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Dynamic Expression of *Broad-Complex* Isoforms Mediates Temporal Control of an Ecdysteroid Target Gene at the Onset of *Drosophila* Metamorphosis

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Metamorphosis in *Drosophila melanogaster* is orchestrated by the steroid hormone ecdysone, which triggers a cascade of primary-response transcriptional regulators and secondary effector genes during the third larval instar and prepupal periods of development. The early ecdysone-response *Broad-Complex* (*BR-C*) gene, a key regulator of this cascade, is defined by three complementing functions (*rbp, br,* and *2Bc*) and encodes several distinct zinc-finger-containing isoforms (Z1 to Z4). Using isoform-specific polyclonal antibodies we observe in the fat body a switch in *BR-C* isoform expression from the Z2 to the other three isoforms during the third instar. We show that the $2Bc^{+}$ function that corresponds presumably to the Z3 isoform is required for the larval fat body-specific expression of a transgenic construct (*AE*) in which the *lacZ* gene is under the control of the ecdysone-regulated enhancer and minimal promoter of the *fat body protein 1* (*Fbp1*) gene. Using *hs(BR-C)* transgenes, we demonstrate that overexpression of Z1, Z3, or Z4, but not Z2, is able to rescue *AE* activity with faithful tissue specificity in a *BR-C* null (*npr1*) genetic context, demonstrating a partial functional redundancy between Z1, Z3, and Z4 isoforms. We also show that continuous overexpression of Z2 during the third instar represses *AE,* while conversely, expression of Z3 earlier than its normal onset induces precocious expression of the construct. This finding establishes a tight correlation between the dynamic pattern of expression of the *BR-C* isoforms and their individual repressive or inductive roles in *AE* regulation. Altogether our results demonstrate that the balance between *BR-C* protein isoforms in the fat body mediates, in part, the precise timing of the ecdysone activation of the *AE* construct but does not modulate its tissue specificity.

Key Words: **larval fat body; ecdysone; fat body protein 1 gene; temporal regulation; zinc fingers.**

INTRODUCTION

Metamorphosis in holometabolous insects is a remarkable developmental process leading to the remodeling of the larva into the adult. During this period, initiation of new programs of gene expression lead to important morphological and physiological modifications within larval and imaginal tissues. Larval tissues are histolyzed or reorganized while imaginal tissues develop into adult structures. In *Drosophila,* this metamorphic transformation is orchestrated by successive waves of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) (Riddiford, 1993). How the hormonal signal is correctly interpreted in each target tissue remains an open question. Cytological studies of ecdysone-induced puffing of polytene chromo-

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somes led Ashburner and his colleagues (1974) to propose a model of action of ecdysone in larval salivary glands that was further refined and extended to include a variety of ecdysone target tissues (Ashburner *et al.,* 1974; Russell *et al.,* 1996; Thummel, 1990, 1996). According to this model, the release of the hormonal signal during the third larval instar represses intermolt genes and induces a small set of early regulatory genes simultaneously. The products of the early genes subsequently repress their own expression and activate a large set of late genes allowing tissues to ensure the correct execution of their metamorphic program.

To date four early genes have been characterized at the molecular level, *Broad-Complex* (*BR-C*), *E74*, *E75*, and *E63-1* (Andres and Thummel, 1995; Burtis *et al.,* 1990; DiBello *et al.,* 1991; Segraves and Hogness, 1990). Consistent with their regulatory function, the first three of these genes direct the synthesis of putative transcription factors. The *BR-C*, located in the 2B5 region of the X chromosome, plays a key role in the genetic control of metamorphic responses to ecdysone because it is implicated in the regulation of intermolt, early, and late gene activities (Belyaeva *et al.,* 1981; Guay and Guild, 1991; Karim *et al.,* 1993; Zhimulev *et al.,* 1982).

The *BR-C* is organized in overlapping transcription units (Bayer *et al.,* 1996; DiBello *et al.,* 1991) that give rise to an extensive family of RNA transcripts (Chao and Guild, 1986; Karim *et al.,* 1993; Karim and Thummel, 1992) encoding several related protein isoforms. These isoforms share a common amino-terminal "core" region containing a BTB/POZ domain likely involved in protein/protein interactions (Bardwell and Treisman, 1994; Zollman *et al.,* 1994). The core exons are alternatively spliced to one of four isoform-specific exons encoding pairs of zinc-finger motifs (Z1 to Z4) (Bayer *et al.,* 1996; DiBello *et al.,* 1991).

The *BR-C* locus encodes three genetic subfunctions each represented by a complementation group: *broad* (*br*), *reduced bristles on the palpus* (*rbp*), and *2Bc*. All mutations affecting these functions fail to complement the *nonpupariating* mutations (*npr1* alleles) that are phenotypically indistinguishable from deletions of the locus (Belyaeva *et al.,* 1980; Kiss *et al.,* 1988). Although several studies have found converging results supporting a plausible correlation between *BR-C* isoforms and complementing genetic functions, there is not a simple one-to-one correspondence between *BR-C* proteins and *BR-C*$^+$ genetic functions (Bayer *et al.,* 1997; Emery *et al.,* 1994). Using heat-inducible *BR-C* transgenes (*hs(BR-C)*) to rescue the lethality associated with each *BR-C* subfunction and restore transcriptional activity of tissue-specific *BR-C*$^+$-dependent target genes, Bayer *et al.* (1997) demonstrated that the *br*$^+$ function is provided by the Z2 isoform only. In contrast, although the *rbp*$^+$ function was fully supplied by the Z1 isoform, it was also partially provided by Z4, indicating functional redundancy between these two isoforms (Bayer *et al.,* 1996; Sandstrom *et al.,* 1997). Finally, the *2Bc*$^+$ function was provided by the Z3 isoform. However, the other *BR-C* isoforms were able to at least partially compensate for the absence of this function. This last observation also indicated a functional redundancy between Z3 and the other *BR-C* isoforms, but further evidence to confirm this hypothesis is lacking. Alternatively, these results could suggest a role of Z3 in the autoregulation of *BR-C* expression, either directly or indirectly through the modulation of the activity of other genes regulating this locus.

The *npr1* mutations lead to lethality at the onset of metamorphosis without puparium formation. Alleles belonging to the *br*, *rbp*, and *2Bc* complementing functions result in lethality somewhat later in development, with severe morphological defects (Belyaeva *et al.,* 1980; Fristrom *et al.,* 1981; Kiss *et al.,* 1988; Restifo and Merrill, 1994; Restifo and White, 1991, 1992). Several lines of evidence demonstrate that these developmental failures are not due to an ecdysteroid deficiency, but rather to defects in the response to the hormonal signal (Fristrom *et al.,* 1981; Kiss *et al.,* 1976, 1978; Murphy *et al.,* 1977; Stewart *et al.,* 1972). Together these data point to the *BR-C* as essential for the proper response of tissues to the ecdysone signal during metamorphosis.

The regulatory role and mode of action of the proteins encoded by the *BR-C* remain to be thoroughly determined. *BR-C* isoforms and *BR-C* mRNAs are expressed differentially in a number of tissues around the time of metamorphosis (Emery *et al.,* 1994; Huet *et al.,* 1993). This supports the postulate of the tissue coordination model (Burtis *et al.,* 1990; Thummel, 1990) that the relative levels of *BR-C* isoforms and other early ecdysone-induced gene products, in hormone target tissues, may contribute to the tissue specificity of the ecdysone response. However, several studies support the idea that the *BR-C* is an important stage regulator of the transcriptional response to ecdysone. The study of the effect of *BR-C* mutations on a series of ecdysone-inducible genes in salivary glands led Karim *et al.* (1993) to conclude that the *BR-C* is implicated in both the timing and the level of transcription of these genes. Crossgrove *et al.* (1996) have suggested that the sequential expression and antagonistic activities of *BR-C* protein isoforms may be responsible for the timing of *L71* late gene expression in salivary glands.

With the aim of further investigating this role of the *BR-C* protein isoforms as temporal regulators, we examined the requirement for and function of individual *BR-C* isoforms in the expression of the *fat body protein 1* gene (*Fbp1*) as a model target gene. *Fbp1* is a primary ecdysone-responsive gene which is expressed exclusively in the fat body during the second half of the third larval instar (Andres *et al.,* 1993; Lepesant *et al.,* 1982, 1986; Maschat *et al.,* 1990). Analysis of its *cis*-regulatory sequences led to the characterization of a 70-bp enhancer (−69 to −138) (referred to as element E) (Laval *et al.,* 1993) and a 32-bp amplifier (−194 to −162) (referred to as element A) (Lapie *et al.,* 1993) that are sufficient to confer the correct temporal and spatial patterns of ecdysone-induced expression of the *Fbp1* gene when they are fused to the *Fbp1* (−68/+80) minimal pro-

TABLE 1
Drosophila Stocks

	Chromosome/balancer	Nature of *BR-C* lesion	Reference
Mutant stocks			
br^{28}	br^{28}/FM6, $l(1)69^{j}$*	P-element insertion in Z2 zinc-finger exon	(a)
$2Bc^{2}$	y $2Bc^{2}$/Binsn	Unknown	(b)
rbp^{5}	y rbp^{5}/Binsn	Stop codon in Z1 linker region	(b, c)
$npr1^{6}$	y $npr1^{6}$/FM6, $l(1)69^{j}$	Unknown	(b)
Df(1)S39	Df(1)S39/FM6, $l(1)69^{j}$	Df(1)1E1-2;2B5-6	(b)
Dp (1; Y)Sz280	y l(1)/FM6, $l(1)69^{j}$/Dp(1;Y)Sz280	Dp(1;Y)1A;2C1-2 with internal deficiency Df(1)2B3-4;2B7-8.	(b)
Transgenic stocks			
AE	w^{1118}; +; AE		(d)
AEhsZ1	w^{1118}; hsp70-dm708; AE		This study
AEhsZ2	w^{1118}; hsp70-cD5; AE		This study
AEhsZ3	w^{1118}; hsp70-dm797; AE		This study
AEhsZ4	w^{1118}; hsp70-28.I; AE		This study

Note. (a) DiBello *et al.* (1991), (b) Lindsley and Zimm (1992), (c) Bayer *et al.* (1997), (d) Antoniewski *et al.* (1996); Brodu *et al.* (1999).
* FM6, $l(1)69^{j}$ was described in Belyaeva *et al.* (1980).

moter driving the *lacZ* reporter gene in the *AE* construct (Antoniewski *et al.*, 1996; Brodu *et al.*, 1999).

