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Ribozyme Gene Therapy for Autosomal Dominant Retinal Disease

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Gene delivery to cells of the retina, particularly to photoreceptor cells, has broad potential both for answering basic questions of retinal biology and for more applied therapeutic purposes. The use of ribozymes as therapy for autosomal dominant retinal diseases is a promising technique, and the theoretical and practical basis for their use is discussed. The process involves designing and testing ribozymes first *in vitro* and then in animal models of retinal disease. Viral vectors based on the nonpathogenic human adeno-associated virus, when coupled with the strong, rod photoreceptor specific opsin promoter, offer an efficient and nontoxic way to deliver and express ribozymes in photoreceptor cells for long time periods of time. Effective ribozyme-mediated therapy also demands careful *in vitro* analysis of a ribozyme's ability to efficiently and specifically distinguish between mutant and wild type RNAs.Finally, effective demonstration of therapy in an animal model requires careful analysis of any rescue effect in the retina using multiple criteria, including biochemical, structural and physiological assays. For this purpose, ribozyme therapy in a transgenic rat model of retinitis pigmentosa containing a dominant rod opsin mutation (proline-to-histidine change at position 23) is discussed in detail.

Key words: Gene therapy; Autosomal dominant; Retina; Ribozyme; Virus; RNA.

Abbreviations: AAV adeno-associated virus; ADRP autosomal dominant retinitis pigmentosa; BOPS bovine opsin promoter; ERG electroretinographic; *gfp* green fluorescent protein gene; ITR inverted terminal repeat; ONL outer nuclear layer; PBS phosphate buffered saline; PR photoreceptor; rAAV recombinant adeno-associated virus; RIS rod inner segments; ROS rod outer segments; RP retinitis pigmentosa; RPE retinal pigment epithelium.

Introduction

A rational approach to genetic therapy for any disease caused by a single defective gene requires four basic elements. First, it is advantageous if the genetic basis of the disease is well characterized. Second, an efficient and nontoxic gene delivery system must be available. Third, a means must be available for properly controlling expression of the therapeutic gene, both insofar as levels of the gene product are concerned as well with regard to which tissues support or do not support expression. Finally, it is highly desirable, but not always necessary, to have an experimental animal model of the disease available for preclinical testing of the therapeutic modality. Each of these prerequisites has been satisfied for retinitis pigmentosa (RP), particularly for autosomal dominant forms of retinitis pigmentosa (ADRP), in which rod cells are usually lost progressively over several decades starting in the second or third decade of life. A gene therapy approach for ADRP will be described using ribozymes, RNA molecules with catalytic RNA-cleaving activities, that prolongs survival of photoreceptor cells in animal models of RP.

Results

It was initially necessary to decide on a method for efficiently introducing the therapeutic gene into photoreceptor cells of the retina. Because of its propensity to establish latency and the fact that it has not been implicated as a pathogen, adeno-associated virus (AAV) has been of considerable interest as a potential vector for human gene therapy (1). Most current AAV vectors lack the two normal viral genes (*rep* and *cap*) and contain in their place the passenger gene under control of a non-AAV promoter. This construct is flanked by the 145bp AAV inverted terminal repeat (ITR). Thus, because the viral *rep* gene is missing, site specific integration does not occur. In spite of this, vector DNA integration still occurs, albeit at random locations (2). Recombinant AAV (rAAV) mediated gene delivery results in long term expression in a wide variety of tissues, including various cell types in the retina (3, 4) , as well as in the lung (5), muscle (6–8), brain (9, 10), spinal cord (11), liver (12–14) and blood vessels (15). Additionally, AAV vectors appear to be remarkably non-toxic with only minimal host response (16). Thus, AAV vectors appear to have significant advantages over most other commonly used viral vectors. Early problems in the development of AAV as a vector have included difficulties in attaining high vector titers and the limited insertional capacity (room for the transgene and promoter) of the vector. However, these

issues have not proven to be insurmountable, and recent improvements in rAAV production now yield preparations in short order that are of high biological titer, free of contaminating infectious agents (17).

