Hoxa1 and Hoxb1 synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch

Anthony Gavalas 1,*, Michèle Studer 2,†, Andrew Lumsden 3, Filippo M. Rijli 1,‡, Robb Krumlauf 2 and Pierre Chambon 1,‡

1 Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163 - 67404 Illkirch Cedex, CU de Strasbourg, France
2 Division of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
3 Department of Developmental Neurobiology, UMDS, Guy’s Hospital, London SE1 9RT, UK
* These authors contributed equally to this work
† Present address: Department of Developmental Neurobiology, UMDS, Guy’s Hospital, London SE1 9RT, UK
‡ Corresponding authors (E-mail: igbmc@igbmc.u-strasbg.fr; rijli@igbmc.u-strasbg.fr)

Accepted 22 December 1997: published on WWW 17 February 1998

SUMMARY

The analysis of Hoxa1 and Hoxb1 null mutants suggested that these genes are involved in distinct aspects of hindbrain segmentation and specification. Here we investigate the possible functional synergy of the two genes. The generation of Hoxa1 3RARE/Hoxb1 3RARE compound mutants resulted in mild facial motor nerve defects reminiscent of those present in the Hoxb1 null mutants. Strong genetic interactions between Hoxa1 and Hoxb1 were uncovered by introducing the Hoxb1 3RARE and Hoxb1 null mutations into the Hoxa1 null genetic background. Hoxa1null/Hoxb1 3RARE and Hoxa1null/Hoxb1null double homozygous embryos showed additional patterning defects in the r4-r6 region but maintained a molecularly distinct r4-like territory. Neurofilament staining and retrograde labelling of motor neurons indicated that Hoxa1 and Hoxb1 synergise in patterning the VIIth through XIth cranial nerves. The second arch expression of neural crest cell markers was abolished or dramatically reduced, suggesting a defect in this cell population. Strikingly, the second arch of the double mutant embryos involuted by 10.5 dpc and this resulted in loss of all second arch-derived elements and complete disruption of external and middle ear development. Additional defects, most notably the lack of tympanic ring, were found in first arch-derived elements, suggesting that interactions between first and second arch take place during development. Taken together, our results unveil an extensive functional synergy between Hoxa1 and Hoxb1 that was not anticipated from the phenotypes of the simple null mutants.

Key words: Hindbrain, Patterning, Segmentation, RAREs, Cranial nerves, Neural crest, Ear development

INTRODUCTION

Extensive mutational analysis in the mouse has demonstrated the role of Hox genes in patterning the body of the developing embryo along the antero-posterior axis of the body and the proximo-distal axis of the limb (for reviews see Favier and Dollé, 1997; Rijli and Chambon, 1997). The anteriormost boundaries of vertebrate Hox gene expression lie in the hindbrain, the patterning of which proceeds through a segmentation process that is highly conserved in vertebrate evolution (Keynes and Lumsden, 1990; Keynes and Krumlauf, 1994; Wilkinson, 1995). This region of the neural tube is transiently divided, along the anteroposterior axis, into a series of 7-8 lineage-restricted compartments, the rhombomeres (r) (Fraser et al., 1990; Wingate and Lumsden, 1996; Lumsden and Krumlauf, 1996).

Loss-of-function mutants have been generated to analyse the genetic control of hindbrain segmentation and specification. Functional inactivation of the Hoxa1 gene results in a complete deletion of rhombomere 5 (r5) (Lufkin et al., 1991; Mark et al., 1993; Dollé et al., 1993) and a severe reduction of r4, thus suggesting that Hoxa1 is acting in maintenance and/or generation of hindbrain segments, rather than in conferring a specific fate to them (Lufkin et al., 1991; Chisaka et al., 1992; Mark et al., 1993; Carpenter et al., 1993; Dollé et al., 1993). In contrast, Hoxb1 appears to be involved in conferring specific identity to r4 cells (Studer et al., 1996). Krox20, a zinc finger transcription factor (Chavrier et al., 1988), is required for the maintenance of the r3 and r5 territories (Schneider-Maunoury et al., 1993, 1997; Swiatek and Gridley, 1993). Accordingly, Krox20 has been shown to be a direct or indirect regulator of Hoxa2, Hoxb2, follistatin, Hoxb3 and Sek1 in r3 and/or r5
(Sham et al., 1993; Nonchev et al., 1996; Vesque et al., 1996; Seitaniidou et al., 1997). Kreisler (kr), a bZIP transcription factor (Cordes and Barsh, 1994), is necessary for establishment of r5 and r6 territories (Frohman et al., 1993; McKay et al., 1994) and appears to be a direct regulator of Hoxb3 in r5 (Manzanares et al., 1997).

The hindbrain metameric organisation is reflected in the organisation of the associated cranial nerves. The trigeminal (Vth) nerve collects axons from motor nuclei lying in rhombomeres r1, r2 and r3, exits the brain from r2, and innervates the first branchial arch. The facioacoustic (VIIth/VIIIth) and glossopharyngeal (IXth) nerves collect axons from motor nuclei lying in r4/r5 and r6/r7, respectively, exit the brain from r4 (VIIth/VIIIth) and r6 (IXth) and innervate the second and the third branchial arches, respectively (Lumsden and Keynes, 1989; Marshall et al., 1992; Carpenter et al., 1993). The role of Hox genes in establishing the segmented, stereotyped pattern of motor neuron axonal projections and the organisation of cranial nerves has been investigated indirectly in mouse (Marshall et al., 1992; Kessel, 1993) and chick embryos (Gale et al., 1996; Nittenberg et al., 1997) by retinoic acid (RA) treatment, which induces ectopic Hox gene expression. Furthermore, the analysis of Hox loss-of-function mutants has shown that these genes influence the patterning of cranial nerves (Carpenter et al., 1993; Mark et al., 1993; Goddard et al., 1996; Studer et al., 1996; Barrow et al., 1996; Gavalas et al., 1997).

Rhombomere-specific generation of neural crest (NC) cells is observed along the dorsal part of the hindbrain, resulting in a segmental pathway of migration (Lumsden et al., 1991; Sechrist et al., 1993). The even-numbered rhombomeres and r1 generate the vast majority of hindbrain crest cells, whereas r3 and r5 are massively depleted from NC cells through apoptosis (Graham et al., 1993, 1994), generating small subpopulations that migrate rostrally and caudally into the hindbrain, cranial nerves and derivatives of the pharyngeal arches. The late-migrating subset of NC cells, which are derived exclusively from even-numbered rhombomeres and populate the prospective exit points of the cranial nerves, may provide chemoattractive signal(s) for outgrowing axons (Bender and Lumsden, 1996). Transplantation experiments suggest that the pharyngeal arch NC is responsible for specifying the fate of mesoderm (Noden, 1988; Trainor et al., 1995) and determining the muscle attachment points to the skeleton of the head (Köntges and Lumsden, 1996).

Hox genes play a major role in patterning the mesenchyme of the pharyngeal arches. The targeted inactivation of Hoxa2 results in a homeotic transformation of the second arch neural crest-derived skeletal elements into first arch derivatives (Gendron-Maguire et al., 1993; Rijli et al., 1993), revealing the existence of a skeletogenic ground patterning program common to the NC of the second and part of the first arches (Rijli et al., 1993). Hoxa3 null mutants show specific deletions or hypoplasias of structures derived from the third pharyngeal arch (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995).

