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Control of expression of the homeotic *labial* (*lab*) locus of *Drosophila melanogaster*: evidence for both positive and negative autogenous regulation

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Summary

The homeotic gene *labial* (*lab*) is required for proper development of the embryonic and adult head in *Drosophila melanogaster*. The *lab* gene product accumulates in a complex pattern in both embryonic and imaginal tissue. During embryogenesis, *lab* is expressed in the endodermally derived cells of the midgut, in ectodermally derived cells of the procephalon and dorsal ridge, and in a small subset of progenitor sensory cells. Imaginal expression is restricted to a narrow region of the peripodial membrane of the eye–antennal disc. As part of our continuing effort to understand the role of *lab* in development, we have begun a dissection of the regulatory elements of the *lab* transcription unit and used germ line transformation experiments to determine which aspects of the observed expression pattern are essential for proper head development and viability. Transgenic embryos harboring an abridged *lab* gene are able to overcome the embryonic lethality associated with the loss of *lab* function and survive to adulthood. Interestingly, in these transgenic lines the *lab* protein accumulates only in a subset of those embryonic cells that normally express the gene, namely the procephalon and the anterior midgut. We also find that, once initiated, *lab* expression is maintained by positive autoregulation. Although *lab* minigene activity is sufficient to rescue the embryonic lethality of *lab* mutations, the transgenes fail to rescue defects in the adult head capsule. However, the defects observed in this study encompass a broader domain than those seen using somatic recombination to generate lab^- clonal tissue. The failed rescue and observed cuticular defects are, at least in part, explained by the observation that the transgenes, rather than failing to be expressed, are associated with ectopic accumulation of *lab* protein in the peripodial membrane of the antennal disc. Moreover, this aberrant expression pattern is correlated with the abnormal expression of two other homeotic genes, *Deformed* (*Dfd*) and *Sex combs reduced* (*Scr*) in the eye–antennal disc. These results are only observed when the transgene is resident in a lab^- genotype and ectopic expression of *lab* and misregulation of *Dfd* and *Scr* are not seen in a lab^+ background. This result suggests that the wild-type *lab* gene product is necessary for the normal regulation of the locus in the imaginal discs, but unlike the case in the embryo, the event is negative. We discuss the biological implications of these results in relation to the role of *lab* in development.

Key words: autogenous regulation, head development, homeotic, *labial*, Antennapedia Complex, *Drosophila*.

Introduction

The segmentally organized body plan of *Drosophila melanogaster* is determined at the cellular blastoderm stage of development. The homeotic genes, which are transcriptionally and, in at least one case, translationally active at this stage (Levine *et al.* 1983; Akam and Martinez-Arias, 1985; Jack *et al.* 1988) are involved in generating segmental identity, but not segmentation itself. The role of the homeotics in the specification of segmental identity is revealed by mutations in these genes which elicit segmental transformations (for a review, see Mahaffey and Kaufman, 1987*a*). The loci of the Bithorax Complex (BX-C) are involved in specifying posterior thoracic and abdominal segmental identity (Lewis, 1978; Sanchez-Herrero *et al.* 1985). A second cluster of homeotic genes, the Antennapedia Complex (ANT-C), comprises genes required for proper head and anterior thoracic development (for a review, see Kaufman *et al.* 1990). All of the homeotic genes of the ANT-C – *Antennapedia* (*Antp*), *Sex combs reduced* (*Scr*), *Deformed* (*Dfd*), *proboscipedia* (*pb*), and *labial* (*lab*) – are required for proper adult development (Kaufman, 1978; Struhl, 1981; Hazelrigg and Kaufman,

1983; Merrill *et al.* 1987, 1989). In addition, all of these genes, with the exception of *pb*, are required for proper embryonic and larval development. More specifically, loss-of-function mutations in the loci of the ANT-C result in cephalic or thoracic segmental transformations and/or failure in head involution (Wakimoto and Kaufman, 1981; Sato *et al.* 1985; Martinez-Arias, 1986; Merrill *et al.* 1987; Regulski *et al.* 1987; Pultz *et al.* 1988; Merrill *et al.* 1989).

Developmental genetic analyses have demonstrated that *lab* function is required for proper development of the embryonic and adult head. During embryogenesis, *lab* is necessary for the proper morphological movements associated with head involution, whereas in the absence of *lab* this process fails (Merrill *et al.* 1989). In this same study, induction of somatic clones demonstrated a role for *lab* in the formation of the adult head capsule – in the absence of *lab* function there is an apparent head-to-thoracic transformation. Recent molecular analyses of the proximal portion of the ANT-C has determined that an ~17 kb transcription unit in this region is that of *lab* (Diederich *et al.* 1989). Transcript and protein localization studies have shown that *lab* gene products accumulate in complex temporal and spatial patterns in the embryo including endodermal regions of the midgut, epidermal regions of the procephalic lobe and the dorsal ridge, the central nervous system (CNS), and in presumed sensory anlagen of the clypeolabrum, thorax and tail region (Hoey *et al.* 1986; Diederich *et al.* 1989). Additionally, *lab* is expressed in a narrow region in the peripodial membrane of the eye–antennal disc (Diederich *et al.* 1991).

In this study, the *cis*-acting regulatory elements necessary for the proper spatial and temporal expression of *lab* were determined by assembling a series of constructs containing an abridged *lab* transcription unit plus flanking upstream and downstream regions. These transgenes rescued the embryonic lethality associated with the loss of *lab* function despite the absence of transgenic expression in the dorsal ridge, posterior midgut (pmg), and the peripheral nervous system (PNS). This suggests that *lab* expression in these cells is dispensable for viability and defines the portion of the expression pattern that is sufficient for head involution and viability. We also show that the *cis*-acting regulatory elements responsible for *lab* expression in the dorsal ridge and pmg are located in the first intron, which is absent from the transgenes. Additionally, we have shown that *lab* is positively autogenously regulated in the embryo and that the *cis*-acting sequences required for autogenous regulation are present in the minigene constructs. Furthermore, transgenic adults display defects reminiscent of, but not identical to, the somatic clones produced by Merrill *et al.* (1989), again suggesting the absence of certain *cis*-acting regulatory elements. However, rather than failing to be expressed, the transgene is associated with ectopic accumulation of *lab* protein in the peripodial membrane of the antennal disc. This ectopic expression of *lab* is accompanied by an alteration in the pattern of expression of the other two ANT-C members expressed in this disc, i.e. there is a truncation of *Dfd* expression and an extension of the domain of *Scr* accumulation. Together, these results explain why the defects observed for the transgenic adult flies are more severe than those generated by somatic recombination. Interestingly, the ectopic expression of the transgene is not seen in the presence of a wild-type copy of *lab*, indicating the possibility of negative-autoregulation of this gene. A description of the transgenes, transgenic protein distribution and adult head capsule defects is presented here.

Materials and methods

Fly stocks

Flies were maintained at 23°C on standard *Drosophila* media supplemented with Baker's yeast. Transgenic stocks were established with the minigenes in various *lab* allelic backgrounds. The deficiencies and alleles used in this study are shown in Table 1. The mutated chromosomes were maintained over the balancers TM3,*Sb* and/or TM6B,*Hu Tb* (Lindsley and Grell, 1968; Craymer, 1984; Lindsley and Zimm, 1991).

Table 1. *Transgenic rescue analysis of* labial *alleles*

	lab^1		lab^2		lab^3		lab^4		lab^6		lab^{14}		lab^{15}		lab^{18}	
	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A
Df(3R)*Scr*	−	−	+	mv	NT		+	mv	+	mv	+	mv	+	mv	+	mv
lab^1 [r9]	−	−	NT		NT		+	mv	+	mv	+	mv	NT		NT	
lab^2 [k3]			−	−	NT		+	mv	+	mv	+	mv	NT		NT	
lab^3 [f7]					−	−	NT		+	mv	+	mv	NT		NT	
lab^4 [f8]							+	mv	+	mv	+	mv	+	mv	+	mv
lab^6 [f33]									+	mv	+	mv	+	mv	+	mv
lab^{14} [vd1]											+	mv	+	mv	+	mv
lab^{15} [vd2]													+	mv	NT	
lab^{18} [vd35]															+	mv

Allele designations are from Lindsley and Zimm (1991). The allele designations in brackets in the vertical column are from Merrill *et al.* (1989). Df(3R)Scr is deleted for the polytene interval 84A1-8B1,2 (Kaufman *et al.* 1980). Null alleles of *labial* are *4* and *14*, the remaining mutations are hypomorphs. The resported results were obtained using P[w^+,$lab^{2.5}$]. The other minigene constructs were only tested using the above null alleles. E, Embryonic; A, Adult; +, rescue; −, failure to rescue; mv, mutant viable; NT, not tested.

Construction of plasmids

Minigene constructs

All minigenes were originally assembled in pHSS7 (Seifert *et al.* 1986) and subsequently shuttled into appropriate transformation vectors. Due to the relatively large size of the *lab* transcription unit a 280 bp *Sma*I fragment, derived from a partial cDNA clone, was used to link two genomic fragments, thus spanning the 14 kb interval created by the first intron. The 3′-genomic fragment used extends ~2.5 kb downstream of the poly(A) site and leaves intact the second intron, which interrupts the homeobox (Mlodzik *et al.* 1988; Diederich *et al.* 1989). The 5′-element includes sequences extending ~5.2 kb upstream of the transcription start site to the proximal breakpoint of *Df*(3R)*MAP8*, a small deficiency that complements *lab* function (Fig. 1; see also Diederich *et al.* 1989). The total construct, which is ~10.4 kb in length, was removed as a *Not*I fragment from pHSS7 (Seifert *et al.* 1986) and inserted into the transformation vector CoSpeR *Not*I (supplied by John Tamkun) creating P[w^+,$lab^{2.5a}$] (2.5a) (Fig. 1). Two additional constructs were generated by deleting the upstream 1.6 kb *Eco*RI fragment. These two constructs, P[w^+,$lab^{2.4a}$] (2.4a) and P[w^+,$lab^{2.4b}$] (2.4b), are identical except that the 5′-most 1.2 kb *Eco*RI fragment is inverted relative to its normal orientation in construct 2.4b (Fig. 1). A fourth construct, P[w^+,$lab^{2.3b}$] (2.3b), was generated in which the 1.2 kb *Eco*RI fragment is replaced with the 5′ 1.6 kb *Eco*RI fragment, which is inverted relative to its normal orientation (Fig. 1).

Reporter gene fusion constructs

The *lab–lacZ* reporter gene fusion constructs were made by replacing the *ninaE* sequences from pDM66A (supplied by D. Mismer, modified from Mismer and Rubin, 1987) with sequences upstream of the *lab* transcription unit. This was accomplished by digesting pDM66A with *Kpn*I, blunt-ending with T4 DNA polymerase, and then digesting with *Bam*HI. A 7.0 kb *Bam*HI fragment containing 6.0 kb of sequence upstream of *lab* and 995 bp downstream of the transcription start site cloned in Bluescribe (Stratagene), was digested with *Bss*HII which cuts 10 bp downstream of the transcription start in the untranslated leader. This digest was blunt-ended with Klenow (NEB), and digested with *Bam*HI. The resulting 6.0 kb fragment containing the 5′ end of the gene was then subcloned into pDM66A creating a gene fusion construct containing 10 bases of the untranslated *lab* leader, the *lab* transcription start site, and ~6.0 kb of upstream sequences. This plasmid was named p6.0*lab*66A and is shown in Fig. 4. A variation of this *lab–lacZ* gene fusion was made by digesting the above construct with *Bam*HI and *Hin*dIII, blunt-ending with Klenow, and ligating with T4 DNA ligase. This results in a deletion of the distal-most ~2.35 kb upstream fragment and creates a gene fusion with ~3.65 kb of *lab* 5′ sequences called p3.65*lab*66A (Fig. 4). These two gene fusions were subcloned into the *Not*I site of CoSpeR *Not*I for transformation. A variation of the larger reporter gene fusion was constructed as a protein fusion rather than a leader fusion in the following manner. The 7.0 kb *Bam*HI fragment described above was inserted as a *Bam*HI fragment into the pCaSpeR-β-gal vector (Thummel *et al.* 1988) resulting in a protein fusion construct containing sequences coding for the first 252 amino acids of *lab* fused in frame with β-galactosidase coding sequences (Fig. 4). This last reporter is referred to as p7.0*lab*Th and is shown in Fig. 4.

