Development 125, 1025-1036 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV2250

Genetic interactions between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning

Michèle Studer[1,*,†], Anthony Gavalas[2,*], Heather Marshall[1], Linda Ariza-McNaughton[1], Filippo M. Rijli[2], Pierre Chambon[2] and Robb Krumlauf[1,‡]

[1]Division of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[2]Institut de Génétique et de Biologie Moléculaire and Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163 – 67404 Illkirch Cedex, CU de Strasbourg, France
*These authors contributed equally to this work
†Present address: Department of Developmental Neurobiology, UMDS, Guy's Hospital, London SE1 9RT, UK
‡Corresponding author (E-mail: r-krumlauf@nimr.mrc.ac.uk)

Accepted 18 December 1997: published on WWW 17 February 1998

SUMMARY

In the developing vertebrate hindbrain *Hoxa1* and *Hoxb1* play important roles in patterning segmental units (rhombomeres). In this study, genetic analysis of double mutants demonstrates that both *Hoxa1* and *Hoxb1* participate in the establishment and maintenance of *Hoxb1* expression in rhombomere 4 through auto- and para-regulatory interactions. The generation of a targeted mutation in a *Hoxb1* 3′ retinoic acid response element (RARE) shows that it is required for establishing early high levels of *Hoxb1* expression in neural ectoderm. Double mutant analysis with this *Hoxb1*$^{3'RARE}$ allele and other targeted loss-of-function alleles from both *Hoxa1* and *Hoxb1* reveals synergy between these genes. In the absence of both genes, a territory appears in the region of r4, but the earliest r4 marker, the Eph tyrosine kinase receptor *EphA2*, fails to be activated. This suggests a failure to initiate rather than maintain the specification of r4 identity and defines new roles for both *Hoxb1* and *Hoxa1* in early patterning events in r4. Our genetic analysis shows that individual members of the vertebrate *labial*-related genes have multiple roles in different steps governing segmental processes in the developing hindbrain.

Key words: *Hox* genes, Regulation, Synergism, Hindbrain, Gene targeting, RARE

INTRODUCTION

Regional diversity in the vertebrate hindbrain is achieved through a process of segmentation, whereby a series of lineage-restricted cellular compartments, termed rhombomeres (r), are formed during early neural development (Fraser et al., 1990; Lumsden and Krumlauf, 1996). These rhombomeric segments are correlated with the periodic organisation of neurons (Clarke and Lumsden, 1993; Lumsden and Keynes, 1989) and the migration of cranial neural crest cells into specific branchial arches where they differentiate to form distinct skeletal and neurogenic components (Köntges and Lumsden, 1996; Lumsden et al., 1991; Sechrist et al., 1993). Coupled to these morphogenetic events, *Hox* genes are involved at the molecular level in regulating multiple aspects of segmental patterning (reviewed by Krumlauf, 1994; Lumsden and Krumlauf, 1996).

Expression and genetic mutant analyses have shown that the precise anterior domains of *Hox* expression are closely associated with their functional roles (reviewed in Duboule, 1993; Krumlauf, 1993, 1994) and that these patterns are generated in two distinct phases, establishment and maintenance (Deschamps and Wijgerde, 1993). In this regard, the vertebrate *labial* orthologs (paralog group 1) are interesting because they are the first members of their complexes to be activated and they display the highest sensitivity to exogenous retinoic acid (RA) (reviewed in Conlon, 1995; Maconochie et al., 1996; Marshall et al., 1996). In the establishment phase, expression of *Hoxa1*, *Hoxb1* and *Hoxd1* is initiated during early gastrulation in primitive streak mesoderm, but subsequently only *Hoxa1* and *Hoxb1* are activated in the overlying neural ectoderm (Frohman and Martin, 1992; Hunt et al., 1991; Murphy and Hill, 1991). By headfold stage, both genes have reached a sharp anterior boundary in neuroectoderm coinciding with the presumptive r3/r4 border, but in early somite stages this expression begins to regress caudally, later becoming localised to the tailbud (Frohman et al., 1990; Murphy and Hill, 1991; Wilkinson et al., 1989b). In the maintenance phase, anterior expression of *Hoxa1* within the hindbrain does not persist during rhombomere boundary formation, while *Hoxb1* is maintained in r4 at high levels until

the disappearance of rhombomere boundaries (Frohman et al., 1990; Godsave et al., 1994; Murphy et al., 1989; Sundin and Eichele, 1990; Wilkinson et al., 1989b).

These dynamic expression domains correlate with the diverse roles of the genes in hindbrain patterning. Functional inactivation of *Hoxa1* results in segmentation defects leading to the partial deletion of rhombomeres, suggesting a role of *Hoxa1* in generating and/or maintaining segmental compartments (Carpenter et al., 1993; Chisaka et al., 1992; Dollé et al., 1993; Lufkin et al., 1991; Mark et al., 1993). In contrast, *Hoxa1* gain-of-function induces a transformation of r2 into an r4 identity (Alexandre et al., 1996; Zhang et al., 1994) and *Hoxb1* loss-of-function produces an alteration in r4 identity (Goddard et al., 1996; Studer et al., 1996), suggesting both genes may have a role in maintaining rhombomere identity. These studies illustrate that *Hox* genes are involved in regulating several distinct steps in hindbrain segmentation. However, the different roles specifically attributed to *Hoxa1* and *Hoxb1* in controlling segmentation and segmental identity, respectively, might represent only a subset of their function and do not exclude them from having additional inputs into rhombomere patterning. Synergy or functional compensation has been observed between paralogous *Hox* genes in a number of tissues revealing added complexity to the roles of *Hox* genes in patterning processes (Condie and Capecchi, 1994; Davis et al., 1995; Favier et al., 1996; Horan et al., 1995; Zakany et al., 1996). Therefore, it is important to know whether there are genetic interactions between *Hoxa1* and *Hoxb1* that could have regulatory and patterning implications in early hindbrain segmentation.

To more fully understand the cascade of regulatory events governing early hindbrain patterning it is also important to understand the basis for generating the restricted domains of *Hox* expression associated with their functional roles. In this regard, mapping of *cis*-regulatory elements has begun to identify regulatory hierarchies controlling the different phases of *Hox* expression (reviewed in Maconochie et al., 1996). For example, a neural regulatory region from the *Hoxb4* locus functions as an *in vivo* target for multiple *Hox* genes, whereby members of the group 4 paralogs and of groups 5-7 stimulate expression from this site in a para-regulatory and cross-regulatory manner, respectively (Gould et al., 1997). Furthermore, the expression of *Hoxb2* in r4 is maintained through cross-regulation by its 3′ neighbour *Hoxb1* (Maconochie et al., 1997), and the r4-restricted domain of *Hoxb1* itself is maintained through a direct and positive auto-regulatory loop, which is repressed in adjacent segments (Pöpperl et al., 1995; Studer et al., 1994). Therefore, in addition to genetic synergy on downstream target genes, auto, cross and para-regulatory interactions among *Hox* genes themselves are important in the maintenance of their spatially restricted patterns of expression.

In contrast, little is known about the *cis*-elements and mechanisms that initially establish *Hox* expression at appropriate levels along the embryonic axis. Several lines of evidence have suggested that retinoids not only modulate *Hox* expression but are also implicated in establishing their expression domains (reviewed in Marshall et al., 1996). Increased RA signalling in vertebrate embryos results in anterior shifts of *Hox* gene expression (Blumberg et al., 1997; Conlon and Rossant, 1992; Dekker et al., 1992; Papalopulu et al., 1991b; Ruiz i Altaba and Jessell, 1991; Simeone et al., 1995; Sive and Cheng, 1991), and influences segmental identity in the hindbrain (Hill et al., 1995; Kessel, 1993; Marshall et al., 1992; Papalopulu et al., 1991a). In *Xenopus* embryos, dominant negative variants of α-retinoic acid receptor (αRAR) isoforms cause posterior shifts and a loss of endogenous *Hox* expression (Blumberg et al., 1997; Kolm et al., 1997) and RA-deficient quail embryos have rhombomere deletions and altered *Hox* expression (Maden et al., 1996). Furthermore, retinoic acid responsive elements (RAREs), which represent canonical consensus sequences for the direct binding of retinoid receptors (reviewed by Mangelsdorf et al., 1995), have been mapped in the vicinity of *Hoxa1* (Frasch et al., 1995; Langston and Gudas, 1992), *Hoxb1* (Langston et al., 1997; Marshall et al., 1994; Ogura and Evans, 1995a,b; Studer et al., 1994) and *Hoxd4* (Moroni et al., 1993; Pöpperl and Featherstone, 1993). Using *lacZ* reporter genes in transgenic mice, mutational analysis of specific RAREs 3′ of both *Hoxb1* and *Hoxa1* has suggested that these elements are involved in regulating aspects of early neural expression. Germline mutations in the *Hoxa1* 3′ RARE result in lower levels of *Hoxa1* expression and a temporal delay in establishing its normal anterior boundary, strengthening the hypothesis that RA plays an important role in controlling early *Hox* expression.

Triggering the *Hoxb1* auto-regulatory loop is essential for maintaining r4 identity and facial motor neuron patterning (Goddard et al., 1996; Pöpperl et al., 1995; Studer et al., 1996). In this study we have examined the involvement of *Hoxa1* in establishing and maintaining the r4 expression domain of *Hoxb1*, as a model to investigate any potential para-regulatory interactions between the two genes. Furthermore, the observations that there is only a partial transformation of r4 to an r2 identity in the *Hoxb1* mutants (Studer et al., 1996), and that there are changes in the size of the r4 territory in *Hoxa1* mutants, led us to investigate a potential functional synergy between these two genes in early hindbrain patterning. To address these regulatory and functional issues, we have generated a mouse line carrying a point mutation in the 3′ RARE of *Hoxb1* and used it in combination with other targeted loss-of-function alleles from both *Hoxb1* and *Hoxa1* in double mutant analysis. Our results demonstrate that the *Hoxb1* 3′ RARE is required for early neuronal expression and that *Hoxa1* and *Hoxb1* work synergistically in initiating the r4-restricted expression of *Hoxb1*. In addition to its role in maintaining r4 identity, we show in this report and in the accompanying paper (Gavalas et al., 1998) that *Hoxb1*, together with *Hoxa1*, has an earlier role in patterning hindbrain structures and neural crest derivatives. We therefore conclude that the establishment of *Hoxb1* in r4 is dependent on the early activation of both *Hoxb1* and *Hoxa1* by endogenous retinoids and that together both these *labial*-related genes are essential for patterning the r4 territory.

