JOURNAL OF
MEDICINAL
CHEMISTRY

© Copyright 1995 by the American Chemical Society

Volume 38, Number 12 June 9, 1995

Perspective

Ribozymes as Human Therapeutic Agents

Ralph E. Christoffersen* and J. Joseph Marr

Ribozyme Pharmaceuticals, Inc., Boulder, Colorado 80301

Received November 22, 1994

I. Introduction

The protein encoded by a particular gene normally corresponds to an RNA sequence considerably shorter than the sequence transcribed from that gene. This is due to the fact that genes typically contain several exons (expressed sequences) separated by a series of introns (intervening sequences). When the RNA is transcribed from a gene, the corresponding intron sequences are spliced out and the exons are ligated in a transesterification reaction. The newly spliced series of exons is then translated into the appropriate proteins.

In the early 1980s Cech and his colleagues discovered that certain RNA splicing reactions are catalyzed by RNA. It was unequivocally demonstrated that certain intervening sequences (Group I) were inherently capable of catalyzing RNA splicing reactions to give rise to mature RNA. Cech termed such RNA molecules, possessing enzymatic activity, "ribozymes".[1-3] These RNA enzymes have now been found in a wide variety of biological systems. By suitable chemical or molecular manipulation, ribozymes can be engineered either to bind specifically to external desired RNA sequence targets and cleave them, thereby inhibiting a gene function, or to ligate new pieces of RNA onto the target by trans splicing to create a new gene function. Therein lies their therapeutic potential.

In less than 15 years since the initial discovery by Cech and Altman,[1-3] the fundamental importance of catalytic RNA (ribozymes) in chemistry and biology has become apparent. The demonstration that RNA plays an active catalytic role in the production of proteins from DNA and is not merely a "passive" participant has caused a major paradigm shift in the role of RNA in chemistry and biology. Furthermore, the availability of "catalytic RNA" to carry out processes previously reserved only for protein enzymes has caused a rethinking of the role RNA may have played in evolution.[4-10]

Ribozymes provide a broad and enabling technology applicable to human disease diagnosis and therapy, agriculture, and animal health.[11] Correspondingly, research interest in ribozymes has grown exponentially over the past several years (Figure 1). Over 500 articles were published through the end of 1993 on various aspects of ribozymes and their role in chemistry, biology, and medicine.

The broad potential of ribozyme technology is due to the fact that a ribozyme will, in principle, selectively bind and cleave any target RNA. Thus, highly specific control of gene expression by ribozyme cleavage and consequent nuclease destruction of mRNA fragments can be contemplated, as illustrated in Figure 2.

In the diagnosis and treatment of human diseases, the sequence-specific enzymatic activity, and the relative ease with which a lead ribozyme can be designed have substantial advantages that may translate into low side effects, high potency, and substantially reduced drug discovery time. Ribozymes are applicable in principle to any disease where a specific protein or virus can be linked to disease etiology.

Translation of this potential into a new class of human therapeutic agents is coupled with technical challenges. In this review, the current status of efforts to demonstrate how ribozymes can be used to treat human diseases will be considered along with identification of remaining issues and possible future directions.

II. Chemistry and Biology of Ribozymes

A. Types of Ribozymes. Ribozymes were identified first by their ability to splice introns out of mRNA when an RNA precursor of *Tetrahymena thermophila* was

0022-2623/95/1838-2023$09.00/0 © 1995 American Chemical Society

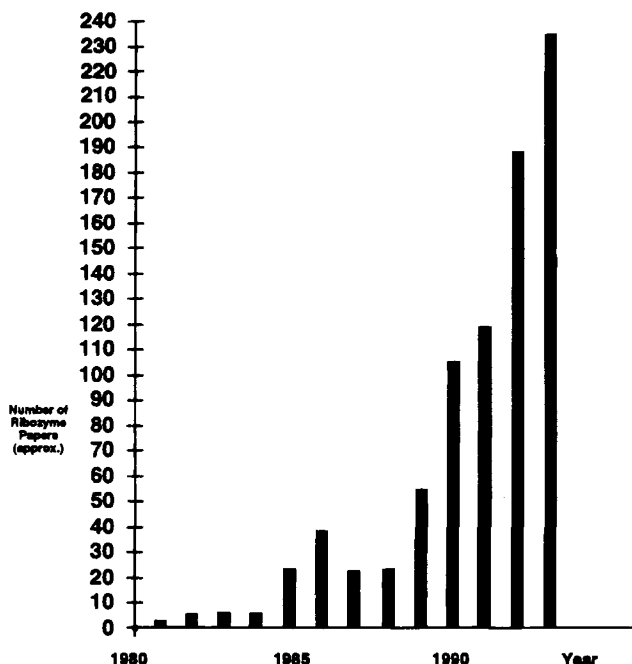


Figure 1. Annual growth of ribozyme publications.

found to be self-splicing.[1,2] Additional studies extended the kinds of self-splicing ribozymes from Group I to Group II introns[12-15] found predominantly in fungal mitochondria. Modification of the *Tetrahymena* self-splicing intron by deleting the first 19 nucleotides created an important change; it converted the self-cleaving ribozyme into one acting on an *external* RNA (or DNA) substrate. The ribozyme was acting "in *trans*". Thus, the group I intron can be engineered to catalyze a sequence-specific reaction. Group I introns also have been found in cyanobacteria[19] and slime mold[20] (Figure 3).

Group I ribozymes range in size from 200 to 1000 nucleotides (Figure 4). They require a U in the target sequence 5′ to the cleavage site, and bind 4–6 nucleotides at the 5′ side.

RNAse P is a ribonucleoprotein consisting of approximately 375 nt of RNA plus a small polypeptide. The RNA portion cleaves tRNA precursors to produce the mature tRNA.[3]

The VS ribozyme is derived from a satellite RNA (VS) of certain natural isolates of *Neurospora*. This ribozyme differs from the others shown in Figure 4 in that it will cleave double-stranded RNA.[21] These three ribozymes are all large, relative to the others, and any applications involving these large ribozymes must necessarily be those which utilize gene therapy methods.

The "Hammerhead" (HH) and "Hairpin" (HP) ribozyme motifs were originally identified in plant viroids and virusoids;[22-26] the Hepatitis Delta Virus (HDV) ribozyme was found in a satellite RNA of human hepatitis B virus.[27,28] Each of these was identified in a self-cleaving form and has been converted to cleave external RNA substrates as well.[25,29-34]

The ability to design ribozymes against selected external RNA targets has expanded their therapeutic potential. Hammerhead ribozymes can be modified in their binding arms to be complementary to any target RNA which contains a UH (where H is any nucleotide but guanosine). The optimum length of the binding arms appears to be 7/7 nucleotides on the 3′- and 5′-ends of the ribozyme. For the HP ribozyme, four nucleotides on the 5′ side and a variable number on the 3′ side of the cleavage site can be modified. In the HDV ribozyme only 7 nucleotides at the 5′ side of the cleavage site need be changed to alter the specificity (Figure 4). Since these ribozymes are relatively small, they can be made by chemical synthesis or produced by viral or nonviral vectors. This ability to modify the binding arms of ribozymes to complement the sequences within a given RNA potentially endows ribozymes with a therapeutic specificity heretofore not possible.

Increasing the length of the complementary binding sequences in the ribozyme should improve the specificity of binding. A recognition sequence of approximately 15 nucleotides—7 in the 3′- and 5′-arms and one at the catalytic core—should be optimal for recognition, specificity, and turnover. This nucleotide length may be unique in the human genome; however, this does not take into account the bias in the genetic code, particularly in regions that are transcribed as mRNA. Nevertheless, this target size of 15 nucleotides will assure reasonable uniqueness and, therefore, therapeutic specificity, that will be assessed as candidate ribozymes appear in therapeutics.[35,36] It may not be desirable to extend the recognition sequence beyond 15 nucleotides since, beyond that point, the binding affinity increases to the point where the off-rate of the cleaved product from the enzyme is too slow to permit efficient catalysis and nonspecific effects also may occur.[37]

B. Mechanism of Action. Ribozymes cleave their target using either a transesterification or hydrolysis mechanism (Figure 3). In the cleavage reaction the formation of a 2′,3′ cyclic phosphate and 5′-hydroxyl are typical products. If the ribozyme is present in excess, as is often the case in practice, but at a concentration that is not saturating with respect to the substrate, the cleavage rate is determined by the second-order rate constant k_{cat}/K_m. Values of approximately 10^8 M^{-1} min^{-1} are frequently observed.[30] If the substrate is in excess, and at saturating concentrations, ribozymes are rate-limited by release of the product.[38] Improvements in ribozyme turnover may result from research to modify ribozymes in a way to increase product release from the complex.

C. Ribozyme Evolution. Techniques to alter or improve ribozyme function, generally known as directed *in vitro* evolution, have been devised.[39-45] By maintaining diversity throughout multiple generations and selectively amplifying those ribozymes with a specific property of interest, the *Tetrahymena* ribozyme was evolved into a Group I intron that cleaved DNA targets in addition to RNA.[39]

In a different application of *in vitro* evolution, Lorsch and Szostak[45] created a random pool of 10^{15}–10^{16} RNA molecules containing an ATP-binding domain and identified five RNAs capable of acting as 5′-kinases and two 2′-kinases. This broadened the scope of ribozyme formation further to include polynucleotide kinase activity. The inherent potential of ribozymes to bind and act on many kinds of substrates coupled with selective methods to amplify and refine these new activities will lead to the development of many new ribozyme functions.

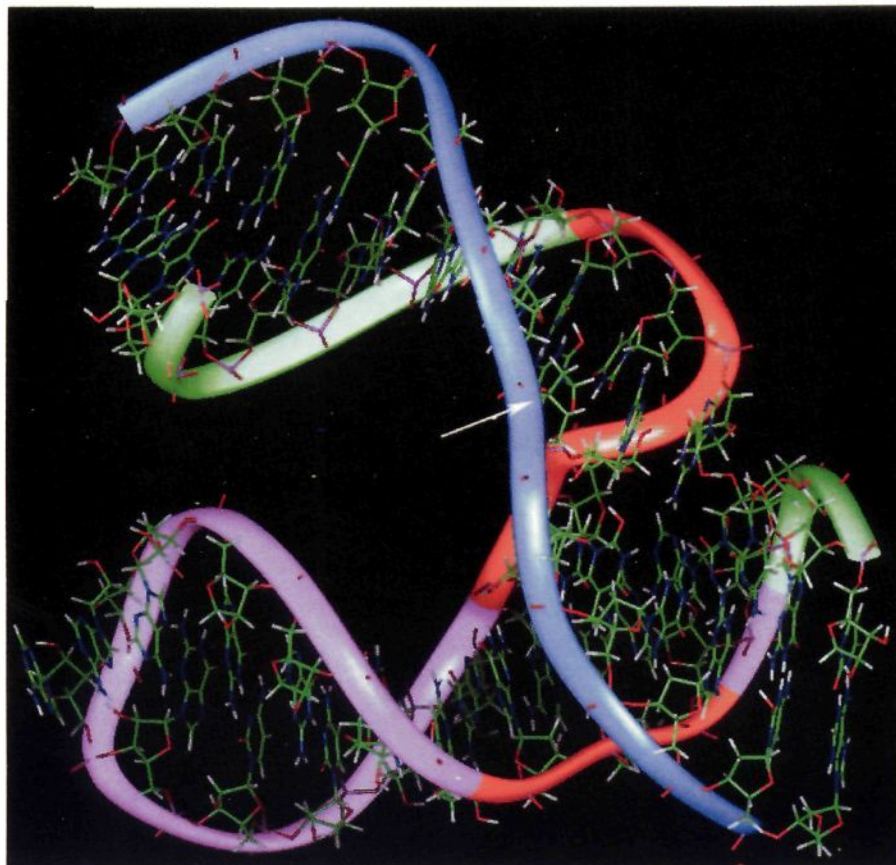


Figure 2. Crystallographic representation of a ribozyme binding to its substrate. This model was constructed from the coordinates determined by Pley, H. W.; Flaherty, K. M.; McKay, D. B. *Nature* **1994**, *372*, 68–74. The figure was kindly supplied by Dr. James McSwiggen, RPI, Inc. The cleavage site occurs on the substrate at the point designated by the arrow. The substrate lies in a vertical orientation in apposition to the binding arms of the ribozyme.

