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The human blue opsin promoter directs transgene expression in short-wave cones and bipolar cells in the mouse retina

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ABSTRACT **Transgenic mouse lines were generated using either 3.8 or 1.1 kb of 5′ upstream flanking sequence from the human blue opsin gene fused to the *lacZ* or human growth hormone reporter gene. Mice were analyzed for appropriate cell-specific and developmental expression patterns. In 13 independently derived lines of animals, transgene expression was limited to photoreceptor and inner nuclear layer cells. Photoreceptors were identified as cone cells based on morphological criteria and colocalization of transgene expression with the cone-associated marker, peanut agglutinin lectin. More specifically, transgene-positive photoreceptors were identified as short-wave cone cells (S-cones) by using the short-wave color opsin-specific antibody, OS-2. Reporter-gene-positive cells of the inner nuclear layer were identified as bipolar cells based on morphological criteria. Transgenes and the endogenous mouse short-wave opsin gene were transcriptionally coactivated at embryonic day 13. These results show that 3.8 or 1.1 kb of human blue opsin upstream flanking sequences are capable of directing expression in short-wave cone cells in a spatially and temporally appropriate fashion and that the human blue opsin gene is the homologue of the short-wave-sensitive pigment, S-opsin, in the short-wave cones of the mouse retina. Expression in the bipolar cells may reflect regulatory mechanisms that are common to these cells and to the cone photoreceptors.**

The vertebrate visual system is composed of two functionally related classes of photoreceptor cells, the rod and cone cells. Rod cells are sensitive to dim light, and cone cells are spectrally tuned to different wavelengths of bright light. These two classes of cells derive from progenitor cells arising at different times during development (1, 2) and express functionally similar but cell-type-specific opsins (3, 4), transducins (5–7), and cGMP phosphodiesterases (8–10).

The color visual system and its associated photopigments is best described in primates. Cones of human and other primates contain three visual pigments maximally sensitive to red (558 nm), green (531 nm), or blue (420 nm) light (11, 12). Genes encoding the red and green pigments are highly similar (13) and reside in tandem array on the X chromosome in humans (14). The blue opsin gene, however, is autosomal and has been mapped to human chromosome 7 (11). It shows a much lower degree of homology with the red and green opsins.

In contrast to the primate color visual system, mouse color vision is poorly characterized. Rodent species have rod-dominant retinas and have been considered to be essentially monochromats (15). Carter-Dawson and Burroughs (16) observed that cone cells make up ≈5% of photoreceptor cells. However, until recently, little was known about the spectral sensitivities or spatial distribution of cone photoreceptor cells. There is evidence for at least two distinct cone types in the mouse retina. Jacobs *et al.* (17) have shown that the mouse retina demonstrates peak sensitivities in the middle-wave (510 nm) and the near-ultraviolet (370 nm) ranges of the spectrum. Immunocytochemical studies with color-specific anti-opsin antibodies have further demonstrated the presence of two classes of cone photoreceptors in the mouse retina (18).

We have generated transgenic mice using 5′ flanking sequences from the human blue opsin gene fused to either the *Escherichia coli lacZ* or the human growth hormone (hGH) reporter gene to study the color visual system in mice. Three fusion constructs were used. The *lacZ* reporter gene was fused to 1.1 kb and 3.8 kb of 5′ human blue opsin sequence and the hGH reporter gene was fused to the 3.8-kb fragment of human blue opsin upstream sequence. Transgenic mice were examined for appropriate cell-specific and developmental onset of gene expression.

Our studies show that human blue opsin upstream regulatory sequences are capable of appropriately directing reporter gene expression in short-wave cone photoreceptor cells (S-cones). In addition, transgene expression was observed in bipolar cells of the inner nuclear layer (INL). We describe the morphology of these cells and the temporal and spatial expression patterns of the transgene in the developing mouse retina.

EXPERIMENTAL PROCEDURES

Plasmid Construction. A human genomic clone (pJHN23) containing the blue opsin gene (11) was kindly provided by J. Nathans (Johns Hopkins University School of Medicine), and 3.8 or 1.1 kb of 5′ upstream regulatory sequence was inserted into pGal-5 (19). The 3.8-kb *Hin*dIII–*Bam*HI 5′ fragment of the human blue opsin gene was also cloned into pOGH (Nichols Institute, San Juan Capistrano, CA).