MATERIALS AND METHODS

Mouse lines, mating and analysis

For staging embryos, midday following observation of the vaginal plug was designated as 0.5 dpc. Lines used were: *Hoxb1*^{null} (Studer et al., 1996), *Hoxb1/HPAP* (Itasaki et al., 1996; Studer et al., 1996), *Hoxa1*^{null} (Lufkin et al., 1991) and *Hoxa1*$^{3'RARE}$ (Dupé et al., 1997),

and genotyping of embryos was performed by PCR using yolk sac tissue and with primers as described in the respective references above. Analysis of embryos carrying the *lacZ* or the *HPAP* reporter genes was performed as previously described (Itasaki et al., 1996; Whiting et al., 1991). Whole-mount in situ hybridization with digoxigenin-labelled probes and serial sectioning were carried out as described (Morrison et al., 1995; Wilkinson and Green, 1990). Probes were: *Krox20* (Wilkinson et al., 1989a), *EphA2 (Sek2)* (Becker et al., 1994), *Hoxb1* (Wilkinson et al., 1989b) and *Otx2* (Ang et al., 1994).

Generation of mice carrying a point mutation in the *Hoxb1* 3′ RARE

The point mutations in the RARE were generated by inverse polymerase chain reaction (PCR) internally of an 800 bp *Eco*RV-*Hin*dIII fragment deriving from the 3′ flanking region of *Hoxb1*, which introduced a *Sal*I or *Acc*I site in place of the RARE (see also Marshall et al., 1994). The construct containing the mutation was called *HRVmRARE*. A 3.9 kb genomic fragment spanning the whole coding region of *Hoxb1* and part of its 5′ flanking region was subcloned into the *Spe*I-*Eco*RV sites of *HRVmRARE* and resulted in *SRVmRARE*. A 3.85 kb fragment, containing the herpes simplex virus thymidine kinase under its own promoter (MC1TK) and a bacterial neomycin phosphotransferase gene driven by the phosphotransferase promoter (pgkneo), was cut with *Bam*HI, blunt-ended, cut with *Xho*I and inserted into the blunt-ended *Cla*I and *Xho*I sites of the multiple cloning site of pBluescript KS+ of *SRVmRARE*. An additional 1.2 kb *Hoxb1* genomic *Spe*I fragment was placed into the *Spe*I site at the 5′ end of the previous construct (*SSRVmRARE*) in order to increase the length of homology between the targeting construct and the endogenous *Hoxb1* locus. The final *Hoxb1* 3′ RARE targeting construct (Fig. 3A) was linearised within the region of homology at the *Hin*dIII site and electroporated into AB2.2 embryonic stem (ES) cells according to Hasty et al. (1991). DNA from 600 individual G418-resistant colonies was digested with *Acc*I and analysed by mini-Southern blot analysis using 5′ (probe 1) and 3′ (probe 2) external probes (Fig. 3). Probe 1 corresponds to a *Hinc*II-*Spe*I 250 bp fragment and probe 2 to a *Rsa*1 600 bp fragment, derived from the 5′ and 3′ flanking regions of the *Hoxb1* locus, respectively. Out of 17 independent clones that contained the 3′ RARE mutation in the 5′ duplicate, two clones (B300 and B349) were chosen for further selection against the presence of TK by adding FIAU to individual replica plates every day for 5 days as described (Ramirez-Solis et al., 1993). After 10 days of selection, FIAU-resistant colonies were picked and screened for the reversion event. Clone B300 gave 53 wild type and 13 mutant revertants, while clone B349 gave 11 wild type and 5 mutant revertants. Four mutant clones from B349 were injected into C57Bl6 blastocysts and a total of 17 chimeras were obtained. Out of nine male chimeras that were crossed to C57Bl6 females, two transmitted the mutation to their offspring.

The genotype of the *Hoxb1*$^{3'RARE}$ mice and embryos was performed either by Southern blot analysis by using probe 1, or by PCR (Fig. 3B). The oligonucleotides 5′-GTGGGTAAGGAGGTGCCTGG-3′ and 5′-TCTCTCCAGCTGAGCTGTGC-3′ were used to amplify a 600 bp fragment in conditions previously described (Whiting et al., 1991). The PCR product was subsequently digested with *Sal*I (Fig. 3B) and two digestion products of 480 bp and 120 bp were obtained in the presence of the mutation.

RESULTS

Hoxb1 regulation in *Hoxb1*^{null} mutant embryos

Our previous analysis in transgenic mice together with other reports in *Drosophila* suggested that expression of the *labial* group is controlled in part by auto-regulatory interactions (Bienz, 1994; Chouinard and Kaufman, 1991; Pöpperl et al., 1995). To investigate at the genetic level whether the r4-restricted domain of *Hoxb1* expression was maintained by an auto-regulatory circuit, we recently generated a loss-of-function mutation of *Hoxb1* and crossed these mutant mice to a transgenic line carrying an alkaline phosphatase reporter construct *(HPAP)* under the control of the *Hoxb1* r4 regulatory region (Studer et al., 1996). Loss of HPAP reporter staining in *Hoxb1*^{null} homozygous embryos at 9.5 days post coitum (dpc) indicated that the r4 expression of *Hoxb1* is dependent upon its own product (Studer et al., 1996). Here we have extended this analysis by examining embryos at multiple stages of development. Consistent with our previous findings, between 9.25-9.5 dpc, 84% (16/19) of homozygous *Hoxb1*^{null} mutant embryos carrying the *HPAP* transgene lost reporter expression specifically in r4 (Fig. 1A,C). However in the three remaining embryos (16%) we noted a low level of *HPAP* reporter staining in r4, which even in the strongest case never reached wild-type levels (Fig. 1B). By 10.5 dpc, we never (0/12) detected *HPAP* staining in r4 (Fig. 1D,E), indicating that the ability to maintain even low levels of reporter expression had been lost by this stage.

To determine whether these changes in *Hoxb1/HPAP* transgene expression were mirrored by the endogenous gene itself, we took advantage of the *Hoxb1/lacZ* fusion protein created by targeted mutagenesis in our *Hoxb1*^{null} recombinant allele (Studer et al., 1996). At 8.5 dpc there was little difference in the r4 staining pattern generated by the fusion protein between heterozygous and homozygous mutant embryos (Fig. 2A,B; and data not shown), indicating that some level of r4 expression can be maintained from the endogenous *Hoxb1* promoter in the absence of functional Hoxb1 protein. This *lacZ* expression even persisted in r4 in 9.5 dpc embryos, unlike the *HPAP* transgene expression, which becomes down-regulated. We attribute this persistence of *lacZ* expression to the higher sensitivity of the β-galactosidase assay compared with alkaline phosphatase staining observed in our hands. However, we cannot exclude the possibility that differential behaviour of the transgene versus the endogenous locus contributes to the variability. These data show that in addition to Hoxb1 protein other components stimulate *Hoxb1* expression in r4 and contribute to its segmental regulation.

Synergy in r4 between *Hoxa1 and Hoxb1*

Next we investigated the nature of these additional components to understand the factors contributing to the establishment of the *Hoxb1* auto-regulatory loop. The previous findings that the *Hoxb1* r4 enhancer was dependent upon *labial* for activity in *Drosophila* embryos (Chan et al., 1996; Pöpperl et al., 1995) and was activated by multiple *labial*-related genes in transgenic mice (Pöpperl et al., 1995; Zhang et al., 1994), prompted us to investigate the role of other group 1 paralogs in the activation of *Hoxb1* expression in r4. We focused on *Hoxa1*, which is the only other paralog expressed in the CNS (Frohman and Martin, 1992; Hunt et al., 1991; Murphy and Hill, 1991), and analysed homozygous mutant embryos for both *Hoxa1* and *Hoxb1* to look for genetic interactions between the genes (Fig. 2). In this work we used the *Hoxa1*^{null} mutants generated by Lufkin et al. (1991), which have a slightly different hindbrain phenotype compared to those (*Hoxa1*^{null}) generated by Chisaka et al. (1992). The main difference between these two alleles is that, based on *Krox20* expression, one *Hoxa1*^{null} mutant retains a vestige of r5 (Dollé et al., 1993; Mark et al., 1993), whereas a

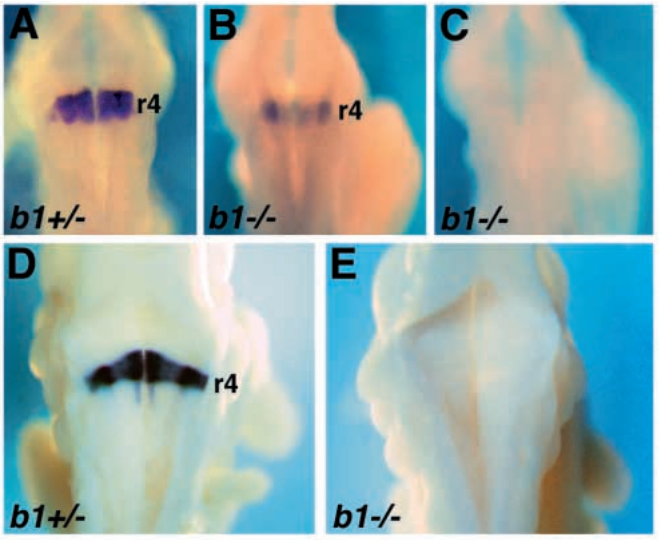

Fig. 1. Alkaline phosphatase reporter staining in *Hoxb1*^{null} single mutant embryos. All the embryos showed in this panel are transgenic for an alkaline phosphatase reporter construct under the control of the *Hoxb1* r4 regulatory region. Dorsal views of 9.5 dpc (A) and 10.5 dpc (D) *Hoxb1*^{null} heterozygous mutants showing high levels of reporter staining in r4. In homozygous mutant embryos either low levels (B) or no reporter staining (C,E) are detected at equivalent stages. The region or rhombomere 4 is labelled r4. Genotypes of the embryos are indicated in the respective panels.

complete deletion of r5 has been reported in the case of the other *Hoxa1*^{null} (Carpenter et al., 1993). Homozygous mutant embryos for both these alleles maintain *Hoxb1* expression in r4, although there is a reduction in its size.

