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 \textit{L71} late genes are dependent on the BR-C \textit{rbp} \(^1\) genetic function. Transgenic animals expressing individual BR-C isoforms were tested for their ability to provide the BR-C \textit{rbp} \(^1\) genetic function by monitoring the transcriptional activation of the \textit{L71} genes. We found that the BR-C Z1 isoforms could complement the transcriptional defects seen in \textit{rbp} mutants but the Z2, Z3, and Z4 isoforms could not. We conclude that the BR-C \textit{rbp} \(^1\) function is provided by the BR-C Z1 isoform in prepupal salivary glands. \textit{L71} gene rescue was restricted to the prepupal salivary gland, suggesting the involvement of additional factors in \textit{L71} gene regulation. Interestingly, we found that the overexpression of Z3 or Z4 isoforms in BR-C \(^2\) salivary glands repressed \textit{L71} expression, indicating that BR-C proteins might also function as transcriptional repressors. Molecular mapping and characterization of the regulatory sequences that control \textit{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.

\section*{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.

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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 285 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,H2 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; Hoye 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.

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 isoform 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, 1 71-7, is located within the late gene cluster between L71-4 and L71-5. All L71 gene transcripts are severely down-regulated in rbp 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 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 isofom 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.

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**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.
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 rbp2, rbp3, rbp5 (Belyaeva et al., 1980) and npr1 (Kiss et al., 1988) have been described (see Emery et al., 1994). Mutant BR-C alleles were maintained in females in combination with the Binsen balancer X chromosome. Animals were raised on standard corn-fruit food (Elgin and Miller, 1978) at 25°C.

To examine the effects of various transgenes in BR-C mutant backgrounds, y BR-C mutant/Binsen females were mated to w118 males homozygous for the transgene of interest on the second or third chromosome. BR-C mutant male larvae were differentiated from their BR-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 digestive tract during the wandering stage after the larva stops 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 edcsyne 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 edcsyne pulse. Other animals were synchronized at the onset of pupariation by collection of 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 EcoRI site as shown in Fig. 2. The cDNAs were derived from clones dm527 (BRcore-Q1-Z1), dm708 (BRcore-TNT-Q1-Z1), dm796 (BRcore-Z2), dm797 (BRcore-N-S-Z3), and 281 (BRcore-Z4) (Di Bello et al., 1991; Bayer et al., 1996). P element transformation was carried out essentially as described by Rubin and Spradling (1982). The BRcore-Q1-Z1 construct was injected into y w; rys065 Sb1 P^f(+); y^2 3996/ 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 w118. All other transgene constructs were injected into w118 embryos with the p25.7hc helper plasmid (Kares 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 Smal–Xbal fragment containing the lacZ gene inserted into a filled-in HindIII site within a 3.2-kb SalI–NheI 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 HindIII–BamHI 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 BamHI site came from within the lacZ fragment. A 424-bp XhoI–BamHI fragment from pBS415(wt) (containing the entire insert) was used to replace a 2.6-kb Xhol–BamHI fragment (containing all of the sequence upstream of the original fusion site) from the original construct. Because the lacZ BamHI 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 XhoI–NotI fragment containing the entire fusion construct was then cloned into pCaSpeR-4 (Thummel and Pirotta, 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 HindIII–BamHI 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 fluorescence-tagged DNA sequencing (ABI). The site 1–2 and site 3 mutations were introduced using the following oligonucleotide pairs: (1-2.a) 5’-GGTTGGAACggGGAAGTGTATTGGTCCggTATAGGACCACATC-3’; (1-2.b) 5’-GATGTGTTCTATccGGACACAAATACCTTCCGGTTTCAAC-3’; (3.a) 5’-GAATCTGACCTA-speciﬁed times; (3.b) 5’-TAACACTTCCccGTTTCCAAC-3’.

