Roles of *Hoxa1* and *Hoxa2* in patterning the early hindbrain of the mouse

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SUMMARY

Early in its development, the vertebrate hindbrain is transiently subdivided into a series of compartments called rhombomeres. Genes have been identified whose expression patterns distinguish these cellular compartments. Two of these genes, *Hoxa1* and *Hoxa2*, have been shown to be required for proper patterning of the early mouse hindbrain and the associated neural crest. To determine the extent to which these two genes function together to pattern the hindbrain, we generated mice simultaneously mutant at both loci. The hindbrain patterning defects were analyzed in embryos individually mutant for *Hoxa1* and *Hoxa2* in greater detail and extended to embryos mutant for both genes. From these data a model is proposed to describe how *Hoxa1*, *Hoxa2*, *Hoxb1*, Krox20 (Egr2) and *kreisler* function together to pattern the early mouse hindbrain. Critical to the model is the demonstration that *Hoxa1* activity is required to set the anterior limit of *Hoxb1* expression at the presumptive r3/4 rhombomere boundary. Failure to express *Hoxb1* to this boundary in *Hoxa1* mutant embryos initiates a cascade of gene misexpressions that result in misspecification of the hindbrain compartments from r2 through r5. Subsequent to misspecification of the hindbrain compartments, ectopic induction of apoptosis appears to be used to regulate the aberrant size of the misspecified rhombomeres.

Key words: Hindbrain patterning, *Hoxa1*, *Hoxa2*, *Hoxb1*, Krox20, *kreisler*, Mouse, Rhombomere

INTRODUCTION

A recurring theme in embryogenesis is the use of transient compartments to segregate and organize cells en route to the formation of more complex tissues and organs. The embryonic vertebrate hindbrain, for example, is temporarily subdivided into 7 or 8 compartments called rhombomeres which play pivotal roles in the differentiation and segregation of neurons and neural crest along the anteroposterior (AP) axis of the hindbrain (Lumsden and Keynes, 1989). The periodicity of the hindbrain, conferred by segmentation also provides a reference system to coordinate the formation of more peripheral tissues of the head, such as the cranial nerves, craniofacial musculature, and bones. Cells within individual rhombomeres are lineage restricted (i.e., are not free to mix with those of neighboring compartments; Fraser et al., 1990). This segregation provides localized environments for cell-cell interaction and gene activation such that each compartment can then diversify by activation of unique compliment of genes (Hunt et al., 1991a, b).

The fact that the expression patterns of *Hox* genes respect presumptive rhombomere boundaries suggests that, as in *Drosophila*, these genes function to establish the identities of compartments. Indeed, the disruption of *Hoxb1* in the mouse does not cause abnormalities in segmentation of the hindbrain but alters the identity of neurons originating in rhombomere (r) 4 (Goddard et al., 1996; Studer et al., 1996). In addition, *Hoxa2* appears to specify the identity of the mesenchymal neural crest derived from r4. In the absence of *Hoxa2* function, r4-neural crest cells acquire the fate normally associated with neural crest from the anterior segments, r1 and r2 (Gendron-McGuire et al., 1993; Rijli et al., 1993; see also Köntges and Lumsden, 1996). However, unlike the situation in *Drosophila*, *Hox* genes appear to be also involved in the establishment and/or maintenance of segmentation. For example, *Hoxa2* is required to set up the r1/2 boundary as well as to regulate the size of r3 (Gavalas et al., 1997; and herein). *Hoxa1* functions to establish the correct boundaries from r3 through r5 (Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Mark et al., 1993; Gavalas et al., 1998; and herein). We will argue that the effects of *Hoxa1* mutations on segmentation can be understood in terms of misspecification of rhombomeres.

To further examine the roles of *Hoxa1* and *Hoxa2* during hindbrain segmentation and patterning, we generated mice with a mutant allele that simultaneously disrupts both genes (Barrow and Capecchi, 1999). From an analysis of hindbrains from embryonic day (E) 8.0-E10.5 double mutants, as well as from those individually mutant for *Hoxa1* or *Hoxa2*, a more detailed picture has emerged of how these two genes interact to pattern the rhombencephalon. From these data, a model is developed to explain how *Hoxa1*, *Hoxa2*, *Hoxb1*, Krox20 and *kreisler* function together to pattern the hindbrain.
MATERIALS AND METHODS

Generation of genotypes

Four different mutant alleles were utilized to generate the mouse embryos described in this study. Hoxa1neo (Chisaka et al., 1992) carries an MC1 neoA cassette in a BglII site in the Hoxa1 homeobox. The Hoxa1GFP allele used in this study is described by Godwin et al. (1998). The green fluorescent protein (GFP) encoding sequences were inserted inframer into the first exon of Hoxa1. The neo gene, used to select for the ES cell containing the targeted insertion of GFP, was subsequently removed from the Hoxa1 locus by CRE/loxP mediated site-specific recombination (Schwenk et al., 1995). The mutant phenotype observed in embryos homozygous for this allele is indistinguishable from those of our Hoxa1neo allele (see Chisaka et al., 1992, and Carpenter et al., 1993). The Hoxa2 mutant allele carries an MC1 neoA cassette inserted into the Hoxa2 homeobox (Barrow and Capecchi, 1999). The fourth allele contains mutations at both the Hoxa1 and Hoxa2 loci (Hoxa1ela/Hoxa2neo). This allele is identical to Hoxa2neo, with the addition of a ClaI linker, frameshift mutation in the Hoxa1 homeobox at the BglII site. The PCR conditions and primer used for genotyping are described by Barrow and Capecchi (1999).

RNA in situ hybridization

RNA in situ hybridization was performed as described previously (Manley et al., 1995). Probes were as follows: Hoxb2, a 900 bp fragment that extends from a site 130 bp upstream of the stop codon into the 3'UTR; EphA4, a 1.2 kb cDNA clone (Gilardi-Hebenstreit, 1992); neuregulin, a 2.2 kb cDNA clone (Meyer et al., 1997); follistatin, a 300 bp BamHI/HincII fragment (Albano et al., 1994).

