

Puffs and PCR: the in vivo dynamics of early gene expression during ecdysone responses in *Drosophila*

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SUMMARY

The steroid hormone ecdysone orchestrates insect development by regulating gene networks. In *Drosophila* the most detailed description of ecdysone action is the sequential activation of early and late puffs in the polytene chromosomes of the late larval salivary gland. A number of these early puffs (2B5, 74EF and 75B) contain complex transcription units (*Broad-Complex*, *E74* and *E75* respectively) encoding families of regulatory proteins which are expressed in most if not all tissues. In vitro, transcripts of the different isoforms of these early genes as well as the ecdysone receptor (*EcR*) present varying dose response characteristics (Karim and Thummel, 1992, *EMBO J.* 11, 4083-4093). We have developed an in vivo approach using a reverse transcription-polymerase chain reaction assay (RT-PCR) so as to visualise these transcripts in the RNA extracted from a single salivary gland. Using one salivary gland lobe for developmental puff staging and the sister lobe for RT-PCR, we have obtained precise developmental profiles for these transcripts and have extended our study to other tissues and stages where puffing studies

were not possible. In the salivary gland we have characterised three distinct ecdysone responses. For the mid and late third larval instar responses our results confirm and extend the conclusions of the in vitro studies concerning the temporal expression of the early gene isoforms. The relatively brief prepupal response contains elements in common with each of the larval responses and all three can be explained by the profiles of the respective ecdysone peaks. Interestingly *EcR* transcripts respond differently during each response. The analysis of different tissues of the same animal reveals subtle differences in the timing of the ecdysone response and isoform expression and suggests that this may reflect tissue differences in the ecdysone profiles. As these molecules have homologues in vertebrates, our analysis may have general implications for the organisation of hormonal responses in vivo.

Key words: puffs, RT-PCR, ecdysone, steroid regulation, *Drosophila*, developmental profile, gene networks, metamorphosis, early gene, transcription factors, isoforms, in vivo

INTRODUCTION

Hormones coordinate the regulation of networks of gene expression that characterise the development and differentiation of higher organisms. In insects, the steroid ecdysone regulates gene expression in many stages and tissues, however the most detailed description of its effects is the sequence of puffs induced in the giant polytene chromosomes of the larval and prepupal salivary glands of *Drosophila melanogaster*. The early in vivo studies of Becker (1959) were later extended by Ashburner (1967) and Zhimulev (1974) to include some 340 puff sites that change their activity in a strictly defined temporal pattern between 110 and 132 hours of development, a period that ends with the histolysis of the larval gland. Ashburner (1971) initiated a series of in vitro experiments with 20-OH ecdysone that reproduced all of the essential features of the late larval response in cultured salivary glands and gave rise to a formal model (Ashburner et al., 1974) which has stimulated

molecular studies. Recent results confirm the similarities between the ecdysone receptor (*EcR* - Koelle et al., 1991) and vertebrate steroid receptors and add a level of complexity to the model with the demonstration that heteromeric receptors mediate the ecdysone response in *Drosophila* (Yao et al., 1992; Thomas et al., 1993). If this response requires two (or more) members of the steroid receptor superfamily, the immediate targets, the early responding puffs of the salivary gland are equally complex. Three of these puffs (2B5, 74EF and 75B) each encode a family of proteins related to known vertebrate transcription factors and are expressed in many tissues (see Andres and Thummel, 1992 for review). Thus the salivary gland remains an interesting model for our understanding of the regulation of steroid coordinated networks of gene activity.

In vitro puffing studies (Richards, 1976a,b) revealed two further phases in the salivary gland ecdysone response following the late larval response: a first period from 3 to 8 hours after pupariation (which occurs at 120 hours) where

ecdysone titres must fall dramatically for the progression of puffing, followed by a second increase in ecdysone titre which regulates the late prepupal ecdysone response from 8 to 14 hours after pupariation. Interestingly, although some of the early puffs of the late prepupal response are in common with those of the late larval response, others are stage specific as are the global responses of the tissue to hormone (glue secretion and gland histolysis respectively). Later puffing studies (Richards, 1982) compared the ecdysone response in chromosomes of the fat body to those of the salivary gland and showed once more that although many early puffs were common to the two tissues there were differences in their response which might find their counterparts at the molecular level. This idea has been developed in the tissue coordination model (Burtis et al., 1990; Thummel et al., 1990) which suggests that one aspect of the stage and tissue-specific response to hormone may consist of differences in the levels of the different isoforms of the transcripts of the early puffs induced by ecdysone in each response. The specificity may lie in the direct action of these factors on target genes or be further elaborated by subsequent cross-talk between the products of these regulatory genes.

The strength of the original cytological studies lay in the fact that they were based on the simultaneous analysis of gene activity at many loci in individual nuclei of a given salivary gland which led to the assignment of larvae to a puff stage (PS, Becker 1959) thus defining the developmental age of the individual. Because of the rapidity of the response to hormone there is necessarily a heterogeneity in the developmental age of late third instar larvae, which has obscured the fine detail of the *in vivo* response at the molecular level, although elegant *in vitro* studies with *E74* (encoded by the 74EF puff), *E75* (75B), *BR-C* (2B5) and *EcR* have revealed the precision of the response (Karim and Thummel, 1991, 1992). From the culture of mass isolated larval organs with different concentrations of 20-OH ecdysone, Karim and Thummel (1992) have proposed two general categories of early gene transcript based on their ecdysone dose-response characteristics: Class I transcripts (e.g. *EcR* and *E74B*) respond to low levels of hormone (first induced from 2×10^{-9} M, 50% activity at 2×10^{-8} M) while Class II transcripts (e.g. *E74A*, *E75A* and *E75B*) respond to higher levels of hormone (induced from 10^{-8} M, 50% activity between 1 and 3×10^{-7} M). *E75A* is the most sensitive of the Class II transcripts being some three-fold more sensitive to hormone than *E74A* and *E75B*. Their analysis of *BR-C* RNAs suggested transcripts with both Class I and Class II characteristics. Note that we use the term ecdysone for 20-OH ecdysone when referring to *in vitro* experiments but for all active ecdysteroids *in vivo*.

While extractions of RNA from 5-10 staged prepupae may be sufficient for the conventional northern analysis of early gene transcripts, our conviction was that for *in vivo* studies, an assay based on the analysis of tissues from individual larvae or prepupae was indispensable. We describe here a RT-PCR based assay and show its application to the salivary gland, where we have followed transcripts of *EcR* and those of the isoforms of *E74*, *E75* and *BR-C* (grouped by their zinc-finger domains) together with those of the gland-specific target gene *Sgs-3* from 86 to 132 hours of

larval and prepupal development. We then used the assay to establish profiles of early gene transcripts in a number of tissues.

We confirm the precision of the biological system as shown by the studies of Karim and Thummel (1991, 1992) and reveal unsuspected detail in both the temporal and spatial response. We show that *EcR* transcript levels in the salivary gland decrease at least 50-fold during the classic late larval puffing response but increase again prior to the late prepupal response. In both responses *E74* isoform switching occurs prior to morphological changes in the 74EF puff. Although we detect transcripts from all of the early genes in the tissues we have examined, *E75* shows the most significant differences between tissues and the most dynamic patterns within tissues. In particular the *E75B* 'one-fingered receptor' isoform (Segraves and Hogness, 1990) is rapidly but transiently induced during the salivary gland responses to hormone. The late larval response includes a novel induction of *E75B* in early prepupae. As suggested by the analysis of cDNAs (DiBello et al., 1991), *BR-C* shows a complex pattern of expression throughout this period. The developmental profiles of *BR-C* transcripts reveal combinations of isoforms encoding Z1, Z2 or Z3 zinc-finger domains at all stages in the salivary gland and in all tissues during the late larval response to ecdysone. Our results suggest that for *BR-C* both promoter usage and alternative splicing are developmentally regulated. This *in vivo* analysis of early gene isoform transcripts confirms that the early response to hormone is stage and tissue specific and suggests new elements that may contribute to this specificity.

MATERIALS AND METHODS

Staging of animals

The *Drosophila melanogaster* wild-type Oregon-R line, maintained at 25°C on a standard cornmeal medium, was used throughout. In these conditions, the first embryos hatch around 22 hours after egg laying, the second to third larval moult is at 72 hours, pupariation occurs at 120 hours and adults emerge at 240 hours. For the mid to late third instar samples, females were placed in split bottles for a 1-2 hour laying period. Larvae were selected at the second to third moult by size and anterior spiracle morphology, placed in 1.5 cm diameter tubes (approx. 20 larvae per tube) and returned to 25°C until they reached the desired age. Larvae synchronised in this way leave the food at approx. 112 hours and pupariate in a 4-8 hour period around 120 hours. Wandering larvae (112-120 hours) were staged by puff analysis of at least five nuclei from one salivary gland. For later stages animals were selected as white prepupae, a stage which lasts approximately 15 minutes, and then aged at 25°C in fresh food tubes for 0-14 hours. These animals were also analysed for their puff stage (see text).

Extraction of RNA

Larvae and prepupae were dissected in insect Ringer. One salivary gland lobe was transferred to cytological fixative (3:1, ethanol:propionic acid) while the sister lobe was placed in 25 µl of ice-cold extraction buffer (3 M LiCl, 6 M urea, 10 mM sodium acetate, pH 5.0, 0.2 mg/ml heparin, 0.1% SDS) and immediately frozen in liquid nitrogen. Where appropriate, further tissues of the same larva were treated in parallel in 25 µl aliquots of extraction buffer. All samples were stocked at -80°C until the first salivary

gland lobe had been analysed cytologically following propionic-carmin-orcein staining (see Ashburner, 1967).

The RNA preparation is derived from the method of Richards et al. (1983). Tubes were allowed to thaw on ice and then vortexed vigorously, which was as effective for the extraction of RNA as conventional grinding, and reduced variability resulting from debris sticking to the piston (data not shown). The tubes were then stored at least 1 hour at 4°C before the addition of 25 µl of 4 M LiCl, 8 M urea. After vortexing followed by centrifugation for 15 minutes at 4°C, the pellet was resuspended in 25 µl of 0.1 M sodium acetate (pH 5.0), 0.1% SDS, by shaking 5 minutes at room temperature. Phenol (25 µl) and chloroform (25 µl) were added and the tube shaken for a further 20 minutes at room temperature. After 5 minutes in a microfuge, 25 µl of the supernatant were transferred to a fresh tube, adjusted to 0.2 M sodium acetate (pH 5.0) and precipitated with 2 volumes of ethanol for 1 hour at -80°C with two agitations at 20 minute intervals. The RNA was recovered by centrifugation 15 minutes in a microfuge at 4°C and resuspended in 10 µl of distilled water. Dilutions were made in distilled water and stocked at -80°C as necessary.

