Developmental Effects of a Chimeric *ultraspiracle* Gene Derived From *Drosophila* and *Chironomus*

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Abstract:

The *ultraspiracle* (*usp*) gene encodes a nuclear receptor that forms a heterodimer with the ecdysone receptor (EcR) to mediate transcriptional responses to the insect steroid hormone, 20-hydroxyecdysone (20HE). The responses ultimately elicit changes associated with molting and metamorphosis. Although Ultraspiracle (USP) is required at several developmental times, it is uncle: whether USP plays stage-specific roles in *Drosophila*. A chimeric transgene (*d/cusp*), produced by replacing the ligand-binding domain (L11311)) of *Drosophila* USP with the equivalent domain from another 11)iptera, *Chironomus tentans*, was tested for its ability to rescue *Drosophila usp* mutants from early larval lethality. A single copy of the *d/cusp* was sufficient to rescue transformants from several lines through larval development but they died suddenly during the late third instar. Additional doses of *d/cusp* were required to allow survival through the adult stage, but they did not restore a normal prepupal contraction. Thus, the arrest at the onset of metamorphosis apparently is caused by the impaired ability of the chimeric USP to mediate a stage-specific function associated with the L11311).

Keywords: ecdysteroid; nuclear receptor; heterodimerization; metamorphosis; chimera; retinoid X receptor (RXR)

Article:

The transcriptional response to ecdysteroids in *Drosophila melanogaster* and other insects requires the action of two nuclear receptor superfamily members (Thomas *et al.*, 1993; Yao *et al.*, 1993), the ecdysone receptor (EcR) and Ultraspiracle (USP). Simplistically, the entry of 20-hydroxyecdysone (20HE) into the cell stabilizes the formation of the EcR/USP heterodimer, which in turn, binds to ecdysone response elements (EcREs) scattered among several gene promoters, although the timing and nature of ecdysteroid-inducible expression is highly variable among cell types (Andres *et al.*, 1993; Huet *et al.*, 1993). At the organismal level, these differences are manifested as the diversity of cellular responses associated with metamorphosis. How this single hormonal signal induces both stage- and cell-specific responses is a major current focus of investigation.

EcR contributes to this diversity through multiple isoforms that are differentially expressed and perform distinct developmental functions (Bender *et al.*, 1997; Talbot *et al.*, 1993), and also interacts with other nuclear receptors to regulate downstream aspects of ecdysteroid-induced transcription (White *et al.*, 1997). Unlike EcR, USP expression varies only modestly during larval development in *Drosophila* and so far, only one form has been identified (Henrich *et al.*, 1994) though multiple forms and more complex regulation have been observed in other insects (Hiruma *et al.*, 1999; Lan *et al.*, 1999; Vogtli *et al.*, 1999).

Drosophila USP participates in both the activation and repression of gene expression (Schubiger and Truman, 2000) and is necessary for both larval and metamorphic development (Hall and Thummel, 1998; Oro *et al.*, 1992). A potential repressive role for USP in eye and neuronal development has been observed (Schubiger and Truman, 2000; Zelhof *et al.*, 1995b), and USP shows biochemical properties suggesting that it is a juvenile hormone receptor (Jones and Sharp, 1997). In *Manduca*, feedback inhibition of ecdysteroid biosynthesis has been associated with phosphorylation of USP (Song and Gilbert, 1998). It is unclear whether USP simply participates in these regulatory and developmental processes or whether USP plays specific and diverse roles for mediating these events. Like its RXR orthologue, USP forms heterodimers with at least two other orphan receptors in *Drosophila*, DHR38 (Sutherland *et al.*, 1995) and Sevenup (SVP; Zelhof *et al.*, 1995a), inferring that USP has specific roles.

Table 1
*Summary of Individual Lines Transformed With
Drosophila/Chironomus usp Transgene**

Line identification	Insertion chromosome	Homozygotic lines?	Lethal stage in *usp* mutants
12B	2nd	Yes	Late larval
19C	2nd	Yes	Late larval
65J	X	Yes	Late larval
67A	X	Yes	Early larval
71D	2nd	Yes	Late larval
72B	X	Yes	Early larval
83C	2nd	Yes	Late larval

*All lines were derived from independently injected embryos as described in Materials and Methods.

Genetic studies have been limited because all of the reported *usp* mutations involve disruptions of the DNA-binding domain and cause early larval death (Henrich *et al.*, 1994; Oro *et al.*, 1990). So far, no in vivo mutations that disrupt the function of the USP ligand-binding domain (LBD) have been reported, although subregions within this nuclear receptor domain are important for ligand binding, dimerization, and cofactor interactions (Mangelsdorf and Evans, 1995; Nagy *et al.*, 1999). Numerous experiments have previously demonstrated that RXR's interaction with different partners is not equivalent (Miyamoto *et al.*, 1997), indicating that RXR, and by analogy, USP, plays multiple and distinct molecular roles mediated through structural features of the LBD that may ultimately be manifested as specific developmental roles (Botling *et al.*, 1997; Mangelsdorf and Evans, 1995).

