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The *Drosophila Broad-Complex* Early Gene Directly Regulates Late Gene Transcription during the Ecdysone-Induced Puffing Cascade

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The ensemble of tissue-specific changes that drives *Drosophila* metamorphosis is initiated by the steroid hormone ecdysone and proceeds through a transcriptional cascade comprised of primary response transcriptional regulators and secondary response structural genes. The *Broad-Complex* (*BR-C*) primary response early gene is composed of several distinct genetic functions and encodes a family of related transcription factor isoforms. Our objective in this study was to determine whether individual BR-C isoforms directly regulate secondary response target genes. A cluster of 10 salivary gland-specific secondary response *L71* late genes are dependent on the *BR-C rbp*$^+$ genetic function. Transgenic animals expressing individual BR-C isoforms were tested for their ability to provide the *BR-C rbp*$^+$ genetic function by monitoring the transcriptional activation of the *L71* genes. We found that the BR-C Z1 isoforms could complement the transcriptional defects seen in *rbp* mutants but the Z2, Z3, and Z4 isoforms could not. We conclude that the *BR-C rbp*$^+$ function is provided by the BR-C Z1 isoform in prepupal salivary glands. *L71* gene rescue was restricted to the prepupal salivary gland, suggesting the involvement of additional factors in *L71* gene regulation. Interestingly, we found that the overexpression of Z3 or Z4 isoforms in *BR-C*$^+$ salivary glands repressed *L71* expression, indicating that BR-C proteins might also function as transcriptional repressors. Molecular mapping and characterization of the regulatory sequences that control *L71-6* expression revealed several Z1 isoform binding sites. Mutagenesis of these Z1 binding sites resulted in the failure to activate late gene expression *in vivo* when measured by transgenic reporter genes. We conclude that the *BR-C* early gene directly activates late gene transcription by interacting with late gene *cis*-acting regulatory elements and that this interaction is responsible for the temporal linkage of early and late ecdysone-induced gene expression.

INTRODUCTION

Steroid hormones regulate transcriptional programs that control developmental processes in most, if not all, multicellular organisms. In *Drosophila,* the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) initiates developmental changes throughout larval and pupal life (reviewed in Riddiford, 1993). In the most dramatic example, an increase in the titer of ecdysone at the end of the third larval instar induces a cascade of gene expression that culminates in the metamorphosis of the larval form of an insect into an adult.

The transcriptional events associated with the onset of metamorphosis have been extensively studied. Observations of the puffing patterns of polytene chromosomes *in vivo* and in cultured salivary glands (summarized in Ashburner *et al.,* 1974) suggested a model of ecdysone-regulated gene expression. Briefly, as the hormone titer increases at the end of third instar development, ecdysone, complexed with a receptor, activates a small set of primary response ''early'' genes that encode transcriptional regulatory proteins. These genes transduce and amplify the original hormonal signal by activating a large number of secondary response ''late'' genes that are thought to encode tissue-specific effector proteins necessary for the developmental events that drive metamorphosis. The early gene products also negatively autoregulate their own expression, thus attenuating the response. An additional level of control is

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provided by the ecdysone–receptor complex, which prevents premature late gene expression until sufficient early gene products accumulate.

The molecular characterization of several early genes provides support for the Ashburner model. The *Broad-Complex* (*BR-C*), *E74*, and *E75* genes are all large (60–100 kb) ecdysone-inducible genes driven by multiple promoters (Burtis *et al.*, 1990; Thummel *et al.*, 1990; Segraves and Hogness, 1990; DiBello *et al.*, 1991; Bayer *et al.*, 1996). Alternatively spliced transcripts from each gene encode families of DNA-binding protein isoforms. These genes are expressed throughout the organism during metamorphosis, with an isoform-specific tissue distribution (Boyd *et al.*, 1991; Huet *et al.*, 1993; Emery *et al.*, 1994) suggesting that early gene isoform combinations can coordinately prescribe tissue-specific gene expression programs during metamorphosis (Burtis *et al.*, 1990; Thummel *et al.*, 1990).

The *BR-C*, located at cytogenetic position 2B5 on the X-chromosome (Belyaeva *et al.*, 1980), encodes a family of protein isoforms that share an identical N-terminal 431-amino-acid region (BRcore) and are distinguished by the presence of one of four pairs of C_2H_2 zinc fingers (Z1, Z2, Z3, and Z4) (DiBello *et al.*, 1991; Bayer *et al.*, 1996) which function in site-specific DNA recognition and binding (von Kalm *et al.*, 1994). The N-terminal 115 amino acids constitute a BTB, or POZ, domain conserved among many zinc finger-containing transcription factors, including those encoded by the *Drosophila bric-a-brac*, *tramtrack*, and *GAGA* factor genes (Zollman *et al.*, 1994). This domain can function as a protein–protein interaction domain and, in some cases, appears to reduce the affinity of associated zinc fingers for DNA (Bardwell and Treisman, 1994; Chen *et al.*, 1995). Some BR-C isoforms contain domains between the BRcore and zinc finger regions that are enriched in specific amino acids (e.g., Q, TNT, NS). Domains such as these can be found in transcription factors and coactivators (Mitchell and Tijan, 1989; Colgan *et al.*, 1993; Hoey *et al.*, 1993). Thus, the BR-C proteins contain site-specific DNA-binding domains, protein–protein interaction domains, and potential transcriptional activation domains, all consistent with their functioning as transcriptional regulatory proteins.

The transcriptional and coding complexity of the *BR-C* is reflected by its genetics in that it has three complementing, genetically defined functions: *reduced bristles on the palpus* (*rbp*), *broad* (*br*), and *2Bc*. In addition, a nonpupariating class of alleles (*npr1*) appears to be deficient in all three functions. Because *npr1* mutant animals are unable to initiate metamorphosis and die as wandering third instar larvae (Stewart *et al.*, 1972; Belyaeva *et al.*, 1980; Kiss *et al.*, 1988) these mutants demonstrate that the *BR-C* is essential for metamorphosis. Most loss-of-function *rbp*, *br*, and *2Bc* alleles result in lethality somewhat later during metamorphosis and exhibit developmental defects that represent subsets of those seen in *npr1* mutants (Belyaeva *et al.*, 1980; Kiss *et al.*, 1988; Restifo and White, 1991, 1992). In addition, studies showing that *BR-C* defects cannot be rescued by exogenous ecdysone (Kiss *et al.*, 1978) and that the *BR-C* functions in a cell-autonomous manner (Vijay Raghaven *et al.*, 1988) lend further support for the role of these proteins as transcriptional regulators acting downstream of ecdysone.

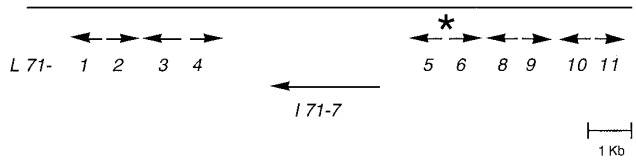


FIG. 1. The *Drosophila* 71E gene cluster. A 14-kb genomic region containing 11 developmentally regulated genes from the 71E salivary gland late puff is shown (Wright *et al.*, 1996). The transcript limits and orientations (5′ → 3′) of 10 late genes expressed during the prepupal stage (prefix: *L71-*) and 1 intermolt gene expressed during the late third instar stage (*I 71-7*) are indicated. The intragenic region located between the *L71-5* and *L71-6* genes and used in DNA-binding and transgenic experiments is indicated by an asterisk.

The *BR-C* functions as an ecdysone primary response gene (Chao and Guild, 1986; Karim and Thummel, 1992) and is essential for transducing the ecdysone signal to secondary response late genes. In particular, the rbp^+ function is required for activation of late gene transcription (Guay and Guild, 1991; Karim *et al.*, 1993). Very little is known about the mechanisms by which early gene products regulate late gene expression. Such evidence is crucial for a mechanistic understanding of the ecdysone-regulated puffing cascade during early metamorphosis, in particular, and steroid hormone-regulated genetic cascades, in general.

We have characterized a cluster of 10 coordinately regulated salivary gland-specific late genes located in the 71E cytogenetic region on the third chromosome (Restifo and Guild, 1986a; Wright *et al.*, 1996). The *L71* genes are arranged as divergently transcribed pairs and encode a family of related proteins of unknown function (Fig. 1). In addition, an intermolt gene expressed during late third instar development, *I 71-7*, is located within the late gene cluster between *L71-4* and *L71-5*. All *L71* gene transcripts are severely downregulated in rbp^5 mutants, indicating that transcription of the *L71* genes is dependent on the *BR-C* rbp^+ function (Guay and Guild, 1991; Karim *et al.*, 1993). In *2Bc* mutants, transcription of individual *L71* genes is slightly reduced, delayed, and prematurely repressed to varying degrees. However, this may be a secondary response to the effects of *2Bc* mutants on *E74A* transcription (Karim *et al.*, 1993) since similar effects are seen in *E74A* mutants (Fletcher and Thummel, 1995a). As expected, no *L71* gene expression is detected in $npr1^3$ mutants, which lack all *BR-C* functions (Karim *et al.*, 1993).

