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Ribozyme gene therapy: applications for molecular medicine

Alfred S. Lewin and William W. Hauswirth

RNA enzymes – ribozymes – are being developed as treatments for a variety of diseases ranging from inborn metabolic disorders to viral infections and acquired diseases such as cancer. Ribozymes can be used both to downregulate and to repair pathogenic genes. In some instances, short-term exogenous delivery of stabilized RNA is desirable, but many treatments will require viral-mediated delivery to provide long-term expression of the therapeutic catalyst. Current gene therapy applications employ variations on naturally occurring ribozymes, but *in vitro* selection has provided new RNA and DNA catalysts, and research on *trans*-splicing and RNase P has suggested ways to harness the endogenous ribozymes of the cell for therapeutic purposes.

There are two basic modes for therapy that targets the genetic basis of disease: replace or resect. For diseases caused by recessive mutations, gene therapists try to complement the defective gene. For dominant disease mutations, however, introducing a normal gene will not work. Many of these diseases are associated with hyperactivation (in the case of oncogenes), or aggregation of a mutant protein (in the case of neurodegenerative diseases), with a normal copy of the same gene being present on the partner chromosome. In these cases, expression of the defective gene must be silenced or at least limited. For such autosomal dominant genetic diseases, ribozymes are a particularly appealing tool: they can be used to reduce expression of a pathogenic gene by digesting the mRNA it encodes.

RNA catalysts

Ribozymes are RNA enzymes that catalyze a variety of reactions in cells. The most complex ribozyme is undoubtedly the ribosome, which

synthesizes sequence-specific peptide bonds in an RNA-dependent reaction. Structural analogies with the self-splicing group II introns indicate that the spliceosome, which comprises five RNA molecules and over 50 proteins, might also be a ribozyme. The catalysts under development for therapy, however, are much simpler than these organelle-sized ribozymes (Fig. 1). Such catalysts include small nucleolytic activities, such as the hammerhead and the hairpin, derived from plant virus satellite RNA, the tRNA processing activity ribonuclease P (RNase P), and group I and group II ribozymes, which occur as introns in organelles and bacteria but can be engineered to act in *trans* on RNA or DNA (Table 1).

In addition to these naturally occurring ribozymes, novel ribozymes have been developed by random sequence *in vitro* selection¹. This technique relies on the prospect that a large population of molecules will include some that can perform a given task, for example, cutting a specific sequence of RNA. The challenge is to isolate these molecules from the remainder of the pool and subsequently amplify them for additional rounds of selection. Fortunately, several research groups have developed strategies for isolation of new ribozymes from pools of random sequences². This method is not limited to RNA catalysts, and in fact, some of the best site-specific ribonucleases are made of DNA (Ref. 3).

Despite their structural diversity, ribozymes and DNA enzymes catalyze only a few reactions: primarily, site-specific cleavage or ligation of

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Table 1. Ribozyme activity in nature and therapy

Ribozyme	Catalytic activity	Relevant role in nature	Therapeutic applications ^a
Hammerhead	Sequence specific ribonuclease	Self-cleaving RNA	Digestion of viral, oncogene or mutant mRNA
Hairpin	Sequence specific ribonuclease	Self-cleaving RNA	Digestion of viral, oncogene or mutant mRNA
RNase P	Structure specific ribonuclease	tRNA processing	Digestion of viral mRNA
Group I intron	RNA cleavage and ligation	Splicing	RNA repair of mutant mRNA or oncogenes
Group II intron	RNA and DNA cleavage and ligation	Splicing and transposition	Gene disruption of viruses and mutant mRNA
Spliceosome	RNA cleavage and ligation	Splicing	Repair of mutant mRNA
DNA enzymes ^b	Sequence specific ribonuclease	None	Digestion of viral, oncogene or mutant mRNA

^aMost of these applications are at the pre-clinical stage. For a detailed list of pre-clinical tests of ribozymes see Ref. 54.
^bNot actually ribozymes.

