Cone-Specific Promoters for Gene Therapy of Achromatopsia and Other Retinal Diseases

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Adeno-associated viral (AAV) vectors containing cone-specific promoters have rescued cone photoreceptor function in mouse and dog models of achromatopsia, but cone-specific promoters have not been optimized for use in primates. Using AAV vectors administered by subretinal injection, we evaluated a series of promoters based on the human L-opsin promoter, or a chimeric human cone transducin promoter, for their ability to drive gene expression of green fluorescent protein (GFP) in mice and nonhuman primates. Each of these promoters directed high-level GFP expression in mouse photoreceptors. In primates, subretinal injection of an AAV-GFP vector containing a 1.7-kb L-opsin promoter (PR1.7) achieved strong and specific GFP expression in all cone photoreceptors and was more efficient than a vector containing the 2.1-kb L-opsin promoter that was used in AAV vectors that rescued cone function in mouse and dog models of achromatopsia. A chimeric cone transducin promoter that directed strong GFP expression in mouse and dog cone photoreceptors was unable to drive GFP expression in primate cones. An AAV vector expressing a human *CNGB3* gene driven by the PR1.7 promoter rescued cone function in the mouse model of achromatopsia. These results have informed the design of an AAV vector for treatment of patients with achromatopsia.

INTRODUCTION

CONE PHOTORECEPTOR CELLS in the human retina are responsible for daylight vision, high visual acuity, and color discrimination. Retinal diseases that affect the cone system, such as achromatopsia and cone or cone/rod dystrophies, lead to severe visual impairment. Achromatopsia is an inherited cone dysfunction characterized from birth by markedly reduced visual acuity, nystagmus, severe photophobia, and complete loss of color discrimination.¹ Approximately 50% of cases are caused by mutations in the cone photoreceptor-specific cyclic nucleotide-gated channel β subunit (*CNGB3*) gene² and 25% are caused by mutations in the cone-specific cyclic nucleotide-gated channel α subunit (*CNGA3*) gene,³ although the prevalence of *CNGA3* mutations is higher in the Middle East and China.^{4,5} A small percentage of cases are caused by mutations in the α subunit of the cone-specific transducin (*GNAT2*) gene,⁶ the cone cGMP-specific 3',5'-cyclic phospho-

diesterase subunit α (*PDE6C*) gene,^{7,8} or the inhibitory (γ) subunit of the cone-specific cGMP phosphodiesterase (*PDE6H*) gene.⁹ Each of these types of mutations results in a loss of cone photoreceptor function in humans with achromatopsia and in animals with mutations in the homologous genes. There is currently no effective treatment for achromatopsia.

The success of gene therapy using adeno-associated viral (AAV) vectors expressing the *RPE65* gene in patients with Leber congenital amaurosis^{10–13} offers promise for treatment of other types of previously incurable retinal diseases including achromatopsia. Restoration of cone function and improvements in photopic vision with AAV vectors have been achieved in mouse and dog models of achromatopsia.^{14–18} Levels of photopic electroretinography (ERG) recovery in these studies varied from 10% to nearly 100% of the wild-type levels, depending on the AAV serotype, promoter, fraction of retina treated, and animal model

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selected. Near wild-type levels of photopic ERG restoration were observed in mouse models of achromatopsia caused by deletion of the *Gnat2*, *Cnga3*, or *Cngb3* gene after treatment with an AAV5-*Gnat2* vector containing a 2.1-kb human cone L-opsin (PR2.1) promoter,¹⁵ an AAV5-*Cnga3* vector containing a mouse S-opsin promoter,^{14,19} an AAV8-*CNGB3* vector containing a human cone arrestin (hCAR) promoter,¹⁸ and an AAV5-*Cnga3* vector containing a hybrid cytomegalovirus (CMV) enhancer/chicken β -actin (CBA) promoter.¹⁷ The hCAR and CBA promoters are not cone-specific. In addition to cones, the hCAR promoter also directs expression in rod photoreceptor cells²⁰ and the CBA promoter directs expression in almost any cell type.²¹ There is limited information on the specificity and efficiency of these promoters for transducing cone photoreceptors in primates.

We constructed a series of rationally designed smaller promoters based on the 2.1-kb human L-opsin promoter and evaluated them for their efficiency and specificity in driving *GFP* reporter gene expression in cone photoreceptors in normal mice and cynomolgus macaques and for rescuing cone function in *CNGB3*-deficient mice. We report here that a shorter version of the L-opsin promoter (PR1.7) was more efficient than the PR2.1 promoter, driving strong and specific green fluorescent protein (GFP) expression in primate cone photoreceptors. Subretinal injection of an AAV-*CNGB3* vector containing the PR1.7 promoter achieved robust and long-term rescue of cone function in *CNGB3*-deficient mice.

