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The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor

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SUMMARY

Pulses of the steroid hormone ecdysone function as key temporal signals during insect development, coordinating the major postembryonic developmental transitions, including molting and metamorphosis. In vitro studies have demonstrated that the EcR ecdysone receptor requires an RXR heterodimer partner for its activity, encoded by the *ultraspiracle* (*usp*) locus. We show here that *usp* exerts no apparent function in mid-third instar larvae, when a regulatory hierarchy prepares the animal for the onset of metamorphosis. Rather, *usp* is required in late third instar larvae for appropriate developmental and transcriptional responses to the ecdysone pulse that triggers puparium formation. The imaginal discs in *usp* mutants begin to evert but do not elongate or differentiate, the larval midgut and salivary glands fail to undergo programmed cell death and the adult midgut fails to form. Consistent with these developmental phenotypes, *usp* mutants show pleiotropic defects in ecdysone-regulated gene expression at the larval-prepupal transition. *usp* mutants also recapitulate aspects of a larval molt at puparium formation, forming a supernumerary cuticle. These observations indicate that *usp* is required for ecdysone receptor activity in vivo, demonstrate that the EcR/USP heterodimer functions in a stage-specific manner during the onset of metamorphosis and implicate a role for *usp* in the decision to molt or pupariate in response to ecdysone pulses during larval development.

Key words: Nuclear receptor, Gene regulation, Metamorphosis, Juvenile hormone, *Drosophila*, Ecdysone receptor, RXR, Ultraspiracle

INTRODUCTION

Retinoid X receptors (RXRs) function as central regulators of hormone responses in higher organisms. Many nuclear receptors, including the receptors for thyroid hormone and retinoic acid, must heterodimerize with RXR to exert their regulatory functions (Mangelsdorf and Evans, 1995). These receptors bind to specific response elements in the genome and control target gene transcription in response to the appropriate hormone. The combinatorial complexity provided through heterodimerization with RXR is further enhanced by the presence of three RXR genes in the vertebrate genome, each encoding multiple protein isoforms (Leid et al., 1992). Although this multiplicity of RXR heterodimers provides a mechanism for achieving transcriptional diversity, it also complicates studies of RXR function during development (Kastner et al., 1995).

The *Drosophila* genome encodes a single known RXR homolog, USP, providing a relatively simple system for defining RXR function during development (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990). Like its vertebrate homolog, USP can heterodimerize with mammalian nuclear receptors, including retinoic acid receptor, thyroid hormone receptor and vitamin D receptor (Khoury Christianson et al., 1992; Yao et al., 1993). USP can also heterodimerize with at least two *Drosophila* nuclear receptors: EcR and DHR38 (Yao et al., 1992; Sutherland et al., 1995). Heterodimerization between EcR and USP is required for binding of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone), sequence-specific interactions with DNA and reporter gene transcription in transfected tissue culture cells (Koelle, 1992; Yao et al., 1992; Thomas et al., 1993; Yao et al., 1993). EcR and USP also co-localize to ecdysone-regulated puffs in the salivary gland polytene chromosomes, suggesting that they function together in vivo (Talbot, 1993; Yao et al., 1993).

The *EcR* gene is over 70 kb in length and encodes at least three protein isoforms, EcR-A, EcR-B1 and EcR-B2, differing in their N-terminal sequences (Talbot et al., 1993). Each EcR isoform can heterodimerize with USP to form an ecdysone receptor (Koelle, 1992). EcR-A is predominantly expressed in adult progenitor cells that proliferate and differentiate during metamorphosis, while the EcR-B isoforms are predominantly expressed in larval cells fated to die. This observation led to the proposal that different EcR isoforms dictate at least part of the tissue specificity of ecdysone responses (Talbot et al., 1993). Consistent with this hypothesis, leg imaginal discs elongate in *EcR-B* mutants, while larval tissues fail to die (Bender et al., 1997; Schubiger et al., 1998). Furthermore,

EcR-B isoforms, but not EcR-A, can rescue defects in the polytene chromosome puffing response of *EcR-B1* mutants (Bender et al., 1997).

In contrast to the complexity of *EcR*, USP is encoded by a 2.3 kb gene with no introns and is widely expressed, both temporally and spatially (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990; Henrich et al., 1994). Three *usp* mutations have been identified, all of which appear to be recessive loss-of-function alleles (Perrimon et al., 1985). The usp^2 mutation is caused by a breakpoint that truncates the *usp* coding region just after the DNA-binding domain (Oro et al., 1990), while usp^3 and usp^4 are point mutations that change highly conserved arginine residues within the DNA-binding domain (Henrich et al., 1994). The usp^2 mutation is thought to be a null allele because it removes the ligand-binding domain, a region required for heterodimerization with EcR and hence both hormone- and DNA-binding activities of the receptor. Loss of maternal *usp* function leads to embryos with a defective chorion and lethality during late embryogenesis with cuticular scarring in posterior abdominal segments (Perrimon et al., 1985; Oro et al., 1992). In contrast, loss of zygotic *usp* function leads to early larval lethality with some surviving second instar larvae carrying an extra set of posterior spiracles, suggesting a defect in molting of the first instar cuticle (Perrimon et al., 1985; Oro et al., 1992). *usp* is also required for morphogenetic furrow movement during adult eye development (Zelhof et al., 1997) and may play a role in fusion of the wing imaginal discs during metamorphosis (Henrich et al., 1994). No studies performed to date, however, have addressed the role of USP as a functional component of the ecdysone receptor during development.

