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Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*

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SUMMARY

The understanding of the molecular basis of the endocrine control of insect metamorphosis has been hampered by the profound differences in responses of the Lepidoptera and the Diptera to juvenile hormone (JH). In both *Manduca* and *Drosophila*, the *broad* (*br*) gene is expressed in the epidermis during the formation of the pupa, but not during adult differentiation. Misexpression of BR-Z1 during either a larval or an adult molt of *Drosophila* suppressed stage-specific cuticle genes and activated pupal cuticle genes, showing that *br* is a major specifier of the pupal stage. Treatment with a JH mimic at the onset of the adult molt causes *br* re-expression and the formation of a second pupal cuticle in *Manduca*, but only in the abdomen of *Drosophila*. Expression of the BR isoforms during adult development of *Drosophila* suppressed bristle and hair formation when induced early or redirected cuticle production toward the pupal program when induced late. Expression of BR-Z1 at both of these times mimicked the effect of JH application but, unlike JH, it caused production of a new pupal cuticle on the head and thorax as well as on the abdomen. Consequently, the 'status quo' action of JH on the pupal-adult transformation is mediated by the JH-induced re-expression of BR.

Key words: Broad, Juvenile hormone, Metamorphosis, Pupal development, Pupal-adult transformation, *Drosophila*, *Manduca*, *Edg78E*, *Acp65A*, *Lcp65A-b*

INTRODUCTION

Molting and metamorphosis in insects are under the control of two hormones, the steroid ecdysone and the sesquiterpenoid juvenile hormone (JH) (Riddiford, 1994) [We use the term 'ecdysone' as a generic term to refer to the natural ecdysteroids that cause molting and metamorphosis, primarily α-ecdysone and its metabolite 20-hydroxyecdysone (Riddiford et al., 2001).] Ecdysone induces and coordinates molting, but the character of the molt is determined by JH. In the presence of JH, there is no change in form; in the absence of JH, ecdysone causes the switching in gene expression necessary for metamorphosis, first to the pupa, then to the adult. JH therefore prevents this switching action of ecdysone and thus maintains the 'status quo' during a molt (Williams, 1961). In the Coleoptera and in Lepidoptera such as the tobacco hornworm, *Manduca sexta*, where the epidermis makes sequentially several larval cuticles, the pupal cuticle and finally the adult cuticle, JH prevents each of the metamorphic transitions (Riddiford, 1995; Willis, 1996). By contrast, in *Drosophila* and the other higher flies, the pupal epidermis, except for the abdomen, is derived from imaginal discs, and exogenous JH does not prevent the larval-pupal transformation, even when given throughout larval life (Ashburner, 1970; Postlethwait, 1974; Riddiford and Ashburner, 1991). Nor does JH have any effect on the subsequent adult differentiation of the head and thorax externally, although it disrupts metamorphosis of the nervous and muscular systems when given during the prepupal period (Restifo and Wilson, 1998). JH application during the final larval instar or during the prepupal period, however, prevents the normal adult differentiation of the abdomen (Ashburner, 1970; Postlethwait, 1974; Riddiford and Ashburner, 1991), whose cells arise from proliferation of the histoblasts during the prepupal period (Madhavan and Madhavan, 1980).

In *Drosophila melanogaster* the ecdysone-induced Broad (BR; previously called the Broad-Complex or BR-C) transcription factors are essential for the onset of metamorphosis since the amorphic *broad* (*br*) mutant *npr* can develop normally to the final larval instar but cannot undergo metamorphosis (Kiss et al., 1976; Kiss et al., 1988). The BR proteins are members of the Broad-Tramtrack-Bric-a-brac (BTB) family of transcription factors that share a common N-terminal domain thought to be important in protein-protein interactions (Zollman et al., 1994). The alternately spliced C terminus contains one of four pairs of C_2H_2 DNA-binding zinc fingers (Z1, Z2, Z3, Z4), and there are three variants of the Z1 isoform generated by alternative splicing in the linker between

the core and the zinc finger (DiBello et al., 1991; Bayer et al., 1996a). BR proteins appear at the onset of metamorphosis in response to ecdysone and have been shown to play a complex role in activation and/or suppression of genes involved in the larval-pupal transformation (Karim et al., 1993; von Kalm et al., 1994; Hodgetts et al., 1995; Crossgrove et al., 1996; Mugat et al., 2000; Renault et al., 2001). Although generally all the different isoforms are found in the various tissues, there is both temporal and tissue specificity as to the predominant isoform present (Huet et al., 1993; Emery et al., 1994; Bayer et al., 1996a; Mugat et al., 2000; Brennan et al., 2001). Mutant rescue experiments have shown that each isoform is associated with a particular class of developmental anomalies at metamorphosis, although there also is some functional redundancy among the four isoforms (Bayer et al., 1997).

In *Manduca*, three BR isoforms have been found – Z2, Z3 and Z4 (Zhou et al., 1998). These BR proteins are first induced in the epidermis when ecdysone acts in the absence of JH to cause pupal commitment at the onset of metamorphosis, then persist during the prepupal period (Zhou et al., 1998; Zhou and Riddiford, 2001). Application of JH at this time prevents the appearance of BR and the larval-pupal transformation. Thus, as in *Drosophila*, BR seems to be a key regulator of the onset of metamorphosis in *Manduca*.

Little is known about the molecular basis of the ecdysone-induced switch involved in the pupal-adult transformation. As discussed above, JH given at the outset of adult development shortly after pupal ecdysis in *Manduca* causes the formation of a second pupal cuticle (Riddiford and Ajami, 1973). In *Drosophila*, the presence of JH slightly earlier during the prepupal period has a similar effect only on the abdomen (Postlethwait, 1974). We now show that JH given at the critical time of the onset of the pupal-adult transformation causes *br* re-expression in response to ecdysone throughout the epidermis of *Manduca* and in the abdomen of *Drosophila*. Furthermore, a second pupal cuticle can be induced in all regions of *Drosophila* without JH treatment by misexpression of BR-Z1 at the time of cuticle formation during the adult molt. Thus, the continued expression of the BR proteins during the pupal-adult transformation appears to be sufficient to explain the 'status quo' action of JH at this time. We also find that premature expression of BR-Z1 in mid-second instar larvae of *Drosophila* causes premature initiation of the pupal cuticle program. Hence, BR is an important specifier of pupal development.

MATERIALS AND METHODS

Animals

Tobacco hornworm (*Manduca sexta*) larvae were reared on an artificial diet at 26°C in a 17L:7D photoperiod that allows continuous development without a pupal diapause (Truman, 1972; Bell and Joachim, 1976). On the day of pupal ecdysis, the pupae were placed in a 12L:12D photoperiod at 25.5°C. Animals were staged individually according to days after pupal ecdysis and/or by various morphological markers as described (Jindra et al., 1997).

