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Sequence and expression pattern of *pax-6* are highly conserved between zebrafish and mice

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Summary

Despite obvious differences in the patterns of early embryonic development, vertebrates share a number of developmental mechanisms and control genes, suggesting that they use similar genetic programs at some stages of development. To examine this idea, we isolated and characterized one such gene, *pax-6*, a member of the pax gene family, from the zebrafish *Brachydanio rerio* and determined the evolutionary conservation in the structure and expression of this gene by comparison to its homolog in mice.

We found two alternatively spliced forms of the zebrafish *pax-6* message. Sequence and expression pattern of the zebrafish *pax-6* gene are remarkably similar to its murine homolog. *pax-6* expression begins during early neurulation. A stripe of cells in the neuroectoderm, including the prospective diencephalon and a part of the telencephalon, expresses *pax-6* as well as the hindbrain and the ventral spinal cord extending from the level of the first rhombomere to the posterior end of the CNS. During later development more limited regions of the brain including the eye, the olfactory bulb and the pituitary gland express *pax-6*. Cells at the midbrain-hindbrain junction express *eng* genes and are separated from the neighboring *pax-6* regions by several cells that express neither gene, indicating a complex subdivision of this region. *pax-6* expression appears during processes when cell-to-cell signalling is thought to be important, for example during induction of the eye and regionalization of the spinal cord and brain, suggesting that it may be one component mediating the response to inductive interactions.

Key words: brain, engrailed, evolution, neurulation, paired-box, spinal cord.

Introduction

Many features of developmental control genes are highly conserved among organisms as diverse as nematodes and humans (Boncinelli et al., 1988; Bürglin et al., 1991; Dressler and Gruss, 1988; Duboule and Dolle, 1989; Graham et al., 1989; Kessel and Gruss, 1990; Scott et al., 1989). Most of the these genes are members of multigene families that share conserved domains such as homeo-boxes, paired-boxes, zinc-fingers, or POU-boxes (Bopp et al., 1986; Dressler et al., 1988; Dressler and Gruss, 1988; Gehring and Hiromi, 1986; Herr et al., 1988; Kessel and Gruss, 1990; Struhl, 1989; and references therein). Recently evidence has been obtained for an essential function during vertebrate development for some of these genes (Balling et al., 1988, 1989; Chisaka and Capecchi, 1991; Cho et al., 1991; Harvey and Melton, 1988; Kessel et al., 1990; McMahon and Bradley, 1990; Ruiz i Altaba and Melton, 1989; Wright et al., 1989). The structural and functional conservation of these genes suggests that despite different kinds of cell movements during gastrulation and neurulation, vertebrates share a common genetic program of development (Dressler and Gruss, 1988; Kimmel, 1989).

One particularly interesting class of genes is the pax gene family which presently contains eight members in vertebrates (Burri et al., 1989; Dressler et al., 1988; Walther et al., 1991). All pax genes analysed thus far are expressed during early development of the mouse in a temporally and spatially restricted manner (Kessel and Gruss, 1990). With the exception of *pax-1*, pax genes are expressed in the developing nervous system along the entire length of the hindbrain and spinal cord and in specific parts of the brain. The various pax genes are expressed in specific dorsoventrally restricted domains (Deutsch et al., 1988; Dressler et al., 1990; Goulding et al., 1991; Jostes et al., 1991; Nornes et al., 1990; Walther and Gruss, 1991). Certain features of their expression patterns, like expression of *pax-2* and *pax-8* in the embryonic kidney and the developing eye (Dressler et al., 1990), suggest that pax genes may function in inductive interactions. To examine the degree of evolutionary conservation of the pax genes and as a first step towards analyzing their functions, we isolated pax genes from the zebrafish, *Brachydanio*

rerio. Here we report the sequence and embryonic expression pattern of the zebrafish *pax-6* gene. Our comparative analysis with its murine homolog demonstrates that this gene has been more conserved both in sequence and expression pattern than the *wnt-1* (Molven et al., 1991), *engrailed*-related (*eng*) (Fjose et al., 1988; Holland and Williams, 1990) or *hox* genes (Njølstad et al., 1990).

Materials and methods

Animals

All embryos used in this study were obtained from the Oregon AB line. Embryos and zebrafish were maintained as described previously (Westerfield, 1989). Embryos were staged by hours postfertilization at 28.5°C (h).

Probes

The following fragments were used to screen the cDNA library: a 2 kb *Bam*HI *pax-3* cDNA fragment (Goulding et al., 1991), a 487 bp *Eco*RI fragment from a *pax-2* cDNA, pC31A, containing paired-box sequences (Dressler et al., 1990; provided by H. Fickenscher), a genomic 320 bp *Hin*dIII - *Xba*I fragment containing paired-box sequences of *pax-4* (Walther et al., 1991), and a 1.7 kb *Eco*RI *pax-6* cDNA fragment (Walther and Gruss, 1991). Subclones of cDNA n108 (Fig. 1A) cloned into the *Eco*RV site of the Bluescript vector (Stratagene) were used for Northern-blot analysis and in situ hybridization (Fig. 1A). Probe A is a 436 *Bam*HI - *Eco*RI fragment from the 5′ end. Probe B is a 42 bp oligonucleotide with the sequence of the paired-box insertion of zf*pax-6b* (synthesized by the Biotechnology laboratory, University of Oregon). Probe C is a 140 bp *Hin*dIII - *Nde*I fragment from the 3′ end of the coding region. A 250 bp *Sau3A* cDNA fragment for *eng-1* and an 890 bp *Eco*RI cDNA fragment for *eng-2* were used for in situ hybridization (Ekker and Westerfield, unpublished data). Digoxigenin-labelled RNA-probes were synthesized with the Genius-Kit (Boehringer) according to the manufacturer's specifications.

Screening of cDNA library

Approximately 1.6×10^6 clones of a λZAP library prepared from 20 to 28 h zebrafish embyros (made by R. Riggleman and K. Helde, kindly provided by D. Grunwald) were screened under conditions of low stringency (43% formamide, 37°C) with probes from the mouse paired-box containing genes *pax-2*, *pax-3*, *pax-5* and *pax-6* as described previously (Breier et al., 1988; Colberg-Poley et al., 1985). 140 clones were isolated and after three rounds of purification 80 clones remained positive. 21 of these clones were identified as cDNAs corresponding to the *pax-6* gene by sequencing, restriction mapping and hybridization with each other at conditions of high stringency. Positive clones were excised from the phage by the protocol recommended by the manufacturer (Stratagene).

