The metamorphosis of holometabolous insects leads to the dramatic reorganization of the entire body plan. In *Drosophila melanogaster*, divergent morphogenetic pathways are initiated at the end of larval development in response to the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone). Nearly all larval tissues, including the salivary glands, muscles, gut and hypodermis, are histolyzed, although a few are retained by the adult fly (Robertson, 1936; Bodenstein, 1965). Concurrently, the imaginal discs and abdominal histoblasts differentiate into adult cuticular structures while clusters of imaginal cells form the internal organs that replace their larval counterparts. Several ecdysone pulses are required for complete transformation of the insect from the larval to the adult form (Richards, 1981a; Riddiford, 1993). A high titer ecdysone pulse has been reproducibly measured at the end of third instar larval development, triggering puparium formation (Richards, 1981b; Handler, 1982). This is followed by a pulse 10-12 hours after pupariation that causes head eversion and the prepupal-pupal transition (Handler, 1982; Sliter and Gilbert, 1992).

The morphogenetic events that take place during the onset of metamorphosis are accompanied by ecdysone-triggered changes in the puffing pattern of the larval salivary gland polytene chromosomes. The products of these genes appear to function as regulators that both repress their own expression and induce a large set of secondary-response genes. We have identified recessive loss-of-function mutations in the early gene *E74*, a member of the *ets* proto-oncogene family that encodes two related DNA-binding proteins, *E74A* and *E74B*. These mutations cause defects in pupariation and pupation, and result in lethality during metamorphosis. Here we extend our phenotypic characterization of the *E74A* and *E74B* mutant alleles to the molecular level by examining their effects on the transcription of over 30 ecdysone-regulated genes. We show that the transcription of most ecdysone primary-response genes during late larval and prepupal development is unaffected by the *E74* mutations. Rather, we find that *E74* is necessary for the appropriate regulation of many ecdysone secondary-response genes. *E74B* is required for the maximal induction of glue genes in mid third instar larval salivary glands, while *E74A* is required in early prepupae for the proper timing and maximal induction of a subset of late genes. *E74* activity is also necessary for the correct regulation of genes expressed predominantly in the fat body, epidermis or imaginal discs. These observations confirm that *E74* plays a critical role in regulating transcription during the early stages of *Drosophila* metamorphosis. In addition, the widespread effects of the *E74* mutations on transcription indicate that *E74* functions in regulatory hierarchies not only in the larval salivary gland, but throughout the entire organism.

Key words: *Drosophila*, *E74*, ecdysone, metamorphosis, ETS, gene regulation
(Beckendorf and Kafatos, 1976; Korge, 1977; Muskavitch and Hogness, 1980; Velissariou and Ashburner, 1980; Meyerowitz and Hogness, 1982; Crowley et al., 1983). Three early puffs contain genes that respond directly to ecdysone: the Broad-Complex (BR-C) from the 2B5 puff, E74 from the 74EF puff, and E75 from the 75B puff (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). These primary-response genes encode sets of related DNA-binding proteins that are proposed to play regulatory roles during metamorphosis (Chao and Guild, 1986; Segraves, 1988; Urenz and Thummel, 1990; Boyd et al., 1991; Huet et al., 1993; von Kalm et al., 1994).

Two late puffs have also been studied at the molecular level. One transcription unit has been identified from the 4F puff (Wolffner, 1980), while the 71E puff contains a cluster of at least three divergently transcribed pairs of genes (Restifo and Guild, 1986a), designated L71-1 through L71-6 (These genes have previously been referred to as Genes I-VI).

Not all ecdysone-regulated genes are encoded by puffs, however, nor is their expression restricted to the salivary gland. Fbp-1 is a primary-response gene expressed predominantly in the fat body (Lepesant et al., 1982), Ddc (Dopa decarboxylase) and Gld (Glucose dehydrogenase) are expressed primarily in the epidermis (Cavener et al., 1986; Clark et al., 1986), and the IMP primary- and secondary-response genes are expressed primarily in the imaginal discs (Natze et al., 1986; Natze, 1993). Unlike the early genes, these genes do not encode DNA-binding proteins and appear to function in tissue-specific responses to ecdysone (Natze, 1993).

We are studying ecdysone regulatory responses at the molecular level by focusing on E74, a complex ecdysone primary-response gene contained within the 74EF early puff. E74 consists of two overlapping transcription units, E74A and E74B, that encode related proteins containing a common carboxyl-terminal ETS DNA-binding domain (Burtis et al., 1990; Karim et al., 1990). The transcription of E74A and E74B is associated with each ecdysone pulse during development (Thummel et al., 1990), and late third instar larvae express E74A and E74B transcripts in both larval and imaginal tissues (Boyd et al., 1991; Karim and Thummel, 1991; Huet et al., 1993). The widespread spatial and temporal patterns of E74 expression suggest that this gene plays multiple roles during development, beyond the third instar larval salivary gland puffing hierarchy.