The wild-type Canton S strain of *Drosophila melanogaster* was used. In order to express BR isoforms at a designated time, transgenic w^{1118} fly lines carrying each BR isoform cDNA under the control of the *hsp70* promoter were used (Crossgrove et al., 1996; Bayer et al., 1997) (generous gift from Dr Cynthia A. Bayer). The three splice variants of the Z1 isoform are denoted by the groups of amino acids in the linker between the core and the zinc-finger region with the longest containing a threonine-asparagine-threonine-rich and two glutamine-rich regions (TNT-Q^1-Q^2-Z1) and the other two being progressively shorter (Q^1-Q^2-Z1 and Q^2-Z1). The isoform-specific transgenic fly lines were homozygous with an insertion in either one or both chromosome 2 and 3. Flies carrying two copies of the hs transgenes were: Z1/Z1 [*708-1* (the TNT-Q^1-Q^2-Z1 variant) and *527-5* (the Q^1-Q^2-Z1 variant)], Z2/Z2 (*cD5*-1), Z3/Z3 (*797-3*) and Z4/Z4 (*Z4-11*). Flies carrying four copies were: Z1/Z1; Z1/Z1 [*708-14; 708-1* (four copies of TNT-Q^1-Q^2-Z1) and *527-5; 708-1* (two copies each of the Q^1-Q^2-Z1 and the TNT-Q^1-Q^2-Z1 variants)], Z2/Z2; Z2/Z2 (*cD5-4C; cD5-1*), Z3/Z3; Z3/Z3 (*797-3; 797-E8*) and Z4/Z4; Z4/Z4 (*Z4-13; Z4-11*). Animals were reared at 25°C and for the studies on pupal and adult development staged at the time of formation of the white puparium (0 hour APF). For the larval studies, eggs were collected at 30 minute intervals, then 24 hours later hatchlings were put on food followed by restaging at ecdysis to the second instar (0 hour L2). To induce BR, transgenic lines were heat-shocked either in vials or on glass slides in 9 cm plastic Petri dishes for 30 minutes at 37°C in a water bath at the beginning of designated time. The wild-type Canton S and w^{1118} lines were also heat-treated similarly to serve as controls.

JH application

Cyclohexane (Aldrich) (10 μl) with or without 10 μg of the JH mimic pyriproxifen (Sumitomo) was applied topically to *Manduca sexta* prepupae at the onset of tanning of the pupal bars on the dorsal metathorax, about 19 hours before pupal ecdysis. *Drosophila* were given 100 ng pyriproxifen topically in 0.2 μl acetone (J. T. Baker) or acetone only at the time of puparium formation.

Dissection and tissue culture

For culture experiments, the *Manduca* pupal fore-wings together with their cuticle were removed at 18 hours after pupal ecdysis. The pupal wings were then cultured on the surface of 0.5 ml Grace's medium (Gibco) at 25°C in a 95% O_2/5% CO_2 atmosphere (Hiruma and Riddiford, 1984). Media containing 5 μg/ml (10^{-5} M) 20E (Daicel Chemical Industries) and/or 1 μg/ml (3×10^{-6} M) JH I (Sci Tech, Praha, Czech Republic) were prepared in polyethylene glycol (PEG)-coated containers as described elsewhere (Riddiford et al., 1979). The wing tissue was precultured for 24 hours, either in hormone-free medium (for the control and for the 20E treatment) or with JH I (for the JH treatments), then transferred either to medium containing 20E or to the same medium as used for preculture. The time of transfer was designated as 0 hours.

RNA extraction and analysis

Total RNA was extracted from *Manduca* pupal wing or the entire anterior (head and thorax) region or the entire posterior region (abdomen) of *Drosophila* by the proteinase K/phenol method (Andres et al., 1993). This RNA (10 μg) was separated on a 1% agarose gel containing 2.2 M formaldehyde, transferred to Duralon u.v. membrane (Stratagene) and cross-linked in an u.v. Stratalinker (Stratagene). Approximately equal loading of the lanes was confirmed by Ethidium Bromide staining of the ribosomal RNA. The 156 bp *Manduca br* core cRNA probe was prepared as described by Zhou et al. (Zhou et al., 1998). The probe against the *Drosophila br* core exon was a 2 kb *Eco*RI/*Bam*HI restriction fragment of cDNA subclone dm796 that included some 5′ noncoding sequence through most of the core (DiBello et al., 1991). Hybridization to prepupal RNA showed that this probe hybridized only to the same size RNAs that were detected by a 511 bp probe from the core coding region used by Bayer et al. (Bayer et al., 1996a). The 0.9 kb *Edg78E* (Apple and Fristrom, 1991), 0.6 kb *Pcp* (Henikoff et al., 1986), 1.3 kb *Edg84A* (Fechtel et al., 1988), and the 0.9 kb *Acp65A* and 0.5 kb *Lcp65A-b* (Charles et al., 1997) cDNA probes were generated as described. The cDNA probes

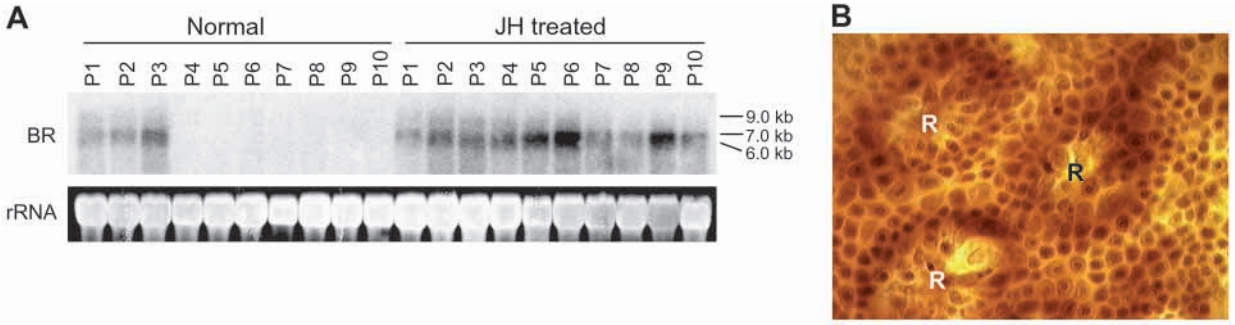

Fig. 1. JH causes re-expression of BR in *Manduca* pupal epidermis. (A**)** Northern analysis of 10 μg total RNA from pupal forewings for the first 10 days after pupal ecdysis, using the *Manduca br* probe. Either 10 μl cyclohexane (normal) or 10 μg of the JH analog pyriproxifen in 10 μl cyclohexane was applied topically at 19 hours before pupal ecdysis. The Ethidium-stained ribosomal RNA indicates equal loading. (B) BR protein in a JH-treated pupal abdomen 5 days after pupal ecdysis as detected by immunocytochemistry with the *Manduca* BR core antibody. The JH treatment was as in A. Note the rosettes (R) of cells forming the pock marks of the second pupal cuticle.

were labeled by random priming using Prime-It II kit (Stratagene) and α-[^{32}P]-dATP (Amersham). Hybridization was for 24 hours in 50% formamide, 0.1% SDS, 5×SSC, 5×Denhardt's solution, 50 mM sodium phosphate (pH 7.0), 100 μg/ml herring sperm DNA, at 42°C for the cDNA probes and at 65°C for the cRNA probe. The membranes were then washed at 65°C for 20 minutes each in 2×SSC and 0.2×SSC containing 0.1% SDS. The northern blots were visualized with a GS 505 Molecular Imaging System (BioRad) and images processed using Canvas software.

Immunocytochemistry and microscopy

The epidermis of *Manduca* was immunostained with the *Manduca* BR core antibody (1:8000) and visualized with diaminobenzidine as described previously (Zhou and Riddiford, 2001). Images were captured with a Sony video camera on a Nikon Optiphot microscope and processed with Adobe Photoshop.

The *Drosophila* abdomen was isolated and the fat body removed, then the abdomen was fixed in 3.7% formaldehyde and processed as for *Manduca* (Zhou and Riddiford, 2001). The abdominal epidermis was stained with a monoclonal antibody against the *Drosophila* BR core region (Emery et al., 1994) (1:250) followed by 1:1000 FITC-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch). Propidium Iodide (PI) was used as a nuclear counterstain. Fluorescent visualization was with a BioRad MRC-600 confocal laser scanning microscope and images processed with NIH Image and Adobe Photoshop.

