Direct crossregulation between retinoic acid receptor β and Hox genes during hindbrain segmentation

Patricia Serpente[1], Stefan Tümpel[2], Norbert B. Ghyselinck[3], Karen Niederreither[3,*], Leanne M. Wiedemann[2,4], Pascal Dollé[3], Pierre Chambon[3], Robb Krumlauf[2,5] and Alex P. Gould[1,†]

[1]Medical Research Council, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK
[2]Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MI 64110, USA
[3]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS/INSERM/ULP/Collège de France, BP10142, 67404 ILLKIRCH Cedex, France
[4]Department of Pathology and Laboratory Medicine, Kansas University Medical Center, Kansas City, KS 66160, USA
[5]Department of Anatomy and Cell Biology, Kansas University Medical Center, Kansas City, KS 66160, USA
*Present address: Departments of Medicine and Molecular and Cellular Biology, Center for Cardiovascular Development, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
†Author for correspondence (e-mail: agould@nimr.mrc.ac.uk)

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Summary

During anteroposterior (AP) patterning of the developing hindbrain, the expression borders of many transcription factors are aligned at interfaces between neural segments called rhombomeres (r). Mechanisms regulating segmental expression have been identified for Hox genes, but for other classes of AP patterning genes there is only limited information. We have analysed the murine retinoic acid receptor β gene (*Rarb*) and show that it is induced prior to segmentation, by retinoic-acid (RA) signalling from the mesoderm. Induction establishes a diffuse expression border that regresses until, at later stages, it is stably maintained at the r6/r7 boundary by inputs from *Hoxb4* and *Hoxd4*. Separate RA- and Hox-responsive enhancers mediate the two phases of *Rarb* expression: a regulatory mechanism remarkably similar to that of *Hoxb4*. By showing that *Rarb* is a direct transcriptional target of *Hoxb4*, this study identifies a new molecular link, completing a feedback circuit between *Rarb*, *Hoxb4* and *Hoxd4*. We propose that the function of this circuit is to align the initially incongruent expression of multiple RA-induced genes at a single segment boundary.

Key words: Segmentation, Rhombomere, Hindbrain, Hox, Retinoic acid receptor

Introduction

During embryogenesis, the vertebrate hindbrain is transiently segmented into seven lineage-restricted units termed rhombomeres (Vaage, 1969; Fraser et al., 1990; Birgbauer and Fraser, 1994). These underlie the subsequent metameric arrangement of sensory and motor nerves within the adult brainstem. Early in hindbrain development, neuroepithelial cells are assigned a specific anterior-to-posterior (AP) identity, depending on their rhombomere of origin. This AP positional identity influences final cell fate and is regulated, at least in part, by the segmental expression of the Hox gene family of homeodomain transcription factors (reviewed by McGinnis and Krumlauf, 1992; Lumsden and Krumlauf, 1996; Capecchi, 1997). To pattern the normal repertoire of cell fates along the AP axis of the hindbrain, the anterior expression borders of at least eleven Hox genes from paralogue groups 1-4 must be accurately aligned at specific interfaces between rhombomeres.

Many different genetic inputs are known to regulate the multigene complexes in which Hox genes reside. These are integrated in a stage-dependent manner by multiple enhancer elements, some of which act on only one Hox gene, while others are shared between several members of a complex (reviewed by Trainor et al., 2000; Kmita and Duboule, 2003). Within the presegmented hindbrain, initiation of Hox gene expression requires a key intercellular signal derived from vitamin A, called retinoic acid (RA) (reviewed by Marshall et al., 1996; Eichele, 1997; Gavalas, 2002). This small lipophilic molecule is synthesized by retinal dehydrogenase 2 (Raldh2/Aldh1a2) within the developing paraxial mesoderm and induces Hox expression in the adjacent neural tube (Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999). The timing of *Raldh2* expression correlates well with the stages at which grafted chick somites are known to possess neural Hox-inducing activity (Grapin-Botton et al., 1995; Itasaki et al., 1996). Moreover, loss of *Raldh2* function produces AP patterning abnormalities and disrupted Hox expression patterns similar to those resulting from retinoid deficiency (Maden et al., 1996; Gale et al., 1999; Niederreither et al., 2000; White et al., 2000; Begemann et al., 2001). RA concentration is not only spatiotemporally regulated at the synthetic level but also via its local inactivation by cytochrome P450 monooxygenases (McCaffery and Drager, 2000; Abu-Abed et al., 2001; Niederreither et al., 2002). The importance of regulating RA availability during hindbrain development is underscored by several studies showing that the addition of exogenous RA produces dramatic rostral shifts in Hox expression boundaries

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(Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Dekker et al., 1992; Marshall et al., 1992). These and more recent studies (Gould et al., 1998; Dupé and Lumsden, 2001; Nolte et al., 2003) are consistent with the hypothesis that Hox genes are differentially sensitive to the level and/or timing of RA signalling and that this contributes to specifying their diverse expression borders within the hindbrain.

Specific enhancers containing retinoic acid response elements (RAREs) mediate RA induction of Hox gene transcription within the hindbrain. The in vivo characterization of these short DNA sequences from two genes of Hox paralogue group 1 (*Hoxa1* and *Hoxb1*) and two from group 4 (*Hoxb4* and *Hoxd4*) has revealed that they function by binding retinoic acid receptors (RARs) and retinoid X receptors (RXRs), probably as RAR/RXR heterodimers (Marshall et al., 1994; Frasch et al., 1995; Morrison et al., 1996; Dupé et al., 1997; Kastner et al., 1997; Gould et al., 1998; Mascrez et al., 1998; Studer et al., 1998; Zhang et al., 2000). Both classes of receptors interact directly with RA: the three RARs (α, β and γ) are capable of binding all-trans and 9-cis forms, whereas the α, β and γ RXRs bind 9-cis RA only (reviewed by Chambon, 1996). Owing to extensive functional redundancy, the crucial role of RARs in hindbrain segmentation and also in Hox regulation only becomes apparent when two are removed simultaneously or when the activities of all three RARs are attenuated using a chemical antagonist (Dupé et al., 1999; Dupé and Lumsden, 2001; Wendling et al., 2001). Thus, although RARβ is unique amongst RARs in having an anterior border of expression at the r6/r7 junction, its inactivation has no apparent effect on caudal hindbrain segmentation or Hox transcription (Ruberte et al., 1991; Ghyselinck et al., 1997; Dupé et al., 1999; Folberg et al., 1999). However, double mutants for *Rarb* and *Rara* do display a severely enlarged r5, loss of the r6/r7 boundary and absence of hindbrain *Hoxd4* expression (Dupé et al., 1999). On the basis of these and other observations, it has been proposed that the normal role of *Rarb* is to mediate a caudal increase in RA signalling, which is necessary for *Hoxd4* activation (Ghyselinck et al., 1997; Wendling et al., 2001).

Although much progress has been made in understanding how hindbrain patterns of Hox expression are generated, less is known about the regulatory mechanisms that govern the neural expression of their key upstream regulators, the RARs. We focus on *Rarb,* and use a combination of genetic, transgenic and biochemical analyses in the mouse and chick to dissect the mechanism generating its segmental expression border. These studies reveal that *Rarb* uses a two-step transcriptional regulatory mechanism: induction by a mesodermal RA signal and maintenance by *Hoxb4* and *Hoxd4*. They also show that *Rarb* is a direct transcriptional target of *Hoxb4*, thus providing evidence that transcriptional regulation between the Hox and RAR gene families is bidirectional. We describe how these results imply the presence of a self-organizing feedback circuit that corrects for small initial differences in the anterior expression borders of multiple Hox and RAR genes, bringing them into register at a rhombomere boundary.

