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A conditional rescue system reveals essential functions for the *ecdysone receptor* (*EcR*) gene during molting and metamorphosis in *Drosophila*

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SUMMARY

In *Drosophila*, pulses of the steroid hormone ecdysone trigger larval molting and metamorphosis and coordinate aspects of embryonic development and adult reproduction. At each of these developmental stages, the ecdysone signal is thought to act through a heteromeric receptor composed of the EcR and USP nuclear receptor proteins. Mutations that inactivate all EcR protein isoforms (EcR-A, EcR-B1, and EcR-B2) are embryonic lethal, hindering analysis of *EcR* function during later development. Using transgenes in which a heat shock promoter drives expression of an *EcR* cDNA, we have employed temperature-dependent rescue of *EcR* null mutants to determine *EcR* requirements at later stages of development. Our results show that *EcR* is required for hatching, at each larval molt, and for the initiation of metamorphosis. In *EcR* mutants arrested prior to metamorphosis, expression of ecdysone-responsive genes is blocked and normal ecdysone responses of both imaginal and larval tissues are blocked at an early stage. These results show that *EcR* mediates ecdysone signaling at multiple developmental stages and implicate *EcR* in the reorganization of imaginal and larval tissues at the onset of metamorphosis.

Key words: *Drosophila*, Ecdysone, *ecdysone receptor*, *ultraspiracle*, Molting, Metamorphosis, Imaginal disc

INTRODUCTION

During *Drosophila* development, pulses of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) act as temporal signals to coordinate the transition between distinct developmental stages. Ecdysone pulses trigger molts to the second and third larval instars and a series of three prominent pulses drive the transition between larval, prepupal, pupal and adult stages. In addition, a mid-embryonic pulse of ecdysone is detected at the time of germ-band retraction and dorsal closure and a small pulse detected at the mid-point of the third instar may trigger larval wandering, a behavioral change that takes place prior to metamorphosis (Richards, 1981; Riddiford, 1993).

Stage- and tissue-specific actions of the ecdysone signal are thought to be mediated by the ecdysone receptor, which is a heterodimer of two nuclear receptors, namely EcR and USP (Koelle, 1992; Yao et al., 1992, 1993; Thomas et al., 1993). The *EcR* gene produces three protein isoforms (EcR-A, EcR-B1 and EcR-B2) through the use of two promoters and alternative splicing (Koelle et al., 1991; Talbot et al., 1993). The EcR isoforms share a common carboxy-terminal region that includes the DNA- and ligand-binding domains, but differ in their amino-terminal domains. The *usp* gene encodes a single protein product homologous to the vertebrate RXR nuclear receptor (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990). The heteromeric receptor is believed to transduce the ecdysone signal by activating a genetic regulatory hierarchy, including a small set of early genes directly induced by ecdysone and a larger set of late genes activated by the early genes and comprising a secondary response to the hormone (Ashburner et al., 1974; Burtis et al., 1990; Thummel et al., 1990). A small set of early-late genes also participates in this hierarchy (Ashburner and Richards, 1976). These genes are directly induced by ecdysone, although they require protein synthesis for their full expression (Stone and Thummel, 1993; Horner et al., 1995).

Identification of mutations that inactivate individual EcR isoforms or subsets of EcR isoforms have been informative in defining functions of particular EcR proteins. Mutations that inactivate only the EcR-B1 isoform block ecdysone responses at metamorphosis in larval and imaginal tissues that normally express high levels of EcR-B1, but allow initiation of normal ecdysone responses in tissues that predominantly express EcR-A (Bender et al., 1997). Mutations that inactivate both EcR-B1 and EcR-B2 are defective in larval molting and in the early stages of neuronal remodeling that take place during metamorphosis (Schubiger et al., 1998). Mutations that inactivate all EcR isoforms show that *EcR* is required to complete embryogenesis (Bender et al., 1997). Mutants lacking *usp* zygotic functions die as first or second instar larvae (Perrimon et al., 1985; Oro et al., 1992), whereas those lacking both maternal and zygotic usp functions die during late embryogenesis, with cuticle defects at the posterior of the embryo (Oro et al., 1992).

Because ecdysone acts at multiple times during development and influences a wide variety of tissues, it has been particularly important to develop conditional mutations so that the function of *EcR* can be studied at any developmental stage. Here we have rescued *EcR* common-domain mutants via heat-shock induced expression of EcR protein and used this system to determine *EcR* requirements during later stages of development. We find that *EcR* is required to complete each larval molt and to initiate metamorphosis. *EcR* mutants arrest during the larval molts with two pairs of larval cuticular derivatives including mouthhooks and spiracles. We have re-examined phenotypes exhibited by loss-of-function *usp* mutants and find that these are identical to those seen in *EcR* mutants arrested at the first larval molt, suggesting that EcR and USP function together in vivo to mediate larval molting. In *EcR* mutants arrested at the onset of metamorphosis, normal imaginal and larval tissue responses to ecdysone are blocked at an early stage. In addition, expression of ecdysone-responsive genes is blocked at this time. These results indicate that *EcR* mediates ecdysone signaling at multiple developmental stages and in multiple tissues during metamorphosis.

