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Zebrafish ultraviolet visual pigment: Absorption spectrum, sequence, and localization

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ABSTRACT **In many vertebrates, UV-sensitive photoreceptors have been identified by microspectrophotometry and UV–visual sensitivity has been identified by behavioral studies, but as yet no vertebrate UV-sensitive pigment gene has been isolated. We have sequenced a cDNA clone that hybridizes to short single cone cells in the zebrafish (*Brachydanio rerio*). These cells, which make up 25% of the cone population in zebrafish retinae, are UV-sensitive ($\lambda_{max} \approx 360$ nm). The visual pigment encoded by this gene is unusual in that its amino acid sequence is more homologous to the rod pigment rhodopsin (up to 89%) than to other cone pigments (35–83%). Like all other vertebrate visual pigments, it contains a lysine residue at position 296, the presumptive retinal binding site, and a glutamate residue at position 113. However, it is unique in possessing a lysine residue at position 126, which may account for the UV-sensitivity of the pigment.**

Photoreceptors sensitive to ultraviolet (UV) have been identified in a number of invertebrates and in most classes of vertebrates, including teleost fish, amphibians, reptiles, birds, and even some mammals (1–6). In vertebrates, the UV–visual pigments are found in cone photoreceptors and presumably contribute to color vision. Indeed, it is now believed that color vision in many vertebrates is tetrachromatic, mediated by red, green, blue, and UV-sensitive cones (7). Genes encoding cone visual pigments have been cloned in a variety of vertebrate species enabling the amino acid sequences to be deduced, but to date no vertebrate UV–visual pigment gene has been isolated (8–16). All visual pigments consist of a transmembrane protein, opsin, to which is bound a chromophore, 11-cis retinal, but they absorb maximally over a wide range of wavelengths from ≈350 to ≈640 nm. There is much interest in understanding the mechanisms underlying spectral tuning of the visual pigments, and UV-sensitive visual pigments may provide special insights in this regard. We report the sequence of a vertebrate UV–visual pigment gene.§

MATERIALS AND METHODS

Microspectrophotometry. The spectral characteristics of the cone visual pigments were determined with a dichroic microspectrophotometer. The microscope optics consisted of two Ultrafluar (Zeiss) objectives, one (32×/0.4) used as a condenser and the other (100×/1.20) used as the objective lens. Both were color-corrected for the range 230–750 nm. With a beam of about 0.6 × 2 μm cross section, individual absorption spectra of outer segments were measured in side-on orientation over the range of 275–645 nm. Multiple reference scans (usually 16) were taken through cell-free areas in the preparation, whereas sample measurements were the average of 8–24 scans. Absorption spectra were calculated from reference and sample transmittance values at 5-nm intervals. Additional calculations, averaging, and smoothing were performed off-line as described (17).

Isolation of cDNA Clone. The cDNA clone was isolated from a zebrafish eye cDNA library (courtesy of Han Chang, Harvard University). The library was screened with a fragment of the human blue cone pigment gene [*BCP*; hs37: J. Nathans (15)], which contained the entire coding region. Nitrocellulose plaque filters were hybridized for 24 hr at 42°C with a probe labeled with ^{32}P in 10% formamide/10% dextran sulfate/1.0 M NaCl/50 mM NaH_2PO_4, pH 7.5/5 mM EDTA/1% SDS and then washed with several changes of the same buffer (without the probe and dextran sulfate). Putative positives were subcloned into pBluescript (Stratagene) and subjected to DNA sequencing by using the enzymatic chain-termination method (Sequenase version 2.0; United States Biochemical).

