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EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue

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SUMMARY

The three *Drosophila* EcR isoforms differ only at their N termini; thus, they share the conserved ligand-binding domain transcriptional activation function (AF2) and only differ in the unconserved A/B region, which contains a second, isoform-specific, activation function (AF1). We have developed a dominant-negative mutant EcR (EcR-DN), expressed it in flies with the GAL4/UAS system, and used it to block ecdysone signaling in eight tissues or groups of tissues. Localized EcR-DN arrests ecdysone-dependent development in the target cells and often – because of a molting checkpoint – arrests development globally. Simultaneously expressing individual wild-type EcR isoforms in the same target tissues suppresses the EcR-DN phenotype and identifies the rescuing isoform as sufficient to support the development of the target. Every isoform, and even an N-terminal truncated EcR that lacks any AF1, supports development in the fat body, eye discs, salivary glands, EH-secreting neurosecretory cells and in the *dpp* expression domain, implying that AF1 is dispensable in these tissues. By contrast, only EcR-A is able to support development in the margins of the wing discs, and only EcR-B2 can do so in the larval epidermis and the border cells of the developing egg chamber. In light of our results, the simplest explanations for the widespread spatial and temporal variations in EcR isoform titers appear untenable.

Supplemental data available on-line

Key words: EcR, AF1, Ecdysone, Nuclear receptors, Isoforms, Dominant-negative

INTRODUCTION

In comparison with most other transcriptional regulators, the nuclear receptors are remarkably complex. We are concerned with one aspect of that complexity: the alternative pathways by which a nuclear receptor can activate transcription. A typical nuclear receptor contains two activation surfaces: a strongly conserved activation function (AF2) in its ligand-binding domain (LBD or E region; Fig. 1) and a second activation function (AF1) within its unconserved N-terminal A/B region. A great deal is known about the role of AF2 in seeding coactivator complexes (Rosenfeld and Glass, 2001), rather less about AF1. Nonetheless, it is clear from assays in different settings that the two AFs play separable, though often complementary, roles. Thus, depending upon the promoter being tested, its chromatin structure and the host cell, full activation may require either AF or both (Tzukerman et al., 1994; Taneja et al., 1997; Sheldon et al., 1999; Gao et al., 2000; Rochette-Egly et al., 2000; Keeton et al., 2002).

An attractive inference is that these differing AF requirements reflect the diversity of the rate-limiting steps that can be involved in the assembly of functional transcription complexes. If so, it is interesting to consider how these requirements are organized globally. One plausible hypothesis is that AF requirements are simply promoter specific. According to this idea, the set of hormone-responsive promoters in any given cell type may approximate a random sample of AF requirements. A corollary is that, if AF1-requiring promoters are prevalent, every cell type should require AF1, and if the receptor exists in multiple isoforms with different AF1s, every cell should require multiple isoforms.

A very different hypothesis is suggested by observations on receptors with alternative, isoform-specific AF1s and particularly by the behavior of such receptors in developmental systems. Many nuclear receptors exist as functionally distinct isoforms differing only in their N-terminal domains [e.g. RARs α, β and γ (Zelent et al., 1991), RXRs α, β and γ (Fleischhauer et al., 1992; Liu and Linney, 1993; Nagata et al., 1994), TRβ (Langlois et al., 1997), GR (Yudt and Cidlowski, 2001), PR (Kastner et al., 1990; Giangrande and McDonnell, 1999; Shyamala et al., 2000), ERα (Flouriot et al., 2000) and EcR (Talbot et al., 1993)]. In addition, receptor isotypes, generated from distinct genes, most often differ in their N-terminal domains [e.g. TR (Hollenberg et al., 1995) and ER (Delaunay et al., 2000; Saville et al., 2000; Weatherman and Scanlan, 2001)]. Where a receptor exists in several isoforms or isotypes, these are generally distributed in a strongly tissue-specific manner (Hodin

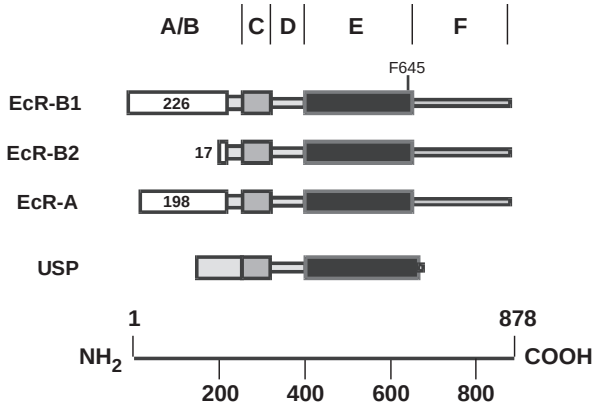


Fig. 1. Domain structures of the ecdysone receptor components. The three isoforms of *Drosophila* EcR and the single *Drosophila* USP are shown, with the standard nuclear receptor regions indicated. Region C is the DNA-binding domain (DBD); region E, the ligand-binding domain (LBD). The three EcR isoforms are identical in sequence except in the A/B regions that are unrelated. The number of isoform-specific residues is shown for each EcR. The scale underneath (in residues) is aligned to EcR-B1. The residue mutated in EcR-F645A is indicated.

et al., 1989; Zelent et al., 1991; Giangrande and McDonnell, 1999; Flouriot et al., 2000; Mollard et al., 2000). Differences in isoform titers can be quite striking, and for some nuclear receptors there is a strong and unmistakable correlation with developmental fate (e.g. Zelent et al., 1991). Given that alternative AF1s have distinct transcriptional effects and that they can activate distinct sets of target genes (Richer et al., 2002), it is reasonable to suppose that the availability of particular AF1s (in specific receptor isoforms or isotypes) may regulate tissue-specific gene expression. Taken to the extreme, this hypothesis would imply that promoters that are induced in all tissues require only AF2, while promoters whose hormone response is tissue-specific require AF1, and in any given cell type the AF1-specific promoters share a requirement for a specific receptor variant.

These alternatives lead to the following question: where isoform (hence AF1) titers vary in a tissue-specific way, are they strongly correlated with tissue-specific requirements for the corresponding AF1s? This question has been addressed using mutant animals. For example, the phenotypes of RXRα-null mice and mice homozygous for a deletion of the AF1 region of RXRα were compared (Mascrez et al., 2001). Animals that lacked AF1 exhibited localized defects, suggesting specific AF1 requirements, though most RXRα functions occurred normally. In a parallel approach, the roles of individual isoforms have been assessed by using isoform-specific mutations (Mulac-Jericevic et al., 2000; Bender et al., 1997; Schubiger et al., 1998). In all these instances, the use of mutant organisms creates daunting interpretive challenges, for it is difficult to decipher the direct, local consequences of receptor failure in the face of numerous organism-wide defects. We have adopted a different approach, arresting receptor function in targeted developmental domains and then testing the abilities of particular receptor isoforms to rescue development when expressed in those domains.

Our experiments involve components of the *Drosophila* ecdysone receptor. Ecdysone triggers molting and metamorphosis in insects. At metamorphosis, virtually every cell is a hormone target, with cells of different tissues proceeding down profoundly different developmental pathways. In addition, ecdysone plays an important role in oogenesis.

The ecdysone receptor is a heterodimer of two nuclear receptors: EcR and the RXR ortholog USP (Koelle et al., 1991; Yao et al., 1992; Yao et al., 1993; Thomas et al., 1993). In *Drosophila*, there are three EcR isoforms that differ only in their N-terminal regions, the three isoforms being derived from a single structural gene by both alternative promoter usage and alternative splicing (Talbot et al., 1993). The potencies of the three isoforms have been tested in an EcR-deficient *Drosophila* cell line (X. H., L. C. and P. C., unpublished), in yeast (Dela Cruz et al., 2000) and in mammalian cells (Mouillet et al., 2001). These studies confirm that the individual isoforms (and their isolated N-terminal regions tested as fusions) differ markedly in their abilities to activate particular test promoters, and that each A/B region contains an AF1 that can activate transcription in an appropriate experimental setting. For example, AF1s from isoforms B1 and B2, but not from A, are active when tested in *Drosophila* cells with an artificial promoter derived from the *Drosophila Eip71CD* gene. It is important to note here that these specific isoform requirements are absolute; that is, increasing the titer of an inactive isoform does not increase its ability to activate transcription (X. H., L. C. and P. C., unpublished).

