Cloning and Expression of Goldfish Opsin Sequences[†,‡]

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ABSTRACT: Five opsin cDNA clones were isolated from a goldfish retina cDNA library and sequenced. On the basis of homology to previously characterized visual pigments, one clone was identified as goldfish rod opsin and a second as a goldfish red cone opsin. Two rhodopsin-like clones were found to be similar to the chicken green opsin, a pigment which shares properties with both rod and cone pigments. A fifth clone was equally homologous to human blue cone opsin and human rod opsin. In order to characterize the spectral properties of the encoded pigments, the five clones were expressed in tissue culture cells and the apoproteins reconstituted with 11-*cis*-retinal. The wavelength of maximal absorption for goldfish rhodopsin is 492 nm and for the fifth pigment, identified as the goldfish blue pigment, 441 nm. Pigments encoded by the two rhodopsin-like clones absorb at 505 and 511 nm and are likely to correspond to the goldfish green pigment previously characterized by microspectrophotometry. The putative red cone opsin cDNA may encode a pigment that is a polymorphic variant of goldfish red since it absorbs maximally at 525 nm.

Visual pigments are G-protein-coupled receptors that consist of a covalent complex of an apoprotein (opsin) and a small chromophore (11-*cis*-retinal). The 11-*cis*-retinal is bound to the opsin at a lysine residue through a protonated Schiff base. Photoisomerization of retinal from 11-cis to all-trans constitutes the primary visual event in a wide variety of organisms. This isomerization induces a conformational change in the protein that leads ultimately to the generation of a neural signal. Regulation of the absorption characteristics of the 11-*cis*-retinal by the protein determines the region of the visual spectrum where a particular visual pigment functions most efficiently. The diversity of environments occupied by vertebrates has led to the evolution of a large number of different pigments, combinations of which are suited to the particular requirements of a given animal. Strong homology between all opsin sequences suggests that their multiplicity arose from an ancient precursor by sequential gene duplication and subsequent functional divergence (Nathans et al., 1986). Pigments found in unicellular algae and halobacteria that have coexisted for 1.5 billion years point to the existence of an opsin-like protein at a very early phase in the evolution of life (Yokoyama & Yokoyama, 1989).

Traditionally, a visual pigment is classified based on the retinal cell type in which it is found. Rod cell pigments, called rhodopsins, mediate vision in dim light. They are a highly homologous class of proteins which absorb maximally at approximately 500 nm. Members of this class are believed to have restricted access to the retinal binding site, on the basis of the relatively slow reconstitution with retinal and slow reaction between bound retinal and hydroxylamine. Color vision is mediated by cone cell pigments, a more diverse group with individual absorption maxima distributed throughout the visible and near-ultraviolet range. Members of this class have more accessible retinal binding sites, as determined by their rapid reconstitution with retinal and sensitivity to hydroxylamine (Wald et al., 1955; Fager & Fager, 1981; Okano et al., 1989).

In humans, four opsins have been cloned (Nathans & Hogness, 1984; Nathans et al., 1986). These include a rhodopsin which absorbs at 498 nm (Bowmaker & Dartnall, 1980) and three cone pigments absorbing at 426, 530, and 557 nm, respectively (Oprian et al., 1991; Merbs & Nathans, 1992). These four pigments are necessary and sufficient to explain normal visual perception in humans (Nathans et al., 1986).

In fish, each visual system has evolved to function in a particular photon environment (Loew & Lythgoe, 1978). Goldfish (*Carassius auratus*) feed near the water's surface, where the light is bright and spectrally broad. It is therefore not surprising that the goldfish retina possesses several spectral sensitivities. Microspectrophotometric studies (MSP) on goldfish photoreceptors have identified a rod pigment and three cone pigments with absorption properties similar to the human pigments (Marks, 1965). Both behavioral and MSP studies have demonstrated the presence of goldfish retinal cone cells which are sensitive to ultraviolet light (Neumeyer, 1985; Bowmaker et al., 1991). In addition, goldfish have been shown to be sensitive to polarized light (Hawryshyn & McFarland, 1987). To date, no full-length opsin sequences have been cloned from this organism.

The determination and comparison of new visual pigment sequences serve as a foundation for addressing questions of visual pigment structure, function, and evolution. Here we report cloning and expression of cDNAs that encode goldfish rod opsin, goldfish blue cone opsin, and a possible polymorphic variant of the goldfish red cone opsin. Two goldfish green opsins show strong homology to the chicken green opsin, a visual pigment which has features of both rod and cone pigments (Wang et al., 1992; Okano et al., 1992).

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[‡] The nucleotide sequences in this paper will be submitted to GenBank.

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GFgr-1      MNGTEGKNFYVPMSNRTGLVRSPFEYPQYYLAEPWQFKILALYLFFLMSM   50
GFgr-2      MNGTEGNNFYVPLSNRTGLVRSPFEYPQYYLAEPWQFKLLAVYMFFLICL   50
CNgr        MNGTEGINFYVPMSNKTGVVRSPFEYPQYYLAEPWKYRLVCCYIFFLIST   50
Hrod        MNGTEGPNFYVPFSNATGVVRSPFEYPQYYLAEPWQFSMLAAYMFLLIVL   50
GFrod       MNGTEGDMFYVPMSNATGIVRSPYDYPQYYLVAPWAYACLAAYMFFLIIT   50
Consensus   MNGTEG..FYVP.SN.TG.VRSP..YPQYYL..PW.......Y.F.L...

