The Ecdysone-Inducible *Broad-Complex* and *E74* Early Genes Interact to Regulate Target Gene Transcription and Drosophila Metamorphosis

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ABSTRACT

Pulses of the steroid hormone ecdysone initiate Drosophila metamorphosis by inducing widespread changes in gene expression. The *Broad-Complex* (*BR-C*) and *E74* are induced directly by ecdysone and encode families of transcription factors that regulate ecdysone primary- and secondary-response genes. Genetic analyses have revealed that mutations in the *BR-C* and *E74* are lethal during metamorphosis and that these mutations cause some similar lethal phenotypes and alterations in secondary-response gene transcription. To examine whether the *BR-C* and *E74* function together during development, we have combined representative alleles from each *BR-C* and *E74* complementation group. Analysis of the morphological and molecular phenotypes of the double-mutant animals reveals that *BR-C* and *E74* alleles act together to produce both novel and synergistic effects. We find that the *BR-C* and *E74* share functions in puparium formation, pupation and early gene induction. In addition, our evidence suggests that the BR-C and E74 transcription factors may directly interact to regulate the expression of salivary gland glue and late genes. This data is consistent with current models which propose that combinations of ecdysone primary-response genes regulate common morphogenetic pathways during insect metamorphosis.

THE steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) coordinates multiple developmental events in holometabolous insects, including molting and metamorphosis (RICHARDS 1981b; RIDDIFORD 1993). During the third larval instar of *Drosophila melanogaster* development, several low titer ecdysone pulses are thought to signal the animal to cease feeding and begin to wander in search of a suitable place to pupariate (reviewed in ANDRES *et al.* 1993; RIDDIFORD 1993). A high titer hormone pulse then triggers pupariation, the formation of the hard pupal case within which the adult structures undergo terminal differentiation (RICHARDS 1981a; HANDLER 1982). During metamorphosis, larval and imaginal tissues adopt opposite fates. Within a few hours after puparium formation, larval tissues begin to undergo either histolysis or extensive remodeling, while predetermined clusters of imaginal progenitor cells initiate morphogenesis into adult tissues and structures (ROBERTSON 1936; BODENSTEIN 1965). After ~10 hr of prepupal development, another ecdysone pulse triggers head eversion and pupation (HANDLER 1982; SLITER and GILBERT 1992). A subsequent broad hormone peak over the next day accompanies the terminal stages of metamorphosis.

The onset of metamorphosis is driven by widespread changes in gene expression, which are triggered by ecdysone via its association with the EcR/Usp receptor heterodimer (KOELLE 1992; YAO *et al.* 1992; THOMAS *et al.* 1993). In the larval salivary gland, the behavior of genes responding to ecdysone can be monitored by examination of puffs on the polytene chromosomes (ASHBURNER *et al.* 1974), which represent sites of active transcription (BEERMAN 1972). A small number of early puff genes respond directly to the hormone, at least three of which encode families of transcription factors. The *Broad-Complex* (*BR-C*) is located within the 2B5 early puff and directs the synthesis of related zinc-finger proteins (DIBELLO *et al.* 1991), while the *E74* gene from the 74EF early puff encodes two proteins that share a common ETS DNA-binding domain (BURTIS *et al.* 1990). *E75* is located within the 75B puff and encodes members of the nuclear hormone receptor family (SEGRAVES and HOGNESS 1990). These early ecdysone-inducible regulatory genes are expressed in complicated spatial and temporal patterns at the onset of metamorphosis and have been proposed to play roles in transducing the systemic hormonal signal into tissue-restricted patterns of gene expression that direct each tissue down its appropriate developmental pathway (SEGRAVES 1988; THUMMEL *et al.* 1990; EMERY *et al.* 1994).

The *BR-C* and *E74* have been extensively characterized at the genetic and molecular levels, and they share similar expression patterns and mutant phenotypes that suggest that these two genes may function together in common developmental pathways during metamorphosis. *BR-C* transcripts are induced early in the third larval instar and reach maximum levels just before puparium formation (ANDRES *et al.* 1993; HUET *et al.* 1993). Each

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BR-C protein isoform contains one of four pairs of C_2H_2 zinc fingers, designated Z1-Z4 (DiBello *et al.* 1991; C. Bayer and J. Fristrom, personal communication). During the onset of metamorphosis these proteins accumulate in both larval and imaginal tissues, in different combinations and with distinct temporal patterns (Emery *et al.* 1994).

The *BR-C* is a complex genetic locus consisting of three lethal complementation groups and an additional class of noncomplementing alleles. The noncomplementing *nonpupariating* (*npr1*) alleles are null for the entire locus, and *npr1* mutants arrest development at the end of the third instar without initiating metamorphosis (Kiss *et al.* 1978, 1988; Belyaeva *et al.* 1982). Alleles of the other three complementation groups are lethal later in metamorphosis and appear to define discrete functional domains. The br^+ (*broad*) and $2Bc^+$ functions are required for imaginal disc evagination and fusion (Kiss *et al.* 1988), respectively, and are involved in optic lobe organization during CNS repatterning (Restifo and White 1991). The rbp^+ (*reduced bristles on palpus*) function is necessary for the histolysis of the larval salivary gland and for the development of specific thoracic muscles (Restifo and White 1992). Molecular and biochemical studies have correlated the rbp^+ function with the Z1 protein isoforms and the br^+ function with the Z2 isoforms. There is currently no strong evidence correlating the $2Bc^+$ function with any BR-C protein isoform (Di Bello *et al.* 1991; Emery *et al.* 1994).

The *E74* transcripts are also widely expressed as metamorphosis begins. *E74B* is induced early in the third instar, coordinately with the *BR-C* transcripts, and is transcribed in both larval and imaginal tissues throughout most of the instar and again in mid prepupae (Karim and Thummel 1991; Andres *et al.* 1993; Huet *et al.* 1993). *E74A*, in contrast, is transcribed in a narrow window just before pupariation, when *BR-C* mRNA accumulation peaks, and again just before pupation. Like the BR-C proteins, E74A is expressed in both polyploid and diploid tissues of third instar larvae and prepupae (Boyd *et al.* 1991). Mutations in *E74A* or *E74B* are lethal during prepupal and pupal development, indicating an essential role for *E74* in metamorphosis (Fletcher *et al.* 1995). *E74A* mutants display tanning defects, reminiscent of *br* mutants, and die as prepupae or pharate adults. *E74B* mutants form misshapen puparia and arrest development predominantly during the prepupal period. A subset develop cryptocephalic phenotypes and display short, twisted legs, identifying a role for *E74B* in head eversion and appendage elongation.

Mutations in the *BR-C* and *E74* also affect the transcription of ecdysone-inducible genes in late larvae and prepupae, consistent with their effects on the early stages of metamorphosis. The induction of glue gene mRNAs in mid third instar larval salivary glands is dependent on both *E74B* and the *BR-C* (Guay and Guild 1991; Karim *et al.* 1993; Fletcher and Thummel 1995), defining a mid third instar ecdysone regulatory hierarchy. The $2Bc^+$ function is necessary at the end of larval development for glue gene repression and for the maximal ecdysone-induction of a subset of early mRNAs (Karim *et al.* 1993). Induction of the *4F* late gene and three pairs of *L71* late genes specific to the prepupal salivary gland is wholly dependent on the rbp^+ function (Guay and Guild 1991; Karim *et al.* 1993), and both $2Bc^+$ and $E74A^+$ regulate the induction and accumulation of the *L71* transcripts (Karim *et al.* 1993; Fletcher and Thummel 1995).

