was isolated from fat body dissected from previtellogenic female mosquito 0-1d, 1-2d, or 3-5d after eclosion (columns 1-3), or vitellogenic mosquito 1h, 3h, 4h, 6h, 15h, 24h , 36h or 48h PBM (columns 4-11). (B). Ovary profile. Total RNA was isolated from ovary dissected from previtellogenic female mosquitoes, 0-1d, 2-3d or 4-5d (columns 1-3) or vitellogenic mosquito 1h, 6h, 12h, 24h,36h, or 48h PBM (column 4-9). Five micrograms of total RNA form each time point was subjected to reverse transcription and PCR amplification using either one pair of primer specific to AaEcR or two pairs of primer specific to USPa and USPb respectively. The PCR product was resolved by agarose gel, followed by Southern Blotting and autoradiography.

PCR products were subjected to southern blot analysis probed with labeled isoform specific probes. USPa mRNA was highly expressed right after emergence, suggesting it may play an important role in the pupae stage (Fig. 1A, lane 1). These mRNA transcripts decreased in previtellogenic period (Fig 1A, lanes 2 and 3). A blood meal induced more dramatic decline of this transcript (Fig1A, lane 4). It became barely detectable 3-hour PBM (Fig. 1A, lane 5), and it remained at low level during the entire active vitellogenic period from 3-hour to 24-hour PBM (Fig 1A, lanes 6-9). Yet, it rose again at 36-hour PBM (Fig. 1A, lane 10), corresponding to the termination of vitellogenic period. These results suggest that USPa plays an important role for the preparation phase of the previtellogenic stage and late-vitellogenic stage

In contrast with the transcription of USPa mRNA, USPb transcript levels were lower in previtellogenic stage (Fig 1A, lanes 1-3). After a blood meal, USPb transcription was slightly enhanced at early times of the vitellogenic stage (1- to 6-hour PBM, Fig 1A, lanes 4-7). This transcript drastically increased at 24-hour PBM (Fig. 1A, lane 9), corresponding to the peak of 20E titer activating high level expression of yolk protein precursor genes. Then this transcript declined at 36-hour PBM (Fig. 1A , lane 10). These results suggested that USPb was the major partner for mosquito EcR to mediate ecdysteroid transactivation in vitellogenesis. Overall, USPa transcripts were much more abundant than USPb in previtellogenic and late vitellogenic periods in the fat body, which was consistent with data I reported previously.

Next, I investigated the mRNA expression profiles of both USP isoforms in the ovary. Total RNA isolated from female mosquito ovaries was subjected to RT-PCR and

southern blot analysis. USPa mRNA was abundant in newly eclosed females (Fig 1-B, lane1), dramatically increased and reached its peak the next day (Fig. 1-B, lane 2), and slightly decreased at 4-5 day old previtellogenic females (Fig.1-B, lane 3). A blood meal drove plummeting of USPa expression (Fig. 1-B, Lane 4) and the decrease persisted with the proceeding of vitellogenesis (Fig. 1-B, lanes 5 and 6) to be below discernible level at 24-hour PBM (Fig.1B, lane 7). At 36-hour PBM, an increase of USPa mRNA was observed (Fig.1B, lane 8), however, it decreased again at 48-hour PBM (Fig.1B, lane 9). Thus the USPa transcription peak coincided with JH titer in the previtellogenic mosquito.

Newly emerged female mosquitoes also contained detectable ovarian USPb mRNA (Fig. 1-B, lane 1), which was enhanced in 2- to 3-day old ovaries and slightly decreasd in 4- to 5- day old ovaries (Fig 1-B, lanes 2-3). A blood meal triggered dramatic increase of USPb message within one hour (Fig.1-B, lane 4) and reached its peak at PBM6 (Fig.1-B, lane 5). Surprisingly, this transcript decreased at active vitelogenic stage 12- to 24-hour PBM, (Fig.1-B, lanes 6 and 7) and rose again at 36-hour PBM and before dropping again at 48-hour PBM. Interestingly, although USPb expression did not exactly parallel the 20E peak at 18-hour PBM, it did coincide with the two small peaks at early 3-hour PBM and late 36-hour PBM, stages of vitellogenesis. This lends support to the conclusion I drew from the fat body profile which described USPb as the major partner for EcR in the mediation of ecdysteroid response.

My results clearly demonstrated that the expression of the two USP isoforms was differently regulated during vitellogenesis. Interestingly, the mRNA expression profiles of two isoforms complemented each other. USPa transcripts were highly expressed in the

previtellogenic fat body and ovary, paralleling JH titer, while USPb transcripts were highly expressed in vitellogenic tissues, correlating with 20E titer.

As EcR dimerizes with USP to exert its function, I then investigated the transcription profile of EcR mRNA utilizing RT-PCR followed by southern blot. In the female fat body, EcR transcript level was very high right after eclosion (Fig.1A lane 1). EcR transcription declined with the rising of JH titer in 1-5 day old female (Fig. 1A, lanes 2 and 3). Following a blood meal, EcR mRNA level increased within 3 hours (Fig. 1A, lanes 4 and 5) corresponding to the small peak of ecdysteroid at 4-hour PMB . It declined gradually after its initial surge, and remained in low level during the active yolk protein production period PBM 16-24 (Fig. 1A, lanes 6-9). EcR transcript increased again at 36-hour PBM to parallel the other small peak of ecdysteroid at late vitellogenic stage (Fig 1A, lane 10) and then declined again at 48-hour PBM.

The ovarian EcR transcription was distinct from that in the fat body. Namely, newly eclosed females contained lower levels of EcR transcript than 2-5 day old females (Fig. 1B, lanes 1-3). After a blood meal, EcR transcription increased more drastically than in the fat body, with prominent increase within 1-hour PBM (Fig. 1B, lane 4), and remained in high level until 6-hour PBM (Fig. 1B, lane 5). However, it declined in active stages of vitelogenesis 12-24 hours PBM (Fig. 1B, lanes 6 and 7). At late stage of vitellogenesis, EcR transcription in the ovary matched its profile in the fat body, with a increase at 36-hour and decrease at 48-hour PBM (Fig. 1B, lanes 8 and 9).

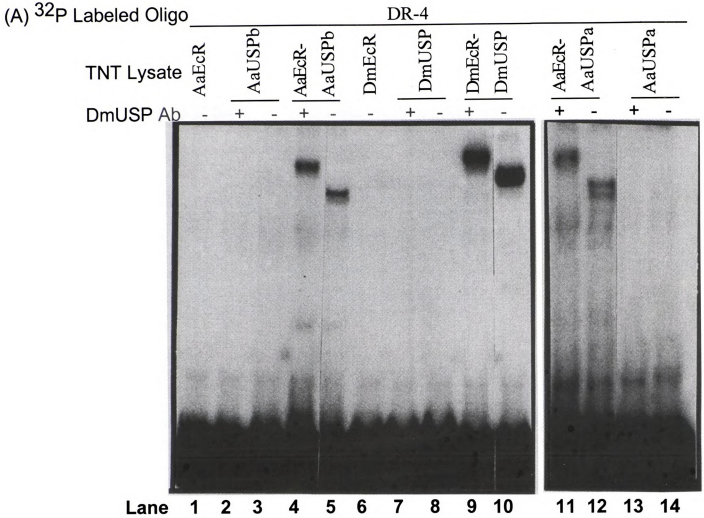
These results indicated EcR transcription was stimulated by low titer of ecdysteroid in early and late stage of vitellogenesis, but repressed by high titer of ecdysteroid at the active stage of vitelogenesis.

Binding to various EcREs by EcR-USPs

I have shown before that the two heterodimers, EcR-USPa and EcR-USPb, bound to the hsp27 response element IR^{hsp}-1 (Kapitskaya et al., 1996). EcR-USPb displays promiscuous binding activities to DNA elements derived from the AGGTCA half-site arranged either as inverted repeats or as direct repeats. One-bp is the optimal spacer for inverted repeats and 4-bp optimal for direct repeats (Wang et al., 1998). I then investigated whether EcR-USPa heterodimer could bind to these various elements.

I first conducted EMSA for DR-4 element, which has been shown to have an optimal spacer among direct repeats for EcR-USPb and DmEcR-DmUSP. As negative controls, TNT lysate programmed with either *Aedes* EcR, USPa, USPb, DmEcR or DmUSP cDNAs did not exhibit binding to DR-4 (Fig. 2A, lanes 1-3, 6-8, 11 and 12). Conversely, combination of EcR and USPb lysates yielded efficient binding, whose specificity was confirmed by supershift with DmUSP antibodies (Fig. 2A, lanes 4 and 5). Similar binding activity was detected for the DmEcR-DmUSP heterodimer (Fig. 2A, lanes 9 and 10). Likewise, EcR-USPa dimer displayed specific binding to the DR4 element as illustrated with a supershift assay (Fig 2A, lanes 13 and 14). Interestingly, longer exposure revealed that DmUSP alone bound to DR-4, while only trace activity was detected for USPa or USPb alone, suggesting DmUSP monomer possessed higher binding activity than the *Aedes* counterparts (data not shown).

I then investigated whether EcR-USPa could bind to the widely spaced element, DR-12, which displays robust binding activity to EcR-USPb and DmEcR-DmUSP heterodimers (D'Avino et al., 1995; Wang et al., 1998). EMSA revealed EcR-USPa bound DR-12 efficiently, and the specificity was demonstrated by supershift assay with


(A) 32P Labeled Oligo
DR-4
TNT Lysate
AaEcR
AaUSPb
AaEcR-AaUSPb
DmEcR
DmUSP
DmEcR-DmUSP
AaEcR-AaUSPa
AaUSPa
DmUSP Ab
- + - + - - + - + - + - + -
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

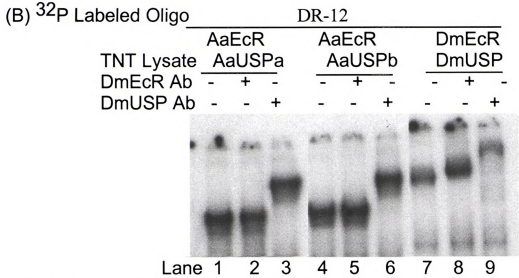


Fig. 2. EcR-USP binding to direct repeats. (A) Binding to DR-4. 32P labeled DR-4 EcRE was incubated with in vitro synthesized AaEcR (lane 1), AaUSPb (lanes 2 and 3), AaEcR and AaUSPb (lanes 4 and 5), DmEcR (lane 6), DmUSP (lanes 7 and 8), DmEcR and DmUSP (lanes 9 and 10), AaUSPa (lanes 13 and 14) or AEcR and AaUSPa (lanes 11 and 12) and subjected to EMSA. Anti-DmUSP antibodies were used for supershift assays to verify binding specificity (lanes 4, 9 and 11). (B) Binding to DR-12. 32P labeled DR12 were incubated with in vitro synthesized AaEcR and AaUSPa (Lanes 1-3), AaEcR and AaUSPb (lanes 4-6) or DmEcR and DmUSP (Lane 7-9) and subjected to EMSA. Supershift assays were conducted with anti-DmEcR antibodies (Lanes 2, 5 and 8) or anti-DmUSP antibodies (Lanes 3, 6 and 9). Oligonucleotides used in this experiment:
DR-4: aagcgaaAGGTCAaggaAGGTCAaggaaaat
DR-12: aagcgaaAGGTCAagaggccaaagaAGGTCAggaaaat

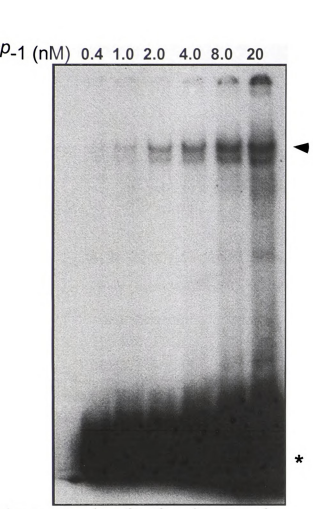
DmUSP antibodies (Fig 2B, lanes 1 and 3). The failure of DmEcR antibodies to supershift EcR-USPa heterodimer indicated these antibodies did not recognize *Aedes* EcR (Fig 2B, lane 2). As positive controls, EcR-USPb and DmEcR-DmUSP bound the DR12 elements potently (Fig 2B, lanes 4 and 7). Binding specificity was confirmed by supershift assays with DmUSP antibodies (Fig 2B, lanes 6 and 9). Although DmEcR antibodies supershifted the DmEcR-DmUSP heterodimer, they failed to supershift the EcR-USPb heterodimer (Fig. 2B, lanes 5 and 8).

Taken together, EcR-USPa bound to inverted repeats, IR^{hsp}-1 (Kapitskaya et al., 1996 and Fig. 3), direct repeats, DR-4 and widely spaced repeats like DR-12 (Fig.2). These results indicated that USPa could function as an efficient partner for EcR resembling the USPb isoform.

Estimation of equilibrium dissociation constant (K_d); EcR-USPb bound EcRE twice stronger than EcR-USPa

Similar binding properties of EcR-USPa and EcR-USPb heterodimers prompted us to investigate whether they possess differential binding affinities for EcREs. EcR-USPb heterodimer binds various EcREs with different binding affinities. The equilibrium dissociation constants (*Kds*) for IR^{hsp}-1, IR^{per}-1 and DR-4 are 3.73nM, 0.326nM and 2.21nM, respectively (Wang *et al.*, 1998). To evaluate whether combinations of EcR with different USP isoforms could exhibit different binding affinities for these DNA elements, I calculated the K_d of EcR-USPa to IR^{hsp}-1, IR^{per}-1 and DR-4. The K_d for DmEcR-DmUSP DNA binding was estimated as well. For each K_d estimation, the EcR-USP heterodimer was incubated in the presence of optimal 20E concentration ($5x10^{-7}$ M), with

Fig.3. Direct measurement of equilibrium dissociation constants (*Kd*). Increasing amount of ^{32}P labeled IR^{hsp}-1 EcRE probe ranging from 0.4nM-20nM was incubated with *in vitro* synthesized AaEcR and AaUSPa and subjected to EMSA. Binding probe and free probe were quantified by phosphor-image analysis. Saturation curve (B) and Scatchard plot (C) were conducted to calculate the *Kd*. This experiment was repeated three times and the mean value taken as the *Kd*.


(A)
32P-IRhsp-1 (nM) 0.4 1.0 2.0 4.0 8.0 20
Lane 1 2 3 4 5 6
Kd=7.36±0.81

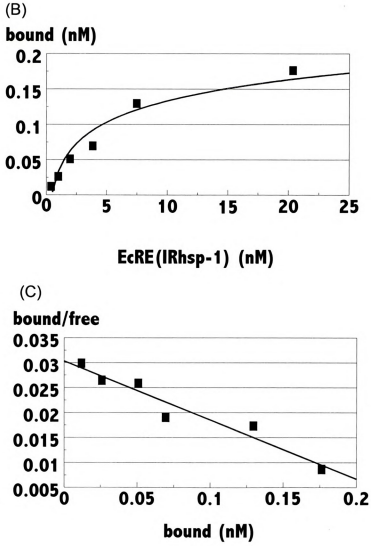

(B)
bound (nM)
0.2
0.15
0.1
0.05
0
0 5 10 15 20 25
EcRE(IRhsp-1) (nM)
(C)
bound/free
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0 0.05 0.1 0.15 0.2
bound (nM)

Table 1. The equillibrium dissociation constants (Kd) of different DNA sequences binding to AaEcR-USPa and AaEcR-USPb. To measure the Kd values, in vitro synthesized AaEcR and AaUSPa were incubated with increasing concentration of 32P labeled IR^{hsp}-1, DR-4 or IR^{per}-1 ranging form 0.4nM to 20nM and subjected to EMSA. Bound and free probe were quantified by phosphor-image analysis, permitting the construction of saturation curve and Scatchard plot (Fig. 3). EMSA and quantification for each element were performed for more than three times and the mean value were taken as the Kd. For comparison, Kd values for EcR-USPb to various elements were also listed (Wang et al., 1998, JBC,273: 27531-27540)

ECRE	IR^{HSP}-1(NM)*	DR-4 (NM)*	IR^{PER}-1(NM)
AaEcR-AaUSPa	7.36±0.81	4.06±0.05	0.476±0.018
AaEcR-AaUSPb	3.73±0.85	2.21±0.36	0.326±0.026
DmEcR-DmUSP	8.24±2.05	4.76±0.88	

* Mean of three determinations ± SE

increasing concentrations of radioactive probes, IR^{hsp}-1, IR^{per}-1 or DR-4. Saturation binding analyses and Scatchard plots (Fig. 3) were used to estimate K_d values for all three heterodimeric pairs for various EcREs (Table 1). Kds of EcR-USPa with IR^{hsp}-1, IR^{per}-1 and DR-4 are 7.36nM, 0.476nM and 4.06nM, respectively. The differences in K_d values for these EcREs indicated that EcR-USPa binding affinity to DR-4 was about 2-fold higher than to IR^{hsp}-1, yet ten times weaker than to IR^{per}-1 in accordance with EcR-USPb binding affinity to these elements. More importantly, these results demonstrated EcR-USPb bound twice stronger to EcREs than EcR-USPa, indicating USPb as a more potent heterodimerization partner for EcR.

