Ecdysone coordinates the timing and amounts of *E74A* and *E74B* transcription in *Drosophila*

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Pulses of the steroid hormone ecdysone function as temporal signals to coordinate the development of both larval and adult tissues in *Drosophila*. Ecdysone acts by triggering a genetic regulatory hierarchy that can be visualized as puffs in the larval polytene chromosomes. In an effort to understand how the ecdysone signal is transduced to result in sequential gene activation, we are studying the transcriptional control of *E74*, an early gene that appears to play a regulatory role in the hierarchy. Northern blot analysis of RNA isolated from staged animals or cultured organs was used to characterize the effects of ecdysone on *E74* transcription. Ecdysone directly activates both *E74A* and *E74B* promoters. *E74B* mRNA precedes that of *E74A*, each mRNA appearing with delay times that agree with their primary transcript lengths and our previous transcription elongation rate measurement of ~ 1.1 kb/min. The earlier appearance of *E74B* transcripts is enhanced by its activation at an ~ 25 -fold lower ecdysone concentration than *E74A*. *E74B* is further distinguished from *E74A* by its repression at a significantly higher ecdysone concentration than that required for its induction, close to the concentration required for *E74A* activation. These regulatory properties lead to an ecdysone-induced switch in *E74* expression, with an initial burst of *E74B* transcription followed by a burst of *E74A* transcription. We also show that the patterns of ecdysone-induced *E74A* and *E74B* transcription vary in four ecdysone target tissues. These studies provide a means to translate the profile of a hormone pulse into different amounts and times of regulatory gene expression that, in turn, could direct different developmental responses in a temporally and spatially regulated manner.

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Steroid hormones provide higher organisms with a systemic signaling system that coordinates the growth and development of different tissues. In the fruit fly, *Drosophila melanogaster*, this function is provided by the steroid hormone 20-hydroxyecdysone (henceforth referred to as ecdysone), which acts throughout the life cycle to synchronize tissue-specific developmental changes (Richards 1981a). Pulses of ecdysone are released into the hemolymph during each of the six developmental stages of *Drosophila*: (1) in the middle of embryogenesis, (2,3) preceding the first and second-instar larval molts, (4) at the beginning of metamorphosis in late third-instar larvae, (5) preceding head eversion in prepupae, and (6) during pupal development (Richards 1981b). The most extensively studied pulse of ecdysone, at the onset of metamorphosis, serves as a temporal cue to synchronize a complex pattern of behavioral, genetic, and morphological changes that culminates in the formation of the adult fly. In triggering metamorphosis, ecdysone activates two divergent developmental pathways: The larval tissues are histolyzed, having served their function during the three larval instars, whereas the imaginal tissues

proliferate and differentiate into their predetermined adult structures.

Insights into the mechanisms whereby ecdysone coordinates these developmental changes at the onset of metamorphosis have been gained by studying the puffing patterns of the giant larval salivary gland polytene chromosomes. Six puffs appear within minutes after the addition of ecdysone. These so-called early puffs display similar temporal profiles, remaining active for several hours, after which they regress. As the early puffs reach their maximum size, a much larger set of >100 late puffs begins to appear. The late puffs are induced in sequence over a 10-hr period, with each puff displaying a characteristic profile of induction and regression. Activation of the early puffs is unaffected by drugs that inhibit protein synthesis and, thus, is a primary response to the steroid hormone. However, both regression of the early puffs and formation of the late puffs are effectively blocked under these conditions, implying a role for ecdysone-induced proteins in these regulatory functions (Ashburner et al. 1974). Ecdysone dose-response studies revealed that the early puffs respond incrementally over an

~600-fold range of ecdysone concentrations, consistent with their direct induction by the hormone. The late puffs, on the other hand, display a threshold response over only a four- to fivefold range, suggesting that ecdysone acts as a trigger, rather than as a sustained stimulus, for late gene activation (Ashburner 1973). Ecdysone also appears to directly repress a subset of the late puffs, as its premature withdrawal triggers the early appearance of some late puffs (Ashburner et al. 1974).

These observations form the basis of the hierarchical model for the genetic control of puffing by ecdysone, proposed by Ashburner et al. (1974). According to this model, the ecdysone–receptor protein complex has two antagonistic functions: to induce directly the early puffs (genes) and to repress directly a subset of the late puffs (genes). The early genes encode regulatory proteins that both repress early gene expression and induce the large battery of late genes. By repressing their own expression, the early genes determine the duration of their activity and, thus, the amounts of early gene products that can accumulate in response to a pulse of ecdysone. In addition, the antagonistic regulation of late gene expression (repression by the ecdysone–receptor protein complex vs. induction by the early gene products) leads to the properly timed sequential activation of the late genes. Burtis et al. (1990) have extended the Ashburner model to account for the diverse effects of ecdysone on target tissues other than the larval salivary gland, and for other times during development that are characterized by a pulse of ecdysone. This tissue coordination model proposes that ecdysone activation of overlapping sets of early regulatory genes directs unique tissue-specific patterns of late gene expression that define the morphological and functional properties of each target tissue at each stage in its development.

We are testing these models of ecdysone action by studying the regulation and function of ecdysone-inducible genes that correspond to characterized early and late puff loci. Our present effort focuses on *E74*, an ecdysone-inducible gene that is responsible for the early puff at position 74EF in the polytene chromosomes. Mutations in *E74* are lethal during pupal development, consistent with this gene playing an essential role in metamorphosis (Burtis 1985). *E74* is a complex gene encoding three overlapping mRNAs that vary at their 5' ends (Fig. 1). The distal promoter directs the synthesis of a 60-kb primary transcript that is spliced to form the 6-kb *E74A* mRNA. Approximately 40 kb downstream from the *E74A* promoter are two *E74B* transcriptional start sites, 300 bp apart, designated *E74B1* and *E74B2*. These two start sites direct the synthesis of 4.8- and 5.1-kb mRNAs that encode the same protein product. The *E74A* and *E74B* transcripts each contain unique 5' exons joined to a common set of three 3' exons (Fig. 1). This nested arrangement of the *E74* mRNAs leads to the synthesis of two related proteins that have unique amino-terminal regions joined to a common carboxy-terminal region. A portion of the carboxy-terminal region resembles that of the protein encoded by the E26 avian erythroblastosis virus *ets* oncogene (Janknecht et al. 1989; Burtis et al.

Figure 1. *E74* gene structure. The approximate sizes and locations of the *E74A* and *E74B* exons are depicted along with a genomic *EcoRI* restriction map from the 74EF region. The solid black boxes represent noncoding 5'- and 3'-flanking regions; the open boxes represent protein-coding regions. The different *E74B* start sites are not distinguished. The hatched portion of exon 8 contains sequences that encode the *E74* ETS domain (Burtis et al. 1990). The arrow marks the location of the X1001 translocation breakpoint (Burtis 1985). Brackets above the genomic map show the location of the three adjacent binding sites for the *E74A* and *E74B* proteins (Urness and Thummel 1990; F.D. Karim and C.S. Thummel, unpubl.). Also shown above the genomic map are the locations of the Northern probes used to detect specific *E74* transcripts.

1990). This 85-amino acid sequence, designated the ETS domain, is shared with a variety of other proteins and has been shown to function as a site-specific DNA-binding domain (Karim et al. 1990). As expected for two proteins that share identical DNA-binding domains, both *E74A* and *E74B* proteins specifically recognize the same 312-bp fragment located 11 kb upstream from the *E74B* start sites (Fig. 1; Urness and Thummel 1990; F.D. Karim and C.S. Thummel, unpubl.). The regulatory function of these sequences, if any, is unknown. In addition, antibody staining of polytene chromosomes revealed that *E74A* protein binds to many early and late ecdysone-induced puffs, suggesting that *E74A* plays a central role in the ecdysone regulatory hierarchy (Urness and Thummel 1990). Thus, *E74* appears to encode two proteins that can bind the same target DNA sequences but may exert unique regulatory functions by virtue of their different amino-terminal amino acid sequences.

