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Gene expression pattern

Six3, a medaka homologue of the Drosophila homeobox gene *sine oculis* is expressed in the anterior embryonic shield and the developing eye

Felix Loosli[1], Reinhard W. Köster[1], Matthias Carl, Annette Krone, Joachim Wittbrodt*

SFB Junior Group, Institute for Human Genetics, University of Göttingen, c/o Max-Planck Institute for Biophysical Chemistry, Am Fassberg, 37077 Göttingen, Germany

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Abstract

The conserved transcription factor *Pax6* is essential for eye development in Drosophila and mammals (Hill, R.E., Favor, J., Hogan, B.L.M., Ton, C.C.T., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D., van Heyningen, V., 1991. Mouse small eye results from mutations in a paired-like homeobox containing gene. Nature 354, 522–525; Ton, C., Hirvonen, H., Miwa, H., Weil, M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N., Meijers-Heijboer, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L.C., Saunders, G.F., 1991. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. Cell 6, 1059–1074; Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo, N., Toniguchi, S., Dari, H., Jseki, S., Ninomiya, Y., Fujiwara, M., Watanabe, T., Eto, K., 1993. A mutation at the Pax-6 gene in rat small eye is associated with impaired migration of midbrain crest cells. Nature genet. 3, 299–304; Quiring, R., Walldorf, U., Kloter, U., Gehring, W.J., 1994. Homology of the eyeless gene of Drosophila to the small eye gene in mice and aniridia in humans. Science 265, 785–789). These findings led to the hypothesis that additional genes involved in invertebrate and vertebrate eye development are structurally and functionally conserved (Halder, G., Callaerts, P., Gehring, W.J., 1995. New perspectives on eye evolution. Curr. Opin. Gen. Dev. 5, 602–609; Quiring, R., Walldorf, U., Kloter, U., Gehring, W.J., 1994. Homology of the eyeless gene of Drosophila to the small eye gene in mice and aniridia in humans. Science 265, 785–789). Candidates for such conserved genes are the Drosophila homeobox gene *sine oculis* (Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V., Zipursky, S.L., 1994. The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron l2, 977–996) and its murine homologue *Six3* (Oliver, G., Mailhos, A., Wehr, R., Copeland, N.G., Jenkins, N.A., Gruss, P., 1995. Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121, 4045–4055). *sine oculis* (*so*) is essential for the development of the larval and adult visual system (Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V., Zipursky, S.L., 1994. The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron l2, 977–996). *Six3* is expressed in the anterior neural plate and optic vesicles, lens, olfactory placodes and ventral forebrain (Oliver, G., Mailhos, A., Wehr, R., Copeland, N.G., Jenkins, N.A., Gruss, P., 1995. Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121, 4045–4055). Overexpression of mouse *Six3* gene in medaka fish embryos (*Orvzias latipes*) results in the formation of an ectopic lens, indicating that *Six3* activity can trigger the genetic pathway leading to lens formation (Oliver, G., Loosli, F., Koster, R., Wittbrodt, J., Gruss, P., 1996. Ectopic lens induction in fish in response to the murine homeobox gene Six3. Mech. Dev. 60, 233–239). We isolated the medaka *Six3* homologue and analyzed its expression pattern in the medaka embryo. It is expressed initially in the anterior embryonic shield and later in the developing eye and prosencephalon. The early localized expression of *Six3* suggests a role in the regionalization of the rostral head. © 1998 Elsevier Science Ireland Ltd. All rights reserved

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1. Introduction

To isolate the medaka *Six3* homologue, we designed

* Corresponding author. Tel.: +49 551 2011671; fax: +49 551 2011755; e-mail: jwittbr@gwdg.de

[1] These authors contributed equally to this work.

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degenerate primers specific for two regions conserved in the *Six/sine oculis* subclass of homeobox genes. The deduced amino acid sequence of the isolated cDNA clone shares the highest sequence similarity with the murine *Six3* gene. This is true for the respective homeodomains that are 95% identical and also for the *Six* subclass-specific regions of about 130 residues amino terminally to the homeodomain (Oliver et al., 1995; Kawakami et al., 1996), where mouse and medaka *Six3* are 74% identical. This indicates that the gene isolated is the medaka *Six3* homologue.

