The *Drosophila Broad-Complex* plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis

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**SUMMARY**

During *Drosophila* third instar larval development, one or more pulses of the steroid hormone ecdysone activate three temporally distinct sets of genes in the salivary glands, represented by puffs in the polytene chromosomes. The intermolt genes are induced first, in mid-third instar larvae; these genes encode a protein glue used by the animal to adhere itself to a solid substrate for metamorphosis. The intermolt genes are repressed at puparium formation as a high titer ecdysone pulse directly induces a small set of early regulatory genes. The early genes both repress their own expression and activate more than 100 late secondary-response genes. The *Broad-Complex (BR-C)* is an early ecdysone-inducible gene that encodes a family of DNA binding proteins defined by at least three lethal complementation groups: *br, rbp*, and *l(1)2Bc*. We have found that the *BR-C* function is also required for the complete ecdysone induction of some early mRNAs (*E74A, E75A*, and *BR-C*) and efficient repression of most early mRNAs in prepupae. Like the intermolt secondary-response genes, the late secondary-response genes are absolutely dependent on *rbp* for their induction. An effect of *l(1)2Bc* mutations on late gene activity can also be detected, but is most likely a secondary consequence of the submaximal ecdysone-induction of a subset of early regulatory products. Our results indicate that the *BR-C* plays a key role in dictating the stage-specificity of the ecdysone response. In addition, the ecdysone-receptor protein complex alone is not sufficient for appropriate induction of the early primary-response genes, but requires the prior expression of BR-C proteins. These studies define the *BR-C* as a key regulator of gene activity at the onset of metamorphosis in *Drosophila*.

Key words: steroid hormones, gene regulation, ecdysone, *Drosophila development*

**INTRODUCTION**

Pulses of the steroid hormone 20-hydroxyecdysone (henceforth referred to as ecdysone) function as temporal signals that trigger the major postembryonic developmental transitions during the life cycle of the fruit fly, *Drosophila melanogaster*. The most dramatic of these transitions, the larval-to-adult metamorphosis, is initiated by one or more pulses of ecdysone during third instar larval development (Richards, 1981). Observation of the giant salivary gland polytene chromosomes revealed dramatic changes in the puffing pattern, reflecting the massive genetic reprogramming triggered by ecdysone (Clever and Karlson, 1960; Becker, 1962; Ashburner et al., 1974). Approximately ten intermolt puffs can be seen in the polytene chromosomes of mid-third instar larvae. At least some of these puffs represent the transcription of a family of tissue-specific genes that synthesize a protein glue used by the animal to adhere itself to a solid substrate for pupariation (Fraenkel and Brookes, 1953; Zhimulev and Kolesnikov, 1975; Beckendorf and Kafatos, 1976; Korge, 1977). The intermolt puffs regress as the ecdysone titer peaks at the end of larval development and a small set of so-called early puffs are induced rapidly, in direct response to the hormone. These puffs encode proteins that exert two opposing regulatory functions - they repress their own activity, self-attenuating the regulatory response, and they induce more than 100 late ecdysone-inducible puffs (Ashburner et al., 1974). It is this large set of secondary-response genes, represented by the late puffs, that are thought to direct the tissue-specific developmental pathways that characterize the early stages of metamorphosis.

Three early puffs have been studied at the molecular level: 2B5, at the tip of the X chromosome, and 74EF and...
75B on the left arm of the third chromosome. The genes responsible for these puffs are designated the Broad-Complex (BR-C; Chao and Guild, 1986; DiBello et al., 1991), E74 (Janknecht et al., 1989; Burtis et al., 1990), and E75 (Feigl et al., 1989; Segraves and Hogness, 1990), respectively. In addition, the EcR component of the ecdysone receptor maps to an ecdysone-regulated puff at 42A (Koelle et al., 1991). These four genes are remarkably similar. First, they are unusually long, extending 60-100 kb. Second, these genes contain multiple nested ecdysone-inducible promoters that direct the synthesis of related mRNA isoforms. E74 contains two promoters, E74A and E74B, that direct the synthesis of mRNAs that have unique 5' sequences joined to a common 3' region (Burtis et al., 1990). In a similar manner, E75 consists of three promoters, E75C, E75A, and E75B, which direct the synthesis of mRNAs with identical 3' sequences, but unique 5' exons (Segraves and Hogness, 1990). The BR-C and EcR genes also contain multiple nested promoters as well as structural complexity at the level of differential splicing (DiBello et al., 1991; Koelle et al., 1991). Finally, all of these genes encode site-specific DNA binding proteins, consistent with their proposed regulatory function. Both EcR and E75 encode members of the steroid receptor superfamily (Segraves and Hogness, 1990; Koelle et al., 1991). The E74 proteins share identical ETS DNA binding domains (Burtis et al., 1990) and the BR-C encodes at least three proteins that are defined by unique pairs of C2H2 zinc fingers. In addition, each of the BR-C mRNAs contains a common core exon that encodes a protein sequence related to several eukaryotic transcription factors (DiBello et al., 1991; Koonin et al., 1992). The BR-C, EcR, E74, and E75 mRNAs are expressed in many tissues of late third instar larvae (Chao and Guild, 1986; Segraves, 1988; Thummel et al., 1990; Koelle et al., 1991).

