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The molting hormone, 20-hydroxyecdysone (20E), orchestrates *Drosophila* development through the actions of the ecdysteroid receptor, which is a heterodimer of two members of the nuclear hormone receptor superfamily, EcR and Ultraspiracle (USP). The EcR/USP heterodimer mediates ecdysteroid response by inducing or repressing the expression of target genes. In this study a chimeric USP whose ligand binding domain (LBD) was replaced by that of a *Chironomus* LBD was used to test ecdysteroid responsiveness *in vitro* and *in vivo.* This was done to determine if the late larval lethality observed in *d/cusp* mutant flies is a result of the EcR/*d/c*USP heterodimer's inability to mediate 20E response. The chimeric USP had transcriptional capabilities comparable to those of *Drosophila* USP with all three *Dm*EcR isoforms *in vitro*, while the ΔDBD versions of the chimeric USP and *Ct*USP constructs did not show the 20E-inducibility with *Dm*EcRB1 that is seen with *Dm*USP ΔDBD (Beatty *et al.*, 2006). RT-PCR was used to test the ability of EcR/*d/c*USP to induce the expression of the 20E-regulated genes, E74A, E74B and BRC-Z1 in larval salivary glands. The expression level of these genes in mutant salivary glands was comparable to the levels seen in salivary glands extracted from wild-type animals. These results suggest the chimeric USP phenotype is not the result of impairment of 20E-inducibility, and that USP may have some function outside its classically understood role as the heterodimeric partner of EcR.

FUNCTIONAL ANALYSIS OF A *DROSOPHILA/ CHIRONOMOUS* ULTRASPIRACLE CHIMERA TO EXAMINE THE ROLE OF USP IN ECDYSTEROID-INDUCIBLE GENE EXPRESSION

By

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Approved by

_____
Committee Chair

To my parents, Bill and Karen Clement, for their limitless support

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Background

Steroid hormones initiate changes in gene transcription that lead to cellular changes and ultimately, changes in whole organisms. Many of these responses occur via nuclear hormone receptors. In *Drosophila*, 20-hydroxyecdysone (20E) orchestrates these complex physiological changes in distinct phases of development through the actions of the ecdysteroid receptor. The molting hormone, 20E, is produced from its precursor, ecdysone, which is the primary steroid product of the ring gland. The functional ecdysteroid receptor is a heterodimer of EcR and Ultraspiracle (USP), which responds to the varying levels of 20E in developing *Drosophila* by inducing or repressing the expression of target genes (Yao *et al*., 1993).

Nuclear hormone receptor structure

EcR and USP are both members of the nuclear hormone receptor superfamily and as such contain distinct functional domains (designated A through F). The A/B domain is the N-terminal region and is variable in length among different nuclear receptors and among isoforms of the same receptor. This domain interacts with proteins involved in transcription and is capable of some ligand independent transcriptional capabilities (activation function-1; AF-1). It has also been shown to have an effect on ligand dependent activation function (AF-2). For instance, a receptor with multiple isoforms that

differ only in its N-terminal domain can show different levels of response to hormone (Beatty *et al*., 2006, Hu *et al*., 2003). The C domain or DNA binding domain (DBD) is a highly conserved region that contains two cysteine-cysteine zinc fingers. This domain defines a protein as a member of the nuclear receptor superfamily and is responsible for recognition of a specific hormone response element (HRE). The D domain or hinge region has been implicated in nuclear localization and response element recognition. The E domain or ligand binding domain (LBD) has a ligand dependent activation function (AF-2) and dimerization interface with other nuclear receptors. The LBD is composed of twelve α-helices that form a ligand binding pocket. Some nuclear receptors have C-terminal F domains, but they are variable and it is unclear what their function is. Loss of this region in *Dm*EcR has no effect on transcriptional capability (Hu *et al*.,2003). The EcR/USP heterodimer is stabilized by 20E allowing it to bind to ecdysone response elements (EcREs) within the promoter regions of genes. It has the highest affinity for a palindromic inverted repeat sequences separated by a single nucleotide characterized by the hsp27 EcRE, but it can also recognize direct repeats separated by one to five nucleotides (Vogtli *et al*., 1998).

Genetic response to 20E

Ecdysone concentrations fluctuate throughout development with the first peak occurring during embryogenesis. This is followed by smaller peaks at the first and second larval instars with only moderate variation during the third instar (Richards 1981, Handler 1982). There is a high peak prior to pupariation and the largest pulse of ecdysone, which is converted to 20E, occurs during metamorphosis to the adult stage

(Andres *et al*., 1993). These peaks trigger signal cascades that reorganize tissues and various concentrations of 20E are associated with different sets of genes. Ashburner's observations of polytene chromosomes in larval salivary glands of *Drosophila melanogaster* led him to develop a model to explain the capability of 20E to induce such complex and varied genetic responses during development (Ashburner, 1974). He suggested that 20E induces the expression of a set of early genes that begin to regress after approximately three hours. At this point a larger set of late genes is induced. Based on *in vitro* studies of salivary glands treated with 20E and the protein synthesis inhibitor cyclohexamide, Ashburner showed that early gene expression was not affected, but that blockage of protein synthesis prevents late gene expression and the normal regression of early genes. This indicated that the protein products of early genes were regulating the expression of late genes. It also showed that the products of at least some of the 20E-inducible genes were repressing the expression of early genes. When 20E was removed from the cultured glands there was an immediate regression in early gene expression while a subset of late genes were prematurely induced. This revealed another aspect of 20E regulated gene expression through the repression of certain late genes.

Another form of temporal regulation can be seen in the structure of the early genes E74, E75 and Broad-Complex (BR-C) all three of which encode DNA binding proteins. These genes are much longer than an average gene (>50kb), and all encode multiple isoforms through differential splicing and/or alternate 20E inducible promoters within the gene (Karim and Thummel, 1991). The alternate promoters within these early genes respond to different concentrations of 20E, which introduces another level of

regulation in response to 20E (Figure 1). Based on this sensitivity the early gene transcripts can be divided into two classes; class I transcripts that respond to low doses of 20E (~2 x 10^{-9} M) and class II that respond to higher concentrations (~1 x 10^{-8} M) (Karim and Thummel, 1992). Class I mRNAs such as E74B and EcR are also defined by their regression as 20E titers increase, while E74A, E75A and E75B of Class II do not regress with additional 20E. BR-C, the largest of the early genes (>100kb), expresses four main isoforms (Z1-Z4) with a total of 12 transcripts or more (Dibello *et al*., 1991). It has a complex expression pattern responding to a wide range of 20E concentrations that could indicate both Class I and Class II promoters within the gene (Karim and Thummel, 1992). In the salivary gland, BRC-Z1 is predominantly expressed and is necessary for glue gene production and the normal degeneration of the salivary gland prior to metamorphosis (Restifo and White, 1992; Costantino *et al*., 2008).

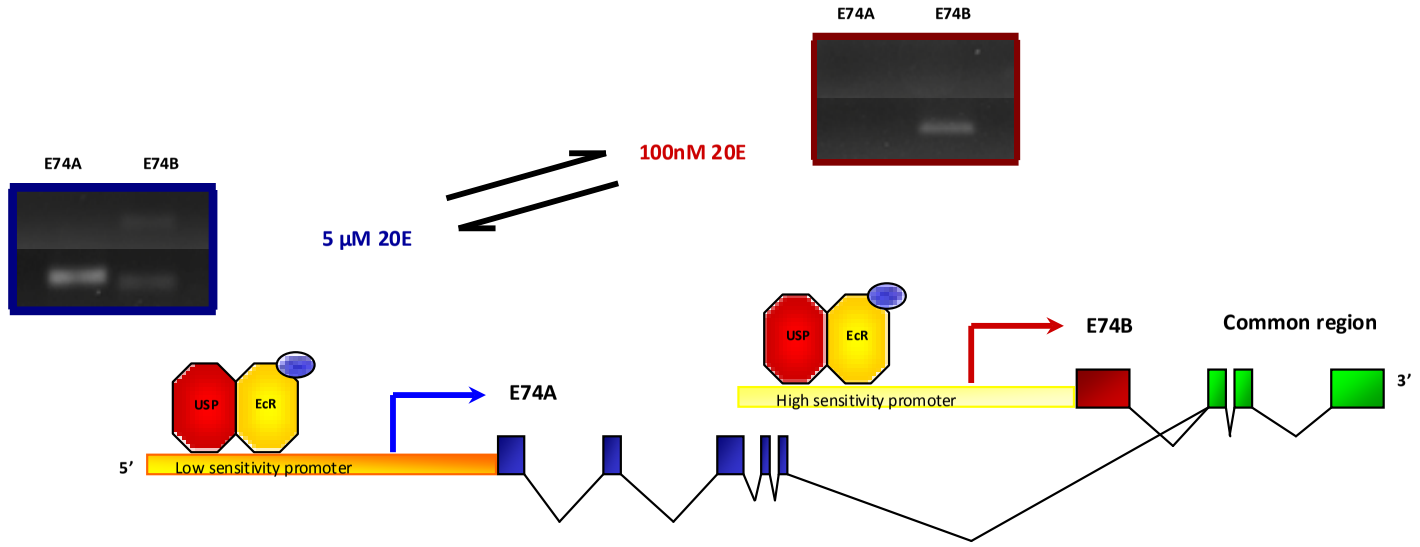


Figure 1. E74 gene schematic illustrating that alternate promoters within this gene respond to different 20E titers. The high sensitivity promoter (yellow bar) initiates the transcription of E74B at low doses while the low sensitivity promoter (orange bar) activates the transcription of E74A at higher doses. Inserts of agarose gel pictures show products of rt-PCR from the total RNA extracted from paired salivary glands of a wild-type animal. One of each pair was left untreated while the second was incubated with either 100nM or 5µM 20E. The low dose insert shows increased expression levels of E74B with 100nM 20E with no discernable E74A expression. The high dose insert shows the ecdysone-inducibility of E74A at 5µM 20E, while E74B expression levels remain the same as, or are lower than those seen in the untreated gland.

As mentioned above, EcR is the product of an early gene and begins to accumulate at low doses of ecdysone. It is one of two *Drosophila* nuclear hormone receptors that has a known ligand and three isoforms exist (A, B1 and B2) that differ only in their N-terminal domain. EcR A and the B isoforms arise through different promoters, while B1 and B2 are generated through differential splicing (Talbot *et al*., 1993). *In vivo* studies with mutant EcR isoforms have shown that different phenotypes result from mutations in specific isoforms indicating tissue and stage specific functions of EcR A, B1 and B2 (Bender *et al*., 1997). EcRA expression predominates in tissues that develop through metamorphosis and become part of adult structures such as the imaginal discs and prothoracic gland cells. EcRB1 tends to be expressed in greater concentrations in larval tissues that degenerate during metamorphosis. *In vitro* studies have shown that EcRA may have an inhibitory function in its A/B domain that is not found in EcRB1 or EcRB2 (Mouillet *et al*., 2001).

