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# Coordination of *Drosophila* Metamorphosis by Two Ecdysone-Induced Nuclear Receptors

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The functions of the ecdysone-induced DHR3 and E75B orphan nuclear receptors in the early stages of *Drosophila* metamorphosis were investigated. DHR3 represses the ecdysone induction of early genes turned on by the pulse of ecdysone that triggers metamorphosis. It also induces  $\beta$ FTZF1, an orphan nuclear receptor that is essential for the appropriate response to the subsequent prepupal pulse of ecdysone. The E75B receptor, which lacks a complete DNA binding domain, inhibits this inductive function by forming a complex with DHR3 on the  $\beta$ FTZF1 promoter, thereby providing a timing mechanism for  $\beta$ FTZF1 induction that is dependent on the disappearance of E75B.

The early stages of *Drosophila* metamorphosis are initiated and controlled by two successive pulses of the steroid hormone 20-hydroxyecdysone, referred to here as ecdysone (1). The first pulse begins some 6 hours before the larval-to-prepupal transition that is marked by puparium formation (pupariation). It peaks at pupariation and is followed by a smaller prepupal pulse that peaks 10 hours after pupariation, just 2 hours before the prepupal-to-pupal transition (Fig. 1B). The late-larval pulse induces a set of metamorphic responses that includes eversion of the imaginal discs to form rudimentary adult appendages, larval tissue histolysis, and the apolysis of the larval cuticle as it forms the puparium. The prepupal pulse is associated with another set of metamorphic responses that includes emergence of the adult head, proliferation of the imaginal histoblast cells to form the epidermis of the adult abdomen, and further histolysis of larval tissues (2). Our interest is in the ecdysone-activated genetic regulatory hierarchies that underlie these two sets of responses. Here we identify multiple functions for ecdysone-inducible nuclear receptors that coordinate these two distinct hierarchies.

An outline of each of these hierarchies was first obtained from observations of the polytene chromosome puffing response to the late-larval (3) and prepupal (4) ecdysone pulses in the larval salivary glands. The principal players are (i) an ecdysone-receptor complex, (ii) a small set of "early" genes that are activated by this receptor complex, and (iii) a large set of "late" genes

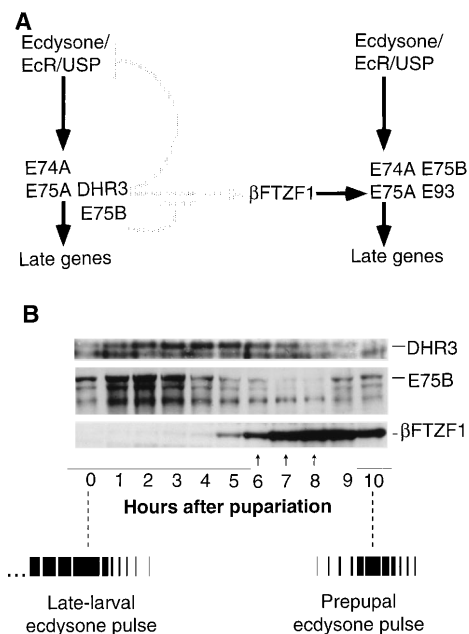
that are targets of transcription factors encoded by the early genes and are assumed to encode proteins that effect the metamorphic response (Fig. 1A). An additional small gene set, the "early-late" genes, also are direct targets of the ecdysone-receptor complex but for maximum response appear to require an ecdysone-induced protein, and thus reach maximal expression after the early genes (3, 5). This outline also included early gene repression by one or more early, or early-late, gene products (Fig. 1A). In confirmation of this outline, transcription factors are encoded by the *E74* (6) and *E75* (7) early genes; their expression is induced as a primary response to ecdysone, and they are subsequently repressed by proteins encoded by ecdysone-induced genes. *E74* and *E75* encode multiple isoforms from

overlapping, differently regulated transcription units, of which *E74A* and *E75A* exhibit these early gene properties (Fig. 1A).