In this study, the *Drosophila usp* gene under the control of its own promoter was modified by replacing its LBD with the equivalent portion of the *Chironomus usp* gene (Vogtli *et al.*, 1999). Although there is considerable similarity between the two USP sequences in their LBD, there are sufficient differences that the resultant chimeric USP can be viewed as a structural "mutation" of the *Drosophila* USP LBD. Among several transformant lines, a single dose of *d/cusp* in *usp* mutants restores a vital function during larval stages, but multiple doses are required for survival through metamorphosis. By contrast, a single dose of the wild-type *usp* gene rescues the entire life cycle (Oro *et al.*, 1990).

RESULTS
Recovery of Individual Transformant Lines
Seven transformant lines of *Drosophila melanogaster* were recovered and stabilized that carried the *Chironomus/Drosophila* chimeric *usp* gene (*d/cusp*) as noted in Table 1. None of the transformed *d/cusp* transgenes exerted a discernible impact on development when maintained in a wild-type background. In other words, there was no indication that the chimeric USP behaves as a dominant negative by interfering with functions of the *Drosophila* USP. The *d/cusp* transgene was then tested for its ability to rescue mutant *usp* larvae, which normally die in the first instar (Perrimon *et al.*, 1986). In five of the transformant lines, *usp* mutants survived normally through larval stages when carrying a single copy of *d/cusp*, but their

development stopped suddenly at the late larval/prepupal transition. Two of these *usp* mutant lines carrying the *d/cusp* transgene (19C and 71D) were selected for more thorough study.

Larval Rescue of *usp* Mutants by a Single Dose of *d/cusp*

A single copy of the wild-type *usp* gene under the control of its own promoter is sufficient for rescue of *usp* mutants through the entire life cycle (Oro *et al.*, 1990). The chimeric *d/cusp* gene was similarly tested in *usp* mutant larvae for its ability to replace mutated function caused b three different alleles (usp^2, usp^3, and usp^4); the usp^2 allele is a presumed null mutation (Oro *et al.*, 1990). During larval development, all mutants carrying a single copy of *d/cusp* developed at a normal temporal rate and reached the late third instar as fully sized larvae. However, in all mutational backgrounds, these larvae suddenly experienced a developmental arrest in the late third instar. The arrest was accompanied by a sudden cessation of movement resemblant of the stationary phase described for *usp* mutants who had been rescued by heat-shock induced expression of dUSP during early larval stages. In that regime, late larval arrest is caused by the dissipation of USP gene product as the onset of metamorphosis approaches (Hall and Thummel, 1998).

The morphology and behavior of arrested larvae was allele-dependent. Mutants carrying the usp^2 null allele and a single dose of *d/cusp* failed to wander off the food and showed no signs of prepupal contraction, although the larval cuticle became partially tanned (Fig. 1). For usp^3 and usp^4 mutants carrying a single copy of *d/cusp*, the cessation of movement also occurred in the late third instar, but larvae showed no sign of prepupal tanning except along the denticle belts of the midsegments. In other words, these mutations exerted a more severe effect than the null allele on cuticular tanning, though the stage of arrest was invariant for all *usp* alleles. The relative severity can be attributed to dominant negative characteristics of usp^3 and usp^4, because these mutant gene products maintain the ability to dimerize normally with EcR and retain partial ability to interact with an *hsp27* EcRE (Henrich *et al.*, 1994).

Multiple Doses of *d/cusp* Rescue *usp* Mutants from Lethality But Do Not Rescue Prepupal Contraction

The developmental phenotypes among *usp* mutants carrying two doses of *d/cusp* is summarized in Table 2. Mutant males carrying the usp^2 allele and two copies of the *d/cusp* often reached the adult stage (female *usp2* mutants cannot survive because the mutation-bearing chromosome is homozygous lethal), but the proportion of survivors differed between the two lines. The survival rate of *usp* mutant and non-usp^2 male siblings was statistically equal in the 71D line through adult eclosion, although most of the *usp2*/Y; 71D/71D males died at or shortly after eclosion. In the 19C line, mutant male survival to adult eclosion was as low as 20% of non-*usp* sibling males. Most of the nonsurviving usp^2 males in this line had died during the prepupal period before head eversion. In both lines, a few of the surviving usp^2 males rescued with two doses of *d/cusp* also developed slightly bent and twisted legs, a phenotype previously associated with mutations of the early puff gene, the Broad Complex, and EcR (Bender *et al.*, 1997; Kiss *et al.*, 1988).

