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The *Drosophila E74* gene is required for the proper stage- and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis

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SUMMARY

The steroid hormone ecdysone directly induces a small set of early genes, visible as puffs in the larval salivary gland polytene chromosomes, as it signals the onset of *Drosophila* metamorphosis. The products of these genes appear to function as regulators that both repress their own expression and induce a large set of secondary-response late 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

INTRODUCTION

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 (Becker, 1959; Clever and Karlson, 1960; Ashburner, 1967; Ashburner et al., 1974). Near the end of larval development, the rising ecdysone titer represses a small set of intermolt puffs and directly induces the formation of fewer than 10 early puffs. Based on the behavior of these puffs in salivary glands cultured under different conditions, Ashburner et al. (1974) proposed two functions for the early puff products – to repress their own expression and to induce the formation of more than 100 late puffs as a secondary response to the hormone. The late puff products, in turn, are thought to be additional regulators and effector molecules that direct salivary gland function and morphogenesis. It has since been proposed that similar regulatory hierarchies coordinate events in tissues throughout the organism at each developmental stage characterized by an ecdysone pulse (Burtis et al., 1990; Thummel et al., 1990).

Ecdysone-regulated genes have been isolated that correspond to each set of polytene chromosome puffs. The intermolt puffs contain genes that encode components of the polypeptide glue used by the larva to immobilize itself for pupariation

(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; Urness 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 (Wolfner, 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 (Natzle et al., 1986; Natzle, 1993). Unlike the early genes, these genes do not encode DNA-binding proteins and appear to function in tissue-specific responses to ecdysone (Natzle, 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 prepupae. Many late puffs are sub-maximally 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 (Urness 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 $E74^{P[neo]}$ and $E74^{DL-1}$ 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 puparium formation (–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 ether 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 *BR-C* 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 1× SSC, 0.1% SDS, 45 minutes in 0.3× SSC, 0.1% SDS and 30 minutes in 0.1× 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

Gene	Locus bound by E74A	Effect in mutant background	
		$E74^{P[neo]}$	$E74^{DL-1}$
Primary-response			
BR-C	–	-	-
EcR	+	-	-
E74A	+	no mRNA	a
E74B	+	-	-
E75A	+	f	f
E75B	+	-	-
E75C	+	-	-
E78B	+	a	-
DHR3	+	f	f
DHR39	–	-	-
Fbp1		g	g
IMP-E1		d	d
IMP-E2		-	-
IMP-E3		-	-
Eip28/29		-	-
Secondary-response			
Sgs-3	–	-	a
Sgs-7	–	-	a
Sgs-8	–	-	a
Sgs-4	+	-	a
Sgs-5	–	-	a
Gene VII	+	-	a
4F	+	-	-
L71-1 – L71-6	+	a,c	b,e
IMP-L1		b,e	b,e,g
IMP-L2		g	g
IMP-L3		g	g
Other			
β*FTZ-F1*	+	-	-
Edg78E		b,e	b
Edg84A		b,e	b
Ddc	+	g	g
Gld		g	g

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 E74A protein in white prepupae (Urness 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

ecdysone-regulated transcription during the onset of metamorphosis, we collected late third instar larvae and prepupae hemizygous for either an *E74A* ($E74^{P[neo]}$) or an *E74B* ($E74^{DL-1}$) loss-of-function allele. The $E74^{P[neo]}$ mutation is caused by a P element insertion into the *E74A* promoter, and the $E74^{DL-1}$ mutation consists of a 14 bp deletion in the *E74B*-specific exon that leads to a premature stop codon (Fletcher et al., 1995). Hemizygotes were collected as dark blue gut mid third instar larvae (~18 hours before puparium formation, see Materials and Methods), clear gut stationary third instar larvae (~4 hours before puparium formation), and at 2 hours intervals throughout prepupal and early pupal development synchronized from the 0 hour white prepupal stage. Animals carrying the $E74^{DL-1}$ parental chromosome *st* p^p e^{11} over *Df(3L)st-81k19*, a deficiency for *E74*, were collected as controls. Total RNA was isolated and analyzed by northern blot hybridization, using radiolabelled probes derived from the ecdysone-regulated genes listed in Table 1. In all cases, the transcription patterns that we observe in the parental stock are similar to those reported in Canton S animals (Andres et al., 1993).

