

## ***Ol-Prx 3*, a member of an additional class of homeobox genes, is unimodally expressed in several domains of the developing and adult central nervous system of the medaka (*Oryzias latipes*)**

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**ABSTRACT** Large-scale genetic screens for mutations affecting early neurogenesis of vertebrates have recently been performed with an aquarium fish, the zebrafish. Later stages of neural morphogenesis have attracted less attention in small fish species, partly because of the lack of molecular markers of developing structures that may facilitate the detection of discrete structural alterations. In this context, we report the characterization of *Ol-Prx 3* (*Oryzias latipes-Prx 3*). This gene was isolated in the course of a large-scale screen for brain cDNAs containing a highly conserved DNA binding region, the homeobox helix-three. Sequence analysis revealed that this gene belongs to another class of homeobox genes, together with a previously isolated mouse ortholog, called OG-12 [Rovescalli, A. C., Asoh, S. & Nirenberg, M. (1996) *Proc. Natl. Acad. Sci. USA* 93, 10691–10696] and with the human *SHOX* gene [Rao, E., Weiss, B., Fukami, M., Rump, A., Niesler, B., et al. (1997) *Nat. Genet.* 16, 54–62], thought to be involved in the short-stature phenotype of Turner syndrome patients. These three genes exhibit a moderate level of identity in the homeobox with the other genes of the *paired-related* (*PRX*) gene family. *Ol-Prx 3*, as well as the *PRX* genes, are expressed in various cartilaginous structures of head and limbs. These genes might thus be involved in common regulatory pathways during the morphogenesis of these structures. Moreover, this paper reports a complex and monophasic pattern of *Ol-Prx 3* expression in the central nervous system, which differs markedly from the patterns reported for the *PRX* genes, *Prx 3* excluded: this gene begins to be expressed in a variety of central nervous system territories at late neurula stage. Strikingly, it remains turned on in some of the derivatives of each territory during the entire life of the fish. We hope this work will thus help identify common features for the *PRX 3* family of homeobox genes.

The Japanese medaka, *Oryzias latipes*, is a daily spawning aquarium fish studied for more than seven decades, with cancer biology, sex determination, and reproductive processes as major foci (1). Classical genetic work in the medaka has given rise to a unique collection of highly inbred lines and natural mutant strains (2). Like the zebrafish, the medaka has a short life cycle and produces numerous transparent embryos. Its genome is smaller, and its genetic map has reached high resolution (3). The number of genes and induced mutants isolated in the medaka is still far smaller than in the zebrafish, but studies of medaka gene function and regulation will benefit from a unique method of gene transfer by nuclear injection (4), recent progress on the long-term culture of embryonic stem cells (5, 6), and the discovery of an apparently intact trans-

posable element inserted at various positions in the tyrosinase gene of medaka albinos (7).

However, few medaka developmental genes have been cloned to date. Our interest in the development of the vertebrate central nervous system (CNS) led us to undergo a search for new types of homeobox genes expressed in the medaka brain. Most homeobox genes have been isolated in vertebrates by homology cloning on the basis of a strong conservation of the whole homeobox among distantly related species. We have chosen to screen an adult medaka brain cDNA library with a mixture of oligodeoxynucleotide probes corresponding to the third  $\alpha$ -helix of most homeoboxes [amino acid residues 46–52 (8)]. Such a strategy was used in various species and has already led to the isolation of previously unknown families of homeobox genes (refer, for example, to refs. 9 and 10). One among the genes resulting from this screen has retained all our attention, because its sequence shows (i) a strong homology in the whole open reading frame with the mouse OG-12 gene (10), the original member of a recently discovered class of animal homeobox genes, expressed in the brain and other tissues of the mouse following a so-far-undescribed pattern, and (ii) a strong homology restricted to the homeobox with a family of *paired-related* genes (*PRX*) identified in higher vertebrates, whose members are clearly involved in head skeleton and limb morphogenesis (11). Because of the small size of medaka brain, we were able to characterize the expression in the CNS from early embryonic stages of development to adulthood. Moreover, the expression pattern was thoroughly described, using a recently established medaka brain atlas (12). This study thus provides comparative data concerning the domains of expression of the *PRX 3* vertebrate genes. Interestingly, a human homolog of *Ol-Prx 3* (currently named *SHOX*) is thought to be involved in the short-stature phenotype of Turner syndrome patients (13). It was also shown to be expressed in various human tissues, including brain. We thus believe that analysis of the role of vertebrate *PRX 3* genes in different developmental processes will benefit from various approaches allowed by the use of several biological models.

