Ribozyme rescue of photoreceptor cells in P23H transgenic rats: Long-term survival and late-stage therapy

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Ribozyme-directed cleavage of mutant mRNAs appears to be a potentially effective therapeutic measure for dominantly inherited diseases. We previously demonstrated that two ribozymes targeted to the P23H mutation in rhodopsin slow photoreceptor degeneration in transgenic rats for up to 3 months of age when injected before significant degeneration at postnatal day (P) 15. We now have explored whether ribozyme rescue persists at older ages, and whether ribozymes are effective when injected later in the degeneration after significant photoreceptor cell loss. Recombinant adeno-associated virus (rAAV) vectors incorporating a proximal bovine rod opsin promoter were used to transfer either hairpin or hammerhead ribozyme genes to photoreceptors. For the study of long-term survival, rAAV was administered by subretinal injection at P15, and the rats were allowed to live up to 8 months of age. For the study of late-stage gene transfer, rAAV was administered at P30 or P45, when 40–45% of the photoreceptors already had degenerated. Eyes were examined functionally by the electroretinogram and structurally by morphometric analysis. When injected at P15, expression of either ribozyme markedly slowed the rate of photoreceptor degeneration for at least 8 months and resulted in significantly greater electroretinogram amplitudes at least up to P180. When injected at P30 or P45, virtually the same number of photoreceptors survived at P130 as when injected at P15. Ribozyme rescue appears to be a potentially effective, long-term therapy for autosomal dominant retinal degeneration and is highly effective even when the gene transfer is done after significant photoreceptor cell loss.

Retinal diseases offer particular promise as targets for gene therapy, because more than 120 different genes have been identified as the cause of retinal disorders (www.sph.uth.tmc.edu/RetNet/), retinitis pigmentosa alone affecting as many as 1 in 3,500 people worldwide (1, 2). Even the much more prevalent age-related macular degeneration, affecting as many as 1 in 4 people by the age of 75, almost certainly has genetic components (3, 4). Naturally occurring, transgenic and gene knockout animal models of several species are readily available that are identical or similar to many of the human disorders (5–7). Moreover, the retina is one of the most readily accessible tissues for experimentation, with relatively straightforward methods of delivery to the subretinal space and its adjacent photoreceptors and retinal pigment epithelial cells. Indeed, gene-based therapy of several types already has been attempted in animal models with retinal degenerations, including the replacement of missing enzymes in recessive disorders (8–11), gene-based delivery of protective neurotrophic factors (12–15), and the introduction of antiapoptosis genes such as *bcl-2* (16).

The problem of dominantly inherited mutations is particularly vexing because production of inappropriate gene products disturbs metabolism or causes the death of cells, as seen by the degeneration of photoreceptors in many different forms of autosomal dominant retinitis pigmentosa. One approach to such disorders is the use of ribozymes, small RNA molecules that cleave mutant transcripts in an allele-specific manner while leaving wild-type transcripts intact. Ribozyme-catalyzed inhibition of gene expression has been shown to occur in several *in vitro* systems (17, 18) and *in vivo* (19–23). In a demonstration that ribozymes may be used therapeutically with dominant negative alleles, we recently found that ribozymes delivered to photoreceptors by using recombinant adeno-associated virus vectors (rAAV) cannot only reduce the mutant transcripts *in vitro* (24), but also *in vivo* (25), thereby slowing the progression of photoreceptor degeneration in P23H (histidine substituted for proline at position 23) mutant rhodopsin transgenic rats (25). In these experiments, both hairpin (Hp11) and hammerhead (Hh13) ribozymes were designed to target the mutant transcript, a model for the most common form of autosomal dominant retinitis pigmentosa in the United States (26). rAAV vectors contained the ribozyme genes under the control of a proximal bovine opsin promoter. We injected these viruses subretinally at postnatal day (P) 15, before photoreceptors had degenerated appreciably, and examined the eyes at different times from P60 to P90 when many photoreceptors had degenerated. At these ages, the uninjected control eyes had only about 60% of the number of photoreceptors of age-matched, normal wild-type retinas because of expression of the mutant opsin, whereas the ribozyme-treated eyes still had about 83–88% of the normal number. This protection or "rescue" was accompanied by significantly greater a and b waves of the electroretinogram (ERG), indicating a functional rescue, as well (25).

