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Does recombinant adeno-associated virus-vectored proximal region of mouse rhodopsin promoter support only rod-type specific expression in vivo?

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Purpose: We have previously found that the -385 to +86 portion of the mouse rod opsin promoter (mOP500) can limit recombinant adeno-associated virus (rAAV)-mediated transgene expression to photoreceptor cells when delivered subretinally [1]. However, the photoreceptor (PR) subtype-specificity of expression remains unclear. Here, we evaluated whether the presence of certain cis-elements in this proximal promoter, such as the rod-specific, neural retina leucine zipper protein (NRL) response element (NRE), can render it a driver of rod-specific expression.
Methods: Subretinal injections of a serotype 5 rAAV vector carrying the green fluorescent protein (GFP) cDNA, driven by mOP500, were administered to male Sprague-Dawley rats at postnatal day (P) 40-48. Two weeks to eight months later, the distribution of GFP-expressing cells in the retina was characterized by GFP-, cone-specific alpha-transducin-immuno-, and peanut agglutinin-lectin histochemistry and by morphological criteria. The same viral suspension was also injected sub-retinally into rhodopsin-knockout rho (-/-) mice either at P18 or P78, and retinas were analyzed by immunohistochemistry and PNA lectin histochemistry two weeks later.
Results: GFP reactivity was found exclusively in the outer nuclear layer (ONL) of rat retinas two weeks after treatment, with abundant reporter gene expression observed in both rods and cones. GFP-positive cones, defined by their typical morphology and the co-linearity of PNA-lectin labeling with GFP-immunoreactivity, were found in all regions of the transduced retinas. GFP-positive cones constituted up to 6% of the total GFP-positive photoreceptors. By eight months post-injection, a low level of GFP-reactivity was additionally observed in the inner nuclear layer (INL) and ganglion cell layer. Photoreceptor-specific GFP expression was also seen in the rho (-/-) mice at both ages tested. In pups injected at P18, costaining with PNA-lectin revealed that up to 15% of the GFP-positive photoreceptors were cones. Despite only a single row of photoreceptors remaining in these knockout mice by P90, numerous GFP-positive cones were still present.
Conclusions: Subretinal delivery of rAAV5 harboring a reporter gene driven by mOP500 results in passenger gene expression in both rod and cones, indicating that this promoter is photoreceptor-specific but not rod-specific. The lack of photoreceptor subtype-specificity suggests that although cones do not express the NRL and NR2E3 trans-factors considered necessary for activation of mOP500 [2-5], other general transcription factors in cones may compensate.

Light detection in vertebrates is mediated through retinal rod and cone photoreceptor cells that are able to transduce light signals into electrical responses via a series of biochemical signal amplification events. In general, rod photoreceptors are sensors of dimlight, while cones are specialized in bright light and color sensing [6]. Rhodopsin is the highly specialized G protein-coupled receptor that detects photons in the rod photoreceptors of the vertebrates. Results obtained from comparisons of promoter sequences and patterns of rhodopsin-specific expression in transgenic animal models provide evidence that rhodopsin gene expression is highly conserved in mammals [7-9] and probably in vertebrates [10].

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The rod cell-specific expression of rhodopsin is tightly regulated and temporally controlled, primarily at the level of transcription [11,12]. As is true for most cell type-specific promoters, transcriptional regulation occurs through a combination of different protein trans-factors binding to cis-elements within the rhodopsin promoter. Two distinct cis-regulatory regions are implicated in the control of rhodopsin expression. The distal element is the rhodopsin enhancer region (RER), a 100 bp sequence located approximately 2 kb upstream from the mRNA initiation site, which is responsible for high levels of rhodopsin expression [8]. The other element, known as the rhodopsin proximal promoter region (RPPR), is located within the -225 to +70 (Figure 1) [9,11,13-19]. Much interest surrounds the RPPR as this sequence is sufficient for exclusive PR expression and includes a number of control elements, such as the photoreceptor conserved element-1 (PCE-1/Ret-1) [20,21], BAT-1 [11], eopsin-1 (E-box) [19], Ret-4 [9], and neural retina element (NRE) [18]. Multiple transcription activators bind to the RPPR and are involved in the regulation of rhodopsin expression, PR cell differentiation and morphogen-

esis [2,22-24]. As it is currently understood, rod-type specific expression is achieved by combination and synergistic action of four main specific activator proteins, neural retina leucine zipper protein (NRL) [18], photoreceptor-specific nuclear receptor N2E3R [22,25-27], orphan nuclear receptor NR1D1 [3], and cone rod homeobox protein CRX [23,28,29] which together efficiently recruit the basal transcriptional machinery to the promoter region [3,30] (Figure 1). Expression of NR2E3 is undetectable in the Nrl (-/-) retina and the absence of NRL or NR2E3 results in loss of rod function and rod-specific phototransduction proteins, with a concomitant increase in S-(short wavelength or blue) opsin and other cone-specific proteins [2,5,22,24,31-33]. This phenotypic conversion of rods to a cone-like morphology occurs in Enhanced S-Cone Syndrome (ESCS) and Goldmann-Favre Syndrome in humans and in the rd7 mice, both characterized by a lack of these transcription factors [22,26,31-33].

Phenotypic similarities between retinas lacking NRL or NR2E3 support the notion that NR2E3 functions in rods and is downstream of Nrl in the transcriptional regulatory hierarchy. NRL appears to act as a molecular switch during photoreceptor (PR) differentiation by promoting rod development while simultaneously suppressing cone identity [2]. The expression of NR2E3 is one of the earliest events in the pathway of rod specific photoreceptor development and it acts in concert with CRX either directly or indirectly, as a repressor of cone-specific genes in rod photoreceptor cells [4,27,31]. Consistent with these observations, NRL and NR2E3 transcription factors are undetectable in cone photoreceptors [2-4]. Less is known about negative regulation of rod-specific transcription. Fiz1, a zinc-finger protein, attenuates transactivation of rhodopsin promoter by interaction with NRL [34] and KLF15, a member of the Sp/Kruppel-like factor family of zinc-finger transcriptional factors that binds to multiple consensus sites in the rhodopsin promoter including SRS-1 and G-rich repressor elements [35].

The use of rAAV vectors for gene delivery to cells of the retina is efficient and safe because AAV can infect nondividing cells is replication defective and lack viral coding sequences that induce immune responses to viral proteins [36-38]. Although rAAV transgene expression specificity can be modulated by utilizing a cell-specific promoter [39], the contribution of the AAV capsid is also significant [37,40,41]. The capsid protein sequences of the 11 AAV serotypes identified so far have considerable differences, and vectors constructed from these show variability in their capacity to transduce different cells. This implies distinct receptor and co-receptor requirements for cell entry [42,43] and possibly diverse interactions with host factors post-entry that may affect the conversion of single-stranded vector genomes into transcriptionally active forms [44-48].