Immunohistochemistry

Krox20 and Hoxb1 double labeling was performed with Hoxb1 and Krox20 antibodies described previously (Manley and Capecchi, 1995 and Goddard et al., 1996). Embryos were preblocked in PBSTM (2% powdered milk, 0.5% Triton X-100 in PBS) at room temperature for 2×1 hour. The embryos were incubated with a 1:150 dilution of rabbit anti-Hoxb1 antibody in PBSTM at 4°C overnight followed by washes as described above. The embryos were then simultaneously incubated overnight but with a Texas Red-conjugated donkey anti-rabbit secondary antibody (Molecular Probes, Eugene Oregon). The embryos were washed and again incubated O/N with a 1:200 dilution of horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody. This incubation was performed in order to saturate the Hoxb1 antibodies with anti-rabbit antibodies. The embryos were washed and incubated overnight with a 1:100 dilution of rabit Krox20 antibody, washed, and incubated overnight with a 1:200 dilution of FITC-conjugated, donkey anti-rabbit secondary antibody (Jackson Immunolabs). Finally, the embryos were washed, rinsed, and stored in PBS. The Texas Red- and FITC-conjugated antibodies were visualized through rhodamine and fluorescein filter sets, respectively, with a laser scanning BioRad MRC1240 confocal microscope. The Hoxa1gfp/Hoxb1 immunohistochemistry experiments were visualized in precisely the same manner.

The positions of rhombomere boundaries in Fig. 3 were defined by the following means: 3 control embryos that had been doubly stained for Krox20 and Hoxb1 were imaged as described above; the rhombomere boundaries, as defined by these two markers, were drawn on the images (in Photosop®) as well as the anterior boundary of the first somite for each of the embryos. Using the somite boundary as a reference point, these measurements were superimposed and then averaged. Similar measurements were taken except using the flexure in the menencephalon as an external reference point. Both of the averaged measurements were superimposed on the mutant embryos shown in Fig. 3.

TUNEL analysis

Embryos were fixed in 4% paraformaldehyde PBS for 2 hours at room temperature and washed 3×30 minutes in PBS containing 1% Triton X-100 (PBST). The embryos were preincubated for 30 minutes at 37°C in 1X terminal transferase buffer (Boehringer Mannenheim). The buffer was removed and replaced with 1X terminal transferase buffer, 0.5 units terminal transferase/μl, and 10 μM dUTP (2:1 ratio of dUTP:dUTP-biotin). The reaction in this mixture was allowed to proceed for 3 hours at 37°C. The terminal transferase product was quantified by reaction with Texas Red-conjugated streptavidin and viewed through confocal microscopy. Following the TUNEL procedure, some of the embryos were embedded in paraffin and sectioned at 7 μm. The sections were mounted on Superfrost + (Fisher) slides and cleared in 10% glycerol/PBS.

RESULTS

Both the Hoxa1 and Hoxa2 single mutants exhibit defects in the organization of rhombomeres in the developing hindbrain (Carpenter et al., 1993; Mark et al., 1993; Gavalas et al., 1997, 1998; Studer et al., 1998). We examined the organization of rhombomeres in each of the single mutants in greater detail to allow comparison with the Hoxa1/Hoxa2 double mutants. By crossing mice carrying the Hoxa1 or Hoxa2 mutations to those harboring the double mutant allele, we generated mice with intermediate genotypes (Hoxa1+/−/Hoxa2+/− and Hoxa1+/−/Hoxa2−/−). The hindbrain patterning defects of mutants
possessing these intermediate genotypes were not distinguishable from those of mice carrying only the respective single mutations (i.e., Hoxa1<sup>-/-</sup> mice were indistinguishable from Hoxa1<sup>+/</sup>-/Hoxa2<sup>+/</sup>- mice; Hoxa2<sup>-/-</sup> mice were similar to Hoxa1<sup>+/</sup>-/Hoxa2<sup>-/-</sup> mice).
2-3 somite stage, the Hoxb1 expression level increases in the presumptive r4 region, and the Krox20 expression domain extends anteriorly (Fig. 1B,C). The anterior limit of Krox20 expression corresponds to the position of the preotic sulcus (Fig. 1, arrowhead), an indentation in the hindbrain that delineates the r2/r3 boundary (Trainor and Tam, 1995). Hoxb1 expression in r4 coincides with a bulge in the hindbrain (Figs 1A-C, 3A,B). Using the positions of the preotic sulcus and the ‘r4 bulge’ as points of reference in Hoxal mutants, however, the anterior limit of Hoxb1 expression is shifted caudally, to the posterior end of this bulge (Fig. 1D). Krox20 expression was also shifted caudally such that it was found at the level of r4, and only a few cells expressing Krox20 were observed in the region just posterior to the preoptic sulcus, which in wild-type embryos would correspond to r3. Thus, in the absence of Hoxal, it appears that the anterior limit of Hoxb1 is established at a more posterior level. Perhaps as a consequence of this posterior shift in Hoxb1 expression, Krox20 expression is also found at a more caudal level.

To directly confirm the posterior shift of Hoxb1 expression in Hoxal mutants, we took advantage of a second allele of Hoxal that has a GFP cassette inserted in frame into the Hoxal encoding unit. In this allele, GFP-fluorescence recapitulates, with high fidelity, Hoxal expression in embryos either heterozygous or homozygous for the Hoxal mutation (Godwin et al., 1998). In heterozygous control, E8.25 embryos, the anterior limits of Hoxal (GFP-green fluorescence) and Hoxb1 (immunohistochemistry-red fluorescence) expression coincide at the r3/r4 presumptive boundary (Fig. 2A-C). However, in Hoxal mutant homozygotes, Hoxb1 expression does not reach the same anterior limit of expression as Hoxal, and is instead observed to be expressed almost one rhombomere caudal to this boundary (Fig. 2D-F). Thus, Hoxal gene activity is required to establish the normal anterior limit of expression of Hoxb1 at the r3/r4 presumptive boundary. Note that in Hoxal mutants, within the region that no longer expresses Hoxb1, neither paralogous gene is functional, placing this region in double jeopardy.

