

Retinoic acid establishes ventral retinal characteristics

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SUMMARY

The developing eye is known to be rich in retinoic acid (RA), and perturbations in RA levels during formation of the optic primordia, as well as RA receptor mutations, cause retinal malformations, especially in ventral eye regions. To test the hypothesis that RA plays a role in the establishment of ventral retinal characteristics, we examined several dorsal and ventral ocular markers in RA-treated zebrafish. The optic stalk represents the ventral-most region of the early eye field. During normal development, the optic stalks constrict, decreasing in width and are gradually replaced by the optic nerve. Systemic high RA levels cause an expansion in the optic stalk with an increased cell content and a patent lumen. In addition, the stalks do not constrict and persist into later stages of development indicating an enhancement of early ventral eye

characteristics. Expression of the transcription factor *pax[b]*, normally confined to the ventral retina, expands into the dorsal retina following RA treatment, whereas *msh[c]*, normally expressed in the dorsal retinal pole, disappears. Activity of an aldehyde dehydrogenase that normally occupies the dorsal third of the retina is reduced or abolished following high systemic RA. When a localized RA source, an RA-soaked bead, is placed next to the developing eye, a fissure resembling the choroid fissure appears in the eye facing the bead. Taken together, these observations suggest that RA is involved in the determination of the ventral retina.

Key words: retinoic acid, optic stalk, choroid fissure, *pax[b]*, *msh[c]*, zebrafish

INTRODUCTION

The vitamin A derivative retinoic acid (RA) has been implicated in the specification of axial patterning in several developing organs, and morphological changes induced by RA have been correlated with alterations in the expression domains of several transcription factors. RA-soaked beads implanted into anterior regions of the limb bud cause mirror-image duplications of posterior limb structures (Tickle et al., 1982). The same treatment results in an aberrant expansion of the expression of genes of the *Hox-d* cluster, normally confined to the posterior region of the limb bud, into anterior limb regions (Nohno et al., 1991; Izpisua-Belmonte et al., 1991; reviewed in Tabin, 1991). In *Xenopus laevis*, systemic RA treatment alters the patterning of anterior neural structures, apparently extending hindbrain and spinal cord regions anteriorly (Durst et al., 1989). These morphological changes are accompanied by a posterior-to-anterior shift in the anterior expression boundaries of several homeobox-containing genes (Cho and DeRobertis, 1990; Sive and Cheng, 1991). RA-treated mouse embryos show a reduction of anterior head structures, a loss of hindbrain segmentation, anterior displacement of the somites (Morriss-Kay et al., 1991) and alterations in the axial skeleton (Kessel and Gruss, 1991). These morphological transformations are accompanied by ectopic anterior expression of the 3' genes of the *Hox-b* cluster (Morriss-Kay et al., 1991), and alterations in the expression

domains of several genes of the *Hox-a* and *Hox-c* cluster (Kessel and Gruss, 1991). Both for the posterior body axis, as well as the limb, these results indicate that RA induces posteriorizing transformations.

The eye occupies a central position in retinoid dependency. In the mature visual system, the dependency is due largely to retinaldehyde serving as chromophore of the visual pigments (Wald, 1935, 1968; Dowling and Wald, 1960). The developing eye, in contrast, is one of the most vulnerable organs to retinoid deficiency: partial vitamin-A deprivation of pregnant pigs and rats results in microphthalmia or anophthalmia in otherwise normal offspring (Hale, 1937; Warkany and Schraffenberger, 1946). Conversely, vitamin-A excess during embryonic development causes severe ocular deformities in rats and humans (Giroud and Martinet, 1961; Giroud et al., 1962). The easy access to early embryonic stages in zebrafish allowed a spatial and temporal analysis of RA-related ocular teratology (Hyatt et al., 1992; Marsh-Armstrong et al., 1994). As in other animals (Shenfeldt, 1972; Wilson et al., 1953), the most vulnerable stage to RA perturbations in the zebrafish eye coincides with formation of the optic primordia. Application of an RA excess during this stage causes proliferation of cells in the ventral region of the eye, resulting in a duplication of the retina (Hyatt et al., 1992). Inhibition of endogenous RA synthesis during the same stage results in half-eyes that lack a ventral retina (Marsh-Armstrong et al., 1994).

The developing eye expresses a diverse range of RA receptors and null-mutations in three of these, alone or in double-mutants, result in characteristic ocular malformations. With increasing mutant dosage, the ventral retina and eye are increasingly reduced in size (Kastner et al., 1994). All of the RA receptors are expressed uniformly in the normal eye, with no apparent asymmetries along the dorsal-ventral axis. Thus, the dorsal-ventral asymmetry appears to originate at the level of RA synthesis. In the retinas of all embryonic vertebrates so far tested, RA synthesis in the dorsal part is mediated by a class-1 aldehyde dehydrogenase, and in the ventral part by a different, much more effective retinaldehyde dehydrogenase. This arrangement results in substantially higher RA levels in ventral, as compared to dorsal embryonic retina (McCaffery et al., 1992, 1993). Furthermore, in zebrafish only the ventral dehydrogenase is active during the first 24 hours of development (Marsh-Armstrong et al., 1994). These observations point to a role of the retinoid system in dorsal-ventral axial patterning of the developing retina. Axial differences of the embryonic retina along the dorsal-ventral and anterior-posterior axes had been predicted to form the basis of the topographical visual projections (Sperry, 1963), but few defined biochemical differences have been detected. Recently, axial differences in two transcription factors were described: *pax[b]*, and its mammalian homolog *pax-2*, is confined to the ventral embryonic retina (Normes et al., 1990; Krauss et al. 1991b), and *msh[c]* to the dorsal retinal pole (Ekker et al., 1992). In addition, the early ventral retina is distinguished by two obvious morphological characteristics: the choroid fissure, which forms the entrance route for blood vessels and exit route for optic axons, and the optic stalk, many cells of which eventually become incorporated into the ventral retina (Holt, 1980). By exposing early zebrafish embryos to high RA levels, we tested how far expression of dorsal-ventral markers can be altered. The results are consistent with the notion that RA is instrumental in establishing the dorsal-ventral retinal asymmetry.

MATERIALS AND METHODS

RA treatment

Breeding fish were maintained and embryos collected as previously described (Hyatt et al., 1992). All embryos were raised at 28°C. Frozen stocks of RA in dimethyl sulfoxide (DMSO) were thawed immediately before use and diluted in tank water; RA stocks were prepared and administered in the dark. Embryos were treated with 1 µM RA for 2 hours starting at the bud stage, or at about 10.5 hours postfertilization (hpf), unless indicated otherwise; other RA concentrations are indicated below. Both all-*trans* or 9-*cis* RA isomers were tested separately, with no obvious qualitative differences in the effects. After treatment, embryos were washed three times in tank water. Control embryos received mock treatments with equivalent concentrations of DMSO. Embryos that developed to 24 hpf were raised in tank water containing 0.2 mM phenyl-thiocarbamide (Sigma) to inhibit pigment formation.

