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Juvenile hormone modulates 20-hydroxyecdysone-inducible ecdysone receptor and ultraspiracle gene expression in the tobacco hornworm, *Manduca sexta*

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Abstract Insect molting and metamorphosis are orchestrated by ecdysteroids with juvenile hormone (JH) preventing the actions of ecdysteroids necessary for metamorphosis. During the molt and metamorphosis of the dorsal abdominal epidermis of the tobacco hornworm, *Manduca sexta*, the isoforms involved in the ecdysone receptor (EcR)/Ultraspiracle (USP) complex change with the most dramatic switch being the loss of USP-1 and the appearance of USP-2 during the larval and pupal molts. We show here that this switch in USP isoforms is mediated by high 20-hydroxyecdysone (20E) and that the presence of JH is necessary for the down-regulation of USP-1 mRNA. The decrease of USP-1 mRNA in day 2 fourth instar larval epidermis in vitro required exposure to a high concentration (10^{-5} M) of 20E equivalent to the peak ecdysteroid concentration in vivo, whereas the increase of USP-2 mRNA occurred at lower concentrations (effective concentrations, $EC_{50}=6.3\times10^{-7}$ M). During the pupal molt of allatectomized larvae which lack JH, USP-2 mRNA increased normally with the increasing ecdysteroid titer, whereas USP-1 mRNA remained high until pupation. When day 2 fifth instar larval epidermis was exposed to 500 ng/ml 20E in the absence of JH to cause pupal commitment of the cells by 24 h, USP-1 RNA remained at its high preculture level for 12 h, then increased two- to threefold by 24 h. The increase was prevented by the presence of 1 µg/ml JH I which also prevents the pupal commitment of the cells. By contrast, USP-2 mRNA increased steadily with the same EC_{50} as in fourth stage epidermis, irrespective of the presence or absence of JH. Under the same conditions, mRNAs for both EcR-B1 and EcR-A isoforms were up-regulated by 20E, each in its own time-dependent manner, similar to that seen in vivo. These initial mRNA increases were unaffected by the presence of JH I, but those seen after 12 h exposure to 20E were prevented by JH, indicating a difference in response between larvally and pupally committed cells. The presence of JH which maintained larval commitment of the cells also prolonged the half-life of the EcR proteins in these cells. These results indicate that both EcR and USP RNAs are regulated by 20E and can be modulated by JH in a complex manner with only that of USP-2 apparently unaffected.

Key words Ecdysone receptor · Juvenile hormone · Ultraspiracle · *Manduca*

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Introduction

Ecdysteroid action at the molecular level has been well studied in insects, especially *Drosophila melanogaster*. The primary biologically active ecdysteroid, 20-hydroxyecdysone (20E), binds to the ecdysone receptor (EcR) when it forms a heterodimer with the Ultraspiracle protein (USP; Yao et al. 1992, 1993; Swevers et al. 1996; Kapitskaya et al. 1996), a homolog of the vertebrate retinoid X receptor (RXR; Mangelsdorf et al. 1992, 1995). The hormone-receptor complex then binds to either a palindromic (Cherbas et al. 1991; reviewed in Cherbas and Cherbas 1996) or a direct repeat (Antoniewski et al. 1996) ecdysone response element (EcRE) to activate or inactivate gene transcription. In the absence of ligand, the EcR/USP complex also may bind and down-regulate gene transcription (Cherbas et al. 1991). EcR isoforms, which differ structurally only in their transactivating A/B domains, are found in *Drosophila* (Talbot et al. 1993; Robinow et al. 1993; Truman et al. 1994), the lepidopterans, *Manduca sexta* (Jindra et al. 1996) and *Bombyx mori* (Kamimura et al. 1997), and the beetle, *Tenebrio molitor* (Mouillet et al. 1997) and show independent temporal and spatial expression patterns. In *Drosophila* there is apparently only one isoform of USP (Henrich et

al. 1994; Huet et al. 1995), but two isoforms have been found in both *Manduca* (Jindra et al. 1997) and the mosquito *Aedes aegypti* (Kapitskaya et al. 1996).

In the polymorphic epidermis of *Manduca sexta*, both isoforms of EcR appear at every molt, with EcR-B1 present during the commitment and predifferentiative phases and EcR-A prevailing at the onset of cuticle synthesis (Jindra et al. 1996). Additionally, EcR-A is present during the switching and predifferentiative events necessary for a new synthetic program at the onset of metamorphosis. The expression of both EcR-B1 and EcR-A genes are induced by 20E in cultured day 2 fifh (final) instar larval epidermis (Hiruma et al. 1997). The two isoforms of *Manduca* USP show an intriguing developmental pattern in the epidermis with USP-1 mRNA and protein present during the intermolt period, then disappearing during the larval and pupal molts when USP-2 mRNA and protein appears (Jindra et al. 1997; Asahina et al. 1997; Fig. 1). Thus, USP-1 is present at the time that EcR-B1 is high during both the larval molt and at the time of pupal commitment in the final larval instar. By contrast, USP-2 appears as the ecdysteroid titer increases in the presence of juvenile hormone (JH) during the wandering stage, but is present only in trace amounts during pupal commitment. Transfection studies with the *Manduca* GV1 cell line have shown that the 20E-induction of the transcription factor MHR3 requires the EcR-B1/USP-1 heterodimer and that the EcR-B1/USP-2 does not bind to the MHR3 promoter although both heterodimers bind ponasterone A with equal affinity (Lan et al. 1998). Thus, the changing combinations of isoforms of this heterodimeric complex during the molt are critical for the transcription factor cascade and other genes regulated directly by ecdysteroids to orchestrate the various developmental events that must occur.

JH is normally present during larval life and prevents metamorphosis by modulating ecdysteroid action at the outset of the ecdysteroid rise for the molt (Riddiford 1996). If JH is present, the molt will be to another larva; if absent, metamorphosis ensues. The molecular basis of this action of JH is not yet clear although recently JH has been found to bind to USP with low affinity and to promote its oligomerization in a yeast system (Jones and Sharp 1997). Therefore, it is important to know how JH may regulate the responses of both USP and EcR to changing ecdysteroid levels.

In this study we show that JH modulates the response of USP-1 but not of USP-2 to 20E, both in vivo and in vitro. Also, JH has no effect on the initial responses of the EcR isoforms to low amounts of 20E, but prevents their up-regulation by high amounts. In addition, JH was found to prolong the half-life of the EcR-B1 protein in the nucleus in the absence of its mRNA.

Materials and methods

Experimental animals and culture method

Larvae of the tobacco hornworm, *Manduca sexta*, were reared on an artificial diet at 25.5°C in a 12 h light:12 h dark photoperiod as described by Truman (1972) and Bell and Joachim (1976). Lights off, the beginning of a new day, was set at 00:00 Arbitrary Zeitgeber Time (AZT; Pittendrigh 1965). Gate II day 2 fourth instar larvae were allatectomized (removal of corpora allata, the source JH) 5–6 h before the head capsule slippage (HCS) stage as described by Hiruma (1980).

The dorsal abdominal integument of day 2 fourth (one segment; 3.5×7.0 mm; 14:00 AZT) and day 2 fifth instar larvae (half segment; 7×7 mm; 14:00 AZT) was cultured on the surface of 0.5 ml Grace's medium (GIBCO) per culture well (Linbro Disposotrays; Flow Laboratories) at 25.5°C in a 95% O_2– 5% CO_2 atmosphere (Hiruma and Riddiford 1984, 1990). For the JH studies, glass wells (12 mm diameter, 10 mm high) coated with 1% polyethylene glycol 20,000 were used (Riddiford et al. 1979) to decrease the possible adsorptive loss. Adhering fat body was completely removed from the epidermis.

20E was purchased from Rohto Pharmaceutical Co., Japan, or was a gift of Dr. Takeshi Matsumoto (99% pure; R. Lafont, personal communication; Daicel Chemical Co., Japan). JH I was purchased from SciTech, Czech Republic. All the media containing JH I were sonicated (Riddiford et al. 1979) and prepared one day before the culture, then they were thoroughly vortexed shortly before the culture. Whenever the pieces of integument were transferred to different medium, they were rinsed with plain Grace's medium at least twice. The concentrations were determined spectrophotometrically [ε_{240}=12,677 in EtOH for 20E (Lafont and Wilson 1992), and ε_{217}=14,800 in MeOH for JH I (Goodman et al. 1978)].