RESEARCH DESIGN AND METHODS

Materials and Methods

Vector plasmid construction. The PR2.1 promoter, which contains a simian virus 40 (SV40) splice donor/splice acceptor sequence, was synthesized on the basis of published sequence information²² with appropriate restriction sites introduced at each end to facilitate cloning. The PR1.7, PR1.5, and PR1.1 promoters were generated by PCR amplification of relevant portions of the synthesized PR2.1 promoter. Vector plasmid pTR-CMVenh-*GFP* (containing AAV terminal repeats, a CMV enhancer sequence, *GFP* cDNA, and an SV40 polyadenylation sequence) was constructed by replacing the *NcoI*-*NotI* fragment (containing a CBA promoter and hRS1 cDNA) in pTR-UF-SB-hRS1 with a *GFP* cDNA amplified by PCR. pTR-CMVenh-*GFP* was then used to construct vector plasmids pTR-PR1.1-*GFP*, pTR-PR1.5-*GFP*, pTR-PR1.7-*GFP*, and pTR-PR2.1-*GFP*, either by replacing the *Acc65I*-*HindIII* fragment that contains the CMVenh sequence of pTR-CMVenh-*GFP* with

the PCR-amplified PR1.5, PR1.7, or PR2.1 sequence, or by inserting the PR1.1 promoter sequence between the CMVenh and *GFP* sequences. Vector plasmids were grown in SURE 2 cells (Agilent Technologies, Santa Clara, CA), and purified with an EndoFree plasmid giga kit (Qiagen, Valencia, CA).

AAV vector production. AAV vectors were produced by transient plasmid transfection of HEK 293 cells and purified by double iodixanol step gradient centrifugation.^{23,24} Briefly, a Nunc Cell Factory (Thermo Scientific, Waltham, MA) containing 1×10^9 HEK 293 cells was cotransfected with a vector plasmid and a helper plasmid encoding AAV *rep* and *cap* genes and the adenoviral genes required to package the expression cassettes into AAV capsids. Transfected cells were harvested 60 hr post-transfection, and the vector-containing clarified cell lysate was subjected to two rounds of iodixanol step gradient centrifugation with collection of the 60–40% step gradient interface. The purified vectors were concentrated and buffer was exchanged for balanced salt solution (BSS; Alcon, Fort Worth, TX) containing 0.014% Tween, passed through a 0.22- μ m (pore size) filter, and dispensed in small aliquots. The concentration of each vector was determined by quantitative PCR.²⁵

AAV vector administration. The design of a promoter comparison study using AAV-*GFP* vectors in C57BL/6 mice is shown in Supplementary Table S1 (supplementary data are available online at www.liebertpub.com/hum), and the design of a promoter comparison study using AAV-*CNGB3* vectors in *CNGB3* knockout mice is shown in Supplementary Table S2. C57BL/6 mice at approximately 6–8 weeks of age or *CNGB3* knockout mice at 30 days of age were anesthetized by intramuscular injection of ketamine–xylazine (1:1 mixture) and pupils were dilated with 2.5% phenylephrine. Subretinal injection was performed by inserting a blunt 32-gauge needle between the retina and retinal pigment epithelium and injecting 1 μ l of AAV vector at a concentration of 2×10^{12} vector genomes (VG)/ml or 5×10^{12} VG/ml into the subretinal space under direct visualization using a dissecting microscope.²⁶

For primate studies, female cynomolgus macaques (*Macaca fascicularis*), 3 years of age and weighing approximately 3 kg, were anesthetized by intramuscular injection of ketamine (2–10 mg/kg), dexmedetomidine (0.025 mg/kg), and glycopyrrrolate (0.01 mg/kg) and pupils were dilated with a topical mydriatic agent. Using a trocar, two cannulas (23- or 25-gauge) were placed through the conjunctiva and sclera approximately 3 mm posterior to the

corneal limbus, one at the 10 o'clock and the other at the 2 o'clock meridian. A fiberoptic light was passed through the 25-gauge cannulas to illuminate the retina. A plano-convex corneal contact lens was used to visualize the retina through a surgical microscope. A 23-gauge injector (D.O.R.C., Exeter, NH) with a 41-gauge extension was then passed through the 23-gauge cannula and the cannula tip was extended and brought in contact with the retina. The vector (100 μ l at a concentration of 5×10^{11} VG/ml) was injected through the retina into the subretinal space, resulting in a subretinal bleb. The 41-gauge cannula tip was retracted and the injector tip was repositioned for the second injection. After the second subretinal injection, the instruments and cannulas were removed from the eye. The sclerotomy was cauterized. A topical antibiotic (0.3% tobramycin/0.1% dexamethasone ophthalmic ointment) was instilled in each eye post-injection. To minimize animal-to-animal variability, different vectors were injected in the two eyes of each animal, using a Latin square design so that a total of four eyes were injected with each vector (see Supplementary Table S3).

