The Murine Cone Photoreceptor: A Single Cone Type Expresses Both S and M Opsins with Retinal Spatial Patterning

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Summary

Mice express S and M opsins that form visual pigments for the detection of light and visual signaling in cones. Here, we show that *S opsin* transcription is higher than that of *M opsin*, which supports ultraviolet (UV) sensitivity greater than midwavelength sensitivity. Surprisingly, most cones coexpress both S and M opsins in a common cone cell type throughout the retina. All cones express M opsin, but the levels are graded from dorsal to ventral. The levels of S opsin are relatively constant. However, in the far dorsal retina, S opsin is repressed stochastically, such that some cones express M opsin only. These observations indicate that two different mechanisms control M and S opsin expression. We suggest that a common cone type is patterned across the retinal surface to produce phenotypic cone subtypes.

Introduction

Rod and cone photoreceptor cells of the retina enable the detection of light and initiation of visual signaling. The spectral range of detection is controlled by the selected expression of visual pigments differing in absorption spectra. The threshold and amplitude of response to light are influenced by the levels of pigment expression within these cells. In addition, the retinal topography, or retinal mosaic, of photoreceptors contributes the potential for color vision, spatial resolution, and orientation of visual scenes (Rodieck, 1998). These attributes vary widely from animal to animal. Evolutionary adaptation to differing environments has affected the selection of pigment isoforms, the level of pigment expression, the number of photoreceptor cell types, and the spatial organization of photoreceptors (Jacobs, 1993).

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In the mouse, the classic work of Carter-Dawson and LaVail (1979a, 1979b) best documents the rod and cone cell types. The retina is rod dominated. Cones constitute only 3% of the photoreceptors and are evenly spaced across the retina. While the cell morphology distinguishes cones from rods, it does not reveal different cone cell types. At the time of the Carter-Dawson and LaVail work (1979a, 1979b), there were no good markers for cone subtypes. Szél, Röhlich, and colleagues initiated studies of cone subtypes in the murine retina and explored their topography (Szél and Röhlich, 1992; Szél et al., 1992, 1994). Their work with the C57BL mouse, using monoclonal antibodies to chicken opsins, suggested that S cones were primarily in the ventral retina and M cones were only in the dorsal retina, thus accounting for the even distribution of cone number across the retina. Additional studies of cone topography in different species of *Mus* and *Apodemus* indicate that both S and M cones can vary in pattern; depending upon the species, patterns exhibit uniform, gradient, or no expression of S or M opsin (Szél et al., 1994). Mechanisms by which patterns might vary among genera have not been considered.

Rod and cone photoreceptors are generally considered to express a single opsin, which covalently binds 11-*cis* retinaldehyde to produce a visual pigment with a unique absorption spectrum. While the S and M cones are considered to be the two cone types in the mouse, several studies challenge the strict interpretation that they are exclusive cell types. A *lacZ* reporter driven by a human upstream M cone control region is not restricted to M cones but is expressed in both cone types (Wang et al., 1992). Likewise, the full human *L opsin* gene (Shaaban et al., 1998) and reporter transgenes for the full human X chromosome tandem L/M gene array (Wang et al., 1999) are expressed in both cone types in transgenic animals. Moreover, the mouse is one of a growing list of animals in which some form of dual expression of opsins in a common photoreceptor type is documented. Rölich and colleagues (1994) indicate that S and M opsins are coexpressed in one cone type in a narrow horizontal "transition zone" near the horizontal midplane of the retina. Physiological studies of mouse electroretinograph (ERG) signals confirm that some proportion of cones express both S and M opsins (Lyubarsky et al., 1999). These observations call for clarification of the nature of the cone types and their topographical organization, since these parameters affect our considerations for mechanisms of cell differentiation, control of opsin expression, and properties of color vision.

In the course of characterizing transgenic animals, we developed probes specific for murine rod and cone opsins and examined the opsin expression and the cone topology in the normal mouse. We have mapped levels of expression of S and M cone opsins, assessed the spatial distribution of opsins across the retinal surface, and characterized the cone cell types. To our surprise, we found that the vast majority of cones express both opsins throughout the retina in a common cell type. The patterns of expression dictate that two different mechanisms control the S and M opsin expression. We suggest a model for transcriptional control that explains our observations and predicts mechanisms that may regulate cone topography.
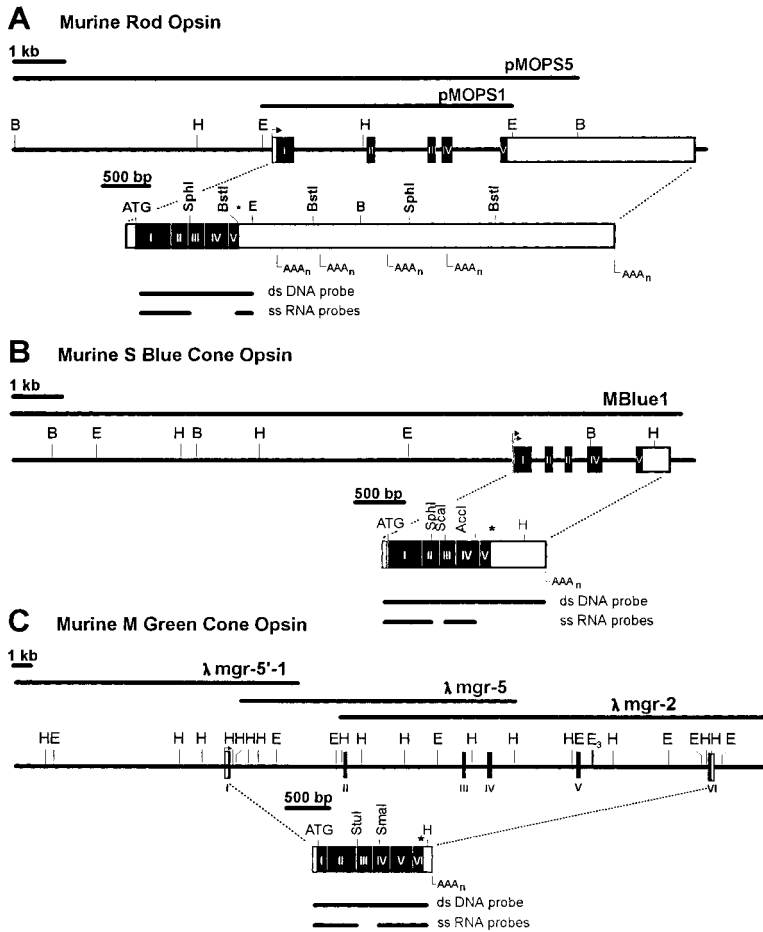
Figure 1. The Murine Rod and Cone Opsin Genes and Transcription Units

Opsin genomic clones, genes, transcript diagrams, and probes used in this work are shown as murine *rod opsin* (A), murine *S opsin* (B), and murine *M opsin* (C). Restriction sites indicated include BamHI ("B"), HindIII ("H"), and EcoRI ("E"). Boxes and lines represent exons and introns, respectively. The closed and open boxes indicate coding and untranslated regions, respectively. The sites for translational initiation (ATG) and termination (TAA or TGA) are indicated. Two transcription start sites were mapped for the *S opsin* gene. A more proximal site at −14 from the ATG translation site is ∼30 bases downstream from a TATA box; a more distal site at −60 is flanked between a TTTAAA site and the TATA box. The start site at −60 seems preferred, since all cDNA clones extended beyond the proximal site. For the *M opsin* gene, a single transcription start site is present 44 bp (±2 bp) upstream from the ATG translation site, ∼22 bp downstream from a TATA box.