Mutations in the BR-C, E74, and E75 result in lethality during larval, prepupal, and pupal development, consistent with these genes playing key roles during metamorphosis (Belyaeva et al., 1980; Burtis, 1985; Kiss et al., 1988; Segraves, 1988). Of these genes, the BR-C has been most extensively characterized by classical genetic studies. The BR-C is defined by at least three lethal complementation groups: }br{ (broad), }rpb{ (reduced bristle number on the palpus), and }l(1)2Bc{. Mutations in each of these classes complement one another, but fail to complement mutations of the nonpupariating (}npr1{) class (Belyaeva et al., 1980; Belyaeva et al., 1982; Kiss et al., 1988). Each of the BR-C functions defined by these three complementation groups are required for distinct aspects of imaginal disc morphogenesis during prepupal development (Fristrom et al., 1981; Kiss et al., 1988), complete metamorphosis of the salivary gland, fat body, gut, and dorsal flight muscles (Kiss et al., 1978; Fristrom et al., 1981; Restifo and White, 1992), and proper remodeling of the central nervous system (Restifo and White, 1991). These alterations in the developmental program are not due to ecdysone deficiencies, but rather result from the cell-autonomous loss of some or all BR-C functions (Kiss et al., 1976a,b, 1978; Fristrom et al., 1981; Vijay Raghavan et al., 1988).

The pleiotropic effects of BR-C mutations on prepupal and pupal development are consistent with the effects of these mutations on ecdysone regulated gene expression at the onset of metamorphosis. The intermolt puffs are evident in the polytene chromosomes of }l(1)2Bc{ or }npr1{ third instar larvae, but some of these puffs either regress more slowly than normal or do not regress at all. In addition, the early puffs 74EF and 75B are induced to only approx. 30% of their normal size in }BR-C{ mutants, and the late puffs never appear (Belyaeva et al., 1981; Zhimulev et al., 1982). Consistent with these puffing phenotypes, }npr1{ and }rpb{ mutations, but not }br{ or }l(1)2Bc{, block the prepupal induction of a cluster of six genes located within the 71E late puff (Guay and Guild, 1991). Furthermore, these mutations prevent the induction of the intermolt glue genes in mid-third instar larval salivary glands (Crowley et al., 1984; Galcerán et al., 1990; Georgel et al., 1991; Guay and Guild, 1991). The activation of BR-C transcription in early third instar larvae, in response to low ecdysone concentrations, is consistent with its early role in inducing glue gene transcription (Karim and Thummel, 1992).

In this study we provide a molecular definition of the effects of BR-C mutations on the late larval ecdysone regulatory hierarchies. We examine the activity of four intermolt genes, three early genes, }EcR{, and seven late genes in }npr1{, }br{, }rpb{, and two }l(1)2Bc{ mutants. In agreement with previous studies, BR-C mutations block the induction of the intermolt genes }Sgs-3{, }Sgs-4{, }Sgs-5{, and }71E{ gene }VII{ in mid-third instar larvae (Guay and Guild, 1991). In addition, the intermolt genes are not repressed at puparium formation in }l(1)2Bc{ mutants, and are dramatically reinduced in apparent response to the prepupal ecdysone pulse. This suggests that the }l(1)2Bc{ function is normally responsible for repressing the mid-third instar regulatory response to ecdysone during prepupal development. Mutations in both }npr1{ and }l(1)2Bc{ result in reduced levels of ecdysone-induced E74A, E75A, and }BR-C{ transcription. This is consistent with the submaximal induction of the 74EF and 75B puffs in }npr1{ and }l(1)2Bc{ mutant animals (Belyaeva et al., 1981; Zhimulev et al., 1982). The }l(1)2Bc{ mutations also appear to reduce the levels of the hypothesized ecdysone-induced repressor of the early genes, since some early mRNAs persist for many hours following pupariation. Finally, we confirm that the }rpb{ function of the BR-C is essential for transcription of the }71E{ (Guay and Guild, 1991) and }4F{ late genes. In addition, we detect a delay in late gene induction in }l(1)2Bc{ mutants, as well as a more rapid repression. Some of these phenotypic effects could be an indirect consequence of the }l(1)2Bc{ mutation, due to the submaximal induction of the early mRNAs. These studies indicate that the BR-C acts as a central regulator in the ecdysone genetic hierarchies at the onset of metamorphosis.

**MATERIALS AND METHODS**

**Mutant and control stocks**

The BR-C mutant alleles and chromosomes used in this study are listed in Table 1. The former designations of the BR-C mutant alleles are also shown (Kiss et al., 1988). The }br{, }rpb{, }l(1)2Bc{ and }l(1)2Bc{ X chromosomes were marked with }yellow{ (Belyaeva et al., 1980), while the }npr1{ chromosome was marked with }yellow{ and }white{ (Kiss et al., 1976a). All of these mutant chromosomes were balanced with the }Binsn{ X chromosome. The BR-C mutants...
that carried a duplication of the 2B5 region (y npr1<sup>1</sup> w/Dp(1;Y)y<sup>2</sup>Y67g) were maintained in stocks with attached-X females (C(1)Dx y f/Dp(1;Y)y<sup>2</sup>Y67g/y npr1<sup>1</sup> w). This duplication (Dp(1;Y)y<sup>2</sup>Y67g) consists of a small portion of the X chromosome (cytogenetic regions 1A-2B17,18) translocated to the Y chromosome (Craymer and Roy, 1980). Strains were maintained at 25°C on cornmeal, yeast and agar in bottles or vials fitted with cotton plugs.

