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Cone-specific expression using a human red opsin promoter in recombinant AAV ☆

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Abstract

Purpose: To determine the feasibility of targeting gene expression specifically to cone photoreceptors using recombinant adeno-associated virus (rAAV) as the vector.

Methods: An rAAV vector was constructed that contains a 2.1 kb upstream sequence of the human red opsin gene to direct green fluorescent protein (GFP) expression. A control construct containing a 472 bp mouse rod opsin promoter, previously shown to drive photoreceptor-specific expression, was also used. Each recombinant virus was injected into the subretinal space of rat, ferret or guinea pig eyes. GFP expression was analyzed 4–6 weeks after injection microscopically.

Result: The human 2.1 kb cone opsin gene upstream sequence targeted GFP expression only to a subset of photoreceptors. Cone-specific expression was shown by co-localization of GFP fluorescence and cone-specific opsin antibody staining. Additionally, in rats, expression was specific for L/M-cones whereas no S-cones exhibited GFP fluorescence. The efficiency of rAAV mediated cone transduction surrounding the injection site was high since every L/M-cone antibody-staining cone was also positive for GFP expression.

Conclusion: The human red/green opsin gene promoter used in this study is sufficient to direct efficient cone-specific gene expression in several mammalian species, suggesting that key cell-type specific regulatory elements must be broadly conserved in mammals. These observations have significance in devising gene therapy strategies for retinal dystrophies that primarily affect cones and point toward a way to functionally dissect the cone opsin promoter in vivo.

Keywords: Cone; Gene therapy; Photoreceptors; Promoter; rAAV; Retina; Retinal degeneration

1. Introduction

Old world primates possess trichromatic vision while new world primates and other mammals are dichromats (Jacobs, 1993). Although some mammals exhibit a level of co-expression, the S (blue), M (green) and L (red) photopigment (cone opsin) genes appear to be expressed mostly mutually exclusively in each cone photoreceptor, thus defining three cone subtypes in primates (Schnapf, Kraft, & Baylor, 1987) and two (L/M and S) cones in other mammals. (Note: to allow cross-reference between human and other mammalian photopigments, the "L/M" and "S" cone designations in rodents refer to the equivalent human red/green and blue photopigments, respectively.) Human red and green cone opsin genes are tandemly arrayed head-to-tail on the X-chromosome (Feil, Aubourg, Heilig, & Mandel, 1990) and are highly homologous in sequence (Nathans, Thomas, & Hogness, 1986). This suggests a

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recent duplication of a common red/green ancestor to create separate genes (Ibbotson, Hunt, Bowmaker, & Mollon, 1992; Jacobs & Neitz, 1987). In contrast, the autosomal blue cone opsin gene diverged over 10^8 years earlier, before the vertebrate radiation. The similarity between human red and green gene sequences extends upstream to about −195 bp, thus highlighting this small upstream domain to likely contain important element of the cone promoter.

In the initial sequence analysis and in subsequent transgenic mouse assays of cone-promoter function, a highly conserved 37 bp element was noted at about −3.5 kbp in the human red opsin gene (Nathans et al., 1986; Wang et al., 1992). Since a DNA region containing this element was required in transgenic mice for transgene expression independent of integration site, it was termed a locus control region (LCR) according to convention (Kioussis & Festenstein, 1997). In addition to the LCR, a proximal region of red or green opsin gene upstream sequences was also required for measurable expression. When a human red opsin gene that included the LCR in 9 kbp of upstream sequence was inserted into transgenic mice, mouse cones expressed the human transgene at levels equivalent to the endogenous M opsin gene and the protein appeared to function normally (Jacobs et al., 1999; Shaaban et al., 1998). This suggested that (1) all *trans*-elements necessary for cone opsin expression are in sufficient excess in mouse cones to support transcription from additional copies of a cone promoter and that (2) mouse *trans*-elements can recognize human cone *cis*-elements. Interestingly, in these transgenic animals the human red photopigment was not only found in mouse M-cones as expected, but in S-cones as well. It therefore appears that either the human red gene does not contain regulatory regions recognized by mouse cone elements for cone subtype restriction or that transgene chromosome position effects had negated cone subtype restriction in the transgenic lines analyzed. Resolve of this issue provided one impetus to somatically deliver genes regulated by cone-promoter elements in rodent cones.

