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Original Article

Expression of *Hoxb-1* during gastrulation and segmentation stages of carp *(Cyprinus carpio)*

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ABSTRACT This report describes the cDNA sequence and embryonic RNA expression pattern of carp *Hoxb-1*. Carp *Hoxb-1* is a *labial*-like, homeobox-containing gene of the 3' end of the *Hox* gene cluster. The expression pattern in carp is compared to that of homologs in other vertebrates. As holds for other *Hox* genes, carp *Hoxb-1* is expressed with highest intensity at a sharp anterior boundary, and expression fades out towards posterior. At later stages, gaps were found in the domain. The gene is expressed from late gastrulation onwards, first mainly in the hypoblast but later in all germ layers. Its most prominent expression area is rhombomere 4 (r4) of the hindbrain. Transcripts were also found in the neural tube, mesoderm (lateral, head and presomite), epidermis and neural crest. At 30 hours post fertilization, *Hoxb-1* was still expressed in r4, in the anterior trunk neural tube and in the branchial arches posterior to r4. *Hox* genes are thought to be involved in the specification of positional values along the embryonic anterior-posterior axis, and *Hoxb-1* expression in r4 is supposed to be important for specifying the unique identity of this hindbrain segment. The conserved expression in r4 suggests that this is also true for carp *Hoxb-1*.

KEY WORDS: *carp, zebrafish, homeobox gene, Hoxb-1, development*

Introduction

Homeobox-genes are widely assumed to play an important role in creating regional diversity along the embryonic a-p (anterior-posterior) axis by providing positional values (see reviews by Gehring *et al.*, 1994; Keynes and Krumlauf, 1994; Krumlauf, 1994). Genes belonging to this family encode proteins with a homeodomain, a DNA-binding motif of 60 aa (amino acids) that regulates the transcription of target genes (Gehring *et al.*, 1994). After having been discovered in the Bithorax and Antennapedia gene complexes of the fruitfly, homeobox-genes were found in many other species (reviewed in Ruddle *et al.*, 1994). The vertebrate homologs of these genes are the *Hox* genes, present as four paralogous clusters: *Hoxa*, *Hoxb*, *Hoxc* and *Hoxd* (see Krumlauf, 1994), each cluster containing up to 13 genes.

The spatial and temporal order of expression of *Hox* genes along the a-p axis of the embryo correlates with their order along the chromosome (colinearity rule, see Duboule and Morata, 1994). In the embryonic nervous system, a prominent site of expression of *Hox* genes, the anterior border of expression is sharp and lies in the spinal cord for the paralogous groups 5-13 (5' parts of the clusters) and in the hindbrain for the groups 1-4 (3' parts of the clusters) (Krumlauf *et al.*, 1993).

Hox genes were also identified in fishes (Ericson *et al.*, 1993; Molven *et al.*, 1993; Marshall *et al.*, 1994; Pöpperl *et al.*, 1995; Alexandre *et al.*, 1996), but of the anteriorly expressed *Hox* genes only the expression pattern of *Hoxa-1* (Alexandre *et al.*, 1996) has been described until now. In a search for *Antennapedia*-related homeobox genes (Stroband *et al.*, 1995) in carp (*Cyprinus carpio*), a cyprinid teleost related to the zebrafish, we isolated *Hoxb-1*. This paper describes the sequence and expression pattern of the gene during gastrulation and segmentation of carp. As in chicken (Sundin and Eichele, 1990; Sundin *et al.*, 1990), mouse (Murphy *et al.*, 1989; Frohman *et al.*, 1990) and *Xenopus* (Godsave *et al.*, 1994), the anterior-most expression of the gene is found in a restricted region of the hindbrain. Thus, *Hoxb-1* might be a useful marker in future studies of hindbrain segmentation in fish.

Results

Sequence analysis of carp Hoxb-1

An Antennapedia-derived homeobox-specific probe was used to screen a carp early segmentation stage cDNA library (Stroband *et al.*, 1995). One of the isolated clones had a length of 1852 bp (base pairs), with an open reading frame. It encoded a 316 aa protein related to *Drosophila labial*. The nucleotide and

Abbreviations used in this paper: aa, amino acids; a-p, anterior-posterior; UTR, untranslated region; ISH, *in situ* hybridization; YSL, yolk syncytial layer; r, rhombomere; DLT, dorsal longitudinal tract; p.f., post fertilization; CNS, central nervous system.

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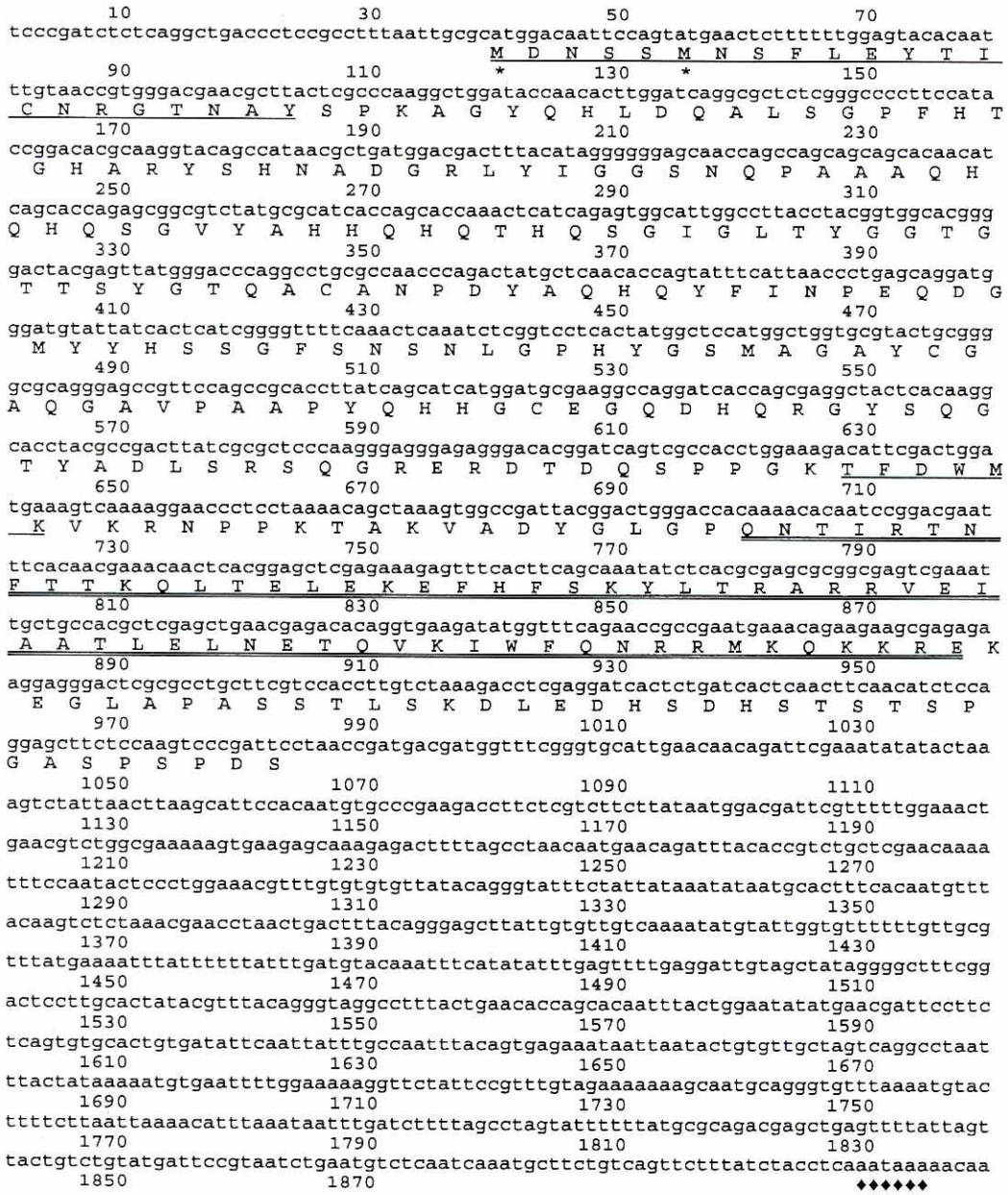