In addition to the *BR-C*, *E74* and *E75* genes, four members of the nuclear hormone receptor family map to polytene chromosome puff loci and are directly inducible by the hormone: *EcR* (Koelle et al., 1991), *E78* (Stone and Thummel, 1993), *DHR3* (Koelle et al., 1992; Horner et al., 1995) and *DHR39* (Ohno and Petkovich, 1992; Ayer et al., 1993; Horner et al., 1995). E74A protein binds to all but the *BR-C* and *DHR39* puff loci, suggesting that *E74* may regulate the transcription of many of these genes 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 $E74^{P[neo]}$ and $E74^{DL-1}$ 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 $E74^{P[neo]}$ and $E74^{DL-1}$ 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 $E74^{P[neo]}$ and $E74^{DL-1}$ 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 $E74^{P[neo]}$ and $E74^{DL-1}$ 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^p e^{11}/*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 $E74^{P[neo]}$ 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 $E74^{X1001}$ mutation (Karim and Thummel, 1991). In $E74^{DL-1}$ 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 $E74^{DL-1}$ 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 $E74^{P[neo]}$ mutants, the prepupal peak of *E75A* mRNA is expanded by 2 hours, while in the $E74^{DL-1}$ mutants

it is compressed by 2 hours (Fig. 1). *DHR3* transcription also displays subtle temporal shifts in the *E74* mutants. In both $E74^{P[neo]}$ and $E74^{DL\text{-}1}$ mutant prepupae, the peak of *DHR3* expression is shifted 2 hours earlier, toward puparium formation; this effect is most evident in $E74^{DL\text{-}1}$ mutant prepupae (Fig. 1). Contrastingly, the timing of *E78B* transcription appears unchanged in the *E74* mutants, but the level of *E78B* mRNA accumulation is reduced in $E74^{P[neo]}$ 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 $E74^{P[neo]}$ and $E74^{DL\text{-}1}$ 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 $E74^{P[neo]}$ mutants while, in the $E74^{DL\text{-}1}$ mutants, *Fbp1* transcripts appear to be briefly reinduced 14 hours after puparium formation. These observations 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 *rbp* and *l(1)2Bc* functions of the *BR-C* (Guay and Guild, 1991; Karim et al., 1993). *E74B* is coordinately 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

