

Recombinant adeno-associated virus targets passenger gene expression to cones in primate retina

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Recombinant adeno-associated virus (rAAV) is a promising vector for gene therapy of photoreceptor-based diseases. Previous studies have demonstrated that rAAV serotypes 2 and 5 can transduce both rod and cone photoreceptors in rodents and dogs, and it can target rods, but not cones in primates. Here we report that using a human cone-specific enhancer and promoter to regulate expression of a green fluorescent protein (GFP) reporter gene in an rAAV-5 vector successfully targeted expression of the reporter gene to primate cones, and the time course of GFP expression was able to be monitored in a living animal using the RetCam II digital imaging system. © 2007 Optical Society of America

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1. INTRODUCTION

Recombinant adeno-associated virus (rAAV) has been studied extensively as a vector for delivering therapeutic genes to the retina in order to treat inherited retinal degenerations.^{1–15} Key features of rAAV are that it is generally nonpathogenic, it can infect nondividing cells, and it lacks sequences encoding viral proteins that induce immune responses.^{2,16,17} It has been shown to be safe when delivered subretinally in rats, dogs, and nonhuman primates.^{18–20} Long-term stable expression of rAAV-delivered reporter genes has been observed in the retina; for example, expression of rAAV-vectored green fluorescent protein (GFP) in photoreceptors was shown to be sus-

tained for at least 2 years in primates, for the lifespan of rodents,^{2,4} and for more than 4 years in dogs.¹² Thus, rAAV-mediated gene therapy holds promise for treating and potentially curing human retinal disease and restoring inherited vision loss.

Although vision is mediated by two types of photoreceptors—rods and cones—humans rely most heavily on cones for carrying out daily activities. Thus, disorders that affect cone function have a greater negative impact on the quality of life. An important application of rAAV-mediated gene therapy in translational medicine is rescuing cone-based vision disorders in humans. A more fundamental application is the potential for

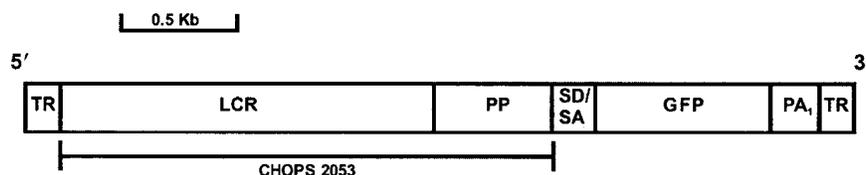


Fig. 1. Molecular map of rAAV.CHOPS2053.GFP. TR=terminal repeats; LCR=locus control region from upstream of the human L opsin gene; PP=proximal promoter of the human L opsin gene; SD/SA=mini-intron with splice donor (SD) and acceptor sites (SA) required for efficient transcription and translation in eukaryotic cells; GFP=cDNA encoding green fluorescent protein; PA1=an SV40 polyadenylation signal for efficient translation in eukaryotic cells.

developing novel approaches to investigate plasticity of the adult primate visual system. For example, some squirrel monkeys have a form of color vision deficiency that serves as a model for human color blindness. Humans normally have short- (S), middle- (M), and long-wavelength (L) sensitive cones. Most often, color vision deficiency is caused by the absence of M or L cone contribution to vision resulting from the deletion of the corresponding cone opsin gene. Using rAAV to deliver an L cone opsin gene to a subset of M cones in color-blind squirrel monkeys that have only M and S cones might allow investigation of the retinal and cortical circuitry for color vision. Recent experiments have demonstrated that using an rAAV vector with the GFP gene controlled by regulatory elements from a gene specifically expressed in human M and L, but not S, cones preferentially targets GFP expression to M cones in rodents.²¹ Thus, if rAAV can transduce primate cones, such an approach could be used to determine the extent to which the adult visual system is capable of extracting and making sense of information added through alteration of the sensory inputs via gene therapy.

Recombinant AAV has been shown to efficiently transduce both rod and cone photoreceptors in rodents and dogs,^{1,3,4,14,22} but cone transduction has not yet been demonstrated in primates. Two different serotypes of rAAV vectors have been studied for their ability to target retinal cells in primates. Serotype-2 (Ref. 2) or serotype-5 (Ref. 10) vectors carrying the gene for GFP under control of the cytomegalovirus (CMV) promoter were injected subretinally in macaque monkeys, and both serotypes produced GFP fluorescence in rods but not cones. Here we tested the hypothesis that regulating the expression of an rAAV-vectored GFP gene using an enhancer and promoter from a gene that is normally expressed specifically in human cones would result in the preferential expression of GFP in cones of virus-treated animals, and that the time course of GFP gene expression can be followed in the living animal.

