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(54) Title: CHIMERIC PROMOTER FOR CONE PHOTORECEPTOR TARGETED GENE THERAPY

(57) Abstract: The subject invention concerns materials and methods for providing for cone cell specific expression of a polynucleotide in a human or animal. One aspect of the invention concerns a polynucleotide promoter sequence that directs expression of an operably linked polynucleotide in cone cells. In one embodiment, a polynucleotide of the invention comprises a nucleotide sequence of an interphotoreceptor retino id-binding protein (IRBP) gene that is positioned upstream of a promoter nucleotide sequence of a cone transducin alpha-subunit (GNAT2) gene. Another aspect of the subject invention concerns methods for expressing a selected polynucleotide in cone cells. The selected polynucleotide can be provided in a polynucleotide of the invention wherein the selected polynucleotide is operably linked to a polynucleotide promoter sequence of the invention. In one embodiment, the selected polynucleotide sequence is provided in a polynucleotide vector of the invention. The vector comprising the selected polynucleotide is then introduced into a cell. The selected polynucleotide is expressed only in cone cells, with very little, if any, expression in rods or other cells. A selected polynucleotide can be one that encodes, for example, a therapeutic protein or a functional protein that is defective or underexpressed in the targeted cone cells.

DESCRIPTION

CHIMERIC PROMOTER FOR CONE PHOTORECEPTOR TARGETED GENE THERAPY

GOVERNMENT SUPPORT

This invention was made with government support under grant number EY008571 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Cone photoreceptors are responsible for high acuity, central daylight and color vision. In humans there are 3 distinct subclasses of cone photoreceptors, each named for the specific wavelength of light to which they respond. Spectral sensitivity it mediated by the specific form of cone opsin that each cone subclass expresses. Cones that express S opsin respond to short wavelength light (blue: 420-440nm) are referred to as "S" cones. Cones that respond to medium wavelength light (green: 534-545nm) express M opsin and are referred to as "M" cones, and finally cones that express L opsin respond to long wavelength light (red: 564-580nm) are referred to as "L" cones. Gene therapy based treatments for a number of diseases affecting cone photoreceptors are currently under development. One such disease, Achromatopsia, is characterized by an inability to see color, blindness in full sunlight (or at high light levels) and very poor visual acuity. Although congenital achomatopsia (ACHM) is a relatively rare disorder, it is a good target for gene therapy as the causative genes are known and proof-of-concept gene replacement studies in animal models have clearly shown success (Pang *et al.* (2010)). ACHM affects all classes of photoreceptors, including S cones. Recent evidence from case studies of patients with ACHM suggests that ACHM is progressive, with cones degenerating over time. Therefore, early intervention with a therapy that targets all cone photoreceptors would be ideal. Additionally, any disease that broadly affects cone

photoreceptors, such as progressive cone dystrophy, would benefit from a gene therapy approach that was capable of targeting all cones.

In order to effectively and safely deliver genes to cone photoreceptors of ACHM affected individuals, gene therapy vectors must utilize promoters that meet the following criteria 1) the promoter must drive transgene expression both efficiently and selectively in cones, with no off-target expression in rod photoreceptors or other non-photoreceptor cell types, such as the retinal pigment epithelium (RPE), 2) the promoter must be capable of driving gene expression in all subclasses of cone photoreceptors, and 3) the promoter should be small, thereby allowing for sufficient carrying capacity of the vector to accommodate transgene DNA. To date, cone targeting promoters used in gene therapy proof-of-concept experiments of ACHM have been deficient in one or more of these criteria. In gene therapy studies by Alexander *et al.* (2007) and Komaromy *et al.* (2010) a 2100 base pair version of the human red/green opsin promoter (PR 2.1) was used to drive therapeutic transgene expression. In humans, the genes for M and L opsin are arranged in tandem on the X chromosome and therefore share a common promoter. In mouse, expression was limited to cones and some rod photoreceptors (Alexander *et al.* (2007)). In dogs, expression was limited to M and L cones (Komaromy *et al.* (2010)). While highly selective for M/ L cone photoreceptors in dogs, PR2.1 mediated expression was not observed in S cones. Additionally the PR2.1 promoter is relatively large and in the case of the CNGB3 form of ACHM, AAV vectors (packaging size limitation of <5KB) are barely able to accommodate promoter and cDNA, and this in turn reduces vector manufacturing efficiency. Promoters isolated from either the human or mouse blue cone (S) opsin gene have also been characterized for AAV mediate expression and gene replacement studies in ACHM animal models. These promoters are from nearly identical regions of the blue cone opsin genes of each respective species (*i.e.*, are homologous). In rat, the human blue cone opsin promoter (HB569) drove reporter gene expression in all cone subclasses; however expression was weaker relative to the PR2.1 promoter (Glushakova *et al.* (2006)). In dog, HB569 performed poorly in terms of both specificity and efficiency, with relatively few L/M cones expressing transgene, rods and RPE positive for expression and overall weak expression. The mouse blue cone opsin promoter (mBP) has been tested in the context of gene replacement for the CNGA3 form of ACHM and performed well, however the likelihood is that like the closely related

HB569, this promoter, will perform poorly in higher order mammals, such as dog and human. Finally, both human and mouse cone arrestin promoters have been utilized in AAV transduction experiments and later in gene replacement studies in ACHM animal models. As with the blue cone promoters, the human and mouse versions of cone arrestin promoters are homologues. In experiments performed in mice aimed at characterizing gene expression mediated by both the mouse cone arrestin promoter (mCAR) and the human cone arrestin promoter (hCAR), strong expression was observed. However specificity was poor, with rods and RPE clearly being transduced. In experiments utilizing mCAR that were performed in dog, the same general expression pattern was seen, with strong expression observed in all classes of cones and off-target expression in rods and RPE. Table 1 summarizes results of cone targeted promoter that have been used (to date) in AAV mediated gene delivery to the retina.

It has been our experience when utilizing photoreceptor specific promoters with AAV that specificity increases when moving from rodent (mouse and rat) to dog, and that the source organism from which the promoter sequence originated has little effect. In the few cases where photoreceptor promoters have been tested in primates, the results have been consistent with those obtained in dog. Therefore, in terms of predicting promoter activity in humans, we place emphasis on results obtained in dog experiments.

Ying *et al.* created a transgenic mouse line in which position -151 to +126 of human cone transducin alpha-subunit (GNAT2) gene was fused to chloramphenicol acetyltransferase gene followed by position -1622 to -1409 of the interphotoreceptor retinoid-binding protein (IRBP) gene (Ying *et al.* (1998)). Later, Ying *et al.* used the same general arrangement of elements to create a transgenic mouse line in which the goal was to ablate cone photoreceptors (Ying *et al.* (2000)). See Figure 1 of Ying *et al.* (2000) for the arrangement of elements used by Ying *et al.* A resulting transgenic mouse line was characterized by Fong *et al.* and found to lack cone photoreceptors, and in ventral retina rod photoreceptors were also absent (Fong *et al.* (2005)). The region-specific absence of rod photoreceptors was reported as a consequence of developmental defect due to lack of cones. However, given that only 2.5% of photoreceptors are cones, loss of rods may have been due to mis-expression of the diphtheria toxin in rods.

BRIEF SUMMARY OF THE INVENTION

The subject invention concerns materials and methods for providing for cone cell specific expression of a polynucleotide in a human or animal. One aspect of the invention concerns a polynucleotide promoter sequence that directs expression of an operably linked polynucleotide in cone cells. In one embodiment, a polynucleotide of the invention comprises an enhancer nucleotide sequence of an interphotoreceptor retinoid-binding protein (IRBP) gene that is positioned upstream (5') of a promoter nucleotide sequence of a cone transducin alpha-subunit (GNAT2) gene. In a specific embodiment, the nucleotide sequence of IRBP comprises sequence -1619 to -1411 of the IRBP gene (SEQ ID NO:2) and the nucleotide sequence of GNAT2 comprises sequence -151 to +126 of the GNAT2 gene (SEQ ID NO:3). In one embodiment, there is no intervening sequence between the IRBP and GNAT2 sequences of the polynucleotide. In an exemplified embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:4, or a functional fragment and/or variant thereof. In another embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, or a functional fragment and/or variant thereof.

Another aspect of the subject invention concerns methods for expressing a selected polynucleotide in cone cells. The selected polynucleotide can be provided in a polynucleotide of the invention wherein the selected polynucleotide is operably linked to a polynucleotide promoter sequence of the invention. In one embodiment, the selected polynucleotide sequence is provided in a polynucleotide vector of the invention. The vector comprising the selected polynucleotide is then introduced into a cell. The selected polynucleotide is expressed only in cone cells, with very little, if any, expression in rods or other cells. A selected polynucleotide can be one that encodes, for example, a therapeutic protein or a functional protein that is defective or underexpressed in the targeted cone cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B. Chimeric IRBP/GNAT2 promoter (Figure 1A) and an AAV vector construct incorporating chimeric IRBP/GNAT2 promoter driving the reporter gene hGFP (Figure 1B).

Figures 2A and 2B. Figure 2A shows a section of mouse retina treated with AAV5-IRBP/GNAT2-GFP at 20X magnification. Figure 2B shows a section of mouse retina treated with AAV5-IRBP/GNAT2-GFP at 40X magnification.

Figure 3 shows a section of mouse retina treated with AAV5-IRBP/GNAT2-GFP + AAV5-PR2.1-mCherry at 20X magnification.

Figures 4A and 4B. Figure 4A shows fundus analysis 4 weeks post subretinal infection of AAV5-IRBP-GNAT2-hGFP in the NRL -/- (all cone) mice. Figure 4B shows retinal section from eye 2 immunostained for GFP at 20x magnification.

Figures 5A and 5B. Figure 5A shows a section of dog retina treated with AAV5-IRBP/GNAT2-hGFP immunostained for L/M opsin and GFP at 40X magnification. Figure 5B shows a section of dog retina treated with AAV5-IRBP/GNAT2-hGFP immunostained for S opsin and GFP at 40X magnification.

Figures 6A and 6B. AAV5-IRBP/GNAT2-hCNGB3 gene therapy in CNGB3 KO mouse.

Figures 7A-7C. Immunohistochemistry showed human CNGB3 staining (green) in the outer segments of many cones (red: cone-specific PNA staining) in AAV5-IRBP/GNAT2-hCNGB3 treated eyes but not in cones from partner untreated eyes of Cngb3 KO mice.

Figures 8A and 8B. Anti-M- (Figure 8B) or S-opsin (Figure 8A) staining showed that S-cones were preserved in P14 + 6-month AAV5-IRBP/GNAT2-hCNGB3 treated eyes but not in contralateral untreated eyes of *Cngb3* KO mice. Most M-opsin containing cones remain at 6.5 months untreated *Cngb3* KO mice.

Figures 9A-9D. Representative ERGs from *Cngb3* KO mice either treated with AAV5-IRBP/GNAT2-hCNGB3 or AAV5-mCAR-pro-hCNGB3 at 6-week or 6-month following treatment at P14. Only left eyes were treated in each mouse.

Figures 10A-10C. GFP reporter gene expression mediated by AAV5 containing a IRBP/GNAT2 promoter. Figure 10A: GFP expression in photoreceptors of C57 BL/6J at 2 months of post-injected retina. Figure 10B: PNA staining of all the cone cells in 2 months post-injected C57 BL/6J transverse retina section that expressing GFP. Blue: DAPI. Figure 10C: PNA staining of cone cells in retinal section from injected C57 BL/6J mice RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer. Bars=50μm.

Figures 11A-1 – 11A-3, 11B-1 – 11B-3. Comparison of targeted GFP gene expression in M- and S-cones. Figures 11A-1 – 11A-3: GFP expression driven by IRBP/GNAT2 promoter in M- and S-cones. Figure 11A-1: GFP gene expression directed by IRBP/GNAT2 promoter; Figure 11A-2: cone cells immunolabeled by M- and S-cone opsin; Figure 11A-3: S+M-opsin labeling (red) co-localizes with GFP gene expression (green) positive cells, the cell nuclei are shown in blue with DAPI; Figures 11B-1 – 11B-3: GFP expression driven by mCAR promoter in M- and S-cones. Figure 11B-1: GFP gene expression directed by mCAR promoter, not only in cones, but also in RPE cells; Figure 11B-2: cone cells immunolabeled by M- and S-cone opsin; Figure 11B-3: S+M-opsin labeling (red) co-localizes with GFP gene expression (green) positive cells, the cell nuclei are shown in blue with DAPI. GFP fluorescence was clearly visualized also in RPE cells. Bars=50μm. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is a chimeric IRBP/GNAT2 polynucleotide of the invention that provides for cone cell specific expression.

SEQ ID NO:2 represents nucleotides -1619 to -1411 of a human IRBP gene.

SEQ ID NO:3 represents nucleotides -151 to +126 of a human GNAT2 gene.

SEQ ID NO:4 is a chimeric IRBP/GNAT2 polynucleotide of the invention that includes 5' EcoR1 and 3'Xba1 and Xho1 restriction sites.

SEQ ID NO:5 is a human CNGB3 polypeptide.