### MATERIALS AND METHODS

**Fish Strains and Embryo Collection.** Medaka embryos and adults of an Orange-Red strain (kindly provided by A. Shima, Tokyo University) were used in most experiments. Albino embryos of an inbred line (i3BR1, gift of Y. Hyodo-Taguchi, National Institute of Radiological Sciences, Chiba, Japan) were also used for unambiguous identification of otherwise

Abbreviation: CNS, central nervous system.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF001393).

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homolog encoded by the OG-12 gene (see below). Such conserved amino-terminal regions are found in most homeodomain proteins (20), but their functions are still unclear.

*Ol-Prx 3* cDNA contains an unusually long 3' untranslated region of more than 3 kb, which is not found in OG-12 cDNAs isolated from 12-day-old mouse embryos (10). It was already reported that brain mRNAs have such an unusually long 3' untranslated region (UTR) (21). Translational efficiency was found to be positively regulated by the length of 3' UTR (22). A putative stabilizing effect of this region remains also to be assayed.

**Comparison Between Medaka Ol-PRX 3 and Mouse OG-12 Proteins.** Strikingly, all amino acids in the Ol-PRX 3 homeodomain are identical to those found in the homeodomain encoded by the OG-12 gene (Fig. 2). This gene was isolated by a screening strategy (using the same oligonucleotide probe) similar to that reported in this paper (10). In contrast, no closely related homeobox gene was reported in *Drosophila*. The *aristaleless* gene [Fig. 2 (23)] has the most nearly identical homeobox, but a primary structure highly divergent outside the homeobox and nonhomologous expression domains. Therefore, the question of the evolutionary conservation of OG-12 genes outside the vertebrate phylum remains open.

Except for the presence of a long polyglycine repeat in the mouse protein, OG-12 proteins exhibit a strikingly high structural similarity (Fig. 1B). No alternative form with 12 additional amino acids such as those found in mice (10) was identified. Similarly, a second type of transcript encoding a shorter protein in the carboxyl-terminal domain was identified in humans (13). No such transcript was detected in medaka. Repeats of such amino acids were found scattered over a large number of homeodomain proteins with apparently no correlation with the species or the family of homeodomain protein (20).

When the mouse protein, with its polyglycine tail deleted, is compared with the fish protein, 72.5% of the amino acids are found to be identical (Fig. 1B). Such a high conservation is not unexpected in homeodomain proteins, and certainly corresponds to the presence of domains with conserved functions. Rovescalli *et al.* (10) identified sequences forming a P-loop domain (24) and a domain related to SH3-binding domains (25) in the carboxyl-terminal region of the proteins that are also found in the Ol-PRX 3 protein (Fig. 1B).

**Ol-PRX 3 Homeodomain Compared with *paired-related* Homeodomains.** The homeodomain encoded is clearly not a *hox/Antp* type homeodomain but is related to the fly *paired* homeodomain (Fig. 2). When examined in the amino-terminal

half of the homeodomain, Ol-PRX 3 homeodomain is most closely related to the *Pax-3* gene homeodomain (76% amino acids in common). However, in the recognition helix at the carboxyl-terminal part of the homeodomain, a glutamine is found at position 50 instead of a serine in the canonical *paired-type* genes. This substitution is characteristic of a subclass of *paired-type* homeobox genes, called *paired-related (PRX)* genes (26). Only 43 or 44 amino acids of the Ol-PRX 3 homeodomain are identical to the mouse *PRX 2* (26) and *Phox2* genes (27), respectively. These two genes are the PRX subclass homologs with the highest identity to Ol-PRX 3 in the homeodomain. However, no homology between Ol-PRX 3 and other PRX proteins is found outside of the homeodomain. In conclusion, *Ol-Prx 3* belongs to a new class of homeobox genes with loose identity, but still common structural features.