USP in development

USP is necessary for multiple stages of development including embryogenesis, larval development and pupation and seems to play a distinct role in morphogenesis (Hall and Thummel 1998, Henrich *et al*., 2000), though its expression varies only modestly throughout development. This variability in function could be attributed to variant interactions between USP and multiple EcR isoforms or the ability of USP to form heterodimers with other nuclear receptor partners such as DHR38 and Sevenup (SVP) (Zehlhof *et al*., 1995, Sutherland *et al*., 1995, Baker *et al*., 2003).

USP has been classified as an orphan receptor because it has no undisputed ligand, although recently it has been proposed as the receptor for methyl farnesoate (Jones *et al*., 2006). Methyl farnesoate is one of three hormones produced by the ring gland (including methyl epoxyfarnesoate and juvenile hormone III bis-epoxide) involved in regulating development, and mutant animals in which farnesoid synthesis is blocked with RNAi shows some phenotypic similarities to USP mutant animals (Jones *et al*., 2009).
USP has a large ligand-binding cavity caused by a loop between helices 1 and 3 that is atypical of both nuclear receptor LBDs in general and USP in other insects. This loop also affects the conformation of the USP LBD by holding Helix 12 in a position that resembles a nuclear receptor bound to an antagonist (Clayton *et al*., 2001). A phospholipid is found within the ligand binding pocket of USP after crystallization of USP. It partially fills the ligand cavity and contributes to this inactive conformation (Billas *et al*., 2001). When Helix 12 is removed, the EcR/USP heterodimer is no longer able to bind DNA or activate transcription, and it interferes with EcR's ability to bind hormone (Przibilla *et al*., 2004). Recently it has been shown that 20E can induce a transcriptional response in the salivary glands of mid-third instar larvae in the absence of USP, although it is not certain whether EcR is forming homodimers or heterodimers with another receptor (Costantino *et al*., 2008).

Not only is USP required for the normal activation of most 20E-inducible genes in the presence of hormone, it is also necessary for the repression of certain genes in the absence of 20E. In differentiating cells, such as sensory neurons and eye discs, it has been shown that the absence of USP causes the premature expression of BRC-Z1 which

normally appears at mid-third instar. The development of these mutant usp tissues resembles that of normal imaginal tissues treated with 20E, suggesting that the normal function of USP is repressive in these cells at this time (Schubiger and Truman, 2000; Gheish and McKeown, 2002; Schubiger *et al.*, 2005).

Drosophila/Chironomus chimeric USP

The midge (*Chironomus tentans*), like Drosophila, is a Dipteran and shares a similar pattern of development with an additional larval-larval molt before metamorphosis. The ecdysteroid receptor in *Chironomus tentans* is also composed of EcR and USP, but unlike *Drosophila*, midges express only one EcR isoform with two USP forms that differ in their N-terminal region. The *Ct*usp gene has been localized to a late ecdysone-induced puff in the *Chironomus* salivary gland suggesting a single gene that is alternately spliced into *Ct*USP-1 or 2 (Vögtli *et al*., 1999). The DBD of *Ct*USP shares almost 100% similarity to *Dm*USP. The LBD is less highly conserved but nonetheless shows much sequence similarity through the first 11 helices. The major differences lie in the two glycine rich regions that appear only in *Drosophila* USP before the first helix and in between helices 5 and 6. Helix 12 shows almost no similarity between the two USP sequences, except for a glutamic acid in the middle of the helix. This acidic residue has been implicated in AF-2 function in the USP homolog RXR (Westin *et al*., 1998; Vogtli *et al*., 1999).

The *Ct*EcR isoform has been shown to be inactive or to have repressive function in cell culture similar to that of *Dm*EcRA. Both *Ct*USP forms appear to heterodimerize equivalently with *Ct*EcR and there is no apparent difference in their ability to bind DNA.

Both forms with *Ct*EcR are also able to bind ligand (ponasterone A) with *Ct*USP-2 showing 2.5 fold higher affinity, but unlike *Dm*EcR, *Ct*EcR in the absence of *Ct*USP was unable to bind detectably to ligand. The *Chironomus* ecdysone receptor was also tested with non-steroidal ecdysone agonists (Smagghe *et al*., 2002). These studies showed that the *Ct*USP/*Ct*EcR complex has greater affinity to these agonists than the *Drosophila* ecdysone receptor.

An ultraspiracle gene was cloned into a fly transformation vector that contained the *Drosophila* 5' untranslated region (UTR) and DNA-binding domain with a repeat polylinker region connected to the *Chironomus* ligand-binding domain (Henrich *et al*., 2000). By exchanging the LBDs of two related species, a chimeric 'mutation' is created and specific functions can be investigated within the LBD. The T-box is an element of 10 amino acids downstream from the second zinc finger and depending on the source, can be considered part of the DBD or hinge (D) domain. It has been implicated in response element recognition and contact with the minor groove of the DNA helix. The T-box amino acid sequence for *Drosophila* and *Chironomous* USP is identical (KREAVQEERQRG), and an unanticipated additional 'mutation' arose during the cloning process that led to a duplication of part of the T-box (EAVQEER) and a polylinker sequence (RSP) between them.

This chimeric USP was cloned into a transformation vector (pMVZ18; Figure 3), flies were transformed with the chimeric USP (*d/c*USP), and the transgene tested in larval rescue studies with usp^2, usp^3 and usp^4 mutant fly lines. These <u>*usp*</u> mutations contain amino acid substitutions in the DBD and all cause early larval death (Oro *et al*., 1990,

Henrich *et al*., 1994). In a wild-type background the chimeric USP does not affect development, which means that it is not interfering with normal USP activity. A single dose of *d/c*USP is able to rescue *usp* mutants through larval development until a sudden arrest occurs at the late third instar. Prior to this arrest, the full-sized larvae failed to wander off the food and become motionless. Multiple doses of the chimeric USP are able to rescue some lines through adulthood, but in all cases the mutant larvae fail to contract, do not evert their anterior spiracles, and have incomplete cuticular tanning.

To rule out the possibility that the chimeric USP phenotype was the result of an unrelated issue, follow up experiments were performed to further characterize the basis for the *d/c*USP lethality. Western blots showed chimeric USP protein levels comparable to those of wild-type, so the phenotype is not caused by low USP expression. Electrophoretic mobility shift assays (EMSAs) were used to test the chimera's ability to interact with *Drosophila* EcR (A and B1) and a palindromic and a direct repeat response element (DR3). These results indicate that *d/c*USP and wild-type USP have very similar behavior when tested with DmEcR A and B1 and an ecdysone response element. Finally, ligand-binding studies were done with ponasterone A to compare the affinity of heterodimers of the chimeric USP, wild-type USP and *Chironomus* USP with *Dm*EcR B1 for this ligand. All of the heterodimers appear to have a similar affinity to ponasterone A, suggesting the chimeric USP does not interfere with the receptor's ability to mediate ecdysteroid response.

The phenotypes resulting from the expression of a chimeric USP in *usp* mutant flies were very similar to those found when *usp* mutants were rescued through the early

larval lethal stage with the addition of *usp* fused to a heat-inducible promoter, and results in larvae that lack USP at metamorphosis (Hall and Thummel, 1998). As noted with the chimeric USP, the larvae failed to wander off their food and experienced a 'stationary stage' prior to arrest. There was a failure to evert anterior spiracles and abnormalities in cuticle development, as well as incomplete prepupal contraction. Interestingly, these USP mutant phenotypes are comparable to those found with EcRB mutant flies which fail to wander normally and contract, and also reach a lethal stationary phase. EcRB1 mutants at this stage show an abnormal response to ecdysone affecting the transcription of some early genes such as BR-C, E74 and E75 (Bender *et al*., 1997). It was also shown that these genes are not being expressed at the late third instar ecdysone peak in the mutants lacking USP altogether (Hall and Thummel, 1998).

In summary, chimeric USP mutant larvae have a phenotype similar to that seen with USP null mutants as well as EcRB mutant animals. While the chimera is able to form a functional heterodimer with EcR, interact with an ecdysone response element, and respond to the ecdysone agonist ponasterone A, the chimeric USP lacks some vital function that would enable it to develop through metamorphosis. USP null mutants showed a normal ecdysone inducible response during early to mid-third instar when ecdysone levels are low. However, both USP null and EcRB mutants are incapable of initiating the genetic hierarchy that is normally induced by the increasing levels of ecdysone just prior to pupariation.

Specific aims of this project

The chimeric USP is capable of performing the functions necessary for development through the larval stages when ecdysteroid titers are low. Based on the phenotypic similarity caused by EcR mutations, the dissipation of USP in the late third instar, and the functional failure of the chimeric USP in the late third instar- it is plausible to hypothesize that the chimeric USP, like the other mutants, is unable to process 20E-induced transcriptional changes in the late third instar. On the other hand, a *Dm*EcRB1/*d/c*USP heterodimer can be seen on EMSA that interacts with an EcRE, suggesting that the metamorphic failure involves a different and unknown failure of the *d/c*USP. Therefore, the experiments described here are intended to determine the capabilities of the chimeric USP for regulating ecdysteroid-inducible gene transcription.

The first aim of this project has been to use a mammalian cell culture system to measure the transcriptional capabilities and differences in activity of the original chimera and a new chimera constructed to remove the repeat/polylinker region, along with a wild-type *Dm*USP when each is tested with *Dm*EcRA, B1 and B2. Comparisons have also been made between *Drosophila* and *Chironomus* USP with all three *Dm*EcR isoforms. ΔDBD versions of each clone were constructed to determine if there is any isoform specific activity such as that shown by *Dm*USPΔDBD with *Dm*EcRB1 (Beatty *et al*., 2006).

The second aim will be to examine the chimeric USP *in vivo* in a USP mutant background during the late third instar to determine the effect, if any, on transcriptional response during third instar leading up to the arrest at late third instar. Cultured salivary

glands from mutants expressing the chimeric USP will be used to test the chimeric USP's ability to respond to low and high doses of ecdysone as well as any possible impact on the normal repressive function of USP. Preliminary results from the cell culture study indicated that the old chimera is able to mediate a transcriptional response to ecdysteroids, though its repressive capabilities cannot be measured within the cell culture system used.