Results

Structures of the Murine Opsin Genes, mRNAs, and Proteins

S, M, and L are used here to denote the three families of opsins, which upon covalently binding 11-*cis* retinaldehyde generate the blue/UV- (or short wavelength–sensitive, SWS), green- (or midwavelength, MWS), and red- (or long wavelength–sensitive, LWS) absorbing visual pigments, respectively. The cones in which these opsins are expressed are noted as S, M, and L cones.

To study gene expression in murine photoreceptor cells, we cloned and characterized genes and cDNAs encoding mouse rod and cone opsins. The gene, transcript, and protein structures and the probes generated are summarized here briefly. The *rod opsin* clones (Figure 1A) were described earlier (Baehr et al., 1988; Lem et al., 1991) and are used here as a reference for cone opsin expression. The *S opsin* gene is encompassed within a 14 kb fragment containing a compact 3 kb region of exons and introns (Figure 1B). Although two transcription initiation sites were identified (Figure 1B), the more distal seems preferred. A mRNA of 1655 bp, to which poly(A) is added, is consistent with the product size of ∼1.7 kb identified by Northern blotting (Figure 2A). In contrast, the murine *M opsin* gene (Figure 1C) spans a 24 kb region. It is considerably larger than the human ∼13 kb green or ∼15 kb red opsin genes

(Nathans et al., 1986). A mRNA of 1216 bp, with addition of poly(A), is consistent with a single product of ∼1.3 kb identified by Northern blotting (Figure 2A).

The S opsin protein, as deduced from the gene and cDNA codons, is a typical heptahelical opsin receptor. It uses lysine K291 to form a covalent Schiff base with 11-*cis* retinal, thereby generating a visual pigment. The S opsin is >85% identical to other mammalian blue cone opsins and is affiliated with the SWS1 subfamily of visual pigments (Max et al., 1995; Yokoyama et al., 1998). Yokoyama and colleagues (1998) expressed this opsin and showed that upon reconstitution with 11-*cis* retinal, it generates a pigment with a λmax of 359 nm. Thus, the mouse S opsin forms an ultraviolet- (UV-) absorbing visual pigment. The mouse M opsin is only 46% identical with the mouse S opsin but shows high similarity to other mammalian red and green cone opsins and belongs to the L/M pigment family cluster (Max et al., 1995; Yokoyama and Radlwimmer, 1998). M opsin was expressed in CV1 cells using a vaccinia expression system (Ausubel et al., 1999) and shown to form a pigment with a λmax of 505 nm upon reconstitution with 11-*cis* retinal (data not shown). The λmax agrees with that reported for murine visual sensitivity (Jacobs et al., 1991) and other reports of expression and regeneration (Sun et al., 1997; Yokoyama and Radlwimmer, 1998). Thus, the mouse M opsin forms a midwavelength-absorbing visual pigment. These opsins, expressed only in photoreceptors, are definitive markers of cell type.
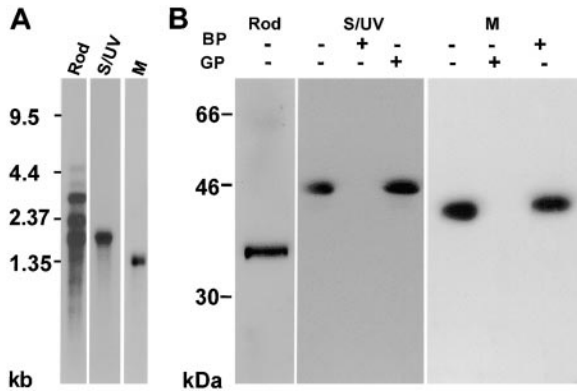
Figure 2. Characterization of Murine Rod and Cone Opsin mRNA and Protein

(A) Northern analysis of mouse opsin mRNA. Five micrograms of poly(A) RNA were loaded per lane. The *rod opsin* mRNA, *S opsin* mRNA, and *M opsin* mRNA were probed with 1.1 kb DNA fragments from each opsin cDNA, respectively, prepared to equal specific activity. Exposure time was 72 hr. The sizes of molecular weight markers (Kb) are indicated at the left.

(B) Western blot of total mouse retinal protein. Lane 1, 1.0 μg total murine retinal protein probed with rod Ret-P1 antibody (1:10,000); lanes 2–4, 10 μg protein probed with S opsin antibody (1:10,000), and lanes 5–7, 20 μg protein probed with M opsin antibody (1:100). BP, N-terminal S opsin peptide (Ac-MSGEDDFYLFQ-[aminohexanoate]) and GP, N-terminal M opsin peptide (Ac-QRLTGEQTLDHY EDSTC-amide), were used at 100-fold excess of the primary antibodies. The S opsin mobility corresponds to a 46,000 Da protein and the M opsin to 42,000 Da.

S Opsin mRNA Is Abundant Compared with M Opsin mRNA
mRNA Levels in the Murine Retina

To characterize the opsin transcripts and evaluate the levels of rod and cone opsin mRNAs in the murine retina, Northern blotting was performed with probes of equivalent size ($\sim$1.1 kb) and specific activity for the *rod*, *S*, and *M opsins*. As indicated in Figure 2A, the mouse *rod opsin* transcript is processed as mRNAs of five different lengths due to a series of polyadenylation sites (Al-Ubaidi et al., 1990), but the cone opsin mRNAs are single transcripts. Quantitation by densitometry indicates a ratio of $\sim$1000:30:10 for the *rod:S:M opsin* mRNAs. In contrast, bovine retinal mRNA generates ratios of 1000:1:30 for *rod:S:M opsin* mRNAs (data not shown). The latter ratio is more consistent with reports of the number of rods compared with S and M cones in other mammals, where S cones are generally the least abundant cone type (Mollon and Bowmaker, 1992; Szél et al., 1996). Thus, the unusually high ratio of *S:M opsin* mRNA in mice may indicate that levels of *S opsin* mRNA per cell are higher than those of *M opsin* mRNA per cell and/or that there are more S cones than M cones.

