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DEVELOPMENTAL BIOLOGY

Developmental Biology 258 (2003) 76–90

www.elsevier.com/locate/ydbio

E74 exhibits stage-specific hormonal regulation in the epidermis of the tobacco hornworm, *Manduca sexta*

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Received for publication 2 December 2002, revised 31 January 2003, accepted 4 February 2003

Abstract

The transcription factor E74 is one of the early genes induced by ecdysteroids during metamorphosis of *Drosophila melanogaster*. Here, we report the cloning and hormonal regulation of E74 from the tobacco hornworm, *Manduca sexta* (*Ms*E74). *Ms*E74 is 98% identical to that of *D. melanogaster* within the DNA-binding ETS domain of the protein. The 5′-isoform-specific regions of *Ms*E74A and *Ms*E74B share significantly lower sequence similarity (30–40%). Developmental expression by Northern blot analysis reveals that, during the 5th larval instar, *Ms*E74B expression correlates with pupal commitment on day 3 and is induced to maximal levels within 12h by low levels of 20-hydroxyecdysone (20E) and repressed by physiologically relevant levels of juvenile hormone I (JH I).

Immunocytochemical analysis shows that *Ms*E74B appears in the epidermis before the 20E-induced Broad transcription factor that is correlated with pupal commitment (Zhou and Riddiford, 2001). In contrast, *Ms*E74A is expressed late in the larval and the pupal molts when the ecdysteroid titer has declined to low levels and in the adult molt just as the ecdysteroid titer begins to decline. This change in timing during the adult molt appears not to be due to the absence of JH as there was no change during the pupal molt of allatectomized animals. When either 4th or 5th instar larval epidermis was explanted and subjected to hormonal manipulations, *Ms*E74A induction occurred only after exposure to 20E followed by its removal. Thus, *Ms*E74B appears to have a similar role at the onset of metamorphosis in *Manduca* as it does in *Drosophila*, whereas *Ms*E74A is regulated differently at pupation in *Manduca* than at pupariation in *Drosophila*.

Keywords: *Manduca sexta*; Metamorphosis; Ecdysone-inducible early gene; Epidermis; Juvenile hormone; Pupal commitment; Gene regulation; Transcriptional regulation

Introduction

Metamorphosis, the transition from the larval to the adult form, involves widespread molecular and cellular changes that result in dramatic morphological and physiological alterations within the animal. These changes encompass the reprogramming or differentiation of certain tissues, histolysis of others, and the de novo formation of certain structures. Metamorphic events are driven and coordinated by the actions of two hormones. The steroid 20-hydroxyecdysone (20E) triggers and orchestrates molting processes, including those that occur during metamorphosis, and the sesquiterpenoid juvenile hormone (JH) determines the nature of the molt (larval–larval or larval–pupal) (Riddiford, 1996). The actions of the ecdysteroids were first shown to affect transcriptional puffing patterns of the polytene chromosomes in the salivary glands of *Chironomus tentans* (Clever and Karlson, 1960). Later, work by Ashburner in *Drosophila melanogaster* led to the model that a liganded ecdysone receptor complex would initiate a hierarchy of gene activation and repression events resulting in the tissue- and cell-specific changes observed during metamorphosis (Ashburner et al., 1974). As predicted, mutant analysis and molecular cloning have shown that several of the early genes directly induced by 20E—E74, E75, and Broad (BR)—are transcription factors that play critical roles at the time of metamorphosis (Burtis et al., 1990; Segraves and

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doi:10.1016/S0012-1606(03)00105-2

Hogness, 1990; DiBello et al., 1991; Karim and Thummel, 1991, 1992; Karim et al., 1993; Fletcher and Thummel, 1995; Fletcher et al., 1995, 1997; Thummel, 1996). BR is a key gene for metamorphosis to the pupa as *Drosophila br* null mutant larva cannot pupariate (Kiss et al., 1988; Bayer et al., 1997), yet its absence is required for the second metamorphic molt of the pupa to the adult (Zhou and Riddiford, 2002).

The E74 gene, located within the 74EF chromosome puff of *D. melanogaster*, produces two splicing variants comprising alternative N termini and a common C terminus that contains an ETS DNA-binding domain (Burtis et al., 1990). This highly conserved motif is widespread throughout the animal kingdom and includes greater than 60 genes (Graves and Petersen, 1998). All members of this family have similar binding specificities and recognize the canonical consensus sequence AA(C/T)C(C/A)GGAAGT (Urness and Thummel, 1990). Although the actions of *D. melanogaster* E74A and E74B (*Dm*E74A, *Dm*E74B) are potentially mediated through binding to the same *cis*-regulatory elements, the two isoforms appear to play different roles in mediating transcriptional responses. *Dm*E74B can act as a repressor of late gene expression, while *Dm*E74A acts as an activator for several identified late genes (Fletcher et al., 1997). At the onset of metamorphosis, *Dm*E74B is expressed in response to low levels of 20E and is thought to play a critical role in repressing late gene activation; it also is important for full induction of *Dm*E74A when circulating 20E titers increase to high levels (Fletcher et al., 1995, 1997). *Dm*E74A then activates a subset of late genes. This relationship between isoforms therefore mandates a critical role for the temporal and spatial patterns of *Dm*E74 expression in coordinating molecular events associated with metamorphosis.

The insect epidermis is responsible for synthesizing and secreting components of the cuticle, and this cuticle changes dramatically in composition during metamorphosis from a larva to a pupa to an adult. Unlike *Drosophila*, in which the adult epidermis is derived from imaginal precursor cells that remain undifferentiated during larval life, the abdominal epidermis of the lepidopteran *Manduca sexta* is polymorphic in that the cells switch to a new program of differentiation at each metamorphic transition. During larval life in response to high ecdysteroids in the presence of JH, this epidermis makes a new larval cuticle for each molt. Then, at the onset of metamorphosis, exposure to low ecdysteroid in the absence of JH irreversibly commits the larval epidermis to a program of pupal differentiation (Riddiford, 1976, 1978; Mitsui and Riddiford, 1978). The pupal cuticle is produced when the epidermis is next exposed to high ecdysteroid, irrespective of the presence or absence of JH (Kiguchi and Riddiford, 1978; Hiruma et al., 1991). However, if JH is present during the initial exposure to the low ecdysteroid, the epidermis remains committed to larval differentiation (Truman et al., 1974; Riddiford, 1976, 1978; Mitsui and Riddiford, 1978).

Here, we report the cloning and characterization of temporal expression patterns for *Manduca* E74A and E74B (*Ms*E74A, *Ms*E74B) during the penultimate and final larval instars and at the onset of adult development. Our data show that *Ms*E74A is expressed relatively late in the larval and pupal molts, and this expression requires exposure to 20E followed by its removal. In the adult molt, *Ms*E74A appears just as the adult cuticle is being deposited. By contrast, *Ms*E74B appears primarily at the time of pupal commitment shortly before the appearance of BR and can be induced by low concentrations of 20E when JH is absent.

Materials and methods

Animals

Tobacco hornworm (*M. sexta*) larvae were individually reared on artificial diet at 25.5 ± 1.0°C in a 12L:12D or 17L:7D photoperiod as previously described (Bell and Joachim, 1976; Truman, 1972). Lights-off is designated as 00:00 Arbitrary Zeitgeber Time (AZT). Developmental staging used time after ecdysis and morphological landmarks during development when available (Truman, 1972; Truman and Riddiford, 1974; Truman et al., 1980; Curtis et al., 1984; Jindra et al., 1997; Langelan et al., 2000).

Fourth instar larvae were allatectomized (removal of the corpora allata, the source of JH) 5–6 h before head capsule slippage during the molt to the 5th instar as previously described (Hiruma and Riddiford, 1984). These animals form black 5th instar larvae (Truman et al., 1973; Hiruma and Riddiford, 1984), then metamorphose to pupae with some adult characteristics in the eye and wing (Kiguchi and Riddiford, 1978).

Isolation of E74 clones from M. sexta

Degenerate PCR from several *M. sexta* cDNA libraries was used to selectively amplify a short segment of the conserved E74 *ets* domain. PCR primer sequences were as follows: outside forward primer: 5′-AYNTAYYTNT-GGSARTTYYT-3′; nested forward primer: 5′-CMN-MGNTTYATHAARTGGAC-3′; outside reverse primer: 5′-TCRTARTTCATNTYNGGYTT-3′; nested reverse primer: 5′-RTTYTTNYKCTANCCCCA- 3′. Amplification occurred with annealing temperatures of 44°C for 30 s, extensions of 72°C, 60 s and denaturing steps of 93°C for 20 s for 38 cycles. The resulting products generated from F1, R1 combinations were used as template DNA for a second round of PCR using primers F2 and R2. Following cloning and sequencing, a short fragment of *Ms*E74 was then used to screen *Manduca* cDNA libraries generated from either a mixture of pupal wing and epidermis (days 8 and 9 pupae) (Jindra et al., 1997) or mixed tissues of day 2, 4th instar and wandering +1 day larvae (Jindra et al., 1996)

under high-stringency screening conditions (Sambrook et al., 1989). Phage DNAs were purified by using the Lambda DNA isolation kit (Qiagen), subcloned into Bluescript (Stratagene), and sequenced using BigDye Terminator reactions (ABI) and automated sequencing.