Using AAV as the vector we have developed a general approach for assaying photoreceptor (PR) promoter constructs *in vivo* by employing rAAV to achieve efficient, cell type-specific expression of exogenous genes in PR cells of the mammalian retina (4). Recombinant AAV vectors (18) were used to transfer a synthetic green fluorescent protein gene (*gfp*) to mouse or rat retinas following injection into the subretinal space (Figure 1). Employing a proximal murine rod opsin promoter (+85 to –386bp, termed "mops500") to drive *gfp* expression, reporter gene product was found exclusively in rod photoreceptors, not in any other retinal cell type or in the adjacent retinal pigment epithelium after subretinal delivery in the adult rat. No pathology was associated with the injections or gene expression for the subsequent 2.5 years (W.W. Hauswirth and J.G. Flannery, unpublished). Transduction efficiency for the mops500 *gfp*-containing rAAV virus in rod PR's was high, 5–10 infectious particles giving one GFP-positive rod cell. GFP-expressing PR's were quite evident in 25–30% of the total retinal area surrounding a single 2µl injection (Figure 1a). Photoreceptors were transduced with nearly 100% efficiency in this region (Figure 1e). Additionally, a lower but substantial GFP signal extended to nearly the retinal periphery (Figure 1b). In total, 2 to 2.5 million rat PR's were transduced as a result of the single subretinal inoculation. Extrapolating to the larger human retina, this level of gene transfer and expression suggests the feasibility of efficient gene delivery as therapy for retinal disease. The *gfp*-containing rAAV stock used in these studies was found to be free of both adenovirus and wild-type AAV, as judged by plaque assay and infectious center assay, respectively. Hence, these experiments establish that purified, helper virus-free rAAV vectors can achieve high efficiency, tissue-specific transduction of PR cells without associated pathological side effects. These studies also validate the use of rAAV vectors as a reproducible way to efficiently introduce DNA constructs into animal PR cells.

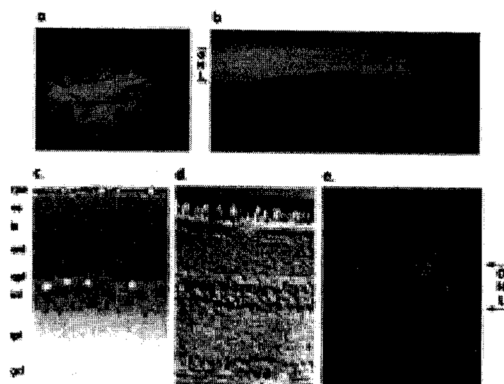

Fig. 1 GFP expression in rat retina injected with a proximal rod opsin promoter-*gfp* construct in rAAV.

a. Whole mount of rat retina (about 1.5 cm in diameter) at 6 weeks after subretinal injection. An area about 30% of the total retina is GFP-positive. The fraction of GFP-positive cells decreases radially surrounding the injection site.

b. Transverse section of the retina shown in panel a. GFP fluorescence is seen throughout the outer nuclear layer and exhibits a gradient of positive cells towards the retinal periphery (ora seratta). No GFP signal is observed in the inner retinal layers.

c. Bright-field micrograph of an untreated rat retina for reference.

d. Phase-contrast micrograph of the section shown in Panel b.

e. Laser scanning confocal image of the retina in panels a, b, and d. GFP fluorescence fills the inner segments and photoreceptor cell bodies, and extends from the synaptic terminal (lower limit of visible green fluorescence) to the region of the external limiting membrane. Abbreviations: ONL, outer nuclear layer; RPE, retinal pigmented epithelium; OS, outer segments; IS, inner segments; OPL, outer plexiform layer; INL, inner plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

The second decision concerned which of the more than 100 gene defects known to cause RP should be our initial target. As of May 1999, there were 108 human loci in the human genome associated with one form or another of retinal disease. Of those, 47 have been characterized at the gene level and 27 have been documented to cause RP. Of those 27 RP-causing genes, 12 are photoreceptor (rod or cone cell)-specific, and half are genes directly involved in the phototransduction cycle, the process initiated by photon absorption in rods or cones that leads to neural signaling. By far the best studied among these 6 genes is rod opsin, whose gene product, when associated with the 11-cis-retinaldehyde chromophore, is responsible for light absorption in rods. Defects in the rod opsin gene are the single most common causes of RP, accounting for about 25% of all forms. A cytosine-to-adenosine base change in the 23rd codon converting a histidine to a proline residue in the protein apparently leads to a misfolded opsin molecule and ADRP. This P23H mutation accounts for about 12% of RP patients in North America. For these reasons, P23H ADRP was chosen as our initial RP type against which to develop a gene therapy strategy.