Materials and methods

Breeding, genotyping and RA-treatment

At least three mouse embryos of the appropriate genotype were analysed for each data point. *Rarb*$^{-/-}$ embryos were generated and identified from heterozygous siblings by Southern blotting as described (Ghyselinck et al., 1997; Niederreither et al., 1999) and *Raldh2*$^{-/-}$ embryos were identified by a characteristic 'squashed somite' phenotype. The *Rarb1lacZ* and *Rarb2lacZ* lines described here correspond to *Rarb1/lacZ2* and *Rarb2/lacZ* respectively (Mendelsohn et al., 1994). *Hoxb4*$^{-/-}$*; Hoxd4*$^{-/-}$ double mutant embryos were generated as previously described (Gould et al., 1997). Yolk sacs of *Hoxb4*$^{-/-}$*; Hoxd4*$^{-/-}$ or *Hoxb4*$^{-/-}$*; Hoxd4*$^{-/-}$; *Rarb1lacZ* /+ or *Hoxb4*$^{-/-}$*; Hoxd4*$^{-/-}$; *Rarb2lacZ* /+ embryos were genotyped by PCR using the following primer combinations.

Hoxb4: CGGCTGGAAGCCGCTCTCTCGC (+ allele), CTGCATCCATGACACAGGCAAACC (– allele) and GAGCCCTATGTAAATCCTGGTGTTG (common).

Hoxd4: CCTACACCAGACAGCAAGTCCTAG (+ allele), CCCGTGATATTGCTGAAGAGCTTGG (– allele) and CTCGGGCAGGAAGGTAACCTAGTC (common)

lacZ: GCGACTTCCAGTTCAACATC (LZ3) and GATGAGTTTGGACAAACCAC (ZT4).

All-*trans* RA (25 mg/ml stock in dimethylsulphoxide) was diluted 1:10 in sesame oil and 200 μl per pregnant dam administered by gavage at E9.25 (~25 mg/kg bodyweight) as previously described (Gould et al., 1998).

X-gal and antibody staining and in situ hybridization

Staining with X-gal or a monoclonal antibody directed against murine Hoxb4 was as described (Gould et al., 1997). We have deposited anti-Hoxb4 at the Developmental Studies Hybridoma Bank (http://www.uiowa.edu/~dshbwww/). Rabbit anti-Krox20 antibody (Cambridge Bioscience) was used at a dilution of 1:100. For analysis by confocal microscopy, specimens were stained with Alexa Fluor 488 secondary antibodies (Molecular Probes), flatmounted in Vectashield with or without propidium iodide (Vector Laboratories), and images prepared using projections of several sections. In situ hybridization was performed as described (Wilkinson, 1992) except that, for *Rarb*, hybridization was at 70°C. Antisense riboprobes incorporating either digoxigenin or fluorescein were made using *Rarb* (Zelent et al., 1991), *Krox20* (Wilkinson et al., 1989a) or *Hoxb4* (Graham et al., 1988) templates.

Sequence analysis

RARβ intron sizes were determined by BLAST-like Alignment Tool (BLAT) (Kent, 2002) comparisons of known RARβ mRNAs (Zelent et al., 1991; Mendelsohn et al., 1994) with the UCSC Genome Browser Databases (Karolchik et al., 2003) (see http://genome.ucsc.edu) using the following genome builds: human May 2004 (hg17), chimp Nov 2003 (pan Tro1), mouse May 2004 (mm5), rat Jun 2003 (rn3) and dog Jul 2004 (canFam1). The distance between the HP site of the distal enhancer and the ATG of the first coding exon (E2) of the RARβ1 mRNA isoform is at least 241 kb in chimp and 215 kb in rat, although gaps in these genome builds indicate that these distances may be larger. There are no gaps in human (240 kb interval) and only a small number of gaps in dog (~232 kb interval) and mouse (~284 kb interval). The position of the P1 promoter is not yet clear in rat, chimp and dog, as 5′ untranslated exon(s) of RARβ1 have yet to be identified. For human, however, Toulouse et al. (Toulouse et al., 1996) have reported the 5′ end of the RARβ1 isoform (Genbank Accession Number U49855), which our BLAT comparisons show to be encoded by six non-coding exons, three of which lie upstream of the HP site. Multispecies HP site comparisons used MULTIZ (Blanchette et al., 2004).

Electrophoretic mobility shift assays (EMSA)

GST-Hoxb4 and Hoxb4 antibody production and EMSA were performed as previously described (Gould et al., 1997), except that Cy5 oligonucleotide labelling was used and gels were imaged on a Typhoon 8700 (Amersham Biosciences). Oligonucleotide sequences were as described (Gould et al., 1997) or as follows (Hox/Pbx sites

in bold, mutated residues underlined): HS1+HS2 WT (*Hoxb4*), GAGAATTATACAGAAAA**CCATTAATCA**CTT; HP WT (*Rarb*), TTTGAGGAGCAGGG**TGATAAATAA**TGGGGCTTTTCCA; and HP MUT (*Rarb*), TTTGAGGAGCAGGGTGGGCCCGCCGGG-GGCTTTTCCA.

Chick electroporation

The distal enhancer (a 2.3 kb *Nhe*I fragment) of *Rarb* was isolated by PCR from mouse genomic DNA using primers AGAATGTGTGT-GCTGACTCTGC and AAGCAGTCTTACCAGGAGGG, cloned into pGEM-T (Promega) and then transferred as a *Sac*II-*Not*I fragment into the BGZ40 vector (Maconochie et al., 1997). The HP site was mutated using the Quick Change kit (Stratagene) with the following 61 mer: CCTTGTGAAGTCCCCTTTGAGGAGCAGGGTGGGGC-TTTTCCAATTGTTATTTGCCAAAAGG. For the 3x HP construct, annealed oligonucleotides GGCCGCAAGCTTGAGCAGGGT-GATAAATAATGGGGCTTGAGCAGGGTGATAAATAATGGGG-CTTGAGCAGGGTGATAAATAATGGGGCTTCCGC and GGAAG-CCCCATTATTTATCACCCTGCTCAAGCCCCATTATTTATCACC-CTGCTCAAGCCCCATTATTTATCACCCTGCTCAAGCTTGC were cloned into *Not*I-*Sac*II cut pBSKS and then transferred into BGZ40. All constructs were confirmed by DNA sequencing.

Hamburger-Hamilton stages 9-11 chick embryos were electroporated as described (Itasaki et al., 1999). BGZ40 derivatives (1 μg/1μl) were co-electroporated with Fast Green and CMV-GFP. Only embryos showing strong GFP expression throughout the neural tube, were analyzed further.

Results

The early borders of *Rarb* and *Hoxb4* expression are different but both require *Raldh2* function

The murine hindbrain becomes segmented into morphologically visible rhombomeres between embryonic day 8.5 (E8.5) and E9.5. By E9.5, the anterior borders of expression of *Hoxb4*, *Hoxd4* and *Rarb* are all congruent at the r6/r7 junction (Fig. 1A,B) (Wilkinson et al., 1989b; Hunt et al., 1991; Ruberte et al., 1991).