Fig. 1. Nucleotide and deduced amino acid sequence of carp *Hoxb-1*. *The first underlined sequence represents the conserved N-terminus and the second underlined sequence is the conserved hexapeptide. The two translation initiation sites are marked by asterisks. The homeodomain is double underlined. The polyadenylation signal is indicated by ◆. The sequence is available from the EMBL database under Accession number X91079.*

deduced aa sequences are shown in Figure 1. The clone had a short 5' UTR (untranslated region) defined by two potential translation initiation sites in frame with the homeodomain. The first start site was located in a suboptimal context (a C at position -3; Kozak, 1986) and was not a likely initiator of translation. In this sense it resembled the first start site of the mouse *Hox1.6 (Hoxa-1)* gene (Larosa and Gudas, 1988). The second start site (5 aa downstream) in the carp gene contained an A at the -3 position, which created a favorable context for translation (Kozak, 1986). The 3' UTR of 865 nucleotides showed a polyadenylation signal sequence (aataaa; Fig. 1) indicating that the 3' UTR was complete.

Sequence analysis revealed that the clone was most closely related to the *labial* class of genes of the vertebrate *Hox* cluster, paralogous group 1. The deduced homeodomain of the carp protein had 92% identity to the *labial/Hox-1* homeodomain consensus sequence (Fig. 2A). The presence of a T at position 37, and an E instead of a Q at position 39 of the homeodomain (Fig. 2A) suggested that the carp gene belongs to the *Hoxb* complex (see Gehring *et al.*, 1994). We therefore named this clone *Hoxb-1*, in accordance with the nomenclature suggested by Scott (1992). The homeodomain of carp *Hoxb-1* was highly similar to that of other species (Fig. 2A), especially chicken and *Xenopus* (97% aa identity). Mouse *Hoxb-1* and human *HOXB-1* homeodomains were both 88% identical to the carp *Hoxb-1* homeodomain. Outside the homeodomain, two other regions of high similarity between *Hoxb-1* of carp and other species existed. At the amino terminus (aa 1 to 22), fourteen aa were conserved between the fish gene product and the chicken, mouse and human proteins (Fig. 2B). Upstream of the homeodomain, 100% identity was

A.

labial/Hox-1 consensus	PNAIRTNFTT KQLTELEKEF HFNKYLTRAR RVETAATLQL NETQVKIWFQ NRRMKQKKRE
carp *Hoxb-1*	Q-T------- ---------- --S------- ---I----E- ---------- ----------
Xenopus Hoxb-1	Q-I------- ---------- ---------- ---I----E- ---------- ----------
chicken *Hoxb-1*	--T------- ---------- ---------- ---I----E- ---------- ----------
mouse *Hoxb-1*	-GGL------ R--------- ------S--- ---I----E- ---------- ----------
human *HOXb-1*	-SGL------ R--------- ------S--- ---I----E- ---------- ----------

B.

carp *Hoxb-1*	MDNSSMNSFL EYTICNRGTN AY
chicken *Hoxb-1*	---TR----- --A------G --
mouse *Hoxb-1*	--YNR-S--- --PL----PS --
human *HOXb-1*	--YNR----- --PL----PS --

C.

carp *Hoxb-1*	TFDWMKVKRN PPKTAKV
chicken *Hoxb-1*	---------- -------
mouse *Hoxb-1*	---------- -------
human *HOXb-1*	---------- -------

Fig. 2. Comparison of conserved regions in *Hoxb-1* sequences. (A) *Comparison of the deduced homeodomain sequences of Hoxb-1 from carp, Xenopus (Dekker et al., 1992), chicken (Sundin et al., 1990), mouse (Frohman et al., 1990), human (Acampora et al., 1989), and the labial/Hox-1 homeodomain consensus sequence (Gehring et al., 1994).* **(B)** *Comparison of the deduced amino-terminal regions of carp, chicken, mouse and human Hoxb-1.* **(C)** *Comparison of the deduced hexapeptide regions of carp, chicken, mouse and human Hoxb-1.*

found in the *labial* class hexapeptide (TFDWMK: Bürglin, 1994; encoded by nucleotides 628 to 645 in carp) and the following 11 aa (Fig. 2C).

Early Hoxb-1 expression

During the process of gastrulation in carp and zebrafish, the blastoderm, originally located at the animal side of the yolk cell, thins and expands over the yolk cell by epiboly. After 50% epiboly, cells at the margin of the blastoderm involute and form the hypoblast layer. The outer cells form the epiblast. During completion of epiboly, involution continues while cells in the hypoblast and epiblast converge towards the midline thereby causing the embryonic axis to extend along the a-p axis. At the end of gastrulation, the epiblast layer is the future ectoderm. The hypoblast cells will give rise to the mesendoderm.