Enhancer test vector constructs

Genomic DNA fragments from the first intron were initially subcloned into pHSS7 and then removed as *Not*I fragments and subcloned into the *Not*I site of the polylinker in the enhancer test vector HZ50 (Hiromi and Gehring, 1987). The gross molecular organization of the first intron and relative positions of the subcloned fragments are shown in Fig. 7. The upstream 1.2 kb *Eco*RI fragment mentioned above was also

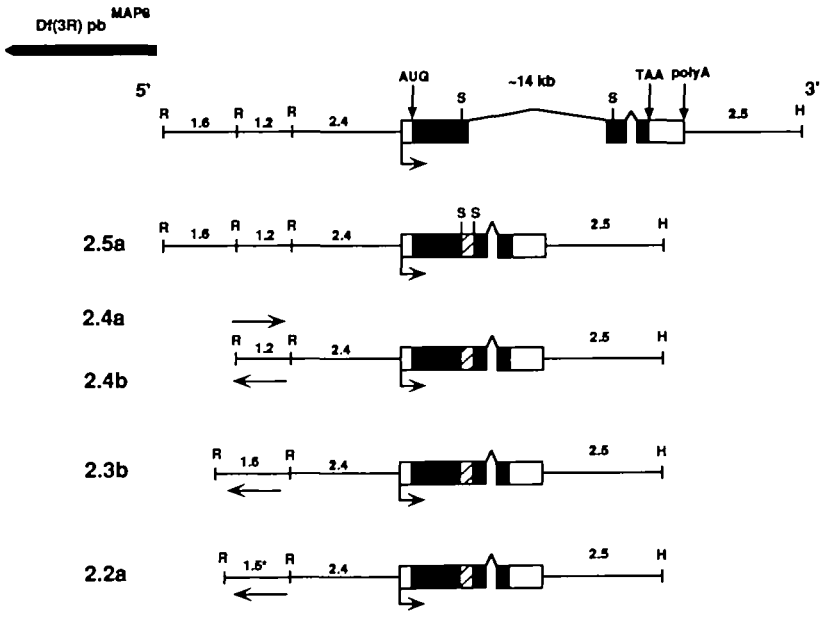


Fig. 1. Structure of *lab* minigene constructs. The *lab* transcription unit is shown at the top. The breakpoint of the mutant chromosome *Df*(3R)*MAP8* defines the 5′-most extent of the *lab* locus. Shaded boxes represent coding regions while open boxes indicate untranslated sequences. Thin horizontal lines represent intronic and flanking sequences. The right-angle arrow below the figure indicates the initiation point and direction of transcription. Distances between relevant restriction sites are given in kilobases (kb). The distance from transcription start to the first upstream *Eco*RI site is 2.4 kb and the distance from the poly(A) site to the *Hin*dIII site is 2.5 kb. Restriction enzymes: H, *Hin*dIII; R, *Eco*RI; S, *Sma*I. Minigene P[w^+,$lab^{2.5a}$] (2.5a) is identical to the transcription unit above except the first intron was eliminated using a 280 bp *Sma*I fragment isolated from a partial cDNA clone (cross-hatched box). Minigene P[w^+,$lab^{2.4a}$] (2.4a) is the same as 2.5a except the 5′ 1.6 kb *Eco*RI fragment is deleted. Minigene P[w^+,$lab^{2.4b}$] (2.4b) differs from 2.4a in that the 5′ 1.2 kb *Eco*RI fragment is in the reverse orientation (horizontal arrows above and below diagrams depict orientation). Minigene P[w^+,$lab^{2.3b}$] (2.3b) has the 1.6 kb *Eco*RI fragment in place of the 1.2 kb *Eco*RI fragment. Minigene P[w^+,$lab^{2.2a}$] (2.2a) is the same as 2.3b except that a 1.5 kb *Eco*RI fragment from the first exon/intron border is substituted for the 1.6 kb *Eco*RI fragment. These five constructs were subcloned as *Not*I fragments into the transformation vector CoSpeR *Not*I (Materials and methods).

subcloned into HZ50. These subclones and plasmids are designated by the test fragment size in kb followed by the vector name, eg, P[ry^+, 1.2*lab*HZ].

Germ line transformation

Germ line transformation was carried out as described in Robertson *et al.* (1988) using a 500 μg ml^{-1} solution of each minigene or gene fusion construct. The minigene and reporter genes were injected into *w*; P[ry^+,Δ2–3] hosts. The enhancer test vectors were co-injected with the P-element helper plasmid P(π)25.7wc (Karess and Rubin, 1984) into ry^{506} embryos. Transformed G1 flies were identified by complementation of the *white*$^-$ phenotype for the minigene and gene fusion constructs, or of the *rosy*$^-$ phenotype for the enhancer test vectors. At least two independent lines were established for each of the constructs except 2.3b. We were only able to obtain a single line of this truncated minigene. However, we have recovered an additional line of a nearly identical construct P[w^+, $lab^{2.2a}$] (2.2a) (Fig. 1) which contains 2.4 kb of upstream sequence plus a 1.5 kb intronic fragment that has no detectable enhancer activity, i.e. it is functionally equivalent to the 1.6 kb upstream fragment in 2.3b. Stable homozygous transgenic stocks were established in various *lab* allelic backgrounds using standard crossing protocols.

Immunological staining

Transgenic embryos were fixed and stained essentially as described in Mahaffey and Kaufman (1987*b*) using the *lab* antisera described in Diederich *et al.* (1989). Embryos were dehydrated in ethanol, cleared in methylsalicylate, and photographed using Nomarski optics on a Zeiss Axiophot microscope. Horse-radish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from BioRad. Imaginal disc staining was performed as described in Pattatucci and Kaufman (1991). The *Dfd* antisera is described in Diederich *et al.* (1991), and the *Scr* antisera is described in Mahaffey and Kaufman (1987*b*). The polyclonal rabbit β-galactosidase antisera was a gift from David Miller and the monoclonal anti-β-galactosidase was obtained from Marie Mazzula. Both are members of this laboratory.

Scanning electron microscopy (SEM)

Transgenic stocks were established in which the *lab*$^-$ alleles were balanced over either TM3,*Sb* or TM6B,*Hu Tb*. Adult flies that eclosed bearing either Sb^+ or Hu^+ phenotypes were stored in 70% ethanol and prepared for SEM as described in Merrill *et al.* (1987).

Results

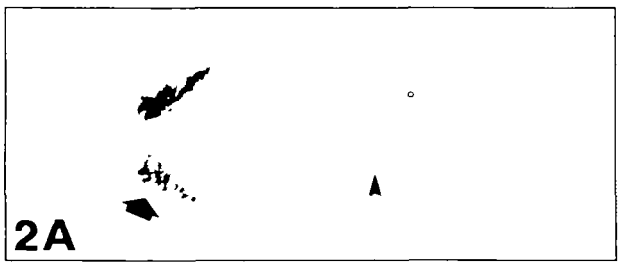
Rescue of the lab$^-$ *phenotype with a minigene construct*

The large size of the *lab* transcription unit, plus the need to include possible upstream and downstream regulatory elements, necessitated the construction of minigenes. We decided initially to remove the 14 kb first intron by using a cDNA fragment to bridge this region. Transgenic lines carrying the minigenes P[w^+,$lab^{2.5a}$] (2.5a), P[w^+,$lab^{2.4a}$] (2.4a), and P[w^+,$lab^{2.4b}$] (2.4b) (Fig. 1), were established and tested for their ability to rescue the null and hypomorphic alleles of *lab* listed in Table 1. The various *lab*$^-$ chromosomes were balanced with either TM3,*Sb* or TM6B,*Hu Tb*. Transgenic rescue was scored by the emergence of adults bearing neither the *Sb* nor *Hu* markers and these results are summarized in Table 1. Adults harboring one or two copies of the 2.5a, 2.4a or 2.4b transgenes in a *lab*$^-$ background emerged and exhibited various head capsule defects reminiscent of those described by Merrill *et al.* (1989). One copy of the transgene rescued to the same extent as two copies. This suggests that regulatory elements necessary for embryonic viability are present in the transgenes while those needed for adult function are absent and presumably located in the first intron.

Every interallelic combination tested yielded transgenic adult survivors. In addition, many homozygous combinations produced rescued adults. Accessory lethal mutations associated with the mutagenized *lab* chromosomes probably accounts for the failure of a few alleles to be rescued as homozygotes (Table 1). However, in all cases, the rescued class did not represent the expected 25% of the adults scored. Lethal phase analysis demonstrated a polyphasic period of lethality occurring throughout the larval and pupal

Fig. 2. Transgenic expression of *lab* during embryogenesis. Embryos are oriented with the anterior end on the left. (A–E) Expression of *lab* in wild-type embryos. (F–J) Transgenic expression of *lab* in *lab*$^-$ embryos. (A,F) Ventral view of a stage-9 embryos (embryonic stages are from Campos-Ortega and Hartenstein, 1985). Accumulation of transgenic *lab* protein is absent from the posterior midgut rudiment (pmg) in F (arrowhead points to the pmg). (B,G) Lateral view of stage-10 embryos. Arrowheads point to the pmg. Transgenic expression in the procephalon (arrow) of the embryos in F and G is normal (compare to A and B). (C,H) Lateral view of stage-14 embryos. Accumulation of *lab* protein in the dorsal ridge is observed in wild-type but not transgenic embryos (open arrow). The small patch of *lab*$^-$ expressing cells below the arrow (H) are located in the procephalon (compare to C). (D and I) Dorsal view of stage-14 embryos. Notice the accumulation of *lab* protein in the cells of the midgut (arrowhead) in I. These cells are derivatives of the anterior midgut (amg). (E and J) Dorsal view of stage-17 embryos. Transgenic accumulation of *lab* protein in the head is greatly diminished (boxed area) when compared to wild-type levels (E). Accumulation in the midgut is still detected (arrowhead).

