The RXR ortholog USP suppresses early metamorphic processes in *Drosophila* in the absence of ecdysteroids

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SUMMARY

The steroid hormone 20-hydroxyecdysone (20E) initiates metamorphosis in insects by signaling through the ecdysone receptor complex, a heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP). Analysis of *usp* mutant clones in the wing disc of *Drosophila* shows that in the absence of USP, early hormone responsive genes such as *EcR, DHR3* and *E75B* fail to up-regulate in response to 20E, but other genes that are normally expressed later, such as *β-Ftz-F1* and the Z1 isoform of the Broad-Complex (*BRC-Z1),* are expressed precociously. Sensory neuron formation and axonal outgrowth, two early metamorphic events, also occur prematurely. In vitro experiments with cultured wing discs showed that *BRC-Z1* expression and early metamorphic development are rendered steroid-

independent in the *usp* mutant clones. These results are consistent with a model in which these latter processes are induced by a signal arising during the middle of the last larval stage but suppressed by the unliganded EcR/USP complex. Our observations suggest that silencing by the unliganded EcR/USP receptor and the subsequent release of silencing by moderate steroid levels may play an important role in coordinating early phases of steroid driven development.

Key words: Ecdysone, Ecdysone receptor, Nuclear receptors, Transcriptional silencing, Imaginal discs, Broad-Complex, *Drosophila*

INTRODUCTION

Hormones, working through their receptor proteins, activate complex signaling pathways that have profound effects on development, physiology and behavior. In *Drosophila* the steroid hormone, 20-hydroxyecdysone (20E), initiates metamorphosis. Early metamorphic events such as chromosome puffing in salivary glands (Ashburner et al., 1974) and the elongation and eversion of the imaginal discs (Fristrom and Fristrom, 1993) have been shown to depend on the presence of 20E. Studying the puffing patterns of the salivary gland chromosomes, Ashburner et al. (1974) proposed a model in which a small set of genes is directly activated by the steroid hormone. The proteins encoded by these 'early' genes act to both activate a second set of genes, the so called 'late' genes, and repress the early genes themselves. Much research effort has focussed on how the hormone activates this transcriptional cascade (the Ashburner cascade) and on the interactions of these hormone-responsive genes (Thummel, 1996). More recently it has become evident that different concentrations of 20E can induce different sets of early genes. Karim and Thummel (1992) showed that one set is expressed at low levels of 20E (2×10^{-8} M), whereas others require 2×10^{-7} M for a 50% induction. Similarly, in the moth *Manduca sexta*, Champlin and Truman (1998) demonstrated that different levels of steroid can mediate qualitatively different types of developmental

responses. For example, in the developing eye anlage of *Manduca*, low to moderate titers of 20E support prematurational events such as the movement of the morphogenetic furrow and specification of cell types in the forming retina. High titers of 20E are needed for final differentiation of the eye (cellular maturation), such as the formation of rhabdomeres, the crystalline cone, the lens, and the screening pigments.

Many studies in *Drosophila* (Yao et al., 1992; Koelle et al., 1992; Thomas et al., 1993; Yao et al., 1993) have demonstrated that the functional receptor complex for ecdysteroids is a heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and ultraspiracle (USP). Evidence for this conclusion comes from DNA gel shift assays as well as cell transfection assays (Koelle, 1992; Yao et al., 1992; Thomas et al., 1993; Yao et al., 1993). Several observations indicate that USP functions together with EcR in hormone-dependent processes in vivo as well. EcR and USP have been shown to bind to 20E regulated puffs on the salivary gland chromosomes (Yao et al., 1993). Also, *usp* mutants die at the time of the first larval molt (Perrimon et al., 1985; Oro et al., 1992). Recently Hall and Thummel (1998) have looked at *usp* mutant larvae which bypassed the early lethal phase by ectopic expression of a heat shock driven *usp*[+] gene during the first larval instar. These animals are arrested at the end of the third larval instar but fail to initiate metamorphosis. Many of the early genes show no or

reduced levels of RNA, and the glue genes also fail to switch off.

Some nuclear receptors, such as the retinoic acid receptor, the thyroid hormone receptor and the ecdysone receptor in insects, have dual action. They bind to hormone response elements in the presence or absence of ligand, but in the presence of ligand the receptor activates transcription, whereas in the absence of ligand, basal transcription is repressed (Damm et al., 1989; Graupner et al., 1989; Cherbas et al., 1991; Dobens et al., 1991). In the case of the thyroid hormone receptor, mutated receptors that fail to bind the hormone do not activate transcription (Damm et al., 1989; Sap et al., 1989) but can still bind co-repressors and constitutively silence transcription (Chen et al.,1995; Hörlein et al., 1995). Lesions of this type lead to neoplastic transformation. If second site mutations are introduced, which prevent the binding of co-repressors, neoplasms no longer form. This indicates that silencing itself is resposible for transformation (Chen et al., 1995). However, the biological role of transcriptional silencing by the unliganded receptor during normal development is poorly understood.

Here we investigate the effects of disrupting the function of *usp* on a cell by cell basis by looking at *usp* mutant patches in an otherwise normal animal. This allows us to directly compare the response of mutant cells at a particular developmental time point with the response of the wild-type cells in the same animal at the same stage. We found that *usp* function is necessary both for activation and repression of a sample of primary response genes, supporting the hypothesis that ecdysteroid action is mediated through the EcR/USP heterodimer in vivo. In addition we have tested if USP is required for early events of adult differentiation at the onset of metamorphosis; in particular, we have analyzed the differentiation of the precursors which give rise to the chemosensory neurons along the wing margin of the adult fly. We show that the hormone-dependent differentiation of wing sensory neurons occurs precociously and becomes independent of the ligand in the absence of USP. We interpret these findings as suggesting that during normal development silencing by the unliganded EcR/USP complex inhibits neuron differentiation, but that this block is subsequently released by moderate steroid levels. We propose that such silencing may play an important role in coordinating early phases of steroid driven development.