*E75A*, which encodes a nuclear receptor, provided the means for cloning two additional nuclear receptor genes: *EcR* (8–11), which encodes the ecdysone receptor isoforms; and *DHR3* (7, 9), an early-late gene (Fig. 1). The *E75B* transcription unit, like an early-late gene, is maximally expressed shortly after pupariation (7, 8) (Fig. 1B) and encodes an aberrant nuclear receptor that lacks the first of the two zinc fingers that typify nuclear receptor DNA binding domains (DBDs) (7). Of 13 additional *Drosophila* genes known to encode nuclear receptors, 7, including *USP* and  $\beta$ FTZF1, are involved in the ecdysone response (13). The *USP* protein heterodimerizes with, and thereby activates, the *EcR* isoforms (10, 14) (Fig. 1A).  $\beta$ FTZF1, induced shortly after *DHR3* expression (15) (Fig. 1B), regulates early gene response to the prepupal ecdysone pulse (16).

We show here that the *DHR3* receptor represses the early genes activated by the late-larval ecdysone pulse and, with the help of *E75B*, provides a temporal linkage between the two ecdysone responses by controlling the expression of  $\beta$ FTZF1. *DHR3* is a likely candidate to down-regulate early genes because it is expressed at an appropriate time (Fig. 1B) and because a *GAL4* yeast two-hybrid test for protein-protein interactions revealed a *DHR3*/*EcR* interaction, as well as the expected *EcR*/*USP* interaction (Fig. 2B). Although the *EcR*-B1 isoform was used in these experiments, it is probable that the other two

**Fig. 1. (A)** The ecdysone regulatory hierarchy that initiates and controls early metamorphosis. The active ecdysone receptor (ecdysone/*EcR*/*USP*) activates a small set of genes (early and early-late genes), some of which encode transcription factors. These gene products in turn negatively autoregulate, turn on a large set of effector genes (late genes), and control the expression of the mid-prepupal factor  $\beta$ FTZF1. This factor then specifies the appropriate early gene response to the prepupal pulse of ecdysone (16). **(B)** Temporal expression of *DHR3*, *E75B*, and  $\beta$ FTZF1 proteins during the prepupal stage. Pupariating animals were incubated on grape agar plates at 25°C for up to 10 hours. Eight to ten individuals were collected at hourly time points and used for protein immunoblots. Each lane represents two larval equivalents. Ponceau staining revealed equal loading between lanes. *DHR3* is recognized as a doublet at 65 kD by rabbit polyclonal antibodies (19).  $\beta$ FTZF1 protein is recognized at 95 kD by rabbit polyclonal antibodies (15, 20). *E75B* is recognized at 190 kD by the mAb 10E11; breakdown products are also observed (19). The pattern of expression of these proteins shows an inverse relation between the presence of *E75B* and the onset of  $\beta$ FTZF1 expression. Arrows mark where  $\beta$ FTZF1 mRNA peaks in expression (15). Ecdysone pulses are represented below, with the late-larval pulse peaking at 0 hours and the prepupal pulse at 10 hours after pupariation.



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isoforms (EcR-A and EcR-B2) would interact with DHR3 in this assay because the NH<sub>2</sub>-terminally truncated Δ361 EcR (Fig. 2A) also interacts with DHR3 (Fig. 2B). Δ361 EcR lacks both the DBD and all of the isoform-specific sequences located NH<sub>2</sub>-terminal to the DBD (11) (Fig. 2A). The EcR isoforms are distributed in a tissue-specific manner (11), and such an isoform-independent mechanism for EcR inactivation would be applicable to all target tissues. EcR-B1 and Δ361 EcR also interact with Δ83 DHR3 and Δ105 DHR3, which lack the first or both zinc fingers of the DBD, respectively, along with the sequences NH<sub>2</sub>-terminal to the DBD (Fig. 2A). Thus the sequences required for the DHR3/EcR interaction lie downstream of the DBD in regions that include the receptor's ligand-binding/heterodimerization domain (LBD), which in other nuclear receptors has been shown to contain sequences required for heterodimerization or homodimerization (17).

