Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*

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Correct regulation of the segment-restricted patterns of *Hox* gene expression is essential for proper patterning of the vertebrate hindbrain. We have examined the molecular basis of restricted expression of *Hoxb2* in rhombomere 4 (r4), by using deletion analysis in transgenic mice to identify an r4 enhancer from the mouse gene. A bipartite Hox/Pbx binding motif is located within this enhancer, and in vitro DNA binding experiments showed that the vertebrate labial-related protein Hoxb1 will cooperatively bind to this site in a Pbx/Exd-dependent manner. The *Hoxb2* r4 enhancer can be transactivated in vivo by the ectopic expression of *Hoxb1*, *Hoxa1*, and *Drosophila labial* in transgenic mice. In contrast, ectopic *Hoxb2* and *Hoxb4* are unable to induce expression, indicating that in vivo this enhancer preferentially responds to *labial* family members. Mutational analysis demonstrated that the bipartite Hox/Pbx motif is required for r4 enhancer activity and the responses to retinoids and ectopic *Hox* expression. Furthermore, three copies of the *Hoxb2* motif are sufficient to mediate r4 expression in transgenic mouse embryos and a *labial* pattern in *Drosophila* embryos. This reporter expression in *Drosophila* embryos is dependent upon endogenous *labial* and *exd*, suggesting that the ability of this Hox/Pbx site to interact with labial-related proteins has been evolutionarily conserved. The endogenous *Hoxb2* gene is no longer upregulated in r4 in *Hoxb1* homozygous mutant embryos. On the basis of these experiments we conclude that the r4-restricted domain of *Hoxb2* in the hindbrain is the result of a direct cross-regulatory interaction by Hoxb1 involving vertebrate Pbx proteins as cofactors. This suggests that part of the functional role of *Hoxb1* in maintaining r4 identity may be mediated by the *Hoxb2* gene.

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Segmentation in the developing vertebrate hindbrain generates repeated morphological units, termed rhombomeres. These segmental units are lineage-restricted cellular compartments that provide a means of allocating blocks of cells that have distinct properties (for review, see Lumsden 1990; Wilkinson 1993; Keynes and Krumlauf 1994). Underlying this cellular organization, the patterns of expression of a number of transcription factors, growth factors, tyrosine kinase receptors, and their ligands have boundaries of expression that are tightly linked to specific hindbrain segments (for review, see Keynes and Krumlauf 1994; Lumsden and Krumlauf 1996). Prominent among these are the *Hox* genes whose expression patterns form an ordered set of overlapping domains that correlate with their gene order along the

chromosome (Hunt et al. 1991; McGinnis and Krumlauf 1992; Keynes and Krumlauf 1994). Mutational analyses and ectopic expression studies in vertebrates and *Drosophila* (for review, see McGinnis and Krumlauf 1992; Krumlauf 1993b) have clearly demonstrated that the *Hox/HOM-C* genes are key regulators of patterning along the anteroposterior (A-P) axis. In the vertebrate hindbrain, for example, ectopic expression of *Hoxa1* (Zhang et al. 1994; Alexandre et al. 1996) or induction of *Hoxa1* and *Hoxb1* by retinoids (Marshall et al. 1992; Kessel 1993; Hill et al. 1995) in mouse and fish embryos have led to a rhombomere 2 (r2) to r4 transformation and strongly imply that *Hox* genes are involved in specification of rhombomere identity. Conversely, loss-of-function experiments of mouse *Hoxb1* (Goddard et al. 1996; Studer et al. 1996) and *Hoxa1* (Carpenter et al. 1993; Dolle et al. 1993; Mark et al. 1993) have led to anterior shifts in rhombomere identity or rhombomere deletions, respectively. Thus, although the role of *Hox* genes in the hindbrain is important, we have a rather limited understanding of the upstream molecular cas-

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cade that regulates the establishment and maintenance of their rhombomere-restricted domains of expression.

The zinc finger-containing gene *Krox20*, which is specifically expressed in r3 and r5 (Wilkinson et al. 1989) is an important upstream component in the control of hindbrain patterning, as loss of *Krox20* results in the progressive deletion of r3 and r5 (Schneider-Maunoury et al. 1993; Swiatek and Gridley 1993). Furthermore, in the hindbrain the group 2 paralogous genes *Hoxa2* and *Hoxb2*, related to *Drosophila proboscipedia*, are up-regulated in r3 and r5, and this aspect of their segmental expression is directly regulated by *Krox20* (Sham et al. 1993; Nonchev et al. 1996a,b; Vesque et al. 1996). Because these two genes arose by duplication and divergence from a common ancestor, it is not surprising that they are controlled by the same upstream components if *cis*-regulatory regions have also been conserved during evolution.

Despite the similarities in expression and regulation between paralogs there is frequently variation in their relative levels within specific segments (Krumlauf 1993a; Keynes and Krumlauf 1994). Thus for group 2 genes in r4, *Hoxb2* is up-regulated and *Hoxa2* is not, whereas in r2 only *Hoxa2* is expressed (Krumlauf 1993a). This type of differential expression suggests that in even-numbered rhombomeres, these two genes have very different modes of regulation.