Photos of pharate adult flies were taken with a Cool Snap camera on a wild dissecting microscope and processed with OpenLab software. Whole mounts of abdominal cuticle were prepared by cleaning the cuticle, followed by dehydration, clearing in xylene and mounting in DPX (Fluka). Images were captured and processed as for the *Manduca* abdomen.

RESULTS

Effects of JH on *br* re-expression during development of a second pupa of *Manduca*

In *Manduca*, BR is normally found in the epidermis beginning at the time of pupal commitment and extending through the prepupal period of pupal cuticle formation (Zhou et al., 1998; Zhou and Riddiford, 2001). After pupal ecdysis, low levels of BR mRNA were found in both pupal wings (Fig. 1A) and abdomen (data not shown) for the first 3 days after pupal ecdysis, but then the transcripts were absent during adult differentiation (beginning day P4) and cuticle formation (beginning day P8). When 10 μg of the JH mimic pyripoxifen was applied to pharate pupae 19 hours before pupal ecdysis, normal pupae were formed, but they subsequently deposited a second pupal cuticle between days 5 and 7 (Fig. 1B) (Riddiford and Ajami, 1973). These JH-treated animals showed a re-induction of BR mRNA and protein with high levels evident on days P5 and P6, the time when the second pupal cuticle is being deposited (Fig. 1A,B). Thus, the presence of JH at the onset of the pupal-adult transformation both prevents the normal turning off of *br* transcription that normally occurs at the onset of adult development and causes the formation of another pupal cuticle.

To determine whether JH acted directly on the epidermis to regulate *br* expression, we cultured day 0 pupal wing epidermis with or without hormones and assessed the effect on BR mRNA. Fig. 2 shows that BR mRNA persisted through the 24 hour preculture period in either hormone-free or JH-containing medium, but then declined to trace levels during the next 24 hours. These trace levels were seen throughout the remainder of the culture period in hormone-free medium, but disappeared after 72 hours in the JH-containing medium. Similarly, when the wing was pre-cultured in hormone-free medium for 24 hours, then exposed to 5 μg/ml 20E (10^{-5} M), there was a rapid decline in the BR mRNA to trace levels by 24 hours and to undetectable by 72 hours (Fig. 2). By contrast, when the wing was precultured with 1 μg/ml JH I and then exposed to both 20E and JH I, BR mRNA initially declined, then reappeared at 72 hours and persisted for at least 120 hours (Fig. 2). Thus, the presence or absence of JH determines whether *br* will be induced or suppressed when pupal epidermis is exposed to 20E.

Effects of JH on *br* re-expression during adult development of *Drosophila*

Drosophila shows a pattern of BR expression that is similar to that seen in *Manduca*. Levels of BR mRNA are high at pupariation and through most of the prepupal period, declining to low levels at 12 hours after puparium formation (APF) (the time of head eversion) that persist until about 24 hours APF (Fig. 3A and data not shown). BR mRNA is then absent during adult development that begins with the onset of adult bristle differentiation about 30 hours APF (Fristrom and Fristrom, 1993).

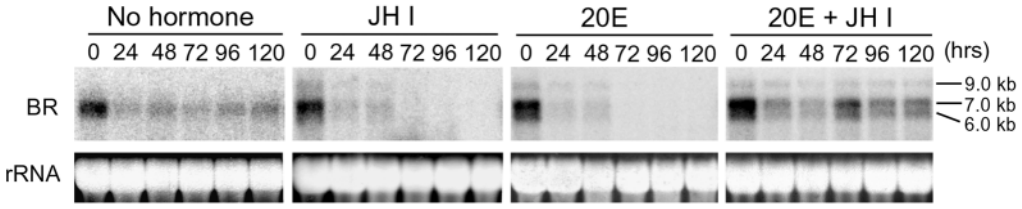

Fig. 2. JH causes re-expression of BR mRNA in *Manduca* pupal wings in vitro. Eighteen-hour-old pupal wings were precultured for 24 hours in either hormone-free medium (no hormone or 20E) or 1 μg/ml (3×10^{-6} M) JH I (for those being exposed to JH). At time 0 they were transferred to medium without hormone or with 1 μg/ml JH I, 5 μg/ml (10^{-5} M) 20E, or both, and cultured for various times, then the RNA extracted and analyzed as in Fig. 1.

JH applied at the time of pupariation causes the formation of a pharate adult with a normal appearing adult head and thorax, but with a pupal-like abdomen (see Fig. 3B) (Ashburner, 1970; Postlethwait, 1974; Riddiford and Ashburner, 1991). When 100 ng pyriproxifen was applied at the white puparium stage, BR mRNA declined normally in the head and thorax by 24 hours APF, with trace levels reappearing between 48 and 72 hours APF (Fig. 3B). By contrast, in the abdomen BR mRNA persisted at low levels through 24 hours APF, then increased to high levels by 36 hours that only declined significantly between 60 and 72 hours APF (Fig. 3B). Particularly prominent during this latter phase was the 4.4 kb Z1 isoform (Fig. 3B) that is also predominant during pupal cuticle formation at 10-12 hours APF (Bayer et al., 1996a).

Using antibodies against the common core region of BR, we found no difference between the JH-treated and the control abdomens in the level of BR protein in the larval cells, the histoblasts, or the imaginal cells between 6 and 24 hours after pupariation (Fig. 4 and data not shown). By 30 hours APF, BR protein has disappeared from the imaginal epidermis in the control abdomens, whereas it remained in the imaginal cells of the JH-treated abdomens. BR persisted in the latter imaginal cells until at least 72 hours APF (Fig. 4).

Effects of JH on cuticle formation during adult development of *Drosophila*

To determine the nature of the abdominal cuticle produced in the JH-treated animals, we used the pupal cuticle genes *Edg78E*, *Edg84A* (Fechtel et al., 1988; Apple and Fristrom, 1991) and *Pcp* (originally described as *Pcp-Gart*) (Henikoff et al., 1986), and the adult cuticle gene *Acp65A* (Charles et al., 1997) as molecular markers. Fig. 3A shows that in acetone-treated controls, the pupal cuticle gene *Edg78E* was expressed at high levels at 12 hours APF and at trace levels up to 24 hours APF, then again at trace levels at 72 hours APF. In data not shown, Edg84A mRNA showed a similar developmental pattern of expression, whereas Pcp mRNA was also present at 0 hours APF, as it first appears in the late third instar (Henikoff and Eghtedarzadeh, 1987). Acp65A mRNA was not detected during pupal cuticle formation, but later appeared at 60 hours APF in the head and thorax and at 66 hours APF in the abdomen (Fig. 3A) during the time of adult cuticle deposition.