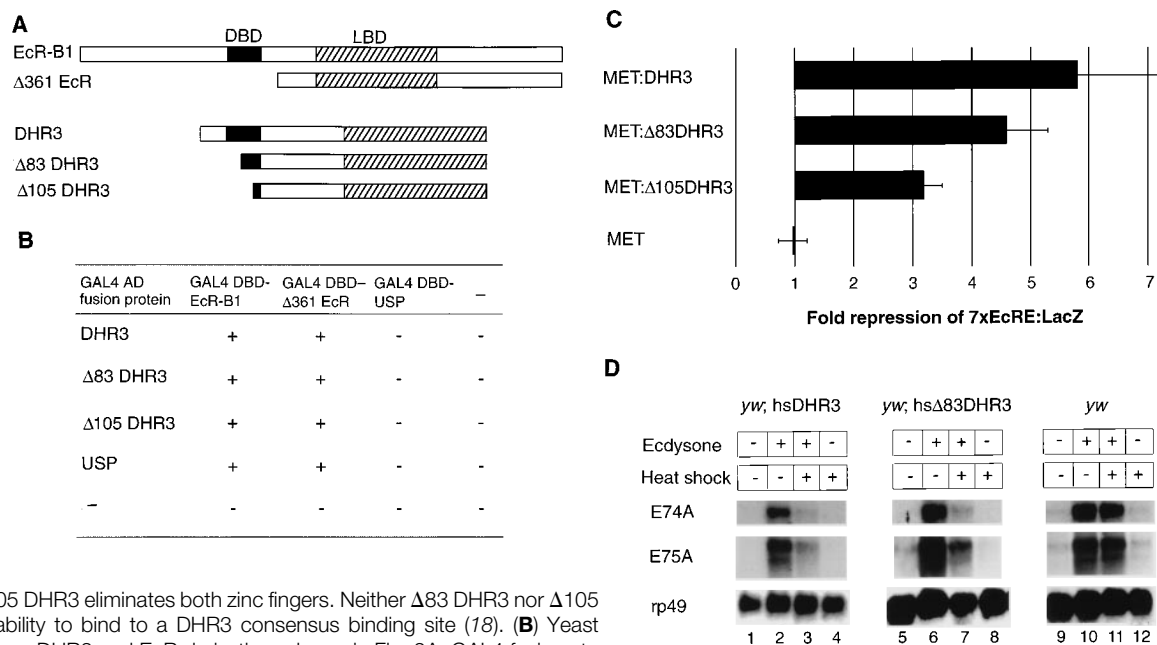
To determine whether DHR3 can inhibit ecdysone induction, we used *Drosophila* S2 cell lines that contain endogenous EcR and USP and two transgenes: an ecdysone-inducible *lacZ* reporter gene controlled by a heptamerized ecdysone response element

[7×EcRE:LacZ (8)] and a Cu<sup>2+</sup>-inducible DHR3 expression gene controlled by a metallothionein promoter (MET:DHR3, MET:Δ83DHR3, or MET:Δ105DHR3). Expression of DHR3 inhibits the ecdysone inducibility of the *lacZ* reporter gene (Fig. 2C).

**Table 1.** Strong DHR3 binding sites to polytene chromosomes from salivary glands prepared 2 to 3 hours after pupariation. A total of 133 sites were detected with two mAbs to different DHR3 epitopes (19). Of these, the 34 sites listed in this table as binding to the X chromosome and to the left (L) and right (R) arms of the second and third chromosomes stained most strongly. Sites corresponding to the *DHR3*, *E74*, *E75*, and *FTZF1* genes are labeled; asterisks indicate other puff sites.

X	2L	2R	3L	3R
2B10-C2*	22CD*	42A8-16*	69B	83EF*
2C	26B2-11*	42C6-9	74B	84C
3E*	30A2-7*	43A	74D <sup>E74</sup>	85C
8EF*	33B*	44A*	75B <sup>E75</sup>	91B
9DF*	33DE*	46F <sup>DHR3</sup>	75D <sup>FTZF1</sup>	98E
10B11-17		48B*	78C*	99B5-C2*
11B14-C2*		54B*		
13B*		58F		
		60A11-B5*		