At early gastrulation stages, no RNA expression of *Hoxb-1* was observed. When 80% of the yolk was covered by the blastoderm (80% epiboly), weak *Hoxb-1* expression was detected near the equator of the egg, in two bands separated by the non-expressing midline. Since the YSL (Yolk Syncytial Layer) was heavily stained at many stages of carp development (Fig. 4E,F), the early expression (whole-mount) could more easily be visualized in zebrafish (Fig. 3A). In sections of carp embryos we observed that the *Hoxb-1* expressing cells were found primarily in the hypoblast layer at this early stage (Fig. 4A). During the progression of gastrulation, epiblast expression increased while the overall expression domain extended in a-p direction (Fig. 3B). At the bud stage (Kimmel *et al.*, 1995), the *Hoxb-1* domain occupied the middle third region of the body axis. It showed a sharp anterior boundary and decreasing expression towards posterior. The most intense labeling at this stage was found in the epiblast, at the lateral borders of the expression domain (Fig. 3B).

Expression during segmentation

During carp segmentation, epiblast and hypoblast cells continue to accumulate in the midline, due to convergent extension movements as have been described for *Fundulus* (Trinkaus *et al.*, 1992). As a result, the *Hoxb-1* expression was gradually gathered in a narrower region. Early in segmentation, the neural plate develops into the neural keel and subsequently into the neural rod by convergent movements of neural progenitor cells (described for zebrafish by Papan and Campos-Ortega, 1994). In the hypoblast layer, the notochord becomes distinct and somites form in the paraxial mesoderm in an anterior to posterior order. The first pair of somites arises at approximately 12 h after fertilization at a position approximately halfway along the a-p axis in carp. The anterior half of the carp embryo is occupied by the developing head.

At the onset of segmentation, the *Hoxb-1* pattern was like that of the bud stage, except the anteriormost expression. At the anterior border of the *Hoxb-1* domain, intensely stained epiblast cells formed a transverse band (Fig. 3C). The band was situated at the level of the future hindbrain and it is supposed that cells in the band are probably neural progenitor cells based on the localization of these cells in zebrafish (Schmitz *et al.*, 1993). In other vertebrates, *Hoxb-1* is expressed in r4 (rhombomere 4) (see references in the discussion). To confirm that this is also true for the carp, we performed double ISHs (*in situ* hybridizations) with zebrafish *Krox-20*, which is expressed in r3 and r5 (Oxtoby and Jowett, 1993). From the 1 somite stage up to late segmentation (Fig. 3F), *Hoxb-1* was expressed between the *Krox-20* r3 and r5 bands, as expected.

At the 8 somite stage, the dorsalmost cells in r4 appeared negative (Fig. 4B) whilst a small area of expression was present in the dorsalmost part of r3 (Figs. 3D, 4B). By the 15 somite stage, this latter expression had disappeared (compare Figs. 3D and 3E, 4B and 4F). The distribution of transcripts in r4 was homogeneous at the 8 somite stage, but at 15 somites the dorsal part of r4 expressed *Hoxb-1* at a higher level than ventral areas (Fig. 4E), including the floor plate. Ventrally, only a paired laterally located group of cells was intensely stained (Fig. 4E). This group may represent developing primary neurons, which

