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The *Drosophila E74* gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone

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SUMMARY

The steroid hormone ecdysone initiates *Drosophila* metamorphosis by reprogramming gene expression during late larval and prepupal development. The ecdysone-inducible gene *E74*, a member of the *ets* proto-oncogene family, has been proposed to play a key role in this process. *E74* is encoded within the 74EF early puff and consists of two overlapping transcription units, *E74A* and *E74B*. To assess the function(s) of *E74* during metamorphosis, we have isolated and characterized recessive loss-of-function mutations specific to each transcription unit. We find that mutations in *E74A* and *E74B* are predominantly lethal during prepupal and pupal development, consistent with a critical role for their gene products in metamorphosis. Phenotypic analysis reveals that *E74* function is required for both pupariation and pupation, and for the metamorphosis of both larval and imaginal tissues. *E74B* mutants are defective in puparium formation and head eversion and die as prepupae or cryptocephalic pupae, while *E74A* mutants pupariate normally and die either as prepupae or pharate adults. We have also investigated the effects of the *E74* mutations on gene expression by examining the puffing pattern of the salivary gland polytene chromosomes in newly formed mutant prepupae. Most puffs are only modestly affected by the *E74B* mutation, whereas a subset of late puffs are sub-maximally induced in *E74A* mutant prepupae. These observations are consistent with Ashburner's proposal that early puff proteins induce the formation of late puffs, and define *E74A* as a regulator of late puff activity. They also demonstrate that *E74* plays a wide role in reshaping the insect during metamorphosis, affecting tissues other than the salivary gland in which it was originally identified.

Key words: *Drosophila*, *E74*, ecdysone, metamorphosis, cryptocephalic pupa, ETS, polytene chromosome

INTRODUCTION

The steroid hormone 20-hydroxyecdysone (henceforth referred to as ecdysone) functions as a temporal signal to coordinate multiple developmental events during the life cycle of the fruit fly *Drosophila melanogaster*. Increases in ecdysone titer accompany each of the six stages of *Drosophila* development (Richards, 1981a, b). The first pulse, of unknown function, occurs during mid-embryogenesis. Two succeeding pulses trigger larval ecdysis, the molting of the larval cuticle that defines the end of the first and second larval instars. These are followed by a high titer pulse at the end of the third larval instar that signals puparium formation and the onset of metamorphosis. During metamorphosis the body plan is reorganized: adult structures are generated from clusters of imaginal cells, while larval tissues are eliminated or remodeled into adult forms. About 12 hours after pupariation, a brief ecdysone pulse triggers head eversion and the prepupal-pupal transition. A subsequent broad peak of ecdysone during pupal development accompanies the terminal stages of differentiation into the adult form.

Insights into the mechanisms whereby ecdysone initiates *Drosophila* metamorphosis have come from observations of the effects of the hormone on the puffing pattern in the larval salivary gland polytene chromosomes (Becker, 1959; Clever and Karlson, 1960; Ashburner, 1972). These studies revealed that the late third instar larval ecdysone pulse directly induces a small set of early puffs that peak in size just prior to pupariation. At this time, a highly reproducible sequence of more than 100 late puffs is initiated and continues through the prepupal period. Based on these and other studies, Ashburner et al. (1974) proposed a hierarchical model for the genetic regulation of polytene chromosome puffing by ecdysone. According to this model, ecdysone forms a complex with its receptor and acts directly to induce early puff formation and to suppress late puff induction. The protein products of the early puffs then activate the late puffs in a precisely timed manner and repress their own expression, thus limiting the duration of the regulatory response.

Evidence confirming this model at the molecular level came first from the isolation of three early genes, each responsible for a different early puff: *E74* from the 74EF puff (Burtis, 1985; Burtis et al., 1990; Thummel et al., 1990), *E75* from the

75B puff (Segraves, 1988; Segraves and Hogness, 1990), and the *BR-C (Broad-Complex)* from the 2B5 puff (DiBello et al., 1991). Mutational analysis of these genes has largely been limited to the *BR-C* – indeed, it is the only one of these genes that was originally defined by that analysis (Belyaeva et al., 1980, 1981). *BR-C* mutations are lethal during prepupal and pupal development, affect both larval and imaginal tissues, and greatly abbreviate the puffing response to ecdysone (Kiss et al., 1978; Belyaeva et al., 1981; Fristrom et al., 1981; Kiss et al., 1988; Restifo and White, 1991, 1992). In contrast, genetic analysis of *E74* and *E75* has been limited to the effects of aneuploidy for a region of 59 polytene bands that includes both the 74EF and 75B puff loci (Walker and Ashburner, 1981). The presence of additional copies of this region leads to a more rapid induction of a subset of late puffs, as well as an increase in their size, whereas the presence of only one copy of this region leads to a corresponding delay in the induction of these late puffs and a reduction in their size. These results suggest that the products of one or both of these early puffs control the activity of some late puff loci, thereby supporting one of the predictions of the Ashburner model.

We have performed a mutational analysis of the *E74* locus in order to expand our understanding of early gene function. Fig. 1 shows the overlapping *E74A* and *E74B* transcription units that make up the *E74* gene (Burtis et al., 1990). Both units are ecdysone-inducible in late third instar tissues by direct activation of their promoters, and each is transcribed in association with all six major ecdysone pulses during development (Thummel et al., 1990). They are also widely expressed in late third instar larvae (Thummel et al., 1990; Karim and Thummel, 1991; Huet et al., 1993), providing the capacity to transduce the ecdysone signal in a variety of target tissues. The E74A and E74B proteins are composed of distinct N-terminal regions and a common C-terminal region containing an ETS DNA-binding domain (Burtis et al., 1990). E74A protein binds to a subset of late puff loci on the polytene chromosomes (Urness and Thummel, 1990), suggesting that E74A may directly regulate the corresponding late genes during metamorphosis.

Here we use *E74* loss-of-function mutations to examine the roles played by the E74A and E74B proteins during metamorphosis, including their roles in the salivary gland polytene chromosome puffing hierarchy. Mutations in *E74* are predominantly lethal during prepupal and pupal development, indicating that E74A and E74B have essential functions during metamorphosis. *E74B* mutants are unable either to form a normal puparium or to evert their cephalic complexes, and die as prepupae or early pupae. In contrast, many *E74A* mutants survive the prepupal period and die as pharate adults. *E74A* activity is essential for the maximal induction of a subset of late puffs at puparium formation, confirming the results obtained by Walker and Ashburner (1981) and implicating *E74A* as one function in the 74EF/75B interval that is necessary for proper late puff regulation. Taken together, these results are in accordance with the predictions of the Ashburner model, and support the extension of this model to other tissues in the developing insect (Thummel et al., 1990).

MATERIALS AND METHODS

Drosophila stocks

Abbreviations of genetic loci are according to Lindsley and Zimm (1992). The isolation of recessive lethal mutations in the *E74* gene has been described in part by Burtis (1985) and will be described in detail elsewhere (K. C. B., unpublished data). Mutations were isolated on an isogenic *st* p^p e^{11} chromosome either by mutagenesis with ethyl methanesulfonate (EMS) using the conditions of Lewis and Bacher (1968) or by X-irradiation (5000 rad).