Loss-of-function mutations specific to either E74A or E74B are predominantly lethal during prepupal and pupal development, consistent with a requirement for these functions during metamorphosis (Burtis, 1985; Fletcher et al., 1995). These mutations also perturb gene expression in the salivary glands, as assayed by measuring polytene chromosome puff diameters in newly formed mutant pupae. Many late puffs are submaximally induced in E74A mutants, while other late puffs are prematurely induced in E74B mutants (Fletcher et al., 1995). All but one of the late puffs affected by the E74A mutation is bound by E74A protein (Urenz and Thummel, 1990), indicating that E74A may directly regulate their expression. Taken together, these observations suggest that proper progression through the early stages of metamorphosis is dependent on the correct regulation of E74 target genes.

In this study, we extend our analysis of the E74 alleles by examining the transcriptional activity of over 30 ecdysone primary- and secondary-response genes in E74A and E74B hemizygous mutant larvae and prepupae. We show that the transcription of some primary-response genes is modestly affected by these mutations during late larval and prepupal development. We also find that E74 is necessary for the appropriate regulation of secondary-response genes restricted in their expression to either larval or imaginal tissues; some of these genes are also stage-specific. E74 therefore regulates the transcription of genes active during different periods of late larval and prepupal development, in tissues with vastly different developmental fates, indicating a role for this gene in multiple ecdysone-regulated morphogenetic processes at the onset of metamorphosis.

**MATERIALS AND METHODS**

**Drosophila stocks**

Abbreviations of genetic loci are according to Lindsay and Zimm (1992). The E74Pneo and E74DL-1 alleles have been described in Fletcher et al. (1995). The E74 alleles and Df(3L)st-81k19, a deficiency for the 73A3 to 74F region (Burtis, 1985), were maintained over the balancer chromosome In(3LR)TM6B Hu e Tb ca. Hemizygous E74 mutant larvae and prepupae were generated by crossing Df(3L)st-81k19/TM6B females with mutant E74/TM6B males at 25°C, and were identified by their Tb+ phenotype. Stocks were maintained at 25°C or 18°C on standard cornmeal/molasses media.

**Staging of E74 mutant larvae and prepupae**

Third instar larvae were raised on food supplemented with 0.05% bromophenol blue (Maroni and Stamey, 1983). Wandering and stationary third instar larvae were collected and staged based on the amount of bromophenol blue in their intestines (Andres and Thummel, 1994). Wandering larvae with dark blue guts are at puff stage 1 (Ashburner, 1967), approximately 12-24 hours from pupariation (~18 hours in each figure). Stationary larvae with clear guts are estimated to be 1-6 hours from pupariation (~4 hours in each figure). For the prepupal time points, stationary larvae were placed on a damp piece of black filter paper in a Petri dish at 25°C and newly formed prepupae were selected as described in Karim et al. (1993). Eleven or twelve animals were collected per time point.

**Northern blot hybridizations**

RNA was extracted following a protocol for RNA isolation using SDS lysis buffer (Andres and Thummel, 1994), with the chloroform extraction step replaced by three sequential other extractions. RNA was fractionated by formaldehyde agarose gel electrophoresis and transferred to nylon membranes as described (Karim and Thummel, 1991), omitting the high molecular weight RNA transfer step. Each loaded sample contained 10 µg of total RNA, and eight identical blots were prepared from each set of RNAs. These blots were sequentially stripped and hybridized with each radioactive probe, as described (Karim and Thummel, 1991). The probes for E74, E75A, E75B, E75C, EcR and BC RNAs were prepared by asymmetric PCR, as described (Karim and Thummel, 1992). All other probes were prepared by random labeling using a Prime-It II kit (Stratagene), and each probe was purified by chromatography through a NucTrap Push column (Stratagene). Following hybridization, the membranes were washed at 55°C for 45 minutes in 1x SSC, 0.1% SDS, 45 minutes in 0.3x SSC, 0.1% SDS and 30 minutes in 0.1x SSC, 0.1% SDS, and exposed on film (Kodak X-Omat). Film exposed with an intensifier screen was preflashed.