In view of the importance of Hox genes in all aspects of hindbrain development, it becomes important to understand the regulatory mechanisms establishing and maintaining their expression as well as the degree of their functional redundancy. In the accompanying paper (Studer et al., 1998) we show that, similarly to Hoxa1 (Dupé et al., 1997, and references therein), a retinoic acid response element (RARE) is necessary for timely establishment of the early expression domain of Hoxb1. In turn, Hoxal and Hoxb1 are essential for establishing Hoxb1 expression in the presumptive r4 through activation of a Hoxb1 autoregulatory loop (Pöpperl et al., 1995). Simultaneous lack of Hoxal and Hoxb1 expression in the presumptive hindbrain leads to early patterning defects, not seen in any of the simple Hoxal and Hoxb1 null mutants (Studer et al., 1998). In this report, we investigate the late phenotypic alterations of double mutants lacking both Hoxal and Hoxb1 expression. In addition, we study the morphological consequences of impairing the early activation of both Hoxal and Hoxb1 by generating Hoxal^{1/3}\text{RARE}/Hoxb1^{1/3}\text{RARE} double homozygous mutants. Our findings show that Hoxal and Hoxb1 synergize in patterning the hindbrain, cranial nerves and derivatives of the pharyngeal arches.

MATERIALS AND METHODS

Whole-mount in situ hybridisation and immunostaining

Whole-mount in situ hybridisation was performed as described (Dupé et al., 1997), with the following modifications. Proteinase K treatment was for 5.5 minutes for 8.5 dpc embryos, 10 minutes for 9.5 dpc embryos, 15 minutes for 10.5 dpc embryos, and embryos were immediately fixed. The reaction for the colour development was done in 10% polyvinyl alcohol (31,000-50,000 kDa), 150 mM NaCl, 100 mM Tris, pH 9.5, and 25 mM MgCl2.

Whole-mount immunostaining using the anti-neurofilament monoclonal antibody 2H3 (Developmental Studies Hybridoma Bank), the anti-Krox20 polyclonal antibody (Berkeley Antibody Company PRB-231P-100) and genotyping were done as described (Mark et al., 1993; Studer et al., 1998).

Retrograde labelling

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and injected with the carbocyanine tracers DiI and DiO (Molecular Probes) in dimethyl-formamide (6 mg/ml). Injections, flat-mount preparations and analysis of retrogradely labeled specimens under a confocal microscope fitted with a 10x lens, were as described (Marshall et al., 1992), using fluorescein and rhodamine filters to visualise the DiO and DiI labelling, respectively. Two overlapping series of images, taken for wild-type (wt) specimens, were subsequently superimposed using Photoshop. A single series of images was taken for all mutants presented.

Scanning electron microscopy

Embryos were dissected in cold PBS, fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), washed in cacodylate buffer for 30 minutes, dehydrated through a graded series of ethanol (50%, 70%, 90%, 100% in ddH2O) for 30 minutes in each solution, and dried with a critical-point drying apparatus. Embryos were mounted on aluminum stubs and coated with palladium-gold using a cold sputter-coater, before photographing with a Philips Electron XL20 scanning electron microscope.

Histological and skeletal analysis

Mouse fetuses were fixed in Bouin’s solution, dehydrated, cleared and embedded in paraffin. Serial histological sections, 10 µm thick, were
stained with Groat’s hematoxylin and Mallory’s trichrome (Mark et al., 1993). For whole-mount analysis of skeletons and inner ears, fetuses were processed as described (Lufkin et al., 1991).

RESULTS

Lowering the response of both Hoxa1 and Hoxb1 to endogenous retinoids results in malformations of the facial nerve

Homozygous Hoxa1^{3′RARE} embryos exhibited an initial delay in Hoxa1 activation and subsequent mild segmentation and cranial nerve defects with a low penetrance. However, these defects were eventually compensated for, and homozygotes, born in the expected Mendelian ratio, were viable and fertile (Dupé et al., 1997). In homozygous Hoxb1^{3′RARE} embryos there was a specific down-regulation of early Hoxb1 neuroectodermal expression (Studer et al., 1998). Homozygotes were born in the expected Mendelian ratio and exhibited a low (5%) perinatal lethality (Fig. 1A). Dead newborns had no milk in their stomach, which indicated a feeding disability. A small fraction (5%) of the surviving homozygotes died at weaning (3rd-4th week) due to excessive growth of the incisor teeth, which block the oral cavity and prevent feeding (Fig. 1B). These mice survived when put on a semi-dry diet and had their teeth clipped regularly. The remainder of the Hoxb1^{3′RARE} homozygotes were viable and fertile.

As Hoxa1 appears to be necessary for the early establishment of Hoxb1 expression (Studer et al., 1998), we asked whether impairing the early activation of both Hoxa1 and Hoxb1 would exacerbate the Hoxb1^{3′RARE} defects. Indeed, the low penetrance of these defects was increased significantly as the two copies of the Hoxa1^{3′RARE} were mutated (Fig. 1A). Given that similar defects were seen in the Hoxb1^{null} homozygotes (Goddard et al., 1996) and that the facial motor nerve of these mutants failed to develop normally (Goddard et al., 1996; Studer et al., 1996), the facial region of affected and unaffected animals was dissected and examined for the presence of the major facial motor nerve branches (Goddard et al., 1996; Fig. 1C). The hyomandibular branch was specifically missing in the affected animals, either unilaterally (in four Hoxa1^{3′RARE}/Hoxb1^{3′RARE} double homozygous and two Hoxa1^{3′RARE}/+Hoxb1^{3′RARE}/−/− compound mutants) or bilaterally (in two Hoxa1^{3′RARE}/Hoxb1^{3′RARE} double homozygous and one Hoxa1^{3′RARE}/+/Hoxb1^{3′RARE}/−/− compound mutants) (Fig. 1D). The inability of newborns to feed could also reflect facial nerve defects, as this nerve innervates the muscles responsible for suckling. However, in contrast to the Hoxb1^{null} homozygotes (Goddard et al., 1996), affected animals did not show signs of facial paralysis but defects in the facial muscles have not been ruled out. Subsequent inbreeding of the surviving double homozygotes produced offspring in which the frequency of the perinatal lethality and the excessively long incisor teeth was reduced. This may reflect the mixed genetic background of the colony and the selection of animals with a genetic makeup compensating for the effect of the RARE mutations.

Skeletal analysis of simple and double mutant newborns, including affected animals, did not reveal any skeletal abnormalities. Hindbrain segmentation of Hoxb1^{3′RARE} homozygous and Hoxa1^{3′RARE}/Hoxb1^{3′RARE} double homozygous embryos was normal, with the exception, in the latter, of a low penetrance and stage-dependent slight reduction of rhombomere 5 (data not shown), which was also observed in the Hoxa1^{3′RARE} homozygous mutants (Dupé et al., 1997).

---

**Fig. 1.** Lethality and facial motor nerve malformations in Hoxa1^{3′RARE}/Hoxb1^{3′RARE} compound mutants.

(A) Frequencies of the perinatal (up to 1 day after birth) lethality and long incisor teeth (arrows in B), in the compound mutants generated. (C,D) Diagrams of the facial motor nerve of wild type and affected animals, respectively (adapted from Goddard et al., 1996), eam, external auditory meatus; sf, stylomastoid foramen. Scale bar, 1 mm.
Cranial nerve development of Hoxb1<sup>3RARE</sup> homozygous and Hoxa1<sup>3RARE</sup>/Hoxb1<sup>3RARE</sup> double homozygous embryos was examined at 10.5 dpc by whole-mount immunostaining and retrograde labelling of the trigeminal (V) and facial (VII) motor neurons. No defects were found with the exception, in the Hoxa1<sup>3RARE</sup>/Hoxb1<sup>3RARE</sup> double homozygous embryos, of a low penetrance unilateral loss of the proximal part of the glossopharyngeal (IX) nerve (data not shown), which was also found in the Hoxa1<sup>3RARE</sup> homozygous mutants (Dupé et al., 1997).