Fig. 3. Embryonic expression patterns of minigene constructs P[w^+,$lab^{2.3b}$] (2.3b) and P[w^+,$lab^{2.2a}$] (2.2a). Embryos are oriented with the anterior end to the left. (A,C) Initiation of *lab* expression in the procephalon (arrow) of embryos carrying 2.3b in a *lab*$^-$ background. The absence of *lab* protein accumulation in the pmg (arrowhead) indicates that these are *lab*$^-$ embryos. (B) Transgenic accumulation of *lab* protein in an embryo carrying 2.2a. Minigene expression is detected in the procephalon (arrow). (D) The inability of these minigene constructs to maintain *lab* expression in the procephalon is demonstrated by the complete absence of *lab* protein in this stage-15 embryo carrying 2.3b. Also note the absence of *lab* protein in the cells of the midgut (arrowhead). Compare to Fig. 2I.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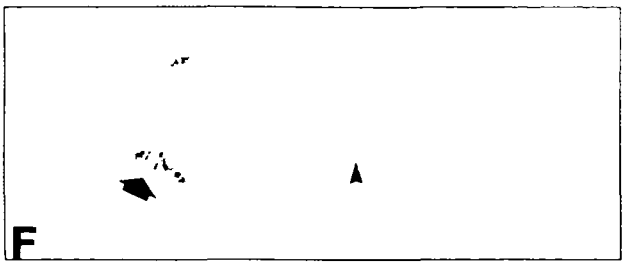
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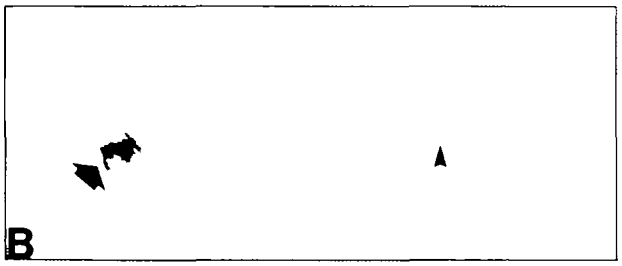
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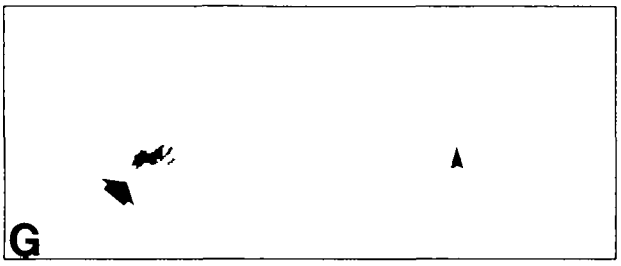
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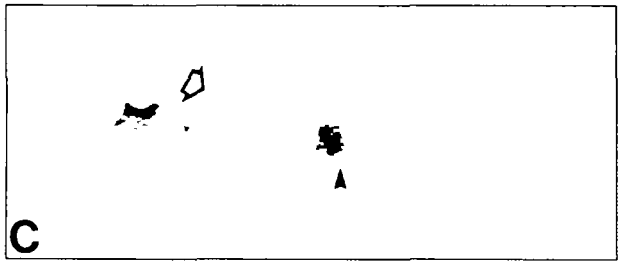
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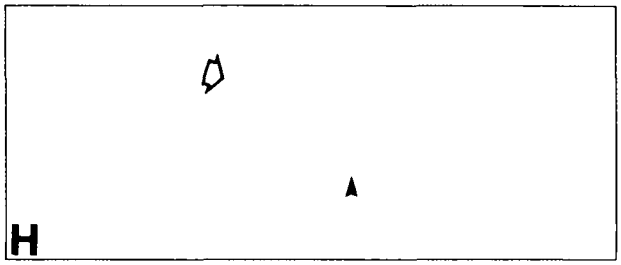
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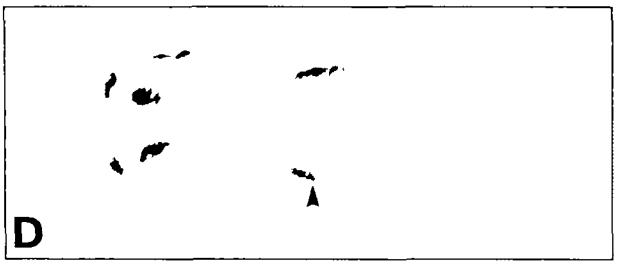
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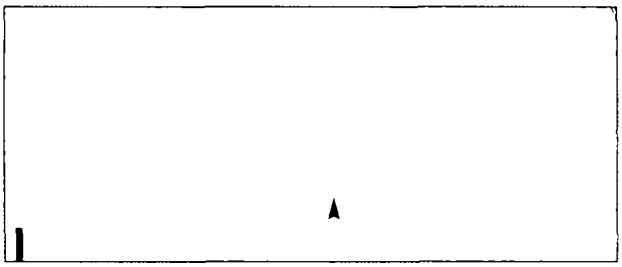
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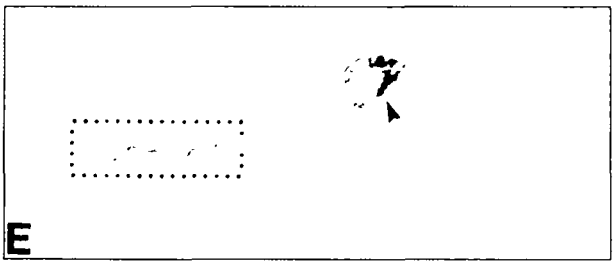
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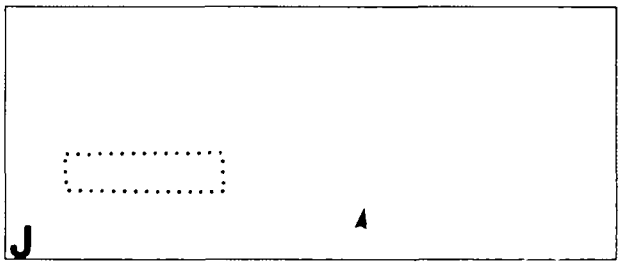
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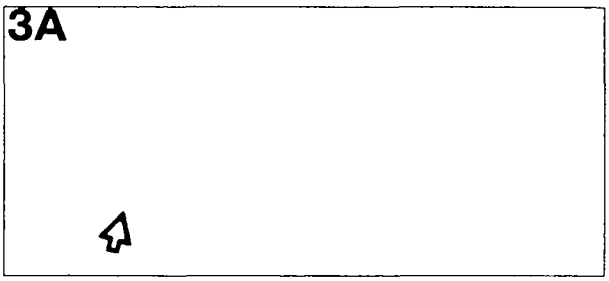
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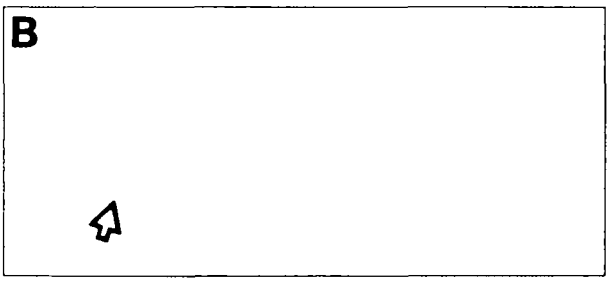
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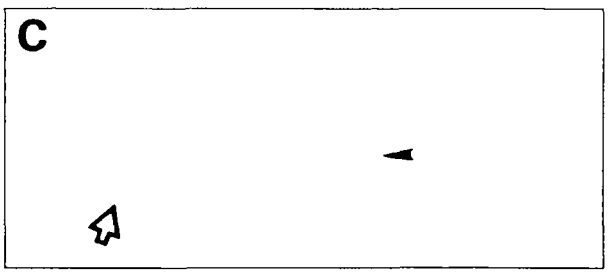
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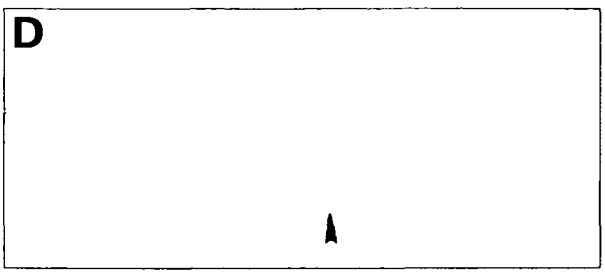
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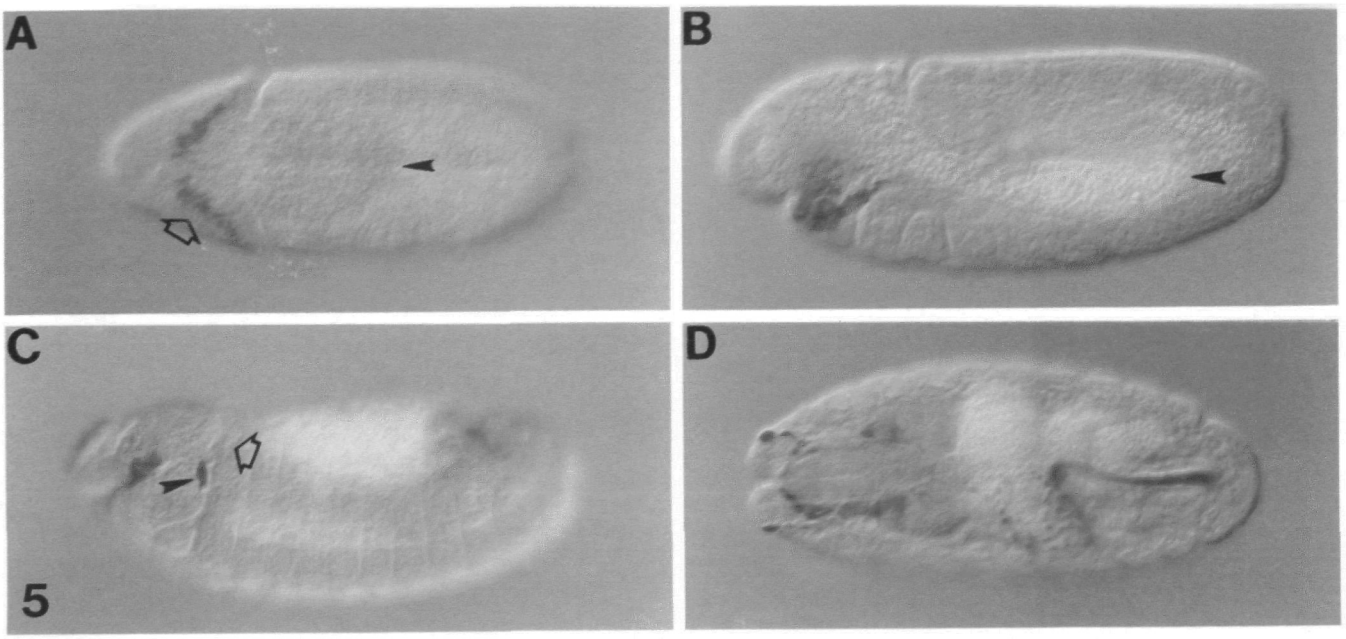


Fig. 5. Transgenic expression of *lab–lacZ* reporter gene constructs. All three reporter gene constructs exhibited the same embryonic expression pattern. The embryos shown here carry the p6.0*lab*66A reporter gene. All embryos are oriented with the anterior end to the left. (A) Ventral view of a stage-9 embryo. Accumulation of β-galactosidase (β-gal) is detected in the procephalon (arrow), however, it is absent from the pmg (arrowhead). This pattern is identical to that seen for the minigene constructs at the same stage (compare to Fig. 2F). (B) Lateral view of a stage-11 embryo showing β-gal accumulation in the procephalon but not in the pmg (arrowhead). (C) Lateral view of a stage-14 embryo. Note the absence of β-gal accumulation in the dorsal ridge (arrow). The arrowhead points to a small cluster of *lacZ*-expressing cells in the procephalon (compare to Fig. 2C,H). (D) Dorsal view of a stage-17 embryo. This pattern is different than that observed for the minigene constructs (Fig. 2J) in that there is detectable β-gal accumulation in the procephalon at this stage. This is likely due to the increased perdurance of the β-gal polypeptide relative to the *lab* protein. Non-specific staining of the hindgut is seen in embryos in which polyclonal anti-β-gal antibody was used. This hindgut staining pattern is also observed in non-transgenic embryos.

