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Homeobox genes in axolotl lateral line placodes and neuromasts

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Abstract Gene expression has been studied in considerable detail in the developing vertebrate brain, neural crest, and some placode-derived organs. As a further investigation of vertebrate head morphogenesis, expression patterns of several homeobox-containing genes were examined using whole-mount in situ hybridization in a sensory system primitive for the vertebrate subphylum: the axolotl lateral lines and the placodes from which they develop. Axolotl *Msx-2* and *Dlx-3* are expressed in all of the lateral line placodes. Both genes are expressed throughout development of the lateral line system and their expression continues in the fully developed neuromasts. Expression within support cells is highly polarized. In contrast to most other observations of *Msx* genes in vertebrate organogenesis, expression of *Msx-2* in developing lateral line organs is exclusively epithelial and is not associated with epithelial-mesenchymal interactions. A *Hox*-complex gene, *Hoxb-3*, is shown to be expressed in the embryonic hindbrain and in a lateral line placode at the same rostrocaudal level, but not in other placodes nor in mature lateral line organs. A *Hox* gene of a separate paralog group, *Hoxa-4*, is expressed in a more posterior hindbrain domain in the embryo, but is not expressed in the lateral line placode at that rostrocaudal level. These data provide the first test of the hypothesis that the neurogenic placodes develop in two rostrocaudal series aligned with the rhombomeric segments and patterned by combinations of *Hox* genes in parallel with the central nervous system.

Key words Sense organ embryology · Ambystoma embryology · Homeobox genes · Vertebrate genetics · Evolution

Introduction

There is now an extensive literature on the involvement of homeobox-containing genes in the morphogenesis of the vertebrate head and of organs specific to vertebrates. The embryonic central nervous system, neural crest mesenchyme, and tissues in later organogenesis are known to express genes of the *Hox*, *Msx* and *Dlx* families during morphogenesis, and genes of all of these groups have been characterized in a number of animal lineages (Akimenko et al. 1994, 1995; Holland 1992; Krumlauf 1994; Ma et al. 1996; Robinson and Mahon 1994). The phylogenetic distribution of conserved sequences within and outside of the homeobox suggests that these genes may have functions important in animal development generally, and the similarities of expression patterns in various vertebrates may be indicative of some developmental roles retained in the different lineages from their common chordate ancestor (Holland et al. 1994; Sharman and Holland 1996). Thus these same genes might also be expected to be associated with the development of a sensory system primitive for the vertebrates, such as lateral lines (Northcutt 1989).

Genes of the *Hox* complex are known to be instrumental in patterning two major constituents of the vertebrate embryo, the central nervous system and the neural crest (Godsave et al. 1994; Keynes and Krumlauf 1994). The lateral line system develops from a third component of the vertebrate embryo, the ectodermal placodes (Northcutt and Gans 1983; Northcutt et al. 1994; and see Fig. 1). The lateral line placodes develop in a dorsolateral series parallel to the main body axis, and it has been hypothesized (Northcutt 1996) that the dorsolateral and ventrolateral placode series may be patterned by a mechanism similar to the *Hox* code described for the head and branchial regions of amniote embryos (Hunt and Krumlauf 1992).

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While a critical test of this placode patterning hypothesis will ultimately require extensive investigation of numerous *Hox* genes in the developing ectodermal placodes, the data presented here offer the first evidence for *Hox* gene expression associated with lateral line placodes. We examined expression of *Hoxb-3* and *Hoxa-4* in the axolotl lateral line placodes, and we discuss some refinements of Northcutt's hypothesis. We also describe the expression of two non-*Hox* homeobox genes, *Msx-2* and *Dlx-3*, in the placodes, and their continued expression in mature lateral line sensory organs.

Materials and methods

Specimens

Axolotls, *Ambystoma mexicanum*, were spawned either at UCI or at the Indiana University Axolotl Colony and were maintained at 20–22°C in 20% Holtfreter's solution. All embryos and early larvae used for in situ hybridization were albinos staged according to the tables of Bordzilovskaya et al. (1989). Prior to fixation animals were anaesthetized in 0.1% MS222 (tricaine methanesulfonate; Sigma).