Generation of Transgenic Mice. Linear fragments of the constructs were injected into fertilized B6D2F1 one-cell embryos (The Jackson Laboratory). Injected embryos were transplanted into the oviducts of pseudopregnant females and offsprings were analyzed for transgene integration. Transgene-positive mice carrying *lacZ* were identified by slot blot analysis of tail DNA using a radiolabeled *Hin*dIII–*Eco*RV *lacZ* fragment (20). Quantitative determination of integrated copy numbers was performed by serial dilutions using purified transgene inserts as control in a Southern blot analysis with digested tail DNA.

Histochemistry and Immunocytochemistry. The eyecups were fixed and prepared for immunohistochemistry and 5-bromo-4-chloro-3-indolyl β-D-galactoside staining as de-

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Abbreviations: INL, inner nuclear layer; hGH, human growth hormone; β-gal, β-galactosidase; E, embryonic day; PNA, peanut agglutinin.
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scribed (19). Sections were stained with anti-β-galactosidase (anti-β-gal) rabbit polyclonal antibody (5 Prime–3 Prime, Inc., 1:500 dilution in blocking solution) for 1 hr, washed three times with PBS containing 0.1% Triton X-100 and 1% normal goat serum, followed by incubation with affinity-purified goat anti-mouse fluorescein isothiocyanate-conjugated IgG antibody (1:40 dilution, Cappel). Colabeling of OS-2-positive outer segments on serial tangential sections was done as described (21).

For semithin sections, eyecups were fixed in 4% (wt/vol) paraformaldehyde for 1 hr and rinsed in 0.1 M sodium phosphate (pH 7.2). Eyecups were incubated in hGH antibody (1:100 dilution, National Hormone and Pituitary Program, Baltimore) overnight, rinsed in buffer, treated with biotinylated goat anti-rabbit antibody followed by an avidin–biotin horseradish peroxidase-conjugate (Vectastain ABC Elite Kit, Vector Laboratories). Eyecups were further fixed in 2% paraformaldehyde/2.5% (vol/vol) glutaraldehyde overnight, then postfixed in 1% osmium tetroxide for 1 hr, and embedded in Epon.

Temporal Expression of the Short-Wave Opsin (S-Opsin) and hGH. Embryonic day 0 (E0) was designated as the day of plug discovery. The RNA from retina was isolated (22) and reverse-transcribed into cDNA, and specific cDNAs were PCR-amplified by the protocol of Rappolee *et al.* (23). The 5′ (5′-CCGACACCCTCCAACAGGGAG) and 3′ (5′-CCCCATCAGCGTTTGGATGCC) PCR primers were used to identify hGH-positive transgenic mice. The hGH primers were designed to span intron 3, so the expected product from genomic DNA template would be 93 bp larger than the cDNA template. The primers used to detect S-opsin were 5′-CCTTCTGTCTCTGCTACGTGCCC-3′ and 5′-GAGAGCCAGACACGTCAGATTCG-3′. These two oligonucleotides span intron 4. PCR products from genomic DNA and cDNA amplification were 890 bp and 240 bp, respectively. The positive control used for the reverse transcription–PCR was β-actin. The primer pair used was 5′-GTGGGCCGCTCTAGGCACCA and 5′-TGGCCTTAGGGTGCAGGGGG.

RESULTS

Thirteen independently derived lines of transgenic mice were produced. Three lines expressed β-gal from 3.8 kb of human blue opsin upstream sequence and two lines expressed the reporter gene from a shorter 1.1-kb upstream fragment. In addition, eight lines of transgenic mice directed the hGH reporter gene from 3.8 kb of blue opsin regulatory sequence (Fig. 1). Examination of adult transgenic animals containing

