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Catalytic Nucleic Acids: From Lab to Applications

L. Q. SUN,[1] M. J. CAIRNS, E. G. SARAVOLAC, A. BAKER, W. L. GERLACH

Johnson and Johnson Research Laboratories, Australian Technology Park, Eveleigh, New South Wales, Australia

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***Abstract*——Since the discovery of self-cleavage and ligation activity of the group I intron, the expansion of research interest in catalytic nucleic acids has provided a valuable nonprotein resource for manipulating biomolecules. Although a multitude of reactions can be enhanced by this class of catalyst, including *trans*-splicing activity of the group I intron (which could be applied to gene correction), RNA-cleaving RNA enzymes or "ribozymes" hold center stage because of their tremendous potential for mediating gene inactivation. This application has been driven predominantly by the "hammerhead" and "hairpin" ribozymes as they induce specific RNA cleavage from a very small catalytic domain, allowing delivery either as a transgene expression product or directly as a synthetic oligonucleotide. Although advances in the development of RNA modifications have improved the biological half-life of synthetic ribozymes, their use is restricted by the mechanistic dependence on conserved 2′OH-moieties. Recently a new class of catalytic nucleic acid made entirely of DNA has emerged through in vitro selection. DNA enzymes or deoxyribozyme with extraordinary RNA cleavage activity has already demonstrated their capacity for gene suppression both in vitro and in vivo. These new molecules, although rivaling the activity and stability of synthetic ribozymes, are limited equally by inefficient delivery to the intracellular target RNA. The challenge of in vivo delivery is being addressed with the assessment of a variety of approaches in animal models with the aim of bringing these compounds closer to the clinic.**

[1] Address for correspondence: Dr. Lun-Quan Sun, Johnson and Johnson Research Laboratories, Locked Bag 4555, Strawberry Hills, NSW 2012 Australia. E-mail: lsun2@medau.jnj.com

I. Introduction

Concepts for a genetic type of therapy were developed almost 20 years ago and have recently been transformed (albeit with limited success) into clinical reality. In 1990, the first gene therapy study for the treatment of ADA deficiency began (Blaese et al., 1995), followed by a rapidly growing number of clinical gene therapy trials, across diseases caused by genetic disorders, viral infections, and malignancies (Miller, 1992; Anderson, 1998). Current strategies are aimed at either the replacement of defective genes or suppression of a pathological gene target. At present, there are several well documented genetic approaches being pursued to affect the ablation of defective gene production. Strategies exemplified by the use of antisense RNA or deoxyoligonucleotides (Milligan et al., 1993; Crook and Bennett, 1996), expression of mutant structural or regulatory genes with dominant repressor activity (Woffendin et al., 1996) and over-expression of competitive RNA sequences (Sullenger et al., 1990; Berkhout and van Wamel, 1995) vary widely in their degree of specificity and serve primarily to block gene expression by interfering with RNA transcription or translation. Unique among these approaches is the use of catalytic nucleic acids, RNA (ribozyme) or DNA (deoxyribozyme, DNA enzyme or DNAzyme) oligonucleotides capable of cleaving a target RNA molecule in a highly sequence-specific manner (Rossi, 1992; Sun et al., 1997, 1999; Welch et al., 1998).

Ribozymes, the most extensively studied of the catalytic nucleic acids, exist in a range of distinct categories of naturally occurring catalytic RNA. These include a series of small ribozymes important for the rolling circle replication of viroid genomes, such as hammerhead and hairpin ribozymes (Haseloff and Gerlach, 1988; Hampel and Tritz, 1989; Rossi, 1992), group I introns (Cech et al., 1981, 1990, 1992), the RNA component of RNase P (Guerrier-Takada et al., 1983; Frank and Pace, 1998), and hepatitis delta virus ribozyme (Branch and Robertson, 1991). In addition to naturally occurring ribozymes, the number of entirely synthetic RNA molecules with identified novel catalytic activities have increased dramatically over the past few years as a result of the development of in vitro selection and evolution techniques. DNAzymes, unlike their naturally occurring counterpart, are a recent development in catalytic nucleic acid technology. Their catalytic activity is exclusively derived from in vitro selection procedures in which deoxynucleotides have been "trained" for activities as diverse as cleavage of RNA or DNA (Carmi et al., 1998), DNA oligonucleotide ligation, and phosphorylation of DNA (Cuenoud and Szostak, 1995; Li and Breaker, 1999a). A new class of DNA enzyme the "10-23" DNAzyme was selected from a combinatorial library of DNA sequences for its ability to cleave a short HIV target RNA (Santoro and Joyce, 1998).

The catalytic activity and specificity of both ribozymes and DNAzymes has been extensively characterized in vitro and in cell culture systems. A wide range of chemical modifications has allowed the synthesis of oligonucleotides with in vivo stability approaching most conventional drugs. Efficient delivery and distribution of oligonucleotide compounds both intracellularly and in vivo remains, however, a critical challenge for a successful transition from the laboratory to the clinic: a limitation common to virtually all aspects of nucleic acid-based therapy. Our discussion will cover a range of issues from structure and catalytic mechanism to delivery and pharmaceutical development. Given the broad scope of this field, we have chosen a limited number of recent references focusing primarily on the investigation of catalytic nucleic acids with potential clinical application.

