Expression of the zebrafish gene hlx-1 in the prechordal plate and during CNS development

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SUMMARY

The zebrafish hlx-1 gene belongs to the H2.0 subfamily of homeobox genes and is closely related to the mouse Dbx gene with respect to both homeodomain homology (96.7%) and neural expression during embryogenesis. Analysis of hlx-1 expression by in situ hybridization reveals several particularly interesting features. In late gastrula embryos, hlx-1 transcripts are detected within a circular area in the region of the presumptive rostral brain. Subsequently, the expression domain becomes restricted to the hypoblast and undergoes dynamic changes involving conversion into a longitudinal stripe which elongates and retracts following a temporal sequence. The site of transient hlx-1 expression along the ventral midline of the rostral neur ectoderm, which in part corresponds to the prechordal plate, suggests a role in the determination of head mesoderm as well as in patterning of the rostral brain. As the midline stripe gradually disappears, the hlx-1 gene becomes regionally expressed within the diencephalon and at a specific dorsoventral level along the hindbrain and spinal cord. In the hindbrain, expression is initiated in dorsoventrally restricted transversal stripes which correlate with the segmental pattern of rhombomeres. The stripes fuse into bilateral columns that are later converted to two series of paired transversal stripes at the rhombomere borders. This pattern is consistent with the proposed subdivision of hindbrain segments into rhombomere centers separated by border regions.

Key words: neurulation, prechordal plate, rostral brain, spinal cord, hindbrain segmentation

INTRODUCTION

A wide range of different transcription factors are expressed during development of the vertebrate central nervous system (CNS). One important class consists of the homeodomain proteins encoded by genes in the Hox complexes, which probably specify anteroposterior positions of cells in the nervous system and other tissues (McGinnis and Krumlauf, 1992). Similar to the homologous genes of the Drosophila homeotic gene complexes (HOM-C), the various Hox genes have expression domains that extend from specific anterior boundaries in the embryonic CNS. However, the expression of individual Hox genes does not seem to be restricted to specific cell types within the spinal cord region and/or hindbrain rhombomeres. As each hindbrain segment can be tentatively subdivided into a rhombomere center and two border regions (Trevarrow et al., 1990), it will be of interest to identify the genes potentially responsible for generating these subdivisions.

Other groups of transcription factors are encoded by Pax genes and several members of the vertebrate homeobox subfamilies related to the Drosophila genes engrailed, even-skipped and Distal-less. These factors appear to have important roles in regionalization and/or dorsoventral patterning of the neural tube (Bastian and Gruss, 1990; Davis et al., 1988; Fjose et al., 1992; Gruss and Walther, 1992; Krauss et al., 1991a; Price et al., 1991). Several members of these groups are expressed within defined domains in the rostral brain during early stages of embryonic development, when regional identities are established. The majority of these genes are also expressed at specific dorsoventral levels in the epichordal division of the neural tube. In the case of murine Pax genes, expression has been observed in both dividing cells, in the ventricular zone, and in specific postmitotic cells (Gruss and Walther, 1992). Similar observations have been made in zebrafish where transcripts and proteins derived from the Pax2 and Pax6 homologues are present in identified interneurons and the basal plate ventricular zone, respectively (Krauss et al., 1991b,c; Mikkola et al., 1992; Püschel et al., 1992).

The establishment of Pax gene expression domains in the hindbrain and spinal cord is probably controlled by the notochord-floor plate signal cascade which is thought to determine the dorsoventral organization of the neural tube (Goulding et al., 1993; Placzek et al., 1991). A different regulatory system is likely to operate in the rostral brain, where both notochord and floor plate are lacking. However, studies of the zebrafish cyclops-1 (cyc-1) mutant in which tissues along
the entire ventral neuraxis do not form, have provided evidence that, also in the rostral brain, neural tissues located at the ventral midline have organizational functions (Hatta et al., 1991; Hatta, 1992). Moreover, analyses of genetic mosaics of cyc-1 indicate that mutant cells of the ventral midline, in the regions of the rostral brain and hindbrain/spinal cord, are not able to respond to inductive signals from the prechordal plate and notochord, respectively (Hatta et al., 1991). In this context, identification of transcription factors whose expression patterns are restricted to the prechordal plate and ventral midline tissues of the rostral brain would be of interest.