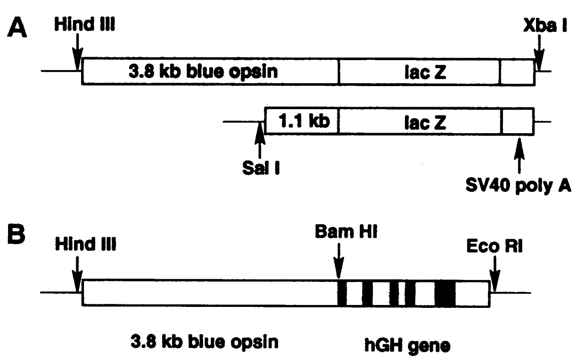

FIG. 1. Transgene fusion constructs. (*A*) Blue opsin upstream regulatory sequences fused to the *lacZ* reporter gene. Two fusion constructs were made containing 3.8 kb or 1.1 kb upstream of the ATG start site. A simian virus 40 polyadenylylation (SV40 poly A) sequence was placed 3′ of the *lacZ* reporter gene. (*B*) The full-length 3.8-kb blue opsin upstream sequences shown in *A* was fused to the hGH reporter gene. The blue opsin–reporter gene fusions were created by blunt-end ligation.

any one of the three constructs revealed expression in two regions of the retina: cells of the photoreceptor layer and of the INL (Fig. 2*A*). A gradient of expression was observed in all five of the β-gal-expressing mice independent of the length of the 5′ upstream sequence and in six of eight hGH transgenic mice. Expression was predominantly localized to the inferior hemisphere, as demonstrated in adult whole-mount retinas stained for β-gal (Fig. 2*B*). Concentration of transgene-expressing photoreceptor cells in the inferior retina was further confirmed in serial sections from both *lacZ* and hGH transgenic mice. Both hGH- and β-gal-expressing transgenic animals also showed that cells of the INL expressed the transgene, albeit in a less prominent gradient than seen in the photoreceptor layer (data not shown). In two transgenic mouse lines expressing hGH, the transgene expressed uniformly throughout the retina in the photoreceptor layer and the INL.

Transgene Expression in Photoreceptor Cells. Transgene-positive photoreceptor cells were identified as cone cells based on morphological criteria and colabeling with the cone-specific markers, peanut agglutinin (PNA) lectin (24) and OS-2 antibody (18). Morphological characterization of photoreceptor cells was based on topological localization and nuclear staining pattern. Nuclei of mouse cone cells have been reported to reside along the sclerad margin of the outer nuclear layer (25). Both β-gal- and hGH-staining cells showed this topological localization (Fig. 2 *A* and *C*). Furthermore, light and electron microscopic examination of transgene-expressing photoreceptor cell nuclei revealed a heterochromatic staining pattern characteristic of cone cell nuclei that is quite distinct from the densely staining nuclei of rod photoreceptor cells (Fig. 2*C*). Thus, the observed morphological characteristics of these photoreceptor cells were consistent with that of cone cells.

Transgene-positive cone cells were also characterized by immunohistochemistry using the cone-specific lectin, PNA (24) (data not shown). PNA lectin exhibited a uniform distribution of cone cells throughout the mouse retina, consistent with previous reports. In five hGH transgenic lines demonstrating a gradient of transgene expression, the inferior retina showed a one-to-one correspondence between hGH and PNA. In the superior retina, only a few PNA-positive photoreceptor cells expressed hGH. Similarly, all five *lacZ* transgenic mouse lines exhibited the superior–inferior gradient, although only 15–25% of PNA-positive cells were β-gal-positive in the inferior region. These findings demonstrate that blue-opsin-driven transgene expression occurs primarily in a subset of cones localized to the inferior hemisphere of the retina. In the two hGH transgenic mouse lines where hGH expression was uniform, one-to-one transgene expression with PNA lectin was observed across the entire span of the retina, indicating that all cone photoreceptor cells in these animals expressed the blue opsin transgene. Southern blot analysis of hGH transgenic animals showed highest copy numbers of the transgene in these lines, demonstrating a correlation between expression level and DNA copy number.

Cone cells were further characterized in an hGH transgenic mouse line by immunohistochemical colocalization with the short-wave opsin antibody, OS-2. This line showed the superior–inferior gradient of transgene expression. Serial tangential sections showed a mosaic of OS-2-positive cells at the depth of the outer segments. At the depth of the inner segments, the same cells stained positively for hGH (Fig. 3). The few hGH-positive cells observed in the superior hemisphere also stained with the OS-2 antibody (data not shown). Thus, colocalization of two cone-specific markers, PNA lectin and OS-2 antibody, and morphological features of transgene-expressing photoreceptor cells support the identity of these cells as cone photoreceptors and, more specifically, as short-wave cone cells.

