

Analysis of transcriptional activity mediated by *Drosophila melanogaster* ecdysone receptor isoforms in a heterologous cell culture system

J. Beatty*†, T. Fauth*†, J. L. Callender*,
M. Spindler-Barth† and V. C. Henrich*

*Center for Biotechnology, Genomics and Health Research, University of North Carolina-Greensboro, Greensboro, NC, USA; and †Department of General Zoology and Endocrinology, University of Ulm, Ulm, Germany

Abstract

Ecdysteroid regulation of gene transcription in *Drosophila melanogaster* and other insects is mediated by a heterodimer comprised of Ultraspiracle (USP) and one of three ecdysone receptor (EcR) isoforms (A, B1 and B2). This study revealed that the EcR/USP heterodimer displays isoform-specific capabilities. EcRB1 is normally induced with a form of USP that is missing its DNA-binding domain (DBD), although potentiation by juvenile hormone (JH) III is reduced. The EcRA and B2 isoforms, however, display almost no response to ecdysteroids with the DBD⁻ USP. A mutation, K497E, in the shared ligand-binding domain of the EcR isoforms caused elevated EcRB2-specific affinity for a canonical ecdysone response element. The effects of directed modification and mutagenesis offer a strategy for developing hypotheses and considerations for studying *in vivo* function.

Keywords: Juvenile hormone, mutagenesis, Ultraspiracle, retinoid X receptor, repression.

Introduction

The insect ecdysteroid receptor is a heterodimer comprised of two nuclear receptors, the ecdysone receptor

(EcR; Koelle *et al.*, 1991) and Ultraspiracle (USP; Henrich *et al.*, 1990; Oro *et al.*, 1990; Shea *et al.*, 1990), which are stabilized by the presence of the insect moulting hormone, 20-hydroxyecdysone (20E; Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993). The heterodimer interacts with several defined DNA sequence elements to regulate the transcription of target genes (Antoniewski *et al.*, 1993; Vogtli *et al.*, 1998; Devarakonda *et al.*, 2003). Both EcR and USP are required for the normal progression of premetamorphic development in *Drosophila melanogaster* (Henrich *et al.*, 1994, 2000; Hall & Thummel, 1998; Bender *et al.*, 1997; Li & Bender, 2000). Ecdysteroids are the only endogenous class of steroid hormones in *D. melanogaster* and other insects, and these trigger both general and cell-specific transcriptional responses (Riddiford *et al.*, 2000; Thummel, 2002).

The diversity of ecdysteroid responses found among developing *Drosophila* tissues involve three natural isoforms of EcR (A, B1 and B2), which differ only in their N-terminal *trans*-activation domain (Talbot *et al.*, 1993) and exhibit different transcriptional capabilities (Hu *et al.*, 2003). Isoform-specific mutations lying within this domain disrupt distinct and essential processes during premetamorphic development. The B isoforms have generally been associated with larval processes (Bender *et al.*, 1997; Schubiger *et al.*, 1998, 2003), and the B2 isoform most efficiently rescues larval development in EcR mutants (Li & Bender, 2001). EcRB2 is also required for proper development of the larval epidermis and the border cells of the developing egg chamber (Cherbas *et al.*, 2003). The A isoform has been implicated in the remodelling of neurones during metamorphosis (Robinow *et al.*, 1993; Truman *et al.*, 1994; Davis *et al.*, 2005), is required for normal development of wing disc margins (Cherbas *et al.*, 2003) and is essential for normal metamorphosis (Davis *et al.*, 2005). Mutations of EcR coding regions shared by the three isoforms arrest development at allele-specific times during the interval from embryogenesis to the onset of metamorphosis (Bender *et al.*, 1997) and disrupt normal oogenesis (Carney & Bender, 2000). However, the *in vivo* distribution of isoform expression is not clearly correlated with developmental

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Received 27 February 2006; accepted after revision 19 June 2006. Correspondence: Vincent C. Henrich, Center for Biotechnology, Genomics and Health Research, 3701 Humanities Hall and Research Administration Building, University of North Carolina-Greensboro, Greensboro, NC 27402–6170, USA. Tel.: +1 336 334 4775; fax: +1 336 334 4794; e-mail: vincent_henrich@uncg.edu

requirements. Several tissue-specific processes do not require a specific isoform, and in some instances do not even require the EcR N-terminal domain (Cherbas *et al.*, 2003). This complex relationship between EcR disruption and resultant phenotype belies the fact that each EcR isoform has both redundant and isoform-specific properties.

Drosophila USP also contributes to the complexity of ecdysteroid receptor function. USP is essential for normal metamorphosis (Hall & Thummel, 1998) although *usp* mutations generate phenotypes that are distinct from those caused by *EcR* mutations (Li & Bender, 2001). USP also fulfills distinct larval and metamorphic functions (Henrich *et al.*, 2000) and possesses both inducible and repressive transcriptional properties (Schubiger & Truman, 2000; Ghbeish *et al.*, 2001; Ghbeish & McKeown, 2002). Experimental studies with mammalian and insect cultures have further raised the possibility that USP's cognate ligand is juvenile hormone (Jones *et al.*, 2001; Sasorith *et al.*, 2002), although this possibility has not been reconciled with *in vivo* responses to exogenous application of juvenoids in *Drosophila* (Zhou & Riddiford, 2002; Dubrovsky *et al.*, 2004; Wilson *et al.*, 2006).

Heterologous mammalian cell cultures have been utilized frequently to assess the function of the EcR/USP dimer because such cells have no endogenous response to ecdysteroids, and become responsive to ecdysteroids only when cotransfected with EcR and either USP or its mammalian homologue, the retinoid X receptor (RXR; Christopherson *et al.*, 1992; Palli *et al.*, 2003). Such studies have already demonstrated that the individual *Drosophila* EcR isoforms are not equivalent in their performance, although all can mediate transcriptional activity in response to ecdysteroids (Mouillet *et al.*, 2001). In cell cultures, ecdysteroid response can further be potentiated by the simultaneous presence of juvenile hormone (JH) III, thus reducing the ecdysteroid dosage necessary for maximum receptor induction by about 10-fold (Henrich *et al.*, 2003). Synergistic ecdysteroid/JH effects have also been observed in insect cells (Fang *et al.*, 2005) and *in vivo* (Dubrovsky *et al.*, 2004). The effects of structurally altered receptors, individual ligands and different DNA response elements have also been tested and compared in heterologous cell culture systems (Vogtli *et al.*, 1998; Henrich *et al.*, 2003).

