Expression of Hox 2.1 Protein in Restricted Populations of Neural Crest Cells and Pharyngeal Ectoderm

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ABSTRACT A polyclonal antibody, aHox 2.1a, was used to localize Hox 2.1 protein in presumptive neural crest cells and nodose ganglion of 8.5–10.0 day p.c. mouse embryos. The following results were obtained: (1) The nodose placode, in its epithelial state, first expresses Hox 2.1 protein at 9.0 d.p.c. By 9.5 d.p.c. presumptive migrating neuroblasts between the nodose placode and ganglion primordium also express Hox 2.1 protein. (2) At 9.5 d.p.c., presumptive crest cells lateral to the cephalic cardinal vein and within pharyngeal arches 4 and 6 are immunoreactive for aHox 2.1a. In the arch 6 region, positive cells extend medially to a mesenchymal cell population on the lateral aspect of the foregut wall. (3) At 10.0 d.p.c., Hox 2.1 protein expression in putative crest cells is restricted to the arch 6 cell population. A similar staining pattern is seen using aHox 2.1a with chick embryos. Comparison with the chicken embryo suggests that the Hox 2.1 positive cells in the pharyngeal arch and those on the lateral aspect of the foregut in the mouse embryo correspond to the caudalmost subpopulation of the circumpharyngeal crest (Kuratani and Kirby: Am. J. Anat. 191:215-227, 1991; Anat. Rec. 234:263-280, 1992). These results are consistent with a role for Hox 2.1 in pattern formation in the caudalmost region of the vertebrate head.

Key words: Hox 2.1, Homeobox genes, Neural crest, Nodose ganglion, Vagus nerve, Mouse embryo, Chick embryo, Gene expression

INTRODUCTION

Homeobox genes encode DNA-binding proteins and are believed to play a role in regulating the morphogenesis and patterning of the vertebrate body. They show region- and stage-specific expression patterns, and specific morphological defects are associated with inactivation or misexpression of Hox genes in the mouse (Balling et al., 1989; Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992). Four clusters of genes in mammals show a strong resemblance to the Antennapedia-Bithorax Complex of Drosophila and are called the Antp class Hox genes (reviewed by McGinnis and Krumlauf, 1992). Genes of the Hox-2 cluster are believed to be the most highly conserved of the Antp class Hox genes and 3' members of this cluster, Hox-2.8, -2.7, and -2.6, are expressed in the hindbrain and neural crest cells in a segmentally restricted fashion (reviewed by Hunt et al., 1991a,b). They are also expressed in epibranchial placodes.

Figure 1 illustrates expression of Hox-2.1 protein in mouse embryos. Expression of Hox-2.1 protein is first detectable in the nodose placode at 9.0 d.p.c., and by 9.5 d.p.c. presumptive migrating neuroblasts between the nodose placode and ganglion primordium also express Hox-2.1 protein. At 9.5 d.p.c., presumptive crest cells lateral to the cephalic cardinal vein and within pharyngeal arches 4 and 6 are immunoreactive for aHox-2.1a. In the arch 6 region, positive cells extend medially to a mesenchymal cell population on the lateral aspect of the foregut wall. At 10.0 d.p.c., Hox-2.1 protein expression in putative crest cells is restricted to the arch 6 cell population. A similar staining pattern is seen using aHox-2.1a with chick embryos. Comparison with the chicken embryo suggests that the Hox-2.1 positive cells in the pharyngeal arch and those on the lateral aspect of the foregut in the mouse embryo correspond to the caudalmost subpopulation of the circumpharyngeal crest (Kuratani and Kirby: Am. J. Anat. 191:215-227, 1991; Anat. Rec. 234:263-280, 1992).