**RESULTS**

**E74 mutations have minor effects on the transcription of ecdysone primary-response genes**

In order to investigate the effects of mutations in E74 on
Table 1. Summary of effects of the E74 mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus bound by E74A</th>
<th>Effect in mutant background</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E74P[neo]</td>
</tr>
<tr>
<td>Primary-response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR-C</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EcR</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E74A</td>
<td>+</td>
<td>no mRNA</td>
</tr>
<tr>
<td>E74B</td>
<td>+</td>
<td>f</td>
</tr>
<tr>
<td>E75A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E75B</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E75C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E75B</td>
<td>+</td>
<td>a</td>
</tr>
<tr>
<td>DHR3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DHR39</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fbp1</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>IMP-E1</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>IMP-E2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IMP-E3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Eip28/29</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Secondary-response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sgs-3</td>
<td>–</td>
<td>-</td>
</tr>
<tr>
<td>Sgs-7</td>
<td>–</td>
<td>-</td>
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<tr>
<td>Sgs-8</td>
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<td>-</td>
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<tr>
<td>Sgs-4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sgs-5</td>
<td>–</td>
<td>-</td>
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<tr>
<td>Gene VII</td>
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<td>-</td>
</tr>
<tr>
<td>4F</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L71-1-6</td>
<td>+</td>
<td>a,c</td>
</tr>
<tr>
<td>IMP-L1</td>
<td>b,e</td>
<td>b,e,g</td>
</tr>
<tr>
<td>IMP-L2</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>IMP-L3</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>btFtz-F1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Edg78E</td>
<td>b,e</td>
<td>b</td>
</tr>
<tr>
<td>Edg84A</td>
<td>b,e</td>
<td>b</td>
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<tr>
<td>Ddc</td>
<td>+</td>
<td>g</td>
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<tr>
<td>Gld</td>
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</tr>
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</table>

The genes examined in this study are divided into primary- and secondary-response categories based on their reported response to ecdysone. Those genes that require a period of low ecdysone titer and those for which ecdysone regulation is unclear have been placed in a third category. Each gene is marked with respect to whether its polytene chromosomal locus is bound (+) or not bound (−) by E74 protein in white prepupae (Urnnes and Thummel, 1990), and coded with respect to its response in each E74 hemizygous mutant genotype. References for each gene listed are in the text.

- a, submaximal induction
- b, premature induction
- c, delayed induction
- d, premature repression
- e, delayed repression
- f, shifted peak of expression
- g, reinduction
- h, normal
- i, no difference
- j, no effect
- k, equal
- l, less
- m, more
- n, same
- o, different
- p, predominant
- q, secondary
- r, initial
- s, late
- t, transient
- u, persistent
- v, temporary
- w, steady
- x, fluctuating
- y, oscillating
- z, stable

The expression of many of these genes is programed during early metamorphosis. Primary-response genes have also been isolated that do not appear to encode DNA-binding proteins, including Eip28/29, IMP-E1, IMP-E2, IMP-E3 and Fbp1 (reviewed in Andres et al., 1993). To determine whether the expression of any of these genes is affected by mutations in E74A or E74B, we analyzed their transcription in animals hemizygous for the E74P[neo] and E74DL-1 mutant alleles (Table 1).

The Eip28/29, IMP-E2, IMP-E3, E75B, E75C, BR-C, EcR and DHR39 early genes are not affected by the E74 mutations at this level of resolution (Table 1). The behavior of these mRNAs in E74P[neo] and E74DL-1 mutant animals is a strong indicator that the E74 mutations do not affect the overall timing of late larval and prepupal development, and that our staging of mutant animals during the prepupal period is accurate. These mRNAs are transcribed throughout the onset of metamorphosis, the BR-C responding to the late larval pulse of ecdysone and E75B to the 10 hour prepupal ecdysone pulse exactly as they do in Canton S animals (Andres et al., 1993). The latter hormone peak is a good indicator of time after puparium formation. Some E74P[neo] and E74DL-1 mutants arrest development during the prepupal period, prior to the prepupal ecdysone pulse (Fletcher et al., 1995); however, the fact that the some early genes respond normally to this hormone pulse and that intact total RNA is present in E74P[neo] and E74DL-1 mutant prepupae and early pupae indicates that these animals survive for some time after their development is arrested.

E74A and E74B transcription in st p e1/Df(3L)st-81k19 hemizygous larvae and prepupae (Fig. 1) is virtually indistinguishable from that seen in wild-type Canton S animals (Karim and Thummel, 1991). The absence of detectable E74A mRNA in the E74P[neo] mutants is consistent with the insertion of a P element into an essential region of the E74A promoter and the amorphic nature of this allele (Fletcher et al., 1995). The timing and levels of E74B mRNA are virtually unchanged, as they are in E74A mutant prepupae carrying the E74X1001 mutation (Karim and Thummel, 1991). In E74DL-1 mutants, E74A mRNA is submaximally induced by the prepupal ecdysone pulse, and its expression persists 14 hours after puparium formation (Fig. 1). The apparent increase in E74A transcription in E74DL-1 mutant late third instar larvae is not reproducible.

