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Interphotoreceptor retinoid-binding protein gene structure in tetrapods and teleost fish

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Purpose: The interphotoreceptor retinoid-binding protein (IRBP) gene possesses an unusual structure, encoding multiple Repeats, each consisting of about 300 amino acids. Our goals were to gain insight into the function of IRBP, and to test the current model for the evolution of IRBP, in which Repeats were replicated from a simpler ancestral gene.

Methods: We employed a bioinformatics approach to analyze IRBP loci in recently completed or near-complete genome sequences of several vertebrates and nonvertebrate chordates. IRBP gene expression in zebrafish was evaluated by reverse transcriptase PCR (RT-PCR) and in situ mRNA hybridizations with gene-specific probes.

Results: Patterns of exons and introns in the IRBP genes of tetrapods were highly similar, as were predicted amino acid sequences and Repeat structures. IRBP gene structure in teleost fish was more variable, and we report a new gene structure for two species, the Japanese puffer fish (Takifugu rubripes) and the zebrafish (Danio rerio). These teleost genomes contain a two-gene IRBP locus arranged head-to-tail in which the first gene, Gene 1, is intronless and contains a single large exon encoding three complete Repeats. It is followed by a second gene, Gene 2, which corresponds to the previously reported gene consisting of two Repeats spread across four exons and three introns. Each of the two zebrafish genes is transcribed. Gene 2 is expressed in the photoreceptors and RPE, and Gene 1 is expressed in the inner nuclear layer and weakly in the ganglion cell layer.

Conclusions: The tetrapod IRBP gene structure is highly conserved while the teleost fish gene structure was a surprise: It appears to be a two-gene locus with distinct Repeat organization in each open reading frame. This gene structure and gene expression data are consistent with possible neofunctionalization or sub-function partitioning of Gene 1 and Gene 2 in the zebrafish. We suggest that the two-gene locus in teleost fish arose as a consequence of either the known whole genome duplication or single gene tandem duplication.

Vertebrate interphotoreceptor retinoid-binding protein (IRBP) is the most abundant soluble protein found in the interphotoreceptor space (IPS) between photoreceptors and the retinal pigmented epithelium (RPE) [1]. It has been proposed that IRBP functions in this space to solve unique difficulties in maintaining retinoid isomerization and chemical form while retinoids cross back and forth between the RPE cell and the photoreceptor cell (PhR) [2,3]. This hypothesis is consistent with the corresponding lack of an IPS, the use of a different retinoid isomerization approach, and the absence of IRBP in invertebrates.

IRBP is expressed in developing PhRs, being turned on embryonically in the mouse at a low level [4]. Then, at birth, IRBP expression is markedly increased, and the mRNA for IRBP rapidly accumulates, preceding the activation of many photoreceptor-specific genes [5]. A similar pattern is found in bovine eyes [6]. In zebrafish, IRBP mRNA is detected in the retina at 50 h post fertilization (hpf) [7]. In zebrafish, the pattern of cell type expression is different from that of other vertebrates, with IRBP mRNA and protein detected in both PhRs and RPE cells [7]. In PhRs, IRBP mRNA precedes the expression of cone opsins and rod opsin [7]. This precocious expression of IRBP, before many vision-specific proteins, is consistent with a possible role for IRBP in developmental, cell survival, or maintenance in the visual system [8], although it may simply reflect differential gene transcription requirements [9,10].

It is not clear how IRBP functions in the visual system, although its retinoid [11-13] and fatty acid binding [14-16] properties are well characterized in experiments using human, bovine, and Xenopus material [14,15,17-23]. The absence of IRBP has no apparent deleterious effect (but see Pugh and Lamb [24]) on the rate of retinoid shuttling between the RPE and the photoreceptor cell, an essential process known as the Visual Cycle [8,25]. This is not to imply that IRBP knockout mice are normal. On the contrary, the absence of IRBP yields a visual system with reduced a-wave magnitude by electroretinography, corresponding proportionally to a histologically reduced thickness of the ONL. Initially, IRBP knockout mice appear to have half the a-wave signal and about half the number of rod photoreceptor cells [8,25]. The outer segments (OS) appear disorganized, and small vacuoles are found between OS [25]. These knockout mouse studies suggest a function for IRBP in the development and maintenance of the PhR.

The mammalian IRBP gene has an unusual gene structure [26,27]. This structure has provoked interest in its evolution from a predicted simpler ancestral gene implied by the
existence of distantly related protein family members. The sequence of the IRBP gene has been used to study the phylogeny of the vertebrates [28,29], and as a consequence, part of the gene sequence of IRBP is known in many (more than 600) species. In mammals, the IRBP protein contains four Repeats, each consisting of about 300 amino acids, and the three-dimensional (3D) structure of this unit, the 300 amino acid long Repeat has been solved [30,31]. A comparison of this structure to other 3D structures revealed that the IRBP Repeat is a member of a large family of proteins including enoyl-coenzyme A (CoA) hydratase [32], dienoyl CoA isomerase [33], 4-chlorobenzoyl CoA dehalogenase [34], and C-terminal protease [35]. The comparison of the primary sequences of IRBP Repeats and other family members shows weak but statistically significant similarities [36]. These family members are synthesized as monomeric polypeptides having only a single Repeat. These monomeric polypeptides can form quaternary structures of three or six polypeptides to provide a functional protein. Many of the family members act as enzymes that modify or digest hydrophobic molecules. It is not understood why there are four Repeats in a single mammalian IRBP polypeptide chain. Any enzymatic activity of IRBP remains unknown, but it probably does not include general protease activity [37] or activities with acyl-CoA substrates [38].

To begin to understand how IRBP functions, we sought to examine variably in the gene structure, with the rationale that if the gene structure varied widely, then the fundamental functional unit for vision within IRBP might correspond to a single part or subset of the full protein. Simpler IRBP orthologs might also highlight which of the protein components is the functional unit in the visual system.

An important concept in evolution [39] is the principle that following gene duplication, both copies of duplicate genes tend to be retained if multiple functions of the protein are subdivided between the duplicates. This partitioning of functions between the two genes is called sub-functionalization [40], and it is often detected in the teleost (bony) fish, which underwent a whole genome duplication (WGD) prior to or coincident with the great radiation of the teleost fish about 350 Mya [41,42]. Under this model, we hypothesize that two IRBP-like genes, each with a different gene structure, different promoter elements, and perhaps a different set of Repeat(s), would exist in teleost genomes. We would further expect a different spatiotemporal expression profile for IRBP gene duplicates. In this study, we sought to test the potential of sub-functionalization in the IRBP locus of teleosts by examining gene structure and putative differential gene expression.

Our approach was to employ bioinformatics to analyze recently completed or near-complete genomes of several tetrapods, teleost fish, and two nonvertebrate chordates. We compared gene structure, predicted protein structure, and used these comparisons to evaluate the current model for IRBP gene evolution [26].

Here we report a high degree of conservation of IRBP gene and protein structure among the tetrapods, but also report a new locus structure for the IRBP gene in two teleost fish: Japanese puffer fish (Takifugu rubripes) and the zebrafish (Danio rerio). These two species share a pattern of exons and introns differing from that predicted for the IRBP gene in the teleost fish. Furthermore, each species contains a two-gene locus, opening the possibility of sub-function partitioning or neofunctionalization [39]. Consistent with this hypothesis, we demonstrate differences in the temporal, spatial, and cell-type expression of the two different IRBP genes in zebrafish.

