

## Functional equivalence of Hox gene products in the specification of the tritocerebrum during embryonic brain development of *Drosophila*

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### SUMMARY

Hox genes encode evolutionarily conserved transcription factors involved in the specification of segmental identity during embryonic development. This specification of identity is thought to be directed by differential Hox gene action, based on differential spatiotemporal expression patterns, protein sequence differences, interactions with co-factors and regulation of specific downstream genes. During embryonic development of the *Drosophila* brain, the Hox gene *labial* is required for the regionalized specification of the tritocerebral neuromere; in the absence of *labial*, the cells in this brain region do not acquire a neuronal identity and major axonal pathfinding deficits result. We have used genetic rescue experiments to investigate the functional equivalence of the *Drosophila* Hox gene products in the specification of the tritocerebral neuromere. Using the Gal4-UAS system, we first demonstrate that the *labial* mutant brain phenotype can be rescued by targeted expression of the Labial protein under

the control of CNS-specific *labial* regulatory elements. We then show that under the control of these CNS-specific regulatory elements, all other *Drosophila* Hox gene products, except Abdominal-B, are able to efficiently replace Labial in the specification of the tritocerebral neuromere. We also observe a correlation between the rescue efficiency of the Hox proteins and the chromosomal arrangement of their encoding loci. Our results indicate that, despite considerably diverged sequences, most Hox proteins are functionally equivalent in their ability to replace Labial in the specification of neuronal identity. This suggests that in embryonic brain development, differences in Hox gene action rely mainly on *cis*-acting regulatory elements and not on Hox protein specificity.

Key words: *labial*, Hox proteins, Brain development, Genetic rescue, *Drosophila*

### INTRODUCTION

The homeotic/Hox genes encode a network of evolutionarily conserved transcription factors that are involved in the specification of segmental identity along the anteroposterior body axis of animals as diverse as insects and vertebrates. This specification of identity is thought to be directed by differential Hox gene action, based on differential spatiotemporal expression patterns, protein sequence differences, interactions with co-factors and regulation of specific downstream genes (Carroll, 1995; Graba et al., 1997; Gellon and McGinnis, 1998; Mann and Morata, 2000). The functional roles of Hox genes in insect development have been studied extensively in *Drosophila*. In *Drosophila*, these genes are arranged along the chromosome in two gene clusters known as the Antennapedia and Bithorax complexes. There is a correlation between the relative position of the Hox genes in the clusters and their spatial and temporal expression pattern in the body; genes located towards the 3' end are expressed more anteriorly and earlier than genes towards the 5' end (spatial and temporal co-

linearity) (Manak and Scott, 1994; Duboule and Morata, 1994; Maconochie et al., 1996).

Hox genes are expressed in the developing brain and ventral nerve cord of *Drosophila* in an ordered set of domains. In the embryonic brain, specific Hox genes are expressed in the posterior half of the tritocerebrum (and to a small extent in the deutocerebrum) as well as in the three subesophageal neuromeres. The tritocerebrum is the posterior neuromere of the supraesophageal ganglion and consists of two bilaterally symmetric hemiganglia that are bounded anteriorly by the deutocerebrum and are linked by the tritocerebral commissure that runs across the midline beneath the gut (Burrows, 1996; Reichert and Boyan, 1997). The tritocerebrum is connected to more posterior parts of the brain through longitudinal connectives, and forms projections to the frontal ganglion via the frontal connectives. The Hox gene that is specifically expressed in the posterior half of the tritocerebral neuromere is *labial* (*lab*). Loss-of-function *lab* mutations cause profound defects in the establishment of the tritocerebral neuromere (Hirth et al., 1998). In *lab* mutants, the tritocerebral commissure is missing and the

longitudinal connectives are reduced or absent. Moreover, the cells in the *lab* mutant domain do not acquire a neuronal identity as exemplified by the lack of expression of neuronal markers indicating that *lab* is required for the specification of neuronal identity in the tritocerebrum. Comparable effects are seen in *Deformed* mutants, the only major difference being that these effects were observed in the mandibular and anterior maxillary brain neuromere, which is the expression domain of *Deformed*. None of the other Hox gene mutants show comparable brain defects (Hirth et al., 1998).

We have used genetic rescue experiments to investigate the functional equivalence of all of the *Drosophila* Hox genes in specifying the neuronal identity in the tritocerebral neuromere. For this we use the Gal4-UAS system (Brand and Perrimon, 1993) for targeted misexpression of Hox genes in the posterior tritocerebral domain (in which *lab* is normally expressed) of *lab* null mutants. As expected, we find that the *lab* mutant brain phenotype can be rescued by targeted expression of the Lab protein under the control of CNS-specific *lab* regulatory elements. We then demonstrated that under the control of these CNS-specific regulatory elements most of the other *Drosophila* Hox gene products are also able to replace the Lab protein in the specification of the tritocerebral neuromere. Only the Abdominal-B protein does not efficiently rescue the *lab* mutant phenotype in the brain. For the other Hox proteins, we observe a correlation between their efficiency of rescue the *lab* mutant brain phenotype and the chromosomal arrangement of their encoding loci. Our results indicate that, despite considerably diverged sequences, most Hox proteins are functionally equivalent in their ability to replace Labial in the specification of neuronal identity in the brain. This suggests that differences of Hox gene action in brain development rely mainly on *cis*-acting regulatory elements and not on Hox protein specificity.

## MATERIALS AND METHODS

### Fly strains and genetics

The P{w<sup>+</sup> *lab*::Gal4}K5J2 driver was generated by cloning a genomic fragment from *labial* that extends from the *Hind*III site 3.6 kb upstream of the transcriptional start site downstream to the *Bss*HII site at +10 bp (Chouinard and Kaufman, 1991). The downstream site was converted using a *Hind*III linker creating a 3.6 Kb *Hind*III fragment that was cloned into this site in pGaTN (Brand and Perrimon, 1993). This plasmid was then cut with *Not*I to remove the *lab*::Gal4 cassette, cloned into pCasperNot (supplied by John Tamkun) and used to generate the transgenic line P{w<sup>+</sup> *lab*::Gal4}K5J2.

