Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9920–9925, August 1999
Neurobiology

Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina

JEAN BENNETT*†‡, ALBERT M. MAGUIRE*, ARTUR V. CIDECIYAN*, MICHAEL SCHNELL†§, ERNEST GLOVER†, VIBHA ANAND*, TOMAS S. ALEMAN*, NARENDRA CHIRMULE†§, ABHA R. GUPTA*, YIJUN HUANG*, GUANG-PING GAO†, WILLIAM C. NYBERG*, JOHN TAZELAAR†, JOSEPH HUGHES†, JAMES M. WILSON†§, AND SAMUEL G. JACOBSON*

*Department of Ophthalmology, F. M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania, 51 North 39th Street, Philadelphia, PA 19104; and †Institute for Human Gene Therapy and §Department of Cellular and Molecular Engineering, University of Pennsylvania, 204 Wistar, Philadelphia, PA 19104

Edited by Jeremy Nathans, Johns Hopkins University School of Medicine, Baltimore, MD, and approved June 22, 1999 (received for review May 10, 1999)

ABSTRACT **Recombinant adeno-associated virus (rAAV) is a promising vector for therapy of retinal degenerative diseases. We evaluated the efficiency, cellular specificity, and safety of retinal cell transduction in nonhuman primates after subretinal delivery of an rAAV carrying a cDNA encoding green fluorescent protein (EGFP), rAAV.CMV.*EGFP*. The treatment results in efficient and stable *EGFP* expression lasting >1 year. Transgene expression in the neural retina is limited exclusively to rod photoreceptors. There is neither electroretinographic nor histologic evidence of photoreceptor toxicity. Despite significant serum antibody responses to the vector, subretinal readministration results in additional transduction events. The findings further characterize the retinal cell tropism of rAAV. They also support the development of studies aimed ultimately at treating inherited retinal degeneration by using rAAV-mediated gene therapy.**

Retinal degenerative diseases are the most common human inherited eye disorders causing blindness. This broad group of diseases includes age-related macular degeneration, affecting 1 in every 10 people over the age of 60, retinitis pigmentosa, which affects ≈1 in 3,000 people in all ethnic groups (1–4), and conditions that are more rare but that cause blindness in infancy or childhood (such as Leber congenital amaurosis and Stargardt disease (5, 6). Retinal degenerative diseases are costly in terms of lost work productivity, need for social support, and individual suffering. There is no treatment available for the vast majority of patients with retinal degeneration.

Progress in understanding the pathogenesis of retinal degenerative diseases has been aided by the discovery of naturally occurring animal strains with retinal degeneration and creation of genetically engineered animal models of the human diseases. Gene therapy approaches have been used successfully to treat retinitis pigmentosa-like disease in a number of these animals (7–13).

As in all gene therapy studies, a critical factor appears to be the vector. Different vectors vary in their ability to target specific cell types efficiently, their ability to deliver genes in a stable fashion, their toxicity, and their elicitation of immune response. One of the most promising vectors for gene therapy aimed at retinal degenerative disease is recombinant adeno-associated virus (rAAV). Although there is a significant time delay between exposure to this virus and onset of transgene expression, rAAV transduces photoreceptors and retinal pigment epithelium (rpe) cells efficiently and in a stable fashion (14–16).

One drawback of the available animal models for inherited retinal degenerations is that their ocular and retinal anatomy differ substantially from those of the human. The nonhuman primate (monkey, for example), however, possesses ocular anatomic features virtually identical to those of the human. The monkey eye is of similar size as a human eye, its components are of similar proportion, and it possesses a macula. There are two main reasons why it is important to evaluate promising gene transfer techniques in the eye of a monkey: (*i*) It is essential to demonstrate that neither the treatment nor the vector result in toxicity to this human-like retina; and (*ii*) it is important to demonstrate that the vectors under consideration for human gene therapy clinical trials deliver transgenes efficiently and in a stable fashion to human-like retina.

This report describes the ability to deliver foreign genes specifically to the retina of a primate. The procedure produces no long-term toxicity and results in transgene expression in up to 100% of the retinal rod photoreceptors at the site of administration lasting >1 year. Subretinal injection of rAAV can be repeated in the same animal to obtain additional transduction events. The results indicate that rAAV is an ideal vector for delivery of genes to rod photoreceptors and for development of gene therapy approaches for treatment of human retinal disease.

METHODS

Preparation of Virus for Injection. pAAV.CMV.*EGFP* contains the "enhanced" version of the green fluorescent protein (EGFP)-encoding cDNA (CLONTECH) driven by the immediate-early cytomegalovirus (CMV) enhancer-promoter and contains a simian virus 40 splice site donor-acceptor and polyadenylation signal. High titer virus free of replication-competent AAV was produced by using a rep-cap expressing cell line and an adenovirus (Ad)-AAV hybrid virus as described (17, 18). In brief, B-50 cells, cells that contain the p5 promoter driving expression of a rep-cap gene, were infected with Sub100r, an E2b-defective Ad5 mutant, at an MOI of 10 for 24 hr. This served to induce high levels of rep-cap expression. The cells then were infected with the Ad.rAAV.CMV.*EGFP* hybrid vector at an MOI of 10 for an additional 48 hr. The cells were harvested, and CsCl gradient purification through three successive gradients was performed to isolate and purify the rAAV. The hybrid virus, Ad.rAAV.CMV.-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: rAAV, recombinant adeno-associated virus; EGFP, enhanced green fluorescent protein; rAAV-EGFP, rAAV carrying cytomegalovirus-driven EGFP; rpe, retinal pigment epithelium; CMV, cytomegalovirus; GCs, genome copies; ERG, electroretinogram; RT, reverse transcriptase; NAbs, neutralizing antibodies; Ad, adenovirus.

