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Segmentation and specification in the branchial region of the head: the role of the *Hox* selector genes

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ABSTRACT ***Hox* genes are segmentally expressed in the developing vertebrate hindbrain, neural crest cells and pharyngeal arches suggesting an important role in patterning these structures. Here we discuss the cellular and molecular mechanisms controlling segmentation and specification in the branchial region of the head. In addition, based on the recent phenotypical and molecular analysis of loss-of-function mutants in the mouse, we speculate that *Hox* genes may act like *Drosophila* selector genes in this system.**

KEY WORDS: *homeotic genes, compartments, hindbrain, pattern formation, Hoxa1, Hoxa2*

Introduction

The homeotic genes of the *Hox* complexes are transcriptional regulators encoding a 60-amino acid DNA-binding motif, the homeodomain, which are homologous to the homeotic genes of the *Drosophila Antennapedia* (ANT-C) and *Bithorax* (BX-C) complexes (reviewed in McGinnis and Krumlauf, 1992). Genetic analysis of *Hox* gene function both in invertebrates and vertebrates has shown that these genes are key developmental regulators which control morphological differences along the anteroposterior (A/P) body axis (reviewed in McGinnis and Krumlauf, 1992; Krumlauf, 1994; Favier and Dollé, 1997). In the mouse, *Hox* genes of paralog groups 1 to 4 (Fig. 1) have segmental expression domains in rhombomeres, rhombencephalic neural crest cells (NCC) and pharyngeal arch mesenchyme (reviewed in Krumlauf, 1993; Mark *et al.,* 1995; Lumsden and Krumlauf, 1996) predicting an important role in patterning these structures. In this review, we discuss the cellular and molecular mechanisms controlling segmentation and specification in the branchial region of the head of vertebrates, with a focus on hindbrain development, and we draw a parallel with the establishment of compartments in *Drosophila* wing imaginal disc. In addition, based on the analysis of loss-of-function mutants in the mouse, we speculate that murine *Hox* genes may act similarly to *Drosophila* selector genes.

Learning from *Hox* gene function in *Drosophila*: 'selectors' of segmental identities

How a single protein present in a cell can change it from belonging to one pattern (e.g., the wing) to being part of another (a haltere)? The discovery of compartments in the wing and the study of the phenotype of certain mutations in the BX-C complex led García-Bellido (1975,1977) to propose the concept of homeotic 'selector' genes as a rationale to explain *Hox* gene function. The fly wing originates from an initial group of about 50 cells, the imaginal disc, which undergo intensive proliferation throughout the larval period and pupation, after which visible cell differentiation begins. During differentiation, about 50,000 cells give rise to the cuticular processes typical of the adult wing pattern. One important feature, which was discovered by clonal analysis (García-Bellido *et al.,* 1973), is that clones generated after a given time in development do not cross certain lines (boundaries) of the cuticular landscape, i.e., the wing disc become subdivided in compartments as proliferation proceeds (García-Bellido *et al.,* 1973; see also for review Lawrence and Struhl, 1996). Compartments are sequentially generated by a binary partition of a previously homogeneous cell population, unlike segmentation of the embryo in which segments are simultaneously generated on the head, thorax and abdomen. The basic property of cells segregating in different compartments is that they never mix with the cells of adjacent compartments, suggesting cell adhesion differences acting throughout development (e.g., García-Bellido and Lewis, 1976). This conclusion is also supported by the results of cell-aggregation experiments of dissociated cells from different disc regions. Therefore, cells in each compartment express properties of specific cell differentia-

Abbreviations used in this paper: ANT-C, Antennapedia Comples; BX-C Bithorax Complax; A/P, anteroposterior; NCC, neural crest cells; Ubx, ultrabithorax; bx, bithorax; pbx, postbithorax; CNS, contral nervous system; CAMs, cell adhesion molecules; cadó, cadherin 6; RTKs, receptor tyrosine kynases; CVA, contralateral vestibuloacoustic; EGL, esternal germinal layer; PDGF-A, platelet-derived growth factor A; ET-1, endothelin-1.

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tion long before cuticular pattern is visible. Each compartment will follow distinct developmental pathways giving rise to different parts of the wing. In this respect, there is a striking analogy between the compartimentalization and subsequent differentiation of the imaginal disc of *Drosophila* and the development of the hindbrain of vertebrates (see below).