METHODS Identification and analyses of IRBP loci: Recently, the complete or nearly complete genome sequences of human (Homo sapiens), chimpanzee (Pan troglodytes), domestic dog (Canis familiaris), domestic cow (Bos taurus), mouse (Mus musculus), rat (Rattus norvegicus), opossum (Monodelphis domestica), chicken (Gallus gallus), the western clawed frog (Xenopus tropicalis), zebrafish (Danio rerio), pufferfish (Tetraodon nigroviridis), fugu (Takifugu rubripes), medaka (Oryzias latipes), and of two urochordates (Ciona intestinalis and Ciona savignyi), have become publicly available. In addition, partial genome information is available for Rhesus monkey (Macaca mulatta), African elephant (Loxodonta africana), domestic cat (Felis catus), domestic sheep (Ovis aries), and domestic pig (Sus scrofa). Expressed sequence tag (EST) profiles are available for goldfish (Carassius auratus), threespine stickleback (Gasterosteus aculeatus), and fathead minnow (Pimephales promelas). The public databases used in this study include GenBank (National Center for Biotechnology Information, Bethesda, MD), UCSC Genome Browser (UCSC Genome Bioinformatics Group, University of California, Santa Cruz), Wellcome Trust Sanger Institute (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK), the National Institute of Genetics, Mishima, Japan, and The Broad Institute, Cambridge, MA. GenBank accession or scaffold numbers for the sequences used in this article are provided in Table 1.

We analyzed IRBP gene orthologs in each of the above species with a combination of commercial software including MacVector (TM) versions 7.0 and 9.0 (Accelrys Software, San Diego, CA), and the Vector NTI suite (build 194, Invitrogen Corporation, Carlsbad, CA) implemented on a Macintosh (Apple Computer, Cupertino, CA) desktop computer, and several web-based packages (each of which is specified in the figure legends or the body of the text).

Cross-species identification of IRBP gene orthologs was based on TBLASTN searches [43] using the human IRBP amino acid sequence as the query. In most cases, the BLOSUM62 matrix and a cutoff E-value of 0.01 were employed. The gene sequence locus was bounded on 5' and 3' ends by identification of genes immediately upstream and downstream of IRBP (usually GDF2 and Annexin 8, respectively). If there were more short exons upstream or downstream of the bounding genes, these would not be considered in the analysis of the individual locus. The likelihood of large (or even small) IRBP gene fragments beyond the immediate vicinity of the present analyses seems unlikely as no other strong sequence similarities were found by Blast searches of nonredundant or species-specific genomic DNA or EST data-
All the sequences of the tetrapods are readily obtained by Blast searches of the public databases. Four of the fish (fugu, zebrafish, medaka, and Tetraodon) are similarly readily obtained from the latest versions of the whole genome sequences. The goldfish sequence was obtained from GenBank. We assembled a sequence from stickleback and fathead minnow sequences of several EST sequences, which are identified by accession number. We emphasize that we did not conduct the sequencing of stickleback or the fathead minnow ourselves.

bases. This is supported by previous Southern blot analyses suggesting that the IRBP gene is found in a single locus in all examined species [44].

The Pustell protein and DNA dot matrix programs from MacVector were used to map the approximate positions of intron-exon boundaries of newly identified IRBP genes. Closer inspection of the DNA sequences applying the consensus splice sites [45] were used to identify the precise boundaries. In most cases the splice site boundaries were confirmed by comparison to cDNA sequences from companion public databases. The AUGUSTUS program [46] was used to predict all protein encoding genes within IRBP loci. Pairwise amino acid sequence comparisons were performed using BLASTp of the Biology Workbench, version 3.2, implemented at the San Diego Supercomputer Center, University of California, San Diego, CA Workbench. Matches to subsequence motifs, such as glycosylation sites, were identified by ad hoc grep pattern searching in a text processor (BBEdit, version 7.1.4; Bare Bones Software, Inc., Bedford, MA).

Reverse transcriptase-coupled polymerase chain reaction: Reverse transcriptase-coupled Polymerase Chain Reaction (RT-PCR) was used to verify mRNA expression in zebrafish. Zebrafish were from the AB line (Eugene, OR; kindly provided by Dr. A. Fritz, Biology Department, Emory University). Embryos were raised at 28.5 °C, and maintained according to standard procedures in 14:10 light-dark cyclic lighting [47]. RNA from whole zebrafish larvae (96 h after fertilization) was isolated using a Trizol kit (Invitrogen). We used a Qiagen OneStep RT-PCR kit, employing a 50 µl final volume, and performed the reverse transcriptase (RT)-PCR am-
plifications according to the manufacturer’s instructions. Gene-specific primers (Table 2) were used at final concentrations of 0.5 µM, and Mg²⁺ was at 2.5 mM. Total RNA (100 ng) was heated for 3 min at 95 °C and rapidly cooled to 4 °C to reduce secondary structure, and then 1 µg RNA was added to the reaction mix. The reverse transcription reaction was conducted at 50 °C for 30 min. Thermocycling included a single initial heat inactivation and denaturation incubation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 45 s, 63 °C for 30 s, and 72 °C for 2 min. The final incubation at 72 °C was for 10 min to allow extension of partially completed PCR products. The sample was held at 4 °C until it was removed from the cycler. RT-PCR products were analyzed by 1% agarose gel electrophoresis, and stained with 1X SYBR green in water. Gel images were captured with a BioRad Gel Doc 1000 system, and cropped in Photoshop version 6.0 without any image enhancements. The PCR products were subcloned into pCR4-TOPO with a TOPO TA Cloning Kit for Sequencing (Invitrogen).

Reverse transcription and touchdown thermocycling: RNA, prepared as described above, from larval and adult zebrafish was employed, typically 100 ng per reaction. A Qiagen OneStep RT-PCR kit was used as recommended by the manufacturer. In a 50 µl reaction, 0.5 µM primer concentrations were used. The RT incubation was at 50 °C for 30 min. The RT was heat inactivated by incubating at 95 °C for 15 min. PCR was conducted using the touchdown approach. The reaction tubes were subjected to 40 cycles of 94 °C for 15 s to denature the DNA; 69 °C (decrementing the annealing temperature by 1 °C per cycle over the first 7 cycles to 63 °C) for 30 s, and an elongation step of 72 °C for 2.0 min. At the conclusion of cycling, the samples were incubated at 72 °C for 10 min as a final elongation step, and the samples were held at 4 °C until they were collected for analysis by gel electrophoresis or for subcloning and sequence analysis.

In situ hybridization: Zebrafish of the Tuebingen (Tue) strain or the albino (albb4) strain were maintained and bred at 28.5 °C, on a 14:10 light/dark cycle, in aquatic housing units in monitored recirculating system water. All procedures involving animals were approved by the University of Idaho Animal Care and Use Committee and conformed to the ARVO statement on the use of animals in research. Tissue from embryonic, larval, and adult zebrafish was processed for in situ hybridization as previously described [7]. In brief, adult zebrafish were anesthetized in 0.2% MS-222 (Sigma, St. Louis, MO), and decapitated. Eyes were enucleated, and then corneas were perforated and lenses removed, and eyes were placed in phosphate-buffered (pH 7.4) 4% paraformaldehyde (PFA), 5% sucrose. Larval zebrafish (99 and 155 hpf) were anesthetized in 0.02% MS-222, then immersed whole into PFA. Embryos (74 hpf) were immersed whole into PFA. All tissues were fixed for one hour, and then washed in phosphate-buffered 5% sucrose, followed by sequential washes with increasing concentrations of sucrose. Tissues were cryoprotected overnight at 4 °C in phosphate-buffered 20% sucrose, then embedded and frozen in a 2:1 solution of phosphate-buffered 20% sucrose/OCT (optimal cutting temperature embedding
medium; Sakura Finetek USA, Torrance CA) and then sectioned at 3 μm.