Morphological analysis

Control and treated embryos were dechorionated and staged at 24 or 36 hpf. Embryos were immediately immersed in a primary fixative of 1% paraformaldehyde, 2.5% glutaraldehyde in 0.06 M sodium phosphate buffer (pH 7.4) containing 3% sucrose. After fixation for 2-3 hours in the cold (6°C), embryos were rinsed and postfixed for 1

hour at 6°C in 1% osmium tetroxide made up in 0.06 M sucrose-free sodium phosphate buffer (pH 7.4). Following dehydration in a graded series of ethanol-water mixtures, embryos were infiltrated with an Epon-Araldite plastic mixture overnight. 6-8 embryos of each treatment group were oriented and embedded for either transverse, horizontal or sagittal sectioning through the head (see Figs 1D, 2B). Serial 1 µm sections were cut with a LKB ultramicrotome, stained with 1% methylene blue/ 1% azure II in 1% borax buffer and photographed under the light microscope.

In situ hybridization

The hybridization probes used were to regions of cDNA clones for which Krauss et al. (1991a,b) and Ekker et al. (1992) had found optimal hybridization signals in zebrafish whole-mount preparations: for *pax[b]*, a 700 bp *HindIII-EcoRI* fragment, and for *msh[c]*, a 445 bp *EcoRI-SacII* fragment; the *pax[b]* clone was provided by S. Krauss; and the *msh[c]* clone by M. Westerfield. Restricted fragments were gel purified using the Magic PCR Preps purification system (Promega). The fragments were labeled with digoxigenin by nick translation for 2.5 hours at 15°C as described by Krauss et al. (1991a,b).

The whole-mount in situ hybridization protocol used was similar to that described by Krauss et al. (1991a). Hybridization temperatures were 55°C. Following in situ whole-mount staining, embryos were dehydrated and embedded in an Epon-Araldite mixture. 6 µm sections were cut on an LKB microtome and stained with a 0.5% eosin solution.

Immunohistochemistry

Whole-mount zebrafish embryos were prepared for the detection of the *pax[b]* protein, as described by Puschel et al. (1992), using horseradish peroxidase as a marker. Embryos were cleared in a glycerol series (50, 70, 95, 100%) and mounted for photography in glycerol within deep-well slides without coverslips.

Retinaldehyde dehydrogenase measurements

Embryos (~50 embryos, 100 eyes) were dissected with fine insect needles in Leibovitz's L-15 medium containing 10% bovine calf serum. Tissues were transferred into microcentrifuge tubes, all liquid was removed, the samples were frozen in liquid nitrogen and stored at -80°C. Retinaldehyde dehydrogenase activities were measured by a zymography bioassay described previously (McCaffery et al., 1992), using F9 teratocarcinoma cells transfected with the RA response element from the RA receptor b driving the β-galactosidase reporter gene (Wagner et al., 1992). Enzyme activities, as reflected in the intensity of the β-galactosidase reaction product in the reporter cells, were quantified as colorimetric readings by ELISA.

Local delivery of RA

For local release of RA, styrene-divinyl benzene co-polymer anion exchange resin beads, 200-400 dry mesh size, in formate form (Biorad) were used as described by Tickle et al. (1982). Because zebrafish are substantially smaller and develop much more rapidly than chick embryos, AG1-X8 beads were chosen, as they release RA over a shorter time period than the AG1-X2 beads used in chick (Eichele et al., 1984), and because AG1-X8 beads are available in smaller sizes. Beads ranging in size between 50 and 75 µm were manually selected and incubated for 30 minutes in 1 µM-100 µM all-*trans* RA in DMSO. The beads were rinsed for 10 minutes in a filter-sterilized Ringer solution containing 116 mM NaCl, 2.9 mM KCl, 3.6 mM CaCl₂, 5 mM Hepes, 100 units penicillin/ml, and 100 µg streptomycin/ml; they were implanted within 50 minutes from the completed rinse. For bead implantation, zebrafish embryos were mounted on a glass slide in a drop of molten 1.4% agar (Difco) in Ringer solution and then overlaid with additional Ringer solution. A wedge was cut in the agar, making the eye accessible to manipulation. To open a small hole in the skin, a drop of mineral oil was

applied onto the epidermis covering the eye and, after ~30 seconds, the skin was mechanically removed by aspirating the oil droplet. The beads were manually inserted using tungsten wire tools. The embryos were allowed to recover for several hours in the Ringer solution, after which they were removed from the agar and transferred to tank water for rearing.

RESULTS

Brief RA treatments reliably induce a retinal duplication in zebrafish (Fig. 1B) when the treatment is confined to a 2-hour interval between 9.5 hpf, the end of zebrafish gastrulation at 100% epiboly, and 11.5 hpf, the 5-6 somite stage of development (Hyatt et al., 1992). For example, exposure of embryos to 1 μ M RA during this period results in retinal duplication in greater than 90% of the animals (unpublished results). We refer to this 2-hour time interval as the critical period of ocular development. Embryos treated at earlier stages, during gastrulation or at mid- and late-blastula stages, lack anterior head structures including the eyes, and animals treated after the critical period do not display complete retinal duplications (Hyatt et al., 1992).

Optic stalk effects

During normal development, the optic stalks constrict extensively beginning at about 18 hpf when they are ~50 μ m in diameter, so that by 24 hpf they are less than 20 μ m in diameter (Schmitt and Dowling, 1994). By 36 hpf, the stalks are virtually eliminated and only remnants of discontinuous spindle-shaped cells extend along the initial axons of the optic nerves. Between 24 and 36 hpf, the choroid fissure, a marker of the eventual

ventral pole of the eye, shifts its orientation from roughly parallel to the surface of the yolk sac (Fig. 1D) to become nearly perpendicular to the yolk sac (Fig. 2A). Sagittal sections reveal that this shift reflects a 45° rotation of the eye in relation to the hindbrain (not shown). It is likely that this rotation occurs passively as a result of the formation of the cephalic flexure, a prominent ventral bend in the neural axis which causes the rostral surface of the neural tube to point towards the yolk sac (Ross et al., 1992). As a result of the reorientation of the eyecups, the optic stalks, as well as the choroid fissures, are gradually repositioned from a roughly rostral/anterior location to a final ventral position in relation to the hindbrain.

