Development 124, 1757-1769 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV6277

Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis

Geanette T. Lam[1], Changan Jiang[2] and Carl S. Thummel[1,2]

[1]Howard Hughes Medical Institute, [2]Department of Human Genetics, 5200 Eccles Institute of Human Genetics, University of Utah, Salt Lake City, UT 84112, USA

SUMMARY

The *DHR3* orphan receptor gene is induced directly by the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *DHR3* expression peaks in early prepupae, as the early puff genes are repressed and *βFTZ-F1* is induced. Here we provide evidence that *DHR3* directly contributes to both of these regulatory responses. DHR3 protein is bound to many ecdysone-induced puffs in the polytene chromosomes, including the early puffs that encode the *BR-C* and *E74* regulatory genes, as well as the *E75*, *E78* and *βFTZ-F1* orphan receptor loci. Three DHR3 binding sites were identified downstream from the start site of *βFTZ-F1* transcription, further indicating that this gene is a direct target of *DHR3* regulation. Ectopic expression of *DHR3* revealed that the polytene chromosome binding pattern is of functional significance. *DHR3* is sufficient to repress *BR-C*, *E74A*, *E75A* and *E78B* transcription as well as induce *βFTZ-F1*. *DHR3* thus appears to function as a switch that defines the larval-prepupal transition by arresting the early regulatory response to ecdysone at puparium formation and facilitating the induction of the *βFTZ-F1* competence factor in mid-prepupae. This study also provides evidence for direct cross-regulation among orphan members of the nuclear receptor superfamily and further implicates these genes as critical transducers of the hormonal signal during the onset of *Drosophila* metamorphosis.

Key words: nuclear hormone receptors, gene expression, *Drosophila* metamorphosis, competence, ecdysone

INTRODUCTION

Small lipophilic hormones, including steroids, retinoids and thyroid hormone, control a wide range of developmental and physiological responses in higher organisms. These molecules are bound by members of the nuclear receptor superfamily which act as ligand-dependent transcription factors that reprogram gene expression in target cells. Extensive studies have provided a detailed understanding of receptor structure and function (Tsai and O'Malley, 1994; Mangelsdorf et al., 1995). In contrast, little is known about the events that occur downstream from the receptor. Relatively few target genes have been identified and it remains unclear how these genes propagate the hormonal signal to direct the appropriate growth and development of the organism. In addition, the isolation of new nuclear receptor genes has outpaced our ability to identify their corresponding ligands, such that more than 100 of these so-called orphan receptors have now been identified (Gronemeyer and Laudet, 1995; Mangelsdorf and Evans, 1995).

We are studying the regulation of *Drosophila* metamorphosis by the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) as a model system for understanding how hormonal signals are transduced into stage- and tissue-specific developmental responses. Several successive pulses of ecdysone direct the onset of metamorphosis (Riddiford, 1993). An ecdysone pulse at the end of larval development triggers puparium formation and the beginning of prepupal development, followed 10 hours later by another pulse that signals the prepupal-pupal transition (Fig. 1, top). Most larval tissues are destroyed during prepupal and early pupal development, as adult structures grow and differentiate from clusters of imaginal progenitor cells (Robertson, 1936). The net result of these divergent developmental pathways is the remarkable transformation of a crawling larva to a highly mobile, reproductively active adult fly.

Observation of the puffing patterns of the larval salivary gland polytene chromosomes has provided critical insights into the mechanisms by which ecdysone directs these complex developmental responses (Clever, 1964; Ashburner et al., 1974). Ecdysone binds to a heterodimer of two nuclear receptors, EcR and USP (Koelle et al., 1991; Koelle, 1992; Yao et al., 1992; Thomas et al., 1993; Yao et al., 1993). This hormone-receptor complex directly induces the transcription of target genes, including approximately six early puff genes in the polytene chromosomes. Some early genes encode transcription factors that transduce and amplify the hormonal signal by inducing large batteries of secondary-response late genes (Ashburner et al., 1974). Two such early regulatory genes have been studied in detail – the *Broad-Complex* (*BR-C*) and *E74*, which encode families of transcription factors that contain zinc finger and ETS DNA-binding domains, respectively (Burtis et al., 1990; DiBello et al., 1991). Two transcript isoforms arise from *E74*, designated *E74A* and *E74B*, of which *E74A* is responsible for puff formation. The *BR-C* and *E74* are essential for critical

developmental responses to ecdysone, and function together to regulate overlapping sets of secondary-response target genes (Kiss et al., 1988; Guay and Guild, 1991; Karim et al., 1993; Fletcher et al., 1995; Fletcher and Thummel, 1995).

Interestingly, at least eight orphan receptor genes are also expressed during the onset of *Drosophila* metamorphosis, providing an ideal system for defining orphan receptor function in the context of a developing animal (Thummel, 1995). Most of these genes are regulated directly by ecdysone and correspond to well-characterized puffs in the polytene chromosomes. Transcripts from four of these genes are expressed for brief intervals during late larval and prepupal development, in response to changes in ecdysone titer: *E75A* and *E78B* (Rev-erb homologs; Segraves and Hogness, 1990; Stone and Thummel, 1993), *DHR3* (RORα homolog; Koelle et al., 1992) and *βFTZ-F1* (SF-1 homolog; Ohno and Petkovich, 1992; Ayer et al., 1993; Lavorgna et al., 1993). *E75A* is induced directly by both the late larval and prepupal ecdysone pulses, in parallel with the *BR-C* and *E74A* (Fig. 1) (Segraves and Hogness, 1990; Karim and Thummel, 1992; Huet et al., 1993). Induction of these early mRNAs in late larvae is followed by *E78B* and *DHR3* early-late gene expression (Fig. 1) (Stone and Thummel, 1993; Horner et al., 1995; Huet et al., 1995; Russell et al., 1996). The *βFTZ-F1* gene is then induced in mid-prepupae as *E78B* and *DHR3* are repressed (Fig. 1). *βFTZ-F1* is repressed both by ecdysone and its own expression, defining a narrow window of activity during the period of low hormone titer in mid-prepupae (Woodard et al., 1994). *βFTZ-F1* appears to function as a competence factor that facilitates the re-induction of the *BR-C*, *E74A* and *E75A* by ecdysone in late prepupae. In addition, *βFTZ-F1* is sufficient to direct the stage-specific ecdysone induction of *E93* in late prepupal salivary glands (Fig. 1) (Woodard et al., 1994). *E93* expression immediately precedes the onset of salivary gland histolysis, suggesting that it may function to direct this response (Baehrecke and Thummel, 1995).

In this paper, we describe the expression and function of the *DHR3* orphan receptor gene during the onset of metamorphosis. *DHR3* 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 (Stone and Thummel, 1993; Huet et al., 1995). This delay appears to be due to a requirement for ecdysone-induced protein synthesis in order to achieve maximal levels of *DHR3* transcription (Horner et al., 1995). A similar mechanism of ecdysone regulation has been reported for the *Manduca sexta* homolog of *DHR3*, *MHR3* (Palli et al., 1992). As a result of this delay, *DHR3* is expressed at high levels in early prepupae, as the early genes are repressed and before *βFTZ-F1* is induced (Fig. 1). Like its vertebrate homolog, RORα, DHR3 can bind as a monomer to a single AGGTCA core sequence that is preceded by an AT-rich sequence (Giguère et al., 1994; Horner et al., 1995).

We show here that DHR3 protein is widely expressed in embryos and early prepupae, at times that parallel its transcription. Antibody staining of salivary gland polytene chromosomes revealed several hundred sites that are specifically bound by DHR3 protein, many of which correspond to known ecdysone-regulated puffs. DHR3 binds to all of the classic early and early-late puff loci, including those that encode the *BR-C*, *E74*, *E75* and *E78* regulatory genes. DHR3 also binds strongly to the 75D puff locus that contains the *βFTZ-F1* gene. Ectopic expression of *DHR3* in transformed late larvae is sufficient to repress *BR-C*, *E74A*, *E75A* and *E78B* transcription and induce *βFTZ-F1*, suggesting that the polytene chromosome binding pattern is functionally significant. DNA binding studies revealed three DHR3 binding sites located downstream from the start site of *βFTZ-F1* transcription, consistent with the direct regulation of this target gene by DHR3. These observations lead to the model that *DHR3* directs the larval-to-prepupal transition during metamorphosis and also provide evidence that orphan receptors function together to direct the appropriate order and stage-specificity of the genetic responses to ecdysone during metamorphosis.

MATERIALS AND METHODS

Molecular cloning

Four constructs were made in order to express DHR3 protein in bacteria. A DHR3-β-galactosidase fusion construct (pWR590-DHR3) was made by inserting a 1559 bp *Hinc*II fragment containing most of the *DHR3* coding region into the *Sma*I site of pWR590 (Guo et al., 1984). A construct expressing full-length DHR3 protein was made by engineering an *Nde*I site upstream from the *DHR3* start codon by PCR using the following oligonucleotide: GCAGTCTAGACATATGTATACGCAACG (the *Nde*I site is underlined). The resultant *Nde*I-*Sca*I fragment, containing the entire *DHR3* coding region and approx. 500 bp of 3′ untranslated region, was then inserted between the *Nde*I-

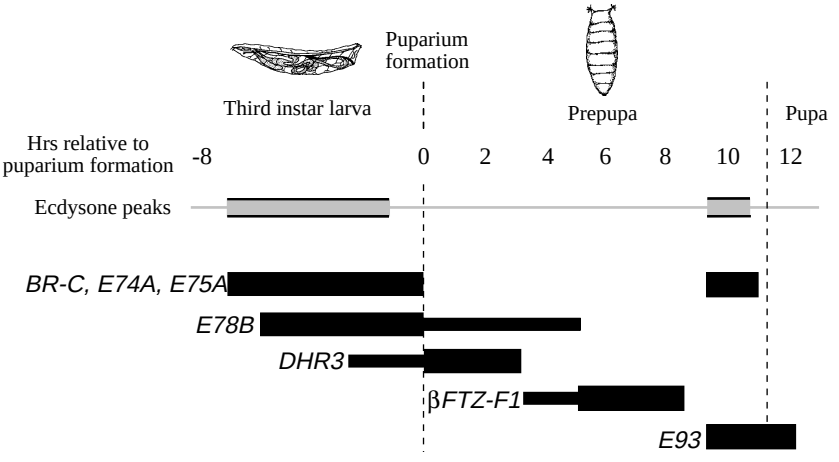


Fig. 1. Schematic representation of ecdysone-regulated transcription during the onset of *Drosophila* metamorphosis. Late third instar larval and prepupal development are represented at the top, above a time course in hours relative to puparium formation and a schematic representation of the late larval and prepupal ecdysone peaks (Riddiford, 1993). The two dotted lines mark the larval-prepupal and prepupal-pupal transitions. The black boxes at the bottom represent the times of ecdysone-regulated transcription in the larval salivary gland. The width of the bar represents approximate levels of mRNA accumulation (Woodard et al., 1994; Baehrecke and Thummel, 1995; Horner et al., 1995; Huet et al., 1995).