Northerns

Northern blotting protocols were as previously described (Sambrook et al., 1989). Briefly, RNA from abdominal epidermis, either freshly dissected or from primary culture, was isolated by using a modification of the guanidinium isothiocyanate method (Hiruma et al., 1997; Chomczynski and Sacchi, 1987). Isolated preparations were transferred directly to the solution of 4M guanidinium isothiocyanate, 0.3 M sodium acetate, pH 4.0, 1 M β- mercaptoethanol, then extracted with 6:1 phenol (pH 4.0):chloroform. After two ethanol precipitations, the pellet was dried, then resuspended in DEPC-treated H_2O.

For each sample, 12 μg total RNA was loaded per lane and electrophoresed through 1% agarose gels containing 2.2 M formaldehyde. Following separation, RNA was transferred onto Duralon UV nylon membranes (Stratagene) by capillary blotting overnight (Sambrook et al., 1989). Membranes were crosslinked (Stratalinker), prehybridized in 50% formamide, 0.1% SDS, 5× Denhardt's solution (Denhardt, 1966), 5× SSC (1× SSC: 0.15 M NaCl, 0.01 M Na citrate) for 3 h. Hybridizations used the same conditions for 16 h in the above solution at 42°C with ^{32}P-labeled antisense RNA generated via in vitro transcription reactions utilizing T7 RNA polymerase (NEB) from a linearized 1.3-kb *Ms*E74A clone. This template fragment comprises the 5′ *Ms*E74A-specific region as well as the common region shared by both *Ms*E74A and *Ms*E74B; as such, this probe hybridizes to both *Ms*E74A and *Ms*E74B transcripts. Washes were performed at 65°C for 20 min each in solutions of 2× SSC, 0.2% SDS; 0.5× SSC, 0.2% SDS; and 0.1× SSC, 0.2% SDS. The resulting signals were visualized through phosphoimaging. Where specified, quantitation of the *Ms*E74A and *Ms*E74B transcripts was accomplished by using the GS-363 Biorad Molecular Imaging System software as previously described (Zhou et al., 1998).

Primary cultures of Manduca epidermis

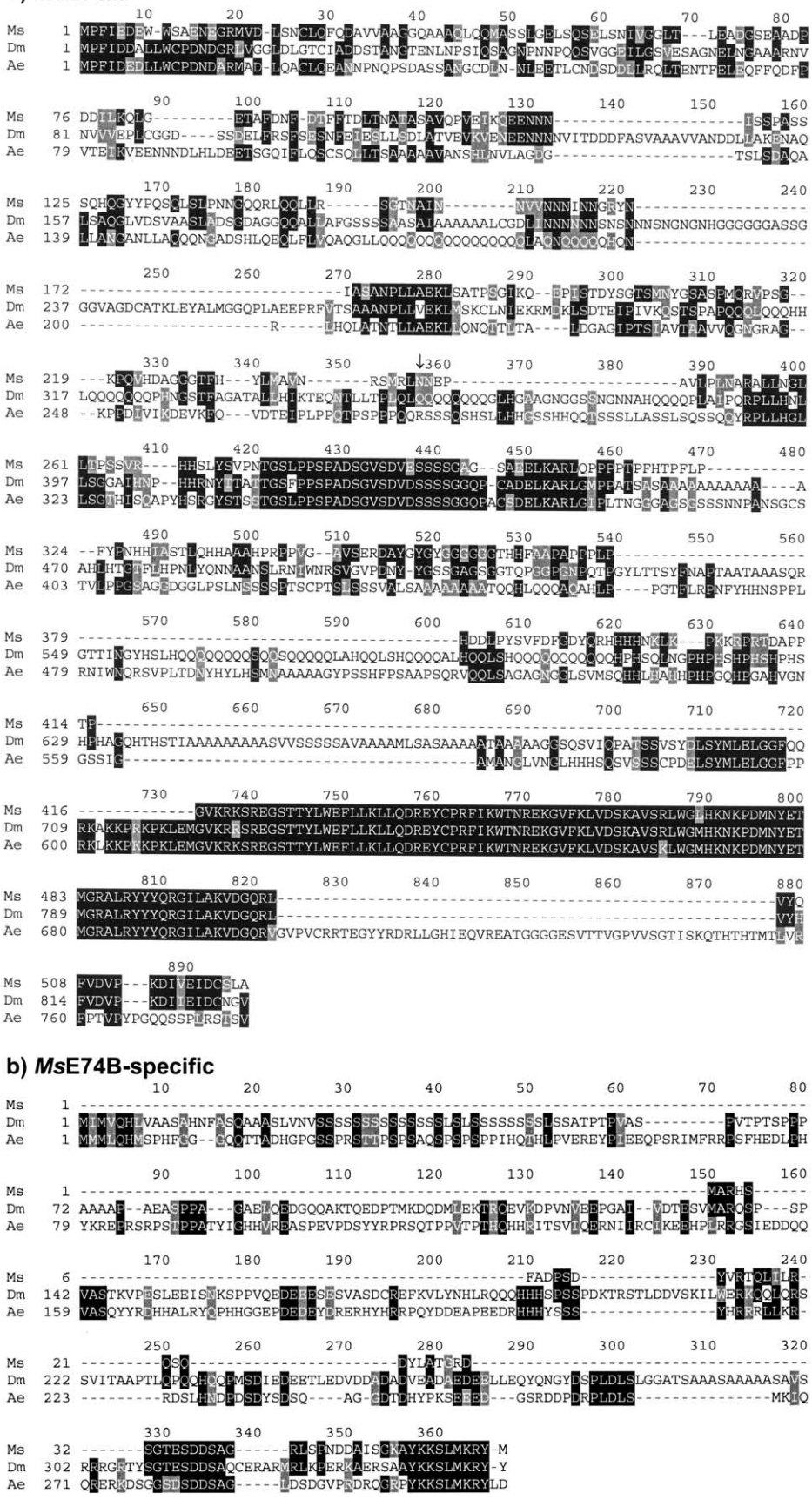
Primary cultures of *Manduca* epidermis were performed according to Riddiford et al. (1979) and Hiruma and Riddiford (1984). All dissections were done in *Manduca* saline (Riddiford et al., 1979), and the dorsal regions of the abdomen were cleaned of fat body, muscles, and trachea, leaving the epidermis attached to the cuticle. Segments A2–A7 were individually isolated by cuts near the anterior and posterior segment boundaries. Each segment was then further dissected into two equal halves, and the resulting pieces were cultured in 0.5 ml Grace's medium (Gibco) per culture per culture well at 25.5°C under 95% O_2–5% CO_2 conditions. 20 Hydroxyecdysone (20E) (Daicel Chemical Co., Japan) was added by dissolving in Grace's medium as previously described (Hiruma et al., 1991). Juvenile Hormone I (SciTech, Praha, Czech Republic) was stored at −20°C as a concentrated stock solution in cyclohexane. Working concentrations were dried under nitrogen and resuspended in Grace's medium 12 h before addition to the cultures. Just prior to use, JH solutions were vortexed vigorously and added to culture wells coated with polyethylene glycol (20,000 MW; Sigma, St. Louis) as previously described (Riddiford et al., 1979). Cultures containing JH I were incubated in JH alone for 1 h before transferring to media containing various hormones (JH, JH + 20E, 20E) as previously described (Zhou et al., 1998).

Immunocytochemistry

His-tagged fusion proteins of *Ms*E74A- and *Ms*E74B-specific regions were prepared from the respective cDNAs by using the *Bam*HI–*Nde*I fragment encoding amino acids 119–160 in *Ms*E74A and 1–64 in E74B, respectively, in the PET-16b vector (Novagen) according to established procedures from Novagen. The protein was purified from an urea-denatured lysate of induced cells using nickel–NTA agarose (Qiagen) (4:1 lysate:50% Ni-NTA slurry). The lysate/slurry mixture was loaded on columns and washed with 100 mM NaH_2PO_4, 8 M urea, 10 mM Tris–HCl, pH 6.3. The fusion protein was eluted with 100 mM NaH_2PO_4, 8 M urea, 10 mM Tris–HCl, pH 5.0, and checked for purity by electrophoresis on a 16.5% Tris–Tricine gel (BioRad). Fractions containing a single band of the correct size were then pooled and stored at −80°C.