In the abdomens of JH-treated animals, the mRNAs for the

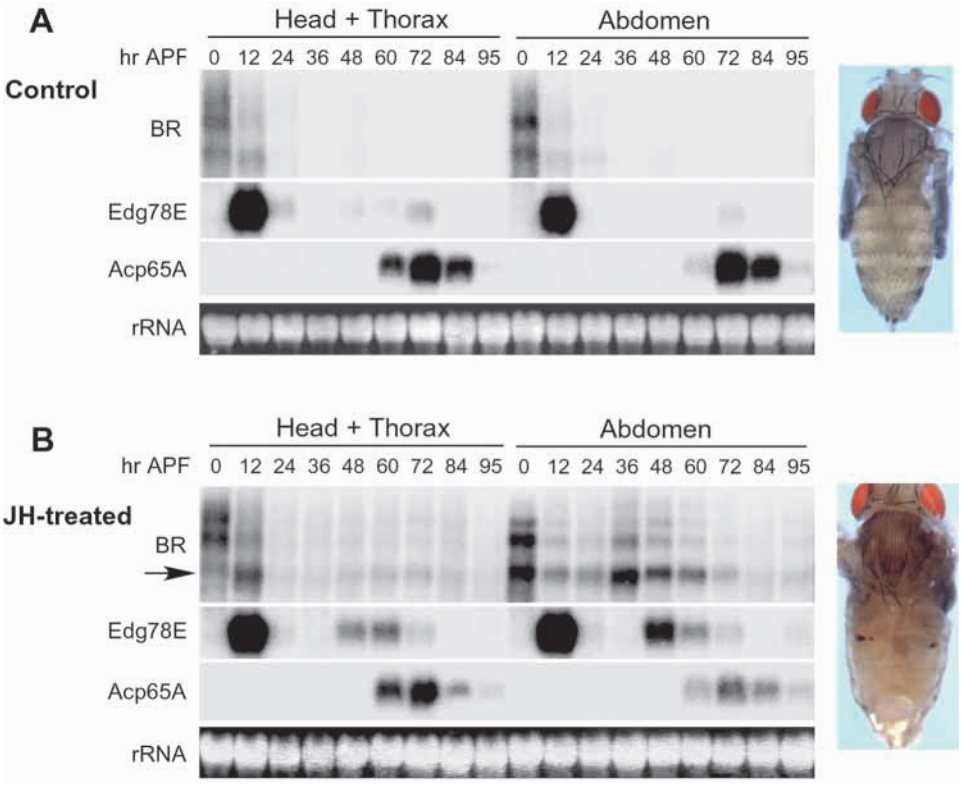

Fig. 3. JH causes re-expression of mRNAs for BR and a pupal cuticle protein Edg78E and suppression of mRNA for the adult cuticle protein Acp65A in the *Drosophila* abdomen during adult development. Northern analysis of 10 μg total RNA from separated anterior (head and thorax) and posterior (abdomen) regions after topical application of (A) 0.2 μl acetone (control) or (B) 100 ng pyriproxifen (JH mimic) in 0.2 μl acetone to the white puparium, using the probes described in the Materials and Methods. Note the predominant *br* transcript re-expressed during 36-72 hours APF is that of the 4.4 kb Z1 isoform (arrow). The Ethidium-stained ribosomal RNA indicates equal loading.

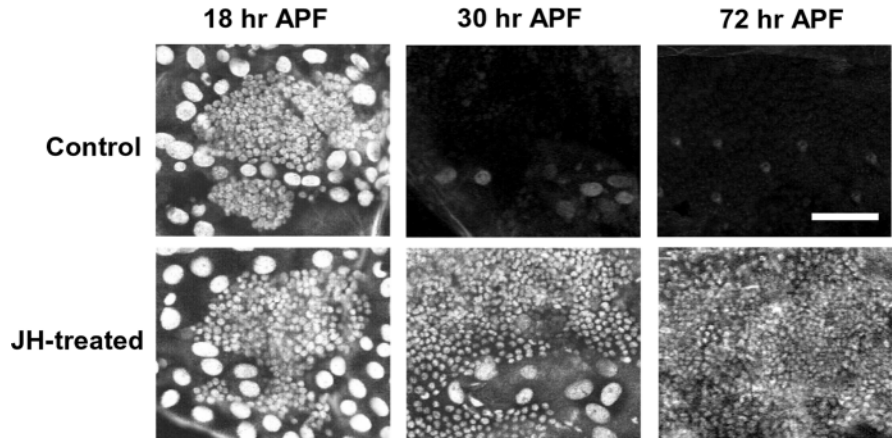

Fig. 4. BR immunostaining of abdominal epidermal cells in control and JH mimic-treated *Drosophila* that were treated at puparium formation (as in Fig. 3) and at various times thereafter. The large nuclei are in the larval cells, whereas the small nuclei are in the adult cells that are derived from the histoblasts. The wild-type Canton S strain reared at 25°C was used. Scale bar: 50 μm.

pupal cuticle genes *Pcp* (data not shown) and *Edg78E* were re-expressed at high levels at 48 hours APF and to a lesser extent later, whereas the mRNA for the adult cuticle gene *Acp65A* was dramatically reduced (Fig. 3B). By contrast, JH treatment had relatively little effect on the normal expression patterns of *Edg78E* and *Acp65A* in the developing head and thorax (Fig. 3B). Thus, the abdomen appears to be making a bona fide second pupal cuticle in response to JH treatment.

Effects of misexpression of *br* during the pupal-adult transformation of *Drosophila*

The above data suggest that the induction of a second pupal cuticle in the abdomen by JH may be mediated through the BR transcription factors. If so, we should be able to mimic the JH effect by re-expression of BR during adult development. We therefore used heat-inducible transgenic fly lines for each of the BR isoforms to examine their effects during this time period (Crossgrove et al., 1996; Bayer et al., 1997).

Misexpression of the BR-Z1 isoform

When the Z1/Z1; Z1/Z1 transgenic line carrying four copies of the TNT-Q^1-Q^2-Z1 isoform was heat-shocked at 48 hours APF for 30 minutes, BR mRNA was high within an hour (Fig. 5A) and persisted for 4-5 hours (data not shown). Under these conditions, the mRNA for the pupal cuticle gene *Edg78E* was detected 12 hours later both in the head and thorax and in the abdomen and was maximal at 66 hours APF (Fig. 5A). By contrast, the adult cuticle gene *Acp65A* was almost completely suppressed in both portions of the animal (Fig. 5A). Similar effects were found when using the Z1/Z1; Z1/Z1 transgenic line carrying two copies of the TNT-Q^1-Q^2-Z1 isoform and two of the Q^1-Q^2-Z1 isoform (data not shown).

To determine the period of sensitivity of these cuticle genes to BR-Z1, we gave a single heat shock at different times between 36 and 60 hours APF, then assessed pupal cuticle gene expression at 66 hours APF and adult cuticle gene expression at 72 hours APF during the time of normal cuticle deposition. Fig. 5B shows that the pupal cuticle genes *Edg78E* and *Pcp* were activated when BR-Z1 was misexpressed at any time between 40 and 60 hours APF, with the strongest effects seen after heat shock at 48 and 52 hours for *Edg78E* and at 48 hours for *Pcp*. The pupal cuticle gene *Edg84A* was also weakly expressed after BR-Z1 expression at 48 and 52 hours (data not shown). The apparent decrease of the expression of the pupal cuticle genes after expression of BR-Z1 at 60 hours APF is probably due to the short 6 hour period between heat shock and sacrifice. When the heat shock was given at 64 hours APF and the animal sacrificed 8 hours later, the pupal cuticle mRNAs were present at levels similar to those found after BR-Z1 expression at 56 hours APF (compare Fig. 5C with Fig. 5B). Suppression of the adult cuticle gene *Acp65A* was complete or nearly so after BR-Z1 expression at 48 hours APF or later (Fig. 5B). Importantly, expression of BR-Z1 at 64 hours APF after transcription of *Acp65A* had begun was still able to suppress its mRNA accumulation (Fig. 5C). Thus, the presence of the BR-Z1 transcription factor beginning about 44-48 hours APF is sufficient to cause the re-expression of several pupal cuticle genes and the suppression of an adult cuticle gene.