This study examines the relative performance of the three *Drosophila* EcR isoforms in terms of their activation by ecdysteroids via a canonical 27 KDa heat shock protein (*hsp27*) ecdysone response element (EcRE; Riddihough & Pelham, 1986), the ability of juvenile hormone to potentiate transcriptional activity of the receptor complex and EcR interaction with *Drosophila* USP in Chinese hamster ovary (CHO) cells. We also examined specific site-directed mutations in the EcR ligand-binding domain (LBD) that were previously shown to perturb the normal function of EcR in a yeast two-hybrid system (Lezzi *et al.*, 2002; Grebe *et al.*,

2003; Bergman *et al.*, 2004; Przibilla *et al.*, 2004). In the present study, the three EcR isoforms displayed properties in the cell culture system that have implications for *in vivo* analysis.

Results

Functional differences among the three *D. melanogaster* EcR isoforms were inferred by previous studies in both *Drosophila* cell cultures (Hu *et al.*, 2003) and heterologous cell culture systems (e.g. Mouillet *et al.*, 2001; Henrich *et al.*, 2003). These studies have already demonstrated isoform-specific capabilities for JH III-mediated potentiation of transcriptional activity, although JH III alone exerts no effect on receptor activity. Further, EcR/mammalian fusion proteins are responsive to ecdysteroids, but only the *Drosophila* EcRB1 isoform induces ecdysteroid-dependent transcription when partnered with RXR, the mammalian orthologue of USP (Henrich *et al.*, 2003).

Effect of DmUSP on EcR isoform activity

Three fusion proteins derived from *DmUSP* (VP16-USPI, VP16-USPII and VP16-USPIII) were used to investigate the properties of the natural *Drosophila* EcR isoforms with *DmUSP*. *DmUSP* forms a heterodimer with EcR in mammalian cell cultures, but is not capable of inducing ecdysteroid-dependent transcription. When the N-terminal domain of *DmUSP* was replaced with the VP16 activation domain, ecdysteroid-responsive transcriptional activity was observed. To measure induction, cells were incubated with 1 μ M muristerone A (murA) for 24 h, a regimen that evoked a maximal transcriptional response from all three EcR isoforms in preliminary ligand dosage studies.

The VP16-USPI fusion protein includes six amino acids in the amino-terminal domain (aa 98–103) that are conserved among all insect USP sequences along with the DNA-binding domain (DBD), hinge region and LBD of *DmUSP* (aa 104–507). The VP16-USPII construct is identical to VP16-USPI, except that these six conserved amino acids are not included. In preliminary studies, the VP16-USPI and VP16-USPII exhibited only minor differences in transcriptional activity when partnered with EcR (data not shown), indicating that the conserved portion of the N-terminal region has no measurable effect upon *hsp27*-EcRE-mediated gene expression. Therefore, VP16-USPII was used for other experiments reported here, except as noted.

When tested with the VP16-USPII fusion protein, the EcRB1 isoform showed basal and murA-induced levels of transcription that were about four-fold higher than those of the other two isoforms (Fig. 1), even though EcRB1 generated a much weaker immunostaining signal than EcRB2 on Western blots (Fig. 2a). Basal transcriptional levels of EcRA were comparable to those generated by EcRB2 but the fold-induction induced by murA was relatively

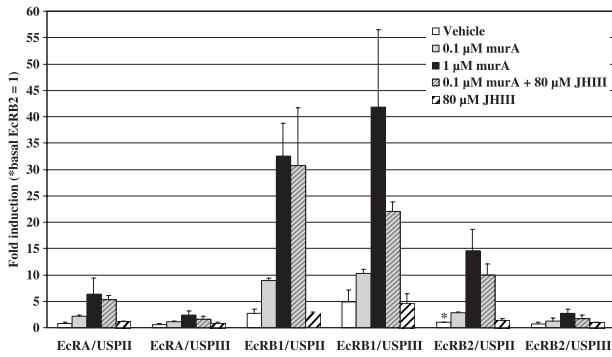


Figure 1. Transcriptional activity of three *Drosophila melanogaster* ecdysone receptor (DmEcR) isoforms (A, B1 and B2) and two VP16-*Dm* Ultraspiracle (USP) (II and III) fusion proteins. Relative luciferase unit levels were normalized with respect to β-galactosidase levels and transformed relative to the mean level of basal EcRB2 transcriptional activity (equal to 1.0, indicated by *). Standard deviations indicated are based on a sample size of 3 for each data point.

