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Multiple Origins of the Green-Sensitive Opsin Genes in Fish

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Abstract. Vertebrate opsins are divided into four major groups: RH1 (rhodopsins), RH2 (rhodopsinlike with various absorption sensitivities), SWS (short-wavelength sensitive), and LWS/MWS (long and middle-wavelength sensitive) groups. The green opsin genes ($g101_{Af}$ and $g103_{Af}$) in a Mexican characin *Astyanax fasciatus* belong to the LWS/MWS group, whereas those in goldfish belong to the RH2 group (Yokoyama 1994, *Mol Biol Evol* 11:32–39). A newly isolated opsin gene ($rh11_{Af}$) from *A. fasciatus* contains five exons and four introns, spanning 4.2 kilobases from start to stop codons. This gene is most closely related to the two green opsin genes of goldfish and belongs to the RH2 group. In the LWS/MWS group, gene duplication of the ancestral red and green opsin genes predates the speciation between *A. fasciatus* and goldfish, suggesting that goldfish also has an additional gene which is orthologous to $g101_{Af}$ and $g103_{Af}$.

Key words: Opsin gene — Visual pigment — Molecular evolution — *Astyanax fasciatus*

Introduction

Many vertebrate eyes contain two types of photoreceptor cells—rods and cones. Rods function in dim light and do not perceive color, whereas cones are responsible for color vision. Photoreceptor molecules, or visual pigments (VPs), are present in the outer segments of these photoreceptor cells. Each VP consists of a transmembrane protein, opsin, and a chromophore that are covalently linked with each other. Many freshwater fishes and amphibians utilize both retinal (vitamin A_1 aldehyde) and 3-dehydroretinal (vitamin A_2 aldehyde) as chromophores. When coupled to vitamin A_2, a VP with the same opsin shifts its absorption maximum (λmax) toward a longer wavelength (e.g., see Dartnall and Lythgoe 1965). The photoreceptor cells in the Mexican characin *Astyanax fasciatus* contain a fairly even mixture of VPs with vitamin A_1 and A_2 chromophores (Kleinschmidt and Harosi 1992). The VPs in the rods, single cones, and the first and second members of double cones in this species have λmax of 520 nm, 453 nm (blue- or short-wavelength sensitive; SWS), 554 nm (green- or medium-wavelength sensitive; MWS), and 596 nm (red- or long-wavelength sensitive; LWS), respectively (Kleinschmidt and Harosi 1992), showing that they are trichromatic like many other vertebrates. Opsins in these VPs are encoded by distinct opsin genes.

Currently known vertebrate opsins have been classified into four distinct phylogenetic groups: (1) an RH1 group consisting of rhodopsins; (2) an SWS group with SWS VPs; (3) an LWS/MWS group with LWS and MWS VPs; and (4) an RH2 group with a mixture of opsins of various absorption sensitivities (Yokoyama 1994; see also Okano et al. 1992). Curiously, two green opsins each in *A. fasciatus* (Yokoyama and Yokoyama 1990a,b) and goldfish (Johnson et al. 1993) belong to the LWS/MWS and RH2 groups, respectively, whereas the red opsins from *A. fasciatus* (Yokoyama and Yokoyama 1990b; Yokoyama et al. 1993) and goldfish (Johnson et al. 1993) belong to the same LWS/MWS group.

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Until now, neither the *A. fasciatus* gene which is orthologous to the two goldfish green opsin genes nor the goldfish gene which is orthologous to the *A. fasciatus* green opsin genes has been reported.

In the present paper, we report the complete sequence of an *Astyanax* opsin gene which belongs to the RH2 group and is most closely related to the two green opsin genes in goldfish. Evolutionary analysis strongly suggests that goldfish has an additional opsin gene which is orthologous to the *Astyanax* green opsin genes.

Materials and Methods

Genomic Library Screening and DNA Sequencing. A genomic library was constructed by using the high-molecular-weight DNA made from one blind cave fish, *Astyanax fasciatus* (Yokoyama and Yokoyama 1990a,b). Thirty-eight positive clones were obtained by using the bovine rhodopsin cDNA (bd20) as a probe (Yokoyama and Yokoyama 1990a,b). Four of these positive clones were identified as overlapping clones of one contiguous region by restriction mapping and Southern blot analyses. One of these clones, λ11, was found to contain a gene (designated $rh11_{Af}$) with the entire coding region and was chosen for further characterization.