***Ol-Prx 3* Expression Pattern in the Adult CNS.** To study the expression of the *Ol-Prx 3* gene, two probes (described in *Materials and Methods*) were used, one containing the homeobox and the other not. They provided identical hybridization patterns at all stages (data not shown). To test for the specificity of the homeobox-containing probe, Northern hybridization was performed. A single transcript size ( $\approx 4$  kb; as attested from the cDNA lengths) was observed after hybridization of hatching embryo or adult brain total RNAs with the homeobox-containing probe (not shown).

Analysis of serial Vibratome and paraffin-embedded sections of medaka CNS hybridized with digoxigenin probes allowed the identification of most *Ol-Prx 3*-expressing structures. A description of the main features follows (from rostral to caudal). No staining was ever observed in the telencephalon (including olfactory bulb; Fig. 3 B and I). In the dorsal and medial diencephalon, several nuclei were labeled, most notably the thalamic nuclei centralis posterioris and dorsalis posterioris (Fig. 3 B and C). Some cells expressed *Ol-Prx 3* in the hypothalamic nucleus diffusus of the inferior lobe (not shown). The most prominent *Ol-Prx 3* expression was found in the dorsal midbrain [periventricular gray zone layer 3 and central zone of the tectum, and layer 2 of the torus semicircularis; Fig. 3 B, D, H, and I]. Other mesencephalic structures were also labeled, albeit less intensively, such as scattered cells in the torus lateralis (Fig. 3C) or in the periventricular central gray (Fig. 3 B and D). Strong expression was also seen in the rhombencephalon, dorsally in scattered cells of the nucleus of the solitary fascicle (Fig. 3 B, F, and I) and ventrally in the reticular formation (Fig. 3 B, E, and F). No expression was detected in the cerebellum (Fig. 3 B, D, and I).

To our knowledge, this expression pattern in medaka CNS is not reminiscent of that of any known homeobox gene of the *PRX* family. It would therefore be of particular interest to have more data about the expression patterns of *Ol-Prx 3* homologs in higher vertebrates.

***Ol-Prx 3* Expression Pattern During CNS Development.** By whole-mount *in situ* hybridization (same probes as described above), we followed *Ol-Prx 3* expression in medaka embryos from neurula onwards. The first signal was detected at stage 24 (15 somites; heart beating) in the hindbrain as three bilateral masses of cells (not shown). Quickly after, at stage 25, five (Fig. 4 A, E, and H, arrowheads) such masses of cells located in the laterodorsal aspect of the neural tube, in close vicinity to the otic vesicles, are strongly labeled. This area remains *Ol-Prx 3*-positive from this stage to the adult, and therefore most likely overlaps the presumptive territory of the nucleus of the solitary fascicle.

At stage 25–26, other domains of *Ol-Prx 3* expression become detectable: (i) two stripes in the ventral aspect of the rhombencephalon/caudal mesencephalon (Fig. 4, arrows), which might represent the developing reticular formation; (ii) a strongly positive domain in the dorsal midbrain area (Fig. 4, stars). We suggest that this latter domain corresponds to the developing tectum and torus semicircularis. Interestingly, at

<i>Ol-Prx 3</i>	KQRRSRNTFLEQLNELERLFDETHYPDAMFREELSQLGLSEARVQVWFQNRRAKCRKQE	
OG-12	-----	100
<i>MHox/Prx 1</i>	---N--T-NSS--QA--V-ER-----V--D-AR-VN-T-----F-RN-	68
<i>S8/Prx 2</i>	---N--T-NSS--QA--V-ER-----V--AR-VN-----F-RN-	71
<i>Pax-3</i>	-----T-A--E--A-ER-----IYT--A--AK-T-----S--RW--A	75
<i>Pax-7</i>	-----T-A--E--KA-ER-----IYT--A--TK-T--F--S--RW--A	71
<i>Pax-6</i>	-LQRN--S--Q--IEA--KE-ER-----V-A--R-AAKID-P--I--S--W-RE-	56
<i>Phox2</i>	-----I--T--SA--K--V-A-----IYT--ALKID-T-----F--	73
<i>DRG11</i>	---N--T-A-Q--EA-AV-AQ--T-V-T--AMKIN-T-----W--T-	66
<i>Otp</i>	-K-H--R--PA-----S-AK--I--AL-I--T--S-----WK-RK	71
<i>Otx-1</i>	---T--RA--DV--A--AK-R--I--V--V--LKKIN-E-S-----K--Q-65	65
<i>Otx-2</i>	---T--RS--DV--A--AK-R--I--V--V--LKKIN-E-S-----K--Q-68	68
<i>Hoxa-7</i>	-RK-GRQTY-RY-TL--KE-HFNRLTRRR-I-IAHA-C-T-RQIKI-----N-WK-EH	36
<i>Hoxb-7</i>	-RK-GRQTY-RY-TL--KE-HYNR-LTRRR-I-IAHT-C-T-RQIKI-----N-WK-EN	36
<i>Al</i>	---Y--T--SF--E--KA-SR-----V-T--AMKI--T--I-----W--	75
<i>Paired</i>	-C--T-SAS--D--A-ER-Q--IYT--A--TN-T--I--S--RL--H	65
<i>Drepo</i>	-KKKT--T--AY--E--A-ERAP--V-A--AIK-N--S-----W--H-	65