Electroretinography evaluation. Full-field scotopic and photopic ERG studies were conducted in CNGB3 knockout mice 3 months after vector administration. Mice were dark adapted for 4 hr and anesthetized with a mixture of ketamine–xylazine, and scotopic responses were elicited using a series of white flashes of three increasing intensities (–20, –10, and 0 dB). Interstimulus intervals were 2.5, 5.0, and 20.0 sec, respectively. Mice were then light adapted to a 30-cd·sec/m² white background for 2 min and photopic responses were elicited, with intensities ranging from –3 to 10 dB. Maximum photopic b-wave amplitudes generated at 10 dB within each cohort were averaged and used for analysis.

Retinal tissue collection and processing. For mouse tissues, eyes were surgically removed, fixed in 4% paraformaldehyde, and then treated with sucrose buffer of increasing concentrations of sucrose (from 5 to 20%) in phosphate-buffered saline (PBS). After removing the cornea and lens, the remaining eye cups were embedded in O.C.T. medium (Sakura Finetek, Torrance, CA) and snap frozen in a bath of dry ice–ethanol for cryosectioning.

For nonhuman primate (NHP) tissues, the eyes were enucleated immediately after animal sacrifice, placed into cold (4°C) 0.1 M phosphate buffer, and promptly placed on a cold plate under a dissecting microscope. Excess tissue was removed and a coronal cut was made to remove the anterior segment

(cornea, iris, and lens). Four radial cuts, one in each quadrant, were made through the retina and sclera extending from the peripheral cut edge to just posterior to the equator in order to flatten the posterior segment. Punches of the retina were made in the superior bleb using a 6 mm trephine placed in a specimen tube, and frozen and stored at –80°C for reverse transcription quantitative PCR (RT-qPCR) analysis of transgene expression. The remainder of the eye was placed into 4% paraformaldehyde in phosphate buffer at 4°C for 2 days and then transferred to 0.1 M phosphate buffer and stored at 4°C. A parasagittal strip of retina/sclera was dissected from each eye that included the blebs, macula, and periphery and embedded in paraffin for immunohistochemistry.

RT-qPCR analysis of retinal transgene expression in primate eyes. The retinal tissue (superior bleb area) from each AAV-injected primate eye or two untreated eyes (negative control) was homogenized in 1 ml of TRIzol and total RNA was extracted with 0.2 ml of chloroform followed by centrifugation at 10,000×g for 18 min at 4°C. The top aqueous phase was transferred to a new tube and an equal volume of 100% RNA-free ethanol was added. Samples were loaded onto an RNeasy column (Qiagen) and processed according to the manufacturer's instructions. Purified total RNA was eluted in 40 μ l of RNase-free water and cDNA synthesis was performed from 1 μ g of total RNA, using a Sprint RT complete-double prePrimed kit (Clontech, Mountain View, CA) according to the manufacturer's instruction. qPCR assays were performed on a MasterCycler RealPlex (Eppendorf, Hauppauge, NY) using SYBR qPCR premix (Clontech) with primers specific to the *GFP* transgene (*GFP-F*, TCGGCCACAAGCTGGAATA; and *GFP-R*, TGCTTGTCGGCCATGATGT), and universal primers for 18S ribosomal RNA (TaqMan ribosomal RNA control reagents; Applied Biosystems, Foster City, CA). PCR cycling parameters were 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 40 sec. The dissociation curve step included 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec. Relative *GFP* mRNA expression was normalized to the simultaneously amplified 18S rRNA housekeeping gene for all samples.

Immunohistochemistry. Mouse retinal cryosections (10 μ m) were stained with rabbit polyclonal GFP antibody, peanut agglutinin (PNA) lectin conjugated to Alexa Fluor 594, and 4',6'-diamidino-2-phenylindole (DAPI) and analyzed by confocal microscopy as previously described.²⁰