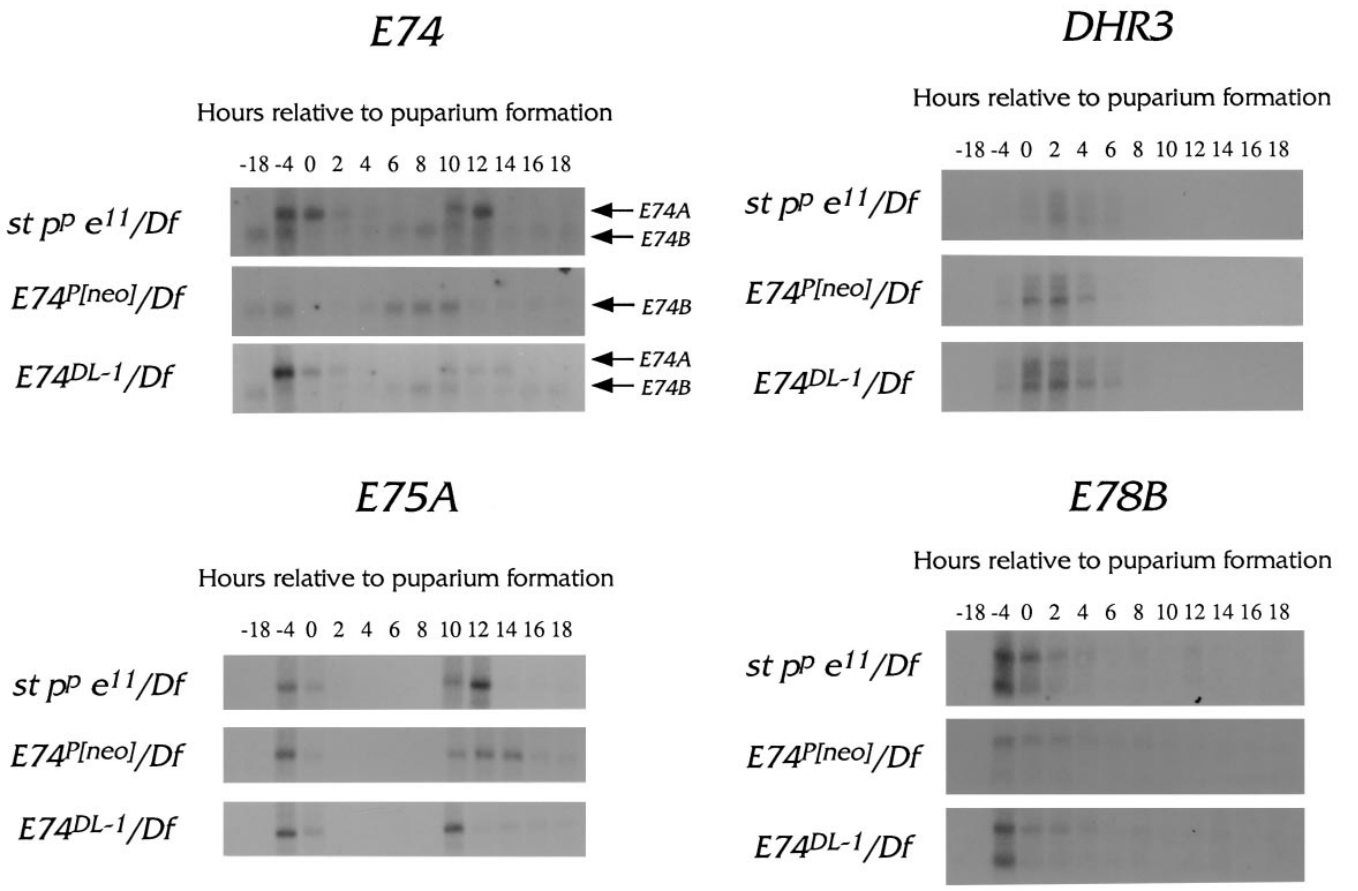

Fig. 1. Temporal profiles of *E74*, *E75A*, *DHR3* and *E78B* transcription in *E74* mutants during late larval and prepupal development. Developmental times are given in hours relative to puparium formation (see Materials and methods for a description of larval staging). Total RNA was isolated from *st* p^p e^{11}*/Df(3L)st-81k19* control animals and from $E74^{P[neo]}$ and $E74^{DL\text{-}1}$ hemizygous mutant animals, and fractionated by formaldehyde gel electrophoresis. Eight sets of control, $E74^{P[neo]}$ and $E74^{DL\text{-}1}$ 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^p e^{11}*/Df(3L)st-81k19* and in $E74^{P[neo]}$ 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 larvae, and is abruptly repressed in white prepupae. In contrast, the overall levels of glue gene mRNA accumulation are reduced in $E74^{DL-1}$ mutant third instar larvae. *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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$ 0 hour time point.

In the $E74^{DL-1}$ 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 $E74^{DL-1}$ mutant prepupae is reduced compared to the control, although not as severely as in the $E74^{P[neo]}$ mutants, while the levels of the other mRNAs are not significantly affected. Thus the *E74A* mutation, and to a lesser extent

