Isolation of a Zebrafish Rod Opsin Promoter to Generate a Transgenic Zebrafish Line Expressing Enhanced Green Fluorescent Protein in Rod Photoreceptors*

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To exploit zebrafish as a transgenic model, tissue-specific promoters must be identified. We isolated a 20-kilobase (kb) zebrafish rod opsins genomic clone, which consists of 18 kb of 5′-flanking region, the entire coding region, and 0.5 kb of 3′-flanking sequence. Polymerase chain reaction, Southern blotting, and DNA sequencing revealed the rod opsins gene lacks introns. The transcription start site was localized by 94 nucleotides upstream of the translation initiation site. Sequence alignment with orthologous promoters revealed conserved cis-elements. This 1.2-kb promoter fragment was cloned upstream of the enhanced green fluorescence protein (EGFP) cDNA and microinjected into 1- to 2-cell stage zebrafish embryos. EGFP expression was detected in the ventral-nasal eye at 3 days postfertilization and spread throughout the eye. Progeny of the positive founder fish, which were identified by polymerase chain reaction amplification of fin genomic DNA, exhibited EGFP expression in the retina, confirming the germline transmission of the transgene. Frozen eye sections demonstrated the EGFP expression was rod-specific and exhibited a similar developmental expression profile as the rod opsins protein. This stable transgenic line provides a novel tool for identification of genes regulating development and maintenance of rod photoreceptors.

The zebrafish, Danio rerio, has emerged as a novel vertebrate model system that is amenable to mutagenesis and transgenesis. High fecundity, rapid oviparous development, and a translucent embryo make zebrafish a prolific experimental model (1). Furthermore, the zebrafish eye possesses distinct advantages for studying the development, function, and inherited diseases of the retina. Eye ontogenesis proceeds rapidly, completing the laminae of the adult retina by 3 days postfertilization (dpf); Ref. 2). The zebrafish eye is relatively large and accessible, and the position and morphology of the rod and cone classes are readily distinguishable (3). Finally, the integrity of visual system structure and function can be evaluated by morphological, behavioral, and electrophysiological methods (4–6). Chemical mutagenesis screens previously identified over 65 zebrafish visual system mutants exhibiting either behavioral (4, 6, 7) or morphological abnormalities (5). Insertional mutagenesis screens generated additional eye mutants (8, 9). Overall, ~35 mutants possess visual system-specific defects (4–7). Transposition, random insertion, and retroviral integration provide the means for introducing transgenes into the zebrafish genome (10–12). Accurate tissue-specific and developmental expression has been recapitulated in nonretinal tissue using endogenous zebrafish promoters (13, 14).

Rhodopsin, comprising the rod opsins protein bound to 11-cis-retinaldehyde, constitutes the photosensitive visual pigment of vertebrate rod photoreceptors (15). Rhodopsin is concentrated in the outer segments of the rod photoreceptor cells, which mediate vision in dim light. Mutations in the human rod opsins gene (RHO) can result in retinitis pigmentosa, a slow retinal degeneration resulting in blindness (16). Drosophila melanogaster, Mus musculus, and Xenopus laevis have served as genetic models for studying rhodopsin function and generating models of inherited human visual disease (17–19). However, none of these models combines the vertebrate eye with features of amenability to mutagenesis screens and transgenesis, as found in zebrafish. The developmental expression of zebrafish rod opsins initiates in two phases. It is expressed initially in a ventral-nasal patch of cells, and subsequently rod opsin-expressing cells arise in a scattered pattern throughout the remainder of the retina (20, 21). Furthermore, rod photoreceptors are added to the fish retina throughout life from two retinal sources: stem cells in the circumferential germinal zone and rod precursor cells, which are found in the outer nuclear layer (22–24).

