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During insect development, steroid hormones interact with two members of the nuclear receptor superfamily, ecdysone receptor (EcR) and Ultraspiracle (USP) to bring about cellular changes. To determine the functional properties of mutations of serine and threonine residues that are possible targets of phosphorylation by protein kinase C (PKC) in the gene that encodes the *D. melanogaster* USP, we developed a heterologous mammalian cell culture system. When tested with the three natural isoforms of *D. melanogaster* EcR, one of the five mutations, S112A in the DNA-binding domain of USP, reduced basal and induced transcriptional activity, by itself and in most combinations with other mutations. Treatment with a PKC inhibitor, chelerythrine chloride (CC), itself had no effect on the transcriptional activity with wild-type USP and with any of the combination of mutations suggesting that the difference in activity caused by S112A does not involve phosphorylation. These mutational studies create a foundation for future *in vivo* experiments.

THE EFFECT ON TRANSCRIPTIONAL ACTIVITY OF MUTATIONS THAT ALTER POSSIBLE PHOSPHORYLATION SITES IN *DROSOPHILA MELANOGASTER* ULTRASPIRACLE (USP)

By

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Approved by

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To the memory of my grandmothers, Marie “Sweetie Pie” Easterday and Marge Clifton

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 Information

Steroid hormone levels fluctuate during developmental, reproductive and metabolic events. Hormone titer peaks trigger and regulate cellular changes associated with these events. During development in *Drosophila melanogaster* and other insects, peaks of the insect steroid hormones, the ecdysteroids, trigger cellular events that lead to developmental changes such as larval molting, puparium formation, imaginal disc and eye differentiation, and metamorphic development (Henrich 2005; Laudet and Bonneton 2005).

Steroid induced developmental processes begin when the steroids enter the cell by passing through the membrane and then bind to a nuclear receptor. Once a ligand binds to its cognate receptor, a functional complex is formed with either an identical receptor (homodimer) or another receptor (heterodimer). The dimer then binds to a specific sequence in the promoter region of a target gene that is known as the hormone response element (HRE; Evans 1988). When bound to the HRE, the dimerized nuclear receptor recruits comodulators to create a complex that includes RNA polymerase to transcribe the target genes. Then the mRNA encoded by the target genes is synthesized into new proteins for cell processes such as differentiation.

DNA binding and transcriptional regulation of target genes are key functions of nuclear receptors (Yao et al., 1993; Lezzi et al., 2002; Grebe,et al., 2003; Henrich 2005), defined by the distinct functional domains characteristic of the protein class. The N-terminal or A/B domain interacts with other transcription factors (Robinson-Rechavi et al., 2003).The highly conserved C domain or DNA binding domain (DBD) contains two cysteine-cysteine zinc fingers which interact with the HRE in the promoter of hormone-responsive genes. In fact, these zinc finger sequences typically define most members of the nuclear receptor superfamily, such as thyroid receptor (TR), glucocorticoid receptor (GR), retinoic acid receptor (RAR), farnesoid X receptor (FXR), and retinoid X receptor (RXR). The insect homologs of FXR and RXR are the ecdysone receptor (EcR) and Ultraspiracle receptor (USP) respectively (Oro et al., 1990; Henrich et al., 1990; Forman et al., 1995). The D domain, or the hinge region, is important for nuclear localization and DNA recognition. This region has been implicated in ligand dependent heterodimerization along with the moderately conserved E or ligand binding domain (LBD; Robinson-Rechavi et al., 2003; Przibilla et al., 2004). The LBD is involved in the recognition of the specific ligand, dimerization between the receptor and other proteins including other nuclear receptors, regulation of hormone dependent transcriptional activity and interaction with protein comodulators. Generally, nuclear receptors are able to stimulate gene transcription without ligand, but induce higher transcriptional levels in the presence of the cognate ligand that the LBD recognizes (Yao et al., 1993). When the ligand binds to the nuclear receptor protein, a conformational change occurs that enables the protein to bind with a higher affinity to the promoter of the target gene.

Ecdysteroids were originally known as the molting hormones. During mid-embryogenesis in *D. melanogaster* EcR/USP dimerization presumably occurs in response to an ecdysteroid peak (Zitnan and Adams 2005). The activated complex triggers processes in larval development that cause *D. melanogaster* to molt. The pulses of the hormone 20-hydroxyecdysone (20E) coordinate the transition of the developmental stages, including the molts to the second and third larval instars and the transition between larval, prepupal, pupal and adult stages (Li and Bender 2000).

Ecdysteroids are released into the hemolymph and are transported to target cells, such as salivary glands. The ecdysteroid then passes through the membrane to enter the cell, binds to its receptor, and finally, regulates transcription. In the salivary gland, a puff occurs on a region of the chromosome that appears as swelling of a chromosomal band as a result of the increasing level of transcription of a gene that is induced by the 20E (Ashburner et al., 1974).

The puffing of individual genes occurs over time. Some puffs occur within minutes after application of 20E, other puffs appear after several hours, and as the first puffs regress the next sequence of puffs start to increase in size. The early puffs respond to a peak of 20E and induce the expression of a set of regulatory genes. Then, with the induction of these regulatory genes as the ecdysteroid peak subsides, the late genes are induced by the early puff gene products, and the late puffs encode proteins that regulate subsequent biological responses (Thummel 2002), as early puffs regress. Therefore the initiation of the puffs is dependent on the presence of ecdysteroids, but once triggered, changes continue to occur that are not dependent on ecdysteroids.

EcR and USP function as a heterodimer. EcR and USP alone are unable to achieve high affinity binding or transcriptional activation, and their activity is dependent on dimerization (Yao et al., 1993). Once this heterodimer is formed, the complex is able to interact with a palindromic sequence in heat shock protein 27 ecdysone response element (*hsp27*-EcRE) (Riddihough and Pelham 1986).

Ecdysteroids bind to EcR to regulate puff gene expression, but less is known about EcR's heterodimeric partner, Ultraspiracle (USP). Studies have demonstrated that 20E binds with EcR, and there is only indirect evidence that juvenile hormone is the ligand that interacts with USP. Some studies have shown that USP physically interacts with juvenile hormone (JHIII), also known as sesquiterpenoid methyl epoxyfarnesoate, and that an USP homodimer can be induced by JHIII with a specific promoter element (Xu et al., 2002).

The *usp* gene maps to 2C1-3 on the distal portion of the X chromosome and has a 2.7kb transcript with no introns (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990; Henrich et al., 1994; Hall and Thummel 1998). The USP protein is homologous to vertebrate retinoid X receptor (RXR) with the LBD (49% identity between RXR and USP) and DBD (86%) showing several regions of amino acid identity. The proteins would be even more similar in sequence except for two glycine-rich spacers that only appear in the USP LBD and are not found in RXRs (Henrich et al., 1990; Oro et al., 1990; Yao et al., 1992). Like mammalian RXR, USP can heterodimerize with the mammalian nuclear receptor retinoid acid receptor (RAR) (Yao et al., 1992). USP *in vitro* achieves high affinity DNA binding to response elements as a dimer with thyroid

receptor (TR), peroxisome proliferator activated receptor (PPAR), and vitamin D receptor (VDR) (Yao et al., 1992, Song et al., 2003).

The USP protein is present throughout embryogenesis, and is required in ecdysone-dependent processes, such as larval molting, growth and pupation (Oro et al., 1992; Hall and Thummel 1998). The loss of function *usp* result in death at the end of the first larval instar (Oro et al., 1990; Henrich et al., 1994). These mutations include three missense substitutions, usp^3, usp^4, usp^5 and a nonsense mutation, usp^2, within the DBD. The missense substitution usp^3 is amino acid R160H and is found in the 5' section of the second zinc finger, usp^4 is the amino acid R130C which is found in the linker between the two fingers, usp^5 is the amino acid R153K in the second zinc finger and the usp^2 mutation truncates the DBD (Oro et al, 1990; Henrich et al., 1994; Lee et al., 2000). Also, the absence of USP in the late third instar prevents a response to 20E and leads to a developmental arrest before metamorphosis (Hall and Thummel 1998).

The *usp* mutations produce different phenotypes at various developmental stages. Also, *usp* appears to be required both maternally and postzygotically (Perrimon et al., 1985; Oro et al., 1992; Henrich et al., 1994). The offspring that lack normal maternal *usp* function die as embryos and have cuticular scarring in the posterior abdominal segments (Perrimon et al., 1985). Lethal mutation of *usp* postzygotically prevents molting at the end the first larval instar and mutants lacking normal *usp* die during the molt from the first to second instar. Extra posterior spiracles form during the incomplete molt, thus the mutation's name, *ultraspiracle* (Perrimon et al., 1985). When a fly obtains an increased dose of mutant *usp* alleles along with a single wild-type allele, the progeny show an

increased frequency of cleft thorax (Henrich et al. 1994). Therefore, *usp* appears to be required both maternally and postzygotically (Perrimon et al., 1985; Oro et al., 1992; Henrich et al., 1994).

USP is necessary during adult eye movement for regulating the progression of morphogenetic furrow movement. The morphogenetic furrow is a monolayer between undifferentiated cells and the cells that form the ommatidia, or eye units. (Oro et al., 1992; Zelhof et al., 1997). The loss-of-function *usp* mutations have the same phenotypes as some EcR mutations, in that they both arrest at the first larval molt and are unable to shed the cuticle. In addition to being unable to shed the cuticle, EcR mutants are unable to shed their mouth hooks between the first and second instars and the second and third instar (Schubiger et al., 1998).

