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Temporal coordination of regulatory gene expression by the steroid hormone ecdysone

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In *Drosophila*, pulses of the steroid hormone ecdysone function as temporal signals that trigger the major postembryonic developmental transitions. The best characterized of these pulses activates a series of puffs in the polytene chromosomes as it triggers metamorphosis. A small set of early puffs is induced as a primary response to the hormone. These puffs encode regulatory proteins that both repress their own expression and activate a large set of late secondary response genes. We have used Northern blot analysis of RNA isolated from staged animals and cultured organs to study the transcription of three primary response regulatory genes, *E75*, *BR-C* and *EcR*. Remarkably, their patterns of transcription in late larvae can be defined in terms of two responses to different ecdysone concentrations. The class I transcripts (*E74B* and *EcR*) are induced in mid-third instar larvae in response to the low, but increasing, titer of ecdysone. As the hormone concentration peaks in late third instar larvae, these transcripts are repressed and the class II RNAs (*E74A*, *E75A* and *E75B*) are induced. The *BR-C* RNAs appear to have both class I and class II characteristics. These data demonstrate that the relatively simple profile of a hormone pulse contains critical temporal information that is transduced into waves of primary response regulatory gene activity.

Key words: Drosophila/ecdysone/metamorphosis/steroid hormones/transcription

Introduction

Each phase in the *Drosophila* life cycle is characterized by a pulse of the steroid hormone ecdysone (20-hydroxyecdysone). These pulses act as temporal signals that trigger progressive developmental changes in each ecdysone target tissue, directing them to assume the morphological and functional properties that are appropriate for each stage in development. Programming this morphogenetic progression requires changes in ecdysone concentration, with peak titers establishing synchrony in the response of the various target tissues and very low hormone levels during the intervals between each ecdysone-triggered step in the developmental pathway.

Pulses of ecdysone occur during each of the three larval instars. The ecdysone pulses during the first and second larval instars trigger molting of the cuticle. This apparently tissue-specific response contrasts with the pleiotropic effects of ecdysone during the third larval instar, when one or more successive hormone pulses lead to a complex series of behavioral and developmental changes that culminate in puparium formation and the onset of terminal differentiation into the adult fly (Richards, 1981a,b). In initiating metamorphosis, ecdysone activates two divergent developmental programs—the larval tissues are histolyzed while the imaginal tissues proliferate and differentiate to form their predetermined adult structures. Puparium formation is followed by at least two more peaks in ecdysone titer (Handler, 1982). One, at ~10 h of prepupal development, triggers head eversion and signals the transition from prepupa to pupa (Sliter and Gilbert, 1992). A final, broad high titer pulse of ecdysone accompanies the last stages of terminal differentiation during pupal development (Richards, 1981a,b).

The remarkable effects of ecdysone during metamorphosis are reflected by dramatic changes in the puffing patterns of the salivary gland polytene chromosomes (Becker, 1959; Clever and Karlson, 1960; Ashburner *et al.*, 1974). Many puffs form and regress in successive waves of activity, triggered by the rising ecdysone concentration several hours before puparium formation. As demonstrated by Ashburner (1972), this precisely orchestrated genetic response to the hormone can be faithfully reproduced in explanted salivary glands. Within minutes following the addition of hormone to the culture medium, a few so-called early puffs are induced. These puffs (including 2B5, 74EF and 75B) appear to be coordinately regulated, peaking ~4 h after the addition of ecdysone and then rapidly regressing. As the early puffs reach their maximum size, the first of more than 100 so-called late puffs appear, each with a characteristic and reproducible profile of activity. In addition to their distinct temporal responses to ecdysone, the early and late puffs can be distinguished by two features of their regulation. First, the early puffs are induced normally in the presence of protein synthesis inhibitors, but fail to regress. In contrast, protein synthesis is required for late puff induction (Ashburner, 1974). Second, the early puffs are induced incrementally over a broad ~600-fold range of ecdysone concentrations while the late puffs are induced with a threshold response over an ~6-fold range of hormone concentrations (Ashburner, 1973).

These and other results were incorporated by Ashburner and his colleagues into a model for the genetic control of polytene chromosome puffing by ecdysone (Ashburner *et al.*, 1974). According to this model, the ecdysone–receptor protein complex has two functions—first, to directly activate the early genes and, second, to repress late gene activity. The proteins encoded by the early genes also exert two regulatory functions. They activate the late genes while they repress their own expression, self-attenuating the regulatory response. The interplay between the early activator proteins and the repressive effects of the ecdysone–

receptor protein complex could contribute to the precise timing of late gene induction. The late genes, in turn, are thought to play a more direct role in the initiation of metamorphosis, or could induce subsequent waves of genetic activity.

Three early puffs have been analyzed at the molecular level: the Broad-Complex (*BR-C*) within the 2B5 puff (DiBello *et al.*, 1991), the *E74* gene within the 74EF puff (Janknecht *et al.*, 1989; Burtis *et al.*, 1990) and the *E75* gene within the 75B puff (Feigl *et al.*, 1989; Segraves and Hogness, 1990). These early genes are remarkably similar in structure. They are all unusually long, extending up to 100 kb in length, they all contain multiple nested ecdysone-inducible promoters and they all encode site-specific DNA binding proteins, consistent with their proposed regulatory function (Figure 1). Furthermore, mutations in these early genes lead to lethality during late larval, prepupal and pupal development, indicating that they play critical roles in metamorphosis. A variety of genetic and molecular studies of these early genes have provided strong support for the Ashburner model (Ashburner, 1990; Thummel, 1990).

E74 consists of two overlapping transcription units, designated *E74A* and *E74B*, that have unique start sites but share a common 3′ end (Burtis *et al.*, 1990). The nested arrangement of these two transcription units leads to the synthesis of two related proteins that share an identical ETS DNA-binding domain. Immunostaining revealed ~70 E74A protein binding sites in the polytene chromosomes, almost all of which correspond to early and late ecdysone-inducible puffs (Urness and Thummel, 1990). Genetic studies suggest that *E74A* function is required for the proper timing of late gene induction (J.Fletcher, K.Burtis and C.S.Thummel, unpublished results).

The structure of *E75* is similar to that of *E74* (Figure 1). *E75* spans >50 kb and consists of three nested ecdysone-inducible promoters that direct the synthesis of three related mRNAs, designated *E75C*, *E75A* and *E75B* (Segraves and Hogness, 1990). Like *E74*, these mRNAs have unique 5′ exons joined to a common set of 3′ exons, leading to the synthesis of three related proteins that share their C-terminal sequences. These proteins are all members of the steroid receptor superfamily, although the E75B protein lacks one of the two zinc fingers required for DNA binding. A ligand for the E75 proteins has not yet been identified (Segraves and Hogness, 1990; Segraves, 1991).

The *BR-C* is the most complex of the early genes analyzed to date. It spans 100 kb and directs the synthesis of more than a dozen distinct mRNAs by a combination of differential splicing and the use of multiple promoters (DiBello *et al.*, 1991). The *BR-C* transcripts can be divided into three classes, each defined by a unique exon that encodes a pair of C_2H_2 zinc fingers. The *BR-C* mRNAs also appear to have one exon in common, designated the 'core' exon.

The *BR-C* appears to be a general regulator of the ecdysone response that is required throughout the early stages of metamorphosis. In mid-third instar larvae, *BR-C* activity is required for glue gene induction, one of the first developmental changes in preparation for puparium formation (Crowley *et al.*, 1984; Hansson and Lambertsson, 1989; Georgel *et al.*, 1991; Guay and Guild, 1991). *BR-C*

