Patterning the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest

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Summary

The structures of the face in vertebrates are largely derived from neural crest. There is some evidence to suggest that the form of the facial pattern is determined by the crest, and that it is specified before migration as to the structures that it is able to form. The neural crest is able to control the form of surrounding, non-neural crest tissues by an instructive interaction. Some of this cranial crest is derived from a region of the hindbrain that expresses Hox 2 homeobox genes in an overlapping and segment-restricted pattern.

We have found that neurogenic and mesenchymal neural crest expresses Hox 2 genes from its point of origin beside the neural plate, during migration and after migration has ceased and that rhombomeres 3 and 5 do not have any expressing neural crest beside them. Each branchial arch expresses a different combination or code of Hox genes in a segment-restricted way.

The surface ectoderm over the arches initially does not express Hox genes, and later adopts an expression pattern that reflects that of neural crest that has come to underlie it. We suggest that initially the neural plate and neural crest are spatially specified, while the surface ectoderm is unpatterned. Subsequently some positional information could be transferred to the surface ectoderm as a result of an interaction with the neural crest.

Given that the role of the homologous genes in insects is position specification, and that neural crest is imprinted before migration, we suggest that Hox 2 genes are providing part of this positional information to the neural crest and hence are involved in patterning the structures of the branchial arches.

Key words: homeobox gene, Hox 2, cranial neural crest, branchial arches, mouse embryology, head development, cranial ganglia, neural plate, hindbrain.

Introduction

The development of the mesoderm of the vertebrate head involves different mechanisms from that of the trunk. These differences may be related to the presence of a tissue, the neural crest, which is able to undertake roles that in the rest of the body are played by the paraxial mesoderm. Neural crest cells originate from the boundary between the neural plate and the surface ectoderm (Verwoerd and van Oostrom, 1979; Nichols, 1981). The crest migrates ventrally and in the hindbrain region of all vertebrates populates a series of repeating structures, the branchial arches (Morris-Kay and Tan, 1987; Le Douarin, 1983; Noden, 1988; A. Lumsden, personal communication). In the trunk, there are subpopulations of crest that follow different temporal and spatial migration pathways, and it is possible that, in the head, there are different populations as well (Lumsden, 1989). The early migrating cells in the head reach the branchial arches and adopt a mesenchymal fate, while later emerging cells remain closer to the neural tube and adopt a neural fate (Nichols, 1981, 1986).

Interactions with head epithelia such as the neural tube and surface ectoderm seem to be important in neural crest differentiation and some aspects of head morphogenesis in chick (Bee and Thorogood, 1980; Thorogood et al. 1986; reviewed in Hall, 1987), Xenopus (Seufert and Hall, 1990) and mouse (Lumsden, 1987). However, evidence suggests that while the neural crest is extensively dependent on surrounding tissue to allow differentiation, some patterning information resides within the crest itself. This has been shown in experiments where sections of chick midbrain neural plate, whose crest normally colonises the first (maxillary and mandibular) arch, have been grafted to the second (hyoid) arch level (Noden, 1983, 1988). The neural crest migrated into the hyoid arch, but there formed mandibles, which are first arch structures. In addition, these ectopic mandibles had a set of muscles attached to them that were derived from the second arch paraxial mesoderm, but resembled first arch muscles (Noden, 1988). Duplicate beaks were also formed on the surface, suggesting that the differentiation pattern of second arch paraxial mesoderm and surface ectoderm was controlled by the neural crest.

Another aspect of head development that is unique is
illustrated by hindbrain formation. Early in development the hindbrain is composed of a repeating pattern of bulges, the rhombomeres. Single cell marking in chick has shown that the rhombomeres are compartments that show lineage restrictions (Fraser et al. 1990), and conservation of their number and position in all vertebrates suggests that they are true segments which play an important part in the development of the head (for refs see Lumsden, 1990).

Much recent work has centred on the isolation of families of transcription factors potentially involved in early developmental decisions such as establishing the basic body plan. Important examples are the Drosophila (HOM-C) and vertebrate (Hox) Antennapedia class homeotic genes, which are organised in tight clusters (Akam, 1989). The extensive similarities between the clusters of these two organisms suggest that HOM-C and the Hox complexes are descendants of an ancestral cluster of genes, whose basic role, specification of position along the body axis, has been retained from a common ancestor (Graham et al. 1989; Duboule and Dollé, 1989; Beeman, 1987; Beeman et al. 1989; Akam, 1987; Kessel et al. 1990).