MATERIALS AND METHODS

Drosophila stocks and crosses

EcR common-domain mutants carrying a heat shock-EcR-B2 transgene were made by mating *yw; EcR^{M554fs}/CyO, y^+; hs-EcR-$B2^{30.1}$/ hs-EcR-$B2^{30.1}$* males to *yw; EcR^{V559fs}/CyO, y^+* females at 25°C in an egg collection apparatus. 12-hour egg collections were made beginning 1 day after mating on 35 mm grape-juice agar plates (Ashburner, 1989) and the eggs were heat shocked at 37°C for 45 minutes immediately after collection. Egg collection plates were subsequently heat shocked once every 12 hours until larvae entered the desired larval stage (1st, 2nd or 3rd instar). Larvae were maintained at 25°C except during heat shock and fed on yeast paste. *EcR* mutant larvae were identified by their *yellow* phenotype and screened for developmental stage by mouthhook characteristics (Bodenstein, 1950) every 12 hours prior to heat shock. EcR^{M554fs} is a null mutation and EcR^{V559fs} is presumed to be a null mutation, based on molecular mapping and a similar lethal period to EcR^{M554fs}. Both mutations are small deletions that result in frameshifts and eliminate a large portion of the EcR ligand-binding domain (Bender et al., 1997). EcR^{M554fs} was induced on a *Canton S* chromosome and EcR^{V559fs} was induced on a *cn, bw* chromosome. In hs-EcR-$B2^{30.1}$ and other heat shock-EcR transgenes, *EcR* cDNAs are driven by the *hsp70* promoter (Bender et al., 1997).

To assess persistence of the EcR-B2 protein following heat shock, larvae heterozygous for *EcR* and carrying two copies of the hs-EcR-$B2^{30.1}$ transgene (*yw; EcR^{M554fs}/CyO, y+; hs-EcR-$B2^{30.1}$/hs-EcR-$B2^{30.1}$*) were maintained at 25°C, collected at the light gut and clear gut stages (approximately 2-12 hours prior to pupariation; Andres and Thummel, 1994) and then heat shocked for 45 minutes at 37°C. Extracts were made from larvae not exposed to heat shock and from animals at six time points between 30 minutes and 18 hours following heat shock. Protein from the equivalent of one half larva was loaded in each lane for polyacrylamide gel electrophoresis and immunoblots were probed with monoclonal antibody DDA2.7, which recognizes a domain common to all three EcR proteins (Koelle et al., 1991).

Microscopy and examination of internal tissues

For observation of larval cuticles, mouth parts and spiracles, larvae were washed in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0), immersed in polyvinyl lactophenol (Gurr) on microscope slides, gently covered with coverslips under a 20 g weight, and heated at 60°C on a slide warmer for 3 days. Slides were observed on a Zeiss Axiophot microscope using DIC optics. For examination of internal tissues, animals were dissected in PBS and fixed in 2% paraformaldehyde in PBS for 30 minutes at room temperature. Tissues were incubated for 15 minutes in 0.5 μg/ml DAPI in PBS, washed in PBS and mounted in 20% glycerol in PBS for microscopy. Wild-type animals (*Canton S*) were staged as dark-gut, light-gut and clear-gut third instar larvae (Andres and Thummel, 1994) and as white prepupae. Staging of the *EcR* mutants is detailed in the Results section.

Western analysis

Protein samples were made by homogenizing 1 larva per 20 μl (except for mid-third instar larvae, which were 10 larvae per 20 μl) of cracking buffer (0.125 M Tris-HCl, pH 6.7, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 4 M urea). Samples were boiled for 5 minutes and centrifuged at 11,000 ***g*** at room temperature. The supernatant was mixed with 2× loading buffer (100 mM Tris-HCl, pH 6.7, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 4 M urea, 15% glycerol, Bromophenol Blue added until dark blue), boiled for 3 minutes, and equilibrated to room temperature before loading on a 7% polyacrylamide SDS gel. The equivalent of one half larva (except for the *EcR* mutant pre-wandering samples, which were five larvae) was loaded in each lane. Loading was controlled by Ponceau S staining of the filter after transfer. Protein was transferred onto nitrocellulose filter, blocked with Blocking Buffer, consisting of 10% nonfat dry milk in TBS/Tween (20 mM Tris-HCl, pH 7.5), 500 mM NaCl, 0.05% Tween20, for 15 minutes, and incubated in primary antibody solution in Blocking Buffer for 1 hour at room temperature. Blots were rinsed twice and washed three times in TBS/Tween for 3 minutes each. A horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Promega) was used for secondary antibody incubation for 1 hour at room temperature (1:2000 dilution for goat anti-mouse or 1:1500 for goat-anti-rabbit) and rinsed and washed after the incubation, as for the primary incubation. Peroxidase activity was detected with a SuperSignal Western detection kit (Pierce) according to the manufacturer's instructions. Blots were stripped by two incubations at 50°C for 30 minutes in stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.7) with mild agitation and reprobed as described above.

The concentrations of primary antibodies used were 1:15 for the anti-EcR common region monoclonal antibody DDA2.7 (Koelle et al., 1991), 1:100 for the anti-Broad complex core region monoclonal antibody 25E9 (Emery et al., 1994), 1:30 for the anti-E75B monoclonal antibody 10E11 (White et al., 1997), 1:15 for the anti-E74A monoclonal antibody 6C5.2 (Munroe, 1995), 1:500 for affinity-purified anti-DHR3 polyclonal antibodies (Lam et al., 1997) and 1:32,000 for anti-FTZ-F1 polyclonal antibodies (Murata et al., 1996).

RESULTS

Heat shock induced expression of EcR-B2 rescues *EcR* common-domain mutants

Mutations that map to common *EcR* exons and therefore inactivate all three EcR isoforms result in embryonic lethality, thus defining the earliest developmental requirements for *EcR* function (Bender et al., 1997). To examine later functions, we rescued *EcR* common-domain mutants (EcR^{M554fs}/EcR^{V559fs}) by expressing EcR proteins under the control of a heat-shock promoter. In Table 1, we show that expression of EcR-B2

Table 1. Rescue of *EcR* common-domain mutants by expression of EcR-B2

	Percentage rescue to each larval instar
Rescue to 1st instar from the embryo	100 (85/74)
Rescue to 2nd instar from 1st instar	68 (50/73)
Rescue to 3rd instar from 2nd instar	59 (61/104)

The numerator in parentheses represents the number of rescued *EcR* mutant larvae (*yw; EcR^{M554fs}/EcR^{V559fs}*). The denominator represents the total number of *EcR* mutant larvae prior to heat shock except for rescue to first instar, in which the denominator is the Mendelian expectation for the number of *EcR* mutant larvae based on a count of wild-type siblings (*yw; EcR^{M554fs}/CyO, y+* and yw; *EcR^{V559fs}/CyO,y+*).

