J Mol Evol (1995) 41:646–656

JOURNAL OF MOLECULAR EVOLUTION

Goldfish Cones Secrete a Two-Repeat Interphotoreceptor Retinoid-Binding Protein

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Received: 21 August 1994 / Accepted: 29 April 1995

Abstract. Vitamin A and fatty acids are critical to photoreceptor structure, function, and development. The transport of these nutrients between the pigment epithelium and neural retina is mediated by interphotoreceptor retinoid-binding protein (IRBP). IRBP, a 133-kDa (human) glycolipoprotein, is the major protein component of the extracellular matrix separating these two cell layers. In amphibians and mammals, IRBP consists of four homologous repeats of about 300 amino acids which form two retinol and four fatty acid-binding sites. Here we show that IRBP in teleosts is a simpler protein composed of only two repeats. Western blot analysis shows that goldfish IRBP is half the size (70 kDa) of IRBP in higher vertebrates. Metabolic labeling studies employing Brefeldin A taken together with in situ hybridization studies and the presence of a signal peptide show that goldfish IRBP is secreted by the cone photoreceptors. The translated amino acid sequence has a calculated molecular weight of 66.7 kDa. The primary structure consists of only two homologous repeats with a similarity score of 52.5%. The last repeats of human and goldfish IRBPs are 69.1% similar with hydrophobic regions being the most similar. These data suggest that two repeats were lost during the evolution of the ray-finned fish (Actinopterygii), or that the IRBP gene duplicated between the emergence of bony fish (Osteichthyes) and amphibians. Acquisition of a multirepeat structure may reflect evolutionary pressure to efficiently transport higher levels of hydrophobic molecules within a finite space. Quadruplication of an ancestral IRBP gene may have been an important event in the evolution of photoreceptors in higher vertebrates.

Key words: Interphotoreceptor retinoid-binding protein — Interphotoreceptor matrix — Vitamin A — Fatty acid-binding proteins

Introduction

The evolution of the visual cycle, the pathway responsible for rhodopsin regeneration, is characterized by increasing separation of the processes of light capture and chromophore reisomerization. In insects, photon capture generates a thermostabile metarhodopsin which regenerates by absorbing a second photon (Hardie 1986). In cephalopods, rhodopsin is contained in the rhabdomes and the retinochrome isomerase is restricted to the myeloid bodies of the inner segments. 11-*cis* and all-*trans* retinal are transported between the rhabdomes and myeloid bodies by retinal-binding protein (Hara and Hara 1967; Seki 1984; Molina et al. 1992). In vertebrates, the

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isomerase is located within the pigmented epithelium (reviewed by Saari 1994).

The separation of opsin and the isomerase necessitates the bidirectional exchange of 11-*cis* retinal and all-*trans* retinol across the interphotoreceptor matrix. This is accomplished by interphotoreceptor retinoid-binding protein (IRBP), a large (145 kDa in bovine) glycolipoprotein, the major protein component of the matrix. IRBP is secreted into the matrix by rods and cones (Gonzalez-Fernandez et al. 1984, 1992; Hollyfield et al. 1985a; Van Veen et al. 1986; Porrello et al. 1991; Carter-Dawson and Burroughs 1992; Yokoyama et al. 1992; Hessler et al. 1995) and removed from the matrix by phagocytic and nonphagocytic endocytic pathways (Hollyfield et al. 1985a; Payman et al. 1994). IRBP facilitates the diffusion of retinoids, promotes the release of 11-*cis* retinal from the pigmented epithelium and protects the isomeric and oxidation state of retinol (reviewed by Carlson and Bok 1992; Crouch 1992; Pepperberg et al. 1993).

IRBP may have a more general role in the transport of hydrophobic molecules, particularly fatty acids. Photoreceptors rapidly and selectively sequester docosahexaenoic acid, which is critical to outer segment structure (Rodriguez et al. 1991; Chen et al. 1992). Interestingly, IRBP carries endogenous fatty acids (Bazan et al. 1985), contains motifs reminiscent of fatty acid-binding proteins (Gonzalez-Fernandez 1993a) and tail-specific protease, an *E. coli* protease which degrades proteins with nonpolar C-termini (Silber et al. 1992). Furthermore, its binding is selective for docosahexaenoic (Chen et al. 1993).

Mammalian IRBP arose from the quadruplication of an ancestral gene (Borst et al. 1989; Liou et al. 1989; Fong et al. 1990). Is there an evolutionary advantage to IRBP's repeat structure? Why does bovine IRBP, which is composed of four repeats, bind only two equivalents of all-*trans* retinol (Fong et al. 1984; Saari et al. 1985; Chen et al. 1993)? One explanation is that two repeats participate in the binding of each ligand molecule. Phylogenetic studies will provide insights into the relationship between repeat number and ligand-binding stoichiometry and predict functional domains within repeats. Preliminary reports of this work have been presented in abstract form (Wagenhorst et al. 1992; Bukelman et al., 1993; Rajenderan et al. 1994).

Methods

Animals. Common goldfish (*Carassius auratus*) 3–4 inches in length were maintained for at least 2 weeks under a 12 h/12 h light-dark cycle before use. Prior to enucleation, goldfish were anesthetized in a 0.1% solution of 3-aminobenzoic acid (Sigma, St. Louis, MO) and euthanatized by decapitation.

Extraction of Interphotoreceptor Matrix. The soluble fraction of the interphotoreceptor matrix was extracted from dark-adapted goldfish retina as described previously (Gonzalez-Fernandez et al. 1984). Following enucleation, residual portions of attached conjunctiva and extraocular muscles were removed. The globes were then washed in chilled phosphate-buffered saline (PBS: 5 mM sodium phosphate, 150 mM NaCl, pH 7.4). An incision was made with a microscapel at the level of the pars plana of the ciliary body. The incision was extended circumferentially with micro scissors and the anterior chamber, lens, and vitreous were removed. Under chilled PBS containing freshly added protease inhibitors (phenylmethylsulfonylfluoride, 2 μM; EDTA, 2.5 mM; *N*-ethyl malamide, 0.125%) the neural retina was carefully detached from the eyecup using a fine glass hook and jeweler's forceps. The retinas were incubated in the PBS for 5–10 min on ice with occasional gentle rocking. The retina and cup were removed and matrix spun at 10,000*g* for 10 min at 4°C. The pellet, which consisted of small tissue fragments, was discarded and the supernatant was subjected to centrifugation at 35,000*g* for 1 h at 4°C.

