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The *Broad-Complex* directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis

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In *Drosophila*, all of the major metamorphic transitions are regulated by changes in the titer of the steroid hormone ecdysone. Here we examine how a key regulator of metamorphosis and primary ecdysone response gene, the *Broad-Complex*, transmits the hormonal signal to one of its targets, the *Sgs-4* glue gene. We show that *Broad-Complex* RNAs accumulate in mid third instar larval salivary glands prior to *Sgs-4* induction, as expected for the products of a gene that regulates the timing of *Sgs-4* activation. The *Broad-Complex* codes for a family of zinc finger transcriptional regulators. We have identified a number of binding sites for these proteins in sequences known to regulate the timing of *Sgs-4* induction, and have used these sites to derive a binding consensus for each protein. Some of these binding sites are required *in vivo* for *Sgs-4* activity. In addition, *rbp*$^+$, a genetically defined *Broad-Complex* function that is required for *Sgs-4* induction, acts through these *Broad-Complex* binding sites. Thus, the *Broad-Complex* directly mediates a temporal and tissue-specific response to ecdysone as larvae become committed to metamorphosis.

Key words: binding consensus/*Broad-Complex*/ecdysone/metamorphosis/zinc finger protein

Introduction

During third instar in *Drosophila* larvae, a series of ecdysone concentration changes triggers metamorphic transitions, such as wandering behaviour and puparium formation. Several studies have identified an increase in ecdysone concentration shortly after the second to third instar molt (Berreur *et al.*, 1979, 1984; Schwartz *et al.*, 1984; Andres *et al.*, 1993). Changes in tissue-specific gene expression in *Drosophila*, as well as studies on other holometabolous insects, suggest that this early third instar change in ecdysone titer signals the onset of metamorphosis (Bollenbacher *et al.*, 1975; Koolman, 1980; Strand *et al.*, 1989; Andres *et al.*, 1993). Shortly thereafter, transcription begins for two key regulators of metamorphosis, the *Broad-Complex* (*BR-C*) and the *E74* gene (Karim and Thummel, 1992; Andres *et al.*, 1993). In organ culture, ecdysone concentrations typical of this period of development rapidly induce *BR-C* and *E74* transcripts (Karim and Thummel, 1991, 1992), suggesting that their activation in early third instar is a primary response to hormone. Later, immediately prior to the larval–prepupal transition, the hormone titer rises again and additional primary response genes are activated [reviewed in Ashburner *et al.* (1974) and Andres and Thummel (1992)]. As the primary response gene products accumulate, they activate a larger set of secondary response genes. The tight temporal regulation required to complete this part of the developmental program and move on to the next stage is achieved in part by a feedback loop in which the products of the primary response genes repress their own activity (Walker and Ashburner, 1981).

The *BR-C*, located within the 2B5 puff on the X chromosome, produces four major classes of proteins with TFIIIA-like zinc fingers (Belyaeva *et al.*, 1980; DiBello *et al.*, 1991; C.Bayer, B.Holley, L.Moran and J.Fristrom, manuscript submitted). The *BR-C* is well-characterized genetically and encodes three complementing, genetically defined functions: *rbp* (*reduced bristle number on the palpus*), *br* (*broad*) and *2Bc*. In addition, a non-pupariating (*npr1*) class of alleles appears to be defective for all three functions of the locus (Belyaeva *et al.*, 1980; Kiss *et al.*, 1988). *npr1* mutants are viable until the end of third instar, at which time they are unable to initiate the prepupal developmental program (Stewart *et al.*, 1972; Belyaeva *et al.*, 1980). Thus, *BR-C* function is essential during the metamorphic period.

Temporal and genetic studies indicate that the *BR-C* coordinates ecdysone-triggered gene expression during third instar and early prepupal development. Synthesis of glue gene RNAs in salivary glands during third instar is severely reduced in two classes of *BR-C* mutants (Crowley *et al.*, 1984; Guay and Guild, 1991; Karim *et al.*, 1993; see below). At the larval–prepupal transition, the *BR-C* also regulates the transcription of other primary ecdysone response genes, apparently by amplifying their response to ecdysone (Belyaeva *et al.*, 1981; Zhimulev *et al.*, 1982; Karim *et al.*, 1993). For example, *E74* and *E75* transcript accumulation is reduced in *BR-C* mutants (Karim *et al.*, 1993). The *BR-C* is also essential for regulation of secondary response gene activity in the prepupal period (Guay and Guild, 1991; Karim *et al.*, 1993). These data demonstrate a central role for the *BR-C* in directing metamorphosis.

Although the importance of the *BR-C* to metamorphosis has long been recognized, nothing is known about the interaction of its protein products with target effector genes. Detailed information of this kind is also lacking for other primary response gene products, yet such knowledge is essential for understanding tissue-specific ecdysone responses. Several possible target genes of the

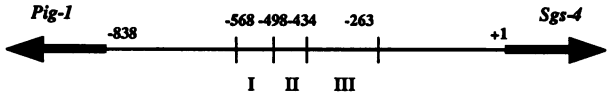


Fig. 1. Diagram of the *Pig-1* and *Sgs-4* genes and the regulatory region between them.

BR-C have been identified, most notably the glue genes induced in mid third instar salivary glands in response to ecdysone (Hansson and Lambertsson, 1983; Crowley *et al.*, 1984). As their name implies, these genes encode components of the larval glue which will be used to affix larvae to a solid surface at the onset of pupariation (Fraenkel and Brookes, 1953; Korge, 1975, 1977; Beckendorf and Kafatos, 1976). The accumulation of glue gene RNA is reduced in two *BR-C* mutant classes, *rbp* and *2Bc* (Guay and Guild, 1991; Karim *et al.*, 1993). However, it is not known if the *BR-C* acts to regulate glue genes directly or through intermediate steps.

Our previous studies of one of the glue genes, *Sgs-4*, enabled us to describe its *cis*-regulation in considerable detail (Shermoen *et al.*, 1987; Mougneau *et al.*, 1993). *Sgs-4* is induced in the middle of third instar through the action of a complex transcriptional switch involving regulatory sequences shared with a nearby divergently transcribed gene, *Pig-1* (Mougneau *et al.*, 1993). As shown in Figure 1, these genes are separated by 838 bp of sequence containing three regulatory elements (elements I–III). Prior to the middle of third instar, elements II and III cooperate to drive transcription from the *Pig-1* promoter. During the middle of third instar, element I becomes active and appears to interfere with the interaction between elements II and III. At this time, element III becomes able to independently direct gene expression in salivary glands, and switches its activity to the *Sgs-4* promoter. Thereafter, *Pig-1* expression is repressed. This knowledge of the molecular events that lead to *Sgs-4* induction in salivary glands offers an excellent opportunity to learn more about the role of *BR-C* proteins in regulating these events.

We describe here evidence for a direct interaction between the protein products of a primary ecdysone response gene and one of its target genes. Our data suggest that the *BR-C* directly mediates the ecdysone-dependent, transcriptional switch that leads to *Sgs-4* induction. We show that *BR-C* proteins bind *in vitro* to sites that are required for the *in vivo* function of at least one element critical for directing this switch. In addition, we show that the genetically defined rbp^+ function of the *BR-C* acts through element III, consistent with the binding of *BR-C* proteins to this element. We also show that both rbp^+ and $2Bc^+$ function are required for *Pig-1* repression, possibly acting through sequences surrounding the *Pig-1* TATA box. A model is proposed which includes a direct role for *BR-C* proteins in regulating *Sgs-4* induction.

Results

The Broad-Complex is required for proper timing of the switch from Pig-1 to Sgs-4

As noted above, the accumulation of *Sgs-4* RNA during third instar is greatly reduced in *npr1*, *rbp* and *2Bc* backgrounds (Crowley *et al.*, 1984; Guay and Guild, 1991; Karim *et al.*, 1993). As a further step towards understanding the action of the *BR-C* at *Sgs-4*, we asked how the switch from *Pig-1* to *Sgs-4* transcription (hereafter referred to as the *Sgs-4* switch) was affected in different *BR-C* mutants. In particular, we wanted to know if the two components of the switch, *Pig-1* repression and *Sgs-4* induction, could be uncoupled.

Wild type and *BR-C* mutant larvae were staged at the second to third instar molt and collected after 10, 24 or 40 h of further development at 25°C (see Materials and methods). These time points correspond to early, mid and late third instar, respectively. Salivary glands were dissected and analyzed for *Pig-1* and *Sgs-4* RNA at each time point using an RNase protection assay (Figure 2). In $BR\text{-}C^+$ animals, the *Pig-1* to *Sgs-4* switch begins by mid third instar and is essentially complete at the 40 h time point. In contrast, in $npr1^3$ mutants the switch fails to occur during this period. These results strongly suggest that $BR\text{-}C^+$ function is required for the timely induction of *Sgs-4* in response to the hormone signal. The *Sgs-4* switch is also affected in rbp^5, br^{28} and $2Bc^1$ mutants (Figure 2). *Sgs-4* RNA accumulation is delayed in all of these mutant classes, with the most severe effect seen in $2Bc^1$ animals, and the least severe in br^{28} where *Sgs-4* RNA accumulates to nearly wild type levels by 40 h. In the rbp^5 and $2Bc^1$ mutant classes, *Pig-1* expression is not fully repressed by 40 h, indicating that both of these genetic functions are required for the proper timing of *Pig-1* repression and *Sgs-4* induction. Thus, the mechanisms used to repress *Pig-1* and induce *Sgs-4* may be coupled.