For *lab*::Gal4-specific targeted misexpression of *proboscipedia* (*pb*), *Deformed* (*Dfd*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) in *lab* mutant embryos, the following UAS::Hox responder lines were used: p[UAS::*pb* 49.1] homozygous on chromosome II (Aplin and Kaufman, 1997); p[UAS::*Dfd*] homozygous on chromosome II (Brown et al., 1999); p[UAS::*abd-A* 21.6] homozygous on chromosome I (Greig and Akam, 1993), supplied by M. Akam; and p[UAS::*Abd-Bm*] homozygous on chromosome II (Castelli-Gair et al., 1994) driving the expression of the Abd-Bm form (Casanova et al., 1986; Zavortink and Sakonju, 1989), supplied by M. Akam.

For *lab*::Gal4-specific targeted misexpression of *labial* (*lab*), *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) in *lab* mutant embryos, p[UAS::*lab*], p[UAS::*Scr*], p[UAS::*Antp*] and p[UAS::*Ubx*] responder lines were generated (Miller et al., 2001). The respective Hox cDNAs were cloned into a polylinker downstream from a minimal *hsp70* promoter of the Gal4 responder plasmid pUAST (Brand

and Perrimon, 1993), which contains a P-element with the *white* mini-gene as a marker. The *hsp70* promoter is activated in the presence of Gal4 because of five upstream Gal4 binding sites (UAS). For generating p[UAS::*lab*], a 2.1 kb cDNA derived from a 2.4a minigene (including the second intron) (Chouinard and Kaufman, 1991) encompassing the entire *lab*-coding region, was digested with the *Ssp*I to generate the 2.1 kb cDNA that was inserted into pBlueScriptKS+ (Stratagene) at the *Eco*RV site. The cDNA was subsequently removed with *Eco*RI(5') and *Kpn*I(3') for insertion into pUAST at the same sites. For generating p[UAS::*Scr*], the 1.2 kb *Bam*HI(5') and *Mlu*I(3') truncated *Scr* L3 cDNA (Mahaffey and Kaufman, 1987) was inserted into pSE280 (Invitrogen) using the same sites. A partial *Scr* cDNA was then removed from pSE280 with *Nco*I(5'), blunted with Klenow and then released with *Xho*I. This modified cDNA was inserted into pUAST at the Klenow blunted *Eco*RI and *Xho*I sites. For generating p[UAS::*Antp*], the entire *Antp* G1100 cDNA (Scott et al., 1983) was inserted into pUAST at the *Eco*RI site. For generating p[UAS::*Ubx*], the previously reported *Ubx* NAB3 cDNA containing isoform 1S, which is the predominant embryonic cDNA (O'Connor et al., 1988), was inserted into pUAST at the *Eco*RI site. All strains, as well as all experimental genotypes, were maintained in standard laboratory cultures at 25°C.

Control experiments verified that the P{w<sup>+</sup> *lab*::Gal4}K5J2 driver is expressed in a spatial pattern, which corresponds to that of endogenous *lab* in the procephalon, and in the tritocerebral neuromere. UAS::transgene activation in the procephalon is delayed for 2.5 hours when compared with earliest presence of endogenous Lab protein (Kaufman et al., 1990), thus under the control of P{w<sup>+</sup> *lab*::Gal4}K5J2, UAS::responder activation starts at late stage 10 (5–5.5 hours AEL) (Campos-Ortega and Hartenstein, 1997). Phenotypic penetrance of the *lab* mutant brain phenotype was 88.6% (*n*=209) when determined with the *lab* null allele *lab*<sup>vd1</sup> (Merrill et al., 1989; Hirth et al., 1998) using flies of the genotype *lab*<sup>vd1</sup>/TM6B-*UbxlacZ*. The ability of the Hox proteins to rescue the *lab* mutant brain phenotype was determined by crossing P{w<sup>+</sup> *lab*::Gal4}K5J2; *lab*<sup>vd1</sup>/TM6B-*UbxlacZ* to either P[UAS::*lab*], *lab*<sup>vd1</sup>/TM3-*AntplacZ* or to flies of genotype P[UAS::*Hox*], *lab*<sup>vd1</sup>/TM6B-*UbxlacZ* where *Hox*=*pb*, *Dfd*, *Antp* and *Abd-B*; or to flies of genotype P[UAS::*Hox*]/+; *lab*<sup>vd1</sup>/+ for *Hox*=*Scr*, *Ubx* and *Abd-A*. All rescue experiments were carried out at 25°C; no significant differences in rescue efficiency were obtained when rescue experiments were carried out at 28°C. To identify rescued *lab*<sup>-/-</sup> cells and their axonal projection pattern, UAS::*tau-lacZ* located on the X chromosome (Callahan and Thomas, 1994) was additionally crossed in.

### Immunocytochemistry and genetic rescue analysis

Whole-mount immunocytochemistry and laser confocal microscopy was performed as previously described (Hirth et al., 1998). In genetic rescue experiments, P{w<sup>+</sup> *lab*::Gal4}K5J2 driven P[UAS::*Hox*] activity in homozygous *lab* null mutants (*lab*<sup>vd1</sup>/*lab*<sup>vd1</sup>) was confirmed by the absence of balancer-specific (TM6B-*UbxlacZ*; TM3-*AntplacZ*) β-gal and/or Labial immunoreactivity, as well as by the presence of corresponding Hox immunoreactivity in the tritocerebral *lab* domain. The criteria used to judge *lab*<sup>-/-</sup> embryos as fully rescued were: (1) the presence of the tritocerebral commissure linking the two tritocerebral hemiganglia; (2) the restoration of the longitudinal pathways between the supra- and subesophageal ganglia; and (3) the expression of neuron-specific molecular labels as assayed by anti-HRP and anti-Elav immunoreactivity (Hirth et al., 1998). Only when all three criteria were fulfilled was the tritocerebrum of a *lab*<sup>-/-</sup> mutant embryo scored as rescued. Additionally, in embryos of the genotype UAS::*tau-lacZ*/+; *lab*::Gal4/UAS::*Hox*; *lab*<sup>-/-</sup>, the specificity of rescue was also determined by the presence of correct axonal projections of rescued *lab*<sup>-/-</sup> cells along the rescued tritocerebral commissure.

