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A Switch in *Broad-Complex* Zinc-Finger Isoform Expression Is Regulated Posttranscriptionally during the Metamorphosis of *Drosophila* Imaginal Discs

Cynthia A. Bayer, Bret Holley,[1] and James W. Fristrom
Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3200

The *Broad-Complex* (*BR-C*) is a key member of the 20-hydroxyecdysone regulatory hierarchy that coordinates changes in gene expression during *Drosophila* metamorphosis. The family of transcription factors encoded by the *BR-C* share a common amino-terminal domain which is fused by alternative splicing to one of four pairs of C_2H_2 zinc-finger domains (Z1, Z2, Z3, and Z4). In this study, we examine the temporal expression of transcripts encoding each *BR-C* zinc-finger isoform—including the newly discovered fourth zinc-finger domain—during the metamorphosis of imaginal discs which form the integumental structures of the adult head and thorax. We find that all *BR-C* zinc-finger RNA isoforms are induced as a primary response to 20-hydroxyecdysone. However, induced *BR-C* RNA isoforms exhibit two divergent expression profiles. The Z2, Z3, and Z4 RNA isoforms accumulate to high levels at the beginning of the ecdysone response and abruptly disappear after several hours. In contrast, the Z1 RNA isoform continues to accumulate while the others decline, resulting in a switch in relative isoform levels. Using probes specific to different regions of the *BR-C*, we show that the switch in *BR-C* RNA isoform expression appears to be posttranscriptionally regulated, presumably by ecdysone-responsive factors. We propose that this switch results from a change in splice acceptor site choice. Finally, we present a model describing how this temporal switch in isoform expression could mediate changes in *BR-C* function, from transcriptional activation to repression and vice versa, that are critical for coordinate downstream target gene expression.

INTRODUCTION

Insect metamorphosis is driven by fluctuations in the titer of the steroid hormone 20-hydroxyecdysone (ecdysone). The sharp rise in the concentration of ecdysone at the end of larval development initiates changes in tissue-specific gene expression through a hierarchy of ecdysone-responsive genes. These events are mediated by the binding of ecdysone in a receptor protein complex which directly activates transcription of the first tier of regulators called ''early ecdysone'' genes. One role of the early ecdysone genes is to coordinate the temporal activation of sets of tissue-specific ''late ecdysone'' genes during metamorphosis. This model of ecdysone-regulated gene expression, first described by Ashburner and co-workers (Ashburner *et al.*, 1974) to explain the sequential puffing activity of *Drosophila* salivary gland polytene chromosomes, has been verified, as well as expanded, over the past several years by the isolation and characterization of many of the early ecdysone regulatory genes (Burtis *et al.*, 1990; DiBello *et al.*, 1991; Koelle *et al.*, 1991; Segraves and Hogness, 1990; Talbot *et al.*, 1993; Thummel *et al.*, 1990) as well as some of their tissue-specific target genes (Fletcher and Thummel, 1995; Guay and Guild, 1991; Hodgetts *et al.*, 1995; von Kalm *et al.*, 1994).

One of the early ecdysone genes, the *Broad-Complex* (*BR-C*), coordinates early metamorphic events (Karim *et al.*, 1993). The class of *BR-C* mutants called *nonpupariating 1*, which lack all function at the locus, demonstrate the essential requirement for the *BR-C* (Belyaeva *et al.*, 1980; Kiss *et al.*, 1976; Stewart *et al.*, 1972). These mutants die as wandering larvae, unable to enter metamorphosis and initiate pupariation. Gene expression is also defective in *BR-C* mutants. The early ecdysone genes *E74* and *E75*, as well as tissue-specific intermolt and late ecdysone genes, such as *Sgs-4* and the *L71* cluster, respectively, are all found to be

[1] Current address: Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020.

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delayed or underexpressed in *BR-C* mutants (Belyaeva *et al.*, 1981; Crowley *et al.*, 1984; Guay and Guild, 1991; Karim *et al.*, 1993). Thus, the *BR-C* has a crucial role in regulating changes in gene expression during metamorphosis.

The molecular structure of the *BR-C* locus, at chromosomal position 2B5, was first described in DiBello *et al.* (1991). The *BR-C* was found to encode a family of DNA-binding proteins that share a common (core) amino terminus fused by alternative splicing to one of three pairs of C_2H_2-type zinc-finger domains, called Z1, Z2, and Z3. DiBello *et al.* (1991) also demonstrated that the core contains a 113-amino-acid sequence that is conserved in the transcriptional regulators encoded by the *tramtrack* gene (Harrison and Travers, 1990; Read and Manley, 1992) as well as a large number of other proteins, primarily zinc-finger proteins (Zollman *et al.*, 1994). This domain, called the BTB domain (Chen *et al.*, 1995; Zollman *et al.*, 1994) or the POZ domain (Bardwell and Treisman, 1994), appears to be involved in protein–protein interactions that may affect binding to DNA.

Consistent with a genetically defined requirement for this gene in metamorphosis, *BR-C* transcripts have been shown to accumulate during larval development in response to ecdysone (Andres *et al.*, 1993; Karim and Thummel, 1992) and in staged salivary glands (Huet *et al.*, 1993; von Kalm *et al.*, 1994). *BR-C* protein products are widely distributed among all tissues examined in the late larval to prepupal periods (Emery *et al.*, 1994; Huet *et al.*, 1993). All tissues studied so far contain all *BR-C* isoforms. Their relative abundance, however, differs greatly from tissue to tissue, suggesting that the various members (or combinations) of the *BR-C* family of proteins function in different developmental pathways.

As might be expected from the wide distribution of *BR-C* proteins, many tissues, including salivary glands, gut, muscle, the central nervous system, and imaginal discs, exhibit defective metamorphic development in *BR-C* mutants (Belyaeva *et al.*, 1980; Fristrom *et al.*, 1981; Kiss *et al.*, 1988; Restifo and White, 1991; Restifo and White, 1992). Kiss *et al.* (1988) have shown that the morphogenetic changes which occur in the imaginal discs that give rise to the adult thoracic integument and appendages are dependent on the *BR-C*. Imaginal discs from the *broad* class of mutants, for example, fail to undergo normal elongation and eversion, the initial phases of appendage morphogenesis.

Here, we examine the ecdysone response of individual *BR-C* zinc-finger RNA isoforms, including a newly isolated Z4 zinc-finger domain, in cultured imaginal discs. These studies reveal a complex temporal pattern of *BR-C* RNA expression. We find that all four zinc-finger RNA isoforms are induced as a primary response to ecdysone in imaginal discs. However, the relative ratios of zinc-finger RNA isoforms change over time. The Z2, Z3, and Z4 RNA isoforms accumulate rapidly in late third instar larval discs in response to ecdysone, but abruptly disappear after 6 hr of culture. In contrast, the Z1 RNA accumulates more slowly, to become the predominant *BR-C* isoform expressed after 6 hr. Regulation of this imaginal disc-specific switch appears to be posttranscriptional. We propose that tissue-specific switches in *BR-C* isoform expression result in changes in *BR-C* function during metamorphosis and that these changes regulate the temporal expression of downstream target genes.

MATERIALS AND METHODS

Imaginal Disc Cultures

Imaginal discs were mass-isolated and cultured as described in Eugene *et al.* (1979). For these studies, larvae were harvested 12–18 hr before puparium formation to ensure that the imaginal discs were not exposed to the *in vivo* rise in ecdysone titer that occurs approximately 6 hr before puparium formation. Imaginal disc cultures were incubated for 3 hr in culture medium (preincubation), to allow dissociation of any endogenous ecdysone from its receptor ($t_{1/2}$ 20 min at 25°C) (Yund *et al.*, 1978), before the addition of 2×10^{-6} *M* 20-hydroxyecdysone (ecdysone) at time 0 hr.