Transactivation of EcR-USP in CV-1 cells: EcR-USPb more potent than EcR-USPa

Transactivation of EcR-USP was studied in the green African monkey kidney CV-1 cell line. This cell line has been shown to contain low endogenous level of RXR and was used to study trans-activation of EcR-USP from various species. I have shown before that transfection of EcR-USPb into CV-1 cells rendered them ecdysteroid responsive. Transactivation activity of various EcREs is correlated with their respective binding activity to EcR-USPb (Wang et al., 1998).

To assess whether EcR-USPa could form functional transactivator, I transfected CV-1 cells with 1200ng reporter plasmid ΔMTV-5xIR^{hsp}-1-CAT, either 400ng EcR, 400ng USPa or combination of these two expression vectors (Fig. 4). Cells transfected with reporter gene alone, or reporter gene with USPa expression vectors did not respond to MurA. Weak responsiveness was detected in cells transfected with reporter gene and EcR expression vector, indicating EcR could heterodimerize with endogenous RXR to

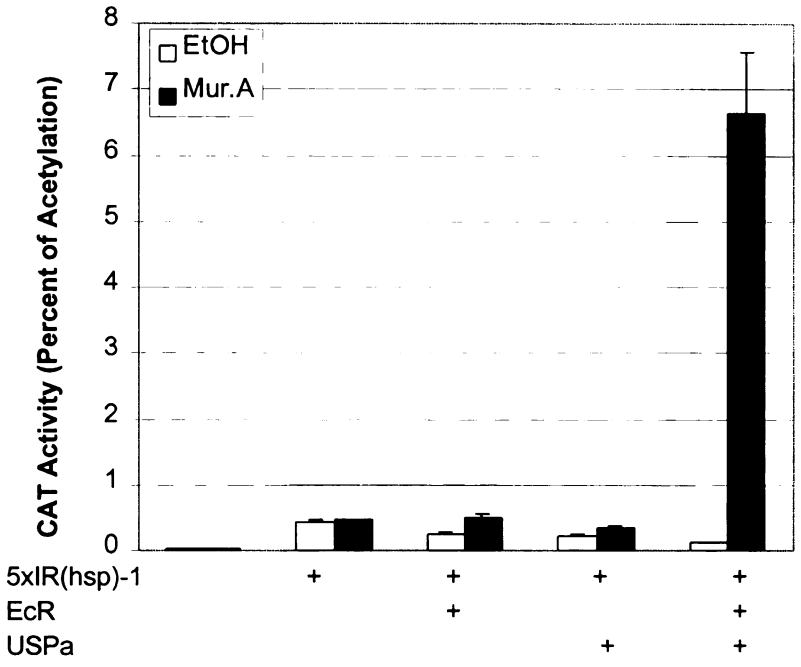


Fig.4. EcR-USPa transactivated a reporter gene (ΔMTV-5xIR^{hsp}-1-CAT) in CV-1 cells. Reporter gene (1.2μg) and 0.4μg CMV-LacZ wree transfected into CV-1 cells with 0.4μg of either AaEcR or AaUSPa plasmid or 0.4μg each of the two expression vectors. The expression vector pCDNA3.1/zeo(+) was used as carrier DNA to equalize the total amount of plasmid so that each well received 2.4μg DNA. Cells acquiring no exogenous DNA were used as a mock control. After transfeciton 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

activate reporter gene. Noticeably, robust reporter gene activity (58-fold) was induced by MurA in cells transfected with EcR and USPa vectors, indicating EcR-USPa heterodimer formed potent transactivator in CV-1 cells.

I reported earlier that EcR-USPb conferred CV-1 cells 40-fold ecdysteroid induction (Wang et al, 1998). However, my DNA binding affinity comparison indicated that USPb as a more potent dimerization partner. This difference led us speculate that over-expression of these receptor proteins in the cells could result in saturated transactivation. I then titrated USP expression vectors in transfection assays. In this experiment (Fig. 5), fixed amount of reporter plasmid (ΔMTV-5xIR^{hsp}1-CAT, 600ng) and EcR expression vector (200ng) were co-transfected into CV-1 cells with increasing amounts of USP expression vectors ranging from 3ng-800ng. Cells transfected with reporter plasmid alone or with EcR expression vector displayed less than 0.5u CAT activity (Fig.4 and data not shown).

Transfecting cells with 3.125ng USPa plasmid with EcR expression vector boosted the CAT activity to 0.93u. CAT activity increased directly with amounts of USPa expression vector, namely 1.19u, 1.91u,, 2.13u, 2.82u, and 3.54u cat activities were detected with 6.25ng, 12.5ng, 12.5ng, 25ng, 50ng and 100ng USPa plasmid respectively, reaching a peak of 3.92u activity with 200ng USPa expression vector. Then further increases of USPa plasmid led to a gradual decrease of CAT activity, 3.81u and 2.27u activities were recorded with 400ng and 800ng USPa expression vectors respectively.

Transfection of cells with EcR and various amount of USPb pasmids created a distinct titration curve. Cells receiving EcR and 3.125ng USPb plasmid showed 1.92u

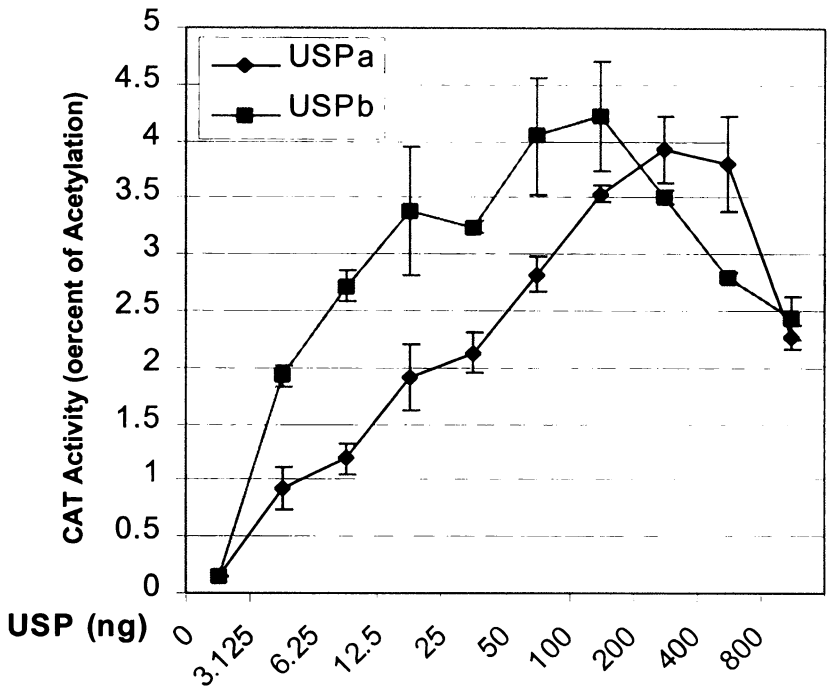


Fig. 5. EcR-USPb mediated more efficient transactivation than EcR-USPa. Reporter plasmid (0.6μg), CMV-LacZ (0.2μg) and AaEcR plasmid (0.2μg) were transfected into CV-1 cells with increasing amount of either AaUSPa or AaUSPb expression vector ranging from 3.1ng-800ng. The expression vector pCDNA3.1/ zeo(+) was used as carrier DNA to equalize the total amount of plasmid so that each well received 2.0μg DNA. Cells acquiring no exogenous DNA were used as a mock control. After transfection, cells were incubated with 1μM MurA at 37^0C for 48 hours and harvested for CAT activity and β-galactosidase activity, and CAT activity was normalized with β-galactosidase activity. The experiments were done in duplicates and repeated three times.

CAT activity, about twice the amount of activity by cells transfected with the same amount of EcR and USPa plasmids (0.93u), in accordance with DNA binding affinities in which EcR-USPb heterodimer displayed twice stronger binding affinity than EcR-USPa (Table 1). Likewise, 6.25ng USPb rendered 2.71u activity which was again around twice the activity conferred by the same amount of USPa (1.19u). Similarly, 3.38u, 3.24u, 4.04u, and 4.22u activities were detected with 12.5ng, 25ng, 50ng and 100ng USPb plasmid respectively, which were consistently higher than the activities detected with corresponding amount of USPa plasmid although they were not exactly two-fold difference. With more than 100ng USPb plasmid, CAT activity started to decline. Namely, 3.51u, 2.79u, and 2.43u activities were detected with 200ng, 400ng and 800ng USPb plasmid respectively. Notably, USPb mediated transactivation reached its peak activity with about half the amount of plasmid compared to USPa (100ng versus 200ng) although the maximum activity mediated by USPb was similar to that of USPa. Before reaching peak activity, USPb mediated transactivation was consistently higher than that of USPa, suggesting transactivation was correlated with DNA binding affinity before saturation. After saturated transactivation, USPb mediated activity dropped faster than USPa, which is consistent with the results in Fig. 4 where 400ng of each receptor plasmid was used and USPa mediated even higher transactivation.

These results further suggested that USPb is a more potent partner for EcR mediating ligand responsiveness in CV-1 cells.

Methoprene could not interact with EcR-USP complex

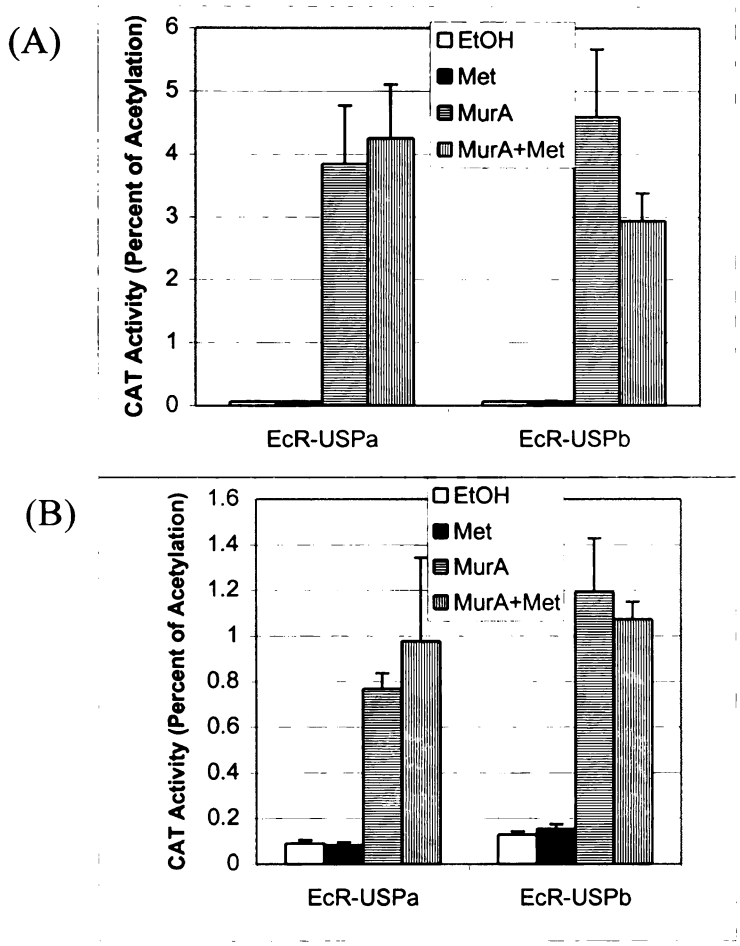


Fig. 6. Methoprene did not affect EcR-USP transactivation. (A). CV-1 cells were transfected with 1.2μg reporter ΔMTV-5xIR^{hsp}-1-CAT, 0.4μg each of CMV-LacZ and AaEcR expression vector with either 0.4μg of AaUSPa or AaUSPb expression vector. After transfection, cells were incubated with vehicle ethanol, 10μM methoprene, 1μM murA or a combination of 10μM methoprene and 1μM murA for 48 hours and harvested for CAT activity and β-galactosidase activity, and CAT activity was normalized with β-galactosidase activity. The experiments were done in duplicates for more than three times. (B). The same as in (A) except ΔMTV-3xDR4-CAT was used as a reporter gene.

Berger et al. (1992) reported that JH and methoprene repressed transactivation mediated by IR^{hsp}-1. Jones and Sharp (1997) have shown that JH interacted with DmUSP in yeast. These reports together with the observation that USPa transcription correlated with JH titer (Fig.1) provoked us to investigate whether JH and methoprene could affect EcR-USP transactivation.

I first used the reporter construct ΔMTV-5xIR^{hsp}1-CAT to address the effect of methoprene. Reporter construct, EcR and either USPa or USPb expression vectors were transfected into CV-1 cells (Fig. 6A). Cells transfected with EcR and USPa vectors exhibited residual CAT activity in the absence of hormone. Addition of methoprene did not affect the CAT activity, which suggests that this ligand exerts no agonistic effect on EcR-USPa. Treatment of transfected cells with MurA induced robust CAT activity, consistent with early results (Fig.4). The MurA induced CAT activity was slightly enhanced by methoprene, yet this potentiation was not statistically significant considering the error bars. Likewise, methoprene alone did not induce EcR-USPb transactivation. It slightly repressed MurA induced EcR-USPb transactivation (Fig. 6A), which was again statistically insignificant, suggesting methoprene functioned neither as an agonist or antagonist for EcR-USPs regarding transactivation via the IR^{hsp}-1 element.

I then utilized another reporter construct with a direct repeat EcRE, ΔMTV-3xDR4-CAT, to investigate methoprene effect. CV-1 cells were transfected with the DR4 reporter, EcR and either USPa or USPb expression plasmids (Fig.6B). For cells transfected with EcR and USPa plasmids, treatment with methoprene alone did not alter CAT activity compared with ethanol controls, indicating methoprene did not function as an agonist for EcR-USPa. In contrast, incubating these cells with MurA elicited robust

CAT activity, indicating that EcR-USPa transactivated the reporter gene via the DR-4 element. MurA induced CAT activity was slightly potentiated by methoprene, albeit statistically insignificant. In a like manner, CAT activity in cells transfected with a DR-4 reporter, EcR and USPb expression vectors was induced by MurA, and not by methoprene. Methoprene insignificantly potentiated MurA induced CAT activity (Fig.6B).

These transfection results indicated methoprene alone did not induce either EcR-USPa or EcR-USPb transactivation, demonstrating this ligand had no agonistic effect on these receptor complexes (Fig.6). Methoprene showed reproducible enhancement on MurA prompted EcR-USPa transactivation via both IR^{hsp}-1 and DR-4 elements. However, the potentiation was not statistically significant. For the EcR-USPb complex, methoprene showed repression via the IR^{hsp}-1 and potention via the DR-4 element. None of these effects, however, were statistically significant. These data suggested that methoprene unlikely functioned as a ligand for the EcR-USP complex.

Importantly, I demonstrated DR-4 as a functional EcRE for EcR-USPa (Fig. 6B) as it functions for EcR-USPb (Fig. 4-B, Wang et al., 1998) and DmEcR-DmUSP (Antonieski et al., 1996). The levels of transactivation mediated by DR4 was lower than that of IR^{hsp}-1, and this was likely due to the copy number of EcREs proceeding the reporter gene (3xDR4 versus 5x IR^{hsp}-1) as DR4 displayed stronger binding affinity than IR^{hsp}-1. For the DR-4 element, EcR-USPb displayed higher binding affinity, yet similar levels of transactivation was achieved for EcR-USPa and EcR-USPb, suggesting the trasactivation assay in this experiment reached saturation as demonstrated earlier (Figs 4 and 5).

DISCUSSION

In this report, I characterized the transcription profile of USP isoforms and EcR during female mosquito reproduction and compared the functionality of EcR-USPa and EcR-USPb employing EMSA and transfection assays. EcR and the two USP isoforms, USPa and USPb, displayed distinct tissue and stage specific transcription profiles. USPa mRNA was highly expressed in previtellogenic and late vitellogenic fat bodies and ovaries, whereas USPb mRNA was enriched in the vitellogenic stage. Moreover, USPa transcription correlated with JH titer especially in the ovary, where both USPa mRNA and JH plateaued at two-day after eclosion, suggesting USPa transcription maybe regulated by JH.

USPb mRNA and 20E plateaued at 20-hour PBM in the fat body. In the ovary, USPb mRNA peaks corresponded with small 20E peaks in early and late vitellogenesis stage, resembling mosquito EcR expression profile. In the fat body, peak USPb transcription occurred 12 hours before that of EcR, suggesting USPb alone or dimerized with another nuclear receptor may function at the active vitellogenic stage. Interestingly, AHR38, the *Aedes* homolog of DHR38 (Sutherland et al., 1995) and vertebrate NGFI-B, is highly expressed at this stage (Zhu, Miura and Raikhel, unpublished results). AHR38 dimerizes with USPb in vitro resembling the DHR38, which interacts with DmUSP to bind the ng-EcRE, DR12 (Crispi et al., 1998). How USPb interacts with other nuclear receptors like AHR38 to function in vitellogenesis remains an open question.

Remarkably, during *Manduca* larval molts, USP1 (counterpart of USPa) disappears and USP2 (counterpart of USPb) is upregulated when the ecdysteroid titer is high (Jindra et al., 1997), resembling fat body USP isoform expression profiles during

mosquito reproduction. Western blot utilizing isoform specific antibodies would provide further evidence defining the roles of USP isoforms in a tissue and stage specific manner.