The accumulation of Broad-Complex RNAs in third instar salivary glands precedes the induction of Sgs-4

If the *BR-C* directly regulates the *Sgs-4* switch, *BR-C* RNAs should be present in salivary glands prior to mid third instar. Therefore, we determined the temporal pattern of transcript accumulation for each class of *BR-C* RNAs in this tissue. The *BR-C* produces four classes of RNAs, designated Z1, Z2, Z3 and Z4, which encode proteins containing different zinc fingers (DiBello *et al.*, 1991; C.Bayer *et al.*, manuscript submitted). When salivary gland RNAs were probed with antisense RNAs complementary to each of the zinc finger classes, all RNA types were detected (Figure 3a). Because the specific activities of these probes and the lengths of their hybridizable sequences vary, the signals were adjusted to determine their relative abundance (Figure 3b). In early third instar, the most abundant *BR-C* RNA is Z3, suggesting that this class of RNA is the first to be induced by ecdysone during this period. Then, the steady state level of Z1, Z3 and Z4 RNA increases significantly between early and mid third instar, prior to the *Sgs-4* switch. Z3 and Z4 transcript accumulation peaks at mid third instar, the approximate time at which the *Sgs-4* switch occurs. In contrast, Z1 RNA continues to accumulate throughout the third instar, while Z2 RNA remains relatively non-abundant at all times and does not appear to be temporally regulated in this tissue. With the exception of the temporal profile of Z4 which is first reported in this study, our data for Z1, Z2 and Z3 agree well with the results of Huet *et al.* (1993). Thus, the temporal profile of *BR-C* RNA expression in

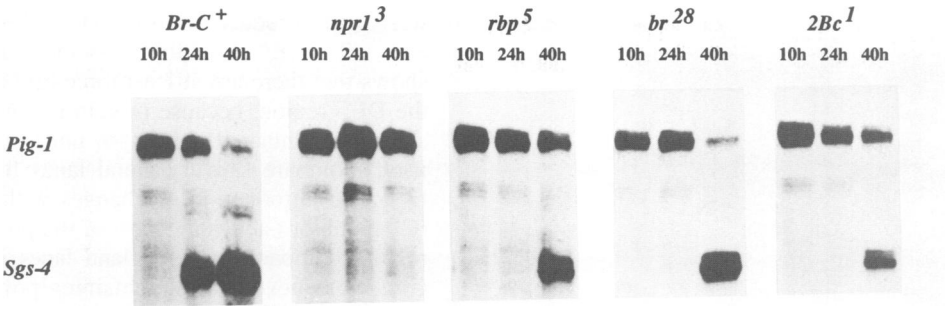


Fig. 2. The role of genetically defined *BR-C* functions in directing the *Sgs-4* switch. Male larvae carrying *BR-C*$^+$ or *BR-C*$^-$ chromosomes were staged at the second to third instar molt and allowed to develop further for 10, 24 or 40 h. At these times, RNA was isolated from salivary glands dissected from representative animals and assayed by RNase protection. Since the amount of DNA per salivary gland increases during third instar (~1.5 replication cycles are observed during this period; Rudkin, 1972), the amount of RNA loaded per lane was adjusted to the amount of DNA. Thus we used RNA equivalent to three salivary glands for 10 h time points, two salivary glands for 24 h time points and one salivary gland for 40 h time points. We have been unable to identify a suitable loading control for salivary gland RNA during third instar. We find that the levels of rp49, actin and tubulin RNAs all decrease in salivary glands after the middle of third instar. Therefore all RNA samples were isolated and assayed at least three times from independently staged animals. Similar results were obtained with each set of samples.

third instar salivary glands is consistent with a direct role for at least Z1, Z3 and Z4 in the timing of the *Sgs-4* switch.

Broad-Complex proteins bind in vitro to elements that direct the Sgs-4 switch

Since all four classes of *BR-C* RNAs are present in third instar salivary glands, we expressed and purified representative isoforms of each class of *BR-C* protein in *Escherichia coli* to assay their ability to bind *Sgs-4* regulatory sequences. We also prepared a partially purified nuclear extract from late third instar salivary glands, and compared its DNA binding properties with those exhibited by the recombinant *BR-C* proteins.

Salivary gland extract protects essentially all of the DNA sequences in elements I and II from DNase I digestion (Figure 4). The Z2 and Z3 proteins each bind to a pair of sites in element I. These binding sites overlap, with the Z2 sites contained within the DNA sequences protected by Z3 protein. In addition to the Z2 footprints in element I, there is also a hypersensitive band in the distal region of this element in lanes bound with Z2 protein (the *Sgs-4* promoter is defined as the proximal end of the intergenic region). This may indicate that there is a third Z2 binding site within element I or, alternatively, there is a conformational change in the DNA close to the Z2 binding sites. We favor the latter hypothesis because there are no protected bands in this region of element I. In contrast to Z2 and Z3, neither Z1 nor Z4 proteins bind to element I (data not shown for Z4).

Hypersensitivity to DNase I digestion in the Z2 protein lanes is also observed at the proximal end of element II (Figure 4), suggesting that Z2 protein may bind to this element. Footprinting of the opposite strand revealed that all four classes of *BR-C* protein bind to a 30 nucleotide region in the proximal half of element II (Figure 5, element III mutant lanes). Because the size of this protected region is large it seems likely that more than one *BR-C* protein binds to element II. We have searched this region for similarity to individual *BR-C* protein binding consensus sites (see below) and find two matches for each protein. This suggests that there are at least two *BR-C* proteins bound to this region of element II.

Element III was examined in more detail. Initial footprinting experiments with salivary gland nuclear extract

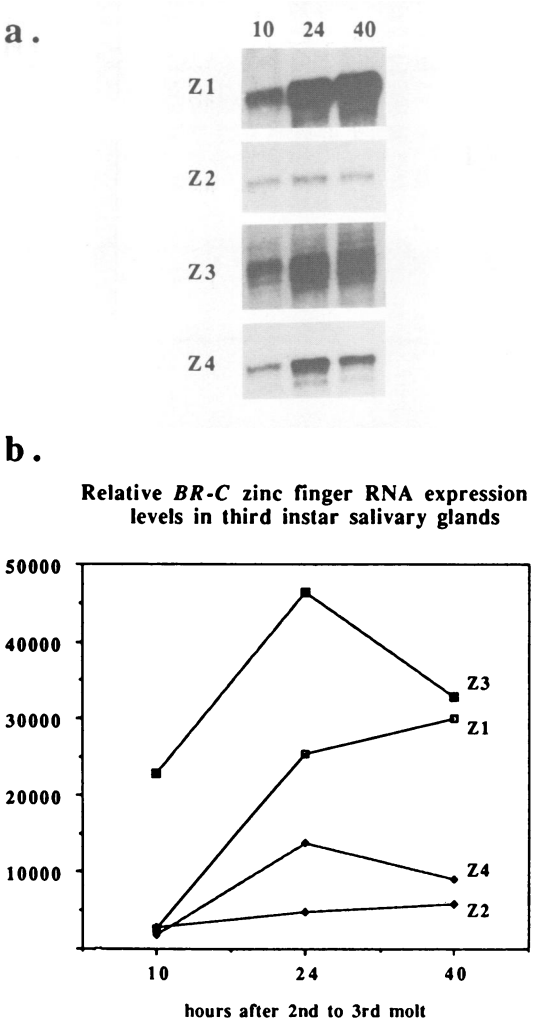


Fig. 3. Temporal expression of *BR-C* RNAs in third instar salivary glands. (**a**) *BR-C*$^+$ animals were staged at the second to third instar molt and allowed to develop further for 10, 24 or 40 h. Salivary gland RNA was isolated and assayed by RNase protection. To correct for changes in polyteny, RNA equivalent to 30, 20 or 10 salivary glands was used for the 10, 24 and 40 h time points, respectively. (**b**) Relative levels of each *BR-C* RNA class were determined using a Molecular Dynamics Phosphorimager. The values obtained were corrected for differences in the specific activities of the probes and the length of their hybridizable sequences. Values on the abscissa are arbitrary units of *BR-C* RNA expression.

