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Original Article

Expression pattern of two *otx* genes suggests a role in specifying anterior body structures in zebrafish

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ABSTRACT We isolated two zebrafish sequences containing a homeobox related to *orthodenticle (otd)*, a gene expressed in the developing head of *Drosophila*. One of these is clearly homologous to *Otx1*, a homeobox gene previously reported to be expressed in the developing rostral brain of the mouse. We termed this zebrafish gene *otx1*. The second gene is not as closely related to *Otx1* and is equally divergent from *Otx2*, a second homeobox gene expressed in the developing rostral brain of the mouse. We termed it *otx3*, even if a corresponding murine *Otx3* gene has not been reported yet. Both genes are expressed in early-gastrula zebrafish embryos in the involuting presumptive anterior mesendoderm. With the extension of the body axis, the expression domain of both genes extends to neuroectodermal regions fated to become fore- and mid-brain. From this stage the expression domains of the two genes differ slightly from each other but both cover the rostral brain with a sharp posterior boundary coinciding with that between midbrain and hindbrain. This late expression closely corresponds to that of the murine *Otx1* gene, whereas the earliest expression of both zebrafish *otx* genes is different from that of *Otx1* and reminiscent of that of *Otx2* in the mouse. In this light, the zebrafish *otx1* and *otx3* genes appear to share some expression features of both murine *Otx1* and *Otx2*. It will be of considerable interest to study the specific role of the various genes of the *otx* family in the development of the zebrafish brain regions. The peculiar spatio-temporal pattern of these genes during early zebrafish gastrulation suggests a role of this gene family in interactions between anterior mesendoderm and neuroectoderm.

KEY WORDS: *gastrulation, zebrafish, Otx, forebrain, midbrain, brain regionalization*

Introduction

We previously reported the isolation and characterization of two murine homeobox genes, *Otx1* and *Otx2* (Simeone *et al.*, 1992, 1993; Boncinelli *et al.*, 1993a,b), containing a homeobox homologous to that of *orthodenticle (otd)* (Finkelstein and Perrimon, 1990; Finkelstein *et al.*, 1990; Finkelstein and Boncinelli, 1994), a gene expressed in anterior regions of the developing head of *Drosophila*. The gene products of the two murine *Otx* genes contain a homeodomain of the *bicoid* class (Driever and Nüsslein-Volhard, 1988) and are able to recognize and transactivate a *bicoid* target sequence (Simeone *et al.*, 1993).

Otx2 is expressed very early in the entire epiblast of prestreak mouse embryos (Simeone *et al.*, 1993). Its expression persists in this tissue even after the formation of the primitive streak in the posterior regions of the embryo. In midstreak embryos its expression becomes progressively restricted to more anterior regions, including anterior mesendoderm and the neuroectoderm corresponding to presumptive fore- and mid-brain (Simeone *et al.*, 1992, 1993, 1995; Ang and Rossant, 1993). In early midgestation mouse embryos *Otx2* is expressed in telencephalic, diencephalic and mesencephalic regions and in developing sense organs (Simeone *et al.*, 1992, 1993).

Thus, in the mouse, *Otx2* expression demarcates anterior regions from pre-streak stages and rostral brain regions from the formation of the headfold until late in gestation. This raises the question of whether the *Otx2* homeodomain protein might play a role in specifying anterior structures, besides being an early marker of their position. With this in mind, we isolated the *Xenopus* (Pannese *et al.*, 1995) and chick (Bally-Cuif *et al.*, 1995) homologues of *Otx2*. Expression analysis in these vertebrate systems and in experimentally manipulated *Xenopus* embryos suggests a role for this gene in specifying anterior body structures and their spatial relationship with posterior trunk structures.

Abbreviations used in this paper: E, embryonic day; hpf, hours post-fertilization.

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otx1  VWFKNRRAKCRQQQQ*SGSSTKTRPAKKKSSPTRESTGSESSGQFTPPAVSS
otx3  ---------------Q-STNS-I------P--S---P------H--------

otx1  AGSSSSSSSSTNNTGIS*****STSTSISTVSSIWSPA*ISPGSAPPSVSLP
otx3  SS-------GS-PSAL-GVGLI-S-S-GTP-P------PV--VP---**---

otx1  EPVAPSNTSCMQRSVSGTASST****YPMPYNQTTGYSQGYPTP*SGSYFSC
otx3  DISP-ASA-----AM-SGGGT-GVPS-------APS-A-----SNAA---G-

otx1  VDCGSYLAPM**HSHHHPH****QLSPMTASSMPTHPHHHISQSSGHHHHHH
otx3  M---------TG-A-----AHHS---TAA-V-GHH--*---G----**--P-

otx1  HQAYSGTGLAFNSSDCLDYKEQTASSWKLNFN**TTDCLDYKDQASWRFQVL
otx3  --G-G--A-P-S----------V--------SSAAA---------A------
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Fig. 1. Alignment of the peptide sequences of zebrafish *otx1* and *otx3* last exons. *The carboxy-terminus of the homeodomain, lying just downstream from a 3' splice site, is boxed. Dashes indicate identity and asterisks indicate gaps. # indicates the stop codon. The sequences shown represent 70% of the protein.*

The murine *Otx1* gene is not expressed as early as *Otx2*. *Otx1* transcripts are first detectable around day 8 of mouse development (E8) in presumptive forebrain and midbrain (Simeone *et al.*, 1992, 1993). *Otx1* expression persists in this region until at least E10.5 and starting from E9 its posterior boundary is very sharp and corresponds to the midbrain-hindbrain border. *Otx1* is also expressed in developing sense organs of the mouse (Simeone *et al.*, 1993).

Zebrafish provides a superb model system for the analysis of embryogenesis (Warga and Kimmel, 1990; Westerfield, 1993; Driever *et al.*, 1994; Mullins *et al.*, 1994). It belongs to the Teleost subphylum and is of particular interest because the organization of the brain is different from that of mammals with regard to the size, the tissue composition and the role of the different structural parts of the brain (Ariens Kappers *et al.*, 1936). The mesencephalon and more precisely the optic tectum, is relatively larger than that of other vertebrates. This region becomes the integrating center of neural activity originating from the optic system. In mammals this integrating center is localized in neocortex. The telencephalon comprises a relatively small portion of the teleost brain and displays a peculiar, evaginated, structure. It does not contain a recognizable archicortex and the two paleocortex regions, termed *epistriata*, are laterally localized and dorsally communicate through an epithelial membrane, the so-called *epithelial lamina*.

To further explore the evolutionary conservation of the expression and function of *otd/Otx* family member, we cloned and characterized two zebrafish homeobox genes, *otx1* and *otx3*. *otx1* is highly homologous to the murine *Otx1* gene, whereas *otx3* is not as similar to *Otx1* and equally divergent from *Otx2*. Recently, Li *et al.* (1994) reported an extensive analysis of the *otx1* and *otx2* genes of zebrafish. Both are already expressed in early gastrula zebrafish embryos in a region located on the dorsal margin of the blastoderm and related to presumptive anterior head structures. When the involution of blastoderm cells is completed, *otx*-expressing cells are found in mesendoderm in the prechordal plate and in neuroectoderm in a region fated to become fore- and mid-brain (Kimmel *et al.*, 1990). From this stage the expression domains of the two genes differ slightly from each other but both cover the rostral brain with a sharp posterior boundary coinciding with that between midbrain and hindbrain.

Results

Screening of the genomic DNA library

We screened a genomic phage library constructed from zebrafish DNA (Molven *et al.*, 1991) using the *Drosophila otd* sequence, previously used for the isolation of the mouse *Otx* genes (Simeone *et al.*, 1992). From the screening of $5x10^5$ phage plaques we obtained several clones belonging to three classes. We report here the characterization of clones belonging to two of these classes. We sequenced the coding region present in these clones. Figure 1 shows the corresponding peptide sequences, representing the carboxy-terminal regions of the homeoproteins, downstream from a splice site. The two sequences are 70% identical. We termed one sequence *otx1* owing to its high overall similarity (78%) to the mouse *Otx1* protein. The second sequence is only 63% identical to murine *Otx1* and 62% identical to murine *Otx2*. We termed it *otx3*, although a corresponding murine *Otx3* gene has not been reported yet. After these experiments were completed, isolation and characterization of the *otx1* and *otx2* genes have been published by Li and collaborators (Li *et al.*, 1994). Their results confirmed that the *otx1* gene whose partial sequence we show in Fig. 1 is identical to the COOH-terminal two-thirds of the reported *otx1* gene. These data support the existence of a new family member, namely *otx3*, since its sequence is distinct from the *otx1* and *otx2* genes.

Expression of otx1 and otx3 during early embryogenesis

We analyzed the expression of the two genes in zebrafish embryos by means of whole-mount *in situ* hybridization. During the course of gastrulation, in a process termed epiboly, the blastoderm spreads over the yolk toward the vegetal pole. The extent of epiboly is used as a staging index (Westerfield, 1993). Results presented in Figs. 2,3 indicate that *otx1* and *otx3* gene expression is highly dynamic and until 80% epiboly (8.5 hpf, hours post fertilization) the expression patterns of the two *otx* genes are indistinguishable. *otx1* and *otx3* transcripts are first detectable at the beginning of gastrulation when or shortly before the deep cells of the blastoderm start involuting around the blastoderm margin (Fig. 2A). At 50% epiboly (5.2 hpf), hybridization first appears as a faint patch of stained cells within the blastoderm margin of the embryo (Fig. 2A). This group of *otx*-expressing cells subsequently involutes and produces a local thickening of the germ ring, called the embryonic shield, which marks the dorsal side of the embryo (Fig. 2B). Observation from the vegetal pole shows that the signal is mostly localized in the deep layer overlying the yolk.