With any potential therapeutic approach, two most relevant questions are how long will the effect last and how late in the disease can the therapy be applied and still have a positive effect? Regarding the duration of action, we would hope that a rAAV-mediated ribozyme effect might be long-lived, because it has now been found that reporter genes (e.g., green fluorescent protein) delivered to photoreceptor cells by rAAV are still expressed after a year in primates (27). In addition, although AAV does lead to a modest humoral immune response, experimental AAV infection in rodents and primates does not lead to a cellular immune response (27, 28). The second question concerns delayed onset of treatment and is relevant to human gene therapy

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Abbreviations: rAAV, recombinant adeno-associated virus; ERG, electroretinogram; P, postnatal day; ONL, outer nuclear layer.

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Table 1. Number of eyes measured histologically in ribozyme experiments

Age injected–age eyes taken	Hp11	Hh13
P15–P130	4	3
P15–P240	5	8
P30–P130	3	3
P45–P130	5	5

Animals were killed at these ages ± 3 days.

in that patients usually will present with symptoms as a consequence of ongoing retinal degeneration. We might expect that the effectiveness of therapy would diminish in proportion to disease progression. Indeed, in some cases, such as retinal ischemia, very little palliative effect is seen when various agents are applied after the insult (29). We now have examined the issues of long-term effect and late-stage gene transfer for ribozyme therapy in the P23H transgenic rats. We have used postinjection intervals to 8 months of age after early injection, or injection at later ages after significant loss of photoreceptor cells.

Materials and Methods

rAAV-Ribozyme Constructs. rAAV constructs are identical to those reported in Lewin *et al.* (25), and the ribozymes have been described in Drenser *et al.* (24). Each of the rAAV-ribozyme virus preparations contained 10^{11}-10^{12} DNase-resistant particles per ml and 10^{9}-10^{10} infectious center units per ml. No helper adenovirus is used in this preparation, so that there is no adenoviral contamination. Wild-type AAV contamination is below levels of detection, one part in 10^{7}.

Animals and Subretinal Injection of rAAV. Transgenic rats with a P23H rhodopsin mutation (produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ on an albino Sprague–Dawley background) of the line TgN(P23H)3 (abbreviated P23H-3) were reared and maintained in a 12 h:12 h light/dark environment of less than 15 ft-c illuminance. At different ages (Table 1) animals were anesthetized by ketamine/xylazine injection, and a local anesthetic (proparacaine HCl) was applied topically to the cornea. Subretinal injections of the rAAV were made with an anterior approach as described (25). This anterior approach results in an 85–90% success rate, but gives a 20–40% incidence of cataracts regardless of the agent injected, including PBS (25), and such animals were not used for ERG analysis. The opposite eye was uninjected because earlier studies showed that PBS-injected surgical control retinas were indistinguishable from those of uninjected eyes (25). All procedures involving the rats adhered to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and the University of California, San Francisco Committee on Animal Research.

Electroretinographic Analysis. Rats were dark-adapted overnight and then in dim red light, were anesthetized with intramuscular injections of xylazine (13 mg/kg) and ketamine (87 mg/kg). Full-field scotopic ERGs were elicited with 10-μsec flashes of white light, and responses from both eyes were recorded simultaneously by using a UTAS-E 2000 Visual Electrodiagnostic System (LKC Technologies, Gaithersburg, MD) as described (25). For quantitative comparison of differences in a-wave and b-wave amplitudes between the two eyes of individual rats, the values from all the saturating stimulus intensities were averaged for a given animal. Means for a given experimental paradigm were compared statistically by using the Student's two-tailed, paired t test.

Retinal Tissue Preparation and Morphometry. At different ages (Table 1) rats were euthanized by overdose of carbon dioxide inhalation and immediately perfused intracardially with a mixture of mixed aldehydes (2% paraformaldehyde and 2.5% glutaraldehyde). Eyes were removed, postfixed in osmium tetroxide, and embedded in epoxy resin, and 1-μm thick histological sections were made along the vertical meridian (30). The thickness of the outer nuclear layer (ONL) was taken as a measure of photoreceptor number (31), and was obtained as described (32). Briefly, 54 measurements of the ONL were made at 18 contiguous fields (each 440 μm in length) around the entire retinal section (three measurements per field; nine fields in each of the superior and inferior hemispheres). The 54 measurements were either averaged to provide a single value for each retina to allow statistical comparison of treatment groups or plotted as a distribution of thickness across the retina. Tissue sections were chosen where the photoreceptor outer segments and Müller cell processes crossing the inner plexiform layer were continuous in the plane of section to assure that the sections were not oblique. For statistical analysis, each treatment group was compared with all of the untreated eyes of the same age taken at the same time by using the Student's two-tailed, unpaired t test.