Polyclonal antibodies were prepared to each of the *Ms*E74A- and *Ms*E74B-specific fusion proteins in rat by R & R Rabbitry (Stanwood, WA). The antiE74A antiserum was used at 1:1000, the antiE74B antiserum at 1:5000, and the Broad antiserum (Zhou and Riddiford, 2001) at 1:6000. Whole mounts of the epidermis were prepared and processed for immunocytochemistry as previously described (Jindra et al., 1996; Asahina et al., 1997). After incubation with the primary antibody, visualization was by a fluorescein-labeled secondary antibody (Jackson Laboratories) using a BioRad laser scanning confocal microscope. The photos were processed with NIH Image and Adobe Photoshop.

Fig. 1. (a) Conceptual translation of *M. sexta* (Ms) E74A open reading frame sequence and comparison to E74A sequences of *D. melanogaster* (Dm) (Burtis et al., 1990) and *Aedes aegypti* (Ae) (Sun et al., 2002). Arrow at residue 243 in the *Manduca* sequence indicates the beginning of the common region between the two isoforms. (b) Conceptual translation of *Manduca* E74B, showing only the N-terminal E74B-specific domain, and comparison to *Drosophila* and *Aedes* as in (a). The sequences were aligned by using Clustal X. Black shading represents identical residues across all three species; gray indicates similar residues. Dashes indicate gaps in sequence and were introduced to facilitate alignment. Stop codons (not pictured) directly follow the last amino acid listed. Numbers on the left indicate the amino acids in each sequence. GenBank Accession Nos. for *Ms*E74A and *Ms*E74B are AY170859 and AY170860, respectively.


a) MsE74A
b) MsE74B-specific

Results

Cloning and sequencing of MsE74A and MsE74B

We used degenerate primers complementary to sequences within the ETS domain of *DmE74* to amplify a 99-bp fragment from two *Manduca* cDNA libraries (see Materials and methods). With this small product as a probe, we then isolated clones that covered the entire open reading frame (ORF) of the *Ms*E74A transcript from the pupal cDNA library. The first *Ms*E74B clones were also isolated from the same pupal library; but these were incomplete and missing the 5′ end of the ORF. To obtain longer clones, we designed further specific primers to the *Ms*E74B-specific sequence and in conjunction with an anchor primer complementary to vector sequence, performed PCR on the combined day 2 4th instar and wandering +1 day larval cDNA library. These products yielded the remainder of the *Ms*E74B ORF. Cloning and sequencing of the two alternative splice variants, *Ms*E74A and *Ms*E74B, cDNAs yielded ORFs of 1572 and 1251 bp, respectively. Sequences are deposited in GenBank (Accession nos. AY170859 and AY170860).

Similarly to E74 from *D. melanogaster* and other closely related drosophilids (Burtis et al., 1990; Jones et al., 1991), *Ms*E74A and *Ms*E74B share common 3′ domains and have splicing-specific 5′ termini (Fig. 1). The highly conserved DNA binding ETS domain is near the C terminus of the common domain (amino acid 416–504) and shares 98 and 97% amino acid identity to the *Drosophila* and *Aedes* (mosquito) E74 genes (Burtis et al., 1990; Jones et al., 1991; Sun et al., 2002). Adjacent to the ETS domain, there are also highly conserved regions among the known insect E74s and other E74-related factors found among vertebrates, including ELK-1 (Karim et al., 1990; Graves and Petersen, 1998). The *Manduca* A- and B- specific domains display less sequence conservation, ca. 20 and 36%, respectively, just as found among the drosophilids (Jones et al., 1991). This lack of conservation for E74 may indicate that the functional domains in these regions are not as critical in activation or suppression as those in the N termini of other ETS proteins, such as *yan* and *pointed* (Graves and Petersen, 1998). The findings that the initiator methionine of *Ms*E74B matches to the 5th methionine in the *Drosophila* E74B and that this residue is a leucine in *Aedes* suggests that we may not have obtained a full-length cDNA clone for *Ms*E74B.

Developmental pattern of MsE74 expression during the final two larval instars and the pupal–adult transformation

We analyzed both mRNA and protein expression of *Ms*E74 in the dorsal abdominal epidermis during the final two larval instars and the molt to the pupa, but only the mRNA expression in the pupal wing during the onset of adult development.

mRNA analysis

Fig. 2 shows Northern blots of the 5-kb *Ms*E74B and 5.5-kb *Ms*E74A transcripts in the abdominal epidermis, beginning with the molt to the 5th instar until pupal ecdysis, then in the pupal wing during the onset of adult development. During the 4th instar, no *Ms*E74 mRNA was detectable during the intermolt feeding stage through day 2 (data not shown) or during the rise of the ecdysteroid titer for the molt, which peaks approximately 6 h prior to head capsule slippage (HCS) (Langelan et al., 2000) (Fig. 2). *Ms*E74A mRNA transiently appeared 6 h after HCS as the ecdysteroid titer was declining followed by *Ms*E74B mRNA that persisted until 10 h before ecdysis (the onset of the tanning of the mandibles). At this time, *Ms*E74A mRNA reappeared at high levels, peaked 3–6 h before ecdysis, declined to trace levels by the time of ecdysis, and was absent during the feeding stage of the 5th instar. *Ms*E74A mRNA reappeared in the abdominal epidermis at high levels during the prepupal period as the ecdysteroid titer declined. In contrast, E74B mRNA disappeared by 9 h before ecdysis to the 5th instar, then reappeared at the time of pupal commitment on day 3 of the 5th instar. Trace amounts of *Ms*E74B mRNA persisted through the onset of the pupal molt on the day after wandering, then disappeared.

During the pupal stage (days P0–P2), trace amounts of the *Ms*E74A transcript were present in the wing epidermis (Fig. 2). Adult development began on day P3 as signaled by retraction of the wing epidermis from the overlying cuticle. *Ms*E74A mRNA transiently appeared at low levels on day P6, then reappeared on days P9 and P10 at high levels coincident with the 20E component of the ecdysteroid titer, then disappeared as the titer declined. During the onset of the pupal–adult transformation, *Ms*E74B mRNA appeared in low amounts on day P4 and persisted through day P5 coincident with increases in ecdysone levels. Then, it disappeared for the remainder of the days studied. We did not assess the levels of either transcript at the end of the adult molt (about day P19–P20).

The ~2.4-kb transcript detected on days P7 and P8 has also been detected on day 2 of the 5th instar and during the 4th instar, although its presence was not always reproducible in these latter stages. This transcript could represent another as of yet uncharacterized E74 isoform or could be an artifact of the protocol. Curiously, in *Drosophila* tissues that were cultured in vitro, a lower molecular weight band similarly appeared that did not correspond to known *Dm*E74A or E74B isoforms and was also not reproducible (Karim and Thummel, 1991).

An extremely abundant ~1.0-kb transcript (not shown) was seen during the intermolt periods when an RNA, but not a DNA, probe for *Ms*E74 was used for hybridization. Moreover, its presence was highly variable, even between repli-