Expression of *Six3* is first detected at late gastrula stages in a U-shaped domain surrounding the anterior end of the axis (Fig. 3A). At early neurula stages, expression is detected in the prosencephalon, optic vesicles and anterior head ectoderm (Fig. 1A). Transient expression is visible at the two-somite stage in the region of the mid/hindbrain boundary (MHB) (Fig. 1B, arrow). At this stage *Six3* is expressed in the entire optic vesicles, the overlaying head ectoderm (Fig. 1C) and weakly in the presumptive lens placode (arrowhead in Fig. 1C). In the prosencephalon, *Six3* is expressed at the level of the optic vesicles, but is excluded from the dorsal most region and the ventral portion (Fig. 1C). At the four somite stage, expression in the presumptive lens placode is upregulated (Fig. 1D, arrowhead). Expression in the optic vesicles is downregulated, starting posteriorly (Fig. 1D, arrows). Thus, at the six somite stage, *Six3* is expressed at low levels in the neuroretina (Fig. 1E, arrowhead), but not in the pigmented retinal epithelium (PRE), (arrow in Fig. 1E). Expression persists in the optic stalk, the ventral diencephalon and transiently in the head ectoderm posterior to the optic vesicles (small arrowhead in Fig. 1E). No expression is detectable in the lens placode (Fig. 1E).