SEQ ID NO:6 is a human CNGA3 protein.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention concerns materials and methods for providing for cone cell specific expression of a polynucleotide in a human or animal. One aspect of the invention concerns a polynucleotide promoter sequence that directs expression of an operably linked polynucleotide in cone cells. In one embodiment, a polynucleotide of the invention comprises an enhancer nucleotide sequence of an interphotoreceptor retinoid-binding protein (IRBP) gene that is positioned upstream of a promoter nucleotide sequence of a cone transducin alpha-subunit (GNAT2) gene. In one embodiment, the

GNAT2 gene sequence of the invention comprises the transcription start site and sequence corresponding to all or part of the 5' untranslated region (5' UTR) of GNAT2. In one embodiment, a polynucleotide of the invention comprises nucleotide sequence from about nucleotide -1650 to about -1350 of the IRBP gene sequence, or a functional fragment and/or variant thereof. In one embodiment, a polynucleotide of the invention comprises nucleotide sequence from about nucleotide -200 to about +200 of the GNAT2 gene sequence, or a functional fragment and/or variant thereof. In a further embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, or a functional fragment and/or variant thereof. In a specific embodiment, the nucleotide sequence of IRBP comprises sequence -1619 to -1411 of the human IRBP gene (SEQ ID NO:2) and the nucleotide sequence of human GNAT2 comprises sequence -151 to +126 of the GNAT2 gene (SEQ ID NO:3). In one embodiment, there is no intervening sequence between the IRBP and GNAT2 sequences of the polynucleotide. In an exemplified embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:4, or a functional fragment and/or variant thereof. IRBP and GNAT2 sequences can be from any mammal, such as mouse, rat, dog, *etc.*, or any primate, including chimpanzee, or human. Polynucleotides of the invention can also comprise a nucleotide sequence encoding a therapeutic protein or a functional protein or a detectable reporter protein of interest (*e.g.*, green fluorescent protein). In one embodiment, the polynucleotide encodes a CNG channel polypeptide. In one embodiment, the CNG channel protein is a mammalian CNG channel protein, such as a human CNG channel protein. In one embodiment, the CNG polypeptide is a CNGA3 or a CNGB3 polypeptide, or a functional fragment or variant thereof. In a specific embodiment, the CNG3B polypeptide comprises the amino acid sequence of SEQ ID NO:5, or a functional fragment or variant thereof. In one embodiment, a polynucleotide of the invention is provided in an AAV vector construct.

Another aspect of the subject invention concerns methods for expressing a selected polynucleotide in cone cells. The selected polynucleotide can be provided in a polynucleotide of the invention wherein the selected polynucleotide is operably linked to a polynucleotide promoter sequence of the invention. In one embodiment, a polynucleotide of the invention used in the method comprises an IRBP gene sequence positioned upstream of a GNAT2 gene sequence. In one embodiment, a polynucleotide

of the invention used in the method comprises nucleotide sequence from about nucleotide -1650 to about -1350 of the IRBP gene sequence, or a functional fragment and/or variant thereof. In one embodiment, a polynucleotide of the invention used in the method comprises nucleotide sequence from about nucleotide -200 to about +200 of the GNAT2 gene sequence, or a functional fragment and/or variant thereof. In a further embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, or a functional fragment and/or variant thereof. In a specific embodiment, the nucleotide sequence of IRBP comprises sequence -1619 to -1411 of the human IRBP gene (SEQ ID NO:2) and the nucleotide sequence of human GNAT2 comprises sequence -151 to +126 of the GNAT2 gene (SEQ ID NO:3). In one embodiment, there is no intervening sequence between the IRBP and GNAT2 sequences of the polynucleotide. In an exemplified embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:4, or a functional fragment and/or variant thereof. In one embodiment, the selected polynucleotide sequence is provided in a polynucleotide vector of the invention. The vector comprising the selected polynucleotide is then introduced into a cell. In the present invention, the selected polynucleotide is expressed only in cone cells, with very little, if any, expression in rods or other cells. A selected polynucleotide can be one that encodes, for example, a therapeutic protein or a functional protein that is defective or underexpressed in the targeted cone cells. A selected polynucleotide can also encode a reporter protein that can be readily detected or identified, such as luciferase, green fluorescent protein (GFP), enhanced GFP, horseradish peroxidase, *etc.*

The subject invention also concerns expression constructs and vectors comprising a polynucleotide of the invention operably linked to an amino acid coding sequence and/or regulatory sequences. In one embodiment, an expression construct or vector of the invention comprises an IRBP gene sequence positioned upstream of a GNAT2 gene sequence. In one embodiment, an expression construct or vector of the invention comprises nucleotide sequence from about nucleotide -1650 to about -1350 of the IRBP gene sequence, or a functional fragment and/or variant thereof. In one embodiment, an expression construct or vector of the invention comprises nucleotide sequence from about nucleotide -200 to about +200 of the GNAT2 gene sequence, or a functional fragment and/or variant thereof. In one embodiment, an expression construct or vector of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, or a functional

fragment and/or variant thereof. In a specific embodiment, the nucleotide sequence of IRBP comprises sequence -1619 to -1411 of the human IRBP gene (SEQ ID NO:2) and the nucleotide sequence of human GNAT2 comprises sequence -151 to +126 of the GNAT2 gene (SEQ ID NO:3). In one embodiment, there is no intervening sequence between the IRBP and GNAT2 sequences of the polynucleotide. In an exemplified embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:4, or a functional fragment and/or variant thereof. In one embodiment, the amino acid coding sequence codes for a protein whose expression in a cone cell provides for treatment of a disease or condition of a cone cell. In one embodiment, the amino acid coding sequence codes for a cone cyclic nucleotide-gated channel (CNG) protein, such as CNGB3 and CNGA3, or a functional fragment or variant thereof. In a specific embodiment, the CNGB3 protein comprises the amino acid sequence of SEQ ID NO:5, or a functional fragment or variant thereof. In one embodiment, the disease or condition is achromatopsia. In another embodiment, the disease or condition is progressive cone dystrophy.

In one embodiment, a vector construct of the present invention is an AAV vector. An AAV vector of the invention can be of any AAV serotype, including, but not limited to, serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and AAV11. In a specific embodiment, an AAV5 serotype is utilized. In one embodiment, an AAV vector of the invention comprises inverted terminal repeats (ITR).

The subject invention also concerns a virus or virion comprising a polynucleotide, expression construct, or vector construct of the invention. In one embodiment, the virus or virion is an AAV virus. Methods for preparing viruses and virions comprising a heterologous polynucleotide or construct are known in the art. In the case of AAV, cells can be coinfected or transfected with adenovirus or polynucleotide constructs comprising adenovirus genes suitable for AAV helper function. Examples of materials and methods are described, for example, in U.S. Patent Nos. 8,137,962 and 6,967,018.

The subject invention also concerns methods for treating or ameliorating diseases and/or conditions that are associated with cone photoreceptors. In one embodiment, a method of the invention comprises administering an expression construct or vector of the invention that also comprises a polynucleotide sequence that codes for a polypeptide that provides for treatment or amelioration of the disease or condition. In one embodiment, a

polynucleotide of the invention used in the method comprises an IRBP gene sequence positioned upstream of a GNAT2 gene sequence. In one embodiment, a polynucleotide of the invention used in the method comprises nucleotide sequence from about nucleotide -1650 to about -1350 of the IRBP gene sequence, or a functional fragment and/or variant thereof. In one embodiment, a polynucleotide of the invention used in the method comprises nucleotide sequence from about nucleotide -200 to about +200 of the GNAT2 gene sequence, or a functional fragment and/or variant thereof. In one embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, or a functional fragment and/or variant thereof. In a specific embodiment, the nucleotide sequence of IRBP comprises sequence -1619 to -1411 of the IRBP gene (SEQ ID NO:2) and the nucleotide sequence of GNAT2 comprises sequence -151 to +126 of the GNAT2 gene (SEQ ID NO:3). In one embodiment, there is no intervening sequence between the IRBP and GNAT2 sequences of the polynucleotide. In an exemplified embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:4, or a functional fragment and/or variant thereof. In one embodiment, a construct or vector of the invention is administered by parenteral administration, such as intravenous, intramuscular, intraocular, intranasal, *etc.* The construct or vector can be administered *in vivo* or *ex vivo*.

In one embodiment, the disease or condition to be treated is achromatopsia. In a further embodiment, the disease or condition to be treated is progressive cone dystrophy. In one embodiment, a polypeptide encoded by the expression construct or vector to be administered is a cone cyclic nucleotide-gated ion channel polypeptide (CNG). In one embodiment, the polypeptide is a CNGA3 polypeptide (see, *e.g.*, GenBank Accession Nos: AAH96300.1, Q16281.2, and AAH96298.1). In another embodiment, the polypeptide is a CNG3B polypeptide (see, *e.g.*, GenBank Accession Nos. AAF86274.1, NP 061971.3, and NM 019098.4). In a further embodiment, a polypeptide encoded by the expression construct or vector is a guanine nucleotide binding protein α-transducing activity polypeptide 2 (GNAT2). In one embodiment, the encoded polypeptide is a mammalian polypeptide. In a further embodiment, the polypeptide is a human polypeptide. In a specific embodiment, a human CNGB3 polypeptide comprises the sequence shown in SEQ ID NO:5. In a specific embodiment, a human CNGA3 protein comprises the sequence shown in SEQ ID NO:6. In a further embodiment, the encoded

polypeptide is an opsin, *e.g.*, M-opsin or L-opsin. Dosage regimes and effective amounts to be administered can be determined by ordinarily skilled clinicians. Administration may be in the form of a single dose or multiple doses. Standard methods for performing gene therapy using polynucleotides, expression constructs, and vectors are known in the art (see, for example, Gene Therapy: Principles and Applications, Springer Verlag 1999; and U.S. Patent Nos. 6,461,606; 6,204,251 and 6,106,826).

The subject invention also concerns a cell comprising a polynucleotide of the invention. In one embodiment, the cell is a cone cell. In another embodiment, the cell is a human cell. In a specific embodiment, the cell is a human cone cell. The cell can express a nucleotide sequence operably linked to a polynucleotide of the invention. In one embodiment, a polynucleotide of the invention is provided in an expression construct and/or vector. In one embodiment, an expression construct or vector of the invention comprises an IRBP gene sequence positioned upstream of a GNAT2 gene sequence. In one embodiment, a polynucleotide of the invention comprises nucleotide sequence from about nucleotide -1650 to about -1350 of the IRBP gene sequence, or a functional fragment and/or variant thereof. In one embodiment, a polynucleotide of the invention comprises nucleotide sequence from about nucleotide -200 to about +200 of the GNAT2 gene sequence, or a functional fragment and/or variant thereof. In one embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, or a functional fragment and/or variant thereof. In a specific embodiment, the nucleotide sequence of IRBP comprises sequence -1619 to -1411 of the human IRBP gene (SEQ ID NO:2) and the nucleotide sequence of human GNAT2 comprises sequence -151 to +126 of the GNAT2 gene (SEQ ID NO:3). In one embodiment, there is no intervening sequence between the IRBP and GNAT2 sequences of the polynucleotide. In an exemplified embodiment, a polynucleotide of the invention comprises the nucleotide sequence shown in SEQ ID NO:4, or a functional fragment and/or variant thereof.

Polynucleotide expression constructs of the invention comprise one or more copies of a polynucleotide of the present invention that directs expression of an operably linked nucleotide sequence in cone cells. As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described wherein the components are in a

relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

Expression constructs of the invention will also generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed. Thus, a person of ordinary skill in the art can select regulatory elements for use in, for example, bacterial host cells, yeast host cells, plant host cells, insect host cells, mammalian host cells, and human host cells. Regulatory elements include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements.

An expression construct of the invention can comprise a polynucleotide promoter sequence of the invention operably linked to a nucleotide sequence encoding a desired polypeptide. Polynucleotide promoters of the invention can be incorporated into an expression construct using standard techniques known in the art. Single or multiple copies of promoters or multiple promoters of the invention can be used in an expression construct of the invention.

Expression constructs of the invention may optionally contain a transcription termination sequence, a translation termination sequence, signal peptide sequence, internal ribosome entry sites (IRES), and/or enhancer elements. Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. Signal peptides are a group of short amino terminal sequences that encode information responsible for the relocation of an operably linked peptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Enhancers are cis-acting elements that increase gene transcription and can also be included in the expression construct. Enhancer elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element. DNA sequences which direct polyadenylation of the mRNA encoded by the structural gene can also be included in the expression construct.

Unique restriction enzyme sites can be included at the 5' and 3' ends of the expression construct to allow for insertion into a polynucleotide vector. As used herein,

the term "vector" refers to any genetic element, including for example, plasmids, cosmids, chromosomes, phage, virus, and the like, which is capable of replication when associated with proper control elements and which can transfer polynucleotide sequences between cells. Vectors contain a nucleotide sequence that permits the vector to replicate in a selected host cell. A number of vectors are available for expression and/or cloning, and include, but are not limited to, pBR322, pUC series, M13 series, and pBLUESCRIPT vectors (Stratagene, La Jolla, CA). Viral vectors include, but are not limited to, retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral (AAV) vectors, herpes viral vectors, *etc.* (see, for example, U.S. Patent Nos. 7,094,604; 6,660,514; 6,165,781).

Polynucleotides, expression constructs, and vectors of the subject invention can be introduced into a cell by methods known in the art. Such methods include transfection, microinjection, electroporation, lipofection, cell fusion, calcium phosphate precipitation, and by biolistic methods. In one embodiment, a polynucleotide or expression construct of the invention can be introduced *in vivo* via a viral vector such as adeno-associated virus (AAV), herpes simplex virus (HSV), papillomavirus, adenovirus, and Epstein-Barr virus (EBV). Attenuated or defective forms of viral vectors that can be used with the subject invention are known in the art. Typically, defective virus is not capable of infection after the virus is introduced into a cell. Polynucleotides, vectors, and expression constructs of the invention can also be introduced *in vivo* via lipofection (DNA transfection via liposomes prepared from synthetic cationic lipids) (Felgner *et al.*, 1987). Synthetic cationic lipids (LIPOFECTIN, Invitrogen Corp., La Jolla, CA) can be used to prepare liposomes to encapsulate a polynucleotide, vector, or expression construct of the invention. A polynucleotide, vector, or expression construct of the invention can also be introduced *in vivo* as naked DNA using methods known in the art, such as transfection, microinjection, electroporation, calcium phosphate precipitation, and by biolistic methods.