We have isolated a zebrafish gene (hlx-1) related to the Drosophila homeobox gene H2.0 (Barad et al., 1988). In early stages of neurulation, the gene displays a highly dynamic expression pattern in the hypoblast of the head region. The expression domain is rapidly transformed from a circular area into a longitudinal stripe, which is transiently detected in the prechordal plate underneath the rostral brain. At later stages, the hlx-1 gene is expressed at multiple sites in all major subdivisions of the CNS. A particularly interesting expression pattern is observed within the developing hindbrain. The individual rhombomere primordia appears to control the temporal activation of hlx-1 and, at later developmental stages, expression is detected near the interrhombomeric boundaries.

**MATERIALS AND METHODS**

**Embyros**

Zebrafish (Brachydanio rerio) were maintained and bred essentially as described in Stuart et al. (1988). Developmental age is given as hours postfertilization (hpf) at 28.5°C, which was the temperature of incubation.

**Isolation and sequence analysis of the cDNA**

A cDNA library was made from poly(A)+ RNA isolated from 12-48 hpf zebrafish embryos. Total RNA was isolated by the guanidinium thiocyanate-CsCl method (MacDonald et al., 1987) and poly(A)+ RNA was purified on an oligo(dT)-cellulose column (Pharmacia). The cDNA was synthesized by random priming according to standard procedures (Sambrook et al., 1989) and 1.5×10⁶ recombinants were obtained by cloning in the phage vector AZAPII (Stratagene). The library was screened at low stringency with a probe mixture of homeobox sequences from class I Hox genes.

**In situ hybridization and sectioning of stained embryos**

In situ hybridization to whole-mount embryos was performed essentially as described by Krauss et al. (1991b,c). Stained embryos were dehydrated in 100% methanol and cleared for 30 minutes in a 1:2 solution of benzyl alcohol and benzyl benzoate. Subsequently, the embryos were mounted in Permount and analysed with DIC optics in a Nikon Microphot-FXA microscope.

To make tissue sections, the stained embryos were dehydrated in ethanol and treated with methyl salicylate for 10 minutes, followed by infiltration with a solution containing one part methyl salicylate and one part resin (Epon 812-Araldite 502) overnight at room temperature. The embryos were then placed in pure resin (2 changes of solution, 2 hours each) and embedded in fresh resin. The blocks were polymerized overnight at 60°C. A tungsten carbide-tipped knife was used to cut 10 μm sections on a 2055 Autocut Rotary Microtome. The sections were dried onto glass slides and mounted in Permount.

**Immunohistochemistry and double staining of in situ hybridized embryos**

Zebrafish embryos were manually dechorionated and fixed for 12 hours at 4°C as described by Krauss et al. (1991b,c). After fixation, embryos were rinsed 3×5 minutes in 0.1 M PO₄ (pH 7.3), 0.5% Triton X-100 and incubated for 30 minutes in a solution containing 2% goat serum, 1% bovine serum albumin (BSA), 1% dimethylsulfoxide (DMSO) and 1×PBS (pH 7.3). The embryos were subsequently incubated for 3 hours with a 1:50 diluted zn-12 antibody (Metcalfe et al., 1990; Trevorrow et al., 1990) in 0.1 M PO₄ (pH 7.3), 1% goat serum, 0.2% BSA and 1% DMSO at 37°C. The embryos were then washed 6×10 minutes in the same buffer followed by incubation overnight at 4°C with a 1:200 diluted horseradish peroxidase (HRP)-conjugated secondary antibody in the same buffer as used for the primary antibody. Embryos were rinsed again 6×10 minutes in the same buffer and incubated for 20 minutes with 1 ml of 0.5 mg/ml 3,3′-diaminobenzidine in 0.05 M PO₄ (pH 7.3). The embryos were stained for about 10 minutes after adding 2 μl of a 3% H₂O₂ solution. After washing 3× in cold 0.1 M PO₄ (pH 7.3) buffer and dehydration in 100% methanol, the stained embryos were cleared in a 1:2 solution of benzyl alcohol and benzyl benzoate before mounting in Permount. For double labelling, the hlx-1-stained embryos were washed 4×5 minutes in 0.1 M PO₄ (pH 7.3) followed by incubation with the zn-12 antibody and immunohistochemical detection as described above.