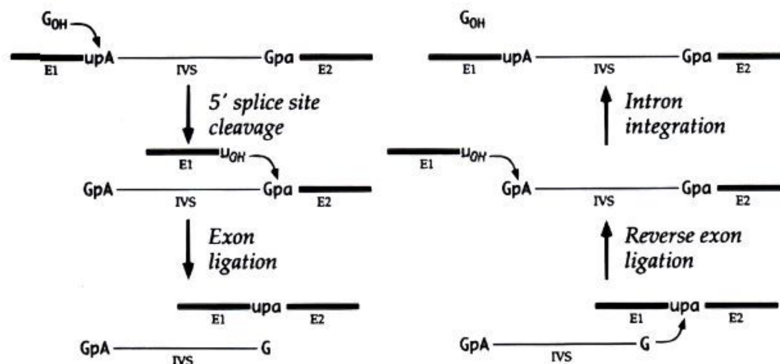


Figure 3. Splicing of group I introns: forward and reverse reactions. The letter E refers to exons and IVS refers to the intervening sequence. The three-letter sequence, such as Gpa or GpA, refers to two nucleotides connected by a phosphodiester bond. In the forward reaction, attack of the required GTP cofactor at the 5′-splice site releases the 5′-exon. The 5′-exon then attacks the 3′-splice site resulting in exon ligation and excision of the IVS. The reverse reaction follows the same pathway in the opposite direction. After binding of the ligated exon RNA in the active site of the linear IVS, attack of the 3′-terminal OH of the IVS on the phosphate at the splice junction results in the addition of the 3′-exon to the 3′-end of the IVS. In the second step, the 3′-hydroxyl of the 5′-exon attacks the phosphate of the first phosphodiester bond of the IVS, releasing G and joining the 5′-exon to the 5′-end of the IVS. Exon sequences are in lower case; IVS sequences in upper case. The authors are indebted to Dr. John Burke of the University of Vermont for this figure.

III. Proof of Principle

To demonstrate the potential of ribozymes, their abilities to cleave mRNA targets must be correlated with biochemical and physiological changes that result from target cleavage. Many studies now have been published which illustrate these proofs of principle.

In a study of HH ribozymes in monkey (COS1) cells,[46] ribozymes designed against chloramphenicol acetyltransferase (CAT) were cloned into a mammalian expression vector and electroporated into the cells. They specifically suppressed CAT expression up to 60% relative to an inactive ribozyme and a corresponding antisense control.

In another study, DNA encoding a ribozyme-tRNA construct was microinjected into the nucleus of frog oocytes.[47] It remained largely in the nucleus, but small

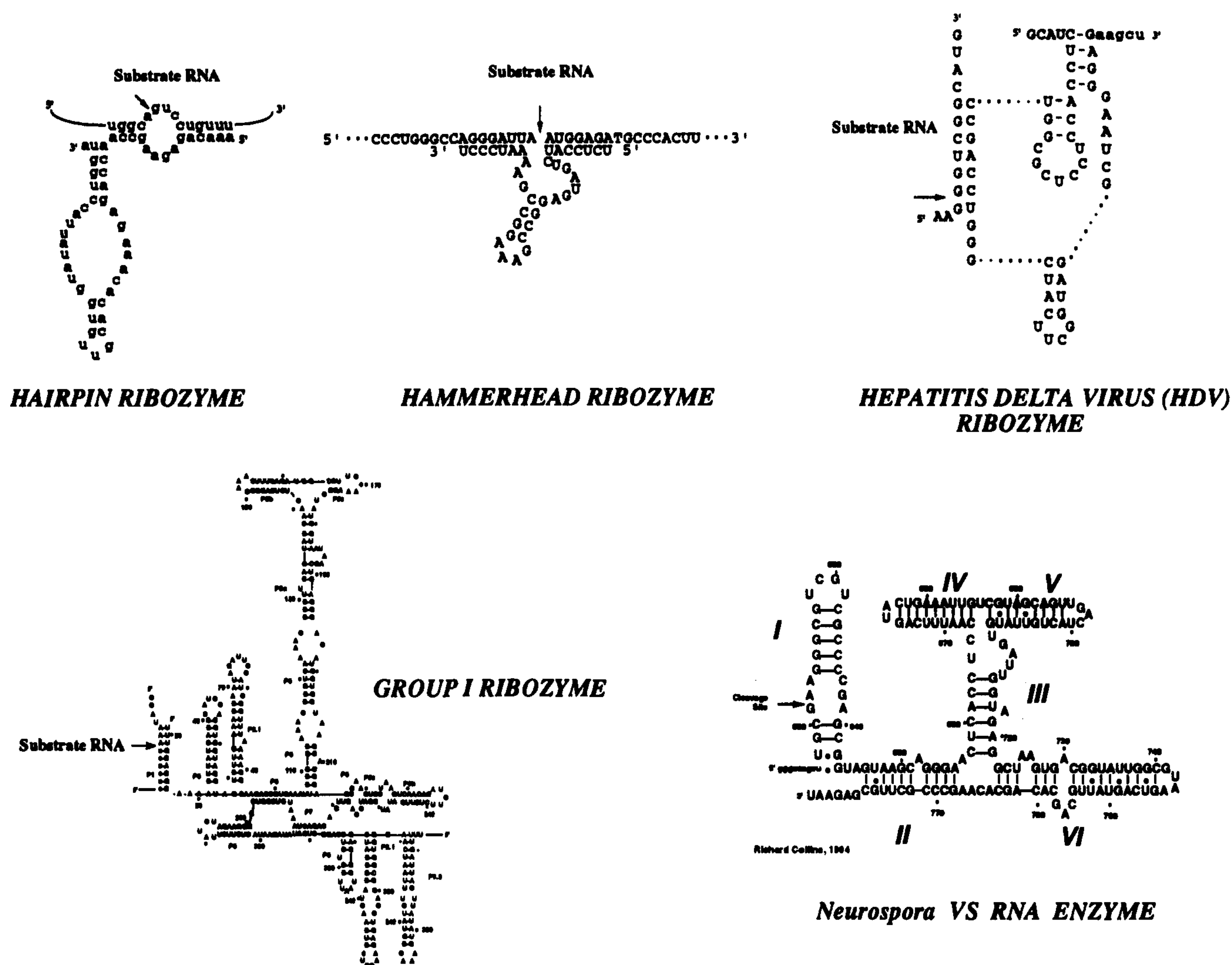


Figure 4. General structures of representative ribozymes. The *Tetrahymena thermophila* self-splicing group I intron structure as proposed by Cech *et al.* (Cech, T. R.; Damberger, S. H.; Gutell, R. R. *Struct. Biol.* **1994**, *1*, 273–280). Arrows indicate the 5′ and 3′ splice-sites. Lower case letters = exons; thick lines = connections where arrowheads show 5′ → 3′; P1, etc. = conserved paired regions; thin lines = tertiary structure. For the Hammerhead, Hairpin, and Hepatitis Delta Virus ribozymes, arrows indicate the site of RNA cleavage. N can be A, U, G, or C; N′ is a nucleotide complementary to N; H is A, U, or C; Y is any pyrimidine. The secondary structure of the Hairpin ribozyme–substrate complex as proposed by Berzal-Herranz *et al.* (Berzal-Herranz, A.; Joseph, S.; Chowrira, B. M.; Butcher, S.; Burke, J. M. *EMBO J.* **1993**, *12*, 2567–2574). The secondary structure of a Hammerhead ribozyme–substrate complex as proposed by Long and Uhlenbeck, (Long, D. M.; Uhlenbeck, O. C. *FASEB J.* **1993**, *7*, 25–30). The secondary structure of a Hepatitis Delta Virus ribozyme-substrate complex as proposed by Perrota and Been, 1992 (supra). The structure of the *Neurospora VS* ribozyme as proposed by Guo, H. C. T. and Collins, R. A. (Guo, H. C. T.; Collins, R. A. *EMBO J.* **1995**, *14*, 368–376).

amounts were transported to the cytoplasm. Reduction of the co-injected U7snRNA target, present in the cytoplasm, was observed after 10 and 20 h.

The studies of L'Huiller *et al.*[48] demonstrated the specificity of a ribozyme directed against α-lactalbumin. Saxena and Ackerman[49] demonstrated that injection of a ribozyme directed against 28S RNA cleaved the 28S RNA but not related RNA. This showed that the ribozyme could detect its substrate among other RNAs present in the cell. Cleavage products for that substrate were found. Other studies at the cellular level now have been reported, and it is clear that the question of cell culture "proof of principle" for ribozymes has been answered affirmatively.[50–52]

Several studies of ribozyme function *in vivo* have been reported. Among the more definitive and dramatic examples is a study in *Drosophila*.[53] Transgenic eggs were generated which carried a ribozyme against the *fushi tarazu* (*ftz*) gene under the control of a heat-inducible promoter. These investigations distinguished the two developmental phases of the *ftz* gene, using timed heat-induction of the ribozyme. Induction of the ribozyme against the first of these development phases, production of a seven-stripe cuticle pattern, created cuticle defects in larvae. Activation of the ribozyme in other eggs later during neurogenesis inhibited central nervous system development without disturbing the antecedent cuticle pattern. These experiments demonstrated that specific induction of ribozymes at different points in *Drosophila* development is possible, that activation of the ribozyme causes the same phenotypic mutations in segmentation and neurogenesis that occur in known *ftz* mutations, and that the presence of the ribozyme caused no other biological or biochemical damage to the organism.

In another study, a plasmid carrying a ribozyme against β_2-microglobulin was injected into the male pronucleus of fertilized oocytes.[54] Seven transgenic

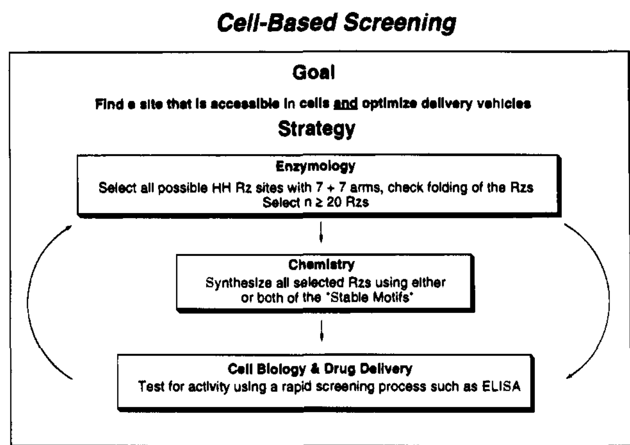


Figure 5. Ribozyme testing scheme.

animals were used to establish three ribozyme-expressing families. Ribozymes were expressed in the lung, kidney, and spleen. Ribozyme expression was accompanied by a reduction of mRNA β_2 microglobulin greater than 90% in the lungs of individual mice; smaller reductions were observed in kidney and spleen. Such a study not only provides convincing proof of principle *in vivo* but also confirms that continued expression of a ribozyme directed against a nonessential function will not result in any obvious toxicity in an animal.

IV. Ribozyme Design and Selection

A. Identification of Active Ribozymes. Identification of a lead ribozyme is a much simpler and shorter process than that required for most chemically synthesized small molecules. Knowledge of the relevant gene sequences and, therefore, its mRNA sequence, is sufficient. Ribozyme targets within the mRNA and the flanking sequences required to assure selectivity can be identified rapidly with existing computer methods. Once these putative ribozyme sites are identified, ribozyme recognition sequences are reviewed to determine whether the arms will fold improperly and bind to one another or to the catalytic core. Those which have the potential to do so are eliminated.[55,56]

At this point one can either determine availability of these potential sites to ribozyme cleavage *in vitro* using an RNAse H assay or move directly to a cell-based tissue culture assay. Before the availability of chemically stabilized ribozymes (see IV.B) *in vitro* screening using the RNAse H assay was employed. In this assay, DNA oligonucleotides are synthesized to be comparable in size to the ribozyme and complementary to the RNA sequences around the putative ribozyme sites. These are mixed with a long RNA substrate, and an excess of RNAse H is added. This enzyme cleaves the RNA strand at the ends of the DNA–RNA heteroduplex. The mixture is analyzed by gel electrophoresis. Sites on the target RNA where the DNA oligomer has bound will be cleaved, and that site is presumed to accessible to a ribozyme. We have found this assay to be have a predictive value of about 80% with respect to the activity of ribozymes in cell culture and animals.

Since the advent of chemically stabilized ribozymes, we have abandoned the RNAse H assay. In its place, we make those ribozymes which are predicted not to fold upon themselves and assay these directly in a tissue culture system in which the ability of the ribozyme to eliminate a biological activity is assessed. This provides a more realistic assessment of the ability of a ribozyme to attack its target mRNA in a cell. This system is more reproducible and has more relevance to the function of that ribozyme in an animal model. We consider it to be the screening method of choice (Figure 5).