GFgr-1      GLPINGLTLVVTAQHKKLRQPLNFILVNLAVAGTIMVCFGFTVTFYTAIN  100
GFgr-2      GLPINGLTLICTAQHKKLRQPLNFILVNLAVAGAIMVCFGFTVTFYTAIN  100
CNgr        GLPINLLTLLVTFKHKKLRQPLNYILVNLAVADLFMACFGFTVTFYTAWN  100
Hrod        GFPINFLTLYVTVQHKKLRTPLNYILLNLAVADLFMVLGGFTSTLYTSLH  100
GFrod       GFPVNFLTLYVTIEHKKLRTPLNYILLNLAISDLFMVFGGFTTTMYTSLH  100
Consensus   G.P.N.LTL..T..HKKLR.PLN.IL.NLA.....M...GFT.T.YT...

GFgr-1      GYFVLGPTGCAVEGFMATLGGEVALWSLVVLAIERYIVVCKPMGSFKFSS  150
GFgr-2      GYFALGPTGCAVEGFMATLGGEVALWSLVVLAIERYIVVCKPMGSFKFSS  150
CNgr        GYFVFGPVGCAVEGFFATLGGQVALWSLVVLAIERYIVVCKPMGNFRFSA  150
Hrod        GYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIERYVVVCKPMSNFRFGE  150
GFrod       GYFVFGRVGCNPEGFFATLGGEMGLWSLVVLAFERWMVVCKPVSNFRFGE  150
Consensus   GYF..G..GC..EGF.ATLGG...LWSLVVLA.ER..VVCKP...F.F..

GFgr-1      SHAFAGIAFTWVMALACAAPPLFGWSRYIPEGMQCSCGPDYYTLNPDYNN  200
GFgr-2      THASAGIAFTWVMAMACAAPPLVGWSRYIPEGIQCSCGPDYYTLNPEYNN  200
CNgr        THAMMGIAFTWVMAFSCAAPPLFGWSRYMPEGMQCSCGPDYYTHNPDYHN  200
Hrod        NHAIMGVAFTWVMALACAAPPLAGWSRYIPEGLQCSCGIDYYTLKPEVNN  200
GFrod       NHAIMGVVFTWFMACTCAVPPLVGWSRYIPEGMQCSCGVDYYTRPQAYNN  200
Consensus   .HA..G..FTW.MA..CA.PPL.GWSRY.PEG.QCSCG.DYYT......N

GFgr-1      ESYVIYMFVCHFILPVAVIFFTYGRLVCTVKAAAAQQQDSASTQKAEREV  250
GFgr-2      ESYVLYMFICHFILPVTIIFFTYGRLVCTVKAAAAQQQDSASTQKAEREV  250
CNgr        ESYVLYMFVIHFIIPVVVIFFSYGRLICKVREAAAQQQESATTQKAEKEV  250
Hrod        ESFVIYMFVVHFTIPMIIIFFCYGQLVFTVKEAAAQQQESATTQKAEKEV  250
GFrod       ESFVIYMFIVHFIIPLIVIFFCYGRLVCTVKEAAAQHEESETTQRAEREV  250
Consensus   ES.V.YMF..HF.IP...IFF.YG.L...V..AAAQ...S..TQ.AE.EV

GFgr-1      TKMVILMVFGFLIAWTPYATVAAWIFFNKGADFSAKFMAIPAFFSKSSAL  300
GFgr-2      TKMVILMVLGFLVAWTPYATVAAWIFFNKGAAFSAQFMAIPAFFSKTSAL  300
CNgr        TRMVILMVLGFMLAWTPYAVVAFWIFTNKGADFTATLMAVPAFFSKSSSL  300
Hrod        TRMVIIMVIAFLICWVPYASVAFYIFTHQGSNFGPIFMTIPAFFAKSAAI  300
GFrod       TRMVVIMVIGFLICWIPYASVAWYIFTHQGSEFGPVFMTLPAFFAKTAAV  300
Consensus   T.MV..MV..F...W.PYA.VA..IF...G..F....M..PAFF.K....