Western Blot Analysis. Proteins from the cytosol or matrix fractions were precipitated with acetone at –20°C and collected by centrifugation at 20,000*g* at 4°C for 30 min. Proteins were treated with dithiothreitol and sodium dodecyl sulfate (SDS) and fractionated by polyacrylamide gel (6–15% gradiant or 10% continuous) in a discontinuous buffer system (Laemmli 1970). For Western blot analysis, proteins were electrophoretically transferred to nitrocellulose. Following transfer, the blot was washed in rinsing solution, TBS (150 mM NaCl, 27 mM KCl, 25 mM, Tris pH 8.0) containing 0.005% nonylphenyl-polyethylene glycol (Nonidet 40, Fluka) and 0.001% Tween 20. The blot was blocked for 2 h in rinsing solution containing 3% bovine serum albumin (BSA, fraction V, Sigma) and 3% powdered milk. This was followed by incubating 4 h in rabbit anti*Xenopus* IRBP serum (Hessler et al. 1995) at a 1:1,500 dilution in TBS containing 3% BSA. The blot was washed in rinsing solution and incubated with a horseradish-peroxidase-conjugated donkey antirabbit IgG at a 1:2,000 dilution in blocking solution and detected by chemiluminescence (Amersham, Arlington Heights,IL).

Metabolic Studies. Dark-adapted neural retinas were incubated at 22°C under dim red light in RPMI medium (GIBCO, Gaithersburg, MD) containing radiolabeled amino acids. As described in the figure legends for individual experiments, the retinas were incubated in the presence of matrix, without matrix, or perfused. For perfusion, 15 retinas detached under saline were transferred to a horizontal cylindrical chamber 1 cm in diameter, 4 cm in length, and closed at both ends by nylon mesh. Oxygenated medium containing 50 μCi [^{3}H]leucine/ml was pumped through the chamber at 1.5 ml/h. Fractions were collected on ice in tubes containing phenylmethylsulfonylfluoride. For experiments involving inhibition of secretion with Brefeldin A (Epicentre Technologies, Madison, WI), retinas washed of matrix were incubated for 2 h in vials containing RPMI with 44 μCi/ml [^{35}S]methionine with or without Brefeldin A. Proteins from the medium were precipitated with acetone, dissolved in Laemmli buffer with dithiothreitol, and subjected to SDS-PAGE (10% acrylamide) and fluorography.

Molecular Cloning. A λgt10 goldfish retina cDNA library (Cauley et al. 1989) was screened under low stringency as described by Gonzalez-Fernandez et al. (1993a) with a full-length human IRBP cDNA (Fong and Bridges 1988) labeled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein 1983). Bacteriophage plaques were immobilized on duplicate Nytran membranes (Schleicher and Schuell, Inc., Keene, NH), lysed by steam using the method of G. Struhl as described by Sambrook et al. (1989), and cross-linked to the membranes by ultraviolet irradiation. Prehybridization was carried out overnight at 42°C in 30% formamide, 1 M NaCl, 100 μg/ml denatured salmon sperm DNA, 1% SDS, 10 mM Tris pH 7.5. Hybridization was carried out overnight under the same conditions with 10^6 cpm/ml denatured probe. Following hybridization, the membranes were washed twice for 30 min in 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1% SDS at room temperature and twice at 50°C. Each wash was 30 min in duration.

The low-stringency screening strategy was successful in isolating a partial-length goldfish IRBP cDNA. An upstream fragment of the initial clone and subsequent clones isolated by high stringency were used to rescreen the library under high stringency. Filters were hybridized overnight at 42°C in 80 mM NaCl, 2 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid], pH 6.5, 50% formamide, 1% SDS, 100 μg/ml denatured salmon sperm DNA, with 10^6 cpm/ml of the goldfish IRBP cDNA fragment. The final wash stringency was 65°C, 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS, pH 7.0.

The filters were exposed to X-AR film at −80°C with an intensifying screen. All bacteriophage clones were purified to homogeneity through at least three platings. The recombinant λDNA was extracted from plaque-purified phage by the method of Chisholm (1989). The portions of the genomic clone which were of interest to the present study were identified by Southern blotting and subcloned into pBluescript (Stratagene). Clones were sequenced using the dideoxy chain termination method with synthetic oligonucleotide primers.

Northern Blot Analysis. Total RNA was extracted from dark-adapted goldfish neural retinas or whole globes by the method of Chomczynski and Sacchi (1987) as described by Gonzalez-Fernandez and Healy (1990). Glyoxal was used to denature RNA before electrophoresis in 1.0% Seakem GTG agarose (FMC, Rockland, ME). The RNA was transferred to Nytran paper (Schleicher and Schuell) and cross-linked by ultraviolet irradiation. Prehybridization was carried out at 42°C in 50% formamide, 5× SSPE (SSPE at a 1× concentration is 0.18 M NaCl; 10 mM sodium phosphate, pH 7.7; 1 mM EDTA), 5× Denhardt's solution (0.1% Ficoll; 0.1% polyvinylpyrrolidone; 0.1% BSA), 1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out overnight in the above buffer with 10^6 cpm/ml of goldfish IRBP cDNA labeled with [α-^{32}P]dCTP by the random primer method. Following hybridization, the 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, at 65°C in 1× SSPE, 0.5% SDS for 30 min, and exposed to X-AR film in the presence of an intensifier screen at −80°C.

In Situ *Hybridization.* Adult goldfish, entrained to a 12-h light/dark cycle for 2 weeks, were enucleated at mid-dark and mid-light. The eyes were immersed in 4% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M phosphate buffer at 4°C. After 1 h, the globes were bisected and fixed for an additional 7 h. After three washes in 0.1 M phosphate buffer, pH 7.4, the tissue was dehydrated through graded ethanols and embedded in diethylene glycol distearate (DGD) (Polysciences, Washington, PA). One-micrometer sections were cut with glass knives, floated onto water, and placed on Probe-On slides (Fisher). Sections were dried overnight at 37°C. Whenever possible, solutions were treated with 0.05% DEPC (diethyl pyrocarbonate; Sigma, St. Louis, MO) and autoclaved before use.