We provide evidence for the direct interaction between an early gene product and a late gene. By inducing Z1 isoform expression in *rbp* mutants at the end of the third larval instar and demonstrating rescue of *L71* gene expression, we show that the BR-C Z1 isoforms can provide the rbp^+

function in prepupal salivary glands. This rescue is tissue- and stage-specific. We have also defined the regulatory elements of one *L71* gene and mapped Z1 protein binding sites. Finally, we show that mutation of these binding sites eliminates *L71*-driven reporter gene activity, demonstrating that the early gene-encoded BR-C Z1 isoform directly regulates induction of this late gene.

MATERIALS AND METHODS

Stocks, crosses, and developmental staging. The *BR-C* mutants rbp^1, rbp^4, rbp^5 (Belyaeva *et al.*, 1980), and $npr1^3$ (Kiss *et al.*, 1988) have been described (see Emery *et al.*, 1994). Mutant *BR-C* alleles were maintained in females in combination with the *Binsn* balancer X chromosome. Animals were raised on standard cornflour food (Elgin and Miller, 1978) at 25°C.

To examine the effects of various transgenes in *BR-C* mutant backgrounds, *y BR-C* mutant/*Binsn* females were mated to w^{1118} males homozygous for the transgene of interest on the second or third chromosome. *BR-C* mutant male larvae were differentiated from their $BR\text{-}C^+$ siblings on the basis of their *yellow* mouth hooks and staged as described below.

Food containing 0.05% (w/v) bromophenol blue (Maroni and Stamey, 1983) was used to stage animals during the late third instar by observing the clearing of the blue dye from the intestine during the wandering stage after the larvae stop feeding (Andres and Thummel, 1994). Larvae with completely blue guts are 12–24 hr away from pupariation (referred to in the text as −18 hr) and have not been exposed to the large pulse of ecdysone at the end of the third instar. Larvae with completely clear guts are 1–6 hr from pupariation (referred to in the text as −4 hr) and have been exposed to the large late third instar ecdysone pulse. Other animals were synchronized at the onset of pupariation by collection at the brief white prepupal stage (Bainbridge and Bownes, 1981) and allowed to develop in humid chambers at 25°C for the specified times.

Construction of transgenic animals carrying BR-C cDNAs under control of heat shock regulatory elements. *BR-C* cDNAs were cloned into the pCaSpeR-hs vector (Thummel and Pirrotta, 1992) at the *Eco*RI site as shown in Fig. 2. The cDNAs were derived from clones dm527 (BRcore-Q^1-Z1), dm708 (BRcore-TNT-Q^1-Z1), dm796 (BRcore-Z2), dm797 (BRcore-NS-Z3), and 28-I (BRcore-Z4) (DiBello *et al.*, 1991; Bayer *et al.*, 1996). P element transformation was carried out essentially as described by Rubin and Spradling (1982). The BRcore-Q^1-Z1 construct was injected into *y w;* ry^{506} Sb^1 *P{ry*$^+$ = *Δ2 − 3}99B/TM6, Ubx* embryos which contain a stable transposase source (Robertson *et al.*, 1988). Transformed animals were identified by expression of the w^+ marker gene, and lines of animals containing single insertions were generated after crossing out the transposase source and replacing the *y w* chromosome with w^{1118}. All other transgene constructs were injected into w^{1118} embryos with the pπ25.7wc helper plasmid (Karess and Rubin, 1984) as described elsewhere (C.A.B., L. von Kalm and J.W.F., submitted for publication).

To induce transgene expression, staged larvae or prepupae were placed in stoppered plastic vials and submerged for the indicated times (heat shock) in a 37°C water bath such that the water level was above the bottom of the vial stopper. Animals were immediately processed as described below or the vials were transferred to a 25°C water bath for the indicated time (recovery), followed by processing.

Construction of transgenic animals containing L71-6:lacZ reporter constructs. A wild-type *L71-6* promoter-driven *lacZ* reporter construct, *L71-6:lacZ*, was generated by modifying a construct containing 2.6 kb of *L71-6* upstream sequence (pBS6.6, Urness and Thummel, 1995). The original construct has a 3.4-kb *Sma*I–*Xba*I fragment containing the *lacZ* gene inserted into a filled-in *Hin*dIII site within a 3.2-kb *Sal*I–*Nhe*I genomic fragment containing the *L71-5* and *L71-6* genes. The *lacZ* gene fragment is inserted into the second exon of *L71-6* such that a fusion protein would be generated containing the first 23 amino acids of L71-6 protein fused in frame to β-galactosidase. The transcript generated from this construct extends into the *L71-6* sequence located downstream from the *lacZ* insert, but a stop codon in the *lacZ* insert limits the expressed protein to an N-terminal L71-6 fusion to β-galactosidase. A 421-bp *Hin*dIII–*Bam*HI fragment containing the *L71-5/6* intergenic region from the construct above was cloned into pBluescript-II (KS^+) (Stratagene) to generate clone pBS415(wt) (a generous gift of L. Urness and C. Thummel). The *Bam*HI site came from within the *lacZ* fragment. A 424-bp *Xho*I–*Bam*HI fragment from pBS415(wt) (containing the entire insert) was used to replace a 2.6-kb *Xho*I–*Bam*HI fragment (containing all of the sequence upstream of the original fusion site) from the original construct. Because the *lacZ Bam*HI site was restored, the fusion remained intact. This pBluescript-II construct contains only 225 bp upstream of the *L71-6* start site (421 bp upstream of the fusion site). A *Xho*I–*Not*I fragment containing the entire fusion construct was then cloned into pCaSpeR-4 (Thummel and Pirrotta, 1992) as shown in Fig. 7.

The mutant binding site construct, *1-3-alt/L71-6:lacZ*, was generated as follows. The wild-type 421-bp *Hin*dIII–*Bam*HI fragment (see above) was used as a template for a series of sequential PCR reactions that introduced mutations at BR-C protein binding sites 1–2 and 3. The sequences of the mutants were confirmed by fluorescent-tagged DNA sequencing (ABI). The site 1–2 and site 3 mutations were introduced using the following oligonucleotide pairs: (1-2.a) 5′-GTTGGAAACggGGAAGTGTTATGTTCCggATAGGACCACATC-3′; (1-2.b) 5′-GATGTGGTCCTATccGGAACATAACACTTCCccGTTTCCAAC-3′; (3.a) 5′-GAATCTGACCTAccGAATccGGAccGTTCATATAAAATTG-3′; and (3.b) 5′-CAATTTTATATGAACggTCCggATTCggTAGGTCAGATTC-3′.

Lowercase letters represent alterations of the wild-type sequence. A 424-bp *Xho*I–*Bam*HI fragment containing mutations in sites 1–3 was used to replace the equivalent wild-type fragment in a fusion with the *lacZ* gene in pBluescript-II to generate paaDm927. A *Xho*I–*Not*I fragment containing the entire fusion was then cloned into pCaSpeR-4 as shown in Fig. 10.

The wild-type *L71-6:lacZ* and the binding site mutant *1-3-alt/L71-6:lacZ* constructs were transformed into the *y w* and the *y w;* ry^{506} Sb^1 *P{ry*$^+$ = *Δ2 − 3}99B/TM6, Ubx* recipient strains, respectively, as described above.

Protein extraction and Western blot analysis. Whole animal protein extracts were prepared as previously described (Emery *et al.*, 1994). Samples of extract equivalent to 0.5 animals were electrophoresed on 7% polyacrylamide SDS gels (Sambrook *et al.*, 1989) and transferred to Immobilon-P membranes (Millipore) using a Genie blotter (Idea Scientific). The membranes were incubated in blocking solution (10% nonfat dried milk, 1× PBS, 0.1% Tween 20) at 4°C overnight. For detection of BR-C proteins, undiluted monoclonal anti-BRcore (25E9) hybridoma supernatant (Emery *et al.*, 1994) was incubated with the membranes at room temperature for 30–60 min. For detection of β-galactosidase, membranes were incubated with monoclonal anti-β-galactosidase antibody (Pro-

mega), diluted 1/5000 in blocking solution, at room temperature for 1 hr. Blots were washed three times (5–10 min each) in 1× PBS, 0.1% Tween 20 followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibodies (Cappel), diluted 1/8333 in blocking solution, at room temperature for 1 hr. Blots were rinsed as before and enzyme-conjugated antibody detection was achieved using the ECL kit (Amersham) and RX-ray film (Fuji).