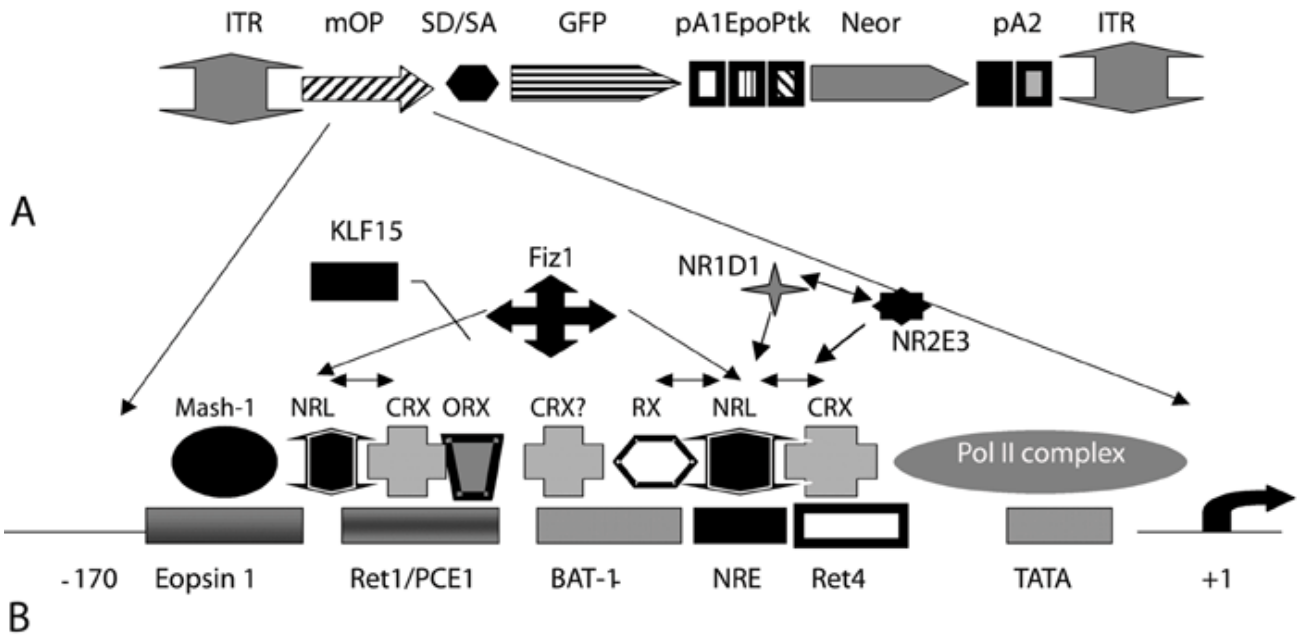

Figure 1. rAAV vector construction. **A**: Schematic diagram of the pTR.mOP500.GFP plasmid DNA used to make the rAAV2/5.mOP500.GFP virus. ITR represents AAV2 internal terminal repeats; mOP500 represents murine rod opsin regulatory sequence from -385 to +86; SD/SA represents SV40 late viral protein gene 16S/19S splice donor and acceptor signal; GFP represents coding sequence for the synthetic green fluorescence gene; Neor represents coding sequence for the neomycin resistance gene; pA1 and pA2 represents polyadenylation signals; Epo represents a tandem repeat of the polyoma virus enhancer region; Ptk represents thymidine kinase promoter of the herpesvirus, Tn5. **B**: Schematic diagram of mOP500 depicting the location of known cis-elements and their corresponding trans-acting factors. Cis-elements include Eopsin-1, PCE-1/Ret-1, BAT-1, NRE, and Ret-4. Transcription factors include Mash-1, CRX, ORX, RX, NRL, NR2E3, NR1D1. Negative trans-elements include Fiz-1, a zinc-finger protein that attenuates transactivation of rhodopsin promoter by interaction with NRL, and KLRF-1, a repressor.

In the retina, AAV2 has been tested more extensively than the remaining serotypes, and subretinal injections of AAV2 in mice consistently transduce the retinal pigmented epithelium (RPE) and some PR cells [37,49-51]. However, pseudotyped AAV5 (an AAV2 genome packaged in an AAV5 capsid, or AAV2/5) is much more efficient at transducing PRs and RPE cells and has faster expression kinetics relative to AAV2 when introduced subretinally [37,41]. This difference is considered important as most retinal degenerative diseases are caused by mutations in genes specifically expressed in PR cells [52].

The ultimate goal of our research is to use gene therapy to cure retinal diseases caused by PR degeneration. To this end, we have used rAAV to investigate the function of opsin promoters in the hope of deliberately targeting therapeutic genes to PRs. A portion of the RPPR (-385 to +86) has previously been reported to direct PR-specific transgene expression following rAAV2-mediated delivery into the retina [1]. However, the PR subtype-specificity of this promoter has not been analyzed. We used rAAV2/5-mediated delivery in the Sprague-Dawley rat and the rho (-/-) mouse [53] in this study to establish if the RPPR, containing rod-supporting transcriptional elements, limits transgene expression to rod PRs only or if it can be extended in a cone-specific environment, as well.

METHODS

Animals: The use of all animals was conducted in accordance with the guidelines established in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the local Institutional Animal Care and Use Committee's regulations.

Male Sprague-Dawley rats were obtained from the Charles River Laboratories (Wilmington, MA), and rho (-/-) mice were received from the Department of Ophthalmology and Genetics at Tufts University School of Medicine (Boston, MA) [53]. All rodents were housed in the University of Florida Health Science Center Animal Care Services Facilities under a 12 h:12 h light-dark cycle.

Recombinant adeno-associated virus production and subretinal injection: The proviral plasmid, pTR.mOP500.GFP,