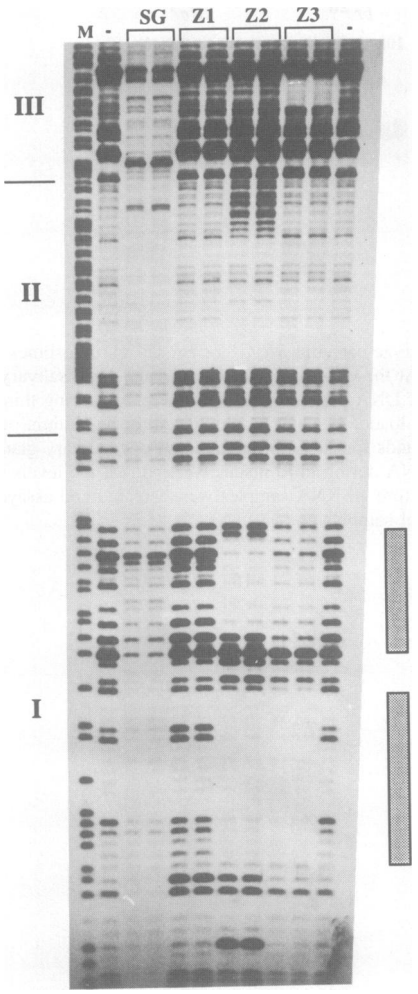


Fig. 4. DNase I footprinting assay of salivary gland and *BR-C* proteins to elements I and II. Salivary gland or *BR-C* protein was incubated with a DNA probe containing elements I and II. Boundaries between the elements are indicated by a horizontal bar. Shaded boxes indicate the approximate limits of each protected region in element I.
M, G + A sizing ladder of the template used in the footprinting assays; −, control lanes with no added protein; SG, 30 μg of salivary gland nuclear protein isolated from late third instar larvae; Z1, 10 μg of BRcore-Q[1]-Z1 protein; Z2, 4 μg of BRcore-Z2 protein; Z3, 2.5 μg of BRcore-NS-Z3 protein. All footprinting reactions included a 1000-fold weight excess (0.5 μg) of poly(dI/C) as a non-specific competitor DNA.

revealed three protected regions within element III (AB, C and DEF in Figure 5; element III, SG lanes). Because these binding sites are large (e.g. site DEF is 55 bp), we suspected that more than three proteins were bound to element III. Further resolution was obtained by introducing mutations into each of the protected regions within element III. Mutations located in the lower half of the AB footprint (B* mutation; changes located between −400 and −411 with respect to the *Sgs-4* transcription start site) abolish binding to only half of this region (compare salivary gland lanes for element III and B*), indicating the presence of at least two proteins within this footprint. This interpretation has been confirmed using a template lacking the A binding site. In this case the B region is still protected in a footprinting assay (data not shown). Specific mutations were also introduced into the DEF region between −320 and −334 (E* mutation). Footprinting of this mutant shows that there are at least three protein binding sites in the DEF region, because protein remains bound to either side of the mutated region but not to the mutated region itself (compare salivary gland lanes for element III and E*). The introduction of changes within the C footprint (C* mutation) abolishes most of the protection within this region (compare salivary gland lanes in element III and C*). We believe that the remaining protection in site C is due to encroachment by the protein bound to site B. We see increased DNase I hypersensitivity in all of the mutated binding sites. This is probably a consequence of distortion of the helix between the proteins bound adjacent to these sites. In summary, these results show that a minimum of six proteins are bound within a 125 bp region of element III.

Next we asked whether individual *BR-C* products bind to the element III sites that were identified using salivary gland extract. When recombinant *BR-C* proteins were incubated with element III, four of these sites (B, D, E and F) were protected (Figure 5).

Similar to *BR-C* protein binding in element I, the *BR-C* proteins are somewhat specific in their choice of binding site in element III. Site B is bound by Z3 and Z4 only (Figure 5; element III), site D is bound by Z1, Z2 and Z4, and site E is protected by Z1 and Z4. Site F is a low-affinity binding site for Z1 and Z4 but appears to have a stronger preference for Z3 (for a better example of Z3 binding to site F, see element III mutants, B* lanes).

Z1 and Z4 proteins appear to bind cooperatively to sites D, E and F. Z1 and Z4 fail to bind to sites D, E or F in the E* mutant (E*, lanes Z1 and Z4), indicating that DNA sequences found within site E are essential for Z1 and Z4 to bind *in vitro* to any of these sites. In contrast, site F is protected by Z3 in the E* mutant (data not shown), indicating that Z3 does not depend on sequences in site E to bind to site F. Z2 protein partially protects site E in the E* mutant (compare E*, lanes SG, Z1 and Z2), suggesting that the DNA sequences recognized by Z2 are not the same as those recognized by Z1 and Z4.

In the E* mutant, site D is protected by salivary gland nuclear protein. However, *BR-C* proteins Z1 and Z4 do not bind to site D in the E* mutant (compare E*, lanes SG, Z1, Z2 and Z4). Therefore, another protein, one that does not require a functional E site to bind to site D, may be present in the salivary gland extract. Alternatively, binding of *BR-C* proteins to site D in the E* mutant may require interactions between different classes of *BR-C* protein (e.g. Z1/Z4 heterodimers), or interactions between *BR-C* proteins and other proteins present in the salivary gland extract.

In contrast to sites D, E and F, binding of *BR-C* proteins to site B does not appear to depend on binding of *BR-C* proteins to other sites in element III. For example, binding of Z4 to site B does not depend on Z4 binding to sites D and E (E*, Z4 lanes), and Z1 and Z4 are bound to sites D, E and F in the B* mutant (data not shown).

In summary, our data show that within element III, salivary gland nuclear extract protects six sites, and *BR-C* proteins bind to four of these sites, B (Z3 and Z4), D (Z1, Z2 and Z4), E (Z1 and Z4) and F (Z1, Z3 and Z4).