Certain mutations in the BX-C *Hox* complex selectively affect disc compartments. For instance, two regulatory mutations in the *ultrabithorax (Ubx)* gene, *bithorax (bx)* and *postbithorax (pbx)*, affect only anterior and posterior compartments respectively, i.e., transform anterior and posterior halves of the haltere in the corresponding parts of the wing (reviewed in García-Bellido, 1975). This suggests the existence of a common underlying 'ground patterning program' between cells of corresponding A/P positions of the wing and the haltere, but the interpretation depends on the presence of the *Ubx* gene which 'selects' differentiation as haltere rather than wing (e.g., by repressing an alternative developmental pathway leading to the wing). Thus, homeotic selector genes are those which control developmental pathways (García-Bellido, 1975). In concomitance with the topographical allocation of compartment founder cells, different combinations of selector genes, among which the *Hox* genes, are activated conferring a specific genetic 'address' or 'code' to each set of cells which is maintained through subsequent DNA replication and cell divisions. Several subsequent binary decisions may be made by a typical group of cells each involving the activation of a 'selector' gene in a subset of the cells and its inactivation in the remainder. Selector genes promote the activation of a large battery of downstream 'realizator' genes which affect the cellular processes relevant to morphogenesis such as, for instance, proliferation rate, mitotic orientation, cell adhesion properties, cell differentiation, etc. Thus, while mutations in 'selector' genes should change the overall pattern of a developmental system (homeosis) without affecting normal cytodifferentiation mechanisms, mutations in 'realizator' genes should affect general properties of cells relevant to morphogenesis. The discovery that *Hox* proteins act as transcription factors and the identification of a few of their direct downstream targets in *Drosophila* (reviewed in Graba *et al.,* 1997) have provided strong support to this idea. Selection of alternative developmental pathways by *Hox* genes may be achieved through a functional hierarchy (Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al.,* 1990; Mann and Hogness, 1990) or competitive interactions (Heuer and Kaufman, 1992; Lamka *et al.,* 1992; Castelli-Gair *et al.,* 1994) among homeoproteins in the binding affinities to a common set of target genes (see also Rijli and Chambon, 1997).

The concept of 'selector' genes provides a conceptual framework to study *Hox* gene function in a number of other different animal systems, including vertebrates, and has been instrumental in the study of the functional role of the murine *Hox* genes in setting up and patterning the hindbrain compartments, the rhombomeres, and the segmented neural crest.

Hindbrain segmentation in vertebrates: of compartments, cell affinities, and cell recognition molecules

During the development of the central nervous system (CNS), a large variety of neurons are generated at appropriate times and locations with respect to the principal axis of the system. An early ordered pattern of cell specification is crucial, given the extraordinary complexity of neuronal functional connections in the mature brain. The possibility that at least part of the CNS is patterned from a reiterated set of repeated units has received much attention in the last few years with the discovery that hindbrain early development proceeds through a transient segmentation process which has been highly conserved in vertebrate evolution (Metcalfe *et al.,* 1986; Hanneman *et al.,* 1988; Lumsden and Keynes, 1989; Fraser *et al.,* 1990; Lumsden, 1990).

The original observation that the neural tube of the chick embryo is progressively subdivided in bulges (rhombomeres) at the level of the rhombencephalon (Vaage, 1969), has been subsequently corroborated at the cellular level by the studies of Lumsden and colleagues (Fraser *et al.,* 1990; Lumsden ,1990). The rhombomeres reflect an intrinsic segmentation of the neural tube which correlates with the subsequent differentiation of neurons in reiterated patterns (e.g., Lumsden and Keynes, 1989; Carpenter *et al.,* 1993; Clarke and Lumsden, 1993). In addition, hindbrain partitioning may underlie segmental specification of NCC contributing to cranial sensory ganglia and branchial arches (Noden, 1983,1988; Lumsden *et al.,* 1991; Serbedzija *et al.,* 1992; Sechrist *et al.,* 1993; Köntges and Lumsden, 1996) (see below).

Several observations, at both the cellular and molecular levels, suggest that rhombomeres behave as compartments of cell-lineage restriction which may share features with the insect compartments. The hindbrain segments are not generated simultaneously from the neural plate but sequentially, by binary partitions of previously homogeneous cell populations, and in an invariant order which does not follow an obvious rostro-caudal progression (Lumsden, 1990). Segmentation is marked by the appearance of a narrow line of specialized cells which form boundaries at the interface of two adjacent rhombomeres (Lumsden and Keynes, 1989; Heyman *et al.,* 1993,1995), similarly to the *Drosophila* wing imaginal disc where, for instance, dorsal and ventral compartments are separated by a band of non-dividing cells (O'Brochta and Bryant, 1985). Boundaries may act as mechanical barriers to cell movements across rhombomere interfaces (Fraser *et al.,* 1990), even though their role in restricting cell mixing has been recently questioned, as cells maintain their rostrocaudal restriction even in the absence of boundaries (Wingate and Lumsden, 1996; Nittenberg *et al.,* 1997). In addition, a few cells do cross segment boundaries at the time of segmentation (Birgbauer and Fraser, 1994). It appears more likely that boundary formation reflects a secondary feature of a segmental organization intrinsic to the different compartments, in which restriction in precursor cell mixing along the A/P axis appears right before, or at the time of, rhombomere formation (Wingate and Lumsden, 1996).