A control cross lacking the *hs-EcR-B2* transgene showed 2% (3/168) first instar survivors, all of which died as early first instar larvae.

protein via heat pulses delivered every 12 hours during development can efficiently rescue *EcR* common-domain mutants to the first, second and third larval instar. Although *hs-EcR-A* and *hs-EcR-B1* transgenes also show efficient rescue of common-domain *EcR* mutants to the first instar (100% (65/62) and 83% (66/80), respectively; see Table 1 legend for explanation of the scoring system), the aggregate rescue rate to the third instar for EcR-A and for EcR-B1 is at least fourfold less than for EcR-B2 (data not shown). The experiments described below were therefore carried out with *EcR* mutants rescued by EcR-B2 expression.

After the withdrawal of heat shock, EcR protein expressed from a *hs-EcR-B2* transgene falls below detectable levels within 12 hours. Fig. 1 shows a western blot containing extracts from larvae heterozygous for *EcR* and carrying two copies of the *hs-EcR-$B2^{30.1}$* transgene (*yw; EcR^{M554fs}/CyO, y+; hs-EcR-$B2^{30.1}$/hs-EcR-$B2^{30.1}$*) probed with a monoclonal antibody (DDA2.7) directed against an EcR common-domain epitope (Koelle et al., 1991). The 80 kDa EcR-B2 protein is detected at high levels within 30 minutes following a 45 minute heat shock at 37°C and is undetectable at 12 hours following heat shock. Thus, rescued *EcR* mutants are predicted to lack detectable EcR protein after 12 hours of incubation at 25°C following heat shock. Analysis of *EcR* mutant phenotypes described below was therefore carried out at time points greater than 12 hours following heat shock.

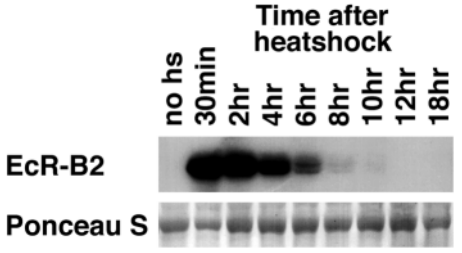

Fig. 1. Turnover of heat shock induced EcR-B2 protein. Extracts from *yw; EcR^{M554fs}/CyO, y^+; EcR-$B2^{30.1}$/EcR-$B2^{30.1}$* larvae without heat shock (no hs) or at times between 30 minutes and 18 hours following a 45 minute heat shock were probed with monoclonal antibody DDA2.7, which recognizes the common domain of the three EcR proteins (Koelle et al., 1991). The 80 kDa EcR-B2 protein is detected at high levels within 30 minutes following heat shock and is undetectable at 12 hours following heat shock. Ponceau S staining of the western blot filter is shown as a loading control.

EcR is required for larval molting and puparium formation

EcR mutants rescued to the first larval instar by expression of EcR-B2 during embryogenesis have normal appearance and movement until the end of the first instar. If no further heat pulses are given, they arrest as first instar larvae. The arrested larvae survive for 1-2 days. Most rescued larvae arrest with both first and second instar mouth parts and posterior spiracles (Fig. 2A,B). Arrested *EcR* mutants appear to retain the first instar cuticle as well as the newly formed second instar cuticle (data not shown). The duplicated posterior spiracle phenotype is similar to that described for mutants in the *usp* gene, which encodes the EcR heterodimer partner USP (Perrimon et al., 1985; Oro et al., 1992). Although a *usp* duplicated mouthpart phenotype has not been described, we find that *usp* mutants also show this phenotype (Fig. 2E), although at a variable frequency depending on the *usp* allele examined.

EcR mutants rescued to the second larval instar by expression of EcR-B2 during embryogenesis and the first instar also have normal appearance and movement until reaching the end of the second instar. If no further heat pulses are given, they arrest as second instar larvae. Most rescued larvae arrest with duplicated larval cuticle (data not shown) and both second and third instar mouthhooks and posterior spiracles (Fig. 2C,D). The arrested larvae survive for 1-2 days. Thus, *EcR* mutants can initiate but not complete larval molts. The similarity between the *EcR* and *usp* mutant phenotypes suggests that EcR and USP function together in larval molting.

EcR mutants rescued to the third larval instar by expression of EcR-B2 during embryogenesis, first instar and second instar appear normal at the beginning of the third instar. Progression of *EcR* mutants through the third instar, however, is significantly delayed. Wild-type third instar larvae typically cease feeding and begin wandering behavior between 24 and 36 hours after the previous molt (Ashburner, 1989; Andres and Thummel, 1994). In contrast, *EcR* mutants generally stop feeding and begin wandering 48-72 hours following the second to third instar molt. Wild-type larvae become stationary within 12-24 hours after initiation of wandering and then pupariate, a process that involves eversion of anterior spiracles, shortening of the larva to form the barrel-shaped puparium, and hardening of the larval cuticle (Fig. 3A). Most *EcR* mutants become stationary within 24-36 hours after beginning wandering, but retain their larval morphology without outward signs of pupariation (Fig. 3B).