In order to test the effect mutations within EcR and USP and without using *D. melanogaster, in vitro* experiments have been utilized. Heterologous mammalian cell cultures have been used for many experiments to examine the transcriptional activity of the EcR/USP heterodimer. The mammalian cells have no endogenous response to ecdysteroids, and therefore will become responsive to ecdysteroids only when cotransfected with EcR and USP, unlike insect cell lines that are naturally responsive to ecdysteroids (Christopherson et al., 1992; Yao et al., 1992; Palli et al., 2003; Beatty et al., 2006). Consequently, in using the mammalian cells transfected with EcR/USP and treated with hormone, the assays produce information about receptor function, and the effect of alterations to the receptor that cause a down-regulation or an up-regulation in the transcriptional activity produced by the receptor. The effects of altered receptors have

been examined using this approach, and this is useful for testing the transcriptional activity of the receptor in its wild-type form and in structurally altered forms.

Mammalian cell cultures cannot ultimately demonstrate how mutations will affect *D. melanogaster*. Nevertheless, cell culture can serve as a gauge of the receptor's capabilities, and can establish a basis for *in vivo* testing. If a mutation has an effect on transcriptional activity *in vitro*, there is a chance the same effect will be seen *in vivo*. Mammalian cell culture does not include the effect the receptor has on different genes and tissues in *D. melanogaster*, but cell culture can test if the receptor and heterodimer is working proficiently to transcribe a gene carrying an EcRE in the promoter. If a mutation limits transcriptional activity *in vitro*, I hypothesize that the mutation will cause a phenotypic change in *D. melanogaster* and/or developmental arrest just as a natural *EcR* or *usp* mutation would because the receptor is not working at a normal level.

Phosphorylation

USP is a phosphoprotein and maybe regulated by phosphorylation (Song et al, 2003). When a molecule is phosphorylated, a phosphate group is added. More importantly, phosphorylation is an essential mechanism for modification of the structure, activity, and lifetime of certain proteins and underlies the regulation of cellular metabolism through many agents including hormones, growth factors, tumor promoters and oncogenes (Weigel 1996; Song et al., 2003).

The first evidence of phosphorylation was from an immunoblot over several developmental stages which showed two bands, though there is only a single *usp* gene (Henrich et al., 1994). Song et al., (2003) also have investigated the effects of

phosphorylation during the developmental stages of *D. melanogaster*. USP phosphorylation is associated with changes in 20E in *D. melanogaster* and it has been suggested that these hormonal level changes are responsible for eliciting USP phosphorylation in early wandering larvae. In *D. melanogaster,* 60% of USP is in the phosphorylated form in the early wandering larval stage, a period that follows a small peak of ecdysteroids. There is a decline of the phosphorylated form in the late wandering stage when 40-50% of USP remains phosphorylated. Even in the absence of 20E, there have been reports of high levels of EcR and USP phosphorylation in *Manduca sexta*, but this has yet to be established in *D. melanogaster* (Song and Gilbert 1998). This suggests that phosphorylation may also be regulated by another unknown signaling pathway, as has been reported in vertebrate systems (Song and Gilbert 1998; Song et al., 2003). Protein kinase C and casein kinase II have been suggested as activators of 20E induced USP phosphorylation.

Four protein bands have been recognized from western blot analysis for *Drosophila melanogaster* USP. These bands include molecular weights of 56kDa, 54kDa, 48kDa and 46kDa, with the 48kDa and 46kDa bands only present in the midgut of the prepupae (Henrich et al., 1994; Song et al., 2003) and raise the possibility that USP exists in a phosphorylated form. In order to test this further, Song et al., (2003) treated salivary glands of *Drosophila melanogaster* with λ-protein phosphatase. The treatment led to a disappearance of the p56 band, and an increase of p54 indicating that p56 is the phosphorylated form of p54. Song et al., (2003) also noted that phosphorylation of p54 is regulated by 20E.

Dephosphorylation did not affect EcR/USP complex formation in *Manduca sexta*, though phosphorylation may play an important role in regulating function of the complex (Song and Gilbert 1998). To assess phosphorylation further, Song et al., (2003) identified via computer generating ten possible phosphorylation sites, seven protein kinase C (PKC) sites and three casein kinase II (CKII) sites. A PKC inhibitor, chelerythrine chloride (CC), blocks USP phosphorylation in glands incubated with the inhibitor for one hour. Also, CC reduces the expression of several 20E induced proteins presumably by inhibiting USP phosphorylation (Sun and Song 2006). When the 20E induced early and late puff genes were incubated with 20E for six hours, the CC almost completely blocks the expression of these genes. Accordingly, Sun and Song (2006) concluded that the PKC-mediated phosphorylation of USP is required for the expression of the 20E induced genes and proteins. These include the early puff genes E74A and E75B and the late puff genes DHR3, E78A, and E78B but not the early puff gene, E74B.

Interestingly, E74B expression was expressed at a higher level in the presence of the PKC inhibitor. The E74B gene is controlled by a low sensitivity promoter with induction occurring at 1×10^{-9} M and E74A is controlled by a high sensitivity promoter with induction occurring at 5×10^{-8} M (Karim and Thummel 1991). Thus the phosphorylation state of USP could affect its relative affinity for the E74A and E74B promoters. These results indicate that PKC mediated phosphorylation is responsible for USP phosphorylation and the inhibition of PKC activity reduces USP phosphorylation and 20E-induced gene transcription and translation.

Song et al., (2003) had not tested these computer generated PKC phosphorylation sites, so there is no evidence that they are true targets of PKC. I hypothesize that these sites are indeed phosphorylation sites targeted by PKC. When USP is unable to be phosphorylated because of one or more PKC mutations, I hypothesize that the transcriptional levels of the EcR/USP complex will be reduced. Also, because it is often necessary to destroy all the phosphorylation sites associated with a protein's activity to see an effect, multiple mutated forms of USP will be made and tested for their activity. I hypothesize that the transcriptional activity will be mostly, if not completely eliminated by the mutation of five PKC sites, including the three in the LBD and the two in the DBD. Further, it is possible that some combinations of mutations for these computer-generated PKC sites will have an effect on transcriptional activity. An important starting point would be to investigate is whether the increase in EcR/USP activity normally caused by ecdysteroids is affected by mutations of the PKC phosphorylation sites in the heterologous cell culture system. I hypothesize that a form of USP carrying all five possible mutated PKC phosphorylation sites will still have a dramatically reduced transcriptional level. I will also test these mutant protein forms to determine if a phosphorylated form of USP exists and what its effect might be.

Specific Aim of Project

This project aims: (1) to examine the effect of one and/or a combination of phosphorylation mutations within the USP DBD and LBD on the transcriptional activity of the EcR/USP heterodimer using Chinese Hamster Ovary (CHO) cells in a mammalian

cell culture system and (2) to determine if there is a phosphorylated form of USP via western blotting procedures.

CHAPTER II

MATERIALS AND METHODS

This study utilizes *in vitro* (cell culture) techniques to gauge the effect of phosphorylation mutations in USP on the EcR/USP heterodimer's function. The mutated pVP16-dUSPII DNA was transfected with the pcDNA3-dEcRA, pcDNA3-dEcRB1 or pcDNA3-dEcRB2 plasmids and a plasmid containing an ecdysteroid responsive reporter gene into Chinese hamster ovary (CHO) cells. The reporter plasmid (pEcREtk-LUC) contains five tandem copies of the *hsp27* EcRE attached to a constitutive thymidine kinase promoter and the luciferase reporter gene (pEcREtk-LUC). The pVP16-dUSPII vector is 4485 base pairs with a herpes simplex virus promoter and the USP coding region from amino acids 104-507 introduced into the EcoRI and HindIII restriction sites of pVP16 (Beatty et al., 2006). The pcDNA3-dEcRA, pcDNA3-dEcRB1 and pcDNA3-dEcRB2 vectors have a cytomegalovirus promoter and with the EcR coding region introduced between the BamHI and EcoRI or XbaI sites respectively (Mouillet, et al., 2001).

Once expressed in the CHO cells, the EcR/USP heterodimer interacts with the EcRE to induce the transcription of the luciferase (LUC) reporter gene. Luciferase activity was measured from the extracted cellular proteins using a luminometer at an absorbance of 562nm, and was normalized by adjusting the activity with that of the β-galactosidase (β-gal) gene expressing plasmid, from the extracted cellular proteins as

measured by its absorbance at 420nm. Once the results from the initial reading of transcriptional activity *in vitro* were obtained, the levels of hormone were then altered in cell culture experiments to see if increasing hormone has an effect on the transcriptional levels of these mutations.

Site Directed Mutagenesis

Site directed mutations of five potential phosphorylation sites have been obtained individually and in all combinations. First, the phosphorylation sites were selected for mutation based on the computer generated sites. The amino acids selected were those PKC sites in the DBD and the LBD that were identified by Song et al., (2003). Site-directed mutations were created by changing one or two nucleotides in the codons so that four serine and one threonine amino acid residues were substituted with alanine (Figure 1). To create these mutations, complementary primers were designed to be centered over the codon of interest in order to replicate the DNA in both the 3' and 5' directions with the mutated codon in place of the original codon. These primers were used to introduce the mutations into the pVP16-dUSPII vector (Beatty et al., 2006) by PCR amplification using the following primers and their reverse complements, with the altered codon underlined. The substituted codons (alanine) are given below the codon that was modified:

S112A: 5' GGG GAT CGG GCC <u>AGT</u> GGC AAG CAC TAC G 3'
(GCT)
T131A: 5' GGC TTC TTT AAA CGC <u>AGA</u> GTG CGC AAG GAT CTC 3'
(GCA)
S393A: 5' GC ATA TTG TCG GAG CTG <u>AGT</u> GTA AAG ATG AAG CCG CT 3'
(GCT)
S468A: 5' C GCT TTG CGA TCG ATC <u>AGC</u> CTG AAG TGC CAG GAT 3'

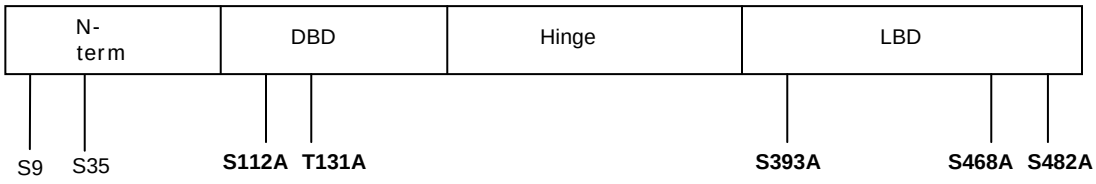


Figure 1: Placement of mutations in domains of USP (bold). The two serine residues in the N-terminal domain were not tested since this portion of USP was replaced with the VP16 activation domain for the cell culture experiments.