The $E74^{P[neo]}$ allele was generated in a screen for P element induced lethal mutations on a *mwh r* e^s chromosome (Cooley et al., 1988), and is revertible to wild type upon excision of the P element (K. C. B., unpublished results). It was obtained from the Bloomington Drosophila Stock Center, where it is designated #P262 P{pUChs:neo}l(3)24. The deficiency *Df(3L)st-81k19*, which lacks the region from 73A3 to 74F, was generated by M. M. Green and obtained from B. Baker. The deficiency *Df(3L)st-ss103*, which lacks the region from 73A to 74A, was generated by M. Ashburner and obtained from B. Baker.

The *E74* alleles and *Df(3L)st-81k19* were maintained over the balancer chromosome *In(3LR)TM6B, Hu e Tb ca.* For analysis of mutant phenotypes and polytene chromosome puffing, *Df(3L)st-81k19/TM6B* females were crossed with mutant *E74/TM6B* males at 25°C. Mutant larvae and prepupae were identified by their Tb^+ phenotype.

Molecular characterization of $E74^{DL-1}$ allele

For sequence analysis of the EMS-induced $E74^{DL-1}$ allele, the entire *E74B*-specific exon was amplified by polymerase chain reaction (PCR). Genomic DNA isolated from late third instar larvae carrying the $E74^{DL-1}$ allele over the deficiency *Df(3L)st-81k19* was amplified using the following overlapping sets of primers spanning exon B: B1 = CATTAACGCAGACATCACGC (intronic) and B6 = ATCCGCGTCATCGACATCCT (complementary to bases 1254-1262; Burtis et al., 1990); B2 = GTTTAAATGCTTGCCAC (intronic) and B4 = TGAAGGAACCAAGACATGCTG (complementary to bases 794-811). Amplified products in a 20 μl total volume were heated for 15 minutes at 99°C, treated with 5 units Klenow DNA polymerase (NEB) in the presence of 20 mM $MgCl_2$ for 30 minutes at 37°C, and purified through 1% NuSieve GTG agarose (FMC BioProducts) gels. The purified fragments were cut from the gels, melted and ligated overnight at room temperature into pBluescript (Stratagene) cut with *Eco*RV. Sequence analysis was performed using a Taq Dye Primer Cycle Sequencing Kit (ABI) on an Applied Biosystems model 373A automated DNA sequencer. Five isolates derived from two independent amplification reactions were sequenced to confirm the presence of the mutation.

Phenotypic characterizations

Crosses for the lethal phase analysis were made to wild-type Canton S flies and were carried out at 25°C as shown in Table 1. A 6-hour egg collection was taken from each cross, approximately 250 eggs counted, and the remainder discarded. After 36 hours, the number of white, unfertilized eggs was subtracted from the original count, and this figure entered into the 'total embryos' column of Table 1. The number of brown, dead embryos was also counted to determine embryonic lethality. After 5 days, wandering third instar larvae were transferred into vials and the number that pupariated was tallied. This number was subtracted from the number of hatched larvae to determine larval lethality. On day 13, flies remaining in their pupal cases were counted to determine pupal mortality.

For phenotypic analysis of mutant pupae, wandering third instar larvae hemizygous for the *E74* mutations were picked in bulk and allowed to wander on a moist black filter paper in a Petri dish for 4 hours, at which time animals that had failed to pupariate were discarded. The remaining prepupae were counted and allowed to continue development at 25°C. Approximately 100 animals of each genotype were followed for 7 days after puparium formation, and each animal was scored for morphological markers representative of successive pupal stages (Bainbridge and Bownes, 1981). Mutant

prepupae and pupae were viewed by phase-contrast dark-field microscopy and photographed with Kodak Ektar 25 color print film.

Cytology

To stage *E74* mutant prepupae as accurately as possible, groups of 10-20 wandering third instar larvae were collected on moist black filter paper in a 150-mm Petri dish and checked every 15 minutes for newly pupariated animals. Salivary glands from newly formed prepupae were dissected in PBS, fixed in 45% acetic acid and stained in a drop of lacto-acetic orcein. Squashes were viewed by phase-contrast microscopy and photographed with Kodak 125 Plus X-pan film. Puffing activity was quantitated as the ratio between the maximum diameters of the puffs and of nearby unpuffed reference bands, measured with a digital filar micrometer. The reference bands used in this study have been previously characterized (Ashburner, 1967; Walker and Ashburner, 1981). Each column and vertical bar in the histograms represents the mean and standard error of at least 19 measurements of puffed chromosomes from five or more pairs of salivary glands.

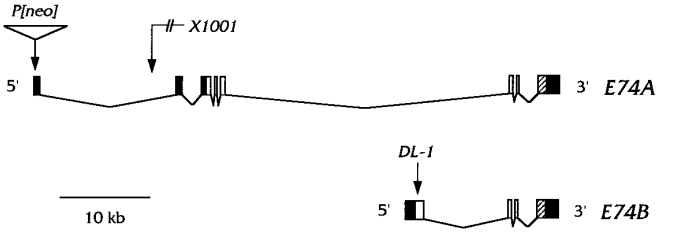

Fig. 1. *E74* gene structure showing the locations of *E74* mutations. The approximate sizes and locations of the *E74A* and *E74B* exons are depicted relative to a 10 kilobase (kb) scale. The solid black boxes represent noncoding 5′- and 3′-flanking regions, while the open boxes represent protein-coding regions. The hatched portion of the last exon shared between *E74A* and *E74B* contains sequences encoding the ETS DNA-binding domain (Burtis et al., 1990). The insertion site in *E74A* sequences of the P element associated with the $E74^{P[neo]}$ allele is indicated with a triangle, and the site of the $E74^{X1001}$ translocation breakpoint is denoted with a broken arrow. The arrow over the *E74B*-specific exon marks the deletion associated with the $E74^{DL-1}$ allele.

RESULTS

Isolation and molecular characterization of *E74* mutations

Mutant alleles of the *E74* gene were recovered from screens for X-ray- and EMS-induced recessive lethal mutations in the chromosomal region 74A to 74F, defined by the overlapping deficiencies *Df(3L)st-ss103* and *Df(3L)st-81k19*. The lethal mutations were subsequently assigned to approximately 30 complementation groups (Burtis, 1985; K. C. B., unpublished data). One mutant allele, $E74^{X1001}$, was found to be associated with a reciprocal translocation having one breakpoint in the first intron of *E74A*, upstream of the coding region (Fig. 1; Burtis, 1985). $E74^{X1001}$ is a member of a complex complementation group, reflecting the known molecular complexity of the *E74* locus (Burtis et al., 1990).