We compared the regulation of *Rarb* and *Hoxb4* by mapping their borders of expression within the presegmented hindbrain at E8.5. At this stage, *Rarb* and *Hoxb4* have anterior limits of expression that are much less sharp than at E9.5. Using the otic sulcus as a morphological landmark for the presumptive r5/r6 boundary, we were surprised to find that the E8.5 expression border of *Rarb* lies significantly more anterior than that of *Hoxb4* (Fig. 1C,D). The rostral borders of both genes were then mapped more accurately by performing double labelling using *Krox20* expression as a landmark for presumptive r5 (Wilkinson et al., 1989a). In this way, neural expression was observed up to the level of the future r5/r6 boundary for *Rarb* but only as far as the approximate location of presumptive r6/r7 in the case of *Hoxb4* (Fig. 1E,F). These results reveal that the neural expression borders of *Hoxb4* and *Rarb* are different at early stages but become congruent at around the time when segmentation into r6 and r7 first becomes morphologically visible.

We then addressed the in vivo mechanisms regulating *Rarb* and *Hoxb4*. For several Hox genes, it is known that hindbrain expression is initiated prior to rhombomere formation by an inductive process involving RA synthesis by the paraxial mesoderm (see Introduction). In the case of *Hoxd4*, RA induction in the hindbrain is abolished in embryos lacking the activity of the RA-synthetic enzyme Raldh2 (Niederreither et al., 2000). We first focused on *Hoxb4* and examined the role of Raldh2 during its induction phase at E8.5 (Gould et al., 1998). At this early stage, the reduction in the amount of tissue in the postotic territory of the hindbrain that occurs progressively from E8.25-E9.5 in *Raldh2*$^{-/-}$ embryos is minimal (Niederreither et al., 2000). We find that both the mRNA and protein products of *Hoxb4* are only very weakly expressed in the presegmented hindbrain of *Raldh2*$^{-/-}$ embryos (Fig. 2A-D). Thus, like *Hoxd4*, the early hindbrain expression domain of *Hoxb4* requires RA and, as the major site of *Raldh2* expression at E8.5 is the paraxial mesoderm, this requirement probably signifies a mesodermal-to-neural induction. At spinal levels, however, a different mechanism of *Hoxb4* regulation

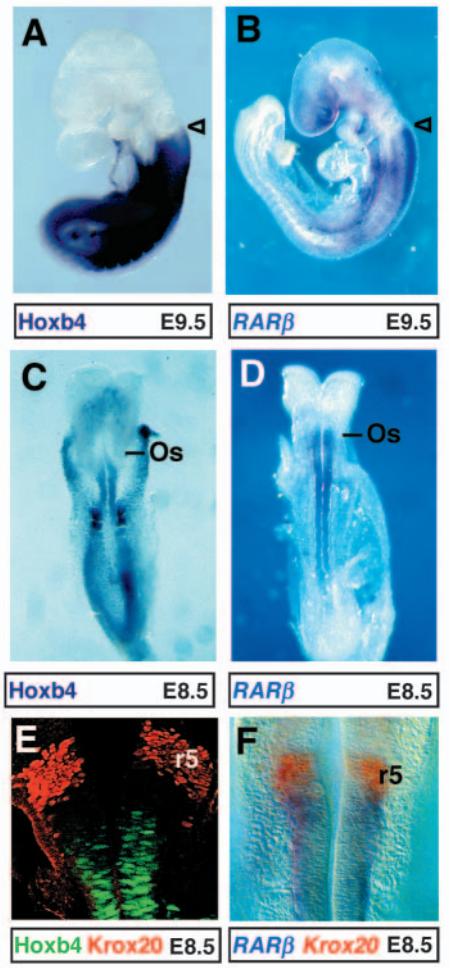

Fig. 1. *Rarb* is expressed more anteriorly than *Hoxb4* in the presegmented hindbrain. The anterior neural expression borders of Hoxb4 protein (A,C,E) and *Rarb* mRNA (B,D,F) are compared at E8.5 and E9.5. (A,B) At E9.5, neural expression of both genes extends up to a straight border at the r6/r7 boundary (arrowhead), lying just caudal to the otic vesicle. (C,D) At E8.5, using the otic sulcus (Os) as a landmark for presumptive r5/r6, it can be seen that neural *Rarb* expression extends more anteriorly than that of Hoxb4. (E,F) At E8.5, with *Krox20* expression marking presumptive r5, it can be observed that *Rarb* is expressed in all of presumptive r6 but Hoxb4 is not. At this stage, the anterior expression border of both genes is diffuse and, for *Rarb*, weak expression may also extend into the posterior part of presumptive r5. Anterior is towards the top in this and all subsequent figures.

must operate as neural expression remains unaffected at the mRNA level, paralleling previous *Hoxd4* findings (Niederreither et al., 2000). As we observe that *Hoxb4* expression in *Raldh2* mutants is dramatically reduced at the protein level in spinal regions (compare Fig. 2A,B with Fig. 2C,D) it is likely that, as yet uncharacterized, post-transcriptional Hox-regulatory mechanisms are involved (Brend et al., 2003; Dasen et al., 2003).

We next sought to identify the mechanism initiating the expression of *Rarb* in the presegmented hindbrain. As with *Hoxb4*, we examined *Rarb* expression in E8.5 embryos lacking Raldh2 activity. Strikingly, we find that *Rarb* mRNA is undetectable within the presumptive hindbrain and spinal cord

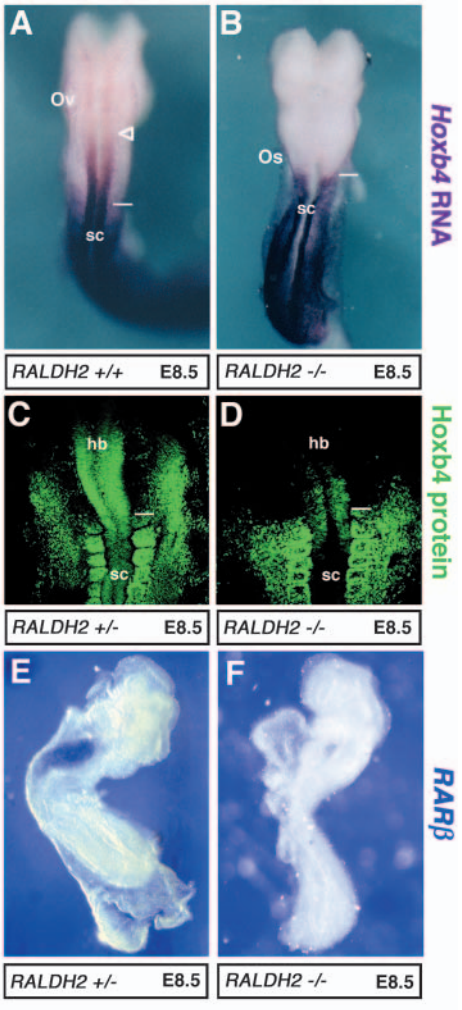

Fig. 2. Early hindbrain expression of *Hoxb4* and *Rarb* requires *Raldh2* activity. The effects of loss of *Raldh2* activity on the early neural expression of *Hoxb4* mRNA (A,B), Hoxb4 protein (C,D) and *Rarb* gene (E,F) are shown. (A,B) Dorsal views of E8.5 embryos showing the *Hoxb4* mRNA distribution in *Raldh2*$^{+/+}$ (A) and *Raldh2*$^{-/-}$ (B) genetic backgrounds. Loss of *Raldh2* function is associated with absence of *Hoxb4* mRNA within the hindbrain but expression within the developing spinal cord (sc) remains unaffected. (C,D) Dorsal views of E8.5 embryos showing Hoxb4 protein expression in *Raldh2*$^{+/-}$ (C) and *Raldh2*$^{-/-}$ (D) embryos. Removal of *Raldh2* activity leads to loss of most Hoxb4 protein within both the developing hindbrain (hb) and spinal cord (sc). However, weak expression is still observed within a few cells at caudal hindbrain and anterior spinal levels. (E,F) *Rarb* mRNA induction within the hindbrain requires *Raldh2* activity. Lateral views of E8.5 embryos of the genotypes *Raldh2*$^{+/-}$ (E) or *Raldh2*$^{-/-}$ (F). Loss of *Raldh2* activity is associated with the absence of all detectable *Rarb* expression within the developing hindbrain and spinal cord. Os and Ov indicate the otic sulcus and otic vesicle, lying adjacent to r5 and r6 in this and subsequent figures, and the approximate position of the paraxial mesodermal limit of *Hoxb4* expression at the somite 6/7 border is also shown (horizontal line in A-D).