FIG. 2. Alignments between the putative homeodomain of medaka Ol-PRX 3 and mouse OG-12 (two first lines) and other mouse (upper frame) and *Drosophila* (lower frame) homeodomains. The amino acid sequence of the previously isolated mouse OG-12, of *Prx* gene-encoded proteins, and of other classes of homeodomain are presented. Amino acids are in single-letter code. The *Ol-Prx 3* sequence is given in the top line and only residues diverging from the *Ol-Prx 3* sequence are represented in the following lines. Gene names are given on the left (italics) and percentages of identity on the right (boldface numbers).



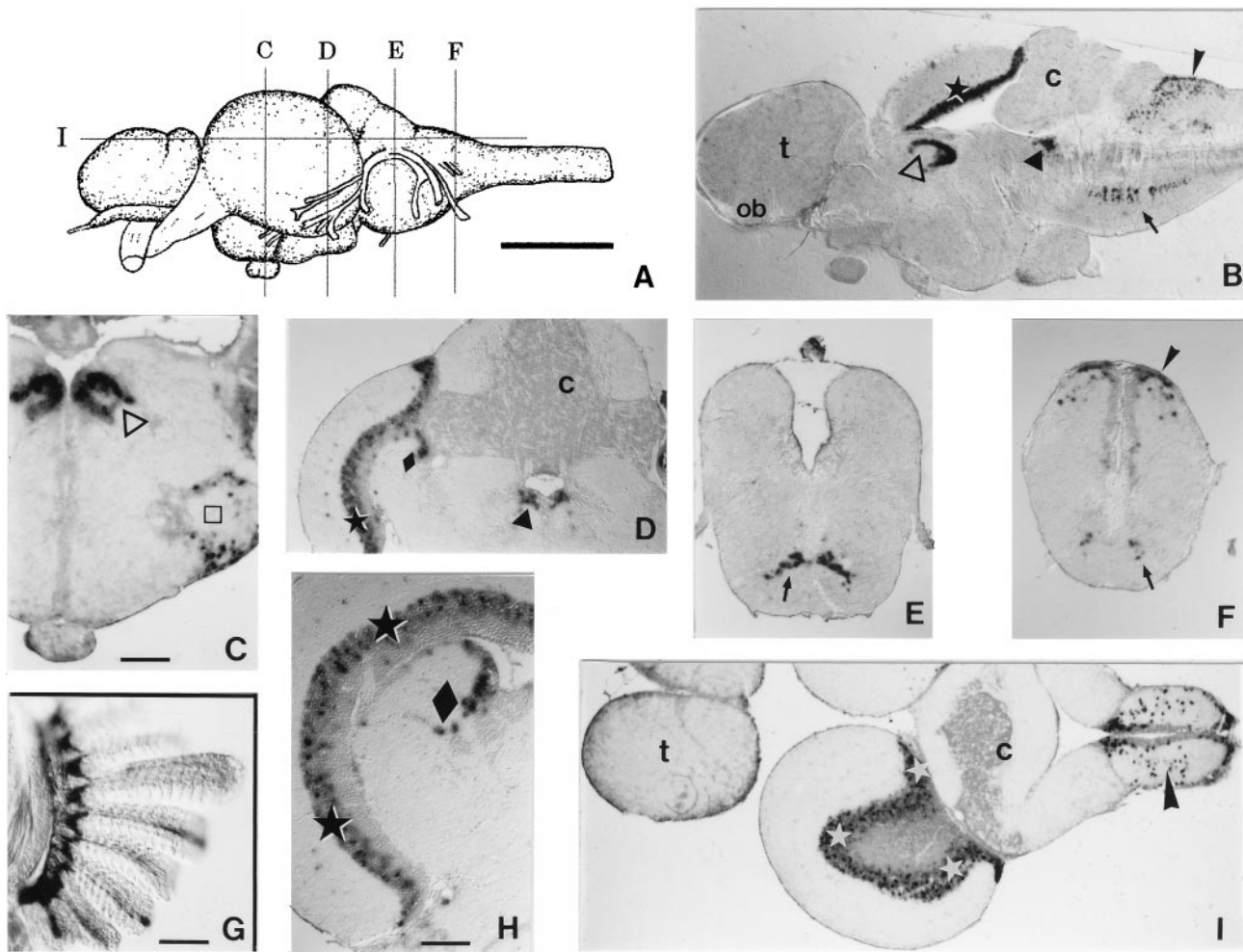


FIG. 3. Localization of *Ol-Prx 3* RNA in adults. (A) Lateral view of an adult medaka brain. The approximate levels of sections C–G are indicated. (B) Midsagittal thick (75- $\mu$ m) Vibratome section of medaka brain, showing the main *Ol-Prx 3* expression domains: dorsal thalamus [nuclei centralis posterioris and dorsalis posterioris ( $\Delta$ )], dorsal midbrain [layer PGZ3 of optic tectum ( $\star$ ) and central gray ( $\blacktriangle$ )], dorsal hindbrain [nucleus of the solitary fascicle ( $\blacktriangleright$ )], and ventral hindbrain [reticular formation ( $\rightarrow$ )]. Note that no cell in the telencephalon (t), olfactory bulb (ob), or cerebellum (c) expresses *Ol-Prx 3*. (C–F) Thin (8- $\mu$ m) paraffin-embedded sections of adult medaka brain (whole-mount hybridized, cut thereafter and counterstained with nuclear fast red). (C) Expression is detected in the nuclei centralis posterioris and dorsalis posterioris of the dorsal thalamus ( $\Delta$ ) and the torus lateralis ( $\square$ ). (D) Expression is seen in the periventricular gray