Murine Cone Opsin–Specific Antibodies

To examine the number of S and M cones and characterize the protein products of gene expression, we raised antibodies to the N termini of opsins. This region provides good specificity and greater accessibility, as compared with the intradiscal C termini. Each antibody shows complete specificity for its corresponding opsin on a Western blot (Figure 2B). No cross-reactivity to rod opsin, the complement cone opsin, or other retinal proteins is observed. Both S and M opsin antibodies are blocked by their respective N-terminal peptides and are unaffected by the
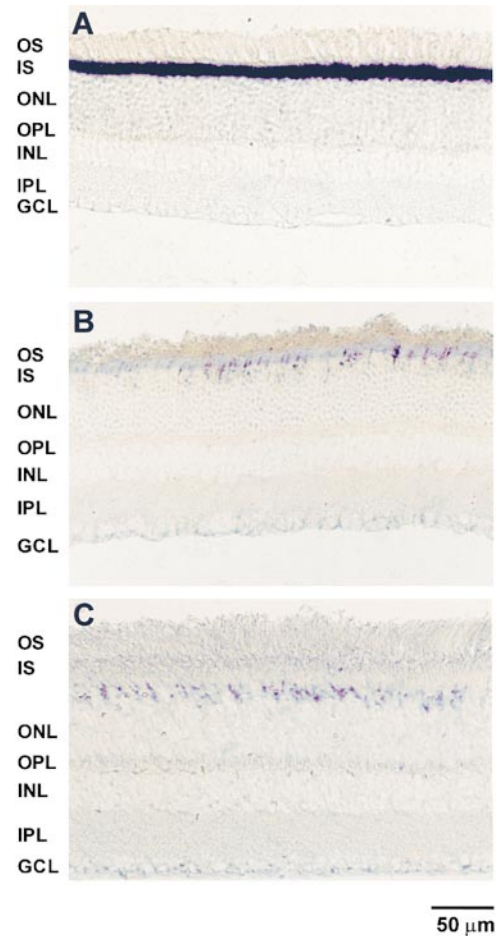

50 μm

Figure 3. In Situ Hybridization of Rod and Cone Opsin mRNAs in the Murine Retina

Serial sections of 7 μm from C57BL/6J retina were hybridized with cDNA probes.
(A) *Rod opsin* cDNA hybridization localizes to the photoreceptor inner segments in the ONL.
(B) S cones labeled by *S opsin* cDNA hybridization.
(C) M cones labeled by *M opsin* cDNA hybridization. Both localize at the interface of photoreceptor inner segments and ONL. The hybridization was visualized with alkaline phosphatase chromogenic substrates NBT and BCIP with 1 hr, 3 hr, and 18 hr development times, respectively.

opposite peptide. Preliminary observations suggest that total S opsin is more abundant than is M opsin. None of the opsins have mobilities proportional to their molecular weight, presumably due to differing posttranslational modifications and affinities for SDS. The specificity of these antibodies makes them suitable markers for S and M cones and studies of cone topography.

Cone Topography Shows a Gradient in S Cone Number and a Gradient in M Opsin Level
In Situ Hybridization of Rod and Cone mRNAs

To evaluate the expression of mRNA in individual photoreceptor cells, DNA and RNA probes (Figures 1A–1C) were used for in situ hybridization. More than 95% of the *rod opsin* mRNA is localized as a continual band running through the inner segments of the retinal photoreceptors (Figure 3A). The *S* and *M opsin* mRNAs are localized to
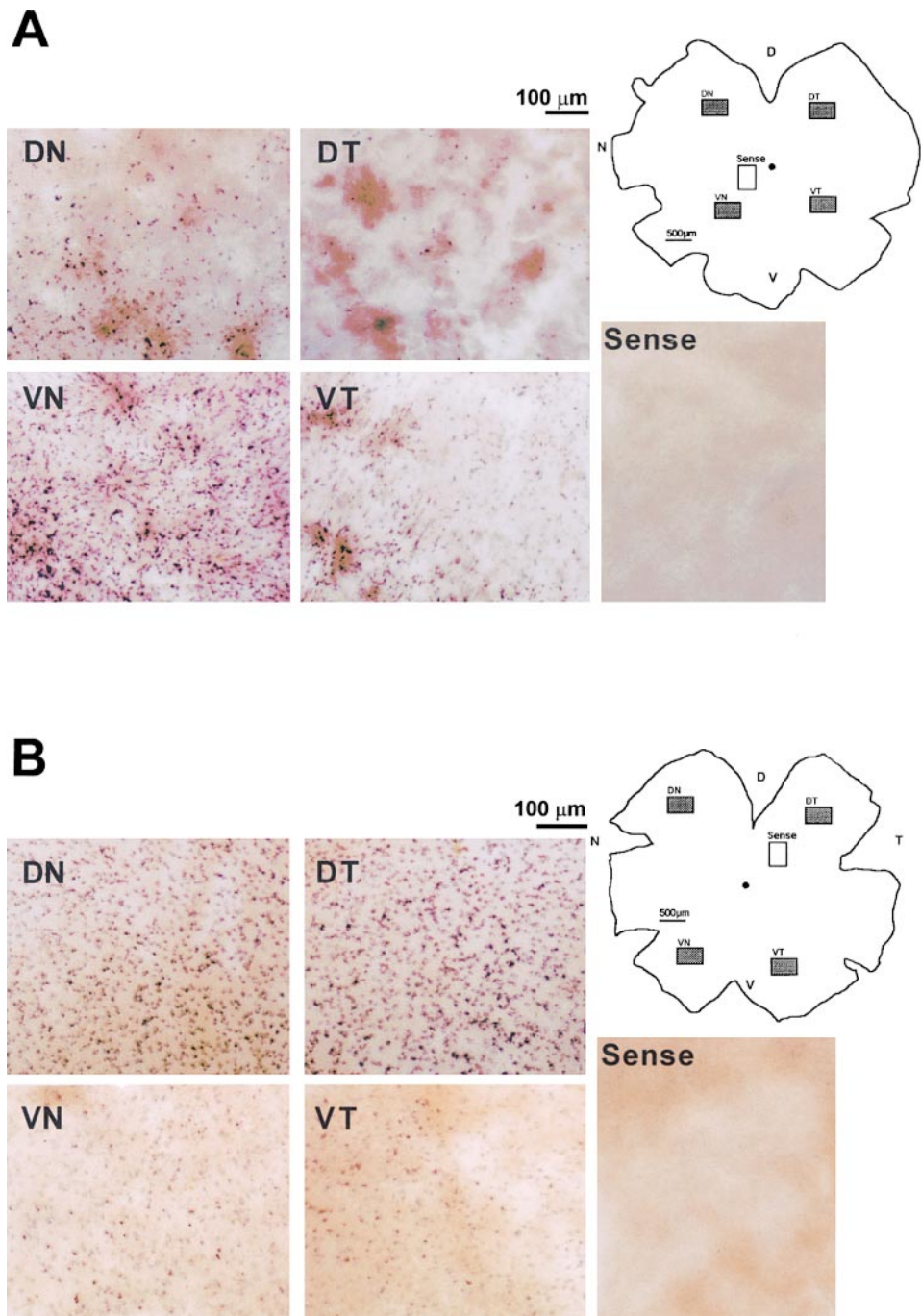
A

100 μm

B

100 μm

Figure 4. In Situ Hybridization of Cone Opsin mRNAs in Murine Retinal Whole Mounts

Whole-mounted retina from adult female DBA/2J-H2D mice hybridized with opsin riboprobes.