We demonstrate here a strong requirement for $BR\text{-}C^{+}$ genetic functions for the normal pattern of expression of the *AE* construct in the larval fat body. We identify which *BR-C* genetic functions are implicated by testing the effects of mutations of each complementation group and the overexpression of individual *BR-C* isoforms. The potential activating or repressing function attributed to individual *BR-C* isoforms from these experiments is supported by the detailed analysis of the expression profile of the four *BR-C* isoforms in the fat body throughout the third larval instar. Finally, the observation of a repression or a temporal deregulation of *AE* expression following heat-shock induction of specific *BR-C* isoforms allows us to assign a role to these isoforms in the timing of the expression of this target construct.

MATERIALS AND METHODS

Drosophila Strains and Crosses

Stocks are listed in Table 1. The br^{28}, $2Bc^{2}$, rbp^{5}, and $npr1^{6}$ mutations and the *Df(1)S39* and *D(1; Y)Sz280* deficiencies and translocations are described in Lindsley and Zimm (1992), except br^{28}, which is described in DiBello *et al.* (1991). The AE stock harbors the transgenic *AE* reporter construct described in Brodu *et al.* (1999). The AEhsZ1–4 stocks, homozygous for the *hsZ1–4* transgenic constructs (Crossgrove *et al.*, 1996) and the *AE* reporter construct were obtained by appropriate crosses. Flies and larvae were reared at 25°C on a standard *Drosophila* medium supplemented with dry yeast. w^{1118} was used as the reference wild-type strain.

To test the effect of *BR-C* mutations on the *AE* construct (Fig. 5), AE males were crossed with heterozygous mutant females carrying *BR-C* alleles depicted in Table 1 to generate male progeny hemizygous for the *BR-C* mutation and heterozygous for the *AE* transgene. These animals were staged as described below and harvested at 114–119 h after egg-laying (AEL).

For the rescue assay (Fig. 6), homozygous males of the AEhsZ1–4 stocks (see Table 1) were crossed to y $npr1^{6}$/FM6 $l(1)69^{j}$ females to generate male progeny hemizygous for y $npr1^{6}$ and heterozygous for both the *hs(BR-C)* and the *AE* transgenes. As a control, male progeny from y $npr1^{6}$/FM6 $l(1)69^{j}$ females crossed to AE males were generated.

Developmental Staging of Larvae

To simplify presentation and comparison of developmental data, all times of development are indicated as hours of development AEL. Under our culture conditions at 25°C, transition from the second to the third instar occurred about 72 h AEL and pupariation at 120 h AEL, and the white prepupal stage was reached about 120–124 h AEL (Bainbridge and Bownes, 1981). It should be noted that the onset of expression of the *AE* transgene takes place after 106 h AEL (Brodu *et al.*, 1999). Two staging methods, as follows, that proved to be reproducible were used.

Test of BR-C mutations and rescue of AE expression assay. w^{1118} and mutant larvae were reared at 25°C in uncrowded vials on the standard medium supplemented with 0.05% (w/v) bromophenol blue. This dye is clearly visible inside the gut of live animals and its clearance is a reliable indicator of the developmental stage of late third-instar larvae. Larvae with lightly stained gut were approximately 5–12 h away from pupariation, i.e., 108–115 h AEL, and those with an almost completely clear gut were approximately 1–6 h away from pupariation, i.e., 114–119 h AEL (Andres and Thummel, 1994). We verified that larvae hemizygous for the *br*, *rbp*, and *2Bc* mutations did not develop any slower than wild-type larvae according to the time of hindgut clearing. Larvae hemizygous for the $npr1^{6}$ mutation never pupariated, but complete gut clearing occurred normally and we relied on this criterion for selecting larvae for assays.

Immunostaining and temporal deregulation experiments with heat-inducible hs(BR-C) transgenes. First-instar larvae from 1-h egg lays were collected within 30 min of hatching, transferred to

standard medium in batches of about 50 larvae, and reared at 25°C. Samples of larvae for immunostaining were taken at 72 (early L3), 96 (middle L3), 118 (Late L3), and 120–124 h AEL (white prepupae) (Fig. 4). For experiments on *AE* deregulation by overexpression of Z3, larvae were taken prior to any detectable expression of the *AE* transgene at 96 h AEL (Fig. 8).

Heat-Shock Protocols

To induce expression of the heat-inducible *hs(BR-C)* transgenes, staged larvae were placed in hermetically closed plastic vials and submerged for 35 min in a 37°C water bath. After heat shocks the vials were placed at 25°C.

Rescue of AE expression. *y npr1*^{6}*/Y; hs(BR-C)/+; AE/+* males were heat shocked at the "light blue gut" stage and then allowed to develop at 25°C for 8 h. "Clear gut" larvae were then collected in batches of three larvae in Eppendorf tubes and frozen on dry ice before storage at −80°C.

Temporal deregulation of AE expression. Heat shocks were performed on AEhsZ2 larvae at 96 h AEL and again at 100 h AEL while AEhsZ1, AEhsZ3, and AEhsZ4 larvae were heat shocked at 73 h AEL and again at 90 h AEL. Larvae were allowed to develop at 25°C until they were collected for β-galactosidase assay.

Generation of Antibodies

Fusion proteins were used as immunogens to produce polyclonal antibodies in New Zealand female rabbits.

Except for Z2, *BR-C* protein-specific regions were chosen outside of the zinc-finger motif. Isoform-specific regions of *BR-C* cDNAs [dm527(Q-Z1), cD5(Z2), dm797(Z3), and 28.I(Z4)] (Bayer *et al.*, 1996; DiBello *et al.*, 1991) were amplified by PCR with primers containing restriction enzyme sites at their ends for subsequent cloning in frame into both pGEX (Pharmacia Biotech) and pMALc2 (Biolabs) bacterial expression vectors in *Escherichia coli* BL21 strain. The amplified regions correspond to nucleotide positions 1899–2107 for the Z1 isoform, 1680–1931 for the Z2 isoform, 2307–2501 for the Z3 isoform, and 1966–2163 for the Z4 isoform. Constructs were verified by sequence determination of the cloned DNA fragments in the resulting pGEXz1 to pGEXz4 and pMALz1 to pMALz4 plasmids.

Bacterial cultures were grown until OD_{600nm} reached 0.5 and induced with 2 mM IPTG for 3 h. GST fusion proteins were purified on glutathione Sepharose 4B (Pharmacia Biotech) while MBP fusion proteins were purified on an amylose resin (Biolabs) in accordance with the manufacturer's specifications.

For the production of anti-Z1, anti-Z2, anti-Z3, and anti-Z4 polyclonal antibodies, 100- to 500-μg aliquots of purified GSTz1 (from pGEXz1), MBPz2 (from pMALz2), GSTz3 (from pGEXz3), and GSTz4 (from pGEXz4) proteins were used to immunize and boost via subcutaneous route two New Zealand female rabbits for each fusion protein. The first injection was in Freund's complete adjuvant and the boost, spaced for at least 3 weeks, was in Freund's incomplete adjuvant. A total of four to five boosts were performed on each rabbit, and sera taken 2 weeks after the last boost were tested for their immunoreactivity against the injected proteins.

Immunoaffinity Purification of Antibodies

About 10 mg of GST, MBP, GSTz1, MBPz2, MBPz3, and MBPz4 proteins were independently immobilized on CNBr-activated Sepharose 4 Fast Flow (Pharmacia Biotech), according to the manufacturer's recommendations.

In the case of the anti-Z1 and anti-Z2 sera, polyreactive antibodies were retained on a GST- or a MBP-coupled Sepharose column and the flowthrough was applied at a flow rate of about 10 ml · cm^{-2} · h^{-1} to a GSTz1- or a MBPz2-coupled Sepharose column, respectively. In the case of the anti-Z3 and anti-Z4 sera, about 20 ml of serum was applied directly to a MBPz3- or a MBPz4-coupled Sepharose column, respectively. Each of the four affinity columns was washed with 1× PBS, 500 mM NaCl until the $OD_{280\ nm}$ reached a null value. The specific antibodies retained were eluted with 100 mM glycine–HCl buffer (pH 2.5) and diluted in 100 mM Tris–HCl (pH 8) in a 1:3 ratio (v/v). About 6 gel volumes of elution buffer were used and the protein content of each collected fraction was determined by $OD_{280\ nm}$ measurement. Fractions with significant amounts of protein were pooled and concentrated on an Amicon concentrator. Purified antibodies were stored in 50% glycerol at −20°C.

In Vitro Translation of BR-C Protein Isoforms

In vitro translation of dm527, cD5, dm797, and 28.I cDNAs was performed using the TNT coupled reticulocyte lysate system from Promega.