While the cells rearrange by convergence and extension movements to elongate the shield along the antero-posterior axis, the hybridization signal consists of a medial-strip cells whose anterior border marks the leading edge of the shield as it narrows and extends toward the animal pole. *otx* transcripts are detectable dorsally as a band of cells probably corresponding to the involuting axial hypoblast (Fig. 2C). Around 70% epiboly (7.5 hpf), significant staining is no longer observed at the level of

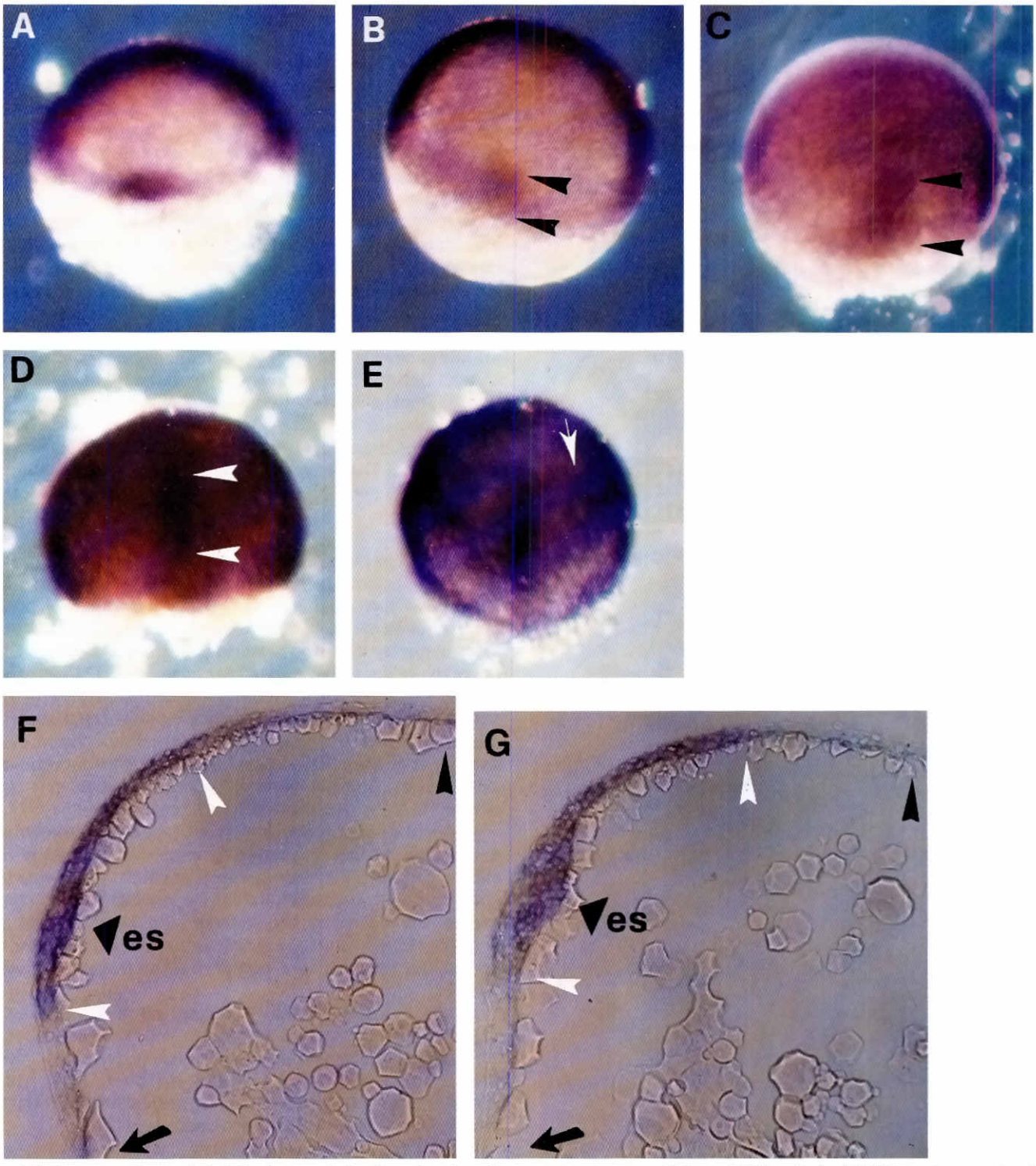


Fig. 2. Whole-mount *in situ* hybridization analysis of *otx1* and *otx3* expression between 50% and 75% epiboly. (A,B,D) *otx3 expression;* **(C,E)** *otx1 expression. Similar results were obtained for the other gene.* **(A)** *50%;* **(B)** *shield stage;* **(C)** *65%;* **(D,F and G)** *70%;* **(E)** *75% epiboly.* **(A-E)** *Dorsal views and* **(F-G)** *sagittal sections of zebrafish embryos. In* **(A)** *expression is detectable as a patch at the marginal zone of the blastoderm.* **(B-D)** *Anterior and posterior limits of the signal are shown by arrowheads; in the embryonic shield* **(B)**, *in the involuting axial hypoblast* **(C)**, *in the anterior axial hypoblast* **(D)** *and some epiblast cells (shown in detail in* **F,G**). *In* **(E)** *otx expression is in the anterior axial hypoblast and some epiblast cells (white arrow).* **(F,G)** *Two sagittal sections through the primordium of the body axis from a 70% epiboly embryo.* **(G)** *Medial section;* **(F)** *parasagittal section. A strong staining is detectable in the embryonic shield, both in the involuting axial hypoblast and in the overlying epiblast, in the region demarcated by the two white arrowheads. Staining is also present in the yolk cell-adjacent layer, extending almost to the animal pole (black arrowhead). An arrow indicates the dorsal blastoderm margin. es, anterior portion of the embryonic shield. 1 cm= 150 μm (A,B,C,D,E); 50 μm (F,G).*

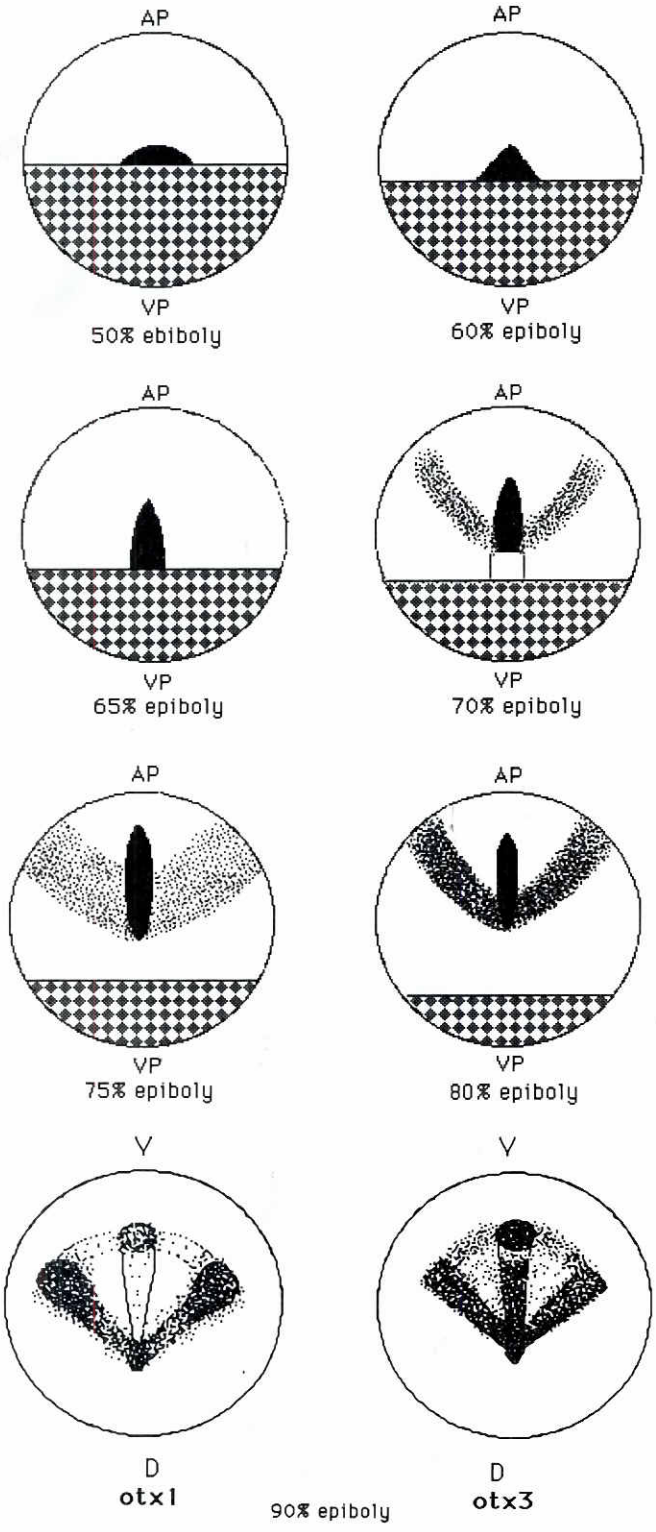


Fig. 3. Schematic representation of the expression patterns of *otx1* and *otx3* during gastrulation. *From 50% epiboly to 80% epiboly, the patterns for otx1 and otx3 are indistinguishable. Dorsal views are shown and the yolk is represented by diamonds. At 90% epiboly the expression pattern of the two genes is different and is shown separately in animal pole view. AP, animal pole; VP, vegetal pole; V, ventral; D, dorsal.*

blastoderm margin and thus *otx* expression only marks the anterior shield (Fig. 2D). From this stage on, transcripts are also detectable in some cells of the epiblast laterally to the dorsal midline. Sections of this stage show that *otx*-expressing cells are localized in the embryonic shield (es), both in the epiblast and in the involuting axial hypoblast. Beyond the anterior edge of the embryonic shield, some yolk cell-adjacent cells extending to the vicinity of the animal pole express both *otx* (Fig. 2F,G) as we found in chick embryos (Bally-Cuif *et al.*, 1995).