Results

Long-Term Ribozyme Therapy. To examine the question of persistence of ribozyme rescue over the long term, we injected the rAAV vectors carrying the Hp11 or Hh13 ribozyme DNAs at P15, before appreciable photoreceptor cell death in the P23H-3 rats, and examined the retinas histologically at P130 and P240. As shown in Fig. 1, representative sections of Hh13-injected retinas illustrate that at these ages the action of the ribozymes still results in the rescue of photoreceptors. For example, at P130 in the posterior retina of the inferior hemisphere in untreated, control eyes, the ONL was reduced from the normal 8–9 rows to about 3–4 rows of photoreceptor nuclei (Fig. 1*A*). The photoreceptor inner and outer segments were still present and their structure was relatively normal and not vacuolated or in structural disarray, although they were only about 30–40% of their normal length (Fig. 1*A*). By contrast, the Hh13 ribozyme-injected retinas from the same animals typically displayed almost twice the number of photoreceptor nuclei (Fig. 1*B*). Equally as important, the cells' inner and outer segments were considerably longer, about 70–80% of normal length. At P240, more photoreceptor degeneration had occurred, with the reduction in the ONL to only 1–2 rows of nuclei in the untreated control eyes, and only short remnants of photoreceptor inner and outer segments surviving (Fig. 1*C*). In the Hh13 ribozyme-treated eyes of the same rats at P240, however, 4–5 rows of nuclei in the ONL survived, and significantly longer photoreceptor inner and outer segments were present (Fig. 1*D*).

When the entire retinal extent was quantified by obtaining the mean ONL thickness for each eye, the morphometric analysis clearly demonstrated that application of either the Hp11 or Hh13 ribozymes at P15 resulted in long-term rescue 115 and 225 days later (Fig. 2). At both P130 and P240, the ONL thickness in each of the ribozyme-treated retinas was significantly greater than in the control eyes ($P < 0.0005$ for each ribozyme at each age). As noted earlier (25), the thickness of the ONL in the ribozyme-treated retinas was about 83–88% of the normal wild-type at the ages of P60-P90 (Fig. 2). In comparison, at P240 the ONL was still about 63–73% of the normal wild type at this older age (Fig. 2).

The distribution of ribozyme rescue in the earlier study between P60-P90 was found to be pan-retinal (25). In the present study, the rescue was also pan-retinal as seen at P130 (Fig. 3*A*) and at P240 (Fig. 3 *B* and *C*). Even though the degeneration is normally more severe in the superior hemisphere of P23H rats, when the means of 27 measurements from each hemisphere were

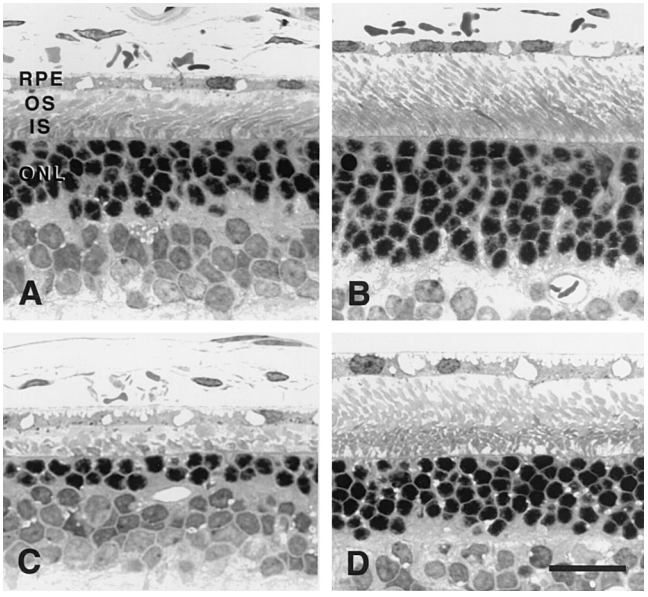

Fig. 1. Light micrographs of plastic-embedded P23H transgenic rat retinas taken at P130 (*A* and *B*) or P240 (*C* and *D*) from the posterior inferior region along the vertical meridian of the eye. (*A*) Uninjected eye from a rat at P130, which shows 3–4 rows of photoreceptor nuclei in the ONL, reduced from the normal 8–9 at this age. Photoreceptor inner segments (IS) and outer segments (OS) are shorter than normal, although still intact. RPE, retinal pigment epithelium. (*B*) Retina from the opposite eye from the same rat as in *A*, which was injected subretinally injected with Hh13 ribozyme at P15. About 6–7 rows of photoreceptor nuclei are present, and the photoreceptor inner and outer segments are about 70–80% of normal length. (*C*) Uninjected eye from a rat at P240, which shows only 1–2 rows of photoreceptor nuclei surviving, and only remnants of inner and outer segments present. (*D*) Retina from the opposite eye from the same rat in *C*, which was injected subretinally injected with Hh13 ribozyme at P15. About 4–5 rows of photoreceptor nuclei are still present and the inner and outer segments are almost as long as at P130 (*B*). Toluidine blue stain. (Bar = 20 μm.)