‡To whom reprint requests should be addressed at: F. M. Kirby Center; 310 Stellar-Chance Labs, 422 Curie Boulevard, University of Pennsylvania, Scheie Eye Institute, Philadelphia, PA 19104-6069. E-mail: jebennet@mail.med.upenn.edu.

EGFP, is an adenovirus in which the E1 region had been deleted and replaced with p.AAV.CMV.*EGFP*.

Genome Titer and Quality Control. Genome titer of the purified virus was determined according to genome copies (GCs) per microliter (18). Transduction titer of the rAAV.CMV.*EGFP* used in the study was assayed on 84-31 cells, an E1/E4-complementing cell line, and was defined as transduction units per milliliter (19). Transduction titer of the purified virus ranged from 1.6×10^{10} to 1.6×10^{11} transduction units/ml.

Quality control studies included evaluation of stocks for replication competent AAV. This was performed by infecting 293 cells with rAAV.CMV.*EGFP* vector in the presence of adenovirus and analyzing total DNA extracted from resulting lysate by Southern blot analysis using a 2.7-kilobase Cap gene fragment as a probe. Cap sequence, reflecting the presence of replication competent AAV, was not detected in 1×10^{11} GCs of rAAV.CMV.*EGFP*, after two rounds of amplification in 293 cells. Purified rAAV.CMV.*EGFP* also was tested for replication competent Ad contamination as described (20). Less than one transducing Ad particle was detected when 1×10^{11} rAAV genomes were tested.

Surgical Procedures. *Gas retinopexy.* After dilating the pupils, 0.1 ml of sterile C_3F_8 gas (Alcon Laboratories, Fort Worth, TX) was injected through the pars plana into the central vitreous by using a 30 gauge needle.

Subretinal injection of rAAV. Anesthesia was achieved by using alphaxalone-alphadolone acetate (Glaxovet, Harefield, Uxbridge, U.K.; 13 mg/kg/hr i.v. infusion). The anterior chamber was tapped with a 30 gauge needle to remove 0.1 ml of aqueous humor. This was stored at −80°C for immunological studies. A 30 gauge anterior chamber cannula (Alcon Laboratories, Fort Worth, TX) was inserted through a sclerotomy and was advanced through the vitreous, and, under microscopic control, 50–100 μl of transfection solution (purified rAAV or vehicle alone) were injected into the subretinal space underlying the central retina. All procedures were done using sterile instruments, surgical fields, and solutions. Inflammation that was observed 3 days after the first set of injections was treated with subconjunctival injection of 2 mg (in 0.5 ml) of decadron phosphate (dexamethasone; Merck Sharp & Dohme) and 12 mg (in 0.3 ml) of kenalog (triamcinolone acetonide; Squibb).

For readministration experiments, identical procedures were used as those for the initial injections except that (*i*) virus was administered to the eyes contralateral to those initially exposed to virus; (*ii*) no control injections were performed; and (*iii*) virus-injected eyes received one subconjunctival injection each of decadron and kenalog immediately after surgery.

Imaging Studies. Ophthalmoscopy was performed at various timepoints posttreatment (3 days, 1 week, and, thereafter, at 1-week intervals). EGFP was identified through illumination with a cobalt blue filter. Fundus photographs were obtained, and digital montages were produced.

Retinal fluorescence was quantified with an imaging fundus reflectometer (21). The animal was anesthetized, and the eye was secured with conjunctival sutures to a metal ring mounted on a head holder. Images were obtained with monochromatic (half-bandwidth 10 nm) green (560 nm) and violet (480 nm) illumination and a high-sensitivity charge-coupled device camera (TEA/CCD-1024TKB2, Princeton Instruments, Trenton, NJ) by using a yellow (50% cut-on at 495 nm) "blocking" filter. Green illumination provided images of fundus features. Violet illumination, optimized to the peak excitation of EGFP, provided images of fluorescence. Preliminary experiments showed excitation and fluorescence spectra to be consistent with EGFP. For large areas of fluorescence, montages were obtained digitally from overlapping views (each ≈20° diameter). Fundus fluorescence is displayed as counts above background and is normalized by exposure time.

Electroretinography. A computer-based system (EPIC-XL, LKC Technologies, Gaithersburg, MD) and Burian-Allen contact lens electrodes (Hansen Ophthalmic Development Lab, Iowa City, IA) were used for recording bilateral simultaneous full-field electroretinograms (ERGs). These experiments followed the imaging studies. Techniques were similar to those used in previous human studies (22, 23). B-wave amplitude and timing were measured conventionally; rod and cone photoresponses were modeled (23). The absolute value of the ERG parameter difference between the two eyes was used to calculate interocular difference statistics (22).

Histological Analyses. *Tissue processing.* One animal was killed 6 months after injection. Aqueous humor was sampled, and the globe was fixed immediately in freshly prepared 4% paraformaldehyde in PBS. After 24 hr, anterior segment structures were dissected from the posterior retina (eye cup). Tissue samples then were isolated for histological and molecular biology studies. The brains and optic nerves were also isolated and fixed.

Tissue was cryoprotected in PBS containing 30% sucrose and then was embedded in optimal cutting temperature compound (Baxter Scientific Products, McGaw Park, IL) and was frozen in dimethylbutane on dry ice. Tissue was sectioned between −19 and −23°C at 8–25 μm. Coverslips were mounted by using citifluor (Ted Pella, Redding, CA). After evaluating EGFP-induced fluorescence, coverslips were removed from selected slides, and sections were stained with hematoxylin and eosin. Alternatively, occasional sections were counterstained with propidium iodide (0.5% in water) to visualize nuclei.