To develop tools for transgene expression in the zebrafish retina, we cloned the rod opsins gene. Characterization of the gene revealed that it lacks introns and contains conserved cis-regulatory sequences. We identified a 1.2-kb promoter fragment that directs enhanced green fluorescent protein (EGFP) expression specifically to rods. Furthermore, this transgene was integrated stably into the germline and transmitted to the subsequent generation. The expression of transgenes in zebrafish photoreceptors will enhance our ability to investigate visual gene function, gene regulation, and gene therapy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T4 polynucleotide kinase, and Taq DNA polymerase were purchased from Fisher. HybNoN+ nylon mem-
pairs; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; bp, base pair(s).
branes were purchased from Amersham Pharmacia Biotech. The Ultra-
spec RNA II isolation system and Hybriol II were obtained from Cinna
Biotec and Intergen, respectively. The zebrafish genomic library was
purchased from Stratagene. The Thermoscript reverse transcriptase
system and ThermoSequenase Radiolabeled Terminator cycle sequenc-
ing kit was used for DNA sequencing.

Polymerase Chain Reaction (PCR) Mapping—Genomic, phage, and
plasmid DNA templates were PCR-amplified using primers spanning
the zebrasfish rod opsin cDNA. PCR parameters were 30 cycles of dena-
turation at 94 °C for 30 s, anneal primers at 65–67 °C for 30 s, and
extension at 72 °C for 5 min using Taq DNA polymerase.

Primer Extension—A primer complementary to the 5'-end of the
zebrafish rod opsin mRNA was end-labeled with 32P using T4
polynucleotidyl kinase. 12 μg of zebrafish eye or body total RNA were
hybridized with the labeled primer for 1 h at either 60 or 42 °C (in 50% 
formamide). After hybridization, the samples were treated with RNase
and denatured by heating at 70 °C for 10 min. The samples were cen-
trifuged and loaded on a 5% polyacrylamide gel, and autoradiography
was performed to detect the labeled product.

Northern Blotting—Total RNA was isolated using the Ultraspec
RNA II isolation system. RNA aliquots (~18 μg) were electrophoresed
in 1% agarose gels containing 6.6% formaldehyde, blotted to Hybond-N
nylon membranes in 20X SSC, and UV cross-linked. Membranes were
hybridized with a probe in Hybriol II containing 35% (v/v) formamide
at 55 °C. Final high stringency washing conditions were 0.08X SSC,
0.1% SDS at 65 °C for 30 min twice.

Genomic Library Screens—Approximately 109 recombinant phage
in a λ FIX phage genomic library were screened with a radiolabeled
zebrafish rod opsin cDNA (26). Escherichia coli strain XL1-MRAP2 was
infected with titrated phage and plated on 150-mm NZY Petri dishes.
Plaques were lifted and UV-crosslinked onto Hybond-N+ nylon mem-
bones (27). Hybridization conditions were as described under “South-
ern Blotting.”

RESULTS

The Zebrafish Genome Contains a Single Rod Opsin Gene—We examined the potential for duplicate rod opsin genes in the
zebrafish genome by Southern blots. The zebrafish rod opsin cDNA typically detects single genomic fragments, al-
though digestion with either ApaI or EcoRI yielded two hybrid-
izing fragments (Fig. 1). The presence of two ApaI fragments is
consistent with an internal ApaI site in the cDNA probe. How-
ever, because no internal EcoRI sites are known, the two EcoRI
fragments are likely due to a sequence polymorphism. Overall,
the patterns indicate the zebrafish rod opsin gene is likely
single copy.

Isolation of a Zebrafish Rod Opsin Genomic Clone—A 20-kbp
rod opsin genomic clone was isolated from a zebrafish library.
The phage clone was mapped by Southern blot analysis using
regions of the rod opsin cDNA as probes (data not shown). The
data suggested that the zebrafish rod opsin gene contained
minimal, if any, intron sequence. A 3-kbp (EcoRI–NotI) frag-
ment detected by the cDNA probe was subcloned into the
pCR2.1 plasmid. We examined this genomic subclone for the
presence of introns by comparing the PCR amplification prod-
ucts from genomic DNA, the genomic clone, and the cDNA
clonne using primers spanning the zebrafish rod opsin cDNA
(Fig. 2). The genomic and cDNA templates consistently prod-
cuced PCR products of the same size (Fig. 2), which further
demonstrates that the zebrafish rod opsin gene lacks introns.

