Rescue of *Drosophila labial* null mutant by the chicken ortholog *Hoxb-1* demonstrates that the function of *Hox* genes is phylogenetically conserved

Beat Lutz, Hui-Chen Lu, Gregor Eichele, David Miller, and Thomas C. Kaufman

*Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030 USA; Howard Hughes Medical Institute, Department of Biology, Indiana University, Bloomington, Indiana 47405 USA*

**Hox** complexes are important players in the establishment of the body plan of invertebrates and vertebrates. Sequence comparison demonstrates a remarkable phylogenetic conservation of key structural features of *Hox* genes. The correlation between the physical order of genes along the chromosomes and their domains of function along the body axis is conserved between arthropods and vertebrates. Ectopic expression experiments suggest that the functions of homeo proteins also are conserved between invertebrates and vertebrates. However, it remains an open question whether vertebrate *Hox* genes expressed under the control of *Drosophila* regulatory sequences can substitute the function of *Drosophila Hox* genes. We have studied this issue with the *Drosophila labial* (*lab*) gene and its chicken ortholog *gHoxb-1*. We fused the entire protein-coding region of *gHoxb-1* with previously identified regulatory sequences of *lab*. This approach places *gHoxb-1* into the normal embryonic spatiotemporal context in which *lab* acts. Ten transgenic lines carrying *gHoxb-1* were established and tested for their ability to rescue *lab* null mutant animals. Eight lines rescued with high efficiency, embryonic lethality, and abnormal head morphogenesis, two defects observed in *lab* null mutant embryos. The rescue with the *gHoxb-1* minigene was close to the efficiency of that obtained with the *Drosophila lab* minigene. This indicates that *gHoxb-1* protein can regulate *lab* target genes and thereby restore embryonic viability. This is striking, as Lab and *gHoxb-1* proteins are divergent except for their homeo domains and a short stretch of amino acids amino-terminal to the homeo domain. Our findings demonstrate a functional conservation of the *lab* class homeo proteins between insects and vertebrates and support the view that function of *Hox* genes resides in relatively few conserved motifs and largely in the homeo domain.

**Key Words:** HOM-C genes; Hox genes; evolution; pattern formation; *Drosophila labial*; chicken *Hoxb-1*; phenotypic rescue

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*Drosophila* homeo box genes from the Antennapedia and Bithorax complexes (*HOM-C*) are master control genes that specify the body plan along the anteroposterior axis (Mahaffey and Kaufman 1987a; McGinnis and Krumlauf 1992; Carroll 1995) and determine cell fate (Hoppler and Bienz 1994). These genes contain a 180-nucleotide motif, the homeo box, which is translated into the homeo domain (McGinnis et al. 1984; Scott and Weiner 1984). Homeo domains contain a helix–turn–helix motif which participates in the specific interaction with DNA sequence elements present in target genes (Capovilla et al. 1994). Homeo box (*Hox*) genes related to those of the *Drosophila* homeotic selector genes have been found in all metazoa, including Hydra, nematodes, Amphioxus, amphibia, birds, and mammals (for review, see Duboule 1994; Krumlauf 1994; Ruddle et al. 1994). On the basis of sequence conservation in the homeo domain, *Hox* genes can be classified into 13 groups (McGinnis and Krumlauf 1992). Order and orientation of *Hox* genes on the chromosomes are largely conserved in all species analyzed (Ruddle et al. 1994). Spatial expression patterns of these genes along the anteroposterior body axis of *Drosophila* and vertebrate embryos are related, suggesting that aspects of the regulation of *Hox* expression are also conserved. Regulatory elements in *labial, Deformed,* and *proboscipedia* groups have been found to be functionally conserved among *Drosophila*, mice and humans (Awgulewitsch and Jacobs 1992; Malicki et al. 1992; Frasch et al. 1995; Pöpperl et al. 1995). Reminiscent of the situation in *Drosophila*, loss- and gain-of-
function mutations in the mouse can result in homeotic transformations [for review, see Krumlauf 1994]. Vertebrate Hox genes, when ectopically expressed in wild-type Drosophila embryos using heat shock promoters, result in phenotypes similar to those observed when the Drosophila orthologs themselves are expressed ectopically [Malicki et al. 1990, 1993; McGinnis et al. 1990; Zhao et al. 1993]. Taken together, these studies emphasize a high degree of structural and functional conservation among Hox genes.

