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ZEBRAFISH INTERPHOTORECEPTOR RETINOID-BINDING PROTEIN: DIFFERENTIAL CIRCADIAN EXPRESSION AMONG CONE SUBTYPES

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Summary

Retinoid trafficking between the photoreceptors and pigmented epithelium is probably mediated by interphotoreceptor retinoid-binding protein (IRBP), a 124–145 kDa glycolipoprotein in mammals and amphibians. In these animals, IRBP is composed of four homologous regions (modules) 300 amino acids in length. We have determined the primary structure of zebrafish IRBP and its expression pattern by northern analysis, reverse transcriptase–polymerase chain reaction and *in situ* hybridization under a variety of lighting conditions. Zebrafish IRBP is half the size (66.3 kDa) of mammalian IRBP because it is composed of only two modules, similar to goldfish IRBP. The first half of the zebrafish protein is most similar to the first module of mammalian IRBP and the second half to the fourth module of mammalian IRBP. This suggests that during the evolution of the ray-finned fish (Actinopterygii), the middle two modules were lost. Each of the modules contains conserved hydrophobic domains which may form the ligand-binding pocket. The expression of zebrafish IRBP mRNA is sevenfold higher in the middle of the light period (at mid-light) than in the middle of the dark period (at mid-dark). This rhythm persists for 2 days under conditions of constant light or constant darkness, then dampens to an intermediate level by 8 days of constant conditions. At mid-light, IRBP mRNA is expressed by all cone types and to a lesser extent by the rods. At mid-dark, the mRNA is restricted to the ultraviolet-sensitive short single cones. These data suggest that IRBP expression is regulated by circadian and light-driven mechanisms that act differentially on the various photoreceptor subtypes in the zebrafish retina.

Key words: interphotoreceptor matrix, vitamin A, zebrafish, *Danio rerio*, circadian biology, molecular evolution, interphotoreceptor retinoid-binding protein.

Introduction

Structural and biochemical changes in the pigment epithelium–photoreceptor complex adapt the retina to environmental lighting. In lower vertebrates, pigment migration and retinomotor movements occur in response to changes in light and dark (Nicol, 1989). Changes in outer segment length, rhodopsin packing density and the expression of visual transduction proteins are also critical to visual adaptation in light and dark (Organisciak *et al.* 1991; Schremser and Williams, 1995*a*,*b*). These morphological and biochemical adaptations are controlled by light (McGinnis *et al.* 1992) and/or by a circadian oscillator located within the photoreceptors (Cahill and Besharse, 1993). The rhythmic expression of phototransduction genes may ensure that optimal levels of the gene products are available at the time of day when they are needed (McGinnis *et al.* 1992; Bowes *et al.* 1988; Craft *et al.* 1990; Pierce *et al.* 1993; Farber *et al.* 1991; Korenbrot and Fernald, 1989).

Light- and dark-induced changes also occur in the interphotoreceptor matrix, which separates the pigmented epithelium and neural retina. The major soluble protein of this matrix in mammals and amphibians is interphotoreceptor retinoid-binding protein (IRBP), a 124–145 kDa glycolipoprotein. IRBP mediates the transfer of all-*trans* retinol and 11-*cis* retinal between the pigmented epithelium and the photoreceptors (reviewed in Pepperberg *et al.* 1993; Saari, 1994). In rats, IRBP is concentrated at the apical surface of the pigment epithelium when exposed to light and around the outer segments during darkness (Uehara *et al.* 1990).

The biochemical activity of IRBP depends on its molar ligand-binding capacity and its concentration in the interphotoreceptor matrix. The binding capacity of the protein is related to its structure. Mammalian and amphibian IRBPs consist of four tandem homologous regions (modules), each

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approximately 300 amino acids in length (Fong *et al.* 1986; Borst *et al.* 1989; Liou *et al.* 1991). The relationship between these modules and the ligand-binding sites of IRBP is not clear. Bovine IRBP binds two equivalents of retinol (Chen *et al.* 1993). The binding sites may be contained within some of the modules since the fourth module binds all-*trans* retinol (Baer *et al.* 1994).

The amount of IRBP in the matrix depends on the balance between the rates of its secretion into and removal from the extracellular compartment. Exposure of mice to constant darkness for 14 days does not change the amount of IRBP in the matrix, but decreases the level of its mRNA (Kutty *et al.* 1994). The amount of IRBP in the matrix is reduced in response to dietary depletion of vitamin A (Katz *et al.* 1993). Factors affecting the rate of removal of IRBP from the matrix may be equally important. In the *vitiligo* mouse, the amount of IRBP in the matrix is increased without an elevation in the level of its mRNA (Smith *et al.* 1994), suggesting a defect in turnover. The above studies suggest that the matrix IRBP concentration is regulated through control of its production and removal. The present study suggests that zebrafish may provide a unique opportunity to study structure/function relationships of IRBP and the mechanisms that control its expression in the interphotoreceptor matrix.

Materials and methods

Animals

Adult, wild-type zebrafish (*Danio rerio*), 3 cm in length, were purchased from a local pet store and maintained under standard conditions (Westerfield, 1993). Experimental protocols were approved by the Institutional Animal Care and Use Committee. Light/dark studies were carried out during the late summer. The fish were entrained for 2 weeks under a 12 h:12 h light:dark (LD) cycle (06:30 h lights on; 18:30 h lights off) before exposure to constant light (LL) or constant darkness (DD). The lighting was supplied by a 15 W fluorescent aquarium light (Penn Plax, Garden City, NY). The maximum irradiance within the aquarium was 10.7 $\mu W\,cm^{-2}$.

Library screening

An adult zebrafish retina cDNA library (Robinson *et al.* 1993) was screened under reduced stringency using a goldfish IRBP cDNA probe (Wagenhorst *et al.* 1995). Duplicate plaque lifts were denatured in NaOH and prehybridized at 32 °C in 0.8 $mol\,l^{-1}$ NaCl, 50 % (v/v) formamide, 1 % (w/v) sodium dodecyl sulfate (SDS), 100 $\mu g\,ml^{-1}$ denatured heterologous genomic DNA, 0.02 $mol\,l^{-1}$ piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.5 (Sambrook *et al.* 1989). The filters were hybridized overnight under the same conditions with approximately 106 $cts\,min^{-1}\,ml^{-1}$ of ^{32}P-labeled goldfish IRBP probe. The cDNA was cloned into pBluescript and sequenced using synthetic oligonucleotides and Sequenase version 2.0 (U.S.B., Cleveland, OH). A 14 kb genomic clone was isolated by screening under high stringency a zebrafish Lambda FixTM II library (Strategene, La Jolla, CA) with a 1.35 kb *Hin*dIII fragment of the zebrafish IRBP cDNA. Hybridization conditions were the same as those described above, except that the hybridization temperature was increased to 42 °C, and the SDS concentration was reduced to 0.5 % (w/v).

Tissue collection and RNA extraction

For the LD, LL and DD experiments, care was taken not to disturb the animals during the time point collections. Multiple fish aquaria were used, each having its own timed light source and box covering the entire tank assembly. Animals were removed from the tanks at intervals of 3 or 4 h. To minimize disturbance, animals were never removed from the same aquarium for consecutive time points. Furthermore, 24 h prior to collection, the animals were segregated within the tanks into 2 l beakers covered with nylon mesh. In this way, the desired number of animals could be easily withdrawn from the tank without exposure to any light and without disturbing the other animals.

To avoid loss of retinal tissue, intact globes were not dissected, but simply flash-frozen separately in liquid nitrogen. The scleral surface was carefully cleaned of extraocular muscles, conjunctiva and orbital tissue. RNA extraction was performed with a Teflon–glass homogenizer using the acid phenol method (Chomczynski and Sacchi, 1987). Each pair of eyes was extracted separately. The average amount of total ocular RNA per eye was determined spectrophotometrically to be 14.5 µg.

Reverse transcriptase–polymerase chain reaction

Standard protocols for reverse transcriptase–polymerase chain reaction (RT-PCR) were followed as described by Perkin Elmer Cetus (Norwalk, CT). In order to obtain a cDNA corresponding to the 3′ portion of the mRNA, total RNA was subjected to reverse transcriptase using the primer 5′-T16 CCGCATGCGGCCGCAGATCTAGATATCGA (5′-T16-*Sph*I/*Not*I/*Bgl*II/*Eco*RV/*Cla*I). It has been shown that the multiple cloning region in this primer can be used during the subsequent amplification (Al-Ubaidi *et al.* 1990). PCR was carried out using primers 5′-CCGCATGCGGCCGCAGATCTAGA, corresponding to the multiple cloning domain of the above primer, and 5′-GACCACATCTATGACAGACC, derived from the third exon of zebrafish IRBP. The amplified 3′ portion of the zebrafish IRBP cDNA was cloned into pCRTMII (Invitrogen, San Diego, CA) and sequenced using synthetic oligonucleotides.

