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DHR3 Is Required for the Prepupal–Pupal Transition and Differentiation of Adult Structures during *Drosophila* Metamorphosis

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Pulses of the steroid hormone ecdysone activate genetic regulatory hierarchies that coordinate the developmental changes associated with *Drosophila* metamorphosis. A high-titer ecdysone pulse at the end of larval development triggers puparium formation and induces expression of the DHR3 orphan nuclear receptor. Here we use both a heat-inducible *DHR3* rescue construct and clonal analysis to define *DHR3* functions during metamorphosis. Clonal analysis reveals requirements for *DHR3* in the development of adult bristles, wings, and cuticle, and no apparent function in eye or leg development. *DHR3* mutants rescued to the third larval instar also reveal essential functions during the onset of metamorphosis, leading to lethality during prepupal and early pupal stages. The phenotypes associated with these lethal phases are consistent with the effects of *DHR3* mutations on ecdysone-regulated gene expression. Although *DHR3* has been shown to be sufficient for early gene repression at puparium formation, it is not necessary for this response, indicating that other negative regulators may contribute to this pathway. In contrast, *DHR3* is required for maximal expression of the midprepupal regulatory genes, *EcR*, *E74B*, and *βFTZ-1*. Reductions in *EcR* and *βFTZ-F1* expression, in turn, lead to submaximal early gene induction in response to the prepupal ecdysone pulse and corresponding defects in adult head eversion and salivary gland cell death. These studies demonstrate that DHR3 is an essential regulator of the βFTZ-F1 midprepupal competence factor, providing a functional link between the late larval and prepupal responses to ecdysone. Induction of *DHR3* in early prepupae ensures that responses to the prepupal ecdysone pulse will be distinct from responses to the late larval pulse and thus that the animal progresses in an appropriate manner through the early stages of metamorphosis.

Key Words: **ecdysone; gene regulation; *Drosophila* metamorphosis; adult differentiation.**

INTRODUCTION

Like other holometabolous insects, the fruit fly *Drosophila melanogaster* undergoes a complete metamorphosis during its life cycle, from a crawling larva to highly motile and reproductively active adult fly (Riddiford, 1993). This transformation is coordinated by successive pulses of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone). A pulse of ecdysone at the end of the third larval instar triggers puparium formation, initiating the prepupal stage of development. The larval midgut and anterior muscles are destroyed in response to this hormone pulse while the imaginal discs evert to form rudiments of their corresponding adult structures (Robertson, 1936; Fristrom and Fristrom, 1993; Jiang *et al.*, 1997). This is followed by another ecdysone pulse, ~10–12 h after puparium formation, that signals the prepupal–pupal transition (Fig. 1). In response to this hormone pulse, the adult head assumes its appropriate position by everting from inside the thorax, the legs and wings elongate, and the salivary glands undergo programmed cell death. A central goal of our studies is to determine how a single hormonal signal can direct these distinct stage- and tissue-specific biological responses.

The ecdysone signal is transduced by a heterodimeric nuclear receptor, encoded by the *EcR* and *usp* genes (Koelle *et al.*, 1991; Yao *et al.*, 1992, 1993). This hormone–receptor complex directly induces the transcription of primary-

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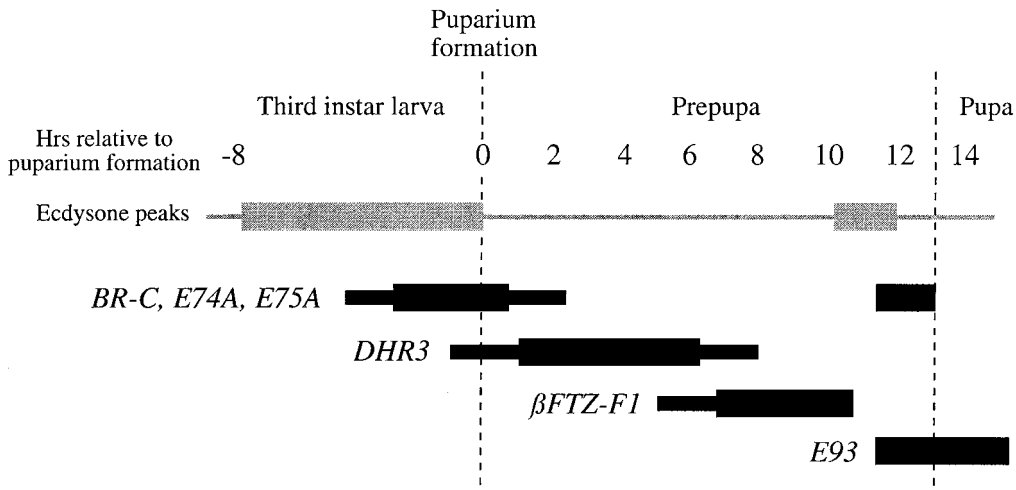


FIG. 1. Schematic representation of ecdysone-regulated transcription during the onset of *Drosophila* metamorphosis. The late-third-instar larval, prepupal, and pupal stages of development are indicated at the top, above a timeline in hours relative to puparium formation. The late larval and prepupal ecdysone pulses are represented by hatched boxes. The timing of *BR-C, E74A, E75A, DHR3, βFTZ-F1,* and *E93* transcription in larval salivary glands is shown by black boxes, where the width of the box represents approximate levels of expression (Woodard *et al.,* 1994; Baehrecke and Thummel, 1995; Horner *et al.,* 1995; Huet *et al.,* 1995).

response genes, including early genes originally defined as ecdysone-inducible puffs in the larval salivary gland polytene chromosomes (Ashburner *et al.,* 1974). The *Broad-Complex* (*BR-C*), *E74,* and *E75* early genes encode families of transcription factors that transduce and amplify the hormonal signal by coordinating the expression of large batteries of secondary-response late genes (Thummel, 1996; Richards, 1997). The *BR-C* encodes a family of zinc finger transcription factors (DiBello *et al.,* 1991), *E74* encodes two isoforms of an ETS domain transcription factor, designated E74A and E74B (Burtis *et al.,* 1990), and *E75* encodes three orphan members of the nuclear receptor superfamily, designated *E75A, E75B,* and *E75C* (Segraves and Hogness, 1990). Genetic studies have demonstrated that *BR-C* and *E74* are required for appropriate developmental responses to ecdysone during metamorphosis (Kiss *et al.,* 1988; Restifo and White, 1992; Fletcher *et al.,* 1995). Furthermore, molecular studies have indicated that these genes can exert their effects through direct regulation of late gene transcription (Urness and Thummel, 1995; Crossgrove *et al.,* 1996). *BR-C, E74A,* and *E75A* are induced directly by ecdysone in late-third-instar larvae, rapidly repressed, and then reinduced by the prepupal hormone pulse that triggers the prepupal–pupal transition (Fig. 1) (Karim and Thummel, 1992; Huet *et al.,* 1993).

In addition to *E75,* a growing number of orphan nuclear receptors have been identified that are regulated by ecdysone and expressed during the onset of *Drosophila* metamorphosis (Thummel, 1995). Orphan nuclear receptors contain both the DNA binding and ligand binding/dimerization domains characteristic of nuclear receptors (Mangelsdorf and Evans, 1995). These receptors are referred to as orphans since their corresponding ligands, if they do exist, have not yet been identified. Indeed, there is no need to evoke a ligand for many of the *Drosophila* orphans, since they are expressed for very brief periods and ectopic expression studies have demonstrated that they can function as constitutive transcription factors.