an antisense RNA of 12 or 13 nucleotides surrounding that site, but skipping the C, which does not form a conventional base pair with the ribozyme (Fig. 2). Because the chemical cleavage step is rapid and the release step is rate-limiting, speed and specificity are enhanced if the hybridizing 'arms' of the ribozyme are relatively short (five or six nucleotides). Ribozymes with longer helices can cut alternative targets if the mismatched nucleotides do

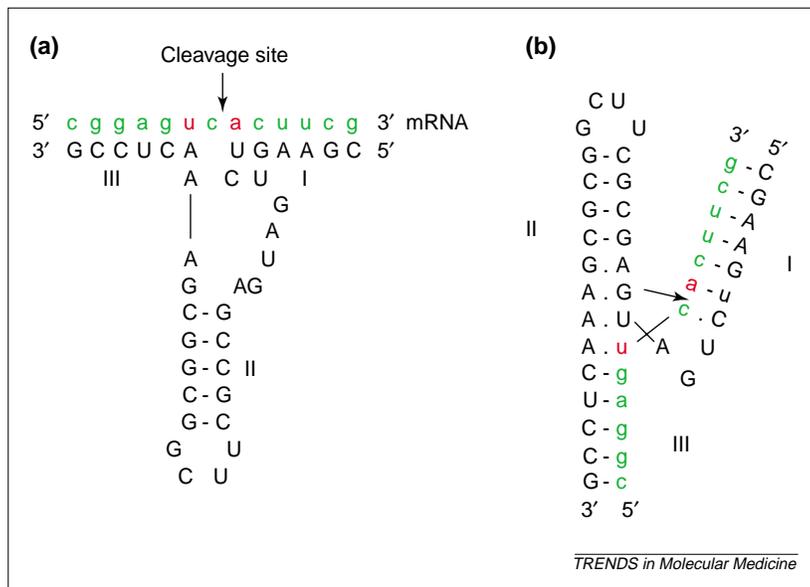


Fig. 2. Two views of a hammerhead ribozyme designed to cleave the P23H rhodopsin transgene present in a rat model of autosomal dominant retinitis pigmentosa (ADRP). Target sequences are in lower-case green and red letters indicate mutated nucleotides in the P23H mRNA. (Wild-type mRNA has cystidines in both positions.) (a) The ribozyme is unfolded to demonstrate base pairing involved in target-site selection. (b) The same ribozyme is presented to illustrate the non-canonical base pairs and tertiary structure of the ribozyme. Helices are numbered as roman numerals.

not affect the immediate environment of the cleavage site. Hairpin ribozymes require a more constrained target mRNA (BNGUC), where B can be any nucleotide except adenosine. By contrast, the minimal sequence requirement for the target of a group I ribozyme is a uridine within a short stem (six–eight nucleotides) formed between the ribozyme and the mRNA. Another important issue in selecting a target is accessibility. Secondary structure in RNA is relatively stable and can interfere with the ability of a ribozyme or antisense RNA to bind to the target site. Although structure-predicting algorithms, such as MFOLD (<http://bioinfo.math.rpi.edu/~mfold/rna/>), could help rule out certain target sites, which sites are accessible must ultimately be determined by experimentation⁵.

Ribozyme delivery

Direct delivery

Strategies for the delivery of therapeutic ribozymes to tissues depend on the disease. Because RNA has a half-life of seconds in bodily fluids, injection or topical application of naked RNA is not practical. Fortunately, the modification of nucleotides, particularly by blocking the 3' end of the molecule and the 2' positions of pyrimidines, stabilizes the ribozyme substantially without severely compromising catalytic activity⁶. Stabilized ribozymes, with half-lives measured in hours, could be provided in repeated doses by intravenous or topical injection (e.g. directly into a solid tumor), a strategy being tested in the development of hammerheads for the treatment of cancer and hepatitis C (Ref. 7). The liver and lung have a tremendous capacity to concentrate intravenously injected polynucleotide–liposome conjugates, which suggests that stabilized ribozymes might accumulate in those organs following intravenous injection⁸. Such ribozymes comprising modified nucleotides do not appear to be acutely toxic but, with the exception of phosphorothioate oligonucleotides, little information is publicly available on the health hazards of long-term exposure to some modified nucleotides.