Z4 cDNA Isolation and Sequence Analysis

λ clones from the 2B5 genomic region (Chao and Guild, 1986) were hybridized at low stringency (29% Formamide/6× SSPE/0.5% SDS/50 μg/ml ssDNA at 42°C) to probes specific for each zinc-finger domain (see below). The final wash was in 6× SSC at 42°C. Z1 and Z3 probes each cross-hybridized with the 6.8-kb *Eco*RI fragment of λ206 at map position 195 kb. After sequence identification of a new zinc-finger (Z4) motif in this fragment, a Z4 PCR amplification product was used to screen the λHORIII larval cDNA library provided by P. Hurban and C. Thummel, as well as a λZAPII imaginal disc cDNA library (described in Appel *et al.*, 1993). Several isolated clones were sequenced using the Sequenase Version 2.0 DNA Sequencing kit (U.S. Biochemical Corp.). Predicted *BR-C* amino acid sequence similarity computations were performed using the BlastP Sequence Homology Search (Altschul *et al.*, 1990) (version 1.3.11) of the nonredundant protein sequence database on the GenomeNet WWW server. The reported similarities are based on the BLOSUM62 scoring matrix. Similar results were found using BLOSUM62 and PAM120 matrices.

RNA Isolation and Hybridization

Total RNA was isolated from white prepupae or imaginal discs using a modification of the Chirgwin *et al.* (1979) guanidinium isolation procedure. For Northern blot analysis, 10 μg of RNA was separated through a 50 m*M* Hepes, pH 7.0/ 2.2 *M* formaldehyde gel and transferred to Magnagraph nylon membrane (Micron Separations, Inc.). Random hexamer primed (Boehringer-Mannheim) ^{32}P-labeled DNA probes were hybridized in 5× SSC/ 40 m*M* $NaPO_4$/ 10% dextran sulfate/ 100 μg/ml ssDNA and washed at high stringency with a final wash of 0.1× SSPE/ 0.1% SDS at 65°C.

To quantify zinc-finger RNA levels, 1 μg of RNA was hybridized to ^{32}P-labeled antisense RNA probes prepared as described by Melton *et al.* (1984). RNase protection assays were performed as described in Mougneau *et al.* (1993) except that RNAse A was omitted from digestions. Reactions were performed with a minimum of fivefold probe excess. All experiments were repeated three times

with similar results. Bands of hybridization on RNase protection gels were quantified using a Molecular Dynamics Phosphorimager.

Probes

For Northern blots, DNA probes specific to *BR-C* promoter regions or introns were prepared from gel-purified restriction fragments of λ clones from the 2B5 genomic region walk (Chao and Guild, 1986). The P_{distal} probe corresponds to a 1.8-kb *Apa*I/*Pst*I restriction fragment within the 4.6-kb *Eco*RI/*Sal*I fragment covering the 539-bp exon 1 at map position 120 kb in λ clone 184. The exon numbers refer to those described in DiBello *et al.* (1991). The P_{prox} probe corresponds to the 500-bp *Eco*RI/*Sal*I restriction fragment (subclone dm813 in DiBello, 1992) covering the 468-bp exon 3 at map position 165 kb in λ clone 193. The core exon probe corresponds to a 511-bp *Pst*I restriction fragment of cDNA cD5. This DNA fragment includes nucleotides 35 through 546 in the sequence shared by all *BR-C* cDNAs (DiBello *et al.*, 1991) and is contained in the core exon located at map position 177 kb (Fig. 1). The 5′ intron probe corresponds to the 2.6-kb *Hin*dIII fragment at map position 125 kb in λ clone 184. The core intron probe corresponds to the 1.4-kb *Xho*I fragment within the 3.7-kb *Sal*I/*Eco*RI fragment at map position 180 kb at the 3′ end of λ clone 193. Strand-specific RNA probes generated from the core intron sequence (data not shown) determined that the hybridization signal shown in Fig. 8 corresponds to RNA produced by the coding strand of the *BR-C* locus.

The core/Z1 intron probe corresponds to the 1.3-kb *Eco*RI fragment at map position 187 kb in λ clone 202. The Z1/Z4 intron probe corresponds to the 1.1-kb *Eco*RI/*Hpa*I fragment within the 5′ end of λ clone 206. This DNA fragment, located at map position 193 kb, lies upstream of the exon containing the Z4 zinc-finger sequence and its linker domain. Sequences within this 1.1-kb *Hpa*I/*Eco*RI fragment are not present in the Z4 cDNA clone 28-I (Fig. 2), but are present at the 5′ end of the incomplete Z4 cDNA clones 5A and 5B (data not shown). The Z4/Z2 intron probe corresponds to the 1.12-kb *Hin*dIII fragment at map position 198 kb in λ clone 206. The Z2/Z3 intron probe corresponds to the 1.85-kb *Xba*I fragment within the 3.7-kb *Hin*dIII fragment at map position 204 kb in λ clone 206. The Z3 3′ UTR probe corresponds to the 1.0-kb *Ava*II fragment within the 3.8-kb *Eco*RI fragment at map position 207 kb in λ clone 207.

Zinc-finger-specific DNA probes were gel-purified from plasmids constructed by PCR-amplifying *BR-C* sequences from cDNAs and cloning into the pCR1000 (Invitrogen) vector. The inserts correspond to the following cDNA sequences: Z1 = 1948–2270, Z2 = 1688–1885, Z3 = 2115–2329, and Z4 = 2631–2863 (DiBello *et al.*, 1991, and Fig. 2). These zinc-finger-specific probes do not cross-hybridize under the Northern conditions described here.

For RNase protection assays of zinc-finger levels, RNA probes were generated using SP6 RNA polymerase to direct synthesis of antisense RNA from the plasmids Z1pSP64, Z2pSP65, Z3pSP64, and Z4pSP64. The same zinc-finger-specific sequences described above were transferred from the pCR1000 vector into the pSP64 or pSP65 vector to generate these plasmids.

RESULTS

Low-Stringency Hybridizations Reveal a New Zinc-Finger Protein Isoform Encoded by the BR-C

The initial molecular analysis by DiBello *et al.* (1991) revealed that the *BR-C* encodes a family of DNA-binding proteins with an amino-terminal core region linked by alternative splicing to one of three pairs (Z1, Z2, or Z3) of C_2H_2 zinc-finger domains. Because all of the cDNAs from the DiBello study were isolated by hybridization to sequences from either the core or the Z1 domain, we investigated whether other zinc-finger domains that were underrepresented in the initial cDNA library screens remained to be identified at the *BR-C* locus. We tested each of the three known zinc-finger domains for hybridization to clones of the *BR-C* genomic region between map positions 180 and 240 kb under low-stringency conditions and found a new zinc-finger sequence at map position 195 kb (Fig. 1). This domain, called Z4, encodes a pair of zinc-finger motifs similar to the Z1, Z2, and Z3 zinc-finger domains (Figs. 2B and 2C). No other DNA sequence with similarity to any of the four zinc-finger domains has been detected by cross-hybridization in the *BR-C* genomic region downstream of the core. From a screen of a larval cDNA library we identified a cDNA that contains the Z4 domain linked in frame to the core sequence, encoding an 877-aa protein of predicted molecular weight 92 kDa (Fig. 2A). Because the entire sequence of the *BR-C* core domain has previously been published in DiBello *et al.* (1991), we show in Fig. 2A only the portion of the Z4 cDNA sequence that is unique to this isoform.