II. Ribozymes

Ribozymes are catalytic RNA molecules possessing, at the very least, enzymatic cleavage and ligation activities (Haseloff and Gerlach, 1988; Hampel and Tritz, 1989; Rossi, 1992). In nature, enzymatic RNA molecules catalyze sequence-specific RNA processing. The specificity is determined by Watson-Crick base-paring between ribozymes and nucleotides near the cleavage site of the target RNA. By altering substrate recognition sequences, several intramolecular *cis*-cleaving ribozymes have been engineered to cleave target RNA in trans (Symons, 1994). Theoretically, these *trans*-cleaving ribozymes can be designed to cleave any RNA species in a sequence-specific manner. Thus, the mRNA coding of any proteins associated with a disease can be selectively cleaved by ribozymes. Consequently, ribozymes have become potentially valuable tools for the inhibition of virus replication, modulation of tumor progression, and analysis of cellular gene function.

A. Catalytic Motifs

At present, there are five major RNA catalytic motifs that are derived from naturally occurring ribozymes: hairpin, hammerhead, group I intron, ribonuclease P and hepatitis delta virus ribozyme. Among those, two RNA catalytic motifs that originate from plant viriod and virusoids have received much attention for their potential use, due to their inherent simplicity, relatively small size, and the ability to be incorporated into a variety of flanking sequence motifs without changing site-specific cleavage capacities. These are hammerhead and hairpin ribozymes, illustrated in Fig. 1. The hammerhead ribozyme model is based on the satellite RNA strand (+) of tobacco ringspot virus (sTobRV[2]). It has

[2] Abbreviations: sTobRV, tobacco ringspot virus; nt, nucleotide(s); PCR, polymerase chain reaction; bp, base pair(s); SMC, smooth muscle cell; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; HDV, hepatitis delta virus; IGS, internal guide sequence; EGS, external guide sequence; TK, thymidine ki-

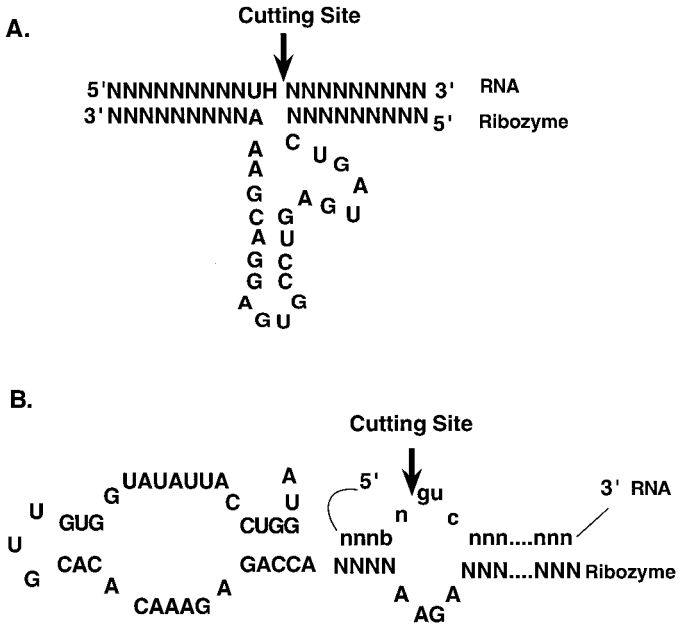

FIG. 1. Structures of hammerhead (A) and hairpin ribozymes (B). Refer to text for details.

three basic components: (i) a highly conserved 22 nucleotide-catalytic domain; (ii) base-pairing sequence flanking the susceptible 3′,5′-phosphodiester bond; and (iii) a recognition sequence on the target RNA such as GUC. The cleavage reaction occurs 3′ to the recognition sequence with formation of a terminus containing a 2′,3′-cyclic phosphodiester and a 5′-hydroxyl terminus on the 3′-fragment (Symons, 1994). The (−) strand of the sTobSV forms a two-dimensional hairpin structure, with four major helical regions possessing catalytic activity. On the basis of this structure, the hairpin ribozyme model consists of four helices and five loop regions formed between a 50-base catalytic RNA and a 14-base substrate RNA. Helices 3 and 4 are within the ribozyme itself, and the ribozyme binds to the target RNA through helix 1 (six base pairs) and helix 2 (four base pairs), separated by a NGUC loop in the substrate strand. The recognition sequence is bNGUC, where b is G, C or U; N is any nucleotide, and cleavage occurs 5′ to the G residue (Hampel and Tritz, 1989). Other catalytic motifs, group I introns that are derived from the self-splicing intervening sequence of *Tetrahymena thermophila*, RNase P, and catalytic domain of human hepatitis delta virus will be discussed in detail in *Section IV*.

B. Kinetics of Ribozymes

A large number of studies have been performed to provide a better understanding of the mechanism of ribozyme-mediated catalysis. In general, a minimal kinetic description for one turnover of hammerhead ribozyme reaction (Fig. 2) involves assembly of ribozyme (E) and substrate (S) into an E · S complex, cleavage of the phosphodiester bond, generating a 5′-product with a 2′,3′-cyclic phosphate terminus (P1) and a product with a 5′-hydroxyl terminus (P2) that remains bound to the ribozyme, and release of the products. A Michaelis-Menten mechanism has been established by using multiple- and single-turnover conditions for the formation of the ribozyme-substrate complex and its subsequent conversion to products (Fedor and Uhlenbeck, 1990; Perreault et al., 1990).