[^{3}H]antisense and sense transcripts were synthesized from the 1.6-kb goldfish IRBP cDNA from the T7 and T3 RNA polymerase promoters following linearization with *Sma*I or *Xho*I endonucleases, respectively. Prior to hybridization, probe size was reduced by alkaline hydrolysis with 0.2 M carbonate, pH 10.2, at 60°C for 20 min followed by neutralization with 3 M sodium acteate, pH 6.0, and 10% glacial acetic acid. Nonradioactive UTP and CTP were substituted with 25 μCi of 5,6-[^{3}H]-UTP (36.6 μCi/mmol; New England Nuclear, Boston, MA) and 25 μCi of 5 [^{3}H]-CTP (22.9 Ci/mmol; NEN) for each 20-μl reaction.

Diethylene glycol distearate was removed from sections with two 10-min changes of xylene and the tissue was rehydrated through RNAase-free graded ethanols to DEPC-treated water. Slides were then immersed in 100 mM Tris-HCl, pH 8.0, 50 mM EDTA at 37°C with 1 μg/ml protease K for 30 min. Slides were then dipped briefly in 0.1 M triethanolamine (Sigma), pH 8.0, and incubated for 10 min in triethanolamine containing 0.25% acetic anhydride. Following acetylation, slides were dipped twice in 2× SSC, dipped in DEPC-treated water, and dehydrated through graded alcohols and air dried. The hybridization solution contained 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1× Denhardt's, 500 μg/ml yeast total RNA, 500 μg/ml poly(A) RNA, and 1.0×10^7 dpm/ml of radiolabled probe. The slide with the tissue sections were paired off, placed face-to-face, and 160 μl of hybridization mixture was placed between each pair by capillary action. The slides were incubated in a humid chamber at 48°C for 15 h. Control and experimental conditions were carried out concurrently.

Following hybridization, slides were washed in 4× SSC for 30 min, in 2× SSC for 10 min, and incubated in 2× SSC containing 20 μg/ml RNAse A at 37°C for 30 min. After RNAse treatment, slides were washed for 1 min in 2× SSC at room temperature and then at 47°C for 30 min in 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC. Slides were dehydrated through graded ethanols and air dried. Slides were dipped in Kodak NTB-2 photographic emulsion (diluted 1:1 with water), and stored in light-tight boxes with dessicant at 4°C. The slides were exposed for 3–4 weeks and developed in Kodak Dektol developer (diluted 1:1 with water) for 2 min at room temperature, fixed for 5 min, and rinsed for 20 min in running water prior to counterstaining with hematoxylin and eosin.

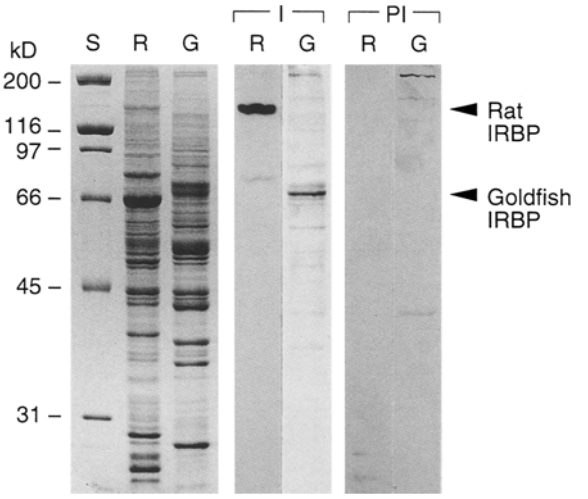

Fig. 1. Comparison of rat and goldfish IRBPs. Rat (*R*) and goldfish (*G*) interphotoreceptor matrix was extracted in PBS containing protease inhibitors and fractionated by SDS polyacrylamide (8%) gel electrophoresis. The Coomassie blue–stained gel is shown in the **left panel.** Identical unstained gels were transferred to nitrocellulose and probed with a rabbit anti*Xenopus* IRBP serum (*I,* **middle panel**) or the preimmune serum (*PI,* **right panel**). The secondary goat antirabbit IgG was conjugated to horseradish peroxidase and detected by chemiluminescence. *Arrows,* rat (144 kDa) and goldfish (70 kDa) IRBP bands; *lane S,* molecular weight standards.

Results

Biochemical and Metabolic Studies

The interphotoreceptor matrix is exposed by gently detaching the neural retina from the retinal pigmented epithelium. Previous experience with mammals and amphibians has shown that virtually all the IRBP in the matrix solubilizes in the saline. In Fig. 1 the size of rat and goldfish IRBPs are compared by Western blot analysis using an antiserum raised against the fourth repeat of *Xenopus* IRBP expressed in *E. coli*. The same

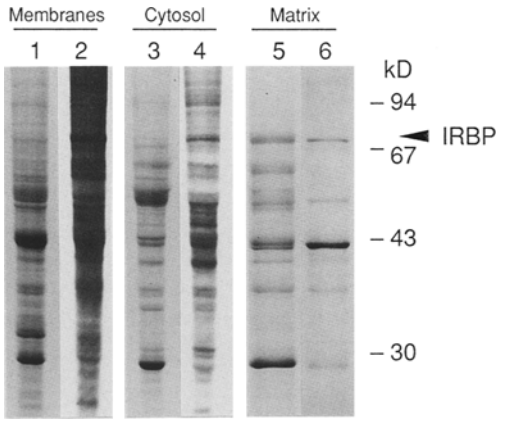

Fig. 2. Short-term organ culture. Unwashed neural retinas were incubated for 2 h in oxygenated RPMI media containing [^{3}H]leucine. Proteins within the membranes, cytosol, and incubation media were subjected to SDS polyacrylamide (10%) gel electrophoresis, stained with Coomassie blue (*lanes 1, 3, and 5*), and analyzed by fluorography (lanes 2, 4, and 6). A prominent radioactive band at 70 kDa corresponds to IRBP.