In this study, we provide genetic evidence that the *BR-C* and *E74* loci function together at the onset of metamorphosis. We have analyzed the morphological and molecular phenotypes of double-mutant animals carrying an *E74A* or *E74B* allele and a representative allele of one of the *BR-C* complementation groups and observe both novel and synergistic effects. Our results indicate that the *BR-C* and *E74* share some functions in controlling morphogenesis and ecdysone-regulated gene transcription, including puparium formation and pupation, early gene induction, and, possibly, eye differentiation. We also observe synergistic effects of some *BR-C* and *E74* alleles on the regulation of glue gene and late gene transcription, suggesting that the transcription factors encoded by the *BR-C* and *E74* may directly interact on these target promoters. Our results provide evidence supporting current models of ecdysone gene regulation, which propose that combinations of ecdysone primary-response genes act in common morphogenic pathways (Burtis *et al.* 1990; Thummel *et al.* 1990; Talbot *et al.* 1993).

MATERIALS AND METHODS

Drosophila stocks and crosses: Abbreviations of genetic loci are according to Lindsley and Zimm (1992). The *BR-C* and *E74* alleles and their maintenance over the *Binsn* X or TM6B *Tb e* balancer chromosome, respectively, have been described (Kiss *et al.* 1988; Karim *et al.* 1993; Fletcher *et al.* 1995). The deficiency *Df(3L)st-81k19* lacks the region from 73A3 to 74F; no haplo-insufficient phenotypes have been associated with this chromosome (Fletcher and Thummel 1995; Fletcher *et al.* 1995). Stocks were maintained at 25 or 18° on standard cornmeal/molasses medium.

To generate *BR-C;E74* double-mutant animals, mutant *BR-C y/w;+/*TM6B *Tb e* females were crossed to *w;* mutant *E74 e/*TM6B *Tb e* males at 25°. Ebony females with wild-type eye color of the genotype mutant *BR-C y/w;* mutant *E74 e/*TM6B *Tb e* were collected and crossed to *w;Df(3L)st-81k19/*TM6B *Tb e* males, yielding male progeny hemizygous for both a *BR-C* and an *E74* mutation. Ebony females with wild-type eye color were collected from among the progeny every generation and backcrossed to *w;Df(3L)st-81k19/*TM6B *Tb e* males to maintain the stock. We saw no effects on the development of compound heterozygous animals carrying either the rbp^5, br^5 or $2Bc^2$ *BR-C* allele and $E74^{P[neo]}$ or $E74^{DL-1}$ allele (data not shown).

Phenotypic characterizations: *BR-C;E74* double-mutant male third instar larvae were distinguished from their siblings by their Tb^+ phenotype and by the yellow color of their mouth hooks and denticle belts. Mutant *BR-C y/w;* mutant *E74 e/Df(3L)st-81k19* third instar larvae were distinguished from their *w;* mutant *E74 e/Df(3L)st-81k19* siblings by their Malpighian tubules, which are colorless in *w* mutants. Control animals

heterozygous for the *E74* and *BR-C* mutations were identified by their *Tb* phenotype, yellow Malpighian tubules and wild-type mouth hook color. The phenotypic analysis of double-mutant pupae was conducted as described (FLETCHER *et al.* 1995).

Northern blot hybridizations: Staging for the developmental northerns, RNA extraction and transfer, northern blot hybridization, and radiolabeled probe preparation were all performed as described by FLETCHER and THUMMEL (1995). Eight animals were collected per time point for RNA extraction, and each lane contained 10 μg of total RNA. Four identical blots were prepared from each set of RNAs. A lane of RNA markers was included on each blot, and a radiolabeled probe directed against the markers was included with each hybridization to obtain equivalent exposures. For mRNA quantitation, autoradiograms of the blots were scanned using a Molecular Dynamics densitometer and the relative amount of RNA per lane was quantitated using their volume integration software.

RESULTS

Many *BR-C;E74* double-mutant combinations cause novel phenotypes during the early stages of metamorphosis: To investigate whether the *BR-C* and *E74* function together during development, we examined the consequences of reducing the effective dose of the rbp^+, br^+ and $2Bc^+$ *BR-C* functions in *E74A* and *E74B* hemizygous mutant animals. The br^5 and $2Bc^2$ alleles used in this study are amorphic for their respective *BR-C* function, and rbp^5 is the most severe allele of the *rbp* complementation group (BELYAEVA *et al.* 1980, 1982; KISS *et al.* 1988). $E74^{P[neo]}$ represents an amorphic allele for the *E74A* transcription unit (FLETCHER *et al.* 1995) and will be referred to here as the $E74A^-$ allele. The $E74^{DI-1}$ loss-of-function mutation is specific to the *E74B* transcription unit and retains little or no *E74B* function (FLETCHER *et al.* 1995). For the purposes of this analysis, $E74^{DI-1}$ will be referred to as the $E74B^-$ allele.

We collected $E74A^-$ or $E74B^-$ hemizygous wandering third instar larvae that were also either heterozygous (a single effective dose) or hemizygous (no effective dose) for the rbp^5, br^5 or $2Bc^2$ *BR-C* allele and followed their progress through development. Unlike animals hemizygous for a single *BR-C* or *E74* allele, which die during prepupal or pupal development, the majority of *BR-C;E74* double-mutant animals of each genotype die before the wandering third instar larval stage. For the purposes of this study, we focused our analysis on the *BR-C;E74* double-mutant animals that survived to the early stages of metamorphosis, when the *BR-C* and *E74* exert critical functions. This enabled us to compare the phenotypes of the *BR-C;E74* double mutants with those described for each individual *BR-C* and *E74* allele and to assess the effect on target gene regulation in the larval salivary gland. Double-mutant animals were scored for two morphological phenotypes: abnormal puparium formation and the stage of developmental arrest (Table 1).

rbp^5 mutant males pupariate normally and die during the pupal period at the beginning of eye development. The phenotype of a representative rbp^5 male is shown in Figure 1A. Each $E74A^-$ female carrying a single effective dose of the rbp^+ function and each $rbp^5;E74A^-$ male analyzed also pupariated normally. Half of each group died during prepupal development (Table 1) and resembled $E74A^-$ mutants with severe phenotypes (FLETCHER *et al.* 1995). A representative $rbp^5/w;E74A^-$ prepupa is shown (Figure 1B). However, 8% of the $E74A^-$ animals carrying a single dose of the rbp^+ function and several $rbp^5;E74A^-$ animals displayed novel phenotypes. These animals survived into the pupal period without undergoing head eversion, developing rudimentary appendages and/or cryptocephalic head structures (Figure 1, C and D). Remarkably, one $rbp^5;E74A^-$ pupa displayed a pigmented eye disc in the upper abdomen (Figure 1D). Such cryptocephalic head structures represent a novel phenotype, as neither the $E74A^-$ or the rbp^5 allele alone affects head eversion (KISS *et al.* 1988; FLETCHER *et al.* 1995).

