Human Blue-Opsin Promoter Preferentially Targets Reporter Gene Expression to Rat S-Cone Photoreceptors

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Purpose. To develop a gene therapy system that specifically targets transgene expression to S-cones of the mammalian retina, the authors coupled recombinant AAV-mediated delivery with the use of a human blue-opsin (HB) promoter to drive expression.

Methods. Two regions of the HB promoter sequence, HB569 and HB996, were amplified from human DNA, cloned into an AAV vector cassette upstream of the green fluorescent protein (*GFP*) gene, and packaged into AAV2 and AAV5 capsids. Eyes of postnatal day (P) 40 to P48 Sprague–Dawley rats were subretinally injected with 2 μL vector. Animals were humanely killed 2 to 3 weeks or 20 months after injection, and the pattern and persistence of GFP expression were analyzed in the treated retinas by immunohistochemistry, Western blotting, and RT-PCR.

Results. AAV5.HB.GFP vectors targeted photoreceptor transduction with an efficiency 20-fold higher than analogous serotype 2 vector. Both AAV5.HB.GFP vectors exhibited similar transduction efficiencies with patterns of GFP expression that did not vary depending on the size of the HB promoter used. Transgene expression was exclusively localized to photoreceptors of retinas treated with either vector. Furthermore, *GFP* expression was observed for at least 20 months. Dual GFP immunostaining with S- or M-opsin antibodies and GFP/PNA labeling revealed that cones coexpressing S-opsin/GFP or M-opsin/GFP constituted 37.5% ± 8% and 13.5% ± 3% of the GFP-positive photoreceptors, respectively, whereas rods constituted 49% ± 5% of the GFP-positive photoreceptors. Because cones constitute approximately 1% of adult rat retinal photoreceptors, it was estimated that the relative transduction efficiency of AAV5.HB.GFP vectors was approximately 100:1 for cones versus rods.

Conclusions. AAV5.HB.GFP vector injected into the subretinal space of Sprague–Dawley rats targeted gene expression to photoreceptor cells with an efficiency approximately 20-fold higher than that for AAV2.HB.GFP. Transgene expression regulated by the human blue cone-promoter persisted at least for 20 months. Cones coexpressing S-opsin and the *GFP* transgene appeared to prevail, confirming that in addition to having properties of the AAV serotype, the promoter choice is key to fine-tuning transgene delivery and expression in specific retinal cells. The system described here may be effective in a therapeutic setting in which strong S-cone transgene expression is required. (*Invest Ophthalmol Vis Sci.* 2006;47:3505–3513) DOI:10.1167/iovs.05-1670

Viewed as promising and powerful tools for the treatment of retinal degeneration, AAV vectors are increasingly used to test gene-based therapies in various animal models designed to mimic such cases of human disease.[1,2] One of the most critical elements in vector design is selection of the promoter driving therapeutic transgene expression. When combined with the appropriate choice of AAV serotype and site of intraocular injection, the type of promoter may allow for specific expression and regulation of the therapeutic gene in only those retinal cells requiring it. Cellular targets of most retinal degenerative diseases appear to be photoreceptors, whereas neurons of the inner retina are relatively preserved.[3–5] Given the obvious importance of photopic vision in humans, preservation or correction of cone function is one central goal of retinal gene therapy. Although the success in AAV gene therapy for retinal degeneration is obvious,[1,2] little has been done to deliver the therapeutic gene(s) primarily to the specific photoreceptors. To this end, we have used AAV vectors to investigate the function of opsin promoters in the hope of developing an efficient cone-targeting system.

Rod and cone opsins, the visual pigments responsible for light absorption, are photoreceptor cell–type specific. Vertebrates have one rod-specific opsin (rhodopsin) and at least four cone-specific opsins,[6] three of which compose human color vision and two that mediate color vision in rodents; blue or S-cone opsin (short-wavelength), green or M-cone opsin (middle-wavelength), and red or L-cone opsin (long-wavelength) have absorption maxima at approximately 430 nm, 530 nm, and 560 nm, respectively.[7–10]

In most adult mammals, rods outnumber cones by at least 20-fold, even though cone pigments evolved long before rhodopsin.[11–13] For example, the rat retinal mosaic includes S-opsin–expressing cones, M-opsin–expressing cones, and rods uniformly distributed across the retina and present at a ratio of 1:16:1983, respectively.[14] Furthermore, immunochemical and electrophysiologic studies provide evidence that cones in some mammals coexpress two visual pigments, S- and M-opsins, in a common cell.[15–22] The current list of mammals exhibiting such coexpression includes rabbit, guinea pig,[15] mouse,[15–17] Siberian hamster,[19] domestic pig,[20] and human.[18,21] Many exhibit differences in the temporal and spatial patterning of the visual pigment coexpression. Thus, it appears that S- and M-opsin coexpression is a common phenomenon among mammals; in fact, the number of species having such dual expression is rapidly becoming larger.[23]

The rat species used in this study was the first to be characterized for dual opsin expression.[24] Using antibodies developed to the C termini of chicken M-/L- and S-opsins, COS-1 and OS-2, and 108B against human blue cone, it was shown that

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cones expressing S-opsin appeared at postnatal day (P) 5 (before M-opsin–expressing cones) and that their densities increase until P11, at that point outnumbering those found in the adult rats. Subsequently, the fraction of S-opsin–expressing cones was reduced by almost 95%. Between P9 and P20, most cones coexpressed S- and M-opsins. By P30, S- and M-expressing cone types were found relatively evenly distributed across the retina. No evidence of cone visual pigment coexpression was reported,[24] and though controversial in view of later studies in other mammalian species and perhaps related to the source of cone opsin antibodies used, it remains the only complete study in the rat.