In summary, despite significant down-regulation of r4 Hoxb1 expression in one third of the Hoxa1<sup>3RARE</sup>/Hoxb1<sup>3RARE</sup> double homozygous embryos examined (Studer et al., 1998), only mild defects in the facial motor nerve were subsequently detected in these mutants (Table 1).

**Defects in the hindbrain of Hoxa1<sup>null</sup>/Hoxb1<sup>3RARE</sup> and Hoxa1<sup>null</sup>/Hoxb1<sup>null</sup> double homozygous embryos**

The combined lack of Hoxa1 and Hoxb1 expression resulted in early loss of the r4 expression of EphA2 (Studer et al., 1998), which was not observed in either Hoxa1 or Hoxb1 simple null mutants. This finding indicated that Hoxa1 and Hoxb1 synergise in early patterning of this territory and raised the possibility that these genes have partially redundant functions in establishing r4 identity.

Krox20 was used as a marker for r3 and r5 territories (Wilkinson et al., 1989a) to examine the hindbrain segmentation of Hoxa1<sup>null</sup>/Hoxb1<sup>3RARE</sup> double homozygous embryos, in which Hoxb1 expression is specifically abolished in the hindbrain (Studer et al., 1998), and Hoxa1<sup>null</sup>/Hoxb1<sup>null</sup> double homozygous embryos. The r3 territory of Hoxa1<sup>null</sup> homozygous embryos appeared enlarged when compared to wild-type embryos (Carpenter et al., 1993; Döllé et al., 1993; Studer et al., 1998; compare Fig. 2B,J with Fig. 2A,I), whereas the r5 territory was severely reduced, as suggested by a patchy, dorsally confined, Krox20 expression at 9.0 dpc (Fig. 2B). Even though Hoxb1<sup>null</sup> homozygous embryos did not show any reduction of r3 and r5 (Goddard et al., 1996; Studer et al., 1996), the combined lack of Hoxa1 and Hoxb1 expression further reduced or eliminated the caudal domain of Krox20 expression (compare Fig. 2B with 2C,D) (Table 2).

Kreisler (kr) was used as a marker to assess the status of r5 and r6 (Cordes and Barsh, 1994; Fig. 2E). In Hoxa1<sup>null</sup> homozygous embryos the domain of kr expression was reduced (Fig. 2F), reflecting the reduction of the r5 territory, whereas it was not affected in Hoxb1<sup>null</sup> homozygotes (Studer et al., 1996). In this respect Hoxa1<sup>null</sup>/Hoxb1<sup>3RARE</sup> double homozygous were not significantly different from Hoxa1<sup>null</sup> homozygotes (Fig. 2G), whereas Hoxa1<sup>null</sup>/Hoxb1<sup>null</sup> double homozygotes showed a further reduction of the kr expression domain (Fig. 2H).

The lack of Hoxb1 expression and the early strong down-regulation of EphA2, in both types of double mutants (Studer et al., 1998), raised the question of whether an r4-like territory was formed at all in these embryos. To address this issue kr and Krox20 probes were used simultaneously on 8.75 dpc wild type, Hoxa1<sup>null</sup> homozygous, Hoxa1<sup>null</sup>/Hoxb1<sup>3RARE</sup> and Hoxa1<sup>null</sup>/Hoxb1<sup>null</sup> double homozygous embryos. In all three
1127Hoxa1 and Hoxb1 synergy

Table 1. Summary of nerve defects

| Hoxa1\textsuperscript{RARE}/Hoxb1\textsuperscript{RARE} double homozygotes (mice at weaning age) | Partially penetrant loss of hyomandibular branch of facial motor nerve. |
| Hoxa1\textsuperscript{null} homozygotes\textsuperscript{†} (10.5 dpc) | Partially penetrant fusion of V and VII/VIII ganglia; facial and trigeminal motor neurons present in overlapping domains; partially penetrant lack of migrating facial branchiomotor neurons (FBMs); reduced r5-like facial motor axon projections. |
| Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{3 RARE} double homozygotes (10.5 dpc) | Fully penetrant fusion of V and VII/VIII ganglia; facial and trigeminal motor neurons present in overlapping domains; partially penetrant exit of facial motor axons from r2; reduced r5-like facial motor axon projections. |
| Hoxa1\textsuperscript{+/-}/Hoxb1\textsuperscript{null} double homozygotes (10.5 dpc) | Partially penetrant loss of IX and X/XI nerves. |
| Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{null} double homozygotes (10.5 dpc) | V and VII/VIII ganglia either fused or VII/VIII not connected with hindbrain; facial and trigeminal motor neurons present in overlapping domains; reduced neurofilament staining of VII/VIII ganglion and inferior IX, X ganglia; dramatically reduced retrogradely labelled facial motor neurons; lack of r5-like facial motor axon projections. |

*Retrograde labelling analysis not performed.

†See also Mark et al. (1993).

Table 2. Summary of hindbrain and pharyngeal arch defects

| Rhombomere 4 | Reduced | r4-like territory present | r4-like territory present |
| Rhombomere 5 | Dramatically reduced | Further reduced or absent | Further reduced or absent |
| Expression of NC markers in second arch (9.25 dpc) | | | |
| AP2 | Reduced | Further reduced | ND |
| CRABPI | Reduced | Absent | ND |
| Second arch at 10.5 dpc | Hypoplastic | Absent | Absent |
| External ear\textsuperscript{‡} | | | |
| Auricle | Normal, occasionally hypoplastic | Absent | Absent |
| External auditory meatus | Normal | Absent | ND |
| Middle ear\textsuperscript{‡} | | | |
| Tympanic ring | Present, but displaced rostrally | Absent | Absent |
| Tympanic membrane | Normal | Absent | ND |
| Stapes | Present, fused to otic capsule | Absent | Absent |
| Malleus | Present, occasionally hypoplastic | Strongly hypoplastic | Strongly hypoplastic |
| Incus | Present | Present | Present |
| Auditory tube | Normal | Absent | ND |
| Stapedius muscle | Normal | Absent or underdeveloped | ND |
| Tensor tympani muscle | Normal | Underdeveloped | ND |
| Inner ear\textsuperscript{‡} | | | |
| Vestibular apparatus | Absent or underdeveloped | Absent | Absent |
| Cochlea | Absent or underdeveloped | Absent | Absent |
| Stylohyoid process\textsuperscript{‡} | Present, sometimes only distally | Absent | Absent |
| Hyoid bone\textsuperscript{‡} | Present, slightly deformed | Lesser horns absent, thinner body | Lesser horns absent, thinner body |
| Stylohyoid muscle\textsuperscript{‡} | Normal | Absent | ND |

*As described (Lufkin et al., 1991; Mark et al., 1993, 1995; Dollé et al., 1993; this report).

†This report.

‡At 17.5 dpc and/or 18.5 dpc.

ND Not determined.

types of mutant embryos a similarly reduced r4-like domain persisted (compare Fig. 2I with 2J-L) (Table 2).

To examine segmentation at a later stage, we used the expression of PLZF, a gene encoding a zinc-finger protein, as a marker for rhombomeric boundaries (Cook et al., 1995) at 10.5 dpc (Fig. 2M). Hoxa1\textsuperscript{null} homozygous embryos showed a reduced r4 territory and a single r5/r6 domain (Fig. 2N), whereas the segmentation pattern of Hoxb1\textsuperscript{null} homozygous embryos was normal at this stage (data not shown). A reduced r4-like territory was still formed in both Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{3 RARE} double and Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{null} double homozygotes (arrows in Fig. 2O,P). Note that PLZF expression in this territory was reduced in these mutants, in agreement with its reduction in r4 of Hoxb1\textsuperscript{null} homozygotes (data not shown). A further reduction of PLZF expression in the caudalmost part of the hindbrain was seen in Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{null} double homozygotes (Fig. 2P).