The RT-PCR protocol

General considerations for designing RT-PCR assays can be found in Wang et al., (1989), Chelly et al., (1990), Makino et al., (1990) and Innis et al., (1990).

(a) Design of primers. In order to standardise reaction conditions, we used 20-mer oligonucleotide primers (with a few exceptions, see Table 1), having a GC content as close to 50% as possible. In each case the RTase-PCR primer was positioned so that a second oligonucleotide could be placed between it and the splice site to serve as a hybridisation probe for confirming PCR products or in subcloning experiments (see below). The 5 primers were placed so that products were in the size range of 150-700 base pairs (bp) to ensure efficient PCR amplification. Primer sequences are given in Table 1 and their position and the size of the RNA derived products are shown in Fig. 2. We used a series of dilutions of a bulk RNA preparation to determine sub-saturation conditions for the PCR products (see for example Fig. 8C). Then, in a first series of glands, two dilutions of template, approximately 10- and 100-fold lower than that leading to saturation were used. In later series we used only the higher concentration as this was clearly sufficient for quantitation. This value is given in Table 1 for each set of oligonucleotides for the salivary gland experiments.

(b) Reverse transcription and amplification protocols. The combined reverse transcription - PCR reaction was set up on ice in 50 mM KCl, 1.5 mM MgCl₂, 25 mM Tris-HCl pH 8.3, 100 µg/ml BSA, 100 µM TMAC (tetramethylammonium chloride), 50 µM of each dNTP with 12.5 pmol/primer plus the RNA or DNA template and distilled water to 49 µl. After the addition of two drops of paraffin oil, denaturing and annealing was performed in the PCR apparatus (DNA Thermal Cycler, Perkin Elmer Cetus) for 3 minutes at 94°C followed by a programmed step (10 minutes) to 55°C. 1 µl of a mix of AMV RTase (2.5 units, Pharmacia) and Taq polymerase (1.25 units, Cetus) was added and the tube left for 20 minutes at 55°C. The subsequent amplification programme was 3 minutes at 94°C followed by 30 cycles of 94°C (1 minute), 55°C (2 minutes) and 72°C (3 minutes) followed by a hold step at 4°C. For the analysis of *rp49* transcripts we used MMLV RTase (50 units per tube, BRL). In this case annealing was programmed to 37°C (13 minutes) and we left the reaction 30 minutes at 37°C before starting the PCR cycles. Both a positive (known template) and a negative (no template) control was included in each set of reactions. For *E75*, the PCR primers were added after the RTase reaction as one of these primers initiated reverse transcription which gave rise to a non-specific PCR product. To confirm that the PCR products were those predicted from earlier studies, they were subcloned into pEMBL plasmids and sequenced on double stranded mini-prep DNA templates by the Sanger dideoxy method.

Discriminating between DNA and RNA templates

If the intron between the PCR primers is short (<1 kb), in addition to the product corresponding to the spliced transcript, we detect a second band that may derive from either pre-mRNA or contaminating DNA (see Fig. 2). To distinguish between these possibilities, for each gene, we compared four aliquots from a mini-extract treated with or without DNase I followed by a standard RT-PCR (with AMV RTase and Taq polymerase) or PCR alone (i.e. Taq polymerase alone). After DNase I treatment (40 units DNase I/RNase-free (Boehringer), 100 mM KCl, 3 mM MgCl₂, 50 mM Tris-HCl, pH 8.3, 200 µM TMAC with 30 units RNasin (Amersham), 30 minutes at 37°C followed by 15 minutes at 65°C to inactivate the enzymes) the reaction volume was doubled with the addition of the remaining components of the RT-PCR reaction as described above.

Table 1. Oligonucleotides used for the RTase-PCR analysis

Gene	RTase/PCR primer	PCR primer	Hybridisation oligo	RNA%
<i>rp49</i>	GTGTATTCGACCACGTTACA	TCCTACCAGCTTCAAGATCAC		10
<i>sgs-3</i>	TGCATGGAGGTTGCGTGGTAGATT	ATGAAGCTGACCATTGCTACCGCCCTA	TTGTCGTAGTTGTGCACGGA	0.1/0.01
<i>EcR</i>	AAGGAAGGTATATTGCGCGC	GGAGAACCAATGTGCGATGA	CACTTGGCCAATATTTTCATC	10
<i>E74A</i>	CGGACTTGTCGATGCTTGA	AAGCTGGAGTACGCCCTCAT		10
<i>E74B</i>	-	TACTCCGGCAGGGAATCCGA		
<i>E75A</i>	ACTGCTGATTCTTGGTGCAC	CAGCAGCAACAACATCACCT		
<i>E75B</i>	-	CAACAACAGGTTGTGGCCAC		30
<i>E75C</i>	-	CAAGGTCAAACAGGCCCTCCA	GTCTAGCTCGATTCTTGATC	
<i>BR-C Z1</i>	CCGAGGTGTTCAATGTTGAG	AACACACAGTTGCAGCAGTC	Q1 : ATGCCGGAGGCACTTGTGAT TNT-Q1 : CTGTAGTTCGAGTTGGAGTT Q2 : CTCCCCTTGGCTTGAGGGGA	10
<i>BR-C Z2</i>	AAATGTGCGTCATGAGCGAG	-	TTTCGAGCAGAGGAGCTTAC	10
<i>BR-C Z3</i>	AGACTCCCTTCAGCGAACT	-		
<i>BR-Ccore</i>			ACCTCGCCGTGTCCCTCAA	

Where products are detected or distinguished by hybridisation (see text), the corresponding internal oligonucleotide(s) is shown. In the right hand column the amount of RNA used in a routine analysis is given as a percentage of the total RNA extracted from one salivary gland lobe. Note that for *Sgs-3* the higher concentration of template was used for the experiments of Figure 3.

RESULTS

Experimental strategy

We have developed an RT-PCR assay for the simultaneous analysis of ecdysone regulated transcripts, in tissues dissected from individual staged larvae and prepupae (see Fig. 1). Our approach implies the processing of a large number of microextracts (approx. 50 ng of total RNA/sample). The general principle is that an oligonucleotide primer is placed 3' to an intron and serves for first strand cDNA synthesis, thereafter DNA amplification proceeds using this primer and a second primer 5' to the intron. This distinguishes, by size, products from spliced transcripts from those resulting from pre-mRNA or DNA contamination of the RNA sample.

We have studied the following genes (see Fig. 2):

(A) The ribosomal protein 49 (*rp49*). These transcripts serve as a standard for RNA extraction and the subsequent RT-PCR assay.

(B) The salivary gland secretion protein ('glue') gene *Sgs-3*. This is a tissue-specific target gene of the ecdysone regulatory network which gives rise to abundant transcripts in the late larval period (110-120 hour) where our assay requires 0.01% of a salivary gland extract. Previous studies (Georgel et al., 1991) suggested its interest as a marker of a mid-third instar larval ecdysone response occurring around 96 hours.

(C) The ecdysone receptor (*EcR*). As Koelle et al. (1991) report the possibility of alternate 5' exons for this gene, our primers are positioned either side of the third intron (see Fig. 2C) so as to measure all isoforms.

(D) The early ecdysone induced gene *E74* which contains an *ets*-related DNA binding domain (see Burtis et al., 1990). This gene gives rise to two major forms of transcripts, *E74A* and *E74B*. *E74B* transcripts have two initiation sites separated by 300 bp (Burtis et al., 1990) which we have not sought to distinguish. A common oligonucleotide primer for reverse transcription is placed in the first shared exon and an *E74A*- and *E74B*-specific primer is positioned in the corresponding 5' exon (Fig. 2D).

(E) The early ecdysone induced gene *E75* which is a member of the steroid receptor superfamily. Three transcripts have been reported, *E75A*, *B* and *C*, which encode distinct protein isoforms (Segraves and Hogness, 1990; Feigl et al., 1989, see Fig. 2E). As for *E74*, a primer for reverse transcription is located in the first shared exon with *E75A*-, *E75B*- and *E75C*-specific primers in the corresponding 5' exons.

(F) The Broad-Complex (*BR-C*), localised in the early ecdysone puff 2B5. In this complex transcriptional unit, transcripts, initiated from one of at least three promoters, contain a common 'core-region' encoding segment that is differentially spliced to one of at least three zinc finger encoding motifs, Z1-Z3 (DiBello et al., 1991). As protein synthesis initiates in the common core region, the finger region is the principal source of functionally distinct protein isoforms and accordingly we have devised a finger-specific strategy to detect Z1, Z2 or Z3 encoding transcripts. For Z1 transcripts, unless otherwise noted, the Z1 product corresponds to the Q¹ variant (Fig. 2F).

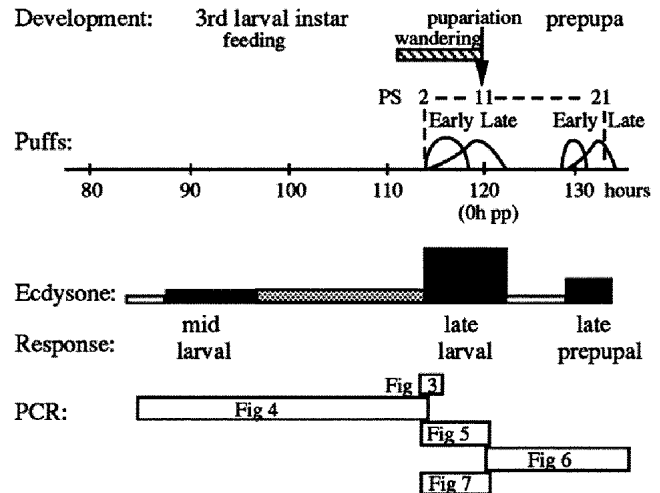


Fig. 1. Ecdysone responses analysed by puffs and PCR in third instar larvae and prepupae of *Drosophila*. The major developmental events are shown on a scale in hours after egg laying at 25°C. The period of puffing studies, puff stages (PS) 2-21 are indicated, the Early and Late responding ecdysone puff classes of the late larval and late prepupal responses are shown schematically. The relative importance of the three titre increases (solid box segments) that initiate the ecdysone responses are suggested below - the exact form and maxima of the peaks is unknown (the relative values are based on ecdysone requirements for Class I and Class II transcript activation (Karim and Thummel, 1992), and radioimmune assays; see Richards, 1981). The titre may be maintained between the mid and late larval responses (stippled box segment) or decrease to basal levels as shown for the early larval and mid prepupal periods (open box segments). The PCR analyses of Figs 3-7 are keyed to this summary.