Morphologically, the pharate adults formed after a single heat shock induction of either Z1/Z1; Z1/Z1 transgenic line between 45 and 52 hours APF had a transparent pupal cuticle on many areas of the head, thorax and abdomen, although the macrochaetes and microchaetes, sometimes slightly tanned, were present (Fig. 6A,B and data not shown). In addition, the dorsolateral areas of the abdomen sometimes showed the beginnings of adult melanization that extended into the anterior region of the tergite where melanization is normally not found (Fig. 6A; 48 and 52 hours). Interestingly, earlier expression of the Z1 isoform between 30 and 39 hours caused truncation of the bristles, first on the head and thorax (30-35 hours) and later only on the abdomen (35-39 hours) (Fig. 6A,B and data not shown), but had no apparent effect on cuticle deposition or bristle pigmentation (Fig. 6A). Effects on abdominal bristle outgrowth were most severe at 39 hours APF, but by 43-45 hours APF there was little effect on outgrowth, only on pigmentation (Fig. 6B). Trichome (hair) formation by the abdominal epidermis was prevented by BR-Z1 expression at 35 or 39 hours APF, but not at later times (Fig. 6B). Heat shock of the w^{1118} parental line at these various times had no effect; the flies emerged and appeared normal (data not shown).

Fig. 6C shows that when the Z1/Z1 transgenic line carrying the TNT-Q^1-Q^2 variant was heat shocked during both of these periods at 36 and at 48 hours, the resultant abdomen mimicked that seen in animals treated with JH at pupariation (Fig. 3B) in that it lacked bristles and remade a transparent pupal cuticle. The head and thorax also remade a pupal-like cuticle, but most bristles were present although mostly unpigmented and sometimes truncated. In some of these animals, the

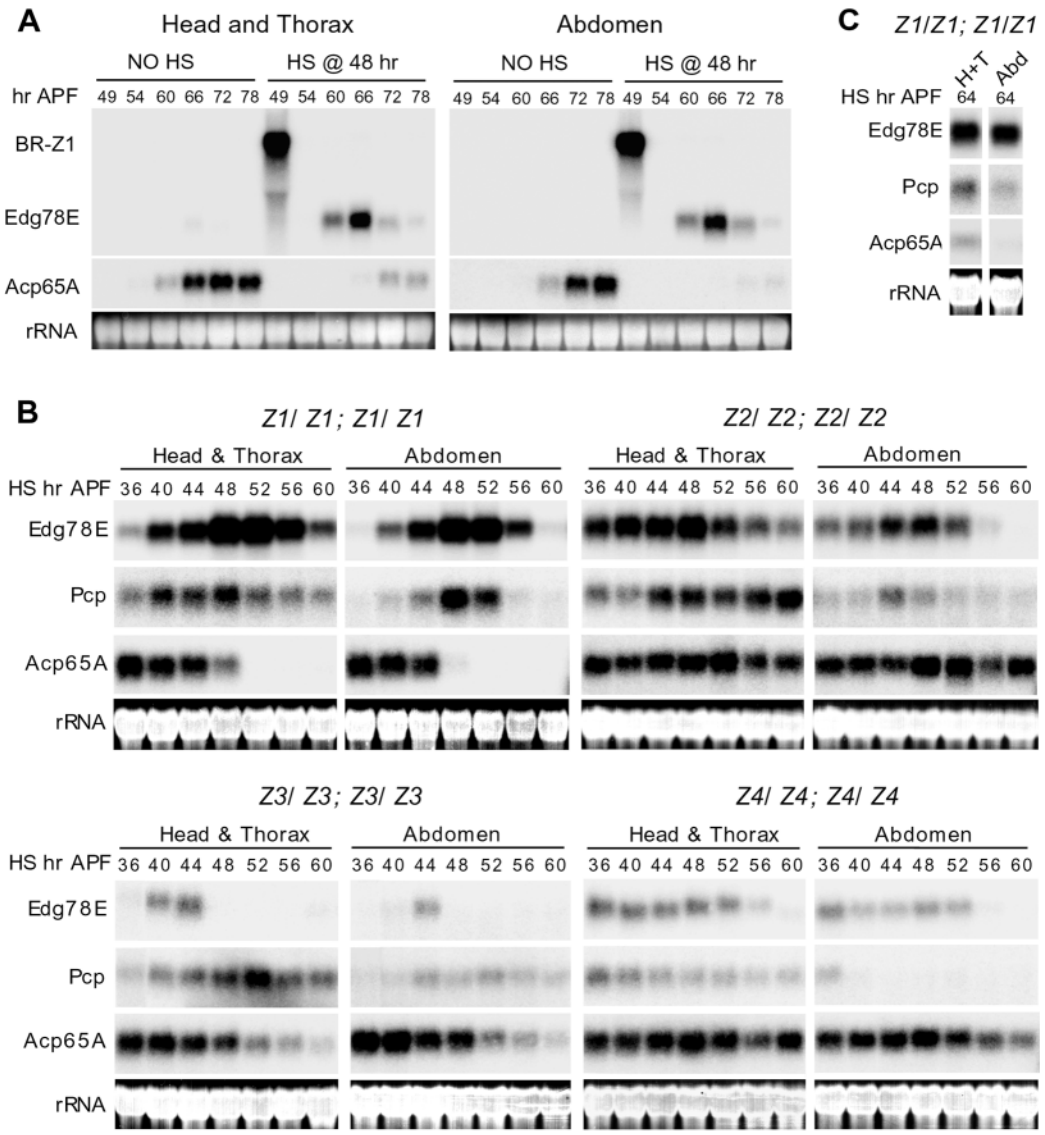

Fig. 5. Effects of misexpression of BR on cuticle gene expression during adult development. (A) Northern analysis of mRNAs for BR-Z1, the pupal cuticle protein Edg78E, and the adult cuticle protein Acp65A in the transgenic fly line (*w*^{1118}*; 708-14; 708-1*) containing four copies of the BR-Z1 isoform variant (TNT-Q^{1}-Q^{2}-Z1) under a heat shock promoter. Pupae were harvested at various times without heat shock or after a 30 minute heat shock starting at 48 hours APF. (B) Misexpression of each of the BR isoforms has different effects on cuticle gene expression. Each of the BR isoforms was heat-induced as in A at the designated time between 36 and 60 hours APF, then the tissue was collected at 66 hours APF for the pupal cuticle genes and at 72 hours for the adult cuticle gene (the time of its peak expression in both regions; see A). We used transgenic lines with four copies of Z1 as in A and of each of the other Z isoforms as described in the Materials and Methods. (C**)** Effects of misexpression of BR-Z1 at 64 hours APF on the expression of cuticle genes at 72 hours APF. In all cases, puparia were maintained at 25°C before and after the heat shock. RNA analysis was carried out as in Fig. 3.

morphogenesis of the wing was also suppressed so that only a pupal-like wing pad was formed rather than the normal adult folded wing (inset, Fig. 6C). Thus, all epidermal cells are capable of responding to BR-Z1 in reiterating their pupal differentiation program provided that the protein is present throughout the period of differentiation.

Effects of misexpression of the BR-Z2, BR-Z3 and BR-Z4 isoforms

Misexpression of four copies of any of the other isoforms of BR during the time of bristle outgrowth also caused truncation of the bristles, but expression of two copies had little effect on the outgrowth (data not shown). By contrast, severe truncation was seen with expression of either two or four copies of the Z1 isoform at this time (Fig. 6A,C and data not shown). Isoform-specific effects however were seen when expression of the four copies was induced during adult cuticle formation. When heat-induced at 44-56 hours APF, BR-Z2 activated *Edg78E* and *Pcp*, but failed to suppress *Acp65A* (Fig. 5B). Morphologically, the resultant pharate adults appeared to be normal (Fig. 7 and data not shown). Apparently, the presence of the two pupal cuticle proteins in an adult cuticle background was unable to cause major morphological abnormalities. By contrast, expression of BR-Z3 at any time between 48 and 60 hours APF partially suppressed *Acp65A* expression and caused the re-expression of *Pcp*, but not of *Edg78E* (Fig. 5B). Pharate adults that had been given a heat shock at 48 hours APF appeared normal (data not shown), but those given a heat shock of BR-Z3 between 52 and 64 hours APF had essentially full-length bristles that were covered with an untanned cuticle rather than the normal melanized cuticle (Fig. 7 and data not shown). The expression of four copies of the Z4 isoform during this period caused some re-expression of *Edg78E* and *Pcp* but little diminution of *Acp65A* expression (Fig. 5B). The uneclosed, pharate adult flies showed bristle characteristics similar to that seen after expression of the Z3 isoform except that the macrochaete sockets were not tanned (Fig. 7). Thus, each isoform has particular effects in directing pupal cuticle deposition, tanning and pigmentation.