Assessment of photoreceptor layer thickness. The status of the photoreceptor layer was graded by counting the number of rows of photoreceptor nuclei and widths of outer and inner segments in injected portions of the retina and comparing the counts with those obtained from adjacent noninjected portions of the retina. Statistical significance was determined by a two sample Wilcoxon Rank sum statistic from the SAS Institute (Cary, NC) (24).

Assessment of green fluorescent protein. Tissue was evaluated in whole mount fashion with an inverted fluorescent Nikon diaphot microscope. Green fluorescent protein was evaluated in tissue sections after illumination with a fluorescein filter. EGFP-induced fluorescence was distinguished from background autofluorescence by illuminating the samples with a rhodamine filter (which shows background fluorescence only) (25).

Analyses of EGFP Expression. *Reverse transcriptase (RT)–PCR amplification of EGFP-specific transcripts.* Sections of retina containing *EGFP*-expressing photoreceptors were microdissected using modifications of techniques described by Fernandez *et al.* (26). Retinal pigment epithelium and inner retinal cells bordering these regions also were isolated. Negative control tissue was obtained from uninjected portions of the same retina or from the corresponding segments in the contralateral (vehicle-injected) eye. Total RNA was extracted and subjected to PCR amplification after or without reverse transcription by using primers specific for the EGFP-encoding cDNA. The details of RT-PCR conditions are as follows: 0.5 mg of retinal total RNA was used as template to synthesize cDNA in a volume of 20 μl using 200 units of Maloney murine leukemia virus reverse transcriptase (1 hr at 37°C). Oligonucleotide primers were EGFP Forward/3 (5′-CAA GTT CAG CGT GTC CG-3′) and EGFP Reverse (5′-TTG TGC CCC AGG ATG TT-3′). Priming of the cDNA was performed with EGFP Reverse. PCR amplification (40 cycles) was performed in a volume of 100 μl. Reactions were performed in an OmniGene thermal cycler (Hybaid, Middlesex, U.K.) with initial denaturation: 10 min, 98°C; denaturation: 94°C, 1 min; annealing: 50°C, 1 min; extension: 72°C, 1 min; final extension: 10 min, 72°C. This produced a product of 344 bp in positive control samples.

Immunological Studies: Enzyme-Linked Immunoabsorbant Assay (ELISA). Serum and anterior chamber fluid samples were analyzed for antibodies to viral capsid proteins by using ELISA. ELISA plates were coated overnight at 4°C with antigen (purified AAV) and then were washed, blocked, and incubated with diluted serum or intraocular fluid. Saline was used as a negative

Table 1. Outline of procedures performed on the eyes of the four monkeys

Animal no.	Gas bubble, −2 weeks	Subret injected at day 0: material; GC × 10^{12}; μl	Expression of EGFP, onset in weeks	Histology	Subret injected at 7 months: AAV; GC × 10^{12}; μl
94B-102					
Eye 1	ND	rAAV; 50; 50	11	6 months	ND
Eye 2	ND	Vehicle; 0; 50	−	6 months	ND
95C-273					
Eye 1	+	rAAV; 25; 50	18	11 months	ND
Eye 2	+	Vehicle; 0; 50	−	11 months	3.84; 80
94B-051					
Eye 1	ND	rAAV; 4.8; 100	12	11 months	ND
Eye 2	ND	Vehicle; 0; 100	−	11 months	4.3; 90
94B-109					
Eye 1	+	rAAV; 3.84; 80	8	ND	ND
Eye 2	+	ND; 0; 0	−	ND	ND

AAV dose is presented as number of GCs. −, absent; ND, not done.

control. Serum from a human patient previously found to have high levels of anti-AAV antibodies was used as a positive control. Samples were incubated with an alkaline phosphatase-conjugated goat-anti-human IgG (Jackson ImmunoResearch, 100 μl/well). The Sigma Fast Paranitrophenyl Phosphate Substrate system was used for the color reaction, and the plates are read at an optical density of 405 nm. Reactions were performed in triplicate.

Identification of Neutralizing Antibodies (NAbs). To determine what fraction of the antibodies induced by treatment with rAAV were neutralizing, 10 μl of serum or intraocular fluid were plated in 96-well round bottom tissue culture plates. Eight serial dilutions of each sample were made in serum-free DMEM. The rAAV.CMV.*EGFP* virus then was added to each of the dilutions in a final volume of 50 μl. The plates were incubated for 1 hr at 37°C, and the samples were transferred to 96-well flat bottom tissue culture plates already seeded with 293 cells at 60% confluence. The next day *EGFP* expression was visualized by using

