Sequential gene activation by ecdysone in *Drosophila melanogaster*: the hierarchical equivalence of early and early late genes

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SUMMARY

Ecdysteroids are key regulators of insect development. In *Drosophila melanogaster* the late larval response to ecdysone is characterised by a precise sequential activation of members of the superfamily of nuclear receptors (*DHR3, DHR39, EcR, E75, E78, FTZ-F1, usp*). Many of these genes are localised in the polytene chromosome puffs of the salivary gland previously classified as intermoult, early or early-late puff loci. Ashburner et al. (Ashburner, M., Chihara, C., Meltzer, P. and Richards, G. (1974) *Cold Spring Harbour Symp. Quant. Biol.* 38, 655-662) proposed a formal model describing interactions between ecdysone, its receptor and the early and late puffs during this ecdysone response. To integrate transcripts from the intermoult and early-late puffs into this model, we have used a micro RT-PCR assay to study their hormonal regulation using salivary gland culture protocols first used in the puffing analyses. We show that transcripts from certain early-late puffs are induced in parallel with the early transcripts and are thus hierarchically equivalent. In vivo the profile of the increase in hormone titre, the sensitivity of different promoters to hormone and the rate of transcript accumulation must contribute to the temporal differences in expression observed between these two classes.

Key words: ecdysone, *Drosophila melanogaster*, early-late genes, regulatory hierarchies

INTRODUCTION

There is a growing awareness of the temporal complexity of hormonal responses in eukaryotes. In insects, this complexity results from the coordinated sequential regulation of ecdysone-responsive genes. In *Drosophila*, studies on the puffing patterns of salivary gland polytene chromosomes at the end of the third larval instar (Ashburner, 1972a for review) gave significant insights into this sequential regulation. Four classes of puffs have been described: the intermoult puffs, which are active at the beginning of the response to ecdysone and thereafter regress, the early puffs, which are induced within minutes by ecdysone, the early-late puffs appearing with a delay of two hours or so, and the late puffs, which appear from three hours onwards. Ashburner et al. (1974) proposed a model for the activation of the early and late puffs. Ecdysone, complexed with a receptor, activates directly and hence rapidly a few early puffs while repressing the late puffs. When the proteins encoded by the early puffs become sufficiently abundant they both repress their own promoters and activate the late genes. Recent molecular studies enable us to integrate the intermoult and early-late genes in this model and, in this study, we determine their hierarchical position in the response.

Molecular analyses have confirmed and extended many aspects of the Ashburner model. The active receptor that binds ecdysone and DNA is now known to be a heteromer containing two proteins, EcR and USP, both of which belong to the nuclear receptor superfamily (Koelle, 1992; Thomas et al., 1993; Yao et al., 1992; Koelle et al., 1994). Furthermore EcR encodes three isoforms (Koelle, 1992) which show tissue- or cell-specific expression patterns (Talbot et al., 1993; Robinow et al., 1993; Truman et al., 1994). EcR and USP colocalize on ecdysone-responsive loci of polytene chromosomes (Yao et al., 1993), including the 74EF, 75B and 2B5 puffs which contain the three early genes, *E74*, *E75* and the *Broad-Complex* (*BR-C*) respectively.

These three early ecdysone-inducible genes are large (>60 kb) and complex, with multiple overlapping transcription units each encoding a family of proteins structurally related to transcription factors (Andres and Thummel, 1992 for review). In particular the E75 proteins are members of the nuclear receptor superfamily. Antibodies directed against E74A and E75A proteins stain early and late responsive loci (Urness and Thummel, 1990; Hill et al., 1993). The early genes are expressed in all tissues studied (Andres and Thummel, 1992; Huet et al., 1993), suggesting that the general features of the Ashburner model apply to other tissues. Although they are potential regulators for the late class of genes or for their own repression, the function of each early gene product remains to be determined.

The position of the early-late puffs in the regulatory hierarchy is ambiguous. They begin to increase when the early
puffs are reaching their maximum size. As their induction is inhibited by cycloheximide they are considered a subclass of the late class of puffs although their behaviour differs from that of the late puffs in that they are not prematurely induced in wash-out experiments (Ashburner et al., 1974). We have studied the expression of the \(E78\) and \(DHR3\) genes, localized at early-late puff sites \(78C\) and \(46F\) respectively, as well as the \(DHR39\) gene, in individual salivary glands, using our micro RT-PCR (reverse transcription-polymerase chain reaction) approach (Huet et al., 1993). All three encode ‘orphan’ (i.e. without a known ligand) members of the nuclear receptor superfamily (Stone and Thummel, 1993; Koelle et al., 1992). We find that these early-late genes are directly induced by ecdysone and are expressed similarly in other tissues. We conclude that they are a general component of ecdysone responses having the same hierarchical status as the early genes in the regulatory cascade. The delay in their induction is not due to a lower sensitivity of their promoters but most likely linked to the kinetics of transcript accumulation.

