

Spatial patterns of ecdysteroid receptor activation during the onset of *Drosophila* metamorphosis

Tatiana Kozlova and Carl S. Thummel*

Howard Hughes Medical Institute, Department of Human Genetics, University of Utah, 15 North 2030 East Room 5100, Salt Lake City, UT 84112-5331, USA

*Author for correspondence (e-mail: carl.thummel@genetics.utah.edu)

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SUMMARY

Ecdysteroid signaling in insects is transduced by a heterodimer of the EcR and USP nuclear receptors. In order to monitor the temporal and spatial patterns of ecdysteroid signaling in vivo we established transgenic animals that express a fusion of the GAL4 DNA binding domain and the ligand binding domain (LBD) of EcR or USP, combined with a GAL4-dependent *lacZ* reporter gene. The patterns of β -galactosidase expression in these animals indicate where and when the GAL4-LBD fusion protein has been activated by its ligand in vivo. We show that the patterns of GAL4-EcR and GAL4-USP activation at the onset of metamorphosis reflect what would be predicted for ecdysteroid activation of the EcR/USP heterodimer. No activation is seen in mid-third instar larvae when the ecdysteroid titer is low, and strong widespread activation is observed at the end of the instar when the ecdysteroid titer is high. In addition, both GAL4-EcR and GAL4-USP are activated in larval organs cultured with 20-hydroxyecdysone (20E), consistent with EcR/USP

acting as a 20E receptor. We also show that GAL4-USP activation depends on *EcR*, suggesting that USP requires its heterodimer partner to function as an activator in vivo. Interestingly, we observe no GAL4-LBD activation in the imaginal discs and ring glands of late third instar larvae. Addition of 20E to cultured mid-third instar imaginal discs results in GAL4-USP activation, but this response is not seen in imaginal discs cultured from late third instar larvae, suggesting that EcR/USP loses its ability to function as an efficient activator in this tissue. We conclude that EcR/USP activation by the systemic ecdysteroid signal may be spatially restricted in vivo. Finally, we show that GAL4-EcR functions as a potent and specific dominant negative at the onset of metamorphosis, providing a new tool for characterizing ecdysteroid signaling pathways during development.

Key words: Nuclear receptor, Metamorphosis, Ecdysteroid, Gene regulation, Steroid signaling, *Drosophila*

INTRODUCTION

Ecdysteroids play a central role in the *Drosophila* life cycle, directing each of the major postembryonic developmental transitions. Ecdysteroid pulses during the first and second larval instars trigger molting of the cuticle, accommodating the dramatic growth that occurs during this phase in the life cycle. A high titer ecdysteroid pulse at the end of the third instar triggers a different response, signaling puparium formation and the onset of metamorphosis. Obsolete larval tissues, including the midgut, muscles and salivary gland, undergo stage-specific programmed cell death during the early stages of metamorphosis while adult structures develop from clusters of progenitor cells and the imaginal discs. The net effect of these divergent ecdysteroid-triggered developmental pathways is the transformation of the basic body plan of the insect, from a crawling larva to a highly mobile and reproductively active adult fly.

The *Drosophila* larval endocrine organ, the ring gland, can synthesize several ecdysteroids, predominantly ecdysone and 20-deoxymakisterone A (reviewed in Riddiford, 1993; Gilbert

et al., 1996). These compounds are considered to be relatively inactive precursors, although ecdysone has been shown to be required for neuroblast proliferation during early pupal development in *Manduca* (Champlin and Truman, 1998). After their release from the ring gland, these precursors are converted by peripheral tissues into more biologically active ecdysteroids, with ecdysone converted into 20-hydroxyecdysone (20E), the major molting hormone in *Drosophila*. Many ecdysteroid precursors and metabolites, however, are present in the hemolymph during each ecdysteroid pulse, and their biological functions, if any, remain unknown (Pak and Gilbert, 1987; Gilbert et al., 1996).

Steroid hormones exert their effects on target tissues by activating ligand-dependent transcription factors that are members of the nuclear receptor superfamily. These receptors are characterized by a highly conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD) that is responsible for hormone binding, dimerization and ligand-dependent transcriptional activation. Nuclear receptors can also contain a highly divergent, ligand-independent transcriptional activation domain at their N terminus, designated the A/B domain.

Several vertebrate nuclear receptors function as heterodimers with the retinoid X receptor (RXR), binding to canonical response elements in the DNA that are arranged as either direct or inverted repeats (reviewed in Mangelsdorf and Evans, 1995).

Similar to the heterodimeric class of vertebrate nuclear receptors, the *Drosophila* ecdysteroid receptor EcR (NR1H1) acts as a heterodimer with the fly RXR homologue, USP (Ultraspiracle, NR2B4) (Koelle, 1992; Yao et al., 1992; Thomas et al., 1993; Yao et al., 1993). Unlike vertebrate nuclear receptors, however, neither EcR nor USP show significant ligand binding without their heterodimer partner. USP is expressed widely during development and is present in all tissues analyzed at the onset of metamorphosis (Henrich et al., 1994). *EcR* encodes three protein isoforms, EcR-A, EcR-B1 and EcR-B2, each with distinct N-terminal A/B domains and an identical C-terminal DBD and LBD (Koelle et al., 1991; Talbot et al., 1993). All three EcR isoforms are able to interact with USP and all can bind ecdysteroids with similar affinity (Koelle, 1992; Yao et al., 1993). EcR is expressed widely throughout development, although EcR-A and EcR-B1 isoforms are present in distinct and largely complimentary patterns at the onset of metamorphosis (Talbot et al., 1993; Truman et al., 1994). Extensive studies have demonstrated that the hormone-bound EcR/USP complex exerts its effects on development by triggering transcriptional cascades of primary- and secondary-response genes, first visualized as changes in the puffing patterns of the giant larval salivary gland polytene chromosomes (reviewed in Russell and Ashburner, 1996; Thummel, 1996). Characterization of *EcR* and *usp* mutants have revealed similar lethal phenotypes, including defects in the destruction of larval tissues and adult tissue morphogenesis during metamorphosis, supporting the conclusion that these factors act together as a heterodimeric ecdysteroid receptor *in vivo* (Bender et al., 1997; Hall and Thummel, 1998; Schubiger et al., 1998; Li and Bender, 2000).

In contrast to our understanding of the transcriptional responses to ecdysteroids, the temporal and spatial specificity of hormone signaling remains poorly understood. For example, it is unclear how the systemic ecdysteroid signal is refined to direct precise spatially restricted biological responses at the onset of metamorphosis. One level at which this specificity could be conferred is through the tissue-specific patterns of EcR isoform expression (Talbot et al., 1993; Truman et al., 1994). Genetic studies have provided some support for this model (Bender et al., 1997; Schubiger et al., 1998); however, there is no strict correlation between the patterns of EcR isoform expression and the different biological responses that are regulated by this receptor.

Temporal and spatial specificity of ecdysteroid signaling may also be modulated through combinatorial interactions between EcR/USP and other *Drosophila* nuclear receptor superfamily members (reviewed in Thummel, 1995). The *Drosophila* NGFI-B homolog DHR38 can heterodimerize with USP, disrupting the EcR/USP interaction and downregulating ecdysteroid-dependent transcription in transfected tissue culture cells (Sutherland et al., 1995). Similarly, EcR can interact with DHR3 in a yeast two-hybrid assay, and both DHR3 and Seven-up can downregulate ecdysteroid-dependent transcription in transfected tissue culture cells (Zelhof et al., 1995; White et al., 1997). USP can bind to some response elements as a homodimer, similar to vertebrate RXR, raising

the possibility that it may be able to act in an EcR-independent manner (D'Avino et al., 1995; Ghbeish et al., 2001).

EcR/USP function may be also modulated by interactions with coactivators and corepressors. Taiman is a transcriptional coactivator for EcR/USP that is required for border cell migration during *Drosophila* oogenesis (Bai et al., 2000). The TIF1 homolog, Bonus, is required for multiple ecdysteroid responses during the life cycle, and can interact with several *Drosophila* nuclear receptors *in vitro*, including EcR and USP (Beckstead et al., 2001). Two corepressors, Alien and SMRTER, can associate with the unliganded EcR/USP heterodimer and mediate repression in tissue culture transfection assays (Dressel et al., 1999; Tsai et al., 1999). Finally, components of the chaperone heterocomplex are critical for activation of the EcR/USP heterodimer both *in vitro* and *in vivo* (Arbeitman and Hogness, 2000). It seems likely that combinatorial interactions between all of these factors will contribute to the stage- and tissue-specificity of ecdysteroid signaling during development.