Anisomycin (Sigma) at 10 μg/ml was used to inhibit >99% of the protein synthesis in the epidermis during culture (Palli et al. 1992). The ecdysteroid concentration in the hemolymph was measured by a radioimmunoassay (Warren et al. 1984). The antiserum was prepared against 20E and has a five-fold higher affinity for 20E than for ecdysone (E) and 3-dehydroecdysone (3DE) (Yokoyama et al. 1996), and 20E was used as a standard. Labeled ecdysone, α-[23, 24, -^{3}H(N)]-E (53 Ci/mmol), was purchased from NEN.

Immunoblotting and immunocytochemistry

Cultured epidermal proteins were extracted in 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS) pH 6.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF) by homogenization followed by centrifugation (12,000 *g*, 5 min). Protein concentration was determined with the BCA protein assay kit (Pierce). Twelve micrograms of total proteins were separated by 9% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, then transferred onto Protran nitrocellulose (Schleicher and Schuell). The filters were blocked overnight in TBST [150 mM NaCl, 50 mM TRIS HCl (pH 8.0), 1 mM ethylene diamine tetraacetic acid (EDTA), 0.1% Tween 20] containing 5% bovine serum albumin and 5% dried nonfat milk. They were then incubated with 6B7 EcR-B1-specific monoclonal antibody (Jindra et al. 1996) in TBST (1:700) for 2 h followed by 1 h in 1:1000 goat antimouse secondary antibody (Jackson ImmunoResearch Laboratories). Detection was performed by chemiluminescence (Renaissance; DuPont NEN) using XAR films (Kodak) or the Molecular Imager System, Model GS-363 (Bio-Rad).

For immunocytochemistry the tissue samples after culture were fixed for 0.5 h in freshly prepared 3.7% formaldehyde (Fisher) in phosphate-buffered saline (150 mM NaCl, 2 mM NaH_2PO_4, 7 mM Na_2HPO_4; PBS), then processed using the 6B7 EcR-B1 antibody as previously described (Jindra et al. 1996). Visualization was with diaminobenzidine (DAB) following incubation with 1:1000 donkey antimouse secondary directly conjugated with peroxidase (Jackson ImmunoResearch Laboratories). Pictures of

these whole mounts were taken with a Sony CCD video camera attached to a Nikon Optiphot microscope and processed with Adobe Photoshop.

RNA isolation and hybridization

Total RNA was extracted from the epidermis by the modified method of Chomczynski and Sacchi (1987; Hiruma et al. 1997). Briefly, after the first precipitation with isopropanol, the RNAs were dissolved in TE (10 mM TRIS pH 8.0, 1 mM EDTA, pH 8.0) followed by ethanol precipitation. The RNA concentrations were determined spectrophotometrically (Davis et al. 1986).

For the detection of EcR-A-, EcR-B1-, USP-1, and USP-2-specific RNAs, cRNA probes were synthesized using RNA polymerase and [α^{32}P]-UTP (3000 Ci/mmol; Amersham) as described by a Promega manual. Hybridization and washing conditions at high stringency were as described (Jindra et al. 1997).

The fluorograms were analyzed by densitometry as previously described (Hiruma and Riddiford 1990). Fluorograms were exposed for varying lengths of times so as to be within a linear range of detection, or the hybridized filters were analyzed by the Molecular Imager System, Model GS-363 (Bio-Rad). Each blot contained standard RNA from day 2 fifth, W0, W1, and W3 epidermis for normalization of different blots.

Results

Regulation of USP gene expression in day 2 fourth (penultimate) larval epidermis by 20-hydroxyecdysone in vitro

The level of USP-1 mRNA in the dorsal abdominal epidermis of the fourth instar larva is relatively high during the feeding stage, then declines to a nearly undetectable level when the ecdysteroid titer increases during the molt to the fifth instar (Jindra et al. 1997; Fig. 1). By contrast, USP-2 mRNA increases with the ecdysteroid titer rise during both the larval and pupal molts (Fig. 1). To determine whether these changes in RNA levels were responses to 20E, we cultured day 2 fourth epidermis shortly before the rise of the ecdysteroid for the molt with 2 µg/ml 20E, a concentration previously determined to be sufficient for induction of a new larval cuticle in vitro (Hiruma et al. 1991). When immediately exposed to 20E after dissection, no exogenous JH was required, indicating that sufficient JH and/or its effects are present in the explanted epidermis to prevent metamorphosis (Hiruma et al. 1991). As seen in Fig. 2A, the level of USP-1 mRNA remained high during exposure to 2 µg/ml 20E and showed no differences from tissue incubated without hormones. Exposure to 5 µg/ml 20E, however, caused about a twofold decline in USP-1 mRNA levels by 24 h (Fig. 2A).

However, 2 µg/ml 20E was sufficient to stimulate a 2,5-fold increase in USP-2 mRNA within 3 h and this elevated level was maintained over the remainder of the 24 h culture period (Fig. 2B). The effective concentration, EC_{50} for the induction of USP-2 by 6 h was about 300 ng/ml 20E (6.3×10^{-7} M; Fig. 2C).

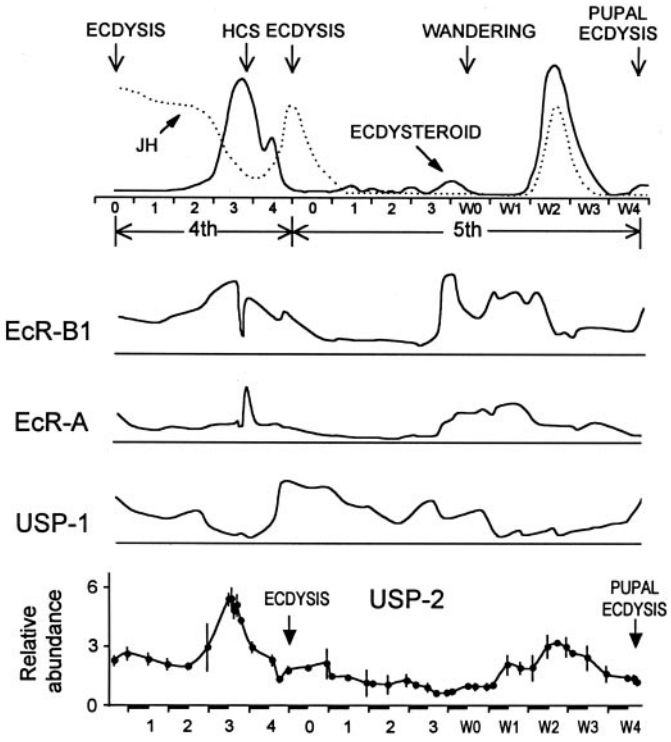

Fig. 1 Diagrammatic fluctuation of the mRNAs for the ecdysone receptors (EcR-B1 and EcR-A; Jindra et al. 1996) and the Ultraspiracle homologs [USP-1 (Jindra et al. 1997) and USP-2] in the dorsal abdominal epidermis of *Manduca* during the final two larval instars. The fluctuation of USP-2 RNA was determined by dot blot analysis using 5 µg total RNA at each time point (n=3–4, mean±SD). The ecdysteroid and juvenile hormone (JH) titers are from Riddiford (1995) except for the ecdysteroid titer in the wandering stage (Fig. 8 in this paper). The relative abundance of USP-2 mRNA was referred to the levels of RNA in the epidermis of day 2 fifth instar larvae as 1. *HCS* onset of head capsule slippage

Regulation of USP and EcR gene expression in day 2 fifth epidermis by 20-hydroxyecdysone and juvenile hormone in vitro

In the fifth larval instar *Manduca*, abdominal epidermis becomes committed to pupal differentiation on the final day of feeding (day 3) in response to the small rise of ecdysteroid in the absence of JH (Riddiford 1978; Fig. 1). Exogenous juvenile hormone prevents pupal commitment if given at the onset of the small rise in ecdysteroid either in vivo (Truman et al. 1974) or in vitro (Riddiford 1976, 1978). Pupally committed epidermis then normally forms a pupal cuticle in response to the prepupal surge of ecdysteroid in the presence of JH (Fig. 1) although it can also form normal pupal cuticle if the JH is removed (Mitsui and Riddiford 1976; Kiguchi and Riddiford 1978).