Another study of cone opsin promoter function employed a Weri RB cell line to assess patterns of transient expression (Shaaban & Deeb, 1998). In addition to noting several fairly ubiquitous SP1 and SP3 sites, one positive-acting DNA region at −93 to −109 bp that footprinted with Weri cell nuclear extract exhibited homology to the rod opsin Ret-1/PCE element (Ahmad, 1995; Morabito, Yu, & Barnstable, 1991) suggesting interaction with an RX-like element. Evidence for CRX-like binding was suggestive but less clear and NRL-DNA binding was concluded to be absent. The strongest footprint (−58 to −80 bp) had about a threefold positive effect in the *in vitro* Weri expression assay. A unique core sequence in this interval is conserved between human red and green opsin promoters and a homologue can be found in the human blue opsin promoter as well, prompting the reasonable suggestion that it may represent a cone-specificity domain. Unfortunately, this cell culture assay system does not act entirely cone like. Inclusion of the human cone LCR in test constructs had no effect on expression efficiency in Weri cells. In view of the LCR's very strong cone-specific enhancer effects in transgenic animals (Wang et al., 1992; Jacobs et al., 1999; Shaaban et al., 1998), this lack of an effect in cultured cells suggests that *in vitro* assay systems may not respond faithfully to important functional aspects of the cone opsin promoter, a second reason for developing an in vivo assay of cone-promoter functions.

Retinal disorders affecting cone photoreceptors, including inherited cone dystrophies, cone–rod dystrophies, rod–cone dystrophies, as well as complex diseases such as age-related macular degeneration (AMD), severely impair visions of millions of people in the world, and there is no effective treatment currently. Recent progress toward safe and effective retinal gene therapies has focused on recombinant viral gene delivery techniques in three general areas. (1) Gene augmentation therapy for lack-of-function mutations such as null mutations in the βPDE gene has shown structural and biochemical rescue in the *rd* mouse (Bennett et al., 1996; Takahashi, Miyoshi, Verma, & Gage, 1999). (2) Allele-specific targeting for dominant-negative mutations, such as anti-P23H rod opsin ribozymes delivered by recombinant adeno-associated virus preserved photoreceptors for more than three months in a transgenic rat model for P23H RP (LaVail et al., 2000). (3) Neurotrophin gene therapy using recombinant adenovirus carrying a CNTF cDNA has lead to structural rescue of photoreceptors for several months in the *rd* (Liang et al., 2001) and *rds* (Ali et al., 2000) mouse models of RP and in the P23H transgenic rat model (Bok, Yasumura, Matthes, & Ruiz, 2002). In each, very encouraging "proof-of-concept" experiments in animal models of RP have been reported (see Dinculescu, Glushakova, Min, & Hauswirth, 2005 for recent review). Although this bodes well for degenerative retinal diseases of rods, cone degeneration due to defects in cone-specific genes have, by comparison, received little experimental attention, yet they encompass an important subset of retinal dystrophies. This, then, provides a third reason: the need for an in vivo assay of cone-specific promoters.

One logical step toward a remedy for this situation and possibly aiding in the development of reagents for cone diseases is to identify promoters that will support efficient and specific expression in cone photoreceptors. We report here cone-specific expression in rats, ferrets and guinea pigs using a relatively short promoter element from the human red cone opsin gene packaged into a recombinant AAV vector.