Alignment of the zinc-finger pairs shows a high degree of similarity among the four *BR-C* protein isoforms (Fig. 2B). In addition to the cysteines, histidines, and hydrophobic residues conserved in all C_2H_2-type zinc-fingers (Miller *et al.*, 1985), similarity is seen throughout the zinc-finger domains, particularly in the region of finger 2 of each pair. The amino acid positions that have been shown to interact with DNA (Fig. 2B,* and P) by crystal-

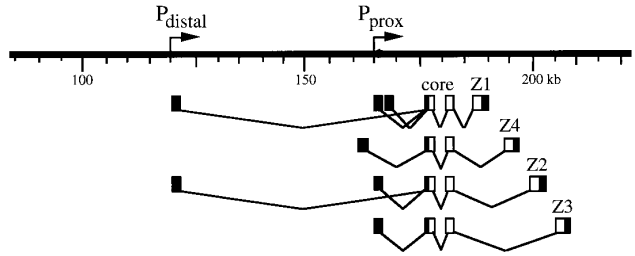
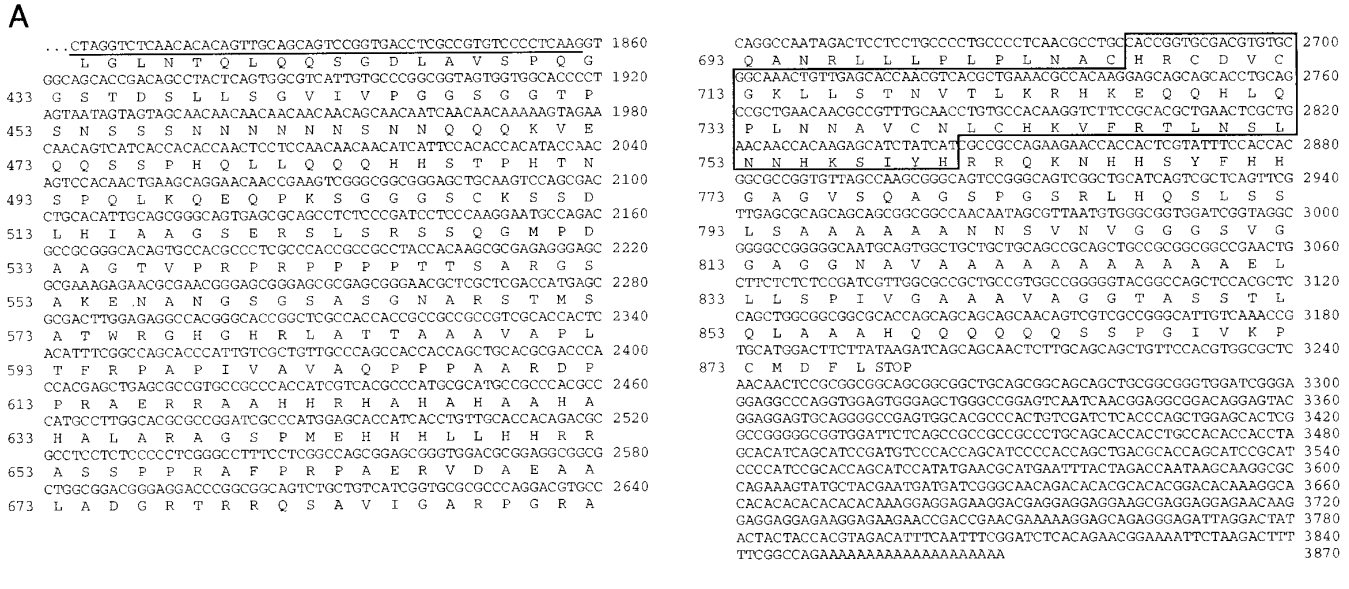


FIG. 1. *Broad-Complex* cDNAs and genomic map. cDNAs representing all forms of differentially spliced *BR-C* transcripts are shown mapped to the Chao and Guild (1986) genomic walk. Note that the 5′ ends of Z1 cDNAs map to 120, 165, or 167 kb and Z2 cDNAs map to either 120 kb or 165 kb (DiBello *et al.*, 1991). Open boxes indicate open reading frames; filled boxes represent untranslated regions in each cDNA. Although three putative promoter regions were described in DiBello *et al.* (1991), we find that only the probes specific to the 120-kb (P_{distal}) and 165-kb (P_{prox}) genomic regions hybridize to *BR-C* transcripts. The 5′ untranslated sequence of the Z4 cDNA that diverges from the core sequence maps to genomic position 163 kb and is not present in any other *BR-C* cDNA.



	Z1	Z2	Z3	Z4	TTK69	TTK88
Z1	100					
Z2	48 (53)	100				
Z3	39 (48)	39 (44)	100			
Z4	52 (68)	37 (52)	41 (54)	100		
TTK69	24 (46)	18 (46)	26 (52)	26 (42)	100	
TTK88	26 (44)	22 (44)	26 (43)	26 (39)	42 (61)	100

FIG. 2. The new *BR-C* zinc-finger pair Z4 is similar to previously identified *BR-C* zinc-finger pairs. (A) Nucleotide and predicted amino acid sequence of Z4 cDNA clone 28-I (GenBank Accession No. U51585). The complete 1680-bp *BR-C* core sequence has been reported in DiBello *et al.* (1991). The 3′ end of the core sequence is shown here underlined, along with the unique Z4 sequence. Numbers at the right refer to the nucleic acid sequence of the entire Z4 cDNA; those at the left refer to the amino acid sequence. The Z4 zinc-finger motif is boxed. No consensus polyadenylation signal is found near the 3′ stretch of adenines. (B) Alignment of *BR-C* zinc-finger pairs. The cysteine and histidine residues that define C_2H_2-type zinc-fingers are shown in bold, and the conserved hydrophobic residues are underlined (Miller *et al.*, 1985). For comparison, the zinc-finger domains of *tramtrack* (*TTK*) proteins are also shown (Harrison and Travers, 1990; Read and Manley, 1992). Six *TTK88* residues are displaced to optimize the alignment in (B) and (C). *TTK69* residues that have been shown by crystal structure analysis (Fairall *et al.*, 1993) to make base or phosphate contacts are marked with * or P, respectively. Identical amino acids as well as conservative matches are shaded (light gray) to highlight similarities among the *BR-C* proteins. Alternative matches among *BR-C* proteins and *TTK* proteins are also shaded (dark gray). (C) The degree of similarity among the *BR-C* family of zinc-finger proteins. The first number in each column represents the percent identical matches across the 54-aa zinc-finger domains shown in the alignments above. The percent positive matches (identities + conservative substitutions) in these regions are shown in parentheses. Similarities to *TTK69* and *TTK88* protein zinc-finger domains are also shown.

lographic analysis of the *Zif268* and *GL1* zinc-finger domains (Pavletich and Pabo, 1991; Pavletich and Pabo, 1993) and finger 2 of the *TTK69* zinc-finger domain (Fairall *et al.*, 1993) are highly conserved in finger 2 of *BR-C* proteins. Somewhat greater diversity is seen among *BR-C* proteins at amino acid positions in finger 1 that correspond to the base-contacting residues defined for finger 1 of the *TTK69* protein (Fig. 2B).

A BlastP database search (Altschul *et al.*, 1990) for sequence similarities across the 54-aa Z4 zinc-finger region

found that the protein with the highest scoring match to Z4 is Z1 with 68% conservative matches ($P = 10^{-16}$) (Fig. 2C). The next highest-scoring matches to Z4 were Z3 with 54% and Z2 with 52% conservative matches ($P = 10^{-7}$). For comparison, non-*BR-C* C_2H_2 zinc-fingers that match Z4 across the entire 54-aa binding domain include the first two of six fingers of *Drosophila SYR*β, a regulatory protein encoded by the *serendipity* locus (Vincent *et al.*, 1985) with 48% conservative matches ($P = 10^{-4}$), and *TTK69*, a 2-fingered protein (Harrison and Travers, 1990) with 42% conservative matches ($P = 10^{-3}$). Although the *TTK69* zinc-finger pair is similar to all four *BR-C* zinc-finger pairs, it is most similar to Z3, with 52% conservative matches ($P = 10^{-7}$) (Fig. 2C). These results show that most *BR-C* zinc-finger domains exhibit similarity to each other and to the zinc-finger domains in other proteins in the range of 40 to 55%. However, the 68% similarity between the Z1 and Z4 domains is unusually high and suggests similar specificity in DNA binding.

These comparisons also suggest that the set of four pairs of closely related *BR-C* zinc-finger domains may have arisen by sequential duplication events at the locus during evolution. Presumably, duplication of an original zinc-finger coding region would allow for subsequent expansion and refinement of the regulatory functions encoded by the *BR-C* locus. The high degree of similarity between Z1 and Z4 zinc-finger domains suggests that they diverged most recently.