Analysis of embryos from crosses between *Hoxa1*^{null} and *Hoxb1*^{null} mutant mice reveals that *Hoxa1* participates in the segmental expression of *Hoxb1*. In double heterozygous embryos reporter staining of the targeted *Hoxb1/lacZ* fusion gene and endogenous *Hoxb1* expression are not altered (data not shown). However in a *Hoxa1*^{null} homozygous background, embryos also heterozygous for *Hoxb1* display a significant decrease of expression in r4 from the *lacZ* targeted *Hoxb1*^{null} allele (Fig. 2C,D). As confirmed by in situ analysis, the levels of endogenous *Hoxb1* expression in r4 are also reduced (Fig. 2H) compared with those in the *Hoxa1*^{null} background alone (Fig. 6F). These changes were confined to the hindbrain as no alteration in patterns of posterior expression was observed (Fig. 2G). This dosage effect indicates that one functional allele of *Hoxb1* is only sufficient to maintain low levels of r4 expression when *Hoxa1* is disrupted. Furthermore, in double *Hoxa1*^{null}/*Hoxb1*^{null} homozygous embryos expression in presumptive r4 from the endogenous *Hoxb1* promoter was completely abolished at 8.5 dpc (Fig. 2E,F) and did not appear at later stages (data not shown). These results demonstrate that synergistic interactions between *Hoxb1* and *Hoxa1* contribute to the segmental regulation of *Hoxb1* expression in the hindbrain.

Generation and analysis of a targeted mutation in the *Hoxb1* 3′ RARE

These experiments show that *Hoxa1* and *Hoxb1* are both required to establish *Hoxb1* expression in r4, but not in posterior domains. Therefore we wanted to investigate the mechanisms involved in the early activation of these genes, which lead to the initiation of the *Hoxb1* auto-regulatory loop. Our previous study in transgenic mice mapped enhancers in the 3′ flanking region of the *Hoxb1* locus that are capable of mediating early neural and mesodermal expression, and comparative analysis of the neuroectodermal enhancer identified a consensus RARE of the DR2 type required for transgene expression (Marshall et al., 1994). To investigate the role played by the 3′ RARE in the control of endogenous *Hoxb1* expression, we generated a germline mutation in this motif (Fig. 3). The 'Hit and Run' targeting strategy (Hasty et al., 1991) was used to introduce the same point-mutations in the endogenous 3′ RARE sequence that inactivated this enhancer in transgenic analysis (Marshall et al., 1994). This strategy completely removed the plasmid sequences and selectable cassettes, generating a mutant allele with only four base pair changes in the RARE motif compared to the wild-type allele (Fig. 3). Heterozygous *Hoxb1*$^{3'RARE}$ embryos were normal and 95% of the homozygous animals

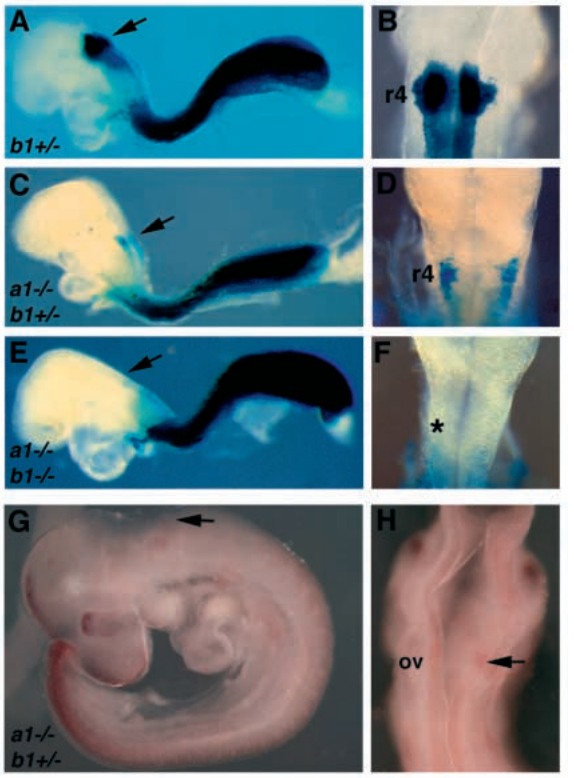

Fig. 2. Reporter staining and endogenous *Hoxb1* expression in double *Hoxb1*^{null}, *Hoxa1*^{null} and compound mutant embryos. Reporter staining originates from the endogenous *Hoxb1* promoter due to the generation of a targeted Hoxb1/lacZ fusion protein (see Studer et al., 1996). Lateral (A,C,E) and dorsal hindbrain (B,D,F) views of β-galactosidase-positive 8.5 dpc single *Hoxb1*^{null}/+ (A,B), *Hoxb1*^{null}/+; *Hoxa1*^{null}/*Hoxa1*^{null} compound (C,D) and double *Hoxb1*^{null}/*Hoxa1*^{null} homozygous (E,F) mutant embryos. Arrows indicate the region of r4 that shows a reduced level of reporter staining and a reduction in size of the r4 territory (C,D); arrow in E and * in F denote complete absence of reporter staining in the r4 region. (G) Lateral and (H) dorsal hindbrain views of a 9.25 dpc *Hoxb1*^{null}/+; *Hoxa1*^{null}/*Hoxa1*^{null} homozygous mutant embryo hybridised with a *Hoxb1* antisense RNA probe. Arrows in G and H indicate low levels of endogenous *Hoxb1* expression in r4. Ov, otic vesicle. Genotypes of the embryos are indicated in A,C,E and G.

were viable and fully fertile when examined in a Sv129 inbred and a C57Bl6/Sv129 hybrid background (data not shown), but 5% of the homozygotes died after birth and showed no milk in their stomach (Gavalas et al., 1998).

To study the role of the 3′ RARE on the spatio-temporal control of *Hoxb1* expression within its normal genomic context, we performed whole-mount in situ hybridisation analysis in headfold stage (7.5-7.75 dpc) embryos (Fig. 4). *Hoxb1* is first expressed at gastrulation within the primitive streak in newly formed mesoderm and then in overlying ectoderm with an anterior boundary in the region of the node (Frohman et al., 1990; Sundin and Eichele, 1990). Double labelling of embryos with *Otx2*, which marks the anterior third of the embryo up to the future midbrain/hindbrain boundary (Ang et al., 1994), and *Hoxb1*, provided a positional marker to aid in evaluating changes in *Hoxb1* expression (Fig. 4A). In homozygous *Hoxb1*$^{3'RARE}$ mutant embryos, *Hoxb1* expression was detected in the posterior two thirds of the embryo (9/11), but the levels were significantly lower than wild-type controls (compare Fig. 4A,C). Transverse sections of mutant embryos from this analysis revealed a strong down-regulation of *Hoxb1* expression in neuroectoderm and reduced levels in the underlying mesoderm in mutant compared to wild-type embryos (Fig. 4B,D). Thus, the 3′ RARE is required in *cis* for regulating proper levels of early neuroectodermal and, to a lesser extent, mesodermal expression of endogenous *Hoxb1*.

Next, we asked whether the lower level of early *Hoxb1* transcripts in the neuroectoderm would affect the later phase of *Hoxb1* expression in r4. No differences in the level of *Hoxb1* expression in r4 were found between mutant (9/9) and wild-type embryos (compare Figs 4E and 5C). Therefore, the up-regulation of *Hoxb1* expression in r4 suggests that either the *Hoxb1* 3′ RARE enhancer is not involved in the onset of *Hoxb1* expression in r4 or that other components are compensating for the loss of the *Hoxb1* 3′ RARE.