(A) S cones labeled by *S opsin* antisense or sense strand hybridization are shown for selected retinal regions as marked.

(B) M cones labeled by *M opsin* antisense or sense strand hybridization. Selected retinal regions are shown as marked in the upper left diagrams. Abbreviations: D, dorsal; V, ventral; N, nasal; T, temporal.

distinct photoreceptor inner segments abutting the nuclei that lie in the outermost region of the outer nuclear stacks (Figures 3B and 3C). The staining is consistent with the position of cone cell bodies, which lie at the top of the outer nuclear layer (ONL) nuclear stack (Carter-Dawson and LaVail, 1979a). For the experiment shown in Figure 3, the probes, of similar size and similar incorporation of

digoxigenin nucleotides, are detected within <30 min for *rod opsin* mRNA, ~3 hr for *S opsin* mRNA, and ~18 hr for *M opsin* mRNA. In later experiments, our ability to detect mRNA more rapidly improved, but the relative times confirm that significantly more *S opsin* mRNA was present per cell than *M opsin* mRNA.
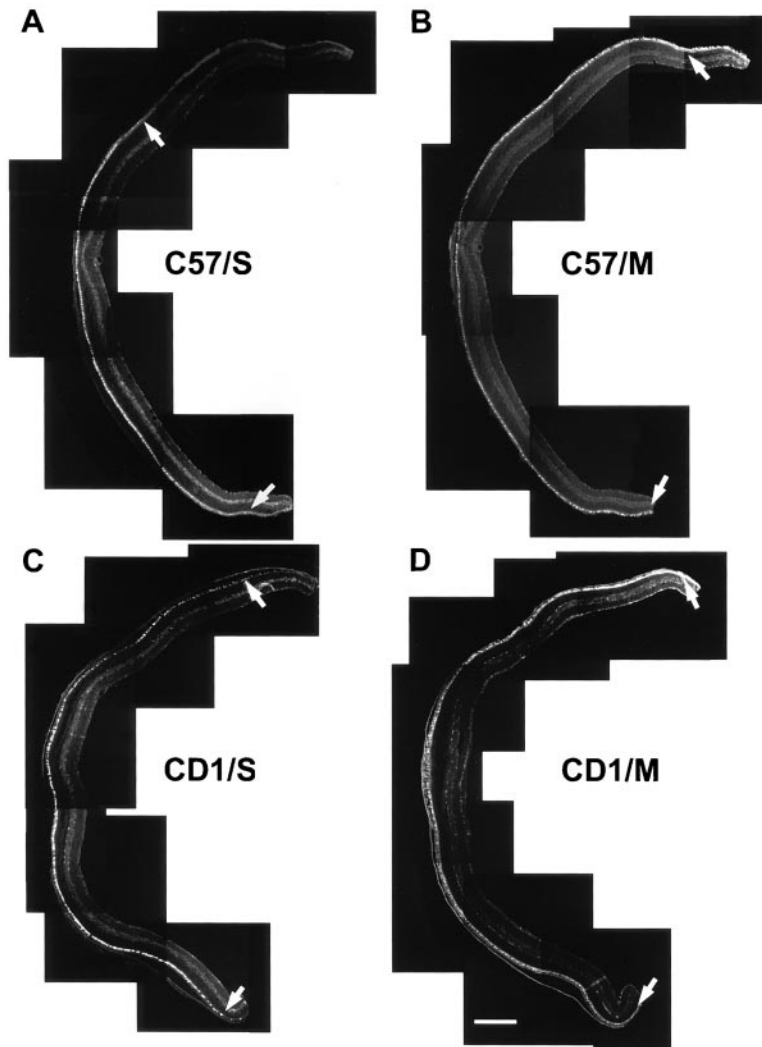
To assess the relative number and spatial arrangement

Figure 5. Dorsal–Ventral Distribution of Cone Opsins in the Murine Retina

Retinal sections were derived from oriented globes within 20 μm of the optic nerve taken from 63- to 90-day-old mice; the dorsal–ventral orientation is from top to bottom. (A and C) C57/S (A) and CD1/S (C) cones in the C57BL/6J and CD1 strains, respectively. (B and D) C57/M (B) and CD1/M (D) cones. The arrows mark the punctate outer segments of cone photoreceptors labeled with S opsin and M opsin antibodies. The S opsin was labeled with a rabbit anti–S opsin antibody (1:40,000 for C57BL/6J sections and 1:20,000 for CD1 sections) combined with a goat anti-rabbit Alexa594 secondary probe (1:125 for both strains). The M opsin was labeled with a chicken anti–M opsin antibody (1:500 for both strains) combined with a goat anti-chicken Alexa594 secondary probe (1:125). Note that the inner nuclear layer and ganglion cell layer show some background staining due to the secondary antibodies. The contrast was set so that this background defined the retinal sections. The montage was compiled from images taken with a 10× objective.

Scale bar, 100 μm.

of rods and cones in the murine retina, in situ hybridization was carried out with whole-mounted retina. *Rod opsin* mRNA is distributed evenly over the surface of the retina (data not shown). For C57BL/6J or DBA/2J-H2D murine retinae, the number of S cones forms a distinct gradient across the retina (Figure 4A). The ventral retina has a higher density of cells expressing *S opsin* mRNA than does the dorsal retina. The ventral:dorsal ratio can be as great as 30:1. However, all cells have similar levels of *S opsin* mRNA when corrected for the plane of focus. M cones are distributed fairly evenly across the retina, yet a gradient is observed in the levels of mRNA (Figure 4B). Distinctly more mRNA is present in cells of the dorsal region than in cells of the ventral region.