Polynucleotides of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58,

59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% or greater as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the polynucleotide sequences of the invention so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis, T. *et al.*, 1982). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25 C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz, G.A. *et al.*, 1983):

Tm=81.5 C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

As used herein, the terms "nucleic acid" and "polynucleotide sequence" refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. The polynucleotide sequences include both full-length sequences as well as shorter sequences derived from

the full-length sequences. It is understood that a particular polynucleotide sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. The polynucleotide sequences falling within the scope of the subject invention further include sequences which specifically hybridize with the sequences coding for a peptide of the invention. The polynucleotide includes both the sense and antisense strands as either individual strands or in the duplex.

Fragments and variants of a polynucleotide or polypeptide of the present invention can be generated as described herein and tested for the presence of function using standard techniques known in the art. Thus, an ordinarily skilled artisan can readily prepare and test fragments and variants of a polynucleotide or polypeptide of the invention and determine whether the fragment or variant retains functional activity that is the same or similar to a full-length or a non-variant polynucleotide or polypeptide, such as cone-specific promoter activity, or formation of ion channels in response to cyclic nucleotides.

As those skilled in the art can readily appreciate, there can be a number of variant sequences of a protein found in nature, in addition to those variants that can be artificially created by the skilled artisan in the lab. The polynucleotides and polypeptides of the subject invention encompasses those specifically exemplified herein, as well as any natural variants thereof, as well as any variants which can be created artificially, so long as those variants retain the desired functional activity.

Also within the scope of the subject invention are polypeptides which have the same amino acid sequences of a polypeptide exemplified herein except for amino acid substitutions, additions, or deletions within the sequence of the polypeptide, as long as these variant polypeptides retain substantially the same relevant functional activity as the polypeptides specifically exemplified herein. For example, conservative amino acid substitutions within a polypeptide which do not affect the function of the polypeptide would be within the scope of the subject invention. Thus, the polypeptides disclosed herein should be understood to include variants and fragments, as discussed above, of the specifically exemplified sequences.

The subject invention further includes nucleotide sequences which encode the polypeptides disclosed herein. These nucleotide sequences can be readily constructed by

those skilled in the art having the knowledge of the protein and amino acid sequences which are presented herein. As would be appreciated by one skilled in the art, the degeneracy of the genetic code enables the artisan to construct a variety of nucleotide sequences that encode a particular polypeptide or protein. The choice of a particular nucleotide sequence could depend, for example, upon the codon usage of a particular expression system or host cell.

Polypeptides having substitution of amino acids other than those specifically exemplified in the subject polypeptides are also contemplated within the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of a polypeptide of the invention, so long as the polypeptide having substituted amino acids retains substantially the same activity as the polypeptide in which amino acids have not been substituted. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ-amino butyric acid, ε-amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ-butylglycine, τ-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form.

Amino acids can be generally categorized in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby a polypeptide having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the polypeptide having the substitution still retains substantially the same biological activity as a polypeptide that does not have the substitution. Table 3 provides a listing of examples of amino acids belonging to each class.

The methods of the present invention can be used with humans and other animals. The other animals contemplated within the scope of the invention include domesticated, agricultural, or zoo- or circus-maintained animals. Domesticated animals include, for

example, dogs, cats, rabbits, ferrets, guinea pigs, hamsters, pigs, monkeys or other primates, and gerbils. Agricultural animals include, for example, horses, mules, donkeys, burros, cattle, cows, pigs, sheep, and alligators. Zoo- or circus-maintained animals include, for example, lions, tigers, bears, camels, giraffes, hippopotamuses, and rhinoceroses.

The polynucleotides contemplated within the scope of the subject invention include the specific polynucleotides exemplified herein as well as equivalent polynucleotides which may be, for example, somewhat longer or shorter than the polynucleotides exemplified herein. For example, using the teachings provided herein, a person skilled in the art could readily make polynucleotides having from 1 to about 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150 or more nucleotides added to, or removed from, either or both ends of the disclosed polynucleotides using standard techniques known in the art. In one embodiment, nucleotides are removed from the 5' or 3' end of the invention. In a specific embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more nucleotides can, independently, be removed from either or both ends of a polynucleotide of the invention, or from either or both ends of an IRBP and/or GNAT2 sequence of a chimeric IRBP/GNAT2 polynucleotide of the invention. In one embodiment, any added nucleotides would be the same as the corresponding nucleotides of the IBRP or GNAT2 gene sequences. Added nucleotide sequences can also provide for restriction sites recognized by one or more restriction endonucleases. The skilled artisan, having the benefit of the teachings disclosed in the subject application, could easily determine whether a variant polynucleotide retained the functional activity of the specific polynucleotides exemplified herein. Such a longer or shorter polynucleotide would be within the scope of the subject invention as long as said polynucleotide retains substantially the same relevant functional activity as the polynucleotides exemplified herein. For example, a longer or shorter variant of an exemplified polynucleotide (*e.g.*, SEQ ID NO:1, SEQ ID NO:2, SEQUENCE ID NO:3, or SEQ ID NO:4) would fall within the scope of the subject invention if the longer or shorter variant polynucleotide had the ability to promote expression in cone cells. In another example, nucleotides can be added or removed between the IRBP and GNAT2 sequences of a chimeric IRBP/GNAT2

polynucleotide of the invention. Similarly, nucleotides can be added or removed from the IRBP and/or GNAT2 sequences of a chimeric IRBP/GNAT2 polynucleotide of the invention as long as the polynucleotide retains substantially the same functional activity, *i.e.*, promotes expression in cone cells, as an exemplified polynucleotide. Methods of identifying whether a fragment of a polynucleotide promoter is capable of initiating gene transcription are well known in the art. U.S. Patent Nos. 6,080,914 and 5,986,174 provide assay systems that can be used for analysis of promoter fragments for activity.

Also within the scope of the subject invention are polynucleotides which have the same nucleotide sequences of a polynucleotide exemplified herein except for nucleotide substitutions, additions, or deletions within the sequence of the polynucleotide, as long as these variant polynucleotides retain substantially the same relevant functional activity as the polynucleotides specifically exemplified herein. Thus, the polynucleotides disclosed herein should be understood to include variants and fragments, as discussed above, of the specifically exemplified sequences.

The subject invention concerns a chimeric promoter for use with viral vectors such as adeno-associated virus (AAV) for the efficient and selective targeting of transgene expression to cone photoreceptors. Constructs of the invention have direct utility as a vehicle for the delivery of therapeutic genes to diseases that affect cone photoreceptors, such as achromatopsia.

A summary of the transduction results of AAV mediated transgene expression utilizing the chimeric IRBP/GNAT2 promoter is given in Table 2.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—Description of chimeric IRBP/GNAT2 promoter

In an attempt to improve upon previous cone targeting promoters used in conjunction with AAV mediated gene delivery, we created a chimeric promoter in which the sequence corresponding to -1619 to -1411 of the interphotoreceptor retinoid-binding protein (IRBP) gene was directly fused to -151 to +126 sequence of human cone transducin alpha-subunit (GNAT2). A depiction of the chimeric IRBP/GNAT2 promoter

is given in Figure 1A. Note that the arrangement of elements differs from those used in Ying *et al.* (1998) and Ying *et al.* (2000) (see Figure 1 of Ying *et al.* (2000)). In this case the IRBP element is located upstream of the GNAT2 and there is no intervening sequence between the elements.

Results: The chimeric IRBP/GNAT2 promoter was incorporated into an AAV vector plasmid containing the reporter gene humanized green fluorescent protein (hGFP) (Figure 1B) and packaged in AAV serotype 5 (AAV5). The resulting vector, AAV5-IRBP/GNAT2-GFP was tested for cone specificity in mouse retina via subretinal injection. The results depicted in Figures 2A and 2B, a retinal section immunostained for GFP and stained with DAPI (stain for cell nuclei, appears as blue), indicate that expression was limited to photoreceptors with no RPE expression observed. All cone photoreceptors appear to be efficiently transduced, with some expression seen in rod photoreceptors.

Example 2

Expression mediated by the chimeric IRBP/GNAT2 promoter was directly compared to that mediated by the PR2.1 promoter. AAV5-IRBP/GNAT2-hGFP vector was mixed at an equal ratio with AAV5-PR2.1-mCherry. Figure 3 is a retinal section from mouse that was treated with the AAV5-IRPB/GNAT2-hGFP + AAV5-PR2.1-mCherry vector mixture and immunostained for GFP and then merged with an image captured for the red channel (mCherry expression is apparent as raw red spectrum fluorescence). All cones cell bodies positive for mCherry also appear to be positive for hGFP expression as indicated by the orange color (overlay of red and green appears as orange). Many cone cell bodies are GFP positive and do not appear to be mCherry positive.

Example 3

The transcription factor neural retina-specific leucine zipper protein (NRL) is required for the development of rod from photoreceptor progenitor cells (Mears *et al.* (2001)). Mice lacking Nrl, *i.e.*, Nrl knock-out mice ($Nrl^{-/-}$), develop retina with an 'all cone' phenotype (Daniele *et al.* (2005)). Furthermore, the photoreceptor-cones of the $Nrl^{-/-}$ mouse most resemble S-cones, with levels of S-cone opsin expression and spectral

sensitivity consistent with being characterized as S-cones (Nikonov *et al.* (2005)). In order to evaluate the ability of the chimeric IRBP-GNAT2 promoter to drive gene expression in S-cones we subretinally injected 6 week old $Nrl^{-/-}$ mice with AAV5-IRBP/GNAT2. Four weeks post injection with vector, fundus images were recorded using the appropriate filters to visualize raw GFP fluorescence (Figure 4A). Strong GFP expression was observed in all eyes treated. Subsequently, eyes were harvested and retinas sectioned and immunostained for GFP and DAPI (Figure 4B). GFP Expression was strong and was restricted to photoreceptor layer.

Example 4

To further characterize the expression pattern of the chimeric IRBP/GNAT2 promoter, AAV5-IRBP/GNAT2-hGFP vector was subretinally injected into dog retina. Figure 5A depicts a section of treated retina that was immunostained for L/M opsin (red) and for GFP (green). The L/M opsin staining is used to identify L/M cones. Results show that GFP signal is limited to cone cells only (as indicated by distinctive morphology of the cone cells). The majority, if not all of the L/M cones are positive for GFP expression. A different section from the same treated dog retina was immunostained with S opsin (red) and GFP (green). In this instance S opsin immunostaining is used to identify S cones (see Figure 5B). S cones are naturally less numerous than L/M cones in dog retina, which is apparent from this image. A majority of S cones in this section are clearly positive for GFP expression.

Example 5

In order to evaluate the ability of the chimeric IRBP/GNAT2 promoter to drive gene expression sufficient to provide therapeutic rescue, an AAV vector construct containing the human gene for CNGB3 under the control of the IRBP/GNAT2 promoter (see Figure 6A) was created and packaged in AAV5. The AAV5-IRBP/GNAT2-hCNGB3 vector was then subretinally injected into the left eye only of CNGB3 Knock-out (CNGB3 KO) mice at 1 month of age. Six weeks later therapeutic efficacy was evaluated by cone specific electroretinogram (ERG). Untreated CNGB3 KO mice exhibit little to no cone ERG as can be seen in the upper panel of Figure 6B. Treatment with

AAV5-IRBP/GNAT2-hCNGB3 resulted in robust improvement in the amplitude of the cone ERG (Figure 6B, lower panel).

Example 6

Mutations in the gene encoding the beta-subunit of the cone cyclic nucleotide-gated channel (CNGB3) cause cone function loss in mammals including humans. We tested two AAV5-hCngb3 vectors with different cone targeting promoters to see if gene replacement therapy would result in restoration of cone function in the Cngb3 knockout mice, a model of human Achromatopsia 1 (ACHM1).

Methods: Human Cngb3 cDNA in conjunction with cone-targeting promoter mCAR-pro or IRBP/GNAT2 was packaged into AAV serotype 5 (AAV5-mCARpro-hCngb3 or AAV5-IRBP/GNAT2-hCngb3 at 10^{13} viral genome-containing particles /ml). At postnatal day 14, 1 µl of either vector was injected subretinally into one eye of groups of 20 Cngb3 knockout mice, respectively. The untreated, contralateral eyes served as controls. Dark- and light-adapted ERGs were recorded periodically from 3 weeks to 6 months after treatment. 6 months after injection, both treated and control eyes were harvested for histochemical studies.

Results: At 3 weeks post-treatment both treated and untreated eyes of Cngb3 knockout mice showed normal rod-derived ERGs. In untreated control eyes, cone-derived ERG signals were nearly unrecordable. In both AAV5-mCAR-hCngb3 and AAV5-IRBP/GNAT2-hCngb3 treated eyes, restored light-adapted cone-derived ERG waveforms were first recorded 3 weeks after treatment and remained stable for at least 6 months (Figures 9A-9D). ERG amplitudes were about 2/3 of those of normal uninjected C57BL/6J mice. Immunohistochemistry showed human CNGB3 staining in the outer segments of many cones in treated eyes but not in cones from partner untreated eyes (Figures 7A-7C). Anti-M-cone or S-cone opsin staining also showed that S-opsins were preserved in treated eyes but not in untreated eyes of Cngb3 knockout mice (Figures 8A and 8B).

Conclusions: Both AAV5-mCAR-hCNGB3 and AAV5-IRBP/GNAT2-hCNGB3 restore cone function and prevent S-cone degeneration for at least 6 months in Cngb3 knockout mice, a model of ACHM1. However additional experiments show that in addition to cones, mCAR-pro also expresses its transgene in the RPE while the

IRBP/GNAT2 promoter is cone-exclusive (see Figures 11A and 11B). Thus, the IRBP/GNAT2 promoter is preferable for use in humans.

Table 1. Summary of results of AAV cone targeting experiments.