Molecular and genetic studies have implicated the DHR3 orphan nuclear receptor as a regulator of the larval–prepupal transition during the onset of metamorphosis. DHR3 is most closely related to the human RORα orphan receptor (75% identity within the DNA binding domain) (Koelle *et al.,* 1992). Consistent with this sequence identity, DHR3 can bind as a monomer to a single AGGTCA core sequence that is preceded by an AT-rich sequence (Lamm *et al.,* 1997). *DHR3* is expressed in parallel with ecdysone pulses throughout development and is induced directly by ecdysone in late-third-instar larvae (Koelle *et al.,* 1992; Horner *et al.,* 1995). Like other early-late genes, however, its peak expression is delayed relative to that of the early genes (Horner *et al.,* 1995; Huet *et al.,* 1995). This delay appears to be due to a requirement for ecdysone-induced protein synthesis to achieve maximal levels of *DHR3* transcription. A similar mechanism of ecdysone regulation has been reported for the *Manduca sexta* homolog of *DHR3, MHR3,* leading to a similar temporal delay in its peak level of expression (Palli *et al.,* 1992). As a result of this delay, *DHR3* is expressed in early prepupae, as the early genes are repressed and *βFTZ-F1* is induced (Fig. 1).

βFTZ-F1 encodes another orphan member of the nuclear receptor superfamily, related to the vertebrate SF-1 orphan receptor (Lavorgna *et al.,* 1993). *βFTZ-F1* is expressed during a brief interval in midprepupae, immediately preceding

the reinduction of the early genes by the prepupal pulse of ecdysone (Fig. 1). Gain-of-function and loss-of-function genetic studies have demonstrated that *βFTZ-F1* is a critical regulator of the prepupal–pupal transition (Woodard *et al.*, 1994; Broadus *et al.*, 1998). *βFTZ-F1* is both necessary and sufficient for maximal reinduction of early gene transcription in prepupae. Induction of the stage-specific *E93* early gene is also dependent on *βFTZ-F1*. *E93* is induced directly by ecdysone in late prepupal salivary glands but shows no response to the same signal several hours earlier, in a late-third-instar larva (Fig. 1) (Baehrecke and Thummel, 1995). Consistent with this regulatory function, *βFTZ-F1* mutants pupariate normally but display defects in the prepupal–pupal transition, including adult head eversion, leg elongation, and salivary gland programmed cell death (Broadus *et al.*, 1998). These observations define *βFTZ-F1* as a critical competence factor that determines the appropriate genetic and biological responses to the prepupal pulse of ecdysone.

Several observations have led to the proposal that *DHR3* functions as both a repressor of the early genes and an inducer of *βFTZ-F1*. Antibody stains of the larval salivary gland polytene chromosomes identified more than 100 sites bound by DHR3 protein, including the puff loci that encompass the *EcR*, *BR-C*, *E74*, and *βFTZ-F1* genes (Lam *et al.*, 1997; White *et al.*, 1997). In addition, the *βFTZ-F1* promoter contains three essential DHR3 binding sites, indicating that DHR3 can directly regulate its transcription (Kageyama *et al.*, 1997). Finally, ectopic expression of DHR3 in late-third-instar larvae is sufficient to repress early gene transcription and induce premature *βFTZ-F1* expression (Lam *et al.*, 1997; White *et al.*, 1997). Taken together, these observations led to the model that *DHR3* determines the transition from a larva to a prepupa by arresting the regulatory response to the late larval ecdysone pulse and facilitating induction of the βFTZ-F1 prepupal competence factor.

Testing this model became possible through the recent isolation of *DHR3* mutations (Carney *et al.*, 1997). Apparent *DHR3* null alleles lead to lethality during embryogenesis with defects in the nervous system and muscles. This early lethality is consistent with the high levels of *DHR3* expression during midembryogenesis (Koelle *et al.*, 1992). None of the 11 *DHR3* mutant alleles, however, allow survival beyond embryogenesis, leaving it unclear what roles *DHR3* might play during later stages of development.

Here we use both a heat-inducible *DHR3* rescue construct and clonal analysis to characterize *DHR3* functions during metamorphosis. These studies demonstrate that *DHR3* is required for adult bristle, wing, and cuticle development as well as the ecdysone-triggered morphological movements that constitute the earliest steps in metamorphosis. The lethal phenotypes of *DHR3* mutants are consistent with a role for *DHR3* in larval muscle function as well as the effects of *DHR3* mutations on ecdysone-regulated gene expression. *DHR3* is not required for early gene repression at puparium formation but is required for maximal *EcR*, *E74B*, and *βFTZ-F1* transcription in midprepupae. The reduction in *βFTZ-F1* expression leads to a corresponding reduction in early gene transcription by the prepupal pulse of ecdysone which, in turn, results in head eversion and salivary gland cell death defects that resemble *βFTZ-F1* mutant phenotypes. These observations indicate that *DHR3* plays an essential role in inducing the βFTZ-F1 competence factor and thus provides a functional link between the late larval and prepupal responses to ecdysone.

MATERIALS AND METHODS

Fly stocks. Three *DHR3* mutant alleles were used in these studies: $DHR3^{G60S}$, $DHR3^{R107G}$, and $DHR3^{22-35}$ (Carney *et al.*, 1997). $DHR3^{G60S}$ and $DHR3^{R107G}$ encode proteins with single amino acid changes at highly conserved positions within the DNA binding domain. In contrast, $DHR3^{22-35}$ is a rearrangement involving the exon that encodes the DHR3 DNA binding domain. These mutations are likely to be null alleles for the *DHR3* locus (Carney *et al.*, 1997). The *P[w⁺; hs-DHR3]* P element (insertion 22C) is located on the third chromosome and is homozygous viable (Lam *et al.*, 1997). Mutant phenotypes were examined in animals carrying a *DHR3* mutation in combination with *Df(2R)12*, a small X-ray-induced deficiency that removes the *DHR3* locus (Weber *et al.*, 1995; R. Burgess and T. Schwarz, personal communication). Two balancers were used to identify mutant larvae: *In(2LR)Bc Gla* and *CyO, y⁺*, scoring for loss of the *Bc* or y^+ markers, respectively. Third-instar larvae were staged by supplementing the food with 0.5% bromophenol blue and scoring for gut staining, while prepupae were staged from puparium formation (Andres and Thummel, 1994).

Rescue of DHR3 mutants. Flies of the following genotypes were crossed: *w;*$DHR3^{G60S}$ or $DHR3^{22-35}$*/In(2LR)Bc Gla* were crossed with *w;(Df(2R)12/In(2LR)Bc Gla; P[w⁺; hs-DHR3]* or *y w;*$DHR3^{G60S}$ or $DHR3^{22-35}$*/CyO, y⁺* were crossed with *y w;Df(2R)12/CyO,y⁺;P[w⁺; hs-DHR3].* Eggs were collected from these crosses for 6 h on small grape agar dishes and allowed to age for 4 h. The dishes were floated in a water bath at 35°C for 30 min after which the embryos were transferred into vials and maintained at 25°C. The vials were heat treated at 35°C for 15 min at four 24-h intervals (28–34, 52–58, 76–82, and 100–106 h after egg laying). *DHR3* mutants [$DHR3^{G60S}$*/Df(2R)12* or $DHR3^{22-35}$*/Df(2R)12*] were identified by looking for larvae without black cells or with yellow mouth hooks, depending on which balancer was used. Siblings from the same vial that were carrying a balancer chromosome were used as internal controls. Control animals began to pupariate ~1 day after the final heat treatment, whereas *DHR3* mutants were further delayed, beginning to pupariate ~2 days after the final heat treatment. Animals were staged relative to puparium formation for phenotypic characterization.

Western blot analysis. Protein extracts were prepared from staged animals by homogenization in sodium dodecyl sulfate (SDS) sample buffer. Protein samples corresponding to approximately one animal/time point were loaded onto each lane of a 6% SDS–polyacrylamide gel, fractionated by electrophoresis, and transferred to a nitrocellulose membrane (Amersham Hybond-ECL). DHR3 protein was detected by incubating the membrane with a 1:500 dilution of affinity-purified anti-DHR3 antibodies (Lam *et al.*, 1997), followed by a 1:1500 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson Labs), and the Amersham ECL detection protocol for chemiluminescence.

