Synergy between *Hoxa1* and *Hoxb1*: the relationship between arch patterning and the generation of cranial neural crest

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**SUMMARY**

*Hoxa1* and *Hoxb1* have overlapping synergistic roles in patterning the hindbrain and cranial neural crest cells. The combination of an ectoderm-specific regulatory mutation in the *Hoxb1* locus and the *Hoxa1* mutant genetic background results in an ectoderm-specific double background, leaving the other germ layers impaired only in *Hoxa1* function. This has allowed us to examine neural crest and arch patterning defects that originate exclusively from the neuroepithelium as a result of the simultaneous loss of *Hoxa1* and *Hoxb1* in this tissue. Using molecular and lineage analysis in this double mutant background we demonstrate that presumptive rhombomere 4, the major site of origin of the second pharyngeal arch neural crest, is reduced in size and has lost the ability to generate neural crest cells. Grafting experiments using wild-type cells in cultured normal or double mutant mouse embryos demonstrate that this is a cell-autonomous defect, suggesting that the formation or generation of cranial neural crest has been uncoupled from segmental identity in these mutants. Furthermore, we show that loss of the second arch neural crest population does not have any adverse consequences on early patterning of the second arch. Signalling molecules are expressed correctly and pharyngeal pouch and epibranchial placode formation are unaffected. There are no signs of excessive cell death or loss of proliferation in the epithelium of the second arch, suggesting that the neural crest cells are not the source of any indispensable mitogenic or survival signals. These results illustrate that Hox genes are not only necessary for proper axial specification of the neural crest but that they also play a vital role in the generation of this population itself. Furthermore, they demonstrate that early patterning of the separate components of the pharyngeal arches can proceed independently of neural crest cell migration.

Key words: Hox genes, Neural crest, Mouse mutants, Hindbrain segmentation, Pharyngeal arches, Cell transposition or grafting, Mouse embryo culture

**INTRODUCTION**

The neural crest is a transient cell population generated at the border between the neural plate and the presumptive epidermis. Their apposition is thought to induce the specification of neural crest cells (ncc) (Dickinson et al., 1995). Neural crest formation is a multistep process in which signals from both the non-neural ectoderm and non-axial mesoderm are involved (for a review see LaBonne and Bronner-Fraser, 1999). Neural crest cells delaminate from the neural tube and migrate along stereotyped routes to populate diverse regions in the embryo, where they give rise to most of the peripheral nervous system, melanocytes, smooth muscles and also, in the head, craniofacial cartilage and bone (Le Douarin, 1983). Neural crest induction takes place at the same time as anteroposterior (AP) patterning events that generate the nested expression domains of the Hox genes along the axis (Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000a).

The hindbrain is a good model system to study the relationship between both regional specification and neural crest induction. The hindbrain is a segmented structure transiently divided in seven lineage-restricted compartments, termed rhombomeres (r) (Fraser et al., 1990; Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996). The anterior limits of Hox gene expression coincide with rhombomere boundaries and display a two segment periodicity (Hunt et al., 1991a; Keynes and Krumlauf, 1994; Lumsden and Krumlauf, 1996; Wilkinson et al., 1989). The segmented structure of the hindbrain underlies the generation of distinct streams of neural crest cells that populate the pharyngeal arches (pa), which are the main metameric components of the developing head (Noden, 1988). Neural crest cells that populate the first (pa1) and second (pa2) pharyngeal arches originate in distinct streams mainly from r1/r2 and r4, respectively. However, r3 and r5 also contribute a small population of ncc that exit the hindbrain at the junction with even-numbered rhombomeres and join the major streams emanating from the even segments (Golding et al., 2000; Köntges and Lumsden, 1996; Lumsden et al., 1991; Sechrist et al., 1993; Serbedzija et al., 1992). The generation of distinct...
ncc streams is attributed to a combination of inter-rhombomeric signalling that leads to cell death in r3 and r5 (Graham et al., 1994; Graham et al., 1993; Graham et al., 1988), exclusion of crest cells from the adjacent mesenchyme (Farlie et al., 1999) and patterning information from the rhombomeres to the adjacent mesenchyme (Golding et al., 2000).

Tissue interactions in the pharyngeal arches are important for proper patterning. The migrating ncc enshroud the cranial paraxial mesoderm in each arch and provide signals that govern muscle identities (Köntges and Lumsden, 1996; Noden, 1983; Noden, 1986; Noden, 1988; Trainor and Tam, 1995; Trainor et al., 1994). Early transplantation experiments suggested that migrating ncc were pre-patterned according to their axial level of origin (Noden, 1983; Noden, 1988), but transplantation of rhombomeric tissue in mouse and chick have revealed the importance of cell community effects and an extensive degree of ncc plasticity in response to mesodermal signals in the arches (Grabin-Botton et al., 1995; Itsaki et al., 1996; Trainor and Krumlauf, 2000a; Trainor et al., 2000). However, ablations of the neural tube in the chick suggest that initial pharyngeal arch patterning may occur in the absence of substantial amounts of ncc (Veitch et al., 1999). Furthermore, studies in chick and mouse have shown that Hoxa2 expression in the ncc is independently regulated from that in the neural epithelium (Maconochie et al., 1999; Mallo and Brandlin, 1997; Nonchev et al., 1996; Prince and Lumsden, 1994).