RNA extraction and Northern blot analysis. Total RNA was extracted from whole animals and dissected tissues using phenol (Guild, 1984; Ashburner, 1989). RNA was fractionated on denaturing formaldehyde–1.4% (w/v) agarose gels as described previously (Restifo and Guild, 1986a) and transferred to GeneScreen nylon membrane (NEN/DuPont) using 20× SSPE at room temperature for >12 hr. Radiolabeled probes were prepared by random hexamer labeling of gel-purified restriction fragments. Prehybridization and hybridization were performed in hybridization buffer (Guild, 1984; Shore and Guild, 1987). Blots were prehybridized for at least 3 hr at 37°C. Radiolabeled DNA probes were hybridized to the blots (2×10^6 CPM/ml) at 37°C for at least 12 hr. To remove excess probe, the membranes were washed for 10 min at room temperature in 1% SDS, 1× SSPE, followed by three 20-min washes at 62–63°C in 0.1% SDS, 0.2× SSPE. The membranes were then exposed to X-ray film (Kodak XAR; Fuji RX) at −70°C with an intensifying screen. Probe was stripped from the blots by boiling 30 min in TE with 0.5% SDS, allowing sequential hybridization with different probes.

DNase I footprinting assays. The *BR-C*-encoded BRcore-Q^1-Z1 isoform was expressed in *Escherichia coli* using the pET-FM vector and purified as described previously (von Kalm *et al.*, 1994). Because of insolubility, purified protein was stored in buffer containing 1 *M* urea. To define Z1 binding sites at *L71-6*, a 449-bp *Eco*RI–*Sal*I fragment containing the *L71-5/6* intergenic region (*Hin*dIII fragment cloned into pBluescript-II, a generous gift of L. Urness and C. Thummel) was radiolabeled at either end and used in DNase I footprinting reactions (Heberlein *et al.*, 1985; von Kalm *et al.*, 1994) with the BR-C Z1 protein. Control reactions were also performed in which probe was incubated with buffer alone or with purified extract from *E. coli* cells containing pET-FM (no insert). In both cases, the final concentration of urea was identical to that in the Z1 reactions. All reactions contained 0.5 μg of poly(dI/dC) (1250-fold weight excess over specific DNA). To test the ability of Z1 proteins to bind altered Z1 sites, identical reactions were performed using *Eco*RI–*Sal*I fragments from the wild-type pBS415(wt) construct (see above) and from another construct containing alterations in sites 1–4 (paaDm931). To generate this construct, paaDm927 (sites 1–3 altered, see above) served as a template for PCR reactions introducing alterations at site 4 using the following oligonucleotide pair: (4.a) 5′GAAGGAGAATACTcAcTTTTATAATTCCAATCC3′ and (4.b) 5′GGATTGGAATTATAAAAgTgAGTATTCTCCTTC3′.

Lowercase letters represent alterations of the wild-type sequence. The altered sequence was confirmed by DNA sequencing. DNaseI footprinting reactions performed with paaDm927 (sites 1–3 altered) gave the same results at sites 1–3 as reactions performed with paaDm931 (sites 1–4 altered).

RESULTS

Only Z1 protein isoforms provide the BR-C rbp^+ function in vivo. *L71* late gene expression is dependent on the *BR-C* rbp^+ genetic function (Guay and Guild, 1991; Karim *et al.*, 1993). The rbp^+ function has been correlated with the BR-C Z1 isoform (Emery *et al.*, 1994). We hypothesized that if the Z1 isoform provides the rbp^+ function, then we might be able to show that this protein directly interacts with the *L71* genes. To test that the Z1 isoform alone provides the rbp^+ function, we induced expression of individual BR-C isoforms in *rbp* mutant animals to see whether any of them could restore *L71* gene expression.

Transgenic flies were constructed in which *BR-C* cDNAs were placed under control of *hsp70* regulatory elements (Fig. 2). To test whether BR-C proteins were synthesized following a brief heat shock, induction of each *hs(BR-C)* transgene in *BR-C* null ($npr1^3$) animals was monitored by Western blot analysis (Fig. 3). Assaying transgene induction in *BR-C* null animals ensures that there is no background from endogenous *BR-C* proteins (Emery *et al.*, 1994). In the case of each transgene, a single protein species of the expected size (Emery *et al.*, 1994) was induced by a 30-min heat shock and persisted for at least 6 hr after induction. On longer autoradiographic exposures, some protein accumulation was evident after 15 min of heat shock (data not shown). The newly synthesized BRcore-Q^1-Z1 ($Z1^a$) proteins decreased in electrophoretic mobility during the recovery period, suggesting a time-dependent protein modification (see Discussion). This time-dependent change also occurs following heat shock induction of proteins from all *BR-C* transgenes tested, but is only apparent when the proteins are fractionated during longer electrophoretic runs (data not shown).

Individual *BR-C* transgenes were tested for their ability to restore *L71* gene expression in *rbp* mutant backgrounds. Male rbp^5 larvae carrying single *hs(BR-C)* transgenes were collected at the end of third instar, at the time of the major premetamorphic ecdysone pulse, and heat-shocked for 30 min. We chose this developmental time for induction of transgene expression in order to mimic the ecdysone-dependent increase in *BR-C* transcript levels prior to *L71* induction (Karim and Thummel, 1992). Following 6 hr of recovery, animals were assayed for *L71* gene expression by Northern blot analysis. As expected, most animals have pupariated by 6 hr of recovery. We find that only BR-C proteins containing the Z1 zinc finger pair were able to rescue *L71* gene expression (Fig. 4A). Both Z1 isoforms tested were able to rescue *L71* gene expression, although the BRcore-Q^1-Z1 isoform ($Z1^a$) allowed *L71* transcripts to accumulate to higher levels, particularly *L71-8* and *L71-9* transcripts. The levels of *L71* transcripts induced by a $Z1^a$ transgene are comparable to *L71* transcript levels in similarly treated *BR-C*$^+$ animals carrying no transgene (e.g., Fig. 5), suggesting that $Z1^a$ induction restores *L71* gene expression to wild-type levels. Heat shock induction for as little as 15 min with a recovery period as short as 2 hr was sufficient for induction of *hs(Z1)*-driven *L71* gene expression in rbp^5 animals (data not shown).

Based on these results, we then tested whether Z1 isoform induction would rescue *L71* gene expression in other *rbp* mutant backgrounds as well. We tested the rbp^1 and rbp^4

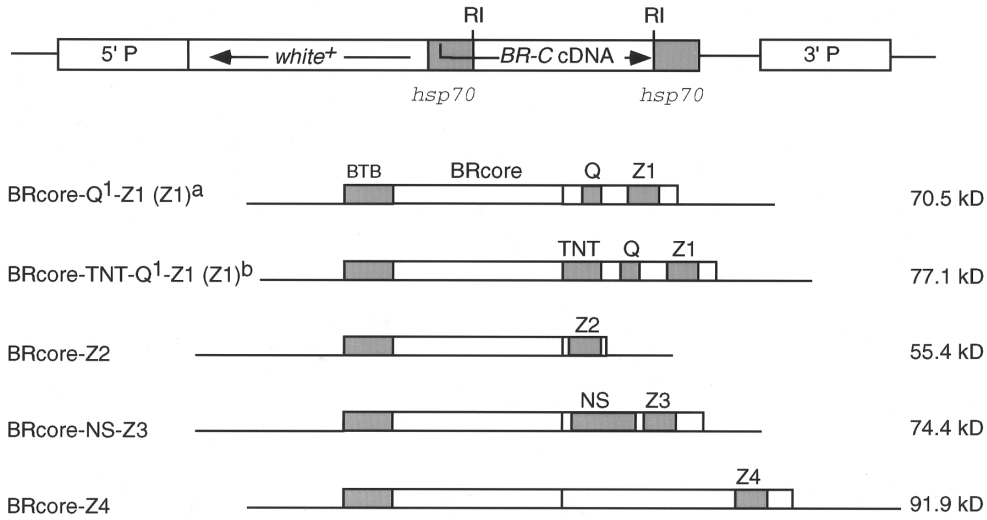


FIG. 2. Heat shock promoter-driven *BR-C* transgenes. *BR-C* cDNAs were cloned into the pCaSpeR-hs P element transformation vector (Thummel and Pirrotta, 1992) at the *Eco*RI site (RI) placing the *BR-C* cDNA under the control of the *hsp70* promoter and 3′ flanking elements. The mini-*white*$^+$ gene allows identification of transgenic animals by eye color phenotype. The 5′ and 3′ P element sequences are also shown. Individual elements in the construct are not drawn to scale. Transcriptional orientations are indicated by the arrows (5′ → 3′). Shown below are the *BR-C* cDNAs used as transgenes and their associated coding regions (boxed). All BR-C proteins contain a common 431-amino-acid BRcore region (DiBello *et al.*, 1991) characterized by the presence of an amino-terminal BTB domain (Zollman *et al.*, 1994). Each isoform is distinguished by a carboxy-terminal C_2H_2 zinc finger pair (Z1, Z2, Z3, Z4). Most isoforms also contain centrally located regions enriched in characteristic amino acids (Q, glutamine; N, asparagine; S, serine; T, threonine). The two Z1 isoforms differ by the presence of a TNT-rich domain between the BRcore and the zinc-finger domains. Calculated molecular weights of the protein isoforms are shown to the right.