GFgr-1      YNPVIYVLLNKQFRNCMLTTIFCGKNPLGDDESS-TVSTSKTEVSSVS--  347
GFgr-2      YNPVIYVLLNKQFRSCMLTTLFCGKNPLGDEESS-TVSTSKTEVSSVS--  347
CNgr        YNPIIYVLMNKQFRNCMITTICCGKNPFGDEDVSSTVSQSKTEVSSVSSS  350
Hrod        YNPVIYIMMNKQFRNCMLTTICCGKNPLGDDE-A-SATVSKTETSQV---  345
GFrod       YNPCIYICMNKQFRHCMITTLCCGKNPFEEEEGA-STTASKTEASSVSSS  349
Consensus   YNP.IY...NKQFR.CM.TT..CGKNP.............SKTE.S.V.--

GFgr-1      ---PA                                               349
GFgr-2      ---PA                                               349
CNgr        QVSPA                                               355
Hrod        --APA                                               348
GFrod       SVSPA                                               354
Consensus      PA
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FIGURE 1: Alignment between human rod opsin (Hrod; Nathans & Hogness, 1984), chicken green opsin (CNgr; Wang et al., 1992; Okano et al., 1992), and the amino acid sequences deduced from goldfish rod opsin cDNA GFrod and goldfish green opsin cDNAs GFgr-1 and GFgr-2.

MATERIALS AND METHODS

Cloning of Goldfish Opsin Sequences. Degenerate primers were designed on the basis of a consensus opsin sequence obtained by aligning all known vertebrate opsin sequences. Primer sequences were as follows: forward primer, 5′-AAGAAGCTNCGNACNCCNCTNAA-3′; reverse primer, 5′-RAANATNACNGGRTTRAA-3′ where N equals G, A, T, or C and R equals G or A. PCR amplification of a λgt10 goldfish retina cDNA library (Cauley et al., 1989) yielded bands of the expected size after 30 cycles of amplification using the following cycling parameters: 94 °C, 1 min; 42 °C, 2 min; 72 °C, 3 min. PCR products were cloned into pUC19 and divided into classes based on sequence. Full-length cDNA clones were obtained by screening the cDNA library with

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GFred       MAEQWGDAIFAARRRGD---ETTRESMFVYTNSNN--TRDPFEGPNYHIA   45
Hred        MAQQWSLQRLAGRHPQDSYEDSTQSSIFTYTNSN--STRGPFEGPNYHIA   48
Consensus   MA.QW.....A.R...D.....T..S.F.YTNSN...TR.PFEGPNYHIA

GFred       PRWVYNLATVWMFFVVVASTFTNGLVLVATAKFKKLRHPLNWILVNLAVA   95
Hred        PRWVYHLTSVWMIFVVTASVFTNGLVLAATMKFKKLRHPLNWILVNLAVA   98
Consensus   PRWVY.L..VWM.FVV.AS.FTNGLVL.AT.KFKKLRHPLNWILVNLAVA

GFred       DLAETLLASTISVTNQFFGYFILGHPMCIFEGFTVSVCGIAGLWSLTVIS  145
Hred        DLAETVIASTISIVNQVSGYFVLGHPMCVLEGYTVSLCGITGLWSLAIIS  148
Consensus   DLAET..ASTIS..NQ..GYF.LGHPMC..EG.TVS.CGI.GLWSL..IS

GFred       WERWVVVCKPFGNVKFDAKWASAGIIFSWVWSAIWCAPPIFGWSRFWPHG  195
Hred        WERWLVVCKPFGNVRFDAKLAIVGIAFSWIWSAVWTAPPIFGWSRYWPHG  198
Consensus   WERW.VVCKPFGNV.FDAK.A..GI.FSW.WSA.W.APPIFGWSR.WPHG

GFred       LKTSCGPDVFSGSEDPGVQSYMIVLMITCCIIPLAIIILCYIAVWLAIRT  245
Hred        LKTSCGPDVFSGSSYPGVQSYMIVLMVTCCIIPLAIIMLCYLQVWLAIRA  248
Consensus   LKTSCGPDVFSGS..PGVQSYMIVLM.TCCIIPLAII.LCY..VWLAIR.

GFred       VAQQQKDSESTQKAEKEVSRMVVVMIFAYCFCWGPYTFCACFAAANPGYA  295
Hred        VAKQQKESESTQKAEKEVTRMVVVMIFAYCVCWGPYTFFACFAAANPGYA  298
Consensus   VA.QQK.SESTQKAEKEV.RMVVVMIFAYC.CWGPYTF.ACFAAANPGYA

GFred       FHPLAAAMPAYFAKSATIYNPIIYVFMNRQFRVCIMQLFGKKVDDGSEVS  345
Hred        FHPLMAALPAYFAKSATIYNPVIYVFMNRQFRNCILQLFGKKVDDGSELS  348
Consensus   FHPL.AA.PAYFAKSATIYNP.IYVFMNRQFR.CI.QLFGKKVDDGSE.S

GFred       -TSKTEVSSVA---PA                                    357
Hred        SASKTEVSSVSSVSPA                                    364
Consensus   ..SKTEVSSV....PA
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FIGURE 2: Alignment between human red opsin (Hred; Nathans et al., 1986) and the amino acid sequence deduced from goldfish red opsin cDNA GFred.

individual PCR clones under high-stringency conditions (Colony/PlaqueScreen protocol, NEN).

Since all sequences obtained by PCR showed homology to rod or red/green visual pigments, the library was screened with a human blue opsin probe (Nathans et al., 1986) at low-stringency to isolate a goldfish blue opsin cDNA.