Analyses in mice with functional knockouts (KO) of Hox genes have suggested that patterning defects in the neuroepithelium or the pharyngeal arches are to a large extent independent (Rijli et al., 1998). The Hoxa2 mutation resulted in re-specification of the second arch neural crest towards first-arch identity in the absence of neural epithelial defects in r4, the segment from which this population is derived (Gavalas et al., 1997; Gendron-Maguire et al., 1993; Rijli et al., 1993). Mutation of Hoxb1 exclusively affected specification of the r4 neuroepithelium, even though Hoxb1 is also expressed in the neurogenic crest of the second arch (Goddard et al., 1996; Hunt et al., 1991b; Murphy and Hill, 1991; Studer et al., 1996). Hypoplasias of ncc-derived elements in the Hox1 mutants were attributed largely to segmentation defects in the hindbrain that resulted in a dramatically reduced r4 territory and a misplaced otocyst that occluded neural crest migration (Chisaka et al., 1992; Lufkin et al., 1991; Mark et al., 1993). The analysis of combined Hoxal and Hoxb1 null mutants revealed extensive synergy between the two genes. Prominent among the defects observed was the loss of all second arch-derived elements, owing to neural crest specification defects (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998).

The mechanisms responsible for generating these ncc defects remained unknown and they could be due to effects of the double mutation on any of the components of the pharyngeal arches, the neuroepithelium or a combination thereof. To address this problem, we have taken advantage of a mutation inactivating a 3' retinoic acid response element (RARE) essential for early expression of Hoxb1 in neural ectoderm (Marshall et al., 1994; Studer et al., 1998). Both Hoxal+/+Hoxb1−/− and Hoxal−/+Hoxb1−/− double mutants lose the ncc-derived second arch elements despite their retaining an r4-like territory. However, Hoxal−/+Hoxb1−/− mutants present additional defects in pharyngeal arch and cranial nerve patterning, suggesting a direct synergistic role of Hoxal and Hoxb1 in other aspects of pharyngeal arch development beyond ncc patterning itself (Gavalas et al., 1998). In order to exclude indirect effects of the mutant second arch environment on ncc development, we focused our analysis on the Hoxal+/−/Hoxb1−/− double mutants. The combination of the Hoxal with the Hoxb1 3'RARE mutation resulted in an ectoderm-specific double mutation, leaving the other germ layers impaired only in Hoxal function. As the migration route and gene expression patterns of the r4 ncc are not qualitatively affected in the Hoxal mutants (Mark et al., 1993; Gavalas et al., 1998; this work), the mesodermal and pharyngeal arch environment remain permissive to ncc migration in this mutant background. Therefore, any additional ncc defects observed in the Hoxal+/−/Hoxb1−/− double mutants should originate at the neuroepithelium itself.

In this study, using molecular and lineage analysis we have demonstrated that while the presumptive r4 territory is reduced but not absent, it is unable to generate ncc in this double mutant background. Graffiti experiments in combination with mouse embryo culture also demonstrate that this is a cell-autonomous defect. These results suggest that Hox genes are not simply necessary for proper axial specification of the ncc, but that they also play a vital role in the generation of the ncc population itself.

### MATERIALS AND METHODS

#### Animals and embryo isolation for cultures

Embryos were obtained from 2-hour morning matings (8:00 am-10:00 am) of heterozygous Hoxal and Hoxb1 3'RARE−/− mutants. The genotype of the embryos was determined using the polymerase chain reaction (PCR). The PCR conditions and primers for the Hoxb1 3'RARE mutation were as described (Studer et al., 1998). For the Hoxal mutation, the conditions were the same and the primers used were 5'GCCATTGGCTGGTAGATGCACGGTG3' (common to both alleles), 5'GATTGGAAGCGGTCTTTGCATCAG3' (specific for the mutant allele) and 5'CATGGGATCGAGGGTTCCTACAG3' (specific for the wild-type allele), which give rise to a 570 bp product for the wild-type allele and a 700 bp product for the mutated allele. Embryo culture conditions were as described previously (Trainor and Krumlauf, 2000b).