Understanding the molecular mechanisms by which a systemic hormonal signal is refined into distinct biological responses requires characterization of the temporal and spatial patterns of nuclear receptor activation during development. A method to achieve this goal was described recently (Solomin et al., 1998) as part of an effort to characterize the patterns of retinoic acid signaling in the mouse central nervous system (CNS). This method involves the establishment of transgenic animals that express the yeast GAL4 DNA binding domain fused to a nuclear receptor LBD, combined with a second transgenic construct that carries a GAL4-dependent promoter driving a *lacZ* reporter gene. The temporal and spatial pattern of β -galactosidase expression in these transgenic animals indicates where and when the LBD has been activated by its ligand, providing a direct means of following the patterns of hormone signaling in the context of a developing organism.

In this paper, we report the adaptation of this system for its use in *Drosophila*, allowing us to follow the patterns of EcR/USP activation at the onset of metamorphosis. We show that this system fulfills the criteria we would expect for an accurate readout of ecdysteroid signaling transduced by the EcR/USP heterodimer. The GAL4-LBD system provides a new direction for defining the molecular mechanisms by which temporal and spatial specificity is conferred in response to ecdysteroid pulses during development.

MATERIALS AND METHODS

Drosophila stocks

hs-GAL4 (P_{w⁺mC=GAL4-Hsp70.PB}89-2-1, stock 1799) and a *UAS-nlacZ* reporter on the third chromosome (P_{w⁺mC=UAS-NZ}J312, stock 3956) were obtained from the Bloomington Stock Center. A *UAS-nlacZ* reporter on the third chromosome, 7.4, was kindly provided by Dr Y. N. Jan. *EcR^{A483T}*, *EcR^{M554fs}*, and the *hs-EcR-B1* stocks were gifts from Dr M. Bender.}}

Generation of *hs-GAL4-LBD* transgenic fly lines

DNA encoding the yeast GAL4 DNA-binding domain (amino acids 1-147) was amplified by PCR from pCMX-GAL4 (a gift from K. Baker and D. Mangelsdorf) using primers flanked by *MfeI* sites (5'-atcaattgtaatacagcactactataggaga and 5'-tacaattgtccttcacaaagatcctcta). A *BamHI* site in the pCMX-GAL4 polylinker, downstream from the GAL4 sequences, was included in the resultant PCR fragment. The

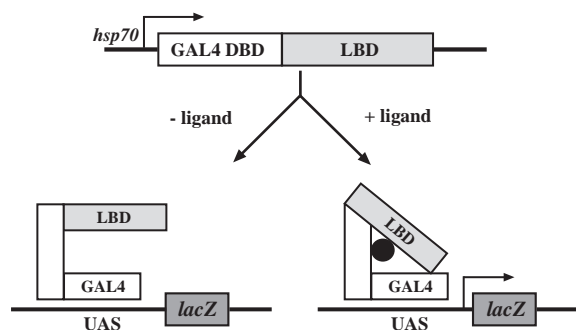


Fig. 1. Schematic representation of the GAL4-LBD system. The upper diagram depicts a fusion gene consisting of the coding region for the yeast GAL4 DNA-binding domain (GAL4 DBD) fused in-frame with the ligand binding domain (LBD) of EcR or USP. This construct is introduced into the *Drosophila* genome under the control of the *hsp70* promoter. Heat-induced GAL4-LBD protein can bind to GAL4 upstream activating sequences (UAS) that are present in a second transgenic construct. In the absence of ligand, the GAL4-LBD protein does not activate *lacZ* reporter gene transcription; however, in the presence of its ligand (black circle) it can switch into an active conformation and induce *lacZ* expression.

region encoding the GAL4 DNA binding domain was excised from this PCR fragment by digestion with *MfeI* and *BamHI*, and this fragment was inserted between the *EcoRI* and *BamHI* sites of the pCaSpeR-hs-act P element transformation vector (Thummel and Pirrotta, 1992). GAL4 sequences in the resulting pCaSpeR-hs-GAL4act construct were verified by DNA sequencing. Two restriction sites, *EcoRI* and *BamHI*, are present in this vector for insertion of sequences encoding nuclear receptor LBDs. DNA encoding the USP LBD and adjoining hinge region (amino acids 170-508) (Oro et al., 1990) was excised from pCMXGAL4-USP (a gift from K. Baker and D. Mangelsdorf) by digestion with *EcoRI* and inserted into the *EcoRI* site of pCaSpeR-hs-GAL4act. DNA encoding the EcR LBD, hinge region and F-domain (amino acids 330-878) (Koelle et al., 1991) was excised from pCMXGAL4-EcR (a gift from K. Baker and D. Mangelsdorf) by digestion with *EcoRI* and *BamHI* and inserted into the corresponding restriction sites of pCaSpeR-hs-GAL4act. The resulting junctions between GAL4 and nuclear receptor sequences were verified by DNA sequence analysis. Each P element construct was introduced into the germline of *w¹¹¹⁸* flies by standard transformation procedures. Two independent homozygous viable transgenic lines were isolated carrying *hs-GAL4-EcR* on the second or third chromosome. Both transgenic lines were used interchangeably in this study. A single homozygous viable *hs-GAL4-USP* insertion was isolated on the third chromosome and was used for all studies reported here. This P element was mobilized using standard techniques (Grigliatti, 1998) and an additional homozygous viable line on the third chromosome with darker eye color was selected and used as the *hs-USP-2* line shown in Fig. 8.

Developmental staging and heat induction of GAL4-LBD fusion proteins

Hs-GAL4-EcR; *UAS-nlacZ* and *hs-GAL4-USP*; *UAS-nlacZ* third instar larvae were staged on food containing 0.5% Bromophenol Blue as described (Andres and Thummel, 1994). All heat treatments were performed by incubating plastic culture vials containing food in a 37°C water bath for 30 minutes. For inducing GAL4-LBD fusions in mid-third instar larvae, animals were maintained on blue food, heat treated, allowed to recover for 6-7 hours at 25°C and selected as dark-blue-gut animals. For inducing GAL4-LBD fusion proteins at the onset of metamorphosis, partial-blue-gut and white-gut third instar larvae were transferred to vials with regular food, heat treated, and allowed to recover at 25°C. Animals that formed white prepupae between 3-4 hours after

heat treatment were selected from this population and allowed to age for an additional 2-3 hours. For analysis of GAL4-USP activation in an *EcR* mutant background, the cross between *yw*; *EcR^{M554fs}/CyO*, *y⁺*; *hs-GAL4-USP/hs-GAL4-USP* females and *yw*; *EcR^{A483T}/CyO*, *y⁺*; *UAS-nlacZ/UAS-nlacZ* males was shifted to a non-permissive temperature of 29°C for 24 hours. *yw*; *EcR^{A483T}/EcR^{M554fs}*; *hs-GAL4-USP/UAS-nlacZ* (*EcR ts*) third instar larvae were distinguished from control siblings (*yw*; *EcR^{A483T}/CyO*, *y⁺*; *hs-GAL4-USP/UAS-nlacZ* or *yw*; *EcR^{M554fs}/CyO*, *y⁺*; *hs-GAL4-USP/UAS-nlacZ*) using the *y⁺* marker. *EcR ts y⁺* larvae and *y⁺* controls were transferred to separate vials, heat treated and staged in parallel as described above.

Histochemical detection of β -galactosidase and immunostaining

β -galactosidase was detected by staining dissected larval tissues with X-gal as described (Kozlova et al., 1998). X-gal stains of larval tissues fixed with 4% formaldehyde (Polysciences) were allowed to develop overnight, while tissues fixed with 1% glutaraldehyde (Sigma) were allowed to develop for several hours. Because of the relatively long staining time, even a low level of background β -galactosidase expression would complicate the interpretation of our results. Consequently, we assayed several independent *UAS-nlacZ* reporter lines at the third larval instar for their basal level of β -galactosidase expression. We found that the *UAS-nlacZ* reporter P{*w⁺mtC=UAS-NZ*}J312 has the lowest level of background expression at this stage in development, with detectable β -galactosidase in the larval salivary glands, cells surrounding the larval mouthhooks, and a few cells in the CNS. This *UAS-nlacZ* reporter was used in all studies reported here. Another reporter line, 7.4, a homozygous viable *UAS-nlacZ* insertion on the third chromosome, gives high background staining in the larval salivary glands and the epidermis of third instar larvae. This stock was used to confirm results obtained with the J312 *UAS-nlacZ* reporter. Attempts to use *UAS-GFP* reporters were unsuccessful due to their reduced sensitivity.