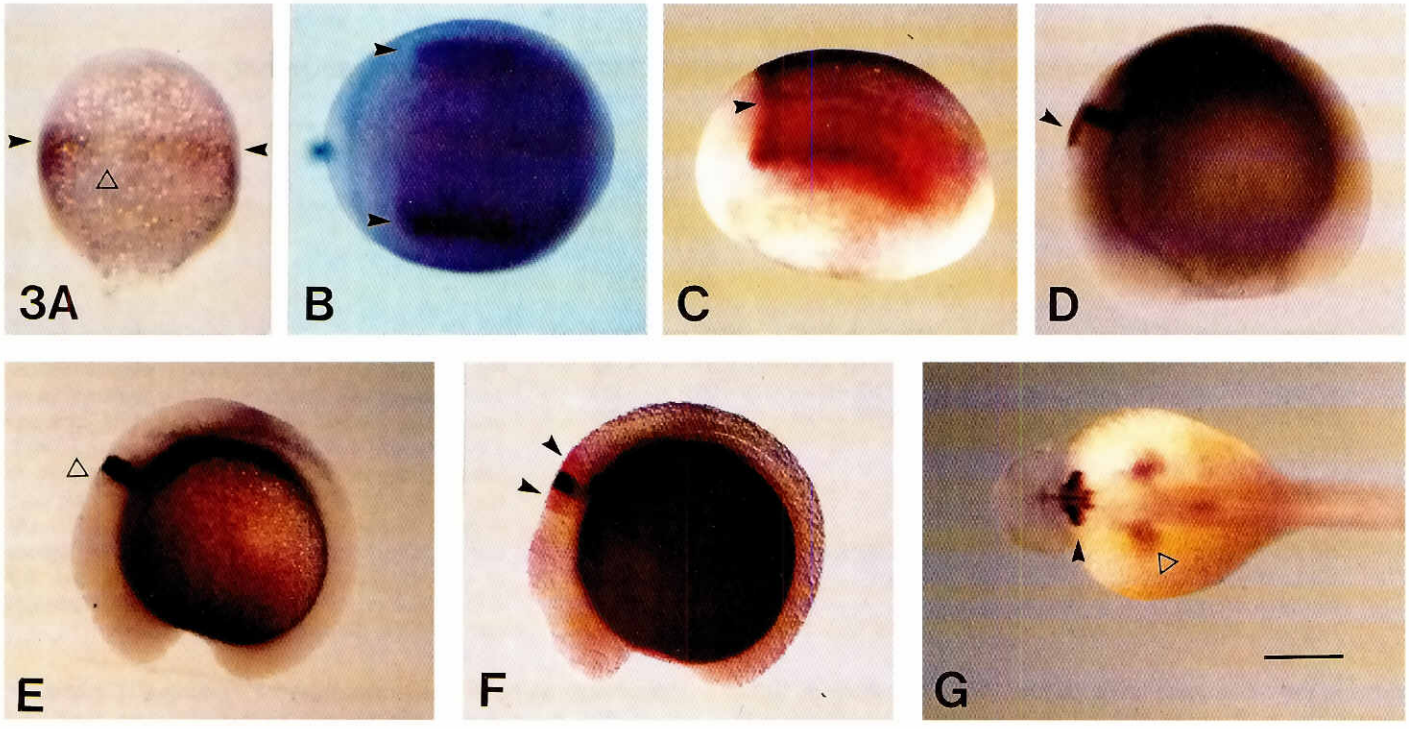

Fig. 3. Expression of carp *Hoxb-1* during late gastrulation, segmentation and early pharyngula stages in whole-mount *in situ* hybridizations (ISH) in carp (B-G) and zebrafish (A). **(A)** *Late gastrulation stage zebrafish embryo with the animal pole to the top. The black arrowheads point at the expression on either side of the midline (open arrowhead points at the midline).* **(B)** *0 somite stage carp, dorsal view with anterior to the left. The arrowheads point at the lateral stripes in the epiblast.* **(C)** *1 pair of somites, dorsolateral view with anterior to the left. The arrowhead points at the transverse band of expression in the epiblast.* **(D)** *8 pairs of somites, lateral view with anterior on the left side. Dorsalmost cells in r3 are stained (arrowhead).* **(E)** *15 pairs of somites, lateral view with anterior on the left. Dorsal cells in r3 no longer express Hoxb-1 (open arrowhead).* **(F)** *As E, double ISH of carp Hoxb-1 (brown staining) in r4, and zebrafish Krox-20 (orange staining) in r3 and r5 (arrowheads).* **(G)** *Dorsal view of carp embryo at 30 h p.f. The black arrowhead points at the r4 expression. The open arrowhead points at the bilaterally symmetrical structures lateral of the neural tube. Bar, 275 μm.*

are found at this position in zebrafish (Hanneman *et al.*, 1988). Dorsolateral to the neural rod in r4, expression was found in a group of cells resembling migrating neural crest cells (Fig. 4E). Posterior to r4, the neural rod in the hindbrain did not express *Hoxb-1* at the stages of 8 and 15 somites (Fig. 4B,C,F). In the trunk the neural rod contained only a weak signal at the 8 somite stage, but at 15 somites this expression was more intense (Fig. 4F). At the level of the first somite (hindbrain-spinal cord transition), it had a sharp anterior boundary and faded out towards the tail. As in r4, the transcripts in the trunk neural rod were distributed in a dorsoventral gradient (not shown). Furthermore, at regular intervals along the a-p axis, as far anterior as r4, cells at lateral positions in the neural rod were intensely stained (Fig. 4F). These cells might be neurons of the dorsal longitudinal tract (DLT), as described for zebrafish by Ross *et al.* (1992). In some sections of the trunk at the 15 somite stage, labeled groups of cells were observed between the somites and the dorsal ectoderm. They could be neural crest cells (see Raible *et al.*, 1992).

At 30 h p.f. (post fertilization) the *Hoxb-1* expression in neural tissue was mainly restricted to r4 and the neural tube in the anterior trunk (Fig. 3G). A dorsoventral gradient could still be observed (not shown). At 52 h p.f., faint labeling was detected only in r4, after prolonged staining.

Expression of Hoxb-1 outside the CNS

Expression of *Hoxb-1* was not confined to neural tissue, but appeared in cells of all germ layers. The strong expression in lateral areas, already seen at the bud stage (Fig. 3B), persisted through segmentation. As cells converged to the body axis, the expression in lateral areas was concentrated in a prominent stripe on both sides of the neural tissue (not shown). In the trunk at 8 somites, this stripe was composed of expression in lateral mesendoderm and in the epidermis above the somites (Fig. 4D). The somites were largely negative, but the presomitic mesoderm contained the signal (not shown). At the level of the posterior hindbrain, *Hoxb-1* expressing cells were present in head mesoderm and epidermis (Fig. 4C). At this stage, the expression pattern showed a gap between the level of r4 and the posteriormost part of the hindbrain (not shown). Transcripts had reappeared again in this region by the 15 somite stage. At the level of r4 at the 8 somite stage, the *Hoxb-1* signal was present in lateral mesoderm, and by 15 somites in the ventrolateral mesendoderm (Fig. 4E).

At 30 h p.f. only two symmetrical structures posterior to the otic vesicle represented expression outside the CNS (central nervous system) (Fig. 3G); sections revealed that these structures were ventrolaterally localized (Fig. 4G). They may correspond to outpocketings of the pharyngeal endoderm in the pha-