*Hinc*II sites of pET23a(+) (Novagen) to create pET23-DHR3. Two $(His)_6$-tagged DHR3 proteins were made for DNase I footprint analysis. Either a 500 bp *Hin*dIII-*Xho*I fragment that spans the 5′ *DHR3* coding region (encoding amino acids 1-163), or a 1.6 kb *Hin*dIII-*Xho*I fragment that contains the full-length *DHR3* coding region, were inserted into the pET-32a(+) polylinker (Novagen). These plasmids were designated pET32-DHR3S and pET32-DHR3L, respectively (for Short and Long DHR3 protein forms). Finally, one plasmid was made to express DHR3 protein in vitro. The 1.6 kb *Hin*dIII-*Xho*I fragment that contains the full-length *DHR3* coding region was inserted between the *Hin*dIII and *Xho*I sites of pBS-SK+ (Stratagene) to create pBS-DHR3.

Antibody preparation

DHR3 protein was expressed in bacteria carrying either pWR590-DHR3 or pET23-DHR3 and isolated from inclusion bodies essentially as described by Rio et al. (1986). These preparations were approx. 95% pure as determined by SDS-PAGE and staining with Coomassie blue. Antiserum from rabbits immunized with the β-galactosidase-DHR3 fusion protein (Berkeley Antibody Company) were assayed by western blot analysis, and serum from one animal was selected for purification. Antiserum was centrifuged and the supernatant was passed over two columns to affinity purify anti-DHR3 antibodies. The first column contained protein extract isolated from bacteria carrying the pWR590 vector, and the second column contained DHR3 protein isolated from bacteria carrying pET23-DHR3. Specific anti-DHR3 antibodies were eluted from the second column as described byCarroll and Laughon (1987) and dialyzed into PBS. Each fraction (approx. 0.5 ml) was tested by western blot analysis using protein isolated from pET23-DHR3 bacteria and from staged prepupae.

Antibody procedures

Embryos collected from a stock of *Df(2R)12/CyO wg-lacZ* flies were stained with antibodies as described by Reuter and Scott (1990). A mixture of 1:100 rabbit anti-DHR3 antibodies and 1:50,000 mouse anti-β-galactosidase antibodies (Promega) was used as a primary antibody, followed by detection with 1:200 lissamine rhodamine (LRSC)-conjugated goat anti-rabbit antibodies (Jackson) and 1:200 fluorescein (FITC)-conjugated goat anti-mouse antibodies (Jackson). The stains were imaged on a BioRad MRC600 confocal laser scanning microscope using dual detector channels to independently visualize the LRSC and FITC signals. Images were generated by Kalman averaging and overlaying 2-3 optical sections of 5.04 μm each.

For detecting DHR3 protein in prepupal organs, animals were staged on blue food, as by Andres and Thummel (1994), dissected, and stained with affinity-purified anti-DHR3 antibodies as described by Boyd et al. (1991).

For western blot analysis, late third instar larvae and prepupae were staged as described above. Protein extracts were prepared from 15 staged animals/time point by homogenization in SDS sample buffer. Protein samples corresponding to 1.5 animals/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 first with a 1:500 dilution of affinity-purified anti-DHR3 antibodies, 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.

DHR3 protein bound to polytene chromosomes was detected as described by Zink and Paro (1989) and Urness and Thummel (1990). Salivary glands were dissected from newly formed white prepupae and fixed in 3.7% formaldehyde. The glands were then squashed on poly-L-lysine coated slides in the presence of 3.7% formaldehyde, 50% acetic acid. Black and white photographs were rapidly taken, after which the chromosomes were flattened, the slides were frozen in liquid nitrogen, and then stored in 1× PBS after popping off the coverslip. Affinity-purified anti-DHR3 antibodies (1:100 dilution) were applied for 30 minutes, followed by several washes and incubation with 1:200 biotinylated goat anti-rabbit secondary antibody (Vector Labs). Slides were then processed with the Vectastain Elite kit (Vector Labs) to detect the immune complexes. Slides were stained with a 1:25 dilution of Giemsa for 30 seconds, rinsed in PBS, mounted, and observed by phase-contrast microscopy.

Ectopic expression of *DHR3*

A 1.6 kb *Eco*RI-*Kpn*I fragment from pBS-DHR3, spanning the entire *DHR3* coding region, was inserted into pUC18 and excised as an *Eco*RI-*Bam* fragment using flanking sites in the polylinker. This fragment was inserted between the *Eco*RI and *Bam* sites in the polylinker of pCaSpeR-hs-act. This vector is derived from pCaSpeR-hs (Thummel and Pirrotta, 1992), but contains the *act5C* 3′ trailer and poly(A) addition sites (C.T. Woodard, personal communication). The resultant P element was injected into w^{1118} embryos, following standard procedures for transformation (Rubin and Spradling, 1982). A total of 8 transformed lines were established. Each line expressed similar amounts of DHR3 protein upon heat shock and recovery, as assayed by western blot analysis (data not shown). One line was selected for further study, designated *w*; P[*hs-DHR3*], carrying the P element as a homozygous viable insertion on the second chromosome.

RNA analysis

w and *w*; P[*hs-DHR3*] animals were maintained on food containing 0.05-0.1% bromophenol blue (Maroni and Stamey, 1983; Andres and Thummel, 1994). Third instar larvae from each stock were transferred to 1.5 ml microcentrifuge tubes with holes punched in the top, immersed into a 37°C water bath for 30 minutes, and then transferred to yeast paste containing 0.05-0.1% bromophenol blue and maintained at room temperature for 2 hours to recover. Newly formed prepupae were selected, as well as larvae that were approx. 4, 8, or 18 hours prior to pupariation, based on their gut color (Andres and Thummel, 1994). RNA was isolated from these staged animals as described by Andres and Thummel (1994). Total RNA samples corresponding to 2 animals/lane were fractionated by formaldehyde agarose gel electrophoresis, transferred to a nylon membrane, and cross-linked by UV irradiation, as described by Karim and Thummel (1991). Blot hybridization and washing was performed as described by Karim and Thummel (1991).

Organ culture

Mid-third instar *w* and *w*; P[*hs-DHR3*] larvae (blue gut crawling larvae) were heat shocked for 30 minutes at 35°C and allowed to recover for 30 minutes at 25°C, as described above. These larvae were then dissected and divided into two wells for each genotype. Organs were cultured as described by Andres and Thummel (1994) and Woodard et al. (1994). One set of organs from each genotype was incubated for 4 hours in the absence of hormone and one set was incubated with 5×10^{-6} M 20-hydroxyecdysone (Sigma) for 4 hours. RNA was extracted and analyzed by northern blot hybridization.

DNA binding studies

The *βFTZ-F1* cosmid clone (a generous gift from P. Reid and C.T. Woodard), containing the entire *βFTZ-F1* gene, was scanned for DHR3 binding sites using the protocol originally described by Hope and Struhl (1985). pBS-DHR3 DNA was linearized by digestion with *Kpn*I and transcribed in vitro using T3 RNA polymerase (Stratagene Transcription kit). The resultant RNA was translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. DNA was digested with *Alu*I, incubated with ^{35}S-labeled DHR3 protein in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 13.3 mg/ml BSA, 5% glycerol), and fractionated on a 4% polyacrylamide gel run in 0.5× TBE, as described by Urness and Thummel (1990). DNA fragments carrying bound DHR3 protein were detected by autoradiography.

Two different sources of DHR3 protein were used for DNase I footprint analysis. Extracts were prepared from BL21(DE3) bacteria carrying either pET32-DHR3S or pET32-DHR3L using 6 M guanidine HCl, 0.1 M $NaPO_4$, 10 mM Tris-HCl, pH 8.0. Following a clearance spin, the protein was allowed to bind to Ni-NTA resin and refold using a linear 6 M to 1 M urea gradient, following the protocol provided by Qiagen. After washing the column, the protein was eluted with 250 mM imidazole in 1 M urea gradient buffer. Each fraction was assayed for DHR3 protein by SDS-PAGE and the peak fractions were pooled and dialyzed against 0.1 M HEMG buffer. The protein from pET32-DHR3S was diluted 4-fold in 0.1 M HEMG before dialysis. Both DHR3 protein preparations were approx. 80% pure, as determined by SDS-PAGE and staining with Coomassie blue. Although both protein forms detected the strong A and C binding sites, only the short DHR3 protein form bound these sites strongly and also protected site B against DNase I digestion. This was due to the propensity of the longer protein form to precipitate.

The 665 bp *Acc*I-*Hin*dIII fragment from the *βFTZ-F1* cosmid was end-filled and inserted into the *Eco*RV site of pBS-KS(−) to provide a template for synthesis of DNA fragments for DNase I footprint analysis. Three pairs of oligonucleotides were used to prime DNA synthesis by PCR: ftzf104 (GTGAGTGACCCAAATATCGATC) and KS3* (CGAGGTCGACGGTATCG); SK5 (TCTAGAACTAGTG-GATC) and ftzf103* (CGCAACGCAGCAAGAGAACATA); SK5 and ftzf102* (GATCGATATTTGGGTCACTCAC). One oligonucleotide of each pair was end-labeled (designated by the asterisk) using [γ-^{32}P]ATP and T4 DNA kinase (Promega). The resultant end-labeled fragment was purified on a low melting point agarose gel, and the amount of labeled fragment was determined by comparison with known standards. The three oligonucleotide pairs described above allowed the PCR amplification of genomic fragments from +156 to +536, −130 to +341 and −130 to +177, respectively. More 5′ sequences, from −131 to −591, were scanned for DHR3 binding sites by DNase I footprinting in a separate set of experiments (data not shown). Only one very weak binding site was found in this interval.