If boundaries do not provide a mechanical barrier that prevent cells from moving across them, what could be the cellular basis of lineage restriction in the developing hindbrain compartments? Grafting experiments in the chick suggest that a potential mechanism of segmental cell lineage restriction may involve the segregation of block of cells with alternating adhesion properties (Guthrie and Lumsden, 1991; Guthrie *et al.,* 1993). In fact, cells from odd-numbered (r3 and r5) rhombomeres mix more easily with each other than they do with cells from even-numbered (r2, r4, r6) rhombomeres and morphological boundaries are formed only at the interface between an odd-even rhombomere pair. Interestingly, there appears to be a hierarchy of differential adhesion (Guthrie *et al.,* 1993) which may reflect the fact that rhombomeres

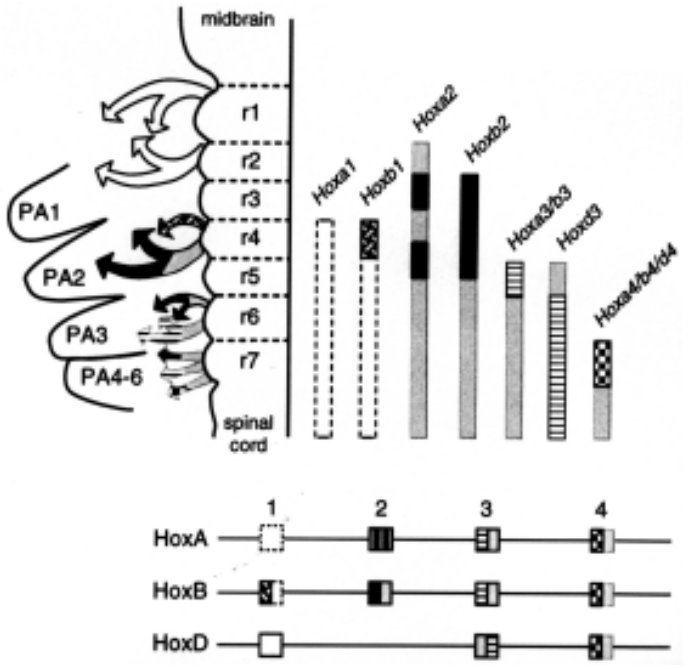

Fig. 1. *Hox* gene expression domains in the mouse hindbrain and rhombencephalic neural crest cells at E9.5. *The rostrocaudal expression patterns of paralogous genes in rhombomeres (r1 to r7) are indicated as bars containing various codes of grey, adjacent to the hindbrain diagram. The Hox paralogs share similar anterior expression boundaries (with the exception of Hoxa2 and Hoxb2), although their expression levels may not be similar and homogeneous along the rostrocaudal axis (hatching or dark shading within each bar correspond to high expression levels, grey shading to lower levels). The expression domains of Hoxa1 and Hoxb1 (dashed bars) are seen at earlier stages, prior to the formation of rhombomere boundaries, and only Hoxb1 (hatching) is maintained at E9.5 in r4. In contrast, Hoxd1 is not expressed in rhombomeres and NCC. Hox gene-expressing neural crest cells (NCC) emigrating to the developing pharyngeal arches (PA1 to PA4-6) and cranial sensory ganglia from distinct rhombomeric levels are represented with filled arrows. Note that NCC emigrating from r1-r2 do not express Hox genes (empty arrows). For simplicity, the small populations of NCC emigrating from r3 and r5 are not indicated. The relationship of the paralogous genes in the Hox complexes is shown at the bottom of the diagram.*

are not generated simultaneously (Lumsden, 1990). In quail/chick grafts, for instance, cells from r3 disperse better in r3/r3 grafts than they do in r3/r5 grafts than they do in r3/r2 graft (Guthrie *et al.,* 1993).

The above experiments suggest that different rhombomeres may express different repertoires of cell surface molecules. Different types of recognition molecules have been shown to be expressed in a restricted manner in the developing hindbrain. Kuratani (1991) described the alternate staining in rhombomeres of the HNK-1 antibody which recognizes a sulfated glucuronic acid-containing carbohydrate epitope shared by several adhesion molecules (including NCAM and L1). Other cell surface antigens, such as peanut agglutinin, are also expressed in a segmental manner in the hindbrain (Layer and Alber, 1990). Two members of the cadherin subfamily of cell adhesion molecules (CAMs), R-cadherin and cadherin 6 *(cad6)* (Ganzler and Redies, 1995; Matsunami and Takeichi, 1995; Redies, 1995; Inoue *et al.,* 1997), also display restricted expression patterns in specific rhombomeres and R-cadherin may be involved in differential segregation of cells (Matsunami and Takeichi, 1995). Another cadherin, F-cadherin, is expressed at boundaries in the *Xenopus* neural tube (Espeseth *et al.,* 1995). The *Eph*-related receptor tyrosine kinases (RTKs) and their membrane-bound ligands are another subclass of cell recognition molecules with segmental expression patterns in the developing brain (reviewed in Wilkinson, 1995; Lumsden and Krumlauf, 1996). Since their ligands are anchored in the plasma membrane, *Eph*-receptors may mediate cell contact dependent-signaling implicated in cell migration, axon pathfinding, and patterning mechanisms (Drescher *et al.,* 1995; Pandey *et al.,* 1995; Winslow *et al.,* 1995; Xu *et al.,* 1995,1996). In the hindbrain, receptors and their ligands are expressed in complementary domains suggesting that they may restrict intermingling of cells of different rhombomeres. Indeed, interference with the normal function of Sek1 by expression of a dominant negative form resulted in cells with r3/r5 identity crossing irregularly into even-numbered rhombomeres (Xu *et al.,* 1995). In addition, *Eph*-receptors and their ligands have been recently involved in restricting migration of specific population of trunk and cranial NCC suggesting a general role in regulating cell movement by a repulsion mechanism (Krull *et al.,* 1997; Smith *et al.,* 1997; Wang and Anderson, 1997).