Conditions for optimal detection of β -galactosidase from the *UAS-nlacZ* reporter were established using an *hs-GAL4* driver. *Hs-GAL4*; *UAS-nlacZ* late third instar larvae were heat treated for 30 minutes, allowed to recover for 4 and 6 hours at 25°C and processed for histochemical staining.

Expression of the GAL4-LBD fusion proteins was determined by heat treating *hs-GAL4-EcR* and *hs-GAL4-USP* late third instar larvae for 30 minutes at 37°C. After 4 hours of recovery at 25°C, these animals were processed for immunostainings with anti-GAL4 polyclonal antibodies (Santa Cruz Biotechnology, Inc) at 1:100 dilution using standard protocols (Patel, 1994). GAL4-EcR and GAL4-USP fusion proteins were detected in both the nucleus and cytoplasm and were at comparable levels in all tissues examined. Immunostainings with a 1:10 dilution of the anti-USP monoclonal antibody AB11 were performed as described above.

Larval organ culture

Third instar larvae reared on blue food were heat treated, then allowed to recover for 2-3 hours at 25°C, and staged animals were dissected in oxygenated Grace's Insect Medium (BRL). Dissected larval organs from 4-5 animals per sample were cultured in 300 μ l of oxygenated Grace's medium using indicated concentrations of 20E (Sigma), essentially as described (Andres and Thummel, 1994). Samples were processed for histochemical staining after 12 hours in culture.

RESULTS

GAL4-EcR and GAL4-USP are activated by the late larval ecdysteroid pulse

In order to monitor the patterns of EcR and USP activation during *Drosophila* development two transgenic constructs were introduced into the fly genome. These constructs carry

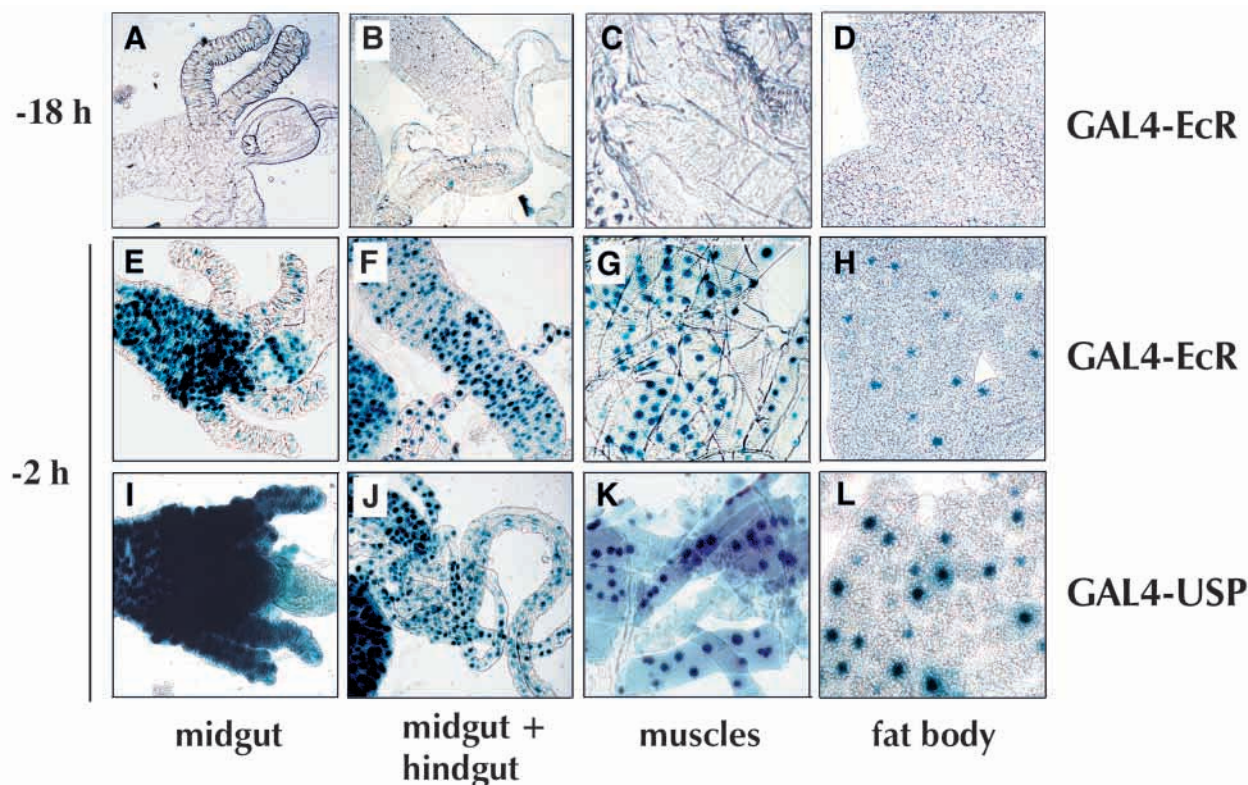


Fig. 2. GAL4-EcR and GAL4-USP are widely activated by the late larval ecdysteroid pulse. *hs-GAL4-EcR; UAS-nlacZ* (GAL4-EcR) or *hs-GAL4-USP; UAS-nlacZ* (GAL4-USP) third instar larvae were heat treated, allowed to recover for several hours, staged at either 12–18 hours before puparium formation (–18 h; A–D) or approx. 2 hours before puparium formation (–2 h; E–L), and processed for histochemical staining. Blue precipitate indicates expression of nuclear β -galactosidase. GAL4-EcR (A–D) and GAL4-USP (data not shown) are not activated in third instar larvae 12–18 hours before puparium formation. In contrast, both GAL4-EcR (E–H) and GAL4-USP (I–L) are widely activated in late third instar larvae, as depicted in the midgut, hindgut, muscles and fat body.

the *hsp70* heat-inducible promoter upstream from the coding region for the yeast GAL4 DNA binding domain, fused to the coding region for either the EcR or USP LBD (Fig. 1). These *hs-GAL4-EcR* and *hs-GAL4-USP* transformants were crossed with flies that carry a GAL4-dependent promoter driving a *lacZ* reporter gene that expresses nuclear β -galactosidase (*UAS-nlacZ*). Expression of β -galactosidase was detected by histochemical staining using X-gal as a substrate. The *hsp70* promoter was selected in order to provide precise temporal control, reducing potential lethality that might be caused by overexpression of the GAL4-LBD fusion proteins. In addition, the *hsp70* promoter should direct widespread expression of the GAL4-LBD proteins upon heat induction. Transcriptional activation by these fusion proteins, however, should only occur at times and in places where the appropriate hormonal ligand is present (Fig. 1).

In order to establish appropriate parameters for *UAS-nlacZ* induction we used a *hs-GAL4* driver, expressing the full-length GAL4 transcriptional activator under the control of the *hsp70* promoter. Expression of β -galactosidase was first detected 4 hours after a 30 minute heat treatment in late third instar larvae of this genotype. This activation was strong in all analyzed tissues by 6 hours after heat induction, making this an optimal time to assay reporter expression (data not shown). Immunostaining with an anti-GAL4 antibody demonstrated that the GAL4-EcR and GAL4-USP fusion proteins are expressed after a single heat treatment of *hs-GAL4-EcR* and *hs-*

GAL4-USP late third instar larvae, as expected. The fusion proteins are present at comparable levels in all tissues examined, including the midgut, muscles, fat body, imaginal discs and ring gland (data not shown).

If activation of GAL4-EcR and GAL4-USP is controlled exclusively by ecdysteroids, then we would expect no activation when the ecdysteroid titer is low and strong widespread activation coincident with ecdysteroid pulses. These conditions are fulfilled during the third larval instar in *Drosophila*, when low ecdysteroid levels are present in mid-third instar larvae, followed by the high-titer late larval ecdysteroid pulse that triggers puparium formation and the onset of metamorphosis. We, therefore, selected these two stages to test the ability of the GAL4-EcR and GAL4-USP constructs to faithfully reflect ecdysteroid signaling *in vivo*.