The coding regions and introns of $rh11_{Af}$ were sequenced by the dideoxynucleotide chain-termination method using double-stranded templates (Sanger et al. 1977; Hattori et al. 1985) of subclones in Bluescript. The subclones were obtained either by isolation of specific restriction fragments and ligation with Bluescript vector or by deletions of some subclones using exonuclease III and mungbean nuclease (Yokoyama and Yokoyama 1990a,b).

Southern Blot Analysis. High-molecular-weight genomic DNA from both American chameleon (*Anolis carolinensis*) and goldfish (*Carassius auratus*) was prepared by following the procedures of Blin and Stafford (1976); 10 μg per lane of genomic DNA was digested by restriction enzymes *Bam*HI, *Hin*dIII, and *Sst*I, electrophoresed on a 0.5% agarose gel, and transferred to a Hybond-N nylon membrane (Amersham) by using the VacuGene vacuum blotting system. The cDNA clone of human red opsin gene (hs7; Nathans et al. 1986) was labeled with [α-^{32}P]-dATP (deoxyadenosine triphosphate) by the random priming method and used as a hybridization probe. Hybridization was carried out at 55°C using the commercial protocol Hybond-N membrane. Hybridized membrane was washed at 55°C four times (30 min each) in 1 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS.

Sequence Analysis. The amino acid sequence deduced from $rh11_{Af}$ ($Rh11_{Af}$) was compared to those deduced from the rhodopsin genes from brook lamprey (Rh_{Lj}; Hisatomi et al. 1991) and goldfish (Rh_{Ca}; Johnson et al. 1993), red opsin genes from goldfish (R_{Ca}; Johnson et al. 1993) and *Astyanax* ($R007_{Af}$; Yokoyama and Yokoyama 1990b; Yokoyama et al. 1993), green opsin genes from goldfish ($G1_{Ca}$ and $G2_{Ca}$; Johnson et al. 1993) and *Astyanax* ($G101_{Af}$ and $G103_{Af}$; Yokoyama and Yokoyama 1990a,b), blue opsin genes from goldfish (B_{Ca}; Johnson et al. 1993) and *A. fasciatus* (B_{Af}; Yokoyama and Yokoyama 1993), and UV-sensitive gene from zebrafish (UV_{Br}; Robinson et al. 1993).

To construct a rooted phylogenetic tree for these opsins, we used the rhodopsins from *Drosophila melanogaster* ($Rh1_{Dm}$; O'Tousa et al. 1985; Zuker et al. 1985; $Rh2_{Dm}$; Cowman et al. 1986; $Rh3_{Dm}$; Zuker et al. 1987; $Rh4_{Dm}$; Montell et al. 1987), octopus (*Paroctopus defleini*) (Rh_{pd}; Ovchinnikov et al. 1988), squid (*Loligo forbesi*) (Rh_{Lf}; Hall et al. 1991), and crayfish (*Procambarus clarkii*) (Rh_{Pc}; Hariyama et al. 1993).

These amino acid sequences were initially aligned by using a multiple alignment program in CLUSTAL V (Higgins et al. 1992) and then adjusted visually to increase their similarity. The number (K) of amino acid substitutions per site for two sequences was estimated by $K = -\ln(1 - p)$, where p is the proportion of different amino acids between the two sequences. Topology and branch lengths of the phylogenetic tree were estimated by using the neighbor-joining (NJ) method (Saitou and Nei 1987) based on the K values. Bootstrap frequencies for branches of the NJ tree were estimated by bootstrap analysis with 1000 replications (CLUSTAL V; Higgins et al. 1992).

Results and Discussion

$rh11_{Af}$ contains five exons and four introns, spanning 4.2 kb from start to stop codons (Fig. 1). Introns are located in exactly the same positions as the vertebrate rhodopsin genes from cow (Nathans and Hogness 1983), human (Nathans and Hogness 1984), and chicken (Takao et al. 1988) and the blue opsin genes from human (Nathans et al. 1986) and *Astyanax* (Yokoyama and Yokoyama 1993). A consensus TATA box sequence (TATAAA) was found 86 bp upstream from the start codon. Splice junction signals (GT/AG) are conserved in all introns and there is no nonsense mutation in the coding region.