2. METHODS

A. Viral Vector

The basic rAAV vector (pTR-UF2) has been described previously,²³ and a modified version was used here. The construct, named rAAV.CHOPS2053.GFP, is illustrated in Fig. 1. This virus, which is serotype 5, contains all of the known regulatory sequences required for efficient transcription and translation, including an enhancer and promoter specific for mammalian L and M cones, a Kozak sequence, a mini-intron with splice donor and acceptor

sites, and a polyadenylation signal. The vector also contains two 145 bp terminal repeats (TR) from an adeno-associated virus. CHOPS2053 is a 2.1 kb fragment that contains two segments of DNA from upstream of the human X-chromosome opsin gene array. The 5' segment is 1557 bp that extends from -4564 to -3007 upstream of the human L opsin mRNA start site and contains the highly conserved 37 bp LCR core element, which has previously been shown to be essential for transcription of the X-chromosome visual pigment genes.²⁴ The 3' segment of CHOPS2053 is 496 bp that lies immediately upstream of the human L opsin translational start site and contains the proximal promoter (PP in Fig. 1) of the L opsin gene.²⁵ A 2.5 kb segment that occurs in genomic DNA between the above-described 5' and 3' segments of CHOPS2053 is deleted in this construct, which is necessary to meet the size restriction for rAAV-based vectors. Immediately downstream of the CHOPS2053 fragment are splice donor and acceptor signals (SD/SA, Fig. 1) from the SV40 late viral protein gene, a recombinant green fluorescent protein (GFP, Fig. 1) cDNA, and the polyadenylation signal (pA1, Fig. 1) from the SV40 genome.

B. Animals

Squirrel monkeys were obtained through advertisements in a newsletter published through the University of Washington Regional Resource Primate Center. These experiments were conducted in accordance with the guidelines of the U.S. NIH regarding the care and use of animals, and adequate measures were taken to minimize pain or discomfort.

C. Injection Procedure

Animal 89S was a 14 year old male squirrel monkey (*Saimiri boliviensis*). The right eye of the animal was injected using a KDS model 210 syringe pump and a 10 cc Becton Dickinson (BD) Luer Lok syringe connected via 88.9 cm of IV tubing to a 3.8 cm long, 30 gauge BD Yale regular bevel cannula (BD Ref. No. 511258). The cannula was manually bent to produce a 135° angle 1.5 mm from the tip. The tubing, syringe, and needle were sterilized prior to use. For the procedure, the tubing and syringe were filled with sterile lactated ringers, and just prior to the moment of injection, 25 μ L of virus mixed with 25 μ L of sterile lactated ringers was withdrawn into the cannula at a rate of 2 mL/min.

The animal was anesthetized with ketamine hydrochloride (15 mg/kg) and xylazine (2 mg/kg), and the right eye was dilated, cleaned with betadine, and treated with cycloheximide and a local anesthetic (0.5% proparacaine drops). Atropine (0.05 mg/kg) was also administered intramuscu-

larly. A subconjunctival injection of 1% lidocaine was given, and the anterior portion of the eye was exposed by performing a limited lateral canthotomy; the lids were held open with a speculum designed for premature infants. A temporal sclerotomy was made 1 mm posterior to the limbus with a 27 gauge needle, through which the injection cannula was inserted. While viewing under a stereomicroscope, the cannula was advanced through the vitreous, and the tip of the needle was placed in the subretinal space, inferior to the fovea. A total volume of 200 μL of virus plus sterile lactated ringers was infused at a rate of 1234 mL/min with a diameter setting of 14.48 mm.

Animal 265750 was a 6 year old male squirrel monkey (*Saimiri sciureus*). The injection procedures used on this animal were identical to those described above with the following improvements. A 500 μL Hamilton Gastight No. 1750 Luer Lock syringe was used in place of the 10 cc syringe, and it was connected to 88.9 cm of 30 gauge tubing with Luer Lock adapters at both ends (Hamilton 30TF double hub). The other end of the tubing was attached to the cannula via a female–female adapter. The syringe, tubing, and needle were filled with 210 μL of sterile lactated ringers, and then 300 μL of undiluted virus was withdrawn through the cannula at a rate of 100 $\mu\text{L}/\text{min}$. Once the cannula had been advanced through the vitreous, two successive 100 μL subretinal injections were made at different locations in the right eye using an infusion rate of 1060 $\mu\text{L}/\text{min}$ with a diameter setting of 3.26 mm. The first injection was made nasal to the optic nerve head, and the second injection was placed inferior and temporal to the optic nerve.