CHAPTER II

MATERIALS AND METHODS

Construction of vectors

The vectors which have been constructed for this project are shown in Figures 2 and 3. The USP clones used in cell culture were constructed by sub-cloning the desired *Drosophila melanogaster*, *Chironomous tentans*, or chimeric inserts into a modified pVP16 vector (Clontech). The first restriction site in the multiple cloning site of pVP16 (EcoRI) was changed to a PstI site using site directed mutagenesis. The PstI site was inserted at the second codon of the MCS using the following primer and its reverse complement: 5'-GTA CGG TGG GGA ACT GCA GGG GAT CCG TCG AC -3' (underlined basepairs indicate mutations introduced). This left 9 nucleotides between the pVP16 AD and each insert, which were later removed with deletion mutagenesis after each insert was verified.

VP16-*Dm*USPF2 and VP16-*Dm*USPF3 (Beatty, *et al*. 2006) also underwent deletion mutagenesis to remove this stuffer region using the forward primer (*d/c*USP del F) 5' CGA GTA CGG TGG GTG CTC TAT TTG CG 3' and it's reverse complement, and the forward primer (*Dm*USPIII delF) 5' GAG TAC GGT GGG AAG CGC GAA GC 3' with its reverse complement. These changes to pVP16 allowed a direct transition from the activation domain to the DNA or ligand binding domain of each USP construct. All clones were made by isolating the region of interest with PCR amplification inserting it

into pVP16 with a PstI restriction site at the 5' end and a HindIII restriction site at the 3' end for unidirectional orientation of each insert.

VP16-*Ct*USPII and *Ct*USPIII

The VP16-*Ct*USPII clone includes the *Chironomous* DNA binding domain (DBD), hinge region and ligand binding domain (LBD) from amino acids 197-553. The region was isolated from FpGEX-*Ct*USP with PCR using a forward primer (*Ct*USPII PstI DBD F) tagged with a PstI site 5'TTTT CTGCAG TGT TCT ATA TGT GGT GAT CGG GCT AG 3' and a reverse primer (*Ct*USPII HindIII 3' R) 5' CGG TGG TGG TGG TGG AAT 3'. VP16-*Ct*USPIII lacks the *Ct*USP DBD coding only the hinge region and LBD (amino acids 263-553). The insert was generated using a forward primer (VP16-*Ct*USPIII F) tagged with PstI 5' TTTT CTGCAG AAG CGC GAA GCT GTG CAG GAA GAG 3' and the reverse primer (New *Ct*USP 3' R) 5' GGG TCG ACT CGA GCT GAA GCT TAA GGA TC 3'. After an initial melting step of 10 seconds at 98°C to denature the template DNA, the following temperature cycles were repeated 29 times: 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 2 minutes (*Ct*USPII) or 1 minute (*Ct*USPIII).

VP16-*d/c*USPII, and VP16-*d/c*USPIII

The insert for d/cUSP in pVP16 was isolated from pC*d/cusp*eR pMVZ18 (Vogtli, M.) using the same methods that were used for the *Chironomous* clones. VP16-*d/c*USPII includes the *Dm*USP DBD along with a portion of the T-box (amino acids 104-178), a polylinker of the sequences 5' CGA TCC CCC 3', and an incomplete section of the

*Ct*USP T-box and LBD from amino acids 266-553. A forward primer (new VP16-PMVZ18 F) tagged with PstI 5' TTTT CTGCAG TGC TCT ATT TGC GGG GAT CGG G 3' and a reverse primer (*Ct*USP3' HindIII R) tagged with HindIII 5' TTTT AAGCTT GCT TGC TGC TAC TGT CCA TCT TAA CCA TC 3' were used to generate this insert. VP16-*d/c*USPIII is the same as *d/c*USPII with the *Dm*USP DBD removed; as such it encodes the short portion of the *Dm*USP T-box (amino acids 170-178), the polylinker, and the *Ct*USP LBD region from amino acids 266-553. The primers for *d/c*USPIII were designed to remove the desired region from VP16-*d/c*USPII rather than pC*d/cusp*eR pMVZ18. The forward primer (PMVZ18III newF) 5' TTTT CTGCAG AAG CGC GAA GCG GTC CAG 3' was tagged with PstI while the reverse primer (PMVZ18III R) 5' CCT CTA CAA ATG TGG TAT GGC TG 3' incorporated the HindIII site introduced in the construction of pMVZ18II. The PCR cycling conditions for ExTaq HS (Takara) were as follows: an initial 10 second denaturation step of 98°C followed by 29 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 2 minutes (*d/c*USPII) or 1 minute (*d/c*USPIII).

The PCR products from *Ct*USPII and III, *d/c*USPII and III, and pVP16 were double digested with PstI and HindIII restriction enzymes to produce 5' and 3' sticky ends. The double digest reactions were incubated at 65°C for 10 minutes to denature the enzymes and then electrophoresed next to a 1kb+ DNA ladder on a 1% agarose gel treated with 1X ethidium bromide. The correct sized bands were excised from the gel and purified with the QiaexII gel extraction kit (Qiagen). Each insert was ligated into pVP16 with T4 DNA ligase (New England Biolabs) at a 3:1 ratio overnight at room temperature.

The ligated clones were then transformed into 45µl of Ultracompetent XL10-Gold cells (Stratagene). The transformed cells were plated onto Luria-Bertani agarose plates inoculated with ampicillin at 50µg/ml. Transformed *E. coli* have ampicillin resistance and colonies form after 16 hours incubation at 37°C. Single colonies were selected and grown in 5ml of LB liquid media with 50µg/ml ampicillin for 8 hours at 37°C with shaking at 250 RPM. The plasmid DNA was purified using Qiagen mini-preps, concentrations determined using a spectrophotometer (Eppendorf) and diagnostic digests were preformed with PstI and HindIII. The clones containing the correct size bands were sequenced using an Amersham MegaBACE and verified by alignment with *Dm*USP, *Ct*USP or pMVZ18 reference sequences. When the insert was verified, deletion mutagenesis was performed to remove the 9 base pair region between the VP16 AD and each insert. The forward primer (delctUSPII F) 5' CGA GTC CGG TGG GTG TTC TAT ATG TGG TG 3' and its reverse complement were used on VP16-*Ct*USPII. The same primers that were used to remove the stuffer region from VP16-*Dm*USPF3 were used on VP16-*Ct*USPIII and VP16-*d/c*USPIII, and the same primers used on VP16-*Dm*USPII were used on VP16-*d/c*USPII. Clones that showed matching sequences with no random mutations, insertions or deletions were then used in cell culture experiments.

VP16-*Dm/Ct*USP (new)

The new chimera was constructed to have a clean fusion of the *Dm*USP DBD (amino acids 104-169) to the *Ct*USP hinge region and LBD (amino acids 263-553). The *Dm*USP DBD was isolated from VP16-dUSPF2 (Beatty, *et al*. 2006) using a forward

primer (*Dm*DBD PstI F) with a 5' PstI tag 5' TTTT CTGCAG GCA GTG CTC TAT TTG CGG GGA TCG G 3' and a blunt end reverse primer (*Dm*DBD bluntR) 5' CAT GCC GCA GGT TAG GCA CTT C 3'. The *Ct*USP LBD was generated using a blunt end forward primer (*Ct*LBD blunt F) 5' AAG CGC GAA GCT GTG CAG GAA GAG AG 3' and the reverse primer (ctUSP HindIII R) used with VP16-*Ct*USPII and *Ct*USPIII. The PCR conditions using Primestar polymerase for *Dm*DBD were as stated above with a 30 second extension time for *Dm*DBD and a 2 minute extension time for *Ct*LBD. The two PCR products were gel purified using the protocol elucidated above and a blunt end ligation was performed. The ligation products were electrophoresed on a 1% agarose gel with 1X ethidium bromide after incubating overnight at room temperature. The appropriate band was gel purified and then amplified using PCR with the forward primer used in the *Dm*DBD isolation and the reverse primer used in the *Ct*LBD isolation. The *Dm/Ct* USP insert was digested with PstI and HindIII and the same ligation and transformation procedure was followed as stated above with the VP16-*Ct*USP and *d/c*USP clones.

Following sequence verification the VP16-*Dm/Ct*USP clone was found to have 2 missing base pairs between the *Dm*DBD and *Ct*LBD. Rather than repeating the procedure above, insertion mutagenesis primers were designed to reinsert the missing base pairs. The following forward primer and its reverse complement were used (*d/c*USP4 insertionF/R) 5' CTA ACC TGC GGC ATG AAG CGC GAA GCT 3' with the underlined region indicating the base pairs inserted. After sequencing to verify the insertion mutagenesis was successful and no other random mutations had occurred,

deletion mutagenesis was performed to remove the 9 base pair stuffer region between the VP16 AD and the *Dm/Ct*USP insert with the following primer and its reverse compliment: (del *d/c*USP F/R) 5’ CGA GTA CGG TGG GTG CTC TAT TTG CG 3’. Again the clone was sequenced to verify that the stuffer region had been removed with no other mutations introduced.

New chimera in fly vector

See Appendix A

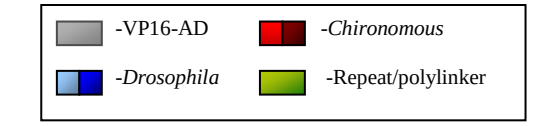
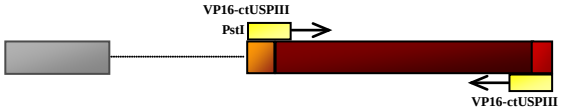
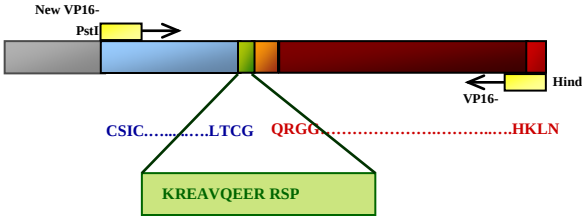
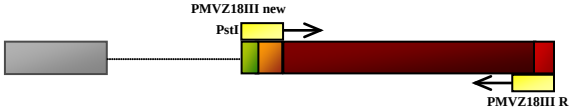
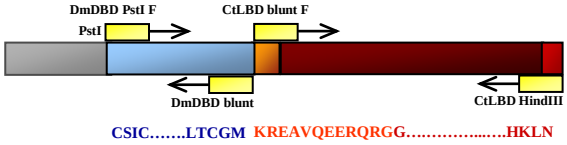
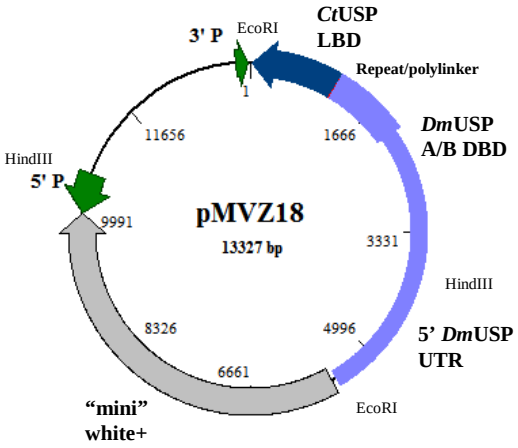
Clone	CONTENT	
pVP16-*Dm*USPII (Beatty *et al.*, 2006)	VP16-AD domain *Drosophila* C/D/E domains	
pVP16-*Dm*USPIII (Beatty *et al.*, 2006)	VP16-AD domain *Drosophila* D/E domains	
pVP16-*Ct*USPII	VP16-AD domain *Chironomus* C/D/E domains	
pVP16-*Ct*USPIII	VP16-AD domain *Chironomus* D/E domains	
pVP16-*d/c*USPII (old)	VP16-AD domain *Drosophila* C domain Repeat polylinker region *Chironomus* E domain	
pVP16-*d/c*USPIII (old)	VP16-AD domain Repeat polylinker region *Chironomus* E domain	
pVP16-*Dm/Ct*USPII (new)	VP16-AD domain *Drosophila* C/D domains *Chironomus* E domain	