***In Situ* Hybridization.** Retinas were dissected from adult zebrafish previously dark-adapted for 60 min. Fish were decapitated, their eyes were removed, and their retinas were dissected under dim red light (filtered by Kodak Wratten no. 29). Retinas were fixed overnight in 4% paraformaldehyde, rinsed in four changes of cold PBST (phosphate-buffered saline containing 0.1% Tween-20; protein grade; Calbiochem), washed briefly in water, and permeabilized in acetone for 5–7 min at −20°C. The retinas were transferred through a series of washes in PBST, a 1:1 (vol/vol) PBST/hybridization buffer [50% formamide, 5× SSC (0.75 M sodium chloride, 0.075 M sodium citrate), 100 μg of salmon sperm DNA per ml, 50 μg of heparin per ml, and 0.1% Tween-20], and finally hybridization buffer for 15 min. Tissue was transferred to fresh Eppendorf tubes for a prehybridization incubation (200 μl) at 55°C for 40 min. Hybridization was carried out at 55°C overnight with digoxigenin (DIG)-labeled riboprobes prepared from template cDNA. After hybridization, the retinas were transferred to 35-mm culture dishes and washed for 60 min in hybridization buffer at 55°C, then for a further 30 min in 1:1 PBST/hybridization buffer, and finally twice for 15 min in PBST alone at room temperature. The retinas were refixed in 4% paraformaldehyde in phosphate-buffered saline and washed in PBST twice for 15 min and then once for 60 min. Pretreated anti-DIG–alkaline phosphatase conjugate antibodies (Boehringer Mannheim) were added to the retinas and left overnight at 4°C; pretreatment of antibodies was performed by diluting the antibody 1:4000 in blocking solution (phosphate-buffered saline containing 0.2% Tween-20, 0.2% Triton-X 100, 0.2% bovine serum albumin followed by a 1-hr preabsorption against paraformaldehyde-fixed/PBST-washed zebrafish retinas. After the overnight incubation in antibodies, mRNA labeled with DIG was then detected by immunoassay with an anti-DIG conjugate (Boehringer Mannheim). A subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-

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Abbreviation: DIG, digoxigenin.

§The sequences in this paper have been submitted to the Protein Identification Resource and have been deposited in the GenBank data base (accession no. L11014).

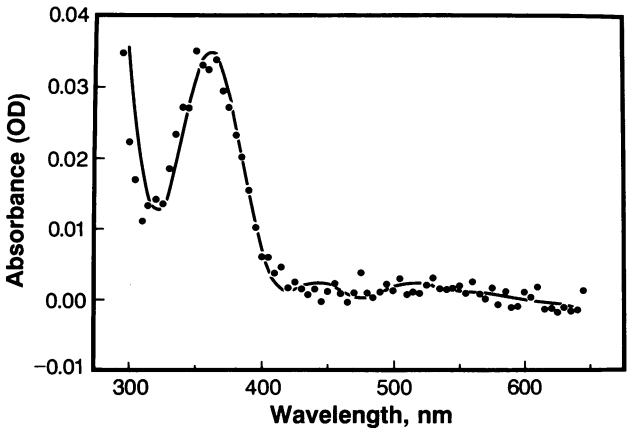

FIG. 1. Mean absorption spectrum for the short single cones in zebrafish. The solid curve represents the Fourier-smoothed spectrum calculated from 6 UV-sensitive cones. Absorbance of the α-band is 0.033. λ_{max} and half-band width are 361 nm and 4200 cm^{-1}, respectively.

indolyl phosphate, and nitro blue tetrazolium salt produces an insoluble blue precipitate, which visualizes hybrid molecules. After *in situ* whole-mount staining, embryos were dehydrated in ethanol, transferred to methyl salicylate for 10–15 min, and then embedded in Epon/Araldite. Sections of 5 μm were cut on a JB-4 microtome and counterstained with a saturated eosin solution.

To generate riboprobes, run-off transcripts from both T3 and T7 promoters were prepared with digoxigenin-UTP according to the manufacturer's specifications ("The Genius System User's Guide for Filter Hybridization," Boehringer Mannheim). The resulting antisense transcripts (complementary to the mRNA target) were utilized for the hybridization.

RESULTS AND DISCUSSION

The zebrafish retina contains four morphologically distinct classes of photoreceptors: rods and three types of cones—two single-cone types and one double-cone type (18). Double cones consist of a long, or principal, member and a short, or accessory, member. We have measured the absorption properties of each class of cone photoreceptor using microspectrophotometry. As in other cyprinids, the principal member of the double cone is red-sensitive ($\lambda_{max} \approx 570$ nm), whereas the accessory member is green-sensitive ($\lambda_{max} \approx 480$ nm).¶ The long single cones are blue-sensitive ($\lambda_{max} \approx 415$ nm), and the short single photoreceptors contain an UV-sensitive pigment with a mean λ_{max} of 362 ± 3 nm ($n = 10$) (Fig. 1). The single cones, containing the UV-absorbing pigment, show linear dichroism from the side (in lateral view) with a similar dichroic ratio to other cones and they "bleach" when exposed to actinic light by the loss of dichroism and creation of a photoproduct that absorbs near 380 nm.