Northern blot hybridiziation. Rescued $DHR3^{G60S}/Df(2R)12$ mutants along with control siblings bearing a balancer chromosome were selected as white prepupae and collected at 2-h intervals throughout prepupal development (see rescue conditions described above). Total RNA was extracted from these staged prepupae as described (Andres and Thummel, 1994), and samples corresponding to approximately two animals were loaded per lane on a formaldehyde–agarose gel. RNA was fractionated by electrophoresis, transferred to nylon membranes, probed, and washed as described (Karim and Thummel, 1991). The probes used in this study have been described (Andres *et al.*, 1993).

Clonal analysis. Mosaics were created using the FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993). Three different *DHR3* mutant alleles, $DHR3^{G60S}$, $DHR3^{22-35}$, and $DHR3^{R107G}$, were recombined onto two different chromosomes bearing FRT insertions at the base of 2R, *P[ry*$^+$*; hs-neo; FRT]42D* and *P[w*$^+$*; FRT]42B,* and FLP recombinase was provided by crossing stocks with *P[ry*$^+$*, hs-FLP]3F; In(2LR)Bc Gla/CyO*. The following crosses were used for mosaic analysis in the absence of *Minute*, scoring for *yellow* (*y*) mutant patches; $y^1\ w^{1118}/Y$; *FRT42D,* $DHR3^x/CyO$ were crossed with $y^1\ w^{1118}$, *FLP3F; In(2LR)Bc Gla/CyO* (using either the $DHR3^{G60S}$ or $DHR3^{22-35}$ alleles). $y^1\ w^{1118}$ *FLP3F/Y; FRT42D,* $DHR3^x/CyO$ males were selected and crossed with $y^1\ w^{1118}$; *FRT42D,* y^+ females. Progeny were heat-treated at 38°C for 30 min to induce recombination, and non-Cy females were scored for mutant patches: $y^1\ w^{1118}$, $P[FLP]3F/y^1\ w^{1118}$; *FRT42D,* $DHR3^x/FRT42D$, y^+. The non-*Cy* males were scored as internal controls (no FLP recombinase): $y^1\ w^{1118}/Y$; *FRT42D,* $DHR3^x/FRT42D$, y^+. FLP recombinase was induced between 24 and 96 h after egg laying. Most clones analyzed were generated between 48 and 96 h after egg laying. These studies were repeated with $DHR3^{G60S}$, $DHR3^{22-35}$, and $DHR3^{R107G}$ alleles linked to FRT42B. Similar crosses were performed for clonal analysis in the wing, scoring *pwn* clones in flies of the following genotype: $y^1\ w^{1118}$, $P[FLP]3F/y^1\ w^{1118}$, *FRT42D, pwn,* $DHR3^x/FRT42D$, y^+ (using either $DHR3^{G60S}$, $DHR3^{22-35}$, or $DHR3^{R107G}$ alleles). Finally, the *FRT42B, DHR3* stocks were used for mosaic analysis in a *Minute* background to increase clone size. *y* clones were scored in flies of the following genotype: $y^1\ w^{1118}$, $P[FLP]3F/y^1\ w^{1118}$; *FRT42B,* $DHR3^x/FRT42B$, y^+, *M(2)53* (using either $DHR3^{G60S}$, $DHR3^{22-35}$, or $DHR3^{R107G}$ alleles). Most animals carrying clones in a *Minute* background died prior to eclosion, although the use of *FRT42B,* which was less efficient than *FRT42D* at inducing clones, result in more survivors.

RESULTS

Ectopic DHR3 Expression Can Rescue DHR3 Mutants to Later Stages of Development

DHR3 protein is widely expressed during midembryogenesis (Lam *et al.*, 1997), raising the possibility that ectopic *DHR3* expression from the *hsp70* promoter might rescue the embryonic lethality associated with *DHR3* mutations. To test this possibility, embryos were collected that carried the *P[w*$^+$*; hs-DHR3]* transgene in combination with either $DHR3^{G60S}/Df(2R)12$ *or* $DHR3^{22-35}/Df(2R)12$, and several heat treatment regimens were tested for their ability to rescue these mutants to the third larval instar. A heat pulse during midembryogenesis followed by four heat treatments at 24-h intervals, approximating the normal peaks of *DHR3* transcription (Koelle *et al.*, 1992), rescued ~7% of the *DHR3* mutants to the end of larval development ($n = 100$). Increasing or decreasing the temperature or frequency of the heat treatments did not increase the efficiency of rescue. Furthermore, the presence of the construct itself, in the absence of heat treatment, was not sufficient to rescue lethality. This frequency of rescue was sufficient to allow a detailed characterization of *DHR3* function during the onset of metamorphosis.

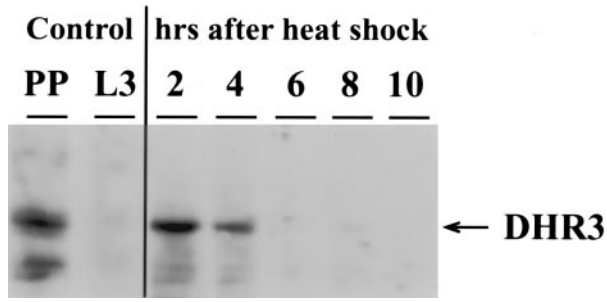
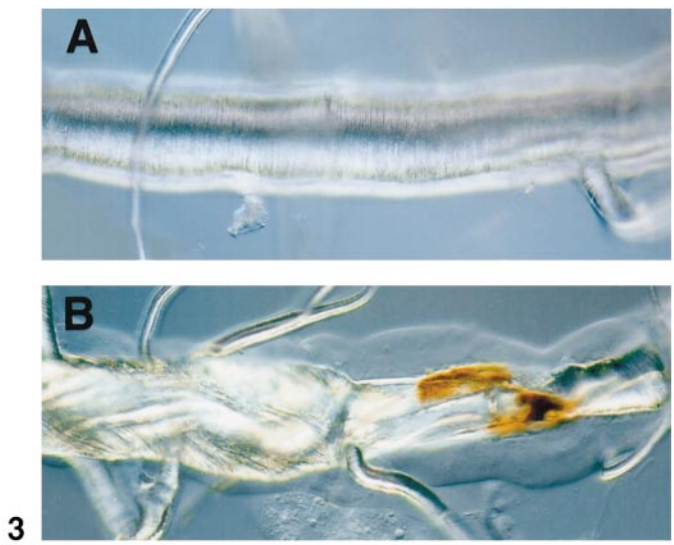


FIG. 2. Ectopically expressed DHR3 protein is unstable. Mid-third-instar larvae carrying the *P[w*$^+$*;hs-DHR3]* rescue construct were subjected to a 30-min heat treatment at 35°C and allowed to recover at 25°C for 2, 4, 6, 8, or 10 h. Protein extracts were prepared and analyzed by Western blotting using a DHR3 polyclonal antibody. A protein extract was prepared from mid-third-instar larvae prior to heat treatment, as a negative control (L3). As a positive control, extracts were prepared from $DHR3^{G60S}/CyO,y^+$ or $Df(2R)12/CyO,y^+$ prepupae (PP). A second gel was run in parallel and stained with Coomassie blue to confirm that approximately equal amounts of total protein were present in each lane (data not shown).