**RESULTS**

**Cloning and characterization of the hlx-1 gene**

The hlx-1 (H2.0-like homeobox; Allen et al., 1991) cDNA was cloned as a part of an effort to isolate homeobox genes with developmental functions (see Materials and Methods). When compared to the homeodomain sequences of other genes (Fig. 1) it is clear that hlx-1 belongs to a vertebrate group related to the H2.0 gene of Drosophila (Barad et al., 1988). Moreover, the level of sequence identity between hlx-1 and the murine Dbx homeodomain (96.7%) is considerably higher than for any of the other known vertebrate genes of this group (Fig. 1). This suggests that hlx-1 and Dbx may be direct homologues. However, to resolve this issue, it will be necessary to obtain the complete DNA sequences of both genes. In addition, a more extensive analysis of the embryonic Dbx expression is required for direct comparison to hlx-1 (see Discussion).

**Dynamic changes of hlx-1 expression in the rostral hypoblast**

To investigate the expression of the hlx-1 gene during embryogenesis, the cDNA was labelled with digoxigenin and used for in situ hybridization to whole embryos. Initially, at 8-9 hpf, expression was seen in dispersed cells within a small circular

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**Fig. 1.** Comparison of the amino acid sequence of the Hlx-1 homeodomain with those of other homeodomains. Dashes indicate positions where amino acids are identical with those in the Hlx-1 sequence. The homeodomains listed are murine Dbx (Lu et al., 1992), chicken CHoxE (Rangini et al., 1991), murine Hlx (Allen et al., 1991), Drosophila H2.0 (Barad et al., 1988) and Antp (McGinnis et al., 1984).
area in the region of the prospective midbrain and forebrain (not shown). Subsequently, this domain became enlarged and stronger hybridization signals were detected in the individual cells (Fig. 2A). During the 9 hour stage (Figs 2A-C, 8A), the expression domain became elongated, probably due to migration of lateral cells towards the midline. Also, a higher and more uniform staining intensity was observed.

As judged from tissue sections of stained 9 hpf embryos (Fig. 3A,B), the positive cells were located in the hypoblast that underlies the rudiment of the rostral brain. Cross-sections in the posterior region of the stripe demonstrated that expression was confined to a flat triangular area that spans about 10 cell diameters ventrally (Fig. 3A). Interestingly, hlx-1 expression was not detected in the most ventral cell layer of the hypoblast which includes prospective endodermal cells. Dorsally, the expression domain seemed also to include some cells of the epiblast. However, at a more rostral position where a weak ventral bending was first observed in whole mounts of this developmental stage (Fig. 2C,D), cross-sections demonstrated restriction of the expression domain within the hypoblast (Fig. 3B). At this level of the body axis, the width and thickness of the stripe were about six and three cell diameters, respectively.

Further elongation and ventral bending was observed at later stages (Fig. 2E-H) and, in 11 hpf embryos, the hlx-1 expression domain appeared as a narrow stripe (about 6 cells wide) located at the ventral midline of the rostral brain (Fig. 4A). The stripe extended up to the anterior end of the forebrain by 12 hpf but the posterior border of the expression domain simultaneously retracted (Fig. 4B). Soon after this stage, further shortening of the hlx-1 stripe was observed (Fig. 4C,D) and, in 22 hpf embryos, it was no longer detected (Fig. 7A).