The seven cell types of the vertebrate neural retina, including rods, S-cones, and M-cones, are generated from multipotent retinal progenitor cells.[25,26] The differentiation of photoreceptor cells can be viewed as a process of selectively turning on and off a given set of genes that ultimately decide whether a cell will become a rod or a cone and, furthermore, an S-cone or an M-cone.[25,27,28]Multiple transcription factors are involved in the regulation of photoreceptor cell–type differentiation and maintenance. Several of these are also known to play pivotal roles in photoreceptor-specific gene regulation. The cone-rod paired-homeobox protein (CRX) binds to and *trans*-activates cone- and rod-specific genes,[29–33] whereas neural retina leucine zipper protein (NRL) is only expressed in rods to activate rod-specific transcription.[34,35] The latter appears to act as a dual-function molecular switch during photoreceptor differentiation and development because it promotes rod differentiation while simultaneously repressing cone identity.[36,37] Suppression of cone fate is achieved through direct or indirect action of the nuclear receptor NR2E3, which is up-regulated by NRL.[28,38] When acting in concert with CRX, NR2E3 also appears to simultaneously promote the rod and repress the cone program during photoreceptor differentiation and maintenance.[28,39] Deletion either of NRL or of NR2E3 results in the loss of rod function and rod-specific phototransduction proteins and increases the expression of S-opsin and a large spectrum of cone-specific proteins. This causes rods to convert to a conelike morphology.[22,38,40–43] In humans, an NR2E3-deficient environment is associated with an enhanced S-cone syndrome (ESCS), Goldmann-Favre syndrome or clumped pigmentary retinal degeneration,[44–47] that resembles the rd7 mouse phenotype.[38,40,48] Patients with NR2R3 mutations have an increased number of S-cones, enhanced S-cone function, and diminished M- and L-cone function and rod function. The specific differentiation and maintenance of M-cones depends on the level of β2 thyroid receptor (TRβ2), a member of the ligand-dependent nuclear hormone receptor family.[27,49] Cones of TRβ2 null mice were found to express only S-opsin, indicating that TRβ2 is critical not only for the expression of M-opsin but also for the repression of S-cone identity.[49] Indeed, the suppression of S-cone identity appears to be achieved through the action of a heterodimer complex of TRβ2 with retinoid X receptor γ (RXRγ), a fellow family member.[27] To summarize, S-cone identity is apparently suppressed in rods by the NR2E3-mediated repression system and in M-cones by the TRβ2-RXRγ system.

The mammalian genes encoding L- and M-opsins have 96% nucleotide identity and reside in a tandem array on the X-chromosome at q28 in humans.[9] The autosomal S-opsin gene has been mapped to the chromosome 7q31.3-32[8] and shows only 43% identity with L- and M-opsins.[7] Nucleotide sequence alignments of the 5′ upstream regions of the human, bovine, and rodent S-opsin genes reveal the region of highest conservation within 400 bp of the translation start site.[50] A functional homology of human and rodent S-cone promoters has been shown in transgenic mouse models.[50,51]

Given the high identity of the rat and human promoters and in conjunction with current knowledge surrounding mammalian photoreceptor differentiation and maintenance, we hypothesized that the human blue-opsin (HB) promoter is likely to be preferentially active in the S-cones of rats. To test this hypothesis, two regions encompassing proximal promoter domains of the human S-opsin (*HB*) gene driving the *GFP* reporter gene were inserted into serotype 2 or serotype 5 rAAV viral particles and were tested in rats for their specificity of expression.

Methods

Animals

Male Sprague–Dawley rats were obtained from the Charles River Laboratories and were housed in the University of Florida Health Science Center Animal Care Services Facilities under a 12-hour light/12-hour dark cycle. All animals were treated in compliance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Florida and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Genomic DNA Isolation and PCR of HB Promoter

To amplify the HB promoter sequence, total genomic DNA was isolated from human hair follicles (Perfect gDNA Mini Sample Kit; Eppendorf Scientific Inc., Westbury, NY), according to the manufacturer's protocol. Two sets of PCR primers were used. One pair was designed to amplify the sequence encompassing nucleotides −557 to +11 (HB569 forward, 5′-CTAGGCATTGTCAAGTTGCCTAA-3′; HB569 reverse, 5′-ATTTTTCTCATGGATGCCCCACA-3′), and the other was designed to amplify the region bounded by nucleotides −992 to +3 (HB996 forward, 5′−TCTCCTGACCTTGTGATCCA−3′; HB996 reverse, 5′−CATGGATGCCCCACACCCCCTCTGAGT−3′). PCR was performed (Expand High Fidelity PCR System; Roche Diagnostics Corp., Indianapolis, IN) under the following cycling conditions: initial denaturation at 94°C for 3 minutes, then 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The integrity of each PCR product was verified by agarose gel electrophoresis with ethidium bromide staining. PCR products were then subcloned into the pCR2.1-TOPO cloning vector (Invitrogen Corp., San Jose, CA) and enzymatically sequenced with M13 primers on an automated DNA sequencer (PE/ABd 373; Perkin Elmer, Applied Biosystems Division, Foster City, CA). Alignment of the deduced nucleotide sequences with the known HB sequence from the Entrez Nucleotide Database at NCBI (accession number L27830) revealed a G-to-A substitution at position –164. This difference was discovered in four independently obtained PCR products from each sample and is likely to be a natural polymorphism.

Construction of rAAV.HB.GFP Vectors

Three rAAV constructs were made, each containing an HB promoter sequence upstream of a *GFP* reporter gene. Briefly, the 569-bp and 996-bp PCR products containing HB promoter regions were directionally cloned into the *Kpn*I and *Xba*I sites of the proviral plasmid pTR-UF5.[52] Recombinant AAV vectors were packaged into serotype 2 and 5 viruses by transient cotransfection of human embryonic kidney 293 cells with helper plasmids that included either the AAV2 REP and capsid genes or the AAV2 REP and AAV5 capsid genes.[53,54] The resultant rAAV2.HB569.GFP, rAAV5.HB569.GFP, and rAAV5.HB996.GFP viruses were purified as previously described[54,55] and were titered to be 4.2×10^{13}, 4.8×10^{13}, and 3.2×10^{12} vector genomes per milliliter, respectively. Two microliters viral suspension was injected into the subretinal space of male Sprague–Dawley rats at P40 to P48.[56]