For each footprinting reaction, 3-5 fmol of end-labeled DNA fragment was incubated with 500 ng purified DHR3 protein and 25 ng of dI-dC (Sigma) in 50 μl binding buffer (see above). Each sample was then treated with dilute DNase I at room temperature for 20 seconds and analyzed by fractionation on an 8% sequencing gel, essentially as described by Galas and Schmitz, (1978); Heberlein et al. (1985).

RESULTS

DHR3 protein is widely expressed during mid-embryogenesis and early metamorphosis

Developmental northern blot analysis revealed two peaks of *DHR3* transcription, during mid-embryogenesis and metamorphosis (Koelle et al., 1992). In an effort to compare this pattern with that of DHR3 protein, rabbit antibodies were raised against DHR3 and affinity purified. Embryos carrying the *Df(2R)12* chromosome were used to determine the specificity of these affinity purified antibodies. *Df(2R)12* carries a small X-ray-induced deficiency that removes the *DHR3* locus (Weber et al., 1995, R. Burgess, T. Schwarz and M. Bender, personal communication). Embryos collected from a stock carrying the *Df(2R)12* chromosome over a *wg-lacZ*-marked balancer chromosome were stained with antibodies to detect both DHR3 and β-galactosidase proteins (Fig. 2). A strong nuclear signal was detected only in those embryos that carry a wild-type copy of the *DHR3* gene on the *wg-lacZ*-marked chromosome (Fig. 2A), while no specific staining was detected in *Df(2R)12* homozygotes at the same stage of development (Fig. 2C). The specificity of this interaction indicates that the affinity-purified antibodies are selectively directed against DHR3 epitopes. DHR3 protein is induced following germ band retraction and can be detected until cuticle deposition late in embryogenesis, paralleling its temporal pattern of transcription (Koelle et al., 1992). DHR3 is widely expressed during mid-embryogenesis, in tissues that include the gut, salivary gland, ventral nerve cord and epidermis. In contrast, the embryonic central nervous system contains little, if any, DHR3 protein.

Western blot analysis was used to determine the temporal profile of DHR3 expression at the onset of metamorphosis (Fig. 3). The 62×10^3 M_r DHR3 protein can first be detected in newly formed prepupae and peaks at 4-6 hours after puparium formation. This pattern of expression closely parallels that of *DHR3* mRNA (Horner et al., 1995). Low levels of *DHR3* mRNA can also be detected in late third instar larvae, but this does not lead to detectable levels of protein by western blot analysis. As expected, DHR3 protein co-migrates with full-length protein synthesized in *E. coli* (data not shown) as well as DHR3 protein expressed in transformed larvae under the control of the *hsp70* heat-shock promoter (Fig. 3).

In order to determine the spatial pattern of DHR3 expression during the early stages of metamorphosis, organs were dissected from late third instar larvae, newly formed 0-hour prepupae and 2-hour prepupae, and stained with anti-DHR3 antibodies (Fig. 4). Low levels of nuclear DHR3 protein can be detected in late

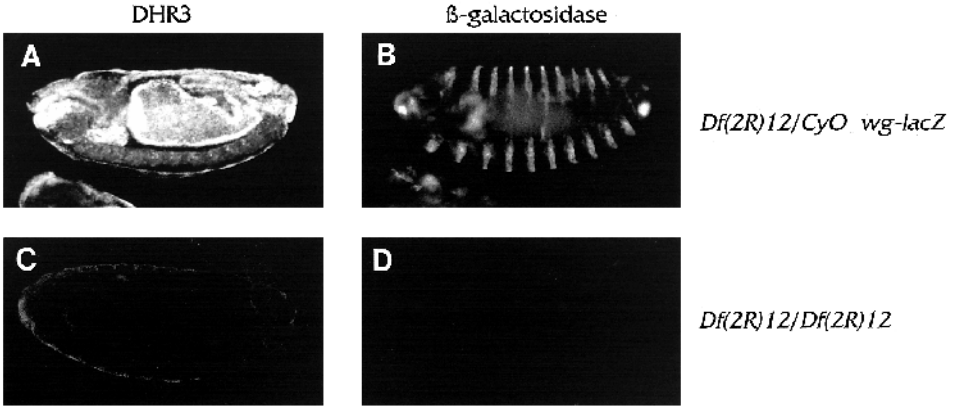


Fig. 2. Anti-DHR3 antibodies selectively detect DHR3 protein during mid-embryogenesis. A 0- to 16-hour collection of embryos from *Df(2R)12*/*CyO wg-lacZ* flies was stained to detect both DHR3 and β-galactosidase proteins. Images were obtained through independent channels on a confocal laser scanning microscope (see Materials and Methods). The *wg-lacZ* pattern indicated embryos carrying an intact *DHR3* gene (A,B), whereas the absence of β-galactosidase indicated *Df(2R)12* homozygotes (C,D). The depicted embryos are at approximately the same stage of development. Only in embryos carrying the marked balancer could an intense nuclear anti-DHR3 signal be detected (A). *CyO* balancer homozygotes (with a distorted pattern of *wg-lacZ* expression) were excluded from this analysis.

Table 1. Polytene chromosome loci stained with anti-DHR3 antibodies

Chromosome				
X	2L	2R	3L	3R
2B5*	21C*	42A*	61A	86E*
8EF*	22C*	43E*	62E*	87F*
9B	23E*	44A*	63A	88A
10EF*	24D	45F	63C	89B*
12B	25A	46A*	63F*	98F*
12DE*	26F	46F*†	65A	99B*
13E*	27C*	47A*	67B*	100E*
14F	28A*	47BC*	70C*	100F
	28E	48B*	70D	
	29F*	49E*	70E*	
		50CD*	72A	
		50F*	72D*	
		51B	73B*	
			74EF*	
			75B*†	
			75D*†	
			76A*	
			76D*	
			78C*	
			79F	

Only sites that could be mapped in at least two independent preparations are listed. Most listed sites stain relatively strongly in at least one preparation. Ecdysone-regulated puffs are marked (*) (Ashburner, 1975), as are the three intensely stained loci (†). Ecdysone-inducible puffs that did not stain include 21F, 63E, 68C, and 71E.

third instar tissues, most clearly in the imaginal discs, consistent with the low levels of *DHR3* mRNA present at this stage. Higher levels of DHR3 protein are present in early prepupae, in both larval and imaginal tissues. DHR3 is expressed in the larval and imaginal cells of the salivary gland, as well as the fat bodies, Malpighian tubules and imaginal discs of 0- and 2-hour prepupae (Fig. 4). Although expression is clearly evident in the peripodial membranes that surround the imaginal discs, DHR3 protein is also present in the disc epithelial monolayer. In addition, DHR3 is expressed in the midgut, garland cells, trachea, ring gland, haemocytes, lymph gland and pericardial cells of early prepupae (data not shown). Only low levels of DHR3 protein are present in the 2-hour prepupal central nervous system and no protein can be detected in the proventriculus (data not shown). The nuclear localization of DHR3 protein in both embryos and prepupae is consistent with its known DNA binding activity (Horner et al., 1995).

DHR3 binds to several hundred sites in the salivary gland polytene chromosomes

The presence of DHR3 protein in larval salivary gland nuclei provided an opportunity to identify the sites of bound DHR3 protein on the giant polytene chromosomes. Polytene chromosomes were spread from 0-hour prepupal salivary glands and photographed to document the banding and puffing morphology. The chromosomes were then treated with anti-DHR3 antibodies, a biotinylated secondary antibody and avidin-conjugated with horseradish peroxidase. Comparison of the peroxidase staining pattern with the original set of photographs allowed the accurate mapping of sites bound by DHR3 (Fig. 5; Table 1).

Several hundred sites in the polytene chromosomes were detected by antibody staining, about half of which were relatively strongly stained. In addition, three sites were intensely stained in a highly reproducible manner, one on 2R and two located next to one another on 3L. The regions that contain these intensely stained sites are shown in Figure 5, along with neighboring loci that are bound by DHR3. A more complete listing is shown in Table 1. Remarkably, the three intensely stained loci each contain an ecdysone-regulated orphan receptor gene: *DHR3* at 46F, *E75* at 75B and *βFTZ-F1* at 75D. About half of the remaining stained loci correspond to ecdysone-regulated puffs that are active during the early stages of metamorphosis. These include the 2B5, 42A, 74EF and 78C puffs, which contain the *BR-C*, *EcR*, *E74* and *E78B*, respectively. DHR3 protein was also detected at the 25A intermolt puff, the 23E and 63F early puffs, the 62E early late puff and the 22C and 29F late puffs. DHR3 protein was not detected at the 68C intermolt puff, or the 21F, 63E and 71E late puffs (Table 1). These observations suggest that DHR3 plays a central role in the ecdysone-triggered regulatory hierarchies that direct the early stages of metamorphosis and raise the interesting possibility that orphan receptors may directly cross-regulate their expression.

Ectopic *DHR3* expression represses early gene transcription and induces *βFTZ-F1*

Given that *DHR3* expression correlates with both early gene repression and *βFTZ-F1* induction (Fig. 1), and that DHR3 protein binds to these puff loci (Fig. 5), we set out to test whether *DHR3* might contribute to these regulatory responses *in vivo*. Flies were transformed with a P element that expresses *DHR3* under the control of the *hsp70* heat-shock promoter, designated P[*hs-DHR3*]. Heat-shocked late larvae that carry this P element express levels of DHR3 protein that are similar to those normally present in mid-prepupae (Fig. 3). Collections of *w* control late third instar larvae and *w*; P[*hs-DHR3*] larvae were heat-shocked and allowed to recover for 2 hours at room temperature. Staged animals were then selected that were approximately 18, 8, or 4 hours prior to pupariation, or newly

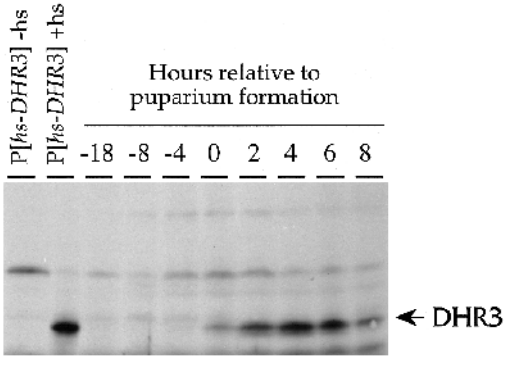


Fig. 3. Temporal profile of DHR3 expression during the onset of metamorphosis. Transformed mid-third instar larvae carrying P[*hs-DHR3*] were either heat shocked for 30 minutes and allowed to recover at room temperature for 2 hours, or maintained at room temperature. Protein extracts prepared from these animals were run alongside extracts prepared from staged wild type late third instar larvae and prepupae. These samples were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and DHR3 protein was detected by chemiluminescence using anti-DHR3 antibodies (see Materials and Methods). Equal amounts of protein were loaded in each lane as determined by Coomassie blue staining of a second gel run in parallel.

