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. Imaging 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, 1987a). 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,Sc and/or TM6B,Hu Tb (Lindsley and Grell, 1968; Craymer, 1984; Lindsley and Zimm, 1991).

<p>| 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 polystyrene interval B8A1-B8B.2 (Kaufman et al. 1980). Null alleles of labial are 4 and 14, the remaining mutations are hypomorphs. The reported results were obtained using P[w- +,lab-2]. 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. |
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| <strong>Table 1. Transgenic rescue analysis of labial alleles</strong> |</p>
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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 Smal 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 NotI fragment from pHSS7 (Seifert et al. 1986) and inserted into the transformation vector CoSpeR NotI (supplied by John Tamkun) creating P[w+, lab25a] (2.5a) (Fig. 1). Two additional constructs were generated by deleting the upstream 1.6 kb EcoRI fragment. These two constructs, P[w+, lab24a] (2.4a) and P[w+, lab24b] (2.4b), are identical except that the 5'-most 1.2 kb EcoRI fragment is inverted relative to its normal orientation in construct 2.4b (Fig. 1). A fourth construct, P[w+, lab23a] (2.3b), was generated in which the 1.2 kb EcoRI fragment is replaced with the 5' 1.6 kb EcoRI 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 KpnI, blunt-ending with T4 DNA polymerase, and then digesting with BamHI. A blunt-ending 7.0 kb BamHI 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 BssHII which cuts 10 bp downstream of the transcription start in the untranslating leader. This digest was blunt-ended with Klenow (NEB), and digested with BamHI. 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 untranslating lab leader, the lab transcription start site, and ~6.0 kb of upstream sequences. This plasmid was named p6.0lab66A and is shown in Fig. 4. A variation of this lab-lacZ gene fusion was made by digesting the above construct with BamHI and HindIII, 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 p3.65lab66A (Fig. 4). These two gene fusions were subcloned into the NotI site of CoSpeR NotI 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 BamHI fragment described above was inserted as a BamHI 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 p7.0lab1Th 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 NotI fragments and subcloned into the NotI site of the polylinker in the enhancer test vector HZ20 (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 EcoRI fragment mentioned above was also

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**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 EcoRI site is 2.4 kb and the distance from the poly(A) site to the HindIII site is 2.5 kb. Restriction enzymes: H, HindIII; R, EcoRI; S, Smal. Minigene P[w+, lab23a] (2.5a) is identical to the transcription unit above except the first intron was eliminated using a 280 bp Smal fragment isolated from a partial cDNA clone (cross-hatched box). Minigene P[w+, lab24a] (2.4a) is the same as 2.5a except the 5' 1.6 kb EcoRI fragment is deleted. Minigene P[w+, lab24b] (2.4b) differs from 2.4a in that the 5' 1.2 kb EcoRI fragment is in the reverse orientation (horizontal arrows above and below diagrams depict orientation). Minigene P[w+, lab23b] (2.3b) has the 1.6 kb EcoRI fragment in place of the 1.2 kb EcoRI fragment. Minigene P[w+, lab22a] (2.2a) is the same as 2.3b except that a 1.5 kb EcoRI fragment from the first exon/intron border is substituted for the 1.6 kb EcoRI fragment. These five constructs were subcloned as NotI fragments into the transformation vector CoSpeR NotI (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",1.2labH2].

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 \(w; P[rY",\Delta2-3]\) hosts. The enhancer test vectors were co-injected with the P-element helper plasmid \(P[\pi]25.7wc\) (Kaeser and Rubin, 1984) into \(w^{stop}\) 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 allele backgrounds using standard crossing protocols.

Immunological staining
Transgenic embryos were fixed and stained essentially as described in Mahaffey and Kaufman (1987b) 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 Patterson and Kaufman (1991). The Dfd antisera is described in Diederich et al. (1991), and the Scr antisera is described in Mahaffey and Kaufman (1987b). The polyclonal rabbit \(\beta\)-galactosidase antisera was a gift from David Miller and the monoclonal anti-\(\beta\)-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 \(P[w^{+},lab^{2-2a}](2.5a), P[w^{+},lab^{2-4b}](2.4a)\), 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 phases of development.
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 p6.0lab66A 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 p3.65lab66A 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⁺,1.2labHZ] (1.2HZ). Surprisingly, no β-gal accumulation was detected in the procephalon. (B) Embryos carrying P[ry⁺,7.6labHZ] (7.6HZ) accumulate β-gal in the pmg (arrowhead) and in the dorsal ridge (arrow). (C) A530 bp EcoRI fragment, located on the 3'-side of the 7.6 kb EcoRI 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+/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+/lab+. (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 $\text{lab}^{-}$ background have lower fecundity than those carrying one wild-type copy of $\text{lab}$. It should be noted that this latter genotype, $\text{lab}^{+/\text{lab}^{-}};\text{P[w+},\text{lab}^\text{minigenes}]$, is morphologically normal at all stages.