hypomorphic alleles and found that Z1 isoform induction was again able to rescue *L71* gene expression (Fig. 4B). Although low levels of the *L71-3* transcripts are seen in the *rbp*^{1} control animals which do not carry a transgene (−), as expected for this weaker *rbp* allele (Guay and Guild, 1991), the level of transcripts is clearly elevated by the induction of Z1 isoform expression. As seen with *rbp*^{5} mutants, expression of Z2, Z3 and Z4 isoforms failed to rescue *L71* gene expression in *rbp*^{1} and *rbp*^{4} backgrounds (data not shown). Together, these results allow us to conclude that the BR-C Z1 isoforms provide the *rbp*$^+$ function in prepupal salivary glands.

The Z3 and Z4 protein isoforms can repress L71 gene expression. Western analyses using anti-BR-C antibody reagents show that the Z1 isoforms are the predominant BR-C proteins present in salivary glands during late third instar and early prepupal development (Emery *et al.*, 1994). These observations are consistent with our rescue results that indicate a role for the Z1 isoform in salivary gland-specific gene expression during these periods. We next investigated whether ectopic expression of the other BR-C isoforms, normally expressed at very low levels in salivary glands, would have any effect on *L71* gene expression. In particular, the Z3 and Z4 isoforms have been implicated in salivary gland gene regulation during the mid–late third instar (von Kalm *et al.*, 1994), prior to *L71* gene expression. We tested late third instar male *BR-C*$^+$ larvae expressing induced *hs(BR-C)* transgenes (*Binsn* siblings of the *rbp*^{5} mutants shown in Fig. 4A) for changes in *L71* gene expression. We found that *L71* gene transcript levels in *BR-C*$^+$ control animals which do not carry a transgene (−), were similar to the levels detected in *BR-C*$^+$ animals expressing induced Z1 and Z2 transgenes (Fig. 5). In contrast, induction of the Z3 or Z4 transgenes caused a reduction in *L71* gene expression. Thus, the low level of Z3 and Z4 expression in mid–late third instar salivary glands may be responsible for repression of *L71* gene expression prior to Z1-mediated induction.

Furthermore, we found that the extent of this reduction exhibited gene pair specificity but varied with different gene pairs. For example, *L71-1/2*, *L71-3/4*, and *L71-5/6* gene pairs showed severe, moderate, and little reduction in transcript accumulation, respectively, when the Z3 or the Z4 transgenes were induced. Note that ectopic expression of the Z3 isoform appears to repress *L71* expression in a *BR-C*$^+$ background to a greater degree than expression of the Z4 isoform. The size of the *L71* transcripts appears to be slightly different between the flies carrying Z3 and Z4 transcripts, most noticeably for *L71-1*. This may be due to shortening of the poly(A) tail, which is known to occur in *L71* transcripts (Restifo and Guild, 1986b). Interestingly, *L71-8* and *L71-9* transcript levels were very low in all *BR-C*$^+$ animals tested (data not shown). In wild-type (Ore-R) animals *L71-8* and *L71-9* are induced to the high levels characteristic

of all of the other *L71* genes (Wright *et al.*, 1996). The expression pattern seen here may be a response to the heat shock regimen, since *L71-8* and *L71-9* gene expression is low even in the absence of a transgene. Alternatively, *L71-8* and *L71-9* transcript levels may be reduced in these hemizygous *Binsn* animals.

Rescue of L71 gene expression by the Z1 isoform is salivary gland-specific. Developmentally regulated *L71* gene expression is salivary gland-specific and restricted to the prepupal period (Restifo and Guild, 1986a). If the Z1 isoform determines the tissue specificity of *L71* gene expression, then heat-induced *hs(Z1)* expression in any tissue may cause ectopic *L71* induction in tissues other than salivary glands. We tested this possibility by assaying *L71* gene expression in both salivary glands and non-salivary gland tissue after heat shocking rbp^5 larvae carrying a *hs(Z1)* transgene (Fig. 6A). We found that when the Z1 transgene was induced, *L71* transcripts were seen in both whole animals and in salivary glands. However, *L71* transcripts were not detected in carcasses after the salivary glands were removed, nor in control animals which do not carry a transgene. These results indicate that rescue of *L71* gene expression is salivary gland-specific and cannot be driven in other tissues by ectopic *BR-C* expression.

Early expression of the Z1 isoform does not cause early L71 gene expression. Because *L71* gene expression is temporally restricted to the prepupal period in wild-type animals (Restifo and Guild, 1986a; Guay and Guild, 1991; Karim *et al.*, 1993; Andres *et al.*, 1993), we tested whether *L71* gene rescue is temporally restricted. Male rbp^5 larvae carrying a *hs(Z1)* transgene were collected either before (−18 hr) or after (−4 hr) the large pulse of ecdysone at the end of the third instar period, as well as at puparium formation (0 hr). After a 30-min heat shock and 6-hr recovery,

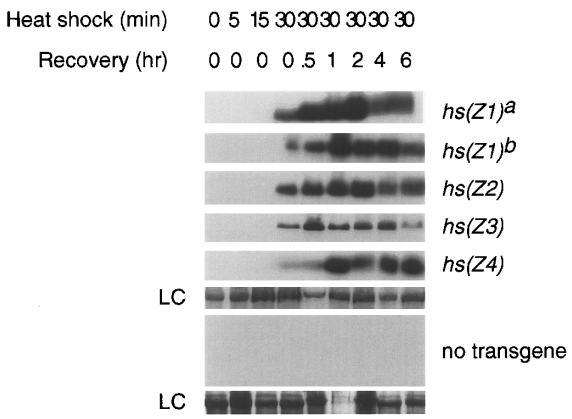


FIG. 3. BR-C proteins are induced following heat shock of *hs(BR-C)* transgenic animals. Male larvae hemizygous for the $npr1^3$ mutation (*BR-C* null) and heterozygous for individual heat shock promoter-driven *BR-C* transgenes, or containing no transgene, were collected approximately 4 hr prior to puparium formation and subjected to a 37°C heat shock followed by a 25°C recovery for the times shown. Protein extracts were fractionated by gel electrophoresis, blotted to nylon membranes, and probed with a monoclonal antibody directed against the BRcore domain. A 200-kDa protein from the Coomassie-stained gel (after transfer) is shown as a loading control (LC) for the *hs(Z4)* and "no transgene" Western blots. All other blots showed a pattern of protein loading similar to the *hs(Z4)* blot. The identity of the transgene used to generate the results in each panel is shown to the right: $Z1^a$ (BRcore-Q^1-Z1), $Z1^b$ (BRcore-TNT-Q^1-Z1), Z2 (BRcore-Z2), Z3 (BRcore-NS-Z3), Z4 (BRcore-Z4).