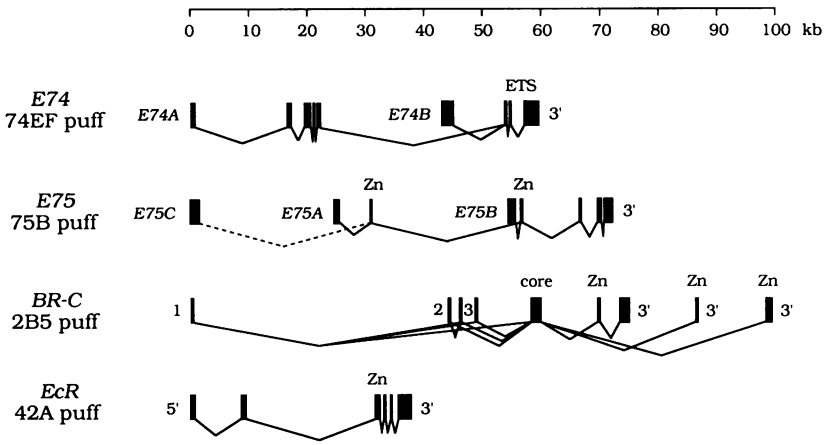

Fig. 1. Early gene structures. The structures of the *E74*, *E75*, *BR-C* and *EcR* genes are depicted. The boxes represent exons and the lines indicate how these exons are joined in the mature mRNAs. *E74* consists of two nested promoters, designated *E74A* and *E74B*, which direct the synthesis of 60 kb and 20 kb primary transcripts, respectively. These RNAs are spliced to form a 6 kb *E74A* mRNA and 4.8 and 5.1 kb *E74B* mRNAs. The *E74B* RNAs derive from two start sites, 300 bp apart, that appear to be coordinately regulated (Burtis *et al.*, 1990). The E74A and E74B proteins share a common ETS-domain sequence that binds a purine-rich core DNA sequence (Urness and Thummel, 1990). *E75* consists of three nested promoters, designated *E75C*, *E75A* and *E75B*, which direct the synthesis of >50 kb, 50 kb and 20 kb primary transcripts, respectively (Segraves and Hogness, 1990; W.A.Segraves and R.Evans, manuscript in preparation). These RNAs are spliced to form the 7.7 and 8.5 kb *E75C* mRNAs, 4.9 and 5.7 kb *E75A* mRNAs, and 5.2 and 6.0 kb *E75B* mRNAs. These RNAs differ in size by 0.8 kb, due to the use of alternative poly(A) addition signals. The E75 proteins are members of the steroid receptor superfamily and, as such, contain two zinc fingers and a conserved ligand binding domain; E75B is an exception since its promoter is located downstream of the coding region for the first zinc finger (Segraves and Hogness, 1990). The *BR-C* gene structure is highly complex. It consists of at least three promoters that direct the synthesis of ~100 kb and ~50 kb primary transcripts. These transcripts are differentially spliced to result in over a dozen mRNAs. All *BR-C* cDNA clones examined to date contain a common core exon. Each *BR-C* mRNA also contains one of three different exons that encode a pair of Cys_2His_2 zinc fingers, suggesting that there are at least three different functional BR-C protein products (DiBello *et al.*, 1991). The *EcR* ecdysone receptor gene encodes at least three mRNA isoforms from two promoters (W.Talbot, E.Swyryd and D.S.Hogness, personal communication). The *EcR* RNA published by Koelle *et al.* (1991) is depicted. This primary transcript spans 36 kb and is spliced to form a 6 kb mRNA. The EcR proteins are members of the steroid receptor superfamily, with two zinc fingers and a conserved ligand binding domain.

activity is also required for a normal puffing response at the end of larval development. The early puffs are significantly smaller in *BR-C* mutant animals and the late puffs are not induced (Belyaeva *et al.*, 1981). Molecular studies have confirmed these observations by demonstrating that a cluster of six secondary response genes derived from the 71CE late puff are not transcribed in *BR-C* mutant animals (Guay and Guild, 1991). Finally, *BR-C* function is required during prepupal development for normal imaginal disc morphogenesis (Kiss *et al.*, 1988), complete metamorphosis of the salivary gland, gut and dorsal flight muscles (Restifo and White, 1992) and proper remodelling of the central nervous system (Restifo and White, 1991).

The recent isolation of another key component in the regulatory hierarchy, the *EcR* ecdysone receptor gene, promises to provide significant insights into the mechanisms by which the ecdysone signal is transduced. The EcR protein is a member of the steroid receptor superfamily and displays all the biochemical and regulatory properties predicted for an ecdysone receptor (Koelle *et al.*, 1991). EcR binds both ecdysone and ecdysone response elements in DNA, and can mediate ecdysone-dependent transcriptional activation of a reporter gene in tissue culture co-transfection assays. The peaks of *EcR* mRNA and protein accumulation during development closely correspond to the known pulses of ecdysone, suggesting that *EcR* may itself be regulated by the hormone. EcR protein is also expressed in all ecdysone target tissues examined, providing the capability for mediating the diverse tissue-specific effects of ecdysone during metamorphosis.

Critical to our understanding of how the early genes transduce the hormonal signal is the definition of their regulation by ecdysone. Our previous studies have shown that both *E74* promoters are directly activated by ecdysone in cultured larval organs. In spite of their simultaneous induction, however, the *E74* mRNAs appear at different times in the cytoplasm, with delay times that are consistent with the lengths of their transcription units (20 kb for *E74B* and 60 kb for *E74A*) and a measured primary transcript elongation rate of ~1.1 kb/min (Thummel *et al.*, 1990). This early appearance of the *E74B* mRNA is enhanced by many hours, *in vivo*, due to its induction by a low ecdysone concentration relative to that required for *E74A* activation. The structure and regulation of the *E74* gene thus lead to an invariant order in the appearance of its two gene products, in which *E74B* always appears before *E74A* in response to a pulse of ecdysone (Karim and Thummel, 1991).

In this paper, we extend our characterization of the ecdysone regulation of early gene activity by determining the temporal patterns of *E75*, *BR-C* and *EcR* transcription, both *in vivo* and in cultured larval organs, as well as their ecdysone dose-response profiles. These genes are all rapidly induced by ecdysone in cultured larval organs, consistent with a primary response to the hormone. Furthermore, each of the three *E75* mRNAs appears with a delay time that is consistent with the length of its transcription unit and a primary transcript elongation rate of ~1.5 kb/min.

Our studies reveal that each transcript, with the exception of *E75C*, can be defined in terms of two distinct regulatory responses to ecdysone. The class I transcripts, including *E74B* and *EcR*, are induced by a low ecdysone concentration ($\sim 2\times10^{-9}$ M) and are repressed at higher ecdysone concentrations. The class II transcripts, including *E74A*, *E75A* and *E75B*, are induced by a higher ecdysone concentration ($\sim 1\times10^{-8}$ M) and their temporal profiles are unaffected by higher ecdysone concentrations. The *BR-C* RNAs appear to have both class I and class II characteristics, suggesting that this complex gene could contain distinct class I and class II promoters. As a result of these modes of regulation, the increasing ecdysone titer during third instar larval development is transduced into an initial burst of class I transcription followed by the activation of the class II promoters. This study thus identifies the class I early RNAs as the initial regulatory response to ecdysone *in vivo*. These results also indicate how the relatively simple profile of a hormone pulse can be transduced into a complex

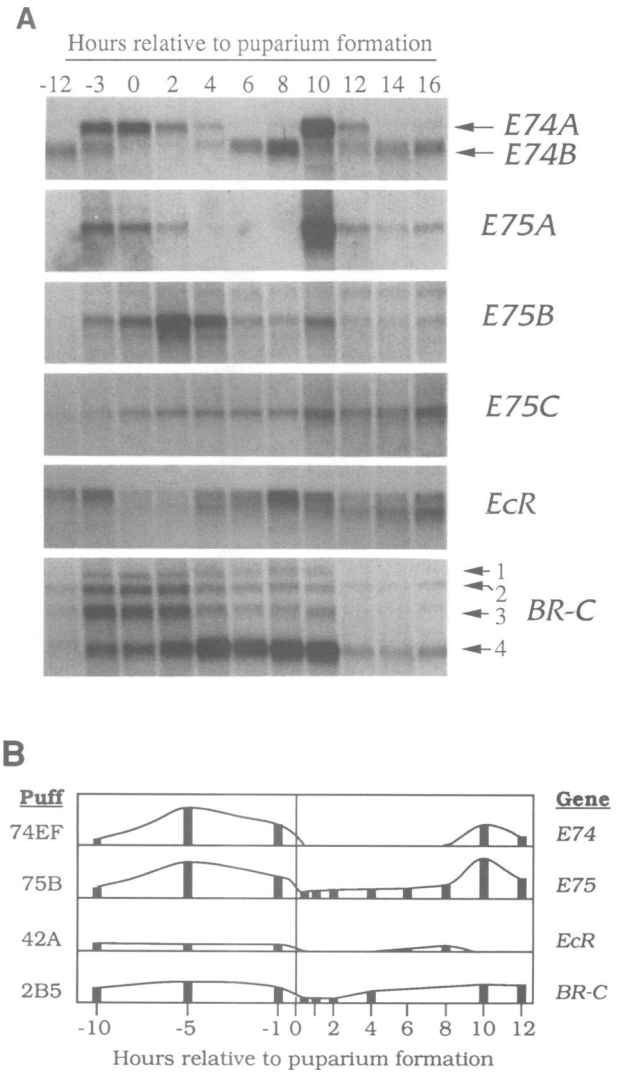

Fig. 2. Temporal profiles of *E74*, *E75*, *EcR* and *BR-C* transcription and puffing during late larval and prepupal development. (**A**) Total RNA was isolated from wandering (>12 h before puparium formation) and stationary (−3 h) third instar larvae as well as from prepupae at 2 h intervals following puparium formation (see Materials and methods). RNA (~20 μg/lane) was fractionated by formaldehyde agarose gel electrophoresis, transferred to uncharged nylon and hybridized with single-stranded radioactive DNA probes, as described in Materials and methods. A nick-translated *rp49* probe (O'Connell and Rosbash, 1984) was included in the hybridization as an internal control for loading (data not shown). (**B**) Time course of 74EF, 75B, 42A and 2B5 puffing during late larval and prepupal development. These data were adapted from Ashburner (1967, 1969).