At the end of the wandering stage, *EcR* mutants display a series of characteristic behaviors. After initiating wandering behavior, wild-type larvae can be staged by clearing of food from the larval gut. Most dark-gut animals will pupariate within 12-24 hours while most clear-gut animals will pupariate in 1-6 hours (Andres and Thummel, 1994). For staging purposes, *EcR* mutants can be classified into three stages: stage 1 (mobile larvae), stage 2 (larvae lack locomotion) and stage 3 (larvae are unresponsive to touch). Stage 1 behaviors are comparable to wild-type behaviors while stage 2 and 3 behaviors are specific to *EcR* mutants. At the beginning of wandering, *EcR* mutants have body size, appearance and patterns of crawling and body movement indistinguishable from wild-type animals at the dark-gut stage (Andres and Thummel, 1994). *EcR* mutants do not feed during wandering and most are able to clear their gut of food. The clear-gut *EcR*

mutants are defined as stage 1. Initially, stage 1 *EcR* mutants crawl actively and move their mouthparts actively and constantly. We describe this as stage 1a. *EcR* mutants then become stationary and cease movement of the mouthparts. If stimulated with a needle, however, they resume crawling and mouthpart movements. This stage is termed 1b.

Stage 2 *EcR* mutants are incapable of resuming mouthpart movement or locomotion when stimulated with a needle. This stage can be subdivided into three parts. Stage 2a mutants wriggle the posterior portion of the body although the anterior tip of the larva appears fixed to the side of the culture vial. Stage 2b mutants have ceased posterior wriggling but will resume wriggling if stimulated by a needle. At this stage, brown spots that may indicate necrosis begin to appear on the cuticle of some mutants. Stage 2c mutants do not resume posterior wriggling when stimulated by a needle. Most stage 2c mutants have brown areas present on the cuticle. Throughout stage 2, larvae continue to exhibit dorsal medial abdominal contraction (i.e. heart pumping; Bainbridge and Bownes, 1981), an activity that is no longer detectable in wild-type animals between 1-7 hours after pupariation. Stage 3 mutants lack any response to stimulation and have ceased dorsal medial abdominal contraction. After entering stage 3, necrotic patches of tissue continue to accumulate and the larvae ultimately deteriorate.

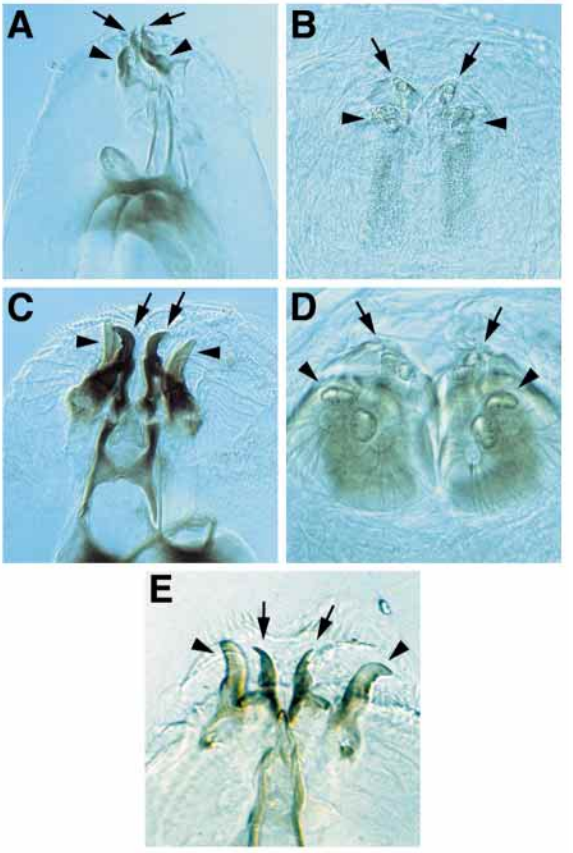

Fig. 2. Phenotypes of EcR mutants defective in larval molting. (A,B) An $EcR^{M554fs}/EcR^{V559fs}$ mutant rescued to the first larval instar by EcR-B2 expression during embryogenesis arrests during the first larval instar. (A) First (arrows) and second (arrowheads) larval instar mouthhooks are indicated. (B) First (arrows) and second (arrowheads) larval instar posterior spiracles are indicated. (C,D) An $EcR^{M554fs}/EcR^{V559fs}$ mutant rescued to the second larval instar by EcR-B2 expression during embryogenesis and the first larval instar arrests during the second larval instar. (C) Second (arrows) and third (arrowheads) larval instar mouthhooks are indicated. (D) Second (arrows) and third (arrowheads) larval instar posterior spiracles are indicated. (E) A *usp* mutant (usp^3/Y) arrested during the first larval instar. First (arrows) and second (arrowheads) larval instar mouthhooks are indicated.

Metamorphic development of imaginal and larval tissues is blocked in nonpupariating EcR mutants

We next examined internal tissues of *EcR* mutants rescued to the third instar. During metamorphosis in wild-type animals, imaginal discs begin a process of elongation and eversion around the time of pupariation that leads to formation of adult structures of the head and thorax (Fristrom and Fristrom, 1993). Discs from mid-third instar (dark-gut) *EcR* mutants (Fig. 4A) are indistinguishable from discs taken from wild-type larvae at this stage. Leg discs initiate elongation in *EcR* mutants (Fig. 4B) but arrest shortly afterwards. Fig. 4C shows a disc taken from a stage-3 mutant. Leg elongation has arrested at a stage comparable to 2 hours after pupariation in the wild type.

Many larval tissues, including the larval midgut and salivary gland, do not contribute to the adult; instead they undergo histolysis at the time of metamorphosis (Robertson, 1936). The destruction of these tissues appears to be a programmed cell death response to the pulse of ecdysone released at this time (Jiang et al., 1997). The course of midgut histolysis can be easily followed in the shortening of the gastric caeca that takes place around the time of pupariation, culminating in the disappearance of these structures by 4 hours after pupariation (Jiang et al., 1997). Histolysis of the larval salivary gland is completed by 15 hours after pupariation (Robertson, 1936; Jiang et al., 1997). Gastric caeca taken from mid-third instar

Fig. 3. Phenotype of nonpupariating *EcR* mutants. (A) A wild-type (*Canton-S*) puparium 2-3 hours after pupariation. (B) A third instar *EcR* mutant larva ($EcR^{M554fs}/EcR^{V559fs}$) in the eighth day after entering the 3rd instar that has retained larval morphology. The mutant larva was rescued to the third larval instar by expression of EcR-B2 during embryogenesis and the first and second larval instars.

