



Novel expression pattern of interphotoreceptor retinoid-binding protein (IRBP) in the adult and developing zebrafish retina and RPE

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Purpose: Interactions between the neural retina and retinal pigment epithelium (RPE) are mediated by the interphotoreceptor matrix (IPM). The transport of retinoids across the IPM is mediated by interphotoreceptor retinoid-binding protein (IRBP). To explore the possibility that IRBP is important during retinal development, we examined its spatiotemporal expression pattern in embryonic zebrafish.

Methods: IRBP mRNA expression was examined using RT-PCR and in situ hybridization. IRBP was localized using antiserum against recombinant zebrafish IRBP. IRBP synthesis and secretion were studied by in vitro metabolic labeling of retinas and RPE-eyecups.

Results: IRBP mRNA was first observed in the pineal at 24 hours post-fertilization (hpf) and in the ventral retina at 50 hpf. Immunoreactive IRBP was first observed at 72 hpf. Remarkably, IRBP was expressed not only by photoreceptors but also by the adult and embryonic RPE. In embryos, expression in both retina and RPE began in a ventronasal patch and spread to involve the entire eye. In general, early IRBP expression was dominated by photoreceptors, but then RPE expression spread beyond the limit of photoreceptor expression. Double in situ hybridizations suggests that cones express IRBP mRNA before they express a specific opsin, while rods may express rod opsin prior to IRBP.

Conclusions: The temporal and spatial patterns of IRBP expression by the RPE and retina are consistent with a role in retinal development and suggest coordination of RPE and photoreceptor differentiation.

The progressive and orderly establishment of cellular phenotypes in the developing central nervous system relies on specific cell-cell interactions. The retinal pigment epithelium (RPE)/neural retina complex provides a model to study such interactions in a region of the central nervous system where cell types are highly defined and accessible. Photoreceptor differentiation and maturation within the neural retina is dependent on the RPE [1-6]. This inductive activity of the RPE is mediated by the extracellular matrix between the RPE and retinal neuroectoderm. This interphotoreceptor matrix (IPM) [7-9] serves many roles that are critical to retinal function, including extracellular transport of retinoids and other nutrients, retinal adhesion, stabilization of the photoreceptor mosaic and sequestration of growth factors (reviewed in [10,11]). The IPM contains S-laminin [12], growth factors [13,14], specific domains enriched in lectin binding glycoconjugates [15-19], metalloproteases [20], and hyaluronan [21].

The major soluble protein component of the IPM is interphotoreceptor retinoid-binding protein (IRBP) [22-28]. In the adult retina, IRBP facilitates the exchange of 11-*cis*retinaldehyde and all-*trans* retinol during the "visual cycle" (reviewed in [29-31]) and solubilizes visual cycle retinoids,

protecting them from isomeric and oxidative degradation [32,33]. Additionally, IRBP promotes both the delivery of all-*trans* retinol from rods to the RPE and the transfer of 11-*cis*retinaldehyde from the RPE to the rods [34-38]. Finally, IRBP binds fatty acids, particularly docosahexanoic acid, which may have a role in modulating its retinoid binding properties [33,39,40].

Amphibian and mammalian IRBPs are composed of four homologous modules, each about 300 amino acids in length [41-46]. The functional significance of this modular structure is not clear, although recent studies suggest that each module has ligand-binding activity [42,47,48]. In contrast, teleost IRBP consists of only 2 modules and is half the size of other vertebrate IRBPs [49,50]. The two-modular IRBP of zebrafish may provide a simpler system for structural and functional studies of this protein. The N- and C-terminal modules of teleost IRBP are most similar to the first and fourth modules of mammalian and amphibian IRBPs [49]. It has been suggested that the middle two modules of an ancestral four-modular IRBP were lost during the emergence of teleosts [49].

Teleost IRBP also shows unique spatial and temporal expression patterns. In mammals [26,51-55] and amphibians [56], IRBP is synthesized and secreted exclusively by retinal photoreceptors and pinealocytes [54,57,58]. In the squirrel [59], *Xenopus* [60] and both normal and neoplastic human retinas [61], IRBP is synthesized by both rods and cones. In zebrafish, IRBP mRNA levels are under circadian regulation and are four to seven times higher at mid-light than at mid-

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dark. At mid-light most or all photoreceptors express IRBP mRNA, while only the UV-sensitive cones express IRBP message at mid-dark [49]. In goldfish, cone photoreceptors express IRBP mRNA, but rods do not, and there is no discernable rhythmicity [50,62].

It has been suggested that IRBP participates in retinal development by facilitating the transport of retinoids and/or other nutrients between the RPE and developing retina. IRBP accumulates in the subretinal space before the visual cycle is operational [60,63-67]. The gene for IRBP is expressed early during rodent retinal development and is upregulated before that of opsin [57,62,65,68,69]. Targeted disruption of *IRBP* results in early photoreceptor degeneration in transgenic mice [70]. In the *Xenopus* embryo, IRBP is first expressed by photoreceptors in the central retina, and a central-to-peripheral gradient of IRBP is established by diffusion of IRBP through the subretinal space [60]. This gradient could allow IRBP to transport retinoids and fatty acids from the RPE to the developing peripheral retina. In contrast to other vertebrates, the zebrafish subretinal space arises not from involution of an optic vesicle but rather through cavitation of a mass of cells emerging from the anterior portion of the neural keel [71]. The unique features of ocular morphogenesis in zebrafish may provide additional insights as to the role of IRBP in retinal development. Portions of the present work have been published in abstract form [72].

METHODS

Animals: All experiments involving animals employed procedures approved by the University of Virginia Animal Care Committee and were performed in accordance with the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Zebrafish (*Danio rerio*) were maintained and bred in the laboratory according to Westerfield [73] at 28.5 °C on a 12h/12h light/dark cycle; maximum irradiance at the water surface was 10.7 μWcm^{-2} . Adult *albino* (*alb*) zebrafish were generously provided by John Dowling (Harvard University).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Southern Blot Analysis: Embryonic zebrafish were collected at the time of fertilization (1-2 cell stage) and allowed to survive for 24-103 hours post-fertilization (hpf) before collection in liquid N_2 . Embryos were homogenized in phosphate buffered saline (PBS) using a glass-teflon homogenizer. Total RNA was extracted from homogenates using the acid-phenol method [74] and treated for 15 min with deoxyribonuclease I (Gibco BRL, Bethesda, MD). RNA samples

were reverse transcribed using random hexamer primers. A 530-base pair fragment of zebrafish IRBP was amplified using forward primer 5'-GATGTTTCATCGCGCTCATCA-3' and reverse primer 5'-GCAGTGTCTGAATGGCTGAT-3'. These primers span introns II and III of the zebrafish IRBP coding sequence [49]. Zebrafish *max* (*zmax*) was used as a control for amplification efficiency and was co-amplified using forward primer 5'-CGACAACGATGATATCGA-3' and reverse primer 5'-ACTGGATGTATTCTGTGGCT-3'. *Zmax*, an inhibitor of the myc family of oncoproteins, has been shown to be present at constant levels throughout zebrafish development [75]. PCR was carried out for 35 cycles in a Perkin-Elmer Gene Amp 2400 (Perkin-Elmer Cetus, Norwalk, CT) thermal cycler. Samples were heated at 95 °C for 2 min before addition of *taq* DNA polymerase. Cycling conditions were: denaturing, 94 °C for 45 seconds; annealing, 63 °C for 45 seconds; extension, 72 °C for 45 seconds. RT-PCR products were separated by 3% MetaPhor (FMC Bioproducts, Rockland, ME) agarose gel electrophoresis and visualized using ethidium bromide.