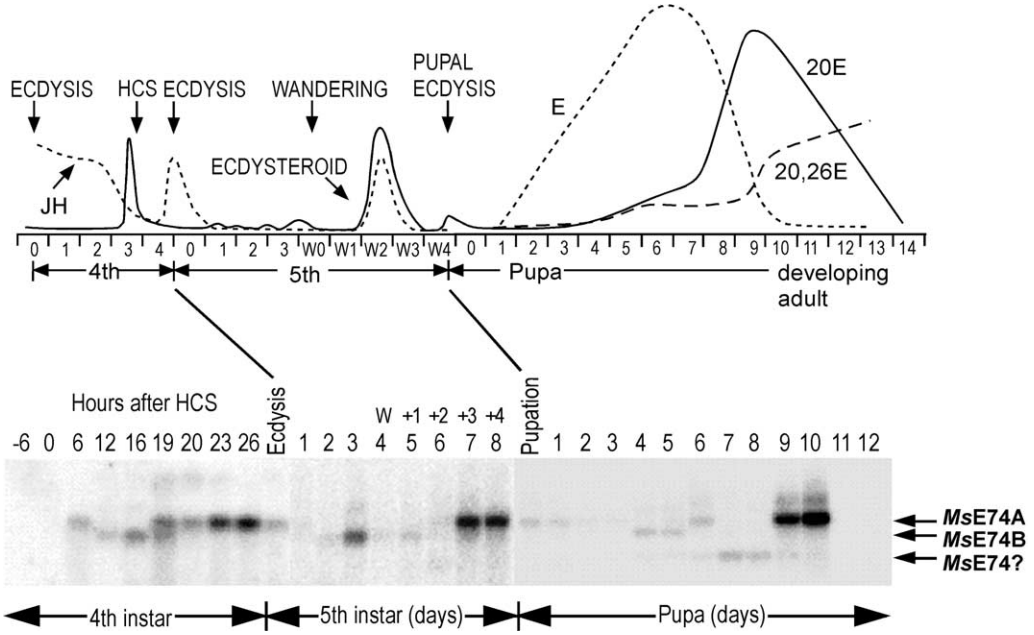


Fig. 2. Profiles for *Ms*E74A and *Ms*E74B mRNAs during the 4th, 5th, and metamorphic stages of development in *Manduca*. For 4th instar larvae, timing was assessed based on spiracle apolysis or head capsule slippage (HCS, ~29 h before larval ecdysis) (Langelan et al., 2000). Fifth instar and pupal stages are depicted in days of development or days after wandering in 5th instar larvae (W+1, 2, 3, and 4). Where applicable, morphological markers were used to confirm timing and developmental staging (Curtis et al., 1984; Truman et al., 1980; Jindra et al., 1997). Each lane has 12 μg RNA derived from dorsal abdominal epidermis (4th and 5th instar larvae) or wing tissue (pupae). Total RNAs were hybridized with an *Ms*E74A RNA probe that included the common region shared with *Ms*E74B; therefore, both the 6.0-kb *Ms*E74A and 5.5-kb *Ms*E74B transcripts were detected. In pupae, an additional 2.2-kb band was consistently detected on days 7 and 8. Shown above the figure are the hormone titers based on Warren and Gilbert, 1986; Riddiford, 1996; Hiruma et al., 1999; and Langelan et al., 2000. Ecdysteroid, total ecdysteroid content of the hemolymph in 20E- equivalents; E, ecdysone; 20E, 20-hydroxyecdysone; 20, 26E, 20, 26-dihydroxyecdysone; HCS, head capsule slippage; JH, juvenile hormone.

cates of similarly staged animals. One possibility is that this very small transcript is not E74 but an unrelated transcript that cross- hybridizes with our RNA probe. Its size, timing, and abundance suggest that it may be a transcript encoding a larval-specific endocuticular protein (Riddiford et al., 1986). The presence of such bands at particular stages have also been seen with RNA probes for the *Manduca* ecdysone receptor (Jindra et al., 1996), Ultraspiracle (Jindra et al., 1997), and E75 (Zhou et al., 1998).

Protein patterns

Fig. 3a shows that both the *Ms*E74A and the *Ms*E74B antibodies are specific for the respective isoforms since immunostaining by each was specifically removed by preincubation with the respective fusion protein, but not by preincubation with the other fusion protein. Both were found to localize in the nucleus as would be expected of this transcription factor.

*Ms*E74A was seen primarily just before larval and pupal ecdysis (Fig. 3b) with none present during the intermolt, the feeding period in either the 4th or the 5th instar or during the upswing of the ecdysteroid titer for the larval or the pupal molt (data not shown). Trace amounts were seen at the time of head capsule slippage during the molt to the 5th instar and 8 h thereafter as the ecdysteroid titer began to decline (data not shown).

In contrast, no *Ms*E74B immunostaining was seen during the 4th instar, the early 5th instar, or the pupal molt (data not shown). A close examination during the molt to the 5th instar showed that *Ms*E74B was transiently present at low amounts between 17 and 19 h after head capsule slippage (data not shown), a time shortly after low amounts of *Ms*E74B mRNA were seen (Fig. 2). High levels of *Ms*E74B, however, were seen in the nucleus on the final day of feeding of the 5th instar (the day of pupal commitment of the epidermis) (Fig. 3c, day 3, 06:00, 14:00, and 22:00). By the morning of the day of wandering, all nuclear staining had disappeared (Fig. 3c, W0, 14:00).

In *Manduca*, Broad appears in a pattern within the abdominal segment as the cells become pupally committed (Zhou and Riddiford, 2001). To determine whether *Ms*E74B also appeared in a similar pattern, we observed different areas within the segment as indicated in Fig. 3c. *Ms*E74B was found to appear first in the lateral and dorsal white stripe (LWS and DWS) areas, then laterally (LAT) (not shown), and finally in the mid-dorsal (MID) region (Fig. 3c, 06:00). By 14:00 on day 3, all areas showed higher levels of staining, but the cells in the area under the black marks on the cuticle next to the LWS and some cells in the mid-dorsal region still lacked E74B (white arrows in LWS and MID, day 3, 14:00). By 22:00, all cells in the segment show approximately equal immunostaining (Fig. 3c).

Since this pattern of appearance of E74B was similar to that of Broad (Zhou and Riddiford, 2001), it was of interest to determine whether they appeared at the same time or sequentially. The ecdysteroid rise that causes pupal commitment occurs either on day 3 (Gate I larvae) or on day 4 (Gate II larvae), depending on the rate of growth of the larvae (Truman and Riddiford, 1974), and occurs earlier in day 4, G II larvae than in day 3, G I larvae (Wolfgang and Riddiford, 1986). Fig. 4 shows that *Ms*E74B and Broad both appear earlier in the day 4 GII larvae than is seen in Fig. 3 for the day 3 GI larvae. Yet in both cases, *Ms*E74B appears in the nucleus before Broad is present. Then, Broad keeps increasing as *Ms*E74B decreases to low levels. Broad declines to low levels by the afternoon of the day of wandering, then reappears late on the day after the onset of wandering and peaks the next day (Zhou and Riddiford, 2001).

Control of MsE74 Expression by 20E and by JH

Induction during a larval molt

Since the developmental pattern of expression seen in Fig. 2 suggested that induction of the E74 isoforms was not part of the early gene response to ecdysteroids during the 4th instar molt, we cultured day 2 4th instar epidermis, explanted before the ecdysteroid rise, with 2 μg/ml 20E, a concentration that can cause the production of larval cuticle in vitro (Hiruma et al., 1991). Fig. 5 shows the results of cultures that have been cultured with 20E for 24 h and then transferred to either 20E-containing medium or to hormone-free medium (NH). Tissue was collected at various times after transfer and assayed for *Ms*E74 mRNA expression. Fourth instar epidermis that remained in 20E-containing medium showed increases in *Ms*E74B mRNA expression beginning 6 h after transfer. Under these hormonal conditions, *Ms*E74A mRNA was not turned on. Yet when tissue was transferred to hormone- free medium, the *Ms*E74A transcript was strongly expressed within 12 h.

Induction of MsE74B mRNA at pupal commitment

To determine whether *Ms*E74B mRNA was induced by 20E at the time of pupal commitment on day 3 of the 5th instar (Fig. 2), we cultured day 2 5th instar epidermis with and without 20E. When 500 ng/ml 20E was added to the culture medium, *Ms*E74B mRNA was maximally induced to approximately sixfold over NH controls by 12 h (Fig. 6a). Under these culture conditions, significant increases in *Ms*E74B transcript levels (approximately a twofold level of induction) can be seen within the 3-h culture period (Fig. 6a). Interestingly, in all cultures, *Ms*E74A was undetectable, even at 48 h (data not shown). Because *Dm*E74A can be induced by high 20E concentrations in *D. melanogaster* (Karim and Thummel, 1991), we also tried 2 μg/ml 20E, a concentration that is sufficient to induce differentiative events in many tissues, including the epidermis (Champlin and Truman, 1998a,b; Champlin et al., 1999; Mitsui and Riddiford, 1976, 1978). Once again, *Ms*E74A expression was undetectable over a 24-h period, while *Ms*E74B mRNA showed an approximate sixfold induction (data not shown).