Successful therapy for any dominant disease requires delivery of an agent that will suppress the action of the dominant allele without damaging the function of the normal gene. Suppression could occur at the DNA level, by correction of the mutated allele. One possible approach for such site-specific gene correction involves the use of hybrid, circular RNA-DNA molecules. While results in liver have been tantalizing (19), delivery of the large amounts of hybrid nucleic acid apparently required for this approach may prove difficult in the retina. Based on current technology, intervention at the level of RNA is more practical than direct gene correction. While antisense oligonucleotides have been successfully employed to inhibit the expression of disease genes, catalytic RNA molecules, ribozymes, are more likely to be allele-specific inhibitors because of their target binding domains can be short (<12 nucleotides) (20). Ribozymes also have the advantage of catalytic turnover – multiple target RNA molecules are cleaved by a single ribozyme. Ribozymes have been

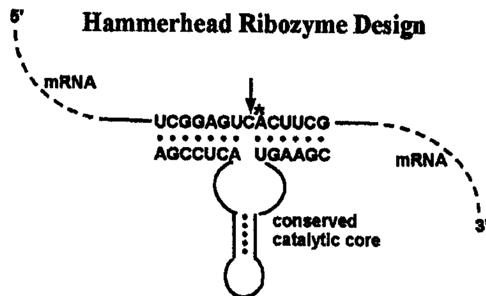

Fig. 2 Schematic structure of the P23H hammerhead ribozyme. The top line represents the target P23H mRNA sequence in the vicinity of the cytosine-to-adenosine mutation marked with an asterisk. The bottom sequence represents the ribozyme RNA showing the sequence of the two targeting arms with the intervening 24 nucleotide conserved catalytic core sequence. The arrow denotes the ribozyme cleavage site in the mRNA.

shown to work in a variety of animal model systems, and we reasoned that they could be used to block the synthesis of mutated proteins that lead to retinal degeneration. Our approach has been to deliver DNA copies of ribozymes specific for the mutant allele using recombinant AAV and the proximal rhodopsin promoter to direct synthesis in rod photoreceptor cells. In this paper we confirm the effectiveness of this approach using a rat model of ADRP caused by a P23H rhodopsin transgene. The design and specificity of our P23H hammerhead ribozyme is shown in Figure 2.

The pTR-UF2 vector (18) provides the AAV terminal repeats needed for packaging the ribozyme constructs. We previously used this vector to direct GFP expression specifically to rat photoreceptors (21). To express ribozymes, we replaced the 472-bp murine rod opsin promoter with a 691 bp fragment of the proximal bovine rod opsin promoter and replaced the *gfp* reporter gene with the ribozyme gene. The bovine promoter fragment contains three proximal promoter elements and the endogenous transcriptional start site at its 3′ end (22). In preliminary experiments, we established that this promoter element would support high efficiency, rat photoreceptor-specific expression *in vivo* (unpublished results). Active and inactive ribozymes were designed, tested and cloned as described by Drenser *et al.* (23). Each ribozyme gene was followed by an internally cleaving hairpin ribozyme sequence derived from plasmid pHC (24) resulting in ribozyme cassettes of 140–152 bp. Self-cleavage by the common downstream ribozyme at the internal cutting site in the primary ribozyme RNA leaves identical 3′ends on each mature ribozyme. An intron and a polyadenylation signal derived from SV40 were included to promote stability of the ribozyme, which may be essential for efficacy (25).

We used a transgenic rat line (TgN(P23H)3) that contained two copies of the wild type rat opsin gene and a mouse transgene expressing the P23H allele of opsin. Expression of the mutated opsin begins at about postnatal day 5 (P5) in rats, and leads to a gradual death of photoreceptor cells. These rats develop an apparently normal retina up to P15, although there are somewhat more pyknotic photoreceptor nuclei in the outer nuclear layer (ONL) than in non-transgenic control rats. Photoreceptor cell death is almost linear until about P60, when about 40% of the photoreceptors have been lost. After P60, the rate of cell loss decreases, until, by one year, the retinas have less than a single row of photoreceptor nuclei remaining.

