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Expression of Rod and Cone Visual Pigments in Goldfish and Zebrafish: A Rhodopsin-like Gene Is Expressed in Cones

Pamela A. Raymond,*† Linda K. Barthel,* Mary Ellen Rounsifer,* Steven A. Sullivan,* and Jennifer K. Knight†
*Department of Anatomy and Cell Biology and †The Neuroscience Program
University of Michigan
Ann Arbor, Michigan 48109-0616

Summary

The primary purpose of the present study was to determine whether a rhodopsin-like gene, which has been postulated to represent the green cone pigment in several species, is in fact expressed in cone photoreceptors instead of rods. The expression patterns of rod opsin and blue and red cone opsins were also examined in both goldfish and zebrafish retinas using colorimetric in situ hybridization. The results demonstrate that the rhodopsin-like gene is expressed in green cones, as predicted. A subset of small cones that do not hybridize with these cRNA probes are tentatively identified as ultraviolet receptors. The results also demonstrate that opsin message in cones is restricted to the perinuclear region, whereas in rods, it is both perinuclear and adjacent to the ellipsoid.

Introduction

Visual pigments have been studied extensively by standard biochemical and spectroscopic methods and, more recently, with molecular techniques (Applebury and Hargrave, 1986; Falk and Applebury, 1987). Rhodopsin (rod opsin) genes have been cloned from several species (Hisatomi et al., 1991; Nathans and Hogness, 1983, 1984; Takao et al., 1988), and they exhibit a high degree of homology (>70%). Among cone pigment genes, those in humans were the first to be identified (Nathans et al., 1986). There are three classes of human color pigments with maximal absorption sensitivity (λ_{max}) at short wavelengths, 426 nm (blue sensitive); medium wavelengths, 530 nm (green sensitive); and long wavelengths, 552–557 nm (red sensitive) (Merbs and Nathans, 1992). The red and green cone opsins are 96% identical, whereas the blue cone opsin is only 43% identical to the red/green pigments, and all three human cone opsins are about 41% identical to human rhodopsin (Nathans et al., 1986). Subsequent to the identification of the human color pigment genes, genomic clones representing red and multiple green cone opsin genes in the cave fish (Astyanax fasciatus) were identified by homology with the human cone pigments (Yokoyama and Yokoyama, 1990a, 1990b). The duplication event that generated red opsin from an ancestral green is thought to be a case of convergent evolution in these fish (Yokoyama and Yokoyama, 1990b).

Among the cone pigments, those in chickens are biochemically the best studied, and within the past two years, the chicken cone opsins have all been cloned. There are four cone pigments in chickens, designated red, green, blue, and violet (Okano et al., 1992; Tokunaga et al., 1990; Wang et al., 1992). The gene coding for the red cone opsin, iodopsin, which is the most abundant visual pigment in chickens, was the first one identified (Tokunaga et al., 1990). Next, an unusual, rhodopsin-like chicken pigment gene was described; the predicted amino acid sequence is 80% identical to chicken rhodopsin, but only 40%–50% identical to the human, chicken, and cave fish cone opsins (Wang et al., 1992; Okano et al., 1992). When the pigment was expressed by transfection of the cDNA into cultured cells, it absorbed maximally at 495 nm and was sensitive to bleaching with hydroxylamine, both characteristic of the chicken green pigment previously identified by biochemical and spectroscopic studies (Wang et al., 1992). Homologous pigments have been identified in gecko (Kojima et al., 1992) and goldfish (Carassius auratus) (Johnson et al., 1993). The chicken blue and violet pigments were the last cloned (Okano et al., 1992). The deduced amino acid sequence of chicken violet (λ_{max} 415 nm) is 80% identical to human blue (λ_{max} 420 nm), whereas chicken blue (λ_{max} 455 nm) shows relatively low similarity (40%–55%) to other mammalian or avian visual pigments (but see below).

Recently, cDNA clones coding for visual pigments in goldfish have been sequenced, and the pigments have been expressed by transfection into cultured cells (Johnson et al., 1993). The clones include a rod opsin (GFrod) and a red (GFred) and blue (GFblu) cone opsin. The GFred clone is 79% identical to human red, whereas GFblu is only 48% identical to human blue (Johnson et al., 1993). A comparision with the chicken blue and violet sequences (not available to the authors at the time of publication) shows that goldfish blue is 68% identical to chicken blue. Two additional clones, 71% and 79% identical to human rhodopsin, were designated as green cone opsins (GFgr-1 and GFgr-2) and are homologous to the chicken and gecko rhodopsin-like green pigments. Since no data have been reported on cellular expression of these rhodopsin-like pigments, their designation as cone pigments remains tentative. If these rhodopsin-like pigments are indeed expressed in green cones, one of the fundamental distinctions between cones and rods—the type of opsin they express—may require reevaluation.

In teleost fish, the different spectral classes of cones are morphologically distinct, and microspectrophotometry has been used to identify the spectral classes of cones in many species (Levine and MacNichol, 1979; Partridge et al., 1989). The high degree of correlation between morphological and spectral classes of cone photoreceptors in fish provides a unique oppor-

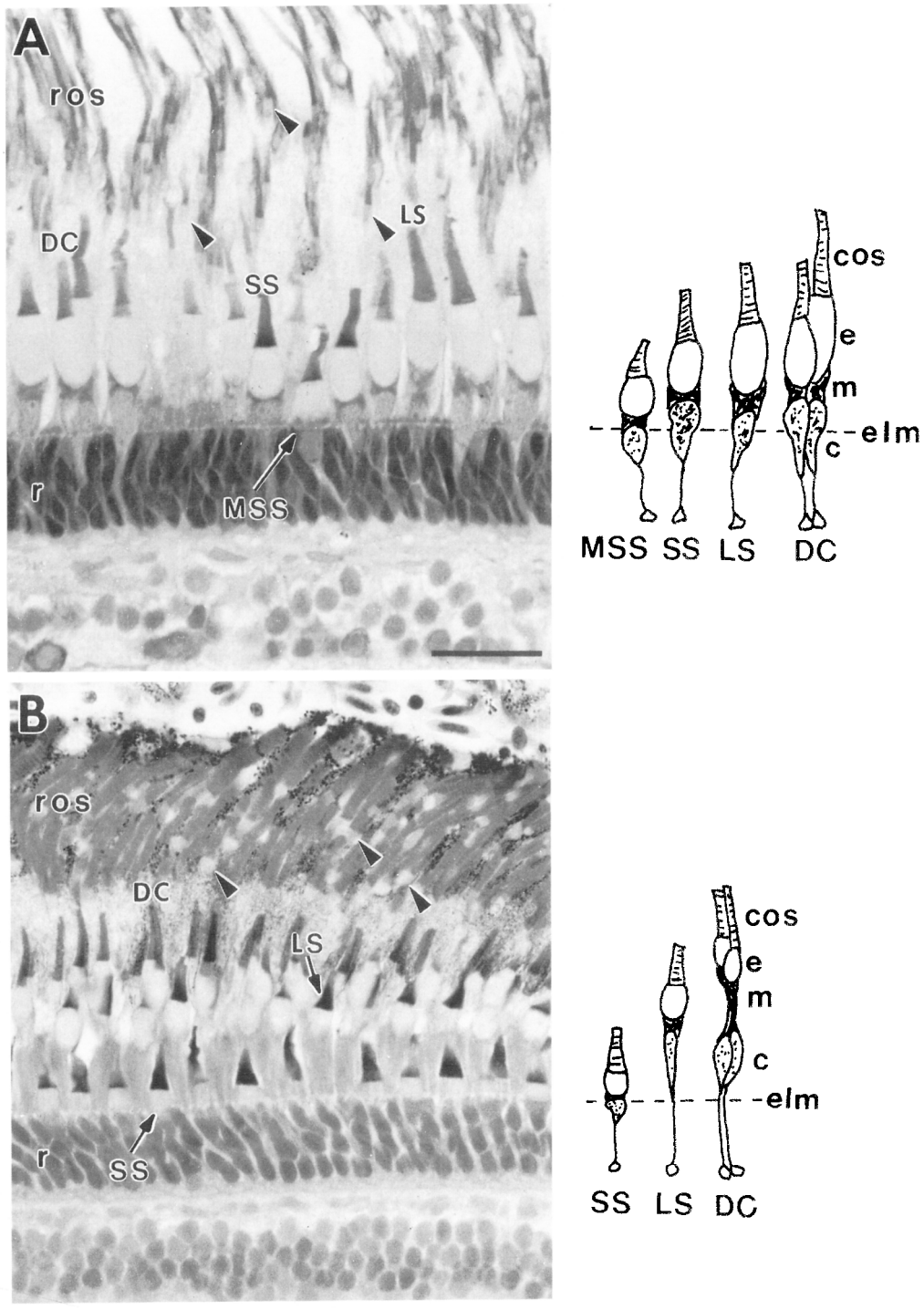

Figure 1. Radial Methacrylate Sections of Light-Adapted Goldfish and Zebrafish Retina

(A) Goldfish retina. (B) Zebrafish retina. ros, rod outer segment; cos, cone outer segment; e, cone ellipsoid; arrowheads, rod ellipsoid; m, myoid; elm, external limiting membrane; c, cone nucleus; r, rod nucleus; DC, double cone; LS, long single cone; SS, short single cone; MSS, miniature short single cone. Bar, 20 μm (A and B).

tunity to test the hypothesis that the rhodopsin-like pigment is expressed in green cones.