Third instar *hs-GAL4-EcR; UAS-nlacZ* and *hs-GAL4-USP; UAS-nlacZ* larvae were subjected to a 30 minute heat treatment and allowed to recover for 6–7 hours, after which animals staged at 12–18 hours before puparium formation were selected and stained for β -galactosidase expression. No GAL4-EcR activation was detected in tissues from these animals, including the gut, muscles, fat body, imaginal discs and ring glands (Fig. 2A–D and data not shown). The only staining detectable, in the salivary glands and cells surrounding the larval mouthhooks, was also detected in larvae carrying only the *UAS-nlacZ* reporter (data not shown). This background expression was

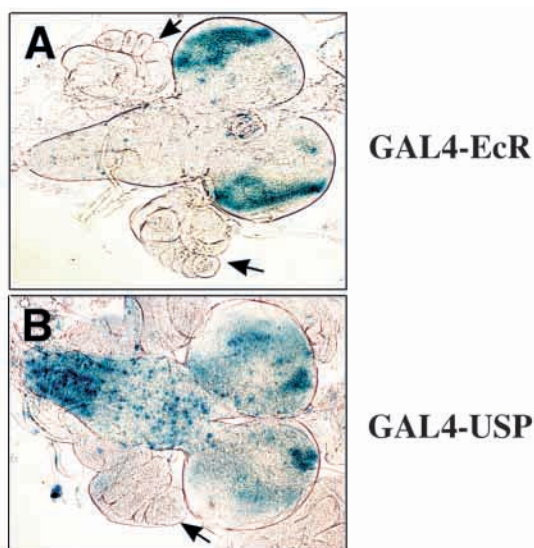


Fig. 3. Distinct patterns of GAL4-EcR and GAL4-USP activation in the central nervous system at the onset of metamorphosis. *hs-GAL4-EcR; UAS-nlacZ* (GAL4-EcR) or *hs-GAL4-USP; UAS-nlacZ* (GAL4-USP) third instar larvae were heat treated, allowed to recover for several hours, staged at approx. 2 hours before puparium formation, and processed for histochemical staining. The CNS and attached leg imaginal discs are shown, with anterior to the right. (A) GAL4-EcR activation is prominent in the optic lobes of the brain. (B) GAL4-USP activation is weaker in this region, but strong in the ventral nerve cord. Neither fusion construct is activated in the leg imaginal discs at this stage (arrows).

disregarded in future experiments. A similar lack of activation was observed with the *hs-GAL4-USP* transformant line (data not shown). Therefore, both GAL4-EcR and GAL4-USP are not activated in third instar larvae at 12–18 hours before puparium formation, consistent with the low ecdysteroid titer at this stage in development.

In contrast, a different pattern emerged when *hs-GAL4-EcR; UAS-nlacZ* and *hs-GAL4-USP; UAS-nlacZ* transformants were analyzed at the onset of metamorphosis. Activation was readily detected in most larval tissues at approximately 2 hours before puparium formation, including the midgut, hindgut, Malpighian tubules, muscles and fat body (Fig. 2E–H for GAL4-EcR and Fig. 2I–L for GAL4-USP). We also detected strong activation of both fusion proteins in larval oenocytes, which have been proposed to be a source of 20E in other insects (Redfern, 1989), as well as above the background of the reporter line in larval salivary glands (data not shown). Therefore, both GAL4-EcR and GAL4-USP are widely activated by the late larval ecdysteroid pulse that triggers the onset of metamorphosis.

It is possible to restrict ecdysteroid signaling to the anterior part of *Drosophila* larvae by using ligature to separate the ring gland from posterior tissues (reviewed in Russell and Ashburner, 1996). When activation of GAL4-EcR and GAL4-USP was assayed in such ligated animals at the onset of metamorphosis, strong widespread β -galactosidase expression was observed anterior to the site of ligature. No activation was detected posterior to the ligature, consistent with direct endocrine activation of the fusion proteins (data not shown).

Tissue-specific patterns of GAL4-EcR and GAL4-USP activation at the onset of metamorphosis

In contrast to the widespread and overlapping patterns of GAL4-EcR and GAL4-USP activation seen in late third instar larval tissues, distinct and spatially restricted patterns of activation were seen in the CNS of these animals (Fig. 3). GAL4-EcR is strongly activated in a medial region of the optic lobes (Fig. 3A), whereas GAL4-USP activation is very weak in this region of the brain and strong in clusters of cells at the anterior end of the optic lobes as well as the posterior half of the ventral nerve cord (Fig. 3B). These activation patterns appear to be dynamic, although the patterns shown in Fig. 3 were observed consistently in animals staged between 3 and 0 hours before puparium formation.

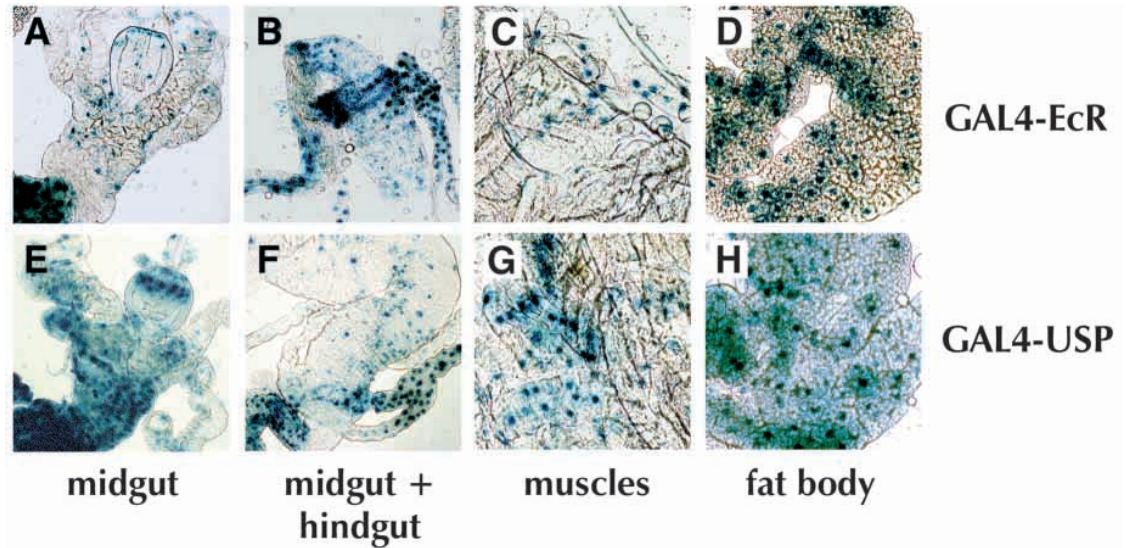
A few tissues also displayed weak or no activation of GAL4-EcR and GAL4-USP at the onset of metamorphosis, including the ring gland (data not shown) and the imaginal discs (arrows, Fig. 3). This observation was unexpected because ecdysteroids are thought to be a systemic signal that activates EcR/USP throughout the animal. This lack of activation cannot be attributed to a lack of endogenous receptor subunits because both EcR and USP are expressed in the ring gland and imaginal discs at the onset of metamorphosis (Talbot et al., 1993; Henrich et al., 1994). The EcR isoform that predominates in imaginal discs, however, is EcR-A, which appears to be a less potent transcriptional activator than EcR-B1 in tissue culture transfection assays (Hu, 1998; Mouillet et al., 2001). We therefore asked if overexpression of EcR-B1 from an *hsp70* promoter could restore GAL4-USP activation in imaginal discs in vivo. No activation, however, was detected in the imaginal discs of these animals at the onset of metamorphosis (data not shown). We thus conclude that, unlike most tissues of a late third instar larva, GAL4-EcR and GAL4-USP are not activated in the ring gland and imaginal discs at this stage in development.

GAL4-EcR and GAL4-USP are widely activated by 20-hydroxyecdysone

A prediction of this system is that GAL4-EcR and GAL4-USP should be activated by the addition of exogenous 20E to cultured larval organs. To test this hypothesis, *hs-GAL4-EcR; UAS-nlacZ* and *hs-GAL4-USP; UAS-nlacZ* third instar larvae were heat treated and selected at 8–18 hours before puparium formation, when the ecdysteroid titer is low and no activation is seen (Fig. 2A–D). Organs dissected from these animals were cultured with a physiological range of 20E concentrations and β -galactosidase expression was monitored by X-gal staining. In the presence of 5×10^{-6} M 20E, widespread activation was seen for both GAL4-EcR (Fig. 4A–D) and GAL4-USP (Fig. 4E–H). Larval organs cultured in parallel in the absence of steroid show no activation of the GAL4-LBD fusions (data not shown, same as Fig. 2A–D). We used 5×10^{-8} , 5×10^{-7} , 5×10^{-6} and 5×10^{-5} M 20E in culture experiments and found that activation was easily detectable at all of these hormone concentrations (data not shown). The activation observed in culture, even with the highest 20E concentrations, however, is less robust than that detected in response to the endogenous late larval pulse of ecdysteroids (Fig. 2E–L). It is likely that this effect is due to the stress inherent in maintaining larval organs in culture, although it may also reflect the state of competence of individual tissues.