Figure 2. Schematic diagrams of the USP inserts for cell culture constructs in the VP16 vector. VP16-*Dm*USPII and III from Beatty *et al.*,2006. Shades of blue represent a *Drosophila* domain, while shades of red represent *Chiromonus*. A dashed line indicates a USPIII construct which does not contain a DBD. Yellow boxes illustrate the position of primers used to amplify each region.

pMVZ18
(from Henrich *et al.*, 2000)

pCasper4 vector
Native *Drosophila* USP promoter
5’ untranslated region (UTR)
Drosophila A/B/C domains
Repeat polylinker region
Chironomus E domain



pCasper4-*dm/ct*USP

pCasper4 vector
Native *Drosophila* USP promoter
5’ untranslated region (UTR)
Drosophila A/B/C domains
Chironomus E domain

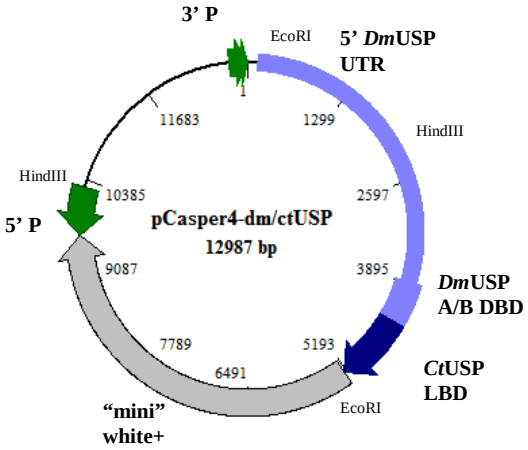


Figure 3. Schematic diagrams of the chimeric USP constructs in fly transformation vectors. pMVZ18 is the original plasmid used in *usp* mutant larval rescue studies (Henrich *et al.*, 2000). pCasper4-*dm/ct*USP cloning was not completed (see Appendix A).

Cell culture

A mammalian cell culture system was used in this study to compare the activity of both the old and new chimera with the wild-type *Dm*USP. Chinese hamster ovary (CHO) cells were co-transfected with pVP16-*Dm*USPII and III, VP16-*Ct*USPII and III, *d/c*USP II and III or the new chimera (*Dm/Ct*USP) along with pcDNA3-*Dm*EcRA, B1 or B2. The cells were also transfected with a luciferase reporter plasmid (pEcRE tk-LUC) containing the hsp27 ecdysteroid response element in its promoter. The transcribed USP and EcR proteins form a heterodimer that interacts with the response element affecting the transcription of the luciferase gene. The pCH111 β-galactosidase plasmid was also introduced and its constitutive activity was used as a measure of transfection efficiency and as a means of normalizing the luciferase activity.

The CHO cells were grown in 15ml of Dulbecco's Modified Eagle's Medium (DMEM/F-12, Gibco) with 5% fetal bovine serum (FBS, MP Biomedicals, Inc.) in a 75 cm^2 cell culture flask. The cells were allowed to grow to confluence in a 37°C water-jacketed incubator with a 5% CO_2 atmosphere. At confluence the cells were prepared for seeding 6-well plates by aspirating the media with a sterile Pasteur pipette within a laminar flow hood. After the removal of the media 3ml of 1X trypsin was added to the flask and allowed to sit for approximately 30 seconds. The trypsin was then removed with care so as not to disturb the cell layer. 5ml of DMEM/F-12 5% FBS was used to separate the cells from the bottom of the flask and resuspend them with out aeration. The cells were transferred to a sterile 14ml Falcon tube and 10μl were removed and added to 90μl Trypan Blue to determine their concentration with a hemocytometer. The plates

were seeded with $3.0x10^5$ cells per well in 2ml DMEM/F-12 5% FBS after pooling in a 50ml tube to insure a consistent density of cells in each well. After 24 hour incubation at 37°C at 5% CO_2 the cells have grown to about 75% confluence which is the appropriate density for transfection.

The cells were co-transfected with pEcRE-tk-LUC, pCHIII, one of the pVP16-USP clones and either pcDNA3-*Dm*EcRA, B1, B2 or *Ct*EcR. Following a 4 hour incubation period the cells were treated with hormone. The treatments were: no hormone, muristerone A (Alexis Corporation, San Diego, CA), JHIII (Sigma, St. Louis, MO), or murA + JHIII. Initial tests were performed at 0 and 1.0 μM murA with all USP clones and *Dm*EcR A, B1, and B2. A dose response experiment was performed with old and new chimera with *Dm*EcR B1 with the following treatment:

0 μM murA

0.1 μM murA

0.5 μM murA

1.0 μM murA

2.5 μM murA

5.0 μM murA

The old and new chimera were also tested for potentiation with *Dm*EcR B1 with the following hormone treatment:

0 μM murA

0.1 μM murA

1.0 μM murA

0.1 μM murA + 80 μM JHIII

80 μM JHIII

24 hours after hormone treatment the cells were harvested, lysed, and assays were performed on the cell extracts. The luciferase activity was measured in a luminometer. The β-galactosidase assay, measured by absorbance at 420nm, was done in duplicate and the average per sample was used to normalize luciferase activity.

Western blot analysis

Western blots were performed on CHO cell culture extracts based on the β-galactosidase activity in the assay described above. The extracts were electrophoresed on a 12% polyacrylamide gel at 150V after which the gel was electroblotted (Mini Trans Blot Module, Bio Rad) on a 0.2μm polyvinylidene difluoride (PVDF) membrane (Immun-Star, Bio Rad) at 300mA. The membrane was soaked in blocking buffer (3% w/v milk powder, 10mM Tris-HCl, 150mM NaCl, 1% v/v NaN_3, 0.1% v/v Tween 20, pH 7.6) for 4 to 6 hours. The USP constructs were detected using the VP16 monoclonal mouse IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that recognizes an N-terminal sequence of VP16 (aa 411-456). The antibody was added at a 1:200 dilution factor in the blocking buffer. A peroxidase-conjugated secondary antibody (goat anti-mouse IgG) diluted 1:1000 (10mM Tris-HCl, 150mM NaCl, 1% v/v NaN_3, 0.1% v/v Tween 20, pH 7.6) was used to detect specific Western signals. The membrane was

incubated in chemiluminescent solution (Biorad) and the image was developed using the Biorad chemiluminescent system.

Drosophila stocks

The chimeric USP construct [yw; 71D/71D] (Henrich *et al*., 2000) was used to rescue *usp*^{4} mutants [FM7/*yusp*^{4}*w*] through early larval lethality. Crosses were raised on standard cornmeal agarose food with the addition of 0.5% bromophenol blue (Sigma, St. Louis, MO), and turned over every 24 hours to maintain a relatively constant supply of emerging larvae. Mutant larvae were determined as males by brown mouthhooks caused by the X-linked mutation, yellow, and closely linked with the recessive lethal *usp*^{4} mutation. Wild-type male and female siblings with black mouthhooks were used as controls. Larval stage was established by the amount of food left in the gut after the larvae ceased feeding and began to wander (Andres and Thummel, 1994). Correctly staged animals had reached third instar size, were still within the food, and had not begun clearing their guts. The excised glands were thinner, slightly shorter than late third instar glands, and had a 'grape cluster' appearance with rounded cell membranes visible along the outside of the gland.

Salivary gland analysis

Paired glands of mutant and control animals were extracted and incubated for one hour in 100 μl Schneider's media (Invitrogen) removing and replacing 50μL of media every 20 minutes. The glands were then separated and placed in a 10μl hanging drop in the lid of a 0.5ml centrifuge tube. One gland of each pair was left untreated while the

second gland was treated with either 100nM or 5μM 20E. The hanging drops were incubated at 25°C for 2 hours after which total RNA was purified using the Qiagen RNeasy Micro kit and protocol.

RT-PCR was performed on extracted RNA using the Qiagen OneStep RT-PCR kit and protocol. Salivary gland extracts were tested for expression of the following genes: rp49 (forward primer 5' GTG TAT TCC GAC CAC GTT ACA 3', reverse primer 5' TCC TAC CAG CTT CAA GAT CAC 3'), sgs-3 (forward primer 5' TGC ATG GAG GTT GCG TGG TAG ATT 3', reverse primer 5' ATG AAG CTG ACC ATT GCT ACC GCC CTA 3'), E74A (forward primer 5' CGG ACT TGT CGA TTG CTT GA 3', reverse primer 5' AAG CTG GAG TAC GCC CTC AT 3'), E74B (forward primer same as E74A, reverse primer 5' TAC TCC GGC ACG GAA TCC GA 3'), and BR-C Z1 (forward primer 5' CCG AGG TGT TCA ATG TTG AG 3', reverse primer 5' AAC ACA CAG TTG CAG CAG TC 3') (Huet *et al.*, 2003). RT-PCR products were electrophoresed on a 1.4% agarose gel and electronic images were analyzed in Quantity One software (BioRad). The density of each band was quantified and normalized relative to the rp49 values. The expression levels of E74A, E74B and BRC-Z1 were reported as percentages of rp49 expression.

CHAPTER III

RESULTS

Cell culture

A heterologous cell culture system was used to examine the transcriptional capabilities of modified USP constructs with *Drosophila* EcR (Beatty *et al.,* 2006; 2009). This system is useful for assessing the ability of a mutated receptor to respond to ecdysteroids, interact with a response element and activate transcription in comparison to a wild-type EcR/USP heterodimer. In this series of experiments, the USPII versions contain the VP16 activation domain with the *Drosophila* DBD and either the *Dm*LBD or *Chironomus* LBD. The old chimera contains the repeat/polylinker region described previously, while the new chimera (VP16-*Dm/Ct*USPII) has a direct transition from the *Dm*DBD to the *Ct*LBD. The repeat/polylinker region was removed to rule out the possibility that the lethal phenotype seen with the USP chimera *in vivo* was the result of this additional sequence. The USPIII plasmids are lacking the DBD.