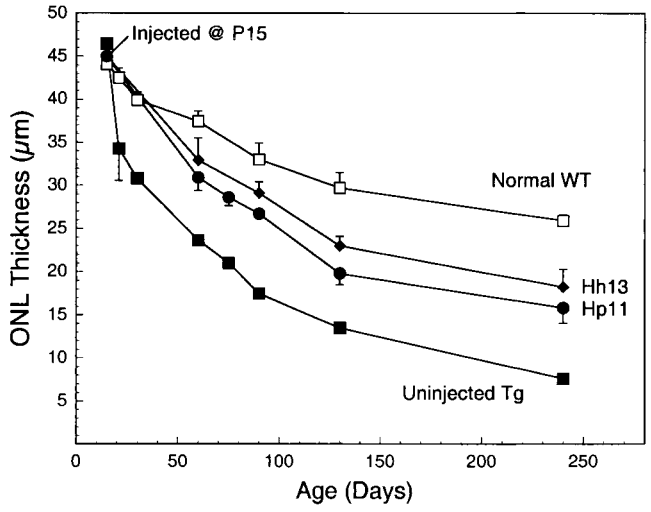

Fig. 2. Measurements of ONL thickness in rats killed at different ages. □ denote normal wild-type (WT), nontransgenic animals. P23H-3 transgenic (Tg) rats were either uninjected (■) or injected subretinally with rAAV vectors carrying either Hp11 (●) or Hh13 (◆) ribozymes. All injections were done at P15. The symbols represent mean ± SD, and error bars were omitted if they fell within the symbol. The number of animals in each group is shown in Table 1. The data points at P60, P75, and P90 are taken from our earlier study (25).

compared, the superior hemisphere of the rats injected with either Hp11 or Hh13 ribozymes was still statistically different from that of the uninjected eyes. An example of the means of three representative retinas from P240 rats is plotted in Fig. 3*B*. However, as shown in Fig. 3*C*, some regions of the superior hemisphere at this age clearly were no longer rescued in some individual retinas. Experiments are continuing to establish the full time course of rescue, particularly in the less severely damaged inferior hemisphere.

To determine whether long-term functional rescue accompanied the photoreceptor structural preservation, ERGs were measured at P180 and P240 in rats scheduled to survive until P240. The Hh13 ribozyme-rescued eyes at P180 showed statistically greater b-wave and a-wave amplitudes than the untreated eyes (Table 2). At P180 and P240 with the Hp11 ribozyme and at P240 with the Hh13 ribozyme, all of the b-wave and a-wave amplitudes were also greater than those of the untreated eyes, but in these groups the differences did not achieve statistical significance (Table 2). This lack of statistical significance resulted in part from the small sample size because of the omission of some rats with cataracts in each group, as well as to a relatively large scatter in the data for the small sample size, particularly at the oldest ages. This lack of significance also may be caused by the fact that, at this more advanced age, only the inferior hemisphere is substantially rescued structurally. Hence, full-field ERG measurements that average the light response across the retina may not effectively monitor local functional rescue in the inferior hemisphere. Thus, at present we can conclude that functional rescue accompanies structural rescue for a substantially longer period than in the previous study (25), but we have not yet been able to determine the exact length of time the functional protection persists. Moreover, the present results preclude a meaningful interpretation of differential structural and functional rescue at late stages of degeneration, which will require additional studies.

Late-Stage Ribozyme Therapy. The second question we addressed was whether ribozymes are effective if introduced after a retinal degeneration has begun. In this case, instead of injecting at P15, before significant photoreceptor degeneration, we injected the ribozymes at 30 or 45 days of age, when 40–45% of the photoreceptors already had been lost. The results are shown in Fig. 4, where the data from the Hp11 (Fig. 4*A*) and Hh13 (Fig. 4*B*) ribozymes are plotted separately. When examined at P130, it can clearly be seen that significant ribozyme rescue occurred after ribozyme treatment at both later ages of P30 and P45. For comparison with the results of injection at P15, before photoreceptor cell death, those data points for the injections at P15 also are shown in Fig. 4. Remarkably, the results with either ribozyme at P130 showed that virtually the same number of photoreceptors survived when injected at the later ages as they did when injected earlier, before the loss of cells. In each case the ONL in the treated retina was approximately 70–82% of the age-matched, normal wild-type value. It therefore appears that even after substantial photoreceptor cell loss, those cells remaining are still responsive to ribozyme therapy.