#### Whole-mount in situ hybridisation and histology

The following mouse cDNA templates were used: Hoxa2, Hoxb2, twist, Sox10, Fgf3, Fgf8, Shh, Bmp7, Ngn2 (Atoh4 – Mouse Genome Informatics), Pax1 and Crabp1. Antisense digoxigenin-labelled riboprobes were synthesised from linearised templates by the incorporation of digoxigenin-labelled UTP (Boehringer) using T3 or T7 or SP6 polymerase. Processing of the embryos and hybridisation with 500 ng ml−1 of the probe were as described previously (Gavalas et al., 1998). The preparation of Haematoxylin and Eosin stained sections was carried out using standard histological techniques.

#### Whole-mount detection of mitosis and apoptosis

Embryos were fixed overnight (o/n) in 4% paraformaldehyde in phosphate-buffered saline (PBS). For whole-mount detection of mitosis, embryos were bleached in 3% H2O2 in PBS for 10 minutes and subsequently washed 4×30 minutes in PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (PBST) followed by a 30 minute wash in PBST containing 5% foetal calf serum (PBSTN). Then, embryos were incubated o/n at 4°C with a rabbit polyclonal
antibody raised against the mitosis-specific phosphorylated histone 3 (Upstate Biotechnology Cat no 06-570) diluted 1:500 in PBST. The next day, embryos were washed seven times for 15 minutes each time with PBST and then 30 minutes with PBSTN. Then, the incubation with a goat anti-rabbit horseradish peroxidase-coupled secondary antibody (DAKO) diluted 1:400 in PBSTN was for o/n at 4°C. The following day embryos were washed seven time for 15 minutes each time in PBST and then incubated in 0.3mg/ml diaminobenzidine (DAB) in PBST for 10 minutes. The embryos were transferred in 35 mm diameter petri dishes and the reaction was carried out by incubating them in 0.3 mg/ml DAB/0.03% H2O2 in PBS. The reaction was followed in a dissection microscope and once complete (5-10 minutes) embryos were washed thoroughly in PBS, fixed in 4% paraformaldehyde in PBS for 2 hours and cleared o/n in 50% glycerol in PBT. Whole-mount detection of apoptosis was carried out using the TUNEL method as described previously (Maden et al., 1997) in PBT. Whole-mount detection of apoptosis was carried out using with the TUNEL method as described previously (Maden et al., 1997) and the final peroxidase reaction was as described above.

**Ncc lineage tracing and transplantation experiments**

Both techniques were carried out as described earlier (Trainor and Krumlauf, 2000b).

**RESULTS**

A territory with new characteristics, rx, forms in place of r4 in the Hoxa1-/Hoxb13 RARE-/- mutants

In previous analysis of Hoxa1 and Hoxb1 double mutants we found that the territory, which would normally form r4, has been altered in a manner not observed in either single mutant, and fails to express the appropriate r4 molecular markers (Gavalas et al., 1998; Studer et al., 1998). Our analysis of the double mutants also showed that defects in the r4-derived neural crest cells led to involution of the second arch and loss of its mesenchymal derivatives (Gavalas et al., 1998). However, the nature of these neural crest defects was unknown and could be related to processes controlling either the generation or the patterning potential of the second arch ncc. To address this issue, we have used a Hoxb1 3 RARE-/- (Studer et al., 1998) mutant to specifically eliminate Hoxb1 function in ectoderm in a Hoxa1-/- genetic background (Lufkin et al., 1991).

The observed loss of second arch neural crest elements in the Hoxa1-/Hoxb1 3 RARE-/- mutants could be due to a loss of r4, the main territory of origin of second arch ncc (Lumsden et al., 1991; Sechrist et al., 1993). In the Hoxa1 mutants, r4 is dramatically reduced when compared with wild type (Mark et al., 1993), but it does not lose the capacity to generate ncc (Mark et al., 1993). We examined whether in the double mutants a distinct territory persisted by performing double in situ hybridisation at 8.5 days post coitus (dpc) with riboprobes for Kroxx20, which marks r3 and r5 (Schneider-Maunoury et al., 1993), and kreisler (kr; Mafb – Mouse Genome Informatics), which marks r5 and r6 (Cordes and Barsh, 1994). A total of seven double mutant embryos were compared with four similarly stained Hoxa1-/- embryos and no differences were found in the extent of the territory not expressing Kroxx20 and kr (Gavalas et al., 1998); compare Fig. 1b with 1c). The boundaries between expressing and non-expressing territories were ill-defined, owing to cell mixing but the presence of non-expressing cells was clear, particularly on the dorsal aspect of the hindbrain where ncc are generated.