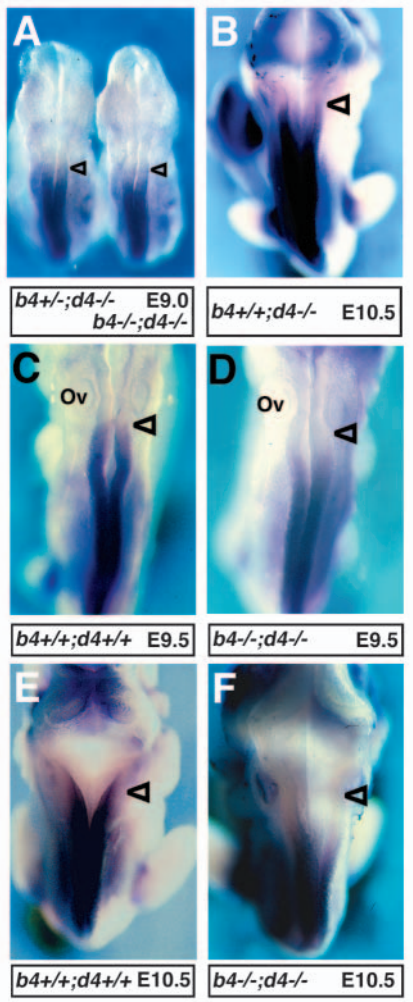

Fig. 3. *Hoxb4* and *Hoxd4* regulate the hindbrain expression of *Rarb* at late but not at early stages. All panels show the expression of *Rarb* mRNA in dorsal views of E9.0-E10.5 embryos lacking from 0 to 4 wild-type alleles of *Hoxb4* and *Hoxd4*. The wild-type position of the neural *Rarb* expression border at r6/r7 (arrowhead) is indicated. (A) E9.0 embryos with *Hoxb4*$^{+/-}$; *Hoxd4*$^{-/-}$ (left) and *Hoxb4*$^{-/-}$; *Hoxd4*$^{-/-}$ (right) genotypes showing wild-type patterns of *Rarb* expression. At this intermediate stage, although the *Rarb* neural border lies in the vicinity of the forming r6/r7 junction, it is not as sharp as at E9.5. (B) E10.5 *Hoxb4*$^{+/+}$; *Hoxd4*$^{-/-}$ embryo with a normal neural border of *Rarb* expression at r6/r7. (C,D) E9.5 embryos that are wild type (C) or *Hoxb4*$^{-/-}$; *Hoxd4*$^{-/-}$ (D) showing abnormal regression of the *Rarb* border associated with loss of function of both *Hox* paralogues. (E,F) E10.5 embryos that are wild type (E) or *Hoxb4*$^{-/-}$; *Hoxd4*$^{-/-}$ (F), showing that, by this late stage, removing the activity of both *Hox* paralogues leads to loss of most *Rarb* expression from the hindbrain.

of these mutant embryos (Fig. 2E,F). This demonstrates clearly that RA, which is produced by the mesoderm, is required to induce the early neural expression of *Rarb*. Hence, the repertoire of RA-inducible genes within the hindbrain extends beyond the Hox family to include *Rarb*. Our combined results demonstrate that, despite initial differences in their rostral expression borders, both *Rarb* and *Hoxb4* are induced in the hindbrain at the mRNA level by the same mesodermal source of RA.

Rarb is regulated by *Hoxb4* and *Hoxd4* at late stages

We next focused attention on later stages of development, to identify the mechanism regulating the expression border of *Rarb* in the segmented hindbrain. During the E8.5-E9.5 time window, the anterior expression border of *Rarb* regresses by approximately one rhombomere in length, matching the sharp segmental border of *Hoxb4* expression at r6/r7. Given this late border congruency, we tested the possibilities that either *Hoxb4* regulates *Rarb* or that *Rarb* regulates *Hoxb4*. Functional overlap among RAR genes results in the overall pattern of *Hoxb4* mRNA appearing normal in *Rarb* single mutants (Folberg et al., 1999). In addition, confocal analysis with single-cell resolution in *Rarb* single mutants provided no evidence for a non-redundant requirement for this particular RAR in specifying the sharpness or positioning of the r6/r7 border of Hoxb4 protein expression at E10 (data not shown). However, a previous study showed that blocking the activities of all types of RAR abolished hindbrain *Hoxb4* induction (Gould et al., 1998). Together with the analysis of mutants lacking multiple RARs (see Introduction), we conclude that, although the set of RARs participating in *Hoxb4* induction does include *Rarb*, other types of RAR can substitute for its role.

To test for the reciprocal regulatory relationship between *Rarb* and *Hoxb4*, we generated double homozygous mice lacking the functions of *Hoxb4* and *Hoxd4*. The activities of both of these paralogues were removed, as previous studies indicated that they play redundant roles in activating a Hox-responsive enhancer in r7 (Gould et al., 1997). In $Hoxb4^{-/-}$; $Hoxd4^{-/-}$ embryos, caudal hindbrain tissue aberrantly expresses several region-specific molecular markers (data not shown). We find that the early phase of neural *Rarb* expression is normal in double homozygous embryos at E8.5 and E9.0 (Fig. 3A and data not shown). Moreover, no alteration in the late phase of *Rarb* expression was observed at E10.5, when only two out of four alleles were mutated ($Hoxb4^{+/+}$; $Hoxd4^{-/-}$ embryos; Fig. 3B). However, in double homozygous embryos, older than E9.0, the position of the *Rarb* border is significantly disrupted. Thus, by E9.5, the *Rarb* expression border in $Hoxb4^{-/-}$; $Hoxd4^{-/-}$ embryos lies approximately one rhombomere in length caudal to the normal r6/r7 location (Fig. 3C,D). In addition, at this stage, the border is less sharply defined than in wild-type embryos. One day later, at E10.5, the caudal shift and diffuseness of the border in $Hoxb4^{-/-}$; $Hoxd4^{-/-}$ embryos have become more dramatic, leaving most of the hindbrain void of detectable *Rarb* expression (Fig. 3E,F). These results demonstrate that *Rarb* expression in the hindbrain is initially independent of group four Hox genes but subsequently becomes positively regulated by overlapping inputs from *Hoxb4* and *Hoxd4*. Late Hox-dependent regulation comes into play at the time of morphological segmentation, serving to sharpen and fix the regressing expression border of *Rarb* at the r6/r7 boundary.

The results thus far are consistent with a two-phase model for the regulation of *Rarb* within the developing hindbrain.