Discussion

It was previously demonstrated that the *in vivo* expression of either a hairpin or hammerhead ribozyme could slow the rate of photoreceptor degeneration for at least 3 months in P23H-3 mutant rhodopsin rats (25). We now have shown that the protective effect lasts for at least 8 months and likely longer. Moreover, ribozyme-protected photoreceptors appear to be significantly healthier based on their substantially longer inner and outer segments, and they give larger functional responses than untreated eyes as measured by the ERG for up to 6 months

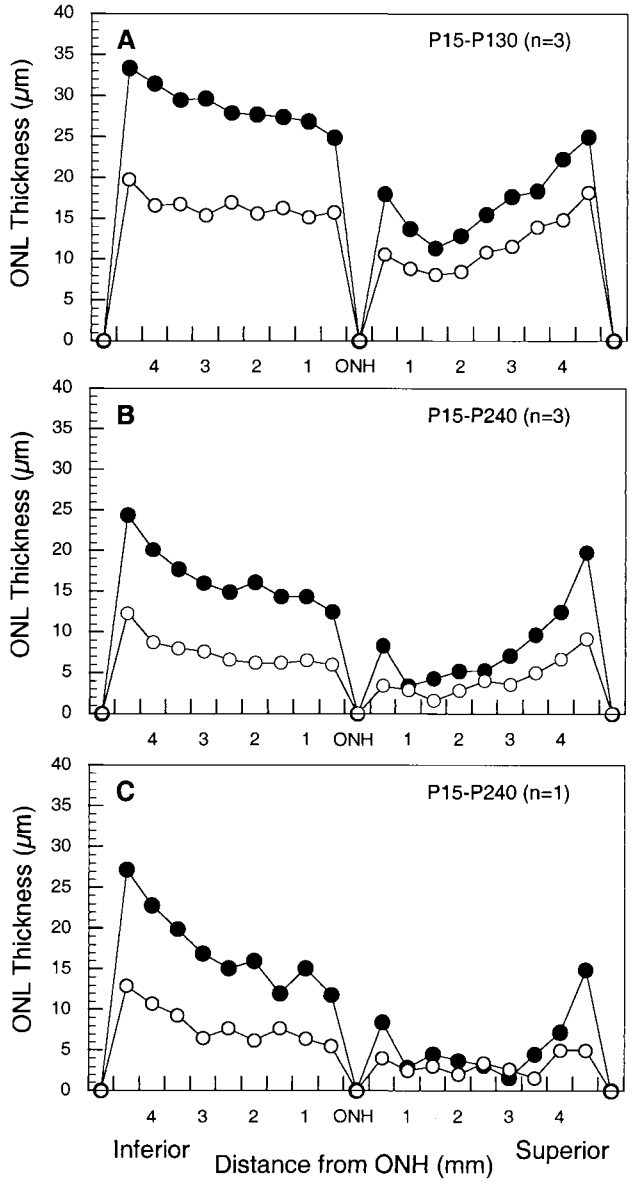

Fig. 3. Measurement of the ONL thickness along the vertical meridian of the eye from the optic nerve head (ONH) to the ora serrata (anterior margin of the retina) of P23H-3 mutant rhodopsin transgenic rats. The rats were either uninjected (○) or injected (●) with Hh13 ribozymes at P15 and the eyes taken either at P130 (*A*) or P240 (*B* and *C*). Means of three representative retinas are shown in *A* and *B*, and the plot of a single rat of those plotted in *B* is shown in *C*. For each rat, three measurements were made in each of nine 440-μm fields in both the superior and inferior hemispheres.

of age, and probably longer. Significantly, the degree of structural protection is relatively greater at the older ages than at the younger ages when compared with the number of surviving photoreceptors in the untreated eyes. At the earlier ages of P60-P90 (45–75 days postinjection), the number of photoreceptors rescued was less than 1.5 times that in the untreated eyes, but at 8 months of age, the ribozyme-treated eyes had 2–2.5 times the number of photoreceptors present in the untreated eyes (Fig. 2).