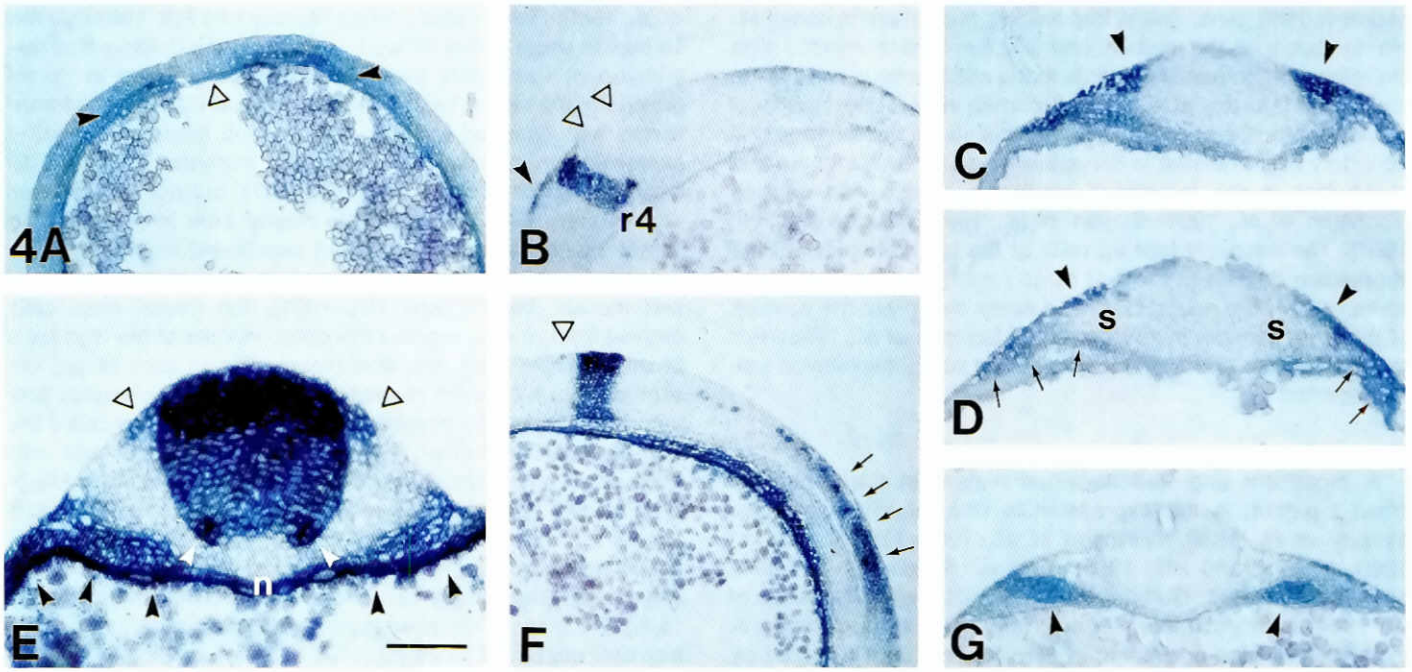

Fig. 4. Expression of *Hoxb-1* in sectioned carp embryos at gastrulation and segmentation stages. **(A)** *Horizontal section at the stage of 80-90% epiboly, dorsal is to the top left. The arrowheads point at the expression in the hypoblast. The midline (open arrowhead) does not express Hoxb-1.* **(B)** *Sagittal section through the neural rod at the 8 somites stage, with anterior to the left. The transverse band of expression is located in the future rhombomere 4 (r4). The black arrowhead points at the expression in the dorsalmost cells in r3. The hindbrain posterior to the future r4 does not express Hoxb-1 (open arrowheads).* **(C-D)** *Cross sections at the 8 somites stage.* **(C)** *Expression at the level of the hindbrain, posterior to r4. The arrowheads point at the expression in the lateral head mesoderm and epidermis, corresponding to the stripes on both sides of the neural rod in the whole-mount expression pattern.* **(D)** *Section at the level of the anterior trunk. The arrows point at the expression in the lateral mesendoderm, the arrowheads indicate the labeled epidermis, s, somite.* **(E)** *Cross section through rhombomere 4 at the stage of 15 somites. The black arrowheads point at the ventrolateral mesoderm; note that the YSL is also stained, but aspecifically. The white arrowheads point at the groups of cells resembling developing primary neurons, the open arrow head indicates the expression in cells resembling the migrating cells of the neural crest, n, notochord.* **(F)** *Sagittal section through the lateral side of the neural tube at the stage of 15 somites. The dorsalmost cells in r3 no longer express Hoxb-1 (open arrowhead). Intensely stained cells might be neurons of the dorsal longitudinal tract (arrows point at some of these cells).* **(G)** *Cross section at 30 h p.f. at caudal position of the otic vesicle. Hoxb-1 expression is found in leaf-like structures at ventrolateral positions (arrowheads). Bar: 65 μm in E, 99 μm in C, D, G, and 128 μm in A, B, F.*

ryngeal arches, as described for zebrafish by Schilling and Kimmel (1994). At 52 h p.f. no expression could be detected outside the CNS.

Discussion

Identification of carp Hoxb-1

Carp *Hoxb-1* could be identified because of the high degree of identity of its nucleotide and amino acid sequences to those of *Hoxb-1* of other vertebrates (mouse *Hox-2.9*: Murphy *et al.*, 1989; Wilkinson *et al.*, 1989; chicken *Ghox-lab*: Sundin *et al.*, 1990; *Xenopus Hoxb-1*: Godsave *et al.*, 1994; man *HOXB1:* Acampora *et al.*, 1989), especially to the homeodomains of chicken and *Xenopus* (97%). The presence of two other highly conserved areas further confirmed the homology to *Hoxb-1* genes of the other vertebrates. Furthermore, in all vertebrates, including carp, *Hoxb-1* has its anterior border of expression in the hindbrain, just like other members of the paralogous groups 1-4 (Krumlauf, 1994), and is specifically expressed in r4 (see references elsewhere in discussion), suggesting analogous functions as well. The *Hoxb-1* gene is a paralog of the *Drosophila labial* gene (Mlodzik *et al.*, 1988) and belongs to the paralogous 1 group (*Hoxa-1, Hoxb-1, Hoxd-1*) of the vertebrate clustered homeobox genes (*Hox* genes). The *Hoxb-1* gene is located at the 3' end of the *Hoxb* cluster, and is among the first *Hox* genes to be transcribed in developing embryos (Krumlauf, 1994). So far, for fishes limited information is available about *Hox* genes with anterior borders of expression in the hindbrain. The sequence and expression pattern of zebrafish *Hoxa-1*, a *labial* paralog like *Hoxb-1*, have been recently described in a study by Alexandre *et al.* (1996). In addition, 3' and 5' regions of the puffer fish *Hoxb-1* gene were identified, that are involved in the establishment of the expression pattern of the gene (Marshall *et al.*, 1994; Pöpperl *et al.*, 1995). This paper is the first to report the complete cDNA sequence and the RNA expression pattern of *Hoxb-1* in fish.

Early Hoxb-1 expression

Carp *Hoxb-1* transcripts were not detected until approximately 80% epiboly, which is slightly later than zebrafish *Hoxa-1*, expressed from 50% epiboly (Alexandre *et al.*, 1996). At this early stage, *Hoxb-1* was found mainly in the hypoblast. During completion of gastrulation, the transcription in the epiblast increased. At the end of epiboly both the hypoblast and the epiblast

expressed the gene, and in that respect the pattern is comparable to those of the mouse and chicken where *Hoxb-1* was expressed in the primitive streak in the epiblast as well as in the mesoderm (Murphy *et al.*, 1989; Frohman *et al.*, 1990; Sundin *et al.*, 1990; Murphy and Hill, 1991). At this stage, the anteriormost boundary of expression in the epiblast was at the same a-p level as that in the hypoblast, similar to mouse and chicken (Frohman *et al.*, 1990; Sundin *et al.*, 1990; Murphy and Hill, 1991). The intensely labeled cells at the lateral borders of the expression domain at the bud stage might correspond to progenitor cells of the neural crest and epidermis, given the position of these progenitors in zebrafish (see Schmitz *et al.*, 1993). For mouse, chicken and *Xenopus* this strong lateral expression was not reported.