Here, we report the expression pattern of goldfish rod and cone opsins in retinas from goldfish and a related member of the cyprinid family, zebrafish (Brachydanio rerio), as determined by colorimetric in situ hybridization. We have prepared cRNA probes to the rod and cone opsin clones from Nakanishi's group (Johnson et al., 1993) and to a partial-length rod opsin cDNA that we cloned (Knight et al., 1992, Invest. Oph-

thal. Vis. Sci. Suppl., abstract). We find that, as predicted, the rhodopsin-like clones are expressed in the class of photoreceptors previously identified as green cones. In goldfish, the expression patterns of the other clones match the microspectrophotometric data, but in zebrafish, we find a discrepancy with regard to the identification of the blue-sensitive cone. However, since the clones used were derived from goldfish, our conclusions from results in zebrafish must be viewed as tentative.

Results

Morphology of Rods and Cones in Goldfish and Zebrafish

The photoreceptors in both goldfish (Stell and Hárosi, 1975) and zebrafish (Branchek and BreMiller, 1984; Nawrocki et al., 1985) have been described previously, and a few distinguishing features are reviewed here to clarify the interpretation of the in situ hybridization results presented below. Both species are members of the cyprinid (e.g., carp and minnow) family of teleost fish, and therefore the structure of their photoreceptors is generally similar. The rod and cone nuclei are segregated into distinct strata within the outer nuclear layer, with the former in multiple rows deep to the external limiting membrane and the latter in a single row along or protruding through the external limiting membrane (Figure 1). The inner segment of the photoreceptor consists of a myoid process and an ellipsoid region. The myoid contains abundant rough endoplasmic reticulum in restricted locations and is therefore where most of the mRNA is found. The myoid region in teleost photoreceptors is contractile and changes in length according to the state of dark and light adaptation in a process known as photomechanical movement (Walls, 1967). The cone myoids contract in light and elongate in dark; rod myoids behave in the opposite fashion. Movements of melanin pigment granules in the processes of the retinal pigmented epithelium are also triggered by light. In the dark, the melanin migrates away from the retina toward the retinal pigmented epithelium cell body, and in the light, pigment moves toward the retina covering the outer segments of the photoreceptors.

Goldfish have five morphological classes of cones—double cones (DCs) with a longer, principal (LD) member and shorter, accessory (SD) member, long single (LS) cones, short single (SS) cones, and miniature short single (MSS) cones (Stell and Hárosi, 1975; Marc and Sperling, 1976a, 1976b; see Figure 1A). The names derive from the overall height of the photoreceptor and the position of the ellipsoid, which on tissue sections are the most distinguishing features of the different cone types. The nuclei of DC, LS, and SS cones protrude partially through the external limiting membrane (Figure 1A). Nuclei of MSS cones always remain below the external limiting membrane and are more rounded in shape. Microspectrophotometric studies have shown that the LD cones and many of the LS cones contain a long wavelength-sensitive red pigment, λ_{max} 579–625 nm, the SD and the remainder of the LS cones contain a middle wavelength-sensitive green pigment, λ_{max} 509–537 nm, and the SS cones contain a short wavelength-sensitive blue pigment, λ_{max} 441–452 nm (Stell and Hárosi, 1975; Tsin et al., 1981). The identity of the pigment in MSS cones is less certain. Originally, it was thought to be blue (Stell and Hárosi, 1975), but more recent studies suggest that the pigment may be maximally sensitive to ultraviolet (UV) light (Hashimoto et al., 1988).

Zebrafish have DCs and two types of single cones (LS and SS) (Branchek and BreMiller, 1984; Nawrocki et al., 1985; see Figure 1B). It has been reported that the principal member of the double cone pair (LD) and the LS cones contain a middle wavelength-sensitive pigment (λ_{max} 480 nm), and the SD cone is maximally sensitive to long wavelengths (λ_{max} 556 nm), which is the reverse of the situation in goldfish (Nawrocki et al., 1985). The SS cone was found to be maximally sensitive to short wavelengths (λ_{max} 417 nm). The vertical separation of ellipsoids belonging to different cone types is more extreme in zebrafish (Figure 1B) than in goldfish (Figure 1A). Zebrafish cones are packed tightly together laterally, so that it is difficult to follow individual cones (especially double cones) from nucleus to outer segment. The nuclei of DCs and LS cones protrude completely beyond the external limiting membrane, but the nuclei of SS cones (like MSS cones of goldfish) remain below. In cryosections viewed with Nomarski interference contrast optics, the most prominent feature is the refractile ellipsoid (see Figure 2B).

Table 1. Goldfish Opsin cDNA Clones Used for cRNA Probes

cDNA Clones	Length	Source	Opsin Gene Family
3.1.4	625 bp	R	Rh
GFrod (3.6)	1324 bp	N	Rh
GFgr-1 (7.3)	1133 bp	N	M_2
GFgr-2 (7.4.2.1)	565 bp	N	M_2
(7.4.2.2)	836 bp		
GFblu (Rb8A−)	600 bp	N	M_1
(Rb8A+)	Not used		
GFred	2047 bp	N	L

The cDNA subclones in pBluescript used to generate cRNA probes are listed by name and number in the first column. Clones GFgr-2 and GFblu contain internal EcoRI sites and are therefore represented by two subclones. The length of the insert is indicated in the second column. The third column gives the laboratory in which the clone was identified (R, Raymond; N, Nakanishi). The fourth column indicates the gene family to which the clone belongs, following the terminology of Okano et al. (1992).

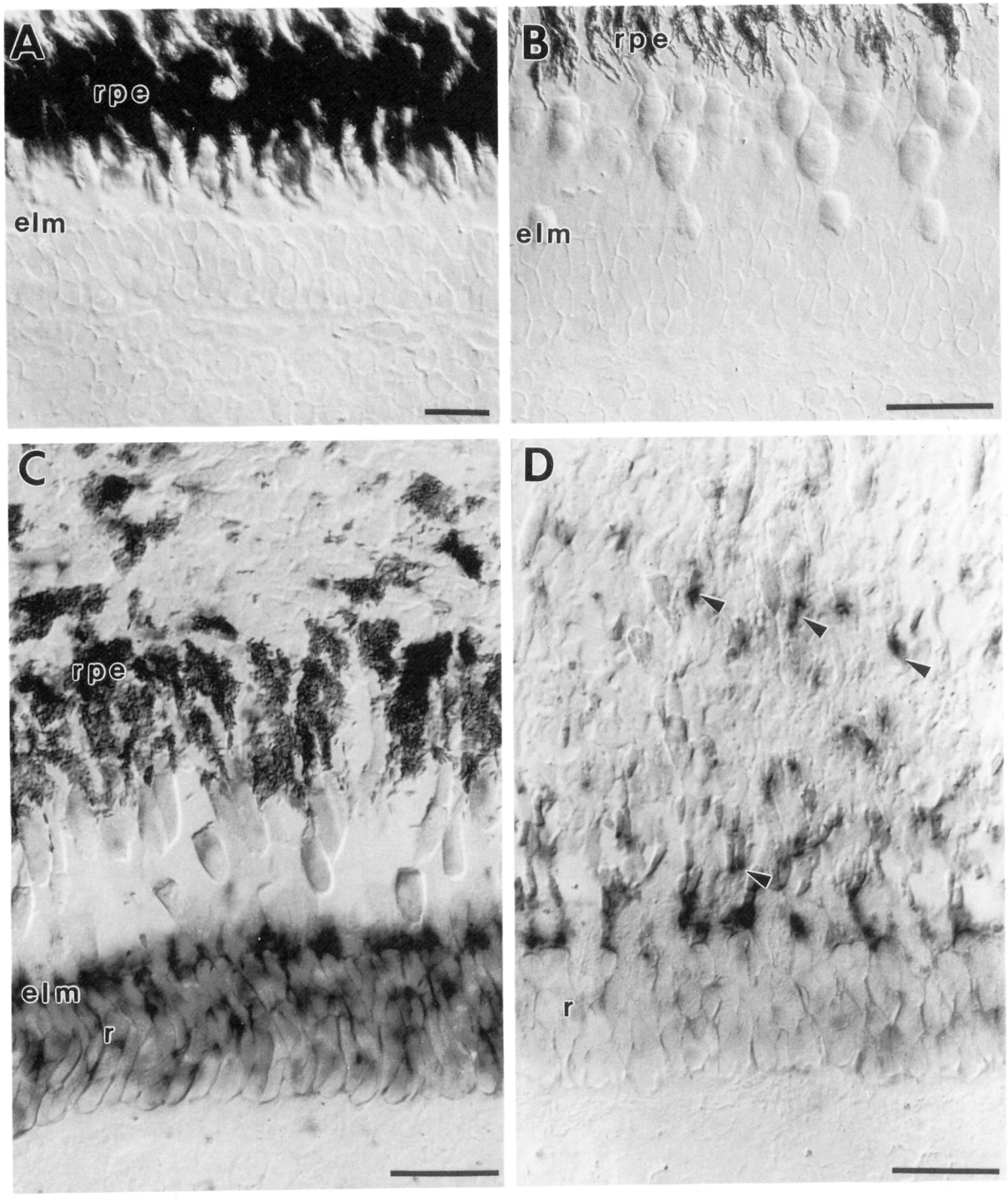

Figure 2. Expression of Rod Opsin in Light- and Dark-Adapted Retina

Sense cRNA probes transcribed from the GFred clone hybridized to cryosections of light-adapted goldfish (A) and zebrafish (B) retina. rpe, retinal pigmented epithelium; elm, external limiting membrane. (C) The antisense cRNA probe transcribed from rod opsin clone 3.1.4 hybridized to a retinal cryosection from a light-adapted goldfish. Signal is restricted to the layer of rod nuclei (r). (D) The same probe hybridized to a retinal cryosection from a dark-adapted goldfish. Punctate staining (arrowheads) is observed at the base of the rod ellipsoids in addition to the layer of rod nuclei. Bars, 20 μm.