In situ hybridizations were conducted as previously described [7]. Gene 1 and Gene 2-specific cDNAs, corresponding to PCR products amplified from zebrafish RNA with gene-specific primers (Table 1) were cloned into pCR4-TOPO-TA (Invitrogen). The plasmid with Gene 1 insert was linearized by digestion with SpeI or NotI and transcribed with T7 or T3 to generate sense and antisense (respectively) digoxigenin-labeled cRNA probes, using components of the Genius Kit (Roche). The plasmid with Gene 2 insert was linearized by digestion with NotI or SpeI and transcribed with T3 or T7 to generate sense and antisense (respectively) probes.

Sections were rehydrated and permeabilized with 10 μg/ml proteinase K for 10 min. This was followed by immersion in 0.26% acetic anhydride. Sections were then dehydrated and hybridized overnight at 56 °C. with sense or antisense Gene 1- or Gene 2-specific probes in a hybridization solution containing 50% formamide. Sections were treated with RNase A and were then incubated overnight at room temperature with anti-dig antibody conjugated to alkaline phosphatase. Hybridization was visualized with the alkaline phosphatase substrates NBT/BCIP; sections were viewed on a Leica DMR compound microscope using Nomarski optics, and were photographed with a Spot Digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Images were arranged in Photoshop CS (Adobe Systems Inc., San Jose, CA).

RESULTS
IRBP genes of tetrapods: A single IRBP locus was identified in the genomes of human, chimpanzee, dog, cow, mouse, rat, opposum, chicken, and frog. The exon-intron structures of these IRBP orthologs are mapped in Figure 1. The mammalian IRBP gene orthologs have exon and intron lengths very similar among the seven species. The exons of the chicken and frog genes are similar in length to those of the mammalian orthologs, while the chicken and frog introns are somewhat longer than their eutherian mammal counterparts. The same number of introns were found in all species, with the possible exception of the domestic dog IRBP gene, which may have an additional 3′ untranslated exon (not illustrated). Gene structure of IRBP in the tetrapods appears to be highly con-

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**Figure 2.** Alignment and comparison of Donor and Acceptor sites from the tetrapods. This is a comparison to the accepted consensus donor and acceptor sites from human genes. All the introns contain invariant GT and AG dinucleotides at the beginning and end of the intron. Most nearby nucleotides closely match the consensus donor or acceptor motif. The lariat consensus sequence is detected in about half the introns within 50 nucleotides of the acceptor site, and all have several A’s, which may function in the absence of a closely matching lariat sequence. These alignments indicate that the site splices are all strongly conserved in position and sequence, and they all appear to function well.

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served. Especially noted was the close similarity of chicken and *Xenopus tropicalis* IRBP genes.

All of the IRBP genes of tetrapods encode a single polypeptide consisting of four homologous Repeats (also referred to as modules), with each Repeat consisting of about 300 amino acids. They all match well to the conserved domain database consensus, pfam02692.11, with bit scores ranging from about 200 to 500, corresponding to E-values of about $10^{-31}$ to $10^{-153}$, where a bit score represents the summed information content. The bit score is derived from the raw alignment score in which the statistical properties of the scoring system are taken into account, and the bit score can be used to compare scores across different tests and types of alignments. A raw alignment score is the sum of the identity and mismatch score at each point in the sequence over a range from which the sum of gap penalties are subtracted. An identity or a substitution score is obtained from the specified weight matrix (usually BLOSUM62 for amino acid alignments).

All tetrapod IRBP orthologs have the same placement of the introns, with exon 1 encoding the first three Repeats and the first quarter of the fourth Repeat, and exons 2-4 encoding the remainder of the fourth Repeat (Figure 1). Exons 1, 2, and 3 are virtually identical in length among orthologs. Exon 4 contains a 3' untranslated region (UTR), and this 3' UTR varies widely in length across species. In general, it is difficult to define the transcription termination site in any eukaryotic gene, strictly based on consensus sequences. However, in well-studied IRBP genes [48], there are multiple transcription termination points as indicated by multiple bands on northern blots, variation in the locations of poly(A) tails in cDNA clones, multiple polyadenylation signals, and multiple lengths of the 3' UTs in sequenced IRBP ESTs containing a poly(A) tract [48].

Figure 2 shows a multisequence alignment near each intron Donor and Acceptor site for the tetrapod orthologs. All the introns contain invariant GT and AG dinucleotides at the beginning and end of the intron. Most nearby nucleotides closely match the consensus donor or acceptor motif. The lariat sequence (not illustrated) matches the consensus in about half the introns within 50 nucleotides of the acceptor site, and all

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**A. Danio rerio IRBP locus**

**B. Tetraodon nigroviridis IRBP locus**

**C. Takifugu rubripes IRBP locus**

**D. Oryzias latipes IRBP locus**

Figure 3. Teleost fish IRBP gene loci. **A:** Zebrafish has two IRBP genes. **B:** *Tetraodon nigroviridis* has a remnant of the upstream gene (Gene 1) and a full copy of Gene 2. **C:** Fugu has two IRBP genes. Panel D: Medaka has a single gene corresponding to Gene 2. In zebrafish and fugu, the two IRBP genes are oriented head to tail, and, across species, the intergenic spacer is about the same size. No intervening genes are found between the two genes in the single locus. The colors originate with the web site computer program called AUGUSTUS [46]. Each exon is marked with two different colors, such that the end of one exon and the beginning of the next exon have the same color. Colors are rotated so that adjacent exons are colored differently (only three colors are needed) and the order of use is blue-red-green, which is then repeated. The first exon of a gene is depicted with an arrow tail at the 5' end, and the last exon is labeled with an arrowhead at the 3' end. For genes that have only a single exon, they are solid blue, with an arrow head and tail. These models are predicted gene structures based on several algorithms. Also, some of the features are small, so that a tiny first exon might not appear to have an arrow tail at the 5' end.
have several adenine bases, which may function in the absence of a closely matching lariat sequence. The position of each intron (relative to the coding sequence of adjacent exons) is invariant among the tetrapods.

There are several whole genome sequencing projects underway in other mammalian species, but we did not detect any matches to human IRBP in them. The African elephant (*Loxodonta africana*) and Rhesus monkey (*Macaca mulatta*) both contained an IRBP ortholog, but the sequence assemblies are incomplete, missing small parts of the IRBP gene. The IRBP genes from these species are not further considered in this report.

**IRBP genes of teleosts:** The genomes of four species of teleost fish have been nearly or completely sequenced. Fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*) genomes are now effectively complete, and those of pufferfish (*Tetraodon nigroviridis*) and medaka (*Oryzias latipes*) are close to completion. IRBP gene orthologs were found in each of these four genomes (Figure 3). Remarkably, in each locus from zebrafish and fugu, there were two predicted genes instead of the anticipated single IRBP gene. Each of the two genes exhibited strong sequence similarity at the amino acid level to the human IRBP sequence, indicating that both of the predicted genes were IRBP genes. The location, orientation, and intron-exon structures were similar in zebrafish and fugu. Two genes were also predicted for the IRBP locus in pufferfish; however, while the second of the genes had a very similar exon-intron pattern to the second of the IRBP genes in fugu and zebrafish, the first gene differed markedly in size. In pufferfish (Figure 3B), this first gene was much smaller and appeared to be a remnant of the first gene, at about one-fifth the size of the corresponding predicted genes from zebrafish and fugu. Despite the small size, it was unmistakable that this gene fragment encoded IRBP amino acid sequence. The medaka IRBP gene locus was different from the loci in zebrafish, fugu, and pufferfish. The medaka locus contained only a single gene corresponding to the second extracellular region of the zebrafish and fugu loci.