Based on the puffing pattern of the polytene chromosome of the *Drosophila* salivary gland, Ashburner et al. (1974) proposed that 20E binds to its receptor to activate a set of early gene expression while repressing another set of late gene expression, and the early gene products in turn stimulate the late gene expression. Recent cloning of the gene regulated by 20E has confirmed and extended the Ashburner model. Low 20E titer in early third instar *Drosophila* larva induces expression of early genes, namely EcR and E74B, and high titer 20E in later third instar represses their expression. Likewise, low 20E titer in mid-prepupa provokes expression of another set of early genes, namely EcR, E74B and βFTZ-F1, and high 20E titer in late prepupa repressed these early gene expression (Thummel, 1997). Strikingly, the 20E regulated gene expression cascade is replayed during mosquito vitellogenesis. A small pulse of 20E in early stage of vitellogenesis stimulated the expression of early genes, namely EcR, ovarian USPb and βFTZ-F1 (Fig. 1, Kapitskaya and Raikhel, unpublised data). High 20E titer in active vitellogenic stage repressed these early genes and low pulse of 20E at late vitellogenic stage reactivated these early genes.

Mutational analysis has revealed that *usp* is required for female reproduction in *Drosophila* (Oro et al., 1992), consistent with the high level expression of USPs during female mosquito reproduction. Most of the usp/Y fly progeny of usp- germline mothers die just prior to hatching. In contrast, usp/Y embryos derived form usp+ germ cells hatch and become first instar larvae, indicating the maternal USP is sufficient to support progeny surval until the first instar. Ovarian usp RNA is synthesize primarily by the

oocyte and nurse cell (Oro et al., 1992). My results indicated that USPb mRNA is predominantly expressed in the vitellogenic *Aedes* ovary, indicating this isoform is deposited in the oocyte to be utilized for later embryogenesis.

Similar to the USPb isoform, USPa dimerized with EcR to bind inverted repeat IR^{hsp}-1, direct repeat DR-4 and the widely spaced EcRE DR-12. The distinct binding affinities was resolved by measuring *Kd*s. For both direct repeats and inverted repeats, EcR-USPb complex consistently displayed twice higher binding affinity than EcR-USPa, suggesting USPb as a more efficient partner for EcR. The functionality of USPa was further verified by transfection assays in CV-1 cells. Both IR^{hsp}-1 and DR4 mediated EcR-USPa transactivation in CV-1 cells. Titration of USP expression vector revealed EcR-USPb transactivated a reporter gene around twice higher than EcR-USPa before researching saturated transactivation, suggesting DNA binding affinity strongly affected transactivation efficiency at low concentration of transactivators. The *in vivo* difference of USP isoforms maybe more profound than disclosed from heterologous CV-1 cells. Different N-terminal A/B domain may result in distinct transactivation domains, as has been well documented for vertebrate nuclear receptors. Techniques like domain mapping would help to define the role of distinct USP N-termini. Northern blots have revealed USP mRNA with distinct size in *Drosophila*, suggesting USP isoforms do occur in this species (Henrich et al., 1994; Shea et al., 1990). However, the lethality of fly USP null mutant can be rescued by ectopic expression of one form of the USP protein, suggesting the function of USP isoforms could be partially redundant and can complement each other, similar to the vertebrate RXR (Kastner et al., 1995) . In support of this conclusion,

the two mosquito USP isoforms dimerized with EcR to bind various EcRE and transactivated a reporter gene in CV-1 cells.

EcREs identified in the *Drosophila* ecdysteroid responsive genes are predominantly inverted repeats with one-bp spacer, IR-1. Horner et al. (1995) first demonstrated that direct repeats with 3-5 bp spacers could bind the fly receptor. The functionality of direct repeats are further verified by transactivation assays. *Drosophila* receptor and *Aedes* EcR-USPb activate reporter gene activity via the DR-4 element. I demonstrated DR-4 could function as an EcRE for EcR-USPa in terms of DNA binding and transactivation. For the inverted repeats, one-bp spacer is very critical, adding or removing a base-pair drastically change the DNA binding activity. By contrast, the spacer length is much more flexible among direct repeats. Direct repeats with 1-5, 11-13 bp spacers bind the EcR-USPb. And these elements exhibited similar activity for the EcR-USPa complex. Interestingly, the mosquito ecdysteroid responsive genes, namely Vg and VCP, contain direct repeat motifs with one or two bp spacers, which display strong binding activity to EcR-USP either from nuclear extracts or from in vitro expression. As USPa mRNA expressed predominantly in the previtellogenic fat body and USPb mRNA in the vitellogenic fat body, it is possible that EcR-USPa (or USPa dimerized with another partner) may bind the yolk protein elements to repress yolk protein gene expression whereas EcR-USPb activate their expression.

Berger et al. (1992) reported that pretreating Schnerder-3 cells with methoprene or JH inhibits 20E activation of the reporter gene, IR^{hsp}-1–CAT , suggesting methoprene antagonizes the effect of ecdysteroid. However, tansfection assays in CV-1 cells indicated that methoprene exerted no statistically significant agonistic or antagonistic

effect on EcR-USP, suggesting that JH activity is not directly mediated through EcR-USP complex. It is also possible that USPa, seemingly heterodimerized with another partner, may serve as a JH receptor. In support of this idea, transfection of farnesoic acid receptor (FXR) and RXR expression vectors into CV-1 cells renders cells responsive to JH (Forman et al., 1995). The JH analog methoprenic acid induces RXR transactivation, yet it does not function for *Drosophila* USP (Harmon et al., 1995). Hence, an insect FXR homolog has yet to be identified to dimerize with USP to form the functional JH receptor.

CHAPTER 4. LIGAND SPECIFICITY OF INSECT STEROID HORMONE RECEPTORS

ABSTRACT

Ecdysteroids play critical roles regulating insect embryogenesis, molting, metamorphosis and reproduction. Ecdysone, the first steroid hormone isolated, has much lower activity than its derivative, 20-hydroxyecdysone (20E) in bioassay studies. This observation together with the identification of ecdysone 20-monooxygenase from various insect species has led to the conclusion that ecdysone was synthesized as a precursor and converted to its active form, 20E. Gene expression modulated by steroid hormones is mediated via nuclear receptors. Functional ecdysteroid receptor complex is a heterodimer consisting of ecdysteroid receptor (EcR) and *Ultraspiracle* (USP) proteins. Bioassays fail to distinguish between the effects of a certain steroid and those of its metabolites, making it difficult to define the functional roles of specific ecdysteroids. Recent cloning of genes encoding EcR and USP proteins from different insect species has provided an opportunity to elucidate the molecular mechanisms underlying the physiological function of various ecdysteroids . To characterize the species specificity of insect steroid hormones, I analyzed the activity of a variety of ecdysteroids using gel mobility shift assays and transfection assays in *Schneider-2* cells. As expected, ecdysone, the commonly believed inactive precursor, did not activate the *Drosophila melanogaster* ecdysteroid receptor complex. In contrast, this steroid functioned as an active ligand for the mosquito, *Aedes*

aegypti, ecdysteroid receptor complex, enhancing DNA binding and transactivating a reporter gene in S2 cells. Subunit swapping experiments indicated that EcR protein, not USP protein, was responsible for ligand specificity. Using domain swapping techniques, I made a series of *Aedes* and *Drosophila* EcR chimeric constructs. The ligand-specific region was mapped near the C-terminal of the ligand binding domain, which was located at the loop connecting Helices 9 and 10 and the N-terminal of Helix 10, as determined by comparison with available crystal structures of homologous nuclear receptors. Site-directed mutagenesis revealed that *Tyr611* in *Drosophila* EcR, whose corresponding residue in *Aedes* EcR is *Phe529*, underlies the ligand specificity. My results demonstrated that ecdysone could function as a *bona fide* ligand in a species-specific manner, opening new avenues for designing environment-friendly and target-specific insecticides.

INTRODUCTION

When mature larvae of the blowfly *Calliphola erythrocephala* are ligated in the middle, only the anterior portions sclerotize and pupate. Yet pupation of the posterior can be induced by injecting hemolymph from larvae which have just undergone pupation (Fraenkel, 1935). This technique, called *Calliphola* test, can be used to quantitate the hemolymph hormone (Becker and Plagge, 1939). Utilizing the *Calliphola* test as a bioassay to monitor hormone activity in extracts from silkworm *Bombyx mori* pupae, Butenandt and Karson isolated the first steroid hormone, ecdysone (Butenandt and Karlson, 1954), whose complete structure was deduced a decade later (Huber and Hoppe, 1965). (The generic term for ecdysone and ecdysone-related compounds is called "ecdysteroid", however, some *Drosophila* scientists still refer to ecdysone as the generic

term). Further fractionation of the silkworm extract led to the isolation of a more polar compound (Karlson et al., 1956), which was characterized as 20-hydroxyecdysone (Hoffmeister and Grutzmacher, 1966), identical to the steroid hormone isolated from the crayfish *Jasus lallandii* named crustecdysone (Hampshire and Horn, 1966) and from the tobacco hornworn *Manduca sexta* (Kaplanis et al, 1966). After these breakthroughs, there have been more than 250 ecdysteroid identified from plants (phytoecdysteroids) and animals (zooecdysteroids) (reviewed by Rees, 1989; Lafont and Horn, 1989).

The abundance of phytoecdysteroids has provided a great resource to purify large amount of ecdysteroids for functional studies in animals. Ecdysteroids were originally isolated as molting hormones, but before long it was discovered that these steroids function in a variety of biological processes in arthropods, namely oocyte maturation, embryonic development, molting, metamorphosis, nervous development and reproduction (Lanot et al., 1989; Hagedorn, 1989). Distinct roles of ecdysteroids were first evidenced by the species-, tissue- and stage- specific distributions. For example, ecdysone occurs in greater concentration than 20E in the pupal stadia of *Bombyx mori*, (Smith, 1985). In contrast, the *Calliphora erythrocephala* pupae contains virtually only 20E (Galbraith et al., 1969). The developmental role in ecdysteroid at metamorphosis has been investigated in great detail in the *Drosophila melanogaster*.(Riddiford, 1993). The major ecdysteroid at fly metamorphosis is 20-hydroxyecdysone, little or no ecdysone is detected (Richards, 1981). Indeed, 20E is up to 100 times more active than ecdysone in eliciting puffing in the Drosophila polytene chromosomes, shortening of the interganglionic ventral nerve cord in *Galleria mellonella*, and evagination and development of the imaginal disks of *Drosophila melanogaster* and *Sarcophafa bullata*

(Reviewed by Smith, 1985). These results led to the conclusion that ecdysone is the prohormone and 20E is the active hormone (Doctor and Fristrom, 1985). In support of this conclusion, the enzyme, ecdysone 20-monooxygenase, which specifically converts ecdysone to 20E has been identified from various species, namely *Locusta migratoria, Manducal Sexta, Schistocerca gregaria and Spodoptera littoralis* (reviewed by Rees 1995).

However, several lines of evidence suggested that ecdysone may have an alternative function besides serving as a prohormone for 20E. First, in some insects, ecdysone concentration exceeds that of 20E in certain developmental stages. Second, ecdysone exerts direct developmental effects such as inducing puffing in *Chironomas tentans* salivary gland polytene chromosome evagination of wing and leg imaginal disks of *Drosophila melanogaster* (Smith, 1985). Thirdly, the expression of ecdysone 20-monooxygenase in some insects including *Manducal Sexta, Schistocerca gregaria and Spodoptera littoralis* does not correlate with the titer of ecdysteroid (Rees, 1995).

Based on the ecdysteroid induced puffing in the giant salivary gland chromosome of the *Chironomus tentans*, Karlson first proposed that steroid hormones function by way of activation of gene expression via the interaction of hormone-receptor complex with chromatin (Clever and Karlson, 1960; Karlson et al., 1963). Under this guideline, many groups detected ecdysteroid-receptor binding activity in various insects (reviewed by Cherbas and Cherbas, 1996) and competition assays revealled the affinity order as ponasterone A>20E>ecdysone (Reviewed by Yund 1989), consistent with the activity they exert in bioassays. Ecdysteroids in *Drosophila* salivary glands associate with the polytene chromosome (Gronemeyer and Pongs 1980).

Recombinant DNA technology has made it possible to isolate genes encoding ecdysteroid receptor (EcR), which was first cloned from the *Drosophila melanogaster.* (Koelle et al., 1991). More detailed analysis revealed that *Ultrspiracle* (USP) protein, the insect homolog of vertebrate retinoid X receptor (RXR), is required to form the functional ecdysteroid receptor in DNA binding, ligand binding and transactivation assays (Yao et al., 1992; 1993, Thomas et al., 1993). Recently, EcR and USP cDNAs have been cloned from a variety of insect species (Henrich and Brown, 1995) including the yellow fever mosquito, *Aedes aegypti* (Cho et al., 1995; Kapitskaya et al., 1996). Resembling the fly counterpart, *Aedes* EcR-USP heterodimer binds EcREs derived from the consensus half site AGGTCA arranged either as direct repeats or inverted repeats, DNA binding activity is correlated with transactivation activity (Wang et al., 1998). Liganded EcR-USP displays enhanced binding activity, suggesting interaction of ecdysteroid with receptor causes significant conformational changes (Kapitskaya et al., 1996.)

In accordance with the multiple functions of ecdysteroids, both EcR and USP proteins exist in multiple isoforms. EcR isoforms have been cloned first from *Drosophila,* (Talbot, et al., 1993) then from other species including *Maduca sexta* (Jindra et al., 1996) and *Bombyx mori* (Kamimura et al., 1997). Although northern and western blots revealed various sizes for *Drosophila* USP mRNA and protein, only one form of *Drosophila* USP has been identified. In contrast, two USP isoforms have been isolated from *A. aegypti* (Kapitskaya et al., 1996) and *M. sexta.*(Jindra et al., 1997). EcR and USP protein sequences exhibit typical characteristics for nuclear hormone receptor, with five distinct domains, namely domains A/B, C, D, E, and F. Based on studies of vertebrate steroid receptors, domain A/B contains transactivation activity. Domain C possesses two C2-C2

zinc modules, which indicates that it is responsible for DNA binding (DBD). Domian D is also called the hinge region as it bridges DBD and LBD. Domain E, which defines ligand binding (LBD) specificity, contains another transactivaiton domain. The function of domain F remains enigmatic. Within the same species, EcR isoforms contain identical amino acid sequences except in their N-terminal A/B domains, suggesting they are derived from alternative promoters and /or splicing variations. Likewise, USP isoforms from the same species display distinct N-termini whereas the rest of the protein sequence is just the same. These results suggest that EcR-USP complex from the same species is unlikely to exhibit different ligand binding specificity. However, receptors from different species may show quite distinct ligand preference as they possess diverse LBDs. The diversity of known EcR LBD protein sequences range from 39-52% (Chapter 1).

Bioassays were originally used to monitor the functional activity of ecdysteroids. Following the *Calliphola* test, other organisms including *Musca domestica, Sarcophaga peregrina, and Chilo suppressalis* have been used for bioassays (reviewed by Smith 1985). Although sensitive, bioassays have an intrinsic problem since they are unable to distinguish a test compound from its subsequent metabolites. Imaginal disks from species including *Drosophila melanogaster* and *Sarcophaga bullata* do not efficiently metabolize steroid hormones. Accordingly, ecdysteroid-induced imaginal disk evagination has been utilized to compare the activity of various ecdysteroid (Smith, 1985). For the different imaginal disks from lepidopteran and dipteran species, the ecdysteroid activity order is ponasterone A>20E>ecdysone (Doctor and Fristrom, 1985), consistent with ligand binding activities. Availability of *Drosophila* embryonic cell lines permitted the investigation of ecdysteroid activity at the cellular level. The ecdysteroid activity based

on morphomogical changes of Kc and BII cells confirmed previous bioassay results (Cherbas et al., 1980; Harmatha and Dinan, 1997). However, there are only limited number of imaginal disk models and cell lines, which are mainly from *Drosophila*, making it difficult to compare the efficiency of ecdysteroid on different species.

Characterization of the ligand species specificity of ecdysteroid receptors is essential to unravel the mechanism underlying ecdysteroid regulated gene expression. In addition, it can shed light on designing target specific and environment friendly pesticides. Although the strategy for insect control utilizing steroid hormone has been proposed for years (Watkinson and Clarke, 1973), the lack of knowledge in ligand specificity has hindered progress in this field. Furthermore, comparison of the functionality of EcR-USP with regard to their respective ligand is indispensable in the identification of the optimal receptor and ligand for suitable induction and minimal toxicity can be achieved in gene delivery for human gene therapy. Recently, ecdysteroid receptor has been successfully applied for controlled gene expression in mammalian cells and mice (Christopherson et al., 1992; No et al., 1996). The ecdysteroid system is one of the most promising gene delivery systems for regulated gene expression for gene therapy (reviewed by Clackson, 1997).

Cloning of genes encoding EcR and USP from various species has enabled us to directly investigate the interaction between an ecdysteroid and its receptor. As a step toward understanding the ligand specificity of insect steroid hormone receptors, I analyzed the effect of various ecdysteroids on DNA binding activities of receptors from two species, *Drosophila and Aedes*. The effect of two major ecdysteroids, ecdysone and 20E, were investigated in great detail. The *Drosophila* EcR responded potently to 20E,

but not to ecdysone, which suggests that ecdysone is indeed a pro-hormone in this species. In contrast, the *Aedes* EcR responded strongly to ecdysone in addition to 20E, demonstrating these two ecdysteroids were functional ligands for the *Aedes* EcR.

MATERIALS AND METHODS

In vitro protein synthesis and electrophoresis gel mobility shift assay (EMSA).