2. Materials and methods

2.1. Recombinant AAV plasmids

The plasmid pR2.1-LacZ containing the 2.1 kb fragment of the upstream sequence of human red opsin gene was generously provided by Dr. J. Nathans. This sequence contains a 1.6 kb BamHI–StuI fragment, extending from −3.1 to −4.6 kb joined to a proximal promoter of 495 bp of the human red pigment gene, and was cloned into the SmaI–NcoI

digested pLacF vector (Wang et al., 1992). The pR2.1-LacZ plasmid was digested with NcoI which was then blunt ended by a Klenow polymerase filling reaction and digested with KpnI to release the 2.1 kb fragment. This fragment was then ligated to a recombinant AAV vector, pTR-UF2, that had been digested with KpnI and XbaI in which the XbaI site was blunted by end-filling, to generate plasmid pR2.1-UF2, a cone promoter driving the GFP gene in an rAAV construct. The control rAAV plasmid mOp-GFP-rAAV contains a 472 bp proximal promoter of the mouse rod opsin gene as described previously (Flannery, Zolotukhin, Vaquero, LaVail, et al., 1997). Plasmid DNA for each construct was isolated using the alkaline lysis method and purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

2.2. Packaging of rAAV

The plasmid DNA, pR2.1-UF-2, or mOp-GFP-rAAV was packaged into recombinant virus by transfection of human 293 cells along with helper plasmid DNA (pDG) (Grimm, Kern, Rittner, & Kleinschmidt, 1998) using the calcium phosphate method (Zolotukhin, Potter, Hauswirth, Guy, & Muzyczka, 1996; Zolotukhin et al., 1999). This helper plasmid contains wild type AAV genes encoding REP and CAP proteins, as well as some early gene product of adenovirus required for packaging rAAV. The transfected cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 37 °C and were harvested 48 h after transfection. The cells were lysed by freeze/thaw three times and the recombinant AAV particles were purified by iodixanol step gradient centrifugation and concentrated through heparin column. The titer of rAAV was determined by quantitative-competitive PCR (QC-PCR) and infectious center assay (McLaughlin, Collis, Hermonat, & Muzyczka, 1988).

2.3. Subretinal injection of rAAV

All animals were treated in accordance with federal, state, and local regulations as well as with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult rats and guinea pigs were anesthetized with ketamine/xylazine (1:1 mixture) through intramuscular injection. The pupils were then dilated with 2.5% of phenylephrine and 1% atropine followed by administration of topical anesthetic (0.5% proparacaine HCl) to the cornea. Subretinal injection was performed by inserting a blunt 32 gauge needle between the retina and retinal pigmented epithelium and injecting 2 μl of the rAAV (5×10^8 infectious units/ml) in rats and 10 μl in ferrets and guinea pigs under direst visualization using a dissecting microscope (Timmers, Zhang, Squitieri, & Gonzalez-Pola, 2001). Contralateral control eyes were injected with rAAV carrying the same GFP reporter gene under the control of the mouse proximal rod opsin gene promoter described above (Flannery et al., 1997).

2.4. Animal tissue preparation

Animals were sacrificed 4–6 weeks after injection and perfused with 4% of paraformaldehyde/0.1 M phosphate buffer (pH 7.2). The eyes were immediately removed and fixed by immersing in the same fixative for two hours at 4 °C after puncturing the cornea to allow penetration of fixative. Following removal of the cornea and lens, eyecups were cryoprotected in 30% sucrose in PBS for several hours or overnight prior to quick freezing in optical cutting temperature (OCT) compound and 14 μm thick sections cut at −20 to −22° C. The sections were mounted on gelatin-coated slides and were either used immediately or stored at −20 °C in the dark.

2.5. Immunocytochemistry

Cone photoreceptor cells were identified by staining tissue sections with FITC-conjugated peanut agglutinin (PNA) and cone opsin specific antibodies. PNA was diluted in PBS according to the instructions of the supplier (Vector Laboratory, Burlingame, CA). A mouse M-cone opsin specific monoclonal antibody, COS-1 (Szel et al., 1986), was diluted 10,000× in PBS solution. Polyclonal antibody JH455 against the mouse blue opsin (kind gift by Dr. Nathans; Chiu & Nathans, 1994) was diluted 5000× in the same buffer. Cryosections were preincubated with blocking reagent for 30 min at room temperature and then incubated with specific antibody overnight at 4 °C. After removal of the primary antiserum and washing with PBS, the sections were incubated with either fluorescein isothiocyonate (FITC) or CY3-conjugated secondary antibody. Slides were coverslipped using Pro-long anti-fade mounting medium (Molecular Probes) and the antibody labeling was examined in a Zeiss microscope equipped with epifluorescence illumination and a high-resolution digital camera.

