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Preservation of Photoreceptor Morphology and Function in P23H Rats Using an Allele Independent Ribozyme

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Abstract

To develop an allele independent ribozyme for the treatment of Autosomal Dominant Retinitis Pigmentosa (ADRP) associated with mutations in the rhodopsin (*RHO*) gene.

A ribozyme targeting dog, mouse, human but not rat rhodopsin (*RHO*) mRNA was designed and tested *in vitro*. Activity of this ribozyme was tested in tissue culture by co- transfection of HEK 293 cells with plasmids expressing opsin mRNA and ribozyme, followed by quantitative RT-PCR to evaluate the level of *RHO* mRNA. For experiments *in vivo*, Rz525 driven by the mouse opsin proximal promoter was inserted in plasmids with AAV 2 terminal repeats (TR) and packaged in AAV serotype 5 capsids. AAV-Rz525 was injected subretinally into the right eyes of P23H rat pups. Left eyes were injected with virus expressing GFP from the identical promoter. Animals were analyzed at 4, 8 and 12 weeks post-injection by full field scotopic electroretinography (ERG). After 12 weeks, animals were sacrificed and retinas were dissected, fixed and sectioned.

Rz 525 had high catalytic activity *in vitro* and led to a 50% reduction of *RHO* mRNA in cells. AAV-Rz525 injection into P23H transgenic rats led to significant preservation (about 50%) of scotopic ERG a- and b-wave amplitudes. Histological analysis showed an increased number of ONL nuclei in the central and superior retina of treated eyes relative to control eyes. RT-PCR analysis revealed 46% reduction of transgenic (mouse) *RHO* mRNA in right eyes relative to left eyes and no change in rat *RHO* mRNA.

AAV5 delivery Rz525 resulted in a partial rescue of the light response and structural preservation of photoreceptors in transgenic rats. This ribozyme may be a useful component of an RNA replacement gene therapy for ADRP

Keywords

Autosomal Dominant Retinitis Pigmentosa; rhodopsin; gene therapy; Adeno associated virus; and ribozyme

1. INTRODUCTION

Retinitis Pigmentosa (RP) is an heterogeneous group of diseases clinically characterized by loss of the night vision followed by progressive loss of peripheral vision (Krauss and Heckenlively, 1982). The disease is caused by heritable defects in rod photoreceptor cells or RPE cells and may be transmitted in an autosomal dominant (ADRP), autosomal recessive (ARRP) or X-linked (XLRP) fashion. However, over 40% of RP cases are simplex, affecting

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no other family member (Humphries et al., 1992). Symptoms of the disease are primarily associated with the death of photoreceptor cells. Rod cells, which are enriched in the periphery of the retina, are most severely affected and die first. Cone cells and central vision degenerate only following the loss of rods.

The first mutation linked to ADRP was in the *RHO* gene which encodes rod cell opsin (Dryja et al., 1991). (We will follow the convention of human genetics and refer to the wild type gene as *RHO* and the mutant gene as *rho*.) The substitution of conservative proline by histidine at residue 23 affects the N-terminal intradiscal domain of the protein. The P23H mutation belongs to Class II of rhodopsin mutations as described by Mendes et al (Mendes et al., 2005). These mutant proteins have reduced binding capability for 11-cis retinal and a defect in glycosylation (Noorwez et al., 2004). In many studies biochemical and molecular mechanisms of the disease have been investigated (Chapple and Cheetham, 2003; Illing et al., 2002 ; Saliba et al., 2002 ; Chapple and Cheetham, 2003). In transfected cells, the mutant protein has a helical content of 50–70% of wild type protein, and the P23H mutation results in the formation of high molecular weight oligomeric species. The accumulation of aggregated rhodopsin in the ER is likely to stimulate the unfolded protein response, leading to apoptosis. However, the behavior of P23H rhodopsin in transfected cells may not reflect the behavior of this protein in photoreceptors. In mice bearing a P23H transgene and also expressing wild-type rhodopsin, at least some of the mutant protein is trafficked to the outer segments of rod cells where it combines with the 11-cis retinal chromophore (Wu et al., 1998) . In addition, increasing the gene dosage of wild-type *RHO* to P23H *rho* in transgenic mice appears to reduce the rate of retinal degeneration (Frederick et al., 2001).