MATERIALS AND METHODS

Fly stocks and generation of clones

We used the *usp²* allele for most of the experiments in these studies. This mutation is caused by a transposition of a third chromosome fragment (66B-67E) leading to a breakpoint in the sequence encoding the DNA binding domain and, thus, disrupting USP function (Oro et al., 1990). In clones homozygous for this allele, cells are also tetraploid for the third chromosome fragment. In order to rule out that the tetraploidy causes the changes we have observed, we analyzed controls carrying a *usp⁺* transformant gene (Oro et al., 1990). The transgene rescues the loss of USP, but the clones are still tetraploid for the third chromosome. For some experiments we have also made clones of two *usp* point mutations (*usp³* and *usp⁴*) which both have substitutions of conserved amino acid residues in the DNA-binding domain, leading to reduced DNA-binding (Henrich et al., 1994). All three alleles have similar lethal phenotypes and are considered to be at least strong hypomorphs. All *usp* alleles and the *usp⁺* transformant line were received from M. McKeown.

To generate clones in the wing discs, we crossed w^{1118} *FRT18A; hs-FLP* females (obtained from the Bloomington stock center) to w^{1118} *usp hs-N-myc FRT18A/Y; <usp⁺>Tb/TM3 Sb* males (Xu and Rubin, 1993). Eggs were collected over an 8 hour period, and clones were induced in the second instar by heat shocking vials containing the larvae for 1 hour in a 37°C water bath. Prior to the dissection of the wing discs, female Tb^+ larvae were heat shocked as above for 30 minutes to induce *myc* expression. Animals were allowed to recover at room temperature for 1-2 hours before dissection. Wing discs were collected from animals of the indicated stages and processed for immunostaining (Schubiger et al., 1998). Wandering larvae were staged according to the food remaining in their gut (Maroni and Stamey, 1983); prepupae were staged in hours after pupariation (APF). For the timing of induction of BRC-Z1, animals were staged after the molt to the third instar (=72 hours after egg deposition; AED). The discs were analyzed with a BioRad 600 confocal microscope, and the images processed with Adobe Photoshop 3.0.

In vitro cultures

In vitro cultures were set up in small Petri dishes (Gibbs and Truman, 1998) in D22 insect medium or Shields and Sang 3M (both from Sigma), supplemented with 7.5% fetal calf serum, a mixture of antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B), and the addition of 0 µg/ml or 1 µg/ml 20E (Sigma). In some cases fragments of fat body were also added. The cultures were kept at 25°C under 95% O_2 and 5% CO_2. After 1 day, the Petri dishes were placed in a 37°C water bath for 30 minutes to induce the MYC marker and then put back at 25°C with oxygenation for 2 hours before fixing and processing as above.

Antibodies

We used monoclonal antibodies 15G1a against EcR-A and AD4.4 against EcR-B1 at a 1:50 dilution, E75B (10E11) at 1:20 and a polyclonal antibody against DHR3 at 1:1000 (all gifts from D. Hogness). Anti-BRC-Z1 was used at 1:100 (a gift from G. Guild), anti-USP antibodies (AB11 and DH9) at 1:100 (gifts from F. Kafatos), anti-β FTZ-F1 (a gift from H. Ueda) at 1:2000, a polyclonal anti-ELAV (Robinow and White, 1991; a gift from S. Robinow) at 1:6000, a monoclonal anti-ELAV (7E8A10, Developmental Studies Hybridoma Bank) at 1:100, and 22C10 (Developmental Studies Hybridoma Bank), at 1:100. Anti-HRP (United States Biochemical) was used at a concentration of 1:1000. For the detection of the MYC marker, we used either a mouse monoclonal antibody at 1:100 (Oncogene) or a rabbit polyclonal antibody (Santa Cruz Biotechnology) at 1:200. We used either FITC- or Texas-Red-conjugated donkey anti-mouse, anti-rat or anti-rabbit secondary antibodies (Jackson ImmunoResearch) at 1:500.

RESULTS

usp² is a protein null

The x-ray induced mutation *usp²* is a transposition of a third chromosome fragment (66B-67E) into the *usp* gene, disrupting its coding region (Oro et al., 1990). This structural change would predict that no functional USP protein is made. To test this, we induced clones during the second larval instar and analyzed the wing discs about 3 days later during the late wandering stages. Discs were labeled with antibodies against 2 different epitopes of the USP protein (Khoury Christianson et al., 1992): AB11, which recognizes the DNA-binding domain, and DH9, which recognizes the most N-terminal

region. Fig. 1 shows a wing disc immunoreacted with AB11. The wild-type clone with 2 copies of *usp*⁺ had high levels of label, the surrounding heterozygous tissue expressed moderate levels, and the homozygous mutant clones showed no detectable USP protein. Using the DH9 mAb, we also could not detect any USP immunostaining in homozygous *usp²* tissue (data not shown). Thus, *usp²* appears to be a protein null. This is in agreement with western blot analyses of mutant larvae (Hall and Thummel, 1998).