Notably, when organs from third instar larvae at 8–18 hours

Fig. 4. GAL4-EcR and GAL4-USP are widely activated by 20E in cultured larval organs. Larval organs dissected from heat treated *hs-GAL4-EcR*; *UAS-nlacZ* (GAL4-EcR) or *hs-GAL4-USP*; *UAS-nlacZ* (GAL4-USP) third instar larvae 18-8 hours before puparium formation were cultured with 5×10^{-6} M 20E and processed for histochemical staining. GAL4-EcR and GAL4-USP are not activated in culture in the absence of 20E (data not shown). Both GAL4-EcR (A-D) and GAL4-USP (E-H) are widely activated by 20E in many larval tissues, including the midgut, hindgut, muscles and fat body.



before puparium formation are cultured with exogenous 20E, activation can be detected in tissues where it was not observed in vivo. In particular, GAL4-EcR and GAL4-USP activation is detectable in both ring glands (Fig. 5B,C) and imaginal discs (Fig. 5E,F) cultured in the presence of 5×10^{-6} M 20E but not without the steroid (Fig. 5A,D). The observation that GAL4-LBD activation can be detected after hormone addition in ring glands and imaginal discs demonstrates that the GAL4-LBD system is functional in these tissues. Interestingly, however, GAL4-EcR and GAL4-USP lose competence to be activated by 20E when imaginal discs are dissected from animals at later stages of development. Thus, whereas GAL4-USP can be activated by 5×10^{-7} M 20E in imaginal discs cultured from third instar larvae at approximately 18 hours before puparium formation (Fig. 6B), no activation can be detected in discs from late third instar larvae, approximately 4 hours before puparium formation, cultured in parallel (Fig. 6D). Activation of GAL4-USP in imaginal discs from earlier stages is quite sensitive, in that it can be detected in response to a 20E concentration as

low as 5×10^{-8} M (data not shown). In contrast, no activation of GAL4-USP can be detected in imaginal discs from late third instar larvae or newly formed prepupae, even when cultured with 5×10^{-6} M 20E, which is likely to exceed the in vivo titer of 20E at the onset of metamorphosis (data not shown). Other tissues cultured from these animals, however, show increased activation of GAL4-USP, as expected. Similar results have been observed with GAL4-EcR in wing and leg imaginal discs from mid- and late third instar larvae cultured in the presence of 5×10^{-6} M 20E (data not shown).

GAL4-USP activation depends on EcR in vivo

We wanted to determine whether GAL4-LBD activation requires the presence of the corresponding heterodimer partner in vivo. It is not possible to assay GAL4-EcR activation in *usp* mutant third instar larvae because these animals die at an earlier stage of development. In contrast, some *EcR* mutants survive to the onset of metamorphosis, allowing analysis of GAL4-USP activation in an *EcR* mutant background. Our attempts to use two different

EcR-B mutant alleles (Schubiger et al., 1998) were unsuccessful because introducing the *hs-GAL4-USP* and *UAS-nlacZ* transgenes into these genetic backgrounds resulted in a significant increase in lethality prior to the onset of metamorphosis. Rather, we assayed GAL4-USP

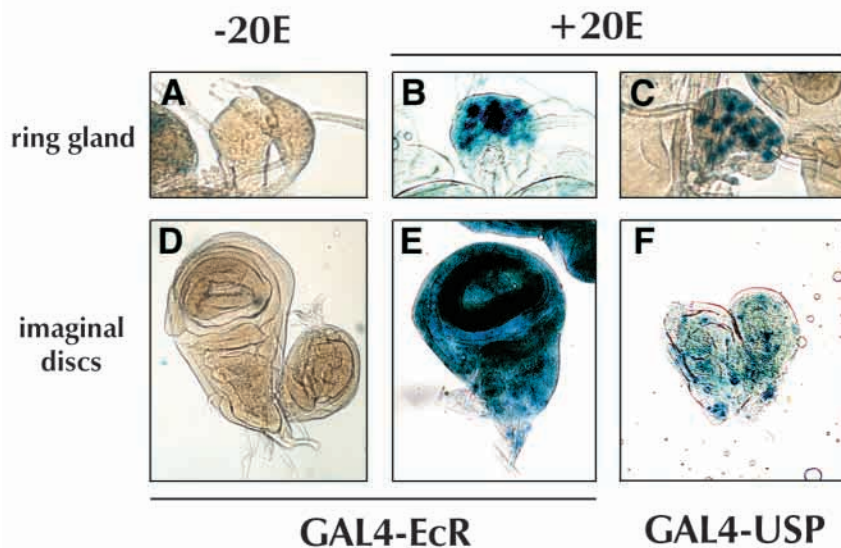


Fig. 5. GAL4-EcR and GAL4-USP can be activated by 20E in the ring gland and imaginal discs. Larval organs dissected from heat treated *hs-GAL4-EcR*; *UAS-nlacZ* (GAL4-EcR) or *hs-GAL4-USP*; *UAS-nlacZ* (GAL4-USP) third instar larvae 18-8 hours before puparium formation were cultured with 5×10^{-6} M 20E and processed for histochemical staining. Both GAL4-EcR and GAL4-USP are activated by 20E in the ring gland (B,C) and imaginal discs (E, wing imaginal disc is shown; F, leg imaginal disc is shown) but not in the absence of hormone (A, ring gland; D, both wing and leg imaginal discs are shown).

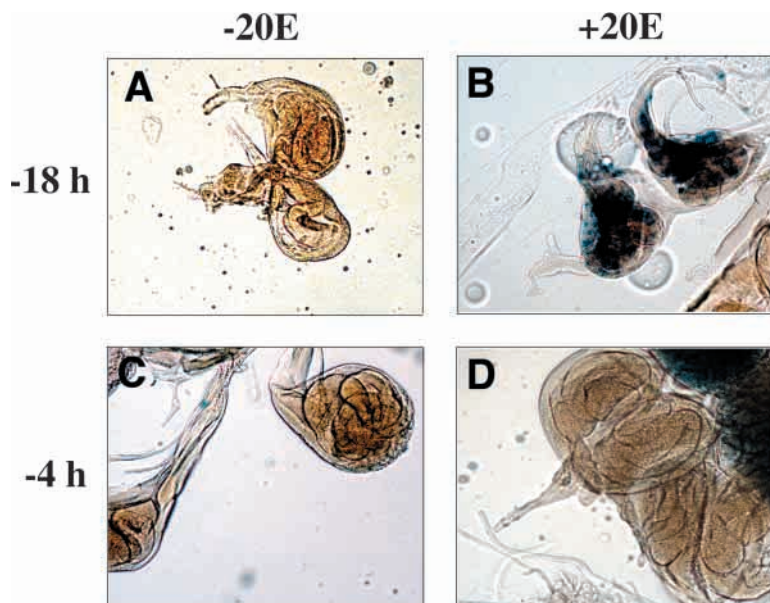


Fig. 6. Stage-specific GAL4-USB activation by 20E in imaginal discs. Larval organs dissected from heat treated *hs-GAL4-USB*; *UAS-nlacZ* third instar larvae at 18 and 4 hours before puparium formation were cultured with 5×10^{-7} M 20E (+20E) or without hormone (-20E). No GAL4-USB activation is seen in the absence of 20E (A,C). In the presence of 20E, GAL4-USB activation is observed in imaginal discs from mid-third instar larvae (B) but not in discs from late third instar larvae (D).

activation in animals heterozygous for a temperature-sensitive *EcR* mutation, *EcR^{A483T}* (Carney and Bender, 2000) and the *EcR^{M554fs}* null allele. A cross between *yw*; *EcR^{M554fs}/CyO*, *y⁺*; *hs-GAL4-USB/hs-GAL4-USB* females and *yw*; *EcR^{A483T}/CyO*, *y⁺*; *UAS-nlacZ/UAS-nlacZ* males was shifted to a non-permissive temperature of 29°C for 24 hours prior to heat treatment. Activation of GAL4-USB in control late third instar larvae (*yw*; *EcR^{M554fs}/CyO*, *y⁺* or *yw*; *EcR^{A483T}/CyO*, *y⁺*) is strong and widespread (Fig. 7A-C), similar to the pattern seen in wild-type late third instar larvae (Fig. 2I-L). This activation is greatly reduced in *yw*; *EcR^{A483T}/EcR^{M554fs}* animals staged and stained in parallel with the controls (Fig. 7D-F). It is not, however, totally

absent, which can be explained by the hypomorphic nature of the *EcR^{A483T}* mutation (Carney and Bender, 2000).