These results suggest that Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{3 RARE} and Hoxa1\textsuperscript{null}/Hoxb1\textsuperscript{null} double homozygous embryos retain a reduced r4-like territory and maintain a hindbrain molecular segmentation pattern similar to that exhibited by Hoxa1\textsuperscript{null} homozygotes. They also suggest that some patterning events in the r5/r6 region require the synergistic action of Hoxa1 and Hoxb1.

**Synergy of Hoxa1 and Hoxb1 in cranial nerve patterning**

Whole-mount immunostaining of the cranial nerves and
associated ganglia and retrograde labelling of the motor neurons were used at 10.5 dpc, to examine the cranial nerve organisation and the segmented pattern of motor neuron axonal projections in Hoxa1null homozygotes (Lufkin et al., 1991) as well as in the Hoxa1null/Hoxb13RARE and Hoxa1null/Hoxb1null double homozygotes. Wild-type embryos as well as Hoxb1null and Hoxb13RARE homozygous embryos from the same colony were used as controls.

Hoxb1null and Hoxb13RARE homozygotes, immunostained with an anti-neurofilament antibody, were indistinguishable from wild-type embryos (Fig. 3A, and data not shown). The degree to which simple Hoxa1null homozygotes were affected was variable, even between the two sides of the same embryo. Six Hoxa1null homozygotes were stained. Two embryo sides were indistinguishable from wild type, two embryo sides had the facioacoustic ganglion (VII/VIIIg) well separated from the trigeminal ganglion (Vg), but with a thinner root connecting it to the rhombencephalon (Mark et al., 1993; Fig. 3B), while the remaining sides showed a proximal fusion of the trigeminal with the facioacoustic ganglion (arrow in Fig. 3C). The removal of a single functional Hoxb1 3' RARE in the Hoxa1null homozygous background was sufficient to cause a shift to the latter phenotype (five Hoxa1null-/Roxb13RARE+/− mutants and one Hoxa1null/Hoxb13RARE double homozygous embryos examined, data not shown).

Three (out of five examined) Hoxa1+/−/Hoxb1null−/− compound mutants lacked unilaterally the proximal part of the glossopharyngeal (IX) nerve (data not shown), and the others unilaterally lacked the proximal part of the vagus (X) and the accessory (XI) nerve as well (Fig. 3D). Hoxa1null/Hoxb1null double homozygotes (Fig. 3E,F) presented an exacerbation of the abnormal phenotypes shown in Fig. 3C,D. The facioacoustic ganglion was still present and apparently normal in size, but its nerve fibers were thinner and the connection to the abnormal inferior ganglia of the IXth and Xth nerves (data not shown).

The organisation of the motor nuclei and of their axonal projections at 10.5 dpc was analysed using retrograde labelling with lipophilic fluorescent dyes. The close apposition or even apparent fusion of the facial and trigeminal ganglia precluded direct injection of the dye into the ganglia or the nerve roots. Multiple DiO injections were therefore performed, starting distally in the mandibular component of the first arch and moving into the distal part of the trigeminal ganglion. Multiple Di injections were carried out in a similar manner in the second arch region. In the wild-type embryos all the motor neurons that can be labelled at this stage by retrograde labelling (Marshall et al., 1992) were indeed detected (Fig. 4A,B). Injections in the trigeminal and the facial motor nerves of Hoxb13RARE homozygous and in the trigeminal motor nerve of Hoxa1null homozygous, Hoxa1null/Hoxb13RARE and Hoxa1null/Hoxb1null double homozygous embryos (Studer et al., 1996) resulted in a pattern of labelled cell-bodies and axons similar to that of wild-type embryos (Fig. 4D-R; and data not shown).

The Hoxa1null homozygotes used in this study (Lufkin et al., 1991), not previously analysed by Di/DiO motorneuron tracing, showed multiple defects in the motor neuron axonal projections and the location of cell bodies (Fig. 4D-I). The presumptive r3 territory appeared to host motor neurons projecting not only towards the trigeminal ganglion through the r2 exit point but also towards the facial ganglion through multiple and ectopic r3 exit points. The degree of mixing

1128 A. Gavalas and others

![Fig. 3. Neurofilament immunostaining of the cranial nerves](image)

(A) Wild-type embryo; note the well individualised (arrow) trigeminal (Vg) and facioacoustic (VII/VIIIg) ganglia. (B) Hoxa1 null mutant presenting a weak phenotype; Vg and VII/VIIIg are still separated (arrow). (C) Phenotype exhibited by the majority of Hoxa1 null mutants and all Hoxa1null/Hoxb13RARE +/− or −/− embryos. Note the fusion between the Vth and VII/VIIIth ganglia (arrow). (D) Hoxa1+/−/Hoxb1null mutants. Note the lack of the IX,X and XI nerves (arrowhead). (E,F) Hoxa1/Hoxb1 double null mutants. Note the weak staining of the VII/VIIIth ganglion and of its projections. In these mutants this ganglion is either fused (arrow) with the Vth (E), or individualised but not connected to the rhombencephalon (F). Asterisks denote the abnormal inferior ganglia of the IXth and Xth nerves in Hoxa1 null mutants (B,C) and Hoxa1/Hoxb1 double null mutants (E,F). Scale bar, 1 mm.
between the facial and trigeminal motor neurons varied from minimal (Fig. 4D-F) to extensive (Fig. 4G-I). In wild-type embryos the facial branchiomotor neurons (FBMs) are formed in r4 and, subsequently, migrate into r5 and r6 along the lateral border of the floorplate (Studer et al., 1996; arrow in Fig. 4B). The r4 territory of the Hoxa1 null homozygotes produced motor neurons that projected normally, but failed to migrate caudally into r5 in some (5/8) mutants (compare Fig. 4A,B and D,E with G,H), thus presenting a Hoxb1 null homozygous phenotype (Goddard et al., 1996; Studer et al., 1996). Furthermore, there was a considerable loss, but not elimination, of motor neurons with axonal projections typical of r5 (i.e. exiting from the vestigial r4) (compare Fig. 4A,B with D,E,G,H).

To determine whether the combined lack of Hoxa1 and Hoxb1 expression in the hindbrain resulted in an abnormal motor neuron phenotype stronger than that of single Hoxa1 null and Hoxb1 null homozygotes, we analysed the Hoxa1 null/Hoxb1 null double homozygotes (Fig. 4J-O). Hoxa1 null/Hoxb1 null double homozygotes were more severely affected than Hoxa1 null homozygotes, since migrating FBM motor neurons were always lacking (Fig. 4J-O) and in two out of six examined Hoxa1 null/Hoxb1 null double homozygotes, r3-residing facial motor axons turned rostrally and exited abnormally through the r2 exit point (Fig. 4M-O). Hoxa1 null/Hoxb1 3'RARE double homozygotes presented a more abnormal phenotype since facial motor neurons were scarce and appeared to exit randomly from the neural tube without fasciculating towards distinct exit points (Fig. 4P-R). The reduced number of motor neurons was in agreement with the scarcity of facial nerve fibers in the periphery (Fig. 3E,F).