The physiological impact of mutated rhodopsin is the loss of the light response detectable by full field electroretinographic analysis (ERG). P23H transgenic mice have a significantly reduced dark-adapted ERG response by 1 month followed by further progressive decline (Goto et al., 1995). Light adapted ERG measurements, which indicate cone function, are normal for the first several months of life, but then they too decline. Three P23H transgenic rat lines carrying a mouse transgene are also available. They differ in their rates of degeneration as measured by ERG and histological analysis. Line 3, which we used in our study, is described as a slowly degenerative line (Machida et al., 2000 ; Organisciak et al., 2003 ; Vaughan et al., 2003). Nevertheless, it loses more than 70% of its photoreceptor ERG response (a-wave) by 29 weeks of age. This erosion of the light response correlates well with the loss of photoreceptors observed by histology (Machida et al., 2000 ; Organisciak et al., 2003).

Although the P23H mutation accounts for 12% of ADRP in North America, over 100 rhodopsin mutations have been described, and most lead to autosomal dominant RP (<http://www.sph.uth.tmc.edu/Retnet>), which is considered an untreatable disease. Two approaches have been taken for gene therapy in ADRP animal models. The first is the delivery of genes for neurotrophic factors such as GDNF (McGee Sanftner et al., 2001), CNTF (Bok et al., 2002) and antiapoptotic proteins such as XIAP (Petrin et al., 2003) . The rationale is that by blocking apoptotic death of photoreceptors, visual function will be maintained. This outcome has sometimes, but not always, been the case, depending on the mutation being treated. Another approach tested in animals has been to block the synthesis of the mutant proteins on the premise that increasing the ratio of normal to mutant rhodopsin will delay the onset of retinal degeneration. An underlying assumption is that the remaining wild type *RHO* will be sufficient to maintain the normal vision (Liang et al., 2004). AAV delivery of ribozymes specific for the P23H transgene mRNA was tested in P23H line 3 rats (Drenser et al., 1998 ; LaVail et al., 2000 ; Lewin et al., 1998). These ribozymes selectively knocked down the mutant mRNA and led to preservation of the photoreceptors infected with the virus for up to 8 months.

The large number of different mutations in rhodopsin that lead to ADRP makes the development of allele-specific ribozymes problematic: a highly active ribozyme cannot be found for each mutation. RNA interference using siRNAs represents an alternative technology. However it will be difficult to come up with potent inhibitors that can discriminate based on single missense mutations. As an alternative, we and others (Millington-Ward et al., 1997 ; Sullivan et al., 2002 ; Gorbatyuk et al., 2005 ; Kiang et al., 2005 ; Cashman et al., 2005) have proposed an RNA replacement strategy in which the levels of both mutant and wild type mRNA will be knocked down using allele non-specific ribozymes or siRNAs. An essential second component of this approach is the delivery of a *RHO* cDNA that is resistant to ribozyme or siRNA mediated degradation. Here we report the implementation of the first part of this strategy. Ribozyme 525 (Rz525) is one of a series of mouse specific ribozymes we have developed that target wild type and mutant rhodopsin. In this paper, we demonstrate that AAV delivery of Rz525 rescues vision in P23H line 3 rats by diminishing the expression of P23H transgene, which was derived from a mouse genomic clone (Naash et al., 1993). These results differ from the earlier demonstration of ribozyme rescue in rat (LaVail et al., 2000 ; Lewin et al., 1998), because Rz525 does not target a specific allele—it simply reduces the expression of mouse *RHO* mRNA in the P23H rats. Consequently, Rz525 is a candidate ribozyme for an RNA replacement gene therapy when combined with a ribozyme-resistant rhodopsin gene.

