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COMPARISON OF THE EXPRESSION PATTERNS OF FIVE DEVELOPMENTALLY REGULATED GENES IN *MANDUCA SEXTA* AND THEIR REGULATION BY 20-HYDROXYECDYSONE *IN VITRO*

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Summary

A previous study, using subtractive hybridization, identified five genes (*esr16*, *esr20*, *Mng10*, *Mng14* and *tps9*) whose transcripts were up-regulated prior to metamorphosis in *Manduca sexta* nervous tissue. The developmental time points chosen for subtraction suggested that expression could be coordinately regulated and should be negatively regulated by the steroid hormones, the ecdysteroids. In the present paper, we present an analysis of the expression patterns of these five genes, using reverse transcription–polymerase chain reaction (RT-PCR), at various times during development and assess the effects of 20-hydroxyecdysone and cycloheximide on their expression *in vitro*. This analysis revealed that with the exception of *esr20* all transcripts were detectable at all times and that all but one of the genes were up-regulated *in vivo* and *in vitro* in the absence or in the presence of low levels of the ecdysteroids. In the absence of ecdysteroids, cycloheximide blocked the accumulation of only two transcripts, *esr16* and *esr20*. These results reveal a much more complex pattern of gene expression in the central nervous system prior to metamorphosis than previously imagined.

Key words: *Manduca sexta*, gene expression, development, 20-hydroxyecdysone, metamorphosis, steroid.

Introduction

Developmental processes are largely governed by temporal and spatial changes in gene expression and are controlled by autocrine, paracrine or endocrine factors. Thus, in order to understand the molecular mechanisms of developmental events, it will be important not only to identify the genes involved in developmental processes but also to determine their expression patterns and their regulation by developmental cues.

A system which has been extensively studied is the regulation of metamorphosis by the ecdysteroids in *Drosophila melanogaster*. In recent years, over 50 ecdysteroid-regulated genes have been identified and studied in detail in *Drosophila*. Simultaneous northern analysis of the expression patterns of many ecdysteroid-regulated genes in third-instar larvae and prepupae revealed that several of them were coordinately regulated, creating four waves of increased gene activity, but also showed that virtually no two expression profiles were identical (Andres *et al.* 1993). Comparison of a subset of the 50 genes using reverse transcription–polymerase chain reaction (RT-PCR) revealed a similar complexity of gene expression patterns both during development and within various tissues (Huet *et al.* 1993).

We are interested in identifying genes expressed at the onset of metamorphosis in the nervous system of another holometabolous insect, the tobacco hornworm *Manduca sexta*, which has been used extensively for studies of developmental events regulated by hormones (Truman, 1988; Riddiford, 1991; Weeks and Levine, 1992). We were particularly interested in genes whose transcripts were up-regulated during the final 24 h preceding pupal ecdysis, as a number of physiological events have been shown to be regulated at this time by the ecdysteroids (e.g. Morton and Truman, 1995). At this time in development, the ecdysteroid titers have peaked and are declining. Previous studies have shown that for pupal ecdysis to take place both the rise and subsequent fall in ecdysteroid titers are required (Truman *et al.* 1983), and we would predict that genes up-regulated at this time should be negatively regulated by the ecdysteroids.

Using subtractive hybridization, we have identified five genes, *esr16*, *esr20*, *Mng10*, *Mng14* and *tps9*, whose transcripts were not detectable or present at low levels 24 h (–24 h) prior to pupal ecdysis and clearly detectable 4 h (–4 h) before ecdysis (Mészáros and Morton, 1996*a*). We have also shown that one of these genes, *esr20*, is negatively regulated by the

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ecdysteroids (Mészáros and Morton, 1994). The initial northern blot analysis also showed that most of the transcripts were relatively rare [constituting 0.001–0.5 % of the poly(A)+ RNA pool; Mészáros and Morton, 1996*a*], which rendered this technique unsuitable for more detailed analysis. To circumvent this problem, we have developed an approach using RT-PCR that allowed the simultaneous comparison of all five transcripts. Here, we describe this approach and present the *in vivo* and *in vitro* expression profiles of the five genes.