In utero exposure of mouse embryos at 7.5 days post-coitum (dpc) to ectopic doses of retinoic acid (RA) induces anterior shifts in the expression patterns of several *Hox* genes, including *Hoxb2* (Morris-Kay et al. 1991; Conlon and Rossant 1992; Marshall et al. 1992; Kessel 1993; Conlon 1995). Under these conditions, RA specifically activates the expression of *Hoxb1* and *Hoxb2* in r2, and associated with this there is a homeotic transformation where r2 adopts an r4-like identity (Marshall et al. 1992). Therefore, these two genes might both be directly involved in mediating this transformation. Previously, we found that the r4-restricted pattern of *Hoxb1* and the late response of *Hoxb1* to RA are controlled by a direct autoregulatory loop, involving a highly conserved bipartite *Hoxb1*(labial)/Pbx-binding site (Popperl et al. 1995; Chan and Mann 1996; Chan et al. 1996).

We were also particularly interested in the mechanism controlling the normal up-regulation of *Hoxb2* in r4 and its response to ectopic doses of RA. Therefore, we used transgenic analysis in mouse and *Drosophila* embryos to characterize *cis*-acting elements and upstream components mediating these patterns of expression. Our findings demonstrate that *Hoxb2* is a target for *Hoxb1* in r4 that emphasizes the importance of cross-regulatory interactions between *Hoxb1* and *Hoxb2* during hindbrain segmentation.

Results

Identification of a *Hoxb2* r4 enhancer

In the developing mouse hindbrain the *Hoxb2* gene is initially expressed in a uniform manner to an anterior

limit at the future r2/r3 boundary (8.0 dpc), but within 12 hr (8.5 dpc) the gene is up-regulated specifically in r3, r4, and r5 (Sham et al. 1993). Elevated expression in r3 and r5 is controlled by the zinc finger transcription factor *Krox20* (Sham et al. 1993; Vesque et al. 1996), and we were interested in determining the molecular mechanisms governing r4-restricted expression of *Hoxb2*. Therefore, *lacZ* reporter constructs in transgenic mice were used to map *cis*-acting elements required in vivo for r4-specific expression.

For deletion analysis we started with a 2.1-kb *Bam*HI–*Eco*RI fragment 5' of the mouse *Hoxb2* gene (Fig. 1A), previously found to direct up-regulation in r3, r4, and r5 (Sham et al. 1993; Vesque et al. 1996). A 1.4-kb subfragment (*Bgl*II–*Eco*RI; construct 1, Fig. 1A), deleting the *Krox20*-binding sites, mediated reporter staining in r4 and its associated neural crest, which migrates into the second branchial arch (Fig. 2A,B). Progressive deletions (constructs 2–9; Fig. 1A,B) identified a 181-bp *Stu*I fragment (construct 9) also able to confer expression of the reporter specifically in r4 and associated crest, in a manner similar to the 1.4-kb subfragment (Fig. 2C–F). Expression of *Hoxb2* is ectopically induced in anterior regions of the hindbrain and midbrain by exposure of embryos to RA (Conlon and Rossant 1992; Marshall et al. 1992), and we found that both the 1.4-kb and 181-bp fragments are able to mediate a response to ectopic RA treatment (Fig. 2G,H). Because all of the fragments were tested on a heterologous promoter in the opposite orientation to that found in the endogenous locus, we conclude that this 181-bp *Hoxb2* region functions as an r4 enhancer capable of mediating a retinoid response.

Sequence analysis of the 181-bp *Hoxb2* r4 enhancer

The sequence of this 181-bp r4 enhancer is presented in Figure 3A, and as a first step toward identifying upstream factors we performed a search of the Eukaryotic Transcription Factor Database (TFD) (Ghosh 1993). There were no consensus RA response elements (RAREs) of the direct repeat class, suggesting that the induction by RA was indirect, and no obvious candidates for the r4 activity were revealed by this approach. However, we noted the presence of a single motif (boxed in Fig. 3A) highly related to a repeated motif identified in the *Hoxb1* locus (Popperl et al. 1995). An alignment of the site in *Hoxb2* with the three sites from *Hoxb1* (Fig. 3B) indicates that this motif is related to a bipartite Pbx/Hox consensus site (Chan and Mann 1996). The part of the *Hoxb2* motif corresponding to the Pbx-binding site (5'-AGATTG-3') closely matches the consensus Pbx/Extradenticle (Exd)-binding site from the *Hoxb1* elements. However, the Hox-binding site (5'-TGATCG-3') in the bipartite motif differs from all of those in *Hoxb1* at position 5 showing that the *Hoxb2* motif is not identical to any of the motifs in *Hoxb1*.