Based on *inter se* complementation analysis, the mutant alleles of the *E74* gene fall into three classes (Burtis, 1985; K. C. B., unpublished data). The first class consists of seven alleles, including $E74^{X1001}$, that fail to complement one another but completely complement a second class consisting of two alleles. The third class of *E74* mutants consists of nine alleles that fail to complement mutations in the other two classes. Given the known molecular structure of *E74*, this data is consistent with the hypothesis that these three classes correspond to mutations in *E74A*, *E74B*, and the *E74* common region, respectively. A more complete description of the *E74* complementation groups and of the putative *E74* common region mutants will be presented elsewhere (K. C. B., manuscript in prep.).

The possibility that $E74^{X1001}$ might retain some *E74A* function, since the breakpoint lies outside of the *E74A* coding region (Burtis, 1985), led us to examine other alleles for molecular defects that might abolish *E74A* activity. $E74^{P[neo]}$, found in a screen for P element-mediated mutations (Cooley et al., 1988), fails to complement $E74^{X1001}$ and is associated with a *P{pUChs:neo}* insertion at position +10 relative to the *E74A* transcriptional start site (Fig. 1). This insertion lies within the minimal sequences necessary to direct *E74A* transcription (Thummel, 1989). We are unable to detect *E74A* transcripts on northern blots of RNA isolated from animals heterozygous for $E74^{P[neo]}$ over a deficiency for the 73A-74F interval, *Df(3L)st-81k19*, but find that *E74B* transcription is unaffected (Fletcher and Thummel, 1995).

$E74^{DL-1}$ and $E74^{PA-9}$ have been classified as putative *E74B* mutations based on their ability to complement the *E74A* alleles but not the putative *E74* common region alleles. $E74^{DL-1}$ appears to be the more severe of these two mutations, since $E74^{DL-1}$ leads to lethality at earlier stages in development than $E74^{PA-9}$, when carried as a *trans*-heterozygote with other *E74* mutations (K. C. B., unpublished data). We therefore chose to analyze the molecular lesion associated with the $E74^{DL-1}$ mutation. The genomic region containing the entire *E74B*-specific exon was amplified by polymerase chain reaction (PCR) from $E74^{DL-1}$/*Df(3L)st-81k19* genomic DNA. A 14 bp deletion was detected from +1118 to +1131 relative to the *E74B* transcriptional start site (Burtis et al., 1990). This deletion alters the reading frame such that a stop codon is introduced 114 bp downstream of the deletion, prematurely terminating the *E74B* ORF in the middle of the first exon. The protein product generated from this ORF is predicted to be one quarter the size of wild-type E74B protein and lacks the ETS DNA-binding domain (Fig. 1).

E74 mutant phenotypes

The effective lethal phases of the $E74^{P[neo]}$ and $E74^{DL-1}$ mutations were determined by crossing females heterozygous for these mutations to males heterozygous for the same allele or for *Df(3L)st-81k19* (Table 1). When hemizygous, the $E74^{P[neo]}$ allele is primarily lethal during pupal development (20%, with an expected maximum lethality of 25%). In homozygotes, the larval lethality increases from 9% to 15%. This increase can be accounted for by the 10% background larval lethality of the homozygous *mwh r* e^s chromosome. Thus, both $E74^{P[neo]}$ homozygotes and hemizygotes arrest development predominantly at the pupal stage, frequently as pharate adults, indicating that $E74^{P[neo]}$ represents an amorphic allele of *E74A*.

The $E74^{DL-1}$ allele is completely lethal during pupal development when hemizygous, but completely lethal during larval

Table 1. Lethal phases of the *E74* mutations

Cross genotypes	Total embryos	% Dead embryos	% Dead larvae	% Dead pupae	Total % dead
mwh r e^s/+ × mwh r e^s/+	250	0	10	2	12
st p^p e^{11}/+ × Df/+	255	1	0	2	3
st p^p e^{11}/+ × st p^p e^{11}/+	210	0	4	1	5
$E74^{P[neo]}$/+ × Df/+	245	0.4	9	20	29
$E74^{P[neo]}$/+ × $E74^{P[neo]}$/+	258	0.4	15	16	31
$E74^{DL-1}$/+ × Df/+	237	0.4	4	27	31
$E74^{DL-1}$/+ × $E74^{DL-1}$/+	258	1	29	0.4	30

A collection of eggs was made from each female × male genotype listed, and the total number of embryos was determined by subtracting the number of unfertilized eggs. The embryos were allowed to develop at 25°C, and the number of dead animals at each stage counted and presented as a percentage of the total embryos scored.
Df=Df(3L)st-81k19.

development when homozygous (Table 1). In this case, the lethal phase shift cannot be accounted for by the 4% background larval mortality due to the *st p^p e^{11}* chromosome, suggesting that the *$E74^{DL-1}$* third chromosome carries an additional lethal mutation. We are therefore unable to determine whether this is a hypomorphic or amorphic allele, although its molecular defect is consistent with *$E74^{DL-1}$* retaining little or no *E74B* activity.

Morphological differences between the *$E74^{P[neo]}$* and *$E74^{DL-1}$* hemizygous mutants could first be detected at puparium formation (Fig. 2; Burtis, 1985). *$E74^{P[neo]}$* hemizygotes appeared to form normal puparia, with everted anterior spiracles and a characteristic smoothed, shortened shape. Hardening of the larval cuticle in *$E74^{P[neo]}$* mutants also proceeded normally, but tanning was delayed by several hours. In contrast, all *$E74^{DL-1}$* hemizygotes failed to evert completely their anterior spiracles and retained their long, segmented larval shapes. Tanning of the elongated puparia proceeded normally in *$E74^{DL-1}$* mutant prepupae, but the darkened cuticle remained somewhat soft.

The lethality of the *$E74^{P[neo]}$* and *$E74^{DL-1}$* alleles was completely penetrant, but each mutant phenotype displayed variable expressivity (Table 2; Fig. 3). We collected approximately 100 individuals, hemizygous for either *E74* allele, as newly formed prepupae, and scored their progress through prepupal and pupal development based on the presence of defined morphological markers (Bainbridge and Bownes, 1981). Approximately half (55%) of the *$E74^{P[neo]}$* hemizygotes died during the late pupal period as pharate imagos. When dissected from their pupal cases 92-95 hours after pupariation, these mutants appeared morphologically indistinguishable from *st p^p e^{11}/ Df(3L)st-81k19* hemizygous controls of the same age (Fig. 4). All three pairs of legs dissected from *$E74^{P[neo]}$* hemizygous mutant pharate adults also appeared morphologically normal, although some instances of curled tarsal segments were observed (Fig. 5, data not shown). Living *$E74^{P[neo]}$* mutant imagos could be dissected from their pupal cases up to 7 days after pupariation; these animals displayed leg twitches and abdominal contractions, but were inviable. *$E74^{P[neo]}$* homozygotes (data not shown), *$E74^{X1001}$* homozy-