The intermoult puffs are at a maximum prior to the late larval ecdysone response and thereafter regress. While the most extensively studied transcripts from intermoult puffs are those of the ‘glue’ genes (see Andres and Thummel, 1992 for review), recent molecular analyses have shown that \(EcR\) and the \(E74B\) and \(E75C\) isoforms can be considered intermoult transcripts (Karim and Thummel, 1992; Huet et al., 1993). We find that they are not repressed directly by ecdysone but may be targets of either early or early-late gene products. Our results provide new elements for the study of interactions in the ecdysone response.

MATERIALS AND METHODS

Developmental analyses

Methods for staging animals have been described in detail as has the micro-RNA extraction from individual salivary glands and the subsequent RT-PCR protocol (Huet et al., 1993). As previously, one lobe was used for the determination of the puff stage and the RNA extracted from the contralateral lobe was first controlled by assaying for \(rtp49\) transcripts. We then undertook RT-PCR reactions using the equivalent of 1.6% of the RNA from a salivary gland per transcript. In the figures, one column is the analysis of the transcripts of one lobe. The salivary glands of this series are different from those of Huet et al. (1993). As primers we use two 20-mer oligonucleotides having a GC content as close as possible to 50%, preferably placed in different exons. Each set of primers allows the amplification of a fragment between 150 and 700 bp whose sequence has been verified after sub-cloning. The amplification products (10 µl final concentration) was added in the \(−10\times\) hybridisation buffer and stored at \(−80^\circ\)C, the second lobe being cultured. The developmental status was verified by an RT-PCR analysis of \(E74\) transcripts using 10% of the RNA from the first lobe. Animals showing \(E74A\) transcripts were rejected. In the figures, the control (or 0 hour) point is derived from such a contralateral lobe. Gland culture at 25°C in modified Grace’s medium (Graces {GibCo}/distilled H\(_2\)O/2.5% ethanol in the ratio 50/9/1) was based on Ashburner (1972b) except that the culture volume was 25 µl per lobe. Where appropriate 20-OH ecdysone (Simes, Milan) was dissolved in the ethanol fraction, while cycloheximide (7×10\(^{-5}\) M final concentration) was added in the distilled water fraction. Glands were cultured in glass depression slides with a grease sealed coverslip to avoid desiccation. For each time point, four glands were analysed in parallel.

RESULTS

Developmental analyses

We have increased the sensitivity of our micro RT-PCR assay (see Materials and methods) so as to compare the temporal profiles of transcripts of \(E74\) and the ecdysone-regulated members of the nuclear receptor superfamily (\(usp\), \(EcR\) isoforms, \(E75\), \(E78\), \(DHR3\), \(DHR39\) and \(FTZ-F1\)) during the late larval and early prepupal response. We have analysed RNAs from individual salivary glands staged by puffing from c. 112 to 126 hours (puff stages 2 to 16 – see Fig. 1). The \(E74\), \(E75\) and \(EcR\) profiles were previously described using ethidium bromide-stained gels and the primers were as given in Huet et al. (1993). Here we focus on \(E78\) and \(DHR3\) (which map at early-late puff sites) and \(DHR39\), so as to define the hierarchical status of the early-late genes. The position of the oligonucleotide primers for these genes are shown schematically in Fig. 2 and their sequence as well as those of the hybridisation probes are given in Table 1 and Material and Methods. Results for the transcripts are grouped by temporal class (receptor, intermoult, early, early-late and mid prepupal) with reference to puffing studies. Fig. 3 shows that there is a tight temporal regulation of expression of 9 isoforms derived from 6 members of the nuclear receptor superfamily in the late larval salivary gland response to ecdysone.

(A) Ecdysone receptor transcripts

We have studied transcripts encoding two components of the ecdysone receptor, \(usp\) and \(EcR\) isoforms (Fig. 3). \(Usp\) transcripts remain essentially unchanged throughout this period. In contrast the profile of \(EcR\) transcripts using amplification of a zone common to all isoforms (\(EcR\) com) confirms that of Huet et al. (1993). Transcript levels decrease dramatically from PS7.
onwards and remain low until PS14. Thereafter they return to maximum levels just prior to the late prepupal ec dysone response. Using an isoform-specific assay (A, B1 and B2 – see Fig. 1) we observe two distinct elements in this profile. B1 and B2 isoform transcripts predominate in the salivary gland while the low levels of A transcripts show little variation throughout this period and are especially responsible for the basal levels seen in early prepupae. Curiously the B2 isoform disappears at PS6 while B1 is maintained until PS7. Note also an exceptional B2 signal seen in the PS11 larva.