DNA Sequencing and Data Analysis. cDNA fragments from hybridizing phage were excised with *Eco*RI and subcloned into vectors suitable for sequencing by the dideoxy chain termination method (Sequenase, United States Biochemical). pBluescript(SK-) templates were sequenced using synthetic oligonucleotide primers while unidirectional deletions were constructed on M13mp19 templates using the Cyclone Biosystem Kit (IBI).

Goldfish opsins were compared to other opsins in the Swiss-Prot database. Sequence alignments and a dendrogram were then constructed using Gene Works Version 2.0 (IntelliGenetics, Inc.) which uses amino acid sequence identity to approximate phylogenetic order. Initiator methionines were identified based on the fulfillment of Kozak criteria for eukaryotic initiation sites as well as relatedness to other opsin sequences.

Northern Blots. Total RNA from headless goldfish and total RNA from goldfish retinae were isolated using the acid/phenol method (Chomczynski & Sacchi, 1987). RNA blots were prepared by standard methods (Sambrook et al., 1989). Filters were probed, and high-stringency washes were performed according to manufacturer's instructions (Zetaprobe, Bio-Rad).

Expression of Goldfish Opsin Sequences. To determine the spectral sensitivities of visual pigments encoded by goldfish clones, the coding regions of particular clones were inserted into the expression vector pCIS (Gorman et al., 1990). The 5′-end of each clone was constructed by PCR so as to replace the goldfish 5′-untranslated region with the ribosomal binding site from bovine rod opsin (Nathans & Hogness, 1983). Constructs were cotransfected with an SV40 T-antigen expression plasmid by the calcium phosphate method into 20–60 10-cm-diameter plates of 293S cells (a suspension-adapted variant of a human embryonic kidney cell line, ATCC CRL 1573). Transfection, membrane isolation, and reconstitution with 11-*cis*-retinal were performed as described previously (Merbs & Nathans, 1992). Reconstituted membranes were resuspended in either 2% CHAPS or 5% digitonin dissolved in 50 mM HEPES, pH 6.5, 140 mM NaCl, 3 mM $MgCl_2$, and 2 mM EDTA.

Absorbance Spectra and Hydroxylamine Reactivity. Solubilized membrane supernatants were maintained at 20 °C in a water-jacketed cuvette holder. A Kontron Instruments Uvikon 860 spectrophotometer was used to record absorption spectra before and after bleaching with a 150-W fiber optic light source. Samples were bleached for 30 s–5 min using a 480-nm short-wave cutoff filter for goldfish blue pigment and either a 530-nm short-wave cutoff filter or a 580-nm narrow-band-pass filter for pigments absorbing at higher wavelengths.

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GFblu      MKQVPEFHEDFYIPIPLDINNLSAYSPFLVPQDHLGNQGIFMAMSVFMFF      50
Hblu       MRKMSE--EEFYL-----FKNISSVGPWDGPQYHIAPVWAFYLQAAFMGT      43
Consensus  M....E..E.FY........N.S...P...PQ.H......F.....FM..

GFblu      IFIGGASINILTILCTIQFKKLRSHLNYILVNLSIANLFVAIFGSPLSFY     100
Hblu       VFLIGFPLNAMVLVATLRYKKLRQPLNYILVNVSFGGFLLCIF----SVF      89
Consensus  .F..G...N......T...KKLR..LNYILVN.S.......IF....S..

GFblu      SFF----NRYFIFGATACKIEGFLATLGGMVGLWSLAVVAFERWLVICKP     146
Hblu       PVFVASCNGYFVFGRHVCALEGFLGTVAGLVTGWSLAFLAFERYIVICKP     139
Consensus  ..F....N.YF.FG...C..EGFL.T..G.V..WSLA..AFER..VICKP

GFblu      LGNFTFKTPHAIAGCILPWISALAASLPPLFGWSRYIPEGLQCSCGPDWY     196
Hblu       FGNFRFSSKHALTVVLATWTIGIGVSIPPFFGWSRFIPEGLQCSCGPDWY     189
Consensus  .GNF.F...HA.......W......S.PP.FGWSR.IPEGLQCSCGPDWY

GFblu      TTNNKYNNESYV--MFLFCFCFAVPFGTIVFCYGQLLITLKLAAKAQADS     244
Hblu       TVGTKYRSESYTWFLFIFCFIVPLSLICFSY--TQLLRALKAVAAQQQES     237
Consensus  T...KY..ESY....F.FCF..............QLL..LK..A..Q..S

GFblu      ASTQKAEREVTKMVVVMVLGFLVCWAPYASFSLWIVSHRGEEFDLRMATI     294
Hblu       ATTQKAEREVSRMVVVMVGSFCVCYVPYAAFAMYMVNNRNHGLDLRLVTI     287
Consensus  A.TQKAEREV..MVVVMV..F.VC..PYA.F....V..R....DLR..TI

GFblu      PSCLSKASTVYNPVIYVLMNKQFRSCMMKMVCGKNIEEDEASTSSQVTQV     344
Hblu       PSFFSKSACIYNPIIYCFMNKQFQACIMKMVCGKAMTDESDTCSSQKTEV     337
Consensus  PS..SK....YNP.IY..MNKQF..C.MKMVCGK.........SSQ.T.V

GFblu      SSVA----PEK                                          351
Hblu       STVSSTQVGPN                                          348
Consensus  S.V........
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FIGURE 3: Alignment between human blue opsin (Hblu; Nathans et al., 1986) and the amino acid sequence deduced from goldfish blue opsin cDNA GFblu.