Hoxb1 and Krox20 expression was then examined at E8.5. In control embryos at E8.5 (4-7 somites), Krox20 expression was observed, as expected, in rhombomeres 3 and 5 and Hoxb1 was found in r4 (Fig. 3A; Wilkinson et al., 1989; Murphy and Hill, 1991). Again, the anterior limit of Krox20 expression in r3 aligns with the preotic sulcus and Hoxb1 expression is restricted to the r4 bulge. The Krox20 expression domain in r5 is at the level of the otic sulcus, a second indentation in the hindbrain that delineates the r5 region (Murphy and Hill, 1991). Thus, as is the case at E8.0, the Hoxb1 and Krox20 expression boundaries correspond to morphological landmarks within the hindbrain. The expression of these genes was not significantly altered in Hoxa2 single mutants (Fig. 3B). In Hoxal mutants, however, the expression patterns are again very different relative to control embryos (Fig. 3C). In spite of these differences in Hoxb1 and Krox20 expression, the Hoxal mutant hindbrains did not differ in length from the hindbrains of age-matched controls and they possessed similar morphological structures such as the preotic sulcus and the r4 bulge. In order to allow the superimposition of the rhombomere boundaries normally associated with wild-type embryos onto Hoxal and Hoxal/Hoxa2 mutant embryos, we measured the distances from the preotic sulcus, the mesencephalic flexure and the first somite to the Hoxb1 and Krox20 expression boundaries in three age-matched (5 somite) wild-type embryos (Fig. 2A and data not shown). These distances were averaged and then superimposed onto age-matched Hoxal and Hoxal/Hoxa2 mutant embryos (Fig. 3C and D and data not shown) using the morphological markers as the points of reference (Materials and Methods).

As in Hoxal mutants at E8.0, those at E8.5 expressed Krox20 in the region of the hindbrain that would have given rise to r4 in control embryos. In contrast to the earlier mutants, however, the Krox20 domain had expanded more significantly into the region immediately caudal to the preotic sulcus or ‘r3’ (Fig. 3C). By E8.75, the Krox20 expression rather than being patchy in the preotic region was more confluent in appearance (Fig. 3E). Thus, the expansion of the Krox20 expression at the level of r3 is reminiscent of that observed in control embryos 12 hours earlier (see Fig. 1A-C). We also observed that the Hoxb1 expression domain was very reduced relative to controls, that its anterior limit was at the level of posterior r4, but that it extended abnormally into r5 and beyond. By E8.75, we observed Krox20 expression in the dorsal neuroepithelium at the level of caudal r5 and r6 (Fig. 3E). In control embryos, we observed similar expression in the dorsal portion of r6 at E8.75 (data not shown; to a lesser extent at E8.5-Fig. 3A). It is therefore possible that these cells in the mutant represent the dorsal r6 expression seen in normal embryos, but may also be a remnant of the r5 Krox20-expressing domain.

At E8.5, Hoxal/Hoxa2 double mutant embryos had a Krox20 staining pattern that is similar to that of Hoxal single mutants, except that it is restricted to the region in the hindbrain that would typically give rise to r4 (Fig. 3D) and very few expressing cells at the level of r3. At E8.75, double mutants had some small patches of expressing cells in the ‘r3’ region (Fig. 3F) demonstrating that the expansion of Krox20 expression into r3 is further delayed relative to Hoxal single mutants. Although the preotic sulcus and r4 bulge are difficult to appreciate in the specimen exhibited in Fig. 3D, it should be noted that other double mutants did possess these morphological landmarks and the expression patterns of Krox20 and Hoxb1 were consistent with those shown here. The specimens shown here were chosen because they were precisely age-matched (5 somites) to the controls used in this study. The Hoxb1 expression domain in double mutants corresponds to the posterior r4/anterior r5 region of age-matched control embryos, similar to the finding in Hoxal single mutants. Interestingly, a similar caudal extension of Hoxal expression has been reported for kreasler mutants (McKay et al., 1994). It is therefore possible that kreasler may be misregulated in mutants that lack Hoxal (see below).

To further explore the perturbations in the organization of rhombomeres in the early hindbrain of Hoxal, Hoxa2 and Hoxal/Hoxa2 mutant embryos the expression patterns of additional molecular markers of the hindbrain were examined.

**Neuregulin and follistatin expression**

In E9.0 control embryos, neuregulin is expressed strongly in rhombomeres 2 and 4 (Meyer et al., 1997) and at lower levels in r1 (Fig. 4A). Hoxa2 mutants show a relatively indistinguishable pattern of neuregulin expression from controls. In both controls and Hoxa2 mutants the r4 expression stripe was in perfect alignment with the second branchial arch.
(Fig. 4A,B) whereas the otic placode was found immediately posterior to this domain at the level of r5. In Hoxa1/Hoxa2 mutants, the nonexpressing region caudal to r2 was much larger than in control embryos, which corresponds to the large Krox20-expressing region observed in embryos at slightly earlier stages of embryogenesis (Fig. 4C). In addition, a thin stripe of neuregulin expression is observed that was posterior to the second branchial arch and adjacent to the otic placode. By these morphologic criteria the second neuregulin stripe corresponds to the region that would normally give rise to r5 in wild-type embryos. Hoxa1/Hoxa2 double mutants exhibit a neuregulin expression pattern reminiscent of Hoxa1 mutants. Again the second neuregulin stripe is posterior to the second branchial arch and at the level of the otic placode. Thus, as appeared to be the case at earlier stages of embryogenesis, the misspecification of r4 identity at the level of r5 is confirmed at E9.0 in mutants lacking Hoxa1. There were two major differences in the double mutants relative to Hoxa1 single mutants. First, the nonexpressing region between the neuregulin-expressing stripes was much smaller in the double mutants. Second, the anterior boundary of this nonexpressing region was patchy. We suggest that the basis for this difference in expression is most likely due to the severe delay in the expression of Krox20 in r3 of the double mutants (see Fig. 3D,F). Hence in the absence of Krox20 expression at the level of r3, these cells take on an r2 identity. Indeed, it has been reported by Helmbacher et al. (1998), that in Hoxa1+/−/Krox20+/− mutant embryos which have patchy Krox20 expression in r3, those cells that do not express Krox20 take on an r2 identity.