USP

During the feeding phase of the fifth instar USP-1 mRNA is high, then declines in the pupally committed cells on the day of wandering and is low during the pre-

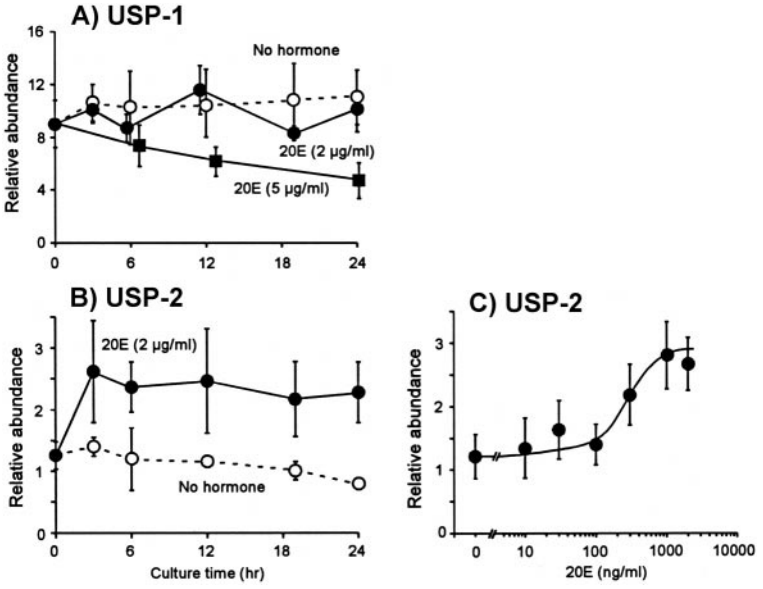

Fig. 2A–C Changes in USP-1 (**A**) and USP-2 (**B** and **C**) mRNA levels in day 2 fourth larval epidermis during exposure to 20-hydroxyecdysone (20E) in vitro. **A** and **B** Time courses during exposure to 2 or 5 µg/ml 20E or to hormone-free medium (*No hormone)* (*n*=4–12, mean±SD). **C** Concentration response of USP-2 mRNA to 20E after 12 h culture (*n*=8, mean±SD). The relative abundances of USP-1 and USP-2 mRNAs were referred to the levels of RNA in the epidermis of day 2 fifth instar larvae as 10 and 1, respectively

pupal period (Jindra et al. 1997; Fig. 1). By contrast, USP-2 mRNA is quite low until the prepupal molting surge of ecdysteroid (Fig. 1). When day 2 fifth instar epidermis was exposed to 500 ng/ml 20E, which causes pupal commitment of all the epidermal cells by 24 h (Riddiford 1978; Riddiford et al. 1986), USP-1 mRNA remained at its preculture level until 12 h (Fig. 3A). It then increased about fivefold by 19 h followed by a slight decline. Northern hybridization detected only a 4.5 kb USP-1 transcript at 19 h, indicating that this increase was due to the same transcript (*n*=4; data not shown). This later increase was sensitive to low levels of 20E with an EC_{50} of about 50 ng/ml (10^{-7} M; Fig. 3C). Importantly, exposure to high concentrations of 20E (2 or 5 µg/ml) for 24 h was unable to cause a decline in USP-1 mRNA, even when 1 µg/ml JH I was present (*n*=4; data not shown).

The presence of 1 µg/ml JH I in the culture medium completely suppressed the later inductive action of 20E on USP-1 mRNA (Fig. 3A). In the absence of 20E, this concentration of JH I had no effect on USP-1 mRNA levels which remained constant for 24 h either in its

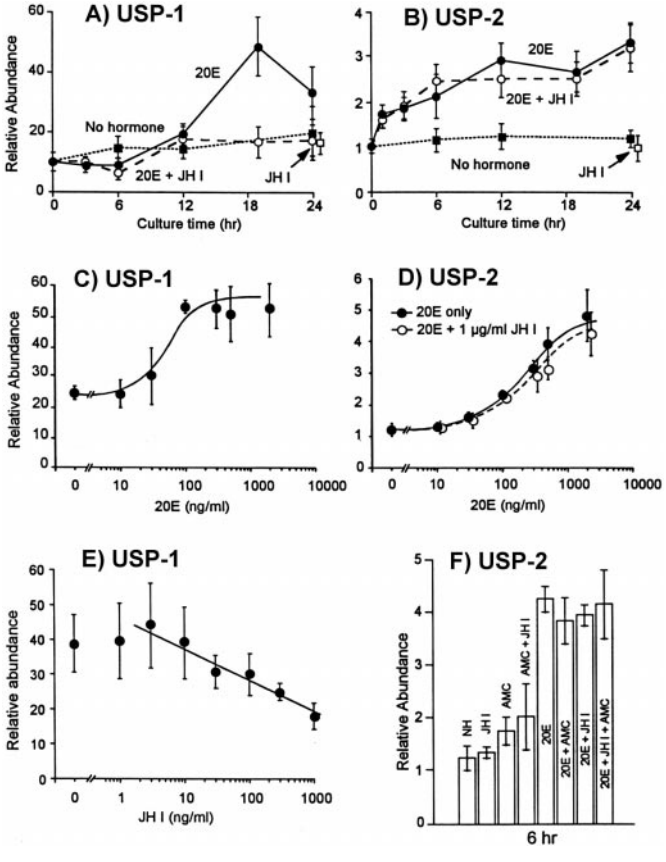

Fig. 3A, B Changes in USP-1 (*n*=16) and USP-2 (*n*=24) (mean±SD) mRNA levels in day 2 fifth instar larval epidermis during exposure to 500 ng/ml 20E, 1 µg/ml JH I, or 500 ng/ml 20E and 1 µg/ml JH I in vitro. The epidermis that was cultured with JH I was pre-incubated with 1 µg/ml JH I for 1–3 h, then transferred to the medium containing both 500 ng/ml 20E and 1 µg/ml JH I. The epidermis that was not cultured with JH I was incubated in plain Grace's medium during this pre-incubation period. **C**, **D** Concentration responses of USP-1 and USP-2 mRNAs to 20E. Day 2 fifth epidermis was cultured with various concentrations of 20E in the presence (only for USP-2) and absence of 1 µg/ml JH I for 18 h. When JH was used, the pieces were pre-treated with JH I as for **A** and **B** (*n*=4, mean±SD). **E** Concentration response of USP-1 mRNA after 18 h exposure to various concentrations of JH I in the presence of 500 ng/ml 20E in vitro (*n*=8, mean±SD). The pieces of day 2 fifth epidermis were pre-cultured for 1–3 h with the same concentrations of JH I that were used for the concentration response. They were then transferred to the medium containing JH I and 20E, and cultured for 18 h. **F** Effect of the protein synthesis inhibitor, anisomycin, on the induction of USP-2 mRNA by 20E in the presence or absence of JH I. Day 2 fifth instar epidermis was cultured in 500 ng/ml 20E±1 µg/ml JH I with or without 10 µg/ml anisomycin (AMC) for 6 h, then the RNA was extracted and analyzed by dot blot hybridization. In this condition, >99% protein synthesis was prevented (Palli et al. 1992). Control levels of USP-2 RNA in epidermis incubated only with anisomycin or in the absence of hormones (NH) are also shown. *Bars* represent averages (*n*=8–12, mean±SD). The relative abundances were as described in Fig. 2