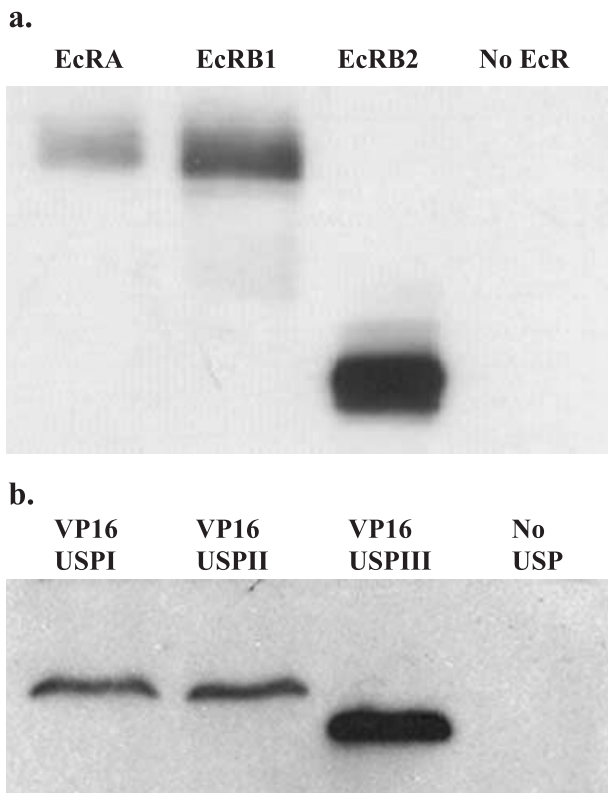


Figure 2. Immunoblots of extracts from Chinese hamster ovary cells transfected with plasmids encoding ecdysone receptor (EcR) and Ultraspiracle (USP). (a) Extracts from cells expressing EcRA, B1 or B2 vectors electrophoresed and immunoblotted with DDA2.7 monoclonal antibody against *DmEcR*. (b) Extracts from cells expressing VP16-USPI, II or III electrophoresed and immunoblotted with VP16 AD antibody (Santa Cruz Biotechnology). Samples were equilibrated based on β-galactosidase activity as a measure of cell mass.

low with the EcRA isoform. The A isoform also generated the weakest immunostaining signal on Western blots, raising the possibility that the weak inducibility could be related to low levels of EcRA expression. When this hypothesis was tested, 250 ng of EcRA-encoding plasmid transfected into cells evoked a maximal level of basal and induced levels of EcRA-induced transcription (Fig. 3). In other words, the relatively weak inductive response of EcRA expression was not caused by a rate-limiting level of available EcRA in the cell, because fold-induction reached a maximal response when as little as 10 ng of EcRA-encoding plasmid was transfected into cells. As in a previous study with a different USP construct, EcRB2 showed the strongest potentiation response to JH III when measured as fold-induction with a 0.1 μM concentration of murA (Henrich *et al.*, 2003).

The VP16-USPIII fusion protein encodes the VP16 AD attached directly to the hinge region and LBD of *DmUSP* and is analogous to the *Choristoneura fumiferana* (*Cf*) USP fusion protein utilized for a previous study (Henrich *et al.*, 2003). The dispensibility of a functional *DmUSP* DBD for EcRB1-mediated, ecdysteroid-inducible transcriptional activity via an *hsp27* EcRE has been demonstrated previously (Ghbeish *et al.*, 2001). Because the *CfUSP* construct had evoked a relatively robust response with all of the three EcR isoforms, it was hypothesized that the equivalent VP16-USPIII construct would behave similarly.

For the A and B2 isoforms, however, VP16-USPIII produced a reduced basal transcriptional level, a severe reduction in responsiveness to murA and the elimination of any potentiation effect of JH III. Only the B1 isoform conformed to predictions by showing an elevated responsiveness to murA when paired with either VP16-USPII or VP16USPIII, although the potentiating effect of JH III with the EcRB1/VP16USPIII dimer was reduced significantly (Fig 1, *t*-test, *P* < 0.05). A reduction of potentiation by about

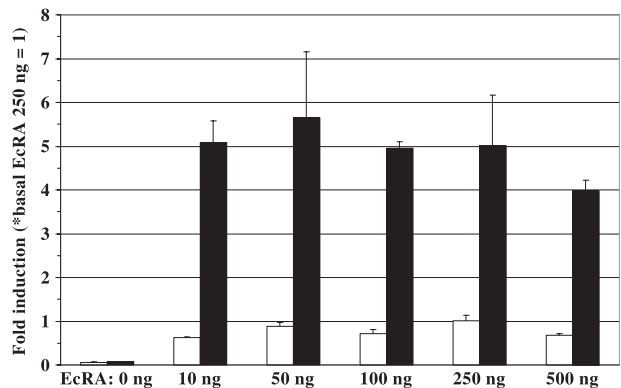


Figure 3. Effects of transfection amounts (0–500 ng) of plasmid vector encoding ecdysone receptor A (EcRA) on basal (unshaded) and muristerone A-induced (1 μM; shaded) levels of transcription with 100 ng VP16-USPII. Fold induction is compared to EcRA in the absence of hormone.

one-half relative to induction by 1 μM murA alone was also observed in two controlled replicates of this experiment.

The activity, as measured by fold-induction, seen with the previously employed VP16-*Cf*USPIII resembled the effects of VP16-USP II in this study, although only the *Cf* form lacks the USP DBD. Western immunostaining of the VP16 domain in cell extracts transfected with the USP constructs generated bands of predicted size and similar density, thus confirming the integrity and expression of the USP constructs used (Fig. 2b).

Effects of site directed mutations on isoform function

Mutant EcR proteins produced by site-directed mutagenesis offer the opportunity to assess the effect of specific structural changes upon receptor function, and potentially can locate specific functional attributes based on the defects caused by the mutation. A fraction of EcR LBD mutations previously tested in a yeast two-hybrid system impair specific receptor characteristics (Lezzi *et al.*, 2002; Grebe *et al.*, 2003; Bergman *et al.*, 2004). Two site-directed mutations described in these studies were tested in each of the three EcR isoforms to determine whether the substitutions evoked the same effect in whole receptors as they did in analogous yeast two-hybrid fusion proteins. Further, these common region mutations were tested in each isoform to explore the possibility that a common region mutation differentially disrupts isoform function.

The K497E mutation. The K497 residue lies in helix 4 and aligns with a consensus cofactor binding site in nuclear receptors. The site has also been implicated in the formation of a salt-bridge with helix 12 to mediate ligand-dependent transcriptional activity (Wurtz *et al.*, 1995a,b). Two different substitutions of this residue, K497A and K497E, result in a dramatically elevated level of ligand-independent transcriptional activity in the yeast two-hybrid system (Bergman *et al.*, 2004). The similar effects caused by two different mutations of K497 strongly suggest that the elevation of basal transcription results from a loss of function normally associated with the K497 residue, although ecdysteroid binding affinity in the mutant fusion protein is actually reduced (Grebe *et al.*, 2003).