Hemizygous BR-C mutant male larvae were distinguished from their wild-type siblings by the yellow phenotype of their mouth hooks and denticle belts. Phenotypically wild-type male larvae that were BR-C mutants/duplication were distinguished from their phenotypically wild-type female siblings by visualizing the testes through their larval cuticles.

**Organ culture**

Wandering third instar npr1<sup>1</sup> and npr1<sup>1</sup>/duplication larvae were staged by raising these animals on food containing 0.05% bromophenol blue (Maroni and Stamey, 1983; Karim and Thummel, 1991). Larvae that have dark blue guts are more than 12 hours from pupariation formation and larvae that have white guts, but are actively crawling, are estimated to be 3 (± 1.8) hours from pupariation formation (L. Boyd, unpublished observation).

Hemizygous npr1<sup>1</sup> males and npr1<sup>1</sup>/duplication males with dark blue staining intestines (48 of each) were collected and washed in water. The two strains were handled in parallel. The larvae were placed in oxygenated Robb’s saline medium at 25°C (Robb, 1969), the cuticle was broken open, and their organs were extruded by squeezing the carcass with forceps. The larval organs and cuticle from each strain were quickly transferred to six wells (8 sets of larval organs per well) of a large well microtiter dish (Corning). A large volume (approx. 1 ml) of fresh oxygenated Robb’s saline medium was added to each well. This was changed twice after approx. 5 minutes. The old Robb’s was removed and 300 µl of fresh oxygenated Robb’s saline medium was added to each well. The microtiter dish was then placed into a Styrofoam box at 25°C, into which a constant flow of oxygen was pumped. The organs were kept in the Robb’s medium for 1 hour to allow them to recover from any previous ecdysone exposure. After the 1 hour recovery, the Robb’s medium was removed and replaced with fresh oxygenated Robb’s. 20-OH ecdysone (Sigma) was added by replacing the old Robb’s with fresh oxygenated Robb’s containing 5x10<sup>-6</sup> M hormone. After the 8-hour time course, the organs plus the Robb’s medium were transferred into microfuge tubes and centrifuged briefly to pellet the organs. The microfuge tubes were then placed on ice to stop the transcriptional activity of the larval organs. The medium was removed and the organs were transferred using forceps to the walls of a 7 ml glass dounce (Kontes). RNA was immediately extracted according to the protocol described by Thummel et al. (1990).

### Table 1. Alleles and chromosomes

<table>
<thead>
<tr>
<th>Mutant class</th>
<th>Alleles</th>
<th>Former designation</th>
<th>Lethal period</th>
<th>Reference</th>
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<tr>
<td>broad</td>
<td>br&lt;sup&gt;5&lt;/sup&gt;</td>
<td>t35</td>
<td>early prepupal</td>
<td>Belyaeva et al., 1980</td>
</tr>
<tr>
<td>reduced bristle number on the palpus</td>
<td>rbp&lt;sup&gt;5&lt;/sup&gt;</td>
<td>t376</td>
<td>late pupal</td>
<td>Belyaeva et al., 1980</td>
</tr>
<tr>
<td>l(1)2Bc</td>
<td>2Bc&lt;sup&gt;1&lt;/sup&gt;</td>
<td>t10</td>
<td>late prepupal or early pupal</td>
<td>Belyaeva et al., 1980</td>
</tr>
<tr>
<td></td>
<td>2Bc&lt;sup&gt;2&lt;/sup&gt;</td>
<td>t76</td>
<td>late prepupal or early pupal</td>
<td>Belyaeva et al., 1980</td>
</tr>
<tr>
<td>nonpupariating</td>
<td>npr1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>npr-1</td>
<td>late third instar larval</td>
<td>Kiss et al., 1976a</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Designation</th>
<th>Chromosome</th>
<th>Cytology</th>
<th>Reference</th>
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<tbody>
<tr>
<td>y&lt;sup&gt;2&lt;/sup&gt;Y67g</td>
<td>Dp(1;Y)y&lt;sup&gt;2&lt;/sup&gt;Y67g</td>
<td>Dp(1;Y) 1A:2B17-18 and 20 A3 to base of X</td>
<td>Craymer and Roy, 1980</td>
</tr>
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</table>

npr1<sup>1</sup>/duplication = y npr1<sup>1</sup> w/Dp(1;Y)y<sup>2</sup>Y67g.

### Northern blot hybridizations

RNA was fractionated by formaldehyde agarose gel electrophoresis and transferred to nylon membranes as described (Karim and Thummel, 1991). Two sets of identical blots were prepared from each set of RNAs. These blots were sequentially stripped and hybridized with each radioactive probe, as described (Karim and Thummel, 1991). Probes for EcR, E74, and E75 RNAs were prepared by asymmetric PCR as described (Karim and Thummel, 1992). All other probes were prepared by random hexamer labeling of gel-purified restriction fragments. The BR-C RNAs were...
detected using the 1.1 kb StuI to BamHI fragment from pcdm527 that contains the entire core region, thus allowing the detection of all BR-C RNA isoforms (DiBello et al., 1991).