Expression in neural tissue

A prominent and well-described feature of the vertebrate *Hoxb-1* pattern is the expression in (the future) r4 (mouse: Murphy *et al.*, 1989; Wilkinson *et al.*, 1989; Frohman *et al.*, 1990; Murphy and Hill, 1991; chicken: Sundin *et al.*, 1990; Maden *et al.*, 1991; Guthrie *et al.*, 1992; *Xenopus*: Godsave *et al.*, 1994; man: Acampora *et al.*, 1989). This characteristic r4-expression is also conserved in carp, as was demonstrated by the double ISH using *Krox-20*, which is expressed in r3 and r5 (Oxtoby and Jowett, 1993). Having been established at the onset of somitic segmentation at the anteriormost border of the expression region, the r4-signal persisted until after 30 h p.f. After the first appearance of the r4-band, the expression in other regions of the presumptive neural tube was low, until the 15 somite stage when *Hoxb-1* was present in the trunk anterior neural rod. A separation between the r4-signal and more posterior CNS expression was previously described for chicken *Hoxb-1* (Sundin and Eichele, 1990; Maden *et al.*, 1991). This separation makes *Hoxb-1* a useful marker for studies of segmentation of this part of the hindbrain. As was reported for the mouse (Frohman *et al.*, 1990), the *Hoxb-1* expression in the future r4 of carp was already established before the segments of the hindbrain were recognized morphologically (for zebrafish at 17 h; Hanneman *et al.*, 1988). The exact role of this and other *Hox* genes in the segmentation process is not clear yet. It has been proposed that *Hox* genes may play a role in the specification of the segment phenotype (Keynes and Krumlauf, 1994). The *Hoxb-1* gene may be involved in specifying the unique identity of r4 (Murphy *et al.*, 1989; Murphy and Hill, 1991; Guthrie *et al.*, 1992).

Recent work shed light on the regulatory mechanism behind the persistent expression in r4 (Studer *et al.*, 1994; Ogura and Evans, 1995; Pöpperl *et al.*, 1995). A conserved regulatory region of the *Hoxb-1* gene is supposed to control the maintenance of the r4-expression through a positive and direct autoregulatory feedback mechanism. In the light of the conserved nature of the mechanism and the conservation of the *Hoxb-1* r4-expression (Pöpperl *et al.*, 1995), it is very likely that this regulatory mechanism also applies to carp.

In the trunk neural tube at the 15 somite stage, the anterior limit of expression was at the level of the first pair of somites, with expression decreasing towards the posterior end of the embryo. In mouse and chicken, the trunk neural tube also showed transcription of the gene (Murphy *et al.*, 1989; Wilkinson *et al.*, 1989; Sundin *et al.*, 1990; Murphy and Hill, 1991). At the 15 somite stage and at 30 h p.f. we observed a dorsoventral distribution of transcripts showing highest expression in dorsal regions of the neural tube. This dorsoventral distribution of transcripts was detected in r4 as well. Such a gradient of *Hoxb-1* expression in neural tissue was also reported for day 9-10 mouse embryos (Murphy and Hill, 1991), but not for *Xenopus* and chicken. Dorsolateral to the neural tube in r4 at the 15 somite stage, carp *Hoxb-1* mRNA was found in the migrating cells of the neural crest. In chicken (Sundin and Eichele, 1990) and mouse (Murphy and Hill, 1991), the neural crest cells derived from r4 also express the gene. In spite of the high level of dorsal expression, the dorsalmost cells in carp r4 did not express *Hoxb-1*, which resembled the mouse expression pattern, that showed no transcripts in a dorsal structure called the roof plate (Murphy *et al.*, 1989). In carp, the ventralmost cells (including the floor plate) contained no or a low amount of *Hoxb-1* transcripts. Similarly, the mouse floor plate did not express *Hoxb-1* (Murphy and Hill, 1991). Not corresponding to this gradient however, groups of cells at ventrolateral positions in r4 (probably developing primary neurons) were intensely stained at the 15 somite stage. This aberration from the dorsoventral pattern was only described for carp.

The dorsoventral expression pattern of carp *Hoxb-1* in the neural tube might be attributed to ventralizing and dorsalizing signals, which originate from the axial mesoderm (Strähle and Blader, 1994) and the non-neural ectoderm (Dickinson *et al.*, 1995), respectively. Mouse *Hoxb* genes display a dorsoventral distribution of transcripts in the neural tube as well (*Hox-2*; Graham *et al.*, 1991). The genes displayed dynamic dorsoventral patterns. Between 12.5 and 14 days post coitum, the expression of all genes was restricted to dorsal positions in the neural tube, similar to carp *Hoxb-1*. It was suggested that the *Hoxb* expression patterns mirror the birth of major classes of neurons in the CNS, and that the *Hoxb* genes provide each successive class of newly-born neurons with anterior-posterior positional information.

Expression outside the CNS

Besides the expression in the CNS, carp *Hoxb-1* was also abundantly expressed in cells of the mesendoderm, epidermis and probably the neural crest. The overall expression pattern in these tissues changed as a result of convergence movements. The expression was initially found in a broad area on the dorsal side of the embryo but narrowed as the embryonic cells concentrated around the axis. A similarly changing expression pattern as a result of cell migrations was described for the zebrafish *engrailed-2* gene (Fjose *et al.*, 1992). In addition, the distribution of *Hoxb-1* mRNA changed as cells differentiated, for example the signal had largely disappeared in the somitic mesoderm.

In general, mouse and chicken *Hoxb-1* transcripts were found in tissues comparable to those in carp, for example in lateral plate and presomite mesoderm, head mesoderm and ectoderm (Frohman *et al.*, 1990; Murphy and Hill, 1991). In contrast to this, Godsave *et al.* (1994) reported for *Xenopus* that *Hoxb-1* was not expressed in mesodermal tissues at any of the developmental stages examined. In this respect *Xenopus* expression resembles that of the ascidian *Halocynthia roretzi Hox-1* gene *(HrHox-1)*

which is expressed exclusively in ectodermal tissues in early embryogenesis (Katsuyama *et al.*, 1995).

Another difference in expression was observed for the gut. In mouse and chicken the gene was expressed in tissues of the gut (Sundin *et al.*, 1990; Murphy and Hill, 1991). Although we observed staining in mesendoderm cells in carp at the 8 somite stage, we detected no transcripts in gut-tissue at 25 h p.f. In addition, the distribution of transcripts in the branchial arches of mouse was studied in detail (Frohman *et al.*, 1990). In 30 h carp and zebrafish embryos we observed *Hoxb-1* in a structure resembling the outpocketing of pharyngeal endoderm in a branchial arch (described by Schilling and Kimmel, 1994).