For EMSA assays, nuclear receptor proteins were synthesized *in vitro* utilizing coupled transcription-translation (TNT) kit from Promega. The *in vitro* expression vectors, pGEM3Z-AaEcR, pGEM3Z-AaUSPb, pGEM7Z-DmEcR, pGEM7Z-DmUSP and pGEM7Z-AaUSPa with entire open reading frames of respective nuclear receptor cDNA open reading frames were constructed as described previously (Kapitskaya et al., 1996; Wang et al., 1998). TNT produced protein was quantified by ^{35}S methionine labeling, SDS-PAGE followed by phosphorimage analysis. Protein yield ranged from 0.1 fmol/μl to 1.6fmol/μl. A parallel TNT reaction with unlabeled methionine was performed to produce the protein for EMSA. Unless otherwise indicated, the amount of DmEcR protein used in each EMSA reaction was at least 10 times more than that of AaEcR protein. Receptor proteins were first incubated with $5x10^{-5}M$ (unless otherwise indicated) ecdysteroid at room temperature for 30 min in a total volumn of 20μl EMSA buffer containing 20mM Hepes pH7.5, 2mM DTT, 100mM KCl, 7.5% glycerol, 1% NP-40 (Boehringer Mannheim), 2μg poly(dI-dC).poly(dI-dC) (Pharmacia Biotech) and 3μg single-strand-DNA (Wang et al., 1998). Then 50fmol ^{32}P labeled probe IR^{hsp}-1 was added

to the mixture followed by incubation at room temperature for another 30min. Bound and free probe were resolved in 5% or 6% native acrylamide gel in 0.5X TBE. The gel was vacuum dried and exposed to either X-ray film (Kodak)or phosphorimage (Molecular Dynamics) for quantification.

Ecdysteroids and purification.

Ecdysteroids, muristerone A (MurA), polypodine B (PolB), 20-hydroxyecdysone (20E), 20-hydroxyecdysone 22-acetate (22A),. 2-deoxy-20-hydroxyecdysone (2DE), and ecdysone were purchased from Sigma. Ponasterone A was purchased form Invitrogen. To ensure the active components in ecdysone was not due to contamination, I used HPLC purified ecdysone, which was provided by Dr. H. H. Rees, The University of Liverpool, UK.

Reporter and insect expression vectors for transfection assays.

The reporter plasmid Eip-Luc and Hsp-Luc were kind gifts from M. Mckeown (Salk Institute, San Diego, CA). The expression vector pAc-DmEcR was provided by W. Segraves (Yale University). The co-reporter pAc5-LacZ (Invitrogen) was used to normalize transfection efficiency. The entire AaEcR cDNA was obtained by digesting pcDNA3.1Zeo(+)-AaEcR (Wang et al., 1998) with BamH1, followed by blunting with Klenow and digestion with Xba1. The AaEcR cDNA fragment was then inserted into the EcoRV and Xba1 sites of pAc5/V5/HisA (Invitrogen), yielding expression vector pAc5-AaEcR. Other expression plasmids including pAc5-AaUSPa, pAc5-AaUSPb and pAc5-DmUSP were constructed by inserting the EcoR1 cDNA fragments from

pcDNA3.1Zeo(+)-AaUSPa, pcDNA3.1Zeo(+)-AaUSPb and pcDNA3.1Zeo(+)-DmUSP (Wang et al., 1998) into the corresponding site of pAc5/V5/HisA, respectively. All these constructs were confirmed by restriction digestion and partial sequencing.

Cell culture and transient transfection assay.

The Schnieder *Drosophila* cells line-2 (S2, Invitrogen) were maintained at 24^{0}C in Schnieder *Drosophila* media supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml pennicillin and 100μg/ml streptomycin (Gibco BRL). Transfection was conducted with LipofectACE™ (Gibco BRL) with a optimal ratio DNA:Lipid=1:20 (weight/weight). Typically, 100ng luciferase reporter gene, 25ng co-reporter pAc5-LacZ, 12.5ng of each receptor and 3μg LipofectACE were mixed in 24-well plate with a total volume of 20μl and incubated at room temperature for 30 min. The expression vector pAc5/V5/HisA was used as carrier DNA so that each well received 150ng total DNA. The transfection cocktail was overlaid with 500μl S2 cells, which was diluted to 10^6 cells/ml in *Drosophila* serum free media (Gibco BRL). Half the amount of DNA, LipofectACE and cells were used for transfection assays in 48-well plates. Transfection was terminated 12 hours later with the addition of 5% fetal bovine serum, together with ecdysteroid to a certain concentration. After 24-hour or 36-hour hormone treatment, the medium was aspirated and the cells in suspension and attachment were combined in 100μl Reporter Lysis Buffer (Promega) and lysed with three cycles of freezing and thawing. Reporter gene assays were conducted as described in the Promega Firefly Luciferase Reporter Systems and β-Galactosidase Systems. A luminometer (Turner

Designs Model TD20e) was used to detect luciferase activity with 10-second delay time and 30-second integration time. The luciferase activity was normalized with β-Galactosidase activity.

Construction of chimeric receptors

Table 1. Chimeric receptor constructed by restriction digestion.

Starting Plasmids	Restriction enzyme	Fragments	New Constructs	Promoter for in vitro expression
pCDNA3.1-AaEcR pCDNA3.1-DmEcR	*BsrG1, Xba1*	6162bp, 2125bp 6074bp, 1939bp	pcDNA3.1/Zeo(+) -AE^{BsrG1}	T7
pCDNA3.1-AaEcR pCDNA3.1-DmEcR	*BsrG1, Xba1*	6162bp, 2125bp 6074bp, 1939bp	pcDNA3.1/Zeo(+) -DE^{BsrG1}	T7
pGEM7Z-DmEcR pCDNA3.1-AaEcR	*Xma3, Xba1*	3435bp, 1364bp, 729bp 3429bp,1869bp,1536bp,680bp,438bp	pGEM7Z-DE^{Xma3}	SP6
pCDNA3.1-AaEcR pCDNA3.1-DmEcR	*Kpn1*	6699bp, 1309bp 5655bp,1508bp, 1183bp	pCDNA3.1- DE^{Kpn1}	T7
pCDNA3.1-AaEcR pCDNA3.1-DmEcR	Bgl2	5959bp, 2323bp, 626bp 6126bp, 3061bp	pCDNA3.1-DE^{Bgl2}	T7
pGEM3Z-AaEcR pGEM7Z-DmEcR	*Nru1, EcoR1*	4890bp, 1303bp 3901bp, 1490bp, 881bp	pGEM3Z-AE^{Nru1}	SP6
pGEM7Z-DmEcR pCDNA3.1-AaEcR	*TthIII1, Xba1*	4920bp, 1409bp, 5437bp,1321bp,1182bp	pGEM7Z-DE^{TthIII1}	SP6
pGEM3Z-AaEcR pGEM7Z-DE^{BsiW1}	**EcoR1, Nru1* **Xba1, Nru1*	4414bp, 1284bp 3931bp, 881bp,729bp	pGEM3Z-AE^{NB}	SP6

*Sites blunted with Klenow
Underlined fragments were used for ligationpcDNA3.1/Zeo(+)-AaEcR was exchanged

Five chimeric receptors, AE^{BsrG1}, DE^{BsrG1}, DE^{Xma3}, DE^{Kpn1} and DE^{Bgl2} were first constructed by swapping at the DBD, boundaries between domains C and D, domains D and E, or domains E and F, respectively. *BamH1* framgment of DmEcR cDNA in pAc5-DmEcR was first subconed into the *BamH1* site of pcDNA3.1/Zeo(+) yielding pcDNA3.1/Zeo(+)-DmEcR. pcDNA3.1/Zeo(+)-AaEcR was constructed in a similar way as described before (Wang et al., 1998). The 1939bp *BsrG1-Xba1* fragment in with the

2125bp *BsrG1-Xba1* fragment in pcDNA3.1/Zeo(+)-DmEcR, yielding two chimeric receptor constructs, pcDNA3.1/Zeo(+)-AE^{BsrG1} and pcDNA3.1/Zeo(+)-DE^{BsrG1}. pGEM7Z-DE^{Xma3} was created by digesting pGEM7Z-DmEcR with *Xma3* and *Xba1*, and the 3435bp fragment containing the vector and 5'-region of DmEcR cDNA was ligated with a 1869bp *Xma3-Xba1* fragment bearing 3'-region of AaEcR cDNA from pcDNA3.1/Zeo(+)-AaEcR. To make the construct pcDNA3.1/Zeo(+)-DE^{Kpn1}, the 1309bp *Kpn1* fragment with a 5'-region of AaEcR cDNA in pcDNA3.1/Zeo(+)-AaEcR was replaced with 1508bp *Kpn1* fragment with a 5'-region from DmEcR cDNA in pcDNA3.1/Zeo(+)-DmEcR. pcDNA3.1/Zeo(+)-DE^{Bgl2} was created by ligating the 5959bp *Bgl2* fragment from pcDNA3.1/Zeo(+)-AaEcR with a 3061bp fragment from pcDNA3.1/Zeo(+)-DmEcR.

Nine chimeric constructs, AE^{Sac1}, AE^{Nru1}, DE^{Bbs1}, DE^{TthIII1}, DE^{Csp1}, DE^{Spe1}, DE^{BsiW1}, AE^{NB} and DE^{SS} by swapping at the LBD using a combination of restriction digestion and PCR amplification techniques. pGEM3Z-AE^{Nru1} was constructed by replacing the 1303bp *Nru1-EcoR1* fragment in pGEM3Z-AaEcR with the 1490bp *Nru1-EcoR1* fragment from pGEM7Z-DmEcR. pGEM7Z-DE^{TthIII1} was constructed by replacing 1409bp *TthIII1-Xba1* fragment in pGEM7Z-DmEcR with the 1182bp *TthIII1-Xba1* fragment from pcDNA3.1/Zeo(+)-AaEcR. To construct pGEM3Z-AE^{Sac1}, a pair of primers DE-*Sac1*-For and DE-*EcoR1*-Rev (See Table 1 for sequence) were utilized to amplify a 836bp fragment from pGEM7Z-DmEcR and this fragment was digested with *Sac1* and *EcoR1* to replace the 991bp *Sac1-EcoR1* fragment in pGEM3Z-AaEcR, yielding the chimera pGEM3Z-AE^{Sac1}. PCR reactions were performed with the polymerase *Pfu* (Promega) with a initial denaturation at 94^{0}C for 2min, followed by 20 cycles of denaturation at 94^{0}C for 45sec, annealing at 60^{0}C for 45sec, and elongation at 72^{0}C for 3min. To make other

chimeric constructs, pGEM7Z-DE^{Bbs1}, pGEM7Z-DE^{Csp1}, pGEM7Z-DE^{Spe1}, pGEM7Z-DE^{BsiW1} and pGEM7Z-DE^{SS}, a pair of forward and reverse primers were annealed with the template either pGEM3Z-AaEcR or pGEM7Z-DmEcR for PCR amplification and the amplified fragments were digested with restriction enzymes to be cloned into the corresponding sites of a target plasmid. The chimeric plasmid pGEM3Z-AE^{NB} was constructed by replacing the 1284bp fragment in *Nru1-EcoR1* fragment with the 729bp *Nru1-Xba1* fragment in pGEM7Z-DE^{BsiW1}, blunting the *EcoR1* and *Xba1* sites.

Table 2. Primers for chimeric receptor construction by PCR

Primers	Template	Amplified Fragment	Replace with	New Construct	Promoter for in vitro expression
DE-*Sac1*-For CACCGAGCTCCGTACGCTGGG CAA DE-*EcoR1*-Rev: GCGAATTCTACTCCAGCAGGA CGTC	pGEM7Z-DmEcR	836bp	991bp *Sac1-EcoR1* fragment in pGEM3Z-AaEcR	pGEM3Z-AE^{Sac1}	SP6
AE-*Bbs1*-For: ACACGATAGAAGACCTGCTGC ACT AE-*Xba1*-Rev: CGGTCTAGAAACCGTGCCCTA CACTAG	pGEM3Z-AaEcR	685bp	1263bp *Bbs1-Xba1* fragment in pGEM7Z-DmEcR	pGEM7Z-DE^{Bbs1}	SP6
AE-*Csp1*-For ATCTTCTCGGACCGGCCCGGA CT AE-*Xba1*-Rev: CGGTCTAGAAACCGTGCCCTA CACTAG	pGEM3Z-AaEcR	596bp	1368 *Csp1-Xba1* fragment in pGEM7Z-DmEcR	pGEM7Z-DE^{Csp1}	SP6
AE-*Spe1*-For GAACTAGTCGAGCACATCCAG AE-*Xba1*-Rev: CGGTCTAGAAACCGTGCCCTA CACTAG	pGEM3Z-AaEcR	563bp	1341 *Spe1-Xba1* fragment in pGEM7Z-DmEcR	pGEM7Z-DE^{Spe1}	SP6
AE-*BsiW1*-For CTCCGTACGCTGGGCAACCAG AE-*Xba1*-Rev: CGGTCTAGAAACCGTGCCCTA CACTAG	pGEM3Z-AaEcR	440bp	1217bp *BsiW1-Xba1* fragment in pGEM7Z-DmEcR	pGEM7Z-DE^{BsiW1}	SP6
AE-*Spe1*-For GAACTAGTCGAGCACATCCAG DE-*Xba1*-Rev TGGTCTAGATGTTGGTGGAGCT GACTC	pGEM3Z-AE^{Sac1}	936bp	1341bp *Spe1-Xba1* fragment in pGEM7Z-DmEcR	pGEM7Z-DE^{SS}	SP6

Construction of site-directed point mutants

DmEcR Site directed mutagenesis was conducted according to the instruction manual in the QuickChange™ Site Directed Mutagenesis (Stratagene). A pair of complementary primers (40 pmol each, see Table 3 for sequence) and 10ng template plasmid pGEM7Z-DmEcR in 100μl total volume were subjected to PCR amplification with *Pfu* (Promega). PCRs were performed with an initial denaturation at 94°C for 2min, followed by 15 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and annealing at 72°C for 14 min. The PCR products were treated with *Dpn1* (Statagene) to remove the methylated template DNA and then gel purified, ligated and transformed in to *E. coli*. Seven site-directed mutants with a single amino acid mutation were constructed this way, namely pGEM7Z-DE^{C602A}, pGEM7Z-DE^{S605P}, pGEM7Z-DE^{M606K}, pGEM7Z-DE^{S607C}, pGEM7Z-DE^{L608S}, pGEM7Z-DE^{F610I} and pGEM7Z-DE^{Y611F}.

Table 3, Primers for site-dirested mutagenesis

Primer Name	Primer Sequence	New Construct
DE-C602A-For	CTCAACCGCCAC**GC**CGGCGACTCAATGAG	pGEM7Z-DE^{C602A}
DE-S605P-For	CACTGCGGCGAC**CC**AATGAGCCTCGTC	pGEM7Z-DE^{S605P}
DE-M606K-For	CTGCGGCGACTCA**AA**GAGCCTCGTCTTC	pGEM7Z-DE^{M606K}
DE-S607C-For	GGCGACTCAATG**TG**CCTCGTCTTCTACG	pGEM7Z-DE^{S607C}
DE-L608S-For	CGACTCAATGAGC**AG**CGTCTTCTACGCAAAG	pGEM7Z-DE^{L608S}
DE-F610I-For	CAATGAGCCTCGTC**A**TCTACGCAAAGCTGC	pGEM7Z-DE^{F610I}
DE-Y611F-For	GAGCCTCGTCTTCT**T**CGCAAAGCTGCTC	pGEM7Z-DE^{Y611F}

The mutagenized codons are underlined and the mutant bases are in bold. Only the forward primer is shown for simplicity.

RESULTS

Ligand enhancement on EcR-USP complex DNA binding activity depended on both protein and ligand concentrations.