Of the other known primary-response genes that encode DNA-binding proteins, E75A, E78B and DHR3 are also moderately affected by the E74 mutations (Fig. 1). Subtle shifts in the timing of E75A transcription are observed in E74 mutant prepupae. In the E74P[neo] mutants, the prepupal peak of E75A mRNA is expanded by 2 hours, while in the E74DL-1 mutants...
it is compressed by 2 hours (Fig. 1). DHR3 transcription also
displays subtle temporal shifts in the E74 mutants. In both
E74\(^{[\text{neo}]}\) and E74\(^{\text{DL-1}}\) mutant prepupae, the peak of DHR3
expression is shifted 2 hours earlier, toward puparium
formation; this effect is most evident in E74\(^{\text{DL-1}}\) mutant
prepupae (Fig. 1). Contrastingly, the timing of E78B tran-
scription appears unchanged in the E74 mutants, but the level
of E78B mRNA accumulation is reduced in E74\(^{[\text{neo}]}\) mutant
animals (Fig. 1). Given the absence of similar temporal shifts
in the expression of other genes, we believe that the subtle
effects on transcription described here reflect real events.

Among the ecdysone primary-response genes that do not
encode putative transcription factors, the down-regulation of
IMP-E1 and Fbp1 transcription is altered in the E74 mutants
(Fig. 2). IMP-E1 mRNA accumulation peaks in mid-prepupae
and then rapidly declines. In E74\(^{[\text{neo}]}\) and E74\(^{\text{DL-1}}\) mutant
prepupae, this down-regulation occurs prematurely, at 6 hours
rather than 8 hours after puparium formation (Fig. 2). The
down-regulation of Fbp1 expression is also affected by the E74
mutations, but with the opposite effect. In the control genotype,
Fbp1 mRNA levels decrease at the prepupal-pupal transition
(Fig. 2). However, Fbp1 transcripts are still detectable 18 hours
after puparium formation in the E74\(^{[\text{neo}]}\) mutants while, in the
E74\(^{\text{DL-1}}\) mutants, Fbp1 transcripts appear to be briefly
reinduced 14 hours after puparium formation. These observa-
tions suggest that E74A and E74B are necessary for the proper
down-regulation of IMP-E1 and Fbp1 transcription in
prepupae.

The E74B mutation affects the induction of intermolt
glue gene transcription

The intermolt glue genes are coordinately induced in mid third
instar larval salivary glands 90-96 hours after egg laying, and
are coordinately repressed at puparium formation (Muskavitch
and Hogness, 1980; Meyerowitz and Hogness, 1982; Guild,
1984; Andres et al., 1993). The induction of these genes
appears to be a secondary response to ecdysone (Hansson and
Lambertsson, 1989) and, in mid third instar larvae, is
dependent on the presence of the \(rhp\) and \(l(1)2Bc\) functions of
the \(BR-C\) (Guay and Guild, 1991; Karim et al., 1993). E74B is
coodinately induced with the \(BR-C\) in early third instar larvae,
several hours before glue gene induction, suggesting that E74B
might also contribute to glue gene regulation.

We tested this hypothesis by examining the effects of the

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**Fig. 1.** Temporal profiles of E74, E75A, DHR3 and E78B transcription in E74 mutants during late larval and prepupal development. Developmental times are given in hours relative to puparium formation (see Materials and methods for a description of larval staging). Total RNA was isolated from \(st\) \(p\)\(^{e11}/Df\)\(3L\)st-81k19 control animals and from E74\(^{[\text{neo}]}\) and E74\(^{\text{DL-1}}\) hemizygous mutant animals, and fractionated by formaldehyde gel electrophoresis. Eight sets of control, E74\(^{[\text{neo}]}\) and E74\(^{\text{DL-1}}\) mutant blots were prepared, and individual sets were
hybridized with radiolabelled DNA probes directed against the E74 common region, the E75A unique exon, the DHR3 common region or the
E78B unique exon (see Materials and Methods). Each blot was also hybridized with a labelled \(rp49\) probe (O'Connell and Rosbash, 1984) to
confirm equivalent loading in each lane (data not shown).
E74 mutations on Sgs-3, Sgs-4, Sgs-5, Sgs-7, Sgs-8 and 71E Gene VII transcription. In both st p/e11/DF(3L)st-81k19 and in E74P[neo] hemizygous mutants, a wild-type pattern of transcription is observed for each glue gene tested (Fig. 3; Table 1). Each is induced in mid third instar larvae, reaches its maximal level of transcription in late third instar larva, and is abruptly repressed in white prepupae. In contrast, the overall levels of glue gene mRNA accumulation are reduced in E74DL-1 mutant third instar larva. Sgs-5 transcripts are barely detectable, while the expression of Sgs-4 is moderately affected (Fig. 3). The responses of 71E Gene VII, Sgs-7 and Sgs-8 resemble that of Sgs-5, while Sgs-3 transcription resembles that of Sgs-4 (Table 1).