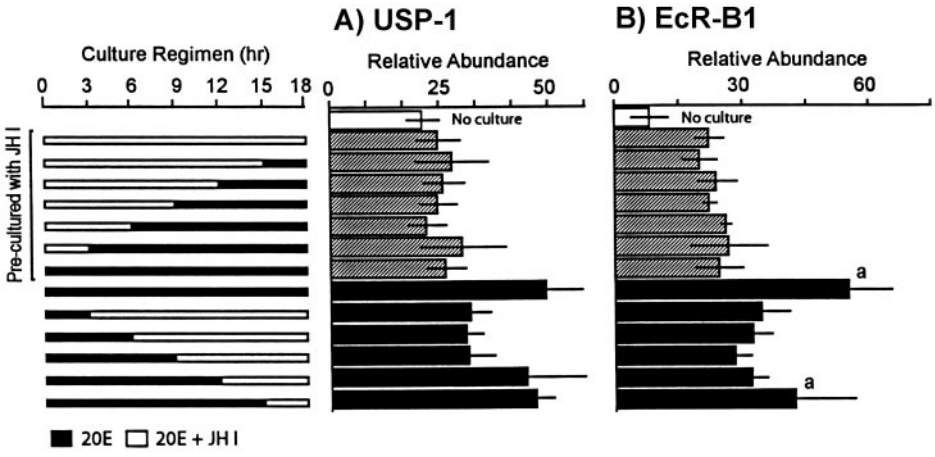

Fig. 4A, B The timing of JH I addition necessary to suppress the expression of USP-1 (**A**) and EcR-B1 (**B**) in day 2 fifth epidermis in vitro. One µg/ml JH I was added to the medium containing 500 ng/ml 20E at various times as indicated in the figure. Pre-culture with 1 µg/ml JH I was performed 1–3 h. When the pre-culture was not performed, the pieces of epidermis were incubated in Grace's medium for the equivalent time. The time of the addition of 20E was set as the start of culture. The relative abundance of USP-1 mRNA was as described in Fig. 2, and that of EcR-B1 is referred to the levels of RNA in the epidermis of day 2 fifth instar larvae as 10 (n=4–8, mean±SD); [a]Not significantly different at P=0.0575

presence or absence (Fig. 3A). The suppressive action of JH I on the later 20E-induced increase in USP-1 mRNA increased with the log of the JH concentration above 10 ng/ml (3×10^{-8} M; Fig. 3E). In all these experiments with JH I, the epidermis was precultured with JH I for 1–3 h before 20E was added and JH was present throughout the exposure to 20E. To determine the critical time for this suppressive action of JH, we transferred the pieces to JH-free medium containing 20E at various times. Figure 4 A shows that the 1- to 3-h pretreatment with JH followed by culture in 20E alone was sufficient for the suppressive effect of JH on the later 20E-induced increase in USP-1 mRNA. No significant difference was found between 1 and 3 h pre-culture with JH I (n=8; data not shown). When the tissue was first exposed to 20E for various times, then transferred to medium containing 20E and JH I, the suppressive effect of JH was lost between 9 and 12 h exposure to 20E (Fig. 4A), approximately the same time when addition of JH was no longer able to prevent the 20E-induced pupal commitment of most of the epidermal cells (Riddiford 1978).

In contrast to USP-1 mRNA, USP-2 mRNA rapidly increased from its initial low levels in day 2 fifth epidermis (Fig. 1) in response to 500 ng/ml 20E, nearly doubling by 1 h followed by a slow steady increase to a tripling of the initial level by 24 h (Fig. 3B). This induction of USP-2 mRNA had an EC_{50} of 300 ng/ml 20E (6.3×10^{-7} M; Fig. 3D), the same as found for the fourth instar epidermis (Fig. 2C). Moreover, this induction of USP-2 mRNA was unaffected by the presence of 10 µg/ml anisomycin, a protein synthesis inhibitor (Fig. 3F), indicating that the induction by 20E was direct rather than through an ecdysteroid-induced transcription factor.

JH I (1 µg/ml) had little effect on the induction of USP-2 mRNA by 20E, either in terms of the time course (Fig. 3B) or the concentration response curve (Fig. 3D). When day 2 epidermis was exposed to JH I alone or to hormone-free medium, no increase of USP-2 mRNA was observed (Fig. 3B). Thus, USP-2 mRNA is directly inducible by 20E but is unaffected by the presence or absence of JH.

EcR

Previous studies showed that culture of day 2 fifth larval epidermis with 500 ng/ml 20E caused increases in both EcR-A and EcR-B1 mRNAs with EcR-B1 RNA showing a biphasic response with peaks at 3 and 18 h (Hiruma et al. 1997). Figure 5A and B confirms this finding with 20E and further shows that the presence of 1 µg/ml JH I had no effect on the initial 20E-induced increases in the EcR mRNAs. The presence of JH I however prevented the second increase in EcR-B1 mRNA (Fig. 5A) and caused a decline in EcR-A mRNA to levels seen in epidermis cultured in hormone-free medium by 12 h (Fig. 5B). This effect of JH I on both EcR-B1 and EcR-A mRNAs was concentration-dependent when assayed after 18 h culture with 20E with suppression first seen at about 1 ng/ml for EcR-A (Fig. 5D) and 10 ng/ml for EcR-B1(Fig. 5C). Exposure to only JH I had no effect on either EcR-B1 or EcR-A mRNA expression after 24 h in vitro which showed similar slight increases in either hormone-free or JH-containing medium over this time period (Fig. 5A, B).

As with the suppressive effect of JH on the later 20E induction of USP-1 mRNA, the initial 1- to 3-h exposure to JH I (with no significant difference between the two preculture periods; n=8) was sufficient for the later inhibitory effect on EcR-B1 expression at 18 h (Fig. 4B). The loss of responsiveness to JH I after exposure to 20E alone was between 12 and 15 h (Fig. 4B), slightly later than seen for USP-1 mRNA (Fig. 4A). Similar effects

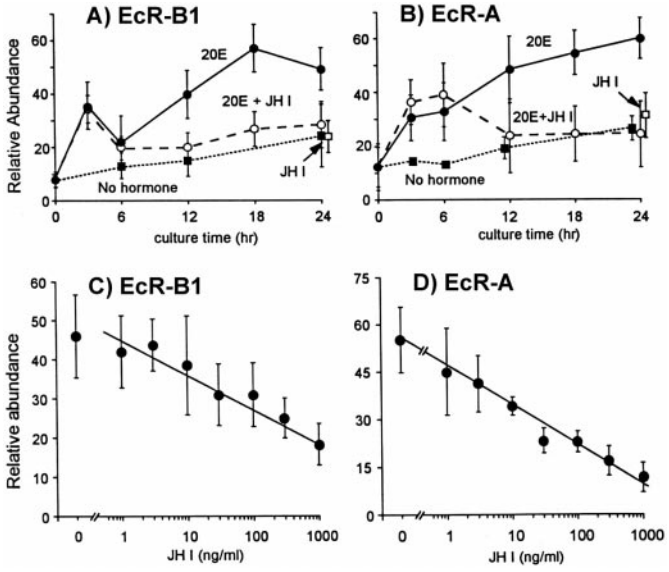

Fig. 5A, B Changes in EcR-B1 (**A**; *n*=20), and EcR-A (**B**; *n*=20, mean±SD) mRNA levels in day 2 fifth instar larval epidermis during exposure to 500 ng/ml 20E, 1 µg/ml JH I, or 500 ng/ml 20E and 1 µg/ml JH I in vitro. The epidermis that was cultured with JH I was pre-incubated with 1 µg/ml JH I for 1–3 h, then transferred to the medium containing both 500 ng/ml 20E and 1 µg/ml JH I. The epidermis that was not cultured with JH I was incubated in Grace's medium during this pre-incubation period. **C**, **D** Concentration response of EcR-B1 and EcR-A mRNA after 18 h exposure to various concentrations of JH I in the presence of 500 ng/ml 20E in vitro (*n*=8, mean±SD). The pieces of day 2 fifth epidermis were precultured for 1–3 h with the same concentrations of JH I that were used for the concentration response. They were then transferred to the medium containing JH I and 20E, and cultured for 18 h. The relative abundances of EcR-B1 and EcR-A were referred to the levels of RNA in the epidermis of day 2 fifth instar larvae as 10

were found for the regulation of EcR-A expression by JH I (*n*=4–8; data not shown).

Thus, the suppressive effects of JH on the later expression of USP-1 and EcR-A and -B1 mRNAs to 20E are specific to these mRNAs and not shared by USP-2 mRNA.