A high titer pulse of ecdysone at the end of larval development triggers puparium formation, initiating the prepupal stage of development and signaling the onset of metamorphosis. Most larval tissues undergo programmed cell death during metamorphosis while adult tissues differentiate from small clusters of imaginal progenitor cells, transforming the larva into an adult fly (Robertson, 1936; Bodenstein, 1965). Ecdysone exerts its developmental effects through the activation of genetic regulatory hierarchies, originally identified as changes in the puffing patterns of the larval salivary gland polytene chromosomes (Becker, 1959; Clever and Karlson, 1960; Ashburner et al., 1974). A small set of early genes are induced directly by ecdysone in these hierarchies, including the *Broad-Complex* (*BR-C*), *E74* and *E75* (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). These genes encode transcription factors that transduce and amplify the hormonal signal by regulating large sets of late secondary-response genes (Ashburner et al., 1974; Urness and Thummel, 1995; Crossgrove et al., 1996). It is the stage- and tissue-specific activation of these target genes that direct the appropriate biological responses associated with metamorphosis.

Studies of the regulation and function of early ecdysone-inducible genes have defined two regulatory hierarchies during the third larval instar that function together to direct the onset of metamorphosis (reviewed by Thummel, 1996; Richards, 1997). The first regulatory hierarchy, activated in mid-third instar larvae, is characterized by the coordinate low-level induction of *EcR*, *E74B* and the *BR-C* in apparent synchrony with a low titer pulse of ecdysone (Andres et al., 1993; Huet et al., 1993). The expression of each of these genes is required for proper puparium formation 1 day later, indicating that the activation of this hierarchy is essential to prepare the animal for metamorphosis (Kiss et al., 1988; Fletcher et al., 1995; Bender et al., 1997). In the salivary glands, the mid-third instar hierarchy is characterized by a switch in secondary-response gene expression, in which *E74B* and the *BR-C* repress the *ng* genes and induce the glue genes (Mougneau et al., 1993; von Kalm et al., 1994; D'Avino et al., 1995b). The glue genes encode a polypeptide glue that is used to affix the animals to a solid surface at puparium formation (reviewed by Meyerowitz et al., 1987). At the end of larval development, the high titer pulse of ecdysone activates the second regulatory hierarchy, which is characterized by higher levels of *BR-C* expression and induction of *E74A* and *E75A* (Andres et al., 1993; Huet et al., 1993). These early genes, in turn, direct a second switch in salivary gland secondary-response genes, repressing the glue genes and inducing the late puff genes, including *L71-6* (Guay and Guild, 1991; Karim et al., 1993; Fletcher and Thummel, 1995).

In this paper, we describe the effects of *usp* mutations on the genetic and biological responses to ecdysone during the onset of metamorphosis. *usp* mutant larvae, rescued past their early lethal phase by ectopic *usp* expression, fail to initiate metamorphosis. The imaginal discs begin to evert but do not evaginate or differentiate, the adult midgut fails to develop and larval tissues are not destroyed. *usp* mutants also display pleiotropic defects in ecdysone-regulated gene expression in late third instar larvae, consistent with the observed developmental phenotypes. Unexpectedly, however, *usp* mutations have no effect on the mid-third instar regulatory hierarchy, a response previously assumed to be dependent on the ecdysone-receptor complex. These studies demonstrate that *usp* is an essential component of the ecdysone receptor in vivo and indicate that this receptor functions in a stage-specific manner at the end of larval development. *usp* mutants also deposit a supernumerary cuticle at the end of larval development, appearing to recapitulate aspects of a larval molt. A similar phenotype is not seen in *EcR* mutants, suggesting that *usp* can function independently of the EcR/USP heterodimer. This phenotype also implicates *usp* as a critical determinant of the decision to molt or pupariate in response to ecdysone pulses during larval development. The similarity of this function with that ascribed to juvenile hormone in other insects raises the possibility that *usp* may play a role in juvenile hormone signaling in *Drosophila*.