In Drosophila, boundaries of gene expression correlate with specific segments (Akam, 1987), which is also true of the rhombomeric segments of the vertebrate head. In the mouse hindbrain, cutoffs of Hox 2 genes correspond to rhombomeric boundaries, with successive genes showing expression limits separated by two rhombomere units (Wilkinson et al. 1989b; Murphy et al. 1989).

The association between branchial arches and specific rhombomeres are conserved in all vertebrates at similar stages of development. Because of the major contribution of neural crest to the branchial arches, and its ability to affect the development of surrounding tissues, the pattern of neural crest differentiation controls the structure of most of this region of the body. Given that the hindbrain crest is a candidate for a vertebrate tissue specified on the basis of its position along the rostrocaudal axis, and evidence of Hox 2 expression in the hindbrain and in neurogenic crest (Holland and Hogan, 1988; Graham et al. 1989; Wilkinson et al. 1989b; Frohman et al. 1990), we have examined the expression of Hox 2 genes in the neurogenic and mesenchymal neural crest of mouse embryos. We find that the limits of expression correspond to the branchial arches, and that each arch has a unique code of gene expression, related to the rhombomeric origin of the crest of that arch. These results suggest that Hox genes may have a role in patterning head structures beyond the neural plate.

**Materials and methods**

*In situ* hybridisation and serial reconstruction was performed as described by Wilkinson and Green, 1990, except that probes were resuspended to a final activity of 1×10⁶ cts min⁻¹ µl⁻¹ in hybridisation buffer. The probes were identical to those of Wilkinson et al. 1989b for Hox 2.7 and Hox 2.9, and Rubock et al. (1990) for Hox 2.8. They were as follows:

- **Hox 2.7**: 0.7 kb BamHI–HindIII fragment
- **Hox 2.8**: 2.2 kb EcoRI–NotI fragment
- **Hox 2.9**: 0.9 kb EcoRI fragment

The mouse strains used were CBA and CBA/C57 crosses, and stage was determined on the basis of timed matings and otic vesicle morphology.

Serial sections were prepared from camera-lucida drawings and reconstructed using the NIMR ‘ssrcon’ package.

**Results**

**Hox 2.8 expression in the branchial arches**

In mice, the neural crest begins to leave the margins of the neural plate at 8 days of development (4 somites) in the cranial region, and by 81 days (11 somites), migration is well under way (Verwoerd and van Oostrom, 1979; Nichols, 1981). Initially we investigated the expression pattern in 9 day embryos in which the migration of cranial crest is complete, to see if there was a correlation between expression of Hox 2 genes in the hindbrain and neural crest.

Fig. 1 shows a series of coronal sections of a 9 day embryo hybridised with Hox 2.8. The planes of section through the embryo are shown in Fig. 1E and the position of the otocyst adjacent to rhombomeres 5 and 6 allows orientation within the hindbrain. Fig. 1A shows hybridisation in the neural tube ending in rhombomere 3, and in the VII/VIII cranial ganglion complex, as previously described (Wilkinson et al. 1989b). Fig. 1B is more ventral, and it is possible to see the beginnings of the first and second branchial arches, the latter adjacent to the cutoff of expression in the neural tube. Hybridisation is seen in the centre of arch 2 and in a more posterior domain, as well as within the neural tube itself. In Fig. 1C, arch 2 is visible as a discrete structure, and it is clear that there is no expression in arch 1, while arch 2 and more posterior regions express Hox 2.8. Fig. 1D crosses the heart, and is ventral to all of the arches except arch 1. Arch 1 is clearly not expressing Hox 2.8, while hybridisation is detected in posterior regions.