Little is known about the distribution of isoform EcR-B2, but it is clear that isoforms EcR-A and EcR-B1 have very different tissue distributions, and their relative titers in different tissues are well correlated with the fates of those tissues during metamorphosis (Talbot et al., 1993; Robinow et al., 1993; Truman et al., 1994; Hodin and Riddiford, 1998). For example, immunohistochemistry shows that in third-instar larvae, B1 predominates in larval tissues that will die during metamorphosis, while A predominates in the imaginal discs.

Genetic studies suggest that the isoforms have overlapping but distinct functions during fly development. Mutations that eliminate all isoforms, only isoform B1, or isoforms B1 and B2 have distinct lethal phases, leading to death at hatching, at pupariation and during larval life, respectively (Bender et al., 1997; Schubiger et al., 1998). It is known that ectopic expression of any single EcR isoform can partially rescue development in *EcR*$^{-}$ animals, with the extent of rescue depending on the isoform (Li and Bender, 2000).

We have used the GAL4 driver system of Brand and Perrimon (Brand and Perrimon, 1993) and dominant-negative mutant EcRs to arrest ecdysone receptor function in selected developmental domains. Then, using the same driver system to express individual EcR isoforms, we have asked which isoforms are sufficient to restore and sustain development. Serendipitously, our experiments have revealed a molting checkpoint – a global block in development induced by local lesions. The developmental arrest associated with that checkpoint is noteworthy in its own right; in the present context it has proven exceptionally useful for our experiments.

MATERIALS AND METHODS

Plasmids

Plasmids for GAL4 driver stocks

Eip$_{657}$-GAL4 was constructed as follows: A *Hin*dIII/*Bam*HI fragment

containing the promoter region –657 to +11 of the gene *Eip71CD* was excised from 657-cc-cat (Cherbas et al., 1991) and inserted between *Bam*HI and *Hin*dIII sites of pUCL1 (Cherbas et al., 1991). The promoter fragment was then re-excised with *Bgl*II and *Bam*HI and inserted into the *Bam*HI site of pGaTB (Brand et al., 1994), to produce a plasmid in which the Eip promoter is followed by a GAL4-coding sequence and an hsp70 transcription terminator. A 4 kb *Kpn*I/*Not*I fragment was excised (containing the promoter, GAL4-coding sequence and transcription terminator) and inserted between the *Kpn*I and *Not*I sites of CaSpeR-4 (Thummel and Pirrotta, 1992) to generate Eip_{657}-GAL4, in which the Eip-GAL4 transcription unit and a w^+ minigene are contained between P element terminal inverted repeats.

Eip_{602}-GAL4 is identical to Eip_{657}-GAL4, except that the promoter fragment was shortened to –602 to +11; this was accomplished by digesting Eip_{657}-GAL4 with *Kpn*I and *Swa*I, blunting the ends with T4 DNA polymerase, and re-ligating the resulting fragment.

Sgs3-GAL4 was a gift from Dr A. J. Andres and is described by Do et al. (T. V. Do, A. Biyasheva and A. J. Andres, unpublished). The promoter fragment is bases –1750 to +20 of *Sgs3*.

Plasmids for UAS responder stocks

The coding sequences from pCMA-EcR-F645A, pCMA-EcR-W650A and pCMA-EcR-C (X. H., L. C. and P. C., unpublished) were excised as *Bam*HI to *Nhe*I fragments and inserted between the *Bgl*II and *Xba*I sites of pUAST (Brand et al., 1994) to make the P element transformation vectors UAS-EcR-F645A, UAS-EcR-W650A and UAS-EcR-C.

Transformed fly lines

Drivers

Sgs-GAL4, Eip_{657}-GAL4 and Eip_{602}-GAL4 were inserted into flies by P element transformation. The Eip_{657} and Eip_{602} drivers had qualitatively similar properties in our assays; Eip_{657}-GAL4 was used to generate the photograph in Fig. 5D; all other Eip driver experiments reported here used Eip_{602}-GAL4.

The GMR, dpp, Ser and act5C drivers were obtained from the Bloomington Stock Center. The Lsp2 driver was a gift from C. Antoniewski. The slbo driver was a gift from D. Montell. See Table 1 for more details about these drivers.

Responders

UAS-EcR-F645A, UAS-EcR-W650A, and UAS-EcR-C were inserted into flies by P element transformation. Multiple UAS-EcR-F645A insertions were tested and found to give qualitatively similar results; the data reported in this paper were derived from an insertion of UAS-EcR-F645A on chromosome II. Two UAS-EcR-C transformants were tested for their ability to rescue the F645A phenotype and were found to have qualitatively similar effects; data are reported for a single insertion on chromosome III. The UAS-EcR wild-type responders (Lee et al., 2000) were a gift from S. Robinow; all were inserted on chromosome III.

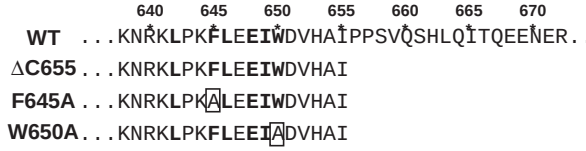
The UAS-*lacZ*.nls responder (chromosome II) (Jacobsen et al., 1998) was a gift from T. Jacobsen. The UAS-GFP.nls responder (chromosome II; B. Edgar, personal communication to FlyBase) was obtained from the Bloomington Stock Center.

lacZ staining

In order to ensure that all tissues of each larva were evenly exposed to the stain, one tip of the larva was cut off and the remainder of the animal was everted like a sock, using a blunt dissecting needle to push the intact end through the longitudinal axis of the animal. The everted larva was then fixed and stained essentially as described (Hazelrigg, 2000).

EcR mutations

The mutations EcR^{M554fs}, EcR^{Q50st}, EcR^{W53st} (Bender et al., 1997), EcR^{31} and EcR^{225} (Schubiger et al., 1998) were obtained from the Bloomington Stock Center. Each was recombined with the UAS-F645A driver to generate a stock of the genotype w^{1118}; UAS-F645A *EcR*/CyO; +. In the experiments reported in Table 2, at least three independent recombinants were used for each *EcR* mutation; results from the different recombinants were indistinguishable and are pooled in the table.



	dimerization with USP	binding to ligand	activation of transcription
ΔC655	wildtype	wildtype	100%
F645A	wildtype	wildtype	1%
W650A	wildtype	undetectable	0%

Fig. 2. Properties of dominant-negative mutant EcRs used in this paper. Sequences are shown only for the region around helix 12 of the LBD; residue numbers are for isoform EcR-B1. Bold type indicates strongly conserved residues; mutated residues are boxed. Functional assays are described by Hu et al. (X. H., L. C. and P. C., unpublished).

Polytene chromosome analysis

Larvae were staged by the morphology of the salivary gland duct (Zhimulev and Belyaeva, 1999). Puff stages were assigned by inspection of all of the known puff sites as described previously (Zhimulev and Belyaeva, 1999).

RESULTS

Dominant-negative EcR mutants (EcR-DNs)

The EcR LBDs are readily aligned with other nuclear receptors. The alignment ends after the highly conserved terminal helix (helix 12) of the LBD; from this point the *Drosophila* EcRs continue through an unusual F region of 223 residues (656-878 in DmEcR-B1). EcRs lacking this region (e.g. EcR-B1-ΔC655) are indistinguishable in ligand binding, affinity for USP, and affinity for DNA (all assayed in vitro) and in transcriptional activation (when tested in the EcR-deficient cell line L57-3-11) (Fig. 2; X. H., L. C. and P. C., unpublished).

Taking EcR-B1-ΔC655 as our starting material, we created EcR-F645A and EcR-W650A by altering highly conserved residues of helix 12. Fig. 2 summarizes their essential properties. When tested in L57-3-11 cells, neither mutant activates transcription in response to ligand. In vitro, both dimerize with USP and bind DNA normally, while F645A but not W650A binds ligand normally. As predicted by these properties, EcR-F645A and EcR-W650A both act as competitive inhibitors of wild-type EcR in cell culture assays (X. H., L. C. and P. C., unpublished); therefore they are dominant-negative mutants (EcR-DNs) that interfere with ecdysone-induced gene activation when expressed at high levels.