There have been conflicting results in the literature concerning the effects of ecdysteroid on receptor binding to its DNA element. Thomas *et al.* (1993) first reported that the ligand muristerone A significantly enhanced DmEcR-RXR heterodimer DNA binding activity, but it had no effect on DmEcR-DmUSP complex. However, results later reported by Yao *et al.* (1993) demonstrated that ecdysteroids including 20E and MurA dramatically enhanced DmEcR-DmUSP heterodimer DNA binding activity. I observed that the ligand effect on *in vitro* translated EcR-USP complex interaction with DNA exhibited a great variability depending on different preparations (data not shown). I speculated that this variability could be the result of variations in protein concentration. Thus I investigated the effect of 20E on EcR-USP complex binding to DNA under different concentrations of receptor proteins. AaEcR and AaUSP proteins were produced *in vitro* with *TNT* rabbit reticulocyte lysate. Equal volumes of lysate programmed with either AaEcR or AaUSP expression plasmid were combined and subjected to EMSA in the presence or absence of $5x10^{-6}$ M 20E. At high receptor protein concentrations with 1µl of each lysate, 20E effect on receptor DNA binding activity was negligible with only 30% enhancement based on Phosphor-image quantification, and strong DNA binding activity was detected independent of ligand. (Fig.1, Lanes 1 and 2). Ligand enhancement increased to 50% when AaEcR and AaUSP lysates were reduced to 0.5µl each (Fig. 1, Lanes 2 and 3). With decreasing amount of receptor lysate, the magnitude of ligand enhancement increased inversely, although the overall binding activity decreased. With 0.25µl, 0.125µl, or 0.063µl each of receptor lysate, 2.7, 3.4 and 80 fold-enhancement was

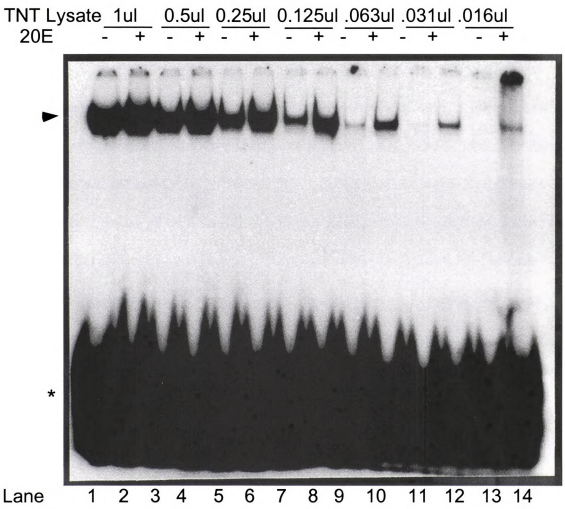


Fig. 1. Protein concentration affects 20E enhancement on receptor DNA binding activity. Equal volume of *in vitro* translated AaEcR and AaUSP proteins, each ranging from 0.016ul to 1ul were incubated with 50 fmol ^{32}P labeled IR^{hsp}-1 EcRE in the absence (lanes 1, 3, 5, 7, 9, 11 and 13) or presence (lanes 2, 4, 6, 8, 10, 12 and 14) of $5x10^{-6}$M ligand 20E. The reaction mixtures were resolved by EMSA and autoradiographed. The DNA-Protein complex is indicated by an arrow head and free probe indicated by an asterisk.

detected respectively (Fig.1 lanes 5-10). When the lysate volume was further reduced to 0.031μl or 0.016μl, the fold-enhancement of 20E approached infinite as the basal level of AaEcR-AaUSP DNA interaction in the absence of ligand became undetectable (Fig. 1, lanes 11-14). These results proved that EcR-USP DNA binding activity depended on receptor protein concentration. The receptor showed little or no binding activity at low receptor protein concentrations in the absence of hormone. As a result, the ligand effect was dramatic as manifested by high fold of 20E enhancement. At high concentrations of receptor protein, EcR-USP exhibited strong DNA binding activity even in the absence of ligand while the effect of ligand inversely decreased. With 0.125μl or 0.25μl of each receptor protein lysate (Fig 1, Lanes 5-8), DNA binding activity was detectable in the absence of hormone and ligand enhancement was conspicuous, hence, 0.1μl or 0.25μl each of AaEcR and AaUSP lysates were used for later experiments.

Next I addressed the question whether ligand enhancement of the receptor-DNA interaction depended on ligand concentration. I first determined the dose response of 20E in EMSA, which is believed to represent an physiologically active hormone. In this experiment, 0.1μl each of AaEcR and AaUSP lysate was incubated with increasing concentration of 20E ranging from $5x10^{-12}$M-$5x10^{-5}$M and subjected to EMSA . As shown before (Fig.1), weak binding was detected in the absence of hormone (Fig. 2A, Lanes 1 and 2) and 20E did not show any detectable enhancement between $5x10^{-12}$M-$5x10^{-9}$M 20E (Fig.2A, Lanes 3-6). Ligand enhancement was detected at $5x10^{-8}$M 20E, and the enhancement steadily increased with increasing 20E concentration from $5x10^{-8}$M-$5x10^{-5}$M (Fig.2A, Lanes 7-10).

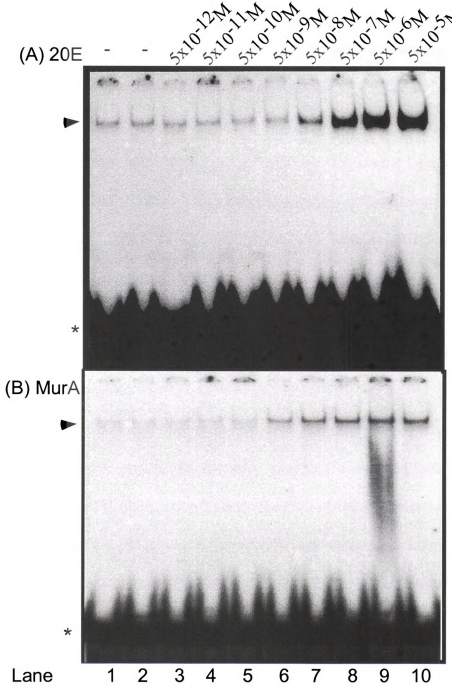


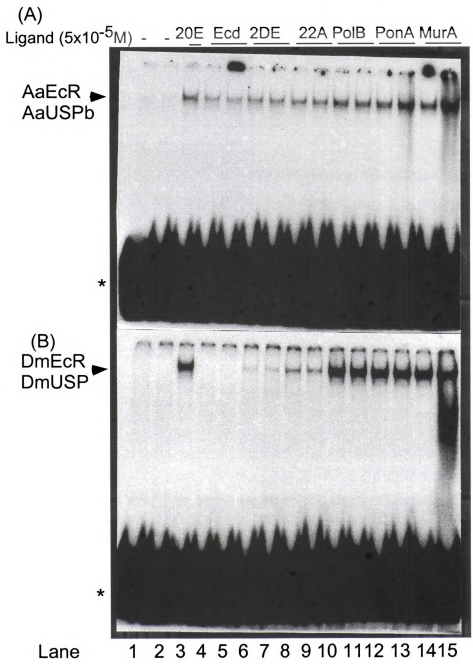
Fig. 2. Ligand dose dependent enhancement on receptor DNA binding activity. (A) *In intro* translated AaEcR and AaUSP proteins, 0.1ul each, were incubated with 50 fmol ^{32}-P labeled IR*hsp*-1 EcRE in the absence ligand (lanes 1 and 2) or in the presence of increasing concentration of ligand ranging from 10^{-12}M-10^{-5}M 20E (Lanes 3-10). The reaction mixtures were subjected to EMSA and autoradiography. (B) The same as A except MurA was used as the ligand. The DNA-Protein complexes are indicated by an arrow heads and free probe indicated by asterisks.

I then tested the dose response of another ligand MurA, which has been extensively used for bioassay and transactivation studies. Unlike 20E, which did not show any detectable enhancement on AaEcR-AaUSP DNA binding activity until its concentration reached $5x10^{-8}$M, MurA displayed noticeable enhancement at a lower concentrations, $5x10^{-9}$M (Fig. 2B, Lanes 1-6). This enhancement continued to grow with increasing MurA from $5x10^{-8}$M-$5x10^{-5}$M (Fig. 2B, Lanes 7-10). These results established that EcR-USP-DNA interaction depended on both receptor protein and ligand concentrations.

Potency of ecdysteroids on EcR-USP interaction with DNA

Taking advantage of the sensitivity of EMSA, I compared the potency of a variety of ecdysteroids, namely MurA, ponasterone A (PonA), polypodine-B (PolB), 20E, 20E-22-acetate (22A), 2-deoxy-20E (2DE) and ecdysone (Ecd). $5x10^{-5}$M of each ecdysteroid was incubated with TNT lysate programmed with receptor expression plasmid and subjected to EMSA. First I compared the effect of ecdysteroids on *Aedes* receptors with 0.1µl each of AaEcR and AaUSP lysate. A weak interaction of receptor-DNA was detected in the absence of any ligand (Fig.3A, Lanes 1 and 2) as shown before (Fig.2). And this interaction was dramatically enhanced with 20E (Fig. 3, Lane 3). Strikingly, ecdysone, which was assumed to be an ecdysteroid precursor, significantly enhanced AaEcR-AaUSP DNA binding activity (Fig. 3, Lanes 4 and 5) although weaker than 20E. Other ecdysoteroids also displayed apparent enhancement with the following decreasing

potency order: MurA>PonA>PolB>20E>22A>2DE>Ecd. I then tested the effect of these ecdysteroids on the *Drosophila* receptor. Due to its low basal DNA binding affinity in the absence of ligand, 1μl each of DmEcR and DmUSP lysate was used for EMSA. Even though ten times more lysate was used, DmEcR-DmUSP did not show discernible basal DNA binding without a ligand (Fig. 3B, Lanes 1 and 2), indicating its basal binding activity was at least 10 times weaker than AaEcR-AaUSP. Since EcR-USP binding to DNA requires both partners, the difference of DNA binding activity most likely reflected different levels of heterodimerization. When 20E was included, DmEcR-DmUSP exhibited higher binding activity than AaEcR-AaUSP (compare Fig 3A, lane 3 with Fig 3B Lane 3). Similarly, PolB, PonA and MurA induced higher DNA binding activity to the *Drosophila* receptor than to the *Aedes* receptor (compare Fig. 3A, Lanes 10-15 with Fig. 3B Lanes 10-15). The ligand 22A induced similar binding activity for receptors from two species (compare Fig. 3A, Lanes 8 and 9 with Fig. 3B Lanes 8 and 9). In contrast, ecdysone and 2DE induced stronger binding activity to *Aedes* receptor than to *Drosophila* receptor (compare Fig. 3A, Lanes 4-7 with Fig. 3B Lanes 4-7). Of particular note, ecdysone, which showed notable enhancement on AaEcR-AaUSP DNA binding activity, did not exert any detectable effect on DmEcR-DmUSP. These results disclosed that ecdysteroids had differential effect on *Drosophila* and *Aedes* receptors, with some ecdysteroids, namely PolB, PonA, MurA and 20E, showing stronger inducibility on *Drosophila* receptor despite the low heterodimerization capacity of DmEcR-DmUSP, while some ecdysteroids, namely ecdysone and 2DE showed stronger inducibility to *Aedes* receptor. The differential effect of ecdysteroids was most notably manifested by


(A)
Ligand (5x10-5M) - - 20E Ecd 2DE 22A PolB PonA MurA
AaEcR
AaUSPb
*
(B)
DmEcR
DmUSP
*
Lane 1 2 3 4 5 6 7 8 9 10 1112 13 1415

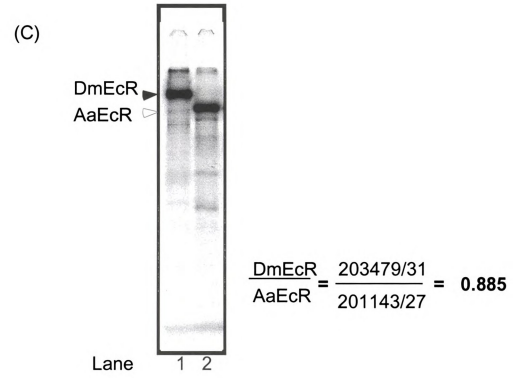


Fig. 3. Differential effects of ecdysteroids on receptor DNA binding activities. (A) *In vitro* tranlated AaEcR and AaUSPb proteins (0.1ul each) were incubated with ^{32}P labeled IR*hsp*-1 EcRE in the absence ligand (lanes 1 and 2) or in the presence 5x10^{-5}M 20E (lane 3), ecdysone (lanes 4 and 5), 2DE (lanes 6 and 7), 22A (lanes 8 and 9), PolB (lanes 10 and 11), PonA (lanes 12 and 13) or MurA (lanes 14 and 15). The reaction mixtures were subjected to EMSA and autoradiography. (B) The same as A except 1ul each of DmEcR and DmUSP lysate were used as receptor proteins. The DNA-Protein complex es are indicated by arrow heads and free probes indicated by asterisks. (C). DmEcR and AaEcR proteins were translated in vitro incorporating ^{35}S-methionine. Two ul each of the translated proteins were resolved through SDS-PAGE and autoradiography. DmEcR and AaEcR proteins contain 31 and 27 methionines, respectively. Phosphor-image quantification normalized with the number of methionines indicated the ratio of DmEcR:AaEcR=0.885

ecdysone, showing apparent enhancement on *Aedes* receptor DNA binding activity, yet no detectable effect on the *Drosophila* receptor. Unlike that of PolB, PonA, MurA and 20E, the weak inducibility of ecdysone and 2DE on the *Drosophila* receptor paralleled the low heterodimerization capability of DmEcR-DmUSP.

In these EMSA experiment, receptor levels were based on the volumes of TNT lysate. I tested whether the volume of lysate corelated with the level of proteins by programming a TNT Transcription-Translation reaction with ^{35}S-Methionine followed by SDS-PAGE and phospor-image quantification. My results indicated the molar ratio of AaEcR to DmEcR proteins was appropriately 1:1 (Fig. 3C), as was the ratio of AaUSP to DmUSP proteins (data not shown).

EcR protein, not USP protein, conferred specific response to ecdysone.

To define the differential effect of ecdysteroids, I took ecdysone and 20E as representatives from each ligand group for further analysis. Functional ecdysteroid receptor is a heterodimer comprised of EcR and USP proteins. Unlike TR, VDR, and RAR, which apparently do not require their heterodimerization partner RXR for ligand binding, ecdysteroid receptor binding to ligand requires both EcR and USP proteins. My EMSA results clearly demonstrated ecdysone can serve as a functional ligand for the *Aedes* receptor, but not for the *Drosophila* receptor. I then conducted subunit swapping experiment to define whether EcR or USP determined ligand specificity. As shown before (Fig 3), AaEcR-AaUSP was activated by both 20E and ecdysone while DmEcR-DmUSP was activated only by 20E, not ecdysone (Fig 4, Lanes 1-6). When AaEcR was paired with DmUSP, its DNA binding activity was highly induced by both 20E and

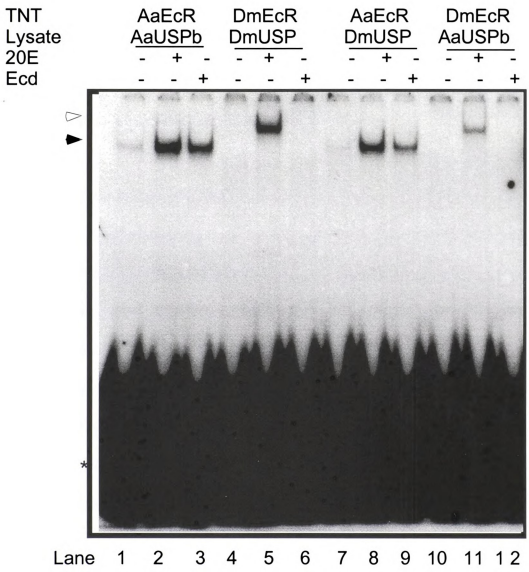


Fig. 4. EcR, rather than USP, conferred specific response to ecdysone. *In vitro* translated proteins, 0.1μl each of AaEcR and AaUSPb (lanes 1-3), 1.0μl each of DmEcR and DmUSP (lanes 4-6), 0.1μl AaEcR and 1μl DmUSP (lanes 7-9), or 1μl DmEcR and 0.1μl AaUSPb (lane 10-12) were incubated with ^{32}P labeled IR^{hsp}-1 EcRE probe either in the absence of hormone (lanes 1, 4, 7 and 10), in the presence 5x10^{-5}M 20E (lanes 2, 5,8 and 11) or ecdysone (lanes 3, 6, 9 and 12). The reaction mixtures were subjected to EMSA and autoradiography. The free probe is indicted by an asterisk and DNA-protein complexes by arrow heads.

ecdysone (Fig. 4, Lane 7-9) analogous to the AaEcR-AaUSP complex. Reciprocally, DmEcR-AaUSP was activated only by 20E, not by ecdysone (Fig. 4, Lanes 10-12) resembling the DmEcR-DmUSP complex. These results substantiated that EcR protein, not USP protein, defined specific response to ecdysone.