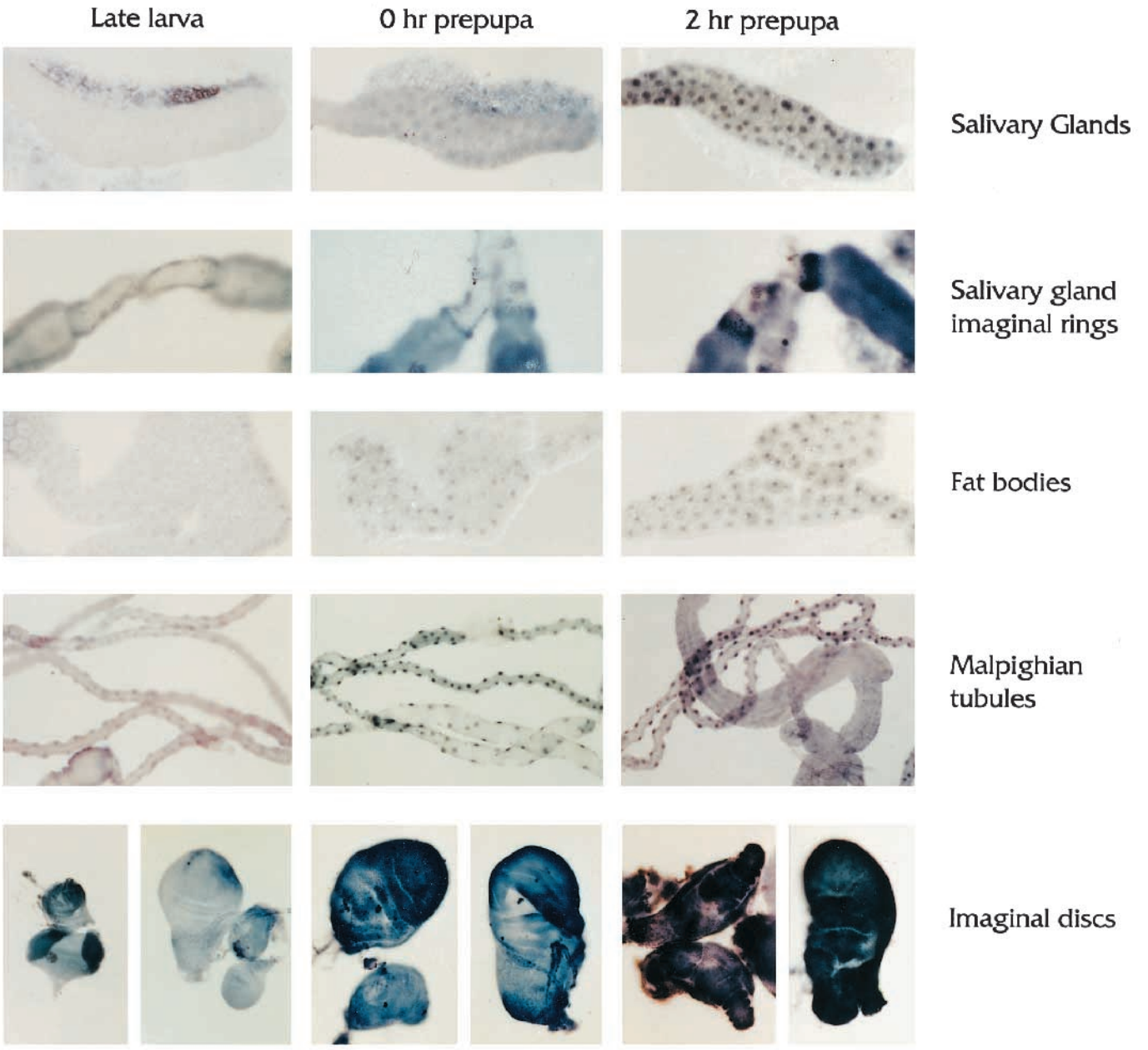


Fig. 4. Spatial pattern of DHR3 expression during the onset of metamorphosis. Organs were dissected from late third instar larvae staged at approximately 4 hours prior to puparium formation (clear gut larvae), newly formed prepupae and 2-hour prepupae. The organs were fixed and stained with antibodies to detect DHR3 protein (see Materials and Methods). Shown, from top to bottom, are salivary glands, the imaginal rings at the anterior end of the salivary glands, fat bodies, Malpighian tubules and imaginal discs. An eye-antennal, wing and haltere disc are shown from late larvae; wing and leg discs are shown from prepupae. Approximately equal staining intensity was observed in eye-antennal, leg, wing and haltere discs at each of these stages (data not shown).

formed prepupae. RNA was extracted from these animals and the levels of early gene and *βFTZ-F1* transcription were determined by northern blot hybridization (Fig. 6).

As expected, *BR-C* and *E74B* mRNAs are present in −18-hour mid-third instar larvae (Fig. 6, leftmost lane). As the ecdysone titer rises in late third instar larvae, *E74B* is repressed and the *BR-C*, *E74A*, *E75A*, and *E78B* are induced (Fig. 6, left four lanes). Interestingly, the levels of all of these mRNAs are reduced in the presence of ectopic *DHR3* (Fig. 6, right four lanes). The efficiency of *BR-C* and *E74B* repression, however, appears to vary with developmental stage. *BR-C* transcription is not affected by ectopic *DHR3* in 0-hour prepupae and *E74B* is not affected in −4-hour late third instar larvae. Nonetheless, this observation supports the hypothesis that *DHR3* contributes to the repression of early gene transcription at puparium formation. Ectopic *DHR3* expression also results in a modest reduction in *EcR* transcription in late third instar larvae (data not shown). In addition, *βFTZ-F1* mRNA can be detected in mid- and late third instar larvae that express ectopic *DHR3*, as much as a day before its normal period of expression in mid-

prepupae (Fig. 6). This observation indicates that *DHR3* is sufficient to induce *βFTZ-F1*, even in the presence of the relatively high ecdysone titer of late third instar larvae. This suggests that *DHR3* can overcome the repression normally mediated by the hormone-receptor complex. The levels of *βFTZ-F1* mRNA accumulation are, however, significantly lower than those seen normally in mid-prepupae (data not shown). Ectopic *DHR3* expression had no effect on *DHR3* or *E75B* transcription (data not shown).

It is possible that the repression of early gene transcription as seen in Fig. 6 could be due to the inherent inaccuracy of our method for staging late third instar larvae. In an effort to test this possibility, we asked if we could achieve a similar result in cultured larval organs where ecdysone responses can be synchronized by the addition of exogenous hormone. Organs were dissected from heat-shocked *w* and *w*; P[*hs-DHR3*] mid-third instar larvae and incubated for 4 hours either in the presence or absence of ecdysone. RNA was then extracted from these organs and analyzed by northern blot hybridization to detect *BR-C*, *E74*, *E75A*, and *E78B* transcription (Fig. 7). Reduced levels of ecdysone induction were observed for each of these genes in the presence of ectopic *DHR3*, identical to the results obtained in vivo. This observation confirms that *DHR3* is sufficient to repress early gene transcription.

DHR3 protein binds to the 5′ end of the *βFTZ-F1* gene

The observations that *DHR3* is sufficient to prematurely induce *βFTZ-F1* transcription and that DHR3 protein binds strongly to the *βFTZ-F1* locus in the polytene chromosomes suggest that *DHR3* may directly regulate *βFTZ-F1* transcription. If this hypothesis is true, then we should be able to identify specific high-affinity DHR3 binding sites in the *βFTZ-F1* gene. As a step toward this goal, we scanned for DHR3 binding sites in an approx. 30 kb cosmid genomic insert that contains the entire 16 kb *βFTZ-F1* gene (P. Reid and C.T. Woodard, unpublished results). Radioactive DHR3 protein was synthesized in vitro and incubated with restriction-digested *βFTZ-F1* cosmid DNA under binding conditions. The DNA-protein complexes were fractionated by low ionic strength polyacrylamide gel electrophoresis and bound DNA fragments were visualized by