Dose response with old and new chimera

The old and new chimera were tested alongside *Dm*USPII with *Dm*EcRB1 and subjected to increasing doses of muristerone A in CHO cells. The basal and induced activity of the old *d/c*USP chimera (analogous to the transformation vector used previously in flies) was consistently higher than that of *Dm*USPII. The new chimera, which lacks the artifactual duplication which was found in the old chimera, showed

higher levels of activity than both *Dm*USPII and the old chimera except with a dose of 0.1 μM where the old and new chimera had a similar level of induction (Figure 4). *Drosophila*EcR/*Drosophila* USP reached maximal levels of activity at 0.5μM murA, while EcR with the old and new chimera both showed maximal levels at 1.0μM murA. However, the actual level of induction at 0.5μM murA was about the same for all USP constructs.

Juvenile hormone potentiation

When the old and new chimera were tested for juvenile hormone potentiation with *Dm*EcRB1, a similar pattern emerged. Both old and new *Dm/*CtUSP chimeras showed greater response to murA than *Dm*USPII. The old chimera and *Dm*USPII showed the same level of potentiation with addition of JHIII to 0.1 μM murA, while the new chimera showed an even higher level of potentiation (Figure 5). The activity of all three USP constructs was at basal levels with the addition of JHIII alone.

Activity of USP constructs with *Dm*EcR isoforms

All of the VP16-USPII (*Dm*USP, *Ct*USP, *d/c*USP (old) and *d/c*USP (new)) were tested with each of the three *Drosophila* EcR isoforms. The results from *Dm*EcRB1 with *Dm*USPII, and both chimeras agreed with earlier experiments (Figures 4 and 6a). The new chimera's ecdysteroid induced activity was higher than that of the old chimera, which was higher than that of *Dm*USPII. The same trend was seen with these clones when co-transfected with *Dm*EcRA. However, the old chimera showed much lower activity with *Dm*EcRB2 than *Dm*USPII while the new chimera's activity was higher than

the old chimera and similar to that of *Dm*USPII (Figure 6a). *Chironomus* USP showed similar levels of activity as *Dm*USP with *Dm*EcR A, but with the *Dm*EcR B isoforms *Ct*USP activity was much lower than *Dm*USP and also lower than both of the chimeric USPs (Figure 6a).

The *Drosophila* ΔDBD USP (*Dm*USPIII) shows isoform specific behavior with little or no activity when co-transfected with *Dm*EcRA or B2, but an increase in activity with *Dm*EcRB1 that was previously identified (Beatty *et al*., 2006). Unlike the behavior of *Dm*USP, neither of the other *Chironomus* ΔDBD clones (*Ct*USPIII and *d/c*USPIII) showed inducible activity with *Dm*EcRB1 (Figure 6b, center set). However, it is evident that *Ct*USPIII and the ΔDBD version of the old chimera showed slightly higher levels of activity with *Dm*EcRB1 than they did with *Dm*EcRA or B2 (Figure 6b). They also showed some inductive capability, but very little when compared to *Dm*USPIII.

Western blot

In order to verify the expression of the VP16-USP constructs in CHO cells, immunoblotting was performed with a VP16 mouse monoclonal antibody that recognizes amino acids 410-456 in the VP16 A/B domain. This is especially important for the ΔDBD *Chironomus* constructs that showed little or no activity with any of the *Dm*EcR isoforms (Figure 6b). Levels of *Dm*USPII and *Dm*USPIII (ΔDBD) agreed with those seen in a previous study (Beatty *et al.*, 2006), and the old and new chimeric USP constructs both showed a strong signal (Figure 7). The *Ct*USPII construct was present at slightly lower levels, even though its measured transcriptional activity with *Dm*EcRA and B1 was

comparable to that of *Dm*USPII (Figure 6a). The *Ct*USPIII (ΔDBD) protein also presented a strong signal, while the ΔDBD version of the old chimera showed the lowest expression levels of all the constructs. The low levels of old chimera III expression complicate the interpretation of its transcriptional activity. Nevertheless, the high expression levels of *Ct*USPIII indicate that the failure to form an active dimer with *Dm*EcR B1 arises from a functional difference in the LBD of *Ct*USP compared to that of *Dm*USP.

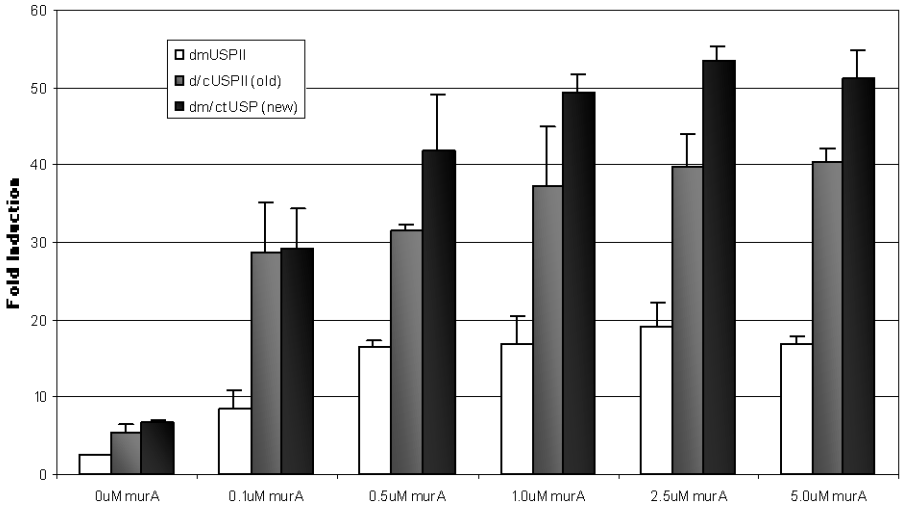


Figure 4. Dose response with muristerone A (murA) from 0-5μM in Chinese hamster ovary (CHO) cells co-transfected with pcDNA3-*Dm*EcRB1, VP16-*Dm*USPII (unshaded), VP16-*d/c*USPII (gray), and VP16-*Dm/Ct*USP (black). Fold induction measured as relative luciferase units normalized to β-galactosidase levels with *Dm*EcRB2/*Dm*USPII at 0μM murA as 1 (not shown).

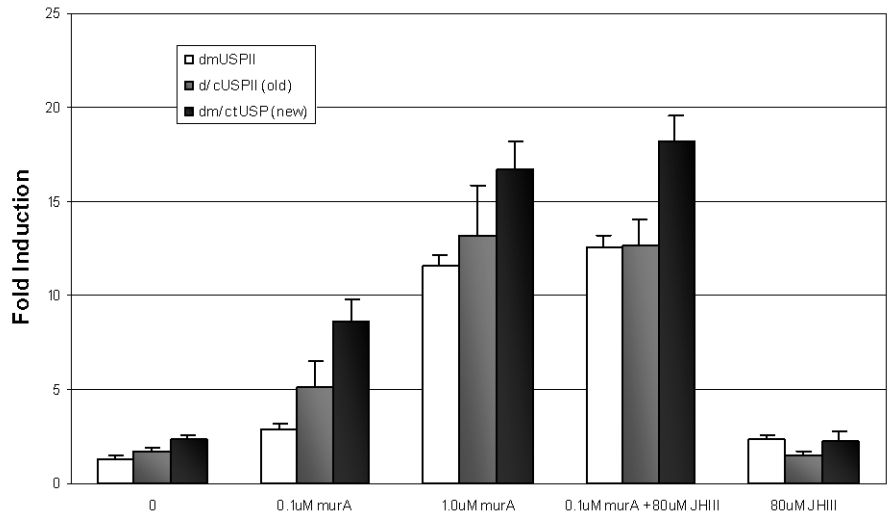
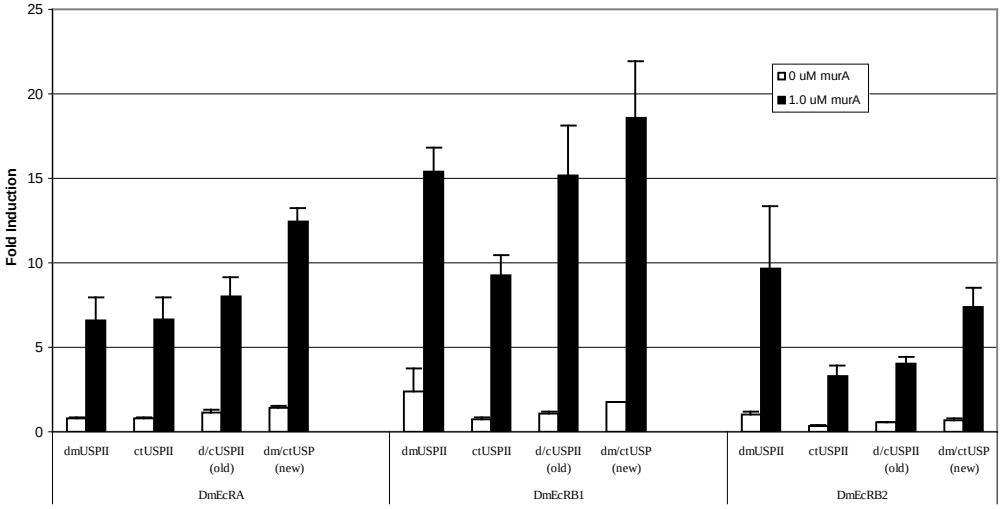


Figure 5. Juvenile Hormone III (JHIII) potentiation by USP constructs and *Dm*EcRB1 with murA at 0μM, 0.1μM and 1.0μM. CHO cells were co-transfected with pcDNA3-*Dm*EcRB1 and VP16-*Dm*USPII (unshaded), VP16-*d/c*USPII (gray), and VP16-*Dm/Ct*USP (black). VP16-*d/c*USPII construct includes polylinker repeat region. VP16-*Dm/Ct*USP is a clean fusion between *Drosophila* DBD and *Chironomus* D domain and LBD.