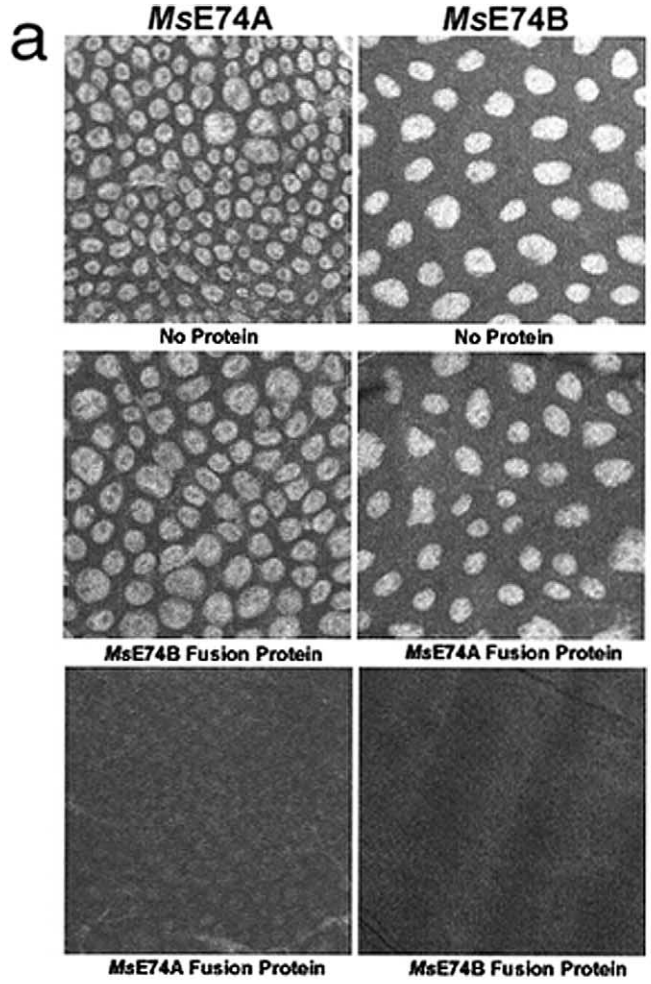
Since both 500 ng/ml and 2 μg/ml 20E gave similar maximal *Ms*E74B expression levels, we determined the 50% effective concentration necessary for induction in day 2 5th epidermis. After culture in medium containing various 20E concentrations, *Ms*E74 mRNA levels were assessed at the time of peak *Ms*E74B mRNA induction (12 h) (Fig. 6a). From a quantitative analysis of the Northern data from these cultures, the EC_{50} concentration of 20E is 30 ng/ml (6×10^{-8} M) and maximal induction is achieved at 100–200 ng/ml ($2–4 \times 10^{-7}$ M) (Fig. 6b). Again, no *Ms*E74A mRNA was seen at any 20E concentration under these conditions.

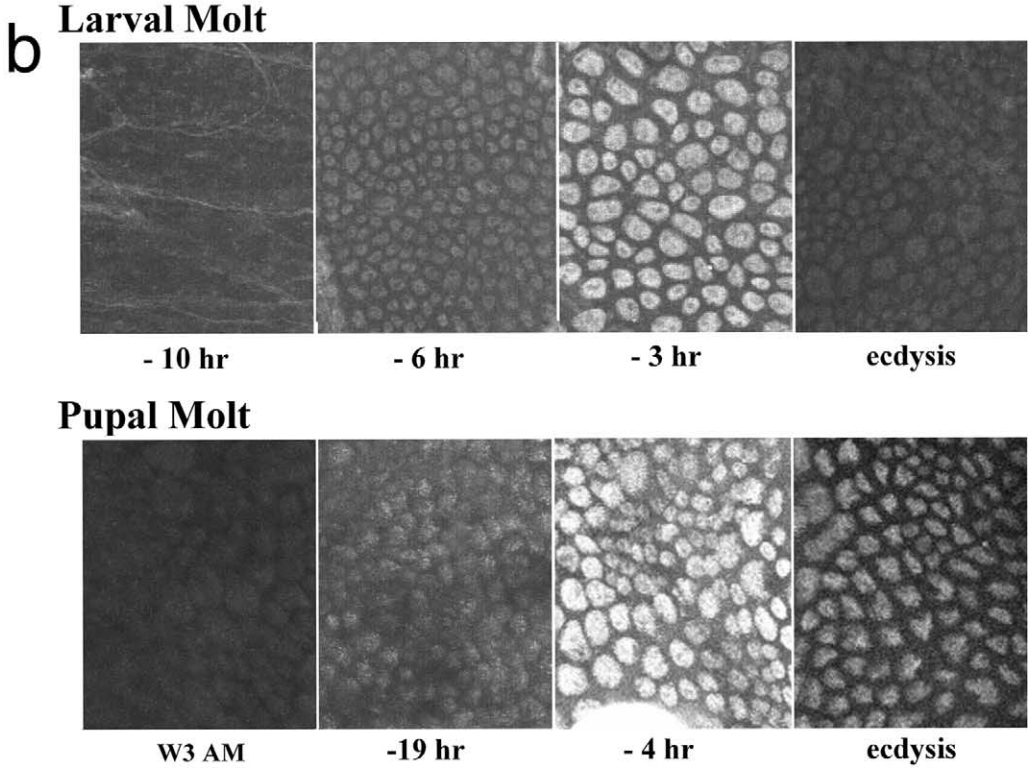
In order to determine whether *Ms*E74B induction in day 2 5th cultures represents a classical early gene response to 20E, we repeated the cultures adding the protein synthesis inhibitor anisomycin. As shown in Fig. 6c, anisomycin in the absence of 20E caused a moderate upregulation of *Ms*E74B mRNA. Yet the addition of 20E with anisomycin caused an increase in this mRNA above that is seen in anisomycin alone. Because this inhibitor is toxic to the epidermis by 10 h (Hiruma and Riddiford, 1990), we only assessed its effects over a 9- h period which is suboptimal for maximal *Ms*E74B expression.

Because JH I effectively blocks pupal commitment both in vivo and in vitro (Riddiford, 1976), we assessed whether the presence of JH I in culture with the 20E influenced *Ms*E74B expression. Fig. 7a shows that 1 μg/ml JH I (3×10^{-6} M) significantly represses *Ms*E74B induction throughout the 48-h culture period. This concentration of JH I is about 100-fold higher than the titer normally seen at the onset of the larval molt (Fain and Riddiford, 1976). We therefore repeated the experiment with different concentrations of JH I in the presence of 500 ng/ml 20E and monitored *Ms*E74B expression at 12 h when maximal induction was seen in the absence of JH. Inhibition occurred over a wide range of JH I concentrations, and the EC_{50} was between 0.1 and 1.0 ng/ml (Fig. 7b). Medium containing 20E + 10 ng/ml JH I or greater resulted in maximal inhibition. Thus, as the epidermis is undergoing pupal commitment in response to 20E in the absence of JH (Riddiford, 1978), it becomes responsive to 20E for the induction of *Ms*E74B, but not of *Ms*E74A, similar to that seen in vivo (Fig. 2). Activation of *Ms*E74B by 20E can be prevented by JH I and correlates with the ability of JH to prevent commitment both in vivo and in vitro (Riddiford, 1976, 1978).

Induction of MsE74 during the pupal molt

Although not expressed when tissues undergo commitment, *Ms*E74A is upregulated to high levels during the pupal molt when ecdysteroid titers are declining. We tested whether the presence of 5 μg/ml 20E followed by its removal from epidermal cultures was sufficient to elicit *Ms*E74A expression in vitro. Day 3 epidermis which is undergoing commitment will complete this process and will


a
MsE74A
MsE74B
No Protein
No Protein
MsE74B Fusion Protein
MsE74A Fusion Protein
MsE74A Fusion Protein
MsE74B Fusion Protein


b
Larval Molt
- 10 hr
- 6 hr
- 3 hr
ecdysis
Pupal Molt
W3 AM
-19 hr
- 4 hr
ecdysis