(dark-gut) *EcR* mutants appear normal (data not shown) and shortening of the gastric caeca is initiated in stage 1b mutants (Fig. 5A). However, gastric caeca shortening is not completed in *EcR* mutants (Fig. 5B). Gastric caeca shortening in *EcR* mutants appears to arrest at a stage comparable to 2 hours after pupariation in the wild type. In addition, the larval salivary glands persist in rescued *EcR* mutants (data not shown).

During metamorphosis, the larval midgut is replaced by the proliferation of imaginal cells present in small clusters called the midgut imaginal islands (Fig. 5C, arrowheads). The midgut imaginal cells begin to proliferate by 2 hours after pupariation and rapidly envelop the larval cells of the gut (Robertson, 1936). The midgut imaginal cells in mid-third instar (dark gut) *EcR* mutants appear normal (Fig. 5C), but do not proliferate in later stage mutants. Fig. 5D shows a stage 3 mutant with no increase in cell number. This phenotype is consistent even in mutants that have persisted in stage 3 for 3-4 days (data not shown).

Expression of ecdysone-response genes is blocked in *EcR* non-pupariating mutants

The phenotypes observed in larval and imaginal tissues in *EcR* mutants suggest that normal response of tissues to ecdysone at the onset of metamorphosis is blocked at an early stage in the absence of the EcR subunit of the ecdysone receptor. To examine the response of ecdysone target genes in *EcR* mutants, we determined the expression pattern of a set of ecdysone response genes for which antibody probes are available. The set tested includes the early genes *E74A* (Burtis et al., 1990) and *BR-C* (DiBello et al., 1991), the early-late gene *DHR3* (Koelle et al., 1992), the *E75B* gene (Segraves and Hogness, 1990), and the *βFTZ-F1* gene, a mid-prepupal ecdysone response gene (Woodard et al., 1994). Fig. 6 shows a series of western blots in which extracts from *EcR* mutants were probed with antibodies that recognize proteins encoded by ecdysone response genes.

Fig. 6 (top) shows that full-length EcR-A and EcR-B1 proteins are not detected in *EcR* mutants rescued to the third instar, consistent with the molecular nature of the EcR^{M554fs} and EcR^{V559fs} deletions. The 80 kDa EcR-B2 protein is not detected in stage 1 or 2 mutants, but low levels of this protein

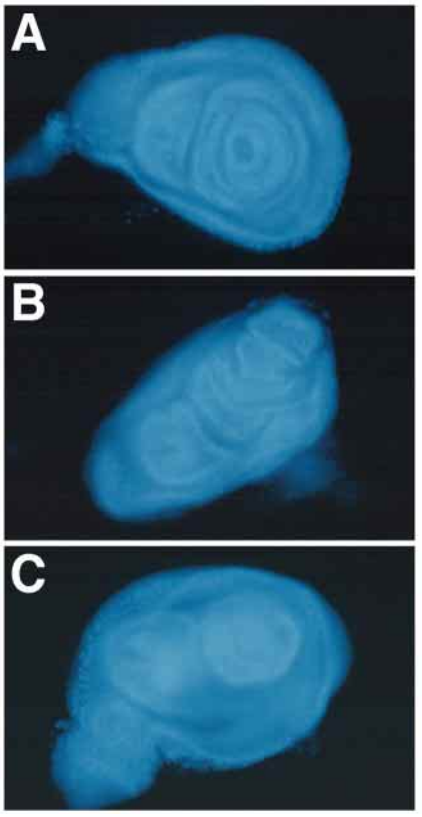

Fig. 4. Phenotype of *EcR* mutant leg discs stained with DAPI. (A) Leg disc from a mid-third instar (dark gut) *EcR* mutant larva ($EcR^{M554fs}/EcR^{V559fs}$) rescued by EcR-B2 expression during embryogenesis and first and second larval instars. This phenotype is identical to those of wild-type larvae at this stage. (B) Leg disc from a stage 1b *EcR* mutant ($EcR^{M554fs}/EcR^{V559fs}$) rescued as described above. Elongation of the leg disc has begun in this animal. (C) Leg disc from a rescued *EcR* mutant ($EcR^{M554fs}/EcR^{V559fs}$) 1 day after entering stage 3. Elongation of the leg disc has arrested at a stage comparable to 2 hours after puparium formation in the wild type.