Table 1. Oligonucleotide primers, hybridisation probes and formamide concentration used for hybridisation and washing buffers

<table>
<thead>
<tr>
<th>Gene</th>
<th>RTAse/PCR primer</th>
<th>PCR primer</th>
<th>Hybridisation oligo</th>
<th>%HCONH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcR A</td>
<td>AACCAGGCACAGCTCCTCTTT</td>
<td>GCACCAACCCACACCTTCCA</td>
<td>GCTGGAAGCCGGAGATCAT</td>
<td>18</td>
</tr>
<tr>
<td>EcR B1</td>
<td>–</td>
<td>GGAATTGATATCCTTACC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EcR B2</td>
<td>–</td>
<td>GCCCATGACCTCTTGGAAG</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DHR39</td>
<td>CCAATGAGGATGAGGATT</td>
<td>GCACACGACATCATCAGCT</td>
<td>GAGATGACCTCTGACATGA</td>
<td>22</td>
</tr>
<tr>
<td>E74B</td>
<td>GACTTACCATCAGATTGGGC</td>
<td>AGAACCTCTCCTTCTTACCG</td>
<td>GCTGGAGACGGCACATTA</td>
<td>22</td>
</tr>
<tr>
<td>DHR3</td>
<td>TATCCGTAGGGCTCCCTT</td>
<td>CTGTAAGGTTCCGAGAGAT</td>
<td>CCACCGTGTTGCGAGTAGA</td>
<td>18</td>
</tr>
<tr>
<td>βFTZ-F1</td>
<td>GAGCTGGGTTTACCGATT</td>
<td>TTAACCTCTCCCTCATCTCT</td>
<td>CGCCTGTGGTGCAGTGA</td>
<td>26</td>
</tr>
</tbody>
</table>

Fig. 1. The ec dysone response in third instar larvae and early prepupae. The late larval ec dysone response analysed by puffs, northern analysis and RT-PCR in third instar larvae and prepupae of *Drosophila*. The time scale is in hours, relative to egg laying at 25°C. The puff stages (PS) 2-16 of the late larval response are indicated as are the periods of activity of the Intermoult, Early, Late and Late puff classes. Ec dysone titres increase between PS1 and PS2 as PS2 is the first cytological sign that the ec dysone response has started. Although the progression PS1 to PS2 occurs in a few minutes when glands are cultured with ec dysone (Ashburner, 1973) this increase could start appreciably earlier in vivo. The ec dysone peak is estimated at 10−6 M close to pupariation (PS11). The profile between PS1 and PS11 is not known. Following PS11 the hormone level declines to a minimum in 2 to 3 hours (Handler 1982). The designations of ec dysone-induced early transcripts as class I and class II are from Karin and Thummel (1992). Their profiles are schematised from results of Huet et al. (1993). Ec dysone levels prior to the late larval response (from 112-114 hours onwards) are unknown, they may be intermediate as indicated (gray box) or may remain at basal levels (white box – mid prepupa), see Richards (1981) and Huet et al. (1993).

(B) Intermoult transcripts

By analogy with intermoult puffs which are abundant at PS1 and regress thereafter, the EcR B1 and B2 transcripts can be classed as intermoult transcripts (Fig. 3). E74B and E75C, which like EcR transcripts are induced in the mid-instar, also regress in late larvae.

(C) Early transcripts

Levels of E74A transcripts mirror early puff activity (Thummel et al., 1990; Huet et al., 1993). The case of E75 is more complex in that both E75B and E75A transcripts are induced (Fig. 3). E75B activation is rapid but shortlived and the 75B puff maximum correlates with the expression of the longer E75A transcripts. Note the reappearance of E75B transcripts in early prepupae (PS12 to PS15) confirming previous suggestions of its reinduction towards the end of the late larval response (see Huet et al. 1993).

(D) Early-late transcripts

These studies led us to class transcripts from three members of the receptor superfamily, E78B, DHR39 (also called FTZ-F1β, Ohno and Petkovich, 1992) and DHR3, as early-late transcripts (Fig. 3).

We observe a significant increase in the level of DHR3 transcripts from PS6 onwards which reach a maximum between PS11 and 13 (an induction estimated between 1- and 200-fold). Thereafter the level decreases and returns to the initial level at PS14 (6h pp).

The E78B and DHR39 transcripts show similar profiles in vivo. An initial induction of a low level of transcripts occurs between PS2 and PS6 and thereafter there is a second period of induction. For E78B, which encodes a receptor isoform without a DNA-binding domain, the maximum is reached by PS8 (an induction estimated as 100-fold) as previously suggested by Stone and Thummel (1993) who used northern analysis of RNA from total larvae. In early prepupae, E78B transcripts return to their initial level, before showing a rein-duction from PS16 (6 hour prepupae) onwards. E78A is not expressed in the larval salivary gland (our data not shown) and in larvae in this period (Stone and Thummel, 1993), and the E78B profile correlates well with the 78C puff profile (previously designated 78D).