The neurofilament staining and the dye injection results (summarised in Table 1) indicate that Hoxa1 and Hoxb1 synergise in patterning the VIIth through XIth cranial nerves. Moreover, the strength of the neurofilament staining and the
number of retrogradely labelled motor neurons appear to be dramatically reduced in the $Hoxa^{1m1l}/Hoxb^{1m1l}$ double homozygotes, as compared to the $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygotes.

**Changes in the NC cells migrating into the second pharyngeal arch correlate with its subsequent involution**

The combined changes in the patterning and molecular identity of the r4 territory in both $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygotes and $Hoxa^{1m1l}/Hoxb^{1m1l}$ double homozygotes (see above, and Studer et al., 1998), prompted us to examine the status of the NC cells of this territory. To that end we used AP2 and CRABPI probes in whole-mount in situ hybridisations. AP2 is a transcription factor expressed in NC cells and their major derivatives (Mitchell et al., 1991). The functional inactivation of this gene results in severe craniofacial defects, demonstrating its essential role in NC development (Schörle et al., 1996; Zhang et al., 1996).

CRABPI, a RA binding protein, is expressed in both the neural epithelium and the migrating NC cells, but not in paraxial mesodermal cells (Ruberte et al., 1992; Maden et al., 1992).

In 9.25 dpc wild-type embryos, both AP2 (Fig. 5A) and CRABPI (Fig. 5D) were expressed in the three distinct streams of cells migrating into the first, the second and the third pharyngeal arches (Lumsden et al., 1991). The reduced intensity of AP2 staining in both $Hoxa^{1m1l}$ homozygous and $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygous embryos (Fig. 5B,C) as compared to wild-type embryos (Fig. 5A), suggests a decrease in the amount of NC cells migrating into the second branchial arch of these mutants. However, whereas $Hoxa^{1m1l}$ homozygotes exhibited a strongly reduced, but still detectable, expression of CRABPI (compare Fig. 5E with F), no CRABPI expression could be detected in the second arch of the $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygous embryos. It should be noted that CRABPI expression was normal in the second pharyngeal arch of $Hoxb^{1m1l}$ homozygotes (Fig. 5G) and that, at this stage (9.25 dpc), there are no obvious morphological differences in the region of the pharyngeal arches between wild-type embryos, $Hoxa^{1m1l}$ homozygotes and $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygotes (Fig. 5D-F). In contrast, the second pharyngeal arch of $Hoxa^{1m1l}/Hoxb^{1m1l}$ double homozygotes was not identifiable at 9.5 dpc (compare Fig. 5G with H).

To look for the second pharyngeal arch at later stages (10.5 dpc), we used both a PLZF probe, as this gene is expressed in the mesenchyme of the first and second pharyngeal arches (Cook et al., 1995), and scanning electron microscopy (SEM). A clear, stepwise loss of PLZF expression was observed in the second pharyngeal arch when $Hoxa^{1m1l}$ homozygotes (Fig. 5J), $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygotes (Fig. 5K) and $Hoxa^{1m1l}/Hoxb^{1m1l}$ double homozygotes (Fig. 5L) were compared to wild-type embryos (Fig. 5I). This reflected the dramatic reduction of the second pharyngeal arch in both types of double mutants, as observed by SEM and paralleled the reduction in CRABPI expression in the second arch. The second pharyngeal arch was hypoplastic in $Hoxa^{1m1l}$ homozygotes and was replaced by a flattened surface in both types of double mutants (Fig. 6A-C and data not shown). It is worth noting that aplasia of the second arch was also evident in both $Hoxa^{1m1l-/-}/Hoxb^{1+/-}$ and $Hoxa^{1m1l-/-}/Hoxb^{13RARE+/-}$ embryos, albeit with a lower penetrance in the latter case (data not shown).

Thus, the combined lack of $Hoxa1$ and $Hoxb1$ expression in the hindbrain of $Hoxa^{1m1l}/Hoxb^{13RARE}$ and $Hoxa^{1m1l}/Hoxb^{1m1l}$ double homozygous embryos results in molecular alterations of the second arch NC cells (summarised in Table 2) with a subsequent early disruption of second arch development. Note that it is not excluded that migratory defects of the second arch NC cells could also contribute to this phenotype.

**Full disruption of ear development and lack of second arch-derived skeletal elements in the $Hoxa^{1m1l}/Hoxb^{13RARE}$ double homozygotes**

The external ear develops from the first and second pharyngeal

---

**Fig. 5.** Expression of molecular markers in the pharyngeal arches. Wild type (A,D,I), $Hoxa1$ null mutants (B,E,J), $Hoxb1$ null mutants (G), $Hoxa^{1m1l}/Hoxb^{13RARE}$ double mutants (C,F,K) and $Hoxa/Hoxb$ double null mutants, (H,L) were hybridised with an AP2 antisense probe at 9.25 dpc (A-C), CRABPI at 9.25 (D-F) and 9.5 (G,H) and PLZF at 10.5 dpc (I-L). Arrowheads in (A-C) mark AP2-positive cells in the second pharyngeal arch (pa2). Brackets mark the second arch in wild type (A,D), $Hoxa1$ null mutants (B,E) and $Hoxb1$ (G) null mutants. Arrows mark the aplasia of the second arch in $Hoxa^{1m1l}/Hoxb^{13RARE}$ double (K) and $Hoxa/Hoxb$ double null mutants (H,L). Bars, 100 μm (A-H), 200 μm (I-L).
arches and also from the first pharyngeal cleft. In *Hoxa*<sup>1</sup> null homozygous newborns the auricle is formed but occasionally hypoplastic (Lufkin et al., 1991; data not shown). Strikingly, the auricle is missing in *Hoxa*<sup>1</sup>null/*Hoxb*<sup>1</sup>3' RARE double homozygotes (Fig. 7A,B), whereas it was rudimentary in *Hoxa*<sup>1</sup>null/*Hoxb*<sup>1</sup>3' RARE+/- mutants (data not shown). The external auditory meatus, which is derived from the first pharyngeal cleft, was also not formed in *Hoxa*<sup>1</sup>null/*Hoxb*<sup>1</sup>3' RARE double homozygotes, in contrast to *Hoxa*<sup>1</sup> null and *Hoxb*<sup>1</sup> null homozygotes (Fig. 7C-E and data not shown).

As the middle ear is derived from the first and second pharyngeal arches as well as from the first pharyngeal pouch, we also examined its development in *Hoxa*<sup>1</sup>null/*Hoxb*<sup>1</sup>3' RARE double homozygous fetuses. In *Hoxa*<sup>1</sup> null homozygotes the tympanic ring, a first arch NC-derived middle ear ossicle, was always fully formed, but rostrally displaced (Lufkin et al., 1991; Fig. 7F). In contrast, it was never present in *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double homozygotes (Fig. 7F) and only partially formed in *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE+/- fetuses (data not shown). The tympanic membrane was also lacking in double mutant fetuses (data not shown). The malleus, which is derived from the NC of the first arch, was either correctly formed or had a slightly smaller neck and processus brevis (in 7/14 middle ears examined) in *Hoxa*<sup>1</sup> null homozygotes, but was markedly reduced in *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double homozygotes (Mark et al., 1995; Fig. 7F). The tensor tympani muscle, which attaches to the cartilaginous part of the auditory

---

Fig. 6. Scanning electron microscopy of the facial region at 10.5 dpc. Arrows mark the first (pa1) and second pharyngeal (pa2) arches. Note the increasing reduction of the second pharyngeal arch from the wild type (A) to *Hoxa*<sup>1</sup> null mutants (B) to *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutants (C). Bar, 200 μm.