Differential response of the *Hoxb2* enhancer to ectopic Hox expression

r4 expression of *Hoxb2* might arise through direct auto-

Figure 1. Transgenic constructs and mapping a *Hoxb2* r4 enhancer. (A–C) At the top is a restriction map of *Hoxb2* 5'-flanking regions, and below are the relevant subfragments cloned into the BGZ40 vector for transgenic analysis. (Right) The construct numbers, domains of expression, and frequency of expression of each construct. The three solid filled ellipses represent the position of the Krox20-binding sites that regulate r3 and r5 expression of *Hoxb2* (Sham et al. 1993); the open ellipse indicates the Hox/Pbx site identified in this study.

or cross-regulatory mechanisms involving Hox and Pbx proteins acting through the *Hoxb2* bipartite motif. In light of the divergence of the *Hoxb2* motif we first wished to address whether the *Hoxb2* r4 enhancer was capable of responding to ectopically expressed Hox genes in transgenic mice. We used a human β -actin promoter/enhancer to generate widespread expression in vivo of a number of Hox genes. Because only members of paralogous groups 1 and 2 are normally expressed in the region of r4 (Krumlauf 1993a) we initially focused on *Hoxb2*, *Hoxb1*, and *Hoxa1*.

In the background of a transgenic line containing the 1.4-kb *Hoxb2* r4 enhancer (construct 1), ectopic expression of *Hoxb1* activates the *lacZ* transgene in regions anterior to the normal r4 domain compared with control transgenic embryos (Fig. 4A,D). *Hoxa1* transactivates the *lacZ* reporter in a manner similar to that of *Hoxb1* (Fig. 4E). Furthermore, we observed that ectopic expression of the *Drosophila labial (lab)* gene also transactivated the r4 reporter (Fig. 4F), suggesting that labial-related proteins in general have conserved the ability to stimulate

this enhancer. In contrast, ectopic *Hoxb2* did not transactivate the r4-*lacZ* reporter (Fig. 4B), although this construct produces functional protein, as evidenced by highly penetrant skeletal phenotypes (data not shown). Therefore, the *Hoxb2* motif is able to discriminate between *Hoxb2* and group 1 Hox proteins in vivo. To determine whether this differential response is unique to *Hoxb2* we tested *Hoxb4* and found that it also failed to activate the reporter (Fig. 4C), although it is capable of activating other target sequences in vivo (Gould et al. 1997). Hence, the *Hoxb2* enhancer mediates a selective Hox response in vivo, which displays a preference for lab-related proteins.

Cooperative binding of *Hoxb1* and *Exd* to the *Hoxb2* motif

These results imply that the r4-specific expression of *Hoxb2* is not a consequence of its own autoregulation but may result from a cross-regulatory interaction with

Figure 2. Identification of a 181-bp r4 enhancer and response to RA in transgenic embryos. (A–F) Lateral or dorsal views of *lacZ* staining patterns in r4 of transgenic embryos generated from a progressive deletion series. Construct numbers are indicated above or below the panels, and anterior is at the top. (F) Staining from a 181-bp r4 enhancer defined by the smallest deletion. (G,H) The *Hoxb2* r4 enhancer responds to ectopic doses of RA and activates expression anteriorly in r2. (RA) Retinoic acid; (NC) neural crest; (OV) otic vesicle; (open arrows) transactivation in r2.

group 1 genes. It seemed likely that the conserved bipartite Pbx/Hox motif in the *Hoxb2* enhancer was involved in the transactivation response and, because of the sequence differences compared with the *Hoxb1* motifs, we tested its ability to bind Hoxb1 as a glutathione *S*-transferase (GST)–fusion protein in vitro by electrophoretic mobility shift assays (EMSAs). The Exd and Hoxb1 proteins alone do not bind to a 21-bp double-stranded oligonucleotide spanning the *Hoxb2* Pbx/Hox consensus site (Fig. 3C; wtB2); however, when added together they generate a slower migrating complex in a manner identical to that observed with repeat 3 from *Hoxb1* (Fig. 3D; Popperl et al. 1995). We have confirmed the presence of Hoxb1 and Exd in the high molecular weight complex by using supershift assays with antibodies against Hoxb1 and an epitope-tagged version of Exd (Fig. 3D; data not shown). Furthermore, the introduction of four point mutations in the core Pbx/Hox motif (Fig. 3C; mB2) abolished cooperative binding of the Hoxb1 and Exd proteins in the assay (Fig. 3D). Therefore, in vitro Hoxb1 is able to bind to the *Hoxb2* motif in an Exd-dependent manner.

The Hoxb2 Pbx/Hox site is necessary for r4 activity, retinoid response, and Hox transactivation of the enhancer in vivo

To address the in vivo significance of the *Hoxb2* motif, we analyzed the function of this site in the context of a 2.1-kb *Bam*HI–*Eco*RI enhancer (construct 10; Fig. 1C), which contains control elements necessary for up-regulation of *Hoxb2* in r3, r4, and r5 (Fig. 5A; Sham et al. 1993). A 7-bp deletion in the core of the *Hoxb2* motif

(construct 11; Fig. 1C) led to a loss of expression of the *lacZ* reporter specifically in r4, whereas the r3 and r5 domains of expression regulated by the Krox20 sites were unaffected (Fig. 5B). In addition, a construct carrying the same 4-bp mutation that abrogated in vitro binding of GST–Hoxb1/Exd to the *Hoxb2* motif (construct 12; Fig. 1C) also led to the loss of expression specifically in r4 (Fig. 5C).