Protein Extracts and Western Blotting

Ten to twenty late third-instar *w*^{1118} or *BR-C* null larvae were homogenized in 40–80 μl cracking buffer [125 mM Tris (pH 6.8), 5% β-mercaptoethanol, 2% SDS, 4 M urea] by vortexing. Samples were boiled (2 min) and centrifuged prior to electrophoresis on a 7% SDS–polyacrylamide gel. Four microliters of extract or 5 μl of *in vitro*-translated *BR-C* protein was loaded in the corresponding lane. Transfers were performed onto nitrocellulose membranes (Schleicher and Schuell) using a Novablot Electrophoretic Transfer Kit (LKB). All subsequent steps were carried out at room temperature. The membranes were blocked in TNT buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.25% Triton X-100] containing 5% nonfat dried milk for 1 h. All antibodies were diluted 1:10^{4} in this blocking buffer. Incubations with these antibodies were performed for 1–2 h. Blots were rinsed three times for 10 min in TNT and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Vector Laboratories) diluted 1:5000. Blots were rinsed as before, followed by three washes with sterile distilled water. Detection of the enzyme-conjugated antibodies was achieved using the ECL kit (Amersham) and X-OMAT AR film (Kodak).

Whole-Mount Immunostaining

Immunostaining of dissected tissues was performed as described in Brodu *et al.* (1999) with the following modifications. Normal goat serum (NGS) (Jackson Immunoresearch) was used instead of BSA. Ten percent NGS was used in the blocking step and 5% NGS was added in PBT to dilute antibodies. The anti-Z1, anti-Z2, and anti-Z3 were diluted 1:3000 and the anti-Z4, 1:4000, and used without preadsorption on embryos.

Histochemical and Spectrophotometric Assays of β-Galactosidase Activity

Histochemical staining and CPRG spectrophotometric assays of β-galactosidase activity were performed as described in Brodu *et al.* (1999).

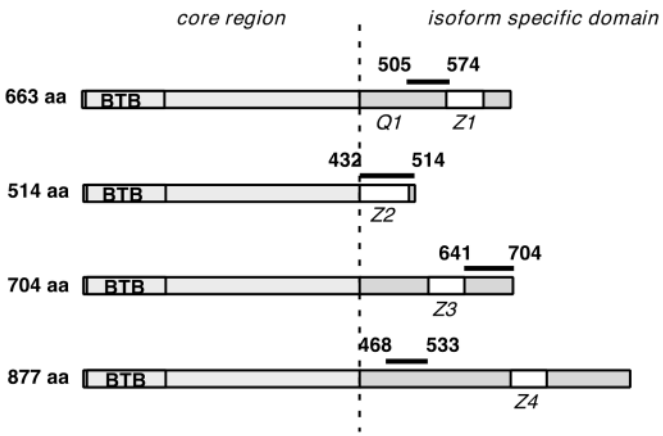


FIG. 1. Schematic representation of the Q1-Z1, Z2, Z3, and Z4 protein isoforms encoded by the *BR-C* locus. The first 431 amino acids defining the core region, common to all *BR-C* proteins, are linked to isoform-specific domains as described in DiBello *et al.* (1991) and Bayer *et al.* (1996). The isoform-specific domains used to produce polyclonal antibodies are each delimited by a line with amino acid coordinates of the peptide sequence indicated. Accession numbers for sequences in GenBank are QZ1, X54663 S79435; Z2, X54665; Z3, X54664 S79445; and Z4, U51585.

RESULTS

Isoform-Specific Anti-BR-C Antibodies

Each *BR-C* isoform is characterized by unique C-terminal zinc-finger and linker domains fused to a common N-terminal core region (Bayer *et al.*, 1996; DiBello *et al.*, 1991). Small portions of these isoform-specific protein regions were expressed in *E. coli* and used as immunogens to generate polyclonal antibodies directed against each *BR-C* protein isoform (Fig. 1). In order to test the specificity of these antibodies, Western blots were performed using *in vitro*-translated *BR-C* proteins and protein extracts from wild-type larvae and larvae carrying a deletion of the entire *BR-C* locus. *In vitro* translation of each of the four *BR-C* cDNAs gave rise to two ^{35}S-labeled bands in SDS–polyacrylamide gel (Fig. 2E). In all four cases, the lower molecular weight band (arrowheads in Fig. 2E) corresponded to the expected size deduced from the cDNA sequence (Bayer *et al.*, 1996; DiBello *et al.*, 1991). We presume that the upper band corresponds to a translational readthrough product. Each anti-*BR-C* antibody recognized specifically the two products of the corresponding *in vitro*-translated *BR-C* cDNA, and no cross-reactivity was observed between the various antibodies (Figs. 2A to 2D, lanes Z1, Z2, Z3, Z4). In each blot, the signal detected in the extracts from wild-type larvae (lanes WT) revealed a group of several bands migrating slower than the lower migrating *in vitro*-translated product (arrowheads in Fig. 2). Because no equivalent signal was detected in the *BR-C* deletion extract (null lanes) we concluded that the antibodies recognized specifically the endogenous *BR-C* isoforms. The multiple specific products detected with each antibody may correspond either to various posttranslational modifications (Bayer *et al.*, 1996; Emery *et al.*, 1994) or to translational products from transcripts resulting from a differential splicing of isoform-specific exons. The isolation of a family of Z1 cDNAs encoding several Z1 protein isoforms differing by the length of the linker between the core region and the zinc-finger domain (DiBello *et al.*, 1991) supports this hypothesis. Similarly, Bayer *et al.* (1996) have identified a family of partially spliced Z4 mRNAs.

The specificity of these antibodies was further tested when we examined the protein defects induced by mutations representative of each *BR-C* complementation group listed in Table 1. The *BR-C* null mutation $npr1^{6}$ results in a total loss of $BR\text{-}C^{+}$ genetic functions and accordingly no *BR-C* protein isoforms were detected in mutant larvae (Fig. 3). The br^{28} mutation results in a loss of br^{+} function associated with a P-element insertion within the Z2 zinc-finger pair (DiBello *et al.*, 1991). We confirmed the previous finding of Emery *et al.* (1994) that this mutation gives rise to the accumulation of truncated Z2 protein products (Fig. 3B) while no other isoform was affected (Fig. 3D and data not shown; see also Bayer *et al.*, 1997). In larvae carrying the $2Bc^{2}$ mutation the multiple Z3-specific bands detected in wild-type larvae were replaced by a single band which migrated slightly faster. This may reflect an alteration of the posttranslational modifications of the Z3 protein (Fig. 3C). A similar effect appeared to take place in the $2Bc^{1}$ mutant larvae (Emery *et al.*, 1994, and our unpublished data). The other *BR-C* isoforms in the $2Bc^{2}$ mutant were detected at the expected molecular weight but appeared to be underrepresented (Fig. 3D and data not shown). The rbp^{5} mutation is associated with a stop codon in the Z1 linker region (Bayer *et al.*, 1997) and as expected no Z1 isoforms were detected with our antibodies (Fig. 3A). The other *BR-C* isoforms were detected at their respective expected molecular weights (Fig. 3D and data not shown).

The Developmental Expression Profile of BR-C Protein Isoforms in the Larval Fat Body Is Dynamic during the Third Instar

Anti-*BR-C* polyclonal antibodies were used to establish a detailed spatial and temporal distribution of *BR-C* proteins in larval and imaginal tissues during the third larval instar (Mugat *et al.*, in preparation). Here, we focused on the characterization of the temporal distribution of *BR-C* proteins in the larval fat body where the *BR-C* target *AE* construct is specifically expressed.

Representative immunohistochemical stainings of dissected fat bodies are presented in Fig. 4. Immunostaining on *BR-C* null larvae carried out in parallel as a control showed no significant specific signal over background (data not shown). A nuclear signal was detected with each antibody in agreement with the role of transcriptional regulator proposed for the *BR-C* proteins. These experiments revealed

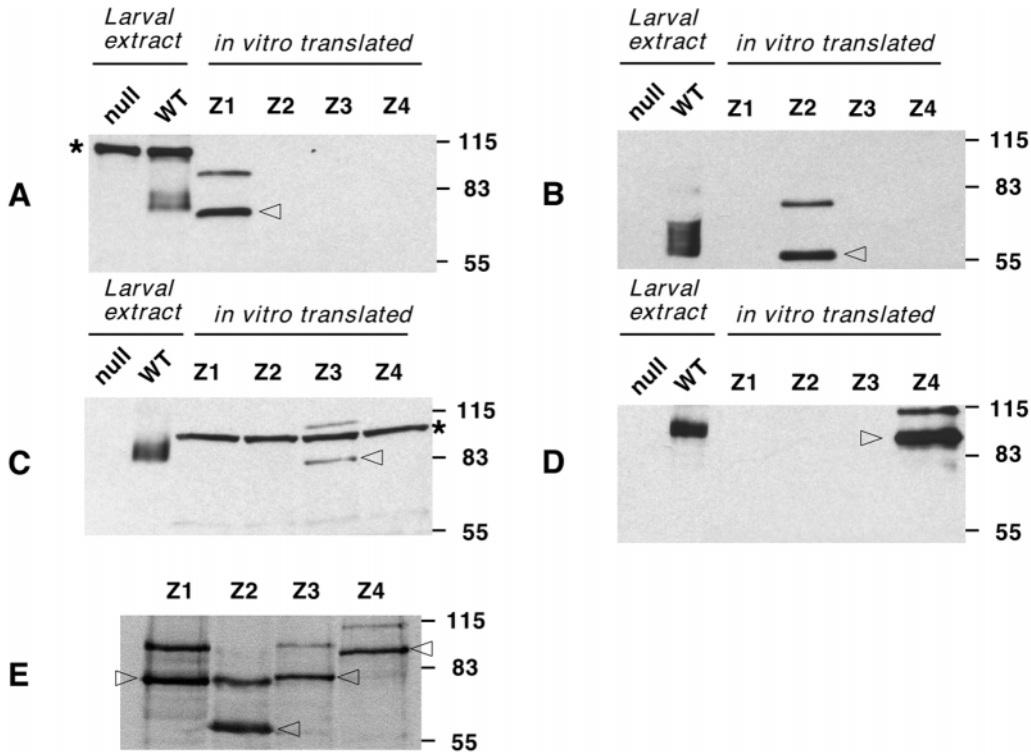


FIG. 2. Test of the specificity of anti-*BR-C* isoforms antibodies. Polyclonal antibodies directed against the Z1 (A), Z2 (B), Z3 (C), and Z4 (D) specific domains were used to probe blots containing protein extract from male third-instar larvae [*Df(1)S39/Dp(1;Y)Sz280* transheterozygotes] carrying a deletion of the *BR-C* locus (null) and from wild-type (WT) late third-instar larvae and each of the *in vitro*-translated *BR-C* isoforms. About one animal equivalent of total extract was loaded in the null and WT lanes. The mobilities of the molecular weight markers are indicated on the right in kDa. Arrowheads indicate the expected molecular weight of the *in vitro*-translated proteins detected with antibodies (A, B, C, D) or radiolabeled (E). (A) A nonspecific high-molecular-weight product (asterisk) was detected with the anti-Z1 in both *BR-C* null and wild-type extracts. (C) A protein from the rabbit reticulocyte lysate was detected with the anti-Z3 (asterisk). (E) Autoradiogram of the ^{35}S-labeled *in vitro*-translated *BR-C* isoforms.