RT-PCR was also used to compare the relative quantity of processed to unprocessed IRBP mRNA at noon and at midnight. 2 µg of ocular RNA pooled from six fish killed either at noon or at midnight was reverse-transcribed using random hexamers, followed by PCR with primers 5′-GATGTTCATCGCGCTCATCA (upstream) and 5′-GGTCTGTCATAGATGTGGTC (downstream). These two primers are located in exons 2 and 3, respectively, and span intron II. The presence or absence of intron II in the IRBP mRNA was confirmed by chemiluminescent (BMB, Indianapolis, IN) Southern blot analysis using a digoxigenin-labeled primer 5′-GCTAGTCCACTACTGGCTAT located within intron II.

Northern analysis

Total ocular RNA was denatured with glyoxal and fractionated

in 1.0 % (w/v) SeaKem GTG agarose (FMC, Rockland, ME) (Feinberg and Vogelstein, 1983). RNA size standards (0.24–9.5 kb BRL, Gaithersburg, MD) were run simultaneously. The RNA was transferred to Nytran paper (Schleicher and Schuell, Keene, NH) and cross-linked by ultraviolet irradiation. For dot blots, RNA was denatured in formaldehyde and applied to Nytran membranes in a dot manifold (Biorad, Richmond, CA). Total RNA corresponding to half an eye (7.25 μg) was applied to each 0.071 cm^2 dot. This quantity of RNA corresponds to half the binding capacity of the Nytran membrane for RNA.

Prehybridization was carried out for 2 h and hybridization overnight. Both were performed at 45 °C in 50 % formamide, 5 × SSPE (1 × SSPE is 0.18 mol l^{-1} NaCl; 10 mmol l^{-1} sodium phosphate, pH 7.7; 1 mmol l^{-1} EDTA), 5 × Denhardt's solution (0.1 % Ficoll; 0.1 % polyvinylpyrrolidone; 0.1 % bovine serum albumin), 1 % SDS and 100 μg ml^{-1} denatured heterologous genomic DNA. Blots were probed with the full-length zebrafish IRBP cDNA (ZF9) labeled with [^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983). Blots were hybridized with 106 disints min^{-1} of radiolabeled probe per milliliter of buffer. Following hybridization, blots were washed twice at room temperature in 6 × SSPE, 0.5 % SDS for 15 min, twice at 37 °C in 1 × SSPE, 0.5 % SDS for 15 min, and finally for 1–2 h at 65 °C in 1 × SSPE, 0.5 % SDS for 90 min. In some experiments, the blots were stripped (5 mmol l^{-1} TrisCl, pH 8.0; 0.2 mmol l^{-1} EDTA; 0.05 % pyrophosphate; 0.1 × Denhardt's reagent for 2 h at 65 °C) and reprobed with an 18S mouse ribosomal cDNA provided by Dr Ramared Guntaka (University of Missouri, Columbia, MO). Blots were exposed against X-AR film (Kodak, Rochester, NY) with an intensifier screen at –80 °C. For quantitative analysis, a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) was used to measure the radioactivity directly from the northern electrophoretic blot or dot blot.

In situ *mRNA hybridization*

For *in situ* hybridization, a probe containing the entire coding region of zebrafish IRBP was prepared. The full-length cDNA (1824 bp) was generated by reverse transcriptase–polymerase chain reaction and cloned into the pCR™II vector. Plasmids were linearized with *Not*I to generate a digoxigenin-labeled cRNA sense probe from the Sp6 promoter and with *Bam*HI to generate an antisense probe from the T7 promoter, by *in vitro* transcription using the Genius System (Boehringer Mannheim, Indianapolis, IN). Whole zebrafish eyes were fixed in 4 % (w/v) paraformaldehyde in 0.1 mol l^{-1} phosphate buffer containing 5 % (w/v) sucrose for 30 min, then bisected along the dorsal/ventral axis and fixed for an additional 30 min (Barthel and Raymond, 1990). The fixed hemispheres were cryoprotected in buffered 20 % (w/v) sucrose overnight, then frozen in a 2:1 mixture of buffered 20 % sucrose/OCT embedding medium (Tissue Tek, Elkhart, IN). 3–5 μm sections were rehydrated, digested with 10 μg ml^{-1} proteinase K, treated with 0.1 mol l^{-1} triethanolamine containing 0.25 % (v/v) acetic anhydride, and dehydrated. Sections were then hybridized overnight at 56 °C with 4–10 μg ml^{-1} cRNA in hybridization solution containing 50 % (v/v) formamide.

For some experiments, opsin riboprobes were also used. Goldfish opsin cDNA clones GFrod, GFblu and GFuv correspond to the entire coding regions of rod opsin, and blue cone opsin and ultraviolet cone opsin, respectively, were the kind gifts of K. Nakanishi (Johnson *et al.* 1993) and F. Tokunaga (Hisatomi *et al.* 1996). These probes selectively hybridize to the appropriate zebrafish photoreceptor sub-types (Raymond *et al.* 1993). Combined *in situ* hybridization was carried out as described above, except that the hybridization solution contained either a combination of zebrafish IRBP cRNA and GFblu cRNA or zebrafish IRBP cRNA and GFuv cRNA (at 4 μg ml^{-1} each).

Following hybridization, the slides were treated with RNAase A and incubated with 1:2000 anti-digoxigenin–alkaline phosphatase (Boehringer-Mannheim) overnight at room temperature. The enzymatic color reaction was carried out overnight with 4-Nitroblue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Slides were coverslipped and viewed under 100 % glycerol with a Leitz Aristoplan microscope (Leica, Heerbrugg, Switzerland) under Nomarski optics. Photographic slides were digitized and printed using Adobe Photoshop (Adobe Photosystems, Mountain View, California) and a Kodak (Rochester, NY) dye-sublimation printer.

Results

Isolation and characterization of cDNA and genomic clones

The cDNA and genomic clones isolated in this study are summarized in Fig. 1. An unprocessed 6.2 kb IRBP cDNA was isolated by screening a zebrafish retinal cDNA library under reduced stringency with a goldfish IRBP cDNA (Wagenhorst *et al.* 1995). A 3′ cDNA fragment was isolated by RT-PCR. The 14 kb genomic fragment was isolated by screening a zebrafish genomic library with a 1.35 kb cDNA fragment of the 6.2 kb cDNA under high-stringency conditions. The genomic clone contains the entire IRBP gene. The lower portion of Fig. 1 summarizes the cDNA structure.

Northern analysis shows that the mRNA for zebrafish IRBP is 2.3 kb in size. The IRBP mRNA message was detected in the eye, but not in brain, skeletal muscle, liver or intestine (Fig. 2). The translated amino acid sequence (Fig. 3) was deduced from the unprocessed cDNA and RT-PCR cDNA. The size of the mRNA for IRBP from the ATG start codon through the poly(A) tail is 2323 bases, which is consistent with the northern blot data in Fig. 2. The translated sequence of the N-terminal 21 amino acids is characteristic of signal peptides (von Heijne *et al.* 1985):

*MAQ***ALVLLVSLLFFSNVA**HCN

The presence of this signal recognition peptide sequence indicates that this protein is inserted into the endoplasmic reticulum and may eventually be secreted. The two amino acids following the initiating Met have a net positive charge (shown in italic). These residues are followed by 15 hydrophobic residues (shown in bold). The final three polar amino acids