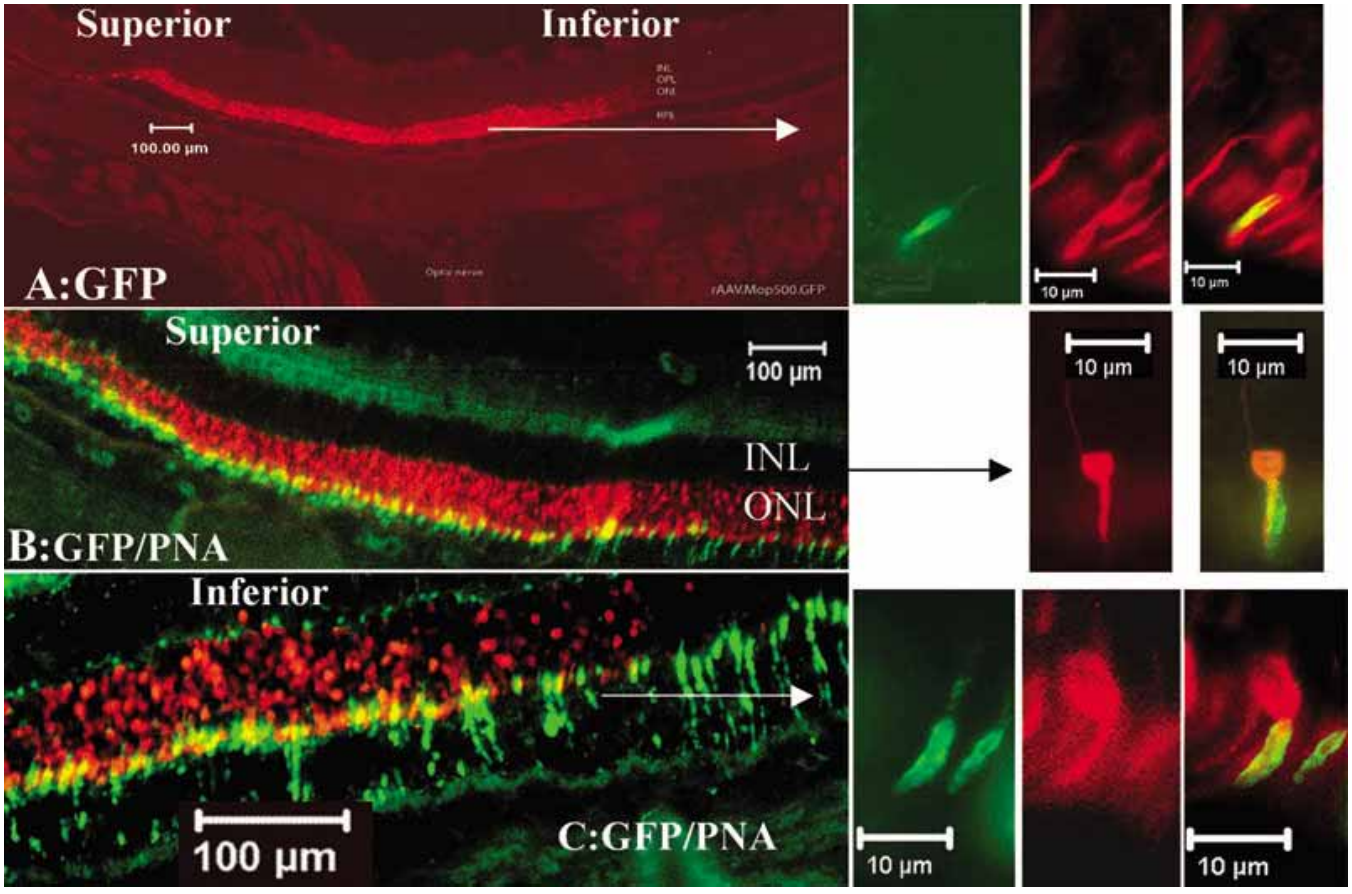

Figure 2. Green fluorescent protein expression is detected in both rods and cones of the Sprague Dawley rat retina two weeks after sub-retinal injection of AAV2/5.mOP500.GFP. Fluorescent micrographs of vertical sections from treated rat eye are presented. GFP expression (**A**; red emission) is exclusively localized to the outer nuclerr layer (ONL). Superior (**B**) and inferior (**C**) retinal fields are shown at increased magnification. Peanut agglutinin (green emission) detected on cone sheaths co-localizes with green fluorescent protein (GFP, red emission). On the right, selected central (**A**), superior (**B**), and inferior (**C**) retinal fields are shown at higher magnifications. All images are oriented with the sclera toward the bottom and the vitreous toward the top. The inner nuclear layer (INL) is identified.

was engineered as described previously in the literature [1] (Figure 1). Pseudotyped AAV5 virus was packaged by transient cotransfection of the human embryonic kidney 293T cell line with pTR.mOP500.GFP, and the helper plasmid, pXYZ5, which includes the AAV2 replication and AAV5 capsid open reading frames [54,55]. Recombinant AAV2/5 was purified using Q-Sepharose (Sigma-Aldrich, St. Louis, MO) anion exchange column chromatography, concentrated, then titered by real-time polymerase chain reaction (PCR) and an infectious center assay as previously described elsewhere [54]. The resulting virus, rAAV2/5.mOP500.GFP, had a biological titer of $2.6x10^{11}$ icu/ml, with the average ratio of physical to infectious particles being approximately 100:1.

Subretinal injections of the rAAV2/5.mOP500.GFP viral suspension (2 µl in P40-P48 rats and 1 µl in P18 and P78 mice) were made in the right eye of all animals as previously described [56]. Control injections were made in the contralateral eye with phosphate buffered saline (PBS) only.

Histological analysis and microscopy: At certain times after injection, the animals were euthanized by CO_2 inhalation. The superior point of the cornea was marked for orientation and the eyeballs were removed, opened by an encircling cut, and the lens and vitreous removed. Eyecups were fixed overnight in 4% paraformaldehyde (PF) and rinsed three times with PBS. Fixed eyecups were cryoprotected by overnight incubation at 4 °C in 30% sucrose solution, embedded in optimal cutting temperature (OCT) mounting medium (Sakura Finetek USA, Inc., Torrance, CA) and frozen in a bath of isopentane (2-methylbutane)-ethanol. Serial vertically oriented sections (10 µm) were taken throughout each entire eyecup on a HM 505 E Cryostat (Microton, Walldorf, Germany) and thaw-mounted onto gelatin-coated slides. The slides were air-dried and stored at -20 °C until processing for immunohistochemistry. For flatmount immunohistochemistry, the retinas were carefully detached from the posterior eyecups, fixed in 4% PF for 24 h at 4 °C and rinsed in PBS for 12 h at 4 °C before processing.

Rabbit anti-GFP polyclonal antibodies and the antirhodopsin monoclonal antibody, B6-3057, were kindly provided by Dr. Paul A. Hargrave (Department of Ophthalmology, University of Florida, Gainesville, FL) [57]. They were diluted to working concentrations of 1:1000 and 1:500 in blocking buffer (1X PBS with 5% bovine serum albumin [BSA] and 0.5% Triton X-100), respectively. GFP and rhodopsin expression was visualized by Cy3 goat-conjugate secondary antibodies (Sigma, St. Louis, MO), diluted 1:100 in blocking buffer, with a red channel (excitation and emission maximum at approximately 580 nm and 615 nm, respectively). The