(GCC)
S482A: 5' C TTC CGC ATT ACC AGC GAC CGG CCG CTG 3'
(GCC)

These mutations were introduced individually and sequentially to produce the mutational combinations. The PCR amplification was cycled according to the following protocol. First, a five minute melting step at 94°C was used to obtain single stranded template DNA. Then, temperature cycling was carried out for a total 30 cycles as follows: 94°C melting for one minute, 58°C annealing for one minute, and 68°C extension for two minutes per kilobase.

The PCR product with the appropriate mutations was then transformed into 45µl Ultracompetent XL10-Gold *E. coli* (Stratagene). The cells were streaked onto Luria-Bertani (LB) agarose plates with 50 µg/ml of ampicillin as a selective marker. The transformed *E. coli* possesses ampicillin resistance and colonies were developed on the LB plate in 16-18 hours of allowed growth time at 37°C. Single colonies were selected and picked using a 10µl pipette tip and then inoculated into 5ml LB liquid cultures with 50 µg/ml of ampicillin and incubated at 37°C with shaking at 250rpm for eight hours.

A mini-prep kit (Qiagen) and protocol was then used to extract the plasmid DNA from the *E. coli.* The plasmid concentrations were quantified spectrophotometrically (Eppendorf). To verify that the correct DNA is indeed what was transformed, 500ng of DNA was digested for one hour at 37°C with EcoRI and/or HindIII restriction enzymes. Once the digestion was complete, the sample was electrophoresed on a 0.7% agarose gel with 1X ethidium bromide and a 1kb standard for size verification.

The clones were then sequenced for verification to ensure that the appropriate mutation was present using commercial protocol (Amersham). The obtained sequences were aligned with the dUSP reference sequence from NCBI to verify that the DNA contained the mutation(s) and no other unwanted mutations were present.

Cell Culture

To test the transcriptional activity, experiments with CHO Cells were performed. The CHO cells were grown to confluence in 15ml Dulbecco's Modified Eagle's Medium-complete (DMEM-C; DMEM/F-12, Gibco) growth medium with 5% fetal bovine serum (FBS, MP Biomedicals, Inc.) in a coated cell culture flask with 75cm^2 of surface area. The flask of cells was then incubated in a 37°C water-jacket incubator with 5% CO_2 atmosphere. When the cells reached confluence the growth medium was aspirated from the flask with a sterile Pasteur pipette in the sanitary environment of a laminar flow hood. Once aspirated, the cells were treated with 3ml of Trypsin (1X) causing the cells to separate from the flask bottom. The trypsin was aspirated with a sterile Pasteur pipette after thirty seconds, with caution taken to avoid disturbing the cells. After the trypsin was removed, the cells were resuspended in 10ml DMEM-C. Fluctuation in pH can kill cells and therefore, it was important not to aerate the medium when resuspending the cells. The resuspended cells were placed into a 50ml tube and a 10µl aliquot was removed and mixed with 90µl Trypan Blue in a 1.5ml tube. The cell dilution created was counted in a hemocytometer and the cell density of the resuspended cells was calculated per milliliter.

Six well plates were seeded with a density of $3.0x10^5$ cells per well in 2ml of DMEM-C. To aliquot the appropriate cell density evenly among each well, the cells and DMEM-C were combined together in a 50ml tube to a total volume of 12ml per plate. The cells were then placed back into the CO_2 incubator to grow for 24 hours. Once the cells reached approximately 75% confluence they were transfected with the experimental plasmids.

The cells were transfected with the reporter plasmids pEcREtk-LUC and pCHIII β-gal. The β-gal is controlled by a constitutively active promoter resulting in the expression of β-gal, which then provides an indirect measure of the cell mass. The plasmids encoding EcR isoforms and USP wild-type and mutants were cotransfected into the cells with the reporter plasmids. After a four hour transfection period, a hormone treatment was applied to the transfected cells. The cells were treated with one or more of the following reagents: the phytoecdysteroid, muristerone A (mur A; Alexis Corporation, San Diego, CA), juvenile hormone III (JHIII; Sigma, St. Louis), and the protein kinase inhibitor, CC (LC Laboratories, Woburn, MA). Hormone treatments were as follows:

0.1 μM mur A

1.0μM mur A

5.0μM mur A

0.1μM mur A + 80μM JHIII

80μM JHIII

10μM CC

10μM CC + 1.0μM mur A

A 1μM and 5μM mur A dose evokes maximum response from a wild-type receptor. A super maximal dosage (5μM) was used to verify that the mutations were not affecting the maximal response of the heterodimer by reducing the affinity of murA for the receptor. Along with the hormone treatment, the PKC inhibitor, CC was added to the vehicle and 1μM MurA treatments to test the effects of the inhibitor in vitro. After 24 hours of the hormone treatment, the cells were harvested, their contents were extracted, and reporter gene assays were performed.

As described earlier, two assays were performed, a LUC assay and a β-gal assay. The LUC assay was performed to analyze the action of the EcR isoforms and the USP wild-type and mutants on the ecdysone responsive LUC reporter gene. The β-gal assay was performed to measure the transfection efficiency in the experiment. The LUC assay was normalized by the β-gal assay. The β-gal was performed in duplicate to obtain results that are more reliable.

Western Blot

The amount of cellular extract was determined from the β-gal activity and 100ng amount was loaded into each lane of an 8% polyacrylamide gel and was then electrophoresed (Invitrogen) at 150 V. The polyacrylamide gel was then electroblotted (Minicell Blot Module, Invitrogen) on a PVDF membrane (0.2μm; Immun-Star, Bio Rad) at 350mA. This 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). Either the VP16 monoclonal mouse IgG antibody (Santa Cruz Biotechnology) or the anti-phosphoserine polyclonal rabbit IgG (Chemicon International) was diluted 1:1000 in

blocking buffer. The VP16 antibody probed the N-terminal domain of USPII wild type and mutant vectors to detect phosphorylation in the cell extracts. The anti-phosphoserine antibody probed for phosphorylated serines with the cell extract. Goat anti-mouse IgG or goat anti-rabbit IgG, peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology), was diluted 1:2500 (10mM Tris-HCl, 150mM NaCl, 1% (v/v) NaN_3, 0.1% (v/v) Tween 20, pH 7.6) to detect specific immunosignals. The membrane was exposed using chemiluminescence (BioRad), and the image was developed.

CHAPTER III

RESULTS

Background

Song et al., (2003) described five phosphorylation sites as potential targets of protein kinase C in the USP DBD and LBD. Previous studies have shown that restriction of PKC phosphorylation via CC, a PKC inhibitor, leads to inhibition of USP phosphorylation and 20E-induced gene expression (Sun and Song 2006). *In vitro* mammalian cell culture can be used to evaluate the effect of the PKC mutations on transcriptional activity in the absence and presence of hormone ligand, and the potentiation of transcription by JHIII. In this study we have examined the effects of CC along with the effects of the mutated five PKC phosphorylation sites on USP *in vitro*.

USP Mutations

Site-directed mutations offer a strategy by which amino acid point mutations can be used to investigate the effects of specific amino acid substitutions in the CHO mammalian cell culture. The mutations studied here were five of the seven PKC induced phosphorylation sites previously identified by Song et al., (2003). Two of the suggested PKC mutations are in the zinc fingers in the DBD (S112A and T131A), and three are in the LBD (S393A, S468A, and S482A). The other two PKC sites are in the N-terminal domain and have not been included because the natural USP N-terminal domain does not produce ecdysteroid activity in mammalian cell culture, and as a result has been replaced with the

VP16 activation domain (Beatty et al., 2006). Mutations at all five sites were produced separately and in twelve different combinations ranging from two mutations to all five mutations combined in the VP16-USPII plasmid (Table 1). Each construct was verified by sequencing.

There are many mutations that disrupt the receptor by impairing receptor functions. All of the mutations that have been chosen, with the exception of S112A, did not impair basal and ligand-dependent transcriptional activity. That is, only S112A had a significant effect on the transcriptional activity in comparison to wild-type USP (Figure 2). The five single USP mutations and twelve combinations of mutations from Table 1 were used to study the transcriptional activation with the three natural *D. melanogaster* EcR isoforms with USPII.

