Phenotypic analysis of EcR-A mutants suggests that EcR isoforms have unique functions during Drosophila development

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Abstract

The steroid hormone ecdysone triggers transitions between developmental stages in Drosophila by acting through a heterodimer consisting of the EcR and USP nuclear receptors. The EcR gene encodes three protein isoforms (EcR-A, EcR-B1, and EcR-B2) that have unique amino termini but that contain a common carboxy-terminal region including DNA-binding and ligand-binding domains. EcR-A and EcR-B1 are expressed in a spatially complementary pattern at the onset of metamorphosis, suggesting that specific responses to ecdysone involve distinct EcR isoforms. Here, we describe phenotypes of EcR-A specific deletion mutants isolated using transposon mutagenesis. Western blot analysis shows that each of these mutants completely lacks EcR-A protein, while the EcR-B1 protein is still present. The EcR¹¹² strain has a deletion of EcR-A specific non-coding and regulatory sequences but retains the coding exons, while the EcR¹³⁹ strain has a deletion of EcR-A specific protein coding exons but retains the regulatory region. In these mutants, the developmental progression of most internal tissues that normally express EcR-B1 is unaffected by the lack of EcR-A. Surprisingly, however, we found that one larval tissue, the salivary gland, fails to degenerate even though EcR-B1 is the predominant isoform. This result may indicate that the low levels of EcR-A in this tissue are in fact required. We identified yet another type of mutation, the EcR⁹⁴ deletion, that removes the EcR-A specific protein coding exons as well as the introns between the EcR-A and EcR-B transcription start sites. This deletion places the EcR-A regulatory region adjacent to the EcR-B transcription start site. While EcR¹¹² and EcR¹³⁹ mutant animals die during mid and late pupal development, respectively, EcR⁹⁴ mutants arrest prior to pupariation. EcR-A mutant phenotypes and lethal phases differ from those of EcR-B mutants, suggesting that the EcR isoforms have distinct developmental functions.

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Introduction

During the life cycle of Drosophila melanogaster, the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) is a critical signal for progression from one life stage to the next. Accordingly, pulses of ecdysone punctuate each stage of the life cycle (Richards, 1981), triggering the transition into the subsequent stage. Without proper cellular responses to this hormone, the animal will not develop normally, and in extreme cases where loss of the hormone has taken place, animals will not transition to the subsequent stage of development and will die prematurely (Berreu et al., 1984; Henrich et al., 1993). Ecdysone has been shown to be required for proper molting between larval instars as well as onset and completion of metamorphosis for pupation and adult development (Borst et al., 1974; Henrich et al., 1993; Sliter and Gilbert, 1992).

Ecdysone tightly coordinates the array of physiological changes that characterize each stage of the life cycle. Interestingly, while all tissues are exposed to the hormone, different tissue types have unique responses to the signal. Perhaps, the best example of these differences is at the onset of metamorphosis when the majority of the larval cells and tissues degenerate by undergoing programmed cell death (Baehrecke, 2000, 2002; Lee et al., 2002; Thummel, 2001),
while the progenitor imaginal cells and discs proliferate and differentiate into pupal structures that ultimately form the adult fly (Riddiford, 1993; Robertson, 1936; Roseland and Schneiderman, 1979).

The ecdysone signal is transduced to target genes in the genome via the ecdysone receptor complex. This complex is made up of a heterodimer of the Ultraspiracle protein (USP) and the Ecdysone Receptor (EcR) proteins (Koelle, 1992; Thomas et al., 1993; Yao et al., 1992, 1993). The EcR–USP complex binds ecdysone and affects transcription of ecdysone target genes following binding of the complex to ecdysone response elements. This molecular interaction is the means by which ecdysone regulates the genes that are responsible for the plethora of physiological changes that are characteristic of the developmental progression through the life cycle.

