Temporal Profiles of Nuclear Receptor Gene Expression Reveal Coordinate Transcriptional Responses during Drosophila Development

APRIL A. SULLIVAN AND CARL S. THUMMEL
Howard Hughes Medical Institute, Department of Human Genetics, University of Utah School of Medicine, University of Utah, Salt Lake City, Utah 84112-5331

The recent completion of the Drosophila genome sequence revealed 21 members of the nuclear receptor superfamily. Many of these genes are transcriptionally regulated by the steroid hormone ecdysone and play a role during the onset of metamorphosis, including the EcR/USP ecdysone receptor heterodimer. As a first step toward a genomic analysis of this gene family, we have characterized the temporal patterns of expression for all detectable nuclear receptor transcripts throughout major ecdysone-regulated developmental transitions in the life cycle: embryogenesis, a larval molt, puparium formation, and the prepupal-pupal transition. We find an unexpected close temporal relationship between DHR3, E75B, and βFTZ-F1 expression after each major ecdysone pulse examined, reflecting the known cross-regulatory interactions of these genes in prepupae and suggesting that they act together at other stages in the life cycle. In addition, E75A, E75B, and DHR4 are expressed in a reproducible manner with DHR3, E75B, and βFTZ-F1, suggesting that they intersect with this regulatory cascade. Finally, we find that known ecdysone-inducible primary-response transcripts are coordinately induced at times when the ecdysteroid titer is low, implying the existence of novel, as yet uncharacterized, temporal signals in Drosophila. (Molecular Endocrinology 17: 2125–2137, 2003)}
late genes. **BR-C** encodes a family of zinc finger transcription factors while **E74** encodes two ETS-domain proteins, **E74A** and **E74B**, from distinct transcripts. **BR-C**, **EcR**, and **E74B** mRNAs are induced by lower concentrations of ecdysone than **E74A** and thus provide a sensitive indicator of hormone titers, as assayed in late third-instar larval organs (24, 25). Higher ecdysone concentrations further up-regulate **BR-C** transcription while repressing **EcR**.

Many *Drosophila* nuclear receptor genes appear to function during metamorphosis, including **EcR**, **usp**, **DHR3**, **DHR38**, **DHR39**, **DHR78**, **E75**, **E78**, and **βFTZ-F1** (23). The **DHR3** orphan nuclear receptor is induced by ecdysone immediately after puparium formation and is required for induction of the βFTZ-F1 competence factor in midprepupae (26–30). **DHR38** is required during pupal development for adult cuticle formation (31). **E75** corresponds to the classic early puff locus at 75B studied by Ashburner and colleagues (17) and encodes three mRNA isoforms, designated **E75A**, **E75B**, and **E75C** (32). **E75A** mutants die primarily during larval stages with a reduced ecdysone titer, while **E75B** mutants are viable, and **E75C** mutants die as adults (33). **DHR78**, **DHR3**, and **βFTZ-F1** are also required during larval development, with mutants displaying defects in ecdysone-triggered molting of the larval cuticle (26, 34, 35). **E78** and **DHR39** are induced directly by ecdysone and are not essential for viability or fertility (35–40).

Some nuclear receptors function during embryogenesis and/or in neuronal development. These include the **tailless** (**tll**) and **knirps** (**kni**) gap genes that contribute to embryonic pattern formation (41–44). **Knirps-related** (**knr**l) functions together with its closely related partner **kni** in both head and wing development (45–48). **FTZ-F1** also encodes an embryonic-specific isoform, αFTZ-F1, that acts as a critical cofactor for the FTZ homeotic transcription factor (49–51), whereas **DHR3** null mutants die during embryogenesis with defects in peripheral nervous system development (52). Three nuclear receptor genes, **dissatisfaction** (**dsf**), **seven-up** (**svp**), and **eagle** (**eg**), are required for neuronal function. **Dsf** is expressed in a subset of central nervous system (CNS) neurons and is required for appropriate adult sexual behavior (53). **Svp** (the fly ortholog of COUP-TF) contributes to a number of developmental pathways, including photoreceptor determination in the adult eye (54–57), whereas **eg** is required for the development of serotonergic neurons in the CNS (58–60). Finally, six nuclear receptor genes have not yet been subjected to specific mutational analysis: **DHR4**, **DHR96**, **dERR** (the fly ortholog of vertebrate **ERR**), **dHNF-4** (the fly ortholog of vertebrate **HNF-4**), **DHR83**, and **CG16801** (the fly ortholog of C. elegans **FAX-1**).

The genome sequence of *D. melanogaster* allows, for the first time, a genomic approach toward studying the nuclear receptor superfamily in this organism.

Comparing and contrasting the regulation and function of *Drosophila* nuclear receptor genes should provide a better understanding of how these factors act together to define specific developmental pathways in the organism. As a first step toward this goal, we have determined the temporal profiles of nuclear receptor gene expression during major ecdysone-triggered developmental transitions in the fly life cycle: embryogenesis, a larval-to-larval molt, puparium formation, and the prepupal-pupal transition. This study reveals a repeated cascade of nuclear receptor transcription, in which a subset of nuclear receptor genes are expressed in a defined sequential pattern in apparent response to each ecdysone pulse examined. A previously uncharacterized orphan nuclear receptor gene, **dERR**, is expressed in embryos and at the onset of metamorphosis in a pattern that correlates with ecdysone pulses, suggesting critical roles at these stages. Finally, **BR-C** and **E74** mRNAs, which were used as markers for ecdysone pulses, are expressed at times during development when the ecdysone titer is low, suggesting that distinct, as yet unidentified, temporal signals may contribute to progression through the *Drosophila* life cycle.

## RESULTS

### Temporal Patterns of Nuclear Receptor Transcription during Embryogenesis

Total RNA was isolated from two independent collections of embryos staged at 2-h intervals throughout the 24 h of *Drosophila* embryonic development. Five Northern blots were prepared using equal amounts of RNA from each time point. These blots were sequentially hybridized, stripped, and rehybridized with radioactive probes derived from each of the 21 nuclear receptor genes encoded by the *Drosophila* genome (Table 1). This approach allowed us to generate time courses of nuclear receptor gene expression that could be directly compared between family members. The transcripts detected are consistent with reported sizes (Fig. 1; Table 1). Transcripts from eight nuclear receptor genes were not detectable during embryonic development: **E75C**, **E78**, **CG16801**, **DHR38**, **DHR83**, **dsf**, **eg**, and **svp** (data not shown).