After each procedure decadron, kenalog, and cephazolin were injected under the conjunctiva to prevent inflammation and infection, and 10 to 20 mL of subcutaneous lactated ringers were given. Subsequent administration of steroids and analgesics were administered as needed for inflammation or discomfort.

D. Fundus Exams

Fundus images were obtained using the RetCam II (Massie Laboratories, Pleasanton, California), a wide field digital pediatric retinal imaging system. Images were taken with either a lens designed for detecting retinopathy in premature infants, which provides a 130° field of view in real time, or with a high-magnification 30° lens. Animals were anesthetized for all exams, and goniosol (hydroxypropyl methylcellulose) was used as an optical coupling between the RetCam lens and the cornea. Images were captured both under standard light conditions and under conditions designed for fluorescein angiography that also allowed visualization of GFP fluorescence. The relative intensity of the GFP fluorescence was determined from the highest intensity pixels in the RetCam images.

E. Histology

Animal 89S was euthanized and enucleated 4 months after receiving a subretinal injection of rAAV.CHOPS2053.GFP in the right eye. The treated eye was injected at the pars plana with 50 to 100 μL of fixative immediately after enucleation and once more during

the first 15 min following enucleation and then placed in a vial of 2% paraformaldehyde overnight. The eye was then transferred to 0.1 M phosphate buffer pH 7.4 then to 0.1 M phosphate buffer pH 7.4 plus 30% sucrose and 0.1% sodium azide. The retina was dissected and cryosectioned serially at 12 μm . Retinal sections were visualized and photographed using a Nikon Optiphot fluorescence microscope.

3. RESULTS

A. rAAV.CHOPS2053.GFP Serotype 5 Transduces Cone Photoreceptors in the Squirrel Monkey

The topography of the squirrel monkey retina has been studied in detail.^{26–28} The primate retina, including that of squirrel monkeys, contains a centrally located, cone-dominated fovea. Results of experiments in squirrel monkey 89S are shown in Fig. 2. Near the injection site, which had been placed just inferior to the fovea, GFP fluorescence was observed in cone photoreceptors. Distal from the injection site, fewer cones showed GFP fluorescence as evidenced by the observation of individual labeled cones (Fig. 2, inset) in a field of photoreceptors that did not exhibit fluorescence.

The GFP-positive cells were easily identifiable as cone photoreceptors based on their morphology, which is very distinct from rods in primates. The majority of the GFP expression was in the cone inner segments, and it was also found occasionally in the outer segments. Because GFP is a soluble protein, this type of expression pattern

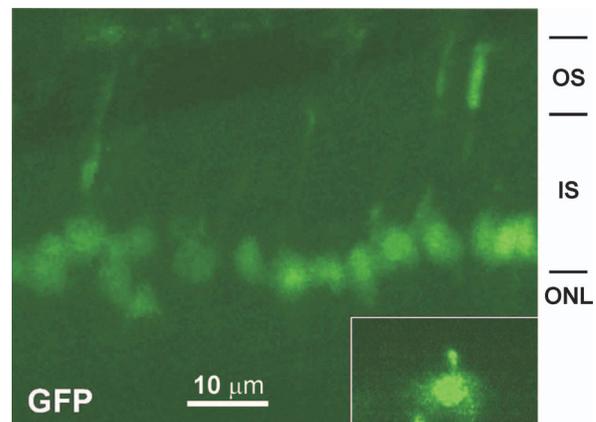


Fig. 2. rAAV.CHOPS2053.GFP transduces cone photoreceptors in the squirrel monkey. Near the injection site, which was placed just inferior to the fovea, GFP fluorescence was observed within cones. At the near peripheral location of the field shown, the cone density is high and there are relatively few rods. At this eccentricity from the fovea in the primate retina, rods and cones are easily distinguishable because of marked anatomical differences, with cone inner segments being 2 to 3 times wider in diameter than rods. At a more peripheral location distal to the injection site (inset), cones are even more distinct with their larger diameters, and fewer cells were transduced as evidenced by isolated cones expressing GFP surrounded by nontransduced cells. In the near periphery, it appears that a majority of cones express GFP. Within the same field, there is also an occasional cell with a narrow profile which expresses GFP. This may be spurious expression of GFP in rods. In locations distal from the injection site where occasional GFP expressing cones are observed, no suspect rods expressing GFP were seen. Abbreviations: OS=outer segment; IS=inner segment; ONL=outer nuclear layer.

within photoreceptors was expected. In those cones in which the transgene expression is particularly robust, GFP has presumably diffused into the outer segments. Some of the fluorescence in the slender structures distal to the cone inner segments may also represent spurious GFP expression in rods.