Control RNA Probes with Sequences Identical to the Opsin mRNAs Gave No Signal

A description of the cDNA clones used to generate cRNA probes is given in Table 1. We verified that all of the control (sense) probes gave no hybridization signal. Examples of GFred sense probes applied to goldfish and zebrafish retinal sections are shown in Figures 2A and 2B, respectively. All other sense probes were similarly negative.

Subcellular Localization of Rod Opsin mRNA

The antisense cRNA probe transcribed from rod opsin clone 3.1.4 hybridized to the outer nuclear layer on retinal sections from light-adapted goldfish (Figure 2C). The patchy appearance of the signal on close examination corresponded to wedge-shaped splotches of reaction product capping individual rod nuclei. This reflects the perinuclear localization of rough endoplasmic reticulum in goldfish rods, as described previously (Raymond, 1985). Identical results were obtained with the rod opsin clone GFrod (data not shown). Both probes also hybridized with a similar pattern on sections of zebrafish retina (data not shown). On retinal sections from dark-adapted goldfish, the rod opsin cRNA probes gave a reduced signal in the outer nuclear layer, but strong, punctate signals were localized to the myoid region adjacent to the ellipsoids (Figure 2D). The retraction of melanin granules in the dark-adapted retina allowed the rod ellipsoids to be easily visualized, whereas in the light-adapted situation, the pigment partially obscured them (Figure 2C), so that it was difficult to determine whether reaction product was also associated with the ellipsoids in the light. Additional experiments are needed to determine whether there is a movement of opsin mRNA associated with light and dark adaptation, as these results seem to suggest.

Rhodopsin-like Genes Are Expressed in Cones

Figures 3A and 3B illustrate the pattern of expression of the rhodopsin-like GFgr-2 cRNA probe. Note that a strong hybridization signal is found in some but not all cones, where it is localized to the neck-like myoid connecting the ellipsoid and nucleus. In favorable cases, the plane of section passes through both members of a double cone pair, and in those instances, the SD cone appears to express the message (Figure 3A). This suggests that the rhodopsin-like gene is expressed in green cones as predicted (Johnson et al., 1993). Further evidence in support of this conclusion is given below in the section on mosaic patterns.

In the light-adapted retina (Figure 3A), the cone myoids are short and squat, but with dark adaptation, the myoid elongates and the ellipsoid and outer segment move away from the nucleus (Figure 3B). The hybridization signal in cones remains associated exclusively with the perinuclear region, however. Therefore, unlike the situation in rods, opsin message is not associated with the base of the ellipsoid in cones. The lack of any hybridization of GFgr-2 probe to rods indicates that there is no cross-hybridization with rod opsin mRNA.

Probes made to the second rhodopsin-like cDNA, GFgr-1, showed a similar pattern of hybridization on sections from goldfish retina (Figure 3C). Both probes also hybridized to some cones in zebrafish retina, and the hybridization pattern of the GFgr-1 cRNA probe is illustrated in Figure 3D. In both goldfish and zebrafish, it was clear that the signal was associated with DCs and that only one of the two members of the DC pair hybridized with GFgr-1 or GFgr-2 probes. In cases in which the plane of section was favorable, the signal could be associated with the SD member of the DC pair in goldfish (Figure 3A). Owing to the tight packing of cones in zebrafish, however, we could not determine whether the hybridization signal was associated with the LD or SD cones. The SS and MSS cones in goldfish and the LS and SS cones in zebrafish were negative. With the stringency conditions used for the in situ hybridizations, the GFgr-1 and GFgr-2 cRNA probes cross-reacted on dot blots (data not shown). At higher stringency conditions on dot blots, the probes were isoform specific. However, in situ hybridizations run at these high stringency conditions have thus far been negative.

Expression of Red and Blue Cone Opsins

The cRNA probes for GFred gave very strong signals on both goldfish and zebrafish retinas (Figures 4A and 4B). Diffusion of the alkaline phosphatase reaction product was quite apparent on these sections, however, and it was difficult to be certain of the identity of the cones expressing the GFred mRNA in goldfish. Tangential sections were much more informative, and the results are described in the next section. In zebrafish, one member of the DC pair hybridized with the GFred probe, and although again we could not ascertain whether the signal was associated with the LD or SD member, from the staining pattern, it was apparent that it was not the same cone type that hybridized with the GFgr probes. The GFred signal exhibited a butterfly-like pattern (Figure 4B), which reflects the bending of the thin myoid processes of the DCs around the ellipsoid of the LS cones (Figure 1B). This pattern was quite unlike that for the GFgr probe, which was block shaped and more closely associated with SS cones (Figure 3D). Tangential sections (data not shown) were helpful, but additional experiments (in progress) are needed to resolve definitively the issue of which DC member expresses which pigment. Like the GFgr probes, the GFred probe never hybridized to SS or MSS cones in goldfish nor to LS or SS cones in zebrafish.

The cRNA probes for GFblu hybridized to the SS cones in the goldfish retina (Figure 4C) and to the LS cones in zebrafish (Figure 4D). The MSS cones in the goldfish and the SS cones in the zebrafish did not hybridize with any of the cRNA probes. Recall that this morphological class of cone was unique in both goldfish and zebrafish in that the nucleus does not