In embryos treated with RA during the critical period, the optic stalks are enlarged and possess a well-formed lumen at 24 hpf (OS in Fig. 1C). As in control embryos at 24 hpf, the optic stalks in RA-treated embryos lie at the anterior of the eyecups and extend laterally ~40 μ m out from the forebrain (compare embryos in Fig. 1D). In the 24 hpf specimen shown in Fig. 1C, the transverse section was taken along the length of the optic stalk and shows that it is continuous with the anterior region of the forebrain (Fb) and possesses a central lumen. The optic stalks, unlike controls, are ~60 μ m wide. The dorsal half of the stalk (*Dos* marked by arrow) is continuous with the dorsal retina (D), whereas the pigment epithelial layer (black arrows) of the ventral retina (V) extends from the ventral half of the optic stalk (*Vos* marked by arrow). The thickened optic stalk in RA-treated animals becomes apparent by 18 hpf, at about the same time as the second retina begins to form. Horizontal and sagittal sections show the reorientation of the eyecups, observed in normal embryos between 24 and

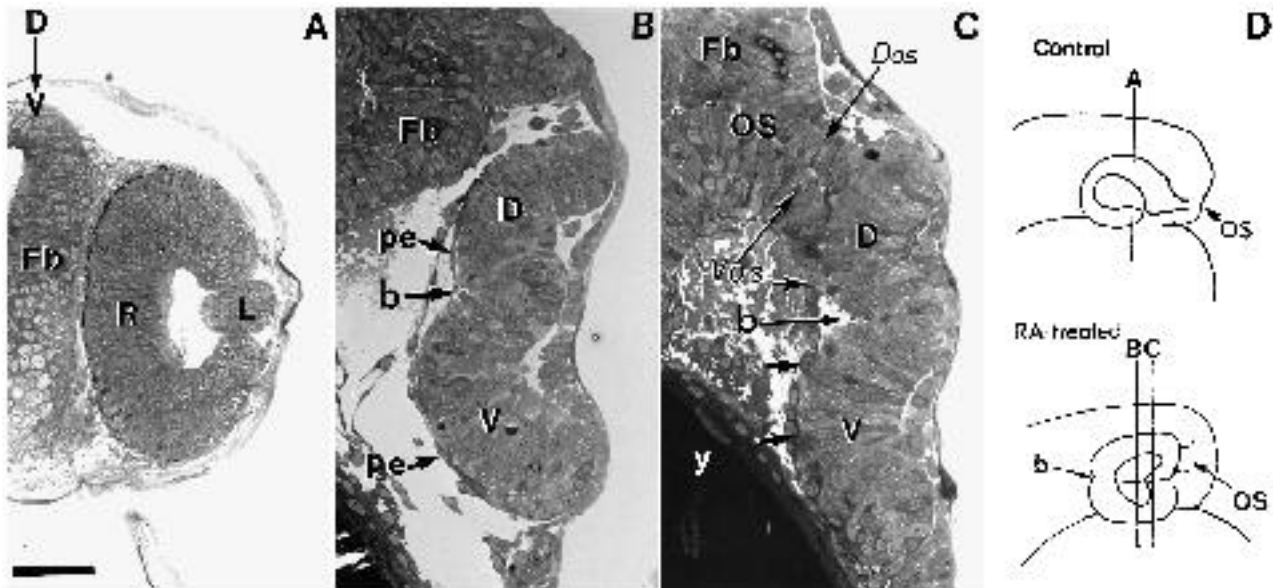


Fig. 1. The level and orientation of transverse sections for control and RA treated embryos are indicated by lines A, B and C, respectively in D. Note the optic stalks (OS) which extend outward from the forebrain are located at the anterior of the eye cups at 24 hpf in both control and RA-treated embryos. A boundary (b), an extension of the optic lumen, divides the dorsal and ventral retinas of RA-treated embryos. (A) The eyecup of a control embryo at 30 hours postfertilization (hpf) with a single pseudostratified retina (R) and lens (L). (B) At 24 hpf, RA-treatment induced an apparent duplicated retina with a dorsal (D) and ventral (V) retina of slightly reduced size. (C) A more anterior section through the duplicated retina along the length of the optic stalk (OS) which extends laterally from the forebrain at 24 hpf (line C in D). The dorsal half of the stalk (*Dos*) is associated with the dorsal retina (D) whereas the ventral half of the optic stalk (*Vos*) is continuous with a layer of pigment epithelium (small arrows) which extends along the ventral retina (V). Note that the optic lumen extends between the dorsal and ventral retinal epithelia which creates a boundary (b) between them at the midline of the eyecup. D-V, dorsal-ventral axis; FB, forebrain; y, yolk; bar, 40 μ m.

36 hpf (Schmitt and Dowling, 1994), does not occur in RA-treated embryos. As a result, the eyes remain oriented with the optic stalks extending from their anterior borders after 36 hpf. This lack of eye rotation is presumed to reflect at least a partial failure in the formation of the cephalic flexure. Sagittal sections reveal that the (ventral) bending of the rostral surface of the forebrain towards the yolk is less pronounced in RA-treated embryos (not shown). In addition, these sections suggest that a portion of the rostral and ventral regions of the forebrain may also be lacking (not shown). Horizontal sections indicate that the lateral surface of the mid- and forebrain regions are excessively broad and distorted and that the ventricles are greatly constricted.

When embryos are treated shortly after the critical period at ~12.5 hpf (during the 7- and 9-somite stage), a partial retinal epithelial layer lies ventral to the original retina and lens by 24 hpf (asterisks in Fig. 2B). This is typically most pronounced adjacent to the anterior-ventral region of the original retina, in the vicinity of the optic stalk. An unusually large number of mitotic profiles is observed ventrally within this region (white arrows in Fig. 2D), indicating that some cells are still competent to respond to RA by forming additional retinal tissue. In addition, the orientation of the optic stalks and choroid fissures does not change between 24 and 36 hpf (compare embryos in Fig. 2A). Sagittal sections show that the ventral bend of the rostral surface of the forebrain towards the yolk sac, i.e. the cephalic flexure, is not as pronounced as that observed in controls (not shown). Horizontal sections taken

through the middle of the eyecups of such specimens show broad optic stalks at 36 hpf, extending from the anterior regions of the eyes across the anterior surface of the forebrain (OS in Fig. 2D). The optic stalks are not evident in comparable sections through the middle of the eyecups in controls, as the reorientation of the eyecups has shifted the remnant of the optic stalks ventrally. The optic stalks in RA-treated embryos are one- to two-cell layers wide and display a central lumen, but are not as broad as those observed in the embryos treated during the critical period (not shown). The distortions within the brain and constrictions of the ventricles, observed in embryos treated with RA at earlier stages, are still present (compare Fig. 2C and D).