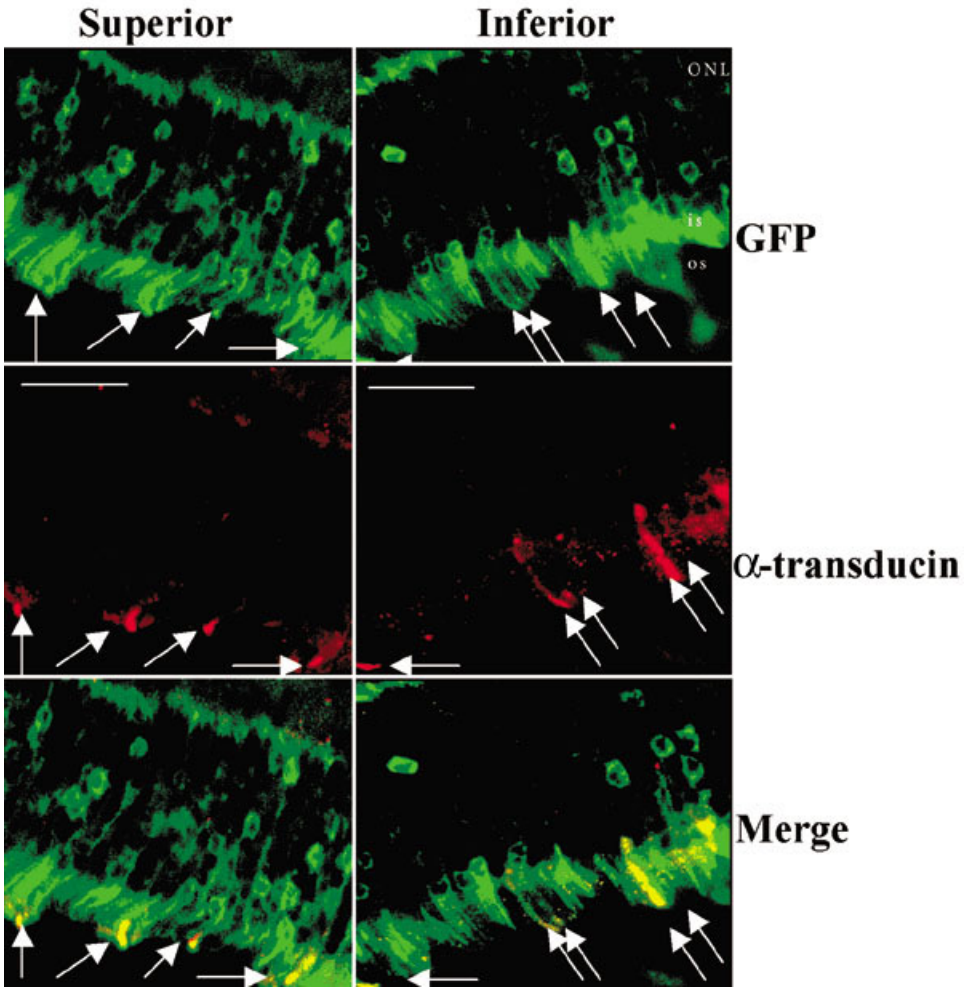

Figure 3. Patterns of cone transduction by AAV2/5.mOP500.GFP vector in treated rat eye: two weeks after sub-retinal injection. Fluorescent micrographs of retinal superior (Superior) and inferior (Inferior) fields are presented. Co-localization (Merge) of cone specific α-transducin (α-transducin) and GFP-transgene expression (GFP) in ONL are pointed by arrows. All cones revealed by α-transducin labeling from either inferior, or superior field are GFP-positive. The scale bar represents 30 µm.

rabbit polyclonal cone's α-transducin antibody (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted 1:100 in 1X PBS with 1% BSA and 0.2% Triton X-100. To identify all cone PRs in retinal sections, a biotinylated peanut agglutinin (PNA) primary (PNA-b, 5 mg/ml, Vector Laboratories, Burlingame, CA) and a fluorescein-streptavidin secondary antibody (st-Fluorescein, 5 mg/ml, Vector Laboratories) with a green emission channel (excitation and emission maximum at approximately 490 nm and 525 nm, respectively) were used at a dilution of 1:200.

Briefly, retinal sections were incubated for up to 1 h in blocking buffer and then incubated for either 4 h at room temperature or overnight at 4 °C with a mixture of antibodies to GFP and PNA-b or GFP and cone-specific α-transducin. For detection in retinal flatmounts, incubation in the above named primary antibodies was increased to 24 h. After three 10 min washes in 1X PBS, sections and flatmounts were incubated for 1 h at room temperature with their corresponding secondary antibody. The sections and flatmounts were then rinsed three more times in PBS and mounted under coverslips in Vectashield medium (Vector Laboratories).

Retinal sections were examined with Axioplan2 Research Fluorescent Microscope (Carl Zeiss Inc., Jena, Germany) using the ARC lamp (100 watt) with broadband FITC and Texas Red filters. The pictures were taken with a digital Spot Camera (software version 3.1, Diagnostic Instruments Inc., Sterling Heights, MI). The following magnification were used: 5x (Zeiss Fluar), 10x (Zeiss Fluax), 20x (Zeiss APO Chromat),