From the deduced amino acid sequence (354 residues long; Fig. 1), several potentially important amino acids can be identified: (1) Lys (299), the site of Schiff base linkage to the chromophore (Bownds 1967; Wang et al. 1980); (2) Glu (116), the Schiff base counterion (Zhukovsky and Oprian 1989; Sakmar et al. 1989; Nathans 1990); (3) Cys (113) and Cys (190), the site for a disulfide bond (Karnik and Khorana 1990); (4) Cys (143), involved in phosphorylation through interacting with the C-terminal tail (Karnik et al. 1993); and (5) multiple serines and threonines in the C-terminal region, potential sites for phosphorylation (Palczewski et al. 1988). These observations, together with no premature termination codon, strongly suggest that $rh11_{Af}$ is a functional gene. Its function, however, remains to be evaluated.

The rooted phylogenetic tree for $rh11_{Af}$ and other fish opsins is shown in Fig. 2. The opsins in fishes are distinguished into four major groups—i.e., RH1, RH2, SWS, and LWS/MWS groups. Among these the RH1 and RH2 groups are most closely related, their common ancestor having diverged from that of SWS opsins, and the common ancestor of all these opsins diverged from that of LWS/MWS opsins before that. (See also Yokoyama 1994.) The groupings of RH1, RH2, LWS/MWS and SWS groups correspond to Rh, M2, L, and a mixture of S and M1 groups in Okano et al. (1992). Note that LWS and MWS opsins belong to two separate groups: (1) $G1_{Ca}$, $G2_{Ca}$, and $Rh11_{Af}$ with unknown function (RH2 group) and (2) $G101_{Af}$, $G103_{Af}$, $R007_{Af}$, and R_{Ca} (LWS/MWS group). When all currently known vertebrate opsins are considered, the latter group also include the red opsins from chicken

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                                                                    GGC TTACCTTCTT GATCACTTTC TGCTAATCCT
CTAAATCCCT TAATCAAGGT ATAAATGCTG CCATTTGGAC ATCGCCTGAG TCACAAGAGG GCAAGGAGCA AGCACTGAAA CCTTTGCCTG ATTTACTGAC AACCACAGAC