Hindbrain segmentation and specification of the segment phenotype: the same set of selector genes at work?

Relatively little is still known about the genetic hierarchy which controls hindbrain development. Clues to the hierarchy of the genetic control of hindbrain segmentation and specification of the segment identity come from the study of the expression patterns and function of the homologues of the fly *Hox* genes.

Paralogous genes in the 3' parts of the vertebrate *Hox* clusters (*HoxA* to *D*) are sequentially and segmentally expressed in the developing hindbrain, with sharp anterior expression boundaries coinciding with rhombomeric borders (Fig. 1) (e.g., Hunt *et al.,* 1991; Murphy and Hill, 1991; Prince and Lumsden, 1994; see also for review Krumlauf, 1993; Keynes and Krumlauf, 1994; Wilkinson, 1995). It is important to note that *Hox* expression domains are established at early neural plate stages (E7.5-8.0 in the mouse), i.e., before the formation of definitive rhombomeres (occurring about one day later), and they are in general maintained up to late stages of hindbrain development well after morphological segmentation has disappeared (with the exception of the *Hoxa1* gene; see below) (Krumlauf, 1993 and refs. therein; Wingate and Lumsden, 1996). A direct correlation exists between *Hox* gene expression and commitment to a rhombomere-specific fate. Grafts of chick neural plate-stage hindbrain neuroepithelium transplanted in more posterior locations express *Hox* genes and display morphological features appropriate for the new location (Grapin-Botton *et al.,* 1995). In contrast, grafts transplanted just before or at the time of rhombomere formation maintain both specific *Hox* expression and their segmental identities (Guthrie *et al.,* 1992; Kuratani and Eichele, 1993; Simon *et al.,* 1995), even though their commitment may still be reversible under certain conditions (Itasaki *et al.,* 1996; Grapin-Botton *et al.,* 1997). Therefore, it appears that, after a period of plasticity, the definitive commitment to a specific segmental fate is accompanied by the establishment of a unique genetic 'address' of *Hox* gene expression in a block of precursors cells

which is maintained through subsequent cell divisions and directs the differentiation program of that segment.

An interesting corollary of these experiments is that definitive commitment to a specific fate coincides with the time of cell-lineage restriction within a specific rhombomere (see above), suggesting that segment formation and specification are temporally linked and may be under the genetic control of the same set of *Hox* selector genes. Should this hypothesis be correct, then one would expect some classes of recognition molecules restricting rostro-caudal cell movement and controlling cell contact-dependent signaling, such as CAMs and RTKs (see above), to be under direct or indirect control of the H*ox* genes. Thus, in the absence of functional compensation, mutations in a *Hox* gene should result in both segmentation and specification problems in the mutant hindbrain, which may be considered as two aspects of the same process. Early precursor cells which are not correctly specified according to their A/P axial level may not be restricted at their appropriate position and/or form a coherent block of cells, therefore not forming a normal segment, but becoming intermingled with cells of similar genetic constitutions at adjacent rostrocaudal positions, eventually acquiring the same fate as their neighbors possibly through a segmental community effect (Gurdon, 1988; Wilkinson, 1995).

The generation of *Hox* mutants in the mouse has provided an invaluable model system for understanding the molecular basis of hindbrain segmentation and patterning and to test some of the above hypotheses. To date, several *Hox* genes with expression domains in the developing hindbrain (paralog groups 1 to 4; Fig. 1) have been knocked out via homologous recombination in ES cells (Capecchi, 1989). Hindbrain alterations have been reported for the Hoxa*1* (Carpenter *et al.,* 1993; Dollé *et al.,* 1993; Mark *et al.,* 1993), *Hoxb1* (Goddard *et al.,* 1996; Studer *et al.,* 1996), *Hoxb2* (Barrow and Capecchi, 1996), and, recently, *Hoxa2* (Gavalas *et al.,* 1997) knockouts. In contrast, *Hoxa3* appears to have primary functions only in mesenchymal neural crest and endoderm (Manley and Capecchi, 1995) (see below). The apparent absence of hindbrain segmentation and/or specification defects in paralog group 4 *Hox* gene mutants (reviewed in Machonochie *et al.,* 1996) may reflect functional compensation by other paralogous and non-paralogous *Hox* genes (e.g., Rijli and Chambon, 1997). The *Hoxb1* (Goddard *et al.,* 1996; Studer *et al.,* 1996) and *Hoxb2* (Barrow and Capecchi, 1996) knockouts resulted in specification defects of distinct subpopulations of neurons originated in rhombomere 4 (r4) (see below). On the other hand, both *Hoxb1* and *Hoxb2* mutations apparently did not affect normal hindbrain segmentation. Although cell lineage restrictions have not been studied in these mutants to reveal subtle segmentation problems, one possibility is that both *Hoxb1* and *Hoxb2* inactivations are functionally compensated at early neural plate stages by the function of their paralogous genes *Hoxa1* and *Hoxa2* .