We conclude that carp *Hoxb-1* can be positively identified by comparing its sequence and expression pattern to those of vertebrate homologs. The largely identical expression patterns suggest similar functions for *Hoxb-1* in the various vertebrate classes, especially in the specification of the fourth rhombomere of the hindbrain, and in the dorsoventral patterning of part of the central nervous system.

Materials and Methods

Embryos

The stripping of carp in order to obtain oocytes and sperm and the subsequent *in vitro* fertilization and culturing of embryos was performed as described by Stroband *et al.* (1995).

Cloning of carp Hoxb-1

Carp *Hoxb-1* was isolated from a carp early segmentation stage cDNA library after screening with an Antennapedia-derived homeobox-specific probe. The production of this probe and the subsequent screening of the library were described in Stroband *et al.* (1995).

DNA sequence analysis

Carp *Hoxb-1* was sequenced using a dideoxy chain termination protocol using sequenase (USB, Cleveland, OH). Both strands were sequenced using vector-specific T3 and T7 primers (Stratagene, La Jolla, CA) and clone-specific primers. Similarity searches were carried out with the Fasta programs (Pearson and Lipman, 1988). The sequence described in this paper is available from the EMBL database under Accession number X91079.

In situ hybridization

Plasmid DNA was digested with EcoRI, phenol-chloroform extracted and subsequently precipitated with ethanol and used as template for the transcription reaction. Dig-labeled antisense RNA probes were synthesized with T7 polymerase using the DIG RNA-labeling kit (Boehringer Mannheim, Germany). The carp *Hoxb-1* probe was diluted 200 times for *in situ* hybridization reactions, and the probe derived from the zebrafish *Krox-20* plasmid (generously provided by G.M. Kelly, University of Western Ontario, Canada) was diluted 4000 times. *In situ* hybridizations were carried out as described earlier (Stroband *et al.*, 1995), with the following modifications; the length of the proteinase K (10 µg/ml) treatment was adjusted for each new batch of proteinase K. The (pre)-hybridization solution contained 50% formamide, 2xSSC, 2% blocking solution (Boehringer Mannheim, Germany), 0.1% Tween-20, 5 mg/ml yeast torula RNA (Boehringer Mannheim, Germany) and 50 µg/ml heparin. The antibody incubation lasted for 3 h up to overnight.

In the double *in situ* hybridization, the hybridization solution contained the carp *Hoxb-1* (DIG-labeled) and the zebrafish *Krox-20* (fluorescein-labeled) probes. The detection of the *Krox-20* transcripts with the alkaline phosphatase conjugated anti-fluorescein antibody (Boehringer Mannheim, Germany), using fast red (Boehringer Mannheim, Germany) as a substrate, was stopped by a 15 min incubation in 0.1 M glycin pH 2.2 in aquadest. Next, the embryos were washed 4 times in PBST (5 min each wash), preincubated in PBST with 1% blocking stock (PBST-BL) (Boehringer Mannheim, Germany) for 3 h and incubated in 1:2000 alkaline phosphatase conjugated anti-DIG Fab fragments (Boehringer Mannheim, Germany) in PBST-BL. After washing, the *Hoxb-1* transcripts were detected using NBT/X-phosphate (Boehringer Mannheim, Germany) as a substrate.

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References

ACAMPORA, D., D'ESPOSITO, M., FAIELLA, A., PANNESE, M., MIGLIACCIO, E., MORELLI, F., STORNAIUOLO, A., NIGRO, V., SIMEONE, A. and BONCINELLI, E. (1989). The human *HOX* gene family. *Nucleic Acids Res. 17:* 10385-10402.

ALEXANDRE, D., CLARKE, J.D.W., OXTOBY, E., YAN, Y-L., JOWETT, T. and HOLDER, N. (1996). Ectopic expression of *Hoxa-1* in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development 122:* 735-746.

BÜRGLIN, T.R. (1994). A comprehensive classification of homeobox genes. In *Guidebook to the Homeobox Genes* (Ed. D. Duboule). Sambrook and Tooze Publication at Oxford University Press, Oxford, pp. 25-71.

DEKKER, E-J., PANNESE, M., HOUTZAGER, E., BONCINELLI, E. and DURSTON, A. (1992). Colinearity in the *Xenopus laevis* Hox-2 complex. *Mech. Dev. 40:* 3-12.

DICKINSON, M.E., SELLECK, M.A.J., McMAHON, A.P. and BRONNER-FRASER, M. (1995). Dorsalization of the neural tube by the non-neural ectoderm. *Development 121:* 2099-2106.

DUBOULE, D. and MORATA, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet. 10:* 358-364.

ERICSON, J.U., KRAUSS, S. and FJOSE, A. (1993). Genomic sequence and embryonic expression of the zebrafish homeobox gene *Hox-3.4. Int. J. Dev. Biol. 37:* 263-272.

FJOSE, A., NJØLSTAD, P.R., NORNES, S., MOLVEN, A. and KRAUSS, S. (1992). Structure and early embryonic expression of the zebrafish engrailed-2 gene. *Mech. Dev. 39:* 51-62.

FROHMAN, M.A., BOYLE, M. and MARTIN, G.R. (1990). Isolation of the mouse *Hox-2.9* gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development 110:* 589-607.

GEHRING, W.J., AFFOLTER, M. and BÜRGLIN, T. (1994). Homeodomain proteins. *Annu. Rev. Biochem. 63:* 487-526.

GODSAVE, S., DEKKER, E.-J., HOLLING, T., PANNESE, M., BONCINELLI, E. and DURSTON, A. (1994). Expression patterns of *Hoxb* genes in the *Xenopus* embryo suggests roles in anteroposterior specification in the hindbrain and in dorsoventral patterning of the mesoderm. *Dev. Biol. 166:* 465-476.

GRAHAM, A., MADEN, M. and KRUMLAUF, R. (1991). The murine *Hox-2* genes display dynamic dorsoventral patterns of expression during central nervous system development. *Development 112:* 255-264.

GUTHRIE, S., MUCHAMORE, I., KUROIWA, A., MARSHALL, H., KRUMLAUF, R. and LUMSDEN, A. (1992). Neurectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transpositions. *Nature 356:* 157-159.

HANNEMAN, E., TREVARROW, B., METCALFE, W.K., KIMMEL, C.B. and WESTERFIELD, M. (1988). Segmental pattern of development of the hindbrain and spinal cord of the zebrafish embryo. *Development 103:* 49-58.