RESULTS

Hox 2.1 Protein Expression in Presumptive Neural Crest Cells in Mouse

While Hox-2.1 protein is found in CNS at 8.5 and 9.0 d.p.c., there was no detectable expression in presumptive neural crest cells (Fig. 1). At 8.5 d.p.c., at the car-
Fig. 1. **A:** Transverse section of an 8.5 d.p.c. mouse embryo immunostained with αHox 2.1a and counterstained with hematoxylin. The postotic pharyngeal area is shown. A presumptive crest cell mass (arrow) which is not stained with the antibody is shown near the wedge of unopened neural plate. Arrowheads indicate the thickening of the pharyngeal ectoderm where the nodose placode is expected. No immunostaining is observed in this section. Bar = 100 μm. Ph, pharynx. **B, C:** Transverse sections of the postotic area of a 9.0 d.p.c. embryo. No structure in the rostral section (B), including the presumptive crest cells (arrowheads), is stained. Several structures in the posterior region shown in (C), such as lateral plate mesodermal cells (arrows), somites, and spinal cord (Som, Sc) are heavily stained. Bar = 200 μm. **D, E:** Another 9.0 d.p.c. embryo slightly more developed than that in B and C. Arrows in D indicate the immunoreactive lateral mesodermal cells surrounding the foregut (Fg). E is enlargement of the boxed area in D. Arrowheads indicate the immunoreactive cells in the surface ectoderm, the future nodose placode. Da, dorsal aorta. Bar = 200 μm.

At the level of the pharyngeal region (i.e., the future postotic level), several clusters of neural crest cells were observed emerging from the edge of the neural plate (Fig. 1A). However, these cells do not stain with αHox 2.1a (Fig. 1A). Some other structures are obviously positive, such as lateral mesoderm, caudal spinal cord, and somites at the trunk level (Fig. 1B,C). By 9.0 d.p.c., neural crest cells should have migrated to the occipital level along the dorsolateral pathway which is just beneath the surface ectoderm. However, no presumptive crest cells staining with αHox 2.1a were seen in this location (Fig. 1D,E). Immunoreactive presumptive crest cells were first
Expression of Hox 2.1 in the Nodule Placode and Nodose Ganglion Primordium in Mouse Embryos

Expression of Hox 2.1 protein in the presumptive nodose placode is first observed in the 9.0 d.p.c. embryo in the postotic pharyngeal area. The precise extent of the stained ectoderm is difficult to determine at this stage since there is no obvious nodose ganglion primordium (Fig. 1B). At 9.5 d.p.c., the immunolabeled area of the pharyngeal ectoderm is expanded (Fig. 2). However, no sign of gangliogenesis is observed at this stage (Fig. 2D,E). There are several immunoreactive cells of undetermined origin in the mesenchyme (Figs. 2D,E,3B).

As development continues, the region of ectoderm expressing Hox 2.1 becomes more defined and at 10.0 d.p.c. is restricted to the area lateral to the nodose ganglion primordium (Fig. 4C,D). All the cells constituting this ganglion primordium show expression of Hox 2.1 protein (Fig. 4C,D), as do individual cells present between the ganglion primordium and nodose placode (Fig. 4D). It is not possible to determine whether these intervening cells are placode-derived, or crest cells, because this small population of immunoreactive cells is continuous at its caudal margin with the putative circumpharyngeal crest cells described above.

Crossreactivity of the αHox 2.1a in the Chick Embryos

Cryostat sections of chick embryos were immunostained with αHox 2.1a. The staining pattern in the chick is almost identical to that of mouse embryos (Figs. 5, 6). However, the anterior limit of expression in the chick was not determined in the present study. These results indicate that the protein recognized by αHox 2.1a is the chicken homologue (Ghox 2.1, Wedden et al., 1989) of Hox 2.1.