B. Stability of Ribozymes in Biological Milieu. Unmodified RNA is unstable in biological systems. This is a significant challenge to establishing ribozymes as human therapeutics.

A number of structural modifications have been applied to synthetic oligonucleotides to enhance nuclease resistance.[57–61] Substitution of the 2′-*O*-Me-modified nucleotide at all positions in a hammerhead ribozyme except G5, G8, A9, A15.1, and G15.2 gives rise to a catalytically active molecule, but with a significantly decreased k_{cat} value.[61] However, these molecules showed a 1000-fold increase in stability as compared to an all-RNA ribozyme. In another investigation, a persubstituted 2′-*O*-allyl-containing ribozyme with ribose residues at positions U4, G5, A6, G8, G12, and A15.1 retained 20% of the catalytic activity of an all-RNA ribozyme. As in the previous case, the stability of this ribozyme was increased, with 30% of the material intact after 2 h compared to a less than 1 min half-life for the all-RNA ribozyme.[62]

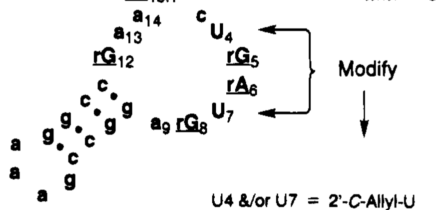
Two phosphorothioate linkages at C3 and U4, with replacement of U7 by adenosine or guanosine in a phosphorothioate-DNA/RNA chimera further stabilize the molecule, but the catalytic activities of these chimeras are significantly reduced.[63] Substitution of all the pyrimidine nucleotides in a HH ribozyme by their 2′-amino or 2′-fluoro analogs resulted in a 1200-fold increased stability in rabbit serum but also gave a 25–50-fold decrease in activity compared to an unmodified ribozyme.

Beigelman *et al.*[64,65] constructed a considerably improved stable HH motif by utilizing selected modification of approximately 32 2′-modified sugars. Most of these were substituted as 2′-*O*-Me residues as described above. The remaining 2′ modifications were introduced at positions U4 or U7. Two of these ribozymes, U4- and U7-2′-amino and U4-2′-*C*-allyl, have wild-type catalytic activity and a 5–8 h half-life in human serum. The addition of a 3′–3′-linked dT or an abasic nucleoside to the 3′ terminus of these ribozymes increases their half-lives in serum to greater than 72 h (Figure 6).

The positions for these particular modifications were determined by analysis of the products of ribozyme degradation in human serum. The presence of 2′-O-Me residues protected their respective bases from exonuclease and endonuclease degradation. The predominant exonuclease in human serum cleaves from the 3′-end of the oligomer. However, there remained some residual exonuclease degradation even after 2′-O-Me substitution. For this reason the inverted T was added to convert the 3′-end into a 5′-"like"-end, which eliminated susceptibility to the 3′-exonuclease. The other vulnerable sites were the two internal pyrimidines in the U4 and U7 positions of the catalytic core, since endonucleases are more effective against pyrimidine linkages than against purine linkages. Modifications at the 2′-positions of U4 and U7 eliminated this susceptibility.

At this time, the best U4/U7 2′-modifications are

NUCLEASE STABLE RIBOZYME



U4 &/or U7 = 2'-C-Allyl-U
2'-NH2-U
2'=CH2-U
2'=CF2-U
2'-dU
2'-F-ara-U
2'-F-ribo-U
2'-O-Me-ribo-U

Lower case = 2'-O-Me
rN = ribonucleotide

U4/U7 (2'/2')	$t_{1/2}$ Activity (min)	$t_{1/2}$ Stability (min)	$\beta = t_S/t_A \times 10$
OH/OH	1	0.1	1
O-Me/O-Me	4	260	650
ara-F/O-Me	5	>500	>1000
O-Me/NH2	5	500	1000
NH2/NH2	2	300	1500
C-Allyl/O-Me	3	>500	>1700
C-Allyl/O-Me + iT	3	4320	14,400
NH2/NH2 + iT	2	4320	21,600

Figure 6. Modifications which confer increased stability to ribozymes. The lower section shows the increase in net activity conferred by the modifications compared to the unmodified (all RNA) ribozyme. The second column indicates the time required to cleave one-half of the substrate in solution under standard conditions (Tris, 50 mM, pH 7.5; $MgCl_2$, 10 mM; substrate, 1 nM; ribozyme at three concentrations: 8, 40, and 100 nM). The third column lists the time for one-half of the ribozyme to be destroyed in human serum at 37 °C. The fourth column is the ratio of the third column to the second. The fourth column is the ratio of the third column to the second which provides a measure of the relative stability–activity values of the ribozymes. The multiplier of 10 is included to relate the ribozymes to a unit stability–activity value for the unmodified molecule.

amino/amino and *C*-allyl/OMe. These provide more than a 1500-fold increase in biological activity and stability as compared to the unmodified ribozyme. An additional increase to 14 000–21 000 for each of these motifs was achieved by introducing 3′-3′-linked T to the 3′-end of the ribozyme (Figure 6). Such extended stability in human serum coupled with the retention of activity should allow use of synthesized ribozymes for exogenous delivery in various therapeutic settings.

V. Delivery of Ribozymes to Cells

There are two general methods of delivering oligonucleotides; incorporation into a viral or nonviral vector of DNA which codes for the oligonucleotide, or use of a chemically-synthesized ribozyme, either coupled physically or chemically to a lipid or other molecule or delivered as a "free" ribozyme. In the former, transfection or infection (plasmid or viral) of the cell depends upon an artificial means of increasing cell permeability or the inherent characteristics of the virus. In the latter, the molecule complexed to the ribozyme makes use of either a nonspecific entry mechanism (as with a lipid carrier) or a somewhat more specific entry method such as receptor-mediated endocytosis.

A. Exogenous Delivery. Many lipid vehicles have been studied for use in exogenous delivery of oligonucleotides. For example, liposomes (bilamellar lipid spheres of approximately 100–500 nm diameter) can encapsulate relatively large quantities of drug molecules either within their aqueous interiors or dissolved into the hydrocarbon regions of their bilayers.[66,67] Liposomes also protect their contents from renal filtration and degradation by serum enzymes.[68] When attached to the proper antibody or other ligand, liposomes can sometimes be directed to a tissue depending on the ability of the ligand to facilitate cell-specific attachment and entry.[69–72]

Despite these advantages, there are obstacles to the use of liposomes as delivery vehicles. Unmodified liposomes do not survive long in systemic circulation since they are removed rapidly by macrophages of the reticuloendothelial system. Such uptake may be avoided partially by modifying the character of the lipids in the bilayer or by a poly(ethylene glycol) coating to prevent nonspecific adsorption of serum proteins and therefore nonspecific recognition of liposomes by macrophages.[73–75] In addition, in general it is not possible to target liposomes to a specific tissue. This has been partially surmounted by the use of receptor-mediated endocytosis where, for example, a liposome has been attached to folic acid *via* the distal end of a few lipid-conjugated poly(ethylene glycol) (PEG) molecules on the liposome surface.[76]

An alternative method which appears to have greater potential is the use of cationic lipids. These are materials which contain a nonpolar long-chain fatty acid, usually C16 or C18, complexed to a cationic polyamine such as spermidine. These lipids, although they aggregate under some conditions when mixed with oligonucleotides, are believed to complex with the oligonucleotide in such a way that it is not internalized into a liposome-type structure. Supporting this concept is the demonstration that oligonucleotides complexed with cationic lipids are susceptible to nucleases whereas those within liposomes are not.

Many investigations to date have utilized DNA–cationic lipid complexes to deliver oligonucleotides to cells.[77–84] It appears that these lipid–DNA complexes have relatively little toxicity if used in low concentrations. At higher concentrations there is evidence of toxicity following intravenous injection in mice.[83] Modification of the lipid carrier reduced the toxicity at lipid and DNA concentrations up to 1000-fold higher than those used previously.[83] Based on these results, DNA–lipid formulations or ribozyme–lipid formulations are being considered as possible delivery vehicles for exogenous oligonucleotide human therapy. Nevertheless, the rules developed for the delivery of DNA via cationic lipids may not apply to small pieces of RNA. Larger circular pieces of replicating RNA behave similar to DNA when delivered with cationic lipids. Smaller pieces of RNA, less than 500 nucleotides, were not taken up under these circumstances (P. Felgner, personal communication). Our internal studies have also dem-

onstrated that a given lipid preparation may serve well in one cell type but not in another.

Intracellular localization after passage across the cell membrane also needs to be considered. For example, even if large numbers of ribozymes are delivered to a particular cell type (as many as several million can be delivered), many of the ribozymes appear trapped within the endosomal compartment of the cell. Certain ribozymes, when microinjected into a cell, will appear in the nucleus almost immediately. When the same ribozymes are delivered with a lipid vehicle, they are trapped in the endosome. This paradox of having large quantities of ribozymes in the cell with little biological activity has led to the conclusion that the choice of lipid vehicles will vary with target cell. Even with cell type optimization, release from the endosome cannot be guaranteed.

Another aspect of intracellular localization also needs to be considered, i.e., co-localization of ribozyme with its intended mRNA target. While this may be possible without intervention, Sullenger and Cech[85] have found that inclusion of a retroviral packaging signal as part of the ribozyme gave 90% reduction of titer of a targeted retrovirus, presumably because in cell culture the ribozyme was co-localized in the nucleus with its viral genomic RNA target. At the same time, the ribozyme had no effect on the same target localized in the cytoplasm. In another method of enhancing ribozyme activity,[86] it was shown in the test tube that the cleavage rate of a HH ribozyme can be enhanced 10–20-fold upon addition of the NC protein of HIV-1, which also enhanced the ability of the ribozyme to discriminate between cleavage of RNA oligonucleotides with differing sequences. Hence, delivery of a ribozyme to the same cellular compartment as the target, either through chemical modification of the ribozyme or otherwise associating it with a "localization" or "enhancement" factor, can be expected to increase the rate at which the ribozyme finds its target and hence increase its effectiveness.

While the use of lipids and other factors to enhance exogenous delivery and effectiveness of oligonucleotides is encouraging, the process for designing such lipids still remains somewhat empirical and must be decided experimentally for each ribozyme in each cell of interest.

B. Endogenous (Vector) Delivery. All studies of viral delivery of ribozymes to date have utilized retroviruses (see below). Other viruses also offer potential for ribozyme gene therapy, e.g., adenovirus, adeno-associated virus, and others.

Retroviral vectors possess several properties of interest. For example, they will transfect and express therapeutic genes in disease-susceptible cells, have no contaminating helper virus, and integrate into the genome of the cell.[87] In viral diseases such as HIV, these characteristics can be used to advantage, i.e., the integrated ribozyme expression unit would be expected to replicate continuously and, when expressed, compete with both infection and replication of wild-type HIV.

Adeno-associated virus (AAV), since it is nonpathogenic and able to transfect diverse cell types, is another attractive vector for ribozyme delivery. A vector consisting only of the *cis*-acting terminal repeats of AAV was established by Samulski.[87] This type of vector has been used to express the TAR antisense from an internal Rous sarcoma virus long terminal repeat, and blocked HIV replication after challenge. A more than 1000-fold reduction in reverse transcriptase activity was seen by day seven post challenge.[87] AAV also has been used to transform $CD34^+$ cells with a transduction efficiency of approximately 80% in the presence of interleukin 3 and granulocyte/macrophage-colony-stimulating factor.[87]

Adenovirus also has been studied as a vector for the delivery of oligonucleotides. It is a DNA virus which enters cells through binding of adenovirus with its cell-surface receptor and transfer into endocytic vesicles.[88–91] Adenoviruses can be used to express genes as well as to enhance delivery of various large molecules such as dextrans, proteins, and plasmid DNA linked to ligands, whether it is replication-competent or replication-deficient.[92,93] The mechanism for enhanced delivery by replication-deficient adenovirus is not clear but involves release of materials from the endocytic vesicle to the cytoplasm. This characteristic has been utilized by Gao *et al.*[94] to deliver genes to airway epithelium *via* an adenovirus–polysine–DNA complex.