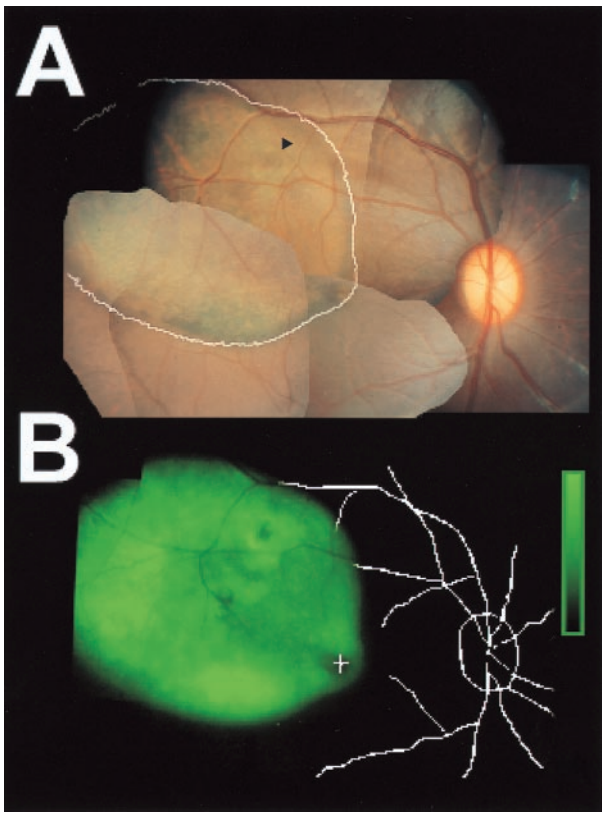

FIG. 1. *In vivo* imaging shows fluorescence localized to the site of subretinal rAAV.CMV.*EGFP* injection at 16 weeks. Montage of color photographs (*A*) and fluorescence intensity (*B*) in eye 1 of animal 94B-109 (Table 1). Extent of fluorescence from *B* is overlaid (white trace) on *A*. Arrowhead, injection site; Fundus landmarks (+, fovea) from *A* are overlaid on *B*. Fluorescence intensity is mapped to increasing intensities of green color; scale bar represents 2.4 log units above background.

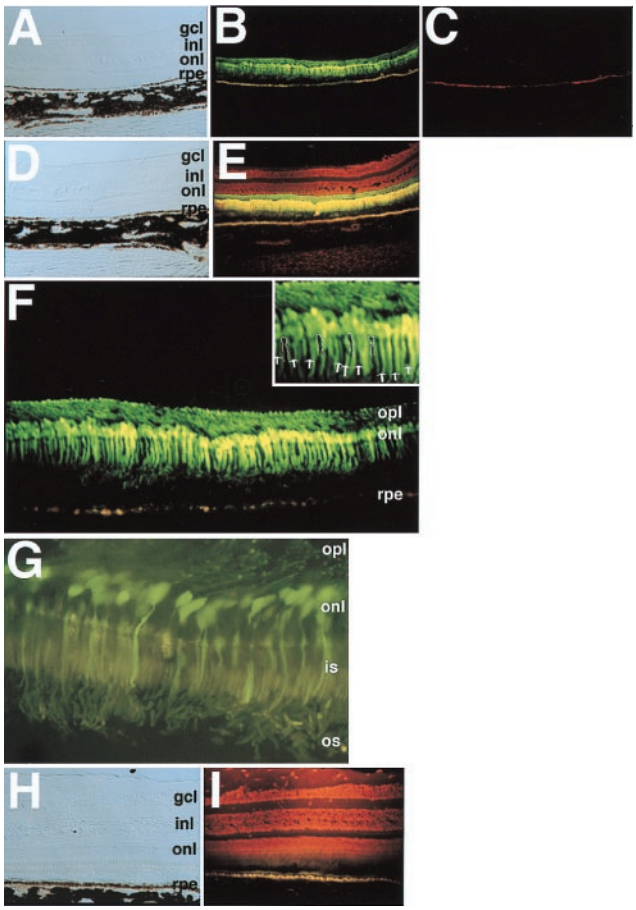

FIG. 2. Histologic evaluation at 6 months postinjection reveals high levels of EGFP in rod photoreceptors. Views are of retinal cross-sections from left eye of monkey 94B-102. (*A*, *D*, and *H*) Light microscopic views. (*B* and *E–G*) Fluorescent views through an FITC filter. (*C*) fluorescent view through a rhodamine filter. (Inset to *F*) Green fluorescent protein is absent in cones (some of which are outlined or indicated with arrows). (*E* and *I*) Sections are counterstained with propidium iodide. (*H* and *I*) Region outside of the injection site lacks EGFP; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium; opl, outer plexiform layer; os, outer segment layer; is, inner segment layer.

Table 2. Effects of subretinal rAAV readministration on serum anti-rAAV NAb levels as measured by ELISA

Day	Animal			
	94B-102	95C-273	94B-051	94B-109
0 First injection	20	20	20	20
28	20	40	20	320
43	20	80	20	320
84	20	80	40	320
107	40	80	80	320
199 Second injection	–	150	ND	ND
235	–	1,280	2,560	ND
285	–	1,280	2,560	ND
325	–	1,380	2,560	ND

Virus was readministered to the second eyes of animals 95C-273 and 94B-051 only on day 199. ND, not done.

a Fluoroimager (Molecular Dynamics). The intensity of EGFP was captured digitally and was inversely proportional to the concentration of NAbs. The NAb titer was defined as the highest dilution that allowed EGFP to be produced to levels 50% of those found in control samples (in which serum had not been added to virus before infection).

Identification of Antibodies to the EGFP Transgene Product. Western blot analysis was performed to determine whether antibodies directed against the EGFP protein were present. For this, 1 μg of purified EGFP protein (CLONTECH) was run on a discontinuous, 1-mm SDS (0.04%) polyacrylamide (10%) analytical gel. After transfer, protein-containing vertical membrane strips were incubated with serum (1:500 diluted in blocking solution) or eye fluid (1:250 diluted in blocking solution) for 12 hr at 4°C. The strips were washed and exposed to the secondary antibody [peroxidase-labeled anti-human IgG (1:1,000)]. The negative control "primary antibodies" consisted of preimmune serum and eye fluid samples. The positive controls were strips incubated with a GFP polyclonal antibody (CLONTECH, diluted 1:250). Subsequently, the blots were washed and the peroxidase reaction was developed by using the ECL Western Detection kit (Amersham Pharmacia).