Three protein isoforms (EcR-A, EcR-B1 and EcR-B2) are produced from the EcR gene by the use of two promoters and alternative splicing (Talbot et al., 1993). These isoforms share the same carboxy terminus, which includes the hormone binding and DNA binding domains, while the amino termini are unique to each isoform. The three EcR isoforms are hypothesized to have unique functions based upon studies that define their distinct temporal and spatial expression patterns (Kim et al., 1999; Robinow et al., 1993; Sung and Robinow, 2000; Talbot et al., 1993; Truman et al., 1994) and the distinct biochemical properties of their unique amino terminal domains (Dela Cruz et al., 2000; Hu et al., 2003; Mouillet et al., 2001). Furthermore, tissues with analogous responses to ecdysone at the onset of metamorphosis express EcR protein isoforms in similar patterns. For example, the EcR-B1 isoform is expressed at high levels in cells with strictly larval functions that do not contribute to the adult structures. In contrast, the EcR-A isoform is expressed at high levels in the imaginal discs, which ultimately differentiate to form the head, thorax and appendages of the adult fly during metamorphosis, and also at low levels in larval tissues and imaginal histoblasts (Talbot et al., 1993). Similarly, the expression of EcR-A is correlated with neuronal maturation while EcR-B1 expression correlates with neuronal regression (Truman et al., 1994).

Mutational analysis of the EcR gene has produced three types of EcR mutants: EcR nulls, in which all isoforms are disrupted; EcR-B mutants, where both EcR-B1 and EcR-B2 are removed; and EcR-B1 mutants, in which only the EcR-B1 isoform is removed (Bender et al., 1997; Schubiger et al., 1998). Phenotypic analysis of these mutant alleles revealed that different lethal phases and mutant morphologies are associated with each type of mutant. EcR null mutants arrest late in embryogenesis. EcR-B1 mutants fail to pupariate and ecdysone responses are inhibited in larval and imaginal tissues that normally express high levels of EcR-B1, while the initiation of ecdysone responses in tissues that normally express high levels of EcR-A is permitted (Bender et al., 1997). EcR-B deletions revealed a requirement for the EcR-B isoforms in larval molting and neuronal remodeling (Schubiger et al., 1998). EcR-A functions have been examined through phenotypic characterization of a strain carrying a P element insertion in an EcR-A intron (D’Avino and Thummel, 2000) and through the use of RNAi strains (Roignant et al., 2003). However, genetic criteria suggest that neither of these approaches allows the complete inactivation of EcR-A.

Here, we describe the phenotypes of three mutant EcR alleles that have been identified from a local transposition mutagenesis screen (Carney et al., 2004). Each of these alleles lacks EcR-A, but retains EcR-B1 expression. Two alleles, EcR112 and EcR139, exhibit a mid-pupal lethality. EcR112 carries a deletion that removes the EcR-A transcription start site and dies during early pupal development. EcR139 carries a deletion that removes EcR-A coding exons A2 and A3 and dies later in pupal development. These mutant alleles reveal a requirement for EcR-A during the pupal-pharate adult transition during metamorphosis. The remaining allele, EcR94, exhibits a phenotype very similar to the EcR-B1 non-pupariating phenotype. The EcR94 allele carries a deletion that removes exons A2 and A3 as well as most of the intervening sequences between the EcR-A and EcR-B transcription start sites. Our results show that EcR-A is required during metamorphic development and suggest that EcR isoforms have distinct developmental functions.

Materials and methods

Western analysis

Whole animal protein extracts were isolated from late third instar (clear-gut) larvae (Andres and Thummel, 1994) using standard extraction protocols (Talbot et al., 1993) and run on a denaturing 6% polyacrylamide gel. After the proteins were transferred to a nitrocellulose membrane, blots were incubated in monoclonal antibody directed against EcR-A (15G1A) at a 1:3000 dilution or EcR-B1 (AD4.4) (Talbot et al., 1993) at a 1:30 dilution for 2 to 4 h. After washing, blots were incubated with HRP labeled goat antimouse secondary antibodies at a 1:5000 dilution for 3 h. A Lumi-light chemi-luminescent detection system (Roche) was used to visualize immunoreactivity.