**Hox 2.8 expression is confined to arch 2 and posterior in regions colonised by neural crest.** The posterior expression is in a region that contains paraxial mesoderm, and it is not possible, because of the lack of cellular resolution with *in situ* hybridisation, to distinguish between tissues expressing Hox 2.8. The branchial arches are largely derived from neural crest, although there is also contribution from paraxial mesoderm in the core of the branchial arch in chick (Noden, 1988). This paraxial contribution to ventral parts of the arch is small and is confined to the core of the arch, so we suggest that the hybridisation seen in arch 2 is largely due to expression in the neural crest.

To confirm that Hox 2.8 expression was consistent with all areas colonised by cranial neural crest, a number of sections of a 94 day embryo frontal to the
branchial arches were hybridised and the embryo reconstructed. The reconstructed embryo is shown in Fig. 2D, with three of the sections used shown in Fig. 2A–C. The first and second branchial arches are clear as discrete structures in this plane of section, and the difference between the expression in first and second arches can be seen. Fig. 2A is the most dorsal section, and the structures in it are shown in Fig. 2A'. A cranial ganglion hybridises, and its position posterior of the otocyst but close to it suggests that it is the superior ganglion of the IX/X ganglion complex. Fig. 1A and previous work (Wilkinson et al. 1989b) suggest that Hox 2.8 is also expressed in the VII/VIII ganglion complex, consistent with the idea that genes are expressed in all cranial ganglia posterior to the cutoff in the neural tube. There may be a slightly increased labelling over the otocyst, but we do not believe it to be labelled above background as it is a tissue more dense than the mesenchyme surrounding it. Fig. 2B and C show first and second branchial arches very clearly, and in both sections surface ectoderm of the second arch is positive in contrast to that of the first arch. In the reconstructed embryo, expression can be seen to be within all areas lateral of the neural tube colonised by the neural crest, and extending ventrally into the second branchial arch. There is no expression in the first arch at any level.

Fig. 1. Expression of Hox 2.8 in coronal hindbrain sections of a 9 day mouse embryo. The relative position of sections is shown in E. A is the most dorsal section. The positions of the three branchial arches that have formed at this stage are shown in A and by the vertical bars on all sections, which indicate the interfaces between branchial arches. Note expression in section D in surface ectoderm terminates before the first arch; no first arch ectoderm expresses Hox 2.8. Sections C' and D' are high power views of sections C and D respectively. (A–D x80, C' and D' x400).
within the embryo, demonstrating that Hox 2.8 expression distinguishes the second from the first arch.

Hox 2.8 is expressed in specific regions of surface ectoderm

The areas of surface ectoderm lateral to the edges of the neural plate are known to produce thickenings or placodes, which generate neural derivatives (D’Amico-Martel and Noden, 1983; Le Douarin et al. 1986). In the light of this and the recent work of Couly and Le Douarin (1990) on the contributions of ectoderm lateral of the neural plate to the head, we were interested to see the extent of Hox 2 expression in the surface ectoderm. In Fig. 1C’ (a high power view of Fig. 1C) and Fig. 2B and 2C, expression of Hox 2.8 is seen on the surface of the second branchial arch, and not on the surface of the first arch. This is confirmed in Fig. 1D’, where surface ectoderm of arch 1 has no signal, but the adjacent posterior ectodermal tissue expresses very strongly. This demonstrates that the surface ectoderm expresses Hox 2.8 at 9 days in the same way as the underlying crest mesenchyme.

Expression of Hox 2.8 in crest at time of emergence

To determine the timing of the onset of Hox 2.8 expression in the crest-derived mesenchyme, we investigated the expression in neural groove stage (8.5 day) mouse embryos in transverse section. A series of sections are shown in Fig. 3, where the plane is not quite perpendicular to the long axis of the embryo; thus one side of the neural plate is more anterior than the other. Sections in the series more anterior than those shown here showed no labelling above background. In the most anterior section (Fig. 3A), only the neural plate is labelled, and only on one side. This section is at the level of the anterior cutoff of expression, between rhombomere 2 (left side, not expressing) and rhombomere 3 (right side, expressing). A near adjacent, more posterior section (Fig. 3B) shows labelling of both sides of the neural plate, with no expression in the other embryonic parts of the section. Fig. 3C shows an additional patch of expression lateral to the dorsal edge of the neural plate. This additional site of labelling is in neural crest (Fig. 3C; nc) (Nichols, 1981, 1986; Hall, 1987; Noden, 1987) and is continuous with the neural plate extending ventrolaterally first on one side and then both sides (data not shown, Fig. 3C). Fig. 3D shows a second region on the right of the section where only neural plate is labelled. We interpret the sections shown to be through the rhombomeres indicated in the diagram on the right of the figure. It indicates both rhombomeres that produce neural crest, and areas that correspond to crest-free zones, based on Hox 2.8 expression and previous studies (Anderson and Meier, 1981; Tan and Morriss-Kay, 1985; Wilkinson et al. 1989b). Thus it seems that where neural crest does arise it expresses Hox 2 genes from time of emergence and that the neural crest migrating into the arches has a Hox 2 label or code.