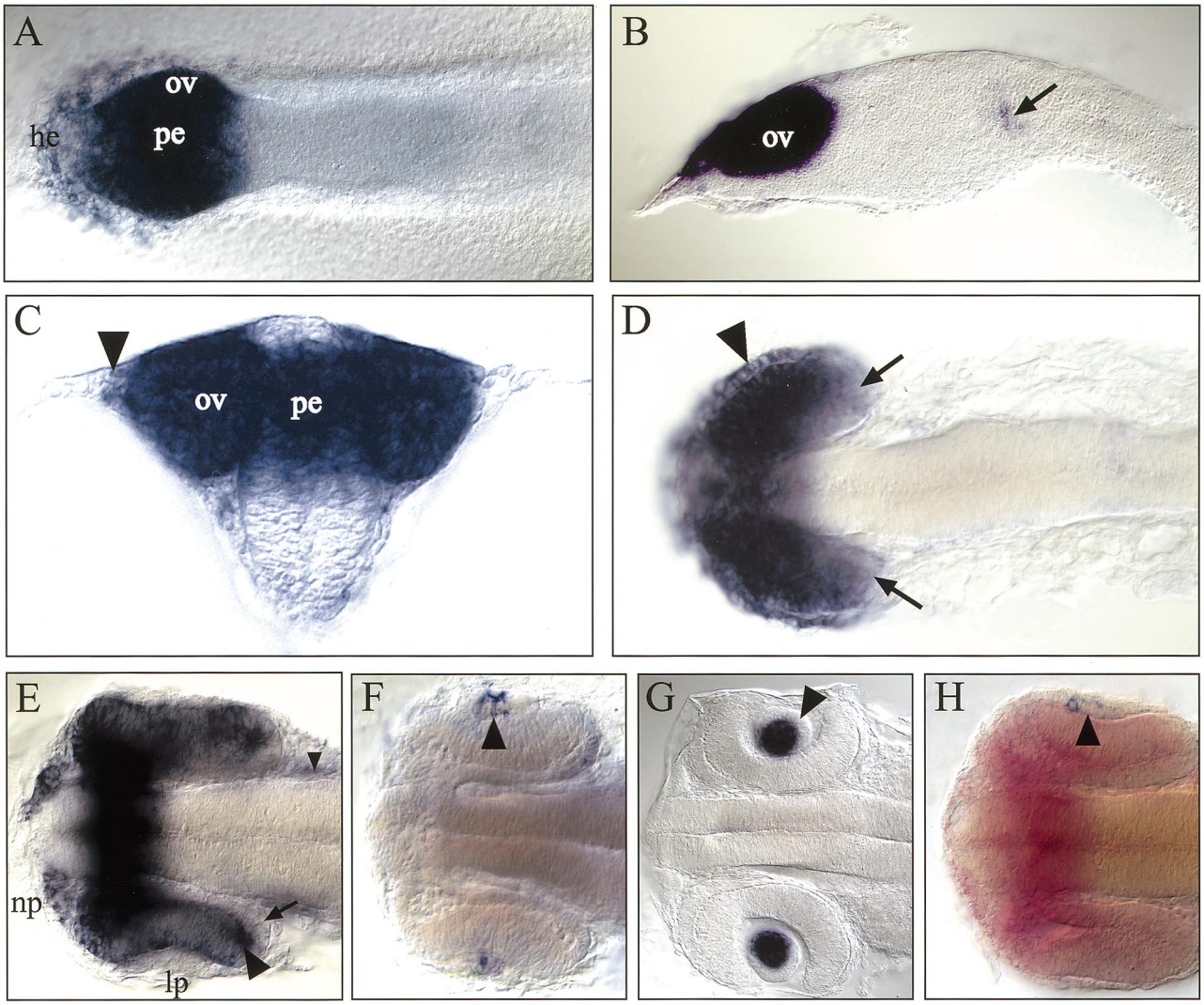

Fig. 1. Whole mount in situ analysis of *Six3* (A–E,H) and *α-crystallin* (F–H) expression. Anterior is to the left in all figures, except indicated otherwise; (A,D,E–H) dorsal views. (A) *Six3* is detected in the prosencephalon (pe), optic vesicles (ov) and anterior head ectoderm (he). (B) Lateral view; arrow indicates expression in the region of the MHB at midneurula stages. (C) Transverse section at the level of the optic vesicle; arrowhead points at lateral head ectoderm. (D) At late neurula stages expression is prominent in the prospective lens placode (arrowhead); downregulation starts in the posterior part of the optic vesicle (arrows). (E–H) At organogenesis stages *Six3* expression is restricted to the neuroretina (E, large arrowhead), *α-crystallin* is expressed in the differentiating lens fiber cells (F–H). Expression of *Six3* (red) and *α-crystallin* (blue) does not overlap in the lens placode (arrowhead in H).