E74 mutations affect the timing and amounts of salivary gland late gene transcription

Seven genes expressed exclusively in prepupal salivary glands have been isolated from the 4F and 71E late puffs (Wolfner, 1980; Restifo and Guild, 1986a). Each gene is induced at puparium formation and repressed 14 hours later. The absolute dependence of all seven genes on the BR-C rbp function for their expression in prepupae suggests that they are induced as a secondary response to ecdysone (Guay and Guild, 1991; Karim et al., 1993), and selective deadenylation appears to play a role in the destabilization of the L71 mRNAs (Restifo and Guild, 1986b). Previous observations that E74A protein binds to both the 4F and 71E late puffs (Urness and Thummel, 1990) and that the 71E puff is reduced in E74P[neo] mutant prepupae (Fletcher et al., 1995), led us to examine whether E74 contributes to the regulation of these late genes.

The induction of 4F gene transcription is not significantly altered in either of the E74 mutants, although its repression is slightly affected (Fig. 4). In contrast, the E74 mutations affect both the timing and levels of L71 transcription. In the E74P[neo] mutants, induction of each L71 gene is delayed by 2 hours and repression occurs 2 hours earlier. In addition, the overall levels of L71 mRNA accumulation are reduced in E74P[neo] mutant prepupae. Fig. 4 depicts the responses of L71-1 and L71-6, which represent the least and most affected genes, respectively. The transcription of L71-4 and L71-5 resembles that of L71-1, while the responses of L71-2 and L71-3 resemble that of L71-6 (Table 1). Prolonged exposure of the autoradiographs reveals no detectable L71 mRNA in the E74P[neo] 0 hour time point.

In the E74DL-1 mutants, in contrast, L71 transcription is prematurely induced in late third instar larvae and continues for 2 hours longer than normal (Fig. 4). The level of L71-6 mRNA accumulation in E74DL-1 mutant prepupae is reduced compared to the control, although not as severely as in the E74P[neo] mutants, while the levels of the other mRNAs are not significantly affected. Thus the E74A mutation, and to a lesser extent...


**E74 mutations affect the transcription of other tissue-restricted ecdysone-regulated genes**

A number of ecdysone-regulated genes have been characterized that are expressed primarily in the imaginal discs or epidermis of third instar larvae and prepupae, tissues in which E74A is also expressed (Boyd et al., 1991). IMP-L1, IMP-L2 and IMP-L3 are transcribed during imaginal disc morphogenesis (Natzele et al., 1986, 1992; Osterbur et al., 1988), and their induction profiles are consistent with a secondary response to ecdysone (Natzele et al., 1987; Natzele, 1993). Edg84A and Edg84A are expressed primarily in the imaginal epidermis of late prepupae and encode pupal cuticle proteins that contribute to the synthesis of the pupal epidermis (Fechtel et al., 1988; 1989). In culture, these genes require first the presence and then the withdrawal of ecdysone for induction (Fechtel et al., 1988). A similar ecdysone response is observed for BFTZ-F1, a member of the nuclear hormone receptor superfamily (Woodard et al., 1994). Ddc and Gld encode enzymes required for the proper formation of the puparium, and both appear to be induced by ecdysone during metamorphosis (Kraminsky et al., 1980; Clark et al., 1986; Murtha and Cavener, 1989). E74A protein binds to the chromosomal region containing the Ddc locus (Urness and Thummel, 1990), making it a candidate for direct regulation by E74.

The temporal profile of each of these genes, with the exception of BFTZ-F1, is altered in the E74 mutant backgrounds (Table 1). The window of IMP-L1 transcription, which is detected in the control genotype between 2 and 8 hours after puparium formation, is expanded in both E74 mutants (Fig. 5). IMP-L1 is prematurely induced at low levels in E74P[neo] and E74DL-1 mutant newly formed prepupae, peaks 2 hours earlier, and is repressed 2 hours later than normal. In the E74DL-1 mutants, IMP-L1 is also briefly reinduced in 14 hour pupae. IMP-L2 transcription in control animals peaks just prior to puparium formation, and then maintains a constant level into the pupal period (Fig. 5). In the E74 mutants, however, IMP-L2 transcription is induced to significantly higher levels 12 hours after puparium formation and continues to be present at high levels during the early pupal period. IMP-L3 transcription, which occurs at low levels in prepupae and is normally repressed 8-10 hours after puparium formation, is also reinduced in E74 mutant pupae (data not shown).

The E74 mutations also affect the expression of Edg78E, Edg84A, Ddc and Gld (Table 1; Fig. 6). Edg78E is prematurely induced in E74P[neo] and E74DL-1 mutant prepupae, and, in the E74P[neo] mutants, it continues to be transcribed into the early pupal period (Fig. 6). Identical results were obtained for Edg84A (data not shown). Ddc expression in late larvae appears to be slightly overinduced in the E74 mutants (Fig. 6). Further, in the absence of E74A, this gene is reinduced in mid prepupae and expressed throughout the late prepupal and early pupal period. Inappropriate reinduction of Ddc is also observed in E74DL-1 mutant prepupae, although the mRNA accumulates to lower levels than in E74P[neo] mutant animals. Identical reinduction in prepupae was observed for Gld, above the low level normally detected in 10-12 hour animals (data not shown). These results reveal an additional role for E74 during metamorphosis, in restricting the expression of imaginal disc and epidermal genes to the appropriate temporal window during prepupal development.