RNA probes

Several homeobox-containing clones isolated from a screen of regenerating axolotl limb blastema cDNA libraries by Gardiner et al. (1995) were used as templates to prepare digoxigenin-labelled RNA probes for in situ hybridization. Information about the probes is given in Table 1.

Probes were synthesized with digoxigenin-labelled uridine triphosphate (UTP) according to the manufacturer's protocol (Boehringer), except that reagents were mixed at room temperature, not on ice, and probes were not hydrolyzed.

Whole-mount in situ hybridization

Our procedure for whole-mount in situ hybridization is as described by Gardiner et al. (1995), with the following modifications to optimize results for embryos and for lateral line organs. Embryos were treated with 10 µg/ml proteinase K at room temperature for 3–15 min. Early larvae (B40–B43) were treated with 10 µg/ml proteinase K at 37°C for 20–30 minutes, and later larvae with 30 µg/ml at 37°C for 30–60 min. Before hybridization, probes were diluted to approximately 10 µg/ml in hybridization buffer and heated to 80°C for 2 min before adding to samples. Hybridization was carried out overnight at 54–65°C. Post-hybridization washes were carried out at temperatures 4–5° higher than the hybridization temperature.

Tissues were blocked in blocking solution (maleic acid buffer with 0.2% bovine serum albumin and 20% heat-inactivated sheep serum) for at least 4 h at 4°C with gentle rotation. Samples were incubated in 500 µl blocking solution with antibody overnight at 4°C with gentle rotation. The next day tissues were washed 6 or more times in MAB over at least 3 h, then twice in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂), all at room temperature. Samples were stained in AP buffer containing a chromogenic AP substrate (340 µg/ml 4-Nitro blue tetrazolium chloride and 175 µg/ml 5-Bromo-4-chloro-3-indolyl-phosphate; Boehringer) for 20 min to 2 h at room temperature. When staining was complete, specimens were rinsed twice in PBS with 0.1% Tween-20 and fixed overnight in phosphate-buffered formaldehyde (3.7%), then stored at 4°C in phosphate-buffered saline (PBS) with 0.2% sodium azide.

Flat skin mounts of whole-mount in situ hybridization specimens

After fixation overnight in 10% formalin (see above), whole-mount specimens were dehydrated to methanol to harden the tissues and render the stain bluer. The head was removed and bisected midsagittally. Internal tissues were then removed using sharp forceps. The remaining skin was rehydrated to PBS with 0.2% sodium azide and any remaining tissues removed. The skin was transferred to a drop of AquaMount on a 24 × 30-mm cover glass and spread flat using a pair of small (no. 505) artist's brushes. A second cover glass containing a drop of AquaMount was placed over the specimen. Flat mounts were allowed to set overnight at room temperature and could then be examined and photographed under a compound microscope.

Sectioning of whole-mount in situ specimens

For cryosectioning embryonic specimens, well-stained embryos were fixed overnight as above and infiltrated with 7% sucrose in PBS, 15% sucrose in PBS, and then 15% sucrose and 7% gelatin in PBS at 37–40°C. Specimens were embedded in Tissue Tek O.C.T. compound, frozen in a dry ice-ethanol bath, and sectioned at 30 µm (nominal) on a Slee HR cryostat at approximately –20°C. Slides were dried for several hours to overnight, then soaked in PBS and mounted in Aqua-Mount.

Later larval tissues were embedded and sectioned in polyester wax as described by Mullen et al. (1996). Blocks were sectioned at 8–12 µm using an AO 820 microtome in an ambient temperature of about 20°C. Slides were dried overnight, dewaxed in two changes of 100% ethanol, and mounted in Aqua-Mount. Sections were counterstained in Safranin O (0.5% aqueous; Kiernan 1989).

Photography and computer enhancement of images

Embryos were photographed whole in PBS using FujiColor 200 print film in a camera mounted on a Wild dissecting microscope. Flat skin mounts and thin sections were photographed with the same camera and film on a compound microscope using Nomarski (flat mounts) or Koehler illumination (thin sections).

All photographs were digitized either by scanning a photographic print on a flatbed scanner (HP ScanJet IICX), or by direct image capture from a microscope-mounted color CCD (charge-coupled device) camera. Images were cropped, enhanced, labelled, and assembled using Adobe Photoshop.