Rescued *DHR3* mutants were selected for phenotypic characterization at least one day after the final heat treatment. To determine if residual DHR3 protein might be present in these animals, we determined the stability of DHR3 protein expressed from the *hs-DHR3* transgene. Mid-third-instar larvae carrying the *P[w*$^+$*; hs-DHR3]* transgene were subjected to a 30-min heat treatment and allowed to recover for different periods, after which protein extracts were assayed for DHR3 protein by Western blot analysis (Fig. 2). High levels of DHR3 protein could be detected by 2 h after heat treatment, comparable to the endogenous levels present in early prepupae. DHR3 protein was, however, undetectable by 6 h after heat treatment. This rapid turnover is consistent with the stability of DHR3 protein observed during normal prepupal development (White *et al.*, 1997). We conclude, therefore, that the rescued mutants lack most, if not all, *DHR3* function by the third larval instar.

DHR3 Mutants Have Defects in the Tracheal Cuticle and Fail to Progress through the Early Stages of Metamorphosis

About half of the *DHR3* mutants rescued to the third larval instar display defects in their tracheal system. Although the formation and branching of the tracheal system



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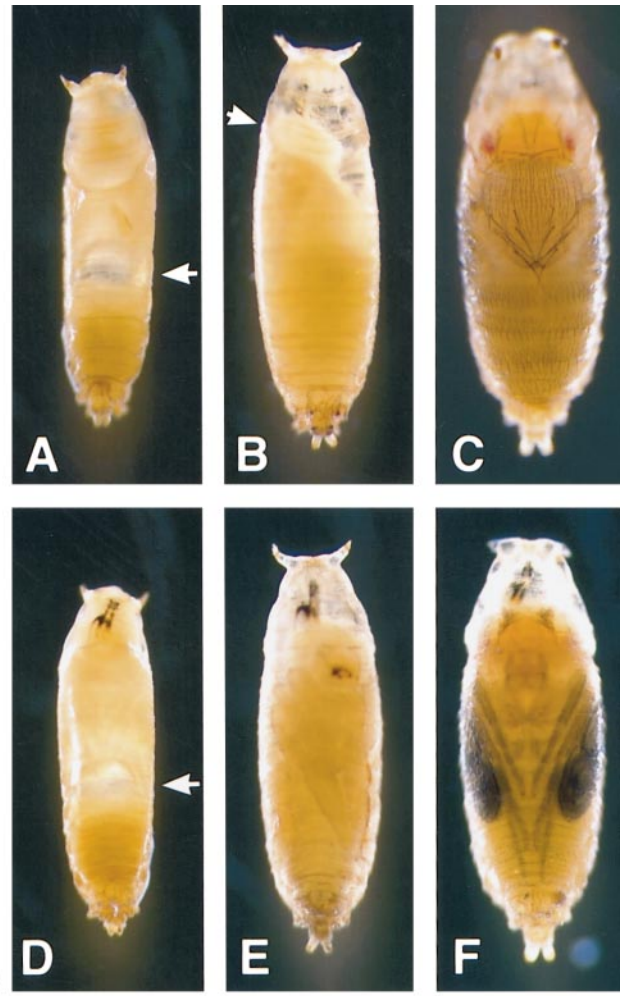

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FIG. 3. Tracheal defects in *DHR3* larvae. Dorsal tracheal trunks were dissected from (A) a control third-instar larva ($DHR3^{G60S}/CyO,y^{+}$; $P[w^{+}$; *hs-DHR3]/+* or *Df(2R)12/CyO*, y^{+}; $P[w^{+}$; *hs-DHR3]/+*) or (B) a $DHR3^{G60S}/Df(2R)12$; $P[w^{+}$; *hs-DHR3]/+* mutant, and mounted in glycerol for photography. The taenidial folds can be seen in (A) as closely spaced parallel ridges that run circumferentially along the internal surface of the tracheal cuticle. The taenidia are severely distorted in *DHR3* mutants (B), resulting in collapse of the tracheal cuticle followed by necrosis.

FIG. 4. Prepupal and pupal lethal phenotypes in *DHR3* mutants. $DHR3^{G60S}/Df(2R)12$; $P[w^{+}$; *hs-DHR3/+* animals were rescued to the third larval instar as described under Materials and Methods. These mutants displayed three distinct lethal phases during prepupal and pupal development. Dorsal (A–C) and ventral (D–F) views of representative animals are shown. *DHR3* mutants die as either a prepupa with a failure in gas bubble translocation (A,D), an early pupa with head eversion defects (B,E) or an externally normal pharate adult (C,F). The gas bubble is marked by arrows in (A) and (D). The arrow in (B) marks the partially everted adult head. Note that the legs in (E) appear fully elongated.

appear normal in *DHR3* mutants, the cuticular structure of the tracheae is defective. The taenidial folds of the tracheal cuticle can be severely distorted (Fig. 3B). In more severe cases, this can lead to collapse of the tracheal cuticle and obstruction of the lumen, followed by necrosis (Fig. 3B). These defects are not present in control siblings subjected to the same heat treatment regime (Fig. 3A).

DHR3 mutants pupariate with a slight delay relative to wild-type siblings that carry a balancer chromosome. These mutants display three distinct lethal phases during metamorphosis: about 25% of the *DHR3* mutants die during prepupal development (Figs. 4A, 4D), 70% die during early pupal development (Figs. 4B, 4E), and 5% survive until the pharate adult stage (Figs. 4C, 4F) ($n = 72$). Some of the dead prepupae are slightly bent at the anterior end and fail to fully evert their anterior spiracles, indicating defects in puparium formation (Figs. 4A, 4D). All dead prepupae also display defects in gas bubble translocation (arrows, Figs. 4A,