Transcripts from nine nuclear receptor genes can be detected at the earliest time point (0–2 h): **usp**, **EcR-A**, **FTZ-F1**, **DHR39**, **DHR78**, **DHR96**, **dERR**, **dHNF-4**, and **tll**. This expression is consistent with the known maternal contribution of **usp**, **EcR**, and **FTZ-F1** (49, 51, 61–64). The observation that transcripts from **DHR39**, **DHR78**, **DHR96**, **dERR**, and **dHNF-4** are undetectable by the next time point examined (2–4 h) suggests that these mRNAs are maternally loaded and rapidly degraded (Fig. 1). **EcR-B** and **usp** transcripts are induced in early embryos, up-regulated at 6–8 h after egg laying (AEL), and maintain expression through the end of
embryogenesis, with down-regulation of EcR-B in late embryos (Fig. 1). EcR-A, in contrast, is expressed for a relatively brief temporal window, at 8–14 h AEL. These patterns of EcR transcription are very similar to those reported by Talbot et al. (64), although earlier repression of EcR-A was seen in our study.

Six nuclear receptor genes are expressed in brief intervals during midembryonic stages. DHR39 and E75A are initially induced at 4–6 and 6–8 h AEL, respectively, and peak at 8–12 h AEL (Fig. 1). This is followed by induction of DHR3, DHR4, and E75B at 8–12 h AEL, followed by βFTZ-F1 expression at 12–18 h AEL. DHR39 appears to exhibit an expression pattern reciprocal to that of βFTZ-F1, with lowest levels of mRNA at 14–16 h AEL and reinduction at 16–18 h as βFTZ-F1 is repressed. This is followed by a second peak of E75A transcription at 18–22 h AEL.

A second group of nuclear receptors, DHR78, DHR96, dHNF-4, and dERR, is more broadly expressed at low levels throughout embryogenesis. DHR78 accumulates above its constant low level of expression between 8 and 14 h AEL. dERR exhibits an apparent mRNA isoform switch between 14 and 18 h AEL. dHNF-4 regulation also appears complex, with two size classes of mRNA induced at approximately 8–10 h AEL. While the 4.6-kb dHNF-4 mRNA is expressed throughout embryogenesis, the 3.3-kb mRNA is down-regulated at 14–16 h AEL. This timing is consistent with work by Zhong et al. (65), who showed that dHNF-4 is expressed primarily in the embryonic midgut, fat body, and Malpighian tubules. Finally, nuclear receptors known to exert essential functions in patterning the early embryo, tll, kni, and knrl, are expressed predominantly during early stages (Fig. 1).

Two genes that are not members of the nuclear receptor superfamily, BR-C and E74, were also examined in this study, as transcriptional markers for ecdysone pulses during development. Unexpectedly, both of these genes are induced late in embryogenesis, several hours after the rise in ecdysone titer at 6 h AEL (66, 67) (Fig. 1). An approximately 7-kb BR-C transcript is induced at 10–12 h AEL and is present through the end of embryogenesis while E74B is induced at 14–16 h AEL and repressed as E74A is expressed from 16–20 h AEL. This BR-C expression pattern is consistent with the identification of the BR-C Z3 isoform in specific neurons of the embryonic CNS (68, 69).

Table 1. Probes for Northern Blot Hybridization

| Transcript | Plasmid/DNA | Probe Template | mRNA Size(s) (kb) | Ref. |
|------------|-------------|----------------|-------------------|-----|
| BR-C       | paaDM527    | Stul/Pvull, 0.5| 10, 8.8, 6.8, 4.4 | 25  |
| CG16801    | Genomic DNA | PCR, 0.9      |                   |     |
| dERR       | GM14739     | PCR, 0.6      | 2.0, 1.6          |     |
| dHNF-4     | pBS/HNF-4   | Bpml, 1.1     | 4.6, 3.3          | 65  |
| DHR3       | pBS/DHR3    | HindIII, 0.9  | 6.3, 5.5, 4.9     | 79  |
| DHR4       | Genomic DNA | PCR, 0.7      | 10                |     |
| DHR38      | pCaSpeRhsGAL4-DHR38 | XbaI/Rl, 1.0 | 6.2, 4.0, 3.6, 1.9 | M&M |
| DHR39      | pBS/DHR39   | Eagl/DralII, 1.1| 5.0              |     |
| DHR78      | pLF5        | Sall, 0.5     | 2.3               | 75  |
| DHR83      | pTOPO/DHR83 | EcoRI, 0.9    |                   |     |
| DHR96      | pLF20       | EcoRV, 1.8    | 2.8               | 75  |
| dsf        | Genomic DNA | PCR, 0.7      | 3.7               |     |
| E74        | pBS/E74     | BgIII/Sall, 0.9| A = 6.0; B = 5.1, 4.8 | 25  |
| E75A       | Genomic DNA | PCR, 0.7      | 5.7, 4.9          | 25  |
| E75B       | Genomic DNA | PCR, 0.3      | 6.0, 5.2          | 25  |
| E75C       | Genomic DNA | PCR, 1.0      | 8.5, 7.7          | 25  |
| E78B       | pcDM304     | Xhol/Rl, 2.3  | 3.5, 2.6          |     |
| eg         | Genomic DNA | PCR, 0.8      | 2.2, 1.9          |     |
| EcR-A      | GB0-EcR-A   | Msci/NotII, 0.7| 5                | 20  |
| EcR-B      | pMK1        | RI, 1.7       | 6K1               | 25  |
| FTZ-F1     | pBS/FTZ-F1  | RI/Xhol, 1.6  | α = 5.2; β = 5.6, 4.8 | 29  |
| kni        | Genomic DNA | PCR, 0.6      | 2.5, 2.2          |     |
| knrl       | Genomic DNA | PCR, 1.0      | 3.8               |     |
| np49       | pBs/rp49    | Ri/HindIII, 0.4| 0.6              | 96  |
| svp        | pC162-2B    | PstI/BspEI, 0.7| 2.3, 1.7         | 76  |
| tll        | N4          | PstI/BgI1I, 1.25| 2.0             | 43  |
| usp        | pZ7-1       | PstI, 0.5     | 2.4               | 97  |

The restriction fragment or PCR product used as a radioactive probe for each transcript is listed. Restriction enzymes indicate those used to cut plasmid DNA. The length of each probe and reported mRNA sizes are listed in kilobases. Lengths for dERR, DHR3, and DHR4 mRNA were determined from size markers. Sizes of CG16801 and DHR83 mRNA are unknown. The EcR-B probe will detect both EcR-B1 and EcR-B2 isoforms. References describe the PCR primers or plasmid DNA used to make each probe, or refer to the Materials and Methods.