Stable transduction

The principal alternative to direct delivery of ribozymes to tissues is the delivery of genes encoding ribozymes using viral gene therapy vectors. Retroviral vectors have been available for over a decade and have many advantages, particularly for *ex vivo* gene delivery to stem cells removed from the body. However, despite recent development of HIV-1-based vectors, retrovirus vectors have several disadvantages, including a limited cell tropism, low viral titer and the potential generation of replication-competent virus during packaging. Of the DNA viruses, vectors based on adenovirus-5 (Ad5) have been used most widely for gene therapy. Ad5 vectors are easy to produce in high titer and purity, and infect a wide variety of dividing and non-dividing cells.

Nevertheless, recombinant adenovirus provokes both humoral and cell-mediated immune responses that restrict its usefulness for human therapy⁹.

Currently the virus vector best suited for ribozyme delivery is adeno-associated virus (AAV), which has all of the advantages of Ad5 vectors but does not stimulate inflammation or a cell-mediated immune response¹⁰. Unlike adenovirus, AAV leads to long-term genetic transduction of infected cells, probably due to integration of the viral genome (including the passenger genes) during latent infections. Only 4.5 kb of therapeutic DNA can be inserted into recombinant AAV. This packaging limit is an impediment for some gene therapies but because ribozymes are small, AAV provides sufficient room for ribozyme genes and the regulatory signals to control their synthesis.

For the expression of ribozymes, DNA oligonucleotides encoding the ribozyme are cloned downstream of RNA polymerase (pol) II or RNA pol III promoters. Pol III normally transcribes small RNAs of high abundance and can produce the large amount of ribozyme that might be required to inhibit expression of a target gene. In most instances, ribozymes are embedded within the transcripts of genes, such as tRNA_{val}, U6 snRNA or the adenoviral VA1 RNA (Refs 11, 12). The downside of using pol III promoters is that they cannot be regulated and will direct the synthesis of large amounts of ribozyme in any infected cell. Consequently, regulated or cell-type-specific pol II promoters have a major advantage. For example, the rhodopsin proximal promoter has been used to achieve high-level transcription of ribozymes that are restricted to rod photoreceptor cells of the retina¹³. Transcription factors that are regulated by the presence of antibiotics or synthetic hormones permit dose-dependent expression of cloned genes, including ribozymes¹⁴. In addition, RNA processing sequences (introns and termination signals) can be included in pol II transcripts, increasing the effective concentration of the ribozymes in the cytoplasm¹⁵. Although expression levels are lower, ribozymes expressed from pol II promoters are frequently more efficient than those expressed from pol III promoters, because pol-II-produced ribozymes co-localize to the cytoplasm with their mRNA targets. The addition of RNA targeting sequences at the 3' end of the transcript could improve performance¹⁶.

Anti-cancer ribozymes

Tumors, which develop as a consequence of a series of genetic abnormalities, are an obvious target for ribozyme therapy. Hammerhead ribozymes have been effective in cutting the mRNA of oncogenes such as *ras* (Ref. 17) or *bcr-abl* (Ref. 18) in the test tube and in transfected cells; however, cultured cells are usually poor predictors of the potency of gene therapy vectors observed in whole tissues. Animal tests employing anti-cancer ribozymes have also been poor models of a human clinical application, because they usually involve pre-treating the tumor with ribozyme before

the cancer cells are introduced into immunodeficient mice. A therapeutically relevant test is to start with tumor-bearing animals and to deliver ribozymes systemically or to individual tumors. A quantitative measure of clinical outcome should include the size and spread of tumors, in addition to survival time. Because all ribozymes also inhibit gene expression by antisense mechanisms, catalytically inactive ribozymes should be tested among the other controls. Such a systematic approach has been taken in only a few cases^{19,20}.