To estimate precisely the location of the hlx-1 domain during early development at a time when morphological markers are not yet visible, embryos were hybridized simultaneously with probes derived from Fig. 2. Expression of hlx-1 in the rostral region of zebrafish embryos during early stages of neurulation. Transcripts were localized by in situ hybridization on a series of whole-mount embryos at 9-10 hpf (A-H). Dorsal views of the rostral region (anterior to the left) of subsequent stages are shown in A,B,E and G. Side views of the same embryos as in C,E and G are shown in D,F and H, respectively. Some embryos were hybridized simultaneously with digoxigenin-labelled probes of both hlx-1 and the pax[6] gene (Krauss et al., 1991b). The double stained embryos shown in I and J are comparable to the stages presented in C and G, respectively. Arrows indicate the location of the presumptive midbrain-hindbrain boundary. Bar, 60 µm. Abbreviations: dc, diencephalon; hb, hindbrain; mb, midbrain; y, yolk.
from hlx-1 and the pax[b] gene. The latter is expressed within two transversal stripes located in the posterior midbrain region which fuse at the dorsal midline in 10 hpf embryos (Krauss et al., 1991b). At later stages, the posterior boundary of the pax[b] expression domain coincides with the furrow separating the midbrain and hindbrain. In double labelled 9 hpf embryos (Fig. 2I), the posterior border of the hlx-1 domain coincided with the site at the dorsal midline where the pax[b] stripes will eventually fuse. This also showed that hlx-1-expressing cells migrate much faster towards the dorsal midline than the neurectodermal, pax[b]-positive cells. At the 10 hour stage, the hlx-1 expression domain showed its maximum posterior extension (Fig. 2J). In these embryos, the posterior border of the hlx-1 stripe was located at the same anteroposterior level as the fusing pax[b] stripes.

At 12 hpf, the hlx-1 gene was expressed within a region of about three cell layers underneath the diencephalon (Fig. 3C). The expressing cells were located in a ventral protrusion (Fig. 3C), called the ‘polster’, which according to Kimmel et al. (1990), may be equivalent to the prechordal plate of amphibians (Meier, 1981; Ballard, 1982). In rostral cross-sections of 16 hpf embryos, hlx-1 transcripts were also detected at the ventral midline (Fig. 3D), but the expression domain mainly included ventral parts of the diencephalon.

### Embryonic expression in the hindbrain and spinal cord

Neural hlx-1 expression in the hindbrain and spinal cord regions was first observed at 10-11 hpf (Figs 4A, 5A). At this developmental stage, two bilateral clusters of cells expressing the hlx-1 gene appeared at a rostrocaudal level which corresponded to the future location of the fifth rhombomere (Mi2; Hanneman et al., 1988; Trevarrow et al., 1990). Two rows of cells which expressed the gene at a somewhat lower level, extended posteriorly from the two hindbrain clusters. By 12 hpf, stronger hybridization signals were detected within a transversal stripe in the region corresponding to the position where the two bilateral clusters were seen earlier (Figs 4B, 5B). Even though this stripe may correspond to a presumptive rhombomere (Mi2), which will eventually become visible at 15-16 hpf (Fig. 8B; Trevarrow et al., 1990), the transcripts were clearly restricted only to particular cells at a specific level in the dorsal part of the basal plate (Fig. 4B). Relatively strong hybridization signals extended posteriorly from the anterior end of the spinal cord in 12 hpf embryos (Fig. 4B,C). This expression domain span the neural keel and included only cells in the dorsal part of the basal plate.

At 15-16 hpf three transversal stripes of different signal intensities were present in the anterior part of the hindbrain (Figs 4D, 5D,E and 8B). Similar to the stripe in the region of the Mi2 rhombomere, expression was restricted to a specific dorsoventral level (Fig. 4D). To determine further whether the anterior hindbrain stripes would correlate with the segmental rhombomeric pattern, hlx-1 stained embryos were double labelled with the monoclonal antibody zn-12. This antibody, which recognizes similar or identical cell surface glycoproteins as HNK-1, labels the second hindbrain rhombomere (Ro2) and the trigeminal ganglia flanking Ro1 at 15-16 hpf (Fig. 5F; Metcalfe et al., 1990; Mikkola et al., 1992; Trevarrow et al., 1990). In double-labelled embryos, the zn-12 stripe (Ro2) did coincide well with the second (and strongest) hlx-1 stripe. As the first (weak) and third stripes were of similar width, they may correspond to Ro1 and Ro3, respectively. The strong transversal stripe first detected at 12 hpf was still present at 15-16 hpf and was separated from the Ro3 stripe by a nonlabelled region which was about one segment wide. Also, the spatial correlation between this stripe and the otic placodes suggested that it was located in the fifth rhombomere (Mi2). However, it is probable that this rather narrow stripe included only the anterior two thirds of Mi2. Further posteriorly, some hlx-1 expression was also present in the anterior part of the sixth rhombomere (Fig. 5D,E).