In preliminary experiments we determined an optimal distribution of the RNA microextract for the various assays (see Materials and methods and Table 1). In general, regulatory gene transcripts showed comparable maximum levels which were at least 100-fold lower than those of *Sgs-3*. In cases where a short intron is involved, we observe a product that derives from contaminating DNA (*rp49*, *EcR* and *E75*) or pre-mRNA in the case of *Sgs-3*, where 100-fold less template is required (data not shown, see Materials and Methods).

Reproducibility of the assay

We established the reproducibility of the assay using late third instar wandering larvae (a period characterised by dramatic puffing changes) by puff staging one salivary gland lobe and analysing the second lobe by RT-PCR. Following protocols derived from puffing studies, we processed at least four glands for each puff stage (PS; see Ashburner, 1967) in each experimental series. An example is given in Fig. 3 for three glands of each of PS1, 2, 3 and 4, taken from a single series. The extraction is clearly reproducible as levels of the *rp49* product are similar for all 12 glands. The products of the two early genes *E74* and *E75* show the precision of this approach. At PS1 the *E74B* product is predominant, by PS2 the ratio of *E74A* to *E74B* is close to 1:1, by PS3 this ratio has become approx. 5:1 and by PS4 *E74B* is visible in just one of the three glands.

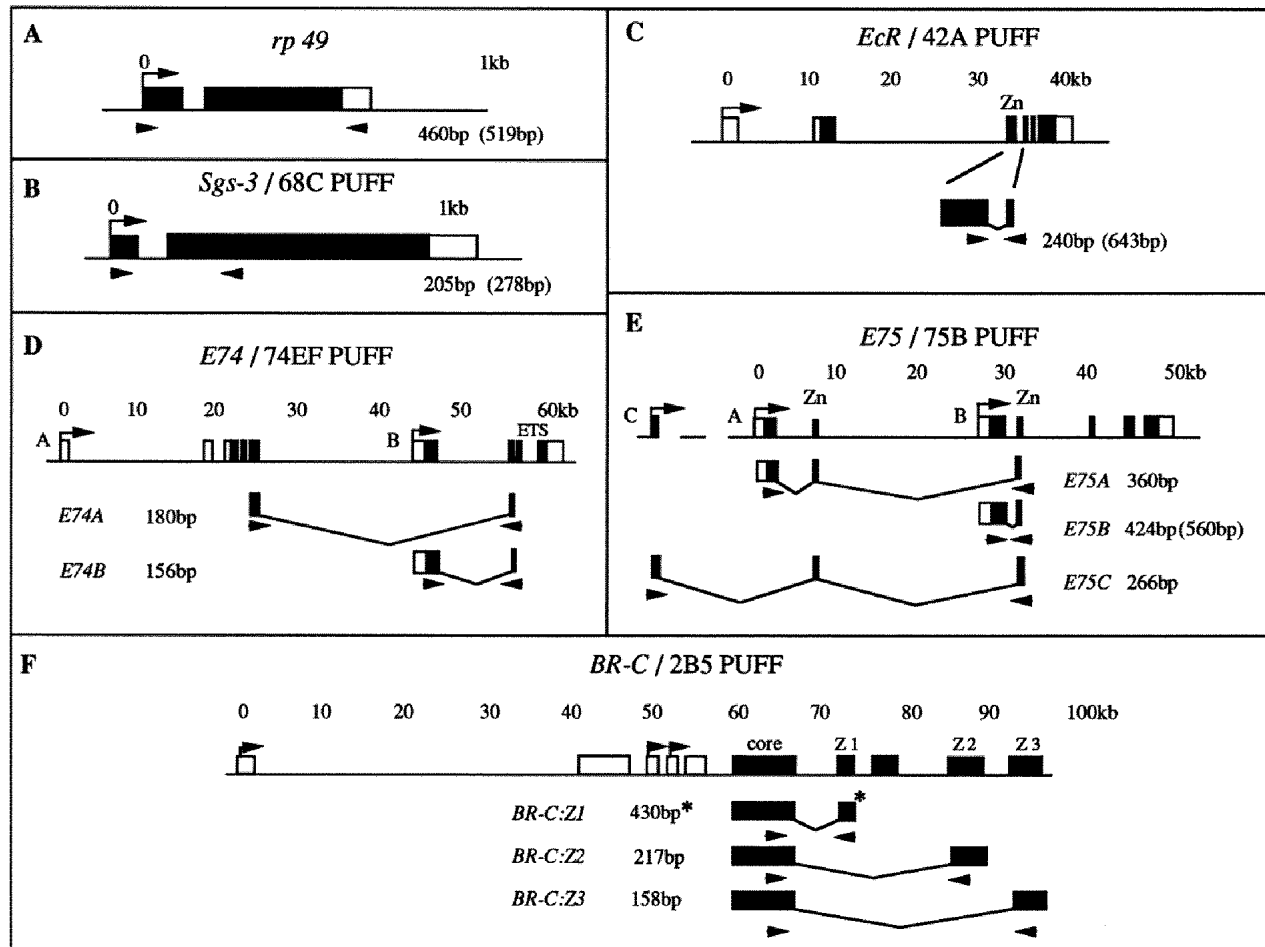


Fig. 2. Gene structure and RT-PCR strategy. For each gene (given with the corresponding chromosomal puff site), transcribed regions are depicted as box segments positioned on the genomic DNA. Coding regions are shown as solid boxes, non-translated regions as open box segments (with the exception of F, see below). The positions of RTase and PCR primers (see Table 1 for sequence) are shown by arrow heads together with the size of the corresponding PCR products. Where DNA or pre mRNA products are detected (see Materials and Methods), their size is given in brackets. (A) Ribosomal protein *rp49*, (O'Connell and Rosbash, 1984), (B) *Sgs-3* (Meyerowitz and Hogness, 1982), (C) *EcR*, the ecdysone receptor gene. The *EcR* RNA of Koelle et al. (1991), including the position of the DNA binding domain (Zn), is shown. The positions of the primers in the third and fourth exon are shown on the enlargement. The *EcR* gene encodes at least three transcript isoforms using two promoters (W. Talbot, E. Swyrd and D. Hogness, personal communication). (D) *E74* (Burtis et al., 1990). The *E74A* and *B* promoters are denoted by arrows and the ETS binding domain (ETS, Urness and Thummel, 1990) is shown. The common and isoform-specific exons recognised in the RT-PCR analysis are depicted below. (E) *E75* (Feigl et al., 1989; Segraves and Hogness 1990; W.A. Segraves personal communication) Three nested promoters give rise to transcripts encoding the A, B and C protein isoforms, orphan members of the steroid receptor superfamily. *E75A* and *E75C* isoforms contain both zinc fingers (Zn) of the DNA binding domain, while *E75B* lacks the first finger. (F) The Broad-Complex. (DiBello et al., 1991). Genomic fragments containing transcribed regions are shown. The splicing of transcripts from the three promoters to the core region (which includes the translation start site) is omitted. The alternative splicing to regions containing pairs of Cys₂His₂ fingers (denoted Z1, Z2 and Z3) is shown. For Z1, the size of the product obtained for the major isoform, Q¹, is given (asterisk). These primers will detect minor isoforms, derived by further alternative processing in the region separating the Z1 fingers from the core-region (DiBello et al., 1991), by products of 379bp (Q²) and 625bp (TNT-Q¹) respectively, which we identify with isoform-specific hybridisation probes (see Table 1).

In parallel, increased *E75B* activity is detected late in PS1 (see below), but while *E75B* persists in PS2 and 3 before diminishing at PS4, *E75A* increases from late PS2 to PS4. Note that RT-PCR is more sensitive than PS analysis. A PS is an approximation as adjacent nuclei in a gland may show slight differences in their puffing pattern, particularly during key phases of the hormonal response (Ashburner, 1967). With RT-PCR we can order glands within a puff stage, for example from the *E75A* profile, the gland PS2c

is a late PS2. Hereafter we will present a single representative gland for each time point. In total for this study, we have analysed 550 microextracts in some 4000 RT-PCR reactions. All of the ethidium bromide stained gels presented were transferred and the membranes hybridised with both isoform-specific and/or common probes (data not shown, see Fig. 8 for examples). We reserve the terms 'absence' or 'disappearance' for cases where products are at least 100-fold lower than their maximum levels as esti-

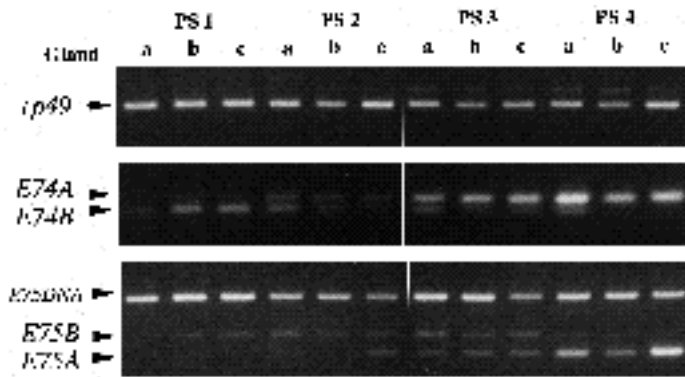


Fig. 3. Correlation of RT-PCR analyses with cytological puff stages. For each puff stage (PS1-PS4), three animals, a, b and c, were staged cytologically using one salivary gland lobe. Each column derives from the RNA of the sister lobe analysed for *rp49*, *E74* (A and B) and *E75* (A and B) transcripts. See Table 1 for the distribution of template RNA for each assay. RT-PCR products (10 μ l of a 50 μ l reaction) were separated on 2% agarose gels and photographed after ethidium bromide staining. *E75* DNA denotes products derived from DNA present in the microextract (see Materials and methods).

mated from serial exposures of the autoradiographs. For convenience, we will refer to these PCR products as transcripts, although of course our assay detects only a specific portion of the transcript. A comparison of our results for *E74A* and *E74B* (see below) with those of Karim and Thummel (1991), who used conventional northern analyses, suggests that we measure full-length transcripts.