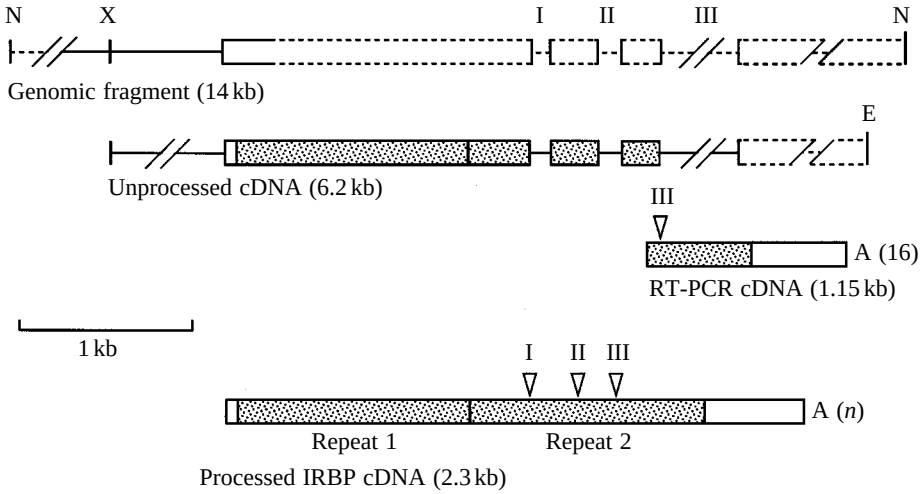

Fig. 1. Zebrafish genomic and cDNA clones. The upper three diagrams represent the cDNA and genomic clones isolated in the present study. The diagram at the bottom is the deduced 2.3 kb mRNA which consists of two modules. Arrowheads locate intron splice sites (I, II, III), which are restricted to the second module. Rectangles are exons; stippled regions are the coding sequences; dashed portions are unsequenced areas. Restriction enzymes: N, *Not*I; E, *Eco*RI; X, *Xho*I. A, poly(A) tail.

(underlined) define the end of the signal peptide. These three residues correspond in position to the pentapropeptide which often remains on human and monkey IRBPs secreted into the interphotoreceptor matrix (Fong *et al.* 1986; Redmond *et al.* 1986). Although it remains to be directly shown that zebrafish IRBP is secreted, it seems likely since goldfish IRBP is secreted in a Brefeldin-A-dependent manner (Wagenhorst *et al.* 1995).

The translated amino acid sequence predicts a 66.3 kDa protein composed of two homologous regions (modules). These regions, which have been referred to in the past as repeats, may be better described as modules (Patthy, 1995) because they are nonidentical and probably arose from a common ancestor. Two putative glycosylation sites are present in the second module (Fig. 3). The second of these two sites (NST) is in a region of high surface probability and is conserved among all the modules of human, bovine, rat, *Xenopus laevis*, goldfish and zebrafish IRBPs (Gonzalez-Fernandez *et al.* 1996; Liou *et al.* 1991). The first glycosylation site in zebrafish IRBP is conserved within the first module of bovine IRBP and the second module of goldfish IRBP. A third glycosylation consensus sequence is found in zebrafish IRBP at four amino acids preceding the stop codon. This site is not conserved in other IRBPs and is not contained in a hydrophilic region. The 3′-UTR is 439 bases in length and ends with a typical polyadenylation signal sequence (AAUAAA) at 17 bases before the poly(A) tail.

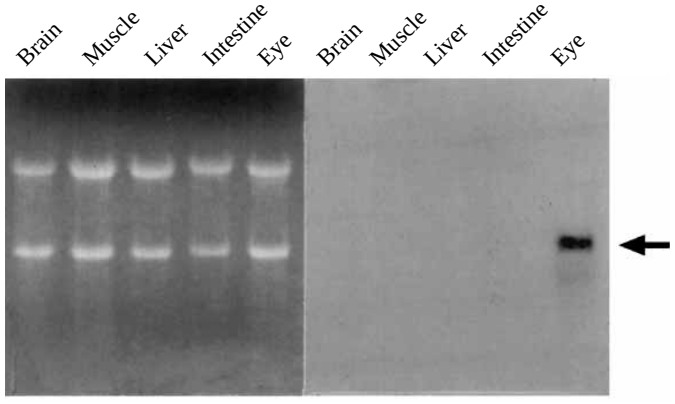

Fig. 2. Tissue distribution of the mRNA for IRBP. Left: ethidium-bromide-stained agarose gel showing the ribosomal RNA subunits. Right: autoradiogram of the northern blot of the same gel. The blot was probed with the zebrafish IRBP cDNA labeled with ^{32}P. The mRNA for zebrafish IRBP is approximately 2.3 kb in size (arrow). 9 μg of total RNA was loaded on each lane from brain, muscle, liver, intestine and eye. The Kodak XAR film was exposed at −80 °C for 51 h with an intensifier screen.

The primary structure of the zebrafish IRBP gene is significantly different from that of amphibian and mammalian IRBPs in that it is half the size, consisting of only two modules instead of four. The goldfish IRBP gene is similar to the zebrafish IRBP gene in having only two modules (Wagenhorst *et al.* 1995). The amino acid sequences of the two zebrafish IRBP modules are approximately 300 amino acids in size and have a 55 % similarity. Between zebrafish and goldfish, the amino acid sequence of the first IRBP module is 92 % similar and that of second module is 88 % similar.

The smaller size of teleost IRBP can be explained by phylogenetic loss of the middle two modules. When zebrafish and human IRBPs are compared, the highest similarity is found between the first and the fourth modules of both proteins (Table 1). In sequencing the unprocessed zebrafish IRBP

Table 1. *Comparison of the modules of zebrafish and human IRBPs*

		Human			
		H1	H2	H3	H4
Zebrafish	Z1	70	56	53	57
	Z2	56	56	51	69

The translated sequence of zebrafish and human IRBPs was used to calculate the percentage of amino acids that are the same between the various modules of zebrafish and human IRBPs. Note that the first and second modules of zebrafish IRBP are most similar to the first and fourth modules of human IRBP, respectively; marked by underlining.