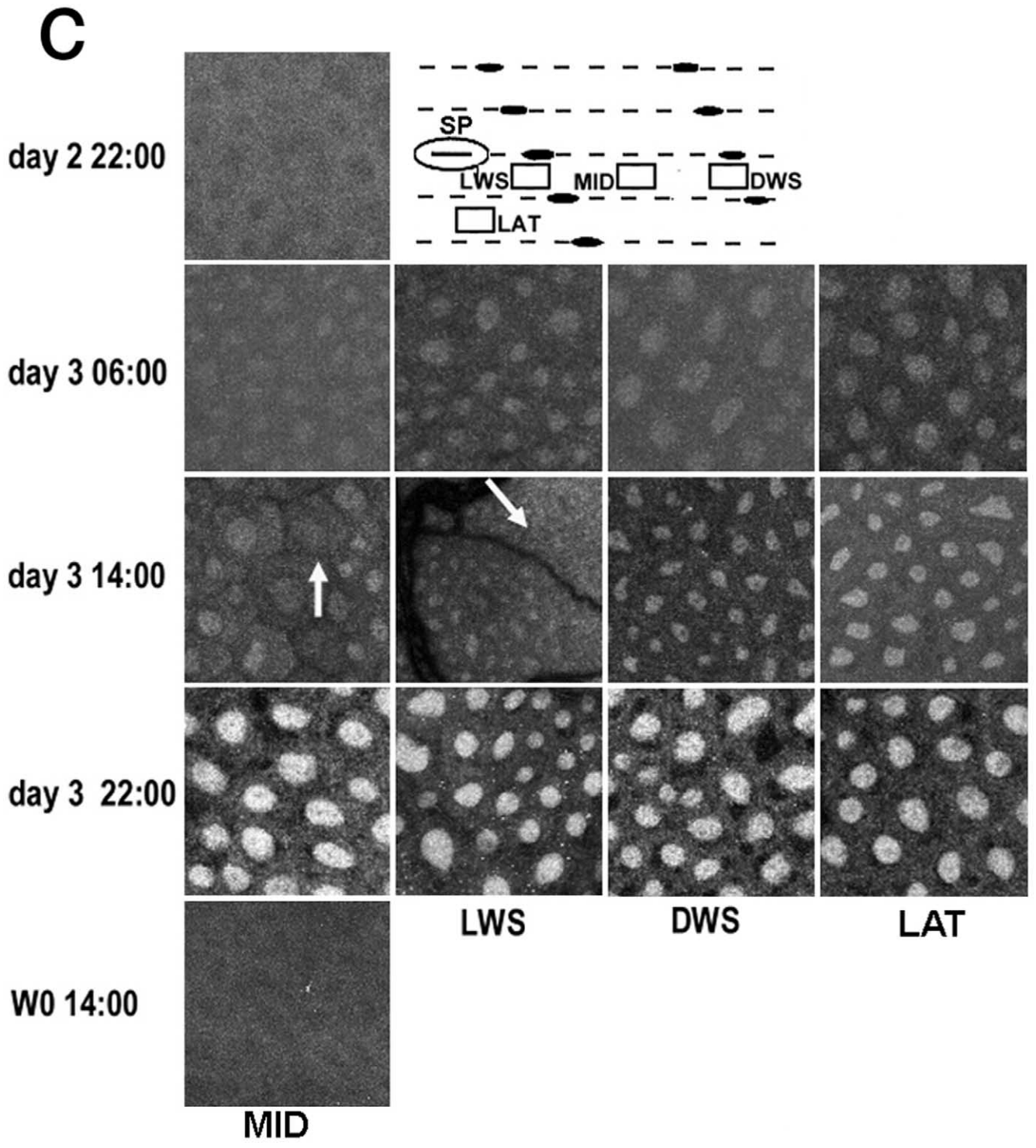


Fig. 3 (*continued*)

Fig. 3. Immunocytochemical analysis of *Ms*E74A and *Ms*E74B expression during the 4th and the 5th instars. (a) Characterization of specificity of the *Ms*E74A and the *Ms*E74B polyclonal antibodies. The antibody was preincubated with no protein or one of the *Ms*E74 fusion proteins (see Materials and methods) before exposure to the tissue. Epidermis from pharate 5th instar larvae 3 h before ecdysis was used for *Ms*E74A, and that from day 3 5th instar larvae was used for *Ms*E74B. (b) Developmental expression of *Ms*E74A during the 4th–5th larval molt and the pupal molt. The times refer to hours before ecdysis. W3, the 3rd day after the onset of wandering (see Fig. 2). (c) The pattern-specific appearance of *Ms*E74B on day 3 of the 5th instar for Gate I animals that are being exposed to the commitment peak of ecdysteroids (see Fig. 2). The inset in the top row shows a hemisegment with the designated areas boxed. DWS, dorsal white stripe; LAT, lateral; LWS, lateral white stripe; MID, mid- dorsal region; SP, spiracle; W0, the day of the onset of wandering. White arrows indicate regions that have not yet acquired *Ms*E74B. Times are AZT and refer to times after lights-off (00:00 AZT).

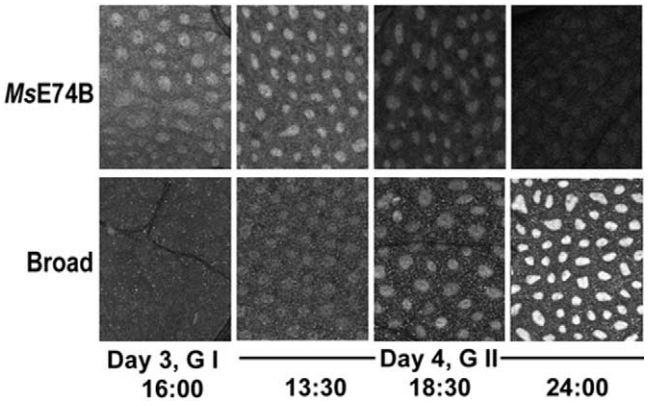


Fig. 4. Comparison of the onset of *Ms*E74B and Broad expression during pupal commitment on day 3 (for Gate I larvae) or on day 4 (for Gate II larvae) of the 5th instar. The ecdysteroids rise earlier in the latter animals (see text for details). Times are as in Fig. 3c.

effectively reproduce many aspects of the pupal molt when cultured with 20E for 72 h followed by exposure to hormone-free medium for 48–72 h (Mitsui and Riddiford, 1976). Under these conditions, *Ms*E74A mRNA was induced to very high levels within 12 h only when 20E was removed from the culture medium. Induction of expression was then followed by a rapid decline in abundance by 24 h (Fig. 8a). In contrast, in the continual presence of 20E assessed through 96 h, *Ms*E74A mRNA first appeared at very low levels at 12 h and remained low through 96 h (Fig. 8b). *Ms*E74B mRNA declined from its initial high level by 12 h, irrespective of whether 20E was removed from the medium (Fig. 8b). After this initial decline in the presence of continuous 20E, *Ms*E74B mRNA then gradually increased, although by 96 h it was still at an intermediate level (Fig. 8b).

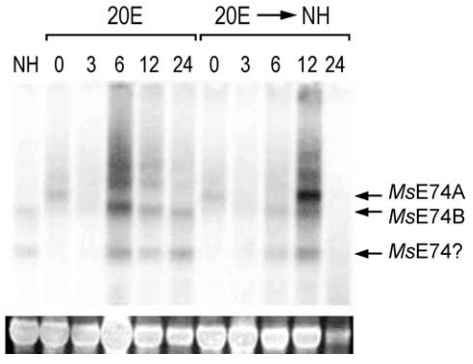


Fig. 5. Induction of *Ms*E74 mRNA in day 2 4th instar dorsal abdominal epidermis *in vitro*. Explanted tissue was cultured in 2 μg/ml 20E for 24 h and then transferred to NH (20E → NH) or transferred to fresh 2 μg/ml 20E (20E) for 0–24 h (0, 3, 6, 12, 24). Control (NH) tissue was harvested immediately following dissection. Total RNA (12 μg) was used for each time point, and blots were hybridized with an RNA probe comprising both common and A-specific domains (see Materials and methods for details). Arrows point to *Ms*E74A, *Ms*E74B, and a potential third *Ms*E74 transcript (*Ms*E74?). Methylene blue-stained rRNA (below) is used as a loading control.

