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), 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

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, 1996a). We have also shown that one of these genes, esr20, is negatively regulated by the...
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, 1996a], 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, 1996b). 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-L1) in the abdominal nervous system (Mészáros and Morton, 1996c). 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 17:7 h L:D photoperiod. The fourth-instar (penultimate) larvae were chosen during the molt after cephalic capsule slippage (approximately 24 h before ecdisis) and 6 h prior to the last larval ecdisis 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 ecdisis. 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 ecdisis. Animals at the onset of the prepupal ecdisis 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 ecdisisory levels (OR stage). Animals prior to pupal ecdisis were staged using the appearance of a pair of sclerotized bars on the dorsal surface of the metathoracic segment (24 h prior to ecdisis, −24 h) and the reabsorption of the molting fluid from the anterior segments (4 h prior to ecdisis, −4 h, Truman et al. 1980). Ecdisising animals were dissected at the onset of the behavior (Reynolds, 1980), and the times after ecdisis 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 (Tm 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>esr20</td>
<td>AGGATGCCTGAAAGGATGAGC</td>
<td>TGGTCCTTACGGTATTCTCGG</td>
<td>101</td>
</tr>
<tr>
<td>esr16</td>
<td>CGTGCTCTCTGTTAGGACG</td>
<td>GGAGGTCCCTCTCAGAGGACG</td>
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</tr>
<tr>
<td>tps9</td>
<td>CAGATTACCCCACGTTACTCGG</td>
<td>AAATTGAAACGGCTACCGGAG</td>
<td>160</td>
</tr>
<tr>
<td>Mng10</td>
<td>GAAAGGTTTCTGGAATTACG</td>
<td>GAGGTAATAAGCTCCTAGGG</td>
<td>173</td>
</tr>
<tr>
<td>Mng14</td>
<td>TTCTGTTCTTAACGCGAGCCCG</td>
<td>AATTTCGAAACTCGCGCCGTC</td>
<td>204</td>
</tr>
<tr>
<td>G3PDH</td>
<td>CGATTAAAGGAACCTGAGGACG</td>
<td>ATAGGAAACCGGATCGAG</td>
<td>232</td>
</tr>
</tbody>
</table>
Expression of Manduca sexta genes

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 MgCl₂, 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 RNAsase 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 DNAsaseI, prior to the RT reaction (50 i.u. of DNAsaseI 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 (NH₄)₂SO₄, 2 mmol l⁻¹ MgCl₂, 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 (ε₂₄₀=12670; 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
ecdysones 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 abdominal nervous systems for 24h in the presence of decreasing concentrations of 20-HE, the major biologically active ecdysteroid, and in the absence of 20-HE and CHX, as we have described previously for northern blot analysis (Mészáros and Morton, 1994), confirming the validity of this method.

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 to previous northern blot analysis clearly showed that levels of Mng10 and tps9 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 ecdisis. 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, 1996a). Another similarity for all the genes is that levels of their transcripts appear to increase first at about -16 h.

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

<table>
<thead>
<tr>
<th>Amount of input RNA (ng RNA)</th>
<th>Amount of PCR product (density of the ethidium-bromide-stained bands in pixels)</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>400</td>
</tr>
<tr>
<td>20</td>
<td>300</td>
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<tr>
<td>30</td>
<td>250</td>
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<td>50</td>
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<tr>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>70</td>
<td>50</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

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 vitro or whether they varied independently. We first looked in more detail at times prior to and just after pupal ecdisis. 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, 1996a). Another similarity for all the genes is that levels of their transcripts appear to increase first at about -16 h.

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<table>
<thead>
<tr>
<th>20-HE (μg ml^-1)</th>
<th>CHX (10 μg ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>0.33</td>
<td>-</td>
</tr>
<tr>
<td>1.00</td>
<td>+</td>
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</tbody>
</table>

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 gyceraldehyde-3-phosphate dehydrogenase (G3PDH). 10 μg of total RNA from -4h 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 24h prior to pupal ecdisis and incubated for 24h in the presence of different 20-hydroxyecdyson (20-HE) concentrations and in the absence of 20-HE without or with 10μg ml^-1 cycloheximide (CHX). Following incubation, total RNA was extracted and 10μg was reverse-transcribed using oligo(dT) primers, for each treatment. Samples of these reactions corresponding to 200ng (G3PDH), 100ng (Mng10), 1 ng (Mng14 and tps9), 200pg (esr16) and 100pg (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.

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 vitro or whether they varied independently. We first looked in more detail at times prior to and just after pupal ecdisis. 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, 1996a). Another similarity for all the genes is that levels of their transcripts appear to increase first at about -16 h.

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

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...
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 eclosion, 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 eclosion 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 in vivo, where expression of some increases and of others declines, and during the onset of pupal development, whereas expression of the other genes stays at relatively high levels until adult eclosion, but declines soon after adult emergence.

There is a broad similarity in the expression patterns in all conditions.

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} \text{ mol} \text{ L}^{-1} for the secondary response genes, 10^{-8} \text{ mol} \text{ 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 eclosion and the threshold 20-HE concentration below which they can be activated (0.01 \mu g \text{ ml}^{-1} or 2.5 \times 10^{-8} \text{ mol} \text{ 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 eclosion 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.

This work was supported by NIH grant NS29740 (D.B.M.), by the Alfred P. Sloan Foundation (D.B.M.) and by a Flinn Foundation Training Grant in Developmental Neuroscience (M.M.). We also wish to thank David M. Rivers for providing us with the Manduca sexta G3PDH clone.

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