In zebrafish, as in other teleost fish, the cones are arranged in a regular geometric array called a "mosaic." Fig. 2 *Top* shows a cross-section of the zebrafish retina, illustrating the regular arrangement of the UV-absorbing short single cones. This section was cut through the inner segments (myoids) of both the double and long single photoreceptors and through the densely staining outer segments of the short single cones. To differentiate the long single-cone inner segments from those of the double cones, sections were stained with a probe specific to the opsin of blue-sensitive cones (see Fig. 2 *Middle*). The long and short single cones alternate in rows between the double cones. The double cones, in turn, alternate their configuration so that the principal member of the double cone (red-sensitive) is always opposite to a short single (UV-sensitive) cone, whereas the accessory (green) member of the double cone is opposite to a long single (blue) cone. This arrangement is shown schematically in Fig. 2 *Bottom*. P. Raymond (personal communication) has proposed an identical cone mosaic for the zebrafish based on colorimetric *in situ*

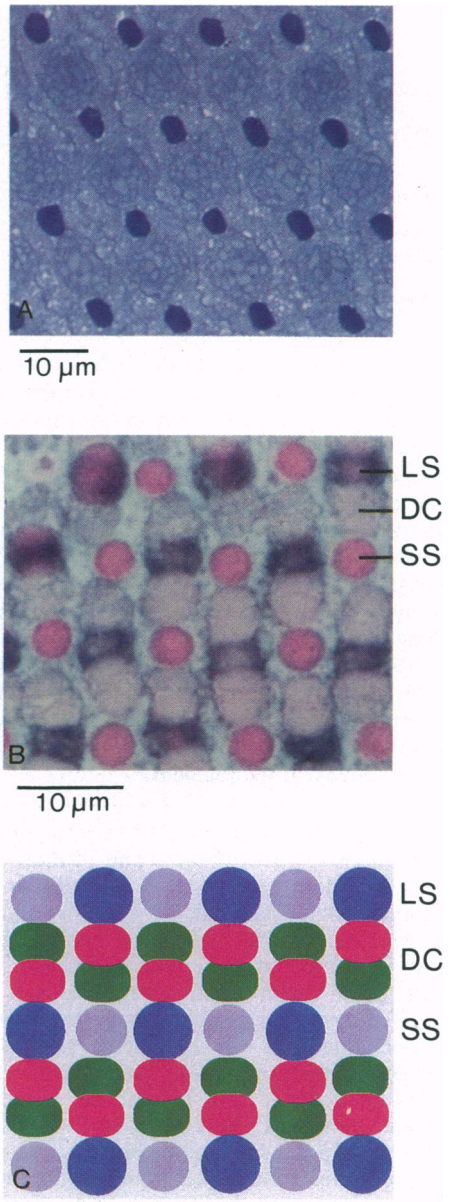

FIG. 2. (*Top*) Light micrograph of zebrafish retinal mosaic. This section is cut through the densely stained outer segments of the short single photoreceptors and shows their arrangement. (1% methylene blue/1% azure blue). (*Middle*) Zebrafish cone mosaic: A 5-μm section stained with eosin and an antisense riboprobe to blue opsin. LS, long single cone; DC, double cone; SS, short single cone. The section is cut through the inner segments of the long single and double cones and through the outer segments of the short single cones. (*Bottom*) Representation of the retinal mosaic in zebrafish. The cones are arranged in rows, resulting in a regular mosaic arrangement. Long single (blue-sensitive) cones are represented by the blue circles; double cones, by the red and green ovals; and the short single cones, by the violet circles.

¶Our microspectrophotometric data disagree somewhat with previously published data by Nawrocki *et al.* (19). We did not find any long single photoreceptors that were green-sensitive as they reported. It is possible that their single green cells were sheared members of double cones. Futhermore, no UV-sensitive cones were observed in the earlier study, perhaps because of the nature of the optics used in their apparatus.