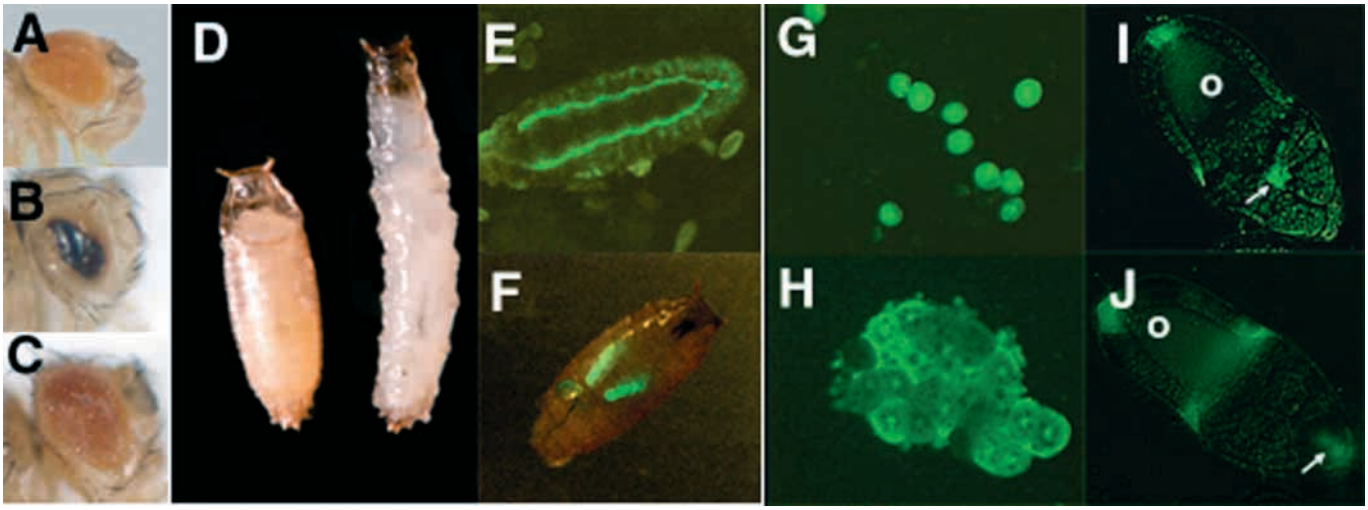


Fig. 3. Effects of EcR-F645A expression in targeted tissues. (A-C) Effects on eye development. Adult eyes are shown from animals containing a *GMR* driver and (A) no responder, (B) an EcR-F645A responder, and (C) responders for both EcR-F645A and EcR-B2. (D) Effects on larval epidermis. The two animals are of equivalent age, and both contain an $Eip71CD_{657}$ driver; the pupa on the left has no responder, the animal on the right has an EcR-F645A responder. (E,F) Effects on glue secretion. The two puparia are of equivalent age, and both carry a transgene expressing fluorescent glue (Sgs-GFP) (Biyasheva et al., 2001). They have an *Sgs3* driver and either no responder (E) or an EcR-F645A responder (F). Note that the green fluorescence in E is entirely external to the puparial case. (G,H) Effects on fat body dissociation. Pupae containing the *Lsp2* driver, a GFP.nls responder and either no EcR responder (G) or an EcR-F645A responder (H) were dissected and viewed by fluorescence; only the fat body cells are visible. (I,J) Effects on border cell migration. Stage 9 egg chambers were dissected from adult females containing the *slbo* driver, a GFP responder, and either no EcR responder (I) or an EcR-F645A responder (J). Arrows indicate border cells; o, oocyte. A-D were photographed under bright field illumination. G,H were photographed with GFP-fluorescence optics. E, F, I and J were photographed with a mixture of bright-field and fluorescence optics.

Table 1. Drivers used in this paper

Driver	Onset of expression	Domain of expression*	Reference
act5C	Embryo	Near ubiquitous	Ito et al., 1997
GMR	Wandering L3	Eye discs, tracheae, gut	Freeman, 1996
dpp	Wandering L3	Discs, salivary glands, gut	Staehling-Hampton and Hoffmann, 1994
Eip_{602}, Eip_{657}	Mid-third instar transition	Epidermis, brain	This paper†
Ser	Mid-third instar transition	Disc margins, proventriculus	Hukriede et al., 1997
Sgs3	Mid-third instar transition	Salivary gland	This paper‡; T. V. Do, A. Biyasheva and A. J. Andres, unpublished
Lsp2	Mid-third instar transition	Fat body	B. Hassad, personal communication to FlyBase
EH	Embryo	Eclosion hormone-secreting neurosecretory cells	McNabb et al., 1997
slbo	Stage 9 egg chambers	Border, polar and centripetal follicle cells	Rørth et al., 1998

*The expression pattern was determined by crossing each driver to a UAS-GFP.nls or a UAS-lacZ.nls responder.

†The drivers contain bases –602 to +11 or –657 to +11 of the *Eip71CD* transcription unit; both drivers have expression patterns that are identical to that described previously for the promoter fragment –657 to +11, which drives a *lacZ* reporter (Andres and Cherbas, 1994). The two drivers are used interchangeably in this paper.

‡The driver contains bases –1750 to +20 of the *Sgs3* transcription unit. Its expression pattern is identical to that described for an Sgs3-GFP fusion containing the same promoter (Biyasheva et al., 2001).

Effects of targeted EcR-DN expression

We generated numerous transformed fly stocks carrying the responder transposons UAS-EcR-F645A and UAS-EcR-W650A, in which a GAL4-dependent promoter expresses the coding sequences of the EcR-DNs. We then selected a panel of driver stocks, each expressing GAL4 in a temporally and spatially distinct developmental domain (Brand and Perrimon, 1993) and crossed the stocks to yield hybrid progeny with targeted EcR-DN expression.

Table 1 summarizes the expression domains of the nine GAL4 drivers we used. For each driver stock, we verified and/or defined its expression domain by crosses to UAS-GFP.nls and (in most cases) UAS-*lacZ*.nls responder stocks. The GFP-expressing progeny were used to define stages of substantial expression, the *lacZ*-expressing stocks to define spatial patterns at higher resolution and sensitivity. Previously undescribed expression patterns for the *dpp* and *GMR* drivers are illustrated in a supplemental on-line figure (see http://dev.biologists.org/supplemental/). Most of the drivers we selected are first expressed during metamorphosis. Some (*Eip*, *Sgs3* and *Lsp2*) first become active at the mid-instar transition – midway through the third larval instar (Andres and Cherbas, 1992; Andres et al., 1993). Others (*GMR*, *dpp* and *Ser*) first show strong expression about 10 hours later, at a stage when

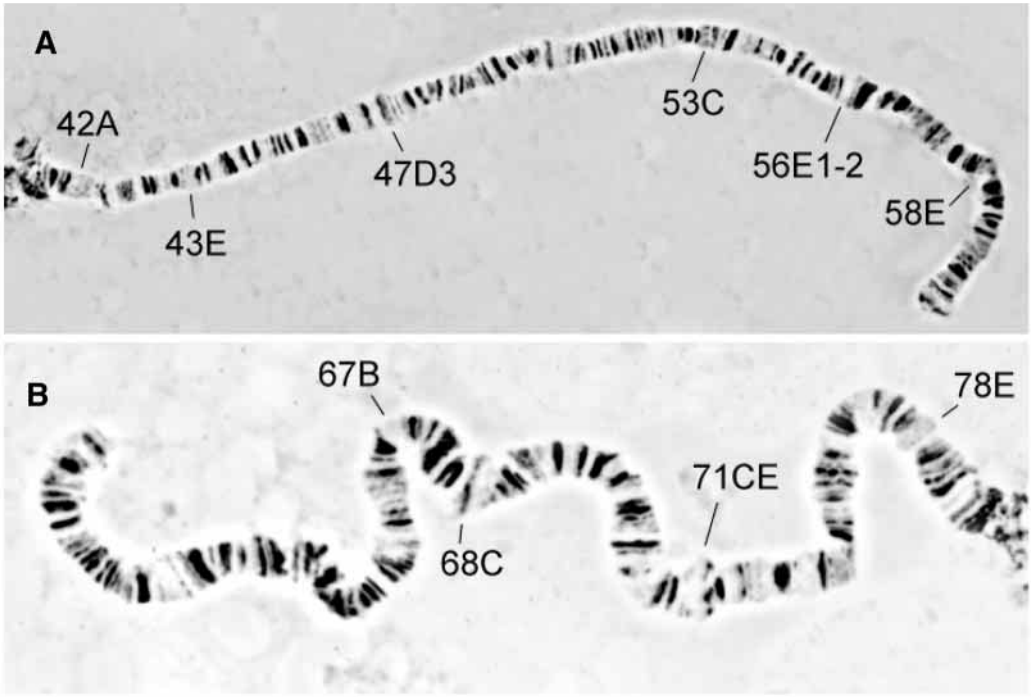


Fig. 4. PS1′ chromosomes from an animal containing the Sgs3-GAL4 driver and UAS-F645A responder. Chromosomes 2R (A) and 3L (B) from a white prepupa are shown. An identical pattern occurs in animals throughout the period from mid-instar transition to white prepupa. See text for discussion of the labeled sites. Note that these chromosomes are of a normal size for white prepupal polytene chromosomes, but that their puffing pattern is similar to that seen in the much smaller PS1′ chromosomes of wild-type early third-instar larvae.

the animals leave the food and the well-known ecdysone-induced puffing cycle begins in the salivary glands.