With the isolation of cDNAs encoding the Z4 isoform we have now identified all major classes of DNA-binding proteins produced by the *BR-C*. Although all *BR-C* proteins are similar to each other throughout the zinc-finger domain, they recognize different target DNA sequences. von Kalm *et al.* (1994) have identified consensus binding sites for each *BR-C* protein isoform in the *Sgs-4* regulatory region. These binding sites contain essentially invariant three nucleotide cores, like those found in *TTK69* consensus binding sites. Binding studies have also shown that the *TTK69* protein requires several residues N-terminal to the finger 1 motif for full binding activity (Fairall *et al.*, 1992). Divergence among *BR-C* zinc-finger domains in the corresponding sequence (Fig. 2B) suggest, by analogy to *TTK69*, that differences in the DNA-binding specificities of *BR-C* proteins may depend largely on the structure of finger 1 and its N-terminal sequence.

The Z1 RNA Isoform Is Distinguishable from Other BR-C Zinc-Finger Isoforms by Size

The RNA expression profile of all early ecdysone genes, including the *BR-C*, was previously described by Andres *et al.* (1993) in their examination of ecdysone-regulated gene activity during metamorphosis. They found that four size classes of *BR-C* RNAs, recognized by a core probe common to all *BR-C* isoforms, were abundantly expressed at the end of larval development, while only the smallest RNA class continued to accumulate into the prepupal stages. Because all four size classes are abundant at the white (0 hr) prepupal stage, we attempted to identify zinc-finger-specific RNA isoforms among these *BR-C* RNAs.

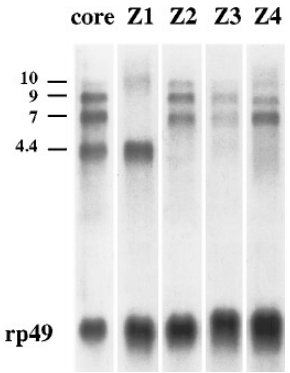


FIG. 3. Zinc-finger-specific RNAs expressed in white prepupae. Total RNA prepared from white (0 hr) prepupae stage animals (10 μg) was hybridized by Northern blot analysis with probes specific to the core or to each zinc-finger exon—Z1, Z2, Z3 or Z4 (as indicated above each RNA panel). The 10-, 9-, 7- and 4.4-kb RNA size classes detected with these probes are indicated at the left of the panels. A probe specific to the ribosomal protein gene *rp49* (O'Connell and Roshbash, 1984) is included as an internal control for loading.

First, total RNA isolated from white prepupae was probed for *BR-C* transcripts with a core sequence by Northern analysis (Fig. 3). This probe detects the same set of four transcripts previously seen by Andres *et al.* (1993) that we measure as 10, 9, 7, and 4.4 kb in size. Samples of white prepupae RNA were also probed with zinc-finger-specific sequences. Using a Z1-specific probe, we detect a predominant 4.4-kb RNA plus a faint, diffuse band in the 10-kb size range. In contrast, the Z2-, Z3-, and Z4-specific probes do not detect the 4.4-kb transcript. Each hybridizes instead to the three large RNA size classes of 10, 9, and 7 kb. Although similar in size, the relative abundance of each RNA size class differs among these three zinc-finger isoforms. The predominant Z2 and Z3 transcripts are the 9-kb and 7-kb forms, while the predominant Z4 transcript is the 7-kb form. These data show that all four *BR-C* zinc-finger isoforms are expressed in white prepupae and that only one zinc-finger isoform (Z1) is specific to any *BR-C* RNA (the 4.4-kb form) detected on a Northern blots. We have been unable to distinguish the other isoforms from each other by size.

All BR-C Isoforms Are Rapidly Induced as a Primary Response to Ecdysone in Cultured Imaginal Discs

The presence of all four *BR-C* zinc-finger isoforms in white prepupal stage animals could result in part from overlapping tissue-specific profiles of *BR-C* expression. The studies of Huet *et al.* (1993) show that relative levels

of *BR-C* zinc-finger isoforms vary among tissues during metamophosis. Therefore, we examined the profile of *BR-C* expression in one tissue type: imaginal discs. We chose imaginal discs, precursors of the adult head and thoracic integument and appendages, for several reasons. First, appendage-forming imaginal discs undergo a series of rapid, tightly coordinated morphogenetic changes in response to the rise in ecdysone titer at the end of the third instar larval period (reviewed in Fristrom and Fristrom, 1993, and von Kalm *et al.*, 1995). Second, these ecdysone-dependent morphogenetic changes, including disc elongation and eversion, have been shown genetically to require *BR-C* function (Fristrom *et al.*, 1981; Kiss *et al.*, 1988). Third, the morphogenetic development of imaginal discs isolated from larvae can be reproduced faithfully in organ culture and equated directly to their development in the animal during metamorphosis. The relation between the developmental timing of imaginal discs cultured *in vitro* and those developing *in situ* has been determined on the basis of morphology (evagination) (Fristrom *et al.*, 1969, Fristrom, 1972), differentiation of the cuticulin layer of the pupal cuticle (Fristrom and Liebrich, 1986), and synthesis and deposition of chitin and pupal cuticle proteins after a 6-hr ecdysone ''pulse'' (Fristrom *et al.*, 1982, Doctor *et al.*, 1985, Fristrom *et al.*, 1986). These results indicate that discs incubated with ecdysone for 6, 12, and 18 hr *in vitro* are approximately equivalent to those developing *in situ* in prepupae at 0–1, 6–7, and 12–14 hr after puparium formation (AP), respectively. This temporal correspondance has also been demonstrated at the level of gene expression. The imaginal disc-specific pupal cuticle protein (PCP) transcripts *Edg78E* and *Edg84A* shown by Fechtel *et al.* (1988) to peak in cultured discs during 12–18 hr of incubation are also shown by Andres *et al.* (1993) to peak *in vivo* in 6- to 12-hr AP prepupae.

Because the pool of mass-isolated imaginal discs is composed of several disc types, approximately 50% wing discs, 30% leg discs, and 5% eye-antennal discs, we first tested each disc type for *BR-C* expression (Fig. 4A). We examined imaginal discs that were cultured for 6 hr in the presence of ecdysone and therefore developmentally equivalent to discs in 0-hr prepupae. We found that both leg and wing discs express the same set of four *BR-C* RNAs found in white (0 hr) prepupae (Fig. 3) or in a mixed population of 6-hr cultured discs (see below). In contrast, in eye discs (removed from the antennal portion) we found only a very low level of the 4.4-kb Z1 isoform. Because of the small contribution of eye discs to the total pool of mass-isolated imaginal discs, these data show that the *BR-C* expression pattern detected in the following experiments primarily reflects expression from appendage-forming discs (wings and legs).

Using cultured, mass-isolated imaginal discs, we examined *BR-C* induction in the presence of ecdysone over time (Fig. 4B). With a core probe, we detect *BR-C* transcripts at low levels in untreated discs (0 hr). However,

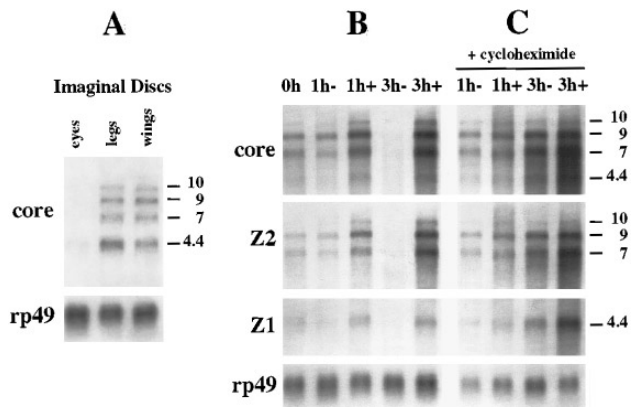


FIG. 4. *BR-C* transcripts are induced in imaginal discs cultured in the presence of ecdysone with or without cycloheximide treatment. (A) Eye discs (antennal portion removed), leg discs, and wing discs sorted from a population of mass-isolated imaginal discs incubated for 6 hr in the presence of ecdysone were used to prepare total RNA. Two micrograms of each sample (equivalent to ~100 eye discs, 50 leg discs, or 25 wing discs) was hybridized by Northern blot analysis with probes specific to the core and to *rp49*. (B) Mass-isolated imaginal discs were cultured for 1 or 3 hr in the presence (+) or absence (−) of 2×10^{-6} *M* ecdysone. (C) A set of identical cultures were treated with 4×10^{-6} *M* cycloheximide added 30 min before the addition of ecdysone. Probes specific to the core, Z1 or Z2 zinc-finger exons, and *rp49* were hybridized sequentially to total RNA (10 μg) on a single Northern blot used in (B) and (C). The Z2 results are representative of Z2, Z3, and Z4 RNA patterns detected in these experiments. Cycloheximide increases *BR-C* RNA accumulation to high levels that cause the 3h+ lanes in (C) to appear overexposed relative to the other lanes.

the levels of these transcripts rapidly increase when imaginal discs are cultured in the presence of ecdysone (2×10^{-6} *M*) (Fig. 4B, 1h+ and 3h+ lanes) and decline when cultured without added ecdysone (Fig. 4B, 1h− and 3h− lanes), clearly showing that accumulation of *BR-C* transcripts is enhanced by ecdysone *in vitro*. We also find that all zinc-finger RNA levels increase in the presence of ecdysone, but that Z2 RNAs (as well as Z3 and Z4 RNAs, data not shown) accumulate to higher levels than Z1 RNAs by 3 hr of incubation.