The two genes predicted in each IRBP locus of fugu and zebrafish differed from the “classical” IRBP gene structure described above for tetrapods. The predicted loci were similar in structure, with the two genes separated by a short (1-2 kb) intergenic spacer. The first gene (Gene 1 hereafter) consisted of a single long exon, which encoded a protein of about 900 amino acids. The second gene (Gene 2 hereafter) had a structure virtually identical to the IRBP gene reported previously by Rajendran and coworkers [27] in zebrafish. Gene 2 in fugu and zebrafish had four exons and three introns, reminiscent of the mammalian, bird, and amphibian IRBP gene structure. The chief difference between the zebrafish and fugu Gene 2 structure and the IRBP gene of tetrapods, was that exon 1 of the teleosts encodes just one full Repeat and a small part of a second Repeat, while exon 1 of tetrapods encodes the first three Repeats and a small part of the fourth Repeat. The remaining exons (exons 2-4) each encode a small part of the fourth Repeat in tetrapods, and in teleosts exons 2-4 each encode an orthologous segment of the second Repeat. The positions of the introns in Gene 2 of teleosts fall almost exactly in the same positions as those in the tetrapod IRBP gene (Figure 4). The sole difference in our predicted structure of Gene 2 (Figure 3A) and the gene structure determined by Rajendran and coworkers [27] was a small exon preceding the previously established exon 1. The small extra exon was predicted by the AUGUSTUS software but appears to be missing from the full-length cDNA sequence previously published [27]. The cDNA clone for the earlier study was obtained from zebrafish retina RNA at an adult stage [27]. While it might be an artifact of the

![Figure 4](http://www.molvis.org/molvis/v12/a180/)

**Figure 4. Alignment and comparison of Donor and Acceptor sites from the teleost fish Gene 2.** This is a comparison to the accepted consensus donor and acceptor sites from human genes. At the beginning and end of each intron, respectively, invariant GT and AG dinucleotides are located. Most nearby nucleotides match the consensus donor or acceptor motif. These alignments indicate that the splice sites are all strongly conserved in position and sequence, and they all appear to function correctly.
AUGUSTUS program, it is worth noting that perhaps under some unusual circumstances, or during development when IRBP is expressed in multiple cell types (including PhRs and RPE cells [7]), this predicted upstream site might serve as a second promoter, offering potential for differential splicing or expression of the same gene in different tissues and at different times.

The predicted amino acid sequences of the teleost IRBP locus showed extensive similarity to the human IRBP amino acid sequence as illustrated in dot matrix comparisons (Figure 5A-C). These comparisons (Figure 5A) revealed five distinct diagonals looking down any column. For example, between positions 200 and 250 on the x-axis. These five diagonals indicate the presence of five Repeats in the zebrafish locus. Subsequent analyses suggest that the five Repeats are divided between two genes with Gene 1 containing the first three Repeats and Gene 2 the remaining two Repeats. A comparison of the amino acid sequence of zebrafish IRBP Gene 1 to human IRBP revealed a major diagonal (Figure 5B), indicating nearly continuous similarity over the entire 900 amino acids of the Gene 1 sequence. Every 300 amino acids an additional diagonal was found, suggesting three 300-amino acid long Repeats. The zebrafish Repeats corresponded to Repeats 1, 2, and 3 from the human sequence, with the greatest similarity to the orthologous Repeat (i.e., Repeat 1 of zebrafish was most similar to Repeat 1 of human, and revealed lesser similarities to the other three human Repeats). No Repeat 4 sequence was detected in zebrafish Gene 1. Similar studies were performed with Gene 1 from fugu and the same patterns and findings were obtained (data not shown). Comparison of the Gene 2 amino acid sequence from zebrafish to the human IRBP amino acid sequence also revealed a series of diagonals (Figure 5C). Along any column, every 300 amino acids another diagonal was found, suggesting sequence similarity to each of the four human Repeats. A major diagonal starting in the upper left corner demonstrated that Gene 2 in zebrafish begins with a Repeat 1-like motif. This diagonal line, though segmented, extends past the end of human Repeat 1 but the diagonal is heavily interrupted beyond this Repeat. Following Repeat 1 of the zebrafish, there was only one more Repeat, corresponding approximately to positions 300 to 600. Therefore, Gene 2 has only two Repeats. Next, it was apparent that the second Repeat in zebrafish Gene 2 was most similar to Repeat 4 of the human amino acid sequence. This is illustrated by the series of four diagonals vertically arranged on the right half of the dot matrix plot. The bottom diagonal was continuous, where the three other diagonals above it had more gaps and discontinuities, showing the greatest similarity to human Repeat 4. Similar results comparing Gene 2 from tetraodon, medaka, and fugu demonstrated that Gene 2 of these fish had the same two-Repeat structure corresponding to human Repeats 1 and 4 (dot plots not shown).

Teleost genomes therefore contain IRBP loci with one or two IRBP genes. Gene 1 from zebrafish and fugu has three Repeats corresponding to Repeats 1, 2, and 3 of the human IRBP gene. Gene 1 in pufferfish contains a short single fragment of Repeat 3 (Figure 3B), while the medaka IRBP locus does not include a Gene 1 ortholog. Gene 2 from zebrafish, fugu, medaka, and pufferfish had two Repeats corresponding to human Repeats 1 and 4.

A few other teleost fish have been subjected to partial EST profiling, and we detected sequence similarities among cDNAs and ESTs from stickleback, goldfish, and fathead minnow. Dotplots comparing a consensus stickleback cDNA to the zebrafish gene locus illustrate evidence for a stickleback two-Repeat protein of the Gene 2-type, matching better to Gene 2 than Gene 1 (data not shown). The percent identity of the stickleback cDNA and Gene 2 from zebrafish is 56% and to Gene 1 is 38% at the nucleotide level. These ESTs and cDNAs

![Figure 5. Dot matrix comparisons showing the existence of a two-gene IRBP locus in zebrafish. A: Dot matrix of human protein against the entire zebrafish gene locus. B: Human protein against translated Gene 1. C: Human protein against Gene 2 protein. The Pustell dot matrix program in MacVector was used employing the pam250 weight matrix and a hash value of 2 in all three panels. Two genes exist in tandem. The genes are oriented head to tail, the genes are close together, the genes are different, with Gene 1 a single exon and three-Repeat long and Gene 2 having four exons, but just two repeats. The optimum alignments to the human IRBP amino acid sequence showed that Gene 1 encodes Repeats 1, 2, and 3, while Gene 2 encoded Repeats 1 and 4.]

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Matrix comparison of fugu and zebrafish (representing the tetrapod and teleost fish IRBP genes). This was shown by dot matrix comparison of the tetrapod and teleost fish IRBP genes at each orthologous splice site boundary, predicted by computer and by hand, as provided in Figure 2 and Figure 4, respectively. The consensus splice site sequences are taken from Zhang [49]. The donor and acceptor sites in IRBP closely match the consensus splice sites, with invariant GT...AG sequences at each orthologous splice site boundary, predicted by computer and hand. The percent identity is indicated in the upper left right of the table. The raw bit score is indicated in the lower left half of each table. The raw bit score is indicated in the lower left half of each table.