As predicted by the Ashburner model, *E74A* transcription is directly induced several orders of magnitude by ecdysone and repressed by ecdysone-induced proteins. Furthermore, the kinetics of this activation and repression, both in vitro and in vivo, parallels the puffing response at 74EF. In support of the tissue coordination model, *E74A* transcription is induced during each of the six developmental stages that are characterized by a pulse of ecdysone. *E74A* is also expressed in most larval and imaginal tissues, suggesting that its presumed regulatory function is not restricted to the larval salivary gland (Thummel et al. 1990; Boyd et al. 1991). Kinetic studies showed that the *E74A* primary transcript is elongated at a rate of 1.1 ± 0.3 kb/min after ecdysone activation of its promoter. This rate measurement, combined with the 60-kb length of the *E74A* transcription

unit, accounts for most of the 1-hr delay seen in the appearance of spliced *E74A* mRNA in the cytoplasm. This observation led to the proposal that the lengths of early ecdysone-inducible regulatory genes may play an important role in determining the timing of their activities. This could, in turn, control the timing of late gene activation as well as contribute toward the time of early gene repression and hence the amount of early gene products that accumulate in response to a hormone pulse (Thummel et al. 1990).

In this study we extend our analysis of *E74* transcriptional regulation by showing that both transcript length and sensitivity to ecdysone concentration play key roles in coordinating *E74* expression. Both *E74A* and *E74B* transcription contribute to 74EF puff formation, accounting for its broad dose-response to ecdysone, as well as indicating that a single early puff locus can encode temporally distinct primary response transcripts. We discuss how different profiles of ecdysone can be translated into different combinations and amounts of early regulatory gene products, providing a potential mechanism for transducing the hormonal signal to direct different developmental pathways.

Results

E74A and *E74B* transcription is coordinately regulated at the onset of metamorphosis

E74A and *E74B* have overlapping but distinct patterns of transcription during *Drosophila* development (Thummel et al. 1990). To better characterize the regulation of *E74B* relative to that of *E74A*, we compared the levels of these transcripts during late larval and prepupal development. RNA was extracted from whole organisms at two late larval stages and prepupae synchronized at 2-hr intervals (± 15 min). Equivalent amounts of total RNA were analyzed by Northern blot hybridization using mixed probes for the *E74A* and *E74B* transcripts (Fig. 2). As shown previously, there are two bursts of *E74A* transcription during this interval, accompanying the peaks of ecdysone in 0- and 10-hr prepupae (Thummel et al. 1990). *E74B* mRNA is present in late third-instar larvae significantly before *E74A*, when the ecdysone titer is low but increasing (Fig. 2; also see Thummel et al. 1990), but can no longer be detected by puparium formation, when the ecdysone titer and *E74A* levels have peaked. As the ecdysone titer rises again during early prepupal development, *E74B* transcripts reappear and continue to accumulate until 10 hr after puparium formation when the ecdysone titer and *E74A* mRNA levels have peaked. After *E74A* repression in 12-hr prepupae, *E74B* is again induced. This temporal profile of *E74B* transcription suggests that it too may be induced by ecdysone, although at a lower concentration than that required for *E74A* activation. In addition, *E74B* repression is coincident with *E74A* induction, suggesting that *E74A*, or another early gene that is expressed in a similar temporal manner, represses *E74B* transcription.

Figure 2. Time course of *E74A* and *E74B* transcription during late larval and prepupal development. Total RNA was isolated from wandering (>12 hr before puparium formation) and stationary (~ 3 hr) third-instar larvae as well as from prepupae at 2-hr intervals after puparium formation (see Materials and methods). RNA (~ 25 μ g/lane) was fractionated by formaldehyde-agarose gel electrophoresis, transferred to uncharged nylon, and hybridized with mixed single-stranded radioactive DNA probes for *E74A* and *E74B* (mixed A and B probes; see Fig. 1), as described in Materials and methods. A nick-translated *rp49* probe was included in the hybridization as an internal control for loading (O'Connell and Rosbash 1984). RNA size markers (BRL) (shown in left lane), were detected by hybridization with nick-translated λ DNA.

E74B, like *E74A*, is induced directly by ecdysone

To determine the effects of ecdysone on *E74B* transcription, mass-isolated late third-instar larval tissues were maintained in culture and exposed to ecdysone for various periods, after which RNA was isolated and analyzed by Northern blot hybridization (Fig. 3). Mature *E74A* mRNA appears with its expected 1-hr delay, consistent with a transcription rate of 1.1 kb/min and the 60-kb length of its transcription unit (Thummel et al. 1990). In further agreement with our previous studies, *E74A* transcripts peak between 4 and 6 hr after ecdysone addition, after which they are repressed. This transcription profile parallels the puffing response of the 74EF locus in cultured salivary glands (Ashburner 1972; Thummel et al. 1990). As expected for organs isolated from late third-instar larvae (Fig. 2), *E74B* mRNA is present in untreated tissues at the 0-hr time point (Fig. 3). *E74B* transcripts begin to accumulate above this basal level between 15 and 30 min after ecdysone addition, in agreement with the delay time expected for a 20-kb ecdysone-inducible transcription unit, and peak at ~ 1 –2 hr after ecdysone addition. After this induction, *E74B* is rapidly repressed such that its transcripts can no longer be detected 3 hr after ecdysone addition. This pattern of *E74B* transcription resembles the *in vivo* pattern in two ways (see Fig. 2): (1) Both *E74A* and *E74B* transcripts accumulate in response to ecdysone, with *E74B* preceding *E74A*; and (2) *E74A* transcriptional induction by ecdysone is accompanied by efficient *E74B* repression. The increase in *E74B* mRNA levels with the expected 20-min delay is consistent with the *E74B* promoter being directly activated by

Figure 3. Time course of *E74A* and *E74B* transcription in cultured larval organs treated with ecdysone. Mass-isolated late third-instar larval organs were maintained in culture and treated with ecdysone for the periods of time shown. Total RNA was extracted and analyzed by Northern blot hybridization (~16 µg total RNA/lane), using a single-stranded radioactive DNA probe directed against the *E74* common region (probe A + B; Fig. 1). Both *rp49* mRNA and the size markers were detected as described in Fig. 2. The 0- and 0.25-hr lanes are slightly underloaded. Several light bands corresponding to breakdown products of the *E74* transcripts can be seen. Detection of these products was not reproducible.

ecdysone. Furthermore, the transcriptional delays of *E74A* and *E74B* seen in Figure 3 demonstrate that primary transcript length acts as a delay timer to coordinate the appearance of the mRNAs in response to ecdysone.

E74B transcription, as with *E74A*, is not inhibited by the addition of cycloheximide, providing further support for its direct induction by ecdysone. Unlike *E74A*, however, treatment with cycloheximide alone results in an accumulation of *E74B* mRNA, although at lower levels than are seen in tissues treated with both ecdysone and cycloheximide (data not shown). The cycloheximide induction of *E74B* can be explained most simply by a stabilization of *E74B* transcripts accompanied by a low level of RNA synthesis and/or the decay of a short-lived repressor of *E74B* transcription.

The three E74B mRNAs are coordinately controlled by ecdysone

The relatively broad band of *E74B* transcripts detected by Northern blot hybridization consists of three mRNAs with unique 5' ends, designated *E74B1a*, *E74B1b*, and *E74B2*. The *E74B1a* and *E74B1b* start sites are 8 bp apart and arise from a tandem duplication located 300 bp upstream from the *E74B2* start site (Burtis et al. 1990). To distinguish the effects of ecdysone on transcriptional initiation and repression of each *E74B* transcript, we performed S1 analysis with a probe designed to distinguish the three start sites (Fig. 4). Consistent with the Northern profile of *E74B* transcript accumulation, all three of the *E74B* transcriptional start sites are activated within 2 hr after ecdysone addition and are significantly repressed by 4 hr. Somewhat fewer transcripts initiate from the

E74B1a start site, whereas more transcripts initiate at the *E74B2* start site. Repression of *E74B2* transcription appears to precede slightly that of *E74B1*. Overall, however, ecdysone induction and subsequent repression from the three *E74B* start sites occurs synchronously, suggesting a common mechanism of regulation by ecdysone. The increased sensitivity of S1 analysis reveals some *E74B* transcripts as late as 8 hr after ecdysone addition. This is consistent with the persistence of *E74B* expression in the anterior gut (see Fig. 8, below), which constitutes a relatively minor proportion of the tissues in our mass-isolated preparations.