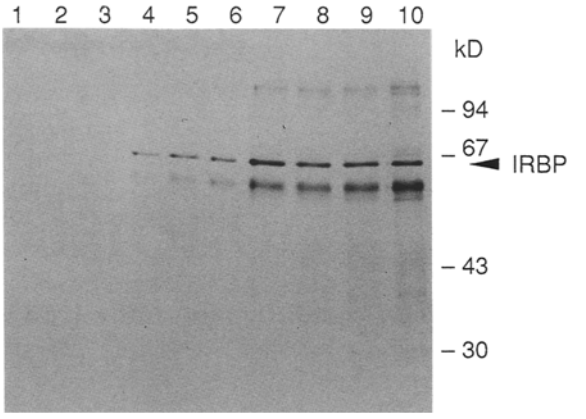

Fig. 3. Secretion of IRBP by perfused goldfish retinas. Isolated retinas were perfused for 10 h in oxygenated medium containing [^{3}H]leucine. *Numbers* above the fluorogram are the hours when the factions were collected. Proteins from each fraction were precipitated with acetone, redissolved in Laemmli buffer with DTT and subjected to SDS-PAGE (10% acrylamide).

result was obtained with an antibovine IRBP serum (data not shown). The M_r of rat IRBP is 144 kDa in agreement with Gonzalez-Fernandez et al. (1984, 1985). In contrast, the immunoreactive band in the goldfish matrix is only 70 kDa. Pre-immune serum failed to cross-react with IRBP from either species.

In Fig. 2, retinas were detached under RPMI medium to which [^{3}H]leucine was added. After 2 h, a prominent radiolabeled protein at 70 kDa was observed in the incubation medium. To obtain further evidence for secretion of IRBP, isolated retinas were perfused with RPMI medium containing [^{3}H]leucine (Fig. 3). A 66-kDa protein was the dominant radioactive band during the first 9 h of the perfusion. To confirm that this protein is secreted, retinas were incubated with and without Brefeldin A, an inhibitor of protein secretion. Secretion of the 66-kDa protein, but not overall protein synthesis nor the profile of radiolabeled proteins, was altered by the presence of the drug (Fig. 4). The smaller size (66 vs 70 kDa) of IRBP in the perfusion and Brefeldin A experiment compared to Figs. 1 and 2 may be due to partial proteolysis of the protein during these incubations since neither protease inhibitors nor the native matrix was present. Taken together, the Western blot analysis and metabolic labeling experiments suggest that an IRBP-like protein is secreted by the goldfish neural retina and that this protein is approximately half the size of IRBP in amphibians and mammals.

Molecular Cloning and mRNA Analysis

By low-stringency screening, we isolated a goldfish IRBP cDNA which is truncated at its 5′ end. An upstream fragment of this cDNA was used to rescreen the library under high-stringency conditions to obtain two nearly full-length clones. A 5′ fragment of one of these cDNAs was used to isolate GF17A, a full-length goldfish IRBP cDNA.

GF17A hybridizes with an mRNA in the neural retina but not in the cerebellum, brain, liver, or intestines (Fig. 5). The mRNA for goldfish and rat IRBPs is compared in Fig. 6. The goldfish message consists of a single transcript of approximately 2.5 kb. The rat mRNA consists of a major 5.2-kb transcript and a minor 6.4-kb mRNA. The longer size of the 6.4-kb transcript is due to 3′ untranslated sequence not contained in the major 5.2 kb mRNA (Gonzalez-Fernandez et al. 1993b). Expression of the mRNA for goldfish IRBP 6 h after light onset and 6 h after lights off was compared by Northern blot analysis and in situ hybridization. No significant difference in the amount of IRBP mRNA could be demonstrated by Northern blot analysis (Fig. 7). At these two time points, retinomotor movements change the morphology of the receptor cells. The diagram to the right of each set of photomicrographs in Fig. 8 depicts the relative position of the cones, rods, and melanin pigment granules within the pigmented epithelium processes. At both time points the cone nuclei lie in the outermost zone of the outer nuclear layer (ONL). During the night, the cone outer segments (COS) with their elipsoids (mitochondria rich portion of the inner segment) move toward the pigment epithelium and the rod outer segments (ROS) move toward the external limiting membrane. During the day, the myoid region of the cones contracts, bringing the ellipsoid and COS inward while the ROS moves outward between the villi of the pigment epithelium. These movements, combined with the migration of the pigment granules, are believed to optimize the positions of the cones and rods for photopic and scotopic conditions, respectively, and perhaps protect the rods from excessive light.

To maximize the spatial resolution of the in situ hybridization, we selected tritium as the isotopic label and

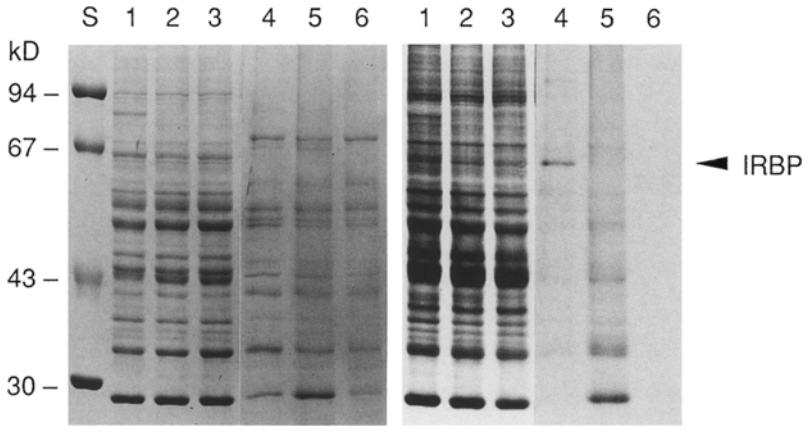

Fig. 4. Inhibition of secretion with Brefeldin A. Washed neural retinas were incubated for 2 h in oxygenated RPMI medium containing [^{35}S]methionine with or without Brefeldin A. **Left:** Coomassie blue–stained SDS gel (10% polyacrylamide) of the soluble proteins within the neural retinal *(lanes 1–3)* and corresponding incubation medium *(lanes 4–6)*. **Right:** Fluorogram of the same gel. *Lanes 1 and 4* (no drug), *lanes 2 and 5* (3 μM Brefeldin), *lanes 3 and 6* (7.2 μM Brefeldin).