Subretinal Injections

Rats were anesthetized by intraperitoneal injection with a mixture of 12.5 mg/kg xylazine (Butler Company, Columbus, OH) and 62.5 mg/kg

ketamine (Phoenix Pharmaceutical, St. Joseph, MO). Pupils were dilated with 2.5% phenylephrine (Akorn, Inc., Decatur, IL), and 0.5% proparacaine (Alcon Laboratories Inc., Fort Worth, TX) was applied for topical anesthesia. The vibrissae were trimmed to get an unobstructed view of the eye and the fundus. A second application of mydriatic and topical anesthetic drops was used as the anesthetics began to take effect. After complete dilation, the anesthetized animal was placed in lateral recumbency under a dissecting microscope (SMZ-1; Nikon, Tokyo, Japan). The rat fundus could be visualized with the application of a drop of 2.5% methylcellulose to the eye. The cornea was carefully punctured nasally with a 28-gauge hypodermic needle (Becton Dickinson, Franklin Lakes, NJ). The needle, with the bevel up, was advanced full thickness through the cornea into the anterior chamber, parallel to the anterior lens face. At least 50% of the bevel was pushed through the cornea to produce a sufficiently large hole to insert a 33-gauge blunt needle (Hamilton Company, Reno, NV). The blunt needle tip was inserted through the corneal puncture and advanced into the anterior chamber, avoiding trauma to the iris and lens. The lens was displaced medially as the needle was advanced toward the desired injection location. Slight resistance to the movement of the needle indicated penetration of the retina and entrance into the subretinal matrix. The syringe was held in place, and the plunger with the contents of the syringe was ejected slowly into the subretinal matrix and created a visible retinal detachment. After subretinal delivery, the needle was gently withdrawn. A small amount of fluorescein (final concentration, 0.1 mg/mL) was administered with the vector to visualize the injection and the bleb formation.

Histologic Analysis and Microscopy

All treated animals were humanely killed at P60 to P72 by CO_2 inhalation and perfused intracardially, initially with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde (PF). The superior point of each cornea was marked for orientation before the eyes were enucleated. They were then fixed for 1 to 2 hours in 4% PF and rinsed three times with PBS. Fixed eyes were cryoprotected by incubation overnight in 30% sucrose before the lens and vitreous were removed. Eyecups were then embedded in Optimal Cutting Temperature compound medium (OCT; Sakura Finetek USA, Inc., San Diego, CA) and frozen in a bath of isopentane (2-methylbutane)-ethanol. Serial vertical sections, each 10-μm thick, were prepared from entire eyecup with the use of a cryostat (HM505E; Microton, Walldorf, Germany) and then thaw mounted onto gelatin-coated slides. The slides were air dried and stored at −20°C until they were processed for immunohistochemistry. For wholemount immunohistochemistry, eyes were removed as described, but the retinas were carefully detached from the posterior eyecups, fixed in 4% PF for 24 hours at 4°C, rinsed in PBS, and stored for up to 12 hours at 4°C before processing.

To identify cone photoreceptors in retinal sections, fluorescein-labeled peanut agglutinin (PNA-F) or biotinylated-PNA (PNA-b; Vector Laboratories, Burlingame, CA) was used at final dilutions of 1:200. Fluorescein-streptavidin (Vector Laboratories) was diluted 1:200 and used to visualize PNA-b reactivity. Rabbit anti–GFP primary antibodies were kindly provided by Dr. Paul A. Hargrave (University of Florida) and were diluted to working concentrations of 1:1000 in blocking buffer (1 × PBS, 5% bovine serum albumin [BSA], 0.5% Triton X-100). GFP expression was visualized with a rabbit primary GFP antibodies and a goat anti–rabbit Cy3 conjugate (Sigma, St. Louis, MO) secondary antibodies, diluted 1:200 in blocking buffer. Primary rabbit polyclonal antibodies generated against the carboxyl terminus of the human blue cone pigment (JH455) and analogous antibodies against human red and green pigments (JH492)[57] were gifts of J. Nathans (The Johns Hopkins University). These polyclonal antibodies were used at a final dilution of 1:1000 to visualize the S- and M-cone segments in rat retinas. Briefly, retinal sections were preincubated for up to 1 hour in blocking buffer, and, for detection all cones expressing GFP, were incubated for 2 to 4 hours at room temperature or overnight at 4°C with a mixture of GFP antibodies and PNA-b. After three 10-minute washes in PBS, sections were incubated for 1 hour at room temperature with their secondary antibodies, a goat anti–rabbit Cy3 conjugate and fluorescein-streptavidin.

To identify GFP and cone-specific opsin coexpression in photoreceptors, sections were initially incubated with GFP primary antibodies for 2 hours at room temperature followed by three 10-minute washes in PBS and then were incubated for 1 hour at room temperature with secondary antibodies, a goat anti–rabbit Cy3 conjugate, followed by the same washing procedure. Sections were then processed with opsin-specific antibodies (JH455 or JH492) for 2 hours at room temperature, washed three times for 10 minutes each with PBS, and incubated with secondary antibodies, a goat anti–rabbit FITC conjugate (Sigma-Aldrich Co., St. Louis, MO), for 1 hour at room temperature. For detection in retinal wholemounts, incubation in the mixture of the GFP primary antibodies and PNA-b was increased to 24 hours. After three 10-minute washes in PBS, wholemounts were incubated for 1 hour at room temperature with their secondary antibodies, anti-rabbit Cy3 conjugate and fluorescein-streptavidin. Finally, sections and wholemounts were rinsed three more times in PBS and mounted under coverslips in a mounting medium for fluorescence (Vectashield; Vector Laboratories).

Immunostained retinal sections were examined (Axioplan2 Research Microscope; (Carl Zeiss Inc., Jena, Germany), and images were analyzed (PhotoShop version 7; Adobe, San Jose, CA). To evaluate reporter gene expression specificity, the total number of GFP-positive cones (identified by GFP immunostaining and PNA-labeling), S-cones (identified by costaining with GFP and JH455 antibodies), and M-cones (identified by costaining with GFP and JH492 antibodies) were counted in at least four treated retinas using seven 10-μm frozen sections for each eye. All sections were cut as near to perpendicular to the horizontal retinal axis as possible, as judged by observing only a single layer of pigmented epithelial cells.