RESULTS

Subretinal Injection of rAAV in the Primate Central Retina. A series of experimental and control subretinal injections of rAAV.CMV.*EGFP* were performed on four rhesus monkeys (*Macaca mulatta*) as listed in Table 1. One concern before initiating the studies was that administration of the experimental and control material could lead to a volume-induced intraocular pressure increase. To minimize this possibility, two of the monkeys were treated with bilateral gas retinopexy 2 weeks before subretinal injection (Table 1).

Doses ranging from 3.8×10^{12} to 5.0×10^{13} GCs (or 2.6×10^{8} to 8.4×10^{9} transduction units) in volumes ranging from 50–100 μl were injected into the subretinal space of the central retinas in one eye only of each of the four monkeys. Control (contralateral) eyes received an injection of vehicle alone. One animal received an injection of vector only (and no control injection) because of anesthesia complications.

All injections were accurately targeted to the subretinal space as assessed ophthalmoscopically. The retinas all had flattened by 24 hr after injection, and media was clear through 48 hr after injection. An inflammatory response developed at 3 days postinjection in virus-injected eyes only. Cultures of the injection solutions were all negative for microbial (anaerobic and aerobic bacteria) contaminants. Inflammation resolved over a period of $\approx$2 weeks after subconjunctival application of corticosteroids. However, there were residual vitreous opacities visible through the remainder of the experiments in the left eye of 94B-051 and a macular hole in one eye (left eye of 94B-102; Table 1).

Full-field ERGs were used to determine whether there was retina-wide toxicity from the vector or the surgical technique; focal ERGs, to evaluate local pathological effects, were not performed. Rod and cone photoreceptor function determined with ERG photoresponses was normal at 4 months, and there was no interocular difference between rAAV.CMV.*EGFP*-injected versus control-injected eyes. Rod and cone ERG b-wave amplitudes, reflecting inner retinal function, were also normal at 4 months in three of four animals. Animal 94B-051 had abnormally reduced b-wave amplitudes. As a group, all animals showed no interocular differences in b-waves. Bilateral symmetry of ERG parameters continued at 11 months in the 2 reinjected animals.

***In Vivo* Imaging Results.** Green fluorescence was not detected in any of the eyes until 8 weeks but was detected in three of four rAAV-injected eyes by 12 weeks (Table 1). By 18 weeks, all four rAAV.CMV.*EGFP*-injected eyes possessed EGFP.

Fundus appearance (Fig. 1*A*) and fluorescence image (Fig. 1*B*) obtained at 16 weeks are shown in a representative animal. The retina is mildly discolored in the injection site (Fig. 1*A*). This site also appears dark in the fluorescence image (Fig. 1*B*). Intensity of fluorescence varies across the region exposed to rAAV.CMV.*EGFP*, and the fovea lacks detectable fluorescence (Fig. 1*B*).

Histologic Results. Six months after injection, the first histological analysis was performed on eyes of monkey 94B-102. High levels of EGFP were observed in the majority of rod photoreceptors within the rAAV injection site (Fig. 2 *B* and *E–G*). Fluorescence was specific to the EGFP protein. Background fluorescence alone was observed after illumination with a rhodamine filter (compare Fig. 2 *C* and *B*). In regions of maximal EGFP fluorescence, there were frequently localized regions of rpe cell-specific pigmentary atrophy. These regions were typically 3–4 rpe cell lengths in width, although a wider region of atrophy is shown in Fig. 2 *B* and *C*. There was no evidence of rpe atrophy in retina injected with control solution.

EGFP-specific fluorescence did not extend beyond the injection site. This is demonstrated in Fig. 2 *H* and *I*, which are from a region 100 μm nasal to the optic disc (nonfluorescent in *in vivo* imaging studies) and lack EGFP-positive cells.

Table 3. Effects of subretinal rAAV readministration on anterior chamber fluid anti-rAAV NAb levels as measured by ELISA

	Animal							
	94B-102		95C-273		94B-051		94B-109	
	Eye							
Day	1	Un	1	2	1	2	1	Un
0	20	20	20	20	20	20	20	20
43	20	20	20	ND	80	80	160	20
84	20	20	20	20	20	20	160	20
285	–	–	40	40	640	2,560	ND	ND
325	–	–	40	40	640	2,560	ND	ND

Eyes are designated 1 or 2 according to the scheme presented in Table 1. Eye 1 was injected at day 0. Eye 2 was injected at day 199. Only one eye was injected in animals 94B-102 and 94B-109. The second uninjected eyes of these animals are designated Un. ND, not done.

EGFP-specific fluorescence was observed in all photoreceptor-specific layers of rAAV.CMV.*EGFP*-exposed retina, including the outer plexiform layer (and Henle fiber layer of the fovea), the outer nuclear layer, and inner and outer segment layers (Fig. 2 *B* and *E–G*). EGFP was conspicuously absent in cone photoreceptors. Positions occupied by some of the EGFP-negative cones are indicated by arrows (and outlines) in the inset to Fig. 2*F*. Only occasional rpe cells possessed EGFP-specific fluorescence. (None are present in Fig. 2.)

Because rAAV can stably transduce retinal ganglion cells and can produce transgene product in the optic nerve and brain (27), we evaluated ganglion cells and their axons. Rare EGFP-positive ganglion cells were observed only in the location of needle entry. EGFP was not detected in the optic nerves or brains nor in anterior segment structures of any of the three nonhuman primates that have been necropsied.