We then examined whether this territory persisted at later stages by staining the hindbrains of double mutant and Hoxa1-/- embryos with Hoxa2 and Hoxb2 riboprobes at 9.5 dpc. We found that, whereas expression of Hoxb2 is continuous throughout the r3-r6 territories in the wild-type and Hoxa1-/- embryos (Fig. 1d,e), there is a clear field of non-expressing cells that corresponds to the presumptive r4 territory in the double mutants. However, this same area expresses Hoxa2 (Fig. 1j), thus defining a new territory of Hoxa2+/Hoxb2- cells, which we designate as rx. Similar to earlier stages, the boundaries of this territory were ill defined and patches of non-expressing cells could be seen in r3. Hoxb2- cells could also

![Fig. 1. A territory with new characteristics, rx, is formed in the place of presumptive r4 in the Hoxa1+/Hoxb1 3 RARE-/- embryos. Wild-type (a,d,g), Hoxa1+/+ (b,e,h) and Hoxa1+/+Hoxb1 3 RARE-/- (c,f,i) embryos were processed for whole-mount in situ hybridisation with kr/Krox20 (a-c), Hoxb2 (d-f) and Hoxa2 (g-i) antisense riboprobes. Double staining with kr/Krox20 shows that the r4 territory of wild-type embryos defined by non-expressing cells (vertical bar in a) was significantly reduced but not eliminated, to similar extents in both Hoxa1+/+ and Hoxa1+/+Hoxb1 3 RARE-/- (vertical bars in b,c, respectively). Hoxb2 expression is continuous throughout the r3-r6 region in both wild-type and Hoxa1+/+ embryos (d,e) but not in Hoxa1+/+Hoxb1 3 RARE-/- embryos (f). There are Hoxb2 non-expressing cells intermingled with r3 cells in both Hoxa1+/+ and Hoxa1+/+Hoxb1 3 RARE-/- embryos (arrowheads in e,f). Expression of Hoxa2 is continuous throughout the r2-r6 region in wild-type (g) as well as Hoxa1+/+ (h) and Hoxa1+/+Hoxb1 3 RARE-/- (i) embryos.]
be observed interspersed in the r3 of the Hoxa1<sup>−/−</sup> embryos (arrowheads in Fig. 1e,f). Hoxb2 remained upregulated in the dorsal edge of the rx territory. This does not necessarily represent r3 or r5 cells encroaching upon rx territory, as such upregulation is observed along the length of the caudal hindbrain in both Hoxa1<sup>−/−</sup> and double mutant embryos (Fig. 2e,f). Interestingly, this upregulation does not occur in the wild-type embryos (Fig. 1d) and it may represent an effect of the Hoxa1 mutation itself.

**Neural crest migratory defects and pharyngeal arch specification defects**

To examine whether this novel rx territory is able to generate ncc, we have performed molecular analysis using general neural crest markers, as well as markers specifically expressed in the neurogenic and mesenchymal ncc.

Hoxa2 and Hoxb2 are expressed in the migrating neural crest of the second arch and are the only Hox genes to be expressed in its mesenchyme (Hunt et al., 1991a; Hunt et al., 1991b). At 9.5 dpc, both genes are expressed in a continuous stream from r4 to the distal tip of the second arch (Fig. 2a,d). In the Hoxa1<sup>−/−</sup> mutants, expression of Hoxa2, as well as Hoxb2, is reduced in both the migrating neural crest and the mesenchyme of the arch (Fig. 2b,e). However, expression of both genes is completely abolished in the second arch of the double mutants (Fig. 2c,f). These results closely parallel the observed changes in the expression of AP-2 and Crabp1 (Gavalas et al., 1998). Interestingly, in the hindbrain of the double mutants expression of Hoxb2 but not Hoxa2 was lost in presumptive r<sub>4</sub> (Fig. 2c,f). This further illustrates the existence of a territory (rx) positioned between the Hoxb2-expressing r3 and r5/r6 territories (Fig. 2c).

Twist encodes a basic helix-loop-helix transcription factor expressed in high levels in the mesenchyme of the pharyngeal arches and regulates the cellular phenotype and behaviour of head mesenchyme cells (Chen and Behringer, 1995). Twist expression is confined mainly at the periphery of the arch core (data not shown), suggesting that it marks mesenchymal ncc. The robust second arch expression seen in wild-type embryos (Fig. 2g) was reduced in Hoxa1<sup>−/−</sup> mutants (Fig. 2h) and it was completely abolished in the second arch of the double mutants (Fig. 2i).

Sox10 is an HMG-box transcription factor expressed in the neural crest cells that contribute to the peripheral nervous system (Kuhlbrodt et al., 1998). At 9.5 dpc, Sox10 is expressed in all neural crest-derived ganglia of the cranial nerves (Fig. 2j). Consistent with a general reduction of the second arch
neural crest cell population in the *Hoxa1* mutants (Mark et al., 1993), expression of *Sox10* was reduced in the facioacoustic ganglion of the second arch (Fig. 2k, arrow). Furthermore, *Sox10* expression was totally abolished in the territories of the neural crest derived superior ganglia of nerves IX and X. This suggests that the loss of the proximal part of these nerves in the *Hoxa1* mutants arises owing to a defect in this population. In the double mutants, *Sox10* expression in the facioacoustic ganglion of the second arch was completely abolished (Fig. 2l).