The rapid induction of a previously activated *BR-C* gene is consistent with the ecdysone response of this locus, the 2B5 puff, in salivary gland chromosomes. The puffing studies of Ashburner (1972) showed that the 2B5 site, unlike the 74EF and 75B sites, has already formed a small puff at PS1 in mid-third instar larvae (~12 hr before pupariation) *in vivo*, as well as within 1 hr of salivary gland culture in the presence of 10^{-6} *M* ecdysone. The timing of this response suggests that *BR-C* induction is a primary response to ecdysone, as is the case with the other early ecdysone genes *E74*, *E75*, and *E63* (Andres and Thummel, 1995; Segraves and Hogness, 1990; Thummel *et al.*, 1990). We therefore determined whether ecdysone can induce all *BR-C* transcripts

in the presence of the protein synthesis inhibitor cycloheximide (Fig. 4C).

We found that cycloheximide treatment itself greatly increases *BR-C* RNA levels. The effect is equivalent for both the Z1 and Z2 RNA isoforms (as well as the Z3 and Z4 isoforms, data not shown). There is substantial evidence from the literature that such increases in transcript accumulation are usually due to the stabilizing effect of cycloheximide on polysomal RNAs. Alternatively, these data could indicate that the *BR-C* is normally repressed in the absence of ecdysone and that this repression requires protein synthesis. However, even in the presence of cycloheximide, the addition of ecdysone to imaginal discs cultures (Fig. 4C, 1h+ and 3h+ lanes) increases *BR-C* RNA levels above the levels found in cultures incubated without ecdysone (Fig. 4C, 1h− and 3h− lanes). This ecdysone-dependent increase, detected with core and zinc-finger-specific probes, indicates that all *BR-C* zinc-finger isoforms are induced as a primary response to hormone in imaginal discs, extending the initial observations of Chao and Guild (1986).

A Switch in BR-C Zinc-Finger Isoform Expression Occurs in Imaginal Discs after 6 hr of Ecdysone Exposure

We next followed the profile of *BR-C* expression in cultured imaginal discs over longer periods of time (Fig. 5A). All zinc-finger RNA isoforms accumulate during the first 3 to 6 hr of culture in the presence of ecdysone, but Z2, Z3, and Z4 transcripts predominate. After 6 hr of culture, however, a switch in isoform abundance occurs. Z1 becomes the predominant *BR-C* isoform. This switch occurs both when imaginal discs are incubated in the continuous presence of ecdysone for 8 or 10 hr and when a 6-hr pulse of ecdysone is followed by further incubation in its absence (conditions that mimick the rise and fall of ecdysone titer *in vivo*) (Fig. 5B, compare lanes 6+/2− and 8+ or lanes 6+/4− and 10+). The Z1 transcript persists for at least 18 hr of culture whether or not the hormone is removed after the first 6 hr of incubation (data not shown). These results indicate that the maintenance of Z1 RNA levels and the reduction in Z2, Z3, and Z4 RNA levels after 6 hr of incubation are independent of ecdysone.

We also measured zinc-finger-specific RNA levels by RNase protection assays (Fig. 6) to determine the total abundance of Z2, Z3, and Z4 zinc-finger RNAs spread over three or more size classes that might be difficult to observe by Northern analysis as their abundance decreases. The Northern blot analysis (Fig. 5A) shows that Z2, Z3, and Z4 RNA levels peak by 3 hr and are already dropping by 6 hr of culture. The RNase protection assays show that Z2 and Z3 RNAs (and to a lesser extent, Z4 RNAs) are clearly the most abundant *BR-C* isoforms at the beginning of the ecdysone response, reaching levels 4- to 6-fold above the Z1 isoform by 6 hr. By 10 hr however, Z2, Z3, and Z4 RNAs have declined to levels 2.5-fold below Z1 RNA levels. These results confirm the switch in zinc-finger isoform accumulation from predominant Z2, Z3, and Z4 levels at the beginning of the ecdysone response to predominant Z1 levels after 6 hr of exposure to ecdysone. Note that the timing of this switch (after 6 hr of culture in the presence of ecdysone) corresponds roughly *in vivo* to puparium formation (0 hr

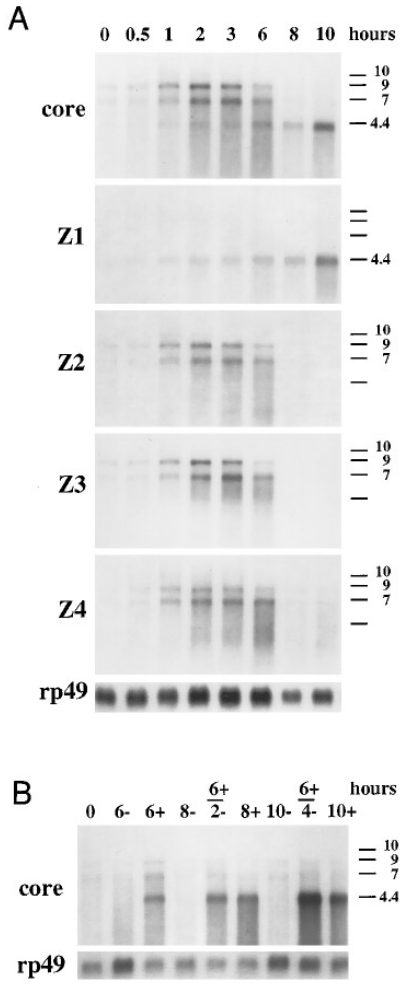


FIG. 5. A temporal switch in *BR-C* zinc-finger RNA expression. (A) Total RNA was prepared from mass-isolated imaginal discs cultured in the presence of 2×10^{-6} *M* ecdysone for 0, 0.5, 1, 2, 3, 6, 8, or 10 hr. Samples from each culture (10 μg) on single Northern blot were hybridized sequentially with probes specific to the core, each zinc-finger exon, and the loading control *rp49*. (B) To compare different culture conditions, imaginal discs were first incubated in the absence (−) or presence (+) ecdysone for 6 hr, after which time the cultures were either harvested (6− and 6+ lanes) or washed and incubated with fresh culture media for another 2 or 4 hr in the absence (8−, 6+/2−, 10−, and 6+/4− lanes) or presence (8+ and 10+ lanes) of ecdysone. Ten micrograms of total RNA prepared from each culture was hybridized by Northern blot analysis with probes specific to the core and to *rp49*. This blot has been slightly overexposed to highlight the disappearance of the 10-, 9-, and 7-kb transcripts after 8 or 10 total hr of incubation.