Drosophila labial (lab) is the most 3' gene in the Antennapedia (Antp) complex. A lab null mutation is embryonic lethal; lab function is required for head involution [Merrill et al. 1989] and for copper cell formation in the larval midgut (Hoppler and Bienz 1994). In mice, three vertebrate orthologs of labial have been identified and are referred to as Hoxa-1, Hoxb-1, and Hoxd-1 [Baron et al. 1987; LaRosa and Gudas 1988; Frohman et al. 1990; Sundin et al. 1990; Murphy and Hill 1991; Frohman and Martin 1992]. Previously, we have reported the isolation and characterization of chicken gHoxb-1 [g for Gallus; Sundin and Eichele 1990; Sundin et al. 1990] whose homeo domain is 85% identical to that of Drosophila lab. Vertebrate Hoxa-1 and Hoxd-1 exhibit similar degrees of conservation with respect to the lab homeo domain. Outside the homeo domain, sequence conservation among lab and Hoxa-1, Hoxb-1, and Hoxd-1 is minimal with the exception of a cluster of ~12 amino acids encompassing the hexapeptide found in all genes belonging to groups 1–8 of the Hox clusters [Bürglin 1994].

A Drosophila lab minigene lab+ m2.4a, which is sufficient to rescue the embryonic lethality of lab mutations, has been constructed [Chouinard and Kaufman 1991]. In this study we have investigated the ability of gHoxb-1 to functionally substitute its Drosophila ortholog by replacing the lab+ m2.4a with a cDNA containing the gHoxb-1 open reading frame. This approach places the vertebrate ortholog into the natural context where labial is normally acting and thus would reveal functional conservation between lab and gHoxb-1 in a physiological situation. Transgenic lines containing the chicken gene were established and crossed with labial null mutant flies. We found that gHoxb-1 rescues embryonic lethality and abnormality in head morphogenesis, two defects in lab null mutant flies [Merrill et al. 1989]. Rescue of the embryonic defects with the gHoxb-1 minigene is almost as efficient as found previously with the Drosophila lab minigene. This is a striking result given that the fly and chicken genes have largely diverged outside the homeo domain and the dodecapeptide.

**Results**

**Phenotypic rescue of Drosophila lab null mutant by gHoxb-1**

As is the case for most paralogous groups, Hoxa-1, Hoxb-1, and Hoxd-1 genes are highly conserved within the homeo domain and in a cluster of ~12 amino acids amino-terminal to the homeo domain [Bürglin 1994]. Although interspecies homologies of vertebrate Hoxb-1 proteins are found throughout the polypeptide chain [Murphy and Hill 1991], homology between Drosophila Lab and vertebrate Hoxb-1 is restricted to the homeo domain and the dodecapeptide (Fig. 1A,B). The amino- and carboxy-terminal regions of Lab and Hoxb-1 are highly divergent, and Lab is approximately twice the size of its vertebrate orthologs. To determine whether such divergence in sequence and size of lab and gHoxb-1 genes would affect their function, the gHoxb-1 cDNA [see Materials and methods] was used in an attempt to rescue Drosophila lab null mutant embryos. The region encoding Lab protein in a minigene construct was replaced by the gHoxb-1 cDNA that contained a complete open reading frame (see Materials and methods and Fig. 2A). The Drosophila regulatory elements retained in this construct are those present in the ~3.6-kb sequence upstream of the lab transcription start site [Chouinard and Kaufman 1991]. These include regulatory sequences necessary to (1) initiate expression in the intercalary segment of the head of the early embryo, (2) control expression in the anterior midgut primordium and to maintain this expression through embryonic and larval development, and (3) maintain intercalary expression until the end of head involution but not to the end of embryogenesis. The up-

![Figure 1. Sequence comparison of Lab class dodecapeptides, homeo domains.](image)

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transgenic genotypes but is undetectable in the wild-type genome. We did not find any defects in the animals carrying P[lgHoxb-1] in a wild-type background. These animals were viable and morphologically normal. Apparently, GHOXB-1 protein does not interfere with normal development.

The various P[lgHoxb-1] transgenic lines were crossed into lab14/TM6B mutant background, and stocks were established. Embryos were collected from these stocks, and the ability of the transgenes to rescue the lab− phenotype was determined. It was found that each of the 10 transgenic lines fell into one of three categories: (1) two lines showed no detectable rescue; (2) seven lines displayed rescue of the embryonic lethality associated with lab− phenotype; and (3) one line (P[lgHoxb-1]23) showed rescue of lab− mutant up to the second larval instar. Table 1 displays the results of the lethal phase analysis of three representative lines. Additionally, the results of control crosses containing no transgene (lab14/TM6B) and the Drosophila lab minigene (P[lab+2.4a]) are included for comparison. As can be seen, the nontransgene control and crosses with P[lgHoxb-1]8.25.2a (category 1) show a similar level of embryonic lethality, suggesting that this line is unable to rescue lab−; whereas the other two crosses show <20% embryonic lethality. Especially in the crosses with P[lgHoxb-1]23, the degree of rescue was 61%, which is almost as good as that of the Drosophila minigene P[lab+2.4a] (75%; Table 1). Note that offspring from crosses with P[lgHoxb-1]9.3.3a or P[lgHoxb-1]23 exhibit an apparent increase in larval lethality relative to the nontransgene and P[lgHoxb-1]8.25.2a crosses (Table 1). This is because the 8.25.2a line shows no significant rescue of the embryonic lethality, whereas the other lines delay the lethal phase to later in development. This is consistent with the observation that Tubby (Tb+) larvae were observed in the P[lab+2.4a] and P[lgHoxb-1]23 crosses. Recall, that Tb is a marker of TM6B; therefore Tb+ larvae are lab14 homozygous (see Materials and methods).