Although the Pbx/Hox site in the *Hoxb2* enhancer is required in vivo for r4 activity, we wanted to determine whether it was also required for the retinoid and Hox responses mediated by the enhancer. Embryos transgenic for construct 11 carrying the deletion of the Pbx/Hox site failed to respond to RA treatment in utero at 7.75 dpc and also to ectopic expression of either *Hoxa1* (0/6) or *Hoxb1* (0/5) (data not shown) under the same conditions that activated the wild-type reporter. This provides evidence that the normal r4 activity, as well as the ectopic responses to lab-related proteins and retinoids, is acting through the same bipartite Pbx/Hox site.

The Hoxb2 motif is sufficient to direct restricted expression in transgenic mice and Drosophila embryos

The three Hoxb1/Pbx-binding sites in the 5' region of *Hoxb1* are not functionally equivalent in vivo and in vitro (Popperl et al. 1995). Because the sequence of the *Hoxb2* motif is different and there is only one copy in the enhancer, we examined whether it was sufficient to direct r4 expression. Three copies of the same wtB2 21-bp oligonucleotide used in the EMSA assay, linked to a *lacZ* reporter gene, were sufficient to confer r4 expression (Fig. 5D,E). Staining was strong ventrally in r4, with a sharp anterior boundary at the junction between r3 and r4, and there was some midline staining in r5 and r6 similar to that observed with the full 181-bp enhancer (cf. Fig. 5, D and E with Fig. 2F).

The *Drosophila lab* gene is able to transactivate the r4 enhancer in transgenic mice (Fig. 4F), and to provide further evidence that lab-related proteins are able to interact on the *Hoxb2* motif in vivo, we tested the ability of the *Hoxb2* motif to direct expression in *Drosophila* embryos. The *lacZ* reporter gene, *3XB2-lacZ*, which has three copies of the *Hoxb2* motif upstream of a minimal promoter driving *lacZ*, was expressed in a *lab*-like pattern in *Drosophila* embryos (Fig. 6). To confirm this we directly compared transgene expression with that of the endogenous labial protein using antibodies in both stage-matched embryos (Fig. 6A–F) and the same embryos (Fig. 6G–I). In stage 11 embryos, expression was observed in the procephalon and posterior midgut primordia (pmg) (Fig. 6A,D). In older embryos (stage 14), expression was also observed in a *lab* pattern in neural and ectodermal cells of the head and in endodermal cells of the midgut (Fig. 6B,C,E–H). The double-stained embryos clearly show the overlap (yellow) between the transgene (green) and *lab* (red) expression (Fig. 6G–I). However, we noted that the *3XB2-lacZ* construct was also expressed in some places where endogenous *lab* is not expressed within the labial and maxillary segments (Fig. 6A–F),

Figure 3. A Hox/Pbx-binding site in the *Hoxb2* r4 enhancer and cooperative binding of Hoxb1 and Exd proteins to the site in vitro. (A) Sequence of the 181-bp r4 *StuI* enhancer. (B) Alignment of the bipartite Hox/Pbx site in the *Hoxb2* enhancer with the three repeats from a *Hoxb1* r4 enhancer and a Hox/Pbx consensus sequence. The boxes in A and B indicate the Hox- and Pbx-binding sites and the bipartite consensus motif. (C) Sequence of wild-type (wtB2) and mutant (mB2) oligonucleotides used for in vitro EMSA and transgenic analysis. (D) EMSA of GST-Hoxb1 and Exd binding to the wild-type (wtB2) and mutant (mB2) *Hoxb2* oligonucleotides and the wild-type *Hoxb1* repeat 3 (B1-R3) Pbx/Hox motifs. (Top) The relevant proteins, antibodies, and pre-immune serum and their concentrations that were incubated with the different double-stranded probes. The respective probes are indicated at the bottom. B1-R3 is the repeat 3 probe from *Hoxb1* as a control as described previously (Popperl et al. 1995). The sequence of the wild-type and mutant *Hoxb2* oligonucleotides used are indicated in C. Hoxb1/Exd (D) indicates the slower migrating complex formed on the sites; SS indicates the supershift of this complex specifically with anti-Hoxb1 antibody (α -B1) and not with pre-immune serum (PI). (FP) Free probe.

suggesting that some *labial*-independent expression is directed by this enhancer.

Hoxb2 reporter expression in *Drosophila* embryos is dependent on *lab* and *exd*

To assess whether endogenous *lab* and *exd* are involved in controlling the overlap between reporter and *lab* expression detailed above, we mated these lines into the appropriate mutant backgrounds. At stage 14 in *lab* mutant embryos carrying the *3XB2-lacZ* construct, reporter expression is specifically lost in all those domains in the endoderm and the labial and maxillary segments showing overlaps with endogenous labial protein but persists in the remaining sites (Fig. 7A,B). Identical results were also observed at stage 16 (data not shown). Furthermore, removal of both maternal and zygotic *exd* led to a more extensive loss of reporter expression (Fig. 7C,D). Virtually all reporter expression is absent in the *exd* mutants and not just the *lab* domain, indicating that there is a common requirement for Exd. These results demonstrate the in vivo requirement of *lab* and *exd* in mediating expression through the *Hoxb2* motif, and together with the mouse experiments suggest that the ability of

the Pbx/Hox site to interact with lab-related proteins has been evolutionarily conserved.