E74B is induced by an ecdysone concentration 25-fold lower than that required for E74A activation

Although primary transcript length contributes to the earlier appearance of *E74B* transcripts (Fig. 3), it alone is

Figure 4. S1 nuclease protection analysis of ecdysone-induced *E74B* transcription. Mass-isolated late third-instar larval organs were maintained in culture and treated with ecdysone for the times shown. Total RNA was purified and analyzed by S1 nuclease protection using a radioactively labeled single-stranded DNA probe that spans all three *E74B* start sites, as described (Burtis et al. 1990). Yeast RNA was included as a negative control. The set of bands below the full-length *E74B2* S1-protected fragment is an artifact caused by occasional melting of four adjacent A–T base pairs (Burtis et al. 1990).

not sufficient to explain the appearance of *E74B* mRNA many hours before *E74A* induction in late third-instar larvae and early prepupae (Fig. 2). Rather, the appearance of *E74B* mRNA with the leading edge of the ecdysone pulse suggests that *E74B* transcription is activated by a lower ecdysone concentration than that required for *E74A* activation. Previous dose-response studies showed that the 74EF puff exhibits a graded response over a 600-fold range of ecdysone concentrations, with the smallest detectable puffing occurring at 1×10^{-9} M and maximal puffing at 5×10^{-7} M ecdysone. The estimated dose for a 50% maximal 74EF puff is 1×10^{-7} M ecdysone (Ashburner 1973).

Figure 5 shows a Northern blot of total RNA isolated from late third-instar larval organs that were incubated for 1.5 hr in the presence of various concentrations of ecdysone, from 1×10^{-11} M to 2.3×10^{-5} M. The amounts of *E74A* and *E74B* mRNA induced at each ecdysone concentration were determined by volume-integration densitometry (see Materials and methods). Two separate preparations of organs yielded similar results. When considered individually, each *E74* transcription unit responds over a 10- to 20-fold range of ecdysone concentrations. The lowest ecdysone concentration that gives detectable *E74A* induction occurs at 5×10^{-8} M, and the maximal response occurs at $\sim 7.5 \times 10^{-7}$ M. *E74B* shows a slight induction above its basal level with 1×10^{-9} M ecdysone and reaches its maximal response at $\sim 5 \times 10^{-8}$ M ecdysone. *E74A* transcription has a 50% maximal response at $\sim 2 \times 10^{-7}$ M ecdysone and *E74B* transcription has a 50% maximal response at $\sim 8 \times 10^{-9}$ M. Considered together, however, *E74A* and *E74B* transcription show a graded response over approximately the

Figure 5. Ecdysone dose-response analysis of *E74A* and *E74B* transcriptional induction. Mass-isolated late third-instar larval organs were maintained in culture and treated with different ecdysone concentrations, as shown, for 1.5 hr. Total RNA was extracted and analyzed (~ 10 μ g/lane) by Northern blot hybridization using a single-stranded radioactive DNA probe directed against the *E74* common region (probe A + B; Fig. 1). The blot was hybridized with a nick-translated rp49 probe as an internal control for loading (O'Connell and Rosbash 1984).

Figure 6. Time course of *E74A* and *E74B* transcription in cultured larval organs treated with different concentrations of ecdysone. Mass-isolated third-instar larval organs were maintained in culture and treated with six different ecdysone concentrations for the periods of time shown. Total RNA was extracted and analyzed (~ 16 μ g/lane) by Northern blot hybridization as described in Fig. 5. RNA isolated from organs treated for 6 hr with ecdysone was used as an *E74A* positive control.

same range of ecdysone concentrations as that required for 74EF puffing. These data also show that the *E74B* promoter is activated by an ~ 25 -fold lower ecdysone concentration than that required for *E74A* promoter activation.

E74 transcription profiles change as a function of ecdysone concentration

The ecdysone dose-response analysis described above was restricted to determining the level of *E74* transcriptional induction after a 1.5-hr incubation with hormone. To confirm and extend this study, six ecdysone concentrations, spanning the range required for 74EF puff formation and *E74* transcriptional induction, were tested in organ culture over an 8-hr time course for their effects on the temporal profiles of *E74* transcription. As before, total RNA was extracted from the organs at each time point and analyzed by Northern blot hybridization using a probe for both *E74A* and *E74B* mRNAs (Fig. 6). Consistent with the dose-response data shown in Figure 5, *E74A* transcription is not detectable below an ecdysone concentration of 5×10^{-8} M and is maximally induced

above 5×10^{-7} M, whereas *E74B* transcription is at its $\sim 50\%$ maximal level at 5×10^{-9} M ecdysone. As expected, the ecdysone concentration does not affect the transcriptional delay determined by the lengths of the *E74* transcription units; thus, *E74B* transcription precedes that of *E74A* at all ecdysone concentrations. The level of *E74A* induction increases with higher ecdysone concentrations, but its overall temporal profile remains unaffected. The profile of *E74B* transcription, however, changes dramatically with different hormone concentrations. At an ecdysone concentration of 5×10^{-9} M there is little, if any, repression of *E74B* transcription, resulting in *E74B* expression for >8 hr. At a maximal dose, *E74B* transcription is confined to a brief 2-hr burst. Whereas the induction level of *E74B* increases slightly at concentrations above 5×10^{-9} M, consistent with its dose response, *E74B* repression occurs earlier and more completely at concentrations above 1.5×10^{-8} M ecdysone. Thus, *E74B* repression, like its induction, occurs in an ecdysone dose-dependent manner. The ecdysone concentration required for 50% maximal *E74B* repression occurs at $\sim 8 \times 10^{-8}$ M, close to the concentration that gives 50% maximal induction of *E74A*. Furthermore, concentrations above 7.5×10^{-7} M ecdysone, the concentration required for maximal *E74A* transcription, do not further attenuate *E74B* expression. This is consistent with *E74A*, or another early gene with a similar pattern of expression, repressing *E74B* transcription. We reserve for the Discussion (below) a more complete consideration of the effects of ecdysone concentration on the timing and levels of *E74* transcription.

E74A does not repress *E74B*

Both our *in vivo* and *in vitro* studies reveal a strict correlation between *E74A* induction and *E74B* repression. The simplest explanation for this observation is that *E74A* represses *E74B*. Direct repression by *E74A* could be mediated at two levels. One possibility is that transcription initiating at the *E74A* start site could block *E74B* transcriptional initiation by promoter occlusion (Adhya and Gottesman 1982). A second model derives from our previous observation that *E74A* protein can bind to three adjacent sites within the *E74* gene, ~ 11 kb upstream from the *E74B* promoter (Fig. 1; Urness and Thummel 1990). By interacting with these sequences, *E74A* protein could repress *E74B* transcription *in trans*. Evidence in favor of a role for ecdysone-induced proteins in *E74B* repression, and against the promoter occlusion model, is the absence of normal *E74B* repression in tissues treated with both ecdysone and cycloheximide (data not shown).

We have employed an *E74A* mutant, X1001, in an effort to determine whether *E74A* protein is required for *E74B* repression. X1001 homozygotes survive through the larval-to-prepupal transition but die by the pharate adult stage. This mutation, an X-ray-induced translocation between the left arms of chromosomes 2 and 3 [T(2,3) 29A-29C;74E-74F], has a breakpoint in the first intron of *E74A* (Burtis 1985), 29 kb upstream from the *E74B* start sites (see Fig. 1). Therefore, X1001 specifically

disrupts the *E74A* transcription unit but leaves the *E74B* transcription unit intact. The *E74A* promoter and first exon, fused to sequences on the second chromosome in X1001 homozygotes, still forms a small ecdysone-inducible puff and generates 1.5- and 3.6-kb transcripts that are properly induced by ecdysone and subsequently repressed (Burtis 1985).