Following the 16 hpf stage, hlx-1 transcripts appeared at a similar dorsoventral level in the remaining part of the hindbrain and the expression level increased. As a consequence, two bilateral columns of expressing cells were detected along the entire hindbrain at 19 hpf (Fig. 4E). Cross-sections and hori-
Horizontal views of the hindbrain of 22 hpf embryos (Fig. 6A,B) showed that the columns (2-3 cell layers), which were located in the dorsal region of the basal plate (Fig. 6B), spanned the walls of the neural tube. At later stages, each longitudinal column started to transform into a series of transversal stripes, generating a repeated pattern of paired stripes by 30 hpf (Figs 6C,E, 8B). The individual stripes were 2-3 cell diameters wide and extended from the luminal side into the marginal zone. A similar segmental expression pattern was observed in the hindbrain at 45 hpf (Fig. 6D). However, at this developmental stage, the stripes had narrowed up to a width of 1-2 cell diameters. Interestingly, the spacing between each pair of hlx-1 stripes correlated well with the width of hindbrain rhombomeres, and the position of the first pair seemed to coincide with the boundary between the two anterior-most rhombomeres (Hanneman et al., 1988; Trevarrow et al., 1990). Consistent with this, the proposed location of Mi2 was directly adjacent to the otic vesicle (Fig. 6E). It is also noteworthy that only a single transversal stripe of lower staining intensity was located at the presumptive boundary between the first rhombomere (Ro1) and the cerebellar anlage (Fig. 6E). Therefore, also with respect to the hlx-1 gene, this border is distinguishable from the interrhombomeric boundaries.

We further confirmed the correlation between the double stripes and rhombomere borders by visualizing the segmental arrangement of reticular neurons in 30 hpf embryos with hlx-1/zn-12 double labelling. The clustered location of the zn-12 labelled neurons in the rhombomere centers (Hatta, 1992; Trevarrow et al., 1990) were consistent with the proposed pattern of paired hlx-1 stripes in the border regions (Fig. 6G).

Similar to earlier developmental stages (Fig. 4C,D), expression of hlx-1 in the spinal cord of 19 hpf embryos was restricted to dispersed cells within a column in the dorsal part of the basal plate (Fig. 4E,F). Moreover, this expression domain, which extended to the tip of the tail, was directly contiguous with the hlx-1 staining localized in the hindbrain. Cross-sections of the spinal cord of both 16 and 22 hpf embryos (Figs 5H, 6I) revealed the presence of hlx-1-expressing cells within a layer of 1-2 cells extending through the wall of the neural tube, indicating that both mitotic and postmitotic cells were included. Clearly, hlx-1 transcripts were present only within a subpopulation of the cells localized in this column (Figs 3F, 6H). Double labelling experiments with hlx-1 and the zn-12 antibody showed that the zn-12-positive Rohon Beard neurons, in the alar plate (Metcalfe et al., 1990), were localized about two cell diameters dorsal to the hlx-1-expressing cells (Fig. 5G).

Fig. 4. Whole-mount in situ hybridization analysis of hlx-1 expression of different developmental stages. Side views (anterior to the left) of 11, 12, 13, 16, 19 and 30 hpf are shown in A, B, C, D, E and G, respectively. The spinal cord of the 19 hpf embryo is shown at high magnification in F. Arrows indicate the location of the midbrain-hindbrain boundary and open triangles mark the position of rhombomere-restricted sites of expression in the hindbrain. Bar, 60 μm. Abbreviations: dc, diencephalon; e, eye; fb, forebrain; fp, floor plate; hb, hindbrain; mb, midbrain; nc, notochord; sc, spinal cord; tc, telencephalon; y, yolk.
Multiple sites of *hlx-1* expression in the rostral brain