The developmental analysis of salivary gland transcripts

We have analysed transcripts in the salivary gland from the mid third instar to the histolysis of the larval gland some 12-15 hours after pupariation, the period of the classical chromosome puffing studies (see Ashburner and Berendes, 1978). This period is most conveniently divided into three phases, each of which includes a distinct response to ecdysone. For the first phase larvae are staged at the second to third larval instar moult (see Materials and methods) and then sampled at 2 hour intervals from 86 hours onwards. The second phase is the post-feeding, wandering phase that ends with pupariation at 120 hours. Here larvae were staged as for Fig. 3. From pupariation (PS11) until the histolysis of the larval salivary gland at PS21 animals were collected as white prepupae and staged both in terms of hours post pupariation (pp) and puff stage. Development is relatively synchronous up until 8 hours pp, thereafter hormone induced changes are rapid and puff staging is a more reliable index (see Richards, 1982).

The period 86-114 hours

While early puffing studies used PS1 to describe the period from the mid-third instar until the late larval ecdysone response in wandering larvae (Fig. 1), later puffing studies (Richards, 1982) and more recent molecular studies (Andres and Cherbas, 1992; Andres and Thummel, 1992 for review) have shown that there are further hormonally regulated

events in this period. Studies in this period are difficult as mid instar larvae are about half the size of late wandering larvae and show considerable developmental variability between individuals (synchronised 24 hours earlier at the second to third instar moult). Fig. 4 shows results from a single series sampled at 4 hour intervals; although differ-

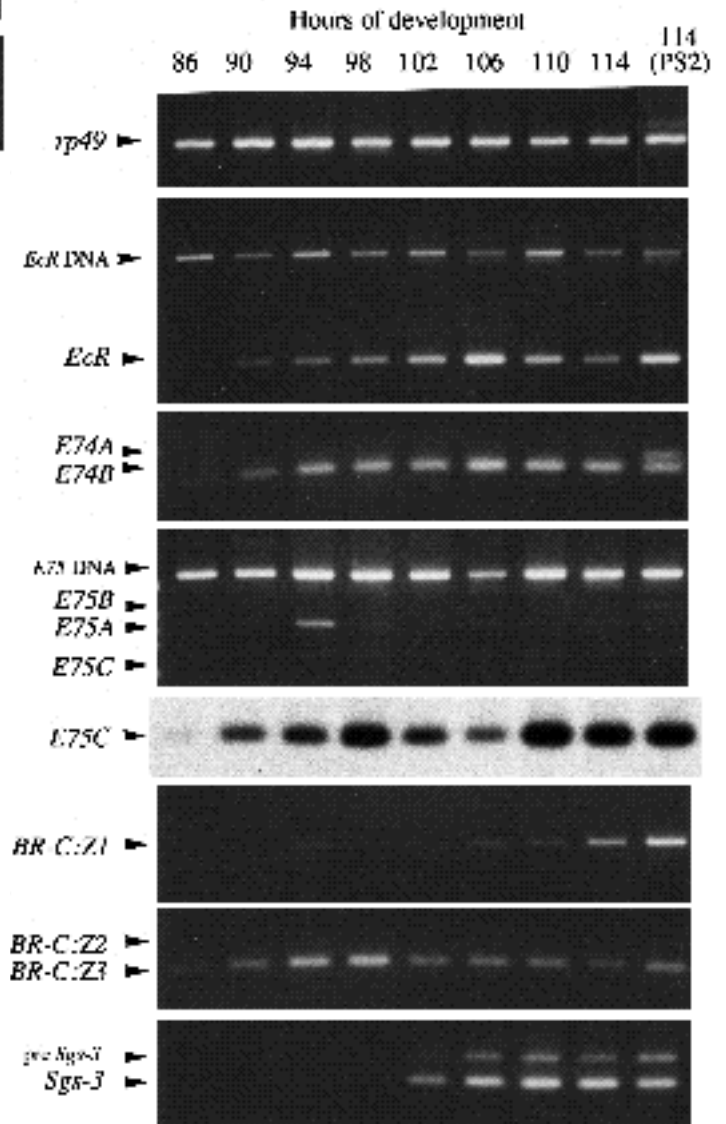


Fig. 4. Analysis of transcripts in the period 86 to 114 hours (after egg-laying). Each column derives from salivary gland RNA from a representative larva of the sample. The products for each gene are described in Fig. 2 and the distribution of template RNA for each assay is given in Table 1. Note that 0.1% template is used for *Sgs-3*. In this period both gland lobes were used for the RNA extract. By 114 hours some animals had already started the late larval response to ecdysone (see Fig. 5) and one such animal, denoted PS2 on the basis of its *E74* and *E75* products (see Fig. 3) is shown. *EcR* and *E75* products that derive from DNA and unspliced *Sgs-3* transcripts (*pre-Sgs-3*) are indicated (see Materials and methods). *E75C* transcripts are of low abundance as seen by ethidium bromide staining and are detected by hybridisation with an isoform-specific probe (see Table 1) after transfer of the same gel (exposure 8 hours). The *E75A* product at 94 hours is an example of sporadic expression (see text).

ent series differ by up to 4 hours in development, the sequence of events is always maintained. Low basal levels of *E75A* are seen throughout this period (see also PS1 glands Fig. 3) but certain individuals showed exceptional levels in the early part of this period (e.g. the gland at 94 hours, Fig. 4). The analyses of the other transcripts revealed less variation between individuals. The Class I transcripts (Karim and Thummel, 1992) *EcR* and *E74B* increase from 86 to 94 hours and then remain relatively constant until the premetamorphic pulse of ecdysone (at approx. 114 hours) which induces the transition to PS2. A similar profile is seen following hybridisation for *E75C* which thus appears with the Class I transcripts. In the salivary gland *E75C* levels are highest during this period but remain low compared to those of *E75A* and *E75B* from PS2 to 10 (see below). For *BR-C*, Z1 containing transcripts increase gradually from 94 hours until 114 hours, while those containing Z3 appear in parallel with *E74B*, peak between 94 and 98 hours and then decrease. This is consistent with the observation that the salivary gland acquires competence to respond to ecdysone by GEBF-I synthesis and *Sgs-3* transcription at approx. 96 hours and that both processes require *BR-C* activity (Georgel et al., 1991). *Sgs-3* is induced some 100,000-fold between 98 and 106 hours (see below).

The late larval period (approx. 112-120 hours)

By 112-114 hours there is heterogeneity in the developmental age of larvae (see Fig. 4, 114h for example), and we staged animals in this period by the PS of one salivary gland lobe. The PS1 and PS2 animals of both series provide the junction between the two periods. It is difficult to determine the duration of each PS (Ashburner, 1967); wandering lasts some 8 hours in our Oregon stock and from the frequency in random sampling, PS1 may continue for 2-3 hours of wandering. The ecdysone induced progression PS2 to PS11 (pupariation) therefore takes 5-6 hours, with an average of some 30 minutes per PS.

The first observation is that *EcR* transcripts, that appear to be induced by a small increase in ecdysone titre between 90 and 94 hours (Fig. 4), may increase at PS2 but diminish dramatically from PS7 onwards (Fig. 5). *EcR* is localised in the region of the 42A puff (Koelle et al., 1991) which is active at PS1 and regresses at PS10 (Ashburner, 1967). This suggests that the puff reflects *EcR* transcription although chromosome refolding may continue after transcription stops.

There is a striking similarity between our RT-PCR results for *E74* and those of Karim and Thummel (1991, their Fig. 2) using northern analyses for *in vivo* studies of whole ani-

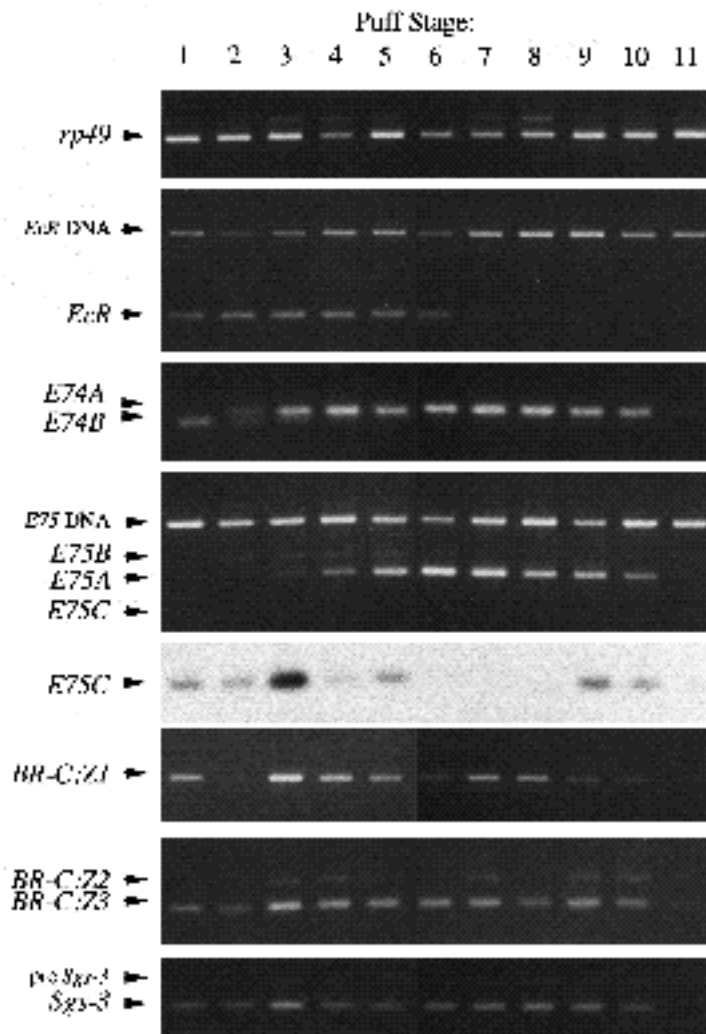


Fig. 5. The late larval ecdysone response from PS1 to PS11. Animals were puff staged using one salivary gland lobe while transcripts were analysed from the RNA of the sister lobe. This is a sequence of events as the duration of each puff stage is unknown (Ashburner, 1967). All symbols and procedures are as in Figs 2-4. Note the abrupt disappearance of many transcripts (excluding *rp49*) between PS10 and PS11. PS11 is the last larval puff stage and continues at puparium formation (120 hours after egg laying - see Materials and methods).

mals prior to pupariation. They showed that at -12 hours *E74B* was abundant, at -3 hours *E74A* and *E74B* were at similar levels and that at pupariation there was essentially only *E74A*. While, as expected, *E74B* is abundant at PS1, we were surprised by the rapidity of the isoform switch as *E74A* transcripts are at the same level as *E74B* by PS2 and predominate by PS3. *E74A* transcripts reach their maximum at the time of the 74EF puff maximum, remain abundant during 74EF regression (PS8-10) and then decrease abruptly at PS11.