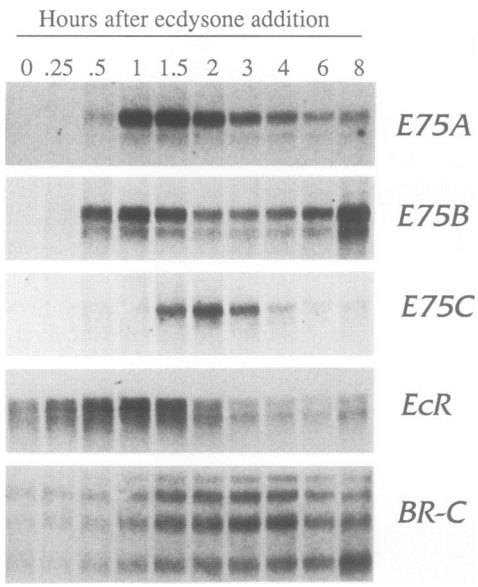

Fig. 3. Time course of *E75*, *EcR* and *BR-C* transcription in cultured larval organs treated with ecdysone. Mass-isolated late third instar larval organs were maintained in culture and treated with 5×10^{-6} M ecdysone for the periods of time shown. Total RNA was extracted and analyzed by Northern blot hybridization (~16 μg total RNA/lane) as described in Figure 2A. Upon longer exposure, a low level of *E75C* RNA can be detected in the early time points (data not shown).

primary regulatory response which, in turn, can function in a combinatorial manner to coordinate the activity of large sets of secondary response genes.

Results

E75, EcR and BR-C have distinct temporal patterns of transcription in vivo

During late larval and prepupal development there are two well characterized pulses of ecdysone, a premetamorphic pulse just prior to puparium formation and a second pulse in 10 h prepupae that triggers head eversion and the prepupal–pupal transition (Richards, 1981b; Handler, 1982; Sliter and Gilbert, 1992). We analyzed *E75*, *EcR* and *BR-C* transcription during these stages in development as a first step towards characterizing their temporal regulation by ecdysone.

RNA was extracted from whole animals at two late larval stages and prepupae were synchronized at 2 h intervals (±15 min). The larval time points (>12 and 3 h before pupariation) are relatively inaccurate due to the asynchrony of *Drosophila* larval development (see Materials and methods). Equivalent amounts of total RNA were analyzed by Northern blot hybridization (Figure 2A). To provide internally consistent results in this and all subsequent Northern blot analyses, a single blot was stripped and hybridized multiple times, using probes directed against *E75A*, *E75B*, *E75C*, *EcR* and *BR-C*. The transcription profiles of *E74A* and *E74B* during this period have been determined previously (Karim and Thummel, 1991) and are included for the purpose of comparison.

The profile of *E75A* transcription in late larvae and prepupae is virtually indistinguishable from that of *E74A*. There are two bursts of *E75A* transcription accompanying the two peaks in ecdysone titer (Figure 2A). The only feature of *E75A* expression that distinguishes it from *E74A* is a slight reinduction in 16 h pupae, as the ecdysone titer begins to rise again (Handler, 1982).

E75B transcription also has two peaks during this period, consistent with earlier observations (Segraves, 1988). *E75B* is induced in −3 h larvae, coincident with *E74A* and *E75A* induction. *E75B* transcripts accumulate during puparium formation and peak at 2 h of prepupal development, several hours later than *E74A* and *E75A*. The second peak of *E75B* activity occurs in 10 h prepupae, paralleling the response of the *E74A* and *E75A* mRNAs (Figure 2A).

The pattern of *E75C* transcription is quite distinct from that of *E75A*, *E75B*, or indeed any other early RNA. *E75C* is detected at low levels in early wandering third instar larvae, >12 h before puparium formation. The level of *E75C* mRNA then rises gradually through prepupal development, appearing to be only minimally affected by the premetamorphic hormone pulse, and peaks in 10 h prepupae, in apparent response to the prepupal pulse of ecdysone. *E75C* mRNA levels drop slightly in 12 h pupae and then increase in 16 h pupae, along with the other *E75* transcripts (Figure 2A).

The probe used to detect *EcR* RNA was derived from sequences that encode the DNA binding domain, allowing the identification of all functional *EcR* mRNA isoforms. Two distinct size classes of *EcR* transcripts can be detected in late larvae and four size classes in early pupae (Figure 2A). To date, three distinct *EcR* transcripts have been defined at the molecular level (W.Talbot, E.Swyryd and D.S.Hogness, personal communication), one of which is shown in Figure 1 (Koelle *et al.*, 1991). The different size classes of *EcR* mRNA appear to be regulated coordinately, with temporal profiles that are nearly identical to that of *E74B* mRNA. *EcR* transcripts can be readily detected in early wandering third instar larvae, >12 h before puparium formation. This observation is consistent with the induction of *EcR* transcription at 96–108 h of development, ~20 h before puparium formation (Koelle *et al.*, 1991), coincident with *E74B* activation (Thummel *et al.*, 1990). *EcR* appears to be induced by the premetamorphic pulse of ecdysone and is rapidly repressed at puparium formation. *EcR* RNA levels rise again gradually during mid-prepupal development, when the ecdysone titer is low but increasing, and peak in 8 h prepupae. The levels of *EcR* mRNA then decrease, between 10 and 12 h after puparium formation, and increase again in apparent response to the hormone titer, paralleling *E74B* and *E75C*. Thus both the late larval and prepupal bursts of *E74B* and *EcR* transcription precede those of *E74A*, *E75A* and *E75B*.

The probe used to detect the *BR-C* transcripts is directed against the common 'core' exon and thus should identify all *BR-C* mRNA isoforms (DiBello *et al.*, 1991). These transcripts resolve into four size classes which, for the purposes of this paper, will be referred to as *BR-C/1* (10 kb size class), *BR-C/2* (8.8 kb size class), *BR-C/3* (6.8 kb size class) and *BR-C/4* (4.5 kb size class; see Figure 2A). These four size classes have not yet been correlated with specific *BR-C* transcription units, although the evidence to date suggests that each RNA size class is represented by more than one mRNA isoform (DiBello *et al.*, 1991; C.Bayer and J.Fristrom, personal communication).

Like *EcR* and *E74B*, all four size classes of *BR-C* RNA can be detected in early wandering third instar larvae, >12 h

before puparium formation (Figure 2A). The RNA levels increase just prior to puparium formation, coincident with the peak concentrations of ecdysone, decline somewhat after 2 h of prepupal development, and then decline to very low levels at the prepupal−pupal transition. This broad peak of activity is similar to the combined responses of the *E74*, *E75* and *EcR* transcripts (Figure 2A). The *BR-C/4* transcript(s) have a unique temporal pattern in that they continue to accumulate in prepupae and form a broad peak between 4 and 10 h after pupariation. A further unique aspect of *BR-C* regulation is the limited effect of the prepupal ecdysone pulse, which appears to repress all *BR-C* RNAs.

E75, EcR and BR-C transcription is rapidly induced by ecdysone in cultured larval organs

Having characterized the temporal profiles of early gene activity at the onset of metamorphosis, we next wanted to determine how much of this pattern could be explained by the increasing ecdysone titer at the end of larval development. Tissues were mass-isolated from late third instar larvae, maintained in culture and treated with a high concentration of ecdysone (5×10^{-6} M) for various periods of time. Total RNA was extracted from these tissues and analyzed by Northern blot hybridization using specific probes for each early gene (Figure 3). This experimental approach provided both a defined ecdysone concentration and a determination of the precise timing of early gene activity following their simultaneous induction by ecdysone.

We have shown previously that the initial appearance of *E74A* and *E74B* mRNA in response to ecdysone is determined by the lengths of their transcription units and the ~1.1 kb/min elongation rate of RNA polymerase II (Thummel *et al.*, 1990; Karim and Thummel, 1991). A similar delay in transcript accumulation is apparent with *E75*, where the *E75B*, *E75A* and *E75C* mRNAs appear sequentially, in an order that is determined by the *E75* gene structure (Figures 1 and 3). *E75B* mRNA appears first, between 15 and 30 min after ecdysone addition. *E75A* RNA appears 30 min after the addition of ecdysone and *E75C* RNA appears between 1 and 1.5 h (Figure 3).

Following their initial appearance, the *E75* transcripts accumulate rapidly and then decrease to lower levels 2−3 h later. *E75B* transcription is distinguished by its biphasic profile, with a second burst of activity 6−8 h after ecdysone addition. As shown by Segraves (1988), this biphasic pattern appears to result from a tissue-specific response to the hormone. Isolated salivary glands and imaginal discs express only a single predominant burst of ecdysone-induced *E75B* mRNA, peaking at 2 h in salivary glands and at 8 h in imaginal discs. *E75C* is expressed in a more discrete burst than either *E75A* or *E75B*. *E75C* mRNA levels peak 2 h after ecdysone addition and then rapidly decline to low levels by 4 h.