In chick, as in mouse, the early migrating crest cells do not stain with the antibody. This is also the case with the early crest derived cells within the circumpharyngeal ridge. Immunostaining with E/C8, a monoclonal antibody which specifically recognizes cells of the circumpharyngeal crest (Kuratani and Kirby, 1992), reveals that at stage 19 crest cells are present in the ridge, but are not recognized by αHox 2.1a in adjacent sections (Fig. 5A–C). However, in situ hybridization studies show that Hox 2.1 mRNA is present in the posterior portion of this ridge as early as stage 17 (Wedden et al., 1989: fig. 5A). However, immunoreactive cells do not appear lateral to the cephalic vein until stage 25 in the present study (Fig. 6A,B). In comparison to Hox 2.1 expression in the mouse embryo, crest cells recognized by αHox 2.1a appear to be located in a more distal area of the migration pathway. It seems likely that, in the chick, the expression of the protein in the crest cells recognized by αHox 2.1a takes place in a more distal area of the migration pathway than in the mouse embryo.
Fig. 2. Whole mount 9.5 d.p.c. mouse embryo stained with αHox 2.1a. A: Lateral view of the embryo. B: Higher magnification of the boxed area in A. Pharyngeal arches are numbered. Note that immunoreactive cells localize within and posterior to pharyngeal arch 4. The presumptive crest cells localize beyond the caudal limit of the pharyngeal apparatus, the cell population making an arch (arrowheads) along the cardinal vein within the lateral body wall. Positive cells dorsoanterior to the arch (arrow) may represent neural crest cells migrating to the arch. Cv, cephalic vein, Ot, otocyst. C: A diagram to demonstrate the distribution of the immunoreactive mesenchymal cells (stippled) and the staining in the ectoderm (stripe). Drawn by the sectioning of the same specimen as A and B and roughly reconstructed and imposed on the contour of the embryo photographed in B. The ectodermal staining implies the development of the nodose placode (Npl) along the circumpharyngeal ridge (Cpr). Note that the area covered by presumptive crest cells is larger than the area covered by the nodose placode. Arrows indicate the planes in which sections D and E have been cut. H, heart; D, E: Transverse sections of a 9.5 d.p.c. embryo. In D Pharyngeal pouch 4 (Pp4) is stained with the αHox 2.1a antibody. Note in both D and E, that there are several immunoreactive cells between the nodose placode (Npl) and the cephalic vein (Cv). Medially, immunoreactive cells (arrowheads) are seen near the foregut (Fg, not shown in E). Da, dorsal aorta. Bar = 200 μm.
Hox 2.1 immunoreactive crest cells are already present in stage 22 chick embryos in the pharyngeal arch area. As is the case in mouse, the αHox 2.1a reactive cells are seen in the pharyngeal arch 6 (Fig. 6A,B). In chick, this population of cells extends into the outflow tract of the heart at stage 25 (data not shown). Although Hox 2.1 positive cells are seen in mesenchymal tissue directly around the foregut (Fig. 6C), continuous with the arch 6 crest cell population, the contribution of these Hox 2.1 positive crest cells to the enteric nervous system (ENS) cannot be established based on these results.

Stage 42 of the chick embryo showed extensive immunoreactivity in the ENS (Fig. 6E,F). Hox 2.1 positive cells are distributed in a patched pattern in two layers of epithelial gut wall, showing the typical morphology of enteric ganglia. Both the myenteric (Awerbach's) and submucosal (Meissner's) ganglia are immunoreactive. However, Hox 2.1 immunostaining is not seen in Remak's ganglion in the hindgut (Fig. 6E). The latter ganglion is believed to originate from the sacral level of the neural crest (Pomeranz and Gershon, 1990; Pomeranz et al., 1991).

Although the expression pattern of Hox 2.1 protein in chick is nearly identical to that of the mouse, there is at least one additional area of immunoreactivity in the chick, i.e., the cervical sympathetic chain. Cells of this structure are derived from neural crest which migrate along the ventrolateral pathway (the pathway for trunk crest cells) and express Hox 2.1 protein at stage 22 (Fig. 6D) (for anatomy see Kuratani, 1990). Hox 2.1 protein expression is not detectable in these areas in chick. In the mouse, all the positive crest cells are presumed to have migrated through the dorsolateral pathway (the cephalic type of crest cells).