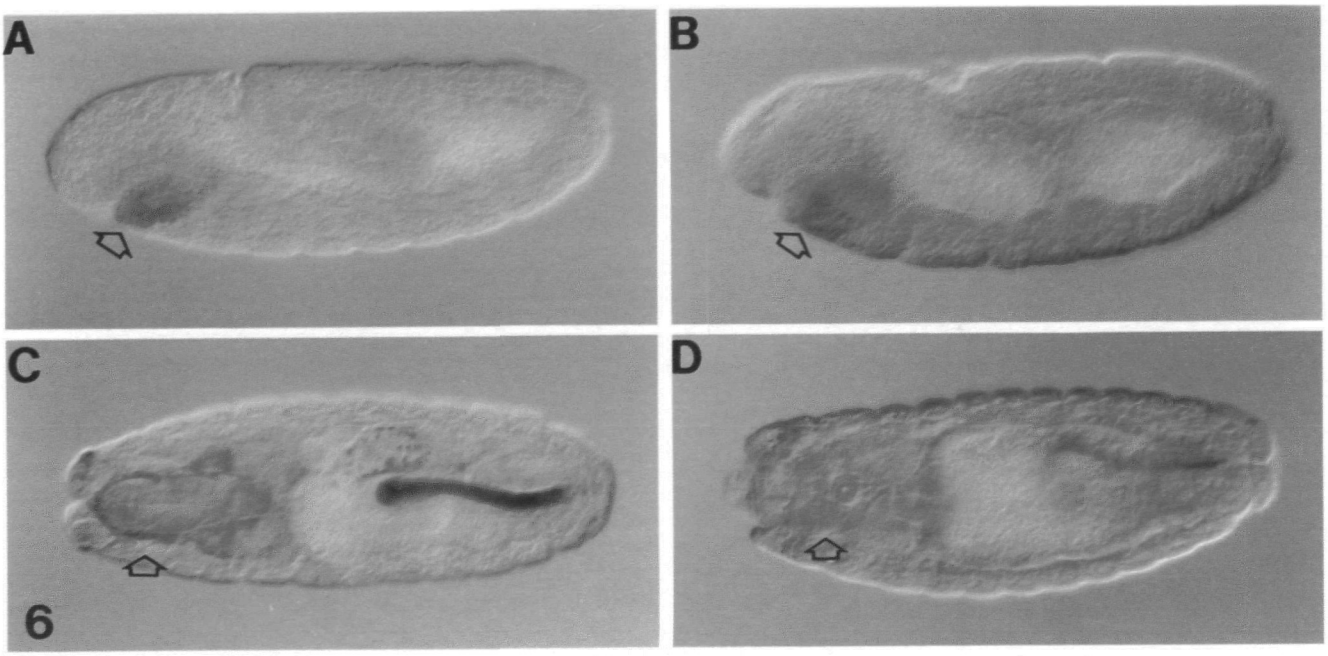
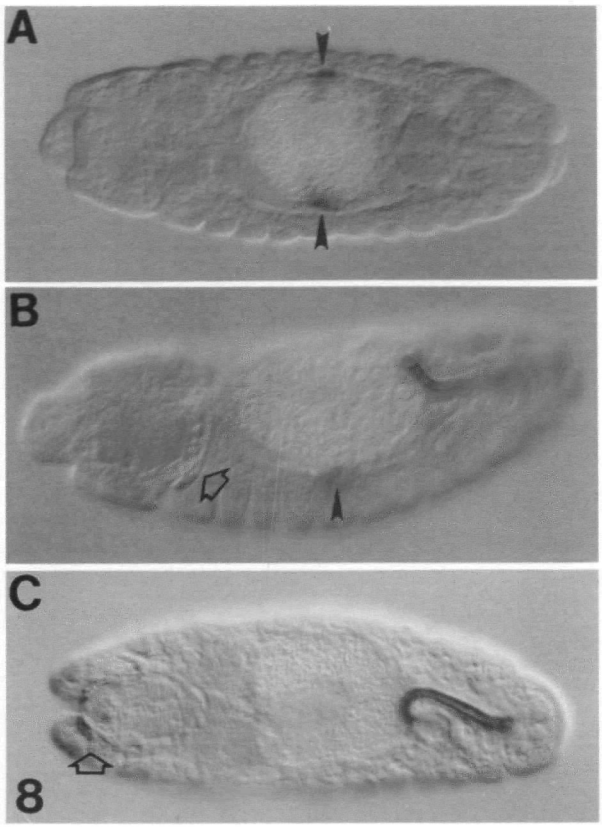


Fig. 6. Failure to maintain reporter gene expression in a lab^- background. Embryos oriented with the anterior end to the left. (A,C) Expression of p3.65*lab*66A in a lab^+ background. Accumulation pattern is identical to that shown in Fig. 5. The arrows point to the procephalic accumulation pattern. (B,D) The same construct in a lab^- background. These embryos were double-stained with antisera directed against β-gal and *lab* protein. Accumulation of β-gal is detected in the procephalon of a stage-9 embryo (arrow in B), but expression of the reporter gene construct is not maintained in these cells as can be seen in D (arrow). Non-specific staining of the hindgut is seen in embryos in which polyclonal anti-β-gal antibody was used. This hindgut staining pattern is also observed in non-transgenic embryos.



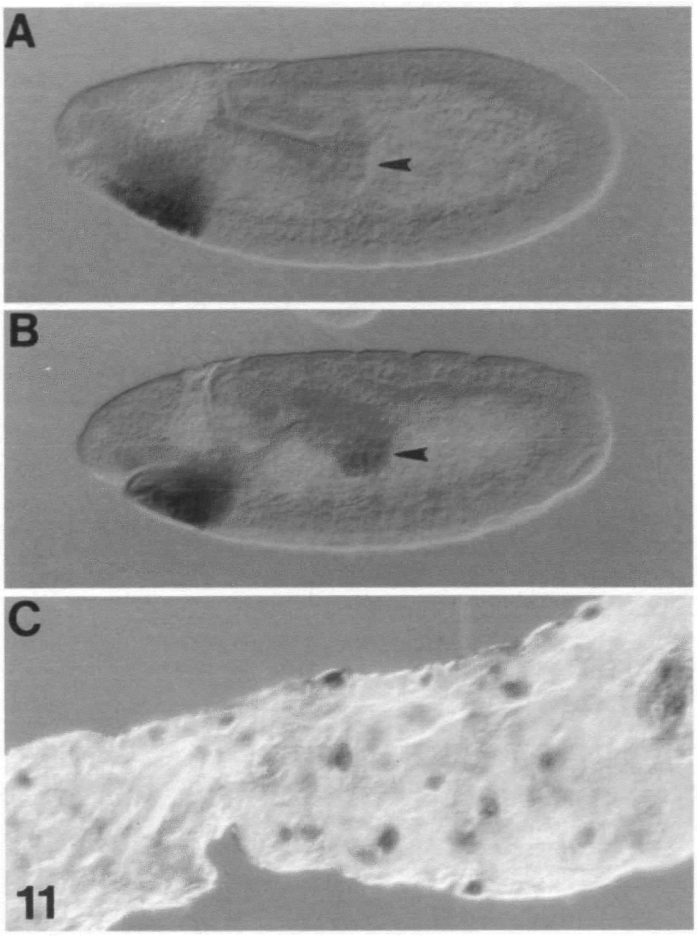
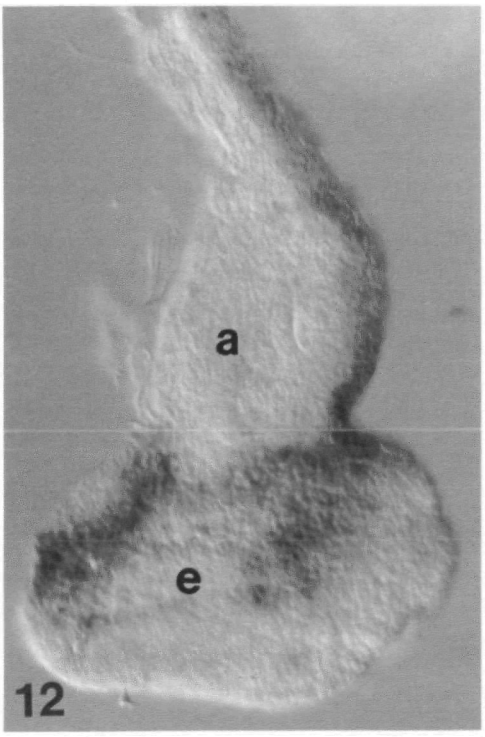


Fig. 8. Localization of *cis*-acting regulatory elements. (A) Accumulation of β-gal is observed in the amg (arrowhead) of embryos carrying the construct P[*ry*$^+$,1.2*lab*HZ] (1.2HZ). Surprisingly, no β-gal accumulation was detected in the procephalon. (B) Embryos carrying P[*ry*$^+$,7.6*lab*HZ] (7.6HZ) accumulate β-gal in the pmg (arrowhead) and in the dorsal ridge (arrow). (C) A550 bp *Eco*RI fragment, located on the 3′-side of the 7.6 kb *Eco*RI fragment in the first intron (Fig. 7), directs the expression of *lacZ* in cells of the procephalon (arrow) late in embryogenesis as seen in this stage-16 embryo. Non-specific staining of the hindgut is seen in B and C in which polyclonal anti-β-gal antibody was used. This hindgut staining pattern is also observed in non-transgenic embryos. The embryo in A was stained using a monoclonal anti-β-gal antibody. Embryos are oriented with the anterior end to the left.

Fig. 11. Accumulation of *Dfd* protein in the posterior midgut of animals heterozygous for *Df*(3R)*MAP11*. (A,B) Lateral view of stage-9 embryos (anterior end is on the left). Accumulation of *Dfd* protein is detected in the pmg of the *Df*(3R)*MAP11* embryo in B (arrowhead; compare to A). This pattern of accumulation persists throughout development as demonstrated by the presence of *Dfd* protein in the pmg of a third instar larva (C).

Fig. 12. Expression of the *lab–lacZ* leader fusion construct in the eye–antennal disc. Accumulation of β-gal in the eye–antennal disc is identical to the pattern observed for *lab* expression (compare to Fig. 10A; Diederich *et al.* 1991). Antennal disc (a); eye disc (e).



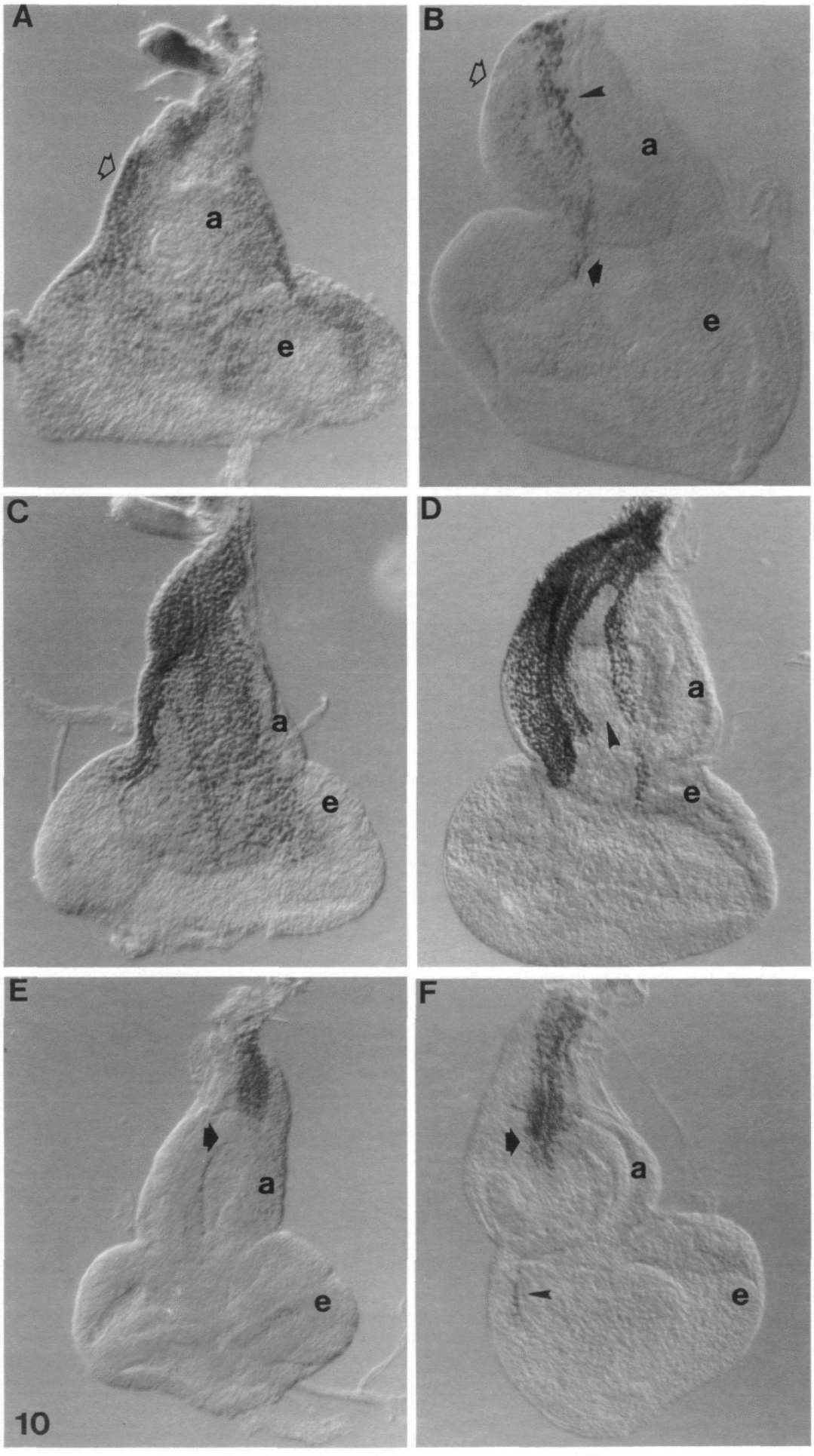


Fig. 10. Imaginal disc expression patterns of *lab*, *Dfd* and *Scr*. (A,C,E) Eye–antennal discs from third instar larvae of the genotype 2.5a/2.5a;lab^{14}/lab^{+}. The staining patterns observed here are identical to those observed in eye–antennal discs from non-transgenic larvae. (B,D,F) Eye–antennal discs from third instar larvae of the genotype 2.5a/2.5a;lab^{14}/lab^{14}. (A) Wild-type pattern of *lab* protein accumulation in the eye–antennal disc. The open arrow points to high levels of *lab* protein accumulation along the lateral region of the antennal disc (a). This preparation was overstained in order to detect the lower levels of accumulation in the eye disc (e). (B) Ectopic accumulation of transgenic *lab* protein (arrowhead) in the antennal disc (a). Low levels of *lab* protein (open arrow) are detected along the lateral edge of this disc (compare to A). Accumulation in the eye disc (e) is detected in a small cluster of cells near the border with a (shaded arrow). (C) Wild-type pattern of *Dfd* protein accumulation in the eye–antennal disc (e and a, respectively). This pattern is altered in D where expression in the eye disc (e) is virtually absent (compare to C) and a large gap in the pattern (arrowhead) is observed in the antennal disc (a). (F) The domain of *Scr* protein accumulation in the antennal disc is extended in the absence of a resident copy of the *lab* gene (shaded arrow) relative to that observed in the presence of a wild-type copy of the *lab* gene (E). Low levels of *Scr* protein accumulation are also observed in the eye disc (e) in the absence of a resident copy of the *lab* gene (arrowhead in F).

stages, with pupal development being the primary stage of death. Pharate adults dissected from the pupal case exhibit the same set of defects observed in eclosed adults. Additionally, transgenic adults in a lab^{-} background have lower fecundity than those carrying one wild-type copy of *lab*. It should be noted that this latter genotype, lab^{+}/lab^{-};P[w^{+},$lab^{minigene}$], is morphologically normal at all stages.