In embryos treated with RA at 14.5 hpf, (11- to 13-somite stage), no hint of retinal duplication is seen. However, similar to the effects of RA treatments at earlier stages, the optic stalks do not constrict, but remain broad and continuous with the anterior surface of the forebrain through 36 hpf (not shown).

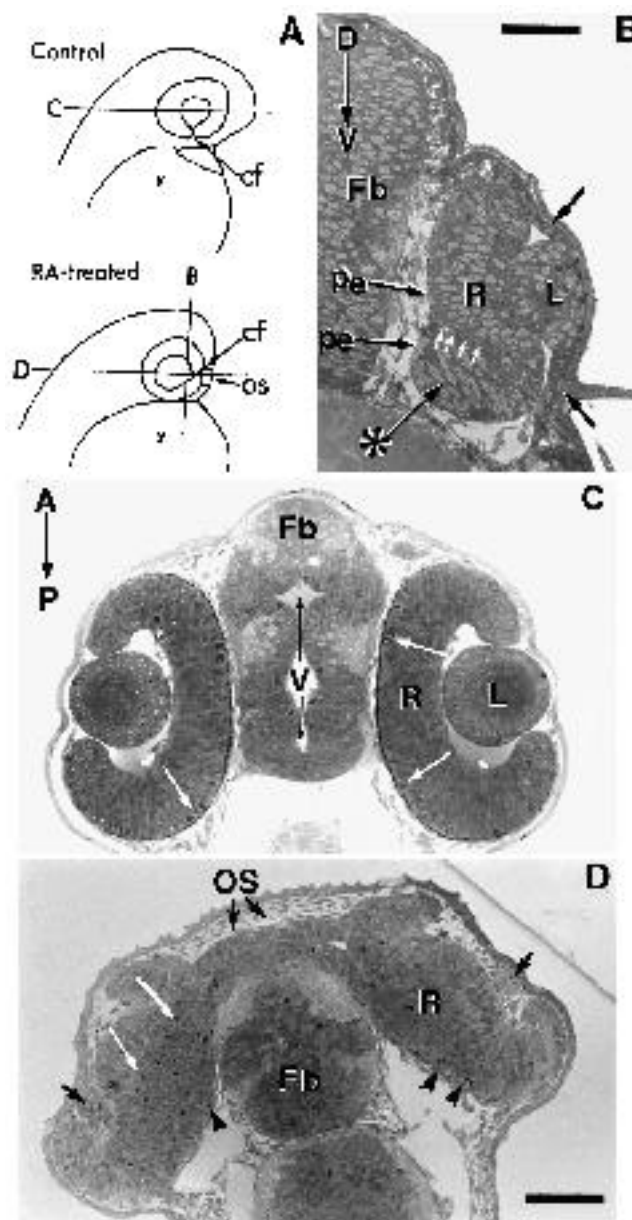


Fig. 2. (A) A control embryo (top) and embryo treated with RA after the critical period (bottom) is shown schematically at 36 hpf. In controls the choroid fissure (cf) is located at the ventral pole of the eyecup. In RA-treated embryos, the optic stalk (OS) and choroid fissure (cf) remains positioned at the anterior of the eyecup due to lack of eye rotation. Lines B, C and D indicate the orientation and level of transverse and horizontal sections shown in B, C and D, respectively. (B) A transverse section along the dorsal-ventral axis (D-V) at 36 hpf after RA treatment between the 7- and 9-somite stages (i.e. shortly after the critical period). A small retinal epithelium (asterisk) lies ventral to the original retina (R) and lens (L). As found in embryos treated during the critical period, an extension of the optic lumen (white arrows) divides the two retinal epithelial layers and the pigment epithelium (pe) extends across the boundary. The lens (L) remains associated with the ectoderm which is unusually thick (arrows). Bar, 40 μ m. (C,D) Sections through the midline of the eyecups at 36 hpf in a control embryo (C) and an embryo treated with RA after the critical period (D). (C) In horizontal sections of controls, a single retina (R) and lens (L) are observed and ventricles (V) are well formed within regions of the forebrain (Fb). Mitotic figures (white arrows) occur along the optic lumen adjacent to the pigment epithelium. The optic stalks which have atrophied are not evident as they lie ventral to the eyecup midline at 36 hpf. (D) An embryo at 36 hpf after a 2 hour RA treatment between the 7- and 9-somite stages. Sections through the midline of the eyecup show the optic stalks (OS) are more than 30 μ m wide and extend from the anterior region of the eyecups across the anterior surface of the forebrain. Distortions in the shape of the eyecups and lenses (black arrows) are observed. Numerous mitotic profiles (white arrows), associated with the partial retinal epithelial layer (see Fig. 2B), are located throughout the deeper regions of the retina (R) in addition to those along the optic lumen (arrowheads). The ventricles within the forebrain are not observed. A-P, anterior-posterior axis; y, yolk; bar, 60 μ m.

The forebrain is still distorted in shape and its ventricles are constricted. Following RA treatments at 18 hpf, (18- to 19-somite stage), the eyes develop normally, but patent optic stalks, although somewhat thinner, persist at 36 hpf (not shown). The optic stalks are now apparent in sections through more ventral regions of the eye and forebrain, indicating the eyes have been reoriented to some extent and suggesting that formation of the cephalic flexure is not as severely inhibited by late RA treatments (not shown).

Pax[b] expression

In control animals, *pax[b]* mRNA expression is first clearly observed at 14 hpf within the optic stalk and the anterior-ventral margin of the eye. At 18 hpf, *pax[b]* continues to be expressed in anterior and ventral regions of the eye and in the optic stalk (Fig. 3A). By 24 hpf, however, expression is limited to the optic stalk and the ventral region of the retina surrounding the choroid fissure (Fig. 3B). By 36 hpf, expression is rarely observed and, when present, is barely visible in the thin remains of the optic stalk. These patterns are similar to those reported by Krauss et al. (1991b). Differences in expression patterns between control and animals treated with RA at 10.5 hpf were first detected at 18 hpf of development, which is at about the time when the second retina begins to form. At this stage, *pax[b]* expression in treated embryos was enhanced significantly within the ventral half of the eye (arrow in Fig. 3C). At 20 hpf, *pax[b]* extended dorsally to include over 80% of the eyecup, and, by 24 hpf, *pax[b]* transcripts were found throughout the eyes of treated fish and expression was robust (double arrow in Fig. 3D). This pattern persisted and was still observed in embryos through 36 hours of development.