The specific loss of expression of all neural crest markers examined in pa2 suggested that neural crest cells emanating from the normal presumptive r4 territory (rx) of the double mutants either failed to emigrate and populate the second arch, or were rapidly eliminated by cell death. Consistent with this idea, histological sections through the arch region of wild-type, *Hoxa1*+/- and *Hoxa1*+/-/Hoxb1 3’ RARE+/- embryos show that despite the reduction (*Hoxa1*+/-) or loss of the second arch ncc (double mutants), mesodermal cells could still migrate and populate pa2 (Fig. 2m-o).

**Neural crest cells are not required for early survival and proliferation of pa2**

In view of the apparent absence or elimination of migratory ncc moving into the second arch, we examined whether this could be manifested by ectopic cell death and/or proliferative defects during early arch development. We investigated cell proliferation by whole-mount immunohistochemistry using a polyclonal antibody against the phosphorylated form of histone 3 (H3), which is present only in mitotic cells (Hendzel et al.,

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**Fig. 3.** Mitotic and cell death patterns in the second pharyngeal arch are not altered despite the loss of ncc. Wild-type (a,d), *Hoxa1*+/- (b,e) and *Hoxa1*+/-/Hoxb1 3’ RARE+/- (c,f) embryos were processed for whole-mount immunohistochemical detection of mitosis (a-c) and cell death (d-f). (a-c) Reduced but considerable mitotic activity was still detected in the second pharyngeal arch (pa2) of both *Hoxa1*+/- and *Hoxa1*+/-/Hoxb1 3’ RARE+/- embryos. (d-f) There was no increase of cell death in the second pharyngeal arch of either *Hoxa1*+/- or *Hoxa1*+/-/Hoxa1+/-/Hoxb1 3’ RARE+/- embryos.

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**Fig. 4.** Signalling molecules are correctly expressed in the second pharyngeal arch despite the loss of ncc. Wild-type (a,d,g,j), *Hoxa1*+/- (b,e,h,k) and *Hoxa1*+/-/Hoxb1 3’ RARE+/- (c,f,i,l) embryos were processed for whole-mount in situ hybridisation with Fgf3 (a-c), Fgf8 (d-f), Shh (g-i) and Bmp7 (j-l) antisense riboprobes. (a-c) Expression of *Fgf3* in the first and second pharyngeal pouches (pp1 and pp2, respectively) was not changed in either *Hoxa1*+/- or *Hoxa1*+/-/Hoxb1 3’ RARE+/- embryos (arrows). (d-f) The weak expression of *Fgf8* on the second pharyngeal arch (pa2) was not abolished in either *Hoxa1*+/- or *Hoxa1*+/-/Hoxb1 3’ RARE+/- embryos (arrows). (g-i) Expression of *Shh* in the second pharyngeal arch (pa2) was not abolished in either *Hoxa1*+/- or *Hoxa1*+/-/Hoxb1 3’ RARE+/- embryos (arrows in). (j-l) Similarly, expression of *Bmp7* in the second pharyngeal arch (pa2) was not abolished in either *Hoxa1*+/- or *Hoxa1*+/-/Hoxb1 3’ RARE+/- embryos. ov, otic vesicle.
Interestingly, we found that proliferation in the second arch of both Hoxa1 and Hoxa1/Hoxb1 3’RARE embryos was reduced but not eliminated (Fig. 3a-c). This reduction in number of mitotic cells in the mutants was consistent with the smaller mesenchymal population in pa2. This demonstrates that even in the absence of ncc there are still mitotically active cells in pa2.

Next, we investigated whether excessive cell death occurred in association with the absence of detectable neural crest cells. TUNEL whole-mount staining revealed that patterns of cell death in both single and double mutant backgrounds were remarkably similar to those of pa2 in wild-type embryo (Fig. 3d-f). The only detectable difference in the patterns of cell death in the mutants was seen around the otic vesicle, consistent with the underdevelopment of the otic vesicle in these mutants (Gavalas et al., 1998; Mark et al., 1993). Therefore, absence of gene expression in markers of migratory second arch ncc is not associated with a major change in mitogenic or survival signals within pa2.