### Laser confocal microscopy

For laser confocal microscopy, a Leica TCS SP was used. Optical sections ranged from 0.4 to 2 μm recorded in line average mode with picture size of 512×512 pixels. Captured images from optical sections

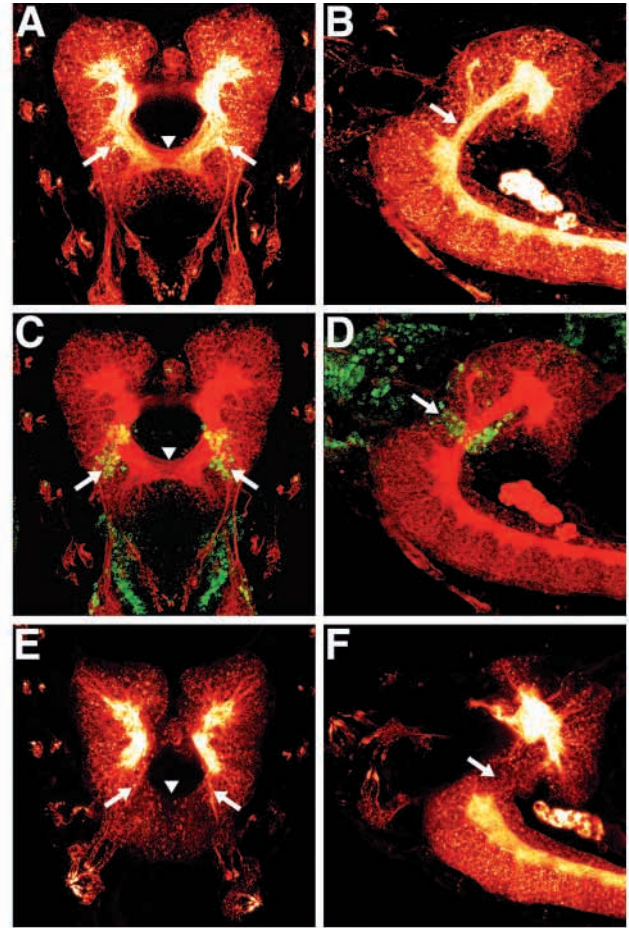
were arranged and processed using IMARIS (Bitplane). Figures were arranged and labeled using Adobe Photoshop.

## RESULTS

In the embryonic brain of *Drosophila*, the *labial* (*lab*) gene is expressed in the posterior half of the tritocerebral neuromere (Fig. 1A–D). In *lab* loss-of-function mutants, regionalized axonal patterning defects occur in the *lab* domain that are due to both cell-autonomous effects and non cell-autonomous effects. Thus, in the absence of *lab*, mutant cells are generated and positioned correctly in the brain, but these cells do not extend axons. Moreover, extending axons from other neighboring wild-type neurons stop at the mutant domains or project ectopically. As a result, dramatic defects in commissural and longitudinal axon pathways occur (Hirth et al., 1998); the tritocerebral commissure, which links the two tritocerebral hemiganglia, is absent and the longitudinal pathways between the supraesophageal and subesophageal ganglia are reduced or absent (Fig. 1E,F). Immunocytochemical analysis demonstrates that cells in the mutant domain do not express any of the numerous neuronal markers such as Elav that positionally equivalent cells express in the wild type, indicating a complete lack of neuronal identity in the *lab* mutant brain domain (Hirth et al., 1998). This strong mutant phenotype is apparent in 88.6% of the cases ( $n=209$ ). These data indicate that *lab* is involved in the specification of tritocerebral neuronal identity in the *Drosophila* brain.

In order to carry out a genetic rescue of the mutant brain phenotype in *lab* mutant embryos, we made use of the Gal4-UAS system (Brand and Perrimon, 1993). For this, a transgenic fly line carrying a Gal4 transcriptional activator under the control of the *lab* promoter together with CNS-specific upstream enhancer elements of the *lab* gene was used (see Materials and Methods). By crossing this *lab::Gal4* line to different UAS responders it is possible to express the responder constructs in a pattern that corresponds to that of the endogenous *lab* gene. To verify this, we first crossed the *lab::Gal4* line to transgenic lines carrying a UAS::*taulacZ* (Callahan and Thomas, 1994) reporter construct. The spatial expression domain of this reporter construct in the embryonic brain mimicked the endogenous *labial* expression domain (Fig. 2A,B). Spatially localized expression domains were seen in the posterior parts of the tritocerebral neuromere. (Ectopic reporter expression was seen in a small number of individual cells in the deutocerebral and mandibular neuromeres.) Double immunostaining experiments using anti- $\beta$ -gal and anti-Lab antibodies confirmed that *lacZ* expression occurred in the axons and cortical cytoskeleton of those cells that showed nuclear Lab expression (Fig. 2C,D).