RESULTS

Some early mRNAs are submaximally induced by ecdysone in npr1 mutant tissues

The npr1\(^3\) mutation causes a late larval developmental arrest that is equivalent to the amorphic phenotype of a deletion of the entire BR-C (Kiss et al., 1976a, 1988). Hemizygous male and homozygous female npr1\(^3\) animals fail to undergo metamorphosis and remain as third instar larvae. When these animals die, approximately two weeks after the final larval molt, there is little or no degeneration of the larval tissues and the imaginal discs have formed bloated vesicles.

In order to investigate the effects of the npr1\(^3\) mutation on early gene expression, newly wandering npr1\(^3\) mutant male larvae were collected, as well as larvae from an npr1\(^3\)/duplication strain that carried a duplication of the distal tip of the X chromosome (including the BR-C) attached to the Y chromosome (npr1\(^3\)/duplication; \(y\) npr1\(^3\) w/Dp[1;Y]\(y^2\)Y67g; see Materials and Methods). The internal organs and cuticle dissected from these animals were washed in ecdysone-free medium and cultured in the presence of 5×10\(^{-6}\) M ecdysone for 0, 1, 2, 4, 6 and 8 hours. Total RNA was then extracted and analyzed by northern blot hybridization using probes directed against E74, E75, the BR-C, and EcR (Fig. 1). The blot was also probed for rp49 mRNA to control for equal loading (O’Connell and Rosbash, 1984).

Each of these regulatory genes is induced by ecdysone in cultured organs isolated from both npr1\(^3\) and npr1\(^3\)/duplication larvae (Fig. 1). The temporal profiles of transcription in cultured organs isolated from npr1\(^3\)/duplication animals are similar to those seen in ecdysone-treated wild-type tissues (Karim and Thummel, 1992). The induction of E74B, E75B and E75C transcription is not significantly affected by the npr1\(^3\) mutation. EcR induction is reduced in the npr1\(^3\) strain while E74A, E75A, and BR-C transcripts are significantly reduced in npr1\(^3\) organs relative to the levels of mRNA that accumulate in npr1\(^3\)/duplication organs. Therefore, the BR-C, as defined by the amorphic npr1\(^3\) allele, is required for the complete ecdysone-induction of some, but not all, early mRNAs.

The l(1)2Bc function, but not br or rbp, is required for maximal early gene induction

In order to determine which BR-C subfunction contributes to the control of early gene expression, we analyzed E74, E75, BR-C, and EcR transcription in each mutant background. Hemizygous br\(^3\), rbp\(^3\), l(1)2Bc\(^1\), and l(1)2Bc\(^2\) mutant males were examined (see Table 1), as well as the corresponding four strains that carry a duplication of the BR-C attached to the Y chromosome (only the results of l(1)2Bc\(^1\)/duplication animals are shown, Fig. 2). The ability of these mutants to survive beyond puparium formation allowed us to determine their in vivo patterns of transcription in response to the late larval and prepupal ecdysone pulses. Animals were collected as wandering third instar larvae (>12 hours before puparium formation), stationary third instar larvae (approx. 3 hours before puparium formation), and at 2-hour intervals throughout preupal development, synchronized at the 0 hour white preupal stage. Total RNA was isolated and analyzed by northern blot hybridization. An RNA sample isolated from Canton S stationary third instar larvae was included as an internal control for RNA transfer and hybridization (‘+’ lane in Figs 2-4).

E74A and E74B transcription in l(1)2Bc\(^1\)/duplication larvae and prepupae is virtually indistinguishable from that seen in wild-type animals (Karim and Thummel, 1991).
E74B is active in crawling third instar larvae and is repressed with the high titer late larval pulse of ecdysone that activates E74A transcription (Fig. 2A). E74A is then repressed in early prepupae and the cycle repeats as the prepupal pulse of ecdysone activates E74B and then E74A. The level of E74A mRNA peaks at approx. 14 hours after puparium formation, 2-4 hours later than the peak levels detected in Canton S animals (Thummel et al., 1990). Similar strain variations have been reported in the timing of the prepupal ecdysone pulse (Richards, 1981).

E74A transcripts are induced to lower levels in l(1)2Bc1 and l(1)2Bc2 mutants, relative to their levels in br5, rbp5 and the l(1)2Bc1/duplication animals (Fig. 2A). The most severe effect is seen in the l(1)2Bc2 mutants, where E74A is reduced approximately 5-fold. This is comparable to the reduction in E74A mRNA levels seen in ecdysone-treated
npr1\textsuperscript{3} mutant tissues (Fig. 1), suggesting that this npr1\textsuperscript{3} phenotype is due to a loss of l(1)2Bc function. In contrast, there is no apparent effect of br\textsuperscript{5}, rbp\textsuperscript{5}, or the l(1)2Bc mutations on E74B induction in late larvae and early prepupa. However, there is an accumulation of high levels of E74B mRNA in rbp\textsuperscript{5} mutant males during the late prepupal stages (Fig. 2A). This result was obtained in two independent analyses of rbp\textsuperscript{5} mutant animals (data not shown).

