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

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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 paracrine 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; Luftkin 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 that 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 Hox4 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 (α-RAR) 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 Hoxal (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 Hoxal 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 Hoxal 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 Hoxal 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 Hoxal in double mutant analysis. Our results demonstrate that the Hoxb1 3′ RARE is required for early neuronal expression and that Hoxal 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 Hoxal, 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 Hoxal and Hoxb1 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<sup>null</sup> (Studer et al., 1996), Hoxb1/HPAP (Itasaki et al., 1996; Studer et al., 1996), Hoxal<sup>null</sup> (Luftkin et al., 1991) and Hoxal<sup>RARE</sup> (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 EcoRV-HindIII fragment deriving from the 3' flanking region of Hoxb1, which introduced a SalI or Accl 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 SpeI-EcoRV 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 BamHI, blunt-ended, cut with XhoI and inserted into the blunt-ended ClaI and XhoI sites of the multiple cloning site of pBluescript KS+ of SRVmRARE. An additional 1.2 kb Hoxb1 genomic SpeI fragment was placed into the SpeI 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 HindIII 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 Accl and analysed by mini-Southern blot analysis using 5' (probe 1) and 3' (probe 2) external probes (Fig. 3). Probe 1 corresponds to a HincII-SpeI 250 bp fragment and probe 2 to a RsaI 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 RARE mice and embryos was performed either by Southern blot analysis by using probe 1, or by PCR (Fig. 3B). The oligonucleotides 5'-GTTGTTAAGGAGGTGCCTTG-3' and 5'-TCTTCCAGGTAGCCTGTG-3' were used to amplify a 600 bp fragment in conditions previously described (Whiting et al., 1991). The PCR product was subsequently digested with SalI (Fig. 3B) and two digestion products of 480 bp and 120 bp were obtained in the presence of the mutation.

**RESULTS**

**Hoxb1 regulation in Hoxb1null 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 Hoxb1null homozgyous 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 Hoxb1null 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 Hoxb1null 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 -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 homozgyous mutant embryos for both Hoxa1 and Hoxb1 to look for genetic interactions between the genes (Fig. 2). In this work we used the Hoxa1null mutants generated by Lufkin et al. (1991), which have a slightly different hindbrain phenotype compared to those (Hoxa1null) generated by Chisaka et al. (1992). The main difference between these two alleles is that, based on Krox20 expression, one Hoxa1null mutant retains a vestige of r5 (Dollé et al., 1993; Mark et al., 1993), whereas a
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⁺RARE embryos were normal and 95% of the homozygous animals complete deletion of r5 has been reported in the case of the other Hoxa1null (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 Hoxa1null and Hoxb1null 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 Hoxa1null homozygous background, embryos also heterozygous for Hoxb1 display a significant decrease of expression in r4 from the lacZ targeted Hoxb1null 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 Hoxa1null 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 Hoxa1null/Hoxb1null 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⁺RARE embryos were normal and 95% of the homozygous animals complete deletion of r5 has been reported in the case of the other Hoxa1null (Carpenter et al., 1993). Homozygous mutant embryos for both these alleles maintain Hoxb1 expression in r4, although there is a reduction in its size.

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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.

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**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, EcoRV; Sp, SpeI; H3, HindIII; Acc, AccI; Sal, SalI; 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 SalI/AccI restriction site that can be used for genotyping. Embryonic stem cell DNA blot hybridised with probe 1 after digestion with AccI and relative to the ‘Run’ targeted allele (left), and PCR products from yolk-sac DNA after digestion with SalI (right). Arrowheads in A indicate the position of PCR primers used in genotyping. kb, kilobases; bp, base pairs.
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, 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 Hoxb13RARE homozygous mutant embryo. me, mesoderm; ne, neuroectoderm; ps, primitive streak; r4, rhombomere 4; A, anterior; P, posterior.