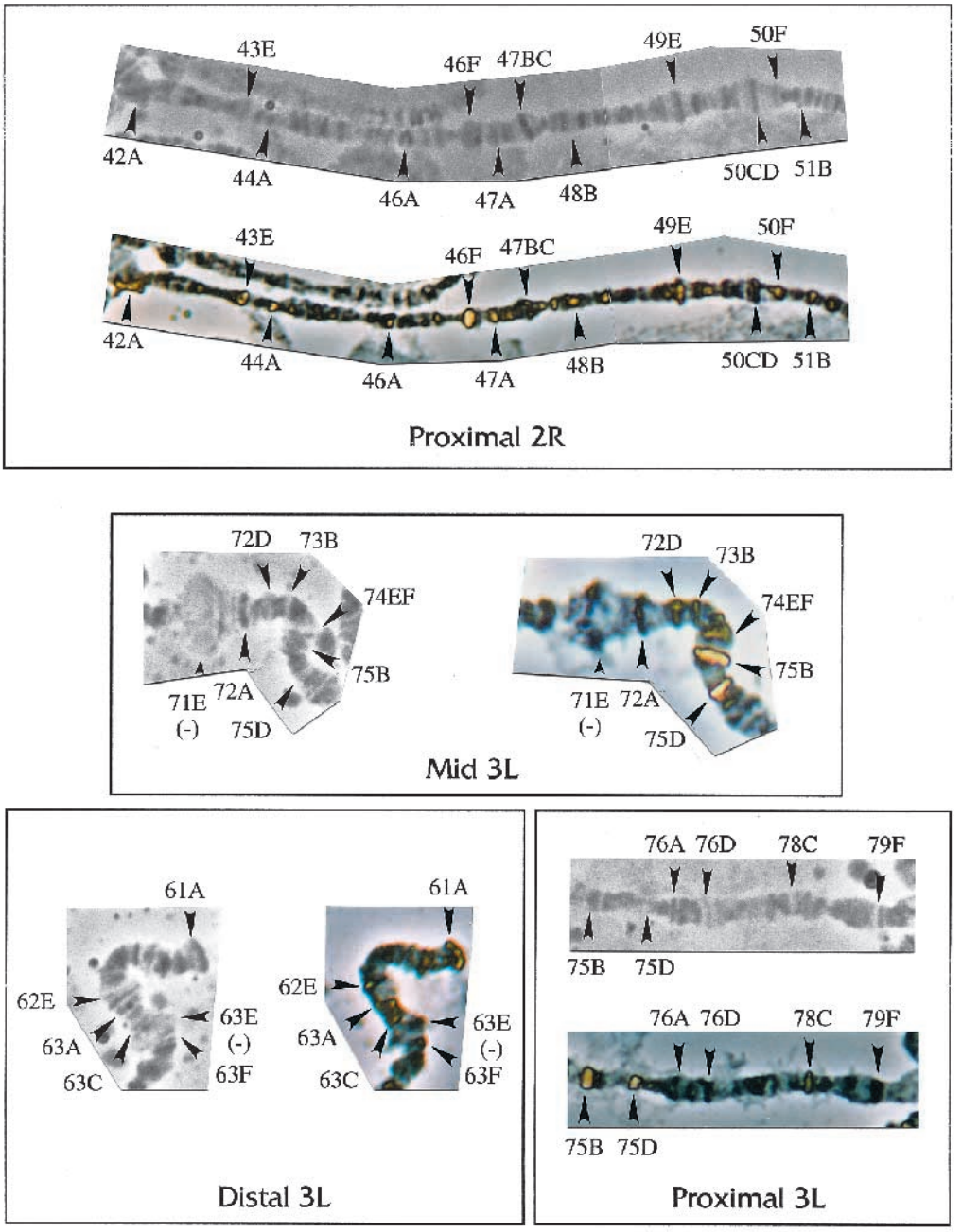


Fig. 5. Localization of DHR3 protein bound to the salivary gland polytene chromosomes. Salivary gland polytene chromosomes from newly formed white prepupae were stained with anti-DHR3 antibodies as described in Materials and Methods. Paired photographs are shown, a black and white photograph taken before the staining procedure and a color photograph taken after the staining procedure, showing the gold refractile stains that mark the bound DHR3 protein. Representative regions of the 2R and 3L chromosomes are shown. These arms contain the most strongly stained sites in the genome, at 46F, 75B and 75D. Many of the stained sites are marked with arrows, most of which puff in response to ecdysone (Table 1). Also shown are two late puffs that do not stain with anti-DHR3 antibodies: 63E and 71E.

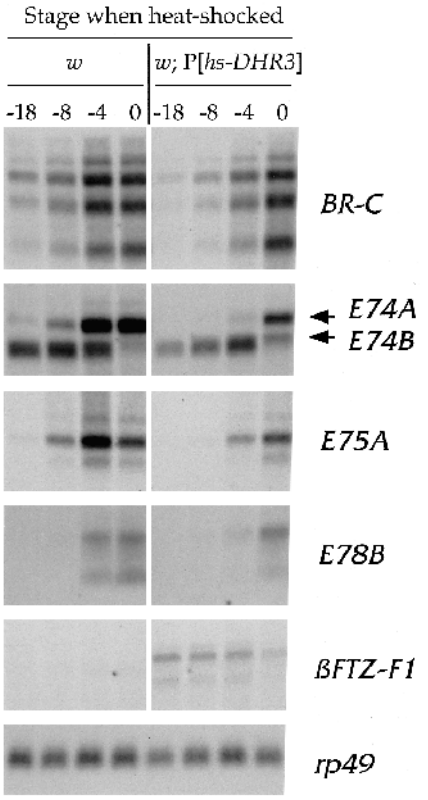


Fig. 6. *DHR3* is sufficient to repress early gene transcription and induce *βFTZ-F1*. A collection of *w* and *w*; P[*hs-DHR3*] crawling third instar larvae were subjected to heat shock and allowed to recover for 2 hours. Animals were then staged, based on gut color, and sorted into four classes: 18, 8, 4 or 0 hours prior to puparium formation (see Materials and Methods). Total RNA was extracted from these animals, fractionated by formaldehyde agarose gel electrophoresis, transferred to nylon, and probed to detect *BR-C*, *E74*, *E75A*, *E78B* and *βFTZ-F1* transcription. A probe from the *BR-C* common region was used to detect all four size classes of mRNA (DiBello et al., 1991). Hybridization to detect *rp49* mRNA was performed as a control for loading and transfer.

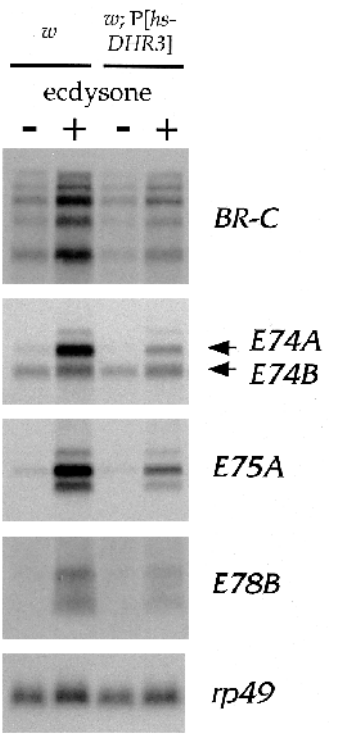


Fig. 7. *DHR3* expression represses early gene induction in cultured larval organs. Mid-third instar *w* and *w*; P[*hs-DHR3*] larvae (blue gut) were heat shocked and allowed to recover for 30 minutes. Organs were then dissected and cultured for 4 hours in the absence (−) or presence (+) of 20-hydroxyecdysone (see Materials and Methods). Total RNA was extracted from these organs and equal amounts were analyzed by northern blot hybridization to detect *BR-C*, *E74*, *E75A* and *E78B* transcription. Hybridization to detect *rp49* mRNA was performed as a control for loading and transfer.

autoradiography (Fig. 8). One fragment from the *βFTZ-F1* cosmid was identified that carried bound DHR3 protein. By repeating this assay with genomic subclones of the cosmid insert, the DHR3 binding site(s) were narrowed down to a 5 kb *Xba*I-*Eco*RI fragment (Fig. 8B). This 5 kb fragment was further subdivided into six smaller restriction fragments shown in Fig. 8A. Three of these fragments contained DHR3 binding sites (a 1.3 kb *Pst*I-*Hin*dIII fragment, 2 kb *Pst*I-*Eco*RI fragment, and 665 bp *Acc*I-*Hin*dIII fragment), whereas three fragments were not bound by DHR3 protein (a 1.8 kb *Nae*I fragment, 0.7 kb *Hin*dIII-*Eco*RI fragment and 2.7 kb *Xba*I-*Pst*I fragment) (Fig. 8C). Taken together, these data indicate that DHR3 is binding to a 603 bp *Nae*I-*Hin*dIII fragment (Fig. 8A). The mobility of the bound fragment was identical to that seen originally in the *βFTZ-F1* cosmid and the 5 kb *Xba*I-*Eco*RI fragment (data not shown). We were thus able to identify binding sites within a 30 kb genomic region and narrow that region down to a 603 bp fragment that spans the start site of *βFTZ-F1* transcription.

Three DHR3 binding sites lie downstream from the start site of *βFTZ-F1* transcription

DHR3 protein was purified from an overproducing strain of bacteria in order to facilitate subsequent DNA binding studies. An initial effort to use full-length DHR3 protein was complicated by the propensity of this protein to precipitate. Overexpression of a truncated form of DHR3 protein in bacteria, extending from amino acids 1-163, resulted in a soluble form of the protein that bound DNA with relatively high affinity. This protein was purified to approx. 80% purity and was used to map DHR3 binding sites in the 603 bp *Nae*I-*Hin*dIII fragment by DNase I protection (Fig. 9A). Three sites were identified, designated A, B and C. These sites span 18-24 bp and are located in the 5′-untranslated region, between 155 and 455 bp downstream from the start site of *βFTZ-F1* transcription (Fig. 9B). The two strongest binding sites, A and C, were also bound by full-length DHR3 protein, although site B was too weak to be detected with this protein (data not shown). In addition, a 460 bp *Acc*I fragment, extending from −131 to −591, was subjected to DNase I footprint analysis using the truncated DHR3 protein. Consistent with our inability to detect DHR3 binding to the 1.8 kb *Nae*I fragment (Fig. 8C), we found no strong binding sites in this interval (data not shown).