zone layer 3 (PGZ3) and central zone layers of the tectum ( $\star$ ), layer 2 of the torus semicircularis ( $\blacklozenge$ ), and the periventricular central gray ( $\blacktriangle$ ). There is no expression in the cerebellum (c). (E) Neurons of the reticular formation express *Ol-Prx 3* ( $\rightarrow$ ). (F) Expression is detected in the reticular formation ( $\rightarrow$ ) and the nucleus of the solitary fascicle ( $\blacktriangleright$ ). (G) *Ol-Prx 3* expression persists in elements of the skeletal axis of the adult gill (but not in its epithelium), as seen in this thick Vibratome section. (H) Transverse paraffin-embedded section of the dorsal midbrain, showing the territories where *Ol-Prx 3* expression is strongest: patches of cells in the PGZ3 of the tectum ( $\star$ ), and layer 2 of the torus semicircularis ( $\blacklozenge$ ). (I) Horizontal paraffin-embedded section of an adult medaka brain. Expression is strong in the PGZ3 of the tectum ( $\star$ ) and the nucleus of the solitary fascicle ( $\blacktriangleright$ ). The telencephalon (t) and cerebellum (c) show no *Ol-Prx 3* expression. (Scale bar in A = 1 mm; in C, 100  $\mu$ m; D–F same magnification as C; in G, 100  $\mu$ m; and in H, 60  $\mu$ m.)

this and later embryonic stages, the label encompasses most of the thickness of the tectum (Fig. 4 F and I). Therefore, *Ol-Prx 3* could provide an useful marker to follow the ontogenesis of the dorsal mesencephalon, especially the neuronal migrations in this area. (iii) Two patches of strongly labeled cells are found in the dorsal thalamic territory, between the optic vesicles (Fig. 4). They probably correspond to the developing nuclei centralis posterioris and dorsalis posterioris.

