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
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 e11 chromosome either by mutagenesis with ethyl methanesulfonate (EMS) using the conditions of Lewis and Bacher (1968) or by X-irradiation (5000 rad).

The E74 Dl-1 allele was generated in a screen for P element induced lethal mutations on a mnh r e1 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 Bloomingston Drosophila Stock Center, where it is designated #P262 P[pUChs:neo]C(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-s313, 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 pupae 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 E74-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 = CATTAAACGAGACATCAGGC (intronic) and B6 = ATCGCGTCATGGACATCCT (complementary to bases 1254-1262; Burtis et al., 1990); B2 = GTTTAAATGCTTGCCAC (intronic) and B4 = TGAAGGAACCAAGACATGCTG (complementary to bases 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 (Unness 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).
preppupae 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.

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**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, E74X1001, 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). E74X1001 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 E74X1001, 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 E74X1001 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. E74Pneo, found in a screen for P element-mediated mutations (Cooley et al., 1988), fails to complement E74X1001 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 E74Pneo over a deficiency for the 73A-74F interval, Df(3L)st-81k19, but find that E74B transcription is unaffected (Fletcher and Thummel, 1995).

E74DL-1 and E74PA-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. E74DL-1 appears to be the more severe of these two mutations, since E74DL-1 leads to lethality at earlier stages in development than E74PA-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 E74DL-1 mutation. The genomic region containing the entire E74B-specific exon was amplified by polymerase chain reaction (PCR) from E74DL-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 E74Pneo and E74DL-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 E74Pneo 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 re e+ chromosome. Thus, both E74Pneo homozygotes and hemizygotes arrest development predominantly at the pupal stage, frequently as pharate adults, indicating that E74Pneo represents an amorphic allele of E74A.

The E74DL-1 allele is completely lethal during pupal development when hemizygous, but completely lethal during larval...
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] homozygotes (data not shown), E74 X1001 homozy-

### Table 1. Lethal phases of the E74 mutations

<table>
<thead>
<tr>
<th>Cross genotypes</th>
<th>Total embryos</th>
<th>% Dead embryos</th>
<th>% Dead larvae</th>
<th>% Dead pupae</th>
<th>Total % dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>mwh r e11/+ × mwh r e11/+</td>
<td>250</td>
<td>0</td>
<td>10</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>st p p e11/+ × Df/+</td>
<td>255</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>st p p e11/+ × st p p e11/+</td>
<td>210</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>E74 P[neo]+ × Df/+</td>
<td>245</td>
<td>0.4</td>
<td>9</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td>E74 P[neo]+ × E74 P[neo]+</td>
<td>258</td>
<td>0.4</td>
<td>15</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td>E74 DL-1/+ × Df/+</td>
<td>237</td>
<td>0.4</td>
<td>4</td>
<td>27</td>
<td>31</td>
</tr>
<tr>
<td>E74 DL-1/+ × E74 DL-1/+</td>
<td>258</td>
<td>1</td>
<td>29</td>
<td>0.4</td>
<td>30</td>
</tr>
</tbody>
</table>

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.

![](image1)!

**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 e11/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.
functions of E74 during Drosophila metamorphosis

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Table 2. E74 mutant phenotypes display variable expressivity

<table>
<thead>
<tr>
<th>Pupal stage:</th>
<th>P1 white puparium</th>
<th>P3 bubble prepupa</th>
<th>P4(ii) head sac evesion</th>
<th>P8 yellow eyes</th>
<th>P11(ii) thoracic bristles</th>
<th>P12(i) tergite bristles</th>
<th>P12(ii) black wings</th>
<th>P14 green meconium</th>
<th>P15(ii) eclosed adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker scored:</td>
<td>st p(^{+}) e(^{1\text{i}}/\text{Df})</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>E74P([\text{neo}])/\text{Df}</td>
<td>100</td>
<td>64</td>
<td>64</td>
<td>59</td>
<td>55</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E74DL-1/\text{Df}</td>
<td>105</td>
<td>105</td>
<td>5</td>
<td>25</td>
<td>24</td>
<td>13</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

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 E74P\([\text{neo}]\) 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 E74P\([\text{neo}]\) mutants arrested development at various stages between pupation and pupal stage P11(ii), which is marked by the pigmentation of the thoracic macrochaetae and microchaetae.

The development of 75% of the E74DL-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 E74DL-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 E74DL-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 macrochaetae. 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 E74DL-1 pupae also displayed the black wing coloration characteristic of P12(ii) pupae, but none eclosed. The E74DL-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 E74DL-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\(^{+}\) e\(^{1\text{i}}/\text{Df}(3L)st-81k19 control prepupa developed normally (left panel), forming fully differentiated head, thoracic and abdominal structures and completely pigmented macrochaetae and microchaetae. Shown in the second panel is an E74P\([\text{neo}]\) mutant hemizygote that has arrested development during the prepupal stage. The abdominal gas bubble of the E74DL-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.](image-url)
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. 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.
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 of the E74^{P[neo]} and E74^{DL-1} hemizygous backgrounds and compared the activities of their late puffs to those from the st p^o 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^o 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 23E, 22C, 21F.

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^o e^{11/Df(3L)st-81k19} pupae are indistinguishable from those of the wild type.

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^o 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.
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\(\times1001\) is a translocation breakpoint mutation that lies within the first E74A intron (Burtis, 1985) and E74\(\times[neo]\) 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\(\times[neo]\) and E74\(\DL-1\) alleles as homozygotes and as hemizygotes, and observed the lethal phenotypes of the E74\(\times[neo]\) 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 dispensable 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

Fig. 7. Puff sizes in E74 hemizygous mutant newly formed prepupae. Comparison of puff sizes in st p\(\times e^{11/Df(3L)st-8lk19}\) 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\(\times[neo]\) mutants. (B) Puff ratios in E74\(\DL-1\) mutants.

78C (Ashburner, 1967), were not affected by the E74\(\times[neo]\) 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\(\times[neo]\) 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).
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)fibrillar dysgenesis [l(1)fdg], lethal polymorph (lpm) and lethal(2)thin [l(2)m] form long, thin puparia and display prepupal or pupal lethality (Benz, 1957; Newman and Wright, 1983; Ball et al., 1985). Mutant l(2)m 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-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-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).

E74P[neo] mutants display lethality during both prepupal and pharate adult development. E74P[neo] mutant hemizygotes form puparia that appear normal, although tanning is delayed. Defective tanning and sclerotization is also found among prepupae carrying br5 mutant alleles of the BR-C (Kiss et al., 1988). Many of the E74P[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. E74P[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 pupariation formation (Robertson, 1936). This phenocritical period follows a peak of E74A protein production at pupariation 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 E74P[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 pupariation formation. A subset of the late puffs that normally peak in size at pupariation are sub-maximally induced in E74P[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 pupariation formation, with the exception of 62E, are unaffected either in E74P[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 large puff subset. All of the affected puffs, with the exception of 21F, are also bound by E74A protein in white prepupa (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 mutation includes 63E, 71E and 82F and thus overlaps the subset affected by the E74P[neo] mutation. These results suggest that the observed effects of the l(1)2Bc 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 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 E74P[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-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 pupariation 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 pupariation 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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