Cell Culture

Numerous mutations and mutational combinations displayed no effect on either basal or ligand-induced transcriptional activity when tested with any of the three EcR isoforms (Figure 2). The individual mutants and combinations that exhibited no effect include the following single mutations: T131A, S393A, S468A, S482A; double mutation combinations: S112A/T131A, S393A/S468A, S393A/S482A, S468A/S482A; Triple mutation combination: S393A/S468A/S482A; and the quadruple mutational combination: T131A/S393A/S468A/S482A. By contrast, a protein carrying the S112A mutation, and most combinations which included S112A, (S112A/S393A, S112A/S468A, S112A/S482A, S112A/S393A/S468A, S112A/S468A/S482A, S112A/T131A/S393A/S468A/S482A) showed a significantly reduced basal level of

Table 1: VP16-USPII showing possible protein kinase C phosphorylation mutations, mutational combinations, and locations in VP16-USPII

Name	DBD	LBD
VP16-USPII (T131A)	T131A	
VP16-USPII (S112A)	S112A	
VP16-USPII (S393A)		S393A
VP16-USPII (S468A)		S468A
VP16-USPII (S482A)		S482A
VP16-USPII (S112A/T131A)	S112A/T131A	
VP16-USPII (S393A/S468A)		S393A/S468A
VP16-USPII (S393A/S482A)		S393A/S482A
VP16-USPII (S468A/S482A)		S468A/S482A
VP16-USPII (S112A/S393A)	S112A	S393A
VP16-USPII (S112A/S468A)	S112A	S468A
VP16-USPII (S112A/S482A)	S112A	S482A
VP16-USPII (LBD PKC)		S393A/S468A/S482A
VP16-USPII (S112A/S393A/S68A)	S112A	S393A/S468A
VP16-USPII (S112A/S68A/S482A)	S112A	S468A/S482A
VP16-USPII (LBD + T131A PKC)	T131A	S393A/S468A/S482A
VP16-USPII (LBD + S112A/T131A PKC)	S112A/T131A	S393A/S468A/S482A

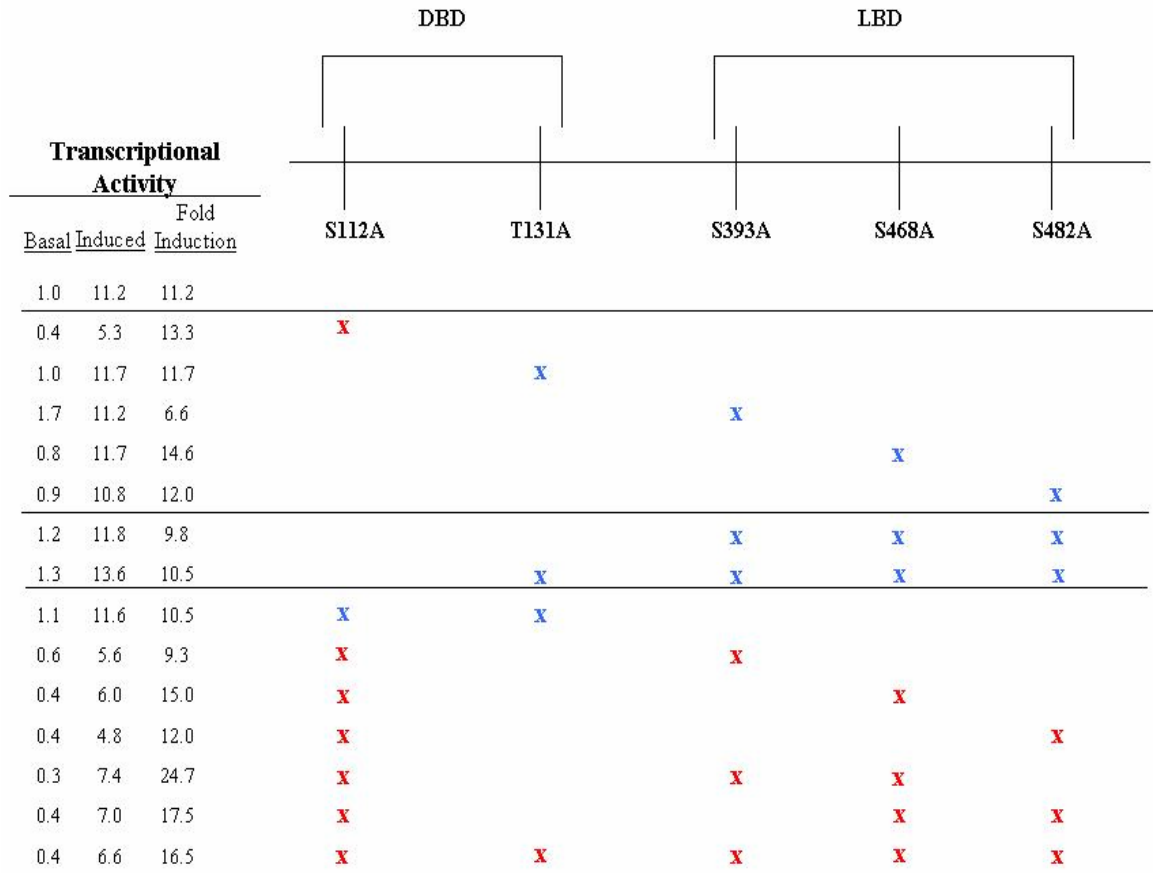


Figure 2: Effects of individual mutations and mutational combinations of VP16-USPII compared to basal and induced transcriptional activity (1.0, 11.2 respectively), with wild-type USP and EcRB1. DBD: DNA Binding Domain; LBD: Ligand Binding Domain. Red x's indicate that the construct had a significant effect ($P<0.05$, t-test) on basal and induced levels of transcription, blue x's indicate no effect.

transcriptional activity and a reduced level in the presence of a supermaximal dose of murA, demonstrating an effect on *hsp27* EcRE mediated transcription (Figure 3). However, fold induction was not reduced. All three isoforms were tested with the S112A mutant USP because in previous studies (Beatty et al., 2006), USP have yielded different results with each isoform. The same pattern was observed with all three of the EcR isoforms with each DBD USP mutant (Figure 4). S112A, T131A, and the combination of the two were also tested with EcRB1 and basal, 1μM, and 5μM murA (Figure 5). The S112A mutation presented with a reduced basal transcriptional activity and reduced ligand-dependent transcription at both 1μM and 5μM murA. T131A and S112A/T131A had a similar transcriptional activity and fold induction with 1μM, and 5μM murA as the wildtype.

Chelerythrine Chloride Treatment

VP16-USPII (S393A/S468A/S482A) and VP16-USPII (S112A/T131A/S393A/S468A/S482A) were treated with CC, a PKC inhibitor. Sun and Song (2006) and treated incubated *D. melanogaster* salivary glands with 100μM CC, and the PKC inhibitor elicited a reduction in 20E responsive genes. The mammalian cells were treated with varying levels of the CC because the chemical can lead to apoptosis, and therefore the level had to be determined which was below the level that would produce programmed cell death. The levels of CC tested were the following: 100μM, 50μM, 10μM, 1μM and 0.1μM. The 100μM and 50μM doses caused apoptosis and therefore no results were obtained via the β-gal assay. The maximal dose that could be

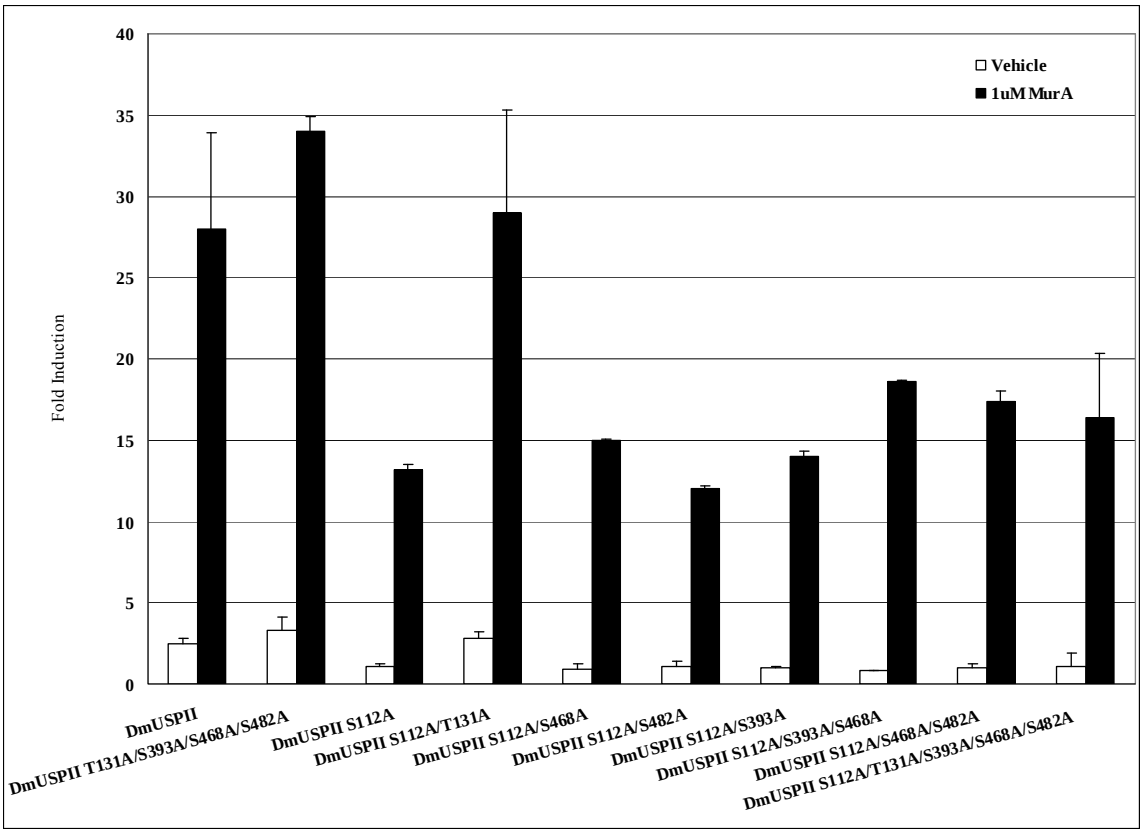


Figure 3: Basal and induced transcriptional activity via an *hsp27* EcRE-regulated luciferase gene using *D. melanogaster* EcRB1 with the USP mutant proteins. Open bars represent relative luciferase activity with no hormone; closed bars represent effects of 1μM murA. (N=4 for each data point) All mutant proteins except T131A/S393A/S468A/S482A have a common mutation, S112A.