For a more detailed analysis of *pax[b]* localization, RA-treated and control embryos, prepared for whole-mount in situ hybridization at 24 hours, were sectioned parallel to the dorsal-ventral axis of the head (see Fig. 4A and D). As noted by Krauss et al. (1991), *pax[b]* expression in control embryos was prominent in the optic stalk (Fig. 4B). In addition, we found *pax[b]* mRNA expression in sections immediately adjacent to the optic stalk, where a sharp band of expression extended across the retina (arrows in Fig. 4C). In RA-treated embryos, *pax[b]* expression was strong within the optic stalk, particularly within its ventral half (Fig. 4E). Furthermore, *pax[b]* was expressed throughout all cells of the neural retina in the experimental animals (Fig. 4F).

For detection of protein, a *pax[b]* antiserum (Puschel et al., 1992) was used. In control animals at 18 hpf, detectable *pax[b]* protein was localized in the optic stalk and the ventralmost regions of the eyecup (Fig. 5A). By 20 hpf, detectable protein was confined along the optic stalk (Fig. 5B), and similar protein patterns were present in control animals at 22 and 24 hpf (Fig. 5C). The *pax[b]* expression in the normal fish confirms the pattern reported by Puschel et al. (1992) and it is similar to the distribution of mRNA.

In RA-treated embryos at 18 hpf, the *pax[b]* protein extended beyond its normal expression in the optic stalk into adjacent ventral eye regions (Fig. 5D) and faint staining was observed in the dorsal retina. By 20 hpf, robust *pax[b]* expression spread from the optic stalk into the ventral half of the eye and into the original dorsal retina (Fig. 5E). At 22 hpf, antibody staining revealed a spread of the protein throughout

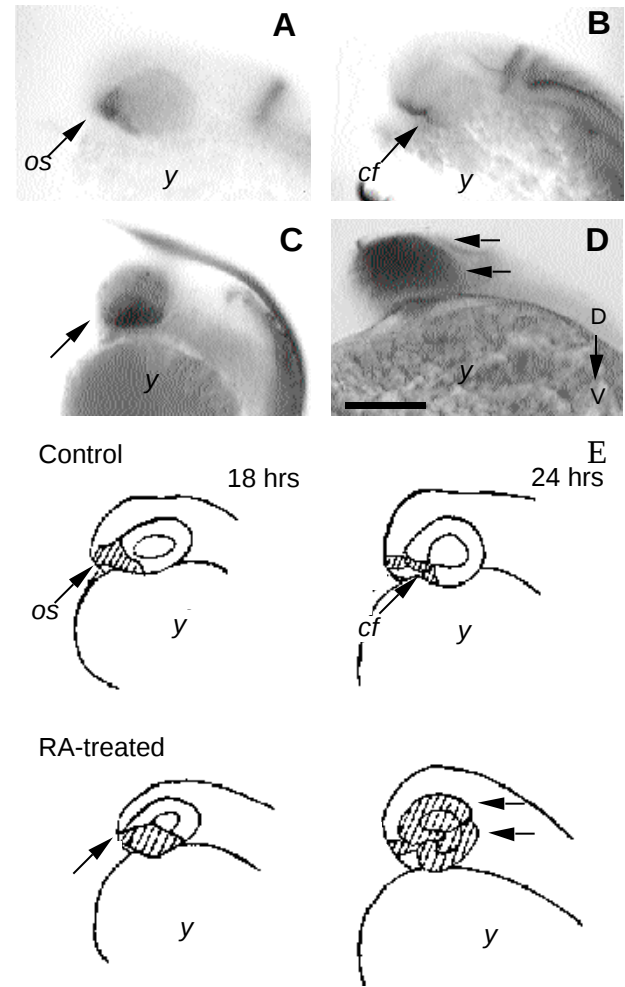


Fig. 3. (A-D) A lateral view of control embryos (A,B) and RA-treated embryos (C,D) showing localization of *pax[b]* mRNA expression by whole-mount in situ hybridization. (A) An 18 hour control embryo with localization of *pax[b]* transcripts within the anterior/ventral region the eye adjacent to the optic stalk (os). (B) At 24 hpf, *pax[b]* transcripts in control embryos have been restricted to the optic stalk and within the choroid fissure (cf). (C) At 18 hpf, a ventral-to-dorsal spread of *pax[b]* transcripts is observed in RA-treated embryos. (D) By 24 hpf, *pax[b]* is expressed throughout the dorsal and ventral regions of the eyecups (double arrow) in treated embryos (arrow). (E) Diagrams of embryos shown above illustrates changes in *pax[b]* expression in control and RA-treated embryos. D-V, dorsal-ventral axis; y, yolk; bar, 210 μ m.

80% of the eye, with only the dorsalmost segment of the eye remaining free (not shown), and by 24 hpf, the protein extended completely throughout both retinas, including all cells of both the original and duplicated retinas (Fig. 5F). The RA-induced alterations of the *pax[b]* protein are similar to its mRNA expression.

To determine if the RA-induced alteration of the mRNA expression pattern of *pax[b]* is correlated with the duplication event, embryos were treated with RA for 2 hours at later times in development and processed for whole-mount in situ hybridization. Whereas eyes of embryos treated at either 12.5 or 14.5 hours did not show fully duplicated retinas, they did display