In initial experiments using the *GMR*, *Eip* and *dpp* drivers, we crossed each driver stock to several independent responder lines for both UAS-EcR-F645A and UAS-EcR-W650A. These tests revealed that each driver yields a qualitatively clear pattern, irrespective of insertion site or choice of EcR-DN, although the results from independent insertions were quantitatively distinguishable. Therefore, in all of the experiments reported here we employed a single F645A responder stock.

The effects of expressing EcR-F645A in each domain are illustrated in Figs 3 and 4, and are summarized below.

act5C

The *act5C* driver is expressed ubiquitously beginning early in embryonic development. Expression of F645A under its control is lethal, either during embryonic development (stages not defined) or soon after hatching.

GMR

Most animals cease development after pupariation. Escapers have defective eyes, with irregular and poorly pigmented ommatidia and large necrotic, melanized patches (Fig. 3B). When the level of F645A expression was varied by using different responder insertions or different temperatures, the efficiency of developmental arrest (measured as the fraction of animals failing to reach adulthood) was well correlated with the severity of the morphological defects in escapers (judged qualitatively, not shown). In short, lethality (arrest) provides a convenient quantitative measure of the EcR-F645A phenotype.

dpp

At 25°C, most animals fail to pupate. At 20°C, some animals arrest before pupation; most pupate, but die during adult development. Escapers are morphologically normal save for the orientation and, sometimes, the length of the posterior scutellar bristles.

Ser

All animals fail to pupate; there are no escapers.

Eip

Pupariation is defective. The animals fail to shorten, and tanning occurs only at the extremities, where the driver is not expressed (Fig. 3D). Their development does not progress beyond the stage shown in the figure and the imaginal discs do not evert. At low temperatures, a few animals escape, becoming morphologically normal adults.

Sgs3

The salivary glands synthesize a small amount of glue (their normal secretory product), but ecdysone-induced glue secretion does not occur (Fig. 3F). The glands histolyse on schedule at pupation and the animals are fully viable. The polytene chromosomes are normal in size, but their puffing pattern remains blocked at puff stage 1′ (PS1′) (Fig. 4) – a stage normally observed only early in the last larval instar, in chromosomes too small to be suitable for cytogenetic analysis (Zhimulev and Belyaeva, 1999). PS1′ is characterized by small puffs at sites encoding glue proteins (3C8-12, 68C, 90D), at other sites that normally regress in the presence of ecdysone (42A4-18, 43E, 53C, 56E1-2), at several sites not correlated with development (47A9-16, 58E, 67B, 71CE, 72D, 88D, 88E) and at the PS1′-specific sites 78E and 80A3 (Becker, 1959, Ashburner, 1967; Ashburner and Berendes, 1978; Zhimulev, 1999). In PS1′, there is no puff at 85F1-6, a region which puffs continuously during the subsequent stages PS1 to PS11.

Lsp2

The fat body cells fail to dissociate at pupation (Fig. 3H). Most

Table 2. Effect of *EcR* mutations on the EcR-F645A phenotype

Driver	EcR genotype: Affected isoforms:	+/+ None	*M554fs*/+ All	*W53st*/+ B1	*Q50st*/+ B1	*225*/+ B1+B2	*31*/+ B1+B2
GMR driver (20°C)	% survival* (*n*)	4.7 (21)	0.12 (2)	11.8 (97)	1.0 (19)	2.4 (11)	1.2 (4)
	Fraction of control†	[1.0]	0.03	2.5	0.21	0.51	0.30
dpp driver (20°C)	% survival* (*n*)	4.2 (33)	< 0.13 (0)	4.4 (19)	4.7 (38)	3.5 (9)	7.7 (17)
	Fraction of control†	[1.0]	< 0.02	1.0	1.1	0.83	1.8
Eip$_{602}$ driver (16°C)	% survival* (*n*)	5.1 (15)	0.6 (5)	3.3 (11)	1.9 (5)	0.6 (3)	n.d.
	Fraction of control†	[1.0]	0.12	0.65	0.37	0.12	
Lsp2 driver (25°C)	% survival* (*n*)	3.7 (12)	< 0.16 (0)	16 (75)	3.6 (35)	0.7 (3)	0.6 (2)
	Fraction of control†	[1]	< 0.03	4.3	1.0	0.19	0.16

Each driver stock was crossed at the indicated temperature to flies of the genotype w^{1118}; UAS-F645A *EcR* / CyO; +, where the *EcR* allele is given in the column headings.

*The frequency of adult progeny carrying the responder chromosome, where 100% is defined as the number of adult progeny carrying the balancer. The number in parentheses is the actual number of non-balancer adult survivors that were scored.

†The ratio of survival in flies heterozygous for the indicated *EcR* mutation to flies homozygous for wild-type *EcR*; all flies carry the driver and responder transposons. For those drivers that were maintained as balanced stocks (*dpp* and *Eip*), only progeny carrying the driver were scored. There was no observable change in results when the genders of the parents were reversed; hence, data are pooled from crosses in the two directions.

animals die late in adult development. Viability is lower in females than in males.

EH

Most animals molt into adults but fail to expand their wings – a phenotype previously observed after genetic cell ablation in the EH domain (McNabb et al., 1997).

slbo

Metamorphosis appears normal, but the resulting females are sterile. In the developing egg chambers, border cell migration is delayed or fails completely (Fig. 3J). The chorions are fragile and eggs collapse soon after oviposition. Dorsal appendages are frequently broad or branched, reminiscent of the *bullwinkle* phenotype (Rittenhouse and Berg, 1995), and operculum structures are poorly developed.

These diverse observations can be summarized as follows: in a cell expressing an EcR-DN, all ecdysone-dependent development is arrested. The complete abrogation of ecdysone-dependent development is shown most clearly by the dramatic arrest of the polytene chromosomes in PS1′. In some domains, EcR-DN expression blocks development globally at the next ecdysone-dependent step. Thus, expression in the Eip, GMR, Ser or dpp domain prevents any sign of pupation. We propose that this blockade illustrates the existence of a ‘molting checkpoint’, which will be described in more detail in the Discussion.

The EcR-DN phenotypes are due to insufficient functional EcR

We have ascribed these phenotypes to arrested ecdysone signaling. In principle, they might be due to any other dominant effect of EcR-DN expression. For example, because crosstalk among nuclear receptors is common, EcR-F645A might disrupt other nuclear receptor signaling pathways. To test whether the phenotypes result from insufficient functional ecdysone receptor we tested the effects of reducing the background concentration of wild-type EcR.

In Table 2, the leftmost data columns compare the effects of targeted EcR-DN expression in wild-type flies and flies heterozygous for the null mutation EcR^{M554fs} (Bender et al., 1997). EcR^{M554fs} is fully recessive; thus, in the absence of EcR-DN expression, the heterozygotes are viable and morphologically normal. Targeted EcR-DN expression reduces viability (a combination of checkpoint effects and more direct lethality). For each of the four drivers tested, reduction in wild-type EcR enhances this phenotype by one to two orders of magnitude. These results are fully consistent with the hypothesis that there is a competition in target cells between EcR-DN and wild-type EcR.

Rescuing EcR-DN phenotypes by expressing wild-type EcR isoforms

In the converse experiment, we sought to suppress the effects of targeted EcR-DN expression by overexpression of single wild-type EcR isoforms. This suppression should succeed only in domains that do not require a mixture of isoforms. Thus, we sought to discover whether such domains exist and, where they exist, to identify the required isoform. We reasoned that if both EcR-DN and a wild-type EcR are expressed from similar GAL4-responsive promoters, the two transgene-derived proteins should be present at comparable concentrations, both far in excess of the normal products; under these circumstances, cell culture data (X. H., L. C. and P. C., unpublished) predict that the EcR-DN will compete poorly.