From stage 28 onwards (and up to adult stage), *Ol-Prx 3* expression remains strong and strictly limited to these areas. No transient domain of expression was ever detected.

To summarize, the expression of this gene is essentially monophasic, several CNS domains being turned on one after the other, and remaining positive up to the adult stage. This type of expression is rather surprising, because most vertebrate homeobox genes have several phases of expression in the CNS [for example, refer to the *even-skipped* family of homeobox genes, (28)]. The molecular mechanisms at work to sustain

such long waves of *Ol-Prx 3* expression in medaka brain remain to be elucidated. Genes involved in the long-term maintenance of homeobox gene expression in *Drosophila* have been isolated (29), and it would be particularly interesting to test whether any vertebrate homologs of these gene families are also found to act during medaka CNS development.

Given the paucity of data on late stages of neural development in teleost fishes on one hand, and the continuity of its expression pattern on the other hand, *Ol-Prx 3* can thus allow molecular identification of specific neuronal territories in a variety of experimental situations.

***Ol-Prx 3* Expression Pattern Outside the CNS.** By whole-mount *in situ* hybridization at various embryonic stages, *Ol-Prx 3* was detected outside the CNS in a variety of chondrifying structures, identified by comparison with previous descriptions of these structures (30). Most prominent is its expression in developing head cartilage: axis of branchial arches, opercles, and mandible (Fig. 4, open arrows). Thus, we propose that