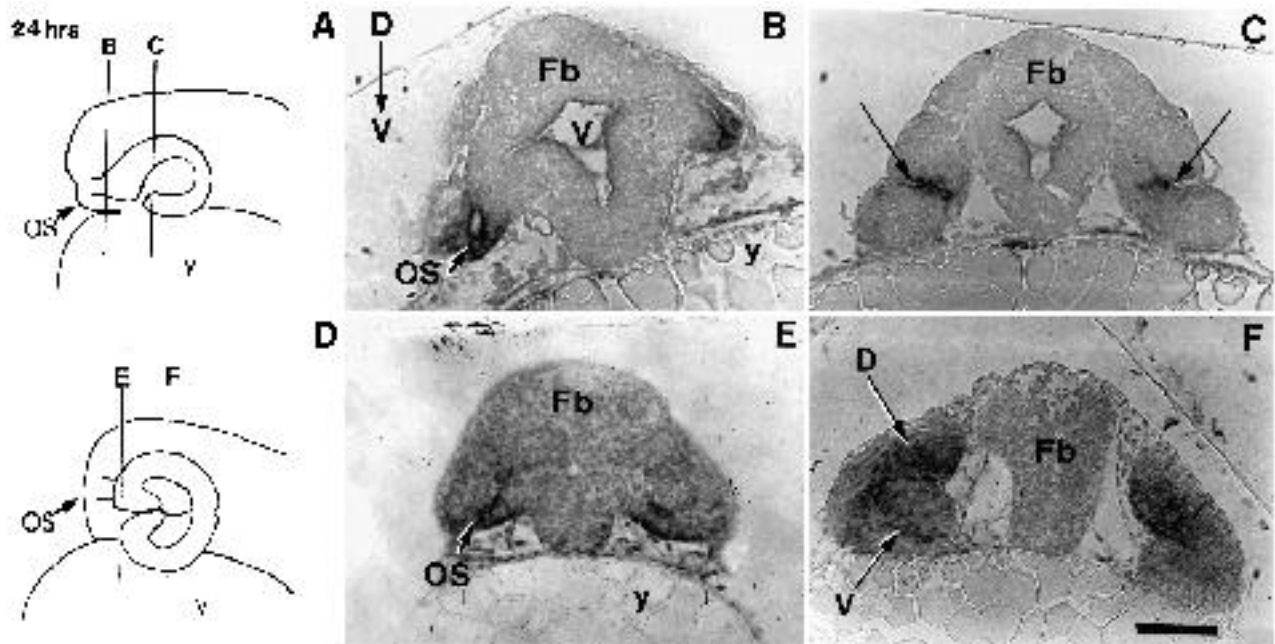


Fig. 4. The localization of *pax[b]* transcripts at 24 hpf in transverse sections (5 µm) of eyes from control embryos (B,C) and embryos treated with all-*trans* RA (E,F). (A) Schematic of a control embryo at 24 hpf indicating the level of transverse sections (lines B and C) shown in B and C, respectively. (B,C) In control embryos, transcripts are localized to the optic stalks (os in B) and to a sharp band extending across the retina (arrows in C) in the anterior region of the eyecup. (D) Schematic of an RA-treated embryo at 24 hpf illustrating the level of transverse sections (lines E and F) shown in E and F. (E,F) In treated embryos, *pax[b]* expression is present within the optic stalks (OS in E) and throughout the neuroepithelium of both the dorsal (D) and ventral (V) retina of the duplication. D-V, dorsal-ventral axis; y, yolk; bar, 65 µm in B, bar, 80 µm in C, E and F.

some enhancement and spread of *pax[b]* expression: a weak *pax[b]* signal was detectable throughout the eyes of these late-treated embryos, and within and adjacent to the optic stalk expression was strong. However, within the neural retina, *pax[b]* was restricted to cells at the vitreal border and at anterior portions adjacent to the optic stalk. Embryos treated at 18 hpf did not display obvious alteration in *pax[b]* expression at 24 or 36 hours of development.

Msh[c] expression

In control fish, *msh[c]* transcripts first became apparent within the dorsal pole of the developing eye at 14 hpf. They remained detectable through 20 hpf, but were no longer observed at 24

hpf. At 20 hpf, *msh[c]* expression was restricted to a small circular area at the dorsal pole of the eyecup (Fig. 6A). Transverse sections from the eyes of 20 hour embryos, as indicated by line D in Fig. 6C, showed *msh[c]* gene expression restricted to a highly localized region of the dorsal retina (arrow in Fig. 6D). In embryos exposed to RA for 2 hours during the critical period (RA treatments starting at 10.5 hpf), no *msh[c]* transcripts could be detected at 14, 16, 18 or 20 hpf (Fig. 6B), and RA treatment at later stages of development (12.5 and 14.5 hpf) also repressed *msh[c]* expression at 20 hpf. This indicates that RA rapidly repressed *msh[c]* expression within 3.5 hours after exposure.

Retinaldehyde dehydrogenase activity

The zebrafish retina contains two retinaldehyde dehydrogenases, one located dorsally and the other in the ventral retina (McCaffery et al., 1993; Marsh-Armstrong et al., 1994). Prior to 24 hpf, only the ventral enzyme is present, but after 24 hpf and persisting into adulthood, both enzymes can be

Table 1. Concentration and location dependence of ectopic fissures induced by retinoic acid soaked beads. Beads were implanted between the 8- and 12-somite stages

Location	µM [RA]	Total number of implants	Number ectopic fissures	% Ectopic fissures
D. retina	0	51	1*	2%
D. retina	1	24	9	37%
D. retina	10	43	38	88%
D. Mes†	10	7	2	14%
D. retina	100	20	20‡	100%

*This embryo had an indentation surrounding the bead that was scored as a fissure, yet it did not have lack of pigmentation that also characterizes retinoic acid induced fissures.

†D. mes represents dorsal mesenchyme.
‡Most of these eyes were also smaller than the control eyes.

Table 2. Stage dependence of ectopic fissures induced in dorsal retina by beads soaked in 10 µM retinoic acid

Stage (somite no.)	Total number of implants	Number ectopic fissures	% Ectopic fissures
5-9	38*	31	82%
10-14	28*	23	82%
15-19	24	3	12%

*43 of these embryos were also counted in Table 1.

distinguished by zymography: the dorsal retinaldehyde dehydrogenase focuses at more acidic pH than does the ventral dehydrogenase (Fig. 7, upper trace). The late-appearing dorsal retinaldehyde activity was used here as a marker of dorsal retinal differentiation. In two experiments, batches of ~100 embryos were exposed to RA starting at 10.5 hpf for 2 hours. In neither experiment could dorsal dehydrogenase activity be detected at 52 hours (Fig. 7, lower trace). In contrast, ventral enzyme activity was unaffected by the RA treatment.

Local application of RA to developing eyes

Anion exchange resin beads readily take up and release RA (Eichele et al, 1984). To test for an effect of local RA delivery on eye development, AG1-X8 beads were loaded either with 1 μ M–100 μ M RA in DMSO, or with DMSO alone, and implanted into the developing eye primordia of 13–15 hpf zebrafish embryos (Fig. 8A). Embryos that received RA-soaked beads could first be distinguished from control fish around 36 hpf. Many of the treated embryos developed a fissure in their eyes that surrounded or was adjacent to the RA-soaked bead. In many cases, the fissure resembled the ventral choroid fissure in size and appearance (Fig. 8C); in others, the fissures were wider than normal. Analysis of histological sections taken within the vicinity of the RA-soaked beads did not reveal any evidence of cell death. Ectopic fissures were never observed in embryos that received only DMSO-soaked beads (Fig. 8B).

To analyze the effect further, beads were soaked in different RA concentrations and implanted at different locations of the eye primordium in 13–15 hpf embryos (Table 1). When beads were loaded with 1 μ M RA, the majority of embryos did not develop fissures; those fissures that did develop were thin in width. With 10 μ M RA beads, the majority of embryos developed fissures, many of which were large. Fissures could be induced in all eye quadrants by altering bead placement: in all cases, fissures developed adjacent to the bead. Fissures were

not observed, however, when the beads were placed at a distance into the mesenchyme surrounding the eye. When the beads were soaked in 100 μ M RA, all of the treated embryos had large fissures and their eyes were smaller than control eyes. At this concentration, non-localized defects, such as swollen pericardial cavities and fin defects, were often observed.