Results

The complete series of ectodermal placodes from which the lateral line system develops, the dorsolateral placodes, can first be recognized in axolotls starting at embryonic stage 35 (about the end of somitogenesis; Bordzilovskaya et al. 1989) as discrete epithelial thickenings lateral to the hindbrain and most anterior portion of the spinal cord (Northcutt et al. 1994; Stone 1922; and see Fig. 1). The expression patterns of five homeobox-containing genes were examined in placode-stage embryos: *Hoxb-3*, *Hoxa-4*, and *Hoxa-5*, *Msx-2*, and *Dlx-3* (see Table 1). *Hoxb-3* is expressed in the hindbrain and middle lateral line placode, which lies adjacent to the region of hindbrain expression; *Hoxa-4* is expressed in a more posterior hindbrain domain, but not in the lateral line placode at that axial level (the posterior placode). Subdivision of the hindbrain into rhombomeres has not been described in axolotls, and classical studies of neurology in amphibians record that *Ambystoma* lacks the

Fig. 1 The six dorsolateral placodes in a stage 35 (Bordzilovskaya et al. 1989) axolotl embryo, which give rise to the lateral line system (five placodes in *black*) and the inner ear (*gray*; no longer a placode by this stage). By stage 35, the anterodorsal and anteroventral placodes have begun to elongate into the sensory ridges from which the supraorbital, suborbital, and mandibular lateral line receptors will differentiate. The middle division of the posterior placode has begun to migrate caudally to form the middle trunk and tail line. (Modified from Northcutt et al. 1995) (Bar 0.5 mm, *ad* anterodorsal lateral line placode, *av* anteroventral lateral line placode, *b* limb bud, *e* eye, *g* gill primordia, *j* lower jaw primordium, *m* middle lateral line placode, *n* olfactory organ, *otv* otic vesicle, *pld* dorsal division of the posterior lateral line placode, *plm* middle division of the posterior lateral line placode, *plv* ventral division of the posterior lateral line placode, *st* supratemporal lateral line placode)

Table 1 RNA probes used for in situ hybridization

Gene ^a	Size	Part of gene ^b
<i>NvMsx-1</i>	~400 bp	5'
<i>Msx-2</i>	~800 bp	5' H 3'
<i>Dlx-3</i>	~500 bp	5' (5)
<i>Hoxb-3</i>	~1.6 kb	5' H 3'
<i>Hoxa-4</i>	~800 bp	5' H 3'
<i>Hoxa-5</i>	~1.1 kb	5' H 3'

^a All probes were made from axolotl clones except *NvMsx-1*

^b Relative to the homeobox; 5' H 3' means the homeobox plus 3' and 5' regions were included in the probe

^c From a newt (*Notophthalmus*) clone, kindly provided by J. Brockes; see Crews et al. 1995

^d M. Carlson et al., in prep.

^e See Mullen et al. 1996

^f See Gardiner et al. 1995

boundary between rhombomeres 2 and 3 (von Kupffer 1906, cited in Kuhlenbeck 1973). Given this uncertainty about rhombomere numbers and identities in axolotls, other landmarks such as the otic vesicle and the gill arch primordia, rather than rhombomeres, were used to locate *Hox* gene expression boundaries in the dorsolateral placodes that develop adjacent to the hindbrain.

Hox gene expression in early placodes

We examined the expression of *Hoxb-3*, *Hoxa-4* and *Hoxa-5* in placodal stage embryos, and all three genes have hindbrain expression patterns consistent with those observed in other vertebrates (Krumlauf 1994).