**DISCUSSION**

Recessive loss-of-function mutations have been isolated that map to either E74A (E74P[neo]) or E74B (E74DL-1), the two transcription units that comprise the E74 early ecdysone-inducible gene (Fletcher et al., 1995). These mutations cause defects in pupariation and pupation, and result in prepupal and pupal lethality. The E74DL-1 pupal lethal mutant phenotype includes cryptocephalic head structures and incomplete
Molecular phenotypes of E74 mutations

appendage elongation, indicating the failure of a number of tissues to complete metamorphosis. In the prepupal salivary gland, a subset of late puffs is submaximally induced in the E74P(neo) mutants, while other late puffs are prematurely induced in the E74 DL-1 mutants. As an initial step toward determining the molecular basis of these phenotypes, we have used northern blot hybridization to examine the transcription of over 30 ecdysone-regulated genes in the E74P(neo) and E74 DL-1 mutant genotypes. Below we analyze the effects of the E74 mutations on ecdysone-regulated transcription and discuss possible interactions between E74 and other regulatory genes.

E74 is required for the proper transcriptional regulation of a subset of ecdysone primary-response genes

Our previous observation that E74B transcription is induced in early third instar larvae raised the possibility that this gene product might be involved in the subsequent ecdysone-induction of other primary-response genes (Andres et al., 1993). Indeed, mutations in the BR-C, which is induced coincidently with E74B, result in reduced levels of ecdysone-induced E74A, E75A, and BR-C transcription in late third instar larvae (Karim et al., 1993). However, our results indicate that E74 plays a relatively modest role in primary-response gene regulation – we detect little or no effect on ecdysone-induced E75B, E75C, BR-C, EcR, DHR39, Eip28/29, IMP-E2 or IMP-E3 transcription in the E74 mutants tested (Table 1).

Analysis of E74 transcription in E74P(neo) hemizygous third instar larvae and prepupae confirms that this mutation is a null mutation that results in no detectable E74A mRNA while leaving E74B unaffected (Fig. 1). The only evidence for E74 autoregulation is the submaximal induction of E74A transcription in E74 DL-1 mutant prepupae (Fig. 1). Three E74A-binding sites have been identified in the middle of the E74 gene (Urness and Thummel, 1990). Since these sites can be bound by E74B protein (Karim, 1992) and E74B transcription immediately precedes that of E74A in late prepupae, we conclude that E74B may directly facilitate the ecdysone-induction of E74A at this stage in development.

We detect effects on the transcription of a subset of primary-response genes in E74P(neo) and E74 DL-1 mutant animals (Fig. 1). It is difficult to determine whether the requirement for E74 for the proper repression of IMP-E1 and Fbp1 transcription in prepupae (Fig. 2) is of importance for development. Similarly, it is difficult to assess the functional significance of the subtle temporal shift in E75A transcription, although the observation that E74A binds to the 75B puff argues that this gene may be a direct regulatory target of E74 function (Urness and

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Fig. 5. Temporal profiles of IMP-L1 and IMP-L2 transcription in E74 mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the IMP-L1 gene or the IMP-L2 gene.

Fig. 6. Temporal profiles of Edg78E and Ddc transcription in E74 mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the Edg78E gene or the Ddc gene.
Thummel, 1990). However, the premature accumulation of DHR3 transcripts in newly formed E74 mutant prepupae provides a molecular basis for the increased size of the 46F puff in E74DL-1 mutant prepupae (Fletcher et al., 1995). In addition to 46F, other puffs that normally peak in size at puff stage 12 (such as 85D) are larger in the E74 mutants, raising the possibility that this subset of puff genes may be coordinately repressed by E74 at puparium formation.

E78B is normally induced several hours after E74A and E75A in late third instar larvae and requires edcsyne-induced protein synthesis for its maximal levels of transcription. Based on these observations, it has been proposed that one or more early edcsyne-inducible proteins are required for maximal E78B induction (Stone and Thummel, 1993). Both E74A and E75A proteins bind to the 78C puff, providing support for this model (Urness and Thummel, 1990; Hill et al., 1993). Our observation that E78B is submaximally induced in E74A mutant late third instar larvae indicates that E74A is at least one critical regulator required for proper E78B transcription (Fig. 1). We did not detect a corresponding reduction in the size of the 78C puff in newly formed E74Pneo mutant prepupae (Fletcher et al., 1995), presumably because the puff has almost completely regressed by this stage of development (Ashburner, 1967). The dependence of E78B transcription on E74A function provides a molecular explanation for the lag in E78B induction seen in wild-type animals – not until a critical level of E74A protein has been synthesized can E78B be fully induced by edcsyne. Recent studies have shown that both BR-C and BFTZ-F1 act in a similar manner to facilitate the subsequent edcsyne-induction of early gene transcription (Karim et al., 1993; Woodard et al., 1994). Such cross-regulation among the early genes may help to ensure that these regulators are induced in the proper temporal order during the early stages of metamorphosis.