As a prelude, we tested whether simply overexpressing single EcR isoforms in small targeted domains deranges development in the absence of any EcR-DN. We used GAL4 drivers to target expression of each EcR isoform in the *GMR*, *dpp*, *Ser* and *Eip* domains and observed no significant effects on morphology or viability (data not shown). The sole exception to this pattern is the *act5C* domain, in which expression of any single isoform causes early lethality. Despite this exception, we find the result surprising and will return to it in the Discussion.

We then co-expressed EcR-F645A with each of the wild-type EcR isoforms (and EcR-C, containing only the sequences common to all isoforms) in targeted developmental domains. The results were domain specific: in numerous cases all of the phenotypes associated with EcR-DN expression are efficiently suppressed (Fig. 3C, Fig. 5, Table 3). Contrary to our expectation, efficient suppression occurs irrespective of

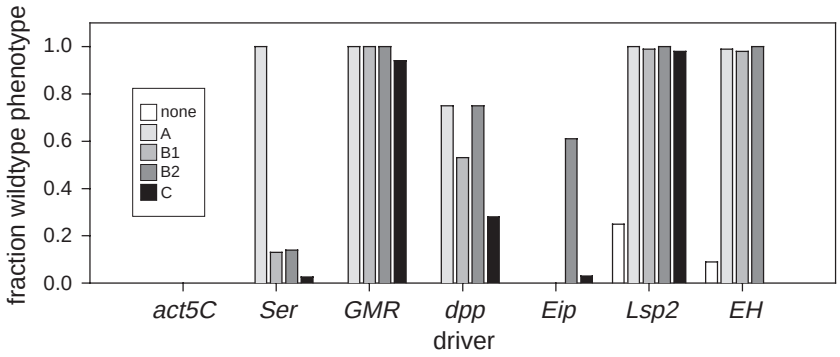


Fig. 5. Effects of targeted expression of individual EcR isoforms in the presence of EcR-F645A. Flies homozygous for both the EcR-F645A responder and a responder corresponding to the indicated wild-type EcR were crossed to each driver stock. EcR-C was not tested with the act5C and EH drivers. All other combinations of drivers were tested; if no bar is visible, there were no progeny with wild-type phenotype. For the EH driver, wild-type phenotype is expanded wings; for the other drivers, wild-type phenotype is survival to adulthood. In the case of the GMR driver, wild-type EcR isoforms restored wild-type eye morphology as well as viability (see Fig. 5C). Data are shown for crosses performed at 25°C, but the results were independent of temperature (data not shown).

isoform in several domains and strict EcR isoform specificity appears to be comparatively unusual.

Thus, any wild-type EcR isoform is able to rescue both normal viability and normal morphology in the *GMR*, *Lsp2* and *EH* domains, and nearly complete viability in the *dpp* domain (Fig. 3C, Fig. 5). In the *Sgs3* domain, each isoform rescued glue secretion partially (Table 3, and below). Given these results, it is not surprising that the common region 'isoform' EcR-C (which lacks any potential AF1) affords substantial, though quantitatively weaker, rescue in the *GMR*, *Lsp2*, *Sgs3* and *dpp* domains.

We observed requirements for specific EcR isoforms in only three domains (Fig. 5, Table 3). In the *Ser* domain, EcR-A appears to be required; it alone rescued completely, yielding fully viable, morphologically normal adults. In the *Eip* domain, only EcR-B2 provided significant rescue: About half of the animals reached adulthood; those that did were morphologically normal. Similarly, only EcR-B2 produced significant rescue of fertility when expressed in the *slbo* domain. In these flies, the border cells migrated normally in most egg chambers and about 25% of the eggs retained a normal shape once laid; a few gave rise to viable larvae. Although EcR-B2 rescue was incomplete in both the *Eip* and *slbo* domains, we note that in those domains neither EcR-A nor EcR-B1 afforded any detectable rescue. In fact, co-expression of EcR-A in the *slbo* domains slightly enhanced the EcR-F645A phenotype, decreasing egg yield and increasing egg fragility.

Remarkably, in every domain we tested (except for the ubiquitous *act5C* domain), a single isoform was able to support development, providing substantial rescue of the EcR-DN phenotypes. We found no evidence of any cell type that requires a mixture of isoforms to support normal development.

Salivary gland puffing under the influence of single EcR isoforms

We also examined the ability of each isoform to rescue the complex ecdysone-induced puffing pathway, expecting that individual early puffs would be supported to different extents by the different EcR isoforms. In fact, each isoform rescues both glue secretion and the puffing sequence – partially. A normal, coordinated progression of puffs occurs at a slower than normal speed until the animal (though not the gland) reaches pupariation (Fig. 6). Thus puffing lags behind duct development, deranging the normally perfect correlation between puffing and duct stage (Zhimulev and Belyaeva, 1999).

In white puparia (normally PS10-11), salivary glands expressing EcR-DN in combination with any of the three isoforms display a puffing pattern characteristic of younger animals, or (more frequently) a mixture of puffs typical of PS10-11 and those of earlier puff stages. The pattern varies among individual animals, and typically includes some PS10-11 puffs (66B, 67B, 71B, 97C2, 85D1-2, 85F1-6) and some puffs characteristic of earlier larval stages (34A5-6, 56E1-2), combined with a reduction or loss of some PS10-11 puffs (47A9-16, 62E, 63E1-3, 71F1-2, 82F). Examples of these aberrant patterns are shown in Fig. 7.

Dominant enhancement of EcR-DN effects as a test of normal isoform functions

When the host animals for targeted EcR-DN expression are heterozygous for EcR^{M554fs}, the EcR-DN phenotypes are exacerbated; i.e. EcR^{M554fs}, which contains a common region frame-shift and is a true *EcR* null (Bender et al., 1997), is a dominant enhancer of the EcR-DN effect. We also tested four more selective *EcR* mutations, the B1-specific nulls EcR^{W53st} and EcR^{Q50st} (Bender et al., 1997), and EcR^{225} and EcR^{31}, in which promoter deletions abolish expression of both B1 and B2 (Schubiger et al., 1998). The results, summarized in Table 2, support the following conclusions:

GMR and dpp drivers

Neither heterozygosity for EcR-B1 nor heterozygosity for both B isoforms has any consistent strong effect on EcR-F645A lethality. As shown in Table 2, the two EcR-B1 mutations exerted opposite effects in the *GMR* domain and had no effect in the *dpp* domain, while the two mutations affecting B1+B2 enhanced EcR-F645A lethality very modestly in the *GMR* domain and exerted small but

Table 3. Rescue of EcR-F645A by individual EcR isoforms: qualitative assays

		Wild-type isoform		
Driver	Phenotype	A	B1	B2
Sgs3	Glue secretion	Partial rescue	Partial rescue	Partial rescue
	Puffing	Partial rescue	Partial rescue	Partial rescue
slbo	Border cell migration	No rescue	No rescue	Partial rescue
	Chorion fragility	No rescue	No rescue	Partial rescue
	Fertility	No rescue	No rescue	Partial rescue

Partial rescue of glue secretion means that in most pupae, fluorescent glue is visible in the glands, in the ducts and on the outside of the puparial case. Partial rescue of salivary gland puffing means that the normal puff progression occurs at a slower than normal pace; see text and Fig. 8 for fuller descriptions. Partial rescue of border cell migration, chorion fragility and fertility are described in the text.