We also studied the early-late transcripts in different tissues (Fig. 4) using the same RNAs as those used in Huet et al. (1993) for the study of early transcripts. Within the limits of these mini-profiles, it is striking that E78B, DHR3 and DHR39 (data not shown) are induced with similar kinetics and to a similar extent in all five tissues studied, although for DHR3 induction is slightly later in the salivary gland and wing discs.
higher concentrations, induction is ecdysone. With 10^{-8} M hormone or -8 M and -8 M ecdysone results in the full induction of both classes (Ashburner, 1973). Hereafter we refer to these concentrations as 10^{-6} M ecdysone. At 1.8 \times 10^{-8} M ecdysone induction is not affected. With 10^{-6} M ecdysone and cycloheximide, E74A is induced and we observe a slight increase in transcript accumulation (data not shown). This induction of E74A is mediated by a promoter having a high sensitivity for

**Analyses of ecdysone regulation in cultured salivary glands**

From their in vivo profiles E78B, DHR3 and DHR39 are early-late genes. To define their hierarchical status, we have adapted the classical culture technique for individual salivary glands (Ashburner, 1972b) in the presence of varying concentrations of ecdysone (plus or minus cycloheximide). The same RNAs were used to study both early and early-late gene regulation. Although series of glands were cultured without hormone or with hormone concentrations ranging from 10^{-8} to 10^{-5} M, for clarity we present results (Fig. 5) from only three series: 0, 1.8 \times 10^{-5} and 1.8 \times 10^{-6} M ecdysone. At 1.8 \times 10^{-8} M ecdysone early puffs are induced suboptimally (c. 20% of maximum activity), but not late puffs, while culture with 1.8 \times 10^{-6} M ecdysone results in the full induction of both classes (Ashburner, 1973). Hereafter we refer to these concentrations as 10^{-8} M and 10^{-6} M, respectively.

**Early transcripts**

E74A is not induced in the absence of ecdysone. With 10^{-8} M hormone or higher concentrations, induction is close to that observed in vivo reaching a maximum in 2 hours. There is little or no effect of dose and the time course of the induction is not affected. With 10^{-6} M ecdysone and cycloheximide, E74A is induced and we observe a slight increase in transcript accumulation (data not shown). This induction of E74A is mediated by a promoter having a high sensitivity for

![Gene structure and RT-PCR strategy](image_url)

**Fig. 2.** Gene structure and RT-PCR strategy. For each gene (given with the corresponding chromosomal site and puff), the exons are depicted as box segments, the introns as lines. Coding regions are shown as solid boxes, non-translated regions as open box segments. The positions of RT-ase and PCR primers (see Table 1 for sequences) are shown by arrowheads together with the size of the corresponding PCR products. Where DNA-derived products are detected, the size is given in brackets. E74, E75 and rp49 are not shown (see Huet et al., 1993). All of these genes belong to the nuclear steroid receptor superfamily. Zn denotes the ‘zinc finger’ DNA-binding domain. (A) EcR : the ecdysone receptor gene. The EcR RNAs derived from the two promoters (A and B) and alternative splicing (B1 and B2) are from Koelle et al., 1991 and Talbot et al., 1993. EcR com. denotes the RT-PCR assay for the region common to all three transcripts (see Huet et al., 1993). (B) usp : ultraspireacle (Oro et al., 1990; Henrich et al., 1990; Shea et al., 1990). In the absence of an intron, RNA samples are pretreated with DNase I to eliminate contaminating DNA (see Huet et al., 1993). (C) E78. Only the B isoform (which lacks the zinc finger domain) is expressed during the period studied (Stone and Thummel, 1993 and our unpublished data). (D) FTZ-F1 : The order of the promoters (Lavorgna et al., 1991, 1993) is unknown (denoted by broken lines and question marks). Only the B isoform is expressed during the period studied here. We detected an intron following the zinc finger domain when using primers positioned in the common region of the α and β isoforms (data not shown). (E) DHR3 (also known as FTZ-F1β) The structure is derived from Ohno and Petkovich (1992). No isoforms have been described. (F) DHR3. The transcript structure is from Koelle et al. (1992). Note however that a number of different sized transcripts are seen in northern analyses.
the ecdysone-receptor complex (strongly induced with a low concentration of hormone) and shows a rapid accumulation of transcripts.

As in normal development between PS2 and 4 (Huet et al., 1993), \( E75B \) transcripts are induced rapidly but transiently in the response (Fig. 5A). While \( E75A \) induction with \( 10^{-6} \) M ecdysone resembles that of \( E74A \), this induction is far more sensitive to hormone dose, both in the timing of the response and transcript levels (see the induction with \( 10^{-8} \) M ecdysone, Fig. 5). Thus the \( E75A \) promoter has a lower sensitivity to the hormonal signal than \( E74A \) and maximum induction requires an important increase in hormone titre.