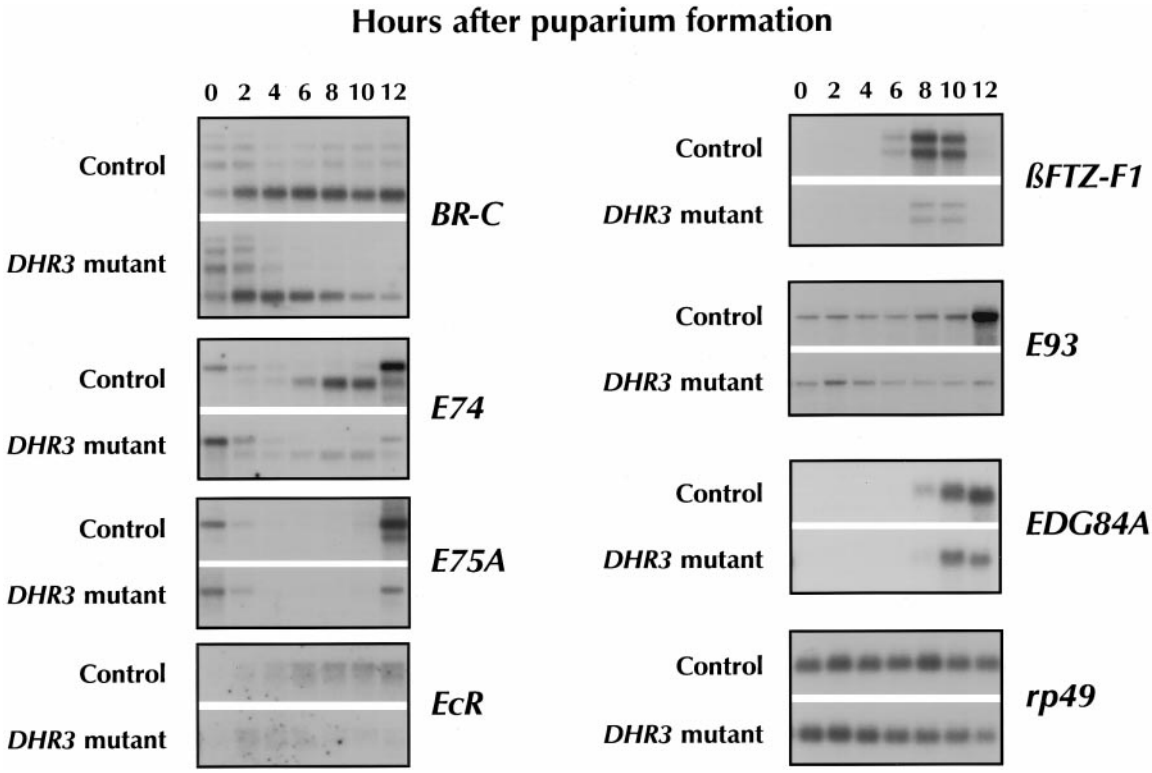

FIG. 5. *DHR3* is required for ecdysone-regulated gene expression in prepupae. *DHR3* mutants and control siblings were staged as prepupae and RNA samples were analyzed by Northern blot hybridization as described under Materials and Methods. Control = $DHR3^{G60S}$/*In(2LR)Bc Gla; P[w*$^{+}$*; hs-DHR3]/+* or *Df(2R)12/In(2LR)Bc Gla; P[w*$^{+}$*; hs-DHR3]/+*. *DHR3* mutant = $DHR3^{G60S}$/*Df(2R)12;P[w*$^{+}$*; hs-DHR3]/+*. Similar results were observed when *CyO, y*$^{+}$ was used as a balancer to select *DHR3* mutant larvae (data not shown). A *BR-C* probe was used to detect all four size classes of RNA (DiBello *et al.*, 1991). Similarly, the *E74* probe detected both the *E74B* (lower band) and *E74A* (upper band) mRNA isoforms. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer (O'Connell and Rosbash, 1984). As expected, the levels of *rp49* mRNA decline gradually following puparium formation (Andres *et al.*, 1993). Mutant and control blots were hybridized, washed, and autoradiographed together to allow a direct comparison of the levels of target gene transcription.

4D). The major lethal phase, during early pupal development, is associated with incomplete eversion of the adult head and defects in gas bubble translocation (Fig. 4B). The legs, however, appear to evert and elongate normally in *DHR3* mutants (Figs. 4D–4F). Identical lethal phenotypes were observed in $DHR3^{G60S}$/*Df(2R)12* and $DHR3^{22-35}$/*Df(2R)12* mutants (data not shown).

Programmed cell death of the larval salivary glands normally occurs near the time of adult head eversion (Jiang *et al.*, 1997). To determine if this response to ecdysone is dependent on *DHR3* function, salivary glands were dissected from five *DHR3* mutant pupae 24 h after puparium formation. Salivary glands were detected in four of these animals, ~10 h after the glands would have normally undergone cell death (data not shown).

DHR3 Is Required for Ecdysone-Regulated Gene Expression during Midprepupal Development

The majority of rescued *DHR3* mutants die during prepupal and early pupal development with defects in adult head eversion and salivary gland cell death. These phenotypes suggest that *DHR3* may be required for the appropriate genetic responses that lead to the prepupal–pupal transition. Furthermore, ectopic expression experiments have indicated a role for *DHR3* in both early gene repression and induction of *βFTZ-F1* (Lam *et al.*, 1997; White *et al.*, 1997). To test these *DHR3* functions, *DHR3* mutants were rescued to the onset of metamorphosis and RNA was extracted from animals collected at 2-h intervals from 0 to 12 h after puparium formation. These RNA samples were analyzed by Northern blot hybridization using a variety of probes directed against ecdysone-regulated genes that are expressed during prepupal development (Fig. 5). All genes examined are expressed normally in control siblings, in patterns that closely parallel those seen in wild-type prepupae (Andres *et al.*, 1993). The expression of many ecdysone-regulated genes, however, is affected in *DHR3* mutants. Slightly higher levels of *BR-C*, *E74A*, and *E75A* early gene transcription can be detected in 0- and 2-h *DHR3* mutant prepupae, consistent with a role for *DHR3* in repression of early gene

transcription. This is, however, a minor effect, indicating that other negative regulators must contribute to this response.

EcR and *E74B* are induced in wild-type midprepupae in parallel with *βFTZ-F1* (Andres *et al.*, 1993). Interestingly, *E74B*, *EcR*, and *βFTZ-F1* are all submaximally transcribed in *DHR3* mutant prepupae, suggesting that they are under coordinate regulation (Fig. 5). Furthermore, the *BR-C*, *E74A*, *E75A*, and *E93* early genes are submaximally induced by the prepupal ecdysone pulse in *DHR3* mutants. These latter effects are most likely indirect, since DHR3 protein is not expressed in late prepupae (White *et al.*, 1997). The *EDG84A* pupal cuticle gene is slightly delayed and reduced in *DHR3* mutants (Fig. 5). In contrast, expression of the endogenous *DHR3* gene as well as *E75B*, *E78B*, the *L71-6* late puff gene, and *Stubble* transmembrane protease gene is unaffected by *DHR3* mutations (data not shown).

Clonal Analysis of DHR3 Mutants Reveals Functions in Bristle, Wing, and Cuticular Development

The majority of rescued *DHR3* mutants (95%) die as prepupae or early pupae, preventing an analysis of *DHR3* requirements during adult development. To assess these functions, we analyzed clones of $DHR3^{G60S}$, $DHR3^{R107G}$, and $DHR3^{22–35}$ mutant cells in adult flies using the FLP/FRT recombination system (Golic and Lindquist, 1989; Xu and Rubin, 1993). Identical phenotypes were observed in all three *DHR3* mutant backgrounds. *DHR3* mutant clones were recovered in the head, thorax, legs, abdomen, and wings and were of the expected size and frequency, indicating that a loss of *DHR3* activity is not cell lethal. Although the markers that were used did not allow the identification of *DHR3* clones in the eyes, no eye defects were observed in mosaic animals, suggesting that *DHR3* is not required for the development of this tissue.

Generation of *DHR3* mutant clones resulted in a high frequency of adults with wings that were either curved or folded and held out from the body (Fig. 6). Clones generated between 72 and 96 h after egg laying resulted in 54% of the flies carrying the $DHR3^{22–35}$ mutation ($n = 68$) or 62% of the flies carrying the $DHR3^{R107G}$ mutation ($n = 76$) having deformed wings. The *pawn* (*pwn*) marker was recombined onto *FRT-DHR3* mutant chromosomes to more accurately determine the role of *DHR3* in wing formation. Both normal and deformed wings were dissected from adult flies carrying *DHR3* mutant clones. Examination of morphologically normal wings revealed that 96% ($n = 26$) contained *DHR3* mutant clones over ≤5% of the wing surface. In contrast, 60% ($n = 20$) of the deformed or severely curved wings contained *DHR3* mutant clones over ~20–40% of their surface, and 40% contained mutant clones over ~50–75% of their surface. Furthermore, in many cases, the locations of curves and folds in the wings correlated with the presence of *DHR3* mutant clones (data not shown). These observations indicate a role for *DHR3* in proper wing development during metamorphosis. In contrast, no phenotypes were associated with *DHR3* mutant clones in the legs (data not shown).

On the head and thorax, *DHR3* mutant clones were associated with short and rough macrochaetae (yellow arrows, Fig. 7A). Of 203 mutant bristles examined in $DHR3^{G60S}$ clones, 96.6% were short and rough. Similarly, 99.7% of 365 mutant bristles in $DHR3^{22–35}$ clones were short and rough. In contrast, *DHR3* mutant clones on the legs had apparently normal bristles (data not shown). Although mutations in the ecdysone-induced *Stubble* gene result in truncated bristles, the phenotype is clearly distinct from the rough bristles observed in *DHR3* mutant clones. Further studies are required to determine how *DHR3* might function in this developmental pathway.