Difference curves were then calculated from pre- and post-bleach spectra. Changes in light scattering during the course of the experiment occasionally produced a sloping background at lower wavelengths. Difference spectra containing this artifact were corrected by subtracting a scaled difference spectrum of detergent-solubilized 293S cell membranes. Hydroxylamine reactivity was determined by adding 800 mM hydroxylamine, pH 6.0, to a final concentration of 50 mM and measuring spectra at specific time intervals. Data were transferred to a MacIntosh computer equipped with CRICKETGRAPH software. To assign λ_{max}, the best-fitting fifth-order polynomial was calculated for a 100-nm portion of each spectrum centered about the approximate peak.

RESULTS

Isolation of Goldfish Opsin cDNAs. On the basis of a comparison of conserved amino acids among vertebrate opsins, degenerate PCR primers were designed corresponding to the first extracellular loop and the C-terminal end of the seventh transmembrane helix in a consensus opsin sequence. In human rod opsin, these regions correspond to amino acids 66–73 and 301–306, respectively. Following low-stringency amplification of goldfish retina cDNA, PCR products from a band of the expected size (750 bp) were cloned and sequenced. The three types of sequence obtained were named PCRJ1, PCRJ2, and PCRJ4. PCRJ1 and PCRJ2 were found to be homologous to human rod opsin while PCRJ4 was found to be homologous to human red cone opsin. To obtain complete sequence information on these clones and to isolate further homologous sequences from goldfish, the cloned PCR fragments were used to screen a goldfish retina cDNA library (Cauley et al., 1989). This screen resulted in full-length clones for PCRJ1 (GFrod), PCRJ2 (GFgr-1), a PCRJ2 homolog (GFgr-2), and PCRJ4 (GFred). The four clones were completely sequenced (Figures 1 and 2).

Since no goldfish sequences similar to a blue opsin sequence were obtained by PCR, the goldfish cDNA library was screened with a probe comprising the coding region of human blue opsin (Nathans et al., 1986). A full-length opsin clone was obtained (GFblu, Figure 3).

Sequence Comparisons. An alignment of clones GFrod, GFgr-1, and GFgr-2 with human rod opsin and chicken green opsin is shown in Figure 1. GFred and the human red opsins are both aligned in Figure 2, and GFblu and human blue opsins are shown in Figure 3. GFrod is 76% identical to human rod opsin and on this basis was tentatively assumed to encode goldfish rod opsin. GFgr-1 is 91% identical to GFgr-2, 72% identical to human rod opsin, and 79% identical to chicken green opsin. GFgr-2 is 91% identical to GFgr-1, 71% identical to human rod opsin, and 77% identical to chicken green opsin. GFred is identified as goldfish red opsin on the basis of two criteria. GFred and the human red opsins are 79% identical. In addition, GFred has three key amino acid residues (serine-180, tyrosine-277, and threonine-285) which are conserved in all red opsins. On the basis of sequence comparison and electroretinography of primate visual pigments, these three amino acids were predicted to be primarily responsible for the spectral shift from 530 to 560 nm (Neitz et al., 1991).

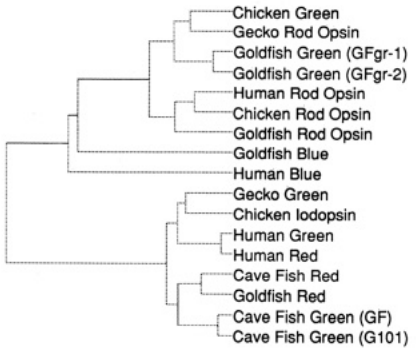

FIGURE 4: Dendrogram illustrating the evolutionary relatedness of visual pigments in the goldfish and other vertebrates: chicken green opsin (Wang et al., 1992; Okano et al., 1992), gecko rod opsin (Kojima et al., 1992), goldfish green opsin GFgr-1, goldfish green opsin GFgr-2, human rod opsin (Nathans & Hogness, 1984), chicken rod opsin (Takao et al., 1988), goldfish rod opsin, goldfish blue opsin, human blue opsin (Nathans et al., 1986), gecko green opsin (Kojima et al., 1992), chicken iodopsin (Tokunaga et al., 1990), human green opsin (Nathans et al., 1986), human red opsin (Nathans et al., 1986), cave fish red opsin (Yokoyama & Yokoyama, 1990a), goldfish red opsin, cave fish green opsin (G_F; Yokoyama & Yokoyama, 1990b), and cave fish green opsin (G101; Yokoyama & Yokoyama, 1990a). Branch lengths approximate the evolutionary distance.