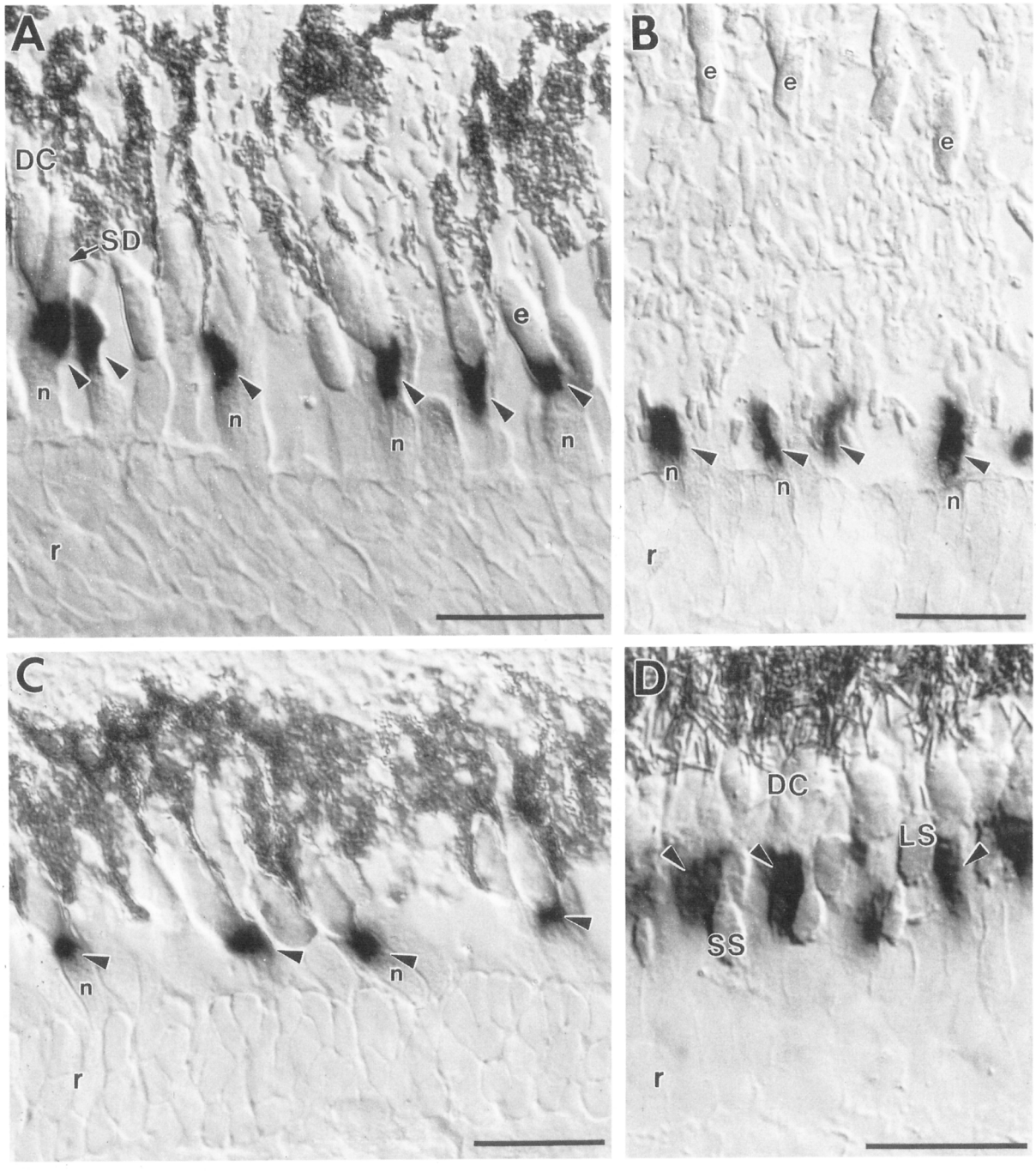

Figure 3. Expression of the Putative Green Cone Opsins

Antisense cRNA probes transcribed from the GFgr-2 clone (A and B) and the GFgr-1 clone (C and D) hybridized to retinal cryosections of light-adapted goldfish (A and C), dark-adapted goldfish (B), and light-adapted zebrafish (D). Intense signal is localized to the myoid region (arrowheads) of some cone photoreceptors between the ellipsoids (e) and nuclei (n), and there is no signal in the rods (r). When the section angle is favorable (A), double cones (DC) are visualized, and only the short double (SD) member hybridizes with the probe. In the dark-adapted retina (B), the signal remains associated with the cone nuclei (n), not the ellipsoids (e). In the zebrafish (D), the signal (arrowheads) is associated with double cones (DC), not long single (LS) or short single (SS) cones. Bars, 20 μm.

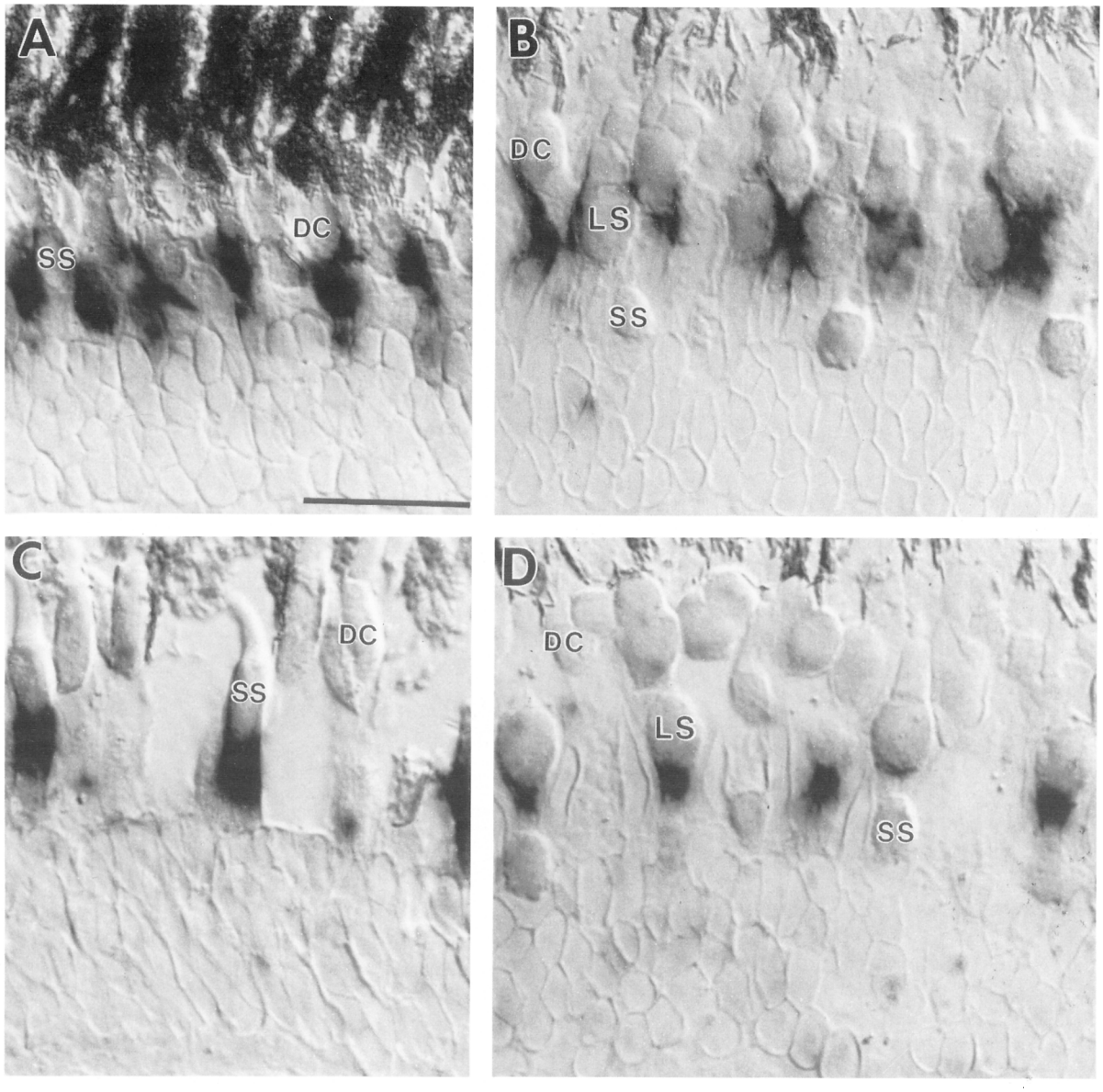

Figure 4. Expression of the Red and Blue Cone Opsins

Antisense cRNA probes transcribed from the GFred clone and hybridized to retinal cryosections of light-adapted goldfish (A) and zebrafish (B), and antisense probes from the GFblu clone hybridized to light-adapted goldfish (C) and zebrafish (D). DC, double cone; LS, long single cone; SS, short single cone. Note that there are no MSS cones in the microscopic fields illustrated in (A) and (C). Bar, 20 μm (A–D).

protrude through the external limiting membrane (Figure 1).

Mosaic Pattern of Spectral Classes of Cones in Goldfish Retina

In the goldfish (Engström, 1960; Marc and Sperling, 1976a, 1976b), as in many other cyprinids (Engström, 1960), the cones are arranged in a square mosaic pattern consisting of four double cone pairs arranged in a cloverleaf pattern around a central SS cone, with an MSS cone positioned at some of the corners (Figure 5A). Because the MSS cone is much shorter than the other cone types, tangential sections passing through the nucleus/myoid region of the DC, LS, and SS cones are at the level of the ellipsoids of the MSS cones, which appear as circular, refractile profiles with Nomarski optics (Figures 5B–5D). The LS cones are randomly distributed, degrading the regular pattern of the cone mosaic in goldfish (Marc and Sperling, 1976a, 1976b).

In situ hybridization with the cone opsin probes on tangential sections revealed that the central single cone in the mosaic hybridized with the GFblu probe (Figure 5B), and one member of each DC pair hybrid-