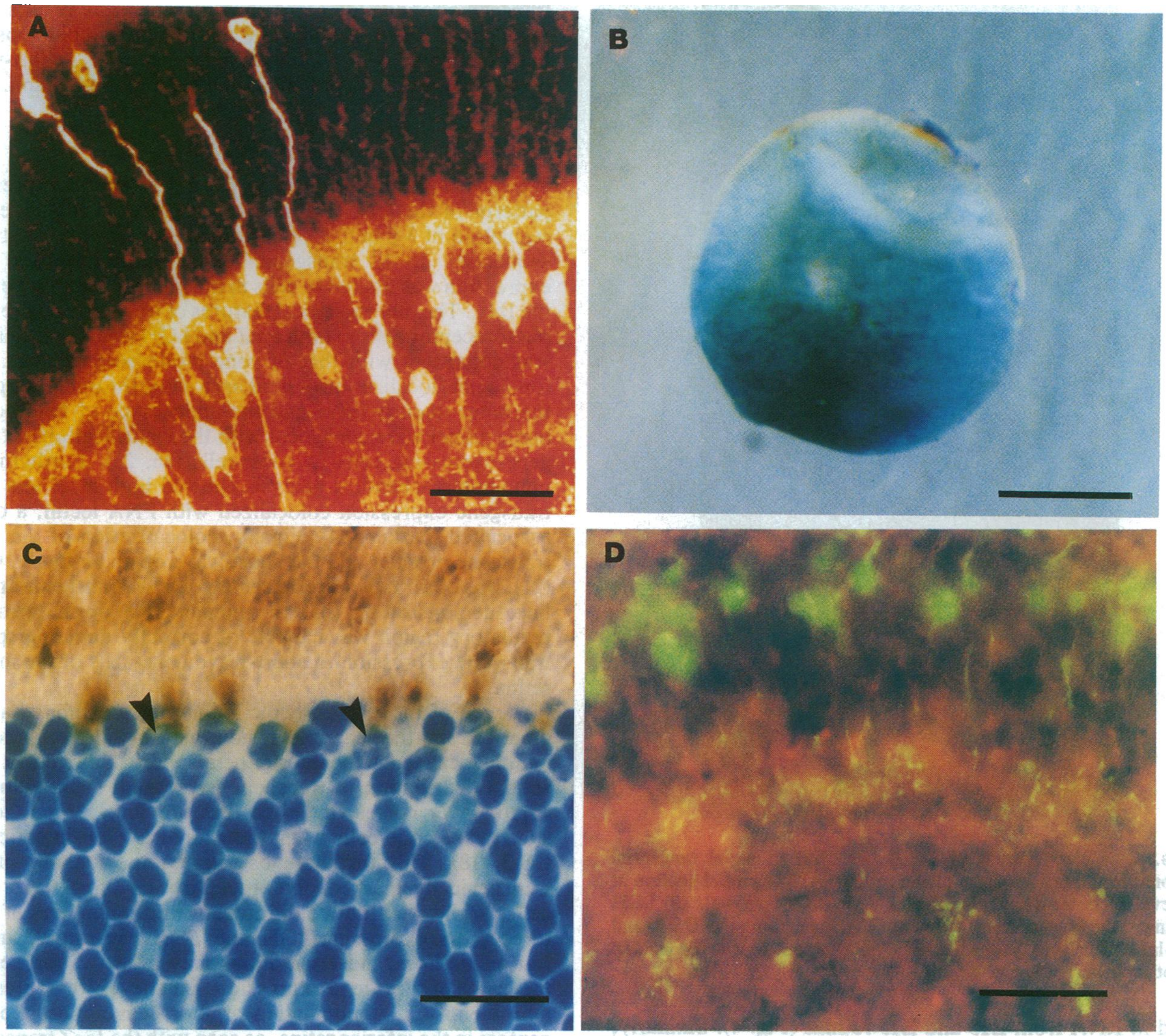

FIG. 2. Transgene expression in transgenic mouse retinas. (*A*) Confocal micrograph of a frozen section stained with a β-gal-specific antibody. Labeled cells are artificially colored a bright yellow. Expression is detected in the photoreceptor layer and the INL of the retina. Note the localization of stained photoreceptor nuclei and the stained cellular processes extending from the photoreceptor and INL cell bodies. (Bar = 50 μm.) (*B*) Whole-mount retina stained for β-gal enzyme activity using 5-bromo-4-chloro-3-indolyl β-D-galactoside substrate. Transgene expression is predominantly localized to the inferior hemisphere (bottom) of the retina. (Bar = 1 mm.) (*C*) The hGH transgene product was detected using an hGH-specific antibody and visualized with diaminobenzidine (DAB; brown stain). Labeled photoreceptor cells localized with heterochromatically stained cone cell nuclei (arrowhead) in 2-μm Epon sections counterstained with Mallory blue and azure II. (Bar = 25 μm.) (*D*) Staining of INL cells in frozen sections from a β-gal transgenic retina. Note the bipolar processes extending from stained INL cells. Descending processes stratify in two layers of the inner plexiform layer. (Bar = 10 μm.)

Transgene Expression in Cells of the INL. Reporter gene expression was also observed in INL cells of all 13 transgenic mouse lines. In both *lacZ* and hGH mice, the gradient of expressing cells at the INL essentially paralleled that observed in cone cells, although the gradient was less prominent than that observed in the photoreceptor layer. In any given area of the retina, the number of transgene-positive INL cells was always greater than the number of cone photoreceptor cells (Fig. 2*A*). The identity of the transgene-expressing cells in this retinal layer was determined based on morphological criteria.