For Southern blot analysis the RT-PCR products were transferred to Nytran paper as previously described [76]. The blots were probed with γP end-labeled oligonucleotides corresponding to sequences internal to those used for RT-PCR (5'-CTGGTGTAGATACTGTCTGG-3' for IRBP; 5'-

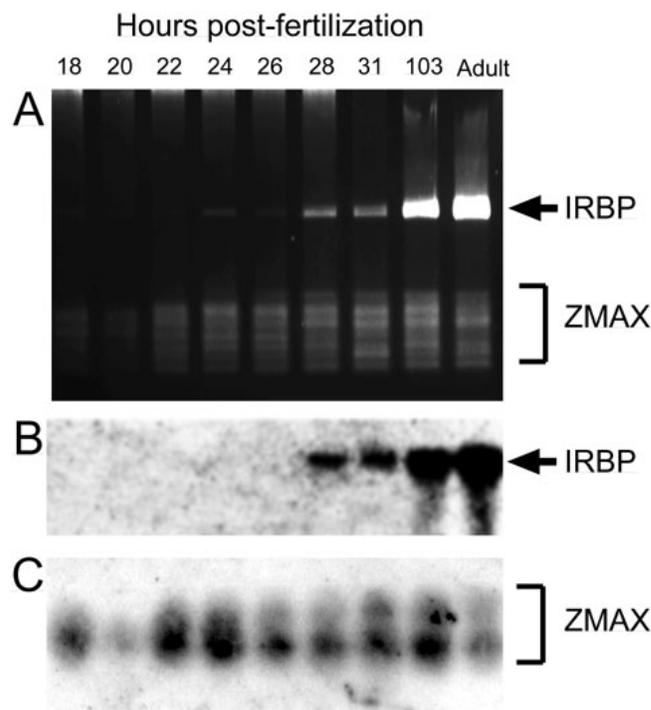


Figure 1. Temporal pattern of IRBP mRNA expression during zebrafish development. (A) RT-PCR amplification of a 530-bp IRBP mRNA fragment from whole zebrafish embryos at various times after fertilization (time indicated above each lane, 3% MetaPhor agarose (FMC) stained with ethidium bromide). IRBP mRNA (arrow) is first detectable at 24 to 28 hours post-fertilization (hpf). Zebrafish *max* mRNA (*zmax*) was co-amplified as an internal standard. (B) and (C): Southern blot analysis of the gel in panel A. IRBP (B) and *zmax* (C) RT-PCR fragments were probed with $[\gamma\text{P}]\text{ATP}$ -labeled oligonucleotides internal to those used for RT-PCR.

TABLE 1. STAGES OF IRBP mRNA EXPRESSION IN RETINA/RPE OF ZEBRAFISH EMBRYOS

Stage	Photoreceptor cell Expression	RPE Expression
0	None	None
1	1-10 cells in ventral retina	None
2	10-25 cells in ventral retina	Weak in ventral region
3	>25 cells in ventral retina, 1-10 cells on the opposite (temporal) side of the choroid fissure, and/or 1-10 cells in nasal retina	Weak in 1/3 of eye
4	1/3 of retina	Stronger in 1/2 of eye
5	2/3 of retina	Strong throughout the eye
6	Entire retina	Strong throughout the eye

TCGGAAGCCCTAAGACAAGG-3' for *zmax*). The blots were prehybridized at 42 °C for 1 h in 6X SSC (1X SSC: 0.15 M NaCl, 15 mM Na citrate, pH 7.0), 1% SDS, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA, and 50 µg/ml heterologous DNA. Hybridization was carried out in the same buffer for 18 h at 68 °C with 10⁶ dpm/ml ^γP end-labeled oligonucleotides. Blots were then washed twice in 2X SSC/0.5% SDS (21 °C) and twice in 1X SSC/1% SDS (37 °C) and exposed to X-ray film.

Tissue preparation for *in situ* hybridization: Embryos were treated at 12 hpf with 0.003% phenothiourea (PTU) to inhibit melanin synthesis, thereby keeping the embryos translucent [73]. Embryos (24–81 hpf) were fixed in 4% paraformaldehyde in phosphate-buffered 5% sucrose and stored in 100% methanol at -20 °C for processing as whole mounts. For sec-

tioning, fixed embryos were embedded in a 2:1 solution of phosphate-buffered 20% sucrose and OCT embedding medium (Tissue Tek, Elkhart, IN), frozen in liquid N₂-cooled isopentane, and sectioned at 3 µm as previously described [77].

For adult animals, lenses were removed from eyes through a small cut in the cornea, and whole eyes were immersed in 4% paraformaldehyde in phosphate-buffered 5% sucrose for 30 min. Eyes were cut in half along the dorsal-ventral axis, then returned to fixative for an additional 30 min. Eyes were embedded, frozen, and sectioned as described above for embryos.

***In situ* hybridization:** Full-length cDNAs (in pBluescript, Stratagene, La Jolla, CA) corresponding to the gene for goldfish rod opsin (GFrod), and goldfish red cone opsin (GFred) were kindly provided by K. Nakanishi (Columbia University

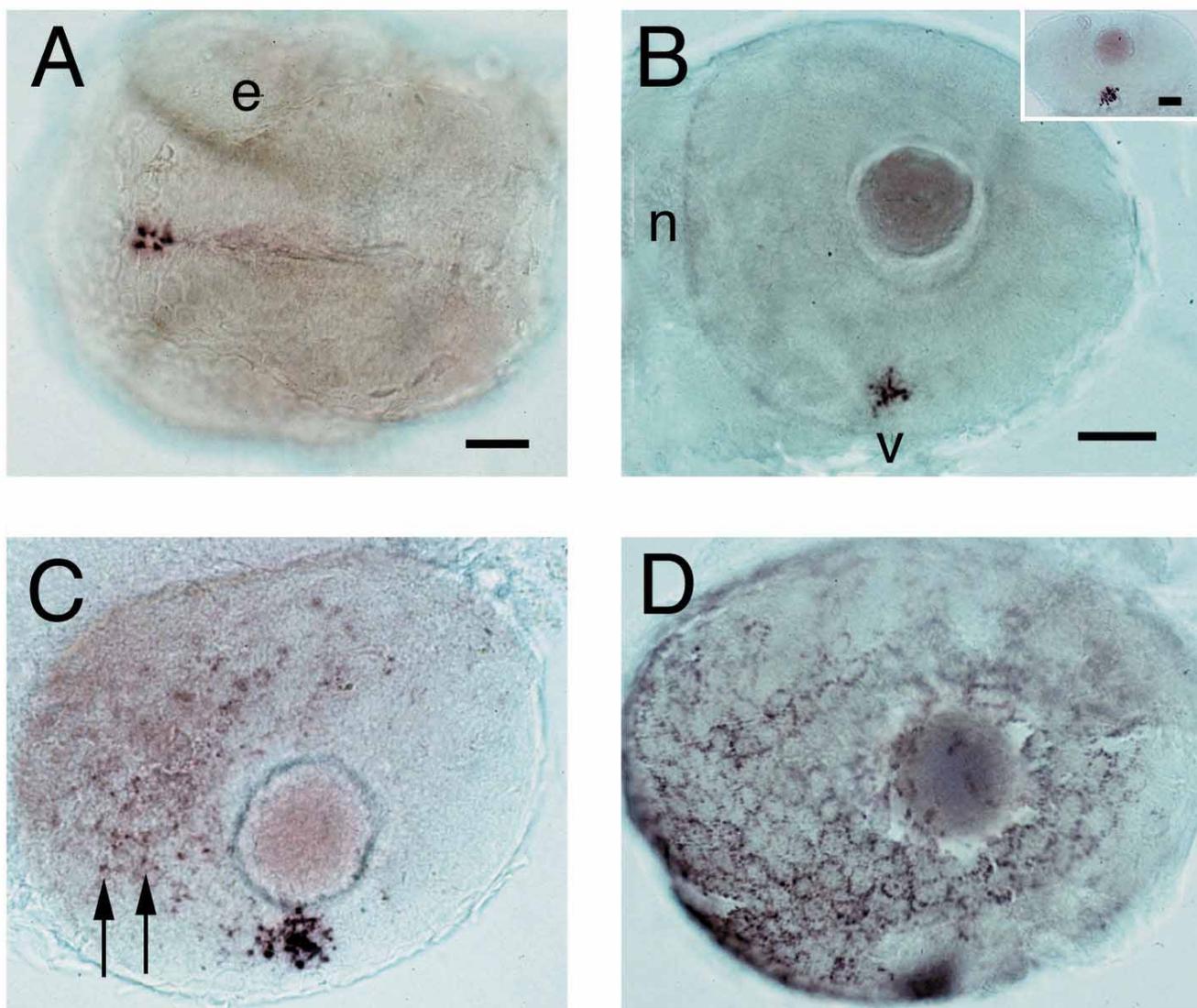


Figure 2. IRBP mRNA expression in embryonic zebrafish whole mounts. (A) 31 hours post-fertilization (hpf) zebrafish hybridized with IRBP cRNA. Five cells on dorsal midline surface of head are labeled; e, eye. (B) 53 hpf zebrafish eye, lateral view, hybridized with IRBP cRNA. Approximately 10 cells in ventral retina are labeled; v, ventral; n, nasal. Inset shows a 53 hpf zebrafish eye, same orientation, hybridized with rod opsin cRNA; approximately 25 cells are labeled. (C) 55 hpf zebrafish eye, same orientation as in B, hybridized with IRBP cRNA. Several additional photoreceptors outside of ventral patch (arrows) are labeled. (D) 60 hpf zebrafish eye, same orientation as B and C, hybridized with IRBP cRNA. The focal plane is at the surface of the eye (superficial to the level of the neural retina), revealing a hexagonal labeling pattern, characteristic of RPE. Scale bars = 50 µm; bar in B applies to C and D.