## USP is required for the up-regulation of the hormone responsive genes *EcR, E75B* and *DHR3*

We analyzed the response of the early genes *EcR-A, EcR-B1, E75B* and *DHR3* in *usp* mutant clones. Since USP and EcR act together as the hormone receptor, we predicted that the up-regulation of these genes would fail in *usp²* clones. Wing discs were harvested between late feeding and early prepupal stages, at times when the tested genes were normally well expressed. The *EcR* gene itself is an early gene and directly activated by 20E (Karim and Thummel, 1992). Fig. 2A shows a mosaic wing disc with strong EcR-A expression in the wild-type cells. In the mutant patches, however, EcR-A was expressed at very much reduced levels, but some expression could still be detected. The EcR-B1 isoform shows an earlier time of activation; it is expressed before EcR-A but then declines during the late wandering phase so that EcR-A becomes the predominant isoform in the imaginal discs at pupariation. Discs with *usp²* clones were taken from late feeding larvae, when EcR-B1 is well expressed. We found that EcR-B1 failed to be up-regulated when USP function was lacking (Fig. 2B). *DHR3* is also directly up-regulated by 20E (Koelle et al., 1992), though its maximal expression depends on protein synthesis (Palli et al., 1992). At 2 hours after puparium formation (APF) DHR3 was expressed in the wild-type cells but severely reduced in the mutant clones (Fig. 2C). We also analyzed the expression of E75B, which is normally expressed strongly during early prepupal stages. Here too, E75B expression was reduced in the cells lacking USP (Fig. 2D). These results demonstrate that USP is required for the induction of these primary response genes, presumably through its dimerization with EcR.

## Loss of USP leads to premature expression of β-FTZ-F1 and the Z1 isoform of the Broad complex (BRC-Z1)

In contrast to *EcR* and *E75B*, *β-Ftz-F1* is not expressed during wandering, but begins being transcibed about 4 hours after pupariation (Lavorgna et al., 1993). *β-Ftz-F1* responds directly to the drop of ecdysteroids during the prepupal stage and is suppressed by rising hormone titers
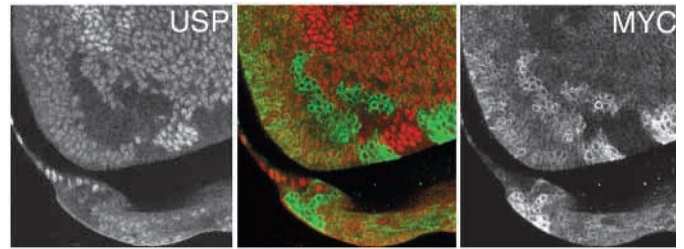
Fig. 1. *usp²* is a protein null. Wing disc from a wandering larva, immunoreacted with an antibody against USP (AB11) (left side) and with anti-MYC (right side). Homozygous *usp²* cells, recognized by their strong anti-Myc labeling show no detectable USP label. In contrast, the homozygous *usp*⁺ cells (negative for the MYC label) show high levels of USP, and the surrounding cells, with one copy of *usp*⁺, show moderate levels. The middle panel is a merged image of the left and right images.
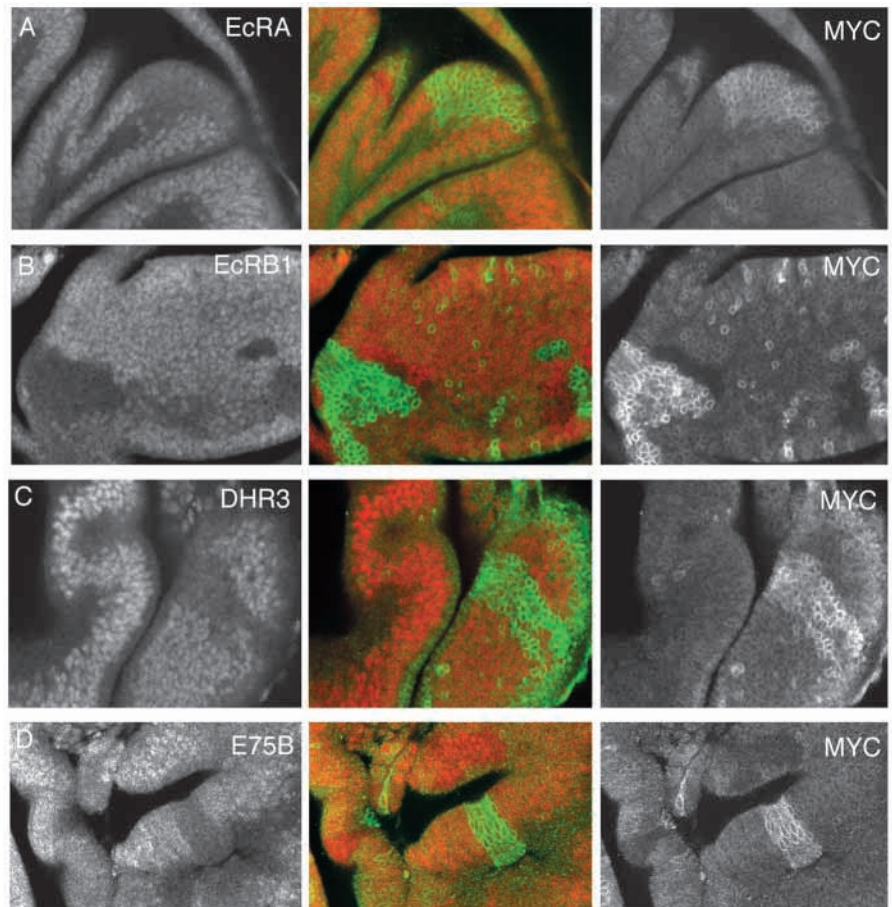
Fig. 2. Activation of hormone responsive genes. (A) Expression of EcR-A in a wing disc from a wandering larva. Nuclear expression is drastically reduced in the *usp²* clone compared to the wild-type tissue surrounding it. (B) EcR-B1 expression in a mosaic disc taken from a late feeding larva. EcR-B1 expression is very low in the *usp²* mutant cells. (C) DHR3 expression in a wing disc 2 hours APF. Cells lacking USP function fail to up-regulate DHR3. (D) E75B expression in a mosaic disc 2-3 hours APF. E75B expression is lower in the *usp²* cells. The right hand panels show MYC expression for the corresponding discs; homozygous *usp²* clones are identified by enhanced anti-MYC staining caused by 2 copies of the *myc* gene. The middle panels are the merged images of the right and left side.
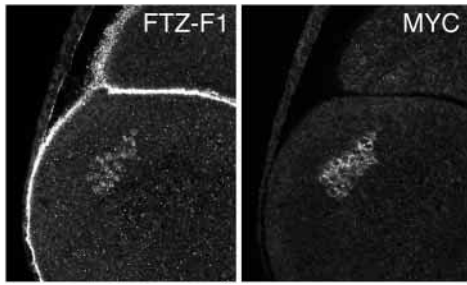
Fig. 3. Repression of the hormone responsive gene *β-Ftz-F1*. Wing disc from a wandering larva shows no β-FTZ-F1 expression in wild-type cells. In contrast, homozygous *usp³* cells are strongly labeled. The right hand panel shows anti-MYC label. The strong anti-MYC label identifies the homozygous *usp³* clone.