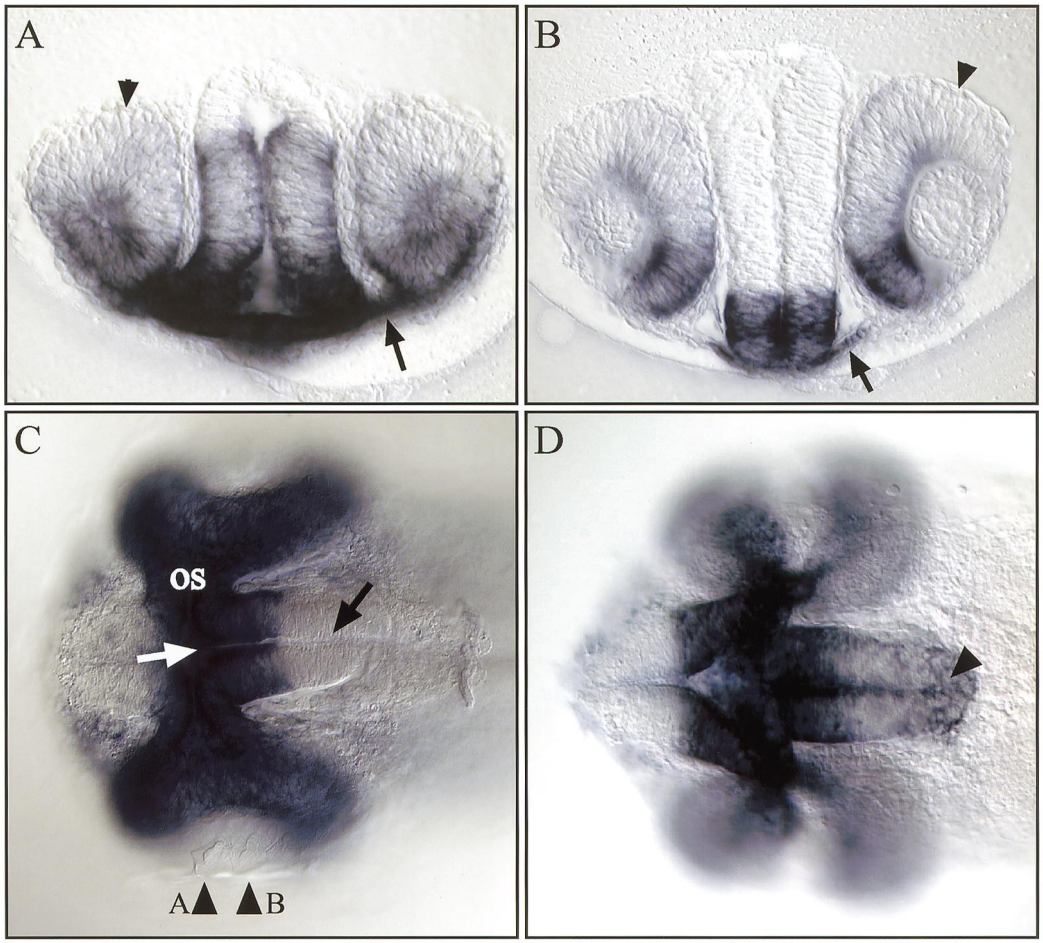

Fig. 2. *Six3* expression at organogenesis stages. (A,B) Transverse sections; (C,D) ventral views. Arrowheads in (C) indicate the level of the sections. (A,B) *Six3* is predominantly expressed in the ventral region of the neuroretina, optic stalks (arrow) and ventral diencephalon. The PRE (arrowhead) does not express *Six3*. (C) Shows expression in the optic stalks (os) and optic chiasm (white arrow); black arrow points to hypophysis. (D) Expression in the adenohypophysis (arrowhead).

To examine whether *Six3* expression temporally overlaps with the onset of lens differentiation, we used α-crystallin expression as a molecular marker for differentiating lens fiber cells (Piatigorsky, 1981; Sax and Piatigorsky, 1994). At the onset of lens differentiation, α-crystallin is expressed in the primary lens fiber cells (Fig. 1F, arrowhead). Later, α-crystallin is expressed in the primary and secondary lens fiber cells, but not in the undifferentiated lens epithelium (Fig. 1G, arrowhead). Double whole mount in situ analysis shows, that *Six3* and *α-crystallin* expression do not overlap (Fig. 1H, arrowhead). Thus, *Six3* expression is restricted to stages prior to the onset of lens differentiation.

During organogenesis, *Six3* expression is predominantly found in the ventral half of the neuroretina, but not the PRE (arrowhead in Fig. 2A,B). In the diencephalon transcripts are detected in hypothalamus, optic stalks and the optic chiasm (Fig. 2A–C). The hypophysis does not express *Six3* (Fig. 2C, black arrow). At the level of the optic stalks, the expression extends dorsally into the epithalamus (Fig. 2A). Later, *Six3* is expressed in the adenohypophysis (Fig. 2D, arrowhead).

We compared the expression patterns of the well established marker genes *Pax6* (Krauss et al., 1991; Püschel et al., 1992) and *Otx2* (Li et al., 1994; Mori et al., 1994) to that of *Six3* (Fig. 3). At the late gastrula stage, the expression domain of *Six3* is located anterior to that of *Pax6*, but partially overlaps (Fig. 3A,B). Consistent with this overlap, both *Six3* and *Pax6* are expressed in the optic vesicles and the prosencephalon at late neurula stages (Fig. 3D,E). In the presumptive lens placode *Six3* is only weakly expressed in contrast to *Pax6* (Fig. 1C and 4A, arrowheads), which is also expressed at later stages of lens differentiation (Fig. 4B).