[78]). These cDNAs, as well as the full-length cDNA (also in pBluescript) corresponding to the gene for zebrafish IRBP [49] were used to prepare digoxigenin (dig)-labeled cRNA probes for nonisotopic *in situ* hybridization, using components of the Genius kit (Boehringer-Mannheim, Indianapolis, IN).

In situ hybridization methods for whole mounts and cryosections have been described [62,77-79]. Whole embryos were cleared in 100% xylene; all remaining steps for *in situ* hybridization were similar for both whole mounts and cryosections. Tissue was rehydrated, digested with 10 $\mu\text{g/ml}$ proteinase K, then treated with triethanolamine-buffered 0.25%

acetic anhydride. Whole mounts were prehybridized at 56 °C for 2 h in a tris-buffered hybridization solution containing 50% formamide, and then hybridized overnight with 4 $\mu\text{g/ml}$ cRNA probe. Tissue was treated with RNase A and incubated overnight with 1:2000 α -dig Fab fragments conjugated to alkaline phosphatase (Boehringer-Mannheim). Hybridization was visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Boehringer-Mannheim). Eyes were removed from whole mounts, mounted under 100% glycerol, and viewed with phase-contrast optics. The pattern and extent of IRBP expression in each eye were

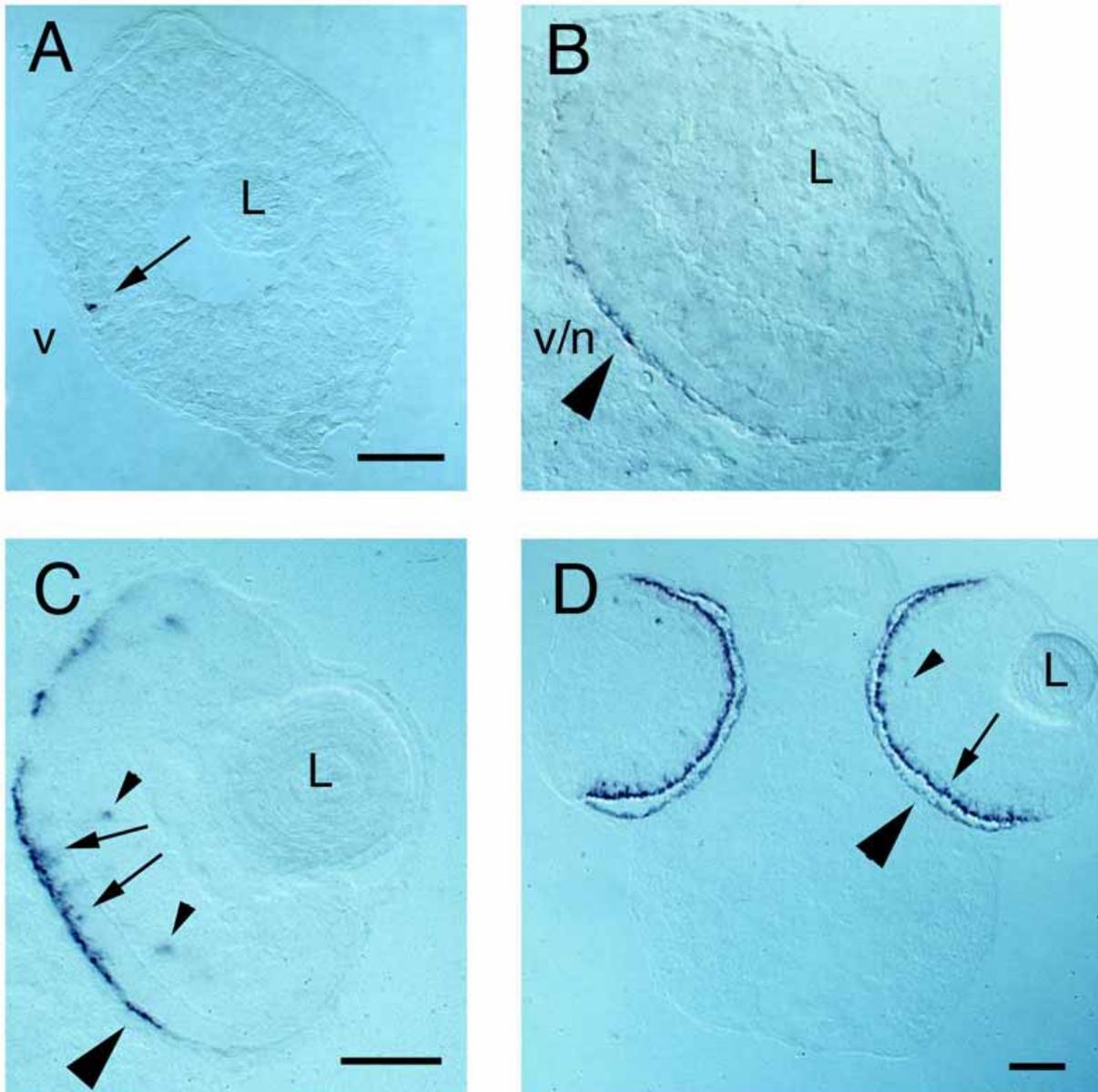


Figure 3. Expression of IRBP mRNA in embryonic zebrafish cryosections. All sections were hybridized with IRBP cRNA. (A) 51 hours post-fertilization (hpf) zebrafish section, arrow indicates a single labeled cell in the photoreceptor layer. The adjacent RPE is not labeled; v, ventral; L, lens. (B) 54 hpf zebrafish section, large arrowhead indicates region of RPE that is labeled; adjacent photoreceptors are not labeled; v/n, ventronasal. (C) 58 hpf zebrafish section, arrows indicate labeled differentiating photoreceptors. Large arrowhead indicates region of RPE that is labeled while adjacent photoreceptor cells are not, small arrowheads indicate labeled INL cells; L, lens. (D) 81 hpf zebrafish section, arrow indicates extensive labeling of photoreceptors, large arrowhead indicates extensive labeling of RPE, small arrowhead indicates labeled INL cell; L, lens. Scale bars = 50 μm ; bar in A applies to B.

assessed using criteria described in Results (and Table 1).

Double in situ hybridization: The nonisotopic procedure for the simultaneous detection of two mRNAs in the same cell (or same tissue) has been described [62]. Hybridization on cryosections was carried out as above, but 2 different cRNA probes were in the hybridization solution: a dig-labeled probe and a fluorescein (FL)-labeled probe. Sections were incubated with a solution containing 1:50 α -dig Fab fragments conjugated to peroxidase and 1:50 α -FL Fab fragments conjugated to alkaline phosphatase. Hybridization of the dig-labeled probe was visualized using diaminobenzidine and H_2O_2 , resulting in a brown color product. Hybridization of the FL-labeled probe was visualized using Vector Blue (Vector Labs, Burlingame, CA), resulting in a blue color product. Presence of both cRNAs results in a characteristic dark precipitate that can be distinguished from either of the two single color products [62].

Immunohistochemistry: Embryonic zebrafish (50-85 hpf) were dechorionated (if they had not already hatched) and fixed in 4% paraformaldehyde in 0.125 M phosphate buffer (pH

7.4) for 12 h at 4 °C. Fixed tissue was dehydrated through graded ethanols and embedded in paraffin. Three μ m sections on polylysine-coated slides were cleared, rehydrated, and incubated for 2 h in blocking solution (0.5% BSA; 2% calf serum; 0.08% Triton X-100 in PBS). Sections were then incubated overnight at 4 °C in rabbit antiserum (1:200 to 1:1000 in blocking solution) directed against recombinant zebrafish IRBP. This antiserum was generated against recombinant zebrafish IRBP-thioredoxin fusion protein expressed in *E. Coli*. Briefly, thioredoxin was selected as the fusion protein because it has been shown to be an effective way to express other IRBPs in a soluble form in *E. coli* [42]. The recombinant zebrafish IRBP was purified by a combination of ion exchange chromatography and arsenical-based affinity chromatography. The antiserum recognizes a single polypeptide of 77 kD on western blot analysis and does not have detectable reactivity for thioredoxin on ELISA (data not illustrated). Sections were washed in PBS and incubated for 2 h in Oregon Green-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR).