Examination of E75A and BR-C transcription revealed a pattern similar to that seen for E74A. The levels of E75A and BR-C mRNAs are significantly lower in l(1)2Bc\textsuperscript{1} and l(1)2Bc\textsuperscript{2} mutant animals, with a more severe effect seen in l(1)2Bc\textsuperscript{2} mutants (Fig. 2B,C). Furthermore, the levels of mRNA accumulation are reduced in response to both the late larval and prepupal ecdysone pulses. In addition, the br\textsuperscript{5} mutation reduces the abundance of the lowest size class of BR-C mRNAs, while leaving the three larger size classes relatively unaffected (Fig. 2C).

None of the mutations has an effect on the in vivo patterns of E75C or EcR transcription (data not shown). The levels of E75B induction, however, are slightly reduced in l(1)2Bc\textsuperscript{2} mutants compared to l(1)2Bc\textsuperscript{1}/duplication animals (data not shown). These results are consistent with the observation that the noncomplementing npr1\textsuperscript{3} mutation has little or no effect on the ecdysone-induction of E75B and E75C transcription in cultured larval organs (Fig. 1).

In addition to the submaximal induction of E74A, E75A, and BR-C transcription in l(1)2Bc mutants, E74A, E74B and E75A mRNA could be detected for many hours following puparium formation in l(1)2Bc\textsuperscript{1} mutants, when their transcription is normally repressed (Fig. 2). This is most evident with E74B which can be detected throughout prepupal development in l(1)2Bc\textsuperscript{2} mutant animals (Fig. 2A). A similar but less severe effect is seen on E74B transcription in the l(1)2Bc\textsuperscript{1} mutant. EcR transcriptional repression also appears to less efficient in l(1)2Bc mutant animals (data not shown).

**BR-C mutations affect both the induction and repression of intermolt gene transcription**

The intermolt genes are coordinately induced in mid-third larval salivary glands, 90-100 hours after egg-laying, several hours after the initial induction of BR-C transcription (Beckendorf and Kafatos, 1976; Georgel et al., 1991, A. Andres, J. Fletcher, F.D.K., and C.S.T., manuscript in preparation). The BR-C appears to be important for intermolt gene induction since the intermolt mRNAs are reduced or absent in npr1 mutant animals (Crowley et al., 1984; Galcerán et al., 1990; Georgel et al., 1991).

To confirm and extend these studies, we examined the effects of BR-C mutations on Sgs-3, Sgs-4, Sgs-5, and 71E gene VII transcription (Muskavitch and Hogness, 1980; Meyerowitz and Hogness, 1982; Guild, 1984; Restifo and Guild, 1986a). All four intermolt genes displayed identical patterns of transcription in ecdysone-treated npr1\textsuperscript{1} cultured organs, and intact br\textsuperscript{5}, rbp\textsuperscript{5}, l(1)2Bc\textsuperscript{1}, l(1)2Bc\textsuperscript{2}, and l(1)2Bc\textsuperscript{1}/duplication late larvae and prepupae (Fig. 3; only the results for gene VII are shown). Consistent with previous observations, no glue mRNA could be detected in npr1\textsuperscript{1} cultured organs treated with ecdysone, whereas transcripts were present in the npr1\textsuperscript{1}/duplication control tissues (data not shown). In l(1)2Bc\textsuperscript{1}/duplication animals, glue mRNA is present in mid-third larval larvae (>12 hours before puparium formation) and repressed at puparium formation, identical to the pattern of transcription seen in wild-type animals. In agreement with the results of Guay and Guild (1991), no glue mRNA can be detected in mid-third instar rbp\textsuperscript{5} mutant larvae. We also see very little, or no, glue mRNA in l(1)2Bc mutant mid-third instar larvae. Unexpectedly, the four intermolt genes are induced to high levels in late third instar larvae (~3 hours) in all BR-C mutant strains tested. They are then repressed in br\textsuperscript{5} and rbp mutant prepupae, although repression is slightly delayed in the br\textsuperscript{5} mutants. Little repression is seen in l(1)2Bc mutant prepupae, resulting in prolonged expression of the intermolt genes into the mid-prepupal stages. Furthermore, the intermolt genes are re-induced in l(1)2Bc\textsuperscript{2} prepupae, in apparent response to the prepupal ecdysone pulse, recapitulating the response normally seen in third instar larvae. These results suggest that the l(1)2Bc function of the BR-C is critical for distinguishing between a third instar and a prepupal regulatory response to ecdysone.