The above results suggest that in the absence of neural crest components other aspects of early arch patterning may not be affected. We examined a number of signalling molecules that...
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are expressed in the surface ectoderm and pharyngeal endoderm encapsulating the arch mesenchyme (Francis-West et al., 1998; Rijli et al., 1998), as reduction or changes in their expression could contribute to the developmental defects found in these mutants. At 9.25 dpc, fibroblast growth factor 3 (\textit{Fgf3}) showed restricted strong expression in part of the second pharyngeal pouch and weak expression in the surface ectoderm of the second arch (Fig. 4a; Mahmood et al., 1995; Wilkinson et al., 1988). This expression pattern did not change appreciably in either \textit{Hoxa1} or \textit{Hoxa1/Hoxb1 3'RARE} mutants (Fig. 4b,c). At the same stage \textit{Fgf8} was also expressed in the surface ectoderm of the second arch (Fig. 4d; Heikinheimo et al., 1994) and this expression was not affected in either of the two mutants examined (Fig. 4e,f). Targeted inactivation of \textit{Shh} in the mouse resulted in cyclopia and loss of pharyngeal arch structures (Chiang et al., 1996), whereas sonic hedgehog (\textit{Shh}) inhibition in the head mesenchyme lead to loss of pharyngeal arch structures (Ahlgren and Bronner-Fraser, 1999). Therefore, we examined \textit{Shh} expression in the mutants but no changes were detected in either \textit{Hoxa1} or \textit{Hoxa1/Hoxb1 3'RARE} mutants (Fig. 4g-i).

Next, we examined the expression of pharyngeal pouch and placode specific markers. \textit{Pax1} belongs to the \textit{Pax1/Pax9} subfamily of paired homeodomain transcription factors (Muller et al., 1996; Peters et al., 1998) and at 9.25 dpc specifically labelled the endoderm of first and second pharyngeal pouches (Fig. 5a). \textit{Pax1} expression in the mutants was indistinguishable from that of the wild-type embryos (Fig. 5a-c). The epibranchial placodes are formed by local
thickenings of the surface ectoderm and they give rise to all the cells of the inferior ganglia of the cranial nerves (Le Douarin et al., 1986). Ngn2 is a bHLH transcription factor necessary for determination of the epibranchial placodes (Fode et al., 1998). We found that all ganglia derived from epibranchial placodes, including the second arch geniculate ganglion, expressed Ngn2 and were formed correctly in both Hoxal and Hoxal/Hoxbl3RARE mutants (Fig. 5d-f). Taken together, these results suggest that important aspects of the signalling and patterning in the second arch have not been altered in the Hoxal/Hoxbl3RARE mutants.

**Lineage analysis of neural crest cell migration in Hox mutant embryos**

As early arch patterning is not affected in the double mutants and we found no evidence for increased cell death, we re-examined whether any neural crest cells that were not detected by molecular markers were migrating from the r4-like territory. To address this problem, we took advantage of our previous fate map of cranial regions (Trainor and Tam, 1995) and performed lineage analysis of neural crest cells migrating from the future r4 or rx territory of cultured wild-type and mutant mouse embryos. At 8.25 dpc (five somite embryos) the pre-rhombomeric territories in wild-type embryos can be distinguished based on the position of the preotic (pos) and otic sulci (os) (Fig. 6a). DiI injections into the dorsal region of future r4 in wild-type embryos, followed by embryo culture for 30-32 hours, revealed extensive labelling of cells emigrating from r4 into pa2 (Fig. 6d).

In the Hoxal and Hoxal/Hoxbl1RARE mutants, the presumptive r4 (rx) territory is reduced in size, the pos is significantly smoother and the os has all but disappeared. Therefore, to investigate cell migration in the Hox mutants, DiI injections were made in a broad enough region to cover the presumptive r4 (rx) territory and the edges of neighbouring territories r3 and r5/6 (area labelled is denoted by brackets in Fig. 6b,c). This approach ensures that despite the loss of morphological landmarks, the r4 (rx) territories and the neighbouring territories r3 and r5 populations, which (in the wild-type) contribute to the ncc populating pa2 (Sechrist et al., 1993), will be labelled. In the Hoxal mutants, neural crest cells migrated into pa2, but they were greatly reduced in number compared with wild type (compare Fig. 6d,g with Fig. 6e,h). In contrast, lineage analysis in the double mutants demonstrated the complete absence of migratory cells derived from rx (Fig. 6f,i). The reduction in migratory cells in the Hoxal mutants and their absence in the double mutants directly correlated with the changes in the expression patterns of neural crest molecular markers. Together, these results strongly suggest that the rx territory in the Hoxal/Hoxbl3RARE mutant background has lost the ability to generate migratory neural crest cells.