In experiments where we delayed the administration of rAAV injection and ribozyme expression until after 40–45% of the photoreceptors had been lost, the ribozyme-mediated rescue remained remarkably effective. Using either Hp11 or Hh13

Table 2. Percent difference in ERG amplitudes between ribozyme-treated and control eyes after injection at P15

Ribozyme	Age analyzed	*n*	b wave	*P*	a wave	*P*
Hh13	P180	3	38.1%	<0.0005	27.7%	<0.005
Hh13	P240	2	45.3%	NS	—	—
Hp11	P240	2	28.5%	NS	7.4%	NS

All values of ribozyme-treated eyes are greater than those of control eyes. NS, not significant.

ribozymes, the number of rescued PR cells at P130 was virtually the same when we administered virus either before (at P15) or after (P30 or P45) the loss of many photoreceptors. These findings are somewhat surprising, because we would have suspected less effective rescue with the later injections simply based on the general therapeutic principle that it is usually more effective to treat at earlier stages of a degeneration. The present finding is even more remarkable when an additional point is considered.

After rAAV infection, expression of reporter genes is typically first seen only after a week or more (33), and full expression is not seen for 3–4 weeks after infection (33–35). If ribozyme expression follows the same delay in onset as the reporter genes, then the injections made in the present study at P30 and P45 would effectively represent the administration of active protective agents even later in the degeneration. This finding adds to the idea that ribozymes are remarkably effective at the later stages of degeneration, at least at this time period in the P23H-3 model, and may halt degeneration completely, at least for the period studied of about 100 days (from P30 to P130).

Why are ribozymes so effective when injected at later stages of the degeneration? One possible explanation noted above for this is that a greater proportion of the rod photoreceptors may be transduced at a later stage than at an earlier stage of degeneration. We know from our previous study that the rAAV viral titer allows about 20–30% of the total rod population to be transduced when all of the rods are present at the time of

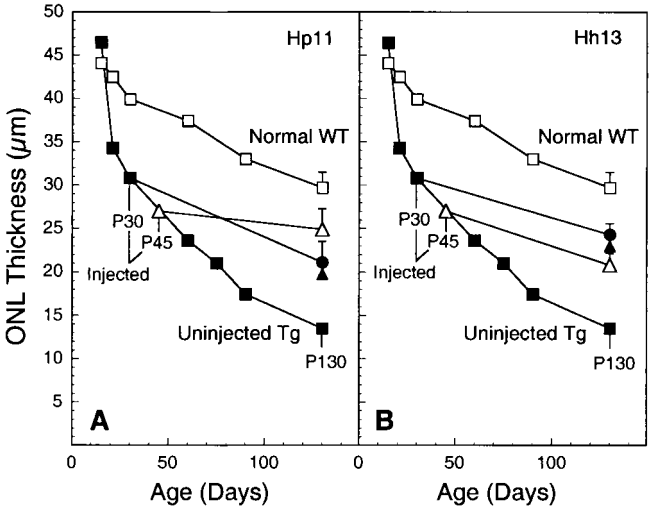

Fig. 4. Measurements of ONL thickness in rats killed at P130 after late-stage ribozyme injection at P30 and P45. Data for the normal wild-type (WT) nontransgenic rats (□) and the uninjected P23H-3 transgenic (Tg) rats (■) are the same as in Fig. 2. P23H-3 rats were injected subretinally with rAAV vectors carrying either Hp11 (*A*) or Hh13 (*B*) ribozymes at either P30 (●) or P45 (△). For comparison, the data point from injection at P15 and killed at P130 is shown (▲). The values represent mean ± SD, and error bars were omitted if they fell within the symbol. The number of animals in each group is shown in Table 1.

Table 3. Uniform preservation of ONL

Age injected	Age analyzed	ONL preserved, μm
P15	P60	8.3
P15	P75	8.8
P15	P90	10.4
P15	P130	7.9
P15	P240	9.4
P30	P130	9.2
P45	P130	9.2

ONL preserved = ONL thickness (μm) of ribozyme-treated retinas (average of Hp11 and Hh13) − ONL thickness of uninjected eyes.

injection at P15 (35). At P30 and P45, only about 55–60% of the photoreceptors are present at the time of injection. Given the same viral titer in the subretinal injections at the younger and older ages, this would mean that the same absolute number of cells could be infected. So instead of 20–30% of the rod photoreceptors potentially being transduced at P15, potentially 50–55% of the rods could be transduced at the older ages. This higher transduction percentage may well be responsible for the overall slower rate of degeneration in the retinas of the rats injected at older ages. If this interpretation is correct, then it argues strongly for the need and advantage of even higher rAAV titers for gene-transfer therapeutic studies.