A good example is an anti-tumor ribozyme (Angiozyme™) targeting the Flt-1 receptor for vascular endothelial growth factor (VEGF)^{21,22}. The rationale for using this ribozyme is that it will block angiogenesis and therefore limit the blood supply to the tumor. Indeed, Angiozyme™ reduced expression of the VEGF receptor in transfected cells and blocked blood vessel proliferation in a corneal model of angiogenesis. The pharmacokinetic properties of this ribozyme have been studied in mice. Although half of this stabilized ribozyme was eliminated within 30 minutes of intraperitoneal or subcutaneous administration, Angiozyme™ could be detected in the serum for over 2 hours and accumulated at therapeutic levels in kidney and skeletal muscle for up to 24 hours following subcutaneous injection. A phase I clinical trial of Angiozyme™ to determine human safety and tolerated dosage is under way.

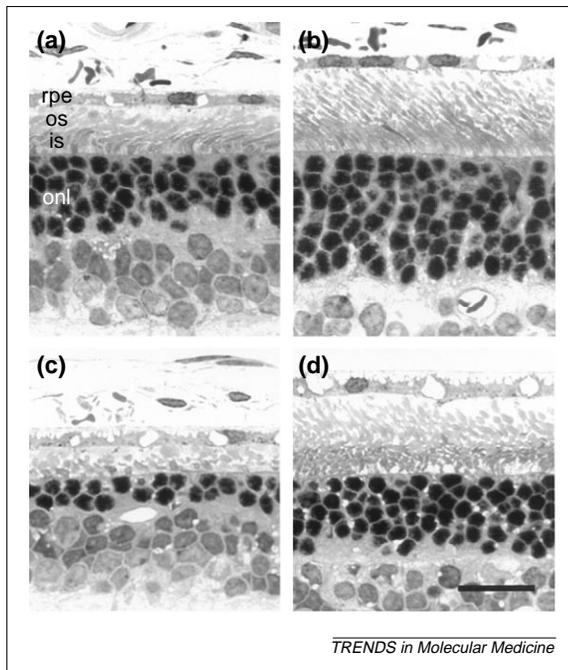
Anti-viral ribozymes

A major focus of research on ribozyme therapy has been on blocking the replication of RNA viruses and retroviruses, specifically HIV-1 (Refs 23,24), hepatitis C virus²⁵ and hepatitis B virus²⁶. These are suitable targets because their genomes contain RNA, and therefore replication can be directly inhibited following uncoating. Both hammerhead and hairpin ribozymes can protect cultured cells from the ravages of HIV-1 infection, but, as no good animal models of HIV-1 infection exist, investigators have been allowed to skip to phase I clinical trials without demonstrating efficacy *in vivo*.

The primary targets for anti-HIV-1 ribozymes are cleavage sites in the 5' leader, the packaging sequence (Ψ) and the *env*, *gag* and *tat* genes^{27,28}. Most of these sites exhibit little or no natural variation among clades of HIV-1. In most instances, ribozymes have been delivered by vectors based on Molony murine leukemia virus (MMLV). Hairpin ribozymes targeted to *tat* and *env* and expressed from a tRNA_{val} cassette have been shown to confer resistance of CD4⁺ T cells to several HIV-1 isolates²⁹. Hammerheads expressed from a pol II promoter and targeted to Ψ or *tat* were also able to block replication of a variety of HIV-1 isolates³⁰.