E75 transcripts are closely correlated with those of *E74* (see also Fig. 3), as would be predicted from puffing studies. The Class II *E75B* transcript is induced in parallel with the *E74A* transcript but is short lived in that it disappears from PS5 onwards while the *E75A* transcript increases from PS3 onwards. From PS5 to PS11 the *E74A* and *E75A* transcripts show a similar profile. As the *E75A* and *E75B* promoters have similar sensitivities to ecdysone (Karim and Thummel, 1992, but see Discussion) the delay in the appearance of the *E75A* transcript relative to *E75B* may be a consequence of primary transcript length (see Fig. 2). In contrast the fact that the *E75B* primary transcript is nearly 40 kb shorter than *E74A* suggests that it is induced somewhat later or that it is not spliced as rapidly as *E74A*. By comparison with *E75A* and *E75B*, the *E75C* transcript is much less abundant in the salivary gland and is rarely detected on the ethidium bromide stained gels. Hybridisation with an *E75C*-specific probe reveals two major periods of activity, the first initiated in mid instar larvae (Fig. 4) continues between PS1 and PS5, the second is at PS9 and 10.

The interpretation of *BR-C* transcripts is complex as the same RT-PCR product may derive from different promoters in the course of the response. Z1-containing transcripts are present at PS1, diminish at PS2 and thereafter are moderately abundant from PS3-PS10. In addition to the Q¹-Z1 transcripts visible on the stained gel, low levels of TNT-Q¹-Z1 and Q²-Z1 transcripts, following a similar profile, are seen by hybridisation and are specific to this period in the salivary gland (data not shown, but see Fig. 7). This is in accord with the frequency of these forms amongst Z1-containing cDNAs (Q¹-Z1, 20/25; TNT-Q¹-Z1, 3/25; Q²-Z1, 2/25 - DiBello et al., 1991). Z2-containing transcripts (barely detectable before 114 hours, see Fig. 4) show a weak activity at PS1 and 2 that increases between PS3 and 10 although there is heterogeneity in these samples. The pattern of Z3-containing transcripts is close to the sum of those of *E74A* and *B*, that is an activity at PS1 and 2 that increases appreciably by PS3, remains abundant until PS10 and diminishes by PS11. If the *BR-C* promoters resemble those of the other early genes this may indicate that a Class I promoter active at PS1 is rapidly repressed by the major increase in ecdysone titre and that simultaneously Class II transcripts are initiated from more distal promoters.

The 68C puff, containing *Sgs-3*, regresses from PS2 onwards in vivo and is rapidly repressed by ecdysone in vitro. Similarly *Sgs-3* transcription is reduced within 15 minutes of hormone treatment in vitro (Crowley and Meyerowitz, 1984), suggesting that the gene should be transcriptionally silent by PS2 or 3. In contrast *Sgs-3* transcripts remain constant and abundant until PS10, suggesting the

presence of a large stable pool of transcripts following puff regression. The level of these transcripts then drops at least ten-fold by PS11 and is a further example of an event which, at pupariation, reduces the levels of most of the transcripts that we have analysed. It may be significant that this decrease appears specific to the salivary gland (see below) at the time of glue expulsion.

The prepupal period (120-135 hours)

The late larval ecdysone response continues beyond pupariation (PS11) until PS14, some 4 hours into the prepupal period. There follows a period of low hormone titre that prepares the gland for its final response to the late prepupal peak of ecdysone at approx. 8 hour pp which ends with the histolysis of the larval gland at 13-15 hour pp. These three phases are marked by changes in the transcripts we have followed (Fig. 6).

Following the global reduction of transcripts at PS11, *E74A* and *E75B* transcripts are detected from PS12 to PS14, a co-expression similar to that seen from PS2 to PS5 (Fig. 5). For *E75* this corresponds to a further isoform switch. Between PS12 and PS14, there is a parallel but less dramatic reinduction of Z2 and Z3 transcripts while Z1-containing transcripts appear later between 4 and 6 hours pp.

EcR transcripts, which are minimal from PS7/8 onwards, increase at PS13/14 at the end of the late larval ecdysone response and thereafter remain relatively stable until PS21. This suggests that *EcR* transcripts are induced following the drop in ecdysone titre, that their induction is a prerequisite to the late prepupal response (see Discussion) and that the smaller late prepupal peak of ecdysone does not lead to the repression of *EcR* transcription. The reinduction of *E74* and *E75* from PS17 to 21 resembles a compression of their larval activity, i.e. Class I transcripts followed by Class II transcripts. However, there is at least one significant difference in the order of induction. *E74B* is induced by PS17, closely followed by *E75B*, an induction that clearly precedes that of *E74A*. The delay in *E75A* induction relative to *E75B* is very similar to that seen in PS1-4. *E75C* transcripts, detected by hybridisation, are even lower between PS11 and PS21 than in the larval period (data not shown). The duration of this response is much shorter than that of the late larval response, confirming results of both puffing studies and prior analyses of *E74A* products (see Boyd et al., 1991).

For *BR-C*, Z2 activity in the salivary gland is highest during the late larval response, although low levels of Z2 can be detected by hybridisation throughout the prepupal period (data not shown). Z1 increases between PS18/19 and PS20, while Z3 transcripts increase during ecdysone responses (PS11-PS14 and PS18-20 in Fig. 6) but have a relatively abundant basal level at most stages.

Tissue differences in the ecdysone response

The above results show that there are differences in both the isoforms of the key regulatory genes that are expressed and the timing of their expression during the responses of the salivary gland to ecdysone at three developmental stages. The prediction from puffing, genetic and molecular analyses is that similar differences exist between tissues and serve to translate a single ecdysone peak into tissue-specific

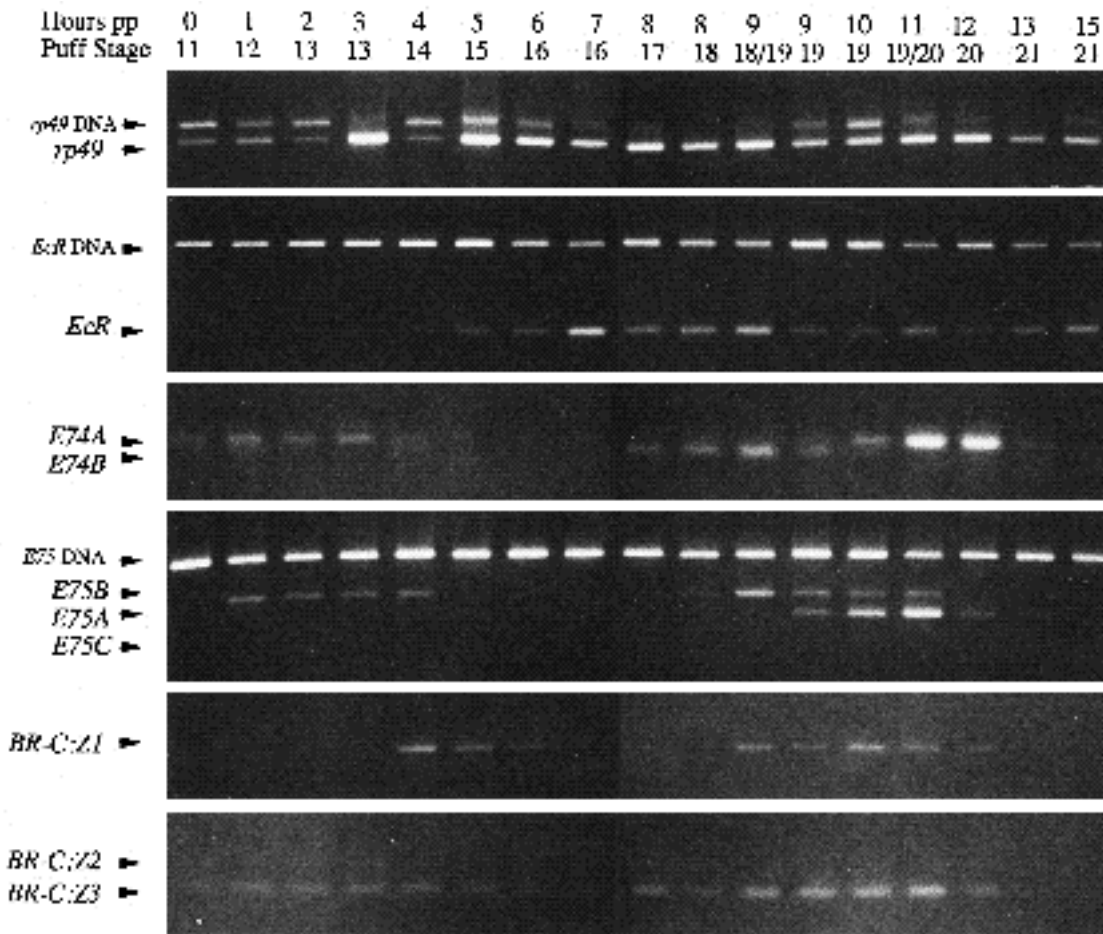


Fig. 6. The prepupal period (PS11-PS21). Animals were staged both by hours post pupariation (pp) and by salivary gland puff stage. RNAs were analysed from the sister lobe as in the samples in Fig. 5. The 0 hour pp corresponds to 120 hours after egg-laying. Puff stages follow those of Ashburner (1967) with the addition of two further intermediate stages PS18/19 and PS19/20 which are clearly distinguished at the cytological level during the late prepupal ecdysone response (see Richards, 1982). In consequence, this analysis emphasises the sequence of events rather than a simple time course (see 8 and 9 hours pp). Gland histolysis occurred at PS21, between 13 and 15 hours pp for different individuals. Variation in *rp49* products most probably derives from the batch of MMLTV RTase used for this series (data not shown). All other transcripts are analysed with AMV RTase (see Materials and methods) and their products are found at levels comparable to those seen between PS1 and PS11 (see Fig. 5).

responses. To test this we have undertaken the simultaneous analysis of transcripts in a number of tissues from individual larvae. An overall picture of the late larval response can be derived from animals at PS1, PS3, PS7 and PS11 (Fig. 7). We present results from five tissues (salivary glands, SG; gut, GT; malpighian tubules, MT; fat body, FB and wing discs, WD) from animals staged by the PS of one salivary gland. As expected from the tissue coordination model we detected transcripts of *EcR*, *BR-C*, *E74* and *E75* in all tissues during this period.