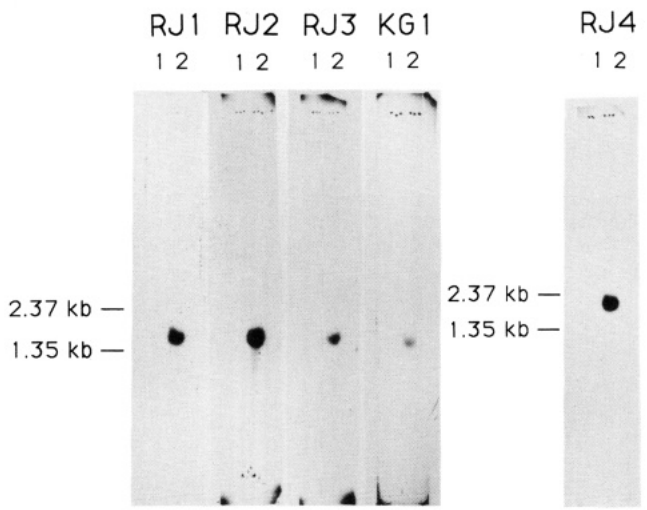

FIGURE 5: Northern hybridizations of goldfish cDNA opsin fragments to total RNA from headless goldfish (lane 1) and goldfish eyes (lane 2). Blots probed with cDNA fragments RJ2 (GFgr-1), RJ3 (GFgr-2), RJ4 (GFred), and KG1 (GFblu) were exposed for 17 h. The blot probed with the RJ1 (GFrod) cDNA fragment was exposed for 1.5 h. Synthetic RNAs were used as size markers (GIBCO/BRL).

Recently, the same residues were substituted into a rod opsin and were shown to red-shift the rhodopsin absorption spectrum (Chan et al., 1992). GFblu is approximately 48% identical to both human rod opsin and human blue opsin and shares limited sequence homology with other visual pigments.

Amino acid sequences deduced from the goldfish cDNA clones were compared to other rod and cone opsin sequences. The results of this comparison are summarized in a dendrogram which is based on percent amino acid identity (Figure 4).

Northern Blots. Northern blots of headless goldfish RNA and eye-derived RNA demonstrate eye-specific expression of all opsin clones (Figure 5). The sizes of transcripts are as follows: GFrod, 1.6 kb; GFgr-1, 1.5 kb; GFgr-2, 1.4 kb; GFred, 1.8 kb; GFblu, 1.4 kb. The relative signal intensity of each transcript is GFrod $\gg$ GFgr-1 > GFred $\gg$ GFgr-2 > GFblu.

Absorbance Properties and Hydroxylamine Sensitivity. The goldfish sequences were subcloned into the eukaryotic expression vector pCIS and expressed in tissue culture cells

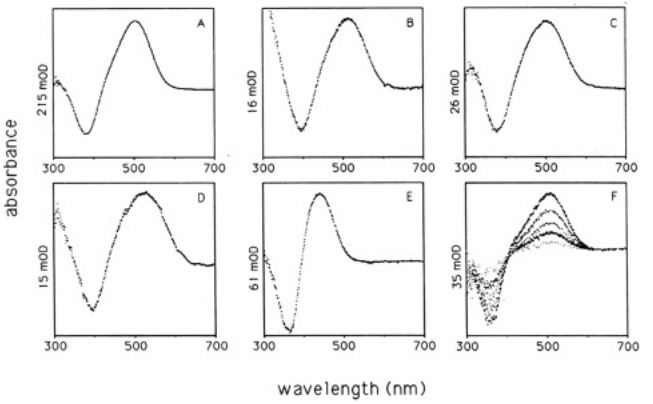

FIGURE 6: (A–D) Photobleaching difference spectra of goldfish pigments GFrod, GFgr-1, GFgr-2, and GFred, respectively. Positive peaks at wavelengths greater than 400 nm correspond to the visual pigment; negative peaks at 389 nm are derived from release of *all-trans*-retinal. (E) Photobleaching difference spectrum of GFblu in the presence of hydroxylamine. The positive peak at 440 nm corresponds to goldfish blue pigment; the negative peak at 367 nm is generated by retinal oxime. (F) Reactivity of GFgr-2 pigment with hydroxylamine at 20 °C. Hydroxylamine was added to a final concentration of 50 mM at $t = 0$ min. Each plot represents the difference between absorption at (top to bottom) $t = 10.3$, 16.3, 22.4, 28.4, and 36.4 min and absorption at $t = 46.5$ min.

in order to confirm the assignment of GFrod and GFred and to identify GFgr-1, GFgr-2, and GFblu. Photobleaching difference spectra of detergent-solubilized cell membranes containing goldfish visual pigments are shown in Figure 6. In all cases, the positive component of the curve is due to the formation of recombinant pigment, and the negative component is due to the release of retinal upon bleaching. Spectra were determined in the presence and/or absence of hydroxylamine. Mean absorption maxima (λ_{max}) are shown in Table I.

The similarity between photobleaching difference spectra taken with and without hydroxylamine shows that there is little accumulation of photoproducts for pigments encoded by GFgr-1, GFgr-2, and GFblu. However, the peak absorbance in the photobleaching difference spectrum of the GFrod pigment is red-shifted from 492 to 502 nm in the absence of hydroxylamine, indicating that under these conditions a long-lived photoproduct exists.