To obtain larger mutant clones on the body, *DHR3* mutant clones were created in a *Minute* background and followed with a y^+ marker. In one experiment, 26 adults were recovered that carried a $DHR3^{22–35}$ mutant clone covering at least one-third of the notum. Of these, 62% carried clones with light, apparently immature, cuticle (Fig. 7B) and 58% displayed indentations in the notum (Fig. 7C). It seems likely that these phenotypes are related in that the dents could arise from a weakened and immature cuticle. Short and rough bristles were also associated with these mutant patches. Similar phenotypes were observed in $DHR3^{R107G}$ mutant clones (data not shown).

$DHR3^{22–35}$ mutant clones in a *Minute* background also revealed defects in the abdomen. Of 37 clones examined in the abdomen, 24% were one to two bristles in size and these microchaetae appeared normal in morphology (although their small size made it difficult to determine if they were rough). Some of these bristles, however, did not point toward the posterior of the body like wild-type abdominal bristles, but rather pointed to lateral positions (single arrow, Fig. 7D). Of the remaining clones examined, 32% covered two bristles at the segmental boundaries and displayed an absence of black pigmentation (Fig. 7E). Finally, 43% of these clones were larger than two bristles and showed distortions in the structure of the abdomen as well as both lost pigmentation and missing bristles (Fig. 7F). Similar phenotypes were observed in $DHR3^{R107G}$ mutant clones (data not shown).

DISCUSSION

The pulse of ecdysone that triggers puparium formation signals a wide range of developmental responses including programmed cell death of the anterior larval muscles and midgut, evagination of the imaginal discs, and abdominal histoblast proliferation (Robertson, 1936; Bodenstein, 1965). The prepupal pulse of ecdysone, 10–12 h after puparium formation, triggers a distinct set of responses including adult head eversion, leg elongation, and destruction of the larval salivary glands. In this paper, we demonstrate that the DHR3 orphan nuclear receptor provides a critical

link between these two ecdysone-triggered developmental pathways, ensuring that the biological responses to the prepupal pulse of ecdysone will be distinct from those induced in late larvae, and thus that the animal progresses in an appropriate manner through the early stages of metamorphosis.

DHR3 Is Required for Patterning and Integrity of the Larval Tracheal Cuticle and Adult Thoracic and Abdominal Cuticles

Respiration in *Drosophila* larvae is achieved via the tracheal system, a tubular epithelial network that provides passive diffusion of gases throughout the animal (Manning and Krasnow, 1993). The epithelial cells of the tracheae secrete a cuticle from their apical surface that lines the lumen and provides structural support. This cuticle is shed at each larval molt, in parallel with replacement of the external larval cuticle. A new cuticle is then secreted and the old cuticle is removed through specialized spiracles (Noirot and Noirot-Thimotèe, 1982).

The abnormal tracheae in *DHR3* mutants appear to result from defects in patterning of the cuticular taenidia. The taenidia form parallel ridges that run circumferentially along the internal surface of the tracheal cuticle (Fig. 3A). In *DHR3* mutants, the taenidia are severely disarranged. As a result, the tracheal cuticle loses its structural integrity, collapses, and undergoes necrosis (Fig. 3B). It seems likely that these defects arise at the molts, when a new layer of cuticle is deposited by the tracheal epithelium. Expression of *DHR3* mRNA at each larval molt, and DHR3 protein in the tracheal epidermis of third-instar larvae, is consistent with this proposal (Koelle *et al.*, 1992; Lam *et al.*, 1997). Moreover, molting of the tracheal cuticle is regulated by ecdysone in parallel with molting of the external larval cuticle (Ryerse and Locke, 1978).

Recent characterization of another ecdysone-inducible orphan nuclear receptor, DHR78, has revealed a similar role in patterning and molting of the tracheal cuticle (Fisk and Thummel, 1998). *DHR78* mutations lead to lethality during the third larval instar with a failure to activate the mid-third-instar regulatory hierarchy that prepares the animals for the onset of metamorphosis. The less severe tracheal phenotypes observed in *DHR78* mutants resemble those seen in *DHR3* mutants, although more severe *DHR78* phenotypes include retention of the tracheal cuticle from earlier molts and breaks in the dorsal tracheal trunks. The similarity between the *DHR78* and *DHR3* tracheal phenotypes suggests that these genes may function in the same pathway to control the molting and patterning of the tracheal cuticle during the larval molts.

Interestingly, *DHR3* is also required for the patterning and integrity of the adult cuticle, as revealed by clonal analysis. The cuticle in a majority of large *DHR3* mutant clones in the notum appears immature (Fig. 7B) and is often weak and indented (Fig. 7C). Similarly, large *DHR3* clones in the abdomen are often associated with distortions in the cuticle, a lack of pigmentation, and missing bristles (Figs. 7E, 7F). Mutations in the *DHR38* orphan nuclear receptor gene are also associated with defects in the adult cuticle, although these are restricted to the apparently specialized cuticle at the leg joints (Kozlova *et al.*, 1998). Deposition of the adult cuticle appears to be dependent on ecdysone, but its regulation is poorly understood (Fristrom and Fristrom, 1993). Taken together, these studies indicate a role for ecdysone-inducible orphan nuclear receptors in tracheal and adult epithelium and indicate that further studies of these receptors may shed light on their roles in cuticle deposition.

DHR3 Mutant Phenotypes Indicate a Role in Larval Muscle Function

DHR3 mutants rescued to the third larval instar display three distinct lethal phases during metamorphosis. The earliest lethal phase is during prepupal development when 25% of *DHR3* mutants die with defects in gas bubble translocation (Figs. 4A, 4D). About half of these prepupae also fail to fully evert their anterior spiracles and do not shorten properly. This shortening defect is relatively mild and is apparent only as a slight bend at the anterior end of the animal (Figs. 4A, 4D). Most *DHR3* mutants (70%) arrest during early pupal development with defects in adult head eversion (Figs. 4B, 4E). Some of these animals also retain gas pockets at the anterior end (Fig. 4B). The remaining 5% die as pharate adults with no apparent developmental defects (Figs. 4C, 4F). It is likely that this small percentage of mutants represent animals that are partially rescued by the *DHR3* transgene. This proposal is supported by the observation that these animals do not show any of the defects associated with *DHR3* mutant clones in the adult.

Interestingly, all of the phenotypes observed in prepupae and early pupae can be explained by postulating a role for *DHR3* in larval muscle function. Contraction of the larval muscles is required to shorten the body segments at puparium formation, forming the characteristic barrel shape of the pupa (Robertson, 1936; Crossley, 1978). Shortly after puparium formation, the anterior larval muscles of the head and thorax are destroyed in response to ecdysone. The abdominal larval muscles, however, persist until after head eversion and contract during prepupal development, driving the movements required for pupation. The first of these movements involves a gas bubble that appears in the center of the prepupa, translocating it to the posterior end of the animal (Chadfield and Sparrow, 1985). Rythymic contractions of the abdominal muscles, accompanied by a swaying motion of the prepupa, then move the gas bubble along the sides of the animal toward the anterior end. As the gas bubble moves, the prepupa withdraws to the posterior of the puparium, creating a space at the anterior end. Contraction of the abdominal muscles then forces the head to evert into the newly created space at the anterior end of the puparium as the gas bubble is expelled, apparently out the anterior spiracles (Robertson, 1936). The resultant pupa