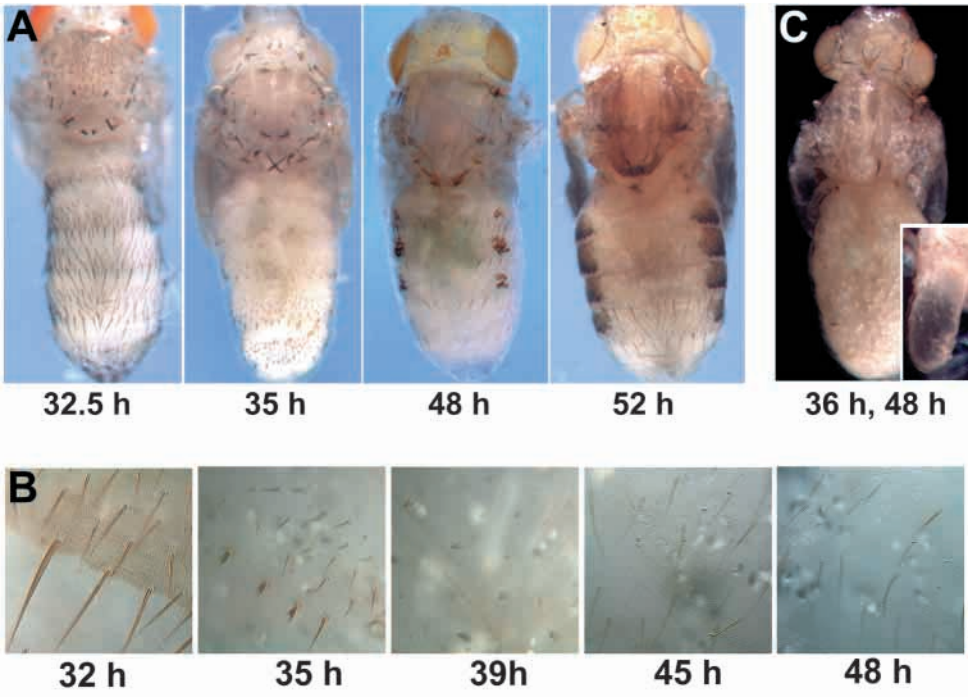

Fig. 6. Effects of misexpression of BR-Z1 on the morphology of the resultant pharate adults. All animals were scored about 120 hours APF, about 24 hours after the expected time of eclosion. (A) Pharate adults of the Z1/Z1; Z1/Z1 line (*w*^{1118}*; 527-5; 708-1*) that have two copies of the TNT-Q^1-Q^2 variant and two of the Q^1-Q^2 formed after expression at various times during adult development. Note the truncated bristles in the head and thorax after misexpression at 32.5 hours, but in the abdomen only later at 35 hours. Note the transparent pupal-like cuticle in the dorsum with little bristle pigmentation except in parts of the abdomen after misexpression at 48 and 52 hours. (B) Whole mounts of abdominal cuticle formed after expression of the same transgene as in A at the designated time APF. Note the truncated bristles after misexpression at 35 and 39 hours and the formation of bristles with little or no pigmentation after misexpression at 45 and 48 hours. **(**C**)** Pharate adult after misexpression of Z1/Z1 (line *w*^{1118}*; 708-1*) at 36 and again at 48 hours APF. Note the thin transparent pupal-like cuticle over the entire animal. Some bristles are present on the head and dorsal thorax but are not pigmented. The inset shows a pupal-like wing found in some of these animals.

Effects of misexpression of BR during the second larval instar

To determine if the expression of BR during larval life could cause premature expression of the pupal cuticle genes, we heat-shocked larvae at various times during the second instar, then analyzed cuticle gene mRNA levels at the time of expected ecdysis to the third instar (24 hours after ecdysis to the second instar). Misexpression of BR-Z1 between 8 and 17 hours after ecdysis to the second instar caused premature expression of the pupal cuticle gene *Edg78E* and suppressed that of the larval-specific cuticle gene *Lcp65A-b* (Charles et al., 1997; Charles et al., 1998) (Fig. 8). These animals formed a new cuticle, but did not ecdyse. By contrast, expression of either the Z2 or the Z4 isoform caused only trace induction of this pupal cuticle gene and that of Z3 had no effect, although the animals died as pharate third instar larvae (Fig. 8). These other isoforms caused variable suppression of the larval cuticle gene that were time and isoform specific. Thus, misexpression of BR-Z1 can cause the premature expression of at least part of the pupal program of cuticle deposition during a larval molt whereas that of the other isoforms primarily suppresses normal larval cuticle gene expression.

DISCUSSION

The *broad* gene is sufficient for specifying pupal development

BR has long been known to be required for the onset of metamorphosis of *Drosophila* because the *nonpupariating* (*npr*) alleles lack all BR proteins and remain as final instar larvae (Kiss et al., 1976; Kiss et al., 1988; Emery et al., 1994). In both *Drosophila* (Karim et al., 1993; Emery et al., 1994; Hodgetts et al., 1995; Bayer et al., 1996a; Bayer et al., 1996b; Mugat et al., 2000; Brennan et al., 2001) and *Manduca* (Zhou et al., 1998; Zhou and Riddiford, 2001), BR transcripts and proteins are expressed prominently during the larval-pupal transformation with different isoforms showing different

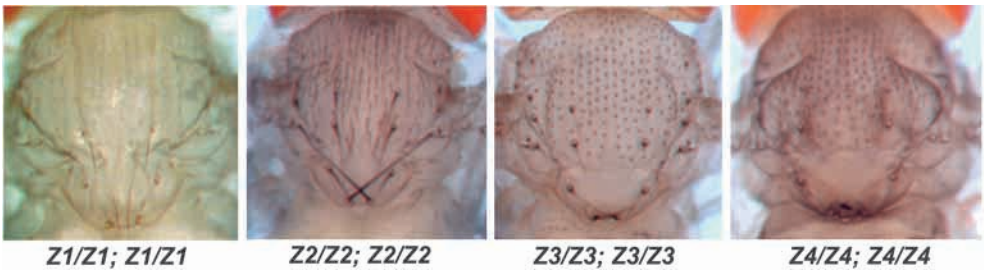

Fig. 7. Thoraces of pharate adults after misexpression of four copies of each of the BR isoforms at 56 hours APF. Note the shiny pupal-like cuticle and thin nonpigmented bristles after expression of the Z1 isoform (the line used in Fig. 5A). The thorax appears normal after misexpression of the Z2 isoform. After misexpression of both Z3 and Z4 isoforms, the cuticle appears normal, but the bristles are nonpigmented with the difference being that the sockets of the macrochaetes are prominent after Z3 but not after Z4 misexpression.