Reverse Transcription–Polymerase Chain Reaction

The presence of the reporter gene in treated retinas was confirmed by PCR, and the persistence of *GFP* mRNA expression was investigated by RT-PCR using *GFP*-specific primers. Briefly, each retina was homogenized in lysis buffer (Roche Diagnostics Corp., Indianapolis, IN), and the lysate was divided into two equal volumes for RNA and DNA purification. Genomic DNA and total RNA were isolated (DNA Isolation Kit for Blood/Marrow/Tissue; High Pure RNA Tissue Kit; Roche Diagnostics Corp.) according to the manufacturer's protocol. First-strand cDNA was generated from RNA (SuperscriptII reverse transcriptase; Gibco BRL, Rockville, MD). Genomic DNA or cDNA was then PCR amplified using *Taq* polymerase (Roche Diagnostics Corp.). The absence of DNA contamination in each RNA preparation was confirmed by PCR with *GAPDH* or β-actin primers. *GFP*-specific primers (GFP sense, 5′-GGCGTGGTCCCAATTCTCGTGGAAC3-′; GFP antisense, 5′-GCGGTCACAAACTCCAGCAGGACCT-3′) were used to generate a 651-bp PCR product under the following cycling conditions: initial denaturation at 94°C for 3 minutes, then 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. *GAPDH* primers were also included in each reaction to serve as an internal control.

Western Blots

GFP protein expression was analyzed in injected retinas by Western blotting. Retinal tissue was homogenized for 10 to 30 seconds in RIPA buffer (150 mM NaCl, 10 mM Tris [pH 7.2], 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA), containing 0.1 mg/mL proteinase inhibitor cocktail (Sigma-Aldrich Corp.). Protein concentrations in samples were measured (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). Samples were normalized by protein concentration and were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories). The PVDF membrane was blocked with 5% BSA in TTBS (30 mM Tris, 150 mM NaCl, 0.5% [vol/vol] Tween-20, pH 7.4)

for 1 hour with gentle agitation at room temperature, then probed overnight at 4°C with GFP and β-actin primary antibodies diluted in 1% BSA/TTBS. After three 10-minute washes in TTBS, the membrane was incubated for 1 hour with horseradish peroxidase–conjugated secondary antibodies (Zymed Laboratories, San Francisco, CA). After three 5-minute washes in TTBS, enhanced chemiluminescence substrate (Pierce, Rockford, IL) was applied to the membrane for 1 minute, and the proteins were visualized by exposure to x-ray film (Kodak, Rochester, NY).

Results

Pattern of Transgene Expression after Subretinal rAVV.HB.GFP Injection

To determine whether the HB promoter could drive cone-specific reporter gene expression, rAAV2.HB.569.GFP, rAAV5.HB.569.GFP, and rAAV5.HB.996.GFP vectors were subretinally injected into the right eyes of rats ($n = 10$ each), and left (control) eyes were injected with PBS. Between 2 to 3 weeks after injection, the eyes were enucleated and the distribution of GFP expression was evaluated in the retina. Western blot hybridization using antibodies specific for GFP confirmed the presence of the reporter protein in retinal extracts prepared from treated eyes (Fig. 1). Retinal wholemounts prepared from five rAAV5.HB.569.GFP–treated eyes were immunohistochemically examined with anti–GFP antibodies. GFP-positive cells were located within all retinal quadrants, though each varied in the distribution and density of transduction. As expected, the central region encompassing the subretinal injection site invariably contained the most GFP-positive cells, with a decreasing gradient of transduced cells extending outward from this site (Fig. 2). High densities of GFP-expressing cells were also found in the superior hemisphere for two of the retinas examined (one in the superior-nasal quadrant and one in the superior-temporal quadrant), the temporal periphery for another retina, and the inferior hemisphere for the remaining two retinas (inferior-periphery and nasal-inferior quadrant). Although we attempted to deliver subretinally to the central retina, this was not always possible because of imperfect surgical control of the injection needle in the small rat eye.

Immunohistochemical analysis of frozen eyecup sections with anti-GFP antibodies demonstrated transgene expression to be exclusively localized to cells of the outer retina (Figures 3–5). Specifically, GFP expression was observed in photoreceptor nuclei, inner segments, and synaptic regions. Given that PNA has a high affinity for the cone sheaths but not for rods,[58] dual GFP/PNA-labeling was performed to determine the proportion of cone photoreceptors transduced by the rAVV5.HB.GFP viruses. Figure 3 shows that approximately half the GFP-expressing cells were cones. The notion that the remaining GFP-positive photoreceptors were rods is partially based on the fact that they differ morphologically from cones[59] in ways that can be seen at the light microscopic level: cones have wider inner segments than rods, their cell bodies are closer to the outer limiting membrane, and their synaptic termini are larger than the spherule-synaptic terminals of rods. Additionally, double staining of sections with anti–GFP and either JH455 or JH492 antibodies further confirmed transgene coexpression with S- or M-opsins (Figs. 4 and 5, respectively), with S-opsin and GFP coexpressing cones appearing to prevail.

Enumeration of Photoreceptor Subtypes Expressing the Transgene

The efficiency of rAAV2.HB.GFP and rAAV5.HB.GFP transduction in the rat retina was evaluated by counting the number of GFP-positive cells in sections transecting the site of injection.

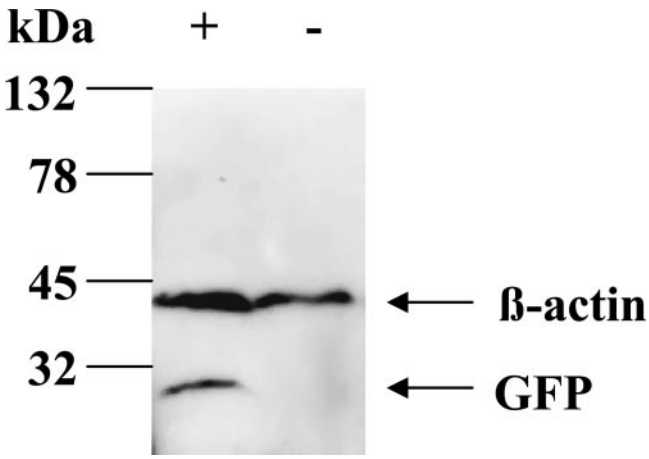

Figure 1. GFP reporter protein is present in the AAV5.HB569.GFP-treated retina (+) and absent in the PBS treated retina (−). *Arrows* indicate the positions of β-actin (loading control) and GFP proteins.