**DISCUSSION**

In the present study, we describe the distribution of Hox 2.1 protein in neural derivatives (CNS, neural crest) and epibranchial placodes of the 9.0–10.5 d.p.c. mouse embryo and compare it to the Hox 2.1 protein distribution in the chick embryo. We identify for the first time the pharyngeal arch 6 subpopulation of crest cells in the mammalian embryo, distinguished by expression of a protein. This is significant since currently there are no reliable antibodies (such as those for the HNK-1/NC-1 antigens in chick) available which can be used to detect neural crest cells in mammals. Although HNK-1 antibodies recognize rat crest cells, they do so for only a short period of time during early migration (Erickson et al., 1989). Other markers, such as ganglioside antibodies for trigeminal crest cells of the mouse embryo (Stainier et al., 1991; Stainier and Gilbert, 1991), are limited to differentiated neurons of trigeminal sensory ganglion and mesencephalic trigeminal nucleus neurons derived from the mesencephalic crest. While vital dye methods are more reliable for lineage tracing than antibody labeling (e.g., wheat germ agglutinin-gold, Prooij et al., 1986; DiI, Serbedzija et al., 1990, 1991), they are limited to identifying only the progeny of labeled cells which may or may not constitute a representative subpopulation. The observations of these labeled mammalian crest cells are exclusively limited for trunk crest cells. Additionally, there are several mRNA markers for early cephalic crest cells in mammals. In situ hybridization studies have shown expression of RARs (Rutherford et al., 1991), CRABP I (Rutherford et al., 1991), Krox-20 (Wilkinson et al., 1989), Hox-2 cluster genes (reviewed by Hunt et al., 1991). Maden et al. (1992) have also shown that cephalic crest cells express CRABP I, but in a larger subset of the cell population (crest cells in pharyngeal arch 2 and posterior). The expression of Hox 2.1 protein observed in the present study is cell type-, segment-, and stage-specific, indicating a morphogenetic role for Hox 2.1 in the development of the vertebrate head.

There have been several descriptive works which dealt with Hox 2.1 RNA (Krulnau et al., 1987; Holland and Hogan, 1988) or its chicken homologue, Ghox 2.1 (Wedden et al., 1989). However, the details of its expression in the neural crest have not yet been intensively studied. In this paper the expression of Hox 2.1 protein has been reported in various tissues in the postpharyngeal region of the mouse embryo. This involves a complicated portion of the vertebrate body, namely the junction between the head and the trunk. In the following discussion, we will deal with the expression of Hox 2.1 protein in presumptive neural crest cells and the nodose ganglion during vertebrate head development in both chick and mouse.

**Circumpharyngeal Crest and Hox 2.1 Expression**

The earliest expression of Hox 2.1 protein in neural crest cells is seen at 9.5 d.p.c. in the mouse embryo. Immunoreactive cells between the presumptive nodose placode and cephalic cardinal vein are likely to be neural crest cells because they are on the dorsoventral cell stream and are situated much more dorsally than the placode at this stage. Hox 2.1 positive cells were also found on the lateral aspect of the foregut in mouse which is continuous with the pharyngeal arch 6 region. In the chick, Hox 2.1 positive cells are also specifically associated with aortic arch artery 6, i.e., within the pharyngeal arch 6 region (Fig. 6A,B). However, the immunoreactivity of the chick crest cells begins later than in mouse (in the more distal migration pathway of the crest cells, Figs. 5A–C, 6A,B) and the topological resemblance to the mouse is more evident by comparison to the distribution of E/C8+ cells in the chick embryo. Since the distribution of Hox 2.1 immunoreactive cells in the mouse at 10.0 d.p.c. resembles that of the postiormost (pharyngeal arch 6 area) population of the circumpharyngeal crest cells in the chick, another antibody, E/C8, was used. E/C8, which recognizes the circumpharyngeal crest in the chick embryo, illustrates the similarity in their topology and demonstrates by comparison that Hox 2.1 expression in mouse is limited to a restricted population of the cephalic crest cells.
Figs. 3–4.
Expression of Hox 2.1 protein

In both species, there is a layer of non-immunoreactive (EC/8 in chick and αHox 2.1a in mouse) mesenchymal cells surrounding the epithelial foregut (Figs. 4E,F, 5D). By comparison with chick it seems that αHox 2.1a positive cells in mouse are of neural crest (Fig. 8). The Hox 2.1 positive mesenchymal cells directly around the foregut in the chick (Fig. 6C) are more likely to be mesodermal than neural crest cells. The latter are expected to migrate along the route of the vagal nerve branch distribution which surrounds the mesenchymal wall of the gut, while the former are directly in contact with the gut epithelium.