**BR-C mutations affect the timing and amounts of late gene transcription**

The 71E late puff contains a cluster of three divergently transcribed pairs of ecdysone-inducible genes, designated genes I-V. These genes encode relatively short mRNAs, from 580 to 825 nucleotides in length. All six of these genes are precisely regulated, both temporally and spatially. They are expressed only in white prepupal salivary glands and repressed 14-16 hours later (Restifo and Guild, 1986a). A similar gene has been isolated from the 4F late puff (Wolfner, 1980). Thus, like the intermolt genes, these genes are coordinately regulated as a tissue-specific and stage-
Molecular phenotypes of Broad-Complex mutations

A wild-type pattern of transcription is seen in both l(1)2Bc\textsuperscript{1}/duplication and br\textsuperscript{5} animals (Fig. 4). In contrast, no RNA can be detected in rbp\textsuperscript{5} mutant animals, confirming that this BR-C subfunction is critical for activating late gene transcription (Guay and Guild, 1991). Quantitation of the levels of late mRNA in l(1)2Bc\textsuperscript{1} mutant animals revealed distinct effects on transcription (Fig. 5). In l(1)2Bc\textsuperscript{2} mutants, induction of all the late genes is delayed by approx. 2 hours and repression occurs 2-4 hours earlier. In addition, the overall levels of mRNA accumulation are reduced in l(1)2Bc\textsuperscript{2} mutant animals, with the exceptions of the 4F gene and gene II. There is no detectable effect of the l(1)2Bc\textsuperscript{1} mutation on the transcription of genes I, II, IV, or the 4F gene. This mutation results in a slight delay and reduction in the levels of gene III transcription and an effect on genes V and VI that is similar to that seen with the l(1)2Bc\textsuperscript{2} mutation. Thus the two l(1)2Bc mutant alleles reveal a graded series of effects on late gene activity. Genes 4F and II are the least affected, genes I, III, and IV show an intermediate effect, and genes V and VI are the most affected (Figs 4, 5).

DISCUSSION

Induction of the BR-C is one of the first regulatory responses to ecdysone during third instar larval development, in preparation for metamorphosis. Consistent with its early activation, this complex gene appears to be critical for the appropriate transcriptional regulation of all three classes of ecdysone-regulated genes defined by the puffing response of the late larval salivary gland polytene chromosomes: the intermolt genes, early genes, and late genes. These observations support and extend the pioneering studies of Belyaeva et al. (1981) who showed that BR-C mutations have pleiotropic effects on the puffing response of the late larval salivary gland polytene chromosomes. Our results are consistent with the identification of a family of DNA binding proteins encoded by the BR-C (DiBello et al., 1991) and suggest that this gene works together with the ecdysone-receptor protein complex to direct the appropriate progression of ecdysone-regulated gene expression at the onset of metamorphosis in Drosophila.

BR-C mutations reveal unexpected complexity in the temporal regulation of intermolt glue gene transcription

We examined the effects of br\textsuperscript{5}, rbp\textsuperscript{5}, l(1)2Bc\textsuperscript{1}, and l(1)2Bc\textsuperscript{2} mutations on Sgs-3, Sgs-4, Sgs-5, and 71E gene VII transcription. The phenotypes are identical for all four genes and reveal unexpected complexity in the mechanisms of intermolt gene regulation by ecdysone.

The br\textsuperscript{5} mutation has a modest effect on intermolt gene expression (Fig. 3). Slightly reduced levels of mRNA are detected in mid-third instar larvae (~12 hour) and some mRNA lingers in 0-hour prepupae, when glue transcripts are normally not detected. The rbp\textsuperscript{5} mutation completely blocks intermolt gene induction in mid-third instar larvae, in agreement with previous observations (Guay and Guild, 1991). In addition, no glue mRNA can be detected in l(1)2Bc\textsuperscript{2} mutant mid-third instar larvae (Fig. 3). Thus, both
rbp and l(1)2Bc functions must be induced early in third instar larval development, most likely as a response to low concentrations of ecdysone (Karim and Thummel, 1992), and are critical for the induction of intermolt gene transcription as a secondary-response to ecdysone (Hansson and Lambertsson, 1989). Although Guay and Guild (1991) also examined the effects of \( l(1)2Bc \) mutations on intermolt gene expression, the time points that they used did not resolve this effect on transcription in mid-third instar larvae.

Interestingly, in all \( BR-C \) mutant backgrounds tested, the four intermolt genes are induced in apparent response to the high titer ecdysone pulse at the end of larval development (−3 hour, Fig. 3). This result suggests that there are two temporally distinct mechanisms of glue gene induction, one in mid-third instar larvae, mediated by the \( BR-C \) in apparent response to an early low titer pulse of ecdysone, and a second mechanism in late third instar larvae that is independent of the \( br \), \( rbp \), and \( l(1)2Bc \) \( BR-C \) subfunctions. For \( Sgs-3 \), this later induction may be due to an, as yet, unidentified \( BR-C \) subfunction, since Crowley et al. (1984) report no detectable \( Sgs-3 \) mRNA in the late stages of \( npr1 \) larval development. \( Sgs-4 \) and \( Sgs-5 \), on the other hand, are present in \( npr1 \) mutant larvae and thus can be induced in a \( BR-C \)-independent manner (Crowley and Meyerowitz, 1984).