The genes and their main characteristics are as follows: *esr20* mRNA is localized to tracheal epithelial cells within and around the nervous system. The coding sequence predicts a 20 kDa protein which has similarity to regions of nucleolin, high molecular mass neurofilament and the *knirps-related* gene product (Mészáros and Morton, 1994). *esr16* encodes a 15 kDa protein which is also specific to the tracheal epithelial cells and shows similarity to a human epididymal protein, HE1 (Mészáros and Morton, 1996*b*). *Mng10* is a single-copy gene which encodes a 26 kDa protein with similarity to the yeast transcription unit Yer082. The transcript is detectable using *in situ* hybridization in 16–20 identified neurosecretory cells (NS-L_1) in the abdominal nervous system (Mészáros and Morton, 1996*c*). *Mng14* is also expressed primarily in large neurosecretory neurons. Its sequence shows no similarity to any known sequence and does not contain any long open reading frame, suggesting that it is untranslated (Mészáros, 1995). The transcript of *tps9* also appears to be untranslated. The sequence shows similarity to a region in the honeybee mitochondrial genome. The mRNA is localized to small, subcellular patches (potentially mitochondria) along the boundary between the neuropil and cell bodies in the abdominal ganglia (Mészáros, 1995).

Materials and methods

Rearing and staging of the animals

Manduca sexta larvae were reared individually on an artificial diet (modified from Bell and Joachim, 1978) at 26 °C in a 17 h:7 h L:D photoperiod. Fourth-instar (penultimate) larvae were chosen during the molt after head capsule slippage (approximately 24 h before ecdysis) and 6 h prior to the last larval ecdysis at the time when the old head capsule was filled with air owing to the reabsorption of the molting fluid (AFBM, Copenhaver and Truman, 1982). Fifth-instar larvae were chosen immediately after ecdysis. At the end of the fifth larval stage, the animals cease feeding, empty their guts and enter the wandering stage (W). Wandering animals were selected on the morning of the day when the dorsal vessel became clearly visible, 5 days prior to ecdysis. Animals at the onset of the prepupal ecdysteroid peak were staged on the basis of the withdrawal of pigment from the ocelli. The ocelli are the simple eyes of the larva, located in five pairs on either side of the mandibles. These eyes contain black pigment which is retracted to the brain 2 days after wandering, which coincides with the beginning of the rise in ecdysteroid levels (OR stage). Animals prior to pupal ecdysis were staged using the appearance of a pair of sclerotized bars on the dorsal surface of the metathoracic segment (24 h prior to ecdysis, −24 h) and the reabsorption of the molting fluid from the anterior segments (4 h prior to ecdysis, −4 h, Truman *et al.* 1980). Ecdysing animals were dissected at the onset of the behavior (Reynolds, 1980), and the times after ecdysis were determined with reference to that time point. Adult development has been divided into 18 stages, each corresponding to approximately 1 day (P1–P18). The stage of the developing adult was determined by observation of morphological changes to the cuticle or to structures visible through the cuticle (Oland and Tolbert, 1987).

RNA purification

Abdominal nervous systems were dissected, rinsed in saline, immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated using TriZol reagent (Gibco BRL) following the manufacturer's instructions, and the amount of RNA was determined spectrophotometrically.

The RT-PCR protocol

Primer design

The PCR primers were chosen with the aid of the primer design function of GeneWorks (IntelliGenetics, version 2.4) such that they would allow identical PCR conditions for all of the genes. The primer sequences and the lengths of the amplified fragments are given in Table 1. The primers were 19–21-mers, with a G+C content of about 50 %, had almost identical melting temperature (T_m 59–62 °C) and amplified fragments that were similar in size (101–232 nucleotides, see Table 1), but separable on high-resolution agarose gels (see below). In addition to the five genes identified by subtractive hybridization, we used the gene for *Manduca sexta* glycerol-3-phosphate dehydrogenase (*G3PDH*) as an internal control.

Table 1. *Sequences of the oligonucleotides used in polymerase chain reaction amplification and length of the amplified regions*

Gene	Forward primer	Reverse primer	Length (bp)
esr20	AGGATGCTGAAGTGGTGAGC	TGTTCCTTACGTCATTCTCGG	101
esr16	CTGTGCTTCTGGTTAGTGCC	TGGAGTTCCTCTTCAGGACG	128
tps9	CGAGTTACCACACGTTAGCG	AATGGAACGTGCTACCGAAG	160
Mng10	GAAGATGTTCTCGGAATCGG	GAGCGTAATAAGCTCTGCAGG	173
Mng14	TTCTGTTCTAAACGCAGCCCG	AATTCGAACACTGGCGGTC	204
G3PDH	CGATTAAGGAACTTGAGGACG	ATAAGGAAGCGGATGCAAGG	232