Up-regulation of *Hoxb2* in r4 is lost in *Hoxb1* mutants

Hoxb1 is the best candidate responsible for the cross-regulation of *Hoxb2* in r4, as it is the only member of paralogous group 1 expressed at a high level in r4 when this up-regulation occurs (Hunt et al. 1991; Murphy and Hill 1991; Krumlauf 1993a). We have recently generated a null mutation in the *Hoxb1* gene (Studer et al. 1996), and here we examined *Hoxb2* expression in these mutants to address the issue of whether *Hoxb1* is required for *Hoxb2* up-regulation (Fig. 8). In wild-type embryos the level of *Hoxb2* expression in r4 steadily increases between 9.0 and 11.5 dpc, whereas the r3 and then the r5 domains regulated by *Krox20* are being progressively down-regulated during this period (Fig. 8A,C; and Wilkinson et al. 1989; Sham et al. 1993). Although up-regulation of *Hoxb2* expression in r4 has started in wild-type embryos at 9.5 dpc (Fig. 8A), this is not observed in *Hoxb1* $-/-$ mutants (Fig. 8B). High-level *Hoxb2* expression in r4 is most apparent at 10.5 dpc, when the r5 domain persists but the r3 domain is reduced to near

Figure 4. Selective transactivation of the *Hoxb2* r4 enhancer by *lab*-related genes in transgenic mice. (A) Reporter staining in a control transgenic embryo carrying construct 1 (Fig. 1A). (B,C) Ectopic expression of *Hoxb2* (B) and *Hoxb4* (C) in this background fails to induce transgene expression. (D–F) However, ectopic expression of the *lab*-related genes *Hoxb1* (D), *Hoxa1* (E), or *lab* (F) transactivate the *Hoxb2* enhancer in regions anterior to r4 (see open arrows). Below each panel is indicated the ectopic expression construct/gene used for transactivation. Transactivation efficiencies (no. of positive transactivations/no. of transgenic embryos): *Hoxb2* (0/8); *Hoxb4* (0/7); *Hoxb1* (4/5); *Hoxa1* (2/4); *lab* (2/2). All embryos are assayed at 9.5 dpc.

background levels (*Hoxb1* +/–; Fig. 8C). However, in homozygous *Hoxb1* mutant embryos up-regulation in r4 is never observed and remains very weak, approximating that seen in r3 (*Hoxb1* –/–; Fig. 8D). This confirms that the r4-restricted domain of *Hoxb2* in the mouse hindbrain is dependent on a cross-regulatory interaction by the *Hoxb1* gene in this segment. Taken together, these data lead us to conclude that *Hoxb2* is a direct target of the *Hoxb1* gene.

Discussion

In the vertebrate hindbrain the regulatory mechanisms underlying the generation and maintenance of rhombomere-restricted patterns of *Hox* expression are slowly beginning to emerge. Genes such as *Krox20* (Sham et al. 1993; Nonchev et al. 1996b) and *kreisler* (Manzanares et al. 1997) are upstream in the pathway, but because of the presence of four related *Hox* complexes encoding proteins with similar DNA-binding abilities, it has been difficult to assess the role of auto- and cross-regulatory cir-

cuits, like those observed for the *Drosophila* *HOM-C* genes in controlling segmental expression. The experiments presented in this study illustrate the importance of positive cross-regulatory interactions among *Hox* genes. A bipartite *Hox/Pbx* motif in the *Hoxb2* locus, both necessary and sufficient for r4 expression, mediates a selective ectopic response to the *lab*-related genes in transgenic mice. Evidence that this site functions in vivo as a *Hox/Pbx* target was provided by the ability of this motif to direct expression patterns in *Drosophila* embryos that were dependent on both endogenous *lab* and *exd*. There is cooperative binding in vitro between *Hoxb1* and *Pbx/Exd* proteins on this *cis*-acting element and an in vivo requirement of endogenous *Hoxb1* for the up-regulation of *Hoxb2* in r4. Together, these findings suggest that positive cross-regulation by *Hoxb1*, through this *Hox/Pbx* site, is responsible for directing the r4-restricted aspect of *Hoxb2* expression during hindbrain segmentation. Therefore, even though there is a continuous domain from r3–r5 where *Hoxb2* is up-regulated, this is not mediated by a single control element and represents the summation of two different mechanisms involving *Krox20* in r3 and r5 and *Hoxb1* cross-regulation in r4.

Figure 5. The *Hox/Pbx* motif is both necessary and sufficient for r4 activity of the *Hoxb2* enhancer. (A) Expression of the wild-type 2.1-kb *Bam*HI–*Eco*RI enhancer in r3, r4, and r5. (B,C) Specific loss of r4 expression if the *Hox/Pbx* site is deleted (B) or mutated (C) in the 2.1-kb enhancer. (D) Three copies of the *Hoxb2* motif are sufficient to direct r4 expression in the mouse hindbrain. (E) Flat mount of the hindbrain of the embryo in D showing staining in r4. There is some midline staining in r5 also seen with the 181-bp enhancer. The nature of the wild-type and mutant sites is indicated in diagrams below each panel. (OV) Otic vesicle.