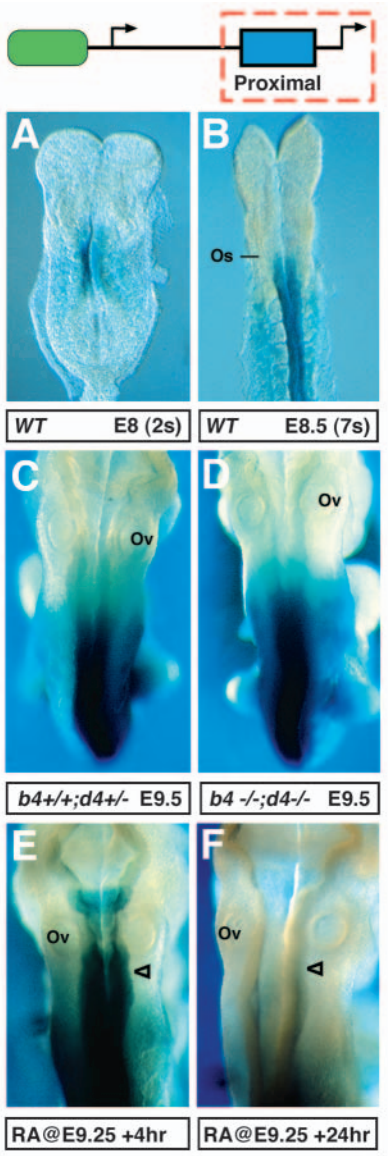

Fig. 4. The *Rarb* Proximal enhancer recapitulates early neural expression and responds transiently to RA. All panels show dorsal views of *Rarb2lacZ* transgenic embryos at E8-E10.25, histochemically stained for β-galactosidase activity. (A,B) E8-E8.5 transgenic embryos at the two-somite (A) and seven-somite (B) stages. At both time points, strong reporter expression is present within the neural plate with weaker staining also visible in paraxial and lateral mesoderm. By the seven-somite stage, a diffuse anterior border maps to the vicinity of the Os. (C,D) E9.5 embryos of the genotype *Rarb2lacZ/+*; $Hoxb4^{+/+}$; $Hoxd4^{+/-}$ (C) and *Rarb2lacZ/+*; $Hoxb4^{-/-}$; $Hoxd4^{-/-}$ (D) showing that, at this late stage, the neural activity of the proximal enhancer is largely confined to the developing spinal cord with only residual low levels remaining within the caudal hindbrain. This late pattern is unaffected when the functions of both *Hoxb4* and *Hoxd4* are removed. (E,F) E9.25 transgenic embryos treated with an exogenous pulse of RA, showing a clear ectopic response within r4-r6 at 4 hours (E), but not 24 hours (F), after treatment. The expression pattern in F is similar to that seen in C.

Expression is first induced within the presegmented hindbrain by an RA signal from the mesoderm, generating a diffuse border at the approximate level of presumptive r5/r6. This early phase of expression at E8.5 is independent of *Hoxb4* and *Hoxd4*. Approximately 1 day later, around the time of rhombomere boundary formation, the border becomes sharpened and keyed onto the r6/r7 junction by overlapping regulatory inputs from *Hoxb4* and *Hoxd4*. Importantly, these results demonstrate clearly that RARs can act downstream of Hox genes. In combination with a large body of published evidence already placing them upstream, our results reveal the existence of bidirectional regulation between the Hox and RAR gene families.

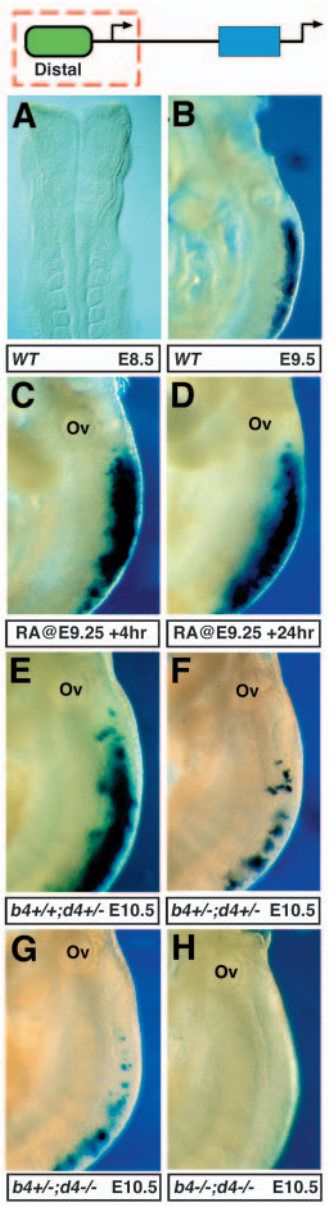

Fig. 5. The *Rarb* Distal enhancer specifies the late r6/r7 border and requires inputs from *Hoxb4* and *Hoxd4*. Panels show dorsal views (A) or lateral views (B-H) of *Rarb1lacZ* transgenic embryos at E8.5-E10.5, histochemically stained for β-galactosidase activity. (A,B) An E8.5 transgenic embryo (A) and a E9.5 transgenic embryo (B), indicating that distal enhancer activity is initiated after E8.5 and by E9.5 is restricted to the dorsal neural tube with a sharp border at r6/r7. (C,D) Transgenic embryos treated with a single dose of RA at E9.25 and allowed to develop in utero for either 4 hours (C) or 24 hours (D). No ectopic response to RA is detected in either case. (E-H) E10.5 transgenic embryos carrying various combinations of *Hoxb4* and *Hoxd4* loss-of-function alleles. Strong distal enhancer activity is seen in *Rarb1lacZ/+; Hoxb4*$^{+/+}$*; Hoxd4*$^{+/-}$ (E), weak distal enhancer activity in *Rarb1lacZ/+; Hoxb4*$^{+/-}$*; Hoxd4*$^{+/-}$ (F) and *Rarb1lacZ/+; Hoxb4*$^{+/-}$*; Hoxd4*$^{-/-}$ (G) and no detectable distal enhancer activity in *Rarb1lacZ/+; Hoxb4*$^{-/-}$*; Hoxd4*$^{-/-}$ embryos (H).

The proximal enhancer mimics early *Rarb* expression and responds rapidly and transiently to RA

To dissect the Hox-RAR feedback mechanism and, in particular, the way in which RA and Hox inputs are temporally coordinated by the *Rarb* gene, we used two transgenic regulatory DNA constructs from this locus (Mendelsohn et al., 1991; Mendelsohn et al., 1994): (1) *Rarb2lacZ*, expressing β-galactosidase under the control of a 3.8 kb genomic fragment (termed the proximal enhancer) that includes the proximal (P2) promoter; and (2) *Rarb1lacZ*, containing a 2.3 kb *Nhe*I genomic fragment (termed the distal enhancer) that encompasses the distal (P1) promoter. At E9.5, *Rarb1lacZ* and *Rarb2lacZ* drive neural expression with anterior borders at r6/r7 and r7/r8, respectively (Mendelsohn et al., 1994). However, in the context of the endogenous *Rarb* gene, transcripts from both the P1 and the P2 promoters are known to be expressed with a r6/r7 boundary at E10.5 (Mollard et al., 2000). This indicates that, when in their normal chromosomal environment, the proximal and distal enhancers may not be selective for one promoter. More specifically, it suggests that the distal enhancer may be capable of regulating both P1 and P2.