that Z2 is the only isoform present in the fat body at the beginning of the third instar (ca. 72 h AEL) (Fig. 4B). Z2 staining decreased to a very low level in mid-third-instar (ca. 96 h AEL) fat body while Z3 expression became detectable (Figs. 4F and 4G). At this time of development, Z2 and Z3 were the only two isoforms detectable in the fat body. In late third-instar fat body (ca. 118 h AEL), Z2 expression was undetectable while Z1, Z3, and Z4 were strongly expressed (Figs. 4I, 4J, 4K, and 4L). The same expression profile was maintained in white prepupae (120 h AEL) (Figs. 4M–4P). Interestingly we observed that the Z2 truncated products detected with our antibody in a br^{28} mutant persisted in the late third-instar fat body (Fig. 4J′).

BR-C Loss-of-Function Mutations Affect the Expression of the AE Construct

We used the *AE* construct, which is expressed in an ecdysone-dependent manner in the fat body of late third-instar larvae (Antoniewski *et al.*, 1996), to test whether this sequential appearance of *BR-C* isoforms was involved in the regulation of *BR-C* downstream target genes in the fat body. To this end, we investigated the effect of representative *BR-C* mutations of the four mutational classes described above (see also Table 1) on the fat body-specific expression of the construct.

All *BR-C* mutations tested but rbp^5 had a strong effect on the level of *AE* expression in late third-instar larvae (114–120 h AEL) (Fig. 5). The $npr1^6$ and $2Bc^2$ genetic context led to a strong decrease of the expression of the construct to only 5 and 0.4%, respectively, of that measured in the wild-type control larvae. In contrast, in an rbp^5 mutant, the level of *AE* expression reached 71% of the level in $BR\text{-}C^+$ control larvae, indicating only a minor effect of the rbp^5 mutation on the construct. Finally, in a br^{28} mutant, the *AE* expression level reached 230% of the corresponding control (Fig. 5) but remained limited to the fat body (data not shown).

Together, these experiments revealed that multiple *BR-C* genetic functions were involved in the activation of the

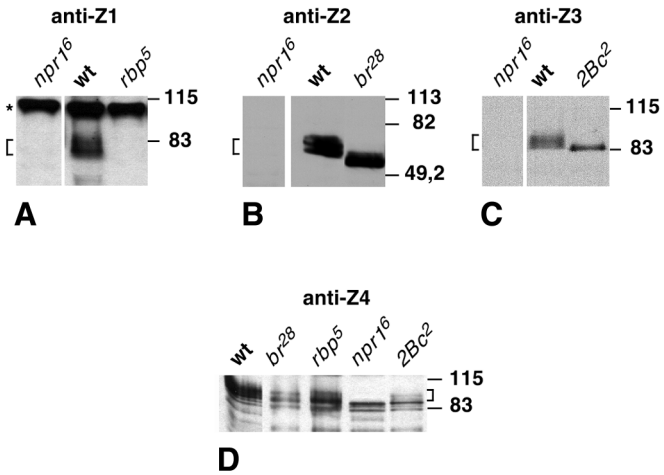


FIG. 3. Western blotting analysis of *BR-C* mutants. Polyclonal anti-*BR-C* isoform-specific antibodies were used to probe blots containing protein extracts from *BR-C* mutants and wild-type animals (wt). The amount of total extract loaded in each lane corresponded to about one animal equivalent. Specific signals are indicated by brackets. The mobilities of the molecular weight markers are indicated on the right in kDa. (A) A nonspecific high-molecular-weight product (asterisk) was detected with the anti-Z1 in rbp^5, $npr1^6$, and wild-type extracts; no Z1 protein was detected in the rbp^5 and $npr1^6$ lanes. Truncated products were detected with the anti-Z2 antibody in the br^{28} mutant and no Z2 isoform was detected in $npr1^6$ mutant larvae (B). A single band migrating slightly faster than the wild-type bands was observed in a $2Bc^2$ mutant with the anti-Z3 antibody while no Z3 isoform was detected in the $npr1^6$ mutant (C). (D) Z4 was undetectable in the $npr1^6$ mutant extract; similar background bands were detected in all lanes.

Fbp1 enhancer and promoter included in the *AE* construct and suggested that the br^+ genetic function, and correspondingly, the Z2 isoform, has a repressive function. These conclusions were further tested by overexpression of individual *BR-C* isoforms in a *BR-C* null genetic context.

Isoform-Specific Rescue of the Expression of the AE Construct

hsZ1, hsZ2, hsZ3, and *hsZ4* constructs in which cDNAs encoding the Z1, Z2, Z3, and Z4 isoforms are expressed under the control of the *hsp70* promoter (Crossgrove *et al.*, 1996) were tested for their ability to restore *AE* expression in a $npr1^6$ mutant background. $npr1^6$ males carrying the *AE* construct and one of the four *hs(BR-C)* transgenes were heat-shocked at late third instar (108–115 h AEL). After an 8-h recovery period, β-galactosidase activity was assayed on extracts from whole larvae. Ubiquitous overexpression of each *BR-C* isoform following heat shock was verified by Western blotting and immunostaining of dissected tissues (data not shown). Results are presented in Fig. 6 in which β-galactosidase activity is expressed as a percentage of the corresponding heat-shocked $BR\text{-}C^+$ control male larvae. Control animals that did not carry a *hs(BR-C)* transgene failed to induce *AE* expression (Fig. 6).

We found that induction of the *hsZ1, hsZ3,* or *hsZ4* transgenes in $npr1^6$ mutant larvae was able to restore 60, 33, and 68% of *AE* expression, respectively, compared to matching control heat-shocked [$BR\text{-}C^+$; *AE; hsZN*] male larvae. Rescued *AE* expression remained limited to the fat body as determined by histochemical staining (not shown). In contrast, *hsZ2* induction was unable to rescue *AE* transgene expression in $npr1^6$ mutant larvae (Fig. 6).

A Switch in BR-C Isoforms Is Responsible for the Proper Temporal Expression of ARE

We find that the Z2 isoform is expressed at the beginning of the third larval instar, before the *AE* construct becomes active at approximately 108 h of development and before the Z3 isoform starts to accumulate. We also find that in the absence of a functional Z2 isoform, i.e., in a br^{28} genetic context, the *AE* construct is significantly derepressed. These observations prompted us to ask whether the sequential appearance of the four *BR-C* isoforms is required for the proper timing of the expression of the *AE* construct during the third instar. To approach this question, we analyzed how *AE* expression was affected if a high level of Z2 isoform were maintained past its normal time of expression, i.e., after 96 h AEL. Conversely, we attempted to deregulate *AE* expression by overexpressing Z1, Z3, or Z4 at the beginning of the third instar (ca. 72 h AEL), i.e., at least 24 h earlier than their normal onset of appearance in the fat body. We took care to determine if the animals were alive after the heat-shock recovery period because previous studies had shown that overexpression of *BR-C* isoforms at a time in development when they are not normally present at high levels leads to a strong lethality (Bayer *et al.*, 1997).

AEhsZ2 larvae (see Table 1) were heat-shocked at 96 and 100 h after egg-laying and animals were collected at 118 h. Under these conditions, the level of β-galactosidase reporter activity was only 25% of that measured in control larvae that were not heat-shocked (Figs. 7A and 7B). We verified that this down-regulation of *AE* expression was due to the overexpression of the *hsZ2* construct and not to a nonspecific effect of the heat shocks by assaying larvae which carry only the *AE* construct. No significant difference in the level of β-galactosidase activity was observed between animals that were heat-shocked and those that were not (Fig. 7B). These results showed that the Z2 isoform, when overexpressed during the second half of the third instar, prevented *AE* from reaching its wild-type level of expression and support the hypothesis that, under physiological conditions, the Z2 isoform maintains *AE* in an inactive state until the middle of the third instar.

In contrast to *hsZ2,* induction of the *hsZ1, hsZ3,* or *hsZ4* transgene at the beginning of the third instar led to different effects on *AE* expression.