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atggctcaagctctagtcctactagtgtcgttgctgttcttcagtaatgtggcgcactgtaacttctct
 M  A  Q  A  L  V  L  L  V  S  L  L  F  F  S  N  V  A  H  C  N  F  S
cccacacttattgcggacatggcaaaaatattcatggacaactactgctcgccagaaaaactcaccggg
 P  T  L  I  A  D  M  A  K  I  F  M  D  N  Y  C  S  P  E  K  L  T  G
atggaagaagccatagatgctgcaagcagcaacacagagatcctcagtatttcagatcccatcatgctt
 M  E  E  A  I  D  A  A  S  S  N  T  E  I  L  S  I  S  D  P  I  M  L
gccaacgttctgactgatgggggttaaaaaaacaatcagcgattccagagtgaaagtcacatacgagcca
 A  N  V  L  T  D  G  V  K  K  T  I  S  D  S  R  V  K  V  T  Y  E  P
gatttgattcttgcggcacctccagccatgcccgacatccctctggagcatctggccgcaatgatcaaa
 D  L  I  L  A  A  P  P  A  M  P  D  I  P  L  E  H  L  A  A  M  I  K
ggcacagtcaaagtggagatcctggaaggcaacattggctacctgaagattcagcacatcattggggag
 G  T  V  K  V  E  I  L  E  G  N  I  G  Y  L  K  I  Q  H  I  I  G  E
gagatggctcagaaagtgggacctcttttgctggagtacatctgggataaaatcctcccgacctctgcc
 E  M  A  Q  K  V  G  P  L  L  L  E  Y  I  W  D  K  I  L  P  T  S  A
atgattctggacttccgtagtacggttaccggcgagctatctggaatcccatacattgtgtcctacttc
 M  I  L  D  F  R  S  T  V  T  G  E  L  S  G  I  P  Y  I  V  S  Y  F
actgatcctgagcctctgatccatatcgattctgtgtacgatcgcactgctgatctcactatagagctg
 T  D  P  E  P  L  I  H  I  D  S  V  Y  D  R  T  A  D  L  T  I  E  L
tggtcaatgccaaccttgttgggaaagagatacggcacctccaaaccactgatcatcctaacaagcaag
 W  S  M  P  T  L  L  G  K  R  Y  G  T  S  K  P  L  I  I  L  T  S  K
gacactcttgggatcgcagaagatgttgcgtattgtcttaaaaaccttaaaagggctaccatcgttggg
 D  T  L  G  I  A  E  D  V  A  Y  C  L  K  N  L  K  R  A  T  I  V  G
gagaacactgctgggggaactgtgaaaatgagcaaaatgaaggtgggcgatactgacttctatgtgact
 E  N  T  A  G  G  T  V  K  M  S  K  M  K  V  G  D  T  D  F  Y  V  T
gtgcctgttgccaagtctatcaacccaatcactggtaaaagctgggaaatcaatggagttgctccagat
 V  P  V  A  K  S  I  N  P  I  T  G  K  S  W  E  I  N  G  V  A  P  D
gtcgacgttgctgcagaagatgcccttgacgctgcaattgcaatcattaaacttcgtgcagaaattcca
 V  D  V  A  A  E  D  A  L  D  A  A  I  A  I  I  K  L  R  A  E  I  P
gcattggctcaggcagccgccacgctgattgcagacaactatgcatttccgagcatcggggaacatgtc
 A  L  A  Q  A  A  A  T  L  I  A  D  N  Y  A  F  P  S  I  G  E  H  V
gcggagaagctggaagctgttgtggccggtggagaatacaacctgatctccacaaaagaagacttggaa
 A  E  K  L  E  A  V  V  A  G  G  E  Y  N  L  I  S  T  K  E  D  L  E
gaaagactctccgaggatctcctaaaactgtcagaggacaagtgtctgaagaccaccagcaacatccct
 E  R  L  S  E  D  L  L  K  L  S  E  D  K  C  L  K  T  T  S  N  I  P
gcacttcctccaatgaaccccacacccgagatgttcatcgcgctcatcaaaagttccttccaaactgat
 A  L  P  P  M  N  P  T  P  E  M  F  I  A  L  I  K  S  S  F  Q  T  D
gttttgaaaacaatattggctaccttcgctttgacatgtttggggacttcgagcatgttgcgaccatt
 V  F  E  N  N  I  G  Y  L  R  F  D  M  F  G  D  F  E  H  V  A  T  I
gctcagatcatagtggaacatgtctggaacaaagtggtggacactgacgcactgatcattgatctgaga
 A  Q  I  I  V  E  H  V  W  N  K  V  V  D  T  D  A  L  I  I  D  L  R
aataacatcggtgggcatgcctcctccattgctggcttttgctcctatttctttgatgctgacaagcaa
 N  N  I  G  G  H  A  S  S  I  A  G  F  C  S  Y  F  F  D  A  D  K  Q
attgttttagaccacatctatgacagaccctccaattccacacgagatcttcagaccctgaacagctc
 I  V  L  D  H  I  Y  D  R  P  S  N  S  T  R  D  L  Q  T  L  E  Q  L
acaggcaggagatatggcagcaagaaaagtgtggtaatcctcaccagtggtgtgaccgctggtgccgct
 T  G  R  R  Y  G  S  K  K  S  V  V  I  L  T  S  G  V  T  A  G  A  A
gaagagtttgttttcatcatgaagaggttgggacgtgcaatgataattggcgagacgacccatggagac
 E  E  F  V  F  I  M  K  R  L  G  R  A  M  I  I  G  E  T  T  H  G  D
tgtcaacctccggaaaccttcgctgtgggcgaaacggacatcttcctttctattccaatcagccattca
 C  Q  P  P  E  T  F  A  V  G  E  T  D  I  F  L  S  I  P  I  S  H  S
gacactgctcagggtccatcttggagggtgctggcattgctcctcatatcccagtgcccgctggtgccg
 D  T  A  Q  G  P  S  W  R  V  L  A  L  L  I  S  Q  C  P  L  V  P
ccctcgacacagcaaagggcatgctcaacaagcatttttccggccaaaaataagttgttcatctgggcc
 P  S  T  Q  Q  R  A  C  S  T  S  I  F  P  A  K  N  K  L  F  I  W  A
tggaacggaagccaaatgatatagtgaatttagattgatggcatatcatgaagaggctgttataatgtg
 W  N  G  S  Q  M  I
tgtagaccaataaggtgatgtttagaagacacagtcaagccttcagttctatttctccattatttctta
agtgttcaatctaaagtatgcaatctctgagaaacagtcagtaaaagagaacgcaaataatctcacagt
gatcatttagaataggcctgttctgaacttgatatactgtataaataccagtgttgggaactattcctt
atgcacattaaaactcatttatgctcatctgtacagtacaagcatatcaacacgcttgtttcaaatgta
actcgacttcgcagtatatatttttgggtttttctgtaagagtgtggagttatactgaaactaaattat
gaagcgtaataaattccacatggtaactgtaaaaaaaaaaaaaaaa
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Fig. 3. cDNA and translated amino acid sequence of zebrafish IRBP. The coding sequence begins with a signal peptide 21 amino acids in length (bold letters with gray background). This is followed by two tandem homologous regions (modules) of 298 and 296 amino acids each (boxed). Two glycosylation consensus sites are present in the second module (white letters with black background). The introns are restricted to the second module (arrowheads). A typical polyadenylation signal (bold and boxed) is present 17 bases before the poly(A) tail. This cDNA sequence is available through the European Bioinformatics Information, GenBank and DDBJ Nucleotide Sequence databases under the accession number X85957 and sequence identification BRRNAIRBP.

clone, we found that the introns are in the second module (Fig. 4). Within the module, the intron positions correspond to that in mammalian IRBPs. The main difference is that the introns are smaller than the corresponding human introns. Taken together, these data suggest that the middle two modules of an ancestral four-modular IRBP were lost during the emergence of teleosts (see Discussion).

The alignment of the two modules of zebrafish IRBP with the four modules of human IRBP is shown in Fig. 5. The most striking finding is the presence of three highly conserved hydrophobic domains. These regions in the zebrafish sequence are virtually identical to the human and amphibian sequences *O*GY*O*R*O*D, *OOO*D*O*R and *OO*GE, where *O* represents a hydrophobic residue (F. Gonzalez-Fernandez *et al.*, 1993; Liou

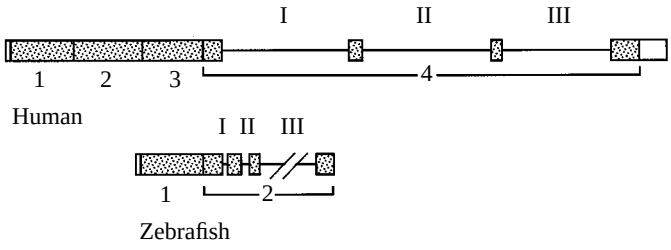

Fig. 4. Comparison of human and zebrafish gene structures. Both genes consist of homologous modules (four in human; two in zebrafish) of approximately 300 amino acids in length. In both species, the introns (I–III) are located at the same relative positions, splitting the last repeat. The first two introns are smaller in zebrafish. The last zebrafish intron is under study (interrupted line).

et al. 1991). Interestingly, the first two of these domains are within the second exon. The alignment shows that not all motifs previously noted in mammalian IRBP are conserved in zebrafish. For example, a stretch of five consecutive prolines in the third module of human IRBP is thought to provide a hinge allowing conformational changes associated with retinol binding (Adler *et al.* 1987). These pentaprolines were apparently lost in teleost fish during the recombination event which eliminated the central modules of an ancestral four-modular IRBP (see Discussion). Despite the loss of the pentaproline region, there appears to be some clustering of prolines near the beginning of both the first and second modules of zebrafish IRBP.

