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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*, *E78B*, 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)

SMALL LIPOPHILIC HORMONES, including retinoic acid, thyroid hormone, and steroids, function as key regulators of development and adult physiology in higher organisms. These signals are transduced by ligand-dependent transcription factors that comprise the nuclear receptor superfamily (1). Nuclear receptors are defined by a highly conserved DNA-binding domain that consists of two zinc fingers, as well as a C-terminal ligand-binding domain that serves multiple functions, including hormone binding, dimerization, and ligand-dependent transactivation. The *Drosophila* genome encodes 21 members of the nuclear receptor superfamily, many of which have close homologs in mammals and *Caenorhabditis elegans* (2–4). The ecdysone receptor (EcR) binds the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) and transduces this signal as a heterodimer with the *Drosophila* retinoid X receptor ortholog, Ultraspiracle (USP) (5–9). DHR38, which encodes the ortholog of vertebrate NGFI-B/Nurr family members, can also heterodimerize with USP and act as an ecdysteroid receptor, although by a novel mechanism that does not involve direct hormone binding (10, 11). USP has been proposed to act as a receptor for the sesquiterpenoid hormone, juvenile hormone (12). The remaining *Drosophila* nuclear receptors are classified as orphan receptors having, as yet, no known ligands (13).

Abbreviations: AEL, After egg laying; APF, after puparium formation; CNS, central nervous system; EcR, ecdysone receptor; βFTZ-F1, β-fushi tarazu factor 1; USP, Ultraspiracle.

Ecdysone functions as a critical temporal signal in *Drosophila*, triggering the major developmental transitions in the life cycle (14). In embryos, ecdysone is required for morphogenetic events as well as cuticle deposition (15). Pulses of ecdysone during the first and second larval instars define the duration of these developmental stages, triggering molting of the cuticle (14). A high titer pulse of ecdysone at the end of the third larval instar triggers puparium formation, signaling metamorphosis, and the onset of prepupal development. This stage is terminated by another ecdysone pulse, approximately 10 h after puparium formation, that triggers eversion of the adult head, marking the prepupal-to-pupal transition. During metamorphosis, ecdysone coordinates the destruction of obsolete larval tissues by programmed cell death and their replacement by adult tissues, directing the formation of the adult fly (16).

Ecdysone exerts its effects on development by triggering cascades of gene expression through the EcR/USP receptor heterodimer (9, 17, 18). *EcR* encodes two protein isoforms, with EcR-B, but not EcR-A, acting as a potent hormone-dependent transcriptional activator in tissue culture assays (19, 20). The hormone-receptor complex directly induces a set of primary-response genes, including the *Broad-Complex* (*BR-C*) and *E74* early puff genes (21, 22) as well as a subset of orphan nuclear receptors (23). These genes encode transcription factors that transduce and amplify the ecdysone signal, regulating the expression of large batteries of downstream secondary-response

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* and *E74B* and inducing *E74A*.

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* (*knrl*) 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

Table 1. Probes for Northern Blot Hybridization

Transcript	Plasmid/DNA	Probe Template	mRNA Size(s) (kb)	Ref.
BR-C	paaDm527	*Stu*I/*Pvu*II, 0.5	10, 8.8, 6.8, 4.4	25
CG16801	Genomic DNA	PCR, 0.9		See M&M[a]
dERR	GM14739	PCR, 0.6	2.0, 1.6	See M&M
dHNF-4	pBS/HNF-4	*Bpm*I, 1.1	4.6, 3.3	65
DHR3	pBS/DHR3	*Hin*dIII, 0.9	6.3, 5.5, 4.9	79
DHR4	Genomic DNA	PCR, 0.7	10	See M&M
DHR38	pCaSpeR/hsGAL4-DHR38	*Xba*I/RI, 1.0	6.2, 4.0, 3.6, 1.9	See M&M
DHR39	pBS/DHR39	*Eag*I/*Dra*III, 1.1	5.0	36
DHR78	pLF5	*Sal*I, 0.5	2.3	75
DHR83	pTOPO/DHR83	*Eco*RI, 0.9		See M&M
DHR96	pLF20	*Eco*RV, 1.8	2.8	75
dsf	Genomic DNA	PCR, 0.7	3.7	See M&M
E74	pBS/E74	*Bgl*II/*Sal*I, 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	*Xho*I/RI, 2.3	3.5, 2.6	83
eg	Genomic DNA	PCR, 0.8	2.2, 1.9	See M&M
EcR-A	GBD-EcR-A	*Msc*I/*Not*I, 0.7	5	20
EcR-B	pMK1	RI, 1.7	6	25
FTZ-F1	pBS/βFTZ-F1	RI/*Xho*I, 1.6	α = 5.2; β = 5.6, 4.8	29
kni	Genomic DNA	PCR, 0.6	2.5, 2.2	See M&M
knrl	Genomic DNA	PCR, 1.0	3.8	See M&M
rp49	pBS/rp49	RI/*Hin*dIII, 0.4	0.6	96
svp	pC162-2B	*Pst*I/*Bsp*EI, 0.7	2.3, 1.7	76
tll	N4	*Pst*I/*Bgl*II, 1.25	2.0	43
usp	pZ7-1	*Pst*I, 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*.
[a] M&M, *Materials and Methods*.