MATERIALS AND METHODS

Fly stocks

The *P*[*hs-neo*^{R}, *hs-usp*] P-element construct was used to rescue either usp^2 or usp^4 mutants past their early larval lethal phase (Oro et al., 1992). *usp* mutant larvae were generated by the following crosses: usp^2/*Binsinscy* X *y*; *P*[*hs-neo*^{R}, *hs-usp*]/TM3 or *y* usp^4 *w*/*Binsn* X *w*; *P*[*hs-neo*^{R}, *hs-usp*]/TM6B. First instar larvae consisting of a mixture of *usp* mutants and wild-type siblings were collected 6 hours after hatching and transferred to vials containing standard cornmeal

agarose food with 0.5% bromophenol blue. The vials were placed in a 37°C circulating water bath for 30 minutes, then transferred to a 23°C incubator for the remainder of development. Mutant animals were distinguished from control siblings by scoring the *yellow* marker. Third instar larvae were staged based on the amount of blue food remaining in their gut following the cessation of feeding (Maroni and Stamey, 1983; Andres and Thummel, 1994). Food supplemented with ecdysone was prepared as described (Furia et al., 1992).

Western blot analysis

Protein extracts were prepared from staged animals by homogenization in SDS sample buffer. Protein samples corresponding to ~0.5 animal/time point were fractionated on 8% polyacrylamide gels and electroblotted onto ECL membranes (Amersham). USP was detected using the AB11 monoclonal antibody, as described (Henrich et al., 1994), followed by a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma) and the Amersham ECL detection kit for chemiluminescence.

Analysis of tissues

For histological analysis of the supernumerary cuticle in *usp* mutants, animals were fixed with glutaraldehyde, embedded in plastic, sectioned, and stained with toluidine blue, essentially as described (Kaznowski et al., 1985). Imaginal discs in *usp* mutants were characterized by dissecting wing and leg discs from either mid-third instar *usp* mutants or mutants 24 hours after the stationary stage. The discs were dissected in PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4]/0.1% Triton X-100, fixed in 2% formaldehyde for 10 minutes, rehydrated in several changes of PBS for 30 minutes, and mounted in 50% glycerol for photography. Larval midguts were dissected from animals at the same stages of development, fixed in 2% paraformaldehyde for 1 hour, incubated with 50 μg/ml DAPI in PBS for 5 minutes, washed several times in PBS and mounted in 50% glycerol for photography.

Northern blot hybridizations

Third instar larvae were maintained on food containing 0.5% bromophenol blue and staged as described above. Control prepupae were synchronized at the white prepupal stage (0 hour prepupae) and *usp* mutants were collected at the stationary stage. These animals were then allowed to age at 23°C for the appropriate time. RNA was extracted from four animals at each developmental stage, fractionated by formaldehyde agarose gel electrophoresis and transferred to nylon membranes as described (Andres et al., 1993). Filters were hybridized, washed and stripped as described (Karim and Thummel, 1991). DNA probes are described by Andres et al. (1993), except the *rpr* probe (Jiang et al., 1997) and a 1.2 kb *Bam*HI-*Hin*dIII fragment from the *Lcp65A b* larval cuticle gene (Charles et al., 1997). Probes were labeled by random priming (Prime-It kit, Stratagene) of gel-purified fragments.

RESULTS

USP protein is undetectable in rescued *usp* mutant mid-third instar larvae

We used a *usp* cDNA under the control of a heat-inducible promoter to rescue *usp* mutants past their early lethal phase. This allowed us to analyze *usp* mutant phenotypes during later stages of development, when ecdysone responses are best characterized. Repeated ectopic expression of *usp* using this construct can successfully rescue *usp* mutants to adulthood and has no effect on the development of wild-type animals (Oro et al., 1992). We found that a single 30 minute 37°C heat pulse early during the first larval instar was sufficient to rescue greater than 70% of the mutant animals to the third instar. Longer heat pulses did not significantly increase the survival rate and the presence of the construct alone was not sufficient to rescue mutant animals (data not shown). Siblings carrying a wild-type *usp* allele were present in all rescue experiments and were used as internal controls. Data are presented for animals carrying the usp^2 mutant allele, although similar phenotypes were seen for usp^4 mutants (data not shown). Examination of USP protein levels 2 hours after a 30 minute heat pulse revealed a significant increase in USP expression relative to control animals (Fig. 1, lanes 1,2). In contrast, no USP protein could be detected in usp^2 mutant mid-third instar larvae, 96 hours after heat treatment (Fig. 1, lane 3). This observation indicates that the rescued mutants are at least strong hypomorphs for *usp* function at the end of larval development. Hereafter, we refer to these rescued animals as *usp* mutants.

usp mutants are defective in puparium formation and deposit a supernumerary cuticle

Normally, near the end of the third instar, a low titer pulse of ecdysone causes larvae to wander from the food in search of a place to pupariate (Berreur et al., 1984; Dominick and Truman, 1985). This is followed several hours later by a high titer pulse of ecdysone that triggers puparium formation – shortening the larval body, everting the anterior spiracles, and tanning and hardening the larval cuticle to form a protective puparial case (Fig. 2A,B). By ~6 hours after puparium formation, apolysis from the larval cuticle is complete and a thin pupal cuticle has been deposited.