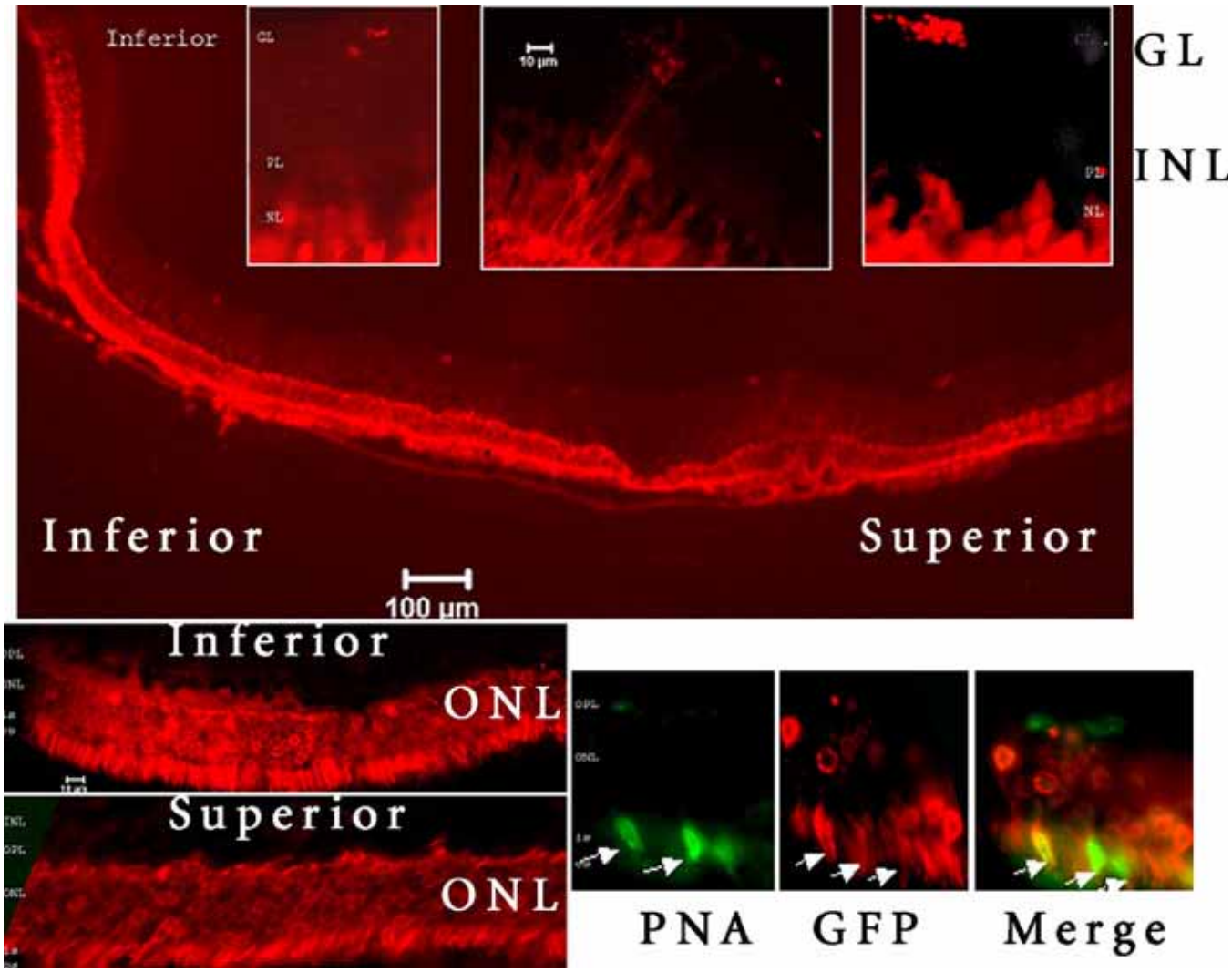

Figure 4. Green fluorescent protein expression persists in the Sprague Dawley rat retina eight months after subretinal injection of AAV2/5.mOP500.GFP. Fluorescent micrographs of frozen retinal section are presented. Green fluorescent protein (GFP) expression (red emission) is predominantly localized to the outer nuclear layer (ONL). The windows of increase magnifications on the top present superior, central and inferior fields of inner retina, and on the bottom, inferior and superior fields of outer retina. The selected field from central retina presents the co-localization (merge) of all cones sheathes (PNA; green emission) and GFP-transgene expression (GFP, red emission) at increased magnification. The arrows indicate PNA-lectin detected on cone sheaths co-localizes with GFP. The ganglion layer (GL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform (OPL), outer nuclear layer (ONL), outer segment (OS), and inner segment (IS) are identified.

40x (Zeiss Plan-Neo Fluar), and oil emersion DIC lenses 60x and 100x and the images were processed using PhotoShop Version 7 image analysis software (Adobe, San Jose, CA).

RESULTS

Opsin promoter confers photoreceptor-specificity of expression in the Sprague-Dawley rat: To evaluate the PR subtype-specificity of the proximal (-385 to +86) mouse rod opsin promoter, 2 µl of rAAV containing a gene for the green fluorescent protein (GFP) driven by this promoter was injected into the subretinal space of 10 male Sprague-Dawley rats at P40. After either two weeks or eight months, the eyes were removed, fixed and the distribution of retinal GFP expression examined by immunohistochemistry on serial 10 µm vertical sections. At two weeks postinjection, six retinas were analyzed and all were found to be efficiently transduced by the rAAV2/5.mOP500.GFP vector. Figure 2 shows the fluorescent micrograph of a typical transverse section taken from the retinal region directly spanning the presumptive injection site. Strong GFP expression was detected in the ONL and extended well into the retinal periphery with almost equivalent intensity. Image analysis of retinal flatmounts immunostained with the GFP antibody revealed that the area of GFP-positive PR cells resulting from a typical injection covered approximately 2/3 of the total rat retinal surface (data not shown). Costaining for PNA-lectin surrounding cone inner segment (IS) indicated that, in addition to rods, cones were also efficiently transduced with the GFP-expressing rAAV (Figure 2B,C). This expression pattern was independent of whether within central, inferior, or superior retinal fields were examined (Figure 2A-C). As both cone subtypes are evenly distributed across the rat retina [58], the even distribution of GFP-expressing cones could not distinguish between transduction of M- or S-cones. However, additional dual-immunostaining of retinal sections with GFP and cone-specific marker, alpha-transducin, that labels both M- and S-cones [59] supported the notion that all cones express the transgene (Figure 3). It therefore appears that vector transduction is independent of cone subtype.

In order to quantify the rod:cone ratio of GFP-positive PRs, we counted all individual cone sheaths associated with a specific GFP-positive PR, in regions that contained no fewer than 100 GFP-positive PRs, across all retinal fields in three sections. Morphological criteria, such as the close positioning of cone cell bodies to the inner limiting membrane and the characteristic structure of their inner segments, were also taken into account. Although rods make up the majority of PRs in the rat retinal ONL and the cone constituent is relatively low at less than 1% [58,60], we estimated that up to 6% of the GFP-positive PRs were cones.

After eight months, treated retinas from the remaining four rats were removed, fixed, and examined for GFP localization. The pattern of transgene expression was similar to that seen in retinas isolated at two weeks postinjection. A strong GFP signal was still detected in the ONL, particularly in the