Under multiple-turnover conditions, the substrate is in excess of the ribozyme so that the ribozyme can catalyze the cleavage of several substrate molecules. The catalytic rate constant or turnover numbers, k_{cat}, is a measure of the rate-limiting step, which can be cleavage, conformational transitions of the ribozyme-substrate complex, or product release. When a ribozyme with six bases in each arm was used in kinetic analysis, k_{cat} and K_M were within typical values of 1–2 min^{-1} and 20–200 nM, respectively. Extension of the helical arms generally results in an increased stability and markedly decreased k_{cat} (Hertel et al., 1994). Under single-turnover conditions, the ribozyme is in excess of the substrate, and such conditions are normally used for cleavage of long mRNA substrate. Cleavage rates are generally several orders of magnitude lower, compared with the rates obtained for short substrate (Heidenreich et al., 1994). In the case of hammerhead and hairpin ribozymes, the presence of Mg^{2+} is essential for the cleavage, and the convincing evidence suggested that Mg^{2+} not only assists in RNA folding but also participates directly in the cleavage mechanism (Dahm and Uhlenbeck, 1991).

C. Optimizing Design

Although mutational, chemical, and physical investigations have advanced our understanding of how ribozymes act in vitro, for a specific target RNA the optimal design of ribozymes for both in vitro and in vivo use still has to be experimentally determined. Here using the hammerhead ribozyme model as an example, some of the basic rules in ribozyme design will be discussed.

Hammerhead ribozymes such as those illustrated in Fig. 1, can cleave any 5′-NUH-3′ triplets of an RNA, where U is conserved, N is any nucleotide and H can be C, U, A, but not G. This cleavage rule has been further extended from the work of several groups (Perriman et al., 1992; Shimayama et al., 1995; Zoumadakis and Tabler, 1995; Ludwig et al., 1998). Comparative studies revealed that the reaction rate (k_{cat}) decreases in the following order: AUC, GUC>GUA, AUA, CUC>AUU, UUC, UUA>GUU, CUA>UUU, CUU. Results from recent studies extend the repertoire of cleavage sites for hammerhead ribozyme application to inhibition of gene

nase; PS, phosphorothioate; PO, phosphodiester; [aPTT], partial thromboplastin time; SALP, stabilized antisense-lipid particles; ODN, oligodeoxynucleotide; HPV, human papilloma virus; DOPE, dioleoylphosphatidylethanolamine; DC-CHOl, 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol; CpG, deoxycytosine-phosphate-deoxyguanosine.