CD34⁺ stem cells would seem to be the appropriate target cells for HIV-1 ribozyme gene therapy because they can produce multiple generations of protected cells in several cell lineages. Even transduction of CD4⁺ T cells could be of benefit, because patients mount a vigorous immune response to HIV-1, and reducing viral

Fig. 3. Adeno-associated (AAV)-delivered ribozymes rescue photoreceptors in a rat model of autosomal dominant retinitis pigmentosa. Micrographs of P23H transgenic rat retinas taken at postnatal day (P)130 (a, b) or P240 (c, d). (a) An untreated rat eye at P130 shows three or four rows of photoreceptor nuclei in the outer nuclear layer (ONL). Photoreceptor inner segments (IS) and outer segments (OS) are shorter than normal. (b) Retina from the opposite eye from the same rat as in (a), which was injected with AAV expressing a hammerhead ribozyme (Fig. 2) at P15. Approximately six or seven rows of photoreceptor nuclei are present, and the photoreceptor inner and outer segments are longer. (c) An uninjected eye from a rat at P240 shows only one or two rows of photoreceptor nuclei and only remnants of inner and outer segments. (d) Retina from the opposite eye from the same rat in C, which was injected with hammerhead ribozyme at P15. About four or five rows of photoreceptor nuclei are still present, and the inner and outer segments are almost as long as at P130 (b). Scale bar, 20 μ m. Reproduced with permission from Ref. 36.



replication could help the immune system destroy the virus. Nevertheless, HIV-1 also replicates in macrophage and other non-T-cells, so that delivery of the ribozyme must also downregulate the virus in these reservoirs. Although both MMLV- and AAV-based vectors have been effective in delivering genes to T cells, safe HIV-1-based vectors with the same cell-tropism as the pathogenic virus would be of great advantage.

Ex vivo delivery of ribozymes for HIV-1 is being studied in clinical trials^{31–33}. All of these trials use MMLV vectors in one of three ways: (1) to infect autologous CD4⁺ T cells from infected individuals; (2) to infect CD4⁺ cells from an uninfected identical twin; or (3) to infect autologous CD34⁺ stem cells from HIV-1 positive volunteers. Treated cells are then re-

infused into the infected individual. Both hammerhead and hairpin ribozymes are under study. The hairpin vectors employ the tRNA_{val} cassette to drive ribozyme expression, whereas the hammerheads have been cloned downstream of the neomycin resistance gene and expressed from the viral LTR. Even though *ex vivo* infection permits wide-spread dissemination of the therapeutic ribozyme to relevant cells, a risk remains of *ex vivo* activation of T cells leading to an actual increase in viral burden. The hairpin ribozyme trial for AIDS therapy has recently been discontinued.

Ribozymes for dominant genetic disease

Cancer and AIDS might not be the best objectives of ribozyme therapy. Gene amplification in tumors and error-prone polymerases in viruses make ribozyme-escape mutants inevitable. Similarly, overexpression of the target gene by transcriptional mechanisms or gene amplification can render perfectly designed ribozymes useless *in vivo*. Delivery barriers also make metastatic tumors and disseminated viruses challenges for ribozyme therapy. By contrast, diseases arising from dominant genetic mutations often affect a single tissue or cell type, and, although clinically devastating, they are at least genetically stable.

Dominantly inherited diseases that arise from the accumulation of malfunctioning or misfolded proteins are ideal targets for ribozyme therapy. (Dominant disease can also arise from haploinsufficiency, in which case ribozymes are counter-productive.) A variety of neurodegenerative, muscular, connective tissue and cardiovascular diseases are associated with dominant gain-of-function mutations. For example, familial amyotrophic lateral sclerosis (ALS) is frequently caused by mutations in *SOD1*, the gene encoding Cu/Zn superoxide dismutase. Amyloid-like aggregates or toxic properties of the protein lead to the death of motor neurons³⁴. Osteogenesis imperfecta, a connective tissue disorder, has been the target of allele-specific ribozymes for α -1(I) collagen mRNA (Ref. 35).

AAV-vectored ribozymes have been used to treat autosomal dominant retinitis pigmentosa (ADRP) in animal models of this blinding disease¹³. Retinitis pigmentosa (RP), which leads to blindness in ~1 in 3500 people, is caused by the death of rod photoreceptor cells of the retina. About 25% of RP is dominantly inherited and most of these cases are associated with mutations in the gene encoding rhodopsin. ADRP is an optimal objective for ribozyme gene therapy for several reasons, including the fact that the disease-causing mutations are known, the tissue is accessible and the number of cells to be treated is limited. AAV has been shown to lead to stable genetic transduction (>30 months in rat) of these cells using the rhodopsin promoter to direct gene expression.