The intermolt genes are coordinately repressed by ecdysone at puparium formation. For \( Sgs-4 \), this repression appears to be mediated directly by the ecdysone-receptor protein complex (Hansson and Lambertsson, 1989). Other glue genes, including \( Sgs-3 \), \( Sgs-7 \), and \( Sgs-8 \), are rapidly repressed by ecdysone (Crowley and Meyerowitz, 1984) and this repression is dependent on ecdysone-induced protein synthesis (Hansson and Lambertsson, 1989). Our results suggest that the ecdysone-induction of the \( BR-C \) \( l(1)2Bc \) subfunction in late third instar larvae mediates this repression of intermolt gene transcription. Thus, in both the \( l(1)2Bc^1 \) and \( l(1)2Bc^2 \) mutants, intermolt mRNA can be detected throughout prepupal development (Fig. 3). These results are consistent with the longer time required for the intermolt puffs to regress in \( l(1)2Bc^1 \) mutant animals (Zhimulev et al., 1982). In the most severe case, in \( l(1)2Bc^2 \) mutant prepupae, the glue genes are reinduced in apparent response to the prepupal ecdysone pulse that triggers head

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**Fig. 5.** Levels of 4F and 71E late mRNA accumulation in \( BR-C \) mutants during late larval and prepupal development. The autoradiograms from the blots described in Fig. 4 were scanned using a Molecular Dynamics densitometer and the relative amounts of each mRNA were quantified using their volume integration software. For the positive control samples (+), both \( l(1)2Bc^1/duplication \) and \( l(1)2Bc^2/duplication \) blots were scanned and the data was averaged. Only the \( l(1)2Bc^2/duplication \) data was used for genes I and IV, since these RNAs were partially run off the bottom end of the \( l(1)2Bc^1/duplication \) blots. An arbitrary value of 100 was assigned to the maximal control value and all other data points were normalized to that value. Only the data for the \( l(1)2Bc^1 \) and \( l(1)2Bc^2 \) mutants are shown. The genes are arranged into three groups based on the similarity of their phenotypes.
eversion and the onset of pupal development (Sliter and Gilbert, 1992). Thus, in this mutant background, the prepupal ecdysone pulse appears to trigger a mid-third instar regulatory response recapitulating an earlier developmental program in a salivary gland that is beginning to undergo histolysis (Restifo and White, 1992). These results indicate the critical importance of the BR-C in dictating a proper stage-specific response to ecdysone. A point mutation has been identified within the Sgs-3 promoter that is required for its repression at puparium formation, thus providing a potential target for this l(1)2Bc activity (Martin et al., 1989).

The BR-C provides the competence for some early promoters to be fully activated by the late larval ecdysone pulse

Consistent with the reduction in the sizes of the 74EF and 75B early puffs in nprl3 and l(1)2Bc alleles (Belyaeva et al., 1981; Zhimulev et al., 1982), the nprl3, l(1)2Bc allele, and l(1)2Bc mutations, but not the br3 or rbp3 mutations, inhibit the ecdysone-induced transcription of E74A and E75A in both late larval and prepupae (Fig. 1). These observations further support the correlation between E74A and E75A transcription and the puffing responses at 74EF and 75B (Karim and Thummel, 1991, 1992). In contrast, however, the reduction in BR-C transcription in nprl3 and l(1)2Bc mutants is not accompanied by a reduction in the diameter of the 2BS puff (Belyaeva et al., 1981; Zhimulev et al., 1982). The br3 mutation appears to have a specific effect on BR-C transcription, reducing the level of the smallest size class (4.5 kb) of BR-C mRNAs in prepupae (Fig. 2C). Indeed, this is the only significant phenotype of the br3 allele noted in this study. These results indicate that an early gene can positively control early gene expression, a form of feedback regulation not predicted by the Ashburner model (Ashburner et al., 1974). EcR transcription is also reduced in ecdysone-treated nprl3 mutant tissues (Fig. 1). However, we do not detect an effect on EcR transcription by the br3, rbp3, or l(1)2Bc subfunctions, suggesting that an, as yet, unidentified BR-C subfunction may contribute to EcR induction (data not shown). Little or no effect on ecdysone-induced E74B, E75B, and E75C transcription was detected in the BR-C mutants tested (Fig. 1), although a slight reduction in E75B transcription was detected in l(1)2Bc allele, when compared to the l(1)2Bc/duplication control (data not shown).

The effects of the nprl and l(1)2Bc mutations are not restricted to the primary-response genes defined by the early puffs in the larval salivary gland polytene chromosomes. These mutations also inhibit the ecdysone-induction of Fbp-1 (Lepesant et al., 1986) and Fbp-2 (Nelson et al., 1991), ecdysone-inducible genes expressed in the late larval fat body. In addition, maximal levels of Ddc transcription in the epidermis of late third instar larvae is dependent on the br subfunction (M. Schous, W. Clark and R. Hodgetts, personal communication). Thus the BR-C facilitates the expression of a subset of genes, modulating their ecdysone-induction in a variety of tissues at the onset of metamorphosis.

The class II ecdysone-inducible RNAs, including EcR, E74B, and at least some of the BR-C transcripts, are induced by low ecdysone concentrations early during third instar larval development. This is followed by the activation of the}

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The phenotypic effects of the BR-C l(1)2Bc mutations on late gene activity are similar, but not identical, to the effects of E74A mutations on these genes. Thus, the induction of each pair of 71E late genes is delayed to different degrees in E74A mutant prepupae, and the levels of genes V and VI mRNA are reduced (J. Fletcher and C. S. T., unpublished results). This suggests that the observed effects of the l(1)2Bc mutations are, most likely, a secondary consequence of a reduction in the levels of E74A and other early regulators that, in turn, activate late gene transcription. The absolute need for the rbp function, which is critical for late gene transcription, is consistent with the ability of the BR-C rbp protein to bind to these late genes and exert a direct effect on their activity (L. Antonio, K. Crossgrove, and G. M. G., unpublished results). It seems likely that the BR-C rbp function, E74A, and other early ecdysone-inducible activators function together in a combinatorial manner to coordinate the transcription of this set of late genes.

