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EMBRYONIC EXPRESSION AND DNA-BINDING PROPERTIES OF ZEBRAFISH PAX-6

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Summary: Zebrafish pax-6 (pax[zf-a]) and its murine homologue are structurally and functionally related to the *Drosophila* paired box gene eyeless, a master control gene for eye development. This report details the zebrafish pax-6 embryonic expression pattern both at the mRNA and protein level. Transcripts are first detected in the presumptive forebrain and hindbrain regions of the neural plate. After formation of the neural keel, Pax-6 protein accumulates within the same two domains. Expression is also observed in the optic vesicles and lens placodes,confirming that the Pax-6 protein is expressed in those areas of the eye where it is assumed to control differentiation. The relative DNA-binding affinity of the zebrafish Pax-6 protein to different categories of Pax recognition sites is shared with the murine homologue.

Developmental control genes containing a paired box (Pax genes) were first identified in *Drosophila* (1) and subsequently a number of structurally related genes have been isolated in mammals and other vertebrates (2-4). The Pax proteins are transcription factors and the paired domain (PD) encoded by the paired box consists of two DNA-binding subdomains with resemblance to the homeodomain (5). In addition, many of these developmental regulatory proteins contain a paired-type homeodomain which also can contribute to the DNA-binding specificity (6).

Most vertebrate Pax genes are, in contrast to Hox genes, not close functional relatives to the structurally homologous group of *Drosophila* genes (7,8). The recent identification of the Pax-6 homologue, eyeless, in *Drosophila*, emphasizes the unique position of this gene within the vertebrate Pax family (9,10). Pax-6 and eyeless were implicated in eye development based on the detection of transcripts in eye primordia and observations that loss-of-function mutations in both genes lead to a reduction or complete absence of all eye structures (7,9,11). Most remarkably, targeted expression of eyeless in various imaginal disk primordia of *Drosophila* induced ectopic eyes and the same was achieved in experiments with Pax-6 (10). These results

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provide strong evidence that eyeless/Pax-6 are functionally strongly conserved master control genes for eye morphogenesis.

The detection of eyeless/Pax-6 transcripts in various regions of the central nervous system in *Drosophila* and mice embryos indicates additional functions in neural development (9,12). Supporting this proposition, notochord grafting experiments were shown to cause early changes in the dorsoventral expression pattern of chicken Pax-6 suggesting this response contributes to neural patterning in the spinal cord (4). In addition, study of the regional expression of the zebrafish Pax-6 homologue in the forebrain led to the proposal that the gene influences the patterning of the first neurons and axons (3,13-15).

This report provides details of the early embryonic expression pattern of the zebrafish pax-6 gene both with respect to mRNA and protein. Consistent with the view that Pax-6 is a master control gene for eye morphogenesis the protein product is detected in the optic vesicle primordia and surface ectodermal cells that form the lens placode. In addition, the study reveals close similarities in the DNA-binding specificity of the zebrafish Pax-6 protein relative to its murine homologue.

MATERIALS AND METHODS

Animals. Adult zebrafish were obtained from commercial suppliers and maintained according to standard methods (16). Embryos were staged at 28.5 °C.

In situ hybridization and immunohistochemistry. Whole-mount in situ hybridization with the entire pax-6 cDNA probe was performed essentially as described by Krauss et al. (13). Procedures for immunohistochemistry and sectioning of stained embryos are according to The Zebrafish Book (16). For primary incubations the anti-Pax-6 antibody was diluted 1:200.

Cloning, expression and purification of PD-GST fusion protein. A 541 basepair PD-containing fragment encoding amino acids 1-180 of the zebrafish Pax-6 protein (13) was amplified by PCR and cloned in the BamHI/SmaI site of the expression vector pGEX 2T (Pharmacia Biotech). Recombinants containing inserts with the appropriate in frame fusion to Glutathion S-transferase (GST) were identified by DNA sequencing. Expression and affinity purification of the fusion protein was performed according to Smith and Johnson (17).