Two copies of the *d/cusp* were never enough to rescue the usp^3 and usp^4 mutant larvae through metamorphosis, and these transformants were arrested at the larval/prepupal transition, just as they were with a single dose of *d/cusp*. At the time of arrest, larvae showed more tanning of the larval cuticle than mutants carrying a single dose of *d/cusp* but they failed to undergo a prepupal contraction and anterior spiracle eversion was incomplete. These observations were also made with *usp* mutants carrying one copy of two different *d/cusp* transgenes (i.e., 19C and 71D), thus demonstrating a dosage effect rather than the inadvertent reduction in survival rate caused by homozygous insertion of the transgene.

The requirement for multiple doses of the *d/cusp* to rescue *usp* mutants beyond the prepupal stage reveals that the chimeric gene product is partially impaired in its ability to perform a vital function at metamorphosis. This possibility was further tested by testing three or four doses of the *d/cusp* in usp^3 and usp^4 larvae (for technical reasons, this could not be performed with usp^2). For these *usp* mutations, three doses of the *d/cusp* was sufficient to rescue at least some flies through the entire life cycle, though many adults failed to eclose successfully. Four doses of the *d/cusp* were sufficient to rescue *usp* mutant flies at an

even higher rate. Ultimately, it was possible to establish a stably reproducing line of flies that was mutant for usp^3 or usp^4, and which carried four doses of a chimeric USP (that is, they were homozygous for two different transgenes simultaneously). Interestingly, although the line is relatively healthy the larvae failed to undergo a complete prepupal contraction, as noted earlier. In other words, this aspect of metamorphosis is not rescuable even with four doses of the *d/cusp* transgene.
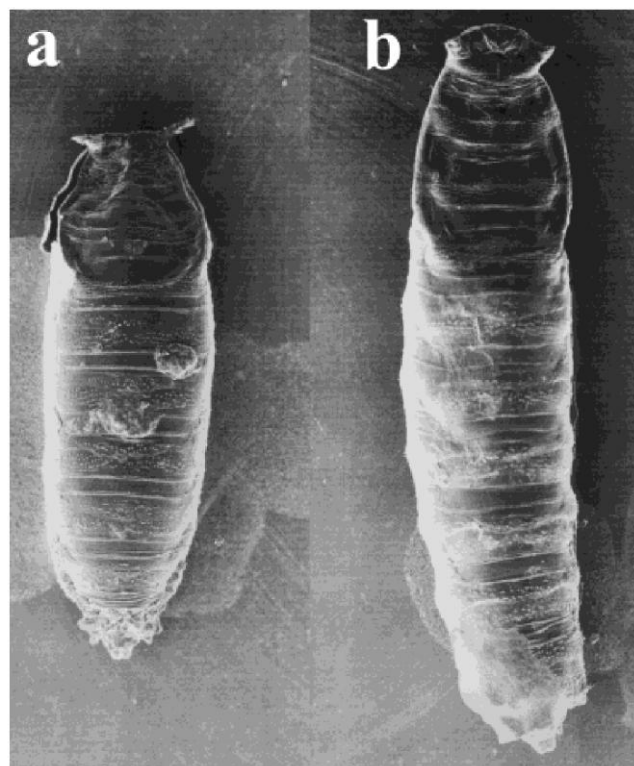

Table 2
A Summary of Developmental Phenotypes Observed Among Mutants Carrying a usp Mutation and Two Copies of d/cusp (19C and 71D line)*

| | Mutant allele | | |
	usp^2	usp^3	usp^4
Lethal stage	White prepupal; Pharate adult	White prepupal	White prepupal
Gut clearing	+	+	+
Wandering behavior	−/+	−	−
Anterior spiracle eversion	−/+	−/+	−/+
Cuticular tanning	+	−/+	−/+
Prepupal contraction	−	−	−

*All genotypes developed normally through the late third instar. A minus sign (−) indicates no evidence of normal phenotype, a plus sign (+) indicates normal phenotype. Double signs (−/+) indicate partially normal phenotype.

FIG. 1. Scanning electron micrographs of (**a**) normal prepupa, and (**b**) a developmentally arrested usp^2 mutant carrying a single dose of *d/cusp* transgene and viewed from the dorsal side. Mutant displays partial anterior spiracle eversion, elongated puparial case, and partial tanning of the outer cuticle. Both images are at the same magnification.