NHP tissues for immunohistochemical (IHC) staining were embedded in paraffin and sections 5 μ m thick were mounted on positively charged glass slides. Some sections were deparaffinized and stained with hematoxylin and eosin. The sections for IHC were deparaffinized by heating in a 60°C oven and washing with xylene followed by graded alcohols, after which they underwent trypsin digestion for 2 min at 37°C. After halting digestion with a series of cold water washes, sections were immersed for 1 hr in PBS containing 5% normal goat serum. Primary antibodies were then added: anti-GFP (MAB3580; Millipore, Bedford, MA), anti-red/green cone opsin (AB5405; Millipore), or anti-blue cone opsin (AB5407; Millipore) in a solution of PBS containing 5% normal goat serum. Sections were immersed in primary antibodies for at least 12 hr. On the next day, primary antibodies were washed off with PBS and secondary antibodies were applied: Alexa Fluor 488 AffiniPure anti-mouse (115-545-146; Jackson ImmunoResearch, West Grove, PA) and Cy3 AffiniPure anti-rabbit (111-165-144; Jackson ImmunoResearch). After incubation for 2 hr, sections were washed thoroughly in PBS and coverslipped with VECTASHIELD hard mounting medium containing DAPI (NC9029229; Fisher Scientific). Images were acquired with an Olympus BS61 microscope and cellSens Dimension 1.4.1 software, using the same exposure time for all slides.

Sections from the 12 study eyes underwent IHC runs with anti-GFP antibodies on four adjacent sections through the subretinal blebs where they extended to the macula. Two of the runs were double-labeled for L/M cone opsin and two were double-labeled for S cone opsin. For the second pair of IHC runs, three sections from control eyes (not exposed to AAV vectors) were also stained for GFP and double-labeled for either L/M-opsin or S-opsin. The intensity of GFP staining was evaluated subjectively by a masked observer from grade 0 (no staining) to grade 3 (marked staining) for each of the four runs on each eye and then averaged.

RESULTS

Design of human cone-specific promoters

Humans and nonhuman primates have three types of cones sensitive to three different spectra, conventionally labeled according to the wavelengths of the peaks of their spectral sensitivities: short (S), medium (M), and long (L) cone types, corresponding roughly to blue, green, and red stimuli, respectively. Lower mammals have only two types of cones, L/M and S. The PR2.1 promoter

is composed of bases spanning positions -4564 to -3009 and -496 to 0 upstream of the human L-opsin gene transcriptional initiation site²⁷ and includes a 0.6-kb locus control region (LCR) that is essential for expression of both L- and M-opsin genes.²² In the AAV5-PR2.1-*hCNGB3* vector used in proof-of-concept studies in the CNGB3-deficient dog model of achromatopsia,¹⁶ the *CNGB3* expression cassette with flanking AAV inverted terminal repeats has a size of 5230 bp, which is larger than the optimal packaging capacity of AAV vectors.²⁸ In addition, the PR2.1 promoter is reported to direct GFP expression only in L/M cones but not S cones in mice and dogs,²⁷ but CNGB3 is normally expressed in all three types of primate cones.

To address these issues, we designed three smaller versions of the PR2.1 promoter (PR1.1, PR1.5, and PR1.7) that retain the 0.5-kb core promoter and a 0.6-kb sequence containing the LCR, but progressively removed other sequences in the promoter (Fig. 1). The 0.6-kb region containing the LCR is known to be essential because its deletion results in absent expression of both L- and M-opsin genes, resulting in blue cone monochromacy, and it is needed for cone-specific expression of a reporter gene in transgenic mice.²² To potentially increase efficiency, a 0.38-kb cytomegalovirus immediate-early enhancer (CMV_{enh}) was added to the 5' end of the shortest of these promoters (PR1.1).

Figure 1. Design of AAV-GFP vectors containing promoters based on the L-opsin promoter. The checkerboard-patterned portion of the four promoters represents nucleotides immediately upstream of the L-opsin coding sequence, and the stippled portion of the PR1.1 promoter represents the cytomegalovirus immediate-early enhancer. Nucleotide sequences of the PR1.7, PR1.5, and PR1.1 promoters are available in GenBank (accession numbers KT886395, KT886396, and KT886397). ITR, inverted terminal repeat; SV40, simian virus 40; SD/SA, splice donor/splice acceptor; polyA, polyadenylation sequence; GFP, green fluorescent protein cDNA; LCR, locus control region.

We also evaluated a small hybrid promoter containing an interphotoreceptor retinoid-binding protein enhancer followed by a GNAT2 that is able to direct cone-specific expression of GFP in L/M and S cones in mice²⁰ and dogs.²⁹

PR2.1-based promoters drive GFP expression in mouse photoreceptors

Expression cassettes containing a *GFP* gene driven by each of four versions of the L-opsin promoter were packaged in AAV5 capsids and delivered bilaterally by subretinal injection of 1 μ l at a concentration of 2×10^{12} VG/ml (2×10^9 VG/eye) in C57BL/6 mice ($n=5$ per group). Mice with successful subretinal injections were euthanized 6 weeks after injection and retinal tissue was processed for IHC staining with polyclonal anti-GFP antibody and PNA lectin, which selectively binds to the sheath of cone outer segments. Vectors containing each of these promoters directed GFP ex-

pression in photoreceptors, and all except the PR1.7 promoter also directed expression in retinal pigment epithelium (RPE) cells (Fig. 2).