Juvenile hormone prolongs the presence of the EcR-B1 protein, but not its mRNA

Day 2 fifth epidermis contains a very low amount of EcR-B1 protein (Jindra et al. 1996). When day 2 fifth epidermis was cultured with 500 ng/ml 20E, the EcR-B1protein as detected by immunoblotting began to increase within 3 h (data not shown; *n*=4) and by 6 h was clearly much higher than that seen in hormone-free medium (Fig. 6A). By 24 h, the level of EcR-B1 in the tissue had increased still further (Fig. 6A). When 1 µg/ml JH I was present with the 20E, EcR-B1 protein increased with the same time course and remained high through the 24 h culture period (Fig. 6A), even though the mRNA declined after the initial increase at 3 h culture (Fig. 5A). No increase of EcR-B1 protein was observed in hormone-free medium (Fig. 6A) despite the slight increase in EcR-B1 mRNA (Fig. 5A). Immunocytochemical studies with the 6B7 mAb specific for EcR-B1 (Jindra et al. 1996) showed that all the EcR-B1 protein was located in the general epidermal nuclei, irrespective of the hormonal conditions, and that after 24 h culture levels were similar between those treated with 20E and with 20E and JH I (Fig. 7, top). Many trichogen and tormogen cell nuclei showed high EcR-B1 levels after 24 h exposure to 20E, but had little or no EcR-B1 if JH was present. Neither the attached tracheae nor the remaining stubs of the intersegmental and intrasegmental muscles showed any nuclear labeling with EcR-B1 during this 24 h culture period (data not shown). EcR-A protein also persisted in the nucleus in epidermis exposed to 20E and JH for 24 h (Fig. 7, bottom) despite the decline in mRNA accumulation (Fig. 5B).

These results suggest that the EcR-B1 protein either has a long intrinsic half life or persisted longer due to the JH, or both. To distinguish between these possibilities, we cultured day 2 fifth epidermis in medium containing both 500 ng/ml 20E and 1 µg/ml JH I for 6 h to allow the EcR-B1 protein to accumulate, then transferred it to either hormone-free or 1 µg/ml JH I-containing medium for various times and analyzed the levels of EcR-B1 RNA and protein. Figure 6B and C shows that after transfer to hormone-free conditions, EcR-B1 protein levels declined rapidly to those seen in tissue maintained continuously in hormone-free medium by 6 h. A slower decline was seen in tissue transferred to JH I-containing medium so that basal levels were not attained until 13 h. By contrast, EcR-B1 mRNA declined rapidly to the basal level within 3 h after transfer to the medium without 20E, irrespective of the presence or absence of JH I (Fig. 6D). Thus, the half life of the protein is longer than that of its mRNA, but in addition, JH somehow causes an additional stabilization.

Effects of allatectomy on prepupal development and EcR and USP expression

Normally during the prepupal surge of ecdysteroid, JH reappears in *Manduca* hemolymph (Baker et al. 1987) and prevents precocious adult differentiation of some of the imaginal tissues (Kiguchi and Riddiford 1978). To determine whether this JH has any influence on the patterns of expression of EcR and USP in the abdominal epidermis at this time, we studied the effects of removal of the corpora allata (the source of JH) and of JH I replacement on prepupal development and on levels of EcR and USP mRNAs. Fourth instar larvae were allatectomized 5–6 h before the head capsule slippage (HCS) stage during the molt to the fifth instar after the critical period for JH for production of a larval cuticle but before the critical period for cuticular melanization (Hiruma and Riddiford 1985). After this operation, JH is undetectable in the hemolymph of day 0 fifth instar larvae (Goodman et al. 1995) and the feeding period of the fifth instar larvae was shortened to 3.65±0.48 days (mean±SD, *n*=123) from 4.38±0.49 days (mean±SD, *n*=63) for intact larvae

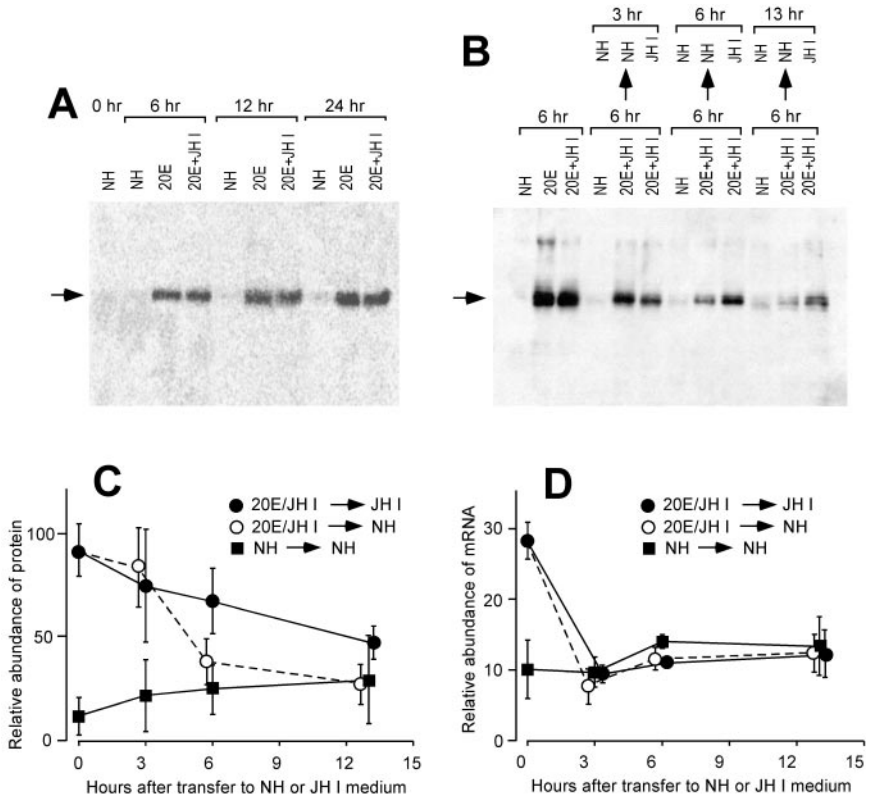

Fig. 6A–D Persistence of the EcR-B1 protein in the presence of JH I as detected by immunoblotting. **A** Day 2 fifth epidermis was cultured with 500 ng/ml 20E with or without 1 µg/ml JH I for 6, 12 and 24 h (a typical result of eight repeats). **B**–**D** Day 2 fifth epidermis was cultured with 500 ng/ml 20E in the presence of 1 µg/ml JH I for 6 h, then transferred to 20E-free medium with or without 1 µg/ml JH I and incubated for the indicated number of hours on the figures. **B** immunoblot typical of four repeats, and **C** shows the average intensities of each band of EcR-B1 protein (n=4). The average abundance of epidermal EcR-B1 protein after culture with 500 ng/ml 20E for 6 h was set as 100. **D** The average abundance of EcR-B1 mRNA (n=4). The relative abundance of the mRNA was as described in Fig. 4. A piece of the cultured integument was bisected, and one half was analyzed for protein and the other for RNA, so that **C** and **D** could be compared in the identically treated epidermis. The *arrows* in **A** and **B** indicate EcR-B1 (*NH* no hormone)

(P<0.0001). These larvae prematurely wandered either on day 3 (Gate 0) or day 4 (Gate I) as previously observed by Kiguchi and Riddiford (1978).

After wandering, ocellar retraction is one of the first major externally visible morphological changes that is caused by the prepupal rise of the ecdysteroid titer (Truman and Riddiford 1974), then pupation occurs 3 days later. Figure 8A and B shows that the timing of ocellar retraction was little affected by allatectomy, but that pupation was delayed by an average of about 18 to 20 h. Moreover, the timing of pupation was not as synchronous in allatectomized larvae as in intact larvae. When 10 µg/ml JH I was applied to the allatectomized larvae on the day of wandering, pupation occurred at the same time as that of intact larvae (Fig. 8B).