Immunohistochemistry of Murine S and M Cones: Strain Dependence

To observe opsin protein expression, we examined oriented retinal sections by immunolabeling with cone opsin–specific antibodies. In the C57BL/6J retina, the spatial organization of cones is consistent with that observed by in situ hybridization. S cones are abundant in the ventral retina, decrease in number in the dorsal region, and become rare in far dorsal retina (Figure 5A). M cones are distributed evenly; however, the cells show a stronger reaction with the M opsin antibody in the dorsal region than

in the ventral retina (Figure 5B). This gradient in protein expression is consistent with the gradient in *M opsin* mRNA expression observed in Figure 4B.

The magnitude of the S cone gradients proves to be strain dependent. In repeating immunolabeling with CD1 (outbred) mice, the gradient, in number of S cones, is less striking than observed in C57BL/6J animals (Figures 5A and 5C). The ventral:dorsal ratio of cells in the CD1 retina is nearer to 4:1, as opposed to 30:1 in C57BL/6J. In contrast, M cones are evenly distributed in both C57BL/6J and CD1 strains (Figures 5B and 5D).

S and M Opsins Are Coexpressed in Cone Photoreceptors

An anomaly arises when the number and topography of S and M cones are considered. A gradient in total cone number, less in dorsal than ventral, is predicted when S and M cones are added in the C57BL/6J retinae. This is inconsistent with the observations of Carter-Dawson and LaVail (1979a), who used clumped chromatin in the nuclei of cells as a cone marker and observed even spacing of cones across the retina. Moreover, cones expressing M opsin are evenly distributed across the retinal surface, and their number ($1.2 \pm 0.2 \times 10^4$ mm^{-2}) is consistent with
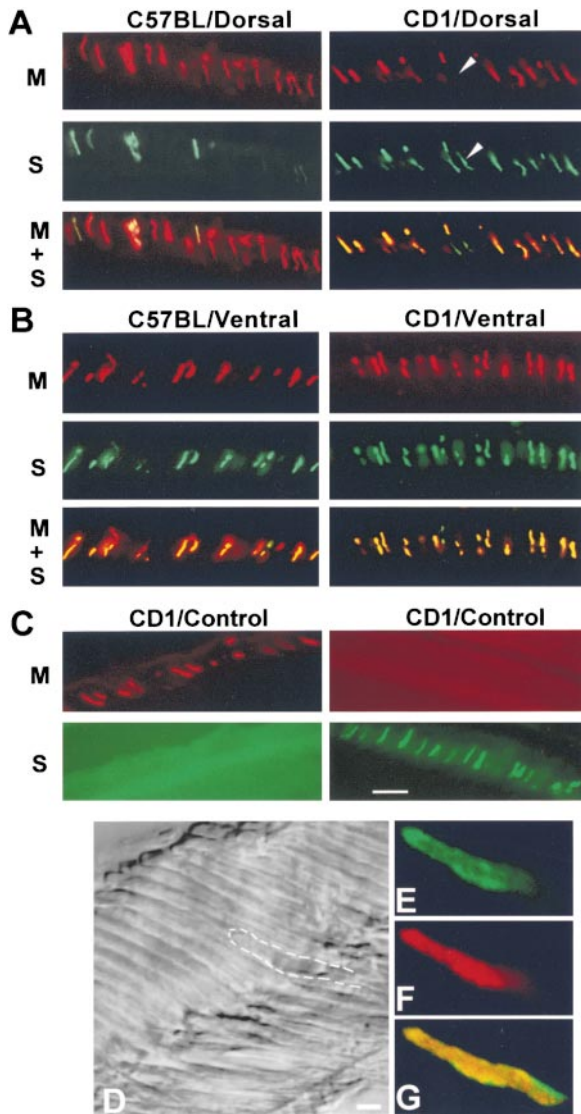
Figure 6. Coexpression of M and S opsins in Cone Cells of the Murine Retina

Retinal sections taken near the optic nerve vertical plane from C57BL/6J and CD1 mice were prepared as noted in Figure 5. The sections were double labeled with a chicken anti–M opsin antibody (1:250) and a goat anti-chicken Alexa594 secondary probe (1:125) combined with a rabbit anti–S opsin antibody (1:20,000) and a goat anti-rabbit Cy2 secondary probe (1:250). M cones are indicated by Alexa594 fluorescence (red emission), S cones are indicated by Cy2 fluorescence (green emission). The superimposed M + S labels were acquired by double exposure of Alexa594 and Cy2 fluorescence.

(A) Regions from the middorsal retinal planes near the optic nerve from C57BL/6J and CD1 mice; arrows indicate a rare cone that appears to express S opsin only.

(B) Regions from the midventral retina.

(C) Control sections from CD1 retinal sections labeled with only one primary but with both secondary antibodies. Left panels, chicken anti–M opsin; right panels, rabbit anti–S opsin. Both left and right panels were stained with goat anti-chicken plus goat anti-rabbit secondary antibodies. The panels lacking cones are overexposed to show lack of cross-reactivity of secondary antibodies. The images were taken with a 63× objective. For confocal microscopy, images were taken with a 100× oil, 1.25 NA objective.

(D) DIC image of the photoreceptor outer segment layer from a CD1 murine retina (age, 1.2 yr) scanned for cone opsin labeling.

the spatial density of total cones in the murine retina as measured using peanut lectin binding on cone cell surfaces (Jeon et al., 1998). If S and M opsin were expressed in separate cells, the density of S plus M cones is estimated to be at least 2-fold greater than reported in these publications. Thus, we carried out double labeling studies with S and M opsin antibodies to compare the cellular localization of these two cone opsins.

Except in the far dorsal retinal region, nearly all murine cone cells are labeled by both S and M opsin antibodies (Figures 6A and 6B). Double labeling was carried out with secondary antibodies coupled to the fluorophores Cy2 (Ex490/Em510) and Alexa594 (Ex594/Em620). Several controls were conducted to validate the labeling experiments. The presence of only one primary antibody in the presence of both secondary antibodies shows that the secondary antibodies do not cross-react with the opposite primary antibody (Figure 6C). Opsins are expressed at very high concentrations ($\sim$3–5 mM) in the specialized outer segments of photoreceptors. Since this cellular region is $\sim$1 μm in diameter, the potential for internal reexcitation is significant. Bleaching the Cy2 chromophore shows no effect on the Alexa594 fluorochrome emission. Controls were carried out with peptide inhibition of each primary antibody. Emission was assessed with S opsin antibody detected with Cy2 IgG and M opsin antibody detected with Alexa594 IgG. The reverse combination of secondary antibodies showed the same results. Cones expressing M opsin only are restricted to the far dorsal retina.