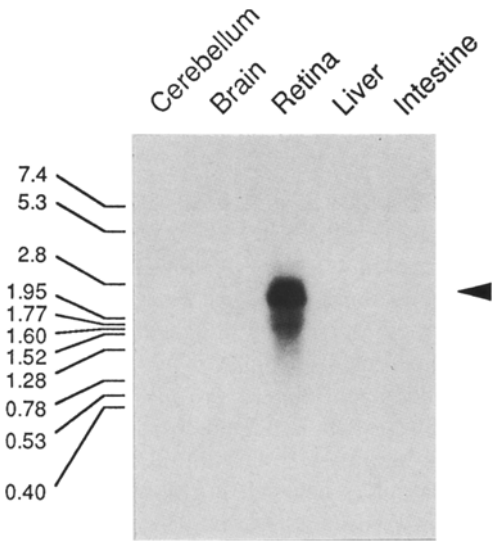

Fig. 5. Northern blot autoradiogram demonstrating the tissue distribution of the mRNA of goldfish IRBP. Eight micrograms of goldfish total RNA from the above IRBP tissues was fractionated through 1% Seakem agarose, transferred to Nytran paper and probed with the ^{32}P-labeled goldfish IRBP cDNA. The molecular weight calibration in kb is shown on the *left*.

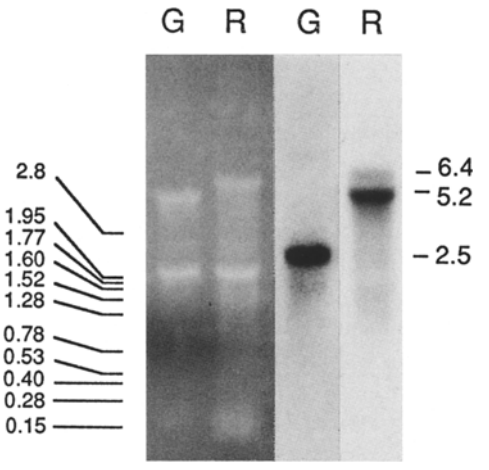

Fig. 6. Northern blot analysis comparing the mRNAs of goldfish and rat IRBPs. Eight micrograms of goldfish (*G*) or rat (*R*) neural retina total RNA was fractionated through 1% SeaKem agarose. In the **left panel,** the ribosomal subunits are visualized with ethidium bromide. The **right panel** shows the Northern blot autoradiogram prepared from the same gel. The blot was probed with a [^{32}P]labeled goldfish or rat IRBP cDNA in lanes *G* and *R*, respectively. The mRNA for goldfish IRBP is 2.5 kD; the major rat IRBP mRNA is 5.2 kb.

used 5-μm paraffin sections instead of frozen sections. Antisense transcripts were generated substituting both UTP and CTP with the corresponding radiolabeled nucleotide in order to enhance the specific activity of the probe. In both the light- and dark-adapted retinas, exposed silver grains were associated with the myoid region of the cone receptors. This inner segment region contains the endoplasmic reticulum. In the dark-adapted retinas the grains clustered at the myoid region of cone photoreceptors. This was particularly striking on sufficiently thin sections where isolated cone nuclei could be clearly identified (Fig. 8). In light-adapted retinas, the grains appeared concentrated in the myoid region compressed between the ellipsoids and cone nuclei. We could not determine which cone subtype(s) is responsible for IRBP synthesis. Future nonisotopic in situ hybridization studies may be able to define the relative contribution of different cone subtypes to IRBP synthesis. Our studies do not rule out a contribution of the rods in the synthesis of IRBP, especially if rod IRBP mRNA expression does not coincide with the two time points examined.

Sequence Analysis

The cDNA and translated amino acid sequence of goldfish IRBP is shown in Fig. 9. The N-terminus is characteristic of natural signal peptides (Von Heijne 1989):

Met-*Ser-Gln*-**Ala-Leu-Leu-Leu-Leu-Ala-Ser-Leu-Leu-Phe-Ser-Ser-Asn-Val-Ala-**
His-Cys-Asp

The two amino acids following the initiating Met have a net positive charge (italic). These residues are followed by 15 hydrophobic residues (bold). The final three amino acids are polar (underlined) and define the end of the signal peptide. These three residues correspond in position to the pentapropeptide often remaining on human

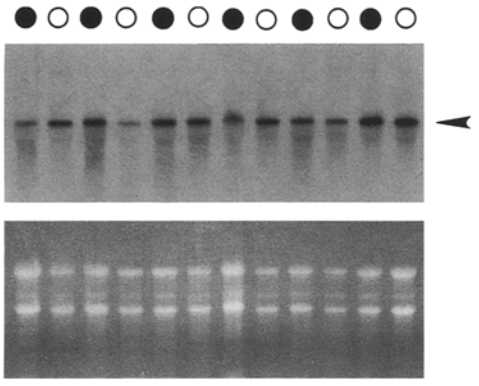

Fig. 7. Northern blot comparing level of the mRNA for goldfish IRBP (arrowhead) at mid-light (○) and mid-dark (●); 10 μg of total ocular RNA extracted from enucleated whole globes was electrophoresed in 1% agarose and stained with ethidium bromide **(bottom panel).** The same gel was transferred to Nytran paper and probed with a goldfish IRBP cDNA labeled with [^{32}P]dCTP and exposed to X-AR film in the presence of an intensifying screen for 12 h **(upper panel).**

and monkey IRBPs within the interphotoreceptor matrix (Fong et al. 1986; Redmond et al. 1986). Whether a propeptide exists on goldfish IRBP awaits amino acid sequencing.

The signal peptide is followed by 612 amino acid residues followed by a TGA stop codon. The computed molecular weight from the translated sequence is 66.7 kDa. This is consistent with the size of IRBP observed in the Western blot and metabolic labeling studies discussed earlier. The higher apparent molecular weight of goldfish IRBP (70 kDa) by SDS-PAGE (Fig. 1) may be due to glycosylation. Two putative glycosylation sites are present in the second repeat (Fig. 9). Goldfish IRBP is approximately half the size of human IRBP, 132.9 kDa (calculated from translated sequence of Si et al. 1989). The coding sequence is followed by 362 nucleotides of 3′ untranslated sequence. This sequence contains a single termination sequence (AATAAA) beginning 22 bases before the polyadenylation tail. The size of the cDNA (2,346 bases) agrees with the Northern blot studies in Figs. 5 and 6.