* M&M, Materials and Methods.
Temporal Patterns of Nuclear Receptor Transcription during a Larval Molting Cycle

First-instar larvae were synchronized as they molted to the second instar, aged and harvested at 4-h intervals throughout second-instar larval development. Two Northern blots were prepared using equal amounts of total RNA isolated from a single collection of animals. Each blot was sequentially hybridized, stripped, and rehybridized to detect nuclear receptor transcription (Fig. 2, left). The following transcripts were not detectable during the second instar: E75C, dERR, CG16801, DHR83, dsf, eg, svp, tll, kn, and knrl (Fig. 2 and data not shown).

EcR-B expression is induced in mid-second-instar larvae, but does not reach maximum levels until 68–72 h AEL, just before the molt (Fig. 2). In contrast, usp is expressed throughout the instar. A sequential pattern of nuclear receptor expression is observed that resembles the pattern seen in midembryogenesis. DHR39 and E75A are expressed in the early second instar. This is followed by induction of E75B, E78B, DHR3, and DHR4, followed by expression of ßFTZ-F1 at the end of the instar. DHR39 again shows a pattern that is approximately reciprocal with ßFTZ-F1, with highest levels during the first half of the instar. Similarly, DHR78, DHR96, and dHNF-4 exhibit broad expression patterns throughout second-instar larval development. E74A, E75A, and DHR38 are coordinately up-regulated with EcR-B at the end of the instar, between 64–72 h AEL. Finally, an approximately 9-kb BR-C transcript is detected throughout the second-larval instar.

Temporal Patterns of Nuclear Receptor Transcription during the Onset of Metamorphosis

We also examined nuclear receptor gene expression throughout the third larval instar and into the early stages of metamorphosis, encompassing the ecdysone-triggered larval-to-prepupal and prepupal-to-pupal transitions. Third-instar larvae were staged relative to the molt from the second instar and harvested at 4-h intervals throughout the 48 h of the instar. Preppupae were synchronized relative to puparium formation (±15 min) and harvested at 2-h intervals up to 16 h after puparium formation (APF). Total RNA was isolated from whole animals and analyzed by Northern blot hybridization. Five blots were prepared from two independent collections of animals. These blots were sequentially hybridized, stripped, and rehybridized to detect nuclear receptor gene expression (Fig. 2). The following transcripts were not detectable during third-instar larval or prepupal stages: CG16801, DHR83, dsf, eg, svp, tll, kn, and knrl (data not shown).

Most nuclear receptor genes show little or no detectable expression in early and mid-third-instar larvae, a time when the ecdysone titer is low (70). Similar to the pattern seen in second-instar larvae, usp is expressed at relatively low levels throughout the instar and up-regulated at puparium formation, while EcR-B is induced at approximately 100 h AEL and rapidly down-regulated at puparium formation (Fig. 2). This is followed by a sequential pattern of nuclear receptor expression similar to that seen at earlier stages.
Fig. 2. Temporal Profiles of Nuclear Receptor Gene Expression during Larval and Prepupal Development

Samples of total RNA were isolated at 4-h intervals throughout second- and third- instar larval development and at 2-h intervals throughout prepupal stages. Equal amounts of RNA from each time point were analyzed by Northern blot hybridization to determine the temporal patterns of nuclear receptor gene expression. Time points of larval development in hours AEL, and hours APF for prepupal and early pupal stages, are shown at the top along with the major ecdysone-triggered developmental transitions represented by lines. The approximate times of peak ecdysone titer are marked with boxes (see text for details). Arrows designate E74A and E74B mRNAs. For BR-C transcripts, the arrow marks the 9-kb RNA (68). The time courses were divided into separate blots at the mid-third instar. The white line down the middle denotes the boundary between these two blots. Although the signal intensity for each gene is approximately equal between the blots, they cannot be directly compared. The apparent increased expression of many genes at 92–96 h AEL is not reproducible. Hybridization to detect rp49 mRNA was used as a control for loading and transfer. The 88- to 92-h AEL, 92- to 96-h AEL, and 14-h APF lanes are underloaded.
DHR39, E75A, and E78B are induced at 116–120 h AEL, in concert with the late larval ecdysone pulse, followed by maximum accumulation of E75B, DHR3, and DHR4 at 0–4 h APF. βFTZ-F1 is expressed from 6–10 h APF, with a pattern that is approximately reciprocal to that of DHR39. EcR-A is expressed in parallel with E75B, DHR3, and DHR4 in midprepupae, similar to their coordinate expression during embryogenesis.

DHR78, DHR96, and dHNF-4 continue to exhibit broad expression profiles throughout third-instar larval and prepupal development (Fig. 2). An E75 isoform not detected in embryos or second-instar larvae, E75C, is also detectable at low levels throughout most of the third instar and up-regulated in correlation with the late-larval and prepupal pulses of ecdysone. DHR38 is detectable at very low levels in early third-instar larvae, in synchrony with the early induction of E74B and BR-C. E74B is repressed, E74A is induced, and BR-C transcripts are up-regulated in late third-instar larvae, in synchrony with the late-larval ecdysone pulse (Fig. 2). The prepupal pulse of ecdysone occurs at 10–12 h APF, marking the prepupal-to-pupal transition. EcR-A, E75A, E78B, DHR4, dERR, E75C, dHNF-4, and E74A are all induced at 10–12 h APF, in apparent response to this hormone pulse. These results are consistent with a microarray analysis of gene expression at the onset of metamorphosis where the temporal profiles of about half of these genes were reported (71).

DISCUSSION

The completion of the *Drosophila* genome sequence has revealed 21 members of the nuclear receptor superfamily, significantly less than the approximately 270 nuclear receptor genes identified in *C. elegans* and 49 nuclear receptor genes in humans (3, 4, 72, 73). The definition of this family allows us, for the first time, to undertake a genomic approach toward studying this class of ligand-regulated transcription factors. This study represents a first step in that direction, determining the temporal profiles of expression for all detectable *Drosophila* nuclear receptor transcripts during each major ecdysone-triggered developmental transition in the life cycle: embryogenesis, a larval molt, puparium formation, and head evisceration. Most nuclear receptors can be divided into one of four classes based on this study: 1) those that are expressed exclusively during early embryogenesis (*kni, knrl, tll*); 2) those that are expressed throughout development (*usp, DHR78, DHR96, dHNF-4*); 3) those that are expressed in a reproducible temporal cascade at each stage tested (*E75A, E75B, DHR3, DHR4, FTZ-F1, DHR39*); and 4) those that are undetectable in our assays (*CG16801, DHR83, dsf, eg, svp*). Below, we discuss these classes and propose new cross-regulatory interactions between orphan nuclear receptors. In addition, we describe unexpected coordinate patterns of gene expression during the life cycle, providing evidence for novel temporal signals that may act independently of the well characterized ecdysone signaling pathway.