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 β*FTZ-F1* expression at 12–18 h AEL. *DHR39* appears to exhibit an expression pattern reciprocal to that of β*FTZ-F1*, with lowest levels of mRNA at 14–16 h AEL and reinduction at 16–18 h as β*FTZ-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).

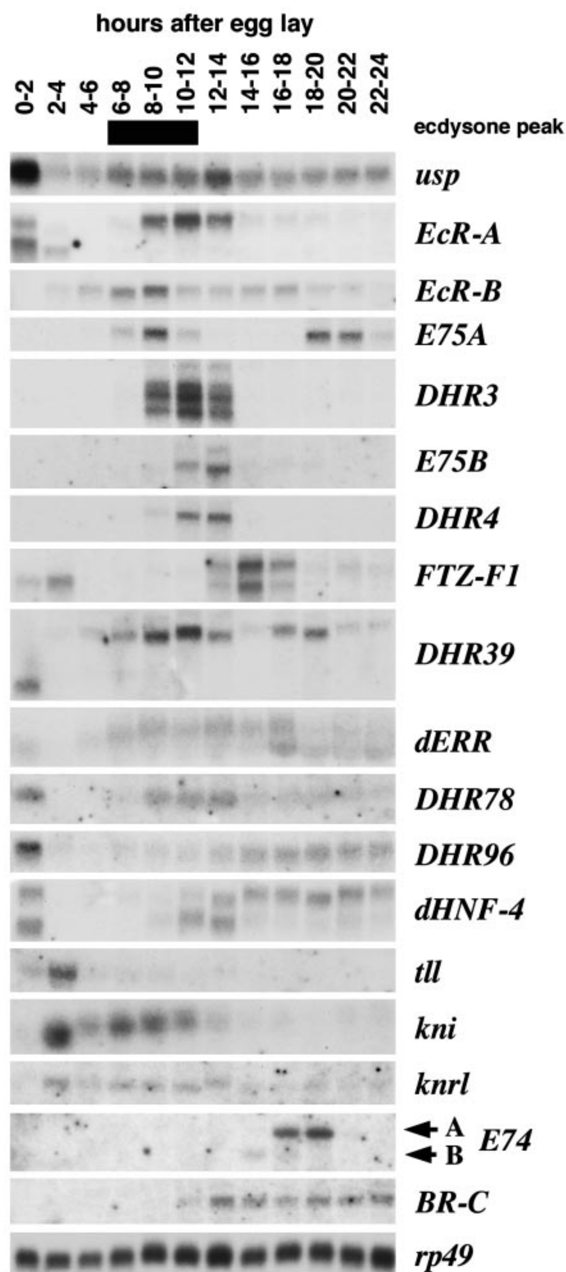

Fig. 1. Temporal Profiles of Nuclear Receptor Gene Expression During Embryogenesis

Equal amounts of total RNA isolated from embryos staged at 2-h intervals were analyzed by Northern blot hybridization to determine the temporal patterns of nuclear receptor gene expression. Time points in hours AEL are shown at the *top* with a *black box* representing the approximate timing of the ecdysone titer peak (see Fig. 3). *Arrows* designate *E74A* and *E74B* mRNAs. Detection of *rp49* mRNA was used as a control for loading and transfer.