usp mutants fail to undergo most of these developmental transitions. usp^2 mutant larvae fail to wander, while many usp^4 mutants wander only a short distance from the food. Rather than forming a puparium, *usp* mutants maintain their larval shape, become motionless at the surface of the food, fail to respond to a touch stimulus and fail to evert their anterior spiracles (Fig. 2C). We have designated this aberrant attempt at puparium formation as the stationary stage. After several

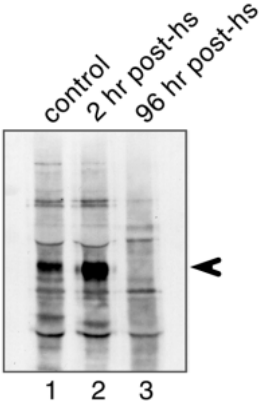

Fig. 1. USP protein is undetectable in usp^2 mutant mid-third instar larvae. Detection of USP (arrow) in wild-type newly formed prepupae was used as a positive control (lane 1). Levels of USP were determined 2 hours post-heat treatment in animals carrying the *P*[*hs-neo*^{R}, *hs-usp*] rescue construct (lane 2), and in usp^2 mutant mid-third instar larvae carrying the rescue construct, 96 hours post-heat treatment (lane 3).

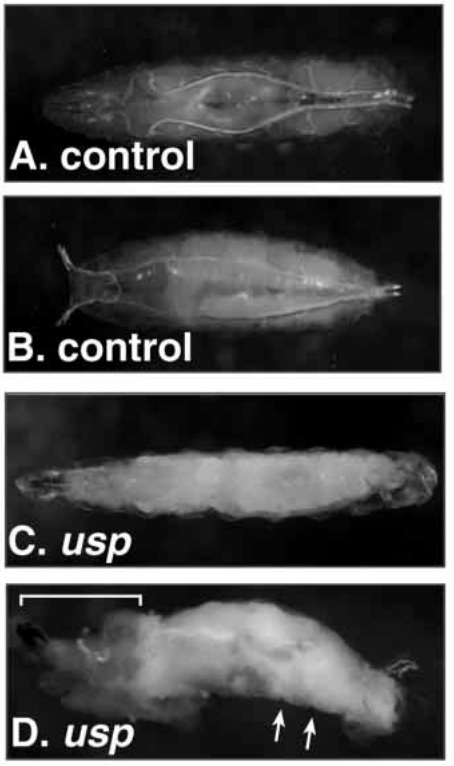

Fig. 2. *usp* mutants are defective in puparium formation. Control wild-type animals as (A) a feeding mid-third instar larva and (B) a newly formed prepupa. usp^2 mutant animals 24 hours after the stationary stage, as an intact animal (C) and with the third instar larval cuticle dissected away (D). Anterior is to the left in all panels. usp^2 mutants (C) fail to shorten their body or evert their anterior spiracles as seen in control animals (B). (D) Imaginal discs and attached larval mouthhooks cluster at the anterior end of a usp^2 mutant (bracket) while the rest of the body is covered by a supernumerary cuticle with apparent segmental ridges (arrows).

hours, *usp* mutants begin to apolyze from their third instar cuticle, as evidenced by retraction of the animals from both the anterior and posterior ends. By 24 hours after becoming stationary, apolysis is complete and the animals easily slip free from the external third instar cuticle. Surprisingly, we found that a supernumerary cuticle covers the posterior two-thirds of the animal (Fig. 2D). This cuticle is thick, well-infiltrated with tracheae and segmentally ridged along the body, hallmarks of a larval rather than a pupal cuticle. Most *usp* mutants die by 72 hours after the stationary phase, as evidenced by the onset of necrosis. All of these phenotypes are fully penetrant.

Larval cuticle consists of two distinct layers, a relatively thick endocuticle surrounded by a thin external epicuticle. Endocuticle is normally deposited continuously throughout the third larval instar (Kaznowski et al., 1985), raising the possibility that the epidermis of *usp* mutants continues to synthesize a third instar endocuticle during the stationary stage rather than depositing a new cuticle. To distinguish between these possibilities, histological sections from *usp* mutants were examined 24 hours after the stationary stage (Fig. 3). Two cuticles could be clearly distinguished in these mutants, each with its own epicuticle and endocuticle, although the most internal endocuticle appears disorganized (Fig. 3B). *usp*

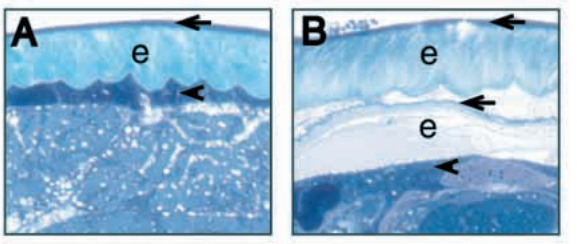

Fig. 3. *usp* mutants synthesize a supernumerary cuticle. Histological analysis was performed as described in Materials and Methods. Sections of a control wild-type third instar larva (A) and a usp^2 mutant 24 hours after the stationary stage (B) are depicted. Epicuticles (arrows), endocuticles (e) and epidermis (arrowheads) are marked.

mutants thus appear to initiate aspects of both a larval molt and puparium formation in response to the high titer late larval pulse of ecdysone.