For each vector treatment, five eyes were isolated 2 to 3 weeks after injection, and 7 to 10 sections were analyzed from each retina. In eyes injected with rAAV5.HB569.GFP, 125 ± 25 GFP-positive cells were counted per section, and 26.5 ± 9 cells per section were counted in eyes treated with rAAV5.HB996.GFP. The lower number of GFP-expressing cells observed in the latter correlated with the lower titer of rAAV5.HB996.GFP vector. Furthermore, cells appeared to be transduced by each vector in a similar spatial fashion (data not shown), suggesting the pattern of GFP expression was independent of the HB promoter region from −558 to −992. These levels of photoreceptor transduction exceeded by approximately a factor of 20 the levels obtained with an analogous serotype 2 vector, rAAV2.HB569.GFP (Fig. 6).

To quantify the populations of cones coexpressing S-opsin/GFP or M-opsin/GFP, two adjacent sections from the central retina were coimmunostained with GFP/JH455 and GFP/JH492 antibodies, respectively. Seven to 10 pairs of adjacent sections from each of four retinas were examined, and 500 to 600 GFP-positive cells were counted. Cone photoreceptors that coexpressed S-opsin/GFP constituted 37.5% ± 8% of these transduced cells, whereas 13.5% ± 3% were M-opsin/GFP-double labeling cones. Hence, approximately half the GFP-positive cells appeared to be cones. To determine whether the remaining cells expressing the transgene were rod photoreceptors, five sections from each of four retinas were immunostained with anti–GFP antibodies and were PNA labeled. PNA-negative rods constituted 49% ± 5% of all GFP-positive cells. This finding supports the idea that rAAV5.HB.GFP transduces only photoreceptors in the rat retina, with 50% of them cones and 50% rods.

Persistence of Transgene Expression in the Retina

To determine the persistence of *GFP* reporter transcription in rAAV5.HB.GFP–treated retinas, total RNA was purified from eyes at 4 months and 20 months after injection and was analyzed by RT-PCR using *GFP*-specific primers. Genomic DNA was also isolated from the 20-month-old retinas to confirm the presence of the transgene by PCR. Figure 7 shows that the 651-bp *GFP* product was amplified in all reactions using treated retinal RNA and DNA templates but not in reactions using the uninjected eye samples. This indicates that under the control of the HB promoter, rAAV-mediated transgene expression is detectable for at least 20 months in the rat retina.

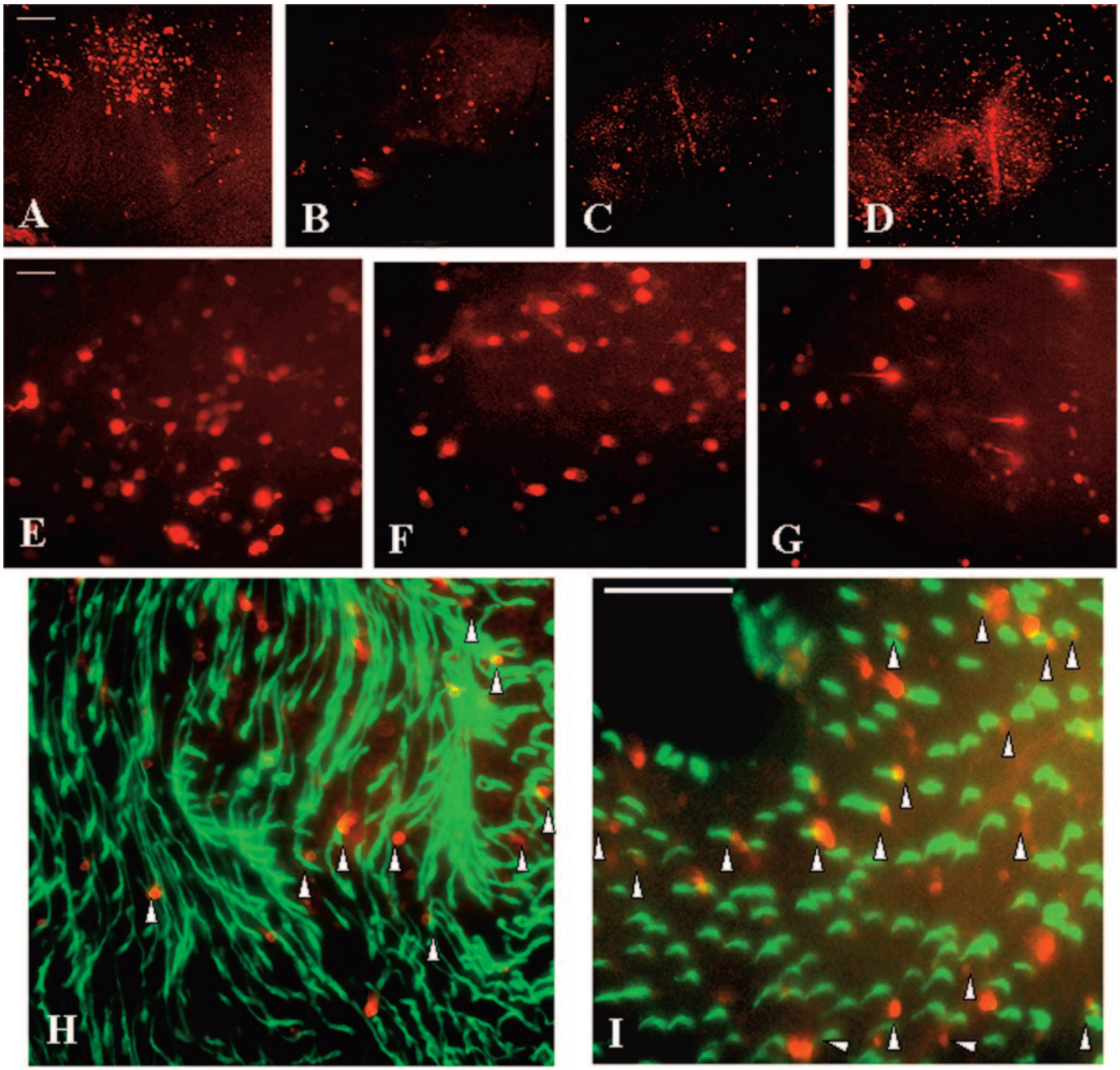

FIGURE 2. GFP-positive photoreceptors are located within all retinal quadrants. Fluorescence micrographs depict wholemounts from the AAV5.HB569.GFP-treated retina. Retinal fields: (**A**) superior, (**B**) inferior, (**C**) nasal, (**D**) central (images were taken with a 5× objective; scale bar, 100 μm). Higher magnifications are shown in (**E**) central, (**F**) superior, (**G**) inferior retina (images were taken with a 20× objective; scale bar, 20 μm). Increased magnifications are shown in (**H**) central and (**I**) superior retina (images were taken with a 40× objective; scale bar, 50 μm). Images show the colocalization of all cone sheaths (PNA, *green*) and GFP-transgene expression (*red*).