The rat model used in these experiments contains a mouse transgene with the P23H mutation¹³. The C→A transversion leading to this substitution does not create a novel ribozyme cleavage site, but the

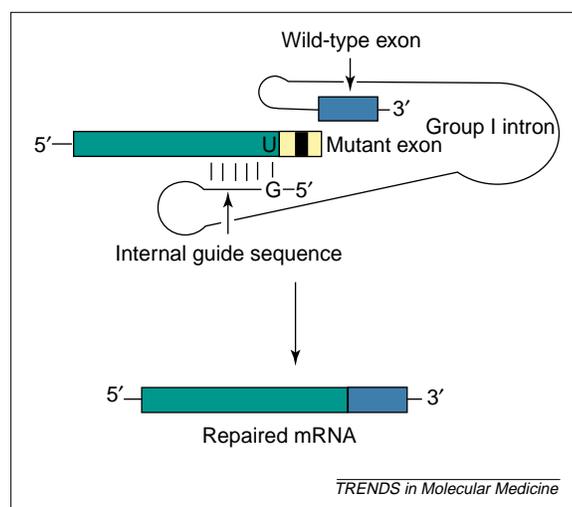
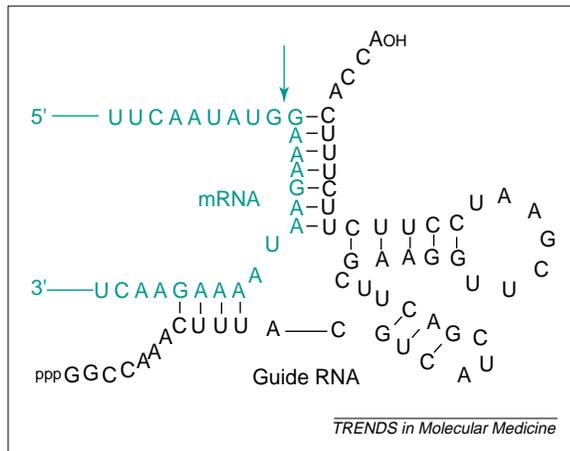


Fig. 4. RNA repair by *trans*-splicing of a group I intron. The intron binds to the mutant mRNA by base pairing with the internal guide sequence. RNA catalyzed *trans*-splicing replaces the mutated downstream sequence with a wild-type sequence fused to the 3' end of the ribozyme. Note that the artificial 3' exon can be extensive, so that this method can make repairs anywhere where that the guide sequence can find purchase.

Fig. 5. An external guide sequence leads to mRNA cleavage by endogenous ribonuclease (RNase) P. The guide sequence (black), when paired to the target RNA (green), forms a structure similar to the tRNA substrate of RNase P. RNase P cuts 5' to the artificial acceptor stem.



preceding codon contains an optimal GUC triplet target for the hammerhead ribozyme used for therapy (Fig. 2). Rats with this mutated gene demonstrate the same pattern of photoreceptor cell loss and progressive blindness as human patients, but the disease course is complete in a year, rather than in several decades as in human RP. Animals were treated in one eye with AAV expressing an active ribozyme and in the other eye with a control virus or a saline injection. Ribozyme treatment provided significant protection of functional vision in treated eyes for periods up to eight months after AAV-ribozyme injection³⁶ (Fig. 3). Retinas of treated eyes were equally preserved if therapy was delayed until substantial rod cell death had already ensued, suggesting that treatment of people with RP could succeed even after symptoms are well advanced.