For double labeling studies, sections were incubated with a combined mixture of the M and S opsin primary antibodies and/or PNA. Separate images of fluorescein or CY3 fluorescence were captured from the same local in a section and digitally overlaid. Control experiments without primary antibody demonstrated that the secondary antibodies did not cross-react with the inappropriate primary antibodies and that fluorescent signals did not bleed through the incorrect channel.

3. Results

3.1. A vector for cone photoreceptor-specific gene expression

To construct a recombinant AAV containing the GFP reporter gene driven by candidate cone-specific promoter, a 2.1 kb fragment spanning about 4.6 kb upstream of the human red pigment gene with a 2.5 kb internal deletion between −3.1 kb and −0.5 kb was inserted into a pTR-UF2 vector (Zolotukhin et al., 1996) to drive GFP reporter gene expression. The resulting construct, pR2.1-GFP-UF2 AAV, is shown in Fig. 1A. As a control, the mouse rod opsin proximal promoter (+86 to −385) was used in the same vector (Fig. 1B). This promoter has been shown to drive photoreceptor-specific expression in mice and rats (Flannery et al., 1997). These constructs were then packaged into rAAV particles by transfecting human 293 cells along with a helper plasmid which provides all the proteins required for packaging in *trans* (Zolotukhin et al., 1996). The rAAV particles were then purified and titered.

The rAAV was injected into the subretinal space of adult animals. For each animal, one eye was injected with

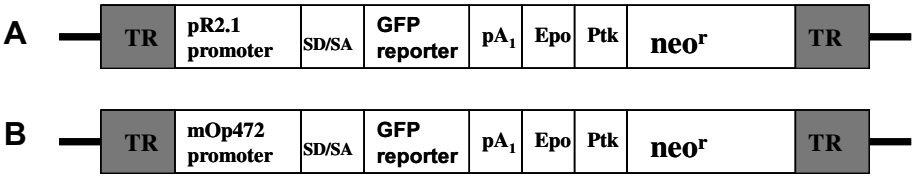

Fig. 1. Schematic diagram of the plasmid constructs used to produce rAAV. (A) Mouse rod opsin proximal promoter driving GFP (mOps-GFP) rAAV cassette. (B) Human L/M cone opsin promoter driving GFP (pR2.1-GFP) rAAV cassette.

the cone-promoter driving GFP-AAV and the other was injected with the control vector (mouse rod opsin proximal promoter driving GFP-AAV). Injected eyes were removed after 4–6 weeks and the retinas fixed and dissected free of RPE and vitreous. Initially, GFP fluorescence was visualized directly from agarose embedded retina sections using an epifluorescence microscope. For subsequent immunostaining and photographing, GFP antibody (Clontech) was employed and subsequently detected using fluorescent dye-conjugated secondary antibody. Fig. 2A shows an