In contrast, the proportion of $E74B^-$ animals displaying either a prepupal or a pharate lethal phenotype was relatively unchanged as the effective dose of the rbp^+ function was reduced (Table 1). No novel or additional phenotypes were observed, and the complete penetrance of the abnormal puparium phenotype among $E74B^-$ prepupae rendered this phenotype uninformative for double-mutant analysis. We were unable, therefore, to detect an interaction between rbp^5 and $E74B^-$ based solely on examination of the double-mutant lethal phenotypes.

br^5 mutant males form a soft, untanned puparium, and fail to develop beyond the prepupal stage (Figure 2A). The majority (58%) of the $E74A^-$ mutants carrying a single effective dose of the br^+ function arrested development during the prepupal period (Table 1), and a representative prepupa is shown in Figure 2B. Unlike either br^5 or $E74A^-$ animals, however, 20% of these $br^5/w;E74A^-$ prepupae displayed a misshapen puparium (Table 1). A novel microcephalic phenotype was also observed among animals of this genotype (Figure 2C). These pupae had partially everted heads and pigmented eyes, but lacked leg and wing structures. $br^5;E74A^-$ animals displayed a more severe lethal phenotype than either br^5 or $E74A^-$ animals, as shown in Figure 2D. The misshapen puparium phenotype was completely penetrant among the $br^5;E74A^-$ prepupae, which arrested development early in the prepupal period (Table 1).

The br^5 allele may also interact with $E74B^-$ during metamorphosis. $E74B^-$ mutants carrying a single effective dose of the br^+ function showed the expected range of lethal phenotypes, from prepupal to cryptocephalic pharate adult (Table 1; Figure 2, E and F). All $br^5;E74B^-$ animals, in contrast, arrested development at the larval-prepupal transition (Figure 2G). The lack of a recognizable puparium among these animals suggests that they died as late third instar larvae. This shift in lethal phase is consistent with an interaction between br^5 and $E74B^-$,

TABLE 1

Phenotypes of *BR-C; E74* double mutant prepupae and pupae

Genotype	Total no.[a]	Puparium shape[b]		Lethal phase[c]			Pharate lethal phenotypes[d]		
		Normal	Misshapen	Prepupal	Pupal	Pharate	Legs/wings	Crypto.	Normal
$rbp^5/w;E74^{P[neo]}/Df$	38	100	—	50	37	13	5	3	5
$rbp^5;E74^{P[neo]}/Df$	13	100	—	54	38	8	—	8	—
$rbp^5/w;E74^{DL\text{-}1}/Df$	43	—	100	49	—	51	41	10	—
$rbp^5;E74^{DL\text{-}1}/Df$	10	—	100	40	—	60	—	60	—
$br^5/w;E74^{P[neo]}/Df$	40	80	20	58	33	9	—	7	2
$br^5;E74^{P[neo]}/Df$	14	—	100	100	—	—	—	—	—
$br^5/w;E74^{DL\text{-}1}/Df$	43	—	100	56	15	29	19	10	—
$br^5;E74^{DL\text{-}1}/Df$	9	—	100	100	—	—	—	—	—
$2Bc^2/w;E74^{P[neo]}/Df$	42	72	28	72	26	2	—	—	2
$2Bc^2/w;E74^{P[neo]}/Df$	19	—	100	84	—	16	—	16	—
$2Bc^2/w;E74^{DL\text{-}1}/Df$	46	—	100	61	11	28	17	11	—
$2Bc^2;E74^{DL\text{-}1}/Df$	21	—	100	100	—	—	—	—	—

w, w^{1118}; *Df*, *Df(3L)st-81k19*.

[a] The total number of newly formed prepupae of each genotype that were collected and allowed to develop at 25°.

[b] The percentage of the total number that formed either a wild type or a markedly misshapen puparium.

[c] The percentage of the total number that arrested development at the stage indicated.

[d] The percent pharate lethality is subdivided into three catagories: leg/wing development in the absence of head structures, a cryptocephalic head phenotype, or a morphologically normal phenotype.

although the possibility that this phenotype represents an additive effect due to an overall reduction in viability cannot be ruled out.

Finally, the $2Bc^2$ mutation may interact with both *E74* alleles. $2Bc^2$ mutant males form a puparium that appears normal, but has an orange cast (Figure 3A). These mutants arrest development shortly after pupation.

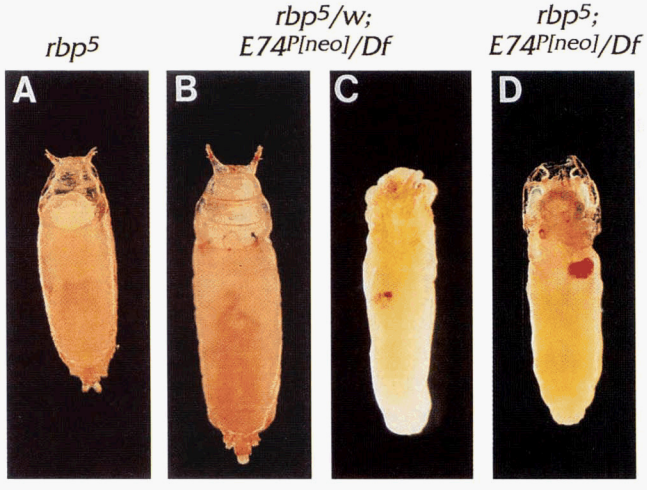


FIGURE 1.—Phenotypes of $E74A^-$ ($E74^{P[neo]}$) hemizygotes carrying different doses of the *BR-C* rbp^+ function. Stationary third instar larvae were collected and synchronized at puparium formation and allowed to develop for 4 days (92–95 hr) at 25° before being photographed. (A) A representative rbp^5 mutant male arrested at the pupal stage. (B) A representative $E74A^-$ hemizygous prepupa carrying a single effective dose of the rbp^+ function. (C) Ventral view of an $E74A^-$ hemizygous pupa carrying a single effective dose of the rbp^+ function, dissected from its pupal case, that displays a novel cryptocephalic phenotype. (D) Ventral view of an $rbp^5;E74A^-$ pupa, dissected from its pupal case, that displays a pigmented eye disc in its abdomen.

More than 25% of the $E74A^-$ animals carrying a single effective dose of $2Bc^+$ formed a misshapen puparium (Table 1; Figure 3B), and the penetrance of this phenotype increased to 100% in $2Bc^2;E74A^-$ prepupae (Table 1; Figure 3, C and D). The vast majority (84%) of the $2Bc^2;E74A^-$ animals arrested development during the prepupal stage (Table 1; Figure 3D), displaying an earlier lethal phenotype than either $2Bc^2$ or $E74A^-$ mutants. Unexpectedly, 16% of the $2Bc^2;E74A^-$ animals displayed a novel phenotype—eye development proceeded to the point where pigmentation could be clearly observed, but the other imaginal discs did not appear to undergo morphogenesis (Figure 3C).

A single effective dose of the $2Bc^+$ function had no discernible effect on the range of $E74B^-$ lethal phenotypes, which extended from the prepupal to cryptocephalic pharate adult stage (Table 1; Figure 3, E and F). However, the lethal phase of the $2Bc^2;E74B^-$ mutants shifted to the prepupal period (Table 1), and the puparium formed by these animals was often extremely curved (Figure 3G). Again, the increased severity of the $2Bc^2;E74B^-$ lethal phenotype suggests an interaction between $2Bc^2$ and $E74B^-$, although an additive effect cannot be conclusively ruled out.