In this series of sections at 8.5 days no labelling above background was detected in surface ectoderm, although it is continuous with the neural plate that expresses high levels of Hox 2.8. Adjacent extraembryonic membranes showed intense labelling, indicating that tissue thickness per se was not the cause of lack of signal (Fig. 3C). Therefore the high level of expression seen in surface ectoderm at 9 days appears to represent a new site of expression. These sections also show that there is no detectable expression in non-crest mesenchyme, consistent with the idea that neural crest and plate, not mesoderm, are the major sites of expression at this stage.

Other genes of Hox 2 are expressed in specific branchial arches

Fig. 4 shows the expression pattern of Hox 2.7 and Hox 2.9 in the branchial arches at similar levels of the body of a 9.5 day mouse embryo to those shown for Hox 2.8. The embryo shown in Fig. 4A and C has been hybridised with Hox 2.7, while that shown in Fig. 4B and D has been hybridised with Hox 2.9.

In Fig. 4A and C, the plane of section is slightly oblique, the lower side of the embryo being more ventral than the upper. Fig. 4A shows the cutoff of expression in the neural tube at the r4/r5 boundary, as shown by Wilkinson et al. 1989b. Fig. 4C shows a more ventral section of the same embryo. The second and third arches are visible only on the upper side of the embryo because of the tilted plane of section. Hybridisation is clearly excluded from the first two branchial arches, and confined to the third and posterior. Hox 2.6 shows an identical background level of expression over the first three branchial arches (data not shown), with a more intense region of expression lateral of the neural tube and posterior of the branchial arches.

Fig. 4B shows a dorsal level of the neural tube. There are three very clear areas of Hox 2.9 hybridisation; the neural tube in rhombomere 4, and either side in the facial-acoustic cranial ganglion complex. This complex is composed of three lobes, which are particularly clear on the upper side of the figure. Three lobes can also be seen in Fig. 1A hybridised with Hox 2.8. Thus both Hox 2.9 and Hox 2.8 label all parts of this ganglion complex. The neurons here are largely derived from the otic placode, but the support cells are a neural crest derivative.

Fig. 4D is a more ventral section of the same embryo, hybridised with Hox 2.9. It is tilted, so that on the upper side the otic vesicle and part of the labelled ganglion complex can be seen, while the lower side is deeper in the embryo, running through the level of the branchial arches. It is clear that while there is specific hybridisation on other parts of this section, there is no expression above background levels in either the first or second branchial arches. This is despite the fact that the second arch is populated by neural crest derived from rhombomere 4, which expresses Hox 2.9. This difference between mesenchymal and neurogenic crest agrees with the observations of Frohman et al. 1990.
Fig. 2. Serial reconstruction of Hox 2.8 expression in the head of a 9.5 day mouse embryo. The age of the embryo was established by the morphology of the otic vesicle. A–C show three sections used in the reconstruction, with A the most dorsal and C the most ventral. D is a reconstruction of the expression. The central nervous system is indicated in green. Expression in areas lateral of the hindbrain colonised in neural crest are shown filled in red. The otic vesicle is shaded in blue. Anterior is to the left. A’ is a drawing to illustrate structures in A. Hybridisation in neural crest is shown in red. Fb, forebrain; B1, first branchial arch; NC, neural crest; Hb, hindbrain; OV, otic vesicle; gIX, part of IX\X ganglion complex. (×100).
Fig. 3. Expression of Hox 2.8 in serial, transverse sections of an 8.5 day mouse embryo hindbrain. The dorsal surface that will become the ventricular surface of the neural tube is uppermost in all sections. A is the most anterior section. The sections are slightly oblique, such that the left-hand side of each section is more anterior than the right-hand side. The arrowheads indicate extraembryonic membranes that are expressing Hox 2.8, and serve as a positive control for hybridisation. At this stage of mouse development, the rhombomeres are only beginning to be visible as morphological units, although their future boundaries are clearly indicated by patterns of gene expression. The areas where neural plate alone expresses Hox 2.8 would correspond to areas suggested to be crest free from other work (Anderson and Meier, 1981; Tan and Morriss-Kay, 1985; Lumsden and Sprawson, 1991). In the light of this, and the known position of the cutoff of Hox 2.8 expression at the r2/r3 boundary (Wilkinson et al. 1989b), we interpret the sections shown to be through the rhombomeres indicated on the diagram on the right of the figure, which also indicates which rhombomeres are producing neural crest. The tissues known to be expressing Hox 2.8 are shaded in grey stipple, and the lobes lateral to the neural plate indicate areas of neural crest that are known to be produced by particular lengths of neural plate. np, neural plate; nc, neural crest; nf, neural fold; ect, surface ectoderm; eem, extraembryonic membrane; fp, floorplate (x200).
Hox 2 expression in cranial neural crest