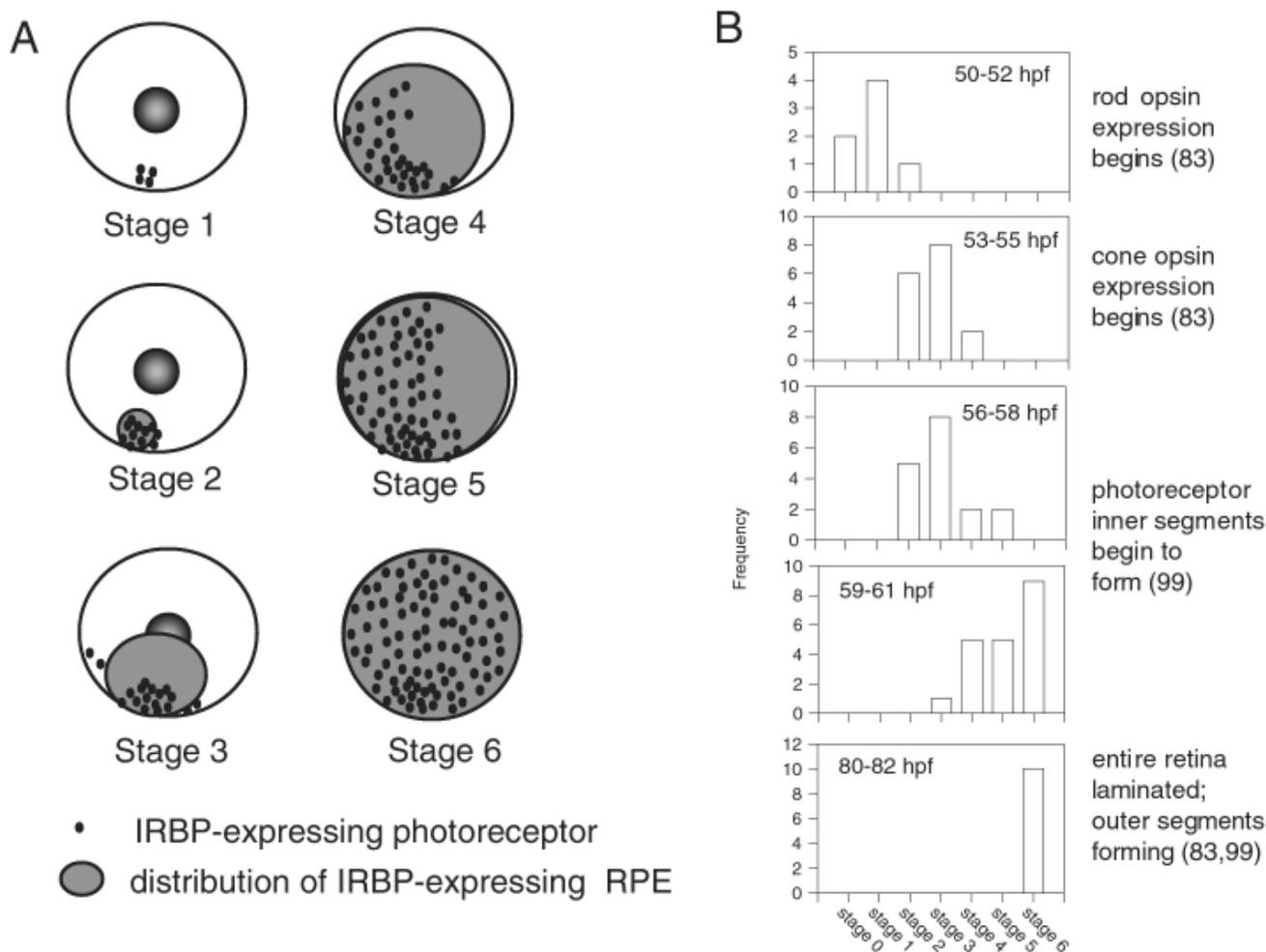


Figure 4. Spatiotemporal patterns of IRBP mRNA expression in differentiating photoreceptors and RPE. (A) Schematic representation of stages of IRBP mRNA expression described in Results. Stages were drawn based on representative whole mounts hybridized with IRBP cRNA (such as those in Figure 2). (B) Histograms showing stages of IRBP mRNA expression as a function of embryonic age. To the right of the histograms are other known events in zebrafish retina development that occur (approximately) at the embryonic ages (hours post-fertilization [hpf]) indicated in the histograms [82,97].

Sections of adult eyes were incubated in 4',6-diaminidino-2-phenylindole (DAPI; Sigma Chemicals, St. Louis, MO) at 0.1 mg/ml in PBS for 20 min. All sections were mounted in Fluoromount-G mounting medium (Southern Biotechnology Associates, Birmingham, AL).

Photography: In situ hybridization preparations were photographed with Ektachrome 160 film (Eastman Kodak, Rochester, NY) under Nomarski differential interference-contrast optics (sections) or phase-contrast optics (whole mounts), using a Leica Aristoplan microscope. The 35 mm color slides were scanned into Adobe Photoshop (Adobe Systems, Mountain View, CA) and adjusted to improve contrast. Fluorescence microscopy was carried out on a Zeiss Axioplan 2 microscope using a Microview CCD camera (Princeton Instruments, Inc., Trenton, NJ) and MetaMorph Imaging software (version 2.7, Universal Imaging, Westchester, PA). Confocal microscopy was carried out using a Zeiss LSM 410 confocal laser scanning microscope.

Metabolic labeling: Corneas from dark-adapted adult zebrafish were perforated using a microscalpel, and the anterior chamber (including the lens) was removed using microscissors and jeweler's forceps. Under fluid (Dulbecco's modified Eagle's medium (DMEM) without methionine, Gibco BRL), retinas were carefully detached and lifted off the RPE using a fine fire-polished glass rod. The optic nerve attachment was cut with microscissors. To confirm that RPE-eyecup preparations did not contain contaminating neural retina, RPE-eyecups were embedded in plastic and examined histologically. We found that the dissection yielded RPE-eyecups devoid of neural retina. Retinas (6 per dish x 2 dishes) and

RPE-eyecups (6 per dish x 2 dishes) were incubated in the dark in 1 ml of DMEM plus 150 μ Ci [35 S]methionine (Pro-Mix in vitro cell labeling mix, Amersham Life Sciences, Arlington Heights, IL) for 4 h at room temperature with gentle agitation. Following incubation, retinas, RPE-eyecups, and incubation media were collected separately and frozen in liquid N₂.

Metabolically labeled IRBP was identified by immunoprecipitation. Retinas and RPE-eyecups were homogenized in 1 ml 1X NET buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.1% nonylphenyl-polyethylene glycol; 0.25% gelatin; 0.02% Na azide) containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1.5 μ g/ml leupeptin, 1.5 μ g/ml pepstatin A, 3.0 μ g/ml aprotinin) using a glass-teflon homogenizer. Insoluble material from homogenates as well as from incubation media was removed by centrifugation at 20,000g for 30 min at 4 °C. The samples were pre-cleared by incubating at 4 °C with 20 μ l rabbit pre-immune serum for 1 h followed by 40 μ l of a 50% suspension of protein A-sepharose CL-4B beads (Sigma) in PBS (pH 8.0) for 30 min. IRBP was immunoprecipitated with 25 μ l rabbit anti-zebrafish IRBP serum at 4 °C for 1 hour followed by 75 μ l of 50% suspension of protein A-sepharose for 1 h. Beads were washed three times in NET buffer and resuspended in Laemmli buffer [80] containing DTT. Samples were subjected to SDS-10%PAGE and exposed in a PhosphorImager (Molecular Dynamics).