GAL4-*EcR* exerts a specific dominant negative effect on ecdysteroid signaling at the onset of metamorphosis

In the course of defining the activation patterns of GAL4-*EcR* and GAL4-USB we noticed that the expression of these fusion proteins occasionally led to lethality, depending on the developmental stage at which the heat treatment was applied. To further characterize these lethal effects, we subjected *hs-GAL4-*EcR** and *hs-GAL4-USB* transformants to 37°C for 30 minutes as either late third instar larvae (when the ecdysteroid titer is high) or 12-20 hour pupae (when the ecdysteroid titer is low) (Pak and Gilbert, 1987). As expected, most control animals subjected to this heat treatment survived normally (Fig. 8). In contrast, all *hs-GAL4-*EcR** transformants and 30-40% of *hs-GAL4-USB* transformants died when subjected to heat treatment as late third instar larvae (Fig. 8). Almost all *hs-GAL4-*EcR** and *hs-GAL4-USB* transformants, however, survived when the heat treatment was applied to early pupae, 12-20 hours after puparium formation (Fig. 8).

In order to characterize the lethal phenotypes of GAL4-*EcR* overexpression at the onset of metamorphosis, *hs-GAL4-*EcR** third instar larvae at 10-12 hours before puparium formation were subjected to two sequential heat treatments separated by a 4 hour recovery period. Under these conditions, 98% of control animals formed normal tanned pupae (Fig. 9A). In contrast, 62% of the *hs-GAL4-*EcR** transformants subjected to this heat regime arrested development as non-pupariating late third instar larvae (Fig. 9B), 28% formed untanned elongated prepupae (Fig. 9C), and the remaining 10% formed partially tanned and misshapen pupal cases (data not shown) ($n=120$). Dissection of arrested non-pupariating third instar larvae aged for approximately 24 hours revealed that the salivary glands failed to undergo programmed cell death in 6 out of 7 animals examined. The larval midguts were also arrested at various stages of cell death,

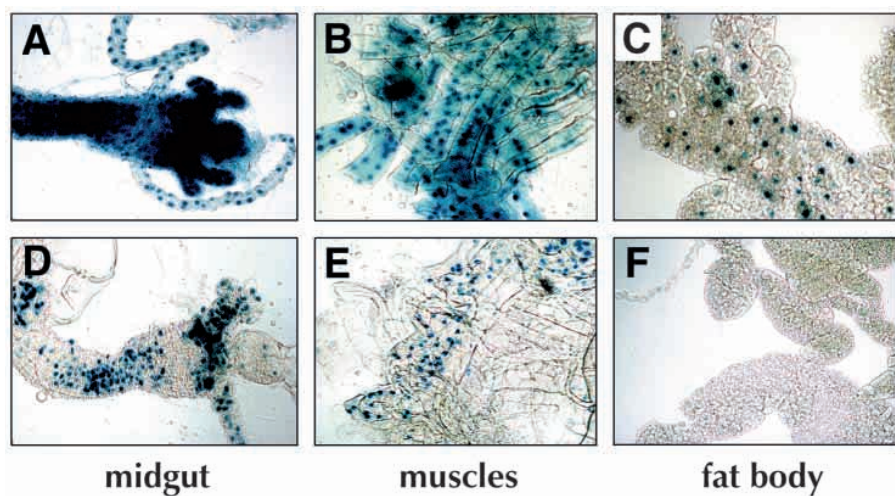


Fig. 7. GAL4-USB activation is significantly reduced in an *EcR* mutant background. GAL4-USB activation was assayed in *yw*; *EcR^{M554fs}/CyO*, *y⁺*; *hs-GAL4-USB/UAS-nlacZ* or *yw*; *EcR^{A483T}/CyO*, *y⁺*; *hs-GAL4-USB/UAS-nlacZ* animals (control) staged in parallel with *yw*; *EcR^{A483T}/EcR^{M554fs}*; *hs-GAL4-USB/UAS-nlacZ* animals (*EcR-ts*) at the onset of metamorphosis. The cross was shifted to 29°C for 24 hours before heat treatment and histochemical staining. GAL4-USB activation is greatly reduced in the midgut, muscles and fat body in the *EcR-ts* mutant (D-F) compared to controls (A-C).

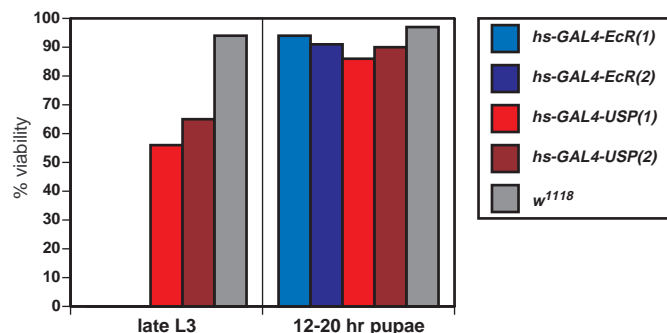


Fig. 8. GAL4-EcR and GAL4-USP expression leads to stage-specific lethality. The graph shows percentage viability after a single 30 minute heat treatment at 37°C of third instar larvae between 8-0 hours before puparium formation (late L3) or early pupae 12-20 hours after puparium formation. Five different stocks were tested: a *w¹¹¹⁸* control (gray bars), two *hs-GAL4-EcR* lines outcrossed to *w¹¹¹⁸* (blue bars), and two *hs-GAL4-USP* lines outcrossed to *w¹¹¹⁸* (red bars) ($n=120-150$ for each line).

with the gastric caecae often persisting. In addition, the imaginal discs failed to undergo morphogenesis in these animals (data not shown). These phenotypes closely resemble those seen in *EcR* and *usp* mutants (Bender et al., 1997; Hall and Thummel, 1998; Schubiger et al., 1998; Li and Bender, 2000) and thus suggest that GAL4-EcR is selectively disrupting ecdysteroid signaling at the onset of metamorphosis.

DISCUSSION

Unlike the majority of transcription factors, which function as constitutive activators, nuclear hormone receptors will only recruit coactivators and induce target gene transcription upon binding their hormonal ligand. The temporal and spatial patterns of nuclear receptor expression thus provide little information about where and when they might exert their regulatory functions. Here, we adapt the GAL4-LBD system devised by Solomin et al. (Solomin et al., 1998) in order to visualize the spatial patterns of ecdysteroid receptor activation at the onset of *Drosophila* metamorphosis. As described below, this system performs as expected. We also detect complex patterns of GAL4-EcR and GAL4-USP activation in the CNS, and distinct differences between the activation of GAL4-EcR and GAL4-USP in most larval tissues that are fated to die and imaginal discs that are fated to form adult cuticular structures, suggesting an additional level for the regulation of these responses. Finally, we show that the GAL4-EcR and GAL4-USP fusion proteins act as specific dominant negative factors that can block ecdysteroid signaling during the onset of metamorphosis. Below, we expand upon these observations and propose that the GAL4-LBD system provides a valuable new tool to characterize nuclear receptor function in the context of a developing organism.