DISCUSSION

In the present study, we have shown that a proximal portion of the HB promoter is capable of driving transgene expression in rat photoreceptor cells for at least 20 months when delivered by rAAV5. The use of the type 5 capsid increased photoreceptor transduction efficiency by approximately 20-fold compared with results seen using rAAV2 harboring the same HB promoters. Furthermore, the pattern of transgene expression did not vary depending on the size of the HB promoter used, demonstrating that the 569-bp sequence is sufficient to preferentially direct transgene expression to S-cones.

Using OS2 and COS1 antibodies to visualize pigments, Szel et al.[14] showed that in adult rats both cone subtypes are uniformly distributed across the retina with no evidence of the visual pigment coexpression. In the current research, we used a different set of antibodies (S-opsin–specific JH455 and M/L-opsin specific JH492, polyclonal, developed against the C termini of human pigments) that might have sensitivities different from those of the antibodies used by Szel et al.[14] To test the possibility of dual-visual pigment coexpression using our cone opsin antibodies, we probed the adult rat retina (wholemounts and cross-sections) with a pair of the JH455/JH492 antibodies but failed to find evidence of S-/M-opsin coexpression in a single cone photoreceptor cell (unpublished observation, 2003). Thus, at least with the reagents we used, dual expression of cone opsins was not detected. This justifies attempting

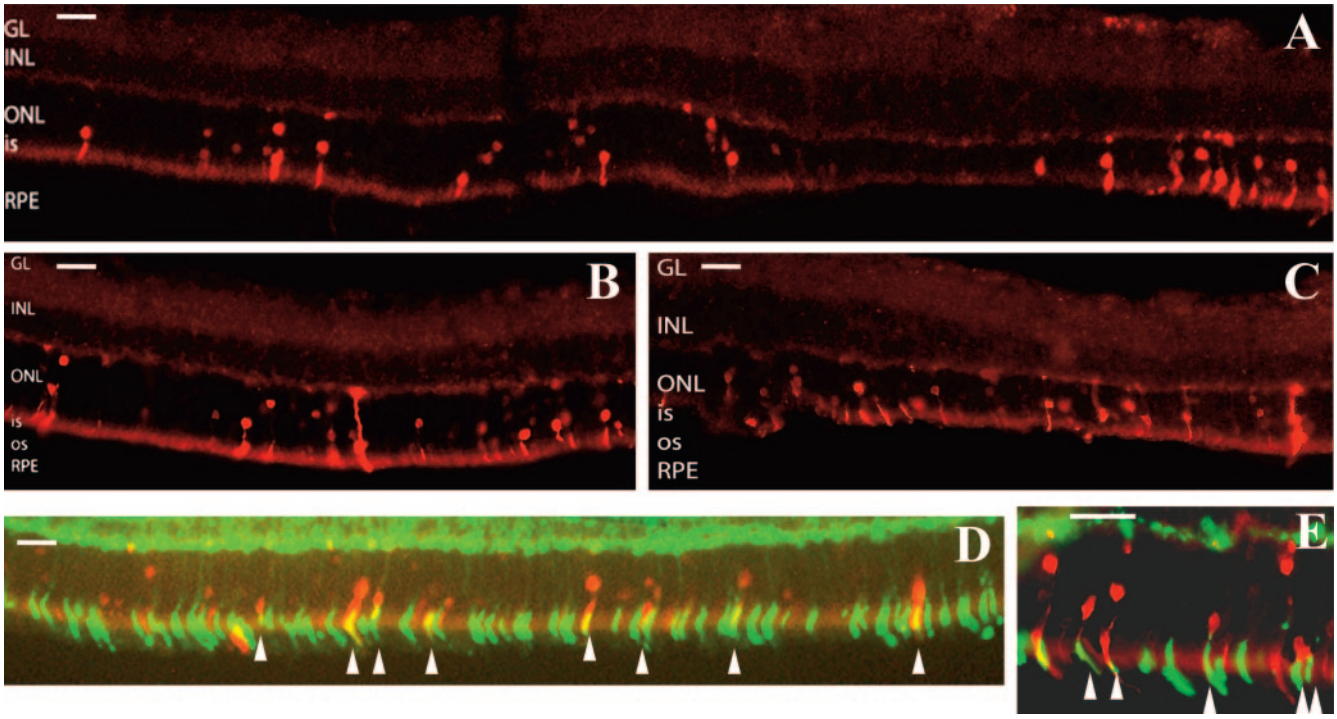

FIGURE 3. Subretinal administration of AAV5.HB569.GFP results in exclusive photoreceptors (**A**–**C**), with most cones expressing the GFP transgene (**D**, **E**). Fluorescence micrographs of cross-sections present fields from the central (**A**), inferior (**B**), and superior (**C**) retina. Correlation of GFP reporter expression (*red*) and cone sheaths (PNA, *green*) is seen in AAV5.HB569.GFP-treated rat retina (**D**, **E** [higher magnification]). *Arrowheads* indicate GFP-positive cones. Scale bar, 10 μm.

to estimate the proportion of transduced cones that express either the S-opsin or the M-opsin.