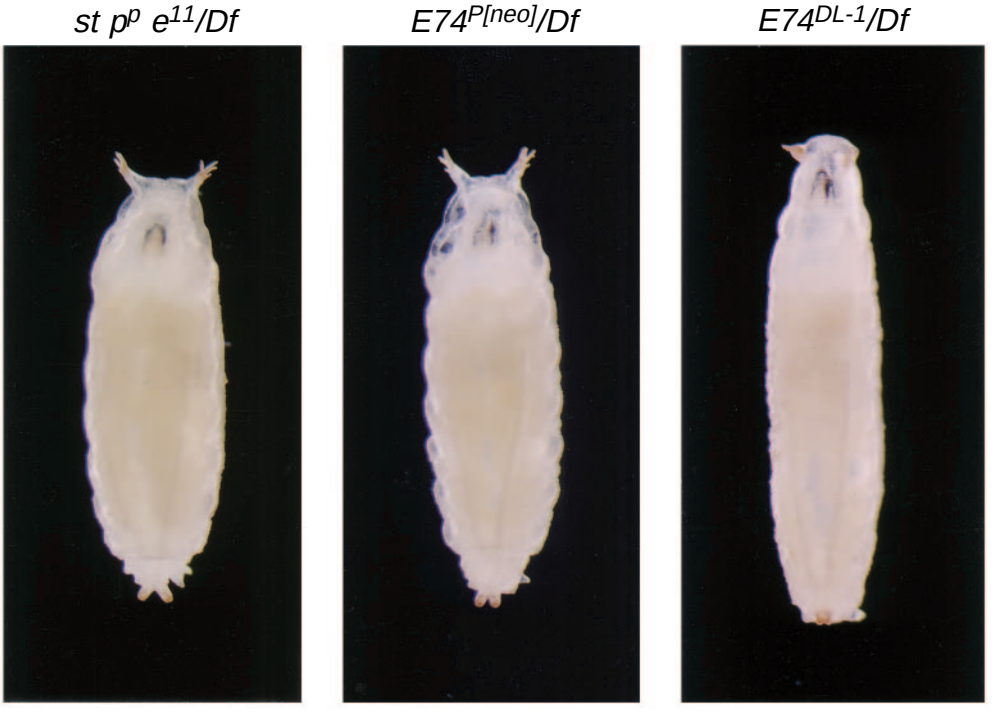

Fig. 2. *E74* hemizygous mutant newly formed prepupae. Shown are prepupae hemizygous for the control genotype or for one of the *E74* alleles. White prepupae of the *st p^p e^{11}/Df(3L)st-81k19* control genotype form normal puparia (left panel). White prepupae hemizygous for the *$E74^{P[neo]}$* allele are indistinguishable from the controls (second panel). Newly formed prepupae hemizygous for the *$E74^{DL-1}$* allele (third panel) fail to completely evert their anterior spiracles and retain an elongated larval shape.

Table 2. *E74* mutant phenotypes display variable expressivity

Pupal stage: / Marker scored:	P1 white puparium	P3 bubble prepupa	P4(ii) head sac eversion	P8 yellow eyes	P11(ii) thoracic bristles	P12(i) tergite bristles	P12(ii) black wings	P14 green meconium	P15(ii) eclosed adult
st p^{p} e^{11}/Df	100	100	100	100	100	100	100	100	100
E74$^{P[neo]}$/Df	100	64	64	59	55	55	55	0	0
E74^{DL-1}/Df	105	105	5	25	24	13	7	0	0

Either 100 or 105 newly formed prepupae from each genotype listed were collected and allowed to develop at 25°C. Each value refers to the number of individuals from the original cohort that displayed the morphological marker characteristic of the pupal stage indicated (Bainbridge and Bownes, 1981). Note that some individuals exhibited characteristics of later stages without undergoing head eversion or displaying eye pigmentation.
Df=Df(3L)st-81k19.

gotes (Burtis, 1985) and *E74A* mutant trans-heterozygotes (K. C. B., unpublished data) all display a similar pharate adult lethal phenotype.

Notably, 36% of the *E74$^{P[neo]}$* hemizygous mutants arrested development during the prepupal period, at stage P3 or P4, having failed to form a gas bubble or undergo pupation (Table 2). Viewed 4 days after puparium formation, these prepupae neither detached from their tracheal and mouth part attachments nor everted their cephalic complexes, and they displayed no signs of imaginal disc evagination (Fig. 3). The remaining 9% of the *E74$^{P[neo]}$* mutants arrested development at various stages between pupation and pupal stage P11(ii), which is marked by the pigmentation of the thoracic macrochaetes and microchaetes.

The development of 75% of the *E74^{DL-1}* hemizygous progeny analyzed was arrested prior to pupation (Table 2, Fig. 3). These mutants formed an abdominal gas bubble and often appeared to have partially undergone larval-pupal apolysis, the retraction of the epidermis from the overlying cuticle that begins 4-6 hours after puparium formation. A significant proportion of the *E74^{DL-1}* hemizygous prepupae also appeared to undertake but not complete pupation. A representative prepupa, shown in Fig. 3, expelled its gas bubble into the posterior end of the puparium and contracted its abdomen along one side. However, this prepupa failed to translocate the gas pocket anteriorly, and the gas remained trapped between the hypodermis and the pupal case.

Of the 25% of *E74^{DL-1}* mutants that survived the prepupal period, 20% failed to evert their cephalic complexes but proceeded to develop some imaginal structures, most notably cryptocephalic head structures (Fig. 4). Red eye pigmentation was clearly visible within the thoraxes of these mutants, under the darkened macrochaetes. About half of these mutants also formed complete or partial bristle patterns on the abdominal tergites, but no segmentation in this region could be discerned. All three pairs of legs dissected from each of the cryptocephalic animals were incompletely elongated, and in several cases lacked the distalmost tarsal segments (Fig. 5). Seven extremely advanced *E74^{DL-1}* pupae also displayed the black wing coloration characteristic of P12(ii) pupae, but none eclosed. The *E74^{DL-1}* mutant with the cryptocephalic phenotype shown in Fig. 4 also displays a cavity in the center of its abdomen where its gas bubble has been retained.