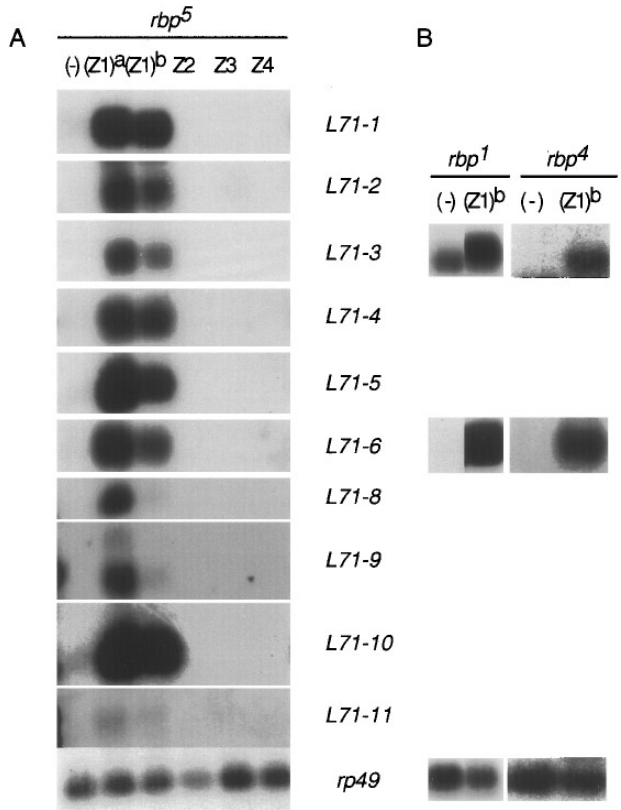


FIG. 4. BR-C Z1 proteins rescue *L71* gene expression in *rbp* mutants. (A) Male larvae hemizygous for rbp^5 and heterozygous for a *hs(BR-C)* transgene, or with no transgene (−), were collected approximately 4 hr prior to puparium formation and subjected to a 30-min 37°C heat shock followed by a 6-hr recovery at 25°C. Total RNA (15 μg per lane) was hybridized on Northern blots with radioactive DNA probes specific for each of the *L71* genes (*L71-1* through *L71-11*). The ribosomal protein gene *rp49* (O'Connell and Rosbash, 1984) served as an internal loading control. Because all *L71* transcripts are of similar size (except *L71-3*), each of three blots was stripped and reprobed three to four times. All blots showed similar patterns of RNA loading as judged by *rp49* hybridization. For comparison, signals from wild-type ($BR\text{-}C^+$) larvae with no transgene can be seen in the leftmost lanes of Fig. 5. Figures 4 and 5 were derived from different regions of a single autoradiogram of the same blot. (B) Male larvae hemizygous for rbp^1 or rbp^4 and heterozygous for a *hs(Z1)* transgene, or with no transgene (−), were treated as described above. Total RNA (10 μg per lane) was hybridized with probes specific for *L71-3*, *L71-6*, and *rp49*.

these animals were assayed for *L71* gene expression by Northern blot hybridization (Fig. 6B). We found that expression of *L71* transcripts failed when the transgene was induced prior to the late third instar pulse of ecdysone (at −18 hr), but *L71* genes were expressed at high levels when *hs(Z1)* transgene induction occurred after the ecdysone pulse (at −4 or 0 hr). Control animals that do not carry a transgene (−) failed to induce *L71* transcripts regardless of the stage at which they were treated. In contrast to these late *L71* genes, *I 71-7*, an rbp^+-dependent intermolt gene located between the *L71-4* and *L71-5* genes was expressed following *hs(Z1)* induction during the period of its normal expression (−18 hr). However, *hs(Z1)* transgene induction at the later developmental times (−4 hr and 0 hr) failed to induce *I71-7* transcription. These results show that Z1 isoform rescue of both *L71* late gene and *I71-7* intermolt gene expression is temporally constrained.

The regulatory elements necessary for L71-6 expression are found in sequences closely linked to L71-6. BR-C proteins bind DNA and directly regulate the *Sgs-4* intermolt gene (von Kalm *et al.*, 1994). To test whether the Z1 isoform directly regulates late gene expression, we defined *cis*-acting *L71* regulatory elements. The arrangement of the *L71* genes as divergently transcribed pairs (Fig. 1) coupled with the observation that subtle differences in the expression profile of these genes in wild-type and mutant backgrounds tend to be shared by gene pairs (Guay and Guild, 1991; Karim *et al.*, 1993; Wright *et al.*, 1996) suggests that regulatory elements might be found in the intergenic regions located between the 5′ ends of each gene pair. To test this hypothesis, we generated transgenic animals that carry an *L71-6* gene containing 225 bp of upstream sequence plus a *lacZ* reporter gene inserted in frame into the second *L71-6* exon

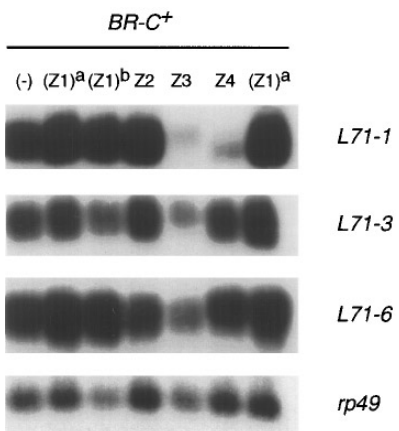


FIG. 5. Ectopic expression of BR-C proteins Z3 and Z4 represses *L71* gene expression. *BR-C*$^+$ male larvae heterozygous for a *hs(BR-C)* transgene, or with no transgene (−), were collected and treated the same way as their rbp^5 siblings used in the experiments described in Fig. 4. Total RNA (15 μg per lane) was hybridized with radioactive DNA probes specific for individual *L71* genes and *rp49*.

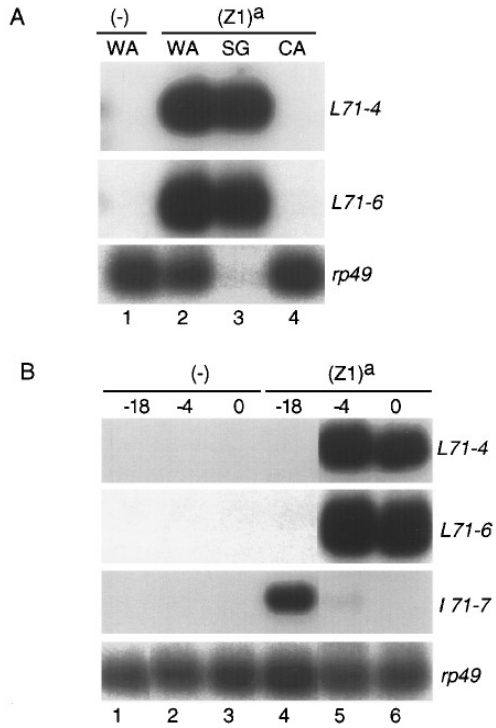


FIG. 6. *L71* gene expression rescue by the Z1 isoform occurs in a tissue- and temporally specific context. (A) Tissue specificity. Male larvae hemizygous for rbp^5 and heterozygous for a *hs(Z1)* transgene, or with no transgene (−), were collected approximately 4 hr prior to puparium formation and subjected to a 30-min 37°C heat shock followed by a 4-hr recovery at 25°C. Total RNA was prepared from whole animals (WA), dissected salivary glands (SG), or the remaining carcasses without salivary glands (CA). The RNA equivalent of five animals was hybridized with probes specific for *L71-4*, *L71-6*, and *rp49* transcripts. (B) Temporal specificity. Male larvae hemizygous for rbp^5 and heterozygous for a *hs(Z1)* transgene, or with no transgene (−), were collected as larvae (−18 hr or −4 hr) or as 0-hr prepupae and subjected to a 30-min 37°C heat shock followed by a 6-hr recovery at 25°C. Total RNA (10 μg per lane) was hybridized with probes specific for *L71-4*, *L71-6*, *I 71-7*, and *rp49* transcripts.

(Fig. 7A). Expression of this reporter construct in a *BR-C*$^+$ background showed that β-galactosidase accumulated to detectable levels by 6 hr after puparium formation and peaked by 18 hr after puparium formation (Fig. 7B). This was true in three independent transgenic lines (data not shown). This profile is similar to that seen for *L71-6* RNA accumulation (Restifo and Guild, 1986a; Karim *et al.*, 1993), though delayed by a number of hours. In fact, we are still able to detect β-galactosidase at 18 and 24 hr after puparium formation, when the salivary glands have undergone histolysis (Mitchell *et al.*, 1977). The L71 proteins are secreted from the salivary glands and can be found in the pupation fluid (L. Wright and G.M.G., unpublished observations). Since the L71-6/β-galactosidase fusion protein contains the puta-