Localization of the Transcription Start Site for Zebrafish Rod
Opsin—We examined the size of the rod opsin mRNA to ap-
proximate the length of the 5'-untranslated sequence. The rod
opsin cDNA clone that we previously characterized was 1,576
nucleotides with 92 nucleotides of 5'-untranslated sequence
(26). We probed zebrafish eye and body total RNA with the rod
opsin cDNA clone on Northern blots. A 1.9-kb transcript was
detected in the eye RNA but not in the body RNA (Fig. 3A). The
size and tissue specificity are consistent with other rod opsin
orthologues (15). The transcription start site of the zebrafish
rod opsin gene was determined by primer extension. Consistent
with the Northern results, primer extension products were
observed with eye RNA templates but not with body RNA (Fig.
3B). DNA sequencing of the 3-kbp genomic subclone with the
identical primer used for primer extension localized the tran-
scriptional start sites to the cytosine and guanine nucleotides

![FIG. 1. Genomic Southern blot of the zebrafish rod opsin gene.](image)
94 and 91 base pairs (bp) upstream of the translation start site, respectively. The presence of more than one primer extension product may be because of differential methylation at the 5' end of the transcript (30).

Sequence of the Zebrafish Rod Opsin Gene—We characterized the 3-kbp rod opsin genomic subclone (pzfrh3CR). This clone contains '1.2 kbp upstream of the transcriptional start site, which is the entire rod opsin coding sequence and 468 bp of 3'-flanking sequence (Fig. 4). Consensus polyadenylation signals (AATAAA) were found at positions 1535 and 1553, located 18–51 bp upstream of two transcription terminator signals (YGTGTTYY) (31). The first polyadenylation signal is in agreement with our cDNA sequence, which deviates from the genomic sequence at position 1559 by the presence of 19 adenine residues. Alignment of proximal promoter sequences of zebrafish, Xenopus, chicken, mouse, and human rod opsin genes identifies conserved cis-elements (Fig. 5) that are implicated in regulating retina-specific gene expression: glass (32, 33); NRE (34, 35); Otx/Bat-1 (36, 37); Ret-1/PCE-1 (38–40), and Ret-4 (41).

A Zebrafish Rod Opsin Promoter Directs EGFP Expression to Photoreceptors—A 1.2-kbp fragment containing the putative zebrafish rod opsin promoter, the transcriptional start site, and 52 bp of 5'-untranslated sequence were cloned upstream of the EGFP cassette to generate pZOP-EGFP. Zebrafish embryos at the 1- to 2-cell stage were microinjected with linearized pZOP-EGFP. Approximately 50% of injected embryos continued developing, with 90% of them hatching and 65% surviving beyond 15 dpf. An average of 6% of the injected embryos expressed the EGFP transgene at variable levels, although expression was always restricted to the eye (Fig. 6, A and B). EGFP expression was detected first at 3 dpf in the ventral-nasal eye and retinal sections localized the EGFP expression to the photoreceptor layer (data not shown). The localization and morphology of the EGFP-expressing cells was consistent with rod photoreceptor-specific expression.

Germline Transmission of the ZOP-EGFP Transgene—

Fig. 2. Mapping the zebrafish rod opsin genomic clone. Primers spanning the rod opsin cDNA were used to PCR amplify the following DNA samples: rod opsin genomic phage DNA (a), zebrafish genomic DNA (b), rod opsin cDNA (c), and the pzfrh3CR plasmid (d). A control reaction that lacked template was also used (–). The primer pairs used in each PCR reaction (RF1-RR1, RF1-RR2, RF2-RR2, RF2-RR3, and RF3-RR4) are shown at the top of the gels. The positions of the primers relative to the cDNA are shown at the bottom of the figure.
fish that were EGFP-positive by fluorescence microscopy survived to adulthood. The EGFP cassette was PCR-amplified from fin genomic DNA in seven of these fish (data not shown). Based on fluorescence microscopy, one of these fish exhibited germline transmission of the transgene to 6% of its progeny. These EGFP-positive G1 progeny were isolated, and their EGFP expression patterns were examined. At 4 dpf, the EGFP expression was limited to the ventral-nasal portion of the retina, although rhodopsin protein expression was detected throughout the retina (Fig. 6C). Careful inspection of the EGFP-expressing cells in the whole-mount-labeled retinas confirmed rhodopsin expression in the outer segments of the EGFP-positive cells (Fig. 6D). At 17 and 54 dpf, EGFP expression in G1 fish spanned the photoreceptor layer of the retina, but with an apparent dorsal-ventral gradient (Fig. 6, E and F, respectively). Close examination of the EGFP-positive cell mor-
The single arrow in C points to an isolated double-labeled cell that is shown at higher magnification in D (arrow points to EGFP-containing cell body; arrowhead points to rhodopsin-positive rod outer segment). E, a 10-μm frozen section of a 17-dpf G1 fish reveals EGFP-expressing cells in the dorsal and ventral aspects of both retinas (arrows point to dorsal retinas of each eye). The arrowhead on the left eye indicates the optic nerve exit point. F, frozen section from a 54-dpf G1 fish reveals EGFP-containing nuclei in the outer nuclear layer (ONL; arrow) and many long rod outer segments that also contain the EGFP (ROS; arrow). G, high magnification image of a single EGFP-positive cell from the section shown in F. The rod outer segment, ellipsoid (E), myoid (M), and nucleus (N) are identified. H, sections were labeled with anti-rhodopsin and detected with Cy3-conjugated secondary antibody. The EGFP signal is present in both the ONL and the layer of ROS, whereas rhodopsin expression was detected in only the ROS. EGFP was not detected elsewhere in the retina. D, dorsal; V, ventral; L, lens; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bars: C and G, 50 μm; D, 10 μm; G, 5 μm; E and F, 100 μm.)