(Woodard et al., 1994). We scored for β-FTZ-F1 expression in *usp* mutant clones during wandering, when the hormone titer is rising and β-FTZ-F1 is normally not expressed. This protein was now expressed in the mutant cells at high levels, at least 12 hours before its expression in wild-type cells (Fig. 3). Thus, USP is required for the suppression of *β-Ftz-F1* during wandering.

We also examined the expression of one of the Broad-Complex isoforms, BRC-Z1. In the imaginal discs, the different isoforms of the BRC show a switch from predomantly expressing Z2, Z3 and Z4 during wandering, to expressing only the Z1 isoform during the prepupal period. *BRC-Z1* is transcribed at gradually increasing levels during wandering but reaches maximum expression during the prepupal period (Emery et al., 1994; Bayer et al., 1996; Fig. 4A). In *usp²*, as well as in *usp³* and *usp⁴* clones, we found that the Z1 isoform was prematurely expressed. We examined this early up-regulation in more detail. The *usp²* clones analyzed from larvae 99 hours after egg deposition (AED) showed no BRC-Z1 (Fig. 4B), but by 102-103 hours AED some *usp²* clones had a few cells expressing BRC-Z1 (Fig. 4C). By the transition to wandering (approximately 110 hours AED), BRC-Z1 was strongly expressed in all cell in *usp²* clones while absent from the surrounding wild-type tissue (Fig. 4D). Wild-type tissue reached BRC-Z1 levels that were similar to those in mutant clones by 4 hours APF (Fig. 4E). These results show that USP does not appear to be necessary for the induction of BRC-Z1 expression but is required for its repression until mid-wandering.

Loss of USP function leads to precocious sensory neuron differentiation

An early sign of adult differentiation in the wing disc is the birth and differentiation of the sensory neurons (Murray et al., 1984; Hartenstein and Posakony, 1989). The dense arrangement and the almost synchronous development of the chemosensory organs along the wing margin provided us with a good area to compare development in *usp* mutant and wild-type cells. As the wing disc undergoes metamorphosis, sensory neuron precursor cells and their progeny can be detected with a variety of different antibodies. The monoclonal antibody 22C10 (Zipursky et al., 1984) recognizes the chemosensory precursors at the time of pupariation, labeling 2 rows of cells in the presumptive anterior wing margin (Fig. 5). Anti-HRP also shows early labeling of the precursor cells, becoming

distinct by 3 hours APF. By 6-7 hours APF *elav*, a neuron-specific gene (Robinow and White, 1991), is expressed in the newly born neurons of the chemosensory organs. At about this time these neurons begin to extend their axons, which label with both 22C10 and anti-HRP. Fig. 6 shows examples of *usp* mutant clones in prospective wing margins. As can be seen using anti-HRP (Fig. 6A), the neurons lacking USP function differentiated precociously, showing strong expression of the HRP-antigen and also having extended their axons. In comparison, the wild-type cells showed weak anti-HRP labeling and there was no sign of axon outgrowth. The precocious axonal growth in the mutant cells often led to abnormal projections with the axons forming loops and branches, frequently extending into the middle of the wing rather than along the path of the first longitudinal vein. We also observed precocious expression of ELAV in *usp* mutant clones, indicating that the neurons were born in the clone well before those in neighboring wild-type tissue (data not shown). In 74 wings analyzed, we found 24 clones in the margin area that showed precocious differentiation detected by anti-ELAV, anti-HRP or 22C10. We also had a few cases of discs from wandering larvae in which clones potentially in the margin region did not show premature differentiation. However, since in these young wing discs we did not have an independent marker to identify the margin region, we do not know if these clones actually extended into the neurogenic region. These cases not withstanding, the characteristic result of a patch of USP null tissue in the wing margin is the premature development of the sensory structures in that region.

Because of the nature of the *usp²* mutation the homozygous clones are also tetraploid for genes in the 66B-67E region of the third chromosome. To rule out an effect of the tetraploidy on neuron differentiation, we analyzed clones in sibs carrying the *usp⁺* transgene on the third chromosome (Oro et al., 1990). In this case the *usp²* clone was also tetraploid for 66B-67E but USP function was restored through the transgene. Thus, any effect we saw in *usp²* mutant clones, which was rescued by the *usp⁺* transgene in the control sibs, is attributable to the lack of USP function and not caused by the tetraploidy. As Fig. 6B shows, we did not observe precocious differentiation in these control animals. In addition, precocious differentiation was also observed in clones of the point mutations *usp³* (Fig. 6C) and *usp⁴* (data not shown). Taking these results together, we conclude that USP functions to repress early sensory neuron differentiation.