In the cross with P[lab+2.4a], there are third-instar larvae, whereas in the case of P[lgHoxb-1]23 larvae survive only to the second instar. Less than 1% of the animals of the P[lgHoxb-1]23 larvae were rescued to the second instar. These animals lived for several days after their TM6B sibs had moulted to third instar and then died. The Drosophila lab minigene is capable of rescuing the adult stage in 3% of the cases (Table 1; Chouinard and Kaufman 1991), but P[lgHoxb-1]23, lab14/1ab14 animals never reach this or the pupal stage. No Tb+ second-instar larvae were found in the P[lgHoxb-1]9.3.3a cross. Based on the similar hatching frequency in this cross to that observed with the P[lab+2.4a] and P[lgHoxb-1]23 crosses, it would appear that the 9.3.3a transgene is capable of rescuing only embryonic lethality.

The unhatched embryos from the above crosses were collected and the cuticles of these animals mounted for examination. There are obvious defects in the formation of the mouthparts and a partial failure in head involution of lab− animals (see Fig. 3C; A shows a wild-type for comparison). In the P[lgHoxb-1]8.25.2a cross individu-

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Figure 2. Structure of gHoxb-1 rescue construct and Southern analysis of transgenic flies. [A] The gHoxb-1 cDNA was placed into P[lab+2.4a] (Chouinard and Kaufman 1991) giving rise to the chimeric transgene GdomHoxb-1lab carried in the construct P[lgHoxb-1]. Noncoding 5' and 3' regions from Drosophila, including the enhancer/promoter region of lab, are indicated with a solid line. The box extending between 3.6 and 6.3 kb represents the gHoxb-1 cDNA and part of the Drosophila 3'-untranslated DNA, including polyadenylation signals. [Inset] Polyclinker sequences from the pHS7A shuttle vector and pBS vector (open boxes), 5' and 3'-untranslated gHoxb-1 sequences (narrow shaded boxes), gHoxb-1 protein-coding region (wide shaded box), homeo domain [solid box], and Drosophila 3'-untranslated region (solid line). The arrow points to the region encoding the conserved dodecapeptide. [B] Southern blot of genomic DNA from wild-type (Ore-R), and from transgenic lines containing the gHoxb-1-specific and chicken-specific probes (Fig. 2B). The presence of the 3.74 kb and gHoxb-l-specific probes. The copy number of the chicken gene, Southern blot analysis was performed on genomic DNA from these lines and an Ore-R control by simultaneously hybridizing with Drosophila and chicken-specific probes (Fig. 2B). The presence of the gHoxb-1 DNA sequences can be seen clearly in the

stream sequences also include autoregulatory elements that require the presence of functional Lab protein [Diederich et al. 1989, Chouinard and Kaufman 1991].

We were able to recover several independent transgenic lines containing the P[w+m2GdomHoxb-1lab] (abbreviated as P[lgHoxb-1]) minigene construct [see Materials and methods]. To confirm that the transgenic lines carried the chicken gene, Southern blot analysis was performed on genomic DNA from these lines and an Ore-R control by simultaneously hybridizing with Drosophila and chicken-specific probes (Fig. 2B). The presence of the gHoxb-1 DNA sequences can be seen clearly in the

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als, defects similar to lab14 were observed [not shown], whereas in the P(lab +m2-a29), P[gHoxb-1]9.3.3a, and P[gHoxb-1]23 crosses, the unhatched animals resembled the wild type [Fig. 3B]. The morphology of the mouthparts is slightly abnormal in that the elements of the head skeleton just posterior to the mouth hooks are poorly organized and the ventral arm is incomplete. Additionally, there is a burr of sclerotized cuticle present at the base of the ventral plate that is reminiscent of a similar [albeit larger] defect at the same position in lab - animals. Taken together, these results indicate that gHoxb-1 is capable of rescuing embryonic lethality and a majority of the morphological defects associated with lab deficiency.

Expression of chicken Hoxb-1 in transgenic flies

To demonstrate that the phenotypic rescue observed resulted from the expression of gHoxb-1, we established the presence of the corresponding mRNA and protein in fly embryos. First, we performed reverse transcriptase (RT)–PCR to examine gHoxb-1 expression in transgenic lines P[gHoxb-1]23, P[gHoxb-1]8.25.2a, and P[gHoxb-1]9.3.3a. PCR reactions performed in the presence of the gHoxb-1-specific primers produced in all three transgenic lines a single PCR product of the same size and sequence as the product generated from chick embryo cDNA and G9 cDNA clone [data not shown; see Materials and methods]. Thus, the transgenic lines expressed the gHoxb-1 gene appropriately.