The PR1.7 promoter drives strong GFP expression in L, M, and S cones in primates

On the basis of the data obtained from the initial testing in mice, and other studies demonstrating that vectors packaged in AAV2tYF capsids (AAV serotype 2 capsids containing three tyrosine-to-phenylalanine mutations) can efficiently target expression in primate foveal cones,³⁰ AAV-*GFP* vectors containing the PR1.7 or PR2.1 promoter or the 0.5-kb IRBP/GNAT2 promoter and packaged in AAV2tYF capsids were evaluated for their efficiency and specificity after subretinal injection into primates. Six cynomolgus macaques received subretinal injections of one vector in each eye (two 100- μ l blebs at a concentration of 5×10^{11} VG/ml in each eye, four eyes per promoter) and had fundus fluorescence images

Figure 2. Evaluation of GFP expression in mouse retinas. Representative images from eyes injected with AAV5-*GFP* vectors containing the PR1.1 promoter (A), PR1.5 promoter (B), PR1.7 promoter (C), or PR2.1 promoter (D) were taken at identical exposure settings at an original magnification of $\times 20$. GFP labeling (green) was seen in the outer nuclear layer (i.e., photoreceptor cell bodies), inner and outer segments, and to a lesser extent in the retinal pigment epithelium. GC, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS/OS, photoreceptor inner segment-outer segment junction; RPE, retinal pigment epithelium. Scale bar: 45 μ m.

obtained 4, 8, and 12 weeks after injection. GFP expression was higher in eyes injected with vectors containing the PR1.7 promoter compared with the PR2.1 promoter, and GFP expression in eyes injected with vectors containing the IRBP/GNAT2 promoter was barely detectable even in overexposed images (Fig. 3).

Twelve weeks after injection, retinal tissue from the superior bleb was harvested for RT-qPCR analysis and the remaining retinal tissue was processed for IHC staining for GFP and L/M or S cone opsins. As shown in Fig. 4 and summarized in Table 1, a clear difference in the intensity of GFP expression was observed in eyes injected with vectors containing each of the three promoters. No GFP-specific labeling was noted in sections from the untreated control eyes, although the commonly observed nonspecific yellow–green autofluorescence was seen in the retinal pigment epithelium (Fig. 4A). No GFP labeling of cones was noted in sections from eyes injected with the rAAV2tYF-IRBP/GNAT2-*GFP* vector (Fig. 4B). GFP staining of variable intensity was seen in cones within the subretinal bleb in sections from eyes injected with the rAAV2tYF-PR2.1-*GFP* vector (Fig. 4C). GFP staining of marked intensity was seen in essentially all cones within the subretinal bleb in all sections from eyes injected with the rAAV2tYF-PR1.7-*GFP* vector, including L and M cones (Fig. 4D) and S cones (Fig. 4E). Because more than 90% of cones in the primate retina are L or M cones, with approximately equal numbers of each, expression in all cones in Fig. 4D implies that both L and M cones were transduced. GFP expression was restricted to cones; the cone cell body, inner processes, and pedicles were GFP-positive, but the abundant rods in adjacent sections were all GFP-negative (Fig. 4F). In eyes from animals injected with the rAAV2tYF-

PR1.7-*GFP* vector, all sections costained with antibodies to S cone opsin demonstrated marked expression of GFP (grade 3) in S cones, but with the rAAV2tYF-PR2.1-*GFP* vector expression of GFP in S cones varied from none (grade 0) to moderate (grade 2) in the eight sections from four eyes examined (Table 1). Additional representative images of GFP expression in the four retinas injected with an AAV2tYF-*GFP* vector containing the PR1.7 promoter or PR2.1 promoter are shown in Supplementary Fig. S1.

RT-qPCR analysis demonstrated minimal GFP mRNA transcription in retinas from eyes injected with the AAV-*GFP* vector containing the IRBP/GNAT2 promoter (mean $\pm$ SD, 0.028 ± 0.016 ng per ng of 18S RNA), confirming the results obtained from in-life fundus fluorescence imaging and IHC staining. GFP mRNA levels were similar in retinas from eyes injected with AAV-*GFP* vectors containing the PR2.1 and PR1.7 promoters (0.138 ± 0.068 ng per ng of 18S RNA and 0.151 ± 0.054 ng per ng of 18S RNA, respectively).