To analyze when fissures could be induced, beads soaked in 10 μ M RA were implanted into dorsal retinas at different stages during neurulation (Table 2). Local RA delivery at this

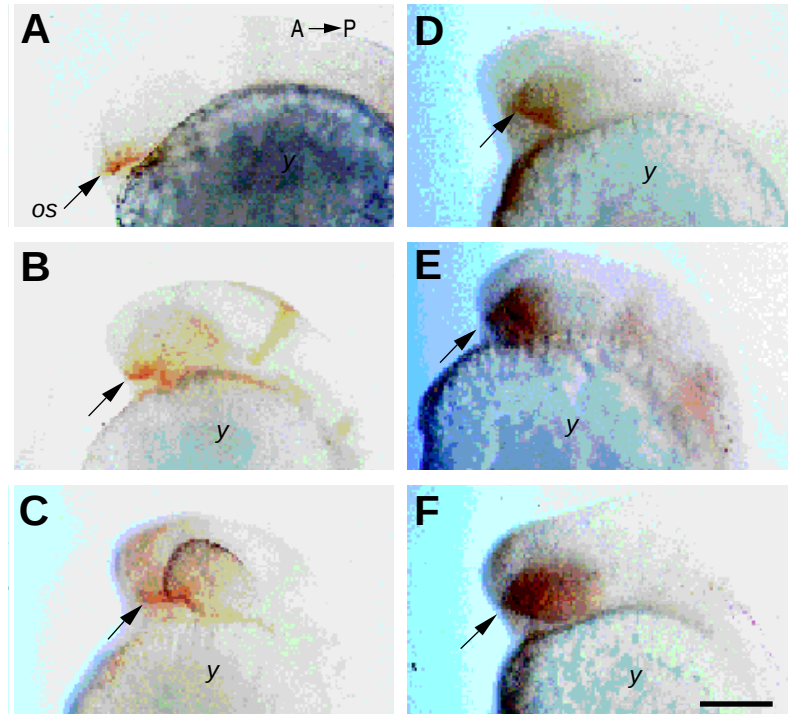


Fig. 5. A lateral view of control embryos at (A) 18 hours postfertilization (hpf), (B) 20 hpf, (C) 24 hpf and of RA-treated embryos during the critical period, at (D) 18 hpf, (E) 20 hpf and (F) 24 hpf, showing localization of *pax[b]* protein by whole-mount antibody staining. Black arrows point to the optic stalk. A-P, anterior-posterior axis; y, yolk; bar, 150 μ m.

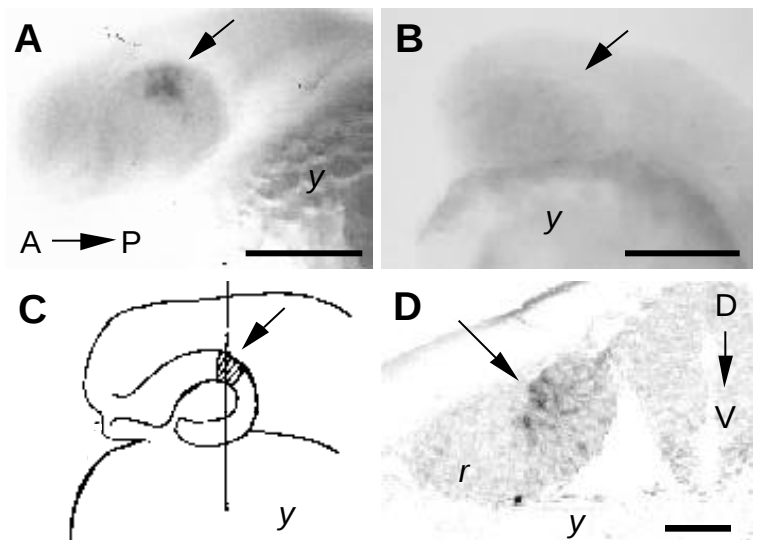


Fig. 6. In situ hybridization of *msh[c]* transcripts at 20 hpf in control embryos (A,D) and embryos treated with RA during the critical period (B). (A) In control embryos, *msh[c]* expression is localized to a small patch in the dorsal/posterior region of the eyecup (arrow) at 20 hpf. (B) No *msh[c]* expression is observed in the eyes (e) of treated embryos. (C) Illustration of a 20 hpf control embryo indicating the level of the transverse section (line D) shown in D. The arrow indicates the patch of *msh[c]* expression shown in A. (D) In transverse section, *msh[c]* expression (arrow) is found in the dorsal region of the neuroepithelium of the retina (R) in sections through the posterior region in the eyes of control embryos. D-V, dorsal-ventral axis; A-P, anterior-posterior axis; bar in A and B, 180 μ m; bar in D, 50 μ m.

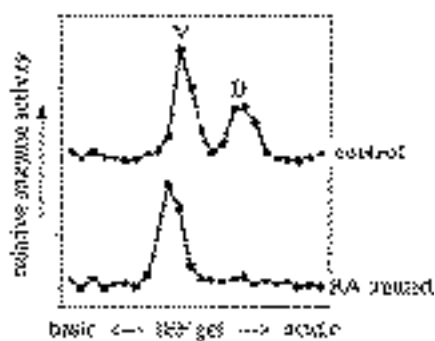


Fig. 7. Zymography bioassay of retinas at 52 hpf in control embryos and embryos treated with RA during the critical period. The retinas of control embryos express two retinaldehyde dehydrogenase activities: an early appearing basic enzyme restricted to the ventral retina and a late appearing acidic enzyme restricted to dorsal retina (Marsh-Armstrong et al., 1994). RA inhibits the expression of the dorsal enzyme.

concentration was found to be equally effective at inducing ectopic fissures at 11.5-13.5 hpf, and at 14-16 hpf, but, after 16.5 hours, very few embryos developed fissures.

DISCUSSION

We have studied the effects of RA application on five markers of dorsal-ventral polarity in the early zebrafish eye: the early optic stalk, the choroid fissure and *pax[b]* as ventral markers, and *msh[c]* and an aldehyde dehydrogenase as dorsal markers. The induced changes in all five markers indicate that early in eye development RA exerts a ventralizing effect on the retina. The optic stalk, a transient structure at the ventral edge of the eye field, is considered as a part of the ventral region of the developing eyecup (Duke-Elder, 1964), as many of its cells will become integrated into the ventral retina (Holt, 1980). Later it obliterates, gradually becoming replaced by components of the optic nerve. Exposure of zebrafish embryos to high RA levels causes an expansion and persistence of early ventral stalk characteristics through 36 hpf. The choroid fissure, an infolding in the eye cup serving as route for axons and blood vessels, marks the ventral pole in normal eyes. In response to an RA-soaked bead placed in the eyefield, an infolding resembling the ventral fissure can be induced at any location around the eye. The transcription factor *pax[b]* is normally expressed in the ventralmost region of the optic primordia; exogenous RA exposure enhances its expression and extends it dorsally. Expression of the two dorsal markers *msh[c]* and aldehyde dehydrogenase is rapidly repressed by exogenous RA.