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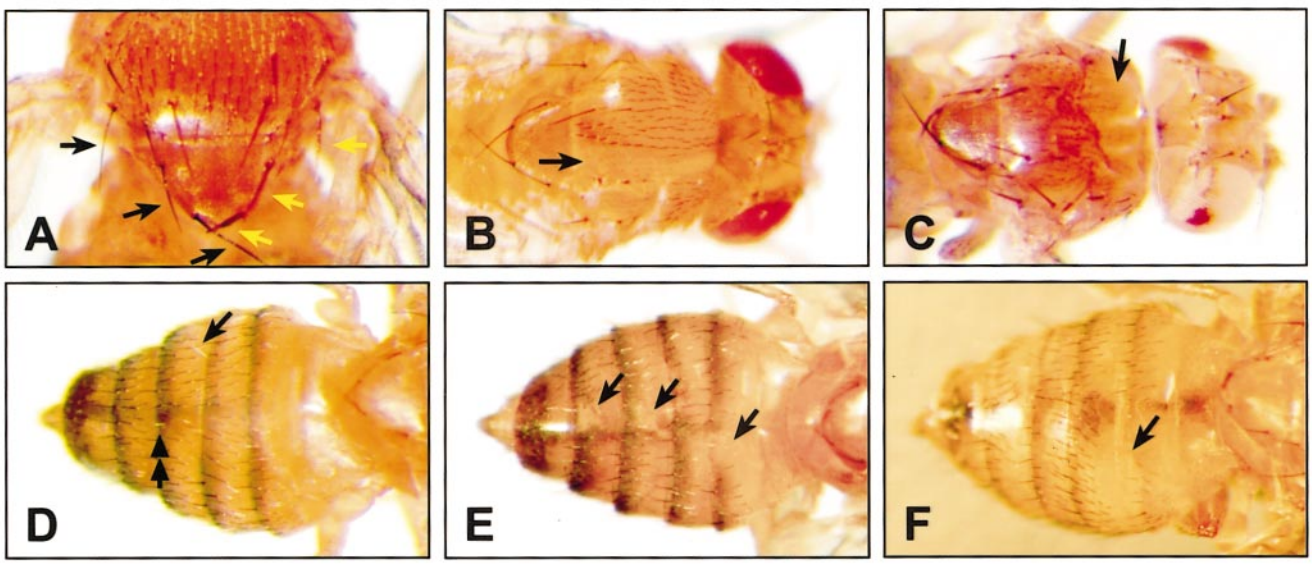

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FIG. 6. *DHR3*$^{22-35}$ mosaic flies have deformed wings that are held out from the body. Most adult flies bearing large *DHR3* mutant clones displayed deformed wings of varying severity. A representative adult with severely deformed wings is depicted. The wings on this fly are also held out away from the body.

FIG. 7. *DHR3* mutant clones are associated with short rough bristles, immature cuticle, and lack of abdominal pigmentation. *DHR3* mutant mosaic animals were generated using the FLP/FRT recombination system in a *Minute* background, as described under Materials and Methods. Clones were identified by the absence of the *yellow*$^{+}$ marker. (A) *DHR3*^{R107G} mutant clones on the thorax contain short and rough bristles. *yellow* mutant bristles are marked by yellow arrows while the contralateral wild-type bristle is marked with black arrows. (B) Some clones contain what appears to be immature cuticle and bristles (arrow) or (C) are associated with dents in the notum (arrow). (D) Abdominal *DHR3*$^{22-35}$ mutant clones show some apparently normal bristles while others point in the wrong direction. The *yellow* mutant bristles in this panel appear white. An abnormal mutant bristle (single arrow) is pointing laterally while a normal mutant bristle (double arrow) points to the posterior. (E) Abdominal *DHR3*$^{22-35}$ mutant clones also show an absence of pigmentation and bristles (arrows). (F) Severe deformations, loss of pigmentation, and missing bristles are associated with large *DHR3* mutant clones (arrow).

contains a distinct head, thorax, and abdomen and continues terminal differentiation to form the adult fly.

Genetic studies also indicate a role for larval muscles in pupariation and pupation. The larval muscle mutants *lethal(1)fibrillardysgenesis [l(1)fdg], lethal polymorph (lpm),* and *lethal(2)thin [1(2)tn]* form long, thin puparia and

display prepupal or pupal lethality (Benz, 1957; Newman and Wright, 1983; Ball *et al.*, 1985). Mutant *l(2)tn* prepupae also show defects in gas bubble translocation and head eversion (Ball *et al.*, 1985), and cryptocephalic phenotypes have been observed among *lpm* mutants (Benz, 1957). Larval muscle mutations also cause some degree of muscular dysfunction that is correlated with sluggish wandering behavior in third-instar larvae (Ball *et al.*, 1985). The absence of this phenotype in *DHR3* mutant larvae suggests that this mutation does not specifically affect larval muscle development. Rather, as discussed below, *DHR3* appears to play a role during later stages of larval muscle function.

EcR, *E74B*, and *βFTZ-F1* are all dependent on *DHR3* for their maximal levels of transcription in midprepupae (Fig. 5). Genetic studies have indicated roles for each of these genes in larval muscle function, suggesting that their submaximal expression in *DHR3* mutants could account for at least part of the observed *DHR3* lethal phenotypes. Prepupal lethal alleles of *EcR* and a *βFTZ-F1* mutation both result in head eversion defects that resemble those seen in *DHR3* mutants (Bender *et al.*, 1997; Broadus *et al.*, 1998). Similarly, *E74B* mutants die during prepupal and early pupal development with defects in gas bubble translocation and head eversion (Fletcher *et al.*, 1995). *E74B* has been proposed to exert its developmental effects by preventing premature destruction of the abdominal muscles. *DHR3* could function in a similar manner, either independently or through *E74B*. Alternatively, *DHR3* could act more directly in larval muscle function. Consistent with this proposal, DHR3 protein is expressed in the larval muscles of midprepupae, indicating that it can directly control the fate of this issue (G.L. and C.S.T., unpublished results). A method is now available that should allow us to distinguish between these possibilities, using green fluorescent protein as a marker to observe larval muscles in living prepupae and pupae (P. Reid and C.S.T., unpublished results). This system should provide a clearer understanding of the regulation of larval muscle function and apoptosis during metamorphosis.

Clonal analysis provides further support of a role for *DHR3* in larval muscle function. Flies bearing large *DHR3* mutant clones in a *Minute* mutant background often have their wings held out away from the body (Fig. 6). This phenotype is indicative of defects in the indirect flight muscles of the adult thorax which are remodeled from persisting larval muscles (Crossley, 1978). The larval muscles of *DHR3* mutants may thus provide a defective template for adult muscle formation, leading to the held out wing phenotype. Studies in *Manduca* have demonstrated that muscle remodeling during metamorphosis is dependent on ecdysone (Hegstrom and Truman, 1996). This pathway appears to be conserved in *Drosophila* because mutations in two ecdysone-inducible genes, *BR-C* and *who*, result in defects in the indirect flight muscles (Baehrecke, 1997; Sandstrom *et al.*, 1997; Bunch *et al.*, 1998). Some *myospheroid* mutations also lead to held out wings and interact genetically with *EcR* and *crooked legs* alleles, providing another link between ecdysone signaling and development of the indirect flight muscles (Wilcox *et al.*, 1989; D'Avino and Thummel, 1998; D'Avino and C.S.T., manuscript in preparation). Further studies of these genes should provide a clearer understanding of how the indirect flight muscles develop in response to ecdysone signaling.

DHR3 Provides a Functional Link between the Late Larval and Prepupal Genetic Responses to Ecdysone

Ectopic expression of DHR3 led to the model that this orphan nuclear receptor directs the larval-to-prepupal transition during the onset of metamorphosis (Lam *et al.*, 1997; White *et al.*, 1997). It was proposed to exert this function by both repressing early genes in newly formed prepupae and inducing the βFTZ-F1 competence factor in mid prepupae, thus arresting the late larval genetic response to ecdysone and preparing for the prepupal response. Our characterization of ecdysone-regulated gene expression in *DHR3* mutants provides confirmation for some, but not all, of these proposed regulatory functions. Contrary to the prediction from ectopic *DHR3* expression studies, the *BR-C*, *E74A*, and *E75A* early mRNAs are repressed at puparium formation in *DHR3* mutants, although the levels are slightly higher than in control animals (Fig. 5). Thus, although *DHR3* is sufficient to repress transcription of the early genes, it is not necessary for this response. We interpret these results by proposing that *DHR3* contributes to repression of the early genes at puparium formation, but that it does so in a redundant manner with one or more other ecdysone-induced transcription factors (Fig. 8). In support of this proposal, DHR3 protein is bound to the early genes in the salivary gland polytene chromosomes of newly formed prepupae and can function as a negative regulator of ecdysone-induced transcription in a transient transfection assay (Lam *et al.*, 1997; White *et al.*, 1997). The E75B/DHR3 heterodimer is a likely candidate for the repressor of the early genes, although the E78B orphan nuclear receptor may also contribute to this response (Stone and Thummel, 1993; White *et al.*, 1997). Both *E75B* and *E78B* mutants are viable, consistent with the proposal that they could function in a redundant manner with DHR3 (Russell *et al.*, 1996; W.A. Segraves, personal communication).