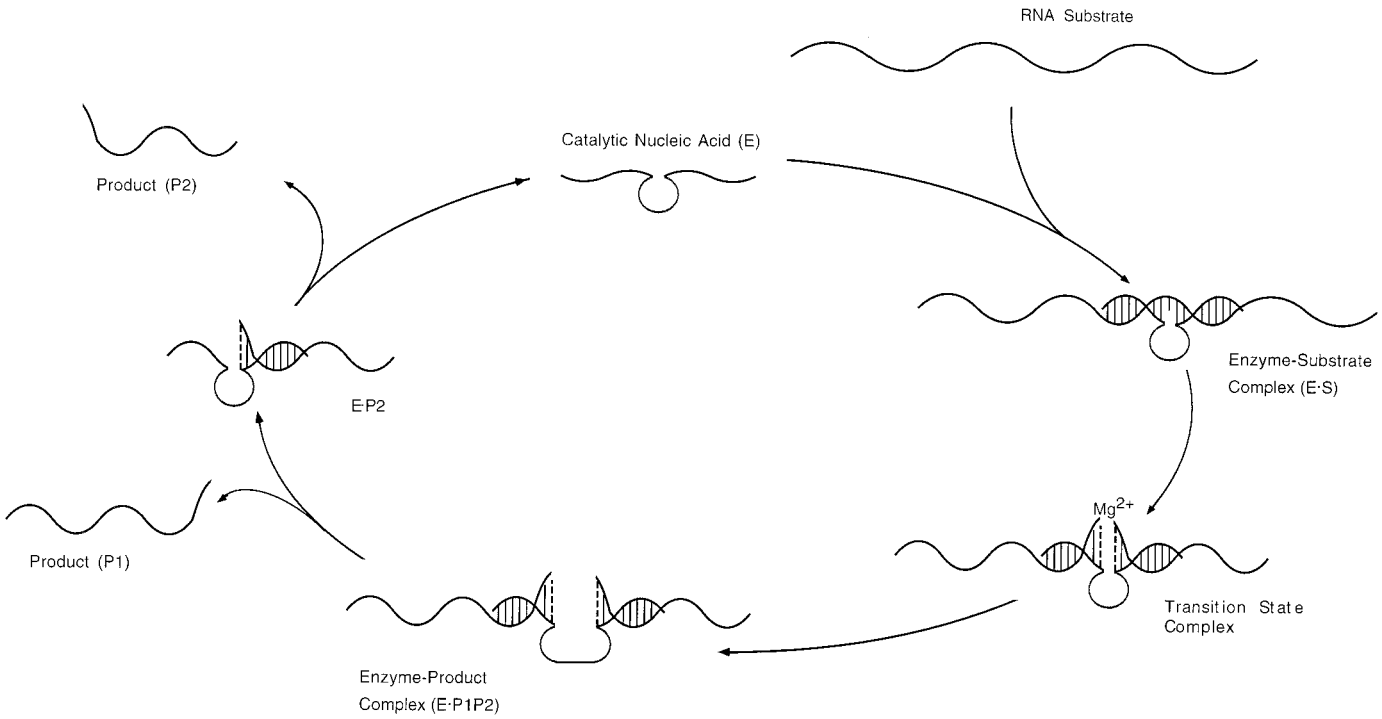

FIG. 2. The catalytic cycle of an RNA-cleaving nucleic acid enzyme. This diagram is a proposed schematic representation of the catalytic cycle typical for small RNA-cleaving nucleic acid enzymes such as the hammerhead ribozyme and the 10-23 deoxyribozyme, which bind to their substrate via Watson-Crick interactions. The description is based on the data from kinetic analysis and X-ray crystal structures (Kumar et al., 1996; Scott et al., 1996; Murray et al., 1998). These reactions are initiated when the binding domains or pairing arms of the enzyme specifically engage the single-stranded RNA substrate by forming two double-helical duplexes (enzyme-substrate complex). Between the binding domains, the catalytic domain forms a catalytically active secondary structure that is capable of positioning an attacking divalent cation (such as Mg^{2+}) in proximity to the cleavage site. In this configuration the reaction between metal ions and RNA is enhanced by many orders of magnitude through stabilization of the transition state (transition state complex). After cleavage of the phosphordiester linkage the enzyme and two cleavage products remain associated (enzyme-product complex) before becoming dissociated (product release). The catalytic nucleic acid is then free to bind more substrate in another cycle of the reaction.

expression. Kore et al. (1998) report that the NUH rule can be modified to the NHH rule, where H is any nucleotide except G. However, whether this extension can be applied to in vivo applications is yet to be tested in a suitable model. This can be further complicated when the target RNA structure in cellular environment is taken into consideration.

In addition to the NHH triplets for cleavage specificity, the ribozyme arm sequence context can also influence cleavage rate significantly. In a simple term, the longer the binding arms, the lower the turnover in cleavage of short substrates. The length of the 3′-arm is apparently more critical for specificity than that of the 5′-arm (Hertel et al., 1996). For a long substrate RNA, intramolecular structures of the target RNA could interfere with the binding of a ribozyme, and for in vivo application, cellular proteins could also affect ribozyme activity. Results from variations in arm length of ribozymes from 5 to 18 nucleotides (nt) have indicated that the ribozyme activity is closely related to the arm length both in symmetric or asymmetric models, and this depended somewhat on the sequence context (Heidenreich and Eckstein, 1992; Scherr et al., 1997; Sioud et al., 1997; Crisell et al., 1993).

D. Target Selection

Given that the biophysical principles governing RNA folding in vivo have not been clearly determined, there is no clear means available to determine the accessibility of a potential RNA substrate cleavage site. As a first approximation the gross topography of the substrate RNA can be simulated by analyzing the region surrounding the cleavage site using an RNA secondary structure folding program. In this way, it may be possible to determine whether or not the target site is buried within an obvious thermodynamically stable region of secondary structure. However, there are limitations to the size of RNA that may be analyzed by this method, and this is clearly a gross approximation of RNA secondary structure that does not take into account tertiary structure nor RNA-protein interaction, which may be present in the intracellular context. Other tools for empirical testing of cleavage site accessibility, such as S1 nuclease or RNase mapping, can supply additional information (Pavlakis et al., 1980; Knapp, 1989). As an alternative, an assessment of biological significance may allow one to predict certain features of the RNA that would indicate accessibility. For example, a number of regions in the HIV-1 genome have been targeted by

different groups using ribozymes, including the 5′-leader region (Weerasinghe et al., 1991; Ojwang et al., 1992), *gag* (Sarver et al., 1990) and *tat* genes (Lo et al., 1992; Sun et al., 1995; Wang et al., 1998), and the ψ packaging site (Sun et al., 1994). These target sites are generally exposed for potential RNA-RNA or RNA-protein interactions as a result of their biological function, and thus should be potentially accessible in cells.

In addition to enzymatic mapping of accessible sites in an RNA, combinatorial approaches may be used to identify ribozyme accessible sites, in which no assumptions regarding the best target sites and corresponding ribozymes are made. This approach may provide a cost-effective mapping technique and could speed up the process of drug discovery. Lieber and Strauss (1995) first reported the use of combinatorial approach for the selection of ribozyme cleavage sites. A library of ribozyme genes with random sequences of 13 nucleotides on both sides of the hammerhead was generated. Using RACE (rapid amplification of cDNA ends) technique, they successfully identified cleavage sites, and subsequently re-amplified and cloned the ribozyme genes. In our laboratory, a similar design was used to construct a synthetic ribozyme library, and sensitivity of detection of cleavage sites was increased significantly by using ligation-mediated polymerase chain reaction (PCR) (Y. Kim and L. Q. Sun, unpublished results). More recently, a simpler approach that requires neither PCR nor cloning was also recently reported by Yu et al. (1998), in which a population of in vitro transcribed hairpin ribozymes with randomized substrate binding arms, was incubated with the substrate RNA, and cleavage positions were determined by primer extension.