A more direct molecular mechanism is revealed by examining follistatin expression in the mutant embryos. At E8.0, follistatin is expressed to an anterior limit at the presumptive r1/r2 boundary down through rhombomere 4 as well as in the paraxial mesoderm (Albano et al., 1994). This large expression domain is broken up by a thin stripe of nonexpression in presumptive r3 which by E8.5 has expanded more completely. Thus, there are two bands of expression in r2 and r4 separated by a nonexpressing region in r3 (see Fig. 4E and Albano et al., 1994). In Krox20 mutants, follistatin is expressed from rhombomeres 2-4, demonstrating that Krox20 directly or indirectly represses the expression of follistatin (Seitanidou et al., 1997). Based on this data, it is not surprising that we find a large region of the hindbrain that does not express follistatin, which corresponds to the large Krox20-expressing domain in Hoxa1 mutant embryos (Fig. 4G). In Hoxa1/Hoxa2 double mutants, there is also a relatively large nonexpressing region, the anterior most portion of which is intermixed with expressing cells (Fig. 4H). Given that Krox20 represses follistatin, we suggest that this patchy follistatin expression in r3 of double mutants would be secondary to the severely affected Krox20 expression at this level of the hindbrain. In mutants lacking Hoxa1, we also remarked that the second follistatin band was very thin (Fig. 4G,H; sometimes difficult to appreciate due to the underlying expression in the paraxial mesoderm). An interpretation for this very thin ‘r4’ band is that follistatin is typically expressed in rhombomeres 2-4 excepting in r3 where Krox20 represses its expression. In mutants lacking Hoxa1, however, we have provided evidence that the Krox20 expression domain extends ectopically into the anterior portion of r4 (Figs 1D, 3D-F).

Thus, follistatin will be repressed in the anterior-most portion of the rhombomere, leaving only a narrow stripe of expression in posterior r4.

**Hoxb2 and EphA4 expression**

To further examine the consequences of the misexpression of Hoxb1 and Krox20 on later hindbrain development, the expression of Hoxb2 and EphA4, which serve as markers of rhombomeres 3-6 at E9.5, were determined. At this stage Hoxb2 is expressed strongly in r3-5, to a lesser extent in r6, and at lower levels in the remaining neural tube (Fig. 5A; Sham et al., 1993; Barrow and Capecchi, 1996). In addition, neural crest cells emanating from r4 and r6 express this gene. In Hoxa2 mutants, Hoxb2 expression is identical to that of control littersmates except that the dorsal-most aspect of r3 is more restricted (Fig. 5B and data not shown). In Hoxa1−/− embryos, r3, or the region defined by the anterior limit of Hoxb2 expression, and the ensuing rhombomere sulcus (designated by the arrowhead in Fig. 5C) appeared only slightly larger than in controls. This observation is in contrast to younger embryos in which an unusually large region of the hindbrain expresses r3 markers. The next segment, which we termed the r4/r6 region, appeared to be one large segment expressing Hoxb2 at high levels (r5 is assumed not to be present due to the fact that at earlier stages in development a region of the hindbrain that expresses r5 markers is not observed in these mutant embryos; see also Carpenter et al., 1993). Neural crest cells expressing Hoxb2 were not detected emanating from this large segment.

Hoxa1/Hoxa2 double mutants showed several significant changes relative to the other mutant classes. First, we observed an ill-defined anterior limit of Hoxb2 expression (Fig. 5D). Interestingly, this limit extended to the bulge in the hindbrain that typically corresponds to the midpoint of r2 in control embryos. In addition, the region of the hindbrain expressing high levels of Hoxb2 anterior to the otic vesicle (presumably r3) were greatly reduced relative to controls and to the other mutant classes. Expression in the r4/r6 region was strong, as in Hoxa1 mutants; however, there was no well-defined posterior limit of expression. Instead, the expression faded in gradient fashion.

In control embryos at E9.5, EphA4 is expressed strongly in r3 and r5 and at lower levels in r2, 4, and 6 (Gilardi-Hebenstreit et al., 1992: Fig. 5E). In Hoxa2−/− mutants, the EphA4 expression pattern is similar to that of controls except that the A-P extent of r3 has been reduced and its anterior limit of expression (r2/3) is ill-defined (Fig. 5F). In Hoxa1 mutants, a single band of expression was seen anterior to the otic vesicle that represents the r3 region (Fig. 5G). As was demonstrated in Hoxa1 mutants in the previous experiment, this r3′ domain is not significantly larger than the r3 band of controls. In addition, the r5 band is not present, presumably due to the fact that r5 markers were never expressed. As was the case in Hoxa1 homozygotes there is a single band of expression anterior to the otocyst in the Hoxa1/Hoxa2 double mutants (Fig. 5H). In the double mutants, however, the EphA4 expression is much more patchy and is restricted to the dorsolateral ridge plate and was found to extend to the mid-point of r2, similar to the Hoxb2 expression pattern (Fig. 5H; arrow). Thus, it is clear from Hoxb2 and EphA4 expression in the double mutants that by E9.5 there is a clear reduction in the size of the r2-r5 region of the hindbrain.
Enlarged rhombomeres in Hoxa1 mutants are regulated by apoptosis

At E8.5, Hoxa1 mutant embryos possess an abnormally large segment of the rhombencephalon that expresses r3 molecular markers, which by E9.5 is reduced to almost normal proportions. Extensive TUNEL assays were performed on Hoxa1 mutants and controls from E8.5 to E10.5 to determine whether apoptosis is used to regulate this abnormally large segment. At E8.5, there were increased, ectopic levels of apoptosis in the anterior hindbrain of Hoxa1 mutants relative to age matched control embryos (data not shown; see also Rossel and Capecchi, 1999). By E9.0-9.5 (20-22 somites), we observed a peak in the level of apoptosis in the hindbrain from approximately r3 and further anterior (Fig. 6B,D). Although more concentrated at the dorsal aspect of the neural epithelium, apoptotic cells were found along the entire dorsoventral axis of the neural tube (Fig. 6D, inset). Following E9.5, the ectopic apoptosis in the anterior hindbrain decreased such that by E10-10.5 the levels were similar to those of wild-type embryos (data not shown). Like Hoxa1 single mutants, we found that Hoxa1/Hoxa2 double mutants also exhibited elevated levels of apoptosis in the hindbrain in early embryogenesis (data not shown).