Lethal phase determination

Twenty five yw; EcR-A/CyO,y+ males were crossed to 25 yw; EcR-M554fs/CyO,y+ virgin females and allowed to mate for approximately 3 days. Eggs were collected on grape juice agar plates and EcR mutant larvae were identified by their yellow phenotype at 36 h after egg laying. Surviving larvae were scored at mid-third instar and at 24-h intervals thereafter and classified using the developmental staging of Bainbridge and Bowes (1981). Percent survival equals the number of animals alive at a
given developmental stage divided by the total number of mutants collected at hatching.

**Immunolocalization**

Monoclonal antibodies specific to EcR-A (15G1A) or EcR-B1 (AD4.4) were used to perform the immunofluorescent staining experiments. EcR-A mutants and wild-type siblings were collected at the developmental stages indicated, dissected and stained simultaneously in separate tubes. A standard protocol for immunostaining was used (Talbot et al., 1993). Tissues were simultaneously treated with DAPI to visualize nuclei.

**Microscopy and photography**

EcR mutants were collected and dissected at developmental stages indicated in the Results section. Photographs were taken using a Zeiss Axiophot compound microscope with attached 35 mm camera or using a Leica MZ6 dissecting microscope with a Hamamatsu model C5810 CCD camera.

**Results**

**Protein expression in EcR-A mutants**

We have previously described the methods for creating the three deletion mutants analyzed here (EcR112, EcR139 and EcR94), which are the result of a local transposon mutagenesis strategy to isolate EcR-A specific deletions caused by transposase induced imprecise excision or male recombination (Carney et al., 2004). The deletion endpoints of these mutants are summarized in Fig. 1. EcR112 is predicted to lack the EcR-A transcript due to a deletion that removes the EcR-A transcription start site. EcR139 carries a deletion that removes the EcR-A coding exons A2 and A3 and is therefore also predicted to lack functional EcR-A protein. Finally, EcR94 carries a large deletion that removes exons A2 and A3. This deletion effectively juxtaposes EcR-A regulatory sequences and the EcR-B transcription start site (Fig. 1).

We first determined whether these mutations in fact removed all EcR-A expression. Western blot analysis of EcR-A mutants heterozygous to an EcR null mutant show that the EcR-A protein is no longer detectable in these animals (Fig. 2). The EcR-B1 protein is, however, still present although there are higher levels of breakdown products apparent. In comparison, a wild-type control at a comparable stage shows the expression of both EcR-A and EcR-B1. These data suggest that EcR112 and EcR139 completely lack EcR-A function while retaining some or all EcR-B1 function.

**EcR-A is required for pupal development**

Our initial lethal phase analysis showed that 73% of EcR112 and 40% of EcR139 mutants survived until the pupal period when heterozygous to the EcR M554fs null mutation while EcR94/EcR M554fs mutant heterozygotes completely failed to pupariate (Carney et al., 2004). Here, we made a more specific determination of the lethal phase of EcR112 and EcR139 mutants when heterozygous to an...
These results confirm our previous observation that EcR112 mutants survive to pupal development at a higher frequency than EcR139 mutants do (Carney et al., 2004). Interestingly, however, more EcR139 than EcR112 mutants survive to later stages of pupal development (Fig. 3). Thus, the predominant lethal period of EcR112 mutants is pupal stage P5 (Bainbridge and Bownes, 1981), subsequent to head eversion but prior to pigmentation of the eyes. In contrast, a significant number of EcR139 mutants reach the P15 pharate adult stage, just prior to eclosion from the pupal case. Notably, however, less than 1% of EcR139 mutant heterozygotes eclose (data not shown).

**Phenotypic analysis of EcR139 and EcR112 mutants**

Fig. 4 shows the early pupal phenotypes of EcR139 and EcR112 mutants when heterozygous to an EcR null mutation. A majority of EcR139 mutants complete pupariation and progress through head eversion, eye development, and leg and wing extension (Figs. 4A,B). However, these animals frequently appear to degenerate in their pupal cases at this stage (Fig. 4G), which may be due to desiccation. Consistent with this idea, EcR139 mutants are often defective in puparium formation, failing to shorten normally and failing to properly position the anterior spiracles (Fig. 4H). These EcR139 animals fail to show darkening of the cuticle normally seen in wild-type animals following pupariation (Fig. 4H), and also fail to exhibit hardening of the cuticle that normally takes place at this time (data not shown). Most EcR112 mutants also form an aberrant pupal case but show an earlier time of lethality than EcR139 mutants do, arresting at pupal stage P5 following head eversion and leg and wing extension (Figs. 4C,D). Thus, the predominant phenotypes of these two EcR-A mutants differ from that shown by EcR-B1 specific mutants (Bender et al., 1997) (Fig. 4E).