Figure 7 shows a Northern blot analysis of RNA isolated from staged prepupae from the parental strain, st p^e e¹¹, or homozygous X1001 mutant individuals. The *E74A* and *E74B* transcription profiles seen in prepupae from the parental strain (Fig. 7) resemble the pattern seen in wild type (Canton S; Fig. 2); however, the prepupal period appears to be significantly extended in the st p^e e¹¹ strain. This delay is evident in both the extended expression of *E74A* after puparium formation and the 6-hr delay in the prepupal ecdysone pulse that reinduces *E74A* transcription, 16 hr after puparium formation. Variability in the timing of the prepupal ecdysone pulse has been observed previously for different strains of *D. melanogaster* (Richards 1980). In addition, both the parental and mutant strains were noticeably delayed, relative to wild type, in the time required to commit to puparium formation and in hardening and tanning of the prepupal cuticle (see Materials and methods). The *E74A* promoter is activated and repressed in homozygous X1001 prepupae at the same times as in the parental strain, indicating that the X1001 mutation does not further affect the timing of the ecdysone pulses (data not shown; Burtis 1985). A common region probe, directed against exon 8 (A + B in Fig. 1), was used to detect *E74B* mRNA, as well as any transcripts that might initiate from sequences within the second chromosome, and extend past the breakpoint through the intact *E74A* open

Figure 7. Time course of *E74A* and *E74B* transcription during late larval and prepupal development in the st p^e e¹¹ parental strain and X1001 mutant strain. Total RNA was isolated from wandering and stationary third-instar larvae, as well as from prepupae at 2-hr intervals after puparium formation. The ages of the two larval samples were estimated to be >12 hr and 3 hr before puparium formation, respectively. There is some variability in the timing of these prepupal stages that might influence the apparent timing of *E74A* and *E74B* expression (see Materials and methods). RNA (~ 25 μ g/lane) was analyzed by Northern blot hybridization as described in Fig. 5.

reading frame. This probe revealed parallel patterns of *E74B* transcription in both the mutant and parental strains, in the absence of clearly detectable transcripts encoding *E74A* protein in X1001 homozygotes (Fig. 7). A low abundance 5.5-kb transcript was detected but did not appear to be ecdysone inducible. An identical pattern of *E74B* transcription was seen when this blot was re-probed with an *E74B*-specific probe (B in Fig. 1; data not shown). Subsequent hybridizations using probes complementary to *E74A* exons A2, A3, A4, and A5 (Fig. 1) showed little, if any, hybridization (data not shown). These data suggest that neither *E74A* transcription nor *E74A* protein plays a role in repressing *E74B* transcription.

E74A and *E74B* are induced by ecdysone in both larval and imaginal tissues

The tissue coordination model predicts that ecdysone activates tissue-specific combinations of early regulatory genes which, in turn, lead to the activation of distinct sets of late genes that define the unique properties of each ecdysone target tissue at each stage in its development. In support of this model, *E74A* transcription is induced by ecdysone in most, if not all, late third-instar larval tissues (Thummel et al. 1990; Boyd et al. 1991). To compare this pattern of expression with that of *E74B*, hand-dissected larval tissues were maintained in culture and treated with ecdysone for 0, 1.5, or 6 hr, after which RNA was isolated and analyzed by Northern blot hybridization (Fig. 8). This experiment was repeated at least three times for each tissue type. Four tissues were examined: imaginal discs (mixed eye–antennal, leg, wing, and haltere discs), salivary glands (with their imaginal rings), larval brains (the paired supraesophageal ganglia and

ventral ganglion with the ring gland removed), and anterior gut (proventriculus, gastric caeca, and midintestine). The discs are strictly imaginal, destined to form different external structures in the adult fly. The larval salivary gland and gut are predominantly larval, and thus destined for histolysis upon metamorphosis, although they also contain clusters of imaginal cells that will expand and differentiate into the corresponding adult organs. The brain is unique in that it is not strictly larval or imaginal but undergoes dramatic remodeling upon metamorphosis.

E74A and *E74B* transcription is induced by ecdysone in all four of these tissues; however, their relative levels and ecdysone induction profiles differ (Fig. 8). The time course of *E74* transcription in imaginal discs and larval salivary glands resembles that seen in total larval tissues (Fig. 3)—*E74A* and *E74B* are induced by ecdysone after a 1.5-hr incubation and, by 6 hr, *E74A* is further induced while *E74B* is fully repressed. The temporal profile of *E74A* and *E74B* transcription in larval brains differs in two aspects from that seen in total tissues: *E74A* is expressed at low levels in the absence of ecdysone and is induced to higher levels than *E74B* after 1.5 hr of ecdysone treatment (Fig. 8). The anterior gut shows a unique temporal pattern of *E74* transcription: *E74B* mRNA is still present by 6 hr after ecdysone addition, as though it were not properly repressed in this tissue, and *E74A* is induced submaximally. This temporal pattern of *E74* transcription is reminiscent of that seen in total tissues treated with a 100-fold lower ecdysone concentration ($\sim 5 \times 10^{-8}$ M; Fig. 6). These results confirm and extend our previous studies of *E74A* transcription and provide further support for the tissue coordination model (Burtis et al. 1990; Thummel et al. 1990). The different patterns of *E74* transcription seen in different tissues suggests that the relative levels and tissue-specific temporal patterns of early gene expression may play a role in directing unique developmental pathways.

Figure 8. Time course of *E74A* and *E74B* transcription in selected larval organs cultured with ecdysone. Brains, anterior gut, imaginal discs, and salivary glands were hand-dissected from 5–10 late third-instar larvae and cultured with ecdysone for the periods of time shown. A complete description of these tissues can be found in Results. The total RNA extracted from these tissues was analyzed by Northern blot hybridization as described in Fig. 5. The 0-hr sample isolated from anterior gut is underloaded and should show a low level of *E74B* mRNA (data not shown).

Discussion

The E74 gene is constructed as a delay timer for the sequential production of alternate DNA-binding proteins

We have determined previously that the *E74A* primary transcript is elongated at a rate of 1.1 ± 0.3 kb/min after ecdysone activation of its promoter. This rate measurement, and the 60-kb length of the *E74A* transcription unit, account for most of the observed 1-hr delay between ecdysone addition and the initial appearance of cytoplasmic *E74A* mRNA (Thummel et al. 1990). The length of the *E74A* unit is also conserved in both *Drosophila pseudoobscura* and *D. virilis* (Jones et al. 1991), suggesting that the 1-hr delay in *E74A* expression may be of functional significance. On the basis of these observations, we proposed that the lengths of the early ecdysone-inducible regulatory genes are important factors in controlling the timing of the genetic response to ecdysone. In support of this proposition, *E74B* transcripts first

appear between 15 and 30 min after ecdysone addition, corresponding to the delay expected for a 20-kb ecdysone-inducible transcription unit (Figs. 3 and 6). Thus, the lengths of the two *E74* transcription units dictate an invariant order in their transcriptional response to ecdysone. At high ecdysone concentrations, when both promoters are activated synchronously, *E74B* mRNA begins to appear when only one-third of the *E74A* unit has been transcribed (Thummel et al. 1990; Fig. 3). Hence, assuming equal rates of transcriptional initiation, the shorter length of the *E74B* transcription unit should lead to the production of *E74B* mRNA at approximately three times the rate of *E74A*. The presence of *E74B* transcriptional start sites, which are 300 bp apart and coordinately induced by ecdysone (Fig. 4), may facilitate the RNA polymerase on-rate by allowing independent formation of two initiation complexes. In addition, the shorter length of the *E74B* unit, relative to *E74A*, provides a more rapid clearance time for unloading RNA polymerase molecules after *E74B* transcriptional repression.