Expression of the Chimeric USP Is Apparently Normal

Although flies carrying two doses of the chimeric gene showed slightly more normal development than those with one dose, it was important to determine whether chimeric USP protein levels are relatively normal in the late third instar as the onset of pupariation approaches. The inability of transformed flies to enter metamorphosis might be influenced by a low level of USP expression at this developmental time when ecdysteroid titers and the requirement for ecdysteroid receptor function reaches unprecedented levels. Also, *usp* mRNA stability and/or translatability might involve regulation through the *Chironomus* 3' UTR. Normally, of course, the USP protein is abundant in the late third instar as it fulfills its role in mediating ecdysteroid responsiveness in the developing larva. As observed on Western blots, larval preparations from transformed lines always showed a 48 KDa protein recognized by a USP monoclonal antibody (kindly donated by F. Kafatos), whose mass corresponded with that predicted for the chimeric USP gene product; this signal was not found in preparations made from nontransformed flies of the same strain (Fig. 2). Moreover, the strength of the chimeric USP signal in all preparations tested was roughly comparable with the one seen for the endogenous copies of USP. Significantly, the level of *d/cusp* detected on the Western blot was dose-dependent, consistent with the effect of dosage on stage and extent of developmental rescue. Therefore, the failure to enter metamorphosis resulted from the inability of the expressed chimeric USP to perform adequately for survival through this developmental time, and not from abnormally low USP titers.
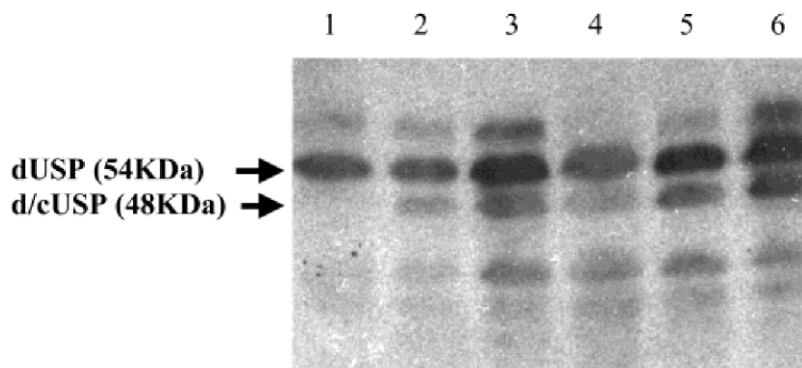
FIG. 2. Western blot of late third larval instar protein samples taken from various *usp* mutant and *d/cusp* transformed lines of *Drosophila melanogaster* using AB11 monoclonal antibody. This antibody recognizes the mutant USP protein produced by *usp^4*. Lane 1: Nontransformed *y ac w*; Lane 2: *usp^4/usp^4* carrying a single dose of p[*d/cusp*]19C; Lane 3: *usp^4/usp^4* carrying two doses of p[*d/cusp*]19C; Lane 4: *usp^4/usp^4* carrying a single dose of p[*d/cusp*]71D; Lane 5: *usp^4/usp^4* carrying two doses of p[*d/cusp*]71D; Lane 6: *usp^4/usp^4* carrying four doses of p[*d/cusp*], two doses of 19C and two doses of 71D.

The Chimeric USP Heterodimerizes Normally With *Drosophila* EcR

The developmental failure of the chimeric USP in the latter portions of the larval stage raises the possibility that a stage-specific arrest follows from an impaired interaction with one or more EcR isoforms. Therefore, *Drosophila* and the chimeric USP were tested on electrophoretic mobility shift assays with both the A and B1 isoforms of EcR. On both a palindromic and direct repeat element, *Drosophila* and chimeric USP showed about the same interaction with each isoform. Surprisingly, however, both the *Drosophila* and chimeric USP showed a greater interaction with the A isoform than the B1 isoform (Fig. 3). Nevertheless, the apparently normal interaction of the chimeric USP with the EcR isoforms indicates that the developmental failure arises from an impaired interaction between the LBD of the chimeric USP and one or more currently unidentified factors.
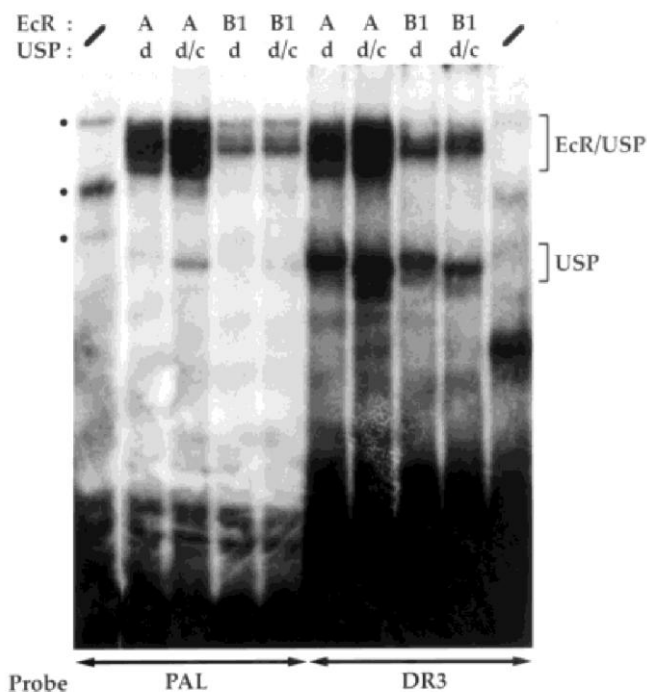

FIG. 3. Electrophoretic mobility shift assay of in vitro translated EcR-A and EcR-B1 with *Drosophila* USP and chimeric USP on hsp27 EcRE and direct repeat EcRE. For USP, d designates *Drosophila* USP, d/c designates chimeric USP. PAL indicates hsp27 EcRE and DR3 indicates direct repeat EcRE with 3 nucleotide spacer. Left lane shows nonspecific bands (indicated with dots) that are seen consistently with hsp27 EcRE and a rabbit reticulocyte lysate containing no in vitro translated product.