Investigations of internal tissues reveal that the EcR112 and EcR139 mutants have salivary glands persisting past the stage in which they should be present. In wild-type animals, the salivary glands normally undergo autophagy (Jiang et al., 1997) approximately 14 h following pupariation. However, of more than 10 EcR112 and EcR139 mutants, around 75-80% have salivary glands that persist beyond this period (data not shown).
mutants examined at late stages (more than 48 h following pupariation), this cell death did not take place, and these animals retain swollen salivary glands (data not shown). Salivary gland degeneration is thought to be under the direction of EcR-B1, as it is the predominant isoform expressed in salivary glands. However, low levels of EcR-A are in fact expressed in the salivary gland (Talbot et al., 1993), and these results indicate that this expression may be required for normal salivary gland cell death. Salivary glands appear to be unique in this respect because other internal structures that predominantly express EcR-B1, such as gastric ceca and larval midgut cells, are unaffected in their developmental progression in EcR112 and EcR139 mutants (data not shown). Alternatively, salivary gland autophagy may be more sensitive to the reduction of EcR-B1 levels compared to developmental progression in other EcR-B1 predominant tissues. In this view, the block in salivary gland breakdown observed in EcR112 and EcR139 mutants would result from the reduction of full-length EcR-B1 levels seen on Western blots (Fig. 2).

A subset of EcR139 and EcR112 mutants survive to later pupal stages (Fig. 3). Fig. 5 shows the late pupal phenotypes of EcR139 and EcR112 mutants when heterozygous to an EcR null mutation. Approximately 15% of the EcR139 mutant animals survive to the P15 pharate adult stage (Figs. 5A,B). These animals rarely eclose, however, and after several days degenerate inside the pupal case (Fig. 5A). About 5% of EcR139 mutants degenerate after the P1 white puparium stage and desiccate inside the pupal case (Fig. 5C). Approximately 2% of the EcR112 mutants survive to the P15 pharate adult stage (Figs. 5E–G). These animals rarely eclose and seem to lack a fully defined operculum (Fig. 5F). The heads of these animals also are
compacted into the anterior portion of the puparium, where there is normally a gap at this point in development (Fig. 5F). In addition, EcR112 pharate adults that are dissected from the puparium have not ejected the larval mouthhooks (Fig. 5G).

EcR139 and EcR112 mutants that survive to the P15 pharate adult stage often exhibit malformed legs. Figs. 6B and C show examples of the malformed leg phenotype seen in EcR112 and EcR139 mutants, respectively, when heterozygous to an EcR null mutation. Typically, kinks are observed in the femur and tarsal segments are shortened and rounded compared to the wild type (Fig. 6A).

Finally, we have observed a behavioral defect during pupariation in both the EcR112 and EcR139 mutants. In the wild type, third instar larvae exit from the food, cease feeding, and wander in search of a suitable pupariation site. Although the data is not as clear for Drosophila, studies in Manduca have shown that these behavioral changes are directly induced by ecdysone (Riddiford, 1993). At the onset of pupariation, wild-type animals become motionless, shorten in length to form the barrel-shaped puparium, attach to a solid surface and harden the cuticle. In contrast, the EcR112 and EcR139 mutants sometimes continue to feed and seem to physically resist the changes of pupariation until the larval cuticle hardens and forms the puparium. These movements then result in the formation of a misshapen puparium (Fig. 5D). In addition, many EcR112 and EcR139 mutants fail to exit the food and instead pupariate in the food, suggesting that they either do not receive or do not respond to the signal that