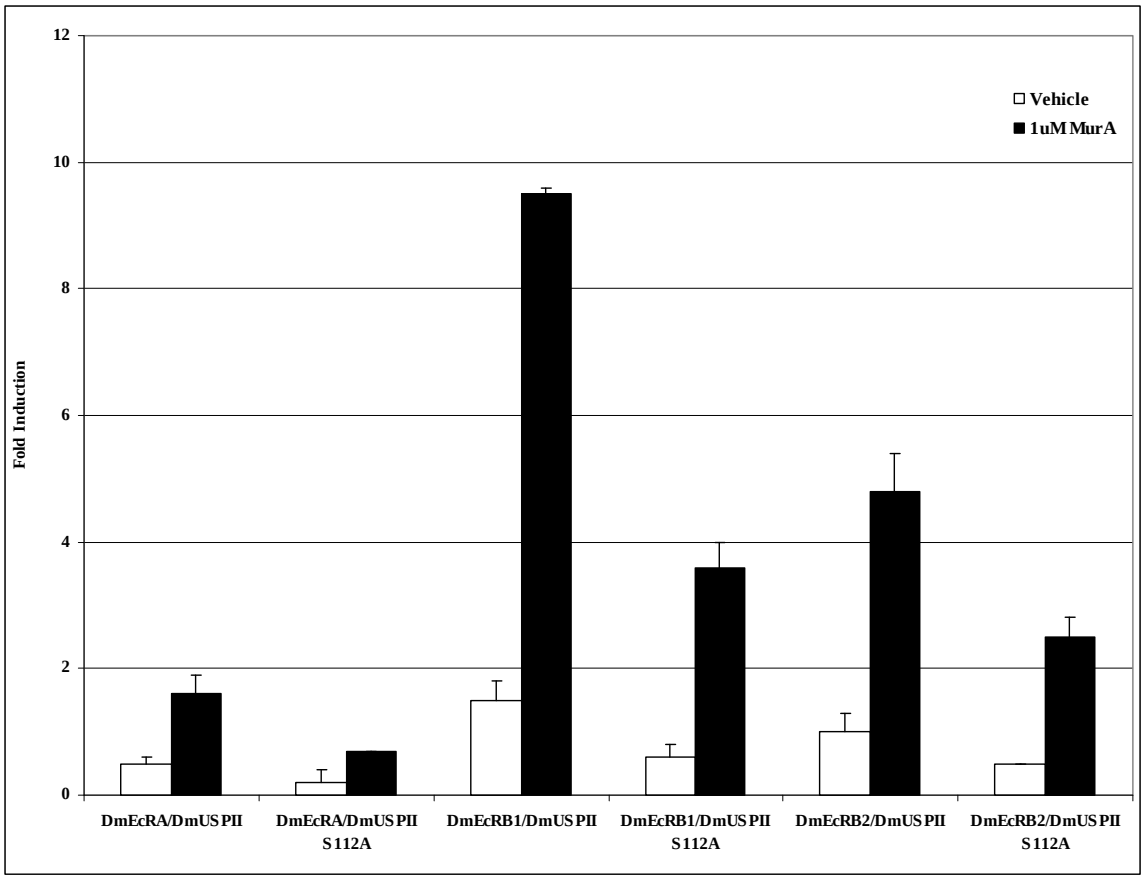


Figure 4: Basal and murA induced transcriptional activity of USP wild-type and USP S112A DBD mutation of *D. melanogaster* VP16-USPII with EcR A, B1 and B2. Open bars represent relative luciferase activity with no hormone; closed bars represent effects of 1μM murA. (N=3 for each data point)

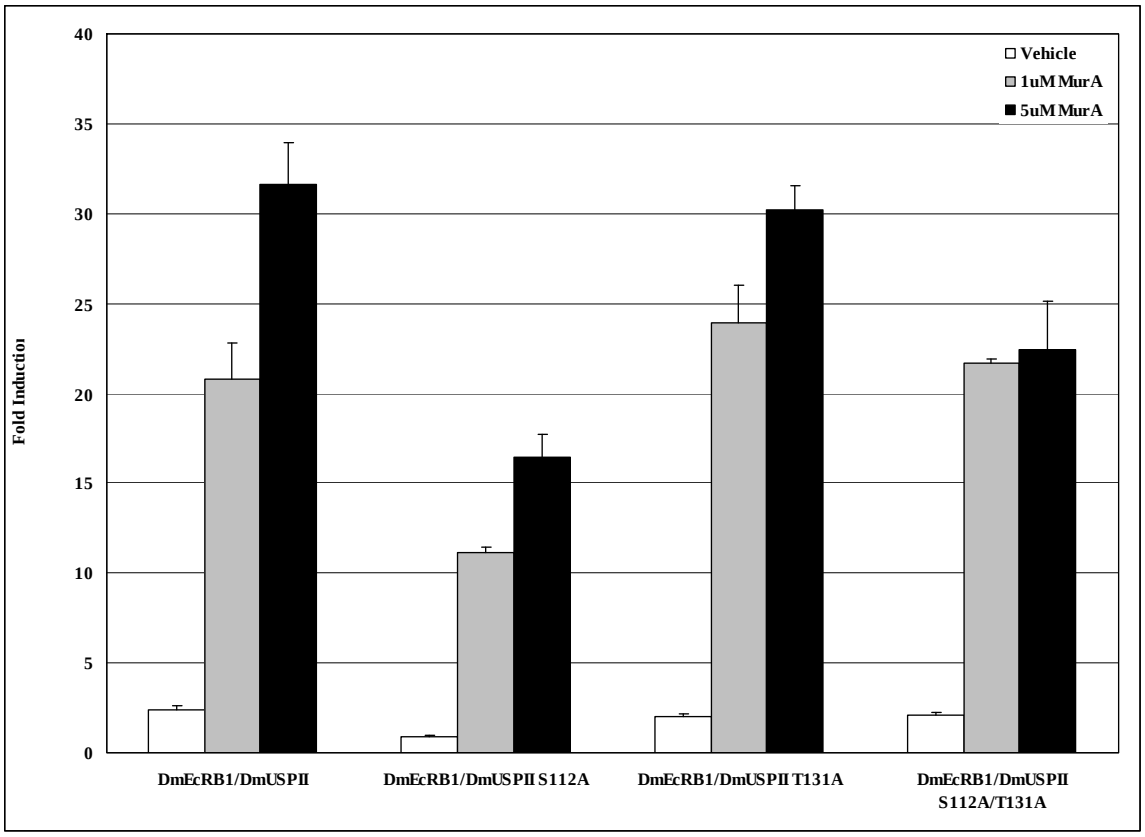
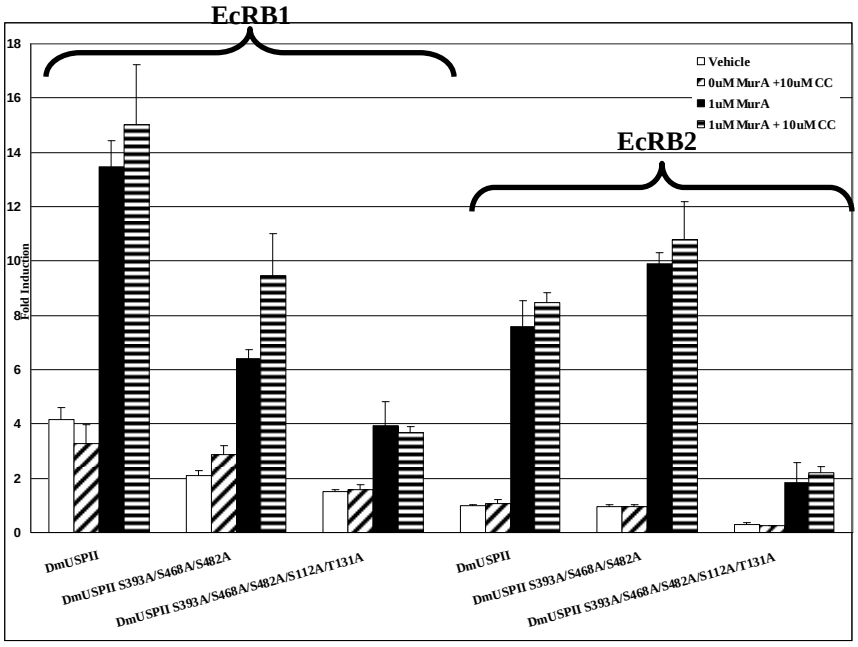


Figure 5: USP S112A, USP T131A, USP S112A/T131A with no hormone, 1μM murA, and 5μM murA. Open bars represent relative luciferase activity with no hormone; gray bars represent effects of 1μM murA; closed bars represent effects of 5μM murA. (N=3 for each data point)

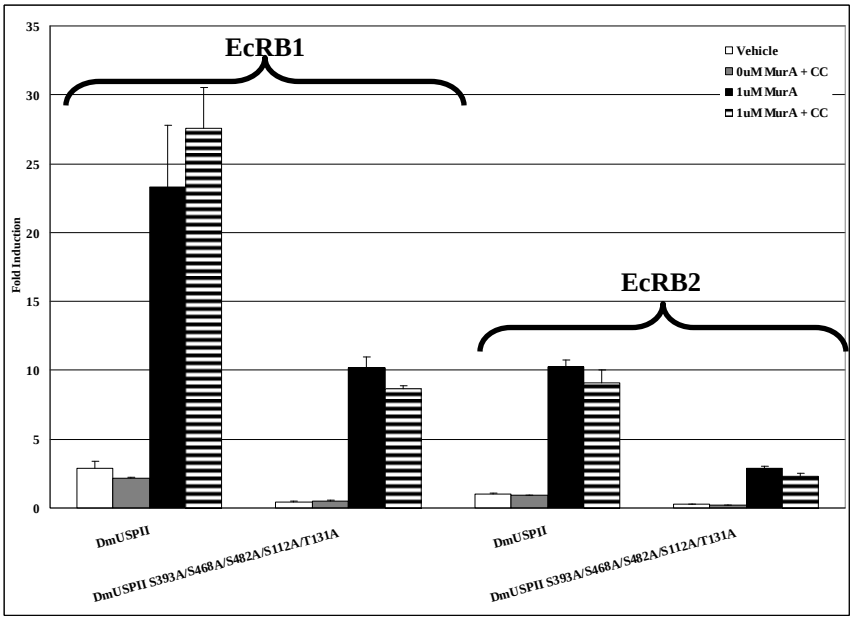
used was 10μM and the mutant USPII forms: S393A/S468A/S482A, and S112A/T131A/S393A/S468A/S482A were treated with CC. Doses aw low as 1μM CC are effective for inhibiting phosphorylation in CHO cells (Zhang et al., 2001) The amounts of plasmid DNA encoding EcR and USP transfected with the cells treated with 10μM was 250ng, the standard amount for cell culture. At this level of transfection, there was no detectable effect on either basal or ligand-induced transcriptional activity (Figure 6a). Nevertheless, it seemed possible that there was a molar excess of EcR and USP for the decreased concentration of CC. To test this possibility, the cell culture was transfected with 25ng of the EcR and USP plasmid vectors. There was still no visible effect on the transcriptional activity of the samples treated with the CC compared to the untreated wild-type and mutant samples with the reduced EcR and USP (figure 6b). Therefore it can be suggested that CC has no effect on EcR/USP transcriptional activity mammalian cell culture at the concentration of 10μM.