The GAL4-LBD system reveals patterns of EcR and USP endocrine activation at the onset of metamorphosis

GAL4-LBD fusion constructs have been widely used to study ligand-dependent activation by nuclear receptor heterodimers

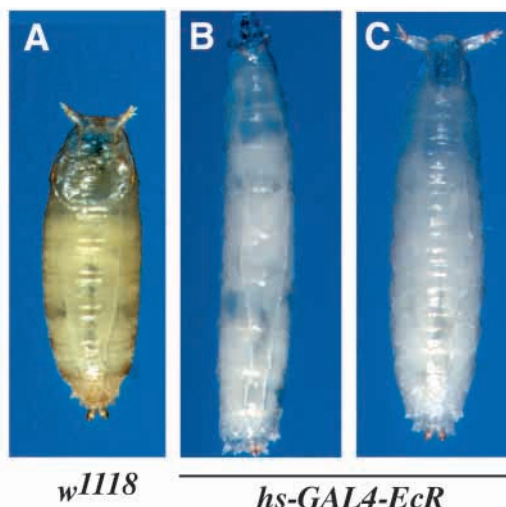


Fig. 9. Lethal phenotypes caused by GAL4-EcR overexpression are similar to *EcR* mutant phenotypes at the onset of metamorphosis. Control *w¹¹¹⁸* and homozygous *hs-GAL4-EcR* third instar larvae were subjected to two sequential heat treatments for 40 minutes at 38°C, separated by a 4 hour recovery period. (A) 98% of the control *w¹¹¹⁸* animals form tanned barrel-shaped pupal cases under these conditions. 62% of heat-treated *hs-GAL4-EcR* animals arrest their development as non-pupariating third instar larvae (B) and 28% form elongated untanned prepupae (C). The remaining 10% form partially tanned and misshapen pupal cases (data not shown) ($n=120$ for each line).

in tissue culture cotransfection assays (Forman et al., 1995; Qi et al., 1995). Similar constructs have also been used in *EcR* mutant *Drosophila* tissue culture cells, showing ligand-dependent activation of reporter gene expression (Hu, 1998). We show here that GAL4-EcR and GAL4-USP can be used to study ecdysteroid-induced activation patterns in the context of a developing organism in vivo. GAL4-EcR and GAL4-USP activation closely parallels the ecdysteroid titer during the onset of metamorphosis. We detect no *lacZ* reporter gene expression in larval tissues 12-18 hours before puparium formation, when the ecdysteroid titer is low, and strong widespread activation in most tissues of late third instar larvae, when the ecdysteroid titer is high (Fig. 2). This activation is restricted to the anterior region of a ligated animal, consistent with its dependence on signaling from the endocrine organ of the insect, the ring gland. Further evidence in favor of endocrine activation of the GAL4-EcR and GAL4-USP fusion proteins derives from our culture experiments, where we see widespread activation in third instar larval organs cultured in the presence of exogenous 20E (Fig. 4). Activation can be readily detected in response to as little as 5×10^{-8} M 20E. This concentration is similar to the K_d of approximately 5×10^{-8} M for 20E determined for the ecdysteroid receptor (Cherbas et al., 1988; Hu, 1998), and close to the minimal concentration of 20E required for EcR/USP activation in transient transfection assays (Dobens et al., 1991; Baker et al., 2000). We therefore conclude that the GAL4-LBD system faithfully recapitulates key aspects of normal activation directed by the full-length EcR and USP proteins in vivo.

It seems most likely that the GAL4-EcR protein dimerizes with endogenous USP, and GAL4-USP protein dimerizes with

endogenous EcR, facilitating both ligand binding and transcriptional activation. This model is consistent with the highly overlapping spatial patterns of GAL4-EcR and GAL4-USP activation reported here. The reduced levels of GAL4-USP activation that we detect in an *EcR* mutant background provide further evidence that GAL4-LBD proteins act in conjunction with their endogenous heterodimer partners (Fig. 7). This observation provides direct evidence that transcriptional activation by USP is dependent on its heterodimer partner *in vivo*, and further supports the critical role of the EcR/USP heterodimer as an ecdysteroid receptor during development.

In conclusion, the overall patterns of GAL4-EcR and GAL4-USP activation in most larval tissues faithfully recapitulates the predicted patterns of EcR and USP activation at the onset of metamorphosis, indicating that this system provides, for the first time, a means of visualizing receptor activation during development. Moreover, our success with GAL4-EcR and GAL4-USP argues that this system will provide a useful new tool for characterizing orphan nuclear receptor function in *Drosophila*. Fusion of the GAL4 DBD to the LBD of an orphan nuclear receptor may allow us to use transgenic animals as a means of ligand discovery, determining when and where ligands are present as well as facilitating their purification and functional characterization.

GAL4-EcR and GAL4-USP are not activated in the ring gland and imaginal discs of late third instar larvae

In contrast to the widespread activation of GAL4-EcR and GAL4-USP seen in most larval tissues at the onset of metamorphosis (Fig. 2E-L), we observe little or no activation in the larval ring gland and imaginal discs at this stage (Figs 3, 6). Several lines of evidence indicate that this tissue-specific lack of activation cannot be attributed to technical problems with the GAL4-LBD system. First, identical results are seen with both constructs, GAL4-EcR and GAL4-USP, suggesting that it reflects effects on both halves of the heterodimer. Second, we detect heat-induced GAL4-EcR and GAL4-USP protein expression in imaginal discs and ring glands from late third instar larvae as well as clear expression of the *UAS-nlacZ* reporter gene in response to a *hs-GAL4* driver (data not shown). Third, both GAL4-EcR and GAL4-USP can be activated by 20E in cultured ring glands and imaginal discs, indicating that the GAL4-LBD system can work in these tissues (Fig. 5). Fourth, this activation is no longer detectable when imaginal discs are isolated from late third instar larvae, even with high 20E concentrations that are likely to exceed the endogenous levels present at the onset of metamorphosis, reflecting the lack of activation seen at this stage *in vivo* (Fig. 5 and data not shown). Taken together, these observations indicate that GAL4-EcR and GAL4-USP lose their ability to be efficiently activated by ecdysteroids in imaginal discs between 18 and 4 hours before puparium formation, and suggest that the endogenous ecdysteroid receptor is regulated in a similar manner.

We have focused most of our effort on characterizing this response in the imaginal discs, which have been so well studied in *Drosophila*, rather than the larval ring gland, which remains less well understood. It is known that the ring gland produces relatively inactive ecdysteroids that are rapidly secreted into the

hemolymph (Redfern, 1989). In addition, the ring gland is unable to convert ecdysone into 20E (King, 1972). Thus, the absence of GAL4-LBD activation in this tissue may simply reflect its low endogenous levels of active ecdysteroids. Alternatively, we might expect to see GAL4-LBD activation in this tissue as a result of possible feedback regulation of its ecdysteroidogenic functions, although it is not known if this regulation might be a direct effect involving EcR/USP in *Drosophila* or whether it might require heterodimer activation. Further studies are needed in order to understand why GAL4-EcR and GAL4-USP are not activated in the ring gland at the onset of metamorphosis. The identification of a key steroidogenic cytochrome P-450 encoded by the *disembodied* gene, as well as the Dare adrenodoxin reductase, provide new directions for understanding how the ring gland controls ecdysteroid biosynthesis in *Drosophila*, and thus may provide answers to some of these questions (Buszczak et al., 1999; Chavez et al., 2000).

There are several possible mechanisms by which the ecdysteroid receptor might not function as an efficient activator in the imaginal discs of late third instar larvae. The first, and most obvious, is the correlation of our GAL4-LBD results in the ring gland and imaginal discs with the high level of EcR-A that is selectively expressed in these tissues at the onset of metamorphosis (Talbot et al., 1993). This correlation becomes even more intriguing when we note that EcR-A has reduced transactivation function in tissue culture transfection assays, while EcR-B1 is a potent activator in these systems (Hu, 1998; Mouillet et al., 2001). These results are consistent with genetic studies in the animal, where ectopic expression of EcR-A is unable to rescue defects in the polytene chromosome puffing pattern or axonal pruning of mushroom body neurons in *EcR-B* mutants (Bender et al., 1997; Lee et al., 2000). Thus, we could propose that the EcR-A/GAL4-USP heterodimer is a poor activator in the ring gland and imaginal discs, resulting in the tissue-specific absence of *lacZ* reporter gene expression in these tissues.

Two lines of evidence, however, challenge this EcR-isoform model. First, we have included a *hs-EcR-B1* transgene along with the *hs-GAL4-USP* transgene and found that this does not rescue *lacZ* induction in the imaginal discs of late third instar larvae (data not shown). Second, if the above model were correct, we might expect to see GAL4-EcR activation in late larval imaginal discs, acting through its endogenous USP partner, independent of EcR-A. This is not seen, however, suggesting that USP might not function as an activator in this tissue; we return to this proposal below. We thus conclude that the tissue-specific expression of EcR-A in the ring glands and imaginal discs of late third instar larvae, although possibly contributing to the reduced activation of EcR/USP in these tissues, is not sufficient to account for our results with the GAL4-LBD system.