The BR-C is a key regulator of the ecdysone genetic hierarchy

Three classes of ecdysone-regulated genes have been defined by the sets of salivary gland polytene chromosome puffs that arise in response to ecdysone during third instar larval development: intermolt, early, and late (Ashburner et al., 1974). The BR-C plays a key role in the regulation of each of these gene classes. Consistent with its global role in these regulatory pathways, BR-C mRNA can be detected throughout most of third instar larval and prepupal development (Karim and Thummel, 1992, A. Andres, J. Fletcher, F. D. K., and C. S. T., manuscript in preparation). We can detect only one role for the BR-C br complementation group; namely, its requirement for the maximal induction of the 4.5 kb BR-C mRNA during prepupal development (Fig. 2C), although we also see a slight effect of the brs mutation on intermolt gene expression (Fig. 3). In contrast, our results indicate multiple stage-specific effects of the rbp and l(1)2Bc mutations and lead to the formulation of a model for the pivotal role of these BR-C subfunctions in controlling ecdysone-regulated salivary gland gene expression at the onset of metamorphosis (Fig. 6). It should be noted that our results are consistent with the prediction of Zhimulev et al. (1982) that early low levels of BR-C expression are required, together with the high titer late larval ecdysone pulse, for proper progression of the puffing response in the polytene chromosomes.

Both the intermolt genes and late genes are induced as stage-specific secondary-responses to ecdysone, during mid-third instar and prepupal stages, respectively. The BR-C rbp function is induced as a primary-response to ecdysone and mediates the induction of these secondary-response genes (Chao and Guild, 1986; Karim and Thummel, 1992). Consistent with a direct role for rbp in intermolt gene and late gene induction is the identification of rbp protein binding sites in these promoters (intermolt: L. Von Kalm, K. Crossgrove, D. von Seggern, S. Beckendorf, and G. M. G., unpublished results; late: L. Antonio, K. Crossgrove, and G. M. G., unpublished results). In addition, a transcription factor has been identified, GEBF-I, that is inducible by ecdysone and interacts with Sgs-3 regulatory sequences at the time of its induction (Georgel et al., 1991). Whether this factor is encoded by the BR-C or works together with the rbp protein, remains unclear. It is also important to note that intermolt gene regulation is distinct from that of the late genes in two ways. First, although the induction of both classes of genes is delayed in l(1)2Bc mutants, induction of the intermolt genes is delayed by as much as a day while the late genes are delayed by only a few hours. Second, the intermolt genes have a second, temporally distinct, mechanism of induction in late third instar larvae, which may or may not be dependent on the BR-C.

In addition to its critical role in inducing secondary-response genes during mid-third instar and prepupal stages, the BR-C also plays a key role in primary-response gene expression induced by the high titer late larval ecdysone pulse. The l(1)2Bc subfunction of the BR-C is required for the maximal ecdysone-induction of a subset of primary-response transcription units, including E74A, E75A, the BR-C, and Fbp-1 (Lepesant et al., 1986). We propose that this defect in l(1)2Bc mutants leads to three distinct alterations in the program of late third instar gene regulation (Fig. 6).

First, the intermolt genes are not repressed at puparium formation, leading to a reinduction in response to the
prepubal ecdysone pulse. In this sense, the BR-C is required for suppressing a mid-third instar regulatory response to the prepubal ecdysone pulse. Second, E74A, E74B, E75A, and EcR mRNAs are not efficiently repressed in early prepupae. Third, the timing and amounts of late gene activity are altered. We propose that all of these effects are a secondary consequence of the reduced ecdysone induction of some early regulatory proteins in l(1)2Bc mutant animals. It is possible that one protein is responsible for repressing both intermolt gene activity and early gene expression. Alternatively, several repressors (as yet, unidentified) could be affected by the l(1)2Bc mutations.

It is important to note that there are two shortcomings to our proposed model. First, we cannot yet prove that the effects of BR-C mutations are due to direct or indirect interactions with target genes. Although, as mentioned above, some studies suggest that the BR-C proteins can bind directly to both intermolt gene and late gene promoters, further studies are needed to define biochemically these interactions and demonstrate their regulatory significance. Second, all of these studies examine RNA isolated from total tissues. Although intermolt and late gene activity is largely restricted to the salivary glands, the early genes are widely expressed in late third instar larvae (Chao and Guild, 1986; Burts et al., 1990; Segraves and Hogness, 1990). Thus these experiments do not address the possible tissue-specific effects of BR-C mutations on early gene regulation. It will be necessary, in future studies, to determine the spatial distribution of BR-C protein expression as well as the effects of BR-C mutations on RNA isolated from individual tissues. Nevertheless, the studies presented here do provide a clear indication of the pleiotropic effects of BR-C mutations and define this gene as a critical regulator of the stage-specific transcriptional responses to ecdysone.

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