Comparison of lab *protein distribution in wild-type and transgenic embryos*

The observation that the minigenes appeared capable of providing the embryonic but not imaginal requirement for *lab* function prompted us to determine whether the minigenes exhibited normal temporal and spatial patterns of *lab* expression during embryogenesis (see Diederich *et al.* 1989 for a description of the wild-type pattern of expression). Embryos from the cross 2.5a/2.5a; lab^{14}/TM3,*Sb*×2.5a/2.5a; lab^{14}/TM6B,*Tb* (lab^{14} is a null allele) were stained using a *lab*-specific antibody (see Materials and methods). All embryos in this preparation exhibited *lab* protein accumulation; however, 23 % (28/123) of the embryos displayed only a subset of the normal *lab* pattern of accumulation. To demonstrate that this result was general, antibody-stained transgenic embryos from the crosses 2.5a/2.5a; lab^{14}/TM3,*Sb*×2.5a/2.5a; *Df*(3R)*Scr*/TM6B,*Tb* and 2.5a/2.5a; lab^{4}/TM3,*Sb*×2.5a/2.5a; lab^{4}/TM6B,*Tb* were examined, and a similar proportion (21 %, 63/296; and 23 %, 37/159, respectively) were seen to have this novel protein accumulation pattern. Comparable ratios and staining patterns were observed for the minigenes 2.4a and 2.4b. Moreover, these upstream truncated constructs were also capable of embryonic rescue demonstrating that the upstream 1.6 kb *Eco*RI fragment is dispensable.

Transgenic expression deviates from the wild-type pattern in four areas. The most intriguing difference is the absence of transgenically generated *lab* protein accumulation in the dorsal ridge (Fig. 2H) as this is a structure intimately associated with head involution, a process that fails in the absence of *lab* function. Normally, *lab* accumulates in these cells during germ-band-retraction and expression is maintained through head involution, after which these cells form the dorsal pouch (Diederich *et al.*1989). The movements of the dorsal ridges appears normal in transgenic null embryos demonstrating that *lab* function in these cells is expendable for head involution.

As can be seen in Fig. 2F through H, a second difference between transgenic and wild-type *lab* expression is the absence of *lab* protein accumulation in cells of the posterior midgut primordium (pmg). In wild-type embryos, *lab* protein begins to accumulate in both the pmg and the anterior midgut primordium (amg) at the early germ-band-extended stages of embryogenesis (Diederich *et al.* 1989; Fig. 2A,B). As the germ band shortens, these two cell groups meet and fuse, forming a continuous midgut primordium (Fig. 2C). Despite the absence of transgenic *lab* expression in the pmg, the formation of the midgut proceeds as in wild-type embryos suggesting that *lab* expression in the pmg is expendable for morphogenesis. Moreover, these animals survive to adulthood and therefore have sufficient gut function for feeding and assimilation. A requirement for expression of *lab* in the midgut in general has been disputed. Diederich *et al.* (1989) report no effects on midgut development in the absence of *lab* function; whereas, Immerglück *et al.* (1990) report that formation of the second midgut restriction is dependent on *lab* expression. Our results suggest, at least for the pmg, that *lab* function is expendable in these cells and that the embryo and larva are viable in the absence of pmg *lab* accumulation.

At the conclusion of embryogenesis (post head involution) transgenic expression of *lab* diminishes relative to wild-type levels in the procephalon as can be seen by comparing Fig. 2E and J. Despite the absence of transgenic *lab* expression in the dorsal ridge, and diminished levels of procephalic transgenic expression at the conclusion of embryogenesis, head involution and development appear to be normal in >95 % of the larvae examined. In addition, transgenic *lab* expression is absent in individual epidermal cells in the clypeolabrum, thorax and tail region (data not shown). These cells are believed to be progenitors of sensory organs unique to these regions. The role of *lab*, if any, in the formation of these cells is therefore not clear as they develop, and likely function, in the absence of *lab* expression (Merrill *et al.* 1989; this study).

The transgenic expression patterns of the remaining minigene constructs, P[w^{+},$lab^{2.3b}$] (2.3b) and P[w^{+}, $lab^{2.2a}$] (2.2a), differs from that of the other three minigene constructs. Additionally both fail to complement the loss of *lab* function. Initially, the patterns of transgenic expression of the five minigene constructs in the procephalon are identical, but this correspondence is short lived. By stage 10 of embryogenesis expression of 2.3b and 2.2a have diminished and eventually *lab* protein is undetectable (Fig. 3). Additionally, expression in the amg is absent in these lines (Fig. 3D). This result suggests that the 1.2 kb *Eco*RI fragment absent from constructs 2.3b and 2.2a contains *cis*-acting regulatory elements for both amg and procephalic expression of *lab*, and that the regulatory elements for the initiation of *lab* expression in the procephalon are separate from the sequences needed to maintain expression in these cells during the crucial stages of head involution.

Transgenic expression is regulated by sequences upstream of the lab *transcription unit and is* lab *dependent*

The regulatory elements required for embryonic viability have been shown to reside within the *lab* minigenes. To determine more precisely the location of these elements, we made *lab–lacZ* reporter gene fusion constructs containing sequences upstream of the *lab* transcription start site. The upstream sequences of the two reporter genes used in this study are essentially the same as those of the transgenes (Fig. 4) and these gene fusions exhibit embryonic protein accumulation pat-

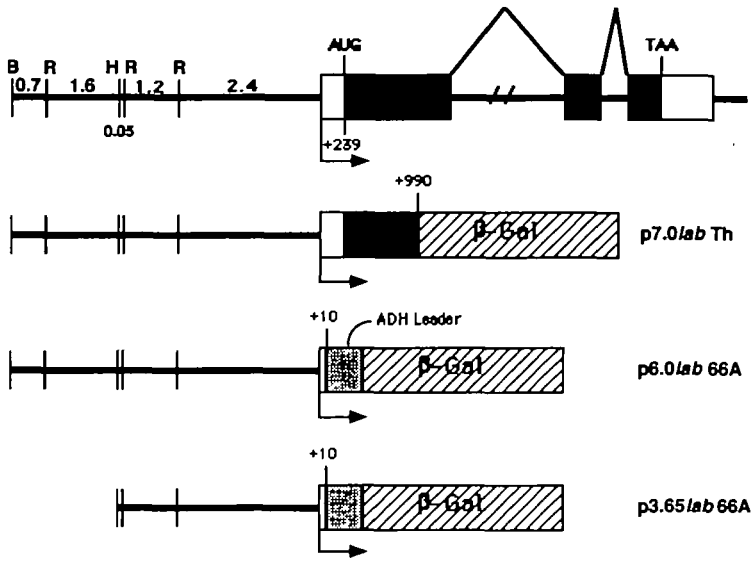


Fig. 4. The *lab–lacZ* reporter gene constructs. The *lab* transcription unit is shown above (5′ is on the left). Shaded boxes represent protein coding regions and open boxes indicate untranslated sequences. The leader is 239 bp in length. The thin lines represent non-transcribed sequences. The distances between restriction sites are given in kilobases (kb) as is the distance from transcription start to the first upstream *Eco*RI site (R). Restriction enzymes: B, *Bam*HI; H, *Hin*dIII. Below the transcription unit are the *lab–lacZ* reporter genes. Construct p7.0*lab*Th, a *lab–lacZ* protein fusion construct, includes the entire untranslated leader (open box) and sequences in the ORF to +995. This portion of the ORF encodes the first 252 amino acids of *lab* (shaded box) which are fused in-frame with *lacZ* coding sequences (cross-hatched box). This construct has essentialy the same upstream sequences as 2.5a (Fig. 1) except for the presence of an additional 750 bp at the 5′-end. Reporter genes p6.0*lab*66A and p3.65*lab*66A are *lab–lacZ* leader fusion constructs. These two constructs include the first 10 bp of the *lab* leader (open box) fused to the *alcohol dehydrogenase* (ADH) leader (stippled box). Sequences upstream of the transcription start site in p6.0*lab*66A are identical to those in p7.0*lab*Th. Construct p3.65*lab*66A was derived from p6.0*lab*66A by removing the 5′ 2.3 kb *Bam*HI–*Hin*dIII fragment (Materials and methods). The right-angle arrow below the constructs indicates the initiation point and direction of transcription.

terns identical to the minigene constructs (Fig. 5). Thus, regulatory elements required for embryonic viability are located upstream of the *lab* transcription start site. The aforementioned results with the minigene constructs in lab^- backgrounds indicate that, once initiated, transgenic expression is maintained during the critical stages of embryonic head development. To test whether the post-establishment phase of *lab* expression involves a feedback loop, as determined previously for the homeotic genes *Ultrabithorax* (*Ubx*) (Bienz and Tremml, 1988) and *Dfd* (Kuziora and McGinnis, 1988), we crossed the reporter gene fusion construct P[w^+,3.65*lab*66A] (3.65*lab*66A) into a lab^- background. If *lab* gene expression is maintained through a positive feedback loop involving the required upstream sequences, we would expect the transgenic accumulation of β-galactosidase to be dependent on the presence of *lab* protein. As can be seen in Fig. 6, this is what is observed. At the early germ-band-extension stage, β-galactosidase accumulation in the procephalon is identical in lab^+ and lab^- embryos. As development proceeds, double-labelling experiments (Fig. 6) demonstrate that the level of β-galactosidase diminishes in those embryos that are lab^-. The low levels of β-galactosidase accumulation observed following retraction of the germ-band are probably due to the perdurance of this protein. This result indicates that the maintenance of *lab* expression, as directed by the upstream sequences, is dependent on the presence of *lab* protein and that the transgenic *lab* protein is capable of fulfilling this function. It should be noted that the 2.3b and 2.2a minigene constructs behave in a similar fashion to the above reporter gene constructs in a lab^- or lab^+ background, i.e. *lab* expression is initiated but not maintained. Thus, we can conclude that necessary *cis*-acting sequences for the autogenous maintenance of *lab* expression must reside in the 1.2 kb *Eco*RI fragment deleted in the 2.3b and 2.2a minigenes (Fig. 1).

Localization of cis*-acting regulatory elements absent from the minigene constructs*

The absence of transgenic *lab* expression in the dorsal ridge, pmg, and the embryonic PNS, and the diminished procephalic accumulation late in embryogenesis, prompted us to investigate the location of the *cis*-acting regulatory elements responsible for these aspects of *lab* expression. There are two possible locations for these missing regulatory elements: the large intron, which was excluded from the minigene constructs, or regions upstream or downstream of the genomic fragments used in the construction of the transgenes. We identified the location of three of the four missing elements by subcloning restriction fragments from the large intron upstream of the *hsp70* promoter–*lacZ* reporter gene in the P-element transformation vector HZ50 (Hiromi and Gehring, 1987). Additionally, a similar construct was generated using the upstream 1.2 kb *Eco*RI fragment, which based on the above results, contains sequences necessary for amg expression and positive autogenous regulation. A diagram of these regulatory constructs is shown in Fig. 7.