Both *EcR* and *BR-C* resemble *E74B* in that their transcripts can be detected at significant levels in untreated late third instar larval organs (Figure 3), consistent with the observation that these RNAs are present in wandering third instar larvae (Figure 2A). *EcR* and *BR-C* transcription is induced above these levels by the addition of ecdysone (Figure 3). *EcR* transcripts peak by 1 h, and then return to their basal level by 2−3 h. This temporal profile is virtually indistinguishable from that of *E74B* (Karim and Thummel, 1991). The *BR-C/1*, *BR-C/2* and *BR-C/3* transcripts display virtually identical temporal responses to ecdysone, with broad peaks of expression between 1.5 and 4 h after hormone addition, reflecting the broad pattern of *BR-C* transcription at puparium formation (Figure 2A). The *BR-C/4* RNA parallels this pattern until 8 h, when it appears to be further induced. This profile is similar to that described above for *E75B*. It is interesting to note that the timing of this apparent reinduction is consistent with the delayed accumulation of both *E75B* and *BR-C/4* RNA *in vivo*, which peak in early prepupae following the premetamorphic ecdysone pulse (Figure 2A). It is possible that there is a peak of *E75B* in late larvae that is obscured by the inaccuracy of the −3 h timepoint.

In summary, the rapid induction of *E75*, *EcR* and *BR-C* transcription indicates that these genes are directly induced by ecdysone. These observations are consistent with earlier studies which have shown that *E75* (Segraves and Hogness, 1990) and *BR-C* (Chao and Guild, 1986) transcription can be induced by ecdysone in the presence of cycloheximide. Furthermore, with the exception of *E75C*, the timing of early gene activity in cultured larval organs is similar to that seen *in vivo* in late larvae and early prepupae.

The early transcription units are activated at one of two threshold ecdysone concentrations

We have shown previously that the *E74B* promoter is activated by a lower ecdysone concentration than the *E74A* promoter, accounting for the appearance of *E74B* mRNA many hours before *E74A* in response to the late larval and prepupal ecdysone pulses (Karim and Thummel, 1991). Based on these observations, we would predict that the low levels of *E75C*, *EcR* and *BR-C* RNA detected in early wandering third instar larvae (>12 h, Figure 2A) are due to their activation by a relatively low ecdysone concentration, similar to the mechanism of *E74B* induction. As a test of this prediction, and as a first step towards determining the effects of different ecdysone concentrations on *E75*, *EcR* and *BR-C* transcription, we performed a dose-response study using cultured larval organs treated with various concentrations of ecdysone, from 10^{-11} M to 10^{-5} M. These organs were incubated for 1.5 h, to allow accumulation of *E75*, *EcR* and *BR-C* RNA to relatively high levels (Figure 3), after which total RNA was extracted and analyzed by Northern blot hybridization. The relative amounts of each RNA were quantified by volume integration densitometry and plotted as a function of the $\log_{10}$(ecdysone concentration) (Figure 4). The data for *E74* were derived from a previous study (Karim and Thummel, 1991).

E75, *EcR* and *BR-C* transcription show graded responses to ecdysone over a ~40- to 400-fold range of concentrations (Figure 4). *E75A* RNA cannot be detected at ecdysone concentrations below 5×10^{-9} M but begins to appear at $\sim7\times10^{-9}$ M, reaches 50% of its maximal activity at $\sim1\times10^{-7}$ M, and peaks above 7.5×10^{-7} M (Figure 4). *E75B* transcription is first induced by $\sim2\times10^{-8}$ M ecdysone, reaches 50% maximal activity at $\sim3\times10^{-7}$ M, and peaks above 1×10^{-6} M (Figure 4). *E75C* is induced above its basal level at the relatively low ecdysone concentration of $\sim2\times10^{-9}$ M, has a 50% maximal response at $\sim1\times10^{-7}$ M, and peaks above 7.5×10^{-7} M (Figure 4). Thus, taken together, the dose-responses of the three *E75* mRNA isoforms span a 500-fold range, from 2×10^{-9} M to 1×10^{-6} M ecdysone, almost identical to that

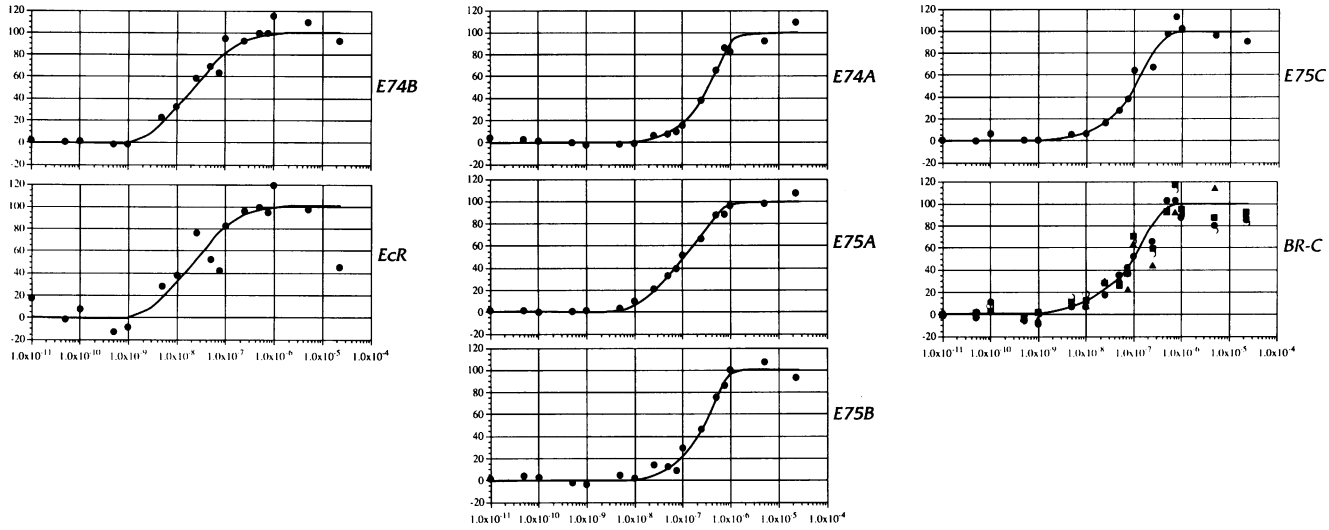

Fig. 4. Ecdysone dose-response analysis of *E75*, *EcR* and *BR-C* transcriptional induction. Mass-isolated late third instar larval organs were maintained in culture and treated with different ecdysone concentrations, as shown, for 1.5 h. Total RNA was extracted and analyzed (~10 μg/lane) by Northern blot hybridization as described in Figure 2A. The levels of RNA in each lane were approximately equal, as determined by hybridization with a radioactive probe for *rp49* mRNA (O'Connell and Rosbash, 1984). The same blot was used for all hybridizations, to provide internally consistent results. The data for *E74* are included for the purpose of comparison, and were derived from the autoradiogram depicted in Figure 5 of Karim and Thummel (1991). The amounts of RNA were quantified by a Molecular Dynamics computing densitometer using their volume integration software. These numbers were normalized to an arbitrary scale of 0–100, representing the lowest and highest levels of accumulated transcript, and plotted as a function of the $\log_{10}$(ecdysone concentration). The class I (*E74B* and *EcR*) and class II (*E74A*, *E75A* and *E75B*) transcripts are grouped together, as are the *E75C* and *BR-C* RNAs which did not fit into either of these classes. The *EcR* data are a summation of all detectable mRNA isoforms; the scatter of these data points is due to the relatively high basal level of *EcR* RNA in untreated larval organs (Figure 3). The four size classes of *BR-C* RNA are plotted on the same graph, since they have essentially identical dose-response curves: *BR-C/1* (●), *BR-C/2* (○), *BR-C/3* (■) and *BR-C/4* (▲).

described for induction of the 75B puff (Ashburner, 1973).

All four *BR-C* transcript size classes appear to respond identically to different ecdysone concentrations; they are initially induced at $\sim 2\times10^{-9}$ M ecdysone, reach 50% of their maximal levels by $\sim 1\times10^{-7}$ M, and peak above 5×10^{-7} M (Figure 4). The ecdysone dose-response profile of *EcR* transcription is virtually indistinguishable from that of *E74B*, with an initial response at $\sim 2\times10^{-9}$ M, a 50% maximal response at $\sim 2\times10^{-8}$ M, and a peak above 2×10^{-7} M (Figure 4).

The early transcripts thus fall into two distinct groups, based on the ecdysone concentration required for their initial induction. *E74B*, *EcR*, *BR-C* and *E75C* are activated at a threshold ecdysone concentration of $\sim 2\times10^{-9}$ M, while *E74A*, *E75A* and *E75B* are induced at a higher threshold ecdysone concentration of $\sim 1\times10^{-8}$ M. These observations are consistent with the temporal order of gene activation *in vivo* (Figure 2A). We reserve for the Discussion a more complete analysis of the ecdysone dose-response profiles and the proposal of a classification system for the regulation of early gene transcription by ecdysone.