**Fig. 2.** DHR3 interacts with EcR in a two-hybrid assay, inhibits transactivation by the ecdysone receptor in tissue culture, and represses early gene induction in vivo. (A) Schematic of the EcR and DHR3 nuclear receptors and their derivatives used in this study. The DBD is black and the LBD is hatched. Δ361 EcR represents an NH<sub>2</sub>-terminal truncation of EcR that eliminates the first 361 amino acids, including the DBD. Δ83 DHR3 represents an NH<sub>2</sub>-terminal truncation of DHR3 that eliminates the first 83 amino acids, including the first zinc finger of the DBD domain. Δ105 DHR3 eliminates both zinc fingers. Neither Δ83 DHR3 nor Δ105 DHR3 proteins retain the ability to bind to a DHR3 consensus binding site (18). (B) Yeast two-hybrid interactions among DHR3 and EcR derivatives shown in Fig. 2A. GAL4 fusions to these nuclear receptors were constructed with either the GAL4 DBD or the GAL4 activation domain (AD). Fusion proteins were expressed in the yeast strain YRG-2, and the resulting strains were tested for growth and production of β-Gal as described (23). A plus sign indicates growth on SDC-leu-trp-his and thus an interaction between the tested fusion proteins. Quantitation of β-Gal activity reveals that the EcR/DHR3 interaction is of the same magnitude as the EcR/USP interaction in this assay. (C) DHR3 can repress transactivation by EcR in tissue culture. S2 tissue culture cells, which contain endogenous EcR/USP, were cotransfected with an ecdysone-inducible 7×EcRE:LacZ reporter gene and a DHR3, Δ83DHR3, or Δ105DHR3 open reading frame under control of a metallothionein promoter (pMET:DHR3, pMET:Δ83DHR3, or pMET:Δ105DHR3) (8, 24). The resulting cell lines were incubated in the presence or absence of 5 × 10<sup>-6</sup> M ecdysone and 80 mM CuSO<sub>4</sub> for 24 hours. Ecdysone induction of the 7×EcRE:LacZ transgene was measured by the o-nitrophenyl β-o-galactopyranoside (ONPG) assay for β-Gal activity. Fold repression by DHR3, Δ83 DHR3, or Δ105 DHR3.80 mM CuSO<sub>4</sub> did not have negative effects on induction of 7×EcRE:LacZ in the absence of DHR3. DHR3, Δ83 DHR3, and Δ105 DHR3 gave comparable levels of expression, as detected with mAb 3D9.1 to DHR3 (18). (D) DHR3 can interfere with the ecdysone induction of early genes in the animal. Transgenic *Drosophila* strains were constructed containing heat shock-inducible DHR3 or Δ83DHR3 (hsDHR3 or hsΔ83 DHR3) with the use of a *yw* strain as the progenitor. Larvae were reared on media containing bromophenol blue to assist in staging by color of the gut, as described (25). Mid-third instar blue-gut larvae carrying these transgenes were collected and either held at 22°C or heat-shocked at 37°C for 40 min. After a brief period of recovery (20 to 30 min), larvae were dissected in Grace's media in the presence or absence of 5 × 10<sup>-6</sup> M ecdysone and were incubated for 4 hours. Total RNA was prepared from these organ cultures with the SDS-lysis method (25). Approximately 25 μg of this RNA was used per lane for Northern (RNA) blots. Northern blots were then probed with radiolabeled sequences unique to the E74A or E75A ecdysone-inducible transcripts (6, 7). Quantitation by phosphorimaging revealed a three- to fourfold repression of ecdysone inducibility of these transcripts by DHR3 or Δ83 DHR3. Ribosomal protein 49 (rp49) was used to standardize for loading.



This effect is specific to the inducibility of the 7×EcRE promoter by the ecdysone/EcR/USP complex, because DHR3 does not exhibit inhibitory effects on a basal promoter driving chloramphenicol acetyltransferase expression nor on a Cu<sup>2+</sup>-inducible promoter driving *lacZ* (18). Δ83 DHR3 and Δ105 DHR3 also repress this ecdysone inducibility (Fig. 2C), which indicates that the DHR3 sequences sufficient for interaction with EcR in the yeast two-hybrid assay are also sufficient for this repression. To test whether DHR3 can inhibit the ecdysone induction of the *E74A* and *E75A* early genes in the ani-

mal, we constructed transgenic *Drosophila* lines that express DHR3 or Δ83 DHR3 in response to heat shock (hsDHR3 and hsΔ83DHR3). We found that both DHR3 and Δ83 DHR3 can repress the ecdysone-induced expression of *E74A* and *E75A* and that heat shock per se does not affect that induction (Fig. 2D).