Western Blots

Western blot analysis is an important tool for observing possible differences between the mutant USP proteins that are not visible in cell culture. All of the mutations and combinations of mutations were treated with the VP16 antibody to probe for the USP protein (Figure 7a). Although S112A and combinations of mutations with S112A demonstrate decreased transcriptional activity, mutant USP proteins did not have a visible shift when compared to USP and the mutant proteins that previously showed no effect on transcriptional activity (Figure 6b). The CC treated USPII, S393A/S468A/S482A, and

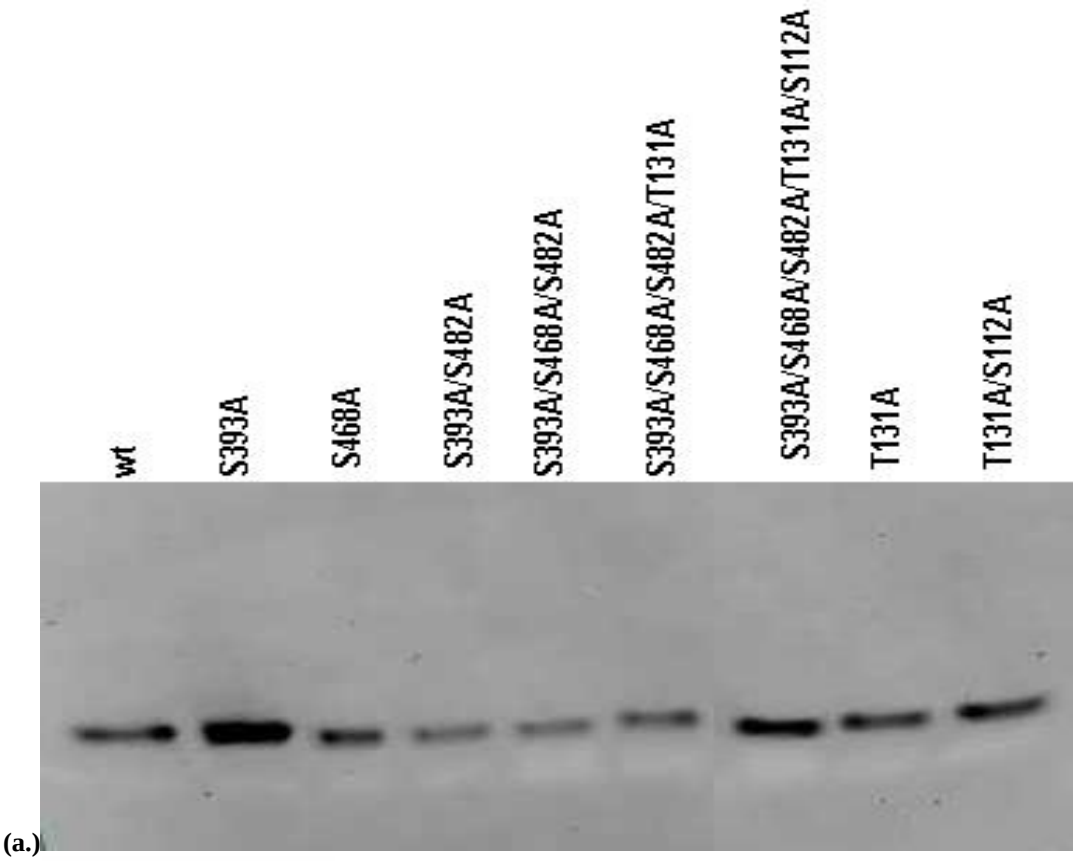


(a.)

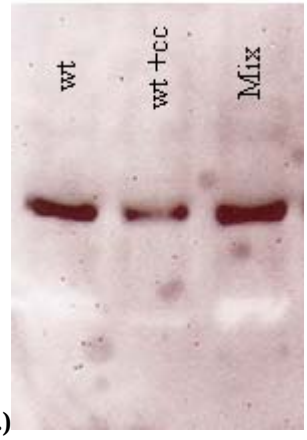


(b.)

Figure 6: Effects of transfection quantity on transcriptional activity with and without CC treatment. (a.)Transfection with 250 ng of USPII, S393A/S468A/S482A and S112A/T131A/S393A/S468A/S482A with EcR isoforms B1 and B2. (b.) transfected with 25ng of EcR and USP. USPII and S112A/T131A/S393A/S468A/S482A with EcR isoforms B1 and B2 treated with CC. Open bars represent no hormone or inhibitor, diagonal hatch marks/gray bars represent no hormone with 10μM CC, closed bars represent 1μM muristerone A with no inhibitor, and horizontal hatch marks represent 1μM muristerone A with 10μM CC. (N=3 for each point)



(a.)



(b.)

Figure 7: Western blots from CHO cell extracts with mouse monoclonal IgG VP16 antibody (a.) various mutant USP proteins on a 6% gel. (b.) wild-type (wt), wildtype with chelerythrine chloride (wt+cc), and a mixture of wild-type and wild-type with chelerythrine chloride (mix) on a 8% gel. Each well's concentration was determined by β-gal activity.

S112A/T131A/S393A/S468A/S482A were also subjected to Western blotting. VP16-USPII, VP16-USPII and CC and a lane with a mixture of the two samples were treated with VP16 antibody and no shift in molecular weight or double banding was seen (Figure 7b). If phosphorylation had been absent there would be a downward shift in molecular weight, or there would be two bands, one of residual phosphorylated USPII protein and also a band of unphosphorylated USPII protein at a reduced molecular weight. Therefore it can be suggested that CC has no effect on mammalian cell culture at the concentration of 10μM.

Next, select mutant proteins were treated with an anti-phosphoserine antibody that recognizes phosphorylated serine residues within proteins. The mutations alter the codon from either a serine or threonine into an alanine and therefore, would not be recognized by an anti-phosphoserine antibody. The anti-phosphoserine antibody produces multiple bands, compared to the one band the VP16 antibody produces, because it detects all proteins containing phosphoserine residues. The mutant proteins that were tested with this antibody include: S112A, T131A, S112A/T131A, S112A/T131A/S393A/S468A/S482A (Figure 8). If there was phosphorylation involved then that the S112A/T131A/S393A/S468A/S482A protein would not be recognized by the antibody, whereas the wild-type protein would be. The USP signal might also disappear when CC was added. The antibody is not specific to USP, and EcR can also be probed for phosphoserines. These mutations were chosen because the USP with all five mutations eliminates all of the computer generated PKC targets. There was no difference observed in the banding pattern and intensity between VP16-USPII and

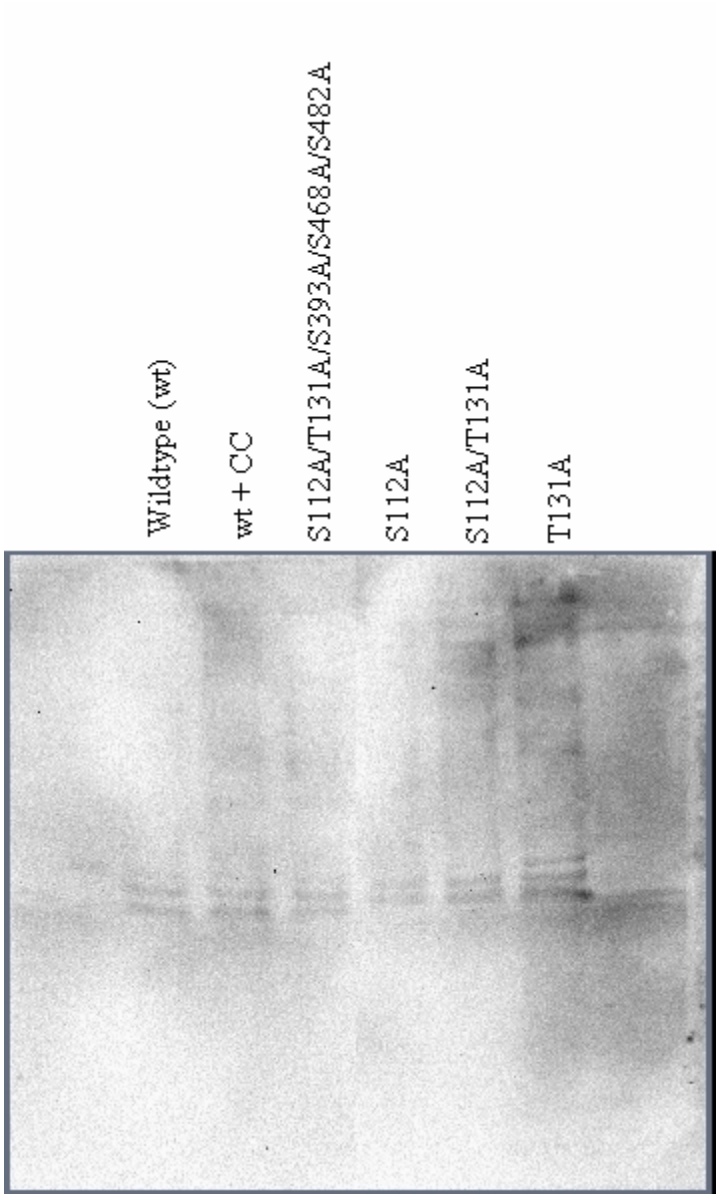


Figure 8: Western blot from CHO cell extracts with a rabbit polyclonal anti-phosphoserine antibody (LC Laboratories). lane 1: VP16-dUSPII (wt), lane 2: VP16-dUSPII with CC (wt + CC), lane 3: S112A/T131A/S393A/S468A/S482A, land 4: S112A, lane 5: S112A/T131A, lane 6: T131A. 8% gel

S112A/T131A/S393A/S468A/S482A. This indicates that there was no difference in the amount of phosphorylated protein.