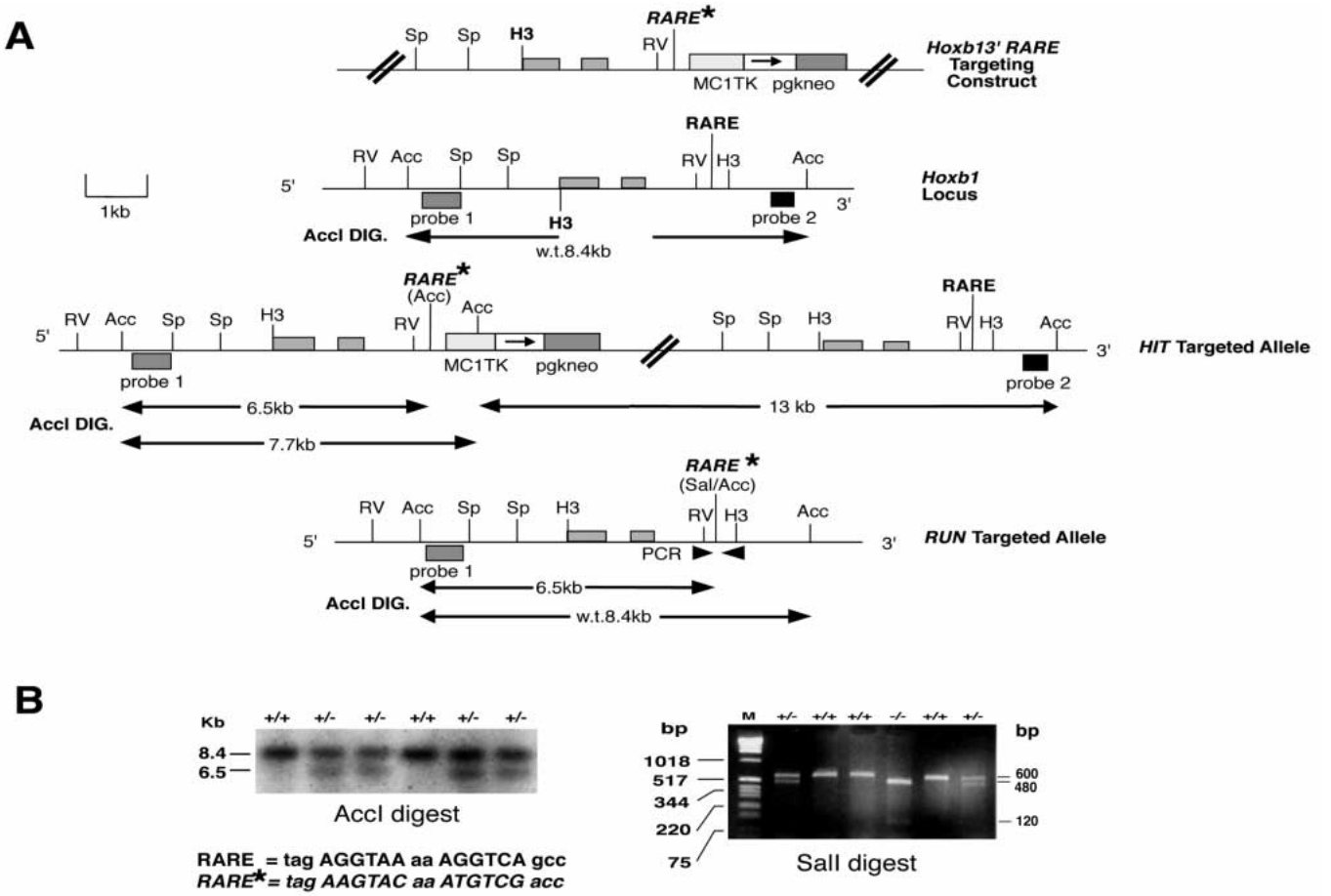

Fig. 3. Targeted disruption of the *Hoxb1* 3′ RARE by homologous recombination using a 'Hit and Run' targeting strategy. (A) Diagram showing the structures of the wild-type *Hoxb1* locus including the position of the wild-type RARE (RARE), the *Hoxb1* 3′ RARE targeting construct, the targeted locus after the 'Hit' event leading to the complete insertion of the targeting construct, and the subsequent 'Run' event after excision of the plasmid backbone, the selectable cassettes and the retention of the point mutations in the RARE sequence (RARE*). The sizes of the restriction fragments required to distinguish between the different recombination events by Southern blot analysis using probes 1 or 2 are listed below each allele. RV, *Eco*RV; Sp, *Spe*I; H3, *Hin*dIII; Acc, *Acc*I; Sal, *Sal*I; DIG, enzyme restriction digestion. Double slash indicates the plasmid backbone. H3 in bold indicates the site in which the integration of the targeting construct has occurred in the genomic locus. (B) The mutations generated in the RARE are indicated at the bottom left. The changes create a new *Sal*I/*Acc*I restriction site that can be used for genotyping. Embryonic stem cell DNA blot hybridised with probe 1 after digestion with *Acc*I and relative to the 'Run' targeted allele (left), and PCR products from yolk-sac DNA after digestion with *Sal*I (right). Arrowheads in A indicate the position of PCR primers used in genotyping. kb, kilobases; bp, base pairs.

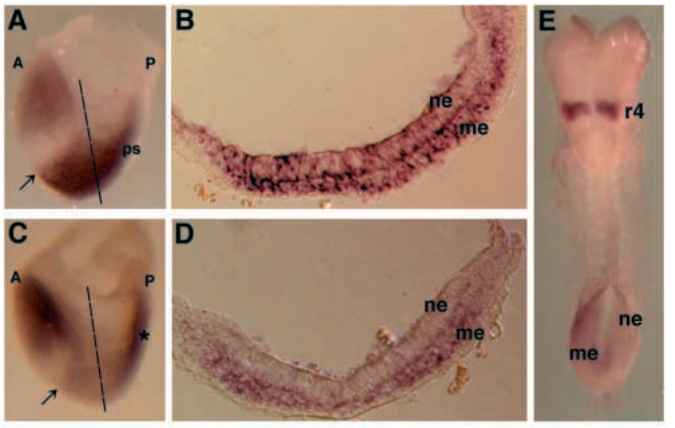

Fig. 4. Endogenous *Hoxb1* expression in *Hoxb1*$^{3'RARE}$ mutant embryos. Whole-mount in situ hybridisation of wild type (A) and *Hoxb1*$^{3'RARE}$ homozygous mutant (C) headfold stage embryos probed with *Otx2* and *Hoxb1*. The arrows indicate the anterior border of *Hoxb1* expression coinciding with the future r3/4 boundary. Note that the overall decreased level of the *Hoxb1* expression domain in C compared to A excluded the primitive streak region, as indicated by *. Transverse sections at the level of the node as indicated in A and C, of wild-type (B) and homozygous mutant (D) embryos, reveal strong down-regulation of *Hoxb1* in neuroectoderm and, to a lesser extent, in mesoderm. The section in D is slightly more anterior than the one in B. (E) Unaltered *Hoxb1* r4 expression in an 8.5 dpc *Hoxb1*$^{3'RARE}$ homozygous mutant embryo. me, mesoderm; ne, neuroectoderm; ps, primitive streak; r4, rhombomere 4; A, anterior; P, posterior.

Double *Hoxa1 and Hoxb1* 3′ RARE homozygous mutants

In view of our finding that both the Hoxb1 and Hoxa1 proteins are involved in establishing r4 expression of *Hoxb1*, we favoured the hypothesis that early expression of *Hoxa1* in neural ectoderm can compensate for the loss of the *Hoxb1* 3′ RARE. In the *Hoxa1* locus there is also a 3′ RARE (Frasch et al., 1995; Langston and Gudas, 1992) and targeted deletion of this motif resulted in a delay in the onset of *Hoxa1* expression in neural ectoderm and mesoderm (Dupé et al., 1997). However, the *Hoxa1*$^{3'RARE}$ mutation did not influence the extent of *Hoxb1* expression in r4 or earlier stages (Dupé et al., 1997; and data not shown). Since the *Hoxa1* and *Hoxb1* 3′ RAREs are required for their respective early neural expression but individually have no effect on *Hoxb1* expression in r4, it is possible that the two 3′ RAREs work synergistically in initiating the auto-regulatory loop in r4. To address this question, we generated double homozygous mutant embryos for the *Hoxa1*$^{3'RARE}$ and *Hoxb1*$^{3'RARE}$ alleles and performed in situ analysis with *Otx2* and *Hoxb1* at different developmental stages (Fig. 5).

At late headfold stage, in five out of six double RARE homozygous mutant embryos examined, the level of *Hoxb1* transcripts was further reduced when compared to single *Hoxb1*$^{3'RARE}$ mutants (compare Figs 5A,B and 4C). In addition, expression in the region of the primitive streak was strongly down-regulated in these embryos when compared to wild type and *Hoxb1*$^{3'RARE}$ mutant embryos (asterisk in Figs 5B and 4A,C). At 8.5 dpc, 10 out of 22 double RARE mutants had either lower levels or a complete absence of *Hoxb1* in r4 compared to wild type (Fig. 5C-E). 1 day later at 9.25-9.5 dpc, 4 out of 12

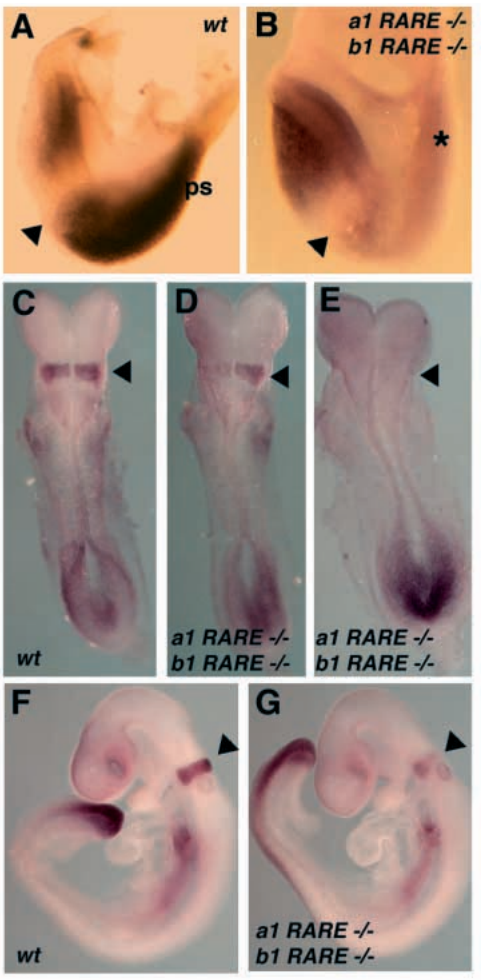

Fig. 5. Down-regulation of *Hoxb1* expression in r4 in *Hoxb1*$^{3'RARE}$/*Hoxa1*$^{3'RARE}$ double mutant embryos. Whole-mount in situ analysis of 7.75 dpc (A,B), 8.5 dpc (C,D,E) and 9.5 dpc (F,G) wild-type (A,C,F) and double RARE mutant (B,D,E,G) embryos hybridised with *Hoxb1* RNA probe. (A,B) Lateral views of headfold stage embryos double-labelled with *Hoxb1* and *Otx2*. Arrowheads show the most anterior border of the *Hoxb1* expression domain. Note in B the overall low level of *Hoxb1* transcript, including the primitive streak region, as indicated by *. (D,G) Transcript levels of *Hoxb1* in r4 are reduced compared to wild-type levels (C,F) (arrowheads). In (E) no *Hoxb1* expression in r4 is detected (arrowhead). ps, primitive streak. Genotypes of the embryos are indicated in the respective panels.

double RARE mutant embryos also showed a decrease of *Hoxb1* transcripts in r4 combined with a specific lack of dorsal expression when compared to wild-type embryos (Fig. 5F,G). Interestingly, the fraction of embryos exhibiting reduced levels of *Hoxb1* expression in r4 correlates with the proportion (33%) of newborn double RARE homozygous mice exhibiting a defect in the mandibular branch of the facial nerve (Gavalas et al., 1998). The clear but variable down-regulation of *Hoxb1* in r4 indicates that the two 3′ RARE enhancers perform common functions in activating the auto/para-regulatory loop of *Hoxb1* expression in r4. Moreover, the diminished expression in the primitive streak region in double mutants suggests a common role in directly or indirectly maintaining *Hoxb1* expression in early primitive streak mesoderm.