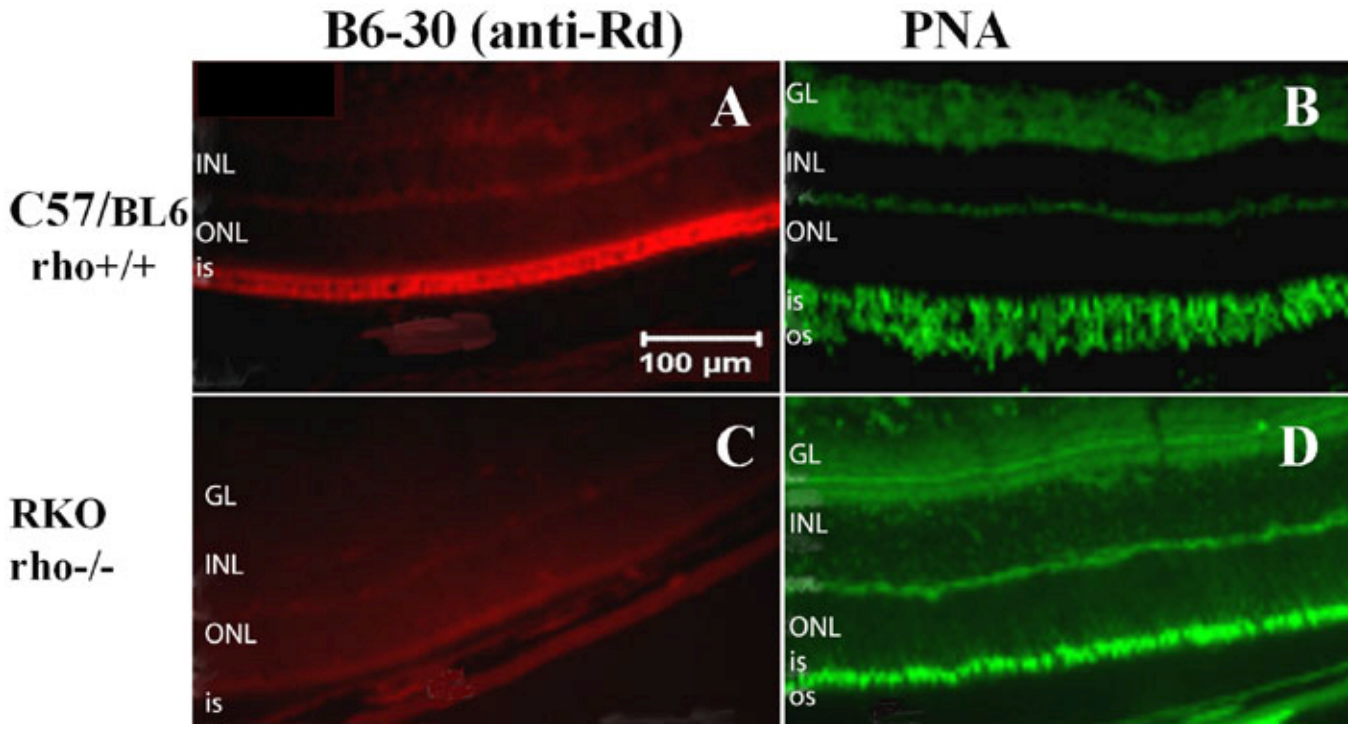

Figure 5. Degeneration of photoreceptors in rho (-/-) mice at P30. Fluorescent micrographs of vertical sections from a rho (-/-) mouse retina (**C**,**D**) and a control C57BL/6J (rho +/+) retina (**A**,**B**) probed with the rhodopsin antibody, B6-30 (**A**,**C**) and the PNA-Fluorescein (**B**,**D**) show the lack of rhodopsin protein and rod outer segments while cone's outer segments are still preserved. The ganglion layer (GL), inner nuclear layer (INL), outer nuclear layer (ONL), outer segment (OS), and inner segment (IS) are labeled. The scale bar represents 100 µm.

PR segments, synapses, and the periphery of the cell bodies (Figure 4). Moreover, the region of positive staining extended sporadically into the INL and ganglion cells, albeit with reduced intensity and frequency (Figure 4). Co-staining transverse retinal sections with the PNA-lectin indicated that most cones, if not all, still expressed the transgene (Figure 4).

Opsin promoter confers photoreceptor-specificity of expression in the rhodopsin knockout mouse: To confirm the PR-specificity of the proximal (-385 to +86) mouse rod opsin promoter in another animal model, we employed mice harboring a null mutation in both copies of the rhodopsin gene. No opsin mRNA and protein is detected in the retina of these rho (-/-) mice and although the retina initially develops normally, the rods fail to develop outer segments [53]. Furthermore, the thickness of the ONL progressively decreases in these animals from the normal 10-12 rows of PR nuclei at P15 to a single, fragmented row of mostly cone nuclei by P90.

Consistent with these observations, immunohistochemistry performed here found that at P30, retinas taken from rho (-/-) mice lacked rhodopsin protein (Figure 5C), although they did exhibit normal cone outer segments (Figure 5D). Hence, the preferential loss of rod PRs over cones in the rho (-/-) mouse model was deemed useful to our study of the PR-subtype specificity of the mOP500. Therefore, 1 µl of the AAV2/5.mOP500.GFP virus was injected subretinally into four rho (-/-) pups at P18 and four adult mice at P78. After two weeks, the eyes were removed, fixed and analyzed for localization of GFP.

Dual GFP-immunocytochemistry and PNA-lectin cytochemistry of retinal flatmounts isolated from treated pups at P30 revealed the transgene to be expressed in most, if not all, cones scattered across the central retina (Figure 6A). Transverse sections of these flatmounts indicated the transgene-expressing cones still contained well developed outer segments (Figure 6B). Furthermore, as seen in rho (-/-) retinas isolated at P30, the retinal ONL still contained numerous rows of rod nuclei, most of which were also efficiently transduced. As the normal mouse ONL is comprised of approximately 97% rods and 3% cones [60,61], transduced rods appeared to constitute the majority of GFP-positive PRs in the young rho (-/-) pups. However, the number of GFP-positive cones were counted as previously described and found to constitute approximately 15% of total GFP-positive PRs. Although the global cone density of the mouse retina is apparently uniform, it does exhibit a dorsal to ventral gradient in M- and S-cone subtype density [61,62]. Since the transduction efficiency appeared to be the