                                    10                                  20                                  30
ATG TCA GGT CTA AAT GGT TTT GAG GGG GAT AAC TTT TAC ATC CCT ATG AGT AAC CGC ACG GGG CTC GTA CGG GAT CCA TTT GTT TAT GAG
Met Ser Gly Leu Asn Gly Phe Glu Gly Asp Asn Phe Tyr Ile Pro Met Ser Asn Arg Thr Gly Leu Val Arg Asp Pro Phe Val Tyr Glu
                                    40                                  50                                  60
CAG TAC TAT TTA GCA GAA CCA TGG CAG TTT AAG CTC CTG GCT TGT TAC ATG TTT TTT CTG ATA TGT CTG GGT CTC CCC ATC AAT GGC TTC
Gln Tyr Tyr Leu Ala Glu Pro Trp Gln Phe Lys Leu Leu Ala Cys Tyr Met Phe Phe Leu Ile Cys Leu Gly Leu Pro Ile Asn Gly Phe
                                    70                                  80                                  90
ACC CTT TTT GTC ACG GCT CAG CAC AAG AAA CTT CAA CAG CCG CTC AAC TTC ATC CTG GTC AAC CTG GCT GTG GCC GGA ATG ATC ATG GTC
Thr Leu Phe Val Thr Ala Gln His Lys Lys Leu Gln Gln Pro Leu Asn Phe Ile Leu Val Asn Leu Ala Val Ala Gly Met Ile Met Val
                                   100                                 110                                 120
TGC TTC GGG TTC ACT ATC ACC ATT AGC TCT GCT GTC AAT GGC TAC TTT TAC TTT GGG CCC ACA GCC TGT GCC ATT GAG GGA TTT ATG GCA
Cys Phe Gly Phe Thr Ile Thr Ile Ser Ser Ala Val Asn Gly Tyr Phe Tyr Phe Gly Pro Thr Ala Cys Ala Ile Glu Gly Phe Met Ala
                                                       130                                 140
ACA CTT GGA G GTGAGCAATG   80bp  TTCTTTAAAG  GT GAA GTG GCT CTC TGG TCA TTG GTG GTG CTC GCC ATT GAG AGA TAC ATT GTG GTC
Thr Leu Gly G                               ly Glu Val Ala Leu Trp Ser Leu Val Val Leu Ala Ile Glu Arg Tyr Ile Val Val
                          150                                 160                                 170
TGC AAG CCC ATG GGA AGC TTT AAA TTC TCA GCC AGT CAT GCA TTA GGT GGG ATT GGT TTT ACC TGG TTC ATG GCA ATG ACC TGT GCT GCT
Cys Lys Pro Met Gly Ser Phe Lys Phe Ser Ala Ser His Ala Leu Gly Gly Ile Gly Phe Thr Trp Phe Met Ala Met Thr Cys Ala Ala
                          18                                  0                                  190
CCT CCT CTT GTT GGA TGG TCC AG GTAAGAATTA  1740bp  TATTAAACAG G TAC ATT CCT GAG GGG CTG CAG TGC TCG TGT GGA CCA GAC TAC
Pro Pro Leu Val Gly Trp Ser Ar                              g Tyr Ile Pro Glu Gly Leu Gln Cys Ser Cys Gly Pro Asp Tyr
                    200                                 210                                 220
TAT ACA CTC AAC CCC AAA TAC AAC AAT GAG TCC TAT GTT ATC TAC ATG TTC GTT GTC CAC TTC ATT GTC CCT GTC ACT GTT ATC TTC TTC
Tyr Thr Leu Asn Pro Lys Tyr Asn Asn Glu Ser Tyr Val Ile Tyr Met Phe Val Val His Phe Ile Val Pro Val Thr Val Ile Phe Phe
                    230                                                                 240
ACT TAT GGA CGG CTT GTG TGC ACA GTC AAA TCG  GTAAGAAACC   500bp  TAAACTCCAG GCT GCA GCA GCA CAG CAG GAC TCT GCA TCC ACT
Thr Tyr Gly Arg Leu Val Cys Thr Val Lys Ser                                 Ala Ala Ala Ala Gln Gln Glu Ser Ala Ser Thr
          250                                 260                                 270
CAG AAG GCA GAA AAA GAA GTC ACT CGA ATG GTC ATC CTG ATG GTG GTG GGA TTT TTG GTG GCC TGG ACC CCC TAT GCC ACC GTT GCC GCC
Gln Lys Ala Glu Lys Glu Val Thr Arg Met Val Ile Leu Met Val Val Gly Phe Leu Val Ala Trp Thr Pro Tyr Ala Thr Val Ala Ala
          280                                 290                                 300
TGG ATT TTC TTC AAC AAA GGT GCT GCT TTC ACT GCC CAG TTC ATG GCC GTT CCT GCC TTC TTC TCC AAA AGC TCA GCT CTC TTC AAC CCC
Trp Ile Phe Phe Asn Lys Gly Ala Ala Phe Thr Ala Gln Phe Met Ala Val Pro Ala Phe Phe Ser Lys Ser Ser Ala Leu Phe Asn Pro
          310                                                 320
ATC ATA TAT GTG CTG CTG AAC AAA CAG  GTTAGTCCAT   860bp  TTGATTACAG TTC CGT AAC TGC ATG CTG ACA ACC CTG TTC TGC GGA AAG
Ile Ile Tyr Val Leu Leu Asn Lys Gln                                 Phe Arg Asn Cys Met Leu Thr Thr Leu Phe Cys Gly Lys
    330                                 340                                 350
AAC CCC CTG GGA GAT GAA GAG TCC TCA ACT GTG TCA ACC AAG ACA GAA GTG TCC ACC GTG TCC AGC GTG TCC CCA GCG TAG  AGGCCTCTGT
Asn Pro Leu Gly Asp Glu Glu Ser Ser Thr Val Ser Thr Lys Thr Glu Val Ser Thr Val Ser Ser Val Ser Pro Ala ***

ATCAACACTG ATTCACCATC AGAAATCTCT GAACTTGACT TGCACTTCGG TTTTGCTTAC ATTGTCTTCT CAACAATCCA CAGTATGTGC TGACTGAGAG
ATTTCTTTTG GTAATGTTTG TATTGTTTGC CTCTGTACCA A
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *Astyanax* $rh11_{Af}$ gene. The deduced amino acid sequence is written below each nucleotide triplet.

(Tokunaga et al. 1990), American chameleon (Kawamura and Yokoyama 1993), and human (Nathans et al. 1986) and the green opsins from gecko (Kojima et al. 1992) and human (Nathans et al. 1986; Yokoyama 1994). It should be noted that when $g1_{Ca}$ and $g2_{Ca}$ are expressed in cultured cells and reconstituted with vitamin A_1, the respective VPs attain λmax of 511 and 505 nm and neither of them exhibit green sensitivity. However, Johnson et al. (1993) suggest that goldfish use these opsins under conditions of vitamin A_2 acclimation to detect green light. More recently, Raymond et al. (1993) have shown that $g1_{Ca}$ and $g2_{Ca}$ are in fact expressed in the green photoreceptor cells of goldfish. $r007_{Af}$ and $g103_{Af}$ are also known to be expressed in the eye in approximately equivalent amounts (Yokoyama and Yokoyama 1993).