Ecdysone, a potent inducer for *Aedes* receptor in S2 cells

To investigate whether ecdysone could function in a cell transactivation assay, the reporter plasmid Eip-Luc was transfected into S2 cells alone or together with expression plasmids harboring either AaEcR, AaUSPa, AaUSPb, DmEcR, DmUSP cDNA or pair-wise combination as indicated. After transfection, cells were incubated either in the absence of hormone or in the presence of $5x10^{-5}$M hormone. Cells obtaining Eip-Luc alone or reporter plasmid together with either AaUSPa, AaUSPb, or DmUSP expression vector became modestly responsive to 20E with around 50% induction which was obviously mediated by endogenous *Drosophila* EcR. Only residual reporter gene activity was detected with ecdysone (Fig. 5, Columns 1, 3, 4 and 6). When AaEcR plasmid was delivered into cells with Eip-Luc, cells became responsive to both 20E and ecdysone(Fig. 5, Column 2). And this response was further boosted by cotransfection with either AaUSPa, AUSPb or DmUSP expression plasmid (Fig.5, Columns 7-9). More importantly, the magnitude of induction mediated through AaEcR by 20E and ecdysone were quite close, 3-5 fold. Cells transfected with DmEcR plasmid turned to be highly responsive to 20E with 5-fold induction while on the contrary only trace response, 50% induction, was detected with ecdysone(Fig.5, Column 5). Cotransfection of cells with DmEcR plasmid and AaUSPa, AaUSPb or DmUSP expression vectors further hoisted

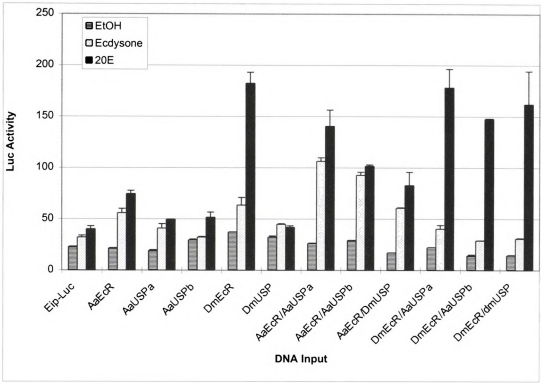
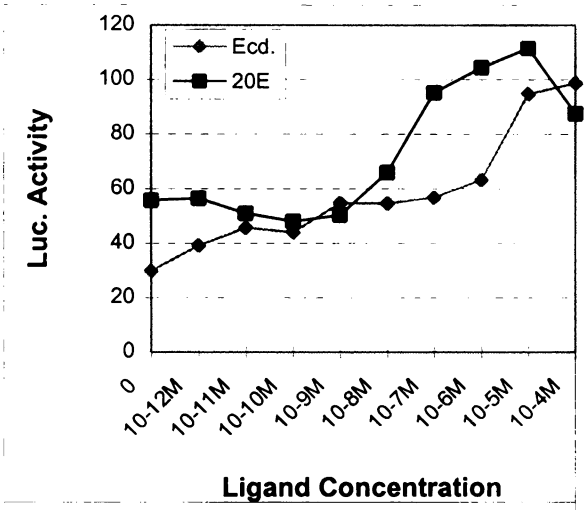


Fig. 5. Ecdysone potently induced Aedes EcR in S2 cells. S2 cells were transfected with 25ng coreporter pAc-LacZ and 100ng reporter plasmid Eip-Luc (columns 1-3), reporter plasmids and 12.5ng each of AaEcR, AaUSPa, AaUSPb, DmEcR or DmUSPb expression vectors (columns 4-18), reporter plasmids and pair-wise combination of receptors, AaEcR and AaUSPa (Column 19-21), AaEcR and AaUSPb (columns 22-24), AaEcR and DmUSP (Columns 25-27), DmEcR and AaUSPa (columns 28-30), DmEcR and AaUSPb (columns 31-33), or DmEcR and DmSP expression vectors(columns 34-36). After transfection, cells were incubated either in the absence of hormone (column 1, 4, 7,10, 13, 16, 19, 22, 25, 28, 31 and 34) in the presence of $5x10^{-5}$M 20E (column 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35) or $5x10^{-5}$M ecdysone (columns 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 and 36) for 36 hours and harvested for β-galactosidase activity and luciferase activity. Luciferase activity was normalized with β-galactosidase activity.

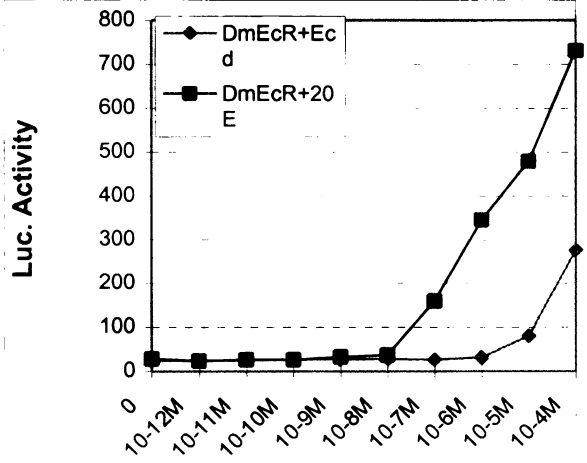
the response to 20E, with 8-11 fold induction, whereas the response to ecdysone remained negligible (Fig.5, Column 10-12). In accordance with EMSA results (Figs 3), 20E performed as a more robust inducer to DmEcR than to AaEcR although it did activated EcR proteins from the two dipteran species, yet ecdysone activated AaEcR more potently than DmEcR. Residual activation of DmEcR by ecdysone could be due to metabolic conversion of ecdysone to 20E or other active ecdysteroids as no activation of DmEcR protein was detected in EMSA. Moreover, in agreement with subunit swapping experiment in EMSA (Fig. 4), transactivation results authenticated that EcR, not USP protein, dictated ligand specificity as AaEcR, paired with either AaUSPa, AaUSPb, or DmUSP responded to ecdysone, whereas DmEcR, paired with either AaUSPa, AaUSPb, or DmUSP did not grant any notable response to ecdysone in contrast to its sturdy response to 20E.

I then titrated 20E and ecdysone in transactivation assays. After transfecting cells with Luc-Eip alone, reporter plasmid and DmUSP plasmid paired with either AaEcR or DmEcR plasmids, cells were incubated either in the absence of hormone or in the presence of increasing concentration hormone ranging from 10^{-12}M-10^{-4}M (Fig. 6). Cells receiving Eip-Luc alone responded to 10^{-8}M 20E with 50% reporter gene induction, and the same magnitude of response required 10^{-6}M ecdysone. Luciferase activity was induced to 2.2, 2.4 and 2.6 fold with 10^{-7}M, 10^{-6}M and 10^{-5}M 20E respectively and then dropped to 2-fold at 10^{-4}M 20E. Reporter gene activity was induced to 2.2 and 2.3 fold

(A)



(B)



(C)

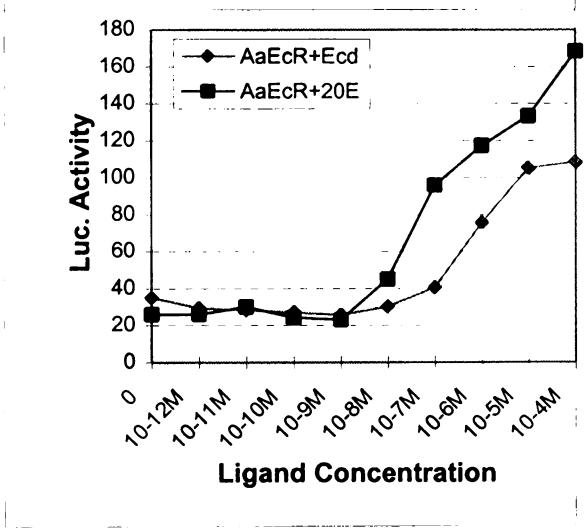


Fig. 6. Dose dependent response of receptors to ecdysteroids. S2 cells ($5x10^5$) were transfected with 25ng co-reporter pAc-LacZ and 100ng reporter plasmid Eip-Luc (panel A), reporter plasmids and 12.5ng each of DmEcR and DmUSP (panel B), or AaEcR and DmUSP expression vectors (panel C). After transfection, cells were incubated in the absence of hormone, in the presence of increasing concentration (ranging from 10^{-12}M to 10^{-4}M)of 20E or ecdysone for 24 hours and harvested for β-galactosidase activity and luciferase activity. Luciferase activity was normalized with β-galactosidase activity.

with 10^{-5}M and 10^{-4}M ecdysone respectively (Fig 6-A). These responses were apparently mediated by endogenous DmEcR-DmUSP protein.

When cells were cotransfected with exogenous DmEcR and DmUSP plasmids, they responded to 10^{-8}M 20E and 10^{-6}M ecdysone, as did with endogenous *Drosophila* receptor (Fig. 6-A), yet the magnitude of the induction dramatically increased, with 1.2, 6, 13, 18 and 28 fold induction detected at 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M 20E respectively. With 10^{-6}M, 10^{-5}M and 10^{-4}M ecdysone, 1.2-, 3- and 10- fold luciferase induction was detected respectively (Fig 6-B). These results denoted that supplementing exogenous DmEcR-DmUSP protein to the cells did not alter its sensitivity rather than the extent of response to 20E. Also of note was that cells did not show decline of luciferase activity at 10^{-4}M 20E.

When cells were cotransfected with AaEcR and DmUSP plasmids, they responded to 20E and ecdysone at the same concentration, 10^{-8}M, with 3.1-, 3.8-, 4.4- and 5.5- fold induction detected for 20E and 1.3, 2.5, 3.4, and 3.5 fold induction for ecdysone at 10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M hormone respectively (Fig. 6-C). These results established that DmEcR-DmUSP was at least 100 times more sensitive to 20E than to ecdysone whereas similar sensitivity to 20E and ecdysone was detected for AaEcR-DmUSP.

Hormone titration assays ascertained that differential effect of ligand on *Aedes* and *Drosophila* receptors was quantitative in transactivation. The induction of DmEcR at supra-physiological concentration of ecdysone could be due to metabolism and/or weak interaction between DmEcR-DmUSP with high level of ecdysone. I concluded that at certain hormone concentration, 10^{-6}M, 20E could activate both *Aedes* and *Drosophila*

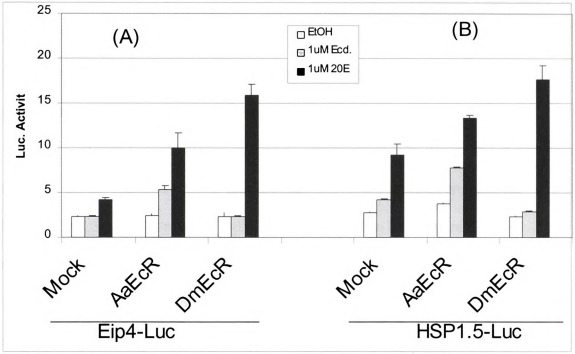
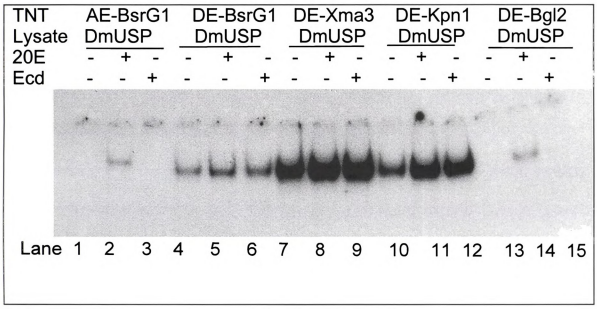


Fig. 7. Ecdysone (10^{-6}M) activated only the *Aedes* receptor not the *Drosophila* receptor. (A) S2 cells ($2.5x10^5$) were transfected with 12.5ng co-reporter pAc-LacZ and 50ng reporter plasmid Eip-Luc (columns 1-3), reporter plasmids and 6.5ng each of AaEcR and DmUSP (column 4-6), or AaEcR and DmUSP expression vectors (column 7-9). After transfection, cells were incubated in the absence of hormone (column 1, 4 and 7), in the presence of 10^{-6}M of Ecdysone (columns 2, 5 and 8) or ecdysone (column 3 , 6 and 9) for 24 hours and harvested for β-galactosidase activity and luciferase activity. Luciferase activity was normalized with β-galactosidase activity. (B) The same as in A except a reporter hsp-Luc was used instead of Eip-Luc.

receptors whereas ecdysone could activate only the *Drosophila* receptor. I then tested the effect of ligand on two reporter constructs, Eip-Luc and Hsp-Luc. These reporter plasmids were transfected into S2 cells either alone or together with DmUSP pasmid paired with AaEcR or DmEcR plasmids and then incubated with or without 10^{-6}M hormone. Cells transfected with reporter alone responded only to 20E, with 1.8 fold induction for Eip-Luc and 3.3 fold induction for Hsp-Luc, indicating endogenous DmEcR-DmUSP did not respond to 10^{-6}M ecdysone. When DmEcR and DmUSP plasmids were delivered into the cells, the magnitude of response to 20E was boosted to around 7-fold induction for both reporters. Eip-Luc did not show detectable induction to ecdysone and Hsp-Luc showed only negligible response to ecdysone. When AaEcR and DmUSP were delivered into cells, induction to 20E was increased to 4-fold for both reporter plasmids. Remarkably, its response to ecdysone was also increased to 2-fold (Fig. 7). These results corroborated 20E as a more robust ligand for DmEcR while on the contrary ecdysone functioned as a potent ligand for AaEcR, and yet almost inert for DmEcR.

Mapping of ligand specific domain in EcRs

EMSA and transfection assays unequivocally proved that ecdysone served as a functional ligand for the *Aedes* receptor.Furthermore, it is the EcR protein, not the USP protein underlay the ligand specificity. Sequence alignments have revealed that EcR protein possessed five putative functional domains, an N-terminal domain A/B, DNA binding domain C, hinge domain D, ligand binding domain E, and C-terminal domain F.



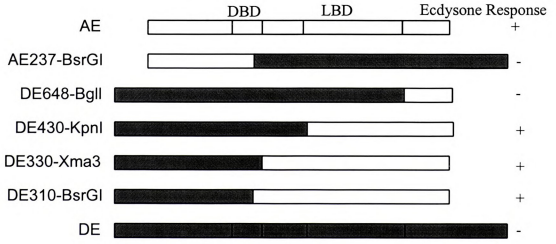


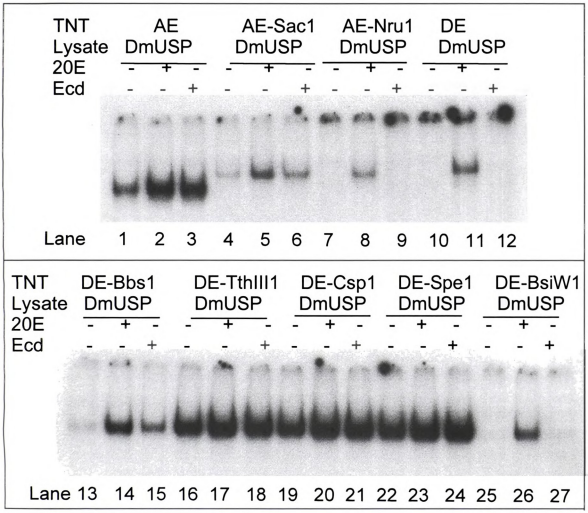
Fig. 8. Localization of the ecdysone-specific region to the LBD. (A) ^{32}P-labeled probe IR^{hsp}-1 was incubated with *in vitro* synthesized DmUSP protein paired with chimeric proteins AE^{BsrGI} (lanes 1-3), DE^{BsrGI}(lanes 4-6), DE^{Xma3}(lane 7-9), DE^{KpnI} (lanes 10-12) or DE^{Bgl2}(lanes 13-15) in the absence of hormone (lanes 1, 4,7 10 and 13) or in the presence of $5x10^{-5}$M 20E (lanes 2, 5,8,11 and 14) or ecdysone (lanes 3, 6, 9, 12 and 15). Bound and free probe were resolved by EMSA followed by autoradiography. (B) Schematic diagram of chimeras and their responsiveness to ecdysone.

Unlike the DBD in domain C, boundaries of domain E is not well defined. To appraise whether ligand specificity determinants were confined to LBD, I made five *Aedes* and *Drosophila* chimeric EcR constructs by swapping the appropriate cDNA sequences. AE^{BsrGI} protein contained A/B domain and most of its domain C from *Aedes* receptor and part of domain C, domains D, E and F from *Drosophila* receptor. Reciprocally DE^{BsrGI} comprised an N-terminal from *Drosophila* receptor and C-terminal from *Aedes* receptor. By swapping at the predicted boundaries between domains C and D, domains D and E, and domains E and F, I created three chimeras with N-terminals from DmEcR and C-terminals from AaEcR, namely DE^{Xma3}, DE^{Kpn1} and DE^{Bgl2}. These chemeric proteins were produced by *TNT in vitro* transcription/translation, paired with DmUSP protein and subjected to EMSA. 0.25µl each of EcR chimera and DmUSP lysate was used per lane. Equivalent to DmEcR, AE^{BsrGI} and DE^{Bgl2} DNA binding activities were amplified by 20E, not by ecdysone. It was noteworthy that these two chimeras did not display any detectable basal DNA binding activity in the absence of 20E (Fig.8, Lanes 1-3, 13-15). In contrast, DE^{Xma3}, DE^{Kpn1} and DE^{Bgl2} chemeric proteins exhibited obvious basal level of DNA binding activity, which was augmented by both ligands, 20E and ecdysone (Fig 8, Lanes 4-12). These results unambiguously narrowed down the ligand specific region to the LBD. Remarkably, the interaction with ecdysone was tightly tied together with levels of heterodimerization as noted earlier (Figs. 3 and 4).