Viral delivery of ribozymes and other oligonucleotides is at an early stage of development, and investigations to date in therapeutic settings are described below. While any of the viral vectors is applicable in principle, more experience is needed. For example, targeting ribozyme-containing vectors to particular tissues or cells may be important. This may be accomplished via a physical method such as an aerosol to the lung epithelium, to a target tissue *ex vivo* such as bone marrow which is then reinfused, by widespread delivery *via* a vector such as adeno-associated virus which would infect many cells and tissues, or by intracellular targeting.[85]

Other nonviral vector approaches also have been used to deliver plasmids and/or DNA. However, it appears that there is no single lipid formulation which will suffice for all cell types, whether it is for a synthetic ribozyme or a vector-delivered ribozyme. For example, San *et al.*[83] showed a significant variation in the amount of DNA expression from one cell type to another. This varied from 1% conversion (transfection efficiency) of MCA205 cells to 15% in human melanoma cells to 55% in 293 cells.

IV. Therapeutic Applications

Therapeutic applications of ribozymes are potentially quite broad but have thus far been applied to situations involving inhibition of overexpression of a gene. The gene target may be foreign, as in a viral infection, or may be a normal gene which has undergone mutation such as an activated protooncogene.

The most obvious areas of therapy at this time are viral infections, both acute and chronic; cancer where an oncogene product is known; and various disease states where overexpression of a particular gene is associated with a disease state. Examples of the latter include restenosis and other cardiovascular diseases, transplant rejection, osteoarthritis, and immunological diseases. This review will concentrate on examples from viral infections and cancer.

A. Viral Diseases. One of the earliest descriptions of the activity of ribozymes against viral diseases was that of Sarver *et al.*[95] In that investigation, cleavage of HIV sequences in a cell-free system by HH ribozymes was demonstrated. Also, in human cells stably express-

ing a HH ribozyme targeted to the *gag* transcript, a substantial reduction in *gag* RNA relative to non-ribozyme-expressing cells was seen. This reduction in RNA was reflected in a reduction in p24 antigen levels of approximately 98%.

To measure long-term effects of ribozyme expression, the growth curve of cells constitutively expressing an anti-HIV ribozyme was followed for 10 days, and no impairment of cell growth was observed.[95] Longer term toxicity studies have shown that ribozyme-containing cells behave as their control non-ribozyme-expressing counterparts when followed for nine months.[96]

A subsequent study[97] used a HH ribozyme designed against a conserved region within the 5′-leader sequence of HIV RNA. This was selected because it is present on all HIV RNAs and is essential for viral transcription, transactivation and translation. Thus, cleavage at this site would be expected to produce a small 5′ fragment to act as a competitive inhibitor of replication, transactivation, and translation. In addition, HIV enhances transcription by a feedback mechanism involving the viral *tat* protein. Therefore, ribozymes were expressed not only in a constitutive manner, but also in a *tat*-inducible manner under the control of a fusion *TK*-transactivation-responsive (TAR) promoter. This allowed the ribozyme to be upregulated in the presence of HIV replication in the cell.

The results of this study demonstrated that stable MT_4 transformants which express the ribozyme under the control of the herpes simplex virus thymidine kinase promoter were only modestly resistant to HIV infection. Virus production was simply delayed. In cells allowing ribozyme expression under control of the simian virus 40 or cytomegalovirus promoter, the rate of HIV multiplication was decreased slightly and virus production was delayed about 2 weeks. The highest level of resistance was observed in MT_4 cells transformed with a vector containing the TAR promoter to allow ribozyme expression in a *tat* inducible manner. No HIV production was observed 22 days after infection of these cells. The results of this study not only corroborated the foregoing and those of others[98] but also illustrated the importance of high expression levels of the ribozyme in HIV-infected cells. An inducible promoter that upregulates the ribozyme in the presence of this infection may enhance efficacy even further.

A similar strategy was described by Yu *et al.*[99] These investigators designed a HP ribozyme to cleave the 5′-leader sequence of HIV. Expression of the HP ribozyme under the control of a β-actin promoter inhibited HIV expression in a transient expression system. In a followup investigation,[100] the human $tRNA^{VAL}$ gene and the adenovirus VA1 gene promoter, both transcribed by RNA polymerase III (pol III), were used as expression cassettes for the HP ribozyme. Because of the small size, high rate of transcription, and broad expression in various tissues, pol III transcription units have been used more broadly thus far for expressing ribozymes than pol II promoters.[101-103] The Yu *et al.*[100] investigation demonstrated that the HP ribozyme expressed from a pol III promoter inhibited HIV expression up to 95% in a transient transfection assay. The authors also showed that the HIV RNA cleavage products were degraded with high specificity.

Both the studies by Weerasinghe *et al.*[97] and Yu *et al.*[100] support the conclusion that, by cleaving at the 5′-leader sequence, the ribozyme removes the RNA cap so that the mRNA is poorly translated and probably more quickly degraded. Moreover, the 5′-leader sequence is highly conserved among most HIV isolates and therefore is a theoretically more important therapeutic target. Of available HIV strains, only "MN" contains one nucleotide substitution in the 5′-leader sequence cleaved by a hairpin ribozyme. There are differences among some HIV strains in other portions of the 5′-leader.[104]

The latter authors also used a retroviral vector to deliver the ribozyme, and demonstrated 70–95% inhibition of several strains of HIV. In these experiments, p24 was analyzed after 24 h, and it is not clear if this particular strategy would be effective in a longer assay. Nevertheless, it supports the earlier work of Weerasinghe *et al.*[97] and adds a second ribozyme motif for potential use against HIV.

In an interesting variation, a ribozyme was created by inserting a 22-nucleotide catalytic HH domain into an antisense RNA of 413 nucleotides directed against the 5′-leader/*gag* region of HIV.[105] This left 129 nucleotides on the 5′-flanking sequence and 284 nucleotides on the 3′-flanking sequence. The rationale for the experiment was to prevent the ribozyme from dissociating after binding and cleaving the target, and thereby discern whether catalysis conferred a significant advantage to antisense RNA. An inactivated ribozyme, which did not cleave the substrate, and the catalytically active ribozyme were transfected into human sw480 cells along with infectious proviral HIV DNA. HIV replication was analyzed by measurement of RNA and by ELISA. The presence of the catalytically active region conferred a 4–7-fold greater inhibition of HIV replication as compared to the antisense and the inactive mutant. Both kinetic and structural studies indicated that the ability to form double strands was not changed in using ribozymes and suggested that the ability to cleave target RNA was a critical prerequisite for the observed increase of inhibition of replication of HIV.

The investigations described above clearly demonstrate the ability of ribozymes to inhibit replication of HIV in cell culture. The inhibition varies from several days to several weeks. One of the possible ways to augment inhibition further is to use promoters which ensure high copy numbers in the cell and/or inducible promoters which are activated by the presence of the viral target.

There are several other methods that may be useful in increasing the activity of ribozymes against HIV, e.g., addition of a protein which facilitates catalysis or the use of multiple ribozymes. For example, a study by Bertrand and Rossi[106] demonstrated that addition of certain RNA binding proteins enhanced the ribozyme cleavage reaction. Some of these activities were dependent upon the ribozyme–substrate hybrid length. Certain proteins, heterogeneous nuclear RNP, A1, and the HIV nucleocapsid protein (NCp7), inhibit the reaction of shorter duplexes. NCp7 also inhibits the cleavage of longer duplexes (17–20bp). Both of the latter enhance the turnover of ribozymes by increasing the rate of product dissociation when the products are bound with 7bp or less. Since A1 is though to interact with

most mRNAs *in vivo*, it may enhance intracellular activity of ribozymes.

Another way to enhance ribozyme effectiveness in solution is through the use of multiple ribozymes.[107] In this investigation, the ribozymes were flanked by *cis*-acting ribozymes at both the 5′- and the 3′-ends so that, upon transcription, multiple ribozymes were trimmed and liberated independently. When levels of ribozyme expression were examined, the amount of transcript was proportional to the number of units connected in tandem. The activities of these ribozymes were also proportional to the number of units. The activities of connected-type ribozymes reached a plateau at values of about $n = 3$. In the tandem *cis*-cleaving ribozymes, $n = 1-10$, their results indicated that the multiple ribozyme expression system could generate independent ribozymes specific for different target sites *in vitro* without sacrificing the activity of any individual ribozyme.

A multiple ribozyme transcript may be more active against HIV in cells than the corresponding individual ribozymes.[108] A transcription unit containing nine ribozymes (a "nonaribozyme") was more active than transcripts with fewer catalytic units. The individual ribozymes were arranged in tandem. The multitarget ribozymes retained the specificity of monoribozymes but were more efficient per ribozyme RNA copy and remained active when part of a large transcript.

In one investigation an anti-HIV ribozyme was found to be less effective than the corresponding antisense.[109] This is the only study which has shown this; the reason is not clear.

The above provides encouragement regarding the potential use of ribozymes in the management of HIV infection. Therapeutic efficacy will require high efficiency of transfection or transduction of the gene coding for the ribozyme. This is attainable in tissue culture but has not yet been demonstrated in humans. An *ex vivo* treatment of hematopoietic stem cells may allow this goal to be realized but will depend to a great degree on the nature of the viral vector. This is a field of science still in its infancy, and the optimum choice of viral vector is not currently apparent. In addition, experiments which have reported activity of ribozymes against HIV have used low multiplicities of infection (the ratio of input virus to cells), usually on the order of $10.^{-3}$ At higher multiplicities, ribozyme protection often disappears. The reduction in virus titer is measured by percent inhibition, but an effective therapeutic will require a reduction of several orders of magnitude. Finally, ribozymes must compete against the best therapeutic agents currently in clinical medicine. Ribozymes and antisense agents have not yet received a thorough comparison with these small molecules. While none of these objections constitute an impassable hurdle, they remain important considerations when evaluating the potential usefulness of ribozymes in this disease.

Ribozyme inhibition of other viruses also has been studied. Xing and Whitton[110] prepared ribozymes which cleave the RNA genome of lymphocytic choriomeningitis virus (LCMV), a prototype arenavirus. Several sites on the LCMV genome were cleaved efficiently in *trans* in solution. The efficiency of the cleavage was site-dependent, and the authors showed that the secondary structure at the target site could abolish ribozyme cleavage. Computer-assisted analysis indicated that much of the LCMV genome may be involved in base pairing which would render it similarly resistant to ribozyme attack. The remaining open regions lacked a GUC target site, but there were several alternative sites available which could be cleaved: AUC, CUC, and AUU. They further demonstrated that an anti-LCMV ribozyme expressed in tissue culture cells diminished viral RNA levels and reduced infectious virus yield approximately 100-fold.[111] This effect was shown to be specific since yields of related arenavirus were not similarly curtailed.

Thus, activity of ribozymes against viruses has been clearly demonstrated in solution and in cells. The challenge now is to arrange appropriate promoters and multiple ribozymes as needed and direct them to highly conserved sequences to achieve the appropriate therapeutic result.

B. Inhibition of Oncogene Function. The crucial differences between normal cells and cancer cells appear to stem from discrete changes in specific genes controlling proliferation and tissue homeostasis. Many cancer-related genes have been discovered which are implicated in the natural history of human cancer because they are consistently found to be mutated in tumors. They fall into two descriptive categories: tumor-suppressor genes and oncogenes.

Oncogenes are evolutionary conserved and have been identified because they induce cellular transformation either when naturally incorporated into a retrovirus or when their DNA is transfected into tissue culture cells. Most of the known oncogenes were originally isolated as viruses containing genes of nonprimate origin. These genes are mutations of protooncogenes which are normally found in cells and are activated during embryogenesis or specific tissue regeneration or cell growth. The mutation, which may be a point mutation, alters the property of the corresponding protein and thereby induces uncontrolled cell growth. Since they are overexpressed and produce RNA which is distinguishable from the protooncogene, oncogenes are potentially excellent targets for ribozyme therapeutic activity.

By delivering phosphorothioate antisense oligomers with the cationic lipid lipofectin, Monia *et al.*[112] demonstrated a 5-fold discrimination between a point mutation in the 12th codon of H-*ras* and the wild-type (WT) H-*ras* target sequence. The authors also showed that discrimination correlated with and was limited by the difference in thermodynamic stability of the hybrids formed between mutants or WT sequences. Because of the sequence requirements of ribozyme cleavage, a ribozyme targeted to this site should have greater discrimination between mutated and wild-type genes than antisense molecules that rely on differences in thermodynamic stability or an RNase H mechanism.