Another possible explanation for the high effectiveness of the ribozyme injections at the older ages is that the rate of photoreceptor cell degeneration and loss is different at the two injection times. For the duration of this study, the degeneration and cell loss rate is biphasic, with a very rapid loss between P15 and about P30 and then a significantly slower rate between about P30 and P130 (Figs. 2 and 4). It may be that ribozymes are more effective in preventing cell loss when fewer cells are entering the apoptotic cell death pathway, or about to do so, either directly or indirectly through neighboring trophic effects (36, 37). Because human retinitis pigmentosa is usually a very slow, progressive disease, ribozyme-mediated protection might be expected to be even more effective than in the animal models. Regardless of the mechanism, these findings of highly effective ribozyme rescue after late-stage gene transfer have very positive implications for the potential for ribozyme therapy for dominantly inherited diseases at many stages of photoreceptor degeneration.

One interesting feature of the ribozyme-mediated preservation of ONL thickness is its quantitative constancy, independent of when therapy was initiated or the survival interval. The direct inference is that the number of photoreceptor cell bodies rescued was surprisingly independent of when or how long therapy ensued. This is clearly seen from the data in Table 3 where the improvement in ONL thickness over the contralateral control eye (average of both Hp11 and Hh13 ribozyme treatments) is calculated as a function of each treatment protocol. It appears that a fixed amount of AAV-ribozyme vector delivered to the rat subretinal space leads to a fixed number of surviving photoreceptors regardless of treatment timing. What aspects of this therapeutic paradigm might account for this sort of behavior? Perhaps most significantly, this AAV vector system is a nonreplicative gene delivery system because all viral genes have been deleted in the recombinant vector. Presumably therefore, only photoreceptors receiving a minimally effective dose of vector at the time of subretinal injection will express a level of ribozyme sufficient to down-regulate P23H opsin mRNA for long-term cell survival. Thus, the question of how many photoreceptors are rescued may not be as intimately tied to the timing of the treatment as it is to how many photoreceptors are effectively transduced at the time of vector injection. Because we attempted to reproduce vector inoculation parameters as closely as possible in all experiments, it is likely that we effectively transduced more or less the same number of photoreceptors in all experiments. This is consistent with observing the 8- to 10.4-μm rescue in ONL thickness in all cases (Table 3) and suggests that the number of photoreceptor cells rescued by ribozyme expression primarily is related to how many target cells are effectively transduced at the time of vector injection. Clearly, the way to experimentally test this conclusion is to introduce more vector through multiple injections in both hemispheres.

In conclusion, ribozyme-directed cleavage of mutant mRNAs appears to be a potentially effective, long-term therapy for autosomal dominant retinal degenerations, and ribozyme rescue can be highly effective even when the gene transfer is done after significant photoreceptor cell loss.

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1. Humphries, P., Farrar, G. J., Kenna, P. & McWilliam, P. (1990) *Clin. Genet.* **38,** 1–13.
2. Berson, E. L. (1993) *Invest. Ophthalmol. Visual Sci.* **34,** 1659–1676.
3. Gorin, M. B., Breitner, J. C. S., De Jong, P. T. V. M., Hageman, G. S., Klaver, C. C. W., Kuehn, M. H. & Seddon, J. M. (1999) *Mol. Vis.* **5,** 29.
4. Zack, D. J., Dean, M., Molday, R. S., Nathans, J., Redmond, T. M., Stone, E. M., Swaroop, A., Valle, D. & Weber, B. H. (1999) *Mol. Vis.* **5,** 30.
5. Wong, F. (1995) *Arch. Ophthalmol.* **113,** 1245–1247.
6. Kohler, K., Guenther, E. & Zrenner, E. (1997) *Klin. Monatsbl. Augenheilkd.* **211,** 84–93.
7. Petersen-Jones, S. M. (1998) *Eye* **12,** 566–570.
8. Bennett, J., Wilson, J., Sun, D., Forbes, B. & Maguire, A. (1996) *Nat. Med.* **2,** 649–654.
9. Jomary, C., Vincent, K. A., Grist, J., Neal, M. J. & Jones, S. E. (1997) *Gene Ther.* **4,** 663–690.
10. Kumar-Singh, R. & Farber, D. B. (1998) *Hum. Mol. Genet.* **7,** 1893–1900.
11. Takahashi, M., Miyoshi, H., Verma, I. M. & Gage, F. H. (1999) *J. Virol.* **73,** 7812–7816.
12. Cayouette, M. & Gravel, C. (1997) *Human Gene Ther.* **8,** 423–430.
13. Cayouette, M., Behn, D., Stendtner, M., Lachapelle, P. & Gravel, C. (1998) *J. Neurosci.* **18,** 9282–9293.
14. Akimoto, M., Miyatake, S., Kogishi, J., Hangai, M., Okazaki, K., Takahashi, J. C., Saiki, M., Iwaki, M. & Honda, Y. (1999) *Invest. Ophthalmol. Visual Sci.* **40,** 273–279.
15. Uteza, Y., Rouillot, J. S., Kobetz, A., Marchant, D., Pecqueur, S., Arnaud, E., Prats, H., Honiger, J., Dufier, J. L., Abitbol, M., *et al*. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3126–3131.
16. Bennett, J., Zeng, Y., Bajwa, R., Klatt, L., Li, Y. & Maguire, A. M. (1998) *Gene Ther.* **5,** 1156–1164.
17. Birikh, K. R., Heaton, P. A. & Eckstein, F. (1997) *Eur. J. Biochem.* **245,** 1–16.
18. Rossi, J. J. (1997) *Ciba Found. Symp.* **209,** 195–204.
19. Lieber, A. & Kay, M. A. (1996) *J. Virol.* **70,** 3153–3158.
20. L'Huillier, P. J., Soulier, S., Stinnakre, M. G., Lepourry, L., Davis, S. R., Mercier, J. C. & Vilotte, J. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6698–6703.
21. Macejak, D. G., Lin, H., Webb, S., Chase, J., Jensen, K., Jarvis, T. C., Leiden, J. M. & Couture, L. (1999) *J. Virol.* **73,** 7745–7751.
22. Abounader, R., Ranganathan, S., Lal, B., Fielding, K., Book, A., Dietz, H., Burger, P. & Laterra, J. (1999) *J. Natl. Cancer Inst.* **91,** 1548–1556.
23. Pavco, P. A., Bouhana, K. S., Gallegos, A. M., Agrawal, A., Blanchard, K. S., Grimm, S. L., Jensen, K. L., Andrews, L. E., Wincott, F. E., Pitot, P. A., *et al*. (2000) *Clin. Cancer Res.* **6,** 2094–2103.