The CC treated cells were also probed with the anti-phosphoserine antibody. The banding produced by the CC treated cells was also at the same intensity as the non-treated cells, therefore these results are inconclusive.

CHAPTER IV

DISCUSSION

A heterologous cell culture system was used to explore the activity of the EcR/USP heterodimer when single and multiple mutations of possible PKC mediated phosphorylation sites were introduced into the gene that encodes *D. melanogaster* USP. The mutations involved amino acids in the LBD and DBD of VP16-USPII (Beatty et al., 2006). While the LBD mutations, either alone or together, had no effect on the transcriptional activity in the cell culture system, one of the DBD mutations, S112A, reduced transcriptional activity by about 50%, both alone and in conjunction with any combination of the three LBD mutations. Also, the reduction caused by S112A was reversed by the effects of T131A, a second mutation in the DBD. None of the LBD mutations, alone or together, affected transcriptional activity. The western blots provided additional insight by showing that the size of USP mutant proteins in comparison to wild-type protein was the same and that the amount of phosphorylated serines in the cell extracts containing mutant proteins, even when treated the CC, were the same as wild-type. The S112A mutation by itself or in combination with LBD mutations caused a reduction in transcriptional activity, but it cannot be concluded that it affects the phosphorylation of the USP protein *in vitro*.

Effects of the DBD Mutations

In the DBD, S112A occurs in the top of the first zinc finger and T131A occurs between the two zinc fingers. Although neither amino acid is involved in an interaction with the DNA itself (Devarakonda et al., 2001), these mutations could possibly affect the capabilities of USP. The DBD is highly conserved and other insects and mammalian RXR's may exhibit the same effect from these mutations. To examine residue conservation among insect USPs, sixteen different insect species representing several different phylogenetic orders and two RXR's (*Mus musculus* and *Homo sapiens*) were compared with the *D.* melanogaster USP sequence. If these mutations involve a conserved residue in *D. melanogaster*, then it is conceivable that these mutations perform an identical role in other species (figure 9). The USP DBD is highly conserved - 86% of the residues are identical among all insect species and only 14% of the residues show any variability in their sequence. All sixteen insect species and the two mammalian RXR's aligned with *D. melanogaster* contain the S112A and T131 residue or its equivalent.

S112A vs. *usp*^{3} and *usp*^{4}

Recessive early larval lethal mutations *usp*^{3} and *usp*^{4} occur in the DBD just as S112A and T131A. The *usp*^{4} mutation is the amino acid R130C and is just before T131A and *usp*^{3} is R160H which occurs in the second zinc finger and are also shared among all the USP and RXR DBD sequences. Ghbeish et al., (2001) tested USP3 and USP4 *in vitro* with EcRB1 and the *hsp27*EcRE. These two mutant proteins increased induction compared to the wild-type EcRB1/USP heterodimer. By contrast, the S112A mutation,

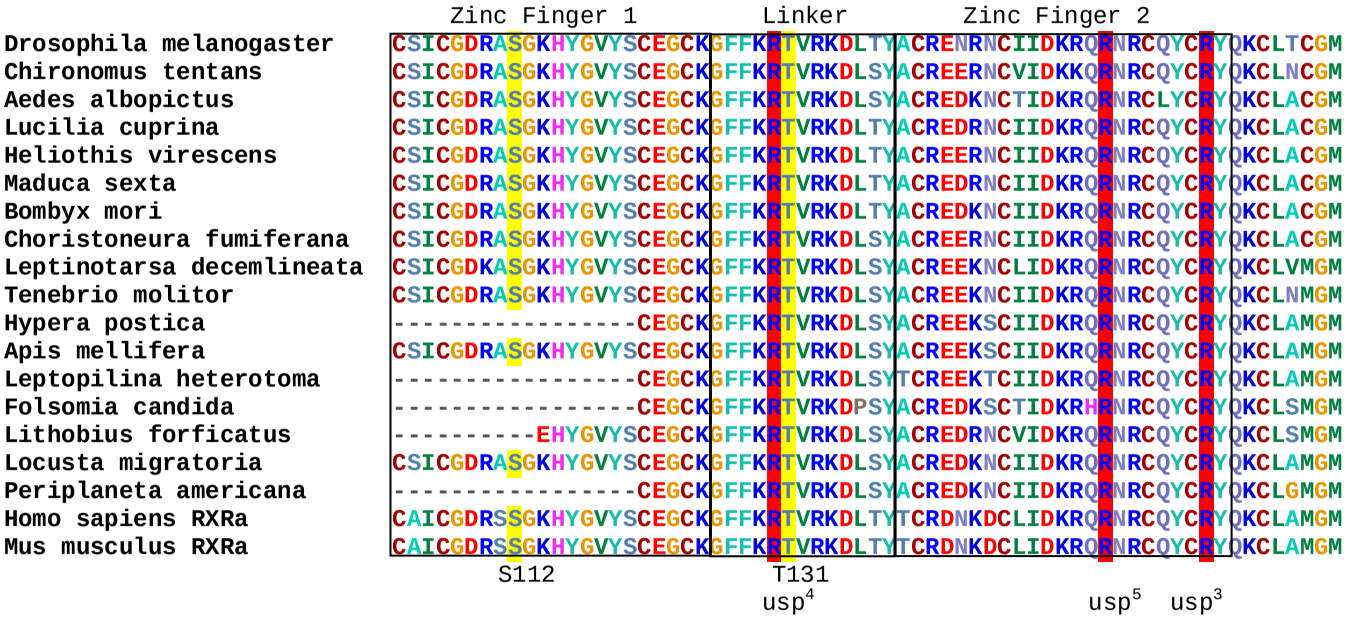


Figure 9: Alignment of DBD depicting S112, T131, *usp*^{3}, *usp*^{4} and *usp*^{5} mutations, zinc fingers and linker between the fingers

which is also conserved, decreased induction by about 50%. This suggests that mutations of conserved amino acids within the same domain could be working through a different mechanism, though it remains unknown what effect S112A would have on USP function *in vivo*. There are a couple of functions the S112A mutation could affect: DNA binding affinity for the receptor to the EcRE and/or USP's dimerization to EcR. The mutation is not affecting the ligand-dependent transcription because the mutation causes a reduction in both basal and induced levels.

Effects of the LBD Mutations

In the LBD the mutations are: S393A is in Helix 7, S468A is in Helix 10, and S482A is just outside of Helix 11 (Figure 10). The LBD is less conserved than the DBD (49%) and consequently, it might be expected that the serine residues within this domain are not as highly conserved among insect species. Out of the three mutations in this domain, S468 is the most conserved with seven of the seventeen total insect species represented conserving the serine with all of these species either in the order Diptera (flies and mosquitoes) or Lepidoptera (moths and butterflies). The S393 position is as conserved as S468, with six of the seventeen insect species sharing a serine at this location, also in Diptera and Lepidoptera. As for S482, there is only one other species, *Aedes albopictus*, that carries the serine at this position. This may simply reflect the fact that S393 and S468 both lie in relatively conserved alpha-helices whereas S482 is just outside of a helix, where the level of conservation is generally lower.