The regulatory element for the pmg is located in a 7.6 kb *Eco*RI restriction fragment as seen by the pattern of β-galactosidase accumulation in Fig. 8B. The temporal pattern of β-galactosidase accumulation in this tissue is identical to that for *lab* protein. Interestingly, the upstream 1.2 kb *Eco*RI fragment directs the expression of β-galactosidase only in cells of the amg and not in the procephalon (Fig. 8A) as might be expected based on the results with the minigene

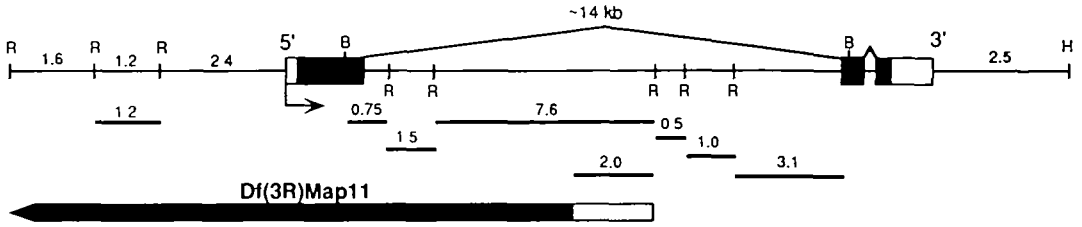


Fig. 7. DNA restriction fragments tested for enhancer activity. Schematic of the *lab* transcription unit is shown above with thin lines representing non-transcribed DNA sequences. Horizontal lines below the transcription unit indicate the size (kb) and position of restriction fragments which were subcloned into the enhancer test vector HZ50 (Materials and methods). Restriction enzymes: B, *Bam*HI; R, *Eco*RI. At the bottom is shown the proximal breakpoint of the mutant chromosome *Df*(3R)*MAP11* (shaded box). The stippled box indicates the region of uncertainty for its proximal endpoint (~2 kb).

constructs. These results not only demonstrate that the regulatory elements required for *lab* expression in the anterior and posterior midgut are separate entities, but that sequences needed for the autogenous maintenance of *lab* expression in the procephalon do not lie solely within the 1.2 kb *Eco*RI fragment, as could be inferred from the results with the minigene constructs.

Dorsal ridge expression is also regulated by sequences in the 7.6 kb *Eco*RI fragment (Fig. 8B). The temporal pattern of β-galactosidase expression in the dorsal ridge is identical to wild-type *lab* accumulation. A 550 bp *Eco*RI fragment located on the 3'-side of the 7.6 kb fragment contains regulatory sequences necessary for expression during the final stages of head involution. Accumulation of β-galactosidase in transgenic lines is observed in the procephalic cells bordering the fused mandibular/maxillary lobes, and in the frontal sac (Fig. 8C). The *cis*-acting regulatory elements in this fragment create the late embryonic procephalic pattern of *lab* expression in the absence of the upstream sequences. This suggests that there is a reiteration of the regulatory elements responsible for this aspect of the *lab* expression pattern. In addition to these positive results, two of the three tested intronic fragments (see Fig. 7) lack any detectable enhancer activity while the third (P[ry^+, 1.0*lab*HZ]) exhibited a non-*lab* pattern in the head of the embryo (data not shown). The regulatory elements required for embryonic PNS expression of *lab* have not yet been identified. One intronic fragment (p3.1*lab*HZ) remains to be tested, thus the PNS elements may reside there (Fig. 7). However, it is possible that the sequences required for this aspect of *lab* expression are located outside of the first intron, most likely in the genomic region downstream of the transcription unit. We are currently testing both of these possibilities.

Failure of the minigene constructs to complement the adult homeotic phenotype

As shown above, the three *lab* minigenes containing at least 3.6 kb of contiguous upstream sequence rescue the embryonic defects associated with the loss of *lab* function, but give rise to adults with defective heads. Previous work in this laboratory by Merrill *et al.* (1989) demonstrated a role for *lab* in the development of the adult head. In that study mitotic recombination was used to demonstrate the requirement for *lab* in the proper development of the anteroventral and posterior regions of the head capsule. The transgenic adults recovered in this analysis have cephalic phenotypes similar to those described by Merrill *et al.* (1989), but the defects encompass a greater area of the head. This extended region includes the ventral aspects of the postgena, which is severely disrupted or absent (see Fig. 9D; for a description of adult head structures see Bryant, 1978). In addition, bristles of the occiput are absent in some adults (data not shown), a region that was never seen to be affected in the mitotic recombination studies. Phenotypic similarities between the adults in these two studies are seen in the morphology and random distribution of the large bristles on the posterior regions of the head. Based on their shape and size, these bristles appear to be ectopically placed thoracic structures (Fig. 9D). Consistent with the findings of Merrill *et al.* (1989), we also see holes in the postgenal region that resemble thoracic spiracles and large, amorphic cuticular protrusions. The severity of the phenotypes was the same for animals bearing one or two copies of the transgene (data not shown). Moreover, although all of the adults exhibited a mutant phenotype, this phenotype was variable, not only among flies, but also between the left and right sides of the head.

Structures in the anteroventral region of the head capsule were also affected in a manner similar to that seen by Merrill *et al.* (1989). In all cases, tissue was deleted from the ventral regions of the eyes with little, if any, effect on the number of ommatidia present (Fig. 9C). Bristles from this region were often absent or duplicated as were the maxillary palps (Fig. 9C). This latter phenotype is most likely associated with and caused by cell death in this region of the eye–antennal disc (Haynie and Bryant, 1986; Merrill *et al.* 1987). In no case were the mouthparts or the occelli and surrounding regions affected. The dysmorphic characters observed in these transgenic animals are reminiscent of the Dfd^- clones in the adult head capsule (Merrill *et al.* 1987).

Imaginal disc expression

The adult phenotypes suggest that the transgenes are failing to be expressed in the eye–antennal disc where

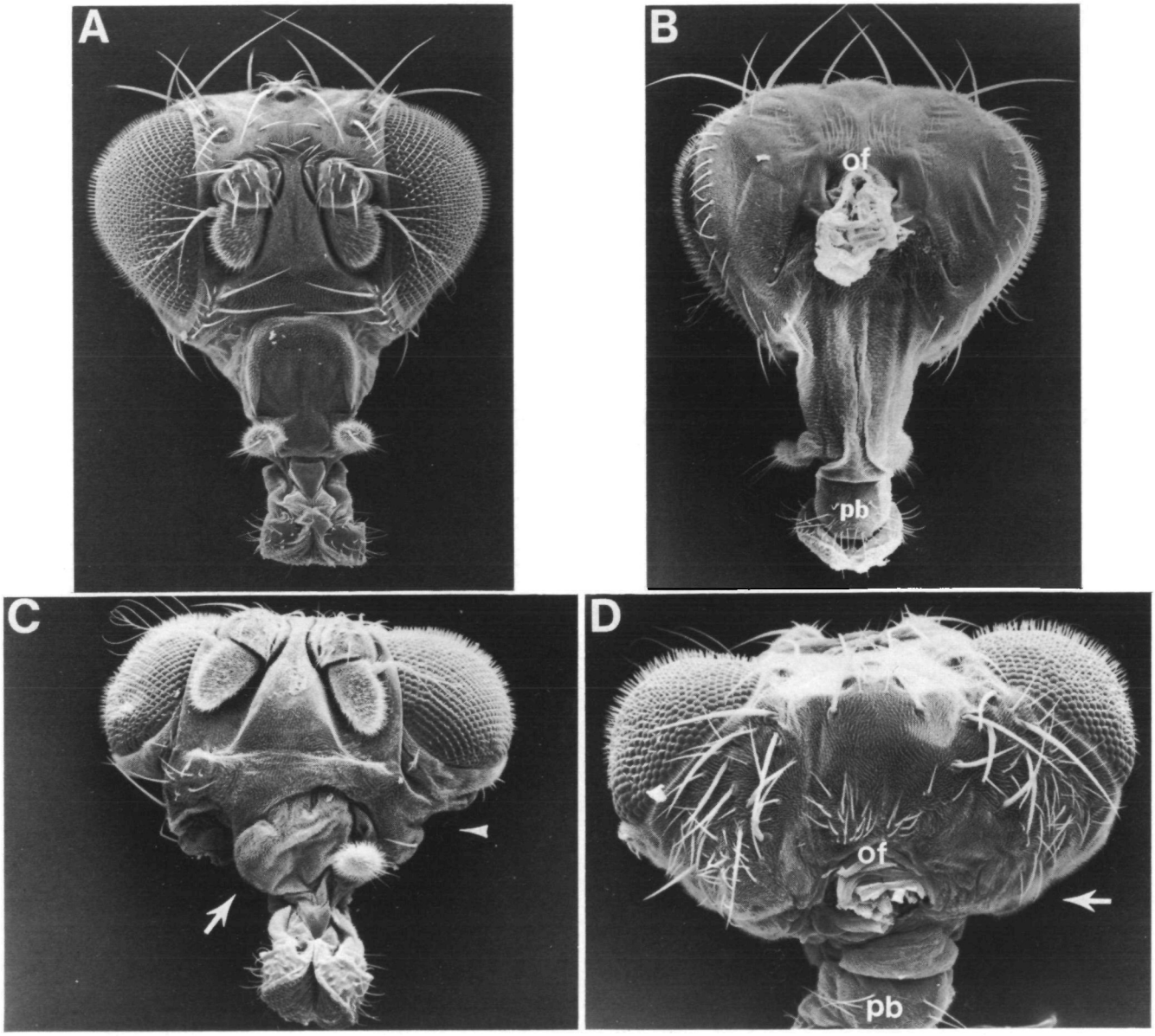


Fig. 9. Scanning electron micrographs (SEMs) of adult heads. (A,B) Anterior and posterior view of wild-type adult heads (of, occipital foramen; pb, proboscis). (C,D) Anterior and posterior view of 2.5a/2.5a;lab^{14}/lab^{14} adult heads. Notice the absence of a maxillary palp (arrow) in C. The arrowhead denotes the ventral region of the head capsule where tissue is deleted. (D) The absence of ventral tissue (arrow) reduces the size of the head capsule resulting in the apparent contraction of the proboscis (pb) relative to the occipital foramen (of). This deletion of tissue also causes a dorsally directed rotation of the eyes. The large, thick bristles are interpreted as ectopically placed thoracic structures.

lab product is normally accumulated. In order to investigate this possibility, third instar larvae (Tb^+) from the cross 2.5a/2.5a;lab^{14}/TM6B,*Tb*×2.5a/2.5a; lab^{14}/TM6B,*Tb* (or 2.4a) were stained with antisera directed against *lab* (Materials and methods). Protein accumulation was occasionally observed in small patches along the lateral regions of the peripodial membrane of the antennal disc where *lab* is normally expressed, but this staining was relatively weak and in many cases absent. Expression in the peripodial membrane overlying the eye disc was absent except for a small region at the lateral juncture of the eye–antennal disc. Normally, *lab* is expressed in a broad domain in this region. Antennal discs from these same animals exhibited ectopic localization of *lab* protein. The pattern of ectopic expression varied among animals and also between discs from the same larva. A typical eye–antennal disc is shown in Fig. 10B, in which the domain of expression is shifted toward the central regions of the antennal disc. The generally observed pattern of accumulation emanates from the stalk of the antennal portion of the disc and extends proximally towards the eye anlagen. The variability of this expression is consistent with the observation that no two animals displayed identical head phenotypes. Accumulation of *lab* protein in the CNS and the amg at

this stage in development is normal while pmg expression is apparently absent.