Ribozyme-mediated suppression of gene expression has proven to be neither straightforward nor routine partly due to the difficulty in defining the most accessible sites. In vitro screening provides the simplest and most direct approach to identifying the accessible sites although there are some concerns in terms of correlation between in vitro and in vivo results. However, a combined approach of cell-based screening systems for target site selection and in vitro screening using the full-length mRNA will provide valuable information for the design of gene-targeted ribozymes.

E. In Vivo Activities and Clinical Applications

The gradual maturation of ribozyme technology from the bench to clinical application involves several major challenges, many of which still need to be resolved. These include extra- and intracellular stability of the ribozyme, delivery of ribozymes to target cells, target accessibility, colocalization of ribozyme and target within cells, and optimal catalytic activity and specificity of the ribozyme. Despite their enzymatic activity, ribozymes must be delivered to target cells in amounts sufficient to both affect a significant proportion of the cell population and to affect a significant proportion of the target mRNA. Although therapeutic application of ribozymes may be achieved by exogenous delivery of chemically produced ribozyme complexed, for example, to a cationic lipid (*Section V*), for certain applications, such as their use in chronic disease or in an antiviral therapeutic settings, they may require permanent availability to be of therapeutic benefit. This can be achieved by transfer of genes encoding for ribozymes using viral vector systems (for review see Sun and Symonds, 1998).

The preclinical and clinical application of ribozyme-based gene therapy has been primarily focused on AIDS, cancers, and other viral infections. This is summarized in Table 1. Here, as an example, we will discuss some of the aspects of ribozyme-based gene therapy for AIDS.

A simple approach to treating HIV-infected patients is the infusion of transduced and "protected" $CD4^+$ peripheral blood lymphocytes. If ribozyme the construct can protect $CD4^+$ T cells from HIV-1 infection and its sequelae in patients, then the decline in the numbers of $CD4^+$ T cells could be halted or even to some extent reversed, and HIV-infected individuals could benefit clinically. It is relevant to note that the half-life of an HIV-infected $CD4^+$ T lymphocyte is of the order of only 2 days. We have initiated two independent Phase I clinical trials to test safety of this approach, the ability to detect ribozyme-containing cells in the bloodstream and the hypothesis that ribozymes can protect $CD4^+$ T lymphocytes from rapid HIV-1-mediated destruction within an infected individual. Both trials use LNL6 vector (a Moloney murine leukemia virus-based expression vector) and recombinant LNL6 containing Rz2 (RRz2), and each trial uses a separate target cell population—$CD4^+$ peripheral blood lymphocytes and $CD34^+$ stem. The first trial involves identical twins, discordant for infection with HIV (Cooper et al., 1999). Healthy $CD4^+$ lymphocytes from the uninfected twin are transduced with a retroviral vector containing the ribozyme gene. These transduced cells are cultured and expanded ex vivo before transfusion into the bloodstream of the corresponding HIV-positive twin. The second clinical trial involves the removal, transduction, and transfusion of $CD34^+$ stem cells within HIV-positive individuals (Rosenblatt

TABLE 1
Preclinical and clinical development of ribozymes

Company	Disease Target	Status
Alza	CNS disease	Preclinical
American Cyanamid	Anti-ras ribozymes	Preclinical
Columbia University	Anti-bcl-2 ribozymes	Preclinical
Gene Shears	Anti-HIV-1 ribozymes	Phase I/II
Immusol	Anti-HIV ribozymes	Phase I/II
	HBV, HCV, restenosis	Preclinical
Innovir	HBV, HCV, CML	Preclinical
Osaka University	Anti-HCV ribozymes	Preclinical
Ribozyme Pharmaceuticals Inc.	HIV-1 infection	Phase I/II
	Angiogenesis	Preclinical
Tokyo University	HCV ribozyme	Preclinical
University of Pittsburgh	Glioma	Preclinical
City of Hope	HIV-1 infection	Phase I/II

CNS, central nervous system; HBV, hepatitis B virus; HCV, hepatitis C virus.

et al., 1999). The rationale being that these transduced stem cells will differentiate and give rise to a variety of lineages that express the ribozyme.

In each trial, separate populations of cells are transduced with the retrovirus vector containing the ribozyme and the vector alone as a control, and equal numbers of these two transduced cell types are then introduced into the recipient patients. This has allowed us to monitor the survival of ribozyme-expressing $CD4^+$ lymphocytes relative to a similar population of transduced $CD4^+$ lymphocytes that have been processed in an identical fashion but do not contain the ribozyme sequence. Cell survival is being monitored by detecting ribozyme and control vector DNA sequences in peripheral blood using quantitative PCR procedures. Preliminary data indicate that transduction of the ribozyme construct into human cells is safe, and the transduced cells can be detected in all the patients.

In summary, a large body of work on ribozyme catalytic efficiency, mode of action, and basic chemistry have accelerated the possibility of using ribozyme human gene therapy to target specific human diseases. With the development of delivery systems well under way, and with much deeper understanding of molecular genetics of human disease, it is hoped that ribozymes will soon emerge as gene-targeted molecular therapies.