Fig. 7. Disruption of ear development. (A,B) The auricle (au) is readily visible in wild-type newborns (A) but is missing (asterisk) in *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutant newborns (B). (C-E) Transverse histological sections of wild type (C), *Hoxa*<sup>1</sup> null (D) and *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutants (E), 17.5 dpc embryos. Note the absence, specifically in the double mutants, of the external auditory meatus (eam), the stapes (st), the auditory tube (at), the tympanic ring (tr) and the hypoplasia of the tensor tympani muscle (ttm). Note also the fusion of the stapes with the otic capsule (oc) in the *Hoxa*<sup>1</sup> null mutants (D), ma, malleus; co, cochlea; bo, basioccipital bone; mb, mandible; Mc, Meckel's cartilage. (F) Dissected middle ear ossicles from wild-type and mutant newborns. Note the lack of the tympanic ring (tr) in the *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutants, and the increasing aplasia of the malleus (ma) from the wild type to the *Hoxa*<sup>1</sup> null mutant to the *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutant newborns. pb, processus brevis of the malleus; mn, manubrium of the malleus; gb, gonial bone. The Meckel's cartilage of the *Hoxa*<sup>1</sup> mutant was cut during dissection, and hence it is represented here with a dashed line. (G,H,I) The medial surface of the right inner ear of a wild type (G), a mildly affected *Hoxa*<sup>1</sup> homozygote (H) and a typical *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutant newborn (I) are shown. Note the absence of the cochlea (co), the styloid (sty), the aplasia of the vestibular apparatus (va) and the lack of any fenestration in the inner ear of the *Hoxa*<sup>1</sup> null/*Hoxb*<sup>1</sup>3' RARE double mutant. ow, oval window; rw, round window. Bars, 2 mm (A,B), 250 μm (C-F) and 400 μm (G-I).
tube and the handle of the malleus, was also underdeveloped in the Hoxa1null/Hoxb13null double homozygotes, but not in Hoxa1null homozygotes (Mark et al., 1995; compare Fig. 7C,D with E). This is not surprising, given the obliteration of the auditory tube and the underdeveloped malleus in these embryos. The incus did not appear to be affected in the examined Hoxa1null homozygotes and Hoxa1null/Hoxb13null double homozygotes. The stapes, a second arch-derived element, is normally attached by its footplate to the labyrinth membrane lining the oval window (Fig. 7C) and is associated with the stapedius muscle. In the examined Hoxa1null homozygotes, both the stapedius muscle and the stapes were present, the latter being fused to the otic capsule (Mark et al., 1993; Fig. 7D). Strikingly, in Hoxa1null/Hoxb13null double homozygotes the oval window was not formed (compare Fig. 7I with H,G) and the stapes was lacking (compare Fig. 7E with C,D) whereas the stapedius muscle was underdeveloped or lacking (data not shown).

The inner ear develops from the ectodermal otic placode, which appears bilaterally alongside r5 and r6 at the time of neural tube closure (Noden and Van de Water, 1986; Keynes and Lumsden, 1990). It has been suggested that inductive signals emanating from the rhombencephalon are crucial for early induction of the otic placode and subsequent differentiation of the otocyst. Inner ear malformations in the cochlea and the vestibular apparatus were variable in Hoxa1null homozygotes (Fig. 7H,I) but fully penetrant in Hoxa1null/Hoxb13null double homozygotes (Fig. 7I). Five out of 14 Hoxa1null homozygous inner ears examined still had a partially formed cochlea and six had recognisable semicircular canals (Fig. 7H), whereas these structures invariably fail to form in Hoxa1null/Hoxb13null double homozygotes (12 inner ears examined) (Fig. 7I).

The styloid process is a second-arch skeletal element (Noden, 1988; Köntges and Lumsden, 1996). In the Hoxa1null homozygotes, the styloid was either correctly formed (9/14 inner ears examined, data not shown) or at least its distal part was present (Fig. 7G,H), whereas it was not identifiable in the Hoxa1null/Hoxb13null double homozygotes (Fig. 7I). The lesser horns and cranial part of the body of the hyoid bone are formed by NC cells of the second arch, whereas its greater horns and the rest of its body are derived from the third arch (Noden, 1988; Köntges and Lumsden, 1996). In the Hoxa1null homozygous fetuses, the hyoid bone was always correctly formed (Lufkin et al., 1991), although it appeared slightly twisted sometimes (Fig. 8B). The hyoid bone of Hoxa1null/Hoxb13null double homozygotes was always missing its second arch-derived elements, as indicated by the lack of the lesser horns and a thinning of the body. The greater horns were fused dorsally with the thyroid cartilage (Fig. 8C). No defects in bones of mesodermal origin (occipital, intraparietal and cervical) were observed in Hoxa1null/Hoxb13null double homozygotes, other than those already described for Hoxa1null homozygotes (Lufkin et al., 1991). The skeletons of Hoxa1null/Hoxb1null double homozygotes showed additional mild abnormalities in the occipital/cervical region only (data not shown).

No ear or pharyngeal arch defects have been reported in Hoxb1null homozygotes (Goddard et al., 1996; Studer et al., 1996). The full disruption of ear development and the loss of all second arch-derived skeletal elements in

DISCUSSION

In this study we have examined the synergistic function of Hoxa1 and Hoxb1 in patterning the hindbrain, the cranial nerves and the pharyngeal arches. Lowering the response of both genes to endogenous retinoids leads to minor, not fully penetrant defects in the development of the facial nerve. In contrast, we have unveiled extensive synergy between the products of the two genes by generating Hoxa1null/Hoxb13null and Hoxa1null/Hoxb1null double homozygotes. Interestingly, these mutants show additional molecular changes in the r4-r6 hindbrain region and in the second pharyngeal arch NC cells, resulting in early disruption of the second arch development, subsequent loss of its derivatives, full disruption of ear development and further patterning defects of the cranial nerves (summarised in Tables 1 and 2).

Overlapping functions of Hoxa1 and Hoxb1 in hindbrain patterning

Detailed analysis of the rhombencephalon of the Hoxa1null homozygotes used in this study by three-dimensional reconstructions (Lufkin et al., 1991) has shown the presence of a single enlarged rhombomeric structure in place of the wt r4/r5/r6 distinct rhombomeres (Mark et al., 1993). However, further analysis suggested that this structure was molecularly divided in distinct territories corresponding to a reduced r4, the
remnant of r5, and r6 (Dollé et al., 1993). Using molecular markers, we have analysed -Hoxa1null/Hoxb13RARE homozygous and -Hoxa1null/Hoxb1null double homozygotes in order to determine whether the combined lack of Hoxa1 and Hoxb1 expression in the hindbrain leads to further alterations of the segmentation pattern.

The caudal domain of Krox20 expression is further reduced or completely eliminated in -Hoxa1null/Hoxb13RARE homozygous and -Hoxa1null/Hoxb1null double homozygotes. This implies that the establishment of Krox20 expression in r5 may depend on signals from r4 and/or the presence of Hoxa1 and Hoxb1 in the presumptive r5 of the presomitic embryo. The combined r5/r6 territory does not appear to be significantly altered in the -Hoxa1null/Hoxb13RARE homozygous, as assessed by kr and PLZF expression, when compared to that of -Hoxa1null homozygotes. However, this territory is further reduced in -Hoxa1null/Hoxb1null double homozygotes. As Hoxb1 mesodermal expression is not abolished (Studer et al., 1998; see also above) in the -Hoxa1null/Hoxb13RARE double homozygotes, this difference could reflect a mesodermal contribution of Hoxb1 in patterning the presumptive r5/r6 region of the neural plate via vertical inductive signals (Frohman et al., 1990).