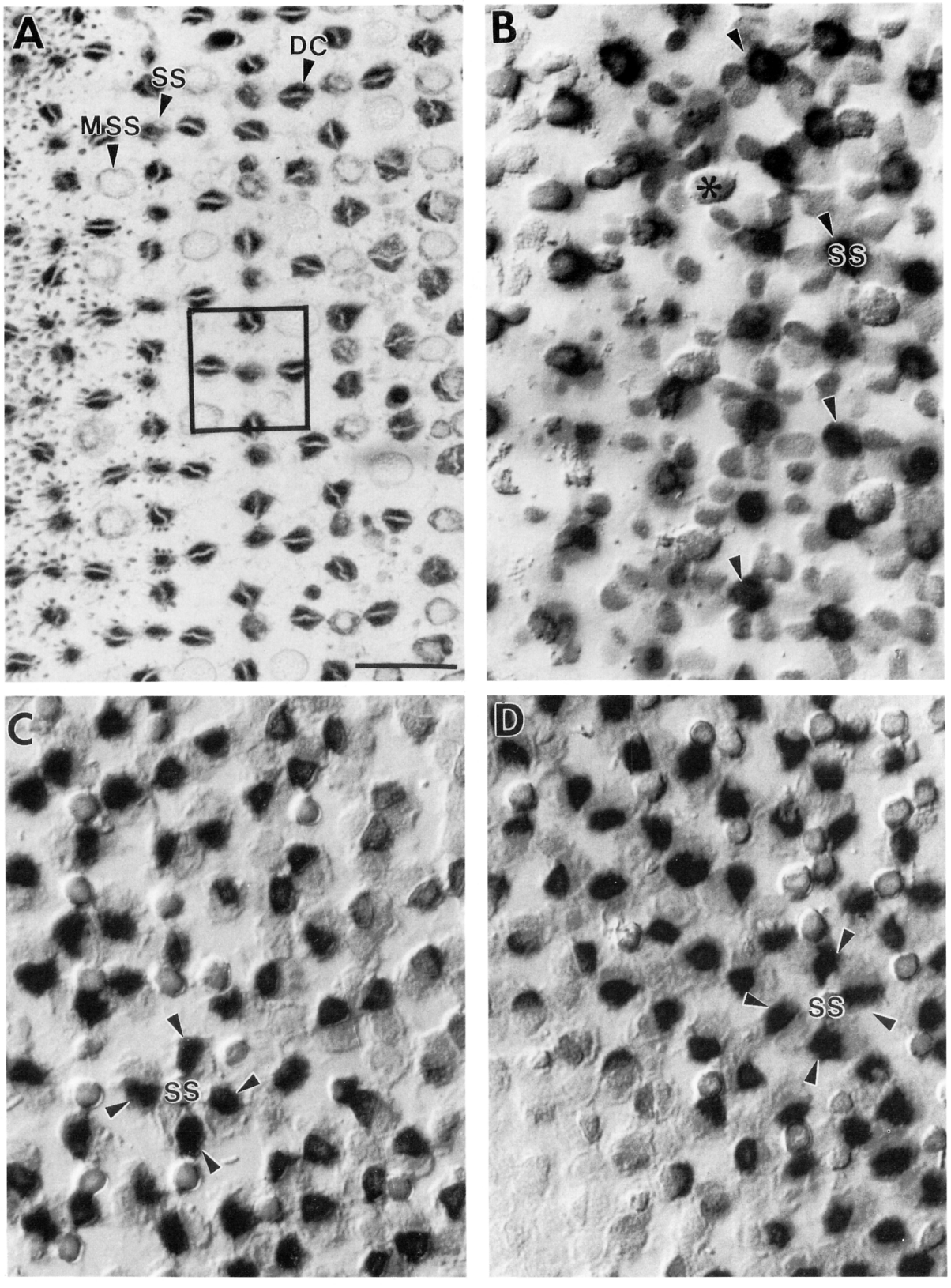

Figure 5. Spectral Organization of the Cone Mosaic in Goldfish

Tangential methacrylate section of light-adapted goldfish retina (A) at the level of the nucleus/myoid process of DC and SS cones and at the level of the ellipsoid of MSS cones. The halos of small, round profiles surrounding the cones at the left are rod myoids near the level of the external limiting membrane (see Figures 2C and 2D). One unit of the square mosaic pattern is outlined. Two of the

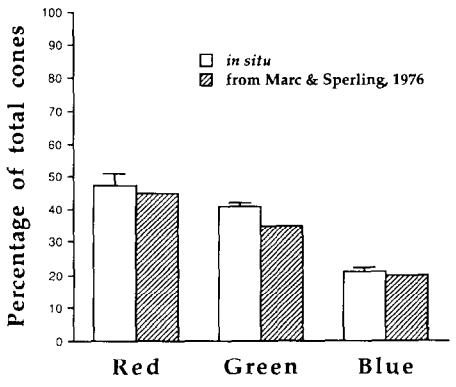

Figure 6. Percentage of Total Cones Hybridizing to Red, Green, and Blue Probes

Hybridization with GFred, GFblu, GFgr-1, and GFgr-2 cRNA probes was done in four separate experiments. At least three separate sections from each experiment were analyzed with oil immersion (63× or 100× objective), and in areas where cones were intact and the plane of section strictly radial, the proportion of labeled and unlabeled cones was determined. Results with the GFgr-1 and GFgr-2 probes were equivalent, and the counts were therefore combined. The total numbers of cones counted were 391 (GFred), 579 (GFblu), and 844 (GFgr). The open bars indicate the averages, and the error bars are 1 SD. The hatched bars are data from Marc and Sperling (1976a). These authors used a histochemical method, in which intact, isolated retinal whole-mounts were exposed to light of different wavelengths in the presence of nitroblue tetrazolium, which was preferentially oxidized to a dark reaction product in those cones maximally excited by the light.

ized with the GFgr-1 and GFgr-2 (Figure 5C) and GFred probes (Figure 5D). The MSS cones were again negative with all probes.

The Proportion of Cone Types in Goldfish Retina Identified with In Situ Hybridization Is Equivalent to Previous Estimates Based on the Spectral Properties of the Opsins

Radial sections of goldfish retina were hybridized with GFred, GFblu, GFgr-1, and GFgr-2 in four separate experiments, and both labeled and unlabeled cones were counted. The average percentages of red, blue, and green cones were 47%, 21%, and 41%, respectively. Because these counts were from separate experiments, and in each experiment only one cRNA probe was used, the percentages were determined independently, which is why they do not add to exactly 100%. Figure 6 illustrates these results and compares them with published data from Marc and Sperling (Marc and Sperling, 1976a, 1976b), who found 45% red cones, 20% blue cones, and 35% green cones.

Discussion

Green Cones Contain a Rhodopsin-like Pigment

The results presented here provide unequivocal evidence that the green cones in goldfish and zebrafish retina express a rhodopsin-like visual pigment gene (Johnson et al., 1993). Members of this new rhodopsin-like gene family have been identified by molecular cloning in chicken (Okano et al., 1992; Wang et al., 1992), gecko (Kojima et al., 1992), and goldfish (Johnson et al., 1993), and in all three species, it has been suggested that the encoded pigment corresponds to the green cone visual pigment previously identified in each of the three species. Araki and colleagues (Araki et al., 1984) noted rhodopsin-like immunoreactivity in single cones of chicken retinas with a polyclonal antibody prepared against bovine rhodopsin, and they speculated that these might be green cones. Since the green cone pigment has a λ_{max} similar to that of rhodopsin, they suggested, correctly as it turns out, that these two pigments might be immunologically similar as well. Immunological cross-reactivity of rhodopsin antibodies with certain classes of cones has also been seen in turtle retina (Ohtsuka and Kawamata, 1990), and in both goldfish and zebrafish, several rhodopsin antibodies, including rho4D2 (Hicks and Molday, 1986) and RET P1 (Barnstable, 1980), cross-react with green cones (P. R., J. K., and L. B., unpublished data). These immunocytochemical results support the identification of the rhodopsin-like pigment as the green cone opsin.

Although these rhodopsin-like pigments are designated as "green," implying that they absorb maximally in the green region of the spectrum, in reality the spectral properties of the designated green cone pigments vary substantially. For example, the λ_{max} of the chicken green pigment is 508 nm (Okano et al., 1989), the gecko green pigment is 467 nm (Crescitelli et al., 1977), and the goldfish green pigment is 509 nm with a retinal-based chromophore (vitamin A_1) and 523 nm with 3-dehydroretinal (vitamin A_2) (Tsin et al., 1981). (In place of or in addition to vitamin A_1, freshwater fish and amphibians often utilize vitamin A_2, which when coupled to the same opsin apoprotein produces a visual pigment with λ_{max} shifted up to 25 nm toward longer wavelengths; this is thought to be an adaptation for aquatic vision [Bridges and Yoshikami, 1970]). It is known that substitution of only a few critical amino acids can have large effects on the spectral characteristics of visual pigments (Neitz et al., 1991), and therefore pigments that are homologous at the molecular level may have very different spectral prop-

MSS corner cones are missing in this unit, which is typical of the degradation of the cone mosaic pattern in the goldfish.

Tangential cryosections hybridized with antisense cRNA probes transcribed from GFblu (B), GFgr-2 (C), and GFred (D). The GFblu probe (B) hybridizes to the central SS cone (arrowheads), producing a daisy-like pattern (B). The ellipsoids of MSS cones (asterisk) appear as large, round refractile profiles spaced at regular intervals in a regular, lattice-like array. The GFgr-2 (C) and GFred (D) probes hybridize to one member of each double cone pair, producing a pinwheel pattern around the SS cones. Bar, 20 μm (A–D).

erties. This point is illustrated by the in situ hybridization results presented here, which reveal that the green cone pigment in zebrafish is very closely homologous to the rhodopsin-like goldfish pigment despite a rather large discrepancy in λ_{max}: the zebrafish green pigment is 480 nm with a retinal-based chromophore (Nawrocki et al., 1985), compared with 509 nm for the corresponding goldfish pigment (Tsin et al., 1981). Until the pigment genes in zebrafish are cloned, however, the precise degree of molecular homology with the goldfish pigment will not be known.

The GFgr-1 and GFgr-2 clones are 91% identical to each other and are thought to represent a gene duplication (Johnson et al., 1993) similar to what has been described for the red/green cone opsins in humans (Nathans et al., 1986) and subhuman primates (Travis et al., 1988). On Northern blots of goldfish retinal mRNA, the relative signal intensity of the GFgr-1 transcript was substantially greater than GFgr-2, and on this basis, Nakanishi and colleagues (Johnson et al., 1993) speculated that GFgr-1 might be expressed in SD cones and GFgr-2 in LS cones, since these two cone types in goldfish exist in a ratio of approximately 3:1 (Stell and Hárosi, 1975). Alternatively, a mixture of the two genes might be expressed in both SD and LS cones. Microspectrophotometric studies in guppies (Poecilia reticulata) have demonstrated that cone photoreceptors in this species sometimes contain mixtures of two pigments, one with λ_{max} of 533 nm and the other 572 nm, which when combined, result in a λ_{max} of 543 nm for an individual cone (Archer and Lythgoe, 1990; Archer et al., 1987). Since the recombinant GFgr-1 and GFgr-2 pigments absorbed maximally at 505 nm and 511 nm, respectively, when reconstituted with 11-cis-retinal (Johnson et al., 1993), whereas microspectrophotometry has shown that both SD and LS cones contained a pigment with λ_{max} of around 509 nm under conditions in which retinal predominated (Tsin et al., 1981), a mixture of two pigments in the green cones seems plausible. Unfortunately, the present results with in situ hybridization cannot distinguish between these alternative possibilities, since at the stringency conditions used, the two cRNA probes showed cross-reactivity on dot blots. Experiments are in progress to design new isoform-specific probes, transcribed from the 3′ untranslated regions of the cDNA clones, which will allow us to address this question explicitly.