(a)



(b)

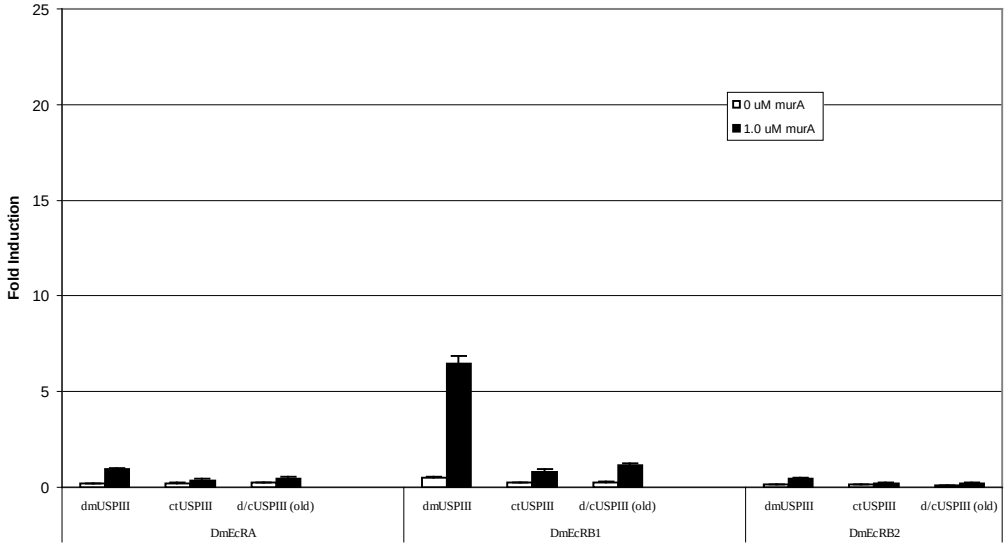


Figure 6. Transcriptional activity of VP16-USP constructs.

(a). USPII constructs are composed of the VP16 A/B domain with *Drosophila* and/or *Chironomus* DBD and LBD. The plasmids were co-transfected with *Dm*EcRA, *Dm*EcRB1, and *Dm*EcRB2. Open bars indicated basal activity while closed bars represent induced activity with 0.1μM murA.

(b). Transcriptional activity of VP16-USPIII (ΔDBD) constructs co-transfected with all three *Dm*EcR isoforms. *Drosophila* and *Chironomus* USP proteins lacking a DBD showed little or no activity with *Dm*EcR with the notable exception of VP16-*Dm*USPIII with *Dm*EcRB1.

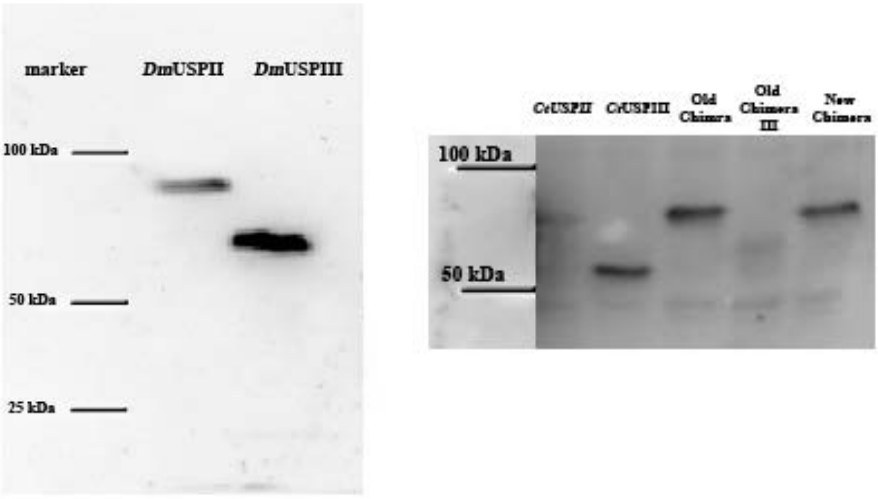


Figure 7. Western blots of *Drosophila*, *Chironomus*, and chimeric USP contructs from the extracts of transfected CHO cells. Immunoblotting was performed with a VP16 mouse monoclonal antibody (Santa Cruz Biotechnology) that recognizes a region within A/B domain of VP16 present in all the VP16-USP constructs. The image on the left contains the signals of VP16-*Dm*USPII and -*Dm*USPIII, while the image on the right showsVP16-*Ct*USPII and -*Ct*USPIII (lanes 1 and 2), the old and new versions of the chimeric USP (lanes 3 and 5), and the ΔDBD version of the old chimera (lane 4).

In vivo response to 20E

In order to test the ability of the chimeric USP to mediate ecdysteroid responsive transcriptional activity, it was tested *in vivo* for its ability to induce gene expression in the larval salivary gland. Salivary glands were dissected in pairs from either mutant or control animals. One gland from each pair was treated with a high (5μM) or low (100nM) dose of 20E, and then tested for transcriptional response to hormone. The other member of the pair was used as a control and incubated with no hormone. The constitutively active rp49 was used as an internal control for the RT-PCR, and the expression of the early low-dose responsive gene E74B, the early high-dose response gene E74A, along with BRC-Z1 was measured relative to rp49 levels.

Low dose response

At 100nM, the late gene E74A shows no response to ecdysone in either the mutant or control animals (Figure 8 (left); Figure 9 (upper)). E74B responds to the low dose of 20E by showing a 2 to 3 fold increase in expression compared to the untreated glands in both mutant and control. BRC-Z1, a gene which is activated by de-repression in *usp* mutant tissues, has little or no activity in the untreated glands and a normal response to ecdysone as compared to the control response. In other words, there are no indications that the chimeric USP is unable to repress BRC-Z1 expression in the absence of hormone.

High dose response

When glands were cultured with 5μM 20E, the mutant animals again showed a normal ecdysone mediated response. The expression of E74A was about 15 fold higher in treated glands than untreated ones in both mutant and control animals (Figure 8 (right); Figure 9 (lower)). While E74A expression levels rose, the expression of the early gene E74B decreased by half in mutant and control glands, indicating normal capabilities in both cases. BRC-Z1 expression, as seen in the low dose experiments, responded normally to 20E in mutant animals by showing little or no activity in untreated glands and increasing to levels comparable to wild-type with treatment.

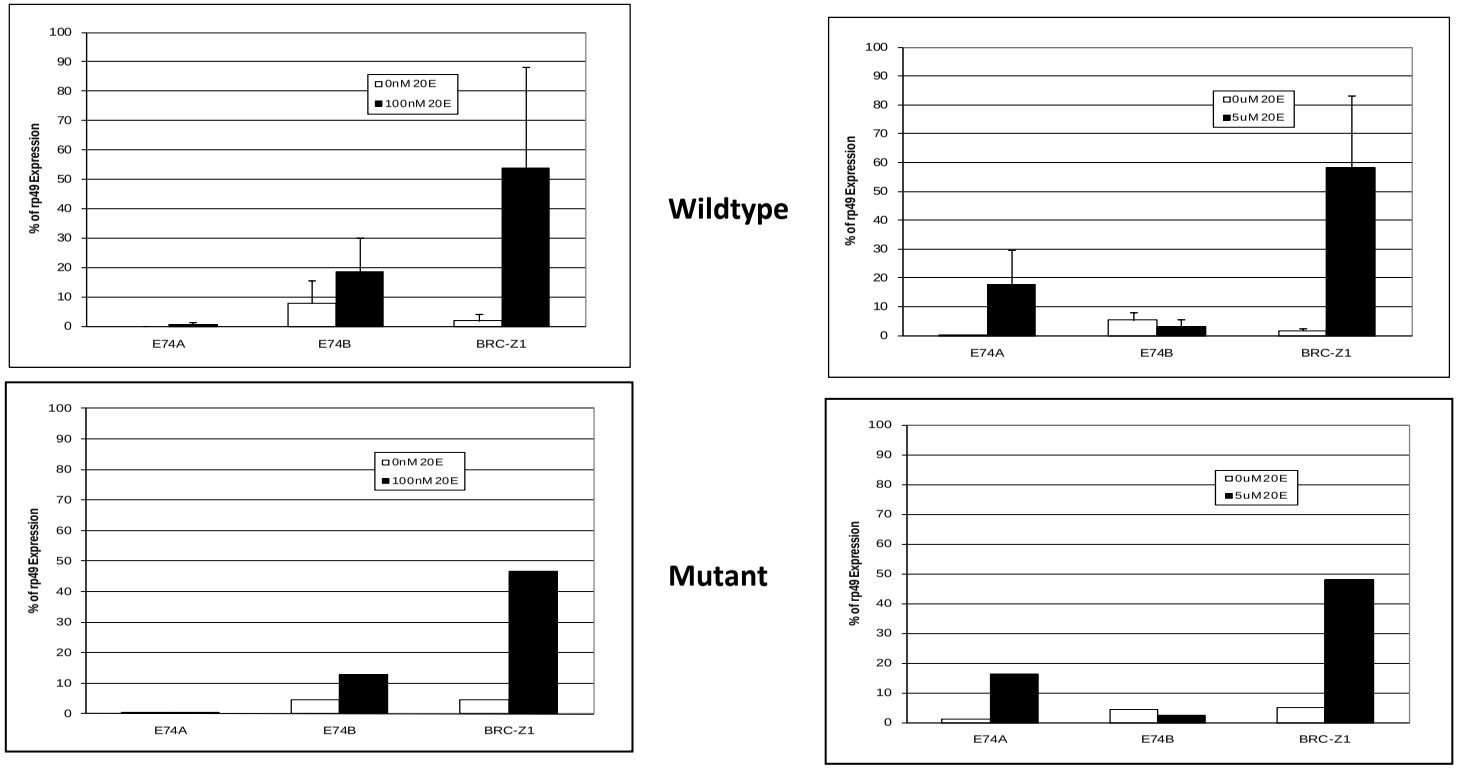


Figure 8. Ecdysteroid-inducible transcription from paired glands dissected from early third-instar wild-type and mutant animals. Mutant animals expressed the original chimeric USP from Henrich *et al.*,2000 in a *usp* mutant background. Glands were separated and one of each pair was left untreated (open bars) while the second was treated with either 100nM or 5μM 20E (solid bars). Total RNA was extracted and reverse-transcriptase PCR was performed to measure the expression levels of rp49, E74A, E74B, and BRC-Z1. rp49 is constitutively expressed in salivary glands and was used as an internal control. PCR products were electrophoresed on a 1.4% agarose gel and band density was quantified using Quantity One software (Biorad). Expression levels of E74A, E74B, and BRC-Z1 are shown as a percent of rp49 expression.