The lengths of the early ecdysone-inducible genes are an invariant parameter that establishes the minimal times of induction and repression, and, hence, the burst size of the encoded gene product. These timing constraints will not vary from one cell to another, or at different times during development. Rather, the flexibility required in the biological response to ecdysone is achieved, at least in part, by the different sensitivities of early gene activation and repression to ecdysone concentration. The dose-response analysis of *E74A* and *E74B* transcriptional induction by ecdysone, shown in Figure 5, demonstrated that *E74B* is induced at an ~25-fold lower hormone concentration than *E74A*. This accounts for the appearance of *E74B* mRNA several hours before *E74A* in late larvae and prepupae (Fig. 2). Furthermore, as seen both in vivo and in vitro, *E74B* repression is coincident with *E74A* induction. This suggests that the *E74* gene is functioning as an ecdysone-regulated switch that directs an initial burst of *E74B* expression followed by a burst of *E74A* expression in response to a high-level pulse of ecdysone. On the basis of these observations, it seems likely that specific combinations of gene length, activating ecdysone concentrations, and timing of repression will dictate the sequence and amounts of early gene expression in response to an ecdysone pulse.

Both E74A and E74B contribute to 74EF puff formation

The kinetics of *E74A* transcriptional activation by ecdysone, and its subsequent repression by ecdysone-induced proteins, parallels the puffing response at 74EF, both in vitro and in vivo. This correlation led to the proposal that ecdysone-induced *E74A* transcription was responsible for the 74EF puff (Thummel et al. 1990). Although our current results support this proposal, it is clear from our studies at lower ecdysone concentrations that *E74B* also contributes to 74EF puff formation. The lowest concentration required for 74EF puff formation, 1×10^{-9} M ecdysone, is the same concentration required for a low,

but detectable, induction of *E74B* transcription (Fig. 5). Similarly, a small 74EF puff is formed at ecdysone concentrations below 5×10^{-8} M ecdysone (Fig. 3a in Ashburner 1973), when *E74B* but not *E74A* is expressed (Figs. 5 and 6). At 6×10^{-8} M and 6×10^{-9} M ecdysone, a submaximal 74EF puff is induced and regresses after several hours (Fig. 6 in Ashburner 1973), following similar kinetics as *E74B* transcription (Fig. 6). An exception is the 2-hr time point at 6×10^{-9} M ecdysone, when the 74EF puff appears to have fully regressed but *E74B* transcripts can still be detected. As the ecdysone concentration increases above the threshold level for *E74A* induction, the 74EF puff is induced to an increasingly larger size until it achieves its maximum size at $\sim 5 \times 10^{-7}$ M ecdysone, the same concentration required for maximum levels of *E74A* transcription (Fig. 5). Thus, although *E74A* and *E74B* are each induced over a relatively narrow 10- to 20-fold range in ecdysone concentration, their combined responses (50% maximal *E74B* induction at 8×10^{-9} M and 50% maximal *E74A* induction at 2×10^{-7} M) span a broad range of ecdysone concentrations, identical to the ~600-fold range required for 74EF puff formation.

The broad ecdysone dose-response of the 74EF puff is shared by at least two other early ecdysone-induced puffs, at positions 23E and 75B in the polytene chromosomes. Whereas 74EF and 75B respond over an almost identical range of ecdysone concentrations, 23E is induced at a significantly lower concentration, with 50% maximal puff induction at $\sim 5 \times 10^{-8}$ M ecdysone (Ashburner 1973). The graded response of the 23E and 75B puffs over an ~600-fold range of ecdysone concentrations suggests that, as with 74EF, these puffs are a composite formed by several ecdysone-inducible transcription units that are activated at different ecdysone concentrations. The lower threshold concentration for 23E puff formation suggests further that, as with *E74B*, it plays one of the earliest roles in the regulatory hierarchy.

Although the 23E early gene has not yet been defined, the gene responsible for the 75B early puff, *E75*, has been cloned and characterized. As with *E74*, *E75* is an unusually complex gene, spanning >50 kb and composed of several nested transcription units. *E75* encodes at least three related proteins, all of which are members of the steroid hormone receptor superfamily (Feigl et al. 1989; Segreaves and Hogness 1990). We predict that each of the *E75* transcripts is activated at different ecdysone concentrations spanning the broad range required for 75B puff formation. Thus, as with *E74*, the *E75* transcripts should be expressed in a reproducible sequence dictated by the lengths of their transcription units and their sensitivities to ecdysone.

Unlike the early puffs, the late ecdysone-induced puffs (22C, 62E, 63E, 78D, and 82F were examined; Ashburner 1973) respond over a narrow four- to fivefold range of ecdysone concentration, from 5×10^{-8} M to 2.5×10^{-7} M ecdysone. These late puffs do not appear at concentrations below 5×10^{-8} M ecdysone, when *E74B* is actively transcribed. This suggests that *E74B*, and other early gene products that are regulated in a similar manner,

cannot independently trigger activation of these late genes. Rather, early genes such as *E74A*, with 50% maximal transcriptional responses at $\sim 2 \times 10^{-7}$ M ecdysone are critical for activating these late genes. The *E74A* protein has been shown to bind a subset of the late puffs, supporting its potential role in late gene activation (Urness and Thummel 1990).

Late larval and prepupal pulses of ecdysone appear to be preceded by a gradual rise in hormone titer that triggers a progressive series of early gene expression

Ecdysone pulses synchronize the progressive developmental changes in each target tissue, directing them to assume the morphological and functional properties appropriate for that stage in development, beyond which they will not progress until triggered to do so by a subsequent pulse of hormone. Programming this morphogenetic progression requires dynamic changes in ecdysone concentration, with peak levels establishing synchrony and very low levels establishing the delays between each ecdysone-triggered step in the developmental pathway. The mid-third-instar larval stage acts as one such period of low ecdysone concentration, preceding the burst of hormone that triggers the onset of metamorphosis. De Reggi et al. (1975) have identified a small ecdysone peak at 8 hr before puparium formation, which could correspond to the small pulse of ecdysone in *Manduca sexta* that initiates larval wandering (Truman and Riddiford 1974). Although other ecdysone titer measurements have not confirmed this peak in *Drosophila* (Richards 1981b), close examination of the salivary gland polytene chromosomes revealed a small 74EF puff at the onset of wandering, ~ 9 hr before puparium formation (Richards 1980). This puff is probably due to transcription of *E74B*, which is induced between 96 and 108 hr of development, from 12–24 hr before puparium formation, significantly earlier than *E74A* (Fig. 2; Thummel et al. 1990). The early induction of *E74B* transcription is consistent with a low pulse of ecdysone preceding the burst that triggers puparium formation. Alternatively, the ecdysone titer may rise gradually, from $<10^{-9}$ M midway through the third-instar larval stage to between 5×10^{-8} M and 1×10^{-7} M ecdysone, preceding the high titer pulse at the end of larval development. These concentrations are consistent with the ecdysone titers of staged third-instar larvae determined by Hodgetts et al. (1977), who detected a gradual increase in the hormone titer beginning at 20 hr before puparium formation.

The presence of a small 74EF puff in early wandering larvae is not unique. Polytene chromosomes from the salivary glands of larvae at a similar stage also contain a small puff at 75B (Richards 1982) and a relatively large 2B5 early puff (Ashburner 1967). Furthermore, mutations in the Broad-Complex (BR-C), the genetic locus from which the 2B5 puff arises, prevent *Sgs-3* glue gene transcription (Crowley et al. 1984; Georgel et al. 1991), reduce the size of the early puffs, and block late puff formation (Belyaeva et al. 1981). This suggests that 2B5, along with 74EF and 75B, provides a very early function

that facilitates a complete regulatory response to ecdysone. We propose that a gradual increase in the ecdysone titer, before the main peak, may be of regulatory significance, directing the sequential induction of very early ecdysone-induced mRNAs. These early–early regulatory components would establish competence for the full spectrum of subsequent regulatory effects as the ecdysone titer peaks and regresses. In addition to facilitating early ecdysone-inducible gene expression, functional consequences of an early–early response to ecdysone could include initiating larval wandering behavior (Truman and Riddiford 1974), as well as inducing salivary gland glue gene expression (Hansson and Lambertsson 1983; Georgel et al. 1991).