At midgastrula, around 75% epiboly (8 hpf), *otx* genes are expressed dorsally in the rostral 1/3 of the embryo as a strongly labeled midline, probably corresponding to the presumptive prechordal plate. Two lateral stripes become clearly visible superficially (white arrow in Fig. 2E). From 75% to 80% epiboly, the expression patterns are modestly rearranged in accordance with the late gastrulation cell movements, namely extension along the anterior-posterior axis and convergence toward the animal pole (Fig. 3).The two lateral stripes are diverging from the dorsal midline by about 45° in each direction.

From this stage on, a slightly different pattern is observed for *otx1* and *otx3*. The entire expression pattern of the two *otx* genes between 50% and 90% epiboly is schematically represented in Fig. 3.

Expression pattern of otx1

At 90% epiboly (9 hpf), *otx1* is already expressed in the prospective fore- and mid-brain. A V-shaped signal pointing towards future caudal regions is clearly visible (Fig. 4A). A particularly strong hybridization signal is detectable in the most anterior portion of the lateral stripes (arrowhead in Fig. 4A,B). The V-shaped signal becomes thicker at tail bud stage (Fig. 4C) and then gives rise to two broad patches by one-somite stage (not shown). The anterior one is localized in the diencephalon, whereas the second lies in the midbrain and its posterior boundary coincides with the midbrain-hindbrain junction. This pattern is shown in Figs. 7A,8 and persists until the 17-somite stage (Fig. 4F).

During the same period, the anterior axial mesendoderm corresponding to the presumptive prechordal plate is also stained (Fig. 4A,B). In the most anterior portion of the embryo, a strong expression appears transiently together with an anterior curved line delimiting the anterior and lateral neural keel (Fig. 4B). At tail bud stage (10 hpf), expression in the medial strip and in the most anterior portion has gradually disappeared (Fig. 4C).

From the 9-somite stage, 14 hpf, a faint dorso-posterior signal in the telencephalon is first visible and could correspond to the *epithelial lamina* which covers the telencephalon of Teleosts (thin arrow in Fig. 4D,E) (Ariens Kappers *et al.*, 1936). Posteriorly, the expression domain appears continuous from the diencephalon to the posterior midbrain (Fig. 4D,E).

Around the 17-somite stage (18 hpf), when the brain is clearly subdivided, the *otx1* hybridization displays the pattern seen in Fig. 4F. Proceeding from anterior to posterior, the first signal is localized in dorso-posterior telencephalon. Then a strong labeling is seen at the level of the epiphysis and around the third ventricle. Posteriorly to it, the hybridization signal extends continuously and ends sharply with a strong ventral labeling just before the midbrain-hindbrain junction (Fig. 4F). Double-staining with *pax2* (Krauss *et al.*, 1991, 1992) indicates that this posterior bor-

der coincides with the anterior *pax2* boundary (Fig. 4F), suggesting that the posterior boundary of the *otx1* expression domain coincides with the posterior boundary of midbrain, as is the case in the mouse (Simeone *et al.*, 1993).

Around 24 hpf, the *otx1* hybridization signal is an anterior-posterior X-shaped signal starting anteriorly in the dorsal telencephalon, extending dorsally in the diencephalon and the midbrain and finishing just before the midbrain-hindbrain junction (Fig. 4G). By squeezing the embryo between slide and coverslip (flat-mounted embryo), it appears that the most anterior signal is localized in the dorso-rostral telencephalon (Figs. 7E, 8). A faint staining is seen in the optic recess. Just caudally to the telencephalon a strong expression is seen in the diencephalon, corresponding to the ventral and dorsal thalamus and slightly fainter in the posterior hypothalamus (see Fig. 7E). In the midbrain, the expression of *otx1* is widespread, with a stronger signal anteriorly in the tectum and posteriorly in the tegmentum. Sections show that in the rostral diencephalon the hybridization signal is very strong in the dorsal roof, probably including epiphysis, and fainter in the floor i.e. the posterior hypothalamus (Fig. 4J). A section around forebrain-midbrain boundary shows that the expression of *otx1* is widespread throughout the neural tube but absent from the floor plate (Fig. 4K).

By 36 hpf, the X-shaped signal is more delineated and sharper (Fig. 4H). Hybridization is particularly detectable in the telencephalon, the epiphysis and the otic vesicles (Fig. 4I). By 48 hpf, expression remains in the same domains but appears fainter (data not shown).

otx1 is also expressed in the developing sensory organs. A faint staining is first detectable in the caudal field of the eyes at 9-somite stage (Fig. 4D). At 24 hpf, the expression is localized in the epithelial layer of the lens, the retinal margin and surface ectoderm (Fig. 4J).

The olfactory region begins to express *otx1* around 19 hpf. Sections of 24 hpf embryos show that the expression is maintained there (data not shown). A signal in the otic vesicles is first visible at 18 hpf and more clearly at 36 hpf (Fig. 4I). The signal appears stronger in the ventrolateral portion of the epithelium surrounding the vesicle (data not shown).

Expression pattern of otx3

otx3 is also expressed in presumptive fore- and mid-brain. At 90% epiboly, the lateral signals describe a large V but their borders are more clearly delineated as compared to *otx1*, probably as a consequence of higher density of *otx3*-expressing cells. While the hypoblast extends to the animal pole, the leading edge of *otx3* hybridization reaches the animal pole. Expression of *otx3* is maintained in the anterior axial hypoblast and appears strong in the very anterior part of the embryo, which forms the leading edge of the prechordal plate (solid arrow in Figs. 5A, 6A). The double staining with *no tail* antiserum, previously termed *zf-T* or *brachyury* (Schulte-Merker *et al.*, 1992, 1994a,b) indicates that the territory of *otx3* expression is anterior to the presumptive notochord and that, anteriorly, the medial *otx3* stripe is continuous to it (Fig. 6A). Sagittal sections of double-stained embryos (not shown) show that *no tail* expressing cells are present in the trunk axial mesoderm, whereas *otx3* expressing cells are localized anteriorly in all three cell layers (see Fig. 6C). First, at 90% epiboly, the two domains are continuous (see Fig. 6A) and then, at tail bud stage, a small gap is seen between the *no tail* expression domain and that of *otx3*. The same observations apply to *otx1* (data not shown).

At tail bud stage, *otx3* hybridization displays the pattern seen in Fig. 5B. This pattern consists of the posterior V-shaped lateral band including two patches symmetrically localized away from the axis (thin arrowheads), the medial stripe and the anterior curved line, which in turn coincides with the rostral and antero-lateral boundaries of the embryo, revealing the outline of the neural keel. Within the medial portion of this curved line abundant transcripts are seen (Figs. 5B,C, 6C). Significantly, while the medial-stripe cells are ventral to the curved line, the hybridization is continuous between the two regions, which are joined through the medial swelling of the curved line (see Figs. 5B, 6B,C). This continuity suggests that the two patterns belong to a single domain of *otx3* expression. Posteriorly the medial hybridization signal persists in all three layers (Fig. 6C).