The EcR/chimeric USP complex showed normal ecdysteroid-binding properties. The sudden failure of developmental processes associated with the chimeric USP might result from its impairment of high affinity ecdysteroid-binding normally associated with the EcR/USP heterodimer. The chimeric USP was therefore compared with *Drosophila* USP and both forms of *Chironomus* USP (1 and 2; Vogtli *et al.*, 1999) for its ability to form a ligand-binding complex with *Drosophila* EcRB1. The competitive binding characteristics for each of these EcR/USP heterodimers revealed no significant differences among them, except that cUSP1 was lower (Fig. 4). This reduction is attributable to differences in the N-terminal domain between the two cUSP forms, because they are identical in all other domains. These experiments cannot discriminate whether the reduced level of ligand binding in cUSP1 was caused by impaired dimerization or whether the 1,BD of EcR is influenced allosterically by the N-terminal domain of cUSP1.

DISCUSSION

The studies reported here reveal that the chimeric USP performs an essential function during larval development that cannot be performed by endogenous USP mutant proteins. The larval function is rescued by a single dose of *d/cusp*, whereas rescue of the metamorphic function requires more doses, indicating that the chimeric USP is impaired in its ability to mediate a function associated with metamorphosis. By comparison, a single dose of the wild-type *Drosophila usp* gene is sufficient for rescue through the entire life cycle (Oro *et al.*, 1990). Thus, not only is USP required at the onset of metamorphosis, as reported earlier (Hall and Thummel, 1998), but its role at this time appears to be distinct from its larval function. The impaired function at metamorphosis is directly attributable to the *Chironomus* LBD of the chimera, because the remaining portion of the transgene, including the promoter, are derived from the endogenous *Drosophila usp* gene. The metamorphic arrest also does not result from subnormal expression. In fact, fewer doses of *d/cusp* were required to rescue the *usp* null allele (which obviously provides no residual function) than was required for the usp^3 and usp^4 mutations that likely retain residual function, further indicating that the developmental failure does not result from a simple deficit of *d/cusp* expression.

Mechanistically, the simplest interpretation of the chimeric USP's effects is that it performs a molecular function inefficiently as the prepupal stage approaches, hence the need for extra copies of the transgene to rescue *usp* mutants. However, several lines of evidence indicate that the arrest at metamorphosis involves one or more specific developmental functions connected with USP function, rather than an impaired interaction between the chimeric USP and EcR. First, the chimeric USP interacted normally with both EcRA and EcRB1 on molecular tests, and the resultant heterodimer interacted normally with ponasterone A. Moreover, though the pupal-adult transition is associated with the largest ecdysteroid peak during the *Drosophila* life cycle, it was not a lethal stage for mutants rescued by the chimeric USP, as might be expected if the failure of the chimeric USP was attributable to the inefficient mediation of an ecdysteroid response.

The most direct indication for a specific failure is the observation that four doses of the *d/cusp* transgene did not result in the contraction of the prepupa, strongly suggesting that the chimera simply lacks a function necessary for normal contraction to occur. We also observed that the prepupal lethal stage was essentially invariant for each combination of *usp* mutant alleles and *d/cusp* dosage, as expected if the arrest involved the failure of one or more specific functions associated with the onset of metamorphosis. By contrast, a nonspecific impairment (but not an elimination) of ecdysteroid response might be expected to produce arrest over a range of time, like those observed for mutations that cause an ecdysteroid deficiency (Henrich *et al.*, 1993; Sliter and Gilbert, 1992). Significantly, the *d/cusp* did not behave as a dominant negative mutation, as expected if the chimeric USP was forming an impaired heterodimer with EcR.