DNA sequencing

Both strands of overlapping subclones of the cDNAs were

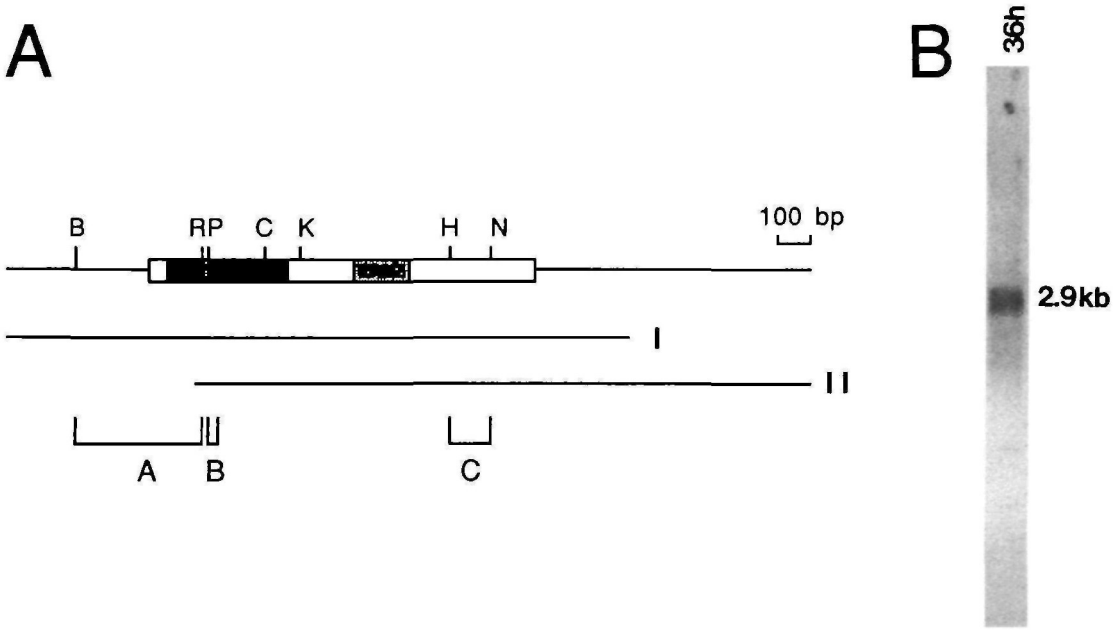


Fig. 1. Structure and expression of *pax-6*. (A) Schematic representation of the pax-6 cDNAs. The box indicates the coding region, the darkly stippled regions the paired domain, the lightly stippled regions the homeodomain and the black region the 14 amino acid insert in the paired domain of zf *pax-6b*. Lines indicate the two classes of isolated cDNAs (I: n9, n16, n27, n108, n130; II: n8, n15). The 5′ ends of class II clones were near position 500 of the sequence shown in Fig. 2. Clone n108 contains an additional sequence 5′ of the insert which most likely represents an intron (data not shown) which is not present in the other partially sequenced cDNA's. Brackets indicate probes used for Northern blot hybridization and in situ hybridization. (A) a *Bam*HI - *Eco*RI fragment, (B) a cloned oligonucleotide containing the 42 bp insert of zf *pax-6b*, (C) a *Hin*dIII - *Nde*I fragment. (B) Northern blot of 36 h embryonic mRNA hybridized with a *pax-6* probe. A Northern blot with 5 μg of poly(A)$^+$ RNA was hybridized with probe A. A message of approximately 2.9 kb was detected. The same result was obtained when using probe C. (B) *Bam*HI, (C) *Cla*I, (H) *Hin*dIII, (K) *Kpn*I, (N) *Nde*I, (P) *Pst*I, (R) *Eco*RI.

sequenced as double-stranded DNA using the sequenase kit (USB). Sequences were analysed using the GCG program package (Devereux et al., 1984).

RNA analysis

For preparation of total RNA, embryos were lysed with 4 M guanidinium thiocyanate and the RNA was isolated with a CsCl gradient as described previously (Püschel et al., 1990). Poly(A)$^+$ RNA was prepared from total RNA with the MicroFastTrack kit (Invitrogen). Northern blots were prepared according to standard procedures and hybridized at 42°C in 50% formamide, 5×SSC, 0.5% SDS and 5×Denhardts. Washes were done as described previously (Breier et al., 1988; Colberg-Poley et al., 1985).

In situ hybridization

In situ hybridization to 8 μm paraffin sections of embryos was done using standard procedures (Wilkinson and Green, 1990; M. Kessel, personal communication). Embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C and dehydrated in a graded alcohol series for 15 minutes at each step and embedded in paraplast plus. Hybridization of whole embryos with RNA probes was done based on established procedures (Tautz and Pfeifle, 1989; S. Schulte-Merker, personal communicaton). Embryos were fixed overnight in 4% paraformaldehyde in PBS and subjected to the following treatments at room temperature, if not stated otherwise: twice 5 minutes in PBS, twice 5 minutes in methanol, 1 hour to several days in methanol at −20°C, 5 minutes in 50% methanol in PBST (1×PBS, 0.1% Tween-20 (Biorad)), 5 minutes in 30% methanol in PBST, twice 5 minutes in PBST, 20 minutes in 4% paraformaldehyde in PBS, twice 5 minutes in PBST, 1 to 20 minutes (depending on the age of the embryos) in 50 μg/ml proteinase K (Boehringer) in PBST, 5 minutes in PBST, 20 minutes in 4% paraformaldehyde in PBS, twice 5 minutes in PBST, 10 minutes in acetylation mix (100 mM triethanoamine, 1/400 volume acetic anhydride), twice 10 minutes in PBST. After this treatment, the embryos were transferred to Eppendorf-tubes with hybridization-mix (50% formamide, 5×SSC, 0.1% Tween-20, 50 μg/ml heparin (Sigma), 100 μg/ml sonicated salmon sperm DNA (Pharmacia), 10 μg/ml yeast tRNA (Sigma)) and prehybridized at 65°C for 1 to 4 hours. Afterwards the hybridization mix was replaced by new hybridization mix containing digoxigenin-labeled RNA probes at 1 to 2.5 ng/μl final concentration and was then incubated overnight. An equal number of embryos, processed in parallel without hybridizing to an RNA probe, were used to preadsorb the anti-digoxigenin antibody. The probe was denatured for 5 minutes at 68°C and chilled on ice. After hybridization, the embryos were transferred to 50% formamide, 2×SSC and washed for 1 hour at 65°C. The following washes were then used: three times 10 minutes in 2×SSC at 37°C, 1 hour in PBST containing 20 μg/ml RNAase A (Sigma) and 100 units/ml RNAase T1 (Boehringer) at 37°C, 10 minutes in 2×SSC at 37°C, 1 hour in 50% formamide/2×SSC at 65°C, 15 minutes in 2×SSC at 55°C., twice 15 minutes in 0.2×SSC at 55°C, 5 minutes in PBST. Embryos were incubated for 1 to 4 hours in PBS containing 2% normal sheep serum, 0.25% Tween-20 and 0.25% Triton X-100. In parallel, embryos were used to preabsorb the anti-digoxigenin antibody - alkaline phosphatase conjugate (Boehringer) in the same buffer at a dilution of 1 to 8000. The embryos were then incubated with the preadsorbed antibody overnight at 4°C. After four 30 minutes washes with the blocking solution at room temperature and three washes for 10 minutes in reaction buffer (100 mM Tris-HCl pH 7.9 or 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, 1 mM levamisol (Sigma), the embryos were stained using 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate and 337.5 μg/ml nitroblue tetrazolium salt in reaction buffer for 5 to 24 hours.