Promoter names	Source	Expression pattern in rodent	Expression pattern in dog	Expression pattern in primate	size	References/Studies
PR 2.1	Human red green opsin promoter	All cones, some rods -Expression strong	-Only L/M cones -Expression strong	-Only L/M cones -Expression strong	2100 bps	Alexander et al. 2007, Komaromy et al. 2008, Komaromy et al. 2010 and Mancuso et al. 2009
HB569 mBP	(HB569) human blue cone opsin promoter (mBP) mouse blue cone promoter	M and S cones and rods -Expression weak	-Few L/M cones, rods and RPE -Expression weak	Not tested	570 bps and 500 bps	Glushakova et al. 2006, Komaromy et al. 2008 and Komaromy et al. 2010 Michalakis et al. 2010
hCAR mCAR	(hCAR) Human cone arrestin promoter (mCAR) mouse cone arrestin promoter	All cones, some rods and RPE -Expression strong	-All cones, rods and some RPE -Expression strong	Not tested	500 bps and 500bps	Li et al. 2002 and Carvalho et al. 2011 Hauswirth and Komaromy unpublished results

Table 2. Summary of AAV5-IRBP/GNAT2-hGFP transduction.

Promoter names	Source	Expression pattern in rodent	Expression pattern in dog	Expression pattern in primate	size	References/ Studies
IRBP/ GNAT 2	Human IRBP gene and human GNAT2 gene	All cones, some rods -Expression strong	-Cones only, L/M and S cones -Expression strong	Not tested	524 bps	Not published

Table 3.	
Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

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CLAIMS

We claim:

1. A polynucleotide that promotes expression in cone cells of an operably linked nucleotide sequence, wherein said polynucleotide comprises an enhancer nucleotide sequence of an interphotoreceptor retinoid-binding protein (IRBP) gene sequence that is positioned upstream of a promoter nucleotide sequence of a cone transducing alpha-subunit (GNAT2) gene sequence.

2. The polynucleotide according to claim 1, wherein said polynucleotide comprises nucleotide sequence from about nucleotide -1650 to about -1350 of the IRBP gene sequence, or a functional fragment thereof.

3. The polynucleotide according to claim 1, wherein said polynucleotide comprises nucleotide sequence from about nucleotide -200 to about +200 of the GNAT2 gene sequence, or a functional fragment thereof.

4. The polynucleotide according to claim 1, wherein said polynucleotide comprises sequence -1619 to -1411 of a human IRBP gene sequence, or a functional fragment thereof.

5. The polynucleotide according to claim 1, wherein said polynucleotide comprises sequence -151 to +126 of a human GNAT2 gene, or a functional fragment thereof.

6. The polynucleotide according to claim 1, wherein said polynucleotide comprises sequence -1619 to -1411 of a human IRBP gene sequence and sequence -151 to +126 of a human GNAT2 gene.

7. The polynucleotide according to claim 1, wherein said polynucleotide sequence comprises the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:4.

8. The polynucleotide according to claim 1, wherein said polynucleotide further comprises a nucleotide sequence encoding a therapeutic protein or a functional protein or a reporter protein.

9. The polynucleotide according to claim 8, wherein said polypeptide is a cyclic nucleotide-gated (CNG) channel polypeptide.

10. The polynucleotide according to claim 9, wherein said CNG polypeptide is a CNGA3 or a CNGB3 polypeptide, or a functional fragment thereof.

11. The polynucleotide according to claim 10, wherein said CNG3B polypeptide comprises the amino acid sequence of SEQ ID NO:5, or a functional fragment thereof.

12. The polynucleotide according to any preceding claim, wherein said polynucleotide is provided in an expression construct.

13. The polynucleotide according to any preceding claim, wherein said polynucleotide is provided in a vector.

14. The polynucleotide according to claim 13, wherein said vector is a viral vector.

15. The polynucleotide according to claim 14, wherein said viral vector is an AAV vector.

16. An expression construct comprising a polynucleotide of any of claims 1-15.

17. The expression construct according to claim 16, wherein said polynucleotide further comprises a nucleotide sequence encoding a polypeptide.

18. The expression construct according to claim 17, wherein said polypeptide is a therapeutic protein or functional protein or a reporter protein.

19. The expression construct according to claim 18, wherein said polypeptide is a cyclic nucleotide-gated (CNG) channel polypeptide.

20. The expression construct according to claim 19, wherein said CNG polypeptide is a CNGA3 or CNGB3 polypeptide.

21. The expression construct according to claim 20, wherein said CNGB3 polypeptide comprises the amino acid sequence of SEQ ID NO:5, or a functional fragment thereof.

22. The expression construct according to any of claims 16-21, wherein said expression construct comprises one or more regulatory elements.

23. The expression construct according to claim 22, wherein said regulatory element is a transcription termination sequence, an enhancer sequence, a polyadenylation sequence, a signal peptide sequence, or an internal ribosome entry site.

24. A vector comprising an expression construct of any of claims 16-23.

25. The vector according to claim 24, wherein said vector is a viral vector.

26. The vector according to claim 25, wherein said viral vector is an AAV vector.

27. A method for treating or ameliorating a disease or condition associated with a cone photoreceptor in a human or animal eye, said method comprising administering to the person or animal a polynucleotide of any of claims 1-15 or an expression construct of any of claims 16-23 or a vector of any of claims 24-26 wherein said operably linked nucleotide sequence encodes a polypeptide that provides for treatment or amelioration of the disease or condition.

28. The method according to claim 27, wherein said disease or condition is characterized by underexpression and/or expression of a defective or non-functional protein,

and wherein said polynucleotide provides for increased expression and/or expression of a functional version of said non-functional protein.

29. The method according to claim 28, wherein the protein is a CNGB3 or a CNGA3 protein.

30. The method according to claim 27, wherein the disease or condition is achromatopsia or progressive cone dystrophy.

31. The method according to claim 27, wherein said polynucleotide, expression construct, or vector is administered by subretinal injection in the human or animal.

32. A method for expressing a polypeptide in a cone cell, said method comprising introducing the polynucleotide of any of claims 1-15 or an expression construct of any of claims 16-23 or a vector of any of claims 24-26 into the cell, wherein said operably linked nucleotide sequence encodes said polypeptide and said operably linked nucleotide sequence is expressed in the cell.

33. The method according to claim 32, wherein the cell is a human cell.

34. The method according to claim 32, wherein said polypeptide is a therapeutic protein or a functional protein.

35. The method according to claim 32, wherein the cell underexpresses and/or expresses a defective or non-functional protein, and wherein said polynucleotide provides for increased expression and/or expression of a functional version of said non-functional protein.

36. The method according to claim 35, wherein the cell underexpresses a CNGB3 or a CNGA3 protein, or the cell expresses a defective or non-functional CNGB3 or CNGA3 protein.

37. A cell comprising a polynucleotide of any of claims 1-15 or an expression construct of any of claims 16-23 or a vector of any of claims 24-26.

38. The cell according to claim 37, wherein the cell is a cone cell.

39. The cell according to claim 37 or 38, wherein the cell is a human cell.

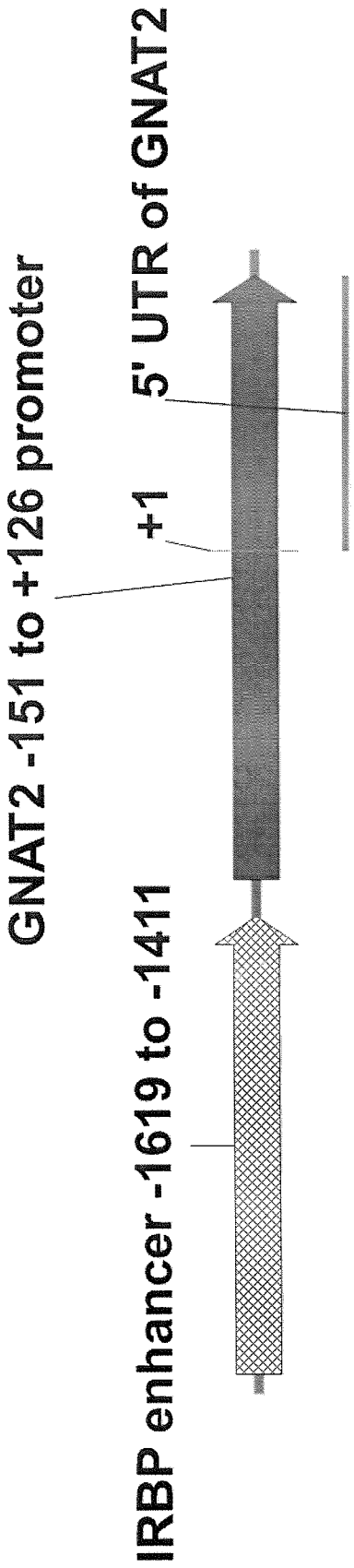
40. The cell according to any of claims 37-39, wherein said operably linked nucleotide sequence encodes said polypeptide and said operably linked nucleotide sequence is expressed in the cell.

41. The cell according to any of claims 37-40, wherein the cell underexpresses and/or expresses a defective or non-functional protein, and wherein said polynucleotide provides for increased expression and/or expression of a functional version of said non-functional protein.

42. A virus or virion comprising a polynucleotide of any of claims 1-15 or an expression construct of any of claims 16-23 or a vector of any of claims 24-26.

43. The virus or virion according to claim 42, wherein said virus or virion is an adeno-associated virus (AAV).

44. The virus or virion according to claim 43, wherein said AAV is serotype 5.



IRBP-GNAT2 promoter

524 bp

FIG. 1A

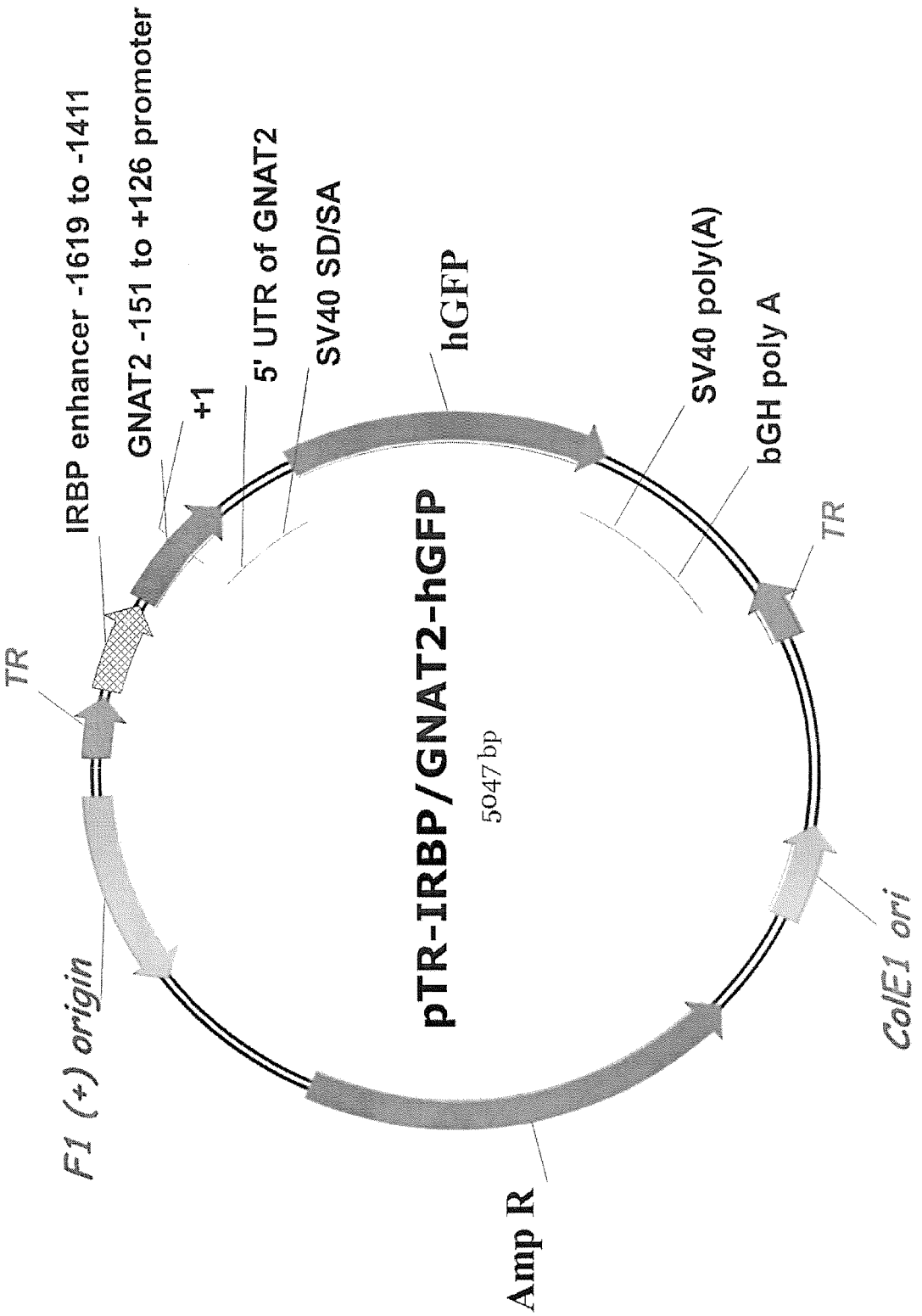

pTR-IRBP/GNAT2-hGFP
5047 bp
TR
IRBP enhancer -1619 to -1411
GNAT2 -151 to +126 promoter
+1
5' UTR of GNAT2
SV40 SD/SA
hGFP
SV40 poly(A)
bGH poly A
TR
ColE1 ori
Amp R
F1 (+) origin