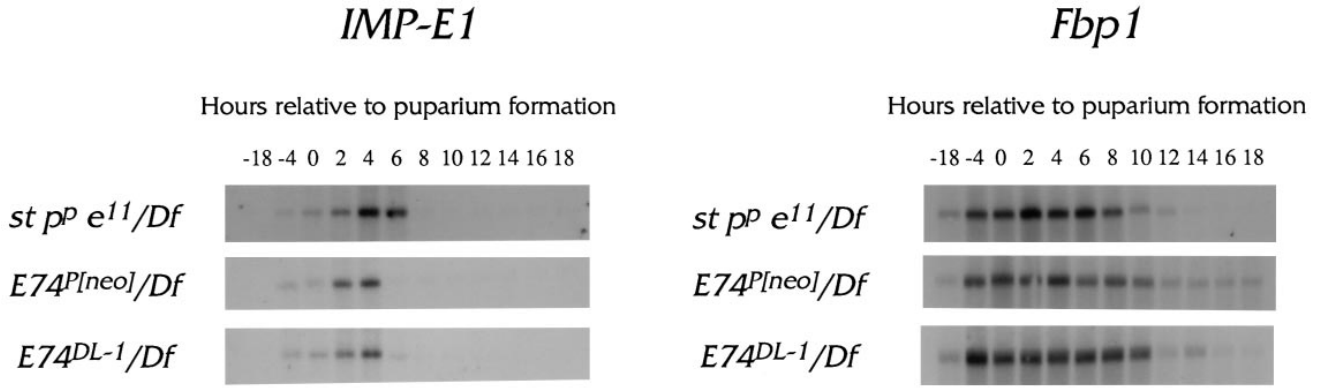

Fig. 2. Temporal profiles of *IMP-E1* and *Fbp1* transcription in *E74* mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the *IMP-E1* gene or the *Fbp1* gene.

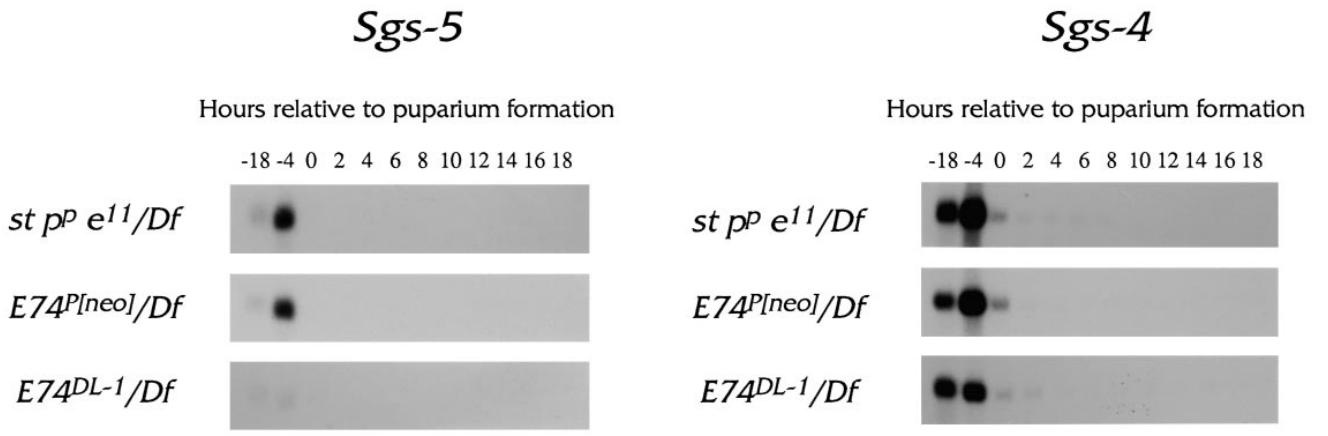

Fig. 3. Temporal profiles of glue genes *Sgs-5* and *Sgs-4* transcription in *E74* mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the *Sgs-5* gene or the *Sgs-4* gene.