Role of 20E and USP in sensory neuron differentiation and BRC-Z1 expression

We further explored the relationship of possible activational and suppressive function of USP to steroid signaling by culturing wing discs from early-mid wandering larvae in vitro in the presence or absence of 1 µg/ml 20E. As shown in Fig. 7A, differentiation of the margin neurons required 20E (58 wings analyzed). Also, as we saw in vivo, neurons in 14 *usp²* clones located in the margin had differentiated at least as far or farther than the surrounding wild-type tissue in the presence of 20E (Fig. 7A). BRC-Z1 expression was also 20E dependent, and after 1 day in culture was strong in both *usp²* and wild-type cells (Fig.7C). The surprising result came from discs cultured in the absence of 20E. In 79 cultured discs, the wild-type tissue arrested its development, but 24 patches of *usp²*

cells that were located in the presumptive margin, underwent neurogenesis and initiated axonal outgrowth. Although we found a similar percentage of margin clones under the two conditions (24% versus 30%), we do not know if all clones containing neurogenic cells showed precocious differentiation in the absence of 20E because we did not have an independent marker for the margin. Interestingly, the extent of neuronal development in the *usp²* clones was essentially the same as that seen for discs cultured with 1 µg/ml 20E. As seen in Fig. 7D, cells in *usp²* clones also showed strong BRC-Z1 expression in the absence of 20E, while the surrounding tissue did not. Thus, 20E is required for neuron differentiation and BRC-Z1 expression. However, in the absence of USP these processes no longer require 20E.

DISCUSSION

USP functions to regulate ecdysteroid-responsive genes in vivo

Since USP and EcR form the ecdysteroid receptor complex (Yao et al., 1993), it is predicted that in the absence of either USP or EcR, ecdysteroid-responsive genes will fail to be properly regulated. Here we have shown that in clones lacking USP protein both hormone-dependent gene activation (*EcR-A, EcR-B1, DHR3* and *E75B*) and repression *(β-Ftz-F1* and *BRC-Z1)* fail, demonstrating that USP function is required to mediate ecdysteroid action in the animal as well as in transfected cell lines (Koelle, 1992; Thomas et al., 1993; Yao et al., 1993). Hall and Thummel (1998) also implicated the EcR/USP heterodimer in hormone receptor function in vivo by their demonstration that in mutant *usp* larvae, genes regulated by the late larval ecdysteroid peak do not up-regulate and these larvae fail to metamorphose. Overall our results based on clonal analysis are in good agreement to theirs, but there are some interesting differences. We found high levels of BRC-Z1 product in the *usp* mutant clones beginning in the mid-third instar. By contrast, in the northern blot analysis of whole mutant larvae, Hall and Thummel (1998) did not find high levels of *BRC-Z1* transcripts. We have also rescued *usp²* mutant larvae in the same manner and found both expression of BRC-Z1 protein and precocious differentiation of sensory neurons in the wing disc (C. Caputo and M. S., unpublished observations). We think it likely that we are seeing a tissue-specific up-regulation of BRC-Z1 that may not be detected in northern analysis of whole animals.

USP as a repressor of gene expression and development

Our analysis of the expression of BRC-Z1 has been especially insightful. In clones lacking USP, BRC-Z1 is still developmentally regulated but it first appears about 103 hours AED rather than at its expected time of expression at mid-wandering. This precocious appearance of BRC-Z1 corresponds to the so called 'mid-third instar transition' (Andres and Cherbas, 1994), which is a time when changes occur in preparation for metamorphosis. Recently Fisk and Thummel (1998) proposed that the gene *DHR78* may play a role in this transition and that these changes may occur independently of the 20E titers. It appears that events at this time serve to activate BRC-Z1 expression but a USP mediated

mechanism suppresses expression until rising ecdysteroid titers remove the inhibition late in wandering.

The development of sensory precursor cells in the wing show a similar response to the loss of USP. As sensory neurons differentiate and project their axons toward the CNS, the timing of these events is crucial. In order for axons to navigate centripetally, guidance cues must be present at the correct place and time. The synchrony of these events, as we have shown for the developing neurons along the presumptive wing margin, is disrupted in *usp* mutant clones. Differentiation and axon outgrowth occur precociously, and, as a consequence, the axons originating from these neurons are misrouted. We have also seen premature differentiation and faulty axon projection for the presumptive campaniform sensilla on the radius of the wing, and Zelhof et al. (1997) reported precocious differentiation of photoreceptors in the eye disc. Thus, USP appears to play a general role of suppressing early sensory neuron differentiation in the imaginal tissue.

Our in vitro experiments show that both the expression of BRC-Z1 and the differentiation of sensory neurons are dependent on exposure to ecdysteroids. Surprisingly, though, the presumed disruption of the ecdysone receptor complex by removal of USP allows these processes to proceed in a hormone-independent fashion. Observations on the developing eye disc showed that 20E is required for the correct progression of the morphogenetic furrow (Brennan et al., 1998), but that loss of USP leads to advancement of the morphogenetic furrow and precocious differentiation of the photoreceptors (Zelhof et al., 1997). We assume that in the case of the eye, the events in the morphogenetic furrow are also rendered hormone-independent by the removal of USP. A working model for this function of USP is developed below (see also Buszczak and Segraves, 1998).

A biological role for silencing by the unliganded receptor?