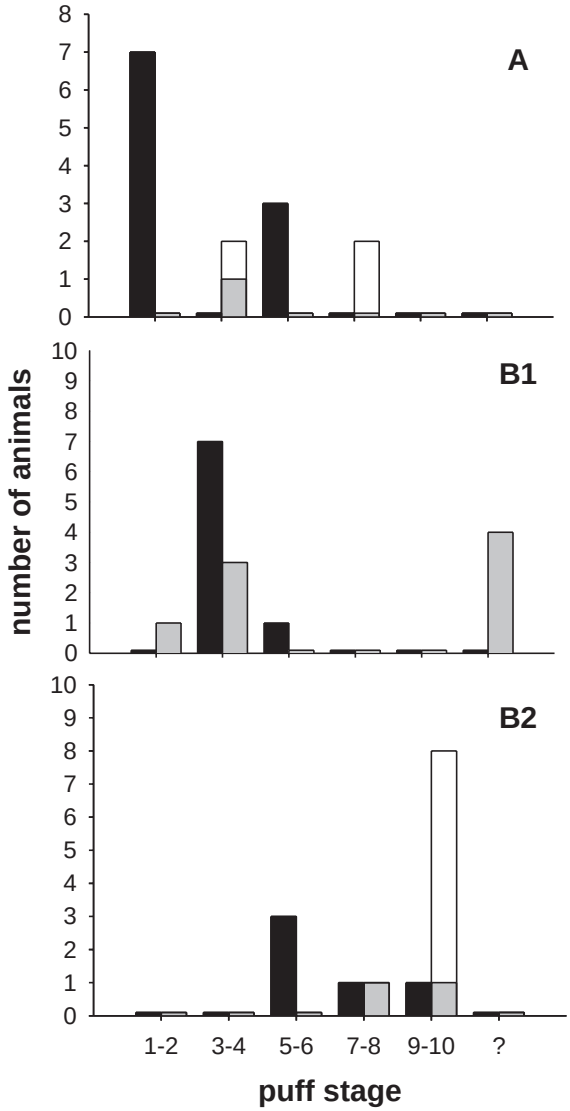


Fig. 6. Partial rescue of polytene puffing pattern by individual EcR isoforms. Each panel represents animals containing the *Sgs3* driver and responders for EcR-F645A and for a wild-type EcR of the indicated isoform. Salivary glands were isolated from post-wandering larvae (black bars) and white prepupae (gray bars), and their developmental stage was confirmed by salivary gland duct morphology (Zhimulev and Belyaeva, 1999). The glands were squashed and puff stages of the chromosomes determined. White extensions of the bars indicate animals selected as white prepupae in which the puffing pattern showed minor deviations from the indicated puff stage. ? indicates animals with a puffing pattern too aberrant to permit assignment of a puff stage. Salivary glands from wild-type post-wandering larvae have PS7-9 polytene chromosomes, and those from wild-type white prepupae have PS10-11 chromosomes.

opposite effects in the *dpp* domain. These results imply that A is the dominant isoform in the *GMR* and *dpp* domains, and are entirely consistent with our observations that all isoforms are capable of supporting normal development in the *GMR* and *dpp* domains (Fig. 5), and with a published report that isoform A is prevalent in both domains (Talbot et al., 1993).

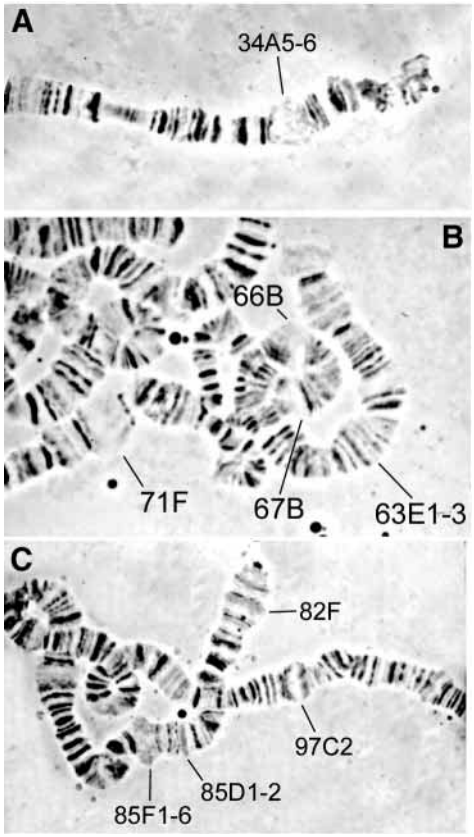


Fig. 7. Polytene chromosomes from a white prepupa bearing an *Sgs3* driver and responders for EcR-F645A and EcR-A. The three panels are different regions of a single chromosome spread. See text for a discussion of the marked sites.

Eip driver

Heterozygosity for EcR-B1 has a very modest effect, but heterozygosity for both B isoforms strongly enhances DN-induced lethality. Although isoform B1 is present at relatively high titer in the larval epidermis (Talbot et al., 1993), only isoform B2 is capable of supporting normal development (Fig. 7). The results suggest that EcR-B2 is the dominant isoform in this domain.

Lsp2 driver

Heterozygosity for loss of both B1+ B2, but not for B1 alone, enhances the EcR-DN effect. This is surprising, as isoform B1 is present at relatively high titer in the fat body (Talbot et al., 1993) and all isoforms are capable of supporting normal development (Fig. 7). These data suggest that EcR-B2 is also present in the fat body at high concentration. Thus, heterozygous loss of EcR-B1 causes at most a modest reduction in the level of functional receptor, while heterozygous loss of both B isoforms causes a severe reduction.

DISCUSSION

Dominant negative EcR mutations

We constructed EcR-F645A and EcR-W650A expecting that, like similar helix 12 mutant TRβ proteins (Collingwood et al.,

1997; Tagami et al., 1998), they would lack the transcriptional activation function AF2 and that they might disrupt signaling by competing with wild-type EcR. Both predictions were confirmed by assays in cultured cells (X. H., L. C. and P. C., unpublished), and the experiments presented here suggest that EcR-F645A expression can block ecdysone signaling in any *Drosophila* cell. We suspect that in vivo EcR-F645A dimerizes with USP and binds to ecdysone response elements, forming unliganded complexes that repress transcription normally, but cannot be converted into activating complexes by ligand (ecdysone). Although still unproven, this scheme conforms to our observations in cultured cells and in vitro and is consistent with the vertebrate precedents (Tagami et al., 1998). This mechanism would account for the interference of EcR-F645A with ecdysone-mediated transcriptional activation. It seems plausible that the mutant EcR also interferes with ecdysone-mediated transcriptional repression (Ashburner, 1973; Andres and Cherbas, 1992), but our experiments do not address this directly.

Our mutant EcRs retain the wild-type B1 N terminus, which has AF1 activity in the wild-type molecule (X. H., L. C. and P. C., unpublished). If AF1 in the mutant EcR mediated transcriptional activation, it would complicate the interpretation of our results. We are confident that this is not the case, for several reasons: (1) co-repressors block AF1 activity; (2) when tested in EcR-deficient cultured cells, under conditions where AF1 is functional in wild-type EcR-B1, EcR-F645A completely fails to activate transcription in response to ecdysone (X. H., L. C. and P. C., unpublished; L. C., unpublished); and (3) In flies, F645A and W650A proteins based on EcR-B2, EcR-A or EcR-C produce results that are qualitatively similar to those we have described here (L. C., unpublished).

Despite residual uncertainties about the molecular details, it is clear that the EcR-DNs block development in individual tissues much as one would expect if ecdysone action had been arrested (details below). Perhaps most telling is the abrogation of all hormone-induced puffing changes in the salivary gland polytene chromosomes. All the phenotypes we observe are associated with a reduction in the level of functional EcR/USP as they are strongly enhanced in a background of lowered wild-type EcR titer and strongly suppressed when extra wild-type EcR is supplied. Analysis in cultured cells (X. H., L. C. and P. C., unpublished) shows that these mutant EcRs interfere with wild-type EcR function only when they are present in substantial excess; we infer that this is the case in the targeted fly tissues. Crucial variables that affect the level of GAL4-mediated expression include temperature and the responder insertion site. In all of the experiments described here, we have used a single insertion of EcR-F645A in polytene division 23C. Alterations in the transposon insertion and in temperature cause variations in the severity of the phenotypes over a wide range (e.g. 0-100% escapers with the *GMR* and *dpp* drivers). This suggests that EcR-DN expression is not far above crucial levels and that the level of expression matters. We will return to these quantitative issues below.

EcR-DN phenotypes: a molting checkpoint

An unanticipated phenotypic consequence of targeted EcR-DN expression is the global block that we refer to as a ‘molting checkpoint’. A full description of this phenomenon awaits further studies, but a brief description is necessary here because it has profound effects on the EcR-DN phenotypes and on our ability to score them.

When EcR-DNs are expressed in some tissues, they cause both local phenotypes and a global effect: metamorphosis stops at the time of the next ecdysone-dependent event. This is most clearly illustrated by EcR-DN expression in the Ser domain; in this case, expression of the EcR-DN is restricted to the margins of the wing and leg discs, but development is blocked in the entire animal. We use the term ‘molting checkpoint’ to describe the global block. A similar phenomenon occurs in all other cases where EcR-DN is expressed in epidermal cells. The expression patterns of the Eip, dpp and GMR drivers are more complex, opening the possibility that the global block results from localized malfunction of crucial tissues such as neurosecretory cells or tracheae; nonetheless, the similarity of the lethal phenotypes makes it attractive to hypothesize that the molting checkpoint is a general consequence of EcR-DN expression in epidermal cells.

A few basic properties of the molting check-point are important here.

(1) Checkpoint arrest is enhanced when EcR levels are reduced and suppressed by rescue constructs; thus, it behaves as a downstream consequence of aborted receptor function.