Identification of regions underlying ligand specificity and heterodimerization

LBDs from *Aedes* and *Drosophila* EcRs are highly conserved with only 28 out of the 220 aa different from each other, 87.3% identical. Using a combination of restriction digestion and PCR amplification, I made seven more chimeric constructs by swapping at the appropriate sites in the LBD. AE^{Nru1} chimera was constructed by replacing the 3' *Nru1* fragment of AaEcR cDNA with the corresponding fragment from DmEcR cDNA. AE^{Sac1} chimera was swapped further down stream utilizing the *Sac1* sit in AaEcR cDNA. AE^{Nru1} and AE^{Sac1} chimera proteins were produced *in vitro*, normalized by ^{35}S-methionine labeling and paired with DmUSP for EMSA analysis. AE^{Sac1} protein, exhibited conspicuous basal level of heterodimerization in the absence of hormone, and its DNA binding activity was enhanced by 20E and ecdysone in a way similar to its AaEcR parental protein (Fig. 9, Lanes 1-6). AE^{Nru1} chimera protein, however, failed to show any basal level heterodimerization and its DNA binding activity was only detected with 20E, paralleling that of DmEcR (Fig. 9. Lanes 7-12). These results implied that the region governing ligand specificity and heterodimerization located between the *Nru1* and *Sac1* sites in the AaEcR cDNA. Accordingly, five more chimeric constructs were produced proceeding from *Nru1* site to *Sac1* site with an N-terminal from DmEcR and C-terminal from AaEcR, namely DE^{Bbs1}, $DE^{TthIII1}$, DE^{Csp1}, DE^{Spe1} and DE^{BsiW1}. Four of these chimeric proteins, DE^{Bbs1}, $DE^{TthIII1}$, DE^{Csp1} and DE^{Spe1} displayed apparent basal DNA binding activity, which was enhanced by 20E and ecdysone (Fig.9 Lanes 13-24). Yet DE^{BsiW1} failed to show basal heterodimerization and its DNA binding activity was not significantly enhanced by ecdysone (Fig.9 Lanes 25-27). Hence, I concluded that protein sequence corresponding to the cDNA region between *Spe1* and *BsiW1* sites

A


TNT Lysate
AE DmUSP
AE-Sac1 DmUSP
AE-Nru1 DmUSP
DE DmUSP
20E
Ecd
Lane 1 2 3 4 5 6 7 8 9 10 11 12
TNT Lysate
DE-Bbs1 DmUSP
DE-TthIII1 DmUSP
DE-Csp1 DmUSP
DE-Spe1 DmUSP
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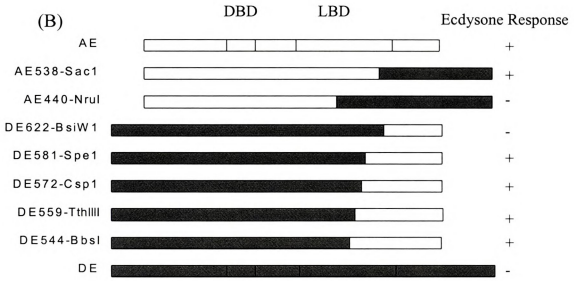

Fig. 9. C-terminal of EcR LBD determined ecdysone binding specificity. (A)^{32}P-labeled probe IR^{hsp}-1 was incubated with *in vitro* synthesized DmUSP protein paired with the wild type proteins AaEcR (lanes 1-3), DmEcR (lanes 10-12), chimeric proteins AE^{Sac1} (lanes 4-6), AE^{Nru1}(lanes7-9), DE^{Bbs1}(lane 13-15), DE^{TthIII1} (lanes 16-18), DE^{Csp1}(lanes 19-21), DE^{Spe1}(lanes 22-24) or DE^{BsiW1} (lanes 25-27) in the absence of hormone (lanes 1, 4,7 10, 13, 16, 19, 22 and 25) or in the presence of 5x10^{-5}M 20E (lanes 2, 5,8,11,14, 17,20, 23 and 26) or ecdysone (lanes 3, 6, 9, 12,15, 18, 21, 24 and 27). Bound and free probe ere resolved by EMSA followed by autoradiography. (B) Schematic diagram of chimeras and their responsiveness to ecdysone.

dictated high level of heterodimerization and specific interaction with ecdysone for AaEcR protein.

Tyr611 in DmEcR was the critical residue defining ligand specificity and heterodimerization.

Comparing AaEcR and DmEcR protein sequences revealed that 8 out of the 40 aa residing between the *Spe1* and *BsiW1* sites were different; and these AaEcR/DmEcR amino acid differences were: His502/Ala584, Ala520/Cys602, Pro523/Ser605, Lys524/Met606, Cys525/Ser607, Ser526/leu608, Ile528/Phe610, Phe529/Tyr611. To identify the critical amino acid conferring ligand specificity, I created seven site directed mutants by converting an amino acid in DmEcR protein to its corresponding residue in AaEcR protein, namely Cys602, Ser605, Met606, Ser607, Leu608, Phe610 and Tyr611 in DmEcR protein were mutated to Ala, Pro, Lys, Cys, Ser, Ile and Phe respectively, yielding DmEcR mutants DE^{C602A}, DE^{S605P}, DE^{M606K}, DE^{S607C}, DE^{L608S}, DE^{F610I} and DE^{Y611F}. These mutant constructs were translated *in vitro,* paired with DmUSP for EMSA analysis. DE^{C602A}, DE^{S607C}, DE^{L608S} and DE^{F610I} proteins did not display any basal heterodimerization and their DNA binding activity was only detected in the presence of 20E similar to the DmEcR parent protein (Fig. 10, Lane 4-9, 16-24). Likewise, DE^{S605P} and DE^{M606K} proteins showed no DNA binding activity in absence of hormone, strong activity with 20E and trace activity with ecdysone (Fig.10 Lanes 10-15), suggesting Ser605 and Met606 in DmEcR protein could be slightly involved with ligand specificity, but not with heterodimerization. Remarkably, DE^{Y611F} protein exhibited apparent basal level of DNA binding activity in the absence of hormone, and this activity was clearly amplified with not only 20E, but also with ecdysone comparable to that of the AaEcR protein (Fig.10,

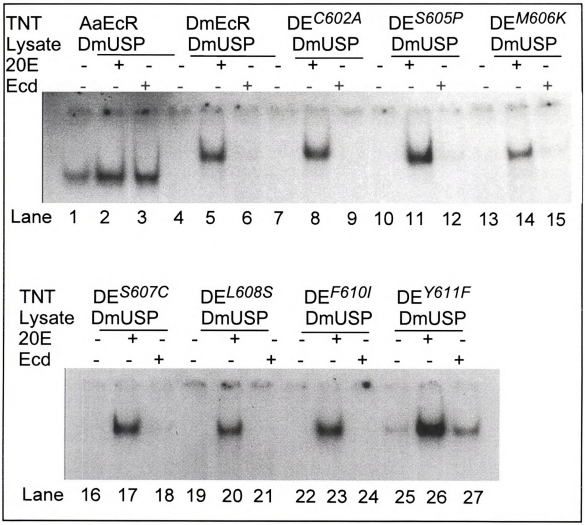

Fig. 10. Tyr611 in DmEcR dictated ligand specificity and heterodimerization efficiency. ^{32}P-labeled probe IR^{hsp}-1 was incubated with *in vitro* synthesized DmUSP protein paired with the wild type proteins AaEcR (lanes 1-3), DmEcR (lanes 4-6), DmEcR point mutants DE^{C602A} (lanes 7-9), DE^{S605P}(lanes 10-12), DE^{M606K} (lane 13-15), DE^{S607C} (lanes 16-18), DE^{L608S}(lanes 19-21), DE^{F610I}(lanes 22-24) or DE^{Y611F}(lanes 25-27) in the absence of hormone (lanes 1, 4,7 10, 13, 16, 19, 22 and 25) or in the presence of 5x10^{-5}M 20E (lanes 2, 5,8,11,14, 17,20, 23 and 26) or ecdysone (lanes 3, 6, 9, 12,15, 18, 21, 24 and 27). Bound and free probe were resolved by EMSA followed by autoradiography.

Lane 1-3, 25-27), indicating Tyr611 in DmEcR was most critical for determining its weak interaction with ecdysone and lower basal level of heterodimerization compared with AaEcR protein.

DISCUSSION

Receptor concentration and localization

My results indicated that receptor protein concentration played an important role affecting the receptor interaction with DNA. At low receptor protein concentrations, conspicuous DNA binding activity can only be detected in the presence of hormone and no or little DNA binding activity could be detected in the absence of hormone. At high protein concentrations, in contrast, receptor exhibited strong DNA binding activity even in the absence of hormone. These results resolved the controversy in the literature regarding the ligand effects on EcR-USP interaction with DNA. Thomas et al (1993) first reported that 20E had no effect on DmEcR-DmUSP binding to DNA, while in contrast, several groups later presented that 20E dramatically enhanced the *Drosophila* receptor binding to an EcRE (Yao et al., 1992; 1993). Based on my results, 20E can exert anything from no effect to strong enhancement on its receptor DNA binding activity depending upon the receptor protein concentration.

Ligand-modulated receptor DNA binding activity has been well documented for vertebrate nuclear receptors. Ligand enhances ER, PR and RXR homodimers, VDR-RXR heterodimer and TR monomer DNA binding activities (Reviewed by Cheskis and Freedman, 1998). Similar controversy was brought out for ER DNA binding activity with

some groups reporting the hormone-independent formation of the ER-ERE complex while others describing ligand-induced ER DNA binding. It was later demonstrated that the ligand effect on ER DNA binding activity depends on the receptor concentration and the hormone is required to promote DNA binding at low but not at high concentrations of ER (reviewed by Cheskis and Freedman 1997).

A large body of information has been accumulated to address the ecdysteroid receptor cellular localization. The receptor was initially reported to be located mainly in the cytosol, as revealed by 3-H ponasterone A binding assays (Maroy et al., 1978). These results were further proven by immunohistochemistry using anti-EcR and anti-USP antibodies. In naive epithelial cell line from *Chironomus tentans*, receptor staining is detected in both the nucleolus and the cytosol. After treatment with 20E, cells lose their cytosol staining while increase their nuclear staining, suggesting 20E stimulate its receptor to migrate from the cytosol to the nucleus (Lammerding-Koppel et al., 1998). In contrast, ^{3}H-ponasterone A binding studies indicates that ecdysteroid receptor in imaginal disk isolated from mid- to late- third instar larvae resides primarily in the nucleus (Yund et al., 1978), these results are confirmed by immunostaining with anti-EcR antibodies (Koelle et al., 1991). However, these conflicting observations do not rule out the possibility that ecdysteroids facilitate the translocation of the receptor from the cytosol to the nucleus. EcRE mediated transactivation is repressed in the absence (Dobens et al., 1991; Cherbas et al., 1991). These results have led to the conclusion that unliganded receptor represses basal transcription. My results indicated localization of receptor in the nucleus does not guarantee its association with target EcRE at low receptor concentration due to its low affinity with EcRE in the absence of hormone. Taken together, ecdysteroid

receptor localization is likely controlled at two steps: translocation from the cytosol to the nucleus and targeting to specific EcREs, the ligand 20E apparently plays stimulary roles in these two processes.

Dose dependent EcR-USP binding to DNA

EMSA results indicated the *Aedes* receptor responded to $5x10^{-8}$M 20E and $5x10^{-9}$ M MurA. Accordingly, MurA was more potent than 20E in stimulating the *Aedes* receptor transactivation in CV-1 cells (Wang and Raikhel, unpublished data). Likewise, MurA is also more effective inducing the *Drosophila* receptor binding to an EcRE and transactivating a reporter gene in CV-1 cells (Yao, et al., 1993). The superior activity of MurA apparently lies primarily in its efficient interaction with the receptor proteins rather than on its metabolic stability. Indeed, 20E is quite stable in cultured *Drosophila* imaginal disks (Maroy et al., 1978). As revealed by partial proteolytic digestion assay, DmEcR alone is protected by MurA, but not by 20E suggesting MurA may employ a distinct mechanism to bind to the receptor (Yao, et al., 1993).

Ligand enhancement on DNA binding activity did not reach saturation at a ligand concentration of $5x10^{-5}$M, which is not surprising considering the DNA binding process involves interaction of four players, EcR, USP, ligand and EcRE.

Differential activity of ecdysteroids

Taking advantage of the sensitivity of the DNA binding assay, I compared the potency of seven ecdysteroids. The potency order is identical for the receptors from two species, *Aedes* and *Drosophila*, which was MurA>PNA>PolB>20E>22Ace>2-

DE>Ecdysone. The potencies of ecdysteroids have been extensively studied utilizing bioassays and extracts from *Drosophila* cells and tissue. Maroy et al. (1978) and Yund et al. (1978) used ^{3}H-PNA as a probe for binding assays with Kc cell and imaginal disks extracts. Their competition assays revealed the affinity order as PNA>20E>Ecd, consistent with our results. Cherbas et al. (1980) utilized the morphological changes in Kc cells to monitor ecdysteroid activity. Recently, Harmatha and Dinan (1997) re-evaluated the ecdysteroid potency using BII cell morphological alteration, with the following order: PNA>PolB>20E>ecd, supporting the results I obtained from EMSA. However, these cell morphology studies are primarily base on *Drosophila* cells, making it difficult to compare the efficacy of ecdysteroid on different species. Availability of the cloned genes from *Drosophila* and *Aedes* enabled us to directly compare the effect of ecdysteroid on these two species. Based on EMSA, the tested ecdysteroids can be defined into three classes, namely high-potency, mid-potency and low-potency ecdysteroids. High potency ecdysteroids, including MurA, PNA, PolB and 20E, induced strong DNA binding activity for both *Aedes* and *Drosophila* receptors. More importantly, the enhancement on the *Drosophila* receptor is significantly higher than that on the *Aedes* receptor, indicating they elicited more profound comformational changes for the fruit-fly receptor. Mid-potency ecdysteroid, namely 22-Ace, induced modest enhancement, and the enhanced DNA binding activity was almost identical for these two receptors. Low-potency ecdysteroids, namely 2-DE and ecdysone, induced weak binding activities for these receptors. In contrast to the high-potency ecdysteroids, low-potency ecdysteroids induced stronger *Aedes* receptor DNA binding activity than it did to the *Drosophila*

receptor, which was most manifested by the ligand ecdysone, with apparent enhancement on the *Aedes* receptor, yet no discernible effect on the *Drosophila* receptor.

Aside from the differential effects elicited by ecdysteroids, the *Aedes* receptor distinguished itself from the *Drosophila* receptor by exhibiting stronger basal level of DNA binding activity in the absence of hormone, even though 10 times each of DmEcR and DmUSP proteins were used in these experiments. Titration experiment using increasing concentration of DmEcR and DmUSP proteins indicated the basal level of *Drosophila* receptor DNA binding activity was detectable only with more than 50 times more each of DmEcR and DmUSP protein. And ecdysone did show trace enhancement on DmEcR-DmUSP DNA binding activity at high protein concentration, yet this enhancement of ecdysone was insignificant compared with that of 20E. For the *Aedes* receptor, although 20E elicited stronger enhancement, the effect of 20E and ecdysone were of similar magnitude.

EcR, not USP, determined ligand specificity.

Taking 20E as the representative for high-potency ecdysteroid and ecdysone as the representative for low-potency ecdysteroid, I proceeded to characterize the ligand specificity. The functional ecdysteroid receptor consists of two subunits, EcR and USP proteins. To identify the subunit conferring ligand specificity, I then conducted subunit swapping experiment. Parallel to AaEcR-AaUSPb dimer, AaEcR-DmUSP responded to ecdysone as revealed by EMSA. In contrast, DmEcR-AaUSPb did not respond to ecdysone simulating DmEcR-DmUSP. These results unequivocally demonstrated that

EcR, not USP, conferred specific response to ecdysone, whereby USP acted as a silent partner.

In vertebrates, TR, VDR and RAR requires RXR to bind DNA elements. Likewise, EcR needs USP to exert DNA binding activity. However, unlike the vertebrate TR, VDR and RAR, which apparently do not require RXR for cognate ligand binding. EcR does not interact with 20E without USP as revealed by partial proteolytic digestion assays (Yao et al., 1993). However, it is unknown whether USP directly interacts with the ligand or merely acts to stabilize the binding pocket. AaEcR showed interaction with ecdysone regardless of the species of USP, suggesting that USP protein functioned solely to stabilize the ligand binding pocket of EcR.

Ecdysone potently activated *Aedes* receptor in S2 cell.

Transfection assays in S2 cells confirmed my EMSA results. At a ligand concentration of 10^{-5}M, AaEcR paired with AaUSPa, AaUSPb or DmUSP conferred S2 cells responsiveness to 20E and ecdysone to similar magnitude. DmEcR pared with AaUSPa, AaUSPb and DmUSP responded drastically to 20E, but only weakly to ecdysone. In accordance with the ligand enhancement on DNA binding activity, 20E induced higher reporter gene activity via the DmEcR than it did via AaEcR. In contrast, ecdysone induced higher reporter gene activity via the AaEcR. More importantly, ecdysone stimulated luciferase activity through DmEcR was insignificantly compared with that of 20E. These results demonstrated that ecdysone did exert only trace activity via the DmEcR.

My understanding of the differential effect of ecdysone was extended by ligand titration assays in S2 cells. Activating AaEcR and DmEcR required 10^{-8}M 20E, and 10^{-8}M ecdysone activated the AaEcR. However, activation of DmEcR required 10^{-6}M ecdysone, verifying that the quantitative difference of ecdysone on AaEcR and DmEcR.

The ecdysone specificity was further confirmed utilizing the two different EcREs, Eip28/29 and hsp EcREs. At a ligand concentration of 10^{-6}M, 20E activated the reporter gene through the two receptors, AaEcR and DmEcR. However, ecdysone activated reporter gene activity only through AaEcR, not DmEcR.

In *A. aegypti*, the ecdysteroid concentration is 10^{-7}M in the previtellogenic stage and increases to $3x10^{-7}$M in vitellogenic stage. The fat body contains predominantly 20E and ovary contains virtually only ecdysone (Hagedorn et al., 1975). Many ovarian genes including EcR and vitelline membrane protein are transcriptionally controlled by ecdysteroid. Hence, I conclude that 20E may act as the functional ligand in the fat body and ecdysone may function in the ovary.

The ecdysone level is below detectable level during the *Drosophila* development (Richards, 1981). The low level of ecdysone in *Drosohpila* is a possible reason why DmEcR has not developed efficient response to this ligand.

AaEcR ligand specific region is located at the I-box.