Only 5% of the *E74^{DL-1}* mutants successfully everted their cephalic complexes. Of these, one animal did not display

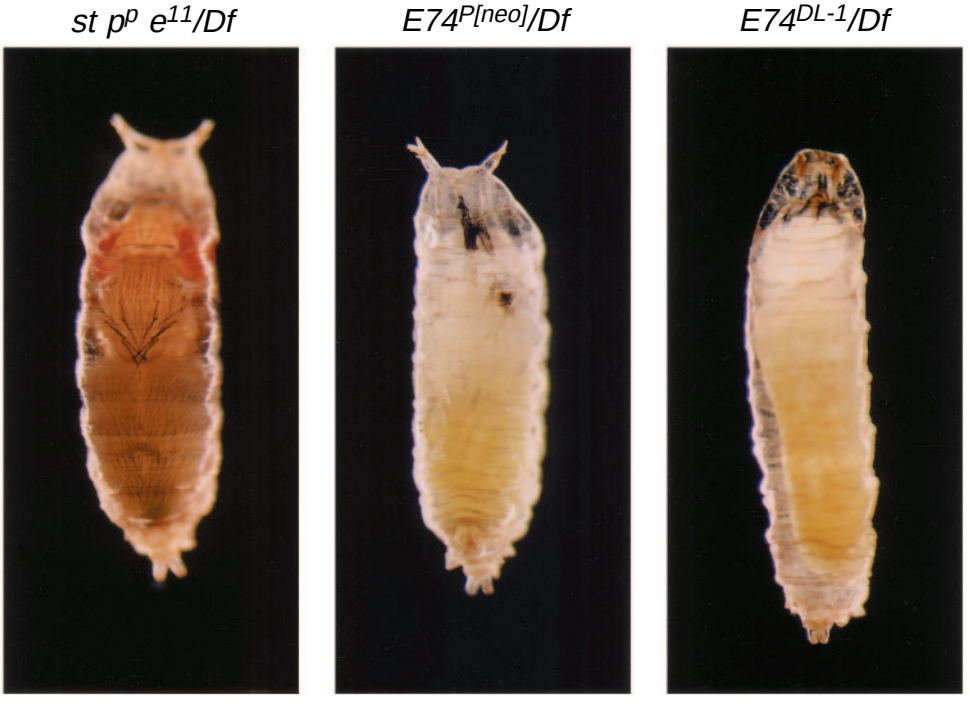

Fig. 3. Phenotypes of *E74* mutant hemizygotes arrested prior to pupation. Prepupae were collected at puparium formation and allowed to develop for 4 days (92-95 hours) at 25°C before being photographed. The *st p^{p} e^{11}/Df(3L)st-81k19* control prepupa developed normally (left panel), forming fully differentiated head, thoracic and abdominal structures and completely pigmented macrochaetes and microchaetes. Shown in the second panel is an *E74$^{P[neo]}$* mutant hemizygote that has arrested development during the prepupal stage. The abdominal gas bubble of the *E74^{DL-1}* mutant hemizygote shown in the third panel has been expelled into the posterior region of the puparium. The abdomen of this prepupa began to contract away from the side of the pupal case, but no further development was observed.

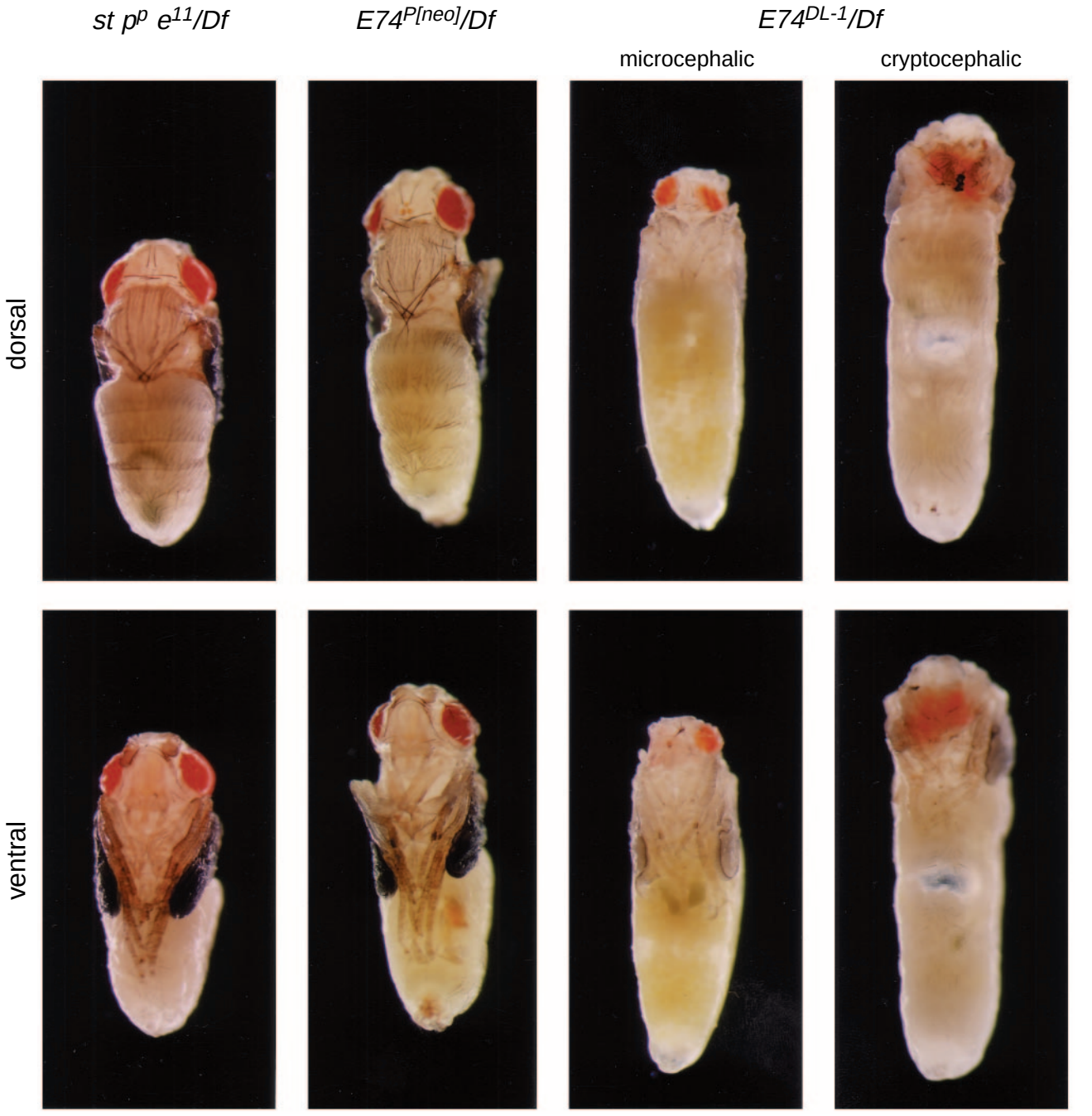

Fig. 4. Phenotypes of *E74* mutant hemizygotes that have undergone imaginal disc morphogenesis. Presented are representative pupae of each of the indicated genotypes, dissected from their pupal cases 4 days after puparium formation (92-95 hours). The upper set of panels present the dorsal view of the pupae and the lower set of panels the ventral aspect. The $E74^{P[neo]}$ hemizygous mutant pupa (second panel) is indistinguishable from the control pupa (left panel), but is inviable when dissected from the pupal case. Two $E74^{DL-1}$ hemizygous mutant pupae are shown, one displaying a rare microcephalic head phenotype (middle panel) and the other the more common cryptocephalic phenotype (fourth panel). The latter pupa also retains its abdominal gas bubble.

further head development, three developed microcephalic phenotypes (Fristrom, 1965), and the other developed into a normal-appearing pharate imago that failed to eclose. An $E74^{DL-1}$ mutant displaying a microcephalic phenotype after 4 days of pupal development is shown in Fig. 4. The head of this imago has only partially everted from the head cavity within the thorax. The thorax itself is malformed, appearing smaller than wild-type and retaining an anterior dorsal cleft where the hypodermis has not completely fused. Both the leg and wing discs have evaginated, but the appendages do not appear fully elongated compared to those of the control. Finally, the abdomen of this $E74^{DL-1}$ mutant is unsegmented and filled with large yellow cells, which appear to be larval adipose cells that have dissociated from the fat bodies.