Aside from occasional spots of rpe atrophy, there was no histological evidence of ocular toxicity after exposure to either rAAV.CMV.*EGFP* or control solution. There were no significant differences in thicknesses of retinal cell layers between equivalent portions of the rAAV-injected and control-injected retinas. There was no significant difference between proportions of cone versus rod photoreceptors in rAAV-exposed versus unexposed regions of the retina. Histopathological evidence of toxicity of either rAAV or control injections on anterior segment structures was not detected. There was no evidence of cellular infiltrate or inflammatory cells on evaluation of hematoxylin and eosin-stained ocular tissue sections.

Transgene Expression Studies. To determine whether EGFP-specific mRNA was still being transcribed long after injection, cells from defined regions of rAAV.CMV.*EGFP*- and control-injected retinas were dissected from frozen tissue sections, and RNA was extracted. Dissection was performed under illumination with blue light so that cells possessing EGFP protein could be identified. The RNA was used in RT-PCR studies designed to amplify an EGFP-specific mRNA product. At 6 months after injection, a 344-bp EGFP-specific RT-PCR product was detected in photoreceptors that possessed histologically detectable EGFP protein. There was no EGFP-specific RT-PCR product identified in portions of the rAAV-injected retina lacking histologically detectable EGFP (i.e., inner retina) or from any portion of the contralateral control-injected retina (not shown).

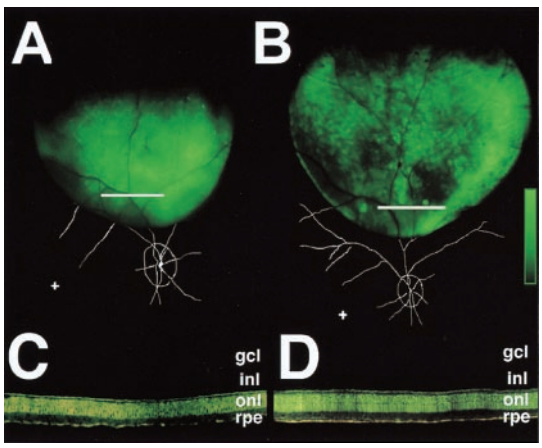

FIG. 3. Subretinal rAAV.CMV.EGFP administration to the eye contralateral to that injected with rAAV.CMV.EGFP 7 months earlier results in high levels of EGFP protein. (*A* and *B*) Fundus fluorescence 7 weeks [95C-273 eye 2 (*A*)] and 16 weeks [94B-051 eye 2 (*B*)] after readministration. Scale bar represents 2.1 log units (*A*) or 2.4 log units (*B*) of fluorescence above background. Fundus landmarks are overlaid (+, fovea). White lines in *A* and *B* indicate the regions shown histologically in *C* and *D*, respectively. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium.

Immunologic Effects and Readministration Studies. Anti-rAAV capsid serum antibodies rose up to 16-fold after subretinal injection of one eye (Table 2). Subretinal injection of rAAV also can result in NAbs in intraocular (anterior chamber) fluid of the injected eye (Table 3). Because rAAV-specific NAbs were identified in animals receiving subretinal injection of rAAV-EGFP, the practical effects of such antibodies were evaluated by readministering the virus. Virus was readministered to eyes contralateral to the previously injected eyes to evaluate the possibility that readministration would lead to toxic immune-mediated changes in the previously (or newly) injected eyes. In addition, the contralateral eyes were evaluated after injection to determine whether additional transduction events were detectable despite the presence of NAbs in the serum.

Readministration of rAAV-EGFP to the eyes contralateral to those that had been injected with virus 7 months earlier resulted in high levels of expression bilaterally (Fig. 3). Transgene expression in the second rAAV-EGFP-injected eyes was detectable ophthalmoscopically earlier than after injection of the first eye (3 weeks versus >8 weeks) (see Table 1) and there was a lack of inflammatory response in the eye. Quantitative fluorescence imaging showed large areas of fluorescence (Fig. 3 *A* and *B*) in the injected regions superior to the optic nerve head. Histological analyses at 11 months (5 months after the second administration) revealed that all of the rod photoreceptors (and no cones) in the injected regions possessed EGFP protein (Fig. 3 *C* and *D*). Although rpe cells positive for EGFP were identified after the second set of injections, these were still not as efficiently transduced as photoreceptors. Optic nerves and brain tissue were negative for EGFP protein.

Subretinal readministration of rAAV resulted in an additional increase in anti-capsid serum NAbs (at least 128 fold over initial values). There was variability in the effect of subretinal injection of rAAV on anti-capsid NAb levels in the anterior chamber fluid, but the first injection was capable of raising the antibody levels of the injected eye by a factor of 8. An increase in intraocular