Fig. 5. Late pupal EcR-A lethal phenotypes. Some EcR139 and EcR112 mutants heterozygous to the null mutation EcR M546 arrest during late pupal development. (A) An EcR112/EcR M546 mutant at the P15 pharate adult stage that has failed to eclose and has undergone degeneration within the pupal case after 24 h. (B) Ventral and dorsal views of a dissected EcR139/EcR M546 pharate adult. (C) An EcR139/EcR M546 mutant that has arrested at the white prepupal stage and undergone dessication within 24 h. (D) An EcR112/EcR M546 mutant that has arrested at the white prepupal stage with a misshapen pupal case. (E, F) A rare EcR112/EcR M546 mutant that has survived to produce a pharate adult shows abnormal operculum formation (arrows). (G) An EcR112/EcR M546 pharate adult extracted from the pupal case. The black circle indicates the larval mouthparts which were not properly ejected during pupal development.


390
stimulates the wandering behavior normally occurring before pupariation. Together, these observations suggest that loss of EcR-A function results in a loss of coordination of larval wandering and pupariation behavior. The failure to exit the food may also allow for longer survival in that the animals that do not leave the food do not show the signs of desiccation we described earlier. This observation suggests that defects in the integrity of the cuticle in EcR112 and EcR139 mutants may be rescued if the animals remain in a moist environment.

**Phenotypic analysis of EcR94 mutants**

Fig. 7 shows the phenotypes of EcR94 mutants when heterozygous to an EcR null mutation. Most EcR94/EcRmutants exhibit a non-pupariating phenotype.
with anterior and posterior gaps similar to that seen in EcR-B1 mutants (Bender et al., 1997) (compare Figs. 7A,B with Fig. 4E). Unlike the EcR-B1 mutants, however, the internal tissues of the EcR94 animals seem to become unstable and degenerate prior to necrosis occurring. It is difficult to define any internal structures within the EcR94 mutants at this stage (Fig. 7C). Approximately 2% of EcR94/EcRM554fs mutants pupariate, but do so improperly. Eversion of the anterior spiracles occurs, but in an abnormal position (compare Fig. 7D to Fig. 4F). EcR94/EcRM554fs mutants of this type undergo desiccation within 24 h of pupariation (Fig. 7E). In addition, some EcR94 mutants of this class form a misshapen pupal case (Fig. 7F).

Immunolocalization of the EcR-B1 protein in EcR94 mutant tissues shows that the larval midgut and salivary glands do not express detectable EcR-B1 (Fig. 8). However, Western blot analysis showed that EcR-B1 is present in whole animals (Fig. 2). This presence of EcR-B1 appears to be due to inappropriate expression in certain tissues that normally express EcR-A. For example, the midgut imaginal rings that form precursors of adult tissues express high levels of EcR-B1 in EcR94 mutants (Fig. 8C), whereas normally these cells express low levels or no EcR-B1 (Fig. 8A). The loss of EcR-B1 in its normal domains of expression, coupled with gain of inappropriate expression in other cell types, is likely due to the juxtaposition of EcR-A regulatory regions to EcR-B1 coding exons and may account for the early lethal phase and distinct phenotype of the EcR94 allele.

Discussion

In this study, we have continued our in vivo genetic dissection of EcR signaling functions (Bender et al., 1997; Carney and Bender, 2000; Li and Bender, 2000; Schubiger et al., 1998) by analyzing the phenotypes of three deletion mutants that affect EcR-A function: EcR112, EcR139 and EcR94. We have previously reported the isolation of these mutants in local transposition and imprecise excision screens (Carney et al., 2004). Western blot analysis (Fig. 2) shows that the EcR-A isoform is no longer produced in any of the three mutants while the EcR-B1 isoform is still present, although higher levels of EcR-B1 breakdown products occur. Two of these strains, EcR112 and EcR139, carry deletions predicted to more specifically affect EcR-A function. EcR112 carries a deletion that removes the EcR-A transcription start site while EcR139 carries a deletion that removes the EcR-A specific coding exons A2 and A3 (Carney et al., 2004, summarized in Fig. 1). EcR112 and EcR139 mutants predominantly arrest during early to mid-pupal development (Fig. 3), indicating a requirement for EcR-A subsequent to formation of the basic pupal body plan but prior to differentiation of most adult structures. The phenotypes exhibited by EcR112 and EcR139 are distinct from those described for EcR-B1 specific mutants (Bender