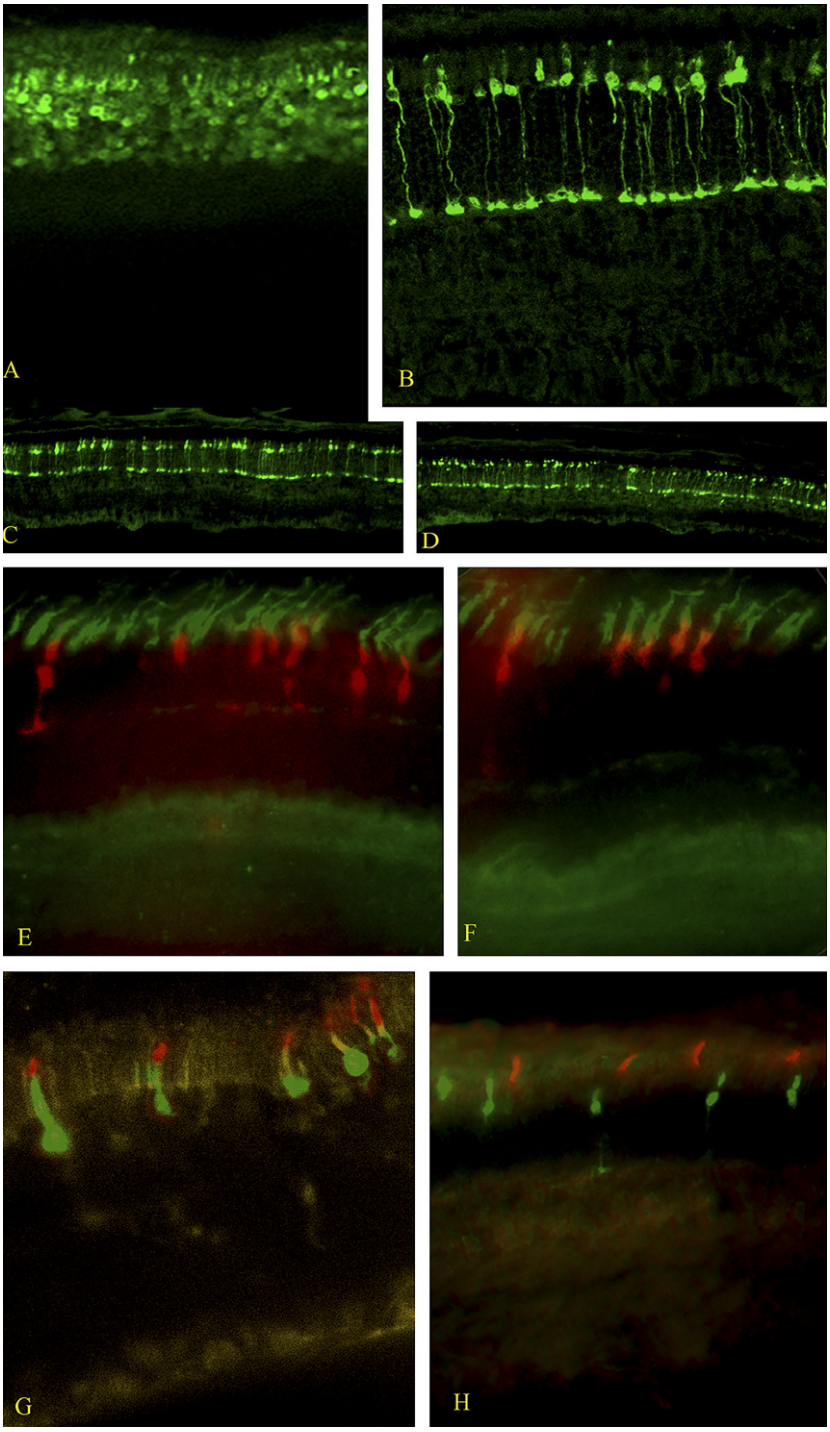

Fig. 2. GFP reporter gene expression in photoreceptor cells of rats and ferrets. (A) GFP expression in rod photoreceptors of rat retina injected with control rAAV containing a mouse rod opsin promoter. (B) GFP expression in photoreceptors of rat retina injected with pR2.1-GFP rAAV. (C and D) GFP expression in photoreceptors of ferret retina injected with pR2.1-GFP rAAV-lower magnification of 2 continuous fields showing GFP expression in photoreceptors of ferret retina injected with pR2.1-GFP rAAV. (E and F) PNA staining of all the cone types in rats in GFP expressed photoreceptor cells. (G) COS-1 monoclonal antibody (L/M cone-specific) staining of the transverse section that expressing GFP. (H) Blue cone specific antibody staining of the retina sections expressing GFP.

example of GFP expression in rod photoreceptors of rat retina injected with control AAV containing mouse rod opsin proximal promoter driving GFP. Panel B show examples of GFP expression in the rat retina (Fig. 2B) and ferret retina (Fig. 2C and D) that had been injected with the pR2.1-GFP-rAAV. The region of GFP-positive photoreceptors extended more than half the retina, equivalent to the transduced area previously noted for the rod promoter (Flannery et al., 1997). GFP protein is localized to the inner segment, cell body and synapse of photoreceptor cells, as expected for a cytoplasmic protein (Fig. 2B). The frequency of GFP-positive photoreceptors however is markedly lower than when the rod opsin promoter vector was used (compare Fig. 2B with Fig. 2A). Approximately one in 50 photoreceptor cells supported GFP expression with the cone opsin promoter. Additionally, a clear gradient in the frequency of transduced cells exists in that near the injection site, the transduction efficiency is much higher than in areas more distal to the injection site. A similar result was obtained when adult ferrets were inoculated with the cone opsin promoter vector.