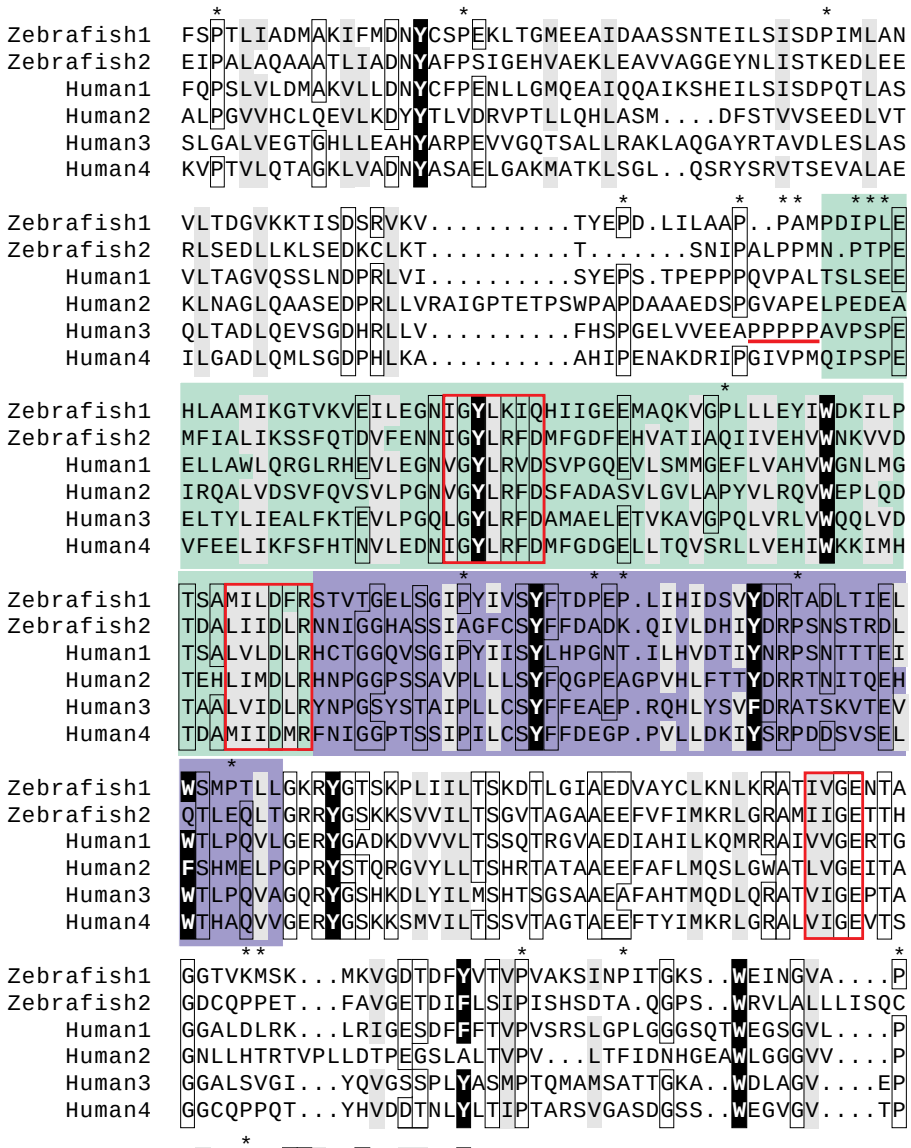

Fig. 5. Alignment of zebrafish and human IRBPs. The boundaries between the two modules of zebrafish IRBP and the four modules of human IRBP were determined using the program LALIGN. All the modules of both proteins were aligned using the program PILEUP (Wisconsin Genetic Package, GCG). Sequences corresponding to the second and third exons are highlighted in green and purple, respectively. Highly conserved regions are boxed in red (see text). A stretch of five consecutive prolines in the approximate center of human IRBP (at the beginning of the third module) is underlined in red. Prolines that are present at the same position in one zebrafish and one human module are marked with an asterisk. Chemically similar residues are indicated when five of the six were similar or identical: F=W=Y (black); I=L=V=M (gray); D=E=*N*=Q, S=T, A=G and R=K=H (boxed).

IRBP mRNA expression at noon and midnight

Northern blot and RT-PCR analysis

The expression of zebrafish IRBP mRNA was compared at mid-light and mid-dark. Fig. 6 shows a northern electrophoretic blot of total RNA extracted from whole eyes enucleated at mid-light and mid-dark of a 12 h:12 h light:dark cycle. Although the same quantity of total RNA was loaded onto the gel, the amount of IRBP mRNA is markedly higher at mid-light than at mid-dark. A lower amount of IRBP mRNA expression could usually be detected at mid-dark. This night-time expression probably corresponds to ultraviolet cone IRBP mRNA expression (see *in situ* hybridization studies below). The relative amounts of

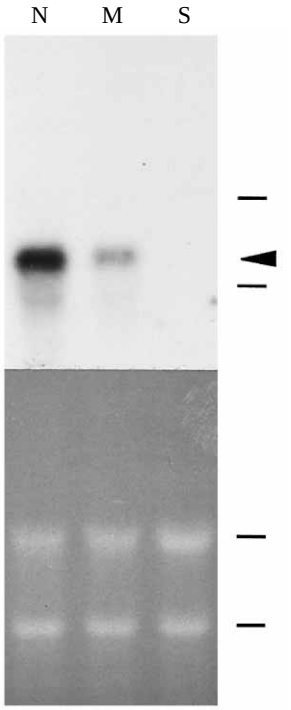

Fig. 6. Northern analysis of IRBP at noon (N) and midnight (M) under cyclic light. Top: autoradiogram of transfer, probed with ^{32}P-labeled full-length zebrafish IRBP cDNA ZF9. On the basis of the phosphoimage analysis, the radioactivity of the IRBP band (arrowhead) at noon is four times greater than at midnight. Bottom: ethidium-bromide-stained agarose gel (1% Seakem) showing the ribosomal subunits (dashes). 7 μg of total RNA was loaded in each lane. Lane S corresponds to skeletal muscle (negative control). The Kodak X-AR film was exposed for 20 h with an intensifier screen at –80 °C.

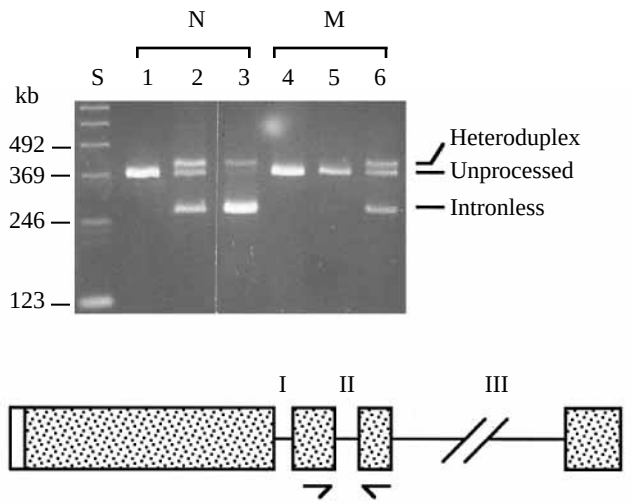

Fig. 7. RT-PCR analysis comparing unprocessed and intronless zebrafish IRBP mRNAs at noon (N) and midnight (M). Total ocular RNA was reverse-transcribed using random hexamers followed by amplification with PCR using primers flanking intron II. The presence or absence of this intron in the amplified message was confirmed by probing the Southern blot analysis of this gel with a labeled intron II oligomer. Lanes 1, 2 and 3 were transcribed from total ocular RNA taken from zebrafish killed at noon; and lanes 4, 5 and 6 were transcribed from total ocular RNA taken from zebrafish killed at midnight. The [$MgCl_2$] was 1.25 mmol l^{-1} (lanes 1 and 4), 3.13 mmol l^{-1} (lanes 2 and 5) and 5 mmol l^{-1} (lanes 3 and 6). At the bottom is a diagram of the zebrafish IRBP gene. Under the gene diagram, the locations of the PCR primers are designated. Lane S corresponds to DNA size standards.

processed and unprocessed IRBP message at mid-light and mid-dark by RT-PCR are compared in Fig. 7. The amount of amplified message depends on the [Mg^{2+}]. At a higher [Mg^{2+}], the intronless mRNA is preferentially amplified (lanes 3 and 6). A slower third band at the intermediate and high concentrations of Mg^{2+} is a heteroduplex cDNA. This interpretation is supported by Southern analysis, which showed that the unprocessed and heteroduplex bands, but not the processed cDNA, hybridize with an intron II oligonucleotide probe (data not shown). Such heteroduplexes are common during RT-PCR of alternatively spliced mRNAs (Zacharias *et al.* 1994). At the same [Mg^{2+}], the ratio of processed to unprocessed message is higher at noon than at midnight. This suggests that there is a daytime enhancement in the stability of the processed mRNA since the total amount of IRBP message is higher during the day than at night.

Localization of zebrafish IRBP mRNA expression

By the use of *in situ* hybridization, zebrafish retinal IRBP expression was localized to photoreceptors; however, the expression pattern seen in retinal tissue fixed at mid-light differed markedly from that in tissue fixed at mid-dark (Fig. 8). In mid-light retinas, IRBP cRNA hybridized strongly with the inner segments of all cone types and weakly with the inner segment myoids and perinuclear region of rod photoreceptors (Fig. 8A). This expression pattern is similar to that seen previously for the rod and cone opsins in fish; cone opsin mRNA is found exclusively in the myoid region of the inner segment, whereas rod opsin mRNA is localized to both the perinuclear region and the inner segment myoid (Raymond *et al.* 1993). Labeling of rod myoids is difficult to distinguish in the case of IRBP, since adjacent cone inner segments are also heavily labeled; an example of the rod opsin expression pattern is shown for comparison (Fig. 8B).