Rescue of cone ERG responses in CNGB3-deficient mice after administration of AAV-hCNGB3 vectors containing the PR1.7 promoter

AAV-*hCNGB3* vectors containing the PR2.1, PR1.7, or IRBP/GNAT2 promoter were packaged in AAV capsids of serotype 5, 9, or 2tYF and evaluated for rescue of cone function in CNGB3-deficient mice. In addition to comparing the three promoters, the study was designed to compare a *CNGB3* cDNA that was codon-optimized for expression in human cells versus a non-codon-optimized cDNA. Each animal received a subretinal injection of $1 \mu\text{l}$ of an AAV vector in one eye at a concentration of 5×10^{12} VG/ml, except for animals in group 5 that received $1 \mu\text{l}$ of

Figure 3. Evaluation of GFP expression in primate retinas by fundus fluorescence imaging. AAV-*GFP* vectors containing the PR1.7 promoter (A), PR2.1 promoter (B), or IRBP/GNAT2 promoter (C) were administered by subretinal injection. Areas with GFP expression appear white on a dark background. The camera automatically adjusts the exposure time; the image in (A), with the greatest intensity of GFP expression, has the darkest background color, but for the image in (C), with the weakest intensity of GFP expression, there is little difference between the area of the subretinal bleb and the background.

Figure 4. Evaluation of GFP expression in primate retinas by immunohistochemical staining. Retinal sections were obtained after subretinal injection of an AAV2tYF-*GFP* vector containing the IRBP/GNAT2 promoter (**B**), PR2.1 promoter (**C**), or PR1.7 promoter (**D–F**), or from a control uninjected eye (**A**). Sections were stained with antibodies to GFP (green) and with an antibody to L/M opsin [red in (**A–D**) and (**F**)] or an antibody to S opsin [red in (**E**)] and counterstained with DAPI (blue). (**A**) Background green fluorescence in the retinal pigment epithelium layer. No cone-specific GFP expression was seen in (**B**) and limited GFP expression was seen in (**C**). Strong GFP expression was seen in essentially all cones in (**D**) and (**E**). (**F**) An overexposed image to show strong GFP staining in periphery cones without GFP staining in rods. Only the cones are positive for GFP. Arrows in (**E**) indicate individual S cones. Animal identification numbers are indicated at the bottom left of each panel. OD, right eye; OS, left eye. Scale bars: (**A–D**) 50 μ m; (**E** and **F**) 20 μ m.

rAAV2tYF-IRBP/GNAT2-*hCNGB3co* at a concentration of 2×10^{12} VG/ml. The contralateral eyes either received a subretinal injection of vehicle or were untreated. Photopic b-wave amplitudes generated at

10 dB (318 cd sec/m²) from the treated and the contralateral control eyes within each group were averaged and compared.

Three months after vector administration, five of the seven groups had significant improvement in cone ERG responses in the treated eye compared with contralateral untreated eyes (Fig. 5 and Table 2). Improvement of ERG cone-mediated b-wave amplitudes in these five groups was similar for vectors containing either codon-optimized or non-codon-optimized human *CNGB3* cDNA driven by the IRBP/GNAT2 promoter (groups 4, 6, and 7), or vectors containing codon-optimized or nonoptimized *hCNGB3* cDNA driven either by the PR2.1 or PR1.7 promoter (groups 1 and 3).

DISCUSSION

Important considerations in the design of an AAV vector include the capsid, enhancer, promoter, cDNA, polyadenylation sequence, and overall size of the DNA expression cassette. Using vector DNA larger than about 5 kb leads to inefficient packaging, with resultant low yields, higher empty-to-full particle ratio, lower infectivity, and nonuniform products resulting from variable truncations at the 5' or 3' ends of the packaged DNA.²⁸ Vectors with a larger cDNA require a smaller promoter, and for some indications it is desirable to have a cell-specific promoter to avoid potential off-target effects. For diseases of cone photoreceptors, another consideration is the ability to achieve expression in all three cone subtypes.

For achromatopsia caused by *CNGB3* mutations, the size of the human *CNGB3* cDNA is 2.3 kb, which limits the size of the promoter that can be used. Komáromy and colleagues evaluated three versions of the human L-opsin promoter (PR2.1, PR0.5, and 3LCR-PR0.5) for their specificity and efficiency in targeting *GFP* gene expression to subclasses of cones in the canine retina when used in AAV serotype 5 vectors.²⁷ The PR2.1 promoter contains 2.1 kb of nucleotides, including the LCR and other sequences upstream of the L-opsin coding region, the PR0.5 promoter contains 500 nucleotides immediately upstream of the L-opsin coding region, and the 3LCR-PR0.5 promoter contains three copies of 35 bp from the LCR in addition to the 500 nucleotides immediately upstream of the L-opsin coding region. The PR2.1 promoter directed high levels of GFP expression that was limited to L/M cones of normal and diseased dogs. No GFP expression was detected in S cones of dogs, similar to results with the PR2.1 promoter in rats³¹ but different from the findings in transgenic mice, in

Table 1. Grading of GFP expression in primate retinas after subretinal injection of AAV vectors containing various promoters