Fig. 8. EcR-B1 expression in EcR94 mutant larval midgut and salivary gland tissues. (A, B) Immunolocalization of EcR-B1 (A) and DAPI staining (B) of the anterior portion of the midgut in a wild-type (Canton S) larva. (C, D) Immunolocalization of EcR-B1 (C) and DAPI staining (D) of the anterior portion of the midgut in an EcR94/EcRM554fs mutant larva. White arrows indicate the midgut imaginal ring of the proventriculus. (E, F) DAPI staining (E) and immunolocalization of EcR-B1 (F) in a wild-type (Canton S) larval salivary gland. (G, H) DAPI staining (G) and immunolocalization of EcR-B1 (H) in an EcR94/EcRM554fs mutant larval salivary gland. Panels C and H are overexposed to show lack of EcR nuclear staining in larval cells.
et al., 1997) and those of mutants that inactivate both EcR-B1\(^{112}\) and EcR-B2\(^{112}\) by virtue of deletion of the EcR-B1 transcription start site (Schubiger et al., 1998). We therefore argue below that EcR-A developmental functions are distinct from those mediated by the EcR-B1 and EcR-B2 isoforms. A third deletion mutant analyzed here, EcR\(^R^{94}\), carries a larger deletion that removes EcR-A coding exons A2 and A3 and places EcR-A regulatory sequences adjacent to the EcR-B1 transcription start site. Interestingly, analysis of EcR expression in EcR\(^R^{94}\) mutants suggests that the EcR\(^R^{94}\) mutation results in both loss of EcR-A expression and ectopic expression of EcR-B1 in cell types that normally express high levels of EcR-A.

**Requirements for EcR-A during pupal development**

Both EcR\(^{139}\) and EcR\(^{112}\) mutants predominantly arrest after successful completion of head eversion and wing and leg imaginal disc extension, events that mark the completion of the prepupal stage and transition to the pupal stage (Fig. 4). However, EcR\(^{139}\) and EcR\(^{112}\) mutants of this type fail to undergo differentiation of the adult cuticle (Fig. 4). These observations suggest that EcR-A is required subsequent to the formation of the basic pupal body plan for the differentiation of adult epidermal cells, consistent with the high level expression of EcR-A in imaginal tissues at the onset of metamorphosis (Talbot et al., 1993). Many internal tissues that predominantly express EcR-B1 at the onset of metamorphosis, such as the gastric caecae and the larval midgut cells, continue their normal developmental progression in EcR\(^{139}\) and EcR\(^{112}\) mutants. A notable exception is the persistence of the larval salivary glands in these mutants. This surprising observation suggests that the low levels of EcR-A present in the salivary gland (Talbot et al., 1993) are required for normal salivary gland destruction or alternatively, that the reduction of full-length EcR-B1 levels seen in Western blots is responsible for this phenotype. EcR\(^{139}\) and EcR\(^{112}\) mutants exhibit dessication following puparium formation (Figs. 4G and 5C), suggesting that EcR-A is required for integrity of the puparium. In addition, the defects observed in larval wandering (data not shown) and puparium formation (Figs. 4C,H and 5D) in EcR\(^{139}\) and EcR\(^{112}\) mutants suggest that EcR-A may function to coordinate these simple behaviors.