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 *βFTZ-F1* at the end of the instar. *DHR39* again shows a pattern that is approximately reciprocal with *βFTZ-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. Prepupae 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.

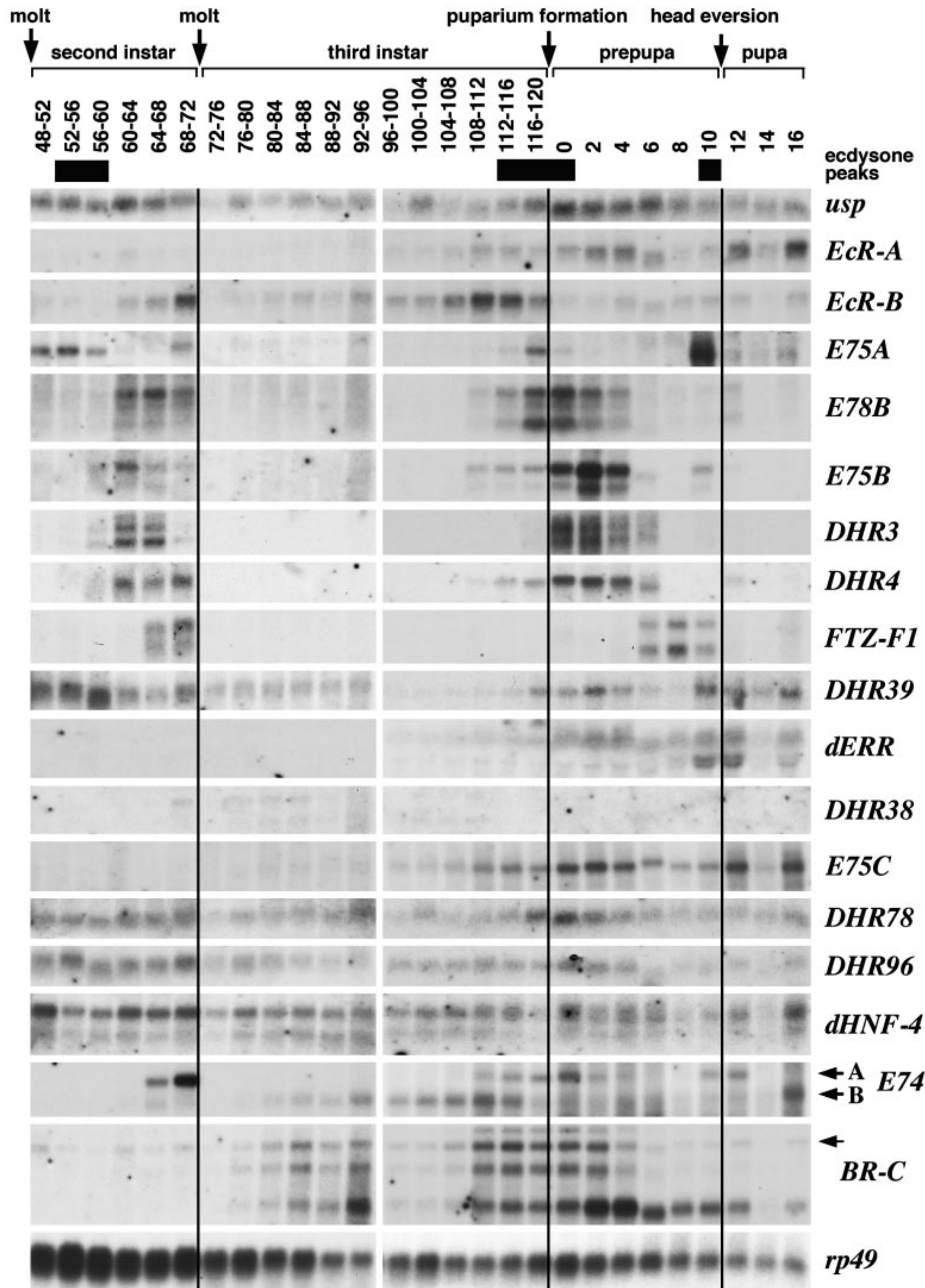

Fig. 2. Temporal Profiles of Nuclear Receptor Gene Expression during Larval and Prepupal Development