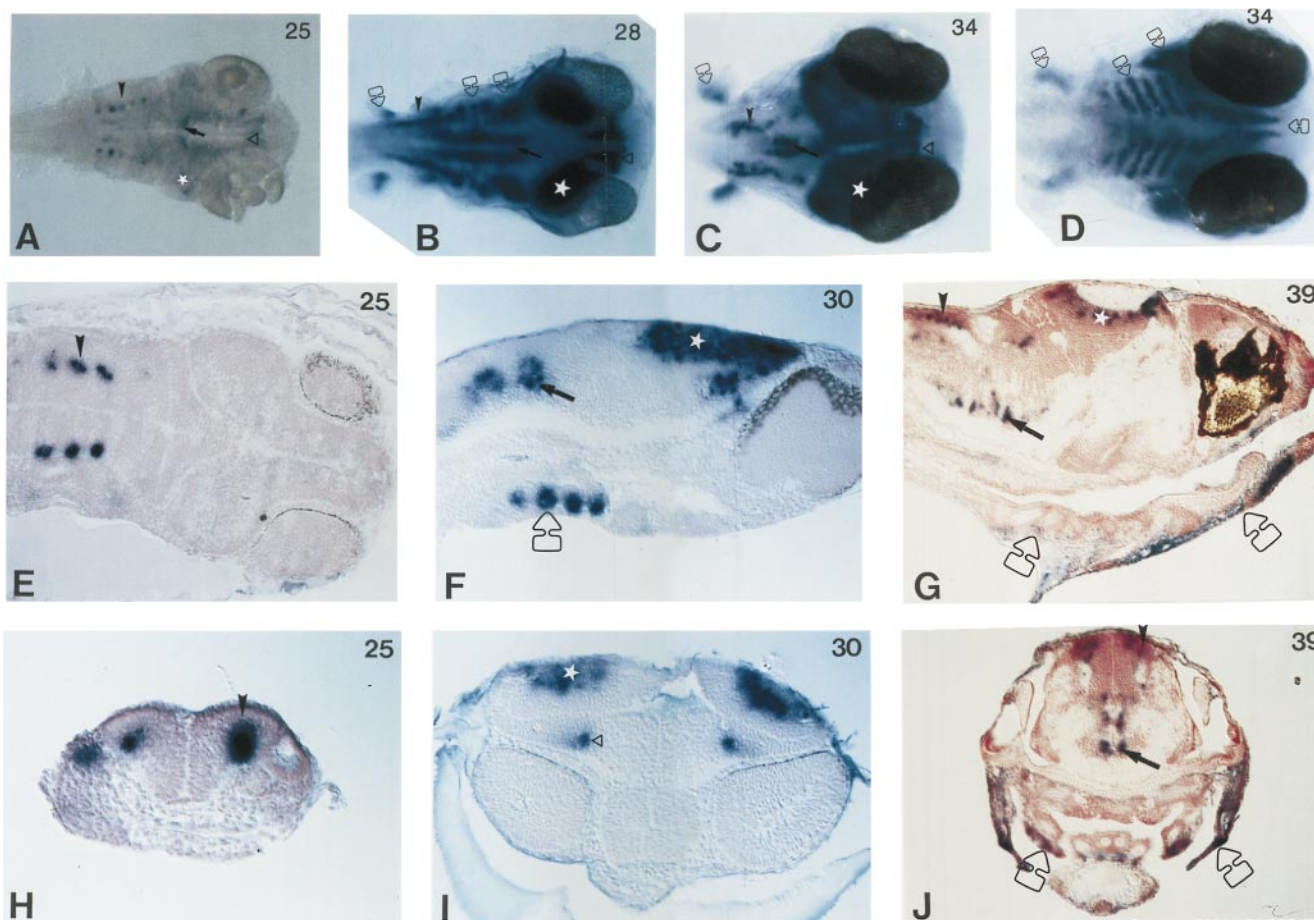


FIG. 4. Localization of *Ol-Prx 3* RNA in embryos. Cells expressing *Ol-Prx 3* appear in dark blue after an alkaline phosphatase chromogenic reaction. Sections were counterstained with nuclear red and photographed using interference contrast optics. Developmental stages according to Iwamatsu (15) are indicated at top right of each picture. In all panels, corresponding domains of expression are pointed out by a symbol, bilateral structures being marked on only one side of the embryo.  $\Delta$ , Anterior domain of expression in the diencephalon.  $\ast$ , Prominent staining in the optic tectum.  $\rightarrow$ , Ventral CNS signal.  $\blacktriangleright$ , Hindbrain positive cells. Open arrow, chondrifying structures. (A–C) Dorsal views of the anterior regions of whole-mounted embryos, cleared in glycerol/PBS, and from which yolk and yolk sac were dissected. (D) Ventral view from an embryo at the same stage as in C. From left to right, indicated by an open arrow: pectoral fins, branchial arches, opercle precursors, and inferior jaw anlage. (E) Horizontal section. Only three of the five pairs of stained cells are visible on this section, due to the plane of section. (F and G) Parasagittal sections of anterior regions of late somitogenesis and hatching embryos. (H) Transverse section through hindbrain; neural keel is V-shaped. (I) Frontal section. Both anterior domains of expression in the diencephalon are visible. (J) Transverse section through otic vesicles, visible on both sides of the embryo.

*Ol-Prx 3* could also be used as a molecular marker to follow chondrification and differentiation of part of the head skeleton in medaka. Outside the head, the only domain of expression was found to be cartilaginous structures in the pectoral fin anlagen (open arrows in Fig. 3 B, C, and D). We have not analyzed in detail adult *Ol-Prx 3* expression outside the CNS, but partial results indicate that it persists at least in the axis of the gills (Fig. 3G).

Whereas *Ol-Prx 3* expression in the CNS does not resemble that of other known *PRX* homeobox genes, its pattern in nonneuronal structures, and notably in cartilages of the branchial arches and pectoral fins, is reminiscent of that of the *PRX* family (26). Together with the fact that homeoboxes of these genes are homologous, this indicates that this latter family and the OG-12/*Ol-Prx 3* family of homeobox genes might be implicated in common regulatory pathways during the development of the head/fin cartilage. Indeed, it is clear from knock-out data in mice, that *MHox*, one member of the *PRX* family, is involved in these developmental events (31). In contrast, these genes appear to be involved in the ontogenesis of wholly different structures in the neuraxis. These speculative points of course await the availability of loss- or gain-of-function mutants in different vertebrate model species. In this respect, it is interesting to note that a recently isolated class of

zebrafish mutants (32, 33) exhibits defects both in head/fin cartilaginous structures and in dorsal midbrain [detected by aberrant axonal migration (34) or tectum defects (32)]. It will be of major interest to test whether some of these lines bear mutations at loci corresponding to the OG-12/*Ol-Prx 3* gene family.

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