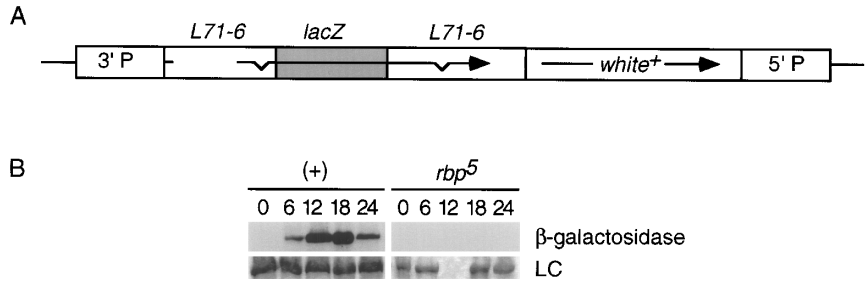


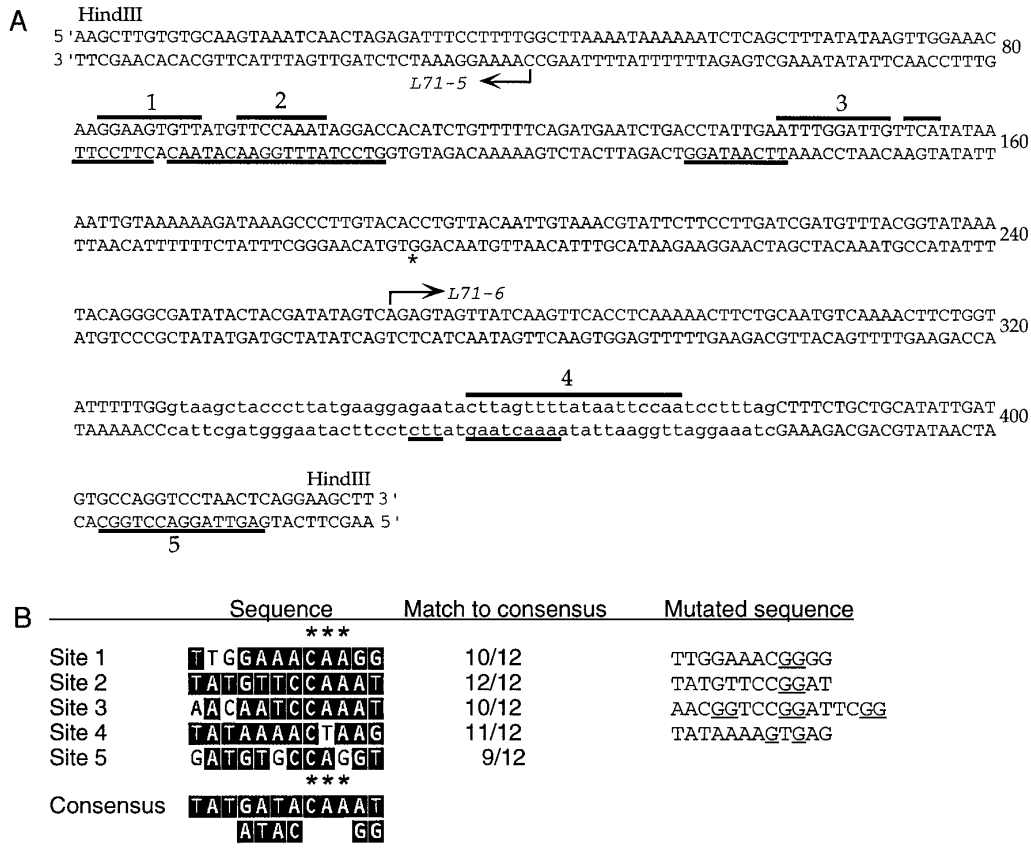
FIG. 7. Regulatory regions of the *L71-6* gene. (A) The P element reporter construct used to define *L71-6* regulatory elements is shown. An *L71-6* gene containing a *lacZ* reporter gene inserted in frame into its second exon synthesizes a fusion *L71-6:lacZ* transcript. Transcriptional orientations are indicated by the arrows (5′ → 3′), and horizontal lines and carets indicate exons and introns, respectively. The transgene extends 225 bp upstream of the *L71-6* start site and contains the entire *L71-5/6* intergenic region. The 5′ portion of the *L71-5* gene is indicated by a short horizontal line upstream of the *L71-6* gene. This transgene was cloned into the pCaSpeR-4 P element transformation vector containing the mini-*white* gene and 5′ and 3′ P element sequences. (B) Male larvae hemizygous for either rbp^5 or $BR\text{-}C^+$ (*Binsn* siblings) and heterozygous for the *L71-6:lacZ* transgene were collected at puparium formation and allowed to age for the times shown (hr). Total protein was probed on Western blots with a monoclonal antibody directed against *E. coli* β-galactosidase. A 200-kDa protein from the Coomassie-stained gel is shown as a loading control (LC). This protein is poorly visible in the rbp^5 12-hr time point, indicating that this lane was underloaded.

tive L71-6 signal sequence (Wright *et al.*, 1996), it is possible that this protein is also secreted and accumulates in the pupation fluid, allowing detection of β-galactosidase in whole animals after the salivary gland has been histolyzed. Consistent with this, staining of *L71-6:lacZ* transgenic animals with X-Gal shows β-galactosidase in the lumen of the salivary gland by 12 hr after puparium formation (data not shown). In addition, we find that β-galactosidase expression from this transgene is completely dependent on the rbp^+ function of the *BR-C* (Fig. 7B). Thus, the *L71* sequences contained within this construct are sufficient for *rbp*-dependent expression of the *L71-6* gene.

L71-6 regulatory elements contain Z1 isoform-binding sites. If the Z1 isoform directly regulates *L71-6* expression, binding sites for the Z1 protein should be located within *L71-6* regulatory sequences. To identify binding sites, we expressed the BRcore-Q^1-Z1 isoform in *E. coli* with an N-terminal 6-histidine fusion, purified the protein using a nickel-chelate resin, and used it in DNase I footprinting experiments with an *Eco*RI/*Sal*I fragment containing all of the *L71-5/6* sequences 5′ to the *lacZ* gene in the *L71-6:lacZ* construct shown in Fig. 7A. The positions of five regions protected by the Z1 protein are indicated on the sequence of the 427 bp *L71-5/6* region shown in Fig. 8A. A typical footprinting gel showing protection of sites 1 and 2 is shown in Fig. 9 (left panel). Sites 1, 2, and 3 are within the *L71-5/6* intergenic region. Site 3 may actually represent several closely linked sites. Site 4 is located in the first intron of *L71-6*, and site 5 is located in the second exon of *L71-6*. The protected sequences can be aligned to generate a loose consensus (Fig. 8B) that is similar to that previously described for Z1 isoform binding sites at *Sgs-4* (TAAT/AT/AG/AACAAG/AT/A, von Kalm *et al.*, 1994). Like the Z1 binding sites at *Sgs-4*, the consensus is centered around a trinucleotide CAA core. Of the three possible alignments at site 3, the single best match is shown. Sites 4 and 5 each have one mismatch within the CAA core. Site 4 matches at all other nucleotides perfectly, while site 5 exhibits the poorest match to the consensus overall.

Because these results show that Z1 protein can bind to specific sequences, we next tested whether these sequences were required for Z1 binding. We introduced alterations by PCR into binding sites 1, 2, and 3 located in the *L71-5/6* intergenic region, as well as site 4 in the first intron. DNase I footprinting showed that the alterations which change the CAA core to CGG in sites 1–3 (see Fig. 8B) eliminate Z1 isoform binding to the intergenic region. Footprinting results from altered sites 1 and 2 are shown in Fig. 9 (right panel). This result indicates that these sequences are important for binding. We also altered site 4 by changing the CTA core to GTG (see Fig. 8B), which did not entirely eliminate Z1 binding (data not shown), suggesting that the surrounding nucleotides may be critical for binding at this site.

Mutation of Z1 isoform-binding sites eliminates L71-6-driven reporter gene expression. If the Z1 isoform directly regulates *L71-6* transcription through the binding sites identified above, then mutation of these binding sites should prevent *L71-6* expression. To test this hypothesis, we cloned a fragment containing alterations in binding sites 1–3 and used it to replace the wild-type intergenic region in the *L71-6:lacZ* reporter construct. We only altered sites 1–3 in this construct since the site 4 alterations did not completely eliminate binding by the Z1 protein. Transgenic animals carrying the P element construct *1-3-alt/L71-6:lacZ* shown in Fig. 10A were generated and assayed for β-galactosidase expression. Protein extracts from staged animals were probed for *lacZ* expression on developmental Western blots. Virtually no re-



B

	Sequence	Match to consensus	Mutated sequence
	***		
Site 1	TTGGAAACAAGG	10/12	TTGGAAACGGGG
Site 2	TATGTTCCAAAT	12/12	TATGTTCCGGAT
Site 3	AACAATCCAAAT	10/12	AACGGTCCGGATTCGG
Site 4	TATAAAACTAAG	11/12	TATAAAAGTGAG
Site 5	GATGTGCCAGGT	9/12	
	***		
Consensus	TATGATACAAAT		
	ATAC GG		

FIG. 8. The BR-C Z1 isoform binds to *L71-6* regulatory sequences *in vitro.* (A) The 427-bp *Hin*dIII fragment encompassing the *L71-5/6* intergenic region used for DNase I footprinting is shown. Areas on each strand protected by the BRcore-Q[1]-Z1 isoform are indicated by lines above or below the sequence. Protected regions are numbered 1 through 5, although site 3 may actually represent more than one binding site. An asterisk at position 191 indicates a hypersensitive site. The *L71-5* and *L71-6* transcription initiation sites are shown, with arrows indicating the direction of transcription (5′ → 3′). The first *L71-6* intron is indicated by lowercase letters. (B) A consensus derived from the five Z1 isoform-binding sites at *L71-6* is shown in the Table. For sites 1 and 5 the sequence used to complete the alignment extends beyond the protected region. In the first column, white letters on black background indicate matches to the consensus. Asterisks indicate the trinucleotide CAA core. Three CAA sequences are found at site 3, but only the one shown matches well with the rest of the consensus. The number of matching nucleotides of the total covered is shown in the second column. In the third column, the alterations (underlined) introduced in these sites to eliminate Z1 isoform binding are shown. All three CAA sequences at site 3 were altered.

porter gene expression was seen in animals carrying *1-3-alt/L71-6:lacZ*, in contrast to animals carrying the *L71-6:lacZ* construct with the wild-type sequence (Fig. 10B). This was true in seven independent *1-3-alt/L71-6:lacZ* transgenic lines. On a long exposure, a very low amount of expression was visible at 6 and 12 hr after pupariation in some *1-3-alt/L71-6:lacZ* transgenic animals (data not shown). These results show that the Z1 isoform directly regulates *L71-6* gene expression through regulatory sequences located in the *L71-5/6* intergenic region.