RESULTS

IRBP expression in whole embryonic zebrafish: The temporal pattern of IRBP mRNA expression in the developing em-

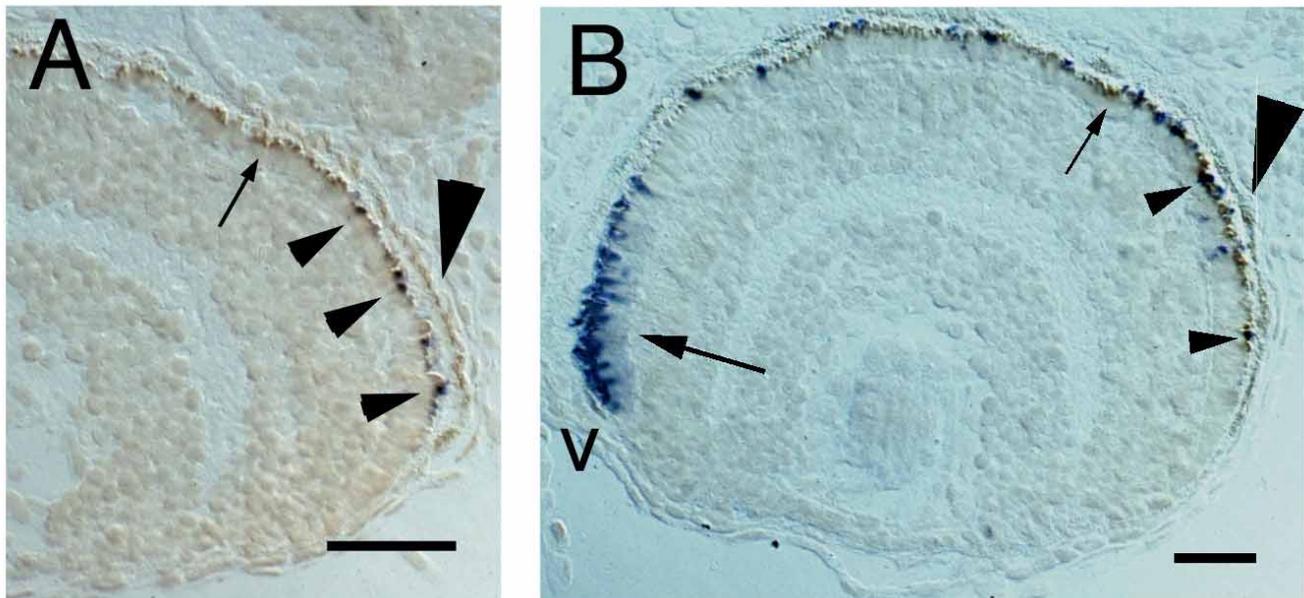


Figure 5. Double in situ hybridization of embryonic zebrafish (80 hpf) cryosections. (A) Section hybridized with dig-labeled IRBP cRNA (brown color product) and FL-labeled red cone opsin cRNA (blue color product). Photoreceptors are labeled with either IRBP cRNA only (brown cells indicated by small arrow) or by both IRBP and red cone opsin cRNAs (black cells indicated by small arrowheads); large arrowhead indicates IRBP-labeled RPE. (B) Section hybridized with dig-labeled IRBP cRNA (brown color product) and FL-labeled rod opsin (blue color product). Photoreceptors are labeled with either IRBP only (brown cells indicated by small arrow), or by rod opsin only (blue cells indicated by large arrow), or by both IRBP and rod opsin (black cells indicated by small arrowheads); large arrowhead indicates IRBP-labeled RPE. Scale bars = 50 μ m.

bryo was followed by RT-PCR using *zmax* mRNA as an internal control for amplification efficiency (Figure 1). Southern blot analysis using [γ P]ATP-labeled probes corresponding to sequence internal to the primers established the identity of the amplified bands (Figure 1B,C). IRBP cDNA was first detected by RT-PCR at 24–28 hpf and by Southern blot analysis at 28 hpf. IRBP mRNA expression was dramatically upregulated between 31 and 103 hpf, corresponding to the period of photoreceptor differentiation and maturation. As previously reported [75], *Zmax* presented as a collection of bands with a similar expression level throughout the developmental stages examined.

The onset of IRBP expression prior to cell birth and differentiation in the zebrafish retina suggests that IRBP was expressed by non-retinal cell types. To evaluate this possibility, embryo whole mounts (18, 26, and 31 hpf) were processed for *in situ* hybridization with the IRBP cRNA probe. Although no specific

hybridization was detected at 18 hpf, IRBP mRNA expression was detected at 26 and 31 hpf in a cluster of cells located near the dorsal midline surface of the embryo's head (Figure 2A) and connected to the CNS by a visible stalk (which is not in the plane of focus in Figure 2A). Cells with this localization have been characterized in teleosts as having photoreceptor morphologies [81] and expressing specific opsin mRNAs [79] (D. Stenkamp, L. Barthel, P. Raymond, unpublished results) prior to the development of these characteristics in retinal photoreceptors. These IRBP-expressing cells were tentatively identified as pineal photoreceptors, although additional characterization is needed to confirm this.

Ocular IRBP mRNA expression:

Older embryos (48 to 82 hpf) were hybridized with the IRBP cRNA0xA3 probe. Beginning at 50 hpf, these embryos showed specific hybridization in cells of the eye (Figure 2B–D) as well as those of the pineal. The first cells in the eye to express IRBP were localized to the ventral pole, near the choroid fissure (Figure 2B), corresponding to the location of the first opsin-expressing photoreceptors ([82] and Figure 2B inset). At later times (54–58 hpf), this patch of IRBP-expressing cells expanded to include cells on the temporal side of the choroid fissure; additional cells elsewhere in the eye began to express IRBP. These additional cells generally showed a nasal distribution with punctate labeling (arrows, Figure 2C). The oldest embryos examined (60–80 hpf) showed conspicuous expression throughout the eye. When the plane of focus was at the surface of the eye, a distinct hexagonal pattern emerged (Figure 2D) that was unlike the pattern seen for photoreceptor-specific gene expression. This hexagonal pattern indicated that cells other than photoreceptors, located superficial to the neural retina, also expressed IRBP. Like the retinal labeling, this superficial hexagonal labeling appeared first in the ventronasal quadrant, then spread throughout the eye (Figure 2D and data not shown).

To determine which cell types in the developing eye expressed IRBP mRNA, fixed embryos (48–82 hpf) were frozen, sectioned at 3 μ m, and hybridized with the IRBP probe. At 50–52 hpf, the labeled cells occupied only the emerging photoreceptor layer (Figure 3A); in slightly older