RT reaction

Total RNA was isolated from five nervous systems for each time point or experimental treatment, yielding approximately 50 μg of RNA. 10 μg of this RNA was used to synthesize single-stranded cDNA in the following reaction: 50 mmol l^{-1} Tris–HCl, pH 8.3, 75 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} of each dNTP, 1 μg of oligo(dT) primer, 0.5 μl of RNAasin (Promega) and 50 i.u. of SuperScriptII (BRL) in 20 μl final volume, for 90 min at 37 °C. To ensure that the RNA templates were eliminated, 2 i.u. of RNAase H was added to each reaction; the reactions were then incubated for a further 15 min at 37 °C, followed by heat inactivation of the enzymes at 70 °C for 15 min.

Because the intron–exon structure of the genes is not known, it was not possible to design primers that would amplify cDNA only. Therefore, it was necessary to ascertain that amplification of contaminating genomic DNA did not contribute to the signal. To test this, we treated a 20 μg sample of the RNA preparations with DNAaseI, prior to the RT reaction (50 i.u. of DNAaseI at 30 °C for 30 min, followed by heat-inactivation of the enzymes), and found no difference between the treated and non-treated samples.

PCR reactions

Samples of the RT reactions were amplified in 60 mmol l^{-1} Tris–HCl, pH 10, 15 mmol l^{-1} $(NH_4)_2SO_4$, 2 mmol l^{-1} $MgCl_2$, 0.125 μg of each primer, 200 μmol l^{-1} of each dNTP and 1 i.u of AmpliTaq polymerase (Perkin Elmer) in 20 μl final volume. The amplification was performed in a Perkin Elmer 9600 thermocycler with the following cycle parameters: 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; followed by 5 min at 72 °C and continuous hold at 4 °C. The amounts of RT reaction to be used for each gene were chosen such that they would yield subsaturation levels of PCR products after 30 cycles (see Fig. 1). The amounts of template were determined using a dilution series of the RT reactions and are given as the amount of initial total RNA: 100 pg for *esr20*, 200 pg for *esr16*, 1 ng for *tps9*, 100 ng for *Mng10*, 1 ng for *Mng14* and 200 ng for *G3PDH*. After amplification, one-fifth of the reactions were separated on a 2 % agarose gel for routine analysis and on a 3.5 % MetaPhor agarose gel (FMC BioProducts) in 1×TAE (0.04 mol l^{-1} Tris-acetate, 0.001 mol l^{-1} EDTA) at 4.5 V cm^{-1} at 4 °C for 6 h. The gel was post-stained with ethidium bromide and photographed for final comparison. With each set of reactions, a control which did not include template was also processed.

To confirm that the amplified fragments were those predicted, the PCR products were cloned using a TA cloning kit (Invitrogen) and sequenced using the Sanger dideoxy method (Sanger *et al.* 1977).

Tissue culture experiments

Abdominal nervous systems were dissected from the animals, rinsed in insect saline (Ephrussi and Beadle, 1936) and incubated in tissue culture medium (TC-100, Gibco) supplemented with 2.5 μg ml^{-1} amphotericin B, 50 μg ml^{-1} streptomycin and 50 i.u. ml^{-1} penicillin and the appropriate amount of 20-hydroxyecdysone (20-HE) or cycloheximide. The concentration of the 20-HE stock solution was checked spectrophotometrically (ε_{240}=12 670; Meltzer, 1971). The incubations were carried out at room temperature (22 °C) for 24 h in small tissue culture dishes placed in a 15 cm Petri dish onto wet Kimwipes in an oxygenated environment with the addition of phenyl thiocarbamide as an antioxidant.

Results

Establishment of the RT-PCR conditions

The basis of non-competitive RT-PCR is the establishment of reaction conditions under which the changes in the quantity of input RNA are reflected in the amount of the PCR product. The amount of PCR product doubles in each cycle until it reaches a plateau. Similar saturation is observed for a given number of cycles when the amount of template is varied. Thus, the utility of this method for the analysis of relative amounts of mRNA depends on finding subsaturation reaction conditions by varying the number of cycles and/or the amount of template in the reaction. Once the appropriate reaction conditions are found, the reliability of the method depends primarily on the integrity of the input RNA. Therefore, it is essential to assay an internal standard in each sample.