At late gastrula stages, the *Otx2* expression domain is cross-shaped, comprising the axis proper and a broad transverse band (Fig. 3C). *Otx2* expression in the midbrain at early neurula stages suggests that this band corresponds to the midbrain anlage (Fig. 3F). The expression domain of *Six3* lies anterior to this band (Fig. 3A,C). *Otx2* expression in the developing optic vesicles is first detected at early somitogenesis in the posterior PRE (Fig. 4C, arrowhead), coinciding with the downregulation of *Six3* expression in

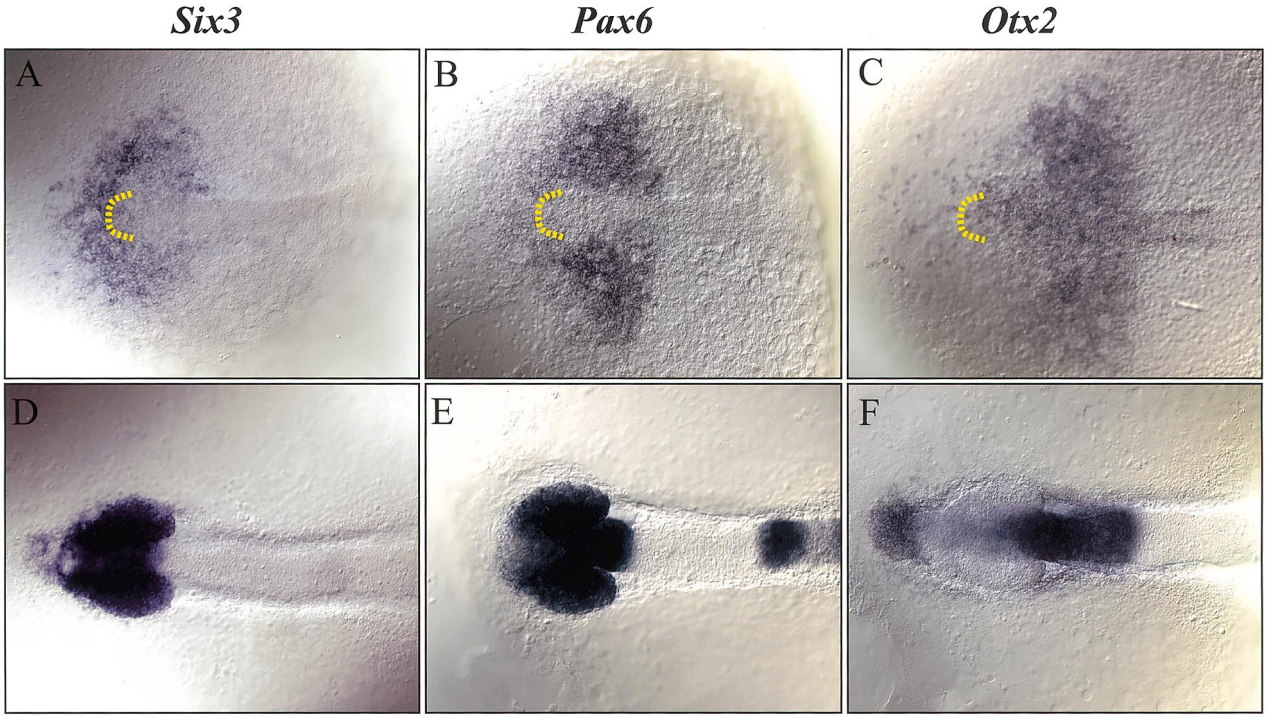

Fig. 3. Comparison of *Six3* (A,D), *Pax6* (B,E) and *Otx2* (C,F) expression at late gastrula stages (A–C) and early neurula stages (D–E). The yellow hatched line in (A–C) outlines the anterior end of the embryonic axis. Note the overlap of the *Six3* and *Pax6*, *Pax6* and *Otx2* expression domains both at late gastrula and early neurula stages.

the optic vesicles (Fig. 1D). Subsequently, *Otx2* is expressed in the PRE but not in the neuroretina, where *Six3* is expressed (Fig. 4D). Thus, both in the optic vesicle and in the optic cup, the expression domains of *Six3* and *Otx2* do not overlap and are mutually exclusive at neurula stages. The expression domains of the medaka *Six3*, *Pax6* and *Otx2* genes suggest a role in regionalization of the rostral head.