Allelic heterogeneity

Designing mutation-specific ribozymes for dominant genetic diseases has an inherent weakness: many

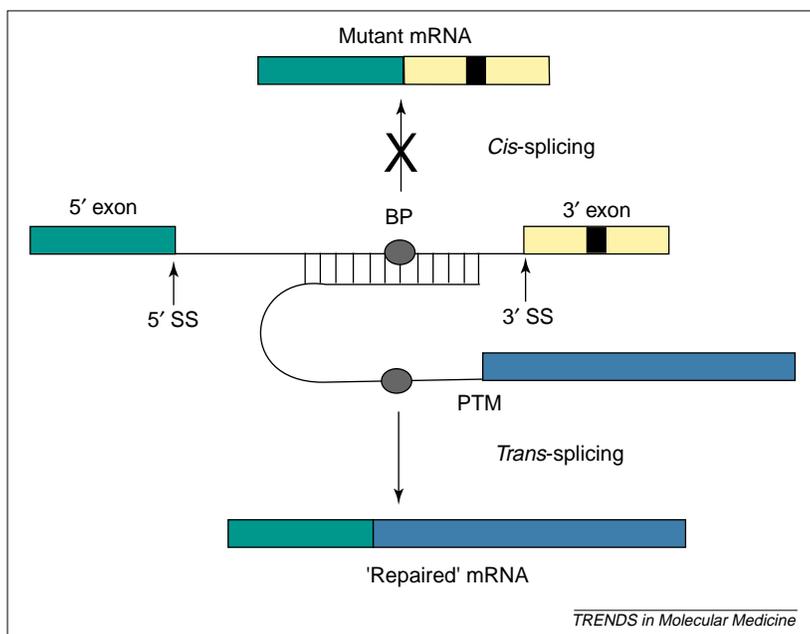


Fig. 6. RNA repair by spliceosomal *trans*-splicing. A pre-*trans*-splicing molecule (PTM) contains a normal branch point (BP) and 3' splice junction (3' SS) upstream of the wild-type exon (blue). The PTM also contains an antisense sequence that pairs with and obstructs the normal branch point for *cis*-splicing.

individual mutations in a gene can lead to the same disease. Over 100 rhodopsin mutations, for example, lead to dominant RP. Designing and testing a ribozyme for each patient or family is not feasible. There is an alternative ribozyme approach termed 'ablate-and-switch', which requires highly active ribozymes designed for the wild-type gene affected by dominant mutations. Also, a 'hardened target' is produced, which is a cDNA clone of the target mRNA in which the cleavage site has been eliminated by silent mutations. Both ribozyme and hardened target are then delivered in the same AAV vector, either as a single transcription unit, with the ribozyme in the 3' untranslated region (UTR), or as separate transcription units. The concept is to remove both mutant and wild-type mRNAs while simultaneously supplying a new 'hardened' wild-type gene. The ablate-and-switch approach has potential problems associated with regulating the correct level of ribozyme-resistant mRNA produced, but this method is currently being developed for several dominantly inherited diseases that display allelic heterogeneity^{37,38}.

Emerging technologies: group I and group II introns

Group I and group II introns, which function both as transposable genetic elements and as self-splicing introns, can be engineered for therapeutic purposes. Unlike hammerheads and hairpins, group I ribozymes can actually repair a defective mRNA. In addition to splicing linked exons, group I introns can catalyze *trans*-splicing of a covalently attached downstream exon to a 'free' upstream exon³⁹. The selection of the splice sites is determined largely by an internal guide sequence of 8–12 nucleotides that anneals with both the upstream and downstream splice junctions. The only sequence requirement is that a uridine ribonucleotide precedes the splice site. Because of this target sequence latitude, ribozymes based on group I introns can be used to repair defective mRNAs by *trans*-splicing (Fig. 4). Sullenger and colleagues⁴⁰ have used this method to repair mutant p53 and sickle-cell hemoglobin (HbS) mRNA in cultured cells. RNA repair by group I ribozymes is inefficient in some sequence contexts, and inappropriate *trans*-splicing can occur⁴¹. However, recent modifications of the method, such as including a binding site for the 3' exon, improve both efficiency and specificity⁴².