DISCUSSION

The BR-C Z1 isoform provides the BR-C rbp$^+$ function. Activation of the *L71* late genes in the prepupal salivary gland is completely dependent on the *BR-C rbp$^+$* function (Guay and Guild, 1991; Karim *et al.*, 1993). A previous observation correlated the BR-C Z1 isoform with the *rbp$^+$* genetic function in that a Z1-specific monoclonal antibody failed to detect any protein in *rbp^5* mutant animals (Emery *et al.*, 1994). If the *rbp$^+$* function is provided by the Z1 isoform, expression of this isoform should be able to restore *L71* gene expression in *rbp* mutant animals. To test this hypothesis, individual BR-C protein isoforms were expressed from transgenes at the time of normal *L71* induction in *rbp* mutant animals. While all of the *hs(BR-C)* transgenic lines expressed protein upon heat shock induction as demonstrated by Western blot analysis (Fig. 3), only the BR-C Z1 isoforms restored *L71* gene expression (Fig. 4) in the

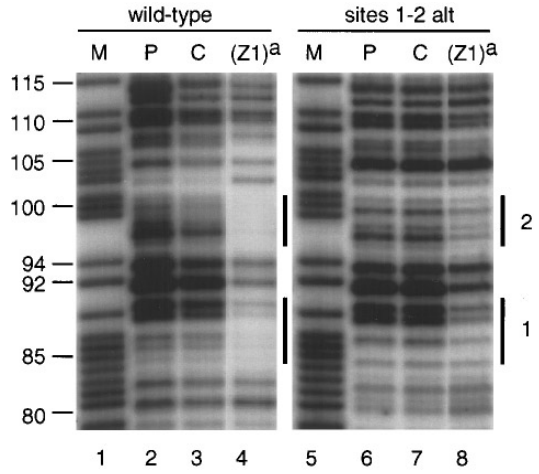


FIG. 9. Footprint analysis of BR-C Z1 protein binding to wild-type and altered sites within the *L71-5/6* intergenic region. A DNA fragment containing the wild-type *L71-5/6* intergenic region (left panel) and a fragment containing alterations (see Fig. 8) in sites 1, 2, 3, and 4 (right panel) were radiolabeled and used in DNase I footprinting assays with the BRcore-Q^1-Z1 isoform. Protected regions 1 and 2 on the sense strand are marked with vertical bars. Nucleotide coordinates corresponding to the sequence shown in Fig. 8 are indicated on the left. Footprinting reactions were carried out with no added protein (P), control extract consisting of purified extract from the *E. coli* expression vector with no insert (C), or 15 μg purified BRcore-Q^1-Z1 protein ($Z1^a$). A Maxam–Gilbert sequencing ladder (G + A reaction) of the labeled DNA provided size standards (M).

three *rbp* alleles tested. We conclude that the BR-C Z1 isoform mediates the rbp^+ function.

Consistent with this conclusion, Z1 isoforms are the predominant *BR-C* isoforms in prepupal salivary glands at the time of *L71* gene induction, at both the RNA (Huet *et al.*, 1993) and the protein level (Emery *et al.*, 1994). In addition, expression of an *hs(Z1)* transgene is able to rescue lethality and bristle defects (C.A.B., L. von Kalm and J.W.F., submitted for publication) and thoracic muscle defects (D. J. Sandstrom, C.A.B., J.W.F., and L. L. Restifo, submitted for publication) in rbp^1 and rbp^5 mutants. The BRcore-Q^1-Z1 isoform ($Z1^a$) was more efficient than the BRcore-TNT-Q^1-Z1 isoform ($Z1^b$) at inducing *L71* gene expression, particularly for the *L71-8* and *L71-9* genes (Fig. 4). Consistent with this, $Z1^a$ transcripts are expressed at 100-fold higher levels than $Z1^b$ transcripts in salivary glands at this time (Huet *et al.*, 1993). Because the heat shock constructs were induced to similar levels (Fig. 3), these isoforms must be functionally different. As expected, their DNA-binding specificity appears to be the same (K.C. and G.M.G., unpublished observations). The only difference between the isoforms is the presence of a TNT domain in the $Z1^b$ isoform. Perhaps this domain alters the specificity of $Z1^b$ isoform interactions with other proteins, for example, by interfering with the glutamine-rich (Q) domain. Glutamine-rich regions have been implicated in interactions with proteins of the transcription initiation complex (Colgan *et al.*, 1993; Hoey *et al.*, 1993).

The BR-C Z1 isoforms directly regulate L71-6 expression. The Ashburner model holds that the early genes directly regulate late gene activity. We identified those *cis*-acting *L71-6* sequences necessary for correct developmental expression of an *L71-6:lacZ* reporter gene construct (Fig. 7). DNase I footprinting analysis showed that the $Z1^a$ isoform specifically bound to five sites, including three sites within the 225-bp upstream sequences located in the intergenic region between *L71-5* and *L71-6* (Fig. 8). When alterations that eliminate Z1 protein binding were introduced into these three sites, reporter gene expression was dramatically reduced (Fig. 10). The relative importance of the individual sites is not known, but can be tested by making constructs in which individual sites are altered. However, the simplest interpretation of these results is that the Z1 isoform directly regulates *L71-6* expression and that this regulation requires sequences adjacent to the *L71-6* promoter within the *L71-5/6* intergenic region.

Additional tissue and temporal factors are necessary for L71 developmental regulation. While the Z1 isoform is essential for *L71* gene induction, other factors are also required. Induction of *hs(Z1)* transgenes in rbp^5 mutants restores *L71* gene expression only in the salivary glands (Fig. 6A), the tissue to which *L71* gene expression is normally confined (Restifo and Guild, 1986a). This suggests that at least one other, probably salivary gland-specific, factor is required for *L71* gene induction. This factor could act either positively in concert with the Z1 isoform, or it could act by relieving a repressive effect. Preliminary evidence suggests that the sequences necessary for tissue-specific *L71-6* expression are retained in the reporter construct containing sequences 225 bp upstream and 700 bp downstream from the *L71-6* start site (K.C. and G.M.G. unpublished observations). The tissue specificity of another salivary gland-specific gene, *Sgs-4*, is thought to be controlled by a factor that binds to the A-site in *Sgs-4* regulatory Element III (discussed in von Kalm *et al.*, 1994). It will be interesting to see whether this factor also acts on the *L71* genes and functions as a general salivary gland tissue specifier.

The *hs(Z1)* transgenes are also temporally constrained in their ability to rescue *L71* gene expression in rbp^5 mutants. *L71* transcripts do not accumulate when a *hs(Z1)* transgene is induced prior to the premetamorphic ecdysone pulse (Fig. 6B). This observation also supports the hypothesis that other factors are needed for *L71* gene induction. One candidate gene that might encode the non-*BR-C* temporal specifier is the E74A product of the *E74* early gene. E74A is an ETS-domain DNA-binding protein that binds *in vitro* to sequences in the *L71-5/6* intergenic region, including a site that overlaps with BR-C Z1 isoform binding sites 1 and 2, and is able to directly regulate *L71-6* gene expression (Urness and Thummel, 1995). In addition, the *E74A* and the *BR-C* functions interact genetically (Fletcher and Thummel, 1995b) in keeping with the idea that ETS-domain proteins often require a protein partner to facilitate DNA bind-