This same 12th codon mutation (GGC to GUC) in H-*ras* creates a consensus HH ribozyme target site.[113] An H-*ras*-dependent cell line was stably transformed with a β-actin expression vector encoding a HH ribozyme. Isolated clones showed reduced H-*ras* expression and reduced rates of cell proliferation. The study demonstrated a decrease in the H-*ras* RNA and a corresponding decrease in the protein derived from H-*ras* expression, p21. The cell lines transformed by the anti-H-*ras* ribozyme were examined for their ma-

lignant potential in athymic (nude) mice. The ribozyme described above was designed to cleave the mRNA of the H-*ras* gene expressed in human bladder carcinoma EJ cells. DNA encoding the ribozyme was cloned into a mammalian expression vector and transfected into these cells. The cell data described above provided convincing evidence *in vitro* that the ribozyme could inactivate the H-*ras* gene product. These same cell lines were then injected by an orthotopic (transurethral) implantation model to recapitulate the invasive potential of bladder carcinoma. The EJ transfected cells preserved the malignant phenotype in these mice and caused highly invasive tumors and death due to high tumor burden.

In contrast, in the EJ clones transfected with the ribozyme-expressing vector there was a dramatic reduction in the malignant phenotype. The tumors displayed limited invasive capacity and there was a significant increase in survival, approximately double that of the control cells (a medium of 75 days *vs* a medium of 47 days). Histology of the bladders demonstrated that control tumors consisted of cells with highly invasive properties with nests of neoplastic cells dispersed throughout the tubules of the normal kidney. In contrast, the ribozyme clones produced tumor nodules that compressed surrounding tubules of the normal kidney but with no evidence of invasion in the vascular spaces. Tumor-bearing mice alive at 86–90 days had ribozymes present as detected by the PCR assay. Thus, this experiment demonstrated the ability of a ribozyme directed against H-ras mRNA to reverse the phenotypic expression H-*ras in vitro* and *in vivo*.

Ribozymes can inhibit the transformation of NIH 3T3 cells by the activated c-Ha-*ras* gene.[114,115] Plasmids containing the ribozyme-encoding genes were expressed under the control of the long-terminal repeats of Rouse sarcoma virus in NIH 3T3 cells transfected with the c-Ha-*ras* gene. These ribozymes inhibited the formation of cell foci by about 50% by cleaving the oncogene mRNA. In addition, when the activated c-Ha-*ras* gene was cotransfected with the ribozyme-encoding gene, colonies were isolated which were morphologically different from those containing only the c-Ha-*ras* gene.

The c-*fos* gene has been implicated in signal transduction, DNA synthesis, and resistance to anti-neoplastic agents.[116] It is one of the earliest known genes activated in response to mitogenic stimuli. Its interaction with the oncogene *jun* and the DNA binding activity of the *fos-jun* heterodimer suggest that fos functions as a transcriptional activator. The linkage between expression of c-*fos* and DNA synthesis genes is supported by data that cisplatin administration leads to a sequential induction of c-*fos* followed by a dTMP synthase and DNA polymerase.[117] A ribozyme was constructed to a site in c-*fos* mRNA, and it was demonstrated that activation of the fos ribozyme resulted in decreased c-*fos* gene expression, increased sensitivity to chemotherapeutic agents (including cisplatin), and a significant decrease in dTMP synthase, DNA polymerase β, topoisomerase I, and hMTII-A gene expression (human metallothionein).[116–119]

Snyder *et al.*[120] demonstrated ribozyme-mediated inhibition of *bcr/abl* gene expression in a cell line which was positive for the Philadelphia chromosome. The Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22 and is found in over 95% of patients with chronic myelogenous leukemia (CML). This translocation results in the transposition of the cellular (c-*abl*) gene from its usual position on chromosome 9 to chromosome 22.[121,122] Transposition of c-*abl* into the *BCR* gene results in the creation of an abnormal fusion gene termed *bcr-abl*. The mRNA transcript resulting from the fusion gene is translated into a p210 protein with augmented tyrosine kinase activity. This $p210^{bcr/abl}$ protein and its corresponding RNA are found in virtually all patients with CML and in about 50% of patients with Philadelphia-chromosome-positive acute lymphoblastic leukemia.

In this study a ribozyme was directed against the junction sequence in *bcr/abl*. The DNA encoding this ribozyme was incorporated into a plasmid and transfected into EM-2 cells (a CML-derived cell line) using a liposome vehicle. The ribozyme decreased levels of detectable *bcr/abl* mRNA in these cells, inhibited expression of the *bcr/abl* gene product, $p210^{bcr/abl}$, completely, and inhibited cell growth by 84%, significantly more than an antisense oligonucleotide. There was no significant inhibition by liposome vector alone, sense oligonucleotide, or unrelated ribozyme. Wright *et al.*[123] also demonstrated ribozyme-mediated cleavage of *bcr/abl*. This ribozyme transcript was tested against a synthetic substrate which covered the translocation sequence. This study confirmed the activity of a ribozyme against the translocation sequence and also showed that cleavage of the normal *bcr* occurred but at a reduced efficiency compared to the *bcr/abl* substrate. A similar study by Shore *et al.*[124] also showed ribozyme-mediated cleavage of the *bcr/abl* oncogene transcript. These investigators designed a ribozyme to cleave a GUU triplet adjacent to the junction of the cBCR and cABL fused genes. The ribozyme efficiently cleaved the RNA transcript *in vitro*. To determine the effect of constitutive expression of the ribozyme on the gene product, the ribozyme cDNA sequence was inserted into different retroviral expression vectors. Introduction of the recombinant retroviruses into the CML blast crisis cell line K562 resulted in the elimination of the p210 protein kinase activity in several single-cell clones infected with the ribozyme expression cassette.

Cantor *et al.*[125] developed a ribozyme that cleaves *rex/tax* mRNA and inhibited bovine leukemia virus expression. The transactivating protein, tax, stimulates the long terminal repeat to promote viral transcription and may be involved in tumorigenesis. Rex is involved in the transition from early expression of regulatory proteins to later expression of viral structural proteins. A ribozyme designed against both target sequences cleaved more than 80% of the target RNA *in vitro*. Synthetic DNA encoding the ribozyme was cloned into an expression vector and transfected into bovine leukemia virus-infected bat lung cells. Intracellular cleavage of the *rex/tax* mRNA was confirmed by reverse transcriptase PCR. In cells expressing the ribozyme, viral expression was inhibited significantly as measured by bovine leukemia virus core protein p24 (61% inhibition) and reverse transcriptase activity (92% inhibition).

Future Aspects

As demonstrated above, control of gene expression using ribozymes holds the potential to provide an

important new paradigm for human therapeutics. In the past several years, substantial progress has been made in translating this potential into reality. Cell culture and *in vivo* efficacy have been demonstrated in a number of systems, and therapeutic applications in viral diseases and cancer have been initiated. However, to complete this process, a number of issues need to be resolved. The current status of these issues, both for ribozymes and other oligonucleotide therapeutic approaches, is discussed below.

A. Specificity. One of the features that distinguishes oligonucleotide-based therapies from other approaches is the high degree of selectivity that is available, at least in principle. Differences are becoming apparent in the translation into practice of ribozyme, antisense, 2′,5′-polyadenosine oligonucleotide and other techniques. (The 2′,5′-polyadenosine guide sequence serves to activate a latent RNAse (RNAse L) in the cell which cleaves double-stranded RNA.[126–128]) One of the particular advantages of ribozymes is, as noted by Herschlag[36] and others,[57] the length of a target sequence of 15 nucleotides which may be unique in the human genome. Herschlag also showed that, for ribozymes and other oligonucleotide therapeutics that act through binding an RNA target followed by cleavage by an additional enzyme, adding bases to a recognition sequence ultimately reduces discrimination, since cleavage occurs virtually every time the target RNA or a mismatched RNA is bound. This occurs despite the weaker binding of the mismatched RNA because dissociation is too slow to allow the ribozyme to discriminate between the target RNA and a mismatched RNA.

The Herschlag conclusions are supported in the study of Goodchild and Kohli.[129] They demonstrated that ribozymes designed to cleave sequences specific to viral RNA (HIV) may be better antiviral agents than larger antisense oligonucleotides. By reducing the base pair formed with the substrate from 20 to 12, they showed that the rate of cleavage *in vitro* increased 10-fold. Deletions within the stem–loop structure in the ribozyme also increased the initial rate of the reaction.

Specificity mismatches near the core not only reduce binding but have an additional negative effect on cleavage. To give an extreme example, matched arms but with a mismatch at the "U" of "UH" site gives no cleavage by a hammerhead ribozyme; the corresponding antisense molecule presumably is not much affected.

Limited specificity is found in most antisense DNA oligomers, which typically contain 20 nucleotides or more.[57] This is particularly true in the case of DNA antisense oligonucleotides comprised of phosphorothioate linkages. To illustrate this point, Woolf and coworkers[126] demonstrated in an oocyte that, as mismatches in an antisense molecule were introduced, the oligomer continued to inhibit its primary target but showed nonspecific inhibition of other targets as well. Since oligonucleotides beyond the length of 15 will increase the number of mismatch recognition opportunities, the appearance of nonspecific effects for oligonucleotides having more than 15 nucleotides is not surprising.

In the case of ribozymes, available internal data on hammerhead ribozymes indicates that the optimum length of recognition arms is 6–7 nucleotides on each side of the catalytic core. Therefore, experimental evidence currently available is consistent with the Herschlag theoretical analysis.[36] Thus, with optimum recognition sequences in ribozymes long enough to recognize a target sequence uniquely but not requiring additional nucleotides for activity, nonspecific effects are minimized.

B. Kinetic Considerations. Kinetic considerations may also be important in the effective translation of oligonucleotide therapeutics into reality. In the case of antisense approaches, the DNA–RNA hybrid may be cleaved by activated RNase H. However, this process requires a trimolecular mechanism involving sequential or simultaneous association of the antisense molecule, the target RNA, and the RNAase in order to achieve cleavage. For this to be an efficient mechanism, the third component of the reaction, i.e., the requisite cleaving enzyme, must be present at saturating concentrations relative to the binary complex. This is difficult to assure in general for all cell types of interest, but studies to date[57] suggest that these enzymes may be present in sufficient abundance to carry out the reactions in some cases. Similar conclusions apply for the 2–5A approach,[127,128] where activation of RNAse L is required to cleave the target in the 2-5A–target complex.

Ribozymes do not require a separate enzymatic component for cleavage of the target. Only the bimolecular complex of ribozyme and target plus Mg^{2+} are needed, since both recognition and cleavage are incorporated into the same molecule. Demonstration of the extent of therapeutic advantage that this provides to ribozymes will presumably become evident as animal and human clinical studies are carried out.

C. Cleavage and Turnover. The ability of ribozymes to cleave their target enzymatically provides at least three significant potential advantages as therapeutics: their mechanism of action which destroys the substrate, multiple turnover to increase potency, and increased specificity when compared to small molecules or antisense therapeutic agents.

To demonstrate the importance of target cleavage, the experiment of Homann and co-workers is relevant.[105] They incorporated the catalytic domain of a HH ribozyme into a 413 nucleotide antisense RNA directed against the 5′-leader/gag region of HIV. The presence of the catalytically active RNA resulted in a 4–7-fold greater inhibition of HIV replication as compared to the parental antisense and the inactive mutant. Using ribozyme turnover to increase potency is at an earlier stage. The study by L'Huiller *et al.*[48] demonstrated cleavage products in cells but also showed a requirement for a ribozyme excess of approximately 1000:1 relative to the substrate. The ribozyme appeared to be functioning as a catalytic agent but was present in substrate quantities. This may be due to a relatively slow turnover rate.

D. Delivery. One of the issues of importance to effective exogenous delivery of ribozymes has been satisfactorily resolved, i.e., the creation of chemically modified ribozymes with both significant activity and long lifetimes in human serum. However, issues of lipid choice, targeting of specific cell types or organs, avoidance of sources of drug elimination such as the kidney, intracellular localization of ribozyme with its target, and increase of effective ribozyme delivery to cells through

improved release from endosomes all remain important challenges.

In the field case of vector delivery, the questions which remain are common to the field of gene therapy. The optimum viral vector is not clear; experience to date is largely limited to retroviral vectors. Optimization of ribozyme activity by modification of the transcription unit also provides opportunities for improvement.[130,131] Delivery of vectors is also an issue; whether *ex vivo* delivery of viral vectors will be sufficient or whether systemic delivery is necessary is not clear. Creation of satisfactory vectors to transfect CD34+ or stem cells is still in its infancy. Whether nonviral vectors can be utilized is not clear. Finally, reduction in production costs and long-term safety questions remain to be answered.