A population of cells molecularly distinct from either r3 or r5/r6 territories (see above) is present at 8.75 dpc and maintained up to 10.5 dpc in the absence of Hoxa1 and Hoxb1. Interestingly, the size of this r4-like segment, as assessed by PLZF staining at 10.5 dpc, is not further reduced in both double mutants, as compared to -Hoxa1null homozygotes. Hoxb2 is the only other Hox gene strongly expressed in r4 (Wilkinson et al., 1989b) and it is likely to be involved, in combination with Hoxa1 and Hoxb1, in establishment/maintenance of this rhombomere, as only its late phase of expression is dependent on Hoxb1 (Maconochie et al., 1997). It is noteworthy that the -Hoxa1null homozygotes generated by Chisaka et al. (1992) lack segmentation in the hindbrain. A key difference may lie in the latter study, in the retention of a truncated Hoxa1 protein, encompassing the N-terminal of the protein but lacking the homeobox (La Rosa and Gudas, 1988). This truncated protein may compete for common interacting factors with other Hox proteins, particularly with Hoxb1 and Hoxb2, thus exacerbating the -Hoxa1null homozygous phenotype. In this respect, it is noteworthy that external and middle ear defects reported for the -Hoxa1null homozygotes generated by Chisaka et al. (1992) are not observed in the -Hoxa1null homozygotes used here (Lufkin et al., 1991), while they are present in the -Hoxa1null/Hoxb13RARE and -Hoxa1null/Hoxb1null double homozygotes.

It has been suggested that inductive signals emanating from the rhombencephalon are crucial for the early induction of the otic placode and differentiation of the otocyst (Noden and Van De Water, 1986). In -Hoxa1null homozygotes, normal development of the inner ear is affected but the penetrance of the defects is not complete, indicating that early inductive events and/or subsequent morphogenesis are not fully disrupted. Consistent with the data indicating that Hoxa1 and Hoxb1 synergise in patterning the r4-r6 region, we find here that the disruption of inner ear development is fully penetrant in the -Hoxa1null/Hoxb13RARE double homozygotes.

In summary, the -Hoxa1null segmentation defects do not appear to be greatly exacerbated in the presented double mutants, apart from further reduction or loss of the caudal Krox20 expression domain. However, the specific loss of EphA2 expression at the 0- to 2-somite-stage in both types of double mutants (Studer et al., 1998) suggests a change in the identity of the prospective r4 territory. A further reduction of the r6 territory is seen specifically in the -Hoxa1null/Hoxb1null double homozygotes. Taken together, these results demonstrate that abrogation of Hoxb1 expression in a -Hoxa1null homozygous background does not further affect segmentation but alters the identity of r4 and repatterns the r5/r6 territory. Thus, Hoxa1 and Hoxb1 appear to functionally synergise for r4 specification but not in hindbrain segmentation.

Hoxa1 and Hoxb1 synergize in patterning the cranial nerves

Results from the analysis of the -Hoxa1null and -Hoxb1null homozygotes have implicated these paralogs in distinct aspects of cranial nerve development (Carpenter et al., 1993; Mark et al., 1993; Goddard et al., 1996; Studer et al., 1996). The analysis of -Hoxa13RARE/Hoxb13RARE, -Hoxa1null/Hoxb13RARE and -Hoxa1null/Hoxb1null double homozygotes indicates that Hoxa1 and Hoxb1 synergise in patterning the cranial nerves (Table 1).

The number of -Hoxa13RARE/Hoxb13RARE double homozygotes lacking specifically the hyomandibular branch of the facial motor nerve correlates well with the percentage of embryos exhibiting a dorsally reduced Hoxb1 expression at 9.5 dpc (Studer et al., 1998), suggesting a causal relationship. This may also imply differential requirements in levels of Hox gene expression for the development of different branches of the facial nerve.

The -Hoxa1null homozygotes further analysed here (Lufkin et al., 1991) show extensive intermixing of V and VII/VIII nerve efferent cell bodies, as do the -Hoxa1null homozygotes generated by Chisaka et al. (1992) (Carpenter et al., 1993). The present analysis, performed at an earlier stage, shows in addition that the VII/VIII nerve efferent axons have multiple exit points rostral to the normal r4 exit point. The intermixing of V and VII/VIII nerve efferent cell bodies and their abnormal axonal projections in both -Hoxa1null homozygotes (Carpenter et al., 1993; this study) might be due to extensive intermixing of r3 and r4 cells, and impaired segmental distribution of NC leading to ectopic exit points able to attract outgrowing motor axons (Niederlander and Lumsden, 1996). This could result from early impaired cell lineage restriction in these mutants (Guthrie and Lumsden, 1991; Guthrie et al., 1993). The apparently normal number of motor neurons labelled by dye injections in -Hoxa1null homozygotes suggests that the reduction or loss of the facial nucleus, detected in these mutants (Lufkin et al., 1991; Mark et al., 1993), occurs at a later stage, possibly due to degenerative processes. The variability seen in the generation of migrating FBM motorneurons in the -Hoxa1null homozygotes may reflect lower levels of Hoxb1 expression in these mutants (Carpenter et al., 1993; Dollé et al., 1993), and subsequently stochastic processes of FBM specification. Accordingly, we have never detected such neurons in -Hoxa1null/Hoxb13RARE homozygotes, which do not express Hoxb1 in the hindbrain (Studer et al., 1998).

The reduced number of r5-like motor neurons in the -Hoxa1null homozygotes and -Hoxa1null/Hoxb13RARE double homozygotes is not surprising given the drastic reduction of
this territory indicated by molecular and histological criteria (Dollé et al., 1993; Mark et al., 1993; see above). On the other hand, some of these neurons may reside in r6 and are now able to project towards r4 due to the reduction of r5 (see also Carpenter et al., 1993; these Hoxa1 null /Hoxb1 3 double homozygotes lack r5 completely). This has been shown to occur in the Krox20 null mutants (Schneider-Maunoury et al., 1997).

The occurrence of facial motor axons exiting from r2 in the Hoxa1null/Hoxb13RARE double homozygotes, may indicate a more extensive rostral spread of r4-like motor neuron precursors in these mutants. It cannot be excluded that molecular changes in the neuroepithelium, similar to those occurring in Hoxa2 null mutants (Taneja et al., 1996; Gavalas et al., 1997), contribute to mis-direction of the motor axons. However, extensive mixing of r3 and r4 cells in Hoxa1null homozygotes and, perhaps to a higher degree, in Hoxa1null/Hoxb13RARE double homozygotes, seems sufficient to account for the cranial nerve abnormal phenotype. It should be noted that, despite the full penetration of the fused ganglia phenotype, the trigeminal and facioacoustic ganglia retain their selective wiring.

The combination of Hoxa1null and Hoxb1null mutations results in further nerve defects. The Hoxa1+/−/Hoxb1null−− mutants fail in many instances to form the proximal part of the IX and X/XI nerves. These defects are reminiscent of those in the Hoxa1null homozygotes, and may be due to failure of the r6-r8 neurogenic crest cells to form the superior ganglia (Mark et al., 1993, and references therein). This further supports the notion that, beyond synergising in r4 patterning, Hoxa1 and Hoxb1 synergise also in patterning the caudal hindbrain.

Earlier studies have suggested that the placode-derived part of the trigeminal ganglion is necessary for trigeminal motor axon outgrowth (Moody and Heaton, 1983). The dramatically reduced number of facial motor neurons and reduced neurofilament staining in the Hoxa1null/Hoxb1null double homozygotes may be due to additional patterning defects of the facioacoustic ganglion. Interestingly, these defects are not present in the Hoxa1null/Hoxb1RARE double homozygotes, despite the loss of Hoxb1 expression in the hindbrain. This suggests that Hoxb1 expression in other tissues (e.g. mesoderm, pharyngeal endoderm) of these mutants is not abolished, rescuing the above defects. In support of this hypothesis, Hoxa1null/Hoxb1null double homozygotes show mesodermal defects not present in the Hoxa1null/Hoxb1RARE double homozygotes.