As noted above, the head defects of transgenic adult flies are more severe than those reported earlier by Merrill *et al.* (1989) and in some aspects resemble defects associated with *Dfd* deficiency (Merrill *et al.* 1987). This can be most readily rationalized if the ectopic *lab* accumulation is affecting the expression of other genes in the peripodial membrane of the eye–antennal disc. This possibility was addressed by examining the expression patterns of *Dfd* and *Scr*, ANT-C members which are also normally expressed in the peripodial membrane of this disc (Chadwick and McGinnis, 1987; Martinez-Arias *et al.* 1987; Glicksman and Brower,1988; Chadwick *et al.* 1990). As can be seen in Fig. 10D, the pattern of *Dfd* expression is dramatically altered in transgenic animals. There is no detectable accumulation of *Dfd* protein in the peripodial membrane overlying the eye disc. Furthermore, large regions of the antennal disc peripodial membrane are devoid of *Dfd* protein as can be seen by comparing Figs 10C,D. Double staining of discs with antisera directed against *lab* and *Dfd* reveals that the absence of normal *Dfd* accumulation in the antennal disc is coincident with the ectopic expression of *lab* in these cells (data not shown). A surprising result was the apparent absence of both gene products in the peripodial membrane of the eye disc where they normally accumulate in a nonoverlapping pattern (Diederich *et al.* 1991). While it is possible that low levels of transgenically produced *lab* protein are present in the peripodial cells of the eye disc, the amount must be sufficiently below wild type to be undetectable by the antibodies and staining protocols used here. We also cannot rule out the possibility that *lab* protein is accumulated in these disc cells at some earlier (i.e. prior to the late third larval instar) stage in imaginal development.

When discs from transgenic animals were stained with antisera directed against *Scr* protein, we observed an expansion of the *Scr* domain of expression in the peripodial membrane of the antennal disc relative to the wild-type pattern of accumulation (Fig. 10E,F). In discs from wild-type animals, *Scr* protein accumulates in a small area of the disc just proximal to the cephalopharyngeal skeleton. In transgenic lab^- discs, we observe an extension of this pattern into what are typically *Dfd*-expressing cells. The apparent effects of ectopic *lab* expression on these two homeotic genes corresponds to the increased severity in head capsule defects seen in this study relative to those obtained using somatic recombination. This suggests that the observed adult phenotype is actually associated with a partial loss-of-function of *lab* and is exacerbated by a similar loss of *Dfd* with a concomitant gain-of-function of *Scr*. Member loci of the ANT-C not expressed in the eye–antennal disc (*Antp* and *pb*) are not affected by ectopic accumulation of *lab* and thus do not contribute to the adult phenotype (data not shown).

The above result indicates that at least one of the ANT-C members involved in head development, i.e. *lab*, has the potential ability either directly or indirectly to cross-regulate other genes in the complex involved in this process. The observed failure of cells in the embryo (Mahaffey *et al.* 1989) and the eye–antennal disc (Diederich *et al.* 1991; this study) to concomitantly express *lab* and *Dfd* led us to investigate the universality (with regard to tissue) of this observation.

In their characterization of the *lab* locus, Diederich *et al.* (1989) describe a deficiency, *Df*(3R)*MAP11*, that fails to complement *lab*. The distal breakpoint of this deletion lies ~28 kb upstream of the *Dfd* locus, while the proximal breakpoint of this deletion is located in the 7.6 kb *Eco*RI fragment of the first intron of *lab* (Fig. 7). The present most salient effect of this lesion derives from the positioning of the *lab* pmg regulatory element upstream of the *Dfd* transcription unit. The effect of this fortuitous juxtaposition is that embryos and larvae heterozygous for this deficiency accumulate *Dfd* protein in endodermal cells of the pmg (Fig. 11). Double staining reveals that these cells also accumulate *lab* protein (data not shown), indicating that co-expression of the two genes is permitted in this tissue, while this is apparently not the case in imaginal tissue from the same developmental stage. It should also be noted that ectopic expression of *Dfd* in the pmg has no apparent affect on viability or the gross morphology of the gut.

During the course of the above analysis, we were struck by a seemingly paradoxical observation: in the presence of a wild-type copy of *lab*, ectopic expression of the transgenes was not observed and, concomitantly, the adult heads were normal in appearance (data not shown). Moreover, in this genotypic background, the pattern of expression of *Dfd* and *Scr* is normal (Fig. 10C,E), again supporting the view that in a lab^- background the ectopic transgenic expression of *lab* is influencing the expression of these two genes. This result was observed for all three rescuing minigenes and in a variety of *lab* mutant backgrounds. Because the transgenes respond to the presence of a resident copy of *lab*, this normal pattern must result from the influence of the wild-type *lab* gene on the minigenes. This suggests that not only is *lab* expression under the control of positive autoregulation in the embryo, but also, possibly, negative autoregulation in imaginal tissue, and that only the *lab* protein produced by the resident gene is capable of negatively regulating imaginal disc expression.

Reporter gene expression in imaginal tissue

Although expression of the minigene constructs in the peripodial membrane of the eye–antennal disc from lab^- animals does not mirror the wild-type pattern of accumulation, it does indicate that tissue-specific *cis*-acting regulatory elements are included in the transgenes. In fact, it is possible that transgenic expression in a wild-type background is normal, but we cannot distinguish this from resident gene product accumulation. To investigate the possibility that the eye–antennal disc-specific *cis*-acting regulatory elements are located upstream of the transcription start site, we used the reporter gene fusion constructs diagrammed in

Fig. 4. All three of these gene fusion constructs have identical patterns of β-galactosidase accumulation in the embryo and this pattern is the same as that observed for the three minigene constructs that complement the *lab*$^-$ embryonic phenotype (Fig. 1). Eye–antennal discs from transgenic larvae carrying p6.0*lab*66A and p3.65*lab*66A have a β-galactosidase accumulation pattern identical to the one observed for *lab* expression (Fig. 12; Diederich *et al.* 1991) indicating that the *cis*-acting regulatory elements necessary for eye–antennal expression are located upstream of the transcription start site. Surprisingly, eye–antennal discs from larvae carrying p7.0*lab*Th, which is a protein fusion rather than a leader fusion, fail to accumulate detectable levels of β-galactosidase (data not shown). A possible explanation for this difference is provided in the Discussion.

Discussion

Embryonic requirements of labial

Genomic DNA rescue analyses of mutations in the homeotic loci of *D. melanogaster* have been hindered by the relatively large size of these genes. To facilitate a rescue analysis of the *lab* locus, a minigene was constructed that eliminated the large first intron (Diederich *et al.* 1989). The five versions of the *lab* minigene used in this study differed in the extent (5.2 kb, 3.6 kb and 2.4 kb), or the orientation, of normally contiguous sequences 5′ of the transcription start site. Germ line transformation experiments using these constructs resulted in the rescue of the embryonic lethality associated with a disruption of the resident *lab* locus when at least 3.6 kb of contiguous upstream sequence was contained in the minigene. This result was surprising in that the transgenes do not exhibit the spatial pattern of *lab* protein accumulation observed in wild-type embryos. Most significant is the absence of protein accumulation in the dorsal ridges, and later the dorsal fold, structures associated with the process of head involution (Turner and Mahowald, 1979). As described in Merrill *et al.* (1989), head involution fails in the absence of *lab* function due to the inability of cells of the procephalon to properly incorporate into the dorsal pouch, which, in turn, blocks the passive migration of the dorsal fold over these structures. Transgenic expression of *lab* is sufficient in overcoming this block in head development, demonstrating that the only requirement for *lab* function during head involution is in the cells of the lateral aspects of the procephalon. Once head involution is complete, the expression of *lab* in the procephalon is expendable since transgenic animals survive despite the absence of procephalic *lab* expression late in embryogenesis. The *cis*-acting regulatory elements necessary for procephalic expression of *lab* late in embryogenesis and in the dorsal ridges are located in the first intron which was excluded from the minigene constructs. A secondary consequence of the disruption of these morphogenetic movements in *lab*$^-$ embryos is the absence of salivary glands (Merrill *et al.* 1989). These structures are present in transgenic animals (data not shown), thus showing that all aspects of embryonic head development are complemented by the transgenes.

The absence of transgenic *lab* expression apparently has no gross effect on the development of a group of larval sense organs, the apparent progenitors of which express *lab* in wild-type embryos. This was also observed in *lab*$^-$ animals as described earlier by Diederich *et al.* (1989) and Merrill *et al.* (1989). While it is not known whether these organs are functional in the transgenic animals, it would appear that, if they are not, viability is not severely compromised. The location of the *cis*-acting regulatory elements necessary for the expression of *lab* in these cells has, to this point, eluded us. In addition, transgenic expression is absent from the endodermally derived cells of the posterior midgut primordium (pmg), but accumulates normally in cells of the anterior midgut primordium (amg). Nevertheless the midgut fuses and differentiates and since larvae survive to adulthood, it would appear that *lab* function may not be critical to gut development. However, it could be that the *lab*-expressing cells of the anterior midgut supply enough of the *lab* function to ensure that midgut development proceeds in a normal fashion. Although we question whether *lab* protein is crucial to the development of the midgut, it is interesting that there are separate *cis*-acting regulatory elements for the amg as compared to the pmg (located upstream and in the 7.6 kb *Eco*RI fragment in the first intron, respectively) (Fig. 13). Interestingly, we also have preliminary evidence that this partitioning of the amg and pmg *cis*-acting regulatory elements is associated with alternate *trans*-activators which recognize and regulate the individual *cis*-acting elements (unpublished observations). These observations suggest that *lab* expression does play a role, or at least at one time was involved in the development of the gut, since the adventitious evolution of independent elements recognized by separate *trans*-acting factors seems unlikely.

Two of the minigene constructs used in this study fail to complement the embryonic lethal phenotype associated with loss of *lab* function. This is due to the absence of sequences needed for the maintenance of *lab* expression in the procephalon once initiated and possibly the absence of *lab* accumulation in the amg. An interesting observation is that the *cis*-acting regulatory elements necessary for the initiation of *lab* expression in the procephalon are separate from those required to maintain expression during the critical stages of head involution. Moreover, the maintenance of *lab* expression in these cells is mediated through a positive feedback loop. This phenomenon of positive autoregulation has been described previously for the homeotic genes *Ubx* and *Dfd* (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990). The sequences required for autogenous maintenance are located upstream of the transcription start site; however, the precise location of these *cis*-acting regulatory elements has not yet been determined. It is possible that multiple *cis*-acting regulatory elements are

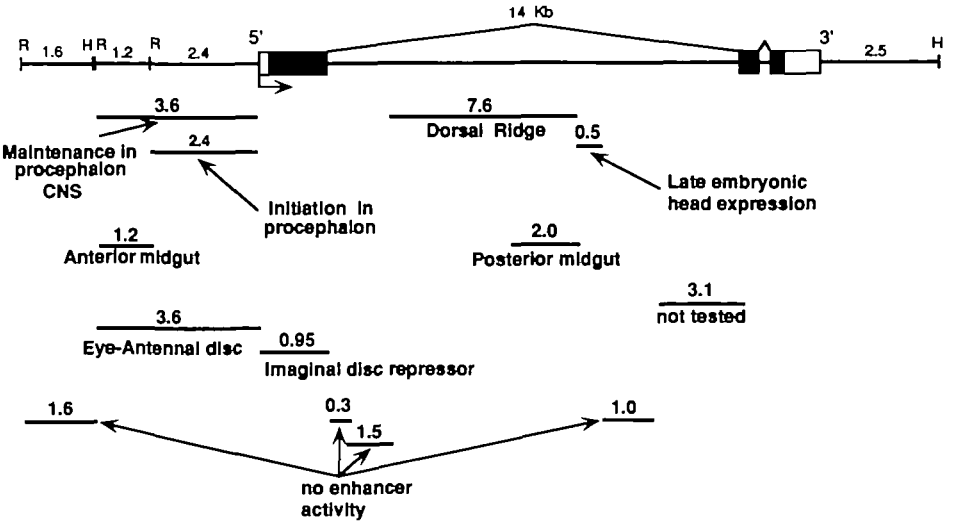


Fig. 13. Summary of the *cis*-acting regulatory elements for the *lab* locus. The *lab* transcription unit is shown at the top. Shaded boxes represent coding regions, open boxes indicate untranslated sequences, and the thin horizontal lines represent non-transcribed regions. The right-angle arrow below the transcription unit indicates the position of the transcription start site and the direction of transcription. Horizontal lines below the transcription unit indicate the position of *cis*-acting regulatory elements within the *lab* locus as determined by minigene and reporter gene fusion constructs. The posterior midgut regulatory element is located within the 3'-end of the 7.6 kb *Eco*RI fragment based on the results with 7.6*lab*HZ and the mutant chromosome *Df*(3R)*MAP11*. Restriction enzymes: R, *Eco*RI; H, *Hin*dIII.

required for the autogenous maintenance of *lab* expression in the procephalon, and that these elements are disrupted in 2.3b, 2.2a (Fig. 1) and 1.2*lab*HZ (Fig. 3) such that each construct contains only a subset of these regulatory elements. This could possibly explain why minigene construct 2.4a and 2.4b, which include the entire upstream region, are expressed during the critical stages of head involution, while constructs 2.3b, 2.2a and 1.2*lab*HZ fail to express in the procephalon. The presence of (and possible requirement for) multiple protein binding sites has been demonstrated for *Dfd* and the pair-rule gene *even-skipped* (Regulski *et al.* 1991; Jiang *et al.* 1991). We are presently defining these sequences and investigating the possibility that *lab* protein directly interacts with the *cis*-acting regulatory elements necessary for maintenance of expression in the procephalon.