Figure 6. The mouse *Hoxb2* motif directs *lab*-like expression patterns in *Drosophila* embryos. (A–C) *lacZ* expression driven by the reporter gene *3XB2-lacZ*; (D–F) expression of the endogenous *lab* gene; (G–I) confocal micrographs showing overlapping expression of *3XB2-lacZ* (green) and *lab* (red). A and D are lateral views of stage 11 embryos; B and E are lateral views of stage 14 embryos; C and F are ventral views of stage 14 embryos. Similarities between the *lab* and *3XB2-lacZ* expression patterns are indicated by open arrows; positions of non-*lab* expression by *3XB2-lacZ* are indicated by asterisks (*). (G–I) Positions of overlapping expression appear yellow. (G) Stage 14 head, focused on the presumptive neural expression (pnr); (H) stage 14 midgut, focused on the endoderm (end); (I) stage 17 head, focused on the V-shaped staining pattern that is characteristic of *lab* expression at this stage (Chouinard and Kaufman 1991). (ect) ectoderm cells of the head; (pmg) posterior midgut primordia.

Regulation of *Hoxb2* in r4

The transgenic experiments strongly suggested that a vertebrate *lab*-related gene is involved in r4 regulation of *Hoxb2*, as transactivation of the *r4-lacZ* reporter was observed with ectopic *Hoxa1*, *Hoxb1*, and *lab* but not with *Hoxb2* or *Hoxb4* (Fig. 4). We favor the idea that *Hoxb1* is normally involved in the r4 regulation of *Hoxb2* because (1) it is the only mouse *labial*-related group 1 gene expressed in r4 at the time of the up-regulation of *Hoxb2* (Hunt et al. 1991; Murphy and Hill 1991); (2) *Hoxb2* expression is unaltered in *Hoxa1* $-/-$

mutant embryos (Dolle et al. 1993); and (3) r4 up-regulation of *Hoxb2* does not occur in *Hoxb1* $-/-$ mutant embryos (Fig. 8). The latter observation is in contrast to a recent study on *Hoxb1* mutants, in which expression of *Hoxb2* at 9.5 dpc was reported to be unchanged (Goddard et al. 1996). However, in our analysis we demonstrate that differences in the up-regulation of *Hoxb2* in r4 are most apparent at 10.5 dpc (Fig. 8), so temporal differences in the stage of analysis are presumably responsible for this discrepancy.

The RA and Hox responses of the enhancer are abolished by mutations in the bipartite Pbx/Hox site, illustrating that this site not only governs r4 regulation but these additional responses. The mapping of these responses to a common site helps to explain the response of the *Hoxb2* enhancer to RA in the absence of consensus RAREs. Previously it has been shown that RA reprograms the hindbrain and induces anterior shifts in both *Hoxb1* and *Hoxa1* expression (Conlon and Rossant 1992; Marshall et al. 1992; Kessel 1993), in part directly through RAREs located at the 3' end of the *Hoxa1* and *Hoxb1* genes (Langston and Gudas 1992; Marshall et al. 1994; Dupé et al. 1997). Therefore, the *Hoxb2* enhancer is stimulated by the ectopically induced *lab*-related genes and responds indirectly to RA through them.

The nature of the site in *Hoxb2*

The response of *Hoxb2* to *lab*-related proteins could be direct or indirect, but we favor the idea that in normal development it is a direct cross-regulation by *Hoxb1* because (1) a single Hoxb1/Pbx consensus site was identified in the *Hoxb2* r4 enhancer, which is both necessary and sufficient for r4-restricted expression; (2) this motif is also required for the ectopic Hox response in trans-

Figure 7. Expression directed by the mouse *Hoxb2* motif in *Drosophila* is dependent on endogenous *lab* and *exd*. (A) Lateral view of wild-type stage 14 embryo stained for *3XB2-lacZ* reporter expression; (B) *3XB2-lacZ* staining in stage-matched *lab* mutant embryos. Black arrows indicate expression in ectodermal cells of the head and endodermal midgut cells (end) that overlap with endogenous *lab* and are specifically lost in the *lab* mutants. White arrowheads indicate additional domains of staining in the head that are unaffected in the *lab* mutants (B). Dorsal view of *3XB2-lacZ* staining in stage 14 wild-type (C) and *exd* mutant embryos (D). Removal of both maternal and zygotic *exd* (D) result in the loss of virtually all reporter expression. (wt) Wild type; (lab-) *lab* mutants; and (exd-) *exd* mutant embryos.