Hoxa1 and Hoxa2 have overlapping functions in the formation of rhombomere boundaries

The rhombomeric boundaries in the hindbrain can be visualized with Nomarski optics (Gavalas et al., 1997). In E10.0-10.5 control embryos, the boundaries separating r1-r6, as well as the otocysts adjacent to the r5/6 region are readily observed (Fig. 7A). Hoxa1 mutants lack two rhombomere boundaries: r1/r2 and r2/r3 (Fig. 7B). This finding is similar yet more severe than data reported by Gavalas et al. (1997) who observed the absence of the r1/r2 and occasionally the r3/4 boundaries with their Hoxa2 allele. It has been previously shown that our Hoxa1 mutants lack r5 (Carpenter et al., 1993). Supporting these data, Hoxa1 mutants were found to have one less rhombomere than control littermates (see Fig. 7C). We also found that the otocyst is positioned at the level of r4 (Fig. 7C; Carpenter et al., 1993; Mark et al., 1993). We suggest that this is likely due to the fact that r4 is specified adjacent to the otocysts (Fig. 4C), rather than to the otocysts being shifted anteriorly to r4. Hoxa1/Hoxa2 double mutants were found to have completely smooth hindbrains devoid of any boundaries at all stages examined (Fig. 7D). Thus, Hoxa1 and Hoxa2 appear to have synergistic roles in the specification of both the anterior and posterior rhombomere boundaries.
DISCUSSION

We have shown that in Hoxal mutants the anterior boundary of Hoxb1 expression does not reach the presumptive r3/r4 boundary. We suggest that as a consequence of the loss of Hoxa1 and Hoxb1 activity at the level of r4, a cascade of gene misexpression ensues that results in misspecification of the hindbrain compartments encompassing r2 through r5. We now review data from previous work, relate it to the results of the present study and propose a model describing how Hoxal, Hoxb1, Krox20, Hoxa2, follistatin, and kreisler contribute to the establishment of the normal pattern of rhombomeres in the developing hindbrain. This model is described in detail below and summarized in Fig. 8.

It has been reported that the establishment of the Hoxb1 expression in r4 depends on both Hoxal and Hoxb1 function (Studer et al., 1998). These authors showed that Hoxb1 expression is initially activated in r4 of both Hoxb1 (normal levels) and Hoxal (weaker and reduced levels) mutant embryos, but was not activated in r4 of Hoxal/Hoxb1 double mutants. We extend these observations by showing that Hoxal is required for the expression of Hoxb1 in the anterior region of r4. We have demonstrated this finding by using both morphological and molecular markers. First, we provided evidence that Hoxb1 expression was shifted posteriorly relative to morphological landmarks (i.e., the preotic sulcus and the r4 bulge). In addition, we demonstrated that in Hoxalgfp mutant homozygotes, Hoxb1 expression is shifted posteriorly relative to the anterior limit of the Hoxalgfp expression domain (i.e.,

Fig. 5. Hoxb2 and EphA4 expression in mutant embryos at E9.5. (A-D) Hoxb2 RNA expression in E9.5 embryos (dorsal views). The arrows point to the flexure marking the midpoint of r2 whereas the arrowheads indicate the sulcus separating r3 and r4. (A) Control embryo (Hoxal+/Hoxa2+/−); strong expression is seen in r3-5 and slightly lower levels in r6. It is also seen in the neural crest emanating from r4 and r6. Note the distance between the midpoint in r2 (arrow) and the r2/3 boundary. (B) Hoxal+/−Hoxa2−/− mutant; expression pattern is indistinguishable from control embryos except that the r2/3 boundary is ill defined and r3 appears reduced in size. (C) Hoxal−/− embryo; there are two segments of Hoxb2 expression: one in r3 which is slightly larger than controls and in the r4/6 region. No neural crest is observed from the r4/6 segment. (D) Hoxal+/−/Hoxa2+/− double mutant embryo. The r3 expression domain (i.e., anterior to the r3/4 sulcus; arrowhead) is much smaller than controls and appears to extend into r2 (arrow). The distance between r2 and the otic vesicle is markedly reduced relative to the other mutant classes and controls. Posterior to the r3/4 sulcus, the expression pattern is similar to Hoxal mutants except that there is no sharp posterior border of expression. (E-F) EphA4 RNA expression in E9.5 embryos (lateral views). (E) Control embryo (Hoxal+/+/Hoxa2+/+) exhibits strong expression in r3 and r5; the anterior limit of expression at the r2/3 boundary is well separated from the midpoint in r2 (arrow). (F) Hoxal+/−/Hoxa2−/− embryo possesses a reduced r3 expression domain. (G) Hoxal−/− mutant with a slightly larger r3 expression domain but lacking the r5 expression stripe. The otic vesicle is adjacent to r4 (or EphA4 non-expressing cells). (H) Hoxal/Hoxa2 double mutant; the anterior limit of expression extends to the midpoint in r2 (arrow). The region between the midpoint of r2 and the otic vesicle has been drastically reduced relative to controls and other mutant classes but does continue to express EphA4, albeit weakly. There is no r5-expressing domain. ov, otic vesicle. Scale bars are 200 μm.

Fig. 6. Apoptosis in the anterior hindbrain of E9.0 embryos. Lateral (A) and dorsal (B) views of a control embryo (+/−); observe the low levels of apoptosis in the anterior hindbrain and moderate levels in r5 and the otocyst. Lateral (C) and dorsal (D) views of a Hoxal+/− embryo; note the extensive apoptosis in the anterior hindbrain. The inset is a transverse section of the embryo in D. The line shows the axial level of the section. Note that the apoptotic cells are found throughout the dorsoventral extent of the neural tube. Scale bar is 200 μm.
Fig. 7. Hindbrain segmentation. (A-D) Hindbrain flat mounts of approx. E10.25 hindbrains visualized by Nomarski optics. (A) Control embryo; note the rhombomere boundaries separating rhombomeres 1-6. The otic vesicle is at the level of r5. (B) Hoxa2 mutant embryo; the r1/2 and r2/3 boundaries are absent. (C) Hoxa1−/− embryo; four boundaries are present. Based on marker analyses, we propose that the missing rhombomere is r5. The otic vesicle is at the level of r4. (D) Hoxa1/Hoxa2 double mutant embryo; note the complete absence of rhombomere boundaries. Otic vesicle (ov). Scale bar is 200 μm.

r3/4; Fig. 2D-F). In contrast, in heterozygous (Fig. 2A-C) and control embryos (Murphy and Hill, 1991) the anterior limit of expression of both paralogs coincide at the r3/4 presumptive boundary. We also found that the Hoxa1gfp expression limit (r3/4) was approximately the same distance from the preotic sulcus in both Hoxa1 mutant homozygous and heterozygous animals, demonstrating that this morphological landmark is at the same axial level in both mutants and controls.