We next determined whether the *labial* mutant brain phenotype could be rescued by transgenic expression of the Lab protein in a *labial* null mutant background. For this, a UAS::*lab* responder was driven by the *lab::Gal4* driver in the tritocerebral *lab* mutant domain. Using this approach, we obtained efficient rescue of all of the tritocerebral defects in the *lab* mutants. Thus, in these rescued embryonic brains, the tritocerebral commissure was present, the longitudinal pathways between the supra- and subesophageal ganglia were restored, and cells in the mutant domain showed correct

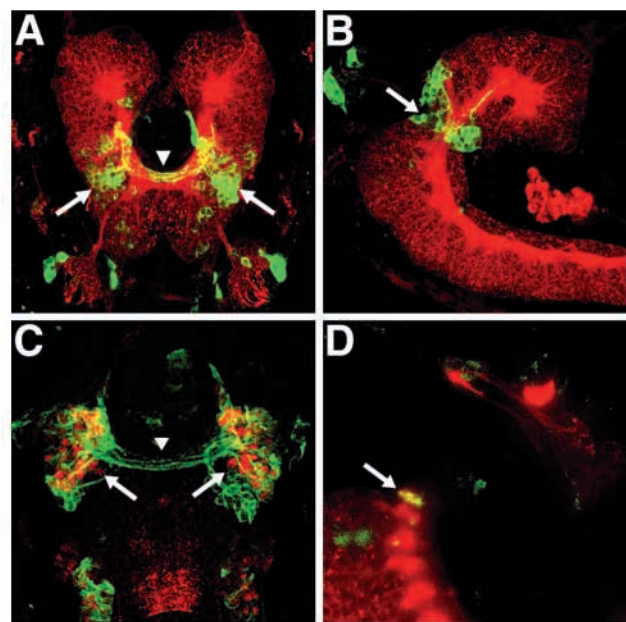


**Fig. 1.** Expression of Labial and *labial* loss-of-function phenotype in the *Drosophila* embryonic brain. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections. (A,C,E) Frontal views; (B,D,F) lateral views. (A,B) Wild-type embryonic brain. Anti-HRP immunolabeling. Arrows indicate circumesophageal connectives, arrowhead indicates tritocerebral commissure. (C,D) Wild-type embryonic brain. Double immunolabeling with anti-HRP (red) and anti-Lab (green). Arrows indicate Lab expression domain, arrowhead indicates tritocerebral commissure. Same embryo as in A,B. (E,F) *lab* loss-of-function mutant embryonic brain. Anti-HRP immunolabeling. Arrows indicate missing circumesophageal connectives, arrowhead indicates missing tritocerebral commissure.

neuron-specific molecular labels, as revealed by anti-Elav (not shown) and anti-HRP immunoreactivity (Fig. 3A,B). A quantification of the rescue efficiency for Lab in these experiments is given in Table 1. The fact that in these experiments Lab protein was indeed expressed specifically in the tritocerebral domain was demonstrated by carrying out anti-Lab immunostaining on these rescued brains (Fig. 3C,D).

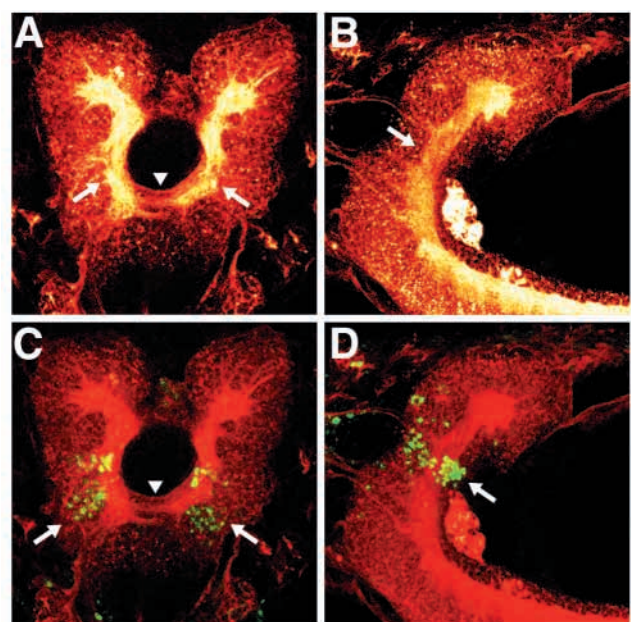
To determine whether other members of the Hox gene complex might also be able to rescue the *lab* mutant brain defects and, thus, be functionally equivalent to Lab in determining the segmental identity of the tritocerebral neuromere, transgenic lines were used in which the coding sequence of each of the remaining seven Hox genes was placed under UAS control (see Materials and Methods) (Miller et al., 2001). As a control, we first determined whether *lab::Gal4* driven misexpression of any of the 8 Hox proteins in a *lab*+





**Fig. 2.** Reporter gene expression phenotype in the wild-type *Drosophila* embryonic brain. Characterization of brain-specific *lab::Gal4* driver K5J2 using P{w<sup>+</sup>; *lab::Gal4*}K5J2-driven *UAS::taulacZ* reporter gene expression (Callahan and Thomas, 1994). Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections. (A,C) Frontal views; (B) lateral view; (D) midline cross-section. (A,B,D) Double immunolabeling with anti-HRP (red) and anti-β-gal (green). P{w<sup>+</sup> *lab::Gal4*}K5J2-driven *UAS::taulacZ* reporter gene expression is seen in the cortical cytoskeleton and axons of cells in the endogenous tritocerebral Lab expression domain (arrows in A,B) of the wild-type embryonic brain. Arrowhead indicates tritocerebral commissure. Ectopic reporter gene expression is seen in a small number of cells in the deutocerebral and mandibular neuromeres. (C) Double immunolabeling with anti-Lab (red) and anti-β-gal (green) shows that reporter gene expression occurs in the cortical cytoskeleton of the cells that also show nuclear Lab expression (arrows in C) as well as in their axons projecting along the tritocerebral commissure (arrowhead indicates the tritocerebral commissure). (D) Reporter gene expression is seen in the midline cross-section of the tritocerebral commissure (arrow).

background had any effects on the development and specification of the tritocerebral lab domain. In none of these experiments did we detect any sign of morphological abnormalities in the tritocerebrum or in any other part of the embryonic brain. Thus, in *lab::Gal4/UAS::Hox; lab*<sup>+</sup> embryos, all labeled structures in the tritocerebral lab domain were normal. Moreover, *lab::Gal4* driven *UAS::taulacZ* reporter



**Fig. 3.** Genetic rescue of the *lab* mutant brain phenotype by transgenic expression of the Lab protein in a *lab*-null mutant background. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections. (A,C) Frontal views; (B,D) lateral views. (A,B) From the same preparation; (C,D) from the same preparation. (A,B) Anti-HRP immunolabeling. Arrows indicate circumesophageal connectives, arrowhead indicates tritocerebral commissure. (C,D) Double immunolabeling with anti-HRP (red) and anti-Lab (green). Arrows indicate targeted misexpression domain of Lab in the *lab* mutant embryonic brain (equivalent to the endogenous expression domain of Lab in the wild-type embryonic brain). Arrowhead indicates tritocerebral commissure.

gene expression in conjunction with Hox gene misexpression in a *lab*<sup>+</sup> background revealed that the tritocerebral *lab*<sup>+</sup> cells showed a wild-type-like axonal projection pattern.