When the *Dm*EcR isoforms carrying the K497E mutation were tested with VP16USP II, the basal rate of transcription was substantially elevated in the EcRB2(K497E) compared to wild-type EcRB2 (*t*-test, $P < 0.001$), as predicted from the yeast two-hybrid study, but no significant effect on basal transcription was observed in either EcRA(K497E) or EcRB1(K497E). MurA-induced levels, however, were similar to wild-type levels in all three isoforms (Fig. 4).

In order to test whether this heightened activity resulted from an abnormally high affinity for *Dm*USP, a competition experiment was devised by which increasing amounts (0–500 ng) of the plasmid encoding EcRB2(K497E) were

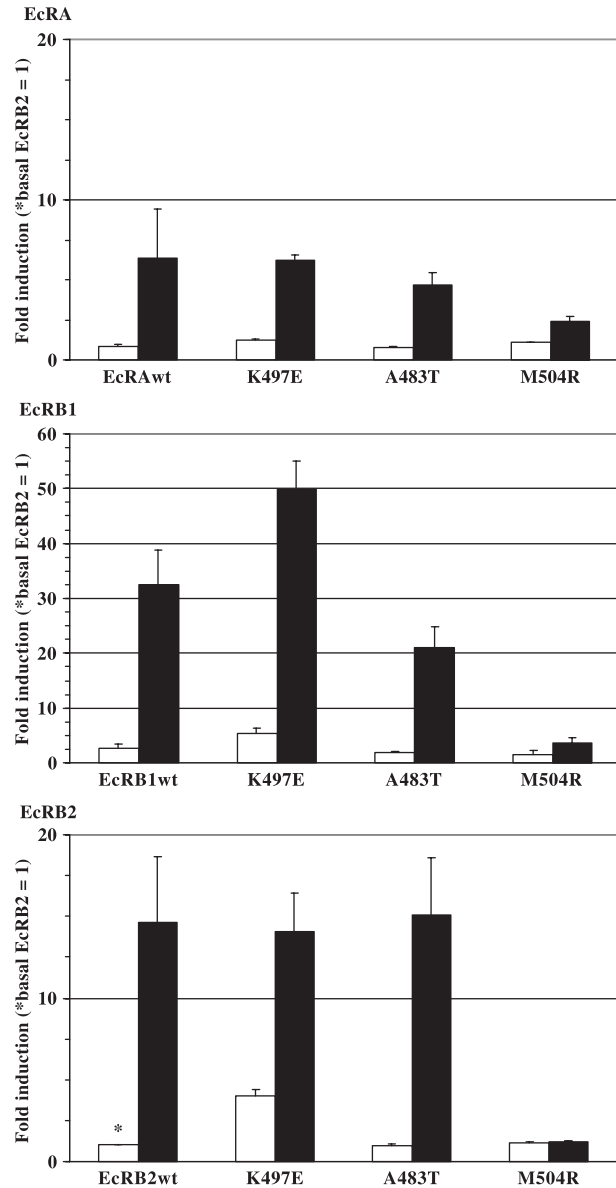


Figure 4. Effects of three site-directed mutations on basal and muristerone A (murA)-induced levels of transcription on three ecdysone receptor (EcR) isoforms of *Drosophila melanogaster*. All relative luciferase unit values were normalized according to β -galactosidase activities and were transformed for all data points relative to mean EcRB2 activity in the absence of hormone. Therefore, fold-induction levels for all three graphs are comparable to a common reference level (basal EcRB2 = 1, indicated by *). EcRB1 scaling is reduced to account for higher quantitative levels of transcription. Each data point shows the standard deviation based on a sample size of 3 or more. Unshaded boxes indicate transcriptional levels in the absence of murA, and darkened boxes indicate transcriptional activity in the presence of 1 μM murA.

transfected along with a fixed amount of the plasmid containing the nonmutated EcB2 isoform (100 ng) and VP16-USP II (100 ng). Transcription was then observed in the absence of murA (Fig. 5). When equimolar amounts of EcR-B2 (K497E) and wild-type EcR-B2 were mixed

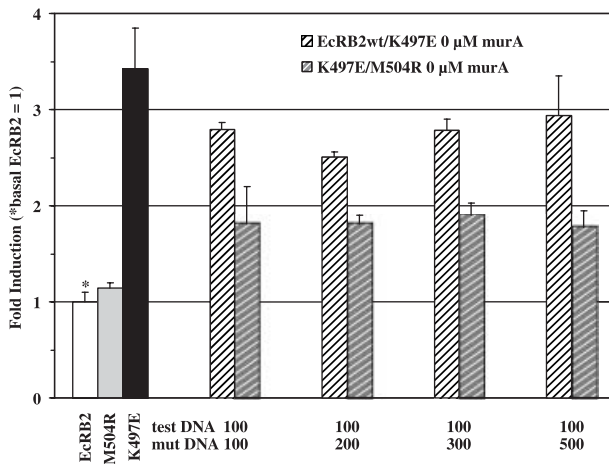


Figure 5. Basal transcription levels resulting from transfection with 100 ng of test ecdysone receptor B2 (EcRB2) and varying levels (100–500 ng) of mutant EcRB2 isoforms. Levels of M504R and K497E mutants are shown in leftmost bars with no test EcR. EcRB2(K497E) was used as a proxy for wild-type EcRB2 to compete with EcRB2(M504R) because the K497E mutation confers a high basal level of activity allowing for competition analysis to be performed. All data points based upon three replicates plotted with standard deviation.

together, the observed basal transcriptional activity was intermediate between the levels produced by each one alone. In other words, EcRB2 displaced EcRB2(K497E) when competing for dimerization with VP16-USP11 (Fig. 4), confirming that the mutant EcR does *not* have a heightened affinity for *Dm*USP.