Some *BR-C;E74* double-mutant combinations affect ecdysone-regulated gene transcription at the onset of metamorphosis: As a first step toward determining the molecular basis for the phenotypes described above and identifying possible interactions between the *BR-C* and *E74*- encoded transcription factors, we examined the effects of the six *BR-C;E74* double-mutant combinations on the transcription of their ecdysone-inducible target genes. We collected staged $E74A^-$ or $E74B^-$ hemizygous third instar larvae and prepupae that were also hemizygous for one of the three *BR-C* alleles, up until the

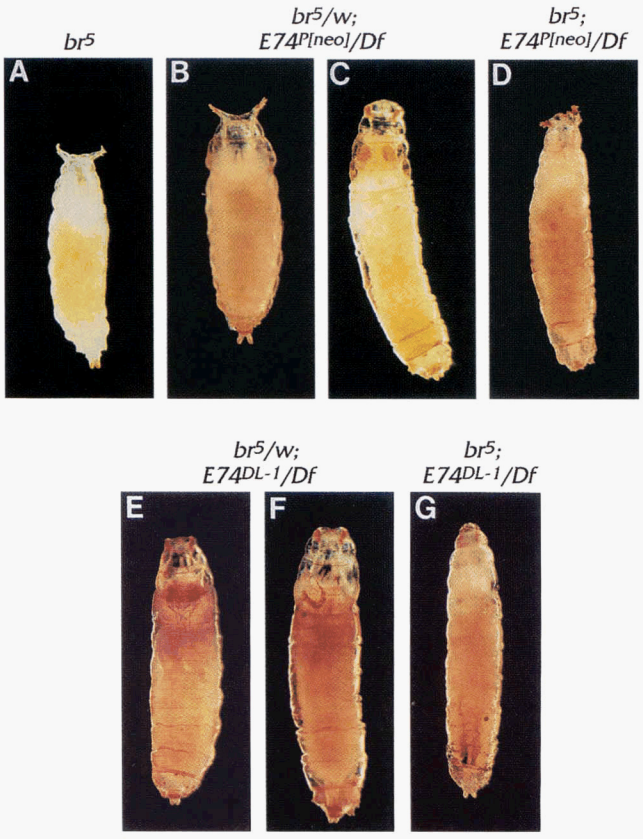


FIGURE 2.—Phenotypes of *E74* hemizygous mutants carrying different doses of the *BR-C* br^+ function. (A) A representative br^5 mutant male arrested at the prepupal stage. (B) A representative $E74A^-$ ($E74^{P[neo]}$) hemizygous prepupa carrying a single effective dose of the br^+ function. (C) A $E74A^-$ hemizygote carrying a single effective dose of the br^+ function that displays a novel head phenotype. (D) A representative $br^5;E74A^-$ prepupa. (E and F) $E74B^-$ ($E74^{DL-1}$) hemizygotes carrying a single effective dose of the br^+ function, representing the range of lethal phenotypes. (G) A representative $br^5;E74B^-$ prepupa arrested at the larval-prepupal transition.

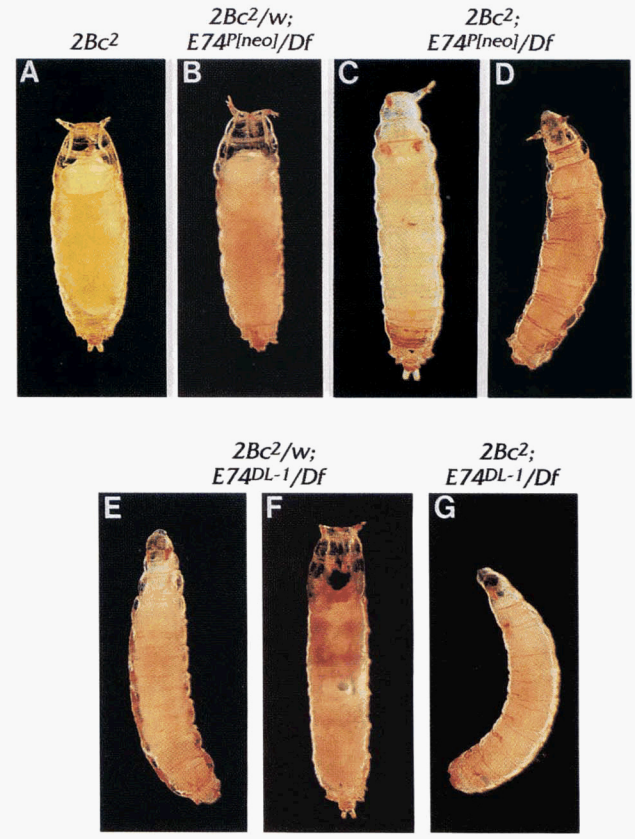


FIGURE 3.—Phenotypes of *E74* hemizygous mutants carrying different doses of the *BR-C* $2Bc^+$ function. (A) A representative $2Bc^2$ mutant male arrested following pupation. (B) A representative $E74A^-$ ($E74^{P[neo]}$) hemizygous prepupa carrying a single effective dose of the $2Bc^+$ function. (C) A $2Bc^2;E74A^-$ animal that displays a novel eye pigmentation phenotype. (D) A representative $2Bc^2;E74A^-$ prepupa. (E and F) $E74B^-$ ($E74^{DL-1}$) hemizygotes carrying a single effective dose of the $2Bc^+$ function, representing the range of lethal phenotypes. (G) A representative $2Bc^2;E74B^-$ prepupa with a severely curved puparium.

midprepupal period when the first mutants arrest their development. Heterozygous $2Bc^2/w;E74A^-$/TM6B or $2Bc^2/w;E74B^-$/TM6B animals were collected as controls. Total RNA was isolated from these animals and analyzed by Northern blot hybridization, using radiolabeled DNA probes derived from glue, early and late ecdysone-regulated genes (Table 2). In all cases, the transcription patterns we observed in the control animals were similar to those reported in Canton S wild-type animals (ANDRES *et al.* 1993).

The glue genes lie within the intermolt puffs on the larval salivary gland polytene chromosomes and encode components of the polypeptide glue used by the larva to affix itself to a substrate in preparation for pupariation (BECKENDORF and KAFATOS 1976; KORGE 1977; MUSKAVITCH and HOGNESS 1980; VELISSARIOU and ASHBURNER 1980; MEYEROWITZ and HOGNESS 1982; CROWLEY *et al.* 1983; GUILD 1984). These genes appear to be coordinately induced in mid third instar larvae as a secondary response to ecdysone (HANSSON and LAMBERTSSON 1989) and are coordinately repressed at puparium formation (CROWLEY and MEYEROWITZ 1984; HANSSON and LAMBERTSSON 1989; ANDRES *et al.* 1993). Maximal induction of the glue genes requires $E74B^+$ as well as the rbp^+ and the $2Bc^+$ functions of the *BR-C* (GUAY and GUILD 1991; KARIM *et al.* 1993; FLETCHER and THUMMEL 1995), while the $2Bc^+$ product is also necessary for their repression (KARIM *et al.* 1993).