Cloning and expression of full-length protein. A cDNA fragment (nucleotide position 466-2701) containing the entire coding region of the zebrafish Pax-6 protein (13) was amplified by PCR. Following addition and digestion of NotI linkers, the fragment was cloned in the NotI site of the eukaryotic vector pRC/CMV (Invitrogen). The construct was transfected into human embryonal kidney cells (293 cells) by the Calcium-precipitation procedure (18) and nuclear extracts were prepared as described by Hoppe-Seyler et al. (19).

Electrophoretic mobility shift assays (EMSA). The assay was performed with 90 ng GST-PD (or 500 ng nuclear extract) and 12,000 cpm probe in a 50 mM KCl buffer (50 mM KCl, 10 mM Tris-HCl [pH 7.5], 5 mM DTT) according to Fried (20). The double-stranded oligonucleotides used for the DNA-binding assays have been described by Czerny et al. (21) and Epstein et al. (22).

RESULTS AND DISCUSSION

Spatial distribution of transcripts at early embryonic stages: The earliest embryonic expression patterns of pax-6 (pax[zf-a]) have not been analysed in detail (3,13,15,23). To gain further insight, we used digoxigenin-labeled DNA probes in whole-mount in situ hybridization experiments on early embryonic stages. At the 10-h stage, when features of the neural plate first become visible, pax-6 transcripts are detectable within a domain

of the prospective forebrain consisting of two triangular areas that are fusing at the dorsal midline during neurulation (Figure 1A,B). A second expression domain appears as a bilateral pair of transverse stripes in the anterior region of the hindbrain primordium (Figure 1A). These bands start to join at the midline similar to the two rostral subdomains, but at a somewhat later stage. Simultaneously they extend parallel stripes of expression posteriorly into the prospective spinal cord region (Figure 1B). The transverse bands extend at a 60° angle relative to the anteroposterior axis and almost parallel to the posterior boundaries of the rostral expression domain. Interestingly, the zebrafish pax[b] and eng-2 genes are at this stage known to be expressed as transverse stripes located in the midbrain-hindbrain border region corresponding to the gap between the two pax-6 domains (24,25). This arrangement suggests a subdivision of the neural plate into segment-like units that in part are determined by the expression of eng-2 and the two pax genes. It is also tempting to suggest an involvement of pax[b] and/or eng-2 in repressing pax-6 expression at the midbrain-hindbrain border.

In agreement with previous analyses based on in situ hybridization with radioactive probes in tissue sections (13,23), pax-6 transcripts are at later stages detected in the hindbrain (not shown) and rostrally in a region that include major parts of the diencephalon and the optic vesicles (Figure 1C,D). At higher resolution in 15-h embryos, pax-6 is strongly expressed near the forebrain-midbrain boundary with transcript levels attenuating in the anterior part of the diencephalon (Figure 1C). In the optic vesicles, staining above background level is detected in most cells but strong hybridization signals are restricted to the regions which face the diencephalon. At later stages of development (19-h postfertilization) a high and uniform level of expression is present throughout the eye cup with the exclusion of the optic stalk region (Figure 1D). Concomitantly, the spatial distribution of pax-6 transcripts has become more differentiated in the forebrain. Consistent with results obtained for 24-h embryos (15), intense staining is detected in the dorsal thalamus whereas expression in the region anterior to the middiencephalic boundary is reduced by more than half (Figure 1D).

Spatial distribution of Pax-6 protein during eye and CNS development: A polyclonal antibody raised against the C-terminus of the zebrafish Pax-6 protein (15) was used for immunohistochemical analysis at early embryonic stages. Following an initial weak