Figure 2 clearly shows that $Rh11_{Af}$ belongs to the RH2 group together with the green opsins from goldfish (Johnson et al. 1993). The RH2 group also includes the chicken green opsin (Okano et al. 1992; Wang et al. 1992) and the blue gecko opsin (Kojima et al. 1992; see Yokoyama 1994). The existence of $rh11_{Af}$ demonstrates that *A. fasciatus* has genes in both RH2 and LWS/MWS groups. Note that all red and green opsins in *Astyanax*

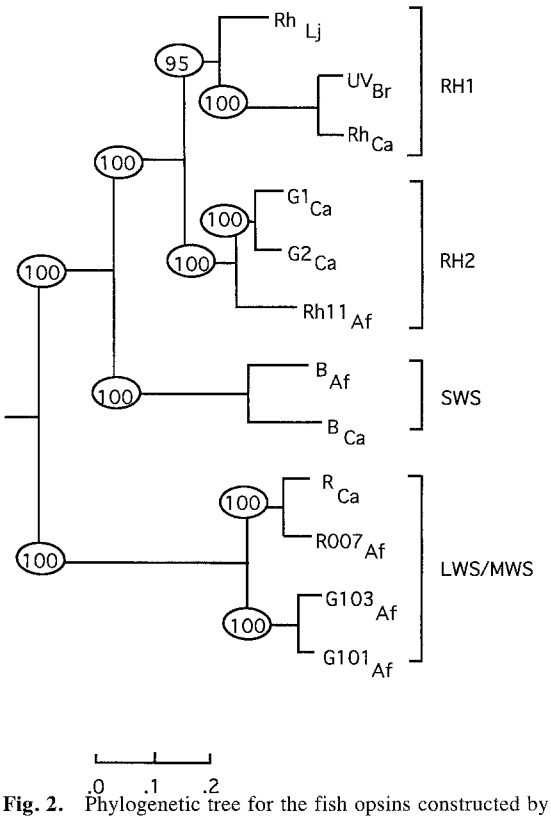

Fig. 2. Phylogenetic tree for the fish opsins constructed by the NJ method (Saitou and Nei 1987) based on *K* values. *Circled numbers* indicate clustering chances generated by bootstrap resampling (CLUSTAL V, Higgins et al. 1992).

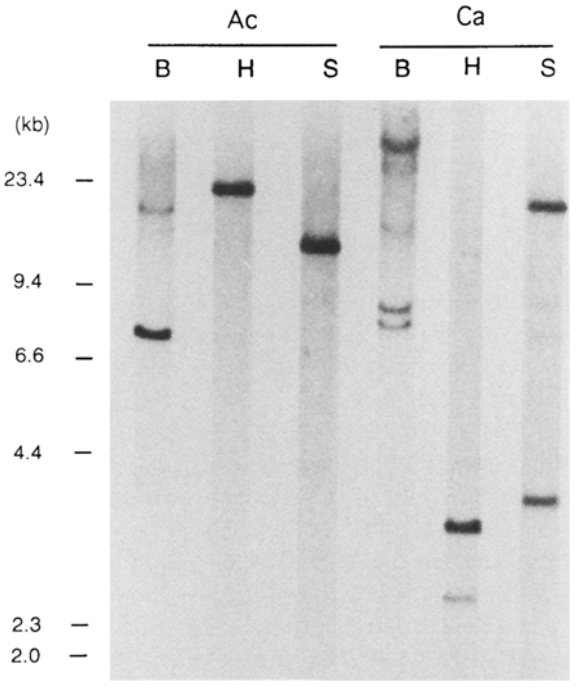

Fig. 3. Southern hybridization of *Bam*HI (*B*)-, *Hin*dIII (*H*)-, and *Sst*I (*S*)-digested genomic DNAs with human red cDNA clone (hs7, Nathans et al. 1986). *Ac* and *Ca* denote American chameleon and goldfish, respectively. λ*Hin*dIII size standards are indicated in kb at the left margin.