3.2. Identification of the cone subtype expressing GFP

Recent studies have shown the presence of two photopic spectral sensitivities and at least two cone types in a number of rodent species by immunocytochemical studies using cone-specific visual pigment antibodies (Szel et al., 1986; Govardovskii, Rohlich, Szel, & Khokhlova, 1992; Szel, Diamantstein, & Rohlich, 1988; Szel & Rohlich, 1992; Szel et al., 1992). Clearly the density of cones relative to rods and the ratio and topographic distribution of the two types of cones are different among different species (Govardovskii, Rohlich, Szel, & Khokhlova, 1992; Szel et al., 1988; Szel et al.,1992). One cone subtype which was recognized by monoclonal antibody specific to middle-to-long wavelength-sensitive visual pigments, contains a visual pigment that is highly homologous to red and green pigments of primates. The other subtype which was recognized by antibody specific to the visual pigment sensitive to short wavelengths, contains a visual pigment highly homologous to blue pigment of primates (Szel et al., 1986).

To further confirm that the GFP expressing cells in Fig. 2B are indeed cones, we performed immunocytochemistry using cone opsin specific antibodies and peanut agglutinin (PNA). PNA labels the matrix sheath of all subtypes of cone photoreceptors, as well as the outer and inner segment of L/M-cone photoreceptors in rodents. Fig. 2E and F shows examples of PNA and GFP antibody double labeling results from two different injected rat retinas. In this case, GFP-positive cells are visualized by CY3-conjugated secondary antibody, and cones by FITC-conjugated PNA. It is clear that the GFP was expressed in the same cone photoreceptors labeled by PNA but in different compartment. To identify the subtypes of these GFP expressing cone photoreceptors, L/M-cone opsin and S-cone opsin specific antibodies were used. Examples of the double labeling of GFP antibody and L/M-cone opsin, and S-cone opsin specific antibodies are shown in Fig. 2G and H, respectively. In this case, GFP-positive cells are green, and cone opsin specific antibody labeled cells are visualized by Cy3-conjugated secondary antibody, and therefore red. GFP obviously was expressed in the same photoreceptors that are labeled by L/M-cone opsin specific antibody COS-1 (Fig. 2G), but not in cones that are labeled by S-cone opsin specific antibody JH455 (Fig. 2H) (Chiu & Nathans, 1994). Therefore these GFP-positive cells are cone photoreceptors, indicating that this 2.1 kb human red/green opsin promoter directed L/M cone-specific expression in rat retinas. Additionally every COS-1 positive cone also showed GFP fluorescence as shown in Fig. 2G, indicating that the transduction efficiency of cone photoreceptor cells by rAAV is very high in the area surrounding the injection site. The different subcellular sites of green fluorescent and antibody-staining signals are clear, indicating that each protein is expressed in the different compartment of the same cone photoreceptor as expected. Opsin protein is localized in the outer segment while the GFP protein is expressed and remains localized in the inner segment of the cone photoreceptor as noted previously for rods (Flannery et al., 1997).

4. Discussion

Effective gene therapy of inherited retinal degenerations depends on a safe and efficient gene delivery system to target the specific cell types affected by the causative mutations. Recombinant AAV vectors provide many advantages over other viral vectors in their broad host range (Buller, Straus, & Rose, 1979; Casto, Armstrong, Atchison, & Hammon, 1967), their ability to infect both mitotic and post-mitotic growth-arrested cells at high efficiency, their ability to accept non-viral regulatory sequences without interference from the packaged viral sequences, and the lack of any associated human disease (Berns & Giraud, 1995). The specificity of targeting to a particular cell type thus is determined by the functionality of the cell-type specific promoter and the site of inoculation.