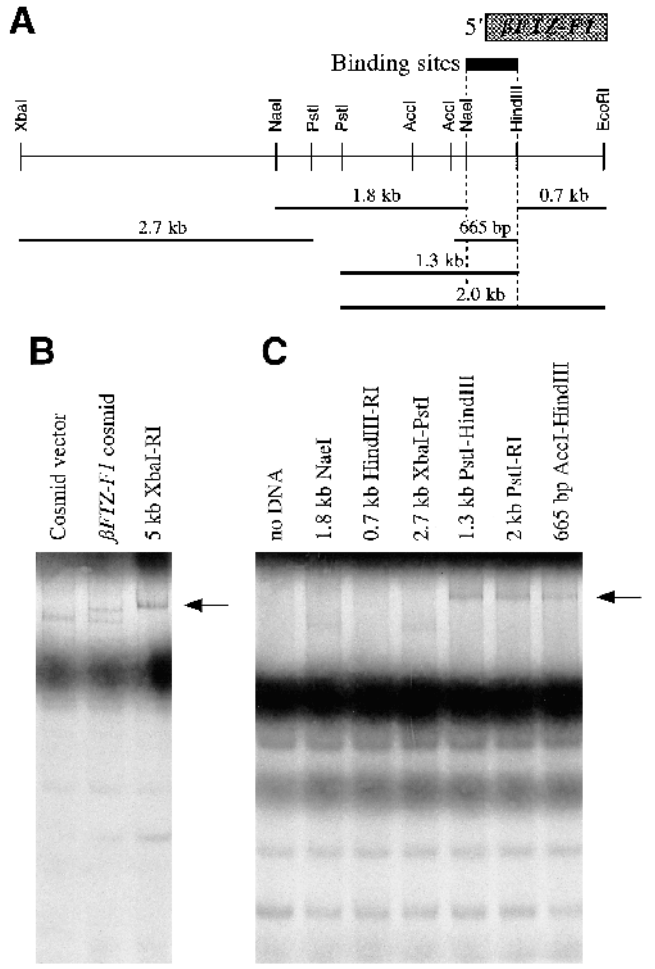
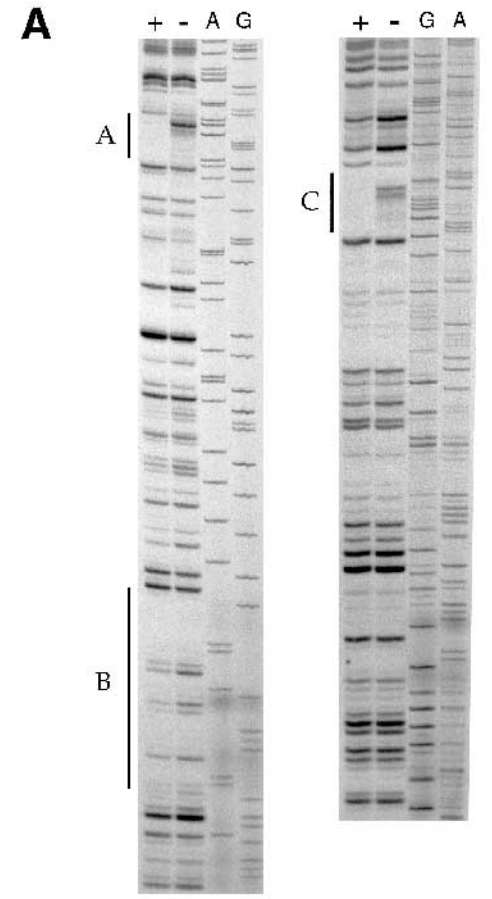


Fig. 8. DHR3 binds to a 603 bp region that spans the start site of *βFTZ-F1* transcription. A cosmid DNA clone encompassing the *βFTZ-F1* gene, as well as restriction fragments isolated from seven plasmid subclones, were digested with *Alu*I and mixed with full-length radioactive DHR3 protein under DNA binding conditions (see Materials and Methods). The DNA-protein complexes were fractionated by low ionic strength polyacrylamide gel electrophoresis and visualized by autoradiography. (A) A restriction map of the 5 kb *Xba*I-*Eco*RI region that contains DHR3 binding sites, as shown in B. The stippled box at top represents the 5′ end of the *βFTZ-F1* gene. Shown below the restriction map are six restriction fragments that were scanned for DHR3 binding sites in C. (B) Three DNAs were scanned for DHR3 binding sites: the NotBamNot CoSpeR vector as a negative control (the cosmid library was a generous gift from J. Tamkun), the *βFTZ-F1* cosmid carrying approx. 30 kb of genomic DNA spanning the *βFTZ-F1* gene and a plasmid subclone carrying the 5 kb *Xba*I-*Eco*RI fragment, shown in A. The arrow marks the *βFTZ-F1* genomic fragment bound by DHR3. The lower band represents a binding site present in the NotBamNot CoSpeR vector. In this experiment, the samples were loaded as the gel was running. This accounts for the apparent slight reduction in mobility of the bound DNA in the 5 kb fragment relative to the *βFTZ-F1* cosmid. A single bound fragment was also identified when the *βFTZ-F1* cosmid was digested with *Hae*III (data not shown). (C) Six restriction fragments from within the 5 kb *Xba*I-*Eco*RI fragment were tested for DHR3 binding sites. The location of these fragments are depicted in A. The arrow marks the fragment bound by DHR3 protein. This DNA co-migrates with the bound DNA present in the 5 kb *Xba*I-*Eco*RI fragment (data not shown).



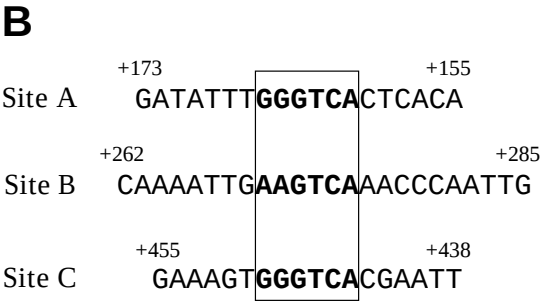


Fig. 9. DHR3 protein binds to three sites downstream from the start site of *βFTZ-F1* transcription. (A) The 603 bp region, corresponding to the *Nae*I-*Hin*dIII fragment identified in Fig. 7, was scanned for DHR3 binding sites by DNase I footprint analysis (see Materials and Methods). The same oligonucleotides that were used to generate the end-labeled fragments for footprint analysis were used for A and G sequence markers, using the Sequenase kit (USB). DNase I digestion was performed either in the presence (+) or absence (–) of 500 ng of DHR3S protein. (B) The DNA sequences of the three binding sites identified by DNase I footprinting. The numbers represent the nucleotide position downstream from the start site of *βFTZ-F1* transcription as determined by 5′ RACE analysis and DNA sequencing (data not shown). The conserved core recognition sequence is boxed.

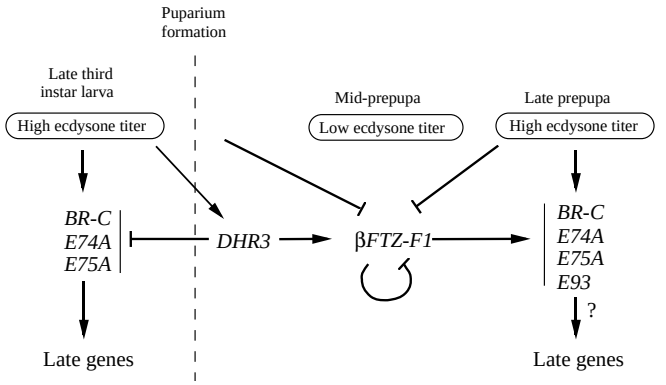


Fig. 10. A model for *DHR3* function during the onset of *Drosophila* metamorphosis. *DHR3* is induced both directly and indirectly by ecdysone, leading to a later period of expression relative to the *BR-C*, *E74A* and *E75A* early mRNAs (Horner et al., 1995). Based on our ectopic expression studies, *DHR3* both represses early gene transcription in newly formed prepupae and facilitates the induction of *βFTZ-F1* in response to the low ecdysone titer in mid-prepupae. *βFTZ-F1*, in turn, provides the competence for the early genes to be re-induced by ecdysone in late prepupae (Woodard et al., 1994). Thus, according to this model, *DHR3* functions as a regulator that defines the end of the larval transcriptional response to ecdysone and the onset of the prepupal program.

DISCUSSION

Two successive pulses of ecdysone trigger distinct stage-specific developmental responses during the onset of *Drosophila* metamorphosis. The late larval ecdysone pulse triggers glue secretion in the salivary gland, larval muscle and gut histolysis, and imaginal disc eversion to form the rudiments of the adult appendages. The ecdysone titer drops following puparium formation and peaks again in late prepupae, triggering pupal cuticle deposition, salivary gland histolysis and head eversion (Riddiford, 1993). These, and other, developmental pathways are coordinated by a complex network of transcription factors that are regulated by changes in ecdysone concentration (Thummel, 1996). Some genes, such as the *BR-C*, *E74A* and *E75A*, are induced in response to both high titer ecdysone pulses, whereas other genes are regulated in a precise stage-specific manner, including *E78B*, *DHR3*, *βFTZ-F1* and *E93* (Fig. 1). Here we describe our characterization of the *Drosophila DHR3* orphan receptor gene. We show that *DHR3* is expressed during early prepupal development and can both bind to, and regulate, genes that direct critical developmental responses at this stage. Below, we present a model for how *DHR3* directs the proper order and timing of regulatory gene activity during the transition from a larva to a prepupa. These studies provide evidence for cross-regulatory interactions among orphan receptors and establish a framework for understanding how systemic hormonal signals are transduced into stage-specific developmental responses.

DHR3 protein is widely expressed during mid-embryogenesis and early prepupal development

Western blot analysis and antibody staining have revealed that DHR3 protein is expressed during mid-embryogenesis and in early prepupae, times that correspond to peak levels of *DHR3* transcription during development (Koelle et al., 1992). The widespread pattern of *DHR3* expression, in virtually all larval and imaginal tissues, suggests that this gene does not perform a spatially restricted function during development, and is reminiscent of the pattern of E74A expression in late third instar larvae (Boyd et al., 1991). In contrast, the BR-C and EcR protein isoforms are expressed in more tissue-restricted patterns during the onset of metamorphosis. EcR-B1 is expressed at higher levels in larval tissues that are fated to histolyze whereas EcR-A predominates in the imaginal discs (Talbot et al., 1993). Similarly, distinct levels and combinations of BR-C protein isoforms are expressed in different tissues, in patterns that reflect the phenotypes of the corresponding *BR-C* mutations (Emery et al., 1994). These data support the tissue coordination model, which proposes that early ecdysone-induced transcription factors function in a combinatorial manner to direct the expression of tissue-specific sets of secondary-response target genes (Burtis et al., 1990; Thummel et al., 1990). Thus, like the ecdysone signal itself, some early regulators are widely expressed and function as temporal cues during development, including E74A and DHR3. These factors act in conjunction with ecdysone-induced proteins that are expressed in a more restricted manner, including the BR-C and EcR isoforms, to provide spatially distinct responses to the hormonal signal.