The substantially elevated transcription associated with B2 (K497E) is not found in any of the isoforms carrying the A483T mutation, which in turn, corresponds to an *in vivo* larval lethal mutation that disrupts a site required for physical interaction between EcR and the corepressor, SMRTER (Tsai *et al.*, 1999; Carney & Bender, 2000; Fig. 4). Interestingly, A483T did not discernibly affect basal transcriptional activity of the B1 isoform, but significantly reduced its ligand-induced transcriptional activity (*t*-test, $P < 0.01$).

The three K497E isoforms were also tested by electrophoretic mobility shift assay (EMSA) to determine whether the elevation of transcription in the mutated B2 is associated with a higher affinity for the *hsp27* response element. The shift revealed that the B2(K497E) isoform has a stronger affinity for the *hsp27* EcRE in the absence of hormone than any of the wild-type isoforms or the mutant A or B1 isoforms (Fig. 6). Unexpectedly, none of the K497E mutated isoforms showed a high level of affinity for the *hsp27* EcRE in the presence of murA, even though wild-type EcR isoforms showed this elevated affinity and transcriptional activity in the mutants approximated the levels found in the equivalent wild-type EcR. This paradoxical result is currently unexplained. Reduced ligand affinity caused by the K497E

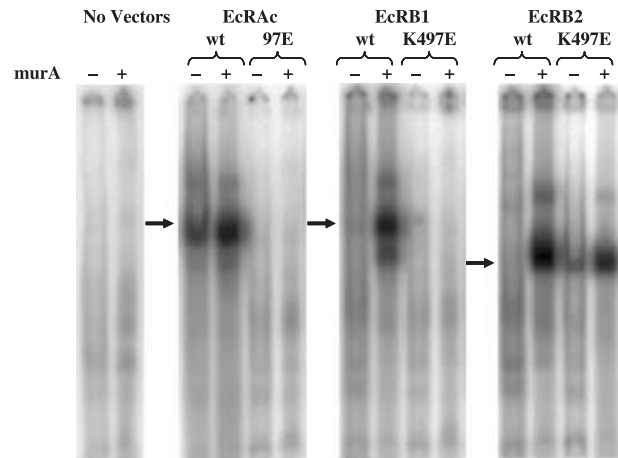


Figure 6. Electrophoretic mobility shift assay using 27 KDa heat shock protein ecdysone response element (EcRE) tested with extracts from Chinese hamster ovary cells transfected with VP16-USP11 and wild-type or EcR (K497E) vectors encoding *Dm*EcRA, B1 and B2 isoforms.

substitution (Grebe *et al.*, 2003) and/or the absence of a contextual sequence surrounding the EcRE element may have obliterated the shift of the mutant EcR complex.

The M504R mutation. Ecdysteroids stabilize the EcR/USP heterodimer which in turn, recognizes promoter elements to regulate transcription. It follows that a receptor unable to bind to a hormone is also unable to be stabilized by the hormone, and ultimately, fails to elevate transcription in the presence of the hormone. Based on alignments of the nuclear receptor superfamily, a mutation was made at a consensus site for ligand binding (M504) in helix 5 of *Dm*EcR (Wurtz *et al.*, 1995a,b). This site corresponds with a predicted ligand-binding site in the retinoic acid receptor (RAR; Bourguet *et al.*, 2000), although this site does not correspond to any that are predicted to contact ecdysteroids in the *Heliothis* EcR model (Billas *et al.*, 2003). When tested for heterodimerization using a yeast two-hybrid assay, the mutant EcR showed normal dimerization affinity for USP in the absence of hormone, but the elevated rate of heterodimerization associated with ligand binding is abolished, and in fact, ligand binding is also eliminated in this mutant (Grebe *et al.*, 2003).

Consistent with those two-hybrid results, basal levels of transcription in this study were unaffected by the mutation in any of the three natural EcR isoforms whereas murA-induced rates of transcription were almost completely eliminated (Fig. 4). In the absence of hormone, EcRB2(M504R) competed with EcRB2(K497E), which in turn, competed with wild-type EcR and served as a proxy for wild-type dimerization. In the absence of hormone, transfection with equal amounts of plasmid encoding K497E and M504R mutant EcRB2 (100 ng of each) reduced the abnormally

high level of transcriptional activity caused by the K497E mutant (Fig. 5). In other words, the EcRB2(M504R) mutant dimerized normally with *Dm*USP in the absence of hormone. On the other hand, the M504R mutant receptor failed to displace wild-type receptor in the *presence* of *murA*. Fold induction was 9.50 ± 1.90 in cells transfected with 100 ng of wild-type EcRB2-expressing plasmid, and did not change significantly even when 500 ng of EcRB2 (M504R)-expressing plasmid was also added (fold induction: 7.35 ± 0.61). This strongly suggests that the ligand-bound, wild-type EcRB2 has an intrinsically higher affinity for USP than the EcRB2 (M504R) receptor (Fig. 5), which is incapable of ligand-binding (Grebe *et al.*, 2003). This result is also consistent with the supposition that when an ecdysteroid binds to EcR, the affinity of EcR and USP is increased.

Discussion

The *in vitro* analysis described here is intended to establish a basis for assessing the *in vivo* function of *D. melanogaster* EcR and USP, with the ultimate aim of understanding how the heterodimer governs individual transcriptional processes underlying development. These studies, along with those reported earlier, have shown that the native EcR isoforms possess both shared and unique characteristics. Further, an analysis of common region EcR mutations led to the identification and characterization of two site-directed mutations in EcR that affected specific molecular functions, one of which also specifically disrupted EcRB2.