Discussion

The cranial neural crest seems to be specified before migration as to the structures it will form, and controls the structures formed by other, non-neural crest tissues (Noden, 1983, 1986). Hox genes in developing systems are thought to be one component of the process of assigning different states to otherwise equivalent groups of cells. The maintenance of a state may be manifested by the continued expression of these genes. We have shown that the neural crest arising from the hindbrain region carries a Hox 2 positional label or combinatorial code from its point of origin to end point of migration. Each branchial arch has a distinct code of Hox 2 expression (with arch one not expressing any Hox gene), and this arch-specific Hox 2 pattern is in the neural crest before it has reached the branchial arches. The cranial ganglia lateral of the neural tube also show the same pattern of expression as the neural tube itself. The expression patterns that we have found are summarised in Fig. 5. Given that Antennapedia class homeobox genes act as positional specifiers (Akam, 1987; Beeman, 1987; Beeman et al. 1989; Kessel et al. 1990), a specific combination of Hox 2 expression could provide part of the molecular mechanism for imprinting of cranial neural crest.

The relationship between expression in neural tube and branchial arches

The pattern of expression in the rhombomeres seems to be out of phase with that of the branchial arches; Hox 2.8 is expressed in rhombomere 3, some of whose motor neurons contribute to cranial nerve V (the trigeminal) innervating arch 1, while in the arch mesenchyme it is confined to arch 2 and posterior. Our data at 8.5 days show that rhombomere 3 and probably r5 do not have hybridising material lateral of them, while the neural plate expresses strongly. This raises the possibility that some areas of the neural plate do not produce neural crest. Consistent with this, Krox 20, which is expressed in neural crest-derived boundary cap cells along the entire neuraxis, is not expressed lateral to rhombomeres 3 and 5 (Wilkinson et al. 1989a). SEM studies of chick and rat embryos at the time of crest emigration suggest that areas of neural tube are crest free
Segmental Expression of Hox 2 Genes

Visceral Arches
Surface Ectoderm
Arch Mesenchyme
Cranial Ganglia
Nervous System
Neural Tube

B1
B2
B3
B4

Hox 2
2.5 2.4 2.3 2.2 2.1 2.6 2.7 2.8 2.9

Fig. 5. Summary of Hox 2 expression found in the hindbrain and branchial arches. The stipple indicates the areas of neural plate where neural crest is produced, and the branchial arch into which it migrates. The ganglion next to rhombomere 2 is the V or trigeminal, that next to rhombomere 4 is the VII/VIII or acoustic-facial complex, and those next to rhombomere 6 are the combined superior ganglia of the IX and X cranial nerves. The shading patterns shown in the cranial ganglia indicate expression of a combination of genes, and do not imply that there is spatial restriction of gene expression within a ganglion. The position in the Hox 2 cluster of the genes discussed in the text are shown at the bottom of the figure.