As shown above (Fig. 5), *EcR* transcripts drop dramatically in the SG from PS7 onwards. This decrease is slightly delayed in the MT, is less dramatic in the FB and GT while in the case of the WD a relatively low level of *EcR* transcripts remains unchanged throughout this period. This may be a consequence of tissue differences in *EcR* isoform expression. The *E74B* to *E74A* switch is slightly faster in the SG than the MT and WD while both the GT and FB have appreciable levels of *E74B* at PS11. This analysis is

in accord with Boyd et al., (1991) who described essentially minor tissue differences in *E74A* expression, notably a slower repression in certain tissues. More significant differences appear with the *E75* analysis, where we distinguish four different profiles. At PS1 *E75A* is active in the GT (as is *E75C*), but not in the other tissues. By PS3 both *E75B* and *E75A* are active in the SG, GT, MT and FB, but only *E75A* is active in the WD. *E75B* transcripts disappear by PS7 in the SG but are still present in the GT, MT and FB. At PS11, both *E75A* and *E75B* are present in the GT and WD, *E75B* persists in both the MT and FB while *E75* transcripts are absent from the SG. In these tissues *E75C* is a minor isoform which decreases in all tissues between PS1 and PS11. At PS1, Z1-containing transcripts from the *BR-C* are abundant only in the GT where they appear to remain constant until PS11 when a second shorter product corresponding to the Q²-Z1 isoform (see above and Fig. 2 legend) is observed. In the SG and MT, Z1 transcripts are seen at PS3 and PS7, but not PS11 while in both the FB

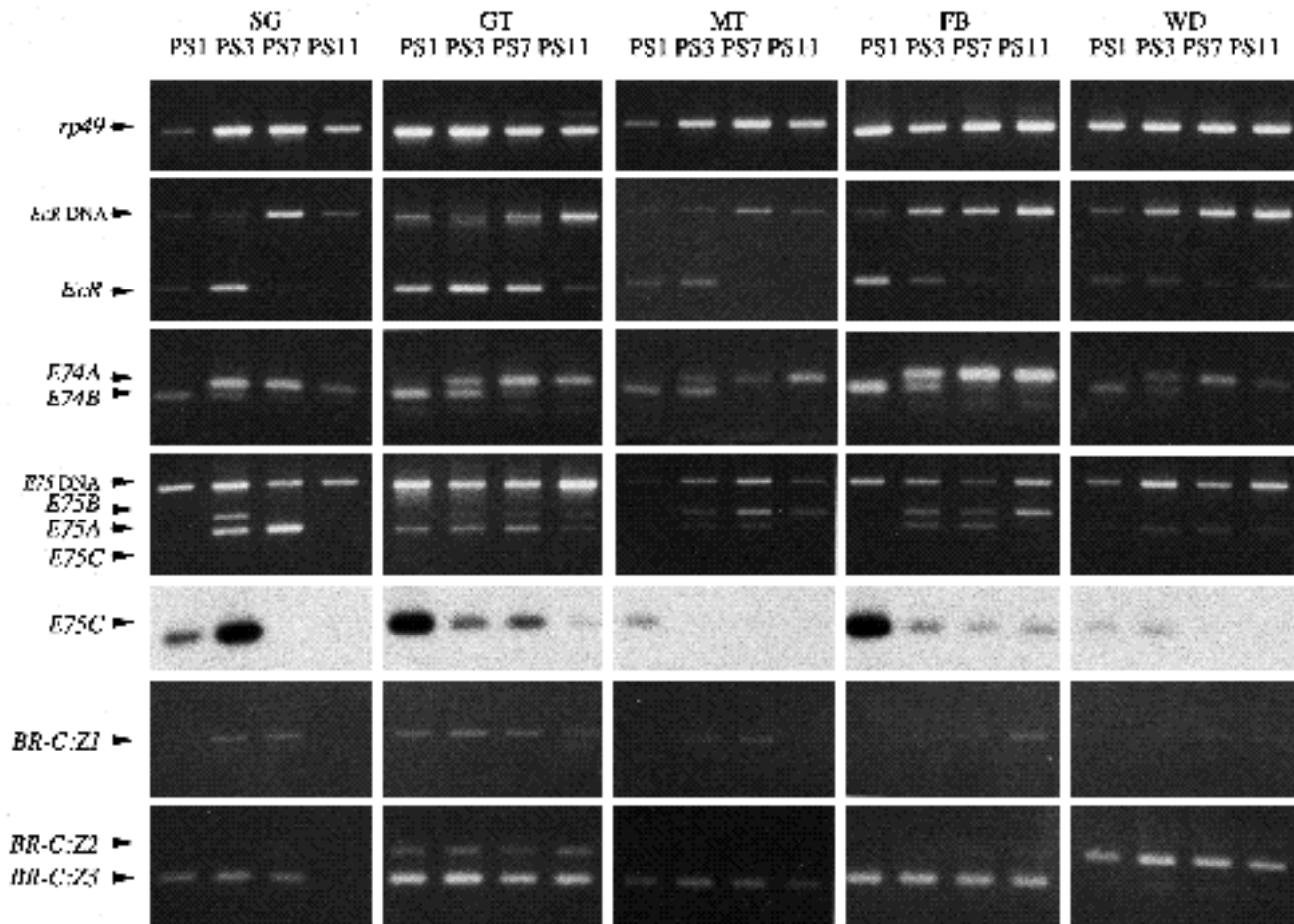


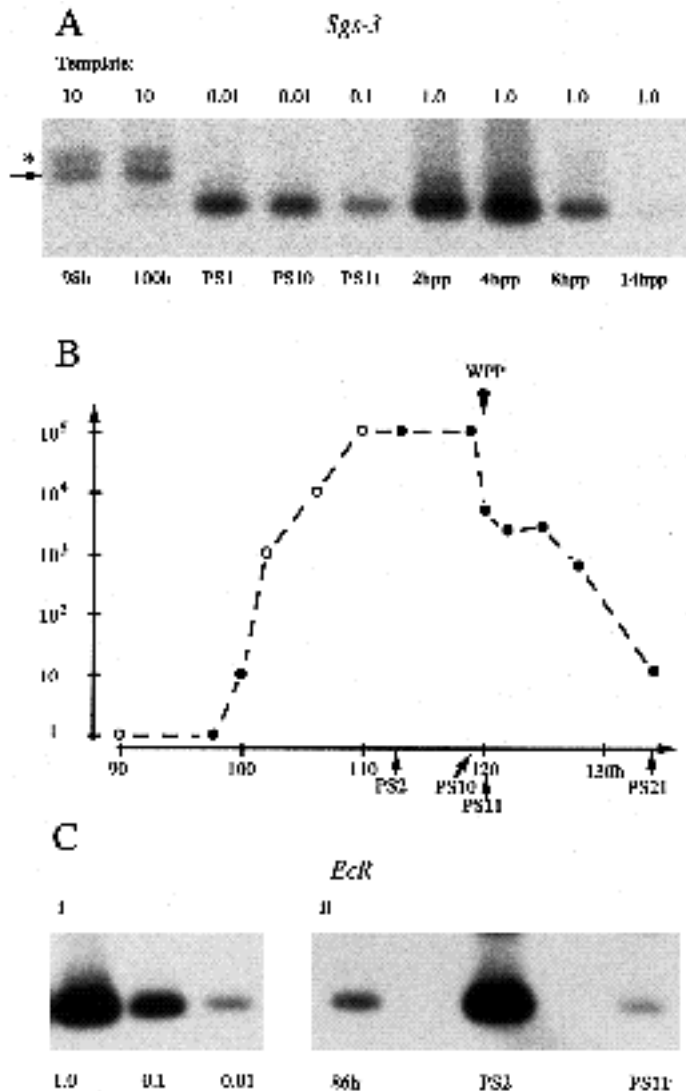
Fig. 7. The late larval ecdysone response in different tissues. Four animals from the late larval period, staged by one salivary gland lobe as PS1, PS3, PS7 and PS11 respectively, were dissected and RNA preparations made from the remaining salivary gland (SG), gut (GT), malpighian tubules (MT), fat body (FB) and the two wing discs (WD). These extracts were prepared and analysed as for the salivary glands of Figs 3-6 except that for the gut, fat body and wing discs the percentage of the RNA extract used as template was reduced by a factor of 2 for each RT-PCR assay. Products from each animal, denoted by its puff stage, are presented as five columns, one for each tissue.

and WD low levels of transcripts increase between PS3 and PS11. Note the presence of low levels of Q^2 -Z1 in the PS7 SG. Results with Z2- and Z3-containing transcripts show important quantitative differences between the tissues. In the SG there are traces of Z2 while Z3 is abundant until PS7, dropping dramatically at PS11. For the other tissues the profiles are constant but with significant differences between the tissues, i.e. MT, GT and FB have mainly Z3 with a trace of Z2 (seen on hybridisation for the MT and SG - data not shown), while the WD have mainly Z2 with a trace of Z3. These results confirm the predictions of DiBello et al., (1991), based on genetic analyses, that Z1 and Z3 should be expressed in salivary glands and Z1 and Z2 in imaginal discs.

Quantitative aspects

There is an important difference in the abundance of *Sgs-3* transcripts compared to those of the regulatory genes such as *EcR*, the former being at least 100-fold more abundant in the late larval salivary gland as determined by template dilutions (see Table 1). A second difference lies in the max-

imum and minimum levels of these transcripts, a problem that we have studied by hybridisation with radioactive probes (Fig. 8). For *Sgs-3* we first compared transcript levels at key stages by serial dilutions of the input RNA, so as to obtain bands of similar intensity on ethidium bromide stained gels (data not shown). We then assembled composite gels, which after transfer and hybridisation gave comparable bands on autoradiographs (Fig. 8A). The combination of the dilution factor and the autoradiographic signal enables us to establish relative levels for this period of development (Fig. 8B). If we set the initial value for *Sgs-3* in 98 hour glands at 1 (although we did not detect a band even on extended exposure of the autoradiograph) we estimate that it reaches 10 by 100 hours, 100,000 in late wandering larvae (PS1 to 10), and then decreases to 5,000 at PS11, 2,000 in 2 hour pp, 1,000 in 8 hour pp and remains between 100 and 10 at the time of gland histolysis in 14 hour pp. For *EcR* (Fig. 8C), unlike *Sgs-3*, we detect transcripts at all stages. The use of serial dilutions of a standard RNA extraction (Fig. 8Ci) confirms both that the assay is quantitative when using sub-saturation levels of template



and establishes a scale for estimating relative levels in the microextracts. From 86 hour, PS2 and PS11 larvae, we obtain values of 2, 100 and 1 respectively (Fig. 8Cii).