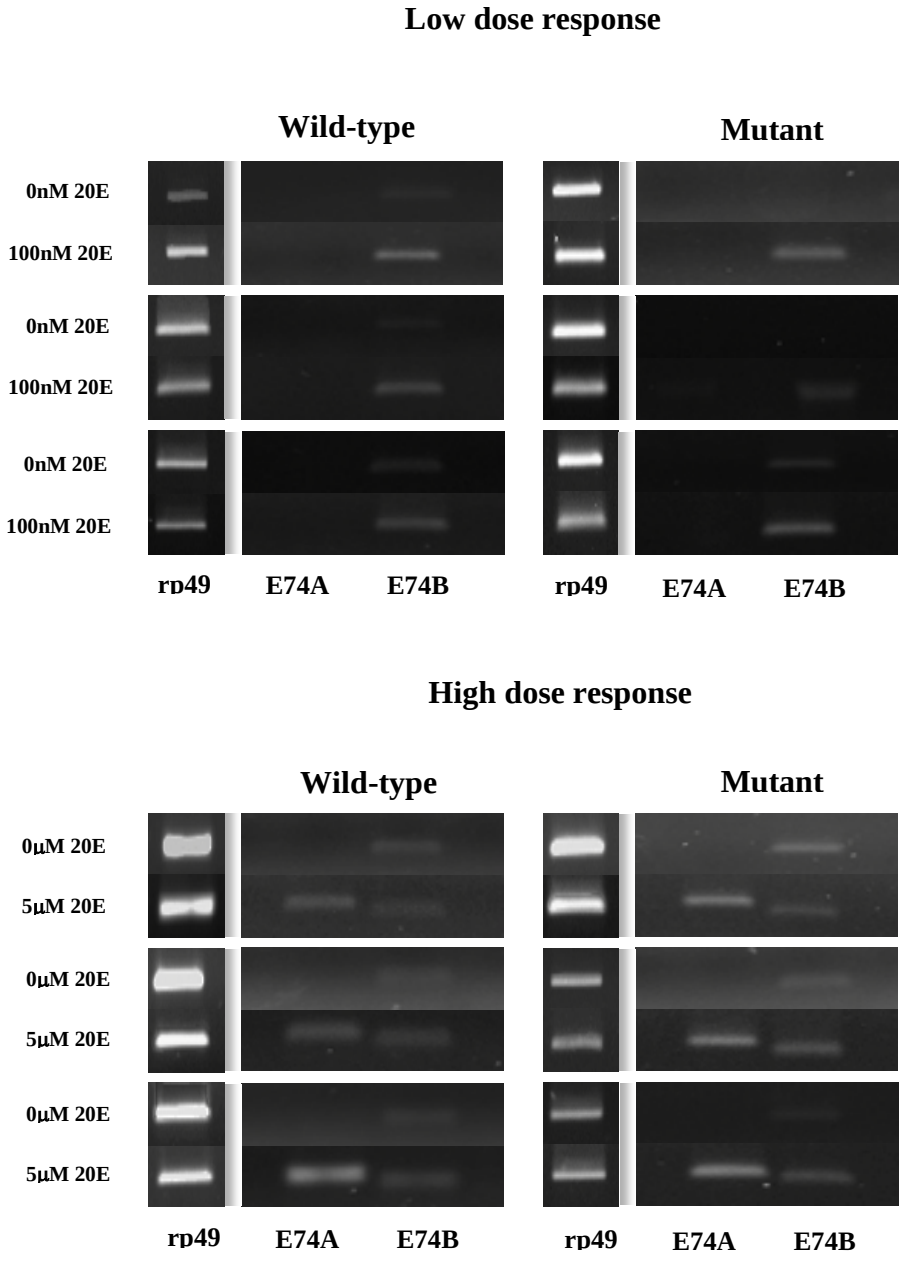


Figure 9. Ecdysteroid-induced response of E74A and E74B at high (5μM) and low (100nM) 20E concentrations. Paired salivary gland experimental technique as described in Figure 8. Low dose response (upper) shows ecdysone-induced expression of E74B with no response in E74A. High dose response (lower) shows activation of E74A while E74B expression remains the same or decreases. A constitutively expressed gene (rp49) was used as an internal control and to normalize the quantified density of each band (data shown in Figure 8) and is placed on the left of each experimental set.

CHAPTER IV

DISCUSSION

This study examined the capability of a *Drosophila/Chironomus* USP chimera to mediate ecdysteroid-inducible response in both a mammalian cell culture system and *in vivo*. The chimeric USP showed comparable basal and murA induced transcriptional activity to *Dm*USP when co-transfected in CHO cells with each of the three *Dm*EcR isoforms. The *d/c*USP/*Dm*EcRB1 heterodimer also showed the same level of potentiation as *Dm*USP/*Dm*EcRB1 when treated with JHIII. In other words, these results reveal no impairment of ecdysteroid response attributable to the chimeric USP within the scope of the cell culture system.

The chimeric USP was then examined *in vivo* using two classic examples of ecdysteroid receptor mediated response; the E74 gene which functions under alternate promoters inducing a high and low sensitivity response, and BRC-Z1 which is an isoform of a complex gene that has been shown to be active through de-repression in the presence of 20E. De-repression occurs prematurely in *usp* mutant tissues, indicating a possible functional failure involving USP mutants. For both genes, the expression patterns from salivary glands extracted from *d/cusp* mutant animals were indistinguishable from those seen in wild type. The chimeric USP is capable of mediating ecdysteroid response at both low and high titers, and does not interfere with the repression of BRC-Z1 in the absence of hormone.

Although this evidence indicates that the chimeric USP is able to function with *Dm*EcR in ecdysteroid-induced regulation, the fact still remains that *d/cusp* mutant flies are unable to develop normally beyond the larval stages. The mammalian cell culture system is a valuable tool for EcR/USP analysis that allows a close examination of an altered ecdysteroid receptor's ability to activate transcription in the presence and absence of hormone. A large number of mutated receptors can be examined relatively quickly and any deviations from normal transcriptional activity can be directly attributed to the mutation. However, only a single type of EcRE was tested in this study and it is possible that the use of alternate response elements known to be active with EcR and USP (Vogtli, *et al.,* 1998) in the reporter gene system in cell culture would reveal a functional abnormality in *d/c*USP.

While examining a modified receptor in a cell culture system has the advantages of testing a receptor in the absence of known comodulators that could affect *in vivo* activity, it is most useful as a means of predicting the effect of mutations to the receptor *in vivo*. In this case, the activity of *d/c*USP *in vitro* suggested it was capable of hormone-induced transcriptional activation. This was borne out with E74 and BRC-Z1 in the salivary gland. However, E74 and BRC-Z1 are just two examples among hundreds of genes regulated by the fluctuating levels of 20E in developing larvae (Guahar *et al.,* 2009). Testing a wider range of ecdysteroid-inducible genes may lead to the identification of the malfunction or malfunctions caused by the chimeric USP that lead to late larval arrest.

This study also focused on the ability of the chimeric receptor to mediate 20E regulation only in the salivary gland, a tissue that predominantly expresses EcRB1 (Talbot *et al.,* 1993). The methods described in this study for salivary gland dissection and hormone treatment could also be used to analyze other tissue types that undergo different genetic changes in response to 20E, most notably the differences between larval tissues that eventually degenerate, and proliferating tissues that will form adult structures, particularly the imaginal discs.

As mentioned above, the *in vitro* transcriptional activity of the full length chimeric USP was comparable to that of *Dm*USP with all three *Dm*EcR isoforms when tested in cell culture. The ΔDBD constructs, on the other hand, revealed a notable difference in activity with *Dm*EcRB1 that is attributable to the *Ct*LBD. As with *Dm*USPΔDBD, the ΔDBD versions of *Ct*USP and the chimeric USP were unable to activate transcription in the presence of hormone with either EcRA or B2. *Dm*USPΔDBD has been shown to activate transcription with EcRB1 (Beatty *et al.,* 2006), but the *Chironomus* and chimeric ΔDBD USP constructs were unable to do so. This suggests that there is some difference between the LBD region of USP and the *d/*cUSP that is responsible for this alternate behavior. The major distinction between the two USP sequences are two glycine rich regions before helix 1 and a β-sheet loop between helices 5 and 6, which so far are unique to *Dm*USP. Interestingly, this indication of a difference in function between *d/c*USP and EcRB1 seen in the cell culture experiments parallels the phenotypic similarities between EcRB1 mutants and the chimeric USP mutants. The chimeric USP is able to rescue *usp* mutant animals through the larval stages, but is unable

to perform some function necessary for metamorphosis. In the same way, EcR null mutants die during embryogenesis, but EcRB1 mutants are able to develop through the larval stages and fail at metamorphosis (Bender *et al.,* 1997). Both the chimeric USP and EcRB1 mutant larvae at late third instar fail to wander, evert their anterior spiracles or contract, and both have abnormal cuticle development. However, an important distinction arises when comparing ecdysteroid-inducible response in the salivary glands. In the EcRB1 mutants puff response was tested and showed an elimination or significant reduction of early and early-late puffs, while RT-PCR analysis of early (E74B and BRC-Z1) and early-late (E74A) gene expression in *d/cusp* mutants showed no deviation from wild type.

A more thorough histological analysis could further elucidate the potential causes of late larval arrest in the *d/c*USP mutants. It has already been shown that the chimeric USP mutants share some phenotypic similarities with USP null and EcRB1 mutants. It would be useful to examine the different tissue types during mid- and late-third *d/cusp* mutant larval to determine if the proliferation of adult tissues is correctly timed and organized, and if larval tissues are degenerating normally.

Mutants with blocked farnesoid synthesis share some phenotypic similarities with USP null mutants, such as the presence of extra mouthhooks and a double cuticle in the larval stages. The farnesoid-defficient mutant late third instar larvae also show abnormal wandering behavior and often fail to wander off the food as is seen with *d/cusp* mutants. In addition, farnesoid-defficient mutants do not fully contract prior to pupariation (Jones *et al.,* 2009). This is a chimeric USP mutant phenotype that persists even in animals that

have been rescued through adulthood with multiple doses of *d/cusp* (Henrich *et al.*, 2000).

It will also be important to determine which region or regions of the *Ct*LBD are responsible for the developmental failure prior to metamorphosis. An efficient strategy would involve creating VP16 constructs with smaller chimeric regions of the *Ct*LBD or point mutations and screening them in the cell culture system. If any of these new USP chimeras showed differences in transcriptional capabilities compared to the original chimera, they could be used in *usp* mutant rescue studies to determine if they salvage USP function through metamorphosis.

The ability of the chimeric USP to function properly through larval development, but fail at metamorphosis illustrates that amino acid sequence similarity between nuclear receptor homologs does not guarantee evolutionary conservation of function. However, these differences in activity caused by minor variations in sequence can be used to analyze the function of individual receptor domains within a relatively simple model organism. Functional capabilities of *Dm*EcR/USP revealed with this system can be used to extrapolate potential function of other members of this highly conserved family of proteins in more complex organisms.

REFERENCES

Andres, A.J., Fletcher, J.C., Karim, F.D., Thummel, C.S., 1993. Molecular Analysis of the Initiation of Insect Metamorphosis: A Comparative Study of *Drosophila* Ecdysteroid-Regulated Transcription. Dev. Biol. 160: 388-404.