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. β*FTZ-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 eversion. 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 α*FTZ-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

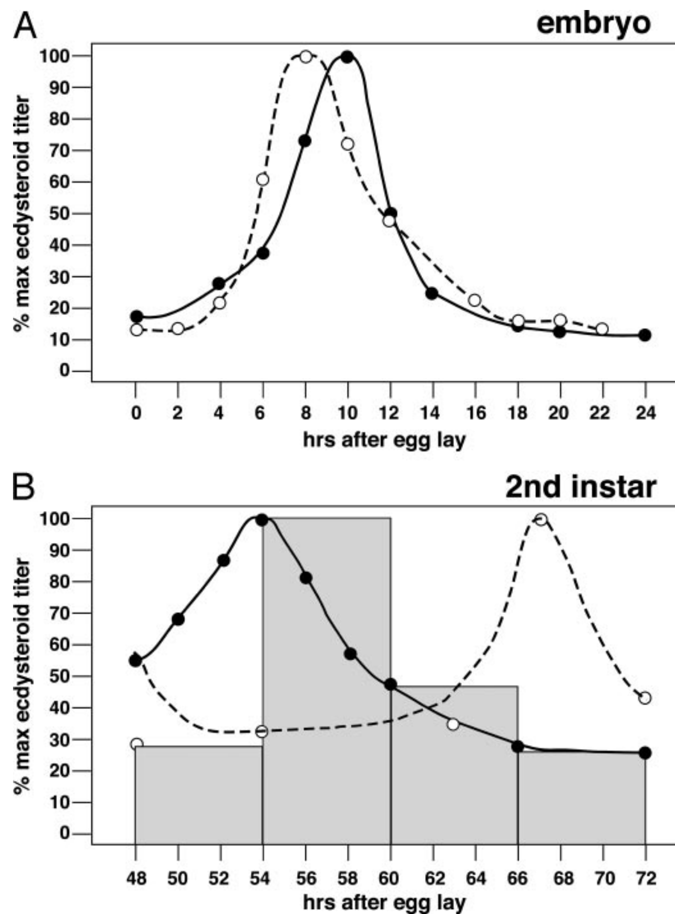

Fig. 3. Ecdysteroid Titer Determinations in Staged *Drosophila* Embryos and Second-Instar Larvae

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.

and play roles in neuronal specification (*eg* and *svp*) (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-