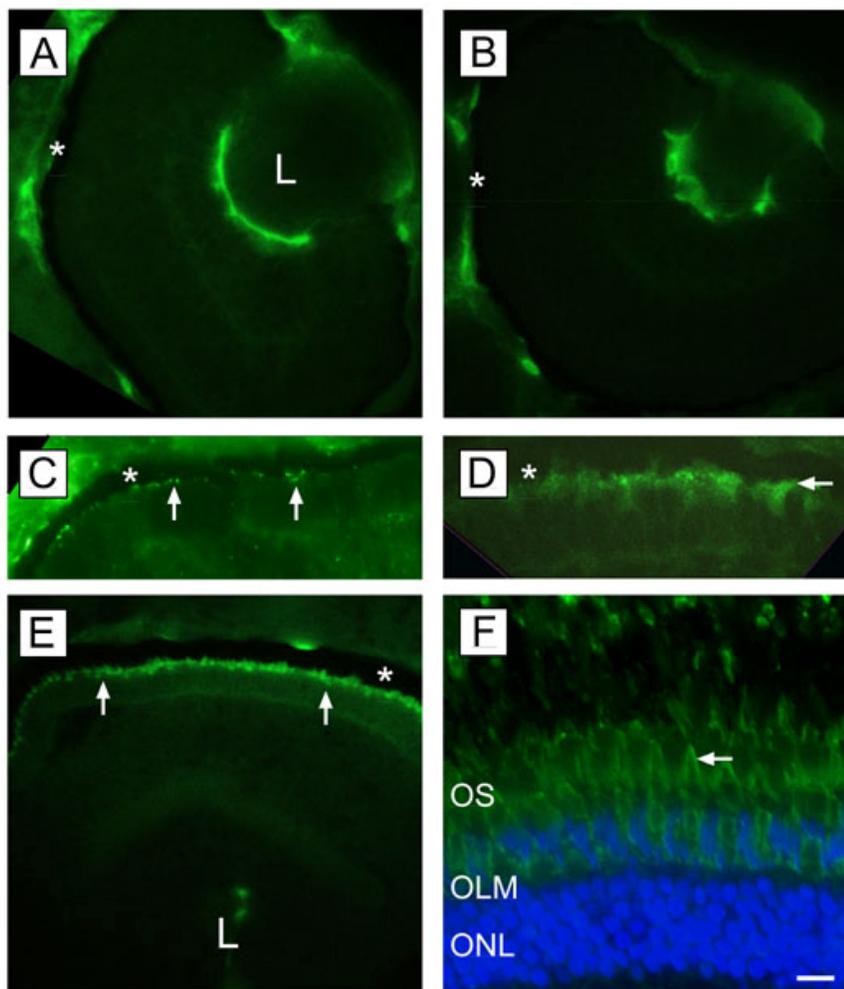


Figure 6. IRBP immunoreactivity in the developing and adult zebrafish eye. (A) 65 hours post-fertilization (hpf) eye. Specific IRBP immunoreactivity is not present in the developing retina or RPE. L, lens. (B) 65 hpf eye. The signal around the developing lens (L) is also seen in eyes reacted with rabbit pre-immune serum. (C) Confocal micrograph of a 72 hpf eye. IRBP immunoreactivity is detectable in a few cells of the photoreceptor layer (arrows). (D) Confocal micrograph of a 77 hpf eye; view of the most mature portion of the retina, near the optic disk. Immunoreactivity is localized to the apical portion of developing photoreceptors (arrow). (E) 79 hpf, IRBP immunoreactivity is detectable across the entire zebrafish retina (arrows). (F) Adult retina at mid-light. IRBP is localized to the region between the outer limiting membrane (OLM) and the RPE. IRBP appears to associate with the outer segments (OS) of cone photoreceptors (arrow). Asterisks (*) in each panel indicate location of RPE/choroid. Scale bar is 50 μ m in Panels A,B,C,E; 20 μ m in D, F. Nuclear stain is 4',6-diamidino-2-phenylindole (DAPI). ONL, outer nuclear layer.

embryos (53-56 hpf), hybridization color product was seen also in the RPE (Figure 3B). Although the RPE does not contain melanin pigment in these preparations (due to treatment of embryos at 12 hpf with PTU), it is still identifiable as a thin layer adjacent to the developing neural retina. It is likely that IRBP expression in the RPE generated the hexagonal labeling pattern seen in the whole mounts, since cells in this epithelial tissue are characteristically packed in a hexagonal array [83]. For example, the hexagonal shape of the RPE cells is clearly illustrated in the pigment mosaic zebrafish described by Streisinger et al. [84]. The young (50-56 hpf) embryos showed IRBP expression in RPE in regions where adjacent photoreceptors did not yet express IRBP (Figure 3B). There were also regions with the opposite pattern: IRBP expression in photoreceptors in regions where the adjacent RPE did not express IRBP (Figure 3A). However, sections of older embryos (58-82 hpf) showed consistent labeling of both cell types, with a similar spatial distribution, although in some cases regions of RPE expressing IRBP extended beyond the limit of photoreceptor IRBP expression (Figure 3C,D). Also in these older embryos, a subpopulation of cells in the inner nuclear layer expressed IRBP (Figure 3C,D). These IRBP-expressing cells were previously described in adult zebrafish [49] and goldfish [62], although they remain unidentified.

Spatiotemporal Distribution of IRBP mRNA: A series of stages was defined to describe the distribution of IRBP expression in the eye during development (Table 1). These stages reflect both total number and distribution of IRBP-expressing photoreceptors in whole mounts (punctate labeling, as distinct from the hexagonal pattern of IRBP expression in RPE), and the distribution of IRBP expression in the RPE. These stages of retinal development, although similar to those defined using opsins [79,82], are unique for IRBP (Figure 4A).

Seventy-two embryos (50-82 hpf) were hybridized as whole mounts with the IRBP probe and assigned one of the above stages (Figure 4B). It should be noted that stages 4-6

were occasionally difficult to distinguish on whole mounts because hybridization color product in the RPE obscured the punctate labeling of the photoreceptors. Therefore, the patterns found in stages 4-6 (Figure 4A) were routinely verified by hybridization of sectioned embryos obtained at the same time as the whole mounts. The rate of progression of embryos through these defined stages was variable, but in general embryos showed a stage 1 pattern of IRBP expression by 50-52 hpf, then progressed through stage 3 by 56-58 hpf. All embryos showed a stage 6 pattern by 80-82 hpf (Figure 4A).

IRBP expression by both rods and cones in embryonic zebrafish: In adult zebrafish, IRBP is expressed by all photoreceptor types, although more weakly by rods than by cones [49]. In embryonic zebrafish, the expression pattern of IRBP by photoreceptors suggested that both rods and cones were expressing IRBP. The earliest opsin-expressing rods and the earliest opsin-expressing cones are co-localized to a small ventral patch on the nasal side of the choroid fissure [82]; the earliest IRBP-expressing photoreceptors were found in this same region. During the intermediate stages of rod recruitment, 52-58 hpf (identified by using rod opsin in situ hybridization [82]), a secondary patch of rod opsin-expressing cells was found on the temporal side of the choroid fissure; during the intermediate stages of red cone recruitment, 58-73 hpf [82], a secondary patch of red cone opsin-expressing photoreceptors was found at the nasal pole. In the present study, secondary patches of IRBP-expressing photoreceptors were found at both locations (stage 3 on Figure 4A).

To verify that both photoreceptor cell types were expressing IRBP in embryos, we performed several double in situ labeling experiments. Cryosections of 80 hpf embryos were simultaneously hybridized with red cone opsin probe (blue color reaction product) and IRBP probe (brown color reaction product). Cells were identified that either (1) expressed only IRBP (brown cells in Figure 5A) or (2) expressed both red opsin and IRBP (black cells in Figure 5A). When sections from

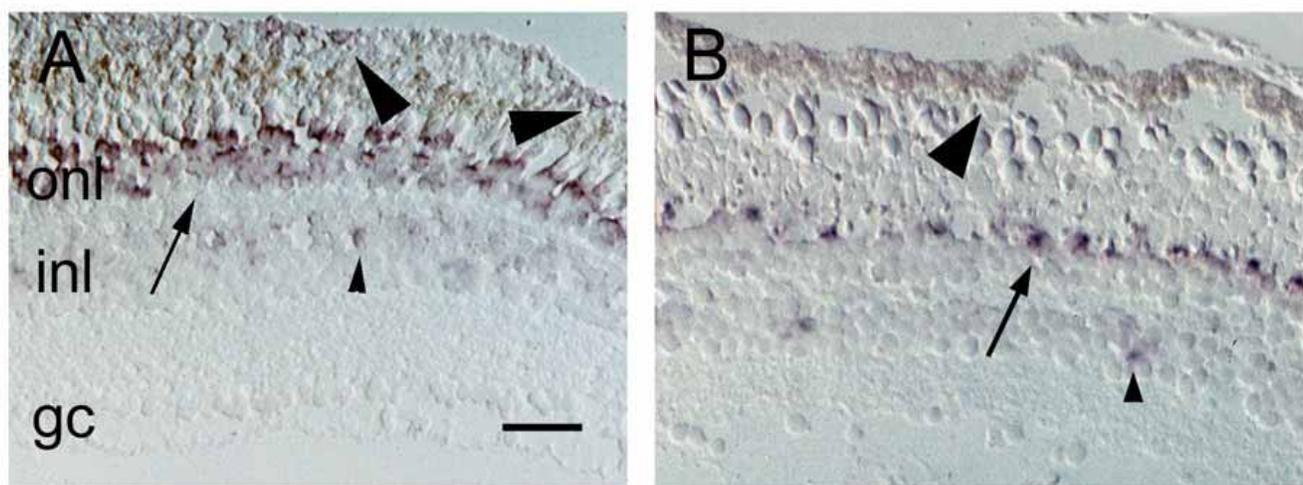


Figure 7. Expression of IRBP mRNA in cryosections from adult albino zebrafish retina and RPE. (A) Light-adapted retina, both rods and cones are labeled (arrow), as are scattered cells in the INL (small arrowhead). The basal region of the lightly-pigmented RPE is also labeled (large arrowheads); scale bar = 50 μ m. (B) Dark-adapted retina, a subset of cones is labeled (arrow), as are some cells in the INL (small arrowhead). The dark-adapted RPE shows a distribution of pigmentation (large arrowhead) that may obscure labeling, making it difficult to discern whether this tissue is expressing IRBP mRNA.