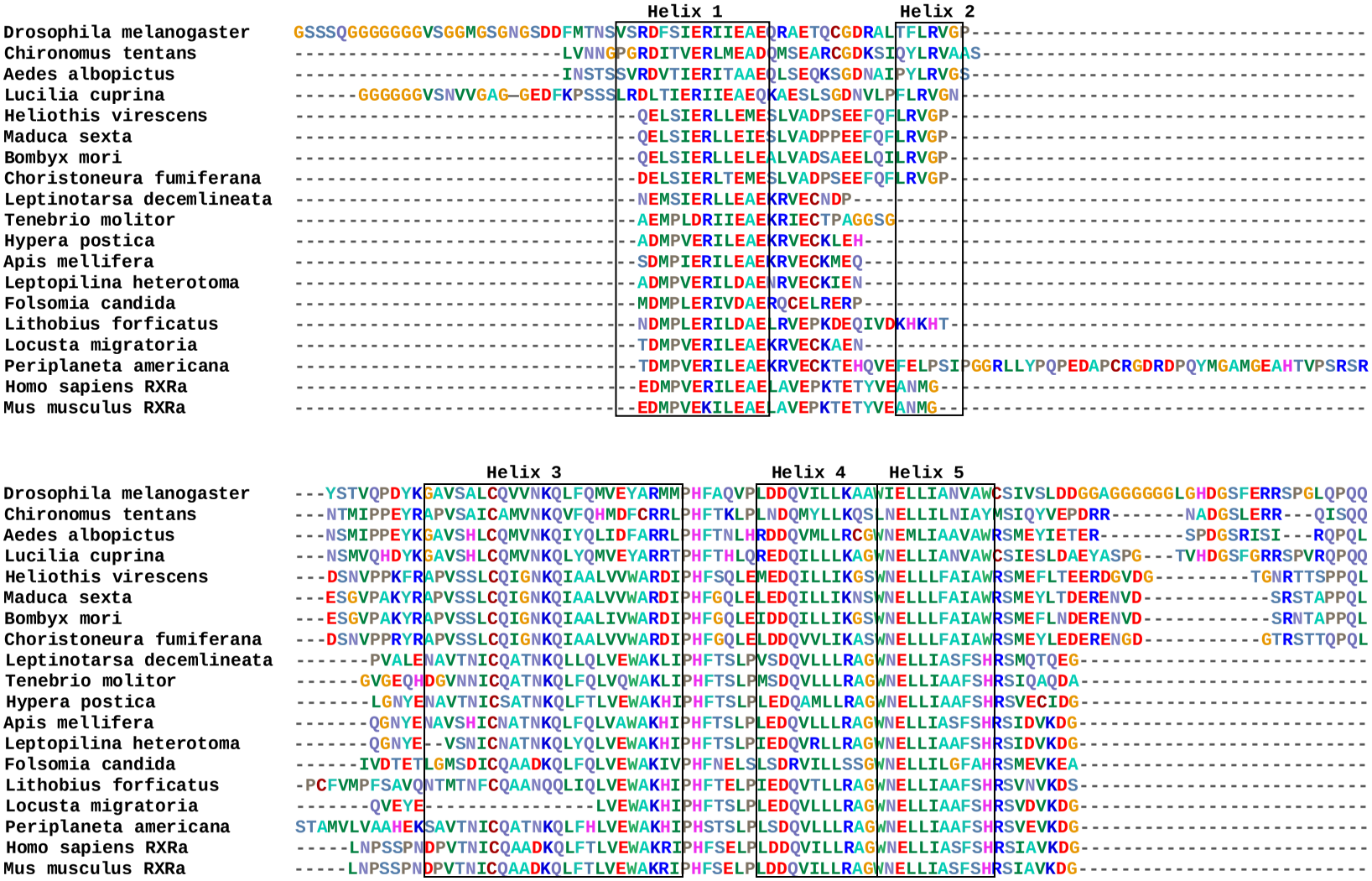
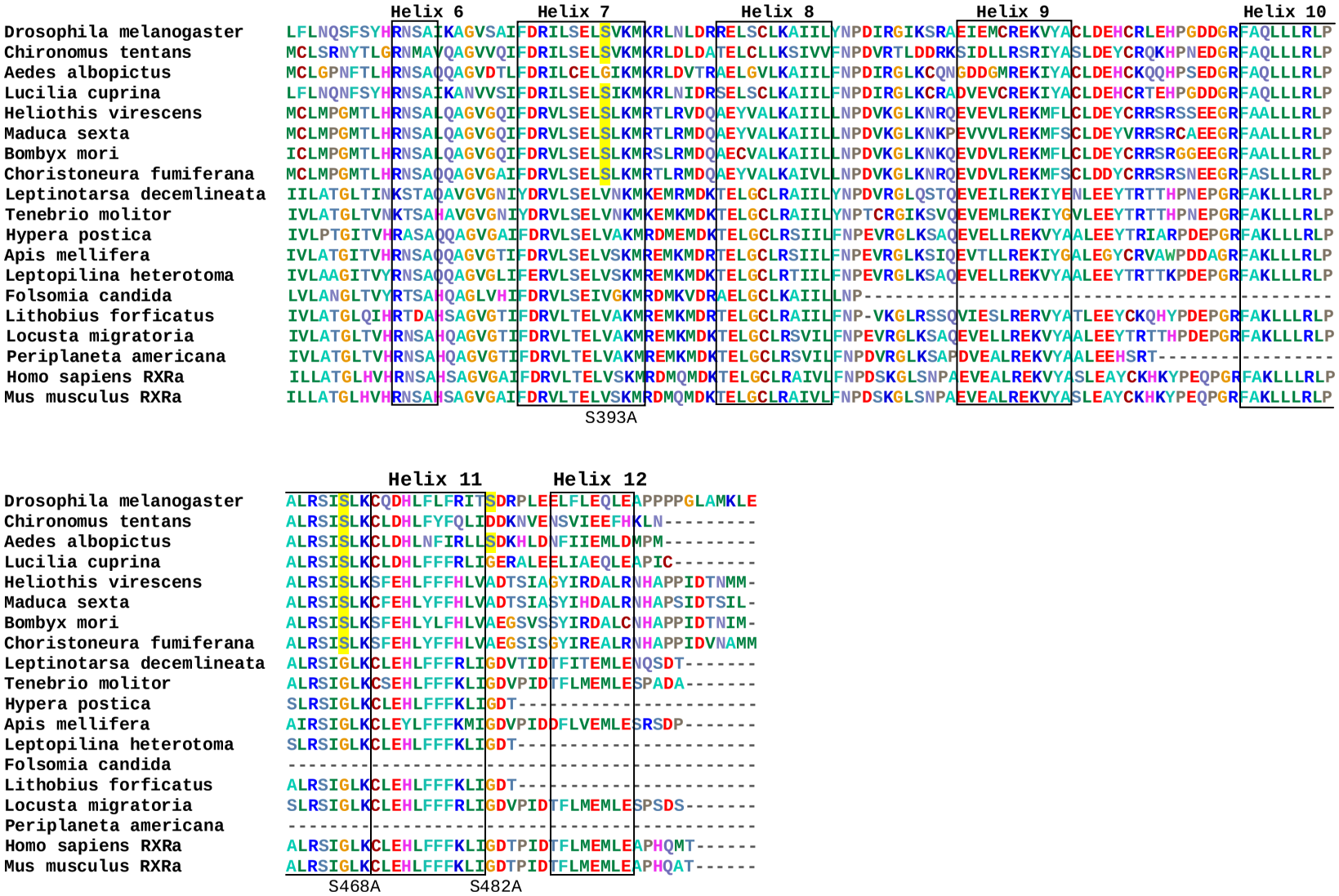


Figure 10: Alignment of the LBD depicting mutation sites S393A, S468A, S482A and the 12 helices


Helix 6
Helix 7
Helix 8
Helix 9
Helix 10
Drosophila melanogaster
Chironomus tentans
Aedes albopictus
Lucilia cuprina
Heliothis virescens
Maduca sexta
Bombyx mori
Choristoneura fumiferana
Leptinotarsa decemlineata
Tenebrio molitor
Hypera postica
Apis mellifera
Leptopilina heterotoma
Folsomia candida
Lithobius forficatus
Locusta migratoria
Periplaneta americana
Homo sapiens RXRa
Mus musculus RXRa
S393A
Helix 11
Helix 12
S468A
S482A

Phosphorylation in Chinese Hamster Ovary cells

The 10μM CC treatment has shown no effect on the transcriptional activity of EcR/USP in the CHO cells for either the wild-type or mutated USP forms. It is left to wonder if these mammalian cells are affected by PKC mediated phosphorylation. With just the results from the cell culture assays, it would signify that the USP protein is not phosphorylated. Through western blotting with the anti-phosphoserine antibody, which probes for serine phosphorylated sites in proteins, there are bands present in CC treated cells that would indicate that there is phosphorylation present but there is no evidence that USP is normally phosphorylated. Therefore, with the treatment of the anti-phosphoserine antibody being more indicative than the cell culture assays, it can be suggested that many proteins in the CHO cells are phosphorylated since the antibody had bound to several unidentified serine phosphorylated proteins.

Possible Phosphorylation Sites of USP

Western blots using the USP-specific AB11mAb in proteins extracted from flies show two bands *in vivo*. A *D. melanogaster/Chironomous tentans* chimera was blotted and the same banding pattern appeared, although this pattern was at 54kDa and 48kDa instead of 56kDa and 54kDa (Henrich et al., 2000). The function of this chimera was to act as a LBD mutation since the entire *D. melanogaster* LBD was replaced by the equivalent *C. tentans* LBD. These results could suggest one of two things. First, it could suggest that the N-terminal domain and DBD are the common target for phosphorylation since this portion is shared by both the wild-type USP and the chimeric USP. It is still possible that some or all of a phosphorylation occurs in the LBD of *Chironomus*.

Through the results obtained in this investigation, the first possibility seems more likely because the mutations in the LBD had no effect on transcription in cell culture but the DBD mutations did.

Other Possible Effects of the Mutations

The mutations may have other possible effects than that were found in this study. These mutations have only been tested with the *hsp27*EcRE, therefore it is not known how they will affect the other direct repeats and palindromic EcREs that have been identified to form a complex with USP/EcR by Vogtli et al., (1998). Sun and Song (2006) noted that when salivary glands are treated with CC, expression of 20E responsive genes is affected. In using cell culture, these genes themselves have not been tested, and therefore it is unknown what effects these mutations could have on the 20E responsive genes. Also, USP can achieve high affinity binding with other receptors (Yao et al., 1992). USP was only tested with EcR in this system. We are unable to speculate on the possible effects that these USP mutations may have on other receptors that bind to USP. S112 is in the first zinc finger, and it is feasible that changing this amino acid to an alanine could have a substantial effect on the zinc finger, but without further testing, we are unable to know what this mutation does.

Future Experiments

The results obtained from this work lead to numerous experiments in the future. Some of these mutations will need to be tested in *D. melanogaster*, in order to investigate if the mutations affect the activity of 20E responsive genes similar so the results from Sun and Song (2006) with the CC. Also, the S112A/T131A/S393A/S468A/S482A USP

mutant will need to be tested in *D. melanogaster* to look for possible phosphorylation effects. These mutations will also need to be compared for developmental effects and compared to those of usp^3 and usp^4. The N-terminal mutations will need to be tested in cell culture with a plasmid that contains the full *usp* sequence rather than the VP16 activation domain, because the mutations could possibly cause this non-transcribing version used in cell culture to produce results. The two N-terminal mutations will also be tested in *D. melanogaster*, because these mutations may also cause an effect by themselves or in combination with the DBD and LBD mutations. Lastly, these mutations should be tested with other direct repeat and palindromic EcREs to examine the response with other elements.

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