In the rostral brain, expression of the *hlx-1* gene was initiated later (13-14 hpf) than in the epichordal division of the CNS (not shown). By the 16 hpf stage, sites of expression were present in both the midbrain and the ventral part of the diencephalon (Fig. 4D). A somewhat longitudinal expression domain was seen in the presumptive tegmental region of the midbrain and this extended further into a column by 19 hpf (Fig. 4E). Thus, at this stage, longitudinal expression domains with similar dorsoventral restrictions were present in both the hindbrain and midbrain. Additional hybridization signals were observed at a site in the rostral midbrain which coincided well with a particular group of cells associated with the posterior commissure (see Discussion).

A small region of *hlx-1* expression was detected near the posterior end of the prechordal stripe at 15-16 hpf (Fig. 4D). By the 22 hpf stage, this domain had expanded into a transversal stripe of high signal intensity in the diencephalon (Fig.
Expression of zebrafish hlx-1 gene

Unlike the other sites of hlx-1 expression in the rostral brain, this domain was sharply defined and included all the cells in the wall of the neural tube (Fig. 7B,D).

After the 22 hpf stage, several features of the hlx-1 expression pattern in the rostral brain were changed. In 30 hpf embryos, expression was still present in the tegmental region of the midbrain, but additional hlx-1 transcripts were detected in cells scattered throughout most of the tectum (Fig. 7E,F).
Moreover, the rostral part of the longitudinal midbrain domain which at 22 hpf also included a small area of the posterior forebrain, had become a regionally restricted expression domain of high signal intensity. The major part of this domain was located in the posterior diencephalon (Fig. 7E) and analysis of cross-sections revealed that this site corresponded to a region in the dorsal thalamus (Fig. 7G). Another noteworthy feature was the direct correlation between its ventral border and the sulcus at the dorsal/ventral thalamic junction (Fig. 7G).

During development from 22 to 30 hpf, the regional hlx-1 domain in the ventral diencephalon had extended along the original anteroposterior axis. However, this extension appeared to be directed ventrally as a consequence of the ventral bending of the hypothalamic tissue. Analysis of cross-sections showed that the diencephalic domain included a part of the thalamus and extended into the dorsal region of the hypothalamus (Fig. 7G,H).

**DISCUSSION**

We have cloned a gene that is expressed in all major subdivisions of the embryonic CNS. A detailed analysis of the spatial distribution of hlx-1 transcripts during embryogenesis by whole-mount in situ hybridization and double labelling with other markers revealed a particularly dynamic expression pattern in the hindbrain and prechordal plate, suggesting multiple functions for this gene during early stages of CNS development.

**Early embryonic expression in the prechordal plate**

In a way similar to the zebrafish pax[b] and eng-2 genes (Krauss et al., 1991b; Fjose et al., 1992), transcripts of the hlx-1 gene are first detected in the presumptive rostral region of late gastrula embryos. However, by contrast to these genes, which are first
expressed as two lateral transversal stripes that converge toward the dorsal midline during neurulation, the hlx-1 expression domain is transformed from a circular area to a longitudinal stripe located in the hypoblast. The formation of the hlx-1 stripe occurs before fusion of the transversal pax[b] stripes at the dorsal midline. Several features of the expression pattern indicate that the hlx-1-positive cells are, at least in part, located in the prechordal plate. Initially, transcripts are detected within a circular area near the rostral end of the embryo which may be the equivalent of the circular prechordal plate observed in higher vertebrates at the corresponding developmental stages (Meier 1981; Ballard, 1982). Although the prechordal plate of zebrafish has not been unequivocally identified as a specific group of cells, the hypoblast beneath the forebrain forms a prominent bulge ("polster") at 11-12 hpf, which probably corresponds to this tissue (Kimmel et al., 1981; Meier, 1981). The ventral bending of the hlx-1 stripe observed in the presumptive midbrain region of 9-10 hpf embryos, seems to reflect a thickening of the brain rudiment and simultaneous formation of the underlying polster. In the region posterior to this bending, the gene appears to be expressed both in the hypoblast and ventral parts of the epiblast. The hlx-1 gene, which after the 15 h stage is expressed along the ventral midline of the diencephalon, may contribute to the patterning of the rostral brain. In support of this suggestion, it has been shown that proper induction of the forebrain in *Xenopus* embryos requires vertical signals from underlying axial mesoderm (Ruiz i Altaba, 1992). Moreover, analyses of the zebrafish cyc-1 mutant have revealed a dependence of ventral midline cells of the rostral brain on signals emanating from the prechordal plate (Hatta et al., 1991). Interestingly, the ventral midline cells of the brain seem to have an expanded organizational function (Hatta et al., 1991; Hatta, 1992) in the rostral region where hlx-1 expression remains until 20 hpf. Thus hlx-1 may be involved in the control of both rostrocaudal and dorsoventral differences in the prechordal division of the brain.