We examined the effects of the six *BR-C;E74* double-mutant combinations on *Sgs-3, Sgs-4, Sgs-5* and 71E gene *VII* transcription. The transcription patterns of all four genes are identical and reveal several synergistic effects, in which the double-mutant phenotype is more severe than the additive effect of the individual mutant phenotypes (Table 2). Glue gene transcription in $br^5;E74A^-$ larvae is identical to that in the controls, while their transcription in $2Bc^2;E74A^-$ larvae (Figure 4A) is the same as in $2Bc^2$ animals (KARIM *et al.* 1993). However, glue gene transcription is reduced 20-fold in $rbp^5;E74A^-$ late third instar larvae compared to the levels in control larvae at the same stage (Figure 4A). In contrast, glue gene mRNA levels in late third instar larvae are relatively

TABLE 2

Summary of effects of the *BR-C;E74* mutations on ecdysone-regulated gene transcription

	$rbp^5;E74^{P[neo]}/Df$	$rbp^5;E74^{DL\text{-}1}/Df$	$br^5;E74^{P[neo]}/Df$	$br^5;E74^{DL\text{-}1}/Df$	$2Bc^2;E74^{P[neo]}/Df$	$2Bc^2;E74^{DL\text{-}1}/Df$
Glue genes						
Sgs-3	a	a	—	b	c	d
Sgs-4	a	a	—	b	c	d
Sgs-5	a	a	—	b	c	d
Gene VII	a	a	—	b	c	d
Early genes						
BR-C	—	—	—	a	c	c
EcR	—	—	—	a	—	—
E74A	—	—	—	a	c	c
E74B	—	—	—	a	—	—
E75A	—	—	—	a	c	c
Late genes						
4F	e	e	f	d	f	g
L71-1, -2, -3, -4	e	e	f	d	f	g
L71-5, L71-6	e	e	f	d	a	g

a, synergistic reduction; b, similar to $E74^{DL\text{-}1}/Df$; c, similar to $2Bc^2$; d, additive effect; e, similar to rbp^5; f, similar to $E74^{P[neo]}/Df$; g, timing unaffected; —, no effect.

unaltered by either the rbp^5 or the $E74A^-$ mutation alone (KARIM *et al.* 1993; FLETCHER and THUMMEL 1995).

Altered glue gene transcription profiles are also observed in $E74B^-$ animals carrying any of the three *BR-C* alleles (Table 2; Figure 4B). Glue gene transcription is reduced in $br^5;E74B^-$ larvae (Figure 4B), to a similar extent as in $E74B^-$ larvae (FLETCHER and THUMMEL 1995). In $2Bc^2;E74B^-$ mutants, glue gene transcription is reduced and not fully repressed, consistent with an additive effect of these two mutations (Figure 4B). Curiously, the levels of glue gene mRNA are transiently reduced in 0 hour $2Bc^2;E74B^-$ prepupae, an effect that is observed with all four glue genes analyzed. Finally, glue gene transcripts are decreased 30-fold in abundance in $rbp^5;E74B^-$ late third instar larvae (Figure 4B), revealing a synergistic effect of these two alleles.

$2Bc^+$ appears to be the only one of the $BR\text{-}C^+$ or $E74^+$ functions that regulates early gene transcription during the third larval instar, when it is required to obtain maximal ecdysone-induction of the *E74A*, *E75A* and *BR-C* mRNAs (KARIM *et al.* 1993). However, *E74B* is induced early in the third instar, coordinately with the *BR-C*, suggesting that *E74* might also contribute to early gene activation (ANDRES *et al.* 1993). To determine whether early gene transcription might require combinations of $BR\text{-}C^+$ and $E74^+$ functions, we analyzed *E74*, *E75A*, *BR-*

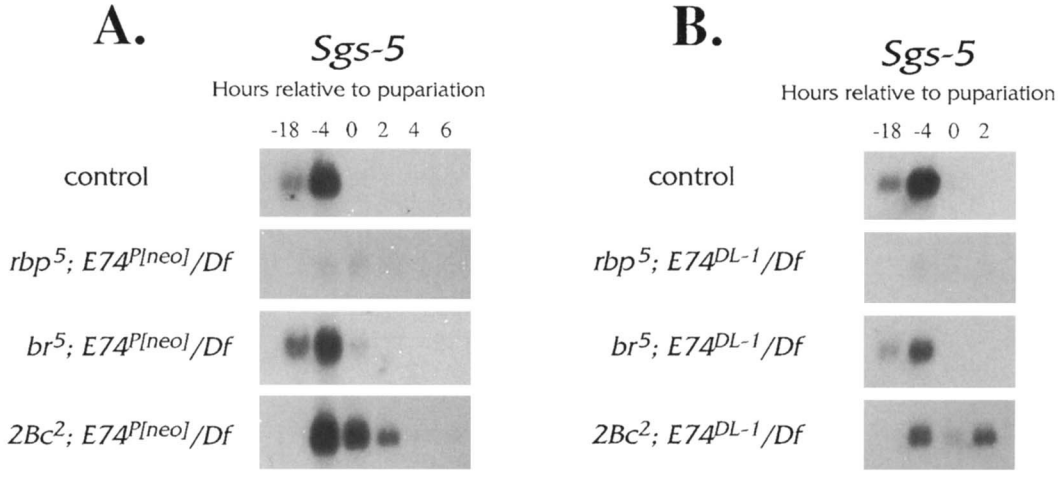


FIGURE 4.—Temporal profile of *Sgs-5* transcription in *BR-C;E74* double mutants during late larval and early prepupal development. Developmental times are given in hours relative to puparium formation. Total RNA was isolated from (A) $E74A^-$ ($E74^{P[neo]}$) hemizygous males or (B) $E74B^-$ ($E74^{DL\text{-}1}$) hemizygous males carrying either the rbp^5, br^5 or $2Bc^2$ allele of the *BR-C*. Total RNA was also isolated from (A) $2Bc^2/w;E74A^-/TM6B$ or (B) $2Bc^2/w;E74B^-/TM6B$ heterozygous females as a positive control. The RNA was fractionated by formaldehyde gel electrophoresis, and four blots were prepared from each of the eight RNA preparations. The set of blots shown was hybridized with a radiolabeled DNA probe directed against the *Sgs-5* mRNA. A radiolabeled probe directed against the *rp49* mRNA (O'CONNELL and ROSBASH 1984) was included in a subsequent hybridization to confirm equivalent loading and transfer in each lane (data not shown). Identical temporal regulation was observed with *Sgs-3*, *Sgs-4* and 71E gene *VII* transcripts (data not shown).

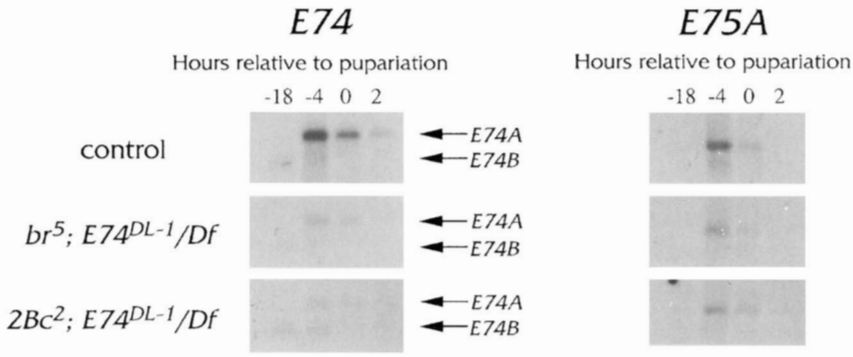


FIGURE 5.—Temporal profiles of *E74* and *E75A* transcription in *BR-C;E74B*$^{-}$ (*E74*$^{DL-1}$) mutants during late larval and early prepupal development. The blots described in Figure 4 were hybridized with radiolabeled DNA probes directed against an *E74* common exon or the *E75A* transcription unit. Identical temporal regulation was observed with *EcR* and *BR-C* transcripts (data not shown).

C and *EcR* transcription in the six *BR-C;E74* double-mutant backgrounds.