The fraction of cones in AAV-treated retinas that expressed GFP and reacted with JH455 antibodies against S-opsin or GFP and reacted with JH492 antibodies against M-/L-opsin allowed an estimate to be made of the subtype specificity of rAAV5.HB569.GFP. Vector-transduced rat photoreceptors exhibited 37.5% ± 8% of S-opsin/GFP–expressing cones compared with 13.5% ± 3% of M-opsin/GFP-expressing cones and 49% ± 5% for rods, a ratio 1:0.36:1.32. Correcting according to the relative frequency of each photoreceptor type, 1:16:1983,[11,14] the relative transduction efficiency of rAAV5.HB569.GFP was 1500:34:1 for S-opsin–expressing cones, M-opsin–expressing cones, and M-opsin–expressing rods, respectively. This assumes equivalent entry of vector into S- and M-cones

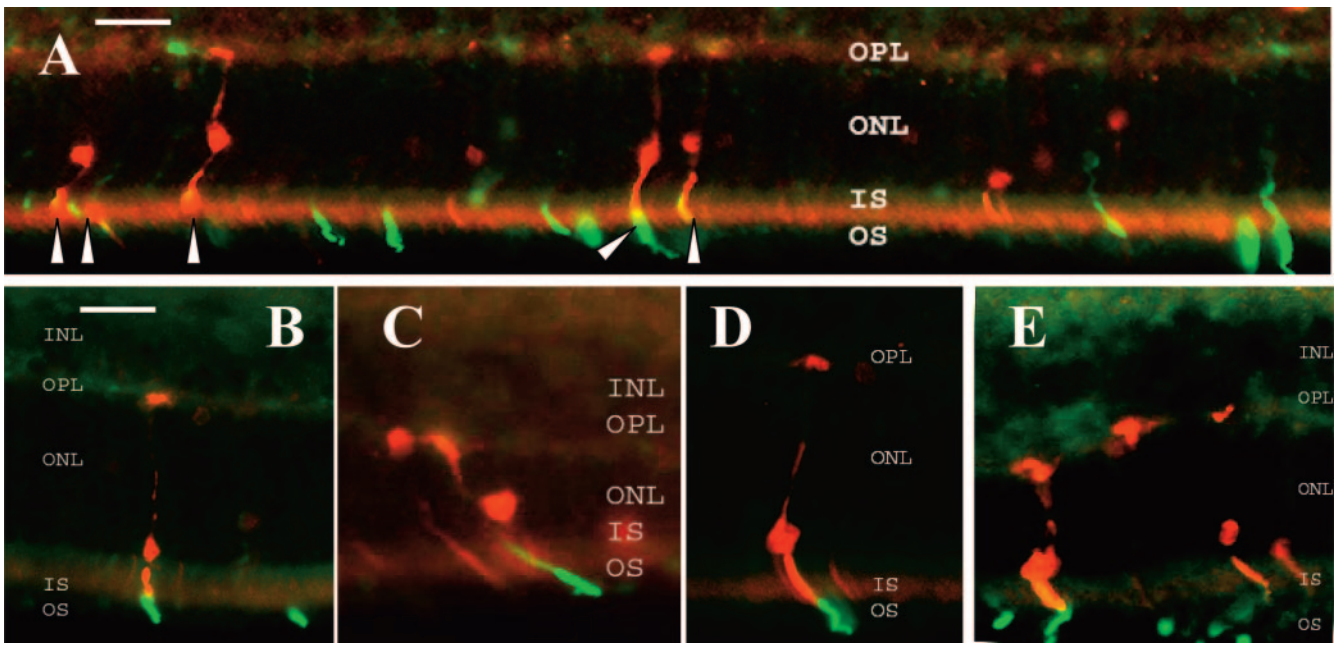

FIGURE 4. Coexpression of S-opsin (*green*) and GFP reporter protein (*red*). (**A**) Central, (**B**, **C**) inferior, and (**D**, **E**) superior retinal fields. *Arrows* indicate cones with colocalization of S-opsin and GFP. Scale bars, 10 μm.

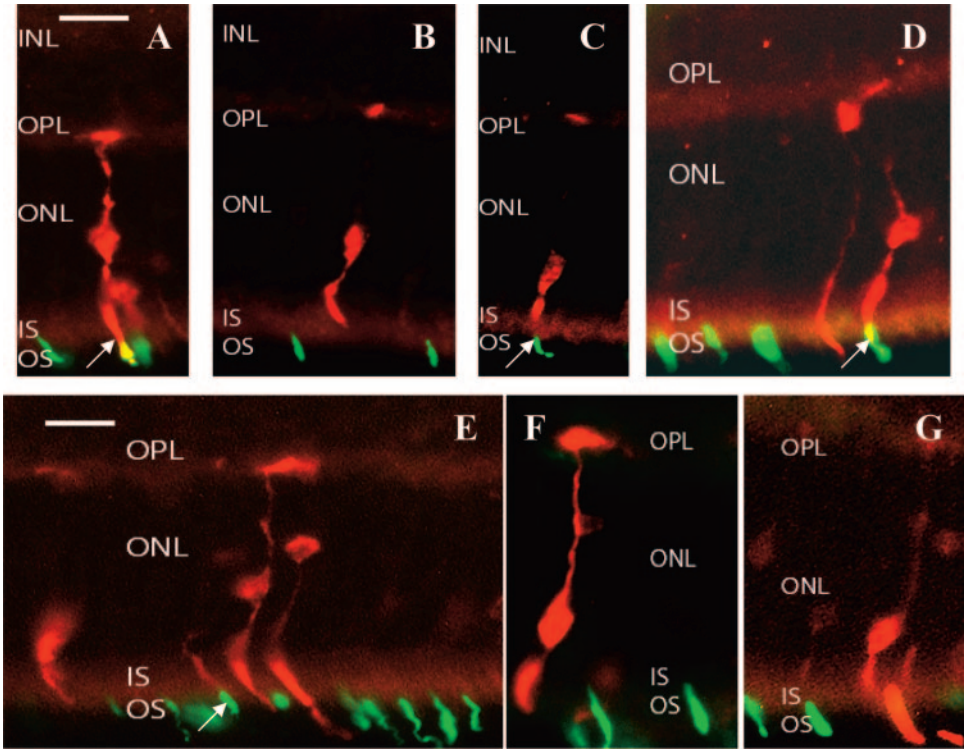

FIGURE 5. Coexpression of M-opsin (*green*) and GFP reporter protein (*red*). (**A**–**D**) Fields from the inferior retina. (**E**–**G**) Fields from the superior retina. *Arrows* indicate cones with colocalization of M-opsin and GFP. Scale bar: (**A**, **E**) 10 μm.