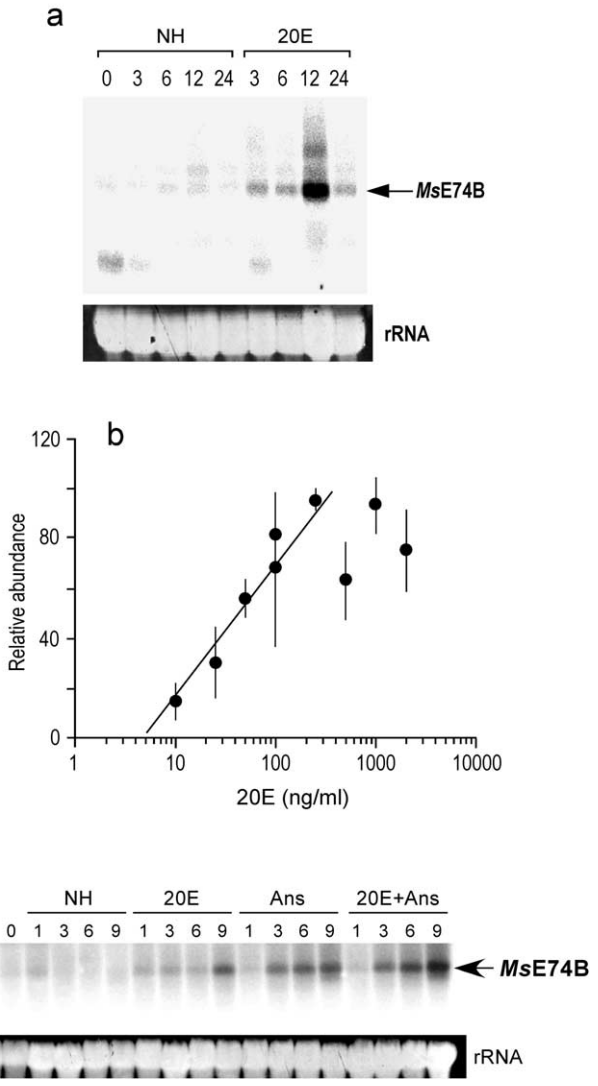


Fig. 6. Induction of *Ms*E74B in day 2 5th instar dorsal abdominal epidermis *in vitro*. (a) Day 2 5th instar larval epidermis was cultured with 500 ng/ml 20E (+20E) or 0 ng/ml 20E (NH) for 0, 3, 6, 12, or 24 h after explantation at 16:00 AZT. Total RNA (12 μg) was run for each time point, and Northerns were hybridized with an *Ms*E74 RNA probe as in Fig. 5. The single 5.5-kb *Ms*E74B transcript is indicated (arrow, *Ms*E74B) along with rRNA loading controls stained with methylene blue (bottom panel). (b) Day 2 5th instar epidermis (16:00 AZT) was exposed to different concentrations (0 to 2000 ng/ml) of 20E in culture for a 12- h period. Total RNA was then analyzed by Northern blot analysis as above, and volume densities were obtained for the *Ms*E74B transcripts by using the Bio-Rad molecular imaging software. Relative abundance, representing the average values ±S.D. of three experiments divided by the control (0 ng/ml 20E) transcript density, was plotted along with standard deviations. (c) Induction of *Ms*E74B mRNA in the presence of the protein synthesis inhibitor anisomycin (Ans). Day 2 5th instar epidermis was cultured with 500 ng/ml 20E in the presence (20E + Ans) or absence (20E) of 10 μg/ml anisomycin or in the presence of only 10 μg/ml anisomycin or in the absence of hormone (NH) for the indicated number of hours, then the RNA was extracted and processed as above.

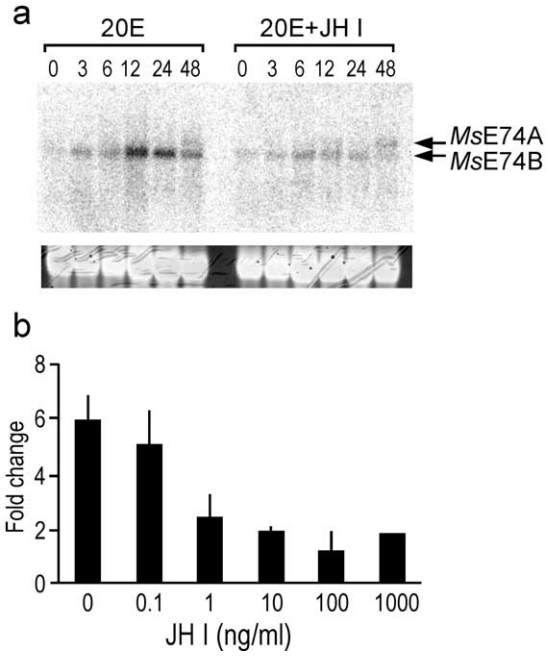


Fig. 7. Suppression of *Ms*E74B mRNA by JH I in day 2, fifth instar animals. (a) Dorsal abdominal epidermis of day 2 5th instar larvae (16:00 AZT) was cultured for 0-48 h in either 500 ng/ml 20E or 20E + JH I (500 ng/ml 20E; 1000 ng/ml, JH I) following a 1-h pretreatment in no hormone or JH I (1000 ng/ml) for the 20E-treated and 20E + JH I-treated samples, respectively. Arrows refer to *Ms*E74B- and *Ms*E74A- (faint at 48 h) specific bands. (b) Day 2 5th instar dorsal abdominal epidermis pieces were precultured for 1 h in Grace's medium containing either no JH I or JH I at concentrations ranging from 0.1 to 1000 ng/ml, then exposed to no hormone, 500 ng/ml 20E, or 500 ng/ml 20E and JH I (0.1 to 1000 ng/ml) and cultured for a 12-h period. Total RNA was then isolated and analyzed as detailed in Materials and methods. Plotted is the fold change along with standard deviations representing the average values ±S.D. of three such experiments divided by transcript densities resulting from 12-h cultures containing 0 ng/ml 20E + 0 ng/ml JH I.

During the pupal molt, *Ms*E74B mRNA is weakly expressed on day W1 but then is absent during and after the rise in the ecdysteroid and JH titers (Fig. 2). To determine whether this lack of *Ms*E74B expression was due to the reappearance of JH at the time of the pupal molt, we allatectomized larvae during the molt to the 5th instar and then assessed the levels of *Ms*E74A and *Ms*E74B mRNAs in the abdominal epidermis during this period. Fig. 9 shows that *Ms*E74B mRNA was present through the afternoon of day W2, then disappeared until pupal ecdysis. *Ms*E74A mRNA was only seen on day W4 just before pupal ecdysis. These allatectomized larvae are delayed in the time of pupal ecdysis about 18–20 h due to a slower rise of the ecdysteroid titer (Hiruma et al., 1999). Consequently, both *Ms*E74A and *Ms*E74B mRNAs appear to be expressed normally relative to the ecdysteroid titer in the absence of JH. The one exception is the reappearance of *Ms*E74B at the time of pupal ecdysis that was not seen in the intact pupa in either the wings (Fig. 2) or dorsal thoracic epidermis (data not shown).

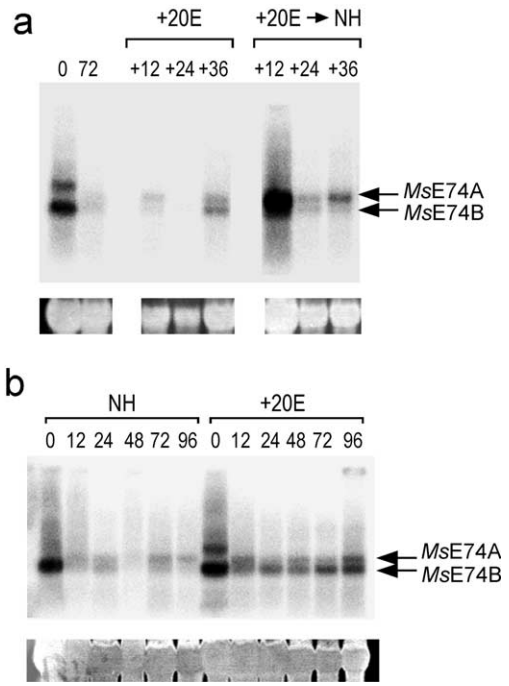


Fig. 8. *Ms*E74 transcript induction in long-term cultures. (a) Northern analysis of day 3 5th instar abdominal epidermal RNA after 72 h of exposure to 5 μg/ml 20E followed by transfer of tissues to either 5 μg/ ml 20E or to 20E-free media (NH). After transfer, the epidermis was incubated a further 12, 24, or 36 h before processing and visualization as described in Fig. 5. (b) Northern analysis of RNA from day 3 5th instar dorsal abdominal epidermis cultured in the presence or absence of 5 μg/ml 20E (20E and NH, respectively). At times from 0 to 96 h, cultures were harvested and resulting total RNAs were analyzed as in Fig. 5. Arrows mark specific *Ms*E74A and *Ms*E74B transcripts. rRNA was used as a loading control (bottom panel) and visualized by methylene blue staining.

Discussion

We report the cloning and characterization of *Ms*E74A and B. The two isoforms are very similar in structure to the

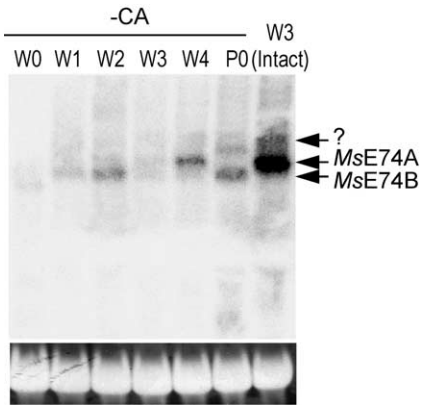


Fig. 9. *Ms*E74 mRNA in the dorsal abdominal epidermis of allatectomized (-CA) larvae from the onset of wandering (W0) to pupal ecdysis (P0). The epidermis was removed at about 16:00 each day. The RNA in the epidermis of an intact larva 3 days after the onset of wandering is shown at the right. rRNA was used as a loading control (bottom panel) and visualized by methylene blue staining.