III. RNA-Cleaving Deoxyribozymes

A. Therapeutic Oligonucleotides

The therapeutic potential of RNA enzymes or ribozymes with RNA-cleaving activity is now well established (Bramlage et al., 1998). The gene suppression activity of these nucleic acid-based agents is probably mediated by a combination of antisense RNA interactions and catalytic destruction of the mRNA target. Although it's possible to manufacture biologically active RNA, its relative fragility in this environment makes it difficult to administer in a direct delivery mode. For this and other reasons, most ribozyme applications rely on transgenic production of RNA in vivo within the context of a gene therapy. Advances in nucleic acid chemistry, however, have led to progress in ribozyme synthesis, which allow the incorporation of modified ribonucleotide analogues with enhanced nuclease resistance (Scaringe et al., 1990; Wincott et al., 1995). This usually involves substitution of the 2′-hydroxyl moiety with some other chemistry such as 2′-deoxy, 2′-*O*-methyl, 2′-amino, or 2′-fluoro derivatives. These 2′-modifications, however, reduce or eliminate catalytic activity when made at conserved positions and thus cannot be applied to the entire ribozyme (Perreault et al., 1990; Pieken et al., 1991; Williams et al., 1992; Yang et al., 1992). The most effective synthetic ribozymes have a chimeric composition that enhances nuclease stability while maintaining cleavage activity (Paolella et al., 1992; Beigelman et al., 1995). Substitution of DNA (and other 2′-modified ribonucleotides) into the helix-forming motifs and specific unpaired positions of the catalytic domain has been shown to produce a substantial improvement in the biological stability; however, these chimeric ribozymes are still relatively vulnerable to endoribonucleases. By comparison, antisense DNA oligonucleotides (ODNs) with their uninterrupted DNA composition have a much greater half-life in vivo. The natural biological stability of DNA compared with RNA can also be readily supplemented by chemistry, which provides even greater resistance to nuclease digestion; the more commonly used modification involves the replacement of phosphodiester linkages in the backbone with phosphorothioate or methylphosphonate moieties. However, despite being more suitable for direct delivery, antisense ODNs are devoid of endogenous RNA cleavage activity and thus can only act as passive inhibitors of translation machinery through their sequence-specific binding activity, or mediate destruction of the RNA target component of an RNA-ODN heteroduplex in the nucleus of cells by activating ribonuclease H.

B. RNA-Cleaving Catalytic DNA

Perhaps an ideal oligonucleotide-based RNA-directed gene inactivation agent is one that could combine the self-sufficient RNA destructive capability of ribozymes, such as the "hammerhead" and the "hairpin", with the biological resilience of the antisense ODN. Although DNA molecules with RNA cleavage activity have not been observed in nature, some are now in existence thanks to an accelerated evolutionary process (in vitro selection) designed specifically to derive DNA sequences with this activity (Breaker and Joyce, 1994, 1995; Santoro and Joyce, 1997). In natural systems the replicability and relative stability of double helical DNA makes it well suited to its role as the custodian of genetic information. In this form DNA secondary and tertiary structure is severely restricted and provides very little opportunity for the exploration of conformations, which might facilitate useful reaction rate enhancement. Despite the dominance of protein catalysts (and a small role for RNA catalysts) in this environment, DNA, which is liberated from its complimentary strand, is also capable of substantial structural diversity and even catalytic activity (Breaker, 1997; Li and Breaker, 1999b).

C. In Vitro Selection

In vitro selection is achieved by combining molecular diversity with the ability to preferentially amplify active molecules. Nucleic acid sequence diversity can be provided in advance by degenerate oligonucleotide synthesis, whereas amplification of the construct is facilitated by PCR. Selection requires some means of partitioning molecules with the desired activity from those in the vast majority, which lack it. In the case of ribozyme and deoxyribozyme selection, active molecules can be separated from their inactive counterparts by the difference

in their respective molecular weights (after self-cleavage reaction) using gel electrophoresis. Alternatively, prospective catalysts are attached to a solid support via their substrate sequence, from which only molecules with self-cleavage activity are released.

The possibility of an RNA-cleaving catalytic DNA was initially explored in selection constructs containing a single embedded ribonucleotide substrate component. Downstream from the substrate, a section of randomized DNA sequence (40–50 bp) provided molecular diversity, whereas fixed sequences on each flank provided stable sites for primer binding. A sample population from these combinatorial libraries (complete library = 10^{13}-10^{14} variants) was immobilized on a solid support containing streptavidin via a biotin tag (Fig. 3). After washing and stripping the complementary template strand in alkali, a buffer containing an appropriate divalent metal ion (Pb^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}) was introduced to generate conditions conducive to a *cis*-cleavage reaction (Breaker and Joyce, 1994, 1995). The release of active members in the library by this self-cleavage allowed them to be selectively amplified and regenerated by PCR for subsequent rounds of selection. After numerous cycles of selection and amplification, the self-cleavage activity of the remaining population was examined by electrophoresis of an end-labeled fraction that was allowed to fold and react in solution. When it was established that the selected pool contained a satisfactory level of self-cleavage activity, the PCR products were cloned and sequenced. The cloned sequences were then used to examine the cleavage activity of individual molecules and predict secondary structures. The secondary structure predictions for the intramolecular configuration of enzyme-substrate complex was then used to separate these two components (of the *cis* arrangement), such that the enzyme and substrate would interact in *trans*. The *trans* cleavage capacity of these DNA enzymes or deoxyribozymes was then examined using a separate oligonucleotide enzyme and substrates. Using this selection protocol in the first instance with Pb^{2+} and then with other buffer systems including Mn^{2+}, Mg^{2+}, and Zn^{2+}, different deoxyribozymes were found that could use each of these divalent metal ions to cleave a single embedded ribonucleotide (Breaker and Joyce, 1994, 1995). In the case of the magnesium-dependent deoxyribozyme, this was accomplished with a rate up to 10^5 times that of the uncatalyzed reaction.