In the vagal region, it is difficult to determine the origin of the Hox 2.1 positive neural crest cell population since crest of both cephalic and trunk origin are present in this area (see Kuratani and Kirby, 1991, 1992). The cephalic subpopulation of vagal crest cells has been called the circumpharyngeal crest, since all of these cells primarily localize within the wall of the pharynx. It has been shown in studies with chick/quail chimeras that these cells are derived from the cardiac neural crest (Kuratani and Kirby, 1991, 1992).

In the chick embryo between stages 17 and 27, the circumpharyngeal crest cells express neurofilament protein and NAPA-73, a protein associated with neurofilaments (Payette et al., 1984; Ciment and Weston, 1985; Ciment et al., 1986; Kuratani and Kirby, 1992). Thus, in the chick it is possible to specifically stain the circumpharyngeal crest in whole (Fig. 7B) and sectioned embryos. In the mouse embryo, the circumpharyngeal ridge is not always visible by morphological observation but its presence is suggested by the development of the pharyngeal arches (compare Fig. 2C with Fig. 5A,B). The caudal extent of the ridge contains Hox 2.1 immunoreactive cells lateral to the cephalic cardinal vein (Fig. 4E,F). It should be noted that the cells within this ridge also express Ghox 2.1, the chick homologue of Hox 2.1 (Wedden et al., 1989). Thus in mouse, the Hox 2.1 positive cells lateral to the cardinal vein show morphological and molecular correlations with the caudal portion of the circumpharyngeal crest cells of the chick embryo. As mentioned above, the crest cells in this area of the chick do not stain well with αHox 2.1a. However, the Hox 2.1 mRNA is detected in this region in stage 17 embryo (Wedden et al., 1989).

Crest cells at the postotic level do not express Hox 2.1 protein while they are migrating from the neural tube. This makes it difficult to determine the precise neuraxial origin of the Hox 2.1 positive crest cells. If the origin of neural crest in the chick embryo is extrapolated to the mouse, Hox 2.1 positive cells would originate from the level of or posterior to rhombomere 7 (Fig. 9). This is in agreement with the Hox 2.1 protein expression pattern seen in the early mouse CNS (Wall et al., 1992). In this study, the anterior boundary of the Hox 2.1 expression in the neural tube was first seen at the level of rhombomere 7 at the 7 somite stage, retracting to the level of somites 3 and 4 by the 9 somite stage. It remains to be solved whether the specific Hox 2.1 expression in arch 6 crest cell subpopulation is already programmed before migration from the neural tube or if environmental cues are necessary to elicit this expression. If the former is the case, it follows that the premigratory crest is already spatially arranged on the neuraxis in a segmental fashion related to the pharyngeal arch arrangement. Quail/chick chimeric studies do not favor this view. Crest cells originating from a region of the neural tube corresponding to the length of 1 somite are often found in all 3 postotic (3 to 6) pharyngeal arches (Miyagawa-Tomita et al., 1991).