Synergy of the *Hoxb1* 3′ RARE with *Hoxa1* in initiating *Hoxb1* expression

The persistence of normal or attenuated r4 expression in the double 3′ RARE mutants, as opposed to the full *Hoxa1*^{null} and *Hoxb1*^{null} double mutants, indicates that other *cis*-elements from these genes apart from the two 3′ RAREs participate in initiating *Hoxb1* expression. Even though *Hoxa1* expression is temporally delayed and levels are reduced in *Hoxa1*$^{3'RARE}$ mutants, the anterior limit of the expression domain is eventually reached at later stages and might then be sufficient to activate *Hoxb1* in r4 (Dupé et al., 1997). Therefore, in order to assess the role of the *Hoxb1* 3′ RARE in the total absence of *Hoxa1*, we generated compound mutant embryos homozygous for both the *Hoxb1*$^{3'RARE}$ and *Hoxa1*^{null} alleles. In single *Hoxa1*^{null} homozygous embryos the overall levels of *Hoxb1* expression including r4 were low at 8.75 dpc, but r4 expression approached wild-type levels at 9.5 dpc even though the overall territory of r4 was smaller (Fig. 6A-F). In contrast, in *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ double homozygous mutant embryos, *Hoxb1* expression was specifically abolished in the r4 domain and reduced in foregut endoderm while tailbud expression was unaltered (Fig. 6G-I). This indicates that *Hoxb1* expression in domains outside of r4 are not dramatically changed in the double mutants compared to single mutant embryos (Fig. 6D,G).These data reveal the key role played by the *Hoxb1* 3′RARE element in establishing *Hoxb1* expression in r4. They confirm that the residual early expression of *Hoxa1*, not controlled by its 3′ RARE, is able to activate *Hoxb1* in r4 and provide further support for the synergistic interactions between *Hoxa1* and *Hoxb1*.

Early patterning of the r4 region in double mutant embryos

In our genetic analysis the absence of up-regulation of *Hoxb1* in r4 seen in both the *Hoxa1*^{null}/*Hoxb1*^{null} and the *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ double mutants could be attributed to a deletion of the r4 territory. Therefore, we used *Krox20* as a flanking marker for r3/r5 to look for the presence of an r4 domain. As previously noted, in *Hoxa1*^{null} single mutants r4 is present but reduced in size. This is evident by the reduced *Hoxb1* expression domain and the shorter distance between the r3 stripe and the thin r5 stripe of dorsally located cells labelled by *Krox20* (Fig. 7C,D) (Dollé et al., 1993). In *Hoxa1*^{null}/*Hoxb1*^{null} double homozygous mutants, we observed a similar *Krox20* expression pattern in r3 and a slight reduction of positive cells dorsally in r5 (Fig. 7E,F), which delineates the presence of an r4-like territory. As shown in the accompanying

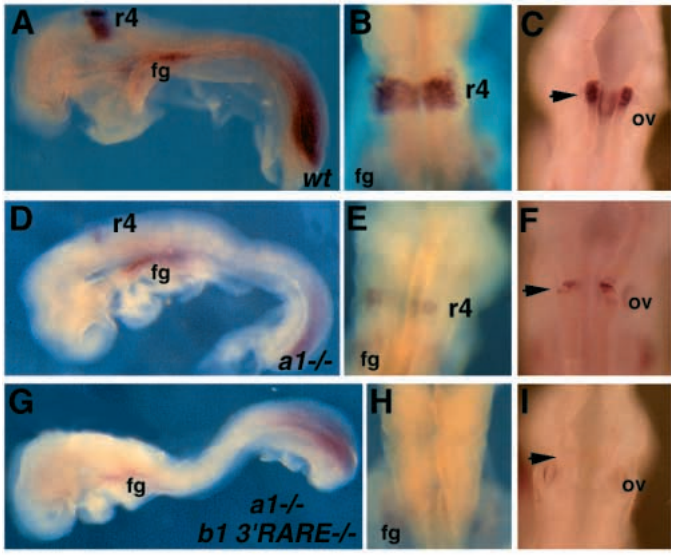

Fig. 6. Up-regulation of *Hoxb1* expression in r4 is lost in *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ double mutant embryos. Lateral (A,D,G) and dorsal views (B,C,E,F,H,I) of 8.75 dpc (A,B,D,E,G,H) and 9.5 dpc (C,F,I) whole-mount embryos hybridised with *Hoxb1* probe. In wild-type embryos (A,B,C) *Hoxb1* expression is localised in presumptive r4 at high levels in the tailbud and in the foregut pocket. In *Hoxa1*^{null} homozygous mutants (D,E), the overall level of *Hoxb1* expression is lower, including in the reduced r4 territory, while at 9.5 dpc (F) *Hoxb1* r4 expression reaches equivalent wild-type levels. However, no *Hoxb1* up-regulation in r4 is observed in double *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ in the two stages examined (G,H,I), and the foregut expression is reduced in these double mutants (G) compared to single *Hoxa1*^{null}(D). The arrows indicate *Hoxb1* expression in the r4 territory in C and F and absence of expression in I. fg, foregut pocket; r4, rhombomere 4; ov, otic vesicle. Genotypes of the embryos are indicated in A, D and G.

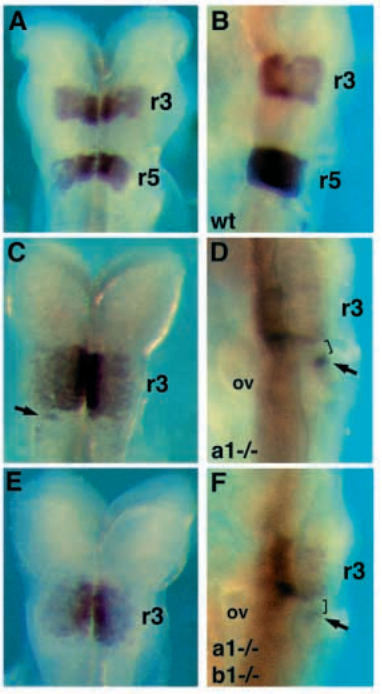

Fig. 7. Persistence of a r4-like territory in double *Hoxb1*^{null}/*Hoxa1*^{null} mutant embryos. Whole-mount in situ analysis of 8.5 dpc (A,C,E) and 9.5 dpc (B,D,F) embryos hybridised with *Krox20* probe. Dorsal view (A) of a 8.5 dpc wild-type embryo showing high level of *Krox20* expression in r3 and r5, while decreased levels in r3 are observed at 9.5 dpc (B). In *Hoxa1*^{null} homozygous embryos there is an enlarged domain of *Krox20* in prospective r3 and a few dorsally located cells in r5 in both stages (C, D). In double *Hoxb1*^{null}/*Hoxa1*^{null} mutant embryos no *Krox20*-positive cells in r5 are detected in 8.5 dpc (E), but patchy labelled cells are still present at 9.5 dpc (F). The arrows in C, D and F indicate the reminant of dorsal *Krox20* labelled cells in r5 and the brackets in D and F the remaining r4-like territory between the r3 and r5 domains of *Krox20* expression. Genotypes of the embryos are indicated in panels B, D and F. ov, otic vesicle.

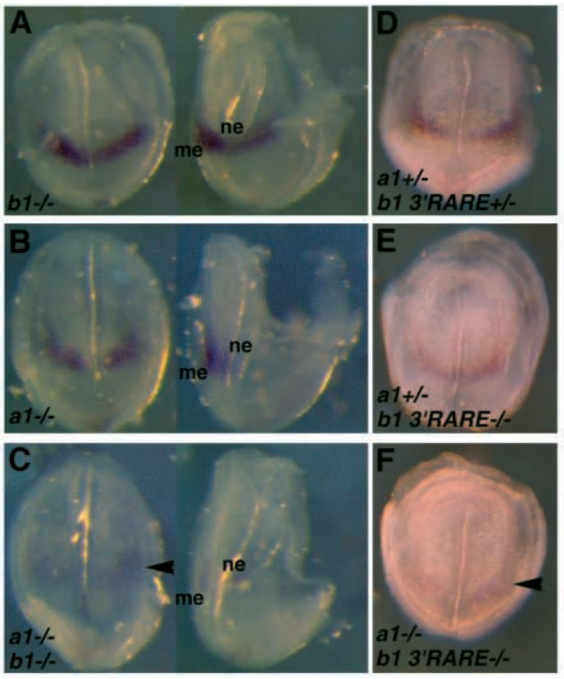

Fig. 8. Down-regulation of *EphA2* expression in prospective r4 in double homozygous mutant embryos. Ventral and lateral views of whole-mount 0-2 somite embryos hybridised with *EphA2* as the earliest prospective r4 marker in mesoderm and neuroectoderm. No dramatic differences in *EphA2* expression are relevant in *Hoxb1*^{null} homozygous (A), *Hoxa1*^{null} homozygous (B) and *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ double heterozygous (D) mutant embryos whereas a slightly lower transcript level is observed in *Hoxa1*^{null} heterozygous/*Hoxb1*$^{3'RARE}$ homozygous double mutants (E). In contrast, strong down-regulation of *EphA2* expression in prospective r4 is detected in *Hoxb1*^{null}/*Hoxa1*^{null} (C) and *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ (F) double homozygous mutant embryos, even if a low level of *EphA2* mesodermal expression is maintained. Arrowheads indicate decreased *EphA2* expression in double homozygous mutants. me, mesoderm; ne, neuroectoderm. Genotypes of the embryos are indicated in the respective panels.

paper (Fig. 2: Gavalas et al., 1998), analysis with additional markers at these stages strongly supports the persistence of an r4-like territory. Therefore, the absence of *Hoxb1* expression in r4 in this region did not reflect a segmental deletion of the r4 territory compared with the single *Hoxa1*^{null} mutation.