To establish an evolutionary relationship between Gene 1 and Gene 2, we performed pairwise comparisons between the indicated two versions of Repeat 1, the only Repeat that occurs in both genes in zebrafish and fugu. There was a relative constancy in the identities and scores regardless of the origin of either Repeat 1 homolog, whether pairs of orthologs or pairs of paralogs, with the ortholog pairs more similar than the paralog pairs. This difference suggested that Genes 1 and 2 diverged before zebrafish and fugu diverged (Table 3). A phylogeny of Repeat 1 is shown in Figure 6 to indicate relative evolutionary distances. This phylogeny suggests that Gene 1 and Gene 2 were created early and simultaneously in tetraspore evolution, well before fugu and zebrafish last shared a common ancestor.

We compared intron locations across the IRBP orthologs. Consensus maps of the tetrapod and teleost fish IRBP genes at each orthologous splice site boundary, predicted by computer and by hand, are provided in Figure 2 and Figure 4, respectively. The consensus splice site sequences are taken from Zhang [49]. The donor and acceptor sites in IRBP closely match the consensus splice sites, with invariant GT...AG sequences at the beginning and end of each intron. There is little variation in the position of introns in the IRBP gene. However, within the intron sequences, except near the splice sites, intronic sequences are not conserved. This was shown by dot matrix comparison of fugu and zebrafish (representing the teleosts) and among several of the tetrapods (data not shown).

We performed multiple sequence alignments of the predicted amino acid sequences of teleost fish IRBP Gene 1 and Gene 2 at the amino acid sequence level (Figure 7). First, we aligned the Gene 1 amino acid sequences from zebrafish and fugu (Figure 7A). Among the 918 aligned positions, only two gaps were inserted totaling 5 amino acids. The sequences are overall about 58% identical. Next, we compared the Repeat 1 amino acid sequence from Gene 2 of several fish. The sequences of six species are shown in Figure 7B, including fugu, goldfish, medaka, stickleback, tetraodon, and zebrafish. Numerous blocks of identical and strongly conserved amino acids are found throughout the entire length of the sequence. There are no long deletions or insertions in any of the aligned sequences, suggesting that no domains have been gained or lost. The Repeat structure in IRBP contains two domains, designated A and B, and both are conserved among all the Repeats in the teleost fish. Potential glycosylation sites are marked and have a conserved location about 200 amino acids from the N-terminal end of Repeat 1, which is similar to the tetrapods. One of the potential hyaluronan binding sites is shared with the mammals, at about positions 220-230 of Repeat 1. This is conserved in all 6 fish (Figure 7A,B).

**Table 3. Percent identities and scores comparing Repeat 1 protein orthologs and paralogs**

<table>
<thead>
<tr>
<th>Repeat 1 gene and species</th>
<th>Z1</th>
<th>F1</th>
<th>Z2</th>
<th>F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>zfish Gene 1 Repeat 1 (Z1)</td>
<td>337</td>
<td>392</td>
<td>343</td>
<td>324</td>
</tr>
<tr>
<td>zfish Gene 2 Repeat 1 (Z2)</td>
<td>302</td>
<td>416</td>
<td>289</td>
<td></td>
</tr>
<tr>
<td>fugu Gene 1 Repeat 1 (F1)</td>
<td>59%</td>
<td>48%</td>
<td>326</td>
<td>312 (bits)</td>
</tr>
<tr>
<td>fugu Gene 2 Repeat 1 (F2)</td>
<td>50%</td>
<td>39%</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td>human Repeat 1 (H1)</td>
<td>53%</td>
<td>42%</td>
<td>51%</td>
<td>47%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeat 1 gene and species</th>
<th>Z1</th>
<th>F1</th>
<th>Z2</th>
<th>F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>zfish Gene 1 Repeat 1 (Z1)</td>
<td>416</td>
<td>404</td>
<td>406</td>
<td>402</td>
</tr>
<tr>
<td>fugu Gene 1 Repeat 1 (Z2)</td>
<td>63%</td>
<td>498</td>
<td>565</td>
<td>408</td>
</tr>
<tr>
<td>Medaka Repeat 1 (M1)</td>
<td>59%</td>
<td>74%</td>
<td>480</td>
<td>379</td>
</tr>
<tr>
<td>Tetraodon Repeat 1 (T1)</td>
<td>63%</td>
<td>89%</td>
<td>759</td>
<td>379</td>
</tr>
<tr>
<td>Goldfish Repeat 1 (G1)</td>
<td>84%</td>
<td>62%</td>
<td>59%</td>
<td>61%</td>
</tr>
<tr>
<td>Stickleback Repeat 1 (S1)</td>
<td>61%</td>
<td>78%</td>
<td>74%</td>
<td>78%</td>
</tr>
<tr>
<td>Human Repeat 1 (H1)</td>
<td>42%</td>
<td>47%</td>
<td>45%</td>
<td>47%</td>
</tr>
</tbody>
</table>

**Nonvertebrate chordate IRBP gene searches:** We were not able to identify an IRBP gene ortholog in _Ciona intestinalis_ or _Ciona savignyi_, two urochordates that lack complex eyes. The most current versions of the genome assemblies of these species were screened for matching sequence on July 8, 2005. Because all vertebrates with “camera-like” eyes have IRBP, and because these two chordates lack IRBP, we suggest that IRBP arose after the divergence of the urochordates from the vertebrates. It will be interesting and useful to search for an ortholog of IRBP in the cephalochordates that have eyes and ciliary photoreceptors; this will be possible when the amphioxus whole genome sequencing project is complete.

**Expression of two IRBP genes in zebrafish:** To determine whether both Gene 1 and Gene 2 of zebrafish were transcribed into stable mRNAs, we performed gene-specific RT-PCRs. A primer pair from Gene 1 was designed to amplify a...
1529 bp band, if an mRNA was transcribed from Gene 1. Because this gene lacks any introns, it was not possible to design a primer pair that spans an intron. However, Gene 2 has three introns, and a primer pair was designed that spanned Introns B and C. If Gene 2 was transcribed and spliced, a band of 532 bp was expected. In both cases, bands of expected size were amplified from 96 h larvae and adult whole eye RNA with each primer set (Figure 8A). In the absence of the reverse transcriptase, no PCR products were detected, suggesting that there was no genomic DNA contamination in the RNA (data not shown). In Gene 2 RT-PCRs, splicing was shown to occur as the RT-PCR product size agreed closely with the expected size based on the removal of the intron from the processed mRNA. These data indicate that both genes in zebrafish are transcriptionally active.

The gene structure of the zebrafish IRBP locus is consistent with the hypothetical formation of a single individual transcript that would include both genes. To determine if this is possible, we performed RT-PCR using a forward primer about 500 bp upstream from the end of the zebrafish Gene 1 and a reverse primer located about 600 bp downstream from the beginning of zebrafish Gene 2. This experiment resulted in amplification of a product of a size lacking the intergenic spacer (Figure 8B). This prominent band was about 1100 bp (expected size of 1098 bp). A less prominent co-migrating band was also amplified from larvae (Figure 8B).