Our data suggest that USP is involved in two distinct types of ecdysteroid controlled responses and that these responses have distinct developmental roles. In some instances USP serves as a hormone-inhibited silencer whereas in others it is a hormone-dependent activator. Early metamorphic events in the wing, including neurogenesis and axonal outgrowth, clearly require ecdysteroids in order to occur, but this requirement is carried out through an ecdysteroid-dependent release of USP-mediated suppression. Thus, in the absence of USP these events occur in a steroid-independent fashion. Importantly, the rate of development in *usp* mutant clones is at least as fast or faster than in wild-type tissue exposed to 20E, suggesting that for these developmental processes the effects of 20E are at best permissive.

A molecular parallel to what we see for early sensory neuron development is illustrated for the expression of BRC-Z1. Even though BRC-Z1 is expressed in the neurogenic regions of the disc, studies with BRC-Z1 mutants show that altered expression of BRC-Z1 does not interfere with the differentiation of the wing or its sensory neurons (Kiss et al., 1988; Bayer et al., 1997; M. S., unpublished observations). Nevertheless the precocious expression of BRC-Z1 in cells lacking USP function provides insight into what we would expect for the genes directly involved in neuronal birth and differentiation.
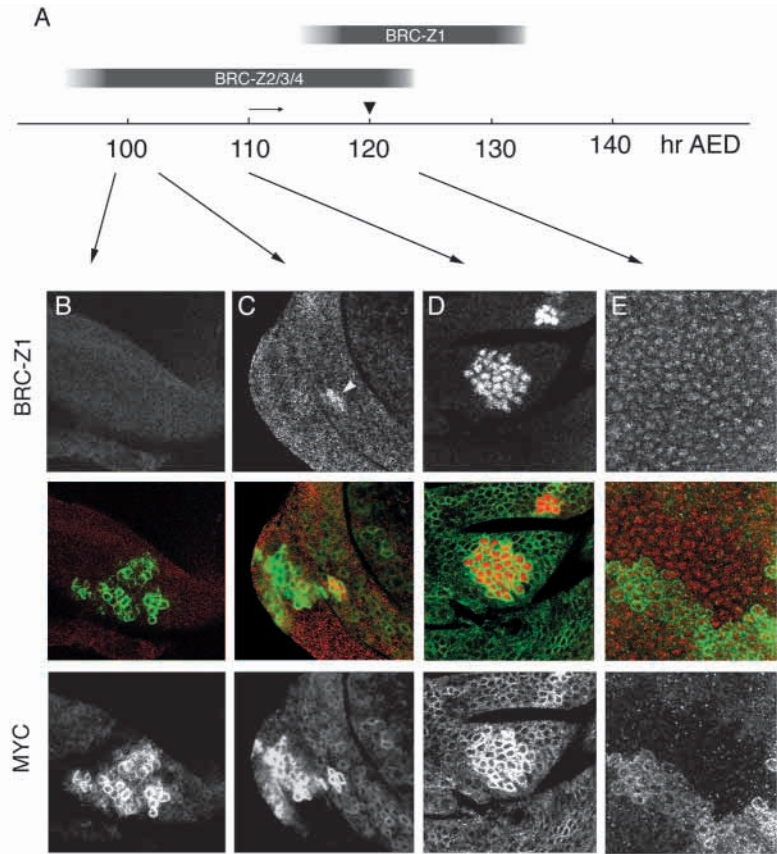
Fig. 4. Onset of BRC-Z1 expression in *usp²* clones in developing wing discs. (A) Time of expression of the four BRC isoforms in imaginal discs. BRC-Z2-4 proteins are expressed during the second half of the third instar and disappear after pupariation. BRC-Z1 is expressed starting at mid-wandering and continuing throughout the prepupal period (data taken from Emery et al., 1994 and Bayer et al., 1996). A small arrow indicates the beginning of wandering; the arrowhead shows time of pupariation. (B) Wing disc 99 hours AED. Neither wild-type nor mutant cells express BRC-Z1. (C) Wing disc 102-103 hours AED. Only a few cells (arrowhead) in the *usp²* clone are expressing BRC-Z1, the surrounding wild-type tissue is suppressed. (D) Wing disc at 110 hours AED. The mutant cells show high expression of BRC-Z1, but the surrounding wild-type cells are still suppressed. (E) Wing at 4 hours APF. Wild-type and mutant cells both express BRC-Z1. Top row shows BRC-Z1 label; bottom row shows MYC label to allow identification of homozygous *usp²* cells based on their high levels of the MYC marker. The middle row is a merged image of BRC-Z1 and MYC label.

BRC-Z1 expression appears to be activated by events during the mid-third instar, but it is suppressed via USP until the titer of 20E is high enough to remove this USP-mediated silencing. In this context the presence of the hormone is permissive in that it allows other factors (induced by the mid-third instar transition?) to take control of gene expression. This de-repression contrasts with the other class of ecdysteroid-dependent events, such as the up-regulation of the early response genes we tested (*EcR, DHR3, E75B*). EcRA, for example, is not up-regulated in the absence of USP, with or without hormone (Fig. 2A and data not shown).

Fig. 8 presents a model to explain the two types of responses. It is based on the ability of the unliganded EcR/USP complex (Cherbas et al., 1991; Dobens et al., 1991) and related nuclear receptors to silence gene transcription (for review, Xu et al., 1999). We have shown two examples which differ in the activational capacity of the liganded receptor. In the first case (Fig. 8A), the liganded receptor acts as a strong activator of transcription. The effects of lack of hormone or lack of a functional receptor would be expected to be the same, namely

the failure to activate target genes. This is what we observed for the up-regulation of *EcR, DHR3* and *E75B*. Interestingly, we found that in *usp²* clones low levels of these proteins are still detectable, possibly as a result of basal transcription in the absence of silencing.