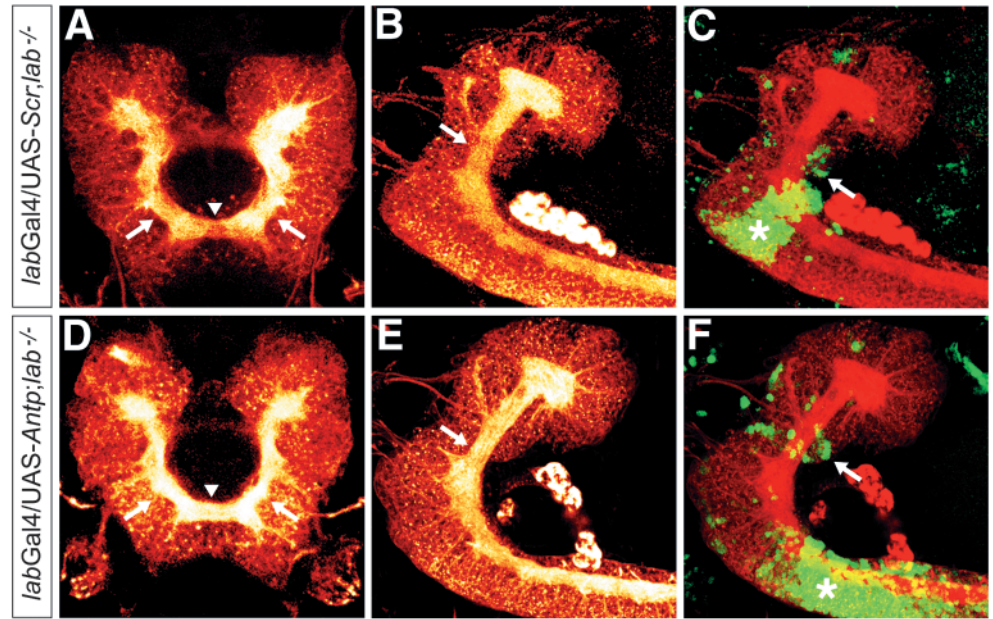
Next, we expressed each of the remaining seven *UAS::Hox* responders under the control of the *lab::Gal4* driver in the *lab* mutant domain. We first investigated the Hox proteins of the Antennapedia-Complex, as in the wild type, all five proteins of this complex are expressed in specific domains of the developing brain (Hirth et al., 1998). Surprisingly, all of the Antennapedia-Complex Hox proteins were able to rescue the *lab* mutant brain defects in these experiments. Examples of the ability of these Hox proteins to rescue the *labial* mutant brain phenotype are shown for Sex combs reduced (Scr) and Antennapedia (Antp) (Fig. 4). In both cases, an efficient rescue

**Table 1. Rescue of brain defects in *lab* mutants by Hox transgene expression**

Hox protein	Lab	Pb	Dfd	Scr	Antp	Ubx	Abd-A	Abd-B
Number examined	132	145	145	142	149	134	138	165
Number rescued	79	77	73	68	69	59	55	12
% rescued	59.8	53.1	50.3	47.8	46.3	44.0	39.8	7.2
% corrected	48.4	41.7	38.9	36.4	34.9	32.6	28.4	0

Quantitative rescue efficiency of *lab* mutant brain defects by the Hox gene products Lab, Pb, Dfd, Scr, Antp, Ubx, Abd-A and Abd-B expressed in the *lab* mutant under the control of the same *lab*-specific *cis*-acting regulatory elements. The number of embryos examined, the number of embryos showing a complete rescue of the tritocerebral brain defects, the percentage of embryos showing a complete rescue of the tritocerebral brain defects (% rescued), and the corrected percentage values for a rescue of the tritocerebral brain defects (% corrected) are shown. Percentage values were corrected in order to take account of the phenotypic penetrance of the *lab* mutation in tritocerebral development (88.6%). Thus, the corrected percentage values were calculated by subtracting 11.4% from the uncorrected percentage values.

**Fig. 4.** Genetic rescue of the *lab* mutant brain phenotype by transgenic expression of the Scr protein (A–C) or the Antp protein (D–F) in a *lab*-null mutant background. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections. (A,D) Frontal views, (B,C,E,F) lateral views. (B,C) From the same preparation; (E,F) from the same preparation. (A,B,D,E) Anti-HRP immunolabeling. Arrows indicate circumesophageal connectives, arrowhead indicates tritocerebral commissure. (C) Double immunolabeling with anti-HRP (red) and anti-Scr (green). Arrow indicates targeted misexpression domain of Scr in the *lab* mutant embryonic brain (equivalent to the endogenous expression domain of Lab in the wild-type embryonic brain). Asterisk labels the endogenous Scr expression domain in the subesophageal ganglion. (F) Double immunolabeling with anti-HRP (red) and anti-Antp (green). Arrow indicates targeted misexpression domain of Antp in the *lab* mutant embryonic brain (equivalent to the endogenous expression domain of Lab in the wild-type embryonic brain). Asterisk labels the endogenous Antp expression domain in the subesophageal ganglion and ventral nerve cord.



of the tritocerebral defects in the *lab* mutants was obtained; the tritocerebral commissure was present, the longitudinal pathways were restored, and cells in the mutant domain showed correct neuron-specific molecular labels. In addition to the *lab::Gal4* driven ectopic expression of Scr and Antp in the tritocerebral *labial* mutant domain, the large endogenous expression domains of these genes were observed unchanged in the subesophageal ganglion for Scr, and in the subesophageal ganglion and ventral nerve cord for Antp (Fig. 4C,F) (Hirth et al., 1998). A quantification of the rescue efficiency for all of the Antennapedia-Complex Hox proteins in these experiments is given in Table 1.