Figure 8. Up-regulation of *Hoxb2* in r4 is specifically lost in *Hoxb1* mutants. (A–D) Flat mounts of embryos hybridized with *Hoxb2* probe. *Hoxb2* is normally expressed at high levels in r4 at 9.5 and 10.5 dpc (A,C), during the period where the r3 domain is progressively down-regulated. In *Hoxb1* mutant embryos (B,D), however, *Hoxb2* expression in r4 is not up-regulated. Note at 10.5 dpc the level of expression in r4 approximates that in r3 in mutant embryos (D). Arrows indicate the r4 domain where expression is altered in mutant and control embryos. Respective genotypes of the embryos are indicated above and below each panel.

genic mice; (3) in *Drosophila* embryos the motif mediates a *lab*-like expression pattern that is dependent on both *lab* and *exd*; and (4) GST-*Hoxb1* binds to the *Hoxb2* motif in vitro in an *Exd*-dependent manner, highlighting the importance of cooperativity between Hox and Pbx proteins in modulating binding specificity (Chan and Mann 1996; Chan et al. 1996; Mann and Chan 1996).

Our transactivation experiments reveal that the Pbx/Hox motif from *Hoxb2* displays a selective ability to respond in vivo to different Hox proteins. Although *Hoxa1*, *Hoxb1*, and *lab* all induced ectopic reporter expression, *Hoxb2* and *Hoxb4* had no effect. Hence, there is a preference for activation by *lab*-related proteins on this site. In contrast, recently a related Pbx/Hox site has been found as part of an auto/cross-regulatory region of the *Hoxb4* gene (Gould et al. 1997). Despite the sequence similarity with the *Hoxb2* Pbx/Hox motif, in vivo this *Hoxb4* element selectively responds very differently, as it is ectopically induced by *Hoxb4* but not *Hoxb1*. Such a change of in vivo specificity is in marked contrast to the general ability of most Hox proteins from paralog groups 1–9 to bind cooperatively with *Exd*/Pbx members in vitro in a graded manner to similar Pbx/Hox sites (Chang et al. 1996; Shen et al. 1996). This suggests that small differences in the Hox or Pbx core-binding sites

can have profound in vivo effects on selectivity of the Hox partner. One of the benefits of detailed in vivo characterization of functionally relevant Hox-binding sites is that it will help in the identification of *Hox* target genes, which is essential for the future understanding of morphogenesis at the molecular level.

Given that *lab* can transactivate the mouse *Hoxb2* r4 enhancer, multimers of the Hox/Pbx site in *Hoxb2* generate many aspects of the *lab* expression pattern in *Drosophila* embryos dependent on endogenous *lab* and *exd*, and the previous observation that *Hoxb1* will rescue *lab* phenotypes in *Drosophila* embryos (Lutz et al. 1996), show the extensive functional conservation of the Hox/*HOM-C lab* family. We noted that the *Hoxb2* motif stimulates *lacZ* reporter staining in additional regions of the *Drosophila* head (Fig. 6), which do not overlap with endogenous *lab* expression. In contrast, this was never observed in *Drosophila* embryos with the *Hoxb1*-repeat 3 transgenic reporter (Chan et al. 1996), suggesting that the *Hoxb2* motif is more promiscuous. However, it is important to note that these additional domains are still dependent on *exd* (Fig. 7D). Comparing repeat 3 with the *Hoxb2* motif, there are two differences in the Hox part of the binding consensus. The general Hox consensus is 5'-NNATNN-3' and the *Hoxb2* motif has a T in position 1 and a C in position 5, where repeat 3 has a G in positions 1 and 5 (Fig. 3A–C). In vitro, differences in the first 2 bp are important for specifying which Hox protein is preferred in the Hox/*Exd* heterodimer (Chan and Mann 1996; Chang et al. 1996; Mann and Chan 1996), but the influence of variations in position 5 have not been examined. One interpretation of the wider expression pattern generated by the *Hoxb2* motif compared with repeat 3 is that other Hox or Pbx proteins might be able to interact on this site. In this regard, we note that the G in position 1 of the repeat 3 *Hoxb1*-binding site is unusual, whereas the T in this position of the *Hoxb2* motif is more typical of Hox proteins in general (Fig. 3B). Thus, having a G at this position may increase the specificity of this binding site for *lab*/*Hoxb1*, accounting for the more *lab*-specific activity of the repeat 3 binding site in vivo.

The Pbx site (5'-AGATTG-3') in the bipartite motif of *Hoxb2* fits the Pbx consensus (5'-TGATNN-3') (Chang et al. 1996) except that in position 1 there is an A. This change might also contribute to the wider expression in *Drosophila* embryos, but there is also an A in position 1 of repeat 1 from *Hoxb1* and it efficiently binds *Hoxb1* and *Exd* in vitro (Popperl et al. 1995). To date there are three vertebrate *Pbx* genes, and they can bind the consensus with comparable affinities (Lu et al. 1994). Because of the widespread expression patterns of the *Pbx* genes and the fact that in *Drosophila* the cellular localization of *Exd* protein is critical for its activity (Mann and Abu-Shaar 1996), it remains to be determined which Pbx protein(s) is the in vivo cofactor involved in regulating r4 expression of *Hoxb2* and *Hoxb1*.

Hoxb1 has an important role in maintaining or specifying segmental identity in r4 (Marshall et al. 1992; Zhang et al. 1994; Goddard et al. 1996; Studer et al.