In the second case (Fig. 8B), gene activation is driven by some other enhancer, induced in the mid-third instar, that interacts with the promoter. However, this enhancer cannot initiate transcription because the unliganded receptor acts as a silencer. In this case the lack of hormone and the loss of receptor function would have opposite effects. The lack of ligand would result in a block of transcription, whereas the lack of receptor function would prevent silencing and allow

Fig. 5. Timing of events in the developing wing disc in hours (hr) after puparium formation (APF). (A) Camera lucida drawing of a wing disc 3 hours APF labeled with 22C10 is shown to indicate the morphology of the developing wing. Sensory precursor cells along the margin have not yet divided to produce the neurons, in contrast to sensory neurons in the interior of the wing. (B) The time of birth of the chemosensory neurons and axon elongation is indicated, as well as time from when 22C10, anti-HRP and anti-ELAV are detected. All times indicated are given only for the precursors of the chemosensory bristles situated along the margin of the wing.
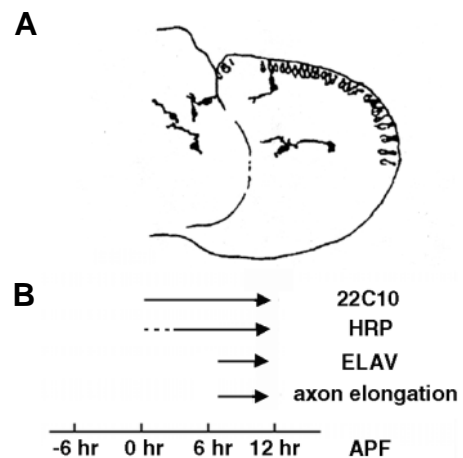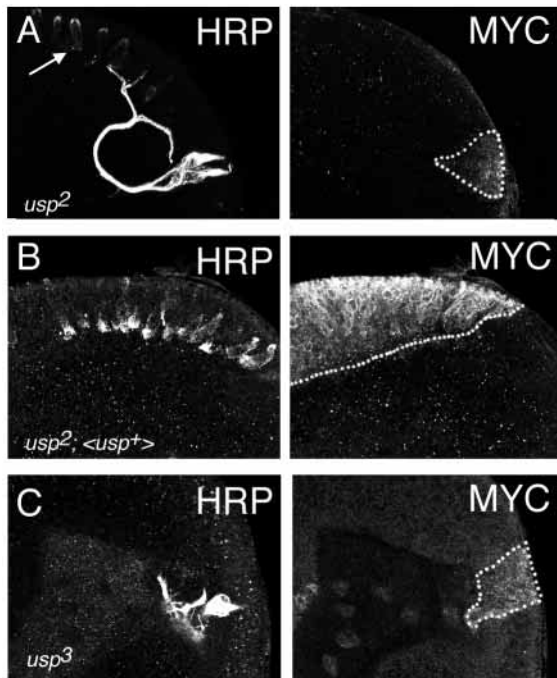
Fig. 6. Sensory neuron differentiation in mosaic wing discs. (A) Wild-type neuronal precursors (arrow) along the margin of a wing disc 5 hours APF show little HRP antigen and have not yet begun axonogenesis. The neurons in the *usp²* clone are advanced in their development and have extended their axons. The axonal projection is abnormal, forming a loop, rather than extending parallel along the margin. (B) Mosaic wing with a *usp²* clone in a genetic background containing a *usp⁺* transgene. The *usp⁺* gene has rescued the precocious differentiation of the cells in the mutant clone. (C) Mosaic wing, 3 hours APF with a *usp³* clone, immunostained with anti-HRP. The mutant cells show precocious neuron differentiation. The right hand panels show MYC labeling to recognize the mutant patches (outlined).

Of course, it is possible that USP suppresses early metamorphic development in association with another, unidentified nuclear receptor family member. For example, it has been demonstrated that USP can heterodimerize with DHR38 (Sutherland et al., 1995). In such a case we would predict that in the absence of high 20E, USP dimerizes with this nuclear receptor and binds to the EcRE to silence transcription. As the titers of 20E rise, USP would then shift to binding with EcR and 20E. However, if the EcR/USP heterodimer functions to silence, we would expect *EcR⁻* clones to give the same results as we reported here.

It has been shown for the thyroid hormone that the lack of ligand produces much more severe and extensive defects than does the lack of receptors (Göthe et al., 1999). Göthe et al. (1999) suggested that chronic repression of thyroid inducible genes by the TR/RXR complex may underlie the more deleterious phenotype of hypothyroid animals. Analogous to the experiments described here, we would expect that some processes, normally under the control of silenced genes, would proceed in receptor-deficient mutants, irrespective of the hormonal titer, thereby alleviating some of the symptomes of ligand deficiency.