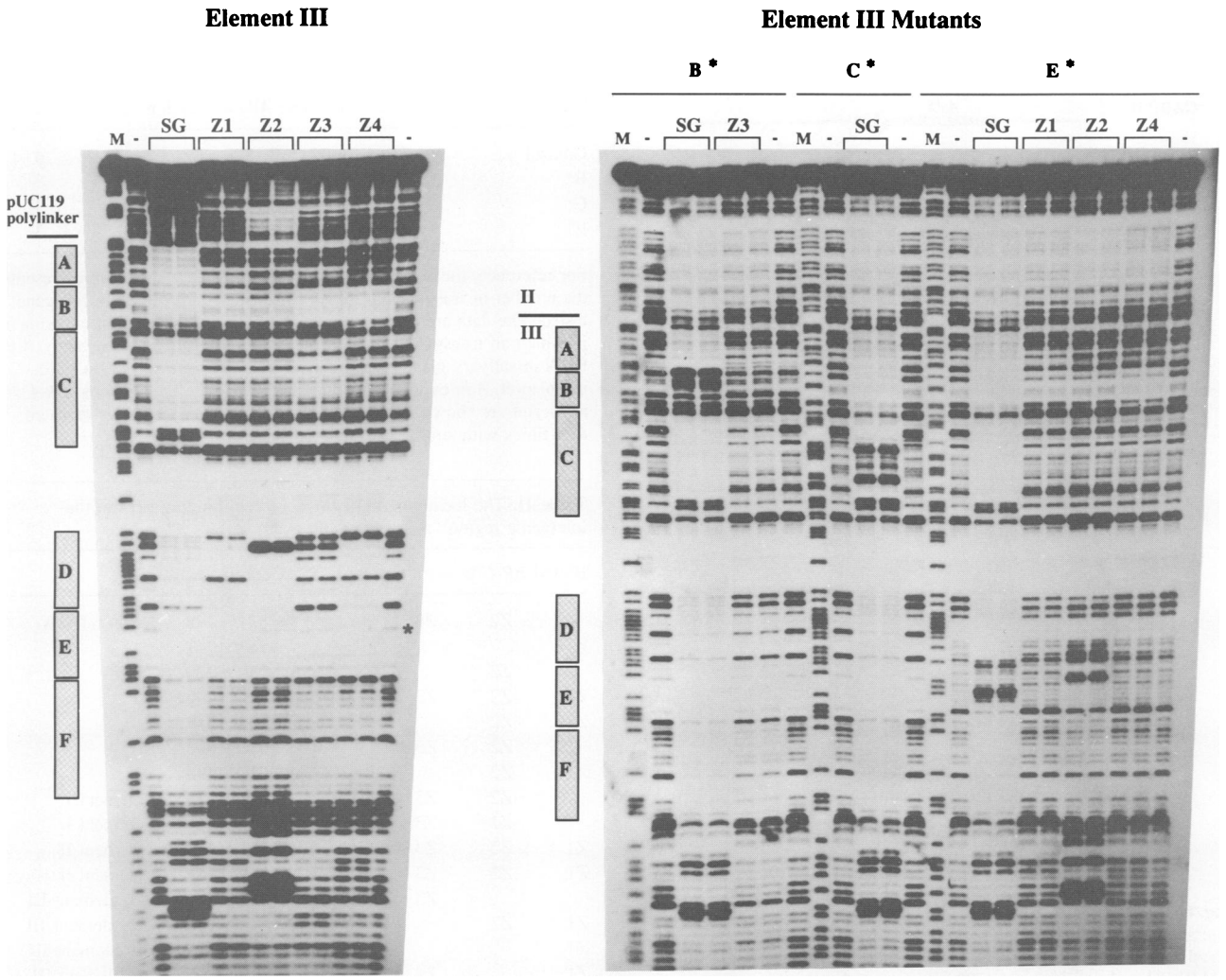


Fig. 5. DNase I footprinting assays of salivary gland and *BR-C* proteins to element III. DNase I footprinting of wild type element III DNA and element III sequences carrying introduced changes (B*, C* and E*) are shown. Shaded boxes indicate the limits of each of the six sites of DNA–protein interaction identified with salivary gland protein. The asterisk indicates the location within site E of a band seen in control digests that is protected by salivary gland extract and Z1 and Z4 proteins. The boundaries between element III and pUC119 polylinker (wild type element III DNA) and elements II and III (element III mutants) are indicated. M, G + A sizing ladder of the template used in the footprinting assays; –, control lanes with no added protein; SG, 30 μg of salivary gland nuclear protein isolated from late third instar larvae; Z1, 10 μg of BRcore-Q[1]-Z1 protein; Z2, 4 μg of BRcore-Z2 protein; Z3, 2 μg of BRcore-NS-Z3 protein; Z4, 10 μg of BRcore-Z4 protein. All footprinting reactions included a 1000-fold weight excess (0.5 μg) of poly(dI/C) as a non-specific competitor DNA.

Broad-Complex binding sites in element III are required for the in vivo activity of an Sgs-4 transgene in late third instar

If *BR-C* protein binding sites within element III are directly involved in the *Sgs-4* switch, then these sites should be required for *Sgs-4* expression *in vivo*. The same mutations used for the *in vitro* footprinting assays were introduced into P element constructs carrying 838 bp of *Sgs-4* upstream DNA fused to an *Adh* reporter gene (−838/*Adh* in Figure 6b). This construct has been shown previously to contain sequences sufficient for correct spatial and temporal control of *Sgs-4* activity (Barnett *et al.*, 1990). Transformed flies carrying −838/*Adh* transgenes with B*, C* or E* mutations were generated, and their *Adh* RNA expression relative to the wild type control assayed in late third instar (Table I).

Adh RNA in salivary glands is consistently at least 20-fold less abundant in animals carrying the B* transgene than the control construct. E* transgenes also express less *Adh* than wild type transgenes, although the decrease in activity (4-fold) is not as severe as that seen in B* mutants. The C* transgene has the mildest effect, reducing transgene activity to approximately half that seen with the control. We conclude that at least the B and E sites are required for proper *Sgs-4* activity *in vivo* during third instar. Thus, two of the sites we identified as *BR-C* binding sites *in vitro* are essential for proper *Sgs-4* activity *in vivo*.

The Broad-Complex acts through element III to regulate Sgs-4 activity

We have shown that *BR-C* proteins bind *in vitro* to sites in element III that are required for *Sgs-4* activity *in vivo*. At least two genetic functions of the *BR-C*, rbp^+ and $2Bc^+$, are required for the proper timing of *Sgs-4* induction

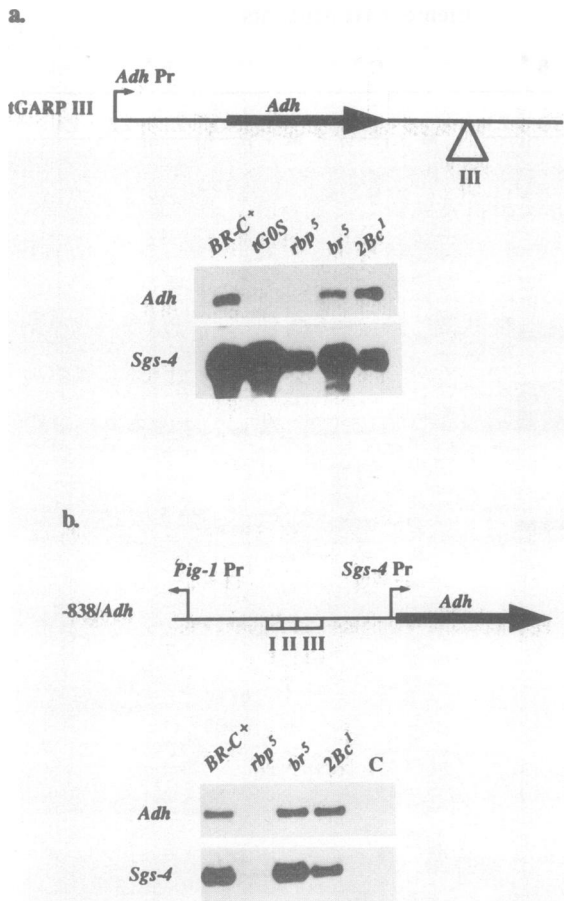


Fig. 6. Requirement for *BR-C* genetic functions in the *Pig-1/Sgs-4* intergenic region. (**a**) rbp^+ function acts through element III. Salivary glands were dissected from *BR-C*$^+$ and *BR-C*$^-$ male larvae carrying tGARP III and the levels of *Adh* transgene and endogenous *Sgs-4* RNA determined by RNase protection assay. tG0S is a control construct containing the same *Adh* reporter gene, but without element III. All sample points were taken 40 h after the second to third molt. The amount of RNA equivalent to one salivary gland was used per lane. The experiment was repeated a total of three times. (**b**) $2Bc^+$ function does not act through the intergenic region to induce *Sgs-4*. The experimental design is identical to (a) except that the −838/*Adh* transgene was used. The relative locations of the *Pig-1* and *Sgs-4* promoters and elements I, II and III are shown. The lane designated C is a control lane in which antisense *Adh* and *Sgs-4* RNAs were mixed and assayed in the absence of salivary gland RNA to test for self- or cross-annealing of the probes. The experiment was repeated a total of three times.

(Guay and Guild, 1991; Karim *et al.*, 1993; Figure 2). Therefore, if the *BR-C* binding sites in element III reflect an *in vivo* requirement for *BR-C* protein binding, at least one of the *BR-C* genetic functions should be required for the activity of element III. We tested this possibility by separating element III from its surrounding sequences in the intergenic region, and assaying the effect of *BR-C* mutations on the activity of this isolated element carried in transformed animals.

We used a construct carrying an *Adh* reporter gene driven by the *Adh* proximal promoter with element III inserted downstream from *Adh* coding sequences (tGARP III in Figure 6a). This transgene exhibits the temporal and spatial expression pattern of *Sgs-4* superimposed on *Adh* activity driven from the proximal promoter, causing *Adh* to be expressed ectopically during late third instar in

Table I. Relative RNA levels of −838/*Adh* transgenes carrying mutations in the B, C or E binding sites

Transgene	Relative *Adh* expression	*n*
Control	1.35 ± 0.38	4
B*	0.03 ± 0.019	3
C*	0.63 ± 0.28	3
E*	0.37 ± 0.17	3

For reference, the activity of a control construct is shown. *n* represents the number of independent transformed lines carrying each transgene tested. The data are from a representative RNase protection experiment in which all transgene-carrying lines were assayed simultaneously. *Adh* RNA in salivary glands was assayed 40 h after the second to third instar molt. *Adh* expression values relative to the endogenous *Sgs-4* transcript are shown in arbitrary units. The experiment was repeated four times with similar results.