**Nodose Placode and Neural Crest Cells**

It has been shown that the inferior ganglia of the cranial nerves V, VII, IX, and X (the nodose ganglion), are formed from two sources of cells; the epibranchial (ectodermal) placode, which contributes the neurons, and the neural crest, which produces supporting cells (Narayanan and Narayanan, 1980; D’Amico-Martel and Noden, 1983; reviewed by Le Douarin et al., 1984). Also, according to D’Amico-Martel and Noden (1983), the nodose placode releases neuroblastos to the ganglion primordium during stages 19 and 20 in the chick em-
Fig. 5. Stage 19 chick embryo. A: A horizontal section of the pharynx stained with αHox 2.1a antibody. Pp4, pharyngeal pouch 4. Sc, Spinal cord. Bar = 200 μm. B: Enlargement of the circumpharyngeal ridge (Cpr), the boxed area in A. No mesenchymal cells are stained within the circumpharyngeal ridge. C: An adjacent section stained with E/C8 showing the same area as in B. Note the immunostained cells in the circumpharyngeal ridge (arrowheads). D, E: Stage 22 chick embryo stained with αHox 2.1a antibody. D: Immunoreactive nodose ganglion. Bar = 100 μm. E: A section ventral to D. Immunoreactive nodose placode (Npl) and putative migrating placode cells (arrows). Pp3, pharyngeal pouch 3. Bar = 200 μm.
Fig. 6. Stage 25 chick embryo stained with αHox 2.1a. A: A horizontal section at the level of the foregut. Three pharyngeal arch arteries are seen (Ao3–6). Note immunoreactive cells are only associated with arch 6. Asterisk shows the position of cardinal vein. Bar = 200 μm. B: Enlargement of the box in A. Asterisk is the cardinal vein. Some cells are positive lateral to the vein. However, they are not stained as heavily as those surrounding the aortic arch 6. C: Lung bud level. Immunoreactive mesenchymal cells (arrowheads) are associated with the foregut (Fg). Lb, Lung bud. Bar = 100 μm. D: Transverse section at the occipital somite level. Cervical sympathetic ganglion stained with αHox 2.1a. Bar = 200 μm. E: Whole-mount specimen of stage 42 chick embryonic hindgut stained with αHox 2.1a. Bar = 1 mm. F: Enlargement of E. Immunostained nuclei form clusters. Bar = 200 μm.
Fig. 7. Comparison between the presumptive crest cells in the mouse and chick. A: A whole mount specimen of 10.5 d.p.c. mouse embryo stained with αHox 2.1a. Note the staining in the postpharyngeal area. The caudal portion of this staining makes an arch which opens anteriorly (arrowheads) and is continuous with the nodose ganglion. Ot, otocyst. B: Lateral view of the stage 19 (4 days) chick embryo stained with E/C8 which specifically recognizes neurons and the circumpharyngeal crest, a subpopulation of the vagal crest cells. This specimen shows the peripheral nervous system as well as the arch-shaped mesenchymal cell mass of the circumpharyngeal crest. The caudalmost portion is shown by arrowheads (Kuratani and Kirby, 1991). This arch of the neural crest cells resembles that of the presumptive crest cells in the mouse (compare with A). C: Section of the chick embryo at a similar stage and stained with E/C8. This section corresponds to the line drawn in B. The immunoreactive cell mass represents the caudal portion of the circumpharyngeal crest. This cell mass includes the vagus nerve (X) and its distribution is almost identical to that of presumptive crest cells in 10.0 d.p.c. mouse embryo in a comparable section (compare with Fig. 3E). Also note the position of foregut wall, pericardium (star), and the anterior cardinal vein (Cv). Fg, foregut; Tr, primitive trachea.

Embryo, which roughly corresponds to 10.0 d.p.c. of the mouse embryo. Therefore, the Hox 2.1 positive cells seen between the presumptive nodose ganglion and placode at 10.0 d.p.c. in mouse (Fig. 3) and in stage 22 in chick (Fig. 5E), may represent placode released neuroblasts. This supports the idea that both the crest cell and the placodal cell components of the nodose ganglion express Hox 2.1 protein.

Hox 2.1 Expression in the ENS

In chick the circumpharyngeal crest contributes to pharyngeal arch 3 and posterior ectomesenchyme (Ku-
Fig. 8. Diagram to summarize the main findings of this study. The presumptive crest cells (black ovals, 1) were first observed at 9.5 d.p.c. between the surface ectoderm and the cephalic cardinal vein (Cv). The distribution of these cells at 10.0 d.p.c. is shown by white circles. This cell population was identified as circumpharyngeal crest cells which pass through the dorsolateral pathway (arrows), populating the circumpharyngeal ridge (2), leading to the lateral aspect of the foregut (Fg) wall and becoming ENS crest cells (4). The stream of cells overlaps the location of the nodose ganglion primordium (Ng) which is contributed to by the nodose placode (Npl) as well as the circumpharyngeal crest cells. At 10.0 d.p.c., cells between the nodose ganglion and placode (3) may represent the cells from the placode which contribute to the formation of the nodose ganglion. In the hindbrain (Hb), an area was found where only the superficial neuroblasts in the external limiting membrane express Hox 2.1 (arrowheads). Da, dorsal aorta; H, heart; Nc, notochord; Pc, pericardiac cavity.