After further development of the in vitro selection protocol, Mg^{2+}-dependent DNA enzymes capable of cleaving a biologically relevant, all RNA substrate, were evolved (Santoro and Joyce, 1997). From this selection, experiment two prototypes were characterized and denoted the "8-17" and the "10-23" RNA-cleaving DNA enzymes (Fig. 4). Both of these molecules are reminiscent of the hammerhead ribozyme in that they contain conserved catalytic domains flanked by variable binding domains. Each binding domain was originally evolved to specifically complement the substrate sequence used in the selection protocol; however, as this was achieved in both of these deoxyribozymes through Watson-Crick base pairing, the possibility existed that the specificity could be altered to suit any target RNA sequence. This was particularly successful in the 10-23 deoxyribozyme, which was found to have a minimum sequence requirement of a purine-pyrimidine pair at the site of cleavage. Despite being less flexible, the 8-17 deoxyribozyme with its requirement for an AG in the substrate, can also be described as a general-purpose endoribonuclease.

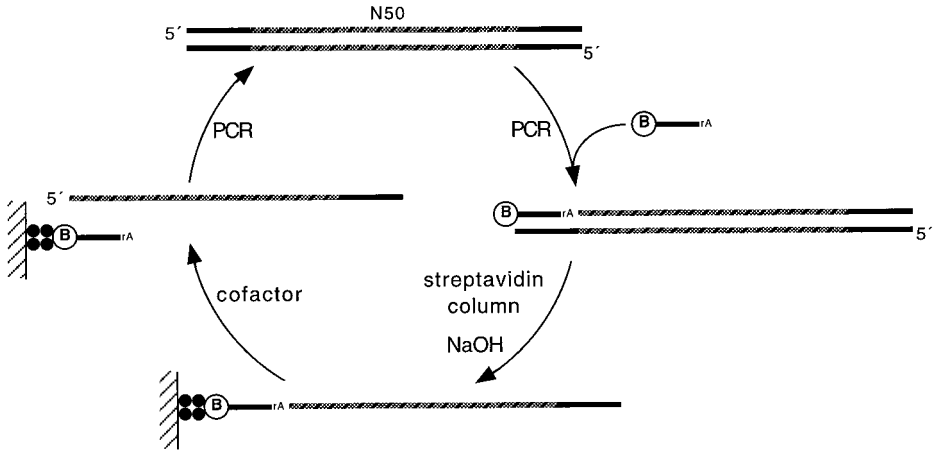

FIG. 3. An in vitro selection scheme for RNA-cleaving catalytic DNA. Adapted from Breaker and Joyce (1994). In this scheme a combinatorial library of different DNA sequences is generated by the synthesis of an oligonucleotide template with a 50 base (N50) stretch of random sequence (hatched lines). To enable amplification and reselection, each molecule from this diverse pool contains a fixed sequence at each end for use as primer binding sites (black). Immobilization is achieved by tethering this assembly of potentially functional sequences through a biotinylated (B) RNA containing substrate (rA) to a solid streptavidin-coated support. After alkali denaturation (to strip away the template strand) the single-stranded residue is allowed to fold around its substrate linkage in the presence of suitable buffer and cofactors. The eluent of this washing (which contains catalytically active molecules) is then used as a PCR template for regenerating the assembly for a second round of selection while the inactive sequences remain tethered to the support. After multiple rounds of selection the relative abundance of each active molecule should be high enough to visualize the activity in a cleavage assay and clone the DNA for sequencing.