No signal was observed with either method when using sense probes as a control (data not shown). Signals observed in whole mount in situ hybridization experiments were also seen when using sections.

Results

Isolation of zebrafish pax-6 *cDNA clones*

We screened a cDNA library prepared from 20 to 28 h zebrafish embyros and isolated 21 clones of the zebrafish gene homologous to the murine *pax-6* gene (Walther et al., 1991; Walther and Gruss, 1991) as shown by sequence analysis (see below). These 21 cDNAs fall into two main classes, which differ at their 3′ ends (I and II, Fig. 1A) based on restriction mapping and sequencing. The difference probably results from internal priming at an A-rich sequence at position 2130 of the sequence (Fig. 2) which is completely contained within clones extending further 3′. No poly(A) addition signal is found at this position. Two forms of cDNAs were found by sequencing the paired boxes of several cDNAs. One corresponds to the described murine cDNA (Walther and Gruss, 1991) and is named zf*pax-6a* in this paper (represented by clones n8, n15, n130, n9, n130), the second, represented by two clones (n108, n16), contained a 14 amino acid (42 bp) insertion after amino acid 45 of the paired box (termed zf*pax-6b*, Figs. 1A, 2). The longest open reading frame codes for a protein of 437 aminoacids which contains both a paired and a homeobox (see below). The complete sequence of 2.8 kb contains 500 bp 5′ nontranslated sequence and a 3′ nontranslated region of 950 bp. Northern analysis of poly(A)$^+$ RNA from zebrafish embryos of 36 h shows a single transcript of approximately 3 kb size (Fig. 1B). Thus our cDNA clones are probably close to full length. Southern blot analysis showed that *pax-6* is a single copy gene (A. Fritz and A.W.P., unpublished results).

pax-6 *is highly conserved*

Comparison of the conceptual translation of the longest open reading frame (ORF) to that of other isolated *pax* genes showed that the 21 isolated cDNA clones were most similar to the murine *pax-6* gene. The coding sequences are 80% identical at the nucleotide level and 97% identical at the amino acid level. Two gaps of 3 and 1 amino acids, respectively, were introduced for the alignment (Fig. 3). The longest ORF of the zebrafish *pax-6* gene contains 19 more amino acids at the amino terminus than its murine counterpart. The first ATG shows a higher degree of similarity to the Kozak consensus sequence than the second (Kozak, 1987). The homolog of the differentially spliced mRNA, zf*pax-6b*, was also found in the mouse. It contains an insert with an identical sequence at the identical position (Walther and Gruss, 1991). At position 82 of the paired domain, the fish gene contains a glycine instead of a serine; the homeodomain is identical. The high degree of conservation also extends outside these