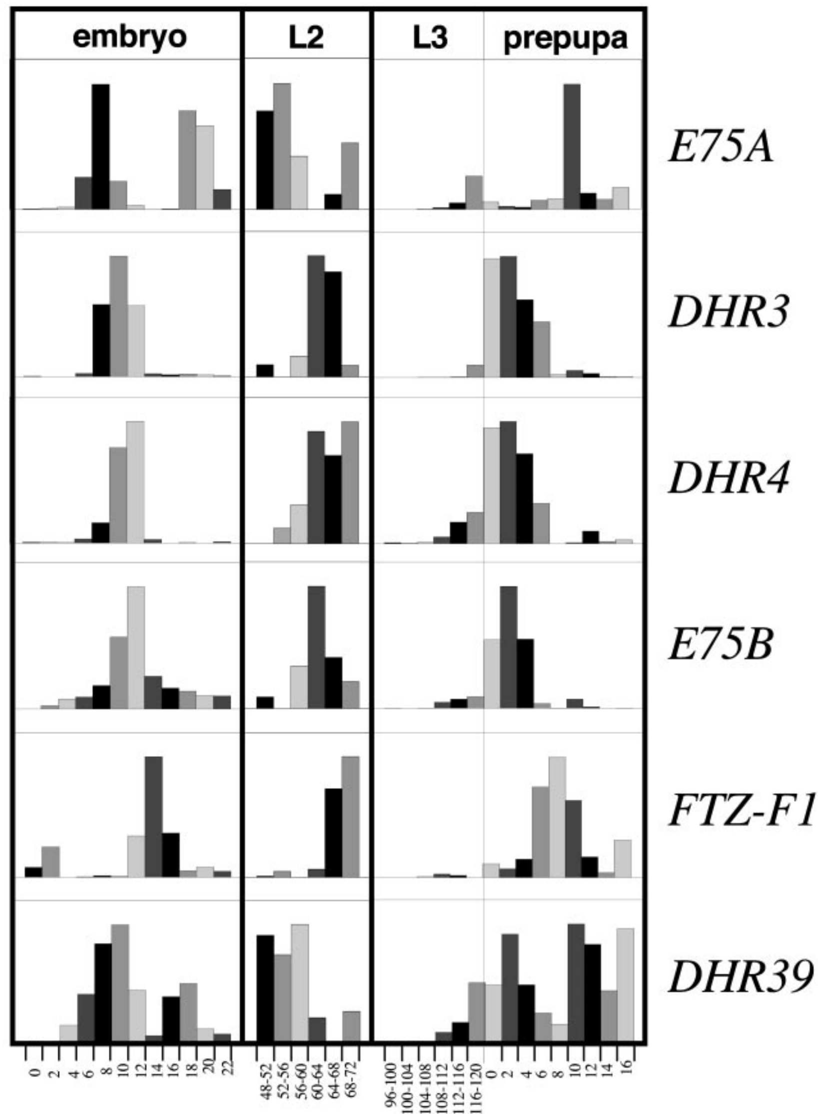

Fig. 4. Six Orphan Nuclear Receptor Genes Are Expressed in a Cascade that is Repeated at Different Stages in the Life Cycle

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).

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 *βFTZ-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 *βFTZ-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 *βFTZ-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 *βFTZ-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

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 ecdysteroid 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 ecdysis, 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 ecdysone 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 ecdysone 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 ecdysone 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

Manduca. This is the first evidence that α-ecdysone is responsible for a specific response in insects. It is unlikely, however, that this signal is transduced through the EcR/USP heterodimer, which shows only very low transcriptional activity in response to this ligand (93). Rather, recent evidence indicates that α-ecdysone may activate DHR38 through a novel mechanism that does not involve direct hormone binding (11).

Studies of ecdysteroid-regulated gene expression in *Drosophila* have also provided evidence for hormone signaling pathways that may act independently of 20-hydroxyecdysone. Several studies have identified a large-scale switch in gene expression midway through the third larval instar, an event that has been referred to as the mid-third-instar transition (94). It is not clear whether this response is triggered by a low-titer ecdysteroid pulse, another hormonal signal, or in a hormone-independent manner (89). Similarly, the *let-7* and *miR-125* micro-RNAs are induced at the onset of metamorphosis in *Drosophila* in tight temporal correlation with the *E74A* early mRNA, but not in apparent response to 20-hydroxyecdysone (95). These studies, taken together with the data presented in this paper, indicate that 20-hydroxyecdysone cannot act as the sole temporal regulator during the *Drosophila* life cycle.

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 all 12 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. Prepupae 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 prepupae) 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-isoamyl alcohol (25:24:1) four times, with a final extraction in chloroform. RNA was ethanol precipitated and resuspended in 16–30 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 (Amersham 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 ATAGATCTGCCACTTAACGAC and TCAGCTGGAGCGTCAGGATCT (a gift from T. Kozlova). The primers used for *CG16801* were GATGTAACCAACGACAACGAGGAGCCGC and AGAGCACCTTTTCCATGGGCGTGTTCCC. The primers used for *dsf* were CCGTCTCGCTGGTGACCAATGTCTCGG and CGCAAATCATGAGAGAGGCGTGGCTGGG. The primers used for *eg* were AGTCTGGAGCAGCTGGCGCGATTACAGC and CCTGTCAACGTTTGTGACCTCACCAGGG. The primers used for *kni* were TACCGGCAGGAGATGTACAAGCACCGCC and CGAATATTCCCCTCATGGCACTAGCCGC. The primers used for *knrl* were TGACGCCCGGCAGACCGCCACAGATGCG and TTCAATGCTGGTCTTTGCTGTCTCTGTGG.

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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.

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