It is curious that the green cone opsin in goldfish and zebrafish is more homologous to rhodopsin than to the red cone opsin, since another member of the cyprinid family, the blind cave fish, appears to have red and green cone opsin genes that are very closely related to each other (Yokoyama and Yokoyama, 1990b), as they are in humans (Nathans et al., 1986). Although the eyes begin to develop in the cave fish embryo, they regress at about the time that retinal differentiation begins, and in adults, the eyes are residual or absent (Zilles et al., 1983). However, two visual pigments with λ_{max} at 494 nm and 525 nm, corresponding to rhodopsin and a green-sensitive pigment, have been detected in the pineal organ of the blind cave fish, indicating the presence of functional visual pigments (Tabata, 1982). It remains to be determined whether cave fish (or their conspecific, eyed, river ancestors) also have a rhodopsin-like green pigment. In the library screen that produced the red/green opsins (which hybridized most strongly to the human red cDNA probe), other genomic clones hybridized with human rod opsin and blue cone opsin cDNA probes, but these have yet to be characterized (Yokoyama and Yokoyama, 1990a).

Rods May Have Evolved from Green Cones

The finding that a gene homologous to rhodopsin is expressed in cone photoreceptors has important implications for understanding the fundamental distinction between rod and cone photoreceptors and the evolution of photoreceptors and visual pigments. The classic definition of rods and cones dates from the observations of Schultze in 1866 (cited in Cohen, 1972). Schultze described two groups of photoreceptors, rods and cones (named for the shape of their outer segments), and he noted that cones predominated in diurnal animals and rods in nocturnal animals. In some species, the morphological distinctions between photoreceptor types are ambiguous, and other properties often serve to distinguish rods from cones, such as the visual pigments they contain, various characteristics of their electrical responses (including kinetics and adaptation properties), ultrastructural details (including organization of outer and inner segments and synaptic terminals), variations in developmental history, and sensitivity to damage (Cohen, 1972). However, there are ambiguities with these criteria as well. A recent discourse on the topic of rod/cone differences argues that because of the "definitional dilemma" that often accompanies the use of ambiguous functional and morphological criteria, the most appropriate distinction between rods and cones is the opsin gene they express (Goldsmith, 1990). With the discovery that a rhodopsin-like pigment is expressed in green cones in fish, this distinction too becomes ambiguous.

With the large number of opsin sequences now known, rapid progress is being made in understanding the molecular evolution of visual pigments. The phylogenetic record suggests that two cone pigments, one sensitive to shorter wavelengths and the other to longer wavelengths, were present at the beginning of vertebrate evolution (Goldsmith, 1990). According to the phylogenetic tree constructed by Okano and colleagues (Okano et al., 1992) on the basis of published sequence data, the long wavelength pigment was ancestral to the family of pigments (group L) that includes human and cave fish red/green and chicken red. The shorter wavelength pigment gave rise to a more diverse set of pigments, including chicken violet/human blue (group S), chicken blue (group M_1), and chicken/gecko green (group M_2). Ac-

cording to their analysis, rhodopsins (group Rh) diverged from the ancestral group M_2 cone pigments somewhat later. The goldfish cDNA clones used in this study (Table 1), the sequences of which were not available to Okano, include representatives from groups L (GFred), M_1 (GFblu), M_2 (GFgr-1 and GFgr-2), and Rh (GFrod and 3.4.1). The grouping of visual pigments into gene families according to their sequence similarity serves to reemphasize the point that neither spectral sensitivities nor color names (red, green, blue, violet, and UV) are accurate reflections of homologies, especially for the pigments sensitive to short and middle wavelengths. Thus, the green opsins in humans and cave fish are in one gene family (group L) and the chicken, gecko, and goldfish green opsins are in another (group M_2). Similarly, chicken and goldfish blue opsins (group M_1) are not in the same family as human blue, which is related to chicken violet (group S).

Several lines of argument, in addition to the visual pigment homologies, support the suggestion that cones are the more primitive photoreceptor and that rods evolved in association with colonization of a nocturnal habitat (Goldsmith, 1990; Vinnikov, 1982). The molecular evidence supports this scheme and further suggests that a specific class of cones, the group M_2 (green) cones, represents the ancestral cone photoreceptor type from which rods evolved.

Cone Pigments and the Cone Mosaic

The results of the in situ hybridization studies with goldfish retina for the most part confirm earlier studies by Marc and Sperling (Marc and Sperling, 1976a, 1976b), who used a histochemical technique for identifying the spectral characteristics of cones at specified positions in the mosaic pattern. In tangential sections (Figure 5B), it is clear that the central single cone hybridizes with the group M_1, GFblu probe and is therefore the SS blue cone. When the group L, GFred or group M_2, GFgr-1 or GFgr-2 probes are used (Figures 5C and 5D), only one member of each DC pair hybridizes with the cRNA probe. The shortest cones, the MSS, do not hybridize with any of the probes and probably correspond to the missing group S opsin, which may correspond to the teleost UV receptor (see below).

The in situ hybridization results on zebrafish agree with some, but not all, of the conclusions from an earlier microspectrophotometric study of visual pigments in zebrafish (Nawrocki et al., 1985). From the distinctive expression patterns, it is clear that one member of the DC pair hybridizes to the group L, GFred probe (Figure 4B) and the other member to the group M_2, GFgr-1 or GFgr-2 probes (Figure 3D). This was also demonstrated on tangential sections (data not shown). It is clear that the group M_1, GFblu probe hybridizes to the LS cones and *not* to the SS cones, which, according to the microspectrophotometry, contain a blue-sensitive pigment (Nawrocki et al., 1985). Microspectrophotometry requires that individual cones be isolated from the retina, and this can be difficult to do while preserving morphological features that allow the cone type to be identified unambiguously. Thus, the LS cones that were identified as green cones in the previous study may have been LD cones detached from their partner, in which case, the shorter LS cones might then have been mistakenly identified as SS cones, especially if there were few or no genuine SS cones in the cell suspension. It is reasonable to suggest that the SS cones might be more difficult to isolate from the retina, since their diminutive size and the placement of the nucleus below the external limiting membrane would render them less susceptible to the shearing forces used to dislodge the cones. A recent microspectrophotometric study of zebrafish photoreceptors found a short wavelength–sensitive pigment in LS cones, in agreement with the present in situ hybridization results (F. Hárosi, E. Schmitt, and J. Robinson, personal communication).

The Putative Ultraviolet Receptor in Goldfish and Zebrafish Has Distinctive Morphological Features

In both goldfish and zebrafish, the shortest cone type failed to hybridize with any of the available probes. These cones, called MSS in goldfish and SS in zebrafish, have several distinctive morphological features in common, as discussed above. Since they did not express any of the four known goldfish visual pigments for which cRNA probes were available (rod opsin, red, green, and blue cone opsins), we suggest that they are homologous to each other, and we further suggest that this short cone may represent the teleost UV receptor. Additional evidence in support of this suggestion follows.

A UV receptor has been described in several species of fish, including goldfish (Hawryshyn, 1991; Hawryshyn and Hárosi, 1991; Neumeyer, 1985, 1986), on the basis of behavioral analysis of wavelength discrimination. Microspectrophotometric measurements in a related cyprinid, the Japanese dace (Tribolodon hakonensis), have documented that MSS cones contain a pigment that is maximally sensitive in the UV region of the spectrum (Hashimoto et al., 1988). The MSS cone in goldfish is located at the corner of the square mosaic pattern, and the UV cone in trout (Salmo trutta) is thought to be located at the corner of the square mosaic (Lyall, 1957), although this latter conclusion is based only on indirect evidence. For example, behavioral studies have shown that UV sensitivity decreases as fish mature (Bowmaker and Kunz, 1987), and correspondingly, the corner cones gradually disappear from the cone mosaic (Lyall, 1957). Tokunaga and colleagues have recently cloned a partial-length cDNA that codes for a goldfish pigment homologous to chicken violet (F. Tokunaga, personal communication) in the pigment family group S (Okano et al., 1992). It seemed reasonable to suggest that this clone may represent the goldfish UV pigment, and we therefore obtained the cDNA fragment coding for the violet pigment from F. Tokunaga. Preliminary results with

a cRNA probe generated from this clone show expression in the MSS cones of goldfish, as predicted. In addition, a full-length cDNA for a putative zebrafish UV pigment has recently been cloned (Robinson et al., 1993, Invest. Ophthal. Vis. Sci., abstract), and in situ hybridization demonstrated that the SS cones of zebrafish express this pigment. Robinson et al. (1993) also found with microspectrophotometry that the SS cones of zebrafish contain a UV pigment with a λ_{max} of 360 nm, contrary to the results of Nawrocki et al. (1985). Taken together, these results lend strong support to the suggestion that the MSS cone in goldfish and the SS cone in zebrafish are UV receptors.