(B) Early-late transcripts

\( DHR3 \) is not induced in the absence of ecdysone. In the presence of hormone, \( DHR3 \) transcript levels increase with ecdysone concentrations and the range of induction is very large (estimated as 1000-fold with \( 10^{-5} \) M ecdysone – not shown). Incubation with \( 10^{-6} \) M leads to a response close to the in vivo situation, with about a 100-fold induction. With both \( 10^{-5} \) M and \( 10^{-6} \) M ecdysone, transcript levels increase until 6 hours of incubation while at \( 10^{-7} \) and \( 10^{-8} \) M ecdysone, the profiles show a maximum at 4 hours. Unlike the early transcripts (see Karim and Thummel, 1992), \( DHR3 \) transcripts are not higher in the presence of ecdysone plus cycloheximide (Fig. 6) than with \( 10^{-6} \) M ecdysone alone. Treatment with cycloheximide alone does not induce \( DHR3 \) (Fig. 6). Thus \( DHR3 \) induction does not require protein synthesis and is a primary response to hormone. The promoter shows a low sensitivity for the ecdysone-receptor complex as \( DHR3 \) induction levels in cultured glands resemble those seen in vivo at \( 10^{-6} \) M but are severely reduced with \( 10^{-8} \) M hormone.

The behaviour of \( E78B \) differs from that of \( DHR3 \). In the absence of hormone we observe a minor induction at 4 hours. In the presence of ecdysone, at all concentrations, we observe a gradual induction, which reaches a maximum at 6 hours that is similar to that observed in vivo (estimated as a 100-fold induction). Incubation with cycloheximide alone does not induce \( E78B \). With \( 10^{-6} \) M ecdysone and cycloheximide we see an increase of \( E78B \) transcripts similar to that seen in the absence of cycloheximide (Fig. 6). This suggests that \( E78B \) induction in glands transferred directly to culture in ecdysone (see Discussion) has little or no dependance on protein synthesis. Thus \( E78B \) is directly induced by ecdysone, and has a high sensitivity for the ecdysone-receptor complex as it is induced in vivo at PS2 immediately following the increase in ecdysone levels. However \( E78B \) transcript accumulation is slower than that of an early gene and the maximum is reached later.

The third early-late transcript, \( DHR39 \), shows a response broadly similar to that of \( E78B \), that is an induction from \( 10^{-8} \) M ecdysone that reaches a peak between 4 and 6 hours and that is largely insensitive to cycloheximide treatments (data not shown).

This analysis of early and early late transcripts shows that they are both directly induced by ecdysone but that the early-late transcripts accumulate less rapidly, irrespective of the ecdysone concentration. We then asked the question: what

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Fig. 3. Transcript profiles during the late larval ecdysone response from Puff Stage 1 to PS16. Animals were puff staged using one salivary gland lobe while transcripts were analysed from the RNA of the sister lobe (see Materials and Methods). Each line derives from the same RT-PCR assay, each column from the RNA of a single salivary gland. With the exception of \( rp49 \) and \( E74 \), the molecules tested are members of the nuclear receptor family. \( rp49 \) transcripts are used to control the micro-extract. \( usp \) transcripts are essentially constant (the low PS3 point is probably an artefact as it is not seen in other glands). The B isoforms of \( EcR \), the second component of the ecdysone receptor, are classed as intermoult transcripts. Therefore transcripts are classed by analogy to puff classes (early, early-late and MPP – mid prepupal puff). In consequence, the various isoforms of a gene are treated separately.
E78B  ↓
DHR3  ↓
E78B  ↓
DHR3  ↓

Fig. 4. Early-late gene expression in different tissues. Four animals from the late larval period, staged by one salivary gland lobe as PS1, PS3, PS7 and PS11 respectively, were dissected and RNA preparations made from the remaining salivary gland (SG), gut (GT), malpighian tubules (MT) and fat body (FB) and the two white discs (WD). The RNA extracts were prepared and analysed as for the salivary glands of Fig. 3. Percentage of RNA used for each tissue: SG, 1%; GT, 0.5%; MT, 1%; FB, 0.5%; WD, 0.5%. These same glands were analysed for early gene expression in Huet et al. (1993).

happens in the first phase of the response? We analysed expression in glands cultured for 15, 30, 45, 90 or 120 minutes in the presence of 10^{-6} M ecdysone (Fig. 7). The results are conclusive, both E78 and DHR3 are induced in parallel with E75B and precede the appearance of mature E74A and E75A transcripts.

We interpret the results of Figs 5A and 7 as well as other glands of the same experimental series (not shown) in Fig. 5B concerning the effects of hormone levels (10^{-6} and 10^{-8} M ecdysone) on the timing and extent of induction. Each gene responds distinctly and the relative order and level of transcripts can be modified by such manipulations of hormone titre.

(C) Intermoult transcripts

The intermoult transcripts EcR, E74B and E75C are at their maximum in late PS1 glands and regress at the end of the third instar following the rise in ecdysone titre.