ratani and Kirby, 1992), but Hox 2.1 expression in neural crest cells of the mouse and chick embryos is seen in a limited subpopulation of circumpharyngeal crest (initially in pharyngeal arches 4 and 6, becoming restricted to the arch 6 area in later development). This implies that Hox 2.1 plays a role in the development of not only pharyngeal arch 6 ectomesenchyme but also of ENS neuron precursors, since in the chick, crest cells which contribute to the formation of the ENS are known to be associated with the ectomesenchyme of pharyngeal arches 3 to 6 (Tucker et al., 1986; Payette et al., 1984; Kuratani and Kirby, 1991, 1992). Furthermore, in vitro and transplantation studies have shown that cultured ectomesenchyme from the postotic pharyngeal arch can give rise to cells of the ENS (Ciment and Weston, 1983). The expression of Hox 2.1 protein in the ectomesenchyme of, and posterior to, pharyngeal arch 4, and the continued expression seen in ENS neuroblasts in older embryos (Fig. 6) (Holland and Hogan, 1988; Wall et al., 1992) indicate that Hox 2.1 protein may be involved in the regulation of neuronal differentiation specific for the ENS. However, whether these Hox 2.1 expressing cells in mouse are of neural crest or lateral plate mesoderm origin is not clear. Additional data will be needed before a conclusion can be reached.

In both chick and mouse another crest cell population, which migrates along the ventrolateral pathway, contributes to the ENS (Noden, 1984, 1988; Serbedzija et al., 1991). However, Hox 2.1 expression was not detected in any of the putative ventrolateral crest cells in the expected area of the trunk.

Homeobox Genes and the Vertebrate Head/Pharyngeometamerism (Metamerism of the Pharyngeal Arch)

Expression of Hox-2 genes in the rhombomeres of the hindbrain demonstrates colinearity between chromosomal and embryonic position, with the most 3' genes of the cluster having the most anterior boundaries of embryonic expression and the most 5' genes of the cluster having more posterior boundaries (reviewed by McGinnis and Krumlauf, 1992). Furthermore, it has recently been shown that many of these genes are also expressed in a colinear fashion in the pharyngeal surface ectoderm, raising the possibility that they play a role in the specification of the developing epibranchial placodes, allowing for interaction with appropriate branchiomeric nerves (Hunt et al., 1991a,b). Hunt et al. (1991a,b) have suggested that there is ectomesenchyme-ectodermal tissue interaction involved in the homeogenetic induction of Hox genes in the epibranchial placode (surface ectoderm). However, ablation experiments have shown that the epibranchial placode can still give rise to neurons in the absence of the neural crest cells that would contribute to these ectomesenchymal-ectodermal interactions (Yntema, 1944; Hamburger, 1961; Kuratani et al., 1991). One alternative hypothesis is that Hox gene expression in the surface ectoderm (placode) is induced by the endodermal pharyngeal pouch (Yntema, 1944). It seems worthwhile to mention that Hox 2.1 is also expressed in pharyngeal pouch 4 epithelium just medial to the nodose placode both in mouse (Fig. 2D) and chick embryos. Another hypothesis is that there are no ectomesenchymal-ectodermal interactions involved in Hox 2.1 expression in the placode and Hox 2.1 protein is expressed in the placode independently of its expression in crest cells.

Hox 2.1 is the caudalmost member of the 3' Hox-2 genes which are expressed in the head and may play a
Fig. 9. A: A model of the development of Hox 2.1 positive crest cells in the mouse. Black arrows indicate the restriction of Hox 2.1 protein expression during development: in the hindbrain and spinal cord, the anterior limit of expression is initially at the level of rhombomere 7 (Wall et al., 1992), then retracts to the junction of the spinal cord and rhombomere 8. Similarly, in Hox 2.1 positive crest cells the anterior limit of expression is first detected in the pharyngeal arch 4 population, then retracts to the arch 6 crest cell population in later stage embryos. The restriction of the anterior limit of Hox 2.1 expression in the CNS and neural crest implies a correlation with neuraxial origin. This is indicated by white arrowheads. Dark areas indicate structures which remain immunoreactive in later stage embryos, such as nodose ganglion, anterior spinal cord, and pharyngeal arch 6 crest cell population. The white arrow indicates the pathway Hox 2.1 positive arch 6 crest cells would follow if they contribute to the formation of the ENS pharyngeal pouches 3 and 4 (pp3, pp4). B: Diagram to show the circumpharyngeal ridge (Cpr) and its relation to the pharyngeal apparatus. Ps3, Ps4, pharyngeal slits 3, 4.