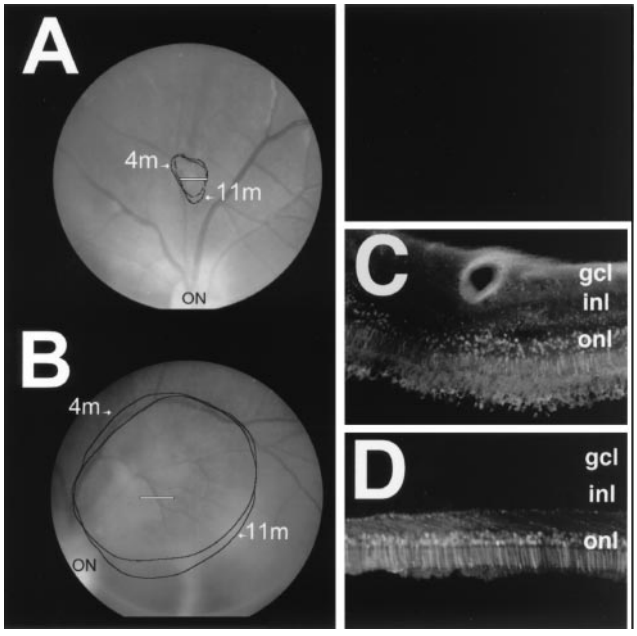

FIG. 4. EGFP fluorescence is stable over time. Shown is the extent of *in vivo* fluorescence at 4 and 11 months (4 m and 11 m) postinjection in 95C-273 eye 1 (*A*) and 94B-051 eye 1 (*B*). White lines in *A* and *B* indicate the regions shown histologically in *C* and *D*, respectively. ON, optic nerve. The section shown in *C* was thicker than that in *D* (30 vs. 18 μm) to enhance detection of the weaker EGFP-labeled photoreceptors in 95C-273 eye 1. Use of the thicker section results in increased retinal autofluorescence. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer.

antibodies was not detected in the control-injected eyes until those eyes themselves were exposed to rAAV. There was no evidence of inflammatory cell infiltrate in any of the eyes. There was no significant difference in humoral response to the transgene protein product (EGFP) comparing pre- and postinjection serum and anterior chamber fluid samples (not shown).

Long-Term Transgene Expression. Transgene expression patterns were maintained through the longest timepoints evaluated. Quantitation of the extent of fluorescence at 4 and 11 months after the first injection in two animals suggested stable levels of EGFP production over a timespan of nearly 1 year (Fig. 4 *A* and *B*). Green fluorescence in every rAAV-injected eye was localized histologically to rod photoreceptors (and occasional rpe cells) (Fig. 4 *C* and *D*). There was no EGFP protein identified in the optic nerves. In another animal being kept for long-term study, ophthalmoscopy showed that transgene expression patterns are maintained for >1 year.

DISCUSSION

The results demonstrate that rAAV can be used to deliver a transgene efficiently, in a stable fashion, and with minimal toxicity to the neurosensory retina of the nonhuman primate. The onset and peak of transgene expression could be monitored in a noninvasive fashion. Clinical–histological correlations demonstrated that fluorescent images viewed in the retina *in vivo* correlated with the fluorescent photoreceptor cells identified microscopically. Photoreceptors were transduced almost exclusively, despite the use of a viral (CMV) promoter that should be functional in a variety of cell types. Although previous studies had demonstrated that photoreceptors can be efficiently transduced by rAAV (14–16, 25, 27, 28), none addressed the possibility that there might be differential targeting of rod and cone photoreceptors. This possibility could be evaluated easily in studies involving the monkey because there is a high percentage of cone photoreceptors in the macula of this species. Of interest, cones were not transduced efficiently by rAAV. The reason for the lack of susceptibility of cones to rAAV-mediated transduction is not clear. Possible explanations include the following: (*i*) Cones lack the surface receptors necessary for rAAV attachment or cellular entry; (*ii*) the virus enters the cone photoreceptors, but these cells do not have the appropriate milieu to allow long-term stability of transgene expression; (*iii*) the CMV promoter is nonfunctional or functions only for a limited time in cone photoreceptors; and (*iv*) the virus or the transgene product is toxic to cone photoreceptors. Experiments in progress aim to evaluate the first three hypotheses. We found no functional (ERG) or histological evidence for rAAV-EGFP-mediated cone photoreceptor toxicity.

One of the most important variables in developing gene-based therapy for human retinal/macular disease is the stability of transgene expression. Ideally, for application to slowly progressive human retinal degenerative diseases, the transgene-expressing cells should persist over years or even decades. We have observed transgene expression in transduced retinal cells at least 1 year after injection with no evidence of diminishing transgene expression over time. Importantly, levels of transgene product also remained constant for >1 year after injection (the latest timepoint).

There were no apparent long-term systemic sequelae caused by exposure to this virus. The only local pathological effect that was observed was occasional pigmentary loss of rpe cells. Experiments in progress aim to evaluate the possibility that rAAV or EGFP itself results in low levels of rpe toxicity. However, even after subretinal readministration of the virus to animals with moderately strong serum NAb titers, there were no significant adverse effects on retinal function or histology. Additional transduction events have not been observed after readministration of rAAV to smooth muscle or airway epithelium (29, 30). Here, we report that rAAV can be readministered to the subretinal space and demonstrate that such a treatment does not cause adverse functional effects. The ability to readminister rAAV to the subretinal space may be attributable to the unique immunological properties of this virus and/or to the immune-privileged nature of the subretinal space.

In summary, the findings presented here, that rAAV can be used to deliver high levels of transgene expression in a stable fashion to rod photoreceptors with minimal toxicity and that this virus can be readministered safely to the eye, are very promising in terms of development of somatic gene therapy for retinal degenerative disease. This virus and/or the present promoter is likely to be of limited use for diseases originating in cone photoreceptors and rpe. However, rAAV-mediated retinal gene therapy is likely to be effective in diseases originating in rod photoreceptors such as the inherited maculopathy Stargardt disease (31) and most forms of retinitis pigmentosa.

We thank C. Baker, N. Syed, and A. Milam for technical assistance. We thank the Cell and Morphology Core of the Institute for Human Gene Therapy for use of microscopes. This work was supported by National Institutes of Health Grants RO1 EY10820 (to J.B.), EY05627 (to S.G.J.), and EY12156 (to J.B.), grants from the Foundation Fighting Blindness (to S.G.J., J.B., and A.V.C.) and the Pennsylvania Lions Sight and Eye Research Foundation (to J.B.), National Institutes of Health Grants P30 DK47757-05AHA (to J.M.W.), F98223E (to V.A.), and T32 EY07131 (to A.R.G.), the P. and V. Mackall Foundation Trust, Research to Prevent Blindness, and the F.M. Kirby Foundation. James Wilson is a founder of and holds equity in Genovo, Inc.