The results presented show that the up-regulation of genes in the ecdysteroid-responsive cascade (the Ashburner cascade) is disrupted in cells lacking USP. In contrast, the birth and early differentiation of sensory neurons occur normally. Thus, genes involved in early neuron differentiation must not be acting through the Ashburner cascade. Consequently, there appears to be at least two lines of ecdysteroid-dependent processes that

transcription to occur. Such a scenario would explain the ecdysteroid dependency for *BRC-Z1* transcription and neuron differentiation, and the premature activation of *BRC-Z1* and the precocious differentiation of sensory neurons in the absence of receptor function.
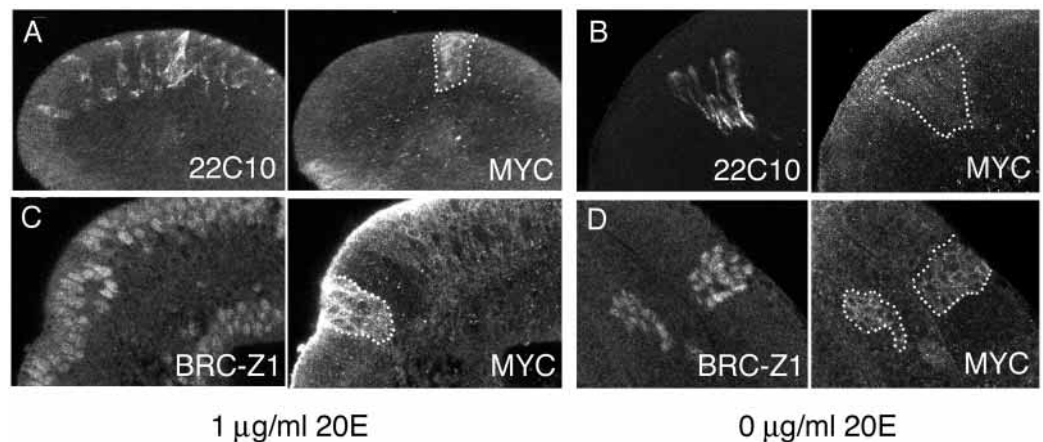
This model predicts that genes involved in these early developmental processes have ecdysone response elements (EcREs) that are effective silencers but lack the ability to activate transcription. Such a 'silencing EcRE' has been found upstream of the EIP 71CD gene (Andres and Cherbas, 1994). The 15 nucleotide core of this EcRE is a potent silencer of transcription in the absence of steroid but a poor activator in the presence of 20E (Cherbas and Cherbas, 1996). In contrast, the other two downstream EcREs for this gene have strong activating and silencing function and would be an example of an inductive EcRE depicted in Fig. 8A. The scheme we have presented is in its simplest form in which the EcR/USP heterodimer functions both as a silencer and an activator.

Fig. 7. In vitro cultures of wing discs from early-mid wandering larvae in the presence or absence of 20E for 1 day. (A) Wing disc cultured with 20E (1 µg/ml). The sensory precursor cells in the margin have developed in wild-type tissue and in a *usp²* clone, but neuronal development is advanced in the mutant cells. (B) Wing disc cultured without 20E. The margin neurons did not develop in the wild-type tissue, but underwent differentiation in the *usp²* clone. (C,D) Expression of BRC-Z1 in cultured wing discs in the presence or absence of 20E.

With 20E exposure (C) both wild-type and *usp²* cells express BRC-Z1, but without 20E (D) BRC-Z1 expression is only found in the mutant cells. The left panels show expression of 22C10 (A,B), or BRC-Z1 (C,D); right panels show MYC labeling marking *usp²* clones (outlined).

Fig. 8. Simple working model for activation and silencing of hormone responsive genes. (A) Inductive EcRE. A 20E inducible gene is repressed when its EcRE is bound by the unliganded receptor, but is strongly activated when the EcRE is bound by the liganded receptor. (B) Permissive EcRE. A signal at the mid-third instar induces an enhancer 'En', which binds to a response element of the gene. However, because of the presence of the unliganded receptor on the silencing EcRE (sil-EcRE), the gene remains suppressed. As the hormone titer rises, the liganded receptor no longer silences and leads to a permissive state, allowing transcription of the gene by the enhancer 'En'.
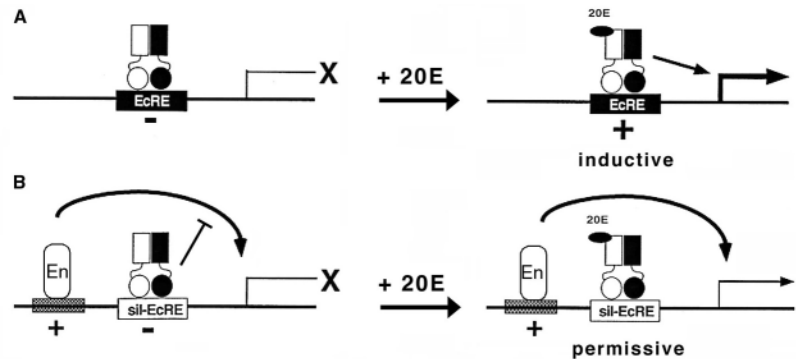
run in parallel as the animal begins metamorphosis. In *Manduca* (Champlin and Truman, 1998) prematurational and maturational processes are clearly separated in time and in the levels of ecdysteroids required for their initiation. The prematurational processes are driven by low levels of 20E that are insufficient to activate the early genes *E75A* and *MHR3*, indicating that the prematurational events do not occur via the Ashburner cascade. Like the neurogenic effects we describe here, we think it likely that in *Manduca* these prematurational processes may also be under the control of silencing EcREs, which would allow these early events to proceed once silencing is lifted by rising titers of hormone. Maturational events would be dependent on the Ashburner cascade and would be predominantly under the control of activating EcREs and require higher hormone titers.

Champlin and Truman's studies have drawn parallels between the prematurational and maturational effects of ecdysteroids in insects and the action of thyroid hormone during amphibian metamorphosis. Low levels of thyroid hormone promote prometamorphic responses that are distinct from those seen at metamorphic climax in response to high hormone titers (Yoshizato, 1996). Such prometamorphic processes might now be interpreted to depend primarily on the release of a developmental block caused by the unliganded receptor. This opens up the possibility that silencing in the absence of hormone may be a general mechanism in normal development to ensure the proper temporal control of early metamorphic events.

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