FIG. 1B

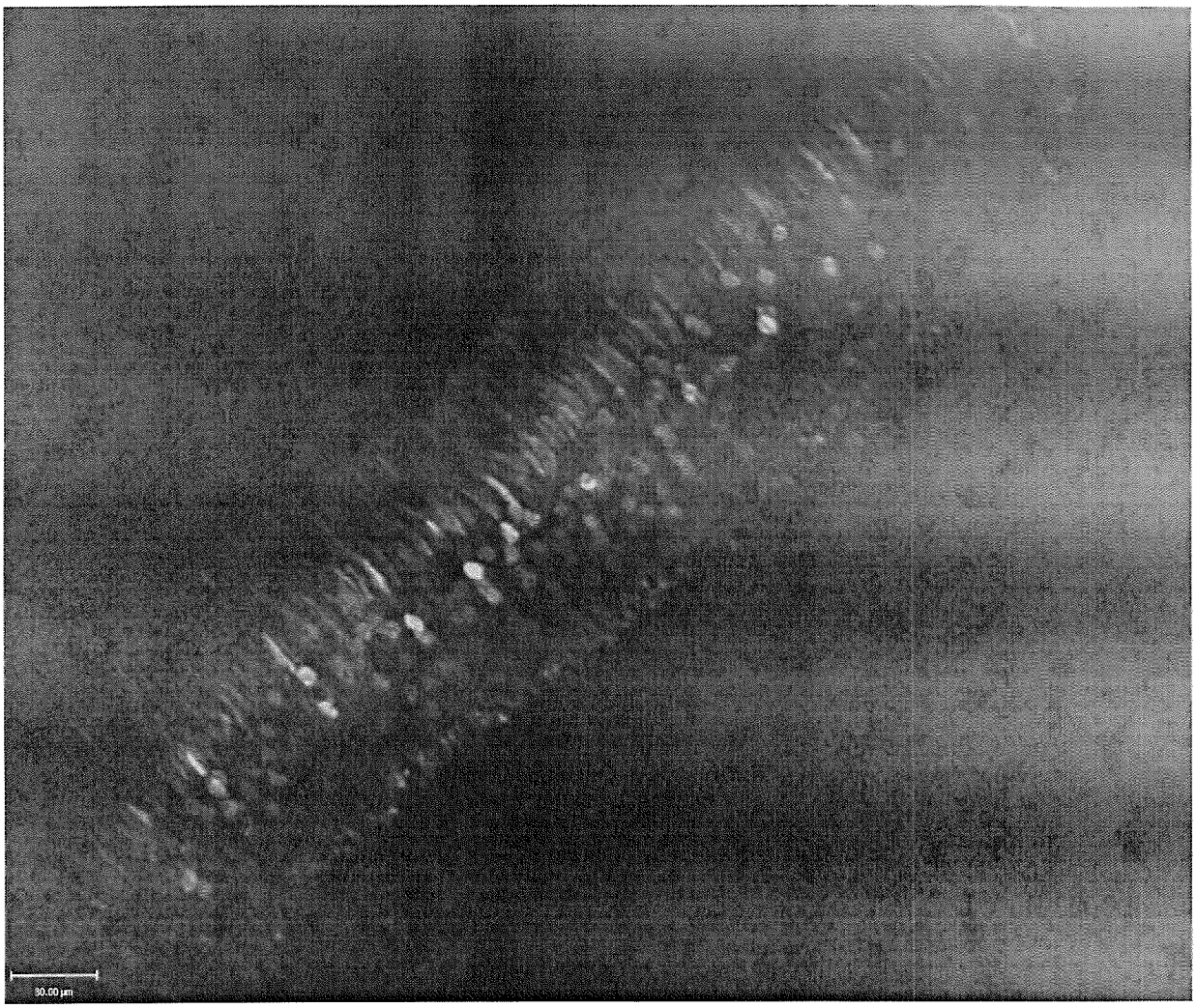


FIG. 2A

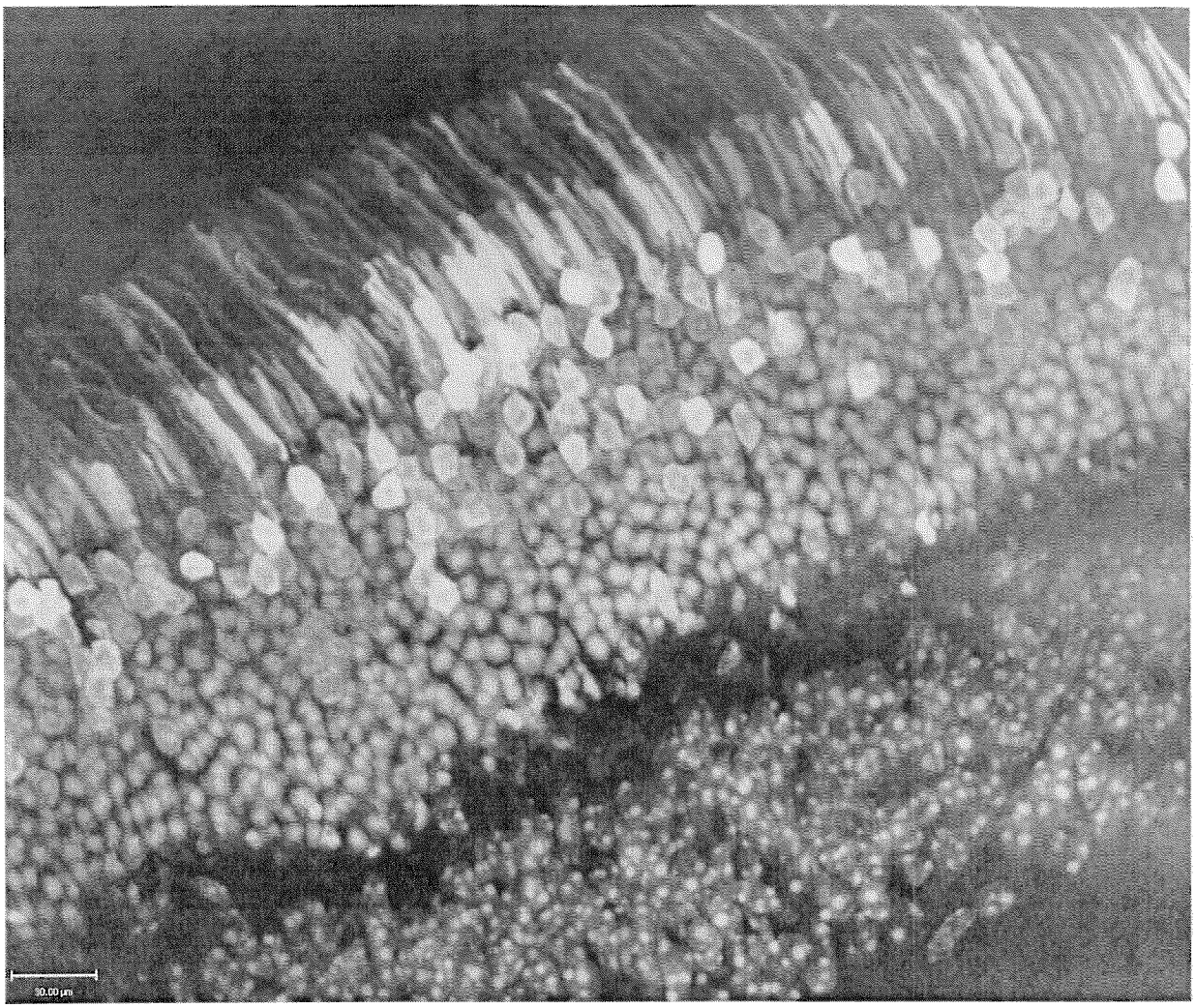


FIG. 2B

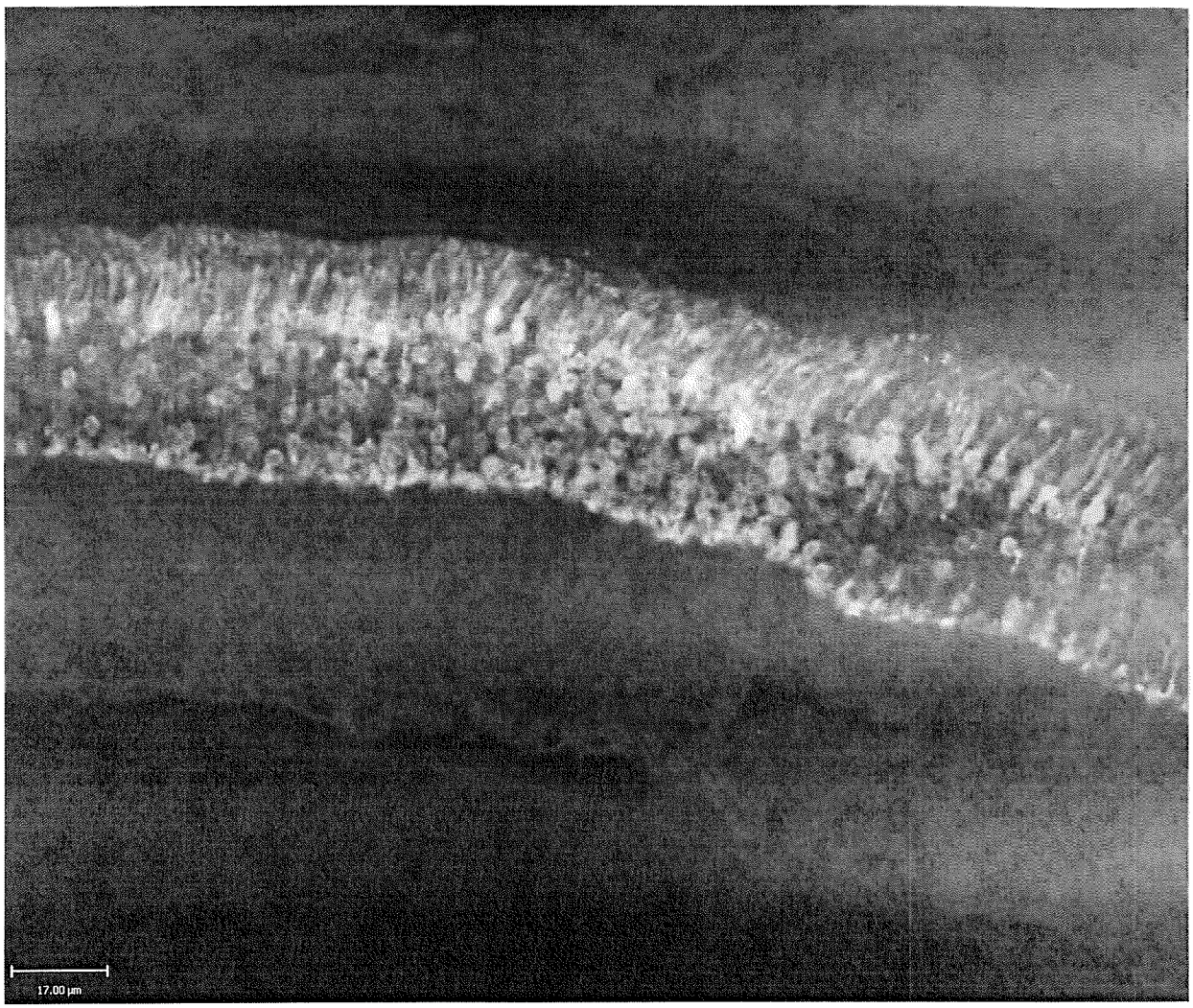


FIG. 3

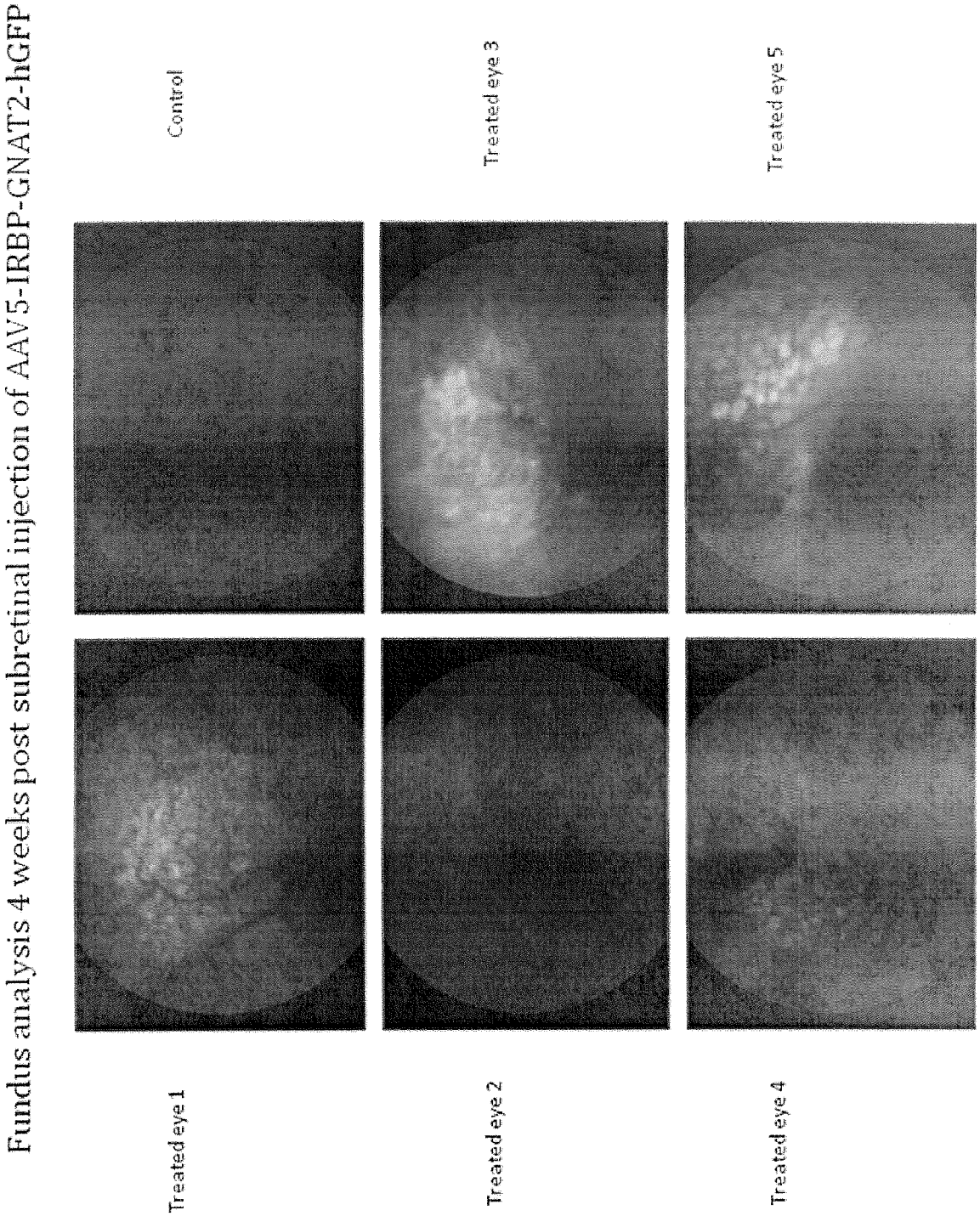

Fundus analysis 4 weeks post subretinal injection of AAV5-IRBP-GNAT2-hGFP
Control
Treated eye 3
Treated eye 5
Treated eye 1
Treated eye 2
Treated eye 4