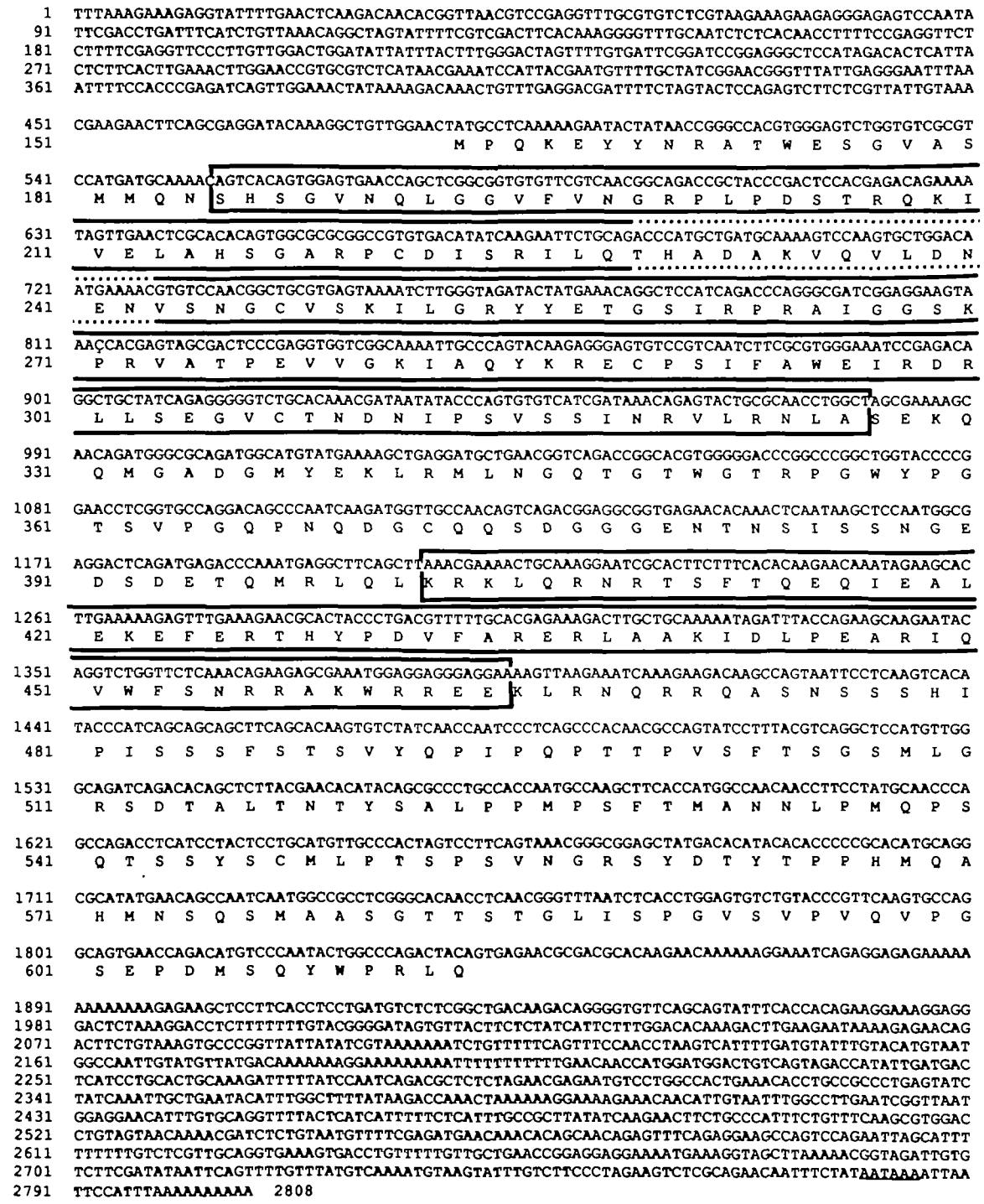


Fig. 2. Sequence of the zebrafish *pax-6*. cDNA n108 and the 3′ nontranslated part of cDNA n8 were sequenced completely. The 5′ and 3′ ends, the paired box and different regions of the coding sequence of 6 clones (n5, n9, n15, n16, n27, n130) were sequenced partially. All sequences were colinear to the one shown. The sequence compiled from all analysed subclones is shown. The first box indicates the paired box, the second the homeobox. The dotted line indicates the insert in the paired box found in clones n16 and n108. The conceptual translation of the longest open reading frame is shown. Several in frame stop codons are present 5′ of the first methionine codon.

domains. Surprisingly, we found that the last 113 bp, including the poly(A) addition signal, are 90% identical to the corresponding sequence of the murine gene (nucleotides 2686 - 2799 in Fig. 2, Walther and Gruss, 1991). This region is separated from the coding sequence by 850 bp which show no appreciable amount of similarity to the murine homolog. Because of the high degree of sequence identity and the almost identical expression pattern (see below), we conclude that we have isolated the zebrafish homolog of the

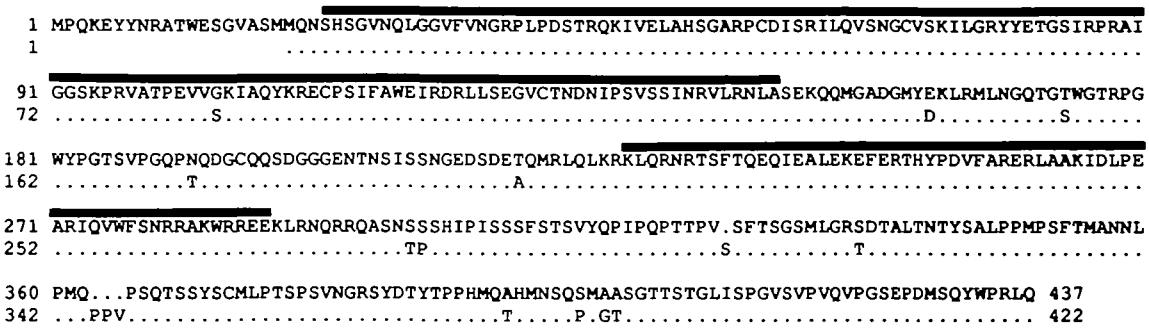


Fig. 3. Sequence comparison of murine and zebrafish *pax-6*. The conceptual translation of the longest open reading frame from the zebrafish *pax-6* cDNA sequence is shown in the top line. Dots in the zebrafish sequence indicate gaps introduced for optimal alignment. The bottom line is the sequence from the murine *pax-6* protein. Dots indicate identical amino acids; paired domains and homeodomains are overlined.

murine *pax-6* gene. The partial sequence of *pax[zf-a]* recently described by Krauss et al. (1991) is identical to our sequence and thus, *pax[zf-a]* probably represents an independent isolate of the *pax-6* homolog.

pax-6 *is expressed during early neurulation*

We analysed expression of the zebrafish *pax-6* gene between 9 h and 3 days of development with in situ hybridization of RNA probes to paraffin sections and to whole embryos. Two different probes derived from sequences common to *zfpax-6a* and *zfpax-6b* produced the same pattern. A probe specific for *zfpax-6b*, containing just the 42 bp insert in the paired box failed to detect expression at any stage analysed (data not shown), probably because of its small size. Thus, the isolation from a cDNA library is the only evidence for expression of this variant. We detected expression first around 10 h (100% epiboly, Fig. 4A-D). Shortly after epiboly is completed and before the tailbud is visible, an oval- shaped domain in the prospective prosencephalon expresses *pax-6* (Fig. 4A-D). It contains a stripe of cells at its posterior margin which express higher levels of *pax-6* than cells farther anterior. Shortly later, additional expression appears in the prospective rhombencephalon on both sides of the midline (Fig. 4A-D). By 14 h (10 - 11 somites; Figs. 4E,F, 5A-C,E) the anterior expression domain includes the optic vesicles, but excludes the optic stalk. Expression in the optic vesicle is highest in the lateral and posterior regions (Figs. 5A,E; 9A, B) which will invaginate to form the optic cup. The anteroposterior and dorsoventral boundaries of expression are identical in the prosencephalon and the optic vesicle (Figs. 4F, 9A,B and not shown). In addition, the overlying ectoderm, which is known in other species to interact with the optic vesicles to form the lens, contains *pax-6* mRNA (Fig. 5E). Most of the hindbrain and spinal cord expresses *pax-6* at this stage excluding only the most dorsal and ventral cells (Fig. 5D). Expression extends along the entire anteroposterior axis (Figs. 4E, 5A,B) and remains essentially the same through 20 h (Fig. 5F and data not shown).

Expression of pax-6 *during neurogenesis*

At 24 h, cells in the ventral part of the hindbrain and spinal cord excluding the floorplate and cells adjacent to it express *pax-6* (Figs. 5G, 6B-C). Expression extends anteriorly to the border between the met- and myelencephalon (Figs. 5F, 6A,B). The anterior boundary of expression in the hindbrain is crescent-shaped when viewed in horizontal sections (Fig. 5F) and extends to the middle of the Ro1 rhombomere near the midline (Fig. 6A,B). A second expression domain includes the diencephalon, parts of the telencephalon and the olfactory bulb (Figs. 5F, 6A,B). In additon, the eyes, including both the optic cup and the lens, express *pax-6* (Fig. 6A, and not shown). Expression in the brain is more restricted at 32 h (Fig. 7A,B) and 36 h (Fig. 7C-H). The dorsal part of the diencephalon contains high levels of pax-6 transcripts up to the border between the di- and mesencephalon (Fig. 7A-H). Two distinct groups of cells at this border (Fig. 7C,D, arrow) and in the pituitary gland (Fig. 7A, B, arrow) also express *pax-6*. These groups are contained within the broader regions that expressed *pax-6* at earlier stages.