and rods by AAV serotype 5 vectors. Hence, the efficiency of S-opsin–expressing cone transduction was approximately 44-fold and 1500-fold higher than that of M-opsin–expressing cones and rods, respectively. This conclusion remains somewhat speculative because of the controversy over whether rat cones express some level of both cone opsins and different sources of antibodies are used for establishing rat retinal mosaic. However, a clear preference remains for targeting S-opsin–positive cones, leading to the conclusion that the human blue opsin promoter drives expression correctly in the analogous rat photoreceptor subtype. Additionally, because the rat retinal mosaic is rod-dominated and has only approximately 1% cones, the efficiency of expression of the vector passenger gene in rods is small, further supporting the cone specificity of this human cone promoter. These ideas are being tested further in the central macula of nonhuman primates, where cones significantly outnumber rods. One question arising from these results is why rods express GFP from the HB promoter at all. An answer may come from the dual-function roles of NRL and NR2E3, the transcription factors regulating photoreceptor differentiation and maintenance. As noted, NR2E3 is up-regulated in rods by NRL, and together they act to promote the rod cell differentiation program while simultaneously repressing S-cone functions.[37,39,41] In normal rods, NR2E3 levels are sufficient to inhibit transcription from the genomic copies of the S-cone promoter. However, when higher copy levels of an extrachromosomal HB promoter are delivered by an rAAV vector (the estimated initial ratio of vector copies to photoreceptors is approximately 10^5 to 10^6 but decreases to 5 to 10 by 30 days after injection; Liu J, Hauswirth WW, unpublished observation, 2004), it is conceivable that the extra *cis* elements in the HB promoter may sequester all free NR2E3 "repressor" molecules. This would allow *GFP* transcription to be initiated from excess, vector-derived copies of the HB promoter. Interestingly, this scenario still must occur infrequently because S-opsin–positive cone transduction was shown to prevail, presumably because NRL and NR2E3 levels are usually sufficient to suppress even vector-derived HB promoters, particularly several months after injection.

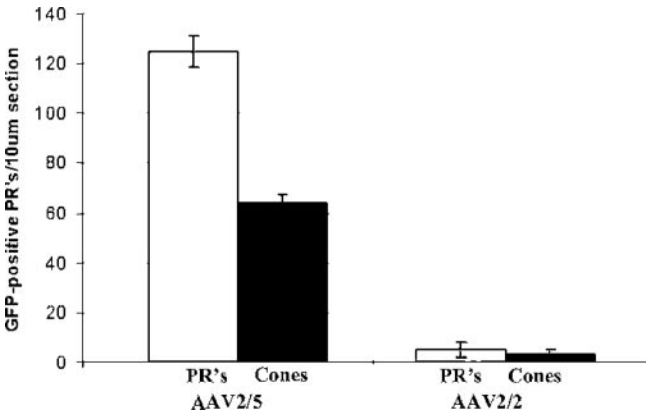

FIGURE 6. Histograms of GFP-positive photoreceptors (*white bars*) and GFP-positive cones (*black bars*) in rat retinas treated with rAAV5.HB569.GFP vector (*left*) and rAAV2.HB569.GFP vector (*right*); 500 to 600 GFP-expressing photoreceptors were counted in each retina.

A related question is why M-opsin–positive cones support expression from the HB promoter. Cones coexpressing M-opsin/GFP appeared to represent a smaller population than seen for cones coexpressing S-opsin/*GFP* transgene. It has been hypothesized that the TRβ2-RXRγ complex in M-cones may act to suppress transcription from genomic copies of the endogenous S-cone promoter. Similar to the insufficiency of the NRL/NR2E3 repression system acting in rods when high copy numbers of AAV-vectored HB promoter *cis* elements are present, levels of the TRβ2-RXRγ complex in M-cones may also be inadequate to suppress *GFP* transcription from the exogenous S-cone promoter.

In conclusion, results presented here demonstrate that a 569-bp proximal region of the HB promoter leads to efficient S-opsin–expressing, cone-preferential transgene expression after rAAV5-mediated delivery. This supports the general concept that in addition to the serotype of the AAV capsid, the

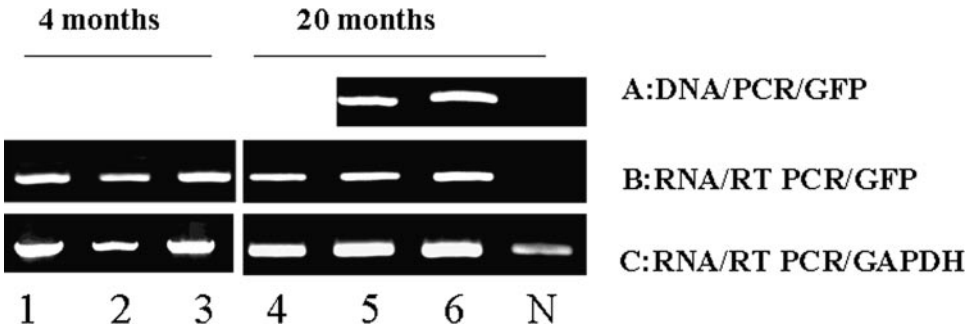

FIGURE 7. Persistence of *GFP*-reporter gene DNA and *GFP* RNA expression in AAV5.BH569.GFP-treated retinas. (**A**) Result of PCR analysis for *GFP* DNA. (**B**) Result of RT-PCR for *GFP* RNA. (**C**) Result of PCR analysis for the *GAPDH* transcript, an internal control to confirm the presence of intact mRNA. Samples 1 to 6 were from injected eyes (1–3, 4 months after injection; 4–6, 20 months after injection), and sample *n* was from an uninjected eye.

promoter is important in fine-tuning transgene delivery and expression in specific cells within the retina. The system described here may have potential in a clinical setting in which strong expression of a therapeutic transgene in S-cones is desired.

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