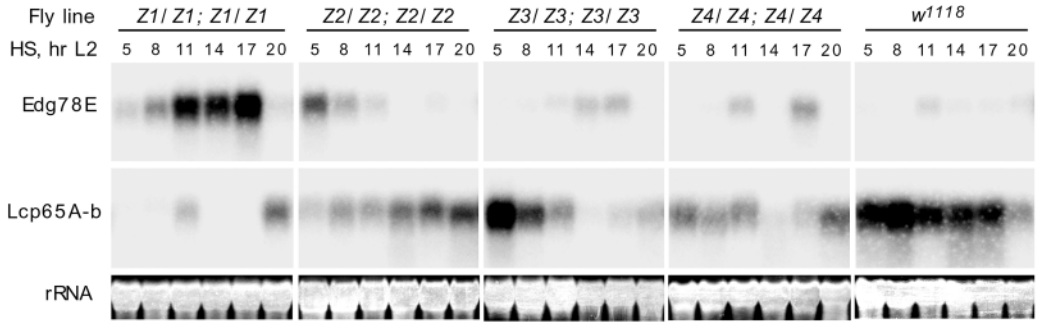

Fig. 8. Premature expression of a pupal cuticle gene *Edg78E* and suppression of a larval cuticle gene *Lcp65A-b* by misexpression of BR during the second larval molt. The heat shock was carried out at the time indicated after ecdysis to the second instar (L2) and the RNA extracted at 24 hours L2. RNA analysis was as in Fig. 3. The Z1 transgenic line used was w^{1118}*; 708-1; 708-14*.

temporal and tissue specificities through this period and causing either activation or suppression of specific genes. For example, in the *Drosophila* salivary gland, the induction of *Sgs-4* and *L71* and the suppression of *Pig-1* during the mid and late third instar require the Z1 isoform (von Kalm et al., 1994; Crossgrove et al., 1996), while the later suppression of *Sgs-4* at puparium formation is due to the downregulation of another transcription factor Forkhead (Fkh) by the Z3 isoform (Renault et al., 2001). By contrast, the Z3 isoform activates the expression of *Fbp1* in larval fat bodies during the second half of the third instar, while Z2 may play a role in repressing its premature expression (Mugat et al., 2000). BR proteins also may play a role in the regulation of chromatin structure, as they are found in over 300 sites on the salivary gland chromosomes including sites in the interband regions and in the heterochromatin (Emery et al., 1994; Gonzy et al., 2002).

BR-Z1 is the predominant isoform during the time of pupal cuticle formation in *Drosophila* (Bayer et al., 1996a). Our studies show that whenever BR-Z1 is expressed during an ecdysone-induced molt, it can direct the epidermis into a program of pupal cuticle production. For example, the molt to the third larval instar in *Drosophila* begins with the rise of the ecdysteroid that peaks about 12 hours after ecdysis to the second instar (Kraminsky et al., 1980). Shortly thereafter, mRNAs for larval cuticular proteins are upregulated (Charles et al., 1998). Expression of BR-Z1 during this time suppressed the larval cuticle gene *Lcp65A-b* and prematurely activated the pupal cuticle gene *Edg78E*. The ability of BR to be a pupal specifier is also evident during an adult molt. This molt begins about 24 hour APF with the rise of the ecdysteroid titer (Handler, 1982), and adult procuticle deposition begins about 53 hours APF during the decline of the ecdysteroid titer (Fristrom and Fristrom, 1993). BR-Z1 was most effective in activating pupal cuticle genes and suppressing an adult cuticle gene when expressed just before the normal onset of adult procuticle gene expression. This temporal restriction suggests that although BR selects which cuticle genes will be expressed, it can only do so within the confines of an ecdysone-induced program that determines the timing of cuticle gene expression at every molt. Therefore, in either a larval or an adult molt, the expression of BR-Z1 is sufficient to redirect that molt towards the pupal program (Fig. 9).

Effects of BR expression on adult differentiation

Adult differentiation of the epidermis can be divided into two developmental phases: cellular morphogenesis followed by cuticle deposition. Morphogenesis of the epidermis begins with the formation and outgrowth of the bristles (macrochaetes, microchaetes) between 30 and 45 hours APF, first in the head and thorax, then in the abdomen (Fristrom and Fristrom, 1993). Trichomes (hairs) are then formed by most of the general epidermal cells, beginning on the wing at 33 hours APF and on the abdomen about 48 hours APF (Mitchell and Petersen, 1983; Mitchell et al., 1983). The general epidermis deposits three cuticular layers: cuticulin, epicuticle and procuticle. The bristle and hair shafts lack the procuticle layer (Fristrom and Fristrom, 1993). Cuticulin formation begins in patches on the wings and legs at 35-36 hours and on the abdomen at 40-45 hours APF, followed by synthesis of a continuous epicuticle once morphogenesis is complete. Adult procuticle synthesis occurs primarily between 53 and 90 hours APF (Reed et al., 1975; Mitchell et al., 1983; Roter et al., 1985; Fristrom and Fristrom, 1993; Qiu and Hardin, 1995). The expression of the adult cuticle gene *Acp65A* is restricted to flexible cuticle regions of the abdomen, the wing hinges, leg joints and the ptilinum and begins about 55-60 hours APF (Charles et al., 1998) (N. Sedano and J.-P. Charles, personal communication) (Fig. 3A).

We find that BR disappears before the onset of adult differentiation in both *Manduca* and *Drosophila*. This disappearance is crucial for normal adult development as the misexpression of BR in *Drosophila* can affect both adult morphogenesis and adult cuticle production. When expressed

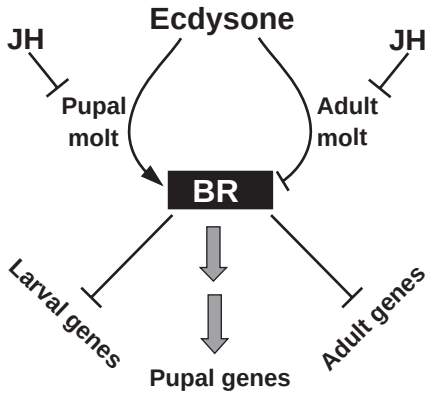

Fig. 9. Summary diagram of the hormonal regulation of Broad (BR) protein expression and its role in the specification of pupal development based on studies of its action on cuticle genes. Juvenile hormone (JH) prevents the pupal molt, by preventing the activation of the *br* gene by ecdysone and prevents the adult molt by preventing the suppression of *br* by ecdysone in JH-sensitive tissues. BR is sufficient to activate the pupal program and to suppress both the larval and the adult programs. See text for details.

between 30 and 40 hours APF, BR caused truncation of the bristles with early times affecting the bristles of the head and thorax and slightly later times affecting those of the abdomen. This timing corresponds to the onset of bristle outgrowth in the different regions (Fristrom and Fristrom, 1993). Suppression of bristle outgrowth occurred with misexpression of each of the BR isoforms, although the Z1 isoform had the strongest effects because the truncation was seen with expression of only two copies as well as with four copies of BR-Z1. Bristle outgrowth occurs by extension of the longitudinal actin microfilament arrays that surround the microtubular core (Fristrom and Fristrom, 1993; Tilney et al., 2000a). These actin filaments are bundled together, then crosslinked to support the elongating bristles, using sequentially the product of the *forked* gene and fascin (Tilney et al., 2000b). Although an occasional forked bristle was seen after misexpression of BR, the primary effect was truncation similar to that seen after exposure to inhibitors of microfilament elongation (Tilney et al., 2000a), indicating BR may be able to interfere, either directly or indirectly, with this process.