**E74 regulates distinct sets of secondary-response genes in the larval and prepupal salivary gland**

The induction of E74B transcription in early third instar larval salivary glands precedes that of the intermolt glue genes by several hours (Andres et al., 1993; Huet et al., 1993). This temporal correlation suggests that E74B might mediate the induction of glue gene transcription as a secondary response to edcsyne. Our observation that the mRNA level of each glue gene tested is reduced or barely detectable in E74DL-1 mutant larvae (Fig. 3; Table 1) supports this hypothesis. A similar phenotype is seen in BR-C rbp and l(1)2Bc mutant larvae (Guay and Guild, 1991; Karim et al., 1993). More recent studies have shown that the effect of rbp is mediated by the Z1 protein of the BR-C, which binds to and regulates the Sgs-4 promoter (von Kalm et al., 1994).

Thus, the coordinate edcsyne-induction of the BR-C and E74B in early third instar larvae directs the subsequent induction of the glue genes in the salivary gland, defining a mid third instar regulatory hierarchy. This hierarchy represents an early developmental response to edcsyne, preceding those predicted by the puffing studies. Furthermore, our identification of a function for E74B in the larval salivary gland complements our previous observation that E74DL-1 mutant hemizygotes are defective in pupariation formation, which implied a role for E74B in the larval epidermis and musculature (Fletcher et al., 1995). Taken together, these results suggest that E74B plays a role in regulating gene expression in many tissues during third instar larval development, preparing the animal for puparium formation in response to the late larval edcsyne pulse.

In contrast to E74B, E74A is expressed in a narrow window at puparium formation, just prior to the induction of the late puffs (Boyd et al., 1991). When Walker and Ashburner (1981) altered the dose of the 74EF75B interval, they found that a subset of late puffs are more rapidly induced and increased in size upon appropriate treatment of salivary glands in culture. Most of these same puffs are also bound by E74A protein (Urness and Thummel, 1990) and significantly reduced in size in E74Pneo mutant prepupae (Fletcher et al., 1995), suggesting that E74A is at least one of the regulators defined by Walker and Ashburner. Our results confirm this hypothesis by demonstrating the effects of the E74 mutations on the transcription of the six genes that map to the 71E late puff.

Mutations in E74 have little effect on 4F late gene transcription; in contrast, the L71 genes are submaximally induced in E74Pneo mutant prepupae, and their induction is delayed by several hours (Fig. 4). The latter observation is consistent with the reduced size of the 71E late puff in E74Pneo mutant white prepupae (Fletcher et al., 1995), and with the behavior of the L71 genes in prepupae carrying the E74A mutation E74x01 (J. C. F., F. Karim and C. S. T., unpublished results). We have recently identified E74A-binding sites in the intergenic region between L71-5 and L71-6 and shown that these sites are critical for appropriate L71-6 regulation during prepupal development (L. Urness and C. S. T., unpublished data). Thus E74A appears to be directly required to regulate a subset of late genes at puparium formation, fulfilling one of the central tenets of the Ashburner model.

**E74 mutations affect the transcription of edcsyne-regulated genes expressed in the imaginal discs and epidermis**

It has been proposed that regulatory hierarchies similar to those seen in the larval salivary gland are induced by edcsyne at different stages of development and in a wide variety of target tissues (Thummel et al., 1990). In support of this hypothesis, the BR-C, E74 and E75 primary-response genes are expressed in both larval and imaginal tissues (Segraves, 1988; Thummel et al., 1990; Boyd et al., 1991; Huet et al., 1993; Emery et al., 1994) and BR-C and E74 mutations have widespread effects on metamorphosis (Burris, 1985; Kiss et al., 1988; Restif and White, 1991, 1992; Fletcher et al., 1995). In this study, we have identified regulatory targets of E74 function in diploid tissues in addition to those in the salivary glands and fat body.

Examination of IMP and pupal cuticle gene transcription in E74Pneo and E74DL-1 mutant prepupae identifies multiple potential roles for E74 in the regulation of imaginal disc morphogenesis. First, IMP-E1, the product of which is associated with early epithelial cell rearrangements in evaciating discs (Natzle et al., 1988), is inappropriately repressed in E74 mutants (Fig. 2). Second, IMP-L2, the transcription of which has been correlated with the spreading and fusion of disc cells to form the continuous adult integument (Osterbur et al., 1988), is induced to higher than normal levels in E74 mutant pupae (Fig. 5). Third, Edg78E and Edg84A, which encode components of the pupal cuticle secreted in part by the peripodial membranes of the imaginal discs (Fechtel et al., 1989), have
expanded transcription profiles in E74 mutant prepupae (Fig. 6; Table 1). In early prepupae, E74A transcripts are abundant in these disc peripodial membranes (Boyd et al., 1991). Together, these observations indicate that E74 has widespread effects on imaginal disc gene transcription, and may help to explain why a significant proportion of E74\textsuperscript{P[neo]} and E74\textsuperscript{DL-1} mutant hemizygotes die between puation and pupation without overt signs of imaginal disc morphogenesis (Fletcher et al., 1995).