Fig. 5. Legs dissected from *E74* mutant hemizygotes that have undergone imaginal disc morphogenesis. Presented are representative first legs of male pupae from each of the indicated genotypes, dissected 4 days after puparium formation (92-95 hours) and photographed at the same magnification. The leg dissected from the $E74^{P[neo]}$ hemizygous mutant pupa (middle panel) is indistinguishable from the wild type leg (left panel), while the leg dissected from the $E74^{DL-1}$ hemizygous mutant pupa (right panel) is incompletely elongated and lacks the two most distal tarsal segments. Legs dissected from *st* p^p e^{11}*/Df(3L)st-81k19* pupae are indistinguishable from those of the wild type.

Cytological analysis of *E74* mutant alleles

Our observation that morphological differences between the $E74^{P[neo]}$ and $E74^{DL-1}$ mutants became apparent at puparium formation led us to investigate whether these mutations had any effect on the polytene chromosome puffs active during this period of development. A large number of secondary-response ecdysone-inducible puffs are normally active in white prepupae, at puff stage (PS) 10-11. The brevity of the white prepupal stage, which lasts only 15-30 minutes at 25°C, enabled us to accurately stage animals to directly compare the sizes of puffs in newly formed prepupae of different genotypes. We dissected salivary glands from newly formed prepupae hemizygous for the $E74^{P[neo]}$ allele or for the $E74^{DL-1}$ allele and compared the activities of their late puffs to those from the *st* p^p e^{11}*/Df(3L)st-81k19* controls (Fig. 6). The degree of puffing was calculated as the ratio between the diameter of the puff and that of a nearby unpuffed reference band (Fig. 7; Ashburner, 1967).

A number of puffs normally active at puparium formation were significantly reduced in size in salivary glands dissected from $E74^{P[neo]}$ hemizygous white prepupae compared to those from the *st* p^p e^{11}*/Df(3L)st-81k19* controls (Fig. 7A). These included late puffs present at loci 21F, 22C, 62F, 63E, 71E, 72D, 82F and 83E, all of which normally peak in size during pupariation (Ashburner, 1967). The 62E early late puff, which is active from PS 6-10, was also significantly reduced in size in the $E74^{P[neo]}$ mutant background. Other early late puffs that are larger at PS 7-8 than in white prepupae, at loci 46F and

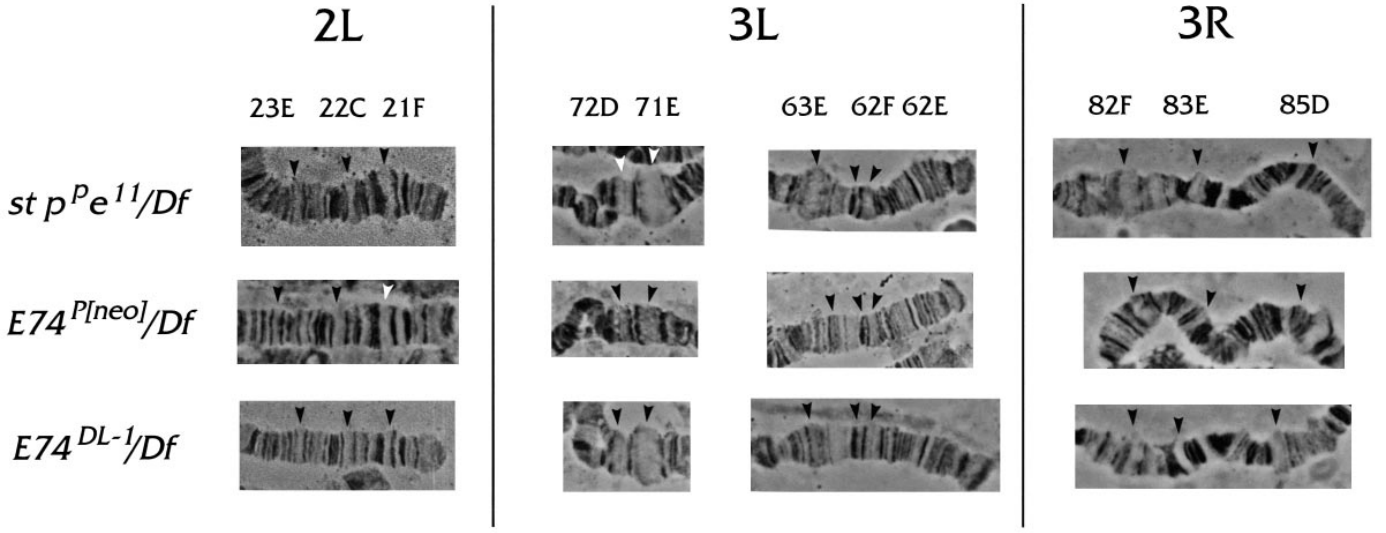

Fig. 6. Polytene chromosome puffing activity in salivary glands from *E74* hemizygous mutant newly formed prepupae. Representative regions of the left arm (2L) of the second chromosome and the left (3L) and right (3R) arms of the third chromosome are depicted. The same chromosomal regions from a *st* p^p e^{11}*/Df(3L)st-81k19* control prepupa (top row), an $E74^{P[neo]}$ hemizygous prepupa (middle row) and an $E74^{DL-1}$ hemizygous prepupa (bottom row) are shown to enable direct comparison. The arrows denote the positions on each chromosome arm of the puff loci listed across the top.