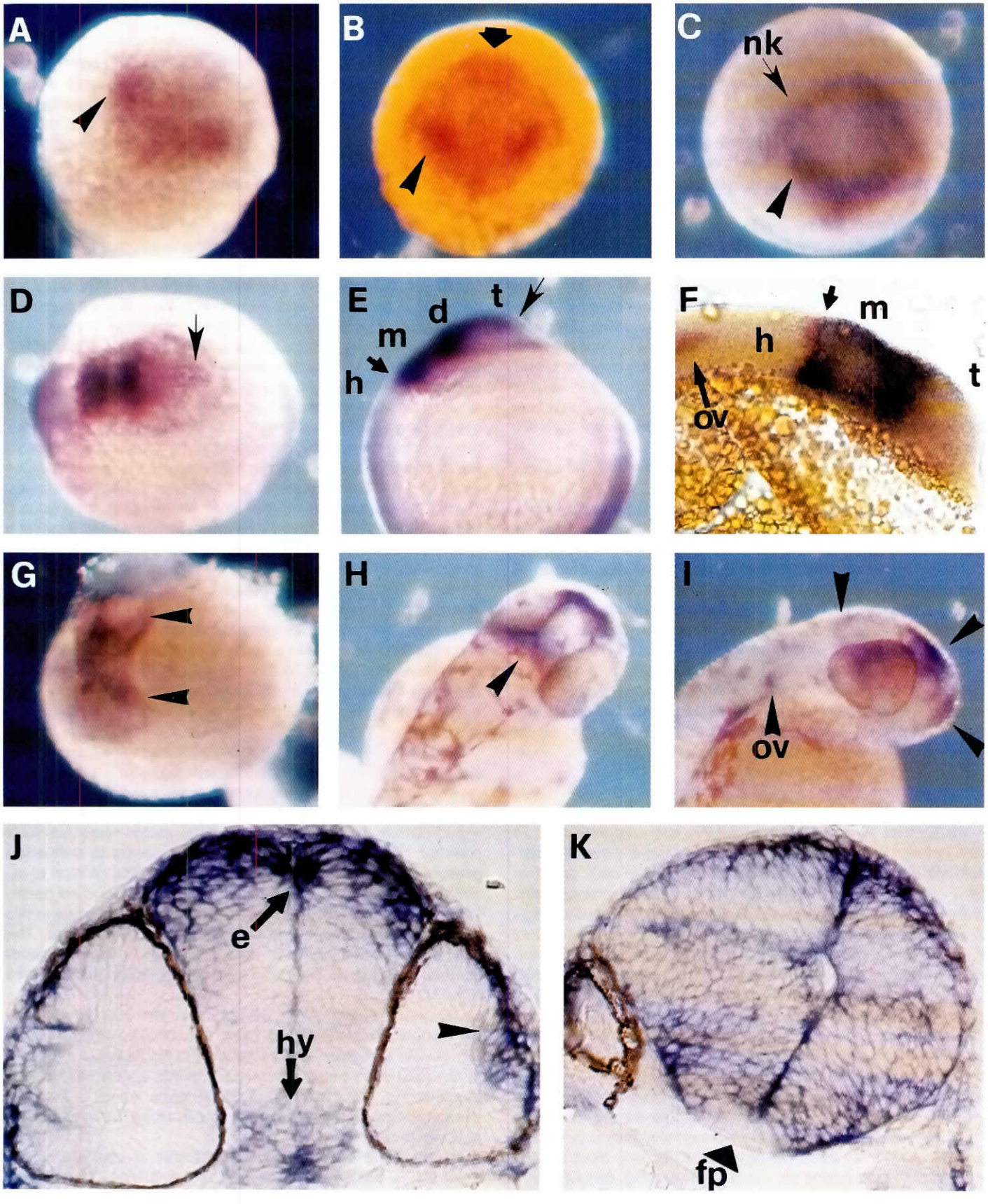
Double staining with *pax2* gene (Krauss *et al.*, 1991, 1992) was performed to estimate the axial level of the posterior V-shaped band. At tail bud stage, *pax2* hybridization describes a large V clearly localized posteriorly to the *otx3* band, partially overlapping with the two longitudinal stretches (Fig. 5C). These data indicate that *otx3* is expressed in the prospective midbrain with a posterior limit overlapping with the anterior one of *pax2*.

By the 1-to-2-somite stage two lateral *otx3* bands are seen. There, the expression in the axial hypoblast is maintained. Instead of the strong signal in the anterior neural keel, a staining is confined to cells scattered along the rostral and lateral borders of the embryo and is maintained over development (Fig. 5D). During later stages the expression in two bands is maintained whereas it decreases in the axial hypoblast. Contrary to *otx1* expression, the two *otx3* expressing regions are well separated and between them there is a faintly stained zone. The most anterior region corresponds to the mid-diencephalon, the posterior one to the caudal midbrain. By flattening the embryo, expression in the presumptive anterior hypothalamus becomes visible (Figs. 7B, 8).

At 9-somite stage, i.e. 14 hpf, the two bands are more distant and show a sharp posterior boundary (Fig. 5E,F). Lateral view reveals that *otx3* is also expressed in the most ventro-anterior part of the embryo (Fig. 5F). This region corresponds to the hypothalamus as seen at the previous stage. More precisely seen by 15-somite stage, its dorsal limit seems to define the position of the future postoptic commissure (POC) (Wilson *et al.*, 1990). The labeling in the diencephalon seems to extend dorso-ventrally along the zona limitans and the caudal boundary in this region to correspond to the future posterior commissure. The expression in the midbrain is more diffuse with a sharp posterior boundary corresponding to the midbrain-hindbrain junction (see Figs. 7D, 8).

Around 17-somite stage, 18 hpf, when the brain is subdivided, posterior to the first thick band, hybridization signal is more diffuse with a sharp posterior boundary in the posterior midbrain. In this area of the midbrain two faint bands appear (thin arrowheads in Fig. 5G). Two antero-lateral spots (arrow in Fig. 5G) along with two symmetrical lateral stripes along the midbrain are now visible.

At 20-somite stage (19 hpf), transcripts of *otx3* are clearly seen in the diencephalon around the epiphysis. The second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band of expression is now well visible. From this region to the posterior border, the signal seems uniform and covers the entire midbrain. The third band persists but remains faint (Fig. 5H).

Around 24 hpf, the expression of *otx3* is faintly visible in the primordia of the optic tecta arising from the midbrain roof while expression is essentially the same as that of the previous stage (Fig. 5I). Flat-mounted embryos reveal that a faint staining is first localized close to the anterior commissure and extends into the diencephalon in the ventral and dorsal thalamus and along the zona limitans intrathalamica. The expression in the anterior hypothalamus has disappeared but persists in its posterior portion. The second expression patch is situated ventrally around the posterior commissure and essentially in the tegmentum. The floor plate does not express *otx3*. Caudally, the signal ends sharply both dorsally and ventrally at the anterior border of *pax2* expression which corresponds to the end of the tectal ventricles (Figs. 7F, 8).

By 36 hpf, the hybridization signal seems to extend uniformly from the telencephalon-diencephalon junction to the tectal ventricles. Figure 6E shows a frontal section through the telencephalon and diencephalon. A strong staining is observed in the anterior ectodermal telencephalon possibly corresponding to the olfactory region along with two thin lines departing from the eyes possibly coincident with the optic nerves. With the exception of these regions, the telencephalon does not express *otx3*, whereas diencephalon does express it strongly. A thick transversal section in the diencephalon shows a strong expression in the geniculate nuclei localized in the ventral thalamus (white arrowheads in Fig. 6F). Finally, embryos of 48 hpf show a faint dorsal signal with a posterior boundary in posterior midbrain (data not shown).

otx3 is also expressed in sensory organs. By 1-to-2-somite stage, a bilaterally paired signal appears as lateral outpockets of the forebrain rudiments which could correspond to the optic pits (arrowhead in Fig. 5D). Later on, in agreement with the previous observation, the developing optic vesicles clearly express *otx3:* at 9-somite stage *otx3* expression is confined to the portion of the optic vesicles which faces the diencephalon, whereas the hybridization signals become more diffuse in the region of the optic stalk (Fig. 5E). At 20-somite stage, medial portions of the eye corresponding to the retina layers are labeled (Fig. 5H). Otic placodes start expressing *otx3* by 14-somite stage. By 24 hpf, the eyes (Fig. 5I), olfactory region and the otic vesicles express *otx3*. By 36 hpf, the ventrolateral portion of the epithelium surrounding the otic vesicles (Fig. 6D) and the olfactory region (Fig. 6E) are clearly positive, whereas the expression in the eyes is less evident (data not shown).

Discussion

The Otx gene family

We isolated two zebrafish homeobox genes clearly belonging to the *Otx* family. Considering the peptide sequence encoded by the carboxy-terminal region (Fig. 1), one of these genes is 78% identical to the mouse *Otx1* protein and only 65% identical to the *Otx2* protein. We termed it *otx1*. The homology of the second gene is only 63% with respect to *Otx1* and 62% with respect to *Otx2*. On the basis of these values we believe that this second gene represents a new member of the *Otx* gene family and we termed it *otx3*. This hypothesis was confirmed by both Li's prior description (Li *et al.*, 1994) and our own recent isolation of the actual zebrafish *otx2* gene, encoding a protein 94% identical to the mouse cognate (our unpublished results). The overall conservation within the *Otx* gene family is striking, as already noted for the *Xenopus* (Boncinelli *et al.*, 1993b; Pannese *et al.*, 1995) and chick (Bally-Cuif *et al.*, 1995) *Otx2* genes. *Otx2* itself appears to be incredibly conserved, whereas *Otx1* is considerably less conserved in zebrafish, *Xenopus* and chick (our unpublished results). The high conservation of *otx2* relative to mouse (94%) is not unique: *pax6* is known to be more strongly conserved (97%) relative to its murine cognate (Püschel *et al.*, 1992). Moreover our data demonstrate the existence of a new member of this family, namely *otx3*, in zebrafish. A similar observation in *Xenopus* (our unpublished results) suggests that in some vertebrate species there are more than one non-*Otx2* members of the *Otx* family. This is at variance with the mouse, where only two *Otx* genes have been found so far. The possible redistribution from species to species of the regulatory tasks among the various members of the *Otx* gene family poses interesting phylogenetic and ontogenetic questions.