During the following 2 days of development, expression levels drop significantly in the spinal cord (data not shown) and become more restricted in the brain to smaller groups of cells in the di- and telencephalon (Fig. 8A-D). By 3 days, expression in the developing eye is restricted to the ganglion cell layer (Fig. 8D). The overall pattern of expression at this stage suggests that particular nuclei of the brain could specifically express *pax-6*. However, since specific brain regions of older zebrafish have yet to be identified, we did not unequivocally assign the detected signals to particular nuclei.

pax-6 *and* eng *expression domains are distinct*

pax-6 is expressed in the prospective brain at a time when the *eng* genes are activated (Hatta et al., 1991). To determine the expression borders of *pax-6* relative to the *eng* genes, we performed double-hybridizations to 14 h embryos with probes for *pax-6* and *eng-1* or *eng-2*. Both *eng* genes are expressed in the gap of *pax-6* activity at the midbrain - hindbrain border (Fig. 9). The *pax-6* and *eng-1* expression domains are adjacent but separated by approximately three to five cells on each side of the *eng-1* stripe (Fig. 8B, C). *eng-2* is expressed

in a narrower stripe in the middle of the *eng-1* expression domain (Fig. 8A, D).

Discussion

Sequence and expression pattern of pax-6 *are highly conserved*

Sequence comparisons of the murine and fish *pax-6* ORFs revealed a remarkably high identity of 97% at the amino acid level. This conservation is considerably greater than that of the fish homologs of *hox-2.2*, *wnt-1*, *eng-1*, *eng-2* and the *hox-7* genes which are all in the range of 70% identical to their murine counterparts (Fjose et al., 1988; Holland, 1991; Holland and Williams, 1990; Molven et al., 1991; Njølstad et al., 1990; Ekker and Westerfield, unpublished data). More importantly, the conservation is seen throughout the entire coding sequence; whereas in the case of the *hox* or *eng* genes, only the homeoboxes and other discrete regions show more than 90% identity to their mouse homologs. In accordance with the conservation of the sequence, the expression pattern of *pax-6* is also nearly identical in terms of tissue and regional specificity in the two species (Walther and Gruss, 1991). Both genes are expressed in the brain, the ventral part of the spinal cord, the olfactory bulb, the pituitary gland and the eye.

Both mice and zebrafish express a variant form of the *pax-6* message, containing a 14 amino acid insertion in the paired domain. The complete conservation of this variant argues for its importance, although the functional consequences of this insertion are presently unclear. Recent biochemical analyses of the paired-box-containing genes showed that they are DNA-binding transcription factors (Chalepakis et al., 1991; Goulding et al., 1991; Treisman et al., 1991) as had been inferred previously from the presence of DNA-binding domains. Since the 14 amino acids are inserted into the paired domain they may affect DNA-binding abilities of the protein, thus providing an additional mode for developmental regulation.

We found an even more striking degree of conservation at the end of the 3′ non-translated region than in the coding sequence (89% compared to 80%). Although the function of this region is unknown, it is possible that it is involved in the regulation of expression, either at the transcriptional or posttranscriptional level.

The high conservation of the *pax-6* gene between mouse and fish, whose ancestors separated 300 million years ago, is remarkable and suggests that this gene serves an important function. In addition to the homeodomains and paired domains, another yet to be identified functional domain in the *pax-6* protein could explain the high degree of homology in the C-terminal half.

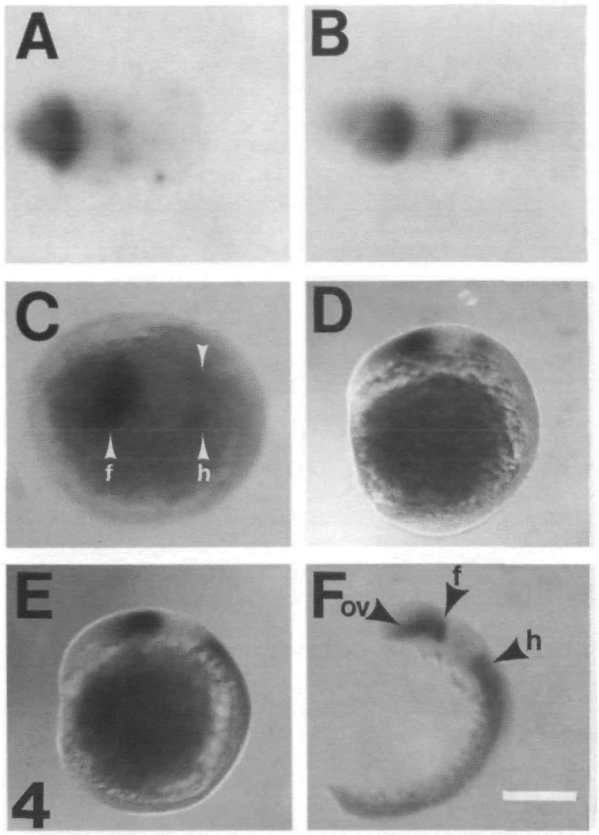
pax-6 *expression and regionalization of the brain*

pax-6 is expressed very early during neurulation in the zebrafish embryo. Initially in the brain (at 10 h) a broad region expresses *pax-6* including the prospective optic vesicle, the telencephalon and the diencephalon. It has been shown that the mesoderm specifies the regional identity of the overlying neuroectoderm in various vertebrates (Frohman et al., 1990; Gurdon, 1987; Hemmati et al., 1990; Sive et al., 1989). Expression of

Fig. 4. *pax-6* is expressed in zebrafish during neurulation. Whole embryos were hybridized with probes A and C, which are specific for pax-6. Hybridization with each probe alone gave identical results. (A, B) Dorsal view of the prospective brain region at 10 h. Two expression domains are discernible. The domain in the prospective forebrain appears earlier (A) than the one in the prospective hindbrain (B). The embryos differ slightly in age and show the gradual appearance of the hindbrain expression domain. Younger embryos show only the forebrain stripe (not shown). (C) Dorsal and (D) lateral view of a 10 h embryo, showing the two expression domains (h, hindbrain; f, forebrain). No expression is seen at the midline of the hindbrain (A, C and data not shown). (E, F) Lateral views of 14 h embryos. Expression is seen in the prospective forebrain (f), hindbrain (h) and the optic vesicle (ov). Anterior is to the left in A - C and up and to the left in D - F. Scale bar is 150 μm in A, B, C and 125 μm in D, E, F.