We next determined the pattern of Lab protein distribution in the rescued lines. We found that anti-Lab antisera recognized gHoxb-1. We recovered rescued second-instar larvae of the genotype P[gHoxb-1]23; lab14/TM6B and defined the distribution of gHoxb-1 protein expression. These animals were recognized by the anti-Lab antibody. As expected, -25% (91/384) of the embryos derived from the lab14/TM6B control cross showed no staining at any stage. All embryos derived from both the P[gHoxb-1]9.3.3a; lab14/TM6B and P[gHoxb-1]23; lab14/TM6B crosses showed staining (n = 243 and 199, respectively). Because of the presence of Lab, heterozygous animals have a normal protein staining pattern [Fig. 5A]. However, at certain stages in embryogenesis ~25% of the embryos showed an abnormal staining pattern, indicating that these individuals were lab14 homozygous. Therefore, the staining in these embryos reflects gHoxb-1 protein. Specifically, prior to head involution (stage 14) there were individuals that had no immunoreactivity in the dorsal ridge [data not shown].

Embryos were also collected and stained with the Drosophila anti-Lab antibody. As expected, ~25% [91/384] of the embryos derived from the lab14/TM6B control cross showed no staining at any stage. All embryos derived from both the P[gHoxb-1]9.3.3a; lab14/TM6B and P[gHoxb-1]23; lab14/TM6B crosses showed staining (n = 243 and 199, respectively). Because of the presence of Lab, heterozygous animals have a normal protein staining pattern [Fig. 5A]. However, at certain stages in embryogenesis ~25% of the embryos showed an abnormal staining pattern, indicating that these individuals were lab14 homozygous. Therefore, the staining in these embryos reflects gHoxb-1 protein. Specifically, prior to head involution (stage 14) there were individuals that had no immunoreactivity in the dorsal ridge [data not shown].

Table 1. Summary of lethal phase analysis for P[lgHoxb-1] rescue of labial null mutations in Drosophila

| Genotype | Eggs (no.) | Unhatched (no.) | Embryonic lethality (%) | Larvae (no.) | Pupae (no.) | Larval lethality (%) | Adults (no.) | Pupal lethality (%) | Total lethality (%) |
|----------|------------|-----------------|-------------------------|--------------|-------------|---------------------|--------------|-------------------|-------------------|
| lab14    | 300        | 85              | 28                      | 215          | 123*        | 31                  | 113          | 3                 | 62                |
| P(lab2.4a) | 300      | 21              | 7                       | 279b         | 129b        | 54                  | 116d         | 10                | 61                |
| 8.25.2a  | 300        | 80              | 27                      | 220          | 117*        | 34                  | 112          | 2                 | 63                |
| 9.3.3a   | 300        | 41              | 14                      | 259          | 150*        | 36                  | 139          | 4                 | 54                |
| 23       | 300        | 34              | 11                      | 266*         | 143*        | 41                  | 114          | 10                | 62                |

*a*All pupae were phenotypically Tb.

*b*Approximately 10% of second- and third-instar larvae were Tb +.

*c*4% of pupae were Tb +.

*d*3% of adults were rescued but had abnormal head morphology.

*e*A small percentage (<1) of second-instar larvae were Tb +.

Figure 3. Cuticle preparations of the late embryonic heads of wild-type (A), P[gHoxb-1]23 lab14/+/lab14 (B) and lab14/+/lab14 (C) individuals. The animal in B shows a partial phenotypic rescue. The rescued embryos are similar to wild type (A). However, they are slightly abnormal in the region posterior to the mouth hooks (MH). This region is poorly developed and broader than normal. Furthermore, the ventral arms (VA) are poorly sclerotized and do not extend posteriorly into the posterior pharyngeal wall (ppw). A knob [*] is present at the junction between the VA and the ventral plate (VP). In C a lab14/+/lab14 embryo illustrates the severe disruption of the mouthparts as a result of loss of lab function. [H] labral; [hys] hypostomal sclerite; [eps] epistomal sclerite; [H] H piece; [H?] presumed H piece; [DBr] dorsal bridge; [DA] dorsal arm.
Figure 4. Midguts of larvae stained for the presence of Lab and gHoxb-1 protein. The guts were removed from Tb [A] and Tb + [B] siblings from P[lgHoxb-1]23, lab14/TM6B parents and are of the same age. Note that the proteins are accumulated in the nuclei of the midgut. Recall Tb + larvae lack a functional lab gene.

shown. Additionally, staining in the head of late embryos was absent (stage 17 and later, Fig. 5B). Embryos containing the lab +mzaa minigene showed the same deficiencies in the protein staining pattern as P[lgHoxb-1],lab14/ lab14 embryos (Chouinard and Kaufman 1991). Because the same regulatory elements are present in the lab minigene and the P[lgHoxb-1] transgene, a similar pattern of expression is expected. All animals showed clear staining in the gut at all stages of embryogenesis, again demonstrating that the IgHoxb-1 minigene has the regulatory element that drives expression in this tissue.