Nuclei of transgene-expressing cells of the INL were localized to a region extending from the center to the distal third of the INL (Fig. 2 *A* and *D*). Three-dimensional reconstruction of serial confocal laser images taken from β-gal-expressing cells showed that these cells extended two diametrically opposed processes, consistent with a bipolar-like morphology (Fig. 2*A*). The ascending process extended to the outer plexiform layer either as a single process or with a single bifurcation. The descending processes terminated in axonal arborizations in at least two strata of the inner plexiform layer (Fig. 2*D*). The topological distribution and cellular morphology of transgene-positive INL cells corresponds to the traits of bipolar cells (26).

In some instances, transgene-expressing cone cell axonal processes appeared to form cellular connections with transgene-expressing INL cells (Fig. 2*A*).

Developmental Time Course of Endogenous Short-Wave Opsin and β-gal and hGH Transgene Expression. To determine whether the onset of transgene expression coincided with the endogenous S-opsin expression, levels of hGH transgene and endogenous short-wave opsin message were measured by reverse transcription–PCR from RNAs prepared from E11, E13, and E15 transgenic retinas. hGH and short-wave opsin transcripts were not detected in RNA preparations from E11 retinas, but both transcripts were

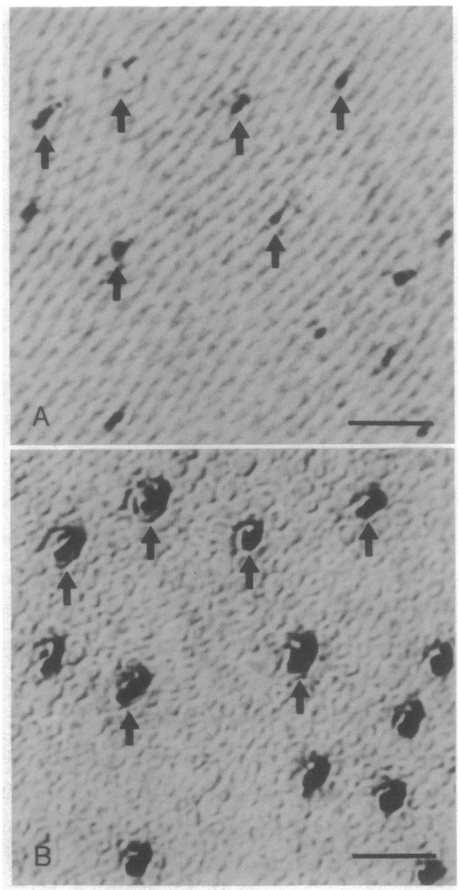

FIG. 3. Colocalization of OS-2 and hGH in serial tangential sections from inferior region of the retina. (*A*) Tangential section at the level of outer segments labeled with OS-2 (arrows). (*B*) hGH labeling at the level of inner segment. The same pattern of OS-2- and hGH-labeled cells indicates the same photoreceptor cells are labeled by both markers. (Bar = 10 μm.)