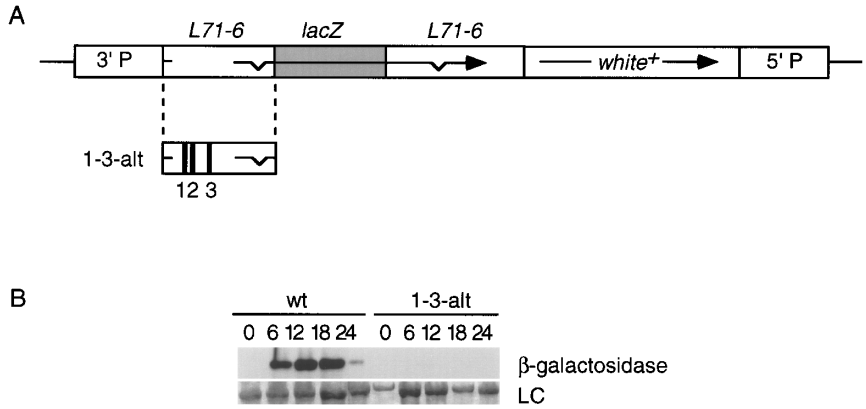


FIG. 10. Alteration of Z1 isoform binding sites eliminates *L71-6*-driven reporter gene expression. (A). The *L71-6:lacZ* reporter gene construct shown in Fig. 7 was modified as shown. Alterations (vertical bands) were introduced into Z1 isoform-binding sites 1, 2, and 3 by PCR. A restriction fragment containing these alterations replaced the corresponding wild-type sequence bracketed by dashed lines in the *L71-6:lacZ* reporter gene construct to yield the *1-3-alt/L71-6:lacZ* construct. (B) Animals homozygous for either the wild-type *L71-6:lacZ* transgene (wt) or the mutated *1-3-alt/L71-6:lacZ* transgene (1-3-alt) were collected at puparium formation and allowed to age for the times shown (hr). Total protein was probed on a Western blot with a monoclonal antibody directed against *E. coli* β-galactosidase. A 200-kDa protein from the Coomassie-stained gel is shown as a loading control (LC).

ing (e.g., Petersen *et al.*, 1995). The E74A protein is induced following the premetamorphic pulse of ecdysone (Boyd *et al.*, 1991), which is consistent with the expression profile of a factor that temporally restricts *L71* late gene expression. Because *L71* gene expression is reduced and delayed, but not eliminated, in *E74A* mutant animals (Fletcher and Thummel, 1995a) and E74A is expressed in tissues other than the salivary gland (Boyd *et al.*, 1991; Baehrecke and Thummel, 1995), it is not likely to function in salivary gland specificity.

L71 gene transcription appears to lag well behind Z1 isoform synthesis in these experiments. While *hs(Z1)* isoforms were readily detected 30 min after heat shock (Fig. 3), *L71* transcripts were not detected until 2 hr after the 30-min heat shock (data not shown). At a transcriptional elongation rate of 1.1 kb/min (Thummel *et al.*, 1990) it should not require 2 hr for the 500- to 800-nucleotide *L71* transcripts to accumulate to detectable levels. Following induction of *hs(Z1)* transgenes, the resulting $Z1^a$ protein slowly increases in apparent size in a time-dependent manner (Fig. 3), suggesting some type of posttranslational modification. If alteration of BR-C isoforms is essential for function, *L71* gene expression may be delayed until enough properly modified protein accumulates. Since inspection of BR-C amino acid sequences reveals potential phosphorylation sites within the BRcore domain (DiBello *et al.*, 1991), protein phosphorylation may play a role in this genetic cascade.

It is also possible that the Z1 proteins must overcome the repressive effects of other proteins on *L71* gene expression. Three candidate repressors come to mind. The Z3 and Z4 containing transcripts also accumulate in salivary glands, but prior to *L71* gene induction, during the mid to late third instar period (von Kalm *et al.*, 1994; Huet *et al.*, 1993). We find that overexpression of these isoforms in a *BR-C*$^+$ background during the period of normal *L71* expression results in *L71* repression. Thus, the sequential production of different BR-C isoforms may be responsible for the physiological timing of *L71* gene expression. Second, the E74B protein may act to repress *L71* gene expression, as suggested by Fletcher and Thummel (1995a,b). Finally, the ecdysone receptor may repress late genes (Ashburner *et al.*, 1974). Accordingly, premature *L71* gene expression could be repressed by one or a combination of these factors until the accumulation of sufficient levels of activating Z1 (and E74A) protein at puparium formation.

The L71 genes are regulated as pairs. The *L71* genes are arranged as five sets of divergently transcribed gene pairs (Restifo and Guild, 1986a; Wright *et al.*, 1996). Subtle differences in the regulation of these genes tend to be shared by gene pairs (Guay and Guild, 1991; Karim *et al.*, 1993; Wright *et al.*, 1996). Therefore, critical regulatory elements may be located in the intergenic regions between the 5′ ends of coregulated genes. Furthermore, these regulatory sequences function bidirectionally. This pairwise regulation is apparent in the sensitivity of the *L71* genes to repression by the *hs(Z3)* and *hs(Z4)* transgenes in *BR-C*$^+$ animals (Fig. 5). In addition, it can be used to illustrate a functional difference between the $Z1^a$ and the $Z1^b$ isoforms. While $Z1^a$ was able to induce high levels of expression of the *L71-8* and *L71-9* gene pair in *rbp*^{5} mutants, much lower levels of induction were observed when a $Z1^b$-expressing transgene was used (Fig. 4).

Further support for the pairwise regulation of *L71* genes comes from the demonstration that Z1 binding sites essential for *L71-6* induction are located in the *L71-5/6* intergenic region. This suggests a mechanism for *L71* gene regulation

in which Z1 proteins binding to the small intergenic regions between these genes simultaneously activate transcription of both promoters. It has been suggested that the BR-C proteins function by altering chromatin structure (Dubrovsky *et al.*, 1994). Thus, binding by Z1 proteins could make the intergenic regions accessible for transcription in both directions. If the gene pairs truly share regulatory elements, a construct containing the *L71-5/6* intergenic region in which a reporter gene is fused to *L71-5* should exhibit the same dependency on functional Z1 binding sites as *L71-6*. We also predict that functional Z1 binding sites are located between all other *L71* late gene pairs.

The only difference we observed between genes within a pair occurred in the *L71-10/11* gene pair. *L71-10* transcripts were always more abundant than *L71-11* transcripts when tested in *hs(BR-C)* transgenic animals subjected to a heat shock regimen. This difference in abundance is not observed in wild-type animals (Wright *et al.*, 1996). In addition, the expression levels of individual *L71* genes in an *E74A* mutant background varies between members of a gene pair (Fletcher and Thummel, 1995a). This suggests a possible difference in the way the BR-C Z1 and E74A proteins interact with the *L71* genes. *BR-C* regulation tends to affect the *L71* genes as pairs, whereas *E74A* appears to affect *L71-1, 4,* and *5* differently than *L71-2, 3,* and *6* (*L71-8* to *11* were not assayed). Sequence analysis of the *L71* genes groups them into two classes designated α and β (Wright *et al.*, 1996). The gene cluster is believed to have evolved as a series of duplication events from an original gene pair such that each resulting gene pair contains one member of each class. Interestingly, the *L71-1, 4,* and *5* genes belong to the α class, while the *L71-2, 3,* and *6* genes belong to the β class. The E74A protein appears to consistently favor the β member of each pair, while the Z1 protein acts equally on both members of a pair, but can vary among pairs.

BR-C function is central to the ecdysone-triggered transcriptional cascade. Based on the puffing patterns displayed by the polytene genome in developing salivary glands, Ashburner *et al.* (1974) made several predictions about the way in which the ecdysone signal is transduced and amplified during metamorphosis. In particular, primary response early genes are thought to encode regulatory products that directly regulate secondary response late gene induction. The general principles of the Ashburner model have withstood the test of time admirably, and in concordance with this model we find that (1) the ecdysone-inducible *BR-C* early gene encodes a family of DNA-binding proteins and (2) the Z1 isoforms directly regulate L71-6 late gene expression by binding to closely linked *cis*-acting sequences.

Of course, the molecular details of this ecdysone-triggered cascade have become more complex now that several early genes have been genetically and molecularly characterized. For example, an extension of the Ashburner model (Burtis *et al.*, 1990; Thummel *et al.*, 1990) suggests that the development of individual tissues during metamorphosis is controlled by unique, but overlapping, distributions of early gene products. Consistent with this idea, *BR-C* and *E74* early gene isoforms are expressed in a tissue-specific manner during metamorphosis (Huet *et al.*, 1993; Emery *et al.*, 1994). In the salivary gland, both the BR-C Z1 and E74A proteins contribute to *L71* late gene activation (Fig. 4; Fletcher and Thummel, 1995a) and directly regulate *L71-6* (Fig. 10; Urness and Thummel, 1995). Because one of the E74A binding sites at *L71-6* overlaps with BR-C Z1 binding sites 1 and 2, it is possible that direct interactions between these proteins are necessary for *L71-6* activation. In fact, phenotypic analysis of *E74A/rbp* double mutants suggests that these products interact (Fletcher and Thummel, 1995b). Thus, the BR-C Z1 and E74A isoforms may act together to define the salivary gland transcriptional hierarchy during metamorphosis.

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