We next investigated the rescue potential of the Hox proteins of the Bithorax-Complex in comparable experiments. In contrast to the Hox proteins of the Antennapedia-Complex, the Bithorax-Complex Hox proteins are not expressed in the developing brain of the wild type, rather their expression domains are restricted to the ganglia of the ventral nerve cord (Hirth et al., 1998). Remarkably, as was the case for the Antennapedia-Complex proteins, both the Ubx and the Abd-A gene products of the Bithorax-Complex were able to rescue the *lab* mutant brain defects in these experiments. Once again, an efficient rescue of the tritocerebral brain defects in the *lab* mutants was obtained; the tritocerebral commissure was present, the longitudinal pathways were restored, and cells in the mutant domain showed correct neuron-specific molecular labels. An example of the ability of these Hox proteins to rescue the *labial* mutant brain phenotype is shown for Ubx (Fig. 5A–C). Note that, in addition to the *lab::Gal4* driven ectopic expression of Ubx in the tritocerebral *labial* mutant domain, the endogenous Ubx expression domain in the ventral nerve cord is also seen (Fig. 5C) (Hirth et al., 1998). In contrast to the other two Bithorax-Complex Hox proteins, use of the Abd-B gene product did not result in an efficient rescue of the tritocerebral defects in the *lab* mutants. In over 90% of the *lab::Gal4/UAS::Abd-B*; *lab*<sup>-/-</sup>

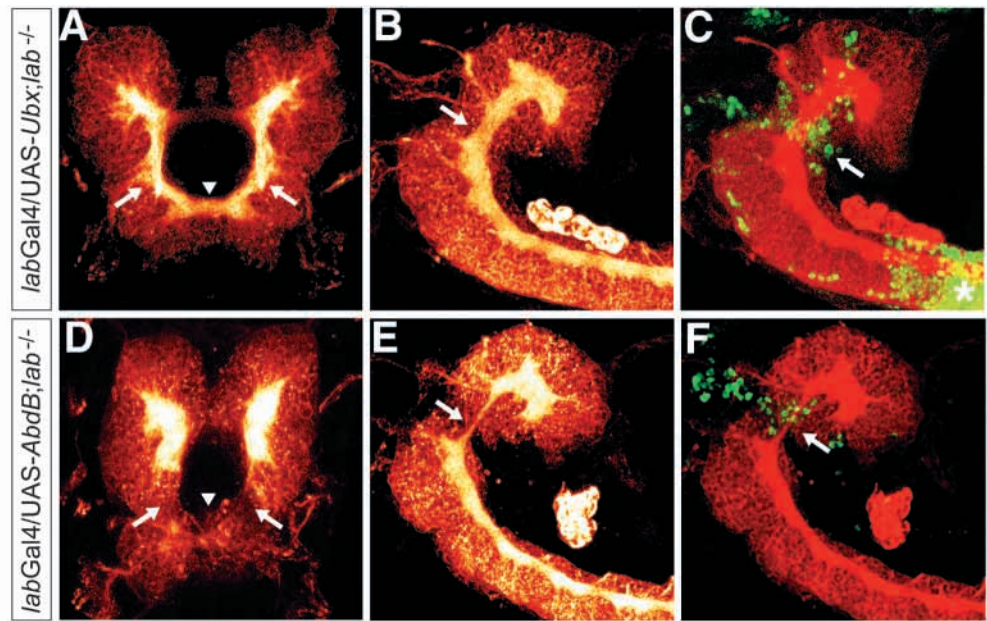
mutant embryos, profound axonal projection deficits were observed in the brain; the tritocerebral brain commissure was absent, the longitudinal brain pathways were reduced or lacking, and cells in the mutant domain lacked correct neuron-specific molecular labels (Fig. 5D–F). A quantification of the rescue efficiency for all of the Bithorax-Complex Hox proteins in these experiments is given in Table 1.

The efficient rescue of the tritocerebral defects in the *lab* mutants, which is achieved by targeted misexpression of seven out of eight Hox genes is striking; in the rescued embryonic brains the tritocerebral commissure was present, the longitudinal pathways between the supra- and subesophageal ganglia were restored, and cells in the mutant domain showed correct neuron-specific molecular labels. However, it is conceivable, that the rescue of all of these neuronal structures might be due to a restoration of generic neuronal properties in the cells of the *lab* mutant domain and not due to the rescue of specific neuronal identities in these cells. To investigate this, we determined whether the rescued cells in the *lab*<sup>-/-</sup> domain project their axons correctly across the rescued tritocerebral commissure, as is the case for *lab*-expressing neurons in the wild-type brain. For this, we co-expressed a *UAS::taulacZ* reporter gene with each *UAS::Hox* responder in the tritocerebral *lab* mutant domain using the *lab::Gal4* driver. This co-expression makes it possible to visualize both the cell bodies and the axonal projections of the rescued *lab*<sup>-/-</sup> cells. For all of the Hox gene products except Abd-B, these experiments demonstrate that the rescued tritocerebral *lab*<sup>-/-</sup> cells are again able to extend axons that projected correctly along the rescued tritocerebral commissure (Fig. 6).