found in RNA from E13 and E15 retinas (Fig. 4). Similarly, β-gal activity was first detected at E13 in tissue sections from

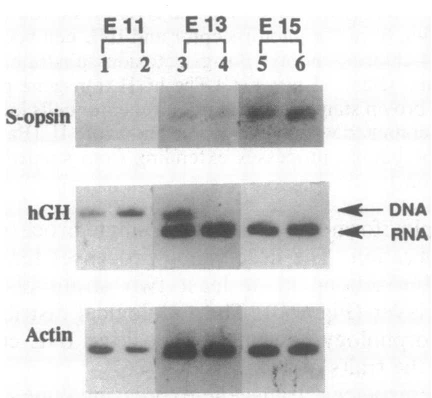

FIG. 4. Developmental time course of blue opsin–hGH transgene and endogenous blue opsin gene transcriptional activation in hGH transgenic mice. RNA was prepared from both retinas of individual transgenic mice at E11, E13, and E15 and reverse-transcribed into cDNA. PCR was performed using primers that distinguished between cDNA and genomic DNA PCR products. Levels of endogenous S-opsin, hGH, and β-actin cDNAs were qualitatively measured. Bands shown represent cDNA PCR products unless otherwise indicated. Lanes: 1 and 2, from E11 animals; 3 and 4, from E13 animals; 5 and 6, from E15 animals. The internal control, actin, was detected at all ages, whereas S-opsin and hGH were not detected at E11 but were present at E13 and E15.

transgenic retinas (data not shown). Thus, the results from β-gal and hGH studies indicate that the human blue opsin 5′ flanking sequences and the endogenous mouse short-wave opsin gene are regulated in a similar temporal fashion.

DISCUSSION

We have investigated the spatial and temporal expression patterns of the *lacZ* and the hGH reporter genes under the regulation of 3.8 or 1.1 kb of human blue opsin 5′ upstream sequences. In 13 transgenic animal lines, reporter gene expression was limited to cells in the photoreceptor layer and the INL. The morphological features of transgene-expressing photoreceptor cells in both *lacZ* and hGH transgenic animals was consistent with that of cone photoreceptor cells. The topological localization of perikarya along the outer margin of the outer nuclear layer and the appearance of heterochromatic nuclei were consistent with the identification of transgene-expressing cells as cone cells (16, 25). Furthermore, immunohistochemical analysis showed both hGH and *lacZ* transgene expression colocalized with PNA lectin, a cone-associated marker (24). Finally, colocalization of the hGH transgene product and the short-wave opsin antibody, OS-2 (21), was observed in a representative hGH transgenic animal line, demonstrating that the human blue opsin upstream regulatory region was capable of correctly directing spatial transgene expression to short-wave cone cells of the mouse retina.

Eleven of the 13 transgenic animal lines exhibited a gradient of reporter gene expression, with expression predominantly localized in the inferior hemisphere of the retina. This spatial distribution of transgene expression in the photoreceptor layer corresponded to that previously reported for short-wave cone cells using the short-wave opsin antibody, OS-2 (18). This further substantiated the identity of the transgenically marked photoreceptors as short-wave cone cells. While quantitative differences in the numbers of photoreceptors expressing hGH and β-gal were noted in the transgenic lines, these variations may reflect differences in the levels of transgene expression. Low levels of expression may identify a subpopulation of short-wave cone photoreceptors in the inferior retina, as seen with the *lacZ* transgenic mouse lines. Intermediate levels of transgene expression may demonstrate a spatial distribution corresponding to that of endogenous short-wave cone cells, as demonstrated by the colocalization of hGH and OS-2 in the representative hGH transgenic line, whereas high levels of expression seemed to drive expression in all cone photoreceptors, as seen in two transgenic lines uniformly expressing hGH. Nathans and collaborators (27) similarly reported transgenic expression in both short-wave and middle-wave cone cells when using human red/green upstream regulatory sequences fused to the *lacZ* reporter gene. They suggested that a negative regulatory element that prevents expression of middle/long-wave pigments in blue cone cells is missing in the human red/green upstream regulatory region. This repressor mode of gene regulation in the photoreceptor cells is also consistent with our data. We observed that the two highest expressing hGH lines that expressed the transgene in all cone photoreceptors contained the highest copy numbers of the transgene. If the middle-wave cone cells contained repressors that prevent expression of short-wave opsin by binding to cis-regulatory sequences, it is possible that high copy numbers of the transgene titrated the repressors, thus allowing for hGH expression in the middle-wave cone cells.