Although the failure of *d/cusp* during the prepupal stage affects specific developmental processes, they apparently include a subset of those associated with ecdysteroid action at this time. The observed phenotypes of *d/cusp* mutants, notably the noncontracting prepupal case, resembled those found among some mutations of EcR (Bender *et al.*, 1997). Other phenotypes associated with terminal prepupal development, such as incomplete anterior spiracle eversion and incomplete larval cuticular tanning also implicate an

impaired response to ecdysteroids (Hall and Thummel, 1998). The ability of some mutants carrying multiples doses of the chimeric USP to survive through the later pupal-adult transition reflects its ability to function normally at this time, or alternatively, that USP is not required for the pupal-adult transition. Although this unanticipated possibility requires more rigorous investigation, the ability of mutant *usp* clones of the wing to undergo a completely normal progression of differentiation into adult tissues (Oro *et al.*, 1992), even in the absence of normal USP function throughout metamorphosis, leaves open the possibility that USP is not required for imaginal disc morphogenesis during the pupal-adult stage. The potential of the chimeric USP to participate in both positive and negative aspects of gene regulation, as the normal USP does, remains to be explored (Schubiger and Truman, 2000), and the suggestion that USP is the *Drosophila* juvenile hormone receptor also provides an interesting possibility for distinguishing USP's larval and metamorphic functions (Jones and Sharp, 1997).
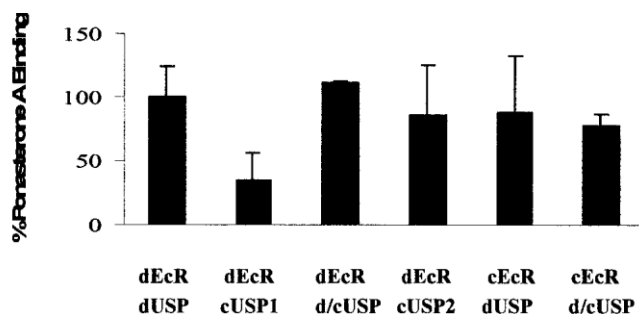

FIG. 4. Ponasterone A binding of EcR and USP complexes relative to *Drosophila* EcR and USP (dEcR and dUSP). Each *Chironomus* isoform (cUSP1 and cUSP2) was tested and d/cUSP designates chimeric USP. *Chironomus* EcR is designated by cEcR. *Chironomus* USP isoforms show virtually no binding in absence of cEcR (data not shown). All points represent the mean and standard deviation calculated from three replicates.

Structurally speaking, there are several regions within the E domain that could account for the impaired ability of the chimeric USP to work as well as wild-type *Drosophila* USP (Wurtz *et al.*, 1996), although the heterodimerization of EcR with the chimeric USP seems to be normal. The specific failure to rescue prepupal contraction raises the possibility that one or more impaired cofactor interactions are associated with this functional deficit (Nagy *et al.*, 1999). Future experiments, involving the use of smaller chimeric regions and site directed mutations should resolve which of these regions contributes to both the rescue of larval development and the failure of metamorphic events, particularly prepupal contraction.

These experiments also illustrate an experimental strategy by which modified forms of *usp* can be introduced into the null usp^2 mutational background and tested for their ability to function during larval and metamorphic phases of development in *Drosophila*. Further, the ability to use the chimeric USP to rescue and examine developmental processes provides a strategy for associating specific structural alterations of USP with potential developmental roles.

MATERIALS AND METHODS
Preparation of Vector
A previously described EcoR1/BamHI fragment that incorporates the entire functional *Drosophila usp* promoter (Oro *et al.*, 1990) and a portion of the *usp* open reading frame was excised from an EMBL4 genomic clone. This fragment was ligated with the BamHI/EcoRI fragment from a cDNA vector (pZ7-1; Henrich *et al.*, 1990) that includes the carboxy-terminal portion of the *Drosophila* USP open reading frame and the 3'UTR of the *usp* gene. The resulting 5.7 kb EcoRI fragment in pBluescript contained the entire *usp* promoter and structural gene (pMVZ10).

To produce the *Drosophila/Chironomus* chimeric USP gene (*d/cusp*), a portion of the previously described pMI20 (Vogtli *et al.*, 1999) that encodes the *Chironomus* USP was excised. A 1. 1 kb fragment extending

from an MvnI site (nucleotide 584) to a SmaI polylinker site of pMI20 was cloned into the SmaI site of pUC18. The 3' end of the *cusp* was adjacent to the EcoRI site of pUC18. The resulting plasmid was linearized with BamHI and the ends filled in by treatment with T4 DNA polymerase. The plasmid was then digested with Asp718 to produce an Asp718/blunt end fragment that includes the entire 3' end of the *Chironomus usp* from nucleotide 584. This fragment was ligated to a 7.2-kb fragment generated by partial digestion of pMVZ10 with HindII and complete digestion with Asp718. This plasmid, designated pMVZ12, was linearized by Asp718 digestion and blunted by treatment with T4 DNA polymerase. After cleavage with BamHI, a 5.4-kb fragment (blunt/BamHI) was cloned into pCaSpeR4 cut with StuI and BamHI to produce pMVZ18. The resulting *d/cusp* includes the 5' end of *Drosophila usp* through position 733 of the pZ7-1 sequence and begins with position 584 of the *Chironomus usp* cDNA sequence.