**Neural crest cells fail to delaminate from the presumptive r4 territory**

The loss of migrating ncc into the second arch could result either from a failure to delaminate or from trapping of the cells in the adjacent mesenchyme. To distinguish between the two possibilities, we examined histological sections of hindbrain preparations. In wild-type embryos at 9.25 dpc, delaminating ncc can be seen both histologically (Fig. 7a) and as CrabpI-expressing cells in two distinct streams adjacent to r4 and r6 (Fig. 7b). In the Hoxal mutants, we detected r4- and r6-derived streams of emigrating ncc (Fig. 7c,d). In the caudal hindbrain the streams are continuous, owing to the drastic reduction of the r5 territory, and delaminating cells can be seen both anterior and posterior to the otic vesicle (Fig. 7c,d; Mark et al., 1993). In contrast, in the Hoxal+/−/Hoxbl13RARE−/− double mutants, there are no delaminating cells from the presumptive r4 (rx) territory (Fig. 7e,f). This situation is very similar to that observed in the Hoxal+/−/Hoxbl13−/− full double mutants (Fig. 7g,h), suggesting that this is a defect arising in the neuroepithelium itself.

The neural crest migratory defects are cell autonomous

The observation that no ncc are delaminating from the hindbrain at the level of the presumptive r4 could arise through either an intrinsic defect in the ability of the neuroepithelium to generate migrating ncc or a non-permissive mesenchymal environment. The latter possibility appears less likely as the Hoxbl3RARE mutation specifically eliminates function in the neural ectoderm and these defects are not seen in the Hoxal mutation alone. To directly investigate whether these defects are intrinsic to the neuroepithelial cells, we performed cell transposition experiments in wild-type and double mutant embryos. Although it would have been desirable to transpose rx cells into wild-type embryos to test their potential, the lack of segmentation and morphological landmarks, and the small size of the territory rx preclude the reliable isolation of rx cells (and, thus, this experiment). Therefore, DiI labelled r4 cells from wild-type embryos were orthotopically grafted into either dorsal r4 of wild-type embryos or the rx region of double mutant embryos at 8.25 dpc (Fig. 8a,b). The migration patterns of these labelled wild-type r4 cells following transplantation into rx or r4 were examined after 24 hours in culture. In both wild-type and Hoxal/Hoxbl13RARE embryos we observed that the grafted cells generated ncc that was able to migrate in the second arch (Fig. 8c,d). This shows that the arch environment in the double mutant embryos is permissive for neural crest cell migration, strongly suggesting that the failure of the neuroepithelium to generate migrating neural crest is caused by cell autonomous defects.

**DISCUSSION**

The combined loss of function of Hoxal and Hoxbl results in loss of second pharyngeal arch derivatives and neural crest specification defects (Gavalas et al., 1998). The loss of these structures could have arisen not only from failure of the ncc to migrate or integrate properly in the arch environment, but also from patterning defects of the arch mesoderm and epithelium. In this study, we addressed this issue genetically by introducing the Hoxbl3RARE mutation in the Hoxal mutant background, which results in loss of function of both Hoxal and Hoxbl only in the neural ectoderm. As Hoxal+/−/Hoxbl1−/− mutants have additional arch and cranial nerve defects, compared with Hoxal+/−/Hoxbl13RARE−/− mutants (Gavalas et al., 1998), this genetic approach allows us to eliminate defects in the arch environment that indirectly affect ncc patterning. Using a combination of molecular markers, lineage analysis and cell grafting, we demonstrated that the neuroepithelium of these...
mutants had cell-autonomous defects that prevent the presumptive r4 (rx) territory from generating neural crest cells. This does not prevent correct early arch patterning from taking place, and suggests that these processes can be uncoupled in vertebrates. Furthermore, our observations imply that Hox genes are not only involved in regulating the specification of axial (AP) identities in the hindbrain and neural crest tissues, but also in controlling the generation of cranial neural crest cells. These results raise several interesting questions with respect to the way in which these processes are related.

Neural crest formation versus AP identity
Simultaneous loss of Hoxa1 and Hoxb1 function does not eliminate the presumptive r4 (rx) territory; however, its precise AP identity is unknown. This cell population does express Hoxa2, but not Krox20, kr or Hoxb2 (Gavalas et al., 1998; Studer et al., 1998). This suggests that rx may have a r2-like character, which is in line with the previous findings that r4 adopts an r2-like identity in single Hoxb1 mutants (Goddard et al., 1996; Studer et al., 1996). However, as demonstrated in this paper, unlike r2, rx in the double mutants lacks the ability to generate migratory neural crest cells. One explanation would be that cells in this territory have been locked in an immature neuroepithelial state; however, we have previously found that motoneurones can be generated from these cells and send projections into the periphery in a even-like segmental pattern (Gavalas et al., 1998). Therefore, in this double mutant context where Hoxa1 and Hoxb1 have been jointly eliminated from only the neuroepithelium, it appears that the formation or generation of cranial neural crest has been uncoupled from segmental identity. Neural crest is still generated at more posterior levels, most probably due to functional compensation from other Hox genes.