In the dorsal retinal regions of C57BL/6J and CD1 retina, the degree of double labeling reflects the limited number of S cones present. Here, S opsin is coexpressed with M opsin, but M opsin is not always expressed with S opsin (Figure 6A). As predicted by single labeling (Figure 5), fewer double labeled cells are observed in the far dorsal retina of C57BL/6J mice than of CD1 mice (Figure 6A). In the middle and ventral regions of the retina, most cells are double labeled independent of strain (Figure 6B). Occasionally, cells labeled by S opsin antibodies only are found. Since the events are rare, these observations may be due to the offset of focal planes of emission from the narrow cell appendages in given sections or to incomplete labeling. This population needs to be more fully examined with confocal spectroscopy.

To confirm the dual expression of S and M opsins, confocal microscopy was carried out. Throughout the retina, the majority of cones expressing S opsin also expressed M opsin. The observations are strain independent except in the far dorsal retina, where single M opsin–expressing cones are observed, more in C57BL than in CD1. High-resolution images of double labeled cells show complete overlap of S and M opsin localization throughout the outer segments of cones (Figures 6D–6G).

(E) Confocal image of M opsin labeled with chicken anti–M opsin antibody (1:500) and goat anti-chicken Cy2 secondary probe (1:250) (green emission); the image is assembled from 26 sections at 0.23 μm scanned with Ar 488 nm excitation.

(F) Confocal image of S opsin labeled with rabbit anti–S opsin antibody (1:20,000) and donkey anti-rabbit Alexa594 secondary probe (1:125) (red emission); the image is assembled as in (E) with ArKr 568 nm excitation.

(G) Layered optical images of (E) and (F) were superimposed to show coexpression. Note that the secondary antibody fluorchromes used for labeling M and S opsins in (E) and (F) are reversed from those shown in (A) through (C).

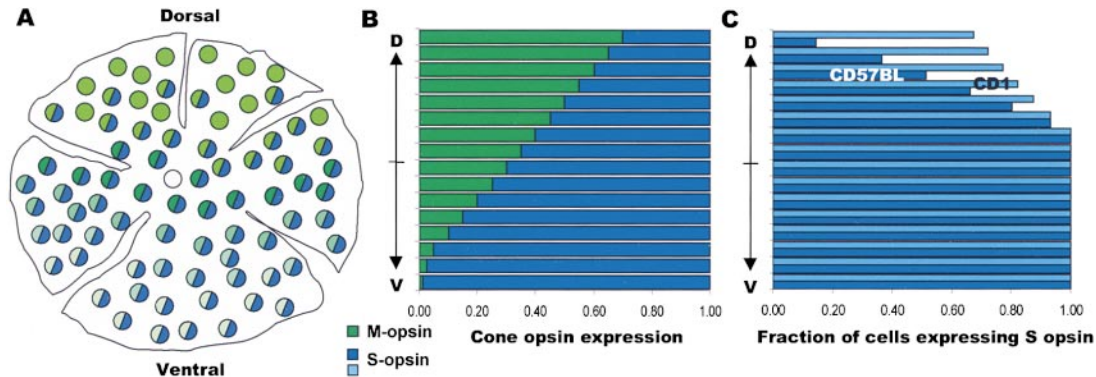Scale bars, 16 μm (A and B); 20 μm (C); 2 μm (D–G).

Figure 7. Two Different Mechanisms Control Expression of M and S opsins in the Murine Retina

(A) Cone photoreceptors in the murine retina are modeled to show coexpression of M and S opsins in cones across the retinal surface. Some cones expressing only M opsin, in which S opsin is repressed, are represented in the dorsal retina. Cones with both opsins express higher levels of M opsin in the dorsal retina than the ventral retina. The actual density of cones $1.2 \pm 0.2 \times 10^4$ mm^{-2} is fairly even across both the dorsal–ventral and nasal–temporal axes but drops at the retinal edge.

(B) The relative amounts of M and S opsin per cone change across the dorsal–ventral axis. *M opsin* may be transcriptionally regulated directly to display a gradient in level, whereas *S opsin* is relatively constant. The plot is scaled to represent the 1:3 ratio of total mRNA observed for *M:S opsin* mRNA measured from Figure 2A.

(C) The fraction of cones expressing S opsin varies across the dorsal–ventral axis. The stochastic expression of S opsin differs for cones in C57Bl/6J and CD1 murine strain. The repression may be achieved during specification of cone fate and prepatterning cone expression. M opsin is expressed in all cones.

Discussion

In the mammalian retina, the cell type, number, and topographical pattern of rod and cone photoreceptors specify the functions of visual sensitivity, contrast, color vision, and spatial resolution. The studies presented above, which explore the level of opsin expression, the occurrence of expression in cone cells, and the spatial topography of cones, argue that there is a common cone type throughout the mouse retina that expresses both S and M opsins. The cones are distinguished spatially by the levels of the two opsins.

Photoreceptors in the Murine Retina

The mouse retina possesses the typical rod scotopic and cone photopic systems for vision (Carter-Dawson and La-Vail, 1979a; Jeon et al., 1998). Short wavelength (λmax 359 nm) and midwavelength (λmax $\sim$510 nm) cone sensitivities have been defined functionally (Jacobs et al., 1991). These provide the dichromatic system typical of nonprimate mammals (Jacobs, 1993). In many mammals, M and L cones usually exceed S cones in number (Mollon and Bowmaker, 1992; Szél et al., 1996). In the mouse, however, the number of S opsin–expressing cones and M opsin–expressing cones is roughly equivalent, except in the far dorsal region of the retina. The total mRNA levels of *S opsin* exceed those of *M opsin* by about 3-fold (Figures 2A, 3B, and 3C). Although more rigorous immunoquantitation must still be carried out, initial observations suggest that protein levels of S opsin also exceed those of M opsin. Thus, on a cell-by-cell basis, the level of S visual pigment is greater than the level of M visual pigment. In accordance, the sensitivity of the S cone system is predicted to be greater than that of the M cone system. In fact, the relative sensitivity measured for the two systems shows that cone receptors in mice are more sensitive to UV light (Jacobs et al., 1991; Lyubarsky et al., 1999). Changes in this ratio that might be affected by diurnal rhythms or light exposure have not been examined.

Coexpression of Visual Pigments

Using fluorescent double labeling with S and M opsin antibodies, the majority of cones throughout the mouse retina are found to coexpress S and M opsins in the same cell (Figure 6). This observation was best confirmed by confocal microscopy (Figures 6D–6G). Our observations extend the findings of Röhlich and colleagues (1994), who noted coexpression in a narrow "transition zone" only. The differences in observations might be explained by the sensitivity of antibodies. The COS-1 antibody used by these investigators is a hybridoma supernatant and may have affinity insufficient to detect lower levels of M opsin, particularly since the mouse M opsin has a two-residue deletion in the C-terminal epitope that the COS-1 antibody detects (Röhlich and Szél, 1993). A report in abstract form suggests that coexpression of S and M opsins extends over the entire ventral region (Glösmann and Ahnelt, 1998). Functional measurements using ERG also support the coexpression of S and M pigments in one cell (Lyubarsky et al., 1999).