role in pharyngeal metamerism. Like other 3' Hox 2 genes, the anterior expression limit of Hox 2.1 in the CNS is found within the hindbrain, and it is expressed in the pharyngeal ectoderm (specifically the nodose placode) and in the cephalic component of the neural crest cells (see above). Hox 2.1 is expressed in the caudalmost component of the cephalic neural crest (circumpharyngeal crest) and the distal ganglion (nodose) of the caudalmost pharyngeal nerve (vagus nerve). The presence of Hox 2.1 protein in these structures of the pharyngeal system suggests that Hox 2.1 has a developmental role in the organization of the caudalmost portion of the "segmental vertebrate head" and may be involved in the formation of part of the ENS.
MATERIALS AND METHODS

The preparation of the antibody has been described previously (Wall et al., 1992).

Pregnant female mice were sacrificed by cervical dislocation and embryos were removed. The embryos were fixed with buffered 4% paraformaldehyde for 20 min, washed with phosphate-buffered-saline (PBS) and stored in 100% methanol at −20°C. Embryos stored in methanol were transferred into DMSO/methanol (1:1) at 0°C for 2 to 3 min. One fourth volume of 10% Triton X-100/distilled water was added and specimens were left at room temperature for 15 to 30 min, then washed with Tris-HCl-buffered-saline (pH 8.0, 150 mM NaCl, abbreviated as TS) containing 0.1% bovine serum albumin (BSA) 3 times for 5 min each and gradually transferred to TS + 0.1% BSA containing 0.5 M sucrose. Embryos were warmed to 37°C and incubated in TS containing 0.5 M sucrose and 7.5% (w/v) gelatin (Sigma, 300 Bloom) for 1 hr. They were chilled on ice for 1 hr and frozen by immersion in n-pentane (dry ice chilled) for 3 min. Sections were cut at 13 μm with cryostat and mounted onto gelatin-coated slides, which were air-dried and kept at −80°C.

In the preparation for immunostaining, the slides were placed at room temperature and gelatin was removed in TS at 37°C for 10 min. After blocking with 5% dry non-fat milk in TS containing 1% Triton X-100 (TSTM) and with 1% periodic acid/distilled water for 20 min, the slides were incubated for 1 hr at room temperature with aHox 2.1a diluted 1/100 in spin-clarified TSTM. Slides were washed in TS + 1% Triton-X 100 (TST) for 10 min, then incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG diluted 1/200 in 5% milk/TST. After washing in TST, HRP-avidin diluted 1:400 in spin-clarified TSTM was added for 30 min. Peroxidase reaction was performed by adding TS containing 0.03% diaminobenzidine (DAB) and 0.03% hydrogen peroxide for 7 min at room temperature. For some specimens (transverse section of 10.0 d.p.c. embryo, Fig. 4), counter staining was performed with fast green.

Chick embryos were immunostained with E/C8 antibody which recognizes NAPA-73, a neuronal protein associated with intermediate filaments (Ciment et al., 1986). Briefly, the embryos were fixed with Carnoy's fixative, embedded in paraffin, and sectioned at 12 μm. After removal of paraffin, the sections were treated with 1% periodic acid/distilled water, washed in PBS containing 1% Triton X-100 (PBST), and incubated with E/C8 diluted 1:20 in PBST-BSA. HRP-conjugated anti-mouse IgM (diluted 1:100 in PBST-BSA) was used as the secondary antibody. Peroxidase reaction was performed by adding PBS containing 0.03% DAB and 0.03% hydrogen peroxide for 7 min at room temperature.

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