The strong lethality of larvae following Z1 overexpression under our heat-shock conditions precluded any conclu-

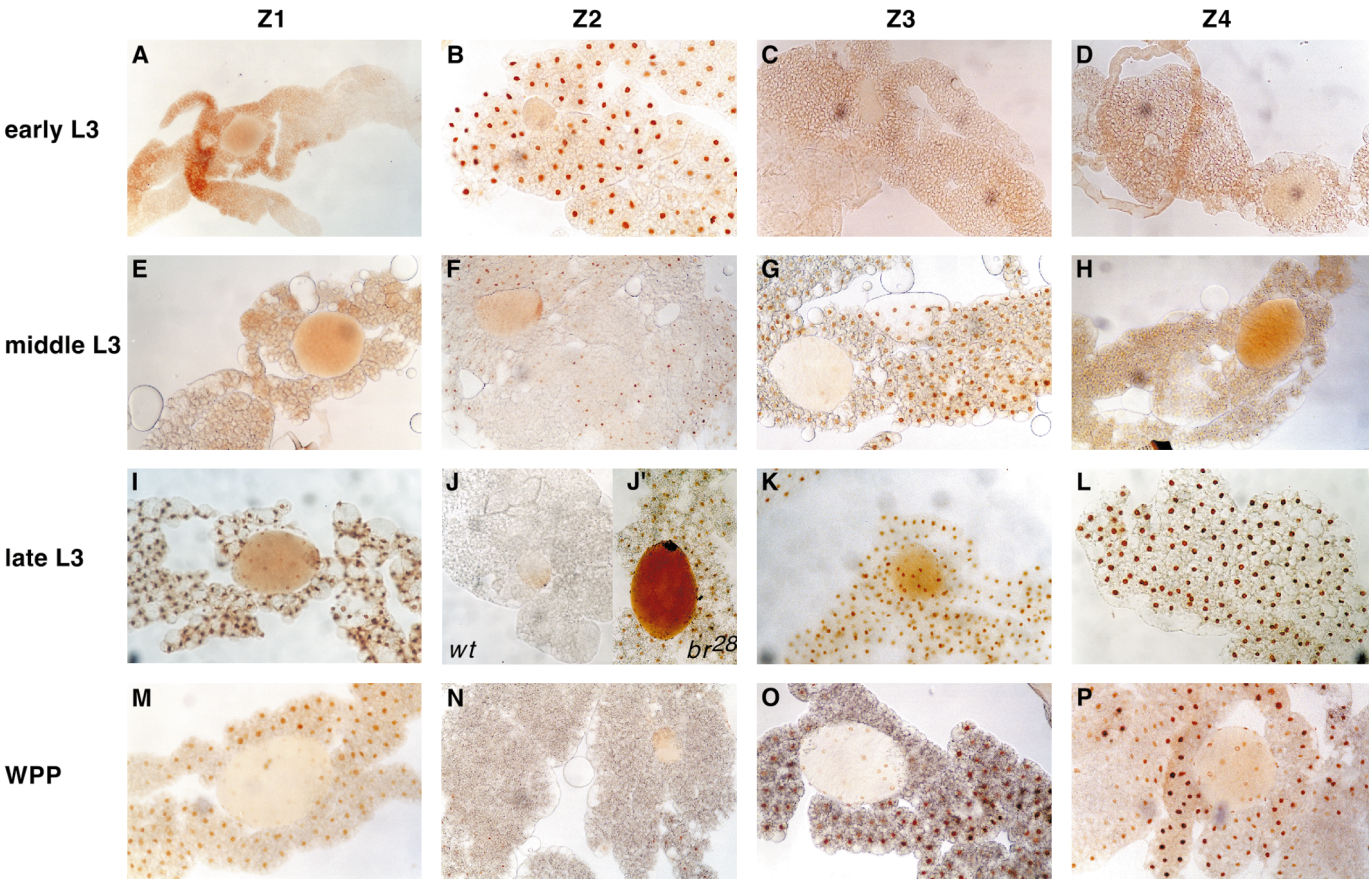


FIG. 4. Dynamic expression profile of *BR-C* protein isoforms in the fat body during the third larval instar. Polyclonal antibodies directed against the Z1, Z2, Z3, or Z4 specific domains were used to immunostain dissected fat bodies, including gonads, from early L3 (72 h AEL) (A, B, C, D), middle L3 (96 h AEL) (E, F, G, H), or late L3 (118 h AEL) (I, J, K, L) larvae or white prepupae (120 h AEL) (M, N, O, P) of the w^{1118} stock. (J′) Late third-instar br^{28} mutant fat body immunostained with anti-Z2; the truncated Z2 isoform persists in the mutant larval tissue while the wild-type Z2 isoform has become undetectable in the wild-type fat body (J). Detection was achieved using horseradish peroxidase-conjugated secondary antibody and diaminobenzidine.

sions from being drawn concerning this isoform. No effect on *AE* expression was observed after *hsZ4* activation. In contrast, induction of *hsZ3* at the beginning of third instar led to the premature expression of *AE* in the middle of the third instar (96 h AEL) exclusively in the fat body (Fig. 8A). In the course of these experiments, we observed that larvae carrying a single copy of the *hsZ3* and *AE* constructs did not show any significant staining in the fat bodies of 96-h larvae after heat shock (Fig. 8B). We asked whether this absence of response was due to a reduction of the dose of Z3 or to a lack of sensitivity of the histochemical assay when only one copy of the *AE* construct was present. Males homozygous for the two transgenes were crossed with females homozygous for *hsZ3* in order to obtain larvae with two copies of *hsZ3* and one copy of *AE* (*hsZ3/hsZ3; AE/+*). When submitted to the heat-shock regimen these animals showed *AE* expression in the fat body at 96 h of development (Fig. 8C). This indicated that two copies of *hsZ3* were required for premature induction of *AE*. In striking contrast, in a br^{28} genetic context, i.e., in the absence of functional Z2 protein, a single copy of the *hsZ3* transgene was sufficient to induce precocious expression of *AE* in the fat bodies of 96-h heat-shocked larvae (Fig. 8D). This result lends further support to our hypothesis that the Z2 isoform represses *AE* in the first stages of the third larval instar.

DISCUSSION

Our study based on the use of specific anti-*BR-C* polyclonal antibodies provides the first evidence for a dynamic expression profile of *BR-C* protein isoforms in the fat body throughout the third larval instar. Although we tested four stages of larval development only, we can establish a sequential order of appearance of these isoforms: Z2 is the first and only isoform detected at the early third-instar

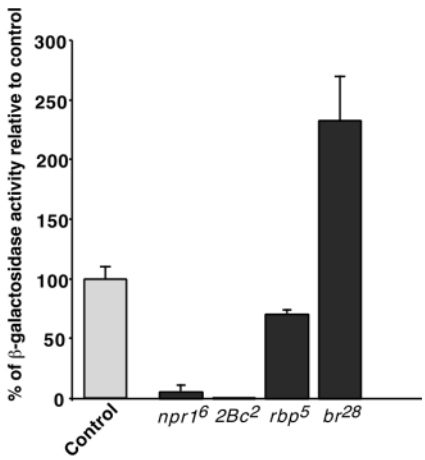


FIG. 5. Effect of *BR-C* mutations on the expression of the *AE* transgene in late third-instar larvae. AE males were crossed to females carrying a balanced *BR-C* mutation (Table 1) to generate male progeny hemizygous for the *BR-C* mutation and heterozygous for the *AE* construct. In each case β-galactosidase activity was assayed on at least five independent batches of three late third-instar larvae at the clear gut stage (i.e., 114–119 h AEL) and the mean was expressed as a percentage of β-galactosidase activity measured in the corresponding heterozygous females (+/*BR-C*$^-$; +/*AE)* (Control). Error bars represent the standard error of the mean.

stage, Z3 appears at mid-third instar, while Z1 and Z4 do not appear until the end of larval development. The *AE* construct's requirement for different *BR-C* functions at different times in third instar development, as tested with *BR-C* loss-of-function mutations and overexpression of individual isoforms, demonstrates that this temporal pattern can be correlated with the regulation of an ecdysone target gene specifically expressed in the fat body tissue.

Activator Role for Z1, Z3, and Z4

The strong negative effect of the *2Bc*^{2} mutation on *AE* expression and the 33% rescue by overexpression of the Z3 isoform in a *BR-C* null genetic context are strong indications that the Z3 isoform is required for activation of the reporter construct. The moderate effect of the *rbp*^{5} mutation suggests that the Z1 isoform is not essential for *AE* expression. This result is in contrast with the strong rescue observed by overexpression of Z1 in the *BR-C* null context. However, the partial functional redundancy between Z1 and Z4 reported previously (Bayer *et al.*, 1997; Sandstrom *et al.*, 1997) and correlated with the high sequence similarity between the Z4 and the Z1 zinc-finger regions could mask the actual effect of the loss of Z1 in the *rbp*^{5} mutant context. The possibility that Z4 may compensate partially for the lack of Z1 is supported by the strong rescue of *AE* expression upon overexpression of Z4 in the *BR-C* null context. Together these results provide further evidence that the Z4 isoform provides a *BR-C* function and suggest that it may have a specific role in the expression of the *AE* target transgene in the fat body. It is thus possible that both Z1 and Z4 are required for correct expression of the *AE* construct. Because Z4 loss-of-function mutations are not available, it is not yet possible to assess further the specific individual roles of Z1 and Z4 in this process.

The level of rescued *AE* expression following induction of Z1, Z3, or Z4 isoforms in a *BR-C* null context is partial in each case and does not exceed 68% of control larvae. A simple explanation is that longer recovery times would be actually necessary to achieve 100%. It has been reported that the increase in Hsp70 protein levels following a heat shock is delayed by several hours in the fat body, in contrast to other tissues (Krebs and Feder, 1997). However, extending the recovery period to 18 h in our experiments gave similar results (data not shown). We are thus led to propose that the simultaneous contribution of more than a single *BR-C* isoform is required to ensure full expression of the target *AE* transgene. The fact that more than one *BR-C* genetic function was required for its correct expression supports this hypothesis.