We speculate that the primary role of *Hox* genes in early hindbrain regionalization is to restrict proliferating neural precursors and their progeny in compartment-like blocks of cells (rhombomeres). Such compartments will follow developmental pathways, controlled by the same set of *Hox* selector genes, appropriate for their A/P axial levels. Thus, mutating a given *Hox* gene may not only cause (partial) respecification to alternative developmental pathways (homeosis), but result in segmentation defects due to altered adhesive and/or signalling properties of group of cells. What is the current evidence for this model? Although still largely speculative, some indirect evidence for a role of *Hox* genes in both segmentation and segment specification comes from the recent analysis of both *Hoxa1* (Lufkin *et al.,* 1991; Chisaka *et al.,* 1992) and *Hoxa2* (Rijli *et al.,* 1993) mutant mice.

The *Hoxa1* case

The expression of *Hoxa1*, together with *Hoxb1*, provides the earliest sign of regionalization in the presumptive hindbrain at headfold stage (E7.5-7.75) (Murphy and Hill, 1991; Dupé *et al.,* 1997). Its expression domain extends from the posterior end of the embryo up the presumptive r3/r4 border and is down-regulated before the formation of boundaries. It is noteworthy that the first constrictions appearing in the chick hindbrain are those delineating the future r4 and r5 territories as a pair (Lumsden, 1990), suggesting that these may be the earliest rhombomeres to be specified. Targeted disruption of *Hoxa1* resulted in segmentation defects, cranial nerve and ganglia, and inner ear abnormalities (Carpenter *et al.,* 1993; Mark *et al.,* 1993; reviewed in Mark *et al.,* 1995). Early molecular analysis (Carpenter *et al.,* 1993; Dollé *et al.,* 1993) revealed that r4, based on *Hoxb1* activity, was severely reduced, while r5, as assessed by *Krox20* expression, was either absent (Carpenter *et al.,* 1993) or very reduced (Dollé *et al.,* 1993). These early defects lead to a dramatic reorganization of the patterning of cranial nerves and sensory ganglia (derived from neurogenic NCC) which appear very similar in both mutants (Carpenter *et al.,* 1993; Mark *et al.,* 1993; Gavalas *et al.,* 1998).

Two main possibilities could account for the apparent depletion of r4 and r5 cells in *Hoxa1* mutant hindbrains (reviewed in Mark *et al.,* 1995; Wilkinson, 1995). In the first scenario, *Hoxa1*, acting as a *Drosophila* gap gene, would be involved in delimiting the future region encompassing r4 and r5. It may activate selector genes which in turn would confer specific rhombomeric identities to r4 and r5. In this case, *Hoxa1* inactivation should result in a lower rate of cell proliferation and/or increased cell death leading to the physical loss of the future r4-r5 region. In the second scenario, *Hoxa1* may act as a selector gene providing an early specification to prospective r4 and r5 cells, e.g., conferring a specific adhesive property to these cells, restricting them at their appropriate axial level. In addition, transient *Hoxa1* expression might be required to activate other *Hox* and/or non-*Hox* selector genes to further specify the r4-r5 region in two distinct rhombomeres. In such a case, prospective r4-r5 cells lacking HOXA1 would not be lost but mix with cells from adjacent rostrocaudal levels and may eventually acquire the fate of their neighbors.

Two recent observations suggest that *Hoxa1* may act as a selector gene conferring rostrocaudal specification to presumptive r4 and r5 cells. Inoue *et al.* (1997) found that the expression of the CAM cadherin 6 *(cad6)* is transiently suppressed in the presumptive r4-r6 region of *Hoxa1* mutant embryos at E8.0-8.5. This result suggests either that early specific adhesive properties of presumptive r4-r6 cells may have been altered, or, alternatively, that there is an early loss of these cells in the mutant hindbrain. The second finding is presented in Figure 2 and is in keeping with the notion that precursor cells may not be properly restricted in the central hindbrain of *Hoxa1* mutants. We analyzed the expression pattern of *Wnt8*, which at about E8.25 is transiently expressed in the presumptive r4 domain (Bouillet *et al.,* 1996; Fig. 2A). *Wnt8*