DHR3 protein binds to many ecdysone-regulated puffs in the polytene chromosomes

A significant advantage of studying ecdysone regulatory hierarchies in the larval salivary gland is the ability to identify potential target genes by antibody staining of polytene chromosomes. Since at least some of these target genes are expressed in other tissues, these studies also provide critical clues to the more general regulatory functions of ecdysone. Localization of DHR3 protein on the polytene chromosomes of newly formed prepupae revealed several hundred bound sites, many of which correspond to ecdysone-regulated puffs (Fig. 5, Table 1). Among these loci are the classic early puffs 2B5, 23E, 63F, 74EF and 75B, the 46F, 62E and 78C early-late puffs, and the 22C and 29F late puffs. With the exception of 2B5, these same sites are bound by the E74A early ecdysone-induced protein (Urness and Thummel, 1990). Unlike E74A, however, DHR3 does not bind to many late puffs, including the well-characterized 63E and 71E puff loci. This observation suggests that *DHR3* function may be more restricted than that of *E74A*, involved primarily in early and early-late gene regulation. Our functional studies support this hypothesis by indicating that *DHR3* functions to arrest the expression of these genes at puparium formation, defining an end to the larval genetic response to ecdysone.

Three sites in the polytene chromosomes are stained strongly by anti-DHR3 antibodies: 46F, 75B and 75D. Remarkably, each of these sites encodes an ecdysone-regulated orphan receptor: DHR3, E75 and FTZ-F1, respectively. A screen through 30 kb of genomic DNA encompassing the *βFTZ-F1* gene revealed three closely spaced DHR3 binding sites downstream from the start site of transcription. This indicates that at least part of the strong DHR3 antibody stain at 75D can be accounted for by direct DNA binding. Ectopic expression of *DHR3* is also sufficient to repress *E75A* and induce *βFTZ-F1* transcription. These observations provide evidence for direct cross-regulation among orphan receptors. It will be interesting

to determine if similar cross-regulatory pathways function in other higher organisms.

The relevance of DHR3 binding to its own puff locus at 46F remains unclear. Although one experiment indicated that ectopic *DHR3* expression led to induction of the endogenous *DHR3* gene, this result could not be reproduced (data not shown). It is also possible that the strong staining of the 46F puff locus could represent binding to a gene other than *DHR3*. The significance of this staining pattern with regard to *DHR3* expression must await the characterization of specific mutations in this gene.

DHR3 protein recognizes canonical RORα binding sites in *βFTZ-F1*

Examination of the DHR3 binding sites in the *βFTZ-F1* gene reveals a close match to the sequences recognized by RORα, its mammalian counterpart (Fig. 9B). Three DHR3 binding sites were identified, extending from +155 to +455 bp relative to the start site of *βFTZ-F1* transcription (Figs 8, 9). The strong binding sites A and C contain a centrally located RGGTCA (where R represents G or A) sequence that matches the core recognition sequence for RORα (Fig. 9B) (Giguère et al., 1994). These sites also contain a precise match to the WWAWNT sequence (where W represents A or T) that lies upstream from the core sequence in a consensus RORα binding site. This sequence is recognized by amino acids that lie immediately C-terminal to the DNA binding domain in RORα, a region that is highly conserved in DHR3 (71% identical) (Koelle et al., 1992; Giguère et al., 1995). In contrast, the weak binding site B has a G at position −1 relative to the core sequence and an A at position +2. Previous studies have detected a G at position −1 in both RORα and DHR3 binding sites (Giguère et al., 1994; Horner et al., 1995). In contrast, no known RORα or DHR3 binding sites contain an A at position +2. This deviation from the consensus most likely accounts for the inability of this site to be completely protected against DNase I digestion (Fig. 9A). The presence of a single hexanucleotide core sequence in the DHR3 binding sites argues that, like its mammalian homolog, DHR3 binds DNA as a monomer. This is consistent with earlier studies of DHR3 protein binding (Horner et al., 1995). Finally, Y. Kageyama, S. Hirose and H. Ueda (personal communication) have shown that a 2.7 kb fragment containing these sites is sufficient for proper *βFTZ-F1* transcription in mid-prepupae, and have independently identified sites A and C as binding sites for a protein present in staged prepupal nuclear extracts. This observation supports our finding and suggests that these sites may be essential for *βFTZ-F1* transcription.

The ability of Rev-erb and RORα to bind the same target sequence raises the possibility that these orphan receptors may function in common regulatory pathways (Harding and Lazar, 1993; Forman et al., 1994; Giguère et al., 1994; Harding and Lazar, 1995). In addition, Rev-erb and RORα can interact with retinoic acid receptors on naturally occurring response elements, suggesting that these orphan receptors may cross-talk with hormone signalling pathways (Harding and Lazar, 1995; Tini et al., 1995). These observations raise the possibility that E75 or E78 proteins may function through DHR3 binding sites to cross-regulate transcription. Furthermore, it is possible that DHR3 may interact with the EcR/USP complex through binding to common response elements. Further studies will be required to determine if these cross-regulatory interactions contribute to ecdysone responses during metamorphosis.

A model for *DHR3* as a regulator of the larval-prepupal transition during *Drosophila* metamorphosis

Studies of puff regulation in cultured larval salivary glands have defined two distinct responses that depend on ecdysone-induced protein synthesis in late third instar larvae: early puff regression and mid-prepupal puff induction. The early puffs normally regress several hours after their induction by ecdysone, but this regression can be effectively blocked by the addition of cycloheximide to the culture medium (Ashburner, 1974). Similarly, the 75CD mid-prepupal puff (*βFTZ-F1*) can be induced in late larval salivary glands that are cultured with ecdysone for 6 hours and then cultured for a further 3 hours in the absence of hormone, recapitulating the changes in hormone titer that normally occur in vivo (Richards, 1976). The 75CD puff, however, is not induced if cycloheximide is present during the initial 6 hour incubation. Taken together, these observations suggest that one or more ecdysone-induced proteins are required to both arrest the larval puffing response to ecdysone and initiate the mid-prepupal puffing response.

Three lines of evidence support the proposal that *DHR3* contributes to these two regulatory functions. First, *DHR3* expression begins to peak in newly formed prepupae, as the early genes are repressed, and *DHR3* is expressed through the mid-prepupal period when *βFTZ-F1* is induced (Figs 1, 3). Second, DHR3 protein is bound to all early and early-late puff loci as well as the 75D region where the *βFTZ-F1* gene resides. DHR3 also binds to sequences near the *βFTZ-F1* promoter, providing the capacity for direct transcriptional regulation. Third, ectopic expression of *DHR3* is sufficient for both early gene repression and *βFTZ-F1* induction.

Based on these observations we propose that *DHR3* plays a critical role during the early stages of *Drosophila* metamorphosis, by ensuring the proper timing and order of regulatory gene expression in response to ecdysone (Fig. 10). We propose that *DHR3* coordinately represses early gene transcription at puparium formation, defining an end to the larval regulatory response to the hormone. The delay in *DHR3* expression is thus of functional significance insofar as it establishes the duration of early gene activity. Our model further proposes that *DHR3* facilitates the induction of *βFTZ-F1* in response to the decrease in ecdysone titer in mid-prepupae. *βFTZ-F1*, in turn, appears to function as a competence factor that directs the appropriate levels and stage-specificity of early gene expression in late prepupae (Woodard et al., 1994). Thus, according to our model, *DHR3* arrests the larval genetic response to ecdysone and allows the appropriate mid-prepupal response to the hormone, directing the transition from a larva to a prepupa. This model also predicts that *DHR3* provides both regulatory functions defined by the puffing studies of Ashburner (1974) and Richards (1976).

Although *DHR3* may play an essential role at the onset of metamorphosis, it seems unlikely that it functions alone in this capacity. Ectopic expression of *DHR3* does not completely repress early gene transcription and leads to relatively low levels of *βFTZ-F1* mRNA accumulation, even under conditions of low hormone titer in mid-third instar larvae (Fig. 6). These

observations suggest that *DHR3* is functioning in concert with other regulators expressed in early prepupae. Two candidates are the E75B and E78B orphan receptors, although neither of these proteins are capable of binding DNA (Segraves and Hogness, 1990; Stone and Thummel, 1993). It is possible that E75B or E78B could function as 'ligands' that interact directly with DHR3 through their conserved ligand binding domains. Recent evidence for these interactions has been obtained by White et al. (1997) who have performed studies on *DHR3* similar to those reported here. White et al. present data indicating that E75B protein may directly inhibit the ability of DHR3 to induce *βFTZ-F1* transcription. This mode of regulation could provide a function for the growing class of orphan receptors that are missing a DNA binding domain (Seol et al., 1996).

A critical test of our model requires the characterization of *DHR3* mutations. We predict that loss-of-function *DHR3* mutations will lead to inefficient early gene repression at puparium formation and reduced levels of *βFTZ-F1* expression. These, in turn, should result in prepupal lethality with defects in head eversion, due to a block in the re-induction of early genes in prepupae (Sliter and Gilbert, 1992). The recent identification of *DHR3* mutants makes these studies feasible, although their highly penetrant embryonic lethality complicates our approach (M. Bender, personal communication). Efforts are currently underway to examine the effects of these mutations on gene expression and development during prepupal stages. Studies of mutations in ecdysone-regulated orphan receptor loci should allow us to further understand how these regulators may be functioning together to direct the early stages of insect metamorphosis.

We thank P. Reid for invaluable assistance with the confocal laser scanning microscope, C. Xu and B. Graves for help with DNase I footprint analysis, P. Reid and C.T. Woodard for the cosmid genomic clone of *βFTZ-F1* and the 5 kb *Xba*I-*Eco*RI subclone, M. Horner for advice on antibody affinity purification, L. Urness for help with the polytene chromosome staining protocol, B. Hall for advice on DNA binding, R. Burgess and T. Schwarz for providing the *Df(2R)12* stock, M. Bender for helpful comments and suggestions during the course of this work and P. D'Avino, G. Fisk, M. Horner and A. Letsou for critical comments on the manuscript.

REFERENCES

Andres, A. J. and Thummel, C. S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. In *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology* (ed. L. S. B. Goldstein and E. A. Fyrberg), pp. 565-573. New York: Academic Press.

Ashburner, M. (1974). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. II. The effects of inhibitors of protein synthesis. *Dev. Biol.* **39**, 141-157.