Discussion

Conservation of function of Lab class homeo proteins

To assess functional conservation of homeo proteins we have replaced the lab protein-coding region of minigene lab +m2.4a (Chouinard and Kaufman 1991) with a gHoxb-1 cDNA. This created a chimeric minigene GdomXt-Ioxb-1 lab, composed of lab regulatory regions and the chicken protein-coding region carried in the P[lgHoxb-1]. Immunostaining of transgenic flies carrying either P[lgHoxb-1] or P[lab +m2.4a] minigenes reveals the same staining pattern encompassing a subset of the domain of expression of the resident locus. Eight of 10 transgenic lines established showed rescue of embryonic lethality (Table 1) and of abnormal head development [Fig. 3] in lab null mutants with an efficiency similar to that seen with the lab minigene lab +m2.4a itself. One transgenic line, P[lgHoxb-1]23, rescued to second-instar larvae at a frequency of ~1%, whereas the other lines died shortly after hatching. Two lines showed no obvious rescue at all. A similar variability in rescue efficiency was observed with transgenic lines carrying the Drosophila P[lab +m2.4a] minigene at different chromosomal locations (Chouinard and Kaufman 1991). Hence, we attribute the differential ability of IgHoxb-1 to rescue the lab14 mutants to effects of the site of integration.

The protein encoded by lab and gHoxb-1 consists of an amino-terminal domain, a dodecapeptide, the homeo domain, and a carboxy-terminal region. The degree of rescue obtained is surprising in view of the extremely low sequence homology in the amino-terminal domain, which constitutes the bulk of the protein. However, strong homology is detected in the homeo domains and dodecapeptides of Drosophila Lab and chicken gHoxb-1 proteins. To assess whether the amino acid substitutions observed could affect the structure of the homeo domain, we examined where these residues are located in the homeo domain structure (Fig. 6). The three-dimensional nuclear magnetic resonance [NMR] structure of the homeo domain of Antp protein reveals that residues participate either in DNA binding or in the hydrophobic core that stabilizes the helix–turn–helix motif characteristic of homeo domains (Billeter et al. 1993). There is near-complete conservation of those residues in Lab and gHoxb-1 which in Antp either interact with the DNA or form the hydrophobic core (Fig. 1B). The only exception is residue 3, which is a conservative substitution. The amino-terminal arm exhibits three substitutions [Figs. 1B and 6], and two conservative and four nonconservative substitutions are found in the rest of the homeo domain [Figs. 1B and 6]. Without exception, these substitutions reside on the surface and changes in the side
Functional conservation of *Hox* genes

Figure 6. Residues that differ between Lab and Hoxb-1 proteins (chick, mouse, and human) are indicated in the three-dimensional structure of the Antp homeodomain. Conformer 1 of the NMR structure is shown associated with the DNA target sequence (gray) (Billeter et al. 1993). Because of the high sequence homology between Antp and Lab, the essential structural features of the homeodomain are presumed to be very similar. α-Helices are shown in yellow; turns and loops in cyan. Location of conservative (magenta) and nonconservative (red) substitutions between Lab and Hoxb-1 are indicated. All substituted residues (1, 2, 3, 4, 10, 11, 24, 27, 32, 36, 39, and 60) reside on the surface of the homeodomain and neither interact with DNA nor are part of the hydrophobic core.

Chains would have little effect on either DNA binding or the structures of hydrophobic core. Therefore, we would predict that the structures of Lab and gHoxb-1 homeodomains are very similar.

Studies with Ultrabithorax (Ubx) and Antp class proteins suggest that a conserved cluster of amino acids amino-terminal to the homeodomain is required for interaction with accessory factors such as Exd (extradenticle protein) (Chan et al. 1994; Chang et al. 1995; Johnson et al. 1995). This cluster is commonly referred to as the hexapeptide but encompasses 12 amino acids in the case of the Lab class of homeo proteins (Fig. 1A; and Bürglin et al. 1995). The high degree of sequence conservation suggests that the structure is also conserved in the Lab class of homeo proteins. Our rescue data support this conclusion, in that the dodecapeptide of gHoxb-1 seems capable of interacting with the same accessory factors as its *Drosophila* cognate.

Lab and its vertebrate cognates are highly divergent in the amino-terminal domain that encompasses the bulk of a homeo protein. Because the chicken gene product rescues embryonic lethality, it seems that these diverged regions are not critical for Hox protein function during embryogenesis. We did not find any defects in those embryos, larvae, and adults that expressed the chicken gene in a wild-type background. These animals were viable and morphologically normal, leading us to conclude that the gHoxb-1 protein, despite its divergent amino-terminal domain, does not interfere with normal development when expressed in the domain of its *Drosophila* cognate.