For each gene, we designed PCR primers that allowed identical PCR conditions and that amplified small fragments of similar size (101–232 bp). The advantage of the small fragments was that they could be separated on high-resolution agarose gels, making it possible to compare all five transcripts in one lane of the gel. By holding the cycle number constant at 30 cycles, we were able to run many samples simultaneously and thus the only variable was the amount of template added. We performed PCR reactions on a dilution series of the product of the RT reaction and chose the amount of starting material that yielded a signal at approximately 80 % of the saturating levels in the −4 h samples. An example of such a dilution series is shown in Fig. 1 for *G3PDH*. At mRNA levels below those at −4 h, the relationship between transcript levels and the signal on ethidium-bromide-stained gels was linear, whereas at mRNA levels above this the comparison was less accurate. When examining the following results, it should also be kept in mind that the abundance of these transcripts is significantly different: *Mng10* is the rarest mRNA, *Mng14* and *tps9* are approximately 100 times more abundant, and *esr16* and *esr20* are 500 and 1000 times more abundant than *Mng10*, respectively, on the basis of the amount of template needed.

Effects of 20-HE and cycloheximide on the expression of the genes in culture

One of our initial predictions was that the subtraction hybridization paradigm would identify genes whose transcripts begin accumulating after a peak of steroid levels had declined (see Mészáros and Morton, 1994). Most genes with such regulation in *Drosophila* have two important features in common. First, they are repressed by high levels of the

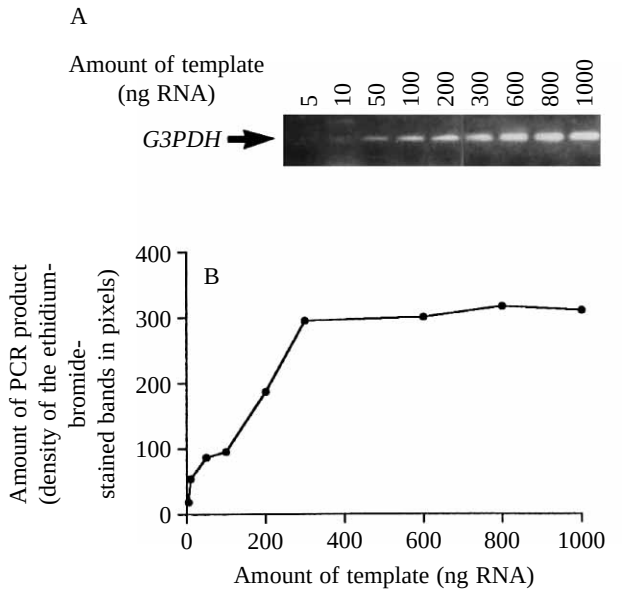

Fig. 1. Establishment of non-saturating conditions for the reverse transcriptase–polymerase chain reaction (RT-PCR) and the relationship between the quantity of input RNA and the intensity of the ethidium-bromide-stained PCR product for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*). 10 μg of total RNA from −4 h abdominal nervous systems was reverse-transcribed and the reaction mixture was diluted to correspond to the amounts of RNA shown in A. Following PCR amplification of the cDNA (30 cycles), one-fifth of the reaction was separated on a 2 % agarose gel and stained with ethidium bromide (A). The density of the bands was then measured using densitometry and NIH image 1.44 and plotted against the amount of input RNA (B).

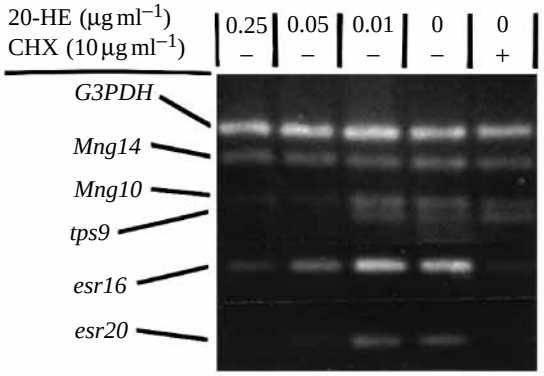

Fig. 2. Regulation of the five genes in cultured nervous systems. Abdominal nervous systems were isolated from animals 24 h prior to pupal ecdysis and incubated for 24 h in the presence of different 20-hydroxyecdysone (20-HE) concentrations and in the absence of 20-HE without or with 10 μg ml^{-1} cycloheximide (CHX). Following incubation, total RNA was extracted and 10 μg was reverse-transcribed using oligo(dT) primers, for each treatment. Samples of these reactions corresponding to 200 ng (*G3PDH*), 100 ng (*Mng10*), 1 ng (*Mng14* and *tps9*), 200 pg (*esr16*) and 100 pg (*esr20*) of total RNA were used as the PCR template. For each condition, one-quarter of each of the six PCR reactions was pooled and loaded together and separated by high-resolution agarose gel electrophoresis.