2. MATERIALS AND METHODS

2.1. Design and test ribozyme *in vitro*

Rz525 targeting mouse *RHO* mRNA was designed to cleave following the G U C triplet in the sequence: G G U G G U C C U G G C. This target was chosen because it was determined experimentally to be accessible for ribozyme cleavage (see below) and contains a mismatch with the rat sequence: The third guanosine in the mouse sequence (underlined) is an adenosine in rat. The C-A mismatch between the ribozyme and the rat target at this position is sufficient to block cleavage of rat *RHO* mRNA (Werner and Uhlenbeck, 1995). *In vitro* cleavage reactions were set up under conditions described earlier (Gorbatyuk et al., 2005), except that the final concentration of MgCl₂ was 2 mM. In these reactions, target concentration exceeded that of ribozyme by 10-fold. Reactions were separated on 10% acrylamide 8M urea gels and analyzed on a Storm Phosphorimager (GE Healthcare, Piscataway, NJ).

2.2. Construction of rAAV-Rz525 to test its activity in tissue culture experiments and *in vivo*

For testing cleavage of *RHO* mRNA in tissue culture, we inserted DNA oligonucleotides for Rz525 in an AAV2 plasmid that contained the CBA promoter to drive the expression of the ribozyme. Rz525 was processed from the primary transcript by using internal hairpin ribozyme located between Rz525 and the SV40 polyadenylation signal (Fig. 2A).

For *in vivo* experiments, we inserted the ribozyme gene into an AAV2 plasmid in which its expression was driven by photoreceptor specific mouse opsin promoter containing proximal 472bp region including 70 nucleotides of the 5' UTR (MOPS500) (Fig 2B). An SV40 derived splice donor/splice acceptor region is present on this plasmid upstream of the hammerhead, and a hairpin ribozyme is present down stream for the release of Rz525 from the 3' end of the transcript. This AAV Rz525 plasmid with type 2 terminal repeats (TR) was packaged in AAV serotype 5 capsids (Zolotukhin et al., 2002). Combination of the MOPS500 promoter and serotype 5 AAV permits specific targeting of ribozyme expression in photoreceptor cells following subretinal injection (Auricchio et al., 2001).

2.3. Testing Rz525 in cell culture

The murine *RHO* cDNA containing the entire reading frame plus 12 nucleotide of the 5' UTR and 135 nucleotides of the 3' UTR was inserted in the pcDNA3Zeo (Invitrogen) plasmid. The

expression of the *RHO* cDNA was driven by CMV promoter. To verify the specificity of Rz 525 to cleave targeted sequence, in our experiment we used rat *RHO* cDNA as well. It was cloned the same way as murine *RHO* gene. HEK 293 cells were transfected at a molar ratio of 1:10 of target to ribozyme. 36 hours following transfection, total RNA was extracted using Trizol Reagent as recommended by its manufacturer (Invitrogen). Samples were treated with RNase free DNase (Ambion), to remove contaminating DNA. Reduction of the target was determined by RT-PCR using AMV reverse transcriptase (GE Healthcare, Piscaway, NJ). The antisense oligonucleotide: GGAAGCCAGCAGATCAGGAAGAAGATGACCATG and TGGCCATCTCCTGCTCGAAGTC were used to prime reverse transcription of *RHO* and β -actin. For amplification of *RHO* and β -actin the sense primers: TTCCTCACGCTCTACGTCACCGTACAGCACAAGAA and TGAGACCTTCAACACCCAGCC respectively were used. Two μ l of the RT reaction was added to the amplification reaction which also contained 200 μ M of each dCTP, dGTP and dTTP, 100 μ M dATP, 2 mM MgCl₂, 20 pM gene specific primers. For measurement of *RHO* mRNA following ribozyme treatment, PCR amplification was stopped at 20 cycles which was verified to be within the linear range of PCR amplification. A non-denaturing 8% polyacrylamide gel was run to detect β -actin and opsin by staining the gel with Sybr Green (Molecular Probes). Bands were quantitated using the Storm Phosphorimager.

2.4. P23H mouse transgenic rat model

These studies were conducted in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. P23H line 3 homozygous animals kindly provided by Professor Mathew LaVail (USCF School of Medicine, Beckman Vision Center) were bred in our laboratory against albino Sprague-Dawley rats (Riverdale) to produce P23H heterozygous rats with a single P23H mouse transgene. All rats were kept in 12/12 light dark cycle of dim light, measuring less than 90 lux at the level of the cages.