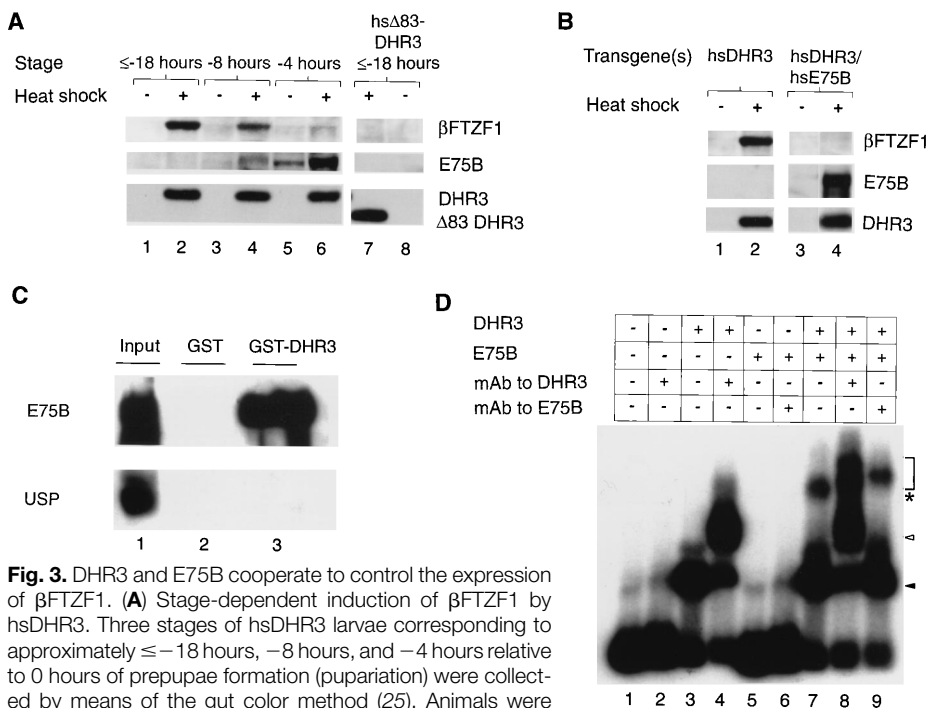
These results indicate that DHR3 acts in a negative feedback loop to repress early genes activated by the late-larval ecdysone pulse. Neither the two-hybrid interaction nor the DHR3 repression activity requires sequences in the DBD or NH<sub>2</sub>-terminal to the DBD.

This leaves the LBD of DHR3 as the probable source of the interactive sequences because of precedents for LBD involvement in nuclear receptor dimerization (17), and suggests that the mechanism for this DHR3 repression may involve an interaction between the DHR3 and EcR LBDs.

The second function of DHR3 provides a temporal linkage between the two ecdysone responses by activating *βFTZF1*. One indication of this activation is the overlap in the temporal patterns of the DHR3 and *βFTZF1* proteins—an overlap that includes the peak of *βFTZF1* mRNA expression (15) (arrows at 6 to 8 hours after pupariation in Fig. 1B). A second indication is the observation that the strongest binding sites of DHR3 protein to polytene chromosomes include the 75D locus, which corresponds to the *βFTZF1* gene (Table 1). These data implicate DHR3 as a candidate activator of *βFTZF1*.

Direct evidence that DHR3 is sufficient for the activation of *βFTZF1* consisted in the premature expression of *βFTZF1* in response to heat-shock induction of DHR3 at different times before pupariation (Fig. 3A), although this expression decreased as the time of induction approached pupariation. The possibility that this decrease resulted from a corresponding increase in *E75B* (Fig. 3A) was enhanced by observations that in the prepupal period, *βFTZF1* is not produced as soon as DHR3 is expressed but only when *E75B* levels plummet (Fig. 1B), and that *E75B* binds to polytene chromosomes at a subset of the DHR3 binding sites, including 75D (19) (Table 1). Furthermore, *E75B* binds to specific DNA sequences in the *E75* gene only in the presence of DHR3 (19). The observation that larvae expressing DHR3 in the presence of *E75B* do not produce *βFTZF1* but do so in the absence of *E75B* (Fig. 3B) provides direct evidence that *E75B* inhibits the DHR3 induction of *βFTZF1* and suggests that *E75B* acts as a repressor of the DHR3 activating function. The observation that Δ83 DHR3 fails to induce *βFTZF1* synthesis when it is expressed at a stage when the full-length protein induces *βFTZF1* (Fig. 3A, lanes 7 and 8) indicates that the DBD of DHR3, and perhaps sequences NH<sub>2</sub>-terminal to it, are necessary for this activating function.