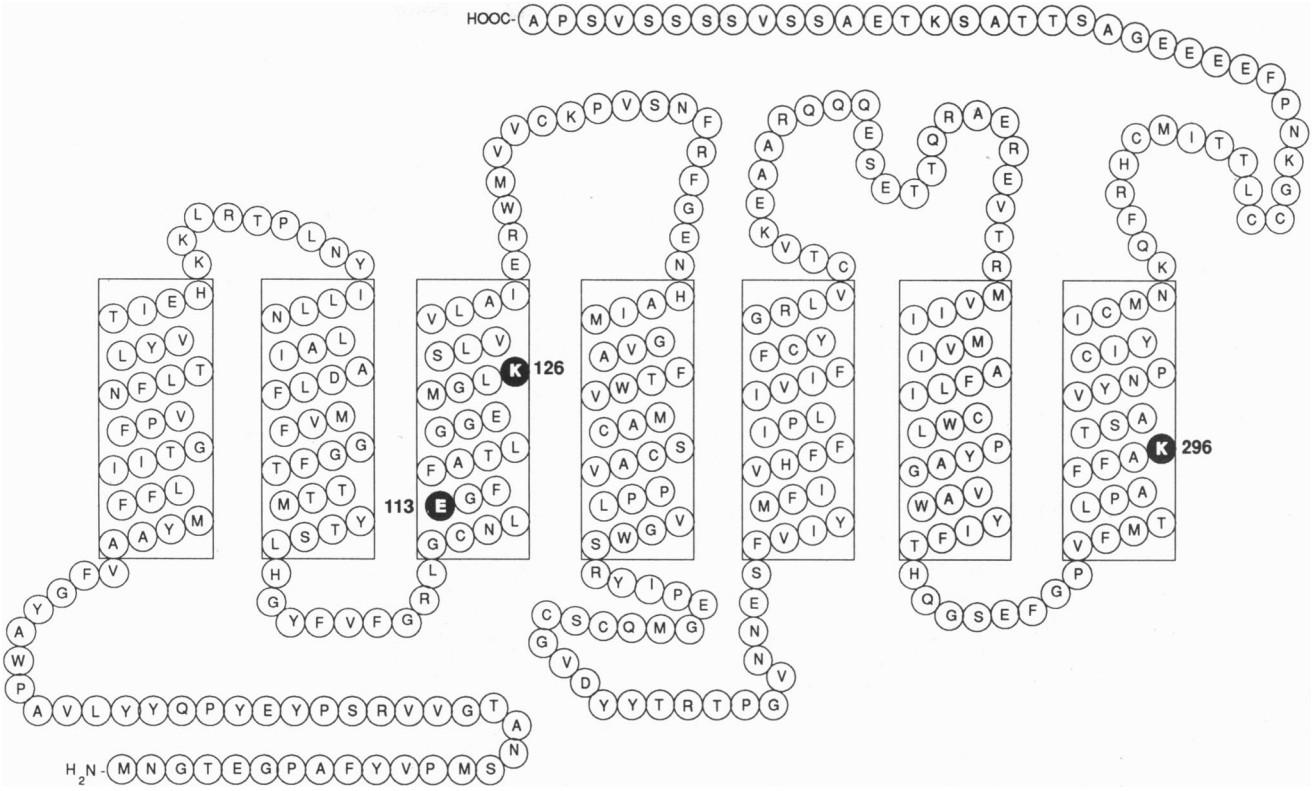

FIG. 3. Proposed structure of the zebrafish UV-sensitive visual-pigment opsin, based on the algorithm of Kyte and Doolittle (40). Amino acids residues are indicated by their single-letter codes. The length of the gene from its mRNA start site to its polyadenylylation site is 1065 base pairs.

hybridization studies using probes generated from blue, green, and red cone-pigment genes of goldfish. In zebrafish, 25% of the cone population is UV-sensitive; furthermore, the UV-sensitive cones are present in adult fish as well as in juveniles, in contrast to some other species (3, 20).

Encouraged by the abundance of UV-sensitive cones in zebrafish, we screened a zebrafish eye cDNA library for genes encoding short-wave length-sensitive visual-pigments and have isolated and sequenced a cDNA clone. Fig. 3 displays the secondary structure prediction of the 355-residue polypeptide encoded by this cDNA. The polypeptide is highly homologous to vertebrate opsins and shares several features common to visual pigments sequenced from a variety of organisms. These include seven hydrophobic transmembrane domains separated by hydrophilic sequences (21, 22), a presumed retinal binding site (Lys-296) in the seventh transmembrane domain (23–25), sites for N-linked glycosylation near the N terminus (26), and a serine- and threorine-rich C-terminal region that may serve as the site for light-dependent phosphorylation (27, 28). On the basis of these criteria, we concluded that this DNA sequence, termed ZFO2, encodes an opsin.