The observed expression pattern of medaka *Six3* is very similar to that reported for the murine homologue *Six3* (Oliver et al., 1995). However, species-specific differences are observed in the developing lens. In medaka, *Six3* expression is restricted to stages prior to differentiation, whereas in the mouse, *Six3* expression is first detected in the differentiating lens where the expression persists. In conclusion the medaka homeobox gene *Six3* is specifically expressed in the anterior embryonic shield and subsequently in the developing eye and prosencephalon suggesting a role for *Six3* in the formation of the respective structures.

2. Experimental procedures

2.1. Medaka stocks

Wild type *Oryzias latipes* from a closed stock at the MPI for Biophysical Chemistry were kept as described (Köster et al., 1997).

2.2. Isolation of the Six3 cDNA

A PCR product of 450 bp was RT-PCR amplified from total RNA isolated from the anterior part of neurula stage embryos using degenerate PCR primers specific for *Six3* (5′-ATIGARMGIYTIGGIMGITTYYTITGG, 3′-TTYTTRAA-CCARTTISMIACYTGIGT). The cycling conditions were: 5 cycles 94°C, 1 min; 47°C, 2 min; 72°C, 4 min and 30 cycles 94°C, 1 min; 52°C 2 min; 72°C 4 min, the resulting PCR product was cloned into the pCRII vector (Invitrogen), sequenced and subsequently used to screen a late neurula medaka λZAP cDNA library at high stringency. Positive phages were plaque purified, converted to Bluescript SK plasmids and sequenced. EMBL database accession number AJ000937.

2.3. Isolation of the Pax6 cDNA

Degenerate PCR primers specific for *Pax6* (5′-AGT-CARAAYWGICAYWSIGGIGTIA, 3′-AACCAIACYT-GIATI-CKIGCYTCIG) were used to RT-PCR amplify a 720-bp fragment from total RNA isolated from neurula stage embryos. The cycling conditions were: 35 cycles 94°C, 1 min; 46°C, 2 min; 72°C, 4 min, the PCR product was cloned as described above and used to screen a late neurula stage cDNA library. Sequence comparison with the RT-PCR product and the known vertebrate *Pax6* homologues revealed that the cDNA clone contains