We analysed early proximal enhancer activity, using E8-E8.5 embryos transgenic for *Rarb2lacZ*. We find that *Rarb2lacZ* is robustly expressed within the neural plate of two-somite stage embryos (Fig. 4A). By the seven-somite stage, reporter activity is observed extending up to the level of the otic sulcus, corresponding to the future r5/r6 boundary (Fig. 4B). This pattern recapitulates the anterior limit of *Rarb* mRNA at E8.5 that we describe here (compare Fig. 1D with Fig. 4B). One day later, at E9.5, proximal enhancer activity has regressed dramatically and is now missing from most of the caudal hindbrain but remains present in the spinal cord (Fig. 4C) (Mendelsohn et al., 1994). This late loss of expression throughout the caudal hindbrain is more extensive than the regression observed for the endogenous gene, indicating that the proximal enhancer accounts for the early but not the late phases of *Rarb* expression. To investigate the factors regulating the proximal enhancer, we initially examined *Rarb2lacZ* expression in *Hoxb4*$^{-/-}$*; Hoxd4*$^{-/-}$ embryos. However, removing the functions of both Hox paralogues does not affect the neural *Rarb2lacZ* expression pattern at E9.5 or E10.5 (Fig. 4C,D; data not shown).

Previous studies have shown that the proximal enhancer region (*Rarb2lacZ* construct) contains a RARE required for its RA responsiveness in cell culture (de Thé et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). Furthermore, in an embryonic context, the *Rarb2lacZ* transgene responds rapidly to a pulse of exogenous RA at E9.25, giving a robust response in the

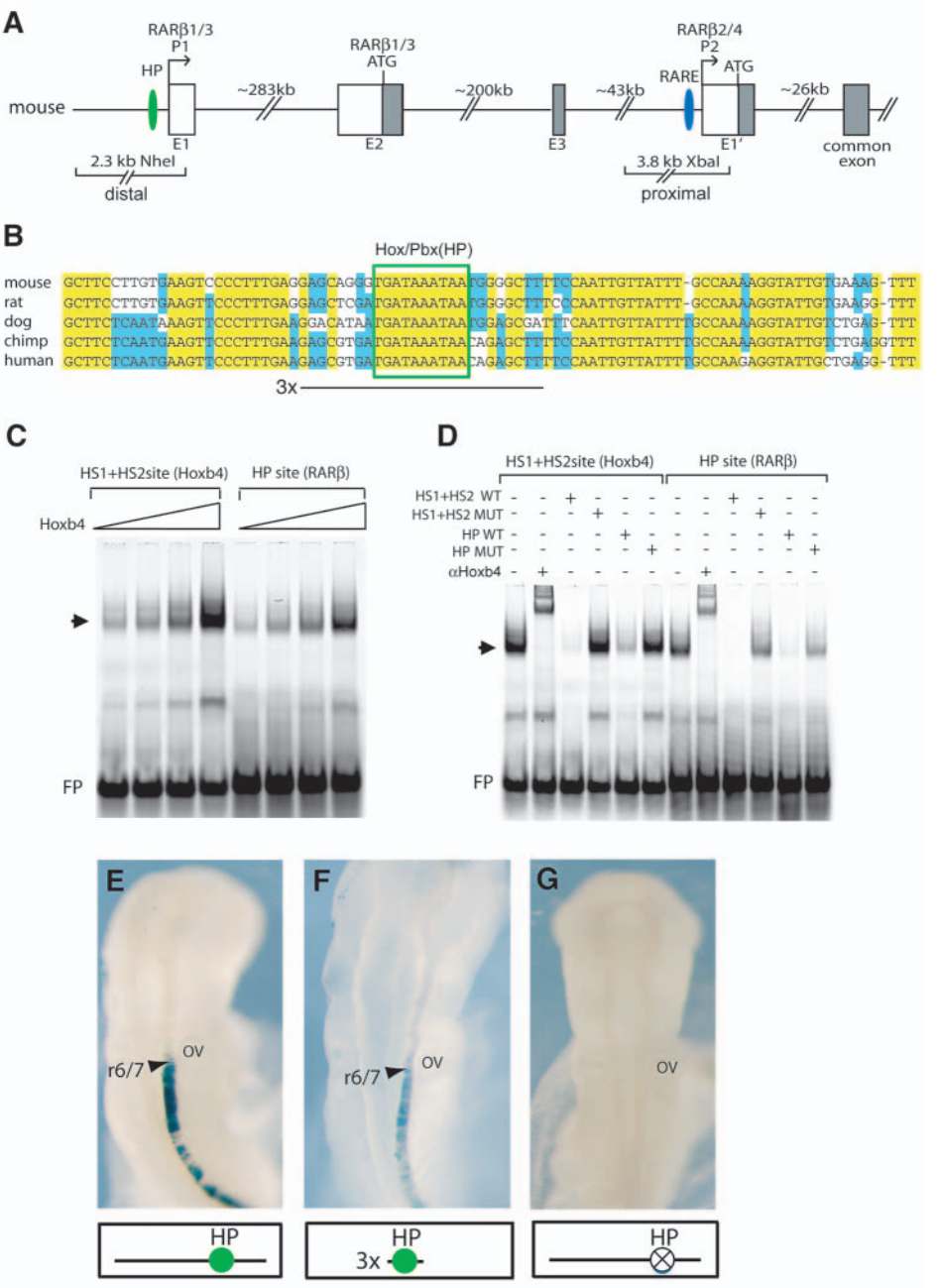

Fig. 6. A conserved Hoxb4-binding site is required for *Rarb* distal enhancer activity in chick embryos. (A) Map of the 5′ end of the *Rarb* gene. Transcripts for the RARβ1 and RARβ3 isoforms initiate at the P1 promoter and those of the RARβ2 and RARβ4 isoforms at the P2 promoter. The positions of the RARE (blue oval; proximal enhancer) and the Hox/Pbx (HP) element (green oval, distal enhancer) are shown. White and grey boxes indicate transcribed non-coding and coding sequences, respectively. The positions of the 2.3 kb distal enhancer and the 3.8 kb proximal enhancer are also shown. Exon E1′, which is equivalent to E4 in previous publications, is relabelled here to emphasize that it is the first exon of *Rarb2/4* transcripts and not contiguous with *Rarb1/3* transcripts. (B) Alignment of the Hox/Pbx (HP) element and flanking sequences in five mammalian species. The HP site is boxed in green and indicated below is the region multimerized for the 3xHP construct used in F. (C,D) Electrophoretic mobility shift assays showing Hoxb4 binding to the HP site. Labelled oligonucleotides containing the HS1+HS2 site (Gould et al., 1997) or the HP site (from *Rarb*) were incubated with increasing amounts of Hoxb4 protein (C) or with a constant amount of Hoxb4 protein in combination with Hoxb4 antibody (αHoxb4) or unlabelled competitor oligonucleotides as indicated (D). (E-G) Dorsal views of chick embryos electroporated with *lacZ* reporter constructs containing the mouse 2.3 kb distal enhancer (E), 3xHP oligonucleotide (F) or 2.3 kb distal enhancer with mutated HP site (G). *lacZ* expression with an r6/r7 boundary can be observed on the electroporated (right) side of the neural tube, except when the distal enhancer containing the mutated HP site (G) was used. The positions of the otic vesicle (OV) and the r6/7 boundary (arrowhead) are shown. Constructs are diagrammed below each panel, the circle indicating intact HP site (green fill) or HP MUT site (cross).

hindbrain within 4 hours (Fig. 4E) (Mendelsohn et al., 1994). Strong ectopic expression is observed up to the r3/4 boundary with levels in r5 much lower than in r4 and r6. To assess whether the proximal enhancer is sufficient to maintain a long-lasting in vivo response to a transient RA signal, we examined *Rarb2lacZ* activity 24 hours after a single RA dose. In contrast to the 4 hour time point, we find that no ectopic *lacZ* expression is detectable after 24 hours (Fig. 4F). At this time, neural *Rarb2lacZ* activity is confined to spinal cord levels, resembling its pattern in untreated E9.5-E10.5 embryos (compare Fig. 4D with 4F). The rapid and transient nature of the RA response indicates that neural activity of the proximal enhancer provides a readout for RA availability and, together with the previous cell culture studies, suggests that this represents a direct response to RARs. The *Rarb2lacZ* analysis also shows that while the proximal enhancer responds to RA and accounts for the early *Raldh2*-dependent phase of *Rarb* expression, it does not contain sequences capable of stably maintaining an RA response and it is not regulated by *Hoxb4* or *Hoxd4*.