Metamorphic changes in internal tissues are blocked in *usp* mutants

Several tissues were examined in order to more accurately assess the developmental status of the stationary animals. Imaginal discs normally begin to evert and elongate to form rudiments of the adult appendages within the first hours after puparium formation, eventually fusing to form a continuous epithelium (Fristrom and Fristrom, 1993). In contrast, the larval midgut initiates programmed cell death, starting with retraction of the four gastric caeca (arrowhead, Fig. 4A) during the first few hours of prepupal development (Jiang et al., 1997). As the larval midgut contracts and dies, it is surrounded by the adult midgut which arises from cells that proliferate and differentiate from small islands of diploid imaginal cells (arrows, Fig. 4C,D). The complete adult midgut is present by 12 hours after puparium formation, encompassing the compacted yellow body of dead larval cells (Jiang et al., 1997). The larval salivary gland is also destroyed during metamorphosis, at ~14 hours after puparium formation.

Imaginal discs look normal in *usp* mutant third instar larvae (Fig. 5A,C) and begin to evert following the stationary stage, but arrest their development at a point normally seen 1 hour after puparium formation (Fig. 5B,D). The gastric caeca also retract in *usp* mutants, although this response occurs gradually over a 24 hour period (arrowhead, Fig. 4B). A slight compaction of the larval midgut can be observed, but the larval cells do not die and the adult midgut does not form. The number of imaginal cells in the midguts of *usp* mutants does not appear to change significantly in the 24 hour period following the stationary stage, indicating that imaginal cell proliferation does not occur (Fig. 4C,D). Larval salivary gland development is also normal until the end of the third instar, even swelling with glue proteins in preparation for puparium formation. Destruction of the larval salivary gland, however, fails to occur and the gland persists until the death of the animal (data not shown). These pleiotropic defects suggest that *usp* mutants are unable to transduce the ecdysone signal that triggers the onset of metamorphosis.

usp mutants display stage-specific effects on ecdysone-regulated gene expression

If USP is an essential component of the ecdysone receptor,

then *usp* mutants should display pleiotropic defects in ecdysone-regulated gene expression at the onset of metamorphosis. To test this hypothesis, RNA was isolated from staged control and *usp* mutant animals and the patterns of ecdysone-regulated transcription were analyzed by northern blot hybridization (Fig. 6). Unexpectedly, activation of the mid-third instar regulatory hierarchy is unaffected by the usp^2 mutation. *EcR*, *E74B* and the *BR-C* are expressed normally in usp^2 mutant mid-third instar larvae, and the *ng* to glue gene switch occurs on time. In contrast, the response to the high titer late larval pulse of ecdysone is blocked. *E74A*, *E75A* and the *BR-C* are not induced in usp^2 late third instar larvae (Fig. 6 and data not shown). Furthermore, the *Sgs-4* glue gene is not repressed at the stationary stage, and the *L71-6* late gene is not induced. These observations indicate that *usp* mutations selectively block the late larval response to ecdysone, consistent with the observed developmental phenotypes. These results are confirmed by analysis of the puffing patterns in the salivary gland polytene chromosomes of usp^2 mutants, where the glue gene puffs fail to regress and the early and late puffs do not form (data not shown).

Destruction of the larval midgut during early prepupal development is accompanied by coordinate induction of the death genes *reaper* (*rpr*) and *hid* (Jiang et al., 1997). These genes are not induced in usp^2 mutant animals (Fig. 6 and data not shown), consistent with the failure of the larval midgut and salivary glands to undergo cell death. In contrast, the *Sb* protease gene (Appel et al., 1993) is expressed normally in usp^2 mutants, although at slightly lower levels, indicating that other factors must contribute to the inability of *usp* mutant imaginal discs to undergo normal eversion and elongation (data not shown).