Early gene transcription in *rbp*^{5}*;E74A*$^{-}$ animals, in *rbp*^{5}*;E74B*$^{-}$ animals and in *br*^{5}*;E74A*$^{-}$ animals is nearly indistinguishable from that in the controls (Table 2). The single exception is *E75A* mRNA, which appears to be slightly reduced in both *rbp*^{5}*;E74A*$^{-}$ and *br*^{5}*;E74A*$^{-}$ larvae and prepupae (data not shown). The level of *E74B* and *EcR* mRNA in *2Bc*^{2}*;E74A*$^{-}$ animals is also the same as in the controls (Table 2), while that of *E74A*, *E75A* and the *BR-C* is reduced as it is in *2Bc*^{2} mutants (KARIM *et al.* 1993). Likewise, accumulation of the *BR-C*, *E74A* and *E75A* transcripts is reduced in *2Bc*^{2}*;E74B*$^{-}$ animals (Figure 5; data not shown) to the same extent as in *2Bc*^{2} animals. However, we observe that the expression levels of all five early mRNAs analyzed are reduced two- to sixfold in *br*^{5}*;E74B*$^{-}$ larvae and prepupae (Figure 5; Table 2). *EcR* and *E74A* represent the least and most severely affected mRNAs, respectively, while expression of the other transcripts is reduced about fourfold. This represents a novel molecular phenotype, as neither the *br*^{5} nor the *E74B*$^{-}$ allele alone affects early gene mRNA levels.

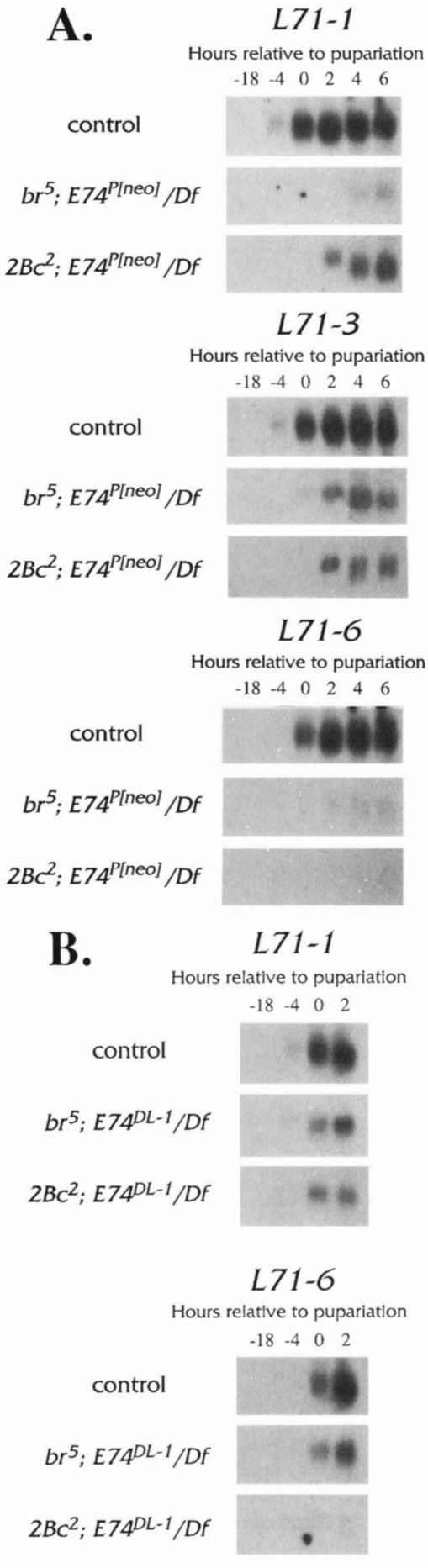
Seven late genes have been described that are restricted in their expression to the prepupal salivary glands. A single late gene has been isolated from the 4F late puff (WOLFNER 1980), while three divergently transcribed, coordinately regulated pairs of late genes have been described within the 71E late puff (RESTIFO and GUILD 1986a): *L71-1* and *L71-2*, *L71-3* and *L71-4*, and *L71-5* and *L71-6*. The *L71* mRNAs are subject to selective deadenylation during the prepupal period, resulting in a gradual decrease in their length (RESTIFO and GUILD 1986b), and are repressed at pupation. These seven genes are not transcribed in *rbp*^{5} prepupae, and their expression is delayed by 2 hr and reduced in both *2Bc*^{2} and *E74A*$^{-}$ prepupae (GUAY and GUILD 1991; KARIM *et al.* 1993; FLETCHER and THUMMEL 1995).

We have examined the transcription profiles of these seven late genes in *BR-C;E74* double-mutant prepupae. As expected, no late gene transcription is detectable in *rbp*^{5}*;E74A*$^{-}$ or *rbp*^{5}*;E74B*$^{-}$ prepupae (Table 2), indicating that *rbp*^{5} is epistatic to the *E74A*$^{-}$ and *E74B*$^{-}$ alleles with respect to late gene induction. In the other mutant genotypes, the late genes show a graded response: *4F* is least affected and *L71-5* and *-6* are most affected in each case, while the other four *L71* genes display an intermediate response (Figure 6, A and B; data not shown). In *br*^{5}*;E74A*$^{-}$ prepupae, *L71* late gene mRNA accumulation is delayed by 2 hr and reduced (Figure 6A), similar to the phenotype observed in *E74A*$^{-}$ mutants (FLETCHER and THUMMEL 1995). *L71* transcription is also reduced in *br*^{5}*;E74B*$^{-}$ prepupae (Figure 6B), an effect that can be attributed to the submaximal induction of *E74A* and *BR-C* transcripts in these animals (Table 2; Figure 5).

In *2Bc*^{2}*;E74A*$^{-}$ prepupae, the *L71-1*, *-2*, *-3* and *-4* mRNAs accumulate after a 2-hr delay and then reach or exceed the levels detectable in *br*^{5}*;E74A*$^{-}$ animals (Table 2; Figure 6A). In contrast, the response of *L71-5* and *-6* is considerably more severe. *L71-5* and *-6* transcription in *2Bc*^{2}*;E74A*$^{-}$ prepupae is delayed by 6 hr, and is reduced 18-fold compared with the controls and fivefold compared with the *br*^{5}*;E74A*$^{-}$ animals (Figure 6A). *L71* gene expression is also reduced in *2Bc*^{2}*;E74B*$^{-}$ prepupae, severely in the case of *L71-5* and *-6* (Table 2; Figure 6B); however, these phenotypes are likely to be a secondary consequence of submaximal *E74A* induction due to the *2Bc*^{2} allele. Surprisingly, the timing of the *L71* late gene induction is unaffected in *2Bc*^{2}*;E74B*$^{-}$ prepupae. *L71-1*, *-2*, *-3* and *-4* mRNAs are readily detected in *2Bc*^{2}*;E74B*$^{-}$ 0 hour prepupae, and a very low level of *L71-5* and *-6* transcription is also detectable upon prolonged exposure (Figure 6B). This phenotype is unexpected because *L71* transcripts are not present in *2Bc*^{2} 0 hour prepupae (KARIM *et al.* 1993).

DISCUSSION

The *BR-C* and *E74* early genes are induced directly by ecdysone in overlapping temporal and spatial patterns during the onset of Drosophila metamorphosis. Mutational analysis of these genes has revealed some similar morphological phenotypes and effects on the transcription of secondary-response target genes, suggesting that the *BR-C* and *E74* might function together in common developmental pathways. Our study of *BR-C;E74* double-mutant animals supports this hypothesis. We describe novel phenotypes in double-mutant prepupae and pupae, implying redundant functions for these



genes in development. In addition, we provide evidence that the *BR-C* and *E74* act together to regulate glue gene, early gene, and late gene transcription, providing a molecular basis for interpreting the observed effects on metamorphosis. Below we discuss instances of functional overlap between the $BR\text{-}C^{+}$ and $E74^{+}$ gene products, and propose that these transcription factors interact directly on the DNA to coordinate the regulation of ecdysone-inducible genes.