The amplified RT-PCR product from 96 h larvae was cloned and sequenced. It was compared to the genomic sequence of the AB strain of zebrafish, which was obtained by cloning PCR amplified genomic DNA from three AB fish. In sequencing the three independent genomic clones of the AB strain, all three gave the identical sequence. The resulting RT-PCR sequence contained, in order, a priming site for primer F1 (Table 2), about 550 nt of Gene 1, about 50 nt of 3’ UTR sequence not homologous to Gene 1, about 500 nt of Gene 2, and a priming site for R3 (Table 2). The overall length and sequence of RT-PCR amplified product is most consistent with an RNA transcript originating in Gene 1, transcribing through the intergenic region into Gene 2, followed by splicing to remove the intergenic spacer. The 50 bp nonhomologous sequence reflects a 50 bp sequence inversion in the Gene 1 3’ UTR, as this inverted sequence was detected when the opposite strands are aligned. It is not clear whether the inversion represents a rare RNA processing error, a cloning or reverse

Figure 6. Phylogenetic analysis of Repeat 1 from IRBP. Evolutionary relationships among fish and human Repeat 1 orthologs and paralogs are illustrated. MacVector version 9.0 was used to build a cladogram of the Repeat 1-like amino acid sequences of the teleost fish and the human Repeat 1. Human Repeat 3 was used as an outgroup for the purpose of rooting this tree. The illustrated tree was built using the neighbor-joining method. Whether using the UPGMA or neighbor-joining method, with uncorrected or Poisson-corrected distances, random or systematic tie-breaking for gaps, in MacVector version 9.0, all the phylogenetic reconstructions gave the same overall structure. A consensus tree from 1000 replications of bootstrapping was calculated, and it was identical to the structure of the best tree, which is illustrated in this image. Among 1000 replications of resampling, all seven relevant nodes occurred frequently, with four appearing in 100% of trees, two nodes occurring in 96% of trees, and one node occurring in 79% of trees. All the nodes are resolved in the illustrated tree. The results suggest a paralogous relationship between the Repeat 1 sequences from Genes 1 and 2 in zebrafish and fugu. The closer relationship of human Repeat 1 to the fugu and zebrafish Gene 1 orthologs than to Gene 2 and the illustrated divergence at the leftmost internal node of the paralogs on this cladogram suggests that the paralogous Genes 1 and 2 arose early or at the origin of the teleosts. This tree is consistent with the already known divergence radiation of the teleost fish about 350 Mya.
Figure 7. Multiple alignment of fish amino acid sequences. A: Gene 1 from zebrafish (zebr1) and fugu (fugu1) are aligned over Repeats 1 through 3, about 930 amino acids. B: Gene 2 Repeats 1 and 2 orthologs from fugu, goldfish, medaka, stickleback, tetraodon, and zebrafish are aligned over about 620 amino acids. Motifs identified: Signal peptide (leader sequence) cleavage sites are shown by the arrow. Glycosylation sites, NX(T|S), are shown in gray boxes with a transparent green fill. Hyaluronan binding sites, (R|K)X7(R|K), are shown in a blue box with a transparent light red fill. Identical amino acids across all aligned positions are shown in blue. Red illustrates identical residues in 3 to 5 of the 6 possible identities. Green illustrates residues with similar chemical properties at a given position.
transcription error, or a rare genomic sequence change within the AB strain of zebrafish. Other than the loss of the intergenic spacer and the short sequence inversion, the RT-PCR and genomic sequences were identical.

The loss of the intergenic spacer from the primary transcript suggests that the spacer may, under some circumstances, function as an intron, when transcription runs beyond the end of Gene 1. This appears to represent aberrant and rare transcription run-through and splicing in the zebrafish IRBP gene locus.

The presence and expression of two IRBP genes in the zebrafish and fugu genomes, but not in other genomes, suggests that in the former species, the two paralogous IRBP genes may have assumed divergent roles [40,50]. Similar neo- and sub-functionalization processes for other duplicated genes have included the emergence of differential expression patterns [39,40]. We performed in situ hybridization with Gene 1- and Gene 2-specific cRNA probes to determine if this was the case for zebrafish IRBP. Consistent with previous findings [7], Gene 2 is expressed in photoreceptors and the RPE, in 74 hpf embryonic, 99 and 155 hpf larval, and adult retinas (Figure 9). In addition, very weak and sporadic expression of Gene 2 was observed [7] in a minor subpopulation of cells in the inner nuclear layer (INL), Figure 9. In contrast, Gene 1 was expressed in a slightly larger proportion of cells residing in the INL, with positive signals weakly detected in embryonic retinas and stronger signals in larval and adult retinas (Figure 9). Expression of Gene 1 was not localized to photoreceptors or to the RPE; however, we occasionally observed expression of Gene 1 in a subpopulation of cells in the ganglion cell layer (data not shown). Both Gene 1 and Gene 2 were expressed in the pineal organ in embryos and larvae, although positive signals were barely detectable for Gene 1 (Figure 9). We did not evaluate IRBP expression in adult pineal organs. The use of sense probes corresponding to Gene 1 or Gene 2 resulted in no labeling (data not shown). These data collectively demonstrate differential spatiotemporal expression of Gene 1 and Gene 2.

DISCUSSION

The structure of the tetrapod IRBP gene: The tetrapod IRBP gene structure is now well established and consistent among multiple taxa. Tetrapod IRBP has four exons and three introns, with a large first exon encoding the first three Repeats and the beginning of the fourth (and last) Repeat. This conclusion is supported by the fact that the genomic sequences of the tetrapod IRBP gene are identical to the RT-PCR results. The presence of two paralogous IRBP genes in the zebrafish and fugu genomes suggests that these genes may have assumed divergent roles, possibly through neo- and sub-functionalization processes. In situ hybridization experiments confirmed the expression patterns of both genes, with Gene 1 expressed in a subpopulation of cells in the ganglion cell layer and Gene 2 expressed in photoreceptors and the RPE. The expression of both genes in the pineal organ also supports their potential roles in vision and other physiological processes. These findings contribute to our understanding of the evolution and function of IRBP genes in different species.
Figure 9. In situ hybridizations. Zebrafish IRBP Gene 1 and Gene 2 are differentially expressed. Retinal cryosections were obtained from 74 hpf (A, B) alb/b4 zebrafish embryos, 99 hpf (C, D) and 155 hpf (E, F) alb/b4 zebrafish larvae, and adult Tuc zebrafish eyes (G, H) and were hybridized with Gene 1- (A, C, E, G) and Gene 2- (B, D, F, H) specific probes. Note that alb/b4 zebrafish do have low levels of pigment in the RPE; dark arrows show Gene 2 hybridization. White arrows show weak expression of Gene 2 sporadically in the INL. Insets in A and B shows Gene 1 and Gene 2 expression in developing pineal; contrast was enhanced to show weak expression of Gene 1. Scale bars represent 50 µm; rpe represent retinal pigmented epithelium, onl represent outer nuclear layer; inl represent inner nuclear layer; gcl represent ganglion cell layer.
supported by the close sequence similarities at donor and acceptor splice sites (Figure 2) and the close amino acid sequence similarities among the different species in each exon. The IRBP gene is highly conserved among the tetrapods, consistent with an important function in the visual system.

There are, however, exceptions to this conserved structure. For example, our analyses predicted that the IRBP gene of the domestic dog may possess an extra intron at the end of the gene. This prediction awaits proof through sequence analysis of the expressed dog IRBP mRNA. An additional 246 nt was also identified in the fourth exon of an inbred jungle fowl IRBP mRNA; this in-frame sequence was not present in the domestic chicken genomic sequence, and appears to be a duplication of a sequence found in the second intron [51]. It is worth mentioning that the opossum and chicken possess long introns. Run-through transcription and multiple polyadenylation sites in the mouse gene, and a large insertion into the 3' UTR of the bovine gene near the 3' end of the gene also reflect variability in the structure of the tetrapod IRBP gene at the 3' end. It does not seem likely though that the Repeat structure of IRBP makes this gene susceptible to run-through or run-on transcription, which seems common among most eukaryotic multicellular organisms.