We also noticed that the degree of rescue was different for each isoform: Z1 and Z4 were able to rescue *AE* activity to higher levels than Z3 could. It is unlikely that this is due to a limiting availability of the Z3 active isoform after heat shock because Western blot analysis (data not shown) showed that overexpression led to the accumulation of a

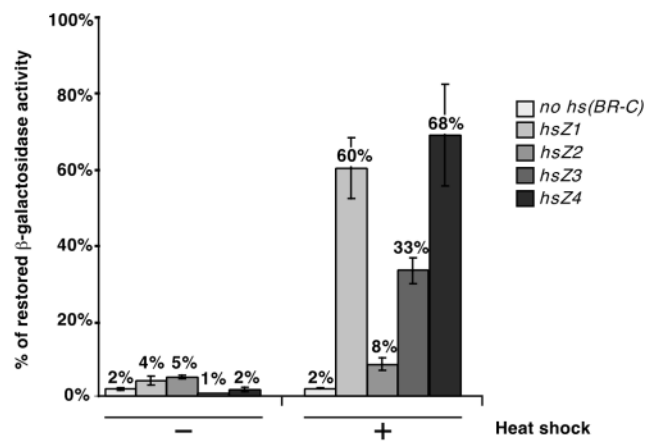


FIG. 6. Rescue of *AE* construct expression in late third-instar larvae by overexpression of *BR-C* isoforms. Light blue gut (i.e., 108–115 h AEL) *y npr1*^{6} male larvae carrying one copy of *AE* and either one copy of an *hs(BR-C)* transgene (*hsZ1*, *hsZ2*, *hsZ3*, or *hsZ4*) or no *BR-C* transgene (no *hs(BR-C)*) were heat-shocked at 37°C for 35 min and then allowed to recover at 25°C prior to harvesting 8 h later as clear gut animals. In each case, β-galactosidase activity was assayed on crude extracts of at least five pools of three heat-shocked (+) or non-heat-shocked (−) larvae and the mean was expressed as a percentage of the β-galactosidase activity measured in the corresponding *BR-C*$^+$ control larvae. Error bars represent the standard error of the mean.

high level of the corresponding protein for each *hs(BR-C)* transgene. The fact that Z3 is normally expressed earlier than Z1 and Z4 in the larval fat body leads us to propose the following hypothesis to account for these differences in rescuing activity. The early expression of the Z3 isoform may prime *AE* induction to an initial plateau level which is further raised to full activation by the later expression of Z1 and/or Z4. This would explain why overexpression of Z3 alone in the *BR-C* null context, i.e., in the absence of endogenous Z1 and Z4, leads to a limited rescue. The observation that overexpression of Z3 in a $2Bc^{1}$ genetic context, i.e., in the absence of functional Z3 and presence of endogenous Z1 and Z4 (unpublished data), leads to a higher level of rescue (63%) than in the *BR-C* null context supports this hypothesis (data not shown). Overexpression of either Z1 or Z4 alone, in the *BR-C* null genetic context, leads to a 60–68% rescue, indicating that these isoforms can act without previous Z3 expression and that their independent overexpression bypasses its absence. The observation that overexpression of either Z1 or Z4 in the $2Bc^{1}$ mutant context leads to 100% rescue confirms this point and indicates that Z1 and Z4 act synergistically to achieve the full expression of the *AE* construct (data not shown).

A Repressor Role for Z2

The finding that overexpressed Z2 is unable to rescue *AE* activity in a $npr1^{6}$ genetic context indicates that either Z2 is not normally required for induction of *AE* or it is not active on this construct. However, the significant elevation of *AE* expression in the absence of br^{+} function indicates that br^{+}, i.e., Z2, has a repressor effect on the expression of this construct. The strong decrease observed in the level of *AE* expression resulting from the continuous overexpression of the Z2 isoform during the second half of the third instar (Fig. 7) and the facilitation by the br^{28} genetic context of the precocious expression of the *AE* construct by premature induction of *hsZ3* (Fig. 8D) strongly support a repressor function for Z2. However, we observed no precocious expression of the *AE* construct in the absence of functional Z2 in the br^{28} context (data not shown) or in the br^{28}/Y; *hsZ3/+; AE/+* context prior to heat shock (Fig. 8D). These results indicate that the absence of the Z2 repressor is not sufficient by itself to allow the expression of the *AE* construct and support the essential requirement for Z3, Z1, and Z4 as activators in this process.

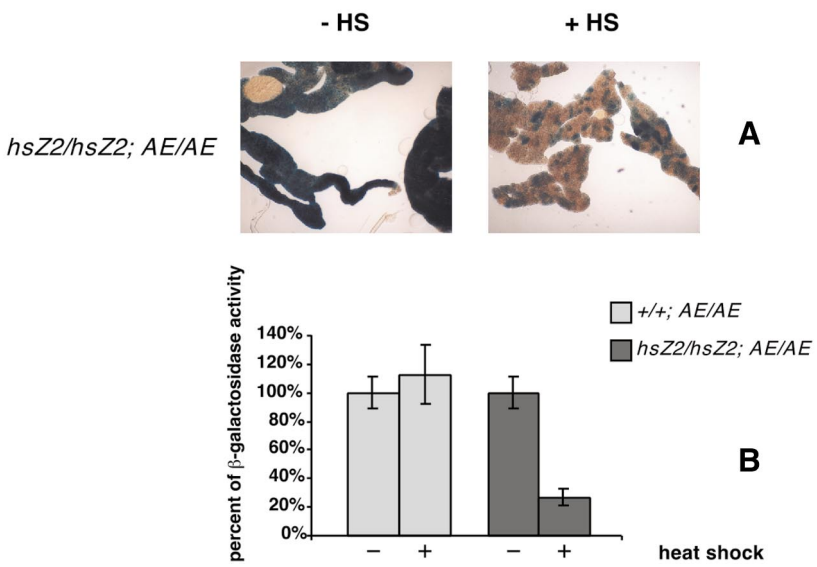
Interestingly, repression of target genes by *BR-C* has been observed previously as well. Karim *et al.* (1993) have shown that the $2Bc^{+}$ function is required for the repression of the 71E gene VII and *Sgs-3*, *Sgs-4*, and *Sgs-5* salivary gland-specific genes at the prepupal stage. The *Sgs-4* gene is repressed by overexpression of Z3 (Bayer *et al.*, 1996) and *L71* genes by Z3 and Z4 (Crossgrove *et al.*, 1996). The component of the *Ddc* gene expressed in the epidermis in late third-instar larvae and white prepupae (Andres *et al.*, 1993; Scholnick *et al.*, 1983) is repressed by induction of *hsZ1* and *hsZ4* in wild-type animals (Bayer *et al.*, 1996). Expression of the *Brg-P9* gene in imaginal discs at puparium formation is repressed by br^{+}/Z2 function (Bayer *et al.*, 1996). The observation that Z2 is expressed in larval tissues that survive metamorphosis (e.g., the ring of diploid imaginal cells at the base of the salivary glands and the small islands of diploid imaginal cells in the larval midgut) (Mugat *et al.*, in preparation) suggests that this isoform could also be involved in the repression of the genes that direct the death response.

BR-C Isoforms Are Involved in the Temporal but Not in the Spatial Control of AE Expression

Together with the sequential appearance of the Z2, Z3, Z1, and Z4 isoforms, our finding that overexpression of the Z3 isoform at the beginning of the third instar in a $BR\text{-}C^{+}$ context results in a premature expression of *AE* with tissue-specific fidelity provides evidence that the *BR-C* is implicated in the temporal regulation of the activation of the *Fbp1* enhancer in the *AE* construct. This leads us to integrate our data into a tentative model of *BR-C* protein action in the regulation of the *AE* construct in the fat body during the third instar (Fig. 9). We propose that at the beginning of the third instar, the predominant Z2 isoform acts as a repressor of *AE* expression. At mid-third instar Z3 may both antagonize Z2 repression and initiate activation of the construct that is then up-regulated to full levels in late third instar by the Z4 and/or Z1 isoforms. It should be noted, however, that overexpression of Z3 did not induce any precocious *AE* activation earlier than 12 h before the normal onset of expression of the construct (data not shown) and that this premature level of expression remained low (Fig. 8A). This strongly suggests that the correct timing of *AE* expression involves additional temporally regulated transactivating factors. One such factor could be the ecdysone receptor itself. We had demonstrated earlier that the EcR/USP ecdysone receptor acts as a hormone-dependent timer for the activation of the *Fbp1* enhancer (Brodu *et al.*, 1999). Along the same line, Schubiger and Truman (2000) have presented recently a model for silencing of hormone-responsive genes at mid-third instar by the ligand-free ecdysone receptor that is released by the rise of the hormone titer. Thus, we propose that the low titer in ecdysone in mid-third instar limits the response of *AE* to Z3 both under normal physiological conditions and in the context of Z3 overexpression (Fig. 9).

It is interesting to note, in this context, that a cooperation of the *BR-C* proteins with stage-specific regulators has been proposed previously by Jiang *et al.* (1997) for the appropriate induction of *reaper* (*rpr*) and *head involution defective* (*hid*) cell death genes in larval tissues that are fated to die.