the same embryos were hybridized with a combination of rod opsin probe (blue color reaction product) and IRBP probe (brown color reaction product), cells were identified that either (1) expressed only rod opsin (blue cells in Figure 5A), (2) expressed only IRBP (brown cells in Figure 5B), or (3) expressed both rod opsin and IRBP (black cells in Figure 5B). Results of both of these experiments were confirmed using the reverse combinations of probe/color reactions. Since double-hybridized cells were found in both cases, we conclude that both rods and cones (at least red cones) express IRBP in the embryo. Therefore, in the first experiment, at least some of the cells expressing only IRBP and not red cone opsin may have been rods. Alternatively, these cells may have been other types of cones or red cones that had not yet begun to express opsin. In goldfish, cones express IRBP before they express a specific opsin [79]. In the second experiment, at least some of the cells expressing only IRBP and not rod opsin may have been either cones or rods that had not yet begun to express opsin. However, the presence of cells that express only rod opsin suggests that rods may express opsin prior to expressing IRBP, or that the level of rod opsin mRNA greatly exceeds that of IRBP.

Spatiotemporal distribution of IRBP immunoreactivity:

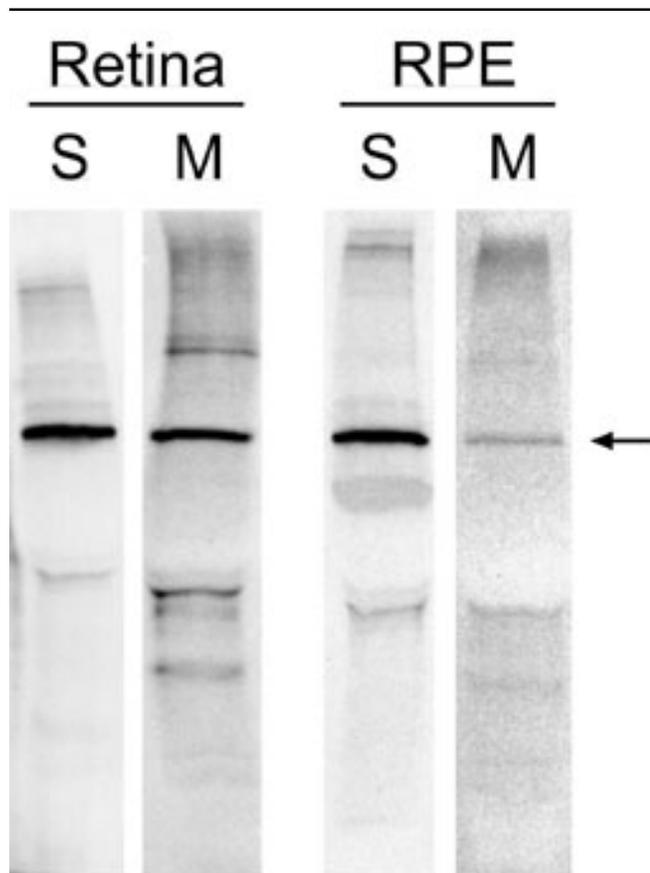


Figure 8. Immunoprecipitation of [³⁵S]IRBP from zebrafish retina and RPE. The isolated retina and RPE-eyecup were incubated in the presence of [³⁵S]methionine for 4 h after which IRBP was immunoprecipitated from the retina and RPE soluble fractions (S) and incubation media (M). Arrow indicates [³⁵S]IRBP. Fluorograms: Lanes S, ~4 days exposure; Lanes M, ~7 days exposure.

The timecourse of IRBP protein expression in the developing zebrafish eye was examined by indirect immunofluorescence (Figure 6). At 65 hpf (Figure 6A) no specific IRBP immunoreactivity was observed in the eyes of 12 animals tested. The apparent signal surrounding the lens was also present in eyes incubated with rabbit pre-immune serum (Figure 6B). IRBP immunoreactivity was first detectable by confocal microscopy at 72 hpf in the more mature regions of the neural retina (Figure 6C) and was localized to the apical surfaces of differentiating photoreceptors (Figure 6D). At 79 hpf IRBP immunoreactivity was visible throughout the outermost portion of the retina (Figure 6E). Because these animals were not treated with PTU, pigmentation in the RPE prevented reliable detection of fluorescent signal in this tissue. In the adult zebrafish retina (Figure 6F) IRBP was localized to the region between the outer limiting membrane and the RPE, where it appeared to surround cone outer segments. IRBP immunoreactivity in the inner nuclear layer was not observed in either adult or embryonic retinas, although a subset of cells in this location express IRBP mRNA (see above).

IRBP expression and secretion by RPE in adult zebrafish:

Expression of IRBP mRNA by the zebrafish RPE has not been previously noted, probably because the dark melanin in the RPE obscures the color reaction for in situ hybridization [49]. Several zebrafish mutant strains are now available that have reduced melanin pigmentation in the RPE; one of these (*alb*) was used to determine whether IRBP is expressed in the RPE of adult zebrafish. In retinal cryosections from light-adapted *alb* adults, the pattern of IRBP mRNA expression was similar to that observed previously [49]: all cone subtypes appeared to be darkly labeled, while rod cell bodies were relatively lightly labeled, and some cells in the inner nuclear layer (INL)

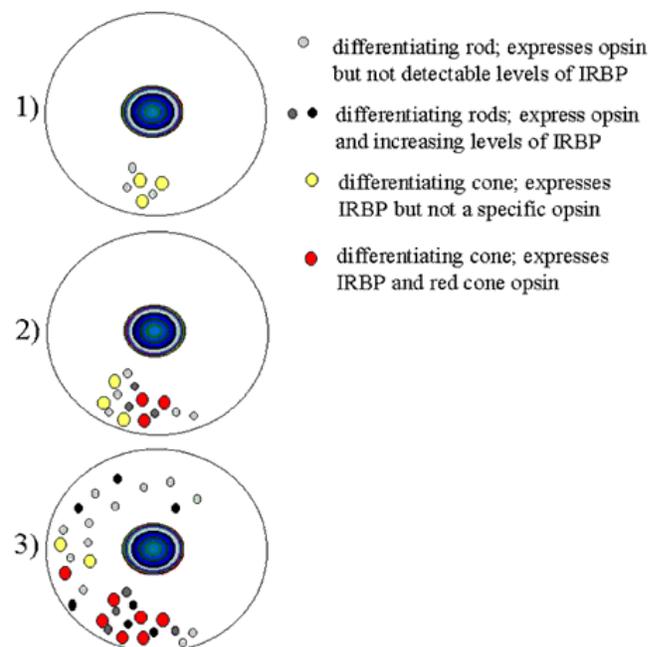


Figure 9. Proposed sequence of events for opsin versus IRBP expression in rods and red cones. (1) 50-52 hpf; (2) 53-58 hpf; (3) 60-80 hpf. See Discussion.

also showed hybridization (Figure 7A). Additionally, the basal region of the RPE was labeled, indicating that, at least in the *alb* strain, IRBP mRNA expression by the RPE is not limited to the embryonic period. In dark-adapted *albadults*, expression of IRBP mRNA was confined to a subpopulation of photoreceptors previously identified as UV-sensitive cones [49]. Expression in the dark-adapted RPE was difficult to discern (Figure 7B). However, the *alb* zebrafish mutant is not completely devoid of melanin granules, and these are redistributed to the basal region of the RPE during dark adaptation. This melanin may have obscured the in situ hybridization color reaction.

Zebrafish retinas and RPE-eyecups were incubated separately in media containing [³⁵S]methionine. [³⁵S]IRBP was immunoprecipitated from the soluble fractions of homogenized retinas and RPE-eyecups as well as from the incubation media (Figure 8). Both retinal homogenates and retinal media contained [³⁵S]IRBP. The RPE-eyecup homogenates contained [³⁵S]IRBP signal comparable in intensity to that of the retinal homogenates. The RPE-eyecup media also contained detectable [³⁵S]IRBP signal. The IRBP band was not present in control immunoprecipitations using rabbit pre-immune serum, while the non-IRBP bands seen in Figure 8 were present in pre-immune serum (data not illustrated).