DISCUSSION

RT-PCR and the ecdysone regulation of genes - general considerations

The study of relative transcript levels shows that regulatory genes may differ from their downstream target genes not only by the overall abundance of their transcripts (the sum of the *EcR* isoform transcripts is between 100- and 1000-fold less abundant than those of *Sgs-3*) but also by their activation/repression characteristics. *EcR* transcripts are always present in this period with levels that vary at most a hundred-fold. We assume that this is reflected in the *EcR* proteins (although post-transcriptional mechanisms may modulate their presence or activity) and that even the lowest level is physiologically important for the autoregulation of *EcR*. Thus, for *EcR*, hormonal regulation appears to be overlaid on a mechanism that ensures basal levels of

Fig. 8. Relative levels of *Sgs-3* and *EcR* transcripts in larval and prepupal salivary glands. (A) *Sgs-3* transcripts from different developmental stages were analysed using template concentrations as shown (expressed in % of RNA extracted from a salivary gland), adjusted to obtain comparable levels of RT-PCR products (see text). Products were separated on a 2% agarose gel and after transfer, hybridised with an internal oligonucleotide probe (see Table 1). Exposure was for 2 hours. The *Sgs-3* transcript is first detected at 100 hours in this experiment. At 98 and 100 hours, DNA templates give rise to the product of the size expected for the *Sgs-3* primary transcript (arrow) plus a second related product (*). Thereafter, with the more dilute templates, the pre mRNA is detected as a minor band. The unexpected product (*), recognised by the internal probe, most probably results from priming 3' to the RTase primer site within the repetitive region of *Sgs-3* (data not shown). (B) The results of A (solid circles) together with time points from other series (open circles) are schematised in terms of the expression of *Sgs-3* relative to its activity in 90-98 hour larvae (set arbitrarily as 1 - see text). Extended exposure (24 hours) of the autoradiograph in A, did not reveal a band at 98 hours (data not shown). Expression (ordinate - note log scale) is estimated by a combination of the autoradiographic signal and the template concentration used to generate the RT-PCR products. Thus for example, the PS1 signal is 10-fold higher than that at 100 hours and derives from a template diluted 1000-fold - hence the overall ratio of 10^4 . Glands staged by PS are placed by arrowheads on the time scale (in hours after egg laying). Glue is released around PS11 at the time of white puparium formation (WPP).

(C) Analysis of minimum and maximum levels of *EcR* transcripts in salivary glands. (i) Aliquots from a serially diluted standard RNA preparation (arbitrary units) were used as templates in RT-PCR, separated on an agarose gel, transferred and hybridised with an internal probe (see Table 1). (ii) A second 10 μ l aliquot from the samples at 86 hours, PS2 (Fig. 4, RNA from two lobes) and PS11 (Fig. 5, RNA from one lobe) were separated on the same gel and treated in parallel with the standards of i. Both were exposed for 16 hours.

activity. Switching of *Sgs-3* is more dramatic. Prior to its activation at approx. 100 hours we do not detect transcripts, which demonstrates that although our RT-PCR assay is highly sensitive it does not detect transcripts at the level of 'illegitimate' or leaky transcription (Chelly et al., 1989). Once *Sgs-3* is activated, there is at least a 100,000-fold increase in expression as would be expected from a strong enhancer. The profile of the decrease in transcripts following pupariation is surprising. The 50-fold drop from PS10 to 2 hour pp corresponds to the image previously obtained by northern analyses (e.g. Richards et al., 1983) but thereafter RT-PCR detects non-negligible levels of transcripts until the destruction of the gland in 13-15 hour pp. This suggests either that RNA that escapes the degradation seen in PS11 salivary glands is relatively stable or that the mechanism of *Sgs-3* repression is not as effective as that which ensures its silence in early larval stages. Hybridisation analyses of *E74*, *E75* and *BR-C* isoforms resemble those of *EcR* (data not shown), and it is possible that the differences between *EcR* and *Sgs-3* in promoter regulation (modulation or on/off switching) are characteristic of regulatory and 'working' genes respectively.

It would be possible, using the relevant in vitro synthesised transcript as a standard for each reaction, to quantify the RT-PCR products in absolute terms (see Wang et al., 1989 for a related approach). An RNA standard is essen-

tial as the quantity of cDNA product obtained from the same transcript but using adjacent oligonucleotide primers may differ several-fold. Thus, within a gene, the use of a single RT primer for *E74* (or *E75*) isoforms facilitates their comparison while the relative levels of Z1, Z2 and Z3 forms of *BR-C* may be distorted by the use of three distinct RT primers.

We have worked with ethidium bromide stained gels not only to develop a non-radioactive approach, but equally so as to detect non-specific PCR products that incorporate primers but are not recognised by an internal labelled probe. In initial experiments we eliminated a number of primers that gave such products. With hybridisation, the sensitivity of the method increases a minimum of ten-fold, so that RNA from a single gland could be analysed for at least one hundred transcripts, if suitable primer pairs have been established.

Ecdysone titres and the activation of Class I and Class II early promoters in salivary glands

Ecdysone studies in the mid to late third larval instar pose problems due to the heterogeneity in the relatively large samples necessary for radioimmune assays (see Richards, 1981 for discussion). The general conclusion is that there are one (or several) small peaks from the mid instar onwards but that the massive increase in titre most probably coincides with the PS1-PS2 transition some 6-8 hours before pupariation. Although the minor peak(s) has biological significance (e.g. inducing glue gene transcripts in the salivary gland, Georgel et al. 1991; see also Andres and Cherbas, 1992), its form remains undetermined. The characteristics of this first response are that it requires only a small titre increase, presumably with a sub-optimal occupation of receptors (so that only Class I promoters with high affinity for the hormone-receptor complex are activated) and that it is very slow in comparison to those of the late larval and prepupal period. Our observation of a few animals with significant levels of *E75A* transcripts suggests that there may be pulses of hormone release that occasionally result in the brief induction of the most sensitive Class II promoter. As mid instar larvae fed high levels of ecdysone (5×10^{-4} M) respond by glue gene transcription but not pupariation we presume that either there exists a mechanism that metabolises excess hormone in this period, and maintains levels sufficient for the sustained activity of Class I promoters only, or that tissues are not yet competent for the late larval response. Although only Class I early transcripts (i.e. *E74B*, *EcR* and presumably one of the *BR-C* promoters) are induced, target genes such as *Sgs-3* are activated as a late response to hormone.

The simplest interpretation of the PS1 to PS2 transition is that an increase in ecdysone titre activates Class II promoters (*E74A*, *E75A*, *E75B* and at least one *BR-C* promoter) and represses directly or indirectly Class I promoters (*EcR*, *E74B* and at least one *BR-C* promoter). The precision of the mechanism is apparent from the synchronous switching of *E74* and *E75* transcripts at this stage. One factor in the timing of isoform appearance is the elongation rate of RNA polymerase II determined for *E74* at approx. 1.1 kb/minute (Thummel et al., 1990; Karim and Thummel, 1991) and estimated at 1.5 kb/minute for *E75* (Karim and Thummel,

1992). *E75B* primary transcripts are 40 kb shorter than those of *E74A* (see Fig. 1) which our RT-PCR assay detects at the earliest 45-60 minutes after activation of the *E74A* promoter (allowing for both transcription and splicing of exon 5 to 6; LeMaire and Thummel, 1990). As we detect these two transcripts simultaneously it suggests that the *E75B* promoter is induced slightly later than the *E74A* promoter despite the similarities in the dose-response curves determined in vitro (Karim and Thummel, 1992). Although *E75A* appears three-fold more sensitive to ecdysone than *E75B* in vitro, we detect *E75A* one PS later than *E75B* suggesting that in the salivary gland a rapid increase in ecdysone titre activates *E75B* shortly after *E75A* and that, as proposed by Karim and Thummel (1992), the timing of the appearance of transcripts is essentially a consequence of the 30 kb difference in primary transcript length.

Although *EcR* repression could be a direct consequence of the rising ecdysone titre, we favour the hypothesis that it involves the protein product of a gene induced following the PS1-PS2 transition, both because the timing coincides with late puff induction and because Karim and Thummel (1992) have shown that, in vitro, high levels of ecdysone lead to a decrease of *EcR* transcripts after some 3-4 hours of culture of larval organs.

While the rising titre and transcript length are sufficient to explain the beginning of the response, the reappearance of *E75B* from PS12 to PS 14 requires further promoter switching which may be *EcR* or ecdysone independent. Although *E74A* transcripts are equally present from PS12 to 14, *E75A* transcripts are not induced at this stage so that not all Class II transcripts are present in the early (0 to 4 hour) prepupae. There are two precedents for this activity from the extended culture of PS1 larval glands with ecdysone. There is a significant plateau in the regression profile of the 74EF and 75B puffs from 8 to 10 hours (Richards, 1976b) and *E75B* transcripts are reinduced at 8 hours of culture (Segraves and Hogness, 1990). Our in vivo analysis adds an unexpected twist in that this occurs in glands with minimal levels of *EcR* transcripts. However *E74A* transcripts are detected in late embryos and late pupae where their regulation may be ecdysone independent (Thummel et al., 1990). The early prepupal *E74A* transcripts are less abundant than those of late larvae and may simply reflect the final phase of the larval response. Similarly, the absence of the *E74A* protein in 2 and 4 hour prepupal salivary glands (Boyd et al., 1991) may indicate that these transcripts are not translated.

The *E75B* isoform is intriguing as it encodes a member of the superfamily of steroid receptors that has a single zinc finger from the DNA binding region and may not be able to bind DNA (Segraves, 1991). In the salivary gland its rapid but transient induction may indicate that it plays a role in heteromer formation between members of the steroid receptor family early in the ecdysone response (see Richards, 1992). Note that all three inductions in the late larval and prepupal period (from PS2 to PS5, PS12 to PS 14 and PS 17 to PS19/20) show a similar time course and level of expression, which may reflect a fundamental aspect of the regulation of *E75B* and its role in the salivary gland.