The distal enhancer mimics late segmental *Rarb* expression and is activated by *Hoxb4* and *Hoxd4*

We analysed the activity pattern of the second transgenic construct, *Rarb1lacZ*, containing the distal enhancer. In contrast to *Rarb2lacZ*, no reporter expression is seen at E8.5 (Fig. 5A). Approximately 24 hours later, activity within the neural tube is initiated with a well-defined limit at r6/r7 that is stably maintained until E10.5 and even later stages (Fig. 5B and data not shown). Therefore, the distal enhancer recapitulates the late but not the early phase of expression of the endogenous *Rarb*

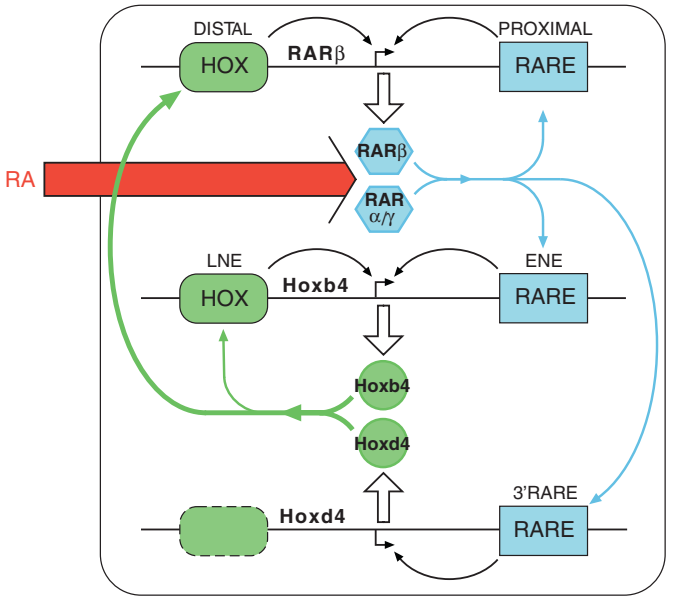

Fig. 7. The Hox-RAR feedback circuit. Cartoon shows the Hox-responsive enhancers (HOX, green) and RA-responsive enhancers (RARE, blue) from *Rarb* (top), *Hoxb4* (middle) and *Hoxd4* (bottom). Enhancer-promoter interactions (black arrows), transcription and translation (unfilled arrows), and transcriptional regulation by Hoxb4 and Hoxd4 proteins (green arrows) and RARs (blue arrows) are shown. The external RA signal that initiates this neural genetic circuit (red arrow) is synthesized by Raldh2 in the adjacent mesoderm. A functional equivalent of the LNE/distal enhancer (HOX element with broken outline) has, thus far, not been identified for *Hoxd4* (Nolte et al., 2003). The correct positioning of the single or multiple transcription start sites of each gene has been omitted for clarity. The central importance of the Hox→RARβ interactions described in this study are emphasized with thick green arrows. This figure also summarizes genetic interactions identified in several other published studies (see text for details).

gene. To assess whether the distal enhancer is regulated in a similar way to its proximal counterpart, *Rarb1lacZ* embryos were treated with a single dose of exogenous RA. As no expression was detected anterior to r6/r7 after 4 or 24 hours, we conclude that the distal enhancer differs from the proximal enhancer in that it does not mediate a direct ectopic response to RA (Fig. 5C,D). As the r6/r7 expression border of distal enhancer activity at E9.5-E10.5 is shared with *Hoxb4* and *Hoxd4*, we next examined *Rarb1lacZ* expression in embryos carrying various combinations of knockout alleles of these two group 4 Hox paralogues. Although loss of one of the four wild-type alleles has no reproducible effect on expression at E10.5, removing two or three copies does lead to significantly reduced neural tube expression (Fig. 5E-G). Removing all four alleles, in *Hoxb4*$^{-/-}$; *Hoxd4*$^{-/-}$ embryos, leads to the complete abolition of all distal enhancer activity with 100% penetrance (Fig. 5H). Therefore the r6/r7 border and all other aspects of distal enhancer activity display an absolute requirement for *Hoxb4* and *Hoxd4* function.

A conserved Hoxb4 binding site is required for distal enhancer activity

To investigate whether the regulation of *Rarb* by *Hox4* genes might be direct, we searched the 2.3 kb distal enhancer sequence for potential Hox response elements. A close match to a bipartite consensus binding site for Hox and Pbx proteins (HP) was identified 83 bp upstream from the characterized murine RARβ1/3 transcription start site (Fig. 6A) (Mendelsohn et al., 1994). This 10 bp element and its surrounding sequences are highly conserved in syntenic regions upstream of *Rarb* in other mammalian genomes such as human, chimp, rat and dog (Fig. 6B).

To determine whether Hoxb4 was capable of direct binding to the HP site, a double-stranded oligonucleotide containing the motif was used in electrophoretic mobility shift assays (EMSA, Fig. 6C). EMSA revealed that the HP site displays concentration-dependent binding of Hoxb4, similar to that seen with a previously characterized Hoxb4-binding site (HS1+HS2) from the *Hoxb4* gene itself (Fig. 6C) (Gould et al., 1997). The specificity of the Hoxb4 protein interaction with HP was confirmed by supershifting the protein-DNA complex with a Hoxb4 antibody and by using various oligonucleotide competition assays (Fig. 6D). Whereas oligonucleotides corresponding to either the HP or the HS1+HS2 sites compete efficiently with one another for Hoxb4 binding, mutated variants (HS1+HS2 MUT and HP MUT: see Materials and methods) do not. Thus, Hoxb4 binds to the conserved HP site within the distal enhancer of *Rarb* in a direct and sequence-specific manner.

To test whether the conserved HP site is functionally relevant in vivo, its activity was monitored by chick in ovo electroporation. We first showed that the 2.3 kb murine distal enhancer can direct *lacZ* reporter expression within the developing chick hindbrain up to the r6/r7 boundary (17/18 embryos, Fig. 6E), the same pattern as observed in transgenic mouse embryos. When present in three copies, a 26 bp oligonucleotide spanning the murine HP site (3xHP) is sufficient to direct a weak r6/r7 pattern (8/12 embryos, Fig. 6F), indicating that rhombomere-specificity information resides within the HP site and/or its immediate flanking sequences. Moreover, within the context of the 2.3 kb distal enhancer, the HP site is required for activity as mutating it to a sequence unable to bind Hoxb4 (HP MUT) results in the complete abolition of *lacZ* expression (0/36 embryos, Fig. 6G). Taken together, the sequence analysis, EMSA and chick electroporation experiments provide strong evidence that Hoxb4 regulates the distal enhancer of *Rarb* in a direct manner via the HP site.

Discussion

Many studies have focused on how one signalling molecule can trigger several different gene expression patterns, as in the morphogen paradigm. Less attention has been directed to the other important problem of how one signal can specify the precisely congruent expression of multiple genes. We have explored this issue in the context of how the expression domains of two RA-induced genes, *Rarb* and *Hoxb4,* become aligned at a segment boundary in the developing hindbrain. We focused on the regulation of *Rarb* and identified a two-step transcriptional mechanism. We now discuss this mechanism and how, in combination with the previously identified *Hoxb4* regulatory mechanism, it provides new insights into the way in which sharp segmental borders of gene expression are generated.