In retinas fixed at mid-dark, only one photoreceptor cell type appeared to hybridize with the IRBP riboprobe (Fig. 8C). This photoreceptor was identified as the short single ultraviolet cone on the basis of three lines of evidence. First, the hybridization signal was in cones with rounded nuclei that were located entirely within the outer limiting membrane. Short single ultraviolet cones are the only cone type with this nuclear position and morphology (Hisatomi *et al.* 1996). A more conclusive identification based on outer segment morphology is not possible because dark-adaptive photomechanical movements have repositioned the outer segments nearer to the retinal pigmented epithelium, where they cannot easily be assigned to the corresponding inner segment/cell body.

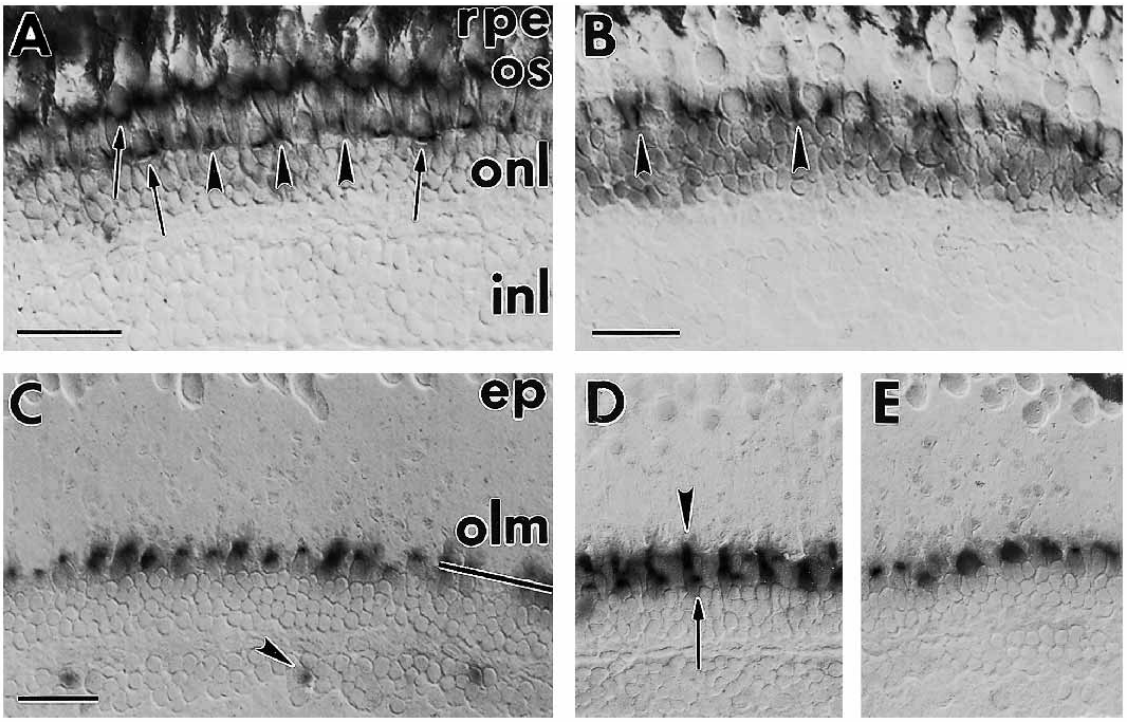

Fig. 8. *In situ* hybridizations of zebrafish IRBP and goldfish opsin cRNAs in zebrafish retinal cryosections. (A) Retina obtained at mid-light, hybridized with zebrafish IRBP cRNA. The reaction product is found in cone photoreceptor inner segment myoids (some examples are indicated by arrows), with weaker labeling of the perinuclear regions of rod photoreceptors in the outer nuclear layer (onl) and the rod myoids (some examples are indicated by arrowheads; compare with B). In this light-adapted retina, heavily pigmented processes of retinal pigmented epithelium (rpe) closely appose most of the cone outer and inner segments (os), but do not contact the outer segments of the short single (ultraviolet) cones, positioned immediately apical to the onl. inl, inner nuclear layer; scale bar, 25 μm. (B) Retina obtained at mid-light, hybridized with goldfish rod opsin cRNA. Reaction product is restricted to the rod perinuclear region and the rod myoids (two examples are indicated by arrowheads). Labeled rod myoids typically appear as narrow vertical bands of reaction product between the cone inner segments. Scale bar, 25 μm. (C) Retina obtained at mid-dark, hybridized with zebrafish IRBP cRNA. Reaction product is found only in one cone photoreceptor type; these cones have nuclei positioned within the outer limiting membrane (olm, delineated by the oblique line segment). In this dark-adapted retina, the retinal pigmented epithelial pigment has migrated basally (and does not appear in this photograph) and cone photoreceptors have elongated such that their outer segments are a substantial distance (50–80 μm) from the corresponding cone nuclei and are not in view. The bases of the refractile inner segment ellipsoids (ep) are seen at the top. Rare inner nuclear layer neurons also hybridize with zebrafish IRBP cRNA (indicated by an arrowhead). Scale bar (applies to C, D and E), 25 μm. (D) Retina obtained at mid-dark, hybridized with a combination of zebrafish IRBP cRNA and goldfish blue cone opsin cRNA. Two distinct populations of cone photoreceptors show reaction product in their inner segments (arrow and arrowhead each indicate a member of one of these cone populations). (E) Retina obtained at mid-dark, hybridized with a combination of zebrafish IRBP cRNA and goldfish ultraviolet cone opsin cRNA. Only one population of cones has hybridized and the pattern matches that of the IRBP cRNA alone (compare with C).

Second, the cones that hybridized with IRBP at mid-dark were regularly spaced. Blue and ultraviolet cones each form regular orthogonal networks in the zebrafish cone mosaic (Johnson *et al.* 1993). The third line of evidence was the most definitive for the identification of the cone types that persisted in generating detectable levels of IRBP mRNA in the dark (Fig. 8D,E). Retinal cryosections hybridized with a combination of IRBP cRNA and GFblu cRNA showed two distinct morphological types of labeled cells, suggesting that IRBP mRNA and blue cone opsin mRNA are not localized to the same cone cell type (Fig. 8D). In contrast, a combination of IRBP cRNA and GFuv cRNA generated a pattern indistinguishable from the pattern seen for IRBP alone (Fig. 8E). Furthermore, the IRBP/GFblu combination labeled twice as many cells as the IRBP/GFuv combination (39.6±1.6 cells 100 μm^{-1} compared with 20.8±2.6 cells 100 μm^{-1}). Taken together, these results indicate that IRBP mRNA is expressed primarily in the ultraviolet-sensitive short single cones in zebrafish retinas at mid-dark. In a small number of cases, a subpopulation of inner nuclear layer neurons was also observed to hybridize with the IRBP riboprobe in sections fixed at mid-dark (Fig. 8C), although hybridization to these cells was far less consistent than hybridization to cones.

Quantitative analysis of IRBP mRNA expression during LD, LL and DD

The expression of IRBP mRNA was measured during LD, LL and DD by northern dot blot analysis. In Fig. 9, the level of IRBP mRNA was measured every 3 h during LD. For each

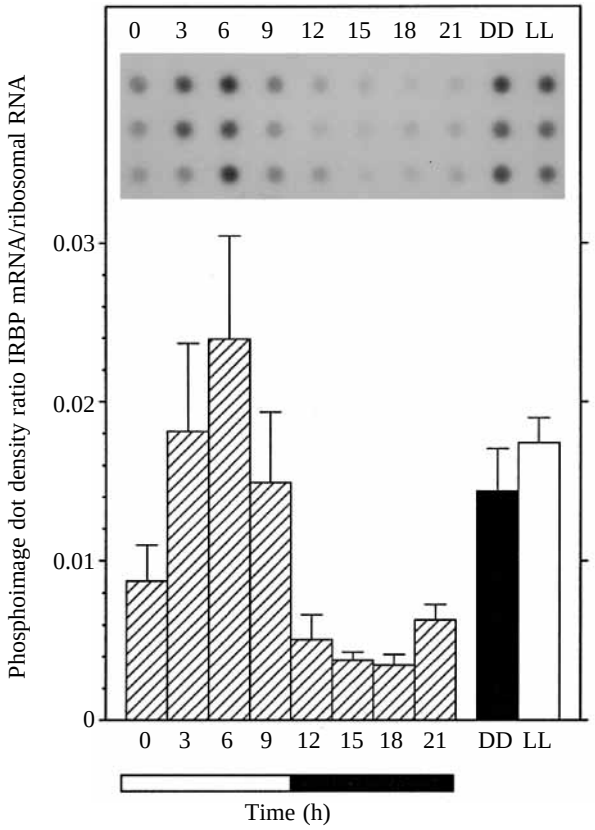

Fig. 9. IRBP mRNA expression under cyclic and constant conditions. Zebrafish were entrained under a 12 h:12 h L:D (06:30 h lights on/06:30 h lights off, LD) cycle and were killed at 3 h intervals beginning at light onset. Other zebrafish, maintained for 8 days in constant dark (DD) or constant light (LL), were killed at subjective noon. For each time point and constant condition, six pairs of eyes were separately extracted and dotted. The ratio of [^{32}P]IRBP cDNA hybridization to that of each dot reprobed with a [^{32}P]18S ribosomal RNA cDNA is compared in each experimental condition. The inset is a representative autoradiogram of dots probed with the IRBP cDNA. Bar under graph: open, light; filled, dark. Error bars are standard errors of the mean, *N*=6.