2.5. Injection animals with rAAA-Rz525

P23H pups were injected with AAV Rz525 or AAV GFP at postnatal day 16 (P16). Animals were anesthetized by intramuscular injection with mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg). The concentration of AAV5Rz525 and AAV5 GFP used for injection was 3.6×10^{12} genome copies per ml. Animals received subretinal injections with of 2 μ l of virus. Right eyes were injected with AAV5 MOPS-Rz525; left eyes served as controls and were injected with AAV5 MOPS-GFP. All injections were administrated in accordance to the procedure described previously (Timmers et al., 2001). The extent of transduction was evaluated by staining retinas with antibodies against GFP (gift of Dr. Paul Hargrave) and FITC conjugated secondary antibody (Sigma-Aldrich Co).

2.6. Electrophoretic analysis

Full-field scotopic (dark adapted) electrophoretic analysis (ERG) was performed on animals injected with rAAV Rz525 and rAAV GFP 4, 8 and 12 weeks after treatment using the UTAS E2000 apparatus with full-field illumination (LKC Technologies, Gaithersburg, MD). Animals were dark adapted for 12 hours before ERG analysis and then anesthetized as described above. Stimuli were presented as 5 flashes at intensities of 0.02, 0.18 and 2.68 cd-s/m² at 30, 60 and 60 second intervals, respectively. For quantitative comparison of differences in a- and b-wave maximum amplitudes between two eyes of individual rats, the values of all stimuli at a given intensity were averaged. Means were compared using Student's t-test for paired data.

2.7. RNA isolation and reverse transcription- polymerase chain reaction

Total RNA was isolated from individual retinas of five P23H rats treated with AAV Rz525 or AAV GFP virus using Trizol Reagent (Invitrogen) following the manufacturer's procedure. After RNA isolation, samples were treated with DNase I (Ambion) to remove genomic DNA contamination. RNA concentration was determined spectrophotometrically. Each RNA sample came from a separate eye. Five animals (10 eyes) were taken for RNA analysis. Quantitative RNA analysis was performed by comparison of *RHO* and β -actin RT-PCR products from right and left eyes RNA of individual rats. Primers and reaction conditions were identical to those described above. The rhodopsin primers amplify both rat and mouse rhodopsin mRNA. After amplification, total *RHO* PCR product (635 bp) was cleaned up with PCR Clean Up Kit (Sigma-Aldrich) to remove nucleotides. Restriction digestion with mouse specific restriction enzyme NcoI (Promega) was then performed. Cleavage of total PCR product with this enzyme gave us three bands that can be recognized as 635 bp (rat *RHO* PCR product), 500 bp and 135 bp (mouse PCR product). Digested PCR products were loaded on 5% polyacrylamide gel for *RHO* PCR products and on 8% gel for β -actin PCR products. The gel was stained with Sybr-green (Molecular Probes) to detect intensities of mouse and rat PCR product bands. Reduction of mouse *RHO* mRNA was measured as a ratio of mouse to rat specific amplification products. Rat amplification products were separately normalized to β -actin, to assure uniform recovery.

2.8. Quantitative Western blot analysis

Differences in rhodopsin protein levels of right and left eyes of individual P23H rats were analyzed by Western blot. Retinal proteins were extracted from the same eyes that were used in RT PCR experiment. By collecting the Trizol Reagent phase containing proteins after removing the aqueous phase containing RNA. Further extraction of protein was performed following the manufacturer's protocol. The protein was dissolved in 50 μ l of Tris-HCl, pH 7.5, 6 M urea. The concentration of protein in each sample was determined using a Bio-Rad DC protein detection kit. Western blot analysis was used to quantify total mouse and rat rhodopsin between right and left eyes of individual animals with β -actin was used as an internal control. The B6-30 antibody used to detect opsin was a generous gift of Dr. Paul Hargrave.