The program LALIGN demonstrated that goldfish IRBP is composed of two ho-mologous repeats (Fig. 10). The first (N-terminal) and second repeat consist of 298 and 293 amino acids, respectively. The second repeat is followed by 21 amino acids comprising the C-terminus. The amino acid sequence of the two repeats is 28% identical and 52% similar. The similarity scores between the repeats of human and goldfish IRBPs range from 52% to 70%. Interestingly, the highest similarity is between the first repeats of both proteins (68%) and the last repeats of both proteins (70%).

Discussion

The unique features of goldfish IRBP are that it is composed of two repeats and is secreted by the cones. In contrast, IRBP in amphibians and mammals is composed of four repeats and is secreted by both rods and cones. Loss of two repeats during the evolution of the ray-finned fish is the most probable explanation at present for the smaller size of IRBP in teleosts. Our observations suggest that two repeats are sufficient for the function of IRBP. Taken together with the known stoichiometry of bovine IRBP, we predict that two repeats bind one retinol equivalent.

Our immunological, metabolic labeling, and cloning studies indicate that both native and newly secreted goldfish IRBP are half the size of IRBP in higher vertebrates. The M_r of IRBP on SDS-PAGE is 140 kDa (human), 144 kDa (bovine and rat), and 124 kDa (*Xenopus*). The apparent differences in the M_r for IRBP among these species may represent different posttranslational modifications, particularly in glycosylation. The present data are consistent with early observations that the size of IRBP in teleosts is 67.6 ± 2.7 kDa (Bridges et al. 1984, 1986). We found no evidence for a high molecular weight precursor. Brefeldin A inhibits the secretion of the goldfish IRBP without affecting protein synthesis. This fungal isoprenoid antibiotic disrupts the dynamic membrane pathway between the endoplasmic reticulum and Golgi (Lippincott-Schwartz et al. 1991). For this effect, Brefeldin A has been useful in the study of post-Golgi trafficking of rhodopsin in the amphibian inner segment (Deretic and Papermaster 1991).

RNA analysis confirms the size of the goldfish gene and suggests that the protein is synthesized by the cones. By Northern blot, the mRNA for goldfish IRBP (2.5 kb) is about half the size of the major transcript for IRBP in rat (5.2 kb). This is further evidence that the smaller size of teleost IRBP is not a consequence of the cleavage of a pre-IRBP. In situ hybridization was performed on animals sacrificed at mid-light and mid-dark to take advantage of retinomotor movements. No significant difference in the level of the mRNA for IRBP was found as has been shown for opsin in the fish *haplochromis* and the toad *Bufo marinus* (Korenbrot and Fernald 1989). Since we examined only two time points, the possibility that the expression of IRBP is circadian cannot be excluded. At mid-light, clusters of exposed silver grains were associated with cone inner segments. At mid-dark, the grains appeared to be restricted to the myoid region of the cone inner segment compressed between the ellipsoid and nucleus. Although rods make up the majority of photoreceptors, a clear association of grains with their inner segments was never convincing. In contrast, the inner segments of both *Xenopus* rods and cones hybridize with antisense IRBP RNA (Hessler et al. 1993, 1995).

Low-stringency hybridization was used to isolate a full-length IRBP cDNA from a goldfish retinal cDNA library. This technique was previously used to isolate a cDNA for *Xenopus* IRBP (Gonzalez-Fernandez et al. 1993a) which led to the isolation of the full-length *Xenopus* IRBP cDNA (Van Niel et al. 1994). *Xenopus*