The maintenance of Hoxb1 expression in r4 after the regression of both Hoxa1 and Hoxb1 expression from the hindbrain is dependent on a Hoxb1 auto-regulatory loop (Pöpperl et al., 1995). Interestingly, kreisler appears to play an important role in ensuring that the autoregulated Hoxb1 expression is restricted to r4. Thus in kreisler mutants, autoregulated Hoxb1 expression is maintained ectopically in r5 (McKay et al., 1994). In mutants that lack Hoxa1, we have also observed that Hoxb1 expression was maintained at the level of r5 suggesting that perhaps kreisler is misregulated in Hoxa1 mutants. Indeed, we have found that in Hoxa1 mutants, the kreisler expression domain has been reduced to the width of single rhombomere corresponding to the level of r6 (J. R. B., unpublished observations; Rossel and Capecchi, 1999; see also Fig. 2 of Gavalas et al., 1998). Thus, Hoxa1 may be required to activate kreisler expression in r5.

Krox20 expression is initially activated in rhombomere 3 at E8.0. It commences as a narrow stripe of cells that expands rostrally with time (Fig. 1A-C; see also Irving et al., 1996). Slightly later in development (approx. E8.5), Krox20 expression is activated in r5 in rostral to caudal fashion (Irving et al., 1996). It appears that the mechanisms that activate Krox20 in rhombomeres 3 are different from those that activate it in r5. For example, previous work by Graham and Lumsden (1996) demonstrate that r5 explants, whether cultured in isolation or transplanted to ectopic regions of the hindbrain, always express Krox20. In contrast, rhombomere 3, whether transplanted to ectopic regions in the hindbrain or cultured in isolation, fail to express it. Only in situations where r3 is adjacent to r4 in explant cultures is r3 found to express Krox20. Furthermore, work by Helmbacher et al. (1998) demonstrates that Hoxa1 (expressed in r4 and more posteriorly) plays a synergistic role with Krox20 in establishing Krox20 expression in r3. Taken together, these results suggest that cells in r5 possess the intrinsic ability to express Krox20, whereas those in r3 appear to require signals from r4 in order to do so. We also have made similar observations in our experiments. For example, we have found that the region of the hindbrain that gives rise to rhombomeres 4 and 5 has the intrinsic ability to express Krox20 provided that neither Hoxa1 nor Hoxb1 is expressed in these rhombomeres at the same time. Thus, in the case of Hoxa1/Hoxb1 double mutants neither Hoxa1 nor Hoxb1 is expressed at the level of r4 or r5 and Krox20 is expressed in these rhombomeres in their absence (Rossel and Capecchi, 1999). In addition, in wild-type embryos, Krox20 is never expressed in r5 until after the retreat of Hoxa1 and Hoxb1 from this region of the hindbrain (Figs 1A-C, 3A).

Interestingly, the activation of Krox20 is in rostral to caudal fashion (Irving et al., 1996) mirroring the anterior to posterior retreat of the Hox1 paralogs from this region. Further evidence of this phenomenon comes from Hoxa1 mutants, where the anterior limit of Hoxb1 expression is shifted to the caudal region of r4. Thus, neither Hoxa1 nor Hoxb1 is expressed in anterior r4 and Krox20 expression is activated in this rhombomere in their absence. Finally, in kreisler and Hoxa1 mutants, which allow ectopic, autoregulated expression of Hoxb1 in r5, Krox20 is never activated at this level (herein; McKay et al., 1994). We conclude, therefore that rhombomeres 4 and 5 are similar with respect to the activation of Krox20 in that they possess the intrinsic ability to activate Krox20 expression in the absence of Hox1 paralogs.

Similar to previous work, we found that activation of Krox20 at the level of rhombomere 3 was dependent on signals from r4. More specifically our work suggests that these signals are downstream from Hoxa1 and Hoxb1. For example, in Hoxa1 mutants we showed that there was a delay in the activation of Krox20 expression in cells just posterior to the preotic sulcus (cells that should give rise to rhombomere 3). Thus at E8.0, in contrast to control embryos, Hoxa1 mutants possess only a few Krox20-expressing cells at the level of r3. By E8.5, this number has expanded and by E8.75 the expansion appears to be complete. We propose that the delay in Krox20 expression is due to the fact that the anterior limit of Hoxb1 expression is found at a more posterior level. Thus, the proposed signal downstream of Hoxb1 is activated in a more posterior domain of the hindbrain and must be propagated a further distance in order to reach the cells in r3 (i.e., the cells just posterior to the
preotic sulcus). In corroboration of this hypothesis, we have observed in Hoxa1/Hoxb1 double mutants that the region just posterior to the preotic sulcus (‘r3’) is completely devoid of Krox20 expression (Rossel and Capecchi, 1999), again demonstrating the requirement of the Hox1 paralogs for the activation of Krox20 in r3. We therefore extend previous experiments (Graham and Lumsden, 1996; Helmabacher et al., 1998) by showing that Hoxal and Hoxb1, two molecules essential for the formation of r4 (Rossel and Capecchi, 1999), are required to activate Krox20 in r3.

We have shown that in Hoxal mutants Krox20 expression, although delayed, expands relatively normally in the r3 territory (Figs 1D, 3C,E). In Hoxal/Hoxa2 double mutants, however, this expansion is severely affected such that even by E8.75 there are very few Krox20-expressing cells at the level of r3 (Fig. 3F). Thus, while Hoxal and Hoxb1 play a role in the activation of Krox20 in r3, Hoxa2 appears to be important for the anterior expansion of this expression domain. It has been demonstrated previously that Krox20 is required for Hoxa2 expression in r3 (Nonchev et al., 1996). It is therefore not surprising that Krox20 mutants appear to show a similar yet more severe defect in the expansion of rhombomere 3 identity (Schneider-Maunoury et al., 1993). In Krox20 mutants, it has been demonstrated that Krox20 is activated in the first few rows of cells in r3, just as in controls at E8.0 (Schneider-Maunoury et al., 1993). In contrast to controls, however, this thin band fails to expand (see fig. 2 of Schneider-Maunoury et al., 1993; fig. 4 of Seitandou et al., 1997) indicating that as was the case with Hoxa2, Krox20 appears to play an important role in the expansion of Krox20-expressing cells in r3.