The anterior boundary of *Hoxb-3* expression in the neural tube is aligned with the rostral edge of the first gill primordium and with the center of the otic vesicle (presumably at the anterior edge of rhombomere 5, based on embryonic anatomy of other hindbrains). By stage 35 and later, *Hoxb-3* expression is also detected as three dorsoventral bands, one in the center of each of the three gill arch primordia (Fig. 2A). Based upon comparison of whole-mount in situ hybridization specimens with serial histological sections of axolotl embryos of the same stage (R. G. Northcutt, unpublished data), the locations of these bands of expression correspond to the positions of migrating neural crest cells in the branchial arches. A patch of *Hoxb-3* expression is detected dorsal to the gill primordia, at the position of the middle lateral line placode (Fig. 2A). Transverse sections through whole-mount in situ specimens show that expression is lacking in the cuboidal epithelium overlying the placode, but that it is present in the columnar cells of the placode itself, and also in the mesenchyme (Fig. 2D). Sections of axolotl embryos after in situ hybridization do not show individual cells clearly. Thus the conclusion that the *Hoxb-3* staining is in the middle placode is based upon: examination of numerous whole-mount in situ hybridization specimens similar to the ones shown in Fig. 2; upon the complete series of sections from which the one shown in Fig. 2D was taken, and from which can be determined the anteroposterior position of that section; and finally upon comparison of those sections with a histological series for the same embryonic stage, from which Fig. 2C was taken (R. G. Northcutt, unpublished). The expression pattern of *Hoxb-3* contrasts with the pattern of *Msx*-

Fig. 2 **A** *Hoxb-3* in a stage 35 embryo. Expression in the hindbrain is consistent with results for other vertebrates, with the anterior border at the level of the otic vesicle. *Hoxb-3* is expressed in the middle lateral line placode (*m*), but not in the posterior placode. **B** *Hoxa-4* in a stage 35 embryo. The posterior lateral line placode is at the same rostrocaudal level as the *Hoxa-4* expression domain, but it does not show *Hoxa-4* expression. **C** Transverse histological section through a stage 35 axolotl embryo, at the level of the second gill arch primordium and the middle lateral line placode. Note the columnar cells of the middle placode (*m*) in the inner layer of skin epithelium and the migratory neural crest cells in the mesenchyme of the gill arch primordium. (glycol methacrylate section, cresyl violet). **D** Transverse cryosection (30 μ m) through a specimen similar to **A**, showing *Hoxb-3* in the neural tube (*nt*), in neural crest mesenchyme (*nc*), and in the middle lateral line placode (*m*). **E** Transverse cryosection through a specimen similar to **B**, showing expression of *Hoxa-4* in the hindbrain but not in the posterior placode (*po*). (For other abbreviations see Fig. 1)

2 (described below), and presumably also of *Dlx-3*, which are in the epithelial layers but not the mesenchyme. Also unlike *Msx-2* and *Dlx-3*, no *Hoxb-3* expression is detected after about stage 37 in cells of the developing or mature lateral line system (data not shown).

Expression of neither *Hoxa-4* nor *Hoxa-5* shows any association with the supratemporal or posterior placodes comparable to that of *Hoxb-3* with the middle placode. The posterior placode originates at an axial level that

corresponds to a region of *Hoxa-4* expression within the neural tube, and it migrates caudally during stages 35 to 40 through axial levels at which *Hoxa-5* is expressed. However, at stage 35 the posterior placode is negative for *Hoxa-4* expression (Fig. 2B, E), and as the dorsal, middle, and ventral divisions (see Fig. 3C) of the posterior

Fig. 3 **A** *Msx-2* expression in an axolotl embryo at stage 35, when the lateral line placodes are present. *Dlx-3* expression is similar at this stage. **B** Transverse cryosection through the embryo in **A**, showing *Msx-2* expression in the posterior placode. Staining for *Msx-2* is in the columnar placodal cells of the inner epithelium as well as in the overlying epithelial layer. **C** Stage 38 embryo (gills trimmed) showing *Msx-2* expression in the three migrating sections of the posterior placode, and in the neuromast primordia of the trunk lateral lines. Dashed line marks the path of the middle posterior lateral line. The middle section of the posterior placode has already reached the tail tip by this stage. **D** Stage 43 feeding larva showing *Msx-2* expression in the neuromasts of all the cephalic lateral lines. Expression is also apparent in the forelimb bud (*b*) at this stage. *Dlx-3* shows a similar pattern of expression in the lateral lines at this stage. **E** Polyester wax section through a primary and secondary neuromast showing *Msx-2* expression (dark blue) in the support cells (*s*), and less in the hair cells (*h*). Cell nuclei are stained red (Safranin O). Based on examination of numerous such sections and flat skin mounts, the cytoplasm of the support cells extends apically over the nuclei of the hair cells, and the staining between the support cell and hair cell nuclei is in the support cell cytoplasm. (For other abbreviations see Fig. 1)

Fig. 3A-E for legend see page 290

placode migrate caudally, they do not express *Hoxa-5* either (not shown). *Hox* gene expression was not detected during differentiation of neuromasts, nor in mature lateral line organs (not shown).