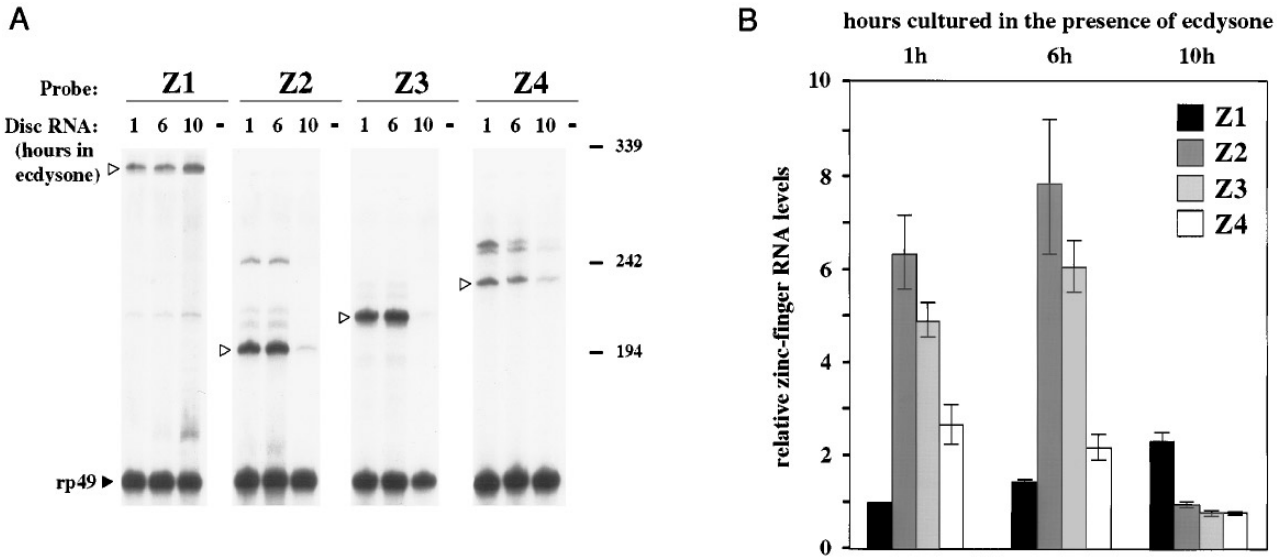


FIG. 6. A temporal switch in *BR-C* zinc-finger RNA expression. (A) Total RNA (10 μg) prepared from imaginal discs cultured for 1, 6, or 10 hr in the presence of 2×10^{-6} *M* ecdysone was assayed by RNase protection. RNA probes (described under Materials and Methods) specific to zinc-finger exon Z1, Z2, Z3, or Z4 protect fragments of 322, 197, 215, or 233 nucleotides, respectively, which correspond in size to the major band (marked by open arrowhead) in each lane. Minor bands of higher molecular weight are due to underdigestion by RNase. These additional bands were included in the quantification of relative RNA levels represented in the graph in (B). An *rp49* RNA probe that protects a 152-nucleotide fragment (marked by closed arrowhead) was also included as a loading control in each RNase assay. In control lanes labeled (−), zinc-finger and *rp49* probes were hybridized in the absence of imaginal disc RNA. The positions of RNA size markers are indicated at the right of the panel. (B) Relative levels of each zinc-finger isoform were measured using a Molecular Dynamics Phosphorimager, corrected for differences in loading by comparison of *rp49* measurements, and adjusted for differences in specific activities of the probes (determined empirically) and lengths of their hybridizable sequences. Error bars indicate the standard deviation of triplicate experiments.

prepupae), a metamorphic stage of dramatic change in the animal.

Two BR-C Promoter Regions Direct Similar Transcription Profiles in Imaginal Discs

Our results show that all *BR-C* zinc-finger RNA isoforms are induced as a primary response to ecdysone; however, Z1 isoform levels are regulated differently from all other zinc-finger isoforms over time. We therefore investigated whether differential usage of the two promoter regions of the *BR-C* could account for the two divergent transcript profiles.

The 5′ ends of *BR-C* cDNAs map to one of four different positions within two regions of the genomic map that are separated by 45 kb (DiBello *et al.*, 1991) (Fig. 1). Transcription start sites within map positions 120, 165, and 167 kb have been identified within these two regions by primer extension and RNase protection (DiBello, 1992). We find that probes specific to exons at map positions 120 and 165 kb detect *BR-C* transcripts in imaginal discs (see below). In contrast to these two regions, a probe specific to map position 167 kb does not hybridize to any stable *BR-C* RNA from imaginal discs or whole animals (data not shown). We do not completely understand the discrepancy between our results and those of DiBello (1992) regarding the 167-kb region. However, because DiBello (1992) mapped this transcription start site using a highly sensitive RNase protection assay and RNA from whole larvae, it is possible that this promoter region is used infrequently in larvae and not at all in imaginal discs. Finally, we find that a probe specific to genomic position 163 kb, where the 5′ end of the Z4 cDNA 28-I maps, does not detect any *BR-C* transcripts either in imaginal discs or whole animals (data not shown). We hypothesize that the cDNA sequence that maps to this genomic position does not represent the 5′ end of a full-length transcript, but corresponds instead to an incompletely processed RNA incorporated into the larval cDNA library. Thus, we can identify only two functional promoter regions: one at genomic map position 120 kb (P_{distal}) and the other at 165 kb (P_{prox}).

We find that both the P_{distal} and P_{prox} promoter regions are responsive to ecdysone and direct similar, though not identical, patterns of *BR-C* transcription (Fig. 7). Steady-state levels of *BR-C* transcripts originating at P_{prox} peak earlier than those originating at P_{distal}. Because of the shorter length of the P_{prox} transcription unit, P_{prox} transcripts could accumulate earlier than P_{distal} even when both promoter re-

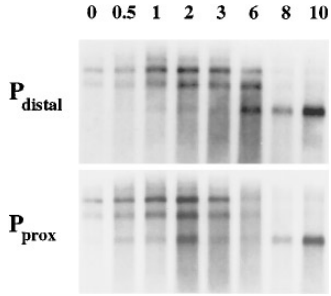


FIG. 7. P_{distal} and P_{prox} direct similar patterns of *BR-C* expression in imaginal discs. The Northern blot shown in Fig. 5(A) was hybridized sequentially with probes specific to the P_{distal} or P_{prox} promoter regions.

gions are simultaneously activated (and repressed). A similar observation has been made for the two widely separated promoters at the ecdysone-inducible *E74* locus (Karim and Thummel, 1991). We also see that Z1 levels associated with P_{prox} are more dynamic, peaking at 2 hr and again by 10 hr of incubation. Despite these small differences, we find that each promoter region synthesizes all four RNA size classes, including the 4.4-kb Z1 isoform and presumably each of the larger isoforms as well, even in the presence of cycloheximide (data not shown). Each promoter region also exhibits the switch in transcript prevalence after 6 hr of culture. Due to their similarity in size, we cannot determine from these data whether each of the Z2, Z3, or Z4 isoforms are transcribed from each promoter region. However, we have isolated Z2 cDNAs that, like Z1 cDNAs, include sequences from either (though never both) the P_{distal} or P_{prox} promoter region (see Fig. 1). Thus, the two different transcript profiles at the *BR-C*, represented by the Z1 and Z2 RNA isoforms, and consequently the isoform switch as well, do not depend on differential promoter region usage.

Posttranscriptional Processing May Contribute to the Large Size and Multiple Forms of BR-C Transcripts

The 7-, 9-, and 10-kb transcripts detected on Northern blots are considerably larger than the 3.5 to 4.4 kb size of the *BR-C* cDNAs isolated during these studies and those of DiBello *et al.* (1991). Therefore, we asked whether any of them might represent incompletely spliced forms of *BR-C* mRNAs. In order to test this hypothesis, we used sequences from intronic regions of the *BR-C* locus to probe for hybridization to ecdysone-induced imaginal disc RNA (Fig. 8). These intronic regions, shown on the genomic map in Fig. 8, are defined by structural analysis of *BR-C* cDNAs.

We find that some intronic sequences are present in the 10-kb RNA size class that is detected by exon probes. For example, sequences from the first intron after the core, the core/Z1 intron, are present in a 10-kb RNA. Although we cannot tell from these data whether all or part of the intron is present in this RNA form, the 10-kb size corresponds to that expected for transcripts containing the Z1 exon linked to the core exon by the entire intervening sequence. The smaller RNAs also detected with this probe may represent splicing intermediates of the core/Z1 intron. The core/Z1 intron must be removed to produce a mature Z1 mRNA. However, these results indicate that sequences from within this intronic region are present in *BR-C* RNAs, even when the levels of spliced Z1 mRNAs increase (i.e., in 10-hr disc cultures, Fig. 8).