It is interesting to note that in Krox20 mutants, all of the cells in the r5 domain express Krox20 (Schneider-Maunoury et al., 1993), again highlighting the fact that cells at the level of r5 possess the intrinsic ability to activate Krox20. These cells, however, do not express Krox20 until E8.5 (after the retreat of the Hox1 paralogs). In contrast, in r3 of the Krox20 mutants, only the first few rows of cells are activated; an expected result given that Hoxal and Hoxb1 are still present in r4 to activate these cells (via downstream signals).

Follistatin is activated very early in the neural plate (Albano et al., 1994). Prior to E8.0 it is presumably expressed as a solid band from r2-r4. Following E8.0, there is a band of nonexpressing cells at the level of r3 which now separates the follistatin expression domain into an r2 and an r4 band. This band of nonexpressing cells corresponds to the Krox20 expression domain. Interestingly, Seitandou et al. (1997) have demonstrated that in Krox20 mutants that follistatin is expressed as a solid domain from r2 to r4, demonstrating that Krox20 represses follistatin in r3. In Hoxal and Hoxal/Hoxa2 mutants the pattern of Krox20 has been significantly altered. It comes as no surprise, therefore, that we see concomitant changes in follistatin expression in these mutant backgrounds. Thus, a large nonexpressing domain separating the two follistatin expression stripes is observed in Hoxal mutants, corresponding to the large Krox20 expression domain in r3 and anterior r4 (Fig. 4G). Not surprisingly, the posterior follistatin-expressing band was very thin, demonstrating that the Krox20 domain extends ectopically into most of rhombomere 4. It is interesting to note that in Hoxal/Hoxal2 double mutants, due to the delay of Krox20 expansion into r3, follistatin is not repressed in this region (Fig. 4H) and thus the expression of r2-specific markers is permitted at this level (see also Fig. 4D).

Neuregulin is expressed in two stripes in mutants lacking Hoxal, similar to controls; however, we find that the second stripe, which typically rests directly above the 2nd arch and anterior to the invaginating otic placode, is now located posterior to the 2nd arch and immediately adjacent to the otic placode (Fig. 4C). This observation provides further evidence that r4 markers are expressed at the level of r5 in Hoxal mutants.

We propose the following model for how Hoxal, Hoxb1, Krox20, Hoxa2, kreisler, and follistatin function together to establish the normal pattern of rhombomeres 2-6 in the early hindbrain (see Fig. 8). Prior to E8.0, Hoxal and Hoxb1 are expressed throughout the neural plate and form a sharp anterior limit at the r3/r4 presumptive boundary, while follistatin is expressed in presumptive rhombomeres 2-4. The expression of Hoxal is required to activate Hoxb1 in the anterior regions of r4. By E8.0, Hoxal and Hoxb1 have activated the transcription of other r4-specific genes including a downstream signal that induces Krox20 expression in the first 3-4 rows of cells in presumptive r3 (Fig. 8A). Once activated, Krox20 activates downstream targets, including Hoxa2 and Hoxb2, while repressing follistatin. The expansion of Krox20 in r3 is dependent on its own expression and that of its downstream target(s), Hoxa2 (and perhaps Hoxb2).

While still expressed in r5 (prior to E8.5), Hoxal not only represses Krox20 but is required to activate strong kreisler expression. By E8.5, the expression of both Hox1 paralogs has repressed from the hindbrain except for the strong autoregulatory Hoxb1 expression in r4. No longer repressed by Hoxal and Hoxb1, Krox20 is now activated throughout r5 (Fig. 8B) commencing with the most anterior cells (see Irving et al., 1996). The net result is strong expression of Krox20 in r3 and r5 and Hoxb1 in r4 (Fig. 8B). In addition, kreisler is expressed in r5 (thanks to the function of Hoxal) and in r6. Finally follistatin, which commenced as a solid band of expression in presumptive r2-r4 prior to E8.0, is now separated into two domains of expression in r2 and r4 due to Krox20 expression in r3.

In the absence of Hoxal, the anterior limit of Hoxb1 is established in the posterior region of r4 (Fig. 8A; al/− and al/a2/−/−). Several consequences result from this defect. First, without Hoxal and Hoxb1 in anterior r4, Krox20 is no longer repressed at this level. Secondly, the putative downstream signaling molecule must be propagated further to reach r3, causing a delay in the induction of Krox20 in r3. In addition, in the absence of Hoxal, kreisler expression is not activated in r5. By E8.5 the expansion of Krox20 in r3, although delayed, has commenced in Hoxal single mutants (Fig. 8B al/−/−). Because Krox20 and its downstream targets are present in the single mutants, the expansion proceeds relatively normally. In Hoxal/Hoxa2 double mutants, Krox20-expressing cells are induced sparingly in the region just posterior to the preotic sulcus (i.e., ‘r3’) (Fig. 8A, al/a2/−/−) similar to Hoxal single mutants; however, due to the absence of Hoxal2, expansion of Krox20-expressing cells in r3 is severely limited (Fig. 8B, al/a2/−/−). Due to the severe delay of Krox20 expression in ‘r3’, follistatin is not repressed and thus this region takes on an r2 identity.