Both a hammerhead and a hairpin ribozyme were designed to cleave the mRNA produced by the P23H transgene. The former is shown diagrammatically in Figure 2. The latter had similar target sequence recognition but contained a "hairpin" core structure. The hammerhead ribozyme (Hh13) cleaved 3′ to the first cytosine residue in codon 23. The hairpin ribozyme (Hp11) cleaved 3′ to the adenosine residue at the first position in codon 22. Control ribozymes (Hp11i and Hh13i, respectively) retained the targeting domains but contained errors in their catalytic domains which precluded activity. The active hammerhead ribozyme (Hh13) digested 20% of the P23H target RNA within 10 min of incubation *in vitro*, and by 5 hours greater than 80% was cleaved. In multi-turnover experiments, both ribozymes exhibited kinetic constants (K_m and k_{cat}) similar to those of naturally occurring ribozymes. The specificity and detailed kinetic properties of these ribozymes *in vitro* have been described elsewhere (23). The two active ribozymes did not digest the wild-type transcript even in the presence of high $MgCl_2$ concentrations. The inactive control ribozymes (Hp11i and Hh13i) were without measurable activity on any substrate. Using total RNA derived from retinas of P23H rats, both the hairpin and the hammerhead ribozymes were able to cleave the mRNA product of the mutant transgene selectively.

At postnatal day 14 or 15 we injected 2 µl of the rAAV-ribozyme vector into the subretinal space between the photoreceptors and the adjacent retinal pigment epithelium. At several time points between P60–P90 animals were sacrificed and their eyes examined. In uninjected control eyes of P23H-3 rats at these ages, the ONL thickness, an index of photoreceptor cell number (26), was reduced to about 60% of normal.

Retinas from ribozyme-injected eyes showed a modest but significant decrease in the accumulation of transcript derived from the P23H transgene. Control retinas (pooled data from 2 uninjected and 6 PBS-injected eyes) exhibited little variation in the level of transgene mRNA (average transgene level was 25.5 ± 3.1% of the wild-type level). Eyes injected with either active ribozyme consistently exhibited lowered transgene mRNA levels relative to total opsin mRNA in the same eye. Retinas receiving the hairpin ribozyme Hp11 showed a 15.3 ± 3.3% decrease in transgene expression, and those with the hammerhead ribozyme Hh13 showed a decrease of 11.1 ± 5.1%. Since only 25–30% of the rods are genetically transduced in rat retinas receiving the amount of rAAV used here, on a per transduced rod cell basis, more than 50% of the mutant mRNA will have been removed in ribozyme-expressing photoreceptor cells. This reduction

of mutant mRNA *in vivo* suggests related attenuation in synthesis of P23H rhodopsin.

To determine the extent of cellular rescue, excised retinas were removed and embedded in epoxy resin, and 1 µm thick histological sections were made along the vertical meridian (27). Tissue sections were aligned so that the rod outer segments (ROS) and Müller cell processes crossing the inner plexiform layer were continuous throughout the plane of section. This ensured that the sections were not oblique. The thickness of the ONL and lengths of rod inner segments (RIS) and ROS were measured as described elsewhere (28). Ribozyme-injected eyes retained significantly more photoreceptors at P60, P75 and P90 than uninjected contralateral control eyes. Retinas injected with AAV expressing the hammerhead ribozyme Hh13 retained 88% of the normal ONL thickness, compared to about 60% in the uninjected controls. An example is shown in Figure 3. Thus, the ONL thickness after Hh13 expression was 40–43% greater than that of uninjected controls, a highly significant difference ($p=0.001$ or less at P60 and P90). Retinal expression of the hairpin ribozyme Hp11 also led to significant rescue when compared to controls, preserving 77–83% of normal ONL thickness. Thus, the ONL thickness after Hp11 expression was 30–39% greater than that of uninjected controls, again a highly significant difference ($p<0.0005$ at all ages). There was no significant effect in PBS-injected control eyes ($p>0.169$ in all cases). This result is consistent with our finding that needle injury during injection to the retina in young rats (P14-P15) does not protect photoreceptors or up-regulate bFGF expression (M.M. LaVail and W. Cao, unpublished observations) as it does in older animals (28, 29). To control for possible rescue by the presence of the bovine opsin promoter (BOPS) or other vector sequences, AAV-BOPS-*gfp* was similarly injected at a titer equivalent to that used for the AAV-ribozymes. Injection of AAV-BOPS-*gfp* did not alter the time course of photoreceptor loss. In contrast, injection of inactive ribozyme resulted in ONL thickness measures greater than uninjected control values, but values consistently less than those from the active Hp11 and Hh13 ribozymes. The partial rescue by delivery of Hp11i and Hh13i is attributable to an antisense effect because these inactive ribozymes can still bind mutant opsin mRNA and, without cleaving the target, could interfere with translation or lead to mRNA turnover. Others have noted that even ribozymes with similarly short targeting arms may have significant antisense effects in cells as well (30).