The lethal period of the EcR-A mutants EcR\(^{139}\) and EcR\(^{112}\) is somewhat heterogeneous, with some lethality observed as early as second instar development (Carney et al., 2004) and some EcR\(^{139}\) and EcR\(^{112}\) mutants surviving to pharate adult stages (Figs. 3 and 5). A similar heterogeneity has been observed for mutations that affect specific isoforms of the E74 and E75 ecdysone target genes (Bialecki et al., 2002; Fletcher et al., 1995) as well as for mutations affecting subsets of EcR isoforms (Bender et al., 1997; Schubiger et al., 1998). These observations likely reflect the very broad spatial and temporal requirements for ecdysone signaling during developmental transitions in *Drosophila*. EcR\(^{139}\) and EcR\(^{112}\) mutants that do survive to pharate adult stages frequently exhibit defects in leg development (Fig. 6). This phenotype is similar to that of the malformed leg syndrome described for Broad-Complex mutants (Kiss et al., 1988) that has been attributed to defects in prepupal leg elongation and eversion. Recently, genetic experiments have linked the Rho1 signaling cascade to ecdysone-driven leg disc morphogenesis (Bayer et al., 2003; Chen et al., 2004; Ward et al., 2003). The leg phenotypes of EcR\(^{139}\) and EcR\(^{112}\) escapers suggest that EcR-A may mediate the ecdysone signal that triggers cell shape changes in the leg epithelium that result in leg disc elongation and eversion.

Although each mutant strain lacks detectable EcR-A expression, EcR\(^{139}\) and EcR\(^{112}\) do not show identical phenotypes. The difference in phenotype does not appear to be a simple difference in the strength of the two alleles because EcR\(^{139}\) reproducibly shows both greater early lethality and later survival relative to the EcR\(^{112}\) mutation. We presume instead that phenotypic differences can be accounted for by differences in the genomic structure of the two EcR deletion strains. The EcR\(^{139}\) deletion removes EcR-A coding exons A2 and A3 while the EcR\(^{112}\) deletion removes the EcR-A transcription start site (Fig. 1). While we have not attempted to distinguish between molecular mechanisms that might account for the observed phenotypic differences, we do not believe that the EcR\(^{139}\) deletion directly affects EcR-B1 expression since the endpoint of this deletion is well upstream of the approximately 9 kb minimal EcR-B promoter element that is sufficient to recapitulate EcR-B1 expression (Li and Bender, unpublished). The phenotypes exhibited by EcR\(^{139}\) and EcR\(^{112}\) mutants are more severe than those reported for a strain in which a P element insertion located downstream of exon A3 affects EcR-A function (D’Avino and Thummel, 2000) or those of a strain in which RNAi was used to specifically inactivate EcR-A (Roignant et al., 2003). This observation suggests that the EcR\(^{139}\) and EcR\(^{112}\) mutations may more completely inactivate EcR-A functions.

**Developmental specificity of EcR-A function**

The lethal period and mutant phenotypes that we have described here for the loss-of-function EcR-A mutant strains EcR\(^{139}\) and EcR\(^{112}\) are distinct from those previously described for mutants that inactivate either EcR-B1 or both EcR-B1 and EcR-B2, suggesting that different EcR isoforms have distinct developmental functions. In contrast to the predominant early to mid-pupal arrest shown here for EcR-A mutants (Fig. 3), EcR-B1 mutants fail to undergo pupariation (Bender et al., 1997). EcR-B deletion mutants that inactivate both EcR-B1 and EcR-B2 primarily arrest during early larval development and escapers also fail to undergo normal pupariation (Schubiger et al., 1998). A prominent aspect of the phenotypes of previously described EcR isoform specific mutants is the loss of coordination of developmental events during metamorphosis. For example,
EcR-B1 mutants fail to undergo early events in pupariation including spiracle eversion, shortening, attachment to a solid surface, and hardening of the cuticle but do undergo the later event of apolysis of the larval cuticle. In addition, normal ecdysone responses are blocked in these mutants in tissues including the larval midgut and salivary gland and the abdominal histoblasts, while other tissues, such as the imaginal discs, initiate their normal response to ecdysone (Bender et al., 1997). These phenotypes are shared by EcR-B escapers that survive to later stages (Schubiger et al., 1998). The EcR139 and EcR112 EcR-A mutants also exhibit some failures of coordination, notably in continued development following defective puparium formation (Figs. 4C,H) and in persistence of the larval salivary glands. In contrast to these examples of unsynchronized development, EcR null mutants rescued beyond earlier embryonic and larval requirements by heat shock induced expression of an EcR cDNA (Li and Bender, 2000) show a distinct non-pupariating arrest phenotype in which all ecdysone responses to the late third instar ecdysone pulse appear to be blocked. These observations suggest that EcR isoforms differentially contribute to tissue-specific ecdysone response during metamorphosis and, with the exception noted earlier of the EcR-A persistent larval salivary gland, are consistent with the distinct patterns of expression observed for EcR-A and EcR-B1 (Talbot et al., 1993; Truman et al., 1994).