No ectopic expression of the *AE* construct was detected following ubiquitous overexpression of individual *BR-C* isoform in $npr1^{6}$ or $BR\text{-}C^{+}$ animals after heat shock. The lack of induction of the construct in tissues other than the fat body suggests that one or several fat body-specific factors are required for *AE* expression in addition to the



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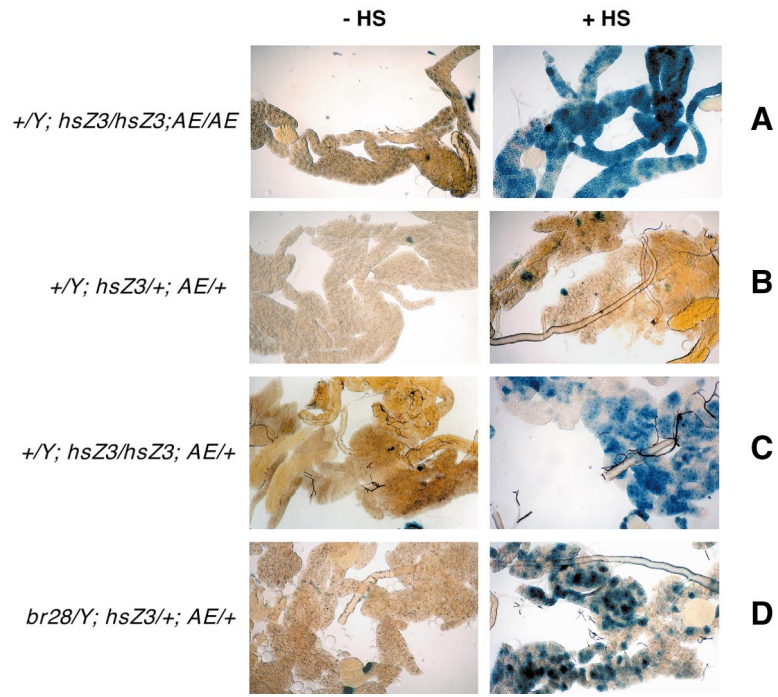

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FIG. 7. Effect of Z2 overexpression on *AE*. AEhsZ2 larvae were heat-shocked (+HS) at 37°C for 35 min at 96 h and again at 100 h of development after egg-laying and allowed to recover for 18 h at 25°C before assaying for β-galactosidase activity by histochemical staining (A) or CPRG spectrophotometric assay (B). As a control, the AE line was treated identically in parallel. (B) Results are expressed in % of β-galactosidase activity measured in non-heat-shocked larvae.

FIG. 8. Temporal deregulation of *AE*. Larvae of the indicated genotypes were staged and separated into two batches. One group was heat-shocked (+HS) at 73 h and again at 90 h of development AEL for 35 min at 37°C and returned to 25°C, the other was not subjected to heat shock (−HS) as a control and maintained at 25°C. Larvae from the two groups were dissected at 96 h AEL and tissues stained for β-galactosidase activity.

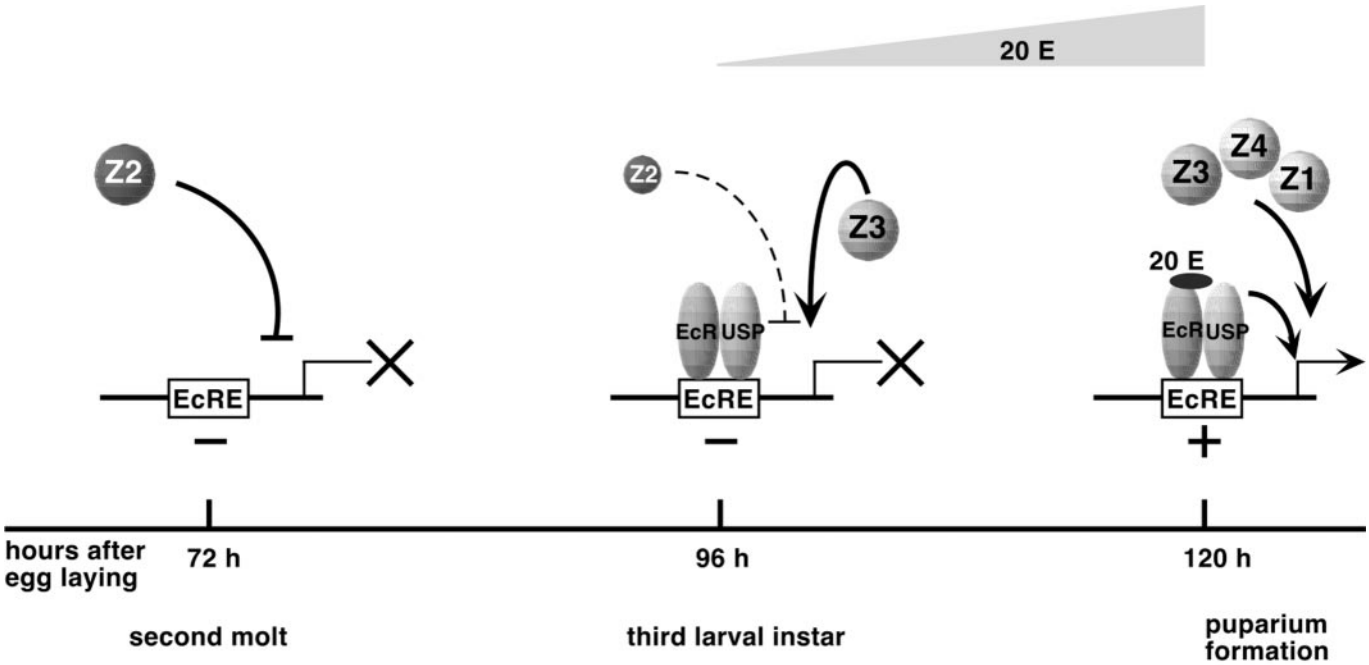

FIG. 9. Tentative model for repression and activation of the *Fbp1* enhancer in the *AE* construct by the *BR-C* isoforms during the third larval instar. In early third instar, Z2 maintains an inactivated state of the enhancer. In mid-third instar, Z3 antagonizes Z2 repression and primes enhancer activation. However, because of the presence of the ligand-free EcR/USP ecdysone receptor bound to the ecdysone response element (EcRE), the expression of the *AE* construct remains suppressed. As the hormone titer rises in late third instar, the ligand-bound receptor no longer silences and together with the Z1, Z3, and Z4 *BR-C* isoforms activates transcription.

BR-C proteins. This is in agreement with our demonstration that the dGATAb/serpent factor plays an indispensable role in the fat body-specific activation of the *Fbp1* enhancer (Brodu *et al.*, 1999; Brodu *et al.*, in preparation).

Mode of Action of the BR-C Proteins

To date the molecular mechanisms of action of the *BR-C* proteins remain unclear. The presence of the zinc-finger motifs suggests that their action is mediated by direct sequence-specific interactions with DNA. Several studies based on the use of recombinant *BR-C* isoforms produced in *E. coli* for *in vitro* footprinting experiments reported a direct *in vitro* binding of these proteins to target sequences in promoter regions of the *BR-C*-regulated *Ddc* (Hodgetts *et al.*, 1995), *Sgs-4* (von Kalm *et al.*, 1994), and *L71* (Crossgrove *et al.*, 1996) genes. Consensus binding sequences for *BR-C* proteins were deduced from these studies but their functional significance has not been tested thoroughly in a transgenic assay. In contrast, comparative mapping of DNase I hypersensitive sites in the vicinity of the small *heat shock protein* genes in salivary glands of wild-type and *BR-C* mutant third-instar larvae led Dubrovsky *et al.* (1994) to suggest that *BR-C* proteins may act more indirectly by opening chromatin to allow efficient binding of DNA by other *trans*-acting factors. In addition, the possibility that the *BR-C* proteins may require the interaction with other proteins for binding to their target sites, *in vivo*, was raised by Lehmann and Korge (1996).

We were unable to observe direct binding of *BR-C* isoforms to *Fbp1* regulatory sequences using larval fat body nuclear extracts (unpublished data). Yet, two observations strongly suggest that the zinc-finger motifs of the *BR-C* proteins are essential for their function. We report here that a truncated Z2 protein product is present throughout the third larval instar in the fat body of br^{28} mutant larvae (Fig. 4J′). The level of *AE* expression is higher in these mutant larvae than in wild-type larvae, which shows that this mutant protein devoid of zinc fingers has lost the repressor activity of the intact Z2 isoform. In addition, a transgenic line containing a Z4 cDNA lacking the zinc-finger region and placed under the control of the hsp70 promoter is unable to rescue *AE* expression in a $npr1^{6}$ genetic context after heat shock (unpublished data).

Regardless of the nature of the molecular interactions of the *BR-C* proteins with chromatin and/or DNA, it is very likely that their activity is modulated by interactions with other *trans*-acting factors as suggested by Lehmann and Korge (1996). The presence of the BTB/POZ domain of protein–protein interaction in the N-terminal region of the core domain common to all isoforms supports this assumption. Additional evidence is provided by the fact that the

individual isoforms can exert opposite effects on different target genes; e.g., Z3 and Z4 are able to activate *AE* in the larval fat body while they can mediate the repression of the *L71* genes in larval salivary glands as shown by Crossgrove *et al.* (1996). Similarly Z2 is unable to activate *AE* in the fat body in our rescue assay while Bayer *et al.* (1997) have shown that it can partially rescue the expression of the *Fbp2* gene in the same tissue in a $2Bc^{1}$ genetic background. How much of these interactions are target-, stage-, and tissue-specific remains to be more thoroughly determined. Identification of protein partners of the *BR-C* proteins would be a major step toward the understanding of the mode of action of these ubiquitous *trans*-acting regulators of the ecdysone response and their integration in the molecular complexes that recruit the stage- and tissue-specific transcription factors that direct the appropriate expression of hormone-regulated genes.

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REFERENCES

Andres, A. J., Fletcher, J. C., Karim, F. D., and Thummel, C. S. (1993). Molecular analysis of the initiation of insect metamorphosis: A comparative study of *Drosophila* ecdysteroid-regulated transcription. *Dev. Biol.* **160,** 388–404.

Andres, A. J., and Thummel, C. S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol.* **44,** 565–573.

Andres, A. J., and Thummel, C. S. (1995). The *Drosophila* 63E early puff contains *E63-1,* an ecdysone-inducible gene that encodes a novel Ca^{2+} binding protein. *Development* **121,** 2667–2679.

Antoniewski, C., Mugat, B., Delbac, F., and Lepesant, J. A. (1996). Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster. Mol. Cell. Biol.* **16,** 2977–2986.