Fig. 3 Retinal morphology in contralateral eyes of a P23H transgenic rat injected subretinally in the right eye with the AAV-hammerhead ribozyme at P15 and analyzed at P89. The left eye was untreated. There is nearly a two-fold preservation of photoreceptor nuclei in the treated eye versus the control eye. Longer photoreceptor inner and outer segments are also apparent in the ribozyme treated retina.

Rescue was relatively uniform across the entire retina and did not parallel the gradient in transduced rods seen with reporter gene constructs (refer to Figure 1). This unexpected pan-retinal extent has several possible explanations. There are approximately 10^7 photoreceptors in the rat retina and the number of rod photoreceptors that were rescued is within the limits predicted from our rAAV titers. Recombinant AAV titers were estimated using both an infectious center assay and a physical particle assay. Together they allow us to construct upper and lower bounds for the number of functional rAAV particles in a single 2µl injection. The upper bound derives from the DNase resistant particle assay, indicating that 2 µl of the rAAV-ribozyme virus preparation contained about $2x10^8$ rAAV. This is an upper estimate because not all particles are expected to be infectious. The lower limit for rAAV titer is generated by the infectious center assay, indicating about 10^7 rAAV per µl. This titer is likely to be an underestimate of the true retinal infectivity, however, because the infectious particle titers were determined *in vitro* in another cell type (HEK 293). In this assay, the initial event, viral adherence to cells attached to culture plates, is diffusion limited (31). Therefore, apparent infectious particle titers can be low by several orders of magnitude depending on viral concentration, transduction time and the volume of virus in the media above the cells (31).

The laminar nature of the retina may also explain the surprising lateral extent of rescue resulting from a single injection. Recombinant rAAV was injected into extracellular space separating the photoreceptor and retinal pigment epithelium (RPE) layers. The initial volume of extracellular space, approximately 0.5 µl, is expected to increase significantly upon a 2 µl subretinal injection. Following injection, the fluid transport property of the RPE should quickly dehydrate this space, reapposing the photoreceptors and RPE and concentrating the vector. Injection-induced detachment of the photoreceptors from the RPE visually resolves within several hours. During this reattachment process, viral particles spread laterally through the subretinal space. An additional factor favoring pan-retinal rescue is the increased transducibility of photoreceptors in neonatal rats compared to adults, as was shown for lentivirus vectors (33). A final alternative explanation is that rescued photoreceptors could support survival of non-transduced photoreceptors by yet unidentified trophic factors (34, 35) giving rise to a positive bystander effect in which genetically rescued photoreceptors can aid in the survival of several rows of neighboring photoreceptors.

In addition to increased survival of photoreceptor cells, injection of the AAV-ribozymes resulted in preservation of the lengths of RIS and ROS. The uninjected control retinas had RIS that were about 90% of normal. Both the active and inactive ribozymes resulted in RIS lengths of 98% or greater of the normal length, and about 10% longer than uninjected controls. Because of the variability and short RIS length, only the Hh13 at P60 and P90, the Hp11 at P75 and the Hp11i at P60 were statistically different from uninjected controls ($p<0.05$). Injection of PBS and AAV-BOPS-*gfp* were indistinguishable from uninjected eyes with regard to RIS length. The ROS lengths were 15–25% longer in the ribozyme-injected eyes compared to those in the uninjected control eyes. However, ROS in the ribozyme-injected eyes were, at the greatest, only 65–75% of normal, compared to the virtually normal RIS lengths. The ROS of the active ribozymes differed significantly from the uninjected controls ($p<0.005$ for all, except <0.02 for Hp11 at P90), as did the inactive Hp11i ($p<0.05$).

We also measured the electroretinographic (ERG) responses from a number of rats to determine whether AAV-ribozyme rescue extended to the physiological response of the retina to light. The b-wave of the ERG, arising from Müller cells and inner retinal neurons, is usually taken as a more reliable measure of retinal function than the photoreceptor-derived a-wave which is often more variable. When recording simultaneously from both eyes of normal rats, the responses measured within 5% of each other for both the b- and a-waves in almost every case (despite significant variation between different animals). Thus, it is noteworthy that the active ribozymes resulted in scotopic (rod-derived) b-waves 30–65% greater than in the contralateral control eyes (Figure 4). The inactive ribozymes, Hp11i and Hh13i, also resulted in greater b-wave amplitudes, but these were less than 25% greater than those of the control eyes, whereas the PBS- and AAV-BOPS-injected eyes were not significantly different from uninjected control eyes (Figure 4; while the a-wave amplitudes were somewhat more variable among the different agents, some showed amplitudes as much as 93% greater than uninjected controls). Neither the PBS- nor the AAV-BOPS-injected eyes yielded a-wave amplitudes greater than controls. Thus, the b-wave amplitude changes for the active and inactive ribozymes reflected the greater survival of photoreceptor cells as measured by ONL thickness. Overall, ERG data demonstrate that ribozyme expression in rods results in functional as well as structural photoreceptor rescue.