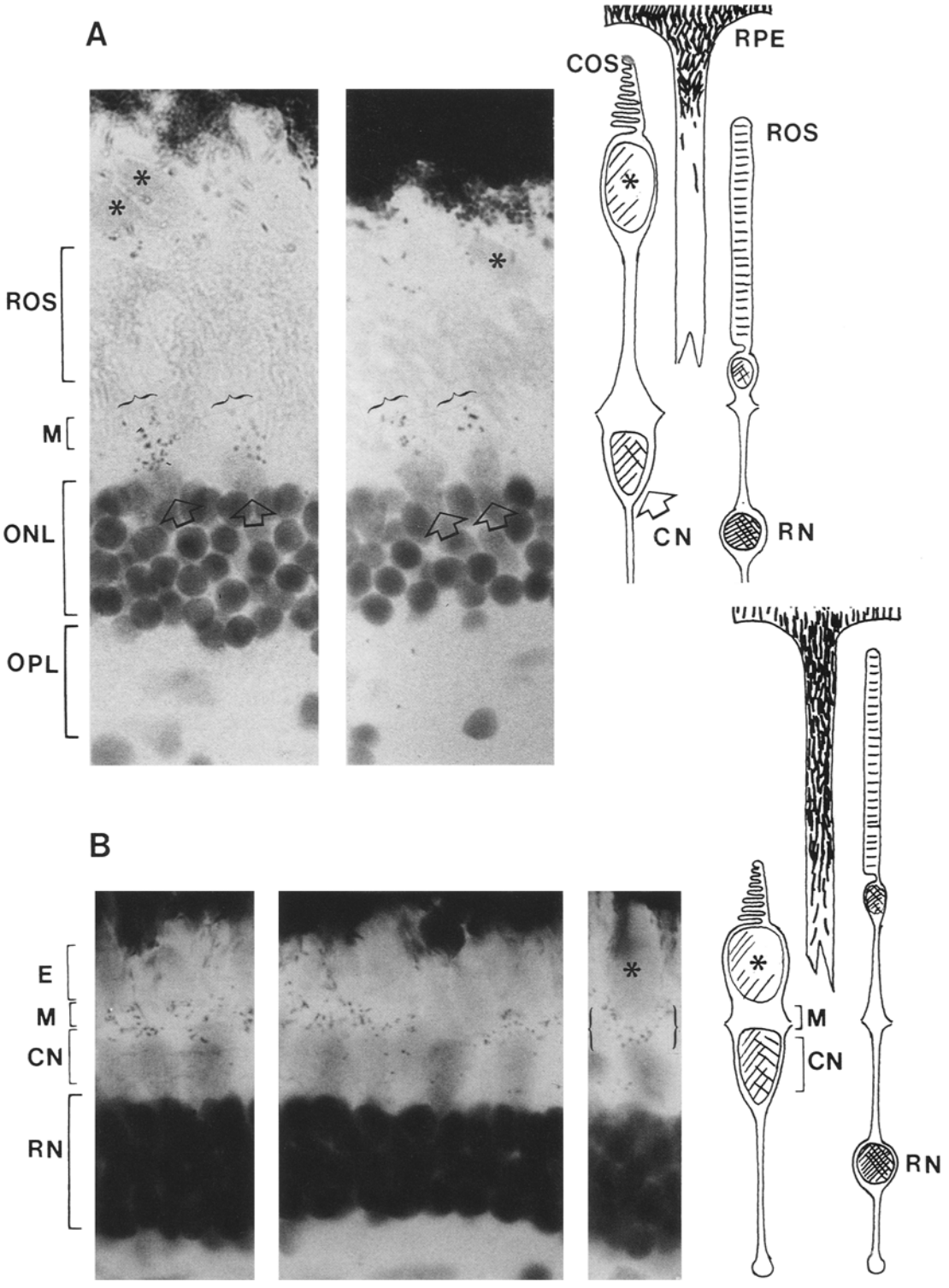

Fig. 8. In situ hybridization using [^{3}H]labeled antisense IRBP transcripts in dark- and light-adapted goldfish retina. Animals were killed at the same times of day as in Fig. 7. **A** Mid-dark: Cones extend placing their outer segments (*COS*) and ellipsoids (*) close to the retinal pigment epithelium (*RPE*); rods retract positioning their outer segments (*ROS*) and ellipsoids inward; melanin granules are sparse in the RPE apical villi. Clustering of silver grains (*brackets*) is apparent over the cone myoid region. Cone nuclei (*CN, arrows*), which are positioned in the outer zone of the outer nuclear layer (*ONL*), are larger than the more abundant rod nuclei. **B** Mid-light: Cones contract bringing the ellipsoid closer to the nucleus; rods extend; melanin granules move into the apical villi. Silver grains localize to the myoid region (*M, brackets* in **third panel**) now compressed between the cone ellipsoid and the nucleus. Silver grains were not present within other layers of the retina nor in any layer when sense transcripts were use (data not shown). *OPL,* outer plexiform layer.

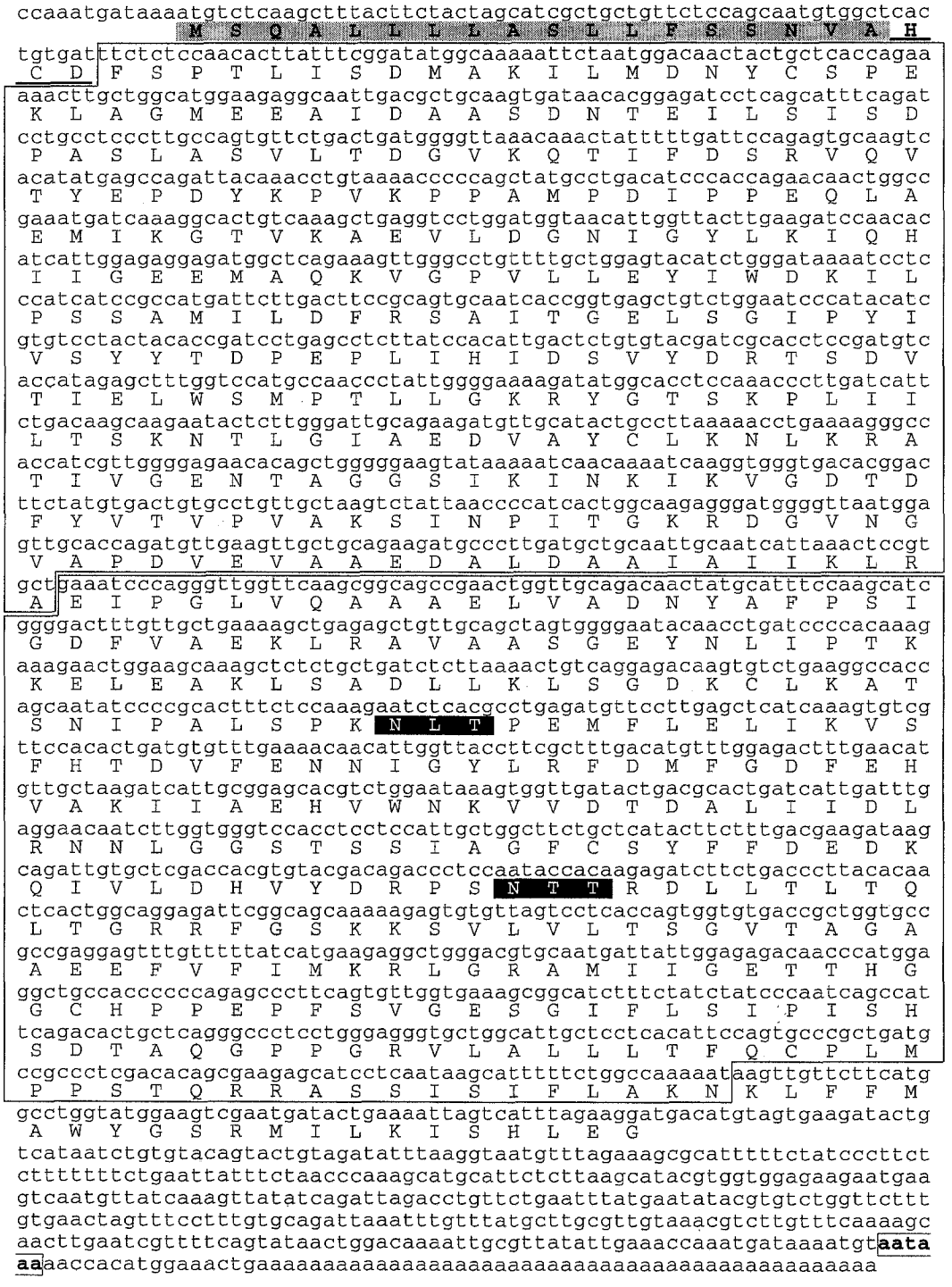

Fig. 9. Translated amino acid of goldfish IRBP. Goldfish IRBP begins with a secretion signal peptide followed by an open reading frame of 612 amino acids. Two glycosylation consensus sites are present in the second repeat. The coding sequence ends with TAG codon and is followed by 362 bases of 3′ untranslated sequence which ends in a typical signal termination consensus sequence 16 bases before a polyadenylation tail. The translated amino acid sequence has a calculated molecular weight of 66.7 kDa (not including signal peptide). This sequence is available through the EMBL Data Library under accession number X80802.