The structure of the IRBP gene locus in teleosts and models of teleost IRBP evolution: The teleost IRBP gene locus varies in structure among the four teleost fish studied here (Figure 3), and has revealed an intriguing evolutionary history of the IRBP gene. There are clear signs of a gene duplication, resulting in a head-to-tail two-gene locus containing two different (but related) IRBP genes. It appears that one of the genes (Gene 1) is in some cases undergoing gene loss or has been relegated to pseudogene status (as observed in medaka and pufferfish) and in other cases potentially has undergone neo- or sub-functionalization (in zebrafish and perhaps in fugu). The second gene in the locus (Gene 2) is uniformly retained in structure in all four species. The two genes contain remarkably different gene structures: Gene 1 consists of a single long exon encoding a 900 amino acid long protein encoding Repeats 1, 2, and 3; Gene 2 is a more typical gene consisting of four exons that encode a polypeptide consisting of two full Repeats, Repeats 1 and 4.

We suggest that the two IRBP genes arose early in the evolution of teleosts, at approximately the time of the whole genome duplication (WGD) that occurred coincident with or just prior to the radiation of the teleosts [39,40,42,50]. The duplication occurred early in the evolution of the teleosts as the two-gene locus is found in the distantly related zebrafish and fugu. This hypothesis is further supported by the presence of Gene 2, preceded by a remnant of Gene 1 in pufferfish, and by the closer sequence similarities of orthologs of Repeat 1 than of paralogs of Repeat 1. We predict that many other teleost fish will have two IRBP genes of similar exon and intron structures in a single locus (Figure 3). The existence of a three-Repeat Gene 1 in fugu, but a Gene 1 remnant in the related pufferfish, and the absence of Gene 1 entirely in the more distantly related medaka, also suggests that the loss or pseudogenation of Gene 1 has occurred as multiple independent events. Perhaps the study of comparative visual requirements in these species may reveal hints as to the function of Gene 1.

It is remarkable that the duplicated IRBP genes have only one Repeat - Repeat 1 - in common. Repeats 2 and 3 are lacking from Gene 2, and Repeat 4 is not present in Gene 1. Pairwise comparisons between any two homologs of Repeat 1 (Table 3), indicated that the Repeat 1 homologs diverged from one another at about the same time. It is tempting to speculate that Repeat 1 may correspond most closely to a putative ancestral single-Repeat IRBP, though Repeat 4 might be the original, as indicated by the presence of introns. The predicted timing of Repeat 1 divergence is consistent with the already known radiation of the teleost fish about 350 Mya. The more important consideration is that roughly the same results were found when comparing the paralogs within one species (Table 3). These comparisons, one in zebrafish and one in fugu, suggest that the two IRBP genes (Gene 1 and Gene 2) arose approximately coincident with the radiation of the teleosts and the proposed whole genome duplication, and the two-gene locus in the euteleosts arose early in the ancestry of these fish. We hypothesize that this event, the creation of the two-gene IRBP locus, may serve as a marker of the euteleosts. A corollary of this hypothesis is that more primitive fish, including coelacanths, lungfish, bow fin, and gar, are predicted to have a single gene IRBP locus, with a four-repeat IRBP gene structure characteristic of the tetrapods. This hypothesis will be tested when whole genome sequencing projects in these and related species are completed.

Mechanisms causing the tandem IRBP gene duplication: Several evolutionary models are consistent with the observed gene structures of the teleost IRBP loci. Here we describe what we believe to be the two most parsimonious models (Figure 10 and Figure 11). For both models, the tetrapods, teleosts, and cartilaginous fish are considered to have a common ancestor having a full four-repeat, four-exon IRBP gene. The evolutionary quadruplication of the even more ancestral single Repeat IRBP gene is still anticipated to occur at about the time when vertebrates first arose during the Silurian period. The other essential requirements are that a model must result in a single locus bearing two IRBP genes in head to tail orientation, with no other IRBP loci in the fish genome.

In the first model (Figure 10), the teleost IRBP two-gene locus is proposed to have arisen as a direct consequence of the teleost WGD, because they (the WGD and the two-gene locus) appeared coincidentally in evolutionary time. The WGD generated two complete four-Repeat IRBP genes, one on each of the duplicated whole chromatids. These duplicates are proposed to have diverged as illustrated in Figure 10, steps 2A, 2B, and 2C, in which each one of the genes loses one or more Repeats and in which there are alterations to the cis-elements in the promoters. Following divergence, a reduction in size of the tetraploid ancestral teleost genome began to occur. The reduction in size is proposed to have occurred by the unequal crossing-over of slightly diverged chromatids and the loss of the chromatin that lacks an IRBP gene (presumably by natural selection). The sequence divergence that had occurred on
the two IRBP loci may have contributed to a crossover event that yielded one new chromatid with two IRBP genes, one gene with three Repeats and a second gene, which has only two Repeats, in head to tail orientation with the first. This is a mechanism that preserves synteny, and it results in only minor revisions to the current model (Figure 12) of IRBP evolution [26].

A second model for the origin of the two IRBP tandem IRBP genes, is a single-gene tandem duplication (Figure 11). This mechanism involves the simple misalignment of two chromatids that are undergoing recombination (Figure 11, Step 1). In Step 1, the unequal cross-over is external to the IRBP gene. In Steps 2 and 3, each of the tandem genes must undergo the loss of internal segments of the gene, with Gene 1 losing Re-
peat 4-encoding DNA, and Gene 2 losing DNA that encodes Repeats 2 and 3. These two steps differ little from similar steps illustrated in the previous model shown in Figure 10. The single-gene tandem duplication model also preserves synteny. This mechanism is well established in many other gene families, and is considered to be the mechanism for the generation of tandem repeats of some of the cone opsin genes [52,53]. Our estimated evolutionary timing of the original gene duplication event predicts that either some teleosts would have two additional IRBP genes in their genomes (if the WGD occurred after the single-gene tandem duplication), or one additional IRBP gene (if the WGD occurred first). We have not found evidence of such additional IRBP genes; however, we have only examined the genomes of four teleosts, and there is evidence that gene loss has eliminated a large proportion of duplicate genes in teleosts [54,55].

It is hypothetically possible to compare the odds of the two models, the tandem duplication (Figure 11) and WGD/compaction (Figure 10) models. Assuming that the probability of retaining a gene duplicate is 24% after the WGD [56], then the two models in Figures 10 and 11 are about equally likely, with the WGD model slightly favored by about 1.6 fold. The complete genome sequences of several particular teleosts should lead to a resolution of which model is responsible for the duplication of the IRBP gene.

Several alternative mechanisms are reasonable to propose, but a detailed discussion is beyond the scope of this paper. We wish to emphasize that, since the IRBP gene duplication cannot be observed directly, we can only make inferences based on limited data and probability. Thus, all these models are to some degree speculative, but they serve as useful hypotheses to test with more sequence data. We hope to build more accurate models and to better distinguish among these models once we obtain whole genome sequence data from several more fish species.