The Subcellular Localization of Opsin mRNA Differs between Rods and Cones

With the increased resolution achieved by the use of digoxigenin-labeled riboprobes, this study has documented a difference between rods and cones in the subcellular distribution of opsin message. The cRNA probes for rod opsin, both the 3.1.4 probe (Figure 2) and the GFrod probe (data not shown), localized to the perinuclear cytoplasm of the rod soma in the outer nuclear layer, as well as the myoid region adjacent to the ellipsoid in the subretinal space. The latter signal was detected in dark-adapted preparations, in which the melanin pigment in the processes of the pigmented epithelial cells had migrated sclerad, leaving the rod myoid/ellipsoid/outer segment region exposed and therefore easily visualized. In light-adapted preparations, it was difficult to discern whether the signal was present near the ellipsoid, whereas the perinuclear signal in the outer nuclear layer was apparently stronger in these preparations compared with the dark-adapted condition. A circadian and light-triggered regulation of opsin mRNA synthesis has been reported in several species (Korenbrot and Fernald, 1989; McGinnis et al., 1992), including fish (Korenbrot and Fernald, 1989), in which synthesis of rod opsin message is stimulated during the light phase. The increased hybridization signal observed in the light-adapted preparations in the present study is consistent with this result.

In contrast with rods, the cRNA probes for cone opsins hybridized only to the perinuclear cytoplasm in both light- and dark-adapted preparations (Figure 3). In the dark, the cone myoids elongate and the ellipsoid/outer segment assemblage moves away from the nucleus, but the opsin message stays behind. The significance of the subcellular localization of opsin message and the implication of the difference between rods and cones is uncertain. At the least, it illustrates yet another morphological feature that distinguishes rods from cones.

Experimental Procedures

Cloning of a Partial cDNA for Goldfish Rod Opsin

A 1126 bp bovine rhodopsin cDNA (rOPps; a gift of C. Barnstable) was used to generate random-primed ^{32}P-labeled DNA probes (Feinberg and Vogelstein, 1983) to screen a goldfish retinal cDNA library (gift of D. Goldman). Hybridization conditions were as follows: 55°C overnight in 5× SSPE, 5× Denhardt's solution, 1% SDS, 1 μg/ml salmon sperm DNA. Wash conditions were as follows: 2× SSC, 1% SDS for 20 min at room temperature, followed by two washes in 0.5× SSC, 1% SDS for 1 hr at 65°C.

Four clones were chosen after secondary screening and subcloned into the EcoRI site of pBluescript KS(+) (Stratgene, La Jolla, CA). Three of these were shown by partial sequencing to contain the same 1329 bp insert, which when compared with GenBank sequences (MacVector, IBI, New Haven, CT), proved most homologous to known rhodopsins. Clone 3.1.4, used to generate cRNA probes for in situ hybridization and dot blots, encodes a fragment that includes transmembrane domains 2–7 and the carboxyl terminus (homologous to amino acids 57–348 of bovine rhodopsin).

Tissue Preparation

Adult goldfish, 4–5 cm total length, and adult zebrafish, 2.5–4 cm, obtained from a local pet store were kept on a 12 hr light:12 hr dark cycle. Fish were anesthetized in 0.02% methane tricaine sulfonate (Sigma, St. Louis, MO) and decapitated. In some cases, goldfish were kept overnight in a dark cabinet and killed the following morning under dim red illumination: these retinas are described as dark-adapted. The tissue was rinsed and prepared for 3 μm cryosections, as described previously (Barthel and Raymond, 1990).

Probe Synthesis

Goldfish opsin cDNA clones GFrod, GFred, GFblu, GFgr-1, and GFgr-2 were the kind gift of K. Nakanishi (Columbia University). Since the GFgr-2 and GFblu cDNA clones contained an internal EcoRI site, subcloning into pBluescript generated two subclones (K. Nakanishi, personal communication). The GFgr-2 subclones are designated 7.4.2.1 and 7.4.2.2, and the GFblu subclones are Rb8A+ and Rb8A− (Table 1). In both cases, the fragments at the 5′ end (7.4.2.1 and Rb8A−) were used to generate cRNA probes for in situ hybridization. For dot blot assays, cRNA probes generated from both 7.4.2.1 and 7.4.2.2 were used. In addition, we used our partial cDNA clone for rod opsin (3.1.4) containing 3′ untranslated sequences. All clones were derived from the same goldfish retinal cDNA library. A list of the seven clones used is given in Table 1.

To generate cRNA probes, run-off transcriptions from both T3 and T7 promoters were done with digoxigenin-UTP, according to the manufacturer's specifications (The Genius System User's Guide for Filter Hybridization, Boehringer Mannheim Corporation, Indianapolis, IN). The resulting antisense transcripts (complementary to the mRNA target) and sense transcripts (identical to the mRNA target, therefore serving as controls) were sized by electrophoresis in a 1.5% formaldehyde gel (Sambrook et al., 1989). The RNA was hydrolyzed for 20 min in 0.05 N NaOH, 15 M NaCl; neutralized in 0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl for 30 min; transferred by capillary action to a Duralose-UV nitrocellulose membrane (Stratagene); and bound to the membrane by UV cross-linking. The digoxigenin-labeled RNA was detected by alkaline phosphatase immunostaining (The Genius System User's Guide for Filter Hybridization, Boehringer Mannheim) with Lumi-Phos 530 (Boehringer Mannheim) as the visualizing agent. The probes contained a mixture of transcripts of varying lengths, generally 700 bp or less.

In Situ Hybridization

A complete description of the methods used for in situ hybridization will appear elsewhere (Barthel and Raymond, submitted), so only a brief description is given here. Slides were treated with 0.01 mg/ml proteinase K (Boehringer Mannheim) at 37°C for 2 min, then incubated at room temperature in 0.1 M triethanolamine (Sigma) (pH 8.0) for 3 min, followed by a 10 min rinse in 0.1 M triethanolamine with 0.25% acetic anhydride (Sigma). The tissue was then dehydrated in graded ethanols and air dried. Approximately 60 μl of hybridization solution, containing 2 μg

or 0.5 μg of the cRNA, was placed on each slide, and the edges of the slide were sealed. Slides were hybridized overnight on a warming tray at 55°-56°C. The next day, slides were washed in 2× SSC followed by 50% formamide in 2× SSC, heated to 65°C, and washed in 2× SSC at 37°C. Tissue was then treated with RNAase A (Boehringer Mannheim) for 30 min. For immunocytochemical detection of cRNA probes, anti-digoxigenin Fab fragment antibodies were visualized with alkaline phosphatase. Photomicrography was done on a Leitz Aristoplan using differential interference contrast illumination.

RNA Dot Blots

Unlabeled sense transcripts were generated following manufacturer's specifications (The Genius System User's Guide for Filter Hybridization, Boehringer Mannheim). Sense transcripts, 1.0 ng and 0.1 ng, were blotted onto strips of Duralon-UV nylon membrane (Stratagene) and bound by cross-linking. Low stringency hybridization was carried out at 55°C overnight in 5× SSC, 50% formamide, 0.02% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent (Boehringer Mannheim Genius Kit 3), 20 mM sodium maleate. For high stringency, hybridization was at 65°C in 0.1× SSC, 50% formamide, 0.02% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent, 20 mM sodium maleate. Digoxigenin-labeled cRNA probes transcribed from clones 7.3 and 7.4.2.1 were diluted to 50 ng/ml. All the strips were processed together in subsequent steps. After hybridization, the strips were washed twice (30 min each time) in 0.1× SSC at room temperature; washed once for 30 min with 50% formamide in 2× SSC at 65°C; and rinsed for 5 min in maleate buffer (100 mM maleic acid [pH 7.5], 150 mM NaCl). Nonspecific binding of the digoxigenin antibody was blocked by incubating with 0.1% blocking reagent in 2× SSC and 0.05% Triton X-100 (Sigma) for 30 min at room temperature. The digoxigenin antibody, conjugated to alkaline phosphatase, was diluted 1:5000, and visualization was done according to manufacturer's specifications (The Genius System User's Guide for Filter Hybridization, Boehringer Mannheim).

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References

Applebury, M. L., and Hargrave, P. A. (1986). Molecular biology of visual pigments. Vision Res. *26*, 1881–1895.

Araki, M., Watanabe, K., and Yasuda, K. (1984). Immunocytochemical localization of rhodopsin-like immunoreactivity in the outer segments of the rods and single cones of chick retina. Cell Struct. Funct. *9*, 1–12.

Archer, S. N., and Lythgoe, J. N. (1990). The visual pigment basis for cone polymorphism in the guppy, *Poecilia reticulata*. Vision Res. *30*, 225–233.