Fig. 5. *pax-6* is expressed in limited regions of the neural keel. Paraffin sections of embryos were hybridized with ^{35}S-labelled RNA probes (probe A, probe C gave the same results). (A,B) Parasagittal sections of a 14 h embryo. Expression is seen in the optic vesicle (A, B), the prospective forebrain (B) and the prospective hindbrain and spinal cord (A, B). Lines in B indicate the plane of section in panels C and D. (C) Cross-section of the prospective forebrain at 14 h. Expression is seen in the dorsal half, excluding the dorsal-most cells of the forebrain. (D) Cross-section of the neural keel at 14 h. Expression is seen in the ventral half. (E) Parasagittal section of the optic vesicle (ov) at 14 h. Expression is seen in the ventral and lateral part of the vesicle. Little or no expression is detectable in the dorsal part. Expression is also seen in the ectoderm (ect) overlying the optic vesicle. (F) Horizontal section of an 18 h embryo. Expression is seen in the diencephalon (di) and the myelencephalon (my). (G) Cross-section through the trunk of a 24 h fish. Expression is detectable in the ventral half of the neural tube, excluding the floorplate and cells adjacent to it as was confirmed by inspecting the corresponding bright-field images (not shown). Anterior is to the left in A, B, E and F and dorsal to the top in C, D and G. Scale bar is 200 μm in A, B, 160 μm in C, D, G and 120 μm in E, F. ce: cerebellum.

Fig. 6. *pax-6* is expressed in the brain and spinal cord. (A) 24 h embryo. Expression is seen in the olfactory bulb (ob), the diencephalon (di), the eye and the myelencephalon (my). (B) Expression in the hindbrain starts at the level of the first rhomobomere (ro1). The line indicates the level of the cross-section in C. (C) A double-exposure of the bright-field (blue) and dark-field (red) images of the same cross-section of the hindbrain at the level of the otocyst (oc). The domain of *pax-6* expression is seen as a red signal. Expression in the hindbrain is restricted to the ventral half excluding the floorplate. Anterior is to the left in A and B and dorsal is up. Scale bar is 100 μm in A; 200 μm in B; and 50 μm in C.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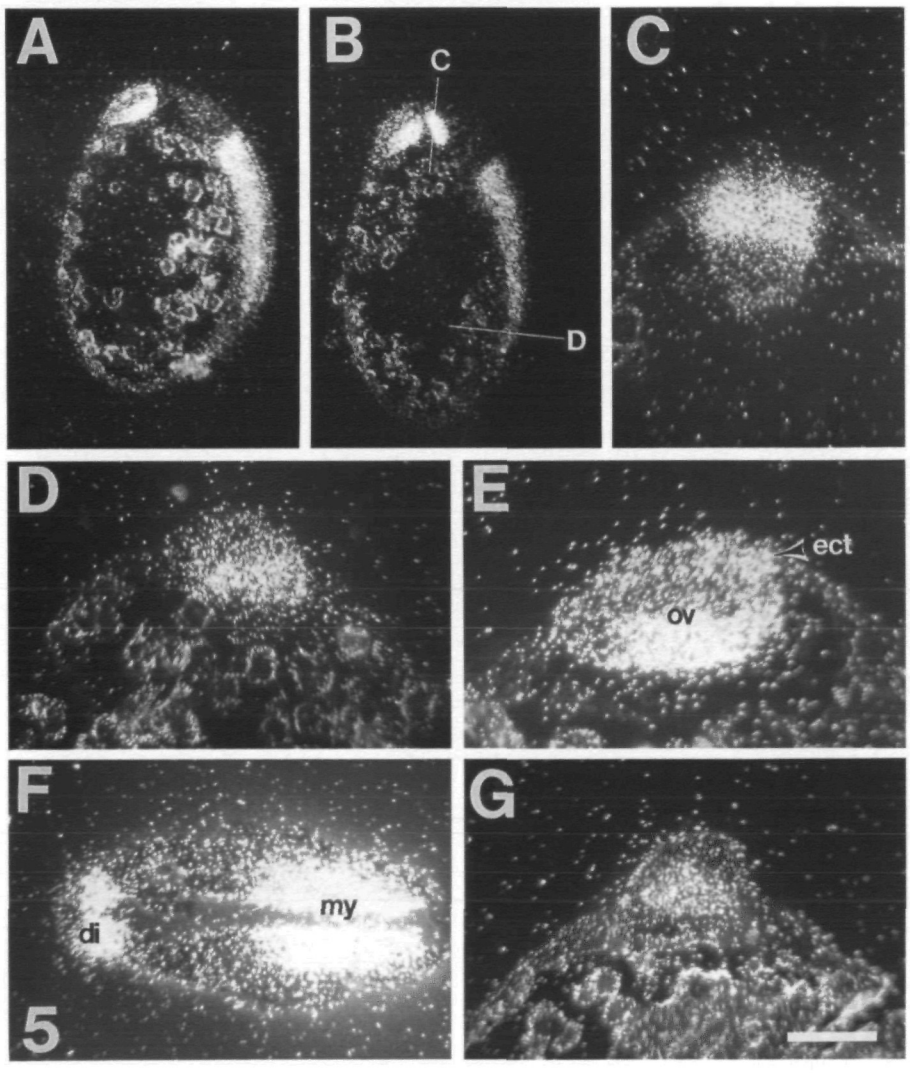
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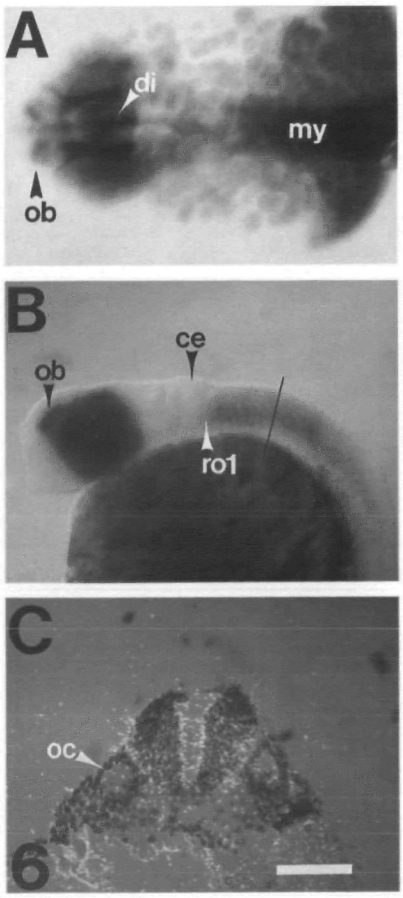
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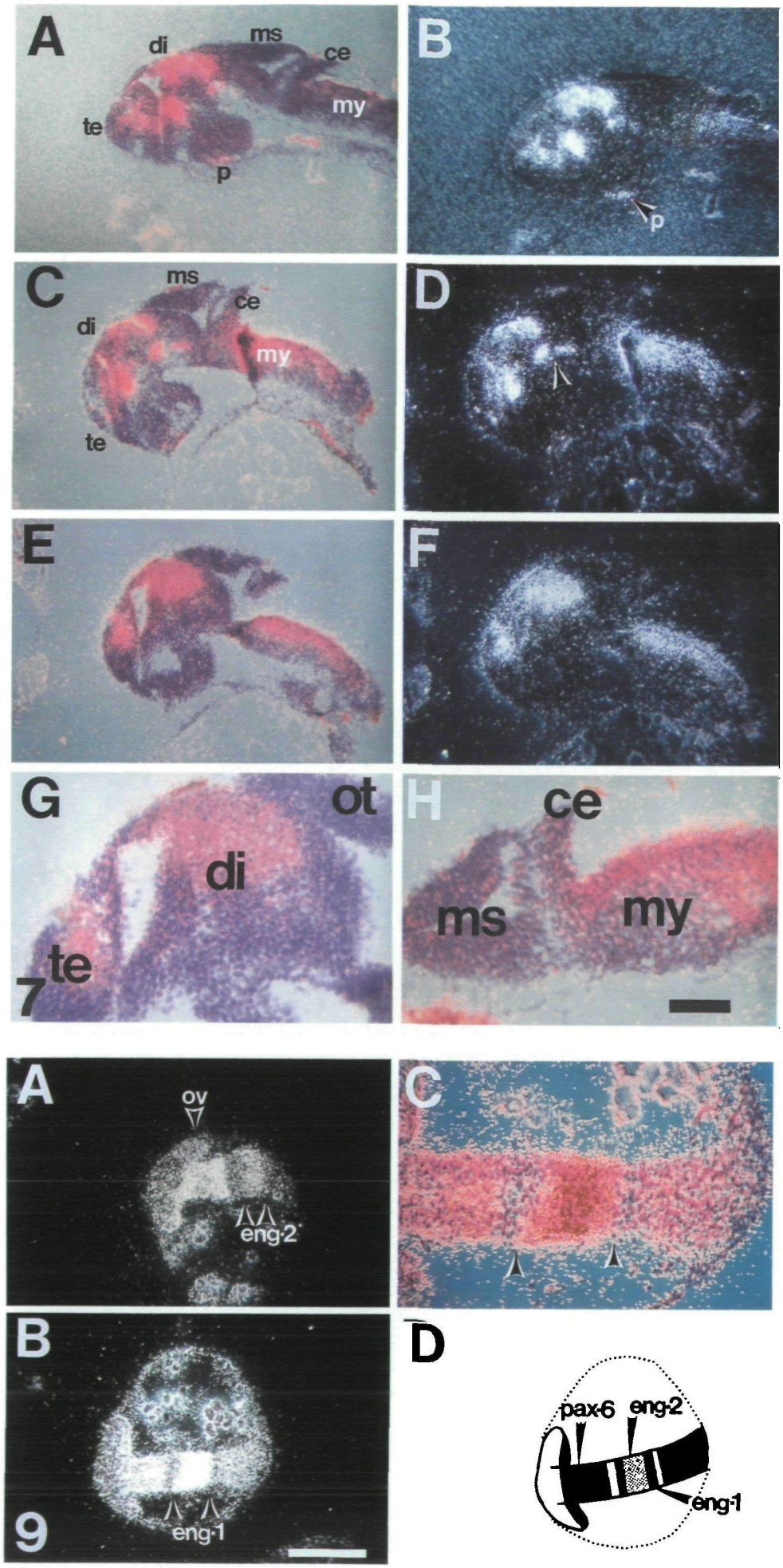