We identified evolutionary conserved sequences in the 5′-flanking region of the rod opsin gene that may control rod opsin expression in zebrafish. The conserved cis-elements (glass, NRE, Ret-1/PC1-1, and Ret-4) include sites for transcription factors (Crx, Erx, Glass, Mash-1, Nrl, and Rx) that regulate retina/photoreceptor/rod-specific expression and that segregate with visual defects (32, 34, 37–41, 44–47). Rod opsin expression is regulated in a combinatorial fashion that depends on the synergistic activities of several transcription factors (34, 39). The presence of these regulatory elements in the ZOP-EGFP transgene likely helped to ensure its robust level of rod-specific expression. Although rod opsin gene expression may be regulated in a complex fashion, its promoter is excellent for expressing transgenes in the rod photoreceptors. Rod opsin promoters have successfully directed gene expression to rod photoreceptors in both mouse and Xenopus (48–51). Also, the promoter in its native state drives a high level of expression during the terminal differentiation of rod cells and maintains that expression level through the life of the cell.

In this study, we identified a 1.2-kbp zebrafish promoter that directs EGFP expression to rod photoreceptors. The expression pattern of the transgene appears to recapitulate the tissue-specific and developmental progression of the zebrafish rod opsin (glass). The single arrow in C points to the fluorescein emerging through the pupil, and the arrow points to the fluorescence emerging through the pupil. The anti-rhodopsin in the rod outer segments was detected with a Cy3-conjugated secondary antibody. The EGFP-expressing cells are located predominately in the ventral-nasal aspect of the retina (double arrows in upper eye; arrowhead points to choroid fissure). The single arrow in C points to an isolated double-labeled cell that is shown at higher magnification in D (arrow points to EGFP-containing cell body; arrowhead points to rhodopsin-positive rod outer segment). E, a 10-μm frozen section of a 17-dpf G1 fish reveals EGFP-expressing cells in the dorsal and ventral aspects of both retinas (arrows point to dorsal retinas of each eye). The arrowhead on the left eye indicates the optic nerve exit point. F, frozen section from a 54-dpf G1 fish reveals EGFP-containing nuclei in the outer nuclear layer (ONL; arrow) and many long rod outer segments that also contain the EGFP (ROS; arrow). G, high magnification image of a single EGFP-positive cell from the section shown in F. The rod outer segment, ellipsoid (E), myoid (M), and nucleus (N) are identified. H, sections were labeled with anti-rhodopsin and detected with Cy3-conjugated secondary antibody. The EGFP signal is present in both the ONL and the layer of ROS, whereas rhodopsin expression was detected in only the ROS. EGFP was not detected elsewhere in the retina. D, dorsal; V, ventral; L, lens; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bars: C and G, 50 μm; D, 10 μm; G, 5 μm; E and F, 100 μm.)