Effects of ecdysone concentration on the temporal profiles of E75, BR-C and EcR transcription

The dose-response study described above examined the effects of ecdysone concentration on a single time point of early gene activity (1.5 h). To confirm and extend these results, we determined the temporal profiles of early gene transcription at six different ecdysone concentrations. Mass-isolated larval organs were cultured for various times with a range of ecdysone concentrations, from 5×10^{-9} M to 5×10^{-6} M. Total RNA was isolated and analyzed by Northern blot hybridization (Figure 5).

As noted earlier, the lengths of the early transcription units impose delays in the ecdysone-induced appearance of their encoded mRNAs. These delay times are built into the gene structure and thus not affected by changes in ecdysone concentration. This can be seen most easily with *E75*, where the order in the appearance of the three *E75* mRNA isoforms is unaffected by the ecdysone concentrations tested (Figure 5).

Like *E74A*, the temporal profile of *E75A* transcription is unaffected by changes in ecdysone concentration; only the level of *E75A* induction increases, consistent with its dose-response. The first peak of *E75B* transcription shows a similar pattern of activity; that is, its temporal profile is unaffected by increases in the ecdysone concentration, but its level of induction increases in a manner that is consistent with its dose-response. Interestingly, the second peak of *E75B* activity, at 6–8 h after ecdysone addition, shows a different dose-response. This peak is first detectable at 1.5×10^{-7} M ecdysone and increases dramatically at higher hormone concentrations (Figure 5). The *BR-C/4* RNA, which shows a similar increase 8 h after ecdysone addition (Figure 3), displays an identical dose-response and thus appears to be regulated coordinately with *E75B* (Figure 5).

E75C transcription differs from that of *E75A* and *E75B* in that it displays a slight shift in its temporal profile with increasing ecdysone concentrations (Figure 5). At low hormone concentrations *E75C* is expressed in a low broad profile, with a peak 3 h after ecdysone addition. At intermediate concentrations, this peak increases and shifts to 2–3 h after ecdysone addition. At high ecdysone

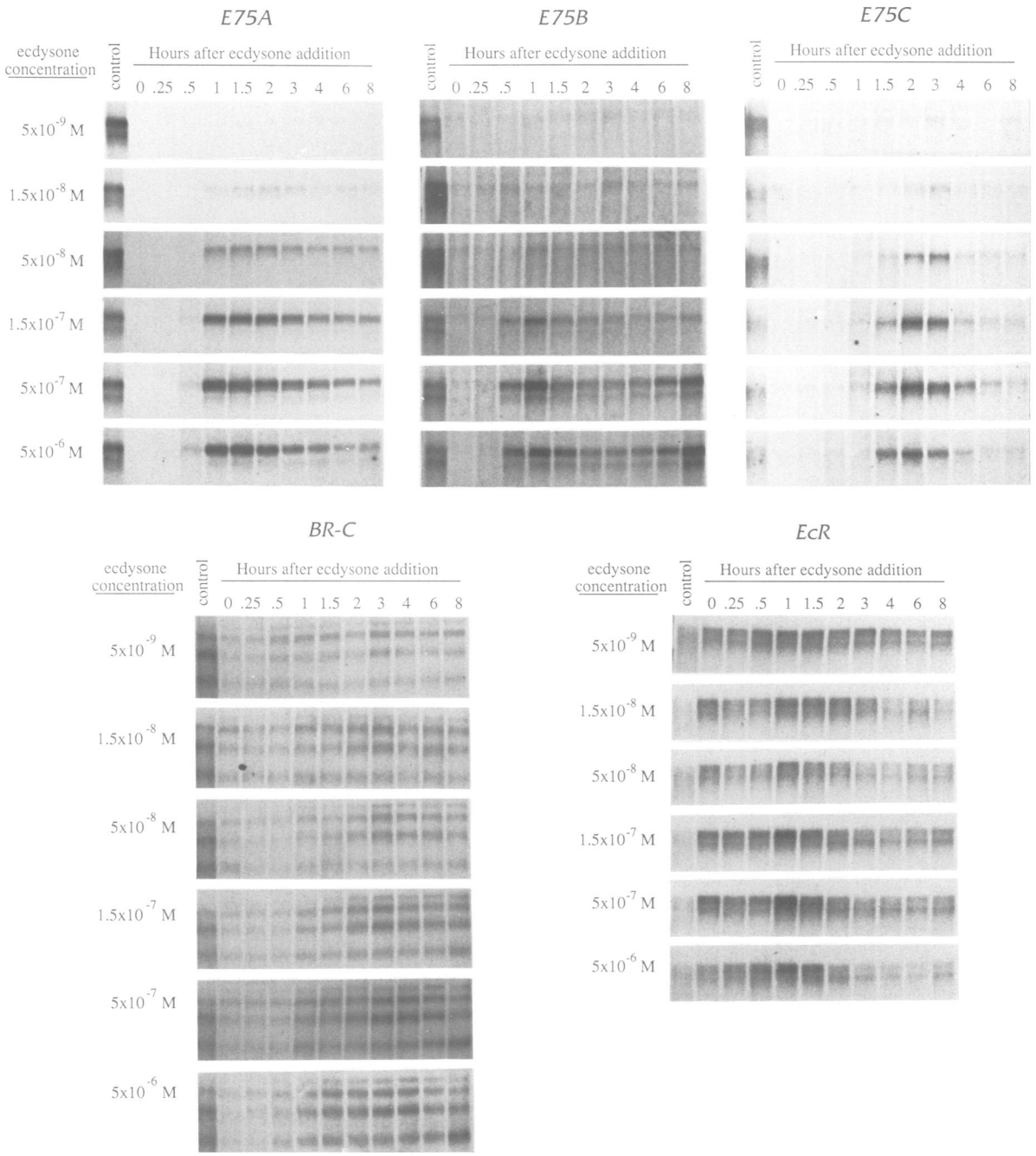

Fig. 5. Time course of *E75*, *EcR* and *BR-C* transcription in cultured larval organs treated with different concentrations of ecdysone. Mass-isolated third instar larval organs were maintained in culture and treated with six different ecdysone concentrations for the periods of time shown. Total RNA was extracted and analyzed (~16 μg/lane) by Northern blot hybridization as described in Figure 2A. RNA isolated from organs treated for 6 h with 5×10^{-6} M ecdysone was used as a positive control (control). The sources of these control RNAs, and hence their amounts, vary from one blot to another.

concentrations, the peak of *E75C* transcription is between 1.5 and 3 h. In this manner, *E75C* transcription resembles that of *E74B*, which displayed an ecdysone dose-dependent temporal shift in its peak of activity (Karim and Thummel, 1991).

Analysis of the temporal patterns of both *BR-C* and *EcR* transcription is complicated by their high basal levels of expression. They, nevertheless, display a subtle temporal shift in their profiles of activity, like *E75C* and *E74B*. *BR-C* transcription shows a slight induction at a low ecdysone concentration (5×10^{-9} M), consistent with its dose-response, and peaks at 3–8 h. This peak sharpens to between 2 and 6 h at intermediate ecdysone concentrations, and, finally, 1.5–4 h at the highest ecdysone concentration (5×10^{-6} M). Similarly, *EcR* transcription is induced by a low ecdysone concentration (5×10^{-9} M) and has a peak of expression between 0.5 and 3 h. At higher ecdysone concentrations, this peak sharpens to between 0.5 and 1.5 h. Thus, the early RNAs that are induced by a low ecdysone concentration (~2×10^{-9} M), *E74B*, *E75C*, *BR-C* and

EcR, display a temporal shift in their peaks of expression with increasing ecdysone concentrations, suggesting that at least some of these transcripts are repressed at higher concentrations of ecdysone in a dose-dependent manner.

Discussion

Molecular studies have defined four regulatory genes, *E74*, *E75*, *BR-C* and *EcR*, that are directly induced by the steroid hormone ecdysone at the onset of metamorphosis. These genes are remarkably similar in structure. They are all unusually long, contain multiple nested ecdysone-inducible promoters, and, consistent with their proposed regulatory function, encode DNA binding proteins. In this study, we have found that these genes also share common mechanisms of transcriptional control by ecdysone. At high ecdysone concentrations, the timing of early gene activity is largely determined by the lengths of their transcription units. The early gene structures thus provide an invariant parameter that establishes the minimal times for the appearance of each early mRNA. These timing constraints should not vary from one cell to another, or at different times during development. Rather, the flexibility in the biological response to ecdysone appears to be achieved by the effects of ecdysone concentration on the activity of each early promoter. Thus, *in vivo*, the rising ecdysone titer triggers the sequential appearance of each early RNA at its characteristic threshold concentration. The early promoters that are activated by low ecdysone concentrations are further induced by higher concentrations and then repressed coincident with the activation of a second set of early promoters. In this manner, the profile of the ecdysone pulse provides critical temporal information that is transduced into waves of DNA binding proteins which, in turn, can regulate the activity of large sets of secondary response genes. In the following sections we discuss these mechanisms of ecdysone regulation and how they might function together to coordinate early gene expression *in vivo*.