(2) The global block to development is invoked by stimuli other than interference with ecdysone signaling. For example, expression of the cell death gene *reaper* in the GMR domain can produce a similar response (L. C., unpublished). If the molting checkpoint is triggered by some general aspect of defective cells the checkpoint is probably closely related to another phenomenon that has been known for many years, i.e. physical injuries, regeneration or genetic disc ablation delay molting (Hadorn, 1937; Madhavan and Schneiderman, 1969; Wigglesworth, 1970).

(3) Imaginal discs from arrested animals can be induced to develop by hormone treatment in vitro; we infer that metamorphosis stops because the ecdysone titer is insufficient (L. C., unpublished).

The temporal progression of the cell cycle must be coordinated and this coordination is achieved by a series of checkpoints responding to aberrant events. Similarly, the complex multicellular events of molting and metamorphosis must be coordinated, and we suggest that the style of this coordination is similar: Progress is delayed by a checkpoint invoked by aberrant development. The checkpoint captured our attention because it revealed itself as a global effect in experiments designed to test purely local phenotypes. It seems likely that the same phenomenon occurs in experiments where targeted expression is not involved; i.e. mutations with diverse, local effects may, because they invoke the checkpoint, reveal themselves by the common phenotype of late larval or pupal lethality. We cannot assess how common this may be.

The checkpoint is important here because it provides a simple quantitative measure of EcR-DN phenotypes. We have used the frequency of adult eclosion as a quantitative indicator of the localized effects of EcR-DN action in several domains. It is the only available assay for the Ser domain, where the checkpoint acts so efficiently that we have observed no escapers under any conditions, and it is a convenient assay in the *dpp* domain. Its validity is clear in the *GMR* domain, where the morphological defects in escapers are proportional to the

frequency of the block to pupation over a wide range of temperatures and responder insertions. We note that lethality caused by EcR-DN expression in the Lsp domain arises from a different mechanism; the animals die late in adult development rather than simply failing to molt.

Morphological phenotypes caused by targeted EcR-DN expression

For those drivers that yield viable, morphologically defective adults, targeted expression of EcR-DNs provides a new way to identify ecdysone-dependent developmental steps. Although we have not investigated any of the EcR-DN-induced morphological phenotypes in detail, our results do support several significant inferences.

In interpreting these data, it is important to consider that the EcR-DNs are effective only when present in large excess over the wild-type EcR (X. H., L. C. and P. C., unpublished); hence, it is unlikely that low-level expression of these drivers, below the level of detection of our reporter assays, plays a significant role in the observed phenotypes. This idea is supported by the fact that specific defects, when they are detected, are always restricted to the tissues in which the driver is known to be expressed.

GMR domain

A morphogenetic furrow traverses the eye disc during the third larval instar, leaving developmentally specified future retinal cells in its wake. GMR expression in the eye disc is confined to these post-furrow cells and precedes overt retinal differentiation, which continues during the pupal period (Ghbeish and McKeown, 2002). Retinal differentiation is known to be ecdysone dependent: In cultured discs of Drosophila (Li and Meinertzhagen, 1997) or Manduca (Champlin and Truman, 1998), hormone is required for the differentiation of both lenses and interommatidial bristles. Mosaic studies have shown that this differentiation requires EcR (Brennan et al., 2001) and USP (Ghbeish and McKeown, 2002). After EcR-DN expression, we observe only abortive lens development, irregular ommatidial arrays and, in most cases, considerable necrosis. The resulting adult eyes are readily distinguishable from those that result when of cell death genes like reaper are expressed from the same driver (L. C., unpublished); hence the phenotype is not simply a consequence of death of the affected cells. More detailed analysis shows an early block to the ordered cell death of excess interommatidial cells (R. Hays, personal communication) and subsequent massive cell death in the retina (K. Moses, personal communication). We infer that EcR/USP signaling is required for the apoptosis that is an essential part of the establishment of the ommatidial array (Rusconi et al., 2000), and we speculate that the subsequent necrosis is a secondary consequence of the disordered array. Though the GMR driver is expressed in a few other tissues, the excellent concordance between morphological rescue of the eyes and checkpoint rescue suggests that it is the defective retinal development that triggers the molting checkpoint.

slbo domain

A variety of evidence suggests that ecdysone plays a role in *Drosophila* oogenesis. Ecdysone signaling in both germline and follicle cells is required for egg chambers to pass stage 8. In particular, *EcR*$^-$ germline clones do not progress beyond stage 8 and stage-specific expression of certain ‘ecdysone hierarchy genes’ (E75, E74,and BR-C) is common to both germline and soma (Buszczak et al., 1999). Our results provide more direct evidence that EcR is, indeed, required in the follicle cells.

The *slbo* driver is expressed, beginning in stage 9 egg chambers, in three discrete groups of follicle cells: border cells, polar cells and centripetal cells (Rørth et al., 1998). At stage 9, the border cells begin to migrate between nurse cells, coming to rest at the anterior end of the oocyte, where they form the opening of the micropyle (Montell et al., 1992). That this migration is blocked by *slbo*-driven EcR-DN expression strongly supports the idea that it is one of the ecdysone-dependent steps associated with the transition described by Buszczak et al. (Buszczak et al., 1999). A role for ecdysone in border cell migration was suggested previously (Bai et al., 2000), because that process requires the p160 co-activator TAI, which can bind EcR in vitro.

In addition, *slbo*-driven EcR-DN expression causes obvious defects in the chorion that cannot be explained by the failure of border cell migration, i.e. defects that do not occur when migration is prevented by *slbo* mutation. They include both visible malformations of the operculum and the nearby dorsal appendages, and generalized chorion fragility. We infer that these chorion defects result from defective ecdysone signaling in the polar and/or centripetal follicle cells. Although EcR has not previously been implicated in choriogenesis, its heterodimer partner USP is known to be a chorion gene transcription factor (Shea et al., 1990), and is thought to function in follicle cells as a heterodimer with an unidentified partner (Christianson et al., 1992). Our data suggest that USP functions in choriogenesis as part of the ecdysone receptor, EcR/USP. It is interesting to note that the BR-C transcription factors, which play a crucial role in the ecdysone response in other tissues, are also known to be essential for the formation of dorsal appendages (Orr et al., 1989; Deng and Bownes, 1997; Tzolovsky et al., 1999).

Eip

Eip driver expression is complex; it is strongest in the larval epidermis and in scattered cells of the brain starting at the mid-instar transition. *Ddc*, a gene crucial for cuticle tanning, is known to be induced by ecdysone (Chen et al., 2002); thus, it is not surprising that *Eip*-driven EcR-DN expression prevents the tanning associated with pupariation (Fig. 5D). Failure of the larvae to contract into the normal puparial barrel was unexpected, as that change probably depends on underlying muscles that do not express the EcR-DN. We consider it most likely that the normal transformation requires both muscle contraction and shape changes in the epidermis, but we cannot eliminate the alternative possibility that competent epidermal cells signal the muscles to initiate the contraction.

Sgs3

The *Sgs3* driver becomes active exclusively in the salivary glands at the mid-third transition. At PS1′, when the development of the EcR-DN-expressing salivary gland stalls, all of the glue puffs, including 68C (the source of the promoter used in the Sgs3 driver), are visible but very small. Normally they become more active before their regression at pupariation.

Both the initial activation and the later repression of the glue genes are thought to be ecdysone dependent (Crowley and Meyerowitz, 1984; Hansson and Lambertsson, 1989). Thus, *Sgs3*-GAL4-driven EcR-DN expression provides an example of EcR-DN expression under the control of an ecdysone-dependent promoter. Using the puff at 68C as a guide, we conclude that both the glue genes and the mutant EcR are expressed at a low steady-state level.

At least three separate ecdysone-responsive events can be monitored in the salivary glands: the puffing sequence, glue secretion (Boyd and Ashburner, 1977; Biyasheva et al., 2001) and histolysis (Jiang et al., 1997). EcR-DN expression arrests the ecdysone-induced puffing sequence at the earliest stage known. It blocks glue secretion. But histolysis of the gland takes place normally in animals expressing EcR-DN from the Sgs3 driver. We attribute this to quantitative insufficiency of EcR-DN expression in this setting. Indeed, histolysis is blocked when EcR-F645A expression in the salivary glands is amplified and prolonged by the inclusion of a UAS-GAL4 transposon (B. Sell and L. C., unpublished).