Msx-2 is expressed in early and migrating placodes

The *Msx-2* expression domains during placode stages correspond well with the location and extent of the dorsolateral placodes as described by Northcutt et al. (1994). *Msx-2* is expressed in the anterodorsal placode as early as stage 30 (data not shown), and expression can be seen in the anteroventral placode and in the neural crest of the first visceral arch at that stage. At stage 35, all five of the lateral line placodes show similar levels of expression (Fig. 3A). The middle division of the posterior placode has begun to migrate caudally at this stage, and *Msx-2* expression continues during its migration. The cephalic sensory organs of placodal origin – the eye, olfactory vesicle, and otic vesicle – also express *Msx-2*.

Transverse sections through stage 35 embryos stained as whole-mounts for *Msx-2* expression show that expression is restricted to the placodal epithelium and is not detected in the underlying mesenchyme (Fig. 3B). At this stage, *Msx-2* is also expressed in the forebrain, dorsal neural tube, jaws and gill arches (not shown). Neural crest expression was not detected at stage 35.

Expression of *Dlx-3* at stage 35 is similar to that of *Msx-2* (not shown). *Dlx-3* appears in the placodal epithelium during the same stages as *Msx-2*, as well as in the dorsal neural tube and branchial arches.

Msx-2 and *Dlx-3* expression continues in the cephalic placodes as they elongate to form sensory ridges at stages 36 to 38, and expression becomes localized to the sites of neuromast differentiation and to the three subdivisions of the posterior placode as they migrate caudally to deposit the neuromast primordia of the trunk and tail lines from stage 35 to about stage 40. The middle division turns dorsally at the level of the anus, then continues caudally to form the tail line (see Fig. 3C). Neuromast primordia first appear as rosettes of cells in the placodal epithelium; sensory hair cells differentiate in the center of the rosette, and their apical ends erupt through the outer epithelial layer starting at stage 37 (Northcutt et al. 1994).

Msx-2 and *Dlx-3* expression continues in mature lateral line organs

Given the associations of *Msx* and *Dlx* genes with the development of many vertebrate organs, it is not surprising that they should also be found in the developing lateral lines. However, two features of the expression of *Dlx-3*, *Msx-2*, and *Msx-1* in the lateral line neuromasts stand out in contrast to their expression in other organs. First, the expression of these genes in other organs is associated mainly with sites of interactions between tissues, where-

as neuromast development takes place entirely within the epithelium and does not involve interactions with mesenchyme. Second, expression of *Msx* and *Dlx* genes in other organs is usually confined to the developmental stages, whereas the expression described here in neuromasts continues in the mature, functioning organs.

Both *Msx-2* and *Dlx-3* are expressed continuously in cells of the lateral lines from placodal stages through differentiation of the neuromast organs. Both genes are expressed at sites of neuromast differentiation in the sensory ridges (Fig. 3C), and expression then becomes localized to the support cells and sensory hair cells of the neuromast organs as they develop (Figs. 3D, E; 4). Expression is similar in all neuromasts, from the cephalic lines to the posterior line neuromasts at the tip of the tail. *Dlx-3* expression is similar in pattern (Fig. 4E, F).

In fully formed primary neuromasts, *Msx-2* and *Dlx-3* transcripts are detected most strongly in support cells, and in the regions of the support cells' cytoplasm that are directly in contact with a central hair cell (Figs. 3E, 4), revealing a polarity in these cells. Expression in mantle cells is lower and less consistent. In some neuromasts, an area of intense expression can be seen in the cytoplasm in the center of the array of hair cells, possibly associated with an extra hair cell disturbing the order of the array (Fig. 4B; both genes show this expression pattern, contrary to an earlier report of differential expression by Metscher et al. 1995). Jones and Corwin (1996) have reported that during regeneration in response to laser ablation of all hair cells in a neuromast, new hair cells differentiate from the remaining support cells. New hair cells in normal neuromasts probably differentiate from the surrounding support cells; this patch of gene expression may correspond with a site of hair cell differentiation.