Therefore the neural crest in an arch expresses a Hox 2 code related to its level of origin along the margins of the neural plate as shown in Fig. 5.

Patterning of facial ectoderm

Recently Couly and Le Douarin (1990) have investigated the fate of cells in this region of the body. At an early stage the surface ectoderm, prospective neural crest and neural plate are continuous, and at this time they made an orthotopic graft of marked (quail) cells, to identify the location of their descendants. On the basis of this they suggest that the neural tube, neural crest and surface ectoderm that will cooperate to form an arch all arise from the same axial level, and that all three have been initially specified as an 'ectomere' on the basis of their axial position.

As long as each tissue has some mechanism for specifying its axial position, the mechanism that specifies the position of part of the neuroepithelium, and hence the branchial arch it belongs to, need not be the same as that which specifies the position of the structures of the arch that it innervates. Thus the nerves and other structures of the same branchial arch need not have the same pattern of Hox 2 expression.

The evidence presented here suggests that a branchial arch is made up of a combination of structures with different positional values in terms of Hox gene expression. For example, rhombomere 3, the most anterior expressing Hox 2.8, gives rise to neurons that contribute to the trigeminal nerve, which innervates the first branchial arch (Lumsden and Keynes, 1989), but neither first arch mesenchyme nor surface ectoderm expresses Hox 2.8.

At 8.5 days the surface ectoderm does not express Hox 2.8 above background levels, while at 9 days, when neural crest migration at this level is complete, the surface expresses Hox 2.8 in a similar way to the underlying mesenchyme. Given the evidence suggesting that mesenchyme can change the fate of overlying ectoderm (Noden, 1988; Richman and Tickle, 1989), perhaps the ectoderm pattern is a result of an interaction with underlying crest mesenchyme, rather than expression maintained from early stages of development. We suggest that initially the neural plate and the neural crest are spatially specified, while the branchial arch identity of surface ectoderm is unspecified. Subsequently some positional information could be transferred to the surface ectoderm as a result of an interaction with the neural crest.

The mechanism of head segmentation

The neural crest migrates from the neural plate, and so it is conceivable that, by patterning the neuroepithelium, Hox 2 genes are part of the process specifying the structures of the head and neck. Given the two segment periodicity of Hox 2 expression, it will be of interest to see how genes of the other three mammalian Hox clusters are expressed here, and whether there is a similar correlation between rhombomere expression and branchial arch coding.

The genes that we have described are unlikely to
provide information such as A-P polarity within an arch, as they are homogeneously expressed there. Information in the head region for skeletal morphogenesis must also come from the crest environment. This is supported by grafts of neural plate in normal and reversed rostrocaudal orientation, in which the structures that form in the second arch are of normal rostrocaudal orientation (Noden, 1983). It is important to note that not all properties of cranial crest are consistent with regional identity being imprinted before migration. McKee and Ferguson (1984) extirpated mesencephalic crest, but found no resulting facial abnormalities, as crest anterior and posterior of the lesion migrated in to fill the defect. This may reflect differences in properties between branchial and more anterior crest, or that crest may become respecified.

The number and size of the repeating units in neural tube and branchial arches is probably established before Hox expression reaches these regions. The neural crest does not appear to be intrinsically segmented despite arising from a segmented structure. Experiments in amphibia involving removal of pharyngeal endoderm, which reduces the number of branchial arches, have shown that the neural crest then migrates down to fill the reduced number of arches that are available (Balinsky, 1981), suggesting that the environment is causing the neural crest to form a series of repeated structures, rather than any intrinsic property of the crest such as its pattern of gene expression. The hindbrain and branchial arches develop in a very similar way in all vertebrates (Romer, 1971; Hall and Horstadius, 1988), and are thought to have been of great importance to primitive vertebrates (Gans, 1989; Langille and Hall, 1989). As the evolution of the neural crest has been suggested to be the key step separating the vertebrates from the other chordates (Gans and Northcutt, 1983), the expression pattern in the anterior regions of non-vertebrate chordates (Holland, 1990), which possess a nervous system but no neural crest, may give insights into how changes in gene expression correlate with the evolution of new aspects of the body plan.

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