Potential neo- and sub-functionalization of IRBP Gene 1 and Gene 2 in zebrafish: Expression patterns and regulation of IRBP in zebrafish have been the subject of considerable investigation. Expression of zebrafish IRBP mRNA is strongly diurnal and has been shown to be circadian in the eye [27,57] and in the pineal [58]. IRBP mRNA is expressed at high levels in light and at low levels in dark, out of phase with CLOCK [57]. IRBP mRNA transcription in the pineal organ is dependent on Otx5 [58]. In addition, zebrafish IRBP mRNA is expressed by the RPE as well as by photoreceptors. This pattern is distinct, as the IRBP of all other vertebrates examined is photoreceptor-specific [7]. Our data introduce an important further consideration in the study of zebrafish IRBP. We provide evidence that two IRBP genes are not only present, but are expressed, in the zebrafish eye. The RT-PCR and in situ hybridization results lend support to the putative neo-functionalization or sub-function partitioning [40,50] of the two IRBP genes. The differential cell-type specific expression from each gene, and the consequent division of the four-
Repeat protein into Repeats 1, 2, and 3 in Gene 1 and Repeats 1 and 4 in Gene 2, now must be considered in re-evaluating expression and regulation of IRBP. In addition, selective gene-specific knockdown strategies in the zebrafish may allow the elucidation of the functions of each IRBP gene. Gene 1 and Gene 2 are both transcribed, and Gene 2 is post-transcriptionally processed as reflected by the loss of at least one intron (Figure 8). These studies also demonstrated that a primary transcript can initiate upstream of Gene 1 and elongate through Gene 2, and is spliced to produce an mRNA lacking the intergenic spacer. It is possible that this spliced mRNA might encode five Repeats. Repeats 1-3 (from Gene 1) may be fused in frame to Repeat 1 and 4 from Gene 2 in a single polypeptide. We have not yet evaluated cell-specific expression of this long, but rare, mRNA.

It was clear from earlier experiments [7] that IRBP was expressed heavily in photoreceptor and RPE cells and pinealocytes [58]. However, Gene 1- and 2-specific expression patterns were not discriminated because Repeat 1-specific sequences are contained in both genes, and the prior probes included Repeat 1. Repeat 1 from Gene 1 exhibits about 70% identity at the nucleotide level to Repeat 1 from Gene 2 (data not shown), which may be sufficient for cross-hybridization. With the use of the gene-specific probes produced here, we tested the hypothesis that Gene 1 and Gene 2 were differentially expressed. The results clearly demonstrated that there was differential expression of the two genes, but we were surprised by the identity of the cell types that expressed Gene 1. First, there was no extensive overlap in expression, with each Gene being expressed predominantly in different cell types. Gene 2 was expressed in the previously detected pattern in both photoreceptor and RPE cells [7]. However, Gene 1 showed an unexpected and novel expression pattern, in a subpopulation of cells in the INL, and occasionally in a subpopulation of cells in the ganglion cell layer. The distinct expression patterns of Gene 1 and Gene 2 are consistent with neo- and sub-functionalization of the two IRBP genes. We have not yet explored whether any of the INL cells that express Gene 1, also express Gene 2. Pursuit of the identity of the Gene 1-expressing cells is underway.

The IRBP gene locus, with two genes having independent expression patterns and therefore presumed independent functions, can now be added to the inventory of examples of neo- or sub-functionalization in light-sensitive tissues. Extra-
The origins of IRBP: It has been known for many years that IRBP is present in vertebrates and absent from the invertebrates. It is also known that only teleost IRBP lacked the four-Repeat structure of the typical vertebrate IRBP [27]. For example, IRBP in the little skate, Leucoraja erinacea, [65] and the dogfish, Squalus acanthias, [49] is a large protein about the same size as tetrapod IRBP. This suggested [27] that the IRBP gene and protein from the elasmobranchi (sharks, skates, and rays; that is, all cartilaginous fish) have the same four-Repeat, 1200 amino acid long polypeptide and the hypothesized four-exon three-intron structure in the most recent common ancestor of all these species. That these species share common ancestors predating the whole genome duplication in the teleosts, suggests that the ancestral form of the vertebrate IRBP gene was the four Repeat structure containing four exons and three introns, similar to that seen in present-day mammals, birds, and amphibians. Proof of this hypothesized gene and protein structure in the rays and sharks awaits the completion of whole genome sequencing projects that are already underway for representative species of the cartilaginous fish [45].

The revised model of IRBP evolution may be further tested by determining the IRBP gene structures in additional taxa of the chordates. In particular we hypothesize that the non-teleost fish (cf., jawless fish, cartilaginous fish, loke fin fish) should contain a gene structure similar to the mammalian structure with four Repeats, roughly encoding a 1200 amino acid long polypeptide (Figure 12).

Summary: The tetrapod IRBP gene structure is tightly conserved. The same number of Repeats and the same gene structure was found in mammals, birds, and amphibians. In the teleosts, the gene and locus structure vary substantially, but in a systematic pattern. In teleost fish, the number of Repeats is different from the mammals in each of the two IRBP genes. In two species, the two-Repeat gene is preceded by an intronless three-Repeat gene. We have revised a model of IRBP gene evolution (Figure 12) to be consistent with these gene structures. The absence of an IRBP gene in urochordates suggests that an original ancestral IRBP gene arose in an interval after the urochordates diverged but before the vertebrates diverged from other chordates. The gene created then was of the tetrapod 4-exon, 3-intron, 4 repeat type. Here we revise the evolutionary history of IRBP in the teleosts, but not in the history of tetrapod or elasmobranch evolution.

The zebrafish gene expression patterns support sub-function partitioning or neo-functionalization of the two teleost IRBP genes. Every teleost that has been previously examined [65] appears to express Gene 2 (based on the size of the protein), and here we showed that in zebrafish, Gene 2 is expressed in PhRs and RPE, establishing that Repeats 1 and 4 may be required for vision. Repeats 2 and 3 were expressed uniquely in INL and perhaps GCL cells and therefore may be necessary for other processes. Teleost fish are different from tetrapods and consequently may differ in their requirements for the types of IRBP Repeats. This difference may reflect different evolutionary pressures, such as genome compaction, or different physiological, environmental, or physical requirements in vision.

ACKNOWLEDGEMENTS

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We thank several major sequencing centers for making whole genome sequences available for these analyses. In accordance with the wishes of these centers, we quote each requested acknowledgment below.

“The zebrafish sequence data were produced by the Zebrafish Sequencing Group at the Sanger Institute and can be obtained from D_reario. The zebrafish draft assemblies were provided by The Wellcome Trust Sanger Institute, Cambridge, UK.”
“The Xenopus tropicalis EST sequence data were produced by the Xenopus tropicalis Sequencing Group at the Sanger Institute and can be obtained from X_tropicalis.”

“The Tetraodon nigroviridis V7 assembly (February 2004) was provided by Genoscope, Evry, France in collaboration with The Broad Institute, Cambridge, MA.”

We thank the Medaka Genome Sequencing Project: “The data has been provided freely by the National Institute of Genetics and the University of Tokyo for use in this publication/ correspondence only.”

“The opossum sequence was made freely available by The Broad Institute, Cambridge, MA.”

“The February 2004 chicken draft sequence was produced by The Genome Sequencing Center at Washington University School of Medicine, St. Louis, MO.”

The fugu IRBP gene locus sequence “has been provided freely by the Fugu Genome Consortium for use in this publication/correspondence only.”

Stickelback and fathead minnow ESTs were obtained from GenBank and the sequences were kindly deposited by the Stanford Human Genome Center, the US EPA, and the DOE Joint Genome Institute Pimephales promelas EST project.

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