The interaction with USP is not equivalent for the three EcR isoforms

These experiments showed that the USP DBD is essential for both ecdysteroid-inducible transcription and JH III potentiation mediated by EcRA and EcRB2 via an *hsp27* EcRE. By contrast, the absence of USP DBD did not impair EcRB1 responsiveness to *murA*. EcR/USP interactions with the *hsp27* EcRE have shown that the USP DBD binds to the 5' half-site and recruits EcR to the 3' half-site (Grad *et al.*, 2001). The ability of EcRB1 to maintain its transcriptional inducibility when USP lacks its DBD is consistent with previously reported results (Schubiger & Truman, 2000; Ghbeish *et al.*, 2001) and suggests that this isoform can interact with an *hsp27* EcRE differently from the A and B2 isoforms. These results further suggest that EcRB1 is specifically responsible for the *in vivo* appearance of ecdysteroid-inducible gene expression in *usp* mutant tissues whose alleles impair USP DBD function (Henrich *et al.*, 1994; Schubiger & Truman, 2000). These same *usp* mutations destroy the normal *in vivo* repression of *Broad-Complex* and β FTZF1 gene expression (Schubiger & Truman, 2000; Ghbeish *et al.*, 2001; Ghbeish & McKeown, 2002). There is evidence that the EcR/USP dimer acts in a ligand-independent fashion to block specific developmental

pathways (Schubiger *et al.*, 2005) and the results reported here further indicate that these interactions with mutant USP products vary among the three isoforms.

While the ability of EcRB1 to respond to *murA* did not require USP to have a DBD, JH potentiation of EcRB1 was reduced by about half when the DBD was removed from USP. This observation argues that ecdysteroid inducibility and JH potentiation of the EcR/USP complex are functionally separable. Finally, the disparity between equivalent USP constructs from different insect species (*C. fumiferana* and *D. melanogaster*) highlights the possibility that species-specific aspects of the USP LBD are important for ecdysteroid receptor function, and by corollary, that the CHO cell culture system can delineate functional differences in ecdysteroid receptor activity for different insect species.

The three isoforms display differences in transcriptional capabilities

The differences observed in the reported characteristics of the three EcR isoforms with a canonical *hsp27* EcRE imply that the three isoforms show discernibly different *in vivo* capabilities. By extension, it is conceivable that the three isoforms also vary in their affinity for other ecdysone response elements and natural ecdysone-responsive promoters. The dimerization interface of EcR and USP likely depends on the nature of the response element, because direct repeat elements require a different relative orientation than the *hsp27* element that was used in this study (Perera *et al.*, 2005). In turn, this may involve important functional differences that can be genetically dissected.

An unexpected indication of isoform-specific function is revealed by the effects of the K497E mutation. The most notable molecular effect of the mutation is that affinity of the B2(K497E) mutant receptor for the *hsp27* EcRE is strongly and specifically elevated in the absence of hormone. While the possibility that this mutation has destroyed a cofactor binding site cannot be formally ruled out, there is no difference in the size of the mutant and wild-type B2 complex assessed by EMSA, as might be expected if a cofactor interaction were involved. Moreover, if a cofactor is involved in this mutational effect, it must occur in mammalian and yeast cells, because the mutation evokes similar effects in both systems. The failure to detect a shift in any of the K497E-mutant isoforms in the presence of hormone, however, leaves open the possibility that other proteins and promoter elements influence transcriptional activity, and that they are unable to interact with the mutant receptor.

Cell culture competition experiments reported here have also shown that K497E has roughly normal dimerization capabilities. Therefore, the high basal transcriptional activity caused by the K497E mutation in a yeast two-hybrid system likely results from a high affinity of the EcR/USP dimer for a response element, rather than a high affinity between the two LBDs. The K497E mutant EcR retained

responsiveness to murA although ligand-binding is impaired (Grebe *et al.*, 2003), which may be the basis for the reduced fold-induction in K497E mutant receptors.

Several possible explanations, which are not mutually exclusive, may account for the isoform-specific effects of the K497E mutation. Most plausibly, the mutation affects the ligand-dependent transcriptional function (AF2) associated with helix 12, which normally interacts with the K497 region in the presence of an activating ligand. The exact nature of this effect is unknown, although the removal of the basic lysine charge caused by either an alanine or glutamic acid substitution conceivably promotes a continuous holo-conformation in helix 12 and quasi-AF2 transcriptional activity. A related possibility is that the mutation disrupts an interaction normally occurring between K497 and the B2 domain. By corollary, the isoform specificity could arise from a steric hindrance that prevents a similar interaction between K497 and the larger N-terminal domains of A and B1.

From a regulatory standpoint, the effect of K497E clearly illustrates that normal transcriptional activity is not synonymous with maximal transcriptional activity for EcR. Whatever the mechanistic basis for the high basal activity, the effects of K497E further indicate that basal transcriptional activity normally includes a repressive process, which is mutationally subverted. The K497E receptor activity profile contrasts with the one previously described for the A483T mutation associated with larval lethality (Carney & Bender, 2000) and *in vitro* corepressor binding (Tsai *et al.*, 1999). Given these distinctions between the two mutational effects, it is conceivable that different molecular mechanisms confer a repression of receptor activity, and that these are genetically separable.

Mutational effects and phenotypic analysis

The mutations described in this study were selected to investigate specific attributes as a basis for subsequent mutational analysis in *Drosophila*. Receptor functionality is actually a composite of numerous subfunctions including interactions with DNA, receptor partners, ligand, transcriptional cofactors and chaperones (Arbeitman & Hogness, 2000), as well as interdomain interactions within each receptor. Therefore, mutations that selectively disrupt specific EcR functions can be employed to assess their effects upon individual developmental processes. By contrast, *in vitro* studies have led to the implication that *in vivo* missense mutations of the EcR LBD (Bender *et al.*, 1997) are hypomorphic, causing partial and nonspecific impairments of dimerization, ligand-binding capability and transcriptional response (Bergman *et al.*, 2004). If so, larval lethality induced by such EcR mutations apparently results from chronically deficient ecdysteroid receptor function.