Lsp

The *Lsp* driver is expressed exclusively in the fat body. Although several genes are known to be ecdysone responsive in the fat body (Lepesant et al., 1978; Brodu et al., 2001), the only obvious morphological correlate of fat body metamorphosis is its dissociation into single, unattached cells at pupation. This dissociation is prevented by *Lsp*-driven EcR-DN expression. The late pupal lethality we observe may be a downstream effect of the failure to dissociate. More likely it occurs because undescribed biochemical defects in the developmentally arrested fat body cells starve the developing adult tissues of nutrients. The gender difference in lethality may be due to differences in the nutritional requirements of the two sexes.

EH

The *EH* driver is expressed throughout development in two neurosecretory cells that are responsible for synthesis of the eclosion hormone. There has been no previous description of a role for ecdysone in the development of these cells; our experiments imply that they require the hormone at some stage for viability and/or development. Indeed, in animals with targeted EcR-DN expression no eclosion hormone-containing cells can be detected in late third-instar larvae (J. Truman, personal communication). Given this, it is not surprising that the EH-driven EcR-DN phenotype is indistinguishable from that observed when the eclosion-hormone-secreting cells are ablated (McNabb et al., 1997).

Identifying isoform requirements by targeted blockade and rescue

We have tested domain-specific isoform requirements by targeted blockade and rescue, using the GAL4 system to direct the expression of both an EcR-DN and a particular wild-type EcR isoform. We reasoned, from cell culture assays, that a wild-type isoform would out-compete the EcR-DN when both were expressed at comparable levels, and that this design would permit us to identify domains where AF1 is dispensable or a single AF1 is sufficient to support normal development.

Certain caveats are appropriate. First, we do not fully understand the competition that is taking place. Intuition suggests that over-expressing a single inappropriate wild-type isoform should interfere with signaling. In some cases it does so. Ubiquitous overexpression of EcR-B1 or B2 a few hours prior to pupariation (using a heat-shock promoter) leads to severe defects in pupation (M. Schubiger, S. Tomita, C. Sung, S. Robinow and J. W. Truman, unpublished), and ubiquitous overexpression of any single EcR isoform throughout development (using an actin5C driver) leads to early lethality (this paper). But most tissues, even those that display isoform-specific rescue of the EcR-DN phenotype, show no apparent developmental abnormality when any single isoform is expressed in the absence of EcR-DN. Though surprising, this result is consistent with observations from our laboratory and others: overexpression of EcR-A in Kc cells does not decrease ecdysone activation of an EcR-B1/B2-responsive promoter (L. C., unpublished). Expression of any single EcR isoform driven by a heat-shock promoter in otherwise wild-type pupae causes no major phenotypic effects (S. Robinow, personal communication). In the experiments reported here, highly expressed EcR-DNs can compete with endogenous EcRs under conditions where wild-type EcR isoforms fail to do so, and the presence of an EcR-DN sensitizes cells to ectopic expression of an inappropriate EcR isoform. As protein-protein interactions at target sites can control the exchange and shuttling of nuclear receptors (Baumann et al., 2001) perhaps an inability of EcR-DN to release co-repressors in response to ligand locks it into position. In addition, feedback effects of EcRs on expression of EcR isoforms from the endogenous EcR gene (M. Schubiger, S. Tomita, C. Sung, S. Robinow and J. W. Truman, unpublished) may reduce the effects of ectopic expression of an inappropriate EcR isoform.

Second, if specific isoform requirements are quantitative rather than qualitative, our experiments might underestimate the differences between domains. We think this unlikely because in a model cell culture system, the isoforms appear to have qualitatively different transcriptional activation capacities (X. H., L. C. and P. C., unpublished). Thus, when there is a requirement for particular isoforms it appears to be absolute rather than relative.

Third, because the rescuing isoforms are independent transgenes, they might be expressed at very different levels and their expression levels could be tissue specific. This could lead us to overestimate the differences in effectiveness of individual isoforms in any one domain and might lead us to overestimate differences between the requirements of different domains. Because of this uncertainty we are most impressed by the surprising result that the EcR isoforms are equivalent in numerous domains.

In the *GMR*, *dpp*, *Lsp2* and *EH* domains any EcR isoform will support metamorphosis, and, for at least the first three, so will EcR-C. This is even more impressive because two of the domains (*GMR* and *dpp*) include diverse cell types. In these four domains, ecdysone-induced transcriptional changes may be mediated by EcR-AF2, by release of EcR/USP-mediated inhibition, or by USP. There is structural evidence to suggest that USP may not be capable of activation (Clayton et al., 2001) and it does not contribute to activation in our cell culture model system (X. H., L. C. and P. C., unpublished), but the first two possibilities are entirely plausible and cannot be distinguished by the experiments described here.

We have also observed the contrary result. In the *Ser* domain, only EcR-A gives full rescue, and in the *Eip* and *slbo* domains only EcR-B2 is effective. Although these effects may be exaggerated by differences in the levels of expression of the responders we used, the clear-cut nature of the differences suggest that they are real. If our interpretation is correct, then in each of these domains at least one crucial promoter requires an isoform-specific EcR AF1.

Salivary gland puffing might be expected to reveal gene-specific isoform requirements with individual isoforms giving different uncoordinated responses. Instead, we can characterize the rescued puffing response, at least during its pre-puparial stages, by a single parameter: its rate. Each of the three isoforms supports the normal, coordinate response. We think it simplest to suppose that the AF1s play no role and that the puffing response is simply a sensitive reporter of the expression levels for the three rescuing transgenes. By contrast, earlier studies using heat shock pulses of single isoforms in an EcR-null background, controlled approximately for protein levels, observed the normal pattern rescued by B1>B2>>>A (Bender et al., 1997). Plainly, it will require a more sophisticated experiment to determine with confidence whether the isoforms differ in their ability to support the puffing pathway.

At pupariation, the rescued puffing responses become uncoordinated. This probably reflects the superimposition of the still-in-progress early response with new gene activities induced by the declining ecdysone titer at pupariation (Richards, 1976).

The results reported here must be put into the context of previous work on the tissue-specific properties of the EcR isoforms. Using different approaches to local rescue, others have demonstrated that isoforms B1 and B2 (but not A) can support the remodeling of mushroom-body γ neurons (Lee et al., 2000) and of the SCP-staining neurons (Schubiger et al., 1998; M. Schubiger, S. Tomita, C. Sung, S. Robinow and J. W. Truman, unpublished). When *EcR* mutations are examined at the level of the whole organism, the effects tend to be widespread. Thus, common region EcR nulls are early embryonic lethals, and EcR-B1 nulls are nonpupariating lethals with defects in the leg discs, the imaginal cells of the midgut islands, the larval gut and the histoblasts (Bender et al., 1997). Animals null for both EcR-B1 and -B2 are early larval lethals (Schubiger et al., 1998). The widespread nature of the defects in these mutant animals makes it impossible to judge the localized requirements for EcR isoforms.

In addition to testing the capacity of individual isoforms to support development in diverse tissues, our experiments contribute to the catalog of isoform distribution in those tissues. The distribution of isoforms is complex both in time and space (Truman et al., 1994; Jindra et al., 1996). Previous experiments used immunostaining to examine the relative levels of isoforms A and B1 in late third-instar tissues (Talbot et al., 1993) and in the CNS (Robinow et al., 1993; Truman et al., 1994). Our measurements of the effects of isoform-specific mutations on EcR-DN phenotypes contribute an estimate of the contribution of individual endogenous isoforms in the particular times and places in which each driver is expressed. These data imply major roles for isoform B2 in the larval fat body and epidermis. In addition, only isoform B2 can rescue the EcR-DN effects in the *slbo* domain, suggesting that it may also be the major isoform in the follicle cells.

We began this paper by posing two alternative models for the distribution of AF1-specific promoters. According to one, individual domains should exhibit specific isoform requirements that can be predicted by their isoform contents. Our results lend no support to this idea. We do not know why isoform titers vary, but they do not appear to be good predictors of isoform requirements. Instead, our results – and the previous results described above – are consistent with the following picture: A small minority of promoters require specific isoforms. As each responding tissue may contain several (or many) critical promoters, specific AF1 requirements are not limited to a small minority of tissues. Still, it is remarkable that tissues lacking even one such critical promoter are not rare. In those tissues – about half of our sample – AF1 is dispensable, and ecdysone effects are mediated by AF2 or by relief of repression. In some cells, at least one critical promoter does require a specific AF1. When many tissues lack a specific isoform, developmental defects are likely and their phenotypes depend on both the missing isoform and the intervention of the molting checkpoint.

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