Ashburner, M. (1975). The puffing activities of the salivary gland chromosomes. In *Handbook of Genetics*, 3 (ed. R. C. King), pp. 793-811. New York: Plenum Press.

Ashburner, M., Chihara, C., Meltzer, P. and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38**, 655-662.

Ayer, S., Walker, N., Mosammaparast, M., Nelson, J. P., Shilo, B. and Benyajati, C. (1993). Activation and repression of *Drosophila* alcohol dehydrogenase distal transcription by two steroid hormone receptor superfamily members binding to a common response element. *Nucl. Acids Res.* **21**, 1619-1627.

Baehrecke, E. H. and Thummel, C. S. (1995). The Drosophila *E93* gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. *Dev. Biol.* **171**, 85-97.

Boyd, L., O'Toole, E. and Thummel, C. S. (1991). Patterns of *E74A* RNA and protein expression at the onset of metamorphosis in *Drosophila*. *Development* **112**, 981-995.

Burtis, K. C., Thummel, C. S., Jones, C. W., Karim, F. D. and Hogness, D. S. (1990). The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell* **61**, 85-99.

Carroll, S. B. and Laughon, A. (1987). Production and purification of polyclonal antibodies to the foreign segment of β-galactosidase fusion proteins. In *DNA cloning*, 3 (ed. D. M. Glover), pp. 89-111. Oxford: IRL Press.

Clever, U. (1964). Actinomycin and puromycin: effects on sequential gene activation by ecdysone. *Science* **146**, 794-795.

DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W. and Guild, G. M. (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* **129**, 385-397.

Emery, I. F., Bedian, V. and Guild, G. M. (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* **120**, 3275-3287.

Fletcher, J. C., Burtis, K. B., Hogness, D. S. and Thummel, C. S. (1995). The *Drosophila E74* gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development* **121**, 1455-1465.

Fletcher, J. C. and Thummel, C. S. (1995). The *Drosophila E74* gene is required for the proper stage- and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. *Development* **121**, 1411-1421.

Forman, B. M., Chen, J., Blumberg, B., Kliewer, S. A., Henshaw, R., Ong, E. S. and Evans, R. M. (1994). Cross-talk among RORα1 and the Rev-erb family of orphan nuclear receptors. *Mol. Endo.* **8**, 1253-1261.

Galas, D. and Schmitz, A. (1978). DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucl. Acids Res.* **5**, 3157-3170.

Giguère, V., McBroom, L. D. B. and Flock, G. (1995). Determinants of target gene specificity for RORα1: monomeric DNA binding by an orphan nuclear receptor. *Mol. Cell. Biol.* **15**, 2517-2526.

Giguère, V., Tini, M., Flock, G., Ong, E., Evans, R. M. and Otulakowski, G. (1994). Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan hormone nuclear receptors. *Genes Dev.* **8**, 538-553.

Gronemeyer, H. and Laudet, V. (1995). Transcription factors 3: nuclear receptors. In *Protein Profile*, 2 (ed. P. Sheterline), pp. 1173-1308. London: Academic Press.

Guay, P. S. and Guild, G. M. (1991). The ecdysone-induced puffing cascade in *Drosophila* salivary glands: a *Broad-Complex* early gene regulates intermolt and late gene transcription. *Genetics* **129**, 169-175.

Guo, L. H., Stepién, P. P., Tso, J. Y., Brousseau, R., Narang, S., Thomas, D. Y. and Wu, R. (1984). Synthesis of human insulin gene VIII. Construction of expression vectors for fused proinsulin production in *Escherichia coli*. *Gene* **29**, 251-254.

Harding, H. P. and Lazar, M. A. (1993). The orphan receptor Rev-ErbAα activates transcription via a novel response element. *Mol. Cell. Biol.* **13**, 3113-3121.

Harding, H. P. and Lazar, M. A. (1995). The monomer-binding orphan receptor Rev-erb represses transcription as a dimer on a novel direct repeat. *Mol. Cell. Biol.* **15**, 4791-4802.

Heberlein, U., England, B. and Tjian, R. (1985). Characterization of *Drosophila* transcription factors that activate the tandem promoters of the alcohol dehydrogenase gene. *Cell* ***41***, 965-977.

Hope, I. A. and Struhl, K. (1985). *GCN4* protein, synthesized *in vitro*, binds *HIS3* regulatory sequences: implications for general control of amino acid biosynthetic genes in yeast. *Cell* **43**, 177-188.

Horner, M., Chen, T. and Thummel, C. S. (1995). Ecdysone regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Dev. Biol.* **168**, 490-502.

Huet, F., Ruiz, C. and Richards, G. (1993). Puffs and PCR: the in vivo dynamics of early gene expression during ecdysone responses in *Drosophila*. *Development* **118**, 613-627.

Huet, F., Ruiz, C. and Richards, G. (1995). Sequential gene activation by ecdysone in *Drosophila melanogaster*: the hierarchical equivalence of early and early late genes. *Development* **121**, 1195-1204.

Karim, F. D., Guild, G. M. and Thummel, C. S. (1993). The *Drosophila*

Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Development* **118**, 977-988.

Karim, F. D. and Thummel, C. S. (1991). Ecdysone coordinates the timing and amounts of *E74A* and *E74B* transcription in *Drosophila*. *Genes Dev.* **5**, 1067-1079.

Karim, F. D. and Thummel, C. S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J.* **11**, 4083-4093.

Kiss, I., Beaton, A. H., Tardiff, J., Fristrom, D. and Fristrom, J. W. (1988). Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* **118**, 247-259.

Koelle, M. R. (1992). Molecular analysis of the *Drosophila* ecdysone receptor complex. Ph.D. thesis, Stanford University.

Koelle, M. R., Segraves, W. A. and Hogness, D. S. (1992). DHR3: a *Drosophila* steroid receptor homolog. *Proc. Natl. Acad. Sci. USA* **89**, 6167-6171.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P. and Hogness, D. S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59-77.

Lavorgna, G., Karim, F. D., Thummel, C. S. and Wu, C. (1993). Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl. Acad. Sci. USA* **90**, 3004-3008.

Mangelsdorf, D. J. and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**, 841-850.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995). The nuclear receptor superfamily: The second decade. *Cell* **83**, 835-839.

Maroni, G. and Stamey, S. C. (1983). Use of blue food to select synchoronous, late third instar larvae. *Dros. Inf. Serv.* **59**, 142-143.

Ohno, C. K. and Petkovich, M. (1992). *FTZ-F1β*, a novel member of the *Drosophila* nuclear receptor family. *Mech. Dev.* **40**, 13-24.

Palli, S. R., Hiruma, K. and Riddiford, L. M. (1992). An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. *Dev. Biol.* **150**, 306-318.

Reuter, R. and Scott, M. P. (1990). Expression and function of the homeotic genes *Antennapedia* and *Sex combs reduced* in the embryonic midgut of *Drosophila*. *Development* **109**, 289-303.

Richards, G. (1976). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*: V. The late prepupal puffs. *Dev. Biol.* **54**, 264-275.

Riddiford, L. M. (1993). Hormones and *Drosophila* development. In *The Development of Drosophila melanogaster*, 2 (ed. M. Bate and A. Martinez Arias) pp. 899-939. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Rio, D. C., Laski, F. A. and Rubin, G. M. (1986). Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. *Cell* ***44***, 21-32.

Robertson, C. W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principle morphological changes. *J. Morphol.* **59**, 351-399.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Russell, S. R. H., Heimbeck, G., Goddard, C. M., Carpenter, A. T. C. and Ashburner, M. (1996). The *Drosophila Eip78C* gene is not vital but has a role in regulating chromosome puffs. *Genetics* **144**, 159-170.

Segraves, W. A. and Hogness, D. S. (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4**, 204-219.

Seol, W., Choi, H.-S. and Moore, D. D. (1996). An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. *Science* **272**, 1336-1339.

Sliter, T. J. and Gilbert, L. I. (1992). Developmental arrest and ecdysteroid deficiency resulting from mutations at the *dre4* locus of *Drosophila*. *Genetics* **130**, 555-568.

Stone, B. L. and Thummel, C. S. (1993). The *Drosophila* 78C early late puff contains *E78*, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone receptor superfamily. *Cell* **75**, 307-320.

Talbot, W. S., Swyryd, E. A. and Hogness, D. S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73**, 1323-1337.

Thomas, H. E., Stunnenberg, H. G. and Stewart, A. F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* **362**, 471-475.

Thummel, C. S. (1995). From embryogenesis to metamorphosis: The regulation and function of Drosophila nuclear receptor superfamily members. *Cell* **83**, 871-877.

Thummel, C. S. (1996). Flies on steroids: Drosophila metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12**, 306-310.

Thummel, C. S., Burtis, K. C. and Hogness, D. S. (1990). Spatial and temporal patterns of *E74* transcription during *Drosophila* development. *Cell* **61**, 101-111.

Thummel, C. S. and Pirrotta, V. (1992). New pCaSpeR P element vectors. *Dros. Info. Serv.* **71**, 150.

Tini, M., Fraser, R. A. and Giguère, V. (1995). Functional interactions between retinoic acid receptor-related orphan nuclear receptor (RORα) and the retinoic acid receptors in the regulation of the γF-crystallin promoter. *J. Biol. Chem.* **270**, 20156-20161.

Tsai, M.-J. and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63**, 451-486.

Urness, L. D. and Thummel, C. S. (1990). Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the *Drosophila* ecdysone-inducible *E74A* protein. *Cell* **63**, 47-61.

Weber, U., Siegel, V. and Mlodzik, M. (1995). pipsqueak encodes a novel nuclear protein required downstream of seven-up for the development of photoreceptors R3 and R4. *EMBO J.* **14**, 6247-6257.

White, K. P., Hurban, P., Watanabe, T. and Hogness, D. S. (1997). Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* (in press).

Woodard, C. T., Baehrecke, E. H. and Thummel, C. S. (1994). A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell* **79**, 607-615.

Yao, T., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P. and Evans, R. M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366**, 476-479.

Yao, T., Segraves, W. A., Oro, A. E., McKeown, M. and Evans, R. M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71**, 63-72.

Zink, B. and Paro, R. (1989). In vivo binding pattern of a transregulator of homeotic genes in *Drosophila melanogaster*. *Nature* **337**, 468-471.

(Accepted 24 February 1997)