To identify which class of photoreceptor expresses ZFO2, a RNA probe was generated, and *in situ* hybridization was performed on whole-mount retinas. Signal was detected in the short single cones, and all short single cones hybridized

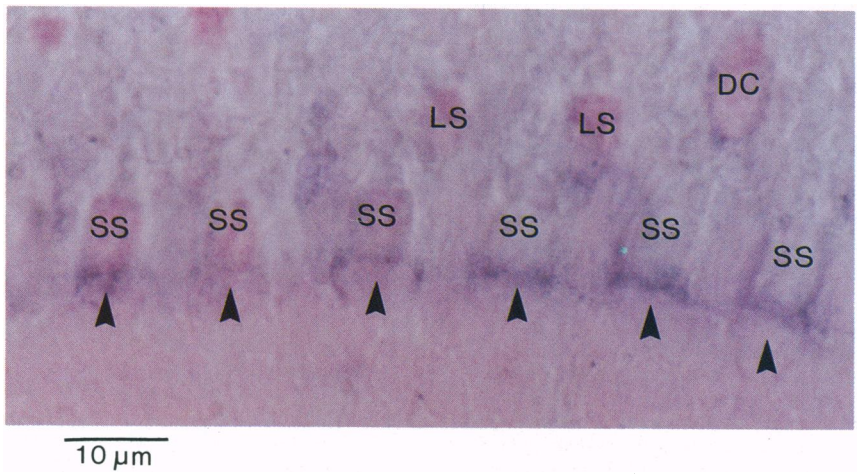

FIG. 4. Longitudinal section (5 μm) of a dark-adapted zebrafish photoreceptor layer, hybridized to a DIG-labeled riboprobe generated antisense to ZFO2. mRNA present in the myoids of all short single photoreceptors (SSC) hybridized to this probe. No signal was detected in either member of the double cones (DC) or in the long single cells (LS). Furthermore, no hybridization to rods was detected. (Bar = 10 μm.)

to this cDNA clone. Hybridization occurred in the myoid region of the short single cones (Fig. 4). Since this type of photoreceptor has its λ_{max} in the UV, we conclude that ZFO2 encodes the opsin for a UV-sensitive visual pigment.

The predicted sequence of our opsin clone is more similar to rod than cone opsins. It is most homologous to lamprey rhodopsin to which it has a protein homology of 89%. ZFO2 has other rhodopsin-like properties: it possesses (*i*) two potential asparagine glycosylation sites, at positions 2 and 15 (26), whereas most cone pigments have a single site; and (*ii*) a conserved histidine at residue 211 that regulates the equilibrium between metarhodopsin I and metarhodopsin II photoproducts (29). ZFO2 is somewhat less homologous to cone opsins (35–83%): it is most homologous to short-wavelength cone pigments including gekko blue (83%), chicken violet (67%), zebrafish blue (61%), and human blue (67%), and it is only about 35% homologous to invertebrate UV-sensitive visual pigments.

The wavelength sensitivity of visual-pigment molecules is believed to be determined by amino acids in the opsin helices, especially in helices 3 and 5, and pigments sensitive in the visible range have an absorption maximum that is red-shifted relative to both free retinal and protonated retinal Schiff bases (30). This red shift in the absorption maximum is thought to result mainly from the presence of a glutamic acid residue (Glu-113) in helix 3 that acts as the Schiff base counterion (31–33). If this glutamic acid residue is mutated to glutamine, the absorption maximum of rhodopsin shifts from about 500 nm to 380 nm (31–33). Whereas invertebrates do not possess a glutamic acid counterion, it has been proposed that a conserved tyrosine serves this role (34–36). Furthermore, this tyrosine is absent in the *Drosophila* UV-sensitive pigments. Thus, the presence of glutamic acid at position 113 in our sequence is surprising. However, an unusual feature of ZFO2 compared with all other vertebrate opsins sequenced to date is the presence of a lysine residue at position 126 in helix 3 rather than a tryptophan. Lysine is a basic amino acid that could perhaps serve as a counter-counterion, negating the effect of Glu-113. It is possible that the lysine residue at position 126 serves to negate the electrostatic charge causing the protonation of the Schiff base. Most models propose that one negatively charged amino acid, glutamic acid (Glu-113), serves as the retinylidine Schiff base counterion. However, the transmembrane regions are thought to be in an α-helical configuration; thus, the distance between the Glu-113 counterion and the lysine residue (Lys-126) would be on the order of 20 Å. If they are this far apart, direct electrostatic contact between Glu-113 and Lys-126 is unlikely to occur. Point charge models, on the other hand, propose that a second negative charge lies along the polyene chain, thus enhancing electron dissociation and inducing the red shift in absorbance maxima (37–39). It is possible that the replacement of Trp-126 by Lys-126 has the opposite effect and thus induces a blue shift in absorbance maximum. This amino acid change may be the reason that the ZFO2 pigment absorbs at ≈360 nm, close to the absorbance of unprotonated retinal Schiff bases. Expression of appropriate mutants of ZFO2, followed by their reconstitution with 11-cis retinal, should provide a test of this hypothesis.

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