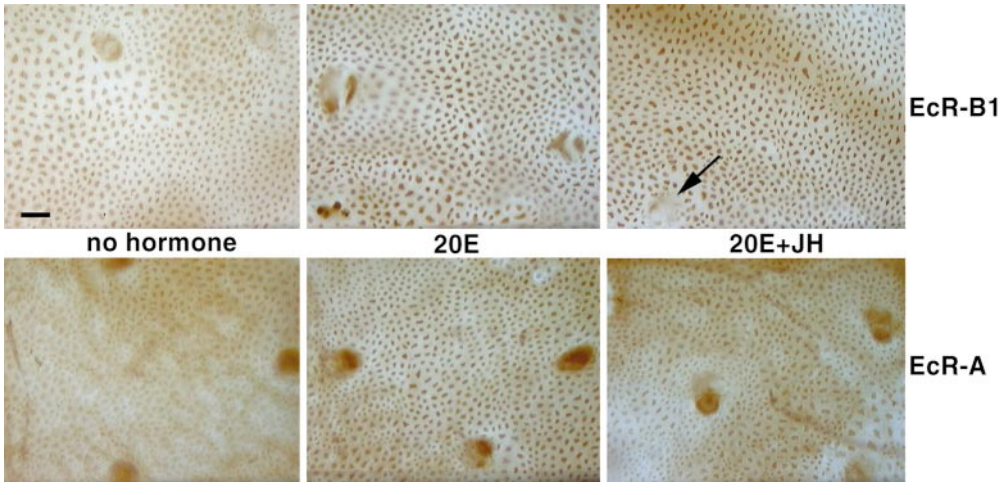

Fig. 7 Immunocytochemical analysis of day 2 fifth epidermis after 24 h in vitro using 1:1000 anti-EcR-B1 monoclonal antibody (6B7; Jindra et al. 1996; *top*) and 1:3000 polyclonal EcR-A antibody (Hegstrom et al. 1998; *bottom*). Day 2 fifth epidermis was cultured as described in Fig. 6A, then pieces were examined at 6, 12 and 24 h. Only 24 h cultures are shown and are representative of six different animals assessed for each treatment. *Arrow* indicates trichogen and tormogen cell nuclei lacking EcR-B1 immunostaining (**Bar** 50 µm)

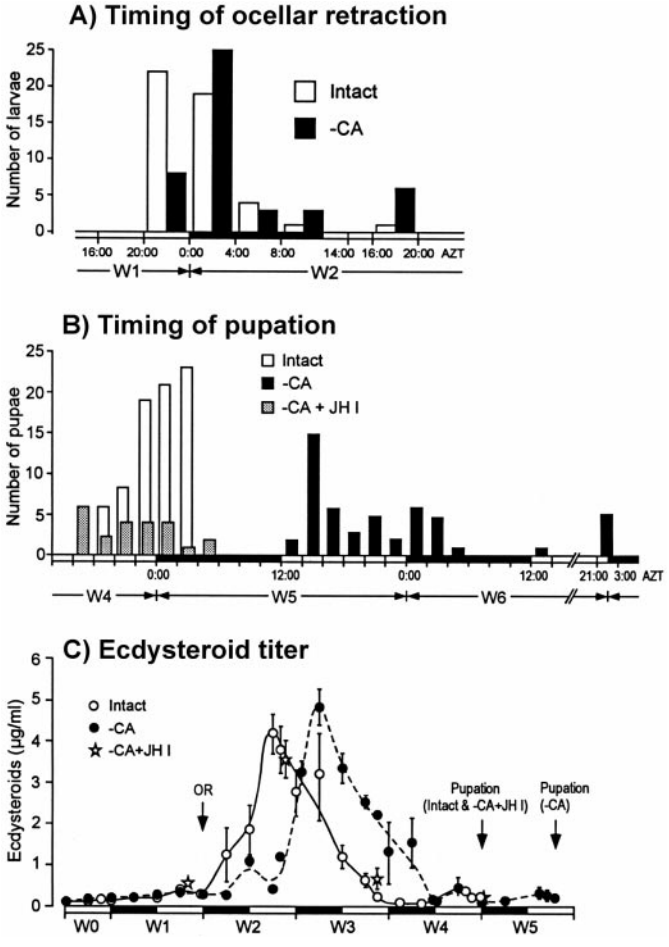

Fig. 8A–C Effect of allatectomy on the timing of ocellar retraction (**A**), pupal ecdysis (**B**), and ecdysteroid titer in the hemolymph (n=3–4, mean±SE). (**C**) during pupal development. In **C**, four points before the pupation in both intact and allatectomized larvae were "tan metathoracic bar" (TM) stage, TM+6 h, onset of molting fluid resorption in the anterior abdomen and posterior abdomen (19, 6, 4, and 2 h before pupal ecdysis), respectively. Allatectomy was performed 5–6 h before head capsule slippage (HCS; see Materials and methods). Under these conditions, all the larvae ecdysed to melanized fifth instar larvae (Hiruma and Riddiford 1985). Ten micrograms of JH I dissolved in 1 µl cyclohexane were topically applied to the dorsal region of the allatectomized larvae on the day of wandering stage [15:00 Arbitrary Zeitgeber Time (AZT), *W0*]

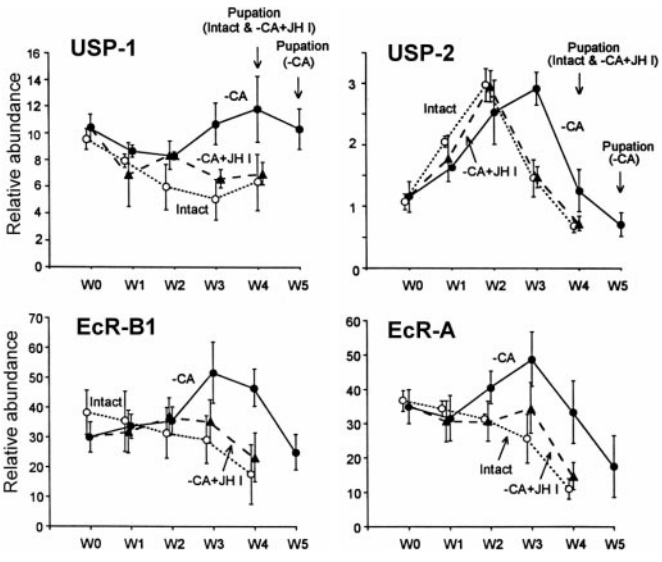

Fig. 9 Effect of allatectomy on the levels of epidermal EcR-B1, EcR-A, USP-1 and USP-2 mRNAs during prepupal development. The RNAs were from larvae at 19:00 to 20:00 AZT in each day except for the final point, just after pupal ecdysis. The experimental manipulations were performed as described in Fig. 8. The relative abundances of RNAs were as described in Figs. 2 and 5 (n=7–8, mean±SD)

In both intact and allatectomized larvae, the ecdysteroid titers in the hemolymph were low until the beginning of the second day after wandering when they began to increase (Fig. 8C). The peak of ecdysteroid was observed in intact larvae at 18:00 AZT on this day and 12 h later in allatectomized larvae. Application of 10 µg/ml JH I on the day of wandering restored the normal timing of the ecdysteroid titer. These results indicate that the delay of pupation in allatectomized larvae is due to the delay in the rise of ecdysteroid in the absence of JH during the prepupal period just as was found in the cabbage armyworm, *Mamestra brassicae* (Hiruma 1986).

Figure 9 shows that the levels of USP-1 and the EcR mRNAs in the dorsal abdominal epidermis of allatectomized larvae were similar to those of intact larvae for the first 2 days after the onset of wandering. Then they all increased in the allatectomized larvae on the third day rather than declined as seen normally in intact larvae. This increase is correlated with the increase of ecdysteroid at this time in the allatectomized larvae (Fig. 8C). The increase in USP-1 mRNA was relatively slight but these significantly higher levels compared to those in intact larvae (P=0.0006) were maintained until pupal ecdysis despite the decline in ecdysteroid titer (Fig. 8C). By contrast, the decline in EcR-A and EcR-B1 mRNA levels on days 4 and 5 paralleled that seen in intact larvae on days 3 and 4 and is likely due to the decline in ecdysteroid. The delayed accumulation of USP-2 mRNA and its subsequent decline in the allatectomized larval epidermis (Fig. 9) clearly mirrored the delayed rise and decline of the ecdysteroid titer in these animals (Fig. 8C), similar to its normal expression in vivo (Fig. 1). The absence of JH had little effect on peak accumulation of USP-2 mRNA. Application of 10 µg JH I to the allatectomized larvae on the day of wandering restored the normal patterns of mRNA expression. Thus, the pattern of expression of USP-2 induced by ecdysteroid during the pupal molt is little changed by the absence of JH whereas those of USP-1 and of the two EcRs are altered such that the rising ecdysteroid causes increases rather than decreases in these mRNAs.