The functional loss of Hoxa1 and Hoxb1 may have interfered with neural crest induction by affecting the competence of ectodermal cells to respond to neural crest-inducing signals such as Bmps and Wnts. Bmp signalling is indispensable for ncc generation, as the combination of Bmp5 and Bmp7 mutations (Solloway and Robertson, 1999), and interference with Bmp signalling resulted in severe depletion of ncc that adopt an r2-like identity in single Hoxb1 mutants (Goddard et al., 1996; Studer et al., 1996). However, as demonstrated in this paper, unlike r2, rx in the double mutants lacks the ability to generate migratory neural crest cells. One explanation would be that cells in this territory have been locked in an immature neuroepithelial state; however, we have previously found that motoneurones can be generated from these cells and send projections into the periphery in a even-like segmental pattern (Gavalas et al., 1998). Therefore, in this double mutant context where Hoxa1 and Hoxb1 have been jointly eliminated from only the neuroepithelium, it appears that the formation or generation of cranial neural crest has been uncoupled from segmental identity. Neural crest is still generated at more posterior levels, most probably due to functional compensation from other Hox genes.

Neural crest cells and the environment
Surprisingly, both the molecular markers and Dil lineage analysis indicates that in the mutants there is no ncc in-filling from adjacent rhombomeres. This is in contrast to the findings in the chick that physical ablation of this population can be compensated for by in-filling of surrounding neural crest populations (Coulby et al., 1996; Hunt et al., 1995; Saldivar et al., 1997). This difference may be due to intrinsic species-specific differences or the in-filling may be a consequence of the experimental procedure itself. Alternatively, head mesenchyme adjacent to the hindbrain can form exclusion zones that help guide ncc in forming distinct migratory streams (Farlie et al., 1999; Golding et al., 2000). As a result of the combined mutations, it could be argued that this exclusion zone has expanded, precluding rx ncc migration. However, the finding that wild-type cells when transplanted into the presumptive r4 territory of the double mutant embryos can generate neural crest cells that migrate into pa2, excludes this possibility.

Fate-mapping studies in the chick have suggested that the odd-numbered rhombomeres contribute ncc to the main streams emanating from the even-numbered rhombomeres (Sechrist et al., 1993). In the double mutants examined here, based on both the molecular and cell-tracing analysis, r3 is unable to contribute ncc that will migrate in the second arch. However, there are patterning defects in r3 of both Hoxa1–/– and Hoxal–/–/Hoxb1 3RARE–/– mutant embryos (Gavalas et al., 1998; Helmbucher et al., 1998), which most probably result from intermingling of misspecified r4 cells with the r3 territory. This may alter the normal ability of r3 to contribute ncc to the second arch.

In the chick, after extensive deletion of the neural tube, arch patterning appears to be unaffected (Veitch et al., 1999). However, the lack of Dil labelling in these experiments left open the possibility of limited ncc in-filling from anterior and posterior regions of the neural tube. Our demonstration of a complete loss of the second arch neural crest cell population has allowed us to assess the direct role of these cells on arch patterning in the mouse. We found that, in its absence, there is no excessive cell death or arrest of proliferation in the arch, implying that this population is not the source of indispensable survival or mitogenic signals. Expression of signalling molecules in the arch epithelium was not affected. The presence of mesoderm/mesenchyme in pa2 in the absence of neural crest cell migration from r3 also implies that ncc are not essential for their migration into the arches. Furthermore pouch formation and ectodermal placode development were normal, highlighting the fact that ncc are not essential for these events. As these processes depend upon proper endoderm patterning (Begbie et al., 1999), it appears that endoderm is relatively normal in the double mutants examined here.

This does not imply that Hoxa1 and Hoxb1 do not have normal roles in endoderm patterning, as they are expressed in this tissue (Hunt et al., 1991b; Murphy and Hill, 1991). However, the Hoxb1 3RARE mutation in the neural enhancer flanking this gene that we used in this study, specifically eliminated expression from only the ectoderm and not the endoderm (Marshall et al., 1994; Studer et al., 1998), which is regulated by a separate control element (Huang et al., 1998). Importantly, endoderm patterning and Hoxa1 and Hoxb1 expression are altered when mouse embryos are treated with a retinoid antagonist, suggesting that these genes do have a role in early endoderm patterning (Wendling et al., 2000).

In conclusion, it is becoming increasingly evident that arch development involves the formation of main components, which are initially patterned in a relatively independent manner, and that subsequent growth and differentiation is
tightly linked to the integration of tissue interactions between these components. This is consistent with the well-established instructive roles of neural crest cells in patterning muscle tissue (König and Lumsden, 1996; Noden, 1988). How the formation and early patterning of the pharyngeal arches are coupled to the regulation of ncc formation and segmental identity will be an important issue to be addressed.

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