After this gradual increase in the ecdysone titer, a pulse of high ecdysone concentration triggers commitment to puparium formation. This is followed by a period of low ecdysone concentration and a second pulse of ecdysone, peaking in 10-hr prepupae (Handler 1982). Both complete pupal cuticle formation (Fristrom et al. 1982) and the prepupal puffing response in the salivary gland polytene chromosomes (Richards 1976a) require these dynamic changes in ecdysone concentration. *E74B* transcription is induced in 4-hr prepupae, followed 4 hr later by *E74A* induction and *E74B* repression as the ecdysone concentration peaks (Fig. 2). The accumulation of *E74B* transcripts for 4 hr before *E74A* induction suggests that, as in late third-instar larvae, the ecdysone titer in early prepupae may rise gradually over the course of several hours. Alternatively, the ecdysone titer could be higher in early prepupae (between 10^{-9} M and 10^{-8} M; Richards 1976b), and *E74B* induction at 4 hr may reflect the turnover of its repressor protein. The genes that are activated with the leading edge of the ecdysone pulse, such as *E74B*, may provide essential functions that facilitate progression to the next ecdysone-triggered developmental stage.

The profile of an ecdysone pulse determines the timing and amounts of E74A and E74B expression

Although dynamic changes in hormone titer are required for synchronizing and coordinating developmental changes, the molecular mechanisms involved in transducing different hormone concentrations to direct unique regulatory responses have been a subject of speculation. Our study of the effects of ecdysone concentrations on *E74* transcription provides a means of translating the profile of a hormone pulse into different times and amounts of regulatory gene expression. This can be demonstrated most clearly by the effects of different hormone concentrations on the time course of *E74B* transcription (Fig. 6). *E74B* is repressed, in a dose-dependent manner, at a significantly higher concentration (50% response = 8×10^{-8} M) than that required for its induction (50% response = 8×10^{-9} M). The 10-fold difference in these threshold concentrations defines a window within which the *E74B* gene products can accumulate. For example, a low broad pulse of ecdysone, above 1×10^{-9} M ecdysone but peaking below 1×10^{-7} M

ecdysone, would result in a long period of *E74B* expression and the accumulation of relatively large amounts of its encoded gene product, with little or no *E74A* expression (Fig. 6). Similarly, as seen in prepupae (Fig. 2), a gradual increase in the ecdysone titer will allow *E74B* to build to high levels over the course of several hours before triggering the switch to *E74A* expression. A sharp high pulse of ecdysone, as seen in our *in vitro* time course (Fig. 3), will direct the minimal amount of *E74B* expression for 1–2 hr. Thus, the duration of *E74B* expression and, hence, the amount of gene product that can accumulate, is determined by the rate at which the ecdysone concentration increases. These results provide some insight into how a complex regulatory response can be triggered by a relatively simple hormonal signal.

E74B is repressed by an early ecdysone-inducible protein other than E74A

The observation that *E74B* is properly regulated in X1001, a chromosomal translocation mutant that disrupts the *E74A* transcription unit but leaves *E74B* intact (Fig. 7; Burtis 1985), argues that *E74A* does not repress *E74B*. Rather, an early ecdysone-inducible gene with an expression profile very similar to that of *E74A* must encode the *E74B* repressor. Furthermore, because *E74B* is repressed after 2 hr at high ecdysone concentration, as *E74A* is induced, we conclude that different ecdysone-inducible proteins may repress *E74A* and *E74B* and that the *E74B* repressor is encoded by a transcription unit that is ~60 kb in length, accounting for the observed 1-hr delay in the onset of *E74B* repression (Fig. 3). Alternatively, a single early ecdysone-induced protein may repress both *E74* transcription units but must do so at different effective concentrations. As we have discussed previously, the *E75* early gene is a good candidate for a repressor of *E74* transcription (Thummel et al. 1990; Urness and Thummel 1990).

We have identified three adjacent binding sites for *E74A* and *E74B* protein, located 11 kb upstream from the *E74B* start sites (Urness and Thummel 1990; F.D. Karim and C.S. Thummel, unpubl.). *E74A* protein is bound to the 74EF puff *in vivo*, suggesting that it occupies the *E74*-binding sites in salivary gland chromosomes (Urness and Thummel 1990). However, *E74A* protein bound to these sequences has no apparent regulatory function. The *E74A* promoter is properly regulated in the absence of these binding sites (Burtis 1985), and *E74B* transcription is not significantly affected by the absence of *E74A* protein (Fig. 7). It remains possible, however, that *E74B* may exert some regulatory function through these sequences, possibly allowing an autocatalytic response to ecdysone.

The pattern of *E74B* expression in X1001 homozygotes also has implications regarding the role of EcR protein in *E74B* induction. EcR protein has all of the properties predicted for an ecdysone receptor (M. Koelle, W. Segaves, W. Talbot, M. Bender, and D. Hogness, unpubl.). Two strong EcR-binding sites have been identified within the first intron of the *E74A* transcription unit,

upstream from the X1001 breakpoint. In addition, several weaker binding sites have been detected at various positions along the *E74* gene, extending downstream from the X1001 breakpoint toward the *E74B* promoter (W. Talbot and D. Hogness, unpubl.). Because *E74B* is induced in homozygous X1001 prepupae, the two strong EcR-binding sites are not essential for *E74B* transcriptional induction. One simple explanation for these results is that *E74B* is activated by either a different ecdysone receptor or a different modified form of the EcR protein. In addition to distinguishing between the *E74A* and *E74B* ecdysone-responsive elements, these different receptor forms may have different binding affinities for ecdysone, accounting for the different ecdysone concentrations required for *E74A* and *E74B* induction.

E74A and E74B are transcribed in unique tissue-specific patterns

The tissue coordination model predicts that regulatory hierarchies similar to that seen in larval salivary glands are activated by ecdysone at other developmental stages and in other target tissues (Burtis et al. 1990). In support of the tissue coordination model, *E74A* and *E74B* are transcribed in four larval and imaginal tissues, with different temporal responses and different relative levels of expression (Fig. 8). The time course of ecdysone-induced *E74* transcription seen in salivary glands and imaginal discs resembles that seen in total tissues (Fig. 3). This is consistent with the high representation of these tissues in our mass-isolated preparations. The temporal pattern of *E74* transcription in the larval brain is also similar to that seen in total tissues, except that low levels of *E74A* mRNA can be detected in the absence of ecdysone and *E74A* is induced to a higher level than *E74B* after 1.5 hr of ecdysone treatment (Fig. 8). The *E74A* mRNA in untreated brains may derive from the proliferation centers. These cells are unique in that they contain high levels of *E74A* RNA in early prepupae, when the total amount of *E74A* mRNA is very low (Boyd et al. 1991). Interestingly, anterior guts treated with a high concentration of ecdysone (5×10^{-6} M) exhibit a unique temporal pattern of *E74* transcription, similar to that seen in total tissues at low ecdysone concentration ($\sim 5 \times 10^{-8}$ M). Although it is possible that the *E74B* repressor is not synthesized in anterior gut, allowing *E74B* transcription to proceed beyond its normal 2-hr duration, the relatively low level of *E74A* mRNA suggests that ecdysone induction may also be altered in this tissue. An intriguing model is that different target tissues require different ecdysone concentrations for *E74A* and *E74B* induction and repression. According to this model, most of the tissues that we mass-isolate from larvae follow the rules defined in the ecdysone dose-response analyses shown in Figures 5 and 6. Anterior guts, however, which represent a relatively minor proportion of our tissue preparations, may require higher ecdysone concentrations to generate the same temporal pattern of *E74* transcription seen in total tissues. Just such a difference in hormone sensitivity was seen when the puffing patterns of fat body polytene chro-

mosomes were compared with those of salivary glands. In fat bodies, the 75B early puff showed a 50% maximal response at $\sim 10^{-8}$ M ecdysone, compared with the 1.5×10^{-7} M ecdysone needed for a similar response in salivary glands. Similarly, the 74EF puff is induced to its maximum size in fat body polytene chromosomes by 8×10^{-7} M α -ecdysone, a concentration that would result in only a minor 74EF puff in the salivary gland (Richards 1982). This can be explained most simply by tissue-specific factors or tissue-specific modifications of the ecdysone receptor(s) that alter receptor–ligand binding. An alternative possibility is that different target tissues have varying permeability to ecdysone or that ecdysone is sequestered at higher local concentrations in certain tissues (Richards 1981b). Dose-response analysis of early gene transcription in isolated larval tissues should allow us to determine whether tissue-specific sensitivities to ecdysone could potentially affect the determination of unique developmental pathways.