Animal/eye	Promoter	Section				Mean	Group mean	GFP expression in:	
		1	2	3	4			L/M cones	S cones
I00253/OS	None (control)	NT	0	NT	0	0	0	NA	NA
IM080058/OS		NT	0	NT	0	0		NA	NA
IM083748/OS		NT	0	NT	0	0		NA	NA
I08050/OS	IRBP/GNAT2	0	0	0	0	0	0	No	No
I08051/OD		0	0	0	0	0		No	No
I08053/OD		0	0	0	0	0		No	No
I08054/OS		0	0	0	0	0		No	No
I08053/OS	PR2.1	1	1	0	0	0.5	1.4	Yes	No
I08050/OD		1	2	1	1	1.25		Yes	Yes
I08052/OD		2	2	1	1	1.5		Yes	No
I08049/OS		3	2	2	2	2.25		Yes	Yes
I08049/OD	PR1.7	3	3	3	3	3	3	Yes	Yes
I08051/OS		3	3	3	3	3		Yes	Yes
I08052/OS		3	3	3	3	3		Yes	Yes
I08054/OD		3	3	3	3	3		Yes	Yes

Note: Values represent the intensity of GFP expression in cone photoreceptors.

AAV, adeno-associated virus; GFP, green fluorescent protein; NA, not applicable; NT, not tested; OD, right eye; OS, left eye.

which the PR2.1 promoter directs reporter gene expression in both L/M and S cones.^{15,22} The PR0.5 promoter was ineffective and the 3LCR-PR0.5 promoter directed only weak GFP expression in L/M cones. The PR2.1 promoter was also the most effective when used for AAV-mediated gene replacement therapy in dogs with CNGB3 achromatopsia. A vector containing the PR2.1 promoter achieved stable rescue of cone function in 12 of 17 injected eyes, but vectors containing the PR0.5 or 3LCR-PR0.5 promoter resulted in transient restoration of cone function in 1 of 2 or 2 of 7 injected eyes, respectively.¹⁶

Although the 5.3-kb expression cassette in the rAAV5-PR2.1-*hCNGB3* vector was effective in the dog model of CNGB3 achromatopsia, it was important to evaluate alternative promoters in order to reduce the overall DNA insert size and to find a promoter that directs expression in L, M, and S cones of primates. We constructed a series of shorter promoters with internal deletions in the PR2.1 promoter (Fig. 1), evaluated them as components of AAV-GFP vectors in mice, and determined that all of these promoters directed strong expression in photoreceptors. We then evaluated the PR2.1 and PR1.7 promoters, and an IRBP/GNAT2 promoter, previously shown to direct transgene expression in mouse and dog L/M and S cones, in cynomolgus macaques. In contrast to a previous study in which 0.5-kb versions of the PR2.1 promoter, with or without adding copies of the locus control region, were inefficient in directing GFP expression in dog cones,²⁷ the PR1.7 promoter that contains sequences surrounding the minimal LCR element was highly efficient in di-

recting GFP expression in L, M, and S cones in the primate retina and was more efficient than the PR2.1 promoter (Fig. 2).

In contrast to mice and dogs, which lack a foveal pit and have dichromatic vision mediated by S cones and L/M cones that have a combined red/green opsin,³² nonhuman primates have a retinal anatomy and physiology nearly identical to humans and have separate populations of L, M, and S cones. The IRBP/GNAT2 promoter was reported to direct high-level expression in mouse and dog cones^{20,29} but was ineffective in primate cones (Fig. 4B). This emphasizes the importance of evaluating components of AAV vectors planned for use in humans in a primate species that is phylogenetically and anatomically closer to humans.

Concurrent with the studies evaluating the PR1.7, PR2.1, and IRBP/GNAT2 promoters for their ability to drive GFP expression in primate cones, we evaluated these promoters for their ability to drive expression of nonoptimized or codon-optimized *hCNGB3* cDNA and rescue the abnormal phenotype in CNGB3-deficient mice. Results of these studies, which included only a subset of all possible combinations of three promoters, two cDNAs, and three capsids, demonstrated that each of the three promoters and both nonoptimized and codon-optimized *hCNGB3* cDNAs were effective in this model of achromatopsia. Rescue of cone ERG responses was seen in five of the seven treatment groups (Fig. 5 and Table 2), the exceptions being the groups administered rAAV2tYF-IRBP/GNAT2-*hCNGB3co* (group 5) or rAAV5-PR2.1-*hCNGB3co* (group 2). However, other vectors containing the IRBP/GNAT2 pro-

Figure 5. Representative photopic electroretinography (ERG) tracings 3 months after subretinal injection of AAV vectors in CNGB3-deficient mice. Red tracings are from the treated eye and black tracings are from the untreated eye, except that the wild-type control red tracings are from the right eye and black tracings are from the left eye.

motor and codon-optimized *hCNGB3* cDNA rescued cone ERG responses (groups 4, 6, and 7), and the same batch of rAAV5-PR2.1-*hCNGB3co* vector was able to rescue ERG cone responses in a dog model of CNGB3 achromatopsia (A.M. Komáromy and G.-J. Ye, unpublished data). The explanation for these divergent results is not apparent.