FIG. 4A

Retinal section from eye 2
Immunostained for GFP
@20X magnification

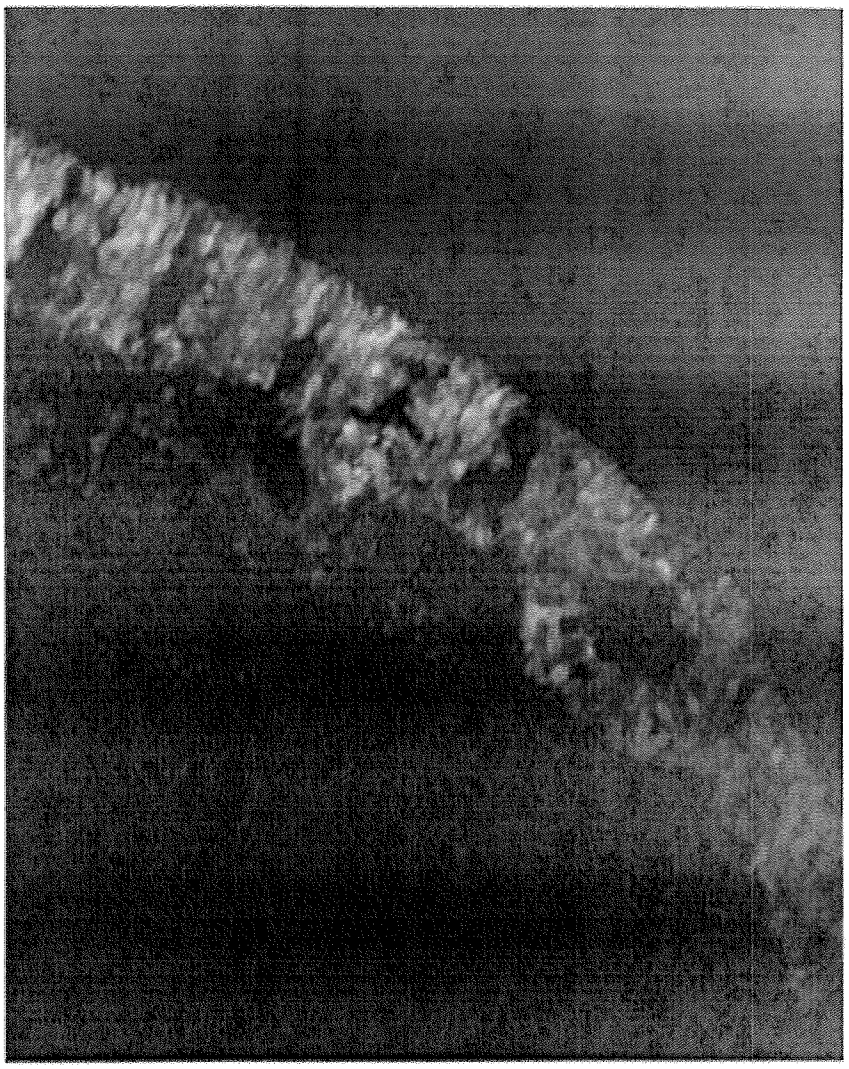


FIG. 4B

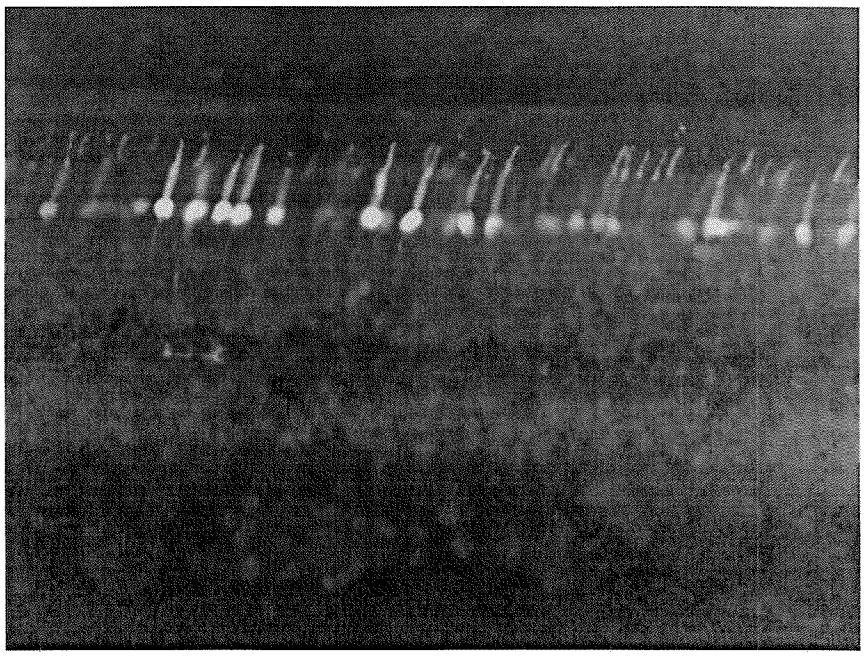


FIG. 5A

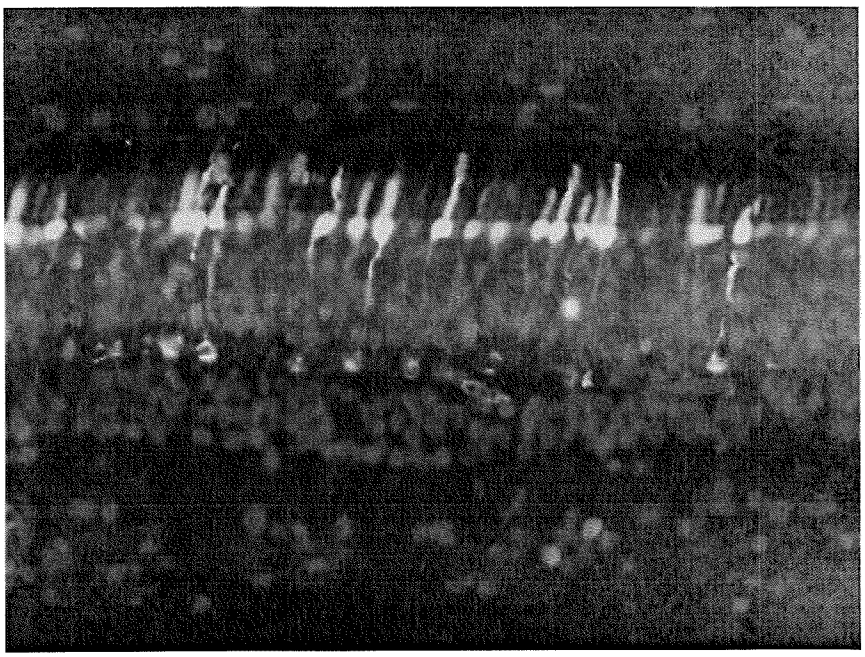


FIG. 5B

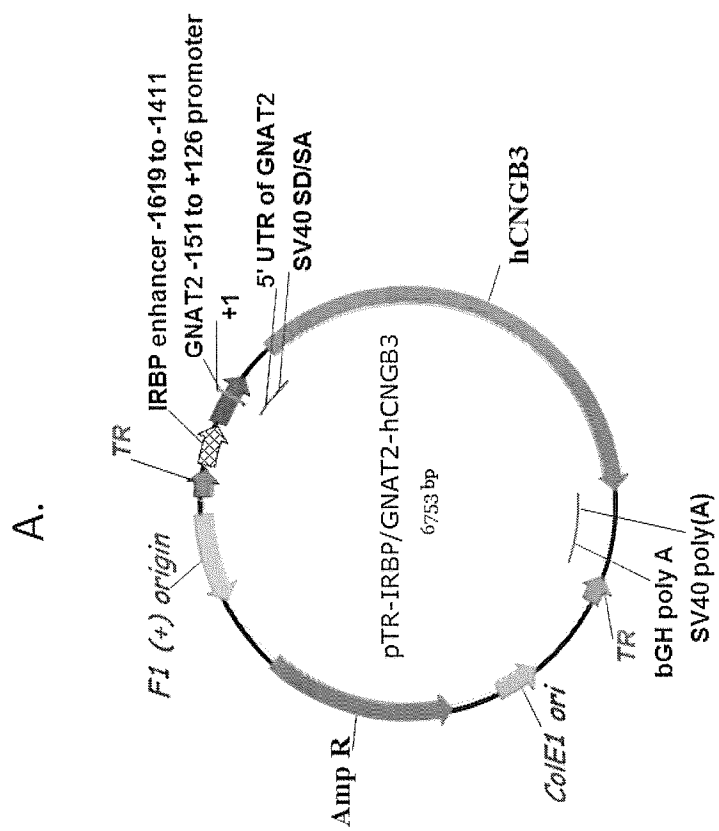

A.
pTR-IRBP/GNAT2-hCNGB3
6753 bp
TR
IRBP enhancer -1619 to -1411
GNAT2 -151 to +126 promoter
+1
5' UTR of GNAT2
SV40 SD/SA
hCNGB3
TR
bGH poly A
SV40 poly(A)
ColE1 ori
Amp R
F1 (+) origin