Early expression

A second major difference between zebrafish and mouse is the early expression of *otx1* (and *otx3*). The mouse cognate,

Fig. 4. Expression of *otx1* between 90% epiboly and 36 h postfertilization (hpf). (A-D) *Dorsal views;* **(G,H)** *dorsolateral views;* **(E,F,I)** *lateral views.* **(A-C,H)** *Anterior is up;* **(D-F,I)** *anterior is to the right;* **(G)** *anterior is down.* **(A)** *otx1 expression in the anterior half of a 90% epiboly embryo; an arrowhead indicates the strong signal in the anterior portion of lateral bands.* **(B)** *Embryo between 90% and 100% epiboly showing the expression in the rostral (thick arrow) and posterior (arrowhead) boundaries.* **(C)** *Tail bud stage showing the expression in the outline of the neural keel (nk) and in the fore- and mid-brain primordium (arrowhead).* **(D,E)** *9-somite embryo showing staining extending from the dorsal telencephalon, in the epithelial lamina (thin arrow), to the midbrain-hindbrain junction (solid arrow). otx1 is expressed in the dorsal diencephalon around the epiphysis and in the dorsal midbrain. Staining in the trunk is non specific.* **(F)** *Flat-mounted preparation of a 17-somite stage embryo after two color whole-mount in situ hybridization using digoxygenin-labeled otx1 probe (blue) and fluorescein-labeled pax2 probe (red). Note the partial overlapping of otx1 and pax2 genes at the m-h boundary (solid arrow). The faint staining with otx1 in the otic vesicles (ov) is covered by the strong staining with pax2.* **(G)** *Embryo around 24 hpf showing the X-shaped signal. Arrowheads demarcate the anterior and posterior limits of the strong hybridization signal. At 36 hpf* **(H,I)**, *the expression in the most rostro-dorsal telencephalon, in the epiphysis and in the otic vesicles (ov) is clearly visible (arrowheads). An additional arrowhead designates the posterior boundary of expression in the brain.* **(J,K)** *Transverse sections of 24 hpf embryos.* **(J)** *Section through the rostral diencephalon showing the strong labeling around and in the epiphysis (e) and in the floor of the diencephalon i.e. the posterior hypothalamus (hy). Note the expression in the region surrounding the lens and precisely in the lens epithelium and in those regions of the retina corresponding to presumptive iris (arrowhead).* **(K)** *Section through the forebrain-midbrain boundary showing the absence of staining in the floor plate (fp). Note otx1 expression in mesenchyme and surface ectoderm in* **(J,K).** *d, diencephalon; e, epiphysis; fp, floor plate; h, hindbrain; hy, hypothalamus; m, midbrain; nk, neural keel; t, telencephalon; ov, otic vesicle. 1 cm= 125 μm (A-E,G); 62 μm (J,K); 100 μm (F,H,I).*

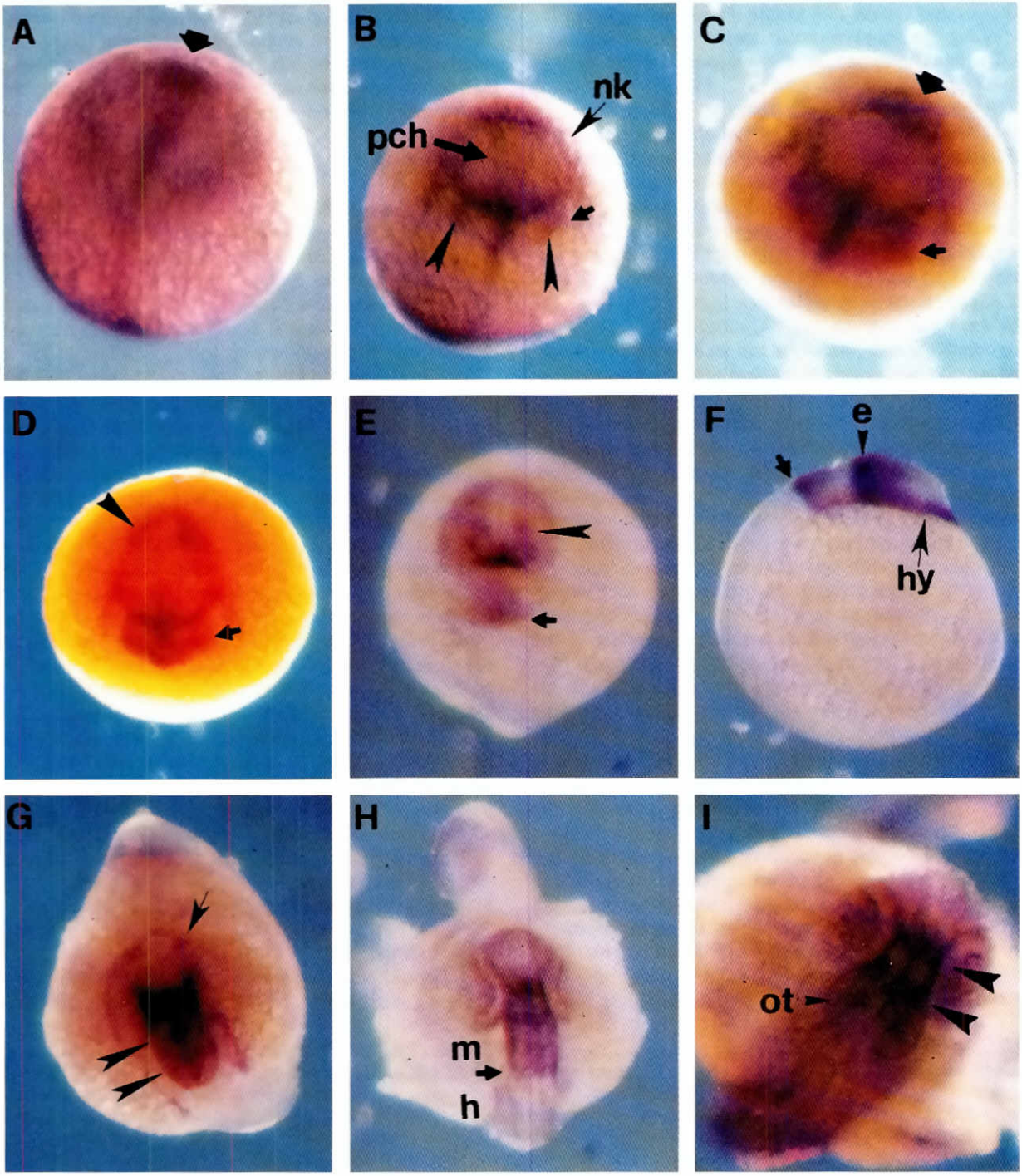

Fig. 5. Expression of *otx3* between 90% epiboly and 24 hpf. (A-E,G-I) *Dorsal views;* **(F)** *lateral view.* **(A)** *90% epiboly embryo showing a strong labeling at the very anterior tip (solid arrow).* **(B,C)** *Tail bud stage embryos showing in* **(B)** *the outline of the neural keel (nk), the prechordal plate (pch), the two lateral stretches (thin arrowheads), the future second band shown in* **(D)** *(solid arrow) expressing otx3 and in* **(C)** *shown to overlap with pax2 expression (red).* **(D)** *1-2-somite embryo showing the optic pits (arrowhead) and the second band which delineates the presumptive midbrain-hindbrain junction (solid arrow).* **(E,F)** *9-somite stage embryos. The optic cups and especially the portions which face the diencephalon show otx3 expression (arrowhead in* **(E)**. *Two bands are detectable; the anterior one is stronger and is localized around the epiphysis (e), whereas the second demarcates the midbrain with a posterior boundary at the midbrain-hindbrain junction (solid arrow). The hypothalamic region is stained (hy).* **(G)** *17-somite embryo showing two faintly labeled regions in the midbrain (thin arrowheads) and two anterior spots (arrow).* **(H)** *20-somite embryo. Now labeling in the retina layers is visible. The boundary between midbrain and hindbrain is indicated.* **(I)** *24 hpf embryo showing the two anterior bands (arrowheads) and a diffuse staining in the optic tectum (ot) and in the eyes. e, epiphysis; h, hindbrain; hy, hypothalamus; m, midbrain; nk, neural keel; ot, optic tectum; pch, prechordal plate. 1 cm= 110 μm (A); 125 μm (B-H); 85 μm (I).*

Otx1, is not expressed before late neurulation, E8-8.25, in the rostral region of the closing neural tube corresponding to prospective fore- and mid-brain (Simeone *et al.*, 1993). Its expression domain shows a posterior boundary approximately at the level of the midbrain-hindbrain boundary. This expression boundary becomes very sharp at E9 (Boncinelli *et al.*, 1993). From this stage on, expression of *Otx1* remains confined to rostral brain regions. We observed an early *otx1* expression also in frog embryos (our unpublished results). However we consider the possibility that an additional member of *Otx* family which is expressed earlier than *Otx1* and *Otx2* might exist in mouse.

otx1 and *otx3* are already expressed at the beginning of gastrulation in zebrafish embryos (Figs. 2, 3). We looked for expression at earlier stages and observed a very faint staining in whole blastoderm for both genes (data not shown). However the intensity of coloration was too weak to unambiguously assess this point. Expression is first clearly detectable prior to any involution as a patch of cells at the blastoderm margin (Fig. 2A). Immediately after the involution of blastoderm cells (Fig. 2B,C), it is difficult to tell whether the signal is present only in the presumptive mesendodermal cells of the forming hypoblast or both in the hypoblast and epiblast. By observing the embryo from the vegetal pole, the signal appears deeply localized. However the unstained superficial region could correspond to the enveloping layer cells (Westerfield, 1993) or to the enveloping layer cells and epiblast together. *otx1* and *otx3* expression is clearly visible in the epiblast at 70% epiboly, less than 2 h later (Fig. 2F,G) whereas *otx* expression in paraxial epiblast is clearly visible around 75% epiboly (Fig. 2E). Transplantation experiments will be necessary to precisely assess whether *otx* expression in the extending epiblast is primary or, alternatively, secondary to its occurrence in the involuting hypoblast.

This early expression of *otx1* and *otx3* is reminiscent of *Otx2* expression in early *Xenopus* (Pannese *et al.*, 1995) and chick (Bally-Cuif *et al.*, 1995) embryos. In these systems *Otx2* is expressed in migrating anterior mesendoderm and slightly later in the overlying anterior neuroectoderm. Manipulation experiments in the frog lead us to suggest that *Otx2* may specify anterior body structures and their spatial relationship with posterior trunk structures. Similar manipulation experiments are possible in zebrafish in order to verify such a role hypothesized for zebrafish genes of the *Otx* family.