While these results demonstrate that a compartmental structure is preserved between r3 and r5 they do not address the nature of its molecular identity. To investigate this issue, we used *EphA2 (Sek2)*, a member of the EpH receptor tyrosine kinase family, as the earliest marker expressed in presumptive r4 (Becker et al., 1994; Ruiz and Robertson, 1994). We previously showed that mesodermal and neural expression of *EphA2* in the region of prospective r4 was not affected in *Hoxb1*^{null} mutant embryos, indicating that in the absence of *Hoxb1* patterning of r4 could be properly initiated but not maintained (Fig. 8A) (Studer et al., 1996). Here, we found that in single *Hoxa1*^{null} homozygous embryos, despite the size reduction in r4 at later stages, expression of *EphA2* in prospective r4 territory was not affected (Fig. 8B). However, both the *Hoxa1*^{null}/*Hoxb1*^{null} and *Hoxa1*^{null}/*Hoxb1*$^{3'RARE}$ double homozygous mutant embryos showed a dramatic down-regulation of *EphA2* expression in both neuroectoderm and mesoderm layers, although a low level of transcripts persisted in the underlying mesoderm (Fig. 8C,F). One functional copy of either *Hoxa1* or the *Hoxb1* 3′ RARE was sufficient to restore expression of *EphA2* (Fig. 8D,E). The requirement for both *Hoxa1* and *Hoxb1* in regulation of *EphA2* links *Hox* genes with the control of cell signalling events and guidance cues mediated by the *Eph* receptor tyrosine kinase family (Orioli et al., 1997). These genetic analyses show that *Hoxb1* and *Hoxa1* synergistically control the early r4 patterning programme and have unmasked a new role for these genes in establishing r4 identity, in addition to their previously described functions in hindbrain segmentation.

DISCUSSION

In this study we have presented genetic analyses that address the mechanisms involved in establishing and maintaining rhombomere-restricted expression of *Hoxb1*. We have shown that auto- and para-regulatory mechanisms are two processes involved in initiating segmental expression of *Hoxb1*. Our findings have revealed synergy between *Hoxa1* and *Hoxb1* in early patterning of the r4 region and have defined a new early role for *Hoxb1*, in addition to its later role in maintaining segmental identity and controlling motor neuron migration. We demonstrated that *Hoxa1* and *Hoxb1* are both required to properly initiate the programme specifying r4 identity, necessary for neural patterning. Furthermore, mutational analysis of the endogenous *Hoxb1* 3′ RARE has highlighted the pivotal role of retinoids acting through this *cis*-element in establishing early *Hoxb1* expression. Our data have important implications for understanding mechanisms involved in early regionalisation of the vertebrate hindbrain.

Initiation and maintenance of *Hoxb1* expression in r4

The genetic experiments presented in this work and data from our previous studies in transgenic mice have identified the basis by which *Hoxb1* expression is generated in r4 and have detailed the specific involvement of *Hoxa1* and *Hoxb1* in this process. Table 1 summarises the effects on both initiation and maintenance of r4 identity in the different genetic backgrounds examined, while Fig. 9 presents a model describing the regulatory interactions between *Hoxa1* and *Hoxb1*. High levels

Table 1. Effects on segmental patterning in different mutant backgrounds

Genotypes[a]	Initiation of r4 identity[b]	Maintenance of r4 identity[c]
Hoxb1^{null}	+	–
Hoxb1$^{3'RARE}$	+	+
Hoxa1^{null}	+	+
Hoxa1$^{3'RARE}$	+	+
Hoxb1^{null}/*Hoxa1*^{null}	–	–
Hoxb1$^{3'RARE}$/*Hoxa1*^{null}	–	–
Hoxb1$^{3'RARE}$/*Hoxa1*$^{3'RARE}$	+/–	+/–

[a]Genotypes indicate homozygous mutants, and in compound heterozygous and homozygous mutants the phenotypes are intermediate.
[b]Initiation is based on early *EphA2* and *Hoxb1* expression in r4.
[c]Maintenance is based on later *Hoxb1* expression in r4.
+, presence of expression; –, absence of expression; +/–, low levels or highly variable expression.

of *Hoxb1* in r4 are maintained through a direct auto-regulatory loop involving the binding of Hoxb1 together with Exd/Pbx proteins as a cofactor on three consensus HOX/PBC sites (Pöpperl et al., 1995; Studer et al., 1996). However, we found that low levels of *Hoxb1* reporter staining can in some cases be observed in r4 at early stages even in the absence of Hoxb1 (Fig. 1B). This indicates that other factors can also initiate or trigger *Hoxb1* transcription, but in the absence of the auto-regulatory loop, expression cannot be stably maintained in r4 (Fig. 1E). Double mutant analysis with *Hoxa1* and *Hoxb1* revealed that *Hoxa1* was responsible for the residual reporter activity (Fig. 2E,F). This illustrates that *Hoxa1* also has a normal role in activating the r4 enhancer through para-regulatory interactions in early stages, but is unable to participate in the long-term maintenance of *Hoxb1* expression because it is not expressed at these later stages (Hunt et al., 1991; Murphy and Hill, 1991). In view of the previous observations that ectopic *Hoxa1* and *labial* are able to transactivate the *Hoxb1* r4 enhancer (Chan et al., 1996; Di Rocco et al., 1997; Zhang et al., 1994), we favour the idea that the *Hoxa1* can activate *Hoxb1* directly through the same bipartite HOX/PBC motifs.

The para-regulatory interactions between *Hoxa1* and *Hoxb1* help to ensure that *Hoxb1* expression is generated in r4, while the *Hoxb1* auto-regulatory function ensures that high levels are maintained. Furthermore, *Hoxb1* works in a direct cross-regulatory manner to up-regulate *Hoxb2* expression in r4 (Maconochie et al., 1997). Therefore, *Hoxb1* utilises auto-, para- and cross-regulatory mechanisms as a part of its role in maintaining r4 identity and regulating facial motor neuron patterning (Barrow and Capecchi, 1996; Goddard et al., 1996; Studer et al., 1996).

The role of the RAREs in establishing early *Hoxb1* expression

With respect to the mechanisms involved in establishing the early expression and triggering the auto-regulatory loop in r4, our targeted mutagenesis of the *Hoxb1* 3′ RARE demonstrated that it is normally required to modulate high levels of early *Hoxb1* expression in the neuroectoderm and, to a lesser extent, in the mesoderm (Fig. 4). A similar situation exists in the *Hoxa1* locus, where a 3′ RARE has previously been shown to be required for the high level of *Hoxa1* expression in both ectoderm and mesoderm (Dupé et al., 1997). There are differences in the influence of these two 3′ RAREs on their respective genes, in that the *Hoxa1* 3′ RARE appears to exert a stronger influence on mesodermal expression and controls the timing of anterior expression (Fig. 4C; Dupé et al., 1997). Together these experiments have shown a genetic requirement for the *Hoxb1* and *Hoxa1* 3′ RAREs in mediating proper early expression of the endogenous genes and strongly suggest that retinoids acting through these elements play an important role in activating *Hox* expression.

In single mutants the roles of these RARE motifs in the regulation of *Hoxb1* were masked due to the synergy or ability of either *Hoxa1* or *Hoxb1* to activate the auto-regulatory loop. However, our analysis in double homozygous mutants has also shown that these RAREs are a major component of the *cis* mechanisms required to trigger *Hoxb1* expression in r4. There is an absolute requirement for the *Hoxb1* RARE in double mutants as compared to the *Hoxa1* motif, revealing that additional *cis*-elements in *Hoxa1* can make a contribution to activation of *Hoxb1*. This could be through additional RA-dependent or RA-independent elements in the *Hoxa1* locus. Furthermore, our model for the role of the 3′ RAREs in modulating the endogenous expression of these genes provides insight into the nature of their response to exogenous doses of RA (Fig. 9). There are distinct temporal windows of competence in the ability of *Hox* genes to respond to RA treatment, and the timing of the early expression mediated by the *Hoxa1* and *Hoxb1* 3′ RARE enhancers corresponds to the

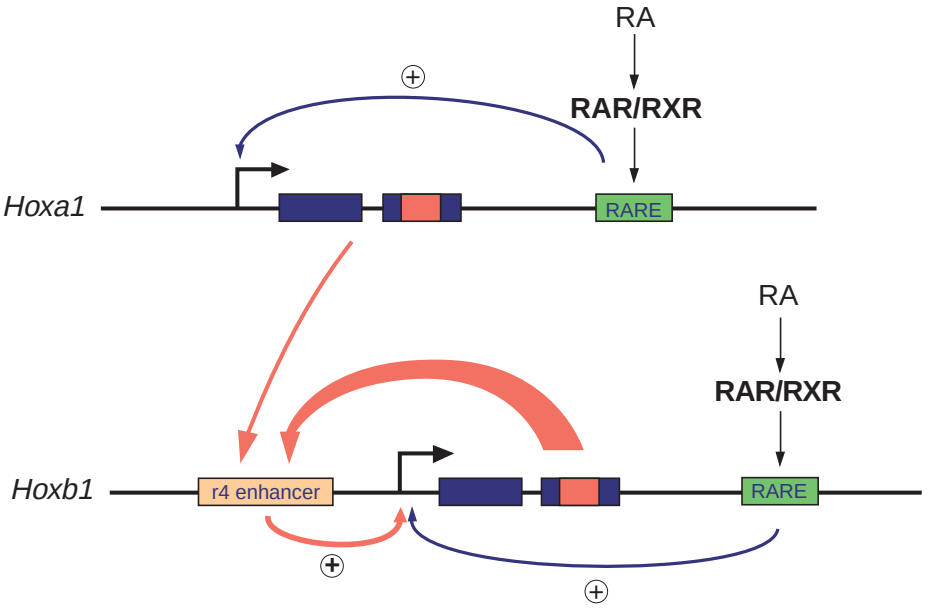

Fig. 9. Genetic interactions between *Hoxa1* and *Hoxb1* in modulating high expression of *Hoxb1* in r4. Schematic representation of the proposed model (see also Marshall et al., 1996). The *Hoxa1* and *Hoxb1* genes are represented by two boxes and the arrows 5′ to the boxes denote the start site for transcription. The r4 enhancer and the RAREs are indicated by coloured rectangles on the schematic *Hoxb1* and *Hoxa1* loci. The curved arrows above the *Hoxa1* locus and below the *Hoxb1* locus, together with the + sign, represent the positive effect of the enhancers on the transcription of the genes. The arrow from *Hoxa1* towards the r4 enhancer of *Hoxb1* indicates the binding of Hoxa1 to the *Hoxb1* enhancer. A thicker arrow from *Hoxb1* towards the r4 enhancer denotes a larger contribution of Hoxb1 compared to Hoxa1 in the maintenance of *Hoxb1* expression in r4. Retinoids, and in particular RA, are bound by retinoid receptors, and activate *Hoxa1* and *Hoxb1* early expression through their respective RAREs located 3′ to the genes. Hoxa1 protein binds subsequently to the motifs embedded in the r4 enhancer, and together with Hoxb1 itself initiates expression of *Hoxb1* in r4. A direct positive feedback circuit represented by the auto-regulatory loop maintains high level of *Hoxb1* expression in r4.