**Nuclear Receptor Expression Patterns Reveal Distinct Temporal Classes**

Three nuclear receptor genes appear to be expressed exclusively during early embryogenesis: *kni, knrl*, and *tll*. This restricted pattern of expression fits well with the functional characterization of these genes, which have been shown to act as key determinants of embryonic body pattern (41, 43, 44, 47, 48). We have also identified eight genes (*usp, EcR, FTZ-F1, DHR39, DHR78, DHR96, dERR, and dHNF-4*) that appear to have maternally deposited transcripts and thus possible embryonic functions. Indeed, maternal functions have been defined for *usp, EcR*, and *dFTZ-F1* (15, 49, 51, 61–63).

Four nuclear receptor genes are broadly expressed through all stages examined: *usp, DHR78, DHR96*, and *dHNF-4*. This is consistent with an analysis of *usp* expression by Henrich et al. (74), as well as the profiles of *DHR78* and *DHR96* expression during the onset of metamorphosis (75). *dHNF-4* mRNA is first detectable at 6–10 h AEL (Fig. 1), as the ecdysone titer begins to rise (Fig. 3A). In addition, peaks of *dHNF-4* expression are seen at 0, 12, and 16 h APF, in synchrony with the *E74* and *E75C* early ecdysone-inducible genes. These observations raise the interesting possibility that this orphan nuclear receptor is regulated by ecdysone.

*DHR38* transcripts are difficult to detect in our assays. This is consistent with earlier studies which used RT-PCR or riboprobes for this purpose (31, 75). Nonetheless, we can detect *DHR38* mRNA during third-instar larval development, consistent with the widespread expression reported in earlier studies. Kozlova et al. (31) have shown that *DHR38* expression peaks at late pupal stages, consistent with its essential role in adult cuticle formation.

*dERR* and *E75C* display related temporal profiles of expression that do not fit with other nuclear receptor genes described in this study. Both of these genes are specifically transcribed during prepupal development, with increases in expression at 0 and 10–12 h APF. dERR, but not E75C, is also expressed during embryogenesis, with an initial induction at approximately 6 h AEL. These increases occur in synchrony with ecdysone pulses, suggesting that these orphan nuclear receptor genes are hormone inducible, although in a stage-specific manner. Further studies of dERR regulation, as well as a genetic analysis of this locus, are currently in progress (Sullivan, A. A., and C. S. Thummel, unpublished results).

Finally, we did not detect transcripts from five nuclear receptor genes during the stages examined: *CG16801, DHR83, dsf, eg*, and *svp*. Interestingly, these genes are implicated in specific neuronal functions during development. Three of these genes, *dsf, eg*, and *svp*, are expressed in small subsets of neurons.
and play roles in neuronal specification (eg andsvp) (57–60, 76) or adult behavior (dsf) (53). It is likely that these highly restricted patterns of expression preclude the detection of their corresponding transcripts by Northern blot analysis.

**CG16801 and DHR83** were identified by virtue of the genome sequence (2). Both of these genes are most similar to C. elegans FAX-1, which is required for neuronal pathfinding and neurotransmitter expression (3, 77). This gene is expressed in specific neurons during development, consistent with the possibility that its fly homologs may also be expressed in a highly cell type-specific manner.

### A Recurring Cascade of Orphan Nuclear Receptor Gene Expression Follows the Midembryonic, Second-Instar, and Third-Instar Ecdysone Pulses

Interactions between the DHR3 and E75B orphan nuclear receptors contribute to appropriate βFTZ-F1 regulation during the onset of metamorphosis. DHR3 is both necessary and sufficient to induce βFTZ-F1 and appears to exert this effect directly, through two response elements in the βFTZ-F1 promoter (26, 27, 78, 79). E75B can heterodimerize with DHR3 and is sufficient to block the ability of DHR3 to induce βFTZ-F1 (27). These three factors thus define a cross-regulatory network that contributes to the timing of βFTZ-F1 expression in midprepupae. βFTZ-F1, in turn, acts as a competence factor that directs the appropriate genetic and biological responses to the prepupal pulse of ecdysone (28, 80, 81). The patterns of DHR3, E75B, and βFTZ-F1 expression that we observe at the onset of metamorphosis are consistent with these regulatory interactions as well as the expression patterns reported in earlier studies (27, 29, 71, 82).

Unexpectedly, the tight linkage of DHR3, E75B, and βFTZ-F1 expression seen at the onset of metamorphosis is recapitulated at earlier stages, after each of the major ecdysone pulses examined, in midembryogenesis and second-instar larval development (Fig. 4). This observation suggests that the regulatory interac-

---

**Fig. 3.** Ecdysteroid Titer Determinations in Staged *Drosophila* Embryos and Second-Instar Larvae

A, Ecdysteroid titer measurements from staged embryos adapted from Maroy et al. (66) (●—●) and Kraminsky et al. (67) (○—○). The percent of the maximum ecdysteroid titer achieved at this stage is plotted vs. the time, in hours AEL. B, Ecdysteroid titer measurements from staged second-instar larvae adapted from Maroy et al. (88) (●—●), Kraminsky et al. (67) (○—○), and Bialecki et al. (33) (bar graph). Not all data points are shown for the curve from Kraminsky et al. The percent of the maximum ecdysteroid titer achieved at this stage is plotted vs. the time, in hours AEL.
tions between these receptors is not restricted to metamorphosis, but rather may recur in response to each ecdysone pulse during development. It is possible that this regulatory cascade contributes to cuticle deposition, which is dependent on ecdysone signaling in embryos, larvae, and prepupae (14, 15). In support of this proposal, DHR3 and \( /H9252 \) FTZ-F1 mutants exhibit defects in larval molting, suggesting that they act together to regulate this early ecdysone response.

Three other orphan nuclear receptor genes, E75A, DHR4, and DHR39, are expressed in concert with DHR3, E75B, and \( /H9252 \) FTZ-F1, after the embryonic, second-instar, and third-instar ecdysone pulses (Fig. 4). A peak of E75A expression marks the start of each genetic cascade, correlating with the rising ecdysone titer in 6- to 8-h embryos, the first half of the second instar, and in late third-instar larvae (Figs. 1, 3, and 4). This is followed by DHR3, E75B, and DHR4 expression which, in turn, is followed by a burst of \( /H9252 \) FTZ-F1 expression (Fig. 4). E78B is expressed in synchrony with DHR4 in late second and third-instar larvae (Fig. 2), but not in embryos. These patterns of expression raise the interesting possibility that E75A, DHR4, and E78B may intersect with the cross-regulatory network defined for DHR3, E75B, and \( /H9252 \) FTZ-F1. E75B and E78B are related to the Rev-erb vertebrate orphan nuclear receptor and are both missing their DNA binding domain (32, 40, 83). E75B and E78B null mutants are viable and fertile, suggesting that they exert redundant regulatory functions (33, 40). E75A mutants die during larval molting.