**Comparison of $\text{lab}$ protein distribution in wild-type and transgenic embryos**

The observation that the minigenes appeared capable of providing the embryonic but not imaginal requirement for $\text{lab}$ function prompted us to determine whether the minigenes exhibited normal temporal and spatial patterns of $\text{lab}$ 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; $\text{lab}^{+/\text{TM3,Slb}}\times 2.5a/2.5a$; $\text{lab}^{+/\text{TM3,Slb},Tb}$ ($\text{lab}^{+}$ is a null allele) were stained using a $\text{lab}$-specific antibody (see Materials and methods). All embryos in this preparation exhibited $\text{lab}$ protein accumulation; however, 23% (28/123) of the embryos displayed only a subset of the normal $\text{lab}$ pattern of accumulation. To demonstrate that this result was general, antibody-stained transgenic embryos from the crosses 2.5a/2.5a; $\text{lab}^{+/\text{TM3,Slb}}\times 2.5a/2.5a$; $\text{DF(3R)Sc}]/\text{TM6B,Tb}$ and 2.5a/2.5a; $\text{lab}^{+/\text{TM3,Slb}}\times 2.5a/2.5a$; $\text{lab}^{+/\text{TM6B,Tb}}$ 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 EcoRI fragment is dispensable.

Transgenic expression deviates from the wild-type pattern in four areas. The most intriguing difference is the absence of transgenically generated $\text{lab}$ protein accumulation in the dorsal ridge (Fig. 2H) as this is a process that fails in the absence of $\text{lab}$ function. Normally, $\text{lab}$ 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 $\text{lab}$ 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 $\text{lab}$ expression is the absence of $\text{lab}$ protein accumulation in cells of the posterior midgut primordium (pmg). In wild-type embryos, $\text{lab}$ 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 $\text{lab}$ expression in the pmg, the formation of the midgut proceeds as in wild-type embryos suggesting that $\text{lab}$ 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 $\text{lab}$ in the midgut in general has been disputed. Diederich et al. (1989) report no effects on midgut development in the absence of $\text{lab}$ function; whereas, Immergliick et al. (1990) report that formation of the second midgut restriction is dependent on $\text{lab}$ expression. Our results suggest, at least for the pmg, that $\text{lab}$ function is expendable in these cells and that the embryo and larva are viable in the absence of pmg $\text{lab}$ accumulation.

At the conclusion of embryogenesis (post head involution) transgenic expression of $\text{lab}$ diminishes relative to wild-type levels in the procephalon as can be seen by comparing Fig. 2E and J. Despite the absence of transgenic $\text{lab}$ 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 $\text{lab}$ 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 $\text{lab}$, if any, in the formation of these cells is therefore not clear as they develop, and likely function, in the absence of $\text{lab}$ expression (Merrill et al. 1989; this study).

The transgenic expression patterns of the remaining minigene constructs, [P[w+,$\text{lab}^{2.3b}$]] (2.3b) and [P[w+,$\text{lab}^{2.2a}$]] (2.2a), differs from that of the other three minigene constructs. Additionally both fail to complement the loss of $\text{lab}$ 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 $\text{lab}$ 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 EcoRI fragment absent from constructs 2.3b and 2.2a contains cis-acting regulatory elements for both amg and procephalic expression of $\text{lab}$, and that the regulatory elements for the initiation of $\text{lab}$ 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 $\text{lab}$ minigenes. To determine more precisely the location of these elements, we made $\text{lab}^{--}\text{lacZ}$ reporter gene fusion constructs containing sequences upstream of the $\text{lab}$ 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 EcoRI site (R). Restriction enzymes: B, BamHI; H, HindIII. Below the transcription unit are the lab–lacZ reporter genes. Construct p7.0labTh, 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 essentially the same upstream sequences as 2.5a (Fig. 1) except for the presence of an additional 750 bp at the 5'-end. Reporter genes p6.0lab66A and p3.65lab66A 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 p6.0lab66A are identical to those in p7.0labTh. Construct p3.65lab66A was derived from p6.0lab66A by removing the 5' 2.3 kb BamHI–HindIII fragment (Materials and methods). The right-angle arrow below the constructs indicates the initiation point and direction of transcription.

Patterns 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– background 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+,3.65lab66A] (3.65lab66A) into a lab– background. If lab 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 EcoRI 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 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 EcoRI 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 EcoRI 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 EcoRI 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.
adult head. In that study mitotic recombination was demonstrated a role for in the development of the head.

Previous work in this laboratory by Merrill et al. (1989) showed that at least 3.6 kb of contiguous upstream sequence rescue the adult homeotic phenotype. However, it is possible that the sequences required for the autogenous maintenance of lab expression in the procephalon do not lie solely within the 1.2 kb EcoRI 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 EcoRI fragment (Fig. 8B). The temporal pattern of β-galactosidase expression in the dorsal ridge is identical to wild-type lab accumulation. A 550 bp EcoRI 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 (p3.1labHZ) 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 (p3.1labHZ) remains to be tested, thus the PNS elements may reside there (Fig. 7).

Arguing for 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 posterior, 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 postgena, which resemble thoracic spiracles and large, amorphous 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 cases 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...
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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+/lab+ 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+/TM6B,Tb × 2.5a/2.5a;lab+/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 (Anip 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 EcoRI 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 genotype 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 $\beta$-galactosidase accumulation in the embryo and this pattern is the same as that observed for the three minigene constructs that complement the lab embryos (data not shown). 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 EcoRI 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 Trembl, 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 untranscribed 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 EcoRI fragment based on the results with 7.6labHZ and the mutant chromosome Df(3R)MAP11. Restriction enzymes: R, EcoRI; H, HindIII.

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 1.2labHZ (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 1.2labHZ 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 transgen 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, p6.0lab66A and p3.65lab66A 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 p6.0lab66A. 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 accommodate 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-
ulation 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 hypothesize 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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