E. Therapeutic Potential. The therapeutic possibilities for ribozymes in clinical medicine are many. The mRNA for any protein which is causative in a disease is a potential ribozyme target. Similarly, microorganisms, especially viruses, since they are the pathogenic agents in infectious processes are likely targets for ribozyme therapy. In each of these general examples, it is the specificity of the ribozyme which is so important in therapy. The ability to eliminate a specific mRNA in a cell without damage to other normal cellular RNA molecules is a significant advance in therapeutics and one which may not apply to other therapeutic modalities.

Chemically synthesized ribozymes can be delivered to a variety of target organs or tissues topically. For example, the direct application of a ribozyme to the arterial wall may be used to modify the restenotic process after angioplasty. The target in this instance would be one of the gene products associated with smooth muscle cell proliferation which is activated by the angioplasty procedure. One could also inject a synthetic ribozyme into a joint space to suppress an inflammatory process by eliminating a particular cytokine or enzyme in that process. A viral infection in the lung, especially one confined to the bronchial epithelium, could be treated by aerosolization of a chemically synthesized ribozyme. The duration of action of the stable ribozyme should be sufficient to treat a viral infection of 4–6 days.

Ribozymes delivered by a vector will be of more use in systemic diseases, infectious or noninfectious, and diseases of a chronic nature, local or systemic, where long-term expression of the ribozyme is important. The most prominent example is the use of a ribozyme against HIV to eliminate the virus from the stem cells in the bone marrow. This could permit the reconstitution of the immune system with cells resistant to HIV. A leukemic process due to the presence of a new gene product, such as *bcr–abl* in chronic myelogenous leukemia, is a natural target for a ribozyme since the RNA product of the fusion gene is not found in normal cells and is known to be causative in the disease. Oncogenes associated with other types of neoplasia, which result from the mutation of a protooncogene, similarly are good potential targets since the new gene product also is not found in the normal adult cell. The list of potential applications is as long as the list of new gene products associated with viral diseases, neoplastic diseases, chronic inflammatory conditions, cardiovascular diseases, genetic diseases, and others where there is overproduction of an aberrant protein.

Acknowledgment. The authors would like to express their appreciation to T. Cech, N. Usman, B. Chowrira, and D. Stinchcomb for helpful comments and suggestions.

Biographies

Ralph E. Christoffersen obtained his Ph.D. degree in Physical Chemistry from Indiana University, Bloomington, IN, in 1964. After postdoctoral experience at the University of Nottingham, England, and Iowa State University in Ames, he joined the faculty of the Department of Chemistry at the University of Kansas. In 1972 he was appointed Professor of Chemistry and in 1979 became Vice Chancellor for Academic Affairs. In 1981, he joined Colorado State University as President, and when he left in 1983 to join the Upjohn Company in Kalamazoo as Director of Biotechnology, was Professor of Chemistry. In 1989, he left the position of Vice President, Discovery Research at Upjohn, to join SmithKline Beecham as Senior Vice President, Research. In 1992, he became President and CEO of Ribozyme Pharmaceuticals in Boulder, CO.

J. Joseph Marr obtained his M.D. degree from Johns Hopkins in 1964 and completed an M.S. in Research in Microbiology at the St. Louis University Graduate School. After six years at Washington University School of Medicine, he became Professor in Medicine and Microbiology at the St. Louis University School of Medicine and reached the rank of Vice Chairman. In 1982, he joined the University of Colorado Health Sciences Center as Professor of Medicine and Biochemistry, Director of the Clinical Laboratories, and Head, Division of Infectious Diseases and the Division of Laboratory Medicine. In 1989 he joined G. D. Searle as Senior Vice President, Discovery Research. Since 1993, he has been Vice President, R & D, and CSO at Ribozyme Pharmaceuticals in Boulder, CO.

References

(1) Cech, T. R.; Zaug, A. J.; Grabowski, P. J. *In vitro* Splicing of the Ribosomal RNA Precursor of *Tetrahymena*: Involvement of a Guanosine Nucleotide in the Excision of the Intervening Sequence. *Cell* **1981**, *29*, 487–496.
(2) Kruger, K.; Grabowski, P. J.; Zaug, A. J.; Sands, J.; Gottschling, D. F.; Cech, T. R. Self-splicing RNA: Autoexcision and Autocyclization of the Ribosomal RNA Intervening Sequence of *Tetrahymena*. *Cell* **1982**, *31*, 147–157.
(3) Guerrier-Takada, C.; Gardiner, K.; Marsh, T.; Pace, N.; Altman, S. The RNA Moiety of Ribonuclease P Is the Catalytic Subunit of the Enzyme. *Cell* **1983**, *35*, 849–857.
(4) Cech, T. R. Self-splicing RNA: Implications for Evolution. *Int. Rev. Cytol.* **1985**, *93*, 3–22.
(5) Darnell, J. S.; Doolittle, W. F. Speculations on the Early Course of Evolution. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1271–1275.
(6) Cech, T. R. A Model for the RNA-catalyzed Replication of RNA. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4360–4363.
(7) Joyce, G. F.; Schwartz, A. W.; Miller, S. L.; Orgel, L. E. The Case for An Ancestral Genetic System Involving Simple Analogues of the Nucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4398–4402.
(8) Cech, T. R. RNA Chemistry. Ribozyme Self-replication? *Nature* **1989**, *339*, 507–508.
(9) Doudna, J. A.; Szostak, J. W. RNA-catalyzed Synthesis of Complementary-strand RNA. *Nature* **1989**, *339*, 519–522.
(10) Cech, T. R. The Efficiency and Versatility of Catalytic RNA: Implications for An RNA World. *Gene* **1993**, *135*, 33–36.
(11) For a review of ribozyme development, see: Cech, T. R. Ribozyme Engineering. *Curr. Opin. Struct. Biol.* **1992**, *2*, 605–609.
(12) Cech, T. R. The Generality of Self-splicing RNA: Relationship to Nuclear mRNA Splicing. *Cell* **1986**, *44*, 207–210.