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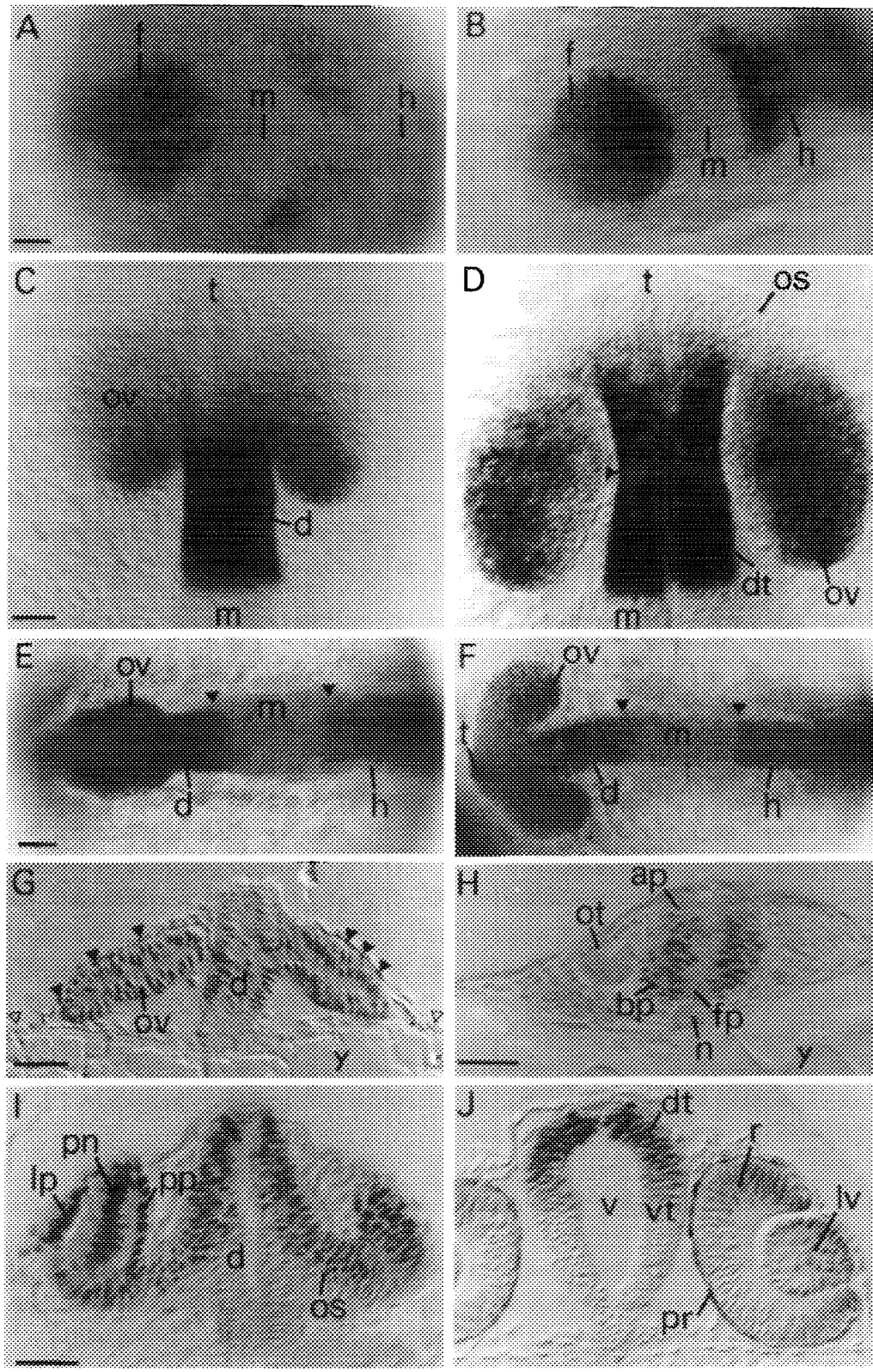
Figure 1. Spatial distribution of zebrafish pax-6 transcripts (**A-D**) and proteins (**E-J**) at early embryonic stages. (**A**) and (**B**) Dorsal views of the rostral regions in 10-h and 11-h embryos, respectively (anterior to the left). (**C**) and (**D**) The dorsal forebrain regions in 15-h and 19-h embryos, respectively (anterior is up). An arrowhead indicates the middiencephalic boundary. (**E**) and (**F**) Dorsal views of the rostral regions in 12-h and 16-h embryos, respectively (anterior to the left). Arrowheads mark the expression boundaries at the midbrain-hindbrain border and in the anterior hindbrain. (**G**) and (**H**) Cross-section from the diencephalic region of a 16-h embryo. Arrowheads mark cells with nuclear labeling in the overlying surface ectoderm. The nonexpressing, lateral surface ectodermal cells are indicated by open triangles. (**H**) Cross-section of the hindbrain in a 20-h embryo at the level of rhombomere 5. (**I**) and (**J**) Cross-sections from the diencephalic regions of embryos at the 19-h and 24-h stage, respectively. Abbreviations: ap, alar plate; bp, basal plate; d, diencephalon; dt, dorsal thalamus; f, forebrain; fp, floor plate; h, hindbrain; lp, lens placode; lv, lens vesicle; m, midbrain; n, notochord; ov, optic vesicle; os, optic stalk; ot, otic vesicle; pn, prospective neural retina; pp, prospective pigmented retinal epithelium; pr, pigmented retina; r, retina; t, telencephalon; v, third ventricle; vt, ventral thalamus; y, yolk. Bar, 40 μm.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staining at the 11-h stage (not shown), Pax-6 specific labelling is clearly seen in cells (mainly in the nuclei) of the putative forebrain and hindbrain regions of 12-h embryos (Figure 1E). Similar staining intensities are also detected at 17-h postfertilization (Figure 1F) indicating that there is no differential regulation of pax-6 at the translational level in the diencephalic and hindbrain regions. In cross-sections of the hindbrain at a later developmental stage (20-h) Pax-6 protein is clearly restricted to the ventral part of the neural tube (Figure 1H). As reported earlier for the dorsoventral distribution of pax-6 transcripts in zebrafish, mice and chicken (4,12,13), protein is detected mainly in a region including the basal- and intermediate plate but excluding the floor plate. This result confirms the expected correlation between the spatial distribution of Pax-6 protein and transcripts, and it supports the proposed role for the Pax-6 gene in dorsoventral patterning (4).