The *Drosophila* P*([lab] -mdt-a2)* minigene rescued 3% of the mutant flies to adulthood, albeit these animals were morphologically abnormal (Chouinard and Kaufman 1991). In contrast, P*([ghoxb]-1)* rescued embryonic lethality and abnormal head development associated with *lab* deficiency, but we did not observe survival beyond the second larval instar (Table 1). We do not think that this difference can be attributed solely to position effects. More likely, it could either be attributable to different levels of expression or the divergence of sequence in the amino-terminal domain. Perhaps there are critical structural features in this domain that are not conserved but required for later development. It is unclear why development is blocked at the second larval instar. One possibility is that the functioning of the midgut is impaired and that the transgenic animals are unable to assimilate food and starve. Consistent with this notion is our observation that the fat bodies of P*([ghoxb]-1)*23, *lab*14/*lab*14 animals are quite small and the larvae appear lethargic. Additionally, it has been shown previously that *lab* expression is important for aspects of metabolic function of the gut at this stage (Hoppler and Bienz 1994).

Conservation of *lab* autoregulation

Several studies show that certain autoregulatory elements of *Hox* genes are conserved and can be interchanged between insects and mammals (Awgulewitsch and Jacobs 1992, Frasch et al. 1995, Pöppperl et al. 1995). Both in the fly (Chouinard and Kaufman 1991; Tremml and Bienz 1992) and in vertebrates (Ogura and Evans 1995, Pöppperl et al. 1995) autoregulation of *lab* class genes has been demonstrated. Pöppperl et al. [1995] showed that the rhombomere 4 enhancer of *Hoxb-1* is able to confer autoregulation on a reporter transgene in *Drosophila*. Moreover, Pbx/Exd binding sites are present in the rhombomere 4 enhancer and autoregulation of this enhancer requires *exd* and *lab* function. Our rescue construct contains *Drosophila lab* autoregulatory sequences (Chouinard and Kaufman 1991) displaying three Pbx/Exd binding sites. Thus gHoxb-1 protein produced in the fly may enhance its own production through these
sites. This may imply that the gHoxb-1 protein interacts with Drosophila Exd possibly mediated through the conserved dodecapeptide [Chang et al. 1995; Johnson et al. 1995], and in this manner exerts autoregulation via the Drosophila pbx/exd elements. In conclusion, Pöpperl et al. (1995) showed that Drosophila Lab can regulate, in conjunction with Drosophila accessory factors, a reporter gene driven by the rhombomere 4 enhancer. Our study implies that vertebrate gHoxb-1 homeo protein can function in conjunction with Drosophila accessory factors on a Drosophila autoregulatory element.

This study establishes that gHoxb-1, when placed under the control of the regulatory region of Drosophila lab, can carry out all functions required during embryogenesis. This suggests that gHoxb-1 binds to lab target genes and is able to interact with the same accessory factors. It is generally assumed that the function of Hox genes resides predominantly in the homeo domain [for review, see Gehring et al. 1994]. It is possible that rescue of later developmental functions falls because of the divergence in the amino-terminal domains. If so, then regions outside the homeo domain may also be important for function.

Materials and methods

Isolation and characterization of gHoxb-1 cDNAs

Phages (3.8 x 10^6) of a Lambda ZAP II cDNA library from Hamburger–Hamilton stage 14–17 [Hamburger and Hamilton 1951] were screened under high stringency conditions (Sambrook et al. 1989) using probes derived from the genomic gHoxb-1 clone (Sundin et al. 1990). Twenty-six clones were characterized by restriction site mapping and by sequencing. One of the cDNAs isolated (referred to as G9) was 2130 bp in length. To directly demonstrate that G9 encodes the same protein as predicted previously on the basis of genomic DNA sequence (Sundin et al. 1990), immunoprecipitation experiments were performed. gHoxb-1 cDNA G9 subcloned in pBS SK–[pBS SK–G9] was linearized with XbaI. One microgram of DNA was transcribed in vitro with T7 RNA polymerase using the Stratagene transcription kit supplemented with 0.5 mM Cap nucleotide m’G[5]ppp[5]A and translated in vitro with reticulocyte lysate [Promega] in the presence of [3H] leucine. gHoxb-1 protein was immunoprecipitated [Davis and Blobel 1986] using affinity-purified anti-gHoxb-1 antibodies [Sundin and Eichele 1990]. The immunoprecipitate was analyzed by SDS-PAGE, revealing a gHoxb-1 protein band of ~32 kD, as predicted by the sequence.