Adult requirements of labial

The *lab* minigene constructs that we have assembled fail to complement the adult phenotype associated with amorphic and hypomorphic *lab* alleles, as surviving adults display cuticular disruptions of the anterior, ventral, and posterior regions of the head capsule. These results are consistent with the work of Merrill *et al.* (1989) in which a homeotic role for *lab* in the development of the head was demonstrated. The defects observed in this study are found over the entire posterior head extending from the ventral rostrum to, but not including, the vertical and postvertical bristles. It is interesting that the phenotypes seen in this study encompass the entire posterior region of the adult head whereas the defects observed by Merrill *et al.* (1989) were restricted to the central region. Reconciliation of these differences is achieved by the observation that the minigene constructs are ectopically expressed in the peripodial membrane of the antennal disc and that this ectopic accumulation of *lab* protein alters the expression patterns of the homeotic genes *Dfd* and *Scr*. Although this effect may be indirect it is not entirely surprising that ectopic *lab* accumulation alters *Dfd* expression due to the fact that these two genes are apparently not normally co-expressed in the cells of this disc (Diederich *et al.* 1991). Thus suggesting that these two genes may be involved at some level in regulating each others domains of expression in the imaginal anlagen. What is surprising is that this ectopic expression of *lab* is associated with an extension of the domain of *Scr* accumulation in the peripodial membrane since there apparently is no co-expression of these two genes either (data not shown). However, it may be that the *Scr* domain of expression is extended in the absence of *Dfd* expression and is not due to activation by *lab*. Moreover, these results, in conjunction with the observation that *lab* and *Dfd* are co-expressed in the midgut of animals bearing a specific chromosomal rearrangement (*Df*(3R)*MAP11*), suggests that cross-regulatory interactions between these two genes, and possibly other homeotic genes, is highly tissue-specific.

These results not only demonstrate that members of the ANT-C can cross-regulate one another in specific tissues, but that there is an aspect of this ectopic expression which is inherently correct. Diederich *et al.* (1991) have demonstrated that *lab* expression is restricted to the cells of the peripodial membrane of the eye–antennal disc, with no detectable accumulation in the disc epithelium. We observe that ectopic transgenic expression is still confined to the peripodial membrane of this disc but the expression within that area of the disc is not normal. Thus one portion of regulation – 'peripodial-eye-antennal' – remains intact but instruction as to where in that domain is missing. This demonstrates that the transgenes contain regulatory elements for eye–antennal peripodial membrane expression and that ectopic expression is not simply due to

anomalous expression in all the cells of this and other discs. Consistent with this observation is that the adult head capsule defects are restricted to those areas affected by *lab*, *Dfd*, and *Scr* mutations. The expression domains of *Dfd* and *Scr*, like *lab*, are restricted to the peripodial membrane. Furthermore, the two *lab–lacZ* leader fusions are expressed in a wild-type pattern in the peripodial membrane of the eye–antennal disc. This suggests that the minigenes and the *lab–lacZ* leader fusions contain the *cis*-acting regulatory elements for proper imaginal disc expression. That the minigene constructs are aberrantly expressed in a *lab*$^-$ background and the fact that *lab–lacZ* protein fusion fails to accumulate β-galactosidase in a wild-type background indicates that these constructs contain elements necessary for pattern modification that are absent from the *lab–lacZ* leader fusion constructs (see below).

One of the more interesting results from this study is the absence of the ectopic transgenic expression in a *lab*$^+$ background. A plausible explanation is that the resident *lab* gene product, or some gene product regulated by the resident *lab* gene, is repressing the expression of the minigene in these cells of the antennal disc. This interpretation raises two interesting questions: why doesn't the transgene repress itself and how does the resident gene repress the expression of a second gene in a population of cells where it is itself not expressed? An answer to the first question may reside in the fragments used to construct the minigenes. The cDNA fragment used to bridge the first intron represents one of the two potential products that are generated by alternative splicing of the *lab* mRNA (Mlodzik *et al.* 1988). As a result, an alternate *lab* isoform that is six amino acids longer than that encoded by the transgenes would be absent from those cells that normally express this form of the gene product. It is possible that the alternatively spliced longer *lab* gene product acts as the repressor of the transgene and that in a *lab*$^-$ background repression is absent because the minigenes lack the ability to generate this isoform. We are currently testing this possibility.

The second question posed above is more perplexing. The most reasonable explanation is that the resident *lab* gene is expressed in these cells, or more likely the progenitors of these cells, at some point in development and that this initial pattern of expression is refined by the negative regulation of *lab* protein or the product of some other gene downstream of *lab*. This interpretation is based on the premise that during the evolutionary history of the adult head there was a time when *lab* was expressed in a much broader domain than what is currently observed in *Drosophila* (a representative of the higher diptera) and that the changes in the morphology of the head through phylogeny were accompanied by truncations in the pattern of gene expression. In the case of *lab*, this restriction of domain could have been imposed by altering or deleting the *cis*-acting regulatory elements or by the evolution of the hypothesized negative autoregulatory network. The latter possibility would be more economical, for deletions of regulatory elements could also affect the ability to express *lab* in those cells in which it is needed. By utilizing an alternative form of the protein and novel *cis*-acting sites, the original *cis*-acting elements are left intact. This model of course would require that the different cell populations have distinct RNA splicing pathways for *lab*.

As noted above our results suggest not only alternative biological roles for the different isoforms of *lab* protein, but that these isoforms may interact with independent *cis*-acting regulatory elements. This model is based on our investigations of the imaginal-specific expression of *lab* and is most clearly seen in the fact that the two classes of *lab–lacZ* reporter gene fusions examined, the leader fusion and the protein fusion constructs, yielded fundamentally different results. Both exhibited the expected embryonic expression pattern, i.e. they behave like the minigene constructs. However, both lines of the protein fusion construct, which contains the entire untranslated leader of *lab* plus coding sequences (Fig. 4), failed to accumulate detectable amounts of β-galactosidase in the eye–antennal disc while the leader-only fusions are expressed in a normal pattern. This suggests that the leader fusion constructs, p6.0*lab*66A and p3.65*lab*66A are missing a regulatory element needed to negatively regulate the expression of the constructs in this imaginal disc. Not that a positive regulatory element is absent from the protein fusion construct since this latter transgene has the same upstream extent as p6.0*lab*66A. Based on this result and interpretation it appears that a *cis*-acting negative regulatory element lies between +10 and +995 on the molecular map of *lab*, where +1 represents transcription start, and that the longer *lab* isoform possibly interacts with this element (Fig. 13). We are currently investigating this possibility by introducing the various fusion constructs into the appropriate genetic backgrounds.

This model of positive/negative autoregulation needs only minor modification in order to accomodate the minigene expression data. The minigenes, like the protein fusion construct, are properly negatively regulated in the presence of a resident copy of *lab*. This is not surprising since the minigene constructs contain sequences defined as negative *cis*-acting regulatory elements by the protein fusion gene. In fact, it is possible that there is a complete absence of minigene expression in the eye–antennal disc in the presence of the resident *lab* gene, but we cannot test this since we cannot distinguish transgenic from wild-type gene product. Although the minigenes are no longer negatively regulated in the absence of resident *lab* gene product, they are not expressed in the normal domain of *lab* (Diederich *et al.* 1991). This is surprising since the results with the *lab–lacZ* leader fusion constructs demonstrate that the *cis*-acting regulatory elements required for proper eye–antennal disc expression are present in the minigenes upstream of the transcription start site (Fig. 13). This apparent paradox can be reconciled in the following manner. The *lab–lacZ* leader fusion genes, which lack the negative *cis*-acting regulatory element, exhibit β-galactosidase accumu-

lation in the eye–antennal disc due to positive regulation by the gene product from the resident copy of *lab* (directly or indirectly). The *lab–lacZ* protein fusion gene and the minigenes are subject to negative autoregulation by the gene product from the resident copy of *lab* (directly or indirectly) in this imaginal disc, but not in the embryo in the case of the protein fusion. This suggests that negative autoregulation is overriding positive autoregulation in the eye–antennal disc. It also suggests that the minigenes and the protein fusion gene are lacking some element(s) needed to override negative autoregulation in this disc, and that this element(s) is dispensable in the *lab–lacZ* leader fusion constructs which lack the negative regulatory element. The reason that we detect ectopic transgenic accumulation of *lab* protein in the absence of a resident copy of this gene, in addition to some normal expression, may be that tissue-specific expression is activated, but not pattern-specific expression, and once activated normal and ectopic expression are maintained *via* positive autoregulation. As mentioned earlier, we believe this tissue-specific activation occurs early in development, probably during embryogenesis.

It is possible that *lab* protein accumulation in the procephalon late in embryogenesis, which is dispensable during this stage in development, is required for the proper expression pattern of *lab* in the anlagen of the eye–antennal disc. Our results do not rule this out since we see only a reduction in transgenic expression in the procephalon late in embryogenesis, not complete elimination (Fig. 2J). Those remaining cells expressing low levels of the transgenes at this stage may be progenitors of the cells in the eye–antennal disc that we observe expressing the transgene in the absence of the resident *lab* gene. In fact, it may be that the intron-located *cis*-acting regulatory element responsible for late embryonic procephalic expression is the element required to override negative autoregulation by the resident gene in the cells of the peripodial membrane where *lab* is normally expressed. This *cis*-acting element alone confers no imaginal expression (data not shown), but in conjunction with the positive autoregulatory element included in the minigenes may result in a wild-type pattern of expression for the transgenes. We are currently pursuing this possibility.

By dissecting the regulatory elements of the *lab* transcription unit *via* germ line transformation experiments, we have identified those aspects of the *lab* expression pattern that are essential for viability and those that are expendable. Stated from an alternate viewpoint, using minigene constructs, in addition to reporter gene fusions, we were able to address the biological role of this homeotic gene during development in addition to identifying the *cis*-acting regulatory elements that direct this expression. This approach enabled us to show that only the procephalic expression of *lab* during head involution is critical to this process and embryonic viability. On the other hand, expression in the dorsal ridge, pmg, embryonic PNS and late procephalic accumulation are apparently dispensable during embryonic development.

Moreover, these minigene constructs permitted us to demonstrate the apparent ability of *lab* to regulate the expression of other members of the ANT-C in the eye–antennal disc. This possibility now offers an explanation for the observation that there is no co-expression of these genes in the cells of this disc and may disclose the presence of a hierarchal regulatory network by which these genes influence the process of adult head development. Diederich *et al.* (1991) report that although there is no co-expression of *lab* and *Dfd* in the eye–antennal disc, there is co-expression of these two genes in the vicinity of the dorsal ridges during embryogenesis in cells which these authors hypothsize to be progenitors of a portion of the presumptive eye–antennal disc. These authors go on to propose that this early co-expression is followed by a refinement of this pattern to mutually exclusive domains of expression. Our results with the transgenes in wild-type and null backgrounds suggest that negative autoregulation of *lab* may be responsible for the refinement of its expression in this cluster of cells, which, in some fashion specifies the extent of the domain of expression for *Dfd*. In the absence of negative regulation, *lab* protein accumulates ectopically in imaginal cells and negatively influences the expression of *Dfd*, thus altering the developmental pathway these cells follow. This indicates that the establishment of the *lab* expression pattern may be a crucial step in the developmental hierarchy that produces the adult head.

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