time point, six pairs of eyes were separately extracted and dotted. The expression pattern was sinusoidal, with a maximum at mid-light and a minimum at mid-dark. After 8 days of either LL or DD, the expression of the IRBP mRNA measured at subjective noon (Fig. 9) or midnight (data not shown) was intermediate between that at mid-light and mid-dark under LD. To characterize the expression of the IRBP mRNA further under constant conditions, we measured message levels throughout the day during LL and DD for an extended period (3 days in LL and 7 days in DD). Under both LL and DD, the mRNA rhythm persisted for 2 days (Fig. 10A,B). After this period, the rhythm dampened and the mRNA level drifted to the maximal LD peak level.

Discussion

The biochemical activity of IRBP depends on its ligand-binding properties and concentration in the interphotoreceptor matrix. Characterization of the ligand-binding sites of IRBP will be facilitated by the study of the simpler teleost protein. The concentration of IRBP in the matrix depends on both the rate of its introduction and the rate of its removal. In this study, we examined the structure and expression of the gene for IRBP in zebrafish. Our findings are twofold. First, the middle two modules of IRBP were lost during the evolution of the ray-finned fish (Actinopterygii). Second, the IRBP mRNA shows differential circadian expression among cone subtypes.

A remarkable feature of the structure of IRBP in mammals (reviewed in Pepperberg *et al.* 1993; Saari, 1994), amphibians (Gonzalez-Fernandez *et al.* 1993) and probably elasmobranchs (Bridges *et al.* 1986; Duffy *et al.* 1993) is that the protein is composed of four tandem homologous regions (modules). Each module is about 300 amino acids in length. The significance of this modular design is unknown, but may be related to the ligand-binding capacity of IRBP, since an individual module binds one equivalent of retinol (Baer *et al.* 1994). Unlike mammalian and amphibian IRBPs, which are composed of four modules, zebrafish and goldfish IRBPs consist of only two. This bimodular structure may be unique to teleost fish, in which the relative molecular mass of IRBP is 67.6±2.7 (Wagenhorst *et al.* 1995; Bridges *et al.* 1984, 1986). Two models could account for the bimodular structure of teleost IRBP. In the first, the IRBP gene duplicated between the emergence of teleosts and amphibians. This would place the final crossover duplication event relatively late in the evolution of vertebrates (just before amphibians). In the second model, two modules were lost during the emergence of the ray-finned fish (Actinopterygii).

Our data suggest that the bimodular structure of teleost IRBP arose through the loss of the middle two modules during the evolution of the Actinopterygii line (Fig. 11A). Like the mammalian gene, the zebrafish IRBP gene contains three introns which are present in the last module. Therefore, the second module of zebrafish IRBP corresponds to the fourth module of mammalian IRBP, the ancestral gene from which IRBP arose (Borst *et al.* 1989). The higher degree of homology between the first and last modules of zebrafish and human IRBPs (Table 1) is explained by an unequal crossover event deleting the middle two modules (Fig. 11B). Elimination of modules through homologous recombination is a common event in the evolution of complex proteins (Patthy, 1995). Finally, four-modular IRBP was likely to be present early in evolution, well before the emergence of Teleostei, since IRBP in stingrays (Bridges *et al.* 1986) and skates (Duffy *et al.* 1993) is similar in size to mammalian and amphibian IRBPs. Although it remains to be formally shown by molecular cloning, elasmobranch IRBP probably consists of four modules. It is generally believed that Chondrichthyes emerged from a primitive bony placoderm before the emergence of Osteichthyes (Sherwood Romer, 1966). Studies of more primitive representatives of the ray-finned fish (Actinopterygii), Sarcopterygii (the ancestors of amphibians) and jawless fish (Agnatha) will be critical to an understanding of how the IRBP gene evolved.

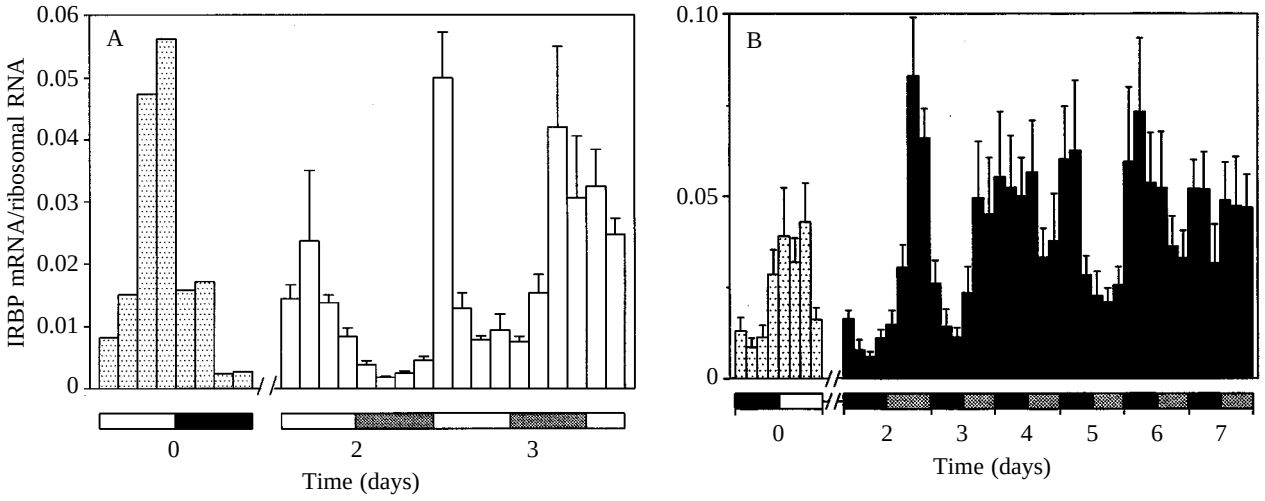

Fig. 10. Expression of the IRBP mRNA during constant conditions. Animals were cycled under 12 h:12 h light:dark (LD) for 2 weeks. On the last day of cyclic light, fish were killed every 3 h. Remaining fish were then exposed to constant conditions (constant light, LL; constant dark, DD). After 1 day of LL or DD, fish were killed every 3 or 4 h for the number of days shown. Error bars are standard errors of the mean. (A) Constant light. The stippled bars represent the mean value from fish during LD (*N*=2). The white bars represent the mean value from fish during LL (*N*=6). Horizontal bar under graph: open bar, light; filled bar, darkness; shaded bar, subjective night. (B) Constant dark. Stippled bars represent the mean value from fish during LD (*N*=4). The black bars represent the mean value from fish during DD (*N*=4). Horizontal bar under graph: open bar, light; filled bar, darkness; shaded bar, subjective day.