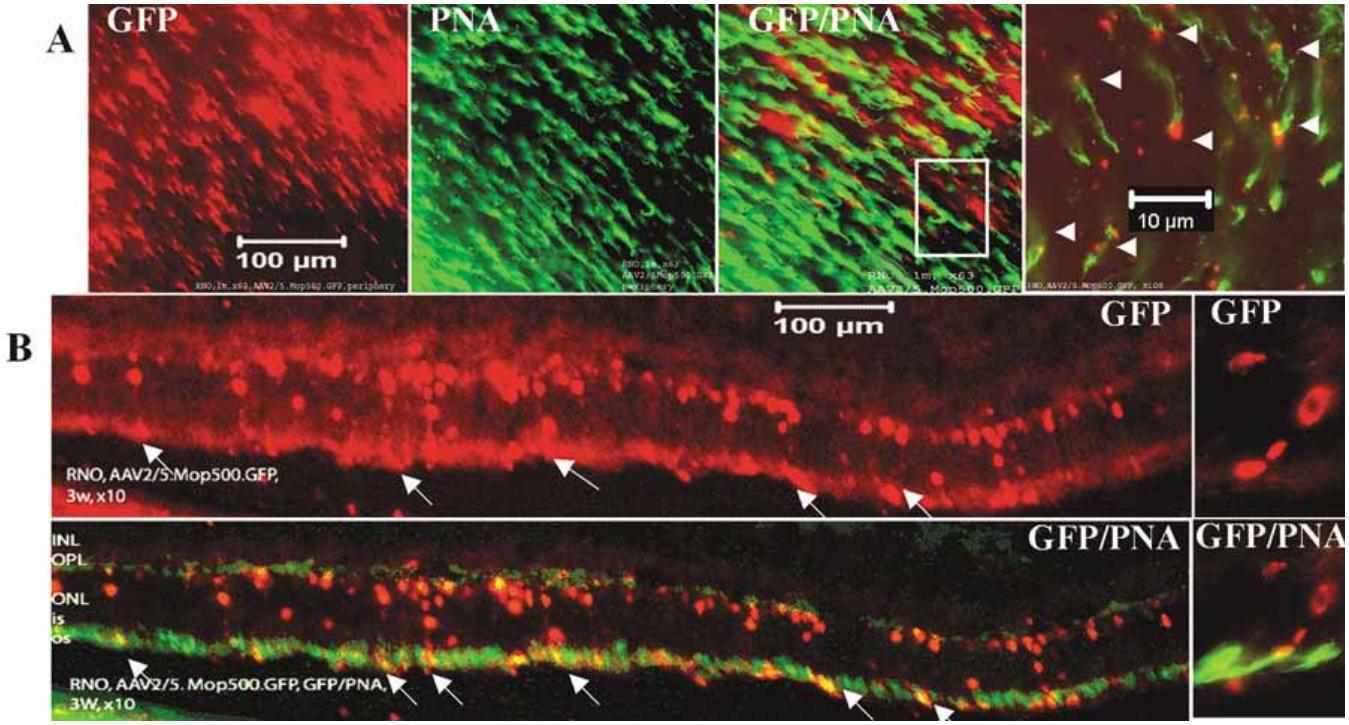

Figure 6. Green fluorescent protein expression is detected in both rods and cones of P30 rho (-/-) mouse retinas two weeks after subretinal injection of AAV2/5.mOP500.GFP. **A**: Fluorescent micrographs showing flatmount of central mouse retina: green fluorescent protein (GFP) expression (red emission), peanut agglutinin (PNA)-lectin binding (green emission); merge, GFP/PNA, detected on cone sheaths co-localizes with GFP. Right: A window depicts higher magnification of a selected field. **B**: Vertical section taken from the central retina. GFP expression (red emission); GFP/PNA co-localization of PNA-lectin binding (green emission) to cone sheaths with GFP-transgene expression. On the right, there is a selected field at higher magnification. The inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and outer segment (OS) are identified. Arrows indicate PNA-lectin detected on cone sheaths co-localizes with GFP. All images are oriented with the sclera toward the bottom and the vitreous toward the top. Scale bars are indicated for each magnification.

same in either superior or inferior retinal fields, there was apparently no preference for M- or S-cone transduction (data not shown).

Retinas isolated from the adult rho (-/-) mice treated with AAV2/5.mOP500.GFP displayed similar ONL degeneration by P90 to that originally described [53], with just one row of mostly GFP-positive cone nuclei (Figure 7). GFP co-labeling with PNA-lectin revealed the outer segments of the remaining cones to be greatly shortened and decayed, although their cell bodies, synapses, and inner segments were still preserved. As for the treated pups, the transduction efficiency appeared similar when viewed from either the superior or inferior retinal fields, indicating no subtype preference in the pattern of cone transduction.

DISCUSSION

Using two rodent models of subretinal rAAV2/5-delivery and the cell-specific mOP500 promoter, we have demonstrated targeted transfer and expression of a reporter gene to both rod and cone PRs. These studies have also validated the utility of rAAV for characterizing interactions between cognate trans-acting factors and the cis-acting response elements in promot-

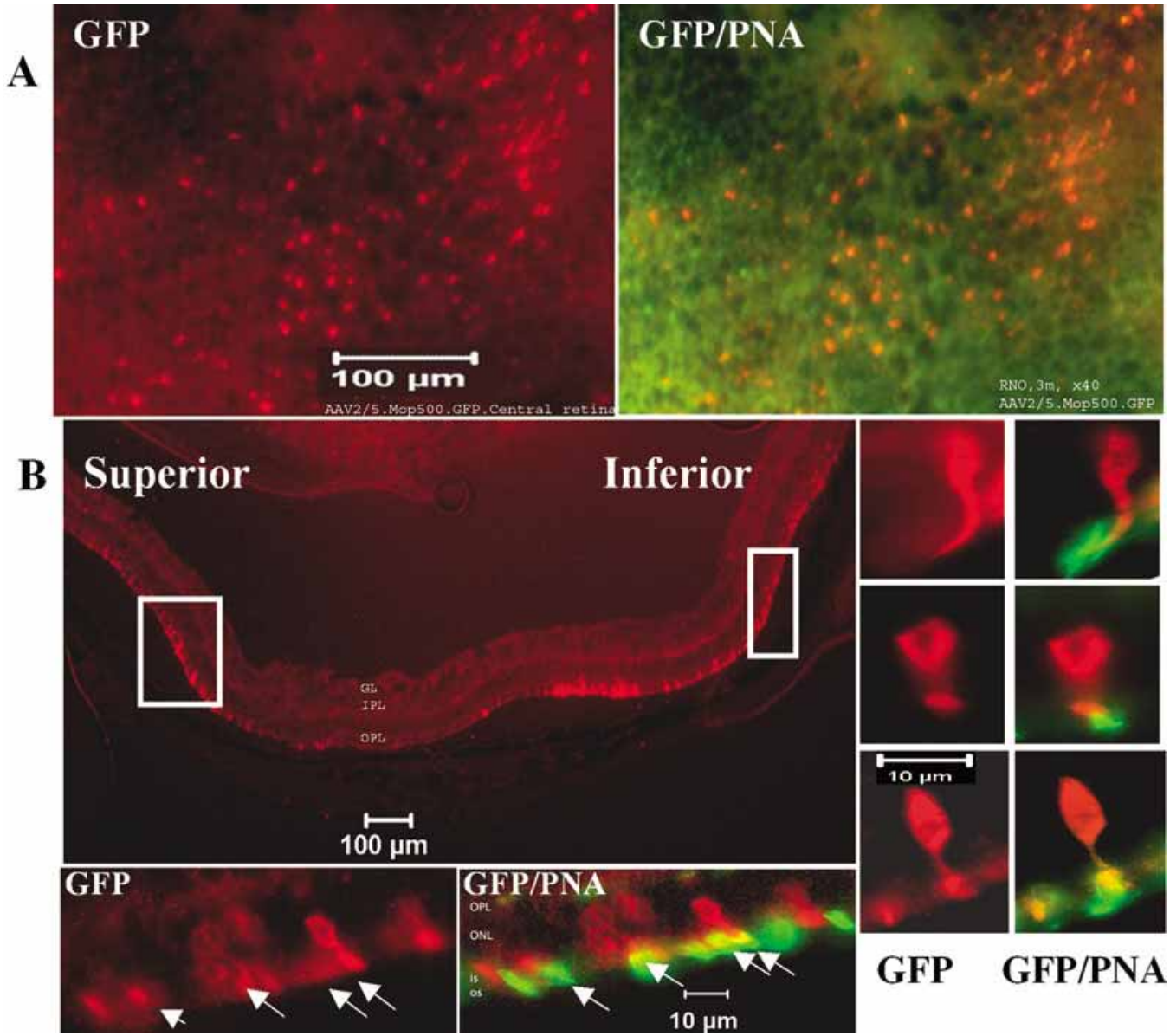

Figure 7. Green fluorescent protein expression is detected in degenerating cones of P90 rho (-/-) mouse retinas two weeks after subretinal injection of AAV2/5.mOP500.GFP. **A**: Fluorescent micrographs showing flatmount of central mouse retina: green fluorescent protein (GFP) expression, red emission, peanut agglutinin (PNA), green emission, and GFP/PNA, co-localization PNA-lectin and GFP-transgene expression. **B**: Fluorescent micrograph of vertical section. The selected fields from Inferior (right) and Superior (bottom) retina are shown at higher magnification. The ganglion layer (GL), inner plexiform layer (IPL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner segments (IS), and outer segments (OS) are identified. Arrows indicate PNA and GFP co-localization.