(Yokoyama and Yokoyama 1990a,b; Yokoyama et al. 1993) and human (Nathans et al. 1986) belong to the LWS/MWS group, whereas the red and green opsins in goldfish belong to LWS/MWS group and RH2 group, respectively (Yokoyama et al. 1993). At present, a goldfish gene, corresponding to $g101_{Af}$ and $g103_{Af}$ is not known. Interestingly, Fig. 2 shows that gene duplication of the ancestral red and green opsin genes predates the speciation between *A. fasciatus* and goldfish, strongly suggesting that goldfish should have a gene which is orthologous to the green opsin genes in *A. fasciatus*. Johnson et al. (1993) have isolated a second red cDNA clone, which differed from r_{Ca} at five nucleotide positions with three nonsynonymous changes. Thus, the number (*K*) of amino acid substitutions per residue between the two red opsins in goldfish is 0.0084. (See Materials and Methods.) These two opsins diverged very recently and their common ancestor and the ancestor of $R007_{Af}$ diverged before that. Thus, the second red opsin in goldfish is very unlikely to be orthologous to the green opsins in *A. fasciatus*.

To evaluate whether goldfish has more than one gene in the LWS/MWS group, the human red cDNA (hs7; Nathans et al. 1986) was hybridized to the genomic DNAs of goldfish and American chameleon *Anolis carolinensis* (Fig. 3). The *A. carolinensis* genome contains only one red opsin gene, which contains six exons and five introns spanning 3.7 kb from start to stop codons (Kawamura and Yokoyama 1993). The *Hin*dIII- and *Sst*I-digested *Anolis* genomic DNAs show one hybridizing band and the *Bam*HI-digested DNAs show two hybridizing bands, which is consistent with the restriction map of the genomic clone for the red opsin gene isolated from *A. carolinensis* (Kawamura and Yokoyama 1993). The length from the initiation codon to the stop codon of $r007_{Af}$ is only 1.6 kb, and this is the shortest vertebrate opsin gene known, to date (Yokoyama et al. 1993). Similarly, the sizes of $g101_{Af}$ and $g103_{Af}$ are about 3.2 kb and 2.8 kb, respectively. Thus, the LWS/MWS gene in goldfish may also be of a similar length. The Southern hybridization using the three different restriction enzyme digests reveals at least two bands for goldfish, strongly suggesting that the goldfish genome contains more than one copy of the LWS/MWS opsin gene.

In goldfish retina, cones have been distinguished into five morphological classes: (1) double cones with a larger, principal (LD) member; (2) double cones with a shorter, accessory (SD) member; (3) long single (LS) cones; (4) short single (SS) cones; and (5) miniature short single (MSS) cones (Stell and Harosi 1975; Marc and Sperling 1976a,b). The LD cones and many of the LS cones have λmax of 579–625 nm; the SD and the remainder of the LS cones have λmax of 509–537 nm; and the SS cones have λmax of 441–452 nm (Stell and Harosi 1975; Tsin et al. 1981). The MSS is suspected to have UV sensitivity (Hashimoto et al. 1988).

When the rhodopsin (rh_{Ca}) and red (r_{Ca}) and blue (b_{Ca}) goldfish opsin cDNA clones are expressed in cultured cells, reconstituted with vitamin A_1, and measured for their absorption spectra, λmax are shown to be 502, 525, and 441 nm, respectively (Johnson et al. 1993). In situ hybridization analyses show that rh_{Ca} and b_{Ca} are expressed in the rods and SS cones, respectively, and also suggest that r_{Ca} is expressed in the LD cones (Raymond et al. 1993). The λmax of 525 nm for the VPs encoded by r_{Ca} is much shorter than 579 nm measured for the LD cells. It remains to be seen if the two red cDNA clones in goldfish represent two alleles or two loci. The present analyses have suggested the existence of more than one goldfish gene in the LWS/MWS group. If these genes are isolated, then the relationship between these genes and the two red cDNA clones also needs to be clarified.

To understand the red and green vision in fish and many other vertebrates, all opsin genes from the LWS/MWS and RH2 groups in different species must be isolated and characterized. It is also essential to evaluate the λmax values of the VPs encoded by these genes. It is now possible to conduct such absorption analyses together with site-directed mutagenesis at specific nucleotide sites (Sakmar et al. 1989, 1991; Zhukovsky and Oprian 1989; Nathans 1990; Chan et al. 1992). Both of these analyses will reveal the evolutionary processes of the red and green opsin genes and the molecular mechanisms involved in achieving the red and green sensitivities in fish and other vertebrates.

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