Lowercase letters represent alterations of the wild-type sequence. A 424-bp Xhol–BamHI 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 XhoI–NotI 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; rys065 Sb1 P^f(+); y^2 3996/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-βCore (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-
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 (Guary 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 (npri\(^{\Delta}\)) 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-Q1-Z1 (Z1\(^{\Delta}\)) 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\(^{-}\) larvae carrying single hs(BR-C) transgenes were collected at the end of third instar, at the time of the major premetamorphic ec dysone pulse, and heat-shocked for 30 min. We chose this developmental time for induction of transgene expression in order to mimic the ec dysone-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 had 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-Q1-Z1 isoform (Z1\(^{\Delta}\)) allowed L71 transcripts to accumulate to higher levels, particularly L71-8 and L71-9 transcripts. The levels of L71 transcripts induced by a Z1\(^{\Delta}\) transgene are comparable to L71 transcript levels in similarly treated BR-C\(^{-}\) animals carrying no transgene (e.g., Z1\(^{\Delta}\) Induction levels from Fig. 5), suggesting that Z1\(^{\Delta}\) 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\(^{-}\) 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\(^{-}\) 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 EcoRI 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 BR-core 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 C2H2 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 BR-core 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 rbp1 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 rbp1 mutants, expression of Z2, Z3 and Z4 isoforms failed to rescue L71 gene expression in rbp1 and rbp4 backgrounds (data not shown). Together, these results allow us to conclude that the BR-C Z1 isoforms provide the rbp1 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 rbp1 mutants shown in Fig. 4A) for changes in L71 gene expression. We found that L71 gene transcript levels in BR-C1 control animals which do not carry a transgene (--), were similar to the levels detected in BR-C1 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-C1 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-C1 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 rbp5 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 rbp5 larvae control (LC) for the hs(Z4) and "no transgene" Western blots. All other blots showed similar patterns of RNA loading as judged by rp49 hybridization. For comparison, signals from wild-type (BR-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 rbp1 or rbp4 and heterozygous for a hs(Z1) transgene, or containing 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 with 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-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.
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, L71-7, an rbp5-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 L71-7 transcription. These results show that Z1 isoform rescue of both L71 late gene and L71-7 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. 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 puation fluid (L. Wright and G.M.G., unpublished observations). Since the L71-6/β-galactosidase fusion protein contains the puta-
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 rbp5 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-Q1-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 EcoRI/SalI 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/AACAAAG/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-
porter gene expression was seen in animals carrying 1-3-alt/L71-6:acrZ, in contrast to animals carrying the L71-6:acrZ construct with the wild-type sequence (Fig. 10B). This was true in seven independent 1-3-alt/L71-6:acrZ transgenic lines. On a long exposure, a very low amount failed to detect any protein in rbp5 mutant animals (Emery et al., 1994). If the rbp5 function is provided by the Z1 isoform, expression of this isoform should be able to restore L71 gene expression in rbp5 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

**DISCUSSION**

**The BR-C Z1 isoform provides the BR-C rbp5 function.** Activation of the L71 late genes in the prepupal salivary gland is completely dependent on the BR-C rbp5 function (Guay and Guild, 1991; Karim et al., 1993). A previous observation correlated the BR-C Z1 isoform with the rbp5 genetic function in that a Z1-specific monoclonal antibody failed to detect any protein in rbp5 mutant animals (Emery et al., 1994). If the rbp5 function is provided by the Z1 isoform, expression of this isoform should be able to restore L71 gene expression in rbp5 mutant animals. To test this hypothesis, individual BR-C protein isoforms were expressed from transgenes at the time of normal L71 induction in rbp5 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
and bristle defects (C.A.B., L. von Kalm and J.W.F., submitted for publication) and the protein level (Emery et al., 1993) and the tissue specificity of another salivary gland-specific factor is required. The tissue specificity of Z1 isoform interactions with other proteins often requires a protein partner to facilitate DNA binding.

**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 Nase footprinting assays with the BRcore-Q1-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 protein from E. coli expression vector with no insert (C), or 15 μg purified BRcore-Q1-Z1 protein (Z1*). 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 (J.J. Sandstrom, C.A.B., J.W.F., and L.L. Restifo, submitted for publication) in rbp1 and rbp3 mutants. The BRcore-Q1-Z1 isoform (Z1*) is more efficient than the BRcore-TNT-Q1-Z1 isoform (Z1B) at inducing L71 gene expression, particularly for the L71-8 and L71-9 genes (Fig. 4). Consistent with this, Z1* transcripts are expressed at 100-fold higher levels than Z1B 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 Z1B isoform. Perhaps this domain alters the specificity of Z1B 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 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 rbp3 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 L71-6:lacZ reporter gene expression (Urness and Thummel, 1995). 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 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.

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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 Z1b 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 Z1a and the Z1b isoforms. While Z1a was able to induce high levels of expression of the L71-8 and L71-9 gene pair in rpb2 mutants, much lower levels of induction were observed when a Z1b-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 (Dubrovslyk 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 dependence 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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