1. Bundey, S. & Crews, S. J. (1984) *J. Med. Genet.* **21,** 417–420.
2. Bunker, C. H., Berson, E. L., Bromley, W. C., Hayes, R. P. & Roderick, T. H. (1984) *Am. J. Ophthalmol.* **97,** 357–365.
3. Kaplan, J., Bonneau, D., Frezal, J., Munnich, A. & Dufier, J. L. (1990) *Hum. Genet.* **85,** 635–642.
4. National Advisory Eye Council (1993) National Institutes of Health Publication 93-3186 (National Institutes of Health, Bethesda, MD).
5. Blacharski, P. A. (1988) in *Fundus flavimaculatus*, ed. Newsome, D. A. (Raven, NY), pp. 135–159.
6. Heckenlively, J. & Foxmann, S. G. (1988) in *Congenital and Early-Onset Forms of Retinitis Pigmentosa*, ed. Heckenlively, J. (Lippincott, Philadelphia), pp. 107–149.
7. Bennett, J., Tanabe, T., Sun, D., Zeng, Y., Kjeldbye, H., Gouras, P. & Maguire, A. (1996) *Nat. Med.* **2,** 649–654.
8. Li, T. & Davidson, B. L. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7700- 7704.
9. Cayouette, M. & Gravel, C. (1997) *Hum. Gene Ther.* **8,** 423–430.
10. Cayouette, M., Behn, D., Sendtner, M., Lachapelle, P. & Gravel, C. (1998) *J. Neurosci.* **18,** 9282–9293.
11. Jomary, C., Vincent, K., Grist, J., Neal, M. & Jones, S. (1997) *Gene Ther.* **4,** 683–690.
12. Lewin, A. S., Drenser, K. A., Hauswirth, W. W., Nishikawa, S., Yasumura, D., Flannery, J. G. & LaVail, M. M. (1998) *Nat. Med.* **4,** 967–971.
13. Bennett, J., Zeng, Y., Bajwa, R., Klatt, L., Li, Y. & Maguire, A. M. (1998) *Gene Ther.* **5,** 1156–1164.
14. Bennett, J., Duan, D., Engelhardt, J. & Maguire, A. M. (1997) *Invest. Ophthalmol. Visual Sci.* **38,** 2857–2863.
15. Flannery, J., Zolotukin, S., Vaquero, M., LaVail, M., Muzyczka, N. & Hauswirth, W. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6916–6921.
16. Grant, C., Ponnazhagan, S., Wang, X.-S., Srivastava, A. & Li, T. (1997) *Curr. Eye Res.* **16,** 949–956.
17. Gao, G.-P., Faust, L. Z., Engdahl, R. K., Qu, G., Nguyen, C. D., Krapf, E. M., Miller, T. J., Xiao, W., Hughes, J. V. & Wilson, J. M. (1998) *Hum. Gene Ther.* **9,** 2353–2362.
18. Fisher, K., Kelley, W., Burda, J. & Wilson, J. (1996) *Hum. Gene Ther.* **7,** 2079–2087.
19. Gao, G.-P., Yang, Y. & Wilson, J. M. (1996) *J. Virol.* **70,** 8934–8943.
20. Fisher, K. J., Jooss, K., Alston, J., Yang, Y., Haecker, S. E., High, K., Pathak, R., Raper, S. E. & Wilson, J. M. (1997) *Nat. Med.* **3,** 306- 312.
21. Jacobson, S. G., Kemp, C. M., Cideciyan, A. V., Macke, J. P., Sung, C.-H. & Nathans, J. (1994) *Invest. Ophthalmol. Visual Sci.* **35,** 2521–2534.
22. Jacobson, S. G., Kemp, C. M., Narfstrom, K. N. & Nilsson, S. E. G. (1989) *Exp. Eye Res.* **49,** 843–852.
23. Cideciyan, A. V., Zhao, X., Nielsen, L., Khani, S. C., Jacobson, S. G. & Palczewski, K. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 328–333.
24. SAS Institute (1989) *SAS/STAT User's Guide, Version 6* (SAS Institute, Cary, NC).
25. Bennett, J., Anand, V., Acland, G. M. & Maguire, A. M. (1999) *Methods Enzymol.*, in press.
26. Fernandez, P. M., Pluta, L. J., Fransson-Steen, R., Goldsworthy, T. L. & Fox, T. R. (1997) *Mol. Carcinog.* **20,** 317–326.
27. Dudus, L., Anand, V., Chen, S.-J., Wilson, J., Fisher, K. J., Acland, G., Maguire, A. M. & Bennett, J. (1999) *Vision Res.* **39,** 2545–2554.
28. Ali, R. R., Reichel, M. B., Thrasher, A. J., Levinsky, R. J., Kinnon, C., Kanuga, N., Hunt, D. M. & Battacharya, S. S. (1996) *Hum. Mol. Genet.* **5,** 591–594.
29. Halbert, C., Standaert, T., Aitken, M., Alexander, I., Russell, D. & Miller, A. (1997) *J. Virol.* **71,** 5932–5941.
30. Xiao, X., Li, J. & Samulski, R. J. (1996) *J. Virol.* **70,** 8098–8108.
31. Sun, H. & Nathans, J. (1997) *Nat. Genet.* **17,** 15–16.