For EcR the essential features of the in vivo profile are seen with 10^{-8} M ecdysone in cultured glands. EcR transcripts remain constant for 2 hours and thereafter decrease to the basal level seen in vivo. With 10^{-8} M ecdysone and cycloheximide, this decrease is inhibited, indicating that it is dependent on protein synthesis and thus indirect. Experiments with 10^{-7} M (not shown) or 10^{-6} M ecdysone reveal a dose effect, as repression is delayed and reduced, suggesting that the promoter of the repressor gene has a low sensitivity for the ecdysone-receptor complex. Repression in the absence of hormone (Fig. 5) is distinct as the decrease begins immediately rather than after a lag of 2 hours. Surprisingly this decrease is not passive as it is blocked by cycloheximide (Fig. 6).

E74B transcripts decrease with 10^{-6} M, 10^{-7} M and 10^{-8} M ecdysone as in vivo and show little or no dose effect (Fig. 5). As for EcR, repression appears to require the synthesis of a repressor which is blocked in the presence of cycloheximide (Fig. 6). However, this repressor may differ from that for EcR as the decrease is evident by 2 hours suggesting a more rapid induction by ecdysone. In the absence of ecdysone or with cycloheximide alone, the same phenomena are observed as with EcR (Figs 5, 6). The differences between EcR and E74B are apparent in the summary diagrams of Fig. 5.

For E75C, repression occurs in the middle of the late larval response (Fig. 3). We observe this repression consistently in glands cultured with ecdysone, and as for EcR and E74B it is blocked by cycloheximide (data not shown).

DISCUSSION

Developmental analyses using RT-PCR

Our assay, using RNA from individual salivary glands, is the molecular extension of the puffing studies that established the complexity of the ecdysone response in Drosophila. It avoids problems of developmental heterogeneity and a set of staged glands can provide transcriptional profiles for some 60 transcripts. Certain of our results differ from those obtained by northern analyses (e.g. Karim and Thummel, 1992; Stone and Thummel, 1993) using RNAs extracted from a mixture of tissues obtained from several animals. This is to be expected as there are tissue differences in early gene activity in the same animal (Huet et al., 1993). For the culture experiments, we dissect glands just prior to the physiological response and transfer them directly to ecdysone containing medium rather than pooling and rinsing tissues in ecdysone-free medium before transfer to hormone. As shown here, incubation of glands without hormone alters transcript profiles (see also discussion in Andres et al., 1993).

Puffs and transcripts

EcR transcript levels correlate with puffing activity at the 42A intermoult puff and, for the early-late puffs, DHR3 (46F) and E78B (78C) transcript levels reflect puffing (but see below for the consequences of cycloheximide treatments). In contrast, the transcriptional activity of DHR39 (maximal between PS6 and 11), which was mapped to 39C (Ohno and Petkovich, 1992) or 39 BC (Ayer et al., 1993), differs from the puffing activity at 39BC (PS10 to PS14 – Ashburner, 1972a), and is closer to that of 39B in this period (PS3 to PS9 – Ashburner, 1978).

The minor activity of FTZ-F1 in late larvae is not predicted from puffing studies as the 75CD puff is not induced by ecdysone in larval glands but is induced after pupariation following a drop in ecdysone titre (Richards, 1976) as reflected in the major increase in βFTZ-F1 transcripts between PS14 and 16 (Fig. 3). Importantly βFTZ-F1 is a prepupal-specific modulator of the ecdysone response (Woodard et al., 1994) and has been localised to ecdysone-induced prepupal puff sites (Lavorgna et al., 1993).

Ecdysone receptor isoforms and tissue specificity

The usp gene encodes a single protein and its expression shows little regulation in this period (Henrich et al., 1994 and results). In contrast EcR expression is regulated during ecdysone responses (Karim and Thummel, 1992; Talbot et al., 1993; Huet et al., 1993) and EcR encodes 3 isoforms, each of which can form a functional heterodimer with USP (Koelle et al., 1994). EcR is thus a potential source of stage or tissue specificity in the receptor as N-terminal isoforms of vertebrate nuclear receptors may have distinct transactivation properties (Nagpal et al., 1992 and references therein). This idea has received
support from studies with antibodies selective for the EcR B1 and A isoforms which discriminate between larval and imaginal tissues of the third instar larvae (Talbot et al., 1993) and between classes of neurons with different developmental fates at metamorphosis (Robinow et al., 1993; Truman et al., 1994).

EcR A transcripts are basal in the salivary gland and the dynamic profiles both in vivo and in cultured glands are those of the B1 and B2 transcripts suggesting that the salivary gland response is essentially mediated by the B isoforms. There is no evidence for isoform switching during the ecdysone response and, as the B1 and B2 profiles are very similar (except perhaps at PS6, see Fig. 3), there is little or no regulation of the alternative splicing in the salivary gland during this period. Although we have observed tissue differences in the relative abundance of EcR isoforms (data not shown) this does not appear to affect the induction of early or early-late transcripts and suggests that tissue specificity is not mediated by these early and early-late transcripts per se.