In conclusion, our results (Table 1) indicate that Hoxa1 and Hoxb1 synergise in patterning the cranial nerves VII through XI. Hoxa1+/−/Hoxb1null−− mutants show malformations in cranial nerves IX through XI not present in either Hoxa1null heterozygotes or Hoxb1null homozygotes. Defects that are not fully penetrant in the Hoxa1null homozygotes become fully penetrant in the Hoxa1null/Hoxb13RARE double homozygotes, and abnormal exit of facial motor axons from r2 occurs only in the latter mutants. In Hoxa1null/Hoxb1null double homozygotes there are further defects in the development of cranial nerves, ganglia and motor neurons.

Early disruption of second pharyngeal arch development in Hoxa1null/Hoxb13RARE double homozygotes

Hoxa1 and Hoxb1 are not expressed in the second arch ectomesenchyme (Frohman et al., 1990; Murphy and Hill, 1991) and there are only minor defects in the mesenchymal neural crest derivatives of Hoxa1null mutants (Lufkin et al., 1991; Mark et al., 1993) and no defects in those of Hoxb1null homozygotes (Goddard et al., 1996; Studer et al., 1996). Strikingly, the combined lack of expression of Hoxa1 and Hoxb1 in the hindbrain synergistically results in aplasia of the second arch and ear defects reminiscent of certain human conditions that have their origin in defects of the first branchial arch and pouch (for a review, see Lambert and Dodson, 1996). The complete lack of second pharyngeal arch cartilage derivatives, the disruption of external and middle ear development, and the apparent lack of any ectopic cartilage elements in Hoxa1null/Hoxb13RARE double homozygous 17.5 dpc fetuses and newborns (Table 2), are consistent with an early disruption of second pharyngeal arch development.

The second pharyngeal arch of the Hoxa1null homozygotes appears to be hypoplastic at 10.5 dpc. This probably results from a reduction in the number of migrating neural crest cells caused either by the reduction in the size of the r4 territory, which provides the bulk of the second arch neural crest (Lumsden et al., 1991; Sechrist et al., 1993), and/or the rostral displacement of the otocyst, which may create a physical barrier (Mark et al., 1993). However, this reduction does not result in loss of second arch-derived elements, whereas the strong hypoplasia of the second arch in the Hoxa1null/Hoxb13RARE double homozygotes has severe phenotypic consequences. Since NC cells are responsible for the patterning of the arch mesenchyme (Noden, 1983), a defect in this population may underlie the second arch involution phenotype. This is consistent with the early patterning defects in presumptive r4 territory of Hoxa1null/Hoxb13RARE and Hoxa1null/Hoxb1null double homozygotes (Studer et al., 1998). Accordingly, expression of two NC markers, CRABPI (Mitchell et al., 1991) and AP2 (Ruberte et al., 1992; Maden et al., 1992) is either abolished or further reduced in the second arch of double mutants at 9.25 dpc, as compared to Hoxa1null homozygotes. Hoxa1null homozygotes and double homozygotes are morphologically indistinguishable in the region of the pharyngeal arches at 9.25 dpc, but 1 day later only the second arch of the double homozygotes has invovled. This observation suggests a proliferative defect and/or excessive cell death of the second arch mesenchyme in the double homozygotes, although an early migratory problem of the second arch cells cannot be ruled out. Use of more NC markers and DiI labelling of migrating NC cells may allow us to distinguish between these possibilities.

A significant number of signalling molecules, such as Fgf3 (Wilkinson et al., 1988; Mahmood et al., 1995), Fgf4 (Niswander and Martin, 1992; Drucker and Goldfarb, 1993), Fgf8 (Crossley and Martin, 1994; Heikinheimo et al., 1994) and endothelin-1 (Kurihara et al., 1994) are expressed in the pharyngeal pouches, and the ectoderm of the pharyngeal arches. Their repetitive expression in the inter-arch regions suggests that they may be part of the signals that control growth and patterning of the pharyngeal arches. It is conceivable that the Hox code of the NC cells migrating from different axial levels (Hunt et al., 1991) enables them to perceive and interpret mitogenic and differentiation signals in order to generate the appropriate structures. The second pharyngeal arch NC cells of the double homozygotes may not be able to perceive these signals, ceasing, therefore, to proliferate, and resulting in the
observed aplasia of the second arch. Patterning of the pharyngeal endoderm may also be affected in the double homozygotes, thus compromising normal development of the second arch and contributing to the involution phenotype. However, the latter possibility is less likely, as *Hoxb1* expression is not abolished in the foregut of *Hoxa1null/Hoxb13RARE* double homozygotes (Studer et al., 1998 and data not shown). It is also worth mentioning that *Hoxa3* null mutants show specific deletions or hypoplasias of structures derived or patterned by mesenchymal NC cells of the third pharyngeal arch (Chisaka and Capeschi, 1991; Manley and Capeschi, 1995). In these mutants, changes in overall generation or migration of NC cells are not observed, arguing for an intrinsic patterning (Manley and Capeschi, 1995) and/or proliferative defect once they have reached their destination.

The unexpected defects found in first arch-derived elements of *Hoxa1null/Hoxb13RARE* double homozygotes are strikingly similar to those found in the gsc null mutants. *Gsc* null mutants lack the external auditory meatus, the tympanic ring, and exhibit hypoplasia of the manubrium and lack of the processus brevis of the malleus (Rivera-Pérez et al., 1995; Yamada et al., 1995). *Gsc* is expressed in the caudal mesenchyme of the first arch and the most rostral portion of the second, after completion of the NC cell migration (Gaunt et al., 1993). A hypothetical signalling centre in this region was postulated to account for the phenotype of *Hoxa2* null mutants (Rijli et al., 1993), which have a set of duplicated first arch elements in place of second arch elements (Gendron-Maguire et al., 1993; Rijli et al., 1993). It is conceivable that the impaired second arch development, in the double mutants analysed here, affects this region as well, leading to first arch defects.

In summary, the early patterning defects in the presumptive r4 region of the double null homozygotes compromise correct specification of the second arch NC cells. This results in involution of the second arch and, consequently, complete disruption of external and middle ear development and loss of all second arch-derived elements. The defects observed in first arch-derived elements indicate that interactions between arches are part of the morphogenetic program in this region.

The authors would like to thank Jean-Luc Vonesch and Nadia Messaddeq for help with the electron and confocal microscopy, Robert Matyas for help with the dissection of the facial nerve and Betty Feret for excellent technical assistance. We are grateful to Manuel Mark and Georgy Köntges for discussions and comments on the manuscript. We thank G. Barsh for the *kr* probe, D. Wilkinson for the *Krox20*, P. Zelent for the *PLZF* and P. Mitchell for the *AP2* probe (Mitchell et al., 1991). The 2H3 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA under contract NO1-HD-7-3263. A.G. was supported by fellowships from EC Human Capital Mobility, Fondation pour la Recherche Médicale (FRM) and a short term EMBO fellowship and M.S. was supported by fellowships from EMBO, EEC Human Capital Mobility and the Medical Research Council (MRC). This work was also supported by grants from the INSERM, CNRS, Collège de France, Association pour la Recherche sur le Cancer (ARC), FRM and LNC.

REFERENCES