The reaction catalyzed by group II introns is even more remarkable⁴³. As part of its mobility function, intron RNA forms a complex with an intron-encoded endonuclease. This ribonucleoprotein catalyzes the cleavage of DNA at the target site for transposition. The selection of cleavage site is determined primarily by base-pairing with the intron RNA, and transposition can be re-targeted by manipulating this internal sequence. Guo *et al.*⁴⁴ used a group II intron from *Lactococcus lactis* to disrupt sites in the HIV-1 provirus and the gene encoding the CCR5 chemokine receptor in mammalian cells, demonstrating the flexibility of the approach. Because the substrate of

transposing group II introns is double-stranded DNA, which has an extended secondary structure, intrastrand folding cannot block the target sites of this ribozyme.

This system has tremendous appeal for the treatment of autosomal dominant genetic diseases. Gene disruption by group II transposition can permanently disrupt pathogenic genes. The target site is long enough (~14 nucleotides) to provide target site specificity but short enough to permit the ribozyme to distinguish mutant from wild-type target genes. However, some mismatches are permitted at the target site and thus, this method might not be useful for single-base mutations. To make their system practical for gene therapy, Guo *et al.*⁴⁴ have cloned the protein subunit of the complex as a separate gene from the intron. Both are small enough to be expressed from a single viral vector. The endonuclease is of bacterial origin, however, and it might be presumed to be antigenic in humans.

Emerging technologies: endogenous ribozymes

Given that cells make their own RNA catalysts, delivery of ribozymes is not necessarily required for ribozyme therapy. For example, the RNA endonuclease activity of RNase P can be harnessed to digest cellular or viral mRNAs (Ref. 45). RNase P, a nuclear ribonucleoprotein complex, normally removes the 5' extension of tRNA precursors, but it can cleave simpler targets that resemble the acceptor stem of a tRNA precursor (a 7 base-pair helix with a 3' CCA extension). The 3' half of this duplex can be provided as a separate molecule, and delivering a short external guide sequence that forms such a stem with a target transcript leads to specific cleavage of that RNA in the nucleus⁴⁶ (Fig. 5). Guerrier-Takada and Altman have tested this approach both in bacteria to block the expression of marker genes and in mammalian cells to destroy influenza virus transcripts⁴⁷.

The spliceosome is another endogenous ribonucleoprotein complex with a ribozyme at its core. It has long been known that the spliceosome can mediate *trans*-splicing, but Mitchell and colleagues⁴⁸ have found a way to exploit this capability to repair defective mRNA. These authors have developed pre-*trans*-splicing molecules (PTMs) that hybridize with the defective mRNA in the intron preceding the mutant exon, thus masking the normal branch site adenosine required for *cis*-splicing (Fig. 6). This arrangement effectively blocks splicing of the damaged downstream exon while positioning the 'corrected' exon to take its place. Investigators at Intronn have successfully tested this technology on a variety of marker and potentially pathogenic genes in cell culture^{49,50}. The pre-*trans*-splicing molecule can, in principle, comprise all of the downstream exons, so that this method can repair mutations in any but the first exon. Although efficiency is still a problem, PTMs hold significant promise as a method to recruit one of the cell's own ribozymes for genetic repair at the RNA level.

Ribozymes as an intermediate technology

Gene correction at the DNA level is the ultimate goal of gene therapy. If a mutation can be corrected to the wild-type sequence at its endogenous chromosomal location, issues of regulated expression or insertional mutagenesis disappear. Despite encouraging preliminary results using viruses⁵¹, chimeric RNA-DNA molecules⁵² and homologous recombination⁵³, efficient gene repair is not currently a reality in mammals. In the meantime, ribozymes provide a precise and powerful way to downregulate or repair defective genes at the RNA level, before toxic or carcinogenic proteins accumulate. This is, in fact, a tactic employed by many of our own tissues to modify transcripts – through RNA editing or alternative splicing – to make the most of the diversity of our 32 000 genes.

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