ecdysteroids both *in vivo* and *in vitro*; second, the accumulation of their transcripts requires protein synthesis (Richards, 1980; Apple and Fristrom, 1991; Woodard *et al.* 1994). To test whether the appearance of any transcript was inhibited by high steroid levels and sensitive to protein synthesis inhibitors, we cultured −24 h nervous systems for 24 h in the presence of decreasing concentrations of 20-HE, the major biologically active ecdysteroid, and in the absence of hormone with or without the addition of cycloheximide (CHX, 10 μg ml^{-1}).

The results are shown in Fig. 2. The signal on the ethidium-bromide-stained gel appeared to be identical in all lanes for G3PDH, suggesting that the RNA was intact and that the efficiency of the reverse transcription reaction was comparable in all samples. The quantity of PCR product for *Mng14* also did not vary in the presence of 20-HE or CHX, although previous northern blot analysis clearly showed that levels of the transcript increase between −24 and −4 h (Mészáros and Morton, 1996*a*). *Mng10* and *tps9* responded similarly to each other: the amount of transcript was negatively regulated in the presence of 20-HE concentrations greater than 0.01 μg ml^{-1}, and transcript accumulation in the absence of 20-HE was unaffected by CHX. The expression of *esr16* and *esr20* was also negatively regulated by 20-HE, with reduced levels of PCR product detectable in the presence of 20-HE concentrations greater than 0.01 μg ml^{-1}, but for these genes expression in the absence of 20-HE was repressed by CHX.

Thus, although all five genes were identified in the same subtractive hybridization protocol and all five have been shown to be up-regulated during the 24 h prior to pupal ecdysis, on the basis of their responses to 20-HE and CHX they fall into three classes: those unresponsive to 20-HE and CHX (*Mng14*), those negatively regulated by 20-HE and unresponsive to CHX (*Mng10* and *tps9*) and those negatively regulated by 20-HE and sensitive to CHX (*esr16* and *esr20*). It is noteworthy that the measurements of abundance obtained for *esr20* are identical to those seen with northern blot analysis (see Mészáros and Morton, 1994), confirming the validity of this method.

Expression of the genes during development

Given these differences in the responses of these genes to 20-HE and CHX, we wanted to determine whether they showed coordinate expression patterns during development *in vivo* or whether they varied independently. We first looked in more detail at times prior to and just after pupal ecdysis. These results are shown in Fig. 3. All of the PCR products showed an increase in levels between −24 and −4 h, as we have described previously for northern blot analysis (Mészáros and Morton, 1996*a*). Another similarity for all the genes is that levels of their transcripts appear to increase first at about −16 h.

At pupal ecdysis and at times shortly afterwards, the expression patterns of the genes differ from one another. The amounts of PCR product for *Mng14*, *Mng10* and *esr16* stay virtually unchanged for the first hour after ecdysis, whereas the amounts of product for *tps9* and *esr20* decline at ecdysis. The

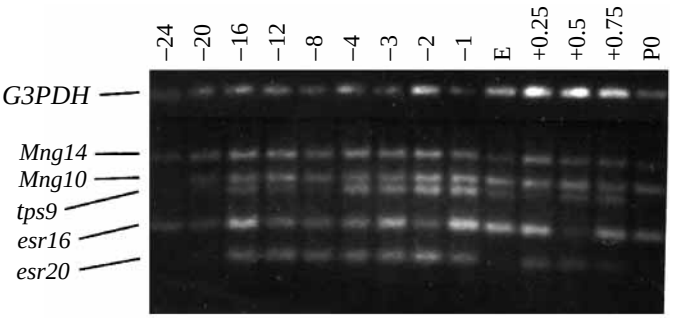

Fig. 3. Analysis of the transcripts between –24 h and the early pupal stage (P0), using RT-PCR. The developmental stages are marked in hours with respect to ecdysis (E), and the bands corresponding to the individual genes are labeled on the left. *G3PDH* was used as a control and was processed in parallel with the rest of the samples, but run on a separate gel. Samples were prepared as described in the legend to Fig. 2.

pattern of expression of *esr20* (an abrupt decline of transcript at ecdysis followed by a transient presence at 0.25 and 0.5 h) is again similar to that seen on northern blots (Mészáros and Morton, 1994).