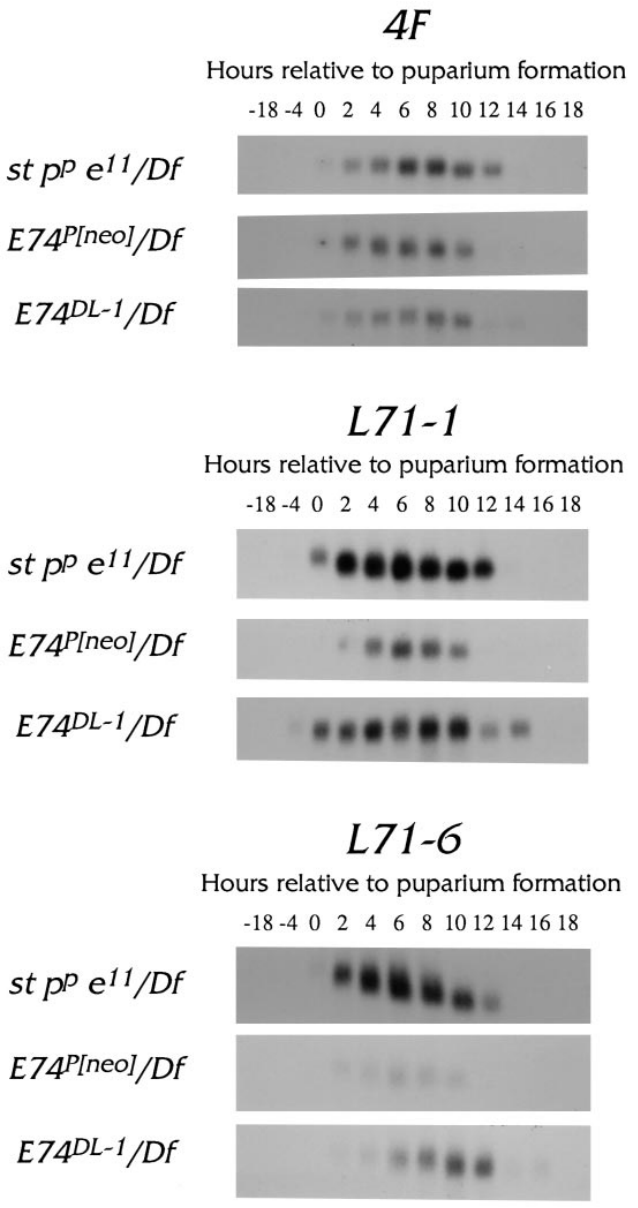

Fig. 4. Temporal profiles of *4F*, *L71-1* and *L71-6* transcription in *E74* mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the *4F* gene, the *L71-1* gene or the *L71-6* gene. The transcription profiles of the other four *L71* genes fall in a range between those of the two genes shown.

the *E74B* mutation, affects both the timing and levels of *L71* gene transcription.

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 (Natzle et al., 1986, 1992; Osterbur et al., 1988), and their induction profiles are consistent with a secondary response to ecdysone (Natzle et al., 1987; Natzle, 1993). *Edg78E* 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 *βFTZ-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 *βFTZ-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 $E74^{P[neo]}$ and $E74^{DL-1}$ mutant newly formed prepupae, peaks 2 hours earlier, and is repressed 2 hours later than normal. In the $E74^{DL-1}$ 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 $E74^{P[neo]}$ and $E74^{DL-1}$ mutant prepupae, and, in the $E74^{P[neo]}$ 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 $E74^{DL-1}$ mutant prepupae, although the mRNA accumulates to lower levels than in $E74^{P[neo]}$ 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* ($E74^{P[neo]}$) or *E74B* ($E74^{DL-1}$), 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 $E74^{DL-1}$ pupal lethal mutant phenotype includes cryptocephalic head structures and incomplete

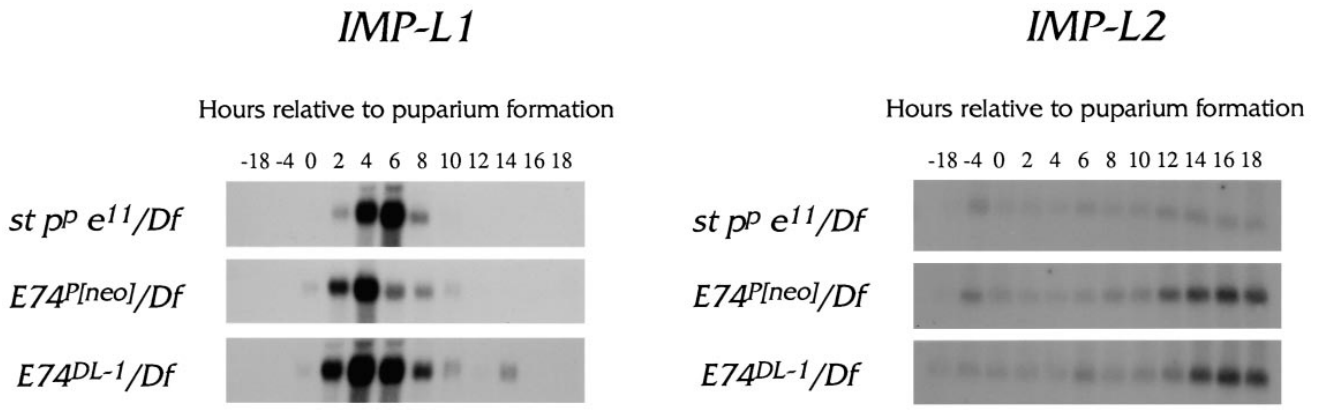

Fig. 5. Temporal profiles of *IMP-L1* and *IMP-L2* transcription in *E74* mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the *IMP-L1* gene or the *IMP-L2* gene.