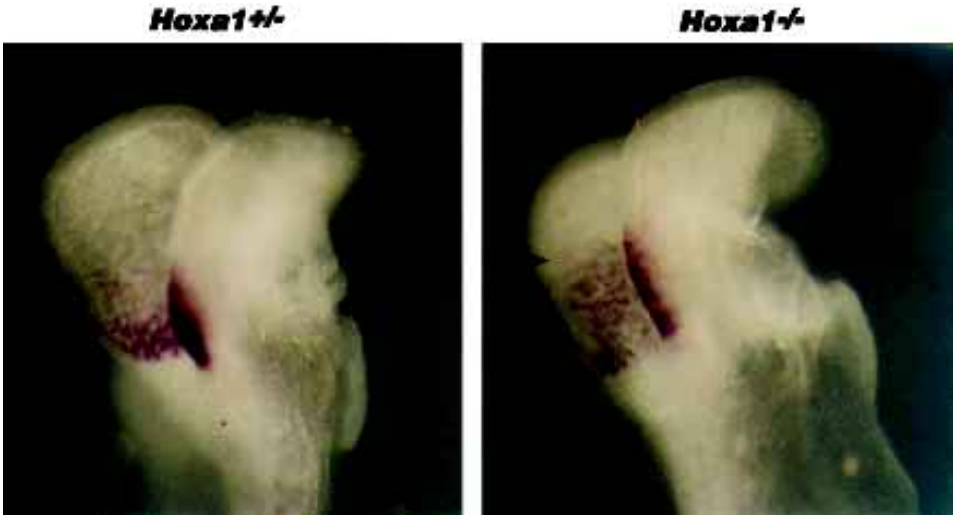

Fig. 2. *Wnt8* expression pattern in the hindbrain of *Hoxa1* mutant embryos. *Lateral views of whole-mount heterozygous* **(A)** *and homozygous* **(B)** *Hoxa1 mutant embryos at about E8.25 probed with Wnt8 for the r4 presumptive domain. Arrowheads denote the rostrocaudal extent of the expression domains in the developing neural tube. In (A), Wnt8 expression is restricted to a compact band of cells with a rather sharp posterior boundary. In the Hoxa1 homozygous mutant (B), the overall Wnt8 expression levels appear reduced, while the expression domain is extended along the rostrocaudal axis displaying less defined anteroposterior restriction, as compared to the heterozygous mutant in (A). Note that heterozygous mutants may already display a partial spread of Wnt8 expression along the rostrocaudal axis, as compared to wild-type embryos (data not shown), particularly on the dorsal part of the neural folds.*

expression in the hindbrain of the *Hoxa1* heterozygous mutant embryo shown in Figure 2A is restricted to a compact band of cells with a rather sharp posterior boundary (arrowheads). In the *Hoxa1* homozygous mutant (Fig. 2B), the overall *Wnt8* expression levels appear reduced. Interestingly, expression in the homozygous mutant appears extended along the rostrocaudal axis displaying less defined anteroposterior restriction (arrowheads in Fig. 2B), as compared to the heterozygous mutant. This result is intriguing, given that the expression of another r4 molecular marker, *Hoxb1*, is severely reduced and spatially restricted in the *Hoxa1* mutants (Carpenter *et al.,* 1993; Dollé *et al.,* 1993), suggesting that a significant portion of the presumptive r4 domain may have been lost. One way to reconcile these observations is to assume that presumptive r4 (and possibly r5) *Hoxa1* mutant cells are not lost at this stage, but just not properly restricted along the A/P axis. Thus, *Wnt8* may provide a useful marker to follow the mixing of mutant presumptive r4 cells with adjacent territories resulting in an apparently enlarged *Wnt8* expression domain. It is interesting to note that the expression domain of *Krox20* in r3 appears posteriorly extended in *Hoxa1* mutants (Carpenter *et al.,* 1993; Gavalas *et al.,* 1998), suggesting that: 1) r3 normal segmentation may indirectly depend on interactions at the interface with r4 *Hoxa1*-positive cells and 2) that r4 *Hoxa1*-mutant cells and r3 *Krox20*-positive cells may intermingle in the mutant background. Support for this interpretation comes from the finding of mixing of facial and trigeminal motor neurons (Carpenter *et al.,* 1993; Gavalas *et al.,* 1998). It will be crucial to study whether some of the r4 mutant cells may be re-specified to an r3 or, perhaps, an r2 fate as a result of the interaction with the new environment. Hints that this may be the case may come from reinterpretation of the *Hoxb1* expression pattern in the mutants. Since r4 presumptive cells may still be present, although redistributed, it follows that *Hoxb1* expression in these cells could depend on *Hoxa1* activity, as is significantly reduced in the absence of *Hoxa1* (Carpenter *et al.,* 1993; Dollé *et al.,* 1993). Indeed, evidence was recently obtained that *Hoxb1* activation *in vivo* requires *Hoxa1* during normal development (Studer *et al.,* 1998). Mutant r4 cells not expressing *Hoxb1* may well be respecified to a more rostral phenotype. In fact, in mice lacking *Hoxb1* activity (Studer *et al.,* 1996), the failure of r4 markers to be upregulated and the associated ectopic expression of r2 markers suggest that r4 may adopt an altered identity. Consistent with this interpretation, the migratory behavior of r4 facial branchiomotor neurons is abnormal, and similar to that of r2 trigeminal neurons (Goddard *et al.,* 1996; Studer *et al.,* 1996). In addition, another population of r4 neurons, the contralateral vestibuloacoustic efferent (CVA) neurons, may be incorrectly specified (Studer *et al.,* 1996).