As is indicated in Table 1, the relative efficiency of rescue of the brain phenotype in *lab* mutants varied systematically for the different Hox proteins. The *lab* responder achieved the best rescue efficiency, while the other Hox responders had slightly



**Fig. 5.** Genetic rescue of the *lab* mutant brain phenotype by transgenic expression of the Ubx protein (A-C), and failure of genetic rescue of the *lab* mutant brain phenotype by transgenic expression of the Abd-B protein (D-F) in a *lab* null mutant background. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections. (A,D) Frontal views, (B,C,E,F) lateral views. (B,C) From the same preparation; (E,F) from the same preparation. (A,B,D,E) Anti-HRP immunolabeling. Arrows indicate location of circumesophageal connectives, arrowhead indicates location of tritocerebral commissure. (C) Double immunolabeling with anti-HRP (red) and anti-Ubx (green). Arrow indicates targeted misexpression domain of Ubx in the *lab* mutant embryonic brain (equivalent to the endogenous expression domain of Lab in the wild-type embryonic brain). Asterisk labels part of the endogenous Ubx expression domain in the ventral nerve cord. (D) Double immunolabeling with anti-HRP (red) and anti-Abd-B (green). Arrow indicates targeted misexpression domain of Abd-B in the *lab* mutant embryonic brain (equivalent to the endogenous expression domain of Lab in the wild-type embryonic brain). The endogenous Abd-B expression domain in the ventral nerve cord is located in posterior neuromeres that are not shown.



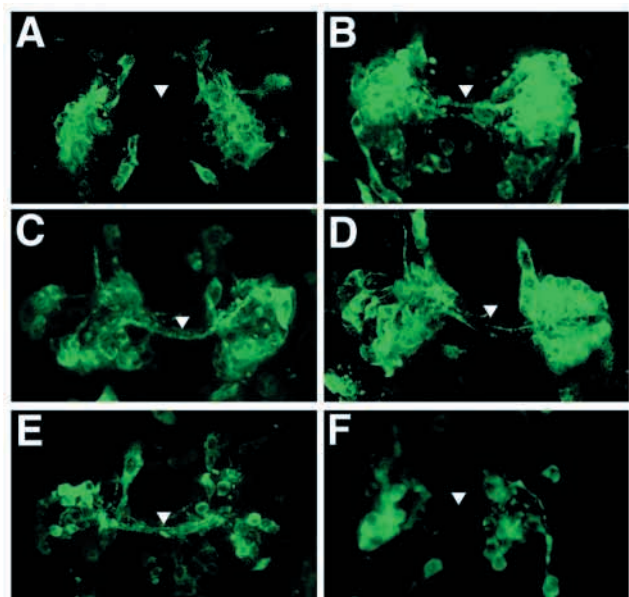
lower rescue efficiencies. Fig. 7 shows the rescue efficiency of all other Hox proteins relative to the rescue efficiency of Lab, which was taken as 100%. Interestingly, the decline in relative rescue efficiency for these other Hox proteins appears to be colinear (Lab>Pb>Dfd>Scr>Antp>Ubx>Abd-A) in that it reflects the proximal-to-distal arrangement of their encoding loci on the chromosome.

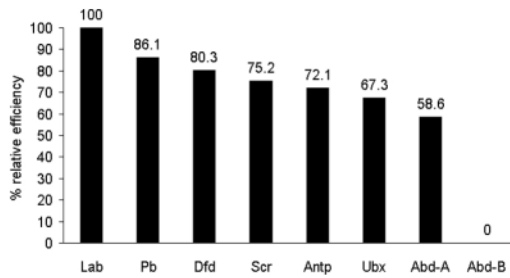
## DISCUSSION

Our findings indicate that Pb, Dfd, Scr, Antp, Ubx and Abd-A, but not Abd-B, are able to substitute efficiently for Lab in

determining the segmental identity of the *Drosophila* brain. Morphological evidence for a homeotic transformation of the tritocerebral neuromere into one of a different segmental identity was not observed in any of these rescue experiments. This suggests, that all of the Hox proteins, with the exception of Abd-B, are to a large degree functionally equivalent to Lab in this aspect of embryonic brain development. This surprising functional equivalence contrasts with the general notion, which is derived from experiments on the specification of other body parts in *Drosophila*, that Hox proteins assign different identities along the anteroposterior body axis by acting as specific selectors of different, alternative developmental pathways

**Fig. 6.** Reporter gene expression shows genetic rescue of commissural axonal projections in the *lab*<sup>-/-</sup> cells of the tritocerebrum by transgenic expression of the Lab, Dfd, Antp and Ubx proteins (B-E), and failure of rescue by transgenic expression of the Abd-B protein (F); also shown is the absence of commissural axonal projections in the tritocerebral *lab*-null mutant domain (A). Laser confocal microscopy of stage 13-15 embryos, reconstructions of optical sections, frontal views. Immunolabeling with anti-β-gal (green). *UAS::taulacZ* reporter gene expression is seen in the cortical cytoskeleton and axons of cells in tritocerebral *lab* mutant. Arrowheads indicate presence or absence of commissural axons of the *lab*<sup>-/-</sup> cells. In A, visualization of cell bodies and axonal projections was by *lab::Gal4* driven *UAS::taulacZ* reporter gene expression in the tritocerebral *lab* mutant domain. In B-F, visualization of cell bodies and genetic rescue of axonal projections of the *lab*<sup>-/-</sup> cells was through co-expression of *UAS::taulacZ* reporter with *UAS::Hox* responders in the tritocerebral *lab* mutant domain by the *lab::Gal4* driver. For all of the Hox gene products except Abd-B these experiments demonstrated that the rescued tritocerebral *lab*<sup>-/-</sup> cells were able to extend axons that projected correctly along the rescued tritocerebral commissure.