Each of the two zebrafish IRBP modules contains a highly conserved region. These regions may form the ligand-binding domains of the protein. Three hydrophobic regions containing arginine residues are conserved between *Xenopus laevis* and mammalian IRBPs (Gonzalez-Fernandez *et al.* 1993; Liou *et al.* 1991). These domains are also present in both modules of

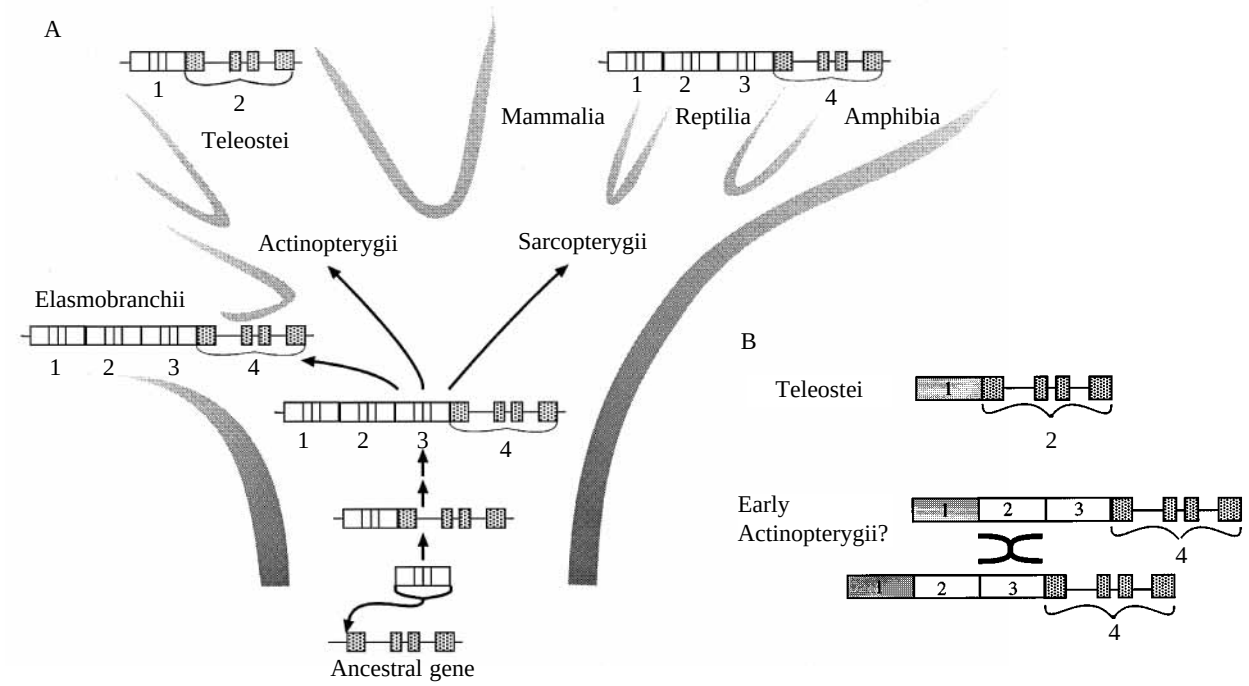

Fig. 11. Model of the evolution of IRBP. (A) IRBP probably arose from an ancestral gene through insertion of a processed gene followed by two unequal crossover events (Borst *et al.* 1989). Mammals, reptiles, amphibians and possibly Elasmobranchii (IRBPs from sharks, skates and rays have not been cloned) have such a four-module IRBP. At some point during the evolution of the Actinopterygii (which gave rise to modern teleosts), the middle two modules of IRBP were lost. (B) On the basis of the observation that the first and last repeats of zebrafish and human IRBPs are the most similar, we envision that during the evolution of the ray-finned fish (Actinopterygii) the middle two modules were lost from an ancestral four-module IRBP through an unequal crossover event.

zebrafish IRBP (Fig. 5). The presence of perfectly conserved arginine residues associated with these hydrophobic domains raises the possibility that they confer specificity for binding fatty acids and retinoic acid by providing an α-guanidinium group to stabilize the carboxyl moiety of these ligands (Cheng *et al.* 1991; Stump *et al.* 1991; Mancini *et al.* 1995). Sequences with a high degree of homology to all three domains are also found in two C-terminal processing proteases: CtpA, a C-terminal protease for the D1 protein of the photosystem II reaction center complex in the eukaryotic green alga *Scenedesmus obliquus* (Anbudurai *et al.* 1994), and Tsp, a tail-specific protease that selectively degrades proteins with nonpolar C termini in *Escherichia coli* (Silber *et al.* 1991). Another homology with the second IRBP domain has been observed in the juvenile-hormone-binding protein of the larvae of *Manduca sexta* (Palli *et al.* 1994). The signficance of these domains will be further understood as IRBP genes are characterized from more vertebrates and eventually from invertebrates.

Our second major finding is that the IRBP mRNA shows differential circadian expression among cone subtypes. The amount of IRBP message is seven times higher at the middle of the light period than at the middle of the dark period. Our RT-PCR data suggest that the enhanced expression represents increased message stability. However, we cannot rule out transcriptional regulation of the IRBP gene. The expression of IRBP mRNA is probably under circadian regulation since the rhythm persists for 2 days under LL or DD. Under constant conditions, the rhythm dampens and the expression of the mRNA appears to be up-regulated. A candidate effector for the circadian regulation of this gene is melatonin. Melatonin is secreted by the zebrafish retina during the night and, under constant conditions, melatonin secretion dampens over a similar time course to the increase in IRBP mRNA expression (Cahill, 1996). This raises the possibility that melatonin inhibits zebrafish IRBP mRNA expression. An alternative mechanism is that IRBP mRNA expression and melatonin synthesis are directly regulated by a circadian clock. A clock located in the photoreceptors (Cahill and Besharse, 1993) regulates the synthesis of melatonin through the transcriptional control of tryptophan hydroxylase (Green and Besharse, 1994; Green *et al.* 1995). The present finding of differential expression of IRBP among cone subtypes suggests that the clock may be restricted to specific photoreceptor classes.

Daytime up-regulation of IRBP mRNA expression is consistent with the role of the protein in the visual cycle. During light, IRBP transports all-*trans* retinol and 11-*cis* retinal between the photoreceptors and the pigmented epithelium while protecting these retinoids from degradation. The circadian expression of IRBP mRNA suggests that much of the protein produced each day is turned over by the following day. Coordination between degradation and synthesis is seen in other photoreceptor proteins (Organisciack *et al.* 1991; Farber *et al.* 1991; Brann and Cohen, 1987), particularly opsins. Opsin and iodopsin mRNA levels rise in the early morning and late afternoon, respectively, to precede disc assembly and shedding of outer segment tips (Korenbrot and Fernald, 1989; Pierce *et al.* 1993). To understand why zebrafish IRBP mRNA expression is elevated during daytime, the kinetics of the turnover of the protein need to be determined.

The rhythmicity of IRBP mRNA expression does not appear to be universal. In *Xenopus laevis*, where a photoreceptor clock has been directly demonstrated (Cahill and Besharse, 1993; Green and Besharse, 1994; Green *et al.* 1995), IRBP mRNA expression has little or no rhythmicity (Green and Besharse, 1995). The expression of the mRNA for IRBP is not rhythmic in the goldfish retina (Rajendran *et al.* 1995). These studies, which relied only on northern blot analysis, do not rule out the possibility that photoreceptor subtypes express IRBP rhythmically in these retinas. Alternatively, the lack of IRBP mRNA rhythmicity may reflect important differences in the mechanisms by which the retinas of different species regulate the concentration of IRBP in the subretinal compartment. For example, the control of the secretion of IRBP may be more important than the regulation of IRBP mRNA expression in species where IRBP message levels do not change rhythmically.

A striking difference in the spatial pattern of IRBP expression in vertebrates studied to date is that in goldfish (like the zebrafish, a member of the cyprinid family) IRBP is expressed primarily in cones (Wagenhorst *et al.* 1995). In contrast, mammalian and amphibian IRBPs are expressed in both rods and cones (reviewed in Hessler *et al.* 1995). We cannot rule out the possibility that teleost rods and cones express different IRBP genes. Previous studies could not further define the photoreceptor subtypes responsible for IRBP mRNA expression because of the limitations of isotopic *in situ* hybridization. In the present study, through the use of digoxigenin *in situ* hybridization, we found that regulation of zebrafish IRBP mRNA expression as a function of light and dark is cell type-specific. IRBP mRNA expression was found in all cone myoids during daytime. In contrast, only ultraviolet-sensitive cones express IRBP mRNA during the night. The relative position of ultraviolet-sensitive cones to that of other cone types suggests a potential role for continuous IRBP expression in these cells. The ultraviolet cone is the shortest photoreceptor and, therefore, has reduced access to the apical surface of the pigmented epithelium. The continued expression of IRBP mRNA in ultraviolet cones in the dark might ensure an adequate local supply of retinoids and/or fatty acids.

The possibility that IRBP may be unique to vertebrates, which require extracellular retinoid transport for rhodopsin regeneration, emphasizes the need for a suitable system for genetic studies of this protein. Genetic approaches that have proved so useful in *Drosophila melanogaster* may soon become available for zebrafish. The characterization of circadian transcriptional regulatory domains in vertebrate genes has been hampered by the lack of an approach to measure gene transcription directly *in vivo.* Zebrafish transgenic technology combined with noninvasive reporter gene monitoring may provide a solution to this problem.

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