A proposed classification scheme for the regulation of early gene activity by ecdysone

Remarkably, the transcripts encoded by *E75* and *EcR*, with the exception of *E75C*, respond to ecdysone in late third instar larvae in a manner that is similar to the response of either *E74A* or *E74B*. This observation allows the definition of two distinct classes of early RNA: the class I transcripts, consisting of *E74B* and *EcR*, and the class II transcripts, consisting of *E74A*, *E75A* and *E75B*. As discussed below, the pattern of *BR-C* transcription can be explained by its containing distinct class I and class II promoters, and *E75C* does not fit into either of these two classes.

The class I transcripts are activated by a low threshold ecdysone concentration of $\sim 2\times10^{-9}$ M. This sensitivity to ecdysone is consistent with the presence of these transcripts in early crawling third instar larvae, when the ecdysone concentration appears to be low but increasing (Hodgetts *et al.*, 1977; Richards, 1981b), and their induction with the leading edge of the premetamorphic ecdysone pulse (Figure 2A). The class I RNAs also appear to be more effectively repressed in an ecdysone dose-dependent manner, at approximately the same hormone concentration as that required for induction of the class II RNAs (Figure 5; Karim and Thummel, 1991). Thus, as the ecdysone titer increases, it signals a transition from class I to class II early gene expression. It is possible that this repression of the class I RNAs is due to one or more of the proteins encoded by the class II transcripts.

The class II transcripts are induced by an ecdysone concentration that is ~10-fold higher than that required for induction of the class I RNAs and require a correspondingly higher ecdysone concentration to reach 50% of their maximal activity (Figure 4). As a result, the class II RNAs are induced later than the class I RNAs *in vivo*, with the peak titers of ecdysone (Figure 2A). The temporal profiles of the class II transcripts are unaffected by higher ecdysone concentrations in cultured organs (Figure 5).

Our intention in proposing this classification system is to emphasize the common features of early gene regulation. We do not, however, intend to imply identical modes of regulation for all RNAs contained within a particular class. For example, *E75A* is ~3-fold more sensitive to ecdysone than the other class II RNAs, *E74A* and *E75B* (Figure 4). We classify *E75A* as a class II transcript based on two properties of its regulation. First, it reaches 50% maximal activity at $\sim 1\times10^{-7}$ M ecdysone, only 3-fold lower than *E74A* and *E75B*, and five times higher than the class I RNAs (Figure 4). As a result, its induction is coincident with the peaks of ecdysone titer *in vivo* (Figure 2A). Secondly, its temporal profile of activity is unaffected by changes in ecdysone concentration in cultured organs (Figure 5).

The *BR-C* is unusual in that its transcripts appear to have both class I and class II characteristics. Like the class I RNAs, *BR-C* transcription is activated by a low threshold ecdysone concentration of $\sim 2\times10^{-9}$ M (Figure 4). This is consistent with its activity in early crawling third instar larvae (Chao and Guild, 1986; Figure 2A). The temporal profile of *BR-C* transcription is also slightly shifted in response to higher ecdysone concentrations (Figure 5).

Along with these class I characteristics, the *BR-C* RNAs reach 50% of their maximal activity at $\sim 1\times10^{-7}$ M ecdysone, similar to the class II RNAs. The *BR-C* thus has a much broader ecdysone dose-response profile than either the class I or class II transcripts—over an ~250-fold range of ecdysone concentrations, as opposed to the ~100-fold range for *E74B* and *EcR*, and the ~45-fold range for the class II RNAs *E74A* and *E75B* (Figure 4). This broad dose-response is reflected by the broad pattern of *BR-C* transcription in late larvae and early prepupae, similar to the combined responses of the other early RNAs (Figure 2A).

The simplest explanation for the complexity of *BR-C* regulation comes from the complexity of the *BR-C* gene itself. Each of the *BR-C* promoters can potentially direct the synthesis of a set of four RNAs of identical size (designated 1–4 in Figure 2A), all of which are detected by the probe used in our Northern blot hybridizations (DiBello *et al.*, 1991). Indeed, removal of the most distal *BR-C* promoter by a chromosomal rearrangement has no effect on the pattern of *BR-C* RNAs that is detectable on Northern blots (C.Bayer and J.Fristrom, personal communication). We postulate that the *BR-C* promoters, like those of *E74*, are under two forms of ecdysone regulation, such that one or more display a class I response to ecdysone and the other(s) display a class II response. Thus the cumulative *BR-C* transcription pattern detected in our Northern blot hybridizations could be due to a superimposition of distinct class I and class II *BR-C*

RNAs. This is, of course, only the simplest explanation of the data. A final definition of *BR-C* regulation by ecdysone must await the identification of promoter-specific probes.

E75C regulation is unique among the early RNAs that we have studied to date. Because the probe used to detect *E75C* RNA is specific for this mRNA isoform, the complexity of *E75C* regulation must be a reflection of the unusual properties of this early promoter. Dose-response analysis of *E75C* transcription revealed a response to ecdysone over a wide 400-fold range of concentrations, 4- to 10-fold greater than that of the other characterized early transcripts (Figure 4). *E75C* RNA can be detected in early crawling third instar larvae (>12 h, Figure 2A), in agreement with its activation by a low ecdysone concentration ($\sim 1 \times 10^{-9}$ M). Its levels, however, are minimally affected by the late larval pulse of ecdysone and only peak in apparent response to the prepupal ecdysone pulse (Figure 2A). This prepupal stage-specificity of *E75C* regulation is somewhat surprising given its ability to be induced by ecdysone in cultured late third instar larval organs (Figure 5). It remains possible that *E75C* is induced by ecdysone in late third instar larvae, but that the very short period of its activity (2–3 h, Figure 3) combined with the inaccuracy of our late larval timepoints (see Materials and methods) obscure this response on our developmental Northern blot (Figure 2A). These complexities of E75C regulation are not consistent with the properties of either the class I or class II RNAs.

Finally, we wish to emphasize that our classification scheme is based on data obtained from intact animals and mass-isolated larval organs, and is not intended to indicate that all tissues respond uniformly to ecdysone treatment. Indeed, a variety of evidence has demonstrated that the early RNAs have unique tissue-specific temporal responses to ecdysone (Richards, 1982; Segraves, 1988; Karim and Thummel, 1991).

The lengths of the early transcription units can function as timers to delay early gene expression in response to high ecdysone concentrations

We have shown previously that, although the *E74A* and *E74B* promoters are activated simultaneously by the addition of a high concentration of ecdysone to cultured larval organs, their mRNAs appear with characteristic delay times of 60 and 20 min, respectively (Karim and Thummel, 1991). These delay times are consistent with the rate of *E74* transcript elongation ($\sim$1.1 kb/min; Thummel *et al.*, 1990) and the lengths of the *E74A* and *E74B* transcription units (60 and 20 kb, respectively). Furthermore, the unusual length of the *E74* gene is conserved in *D.pseudoobscura* and *D.virilis*, suggesting that the delays dictated by this structure may be of functional significance (Jones *et al.*, 1991). Our characterization of *E75* transcription at high hormone concentrations (Figure 3) indicates that similar temporal delays are associated with the expression of other early genes. The three *E75* mRNA isoforms appear with delay times that are determined by the *E75* gene structure—*E75B* RNA can first be detected by 15–30 min, *E75A* by 30 min and *E75C* by 1–1.5 h. These delay times, and the lengths of the *E75* transcription units, suggest that RNA polymerase is moving at a rate of $\sim$1.5 kb/min, close to the 1.3–1.4 kb/min elongation rate estimated for *Ubx* transcription in early embryos (Irvine *et al.*, 1991; Shermoen and O'Farrell, 1991). These results suggest that *E74* transcription may be slow, relative to that of other *Drosophila* genes. Furthermore, transcript elongation rate may contribute to the delay timing determined by primary transcript length.

The profile of an ecdysone pulse coordinates the timing and amounts of early gene transcription

The class I RNAs, *E74B* and *EcR*, along with at least some of the *BR-C* transcripts, are present in early wandering third instar larvae, >12 h before pupariation. These RNAs are further induced with the leading edge of the premetamorphic pulse of ecdysone. As the hormone titer peaks, just prior to pupariation, these transcripts are down-regulated and the class II RNAs, including *E74A*, *E75A* and a possible class II component of the *BR-C*, are induced. *E75B* peaks in 2 h prepupae, perhaps reflecting its second phase of activity in cultured organs (Figure 3) and *E75C* peaks in late prepupae (Figure 2A). In large part, this temporal progression of early regulatory gene activity can be explained by the sensitivities of the early promoters to ecdysone concentration (Figure 4). Thus there is critical temporal information contained within the profile of an ecdysone pulse. The increasing ecdysone titer determines the timing and order of early gene activity. Furthermore, the repression of the class I RNAs as the class II RNAs are induced leads to a switch in regulatory activity, from a mid-third instar pattern of early gene expression to a late larval pattern of expression.