We also find that sequences from the intron between Z1 and Z4 (the Z1/Z4 intron) hybridize to the 10-kb RNA size class (Fig. 8). This could indicate that some Z4 RNAs accumulate as incompletely spliced forms. Consistent with this observation, we have found Z1/Z4 intron sequences in a number of presumably partially spliced Z4-containing cDNAs that were isolated during the library screen (data not shown). These results also raise the possibility that Z1 and Z4 exons could be present in the same partially processed transcript.

Other intronic regions do not appear in any *BR-C* RNA. A probe specific to the 5′ intron (separating the two promoter regions) detects a smear that we assume represents active processing of RNAs of varying sizes (Fig. 8). This smear is also seen in the background of the set of four *BR-C* size classes in the panel probed, for reference, with a core exon sequence. A probe specific to the core intron that splits the core region into two exons does not hybridize to any core-containing *BR-C* transcript, but detects a smaller 3-kb RNA. This RNA may represent the released intron itself. Together, these results indicate that intervening sequences upstream of, or within, the core domain are rapidly removed from *BR-C* transcripts. Finally, in contrast to the Z1/Z4 intron, sequences corresponding to the Z4/Z2 intron and the Z2/Z3 intron are not detected in any *BR-C* RNA (Fig. 8).

We also found evidence suggesting that alternative 3′ untranslated sequences contained in Z2, Z3, and Z4 *BR-C* RNAs, but missing from their corresponding cDNAs, could contribute to the large size of these isoforms. For example, we have identified portions of two alternative 3′ UTR sequences in some partial Z4 clones (data not shown). In addition, we find that a probe sequence immediately downstream of the Z3 coding region is present in the 9-kb, but not in the 10-kb or 7-kb, RNA size class (see Z3 3′ UTR panel in Fig. 8). This result suggests that differences in size among the three Z3-containing RNA classes are due, at least in part, to differences in the size of alternative 3′ UTR sequences.

Together, these results suggest that a fraction of Z1 RNAs may accumulate as large 10-kb incompletely spliced molecules. In contrast, sequences from intronic regions do not appear to contribute to size variations among the large Z2 and Z3 RNAs. Other evidence suggests instead that alternative 3′ UTR sequences are responsible for the large size and multiple forms of Z2 and Z3 RNA isoforms. Finally, while

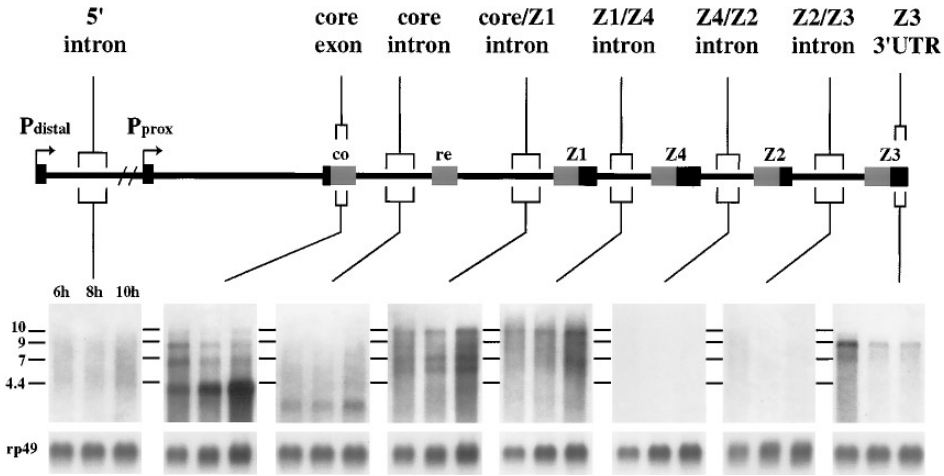


FIG. 8. Some intronic sequences are present in *BR-C* RNAs. Total RNA was prepared from imaginal discs cultured for 6, 8, or 10 hr in the presence of 2×10^{-6} *M* ecdysone. Ten-microgram samples were hybridized by Northern blot analysis with intron-specific probes (described under Materials and Methods) whose positions on the structural map are indicated by brackets above each panel. These probes are restricted to intronic regions which, based on our analysis of *BR-C* cDNA structure, are indicated by a thick line while exons are shown as boxes. The gray regions of boxes indicate open reading frames; the black regions indicate untranslated regions of each exon. *rp49* RNA levels are also shown for each Northern blot. The *BR-C* RNA pattern detected with a core exon probe, shown for comparison, is underexposed ~5× relative to the other Northern blot panels.

some size variations in Z4 RNAs may also be accounted for by differential incorporation of 3′ UTRs, the largest Z4 form might represent a partially spliced RNA. These results suggest that differential zinc-finger isoform expression of the *BR-C* is regulated by posttranscriptional events.

DISCUSSION

Four Broad-Complex Zinc-Finger Isoforms Are Induced by Ecdysone in Imaginal Discs

Transcriptional activation of the *Broad-Complex* is one of the earliest responses to the metamorphic changes in ecdysone titer. Andres *et al.* (1993) have shown that *BR-C* transcripts are initially expressed at low levels in early-third instar larvae and rapidly accumulate with the rising hormone titer during the last several hours of this final larval instar. Although *BR-C* zinc-finger RNAs and protein isoforms have been detected in every tissue examined during metamorphosis (Emery *et al.*, 1994; Huet *et al.*, 1993), the relative ratio of zinc-finger isoforms differs among tissues, implying that differential expression of *BR-C* isoforms may contribute to the diversity of ecdysone responses among tissues. For example, Z1 and Z3 are the predominant isoforms expressed in late third instar larval salivary glands; at the same stage, Z2 predominates in imaginal discs (Huet *et al.*, 1993; von Kalm *et al.*, 1994, and Fig. 6). Thus far, the molecular means by which these differences arise has not been understood. The studies we present here provide the first insights into the regulation of differential *BR-C* zinc-finger expression.

Our studies have concentrated on the ecdysone response of the *BR-C* in cultured imaginal discs consisting mostly of appendage-forming leg and wing discs. We find that all four zinc-finger RNA isoforms are induced in response to ecdysone in imaginal discs. These induction profiles, however, fall into two different classes. The Z2, Z3, and Z4 isoforms are rapidly induced, but disappear after 6 hr of exposure to ecdysone. Their expression profiles are quite distinct from that of the Z1 isoform which accumulates more slowly over a longer period of time (Fig. 5A). This switch in zinc-finger isoform expression does not occur in salivary glands, where Z3 RNAs reappear along with Z1 RNAs after a transient decline at puparium formation (Huet *et al.*, 1993). The imaginal disc-specific switch is, however, detected in whole animals at the beginning of the prepupal period (Andres *et al.*, 1993, and data not shown). This suggests that *BR-C* RNA expression predominates in imaginal discs over other tissues during metamorphosis or that this particular isoform switch is common to the majority of tissue types at this time in development.

This *BR-C* RNA isoform switch is similar to the protein isoform switch detected in imaginal discs by Emery *et al.* (1994). They report a shift from predominant Z2 protein expression in larval discs to Z1 protein expression in prepupae. They did not assay for Z4 expression in these studies. However, they did not detect any Z3 protein in 0-hr prepupal imaginal discs, although we detect Z3 RNA at this time. Although we do not fully understand this apparent discrepancy, Emery *et al.* (1994) were also unable to detect Z3

protein in late-third instar salivary glands where Z3 RNA is known to be abundant. They postulate that Z3 protein levels may have fallen below their level of detection, or that the Z3 isoform may be subject to additional posttranscriptional regulatory events.

A Model for Posttranscriptional Regulation of the Zinc-Finger RNA Switch

The *BR-C* isoform switch we observe in cultured imaginal discs is like that previously observed in whole animals (Andres *et al.*, 1993). Here, however, cultured discs are isolated from any immediate humoral influences imposed by the animal's hormonal physiology. Moreover, the switch occurs whether or not ecdysone is removed after several hours of culture. Thus, the molecular changes we see in these discs, once triggered by the addition of ecdysone, are driven by a preprogrammed tissue-autonomous regulatory mechanism that is independent of subsequent external cues.