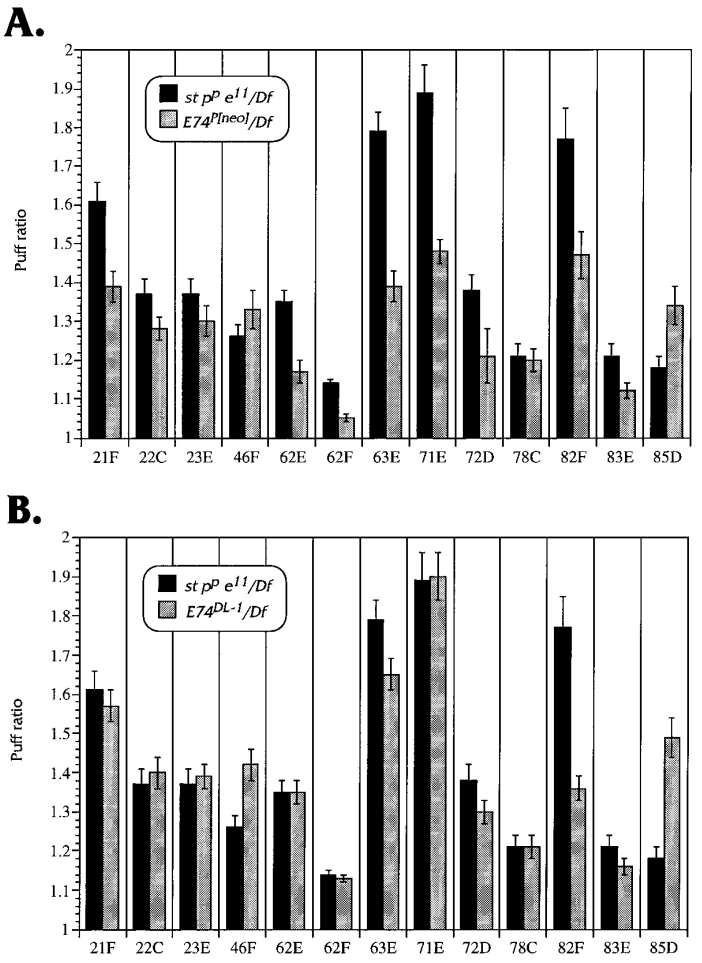

Fig. 7. Puff sizes in *E74* hemizygous mutant newly formed prepupae. Comparison of puff sizes in *st* p^p e^{11}*/Df(3L)st-81k19* control white prepupae (solid columns) and in newly formed prepupae hemizygous for each of the *E74* alleles (stippled columns). The puffs listed on the abscissa fall into three classes: the 23E puff is an early puff, the 46F, 62E and 78C puffs are early late puffs, and the rest are considered late puffs (Ashburner et al., 1974). (A) Puff ratios in $E74^{P[neo]}$ mutants. (B) Puff ratios in $E74^{DL-1}$ mutants.

78C (Ashburner, 1967), were not affected by the $E74^{P[neo]}$ mutation. The 23E early puff, still active in white prepupae, was also relatively unaffected. Finally, the 85D puff, which is normally induced at PS 11 and reaches its maximum size at PS 12 (Ashburner, 1967), appeared to be larger than normal in $E74^{P[neo]}$ hemizygotes.

In $E74^{DL-1}$ hemizygous newly formed prepupae, most of the late puffs were unchanged in size compared to the corresponding puffs in control hemizygotes (Fig. 7B), with only 63E and 82F exhibiting significantly smaller puff ratios than wild type. In contrast, the 46F and 85D puffs were significantly larger in size in the $E74^{DL-1}$ mutant background. Both of these puffs normally reach their maximum sizes at PS 12, 1-2 hours after puparium formation. Other puffs normally peaking in size at PS 12, including those at 34A and 38A, also appeared to be prematurely induced in $E74^{DL-1}$ mutant prepupae (data not shown).

DISCUSSION

E74A and *E74B* play critical roles during metamorphosis

E74 is a complex genetic locus that consists of two lethal complementation groups and a third class of non-complementing alleles (Burtis, 1985; K. C. B. unpublished data). Two alleles in one complementation group have been mapped to the *E74A* transcription unit: $E74^{X1001}$ is a translocation breakpoint mutation that lies within the first *E74A* intron (Burtis, 1985) and $E74^{P[neo]}$ contains a P element insertion that inactivates the *E74A* promoter (Fig. 1). The $E74^{DL-1}$ mutation complements these *E74A* mutations and contains a small deletion in the *E74B*-specific exon that causes premature termination of the *E74B* open reading frame. Based on this data, we conclude that one lethal complementation group consists of *E74A* alleles and the other consists of *E74B* alleles, while mutations in the non-complementing class affect both *E74A* and *E74B*.

We have examined the lethal phases of the $E74^{P[neo]}$ and $E74^{DL-1}$ alleles as homozygotes and as hemizygotes, and observed the lethal phenotypes of the $E74^{P[neo]}$ and $E74^{DL-1}$ mutants during metamorphosis. We find that these mutants arrest their development predominantly during the prepupal and pupal stages, consistent with a critical role for *E74A* and *E74B* in metamorphosis. The expression of *E74A* or *E74B* during the earlier embryonic and larval stages (Thummel et al., 1990) thus appears to be dispensible for development.

E74B function is required for both pupariation and pupation. $E74^{DL-1}$ hemizygous third instar larvae form abnormally long and thin puparia, retaining their larval shape (Fig. 2). Of these, 75% arrest development during the prepupal period and often appear caught in the process of gas translocation (Fig. 3). The rest develop imaginal head and thoracic structures characteristic of pupal stages despite failing to expel their gas bubbles or evert their cephalic complexes (Fig. 4, Table 2). Head eversion is dependent on the ecdysone pulse that occurs 10-12 hours after pupariation (Sliter and Gilbert, 1992). Our results suggest that this pulse of ecdysone exerts its effects, at least in part, through the induction of *E74B* during the prepupal period (Karim and Thummel, 1991).

Failure of the adult head to emerge from the thorax is a phenotype that was first described for the pupal lethal mutant *cryptocephal (crc)*, which maps to the 39BC region (Hadorn and Gloor, 1943). *crc* homozygotes pupariate normally, but display a gradient of severity of head structure positions, including cryptocephalic and microcephalic phenotypes (Fristrom, 1965). Other *crc* pupal phenotypes include shortened legs and wings, and incomplete abdominal differentiation (Chadfield and Sparrow, 1985), defects that we also observe among $E74^{DL-1}$ mutants (Figs 4, 5). The similarity of the *crc* and *E74B* mutant phenotypes suggests that these genes may act in the same morphogenetic pathway during prepupal development.

The $E74^{DL-1}$ prepupal and pupal mutant phenotypes can be explained by postulating a role for *E74B* in the proper functioning of the larval muscles during early metamorphosis. Contraction of larval muscles is required to shorten the body segments at puparium formation (Crossley, 1978). These muscles begin to degenerate several hours after pupariation, a phenomenon that in *Calliphora* has been correlated with

increases in ecdysone titer (Crossley, 1968, 1978; Zachary and Hoffman, 1980). These muscles nevertheless contract during pupation (Robertson, 1936). The movements required for pupation – including gas bubble translocation, withdrawal of the prepupa to the posterior of the puparium and head sac eversion – are all mediated by contraction of the larval abdominal muscles, the majority of which are histolyzed following pupation. One set of dorsal abdominal muscles per segment persist into the pupal period, and are thought to establish the segmentation of the adult abdominal hypodermis before disintegrating (Robertson, 1936).