These temporally distinct waves of early regulatory gene expression appear to be of functional consequence. *EcR* RNA can be detected at all stages, providing a low basal level of receptor protein (Koelle *et al.*, 1991). In response to the low levels of ecdysone at the leading edge of each ecdysone pulse, *EcR* RNA is rapidly induced. This early autocatalytic response to the hormone facilitates the accumulation of receptor protein for later regulatory responses to ecdysone, including the induction of the class II RNAs by the peak hormone concentrations. The early induction of the *BR-C* also appears to be of functional significance. In mid-third instar larvae, the *BR-C* is required for induction of the glue genes, as a secondary response to ecdysone (Crowley *et al.*, 1984; Hansson and Lambertsson, 1989; Georgel *et al.*, 1991; Guay and Guild, 1991). The *BR-C* is also required at puparium formation and during prepupal development for induction of late gene expression (Guay and Guild, 1991) and appropriate morphogenesis (Kiss *et al.*, 1988; Restifo and White, 1991, 1992). In a similar manner, the brief burst of *E74A* activity at puparium formation appears to be critical for timing late gene induction (J.Fletcher, K.Burtis and C.S.Thummel, unpublished results).

Although a gradual increase in ecdysone titer during third instar larval development appears to play a key role in timing early gene activity, there may be times during development when a very rapid rise in the ecdysone titer could trigger the essentially simultaneous activation of the early promoters. Under these conditions, the length of each early transcription unit would be the predominate temporal regulator of early gene activity. It seems likely that these two mechanisms do not function independently of one another, but rather work together to control the timing of early gene activity in the animal. The rate of increase in ecdysone concentration would be the key factor that would determine the relative contributions of each of these mechanisms to the overall temporal coordination of early gene expression.

Finally, the presence of the class I early proteins in late third instar larvae has implications for the cycloheximide inhibition studies used by Ashburner and others to distinguish between primary and secondary ecdysone-response genes (Ashburner, 1974). The addition of cycloheximide to cultured late third instar larval organs locks in a combination of pre-existing regulatory proteins that is sufficient to facilitate a complete primary response to the hormone. Although the EcR ecdysone receptor is obviously a critical member of this protein population, other regulators, such as the *BR-C*, are also required (Belyaeva *et al.*, 1981). Further definition of these class I regulators should provide a clearer understanding of the molecular components that are required to make late larval tissues competent to respond fully to the hormone at the onset of metamorphosis.

Puffing reflects the activity of some, but not all, early transcription units

Although the early puffs respond to ecdysone as a single temporal class, the transcription units that they represent show distinct patterns of activity which could not be predicted based on their puffing response. The puffing patterns of 74EF (*E74*), 75B (*E75*), 42A (*EcR*) and 2B5 (*BR-C*) are depicted in Figure 2B (Ashburner, 1967, 1969). These puffing patterns closely parallel the accumulation of some, but not all, of the early transcripts (Figure 2B).

The puffing profiles of 74EF and 75B are very similar, with the exception of the early prepupal time points, when only 75B is active (Figure 2B). This pattern of 75B puffing resembles the cumulative transcription of *E75A* and *E75C*, the two longer *E75* transcription units. The shortest *E75* unit, *E75B*, peaks in 2 h prepupae and is not represented by a corresponding change in the diameter of the 75B puff. The dose-response profile of the 75B puff (Ashburner, 1973) is also consistent with the combined dose-responses of the *E75A* and *E75C* mRNA isoforms (Figure 4). The 2B5 puff in −10 h larvae (Figure 2B) is a reflection of the low levels of *BR-C* RNA in early crawling third instar larvae (Figure 2A). In contrast to the large size of the 2B5 puff at this stage, however, the 74EF puff is very small, leading to the incorrect impression that this locus is inactive prior to the premetamorphic pulse of ecdysone. Indeed, *E74B* is actively expressed in early crawling third instar larvae, but, perhaps due to its small size, has only a minimal effect on the size of the 74EF puff.

The *EcR* gene maps to an ecdysone-regulated puff at 42A (Koelle *et al.*, 1991). The temporal profile of the 42A puff during late larval and prepupal development (Ashburner, 1967, Figure 2B) closely parallels the pattern of *EcR* transcription at these stages in development (Figure 2A). The expression of *EcR* protein in the larval salivary gland (Koelle *et al.*, 1991) further supports the possibility that the 42A puff represents the ecdysone-induction of *EcR* transcription. Although Ashburner did not detect an increase in 42A puff diameter in response to the premetamorphic pulse of ecdysone (Ashburner, 1967), this effect may have been obscured by the relatively small size of this puff.

In conclusion, although the pioneering puffing studies of Becker (1959), Clever and Karlson (1960) and Ashburner (1974) provided key insights into the mechanisms of ecdysone action during the onset of metamorphosis, the puffing patterns are only a partial reflection of gene activity at those loci. Clearly, the advantage provided by having molecular probes for ecdysone-inducible genes is that their activity can be assayed at any time or place, independent of the puffs that provided the initial clues to their existence. The results presented here indicate that the class I transcripts represent the first regulatory response to the hormone in preparation for metamorphosis. Further definition of their activity during third instar larval development should provide insights into their potential role in a mid-third instar ecdysone regulatory hierarchy (Andres and Thummel, 1992), as well as what roles they might play in facilitating the activation of the class II RNAs at the end of larval development. In addition, phenotypic studies of early gene mutations on both early and late gene activity should reveal the regulatory significance of this precisely orchestrated temporal progression of early gene expression.

Materials and methods

Developmental staging

Late third instar larvae were staged by growth on food containing 0.05% bromophenol blue (Sigma). As the larvae begin wandering, the blue dye gradually clears from their intestine indicating the time since the cessation of feeding (Maroni and Stamey, 1983). Actively crawling larvae with blue intestines were >12 h from pupariation (the −12 h timepoint in Figure 2A; L.Boyd, unpublished results). Larvae that have completely cleared their alimentary tracts pupariate within 3 ± 1.8 h (the −3 h timepoint in Figure 2A; L.Boyd, unpublished results). Prepupae can be staged more accurately, within an error of ~15 min, by synchronizing at the white prepupal stage. These prepupae were collected and allowed to develop at 25°C for the appropriate times after which they were frozen at −80°C.

RNA isolation and Northern blot hybridization

Mass-isolation of larval organs, RNA extraction, and Northern blot hybridization were performed as described (Karim and Thummel, 1991). The developmental Northern blot depicted in Figure 2A was prepared for this study. All other blots were the same as those used by Karim and Thummel (1991) to analyze *E74* transcription. By using the same set of blots we obtained internally consistent results allowing us to compare directly the responses of different early genes to ecdysone. Each blot was probed with radioactive DNA derived from *E75*, *BR-C* or *EcR*, prepared as described below. Upon achieving a series of autoradiographic exposures, the blots were stripped for re-probing by boiling for 20−30 min in 10 mM Tris−HCl (pH 7.8), 1 mM EDTA, 1% SDS. For the dose-response study shown in Figure 4, the levels of early transcript accumulation were determined with a Molecular Dynamics 300S computing densitometer, using their volume integration software. We have recalculated our results for the dose-response of *E74* transcription and obtained slightly different numbers from those reported in Karim and Thummel (1991), as depicted in Figure 4. *E74B* was reported to be activated at $\sim 1\times10^{-9}$ M ecdysone; we now detect this response at $\sim 2\times10^{-9}$ M ecdysone. *E74B* was reported to reach 50% maximal activity at $\sim 8\times10^{-9}$ M ecdysone; we now detect this response at $\sim 2\times10^{-8}$ M ecdysone. *E74B* was reported to achieve a maximal response at $\sim 5\times10^{-8}$ M ecdysone; we now measure a maximal response above $\sim 2\times10^{-7}$ M ecdysone. Our recalculated values for the *E74A* ecdysone dose-response are virtually identical to those reported previously. *E74A* was reported to be activated at $\sim 5\times10^{-8}$ M ecdysone; we now detect this response at $\sim 2\times10^{-8}$ M ecdysone. *E74A* was reported to reach 50% maximal activity at $\sim 2\times10^{-7}$ M ecdysone; we now detect this response at $\sim 3\times10^{-7}$ M ecdysone. Our measurement of a maximal response for *E74A* transcription above $\sim 7.5\times10^{-7}$ M ecdysone is identical to our recalculated value.

Radioactive probes for detecting early RNAs

Single-stranded radioactive DNA probes were synthesized by PCR, essentially as recommended by the manufacturer (Perkin-Elmer/Cetus). As a first step in making radioactive probes, double-stranded PCR products, ~300−1000 bp in length, were synthesized for each early gene using the appropriate primer pair (see below) and either genomic DNA or cDNA as a template. The double-stranded PCR products were gel purified on low melting point agarose and used as templates to generate single-stranded sense templates. Although it is possible to use these double-stranded templates directly for the synthesis of radioactive antisense probes, we have found

that creating a single-stranded sense template as an intermediate step results in probes with a higher signal-to-noise ratio.