24. Drenser, K. A., Timmers, A. M., Hauswirth, W. W. & Lewin, A. S. (1998) *Invest. Ophthalmol. Visual Sci.* **39,** 681–689.
25. Lewin, A. S., Drenser, K. A., Hauswirth, W. W., Nishikawa, S., Yasumura, D., Flannery, J. G. & LaVail, M. M. (1998) *Nat. Med.* **4,** 967–971.
26. Berson, E. L., Rosner, B., Sandberg, M. A. & Dryja, T. P. (1991) *Arch. Ophthalmol.* **109,** 92–101.
27. Bennett, J., Maguire, A. M., Cideciyan, A. V., Schnell, M., Glover, E., Anand, V., Aleman, T. S., Chirmule, N., Gupta, A. R., Huang, Y., *et al*. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 9920–9925.
28. Hernandez, Y. J., Wang, J., Kearns, W. G., Loiler, S., Poirier, A. & Flotte, T. R. (1999) *J. Virol.* **73,** 8549–8558.
29. Unoki, K. & LaVail, M. M. (1994) *Invest. Ophthalmol. Visual Sci.* **35,** 907–915.
30. LaVail, M. M. & Battelle, B. A. (1975) *Exp. Eye Res.* **21,** 167–192.
31. Michon, J. J., Li, Z. L., Shioura, N., Anderson, R. J. & Tso, M. O. M. (1991) *Invest. Ophthalmol. Visual Sci.* **32,** 280–284.
32. Faktorovich, E. G., Steinberg, R. H., Yasumura, D., Matthes, M. T. & LaVail, M. M. (1990) *Nature (London)* **347,** 83–86.
33. Bennett, J., Duan, D., Engelhardt, J. F. & Maguire, A. M. (1997) *Invest. Ophthalmol. Visual Sci.* **38,** 2857–2863.
34. Ali, R. R., Reichel, M. B., De Alwis, M., Kanuga, N., Kinnon, C., Levinsky, R. J., Hunt, D. M., Bhattacharya, S. S. & Thrasher, A. J. (1998) *Hum. Gene Ther.* **9,** 81–86.
35. Flannery, J. G., Zolotukhin, S., Vaquero, M. I., LaVail, M. M., Muzyczka, N. & Hauswirth, W. W. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6916–6921.
36. Huang, P. C., Gaitan, A. E., Hao, Y., Petters, R. M. & Wong, F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8484–8488.
37. Mohand-Said, S., Deudon-Combe, A., Hicks, D., Simonutti, M., Forster, V., Fintz, A. C., Leveillard, T., Dreyfus, H. & Sahel, J. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8357–8362.