The *Hoxa2* case

A recent phenotypical analysis of the *Hoxa2* mutants (Gavalas *et al.,* 1997) shows that this gene may provide an example of a selector gene involved in both restricting cells in compartments and directing their subsequent differentiation. *Hoxa2* is the only *Hox* gene expressed in r2 (Krumlauf, 1993; Prince and Lumsden, 1994) and, in r3, is only coexpressed with its paralog, *Hoxb2* (Krumlauf, 1993; and refs. therein) (Fig. 1). *Hoxa2* is also expressed in more posterior rhombomeres, in migrating NCC (except those derived from r2; Hunt *et al.,* 1991; Prince and Lumsden, 1994) and in the mesenchyme of the second and more posterior pharyngeal arches (Fig. 1) (see below). Targeted inactivation of *Hoxa2* results in lethality at birth and in a homeotic transformation of the skeletal elements of the second pharyngeal arch (Gendron-Maguire *et al.,* 1993; Rijli *et al.,* 1993; reviewed in Mark *et al.,* 1995) (see below). The analysis of hindbrain patterning in these mutants reveals that r2 and r3 segmental identities are altered, both at the molecular and morphological level (Gavalas *et al.,* 1997). The expression patterns of two recognition molecules, members of the *Eph*-subfamily of RTKs, are selectively changed suggesting a modification of rhombomere-specific cell-signaling properties: *Sek1*(Nieto *et al.,* 1992) expression is abolished in r2, and *MDK1* expression is abolished in r3 and altered in r2 and r4 (Taneja *et al.,* 1996). These findings together with the ectopic expression of two r1-specific markers, *En2* (Davis and Joyner, 1988) and *Sax1* (Schubert *et al.,* 1995), in the r2-r3 region of the mutants, suggest a (partial) switch in cell fate of r2-r3 towards an r1 identity, which may therefore represent a 'ground patterning program' for

hindbrain compartments (see above). In support of this hypothesis, morphological changes are observed in the brain of mutant fetuses. The cerebellar surface is enlarged, possibly because of an abnormal recruitment of exterminal germinal layer (EGL) cells, normally derived from r1 (and the isthmic region), from the alar plates of mutant r2 and r3. Concomitantly, the anterior portion of the longitudinal column of cochlear nuclei, normally derived from r3 and more posterior rhombomeres (Marin and Puelles, 1995), is missing in the pons of mutant fetuses. Interestingly, aspects of r2 and r3 motor neuron differentiation are altered as well. Trigeminal motor axons turn caudally and exit the hindbrain from the r4 facial nerve exit point and not from their normal exit point in r2. In addition, the hindbrain segmentation pattern is altered at E10.5: the r1/r2 boundary is missing and the r2/r3 border is affected, even though only partially. One possibility is that, in r3, *Hoxb2* may partially compensate for the loss of *Hoxa2*, even though r3 patterning problems have not been reported for a *Hoxb2* mutation (Barrow and Capecchi, 1996). Analysis of double *Hoxa2/Hoxb2* mutants will be required to reveal potential functional redundancy between these genes. It is noteworthy that some r2 features are conserved in the *Hoxa2* simple mutants, such as the presence of a r2 nerve exit point. Thus, *Hoxa2* may specify the fate of only a subset of cell populations and the remaining r2 territory may not be under the control of *Hox* genes.

Hox genes, neural crest cell specification, and patterning of the branchial arches

Rhombomere-specific production of NCC 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 segmented expression of *Hox* genes in the hindbrain is reflected in the NCC which express a complement of *Hox* genes characteristic of the axial level of their origin (Hunt *et al.,* 1991). The even-numbered rhombomeres and r1 generate the vast majority of hindbrain crest cells, whereas r3 and r5 are massively depleted from crest cells through apoptosis (Graham *et al.,* 1993,1994), generating small subpopulations migrating rostrally and caudally into the arches (Sechrist *et al.,* 1993; Köntges and Lumsden, 1996). The hindbrain NCC migrate ventrally in three distinct streams at the axial levels of rhombomeres 2, 4 and 6, and populate the pharyngeal arches giving rise to cranial sensory ganglia, mesenchyme and contributing to the formation of skeletal and vascular structures (Le Lievre and Le Douarin, 1975; Noden, 1983; Bockman and Kirby, 1984; Couly *et al.,* 1993; Köntges and Lumsden, 1996). In addition, transplantation experiments suggest that the pharyngeal arch neural crest is responsible for specifying the identity of muscles (Noden 1988) and determining the muscle attachment points to the skeleton of the head (Köntges and Lumsden, 1996). The understanding of the nature of the patterning mechanisms of the pharyngeal arch neural crest-derived mesenchyme and the role played by the H*ox* genes is still in an early phase (reviewed in Mark *et al.,* 1995). The main conclusion of Noden's transplantation experiments (Noden, 1983) was that the morphogenetic fate of first arch osteogenic and chondrogenic NCC is specified at the neural plate stage, i.e., before the onset of migration. On the other hand, other experiments have pointed out the importance of the competence of surface ectoderm of the arch in patterning neural crest cell-derived structures (Mina and Kollar, 1987; Lumsden, 1988). The generation and analysis of loss-of-function alleles suggested that *Hox* genes play a major role in conferring segmental identity to the mesenchyme of the pharyngeal arches (see below). In addition, the observation that subpopulations of NCC rapidly change *Hox* gene expression levels upon leaving the neural tube (Prince and Lumsden, 1994; Nieto *et al.,* 1995; Saldivar *et al.,* 1996) argue for an important role of the arch environment in the regulation of *Hox* gene expression in the NCC.