Fig. 7. *pax-6* is expressed in distinct regions at 32 and 36 h. Parasagittal sections of 32 h (A, B) and 36 h (C - H) fish were hybridized with a pax-6 probe (probe A, probe C gave identical results). Double-exposures of bright-field (blue) and dark-field (red) images (A, C, E) and dark-field images (B, D, F) of the same sections. Expression is detectable in the myelencephalon up to the border between the myelencephalon and the cerebellum (A - F, H). The density of grains over the cerebellum and the mesencephalon is the same as that seen throughout the slide and do not represent specific hybridization to *pax-6* transcripts. *pax-6* is expressed in the diencephalon and the telencephalon (A - F, G) and specific subareas within these regions (C, D: arrow, G). Abbreviations: ce, cerebellum; di, diencephalon; ms, mesencephalon; my: myelencephalon; ot: optic tectum; p, pituitary gland; te, telencephalon. Anterior is to the left, dorsal up. Scale bar is 100 μm in A - F and 40 μm in G, H.

Fig. 9. *pax-6* and *eng*-genes are expressed in adjacent regions. Adjacent horizontal sections of 14 h embryos were hybridized with probes specific for *pax-6* or *eng-1* separately or with *pax-6* and *eng-2* (A) or *pax-6* and *eng-1* (B, C) together. Anterior is to the left. Only sections with the double-hybridization are shown. Arrows delineate the limits of *eng-1/2* expression. The signals to the left and to the right of *eng-1* expression are specific for *pax-6*. The *eng* genes are expressed in the gap of *pax-6* activity in the mes- and metencephalon. C is a higher magnification view of B. The *eng-1* expression domain is separated from that of *pax-6* by about 5 to 10 cells which show no activity of either gene. (D) Schematic summary of the superimposed expression domains. Stippled areas express *pax-6*, the dark areas express only *eng-1* and the lightly stippled area expresses both *eng-1* and *eng-2*. The broken line indicates the edge of the yolk. Scale bar is 200 μm in A, B, D and 80 μm in C.