Table II. The locations of 19 *BR-C* protein binding sites in the intergenic region

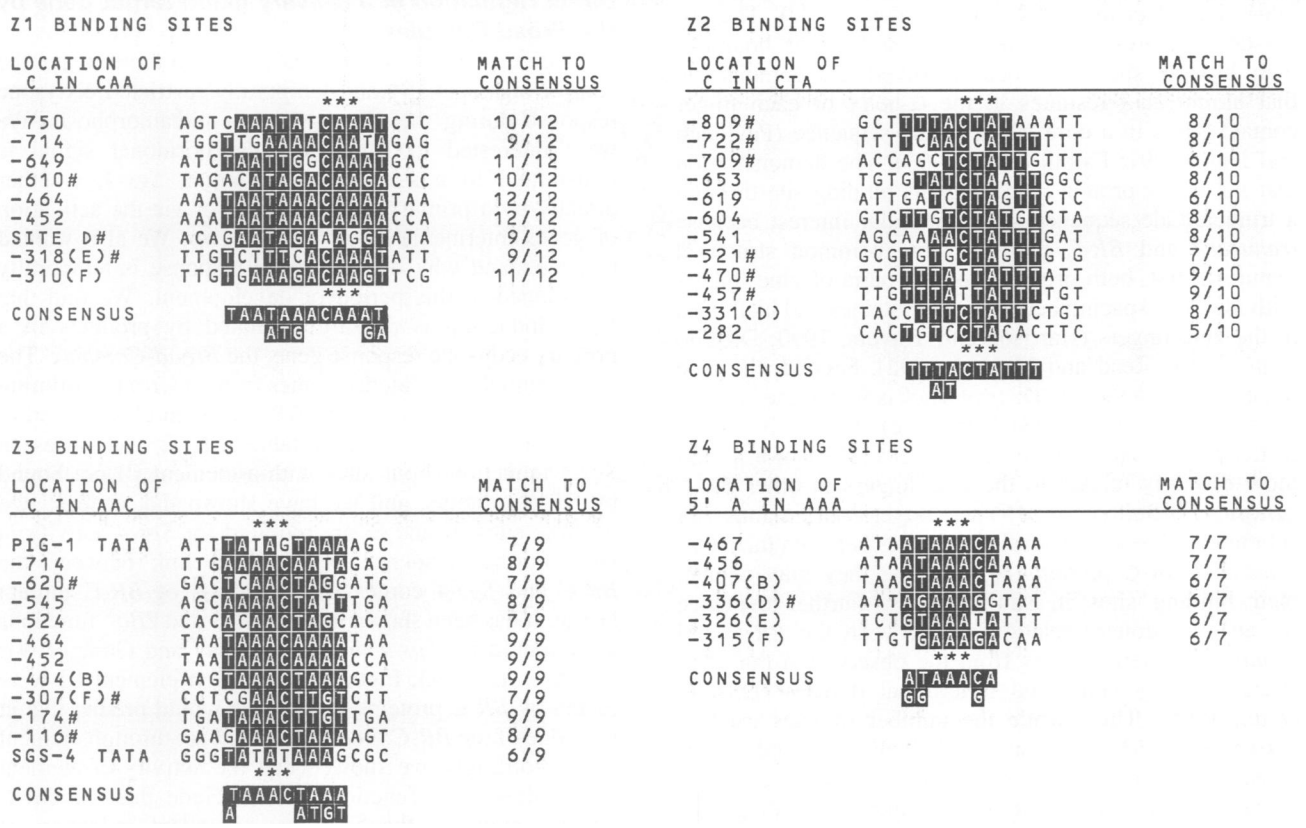
Bound *BR-C* protein				Coordinates	
	Z2	Z3		−821/−798	*Pig-1* TATA
Z1				−753/−741	
	Z2			−734/−720	
Z1	Z2	Z3		−720/−702	
Z1	Z2			−668/−649	
	Z2	Z3		−631/−612	
Z1	Z2			−616/−595	
	Z2	Z3		−553/−533	element I
	Z2	Z3		−530/−514	element I
Z1	Z2	Z3	Z4	−475/−460	element II
Z1	Z2	Z3	Z4	−459/−444	element II
		Z3	Z4	−410/−394	B, element III
Z1	Z2		Z4	−344/−330	D, element III
Z1			Z4	−330/−318	E, element III
Z1		Z3	Z4	−320/−305	F, element III
	Z2			−293/−276	
		Z3		−182/−168	
		Z3		−128/−109	
		Z3		−38/−22	*Sgs-4* TATA

Footprint coordinates are relative to the *Sgs-4* transcription start. Binding sites in elements I, II and III, and at the *Pig-1* and *Sgs-4* TATA boxes are indicated.

salivary glands (Mougneau *et al.*, 1993). After crossing this transgene into different *BR-C* mutant backgrounds, element III activity in late third instar salivary glands was assayed (Figure 6a). The tGARP III transgene is expressed at negligible levels in rbp^5 mutants (0.3% of *BR-C*$^+$). In contrast, the activity of the transgene is relatively unaffected by br^5 and $2Bc^1$ mutations (61 and 134% of *BR-C*$^+$ activity, respectively). Karim *et al.* (1993) showed that glue gene RNA accumulation is delayed in *2Bc* mutants. The approximately wild type level of transgene expression seen in $2Bc^1$ is not attributable to mis-staging of the animals since the activity of the endogenous *Sgs-4* gene is severely reduced in these salivary glands (8.5% of *BR-C*$^+$). We conclude that of the two *BR-C* functions known to be required for *Sgs-4* expression, only one of them, rbp^+, must act through element III.

We were surprised to find that $2Bc^+$ function was not required for element III activity. Therefore, we asked whether this *BR-C* function acts through another part of

Table III. Predicted consensus binding sites for each *BR-C* protein class



Matches to each individual consensus are shown. Coordinates are relative to the *Sgs-4* transcription start. All binding sites are shown 5′ to 3′. (*) indicates the location of conserved trinucleotide cores within individual consensus sequences. (#) indicates matches to the consensus found on the antisense strand. Binding sites in element III (B, D, E and F) are indicated.

the *Pig-1/Sgs-4* intergenic region. In these experiments we used the −838/*Adh* transgene in which 838 bp of *Sgs-4* upstream DNA is fused to an *Adh* reporter gene (Figure 6b). This construct includes all of the DNA required for the *Sgs-4* switch, including the *Pig-1* promoter. The expression of this construct in different *BR-C* mutant backgrounds is shown in Figure 6b. As expected, rbp^5 has a strong effect on the activity of this transgene (10% of *BR-C*$^+$ activity), while in br^5 mutants the −838/*Adh* construct is expressed at essentially control levels (150% of *BR-C*$^+$). Surprisingly, in $2Bc^1$ mutants the transgene is also expressed at a level similar to the control (159% of *BR-C*$^+$). This result indicates that the aspect of $2Bc^+$ function that is essential for *Sgs-4* induction acts through sequences located outside the intergenic region.

Broad-Complex binding sites in the intergenic region suggest individual consensus binding sequences

As part of our effort to understand the role of the *BR-C* in directing the *Sgs-4* switch, we identified additional *BR-C* binding sites in the *Pig-1/Sgs-4* intergenic region. The results of a footprinting analysis across the entire intergenic region are summarized in Table II. We observed a total of 19 binding sites in this 832 bp region, 11 of them located outside elements I, II and III. Of the 19 *BR-C* binding sites in the intergenic region, 13 are recognized by more than one class of *BR-C* protein, suggesting that *BR-C* proteins can cross-recognize the binding sites of other *BR-C* family members *in vitro*. However, only two sites, those in element II, are bound by all *BR-C* protein classes. In contrast to the sites in element II, six other sites are bound by a single protein class, indicating binding specificity for individual isoforms.

By comparing binding sites for each isoform (Table II) we developed a consensus sequence for each protein class (Table III). In all four cases the binding sites can be aligned around related, nearly invariant three nucleotide cores (Z1, CAA; Z2, CTA; Z3, AAC; Z4, AAA), surrounded by slightly more variable sequences. CAA is observed in eight of the nine Z1 binding sites in the intergenic region. The one exception is site D where Z1 binding depends on sequences in the adjacent site E (Figure 5). For Z2, all 12 binding sites have an internal PyPyA and for nine of these the sequence is CTA. This includes three sites that are otherwise a poor match to the consensus (5/10, 6/10 and 6/10). When compared with other *BR-C* protein classes, the sequences surrounding the Z2 trinucleotide core are somewhat more variable. Ten of the Z3 sites contain the sequence AAC. The only exceptions are binding sites at the *Pig-1* and *Sgs-4* TATA boxes which may be atypical cases for Z3 binding (see Discussion). Finally, all six Z4 binding sites contain the trinucleotide AAA.

Trinucleotide sequences have been shown to be important in the binding of at least three members of the C_2H_2

family of zinc finger proteins, *tramtrack*, *Zif268* and the chorion transcription factor *CF2* (Pavletich and Pabo, 1991; Fairall *et al.*, 1992; Gogos *et al.*, 1992). The co-crystal structures of both *tramtrack* and *Zif268* bound to their binding sites have been resolved and demonstrate that amino acid residues in the α-helix of each finger contact bases in a core trinucleotide sequence (Pavletich and Pabo, 1991; Fairall *et al.*, 1993). The demonstration that *tramtrack* protein recognizes its binding site through a trinucleotide sequence is of particular interest because *tramtrack* and *BR-C* proteins have common structural features. First, both proteins contain pairs of zinc fingers with identical spacing between the cysteines and histidines in the zinc fingers (Harrison and Travers, 1990; DiBello *et al.*, 1991; Read and Manley, 1992). Second, the zinc fingers found in the 69 kDa *tramtrack* isoform (the isoform used to derive the co-crystal structure) are closely related to the pair of zinc fingers in the *BR-C* Z3 isoform, and more distantly related to the zinc fingers in other *BR-C* isoforms (DiBello *et al.*, 1991; C.Bayer *et al.*, manuscript submitted). These structural similarities between the *tramtrack* and *BR-C* proteins suggest that they may contact their binding sites in similar ways. Further evidence for an evolutionary relationship between the *BR-C* and *tramtrack* proteins comes from the observation that they share a highly conserved N-terminal domain (DiBello *et al.*, 1991). Thus, while the number of sites used to derive each *BR-C* consensus is relatively small and further experiments are needed to confirm these results, the different trinucleotide cores seen within the binding sites for individual *BR-C* protein classes may be important in determining the distinct binding preferences of these proteins in the intergenic region.