KATSUYAMA, Y., WADA, S., YASUGI, S. and SAIGA, H. (1995). Expression of the *labial* group Hox gene *HrHox-1* and its alteration induced by retinoic acid in development of the ascidian *Halocynthia roretzi*. *Development 121:* 3197-3205.

KEYNES, R. and KRUMLAUF, R. (1994). *Hox* genes and regionalization of the nervous system. *Annu. Rev. Neurosci. 17:* 109-132.

KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMANN, B. and SCHILLING, T. (1995). Stages of embryonic development of the zebrafish. *Dev. Dynamics 203:* 253-310.

KOZAK, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell 44:* 283-292.

KRUMLAUF, R. (1994). *Hox* genes in vertebrate development. *Cell 78:* 191-201.

KRUMLAUF, R., MARSHALL, H., STUDER, M., NONCHEV, S., SHAM, M.H. and LUMSDEN, A. (1993). *Hox* homeobox genes and regionalization of the nervous system. *J. Neurobiol. 24:* 1328-1340.

LAROSA, G.J. and GUDAS, L.J. (1988). Early retinoic acid-induced F9 teratocarcinoma stem cell gene *ERA-1*: alternate splicing creates transcripts for a homeobox-containing protein and one lacking the homeobox. *Mol. Cell. Biol. 8:* 3906-3917.

MADEN, M., HUNT, P., ERIKSON, U., KUROIWA, A., KRUMLAUF, R. and SUMMERBELL, D. (1991). Retinoic acid-binding protein, rhombomeres and the neural crest. *Development 111:* 35-44.

MARSHALL, H., STUDER, M., PÖPPERL, H., APARICIO, S., KUROIWA, A., BRENNER, S. and KRUMLAUF, R. (1994). A conserved retinoic response element required for early expression of the homeobox gene *Hoxb-1*. *Nature 370:* 567-571.

MLODZIK, M., FJOSE, A. and GEHRING, W. (1988). Molecular structure and spatial expression of a homeobox gene from the *labial* region of the Antennapedia-complex. *EMBO J. 7:* 2569-2578.

MOLVEN, A., HORDVIK, I. and NJØLSTAD, P.R. (1993). Sequence analysis of the zebrafish *hox-B5*/*hox-B6* region. *Biochim. Biophys. Acta 1173:* 102-106.

MURPHY, P. and HILL, R.E. (1991). Expression of the mouse *labial*-like homeobox-containing genes, *Hoxb-1* and *Hox 1.6* during segmentation of the hindbrain. *Development 111:* 61-74.

MURPHY, P., DAVIDSON, D.R. and HILL, R.E. (1989). Segment-specific expression of a homeobox-containing gene in the mouse hindbrain. *Nature 341:* 156-159.

OGURA, T. and EVANS, R.M. (1995). A retinoic acid-triggered cascade of *HOXB1* gene activation. *Proc. Natl. Acad. Sci. USA 92:* 387-391.

OXTOBY, E. and JOWETT, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res. 21:* 1087-1095.

PAPAN, C. and CAMPOS-ORTEGA, J.A. (1994). On the formation of the neural keel and neural tube in the zebrafish *Danio (Brachydanio) rerio*. *Roux Arch. Dev. Biol. 203:* 178-186.

PEARSON, W.R. and LIPMAN, D.J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA 85:* 2444-2448.

PÖPPERL, H., BIENZ, M., STUDER, M., CHAN, S.-K., APARICIO, S., BRENNER, S., MANN, R.S. and KRUMLAUF, R. (1995). Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent on *exd/pbx*. *Cell 81:* 1031-1042.

RAIBLE, D.W., WOOD, A., HODSDON, W., HENION, P.D., WESTON, J.A. and EISEN, J.S. (1992). Segregation and early dispersal of neural crest cells in the embryonic zebrafish. *Dev. Dynamics 195:* 29-42.

ROSS, L.S., PARRETT, T. and EASTER Jr., S.S. (1992). Axogenesis and morphogenesis in the embryonic zebrafish brain. *J. Neurosci. 12:* 467-482.

RUDDLE, F.H., BARTELS, J.L., BENTLEY, K.L., KAPPEN, C., MURTHA, M.T. and PENDLETON, J.W. (1994). Evolution of *Hox* genes. *Annu. Rev. Genet. 28:* 423-442.

SCHILLING, T.F. and KIMMEL, C.B. (1994). Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. *Development 120:* 483-494.

SCHMITZ, B., PAPAN, C. and CAMPOS-ORTEGA, J.A. (1993). Neurulation in the anterior trunk region of the zebrafish, *Brachydanio rerio*. *Roux Arch. Dev. Biol. 202:* 250-259.

SCOTT, M.P. (1992). Vertebrate homeobox gene nomenclature. *Cell 71:* 551-553.

STRÄHLE, U. and BLADER, P. (1994). Early neurogenesis in the zebrafish embryo. *FASEB J. 8:* 692-298.

STROBAND, H.W.J., STEVENS, C., TE KRONNIE, G., SAMALLO, J., SCHIPPER, H., KRAMER, B. and TIMMERMANS, L.P.M. (1995). Expression of *carp-cdx1*, a *caudal* homolog, in embryos of the carp, *Cyprinus carpio*. *Roux Arch. Dev. Biol. 204:* 369-377.

STUDER, M., PÖPPERL, H., MARSHALL, H., KUROIWA, A. and KRUMLAUF, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science 265:* 1728-1732.

SUNDIN, O.H. and EICHELE, G. (1990). A homeo domain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev. 4:* 1267-1276.

SUNDIN, O.H., BUSSE, H.G., ROGERS, M.B., GUDAS, L.J. and EICHELE, G. (1990). Region-specific expression in early chick and mouse embryos of *GHox-lab* and *Hox 1.6*, vertebrate homeobox-containing genes related to *Drosophila labial*. *Development 108:* 47-58.

TRINKAUS, J.P., TRINKAUS, M. and FINK, R.D. (1992). On the convergent cell movements of gastrulation in *Fundulus*. *J. Exp. Zool. 261:* 40-61.

WILKINSON, D.G., BHATT, S., COOK, M., BONCINELLI, E. and KRUMLAUF, R. (1989). Segmental expression of *Hox-2* homoeobox-containing genes in the developing mouse hindbrain. *Nature 341:* 405-409.

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