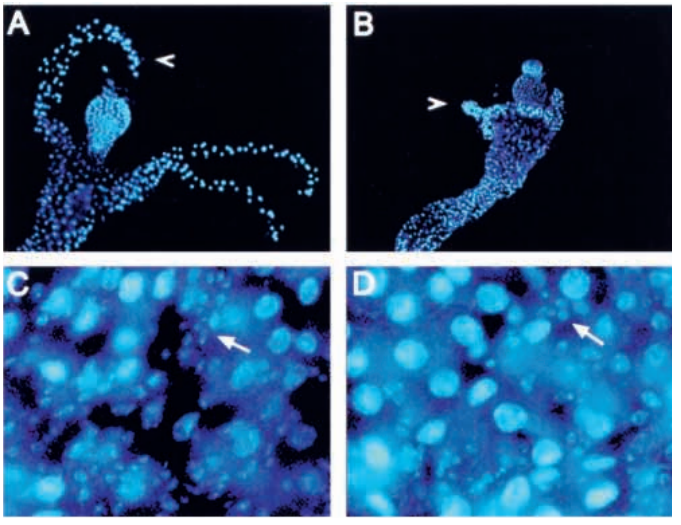

Fig. 4. Midgut metamorphosis fails to occur in *usp* mutants. DAPI-stained larval midguts from usp^2 mutants as (A,C) mid-third instar larvae and (B,D) 24 hours after the stationary stage. The midguts from *usp* mutant mid-third instar larvae (A,C) look identical to those from wild-type animals (Jiang et al., 1997). Low-magnification images show the midgut with four anterior-projecting gastric caeca (arrowheads in A,B) and the bulb-shaped proventriculus at the anterior end of the midgut, while higher magnification images (C,D) reveal the islands of imaginal diploid nuclei (arrows) surrounded by large larval polytene nuclei. The midguts in *usp* mutants 24 hours after the stationary stage have partially contracted, as revealed by the relatively high density of larval nuclei (B,D). These midguts resemble those seen in wild-type animals several hours after puparium formation (Jiang et al., 1997).

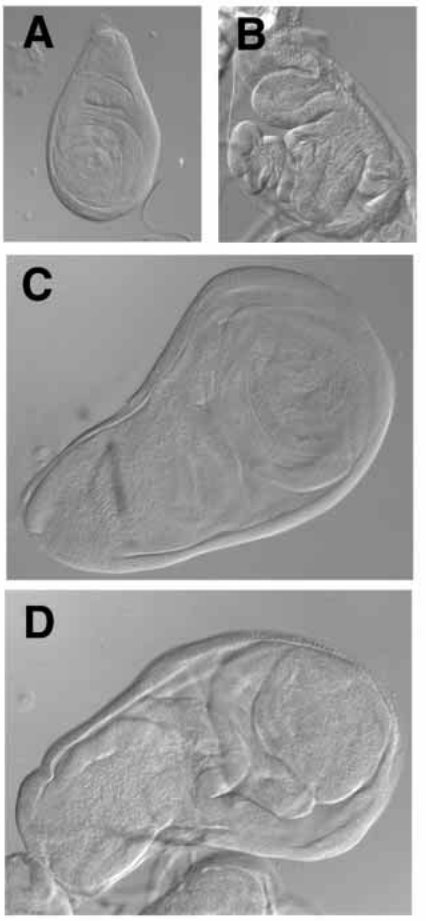

Fig. 5. *usp* mutants fail to fully evert their imaginal discs. Depicted are leg (A,B) and wing (C,D) imaginal discs of usp^2 mutants. Imaginal discs dissected from usp^2 mutant mid-third instar larvae (A,C) are indistinguishable from those in wild-type animals. Discs from stationary *usp* mutants 24 hours after the stationary stage (B,D) appear identical to discs from wild-type animals at ~1 hour after puparium formation (Fristrom and Fristrom, 1993).

The expression of larval and pupal cuticle genes was also studied to more accurately determine the nature of the supernumerary cuticle. Interestingly, all three genes examined are misexpressed in usp^2 mutants (Fig. 6 and data not shown). The *Lcp65A b* larval cuticle gene (Charles et al., 1997, 1998) is expressed long after the stationary stage in usp^2 mutants, and the *Pcpgart* and *Edg78E* pupal cuticle genes (Henikoff et al., 1986; Fechtel et al., 1988) are widely expressed, at both earlier and later times than their normal brief peak of expression in mid-prepupae. These observations indicate that the stage-specificity of cuticle gene expression has been disrupted by the usp^2 mutation. They also indicate that the ecdysone receptor can function as both a repressor and activator of target gene transcription, supporting an earlier study by Apple and Fristrom (1991) which showed that *Edg78E* is repressed by ecdysone.