Goosecoid is an homeobox-containing gene expressed in homologous structures in various vertebrate systems (De Robertis *et al.*, 1994). These structures have organizer activity and control the development of anterior mesendoderm in the head. The expression pattern of zebrafish *goosecoid* (Stachel *et al.*, 1993; Schulte-Merker *et al.*, 1994a) during early gastrulation is very similar to that of *otx1* and *otx3*. The same observation has already been made between *Otx2* and *goosecoid* expression in *Xenopus* (Pannese *et al.*, 1995) and chick (Bally-Cuif *et al.*, 1995) embryos. In agreement with what was observed in these systems, the earliest *goosecoid* expression overlaps with that of the *otx* genes but extends to cells located slightly more superficially than the *otx*-expressing ones. Subsequently, *otx* and *goosecoid* expression transiently coincides in an advancing patch of deep cells and finally in the prechordal plate, anterior to the *no tail* expression domain in the notochord (Schulte-Merker *et al.*, 1994a). At later stages the expression of the two *otx* genes extends to neuroectodermal cells as well, whereas *goosecoid* expression persists exclusively in mesendoderm and progressively fades out (even if *goosecoid* expression in anterior neuroectoderm has been described [Thisse *et al.*, 1994]). It will be of interest to clarify the relationship between *otx* genes and *goosecoid* as clues for inductive events during gastrulation.

No tail (ntl) is the zebrafish cognate of the mouse *T* gene (Herrmann *et al.*, 1990) and of the *Xenopus Brachyury* gene (Smith *et al.*, 1991). In zebrafish *ntl* protein is present in both epiblast and hypoblast cells in the germ ring. After involution, this protein only remains in axial hypoblast cells that will form the notochord (Schulte-Merker *et al.*, 1992). We compared its expression with that of the *otx* genes in order to localize precisely the *otx* expression domains (Fig. 6A). As described previously for *goosecoid* (Schulte-Merker *et al.*, 1994), *otx1*, *otx3* and *ntl* show an initial overlapping expression domain at the dorsal blastoderm margin of the early gastrula. However, *otx*- and *ntl*-expressing cells become progressively separated during gastrulation, with the *otx* genes expressed more anteriorly. At late gastrula, *otx1* and *otx3* are expressed in neuroectoderm and in the anterior axial hypoblast, anterior to the *ntl*-expressing cells of the presumptive notochord. Then, during early somitogenesis, *otx* expression in the anterior axial hypoblast is downregulated (Figs. 4C, 5D).

The expression patterns of *otx1* and *otx3* start to differ at around 90% epiboly. Their domain of expression is similar, extending from the most anterior part of the embryo to the presumptive midbrain-hindbrain boundary. A major difference is seen in the lateral bands at the level of the presumptive midbrain. Actually, *otx1* is less uniformly expressed along these lateral bands, its expression being stronger in the anterior portion (Fig. 4B). This feature is transient and its significance unclear. The second difference resides in the two longitudinal stretches seen for *otx3* (thin arrowheads in Fig. 5B). This signal progressively disappears and seems to correspond to the temporary lateral borders of the midbrain.

otx expression in the developing brain

Zebrafish *otx1* and *otx3* transcripts have a highly dynamic and specific pattern of expression in the head embryonic structures.

During somitogenesis they show first a pattern that is very simple and restricted to diencephalon and midbrain. Around 4-somite stage, *otx1* is continuously expressed from the diencephalon to the midbrain-hindbrain junction, as seen in the mouse. Conversely, *otx3* expression is very early confined to three distinguishable zones: the anterior hypothalamus, the middiencephalon and the posterior midbrain. Expression is faint in the region between the latter two domains (Fig. 7A,B). When the brain starts to be subdivided this pattern becomes progressively more complex. *otx1* alone starts to be clearly detectable in telencephalon. Transcripts are first seen around 9-somite stage and do not cover the entire telencephalon but the most dorso-posterior portion. Later on (around 24 hpf), only the most dorso-rostral telencephalon and the dorsal zone close to the anterior commissure express *otx1*. Conversely, *otx3* is never clearly expressed in telencephalon (Fig. 7E,F). This feature is different from the expression in the telencephalon of the mouse *Otx1* and *Otx2* genes. This discrepancy might be related to the structural diversity between Teleosts and mammalian brain. In fact, some func-

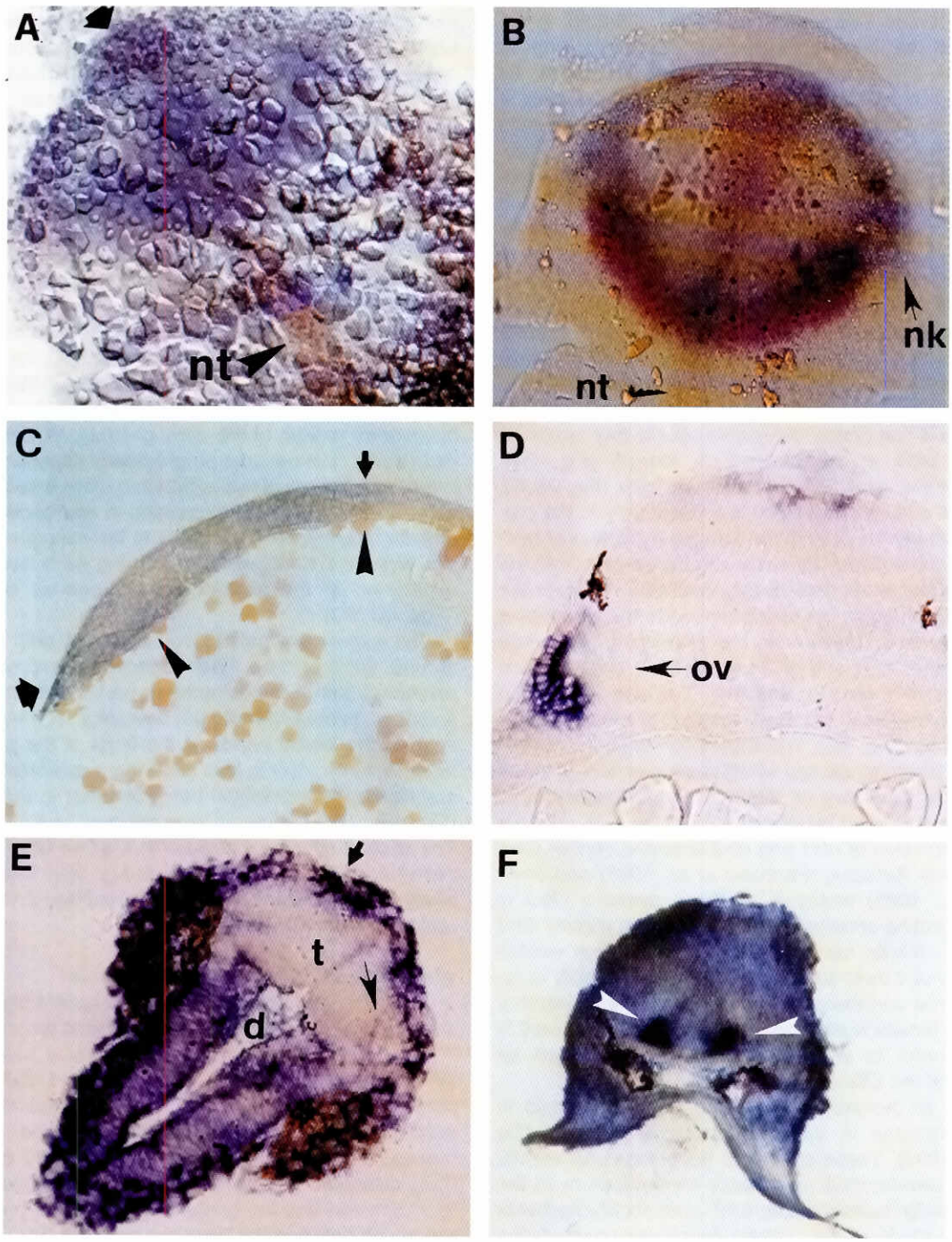

Fig. 6. Expression of *otx3* between 90% epiboly and 36 hpf. **(A,B,D)** *Flat-mounted preparations of a 90% epiboly* **(A)** *and tail bud* **(B)** *stage embryos.* **(C-F)** *Sections of tail bud* **(C)**, *24 hpf* **(D)** *and 36 hpf embryos* **(E,F).** **(A)** *Double staining with the no tail antiserum revealing the presumptive notochord in brown (nt).* **(B)** *Flat-mounted preparation of the embryo in Fig. 5C revealing the notochord, the prechordal plate, the medial swelling of the curved line which delineates the neural keel (nk). Pax2, in red, overlaps with the presumptive posterior midbrain.* **(C)** *Sagittal section of tail bud stage embryo. Anterior is to the left. Note the dorso-ventral labeling at the very anterior tip of the embryo (solid arrow), the signal in the mesendoderm (arrowheads) and the posterior signal in all three layers which corresponds to the medial portion of the V-shaped signal in Fig. 5B. Thin arrow indicates the presumptive midbrain-hindbrain junction.* **(D)** *Transversal section of a 24 hpf embryo showing otx3 expression in the ventrolateral portion of the epithelium surrounding the otic vesicle (ov) and a faint staining at the surface of the hindbrain.* **(E)** *Frontal section of a 36 hpf embryo showing a strong signal in the diencephalon (d) and anteriorly in the olfactory region (solid arrow) and fainter in the telencephalon (t) along the optic nerves (thin arrow).* **(F)** *Thick transversal section (25 μm) of a 36 hpf embryo through the diencephalon revealing a staining in the geniculate nuclei in the ventral thalamus (white arrowheads). d, diencephalon; nk, neural keel; nt, notochord; ov, otic vesicle; t, telencephalon. 1 cm= 63 μm (A,B,D); 50 μm (C); 16 μm (E); 25 μm (F).*

tions which are characteristic of mammalian cortex are shared by the zebrafish optic tectum (Ariens Kappers *et al.*, 1936).