By E8.5 for both mutant classes, Hoxb1 expression has
Fig. 8. Schematic representation of embryonic hindbrains from E8.0-E9.5. (A, wt) Hoxa1 and Hoxb1 (red) are coexpressed up to the presumptive r3/4 boundary. Hoxa1 is required to establish Hoxb1 expression in anterior r4. Hoxa1 and Hoxb1 activate the transcription of r4-specific downstream targets including a signal (upward arrow) which in turn induces Krox20 (K-20; green) expression in cells just anterior to the r3/4 boundary (in cells that are not expressing Hoxa1 or Hoxb1). Krox20 is repressed, however, in r4 and r5 cells that are expressing Hoxa1 and Hoxb1. Hoxa1 is required for kreasler (kr) expression in r5. (A, a1+– and a1/a2−+) Without Hoxa1, the anterior limit of Hoxb1 is established in the posterior region of r4. Because of this posterior shift, neither Hoxa1 nor Hoxb1 is expressed in the anterior portion of r4 and Krox20 is no longer repressed there. Furthermore, the signal downstream of Hoxb1 (upward arrow) must be propagated a longer distance causing a delay in the induction of Krox20 expression in presumptive r3. Due to the absence of Hoxa1, kreasler expression is not activated in r5. (A, a1/b1−−) Without Hoxa1 and Hoxb1 expression, Krox20 expression is no longer repressed in r4 and r5. In addition, the signal downstream from Hoxa1 and Hoxb1 required to induce Krox20 expression in r3 is not activated. (B, wt) By E8.5, Hoxa1 expression has completely retreated from the hindbrain. Hoxb1 also retreated with the exception of the strong autoregulatory expression in r4. Once Hoxa1 and Hoxb1 expression has fully retreated from r5, Krox20 expression commences at this level. Krox20 expression also expands into r3. This expansion requires activation of its downstream target Hoxa2 and possibly Hoxb2. Strong kreasler expression in r5 maintains Hoxb1 autoregulated expression at the r4/r5 boundary. (B, a1−−) Hoxb1 expression retreats from the caudal hindbrain leaving autoregulated expression in caudal r4. Because kreasler is not activated in r5, autoregulated Hoxb1 expression extends into r5 as well. Krox20 expansion into r3 although delayed (due to the fewer number of cells that were induced at E8.0) occurs somewhat normally due to the fact that Krox20 and its downstream targets Hoxa2 and perhaps Hoxb2 are functioning. As a consequence of the larger expression domains of follistatin (r2 and part of r3) and Krox20 (part of r3 and r4), a regulatory event driven by apoptosis (orange dots), commences in these regions of the neural tube. (B, a1/a2−−) The hindbrain is similar to that of Hoxa1 singal mutants except that Krox20 expansion into r3 is severely delayed. Hoxa2 is a downstream target of Krox20 and if absent, cripples the expansion of Krox20-expressing cells into r3. (B, a1/b1−−) Krox20 is never induced in r3 and thus never expands into r3. As a result, follistatin expression extends to the r3/r4 boundary (M. Rossel and MRC, unpublished results). Due to enlarged follistatin and Krox20-expressing domains, apoptosis is activated in the neural tube at this level. (C, wt) A normal hindbrain with neural crest emanating from even numbered rhombomeres to their appropriate branchial arch. (C, a1+−) Due to the apoptosis at the levels of r2 and r3, there is not only a reduction in the number of neural crest (symbolized by the light blue color) that will populate the first arch, but also the abnormally large r3 is reduced to almost normal proportions. There is also a reduction in the number of neural crest that reach the second arch (light red) due to the reduced size of r4 and the fact that the otocyst may act as a barrier to prevent normal migration of the crest. The otocysts do not shift anteriorly to the level of r4, instead r4 is specified more posteriorly (at the level of the otocysts). (C, a1/a2−−) Very similar to Hoxa1 singal mutants except that, due to the lack of Hoxa2, the r4 neural crest has taken on an r1/r2 identity (blue). In addition, the lack of Hoxa1 causes a reduction in r4 neural crest (indicated by light blue) contributing to the second arch. (D, a1/b1−−) r4 is never specified. Therefore, there is no r4 neural crest to populate the second arch.

Without the function of either of the Hox1 paralogs in the developing hindbrain of E8.0 embryos, several consequences would be predicted to ensue. First, Krox20 expression would be derepressed at the level of both r4 and r5 (a1b1−−, Fig. 8A). Second, the signal downstream of Hoxa1 and Hoxb1 would never be activated and thus Krox20 expression would not be induced in the region of the hindbrain that should give
rise to r3 (Fig. 8A). Third, because Krox20 expression is not activated in r3, follistatin expression is not repressed at this level transforming this region to an r2 identity. Finally, as was the case with the other mutant classes lacking HoxA1, kreasler expression would not be activated in r5. All of these predictions have been observed in HoxA1/HoxB1 double mutant embryos (Rossel and Capocchi, 1999 and data not shown).

**Later hindbrain patterning**

Early on, HoxA1 mutants have an extended Krox20 expression domain. We suggest that extension of the r3-Krox20 domain results from part of r4 being transformed to an r3 fate. A similar finding was reported by McKay et al. (1994) for kreasler mutants where at early stages the 5th rhombomere appears to take on an r4 identity. Thus, the region of the hindbrain expressing HoxB1 is larger than normal and by E9.5 they observed an elevation of cell death in r4. They proposed that an intrinsic mechanism exists in the embryo that regulates the larger r3 domain. We suggest that extension of the r3-HoxB1 expression domain in r2 (due to the delay of Krox20 expansion into r3) and that of Krox20 in the r3-4 region (due to the derepression of Krox20 in r4; see Fig. 8B).

**Conclusion**

We have presented evidence that HoxA1 is required to establish the expression of HoxB1 to the normal presumptive r3/4 boundary. Failure to do so not only affects the identity of r4, but also delays the specification of r3. We provide evidence, as have others (Helmischer et al., 1998), that the expansion of r3 identity is also dependent on Krox20 and its downstream targets. We suggest that HoxA1 may be required for kreasler expression in r5. Without kreasler at this level, autoregulatory HoxB1 expression is allowed to extend into r5, changing its fate to an r4 identity as seen in kreasler mutants. Thus, we propose that in mutants lacking HoxA1, the region of the hindbrain that normally gives rise to r4 and r5 has been anteriorly transformed to an r3 and r4 identity, respectively. *Drosophila* embryos lacking HOM-C gene function, also exhibit anterior transformations in the identity of parasegments, demonstrating an evolutionarily conserved role among insects and vertebrates for Antennapedia class transcription factors during embryogenesis (reviewed in McGinnis and Krumlauf, 1992). Unlike the fruit fly, which treats homeotically duplicated segments as separate compartments, the mouse (and perhaps all vertebrates) considers the duplicated area as one unusually large segment and subsequently regulates its size via apoptosis. This apoptotic process may play an important role in regulating the size of rhombomeres during normal development. Finally, we have provided evidence that the early patterning defects and subsequent remodeling of the mutant hindbrains underlie the abnormalities observed in the organization of compartments in the mutant hindbrains.

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