From 4 to 8 hours after pupariation the salivary gland must experience low ecdysone titres for the progression of

puffing patterns and the acquisition of competence (between 5 and 6 hours) for the late prepupal response to ecdysone (Richards, 1976a,b). The simplest interpretation of the present results is that the increase in *EcR* transcripts from 5 hours onwards, is the molecular basis of competence. Although glands are competent by 6 hours, *in vivo* they respond at 8 hours as seen by the PS18/19 transition. During this shorter response, *EcR* transcript levels appear to remain stable, which contrasts with their induction in the mid larval period and their repression during the late larval response. This suggests that *EcR* is not a Class I transcript in this period and may reflect changes in *EcR* isoform expression between the larval and prepupal stages. From 8 hours pp the increasing ecdysone titre sequentially activates both Class I (*E74B*) and Class II (*E74A*, *E75A* and *E75B*) transcripts. This resembles a compression of the activity seen in the larval period, where each class is associated with a distinct biological response.

Puffs and transcripts

At PS1 in wandering larvae there is little sign of puffing activity at 74EF and 75B although *E74B* transcripts are abundant. This may be a consequence of the short *E74B* transcription unit, as puff formation is related to both promoter strength and transcript length (Simon et al., 1985). PS2 is defined by the first cytological appearance of 74EF and 75B induction, which can be detected within 5-10 minutes of ecdysone treatment *in vitro* (Ashburner, 1973). However at PS2, *E74A* transcripts, which we detect at the earliest 45-60 minutes after activation of the *E74A* promoter (see above) are abundant. *In vitro*, full length *E74A* transcripts are detected between 1 and 1.5 hours of culture (Karim and Thummel, 1991). Thus *in vivo* the *E74A* promoter must be activated late in PS1, prior to puff formation. At PS4/5 when the 74EF and 75B puffs have reached their maximum size, levels of the two shorter transcripts have dropped dramatically while the longer transcripts are at their maximum levels. Puff regression is followed by the disappearance of the long transcripts, *E74A* and *E75A*, although *E74A* persists in early prepupae. However, Boyd et al. (1991) detected nuclear *E74A* in salivary glands of late larvae but not in 0 hour or 2 hour prepupae suggesting either that the transcripts are entirely cytoplasmic at this point or below the levels of detection of their *in situ* analysis. In the prepupal response, *E74B* and *E75B* transcripts are detected at PS17 (i.e. just prior to the cytological sign of the reinduction of 74EF and 75B) and thereafter are replaced by the *E74A* and *E75A* forms respectively. As the puff 75B remains partly open throughout the prepupal period it may facilitate the activation of *E75A* which precedes *E74A* in this response. The duration of puffing at 74EF and 75B and expression of the *E74A* and *E75A* transcripts is shorter in the prepupal response than the larval response.

If large puff formation is related to the presence of long Class II transcripts, short pulses of ecdysone in the period 90 to 112 hours might lead to the occasional presence of small puffs at 74EF and/or 75B seen in some nuclei (data not shown). This is consistent with the observation that PS1 glands exposed briefly to high concentrations of hormone respond by a short-lived induction of 74EF and 75B with-

out progression to the late response (Ashburner and Richards, 1976).

BR-C transcripts

In general agreement with the analysis of DiBello et al. (1991), we found that for Z1-containing transcripts, the Q¹ isoform was the most frequent, Q² occasionally reached similar levels (notably at PS11 in the gut, Fig. 7) while the maximum levels of the TNT-Q¹ form (in late larval salivary glands) are about one hundred-fold lower as determined by hybridisation with an oligonucleotide common to all forms (data not shown). Similarly the Z2 isoform, found in both whole animal and imaginal disc cDNA libraries (DiBello et al., 1991), was most abundant in the wing disc. In contrast we observed consistently higher levels of Z3 products than would be predicted from that analysis.

The unravelling of the regulation of the *BR-C* promoters and the function of the protein isoforms will no doubt prove to be complex and an extension of our present assay to include promoter usage will be necessary. The developmental profiles of Z1, Z2 and Z3 in the salivary gland are not identical, which would be the case if the relative (but fixed) strength of the splice acceptor sites determined the splicing of the core-region to the Z1, Z2 and Z3 encoding exons. The changes we observe suggest that ecdysone may regulate *trans*-acting factors, which modify splice site usage. If the hormone induces both promoter switching, as for the other early genes, and changes in the splicing pattern, there may be preferential association of certain promoters with specific zinc finger encoding regions in the *BR-C* transcripts. It is evident that in addition to this developmental regulation there are tissue-specific patterns of *BR-C* splicing (Fig. 7).

The link between *BR-C* transcripts and protein function may not be straightforward. A combination of molecular and genetic analyses suggest that Z1 transcripts correspond to *rbp* function and Z3 to *l(1)2Bc* (see DiBello et al., 1991). Our analysis in the period 88-98 hours reveals a peak of Z3 transcripts that precede *Sgs-3* activation. However, the fact that glue gene transcripts are found in *l(1)2Bc* mutants but not *rbp* mutants (Guay and Guild, 1991) suggests that in this period it is the less abundant Z1 transcripts that are essential for inducing glue gene transcription.

Stage and tissue differences in the ecdysone response in *Drosophila*

These studies, taken with other recent results add considerably to the complexity of the molecular description of the ecdysone response in *Drosophila*. Currently, the basis of stage and tissue-specific early responses may involve differences in the ecdysone titre(s), the isoforms of *EcR*, other members of the steroid receptor superfamily (see Yao et al., 1992; Thomas et al. 1993) and the accessibility of the different promoters of the early genes.

The analysis of transcripts from different tissues of the same animal confirms the predictions of the 'tissue coordination model' (Burtis et al., 1990; Thummel et al., 1990) and excludes problems that derive from the developmental heterogeneity of groups of larvae. For the late larval response alone we can distinguish four major differences between tissues. The first is the timing of the initiation of

the response, which derives from the observation of *E75A* activity at PS1 in the gut, i.e. prior to the hormone response in the other tissues. The second is the rapidity of the response, e.g. both *E74B* to *E74A* switching and the disappearance of *EcR* transcripts occurs more rapidly in the salivary gland than in the other tissues. The third is the difference in profiles of *E75A* and *E75B* transcripts, which show four distinct patterns in our PS1-11 study. Finally although *BR-C* profiles are relatively constant in this period they show clear tissue differences suggesting that tissue-specific factors modulate splice site selection.

The first two points suggest that effective hormone titres or receptor concentrations may differ between tissues. Effective hormone titres depend on the ecdysone-metabolising enzymes present in each tissue, which are not uniformly distributed. Although we have used ecdysone as a synonym for 20-OH ecdysone *in vitro*, at this stage we cannot exclude the possibility that *in vivo* the active ecdysone(s) may differ between tissues. This difference may be increased by differences in *EcR* levels (or isoforms) or the presence or absence of other members of the receptor family leading to tissue-specific heteromers. For *EcR* there are differences between tissues (Fig. 7), which require further quantitative and qualitative (isoforms) analyses. From the activity of *E75* shown in Fig. 7 and the analysis of other animals (data not shown) it appears that the late larval ecdysone response is seen first in the gut followed by the wing disc and fat body and finally in the salivary gland and malpighian tubules. For *E74*, apart from minor differences in the rapidity of the response, the profiles are very similar in the different tissues even if the abundance of *E74* transcripts relative to those of the other early genes differs between tissues (compare transcript levels in the gut and fat body, Fig. 7). Unlike the salivary gland, *E74A* transcripts remain abundant in the fat body at PS11, which as Boyd et al. (1991) have noted would explain the extended activity of the 74EF puff in this tissue (Richards, 1982). *In vitro*, Karim and Thummel (1991) detected differences from this pattern in the ecdysone induction of *E74A* and *E74B* in brains and anterior gut. *In vivo*, we did not observe such differences in brain (data not shown) and whole gut analyses (Fig. 7) and our results support the conclusion, based on the overall similarity of *E74A* expression in most tissues (Boyd et al., 1991), that although *E74* plays a central role in the ecdysone response it is not directly responsible for tissue-specific responses. It remains to be seen if the tissue differences in *E74* transcript profiles are reflected in the levels of the corresponding proteins and, more importantly, have consequences for network regulation.

Results with *E75* suggest two sources of tissue specificity. In the salivary gland the transcript profiles can be explained by the activation of the *E75A* promoter closely followed by the *E75B* promoter with transcript length then determining the order of appearance (Karim and Thummel, 1992). This clearly does not apply to the other tissues, where, in addition, *E75B* repression is also different. One interpretation is that pre-existing tissue-specific factors alter the promoter sensitivities relative to those in the salivary gland. If this is the case the assignment of an early promoter to Class I or Class II may be tissue dependent. Indeed, based on the timing of its induction in the WD,

E75B would be classed as a late transcript. A more interesting possibility is that the ecdysone titre may start to increase earlier but does so less rapidly in other tissues. In particular, the insect gut is important for ecdysone metabolism and excretion (see Isaac and Slinger, 1989) and in consequence much of the hormone delivered by the haemolymph may be inactivated rather than binding to nuclear receptors. If this is the case, a slower increase in ecdysone titre may trigger the *E75A* promoter significantly earlier than the *E75B* promoter so that in spite of the differences in transcript length, it is the *E75A* transcript that appears first in this tissue. Similar arguments will explain *E75* profiles in other tissues. However, the two mechanisms are not exclusive and both may contribute to the tissue differences we observe. In the tissues we have followed, *E75C* appears to be a minor isoform, which may explain the absence of *E75C* in the report of *E75* salivary gland cDNAs (Segraves and Hogness, 1990), the isoform, being first isolated from an adult head cDNA library (Feigl et al., 1989). It remains to be seen whether the corresponding protein plays a regulatory role during this period.

Future directions

By RT-PCR we have obtained a molecular overview of the events underlying the regulation of gene networks first studied by chromosome puffing. The technique provides an *in vivo* characterisation of each ecdysone response in terms of early gene isoform expression and generalises the analysis to tissues and stages where puffing analysis is not possible. Its extension to studies of other genes and other developmental problems is evident. As most of these molecules have homologues in vertebrates and recent results show the high degree of conservation for heterodimerisation and DNA binding amongst invertebrate and vertebrate members of the steroid receptor superfamily (Yao et al., 1992; Thomas et al., 1993), there is an obvious application to the study of hormonal responses in vertebrates. The approach is complementary to studies that use isoform-specific antibodies to establish developmental profiles of such regulatory molecules. As we detect transcripts of a number of known or putative transcription factors in all of the tissues examined, it should prove possible to follow the regulatory molecules of other gene networks (even if they are ten-fold less abundant). This is particularly appropriate where the tissues are relatively homogenous. The ability to work *in vivo* (or in organ culture) with tissues from individual staged larvae will prove useful for the analysis of cross-talk in the ecdysone-induced networks where transcripts of key genes can be analysed either in existing mutants or in transgenic larvae carrying *in vitro* modified alleles.

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