Chimeric Sequence Information

The chimeric USP sequence is composed of amino acids 1 through 178 from the *Drosophila* USP (Henrich *et al.*, 1990) and positions 175 through 451 of the *Chironomus* USP, with a linker of three amino acids between them (Vogtli *et al.*, 1999) as shown in Figure 5. The deduced and aligned amino acid sequences of *Bombyx* USP (Tzertzinis *et al.,* 1994) and the human RXR are also compared. In the resulting vector, the ORF region is flanked by the *Chironomus* 3' UTR and the *Drosophila* 5' promoter, *usp* transcriptional start site, and 5' UTR.

Transformation and Recovery of Transformants

Transfomation procedures were carried out according to standard protocols. Embryos of the genotype *y ac w* were collected for approximately 15 min at 18°C and co-injected with the aforementioned p[*d/cusp*] vector and pπ2.5w^{yco} vector. Injected embryos that later hatched into first instar larvae were transferred to food bottles and reared at 25°C. Adult survivors were then crossed with *y ac w* flies and progeny were screened for the presence of colored eyes, indicative of transformation. Siblings showing *w+* pigmented eyes were then crossed and selected for homozygosity, when possible. Transgenes were also mapped to specific chromosomes by standard crosses with balancer stocks. The transformed flies were later crossed with appropriate *usp* mutant strains. Chromosomes bearing usp^3 and usp^4 are marked with white eyes and yellow body, so that transformants were selectable by the presence of colored eyes, and *usp* mutant larvae were selectable by the presence of brown mouthhooks from appropriate crosses. In the case of usp^2, which carries *y+*, the marking was reversed so that males carrying usp^2 had black larval mouthhooks, whereas mutants showed brown mouthhooks.

Western Blot Procedures

Protein extractions of late third instar larvae were made according to published protocols (Song *et al.*, 1997). The AB1 1 monoclonal antibody (Khoury-Christianson *et al.*, 1992) was used to test for the presence of the *Drosophila* and chimeric USPs in the protein preparation after SDS-PAGE and immunoblotting.

EMSA Studies

Experimental conditions for EcR and USP EMSA studies have been described previously (Henrich *et al.*, 1994). All proteins were generated through a coupled in vitro transcription/translation system in rabbit reticulocyte lysate (Promega). For each plasmid vector used, a mixture of undigested plasmid (1 µg) and lysate (25 µl) was performed in the presence of 35S labeled methionine to estimate translation efficiency. A second mixture without radiolabeled amino acids was prepared for EMSA analysis. The following vectors were used (T3 or T7 RNA polymerase for in vitro transcription is given in parentheses):

p2C *Drosophila usp* (T3; Henrich *et al.*, 1994)

pCA1 *Drosophila* EcR-B1 (T7; Henrich *et al.*, 1994)

pWT57 *Drosophila* EcR-A (T3)