To identify the components conferring ligand specificity, I first used domain swapping technique to locate the critical domain. Not surprisingly, the EcR LBD was identified as the crucial region. Importantly, as discussed earlier, response to ecdysone is interconnected with its basal heterodimerization capacity. Chimeras including DE^{BrG1},

DE^{Xma3} and DE^{kpn1} with LBDs from AaEcR displayed apparent basal DNA binding activity together with their conspicuous response to ecdysone. Suhr et al., (1998) reported the Bombyx EcR hinge region possessed dimerization capacity. DE^{kpn1}, with its hinge region from DmEcR, showed strong basal binding comparable to DE^{BsrG1} and DE^{Xma3} with hinge regions from AaEcR. These results indicated that the heterodimerization activity of AaEcR hinge region is insignificant compared with that in the LBD.

More detailed domain swapping experiments revealed that high level heterodimerization and response to ecdysone were determined by the C-terminal of AaEcR, namely aa502-aa529 in AaEcR, corresponding to aa584-aa611 in DmEcR. This region corresponds to helices 9 and 10 where compared with the available crystal structures for RXR, TR, RAR, PR and ER (Bourguet et al., 1995; Wagner et al., 1995; Renaud et al., 1995; Brzozowski et al., 1997; Williams and Sigler, 1998; Tanenbaum et al., 1998). Interestingly, helices 10 in ER and RXR are located at the homodimeriation interface , whereas helices 11 and 12 occupied the dimerization interface in PR. Using similar domain swapping techniques, Perlmann et al. (1996) located a dimerization box, designated identity-box (I-box). I-Box is critical in the formation of COUP-TF homodimers, RXR-RAR and RXR-TR homodimers, but not RXR-VDR or RXR-PPAR heterodimers. Remarkably, the I-box is identical to the critical region I identified conferring differential heterodimerization for AaEcR and DmEcR. Moreover, I discovered that the I-box was also involved with ligand specificity.

Phe529 specified AaEcR high level heterodimerization and high level response to ecdysone.

There are only eight amino acids in AaEcR different from that in DmEcR in the I-box, seven of which are clustered together. To locate the critical amino acid underlying species specificity, I then constructed seven site directed mutants by converting a residue in DmEcR to its corresponding residue in AaEcR. EMSA revealed that DE^{Y611F} gained response to ecdysone, indicating Phe529 rendered AaEcR high affinity to ecdysone. A less polar residue, Phe529, conferred AaEcR high affinity to ecdysone than DmEcR. Reciprocally, a more polar residue, Tyr611, rendered DmEcR more responsive to 20E than AaEcR. It is not known whether Phe529/Tyr611 directly interacts with ligand or exerts effect through affecting the overall structure of the binding pocket. Among the EcRs cloned form 12 species, only three of them, namely *Bombyx, Choristoneura* and

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                                        I-Box
HvEcR  RPGLEQ PLLVEEIQRYYLNTLRVYILNQ NSASPRGAV IFGEILGILTEIRTLG
MsEcR  RPGLEQ PLLVEEIQRYYLKTLRVYILNQ HSASPRCAV LFGKILGVLTELRTLG
CfEcR  RPGLEQ PQLVEEIQRYYLNTLRIYILNQ LSGSARSSV IYGKILSILSELRTLG
BmEcR  RPGLEQ PSLVEEIQRYYLNTLRIYIINQ NSASSRCAV IYGRILSVLTELRTLG
LcEcR  RPGLEE AELVEAIQSYYIDTLRIYILNR HCGDPMSLV FFAKLLSILTELRTLG
CcEcR  RPGLEK AQLVEEIQSYYIDTLRVYIINR HCGDSMSLV FFAKLLSILTELRTLG
DmEcR  RPGLEK AQLVEAIQSYYIDTLRIYILNR HCGDSMSLV FYAKLLSILTELRTLG
AaEcR  RPGLEQ AELVEHIQSYYIDTLRIYILNR HAGDPKCSV IFAKLLSILTELRTLG
CtEcR  RPGLEK AEMVDIIQSYYTETLKVYIVNR HGGESRCSV QFAKLLGILTELRTMG
LmEcR  RPSLVE GWKVEKIQEIYLEALKAYVDNR R..RPKSGT IFAKLLSVLTELRTLG
TmEcR  RPSLIE GWKVEKIQEIYLEALRAYVDNR R..SPSRGT IFAKLLSVLTELRTLG
CpEcR  RPNLKE LKKVEKLQEIYLEALKSYVENR R..LPRSNM VFAKLLNILTELRTLG
AamEcR RPSLVD PHKVERIQEYYIETLRMYSENH R..PPGKN. YFARLLSILTELRTLG
             Helix 9                          Helix 10
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Fig. 11, I-box in EcR proteins. I-boxes of EcR Protein sequences from 13 arthropod species are aligned by GCG pileup. The putative I-box is indicated by a dashed line. Helices 9 and 10 are underlined. The critical residue defining ligand specificity is in bold italics.

Drosophila, have a Tyr at this position, whereas the other nine species contain a Phe (Chapter 1). It is likely that the heterodimerization is not exclusively dictated by this residue, as *Bombyx* EcR possesses stronger dimerization capacity than the DmEcR,

although both of them contain a Tyr at this position. If specific response to ecdysone is solely dictated by this residue, I can predict that EcRs from the other nine species would highly responsive to ecdysone.

Implications of EcR ligand specificity

I present evidence that ecdysone is a potent ligand for AaEcR, but not for DmEcR. To my knowledge, this is the first direct evidence addressing the ligand specificity of insect steroid hormone receptors. Among the EcRs cloned from twelve insect species, *Drosophila* EcR is only second to *Lucilia* EcR regarding LBD sequence similarity to AaEcR (Chapter 1). LBDs from *Drosophila* and *Aedes* EcRs contain 87.4% identity. Yet, there is apparent ligand specificity for receptors so closely related which suggests that it should be possible to design target-specific and environment-friendly pesticide. Bioassay has long been used to search for ecdysteroid analogs. Indeed, the nonsteroid agonist RH5849 was identified based on its effects on Kc cell morphological changes (Wing, 1988). More detailed studies led to the identification of RH5992 (tebufenozide), which is more potent to Lepidopteran than to dipteran insects, most likely due to its higher retention rate in Lepidopteran cells (Sundaram et al., 1998). RH5992 activated the *Drosophila* and *Aedes* receptor (Wang and Raikhel, unpublished data; Suhr et al., 1998), suggesting it is an effective agonist for dipteran receptor. Bioassays involves complexity due to ligand metabolism and they are technically difficult to compare the species specificity of ecdysteroids. Availability of cloned EcR from different species has brought insecticide research into a new era. Taking advantage of the cloned cDNA from *Aedes* and *Drosophila*, I discovered that these two receptors displayed distinct responses to

ecdystroids, with 20E more effective for DmEcR and ecdysone more effective for AaEcR. Whether ecdysone is an effective ligand for receptors from other species awaits further investigation. Apparently, C-20 hydroxylation plays a great impact on ligand efficacy. Drugs analogous to ecdysone but distinct from 20E may be utilized to target mosquito, the deadly disease vector.

My results also indicated that it is possible to optimize the EcR utilized for the Inducible-Gene-Expression system. Due to low toxicity of ecdysteroid and high inducibility of ecdysteroid receptor, EcR transactivation has been successfully utilized for controlled gene expression in mammalian cells and mouse. Because DmEcR has low heterodimerization capacity, transactivation in some cell lines including CV-1 can not be achieved without cotransfection of USP or RXR cDNA. Suhr et al (1998) demonstrated that Bombyx EcR has higher herodimerztion ability with endougenous RXR in CV-1 cells, enabling transactivation in these cells without exogenous RXR. I found the that AaEcR is a more efficient partner than USP. Whether it is more effective than BmEcR remains an open question. More importantly, I discovered that AaEcR possessed distinct ligand specificity compared with DmEcR. Although ecdysteroid has low toxicity in vertebrates, repeated administration of potent ecdysteroids including 20E cause a marked effect on red blood regeneration in phenylhydrazine induced anemia in rats (Syrov et al., 1997). Utilization of a less potent ligand like ecdysone may reduce the side effects. Thus receptors like AaEcR, which responds to ecdysone, may be indispensable for optimal implementation of the inducible system.

CHAPTER 5. SUMMARY AND FUTURE RESEARCH PROSPECTS

Ecdysteroid responsive elements in target genes.

In this dissertation, I have reported the extensive investigation of the interaction between mosquito EcR-USP with various DNA elements. AaEcR-AaUSP binds elements derived from the consensus half site AGGTCA arranged as either inverted repeats or direct repeats. One-base pair is the optimal spacer among inverted repeats and four-base pair the optimal among direct repeats. Transfection assays in CV-1 cells revealed that DNA binding activity is related to transactivation. Several groups have obtained similar results for the fruit-fly receptor DmEcR-DmUSP. Considering that EcR and USP protein from all the known arthropoda species possesses identical P-box EGcKg, I speculate that all the receptors would prefer similar half sites.

The one-bp spacer is critical in inverted-repeats. Changing the spacer length virtually abolishes binding activity for the IR^{hsp}-1 element. For the perfect palindromes, the stringency for spacer length requirement is reduced, yet IR^{per}-0 and IR^{per}-2 still display dramatically lower binding activity than IR^{per}-1. For the direct repeats, the spacer length is less stringent, with DR-3 to DR-5 displaying similar binding activity. In light of these discoveries, I expect that more EcREs to be identified as direct repeats with variable spacer length. Indeed, two direct-repeat elements, a DR-1 ($_{-333}$AGGCCAaTGGTCG$_{-321}$) and a DR-2 ($_{-422}$GGGTCGttAGGTCA$_{-435}$) elements have been identified from the mosquito ecdysteroid responsive genes Vg and VCP, respectively. These elements bind to proteins in nuclear extracts as well as *in vitro* produced EcR-USP proteins (Martin and

Raikhel, unpublished data). In addition, a DR-3 ($_{288}$AGTTCAttcAAGTCA$_{312}$) and an IR-1 ($_{2649}$AGATCAcTGACTT$_{2661}$) elements are located in the coding region of the Vg gene. The functionality of these elements can be tested by transactivation assays utilizing mammalian or *Drosophila* cell lines. However, organismal transformation has only been well developed for *Drosophila*. Without suitable mosquito cell lines, it would be challenging to prove the *in vivo* relevance of the EcREs.

To verify the *in vivo* functionality of these EcREs in insects, I propose a strategy termed chromatin immunoprecipitation, which has been successfully utilized to investigate the direct interaction of a transcription factor with its chromatin binding site (Bigler, et al., 1994) . In this experiment, isolated nuclei from the mosquito fat body are treated with restriction enzyme and then immunoprecipitated with anti-EcR or anti-USP antibodies. The EcRE in the protein-DNA complex is then purified, cloned and subjected to sequencing. For known responsive genes like Vg and VCP, primers can be designed for PCR amplification to check specific target sites. Moreover, this technique would be employed to identify EcREs in unknown responsive genes.

Transactivation and cofactors.

I showed that AaEcR-AaUSP transactivated a reporter gene in CV-1 and S2 cells. Similar transactivation activity has been reported for receptor from other species including *Drosophila, Lucilia, Bombyx and Amblyomma*. Nuclear receptors regulate gene expression at the transcription level by modulating initiation process at the target promoter (Simons, 1998). They can interact directly with initiation factors. For example, AF-1 and AF-2 in ER and AF-2 in RXR interact TBP *in vitro* while ER, PR, COUP-TF

and VDR interact with TFIIB (Reviewed by Bagchi, 1997). A variety of cofactors including coactivator and corepressor, which bridge the interaction between nuclear receptor and transcription machinery, have been identified by yeast two-hybrid assay or far-western cloning techniques. Coactivators facilitate transcriptional activation while corepressors enhance repression. Recently, it was shown that many of these coactivators including SRC-1, CBP/P300, P/CAF and ACTR possess intrinsic histone acetyl transferase activity (Chen et al., 1997; Jenster et al., 1997). Reciprocally, corepressors interact with histone deacetylase (mSin3A, Nagy et al., 1997). Therefore, the whole scenario of nuclear receptor turns to be in one hand the nuclear receptor recruit basal transcription machinery by interacting with initiation factors like TBP and TFIIB while in the mean time call up the histone acetylase to modify histones so that chromatin can be loosen up for the basal machinery to settle down to initiate transcription.

Identification of these cofactors is essential to understand the mechanism of steroid receptor activated transcription in insects. None of these cofactors has been reported in mosquito. Two strategies can be employed to isolate these cofactor homologues: homology based PCR cloning and yeast two-hybrid assay. Since insect EcR-USP function in mammalian cells, it is likely that the insect receptor interacts with homologous cofactors. Accordingly, PCR primers based on conserved regions can be utilized to amplify their homologue from insects. Alternatively, yeast two hybrid cloning would be a valuable assay to clone these cofactors. The AF-2 core located at helix 12 is an ideal bait. Yet, the functionality of this AF-2 region must first be confirmed by domain mapping techniques.

EcR and USP isoforms have been isolated from several insect species including the mosquito *A. aegypti*. The deduced protein sequences differ in their N-terminal A/B domain (AF-1), suggesting they may contain different transactivation domains. The yeast two hybrid assay can be utilized to identify the putative isoform specific cofactors.

Ligand specificity and application.

Ecdysone has long been postulated as a prohormone. I demonstrated for the first time that this steroid serves as a functional ligand for the EcR-USP receptor. Intriguingly, ecdysone functions much more potently for the *Aedes* EcR receptor than for the *Drsophila* EcR receptor. The responsiveness to AaEcR is tightly associated with its higher basal level of heterodimerization. Domain swap techniques have allowed me to narrow down the ligand specific region to the I-box. Using site directed mutagenesis, I identified a single amino acid, a Phe in AaEcR versus a Tyr in DmEcR, which is most critical for conferring ligand binding specificity and basal level of heterodimarization.

Among the EcR protein sequences cloned from 13 arthropoda species, only three of them namely DmEcR, CfEcR and BmEcR contain a Tyr whereas the other proteins contain a Phe at this critical site. It would be interesting to test the responsiveness of other receptors to ecdysone to see whether this critical site determines ligand specificity in other receptors.

LBD in AaEcR in 87.4% identical to that in DmEcR, yet conspicuous ligand specificity exist for such highly related receptors, suggesting that it is possible to identify species specific ligand. Various techniques including whole animal bioassay, organ and cell culture have been utilized to test ecdysteroid activity and the ecdysteroid agonists and

antagonists have been identified using morphological response of *Drosophila* embryonic cell lines (Wing, 1988; Dinan et al., 1997). Yet it is difficult to compare the ecdysteroid activity for different species using the previous techniques. With the cDNA encoding EcR proteins from various arthropodes available, the EMSA and transfection assays utilized in my experiment can be easily scaled up to test numerous compounds to identify species specific ligand. In fact, transfection assay based screening has led to the identification of ligand for many mammalian orphan nuclear receptors including LXR, SF-1 (Janowski et al., 1996; Lala et al., 1997).

There are two immediate practical application for species-specific ecdysteroids. First, it will facilitate designing target-specific and environment friendly insecticides. Direct utilization of ecdysteroid as insecticides has not been successful due to its poor stability and lack of species specificity. Stable and target specific insecticides can be designed based on species specific ecdysteroid. Secondly, ligand specificity research would help to optimize the ligand and receptor required for the ecdysteroid-inducible system. Due its high inducibility, this system is most promising for regulated expression in human gene therapy. Recent results indicated that repeated exposure to potent ligand like 20-hydroxyecdysone causes anemia in rats and less potent ligand such ecdysone have no discernible site effects (Syrov et al., 1997). Therefore, receptors responsive to ecdysone including AaEcR would be indispensable for successful utilization of this inducible system.

JHR, the receptor for the other lipophilic hormone JH (juvenile hormone)

Insect development and reproduction are orchestrated by two lipophilic hormones, ecdysteroid and JH. During the past decade, rapid progress has been achieved to characterize the mechanism of ecdysteeroid regulated gene expression (Thummel, 1997). Yet the molecular mechanism of JH action remains enigmatic as the JHR has not been identified in insect. Ironically, a nuclear receptor responsive to JH has been cloned in vertebrate rat, farnesoid X activated receptor (FXR). When the expression vectors containing FXR and RXR cDNAs are cotransfected into CV-1 cells with a reporter plasmid harboring the EcRE IR^{hsp}-1, the reporter gene is activated upon treatment with farnesoids, among which JH is the most potent ligand (Forman et al., 1995). However, it is not known whether JH *per se* activated the FXR-RXR heterodimer or its metabolites exerts the function. This question can be easily answered by direct ligand binding assay as the radioactive JH is commercially available. Alternatively, gel shift assays can be performed to see whether JH could influence FXR-RXR DNA binding activity since nuclear receptor DNA binding activity is affected by the ligand.

Identification of JHR is essential to understand the mode of JH action. My results indicated that JH analog methoprene exerted neither agonistic nor antagonistic effect on EcR-USP. The functional JHR is most likely a heterodimer consisting USP and the insect FXR homologue. As FXR is highly related to EcR, homology based PCR cloning would be problematic. The efficient strategy to identify insect FXR homologue would be yeast two-hybrid screening using the USP protein as the bait and the prey cDNA obtained from a stage and tissue with high JH titer like the female mosquito fat body at two-day post eclosion.

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