same period that they display ectopic expression in response to exogenous RA (Conlon and Rossant, 1992; Hill et al., 1995; Marshall et al., 1992; Morrison et al., 1996, 1997; Simeone et al., 1995). Therefore, the ectopic RA-induced activation of these genes early in hindbrain development is most likely a direct response mediated through these RARE motifs. In contrast, the RA-induced transformation of r2 to r4, which occurs following these early treatments (Hill et al., 1995; Kessel, 1993; Marshall et al., 1992), is a consequence of stably triggering *Hoxb1* in r2 by an indirect mechanism, involving the *Hoxb1* auto-regulatory loop.

Synergy between *Hoxa1* and *Hoxb1* and new roles in specifying r4 identity

We have demonstrated a synergy between *Hoxa1* and *Hoxb1* with respect to the establishment of the *Hoxb1* r4 auto-regulatory loop. Furthermore the loss of the earliest known marker of presumptive r4, *EphA2*, extends this synergy to other early patterning events in this region (Fig. 8). These genetic interactions have unmasked new roles for *Hoxa1* and *Hoxb1* in programming the specification of r4 identity. Loss of *Hoxa1* reduces the size of r4 but does not alter its normal identity (Fig. 6D-F); (Carpenter et al., 1993; Dollé et al., 1993), whereas loss of *Hoxb1* has no influence on the size of r4 but fails to maintain its proper identity (Goddard et al., 1996; Studer et al., 1996). The absence of both genes does not lead to a further reduction in r4 size, based on *Krox20* expression (Fig. 7), indicating the presence of a territory between r3 and r5 with an unknown identity. We interpret the absence of all r4 markers in this territory as a failure to initiate rather than maintain the specification of r4 identity. This defines a new role for *Hoxa1* in later events, compared with its early role in establishing segments. Similarly in addition to the requirement of *Hoxb1* in maintaining r4 identity, our genetic analyses have unmasked an earlier role of *Hoxb1* in establishing r4 identity. In summary, these findings demonstrate that individual members of the group 1 genes can have distinct roles in multiple steps governing segmental processes in the developing hindbrain.

Multiple and diverse functions for *Hox* genes are likely to be a common feature in evolution. For example, the *C. elegans Antp*-related *Hox* gene, *mab-5*, can sequentially programme proliferation, differentiation and morphogenesis independently within the same cell lineage (Salser and Kenyon, 1996). In *Drosophila*, *labial (lab)* function is necessary for normal head development in both embryos and adults, but the role of *lab* in the two stages appears to be quite different. In the embryo, *lab* appears to be involved in the process of head involution rather than in the correct specification of the identity of a particular segment (Diederich et al., 1989). In the adult, the absence of *lab* function results in a transformation of posterior head to thorax as well as in deletion and/or duplication of other specific head tissue, behaving more like a traditional homeotic gene (Merrill et al., 1989). Hence, in the process of duplication and divergence of the homeotic complex during vertebrate evolution, multiple functions of the ancestral *labial* gene could have been partitioned between different paralogs.

The presence on an unidentified territory in the place of r4 raises a question as to the long-term consequences that would arise from a failure to initiate the r4 programme. To address this issue, Gavalas et al. (1998) present in the accompanying paper a detailed analysis of morphogenetic defects in different genetic backgrounds. In agreement with the early defects in r4 patterning documented in this study, they find a variety of defects arising from r4-derived neural crest cells and patterning of neural populations in the hindbrain. In the hindbrain some of the defects are observed in segments other than r4, outside the domains of *Hoxb1* and *Hoxa1* expression, suggesting abnormalities in interactions or signalling between adjacent segments.

We thank Allan Bradley and Ramiro Ramirez-Solis for hosting M.S. and for training in ES cell technology and the 'Hit and Run' method; Amanda Hewett for animal husbandry; Elena Grigorieva for discussions on mutant phenotypes; Tom Lufkin for generating the *Hoxa1*^{null} mice; and Andrew Lumsden, Georgy Köntges and members of our groups for useful general discussions. M.S. was supported by fellowships from EMBO, EEC Human Capital Mobility and the Medical Research Council (MRC), A.G. by fellowships from EEC Human Capital Mobility, EMBO (short term) and Fondation pour la Recherché Médicale (FRM). This work was also supported by the MRC and a NATO Collaborative travel grant to R.K.

REFERENCES

Alexandre, D., Clarke, J. D., Oxtoby, E., Yan, Y.-L., Jowett, T. and Holder, N. (1996). Ectopic expression of *Hoxa-1* in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid -induced phenotype. *Development* **122,** 735-746.

Ang, S.-L., Conlon, R. A., Jin, O. and Rossant, J. (1994). Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. *Development* **120,** 2979-2989.

Barrow, J. and Capecchi, M. (1996). Targeted disruption of the *Hoxb2* locus in mice interferes with expression of *Hoxb1* and *Hoxb4*. *Development* **122,** 3817-3828.

Becker, N., Seitanidou, T., Murphy, P., Mattei, M.-G., Topilko, P., Nieto, M. A., Wilkinson, D. G., Charnay, P. and Gilardi-Hebenstreit, P. (1994). Several tyrosine kinase genes of the Eph family are segmentally expressed in the developing hindbrain. *Mech. Dev.* **47,** 3-18.

Bienz, M. (1994). Homeotic genes and positional signalling in the Drosophila viscera. *Trends Genet.* **10,** 22-26.

Blumberg, B., Bolado, J., Moreno, T., Kintner, C., Evans, R. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124,** 373-379.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of *Hoxa-1 (Hox-1.6)* function results in the reorganization of the murine hindbrain. *Development* **118,** 1063-1075.

Chan, S.-K., Pöpperl, H., Krumlauf, R. and Mann, R. S. (1996). An extradenticle-induced conformational change in a Hox protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J.* **15,** 2476-2487.

Chisaka, O., Musci, T. and Capecchi, M. (1992). Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene Hox-1.6. *Nature* **355,** 516-520.

Chouinard, S. and Kaufman, T. C. (1991). Control of expression of the homeotic *labial* (*lab*) locus of *Drosophila melanogaster*: evidence for both positive and negative autogenous regulation. *Development* **113,** 1267-1280.

Clarke, J. D. and Lumsden, A. (1993). Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain. *Development* **118,** 151-162.

Condie, B. G. and Capecchi, M. R. (1994). Mice with targeted disruptions in the paralogous genes Hoxa-3 and Hoxd-3 reveal synergistic interactions. *Nature* **370,** 304-307.

Conlon, R. A. (1995). Retinoic acid and pattern formation in vertebrates. *Trends Genet.* **11,** 314-319.

Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116,** 357-368.

Davis, A. P., Witte, D. P., Hsieh-Li, H. M., Potter, S. S. and Capecchi, M. R. (1995). Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. *Nature* **375,** 791-795.

Dekker, E. J., Pannese, M., Houtzager, E., Timmermans, A., Boncinelli, E.

and Durston, A. (1992). Xenopus Hox-2 genes are expressed sequentially after the onset of gastrulation and are differentially inducible by retinoic acid. *Development* **Supplement,** 195-202.

Deschamps, J. and Wijgerde, M. (1993). Two phases in the establishment of *Hox* expression domains. *Dev. Biol.* **156,** 473-480.

Di Rocco, G., Mavilio, F. and Zappavigna, V. (1997). Functional dissection of a transcriptionally active, target-specific Hox-Pbx complex. *EMBO J.* **16,** 3644-3654.

Diederich, R. J., Merrill, V. K. L., Pultz, M. A. and Kaufman, T. C. (1989). Isolation, structure and expression of labial, a homeotic gene of the Antennapedia Complex involved in Drosophila head development. *Genes Dev.* **3,** 399-414.

Dollé, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D. and Chambon, P. (1993). Local alterations of *Krox-20* and *Hox* gene expression in the hindbrain of *Hoxa-1(Hox-1.6)* homozygote null mutant embryos. *Proc. Nat. Acad. Sci. USA* **90,** 7666-7670.

Duboule, D. (1993). The function of Hox genes in the morphogenesis of the vertebrate limb. *Ann. Genet.* **36,** 24-9.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. (1997). *In vivo* functional analysis of the *Hoxa1* 3′ retinoid response element (3′ RARE). *Development* **124,** 399-410.

Favier, B., Rijli, F. M., Fromental-Ramain, C., Fraulob, V., Chambon, P. and Dollé, P. (1996). Functional cooperation between the non-paralogous genes Hoxa-10 and Hoxd-11 in the developing forelimb and axial skeleton. *Development* **122,** 449-460.

Frasch, M., Chen, X. and Lufkin, T. (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine Hoxa-1 and Hoxa-2 loci in both mice and Drosophila. *Development* **121,** 957-974.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344,** 431-435.