To test whether that repression could occur by direct interaction between the two proteins, *E75B* and glutathione-S-transferase–DHR3 (GST–DHR3) were produced in a baculovirus expression system, and *E75B* was tested for its ability to bind to a column consisting of GST–DHR3 bound to glutathione-linked beads. *E75B* bound to GST–DHR3 but not to GST when it was similarly bound to such a column (Fig. 3C), whereas USP, which was also produced in the baculovirus system, failed to bind to either column. These results indicate that



**Fig. 3.** DHR3 and *E75B* cooperate to control the expression of *βFTZF1*. **(A)** Stage-dependent induction of *βFTZF1* by hsDHR3. Three stages of hsDHR3 larvae corresponding to approximately ≤ -18 hours, -8 hours, and -4 hours relative to 0 hours of prepupae formation (pupariation) were collected by means of the gut color method (25). Animals were either incubated at 22°C or heat-shocked at 37°C for 40 min and were then allowed to recover for 2 hours and 20 min at 22°C. Heat-shocked animals collected at -4 hours had reached pupariation at the time of their preparation, whereas their non-heat-shocked cohorts had not, which suggests that heat shock slightly accelerated their development. Gels were run as above and protein immunoblots were probed for *βFTZF1*, *E75B*, and DHR3. DHR3 exhibits a stage-dependent ability to induce *βFTZF1* in these experiments that inversely correlates with the level of *E75B* (lanes 1 through 6). Control experiments with animals not bearing a transgene show that increased endogenous *E75B* in heat-shocked versus non-heat-shocked animals of the same age group is due to the heat shock (18). hsΔ83DHR3 larvae were collected at ≤ -18 hours and treated as above. Although Δ83 DHR3 is expressed in amounts comparable to that of full-length DHR3, it cannot induce *βFTZF1* expression (lanes 7 and 8). **(B)** *E75B* is sufficient to block *βFTZF1* induction by DHR3. hsDHR3 or hsDHR3/hsE75B larvae were collected and treated as above at ≤ -18 hours relative to pupariation. When *E75B* is co-expressed with DHR3 at this stage, induction of *βFTZF1* is blocked (lanes 3 and 4). When expressed at this stage, *E75B* alone does not appear to have an effect on the animal. **(C)** GST-DHR3 interacts with *E75B* on a glutathione column. Protein immunoblots were performed to detect the baculovirus-produced *E75B* and USP proteins. Crude extracts containing *E75B* or USP are shown in lane 1. Eluted samples from columns containing GST or GST-DHR3 are shown in lanes 2 and 3, respectively. Extracts, columns, and eluted samples were prepared as described (26). **(D)** DHR3 forms a complex with *E75B* on the *βFTZF1* promoter. Sequences corresponding to DNA from the *βFTZF1* promoter were used to perform gel shifts with baculovirus-expressed DHR3 and *E75B* as described (26, 27). The solid arrowhead marks DHR3 shifted complexes whereas the open arrowhead marks DHR3-containing complexes supershifted with the mAb to DHR3 WTG1. The asterisk marks the additional DHR3 shifted complex formed on this DNA in the presence of *E75B*. The brackets show supershifting of this complex in the presence of mAb to DHR3 WTG1 or mAb 10E11 to *E75B*, confirming the presence of both DHR3 and *E75B* in this complex. A similar result was achieved with an approximately 95-bp fragment (Hinc II site to an Sph I site) containing the 5' DHR3 binding site (18).

E75B could repress the activation function of DHR3 by direct interaction.

Finally, we wished to test if DHR3 can interact with E75B on target DNA. Recently, regulatory elements sufficient for reproducing the wild-type temporal expression pattern of  $\beta$ FTZF1 have been identified, and DHR3 has been shown to bind to sequences within these elements (20). Specifically, DHR3 has two high-affinity binding sites approximately 300 base pairs (bp) apart that lie downstream of the transcription start site of  $\beta$ FTZF1 (20). DHR3 appears to bind as a monomer to these sites, because sequencing and footprinting analyses uncover single consensus DHR3 sites at each of these loci (20). To determine if DHR3 can associate with E75B on these elements, we performed gel shift assays using DNA sequences containing one or the other of these high-affinity sites. Results were similar for both sites, and gel shifts using the stronger binding 3' site are shown in Fig. 3D. As previously demonstrated (20), DHR3 can bind sequences containing this site (lanes 3 and 4). E75B failed to bind this DNA element in the absence of DHR3 (lanes 5 and 6). However, when both DHR3 and E75B were present, an additional shifted complex formed (lane 7, asterisk). When monoclonal antibodies (mAbs) to DHR3 or E75B were also present, a super-shifted complex formed, confirming that both DHR3 and E75B were present in this complex (lanes 8 and 9, bracket). These results demonstrate that E75B can associate with DHR3 on regulatory sequences shown to be sufficient for  $\beta$ FTZF1 induction in the animal.