The transcription profiles of Ddc and Glld, which are expressed primarily in the larval epidermis are also altered by the E74 mutations. At the onset of metamorphosis, these genes are transcribed in a narrow window around the time of puparium formation, apparently in response to the late larval ecdysone pulse (Clark et al., 1986; Murtha and Cavener, 1989). Unexpectedly, Ddc and Glld are reinduced in E74 mutant prepupae and continue to be active into the pupal period (Fig. 6; Table 1). It is possible that the effect of the E74 mutations, at least on Ddc transcription, represents its premature induction in imaginal discs. Ddc induction in prepupal imaginal discs, but not in the larval epidermis, requires a period of low hormone titer (Clark et al., 1986). In E74\textsuperscript{P[neo]} mutant prepupae, the transcription profile of Ddc is very similar to those of Edg78E and Edg84A, which also require a period of low hormone titer for their induction (Fig. 6). We thus suggest that all three of these genes may respond in similar fashion to E74 in prepupal imaginal discs. We further propose that the inappropriate induction of imaginal disc genes in mid prepupae is an indirect consequence of the E74 mutations, as E74A transcripts are not present at this time and the reinduction of E74B has just begun.

Our findings support the hypothesis that the widespread morphogenetic changes associated with insect metamorphosis are mediated through ecdysone-regulated gene hierarchies. We show that, during early metamorphosis, E74 is required in the larval salivary gland for the correct regulation of two temporally distinct sets of secondary-response genes, as well as a subset of primary-response genes. E74 is also necessary for the proper transcription of ecdysone-regulated genes in variety of other polyploid and diploid ecdysone target tissues. Further, the multiple stage-specific effects of the E74 mutations are consistent with a regulatory role for E74 at other developmental stages characterized by an ecdysone pulse. These experiments do not address the possibility that the E74 mutations may affect the spatial regulation of target gene expression, since our RNA samples were prepared from whole animals. In addition, minor variations in transcription in a small region of the animal may not be detected by this method. These issues can be addressed in future experiments, most effectively by using specific antibodies directed against different ecdysone-regulated proteins.

**Other regulatory genes may function together with E74 to control gene expression**

Interestingly, we never detect a complete loss of transcription due to the E74 mutations, even in the case of severely affected genes such as Sgs-5 or L71-6 (Figs 3, 4). One possibility is that some residual E74 function is still present in the mutants analyzed, although the genetic and molecular nature of the E74A and E74B mutations makes this unlikely (Fletcher et al., 1995). Alternatively, E74 may interact with one or more auxiliary transcription factors to increase the levels of glue gene and L71 gene transcription. E74A is capable of only an ~2-fold induction of target gene expression in tissue culture cotransfection assays (L. Urness, unpublished results). Similar weak activation has been reported for other ETS domain proteins, many of which require a partner protein for their maximal regulatory activity (Dalton and Treisman, 1992; Gogone et al., 1993; Pongubala et al., 1993; Wang et al., 1994).

Based on the similarity between the BR-C and E74 mutant phenotypes during metamorphosis, the BR-C is a likely candidate to function together with E74 to regulate gene expression in a variety of tissues. BR-C npr mutants, like E74B mutants, affect puparium formation, and several BR-C alleles affect imaginal disc elongation or fusion (Kiss et al., 1978, 1988). BR-C functions are also required to regulate glue gene and late gene transcription in the salivary gland (Guay and Guild, 1991; Karim et al., 1993). Indeed, we have found that the BR-C and E74 functionally interact in several morphogenetic pathways during metamorphosis, and act together to regulate the transcription of other genes in the late larval ecdysone hierarchy (J. C. F. and C. S. T., unpublished data). The BR-C Z1 protein is expressed predominantly in the larval salivary glands and binds both the Sgs-4 (von Kalm et al., 1994) and L71-5/-6 (K. Crossgrove and G. Guild, personal communication) promoters. Although Sgs-4 control sequences have not been examined for E74-binding sites, strong E74A-binding sites lie adjacent to two Z1-binding sites in the L71-5/-6 intergenic region (L. Urness and C. S. T., unpublished data). K. Crossgrove and G. Guild, personal communication). Experiments are currently underway to examine whether Z1 and E74A function together to regulate directly L71 transcription.

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