Several genes have recently been identified which are active both in the prechordal plate and other mesodermal tissues during early stages of development. Among these, the two *Xenopus* transcription factor encoding genes goosecoid and XFKH-1 are of particular interest as they are also active very early in the dorsal lip (Cho et al., 1991; Dirksen and Jamrich, 1992) and may thus be located upstream of hlx-1 in a regulatory cascade.

As far as the closely related murine Dlx and chicken CHoxE genes are concerned, prechordal plate expression has not been reported (Lu et al., 1992; Rangini et al., 1991). This could be due to the reduced size of the prechordal plate in mouse embryos (Tam et al., 1982) and/or that early stages were not analysed in detail by in situ hybridization.

**Dorsoventral restriction of hlx-1 expression in the hindbrain and spinal cord**

The hlx-1 gene is expressed in single cells within longitudinal columns in the spinal cord at early embryonic stages. During the early stages of neurogenesis the zebrafish spinal cord is known to contain a small number of neurons that are organized in a very simple manner (Bernhardt et al., 1990; Kuwada and Bernhardt, 1990). The individual hemisegments in 18-20 h embryos contains approximately 18 postmitotic neurons, and these are identifiable on the basis of their unique locations, sizes and axonal outgrowth (Bernhardt et al., 1990).

Within the spinal cord region, hlx-1 expression appears in dispersed cells at a dorsal level in the basal plate 4-5 hours before the first postmitotic neurons start to project axons (Kuwada and Bernhardt, 1990), suggesting that hlx-1 plays a role in the determination of specific subsets of neurons. A similar function has been proposed for the zebrafish *pax[b]* gene which is known to initiate expression in precursors of commissural secondary ascending interneurons (CoSA) at 13 hpf (Mikkola et al., 1992). However, expression of hlx-1 is initiated earlier than *pax[b]* and the transcripts seem to be present in both mitotic and postmitotic cells. As judged from differences in dorsoventral position and cell numbers per hemisegment, the hlx-1-positive cells do not correspond to any of the other types of neurons which have been described in 20 hpf embryos (Bernhardt et al., 1990). The identities of the additional postmitotic neurons that have not projected axons at this early stage are not known but may include three subpopulations of commissural neurons, which are first visible at 22-23 hpf (Bernhardt et al., 1990). The hlx-1 labelled cells could correspond to precursors of one or more of these three types of interneurons. Alternatively, expression of hlx-1 may be more directly linked to dorsoventral patterning and possibly the formation of the border between basal- and alar plate.
Expression in the embryonic hindbrain and spinal cord is initiated simultaneously at comparable dorsoventral levels in the neural keel of 10 hpf embryos, indicating a response to the same signals. Differences are observed later when the hindbrain becomes subdivided into segmental units. Thus at 12 hpf, a transversal stripe of *hlx-1* expression appears at a specific dorsoventral level in the region of the presumptive Mi2 rhombomere. Similarly, dorsoventrally restricted transversal stripes correlate with the three most anterior hindbrain segments at 15-16 hpf. This pattern shows that *hlx-1* expression follows the maturation of the rhombomeres where it may act as a determinant of the dorsoventral polarity. Several members of other vertebrate gene families, including *Krox20* (Wilkinson et al., 1989), cytoplasmic retinoic acid binding proteins (Ruberte et al., 1992), *seven-up* related nuclear receptors (Fjose et al., 1993) and some *Hox* genes (McGinnis and Krumlauf, 1992), are known to have rhombomere specific expression domains at equivalent embryonic stages. However, in these cases the expression domains span most of the rhombomeric units.