B. Green Fluorescent Protein Fluorescence Imaged in a Living Monkey Using the RetCam II

Figure 3 shows fundus images that were obtained from a living squirrel monkey (265750) using the RetCam II imaging system. Digital fundus images were obtained under white-light illumination prior to viral delivery, and again immediately following the injection procedure [Figs. 3(a)

and 3(b), respectively]. A time course of GFP expression in the living monkey eye was documented using the fluorescein angiography mode of the RetCam II. A green filter was placed between the light source and the camera lens, and fundus images were taken under white light [Fig. 3(d)] and under blue light illumination [Figs. 3(e)–3(h)] at the same retinal locations. Comparison of images using retinal landmarks visible under both lighting conditions and in the pre- and postinjection pictures allowed the locations of the injection sites to be compared with the sites of GFP expression. Fluorescence first became detectable in the squirrel monkey at approximately 9 weeks postinjection. It continued to increase in area and intensity through 24 weeks postinjection [Figs. 3(e)–3(h)]. The Ret-

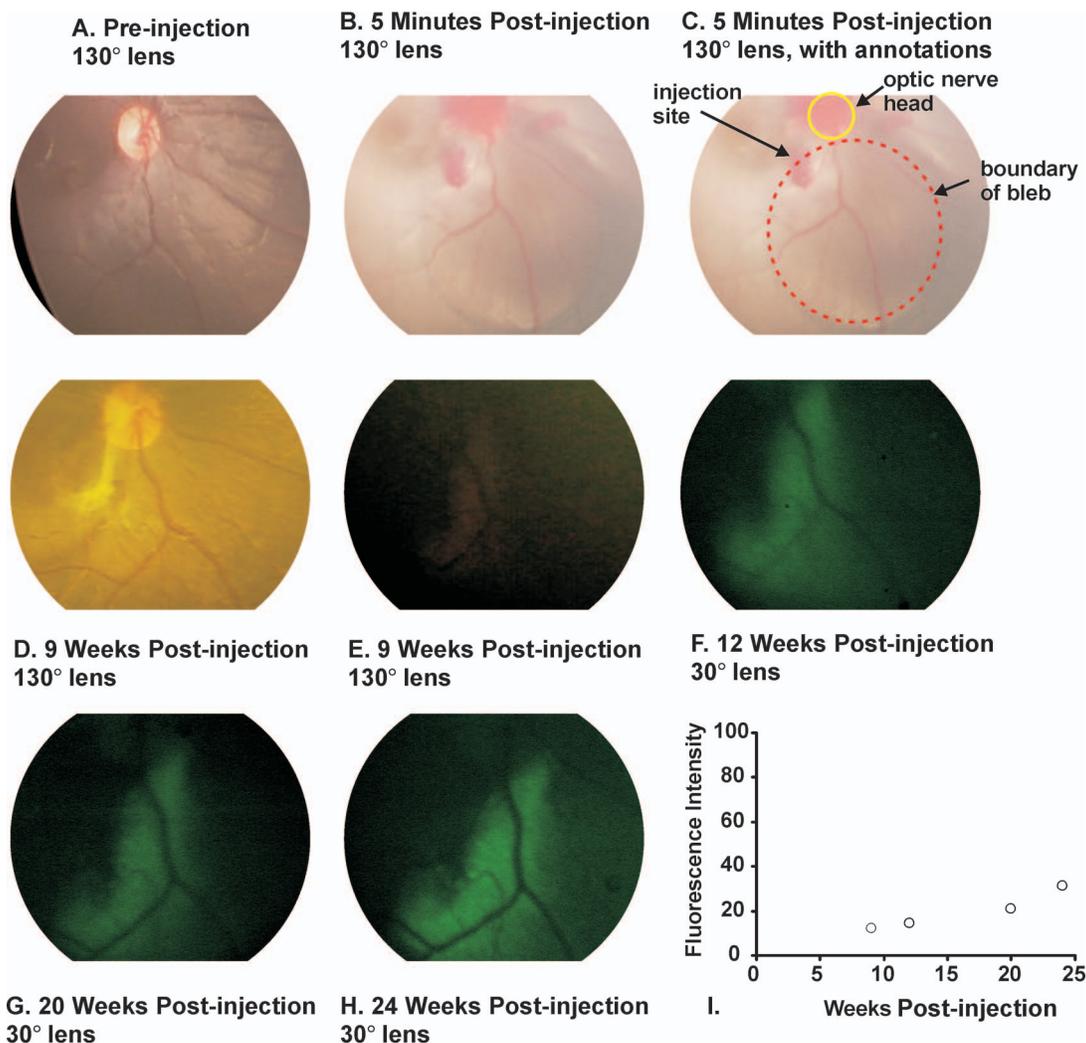


Fig. 3. GFP fluorescence imaged in a living monkey using the RetCam II. All images show the right eye of animal 265750. Images in panels (A)–(D) were obtained using a halogen bulb; images in panels (E)–(H) were obtained using a xenon bulb. In panels (D)–(H), images were obtained with a green filter (510 nm cutoff) placed between the light source and the camera for detecting GFP fluorescence. (A) Fundus image taken immediately before the injection procedure. Blood vessels provide landmarks that allow comparisons to be made across images. (B) Same retinal area as panel (A), which corresponds to the location of the injection that was made inferior and temporal to the optic nerve head, imaged directly following the injection procedure. A large bleb of virus-containing solution that was made underneath the retina is visible. The bleb and retinal landmarks are labeled in (C). (D) Image taken 9 weeks postinjection using a 130° lens. (E) Same retinal area as panel D showing a small, roughly J-shaped area of GFP expression. (F) Image taken 12 weeks postinjection using a high-magnification 30° lens. The smaller lens was found to prevent unfiltered light at the edges, thus eliminating the greenish background seen in the image shown in panel (E). In images (F)–(H), all of the green light is coming strictly from the GFP fluorescence. (G) Same retinal area shown in panel (F), imaged at 20 weeks postinjection. (H) An image taken at 24 weeks postinjection. (I) Graph of the relative intensity of the GFP fluorescence in images (E)–(H). Pixel intensities are plotted on a scale in which the lowest possible intensity equals zero and the highest equals 100.

Cam is not as sensitive at detecting the GFP expression as fluorescence microscopy methods. Thus, it is likely that there are additional cells outside of the bright fluorescent field shown in the RetCam images of Fig. 3 that are also expressing GFP.

4. DISCUSSION