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). This finding indicates the existence of a skeletogenic 'ground patterning program' common to the mesenchymal neural crest of (part of) the first and second arches (Rijli *et al.,* 1993; reviewed in Mark *et al.,* 1995), and that *Hoxa2* acts to 'select' the developmental pathway appropriate to the second pharyngeal arch. The generation of a mirror-image rather than a tandem duplication is consistent with the presence of a signaling center common to first and second pharyngeal arch mesenchymal cells, lying at their interface, and consistent with the hypothesis that *Hoxa2* changes the interpretation of these signals (Rijli *et al.,* 1993).

The *Hoxa3* null mutants show specific deletions or hypoplasias of structures derived from the third arch. They are athymic, aparathyroid, have reduced thyroid and exhibit malformations of the throat cartilage and musculature, the bones of the jaw and the heart (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). In these mutants, changes in overall production or migration of neural crest are not observed, arguing for an intrinsic patterning and/or proliferative defect once they have reached their destination (Manley and Capecchi, 1995). The failure of these mutants to upregulate *Pax1* in the pharyngeal endoderm suggests that *Pax-1* is a likely downstream target of *Hoxa3* (Manley and Capecchi, 1995).

The *Hoxa1* null mutants presented neural crest defects limited to the neurogenic derivatives of rhombomeres 6 and 7 (Carpenter *et al.,* 1993; Mark *et al.,* 1993), whereas *Hoxb1* null mutants do not show any neural crest defects (Goddard *et al.,* 1996; Studer *et al.,* 1996). Strikingly, lack of expression of both *Hoxa1* and *Hoxb1* in the presumptive hindbrain leads to early disruption of the development of the second pharyngeal arch and subsequent loss of the second arch derived mesenchymal structures (Gavalas *et al.,* 1998). Interestingly, the migration pathway and the amount of second arch neural crest migrating in the second arch of double mutants do not appear to be significantly altered, as assessed by analysis with molecular markers, compared to that of the *Hoxa1* mutants. Since neither *Hoxa1*, nor *Hoxb1* are expressed in migrating mesenchymal NCC (Murphy and Hill, 1991), the pharyngeal arch defects may therefore reflect an early specification defect of premigratory NCC, e.g., the lack of expression of a given receptor mediating the response to a mitogenic signal once the NCC have reached their final destination in the arch. Alternatively, the second arch defects may be due to a primary requirement of *Hoxa1* and *Hoxb1* in the pharyngeal endoderm, which in turn may provide patterning signals for the arch mesectoderm, similarly to the case of *Hoxa3* (Manley and Capecchi, 1995). It is interesting to note that *labial*, the closest *Drosophila* homolog of Ho*xa1* and *Hoxb1*, is required to specify the fate of a specific endodermal cell type (Hoppler and Bienz, 1994).

What could be and where are located the signaling molecules to which NCC cells respond for correct patterning of the pharyngeal arches? A constellation of molecules is expressed specifically in the ectoderm and endoderm that encapsulate the pharyngeal arch mesenchyme. The mouse *Fgf-3* (Wilkinson *et al.,* 1988; Mahmood *et al.,* 1996), *Fgf-4* (Niswander and Martin, 1992; Drucker and Goldfarb, 1993) and *Fgf-8* (Crossley and Martin, 1995; Heikinheimo *et al.,*

1994) are detected in the endoderm of the pharyngeal pouches, and the ectoderm of the pharyngeal arches. The repetitive expression of the *Fgfs* in regions between successive pharyngeal arches suggests that they may be part of the signals that control their growth and patterning.

Bmp-4 and *Bmp-7* are expressed in the distal tip of the second pharyngeal arch and near the first pharyngeal cleft and the pharyngeal clefts, respectively, of the developing chicken embryo at stages 14-18 (Wall and Hogan, 1995) whereas, at a comparable stage, expression of a *Type I BMP receptor* has been detected in the mesenchyme of the mandibular part of the first arch and the second arch of the developing mouse embryo (Dewulf *et al.,* 1995).

The *platelet-derived growth factor A (PDGF-A)* and its receptor *(PDGFR-A)* are expressed in a complementary manner in the pharyngeal arches, the receptor been expressed in the mesenchyme and PDGF-A in the cleft regions (Orr-Urtreger and Lonai, 1992). The analysis of fluorescently labeled neural crest tissue in *Xenopus* embryos suggested that NCC are the only source of *PDGFR-A* in the arch mesenchyme (Ho *et al.,* 1994).

Endothelin-1 (ET-1) is also expressed in arch cleft regions and its functional inactivation leads to hypoplasia of both the first and second pharyngeal arches resulting in severe craniofacial abnormalities which include loss of the tympanic ring, all the middle ear ossicles and grossly underdeveloped auricles (Kurihara *et al.,* 1994).

Given the spatial distribution of these signaling molecules it is tempting to speculate that they provide a morphogenetic field into which the mesenchymal cells grow and differentiate into the appropriate structures. In this scenario *Hox* genes would provide the means, by activating a battery of downstream target genes, to perceive and correctly interpret these signals.

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