Among groups in which only the cDNA differed (group 1 vs. group 2 and group 4 vs. group 7), the

nonoptimized *hCNGB3* cDNA was associated with greater increases in cone ERG responses than the cDNA that was optimized based on human codon usage. Similarly, among groups in which only the promoter differed (groups 1 and 7 with nonoptimized *hCNGB3* cDNA and groups 2, 3, and 4 with codon-optimized *hCNGB3* cDNA, all packaged in AAV5 capsids), it appears that the IRBP/GNAT2 promoter was more effective than either the PR1.7 or PR2.1

Table 2. Cone electroretinographic responses 3 months after subretinal injection of AAV vectors in *CNGB3*-deficient mice

Group	Vector	Photopic b-wave amplitude (μ V)			P value	
		Uninjected eyes	Vehicle-injected eyes	Vector-injected eye	Vector-injected vs. uninjected	Vector-injected vs. vehicle-injected
1	AAV5-PR2.1-hCNGB3	47.7 (4)	45.4 (6)	84.7 (10)	0.062	0.003
2	AAV5-PR2.1-hCNGB3co	36.6 (5)	47.7 (6)	39.8 (11)	0.381	0.245
3	AAV5-PR1.7-hCNGB3co	53.2 (7)	55.8 (5)	80.3 (10)	0.002	0.028
4	AAV5-IRBP/GNAT2-hCNGB3co	60.9 (5)	65.4 (7)	107.2 (10)	0.007	0.010
5	AAV2tYF-IRBP/GNAT2-hCNGB3co	83.9 (5)	63.7 (5)	83.4 (10)	0.487	0.131
6	AAV9-IRBP/GNAT2-hCNGB3co	47.5 (4)	65.0 (5)	110.8 (9)	0.0002	0.002
7	AAV5-IRBP/GNAT2-hCNGB3	50.8 (7)	61.5 (5)	117.9 (10)	0.00007	0.0002

Note: Values represent ERG responses to a 10-dB stimulus (318 cd/m²). Numbers in parentheses indicate the number of animals per group with data. *P* values are for a one-sided *t* test (*P* values in boldface indicate a significant difference). The mean $\pm$ SD value in age-matched wild-type C57BL/6 mice (*n*=4) is 133.3 $\pm$ 15.1.

promoter in mice. However, results of the promoter evaluation study in primates shown in Figs. 3 and 4 indicate that the IRBP/GNAT2 promoter is ineffective in primate cones.

On the basis of results with GFP expression driven by the IRBP/GNAT2 promoter in mice and dogs that were available before most of these studies began, we designed the mouse efficacy studies to focus on that promoter. However, on the basis of results of the studies in nonhuman primates, the vector that will be used in patients with achromatopsia will contain the PR1.7 promoter and the *CNGB3* cDNA that was optimized on the basis of human codon usage, and will be packaged in AAV2tYF capsids that are able to deliver a cDNA expression cassette to all three types of primate cones after subretinal injection (Fig. 4.) The rAAV2tYF-PR1.7-h*CNGB3co* vector has been tested in toxicology studies in *CNGB3*-deficient mice (G.-J. Ye, E. Budzynski, P. Sonnentag, T.M. Nork, P.E. Miller, L. McPherson, J.N. Ver Hoeve, L.M. Smith, T. Arndt, S. Mandapati, P.M. Robinson, R. Calcedo, D.R. Knop, W.W. Hauswirth and J.D. Chulay, unpublished data) and normal cynomolgus macaques (G.-J. Ye, E. Budzynski, P. Sonnentag,

T.M. Nork, P.E. Miller, A.K. Sharma, J.N. Ver Hoeve, L.M. Smith, T. Arndt, R. Calcedo, S. Gaskin, P.M. Robinson, D.R. Knop, W.W. Hauswirth and J.D. Chulay, unpublished data) and will soon be evaluated in a phase 1/2 clinical trial.

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AUTHOR DISCLOSURE

G.-J.Y. and J.D.C. are employees and shareholders of Applied Genetic Technologies Corporation (AGTC) and have a conflict of interest to the extent that this work potentially increases their financial interests. W.W.H. and the University of Florida have a financial interest in the use of AAV therapies and are shareholders of AGTC. None of the other authors has a competing financial interest.

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