2.9. Retinal tissue preparation and histological quantification of retinal Outer Nuclear Layer

Eye cups were enucleated, the tissue was fixed and prepared for frozen sectioning as described earlier (Gorbatyuk et al., 2005). 12 μ m sections were obtained by using cryostat. Slides with right and left retinas were used for further histological and immunochemical analysis. To measure the thickness of the Outer Nuclear Layer (ONL) we stained cryostat sectioned retinas with propidium iodide. Digital images of retinas of right and left eyes of individual mice were analyzed in 6 inferior and 6 superior sectors starting from optical nerve head. Images were analyzed using the Image J program (<http://rsb.info.nih.gov/ij>). Ten individual measurements were made in each sector and averaged. In addition, the overall ONL thickness was estimated by averaging the thickness in all sectors. Statistical analysis comparing treated (right) and control (left) eyes was performed using Student's t-test for paired data.

3. RESULTS

3.1. Design and testing of Rz525

Ribozyme 525 (Rz 525) was designed to cleave mouse rhodopsin mRNA at position 461 (Genbank accession number BC013125) and the dog sequence at the position 525 (Genbank accession number X71380) (Fig1 A). The sequence has a single C-A mismatch with rat rhodopsin mRNA at position -4 relative to the cleavage site. The cleavage time course of a synthetic oligonucleotide target by Rz525 was determined under conditions of substrate excess. The rat target sequence, containing a C-A mismatch, was not cleaved by the ribozyme under

the same conditions (Fig1 B). Rz525 had a higher cleavage rate under low magnesium conditions (2 mM MgCl_2) than another rhodopsin ribozyme we have recently described that was active both in tissue culture and *in vivo* (Gorbatyuk et al., 2005) (Fig. 1C). To compare Rz525 to the previous ribozymes we have employed in the past, we performed multiple turnover kinetic analysis and determined the K_m to be 152 nM and the k_{cat} to be 0.78 min^{-1} . These values are similar to those of ribozymes we have tested *in vivo* (Drenser et al., 1998) even though those ribozymes were tested at ten times higher concentration [Mg^{+2}], which stimulated their activity.

3.2. Ribozyme 525 reduces *RHO* mRNA in cultured cells

To test Rz525 on full length mRNA *in vitro*, HEK 293 cells were co-transfected with a plasmid expressing mouse *RHO* or rat *RHO* driven by CMV promoter and a second plasmid expressing the ribozyme driven by the CBA promoter (Fig 2 A). As a control, CMV- *RHO* plasmid was co-transfected with a plasmid expressing an irrelevant ribozyme. After 36 hours, total RNA was extracted and analyzed by reverse transcription PCR as described above. We detected a 47% reduction ($p < 0.03$) of the murine *RHO* mRNA transcript with the AAV-Rz525 vector (Fig.3) indicating that the Rz525 cleavage site is accessible in the mRNA under physiologic conditions. The rat *RHO* mRNA was not digested under the same conditions (data not shown).

3.3. Injection of AAV- Rz525 and the response to the light

For testing the ribozyme in rats, its expression was driven by the mouse proximal opsin promoter (MOPS500) (Fig 2 B). The presence of splice donor/splice acceptor elements upstream and an internally cutting hairpin ribozyme downstream helps to release Rz525 from the primary transcript. This vector contains AAV2 terminal repeats but was packaged into AAV5 capsids. A similar vector expressing GFP rather than ribozyme was used as a control. The extent of retinal transduction following subretinal injection was determined by GFP immunofluorescence detected by FITC conjugated to secondary antibody (Fig. 4). In these experiments, typically 40–50% of the retina was transduced based on visualization of GFP.