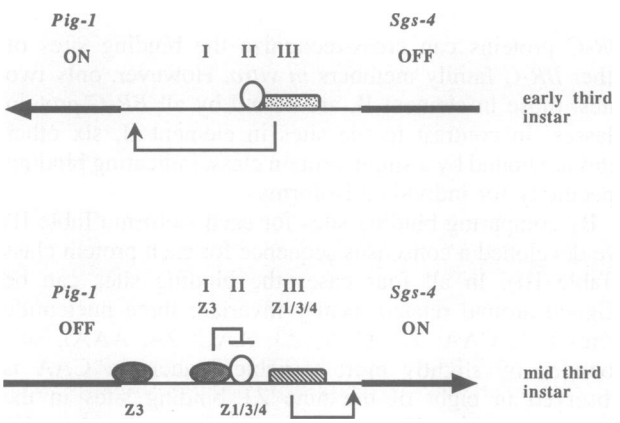


Fig. 7. A model of the switch leading to *Sgs-4* induction in third instar salivary glands showing the proposed involvement of *BR-C* protein products. Because only Z1, Z3 and Z4 RNAs are developmentally regulated in third instar salivary glands and Z2 expression is low, Z2 protein has not been included in the model. We propose that Z1, Z3 and Z4 protein concentrations increase by the middle of third instar to levels that facilitate binding to their target sites in elements I, II and III. Binding of Z3 to element I serves to disrupt the interaction between elements II and III and the *Pig-1* promoter. Z1, Z3 and Z4 protein binding to sites within element III causes this element to redirect its activity to the *Sgs-4* promoter. The choice of promoters available to interact with element III may be further limited by preferential binding of Z3 to sequences surrounding the *Pig-1* TATA box.

Discussion

Direct regulation of a salivary gland target gene by the Broad-Complex

Our primary goal in this study was to investigate the control of a spatially and temporally restricted ecdysone response during the early stages of metamorphosis. We were interested to learn how the hormonal signal is transmitted to a salivary gland target, *Sgs-4*, whether directly by a primary response gene or via the activation of genes intermediate between the two. We also wanted to understand why this particular response is temporally constrained to this period of development. We find that *Sgs-4* induction is directly regulated by products of a primary ecdysone response gene, the *Broad-Complex*. The most compelling evidence comes from *in vitro* footprinting studies. Members of all four *BR-C* protein classes bind to regulatory elements with established, *in vivo* roles in *Sgs-4* induction. Four sites within element III are bound by *BR-C* proteins, and we have shown that at least two of these sites, B and E, are required for *Sgs-4* activity *in vivo*. Further evidence for a direct link between the *BR-C* and *Sgs-4* comes from analysis of *BR-C* mutant larvae. It has been shown that the rbp^+ and $2Bc^+$ functions are required for *Sgs-4* induction (Guay and Guild, 1991; Karim *et al.*, 1993; Figures 2 and 6). If element III were bound by *BR-C* proteins *in vivo*, we would predict that at least one of the *BR-C* functions would act through element III. Accordingly, we showed that the activity of element III requires rbp^+ function. We conclude that the *BR-C* directly mediates the ecdysone-dependent induction of *Sgs-4* in third instar salivary glands.

The kinetics of accumulation of *BR-C* RNAs in third instar salivary glands (Figure 3) suggest an explanation for the timing of *Sgs-4* induction. *BR-C* transcripts accumulate in early third instar animals (Andres *et al.*, 1993) and salivary glands (Figure 3), most likely as a primary response to increased hormone levels (Karim and Thummel, 1992). We suggest that *Sgs-4* is induced when *BR-C* protein products become sufficiently abundant. These observations fit well with previous suggestions that the *BR-C* has a coordinating role in metamorphosis (Karim *et al.*, 1993).

Karim *et al.* (1993) showed that the induction of glue gene RNAs is delayed in rbp^5 and $2Bc^1$ mutants, and we have seen delayed accumulation of *Sgs-4* RNA in $npr1^3$ animals (data not shown; see also Crowley *et al.*, 1984). However, we do not believe that the reduced levels of *Sgs-4* RNA accumulation seen in these mutants are attributable to general developmental delay in these animals. We have examined the *Adh* switch from proximal to distal promoter expression in early third instar fat body (Benyajati *et al.*, 1983) in *BR-C* mutants (L.von Kalm, C.Bayer, S.K.Beckendorf, data not shown). When compared with the severe delay in glue gene induction (Figure 2), the *Adh* switch is only slightly delayed in $npr1^3$ animals, and this delay is identical to that seen in rbp^5, br^{28} and $2Bc^1$ mutants. We also note that in a $2Bc^1$ mutant the expression of our −838/*Adh* transgene is not delayed, even though endogenous *Sgs-4* induction is weak (Figure 6b). Thus, only those genes dependent on *BR-C* products

for their activity exhibit delayed expression patterns in *BR-C* mutants.

The role of the Broad-Complex in directing the Sgs-4 switch

The data presented here suggest that the mechanisms underlying repression of *Pig-1* and induction of *Sgs-4* are likely to have a common feature because protein products of the *BR-C* regulate both events (Figure 2). Our previous work identified the sequences required to direct the timing of the *Sgs-4* switch (Mougneau *et al.*, 1993). During mid third instar, element I becomes active and disrupts the interaction of elements II and III with the *Pig-1* promoter. At this time element III changes, becoming competent to direct salivary gland expression by itself and activating the *Sgs-4* promoter, which completes the switch. This change in the competence of element III is likely to reflect the binding to this enhancer of proteins that first appear in salivary glands at the time of the switch. Based on the data we have described here, we can now propose a model of the *Sgs-4* switch which implicates *BR-C* proteins in all aspects of the switch.

Although *BR-C* proteins bind to 19 sites in the intergenic region (Table II), the *in vivo* relevance of all of these sites to *Sgs-4* induction is unknown. For example, *BR-C* proteins may not be able to compete successfully for all of these binding sites in the presence of other salivary gland DNA binding proteins. Therefore, in developing this model (Figure 7) we included only those *BR-C* proteins temporally regulated in third instar salivary glands (Z1, Z3 and Z4), and only those aspects of our *in vitro BR-C* binding data that can be linked to regulatory elements whose roles have been demonstrated *in vivo*. We propose that there are three separate effects of the *BR-C* on the *Sgs-4* switch. First, during the middle of third instar, Z1, Z3 and Z4 protein levels reach concentrations sufficient to promote their binding to elements I, II and III. Binding of Z3 to element I interferes with the interaction among elements II and III and the *Pig-1* promoter. One potential mechanism for this interference is a direct interaction between Z3 protein bound to element I and the *BR-C* proteins bound to the proximal region of element II. As the II–III interaction is destabilized and Z1, Z3 and Z4 bind to element III, a new interaction between element III and the *Sgs-4* promoter is stabilized. Although other proteins bound to element III are likely to be involved, the binding of *BR-C* proteins to element III at this stage can account for our observation that this element becomes competent to direct salivary gland transcription by itself only after mid third instar.

Second, our footprinting analysis of the *Pig-1/Sgs-4* intergenic region suggests that there may be an additional component to the switch: binding of Z3 to the *Pig-1* TATA region (see Table II). Although Z3 recognizes both the *Pig-1* and *Sgs-4* TATA binding regions *in vitro*, the preference of Z3 for the *Pig-1* TATA box is noticeably stronger (in preliminary experiments we estimate a 5-fold greater affinity of Z3 protein for the *Pig-1* TATA site). This result suggests that *BR-C* proteins may act to repress *Pig-1* by binding to its promoter, thus establishing another level of specificity in the *Sgs-4* switch. Binding to the *Pig-1* promoter would limit the choice of promoters available for interaction with element III after mid third instar. Further experiments are needed to test this promoter-specific role for the Z3 protein class as this may represent a rare example of cell type-specific transcriptional control at the level of a TATA box (McCormick *et al.*, 1991; Stromstedt *et al.*, 1991).

Finally, we have shown that $2Bc^+$ function is not essential for activity of the intergenic region when rbp^+ activity is present. To explain the strong effect of this mutant on *Sgs-4* expression, we must conclude that $2Bc^+$ function acts through sites which are outside the intergenic region. We have measured the activity of the −838/*Adh* transgene in wild type animals at a variety of chromosomal insertion sites and, assuming that the stability of *Adh* and *Sgs-4* RNAs in salivary glands is similar, find that the transgene has only 10–15% of endogenous *Sgs-4* activity in late third instar salivary glands. Thus, sequences outside the intergenic region, which may be targets for $2Bc^+$ activity, are required for wild type *Sgs-4* expression levels.