A comparison of the recombinant pigments with those found in the goldfish retina is complicated by the presence in goldfish of 11-*cis*-3-dehydroretinal under certain conditions of light and temperature (Tsin et al., 1981). Binding of 11-*cis*-3-dehydroretinal results in pigments, called porphyropsins, which are red-shifted compared with their 11-*cis*-retinal-containing counterparts. In this study, recombinant visual pigments were reconstituted only with 11-*cis*-retinal. MSP of goldfish photoreceptors from fish which have been acclimated under conditions where 11-*cis*-retinal is the predominant chromophore has detected a rod pigment with absorption at 499 nm, a green cone pigment at 509 nm, and a blue cone pigment at 441 nm. It is likely that these correspond to GFrod, GFgr-1 or GFgr-2, and GFblu pigments, respectively. No counterpart to GFred has been found by MSP.

The results of hydroxylamine treatment of the GFrod, GFgr-1, GFgr-2, and GFblu pigments are shown in Table I and in Figure 6. Pigments encoded by GFgr-1, GFgr-2, and GFblu react rapidly with hydroxylamine with average $t_{1/2}$ values of 10.9, 8.7, and 9.0 min, respectively. These sensitivities are consistent with those of cone pigments. The pigment encoded by GFrod did not react detectably with hydroxylamine over

Table I: Absorption Maxima and Hydroxylamine Sensitivities of Goldfish Visual Pigments

pigment	λ_{max} (nm)[a]	n^a	λ_{max} (nm)[b]	n^b	$t_{1/2}$ (min)[c]	n^c
GFrod	502.3 ± 2.5	2	492.0	1	nr	2
GFgr-1	511.1 ± 4.1	2	511.3 ± 1.8	2	10.9	1
GFgr-2	504.5 ± 2.1	2	506.0 ± 0.0	2	8.7 ± 0.7	2
GFred	524.7 ± 0.8	3	nd	0	nd	0
GFblu	441.0[d]	1[d]	440.1 ± 2.6	5	9.0 ± 0.4	2

[a,b,c] Errors are given as standard deviations. All measurements were taken at 20 °C. [a] Trials were conducted in the absence of hydroxylamine. λ_{max} values were computed from photobleaching difference spectra obtained by subtracting the average of four postbleach spectra from the average of four prebleach spectra. [b] Trials were conducted in the presence of hydroxylamine. λ_{max} values were computed from photobleaching difference spectra obtained by subtracting single pre- and postbleach spectra. [c] Half-times for reactions between specific goldfish visual pigments and hydroxylamine were computed by plotting the natural log of the absorbance at 510 nm (GFrod, GFgr-1, GFgr-2) or at 450 nm (GFblu) as a function of time for specific time intervals from 10 min up to 46.5 min after the addition of hydroxylamine. (Data collected for the first 10 min were not considered to minimize base-line distortion caused by the reaction of free 11-*cis*-retinal with hydroxylamine.) $t_{1/2} = 0.693/k$ where $-k$ equals the slope of the best fitting straight line drawn through all points. [d] Distortion created by the release of *all-trans*-retinal was removed by the addition of a scaled absorption curve of *all-trans*-retinal to the photobleaching difference spectrum. nd, not determined. nr, no reaction.

the 47-min experiment, consistent with its assignment as goldfish rhodopsin.

DISCUSSION

Biochemical Properties of Goldfish Visual Pigments. Goldfish opsins encoded by cDNA clones GFrod, GFgr-1, GFgr-2, GFred, and GFblu possess several structural features common to other opsins. Lysine-296, which forms a Schiff base linkage to the retinal chromophore (Bownds, 1967; Wang et al., 1980), and glutamate-113, thought to be the Schiff base counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989, 1991; Nathans, 1990), are found in all five goldfish opsins. Other shared features include (i) cysteine residues at positions 110 and 187 that form a disulfide bond necessary for the formation of functional rhodopsin (Karnik & Khorana, 1990), (ii) glutamate-134 and arginine-135, believed to interact directly with the G-protein transducin (Franke et al., 1990), and (iii) numerous C-terminal serine and threonine residues which serve as potential phosphorylation sites for rhodopsin kinase (Hargrave, 1982; Palczewski et al., 1988).

As expected based on the strong homology found among rod opsins from various organisms, the goldfish rod opsin GFrod does not differ substantially from its human counterpart. Moreover, goldfish rhodopsin is the most abundantly expressed pigment in the goldfish eye and is insensitive to attack by hydroxylamine.

The goldfish opsins encoded by clones GFgr-1 and GFgr-2 are homologous to the recently reported chicken green opsin (Wang et al., 1992; Okano et al., 1992). Together they form a new class of visual pigments which shares characteristics of both rod and cone opsins. Rodlike properties include (i) a higher identity to rod opsins (63–76%) than to cone opsins (37–53%), (ii) two potential asparagine glycosylation sites at positions 2 and 15 (Fukada et al., 1979), and (iii) a conserved histidine at residue 211 which regulates the equilibrium between metarhodopsin I and metarhodopsin II photoproducts (Weitz & Nathans, 1992). Like many cone pigments, the goldfish pigments GFgr-1 and GFgr-2 and the chicken green pigment are rapidly inactivated by hydroxylamine.