Single-stranded sense templates were synthesized by asymmetric PCR in which the sense primers were used at a 100-fold excess relative to the antisense primers (1 μg: 10 ng). The PCR products were gel purified on low melting point agarose and used as templates for synthesizing radioactively labeled antisense probes.

The labeled antisense probes were synthesized by asymmetric PCR using a 100-fold excess of the antisense primers (1 μg: 10 ng). The labeling reaction for each probe was done in a final volume of 40 μl. The reaction mix consisted of ~100–250 ng single-stranded sense template, 4 μl of 10× buffer (Perkin-Elmer/Cetus), 6 μl of a mix containing 1 mM each of dATP, dGTP and dTTP, 4 μl of 0.1 mM dCTP, 10 μl of 3000 Ci/mmol [α-^{32}P]dCTP (Amersham), 1 μg of the antisense primer, 0.01 μg of the sense primer, ddH_2O to 39.5 μl and 0.5 μl (2.5 units) of *Taq* DNA polymerase (Boehringer-Mannheim). The reaction mixtures were covered with a small volume of mineral oil to prevent evaporation. All PCR reactions were carried out under the same conditions in an automated thermal cycler (Perkin-Elmer/Cetus) with 25 cycles at 94°C for 1 min, 40°C for 2 min and 72°C for 2 min. At the end of the 25 cycles the samples were incubated for 5 min at 72°C and then the temperature was lowered to 4°C. A small amount of chloroform was then added to the samples to separate the mineral oil from the aqueous phase. The PCR-amplified, radioactively labeled DNAs were ethanol precipitated, resuspended, denatured and run on a low melting point agarose gel. The gel slices containing each radioactive probe were melted at 70°C for 15 min and then adding directly to the hybridization solution at a concentration of 5×10^5 c.p.m./ml.

Some of the oligonucleotides used in the PCR reactions had an *Xba*I restriction site at their 5′ ends, in order to facilitate cloning of the PCR-amplified fragments. The oligonucleotide sequences were chosen to provide specific probes, devoid of repetitive sequences. For each of the following primer pairs, the first oligonucleotide is in the sense orientation and the second in the antisense orientation. A radioactive probe derived from the *E74* 3′ common region was used to detect the *E74A* and *E74B* mRNAs. This probe was synthesized from an *E74B* cDNA clone using the following primer pair: E74×8-1 (GTCGACTCGAAGGCTGTGTC) and E74×8-2 (GATCTCCGTATACATATGTTC) (Burtis *et al.*, 1990). Probes specific for the *E75A* and *E75B* mRNA isoforms were generated from genomic *Drosophila* DNA using the following primer pairs: *E75A*-1 (GCTCTAGACATTGACTAACTGCCACTCGCA) and *E75A*-2 (GCTCTAGACAACACTGCAGTGGGACCATCG); *E75B*-1 (GCTCTAGACACCAAAGCCATGTGCCGATCT) and *E75B*-3 (GGCGCAGGAGATTGGCGATT) (Segraves and Hogness, 1990). A probe directed against the *E75C* mRNA isoform was generated from an *E75C* genomic clone, pBS250 (a gift from W.Segraves) using the following primer pair: *E75C*-5 (CAAACATACTCAGACGCTGC) and *E75C*-4 (GGCTCGCTGTTGCACGTACTCTAC). A probe directed against the coding region for the *EcR* DNA binding domain, and part of the ligand binding domain, was generated from a cDNA library that was enriched for early ecdysone-inducible sequences (a gift from P.Hurban) using the following primer pair: *EcR*-2b (GCTCTAGACGATTCGGTGAATTCTATATCTTC) and *EcR*-6x (GCTCTAGACATACGCAGCATCATCACCTCCGAC) (Koelle *et al.*, 1991). A probe directed against the *BR-C* core exon, which should detect all *BR-C* transcript forms, was synthesized from a *BR-C* cDNA clone, paaDm527 (a gift from G.Guild), using the following primer pair: *BR-C*-1 (GCTCTAGAATAGGAACACCTATAGAC) and *BR-C*-2 (GCTCTAGATGTTGCTGCCACTGCCAAC) (DiBello *et al.*, 1991).

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References

Andres,A.J. and Thummel,C.S. (1992) *Trends Genet.*, **8**, 132–138.
Ashburner,M. (1967) *Chromosoma*, **21**, 398–428.
Ashburner,M. (1969) *Chromosoma*, **27**, 47–63.
Ashburner,M. (1972) *Chromosoma*, **38**, 255–281.
Ashburner,M. (1973) *Dev. Biol.*, **35**, 47–61.
Ashburner,M. (1974) *Dev. Biol.*, **39**, 141–157.
Ashburner,M. (1990) *Cell*, **61**, 1–3.
Ashburner,M., Chihara,C., Meltzer,P. and Richards,G. (1974) *Cold Spring Harbor Symp. Quant. Biol.*, **38**, 655–662.
Becker,H.J. (1959) *Chromosoma*, **10**, 654–678.
Belyaeva,E.S., Vlassova,I.E., Biyasheva,Z.M., Kakpakov,V.T., Richards,G. and Zhimulev,I.F. (1981) *Chromosoma*, **84**, 207–219.
Burtis,K.C., Thummel,C.S., Jones,C.W., Karim,F.D. and Hogness,D.S. (1990) *Cell*, **61**, 85–99.
Chao,A.T. and Guild,G.M. (1986) *EMBO J.*, **5**, 143–150.
Clever,U. and Karlson,P. (1960) *Exp. Cell. Res.*, **20**, 623–626.
Crowley,T.E., Mathers,P.H. and Meyerowitz,E.M. (1984) *Cell*, **39**, 149–156.
DiBello,P.R., Withers,D.A., Bayer,C.A., Fristrom,J.W. and Guild,G.M. (1991) *Genetics*, **129**, 385–397.
Feigl,G., Gram,M. and Pongs,O. (1989) *Nucleic Acids Res.*, **17**, 7167–7178.
Georgel,P., Ramain,P., Giangrande,A., Dretzen,G., Richards,G. and Bellard,M. (1991) *Mol. Cell. Biol.*, **11**, 523–532.
Guay,P.S. and Guild,G.M. (1991) *Genetics*, **129**, 169–175.
Handler,A.M. (1982) *Dev. Biol.*, **93**, 73–82.
Hansson,L. and Lambertsson,A. (1989) *Hereditas*, **110**, 61–67.
Hodgetts,R.B., Sage,B. and O'Connor,J.D. (1977) *Dev. Biol.*, **60**, 310–317.
Irvine,K.D., Helfand,S.L. and Hogness,D.S. (1991) *Development*, **111**, 407–424.
Janknecht,R., Taube,W., Lüdecke,H.-J. and Pongs,O. (1989) *Nucleic Acids Res.*, **17**, 4455–4464.
Jones,C.W., Dalton,M.W. and Townley,L.H. (1991) *Genetics*, **127**, 535–543.
Karim,F.D. and Thummel,C.S. (1991) *Genes Dev.*, **5**, 1067–1079.
Kiss,I., Beaton,A.H., Tardiff,J., Fristrom,D. and Fristrom,J.W. (1988) *Genetics*, **118**, 247–259.
Koelle,M.R., Talbot,W.S., Segraves,W.A., Bender,M.T., Cherbas,P. and Hogness,D.S. (1991) *Cell*, **67**, 59–77.
Maroni,G. and Stamey,S.C. (1983) *Dros. Inf. Serv.*, **59**, 142–143.
O'Connell,P. and Rosbash,M. (1984) *Nucleic Acids Res.*, **12**, 5495–5513.
Restifo,L.L. and White,K. (1991) *Dev. Biol.*, **148**, 174–194.
Restifo,L.L. and White,K. (1992) *Wilhelm Roux's Arch. Dev. Biol.*, in press.
Richards,G. (1981a) *Biol. Rev.*, **56**, 501–549.
Richards,G. (1981b) *Mol. Cell. Endocrinol.*, **21**, 181–197.
Richards,G. (1982) *Wilhelm Roux's Arch. Dev. Biol.*, **191**, 103–111.
Segraves,W.A. (1988) Ph.D. thesis, Stanford University.
Segraves,W.A. (1991) *Cell*, **67**, 225–228.
Segraves,W.A. and Hogness,D.S. (1990) *Genes Dev.*, **4**, 204–219.
Shermoen,A.W. and O'Farrell,P.H. (1991) *Cell*, **67**, 303–310.
Sliter,T.J. and Gilbert,L.I. (1992) *Genetics*, **130**, 555–568.
Thummel,C.S. (1990) *BioEssays*, **12**, 1–8.
Thummel,C.S., Burtis,K.C. and Hogness,D.S. (1990) *Cell*, **61**, 101–111.
Urness,L.D. and Thummel,C.S. (1990) *Cell*, **63**, 47–61.

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