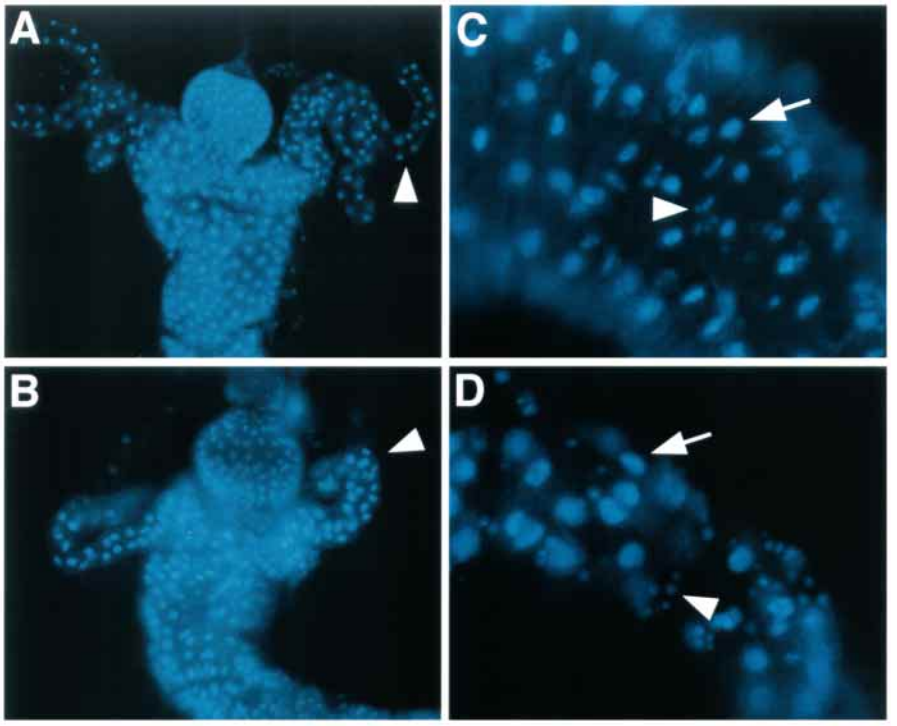

Fig. 5. Phenotypes of *EcR* mutant midgut tissues stained with DAPI. (A) Anterior midgut of a stage 1b *EcR* mutant ($EcR^{M554fs}/EcR^{V559fs}$) rescued to third instar by expression of EcR-B2 during embryogenesis and first and second larval instar. Length of the gastric caeca (arrowhead) is comparable to wild-type larvae at the clear gut stage. (B) Anterior midgut of a rescued *EcR* mutant ($EcR^{M554fs}/EcR^{V559fs}$) 3 days after entering stage 3. The gastric caeca have shortened (arrowhead) but arrest at a stage comparable to 2 hours after puparium formation in the wild type. (C) Midgut imaginal islands (arrowheads) of a mid-third instar (dark gut) *EcR* mutant ($EcR^{M554fs}/EcR^{V559fs}$) rescued as described above. The number and arrangement of the small diploid midgut imaginal cells is comparable to wild-type larvae at this stage. Arrows, large polyploid nuclei of the larval midgut cells. (D) Midgut imaginal islands (arrowheads) of a stage 3 *EcR* mutant ($EcR^{M554fs}/EcR^{V559fs}$) rescued as described above. The *EcR* mutant midgut imaginal cells fail to proliferate. Arrows, large polyploid larval midgut nuclei.

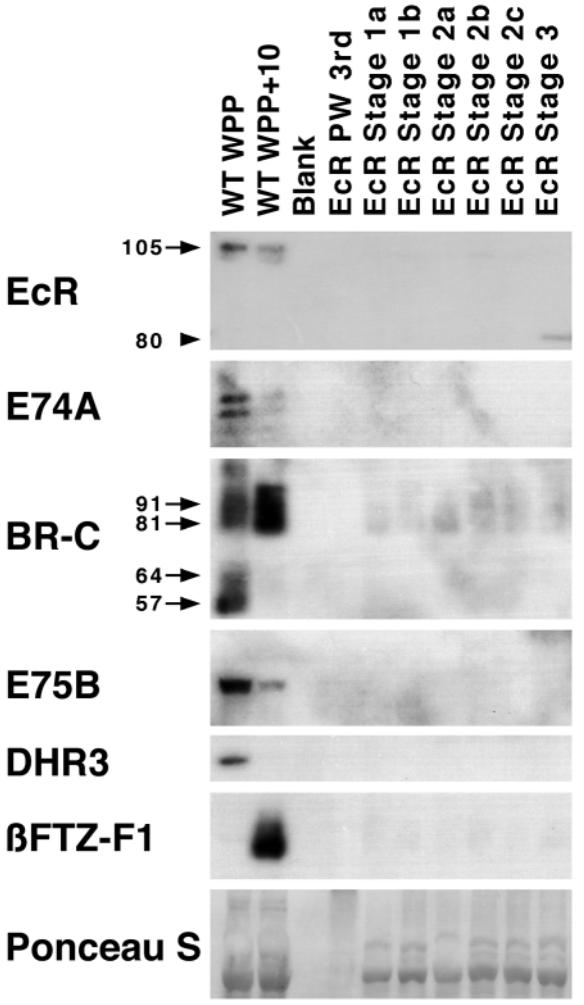

Fig. 6. Expression of ecdysone-responsive genes in *EcR* mutants. Extracts from wild-type animals and *EcR* mutants were assayed for expression of a set of ecdysone target genes by western analysis. Extracts were made from wild-type (*Canton S*) animals at the white prepupal stage (WT WPP) and 10 hours after the white prepupal stage (WT WPP+10). Extracts were made from *EcR* mutants ($EcR^{M554fs}/EcR^{V559fs}$) at seven developmental stages (EcR lanes) following rescue to third instar by EcR-B2 expression through the second instar. An arrow to the left of the EcR panel indicates a band at 105 kDa corresponding to the EcR-A and EcR-B1 proteins and an arrowhead indicates a faint band at 80 kDa in the rightmost lane corresponding to the EcR-B2 protein. The E74A gene encodes multiple proteins due to use of multiple initiator codons (Boyd and Thummel, 1993). Apparent molecular masses (in kDa) are indicated for BR-C proteins by arrows. Each lane was loaded with the equivalent of one half larva except for the *EcR* pre-wandering lane, which was loaded with the equivalent of five larvae. Ponceau S staining of the western blot filter is shown as a loading control.

are detected in stage 3 mutants (Fig. 6). Mutants at this stage show necrotic patches of tissue and it is possible that the low-level expression of EcR-B2 seen may result from induction of the hsp70 promoter in a subset of deteriorating cells.