ers that regulate PR gene expression in the rodent. Until recently, promoters have primarily been investigated via transient transfection of cells in vitro. Although primary retinal cell lines do resemble their in vivo counterparts, such isolated models can never accurately mimic the exchanges that occur within the environment of the eye. The use of rAAV to deliver genes to the highly differentiated cells of the rodent retina clearly provides a superior model to study the regulation of cell-specific promoters such as mOP500.

It has previously been reported that 3-4 rAAV2 particles can transduce one PR cell when the viral titer is $4.6x10^9$ icu/ml [1]. Although the absolute transduction efficiency of the virus used in this study was not determined, by virtue of its unique capsid, rAAV2/5 is known to be more efficient in PR transduction and have an earlier onset of transgene expression compared to rAAV2 [41]. Furthermore, recent improvements to rAAV purification and concentration by our group have resulted in significantly greater rAAV titers. Indeed, the titer of infectious rAAV2/5.mOP500.GFP used here was approximately 50 fold higher than in the earlier study. We have consequently seen by analyses of retinal flatmounts that the infected area of the retina can be doubled by employing the more concentrated rAAV2/5 vector over rAAV2 (data not shown). Additionally, the larger dose of viral DNA used here could also explain the longevity of transgene expression observed in the treated rat retinas. It is likely that a slow but progressive elimination of episomal viral DNA from nondividing retinal PRs occurred, resulting in sustained expression of GFP.

Cones comprise approximately 1% and 3% of PRs in the retina of the rat and mouse, respectively [60]. Yet, we have shown here that the proportion of transgene-expressing cones was relatively higher than that of transduced rods in both models. It is unknown if the cones were actually targeted by rAAV2/5 more efficiently than rods, but one could hypothesize that they have higher densities of the cell surface-receptors and coreceptors required for AAV5 entry. Further study to determine the AAV5 cell surface receptor profiles of rods and cones will be necessary to substantiate this.

The higher virus titer and infective selectivity of the AAV5 capsid are not the only explanations for the discriminate GFP expression observed in rods and cones after treatment with rAAV2/5.mOP500.GFP, as AAV2/5 is also known to transduce RPE cells after subretinal injection [41]. The contribution of the proximal portion of the murine rod opsin promoter (-385 to +86) must also be significant in enhancing targeted transgene expression. However, the inclusion of the RPPR in mOP500, which contains cis-elements believed to be involved in the promotion of rhodopsin expression [9,11,13-21] was not sufficient to restrict expression of the transgene to rods only. This finding was somewhat unexpected as NRL and NR2E3, transcription factors expressed in rods and considered to be important for transcription initiation from the endogenous rhodopsin promoter, are absent in cone PRs [2-4]. Furthermore, NR2E3, which functions in concert with CRX as a transcriptional activator in rods during PR differentiation and maintenance, has been shown to exert opposing effects on the transcription of cone genes [2-4,27]. We can speculate therefore that excess viral DNA copies of the limited rhodopsin promoter might exceed the level of trans-acting factors that normally suppress expression in cones.

It is also plausible that an increase in binding could occur between the rAAV-delivered cis-elements and other nonspecific transcription factors present in cones, leading to unregulated transgene transcription. These nonspecific general factors could be unidentified or known transcription factors common to both rods and cones, such as CRX and OTX. CRX appears to have a broad influence on PR-specific gene expression, since it can bind to and transactivate from a wide range of regulatory elements in several genes with similar affinity. It is believed that in rods, CRX and NRL together with NR2E3 and NR1D1 synergistically transactivate the rhodopsin promoter, presumably by forming a stable enhanceosome complex that provides both specific and efficient DNA binding to their cognate cis-elements in the RPPR [3,4,30]. However, it is conceivable that CRX can individually induce rhodopsin promoter activity by binding to the Ret-4, BAT-1, and possibly Ret-1 regulatory sites [9,17,18,21,29] and by recruiting some additional transcription factors. Currently, RP1, GUCY20 and ABCA4 transactivators are identified as candidates for novel CRX targets [63].

Our results are consistent with observations obtained with two independent transgenic mouse lines carrying bovine rhodopsin promoter fragments extended from -2174 to +70 bp and from -222 to +70 bp [14]. Both showed reporter gene expression in cones and rods, although the level of transgene expression appeared to be less in the cones than in the rods. These results demonstrate that the -2174 to +70 bp and -222 to +70 bp bovine rhodopsin promoter fragments are also not completely rod-specific in transgenic mice [14].

Finally, our current investigation also examined the longevity of transgene expression in the normal rat retina. At eight months postinjection, the GFP-reporter was still abundant in the ONL and was additionally found in the INL and ganglion cells, but at a much lower intensity. The GFP expression detected in the inner retina could merely be a result of leaking from the ONL, the site of high vector accumulation. However, studies of targeted viral delivery in brain tissue have reported that recombinant adenovirus has the ability to transport retrogradely from axon terminals at the primary site of injection to infect cell subpopulations in distant secondary regions [64,65]. Furthermore, vector DNA has been detected in the optic nerve following subretinal injection of rAAV-2/2, -2/4, or -2/5 and in the brain after intravitreal delivery of rAAV-2/2, suggesting anterograde and transsynaptic transport of rAAV from the retina to neuron terminals [66]. Additional experiments will be necessary to confirm our observation and to further elucidate the ability of rAAV vectors to spread by transneuronal transport through PR axons and synapses to the inner retina.

In summary, although the -385 to +86 proximal sequence from the murine rod opsin gene failed to exhibit strict rod-specific gene expression via subretinal rAAV2/5-mediated delivery, these studies suggest that in the absence of NRL and

NR2E3, other unidentified factors present in cones may compensate to transactivate this promoter. Elucidation of these factors should provide important new insights into the control of therapeutic transgene expression in rods and cones. Nevertheless, the mOP500 promoter could still be potentially useful in a therapeutic paradigm where strong transgene expression is required in mammalian PRs. Our data also confirm that the combination of AAV capsid and promoter can be deliberately chosen to fine-tune transgene delivery and expression to specific cells within the retina.

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WWH and the University of Florida own equity in a company, Applied Genetic Technologies Corp., that may commercialize some of the technology described in this work.

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