FIG. 6A

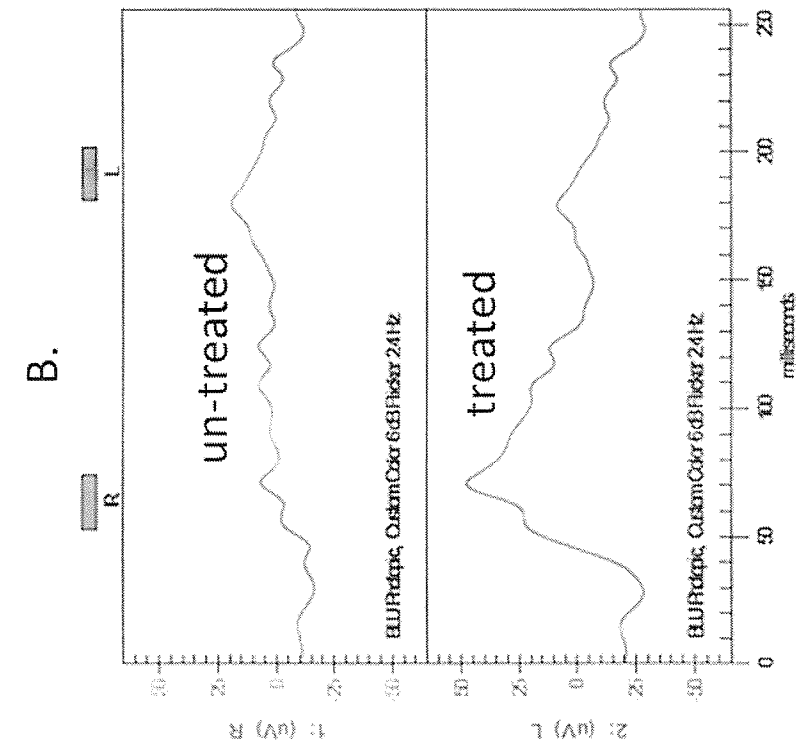

B.
R
L
un-treated
treated
milliseconds

FIG. 6B

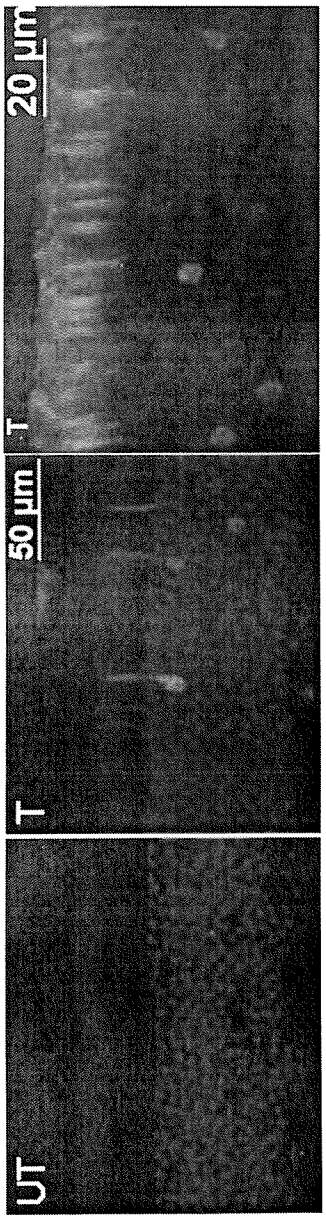


FIG. 7A

FIG. 7B

FIG. 7C

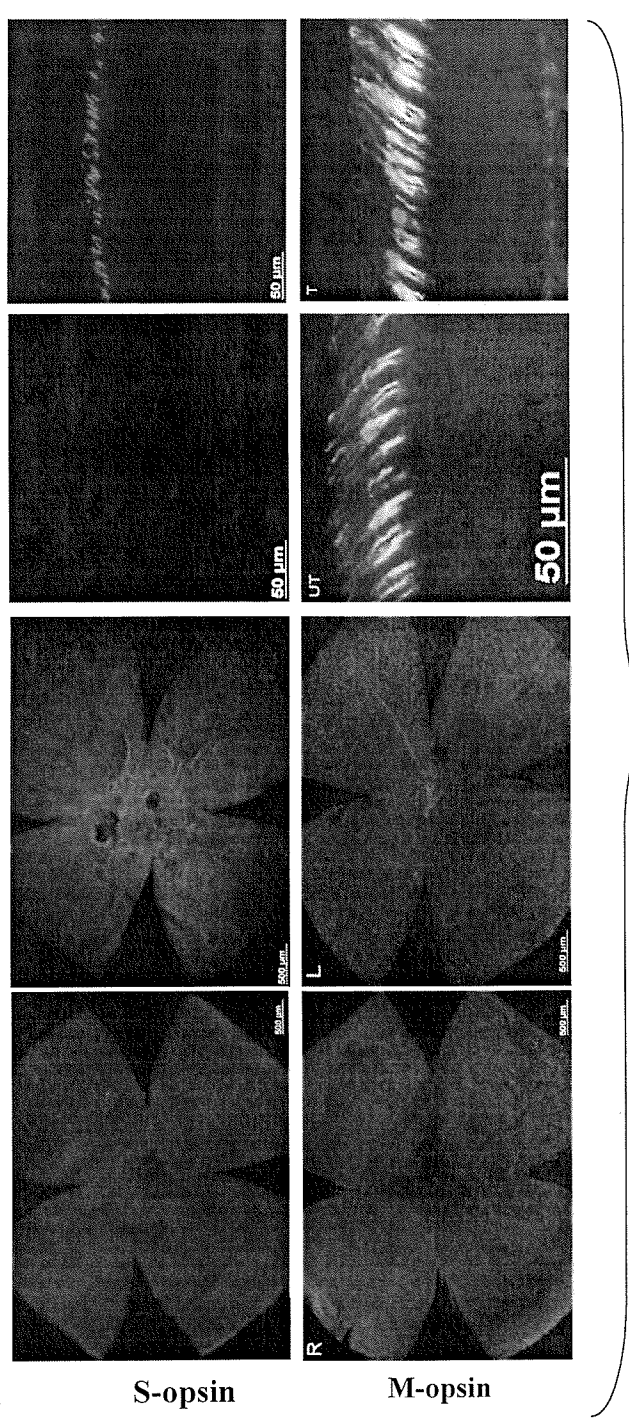


FIG. 8A

FIG. 8B

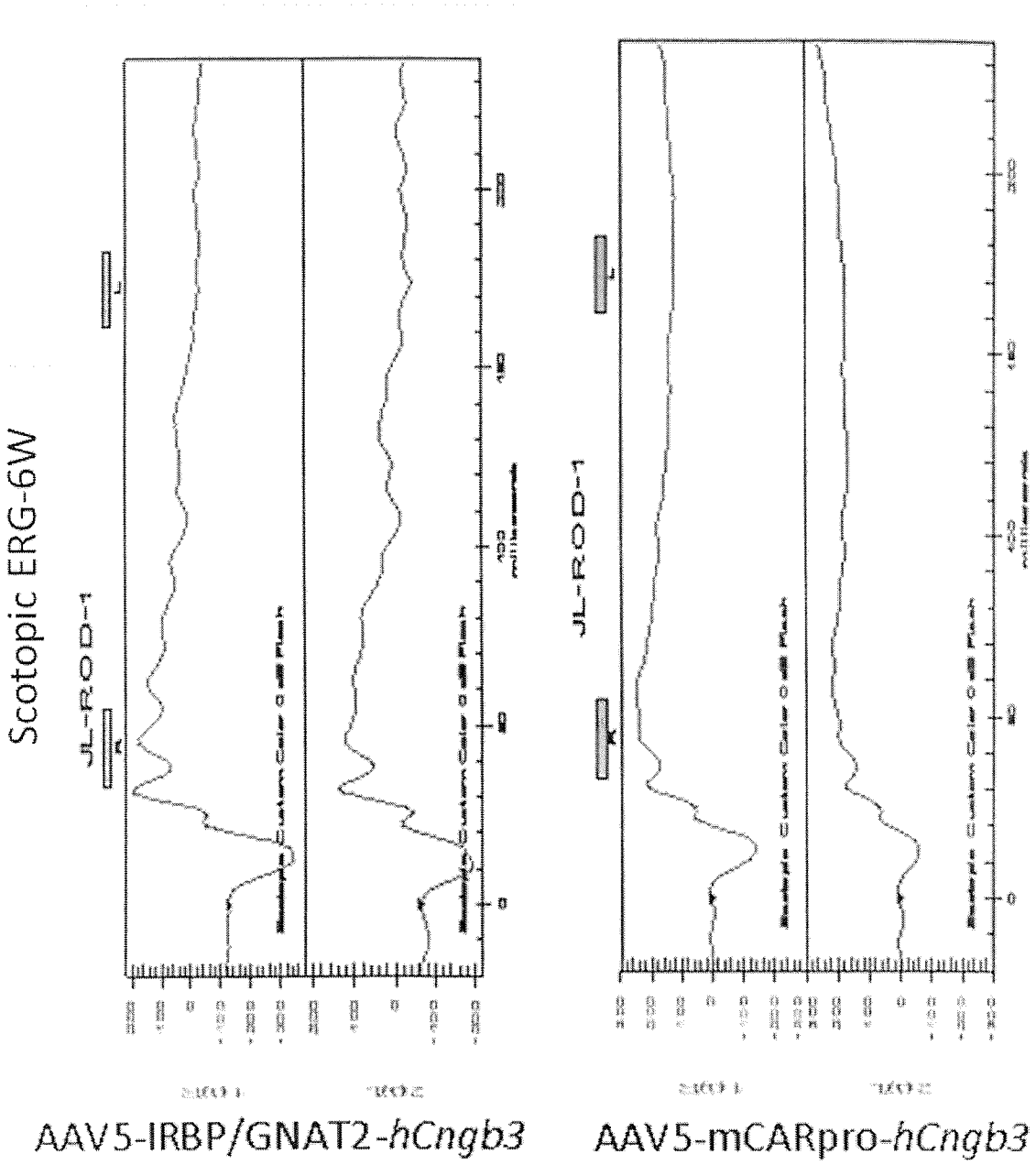

Scotopic ERG-6W
JL-ROD-1
JL-ROD-1
AAV5-IRBP/GNAT2-hCngb3
AAV5-mCARpro-hCngb3

FIG. 9A

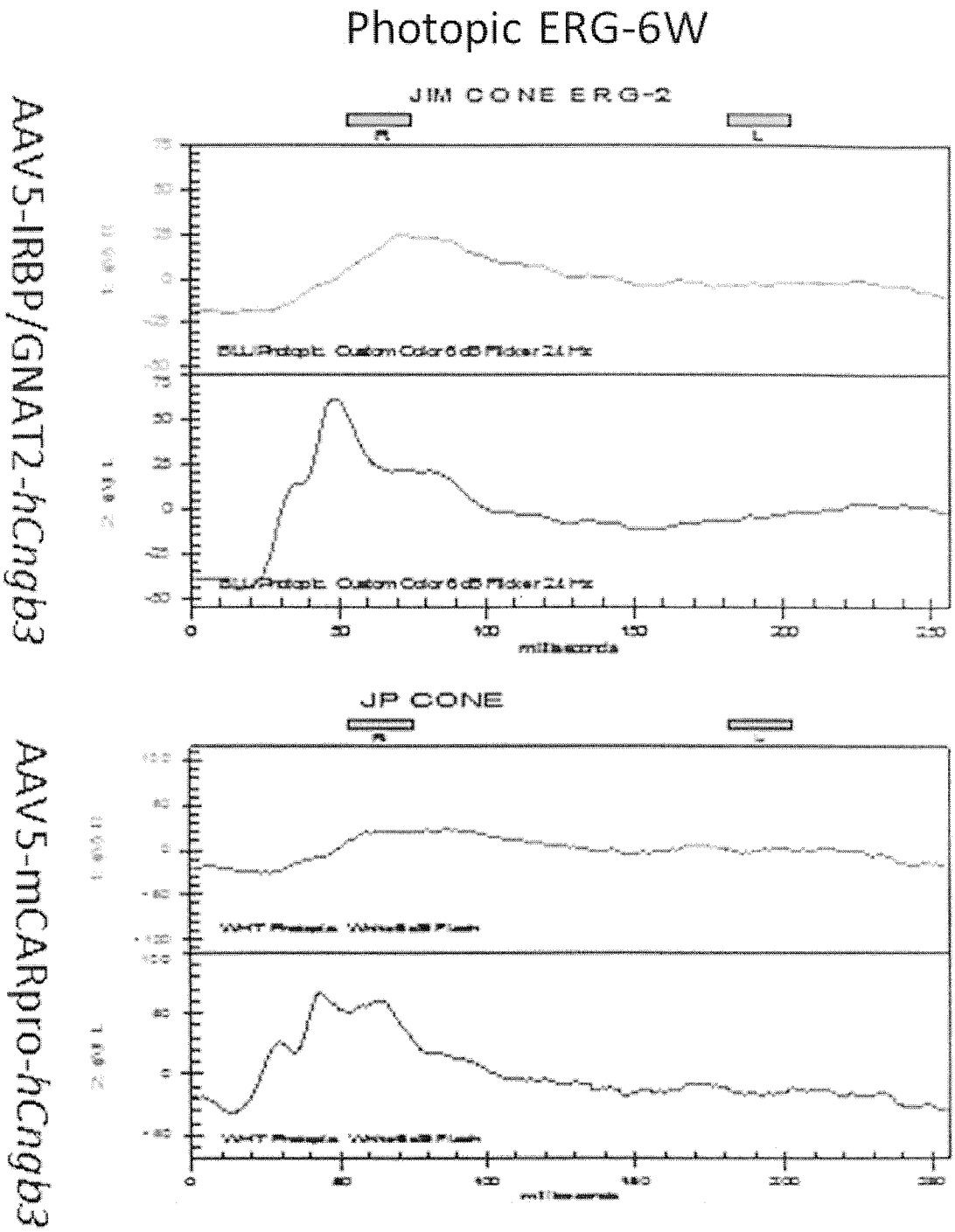

Photopic ERG-6W
AAV5-IRBP/GNAT2-hCngb3
AAV5-mCARpro-hCngb3
JIM CONE ERG-2
JP CONE
milliseconds