At least some of the changes in *BR-C* isoform expression appear to be regulated posttranscriptionally. First, we established that the switch in zinc-finger isoform expression does not result from differential use of the two *BR-C* promoter regions P_{distal} and P_{prox} (Fig. 7). Additional analyses of transcript patterns found in *BR-C* mutants that remove the distal promoter region indicate that each can drive synthesis of the full repertoire of zinc-finger isoforms (C.B. and J.W.F., unpublished results). Therefore, zinc-finger isoform expression does not depend on the choice of promoter region. Second, we found that sequences from intronic regions upstream of the Z1 exon and the Z4 exon are present in *BR-C* RNAs of the largest size class (Fig. 8). These results suggest that splicing of some *BR-C* RNA forms is regulated.

We propose that this switch in zinc-finger transcript accumulation results from a temporal change in splice site choice. An alternative model is one in which the switch is regulated by differential transcript stability, but we have no evidence that addresses this hypothesis. What we do see is that when the *BR-C* is first induced at the onset of metamorphosis Z2, Z3, and Z4 RNAs predominate over Z1. However, the 5′ position of the Z1 exon dictates that synthesis of Z2, Z3, or Z4 exon-containing RNAs must involve transcription through the Z1 exon. The Z1 exon (plus the intronic sequences surrounding this exon) could initially be eliminated from the pool of early *BR-C* RNAs by splicing. We propose that, after several hours of incubation with ecdysone, a switch in splice site choice occurs and the Z1 exon acceptor site is preferentially used. The temporal nature of this switch in splice site choice further suggests that factors regulating selective splicing are themselves ecdysone regulated. Such factors might establish the Z1 exon acceptor site as a preferred splice site, prevent use of the Z2, Z3, and Z4 acceptor sites, or terminate transcription immediately downstream of the Z1 exon, making the Z1 exon acceptor site the default splice. Alternatively, Z1-specific repressive splicing factors might be repressed by ecdysone. Whatever the mechanism determining splice acceptor site choice, the result would be to alter the relative levels of zinc-finger RNAs.

Finally, we postulate that posttranscriptional regulation of *BR-C* zinc-finger RNAs is tissue-specific. The switch from predominant Z2, Z3, and Z4 expression in larvae to Z1 expression in prepupae occurs in imaginal discs. A different kind of switch occurs in salivary glands where Z3 predominates throughout the third instar, while equivalent levels of Z1 accumulate only at the end of this period (Huet *et al.*, 1993; von Kalm *et al.*, 1994). Both Z1 and Z3 continue to predominate in salivary glands during the prepupal period. If *BR-C* zinc-finger RNA expression in salivary glands is also regulated posttranscriptionally, then differences in isoform ratios between glands and discs are presumably be due to tissue-specific differences in factors controlling splice site choice.

The Switch in BR-C Zinc-Finger Expression Restricts the Timing of Downstream Ecdysone Responses

The availability of data on the dynamics of *BR-C* RNA and protein isoform expression, on the nature of the genetic functions, and on the regulation of downstream genes provides, for the first time, an opportunity to understand the major role of the *BR-C* during metamorphosis. The switch in *BR-C* RNA isoform expression that we observe in imaginal discs, also manifested as a dramatic shift in protein expression from Z2 to Z1 (Emery *et al.*, 1994), occurs at puparium formation. The precipitous nature of the switch in zinc-finger RNA isoform expression and the resulting change in *BR-C* protein isoform ratios at a key stage in metamorphosis impels us to believe that alterations in the function of the *BR-C* are critical for the temporal regulation of downstream target genes during metamorphosis.

Genetic analyses have shown that specific target genes have different requirements for *BR-C* function. Three different complementation groups at the *BR-C* locus, *br*, *rbp*, and *2Bc*, have long been recognized genetically (Belyaeva *et al.*, 1980; Kiss *et al.*, 1988). Mutations in any of these complementation groups affect gene expression and consequently the developmental outcome of tissues such as salivary glands and imaginal discs that are destined for different fates during metamorphosis. We originally proposed in DiBello *et al.* (1991) that these complementation groups represented subfunctions corresponding to specific zinc-finger proteins; that Z1 mediates rbp^+ function, Z2 mediates br^+ function, and Z3 mediates $2Bc^+$ function. This notion is now amply supported by current studies (Emery *et al.*, 1994, K. Crossgrove, C.B., J.W.F., and G. Guild, unpublished results; C.B., L. von Kalm, and J.W.F., unpublished results). No genetic correlation has yet been made for the newly described Z4 isoform.

Several genes regulated by the *BR-C* have now been identified. Genetic and transgenic analyses of *BR-C* regulation of downstream genes shows that, in at least three

cases, the Z1 isoform provides a regulatory function opposite to that of other *BR-C* isoforms. One case particularly relevant to the current discussion involves a gene called *Brg-P9* that is expressed in imaginal discs. *Brg-P9* expression, which is induced at puparium formation in wild-type animals, is repressed by br^+ (Z2) function and activated by rbp^+ (Z1) function (J. Emery and G. Guild, personal communication). This implies that just prior to puparium formation, high levels of Z2 protein repress *Brg-P9* expression, while in prepupae, high levels of Z1 protein induce *Brg-P9* expression. Thus, the switch from predominant Z2 to Z1 isoform expression may mediate the precise temporal control of *Brg-P9* expression.

Opposing *BR-C* functions also appear to regulate the expression of salivary gland-specific genes. The intermolt gene *Sgs4* requires rbp^+ function for induction in early larvae (Guay and Guild, 1991; Karim *et al.*, 1993), while the ''late ecdysone'' *L71* genes require rbp^+ function for their expression in prepupae (Guay and Guild, 1991). Recent studies show that expression of a Z1 transgene can rescue defective *Sgs4* or *L71* expression in rbp^- mutants (C.B., L. von Kalm, and J.W.F., unpublished results; K. Crossgrove, C.B., J.W.F., and G. Guild, unpublished results). The repressing function of $2Bc^+$, later required at puparium formation (Karim *et al.*, 1993), also correlates with the observation that Z3 transgene expression represses *Sgs4* in wild-type animals. Thus, as with *Brg-P9*, the Z1 protein isoform performs a regulatory function opposite to that of another *BR-C* isoform (here, Z3). An additional genetic requirement for $2Bc^+$ function in larvae for the initial induction of *Sgs4* is not as easily understood in terms of *BR-C* proteins. However, the demonstration by von Kalm *et al.* (1994) that all *BR-C* proteins can footprint *Sgs4* regulatory sequences and the requirement of these sites for the *in vivo* expression of this gene suggest that *BR-C* proteins such as Z1 and Z3 may compete for control of the *Sgs4* regulatory element.

As other *BR-C* targets are identified and analyzed, we expect to find that their temporal expression is also often controlled by opposing *BR-C* functions. For example, *Dopa decarboxylase* (*Ddc*) transcripts accumulate rapidly at the end of larval development and decline after puparium formation (Andres *et al.*, 1993; Hodgetts *et al.*, 1995). br^+ function is required for the initial expression of *Ddc* in the larval epidermis (Hodgetts *et al.*, 1995). Correspondingly, Z2 transgene expression is found to activate *Ddc* in br^- mutants, while Z1 or Z4 transgenes repress this gene in wild-type animals (L. von Kalm and J.W.F., unpublished results). *In vitro* footprinting studies of Hodgetts *et al.* (1995) show that binding sites for these proteins overlap in the epidermal response element in the first intron of *Ddc* (Shen and Hirsh, 1994). Thus, *BR-C* proteins may compete for control of the *Ddc* epidermal element.

We suggest that the primary role of the *BR-C* is to restrict temporal expression of downstream genes to discrete developmental periods. The results from this and other studies indicate that developmental changes in the ratios of *BR-C* products, particularly at puparium formation, could mediate changes in *BR-C* function. The evidence from DNA-binding studies supports the idea that changes in *BR-C* protein isoform ratios may lead to changes in the regulation of downstream targets.

ACKNOWLEDGMENTS

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