Construction of rescue plasmid

To facilitate cloning of the gHoxb-1 cDNA into the Drosophila P[lab+m2-act] minigene construct [Chouinard and Kaufman 1991], the shuttle vector pHSST7A [Seifert et al. 1986] was used. A Clal–BamHI fragment of clone G9 was excised from pBS SK–G9 and inserted into Clal and BamHI sites of pHSST7A, creating plasmid pHSST7A–G9, which contains unique polylinker sites AscI and SpeI. The AscI–SpeI fragment of pHSS7A–G9 was isolated and used for ligation into P[lab+m2-act]. To replace the complete Drosophila lab protein-coding sequence of lab+m2-act with the gHoxb-1 full-length cDNA, we used one of the BssHII site of P[lab+m2-act] [located 10 bp downstream from the transcription initiation site and 220 bp upstream from the translation initiation initiation site [Diederich et al. 1989]] and the SpeI site [located 200 bp downstream of the translation stop codon]. Because P[lab+m2-act] has four BssHII sites, P[lab+m2-act] was partially digested with BssHII, phenol-extracted, and then cut with SpeI. After phenol extraction, the cleaved plasmid was ligated with the AscI–SpeI fragment of gHoxb-1. Clones were screened by PCR using a 5’ primer located in the promoter of the Drosophila lab gene and a 3’ primer located in the gHoxb-1 cDNA. PCR-positive clones were characterized further by restriction analysis, and two of them (LG 15 and LG 18) were sequenced at the lab:gHoxb-1 junction and were shown to be identical. Southern blots of both clones were hybridized with a lab-specific probe [nucleotides 100–1060, Spel–NcoI, Diederich et al. 1989]. Neither clone hybridized to the lab probe, demonstrating that lab protein-encoding sequences were absent.

We have named the Drosophila::Chicken chimeric minigene GdomHoxb-1 lab and the construct that carries it P[w+m cGdomHoxb-1 lab] [abbreviated as P[lgHoxb-1]]. The results reported here utilized transgenic lines derived from LG 18.

Germ-line transformation

Germ-line transformation was carried out according to Robertson et al. [1988] using a 0.5-mg/ml solution of minigene construct P[lgHoxb-1]. P[lgHoxb-1] was injected into w, P[y+; Δ2–3] hosts. Transformed G1 flies were identified by complementation of the white phenotype. Multiple independent transformant lines were isolated. Chromosomal linkage was determined by segregation with respect to the balancer chromosomes Binsinsey, SM5, and TM3.Sb. Using standard crossing protocols, lines that carried a copy of the transgene on the second chromosome and were homozygous viable were selected to establish transgenic stocks. Ten such lines were obtained and are designated in order of recovery: P[lgHoxb-1]8.25.1b, P[lgHoxb-1]8.25.2a, P[lgHoxb-1]9.2.2a, P[lgHoxb-1]9.2.1b, P[lgHoxb-1]9.2.4a, P[lgHoxb-1]9.2.5a, P[lgHoxb-1]9.3.3a, P[lgHoxb-1]9.28.1a, and P[lgHoxb-1]23. All are independent isolates based on their recovery from different G0 parents and differences in pigmentation levels conferred by the w+m c marker.

Southern analysis

Southern analysis of RT–PCR products was carried out according to Sambrook et al. [1989]. After separation of the DNA on a 1.2% agarose gel, the DNA was transferred onto a Hybond-N+ membrane [Amersham] that was hybridized with [32P]-end-labeled gHoxb-1-specific oligonucleotide C: GACCGCTGCGCTACTGCA (Sundin et al. 1990). Oligonucleotide C hybridized with RT–PCR products from transgenic lines, showing that these lines expressed gHoxb-1 mRNA. Southern analysis of genomic DNA from wild-type [Ore-R] and transgenic adult flies [P[lgHoxb-1]23, P[lgHoxb-1]8.25.2a, P[lgHoxb-1]9.3.3a] was performed as follows: Genomic DNA (10 μg) was cut with EcoRI and fractionated on a 1% agarose gel. The DNA was transferred onto a Nitran membrane [Schleicher & Schuell]. The blot was simultaneously hybridized with random-primer, 32P-labeled probes from lab [nucleotides 100–1060, Spel–NcoI [Diederich et al. 1989]] and gHoxb-1 [nucleotides 157–688, EcoRI–XmaI [Sundin et al. 1990]]. Following prehybridization for 4 hr at 50°C in 5X SSC, 0.1% deionized formamide, 10% dextran sulfate, 1X Denhardt’s solution, 0.1 μm sodium phosphate [pH 8.0], 0.1% SDS, 1 μM EDTA, and 1 mg/ml of yeast total RNA, the blot was hybridized at 50°C overnight in 5X SSC, 0.1% SDS, and 1 μM EDTA. It was then washed four times for 45 min each at 70°C in 0.2X SSC, 0.7% SDS, and 1 μM EDTA. The blot was subjected to autoradiography and was analyzed by PhosphorImaging to determine the transgene copy number.
**Construction of stocks for rescue**