In *EcR* mutants, expression of E74A, BR-C 64 kDa and 57 kDa proteins, E75B and DHR3 is completely blocked (Fig. 6). In addition, expression of BR-C 91 kDa and 81 kDa proteins and of βFTZ-F1 is sharply reduced. Thus, mutants lacking *EcR* fail to induce ecdysone target genes that are normally expressed at the time of pupariation.

DISCUSSION

Ecdysone signaling plays a critical role in coordination of post-embryonic development in *Drosophila*. To determine *EcR* requirements after embryogenesis, we have devised a conditional rescue system in which embryonic lethal *EcR* common-domain mutants are rescued to later stages of development by periodic heat-shock induction of *EcR* expression. Induction of *EcR* expression can be stopped following rescue of *EcR* mutants past a critical period, allowing determination of *EcR* requirements during the following developmental stage. Using this system, we have shown that *EcR* is required for each larval molt and for the initiation of metamorphosis. In animals arrested at the onset of metamorphosis, ecdysone responses are blocked at early stages in both imaginal and larval tissues, as is the expression of known ecdysone-responsive genes. These results show that *EcR* is required for ecdysone signaling at multiple developmental stages and in imaginal and larval tissues for ecdysone response at the onset of metamorphosis.

We have found that expression of any of the three EcR protein isoforms can efficiently rescue *EcR* common-domain mutants to the first instar, suggesting that EcR isoforms may be functionally equivalent in this developmental context. In contrast, however, expression of different EcR isoforms results in different efficiencies of common-domain mutant rescue to later developmental stages, and an earlier study (Bender et al., 1997) demonstrated functional differences between EcR isoforms in rescue of defects in polytene chromosome transcriptional puffing in an *EcR-B1* mutant background. Together, these results suggest that functional requirements for specific EcR isoforms depend on stage- and tissue-specific contexts. Such differences may reflect tissue-specific expression of EcR transcriptional cofactors.

The conditional rescue system described here, along with a temperature-sensitive *EcR* mutation that we have recently used to show that *EcR* is required for normal oogenesis (Carney and Bender, 2000), will allow investigation of *EcR* functions in virtually any tissue at any developmental stage. Preliminary data suggests that these conditional systems will also prove useful in experiments designed to identify *EcR*-dependent target genes using DNA microarray technology (T.-R. L., K. White and M. B., unpublished data).

EcR requirements during the larval molting cycle

Larval molting in insects is an intricate process that requires the input of multiple hormonal factors (Truman, 1990; Nijhout, 1994). Molting involves separation of the larval cuticle from the underlying epidermis (apolysis), subsequent formation of a new cuticle and, finally, shedding of the old cuticle (ecdysis). *EcR* mutants rescued to the first or second instar arrest at the subsequent larval molt (Fig. 2). Most arrested *EcR* mutants exhibit duplicated cuticular structures including larval mouthparts and posterior spiracles. The rescued *EcR* mutants therefore appear to complete the earliest stages of larval molting, including apolysis and new cuticle formation, but are unable to undergo ecdysis.

The endocrine control of ecdysis is particularly well understood in the tobacco hornworm, *Manduca sexta*. In *Manduca*, a drop in the ecdysteroid titer is required for release of eclosion hormone (EH) and the initiation of pupal and adult ecdysis (Truman et al., 1983). In addition to EH, a second peptide hormone, ecdysis triggering hormone (ETH), is also involved in regulation of ecdysis behavior (Zitnan et al., 1996). EH and ETH appear to act in a positive feedback loop that ultimately results in a massive release of EH and ETH and the modulation of a series of pre-ecdysis and ecdysis behaviors that culminate in shedding of the larval cuticle (Ewer et al., 1997; Zitnan et al., 1999). Interestingly, recent experiments suggest that ETH transcription may be induced by ecdysone, suggesting a further link between ecdysone signaling and peptide hormone activation of ecdysis (Zitnan et al., 1999).

Our results show that *EcR* is required for ecdysis in *Drosophila* and suggest that the hormonal regulatory network that controls ecdysis is conserved between *Manduca* and *Drosophila*. This hypothesis is consistent with recent functional studies of EH in *Drosophila* in which EH-expressing cells were ablated (McNabb et al., 1997), as well as a study demonstrating the existence of a peritracheal neuropeptide system in *Drosophila* (O'Brien and Taghert, 1998). Thus the ecdysis defects seen in *EcR* mutants may result from defects in synthesis or release of *Drosophila* EH and/or ETH-like peptides.

Apolysis and new cuticle formation are triggered by an increasing titer of ecdysone (Riddiford, 1993). It is therefore curious that the rescued *EcR* mutants appear to complete this part of the molting cycle. Does this indicate that *EcR* is not required for initiation of the molting cycle? Phenotypic analysis of *EcR* mutants lacking *hs-EcR* transgenes argues against this interpretation. For example, *EcR* null mutant escapers that hatch into first instar larvae (Table 1; Bender et al., 1997) die prior to formation of the second instar larval cuticle. In addition, many arrested *EcR-B* mutant larvae (20-35%) lack duplicated larval cuticular structures (Schubiger et al., 1998). These findings suggest that *EcR* is also required during larval stages at a time prior to new cuticle synthesis. We therefore favor the alternative explanation that the EcR protein induced by the final heat shock during the previous stage perdures long enough to allow synthesis of the new cuticle. In wild-type animals, apolysis and new cuticle synthesis take place at about 10 and 12 hours, respectively, following ecdysis (Kaznowski et al., 1985). The persistence of low levels of EcR protein for up to 10 hours following heat shock (Fig. 1) may be sufficient to permit completion of the earliest steps of the molting cycle.