The *BR-C* and *E74* function together during the onset of metamorphosis: Each of the *BR-C* and *E74* alleles used in this study is lethal after puparium formation (Belyaeva *et al.* 1980, 1982; Kiss *et al.* 1988; Fletcher *et al.* 1995), and thus these genes have critical functions during metamorphosis. In contrast, many *BR-C;E74* double mutants die during embryonic or larval development, before the wandering stage that precedes pupariation. This result indicates that the *BR-C* and *E74* may function during the early stages of the life cycle. Indeed, the *BR-C* is transcribed during late embryogenesis and *E74* is transcribed during embryogenesis and the first and second larval instars, coincident with the ecdysone pulses that occur during these stages in development (Thummel *et al.* 1990; C. Bayer and J. Fristrom, personal communication). Our observations provide the first indication that these early patterns of *BR-C* and *E74* expression may be of functional significance.

We also observe several instances where the *BR-C* and *E74* function together during late larval and prepupal development. Formation of an elongated puparium with incompletely everted spiracles is a novel and completely penetrant phenotype among br^{5};$E74A^{-}$ and $2Bc^{2}$;$E74A^{-}$ prepupae (Table 1, Figures 2D and 3D). This result indicates that in addition to their independent roles in puparium tanning or sclerotization (Kiss *et al.* 1988; Fletcher *et al.* 1995), the br^{+}, $2Bc^{+}$ and $E74A^{+}$ products also have overlapping functions in shaping the pupal case. A subset of $E74A^{-}$ prepupae carrying a single effective dose of rbp^{+} or br^{+} also display defects in head eversion, resulting in cryptocephalic or microcephalic lethal phenotypes (Figures 1C and 2C). These novel phenotypes are not observed among *BR-C* or *E74A* single-mutant animals, suggesting that these alleles genetically interact and indicating that the *BR-C* and *E74* contribute to some of the same developmental

Figure 6.—Temporal profiles of *L71-1*, *L71-3* and *L71-6* transcription in *BR-C;E74* double mutants during late larval and early prepupal development. (A) The *BR-C;E74A*$^{-}$ ($E74P^{[neo]}$) blots described in Figure 4 were hybridized with radiolabeled DNA probes directed against the *L71-1*, *L71-3* or *L71-6* late gene. The transcription profile of *L71-5* is identical to that of *L71-6*, and the profiles of *L71-2* and *L71-4* resemble that of *L71-1* (data not shown). (B) The *BR-C;E74B*$^{-}$ ($E74^{DL\text{-}1}$) blots described in Figure 4 were hybridized with radiolabeled DNA probes directed against the *L71-1* or *L71-6* late gene. The transcription profile of *L71-5* is identical to that of *L71-6*, and the profiles of the other three *L71* genes fall in a range between those of those genes shown (data not shown).

processes. Both puparium formation and head eversion are immediately preceded by high titer ecdysone pulses (RICHARDS 1981b; SLITER and GILBERT 1992), consistent with a role for both the *BR-C* and *E74* in regulating these developmental events. Furthermore, both events are dependent on the contraction of larval abdominal muscles (ROBERTSON 1936; CROSSLEY 1978) suggesting that these genes may exert their effects, at least in part, through the activity of the larval musculature.

Complete or partial eye development was observed among some *2Bc²;E74A⁻* animals (Figure 3C), and a fully pigmented eye formed in the abdomen of one *rbp⁵;E74A⁻* pupa (Figure 1D). This phenotype represents a rescue of eye development, as neither *rbp⁵* nor *2Bc²* hemizygotes survive beyond the midpupal period when pigmentation of the retina begins (BELYAEVA *et al.* 1982; KISS *et al.* 1988). These observations suggest that *E74A* may act together with one or more *BR-C* products to negatively regulate aspects of eye development. Consistent with this proposed function, both E74A protein and the Z1 protein isoform of the *BR-C* are expressed in the imaginal discs (BOYD *et al.* 1991; EMERY *et al.* 1994). Another ETS family member, *yan*, can also negatively regulate eye development, acting as a cell-autonomous negative regulator of R7 photoreceptor development (LEI and RUBIN 1992).

The *BR-C* and *E74* function together to regulate ecdysone-inducible target gene transcription: The *rbp⁵;E74A⁻* allele combination causes a significant reduction in salivary gland glue transcript accumulation in late third instar larvae (Figure 4A). This is a novel molecular phenotype, as the *E74A⁻* allele alone has no discernible effect on glue gene transcription (FLETCHER and THUMMEL 1995) and the *rbp⁵* allele does not alter glue transcript accumulation late in the third instar (KARIM *et al.* 1993). Thus, the *rbp⁺* and *E74A⁺* products appear to play redundant roles in this process. Z1 BR-C protein is present in mid and late third instar larval salivary glands and binds to *Sgs-4* regulatory sequences, consistent with the regulation of glue gene transcription by *rbp⁺* (EMERY *et al.* 1994; VON KALM *et al.* 1994). A role for *E74A*, however, is more difficult to discern. Although *E74A* mRNA is present in late third instar larvae (KARIM and THUMMEL 1991), accumulation of its protein product is delayed by several hours and peaks at puparium formation (BOYD *et al.* 1991). It is possible that the requirement for *E74A* can be provided by the low levels of E74A protein present in late larvae. Alternatively, the contribution of *E74A* may be an indirect effect of its expression at the beginning of the third larval instar, approximately one-half day before glue gene induction (ANDRES *et al.* 1993).

Glue transcript accumulation is also significantly reduced (30-fold) in *rbp⁵;E74B⁻* larvae, revealing a synergistic effect of the two alleles (Figure 4B). The Z1 BR-C isoform and *E74B* transcripts are both expressed in mid third instar larvae (ANDRES *et al.* 1993; HUET *et al.* 1993; EMERY *et al.* 1994), and neither appears to regulate the expression of the other as deduced by their expression patterns in single-mutant animals. This evidence suggests that these two functions do not act sequentially in a pathway leading to glue gene induction, but rather at the same step. Yet, each function has an independent effect on glue gene transcription (Figure 7A). Glue gene induction is delayed in *rbp⁵* mutant larvae but reaches wild-type levels by the end of the instar (GUAY and GUILD 1991; KARIM *et al.* 1993), whereas the overall levels of glue mRNA accumulation are reduced in *E74B⁻* mutants (FLETCHER and THUMMEL 1995). Our observations lead to the hypothesis that *rbp⁺* (Z1) activates glue gene transcription in mid third instar larvae and that E74B interacts directly with Z1 to achieve maximal levels of glue transcription (Figure 7A). Given that the *rbp⁺* function directly regulates the *Sgs-4* promoter (VON KALM *et al.* 1994), a test of our model will be to determine whether these regulatory sequences also contain E74B binding sites.

The combination of the *br⁵* and *E74B⁻* alleles reveals a novel effect on early gene transcription. The *E74, BR-C, EcR* and *E75A* early mRNAs are all submaximally induced in *br⁵;E74B⁻* larvae (Table 2; Figure 5), indicating a redundant role for the *br⁺* and *E74B⁺* products in the regulation of early gene transcription. A global requirement for these products to achieve maximal early gene induction may also account for the early lethality observed among *br⁵;E74B⁻* mutants. These animals die at the larval-prepupal transition, apparently without undergoing pupariation (Figure 2B). A similar lethal phenotype characterizes the amorphic *nonpupariating* (*npr1*) alleles of the *BR-C* (KISS *et al.* 1976, 1978), which also leads to reduced induction of the *BR-C, E74A,* and *E75A* early mRNAs (KARIM *et al.* 1993). Our results suggest that the activation of the *BR-C* and *E74B* promoters in early third instar larvae, apparently by a low titer ecdysone pulse, plays an important and at least partially overlapping role in the subsequent induction of the early mRNAs by the high titer late larval ecdysone pulse (KARIM and THUMMEL 1992; ANDRES *et al.* 1993) (Figure 7B).