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 Hoxa1null and Hoxb1null 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 Hoxa13’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 Hoxb13’RARE and Hoxa1null alleles. In single Hoxa1null 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 Hoxa1null/Hoxb13’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 Hoxa1null/Hoxb1null and the Hoxa1null/Hoxb13’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 Hoxa1null 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 Hoxa1null/Hoxb1null 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 Hoxa1null/Hoxb13’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 Hoxa1null 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 Hoxa1null/Hoxb13’RARE in the two stages examined (G,H,I), and the foregut expression is reduced in these double mutants (G) compared to single Hoxa1null(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 Hoxa1null/Hoxa1null 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 Hoxa1null 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 Hoxb1null/Hoxa1null 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 remnant 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.
dramatic differences in earliest prospective r4 marker in mesoderm and neuroectoderm. No whole-mount 0-2 somite embryos hybridised with double homozygous mutant embryos. Ventral and lateral views of r4 is detected in contrast, strong down-regulation of slightly lower transcript level is observed in EphA2 (F) double homozygous mutant embryos, even if a low level of EphA2 expression are relevant in hindbrain segmentation.

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 Hoxb1null homozygous (A), Hoxa1null homozygous (B) and Hoxa1null/Hoxb13RARE double heterozygous (D) mutant embryos whereas a slightly lower transcript level is observed in Hoxa1null homozygous/Hoxb13RARE homozygous double mutants (E). In contrast, strong down-regulation of EphA2 expression in prospective r4 is detected in Hoxb1null/Hoxa1null (C) and Hoxa1null/Hoxb13RARE (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 Hoxa1null 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 Hoxb1null 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 Hoxa1null 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 Hoxa1null/Hoxb1null and Hoxa1null/Hoxb13RARE 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>
<thead>
<tr>
<th>Genotypes</th>
<th>Initiation of r4 identity</th>
<th>Maintenance of r4 identity</th>
</tr>
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<tbody>
<tr>
<td>Hoxb1null</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hoxb13RARE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hoxa1null</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hoxb13RARE</td>
<td>+</td>
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<tr>
<td>Hoxb1null/Hoxa1null</td>
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<tr>
<td>Hoxb13RARE/Hoxa1null</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hoxb13RARE/Hoxb13RARE</td>
<td>+/-</td>
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Genotypes indicate homozygous mutants, and in compound heterozygous and homozygous mutants the phenotypes are intermediate.

Initiation is based on early EphA2 and Hoxb1 expression in r4.

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 pararegulatory 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 Hoxal 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 Hox2 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 Hoxal locus, where a 3’ RARE has previously been shown to be required for the high level of Hoxal 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 Hoxal 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 Hoxal 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 Hoxal 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 Hoxal motif, revealing that additional cis-elements in Hoxal can make a contribution to activation of Hoxb1. This could be through additional RA-dependent or RA-independent elements in the Hoxal 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 Hoxal and Hoxb1 3’ RARE enhancers corresponds to the

![Fig. 9. Genetic interactions between Hoxal and Hoxb1 in modulating high expression of Hoxb1 in r4. Schematic representation of the proposed model (see also Marshall et al., 1996). The Hoxal 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 Hoxal and Hoxal loci. The curved arrows above the Hoxal 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 Hoxal towards the r4 enhancer of Hoxb1 indicates the binding of Hoxal to the Hoxb1 enhancer. A thicker arrow from Hoxal towards the r4 enhancer denotes a larger contribution of Hoxb1 compared to Hoxal in the maintenance of Hoxb1 expression in r4. Retinoids, and in particular RA, are bound by retinoid receptors, and activate Hoxal and Hoxb1 early expression through their respective RAREs located 3’ to the genes. Hoxal protein binds subsequently to the motifs embedded in the r4 enhancer, and together with Hoxal 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.](image)
The presence on an unidentified territory in the place of r4 function results in a transformation of posterior head to thorax tissue, behaving more like a traditional homeotic gene. Therefore, the 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.

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Dekker, E. J., Pannese, M., Houtzager, E., Timmermans, A., Boncinelli, E. and Kessel, 1993; 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 Kroxi20 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 (Diedrich 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, Gavallas 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.

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