Discussion

Insect molting and metamorphosis are orchestrated by ecdysteroids with JH acting to prevent the switching ac-

tions of ecdysteroids necessary for metamorphosis (Riddiford 1996). In *Drosophila* where most larval tissues are discarded at metamorphosis and the pupa and adult are formed from imaginal discs, this action of JH is limited to a few tissues: namely, the cerebral nervous system (CNS) that shows considerable remodeling during metamorphosis (Truman and Bate 1988; Restifo and Wilson 1998) and the abdominal histoblasts which do not divide until the onset of metamorphosis (Postlethwait 1974). By contrast, in Lepidoptera most tissues are remodeled at metamorphosis for their new adult roles, and these processes are initiated by ecdysteroids acting in the absence of JH. The critical time for this action of JH is at the outset of the ecdysteroid rise (Riddiford 1996), a time when the ecdysteroid-induced transcription factor cascade is being initiated [reviewed for *Drosophila* (Thummel 1996) and for *Manduca* (Riddiford et al. 1998)] and a time when both qualitative and quantitative changes in the EcR/USP heterodimer may occur (Jindra et al. 1996, 1997). In *Manduca* epidermis the presence of JH prevents the 20E induction of the RNAs for the Broad Complex transcription factors which first appear at the time of pupal commitment of the epidermis (Zhou et al. 1998b) and enhances the levels of the RNA for the 20E-induced early E75 A transcription factor (Zhou et al. 1998a). The present studies have concentrated on the effects of JH on the 20E-induced changes in the two members of the EcR heterodimer, EcR and USP.

These studies have shown that the appearance of USP-2 mRNA in *Manduca* abdominal epidermis during both the larval and pupal molts (Jindra et al. 1997) is dependent solely on the increasing ecdysteroid titer, but the normal loss of USP-1 mRNA in response to high ecdysteroid at these times depends on the presence of JH. In the absence of JH, USP-1 mRNA persists at high levels during exposure to ecdysteroid. Larval epidermis also responds immediately to an increase in 20E by accumulating both EcR-B1 and EcR-A mRNAs with differing dynamics, but neither the levels nor the timing is dependent on JH (Jindra et al. 1996; Hiruma et al. 1997; these studies). Once the epidermis becomes pupally committed, however, both EcR mRNAs are up-regulated by 20E in the absence of JH but down-regulated by 20E in its presence.

Control of USP isoform switching during the molt by 20E and JH

Although *Drosophila* apparently has only one isoform of USP, mosquitoes (Kapitskaya et al. 1996), *Manduca* (Jindra et al. 1997), and ticks (Guo et al. 1998) have two isoforms of this heterodimeric partner for EcR. Relatively little is known about the function of these different isoforms. In *Manduca* epidermis there is a switch from USP-1 to USP-2 mRNA (Jindra et al. 1997) and protein (Asahina et al. 1997) during both the larval and pupal molts. Only the EcR-B1/USP-1 heterodimer is able to bind to and activate the 4.5-kb MHR3 promoter in the ecdysteroid-responsive *Manduca* GV1 cell line (Lan et al. 1998). The EcR-B1/USP-2 heterodimer neither binds nor activates this promoter although it binds ponasterone A with equal affinity to that of EcR-B1/USP-1. In the epidermis USP-1 mRNA has declined to low levels (Jindra et al. 1997) by the time that MHR3 is being induced (Palli et al. 1992; R. Langelan, K. Hiruma, S. R. Palli, and L. M. Riddiford, in preparation). Presumably sufficient USP-1 protein is still present at this time to participate in the induction. Since the presently available USP antibody detects both isoforms and the latter differ only slightly in molecular weight, the distinction between the two when both are present is equivocal (Asahina et al. 1997).

Our present studies show that USP-2 mRNA is up-regulated rapidly by concentrations of 20E above 100 ng/ml ($EC_{50}=2\times10^{-7}$ M), hence its appearance in vivo early during the ecdysteroid rise for the molts. By contrast, the down-regulation of USP-1 RNA in fourth instar epidermis in vitro required high levels of 20E ($>4\times10^{-6}$ M 20E). Recent radioimmunoassays with the antibody used here with high affinity for 20E (Yokoyama et al. 1996) show that peak levels of 20E during the fourth instar molt of *Manduca* are about 8×10^{-6} M (F. Malone and L. M. Riddiford, unpublished), a somewhat higher level than the 6×10^{-6} M 20E equivalents previously determined by Curtis et al. (1984). Thus, USP-1 mRNA is down-regulated in vitro by physiologically relevant levels of 20E but not as rapidly as seen in vivo where levels are already basal by several hours before the peak of the ecdysteroid titer (Jindra et al. 1997). The finding that the down-regulation of USP-1 mRNA by rising ecdysteroid during the pupal molt requires the presence of JH suggests that the presence of JH, albeit at declining levels (Fain and Riddiford 1975; Hidayat and Goodman 1994), may also be important in modulating the responsiveness of USP-1 mRNA during the larval molt. Although sufficient JH and/or its effects are present in this epidermis at the time of explantation to allow a larval molting response to 4×10^{-6} M 20E (Hiruma et al. 1991), it is not sufficient for the normal down-regulation of USP-1 mRNA by 20E.

During the pupal molt the up-regulation of USP-2 mRNA by the rising ecdysteroid was unaffected by the presence or absence of JH. The only effect of allatectomy was a delay in the USP-2 peak expression which paralleled the delay in the prepupal ecdysteroid peak. By contrast, the down-regulation of USP-1 mRNA did not occur in the absence of JH in the allatectomized larvae. Instead with the increasing ecdysteroid titer, USP-1 mRNA levels rose slightly and remained high until pupal ecdysis. Whether or not this mRNA is still translated into protein cannot be determined at present as discussed above. The biological consequence of this continued high level of USP-1 mRNA is clear since on a gross level the abdominal epidermis produces an apparently normal pupal cuticle in the absence of JH either in vivo (Kiguchi and Riddiford 1978) or in vitro (Mitsui and Riddiford 1976). An increase in USP-1 mRNA is normally seen in

both pupal wings and dorsal thorax at the time of the peak of the ecdysteroid titer for the adult molt (Jindra et al. 1997). Yet after injection of pyriproxifen, a JH mimic, to cause the formation of a second pupal cuticle, USP-1 mRNA decreases with the rising ecdysteroid titer (F. Malone and L.M. Riddiford, unpublished) as seen in both the normal larval and pupal molts. Thus, the presence of JH seems to be necessary for the down-regulation of USP-1 by 20E in the epidermis.

Influence of JH on patterns of EcR and USP during pupal commitment and subsequent differentiation

Normally the epidermis is pupally committed by exposure to a small transient increase in ecdysteroid in the absence of JH on the final day of feeding (day 3 of the fifth instar; Riddiford 1978; Wolfgang and Riddiford 1986). This pupal commitment occurs on a cell-by-cell basis over the abdominal segment during about a 24 h period as assessed by the ability of the cells to make pupal cuticle despite being challenged with a larval molting environment by implantation into a molting fourth instar larvae (Riddiford 1978) and by their inability to make another larval cuticle if given JH during this period (Truman et al. 1974). The wandering behavior that begins early the following day is also initiated by this transient ecdysteroid in the absence of JH (Dominick and Truman 1985). The day after the onset of wandering, the ecdysteroid titer begins to rise again, slowly at first, then more rapidly to its peak on the second day (Kato and Riddiford 1987; Fig. 8C). Coincident with and caused by the slow rise in ecdysteroid is an increase in mitotic rate in the epidermis (Kato and Riddiford 1987) accompanied by cellular changes necessary to turn a larval abdomen into a pupal abdomen (Locke and Huie 1981; L.M. Riddiford, unpublished) before the pupal cuticle is deposited during the decline of the ecdysteroid titer on the third day after the onset of wandering (Sedlak and Gilbert 1979; Kiely and Riddiford 1985). In the following discussion, the cellular events caused by the rising ecdysteroid prior to pupal cuticle deposition will be grouped together and referred to as “pupal predifferentiative changes”.