Fig. 4. Six Orphan Nuclear Receptor Genes Are Expressed in a Cascade that is Repeated at Different Stages in the Life Cycle

Northern blot data for E75A, DHR3, DHR4, E75B, FTZ-F1, and DHR39 were quantitated by laser scanning densitometry and converted into histograms. The plots are aligned using different gray tones for each time point, with the times listed at the bottom in hours AEL for embryonic (embryo), second-instar (L2), and third-instar (L3) stages, and hours APF for prepupal stages (prepupa).
stages, with no known direct regulatory targets (33). DHR4 mutants have not yet been described, although recent work indicates that this gene exerts essential roles in genetic and biological responses to the late larval ecdysone pulse (King-Jones, K., J.-P. Charles, and C. S. Thummel, unpublished results). Further functional studies of these nuclear receptor genes should provide insight into their possible contribution to the regulatory circuit defined by DHR3, E75B, and βFTZ-F1.

Interestingly, DHR39 displays a reproducible pattern of expression that is inversely related to that of βFTZ-F1, defining possible repressive interactions (Figs. 1, 2, and 4). DHR39 and βFTZ-F1 have a similar DNA binding domain (63% identity) and bind to identical response elements, suggesting that they may exert cross-regulatory interactions (36, 37, 84). Moreover, DHR39 can repress transcription through the same response element that is activated by βFTZ-F1 (36). It would be interesting to determine whether the reciprocal patterns of DHR39 and βFTZ-F1 expression during development is of functional significance.

Recent work has defined a cross-regulatory cascade of vertebrate nuclear receptors that regulate bile acid synthesis, indicating that these interactions are not unique to insects (85, 86). Binding of bile acids to the FXR nuclear receptor directly induces expression of an atypical nuclear receptor that lacks a DNA binding domain, SHP. This factor, in turn, heterodimerizes with an orphan nuclear receptor, LHR-1, inhibiting its transactivation function and thereby down-regulating both LHR-1 and the rate-limiting enzyme in the bile acid synthetic pathway, cholesterol 7α-hydroxylase. Regulatory cascades of nuclear receptor expression may thus provide a more general means of obtaining specificity and feedback regulation in hormone-controlled biological pathways.

Evidence for New Temporal Signals during the Drosophila Life Cycle

The transcription of BR-C, EcR, E74, and E75 has been extensively characterized during the onset of metamorphosis, due to their rapid and direct regulation by the steroid hormone ecdysone at this stage in development (24, 25, 32, 64, 68, 82). Surprisingly, however, their expression appears to be disconnected from the high-titer ecdysteroid pulses during embryonic and second-instar larval stages. As expected, EcR is induced early in embryonic development, in coincidence with the rising ecdysone titer at 4–10 h AEL, with EcR-B transcripts appearing first followed by EcR-A (Figs. 1 and 3A). BR-C mRNA, however, is not seen until 10–12 h AEL and E74B mRNA is induced even later, at 14–16 h AEL, when the ecdysoid titer has returned to a basal level (Fig. 3A). Both EcR-B and E74B are repressed from 16–20 h AEL as E74A and E75A are induced, a switch that has been linked to the high-titer ecdysone pulse in late third-instar larvae (24, 25); however, this response occurs during late embryogenesis when the ecdysteroid titer is low (Figs. 1 and 3A). A similar observation has been made for E75A expression in the Manduca dorsal abdominal epidermis, where a brief burst of E75A mRNA is detected immediately before pupal ec dysis, after the ecdysteroid titer has returned to basal levels (87).

Ecdysteroid titer measurements for the second larval instar in Drosophila (Fig. 3B) are in more dispute than those determined by Maroy et al. (66) and Kraminsky et al. (67) for embryonic development (Fig. 3A). Both Kraminsky et al. and Bialecki et al. (33) report a peak of ecdysteroids between 4 and 12 h after the first-to-second instar larval molt (Fig. 3B). The data from Maroy et al. (88) show a much later ecdysteroid peak, at the end of the instar (Fig. 3B). This conclusion is based, however, on only five data points through the second instar. Moreover, Kraminsky et al. and Maroy et al. (88) synchronized their collections at hatching, rather than the molts, and did not report on the synchrony of their animals as they progressed through the instars. In contrast, Bialecki et al. (33) staged their animals at the first-to-second instar larval molt and followed their progression into the third instar. It thus seems likely that the second instar ec dysone pulse occurs during the first half of the instar. This profile is consistent with the early induction of E75A (Fig. 2). Ecr-B and E74A, however, are not induced until the second half of the second instar, with a peak at the end of the instar. BR-C mRNA levels remain steady throughout the second instar. Finally, as reported earlier, EcR-B, E74B, and BR-C are induced in early to mid-third-instar larvae, a time when one or more low-titer ec dysone pulses may occur (64, 82, 89). It is curious that E74B is poorly expressed relative to E74A during embryonic and second-instar larval stages, disconnecting its expression from that of EcR. This pattern is not seen in studies that focused on the onset of metamorphosis (25, 82, 89). Taken together, the temporal profiles of early gene expression (EcR, BR-C, E74, E75A) during late embryonic and late second-instar larval stages appear to be unlinked to the known ecdysteroid pulses at these stages. This could indicate that these promoters are activated in a hormone-independent manner at these stages in the life cycle. Alternatively, these ec dysone primary-response genes may be induced by a novel temporal signal that remains to be identified.

Several lines of evidence indicate that 20-hydroxyecdysone is not the only temporal signal in Drosophila. A major metabolite of this hormone, 3-dehydro-20-hydroxyecdysone, was shown to be as effective as 20-hydroxyecdysone in inducing target gene transcription in the hornworm, Manduca sexta (90). Similarly, 3-dehydro-20-hydroxyecdysone is more efficacious than 20-hydroxyecdysone in inducing Fbp-1 transcription in the Drosophila larval fat body (91). Champlin and Truman (92) have shown that a high-titer pulse of α-ecdysone, the precursor to 20-hydroxyecdysone, can drive the extensive proliferation of neuroblasts during early pupal development in
**MATERIALS AND METHODS**

**Developmental Staging**

Canton S wild-type flies were maintained at 25 C. Embryos were collected on molasses/agar plates supplemented with yeast paste. After 2 d of conditioning and discarding an initial egg lay, embryos were collected at 2-h intervals and harvested either immediately or after aging for 2–22 h at 25 C. To harvest, embryos were washed with water over a nylon mesh, dechorionated in 50% bleach, rinsed, and frozen at −80 C until 12 h time points were collected. Larvae were staged by placing either first or second instar larvae in petri dishes on moist black filter paper supplemented with yeast paste and maintaining them at 25 C. The plates were examined 4 h later for larvae that had molted. Newly molted larvae were collected immediately or aged in 50-mL glass beakers with fresh yeast paste in a humidified chamber. They were subsequently harvested at 4-h intervals and frozen at −80 C. Pupa were staged relative to puparium formation and aged at 2-h intervals for up to 16 h APF, and then frozen at −80 C. Embryos and third-instar larvae advanced to the next developmental stage at the appropriate time, indicating that the staging was accurate.