### Ecdysone titres and stability of ecdysone receptors

The profile of the ecdysone titre during the late larval puff stages is unknown. The comparison of in vivo profiles of transcripts with those of the culture experiments suggest its importance. From dose-response experiments, the ecdysone titre prior to PS2 should be inferior to $10^{-8}$ M as this level of hormone induces E74A transcripts in culture (Fig. 5). This contrasts with results of Karim and Thummel (1992) which suggested that at least $5 \times 10^{-8}$ M ecdysone was required for this induction. Transplantation of glands into $10^{-6}$ M ecdysone reproduced sequential puff activation (Ashburner, 1973) suggesting that late gene activation was not due to a recruitment linked to a gradually increasing ecdysone titre. However, in gland culture with $10^{-6}$ M ecdysone (Fig. 7) we modify the order of gene activation and induce a number of early and early-late transcripts in parallel. This suggests that the ecdysone profile provides an element of timing in vivo and that a recruitment exists at the beginning of the response for ecdysone-induced transcripts, as there is first activation of the high (E74A and E75A) followed by the low (DHR3 and E75A) sensitivity promoters.

EcR transcripts decrease dramatically (c.100-fold, see Huet et al., 1993) both in vivo (from PS7) and from 2 hours onwards in culture (Fig. 5) and this may be an important element for limiting the duration of the response. If the EcR protein is stable (as is suggested by the presence of EcR B1 in PS11 salivary glands, Talbot et al., 1993), this may have little consequence for the ecdysone-dependent initiation of early-late transcripts during the latter half of the response (PS7-PS14) although receptor levels may decrease in parallel with the ecdysone titre in early prepupae (PS11 onwards). It is also possible that the activity of the receptor complex is modulated by temporal modifications in further co-factors.

### The early-late genes

The early-late puffs are considered a subclass of the late puffs (Ashburner et al., 1974). However, they differ from the late puffs in that they regress immediately after hormonal withdrawal in culture, a characteristic shared with the early puffs. The early-late puffs that we have followed give rise to transcripts that belong to a subclass of the early transcripts. For E78B, ecdysone induction in cultured glands is seen at 15 minutes (Fig. 7) and precedes the appearance of processed E74A and E75A transcripts. Early-late transcripts are induced in glands treated with ecdysone and cycloheximide, indicating that they are directly induced by the hormone-receptor complex. Stone and Thummel (1993) also observed the induction of E78B in the presence of cycloheximide, but considered it suboptimal. This may be related to protocol differ-

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**Fig. 5.** Ecdysone dose-response studies with cultured salivary glands. (A) Intermoult, early and early-late transcript expression in PS1 late larval salivary glands cultured in the presence of $1.8 \times 10^{-6}$ M, $1.8 \times 10^{-8}$ M ecdysone or in the absence of hormone (0M) for 1, 2, 4 and 6 hours. The 0 hour time point derives from a control (non-incubated) gland. For culture conditions see Materials and Methods. All other parameters were those used in the experiments of Fig. 3. (B) An interpretation of the activation and accumulation of early and early-late transcripts (their subsequent regression is not represented) and the repression of intermoult genes, using both the data of A and results derived from further glands. The results of Fig. 7 are included for the early part of the curve (<1 hour).
ences (see above) as they did not observe induction of E78B with $10^{-8} \text{M}$ ecdysone which gave a major induction in our experiments (Fig. 5). The reason for the difference between puffing and transcript analyses is not clear. One possibility is that early-late puff formation requires a further cycloheximide-sensitive component, which serves to distinguish the two puff classes. Note that the profile of DHR3 and E78 transcripts accumulation (from 0 to 6 hours) is not altered in the presence of cycloheximide (data not shown).

As the early-late genes are directly induced by ecdysone, why is there a delay in puff appearance and the maximum levels of transcripts? We can exclude a mechanism that depends exclusively on the sensitivity to the hormone-receptor complex as we have characterised both an early (E74A) and early-late (E78B) gene with high sensitivity as well as an early (E75A) and early-late (DHR3) gene with low sensitivity to ecdysone. Premessenger length has also been suggested as an element of timing in the ecdysone response (Thummel et al., 1990). Transcript elongation can account for the 45 minute delay in the appearance of E74A and E75A messengers with their large 5′ introns (see Karim and Thummel, 1992; see also Fig. 7). However, the appearance of E78B transcripts at 15 minutes (Fig. 7) excludes an explanation based on the rate of polymerase elongation.

In contrast to the early transcripts, which are maximum at 1 or 2 hours, DHR3 and E78B are induced rapidly but only reach their maximum at 4 or 6 hours. Note that in vivo we can find a low sensitivity promoter (E75A) with a rapid accumulation profile, and a high sensitivity promoter (E78B) with a low rate of accumulation thus demonstrating that the phenomena are not coupled. The differences in transcript accumulation may reflect differences in the rate of initiation (i.e. the number of polymerases recruited per minute at the promoter) and/or the stability of the transcript. Such differences may modulate the delay in the response to the same hormonal signal and provide temporal heterogeneity in both the repression of early genes and the induction of the late class of genes.