FIG. 9B

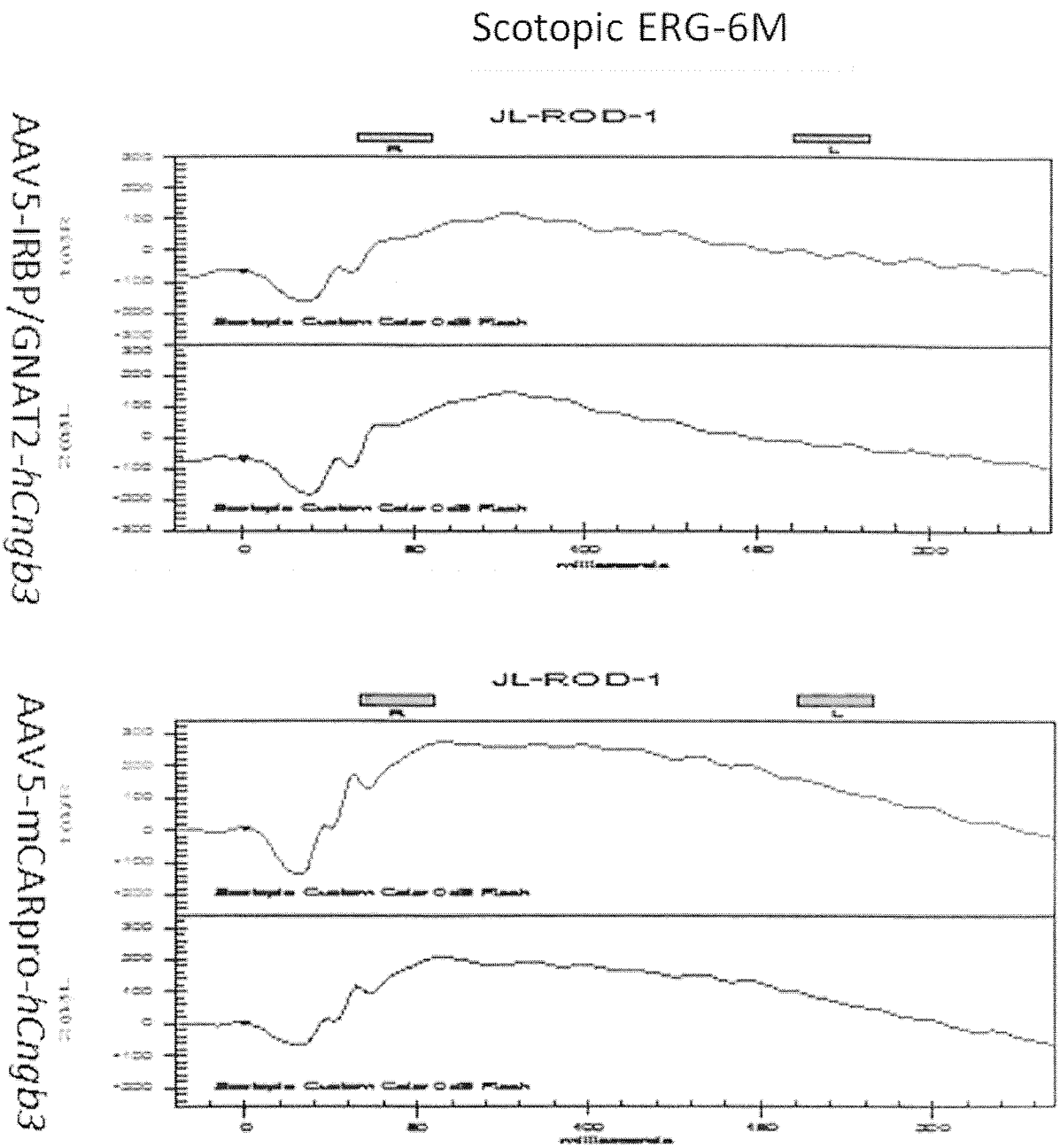

Scotopic ERG-6M
AAV5-IRBP/GNAT2-hCngb3
JL-ROD-1
AAV5-mCARpro-hCngb3
JL-ROD-1

FIG. 9C

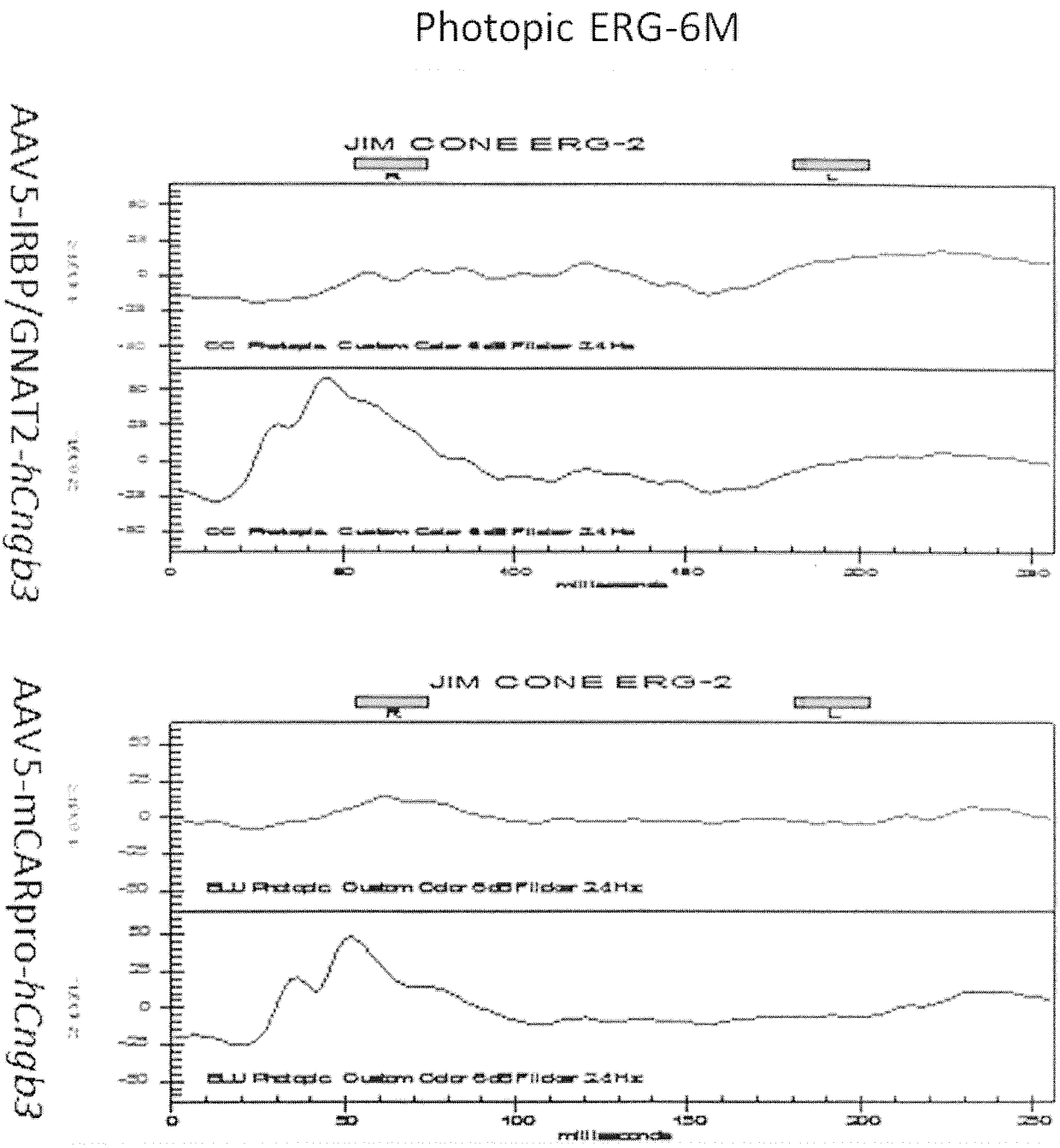

Photopic ERG-6M
AAV5-IRBP/GNAT2-hCngb3
JIM CONE ERG-2
R
L
CC Photopic Custom Color 6dB Filter 3.4Hz
CC Photopic Custom Color 6dB Filter 3.4Hz
0
50
100
150
200
250
milliseconds
AAV5-mCARpro-hCngb3
JIM CONE ERG-2
R
L
BLU Photopic Custom Color 6dB Filter 3.4Hz
BLU Photopic Custom Color 6dB Filter 3.4Hz
0
50
100
150
200
250
milliseconds

FIG. 9D

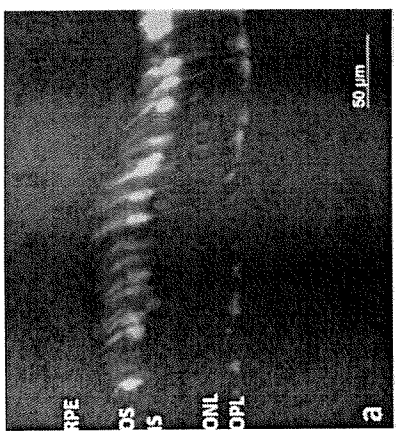


FIG. 10A

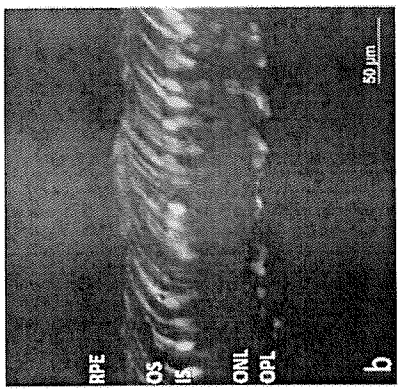


FIG. 10B

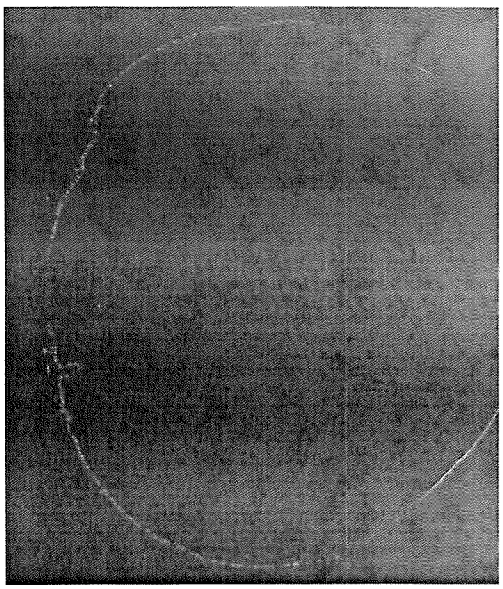


FIG. 10C

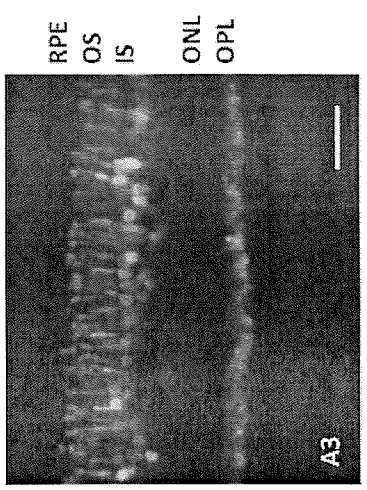


FIG. 11A-3

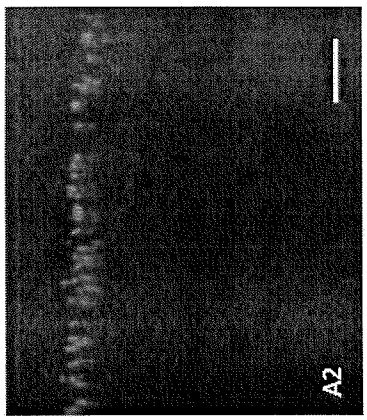


FIG. 11A-2

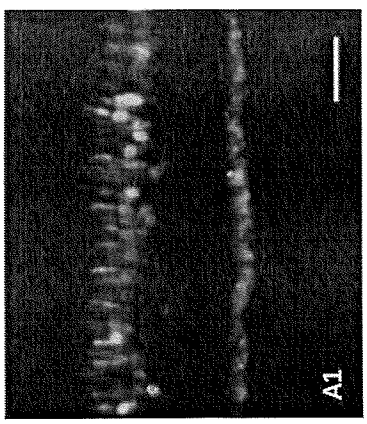


FIG. 11A-1

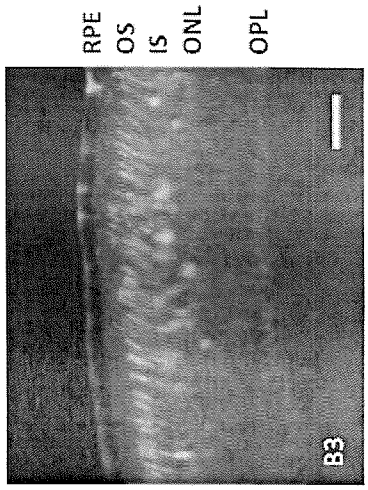


FIG. 11B-3

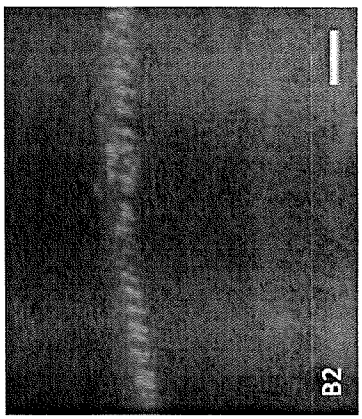


FIG. 11B-2

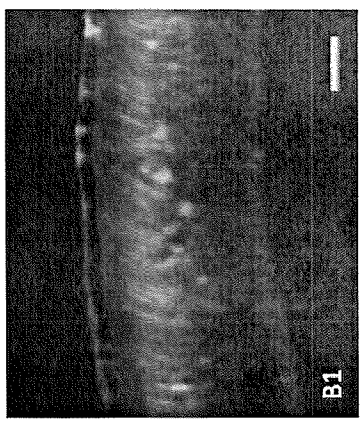


FIG. 11B-1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/062478

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/11(2006.01)i, C12N 15/86(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/11; A61K 48/00; C12N 15/85

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & keywords: interphotoreceptor retinoid-binding protein, IRBP, cone transducing alpha-subunit, GNAT2, cone cells, promoter, enhancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YING, S. et al., `A CAT reporter construct containing 277bp GNAT2 promoter and 214bp IRBP enhancer is specifically expressed by cone photoreceptor cells in transgenic mice`, Curr. Eye Res., August 1998, Vol.17, No.8, pp.777-782. See abstract; p.778, right column; figure 1.	1-12
A	YING, S. et al., 'Retinal degeneration in cone photoreceptor cell-ablated transgenic mice', Mol. Vis., 24 June 2000, Vol.6, pp.101-108. See abstract; p.101, right column; figure 1.	1-12
A	WO 2011-034947 A2 (UNIVERSITY OF WASHINGTON et al.) 24 March 2011 See abstract; claims 1, 5-8, and 18-21.	1-12
A	WO 98-48027 A2 (UNIVERSITY OF FLORIDA) 29 October 1998 See abstract; claims 1-5, 10, 11, 15, and 16.	1-12
A	FONG, S. L. et al., 'Characterization of a transgenic mouse line lacking photoreceptor development within the ventral retina', Exp. Eye Res., October 2005, Vol.81, No.4, pp.376-388. See abstract.	1-12

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 FEBRUARY 2013 (26.02.2013)	**27 FEBRUARY 2013 (27.02.2013)**
Name and mailing address of the ISA/KR Facsimile No. 82-42-472-7140	Authorized officer HEO, Joo Hyung Telephone No. 82-42-481-8150

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2012/062478

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011-034947 A2	24.03.2011	WO 2011-034947 A3	29.09.2011
WO 98-48027 A2	29.10.1998	AU 1998-71406 B2	12.07.2001
		AU 7467598 A	13.11.1998
		BR 9809284 A	03.08.2004
		CA 2287495 A1	29.10.1998
		CA 2287508 A1	29.10.1998
		EP 0977841 A2	09.02.2000
		EP 0977880 A2	09.02.2000
		JP 2001-523959 A	27.11.2001
		JP 2001-527399 A	25.12.2001
		WO 98-48009 A2	29.10.1998

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/062478

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 27-31
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 27-31 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 2, 3, 14, 15, 17-21, 23, 25, 26, 28-31, 33-36, 38, 43, 44
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 14, 15, 17-21, 23, 25, 26, 28-31, 33-36, 38, 43, and 44 are unclear (PCT Article 6) because they refer to claims which are not drafted in accordance with the second and/or the third sentence of Rule 6.4(a).

3. ☒ Claims Nos.: 13, 16, 22, 24, 27, 32, 37, 39-42
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.