**RNA Isolation**

Each collection of animals (~100 μL vol of embryos, 30–40 second-instar larvae, eight to 10 third-instar larvae or prepupa) was homogenized in 400 μL RNA extraction buffer (0.1 M Tris, 0.1 M NaCl, 20 mM EDTA, 1% Sarkosyl). The sample was then extracted with phenol/chloroform-isooamyl alcohol (25: 24:1) four times, with a final extraction in chloroform. RNA was ethanol precipitated and resuspended in 20–30 μL diethylpyrocarbonate-treated water. RNA yield varied from 2–10 μg/μL.

**Northern Blot Hybridizations**

Total RNA (10 or 15 μg) was fractionated on formaldehyde 1% agarose gels for embryonic or larval/prepupal samples, respectively. RNA was transferred to nylon membranes and cross-linked by UV irradiation as described (24). Blot hybridization, washes, and stripping were performed as described (89), except blots were stripped by boiling for 5 min instead of 30 min. Many hybridizations were confirmed by probing an independent blot, including all nuclear receptor genes that gave no detectable signal. The expression patterns of some nuclear receptor genes (see Fig. 4) were quantitated by laser scanning densitometry using ImageQuant software (Amer sham Pharmacia Biotech, Arlington Heights, IL). Values were converted to percentages of the highest value for each autoradiograph and plotted into histograms. Thus, although each histogram represents the relative levels of expression for one transcript at one stage, the relative levels of expression between different histograms are not meaningful.

**Probe Preparation**

DNA templates used to prepare labeled probes are listed in Table 1. Most probes were prepared by gel purifying a restriction fragment and labeling it with random oligonucleotide primers (Prime-It, Stratagene, La Jolla, CA). dERR, CG16801, DHR4, dsf, eg, kni, and knrl probe templates were prepared by PCR amplification from genomic DNA isolated from adult flies, except dERR, which was amplified from a CDNA clone. DHR4 and DHR83 probes were a gift from K. King-Jones. The DHR38 probe was a gift from T. Kozlova. The PCR primers used to generate a probe for dERR were ATAGATCTGCG-CACCTAACGC and TCAGCCTGAGGGCTAGGATCT (a gift from T. Kozlova). The primers used for CG16801 were GATT-GTAAACCAAGGAACAGGAGGAGGCG and AGAGCACCTT-TCCATGGGGCTTCCCG. The primers used for dsf were CCGTCTCGCTGTGACACTAAATTGTG and CGCAAATCAT- CACCACTACCCACAGGG. The primers used for kni were TACCCGACGAGGATGTCAACGACCGCC and CGA- ATATCCTCCCTCATGGCAGCTAACC. The primers used for knrl were TGACGCCGCGACGCGCCGACAGAGGCG and TTCAGTGTGTCTTCTGTCGTCG.

**Acknowledgments**

We thank M. Bialecki for his help and advice on staging larvae and for providing the E75 probes; L. Cherbas for the GBD-EcR-A cDNA; K. King-Jones for providing the DHR4 and DHR83 probes; T. Kozlova for the dERR probe; R. Beckstead for helpful discussions; and R. Beckstead, K. King-Jones, and T. Kozlova for critical comments on the manuscript.

C.S.T. is an investigator with the Howard Hughes Medical Institute.

Received December 18, 2002. Accepted July 15, 2003.

Address all correspondence and requests for reprints to: Carl S. Thummel, Howard Hughes Medical Institute, Department of Human Genetics, University of Utah School of Medicine, 15 North 2030 E, Room 5100, University of Utah, Salt Lake City, Utah 84112-5331.

A.A.S. was supported by an NIH Training Program in Genetics (5T32-GM07464).
REFERENCES

1. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
2. Adams MD, Celinker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SM, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champine M, Pfeiffer BD, et al. 2000 The genome sequence of Drosophila melanogaster. Science 287:2185–2192
3. King-Jones K, Thummel CS 2003 Drosophila nuclear receptors. In: Bradshaw R, Dennis E, eds. Handbook of cell signaling. New York: Academic Press; 69–73
4. Maglich JM, Sluder A, Guan X, Shi Y, McKee DD, Carrick N, Kamdar K, Willson TM, Moore JT 2001 Comparison of complete nuclear receptor sets from the human, Caenorhabditis elegans and Drosophila genomes. Genome Biol 2:research0029.1–0029.7
5. Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, Hogness DS 1991 The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67:59–77
6. Yao T, Segraves WA, Oro AE, McKeown M, Evans RM 1992 Drosophila ultraspirel modulates ecdysone receptor function via heterodimer formation. Cell 71:63–72
7. Yao T, Forman BM, Jiang Z, Cherbas L, Chen JD, McKeown M, Cherbas P, Evans RM 1993 Functional ecdysone receptor is the product of EcR and Ultraspirelle genes. Nature 366:476–479
8. Thomas HE, Stunnenberg HG, Stewart AF 1993 Heterodimerization of the Drosophila ecdysone receptor with retinoic X receptor and ultraspirelle. Nature 362:471–475
9. Riddiford LM, Cherbas P, Truman JW 2000 Ecdysone receptors and their biological actions. Vitam Horm 60:1–73
10. Sutherland JD, Kozlova T, Tzertzinis G, Kafatos FC 1995 Drosophila hormone receptor 38: a second partner for Drosophila USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. Proc Natl Acad Sci USA 82:7966–7970
11. Bialecki M, Shilton A, Fichtenberg C, Segraves WA, Burtis KC, Thummel CS, Jones CW, Karim FD, Hogness DS 1991 The Drosophila hormone receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol Cell 3:143–149
12. Lavorgna G, Ueda H, Clos J, Wu C 1991 FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu. Science 252:848–851
13. Segraves WA, Hogness DS 1992 DHR3: a Drosophila steroid receptor homolog. Proc Natl Acad Sci USA 89:6167–6171
14. Kozlova T, Pokholkova GV, Tzertzinis G, Sutherland JD, Zhimulev IF, Kafatos FC 1998 Drosophila hormone receptor 38 functions in metamorphosis: a role in adult cuticle formation. Genetics 139:1465–1475
15. Segraves WA, Hogness DS 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
16. Ayer S, Walker N, Mosammaparast M, Nelson JP, Shilo B, 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. Nucleic Acids Res 21:1619–1627
17. Thummel CS 1996 Flies on steroids—Drosophila metamorphosis and the mechanisms of steroid hormone action. Trends Genet 12:306–310
18. Mouillet JF, Henrich VC, Lezzi M, Vogtli M 2001 Differential control of gene activity by isoforms A, B1 and B2 of the Drosophila ecdysone receptor. Eur J Biochem 268:1811–1819
19. Hu X, Cherbas L, Cherbas P 2003 Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. Mol Endocrinol 17:716–731
20. DiBello PR, Withers DA, Bayer CA, Fristrom JW, Guild GM 1991 The Drosophila Broad-Complex encodes a family of related proteins containing zinc fingers. Genetics 129:385–397
21. Burgt KC, Thummel CS, Jones CW, Karim FD, Hogness DS 1990 The Drosophila 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two eft-related proteins. Cell 61:85–99
22. Karim FD, Thummel CS 1991 Ecdysone coordinates the timing and amounts of E74A and E74B transcription in Drosophila. Genes Dev 5:1067–1079
23. Karim FD, Thummel CS 1992 Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. EMBO J 11:4083–4093
24. Lam G, Hall BL, Bender M, Thummel CS 1999 DHR3 is required for the prepupal-pupal transition and differentiation of adult structures during Drosophila metamorphosis. Dev Biol 212:204–211
25. White KP, Hurban P, Watanabe T, Hogness DS 1997 Coordination of Drosophila metamorphosis by two ecdysone-induced nuclear receptors. Science 276:114–117
26. Broadus J, McCabe JR, Endrizzi B, Thummel CS, Woodard CT 1999 The Drosophila pFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol Cell 3:143–149
27. Vassalli JS, LaFon GM 1991 The Drosophila Broad-Complex encodes two related proteins of the steroid receptor superfamily containing zinc fingers. Trends Genet 12:306–308
37. Ohno CK, Petkovich M 1992 FTZ-F1β, a novel member of the Drosophila nuclear receptor family. Mech Dev 40: 13–24
38. Horner M, Chen T, Thummel CS 1995 Ecdysone regulation and DNA binding properties of Drosophila nuclear hormone receptor superfamily members. Dev Biol 168: 490–502
39. Horner MA, Thummel CS 1997 Mutations in the DHR39 orphan receptor gene have no effect on viability. DIS 80:35–37
40. Russell SR, Heimbeck G, Goddard CM, Carpenter AT, Ashburner M 1996 The Drosophila Eip78C gene is not vital but has a role in regulating chromosome puffs. Genetics 144:159–170
41. Steingrimsson E, Pignoni F, Liaw G, Lengyel JA 1991 Dual role of the Drosophila pattern gene tailless in embryonic termini. Science 254:418–421
42. Rothe M, Nauber U, Tauber M 1998 Three hormone receptor-like DHR39 genes encode an identical DNA-binding finger. EMBO J 8:3087–3094
43. Pignoni F, Baldarelli RM, Feinberg A, Segre MS, Lengyel JA 1990 The Drosophila gene tailless is expressed at the embryonic termini and is a member of the steroid receptor superfamily. Cell 62: 151–163
44. Nauber U, Pankratz MJ, Kienlin A, Seifert E, Klemm U, Lunde K, Biehs B, Nauber U, Bier E 1998 The knirs and knirs-related genes organize development of the second wing vein in Drosophila. Development 125: 4145–4154
45. Rothe M, Wimmer EA, Pankratz MJ, Gonzalez-Gaitan M, Jackle H 1994 Identical transacting factor requirement for knirs and knirs-related gene expression in the anterior but not in the posterior region of the Drosophila embryo. Mech Dev 46:169–181
46. Oro AE, Ong ES, Margolis JS, Posakony JW, McKeown M, Evans RM 1988 The Drosophila gene knirs-related is a member of the steroid-receptor gene superfamily. Nature 336:493–496
47. Gonzalez-Gaitan M, Rothe M, Wimmer EA, Taubert H, Jackle H 1994 Redundant functions of the genes knirs and knirs-related for the establishment of anterior Drosophila head structures. Proc Natl Acad Sci USA 91: 8567–8571
48. Yu Y, Li W, Su K, et al 1997 The nuclear hormone receptor Ftz-F1 is a cofactor for the Drosophila homeodomain protein Ftz. Nature 385:552–555
49. Schwartz CJ, Sampson HM, Hioue D, Percival-Smith A, Copeland JW, Simmonds AJ, Krause HM 2001 FTZ-Factor1 and Fushi tarazu interact via conserved nuclear receptor motifs. EMBO J 20:310–319
50. Guichet A, Copeland JW, Erdelyi M, Hioue D, Zavorsky P, Ho J, Brown S, Percival-Smith A, Krause HM, Ephrussi A 1997 The nuclear hormone homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. Nature 385:548–552
51. Carney GE, Wade AA, Sapra R, Goldstein ES, Bender M 1997 DHR3, an ecdysone-inducible early-late gene encoding a Drosophila nuclear receptor, is required for embryogenesis. Proc Natl Acad Sci USA 94: 12024–12029
52. Finley KD, Edmonson PT, Foss M, Gross E, Ghbeish N, Palmer RH, Taylor BJ, McKeown M 1998 Dissatisfaction encodes a tailless-like nuclear receptor expressed in a subset of CNS neurons controlling Drosophila sexual behavior. Neuron 21:1363–1374
53. Begemann G, Michon AM, vd Voorn L, Wepf R, Midzick M 1995 The Drosophila orphan nuclear receptor seven-up requires the Ras pathway for its function in the photoreceptor determination. Development 121:225–235
54. Kramer S, West SR, Hiromi Y 1995 Cell fate control in the Drosophila retina by the orphan receptor seven-up: its role in the decisions mediated by the ras signaling pathway. Development 121:1361–1372
55. Pereira FA, Tsiu MJ, Tsi SY 2000 COUP-TF orphan nuclear receptors in development and differentiation. Cell Mol Life Sci 57:1388–1398
56. Hiromi Y, Midzick M, West SR, Rubin GM, Goodman CS 1993 Ectopic expression of seven-up causes cell fate changes during ommatidial assembly. Development 118: 123–135
57. Dittrich R, Bossing T, Gould AP, Technau GM, Urban J 1997 The differentiation of the serotonergic neurons in the Drosophila ventral nerve cord depends on the combined function of the zinc finger proteins Eagle and Huckebein. Development 124:2515–2525
58. Higashijima S, Shishido E, Matsuizaki M, Saigo K 1996 Eagle, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the Drosophila CNS. Development 122:527–536
59. Lundell MJ, Hirsh J 1998 Eagle is required for the specification of serotonin neurons and other neuroblast 7–3 progeny in the Drosophila CNS. Development 125: 463–472
60. Carney GE, Bender M 2000 The Drosophila ecdysone receptor (EcR) gene is required maternally for normal oogenesis. Genetics 154:1203–1211
61. Buszczak M, Freeman MR, Carlson JR, Bender M, Cooley L, Segrevas WA 1999 Ecdysone response genes govern egg chamber development during mid-oogenesis in Drosophila. Development 126:4581–4589
62. Perrimon N, Engstrom L, Mahowald AP 1985 Developmental genetics of the 2C-D region of the Drosophila X chromosome. Genetics 111:23–41
63. Talbot WS, Swyryd EA, Hogness DS 1993 Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73:1323–1337
64. Zhang W, Sladek FM, Darnell Jr JE 1993 The expression pattern of a Drosophila homolog to the mouse transcription factor HNF-4 suggests a determinative role in gut formation. EMBO J 12:537–544
65. Maroy P, Kaufmann G, Dübendorfer A 1988 Embryonic ecdysteroids of Drosophila melanogaster. J Insect Physiol 34:633–637
66. Kraminsky GP, Clark WC, Estelle MA, Gietz RD, Sage BA, O’Connor JD, Hodggets RB 1980 Induction of translatable mRNA for dopa decarboxylase in Drosophila: an early response to ecdysone. Proc Natl Acad Sci USA 77:4175–4179
67. Bayer CA, Holley B, Fristrom JW 1996 A switch in broad-complex zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of Drosophila imaginal discs. Dev Biol 177:1–14
68. Zhou B 2000 E75 and Broad Complex: two JH-regulated genes in the ecdysone signaling pathway, PhD thesis. University of Washington, Seattle, WA
69. Richards G 1981 The radioimmune assay of ecdysteroid titres in Drosophila melanogaster. Mol Cell Endocrinol 21:181–197
70. White KP, Rifkin SA, Hurban P, Hogness DS 1999 Microarray analysis of Drosophila development during metamorphosis. Science 286:2179–2184
71. Robinson-Rechavi M, Carpentier A, Duffraisse M, Laudet V 2001 How many nuclear hormone receptors are there in the human genome? Trends Genet 17:554–556
72. Sluder AE, Maina CV 2001 Nuclear receptors in nematodes: themes and variations. Trends Genet 17: 206–213
73. Henrich VC, Szekely AA, Kim SJ, Brown NE, Antoniewski C, Hayden MA, Lepesant JA, Gilbert LI 1994 Expression
and function of the *ultraspiracle* (usp) gene during development of *Drosophila melanogaster*. Dev Biol 165:38–52