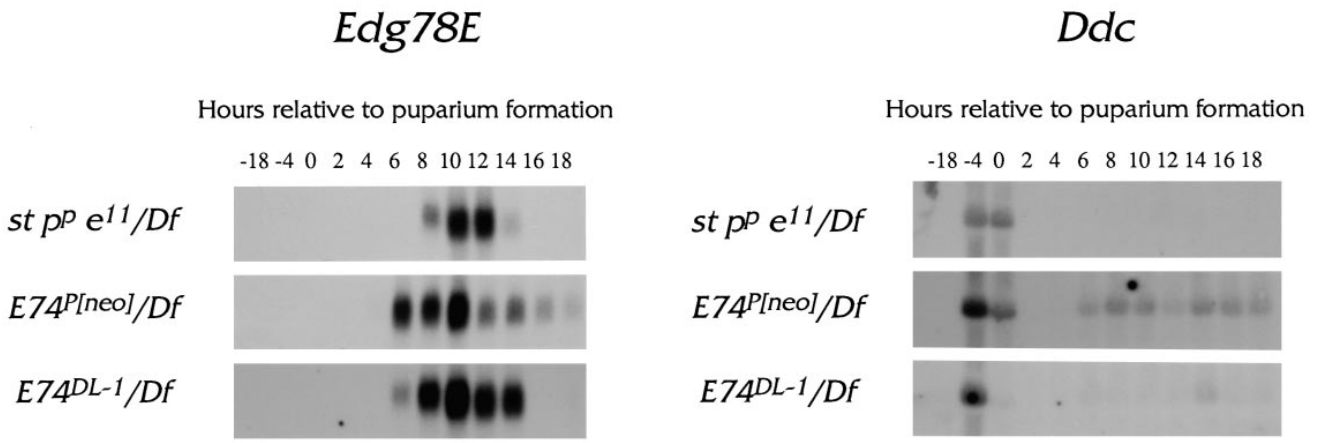

Fig. 6. Temporal profiles of *Edg78E* and *Ddc* transcription in *E74* mutants during late larval and prepupal development. The blots described in Fig. 1 were hybridized with radiolabelled DNA probes directed against the *Edg78E* gene or the *Ddc* gene.

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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$ 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

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 $E74^{DL\text{-}1}$ 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 ecdysone-induced protein synthesis for its maximal levels of transcription. Based on these observations, it has been proposed that one or more early ecdysone-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 $E74^{P[neo]}$ 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 ecdysone. Recent studies have shown that both *BR-C* and *βFTZ-F1* act in a similar manner to facilitate the subsequent ecdysone-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 ecdysone. Our observation that the mRNA level of each glue gene tested is reduced or barely detectable in $E74^{DL\text{-}1}$ 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 ecdysone-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 ecdysone, 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 $E74^{DL\text{-}1}$ mutant hemizygotes are defective in puparium 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 ecdysone 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 74EF/75B 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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$ 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 $E74^{P[neo]}$ mutant white prepupae (Fletcher et al., 1995), and with the behavior of the *L71* genes in prepupae carrying the *E74A* mutation $E74^{X1001}$ (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 ecdysone-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 ecdysone 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 (Burtis, 1985; Kiss et al., 1988; Restifo 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 $E74^{P[neo]}$ and $E74^{DL\text{-}1}$ 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 evaginating 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 $E74^{P[neo]}$ and $E74^{DL\text{-}1}$ mutant hemizygotes die between pupariation and pupation without overt signs of imaginal disc morphogenesis (Fletcher et al., 1995).

The transcription profiles of *Ddc* and *Gld*, 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 *Gld* 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 $E74^{P[neo]}$ 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 co-transfection 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; Gegonne 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 to 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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