Trichome production in the abdominal epidermis was suppressed by BR-Z1 expression between 36 and 39 hours APF. As nearly every epidermal cell normally produces a trichome, this result shows that early BR expression also suppresses morphogenesis of the general epidermis. In this case, the effective time was about 10-12 hours before abdominal trichome production. By 42 hours APF bristle and trichome morphogenesis was no longer affected by expression of BR. Between this time and 60 hours APF, the effects were primarily on the types of cuticle proteins produced. BR-Z1 was most effective in suppressing adult cuticle gene expression and causing re-induction of pupal cuticle gene expression with the resultant formation of a thin, transparent, pupal-like cuticle by the general epidermis. None of the other isoforms had such a dramatic effect on the external appearance of the cuticle, although BR-Z2 caused re-expression of the two pupal cuticle genes, and BR-Z3 caused re-expression of one pupal cuticle gene and suppression of the adult cuticle gene studied, indicating that they normally play a role in production of pupal cuticle. Cuticle is composed of many proteins (Willis, 1996), so a predominance of adult cuticle proteins could maintain the cuticular morphology despite the presence of some pupal cuticle proteins or the absence of specific adult cuticle proteins. Further study is required to resolve this issue.

Although bristle morphogenesis was unaffected by expression of BR during the onset of cuticle formation, bristle pigmentation and sclerotization were subsequently inhibited. Whether this suppression is due to the type of epicuticle deposited or to an inhibitory action of BR on the melanization and sclerotization pathways themselves is unclear. In the case of BR-Z1, this effect was most pronounced when expression was either between 43 and 48 hours APF or later during 54-60 hours APF. Although the pupal cuticle genes used in this study all encode proteins found in the pupal exocuticle (the outer procuticle) (Henikoff et al., 1986; Fechtel et al., 1988; Apple and Fristrom, 1991), BR-Z1 probably also directs pupal epicuticle production. If so, the earlier expression of BR-Z1 may be suppressing the deposition of the proenzymes necessary for tanning and melanization that are normally associated with adult cuticle. Such a suppression would not be unexpected as normal pupal cuticle does not tan or melanize. These proenzymes are often laid down very early in formation of the new cuticle (Hiruma and Riddiford, 1993; True et al., 1999). BR-Z3 or BR-Z4 also suppressed bristle pigmentation but only when expressed late between 52 and 60 hours APF. This effect of later expression of any of these three isoforms is probably due to an interference with the production or deposition of the substrates for these enzymes that normally appear in the cuticle shortly before the proenzymes are activated (Hiruma et al., 1985). However, an effect on the pigmentation process itself that occurs later cannot be ruled out.

These different effects of BR misexpression depending on its timing indicate that BR and/or the unknown proteins whose expression BR regulates must be present to direct the pupal program. Once they disappear, the cells can revert back to the expression of the adult program. In our experiments, BR transcripts had disappeared by 6 hours after the heat shock (e.g. Fig. 5A), but the proteins are present until at least 9 hours (Crossgrove et al., 1996; Bayer et al., 1997). Thus, in order to obtain a second pupal cuticle that lacks bristles and trichomes, one must express BR-Z1 during both the initiation of bristle outgrowth and the onset of procuticle formation.

An important outcome of these studies is the finding that the presence of BR-Z1 at the time of cuticle formation is sufficient to redirect the program of cuticle gene expression into a pupal mode in cells that have completed their adult morphogenesis. This is most clearly seen after expression of BR at 48 or 52 hours APF. The cells of the general abdominal epidermis make the adult hairs but then deposit procuticle that includes pupal cuticle proteins. Thus, cells already committed to and expressing aspects of adult differentiation are plastic and can be caused to re-express pupal products when given the proper transcription factor. Clearly the suppression of *br* through the duration of adult development is essential for the normal completion of metamorphosis.

JH exerts its ‘status quo’ action by regulating BR expression

JH has long been known to prevent metamorphosis without interfering with the molting process itself (Wigglesworth, 1934; Williams, 1961). In both *Manduca* (Riddiford and Ajami, 1973) and *Drosophila* abdomens (Postlethwait, 1974), JH causes the formation of a second pupal cuticle only when given before the onset of the adult molt. Our studies here have revealed that this re-expression of the pupal program in both species is associated with the re-induction and maintenance of BR expression during the molt (Figs 1-4). This renewed BR expression appears to be sufficient to mediate the ‘status quo’ action of JH, as BR can both activate pupal genes and suppress adult genes (Fig. 5). Thus, during the crucial period of adult commitment, ecdysone in the absence of JH must switch off BR so that the adult-specific program of differentiation can occur (Fig. 9).

In *Drosophila* the JH-sensitive period of the abdomen is during the prepupal period with the highest sensitivity being at the time of pupariation and loss of sensitivity after head eversion at 12 hours APF (Postlethwait, 1974). During this time the histoblasts are proliferating rapidly. After this JH-sensitive period is over, beginning about 15 hours APF, these imaginal cells spread over the pupal abdomen and replace most of the larval cells by about 28 hours APF (Madhavan and

Madhavan, 1980). Throughout this period, we showed that both types of cells express BR. JH given at pupariation had no apparent effect on the proliferation or spreading of these cells or on their replacement of the larval epidermis. Nor did it interfere with their normal BR expression during this period. Its effect was only to cause renewed and sustained expression of BR in the imaginal cells during the adult molt up through 72 hours APF.

JH at pupariation has no apparent effect on the adult development of the *Drosophila* head and thoracic structures that are derived from the imaginal discs (Ashburner, 1970; Postlethwait, 1974). We show here that the refractoriness of the head and thorax to the JH treatment is due to the inability of JH to cause BR re-expression in these regions during the adult molt (Fig. 3B). Yet appropriate misexpression of BR during adult differentiation results in pupal cuticle formation in both the head and thorax, showing that BR retains its pupal-specifying function in these regions. Hence, the refractoriness to JH of the head and thorax must be due to a lesion in the pathway from the JH receptor to BR re-induction, possibly the loss of the receptor itself.

In all insects including *Drosophila* (Bownes and Rembold, 1987), JH is present during the larval molts, then declines during the last larval instar (Riddiford, 1994). In both *Manduca* (Zhou et al., 1998) and *Drosophila* (B. Zhou, PhD thesis, University of Washington, 2000) epidermis and imaginal discs, BR is not expressed during the larval molt. Pupal commitment of the polymorphic epidermis of *Manduca* by 20E at the end of the larval feeding period is correlated with the appearance of BR, and both can be prevented by JH (Zhou et al., 1998; Zhou and Riddiford, 2001) (Fig. 9). By contrast, in *Manduca* wing imaginal discs, BR appears earlier in the final larval instar as the discs become competent to metamorphose, and JH cannot prevent this appearance but only delays it (Zhou et al., 1998). In *Drosophila* and the higher flies, the pupa is derived from imaginal discs except for the abdominal cuticle that is produced by the persisting larval epidermal cells and the histoblasts. Although the effect of JH on the appearance of BR in *Drosophila* discs and larval epidermis has not been directly studied, dietary JH throughout larval life delays the onset of metamorphosis but does not prevent pupation (Riddiford and Ashburner, 1991), indicating that these tissues can turn on BR despite the presence of JH. Thus, the derivation of the *Drosophila* pupa from primarily imaginal discs probably accounts for the inability of JH to prevent the larval-pupal transformation although the lack of effect of JH on the abdominal epidermis in its switch to pupal cuticle production remains unexplained. The mechanism whereby JH prevents the switching-on of BR by ecdysone during a larval molt and also prevents its switching-off by ecdysone at adult commitment is still unclear.

These studies demonstrate for the first time that by the misexpression of a single transcription factor of the ecdysone cascade, the BR-Z1 isoform, one can redirect cells undergoing either larval or adult differentiation into a pupal developmental program. They also provide the first molecular basis for the 'status quo' action of JH on the pupal-adult transformation by showing that JH causes the re-induction of BR expression and consequently re-expression of the pupal program during the molt.

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