When exposed to 500 ng/ml (10^{-6} M) 20E in vitro [a concentration 4 times higher than that seen in vivo (Wolfgang and Riddiford 1986)], day 2 fifth larval epidermal cells become pupally committed over a 24 h period with some cells requiring longer exposures than others as is also seen in vivo (Riddiford 1978; Mitsui and Riddiford 1978; Riddiford et al. 1986). Pupal commitment of about half of the cells is complete by 14 h (Riddiford 1978; Mitsui and Riddiford 1978). During such an exposure to 20E, the changes in both EcR-A and -B1 mRNAs (Hiruma et al. 1997; Fig. 5A, B) are similar to those seen in vivo during day 3 and wandering to the beginning of the day after wandering (Jindra et al. 1996; see Fig. 1). Thus, EcR-B1 shows a biphasic response with a rapid initial increase followed by a second increase in the final 12 h. By contrast, EcR-A increases more slowly but steadily. When JH was present, the early responses of both EcRs to 20E were unchanged whereas the responses during the second 12 h were prevented. Similarly, the increase of USP-1 mRNA to 20E during the last 12 h was prevented by JH. By contrast, JH had no effect on the pattern of USP-2 accumulation throughout the culture with 20E. Under these culture conditions, the mRNA for the transcription factor E75A was rapidly induced to maximal levels by 2–3 h exposure to 20E, then declined to low levels by 6 h which were maintained until 24 h (Zhou et al. 1998a). In this case, the presence of JH increased the mRNA abundance nearly twofold but did not change the pattern of accumulation. The RNAs for the BRC transcription factors which in *Manduca* first appear in the epidermis at pupal commitment only appeared in this cultured epidermis after 6 h of 20E, then increased over the next 18 h (Zhou et al. 1998b). The presence of JH prevented their appearance at any time during the 24 h exposure to 20E. Thus, JH affects the response of each of these genes to 20E differently, having no early effects on the responses of EcR and USP, preventing the up-regulation of BRC and quantitatively regulating the level of the E75A mRNA induced. None of these genes show any response to JH alone. These results suggest that the later effects of JH on the expression patterns of EcR-A, EcR-B1, and USP-1 are the results of differences in the transcription factor cascade induced by 20E alone and by 20E in the presence of JH. These differences could be due to the differences in E75A and BRC levels discussed above or to some other factors not yet identified in *Manduca*.

In all the in vitro cultures with JH discussed above, the epidermis was preincubated with JH for 1–3 h before the addition of 20E since this regimen had been found necessary for the prevention of pupal commitment in all the cells (Riddiford 1978). After simultaneous exposure to 20E and a JH mimic epoxygeranylsesamole (EGS), some cells became pupally committed (Riddiford 1978). By 9 h exposure to 20E, the addition of JH was unable to prevent pupal commitment in 60% of the cells and by 16 h all cells had lost their responsiveness to JH. In the present experiments the preincubation with JH I was sufficient to inhibit the later changes in EcR and USP-1 mRNAs in response to 20E. Since the half life of JH I in day 2 epidermis is 21 min (Mitsui et al. 1979), the JH I in the epidermis should be reduced to about 10^{-9} M by about 4 h after transfer to hormone-free medium. However, the cuticle retains about 3–4% of the JH taken up (Mitsui et al. 1979) which likely slowly leaches into the epidermis under the hormone-free conditions so physiological concentrations of JH [2×10^{-8}M at the time of the fourth instar molt (Fain and Riddiford 1975] are probably maintained for some time after the transfer.

The changes seen in the second 12 h of exposure to 20E alone are either simply those of pupally committed cells or possibly associated with the onset of the pupal predifferentiative phase in these cells during the continued exposure to 20E since the 20E is not metabolized by

the epidermis during the culture period (Hiruma et al. 1997). Apolysis but no cuticle production is evident after 24 h exposure to 20E, and wandering stage epidermis must be exposed to the low concentration of 20E (500 ng/ml) used here for 4 days to obtain a new cuticle (Mitsui and Riddiford 1976). The responses of EcR-A, EcR-B1, and USP-1 mRNAs during this time are similar to those seen in epidermis of allatectomized larvae during the pupal molt when no JH is present (Fig. 9). This effect of 20E can be inhibited by JH when added at any time up to 12 h after exposure to 20E, then as over 50% of the cells become pupally committed, JH seems to lose its effectiveness. Yet during the pupal molt in vivo this up-regulation by 20E in allatectomized larvae can be inhibited by JH application during the wandering stage when all the epidermal cells are pupally committed (Riddiford 1978), indicating that pupally committed cells can respond to the JH. The lack of effect of JH in vitro on these mRNA levels at 18 h when added after 12–15 h exposure to 20E likely simply reflects the short time interval between application and analysis since the level of mRNA at the time of application is high.

In contrast to pupally committed epidermis, 50% of the day 2 fifth larval epidermal explants formed a new larval cuticle after exposure to 1 µg/ml 20E and 3 µg/ml JH I for only 24 h (Mitsui and Riddiford 1978) and day 2 fourth epidermis formed a thin new cuticle after exposure to 500 ng/ml 20E for 17 h (Hiruma et al. 1991). In the latter case 1 µg/ml 20E was necessary for the normal thick, sculptured larval cuticle production. In both cases the new cuticle was not seen during the exposure to 20E but only after transfer to hormone-free medium and then after a minimum of 17 h. Therefore, in our cultures in the presence of JH, the day 2 fifth epidermis is likely beginning the predifferentiative phase of the larval molt during the exposure to 500 ng/ml 20E. Except for EcR-A mRNAs, the changes in EcR-B1 and the 2 USPs are consistent with their in vivo changes during the early part of the ecdysteroid rise (Jindra et al. 1996, 1997). The early transient increase in EcR-A mRNA was not detected in vivo (Jindra et al. 1996) which may have been due to lack of sampling during about an 8 h period during the initial rising phase of ecdysteroid.

These studies have concentrated on the changes in the mRNA levels of the EcR and USP isoforms. The developmental patterns of the changes in levels of the USP isoforms (Asahina et al. 1997) and EcR-B1 (Jindra et al. 1996) are similar to those of their RNAs during the fourth and fifth larval instars. Also, in vitro in the absence of JH, EcR-B1 protein levels mirror the changing mRNA levels although with a slight time-lag. By contrast, in the presence of JH, where both EcR-A and EcR-B1 mRNA levels decline after 6 h, the respective proteins are still found in the nucleus after 24 h and presumably remained functional. The further studies with EcR-B1 by immunoblotting showed that the half life of the protein is longer than that of its mRNA, but in addition, the protein persists longer in the presence of JH. Either JH may stabilize the protein or it may increase the translation rate of the low level of mRNA present. Preliminary immunocytochemical experiments with the USP monoclonal antibody that detects both isoforms (Asahina et al. 1997) show similar levels in tissue treated with 20E alone and 20E with JH (Asahina and Riddiford, unpublished). For further analysis an antibody specific to one of the isoforms is needed.

These studies have thus shown that initial responses of EcR-A and EcR-B1 in larval epidermis to 20E are unaffected by JH but only their later responses as the cells are becoming pupally committed are altered. Also, the regulation of USP-2 mRNA by 20E is not affected in either pattern or amount by JH. By contrast, the down-regulation of USP-1 mRNA by 20E appears to require the presence of JH. The basis of this action and its biological significance remain unclear. These results and the absence of any effect of JH by itself on the levels of either USP mRNA indicate that if USP were the biological receptor for JH as proposed by Jones and Sharp (1997), then it behaves much differently than EcR (Karim and Thummel 1992; Jindra et al. 1996; Hiruma et al. 1997) and thyroid hormone receptors (Tata 1996) which are up-regulated by their ligands.

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