Following injection, we monitored animals over a 12 week period using electroretinography (ERG). It should be noted that full-field electroretinograms measure the response from the entire retina: we could not confine the analysis to the 50% we have productively infected with AAV. Ribozyme treatment of the P23H transgenic rats resulted in significant improvement in both a- and b-wave maxima compared to control eyes (Fig. 5 B and C). Four weeks after injection (P_{45}), the a-wave amplitude in AAV-Rz525 treated eyes was rescued by 50% relative to contralateral control eyes treated with AAV-GFP. At 8 (P_{72}) and 12 (P_{100}) post injection weeks the a-wave maxima in eyes treated with Rz525 remained 50% higher than controls eyes, although absolute a-wave amplitudes declined compared to the 4 week measurement. The differences between treated and control eyes were statistically significant, $p < 0.00044$ (4 weeks), $p < 0.05$ (8 weeks), and $p < 0.018$ (12 weeks). Rz525 treatment had a similar impact on the preservation of the b-wave response. Rescue effects were consistently about 50% compared to control treated eyes. P values at 4, 8 and 12 weeks were $p < 0.0019$, $p < 0.00087$, $p < 0.04$, respectively.

3.4. Reduction of mouse P23H *RHO* mRNA levels in Rz525 treated retinas.

Following the last ERG measurement at 12 weeks after injection, mice were euthanized and eyes were analyzed for levels of rhodopsin mRNA and retinal structure. Mouse and rat rhodopsin cDNAs can be distinguished following RT-PCR because of an *NcoI* restriction site difference in the amplified region. Normalizing *RHO* mRNA recovery to levels of β -actin, we observed a 46% reduction of mouse rhodopsin mRNA (Fig.6). This reduction was much greater than that previously reported in P23H line 3 rats using AAV delivery of a P23H specific hammerhead ribozyme (Lewin et al., 1998)

Western blot analysis did not show significant difference in total opsin protein level between right and left eyes of ribozyme treated rats (data not shown). The reduction of mouse rhodopsin mRNA by 46% probably did not lead to a significant reduction of total protein detectable by the B6–30 antibody, which is directed against the N-terminus of opsin and reacts with both the rat and the mouse protein. While ribozyme can be expected to reduce the expression of mutant mouse *RHO*, delivery of the ribozyme also led to an increase in the survival of rod photoreceptors cells in the central retina (see below). The fact that there was no significant change in the level of total rhodopsin protein measured by western blot is most likely related to the comparatively small contribution of the mouse rhodopsin to total rhodopsin in the retinas that were only partially transduced with AAV expressing the ribozyme.

3.5. *Rz525 treatment prevents degeneration of retinal structure.*

In contrast, reducing the expression of the transgene had a major impact on retinal structure as measured by the thickness of the ONL. These are the nuclei of the photoreceptor cells, and the thickness of the ONL is taken as a measure of photoreceptor survival (Faktorovich et al., 1990). To estimate photoreceptor survival, frozen sections of fixed retinas were made along a vertical meridian through the optic nerve head. These sections were stained with propidium iodide and photographed with a fluorescence microscope. We found that the thickness of the ONL was preserved in retinas treated with Rz525 (Fig. 7 A and B). This preservation was evident in the superior hemisphere and in the central retina, site of subretinal vector delivery. Comparison of the ONL lengths in the Rz525 and GFP treated retinas revealed improvement in both hemispheres of the ribozyme treated retinas. However, statistically significant difference was demonstrated only in the superior retina (p value* < 0.018 and p value ** < 0.048).

4. DISCUSSION

Rhodopsin mutations leading to ADRP may be genetically dominant because they lead to the formation of a toxic protein (toxic gain-of-function mutations). An example would be c-terminal mutations which lead to the mislocalisation of rhodopsin to the nerve terminal (Deretic et al., 2005). Alternatively, they may be dominant because they interfere with the normal displacement and function of wild type rhodopsin encoded by the other allele (dominant negative mutations) (Wilson and Wensel, 2003). In either case, reducing the level of the defective protein may be beneficial as part of a gene therapy for this untreatable disease. Dominant RP has not been associated with null mutations, and the dominance is not related to haploinsufficiency. Mice that are hemizygous for a rhodopsin disruption have approximately half the rhodopsin content of normal mice, and their response to light is somewhat attenuated (Humphries et al., 1997; Lem et al., 1999). Therefore, a knockdown of mutant rhodopsin allele using a ribozyme or an siRNA might be therapeutic itself, but for full function it should be accompanied by the replenishment of rhodopsin to its normal level in order to preserve retinal structure and light sensitivity.