Structure–function relationships at the Broad-Complex

To understand the role of the *BR-C* in metamorphosis, it will be necessary to understand the relationship between individual protein isoforms and specific *BR-C* genetic functions. Based on the centromere distal to proximal arrangement of the zinc fingers, genetic mapping of some alleles from each group, and the observation that mutant br^{28} animals have a P element insertion in the Z2 pair of zinc fingers, DiBello *et al.* (1991) proposed the following relationships: Z1 = rbp^+, Z2 = br^+ and Z3 = $2Bc^+$. However, the discovery of a fourth pair of zinc fingers (C.Bayer *et al.*, manuscript submitted) makes a one to one correspondence between zinc fingers and genetic functions less likely. In support of a correlation between Z1 and rbp^+ function, expression of Z1 protein under the control of a heat-inducible promoter has been found to be sufficient to rescue the expression of both *Sgs-4* and a 71E late gene in rbp^5 mutants (K.Crossgrove, C.Bayer *et al.*, manuscript submitted). Preliminary evidence suggests that Z2, Z3 and Z4 cannot provide this rescue for the 71E late gene.

We have shown that rbp^+ function is essential for the activity of element III (Figure 6a). In our experiments *Sgs-4* activity is reduced ~10-fold in rbp^5 mutants, and stronger effects, up to 30-fold, have also been reported (Guay and Guild, 1991). Of the four *in vitro BR-C* binding sites in element III, three of them, sites D, E and F, are potentially bound by Z1 *in vivo*. The effect of a mutation in the E binding site on *Sgs-4* expression is only 4-fold, which is not enough to account for the reduction seen in rbp^5 animals. However, an *Sgs-4*-underproducing strain, Hikone, carries a deletion that removes the DNA which contains sites D, E and F (Muskavitch and Hogness, 1982). In this variant, *Sgs-4* expression is reduced 10- to 50-fold (Muskavitch and Hogness, 1980; Barnett *et al.*, 1990). Thus, loss of Z1 binding to the element III sites D, E and F could account for the reduced *Sgs-4* activity seen in rbp^5 animals.

Site B also has a strong effect on *Sgs-4* activity *in vivo*,

~20-fold in our experiments, similar to the effect of rbp^5 on *Sgs-4* expression. Since Z3 and Z4, but not Z1, bind to the B site, it is possible that rbp^5 animals could be defective in more than one protein (e.g. Z1 and either Z3 or Z4). We are currently testing these possibilities using heat-inducible *BR-C* proteins as described above.

Finally, our observations are consistent with the evidence linking Z2 to br^+ function. Loss of br^+ function has only a modest effect on *Sgs-4* induction and no effect on *Pig-1* repression (Figure 2). Accordingly, Z2 RNA is expressed at low levels in salivary glands throughout third instar, and its activity does not appear to be developmentally regulated (Figure 3).

Salivary gland-specific ecdysone response

One of the primary roles of the *BR-C* during metamorphosis is to coordinate the timing of the hormone response in different tissues (Karim *et al.*, 1993). The kinetics of salivary gland *BR-C* RNA accumulation described here and elsewhere (Huet *et al.*, 1993) tend to support such a role for the *BR-C* in salivary glands. The *BR-C* is expressed in many tissues, although the relative amounts of each class of RNA differ (Huet *et al.*, 1993). Given this expression pattern, it seems likely that the diversity of tissue-specific hormone responses seen during metamorphosis is determined by interactions between the products of primary response genes, such as the *BR-C* and factors with more localized distributions.

Element III is able to direct salivary gland-specific expression after the *Sgs-4* switch has occurred, indicating that one of the proteins bound to this element may be a tissue specifier. Within element III, sites B, D, E and F are bound by *BR-C* proteins *in vitro*, leaving the proteins bound to sites A and C as the most obvious candidates for tissue specifiers. C* transgenes are active in salivary glands during third instar, making it unlikely that this protein is an essential tissue specifier. However, we have assayed the effect of a deletion of site A using a transgene similar to tGARP III (see Figure 6a) but with elements I, II and III inserted upstream of the *Adh* reporter gene (E.Mougneau, D.Von Seggern and S.K.Beckendorf, unpublished results). In this context, a deletion of site A results in at least 100-fold reduced activity in early as well as late third instar. This suggests that the protein binding to site A is required for both *Pig-1* and *Sgs-4* expression, making it a good candidate for a tissue specifier. One of our current goals is to clone the gene encoding this protein. It will be interesting to see whether the A protein interacts with *BR-C* products to regulate *Sgs-4* induction.

Materials and methods

Stocks, crosses and developmental staging

The *Broad-Complex* mutants $npr1^3$, br^5 and $2Bc^1$ (Kiss *et al.*, 1988), rbp^5 (*t376*; Belyaeva *et al.*, 1980) and br^{28} (DiBello *et al.*, 1991) have been described. The *t242* chromosome, which originates from the same progenitor as rbp^5, br^5 and $2Bc^1$, was used as a $BR\text{-}C^+$ control (Figure 2). Mutant *BR-C* alleles were maintained as mutant/*Binsn* or as *C(1)DX, y f/y BR-C* mutant/$Dp(1:Y)y^2$ *Y67g19.1* stocks. The $Dp(1:Y)y^2$ *Y67g19.1* 2B5 duplication chromosome contains the X chromosome cytogenetic regions 1A; 2B17-18 (Belyaeva *et al.*, 1980, 1982; Kiss *et al.*, 1988). The *y t242* control was maintained as *C(1)DX, y f* shi^{ts1}*/y t242/*$Dp(1:Y)y^2$ *Sz280*. The $Dp(1:Y)y^2$ *Sz280* $BR\text{-}C^-$ duplication chromosome contains the X chromosome cytogenetic regions 1A; 2C1-2 with an internal deficiency 2B3-4; 2B7-8 which uncovers the *Broad-Complex* (Belyaeva *et al.*, 1982; Kiss *et al.*, 1988).

BR-C mutant males were generated in two ways. (i) To examine the effect of *BR-C* mutations on the *Sgs-4* switch (Figure 2), *C(1)DX, y f* shi^{ts1}*/Dp(1:Y)*y^2 *Sz280* females were mated to *yBR-C* mutant/$Dp(1:Y)y^2$ *Y67g19.1* or *y t242/Dp(1:Y)*y^2 *Sz280* males and allowed to lay at 25°C for 6 h. Embryos were maintained at 25°C for a further 6 h then transferred to 30°C for 12 h. All *C(1)DX, y f* shi^{ts1}*/Dp(1:Y)* female embryos die after prolonged exposure to 30°C because of the shi^{ts} mutation (Poodry *et al.*, 1973), leaving only *yBR-C* mutant/*Sz280* male larvae as survivors. Newly hatched larvae were collected and transferred to cornmeal plates (200 per plate) at 25°C. Larvae were then staged to 10, 24 and 40 h after the second to third instar molt (Mougneau *et al.*, 1993). Salivary glands were dissected and all attached fat body removed. To ensure that all larvae were carrying the expected mutant *BR-C* chromosome, at least 50 animals were retained and allowed to develop so that the lethal phase of the animals could be determined. In the case of controls, adults were examined to ensure that all were y^2f^+ males.

(ii) To examine the effect of *BR-C* mutations on the activity of the tGARP III and −838/*Adh* transgenes (Figure 6), *y BR-C* mutant/*Binsn* females were mated to ACR males (Adh^{n6} *cn;* ry^{506}; Shermoen *et al.*, 1987) homozygous for one of these transgenes, and allowed to lay at 25°C for 6 h. Embryos were allowed to develop for a further 18 h at 25°C. Newly hatched larvae were collected and transferred to cornmeal plates (200 per plate). Third instar animals were staged to 40 h after the second to third instar molt (Mougneau *et al.*, 1993) and larvae carrying mutant *BR-C* chromosomes identified on the basis of their golden brown mouth parts. tGARP III and −838/*Adh* are described in Mougneau *et al.* (1993).

RNase assays

Plasmid constructs. Sgs-4 RNA levels were assayed using the plasmid pGEM-SGS4 (Mougneau *et al.*, 1993). *Pig-1* RNA was assayed using the plasmid pB′ which is a *Bam*HI fragment (−839/−1123 of *Sgs-4*) cloned into pGEM2. *Adh* RNA was assayed using the plasmid SP6mel (Fischer and Maniatis, 1986). *BR-C* zinc finger antisense RNAs were made from plasmids pCR1000Z1, pCR1000Z2, pCR1000Z3 and pCR1000Z4. Each of these *BR-C* zinc finger plasmids was constructed by PCR amplifying each individual zinc finger and cloning into the vector pCR1000 (Invitrogen). The specific sequences amplified for Z1 (1948–2270 in clone dm334), Z2 (1687–1885 in clone cD5) and Z3 (2115–2329 in clone dm717) are based on DiBello *et al.* (1991). The construction of pCR1000Z4 will be described (C.Bayer *et al.*, manuscript submitted).