IRBP contains the four-repeat structure typical of mammalian IRBPs and its molecular weight calculated from the translated sequence is 132.7 kDa. The calculated weights of human and bovine IRBPs are 132.9 and 137.3 kDa, respectively. In contrast, goldfish IRBP contains only two repeats and is 66.7 kDa.

The model of Borst et al. (1989) accounts for the quadruplication of repeats with introns restricted to the fourth repeat. In this model, a processed gene was inserted within the 5′ end of an ancestral gene containing three introns. Two unequal crossovers subsequently gave rise to the four-repeat gene. Presumably, a species may be found which contains an IRBP composed of only one repeat. It was suggested that retinal-binding protein in

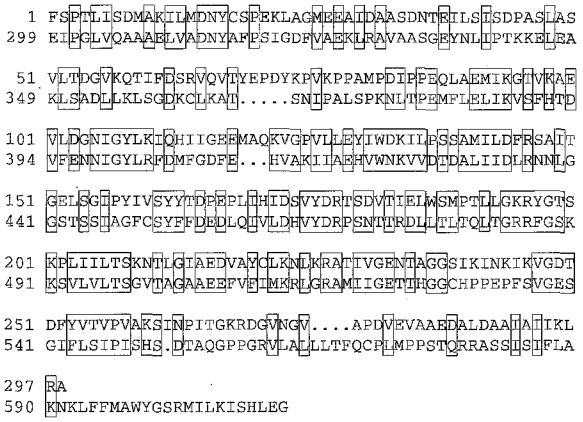

Fig. 10. Internal homology in primary structure of goldfish IRBP. The first (N-terminal) and second repeats are aligned in the upper and lower sequences, respectively. Chemically similar residues are *boxed* (LIVM, KHR, DENQ, ST, AG, and FWY).

cephalopods might represent the ancient one-repeat protein because of its small size and cross-reactivity with antibovine IRBP serum (Fong et al. 1988). However, cloning studies could not find any sequence in common between the squid retinal-binding protein and IRBP (Ozaki et al. 1994). Furthermore, we found no homology between goldfish IRBP and squid retinal-binding protein even when phylogenetically conserved regions within IRBP were used in the comparison. It therefore remains to be determined if a modern species exists which utilizes an IRBP composed of only one repeat.

Of animals studied to date, the two-repeat structure is unique to teleosts. How did teleosts obtain a two-repeat IRBP? Perhaps the two unequal crossovers proposed by Borst et al. (1989) occurred between the emergence of fish and amphibians. However, IRBP in stingrays and skates (Elasmobranchii) probably consists of four repeats since its size is 140 kDa (Bridges et al. 1986; Duffy et al. 1993). Since Chondrichthyes emerged from a primitive bony placoderm (Sherwood Romer 1966), the four-repeat IRBP in modern Elasmobranchii would represent convergent evolution. A more plausible model is that two repeats were lost during the evolution of the ray-finned fish (actinopterygii) before the emergence of teleosts. Studies in progress in our laboratory will distinguish between these two models by examining earlier members of the ray-finned fish, sarcopteryii (the ancestors of amphibians), and jawless fish (agnatha).

What is the physiological significance of IRBP's multirepeat structure? Gene duplication allows for enhancement of the same function or the acquisition of a new function by divergent mutations. Gene elongation, which commonly occurs through intragene duplication, is an important way complex genes evolve from simple ones. Other mechanisms for elongation are usually disruptive (Li and Graur 1991).Domain duplication enhances function by increasing the number of active sites (Creighton 1993). For example, the three-domain structure of trypsin inhibitor ovomucoid arose through two duplication events. Each domain binds one serine protease molecule.

There are several possible advantages to IRBP's multirepeat structure. Intragene duplication may have enhanced the protein's capacity to bind vitamin A. Consistent with this hypothesis is the finding that the fourth repeat of *Xenopus* IRBP possesses vitamin A binding activity (Baer et al. 1994). In this regard it will be interesting to compare the binding capacity of four domain IRBPs with teleost IRBP. Another possibility is that the larger size of IRBP may help to confine it to the subretinal space. Bunt-Milam et al. (1985) found that bovine IRBP is too large to diffuse from the matrix sclerad due to the smaller pore radius of the zonulae adherens that separate photoreceptors from Muller cells. Among the proteins studied, those smaller than albumin (M_r—67; Stokes's radius 36 Å) were able to diffuse through the zonulae adherens. Although we do not know what the Stokes's radius of teleost IRBP is nor the pore size of the retinal zonulae adherens in the goldfish retina, it seems that teleost IRBP is close to the molecular-size cutoff. A one-domain IRBP would probably slip through the junctional complex. A final possible significance of the duplication event is that it may have allowed IRBP to gain new functions in addition to its retinoid transport properties. Consistent with this notion is the observation of Redmond and Nickerson (1992) that the first domain of IRBP does not bind vitamin A. Perhaps the first domain has a separate role, such as interaction with a cell-surface receptor or another yet-unknown function. Comparative studies exploring the relationship between IRBP's domains and its function will allow us to better understand the role of this major component of the interphotoreceptor matrix.

Acknowledgments. This work was supported by The Thomas F. Jeffress and Kate Miller Memorial Trust (F.G.-F); by grant #IN149H from the American Cancer Society (F.G.-F.); by a fellowship from the Howard Hughes Medical Institute (R.R.R.); by an unrestricted developmental grant from Research to Prevent Blindness; by Neuropathology Research Training Grant T32 NS 7236 of the N.I.H. (R.B.H.); and by a National Institutes of Health grant EY09412 (F.G.-F.).

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