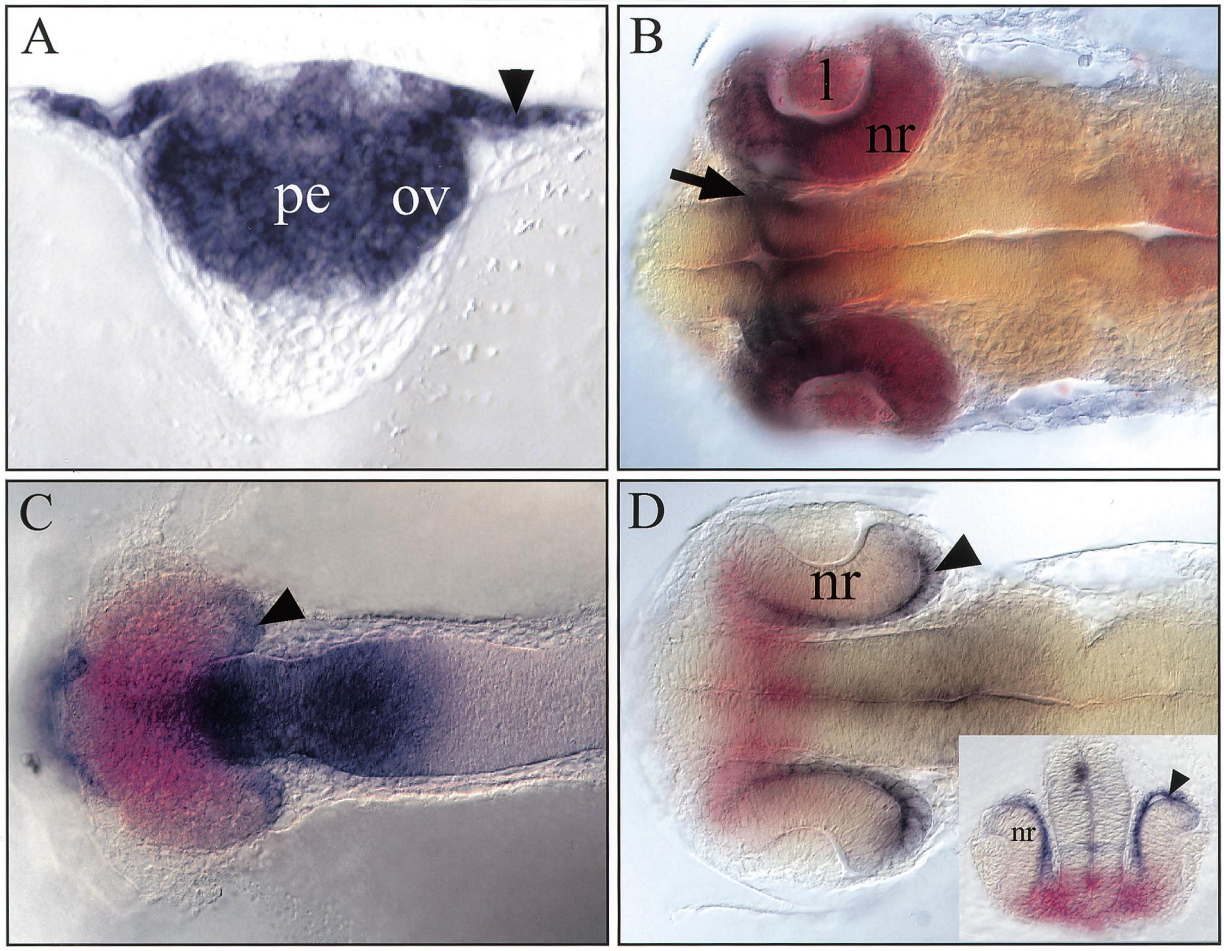

Fig. 4. Comparison of *Six3*, *Pax6* and *Otx2* expression in the developing eye. (B–D) Dorsal view. (A) Transverse section showing *Pax6* expression in the optic vesicle, dorsal prosencephalon and prospective lens placode (arrowhead). (B) Ventral view, *Six3* (blue) and *Pax6* (red) in the eye at late organogenesis stages, arrow indicates optic stalk. Note *Pax6* expression in the neuroretina (nr) and lens (1). (C,D) Dorsal views, *Six3* (red) and *Otx2* (blue) during somitogenesis (C) and organogenesis (D). Arrowhead in (C) indicates the posterior end of the optic vesicle. Arrowhead in (D) (inset shows a transverse section) points at PRE.

putative intronic sequences in the region 5′ to the paired box as well as the paired box itself. EMBL database accession number AJ000938.

2.4. Isolation of the Otx2 PCR fragment

A 540-bp fragment was RT-PCR amplified from total RNA isolated from neurula stage embryos using degenerate PCR primers specific for *Otx 2* (5′-ATGATGWSITAYY-TIAARCARCCICCITA, 3′-GTRTAIGTCATIGGRTAIS-WICKKTGC AT). The PCR conditions were: 5 cycles 94°C, 1 min; 40°C, 2 min; 72°C, 4 min; 3 cycles with annealing at 45°C and finally 35 cycles with annealing at 55°C. The resulting PCR product was cloned as described above. EMBL database accession number AJ000939.

2.5. Isolation of the α-crystallin PCR fragment

To RT-PCR amplify a 440-bp fragment from total RNA of organogenesis stage embryos, the following primers were used: 5′-CGNCTGTTYGAYCARTTYTTYGG and 3′-GGYTTYTCYTCICKISWSACRGGRAT. The cycling conditions were: 5 cycles 94°C, 1 min; 53°C, 2 min; 72°C, 4 min, followed by 35 cycles with annealing at 58°C. The resulting PCR product was cloned as described above. EMBL database accession number AJ000940.

2.6. Whole mount in situ hybridization

Whole mount in situ hybridization was performed using digoxigenin and fluoresceine labeled RNA probes as described by Köster et al. (1997) with the following modification: the temperature of the hybridization and the washing steps was increased to 65°C. For *Six3*, *Pax6*, *Otx2* and *α-crystallin* the entire cDNA was transcribed for the RNA probes. In all double labelings the fast red detection (red) was performed first, followed by the NBT/BCIP (blue) staining.

2.7. Vibratome sections

Vibratome sections were done following standard procedures (Bober et al., 1994).

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