Future considerations

Although the early puffs can be grouped into a single temporal class, they appear to represent a spectrum of early transcriptional responses to ecdysone, of which *E74B* represents an earlier response and *E74A* represents a later response. The molecular mechanisms that dictate these different temporal responses to ecdysone are most likely shared among other early transcription units in the regulatory hierarchy. Thus, we predict that the multiple transcripts encoded by the BR-C and *E75* will be expressed in a temporal pattern determined by the lengths of their transcription units and the ecdysone concentrations required for their activation and repression. Consistent with this, both of these early genes contain nested promoters spanning unusually long transcribed regions, up to 100 kb in length (Chao and Guild 1986; Feigl et al. 1989; Galcerán et al. 1990; Segreaves and Hogness 1990; Thummel 1990). As with *E74*, the *E75* transcripts have distinct temporal responses to a high concentration of ecdysone. *E75* expression, however, has an additional level of complexity in that the *E75A* and *E75B* mRNAs show a biphasic response during continuous culture of salivary glands with ecdysone (Fig. 6 in Segreaves and Hogness 1990).

Furthermore, we predict that EcR is an early ecdysone-inducible gene, based on the observation of Deak et al. (1988), who noted that bursts of ecdysone receptor synthesis follow the pulses of ecdysone during embryonic and larval development. Furthermore, as this proposed autocatalytic loop should be active early in the response to ecdysone to provide high levels of receptor for subsequent steps in the hierarchy, it is likely that EcR, as with *E74B*, is activated by low ecdysone concentrations.

The sequential induction of early transcription units, coupled with their ordered repression by different early ecdysone-induced proteins, should lead to waves of co-expressed early gene products that could act in a combinatorial fashion to coordinate the expression of late genes. Defining the expression profiles of the different

early ecdysone-inducible transcripts will provide clues regarding potential combinatorial interactions among early gene products. These studies should also provide further insights into the molecular mechanisms that control the timing of sequential gene expression in response to ecdysone.

Materials and methods

Developmental staging

Late third-instar larvae were staged by growth on food containing 0.05% bromphenol blue, as described by Maroni and Stamey (1983). Actively feeding larvae, through the mid-third-instar stage, can be identified by their blue alimentary tracts. Larvae that have completely cleared alimentary tracts pupariate within 3 ± 1.8 hr (the -3 -hr time point in Figs. 2 and 8; L. Boyd, unpubl.). Prepupae can be staged more accurately, within an error of ~ 15 min, by synchronizing at the white prepupal stage. Both the *E74* mutant strain X1001 and its parental strain (st p^P e¹¹) have a low level of late larval lethality and show a delay in their commitment to puparium formation. Some individuals appeared to form normal white prepupae, in that they were stationary and had the characteristic barrel shape and everted spiracles, yet would begin to crawl after they were handled. This delay in commitment may be related to the delay in the ecdysone pulses seen by Northern blot analysis (Fig. 7). Both parental and *E74* mutant strains also did not tan properly, due to the *ebony* mutation. To circumvent these problems with staging prepupal development, >100 wandering third-instar larvae were collected on moist paper in a 150-mm petri dish and checked every 15 min for newly pupariated animals. These prepupae were collected and allowed to develop at 25°C for the appropriate times after which they were frozen on dry ice.

Larval organ culture

Late third-instar larval organs were mass-isolated as described by Thummel et al. (1990) and Wolfner and Kemp (1988). Organs obtained using this procedure include salivary glands, imaginal discs, mid- and hind-intestine, and Malpighian tubules. Most of the fat bodies, brains, anterior gut, and cuticle (with attached epidermis and muscle) were removed during extraction. Organs were cultured in 5 ml of Robb's saline (Robb 1969) in 100-mm petri dishes at 25°C. The organs were incubated in a Styrofoam box under a constant flow of oxygen. Incubation in the presence of oxygen was found to increase the efficiency of *E74A* and *E74B* transcriptional repression, presumably by preventing anaerobic stress and its associated effects on protein synthesis (F.D. Karim, unpubl.). Ecdysone [20-OH ecdysone (Sigma)] was stored in 95% ethanol and added to a final concentration of 5×10^{-6} M, unless otherwise specified. Ecdysone concentrations were determined by measuring the absorbance at 240 nm assuming a molar extinction coefficient of 12,400 M/cm (Horn 1971). Transcription was arrested at the end of the incubation period by placing the dishes at -20°C for 5 min and then at 4°C while the organs were harvested. Organs were collected by low-speed centrifugation, and the pellets were frozen at -80°C for storage before RNA extraction. Specific organs were hand-dissected from wandering late third-instar larvae and incubated in 2-ml Dounce (Kontes) containing 400 μl of Robb's saline supplemented with ecdysone. After the incubations, organs were collected at the bottom of the Dounce by a brief centrifugation in an Eppendorf centrifuge and total RNA was extracted.

RNA isolation

Isolation of RNA from intact animals or small amounts of tissue was done as described previously (Thummel et al. 1990). RNA isolation from mass-isolated organs was performed essentially as described by Sambrook et al. (1989), following a protocol provided by A. Andres. Frozen organ pellets were homogenized rapidly with 10 strokes of a B pestle in a 7-ml Dounce (Kontes) containing 1 ml of extraction buffer [0.2 M NaCl, 20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 2% SDS]. The homogenate was transferred to a 10-ml centrifuge tube and incubated with 25 mg of proteinase K (Boehringer Mannheim) for 1 hr at 50°C. Protein was extracted by the addition of 0.5 ml of phenol, vortexing for 10 min, addition of 0.5 ml of chloroform, vortexing for 10 min, and centrifugation. The aqueous phase was re-extracted with 1 ml of chloroform by vortexing for 10 min, after which the RNA was collected by ethanol precipitation. The RNA pellet was resuspended in 100 μ l of water and stored at -80°C . For S1 analysis, contaminating DNA was removed by digestion with RNase-free DNase (Promega), followed by phenol extraction and ethanol precipitation.

Northern blotting and hybridization

RNA (16–25 μ g of total RNA) was fractionated by formaldehyde-agarose gel electrophoresis as described (Thummel et al. 1990). Efficient transfer of high-molecular-weight RNA was achieved by soaking the gel for 40 min in 50 mM NaOH and 100 mM NaCl, rinsing in water, and neutralizing in 100 mM Tris-HCl (pH 7.8) for 30 min. RNA was transferred to uncharged nylon membranes [GeneScreen (Dupont-NEN) or DuralonUV (Stratagene)] by blotting in $20\times$ SSC overnight, and cross-linked to the nylon by ultraviolet irradiation (Stratagene Stratalinker in auto-cross-link mode). The membranes were prehybridized and hybridized according to a Stratagene protocol, at 42°C in 50% formamide, 20 mM PIPES (pH 6.5), 800 mM NaCl, 1% SDS, and 150 μ g/ml of denatured herring sperm DNA (Sigma). Single-stranded antisense DNA probes for *E74* transcripts, radioactively labeled by synthesis from m13 inserts (Burtis et al. 1990), were added to the hybridization mix at a concentration of 5×10^5 cpm/ml. All other probes were radioactively labeled by nick translation (Sambrook et al. 1989). After hybridization, the membranes were washed in $0.7\times$ SSC, 0.1% SDS at 55°C , and exposed on film (Kodak X-Omat). Films exposed with an intensifier screen were preflashed. For the dose-response analysis of *E74* transcription, the levels of *E74A* and *E74B* mRNA were determined with a Molecular Dynamics 300S computing densitometer, using their volume-integration software. Blots were stripped for reprobing by boiling for 20–30 min in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 1% SDS. Some blots have been stripped more than five times with no detectable loss of RNA from the membrane.