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Drosophila USP  CEGCKGFFKRTVRKDLTYACRENRNCIIDKRQRNRCQYCRYQKCLTCGMKREAVQEER  RSP QRGARNAAGRL---SAS192
Chironomus USP  CEGCKGFFKRTVRKDLSYACREERNCVIDKKQRNRCQYCRYQKCLNCGMKREAVQEER ...  QRG-----GK-----SQK182
Bombyx USP      CEGCKGFFKRTVRKDLTYACREDKNCIIDKRQRNRCQYCRYQKCLACGMKREAVQEER ...  QRAARRTEDAHPS----201
Human RXR       CEGCKGFFKRTVRKDLTYTCRDNKDCLIDKRQRNRCQYCRYQKCLAMGMKREAVQEER ...  QRGKDRNENEVESTSS-225
                         DNA-binding domain                                T-box
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Drosophila USP  GGGSSGPGSVGGSSSQGGGGEGGVSGGMGSGNGSDDFMTNSVSRDFSIERIIEAEQRAETQCGDRALTFLRVGPYSTVQ-271
Chironomus USP  -----------------------------GDDMSISSTQSLVNNGPGRDITVERLMEADQMSEARCGDKSIQYLRVAASNTMI-232
Bombyx USP      ----------------------------------SSVQELSIERLLELEALVADSA--EELQILRVGPESGV--237
Human RXR       --------------------------------------ANEDMPVERILEAELAVEPK------TETYVE-------252
                                                                  H1                    H2
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Drosophila USP  -----------PDYKGAVSALCQVVNKQLFQ-MVEYARMMPHFAQVPLDDQVILLKAAWIELLIANVAWCSIVSLDDGG338
Chironomus USP  -----------PEYRAPVSALCAMVNKQVFQHMDFCRRL-PHFTKLPLNDQMYLLKQSLNELLILNIAYMSIQYVEPDR300
Bombyx USP      -----------PAKYRAPVSSLCQIGNKQIAALIVWARDI-PHFGQLEIDDQILLIKGSWNELLLFAIAWRS---MEFL-301
Human RXR       ANMGL-----NPSSPNDPVTNICQAADKQLFTLVEWAKRI-PHFSELPLDDQVILLRAGWNELLIASFSHRSIA-V-KD-338
                          H3                              H4        H5
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Drosophila USP  AGGGGGLGHDGSFERRSPGLQPQQLFLNQSFSYHRNSAIK-----------AGVSAIFDRILSELSVKMKR-LNLD    403
Chironomus USP  RNA-------DGSLERRQ-ISQ-QMCLSRNYTLGRNMAVQ-----------AGVVQIFDRILSELSVKMKR-LDLD    355
Bombyx USP      -N-------D---ER-------ENVDSRNTAPPQLICLMPGMTLHRNSALQAGVGQIFDRVLSELSLKNR-SLRMD    358
Human RXR       --------------------GILLATGLHVHRNSAHS-----------AGVGAIFDRVLTELVSKMR-DMQMD    380
                             H6                                  H7
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Drosophila USP  RRELSCLKAIILYNPDIRGIKSRAEIEMCREKVYACLDEHCRLEHPGDDGRFAQLLLRLPALRSISLKCQDHLFLFRITS483
Chironomus USP  ATBLCLLKSIVVFNPDVRTLDDRKSIDLLRSRIYASLDEYCRQKHPNEDGRFAQLLLRLPALRSISLKCLDHLFYFQLID433
Bombyx USP      QAECVALKAIILLNPDVKGLKNKQEVDVLREKMFLCLDEYCRRSRGGEEGRFAALLLRLPALRSISLKSFEHLYLFHLVP439
Human RXR       KTELGCLRAIVLFNPDSKGLSNPAEVEALREKVYASLEAYCKHKYGEQPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIG460
                   H8                        H9                    H10         H11
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Drosophila USP  DRPLLEELFLEQLEAPPPPGLAMKLE*       509
Chironomus USP  DKNV-ENSVIEEFH---------KLN*       451
Bombyx USP      --RA---------A*                   441
Human RXR       -DTPIDTFLMEMLEAPHQ----MT*         479
                     AF2 domain
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FIG. 5. Amino acid alignment of a portion of Ultraspiracle from *Drosophila* (Henrich *et al.*, 1990), *Chironomus* (Vogtli *et al.*, 1999), and *Bombyx* (Tzertzinis *et al.*, 1994). This region includes the DNA-binding domain and all residues to the carboxy-terminal side. The underlined residues indicate functional motifs and helices. The shaded amino acids indicate the sequence of the chimera, including a three-residue splice junction.

The PMVZ13 vector was prepared by ligating a 3.6 kb HindII/Asp718 fragment of pZ7-1 together with the aforementioned 1.1 kb fragment of PMVZ12. The WT57 vector was generously provided by Dr. David Hogness.

Gel shifts were performed as described in Henrich *et al.* (1994). Two microliters of the protein lysate were mixed for each combination of EcR and USP. Either the hsp27 or the DR3 (Antoniewski *et al.*, 1993, 1996) radiolabeled (^{32P}) oligonucleotide probe (30 fmoles) was added to the mixture. The TNT coupled reticulocyte lysate system (Promega) was used according to the instructions of the manufacturer for in vitro transcription/ translation of the receptor proteins using the T7 promoter for transcription of the cDNAs of dEcR-A, dEcR- B1, dUSP, and the T3 promoter for CtUSP-1 and the chimeric USP.

Ponasterone Binding Tests

Levels of in vitro translated EcR and USP were determined by evaluation of Western blots using the aforementioned monoclonal antibody AB11 (Khoury-Christianson *et al.*, 1992) and an ECL detection kit (Amersham) using methods previously described by Rauch *et al.* (1998). Specific signals on the X-ray film (Biomax, Kodak) were scanned and the intensity of the protein bands were quantified with an image analysis system (PHORETIX, Non-Linear Dynamics, Ltd., New Castle, UK), and these data were later used to normalize the radioactivity associated with individual protein samples.

Ligand binding of the quantified, in vitro translated receptors was tested with [33]H-ponasterone A (specific activity 7.9 TBq/mmol). Each assay contained 5 nM 3 H- ponasterone A (final concentration) and 10 µl of the EcR- and USP-TNT-lysate in a final volume of 40 µl containing 20 mM HEPES, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.9). A cocktail of protease inhibitors (aprotinin, leupeptin, pepstatin in a final concentration of 1 µg/ml) was added immediately before testing. Nonspecific binding was determined in the presence of 0. 1 mM unlabeled 20-hydroxyecdysone in parallel hormone binding assays. The reaction mixtures were incubated for 1 h at room temperature, then unbound ligand was removed by vacuum filtration as described previously by Rauch *et al.* (1998). Radioactivity was counted with a liquid scintillation counter (1600TR, Canberra-Packard).

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