Development

Raldh2-dependent RA synthesis induces *Rarb* and *Hoxb4* within the hindbrain

Raldh2-dependent RA signalling from the paraxial mesoderm induces a diffuse and unstable border of *Rarb* mRNA within the presegmented hindbrain. In the absence of Raldh2, no *Rarb* expression is detected within the neuroepithelium, indicating that initial induction by RA is probably transduced by RARα and/or RARγ and not by *Rarb* itself. This also implies that early hindbrain expression of at least one of these RARs is independent of induction by RA. The presegmental phase of *Rarb* expression is regulated by the proximal RARE-containing enhancer, which responds rapidly and transiently to RA with similar kinetics to a RARE that is a known direct target of RARs (cf. Gould et al., 1998). Together with evidence that the proximal RARE directly binds RARs and mediates an RA-response in cultured cells (de Thé et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990), our findings strongly suggest that the induction of neural *Rarb* expression by RARs is direct. Hence, following initial neural induction via RARα and/or RARγ, RARβ would then be available to augment its own expression via direct binding to the RARE of the proximal enhancer.

Group 4 Hox genes regulate the segmental expression of *Rarb* in a direct manner

Rarb is a well-known regulator of Hox genes, including those of paralogue group 4 but, surprisingly, our study indicates that reciprocal regulation also occurs. Together, our mouse knockout and transgenic analyses reveal that *Rarb* lies downstream of *Hoxb4* and *Hoxd4*. Moreover, the combined EMSA and chick electroporation approaches indicate that regulation is mediated in a direct manner via Hoxb4 binding to the HP site of the *Rarb* distal enhancer. Given the similar homeodomain sequences and DNA-binding specificities of Hoxb4 and Hoxd4, it is likely that the observed genetic requirement for *Hoxd4* is also mediated in the same direct manner.

Rarb and *Hoxb4* use similar two-step regulatory mechanisms

We have shown that the mechanisms regulating both the early and late phases of *Rarb* expression within the hindbrain operate at the transcriptional level. At E8.5, the proximal retinoid-dependent enhancer recapitulates the initial diffuse and transient *Rarb* expression up to the presumptive r5/r6 border. Likewise, at E9.5-E10.5, the distal Hox-dependent enhancer maintains the stable segmental border at r6/r7 via a direct input from *Hoxb4* and probably *Hoxd4*. Thus, the summation of the activities of the proximal and distal enhancers accounts for the establishment and maintenance of hindbrain expression of the endogenous *Rarb* gene.

The mechanism regulating *Rarb* in the presegmented hindbrain is similar to that of *Hoxb4*. Both genes are transcriptionally induced by a Raldh2-dependent RA source and both possess RARE-containing enhancers [for Hoxb4, this is termed the early neural enhancer (ENE) (Gould et al., 1998)] that direct neural expression with borders that recede after E8.5. These caudal shifts presumably reflect regression of the inducing ability of the paraxial mesoderm with increasing embryonic age (Itasaki et al., 1996; Gould et al., 1998). Although there are strong parallels between the RARE enhancers of *Rarb* and *Hoxb4*, there are also some differences. For example, proximal enhancer activity begins at around the two-somite stage, whereas ENE activity begins at the nine-somite stage. In addition, at E8.5, the anterior border of the *Rarb* proximal enhancer is at presumptive r5/6 but that of the *Hoxb4* ENE is at presumptive r6/r7. Although the DNA element responsible for these expression differences is undefined, it may be relevant that the DR5 class of RARE, present in both enhancers, differs at 3/12 nucleotide positions.

The regulatory parallels between *Rarb* and *Hoxb4* also extend to the later phase of segmental expression. Like *Rarb*, *Hoxb4* uses a two-step regulatory strategy of establishment and maintenance within the hindbrain, involving two enhancer elements that are mechanistically and physically separable (Gould et al., 1998). For *Hoxb4*, the late hindbrain element is termed the late neural enhancer (LNE) (Gould et al., 1997). Both the *Rarb* distal enhancer and the *Hoxb4* LNE drive expression with a sharp r6/r7 border and respond to stabilizing inputs from group 4 Hox genes. In both cases, these late Hox inputs serve to halt the caudal regression of diffuse borders that were established by RARE-containing enhancers. However, when the functions of *Hoxb4* and *Hoxd4* are completely removed, *Hoxb4* LNE activity is lost only from r7 (Gould et al., 1997), whereas *Rarb* distal enhancer activity is abolished within the entire neural tube. This suggests that, although group 4-6 Hox paralogues activate the *Hoxb4* LNE (Gould et al., 1997), only some or all of the group 4 Hox genes may be capable of activating the *Rarb* distal enhancer.

A feedback circuit aligning the segmental expression borders of *Rarb, Hoxb4* and *Hoxd4*

We have argued that the early induction and late maintenance mechanisms regulating hindbrain *Rarb* transcription are strikingly similar to those of *Hoxb4*. As both genes are transcriptionally regulated by RARs and Hox proteins, numerous types of feedback loop are possible. Importantly, the Hox-to-*Rarb* direction of regulation that we have uncovered in this study identifies a new molecular link completing a circuit between the two different gene families. Combining our results with those of several previous studies (see Introduction), it can be seen that bidirectional Hox-RAR regulation forms the core of a complex genetic circuit involving direct and indirect interactions between *Rarb*, *Hoxb4* and *Hoxd4* (Fig. 7). It is likely that the feedback circuit we describe also includes a fourth gene, as *Hoxb3* shares the LNE with *Hoxb4* and thus upregulates its expression posterior to r6/r7 at late stages (Gould et al., 1997). One important requirement for the indirect type of feedback loop to occur is that the RAR→Hox and Hox→RAR interactions must overlap in developmental time rather than being strictly sequential. More specifically, RAR→Hox regulation must persist long enough after the initial stage of RA-dependent Hox induction at E8.5 to coincide with the onset of the reciprocal Hox→RAR regulation, first detectable at around E9.5. Consistent with this temporal overlap, the isolated ENE of *Hoxb4* does remain active within the neural tube at E9.5 (Gould et al., 1998).

The complex transcriptional feedback circuit identified here maintains gene expression and has the capacity to align the expression borders of multiple Hox and RAR genes at a single segment boundary. Early positional information conveyed by

RA induction is not sufficient to specify the late segmental expression border of *Rarb* or *Hoxb4*. Furthermore, although both genes interpret the same *Raldh2*-dependent signal, small differences in the responsiveness of their RARE-containing enhancers produce early expression borders that are out of register. Despite this, and some variations in the Hox-responsive late enhancers of *Hoxb4* and *Rarb*, when all of the components are appropriately combined within the in vivo genetic circuit, they function to generate congruent expression at the r6/r7 boundary. In addition to these self-organizing properties, the Hox-RAR circuit is also genetically robust such that overlapping inputs from both gene families ensure that loss of any one gene has a minimal effect on the expression of the others. For example, we found that the r6/r7 expression border of *Hoxb4* is unaffected by loss of all *Rarb* activity and, conversely, that the *Rarb* mRNA pattern remains normal when all *Hoxd4* activity is removed. Finally, the identification of the above border-matching circuit was initiated by the observation that group 4 Hox genes share a segmental expression pattern with *Rarb*. As *Rara* is reported to be expressed up to the r3/r4 boundary (Ruberte et al., 1991), coincident with the expression limits of *Hoxa1* and *Hoxb1*, a related Hox-RAR feedback circuit may also operate at this segment border.

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