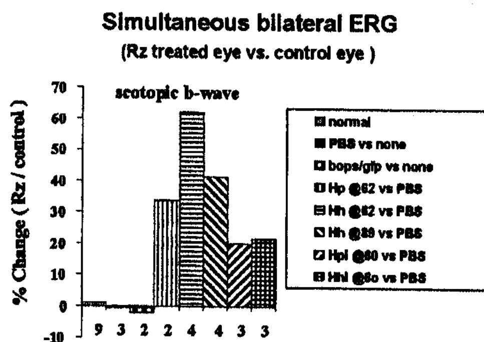

Fig. 4 Comparison of electroretinographic (ERG) scotopic b-wave amplitudes between ribozyme treated and contralateral control eyes in P23H transgenic rats. The comparison is reported as a percentage amplitude change in the treated eye relative to the untreated eye when the ERG is measured simultaneously in both eyes. Below each bar the number of animals analyzed is noted.

Discussion

Although antisense oligonucleotides also have been tested as therapeutic agents for treatment of diseases resulting from expression of toxic gene products, antisense therapy requires rapid delivery of massive quantities of oligonucleotide complementary to the mRNA in the appropriate cell to inhibit translation of the target mRNA. Ribozymes have an advantage in this regard, because multiple copies of specific RNA molecules are cleaved in a catalytic manner. In addition, ribozymes hold promise for clinical applications because they are potentially allele-specific, cleaving mutated transcripts while leaving wild-type transcripts intact. Ribozyme catalyzed inhibition of gene expression has been tested in several *in vitro* systems, including plants (36), mammalian cells (37, 38) and Xenopus oocytes (39). Ribozymes also work *in vivo*. Adenovirus-delivered ribozymes led to 96% reduction in human growth hormone in transgenic mice expressing this protein (40), and crosses between transgenic mice expressing bovine ß-lactalbumin and mice expressing a ribozyme against that mRNA resulted in substantial reductions in mRNA levels in doubly transgenic progeny (41). However, prior to this research, ribozymes had not been tested against a dominant negative allele *in vivo*.

As described here, *in vivo* expression of several ribozymes in a transgenic rat model for P23H RP markedly slowed the rate of rod photoreceptor degeneration for at least 3 months as assessed functionally by electroretinographic analysis, cellularly by preservation of retinal photoreceptors and molecularly by specific reduction in mutant mRNA levels in treated eyes (42). Intracellular production of ribozymes in rod photoreceptors was achieved by transduction with an rAAV incorporating the same rod opsin promoter shown earlier to support rod specific expression (21). This study establishes the initial proof-of-principle that a recombinant AAV vector containing the appropriate photoreceptor-specific promoter can mediate functional rescue of a genetically determined retinal photoreceptor degeneration and that ribozymes can be effective against an autosomal dominant genetic disease.

Several features of our rAAV vectors designed to deliver opsin-specific ribozymes are likely to have been helpful in achieving photoreceptor rescue. First, the rod opsin promoter elements generate photoreceptor-specific expression in a significant portion of the rat retina after a single subretinal inoculation (21). Such

constructs result in persistent photoreceptor expression of the passenger gene (>30 months, WW Hauswirth and JG Flannery, unpublished), consistent with observations of rAAV-transduced skeletal muscle (43, 8). Second, the inclusion of the downstream, self-cleaving ribozyme is likely to have increased the bioavailability of the opsin ribozyme by creating a precise 3′ end containing only sequences complimentary to the target RNA. Finally, the intron and strong polyadenylation sequences in the vector are important for maintaining high levels of intracellular ribozyme.

That photoreceptor degeneration in an animal model of dominant retinitis pigmentosa can be slowed by ribozyme-targeted destruction of P23H mutant RNA using encourages the further exploration and development of ribozymes as therapeutic agents for other forms of ADRP. Beyond retinal diseases, since a complete removal of mutant mRNA is often not necessary to achieve phenotypic rescue, this AAV-ribozyme strategy may be widely applicable to a variety of other dominantly inherited diseases as well.

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