We also analyzed the expression patterns of these genes at the fourth to the fifth larval ecdysis, throughout the fifth instar and during adult development. These results along with the ecdysteroid titers at these times are shown in Fig. 4. To make the interpretation of the results easier, the intensity of the bands from the ethidium-bromide-stained gels (Fig. 4C) was measured by densitometry, normalised to the density of the control gene, *G3PDH*, and plotted (Fig. 4B). This representation shows the relative changes in transcript levels in this particular experiment and does not attempt to quantify the transcripts.

Again, a number of different patterns emerge. All of the transcripts show an increase just prior to both larval and pupal ecdysis. Immediately following either ecdysis, however, different genes behave differently: levels of *Mng14*, *tps9*, *esr16*

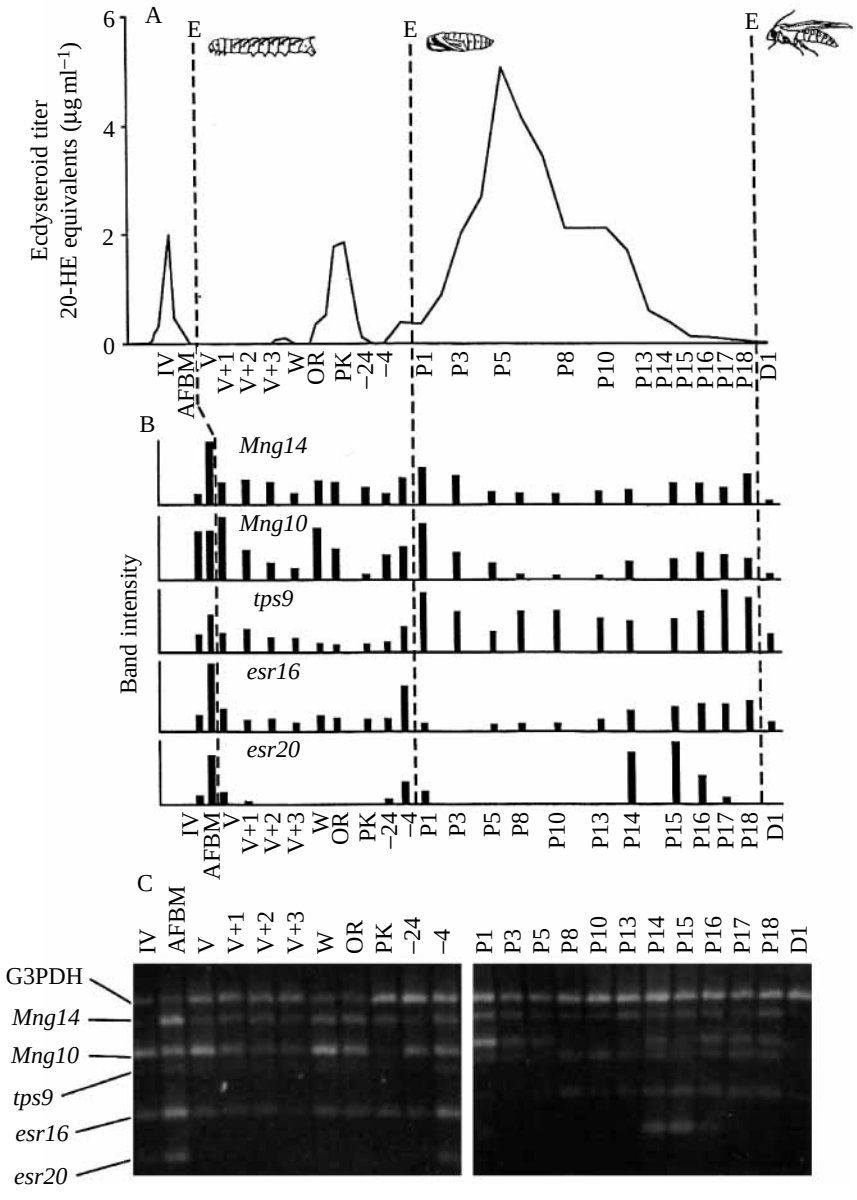

Fig. 4. Comparison of the expression patterns from late fourth-instar larvae to the adult. (A) Ecdysteroid titers. Data for the fourth (IV) and early fifth (V) instar were taken from Bollenbacher *et al.* (1975), for the prepupal peak (PK) from D. B. Morton (unpublished results) and for adult development from L. P. Tolbert (personal communication). (B) Graphical representation of the intensity of bands (measured using densitometry and NIH image 1.44 and normalized to G3PDH) on the gel shown in C). (C) Ethidium-bromide-stained gels of the separated PCR fragments. The gels were prepared as described in Fig. 2. For more details and an explanation of the abbreviations and stages used, see Materials and methods. D1, adult day one; E, ecdysis.

and *esr20* all decline after larval ecdysis, whereas levels of *Mng10* continue to increase. At pupal ecdysis, levels of *Mng14*, *Mng10* and *tps9* increase, whereas levels of *esr16* and *esr20* decline. Just prior to pupal development (at the wandering stage), *Mng14* and *Mng10* show transient increases in expression, whereas levels of the other genes remain constant throughout the fifth instar.

In general, during adult development, most of the transcript levels decline between P1 and P8 and then increase towards the end of adult development, although *tps9* remains at fairly constant levels throughout the pupal and developing adult stages. *esr20* reaches its peak expression first, at P15, and expression then declines towards P18, whereas expression of the other genes stays at relatively high levels until adult ecdysis, but declines soon after adult emergence.

There is a broad similarity in the expression patterns for all the genes: during times of high ecdysteroid titers, before larval and pupal ecdysis and in the middle of adult development, the transcript levels are relatively low. The major differences, however, come immediately after ecdysis, where expression of some increases and of others declines, and during the onset of pupal development, where expression of some stays unchanged and of others shows transient increases. When comparing the expression patterns *in vivo* and those in response to 20-HE and CHX in tissue culture, no two genes show the same expression patterns in all conditions.