Archer, S. N., Lythgoe, J. N., and Partridge, J. C. (1987). Visual pigment polymorphism in the guppy *Poecilia reticulata*. Vision Res. *27*, 1243–1252.

Barnstable, C. J. (1980). Monoclonal antibodies which recognize different cell types in the rat retina. Nature *286*, 231–235.

Barthel, L. K., and Raymond, P. A. (1990). Improved method for obtaining 3-μm cyrosections for immunocytochemistry. J. Histochem. Cytochem. *9*, 1383–1388.

Bowmaker, J. K., and Kunz, Y. W. (1987). Ultraviolet receptors, tetrachromatic colour vision and retinal mosaics in the brown trout (*Salmo trutta*): age-dependent changes. Vision Res. *27*, 2101–2108.

Branchek, T., and BreMiller, R. (1984). The development of photoreceptors in the zebrafish, *Brachydanio rerio*. I. Structure. J. Comp. Neurol. *224*, 107–115.

Bridges, C. D. B., and Yoshikami, S. (1970). The rhodopsin-porphyropsin system in freshwater fishes. I. Effects of age and photic environment. Vision Res. *10*, 1315–1332.

Cohen, A. I. (1972). Rods and cones. In Handbook of Sensory Physiology, Volume VII (2), M. G. F. Fourtes, ed. (New York: Springer-Verlag), pp. 63–110.

Crescitelli, F., Dartnall, H. J., and Loew, E. R. (1977). The gecko visual pigments: a microspectrophotometric study. J. Physiol. *268*, 559–573.

Engström, K. (1960). Cone types and cone arrangement in the retina of some cyprinids. Acta Zool. *41*, 277–295.

Falk, J. D., and Applebury, M. L. (1987). The molecular genetics of photoreceptor cells. Prog. Retinal Res. *7*, 89–112.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6–13.

Goldsmith, T. H. (1990). Optimization, constraint, and history in the evolution of eyes. Quart. Rev. Biol. *65*, 281–322.

Hashimoto, Y., Hárosi, F. I., Ueki, K., and Fukurotani, K. (1988). Ultra-violet-sensitive cones in the color-coding systems of cyprinid retinas. Neurosci. Res. (suppl.) *8*, 81–96.

Hawryshyn, C. W. (1991). Light-adaptation properties of the ultraviolet-sensitive cone mechanism in comparison to the other receptor mechanisms of goldfish. Vis. Neurosci. *6*, 293–301.

Hawryshyn, C. W., and Hárosi, F. I. (1991). Ultraviolet photoreception in carp: microspectrophotometry and behaviorally determined action spectra. Vision Res. *31*, 567–576.

Hicks, D., and Molday, R. S. (1986). Differential immunogold-dextran labeling of bovine and frog rod and cone cells using monoclonal antibodies against bovine rhodopsin. Exp. Eye Res. *42*, 55–71.

Hisatomi, O., Iwasa, T., Tokunaga, F., and Yasui, A. (1991). Isolation and characterization of lamprey rhodopsin cDNA. Biochem. Biophys. Res. Commun. *174*, 1125–1132.

Johnson, R. L., Grant, K. B., Zankel, T. C., Boehm, M. F., Merbs, S. L., Nathans, J., and Nakanishi, K. (1993). Cloning and expression of goldfish opsin sequences. Biochemistry *32*, 208–214.

Kojima, D., Okano, T., Fukada, Y., Shichida, Y., Yoshizawa, T., and Ebrey, T. G. (1992). Cone visual pigments are present in gecko rod cells. Proc. Natl. Acad. Sci. USA *89*, 6841–6845.

Korenbrot, J. I., and Fernald, R. G. (1989). Circadian rhythm and light regulate opsin mRNA in rod photoreceptors. Nature *337*, 454–457.

Levine, J. S., and MacNichol, E. F. (1979). Visual pigments in teleost fishes: effects of habitat, microhabitat, and behavior on visual system evolution. Sensory Processes *3*, 95–131.

Lyall, A. H. (1957). The growth of the trout retina. Quart. J. Microsc. Sci. *98*, 101–110.

Marc, R. E., and Sperling, H. G. (1976a). The chromatic organization of the goldfish cone mosaic. Vision Res. *16*, 1211–1224.

Marc, R. E., and Sperling, H. G. (1976b). Color receptor identities of goldfish cones. Science *191*, 487–488.

McGinnis, J. F., Whelan, J. P., and Donoso, L. A. (1992). Transient, cyclic changes in mouse visual cell gene products during the light-dark cycle. J. Neurosci. Res. *31*, 584–590.

Merbs, S. L., and Nathans, J. (1992). Absorption spectra of human cone pigments. Nature *356*, 433–435.

Nathans, J., and Hogness, D. S. (1983). Isolation, sequence analysis, and intron–exon arrangement of the gene encoding bovine rhodopsin. Cell *34*, 807–814.

Nathans, J., and Hogness, D. S. (1984). Isolation and nucleotide sequence of the gene encoding human rhodopsin. Proc. Natl. Acad. Sci. USA *81*, 4851–4855.

Nathans, J., Thomas, D., and Hogness, D. (1986). Molecular genetics of human color vision: the genes encoding blue, green and red pigments. Science *232*, 193–232.

Nawrocki, L., BreMiller, R., Streisinger, G., and Kaplan, M. (1985). Larval and adult visual pigments of the zebrafish, *Brachydanio rerio*. Vision *25*, 1569–1576.

Neitz, M., Gerald, J., and Jacobs, H. (1991). Spectral tuning of pigments underlying red-green color vision. Science *252*, 971–974.

Neumeyer, C. (1985). An ultraviolet receptor as a fourth receptor type in goldfish color vision. Naturwissenschaften *72*, 162–163.

Neumeyer, C. (1986). Wavelength discrimination in the goldfish. J. Comp. Physiol. A *158*, 203–213.

Ohtsuka, T., and Kawamata, K. (1990). Monoclonal antibody labels both rod and cone outer segments of turtle photoreceptors. Exp. Eye Res. *50*, 483–486.

Okano, T., Fukada, Y., Artamonov, I. D., and Yoshizawa, T. (1989). Purification of cone visual pigments from chicken retina. Biochemistry *28*, 8848–8856.

Okano, T., Kojima, D., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1992). Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proc. Natl. Acad. Sci. USA *89*, 5932–5936.

Partridge, J. C., Shand, J., Archer, S. N., Lythgoe, J. N., and van Groningen-Luyben, W. A. H. M. (1989). Interspecific variation in the visual pigments of deep-sea fishes. J. Comp. Physiol. A *164*, 513–529.

Raymond, P. A. (1985). Cytodifferentiation of photoreceptors in larval goldfish: delayed maturation of rods. J. Comp. Neurol. *236*, 90–105.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Stell, W. K., and Hárosi, F. I. (1975). Cone structure and visual pigment content in the retina of the goldfish. Vision Res. *16*, 647–657.

Tabata, M. (1982). Persistence of pineal photosensory function in blind cave fish, *Astyanax mexicanus*. Comp. Biochem. Physiol. *73A*, 125–127.

Takao, M., Yasui, A., and Tokunaga, F. (1988). Isolation and sequence determination of the chicken rhodopsin gene. Vision Res. *28*, 471–480.

Tokunaga, F., Iwasa, T., Miyagishi, M., and Kayada, S. (1990). Cloning of cDNA and amino acid sequence of one of chicken cone visual pigments. Biochem. Biophys. Res. Commun. *173*, 1212–1217.

Travis, D. S., Bowmaker, J. K., and Mollon, J. D. (1988). Polymorphisms of visual pigments in a callitrichid monkey. Vision Res. *28*, 481–490.

Tsin, A. T. C., Liebman, P. A., Beatty, D. D., and Drzymala, R. (1981). Rod and cone visual pigments in the goldfish. Vision Res. *21*, 943–946.

Vinnikov, Y. A. (1982). Molecular Biology, Biochemistry and Biophysics. Evolution of Receptor Cells. Cytological, Membranous and Molecular Levels, G. F. Springer and H. G. Wittman, eds., N. Bobrov, transl. (New York: Springer-Verlag).

Walls, G. L. (1967). The Vertebrate Eye and Its Adaptive Radiation (New York: Hafner).

Wang, S.-Z., Adler, R., and Nathans, J. (1992). A visual pigment from chicken that resembles rhodopsin: amino acid sequence, gene structure, and functional expression. Biochemistry *31*, 3309–3315.

Yokoyama, R., and Yokoyama, S. (1990a). Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus*, and human. Proc. Natl. Acad. Sci. USA *87*, 9315–9318.

Yokoyama, R., and Yokoyama, S. (1990b). Isolation, DNA sequence and evolution of a color visual pigment gene of the blind cave fish *Astyanax fasciatus*. Vision Res. *6*, 807–816.

Zilles, K., Tillmann, B., and Bennemann, R. (1983). The development of the eye in *Astyanax mexicanus* (Characidae, Pisces), its blind cave derivative, *Anoptichthys jordani* (Characidae, Pisces), and their crossbreds. Cell Tissue Res. *229*, 423–432.