Transgene expression was first seen at E13 in both *lacZ* and hGH transgenic lines. This was coincident with the appearance of endogenous S-opsin transcription, as determined by reverse transcription–PCR. The human blue opsin upstream sequences, therefore, directed transcriptional onset of the hGH and *lacZ* reporter genes with high fidelity. This temporal pattern coincides with the birth of cone cells (1, 2) and may reflect a cellular determination event for S-cones. However, it remains to be determined whether the S-opsin photopigment is present at E13.

Temporal studies of cone formation in the developing mouse retina show that short-wave (blue) cones develop prior to middle-wave (red/green) cones (28). This is quite different from photoreceptor differentiation in primates, where red and green cone formation precedes blue cone development (29). Our transgenic animal studies and those of Wang *et al.* (27) are consistent with the maturation of S-cones prior to middle-wave cones during murine retinal development. Blue opsin transgene activity occurs embryonically, and the onset of human red/green transgene activity is observed much later at postnatal day 6 (27).

Transgene expression was also observed in the INL of all of the transgenic animal lines. These cells resemble bipolar cells based on cellular morphology (26). To our knowledge, opsin expression in cells of the INL has not been reported. A spatial and temporal study of S-opsin expression in the mouse retina using the OS-2 antibody did not show labeling in the INL (28). One possibility is that cells of the INL express S-opsin, albeit at a level below the levels of immunological detection. On the other hand, the transgene expression in the INL may be the result of a lack of appropriate regulatory sequences. For example, the INL cells may share a common set of transcriptional factors with the cone cells to regulate functionally related genes, but the human blue opsin 5′ upstream sequences in our constructs may lack the negative cis-regulatory elements necessary for repressing S-opsin expression in bipolar cells. Supporting evidence for this hypothesis is the presence of a number of common gene products in cone and bipolar cells such as the retina-specific Ret-C1 antigen (30) and the $\beta3$ subunit of transducin (7, 31). Recoverin is present in cone bipolar cells (32) and in both rod and cone photoreceptor cells (33).

Axonal arborization in two bands of the inner plexiform layer as demonstrated by β-gal immunofluorescence in the *lacZ* transgenic retina corresponded with previous observations of cone bipolar axonal stratification (32). Cellular connections observed between β-gal-expressing cone cells and some of the transgene-expressing bipolar cells suggest that at least some of the transgene-expressing bipolar cells may be specific for short-wave cone cells. Based on current knowledge of blue cone bipolar cells in the monkey retina, it is unlikely that the labeled bipolar cells are exclusively blue cone bipolar cells, which are reported to ramify in a single stratum of the inner plexiform layer (34). Thus the presence of two strata of ramification in the inner plexiform layer suggests that other classes of cone bipolar cells express the transgene as well.

In conclusion, we have demonstrated that human blue opsin 5′ upstream regulatory sequences drive the expression of reporter genes in two areas of the retina: in cone photoreceptor and bipolar cells. Spatial and temporal expression are similar to that of the short-wave S-opsin, suggesting that human blue opsin is the homologue of the short-wave S-opsin in cones of the mouse retina. In the 13 transgenic lines generated, a spectrum of transgene expression was seen in the cone photoreceptors, supporting a repressor mode of gene regulation. Also, reporter gene expression in the bipolar cells suggests the presence of common transcriptional activators between these two cell types. These transgenic lines may, therefore, be useful in elucidating the mechanism of gene regulation for the color opsins.

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