While the validity of the *in vitro* capabilities reported here for *in vivo* events will depend upon specific experiments,

some consistencies in performance have been noted. For instance, EcRB1 not only mediates the highest level of transcriptional activity in cell cultures, it is also the only isoform that completely restores polytene puffing in the *Drosophila* larval salivary gland of EcR- mutants (Bender *et al.*, 1997). Alternatively or additionally, the unique EcRB1/USP interaction seen in cell culture may reveal a transcriptional response in salivary glands that the other isoforms are unable to perform as efficiently. Similarly, EcRB2 is the only isoform potentiation by JH using 20E as an agonist as well as the most effective isoform for rescuing embryonic lethality in EcR-null mutations through the larval stages, during which both juvenile hormone and ecdysteroid titres are periodically elevated (Henrich *et al.*, 2003). Taken together, the ability of B2 activation to be maximized by the presence of JH may be particularly important for larval-larval transitions, because ecdysteroid peaks during this developmental period could be too low by themselves to trigger some ecdysteroid-inducible responses. JH itself is derived from the insect mevalonate pathway (Belles *et al.*, 2005), which in turn is driven by the intake of dietary nutrients. The ability of JH to potentiate ecdysteroid activity suggests that this is a functional interface between the nutritional state of feeding larvae and the hormonal processes that trigger moulting processes. When the A and B1 *Manduca* EcR isoforms are expressed together in a *Manduca* cell line, ecdysteroid-responsive gene expression is reduced, suggesting a difference in transcriptional capabilities consistent with those observed for the *Drosophila* EcRA and B1 isoforms in this study (Hiruma & Riddiford, 2004).

In summary, the characterization of the ecdysteroid receptor complex noted here represents a systematic approach for classifying the effects of EcR and USP mutations on specific subfunctions. Transgenic flies expressing these mutant EcR isoforms can now be tested for their ability to rescue essential functions destroyed by endogenous EcR mutations and also, for their effects upon ecdysteroid-dependent transcriptional activity.

Experimental procedures

This study employed Chinese hamster ovary (CHO) cells to evaluate the transcriptional activity mediated by *Drosophila melanogaster* EcR and USP using methods described previously (Tsai *et al.*, 1999; Henrich *et al.*, 2003). Unless noted otherwise, 250 ng of each EcR and USP-encoding plasmid was transfected into 2 ml of cell culture medium for these experiments. As described in Heinrich *et al.* (2003), a reporter gene carrying five tandem copies of the *hsp27* EcRE (Riddihough & Pelham, 1986) is attached to a constitutive thymidine kinase promoter and a luciferase reporter gene (pEcREtk-LUC). Vectors expressing EcR, USP, the reporter gene and a constitutively active CMV promoter fused to a β -galactosidase gene were co-transfected by lipofection (Gene Therapy Systems Inc, San Diego, CA) into the CHO cells following

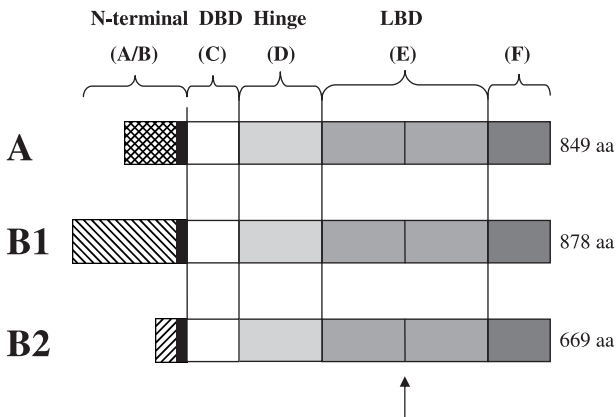


Figure 7. Schematic diagram of the three isoforms of ecdysone receptor (EcR) investigated in this study. A/B indicates isoform-specific N-terminal domain which includes a commonly shared region designated in black. C indicates DNA-binding domain (DBD), D indicates hinge region, E designates ligand-binding domain (LBD), and F designates a carboxy-terminal domain. C–F domains are shared by A, B1 and B2 isoforms. Arrow designates approximate location of K497 residue discussed in text.

the manufacturer's protocols. Transcriptional activity mediated by the *hsp27* EcRE was measured by detecting the luminescence produced by luciferase in extracts from transfected cells and cell mass was determined by measuring β -galactosidase activity in these extracts. The EcR isoform vectors (A, B1 and B2) used for this study have been described previously (Fig. 7; Mouillet *et al.*, 2001; NT033778). Additionally, three different VP16-USP vectors were utilized in this study and their construction is described below.

After a 4 h transfection period, the hormone treatment was added by diluting murA (Alexis Corporation, San Diego, CA), JH I (Scitech, Prague, Czech Republic), and/or JH III (Sigma, St Louis, MO) dissolved in dimethylsulphoxide (DMSO) to a final culture medium concentration of 0.1% DMSO. The cells were harvested 24 h after treatment and the contents of the cells extracted for measuring the luciferase and β -galactosidase activities.

Normalization of data points

Luciferase activity was normalized by weighting its activity relative to the constitutive expression of the β -galactosidase gene, whose enzymatic activity is proportional to cellular mass. These values were expressed as relative luciferase units (RLUs). These data were further transformed so that the mean value of a designated control group for each experiment was assigned a value of 1. Using these normalized values, standard deviations were calculated based on at least three independent samples for all data points. Fold-induction is therefore the ratio of mean normalized RLU values of an experimental group relative to the mean normalized RLU value of a designated control group. Individual values were tested to determine whether significant differences existed between specific comparable groups (*t*-test, $P < 0.05$).

Construction of USP vectors

USP constructs were made by subcloning three different *D. melanogaster* USP inserts into the pVP16 vector (Clontech,

Mountain View, CA). The resulting vector encodes a fusion protein consisting of the viral protein 16 (VP16) activation domain attached to various carboxy-terminal fragments of the USP open reading frame. The USP portion of each fusion gene was isolated by PCR from a plasmid, pZ7-1 (Henrich *et al.*, 1990; AY069393). Each forward primer was tailed with an *EcoRI* restriction site on the 5' end and the reverse primer (dUSPR) was tailed with a *HindIII* site on its 5' end.