Previous experiments showed that exposure of zebrafish embryos to high RA levels causes proliferation of cells at the ventral retinal margin, resulting in a duplication of the retina (Hyatt et al., 1992). The RA-induced hyperproliferation of cells within the ventral segment of the eyefield generates a new layer of neural epithelium separated from the original neuroepithelium by an extension of the optic lumen. This process can be observed at 18 hpf, approximately 7.5 hours after the application of RA to the developing embryo. Each of the neural retinal epithelia develops independently. For example, invagi-

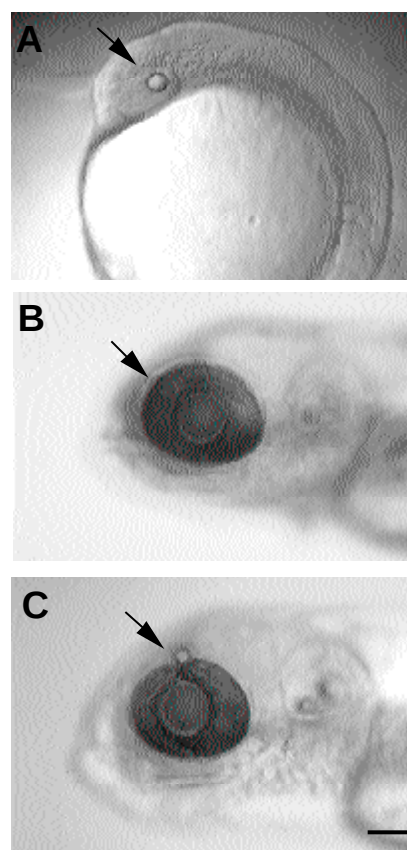


Fig. 8. Effects of RA-soaked beads implanted into the eye anlage of developing zebrafish. (A) Bead implanted into the prospective dorsal retina of zebrafish embryo at 13-15 hpf. (B) 3.5-day-old zebrafish embryo in which a bead soaked in DMSO was implanted into the dorsal-nasal retina at 13-15 hpf. (C) Dorsal fissure in 3.5-day-old embryo in which a bead soaked in 10 µM RA was implanted into the dorsal retina at 13-15 hpf. Arrows point to location of implanted beads; bars, 100 µm.

nation of the dorsal retina occurs before the ventral retina, and cell differentiation within the dorsal retina precedes cellular differentiation in the ventral retina by 4-8 hours. Furthermore, as reported in the earlier work, separate fields of ganglion cells form in the dorsal and ventral retinas, and bundles of ganglion cell axons exit each of the two retinas independently (Hyatt et al., 1992). Finally, recent observations indicate that two distinct patches of precocious photoreceptors form within the eye, one dorsally, the other ventrally, providing further evidence for a retinal duplication (unpublished results). In *Xenopus*, Manns and Frittsch (1991) showed that retinoic acid induced excess retinal tissue to form during early eye development. They did not describe a retinal duplication, but suggested a buckling of the retina in response to RA.

Inhibition of endogenous RA synthesis with citral during the same developmental stages results in fish that lack a ventral retina (Marsh-Armstrong et al., 1994). When citral treatment is combined with exposure to high exogenous RA, the fish embryos are severely deformed overall due to exposure to two teratogens. However, the eyes are more normal, suggesting that RA is an important factor for ventral retina development. Both the earlier and the present observations suggest the existence

of a RA-rich source in the developing eye, which is normally localized in the ventral part of the optic primordia and which directs expression of dorsal-ventral characteristics of the retina. In zebrafish, RA is likely provided by a retinaldehyde dehydrogenase whose expression in the eye region begins at the optic primordial stage, and which is later restricted to the ventral retina (Marsh-Armstrong et al., 1994). In mouse, RA is synthesized ventrally by V2 retinaldehyde dehydrogenase, an enzyme appearing in the eye region during formation of the optic pit, a period that corresponds approximately to the RA-responsive time window identified in zebrafish (McCaffery et al., 1993).

The most extensively studied dorsal-ventral differences in the retina concern the projections of ganglion cells: dorsal ganglion cells project to the lateral optic tectum and ventral ganglion cells to the medial tectum, a pattern established by the earliest optic axons (Stuermer, 1988) and believed to be specified during the optic primordial stages (Sperry, 1963). Except for the dorsal aldehyde dehydrogenase, expression of the axial markers studied here is also limited to early eye development. In adult retinas, only a few dorsal-ventral differences have been described; for instance, bullfrogs synthesize rhodopsin in ventral retinal regions but porphyropsin in the dorsal retina (Reuter et al., 1971) and, in mice, blue cones are restricted to the ventral retina, whereas green cones are found mainly in the dorsal retina (Szél et al., 1992).

It is likely that the period during which the developing eye is most competent to respond to RA (the critical period) corresponds to the initial period of dorsoventral axial determination of the retina, as postulated by work on the retinotectal system. RA is a plausible candidate to be a principle polarizing agent for establishing the dorsoventral retinal axis, with regions of the eye closest to an RA source responding with ventral characteristics and removed regions with dorsal characteristics. The polarizing actions of RA are likely to be mediated by transcriptional alteration of a range of factors, including *pax[b]*, *msh[c]* and other factors yet to be identified. These, in turn, may eventually lead to a dorsoventrally graded expression of cell-surface receptors, which could enable optic axons to search out their appropriate target location in the optic tectum. Whereas specification of the dorsoventral axis becomes increasingly more refractory to RA after the critical period, the actions of the polarizing agent are perpetuated in the developing retina by a ventral-dorsal RA gradient generated by the arrangement of two different RA-producing enzymes. Early in zebrafish eye development, at the 9- to 12-somite stage, only a ventral enzyme is present, whereas a dorsal enzyme is detectable by the 30-somite stage (~24 hpf). The spatial expression of these enzymes is likely to be a direct consequence of the initial polarizing event, as indicated by the observation that RA treatment during the critical period suppresses the dorsal enzyme. A consequence of the perpetuation of the polarizing activity may be in the ordered dorsoventral arrangement of factors expressed long after the initial polarizing event, such as dorsoventral segregation of visual pigments and cone types.

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