DISCUSSION

The development of the neural retina depends on retina-RPE interactions that are mediated by the molecular components of the IPM [1-6]. IRBP is abundant in this matrix, and it is expressed earlier than other visual cycle proteins [57,60,62-69]. The observed expression of IRBP prior to the onset of visual function has led to the suggestion that IRBP may participate in retinal development. Here we have sought to address this possibility by defining the spatial and temporal characteristics of IRBP expression in the zebrafish embryo.

Onset of IRBP Expression in Developing Rods and Cones: A Proposed Series of Events: Does IRBP expression precede opsin expression in both rods and cones? In mammals, the expression of IRBP mRNA is upregulated before that of rod opsin [66,69]. In goldfish, where only cones express IRBP mRNA [50], IRBP expression by presumptive cone photoreceptors at the retinal margin of adult animals occurs before these cells begin to express any specific cone opsin [62]. The double in situ hybridization approach used in the latter study [62] and in the present study allowed IRBP mRNA expression to be visualized simultaneously with rod opsin or red cone opsin mRNA. The results indicate that both rods and cones express IRBP mRNA during zebrafish development, but the timecourses may differ. Although we cannot be certain from the results presented here whether both rods and cones express mRNA for IRBP prior to that for opsin, our results taken together with data from the literature allow the following conclusions to be made: (1) The onset of rod opsin expression precedes the onset of red cone opsin expression [82,85]. It is unlikely that an individual photoreceptor switches from expression of rod opsin to expression of red cone opsin in zebrafish since this does not occur in a closely-related teleost, goldfish [62,79]. (2) The onset of IRBP expression (50-52 hpf)

occurs at the same time as the onset of rod opsin expression but prior to the onset of red cone opsin expression (52 hpf or later) [82]; present study; see also [62]. (3) In double-hybridized preparations, cells are present that express both red cone opsin and IRBP, or only IRBP, but not red cone opsin without IRBP (present study, and see [62]). (4) In double-hybridized preparations, cells are found that express rod opsin and IRBP, or only IRBP, or only rod opsin (present study). (5) The spatiotemporal pattern of IRBP expression during eye development resembles the spatiotemporal patterns of both rod opsin expression and red cone opsin expression (present study; [79,82,86]).

These findings are consistent with the following series of events (illustrated in Figure 9): (1) At 50-52 hpf the first rods to differentiate express opsin. At about the same time the first cones to differentiate express IRBP but do not yet express a specific opsin. (2) At 53-58 hpf more rods express opsin, and the first rods now express IRBP at detectable levels (but at levels insufficient to show double hybridization with rod opsin); the first cones now express a specific cone opsin. (3) At 60-80 hpf IRBP mRNA levels in some rods become high enough to be detectable by double hybridization with rod opsin. Several alternative scenarios are possible, but the only way to verify the sequence of events described above would be to attempt a triple in situ hybridization experiment using rod opsin, red cone opsin, and IRBP probes that would give distinct color products for each possible combination of probes. If such an experiment were technically feasible, it would still be complicated by the existence of cone subtypes that express other cone opsins.

Comparative Spatiotemporal Patterns of RPE and Photoreceptor Development Using IRBP mRNA Expression as the Marker for Both: Identification of differentiated RPE has often been based upon the presence of either melanin or cell type-specific functional properties such as cell polarity and formation of tight junctions (reviewed in [87]). Melanin synthesis occurs at about the time when proliferation and differentiation of the neural retina have just begun [88], while RPE cell polarization is a later and more protracted process that takes place as photoreceptor inner and outer segments are forming [87]. Zebrafish is a relatively new model for eye development, and as such there is little information available on the development of RPE cell polarity and the expression of RPE-specific genes in this species. Furthermore, melanin would not be universally useful as a marker for RPE differentiation in zebrafish since eye development in this species is commonly studied using animals treated with PTU (and therefore unpigmented) or mutants with reduced melanin which allow internal eye structures to be visualized. Although we do not know the functional importance of IRBP synthesis by the RPE in zebrafish, the timing and spatiotemporal pattern of IRBP mRNA expression suggest that it might serve as an alternative (but later) marker for RPE differentiation.

IRBP expression has also been used as a marker for photoreceptor differentiation, since in most species it is photoreceptor-specific. Because the appearance of a labeled RPE cell is distinct from that of a labeled photoreceptor in a zebrafish embryo whole mount, a comparative study of the spatiotem-

poral patterns of both RPE and photoreceptor differentiation in this animal is possible using only IRBP mRNA expression as the marker. The onset of IRBP mRNA expression in each cell type occurs in approximately the same location: ventral retina, on the nasal side of the choroid fissure (Figure 2 and Figure 4). Later, IRBP mRNA expression spreads across the RPE and neural retina such that the ventronasal quadrant is the first region of both the retina and the RPE to fully express IRBP mRNA (Figure 2 and Figure 4). This spatiotemporal pattern corresponds to the patterns described for the appearance of photoreceptor-specific markers [79,82,86,89,90] and markers for ganglion cells [79,86,91]. Recent experiments have shown that two zebrafish *hedgehog* genes are also expressed in the RPE, with similar spatiotemporal patterns [92]. This consistent pattern of differentiation suggests that retinal and RPE cell development are coordinated in the zebrafish, either by cell-cell communication that propagates a wave of differentiation beginning at the ventral initiation site and/or by diffusible signals originating outside the retina and having a defined spatiotemporal distribution.

While the patterns of IRBP mRNA expression in RPE and photoreceptors are similar, they do not match precisely. In some regions IRBP mRNA expression in RPE occurs before expression in photoreceptors, while in other regions expression occurs in the opposite sequence (Figure 3 and Figure 4). This may indicate that the propagation of differentiation in the RPE has some degree of independence from that in the neural retina, or that additional signals that are unique for each tissue also participate in promoting the expression of IRBP mRNA.

A New Role for RPE: Synthesis and Secretion of IRBP:

The RPE has many important functions, including absorption of scattered light, promotion of retinal adhesion, phagocytosis of shed outer segment disks, renewal of the visual pigment chromophore, and promotion of photoreceptor health and development (reviewed in [83,93]). The synthesis and secretion of IRBP into the IPM is a function for RPE that has not been previously described. Although this function may be limited to teleosts (or specifically to zebrafish), mammalian RPE cells are known to synthesize and secrete retinol binding protein [94-96]. This additional capacity of the zebrafish RPE may reflect a difference in the mechanisms by which different species regulate the concentration of IRBP and/or retinoids in the IPM. Teleost retinas have a unique tiered anatomy in which the cone photoreceptors, especially UV-sensitive cones, are located distant from the RPE. This unusually large distance between the RPE and the most distal photoreceptors may create additional need for transport proteins such as IRBP and may explain the need for supplemental expression of IRBP by the RPE.

The timing of IRBP secretion is appropriate for a role in later stages of photoreceptor differentiation and activity, such as the formation and elongation of outer segments (60-80 hpf, [97]), and the onset of visual function (72 hpf, [98]). In transgenic mice lacking IRBP protein, photoreceptors show some structural abnormalities, and many of these cells degenerate over the first postnatal month [70]. Our findings do not rule out a role for IRBP in earlier developmental events, since

the difference in timing of detectable IRBP protein (72 hpf) relative to that of the mRNA (50-52 hpf) may simply reflect differences in the sensitivities of immunochemical detection versus in situ hybridization.

The expression of a "photoreceptor-specific" gene by the RPE may provide hints regarding progressive restriction of cell fate during zebrafish eye morphogenesis. Cells that comprise the zebrafish RPE do not originate from a defined lineage, nor do they "share a common pool of ancestral cells" as they do in mice [84]. Development of the zebrafish eye shows a number of additional distinctions. Rather than forming a spherical optic vesicle, the zebrafish eye primordium initially forms a drooping optic "wing" with a cavitated lumen separating lateral and medial neuroepithelia. It had been inferred that the medial cells form the RPE and the lateral cells proliferate to form the neural retina [71]. However, recent fate-mapping studies have shown that cells from both halves of the eye primordium contribute progeny to the neural retina [99], suggesting that RPE and neural retina lineages remain indistinct until fairly late in eye morphogenesis. It is possible that these late determinative events in the zebrafish eye result in RPE cells sharing a molecular marker (IRBP expression) with cells of the neural retina.

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