The VP16-USPI vector includes the last six amino acids of the N-terminal domain and the remaining carboxy-terminal portion of the *DmUSP* open reading frame (amino acids 98–507; Henrich *et al.*, 1990). This portion also encodes the two cysteine–cysteine zinc fingers of the DBD, the hinge region, and the LBD. The fragment was isolated by PCR using a forward primer, 5'-TTTTGAATTC-AGCGGCAGCAAGCACCTCTGC-3' and the reverse primer (dUSPR): 5'-TTTTAAGCTTTAGAGTCGGGACCCCTACTCC-3' (underlining designate *EcoRI* and *HindIII* restriction sites, respectively). The slightly shorter VP16-USPII vector does not include the N-terminal amino acids, starts at the USP DBD and codes for amino acids 104–507. The USPII insert was isolated using the forward primer, 5'-TTTTGAATTCCTGCTCTATTTGCGGGGATCGG-3' with the aforementioned dUSPR reverse primer. A third vector, VP16-USPIII, codes for amino acids 170–507 and resembles the VP16-USPIII except that it lacks the USP DBD. It was generated using a forward primer with the sequence 5'-TTTTGAATTC-AGCGCGAAGCGGTCCAGGAG-3' and the dUSPR primer. After an initial 5 min melting step at 94 °C, PCR was used to amplify the inserts under the following cycling conditions: 94 °C melting for 1 min, 58 °C annealing for 1 min, 68 °C extension for 2 min, over 30 cycles.

The PCR products and the pVP16 vector were double digested with the *EcoRI* and the *HindIII* restriction enzymes, the samples were electrophoresed in a 1% agarose gel and the appropriate bands were excised from the gel (Qiagen, Valencia, CA). The gel-extracted PCR products were ligated into the pVP16 vector using T4 ligase (New England Biolabs, Beverly, MA) and the ligation mixture was added to 45 μ l Ultracompetent XL10-Gold *E. coli* (Stratagene, La Jolla, CA). The cells were then streaked on to Luria-Bertani (LB) agarose plates and transformants selected with ampicillin and grown with selection in liquid culture. Plasmid DNA was extracted and the vectors verified by restriction analysis and DNA sequencing.

Site directed mutagenesis

Site-directed mutations were produced in each of the three EcR isoform vectors by changing one or two nucleotides in the codon corresponding to a specific amino acid position using a Quik-Change II site-directed mutagenesis kit (Stratagene). The presence of the mutations and the integrity of the remaining coding region of the protein were verified by sequencing. The mutations K497E, M504R and A483T were made in each of the three EcR isoforms using the following primers and their reverse complements:

K497E: 5'-CAG ATC ACG TTA CTA GAG GCC TGC TCG TCG G-3'

M504R: 5'-CTC GTC GGA GGT GAG GAT GCT GCG TAT G-3'

A483T: 5'-G TTT GCT AAA GGT CTA CCA ACG TTT ACA AAG ATA CCC CAG G-3'.

After subsequent transformation of the mutagenesis reaction mixture in the Ultracompetent XL10-Gold *E. coli*, the transformants were cultured as described previously and plasmid DNA extracted using a Qiagen midi-prep kit.

Electrophoretic mobility shift assays

Extracts transfected with the appropriate EcR and USP vectors were prepared according to the method described in Kitareewan *et al.* (1996). A double stranded *hsp27* response element probe was constructed using the forward primer 5'AGCGACAAGGGT-TCAATGCACTTGT-3' and the complementary reverse primer. The probe was end-labelled by fill-in reaction with the Klenow fragment and [α - 32 P]-dCTP according to published protocols (Mouillet *et al.*, 2001). After labelling, the probe was purified by centrifugation, applied to an affinity column and purified following the manufacturer's instructions (Mini Quick Spin Columns, Roche Applied Sciences, Indianapolis, IN).

The binding reactions were prepared as reported previously (Mouillet *et al.*, 2001). Protein extracts were added to the binding reactions after normalization by -galactosidase reporter gene activity. A Bradford assay measured the total protein content for these extracts between 100 and 150 μ g (Bradford, 1976). An 11 μ l volume of cell extract with the lowest -galactosidase reporter gene activity was added to the binding reaction. All other extracts were added proportionally to the reaction based on -galactosidase activity relative to the one with the lowest activity. murA (Alexis Biochemicals, San Diego, CA) dissolved in ethanol to a final concentration of 10 μ M or an equivalent volume of ethanol was then added to each reaction mixture. Finally, approximately 30 000 cpm of the radio-labelled *hsp27* element was added to the binding reaction and incubated with the extract for 20 min at room temperature. A 5% native polyacrylamide gel was used to separate the complexes.

Western blots

Cellular extracts with equal amounts of β -galactosidase activity were loaded on to lanes of a 15% polyacrylamide gel and subjected to electrophoresis (Biometra, Gottingen, Germany) at 15 mA. The gel was then electroblotted (MiniVE Blotter Module, Amersham Pharmacia Biotech, Piscataway, NJ) on to a nitrocellulose membrane (NC 45, 0.45 μ m, Serva, Heidelberg, Germany) at 300 mA and 20 V. The membrane was then soaked in blocking buffer (3% (w/v) milk powder, 1% (w/v) BSA, 20 mM Tris/HCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6). EcR was probed with the monoclonal IgG mouse antibody DDA2.7 diluted 1 : 1000 in blocking buffer; this antibody recognizes the D-domain shared by all three *DmEcR* isoforms (Koelle *et al.*, 1991). A peroxidase-conjugated secondary antibody (anti-mouse IgG, Sigma-Aldrich, Taufkirchen, Germany) was diluted 1 : 1000 (20 mM Tris/HCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6) to detect specific signals. The membrane was exposed to X-ray film (Hyperfilm, Amersham) and the image developed.

For USP Western blots, similar protocols were followed and a monoclonal mouse IgG antibody that detects the N-terminal VP16 domain was utilized (Santa Cruz Biotechnology, Inc; Santa Cruz, CA) after a 1 : 200 dilution in blocking buffer.

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