The results presented here demonstrate several features of the human cone opsin promoter with regard to its therapeutic potential. First, 2.1 kb of the human red/green opsin gene upstream sequence is sufficient to target cone photoreceptor-specific expression of the reporter gene in rats and guinea pigs (data not shown) using recombinant AAV as a vector through subretinal injections. This regulatory sequence extends to −4.6 kb upstream of the human red pigment gene and contains a 2.5 kb internal deletion spanning the region from −3.1 to −0.5 kb. Thus the promoter used contains a proximal (495 bp) and an upstream domain from −3.1 to −4.6. This upstream domain contains a locus control region (LCR) that is essential for expression of both red and green pigment genes in human and is highly conserved among human,

cow, and rats (Wang et al., 1992). It is interesting that even though non-primate mammals have only a single copy of the visual pigment gene, they retain a LCR domain homologous to that of the red/green pigments of human. Second, the expression of the GFP reporter gene is cone specific as demonstrated by cone-specific antibody co-localization. It is interesting to note that this 2.1 kb human red/green opsin gene promoter is capable of directing L/M-cone, but not S-cone-specific reporter gene expression in rats. This is different from the observation by Wang et al. (Wang et al., 1992) in which the same 2.1 kb human red/green opsin gene promoter has been shown to direct reporter gene expression in both L/M- and S-cone photoreceptors in transgenic mice. Such discrepancy could be due to the species difference. It has been shown that the rat retina contains less than 1% cone photoreceptors, the majority of which (93%) are L/M-cones, and the distribution of the two cone types is nearly uniform throughout the rat retina (Szel & Rohlich, 1992). However in the mouse retina, cone photoreceptors, which make up about 3% of all photoreceptors, follow an uneven distribution in which the L/M-cones exist exclusively in the dorsal half of the mouse retina, while most S-cones occupy the ventral half with a small number scattered among the L/M-cones in the dorsal retina (Szel et al., 1992). It could also be due to the different experimental procedures. In transgenic mice, the reporter gene expression could have different patterns of tissue and developmental stage-specific expression due to random integration of target gene with different copy number, whereas in our rAAV mediated delivery system, the reporter gene expression is not influenced by chromosomal structure. It is also possible that different serotypes of AAV also have different trophisms for different cell types, independent of promoter used. In this experiment, we used serotype 2, but recently we and other groups (unpublished and personal communications) have found that serotypes 5 and 9 transduce photoreceptor and RPE much more efficiently. We have not compared different serotypes with this particular promoter construct and we do not know with higher transduction efficiency for photoreceptor with AAV5 and AAV9, whether this promoter supports reporter gene expression in S-cone also.

Cone-specific expression of GFP driven by a human red/green pigment gene promoter in rats and guinea pigs indicates that this promoter is also functional in this species despite the significant differences in color vision perception and significant divergence between the two species. This suggests that many of the key regulatory elements for photoreceptor-specific gene expression are broadly conserved in mammals. Third, the efficiency of transduction of cone photoreceptor cells surrounding the injection site is very high, as demonstrated by the fact that every COS-1 antibody-staining cone is also positive for GFP expression near the injected area of the retina. This implies that high efficient transduction may also be achievable in the cone-rich macula of primates.

In conclusion, we have shown that cone photoreceptor cells can be specifically targeted at high efficiency by using a human promoter of the red/green pigment genes mediated by rAAV and delivered via subretinal injections. This has significant implications for the therapy of retinal degenerations that primarily affect cone photoreceptors. These include macular degeneration, cone dystrophies, and cone–rod dystrophies as well as the later stages of retinitis pigmentosa in which cone photoreceptors also degenerate in response to loss of rod photoreceptors. In addition, this rAAV mediated photoreceptor-specific gene delivery system should provide a direct in vivo functional assay system for identifying important *cis*-regulatory elements in cones. Understanding the regulatory mechanisms that control which cell or cells express a particular retinal gene at specific levels is key to developing an efficient and safe retinal gene therapy.

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