Both genes are expressed very early in the diencephalon, each exhibiting a peculiar pattern. A striking difference is seen in the hypothalamus. During the generation of the tract of the postoptic commissure (TPOC) (15-20-somite stages) dividing the diencephalon into the thalamic and hypothalamic region, *otx3* displays a dynamic expression in the hypothalamus. While *otx3* expression is first localized in the anterior hypothalamus (Fig. 7B, 4-somite stage), it gradually progresses posteriorly, still maintaining a strong expression in anterior regions (Fig. 7D, 15-somite stage). Finally the expression becomes confined to the posterior hypothalamus (Fig. 7F, 28-somite stage). It would be interesting to see whether this expression follows or anticipates the formation of the TPOC. In contrast, *otx1* appears confined to the region of the presumptive posterior hypothalamus (Fig. 7E).

When the commissures and main tracts are formed, both genes show strong expression in the epiphysis and ventrally along the dorso-ventral diencephalic tract (DVDT), as reported for the mouse *Otx1* and *Otx2* (Simeone *et al.*, 1993). Interestingly, this tract and the posterior commissure begin to form between 18 and 19 hpf, several hours after the initiation of *otx1* and *otx3* expression. This data suggests the involvement of these genes in specifying this tract.

Except for the *otx1* expression in the anterior telencephalon, the anterior borders of *otx1* and *otx3* expression appear to be identical, localized around the anterior commissure (Fig. 7E,F).

In midbrain, whereas *otx1* transcripts are mainly found dorsally and along the midbrain-hindbrain junction, *otx3* expression is mainly ventral. A particular strong staining is seen in the tegmentum, in a region which could correspond to the nucleus of medial longitudinal fasciculus (Wilson *et al.*, 1990).

These data are in agreement with the recent publication of MacDonald *et al.* (1994) which have shown that the boundaries of the expression domains of some genes correlate with the positions at which pioneering neurons differentiate and extend their axons (Wilson *et al.*, 1990, 1993; MacDonald *et al.*, 1994).

The posterior boundary of *otx1* and *otx3* expression is well defined from the end of gastrulation, suggesting that the midbrain posterior limit is specified very early. The identification of the location of the most posterior band was made by double staining with *pax2*, known to be expressed at the midbrain-hindbrain boundary (Krauss *et al.*, 1991, 1992). Double staining performed on embryos from 90% epiboly to 24 hpf stages shows that the *pax2* domain overlaps with the *otx* most posterior expression. Thus the posterior boundary of *otx* gene marks the boundary of the midbrain just anterior to the midbrain-hindbrain junction.

Similarly to the *Otx1* and *Otx2* of mouse, *otx1* and *otx3* of zebrafish are expressed differentially in the developing diencephalon and midbrain. Their peculiar distribution suggests an involvement, in cooperation or independently, in the patterning of these brain regions.

Mouse and zebrafish *otx1* are also expressed in a similar manner in the sensory organs. The region giving rise to the iris, the optic nerves, the olfactory epithelium and a ventro-lateral area of the otic vesicles express *otx1* and *otx3*.

Recently Li and collaborators have studied the expression of *otx1* and *otx2* genes of zebrafish (Li *et al.*, 1994). We made a comparison to our results for the *otx1* gene since the *otx1* sequence that we show in Fig. 1 is similar to the corresponding region of *otx1* gene previously reported by them. Their expression data are in agreement with our results, although some differences are seen.

They report first *otx1* expression around 55% epiboly (midgastrula), whereas we observed that expression is first detectable even earlier, around 50% epiboly as a patch of cells in the dorsal blastoderm margin.

According to them, the expression in telencephalon is detectable before tail bud stage and disappears between tail bud and 5-9-somite stages. Conversely, by bud stage we observed a staining in the presumptive diencephalon-midbrain area only. In our hands, the expression in the telencephalon is first visible around 9-somite stage, a time corresponding to the second wave of expression reported by them.

At 24 hpf, they observed a stronger signal in the tegmentum compared to the tectum. In our case the expression is especially strong in the dorsal tectum and in restricted zone of the ventro-posterior tegmentum. These discrepancies could be due to the use of different probes for the whole-mount *in situ* hybridization or/and to the sensitivity of this technique.

In the light of Li's work, we noticed that the early expression pattern of *otx3* is closer to that of *otx1*. In contrast, the late expression pattern of *otx3* is closer to that of *otx2*. Interestingly, the time of appearance for *otx1* and *otx3* is similar and they are both detected before *otx2*. These is in contradiction with what happens in the mouse and should be clarified in the future.

In accordance with Li's description and the previous study in mouse, zebrafish *otx* genes appear to be expressed in regions corresponding to main axon tracts or commissures. Indeed *otx3* and *otx2* as well seem to be expressed along the tract of the postoptic commissure. Moreover, the expression zone of the three genes ventral to the epiphysis seems to correlate with the position of dorso-ventral diencephalic tract, as seen previously in the mouse. In addition we observed for *otx3* alone a strong expression in the zone of the nucleus of medial longitudinal fasciculus.

In conclusion, homeobox genes of the *Otx* family are expressed in a very similar manner in mouse, frog, chick and zebrafish. They are first expressed immediately before or at gastrulation in a population of deep cells fated to migrate anteriorly and give rise to anterior mesendoderm. In late gastrulae, *Otx* expression extends to the overlying anterior neuroectoderm in a continuous region fated to become forebrain and midbrain. This expression domain persists through neurulation with a sharp posterior boundary at the midbrain-hindbrain junction. Finally, at the end of neurulation the continuous *Otx* expression domain becomes subdivided in several more restricted subdomains. Some of these demarcate major anatomical structures in the developing brain. Prominent among these is the zona limitans intrathalamica (zli, termed also dvdt) at the boundary between ventral thalamus and dorsal thalamus. The striking conservation of the expression domain through the various vertebrate species suggests a common role for these genes in brain specification and patterning.

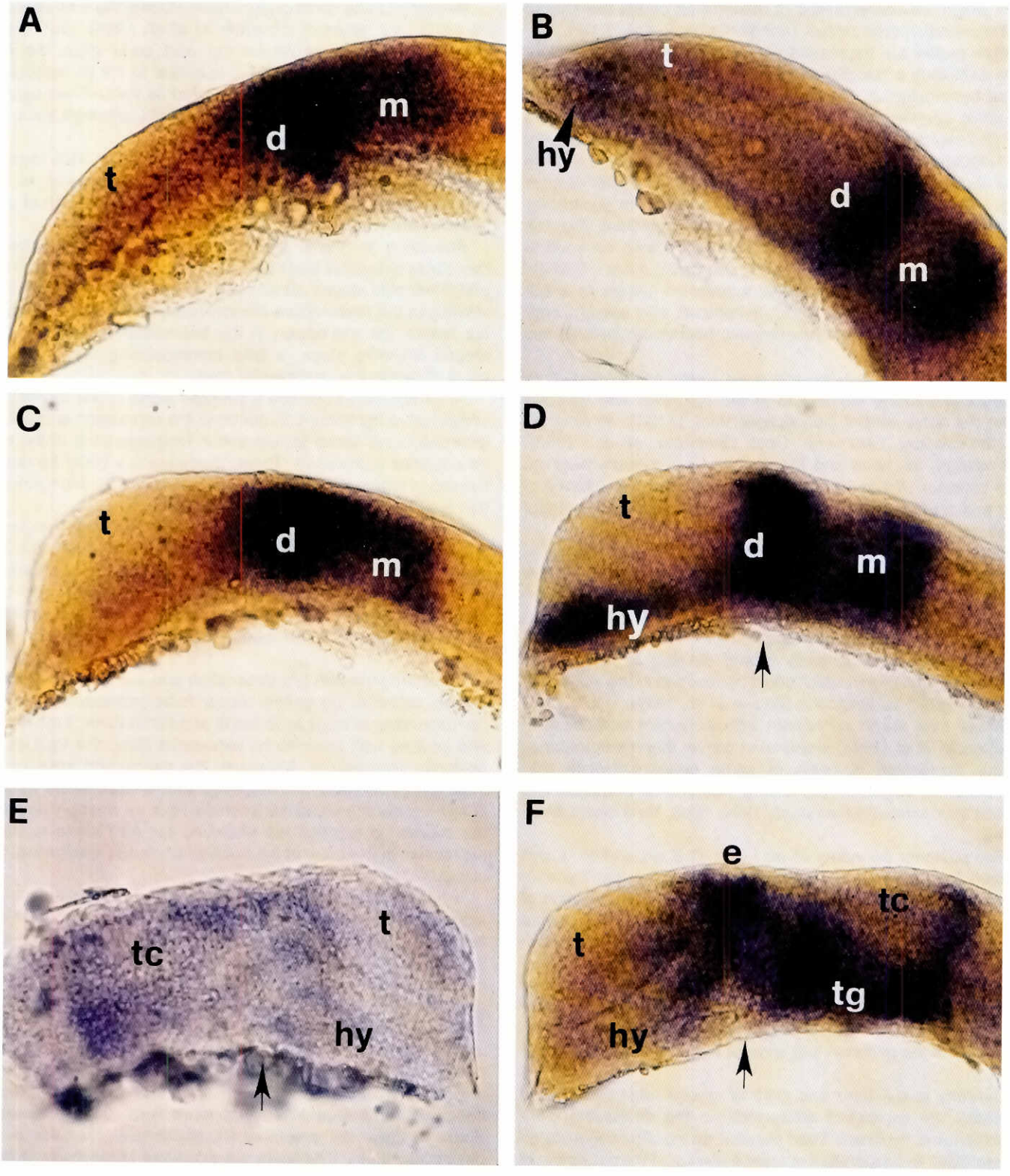

Fig. 7. Spatial and temporal distribution of the *otx1* and *otx3* genes expression during somitogenesis. *Lateral views of whole-mounted embryos labeled with otx1* **(A,C,E)** *or otx3* **(B,D,F)** *probes. Rostral is to the left except in* **(E)**; *dorsal is up. In* **(D,E,F)** *an arrow indicates the position of the dorsoventral diencephalic tract (DVDT).* **(A)** *otx1, <4 som;* **(B)** *otx3, 4 som;* **(C)** *otx1, 13 som. The expression in telencephalon is not visible.* **(D)** *otx3, 15 som;* **(E,F)** *otx1 and otx3 respectively, 28 som. d, diencephalon; e, epiphysis; hy, hypothalamus; m, midbrain; tc, tectum; t, telencephalon; tg, tegmentum. 1 cm= 50 μm.*