EcR is required to initiate metamorphosis

Although initially normal in appearance and behavior when rescued to the third larval instar, *EcR* mutants exhibit substantial delays in progression through this stage and fail to pupariate (Fig. 3). *EcR* mutants are delayed in initiating wandering behavior and in extreme cases may persist in larval form for up to 7 days, rather than the normal 2 days, following the second to third larval molt. Appearance of internal tissues is consistent with an early block in ecdysone responses in all tissues examined. Leg imaginal discs fail to elongate (Fig. 4), gastric caeca shortening is arrested (Fig. 5A,B), and the larval salivary glands persist in *EcR* mutants. In addition, proliferation of midgut imaginal cells is not initiated in *EcR* mutants (Fig. 5C,D). These results suggest that *EcR* is required for most or all ecdysone-regulated developmental events during early metamorphosis, including imaginal disc morphogenesis, destruction of larval tissues through programmed cell death and proliferation of imaginal cells.

The phenotype of rescued *EcR* null mutants reported here differs in several respects from mutants lacking only *EcR-B1* functions. *EcR-B1* mutants fail to pupariate, but successfully complete the larval/pupal apolysis (Bender et al., 1997), an event that is blocked in rescued *EcR* null mutants (Fig. 3). Mutants that lack EcR-B1 and EcR-B2 due to deletion of the *EcR-B* transcription start site show a similar phenotype to *EcR-B1* mutants (Schubiger et al., 1998), suggesting that EcR-A is sufficient to trigger larval/pupal apolysis.

As shown by the elongation and fusion of leg imaginal discs, *EcR-B1* mutants initiate normal ecdysone response in the imaginal discs, a class of tissue that expresses high levels of EcR-A (Talbot et al., 1993), whereas ecdysone-triggered responses are defective in these mutants in tissues that express high levels of EcR-B1 (Bender et al., 1997). These observations, and the finding that disc elongation and eversion in the rescued *EcR* null mutants described here are blocked at an early stage (Fig. 4), are consistent with the model that EcR-A and EcR-B1 trigger distinct developmental responses to ecdysone (Talbot et al., 1993). Interestingly, however, proliferation of midgut imaginal cells is blocked at an earlier stage in *EcR* null mutants (Fig. 5C,D) than in *EcR-B1* mutants (Bender et al., 1997), suggesting that either EcR-A or EcR-B2 may also participate in the control of midgut imaginal cell proliferation in response to ecdysone.

Western analysis of extracts from rescued *EcR* mutants using antibodies directed against products of five ecdysone-responsive genes shows that expression of three early response genes are largely (*BR-C*) or completely (*E74A*, *E75B*) abolished (Fig. 6). Expression of the early-late gene *DHR3* and the mid-prepupal response gene *βFTZ-F1* are also severely affected. These results show that expression of ecdysone-responsive genes early in metamorphosis is dependent on *EcR*. The results confirm and extend to the whole animal our earlier finding from analysis of larval salivary gland polytene chromosomes that transcriptional puffing of ecdysone-responsive genes is blocked in *EcR-B1* mutants (Bender et al., 1997). The retention of low levels of BR-C 91 and 81 kDa products in *EcR* mutants is consistent with the incomplete block to *BR-C* puffing previously seen in *EcR-B1* mutants (Bender et al., 1997).

Do EcR and USP function together in vivo?

It is well established that both EcR and USP are required in vitro for DNA binding and hormone binding and that both proteins are required for transcriptional activation from ecdysone response elements in heterologous cell systems (Koelle, 1992; Yao et al., 1992, 1993; Thomas et al., 1993). However, the extent to which EcR and USP function together in vivo to mediate ecdysone signaling, or indeed, whether EcR and USP are obligate heterodimers in this process, are still open questions. Comparison of *EcR* mutant phenotypes shown here with those described previously for *usp* (Perrimon et al., 1985; Oro et al., 1992; Hall and Thummel, 1998) suggests that EcR and USP function together in vivo to mediate many of the

known signaling functions of ecdysone. Thus the molting defects exhibited by *EcR* mutants rescued to the first instar (Fig. 2A,B) are identical to those seen in *usp* loss-of-function mutants (Fig. 2E; Perrimon et al., 1985; Oro et al., 1992). *usp* functions during later development have been investigated using a rescue system analogous to that described here (Oro et al., 1992; Hall and Thummel, 1998). In rescued *usp* mutants, internal tissues including gastric caeca, imaginal discs and midgut imaginal cells arrest at comparable stages (Hall and Thummel, 1998) to those described here for rescued *EcR* mutants (Figs 4 and 5). The similarity of these phenotypes argues that EcR and USP function together in vivo to mediate ecdysone signaling during larval molting and during the dramatic reorganization of imaginal and larval tissues at the onset of metamorphosis.

The phenotypes of rescued *EcR* mutants and *usp* mutants differ in several interesting respects. First, *usp* mutants fail to undertake wandering behavior in the mid-third instar (Hall and Thummel, 1998), a behavior that is delayed but not blocked in *EcR* mutants. Secondly, non-pupariating *usp* mutants undergo apolysis from the larval cuticle and secrete a supernumerary larval cuticle (Hall and Thummel, 1998). Thus the functions of EcR and USP may diverge during progression through the third instar. Based on transcriptional studies of rescued usp mutants, Hall and Thummel (1998) have suggested that *usp* is not required for activation of the mid-third instar ecdysone regulatory hierarchy, an event that is thought to prepare the animal for metamorphosis (Andres et al., 1993; Huet et al., 1993). Although our western analysis of *EcR* mutants does not allow us to ascertain whether *EcR* is required for this event, transcriptional profiling of *EcR* mutants using DNA microarrays currently in progress (T.-R. L., K. White, M. B., unpublished data) should resolve this question in the future. These studies, coupled with the mutational analysis of *EcR* and *usp*, should prove useful in elucidating the precise biological consequences and molecular mechanisms of mid-third instar ecdysone signaling and the relative contribution of *EcR* and *usp* to this process.

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