Ashburner, M., Chihara, C., Meltzer, P., and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38,** 655–662.

Bainbridge, S. P., and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster. J. Embryol. Exp. Morphol.* **66,** 57–80.

Bardwell, V. J., and Treisman, R. (1994). The POZ domain: A conserved protein–protein interaction motif. *Genes Dev.* **8,** 1664–1677.

Bayer, C. A., Holley, B., and Fristrom, J. W. (1996). A switch in *Broad-Complex* zinc-finger isoform expression is regulated post-transcriptionally during the metamorphosis of *Drosophila* imaginal discs. *Dev. Biol.* **177,** 1–14.

Bayer, C. A., von Kalm, L., and Fristrom, J. W. (1997). Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the *Broad-Complex* during *Drosophila* metamorphosis. *Dev. Biol.* **187,** 267–282. [Published erratum appears in *Dev. Biol.,* 1997, **191,** 311–312]

Belyaeva, E. S., Aizenzon, M. G., Semeshin, V. F., Kiss, I. I., Koczka, K., Baritcheva, E. M., Gorelova, T. D., and Zhimulev, I. F. (1980). Cytogenetic analysis of the 2B3-4–2B11 region of the X-chromosome of *Drosophila melanogaster.* I. Cytology of the region and mutant complementation groups. *Chromosoma* **81,** 281–306.

Belyaeva, E. S., Vlassova, I. E., Biyasheva, Z. M., Kakpakov, V. T., Richards, G., and Zhimulev, I. F. (1981). Cytogenetic analysis of the 2B3-4–2B11 region of the X chromosome of *Drosophila melanogaster.* II. Changes in 20-OH ecdysone puffing caused by genetic defects of puff 2B5. *Chromosoma* **84,** 207–219.

Brodu, V., Mugat, B., Roignant, J. Y., Lepesant, J. A., and Antoniewski, C. (1999). Dual requirement for the EcR/USP nuclear receptor and the dGATAb factor in an ecdysone response in *Drosophila melanogaster. Mol. Cell. Biol.* **19,** 5732–5742.

Burtis, K. C., Thummel, C. S., Jones, C. W., Karim, F. D., and Hogness, D. S. (1990). The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. *Cell* **61,** 85–99.

Chao, A. T., and Guild, G. M. (1986). Molecular analysis of the ecdysterone-inducible 2B5 "early" puff in *Drosophila melanogaster. EMBO J.* **5,** 143–150.

Crossgrove, K., Bayer, C. A., Fristrom, J. W., and Guild, G. M. (1996). The *Drosophila Broad-Complex* early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade. *Dev. Biol.* **180,** 745–758.

DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W., and Guild, G. M. (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* **129,** 385–397.

Dubrovsky, E. B., Dretzen, G., and Bellard, M. (1994). The *Drosophila Broad-Complex* regulates developmental changes in transcription and chromatin structure of the 67B heat-shock gene cluster. *J. Mol. Biol.* **241,** 353–362.

Emery, I. F., Bedian, V., and Guild, G. M. (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* **120,** 3275–3287.

Fristrom, D. K., Fekete, E., and Fristrom, J. W. (1981). Imaginal disc development in a non-pupariating lethal mutant in *Drosophila melanogaster. Wilhelm Roux's Arch. Dev. Biol.* **190,** 11–21.

Guay, P. S., and Guild, G. M. (1991). The ecdysone-induced puffing cascade in *Drosophila* salivary glands: A *Broad-Complex* early gene regulates intermolt and late gene transcription. *Genetics* **129,** 169–175.

Hodgetts, R. B., Clark, W. C., O'Keefe, S. L., Schouls, M., Crossgrove, K., Guild, G. M., and von Kalm, L. (1995). Hormonal induction of Dopa decarboxylase in the epidermis of *Drosophila* is mediated by the *Broad-Complex. Development* **121,** 3913–3922.

Huet, F., Ruiz, C., and Richards, G. (1993). Puffs and PCR: The *in vivo* dynamics of early gene expression during ecdysone responses in *Drosophila. Development* **118,** 613–627.

Jiang, C., Baehrecke, E. H., and Thummel, C. S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* **124,** 4673–4683.

Karim, F. D., Guild, G. M., and Thummel, C. S. (1993). The *Drosophila Broad-Complex* plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Development* **118,** 977–988.

Karim, F. D., and Thummel, C. S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J.* **11,** 4083–4093.

Kiss, I., Beaton, A. H., Tardiff, J., Fristrom, D., and Fristrom, J. W. (1988). Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* **118,** 247–259.

Kiss, I., Bencze, G., Fodor, G., Szabad, J., and Fristrom, J. W. (1976). Prepupal larval mosaics in *Drosophila melanogaster*. *Nature* **262,** 136–138.

Kiss, I., Szabad, J., and Major, J. (1978). Genetic and developmental analysis of puparium formation in *Drosophila melanogaster*. *Mol. Gen. Genet.* **164,** 77–83.

Krebs, R. A., and Feder, M. E. (1997). Tissue-specific variation in Hsp70 expression and thermal damage in *Drosophila melanogaster* larvae. *J. Exp. Biol.* **200,** 2007–2015.

Lapie, P., Nasr, F., Lepesant, J. A., and Deutsch, J. (1993). Deletion scanning of the regulatory sequences of the *Fbp1* gene of *Drosophila melanogaster* using P transposase-induced deficiencies. *Genetics* **135,** 801–816.

Laval, M., Pourrain, F., Deutsch, J., and Lepesant, J. A. (1993). *In vivo* functional characterization of an ecdysone response enhancer in the proximal upstream region of the *Fbp1* gene of *D. melanogaster*. *Mech. Dev.* **44,** 123–138.

Lehmann, M., and Korge, G. (1996). The fork head product directly specifies the tissue-specific hormone responsiveness of the *Drosophila Sgs-4* gene. *EMBO J.* **15,** 4825–4834.

Lepesant, J. A., Levine, M., Garen, A., Lepesant-Kejzlarvoa, J., Rat, L., and Somme-Martin, G. (1982). Developmentally regulated gene expression in *Drosophila* larval fat bodies. *J. Mol. Appl. Genet.* **1,** 371–383.

Lepesant, J.-A., Maschat, F., Kejzlarova-Lepesant, J., Benes, H., and Yanicostas, C. (1986). Developmental and ecdysteroid regulation of gene expression in the larval fat body of *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol.* **Suppl. 1,** 133–141.

Lindsley, D. L., and Zimm, G. G. (1992). "The Genome of *Drosophila melanogaster*." Academic Press, San Diego.

Maschat, F., Dubertret, M. L., Therond, P., Claverie, J. M., and Lepesant, J. A. (1990). Structure of the ecdysone-inducible *P1* gene of *Drosophila melanogaster*. *J. Mol. Biol.* **214,** 359–372.

Murphy, C., Fristrom, J. W., and Shearn, A. (1977). Aspects of development of imaginal discs in a non-pupariating lethal mutant in *Drosophila melanogaster*. *Cell Differ.* **6,** 319–330.

Restifo, L. L., and Merrill, V. K. (1994). Two *Drosophila* regulatory genes, *deformed* and the *Broad-Complex,* share common functions in development of adult CNS, head, and salivary glands. *Dev. Biol.* **162,** 465–485.

Restifo, L. L., and White, K. (1991). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of the central nervous system in *Drosophila*. *Dev. Biol.* **148,** 174–194.

Restifo, L. L., and White, K. (1992). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila:* Salivary glands, muscle, and gut. *Wilhelm Roux's Arch. Dev. Biol.* **201,** 221–234.

Riddiford, L. M. (1993). Hormones and *Drosophila* development. *In* (M. A. Bate, Ed.), pp. 899–939.

Russell, S. R., Heimbeck, G., Goddard, C. M., Carpenter, A. T., and Ashburner, M. (1996). The *Drosophila Eip78C* gene is not vital but has a role in regulating chromosome puffs. *Genetics* **144,** 159–170.

Sandstrom, D. J., Bayer, C. A., Fristrom, J. W., and Restifo, L. L. (1997). *Broad-Complex* transcription factors regulate thoracic muscle attachment in *Drosophila*. *Dev. Biol.* **181,** 168–185.

Scholnick, S. B., Morgan, B. A., and Hirsh, J. (1983). The cloned dopa decarboxylase gene is developmentally regulated when reintegrated into the *Drosophila* genome. *Cell* **34,** 37–45.

Schubiger, M., and Truman, J. W. (2000). The RXR ortholog USP suppresses early metamorphic processes in *Drosophila* in the absence of ecdysteroids. *Development* **127,** 1151–1159.

Segraves, W. A., and Hogness, D. S. (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4,** 204–219.

Stewart, M., Murphy, C., and Fristrom, J. W. (1972). The recovery and preliminary characterization of X chromosome mutants affecting imaginal discs of *Drosophila melanogaster*. *Dev. Biol.* **27,** 71–83.

Thummel, C. S. (1990). Puffs and gene regulation—Molecular insights into the *Drosophila* ecdysone regulatory hierarchy. *BioEssays* **12,** 561–568.

Thummel, C. S. (1996). Flies on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12,** 306–310.

von Kalm, L., Crossgrove, K., Von Seggern, D., Guild, G. M., and Beckendorf, S. K. (1994). The *Broad-Complex* directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J.* **13,** 3505–3516.

Zhimulev, I. F., Vlassova, I. E., and Belyaeva, E. S. (1982). Cytogenetic analysis of the 2B3-4–2B11 region of the X chromosome of *Drosophila melanogaster*. III. Puffing disturbance in salivary gland chromosomes of homozygotes for mutation l(1)pp1t10. *Chromosoma* **85,** 659–672.

Zollman, S., Godt, D., Prive, G. G., Couderc, J. L., and Laski, F. A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **91,** 10717–10721.

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