Based on their individual mutant phenotypes, *2Bc⁺* and *E74A⁺* have been proposed to act sequentially in the late gene induction pathway (KARIM *et al.* 1993). The accumulation of the *L71* transcripts is delayed by 2 hr and reduced in both *2Bc²* and *E74A⁻* prepupae (KARIM *et al.* 1993; FLETCHER and THUMMEL 1995). This phenotype in *2Bc²* animals is most simply explained as a secondary consequence of the submaximal ecdysone-induction of *E74A*, effectively phenocopying *E74A⁻* animals (KARIM *et al.* 1993). The observation that *L71-1, -2, -3,* and *-4* transcripts are reduced and delayed in *2Bc²;E74A⁻* prepupae, identical to the pattern seen in *E74A⁻* animals, provides further support for this model (Figures 6A and 7C).

In contrast, *L71-5* and *L71-6* transcription is delayed and greatly reduced (18-fold) in *2Bc²;E74A⁻* prepupae, revealing a synergistic effect of the two alleles (Figure

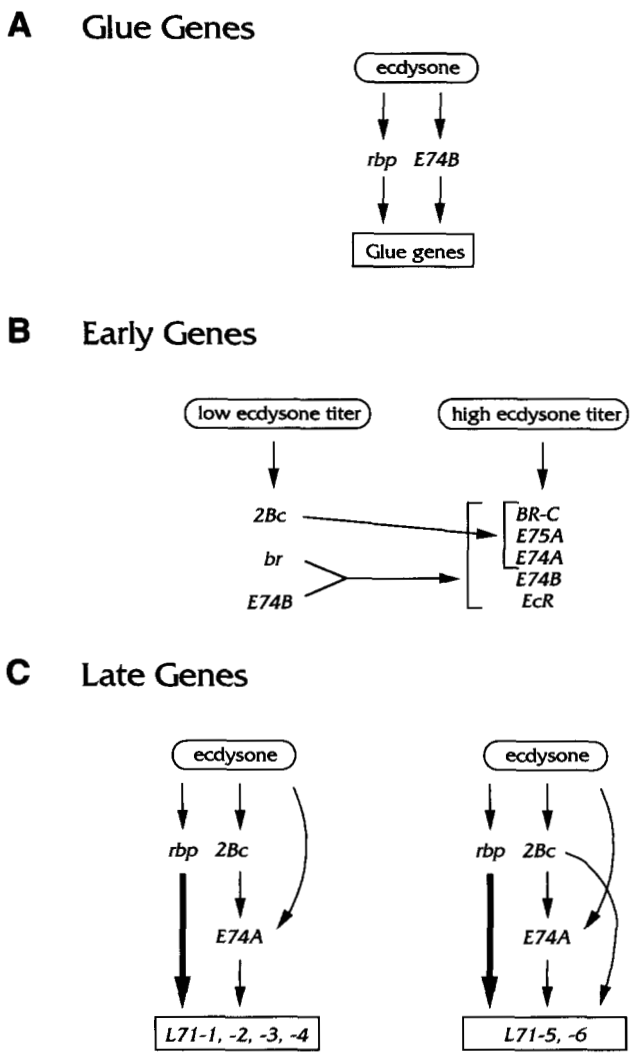


FIGURE 7.—Models for the regulation of glue, early, and late gene transcription by the *BR-C*$^+$ and *E74*$^+$ functions. (A) The *rbp*$^+$ and *E74B*$^+$ functions act together to regulate glue gene transcription in mid third instar larvae. *rbp*$^+$ is required for the proper timing of glue gene induction while *E74B*$^+$ is required for maximal levels of glue gene transcription. In the absence of both functions, glue mRNA is almost undetectable. (B) The *BR-C* and *E74B* have redundant functions in up-regulating early gene transcription in late third instar larvae. It has been shown previously that the early induction of the *2Bc*$^+$ function, apparently by a low titer ecdysone pulse, is required for the maximal induction of the *BR-C*, *E74A*, and *E75A* early mRNAs by the high titer late larval ecdysone pulse (KARIM *et al.* 1993). This study reveals that the *br*$^+$ and *E74B*$^+$ functions play redundant roles in up-regulating the early mRNAs. (C) The *2Bc*$^+$ function may contribute directly to the regulation of *L71-5* and *L71-6* transcription in prepupae. The *rbp*$^+$ function is essential for late gene transcription (heavy arrow). Maximal levels of *E74A*$^+$, achieved through the *2Bc*$^+$ function, are required for the proper timing and levels of *L71-1, -2, -3* and *-4* mRNA accumulation. In addition, almost no *L71-5* or *L71-6* mRNA is detectable in *2Bc*^{2}*;E74A*$^-$ double mutants, suggesting a direct role for *2Bc*$^+$ in regulating this gene pair.

6A). From this observation, we conclude that the regulation of *L71-5* and *L71-6* transcription by the *2Bc*$^+$ function is not mediated solely through *E74A*$^+$. Rather, in addition to the products of *rbp*$^+$ and *E74A*$^+$, the *2Bc*$^+$ product itself, or another target of *2Bc*$^+$ regulation, also contributes to the control of *L71-5* and *L71-6* transcription in prepupal salivary glands (Figure 7C). The responses of *L71-5* and *L71-6* in *2Bc*^{2}*;E74A*$^-$ prepupae distinguish the regulation of this gene pair from that of the other two described *L71* gene pairs, providing evidence that the coordinate regulation of these six late genes can be uncoupled (Figure 7C).

Finally, we observe a surprising effect on late gene transcription in *2Bc*^{2}*;E74B*$^-$ mutant prepupae. The induction of the *L71* transcripts, which normally occurs at puparium formation, is delayed by 2 hr in *2Bc*^{2} prepupae (KARIM *et al.* 1993). Yet, these transcripts are present in *2Bc*^{2}*;E74B*$^-$ 0 hour prepupae (Table 2; Figure 6B). We propose that active repression occurs at the *L71* promoters, and that this repression can be alleviated by the removal of *E74B*$^+$ activity. *E74B*, which is expressed in the larval salivary gland and which is transcribed throughout the late third instar until a few hours before puparium formation (KARIM and THUMMEL 1991), is itself a likely candidate to encode this negative regulator of late gene activity. Additional evidence that *E74B* regulates *L71* expression will be presented elsewhere (J. C. FLETCHER and C. S. THUMMEL, unpublished observations).

The *BR-C* and *E74* early puff genes are induced directly by ecdysone and encode families of transcription factors that are expressed in overlapping spatial and temporal patterns during the onset of metamorphosis. The finding that early puff genes are expressed outside the larval salivary gland led to the proposal that combinations of early regulatory genes coordinate tissue responses at each stage of development characterized by a hormone pulse (BURTIS *et al.* 1990; THUMMEL *et al.* 1990). Our results provide strong support for this model, by demonstrating a functional overlap between the *BR-C* and *E74* early gene products in tissue-specific developmental processes and in the genetic regulatory hierarchies of the larval salivary gland. Similar genetic studies of other ecdysone-inducible early genes should yield further insights into the mechanisms by which steroid signals are transduced into stage- and tissue-specific developmental responses.

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