The coexpression of both opsins in one cell explains why transgenes constructed with human promoters ligated to cone opsins or reporter genes are expressed in both S and M cones in transgenic mice (Wang et al., 1992, 1999; Shaaban et al., 1998; Jacobs et al., 1999). The promoters do not necessarily lack specificity, but rather, the mouse has only one cone type that expresses multiple pigments. Moreover, the expression of two opsins in the cone photoreceptor of the mouse is just one example in a growing list of reports for multiple expression of opsins in photoreceptors. Rabbits, guinea pigs (Röhlich et al., 1994), and probably marsupials (Hemmi and Grünert, 1999) appear to coexpress two opsins per cell. Fish (Archer and Lythgoe, 1990), eels (Wood and Partridge, 1993; Hope et al., 1998), the tiger salamander (Makino and Dodd, 1996), and the gecko (Loew et al., 1996) express two or three opsins in the same cell. Fly photoreceptors often contain a second "sensitizing" pigment, in addition to the primary pigment, that broadens the spectral sensitivity (Nolte and Brown,

1972; Kirschfeld et al., 1983, 1988). Butterfly photoreceptors express two opsins that generate two visual pigments per cell (Kitamoto et al., 1998). Thus, the phenomenon may be fairly common in both invertebrate and vertebrate eyes.

Topographic Gradients of Opsin Expression Define Cone Subtypes

The expression of cone opsins is a late postmitotic event that occurs several days after the initial birthdate of the cone cell; cones are specified as early as embryonic day 11 in the mouse (Carter-Dawson and LaVail, 1979b), and cone opsins are detected postnatally, after postnatal day 1 (P1) (Szél et al., 1993). In mapping cone properties across the murine retina, we noted little difference in M or S opsin expression across the temporal–nasal coordinate. However, in the dorsal–ventral coordinate, we detected two types of spatial gradients that control cone opsin expression and generate cone subtypes (Figure 7). Two different mechanisms must direct patterning of these gradients.

M opsin Expression

All cones express M opsin. However, the level of M opsin mRNA and protein per cell tapers from the dorsal to the ventral retina (Figures 4B, 5B, and 5D). A simple gradient that regulates the transcription of M opsin could explain the graded expression (Figure 7B). For example, a gradient in hormone (ligand) that controls nuclear receptor activity or the graded expression of a limiting transcription factor would achieve this effect. Many candidates are possible in that gradients in transcription factors and ligand regulators are observed from the earliest time of specification of ocular tissue throughout development and in the mature retina (McCaffery et al., 1992; Mangelsdorf et al., 1995; Dräger and McCaffery, 1997; Harris, 1997).

S opsin Expression

In contrast to M opsin, the levels of S opsin mRNA and protein expressed in cones are relatively constant. However, in the far dorsal retina, expression of S opsin is shut off randomly. The number of cones in which S opsin is repressed increases out to the periphery (Figures 4A, 5A, and 5C). While this phenomenon might be explained by a threshold response to some factor, it seems likely that a more complex mechanism is needed to explain the stochastic repression that is graded to differing degrees in C57BL/6J or CD1 animals (Figure 7C). The factors controlling expression might be determined during prepatterning of cones across the retinal surface. For example, as cones are specified by a Delta-Notch-like mechanism association with feedback processes and elaboration of cellular memory, factors controlling transcription may be turned on/off permanently to affect S opsin expression in an all-or-none fashion. Delta-Notch mechanisms specify retinal cellular fates in the Xenopus retina (reviewed by Perron and Harris, 2000). Similar mechanisms specify photoreceptors in the Drosophila eye, as well as coordinate the expression of specific visual pigments in each photoreceptor type (reviewed by Brennan and Moses, 2000). Ultimately, the regional presence, absence, or threshold of appropriate transcription factors must repress S opsin expression or achieve chromatin condensation in some cells but not in their neighbors.

These types of mechanisms are widespread effectors of pattern in tissues (Chan and Jan, 1999; Perrimon and McMahon, 1999; Scott, 2000). The two mechanisms are sufficient to explain cone patterns in the rodent genera,

Mus musculus, Mus spicilegus, Apodemus sylvaticus, and Apodemus microps, as described by Szél and colleagues (1994). In rodents and other mammals, differential patterning of the cone mosaic could be achieved by changes in gradients, alterations in transcriptional response elements, specification of cone number, and setting activation/repression of opsin transcription, thereby controlling the levels of cone opsins. If these mechanisms apply in primates, the additional mechanism of X chromosome inactivation of alleles, in conjunction with the regulation of the tandem L/M opsin locus by the upstream locus control region, can specify alternate M or L opsin expression in cones (Wang et al., 1992, 1999; Deeb and Motulsky, 1996). This subtle reconsideration of the nature of cone subtypes remains to be proven as a general mechanism of cone specification. However, the model directs attention to mechanisms that need to be explored.

Visual Signaling in Mouse

The distribution of photoreceptor cell types and their relative abundance can be expected to reflect the behavioral tasks of a mouse and its nocturnal/crepuscular life style. The rod-dominant retina provides high sensitivity for low levels of light. The most effective role of the cones is to extend the wavelengths of light to which the retina responds. The presence of two visual pigments in a common cone cell broadens the spectral range to which a cone cell is sensitive. This should allow the mouse to view predators across scenes of dim to bright light intensities. The UV pigment should provide better contrast in detecting dark objects against a gray or overcast sky, since the UV sensor extends the range of sensitivity for scattered light (Tovee, 1995). The latter property would be most prominent in the ventral retina, where the number of cones expressing S opsin is greatest.

Finally, whether mice have color vision is an open question. The paradigm for color vision is based on spectral opponency, which is achieved by comparing the output of two cone types with mutually exclusive expression of visual pigments. Thus, it seems unlikely that the laboratory mouse perceives color. Although cones expressing only M opsin are found in the dorsal region, their neighbors express both M and S opsins. Little or no behavioral evidence for color vision in mice has accumulated (Walls, 1942). Even the recent, interesting work by Jacobs and colleagues (1999) failed to find any evidence for wavelength discrimination. Color perception would require differential signaling processes for excitations of different pigments, a concept that conflicts with the generally held principle of univariance in photoreceptors (Rodieck, 1998). Is it possible that the S and M visual pigments signal differentially? If not, the preponderance of data suggests that laboratory mice are color blind but are optimized for sensitive contrast detection in a nocturnal/crepuscular world.