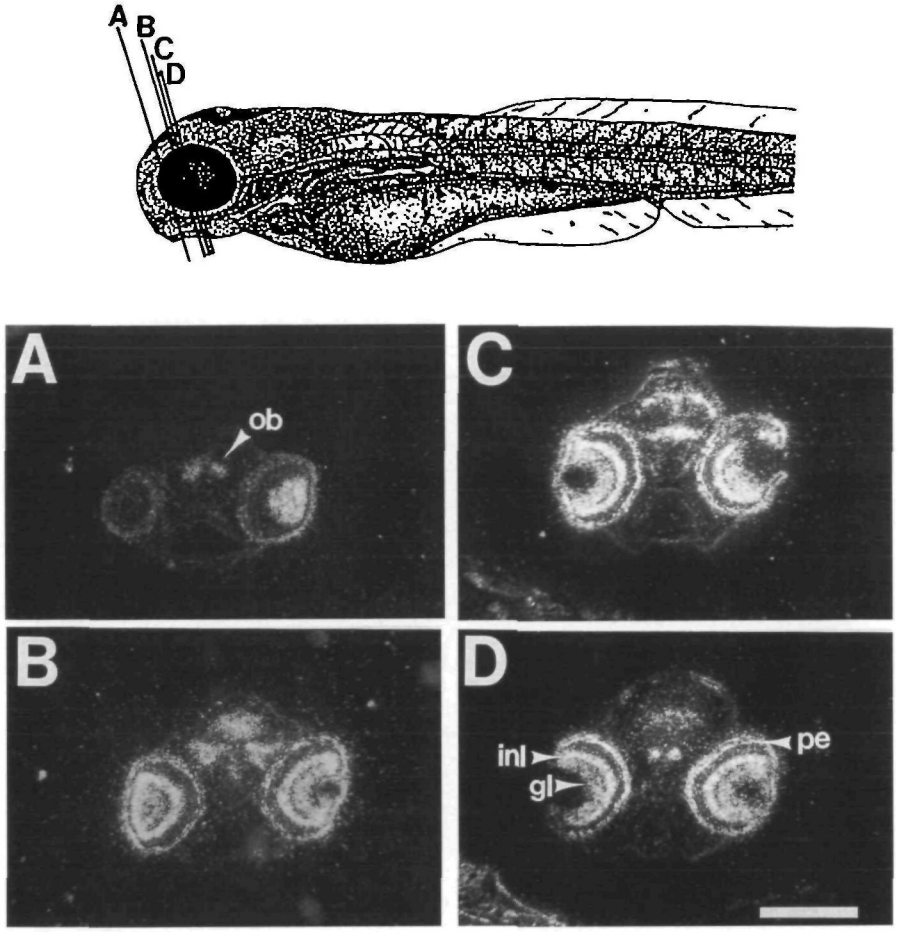

Fig. 8. *pax-6* is still expressed in the head at hatching (72 h). Cross-sections of 3-day fish were hybridized with pax-6 specific probes. Dorsal is up. The sketch in the upper part indicates approximate levels of the sections. Expression is apparent in the olfactory bulb (A, ob), the ganglion cell layer (gl) and the inner nuclear layer (inl) of the eye (A - D). Specific parts of the diencephalon express pax-6 (C - D). The signal in the pigment epithelium (pe) of the eye is due to pigment and does not represent a hybridization signal. Scale bar is 200 μm in A - D and 290 μm in drawing.

pax-6 could be an early response to this induction (Gurdon, 1987). *pax-6* expression is first seen around the time epiboly is complete, at a time when other genes implicated in regional specification of the ectoderm, like the *eng* genes, are activated (Davis et al., 1991; Hatta et al., 1991). Our double labelling demonstrates that both *eng-1* and *eng-2* are expressed in the gap of *pax-6* activity in the mes- and metencephalon. Interestingly, the *eng-1* domain is not directly adjacent to, but separated from, *pax-6* by several cells expressing neither gene. This could indicate a more complex subdivision of this region of the brain than previously suggested by analysis of *Hox-2* and *Krox-20* expression in the hindbrain. The *Hox-2* and *Krox-20* expression domains coincide with neuromere borders (Stern and Keynes, 1988; Lumsden, 1990; Wilkinson, 1990; and references therein) and are, therefore, directly adjacent to one another, whereas the *eng* and *pax-6* domains are separated and seem to cross over neuromere borders.

pax, *eng* and other genes could form overlapping and complementary domains in the forebrain as they do in the hindbrain and spinal cord. Their early expression makes them good candidates for functioning in the regionalization of the forebrain in response to inductive signals from the mesoderm by regulating directly or indirectly other genes during the differentiation of neuronal structures. *pax-6* expression in the brain becomes progessively more restricted during development. Thus *pax-6* could, in addition, be involved in the specification of specific subsets of neurons, as has been shown in *Drosophila* for segmentation genes (Doe et al., 1988).

Induction of pax-6 *expression*

In the spinal cord, the initially broad region of expression becomes restricted to a ventral domain by 24 h. The notochord plays a key role in the dorsoventral patterning of the spinal cord (Yamada, 1990). In the mouse, the *pax* genes have overlapping and complementary expression domains in the spinal cord and are good candidates for genes responding to signals from the notochord and/or the floor plate. Recent evidence, that pax-gene expression is regulated by signals originating from the notochord (Goulding et al., unpublished data), supports this notion.

Two other sites of *pax-6* expression, the pituitary gland and the optic vesicle and eye, are consistent with its role in induction. The floor of the diencephalon and Rathke's pouch interact to form the pituitary gland and the optic vesicle interacts with the overlying ectoderm and induces the formation of the lens (Gurdon, 1987; and references therein). It remains to be determined if the same kind of interactions take place in the zebrafish. The isolation of the *pax-6* gene provides a useful marker to study these processes. As the eye develops, *pax-6* expression becomes progressively re-

stricted; at 24 h both optic cup and lens express *pax-6* whereas at 3 days only the ganglion cell layer shows *pax-6* activity.

Conclusions

The expression pattern of *pax-6* points to several developmental processes in which it may serve a regulatory function. *pax-6* could be involved in the early regionalization of the brain and in dorsoventral patterning of the spinal cord. The expression in specific parts of the brain later in development suggests a function in specifying subsets of cells during neurogenesis. Thus, the pax genes may serve several different functions, as previously described for the segmentation genes in *Drosophila* (Doe et al., 1988). *pax-6* is expressed predominantly in structures where inductive, cell-to-cell interactions regulate development. Thus, it may be activated by inductive signals and may, itself, be part, of a signaling pathway.

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Note added in proof

The nucleotide sequences reported in this paper will appear in the EMBL, Gen Bank, and DDBJ nucleotide sequence databases under the accession number X63183.