(13) Peebles, C. L.; Perlman, P. S.; Mecklinburg, K. L.; Petrillo, M. L.; Tabor, J. H.; Jarrell, K. A.; Cheng, H.-L. A Self-splicing RNA Excises an Intron Lariat. *Cell* **1986**, *44*, 213–223.
(14) Van der Veen, R. T.; Arnberg, A. C.; Van der Horst, G.; Bonen, L.; Tabak, H. F.; Grivell, L. A. Excised Group II Introns in Yeast Mitochondria Are Lariats And Can Be Formed By Self-splicing *In Vitro*. *Cell* **1986**, *44*, 225–234.
(15) Michel, F.; Umesono, K.; Ozeki, H. Comparative and Functional Anatomy of Group II Catalytic Introns – A Review. *Gene* **1989**, *82*, 5–30.
(16) Zaug, A. J.; Kent, J. R.; Cech, T. R. A Labile Phosphodiester Bond at the Ligation Junction in A Circular Intervening Sequence. *Science* **1984**, *224*, 574–578.
(17) Zaug, A. J.; Cech, T. R. The Intervening Sequence RNA of *Tetrahymena* Is An Enzyme. *Science* **1986**, *231*, 470–475.
(18) Zaug, A. J.; Been, M. D.; Cech, T. R. The *Tetrahymena* Ribozyme Acts Like An RNA Restriction Endonuclease. *Nature* **1986**, *324*, 429–433.
(19) Xu, M.-Q.; Kathe, S. D.; Goodrich-Blair, H.; Nierzwicki-Bauer, S. A.; Shub, D. A. Bacterial Origin of Chloroplast Intron: Conserved Self-Splicing Group I Introns in Cyanobacteria. *Science* **1990**, *250*, 1566–1570.
(20) Johansen, S.; Vogt, V. M. An Intron in the Nuclear Ribosomal DNA of Didymium iridis Codes for a Group I Ribozyme and a Novel Ribozyme That Cooperate in Self-Splicing. *Cell* **1994**, *76*, 725–734.
(21) Guo, H. C. T.; Collins, R. A. Efficient *trans*-cleavage of a stem-loop RNA substrate by a ribozyme derived from *Neurospora VS* RNA. *EMBO J.* **1995**, *14*, 368–376.
(22) Prody, G. A.; Bakos, J. T.; Buzayan, J. M.; Schneider, I. R.; Bruening, G. Autolytic Processing of Dimeric Plant Virus Satellite RNA. *Science* **1986**, *231*, 1577–1580.
(23) Hutchins, C. J.; Rathjen, P. D.; Forster, A. C.; Symons, R. H. Self-cleavage of Plus And Minus RNA Transcripts of Avocado Sunblotch Viroid. *Nucl. Acids Res.* **1986**, *14*, 3627–3640.
(24) Uhlenbeck, O. C. A Small Catalytic Oligoribonucleotide. *Nature* **1987**, *328*, 596–600.
(25) Forster, A. C.; Symons, R. H. Self-cleavage of Virusoid RNA Is Performed By The Proposed 55-Nucleotide Active Site. *Cell* **1987**, *50*, 9–16.
(26) Edington, B. V.; Nelson, R. S. Utilization or Ribozymes in Plants. *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson, R. P., Izant, J. G., Eds.; Raven Press: New York, 1992; pp 209–221.
(27) Sharmeen, L.; Kuo, M. Y. P.; Dinter-Gottlieb, G.; Taylor, J. Antigenomic RNA of Human Hepatitis Delta Virus Can Undergo Self-Cleavage. *J. Virol.* **1988**, *62*, 2674–2679.
(28) Wu, H.-N.; Lin, Y.-J.; Lin, F.-P.; Makino, S.; Chang, M.-F.; Lai, M. M. C. Human Hepatitis Delta Virus RNA Subfragments Contain An Autocleavage Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1831–1835.
(29) Haseloff, J.; Gerlach, W. L. Simple RNA Enzymes with New and Highly Specific Endoribonuclease Activities. *Nature* **1988**, *334*, 585–591.
(30) Hampel, A.; Tritz, R.; Hicks, M.; Cruz, P. "Hairpin" Catalytic RNA Model: Evidence for Helices and Sequence Requirement for Substrate RNA. *Nucl. Acids Res.* **1990**, *18*, 299–304.
(31) Feldstein, P. A.; Buzayan, J. M.; Bruening, G. Two Sequences Participating in the Autolytic Processing of Satellite Tobacco Ringspot Virus Complementary RNA. *Gene* **1989**, *82*, 53–61.
(32) Perrotta, A. T.; Been, M. D. A Pseudoknot-Like Structure Required for Efficient Self-Cleavage of Hepatitis Delta Virus RNA. *Nature* **1991**, *350*, 434–436.
(33) Branch, A. D.; Robertson, H. D. Efficient *Trans* Cleavage and A Common Structural Motif for the Ribozymes of the Human Hepatitis Delta Agent. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10163–10167.
(34) Perrotta, A. T.; Been, M. D. Cleavage of Oligoribonucleotides By A Ribozyme Derived from the Hepatitis Delta Virus RNA Sequence. *Biochemistry* **1992**, *31*, 16–21.
(35) Helene, C.; Toulme, J. J. Specific Regulation of Gene Expression by Antisense, Sense, and Antigene Nucleic Acids. *Biochim. Biophys. Acta* **1990**, *1049*, 99–125.
(36) Hearst, J. E. A Photochemical Investigation of the Dynamics of Oligonucleotide Hybridization. *Annu. Rev. Phys. Chem.* **1988**, *39*, 291–315.
(37) Herschlag, D. Implications of Ribozyme Kinetics for Targeting the Cleavage of Specific RNA Molecules *in Vivo*: More Isn't Always Better. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6921–6925.
(38) Young, B.; Herschlag, D.; Cech, T. R. Mutations in a Nonconserved Sequence of the Tetrahymena Ribozyme Increase Activity and Specificity. *Cell* **1991**, *67*, 1007–1019.
(39) Beaudry, A. A.; Joyce, G. F. Directed Evolution of an RNA Enzyme. *Science* **1992**, *257*, 635–641.
(40) Green, R.; Szostak, J. W. Selection of a Ribozyme that Functions as a Superior Template in a Self-Copying Reaction. *Science* **1992**, *258*, 1910–1915.
(41) Lehman, N.; Joyce, G. F. Evolution *In Vitro* of an RNA Enzyme with Altered Metal Dependence. *Nature* **1993**, *361*, 182–185.
(42) Pan, R.; Uhlenbeck, O. C. *In Vitro* Selection of RNAs that Undergo Autocatalytic Cleavage with Pb^{2+}. *Biochemistry* **1992**, *31*, 3887–3895.
(43) Bartel, D. P.; Szostak, J. W. Isolation of New Ribozymes from a Large Pool of Random Sequences. *Science* **1993**, *261*, 1411–1418.
(44) Prudent, J. R.; Uno, T.; Schultz, P. G. Expanding the Scope of RNA Catalysis. *Science* **1994**, *264*, 1924–1927.
(45) Lorsch, J. R.; Szostak, J. W. *In vitro* Evolution of New Ribozymes with Polynucleotide Kinase Activity. *Nature* **1994**, *371*, 31–36.
(46) Cameron, F. H.; Jennings, P. A. Specific Gene Suppression by Engineered Ribozymes in Monkey Cells. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9139–9143.
(47) Cotten, M.; Birnstiel, M. L. Ribozyme Mediated Destruction of RNA *in vivo*. *EMBO J.* **1989**, *8*, 3861–3866.
(48) L'Huillier, P. J.; Davis, S. R.; Bellamy, A. R. Cytoplasmic Delivery of Ribozymes Leads to Efficient Reduction in α-lactalbumin mRNA Levels in C1271 Mouse Cells. *EMBO J.* **1992**, *11*, 4411–4418.
(49) Saxena, S. K.; Ackerman, E. J. Ribozymes Correctly Cleave a Model Substrate and Endogenous RNA *In Vivo*. *J. Biol. Chem.* **1990**, *265*, 17106–17109.
(50) Inokuchi, Y.; Yuyama, N.; Hirashima, A.; Nishikawa, S.; Ohkawa, J.; Taira, J. A Hammerhead Ribozyme Inhibits the Proliferation of an RNA Coliphage SP in *E. Coli*. *J. Biol. Chem.* **1994**, *269*, 11361–11366.
(51) Efrat, S.; Leiser, M.; Wu, Y.-J.; Fusco-DeMane, D.; Emran, O. A.; Surana, M.; Jetton, T. L.; Magnuson, M. A.; Weir, G.; Fleischer, N. Ribozyme-Mediated Attenuation of Pancreatic β-Cell Glucokinase Expression in Transgenic Mice Results in Impaired Glucose-Induced Insulin Secretion. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2051–2055.
(52) Steinecke, P.; Herget, T.; Schreier, P. H. Expression of a Chimeric Ribozyme Gene Results in Endonucleolytic Cleavage of Target mRNA and A Concomitant Reduction of Gene Expression *In Vivo*. *EMBO J.* **1992**, *11*, 1525–1530.
(53) Zhao, J. J.; Pick, L. Generating Loss-of-function Phenotypes of the *fushi tarazu* Gene with a Targeted Ribozyme in *Drosophila*. *Nature* **1993**, *365*, 448–451.
(54) Larsson, S.; Hotchkiss, G.; Andang, M.; Nyholm, T.; Inzumza, J.; Jansson, I.; Ahrlund-Richter, L. Reduced β2-microglobulin mRNA Levels in Transgenic Mice Expressing a Designed Hammerhead Ribozyme. *Nucl. Acids Res.* **1994**, *22*, 2242–2248.
(55) Zuker, M. On Finding All Suboptimal Foldings of an RNA Molecule Science **1989**, *244*, 48–52.
(56) Christoffersen, R. E.; McSwiggen, J. A.; Konings, D. Application of Computational Technologies to Ribozyme Biotechnology Products. *J. Mol. Struct. (THEOCHEM)* **1994**, *311*, 273–284.
(57) Beaucage, S. L.; Iyer, R. P. The Functionalization of Oligonucleotides *via* Phosphoramidite Derivatives. *Tetrahedron* **1993**, *49*, 6123–6194.
(58) Milligan, J. F.; Matteucci, M. D.; Martin, J. C. Current Concepts in Antisense Drug Design. *J. Med. Chem.* **1993**, *36*, 1923–1937.
(59) Pieken, W. A.; Olsen, D. B.; Benseler, F.; Aurup, H.; Eckstein, F. Kinetic Characterization of Ribonuclease Resistant 2′-modified Hammerhead Ribozymes. *Science* **1991**, *253*, 314–317.
(60) Scaringe, S. A.; Franklyn, C.; Usman, N. Chemical Synthesis of Biologically Active Oligoribonucleotides Using β-cyanoethyl Protected Ribonucloside Phosphoramidites. *Nucl. Acids Res.* **1990**, *18*, 5433–5441.
(61) Yang, J.-H.; Usman, N.; Chartrand, P.; Cedergren, R. J. Minimum Ribonucleotide Requirement for Catalysis by the RNA Hammerhead Domain. *Biochemistry* **1992**, *31*, 5005–5009.
(62) Paolella, G.; Sproat, B. S.; Lamond, A. I. Nuclease-resistant Ribozymes with High Catalytic Activity. *EMBO J.* **1992**, *11*, 1913–1919.
(63) Shibahara, S.; Mukai, S.; Morisawa, H.; Nakashima, H.; Kobayashi, S.; Yamamoto, N. Inhibition of Human Immunodeficiency Virus (HIV-1) Replication by Synthetic Oligo-RNA Derivatives. *Nucl. Acids Res.* **1989**, *17*, 239–252.
(64) Beigelman, L.; Karpeisky, A.; Usman, N. Synthesis of 1-Deoxy-D-Ribofuranose Phosphoramidite and the Incorporation of Abasic Nucleotides in Stem-Loop II of a Hammerhead Ribozyme. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1715–1720.
(65) Usman, N.; Beigelman, L.; Draper, K.; Gonzalez, C.; Jensen, K.; Karpeisky, A.; Modak, A.; Matulic-Adamic, J.; DiRenzo, A.; Haeberli, P.; Tracz, D.; Grimm, S.; Wincott, F.; McSwiggen, J. Chemical Modification of Hammerhead Ribozymes: Activity and Nuclease Resistance. *Biochemistry*, in press.
(66) Lichtenberg, D.; Liposomes: Preparation, Characterization, and Preservation. *Methods Biochem. Anal.* **1988**, *33*, 337–468.
(67) Litzinger, D. C.; Huang, L. Phosphatidylethanolamine Liposomes: Drug Delivery, Gene Transfer and Immunodiagnostic Applications. *Biochim. Biophys. Acta* **1992**, *1113*, 201–227.
(68) Mayer, L. D.; Tai, L. C. L.; Ko, D. S. C.; Masin, D.; Ginsberg, R. S.; Cullis, P. R.; Bally, M. B. Influence of Vesicle Size, Lipid Composition, and Drug-to-Lipid Ratio on the Biological Activity of Liposomal Doxorubicin in Mice. *Cancer Res.* **1989**, *49*, 5922–5930.