Baker, K. D., Shewchuck, L. M., Kozlova, T., Makishima, M., Hassell, A., Wisely, B., Caravella, J. A., Lambert, M. H., Reinking, J. L., Krause, H., Thummel, C. S., Willson, T. M., Mangelsdorf, D. J., 2003. The Drosophila Orphan Receptor DHR38 Mediates an Atypical Ecdysteroid Signaling Pathway. Cell. 113: 731-742.

Beatty, J., Fauth, T., Callender, J. L., Spindler-Barth, M., Henrich, V. C., 2006. Analysis of transcriptional activity mediated by Drosophila melanogaster ecdysone receptor isoforms in a heterologous cell culture system. Ins. Mol. Biol. 15 (6): 785-795.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., Hogness, D.S., 1997. Drosophila Ecdysone Receptor Mutations Reveal Functional Differences among Receptor Isoforms. Cell 91: 777-788.

Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., 2001. The structure of the ultraspiracle ligand-binding pocket domain reveals a nuclear receptor locked in an inactive formation. PNAS 98: 1549-1554.

Costantino, B.F.B., Bricker, D.K., Alexandre, K., Shen, K., Merriam, J.R., Callender, J.L., Henrich, V.C., Presente,, A., Andres, A.J., 2008. A Novel Ecdysone Receptor Mediates Steroid-Regulated Developmental Events during the Mid-Third Instar of *Drosophila*. PloS.

Handler, A. M., 1982. Ecdysteroid titres during pupal and adult development in *Drosophila melanogaster*. Dev. Biol. 93: 73-82.

Hall, B.L., and Thummel, C.S., 1998. The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. Development 125: 4709-4717.

Henrich, V.C, Szekely, A.A., Kim, S.J., Brown, N.E., Antoniewski, C., Hayden, M.A., Lepesant, J.-P., Gilbert, L.P., 1994. Expression and Function of the *ultraspiracle* (*usp*) Gene during Development of *Drosophila melanogaster*. Dev. Bio. 165: 38-52.

Henrich, V.C., Vogtli, M.E., Antoniewski, C., Spindler-Barth, M., Przbibilla, S., Noureddine, M., Lezzi, M., 2000. Developmental Effects of a Chimeric *ultraspiracle* Gene Derived from *Drosophila* and *Chironomous*. Genesis 28: 125-133.

Hsieh, J.-C., Jurutka, P.W., Selznick, S.H., Reeder, M.C., Haussler, C.A., Whitfield, G.K., Haussler, M.R., 1995. The T-box near the zinc fingers of the human vitamin D receptor is required for heterodimeric DNA binding and transactivation. Biochem. Biophys. Res. Comm. 215: 1-7.

Hu, X., Cherbas, L., Cherbas, P., 2003. Transcription Activation by the Ecdysone Receptor (EcR/USP): Identification of Activation Functions. Mol. Endocrinology 17 (4): 716-731.

Jones, G., and Sharp, P. A., 1997. Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. PNAS. 94: 13499-13503.

Mouillet, J., Henrich, V.C., Lezzi, M., Vogtli, M., 2001. Differential control of gene activity by isoforms A, B1 and B2 of the *Drosophila* ecdysone receptor. Eur. J. Biochem 268: 1811-1819.

Przibilla, S., Hitchcock, W.W., Szecsi, M., Grebe, M., Beatty, J., Henrich, V.C., Spindler-Barth, M., 2004. Functional studies on the ligand-binding domain of Ultraspiracle from *Drosophila melanogaster*. Biol. Chem. 385: 21-30.

Richards, G., 1981. The radioimmune assay of ecdysone in polytene chromosomes of *Drosophila melanogaster*. Mol. Cell. Endocrinology. 21:181-197

Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., 2003. Isoform specific control of gene activity in vivo by the *Drosophila* ecdysone receptor. Mechanisms of Development 120: 909-918.

Smagghe, G., Dhadialla, T.S., Lezzi, M., 2002. Comparative toxicity and ecdysone receptor affinity of non-steroidal agonists and 20-hydroxyecdysone in *Chironomous tentans*. Insect Biochem. Mol. Bio. 32: 187-192.

Sutherland, J. D., Kozlova, T., Tzertzinis, G., Kafatos, F. C., 1995. *Drosophila* hormone receptor 38: A second partner for Drosophila USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. PNAS. 92: 7966-7970.

Talbot, W.S., Swyryd, E.A., Higness, D.S., 1993. Drosophila Tissues with Different Metamorphic Responses to Ecdysone Expree Different Ecdysone Receptor Isoforms. Cell 73: 1323-1337.

Vogtli, M., Imhof, M.O., Brown, N.E., Rauch, P., Spindler-Barth, M., Lezzi, M., Henrich, V.C., 1999. Functional characterization of two *Ultraspiracle* forms (CtUSP-1 and CtUSP-2) from *Chironomous tentans*. Insect Biochem. Mol. Bio. 29: 931-942.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P., Evans, R.M., 1993. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. Nature 366: 476-479.

Zelhof, A. C., Yao, T. P., Chen, J. D., Evans, R. M., McKeown, M., 1995a. Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. Mol. Cell. Biol. 15: 6736-6745.

Zelhof, A. C., Yao, T., Evans, R. M., McKeown, M., 1995b. Identification and characterization of a *Drosophila* nuclear receptor with the ability to inhibit the ecdysone response. PNAS. 92: 10477-10481.

Zhao, Q., Chasse, S. A., Devarkanda, S., Sierk, M. L., Ahvazi, B., Rastinejad, F., 2000. Structural Basis of RXR-DNA Interactions. J. Mol. Biol. 296: 509-520.

Appendix A.

NEW CHIMERA IN FLY TRANSFORMATION VECTOR

The following strategies were employed in an attempt to construct a *Drosophila/Chironomus* chimeric USP with a clean transition from the *Drosophila* DBD through the *Chironomus* LBD, all of which were ultimately unsuccessful.

I. pVP16-d/cUSP transfer to fly transformation vector

The original chimeric USP (pMVZ18) is in a modified pCasper4 vector under the control of the native *Drosophila* USP promoter. The first strategy was designed to remove the clean chimeric region already constructed in the cell culture vector pVP16 and transfer the discrete region into pMVZ18, replacing the repeat/polylinker region present in the old chimera. The pMVZ18 construct is a large plasmid (13.3kb), and while a unique restriction site (PacI) was found in the *ct*LBD, no viable unique site was present in the *dm*DBD. As such, it became necessary to introduce a unique restriction site using site-directed mutagenesis. The following primers were used to mutate two nucleotides and introduce an Mlu I restriction site to the *dm*DBD pVP16-*d/c*USP and pMVZ18 (primer forward 5' GGA TCT CAC ATA CGC GTG CAG GGA CTG C 3' and its reverse compliment). At the same time mutagenesis primers were designed to remove the Mlu I site upon the transfer of the chimeric fragment. While the site-directed mutagenesis was successfully completed on the pVP16-*d/c*USP construct, and the chimeric fragment was isolated by a double digest with Mlu I and Pac I, the mutagenesis was unsuccessful on pMVZ18. After several attempts this strategy was abandoned.

II. Deletion mutagenesis with megaoligo primers

New primers were designed to flank the repeat polylinker region in pMVZ18, removing the 30 base pair fragment 5' GCG GTC CAG GAG GAG CGT CGA TCC CCC GAA 3' using a modified deletion mutagenesis protocol with a two step PCR process (Wang and Malcolm, 1999). The deletion primers (primer forward 5' CCT GCG GCA TGA AGC GCG AAG CTG TGC AGG AAG AGA GGC 3' along with its reverse compliment) were used in a preliminary single primer extension reaction with a 30 second initial denaturation step at 95°C followed by 10 cycles of: 95°C for 30 seconds, 55°C for 15minutes and 68°C for 8 minutes. After which 25μL of each single primer reaction were combined and cycled under normal mutagenesis cycling conditions (Quik-Change Mutagenesis, Stratagene). Multiple attempts were made to optimize both the single primer extension step and the second standard amplification step without success.

III. Amplification away from repeat/polylinker and re-circularization

Again using pMVZ18 as a template, primers were designed flanking the repeat/polylinker region with a reverse primer (5' GCG CTT CAT GCC GCA GTT TA 3') at the 3' end of the *dm*DBD and a forward primer (5' GAA GCT GTG CAG GAA GAG AGG CA 3') at the 5' end of the *ct*LBD. These primers would amplify away from the repeat region theoretically resulting in an open plasmid, without the repeat/polylinker, that could be re-circularized into a pMVZ18 construct with a clean transition between the *dm*DBD and *ct*LBD. Difficulties optimizing the PCR cycling conditions led to excessive

non-specific amplification and insufficient PCR product available for the subsequent circularization step.

IV. Isolation and ligation of *dm*USP 5'UTR-DBD and *ct*USP LBD

Employing a strategy similar to that which led to the successful construction of the new chimera in the pVP16 cell culture vector, the next attempt involved the amplification of the *dm*USP 5' untranslated region through it's DBD along with the separate amplification of the *ct*USP LBD. Both of these regions would be blunt ligated and then re-amplified to select for those fragments in the correct orientation (see Materials and Methods VP16-*Dm/Ct*USP (new)). Rather than return to using pMVZ18 as a template, p5'*usp*1B was used instead. This plasmid contains the *Drosophila* 5' UTR region through the DBD and is much smaller (7.4kb) than pMVZ18, potentially making it easier to work with. The forward primer (5' TTC GCC AAA TAG CAC AGA GAA TGC G 3') was used in conjunction with the same reverse primer that was used in the VP16-*Dm/Ct*USP strategy mentioned above. The primers used to isolate the *ct*USP LBD were also the same. A 4.3kb dmUSP fragment was amplified from p5'*usp*1B and purified as was the 1.1kb *ct*USP LBD fragment. A blunt ligation was performed and the resulting 5.4kb fragment was gel purified and re-amplified using the forward *dm*USP primer and the reverse *ct*USP LBD primer. It was then attempted to blunt ligate the fragment into a pCasper4 vector. Multiple attempts of blunt ligation were unsuccessful, and the next strategy was to use restriction sites (5' EcoRI and 3' KpnI) inherent in the 5' and 3' regions of the chimeric fragment. As there is an EcoRI site approximately 15bp from the stop codon in the *ct*USP LBD, the *dm*USP and *ct*USP fragments had to be digested

separately before the blunt ligation/re-amplification described above. At the same time a modified strategy was attempted using the two digested fragments and pCasper4 digested with EcoRI and KpnI in a 3-way sticky-blunt-sticky ligation. Both of these strategies failed initially, and due to time constraints were unable to be further pursued.