RNase protection assays. RNA from salivary glands was isolated as described (Barnett *et al.*, 1990). Antisense RNA probes were prepared essentially as described by Melton *et al.* (1984) and RNase protection assays performed as described (Mougneau *et al.*, 1993). In all assays, reactions containing only labelled antisense RNAs were tested for self annealing of the probes. All reactions were performed at a minimum of 5-fold probe excess. For quantification of signals, the dried gels were exposed to a Molecular Dynamics Phosphorimager screen. Signal strength was measured by drawing a box around the band and integrating the volume using the manufacturer's software. These values were corrected for background by subtracting measurements from control boxes of the same size taken from a relevant position on the gel.

Protein purification

Mass isolation of salivary glands. Salivary gland isolation was based on Zweidler and Cohen (1971) and Boyd (1978). Wandering third instar larvae were collected and washed to remove food particles. Larvae were floated on 20% glycerol and suspended in ice-cold organ isolation medium (25 mM β-glycerophosphate, 10 mM KH_2PO_4, 30 mM KCl, 10 mM $MgCl_2$, 162 mM sucrose, 3 mM $CaCl_2$). A typical preparation began with 200–600 ml of larvae. All steps from this point on were carried out at 4°C in a cold room. Larvae were crushed between two stainless steel rollers and examined visually to ensure that most salivary glands were released intact from the carcasses. Organs were separated from carcasses and allowed to settle. The fat body was aspirated and discarded, and the remaining tissues (mostly salivary glands, gut and imaginal discs) were poured into a 1 l graduated cylinder. The cylinder was filled, mixed by inversion and the tissues allowed to settle. The denser tissues (salivary glands, gut and some imaginal discs) settle to the bottom most rapidly in this step. Most of the medium was aspirated off and the cylinder refilled. This settling step was repeated a total of

six times. Organs were then transferred to a specially constructed centrifugal separator (Zweidler and Cohen, 1971) to remove the contaminating gut. The purified salivary glands were concentrated and frozen in liquid nitrogen. A typical preparation yielded 1–3 ml of salivary glands at >80% purity.

Preparation of crude nuclear extracts. Salivary gland nuclear extracts were prepared following the protocol of Soeller *et al.* (1988) for making *Drosophila* embryonic transcription extracts. Typical preparations began with from 15 to 50 g of purified salivary glands. Crude nuclear protein was stored in 0.1 M HEMG (25 mM HEPES−KOH, pH 7.6, 0.1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 100 mM KCl, 1 mM DTT, 0.2 mM PMSF, 1 mM sodium metabisulfite) at −70°C. Nuclear extracts were fractionated on heparin agarose (EconoPak, BioRad) and protein eluted in the 0.1–0.5 M KCl fraction stored in 0.1 M HEMG at −70°C. This fraction typically contained 20–25% of the total crude extract loaded. If necessary the fractionated protein was concentrated in dialysis tubing packed in Ficoll at 4°C.

Plasmid constructs used to make BR-C proteins. BR-C cDNAs were cloned into the bacterial expression vector pDS-MCS (Schindler *et al.*, 1992) or PET-FM (Studier *et al.*, 1990; modified by S.Stevens and T.Kadesch). For the pDS-MCS constructs, PCR was used to generate an *Eco*RV site directly upstream of the ATG initiation codon shared by all *BR-C* proteins (DiBello *et al.*, 1991). Coding sequences specific to each *BR-C* isoform were then inserted downstream of the ATG at a common *Stu*I site. All clones contain nucleotides 387–474 derived from dm708. The expression constructs dm830 (BRcore-Z2), dm848 (BRcore-Q^1-Z1) and dm849 (BRcore-NS-Z3) also contain nucleotides 475–2322 (derived from dm796), 475–2951 (dm527) and 475–2692 (dm797), respectively. For the PET-FM constructs the entire BRcore-Q^1-Z1 coding region (derived from dm527) was amplified by PCR such that an *Eco*RI site was generated immediately upstream of the AUG initiation codon. A BRcore-Z4 expression construct was then generated by replacing a Z1-specific *Xho*I fragment with a Z4-specific *Xho*I fragment.

Purification of BR-C proteins. Plasmids were grown in either *E.coli* M15 cells transformed with pDMI ($lacI^q$) (pDS-MCS-based plasmids) or *E.coli* BL21 cells (pET-FM-based plasmids) and induced with 0.1–1.0 mM IPTG for 2 h. Proteins were solubilized in 6 M guanidine hydrochloride, purified on Ni^{2+} NTA resin (Qiagen) and eluted in decreasing pH steps in the presence of 8 M urea. BRcore-Q^1-Z1 (pDS-MCS) and BRcore-Z2 were eluted at pH 6.3, BRcore-NS-Z3 was eluted at pH 5.9 and 4.5, and BRcore-Q^1-Z1 (pET-FM) and BRcore Z4 were eluted at pH 4.5. Extracts from cells containing pDS-MCS or PET-FM (no *BR-C* insert) were eluted for use as controls. Purified protein was dialysed against 10–40 mM HEPES pH 7.9, 80–100 mM KCl, 0.05–0.10% Triton-X100, 1 mM DTT, 10–60 μM $ZnCl_2$ and 5–20% glycerol in a stepwise manner (1, 0.1 and 0 M urea) at 4°C. In some cases the protein aggregated and precipitated at urea concentrations <1 M. In these cases the protein was not dialysed to lower urea concentrations. Protein concentration was determined by densitometric analysis of Coomassie-stained proteins resolved by SDS−PAGE.

Footprinting assays

Plasmid constructs. The *Sgs-4 DdeI/DdeI* fragment (−568 to −149) was used to footprint elements I and II (Jongens *et al.*, 1988). The sequence from −434 to −263 was used to footprint element III. Constructs carrying mutations in element III (B*, C* and E*) are described below.

Footprinting assays. DNase I footprinting reactions (Heberlein *et al.*, 1985) contained 0.5 μg of poly(dI/C) as non-specific competitor. This represented a 500- to 1000-fold weight excess over specific DNA (1–2 fmol per reaction). Because Z1 and Z4 *BR-C* proteins are difficult to solubilize in footprinting buffer, up to 0.4 M urea was included in some reactions containing these proteins. In both cases the footprints observed in the presence of urea are identical to those seen in its absence. Generally, more protein is required to obtain complete protection from DNase I digestion in the presence of urea. Control extracts from *E.coli* cells containing pDS-MCS or PET-FM (no *BR-C* insert) were also tested in footprinting assays. These extracts have no footprinting activity on the templates used in this study.

Construction and in vivo analysis of transgenes carrying mutations in element III

Plasmid constructs and in vitro mutagenesis. The mutant *Sgs-4/Adh* reporter constructs are derivatives of −838/*Adh* (Mougneau *et al.*, 1993). Site-directed mutagenesis was performed using synthetic oligonucleotides as described by Kunkel (1985), and the sequences of the mutants were confirmed by dideoxy sequencing. The B*, C* and E* mutations were introduced using the following oligonucleotides: B*, 5′-CAACAGCTGCGGTTAcGgcccgggccGCTGGTGCTCGAGAT-3′; C*, 5′-AGCTGGTGCTCGAGAgccGcccGgggccGCCAACCCGCCGGTCCAA-3′; and E*, 5′-GGAAAATATACCTTTCgcggCgGgcccgggcTGTGAAAGACAAGTT-3′. Lower case nucleotides represent changes from the wild type sequence. Beginning with the first mutated nucleotide, the wild type sequences are: B*, 5′-AGTAAACTAAA-3′; C*, 5′-TAAGTTGGTTTAA-3′; and E*, 5′-TATTCTGTAAATATT-3′.

P element transformation. P element transformation (Rubin and Spradling, 1982) was performed using the recipient strain, ACR (Shermoen *et al.*, 1987). Homozygous transformed lines were tested for the presence of unique inserts by Southern hybridization (Southern, 1975). With the exception of one B line, all of the transformed lines generated carried unique single insertions.

Expression assays of the Sgs-4/Adh fusion genes. Animals were staged to 40 h after the second to third molt and expression assayed by RNase protection using the plasmids pGEM-SGS4 and SP6mel (see above). For quantification of *Adh* and *Sgs-4* expression, band intensities were determined using a Molecular Dynamics Phosphorimager. For each band of interest, an identical area just above the band was quantified to control for the variation in background level. The relative expression of the *Adh* transgene was determined as the ratio of the *Adh* transgene signal to the endogenous *Sgs-4* signal as follows: Relative expression = [(*Adh*) − (*Adh* background)]/[(*Sgs-4*) − (*Sgs-4* background)]. These experiments were repeated twice using whole larvae and twice using dissected salivary glands, with essentially identical results.

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