The level of *βFTZ-F1* mRNA in *DHR3* mutants is significantly reduced (Fig. 5), consistent with the ectopic gain-of-function studies (Lam *et al.*, 1997; White *et al.*, 1997) and indicating that *DHR3* is both necessary and sufficient for *βFTZ-F1* induction. Interestingly, White *et al.* (1997) have shown that E75B can heterodimerize with DHR3 and thereby block the ability of DHR3 to induce *βFTZ-F1* transcription (Fig. 8). Thus, the timing of *βFTZ-F1* induction appears to be determined by both accumulation of the DHR3 activator and decay of the E75B repressor. The observation that *E75B* expression is unaffected in *DHR3* mutants (data not shown) indicates that the reduction in *βFTZ-F1* transcription caused by this mutation is not due to

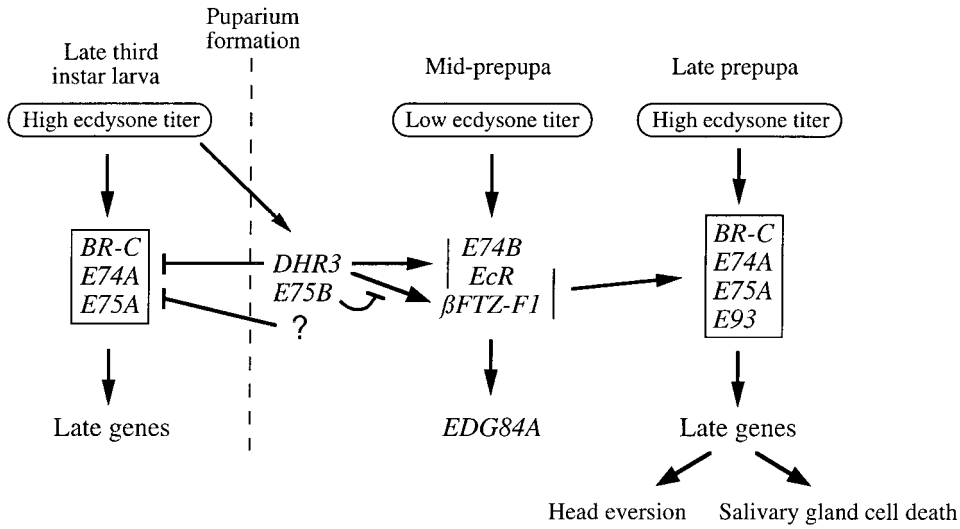

FIG. 8. Model for *DHR3* function during the onset of metamorphosis. *DHR3* is induced both directly and indirectly by the late larval ecdysone pulse, leading to a later period of expression relative to the *BR-C*, *E74A*, and *E75A* early mRNAs (Horner *et al.*, 1995). Although *DHR3* is sufficient to repress early gene transcription, little effect is seen in *DHR3* mutant prepupae (Fig. 5), suggesting that another repressor functions together with *DHR3* to direct this regulation (question mark). *DHR3* is both necessary and sufficient for *βFTZ-F1* induction (Lam *et al.*, 1997; White *et al.*, 1997; Fig. 5). E75B can mask this activation function by heterodimerizing with DHR3; hence the timing of *βFTZ-F1* induction is determined by both the induction of *DHR3* and decay of E75B (White *et al.*, 1997). *DHR3* is also required for maximal levels of *E74B* and *EcR* transcription in midprepupae (Fig. 5). *βFTZ-F1* provides the competence for the early genes to be reinduced by ecdysone in late prepupae and for stage-specific induction of *E93* in salivary glands (Broadus *et al.*, 1998). The head eversion and salivary gland cell death defects observed in *DHR3* mutants are consistent with an attenuation of the prepupal regulatory hierarchy. Cross-regulation of *DHR3* and *βFTZ-F1* thus provides a functional link between late larval and prepupal responses to ecdysone.

a delay in *E75B* expression, but rather elimination of the *DHR3* activator.

The induction of *E74B* and *EcR* that occurs in parallel with *βFTZ-F1* in midprepupae is also dependent on *DHR3*. This indicates that *DHR3* functions more widely than previously thought, as a general regulator of the midprepupal genetic response to ecdysone (Figs. 5, 8). Furthermore, the *BR-C*, *E74A*, *E75A*, and *E93* early mRNAs are submaximally induced by the prepupal ecdysone pulse (Fig. 5). Interestingly, DHR3 protein is not present at this time (White *et al.*, 1997). Rather, this effect appears to be an indirect consequence of reduced levels of *EcR* and *βFTZ-F1* (Fig. 8). Both *EcR* and *βFTZ-F1* are required for maximal transcriptional responses to ecdysone (Bender *et al.*, 1997; Broadus *et al.*, 1998). Similarly, transcription of the *EDG84A* pupal cuticle gene is slightly reduced and delayed in *DHR3* mutants (Fig. 5), similar to the effect seen in *βFTZ-F1* mutant prepupae (Broadus *et al.*, 1998). This model is also consistent with the defects in adult head eversion and salivary gland cell death in *DHR3* mutant pupae (Fig. 4 and data not shown), which resemble lethal phenotypes associated with *EcR* and *βFTZ-F1* mutations (Bender *et al.*, 1997; Broadus *et al.*, 1998). It seems likely that these developmental defects in *DHR3* mutants arise from submaximal early gene induction as a consequence of the reduced levels of *EcR* and *βFTZ-F1* expression (Fig. 8).

βFTZ-F1 has been shown to function as an essential competence factor for stage-specific responses to the prepupal pulse of ecdysone (Broadus *et al.*, 1998). Given that *βFTZ-F1* is dependent on *DHR3* for its maximal expression, this study identifies *DHR3* as an indirect regulator of the prepupal–pupal transition. In this regard it is interesting to note that the legs elongate normally in *DHR3* mutants unlike the short legs observed in *βFTZ-F1* mutants. These results suggest that another regulator must function as an inducer of *βFTZ-F1* expression in this tissue.

The dependence of *βFTZ-F1* transcription on *DHR3* demonstrates that these two orphan nuclear receptors provide a critical link between the late larval and prepupal genetic responses to ecdysone. The stage-specific induction of *DHR3* indicates that the animal has progressed through pupariation formation. One manifestation of this progress is direct repression of the late larval ecdysone hierarchy, possibly the *DHR3* and one or more other negative regulators (Fig. 8). In addition, *DHR3* induces *EcR*, *E74B*, and *βFTZ-F1* in midprepupae, thereby facilitating reinduction of the early genes in late prepupae as well as directing stage-specific induction of early genes such as *E93*. Presumably, it is through the stage-specific early regulatory genes like *E93* that biological response to the prepupal pulse of ecdysone are distinguished from those triggered by the hormone in late-third-instar larvae. Thus, *DHR3* and

βFTZ-F1 ensure that the animal progresses through metamorphosis with appropriate stage-specific responses to each pulse of hormone. This cross-regulatory network provides a mechanism by which a single hormonal signal can be refined into distinct stage-specific pathways during development.

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