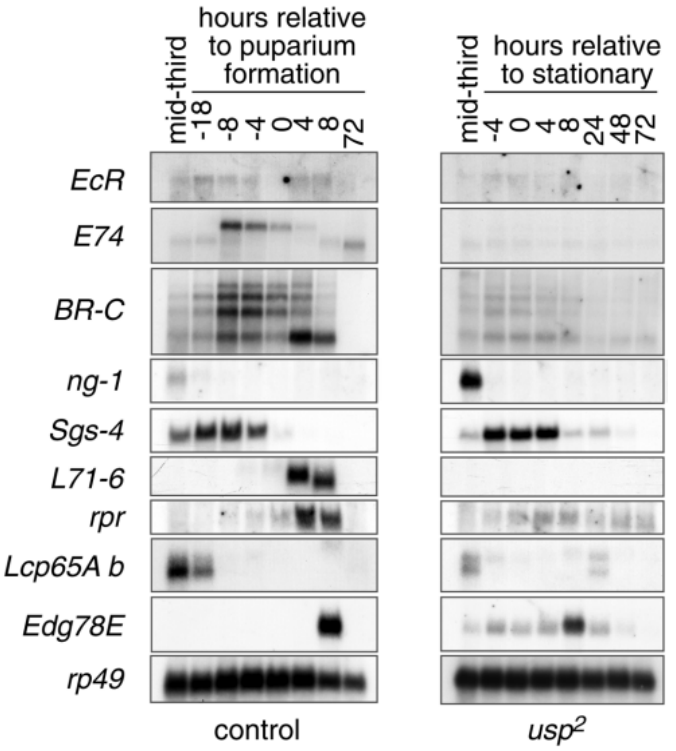

Fig. 6. *usp*^{2} mutants show stage-specific effects on ecdysone-regulated gene expression. Control siblings were subjected to the same heat pulse as *usp*^{2} mutants. Control animals were staged relative to puparium formation while *usp*^{2} animals were staged relative to the stationary phase. Mid-third instar time points were equivalent in both control and mutant animals. Northern blot hybridization was performed as described in Materials and Methods. *E74* encodes A and B isoforms, seen as upper and lower bands, respectively. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer (O'Connell and Rosbash, 1984).

DISCUSSION

USP is an essential component of the ecdysone receptor in vivo

usp mutants form stationary animals that maintain the length and shape of a late third instar larva. The imaginal discs in these mutants begin to evert but fail to elongate or differentiate, the adult midgut does not form, and the larval midgut and salivary glands fail to undergo programmed cell death. *usp* mutants are thus arrested at the larval-prepupal transition, indicative of their inability to respond to the hormonal signal that triggers metamorphosis.

Some *usp* mutant phenotypes are similar to those reported for temperature-sensitive alleles of *ecd*^{1} and *dre4* that lead to a significant reduction in ecdysone titer (Berreur et al., 1984; Sliter and Gilbert, 1992). Under appropriate conditions, these mutant larvae fail to wander, do not properly contract their body or evert their anterior spiracles at the end of the third instar, and are blocked in imaginal disc development – all defects reported here for *usp* mutants. The similarities between these mutant phenotypes argue that *usp* function is required to transduce the ecdysone signal during development.

Consistent with the proposed function of the EcR/USP heterodimer, many *usp* mutant phenotypes also resemble those reported for *EcR-B* mutations (Bender et al., 1997; Schubiger et al., 1998). *EcR-B* mutant larvae do not wander properly, fail to shorten, become stationary and display defects in larval midgut cell death, similar to the phenotypes observed in *usp* mutants. Two *EcR-B* mutant phenotypes are, however, less severe than those reported here for *usp* mutants. The imaginal discs in *EcR-B* mutants elongate (although they do not evert or differentiate) and the imaginal cells in the midgut begin to proliferate, responses that are blocked in *usp* mutants (Fig. 4, 5). The more severe phenotypes in *usp* mutants support the proposal that USP functions as an obligate heterodimer partner for all EcR isoforms (Koelle, 1992).

Taken together, the *EcR-B* and *usp* mutant phenotypes demonstrate that these two receptors are required for ecdysone signaling in vivo and argue that the EcR/USP heterodimer is the functional receptor that triggers puparium formation and the onset of metamorphosis. These conclusions are further supported by the observation that *usp* mutant third instar larvae are unaffected by maintenance on food supplemented with a high concentration of ecdysone. In contrast, control larvae raised under these conditions undergo rapid and premature puparium formation (data not shown). The defect in *usp* mutants, therefore, lies in their ability to respond to ecdysone rather than in the production of the ecdysone signal itself.

A possible role for USP in juvenile hormone signaling

The epidermis of *usp* mutants responds to the late larval ecdysone pulse in a manner that is distinct from the responses of the internal tissues. Whereas the larval midgut and imaginal discs attempt to initiate metamorphosis in *usp* mutants, the epidermis synthesizes a supernumerary cuticle, recapitulating aspects of an earlier genetic program (Fig. 3). The production of a supernumerary cuticle in *Drosophila* is a novel observation. Normally, the larval abdominal epidermis is reprogrammed to produce a pupal cuticle following puparium formation (Fristrom and Fristrom, 1993). The concurrent expression of larval and pupal cuticle genes in both third instar and stationary animals confirms that the epidermal cells are receiving inappropriate cuticle production signals (Fig. 6).

EcR-B mutants secrete a pupal cuticle and form a constriction between the thoracic and abdominal regions, similar to wild-type animals (Bender et al., 1997; Schubiger et al., 1998). The apparent absence of a cuticular phenotype in these mutants could be due to functional redundancy with EcR-A, although this isoform is not detectable in larval epidermal cells (Talbot et al., 1993). Alternatively, *usp* may play a distinct role in programming the developmental switch from larval to pupal cuticle deposition. This function could be mediated by USP homodimers or by heterodimerization with another *Drosophila* nuclear receptor (Khoury Christianson et al., 1992; D'Avino et al., 1995a; Sutherland et al., 1995; Antoniewski et al., 1996).