Taken together, our results demonstrate that DHR3 has dual roles during metamorphosis: gene activation, which is dependent on DNA binding; and gene repression, which is independent of DNA binding. We have provided evidence that DHR3 can interact with the EcR subunit of the ecdysone receptor, albeit in yeast, and that it can attenuate the ability of the ecdysone receptor to activate target genes in the presence of ecdysone. DHR3 is therefore one of the ecdysone-inducible gene products predicted to negatively regulate the expression of genes directly induced by ecdysone (Fig. 1A) (3). This is the first such ecdysone-inducible gene product shown to be capable of this function. We suggest that DHR3 interacts with EcR to effect this regulation. We have also shown that DHR3 can act as an activator of the target gene  $\beta$ FTZF1 and that another nuclear receptor, E75B, can inhibit  $\beta$ FTZF1 induction by DHR3 (Fig. 1A). The mechanism of this inhibition is different from that previously demonstrated for nuclear receptors (17, 21), as E75B acts like a co-repressor with DHR3 rather than like a competitor with DHR3 for DNA binding. The restricted temporal expression of

E75B apparently acts as a precise timer for the onset of  $\beta$ FTZF1 expression. Accordingly, DHR3 appears to act in concert with E75B to provide an important temporal link between the late-larval and prepupal ecdysone responses by tightly controlling the induction of  $\beta$ FTZF1, which is required for the specificity of the prepupal ecdysone response (16).

Some cautionary notes should be added to this simple picture of the dual temporal functions of DHR3. In particular, the proposal that DHR3 represses the ecdysone induction of early genes by interaction with EcR rests solely on the yeast two-hybrid data. (Our attempts to demonstrate direct interaction between GST-DHR3 and EcR according to the method used in Fig. 3C have thus far been negative.) Furthermore, the recent evidence that DHR3-E75B complexes bind to DNA of the E75 early gene (19) suggests an additional mechanism by which early genes may be repressed. Finally, ecdysone appears to have a direct repressive effect on the  $\beta$ FTZF1 gene through an unknown mechanism (16), in addition to the effects of the ecdysone-inducible protein E75B. We expect that studies using DHR3, E75, and EcR mutations will answer or better define such questions.

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- Baculovirus reagents were obtained from Pharmingen. The DHR3, E75B, and USP open reading frames were inserted into the baculovirus transfer vector pVL1393; DHR3 was also inserted into pAcGHLT to produce GST-DHR3, and the empty vector was used to produce GST alone. Crude extracts for chromatography were prepared by harvesting infected cells through brief centrifugation and resuspension in bv150 [150 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM KCl, 50 mM Hepes (pH 7.4), 1 mM dithiothreitol phenylmethylsulfonyl fluoride (100  $\mu$ g/ml) and leupeptin and pepstatin (1  $\mu$ g/ml each)]. Cells were lysed by sonication on ice and cleared of debris by centrifugation at 100,000g for 20 min. Protein was determined by Bradford assay. Five hundred micrograms of crude extracts containing GST-DHR3 or GST were applied to 300  $\mu$ l of prewashed glutathione Sepharose 4B beads (Pharmacia) and allowed to bind for 30 min at 4°C. Columns were washed three times with 5 ml of bv150. Two milligrams of crude extracts containing either E75B or USP were then applied and were incubated for 30 min at 4°C, then washed as above. Bound proteins were eluted by incubation with 300  $\mu$ l of 40 mM reduced glutathione and 50 mM Tris (pH 8.0) for 15 min at 4°C. Crude extracts for mobility-shift assays were prepared in the same way, except that EcR100 buffer [100 mM KCl, 1 mM MgCl<sub>2</sub>, 10% glycerol, and 25 mM Hepes (pH 7.0)] was substituted for bv150.
- Two high-affinity DHR3 binding sites have previously been identified within the sequences used (20). DNA containing these sites was digested with Dra I-Hind III, which released the 3' DHR3 binding site plus flanking DNA. This ~130-bp fragment was gel-purified and end-labeled with [<sup>32</sup>P]dATP. The labeled DNA was then incubated for 45 min on ice with extracts containing DHR3 or E75B or both, or control extracts. A concentration of total protein of 1 mg/ml in 100 mM KCl, 10 mM Hepes, 1 mM MgCl<sub>2</sub>, and 10% glycerol (pH 7.4) was used in the combinations listed. Agarose gels (0.6%) were run in 1× Tris-borate/EDTA or glycine buffer.
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