Expression in secondary neuromasts is similar to that in primary neuromasts. At about 1 week posthatching, secondary neuromasts begin to develop from cells of the mantle zones surrounding primary neuromasts (Stone 1933). The secondaries express *Msx-2* as soon as they are morphologically recognizable, and expression continues in the same pattern as in primary neuromasts (Fig. 4D).

The pit organ mechanoreceptors of the lateral lines, which are developmentally and morphologically similar to neuromasts (Northcutt and Bleckmann 1993), also express *Msx-2* (Fig. 3D, dots) and *Dlx-3* (not shown). The electroreceptive ampullary organs of the lateral line form along the margins of the same placodal epithelium from which mechanoreceptors develop (except the posterior placode, which does not produce electroreceptors), starting after most of the primary neuromasts have already erupted, at about stage 42 (Northcutt et al. 1995). The ampullary organs show some expression of *Msx-2* and *Dlx-3*, but it is less intense and does not show the same localization within the organ as expression in neuromasts (data not shown). The non-sensory ciliated epithelial cells of the body surface also express both genes, and in contrast to expression in support cells of the neuromasts, transcripts appear to be uniformly distributed within the

Fig. 4 **A** Mature neuromast in a flat-mounted skin from a hatching larva showing *Msx-2* expression in the support cells (*s*), with weaker staining in hair cells (*h*). The visible outlines correspond to individual cell nuclei. The cytoplasm of the support cells extends inward toward the hair cells, whose cytoplasm is concentrated directly above the center of the neuromast organ. The staining that appears between the support and hair cell nuclei is in the support cells' cytoplasm. **B** A mature neuromast showing a ring of *Msx-2* expression in the support cells' (*s*) cytoplasm surrounding the hair cells, and a patch of expression in the hair cells themselves (*h*). **C** *Msx-1* expression in mature neuromasts. **D** *Msx-2* expression in a secondary (2) neuromast, showing a similar pattern to that seen in primary neuromasts (1). **E** *Dlx-3* expression in a mature neuromast, at a slightly later stage than **A–C** (note the larger number of hair cells). In this example, *Dlx-3* transcripts are most abundant in the support cells surrounding the sensory hair cells, with only limited expression in the hair cells themselves. Again the stained cytoplasm belongs to the support cells. **F** Section through a neuromast expressing *Dlx-3* both in the support cells and in a patch in the hair cells. (8 μ m polyester wax section, Safranin O counterstain) (For other abbreviations see Fig. 1)

cytoplasm (not shown). The remaining epithelial cells of the body surface of hatching larvae did not express either *Msx-2* or *Dlx-3* at detectable levels.

Expression of a second *msh*-like gene, *Msx-1*, was examined using an RNA probe made from a newt (*Notophthalmus*) *Msx-1* clone, kindly provided by J. Brockes. Since the expression patterns of *Msx-1* and *Msx-2* were different from each other in limb buds (not shown), where the normal expression of homologs of these genes has been described for other vertebrates, it can be concluded that the *Msx-1* probe is capable of detecting *Msx-1* expression specifically. Like *Msx-2*, *Msx-1* is ex-

pressed in hair cells and support cells of mature neuromasts (Fig. 4C). Its expression is not as strong as *Msx-2* and in contrast to both *Msx-2* and *Dlx-3*, it is not localized within the cytoplasm. Therefore the characteristic ring of expression that surrounds the central hair cells is not seen with *Msx-1*.

Discussion

The described pattern of *Hoxb-3* expression is consistent with the hypothesis that the ectodermal placode series are patterned in parallel with the developing central nervous system and by a similar genetic mechanism (Northcutt 1993, 1996). The *Hox*-defined segments of the neurula may form developmental compartments within which the lateral walls of the neural folds and migratory neural crest function to form the structures characteristic of each rostrocaudal level (Couly and Le Douarin 1990; Hunt and Krumlauf 1992). Because the lateral line placodes arise from the lateral walls of the hindbrain neural folds at different rostrocaudal levels, their individual identities might also be specified by differential expression of *Hox* genes (Northcutt 1996). The expression of *Hoxb-3* in the middle lateral line placode and not in more anterior placodes tends to support this hypothesis of placodal patterning.