Because the multiplicity of rhodopsin mutations leading to ADRP or to constitutive stationary night blindness, we have designed ribozymes, like Rz525, that cleave both wild-type and mutant rhodopsin mRNAs. This is a component of an RNA replacement strategy, in which the ribozyme will be partnered with a rhodopsin cDNA that is hardened through silent mutations against cleavage by the ribozyme. We are not unique in this pursuit: others have also tested mutation-independent ribozymes and siRNAs (Kiang et al., 2005; Sullivan et al., 2002). We are the first to test the efficacy of these ribozymes in the retinas of animals. In previous papers, we showed that rhodopsin (Gorbatyuk et al., 2005) and PDE- γ (Liu et al., 2005) specific ribozymes knocked down the expression of their target genes sufficiently to cause retinal degeneration in mice. Those results confirmed that ribozymes could lead to physiological

knockdown of their target proteins *in vivo* and represented the first stage in the cut-and-replace approach to gene therapy at the RNA level.

The combination of ribozyme 525 and the P23H transgenic rats presented a special opportunity to test an allele-independent, species-specific ribozyme. Since this ribozyme cuts mouse, but not rat rhodopsin mRNA, it was able to deplete the level of mutant transcript by 46% resulting in partial rescue of the retinal degeneration phenotype. This indicates that allele independent ribozymes alone can slow the course of retinal degeneration in the correct genetic context. Rescue was not complete, most likely because the entire retina was not transduced, leaving approximately 50% of rods untreated. Therefore, since we followed retinal degeneration by full-field ERG, which measures the light response of the whole retina, we could not expect complete preservation of the response. The relative rescue of ribozyme treated eyes compared to control injected eyes was approximately 50% for both a-wave and b-wave amplitudes, and this level corresponds to the preservation of photoreceptors based on ONL thickness. Machida and co-workers (Machida et al., 2000) reported a tight correlation between the ERG response and the survival of rod photoreceptor cells during the maturation of P23H rats. Our result confirms this relationship for P23H rats which were the subjects of gene therapy.

Despite the observed rescue after treatment, ERG amplitudes continued to decline at a parallel rate in both treated and control eyes. The probable cause for this is the light sensitivity of P23H rats. These albino animals are very sensitive to mesopic light of 40–60 lux (Walsh et al., 2004). Although we have taken special precautions, such as removing bulbs from the overhead lighting and shielding the tops of the racks to reduce their exposure, the erosion of the ERG response was faster in the P23H line 3 animals used in this experiment than we had previously seen in the animals of the same line housed at the University of California, San Francisco (LaVail et al., 2000; Lewin et al., 1998).

There are several advantages of an allele independent approach using ribozymes to knockdown endogenous mRNA. First, because ribozymes can be designed to cleave regions of the mRNA unaffected by the most prevalent ADRP mutations, we can treat a broad spectrum of the rhodopsin mutations. This makes overcoming safety and regulatory issues simpler than if an individual ribozyme was needed to target each *rho* mutation in an allele specific approach. Second, designing allele independent ribozymes is easier. We can use the findings of others (Clouet-d'Orval, B. and Uhlenbeck, O.C. 1997) to design highly active ribozymes without being confined to a particular target site. Indeed, Rz525 was far more active *in vitro*, even at low levels of divalent cation, than other allele-specific ribozymes we have used for gene therapy for P23H in the past (Drenser et al., 1998). As expected, Rz525 was also much more effective *in vivo* than our previously designed allele specific hammerhead and hairpin ribozymes in reducing the level of P23H mRNA (46% reduction versus 11–15% reduction) (Lewin et al., 1998). The reduction was sufficient to make a physiologic impact (Figs. 5 A and B, Fig. 7 A and B).