(69) Litzinger, D. C.; Huang, L. Biodistribution and Immunotargetability of Ganglioside-Stabilized Dioleoylphosphatidylethanolamine Liposomes. *Biochim. Biophys. Acta* **1992**, *1104*, 179–187.
(70) Leserman, L. D.; Weinstein, J. N.; Blumenthal, R.; Terry, W. D. Receptor-mediated Endocytosis of antibody-opsonized liposomes by tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 4089–4093.
(71) Berinstein, N.; Matthay, K. K.; Papahadjopoulos, D.; Levy, R.; Sikic, B. I. Antibody-Directed Targeting of Liposomes to Human Cell Lines: Role of Binding and Internalization of Growth Inhibition. *Cancer Res.* **1987**, *47*, 5954–5959.
(72) Milhaud, P. G.; Machy, P.; Lebleu, B.; Leserman, L. Antibody-targeted Liposomes Containing poly(rl)-poly(rC) Exert a Specific Antiviral and Toxic Effect on Cells Primed with Interferons α/β or γ. *Biochim. Biophys. Acta* **1989**, *987*, 15–20.
(73) Moghimi, S. M.; Patel, H. M. Opsonophagocytosis of Liposomes by Peritoneal Macrophages and Bone Marrow Reticulendothelial Cells. *Biochim. Biophys. Acta* **1992**, *1135*, 269–274.
(74) Papahadjopoulos, D.; Allen, T. M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K.-D.; Woodle, M. C.; Lasic, D. D.; Redemann, C.; Martin, F. J. Sterically stabilized liposomes: Pronounced improvmenets in blood clearance, tissue distribution and therapeutic index of encapsulated drugs against implanted tumors. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11460–11464.
(75) Klibanov, A. L.; Maruyama, K.; Beckerleg, A. M.; Torchilin, V. P.; Huang, L. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. *Biochim. Biophys. Acta* **1991**, *1062*, 142–148.
(76) Lee, R. J.; Low, P. S. Delivery of Liposomes into Cultured KB Cells *via* Folate Receptor-mediated Endocytosis. *J. Biol. Chem.* **1994**, *269*, 3198–3204.
(77) Nabel, E. G.; Plautz, G.; Boyce, F. M.; Stanley, J. C.; Nabel, G. J. Recombinant gene expression *in vivo* within endothelial cells of the arterial wall. *Science* **1989**, *244*, 1342–1344.
(78) Nabel, E. G.; Plautz, G.; Nabel, G. J. Site-specific Gene Expression *In Vivo* By Direct Gene Transfer into the Arterial Wall. *Science* **1990**, *249*, 1285–1288.
(79) Nabel, G. J.; Nabel, E. G.; Yang, Z.-Y.; Fox, B. A.; Plautz, G. E.; Gao, X.; Huang, L.; Shu, S.; Gordon, D.; Chang, A. E. Direct Gene Transfer with DNA Liposome Complexes in Melanoma: Expression, Biologic Activity and Lack of Toxicity in Humans. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11307–11311.
(80) Plautz, G. E.; Yang, Z.; Wu, B.; Gao, X.; Huang, L.; Nabel, G. J. Immunotherapy of malignancy by *in vivo* gene transfer into tumors. *Proc. Natl. Acad. Sci., U.S.A.* **1993**, *90*, 4645–4649.
(81) Stewart, M. J.; Plautz, G. E.; Del Buono, L.; Yang, Z. Y.; Xu, L.; Gao, X.; Huang, L.; Nabel, E. G.; Nabel, G. J. Gene Transfer *in Vivo* with DAN-liposome Complexes: Safety and Acute Toxicity in Mice. *Human Gene Ther.* **1992**, *3*, 267–275.
(82) Zhu, N.; Liggitt, D.; Liu, Y.; Debs, R. Systemic Gene Expression after Intravenous DNA Delivery into Adult Mice. *Science* **1993**, *261*, 209–211.
(83) San, H.; Yang, Z.-H.; Pompili, V. J.; Jaffe, M. L.; Plautz, G. E.; Xu, L.; Felgner, J. H.; Wheeler, C. J.; Felgner, P. L.; Gao, X.; Huang, L.; Gordon, D.; Nabel, G. J.; Nabel, E. G. Safety and Short-Term Toxicity of a Novel Cationic Lipid Formulation for Human Gene Therapy. *Human Gene Ther.* **1993**, *4*, 781–788.
(84) Plautz, G. E.; Wu, B.-Y.; Gao, X.; Huang, L.; Nabel, G. J. Gene Transfer *In Vivo* with DNA-liposome Complexes: Lack of Autoimmunity and Gonadal Localization. *Human Gene Ther.* **1992a**, *3*, 649–656.
(85) Sullenger, B. A.; Cech, T. R. Tethering Ribozymes to a Retroviral packaging Signal for Destruction of Viral RNA. *Science* **1993**, *262*, 1566–1569.
(86) Tsuchihashi, A.; Khosla, M.; Herschlag, D. Protein Enhancement of Hammerhead Ribozyme Catalysis. *Science* **1993**, *262*, 99–102.
(87) Samulski, R. J. Adeno-associated virus: Integration at a Specific Chromosomal Locus. *Curr. Opin. Genet. Dev.* **1993**, *3*, 74–80.
(88) Seth, P.; Rosenfeld, M.; Higginbotham, J.; Crystal, R. G. Mechanism of Enhancement of DNA Expression Consequent to Cointernalization of a Replication-Deficient Adenovirus and Unmodified Plasmid DNA. *J. Virol.* **1994**, 933–940.
(89) FitzGerald, D. J. P.; Padmanabhan, R.; Pastan, I.; Willingham, M. C. Adenovirus-induced Release of Epidermal Growth Factor and Pseudomonas Toxin into the Cytosol of KB Cells During Receptor-mediated Endocytosis. *Cell* **1983**, *32*, 607–617.
(90) Seth, P.; FitzGerald, D.; Willingham, M.; Pastan, I. Pathway of Adenovirus Entry into Cells, in *Virus Attachment and Entry into Cells*; Colwell, R., Lonberg-Holm, K., Eds.; American Society for Microbiology: Washington, D.C., 1986.
(91) Otero, M. J. and Carrasco, L. Proteins Are Co-internalized with Virion Particles During Early Infection. *Virology* **1987**, *160*, 75–80.
(92) Cotten, M.; Wagner, E.; Zatloukal, K.; Phillips, S.; Curiel, D. T.; Birnstiel, M. L. High Efficiency Receptor-mediated Delivery of Small and Large 48 Kilobase Gene Constructs Using the Endosome-disruption Activity of Defective or Chemically Inactivated Adenovirus Particles. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6094–6098.
(93) Curiel, D. T.; Agarwal, S.; Wagner, E.; Cotten, M. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8850–8854.
(94) Gao, L.; Wagner, E.; Cotten, M.; Agarwal, S.; Harris, C.; Romer, M.; Miller, L.; Hu, P.-C.; Curiel, D. Direct *In Vivo* Gene Transfer to Airway Epithelium Employing Adenovirus-Polylysine-DNA Complexes. *Human Gene Ther.* **1993**, *4*, 17–24.
(95) Sarver, N.; Cantin, E. M.; Chang, P. S.; Zaia, J. A.; Ladne, P. A.; Stephens, D. A.; Rossi, J. J. Ribozymes as Potential Anti-HIV-1 Therapeutic Agents. *Science* **1990**, *247*, 1222–1225.
(96) Rossi, J. J.; Sarver, N. Catalytic Antisense RNA (Ribozymes): Their Potential and Use as Anti-HIV-1 Therapeutic Agents. *Innovations in Antiviral Development and the Detection of Virus Infection*; Block, T.; Ed.; Plenum Press: New York, 1992; pp 95–109.
(97) Weerasinghe, M.; Liem, S. E.; Asad, S.; Read, S. E.; Joshi, S. Resistance to Human Immunodeficiency Virus Type 1 (HIV-1) Infection in Human CD4+ Lymphocyte-Derived Cell Lines Conferred by Using Retroviral Vectors Expressing an HIV-1 RNA-Specific Ribozyme *J. Virol.* **1991**, *65*, 5531–5534.
(98) Dropulic, B.; Lin, N. H.; Martin, M. A.; Jean, K. T. Functional Characterization of a U5 Ribozyme: Intracellular Suppression of Human Immunodeficiency Virus Type I Expression. *J. Virol.* **1992**, *66*, 1432–1441.
(99) Yu, M.; Ojwang, J.; Yamada, O.; Hampel, A.; Rapapport, J.; Looney, D.; Wong-Staal, F. A Hairpin Ribozyme Inhibits Expression of Diverse Strains of Human Immunodeficiency Virus Type I. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6340–6344.
(100) Ojwang, J. O.; Hampel, A.; Looney, D. J.; Wong-Staal, F.; Rappaport, J. Inhibition of Human Immunodeficiency Virus Type 1 Expression by a Hairpin Ribozyme. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10802–10806.
(101) Sullenger, B. A.; Lee, T. C.; Smith, C. A.; Ungers, G. E.; Gilboa, E. Expression of Chimeric tRNA-driven Antisense Transcripts Renders HIV3T3 Cells Highly Resistant to Moloney Murine Leukemia Virus Replication. *Mol. Cell. Biol.* **1990**, *10*, 6512–6523.
(102) Sullenger, B. A.; Gallardo, H. F.; Ungers, G. E.; Gilboa, E. Overexpression of TAR Sequences Renders Cells Resistant to Human Immunodeficiency Virus Replication. *Cell* **1990**, *63*, 601–608.
(103) Jennings, P. A.; Molloy, P. L. Inhibition of SV40 Replicon Function by Engineered Antisense RNA Transcribed by RNA Polymerase III. *EMBO J.* **1987**, *6*, 3043–3047.
(104) Myers, G.; Korber, B.; Berzofsky, J. A.; Smith, R. F.; Pavlakis, G. N. *Human Retroviruses and AIDS*; Theoretical Biology and Biophysics; Los Alamos, 1992.
(105) Homann, M.; Tzortzakaki, S.; Rittner, K.; Sczakiel, G.; Tabler, M. Incorporation of the Catalytic Domain of a Hammerhead Ribozyme into Antisense RNA Enhances its Inhibitory Effect on the Replication of Human Immunodeficiency Virus Type 1. *Nucl. Acids Res.* **1993**, *21*, 2809–2814.
(106) Bertrand, E. L.; Rossi, J. J. Facilitation of Hammerhead Ribozyme Catalysis by the Nucleocapsid Protein of HIV-1 and the Heterogeneous Nuclear Ribonucleoprotein A1. *EMBO J.* **1994**, *13*, 2904–2912.
(107) Ohkawa, J.; Yuyama, T.; Nishikawa, S.; Taira, K. Importance of independence in ribozyme reactions: kinetic behavior of trimmed and of simply connected multiple ribozymes with potential activity against human immunodeficiency virus. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11302–11306.
(108) Chen, C.-J.; Banerjea, A. C.; Harmison, G. G.; Haglund, K.; Schubert, M. Multitarget-ribozyme Directed to Cleave at up to Nine Highly Conserved Hiv-1 Env RNA Regions Inhibits HIV-1 Replication – Potential Effectiveness Against Most Presently Sequenced Hiv-1 Isolates. *Nucl. Acids Res.* **1992**, *20*, 4581–4589.
(109) Lo, K. M. S.; Biasolo, M. A.; Dehni, G.; Palu, G.; Haseltine, W. A. Inhibition of Replication of HIV-1 by Retroviral Vectors Expressing *tat*-Antisense and Anti-*tat* Ribozyme RNA. *Virology* **1992**, *190*, 176–183.
(110) Xing, Z.; Whitton, J. L. Ribozymes Which Cleave Arenavirus RNAs: Identification of Susceptible Target Sites and Inhibition by Target Site Secondary Structure. *J. Virol.* **1992**, *66*, 1361–1369.
(111) Xing, Z.; Whitton, J. L. An Anti-Lymphocytic Choriomeningitis Virus Ribozyme Expressed in Tissue Culture Cells Diminishes Viral RNA Levels and Leads to a Reduction in Infectious Virus Yield. *J. Virol.* **1993**, *67*, 1840–1847.
(112) Monia, B. P.; Johnston, J. F.; Ecker, D. J.; Zounes, M. A.; Lima, W. F.; Frier, S. M. Selective Inhibition of Mutant Ha-*ras* messenger RNA Expression by Antisense Oligonucleotides. *J. Biol. Chem.* **1992**, *267*, 19954–19962.

(113) Kashani-Sabet, M.; Funato, T.; Tone, T.; Jiao, L.; Wang, W.; Yoshida, E.; Kashfinn, B. I.; Shitara, T.; Wu, A. M.; Moreno, J. G.; Traweek, S. T.; Ahlering, T. E.; Scanlon, K. J. Reversal of the Malignant Phenotype by an Antl-*ras* Ribozyme. *Antisense Res. Dev.* **1992**, *2*, 3–15.
(114) Koizumi, M.; Kamiya, H.; Ohtsuka, E. Ribozymes Designed to Inhibit Transformation of NIH3T3 Cells by the Activated c-Ha-*ras* Gene. *Gene* **1992**, *117*, 179–184.
(115) Koizumi, M.; Kamiya, H.; Ohtsuka, E. Inhibition of c-Ha-*ras* Gene Expression by hammerhead ribozymes containing a Stable C(UUCG)G Hairpin Loop. *Biol. Pharm. Bull.* **1993**, *16*, 879–883.
(116) Scanlon, K. J.; Jiao, L.; Funato, T.; Wang, W.; Tone, T.; Rossi, J. J.; and Kashani-Sabet. Ribozyme-mediated cleavage of c-*fos* mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10591–10595.
(117) Kashani-Sabet, M.; Wang, M.; Scanlon, K. J. Cyclosporin A Suppresses Cisplatin-induced c-*fos* gene Expression in Ovarian Carcinoma Cells. *J. Biol. Chem.* **1990**, *265*, 11285–11288.
(118) Rauscher, F. J., III; Cohen, D. R.; Curran, T.; Bos, V. J.; Vogt, P. K.; Bohmann, D.; Tjian, T.; Franza, B. R., Jr.; Cyclosporin A Suppresses Cisplatin-induced c-*fos* Gene Expression in Ovarian Carcinoma Cells. *Science* **1988**, *240*, 1010–1016.
(119) Funato, R.; Yoshida, E.; Jiao, L.; Tone, T.; Kashani-Sabet, M.; Scanlon, K. J. The Utility of An Anti-*fos* Ribozyme in Reversing Cisplatin Resistance in Human Carcinomas. *Adv. Enzyme Regul.* **1992**, *32*, 195–209.
(120) Snyder, D. S.; Wu, Y.; Wang, J. L.; Rossi, J. J.; Swiderski, P.; Kaplan, B. E.; Forman, S. J. Ribozyme-mediated Inhibition of *bcr-abl* Gene Expression in a Philadelphia Chromosome-Positive Cell Line. *Blood* **1993**, *82*, 600–605.
(121) Heisterkamp, N.; Stephenson, J. R.; Groffen, J.; Hansen, P. F.; De Klein, A.; Baartram, C. R.; Grosveld, G. Localization of the c-*abl* Oncogene Adjacent to a *trans*location Break Point in Chronic Myelocytic Leukaemia. *Nature* **1983**, *306*, 239.
(122) Bartram, C. R.; De Klein, A.; Hagemeijer, A.; van Agthoven, T.; van Kessel, A. G.; Bootsma, D.; Grosveld, G.; Ferguson-Smith, M. A.; Davies, T.; Stone, M.; Heisterkamp, N.; Stephenson, J. R.; Groffen, J. *Trans*location of c-*abl* Oncogene Correlates with the Presence of a Philadelphia Chromosome in Chronic Myelotic Leukaemia. *Nature* **1983**, *306*, 277.
(123) Wright, L.; Wilson, S. B.; Milliken, S.; Biggs, J.; Kearney, P. Ribozyme-mediated cleavage of the bcr/abl transcript expressed in chronic myeloid leukemia. *Exp. Hematol.* **1993**, *21*, 1712–1718.
(124) Shore, S. K.; Nabissa, P. M.; Reddy, E. P. Ribozyme-mediated Cleavage of the *bcr/abl* Oncogene Transcript: *In Vitro* Cleavage of RNA and *in Vivo* Loss of p^{210} Protein-kinase Activity. *Oncogene* **1993**, *8*, 3183–3188.
(125) Cantor, G. H.; McElwain, T. F.; Birkebak, T. A.; Palmer, G. H. Ribozyme Cleaves *rex/tax* mRNA and Inhibits Bovine Leukemia Virus Expression. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10932–10936.
(126) Woolf, T. M.; Melton, D. A.; Jennings, C. G. B. Specificity of Antisense Oligonucleotides *In Vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7305–7309.
(127) Torrence, P. F.; Maitra, R. K.; Lesiak, K.; Khamnei, S.; Zhou, A.; Silverman, R. H. Targeting RNA for Degradation with a (2′-5′)oligoadenylate-antisense Chimera. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1300–1304.
(128) Maran, A.; Maitra, R. K.; Kumar, A.; Dong, B.; Xiao, W.; Li, G.; Williams, B. R. G.; Torrence, P. F.; Silverman, R. H. Blockage of NF-κB Signaling by Selective Ablation of an mRNA Target by 2–5A Antisense chimeras. *Science* **1994**, *265*, 789–792.
(129) Goodchild, J.; Kohli, V. Ribozymes That Cleave An RNA Sequence from Human Immunodeficiency Virus: the Effect of Flanking Sequence on Rate. *Arch. Biochem. Biophys.* **1991**, *284*, 386–391.
(130) Taylor, N. R.; Rossi, J. J. Ribozyme-mediated Cleavage of an HIV-1 *gag* RNA: The Effects of Nontargeted Sequences and Secondary Structure on Ribozyme Cleavage Activity. *Antisense Res. Devel.* **1991**, *1*, 173–186.
(131) Chowrira, B. M.; Pavco, P. A.; McSwiggen, J. A. *In vitro* and *in vivo* Comparison of Hammerhead, Hairpin and Hepatitis Delta Virus Self-processing Ribozyme Cassettes. *J. Biol. Chem.* **1994**, *269*, 25856–25864.

JM9407872