The phenotypes of several other mutants also indicate a role for larval muscles in pupariation and pupation. The larval muscle mutants *lethal(1)fibrillardysgenesis [l(1)fdg], lethal polymorph (lpm)* and *lethal(2)thin [l(2)tn]* form long, thin puparia and display prepupal or pupal lethality (Benz, 1957; Newman and Wright, 1983; Ball et al., 1985). Mutant *l(2)tn* prepupae also show defects in gas bubble translocation and head eversion (Ball et al., 1985), and cryptocephalic phenotypes have been observed among *lpm* mutant imagos (Benz, 1957). Each larval muscle mutation also causes some degree of muscular disfunction that is correlated with sluggish wandering behavior in third instar larvae (Ball et al., 1985). The absence of this phenotype in $E74^{DL\text{-}1}$ mutant larvae leads us to believe that this mutation does not specifically affect larval muscle development. Rather, our results suggest that *E74B* may contribute to preventing the ecdysone-induced degeneration of larval abdominal muscles until their task of reshaping the larval and prepupal body is complete. Premature histolysis of abdominal muscle groups would account for the defects seen in both pupariation and pupation, as well as for the failure of the adult abdominal hypodermis to undergo segmentation (Fig. 4).

The legs and wings of $E74^{DL\text{-}1}$ hemizygous mutant pupae are not fully elongated or are missing distal segments (Figs 4, 5), indicating that imaginal structures are also affected by the loss of *E74B* function. Similar phenotypes, including incomplete elongation and fusion of imaginal discs, have been observed among pupae that carry mutations in the *BR-C* early puff gene (Kiss et al., 1988). The *BR-C* functions required for imaginal disc morphogenesis are divided between two independent domains: the *broad* function mediates the eversion and elongation of appendages, while the *l(1)2Bc* function is involved in disc fusion (Kiss et al., 1988).

$E74^{P[neo]}$ mutants display lethality during both prepupal and pharate adult development. $E74^{P[neo]}$ mutant hemizygotes form puparia that appear normal, although tanning is delayed. Defective tanning and sclerotization is also found among prepupae carrying br^5 mutant alleles of the *BR-C* (Kiss et al., 1988). Many of the $E74^{P[neo]}$ mutants evert their cephalic complexes and develop into imagos morphologically indistinguishable from wild type (Table 2; Figs 4, 5), although they fail to eclose from the pupal case. $E74^{P[neo]}$ mutants that are arrested during prepupal development fail to evert their imaginal discs or undergo larval/prepupal apolysis; the latter event normally begins about 4 hours after puparium formation (Robertson, 1936). This phenocritical period follows a peak of E74A protein production at puparium formation that occurs in response to the late larval ecdysone pulse (Thummel et al., 1990; Boyd et al., 1991). We plan to test whether ectopic expression of *E74A* at pupariation can rescue this block in $E74^{P[neo]}$ development.

E74A is required in white prepupae for the proper salivary gland puffing response to ecdysone

Cytogenetic analysis of polytene chromosome puffing patterns in *E74* mutant backgrounds reveals that *E74A* function is necessary for the proper regulation of some late puffs at puparium formation. A subset of the late puffs that normally peak in size at pupariation are sub-maximally induced in $E74^{P[neo]}$ mutants (Fig. 7A). Many of these same puffs were also shown to be affected by alterations in the dose of the 74EF/75B region (Walker and Ashburner, 1981). Late puffs that normally peak in size prior to puparium formation, with the exception of 62E, are unaffected either in $E74^{P[neo]}$ mutants or in aneuploids for 74EF/75B. Thus, our results confirm those of Walker and Ashburner, and identify *E74A* as one function within the 74EF/75B region that is necessary for the proper regulation of the late puff subset. All of the affected puffs, with the exception of 21F, are also bound by E74A protein in white prepupae (Urness and Thummel, 1990), indicating that *E74A* may directly regulate their activity.

A number of early and late puffs are also sub-maximally induced in the polytene chromosomes of larvae and prepupae deficient for the *l(1)2Bc* function of the *Broad-Complex* (Zhimulev et al., 1982). Among the underdeveloped early puffs are 74EF and 75B, and molecular studies have shown that the level of *E74A* and *E75A* transcription is reduced in *l(1)2Bc* mutant late third instar larvae and prepupae (Karim et al., 1993). Interestingly, the subset of late puffs affected by the $l(1)2Bc^1$ mutation includes 63E, 71E and 82F and thus overlaps the subset affected by the $E74^{P[neo]}$ mutation. These results suggest that the observed effects of the $l(1)2Bc^1$ mutation on some of the late puffs may be a secondary consequence of the sub-maximal induction of *E74A*. Further, they indicate that the maximal induction of late puffs in the larval salivary gland requires, at the least, a combination of functions encoded within two early puffs, 2B5 and 74EF, providing strong support for the Ashburner model (Ashburner et al., 1974). Double mutant combinations of *l(1)2Bc* alleles with $E74^{P[neo]}$ should help to elucidate interactions between the products encoded by these two functions.

In contrast, most late puffs are not affected by the $E74^{DL\text{-}1}$ mutation (Fig. 7B). *E74B* is expressed earlier than *E74A*, both in third instar larvae and during the mid prepupal period (Karim and Thummel, 1991). A static assessment of puffing patterns during puparium formation might not reveal altered behavior among puffs that are induced prior to or following that time. Supporting the inference that *E74B* may affect puffs not normally active at pupariation, we find that a subset of late puffs is prematurely induced in newly formed prepupae in the absence of *E74B* function (Fig. 7B). This observation suggests that the expression of *E74B* in late third instar larvae is required to keep these late puffs repressed until 1-2 hours after puparium formation.

The *E74* ETS proteins may interact with accessory factors during development

There is growing evidence that ETS domain proteins like E74A and E74B exert their regulatory functions through interactions with accessory transcription factors. The Ets-related protein Elk-1 has been shown to bind cooperatively with serum response factor (SRF) to the serum response element in the *c-*

fos promoter (Hipskind et al., 1991; Dalton and Treisman, 1992), and PU.1 binds to the immunoglobulin κ 3′ enhancer and recruits a second protein, NF-EM5, to an adjoining site (Pongubala et al., 1992). Furthermore, Ets-1 and Core Binding Factor (CBF) have been shown to bind cooperatively to adjacent sites in the T-cell receptor β gene enhancer (Wotton et al., 1994).

Analogously, we propose that E74A and E74B may mediate their regulatory functions through associations with other transcription factors. The best candidates for *E74* partner proteins are those encoded by the *BR-C*, the temporal and spatial expression patterns of which overlap with those of *E74* (Andres et al., 1993; Emery et al., 1994). As described above, the *E74* and *BR-C* mutant phenotypes are similar in many respects, and some *E74* and *BR-C* mutations interact genetically (J.C.F. and C.S.T., unpublished data). Finally, some binding sites for the E74A and BR-C Z1 proteins overlap in the *L71-6* late gene promoter, providing the potential for these proteins to physically contact each other when bound to DNA (L. Urness and C.S.T., unpublished data; K. Crossgrove and G. Guild, personal communication).

The observation that some ETS-related proteins interact with cell-type specific factors (Pongubala et al., 1992) raises the interesting possibility that E74A and E74B may associate with partners that have restricted patterns of expression. Such interactions could provide a means by which widely expressed proteins, such as E74A, could function in a variety of distinct developmental pathways during the early stages of *Drosophila* metamorphosis.

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