Isolation and Characterization of Murine Opsin Clones

Mouse *rod opsin* cDNA and genomic clones were isolated as previously described (Baehr et al., 1988; Lem et al., 1991). Mouse *S opsin* cDNA clones were isolated from a λZAP library prepared from BALB/c retinal mRNA probed with the human blue hs36 cDNA (Nathans et al., 1986) under low stringency at 58°C. *M opsin* cDNA clones were generated by PCR; first strand cDNA was synthesized from BALB/c total retinal RNA with random hexamers (Boehringer Mannheim), followed by PCR using primers (National Biosciences) derived from exons 1 and 6 genomic sequences (5′-CCAGAGAC AGTTTTCTACAG-3′ and 5′-GCTTTGACGGATGGGACAGG-3′). The product was cloned into pCR1000 (Invitrogen).

The *S opsin* gene was obtained from a mouse (strain B10) EMBL3 genomic library (Frischauf et al., 1983) by screening with the human blue cone hs36 cDNA at high stringency at 65°C. Intron 1 and 3′ regions of the *M opsin* gene were obtained from a heterozygotic C57BL/6J+/rd mouse EMBL3 genomic library by screening with a human green cone hs4 cDNA at low stringency at 54°C. The upstream region and exon 1 were obtained by rescreening at high stringency with a 3.5 kb HindIII fragment from intron 1 (Figure 1C). Both strands of all clones were sequenced using the method of Sanger (1977). The transcription start sites for both the *S* and *M opsin* genes were mapped by primer extension and RNase protection assays.

Northern Hybridization

Total mouse retinal RNA (C57BL/6J) was isolated according to Chirgwin (1979). Poly(A) RNA was selected using the Poly(A) Quick Kit (Stratagene), fractionated on 1.2% NA agarose, and transferred to Hybond-N$^+$ membranes (Amersham). The membranes were hybridized with DNA probes (Figures 1A–1C) of equal size, labeled to the same specific activity with [^{32}P]dCTP using a random prime kit (Boehringer Mannheim), washed, and exposed to autoradiographic film for 2 hr to 5 days. The hybridized bands were quantitated by scanning a series of autoradiographs at different exposures with a Pharmacia XYZ densitometer.

In Situ Hybridization of Retinal Sections and Whole Mounts

For retinal sections, eyes from adult C57BL/6J mice were fixed in phosphate-buffered saline (PBS) containing 4% (w/v) paraformaldehyde for 2–4 hr at room temperature, then overnight at 4°C with the same solution plus 0.1% (w/v) sodium deoxycholate and 0.1% (v/v) Triton X-100; the tissue was then dehydrated and embedded in paraffin. Sections (7 μm) were deposited on poly-L-lysine-treated slides, deparaffinized, and rehydrated for hybridization (Ausubel et al., 1999). The tissue was pretreated with 50 μg/ml proteinase K in PBT for 4 min at room temperature, washed three times in PBT, and postfixed for 20 min. Digoxigenin dUTP-labeled DNA probes (Figures 1A–1C) were prepared using the Genius System (Boehringer Mannheim). Prehybridization and hybridization were carried out at 50°C for 1 hr and 18 hr, respectively. The probe was detected with alkaline phosphatase–conjugated antibody to digoxigenin.

For retinal whole mounts, eyes from adult female DBA/2J mice (Jackson) were marked for orientation and enucleated, and the cornea was incised for fixation. Eye cups were fixed for ~3.5 hr with 4% paraformaldehyde in 0.1 M MOPS (pH 7.4), 2 mM EGTA, and 1 mM MgS0$_4$ and then cut along the ora serrata to remove the cornea and lens. After marking the dorsal orientation by incision, retinae were teased out gently and refixed in a microfuge tube for ~30 min. Solution hybridization was carried out on the retinae using a protocol adapted from Harland (1991), Conlon and Herrmann (1993), and Wilkinson (1992). Digoxigenin dUTP-labeled sense and antisense riboprobes (Figures 1A–1C) were prepared, hybridized, and developed using the Genius System. Small cuts were made in the retinae to facilitate flat mounting on silylated slides (Onasco Products, Houston, TX), photoreceptor side up.

Antibody Preparation, Western Blotting, and Immunochemistry

Ret-P1, a monoclonal antibody to amino acids 4–10 of rod opsin (Fekete and Barnstable, 1983; Hargrave et al., 1986), was prepared as an ascites fluid. The murine M opsin antibody was raised in chickens against an N-terminal peptide, Ac-QRLTGEQTLDHYED-cys, coupled to diphtheria toxin (Chiron Mimotopes). Crude IgY

was fractionated from egg yolks (Jensenius et al., 1981; Hassl and Aspock, 1988) and affinity purified with a thiol-linked peptide-Sepharose (QCB). The S opsin antibody was raised in rabbits to an N-terminal peptide, Ac-MSGEDDFYLGQ-(aminohexanoate)-K-amide, coupled to keyhole limpet hemocyanin (QCB). The latter was used either as a high dilution of antiserum or as an affinity-purified preparation, both showing the same results. Secondary antibodies used include horseradish peroxidase–coupled-IgG (Amersham or Jackson), Alexa594-coupled IgG (Molecular Probes), and Cy2-coupled IgG (Amersham and Jackson).

For Western blots, total retinal protein was prepared from C57BL/6J mice. Working at 4°C, frozen retinae were placed in ice-cold 5 mM HEPES (pH 7.5), 250 mM sucrose with Complete protease inhibitors (Boehringer Mannheim), vortexed until clear, and centrifuged at 700 × g for 10 min to remove particles. The protein was fractionated by SDS–polyacrylamide gel (10%) electrophoresis and transferred to Immobilon-P membranes (Millipore) for immunodetection. Blots were visualized using the ECL Western Blotting Detection System (Amersham).

For immunohistochemistry, eyes were fixed in 4% paraformaldehyde/PBS for 2 hr to overnight, cryoprotected with sucrose, and embedded in O.C.T. (Miles) with specific orientation. Serial cryostat sections (12 μm) were prepared. Slides were rinsed in PBS, blocked with blocking buffer (0.5 M NaCl, 0.1 M NaPO$_4$ (pH 7.4), 0.5% Triton X-100, 10% normal goat serum) for 30 min to 1 hr., and incubated at 4°C overnight with primary antibody diluted in blocking buffer. The slides were rinsed in PBS, incubated with secondary antibody in blocking buffer for 1 hr, then rinsed with PBS before coverslipping. Microscopy was carried out with a Zeiss Axioskop using the filter sets HQ FITC 41001 (Chroma) and VIVID filter set XF102 (Omega). Digitized retinal images were recompiled using Adobe Photoshop 5.0.

Confocal Microscopy

Images of retinal sections, prepared as described above, were acquired with a Leica TCS SP spectral confocal microscope. Laser intensities and emission band pass were adjusted to minimize bleaching; little change was observed over 2 hr. Controls in which the Cy2 chromophore was bleached showed that the Alexa594 fluorochrome remained unaffected.

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