1996). Because our results suggest that *Hoxb2* is a direct target of *Hoxb1* in r4, *Hoxb2* itself seems likely to have an integral role in the r4 patterning process and would mediate its influences in a *Hoxb1*-dependent manner. These experiments demonstrate that direct auto- and cross-regulatory interactions between *Hox* genes are integral components of segmental patterning in the vertebrate hindbrain.

Materials and methods

DNA constructs, manipulations, and sequencing

Reporter constructs detailed in Figure 1 were prepared by digestion of *Hoxb2* upstream regions with the appropriate restriction endonucleases and end-filling using T4 DNA polymerase and the four dNTPs, and subcloned into the BGZ40 *lacZ* reporter gene vector (Yee and Rigby 1993) containing a minimal human β -globin promoter. Constructs for the ectopic expression of *Hoxa1* and *Hoxb1* (Popperl et al. 1995) and *Hoxb4* (Gould et al. 1997) have been described previously. The *Hoxb2* and *Drosophila lab* ectopic expression constructs were prepared by subcloning a full-length *Hoxb2* cDNA (M.K. Maconochie, unpubl.) or an end-filled *EcoRI*–*HindIII* fragment from pLabial *SspI* containing the *lab* open reading frame and excluding introns (Chouinard and Kaufman 1991) into the same β -actin expression vector, respectively (Zhang et al. 1994; Popperl et al. 1995). The production of protein from the *Hoxb4* construct was confirmed by immunostaining (Gould et al. 1997).

Enhancer mutations (deletions and substitutions) were generated by site-directed mutagenesis using the Sculptor In Vitro mutagenesis kit (Amersham International Plc). Confirmation of the efficacy of mutagenesis and enhancer sequencing was according to the dideoxy chain termination method with Sequenase enzyme (U.S. Biochemical). The enhancer mutations and three copies of the wtB2 oligonucleotide (sequence in Fig. 3C) were subcloned into the end-filled *SpeI* site of BGZ40 as above. Unless specifically mentioned above, all DNA manipulations were carried out according to standard procedures as described in Sambrook et al. (1989).

Drosophila constructs and transgenic analysis

The *3XB2-lacZ* reporter gene was generated in the CPLZ P-element vector, and transgenic fly stocks were generated exactly as described for the repeat 3 reporter gene *3Xrpt3-lacZ* (Chan et al. 1996). Multiple transgenic fly stocks were examined as described (Chan et al. 1996) and all had very similar, if not identical, *lacZ* expression patterns. Positions of *lab* expression were evaluated according to published studies (Diederich et al. 1989; Chouinard and Kaufman 1991). The *lab* mutant line [*lab(11)*], the *exd* null allele [*exd(xp11)*], and removal of maternal and zygotic *exd* were performed as described previously (Chan et al. 1996).

Generation and analysis of transgenic mice

The DNA constructs generated above were microinjected as linearized inserts with vector sequences removed into fertilized mouse eggs generated from crosses of F₁ hybrids (CBA/CA $\times$ C57BL/10), and *lacZ* reporter activity was assayed as described previously (Whiting et al. 1991). In the transactivation experiments males homozygous for the construct 1 transgene (carrying the *Hoxb2* r4 enhancer) were mated to superovulated F₁ hybrid females to collect fertilized eggs for the subsequent

microinjection of various β -actin/cDNA ectopic expression constructs. F₀ embryos were then harvested to assay for changes in *lacZ* expression. RA treatment of embryos was as described previously where pregnant females were administered all-*trans* RA (Sigma) by gavage with sesame seed oil to provide a dose of ~20 mg/kg of maternal body weight (Marshall et al. 1992). In situ hybridization was as described previously (Wilkinson 1992).

Electrophoretic mobility shift assays

The production of GST–Hoxb1 fusion protein, polyclonal Hoxb1 antisera, and Exd homeodomain polypeptide have been described in detail elsewhere (Chan et al. 1994; Popperl et al. 1995; Studer et al. 1996). Polyclonal antiserum was recovered from rabbits immunized with the GST–Hoxb1 protein. Oligonucleotides used in the electrophoretic mobility shift assay (EMSA) have been described in Figure 3C. These were end-labeled using α -³²P-labeled dGTP and used for addressing in vitro binding activity of GST–Hoxb1 and Exd proteins alone and in combination in the amounts detailed in Figure 3D. The binding reactions were performed in 20- μ l reactions in buffers as described previously (Popperl et al. 1995). Briefly, for binding assays the buffer used was 20 mM HEPES–KOH (pH 7.9), 60 mM KCl, 5 mg/ml of BSA, 1 mM DTT, 0.5 mM EDTA, 12% glycerol, and 200 ng of poly[d(I–C)]. For the supershift demonstration 1 μ l of polyclonal antiserum was used, and 1 μ l pre-immune serum was used as control. Samples were incubated on ice for 20 min, and labeled oligonucleotides were added and incubated for an additional 30 min. The reactions were fractionated through a 6% polyacrylamide gel with 0.5 $\times$ TBE as running buffer for 75 min at 30 mA. Gels were vacuum dried and exposed to autoradiographic film.

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