Before they can be used for human gene therapy, ribozymes such as Rz525 may need to be coupled with a ribozyme resistant *RHO* gene as a replacement for endogenous rhodopsin. Hardening the target through silent mutations is not a major hurdle—the single nucleotide change that discriminates rat from mouse *RHO* mRNA makes the rat mRNA resistant to the ribozyme (Fig. 1). What is a challenge is to express the resistant protein at an appropriate level to replace the wild type protein lost due to ribozyme destruction of its mRNA. Another concern is over-expression of rhodopsin may be toxic to photoreceptors (Olsson et al., 1992; Tan et al., 2001). Because of this possible toxicity and because we cannot infect every photoreceptor cell, it is probably prudent to deliver the ribozyme and the replacement gene in one package, i.e., using the same AAV vector. This should be possible because the coding regions for the ribozyme and for rhodopsin are both short (35 nt and 1046 nt, respectively). Cell type specific

expression can be mediated by a rhodopsin promoter less than 500 bp (Flannery et al., 1997; Zack et al., 1991), so that all of the required elements could fit within the packaging limits of AAV.

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Fig. 1.

Secondary structure and activity of a mouse-specific ribozyme. (A) Structure of Rz525 targeting mouse RHO. The rat RHO sequence has mismatch with the ribozyme 4 residues before the cleavage site to the cleavage site (arrow). (B) Comparison of mouse and rat target oligonucleotides reacted with ribozyme *in vitro*. ^{32}P -labeled target oligonucleotides (10 pmole) were incubated in the presence of Rz525 (1 pmole) at 37° C, 20 mM Tris HCl, pH 7.8, 2 mM MgCl_2 . At 0, 1, 2, 3, 4, 5, 10, 30 and 60 min, 5 μl aliquots were removed, mixed with formamide-dye and loaded on a denaturing polyacrylamide gel to identify cleavage products (6 nucleotides). (C) Quantitation of the percent of mouse target cleaved based on the gel shown in (B).

Fig. 2.

Construction of an AAV vectors to express Rz525 in cultured cells (A) and *in vivo* (B). In cultured cells expression of Rz525 was driven by the hybrid CMV enhancer chicken β -actin (CBA) promoter. Ribozyme was released by the splicing of β -actin intron on 5' end and an internally cleaving hairpin Rz on the 3' flank. *In vivo* expression of Rz 525 was driven by the MOPS500 promoter, which contains 487 bp of the proximal promoter region of the mouse rod opsin gene. The presence of splice donor-splice acceptor element from SV40 aids in trafficking of the ribozyme from the nucleus to the cytoplasm, and a hairpin ribozyme helps release Rz525 from the primary transcript. TR= AAV2 terminal repeats.

Fig. 3. Reduction of murine *RHO* mRNA in cultured cells following co-transfection with pCBA-Rz525. Molar ratio of target plasmid to ribozyme plasmid in the transfection mixture was 1:10. Levels were measured by RT-PCR normalized to β -actin mRNA.

Fig. 4. Immunostaining of retinas injected with AAV5 MOPS500GFP. GFP is shown in green, rhodopsin staining is shown in blue, nuclear DNA staining by propidium iodide is red.

Fig. 5. Preservation of a-wave (A) and b-wave (B) amplitudes of full field ERG analysis in P23H rats injected at p16 in their right eyes with AAV5 Rz525 (black columns) and AAV5 GFP in their left eyes (white columns). (C) Representative wave forms from 3 different animals reflecting a more intense response to light flash in right (Rz treated) eyes at 8 weeks post injection.

Fig. 6.

Specific reduction of mouse *RHO* mRNA in ribozyme treated eyes. Panel A: Right eyes injected with Rz 525 show reduction in mouse *RHO* mRNA content. ($P < 0.025$). To distinguish mouse and rat mRNA, total PCR product was digested with the mouse *RHO*-specific endonuclease NCO1. Panel B: Image of polyacrylamide gel with representative mouse and rat *RHO* RT-PCR products obtained from right eyes injected with Rz525 (Rz+) and left eyes injected with AAV5 GFP (control).

Fig. 7.

Ribozyme treatment preserves the integrity of the outer nuclear layer. Images of retinas were divided into 12 sectors. Length of ONL was measured at 10 loci in each sector under 100X magnification and expressed in relative units. (A) Observed differences in ONL thickness of the superior hemisphere. P value * - 0.018 and ** - 0.048. (B) Representative sections from the central retinas of control (left) and ribozyme treated eyes (right).