Interestingly, the rostral expression domain in 12-h embryos has a uniform staining intensity and it includes the primordia of the optic vesicles that at this stage are distinguished as lateral bulges (Figure 1E). Thus there is no clear difference in Pax-6 expression between cells located inside or outside the 'eye field' of the neural keel. Accordingly, additional gene(s) must be involved in initially defining the boundaries of the optic vesicles. This contrasts the situation in *Drosophila* where ectopic expression of eyeless (or murine Pax-6) is sufficient to initiate eye development (10).

The antibody labels nuclei of most cells in the optic vesicles of 16-h embryos (Figure 1F,G), but as with the observed spatial distribution of transcripts at the 15-h stage (Figure 1C), the level of Pax-6 protein is higher in the regions facing the neural tube. Moreover, cross-sections of the optic vesicles reveal Pax-6 expression in the overlying surface ectoderm whereas ectodermal cells further lateral and dorsal (above the diencephalon) are not labelled (Figure 1G). This demonstration of Pax-6 protein in primordial lens placode cells is consistent with previous observations of Pax-6 transcript localization in zebrafish, mice and chicken (12,13,26,27). The level of Pax-6 protein is increased in subsequent stages of eye morphogenesis and the staining in ectoderm becomes restricted to the lens placode (Figure 1I). This observation provides support to the proposal that Pax-6 is essential for the transition from ectoderm to lens placode (23). In addition to the involuting lens primordium, high levels of Pax-6 protein are also detected in the prospective pigmented retinal epithelium layer and neural retina (Figure 1I). Moreover, optic stalk staining clearly connects the eye expression to the diencephalic domain. At a later stage (24-h) a more differentiated expression pattern is observed in the eye where staining is most predominant at the rim of the retina and in outer parts of the lens vesicle (Figure 1J).