If there is a developmental registry between dorsolateral placodes and hindbrain *Hox* domains, the posterior placode would be expected to express *Hoxa-4*, provided that it originates within the *Hoxa-4* domain. The absence of *Hoxa-4* expression in the placode might be due to its

alignment with a more anterior segment: the posterior placode could originate in the gap between the *Hoxb-3* and *Hoxa-4* expression domains (see Keynes and Krumlauf 1994).

The difference in expression of *Hoxb-3* and the two *Hoxa* genes in placodes suggests another question amenable to experimental testing: the absence of *Hoxa-4* expression in the posterior placode may be indicative of different derived roles for paralogous *Hoxb* and *Hoxa* genes in the mechanism specifying placode identities. The phylogenetic appearance of the lateral line system coincides with the divergence of the vertebrate lineage from non-vertebrate chordates (Northcutt 1989), and therefore with at least part of the expansion of the *Hox* gene complex (Holland and Garcia-Fernandez 1996; Pendleton et al. 1993; Schubert et al. 1993). It should be noted however that the involvement of other *Hox* paralogs and the possibility of *Hox* paralogs having been lost in the ambystomid (or urodele) lineage cannot be ruled out with data available for so few genes (see Maconochie et al. 1996, for a review of paralogous *Hox* genes). The critical tests of these possibilities will have to await cloning of a more complete set of axolotl *Hox* genes and a more extensive study of their expression in the embryonic neural tube and placodal ectoderm.

Msx and *Dlx* genes in lateral line organs

Msx genes have been associated mainly with sites of epithelial-mesenchymal interactions during development (Thesleff et al. 1995, and references therein), and their expression has been shown to be induced by tissue interactions in developing limbs (Davidson et al. 1991; Ros et al. 1994), teeth (Jowett et al. 1993), and other organs (Davidson 1995; Noveen et al. 1995). In contrast to these other organ systems, the axolotl lateral line system develops exclusively from ectoderm, at least once the placodes have formed. Experiments involving transplantation of placodal epithelium to ectopic locations on living embryos (Northcutt et al. 1995; Parichy 1996) have indicated that the development of the lateral line sensory organs from placodes does not depend on epithelial-mesenchymal interactions. Unlike other ectodermal sensory placodes, the lateral line placodes do not invaginate into the underlying mesoderm, and *Msx-2* is expressed only in the placode and overlying epithelial layer, not in the mesenchymal tissue underlying the placode, at least at stage 35. The mature lateral line organs also do not appear to depend on interaction with their underlying tissues, and *Msx-2* expression in neuromasts is also exclusively epithelial. These observations are consistent with a functional role for *Msx-2* in processes within the lateral line ectoderm, rather than in interactions between tissues of different germ layer origins.

Once the individual neuromast organs become recognizable, expression of both *Msx-2* and *Dlx-3* continues in the mature organs, mainly in a ring around the sensory hair cells, with patches of expression in the hair cells themselves. The specific locus of origin of new hair cells

in normal neuromasts is not known, but in axolotl neuromasts in which all the hair cells have been ablated by a microscopic laser beam, new hair cells differentiate from the remaining support cells (Jones and Corwin 1996). It is possible that the patches of expression among the hair cells in unperturbed neuromasts coincide with sites of new hair cell differentiation, either in response to cell death or as a component of neuromast growth.

The heterogeneous gene expression within the cytoplasm of support cells in the neuromasts may reflect involvement in patterning or polarizing the neuromast organs. Hair cells are aligned within each neuromast for maximum sensitivity to particle waves travelling along the direction of the major axis of the neuromast organ (Lannoo and Smith 1989), and secondary neuromasts differentiate among the mantle cells along the major axis of the primary neuromast (Northcutt et al. 1994, and see Fig. 4D). It is unlikely that the *Msx* and *Dlx* genes are themselves responsible for establishing this polarity, since the neuromasts in any line have characteristic alignments and expression correlates with the structure within each neuromast, not with the pattern or alignment of the neuromasts within a line. However, they may be part of the genetic mechanism that regulates growth of the neuromast so as to produce an elongate, polarized organ.

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