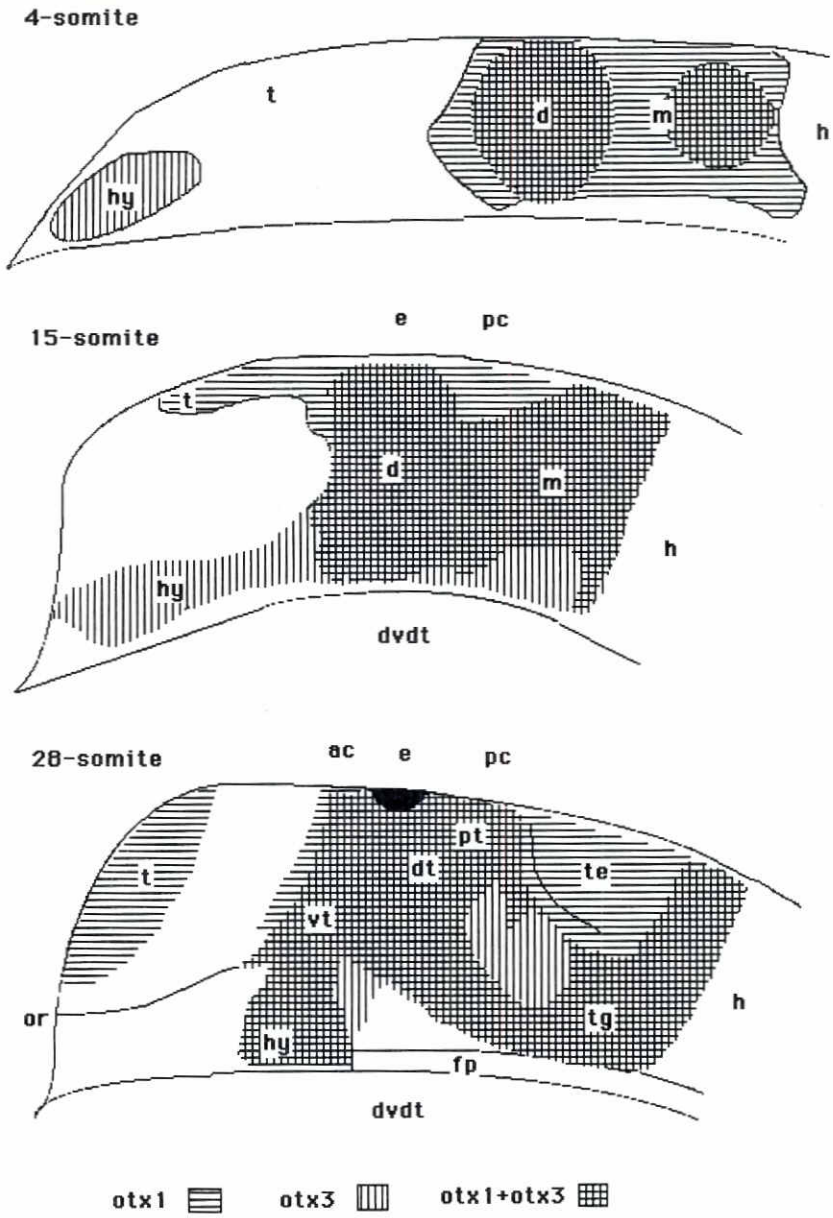

Fig. 8. Summary of embryonic expression domains of *otx1* and *otx3*. *Regions of the embryo expressing only otx1 are indicated by horizontal lines; those expressing only otx3 are indicated by vertical lines, and those expressing both otx genes are indicated by crossed lines. Lateral views of 4-somite, 15-somite, and 28-somite stages embryos. Epiphysis is indicated in black because at this stage its structure is discernible. Anterior is to the left. ac, position of the anterior commissure; dt, dorsal thalamus; dvdt, dorsoventral diencephalic tract (zli); e, epiphysis ; fp, floor plate; h, hindbrain; hy, hypothalamus; m, midbrain; or, optic recess; pc, position of the posterior commissure; pt, pretectum; t, telencephalon; te, tectum; tg, tegmentum; vt, ventral thalamus.*

Materials and Methods

Screening of a zebrafish genomic DNA library

A zebrafish genomic DNA library (Molven *et al.*, 1991) was screened under low stringency hybridization and washing conditions with a *Drosophila otd* sequence, already used by us for the isolation of the mouse *Otx* genes (Simeone *et al.*, 1992). Of the $5x10^5$ phage plaques screened, several independent positive clones were isolated. Three of them were sequenced. Only two genes, termed *otx1* and *otx3*, are described in this paper.

Probes for in situ hybridization

Sense and antisense RNA probes were produced using the DNA labeling Kit from Boehringer Mannheim by incorporating digoxygenin/fluorescein-11-UTP into the transcription products of selected *otx1*, *otx3* and *pax2* subclones inserted into appropriate transcription vectors containing T7 and SP6 promoters.

The *otx1* and *otx3* probes used are shown in Fig. 1. They were cloned into pGEM3 vector. The *pax2 (pax[zf-b])* probe was previously described in Krauss *et al.* (1991, 1992).

Whole-mount in situ hybridization

We used a protocol described in Oxtoby and Jowett (1993) with some modifications. Zebrafish embryos grown at 28.5°C were fixed in 4% PAF in 1xPBS, overnight (ON) at 4°C. After fixation, the embryos were washed at least 3x15 min in PBS and dechorionated. Then they were cleared in methanol 100% (at least 30 min at -20°C), rehydrated through a methanol/PBT series and washed twice in PBT (PBT: 0.1% Tween 20 in PBS). Embryos older than 1 somite were treated with proteinase K at final concentration of 10 µg/ml in PBT, 5 min to 15 min according to stage. After two brief washes in PBT, they were fixed again in PAF 4% in PBS for 20 min at room temperature (RT). The embryos were subsequently extensively washed and prehybridized for at least 4 h at 65°C in hybridization buffer HB (50% formamide, 5xSSC, 500 µg/ml torula yeast RNA, 50 µg/ml heparin, 0.1% Tween 20, 1 M citric acid, pH 6 final).

The prehybridization solution was removed and 200 µl of HB containing 1 µg/ml of digoxygenin-labeled RNA probe was added to the embryos. After the hybridization ON at 65°C, the embryos were washed at 65°C through a HB/2xSSC series, twice in 2xSSC then twice in 0.2xSSC for 30 min and finally through a 0.2xSSC/PBT series, then in PBT. They were preblocked 1 h at RT with 2% sheep serum, 2 mg/ml BSA in PBT and incubated with preadsorbed alkaline phosphatase conjugated antidigoxygenin Ab (1/2,000) for 2 h at RT. After washing 4x20 min in PBT and 3x5 min in BCL (0.1 M Tris/HCl pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 0.1% Tween 20 in water), the enzyme activity was revealed by addition of NBT/BCIP in BCL and stopped by washing in PBT. For observing the staining, embryos were squeezed between slide and coverslip (so-called flat-mounted embryos).

Two color whole-mount in situ hybridizationn.

Double staining was carried out essentially as described in Hauptmann and Gerster (1994). In general, position markers were stained in red and *otx* genes in blue.

Whole-mount immunochemistry

The *no tail* antiserum (kindly given by Schulte-Merker) was used on whole-mount *in situ* hybridized embryos as described in Halpern *et al.* (1993) with some modifications. Briefly, after several washes in PBS and PBT (PBS, 0.1% Triton X-100, 1% BSA, 1% DMSO) embryos were incubated in a blocking reagent mix containing 2% normal goat serum in PBT for 30 min at RT. Then embryos were incubated ON at 4°C in the *no tail* antiserum diluted 1/5,000 in the previous blocking buffer with gentle rocking. After washing in PBT, incubation in biotinylated goat anti-rabbit Ig antibody (1/200) (SB, Southern Biotechnology) for 4 h at RT, washing and incubation in 1/50 streptavidine-peroxidase (SB) for 2h at RT, the embryos were finally washed extensively and the enzyme activity was revealed in DAB 0.5 mg/ml containing 0.001% H_2O_2. The color reaction was allowed to proceed for 15-30 min and stopped by several washes in PBT.

Sectioning of whole-mount hybridized embryos

After washings in PBT and PBS embryos were dehydrated with an ethanol series (30-50-70-85-95-100%) for 20 min each followed by xylene 20 min and embedded in paraffin wax. 10 µm sections were prepared and dewaxed in xylene (twice, 10 min) possibly rehydrated, washed in PBS and counterstained with propidium iodide and finally dehydrated and mounted with Depex.

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