Drosophila homologs and are 98% identical within the ETS domain. Other similarities to *Drosophila* include hormonal regulation at the level of transcription—either through de novo synthesis or stability of the transcripts. At the time of pupal commitment, *Ms*E74B induction is similar to *Drosophila* at the mid-third-instar transition in that activation occurs in response to 20E. In *Manduca*, this induction is inhibited by JH at concentrations similar to that found in vivo during a larval molt. In striking contrast to *Drosophila* E74A regulation, *Ms*E74A mRNA expression during the molts requires decreasing titers of ecdysteroid and is not induced at high ecdysteroid titers such as it is at pupariation in *Drosophila*.

MsE74B regulation and stage specificity

*Ms*E74B expression in *Manduca* epidermis correlates well with the timing and induction parameters necessary to induce pupal commitment. In 5th instar larvae, *Ms*E74B mRNA and protein appear on day 3 as the cells are becoming committed to their pupal fate (Riddiford, 1978). In cultured explants, induction occurred in direct response to 20E in the absence of JH. Although exposure to a concentration of anisomycin that inhibits >99% of all protein synthesis (Hiruma and Riddiford, 1990) caused some increase in *Ms*E74B mRNA levels, the addition of 20E induced a more robust response, indicating that 20E acts directly on the *Ms*E74 gene. A similar conclusion was made in *Drosophila*, where the induction of both *Dm*E74A (Burtis et al., 1990) and *Dm*E74B mRNAs (Richards et al., 1999) by 20E occurred in the presence of cycloheximide, another protein synthesis inhibitor. Richards et al. (1999) also noted the increase in expression of certain intermolt transcripts such as *Dm*E74B in the presence of cycloheximide alone just as we have found here for *Manduca* E74B in anisomycin alone. Presumably, this reflects the stabilization of the low level of mRNA present at the time of explantation or possibly the loss of a short half-life inhibitor (Richards et al., 1999).

The concentration of 20E (20–30 ng/ml) necessary to induce *Ms*E74B mRNA is lower than the EC_{50} necessary to induce pupal commitment in culture (100 ng/ml 20E) (Riddiford, 1976), but corresponds well with the titer of 20E (Wolfgang and Riddiford, 1986) at the time that *Ms*E74B mRNA is first seen in vivo (Fig. 2). Furthermore, *Ms*E74B mRNA appears within 3 h of exposure to 20E and is therefore expressed during the commitment process in culture. The timing of *Ms*E74B mRNA and its responsiveness to low concentrations of 20E at this stage are similar to the regulation of E74B in *Drosophila* at the time of the mid-third instar transition that marks the onset of metamorphosis (Karim and Thummel, 1991, 1992).

In *Drosophila* at the mid-third instar transition, both E74B and BR mRNAs appear (Karim et al., 1993). In *Manduca* epidermis, BR appears at the time of pupal commitment, and this appearance is tightly correlated with the commitment of the cells that occurs in a stereotyped pattern across the segment (Zhou et al., 1998; Zhou and Riddiford, 2001). The present studies have shown that *Ms*E74B appears in these cells before BR, then disappears as the cells are committed and express high levels of BR (Fig. 3B). Thus, it seems likely that *Ms*E74B is acting in the cells to cause pupal commitment and that the manifestation of that commitment is the upregulation of BR. In *Drosophila*, both genes appear to be important for the early events of metamorphosis because *E74B* mutants ($E74^{DL\text{-}1}$) have a complete absence or drastic reduction of salivary gland glue gene expression (Fletcher et al., 1997). Similar phenotypes are seen in *rbp* and *l(1)2BC* mutants of the *br* gene, indicating that BR and E74B are interacting (or collaborating) to coordinate developmental events. Furthermore, *br, E74* double mutants display a more severe phenotype than either mutant alone, showing that there is a genetic interaction between these two genes. Whether this interaction is direct is unknown but seems unlikely based on a lack of identifiable domains that could mediate protein–protein binding for both BR and E74B. In *Manduca*, there is little or no overlap in expression of E74A and BR at least at the time of commitment, which would also rule out direct physical interactions in vivo.

The conclusion that *Ms*E74B is involved in the process of pupal commitment is strengthened by the finding that transcript can only be induced by 20E in the absence of JH just as is true of pupal commitment in vivo (Riddiford, 1976, 1978). The level of JH necessary to block the appearance of *Ms*E74B mRNA (EC_{50} between 3×10^{-10} M and 3×10^{-9} M JH I) is over 10- fold lower than the concentration necessary to prevent BR induction and is below the physiological range of JH titers in *Manduca* during the 4th and early 5th instar (Fain and Riddiford, 1975) and is below the JH levels necessary to block pupal commitment in vitro (Riddiford, 1976). Therefore, physiological levels of JH that can repress commitment are also sufficient to inhibit *Ms*E74B expression in culture.

Regulation of E74A by the ecdysteroids

In *Manduca*, *Ms*E74A mRNA and protein are expressed mainly at the end of the larval and pupal molts as the ecdysteroid titer declines (Figs. 2 and 3b) and in vitro it only appeared after exposure to 20E followed by exposure to hormone-free medium (Fig. 8). Similarly, in *Drosophila* during the molt to the third instar, *Dm*E74A mRNA appears on the decline of the ecdysteroid titer (Bialecki et al., 2002). Thus, its requirement for 20E followed by its removal is similar to that for βFTZ-F1 (Hiruma and Riddiford, 2001) and for dopa decarboxylase (DDC) (Hiruma and Riddiford, 1990).

Yet, during the final larval instar, E74A mRNA appears in *Drosophila* at the peak of the ecdysteroid titer for pupariation and is induced in vitro by a high concentration of 20E (Karim and Thummel, 1991, 1992). We did not detect

any E74A in *Manduca*, either in vivo during the pupal molt at the peak of the ecdysteroid titer on day W2 (Hiruma et al., 1999) or during exposure of pupally committed epidermis to 5 μg/ml 20E in vitro. The reason for this difference is unclear, yet other genes follow a similar paradigm. For instance, in *Drosophila*, DDC normally appears at the ends of the molts shortly before ecdysis (Kraminsky et al., 1980), but also appears at high levels and is induced by 20E at the time of pupariation (Kraminsky et al., 1980; Clark et al., 1986; Hodgetts et al., 1995). In *Manduca*, declining 20E titers are necessary for induction of DDC (Hiruma and Riddiford, 1990). In *Drosophila*, both the Z2 isoform of Broad and 20E are required to induce the high levels at pupariation (Hodgetts et al., 1995). BR-Z2 is present in *Manduca* epidermis at the time that the ecdysteroid titer peaks on day W2 (Zhou and Riddiford, 2001). Possibly either the BR-Z2 response element or the special upstream ecdysone response element that mediates this induction in *Drosophila* (Chen et al., 2002) is lacking in the promoter of the *Manduca E74* gene.

MsE74 expression during the pupal–adult transformation

During the first 2–3 days of the pupal adult transformation, the *Ms*E74A transcript is present at very low amounts. Because circulating hormone titers are extremely low during this time, it is likely that transcript detected here is due to persistence from the end of the pupal molt when, on days W3 and W4, *Ms*E74A mRNA is expressed at high levels. On days P4 and P5, there is a switch in transcript expression and *Ms*E74B mRNA becomes exclusively expressed at our level of detection. This expression disappears by day 6. The timing of *Ms*E74B mRNA correlates very well with the gradually increasing level of circulating ecdysone with a low level of 20E (Warren and Gilbert, 1986). Functionally, ecdysone or low levels of 20E have been shown to play critical roles in driving predifferentiative responses in a variety of tissues including proliferation of myoblasts and of optic lobe neuroblasts (Champlin and Truman, 1998a; Champlin et al., 1999). It is therefore possible that *Ms*E74B is one of the transcription factors involved in driving these responses but further experiments are necessary to test this hypothesis.

*Ms*E74A mRNA is again expressed at high levels on days P9 and P10 as the 20E titer peaks and cuticle is deposited (Nardi and Magee-Adams, 1986; L.M.R., unpublished observations). Thus, *Ms*E74A appears earlier in the adult molt than it does in either the larval or the pupal molts. One possibility is that the absence of JH throughout this stage of development causes a change in the timing of *Ms*E74A expression relative to the peak of the ecdysteroid titer. However, the absence of JH during the pupal molt in the allatectomized animals did not cause the earlier expression of *Ms*E74A relative to the ecdysteroid titer (Fig. 8). Clearly, further studies are needed to resolve this question.

Acknowledgments

This study was supported by grants from NIH (NS 29971 and GM 60122) and NSF (IBN-9817339).

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