Zebrafish Pax-6 DNA-binding properties: In an initial effort to investigate the transcriptional regulatory functions of the zebrafish Pax-6 protein, its DNA-binding properties were compared to those known for the murine homologue (21,22). Both the PD and the full-length protein were assayed for DNA-binding activity to a high affinity Pax protein recognition site (CD19-2Ains) efficiently recognized by murine Pax-6 (21). EMSA with the PD-GST fusion protein revealed strong binding to the CD19-2Ains probe and the specificity of this

interaction was demonstrated by differential competition with unlabeled probes containing various types of binding sites (Figure 2A). While unlabeled CD19-2Ains competes efficiently, the related Pax sites CD19-2 and H2A2.2 show only weak competition. In the case of an unrelated site (AP1) and another Pax site (PRS-4) derived from the *Drosophila* Paired e5 site of the even-skipped gene (28), significant competition is not detected. Studies on the murine Pax-6 PD have revealed similar preferences for these Pax sites (21,22). The results are consistent with the half-site model proposed for the DNA interaction by the two subdomains of the PD (21). According to this model, which was recently confirmed by crystallographic analysis (5), PD recognition sites consist of 3' and 5' half-sites that bind to the discrete N-terminal and C-terminal subdomains, respectively, hence in different recognition sites (class I and class II) the individual half-sites contribute unequally to the binding affinity. As reported for the murine homologue (21,22), the PD of zebrafish Pax-6 prefers the CD19-Ains sequence which optimizes specific interactions between the two half-sites and both subdomains. For the full length protein the pattern of competition with the different recognition sites (Figure 2B) is

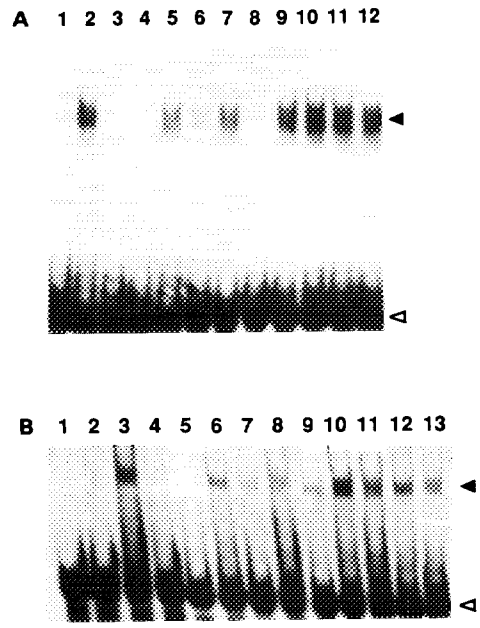

Figure 2. DNA-binding of the zebrafish Pax-6 PD and the full-length protein. In (**A**) and (**B**) different lanes show competition with excess (50x/100x) of various unlabeled sites. (**A**) Analysis of the binding of PD (GST-PD fusion protein) to labeled CD19-2Ains probe (in all lanes). **Lanes**: **1**, labeled probe only; **2**, labeled probe and PD only; **3**, 50x CD19-2Ains; **4**, 100x CD19-2Ains; **5**, 50x CD19-2; **6**, 100x CD19-2; **7**, 50x H2A2.2; **8**, 100x H2A2.2; **9**, 50x AP1; **10**, 100x AP1; **11**, 50x PRS-4; **12**, 100x PRS-4. (**B**) Analysis of the binding of full length zebrafish Pax-6 (from nuclear extracts) to labeled CD19-2Ains probe (in all lanes). **Lanes**: **1**, labeled probe only; **2**, labeled probe and nuclear extracts from cells transfected with the vector; **3**, labeled probe and Pax-6 nuclear extract only; **4**, 50x CD19-2Ains; **5**, 100x CD19-2Ains; **6**, 50x CD19-2; **7**, 100x CD19-2; **8**, 50x H2A2.2; **9**, 100x H2A2.2; **10**, 50x AP1; **11**, 100x AP1; **12**, 50x PRS-4; **13**, 100x PRS-4. In both panels the free labeled probe is indicated by open triangles and arrowheads mark the probe/protein complexes.

almost identical to that shown for the PD alone, emphasizing the predominance of the PD in the interaction with these sites. This study demonstrates for the first time the close correspondence in DNA-binding specificity of homologous Pax proteins in zebrafish and mammals.

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