Direct evidence linking clones GFgr-1 and GFgr-2 to cone cells comes from both spectroscopic and in situ hybridization analyses. Riboprobes made from these clones hybridize exclusively to green cones in goldfish eyes, specifically to the accessory member of double cones (SD) and to some long single cones (LS) (Raymond et al., 1992). MSP shows that all SD and green LS cones absorb at 509 nm under conditions where 11-*cis*-retinal is the predominant chromophore and at 537 nm when 11-*cis*-3-dehydroretinal predominates (Tsin et al., 1981). Since GFgr-1 and GFgr-2 pigments absorb respectively at 511 and 505 nm when reconstituted with 11-*cis*-retinal, it is likely that goldfish use these rhodopsin homologues under conditions of 11-*cis*-3-dehydroretinal acclimation to detect green light. Pigments GFgr-1 and GFgr-2 could be expressed as a mixture in both SD and green LS cones to produce a mean peak absorption between 505 and 511 nm. This would account for the fact that MSP has detected absorption maxima of 509 nm in both cone types. Alternatively, GFgr-1 may be expressed exclusively in SD cones and GFgr-2 in the LS green cone type which exist in approximately a 3 to 1 ratio in goldfish retinae (Stell & Hárosi, 1976; Marc & Sperling, 1976). This possibility is suggested by Northern blot analyses which show a high level of expression of GFgr-1 relative to GFgr-2.

Although goldfish blue pigment GFblu shows limited homology to human blue pigment, it is easily bleached by hydroxylamine and absorbs maximally at 441 nm, near its human counterpart. It is interesting to note that residue 292 is serine in goldfish, human, and chicken (Okano et al., 1992) blue opsins and alanine in all long-wavelength opsins published to date. A possible interaction of this residue with retinal is suggested by an alanine-292-aspartate mutation in bovine rhodopsin that causes a 10-nm blue-shift in the absorption maximum and reduced levels of transducin activation (Nakayama & Khorana, 1991).

No counterpart to the goldfish red cone opsin isolated in this study has been found by MSP. When reconstituted with 11-*cis*-retinal, GFred pigment absorbs at 525 nm whereas the goldfish red pigment detected by MSP absorbs maximally at 579 nm when 11-*cis*-retinal predominates and at 625 nm when 11-*cis*-3-dehydroretinal predominates (Tsin et al., 1981). Nevertheless, GFred is highly homologous to both human and cave fish red opsins. One possible explanation is that the red cDNA clone reported here encodes a polymorphic variant[1] of the normal goldfish red opsin that has not been observed by MSP. Opsin polymorphisms are not uncommon in fish, and sometimes members of a particular species have different opsin sequences. For example, new opsins are expressed in the American eel upon migration from brackish to salt water (Beatty, 1975) and in the juvenile pollack when it moves to lower depths to feed on small fish and crustacae (Shand et al., 1988). Individual guppies have either one or two long-wavelength opsins that are expressed in different cone cells

[1] Subsequently, a second full-length putative red opsin cDNA was isolated from the goldfish retina library and sequenced. The coding region of this new clone was found to differ from GFred cDNA at five nucleotide positions with two silent substitutions and three substitutions that cause amino acid changes. These data support the conclusion that opsin polymorphisms are present in the goldfish.

or as a mixture in a third cone type (Archer & Lythgoe, 1990).

None of the cDNA clones identified in this study appear to encode the goldfish UV-sensitive pigment. Its absence may reflect a low degree of homology with the PCR primers and the human blue opsin probe or a low abundance of its mRNA in goldfish retinae.

Visual Pigment Evolution. The similarity between opsins from distantly related organisms indicates that they have arisen from a common ancestral gene. The dendrogram shown in Figure 4 illustrates the evolutionary relatedness of human, chicken, goldfish, cave fish, and gecko rod and/or cone opsins. The basic relationships suggested by our model, as they relate to mammalian rod opsins and the human cone opsins, are similar to those previously reported (Iwabe et al., 1989). We propose that long- and short-wavelength prototype genes diverged first. The short-wavelength ancestor then gave rise both to blue opsin genes and to a rod opsin-like gene. The latter evolved into two present day phylogenetic clusters, one which includes the human, chicken, and goldfish rod opsins and a second which includes the goldfish green opsin, the chicken green opsin, and gecko rod opsin. The long-wavelength ancestor evolved into human and cave fish red and green opsins, the gecko green opsin, and the chicken and goldfish red opsins. Our data reveal an unexpected multiplicity of rhodopsin-like green opsins. Together with the chicken green opsin (Wang et al., 1992; Okano et al., 1992), the goldfish GFgr-1 and GFgr-2 opsins show that the gene duplication and divergence within the rod opsin branch of the visual pigment tree occurred early in vertebrate evolution. Although the physiological role(s) of the rhodopsin-like green opsins are not understood, these data suggest that they will be found in many vertebrates.

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