A third EcR deletion mutant whose phenotype is described here, EcR94, carries a deletion of approximately 37 kb extending from 158 bp downstream of EcR-A exon A1 to 92 bp upstream of the EcR-B transcription start site (Carney et al., 2004). An examination of EcR expression in this mutant indicates that EcR-B1 expression is lost in larval tissues that normally express high levels of EcR-B1 such as the salivary gland and midgut but that EcR-B1 is now ectopically expressed in certain tissues that normally express EcR-A (Fig. 8). This observation suggests that the juxtaposition of EcR-A regulatory elements to the EcR-B transcription start site in EcR94 mutants brings EcR-B1 expression under the control of at least some EcR-A regulatory elements. The earlier lethality and distinct phenotypes of the EcR94 mutant strain compared to EcR139 and EcR112 are likely to result from a combination of loss of normal EcR-B1 expression and ectopic expression of this isoform in this strain.

As has been noted previously (Cherbas et al., 2003), the inactivation of specific receptor isoforms or groups of isoforms in the whole animal poses several challenges to determining direct consequences of loss of receptor function. These challenges, which extend beyond the difficulties specific to the EcR94 mutant strain, include the possibility of observing indirect, non-autonomous events in other cells or tissues due to disruption of downstream cell–cell signaling in an EcR expressing tissue, the possibility that EcR isoforms regulate the expression or function of one another, and the possibility that EcR may exert feedback control on ecdysone synthesis. Cherbas et al. (2003) have demonstrated non-autonomous developmental arrest phenotypes following the inactivation of EcR functions via the expression of dominant negative forms of EcR in specific tissues. Thus, it will be important to determine whether the EcR-A effects described here are autonomous or non-autonomous. Given the technical limitations on creating FRT generated EcR clones due to the location of the EcR gene very close to the centromere, a particularly powerful approach to separating autonomous and non-autonomous EcR effects may be to transplant EcR mutant tissues taken prior to metamorphosis into a wild-type host and observe responses in mutant tissues following metamorphosis of the host.

As discussed above, analysis of loss-of-function EcR mutations has been useful in assigning distinct developmental functions to the different EcR isoforms. Because many tissues express multiple EcR isoforms and because the relative levels of EcR isoforms vary dramatically between tissues, it is important to note that an analysis of loss-of-function isoform specific mutations does not by itself allow assignment of specific biochemical functions to particular isoforms. EcR isoform-specific mutations, however, have proved useful in rescue experiments designed to ask whether different EcR isoforms are functionally distinct in vivo. Thus, rescue of salivary gland polytene chromosome puffing (Bender et al., 1997) or remodeling of mushroom body neurons (Lee et al., 2000) in EcR-B1 mutants, or rescue of defective remodeling of a set of thoracic neurosecretory neurons in EcR-B mutants (Schubiger et al., 2003) has demonstrated differences in the abilities of the three EcR isoforms to functionally substitute in these cellular contexts. These observations are consistent with biochemical experiments that indicate that the different EcR isoforms have distinct transcriptional activation properties (Dela Cruz et al., 2000; Hu et al., 2003; Mouillet et al., 2001). In an alternative approach, targeted expression of an EcR dominant negative was used to inactivate EcR function in specific tissues and the resulting defects were rescued via the expression of different EcR isoforms in the targeted domains (Cherbas et al., 2003). This study showed that EcR dominant negative induced defects could be rescued in many tissues by the expression of any of the three EcR isoforms while a smaller set of tissues exhibited EcR isoform-specific rescue requirements, prompting the authors to argue that only a minority of ecdysone responsive promoters require specific EcR isoforms. In the future, identification of EcR-A and EcR-B1 dependent ecdysone target genes via whole genome expression analysis using EcR mutants described here or previously isolated should allow a direct test of this proposition.

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