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References

- Adhya, S. and M. Gottesman. 1982. Promoter occlusion: Transcription through a promoter may inhibit its activity. *Cell* **29**: 939–944.
- Ashburner, M. 1967. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. I. Autosomal puffing patterns in a laboratory stock of *Drosophila melanogaster*. *Chromosoma* **21**: 398–428.
- . 1972. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. VI. Induction by ecdysone in salivary glands of *D. melanogaster* cultured in vitro. *Chromosoma* **38**: 255–281.
- . 1973. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. I. Dependence upon ecdysone concentration. *Dev. Biol.* **35**: 47–61.
- Ashburner, M., C. Chihara, P. Meltzer, and G. Richards. 1974. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38**: 655–662.
- Belyaeva, E.S., I.E. Vlassova, Z.M. Biyasheva, V.T. Kakpakov, G. Richards, and I.F. Zhimulev. 1981. Cytogenetic analysis of the 2B3-4-2B11 region of the X chromosome of *Drosophila melanogaster*. II. Changes in 20-OH ecdysone puffing caused by genetic defects of puff 2B5. *Chromosoma* **84**: 207–219.
- Boyd, L., E. O'Toole, and C.S. Thummel. 1991. Patterns of *E74A* RNA and protein expression at the onset of metamorphosis in *Drosophila*. *Development* (in press).
- Burtis, K.C. 1985. "Isolation and characterization of an ecdysone-inducible gene from *Drosophila melanogaster*." Ph.D. thesis. Stanford University, Stanford, California.
- Burtis, K.C., C.S. Thummel, C.W. Jones, F.D. Karim, and D.S. Hogness. 1990. The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell* **61**: 85–99.
- Chao, A.T. and G.M. Guild. 1986. Molecular analysis of the ecdysterone-inducible 2B5 "early" puff in *Drosophila melanogaster*. *EMBO J.* **5**: 143–150.
- Crowley, T.E., P.H. Mathers, and E.M. Meyerowitz. 1984. A trans-acting regulatory product necessary for expression of the *Drosophila melanogaster* 68C glue gene cluster. *Cell* **39**: 149–156.
- Deak, P., P. Zavorszky, and P. Maroy. 1988. Moulting hormone regulates its receptor level in *Drosophila melanogaster*. *Insect Biochem.* **18**: 847–852.
- DeReggi, M.L., M.H. Hirn, and M.A. DeLaage. 1975. Radioimmunoassay of ecdysone. An application to *Drosophila* larvae and pupae. *Biochem. Biophys. Res. Commun.* **66**: 1307–1315.
- Feigl, G., M. Gram, and O. Pongs. 1989. A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in *Drosophila melanogaster*. *Nucleic Acids Res.* **17**: 7167–7178.
- Fristrom, J.W., J. Doctor, D.K. Fristrom, W.R. Logan, and D.J. Silvert. 1982. The formation of the pupal cuticle by *Drosophila* imaginal discs in vitro. *Dev. Biol.* **91**: 337–350.
- Galcerán, J., J. Llanos, J. Sampedro, O. Pongs, and M. Izquierdo. 1990. Transcription at the ecdysone-inducible locus 2B5 in *Drosophila*. *Nucleic Acids Res.* **18**: 539–545.
- Georgel, P., P. Ramain, A. Giangrande, G. Dretzen, G. Richards, and M. Bellard. 1991. *Sgs-3* chromatin structure and trans-activators: Developmental and ecdysone induction of a glue enhancer-binding factor, GEBF-1, in *Drosophila* larvae. *Mol. Cell Biol.* **11**: 523–532.
- Handler, A.M. 1982. Ecdysteroid titres during pupal and adult development in *Drosophila melanogaster*. *Dev. Biol.* **93**: 73–82.

- Hansson, L. and A. Lambertsson. 1983. The role of *su(f)* gene function and ecdysterone in transcription of glue polypeptide mRNAs in *Drosophila melanogaster*. *Mol. Gen. Genet.* **192**: 395–401.
- Hodgetts, R.B., B. Sage, and J.D. O'Connor. 1977. Ecdysone titers during postembryonic development of *Drosophila melanogaster*. *Dev. Biol.* **60**: 310–317.
- Horn, D.H.S. 1971. The ecdysones. In *Naturally occurring insecticides* (ed. M. Jacobson and D.G. Crosby), pp. 333–459. Marcel Dekker, New York.
- Janknecht, R., W. Taube, H.-J. Lüdecke, and O. Pongs. 1989. Characterization of a putative transcription factor gene expressed in the 20-OH-ecdysone inducible puff 74EF in *Drosophila melanogaster*. *Nucleic Acids Res.* **17**: 4455–4464.
- Jones, C.W., M.W. Dalton, and L.H. Townley. 1991. Interspecific comparisons of the structure and regulation of the *Drosophila* ecdysone-inducible gene *E74A*. *Genetics* **127**: 535–543.
- Karim, F.D., L.D. Urness, C.S. Thummel, M.J. Klemsz, S.R. McKercher, A. Celada, C. Van Beveren, R.A. Maki, C.V. Gunther, J.A. Nye, and B.J. Graves. 1990. The ETS-domain: A new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes & Dev.* **4**: 1451–1453.
- Maroni, G. and S.C. Stamey. 1983. Use of blue food to select synchronous, late third instar larvae. *Dros. Inf. Serv.* **59**: 142–143.
- O'Connell, P. and M. Rosbash. 1984. Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12**: 5495–5513.
- Richards, G. 1976a. The control of prepupal puffing patterns in vitro: Implications for prepupal ecdysone titre in *Drosophila melanogaster*. *Dev. Biol.* **48**: 191–195.
- . 1976b. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. IV. The mid prepupal period. *Dev. Biol.* **54**: 256–263.
- . 1980. Ecdysteroids and puffing in *Drosophila melanogaster*. In *Progress in ecdysone research* (ed. J.A. Hoffman), pp. 363–378. Elsevier Press, Amsterdam.
- . 1981a. Insect hormones in development. *Biol. Rev.* **56**: 501–549.
- . 1981b. The radioimmune assay of ecdysteroid titres in *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* **21**: 181–197.
- . 1982. Sequential gene activation by ecdysteroids in polytene chromosomes of *Drosophila melanogaster*. VII. Tissue specific puffing. *Wilhelm Roux's Arch. Dev. Biol.* **191**: 103–111.
- Robb, J.A. 1969. Maintenance of imaginal discs of *Drosophila melanogaster* in chemically defined media. *J. Cell Biol.* **41**: 876–885.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Segraves, W.A. and D.S. Hogness. 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.
- Thummel, C.S. 1990. Puffs and gene regulation—Molecular insights into the *Drosophila* ecdysone regulatory hierarchy. *BioEssays* **12**: 1–8.
- Thummel, C.S., K.C. Burtis, and D.S. Hogness. 1990. Spatial and temporal patterns of *E74* transcription during *Drosophila* development. *Cell* **61**: 101–111.
- Truman, J.W. and L.M. Riddiford. 1974. Physiology of insect rhythms. III. The temporal organisation of the endocrine events underlying pupariation of the tobacco hornworm. *J. Exp. Biol.* **60**: 371–382.
- Urness, L.D. and C.S. Thummel. 1990. Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the *Drosophila* ecdysone-inducible *E74A* protein. *Cell* **63**: 47–61.
- Wolfner, M. and D.J. Kemp. 1988. A method for mass-isolating ecdysterone-inducible tissues of *D. melanogaster*. *Dros. Inf. Serv.* **67**: 104–106.

Ecdysone coordinates the timing and amounts of E74A and E74B transcription in *Drosophila*.

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