**Fig. 7.** Relative rescue efficiency of Hox gene products as related to Lab. The relative efficiency of rescue of the tritocerebral brain defects in *lab* null mutants is shown for the Hox gene products Lab, Pb, Dfd, Scr, Antp, Ubx, Abd-A and Abd-B expressed in the *lab* mutant under the control of the same *lab*-specific *cis*-acting regulatory elements (see Table 1). The rescue efficiency for Lab is taken as 100% and the rescue values (the relative percentage of embryos showing a complete rescue of the tritocerebral brain defects) of the other Hox gene products are shown in percentage relative to this. The relative rescue efficiency of the Hox gene products (Lab>Pb>Dfd>Scr>Antp>Ubx>Abd-A) reflects the proximal-to-distal arrangement of their encoding loci on the chromosome.

(Garcia-Bellido, 1975; Lawrence and Morata, 1994; Gellon and McGinnis, 1998; Mann and Morata, 2000).

There are several possible explanations for this discrepancy. First, the functional role of Lab in the specification of neuronal identity in the brain may differ from the role of other Hox proteins in other parts of the CNS. For example, Hirth et al. (Hirth et al., 1998) have found that the loss-of-function phenotype of *lab* (and *Dfd*) in the embryonic CNS differs from that of the remaining Hox genes. Moreover, in contrast to other domains in the embryonic CNS, there is an absence of overlapping expression with other Hox proteins, so that there is no genetic 'backup' in the tritocerebrum. Similar observations on Lab have been made in epidermal structures (Kaufman et al., 1990; McGinnis and Krumlauf, 1992; Morata, 1993). Second, it is conceivable that all Hox proteins can specify neuronal identity and the generic formation of commissural and longitudinal connections in the CNS. However, this seems unlikely, as the morphology and innervation of the tritocerebral neuromere is unique and highly specific, and unlike that of any other neuromere in the CNS (Burrows, 1996). Similarly, the morphology, mode of formation and gut-specific association of the developing tritocerebral commissure is clearly different from that of the other ganglionic commissures in the embryonic CNS (Wildemann et al., 1997). Third, Hox proteins may indeed be to a larger degree functionally interchangeable in the CNS than hitherto expected. In this respect, two sets of recent functional complementation experiments carried out on mammalian *Hoxa3/Hoxd3* genes and on mammalian *Hox11a/Hox11d* genes are noteworthy because they indicate that paralogous gene products can carry out identical biological functions if they are placed under the control of the appropriate *cis*-acting regulatory elements (Zakany et al., 1996; Greer et al., 2000). Our results extend this notion of functional equivalence of Hox genes from the level of paralogous genes to the level of the entire Hox gene cluster, excepting *Abd-B*. This, in turn, suggests that almost all of the Hox proteins can carry out identical biological functions in the *Drosophila* brain, if

they are under the control of the same *cis*-acting regulatory elements.

In our experiments, all of the Hox responders were expressed in the *lab* mutant under the control of the identical, *lab*-specific regulatory elements. Under these circumstances, the Lab responder achieved the best rescue efficiency, while the other Hox responders (with the exception of Abd-B) had somewhat lower rescue efficiencies that ranged from 86–59% of the rescue values achieved by Lab (see Fig. 7). Interestingly, the relative rescue efficiency of the Hox gene products Lab, Pb, Dfd, Scr, Antp, Ubx and Abd-A reflect their proximal-to-distal arrangement of their encoding loci on the chromosome. It is conceivable that this co-linear correlation of rescue efficiency among these Hox gene products is due to the variability in the Gal4-UAS system, to positional effects of transgene insertions, or to differences in transgene expression levels. However, a more reasonable explanation is that the decline in relative rescue efficiency among these Hox proteins, as well as the qualitative difference between Abd-B and the other Hox proteins in their ability to rescue the *lab* mutant brain phenotype, is due primarily to Hox protein sequence differences. Hox proteins do indeed show sequence differences, the most notable of which reside in the homeodomain, the hexapeptide motif (lacking in Abd-B), and the linker lengths between the homeodomain and the hexapeptide motif (Gehring et al., 1994; Duboule, 1994; Mann, 1995; Chan et al., 1996; Mann and Chan, 1996; Piper et al., 1999; Passner et al., 1999).

We posit that the findings reported here have implications for understanding Hox gene function and evolution. The functional equivalence of almost all of the Hox proteins in brain neuromere specification implies that the specificity of Hox gene action is achieved mainly through regulatory elements that control position, timing and level of Hox gene expression and only to a lesser degree through Hox protein sequence differences. Similar findings have been obtained in studies on Pax gene interchangeability in *Drosophila* (Li and Noll, 1994). Thus, the genes *paired* and *gooseberry*, which have distinct developmental roles in embryogenesis and have considerably diverged coding sequences, can exert the same conserved function in genetic rescue experiments. Comparable findings have recently been reported in mammals (Bouchard et al., 2000), corroborating the idea put forward by Noll that the essential difference among these developmental regulatory genes of the same family may reside in their *cis*-regulatory regions.

The fact that the expression of different Hox genes in the *lab* mutant domain does not cause homeotic transformation of tritocerebral identity, suggests that Hox proteins act as 'mediators' rather than as 'selectors' within the developmental pathway that specifies segmental neuronal identity in the *Drosophila* brain. Recent experiments using both loss- and gain-of-function mutations suggest that this also applies to the specification of other structures along the anteroposterior body axis of *Drosophila*. For example, in haltere development, *abd-A* and to some extent *Abd-B* can substitute for *Ubx* gene action (Casares et al., 1996). Moreover, a comparable lack of Hox gene specificity has been observed in gonad development (Greig and Akam, 1995).

Finally, the high degree of functional interchangeability of Lab and all of the other *Drosophila* Hox proteins, with the exception of Abd-B, is consistent with evolutionary studies that



propose a common origin of all of the Hox genes from a single ancestral progenitor and an early singularity of *Abd-B*-like genes in the ancestral Hox gene cluster (Schubert et al., 1993). Given the striking evolutionary conservation of structure, expression and brain-specific function of *lab* and its mammalian *Hox1* orthologs (Hirth and Reichert, 1999; Reichert and Simeone, 1999), it will now be important to determine whether functional equivalence among non-paralogous Hox gene products is also valid for vertebrate hindbrain development.

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