Later during embryogenesis, a repeated pattern of paired *hlx-1* stripes appears which seems to indicate a subsegmental organization of hindbrain rhombomeres, as already proposed by Trevarrow et al. (1990). According to this proposal, two regions repeat in an alternating pattern along a series of seven segments. While the rhombomere centers contain the first basal plate neurons to develop, the segment boundaries contain the first neurons to develop in the alar plate. This model is partially based on the staining pattern of the monoclonal antibody, zn-5, which starts to label neurons near the segment borders at 22 hpf (Trevarrow et al., 1990). Interestingly, the zn-5 labelling observed in the hindbrain of 48 hpf embryos is very similar to the expression patterns of *pol-1*, a comparable developmental stage suggesting that the same commissural interneurons are stained in both cases. Alternatively, the *hlx-1* gene may be expressed in a different subset of neural cells in the rhombomere border regions. In any case, the *hlx-1* gene is the first identified regulatory gene that reveals the segmental subdivisions proposed for the hindbrain rhombomeres. Although the closely related murine *Dbx* and chicken *CHoxE* genes have similar dorsoventrally restricted expression patterns, correlations with hindbrain segmentation were not observed (Lu et al., 1992; Rangini et al., 1991).

**Regional and cell type specific expression in the rostral brain**

Several different types of *hlx-1* expression domains are present during development of the rostral brain. One of these which initially shares some features with the hindbrain and spinal cord expression patterns, extends throughout the tegmental region of the midbrain. The similarities with respect to the dorsoventral location and the time of induction indicate that control mechanisms responsible for setting up the *hlx-1* expression patterns in these regions are partially the same. Consequently, the *hlx-1* gene could be involved in the determination of similar subsets of neurons in the midbrain, hindbrain and spinal cord. Another possibility is that the expression of *hlx-1* is generally associated with the formation of borders, since the transcripts are detected in cells located near the borders between rhombomeres and alar/basal plate.

An additional site of *hlx-1* expression which includes only a small group of cells, is located dorsally in the anterior part of the midbrain at 19 hpf and older stages. The position of these cells corresponds to the location of the nuc PC (nucleus of the posterior commissure) neurons which at 20 hpf project axons along the posterior commissure located at the forebrain-midbrain border (Chitnis and Kuwada, 1990). The *hlx-1* gene may thus play a role in the ontogenesis of this particular subset of midbrain neurons.

In 22 hpf embryos, the highest level of *hlx-1* expression in the rostral brain is located within a sharply defined region in the ventral part of the diencephalon. This domain which is first detected at 15 hpf, is likely to reflect an early regionalizing function of the *hlx-1* gene. However, partly due to the observed spatiotemporal changes, it is unclear whether this expression domain can also be correlated with the segmental subdivisions which have been proposed for the embryonic chick diencephalon (Figdor and Stern, 1993).

An additional *hlx-1* domain with regional features appears near the forebrain-midbrain border at a later developmental stage. The ventral boundary of this area, which is mainly located in the diencephalon, coincides with the morphological border (sulcus) separating the dorsal and ventral thalampus. This correlation suggests an involvement of *hlx-1* in the formation of the dorsoventral thalamic border.

On the basis of these features, it is likely that the *hlx-1* gene plays a role in several different aspects of regionalization, differentiation and border formation in the rostral brain. Similar functions have been proposed for other homeobox genes including members of the *Pax, Dlx, Emx* and *Otx* sub-families, which also have regional expression patterns in the forebrain (Krauss et al., 1991a,b,c; Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Simeone et al., 1992). Therefore, in the context of rostral brain development, *hlx-1* is likely to be part of a combinatorial system of homeobox genes.

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Expression of zebrafish hlx-1 gene


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