Discussion

The *in vivo* data show that, as predicted by the subtractive hybridization rationale, the mRNA levels of all the genes increase at times of low ecdysteroid titers and that, with the exception of *Mng14*, all of the transcripts are more abundant at low 20-HE levels *in vitro*. The analyses, however, also revealed several unexpected points, such as the lack of sensitivity of three of the genes to inhibition of protein synthesis *in vitro* and an increase in transcript levels for some of the genes after ecdysis and just prior to pupal development.

Two categories of genes with similar properties to those of *esr16* and *esr20* have been described in *Drosophila*: the secondary response genes and the genes corresponding to the mid-prepupal puffs. *In vitro*, the *Drosophila* genes and *esr16* and *esr20* are repressed by high ecdysteroid concentrations. In addition, they are expressed at relatively low ecdysteroid levels both *in vitro* and *in vivo*, and the accumulation of the majority of these transcripts is dependent on protein synthesis. The two major differences between the two classes of *Drosophila* genes appear to be the threshold concentrations of 20-HE below which their transcripts start accumulating (10^{-7} mol l^{-1} for the secondary response genes, 10^{-8} mol l^{-1} for the mid-prepupal genes) and the timing of their expression *in vivo* (Andres *et al.* 1993). The timing of the maximum *esr16* and *esr20* mRNA levels before ecdysis and the threshold 20-HE concentration below which they can be activated (0.01 μg ml^{-1} or 2.5×10^{-8} mol l^{-1}) suggest that they are more similar to the *Drosophila* mid-prepupal genes than to the secondary response genes. Given the differences in the physiology of ecdysis and the changes in ecdysteroid titers in the two species, however, it remains to be seen whether such comparisons are meaningful.

In summary, in contrast to our expectations, although the expression patterns of these genes had some broad similarities to each other, no two of these genes behaved in an identical manner. It will be interesting to analyze the regulation of these genes in more detail and to determine at what level(s) they are regulated. Despite their differences, it is generally true that their mRNA levels are lower in the presence of the ecdysteroids than in the absence of the hormone; thus, they could be excellent tools in understanding the different ways in which the hormone–receptor complex affects gene expression.

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