Frohman, M. and Martin, G. (1992). Isolation and analysis of embryonic expression of *Hox-4.9*, a member of the murine labial-like gene family. *Mech. Dev.* **38,** 55-67.

Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse Hox-2.9 gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110,** 589-607.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F., Krumlauf, R. and Chambon, P. (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* (in press).

Goddard, J., Rossel, M., Manley, N. and Capecchi, M. (1996). Mice with targeted disruption of *Hoxb1* fail to form the motor nucleus of the VIIth nerve. *Development* **122,** 3217-3228.

Godsave, S., Dekker, E.-J., Holling, T., Pannese, M., Boncinelli, E. and Durston, A. (1994). Expression patterns of *Hoxb* genes in the *Xenopus* embryo suggest roles in anteroposterior specification of the hindbrain and in dorsoventral patterning of the mesoderm. *Dev. Biol.* **166,** 465-476.

Gould, A., Morrison, A., Sproat, G., White, R. and Krumlauf, R. (1997). Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping *Hox* expression patterns. *Genes Dev.* **11,** 900-913.

Hasty, P., Ramirez-Solis, R., Krumlauf, R. and Bradley, A. (1991). Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* **350,** 243-246.

Hill, J., Clarke, J. D. W., Vargesson, N., Jowett, T. and Holder, N. (1995). Exogenous retinoic acid causes specific alterations in the development of the midbrain and hindbrain of the zebrafish embryo including positional respecification of the Mauthner neuron. *Mech. Dev.* **50,** 3-16.

Horan, G., Ramirez-Solis, R., Featherstone, M., Wolgemuth, D., Bradley, A. and Behringer, R. (1995). Compound mutants for the paralogous *Hoxa-4, Hoxb-4* and *Hoxd-4* genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. *Genes Dev.* **9,** 1667-1677.

Hunt, P., Gulisano, M., Cook, M., Sham, M., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991). A distinct *Hox* code for the branchial region of the head. *Nature* **353,** 861-864.

Itasaki, N., Sharpe, J., Morrison, A. and Krumlauf, R. (1996). Reprogramming Hox expression in the vertebrate hindbrain: Influence of paraxial mesoderm and rhombomere transposition. *Neuron* **16,** 487-500.

Kessel, M. (1993). Reversal of axonal pathways from rhombomere 3 correlates with extra Hox expression domains. *Neuron* **10,** 379-393.

Kolm, P., Apekin, V. and Sive, H. (1997). *Xenopus* hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

Köntges, G. and Lumsden, A. (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.

Krumlauf, R. (1993). Mouse Hox genetic functions. *Curr. Opin. Genet. Dev.* **3,** 621-625.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78,** 191-201.

Langston, A., Thompson, J. and Gudas, L. (1997). Retinoic acid-responsive enhancers located 3′ of the HoxA and the HoxB gene clusters. *J. Biol. Chem.* **272,** 2167-2175.

Langston, A. W. and Gudas, L. J. (1992). Identification of a retinoic acid responsive enhancer 3′ of the murine homeobox gene Hox-1.6. *Mech. Dev.* **38,** 217-228.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M. and Chambon, P. (1991). Disruption of the Hox-1.6 homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66,** 1105-1119.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337,** 424-428.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274,** 1109-1115.

Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113,** 1281-1291.

Maconochie, M., Nonchev, S., Studer, M., Chan, S.-K., Pöpperl, H., Sham, M.-H., Mann, R. and Krumlauf, R. (1997). Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*. *Genes Dev.* **11,** 1885-1896.

Maconochie, M. K., Nonchev, S., Morrison, A. and Krumlauf, R. (1996). Paralogous *Hox* Genes: Function and Regulation. *Annu. Rev. Genet.* **30,** 529-556.

Maden, M., Gale, E., Kostetskii, I. and Zile, M. (1996). Vitamin A deficient quail embryos half a hindbrain and other neural defects. *Curr. Biol.* **6,** 417-426.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995). The Nuclear Receptor Superfamily: The Second Decade. *Cell* **83,** 835-839.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119,** 319-338.

Marshall, H., Morrison, A., Studer, M., Pöpperl, P. and Krumlauf, R. (1996). Retinoids and Hox genes. *FASEB J.* **10,** 969-978.

Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A. and Krumlauf, R. (1992). Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* **360,** 737-741.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature* **370,** 567-571.

Merrill, V., Diederich, R., Turner, F. and Kaufman, T. (1989). A genetic and developmental analysis of mutations in labial, a gene necessary for proper head formation in *Drosophila melanogaster*. *Dev. Biol.* **135,** 376-391.

Moroni, M., Vigano, M. and Mavilio, F. (1993). Regulation of the human *HOXD4* gene by retinoids. *Mech. Dev.* **44,** 139-154.

Morrison, A., Ariza-McNaughton, L., Gould, A., Featherstone, M. and Krumlauf, R. (1997). *HOXD4* and regulation of the group 4 paralog genes. *Development* **124,** 3135-3146.

Morrison, A., Chaudhuri, C., Ariza-McNaughton, L., Muchamore, I., Kuroiwa, A. and Krumlauf, R. (1995). Comparative analysis of chicken *Hoxb-4* regulation in transgenic mice. *Mech. Dev.* **53,** 47-59.

Morrison, A., Moroni, M., Ariza-McNaughton, L., Krumlauf, R. and Mavilio, F. (1996). In vitro and transgenic analysis of a human *HOXD4* retinoid-responsive enhancer. *Development* **122,** 1895-1907.

Murphy, P., Davidson, D. R. and Hill, R. E. (1989). Segment-specific expression of a homeobox-containing gene in the mouse hindbrain. *Nature* **341,** 156-159.

Murphy, P. and Hill, R. E. (1991). Expression of the mouse labial-like homeobox-containing genes, Hox 2.9 and Hox 1.6, during segmentation of the hindbrain. *Development* **111,** 61-74.

Ogura, T. and Evans, R. (1995a). Evidence for two distinct retinoic acid response pathways for *Hoxb-1* gene regulation. *Proc. Nat. Acad. Sci. USA* **92,** 392-396.

Ogura, T. and Evans, R. (1995b). A retinoic acid-triggered cascade of *Hoxb-1* gene activation. *Proc. Nat. Acad. Sci. USA* **92,** 387-391.

Oroli, D. and Klein, R. (1997). The Eph receptor family: axonal guidance by contact repulsion. *Trends Genet.* **13**, 354-359.

Papalopulu, N., Clarke, J., Bradley, L., Wilkinson, D., Krumlauf, R. and Holder, N. (1991a). Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in Xenopus embryos. *Development* **113,** 1145-1159.

Papalopulu, N., Lovell-Badge, R. and Krumlauf, R. (1991b). The expression of murine Hox-2 genes is dependent on the differentiation pathway and displays collinear sensitivity to retinoic acid in F9 cells and Xenopus embryos. *Nucl. Acids Res.* **19,** 5497-5506.

Pöpperl, H., Bienz, M., Studer, M., Chan, S.-K., Aparicio, S., Brenner, S., Mann, R. S. and Krumlauf, R. (1995). Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent on *exd/pbx*. *Cell* **81,** 1031-1042.

Pöpperl, H. and Featherstone, M. S. (1993). Identification of a retinoic acid repsonse element upstream of the murine *Hox-4.2* gene. *Mol. Cell. Biol.* **13,** 257-265.

Ramirez-Solis, R., Davis, A. and Bradley, A. (1993). Gene targeting in embryonic stem cells. In *Guide to Techniques in Mouse Development*, vol. 225, (ed. P. Wassarman and M. DePamphilis), pp. 855-878. Academic Press.

Ruiz i Altaba, A. and Jessell, T. (1991). Retinoic acid modifies mesodermal patterning in early Xenopus embryos. *Genes Dev.* **5,** 175-187.

Ruiz, J. and Robertson, E. (1994). The expression of the receptor-protein tyrosine kinase gene, eck, is highly restricted during early mouse development. *Mech. Dev.* **46,** 87-100.

Salser, S. and Kenyon, C. (1996). A *C. elegans Hox* gene switches on, off, on and off again to regulate proliferation, differentiation and morphogenesis. *Development* **122,** 1651-1661.

Sechrist, J., Serbedzija, G. N., Scherson, T., Fraser, S. E. and Bronner-Fraser, M. (1993). Segmental migration of the hindbrain neural crest does not arise from its segmental generation. *Development* **118,** 691-703.

Simeone, A., Avantaggiato, V., Moroni, M. C., Mavilio, F., Arra, C., Cotelli, F., Nigro, V. and D, A. (1995). Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system. *Mech. Dev.* **51,** 83-98.

Sive, H. and Cheng, P. (1991). Retinoic acid perturbs the expression of *Xhox.lab* genes and alters mesodermal determination in *Xenopus laevis*. *Genes Dev.* **5,** 1321-1332.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384,** 630-634.

Studer, M., Pöpperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265,** 1728-1732.

Sundin, O. H. and Eichele, G. (1990). A homeo domain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev.* **4,** 1267-1276.

Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P. W. J., Stott, D. and Allemann, R. K. (1991). Multiple spatially specific enhancers are required to reconstruct the pattern of *Hox-2.6* gene expression. *Genes Dev.* **5,** 2048-2059.

Wilkinson, D. and Green, J. (1990). In situ hybridization and three-dimensional reconstruction of serial sections. In *Postimplantation Mouse Embryos: A Practical Approach* (ed. A. J. Copp, D. L. Cockcroft and B. D. Hames), pp. 155-171. IRL Press, Oxford.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989a). Segment-specific expression of a zinc finger gene in the developing nervous system of the mouse. *Nature* **337,** 461-464.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989b). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341,** 405-409.

Zakany, J., Gerard, M., Favier, B., Potter, S. S. and Duboule, D. (1996). Functional equivalence and rescue amongst Group 11 Hox gene products in vertebral patterning. *Dev. Biol.* **176**, 325-328.

Zhang, M., Kim, H.-J., Marshall, H., Gendron-Maguire, M., Lucas, A. D., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R. and Grippo, J. F. (1994). Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. *Development* **120,** 2431-2442.