The above chromosome 2-linked homozygous viable transgene stocks were crossed into a background containing lab^{14} and the balancer TM6B, Hu Tb. The lab^{14} allele is a protein and RNA null (Chouinard and Kaufman 1991). The TM6B balancer was chosen for the presence of the Tb marker, which produces second- and third-instar larvae, pupae and adults that are phenotypically short and fat. Using this marker in a cross of P[ghoxb-1]n, lab^{14}/TM6B flies, it is possible to unequivocally determine if genotypically P[ghoxb-1]n, lab^{14}/lab^{14} animals survive beyond the first larval instar.

**Determination of lethal phase**

Flies of the genotypes P[ghoxb-1]n, lab^{14}/TM6B, P[lab^{+}/m2^{4E}], lab^{14}/TM6B and lab^{14}/TM6B were crossed and allowed to oviposit for 4 hr on food plates. From each of the crosses 300 eggs were transferred to fresh media and allowed to develop. At 26 hr postoviposition, the unhatched eggs were treated as described below for cuticle preparations and the hatched larvae were allowed to continue development. The plates were monitored daily for the presence of Tb^{+} larvae. The number of pupae from each cross was determined and the presence of Tb^{+} again assessed. Finally the number of adults resulting from each cross was ascertained. These counts allowed for the determination of the predominant phase of lethality (embryonic, larval or pupal) and whether the transgenes were capable of rescue.

**Cuticle preparation and antibody staining**

The ability of P[ghoxb-1]n to rescue the null lab phenotype was examined in embryos that were homozygous for both P[ghoxb-1]n and lab^{14}. A 12-hr egg collection from P[ghoxb-1]n, lab^{14}/TM6B parents was obtained. After 24 hr the embryos and unhatched larvae were fixed and prepared as described in Van der Meer (1977) except that they were mounted in polyvinyl lactophenol (Curt, UK). These cuticle preparations were scored for the morphology of their mouthparts, and these were compared to the morphology of animals collected from lab^{14}/TM6B crosses without the transgene construct. Embryos were also collected from the same crosses and subjected to immunohistochemical staining. Whole-embryo fixation and antibody staining using horseradish peroxidase (HRP)-conjugated secondary antibodies was done as described in Mahaffey and Kaufman (1987b). The anti-Lab antibodies used are described in Diederich et al. (1989). In the course of these studies it was found that this polyclonal antisera apparently cross-reacts with and identifies the presence of the gHoxb-1-encoded protein. Whole-mount immunostained embryos and larval guts were examined and photographed using DIC optics on a Zeiss Axiohot microscope; phase contrast was used with embryonic cuticles. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

**RT–PCR**

To isolate total RNA, embryos of transgenic *Drosophila* lines P[ghoxb-1]123, P[ghoxb-1]18.25.2a, P[ghoxb-1]19.33a, and wild-type Ore-R line were collected at 4–14 hr of embryogenesis. Chicken embryos were collected at stage 21. Total RNA was extracted with the RNeasy total RNA kit (Qiagen). To remove contaminating DNA, 30 μg of total RNA was incubated for 30 min at 37°C with 10 units of DNase I [BRL] in a buffer consisting of 40 units of RNasin [Promega], 50 mM KCl, 10 mM Tris-HCl (pH 8.3) 1.5 mM MgCl₂ and 1 mM dNTPs [Promega] (Orly et al. 1994). The reverse transcriptase reaction was terminated by heating for 5 min at 95°C. Four microliters of this reaction was used as template in a 20-μl PCR containing 1× PCR buffer, 2.2 mM MgCl₂, 1 μM of each primer, and 2.5 units of Taq polymerase [Perkin-Elmer]. Forty cycles were performed on a Perkin-Elmer 9600 using a denaturing temperature of 94°C for 1 min, an annealing temperature of 63°C for 45 sec, and an extension temperature of 72°C for 45 sec. PCR products were separated on a 1.2% agarose gel and analyzed by Southern blots. Primer set for gHoxb-1-specific PCR: primer A (forward), CAGCTACTTGC-CGGGGTTTAC (Sundin et al. 1990); primer B (reverse), GAGGCGCGACCTTCCCTTTTC. Primer set for *Drosophila* lab specific PCR: primer D (forward), GCCATCGCAT- CATCCTTTAGTCCCAG (Diederich et al. 1989); primer E (reverse), CTGCTGCTCATCATGAGATCCTGATC. Using primers A and B, there were no PCR products from Ore-R flies, and RT–PCR omitting reverse transcriptase did not yield any PCR products. Control PCRs with primers D and E resulted in expected PCR products indicating that proper cDNA synthesis occurred in all lines analyzed.

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B Lutz, H C Lu, G Eichele, et al.

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