The intermoult transcripts

In culture, the effect of hormone on intermoult puffs is variable (Ashburner, 1973) and most regress in the absence of hormone. Karim and Thummel (1992) showed that the temporal profile of induction of class I transcripts (EcR and E74B) in cultured late larval tissues altered with increasing ecdysone titres and that they were rapidly repressed by high concentrations of ecdysone. Our results with late larval glands confirm an ecdysone-dependent repression (compare results with $10^{-6} \text{M}$ and $10^{-8} \text{M}$ ecdysone, Fig. 5). We also observe a PS1 to PS2 type progression with $10^{-7} \text{M}$ ecdysone (not shown), in agreement with Karim and Thummel (1991) who showed that both E74B regression and E74A induction were 50% maximal with $8 \times 10^{-8} \text{M}$ ecdysone.
Our assay provides a more precise timing of intermoult transcript regression which for E74B, EcR and E75C begins at PS2, PS6 and PS7-9, respectively. In vivo and culture experiments show that both E74B and EcR are induced by low concentrations of edcsyne and later repressed when the edcsyne titre increases (Karim and Thummel, 1992; Huet et al., 1993, the present study). In vivo E74B transcripts disappear more gradually than those of the EcR B isoforms (Fig. 3), which disappear abruptly at PS6-7. This may indicate either that the E74B transcripts are intrinsically more stable or reflect properties of the repressor of E74B. In cultured glands, E74B repression is more sensitive to hormone than that of EcR again suggesting that gene-specific factors are involved. From the sensitivity to hormone and the in vivo timing, it appears that the repressor of E74B could be an early product of the response while that of EcR may prove to be the product of an early-late transcript.

In the absence of hormone, intermoult transcripts start to decrease immediately upon incubation and show a time course that differs from their edcsyne-dependent disappearance (Fig. 6 and data not shown), suggesting a distinct mechanism. As this process is blocked by cycloheximide, it is not passive degradation but rather implies an active mechanism dependent upon factors with a short half-life. This mechanism co-exists with transcriptional regulation and the profiles that we observe both in vivo and in cultured glands result from a combination of these effects.

The hierarchical status of the early-late transcripts and interactions in the edcsyne regulatory cascade

Ashburner et al. (1974) proposed the first regulatory interactions in an edcsyne response to E74B, the early proteins in both the induction of late genes and the repression of their own activity. One attempt to incorporate the early-late genes in this scheme was a suggestion that their activation required the de novo synthesis of receptor subunits (Richards, 1992) and that isoform changes or a sequential synthesis of receptor-like molecules might provide a timing mechanism within the edcsyne response.

Our experiments reveal a rather different hierarchical organisation. The early-late transcripts are directly induced by edcsyne and are distinguishable from the early transcripts only by their rate of accumulation. This is in accord with both the localisation of edcsyne (Gronemeyer and Pongs, 1980) and receptor components (Yao et al., 1993) on early-late puff sites. They are induced to a similar extent and with similar kinetics in all tissues studied (Fig. 4), suggesting a more generalised role in the response than expected based on the time of appearance of the early-late puffs. Thus the early-late transcripts are hierarchically equivalent to the early transcripts and neither subclass shows important tissue-specific differences in their induction by edcsyne. Unlike the early gene transcripts, which accumulate in the presence of edcsyne and cycloheximide (Karim and Thummel, 1992 and our data not shown) as predicted by the autoregulation postulated by Ashburner et al. (1974), DHR3, E78B and DHR39 do not accumulate in the presence of cycloheximide. This may indicate that they are not repressed by an edcsyne-induced factor.

Certain correlations are particularly striking, notably the mirror image of DHR3 and EcR in vivo. Both the activation of DHR3 and the repression of EcR are delayed and show a low sensitivity for the hormone-receptor complex. This suggests that the best candidate for EcR repression is a gene whose regulation is similar to that of DHR3, and this cross-talk may continue later in the prepupal period (see PS15 and 16, Fig. 3). It will be interesting to determine by immunostaining whether the DHR3 protein is localised at the 42A puff site. A further example is the correlation between the gradual activation of E78B and DHR39 with their promoters of high affinity, and the gradual repression of E74B whose repressor shows similar characteristics. As the E78B protein lacks a DNA-binding domain, it would presumably act as a dominant negative factor and is a possible candidate for E74B repression. This can be tested in appropriate mutant strains.

These experiments enable us to integrate the early-late genes into the Ashburner model (Fig. 8). The most important implication is that the response is not only mediated by the six early genes but by a higher number of regulatory molecules than previously thought. At least some of these early-lates are not tissue-specific and may serve both to activate the late genes and/or repress the intermolt class of genes or equally the early class of genes. Immunocytochemical studies with isoform-specific antibodies using staged salivary gland chromosomes should further our understanding of this regulatory network.

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