Here, the progress of gene therapy was monitored in a living primate using a portable digital imaging system, the RetCam II. Advantages of this system are that it is a non-invasive device that is easy to use, and it provides better image quality than that obtained with traditional ophthalmoscopy. The camera itself is contained in a handheld unit, allowing the locations of the injection sites and blebs of virus-containing solution to be quickly documented immediately following each injection procedure. Anatomical locations of the injection sites and blebs were then compared with subsequent fluorescent images within the same animal, thus allowing the area and time course of transgene expression to be evaluated. Fluorescence fundus photography has been used previously to image virus-mediated GFP gene transfer in living animals,^{2,10,29} and it has also been used to follow a time course of rAAV-GFP expression within rodents.³ More recently, the RetCam II was used to image viral-mediated GFP expression in living rodents, and extremely sharp images of the rat retina were obtained.³⁰ Thus, the RetCam technology holds promise for assessing outcomes in gene therapy in the eye. One of the important factors in making advancements in gene therapy for human eye diseases is testing viral vectors in primates. The ability to document the location and extent of the bleb of virus, and then to directly compare it with the area of resultant transgene expression as a function of time in a primate provides valuable information as we move toward developing gene therapies that target cones in humans.

Previous experiments using rAAV in rodents have demonstrated that transgene expression within different retinal cell types is dependent on the promoter used. For example, the CMV promoter has been shown to drive expression of rAAV-GFP almost exclusively in RPE cells, except for the occasional positive cell in the neural retina of the rat.³ In a similar experiment in which rAAV-GFP was driven by a rod opsin promoter, GFP was specifically expressed within rat photoreceptor cells.¹ Most recently, the human short-wavelength sensitive opsin promoter has been used in an rAAV-GFP vector in the rat, and transgene expression was exclusively localized to photoreceptors, with the majority of cells transduced being S cones, while M cones and rods were also targeted.¹⁴ Thus, promoter choice appears to be critical for achieving cell type-specific expression, and the specificity of these promoters remains to be tested in a primate model.

Previous studies that used either rAAV-GFP serotype 2 or serotype 5 did not detect GFP expression in primate cones. In the experiments reported here, GFP expression was mediated by cone-specific regulatory elements instead of by the constitutive viral promoter, CMV, used earlier. Cone-specific regulatory elements may be critical for driving robust levels of viral-delivered genes in primate cone photoreceptors.

The successful transduction of monkey cones demonstrated here opens the possibility of using rAAV vectors to treat cone-based vision disorders in humans. Additionally, these results indicate that rAAV.CHOPS2053 is the vector of choice for experiments in which we are investigating the neural plasticity of the primate visual system by adding a third spectral type of cone in dichromatic monkeys.

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