The lack of GAL4-EcR and GAL4-USP activation in imaginal discs is in agreement with the clonal analysis of *usp* mutants. These studies have demonstrated that USP acts as a repressor in the wing and eye imaginal discs of third instar larvae, and that this repressive function is relieved by addition of ecdysteroids (Zelhof et al., 1997; Schubiger and Truman, 2000). Similar results have been reported in *usp* mutants rescued to later stages of development, where the *Edg78E* imaginal disc-specific cuticle gene is derepressed at the onset of metamorphosis while its stage-specific activation in mid-

prepupae remains relatively unaffected (Hall and Thummel, 1998). Indeed, Schubiger and Truman (Schubiger and Truman, 2000) argue that silencing by the unliganded EcR/USP heterodimer, and later hormone-dependent release of this silencing, might be a critical level at which the receptor exerts its effects in imaginal discs. Importantly, our results are consistent with this model for ecdysteroid receptor function, since the GAL4-LBD system will not detect repressive functions for EcR or USP in our experiments.

It has been shown that EcR/USP can also function as a transcriptional activator in the imaginal discs of late third instar larvae (Huet et al., 1993; Schubiger and Truman, 2000). However, the level of primary-response target gene transcription in imaginal discs is significantly lower than that seen in larval tissues (Huet et al., 1993), where we observe strong activation with the GAL4-LBD system. It is possible that this reduced transactivation function might be below the threshold detectable by the GAL4-LBD system.

There are several levels at which EcR/USP activity might be modulated in imaginal discs. One of these is through selective transport of ecdysteroids such that the intracellular hormone concentration in imaginal discs is reduced relative to that of other tissues in the animal. Interestingly, this function has been proposed for the ABC transporter protein encoded by the *Drosophila E23* early ecdysteroid-inducible gene (Hock et al., 2000). Recent results suggest that *E23* might be induced earlier in the imaginal discs relative to other tissues in the animal, consistent with a possible role in tissue-specific reduction of hormone titer at the onset of metamorphosis (T. Cottrill and D. Garza, personal communication).

Coactivators and corepressors may also play a key role in modulating EcR/USP activity. The expression pattern of the Alien corepressor is particularly intriguing in this regard, as it appears to be restricted to proliferating imaginal tissues that will differentiate into adult structures during metamorphosis, and is not detectable in larval tissues, providing a mechanism for mediating the tissue-specific repressive functions of EcR/USP in imaginal discs (S. Lier and A. Paululat, personal communication).

In conclusion, our results are consistent with a role for the EcR/USP heterodimer as a repressor in imaginal discs, and suggest that much, if not all, ecdysteroid-induced disc development might be mediated by hormone-dependent alleviation of this repression. The reduced transactivation potential of EcR/USP in imaginal discs could be regulated at many levels, including selective ecdysteroid transport, post-translational modification of the EcR and USP, their interactions with other nuclear receptors, and specific cofactor interactions. Considering the complexity of nuclear receptor signaling pathways, it seems likely that a combination of these factors will contribute to refining EcR/USP activity in the imaginal discs and other tissues at the onset of metamorphosis.

GAL4-EcR and GAL4-USP are activated in complex patterns in the CNS

We observe spatially restricted and largely distinct patterns of GAL4-EcR and GAL4-USP activation in the CNS at the onset of metamorphosis. Understanding the significance of these patterns will require more detailed studies that extend beyond the limits of this initial report. Nonetheless, there are several aspects of these activation patterns that are consistent with our

current understanding of the roles of EcR and USP in CNS development. First, the cells where GAL4-EcR is most active at this stage correlate with the location of the optic proliferation zones (White and Kankel, 1978; Truman et al., 1994) (Fig. 3A), consistent with the known role for ecdysteroids in neuronal proliferation during metamorphosis (Champlin and Truman, 1998). It is also interesting to note that the pattern of GAL4-USP activation in the CNS reflects a subset of the EcR-B1 expression pattern at the onset of metamorphosis (Truman et al., 1994; Schubiger et al., 1998; Lee et al., 2000). EcR-B1 is most abundantly expressed in the mushroom body neurons and surrounding cells of the optic lobes as well as the abdominal neuromeres of the ventral nerve cord. GAL4-USP activation is strongest in a cluster of cells at the anterior end of the optic lobes that could correspond to the mushroom body neurons, and is clearly elevated in the abdominal neuromeres (Fig. 3B). In addition, GAL4-USP activation in the CNS is significantly reduced in an *EcR* mutant background, supporting the conclusion that it is acting as a heterodimer with endogenous EcR (data not shown). Interestingly, low levels of GAL4-EcR activation can also be seen in the cluster of anterior neurons in the optic lobes that show high levels of GAL4-USP activation (Fig. 3). Unambiguous identification of these cells, however, will require more detailed studies of the patterns of GAL4-EcR and GAL4-USP activation in the CNS as well as the use of cell-type specific markers.

The restricted activation of GAL4-EcR cannot be attributed to the distribution of endogenous USP in the CNS, which is widely expressed in this tissue at the onset of metamorphosis (Henrich et al., 1994) (T. K., unpublished data). Similarly, many neurons that express EcR-B1 in the optic lobes do not show high levels of GAL4-USP activation (Truman et al., 1994) (Fig. 3B). One possible explanation for these limited patterns of activation is that EcR might function independently of USP in certain cells of the CNS. Alternatively, any of the mechanisms proposed above for the reduced levels of transactivation seen in late larval imaginal discs could account for these complex cell-type specific patterns of GAL4-LBD activation in the CNS.

GAL4-EcR and GAL4-USP provide a new tool to inactivate ecdysteroid signaling pathways

Expression of either GAL4-EcR or GAL4-USP in late third instar larvae leads to a high degree of lethality (Fig. 8). In contrast, expression at 12-20 hours after puparium formation, when the ecdysteroid titer is low (Pak and Gilbert, 1987), has no significant effect on development, suggesting that this lethality is due to defects in ecdysteroid signaling rather than to non-specific effects on viability. Further evidence that GAL4-EcR exerts a dominant negative effect on ecdysteroid signaling derives from our characterization of the lethal phenotypes of these animals. Expression of GAL4-EcR in late third larvae leads to developmental arrest, with the formation of stationary nonpupariating larvae that fail to shorten and tan (Fig. 9). Dissection of these animals reveals that the larval midgut and salivary glands do not undergo their normal programmed cell death and adult structures do not undergo morphogenesis. These defects closely resemble those seen in *EcR* or *usp* mutants at the onset of metamorphosis, providing evidence that GAL4-EcR expression specifically and efficiently disrupts ecdysteroid signaling at this stage in

development (Bender et al., 1997; Hall and Thummel, 1998; Schubiger et al., 1998; Li and Bender, 2000). It is important to note that the timing and levels of GAL4-EcR expression are critical for the efficiency of these phenotypic effects. Not only is the dominant negative activity stage-specific, but we also need two sequential rounds of heat-induced GAL4-EcR expression in order to effectively block ecdysteroid signaling. These observations suggest that the GAL4-EcR protein is unstable, and that appropriate experimental parameters are needed to achieve efficient dominant negative effects with this fusion protein.

Our results are consistent with studies in vertebrate cells which have demonstrated that N-terminal truncations of a nuclear receptor can lead to specific dominant negative effects on hormone signaling. For example, thyroid hormone receptors or RXR that lack their DNA-binding domain can still dimerize with their endogenous partners in the cell, forming inactive complexes that fail to mediate hormone-inducible target gene transcription (Forman et al., 1989; Minucci et al., 1994). We envisage that GAL4-EcR and GAL4-USP function in a similar manner in *Drosophila*, dimerizing with endogenous EcR or USP and thereby reducing the levels of active receptor. It is possible that the levels of USP are lower than those of EcR at the onset of metamorphosis, providing an explanation for why GAL4-EcR expression results in more penetrant lethality than GAL4-USP at this stage in development (Fig. 8).

The use of a heat-inducible promoter to direct GAL4-LBD expression provides an effective means of controlling its dominant negative activity. By using GAL4-LBD expression to disrupt ecdysteroid signaling at different stages in the life cycle we can examine the effects on a wide range of ecdysteroid-triggered developmental responses. This provides one means of overcoming the early lethality associated with strong loss-of-function *EcR* and *usp* mutations, facilitating studies of ecdysteroid signaling at later stages in development. In addition, expression of a dominant negative receptor provides the only known means of inactivating EcR/USP function in the early *Drosophila* embryo, when both EcR protein and mRNA are deposited maternally (T. K., unpublished results). GAL4-LBD constructs thus provide a new and valuable tool for functional characterization of ecdysteroid signaling pathways in *Drosophila*, as well as a range of new directions that should further our understanding of how nuclear receptor superfamily members exert their multiple effects throughout the fly life cycle.

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