These observations raise the interesting possibility that *usp* may regulate responses to juvenile hormone (JH) during development. Physiological studies in a variety of insects have demonstrated a role for JH in maintaining larval stages of development. Pulses of ecdysone in the presence of JH lead to molting of the larval cuticle whereas a pulse of ecdysone in the absence of JH signals the onset of metamorphosis. Consistent with this model, removal of the corpora allata (which

synthesizes JH) can lead to precocious metamorphosis while implantation of the corpora allata, or ectopic JH application, can result in supernumerary larval molts (Wigglesworth, 1976). Similar effects, however, have not been demonstrated in *Drosophila*, raising the possibility that higher insects do not depend on JH for maintaining their larval status (Riddiford and Ashburner, 1991). Nevertheless, the JH titer in *Drosophila* is high during larval stages and drops during the final instar, similar to the pattern seen in insects that respond to JH treatment (Sliter et al., 1987). Furthermore, the production of a supernumerary cuticle in *usp* mutants is consistent with a JH effect in *Drosophila* and suggests that this receptor may be functioning in a JH signaling pathway. A recent study has proposed that USP is a JH receptor, although this binding is not saturable and is of low affinity (Jones and Sharp, 1997). An effect of JH on the transactivation function of USP has also not been demonstrated. Further biochemical and genetic studies should resolve what role, if any, JH plays during preadult *Drosophila* development, and whether *usp* functions in a JH signaling pathway.

The ecdysone receptor functions in a stage-specific manner in third instar larvae

The molecular and developmental phenotypes of *usp* mutants indicate that the ecdysone receptor functions in a stage-specific manner during the onset of metamorphosis. *usp* mutant third instar larvae develop normally in the absence of detectable USP protein until the wandering stage at the end of the instar (Fig. 1 and data not shown). Moreover, the mid-third instar regulatory hierarchy is activated in *usp* mutants while the developmental and genetic responses to the late larval pulse of ecdysone are selectively blocked (Figs 2, 6). Interestingly, *EcR-B1* also appears to function in a stage-specific manner during the onset of metamorphosis. The *BR-C* (2B5) and glue gene puffs are present in the polytene chromosomes in *EcR-B1* mutant third instar larvae, but the glue puffs fail to regress in late larvae, and the *E74* (74EF) and *E75* (75B) early puffs fail to form (Imam, 1996; Bender et al., 1997). This suggests that the *BR-C* and glue genes are induced normally in *EcR-B1* mutants, but that the response to the high titer late larval ecdysone pulse is selectively blocked – a phenotype identical to that described here for *usp* mutants. Furthermore, EcR and USP proteins are expressed at very low or undetectable levels in mid-third instar larvae (Talbot et al., 1993; Henrich et al., 1994). The EcR/USP heterodimer thus appears to function as the receptor that transduces the high titer late larval pulse of ecdysone that signals puparium formation.

This conclusion raises the question of how the mid-third instar regulatory hierarchy is controlled. The mid-third instar hierarchy was discovered through studies of ecdysone-regulated gene expression, which demonstrated that a subset of early genes, *EcR*, *E74B* and the *BR-C*, are induced in mid-third instar larvae, immediately before glue gene induction and about a day before the high titer late larval pulse of ecdysone (Georgel et al., 1991; Andres et al., 1993; Huet et al., 1993). Hormone dose-response studies using cultured late larval organs demonstrated that these early genes are more sensitive in their response to ecdysone (Karim and Thummel, 1992). This observation, combined with the identification of one or more low titer pulses of ecdysone in early and mid-third instar larvae, led to the model that the mid-third instar hierarchy is triggered by a low titer pulse of ecdysone (Karim and Thummel, 1992; Andres et al., 1993; Huet et al., 1993). The results presented here – that the mid-third instar hierarchy is active in the apparent absence of EcR-B and USP – indicate that this response is not dependent on ecdysone. Rather, recent genetic studies have suggested that the mid-third instar response is dependent on a different signaling pathway under the control of the DHR78 nuclear receptor (Fisk and Thummel, 1998). *DHR78* mutants are arrested in their development during the third larval instar and fail to activate the mid-third instar regulatory hierarchy, in apparent response to an unknown hormonal signal.

The picture that is thus emerging is one in which the onset of *Drosophila* metamorphosis is dependent on the activity of more than one receptor and is regulated by more than one hormone. Moreover, USP appears to function like its vertebrate counterpart, RXR, by integrating multiple signaling pathways during development. Further studies should indicate how the interplay between nuclear receptors coordinates the complex developmental pathways associated with insect metamorphosis.

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