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opsin gene (20, 21, 52). At 3 dpf, EGFP-positive cells are clustered in the ventral-nasal retina and begin to appear singly in other retinal regions at 6–7 dpf (data not shown). However, a more critical analysis at the cellular level reveals two subtle divergences from endogenous rod opsin expression. First, the temporal expression of ZOP-EGFP is delayed slightly relative to rod opsin. Rhodopsin expression is observed first in the ventral retina, nasal to the choroidal fissure. Although differentiating rods accumulate rapidly and to a high density in this ventral patch, rods are added at a slower rate and in a random, isolated fashion in other retinal regions (20). In contrast, the EGFP expression pattern at 4 dpf appears like the pattern of rhodopsin expression seen in developing fish at 55–60 h post-fertilization (20). Thus, rods appear to differentiate and express rhodopsin prior to expressing EGFP. This result may suggest that an additional factor necessary for the initiation of rod opsin expression during early differentiation acts beyond our 1.2-kbp cloned promoter fragment. Second, in adults, not all rod photoreceptors express EGFP, and the expression may occur in a dorsal-ventral gradient. A gradient of transgene expression pattern at 4 dpf appears like the pattern of opsin gene expression. Although the expression of EGFP is consistent with percentages previously described (13, 14). The frequency of injected embryos exhibiting germine transmission was 0.25–1.75%. However, the frequency of transmission may have been underestimated because of gene silencing and technical difficulties in detection of EGFP in the eye. In addition to screening for transgene expression by fluorescence microscopy, adult fish were screened secondarily for stable transgene integration by PCR amplification of fin genomic DNA. Adults that typed positive were outcrossed, and their progeny were screened by fluorescence microscopy to verify transmission of the transgene in the germline. As the fish age, microscopic observations of the retina become obscured by the envelope of melanophores and iridophores around the eyes. Treatment of fish with 1-phenyl-2-thiourea inhibits the synthesis of melanin, thus permitting observation of EGFP through the translucent eye at 3 dpf (the onset of rhodopsin expression). However, the 1-phenyl-2-thiourea does not inhibit the production of the gold- or silver-reflecting components of the iridophores so that at 6–8 dpf, EGFP fluorescence is often observed only as it exits through the lens. Thus, proper orientation of the fish, as previously reported for Xenopus (54), is critical to ensure the accurate detection of EGFP expression.

The identification of a functional zebrafish rod opsin promoter will provide several significant applications. The promoter will be used to drive the expression of putative dominant mutations to generate models of human retinal diseases. Also, as candidate genes are identified for zebrafish retinal mutants, the expression of wild-type genes in the mutant will be a powerful method to confirm gene identification. This type of “gene rescue” has been widely exploited in Dro sophila. In addition, transgenic lines that express markers such as EGFP will be useful tools in mutagenic screens, in which subtle phenotypes may be identified more easily by the loss of a readily observable marker like the EGFP. Because the cis-elements in rod opsin promoters are conserved between zebrafish and mammals, transgenic zebrafish may prove useful in characterizing the developmental regulation of rod opsin and other photoreceptor genes. Transgenic zebrafish also may prove useful in elucidating the current controversy over the nature of circadian photoreception. The controversy stems from whether the circadian photoreceptor is initiated in the eye, pineal, or other tissues and whether the photoreceptor is Opsin-based, a cryptochrome, or an unknown photoreceptor (55, 56). Apparent system redundancy and species differences complicate the issue (57, 58). In zebrafish, six opsin genes expressed in four retinal photoreceptor classes and six cryptochrome genes expressed in the body, brain, and eye have been characterized (26, 57). Mutagenesis screens to identify circadian mutants and the ablation of specific retinal cell populations by targeted expression of toxic genes may prove useful to reveal genes that function in circadian photoreception.

Acknowledgments—We thank D. Ponder for helpful discussions, A. Zierer for assistance with microphotography, and Zebrafish Management, Ltd. for providing Tetra AZ baby powder. We also thank D. Quijano, D. Rincon, and D. Atkinson for assistance with the animal care. We thank T. David Ponder for helpful discussions, and A. Zierer for assistance with microphotography, and Zebrafish Management, Ltd. for providing Tetra AZ baby powder. We also thank Emily Cassidy, Rachel Hartzell, and Debbie Bang in the University of Notre Dame’s Zebrafish Facility.

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