75. Fisk GJ, Thummel CS 1995 Isolation, regulation, and DNA-binding properties of three *Drosophila* nuclear hormone receptor superfamily members. Proc Natl Acad Sci USA 92:10604–10608

76. Mlodzik M, Hiromi Y, Weber U, Goodman CS, Rubin GM 1995 Isolation, regulation, and cell fates. Cell 60:211–224

77. Much JW, Slade DJ, Klampert K, Garriga G, Wightman B 2000 The fax-1 nuclear hormone receptor regulates axon pathfinding and neurotrophin expression. Development 127:703–712

78. Kageyama Y, Masuda S, Hirose S, Ueda H 1997 Temporal regulation of the mid-prepupal gene FTZ-F1: DHR3 early late gene product is one of the plural positive regulators. Genes Cells 2:559–569

79. Lam GT, Jiang C, Thummel CS 1997 Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. Development 124:1757–1769

80. Lee CY, Simon CR, Woodard CT, Baehrecke EH 2002 Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. Dev Biol 252:138–148

81. Woodard CT, Baehrecke EH, Thummel CS 1994 A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. Cell 79:607–615

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

83. Stone BL, Thummel CS 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

84. Ohno CK, Ueda H, Petkovich M 1994 The *Drosophila* nuclear receptors FTZ-F1β and FTZ-F1α compete as monomers for binding to a site in the *fushi tarazu* gene. Mol Cell Biol 14:3166–3175

85. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Klewer SA 2000 A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell 6:517–526

86. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwex J, Mangelsdorf DJ 2000 Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 6:507–515

87. Zhou B, Hirma K, Jindra M, Shinoda T, Segraves WA, Malone F, Riddiford LM 1998 Regulation of the transcription factor E75 by 20-hydroxyecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*, during larval molting and metamorphosis. Dev Biol 127–138

88. Maróthy P, Koczka K, Fekete É, Vargha J 1980 Molting hormone titer of *D. melanogaster* larvae. Dros Info Serv 55:98–99

89. Andres AJ, Fletcher JC, Karim FD, Thummel CS 1993 Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. Dev Biol 160:388–404

90. Hiruma K, Bocking D, Lafont R, Riddiford LM 1997 Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta*. Gen Comp Endocrinol 107:84–97

91. Somme-Martín G, Colardeau J, Beydon P, Blais C, Lepeant JA, Lafont R 1990 P1 gene expression in *Drosophila* larval fat body: induction by various ecdysteroids. Arch Insect Biochem Physiol 15:43–56

92. Champlin DT, Truman JW 1998 Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. Development 125:269–277

93. Baker KD, Warren JT, Thummel CS, Gilbert LI, Mangelsdorf DJ 2000 Transcriptional activation of the *Drosophila* ecdysone receptor by insect and plant ecdysteroids. Insect Biochem Mol Biol 30:1037–1043

94. Andres AJ, Cherbas P 1992 Tissue-specific ecdysone responses: regulation of the *Drosophila* genes *Eip28/29* and *Eip40* during larval development. Development 116:865–876

95. Bashirullah A, Pasquinelli AE, Kiger AA, Perrimon N, Ruvkun G, Thummel CS 2003 Coordinate regulation of small temporal RNAs at the onset of *Drosophila* metamorphosis. Dev Biol 259:1–8

96. O’Connell PO, Rosbash M 1984 Sequence, structure, and codon preference of the *Drosophila ribosomal protein 49* gene. Nucleic Acids Res 12:5495–5513

97. Oro AE, McKewon M, Evans RM 1990 Relationship between the product of the *Drosophila ultraspiracle* locus and vertebrate retinoid X receptor. Nature 347:298–301