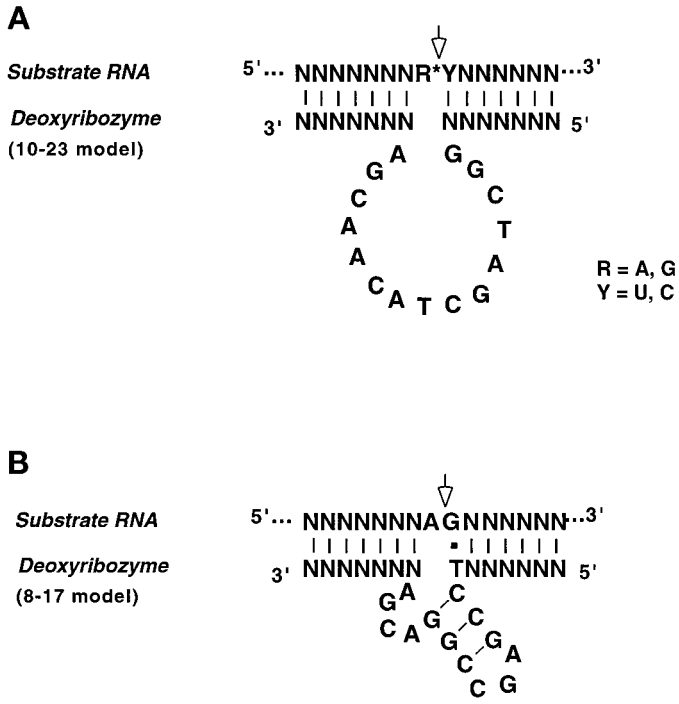

FIG. 4. A schematic representation of the "10-23" and "8-17" general purpose RNA-cleaving deoxyribozymes. Panels A and B contain illustrations of the 10-23 and 8-17 deoxyribozymes, respectively. Watson-Crick interactions for each deoxyribozyme-substrate complex are represented by generic ribonucleotides (N) in the target (top) and the corresponding deoxyribonucleotides (N) in the arms of the deoxyribozyme (bottom) of each. The defined sequences in the loop joining the arms and spanning a single unpaired purine at the RNA target site of each model represent the conserved catalytic motif.

D. The "10-23" RNA-Cleaving DNA Enzyme

The 10-23 DNA enzyme or deoxyribozyme was named from its origin as the 23rd clone of the 10th cycle of in vitro selection (Santoro and Joyce, 1997). This enzyme has a number of features that endow it with tremendous potential for applications both in vitro and in vivo. These include its ability to cleave almost any RNA sequence with high specificity provided it contains a purine-pyrimidine dinucleotide. This can be accomplished at very high kinetic efficiency with rates approaching and even exceeding those of other nucleic acid and protein endoribonucleases (Santoro and Joyce, 1997). This remarkable activity is all the more spectacular when considering that it is achievable at concentrations of magnesium down in the physiological range. After characterization of the 10-23 catalytic motif, more rounds of reselection and amplification were undertaken with a partially randomized sequence (25% degeneracy) with the aim of further optimizing the catalytic activity (Santoro and Joyce, 1997). Surprisingly, this selection did not yield any refinement of 10-23 deoxyribozyme, indicating that the catalytic motif was highly conserved and had very little sequence redundancy.

1. Kinetic Efficiency. The ability of the 10-23 deoxyribozyme to cleave purine-pyrimidine junctions meant that the AUG start codon of any gene could be used as a target. Early kinetic analysis of the 10-23 deoxyribozyme focused on synthetic substrate sequences derived from the start codons of various HIV genes (Santoro and Joyce, 1997). A key point that emerged from this analysis was that the kinetic efficiency of deoxyribozyme-catalyzed cleavage varied substantially from one substrate sequence to the next. This sequence-dependent variability seemed to be closely associated with the thermodynamic stability of the enzyme-substrate heteroduplex as predicted by the hybridization free energy (Sugimoto et al., 1995). In this relationship, DNA enzymes with the greatest heteroduplex stability indicated by a low free energy of hybridization (calculated using the nearest neighbor method), was often found to have the greatest kinetic activity. The sensitivity to heteroduplex stability in most instances can be counterbalanced to some extent by increasing the arm length until the hybridization free energy decreases to a threshold level. At this point the heteroduplex stability is optimal for catalysis, and the enzyme activity can approach its maximum efficiency. Factors other than length that tend to increase the heteroduplex stability include the general GC content and specific pyrimidine's content of the DNA component (Ratmeyer et al., 1994; Sugimoto et al., 1995; Gyi et al., 1998). The influence of heteroduplex stability on the kinetic efficiency of the deoxyribozyme is probably derived from its effect on the K_M of the reaction. The inverse relationship between K_M and enzyme-substrate complex stability can be observed by increasing the substrate binding domain length such that the heteroduplex stability is increased, which usually causes the K_M to fall toward its minimum. The benefit to the overall kinetic efficiency obtained by increasing binding domain length, however, is limited by the adverse effect it has on catalytic turnover (indicated by the k_{cat}), which occurs when the enzymes increased affinity for the products slows down the catalytic cycle by reducing the rate of product release. In a more recent investigation of this behavior in reactions where the substrate binding domain length ranged between 4/4 and 13/13, the maximum overall efficiency (k_{cat}/K_M) under physiological reaction conditions was found with an arm length of between 8 and 9 bp (Santoro and Joyce, 1998).

The majority of kinetic analyses of the 10-23 deoxyribozyme has been performed on molecules with symmetric RNA binding domains, irrespective of each arms contribution to the total thermodynamic stability of the enzyme-substrate complex. There is some evidence to suggest that the optimal design of a 10-23 deoxyribozyme for a given target site should take into account both the sequence and length of the individual binding arm to accommodate the differences in stability of their respective complexes with the substrate. Experiments with different length binding domains have shown that in some instances the rate of deoxyribozyme-catalyzed cleavage can be enhanced by asymmetric arm length

truncation. In the case of deoxyribozymes targeting the c-*myc* translation initiation region, we found that the observed rate was highest in the molecule with a binding arm length ratio (5′/3′-bp) of 6/10 (Cairns et al., 2000a). This relative advantage derived by truncation of the binding domain on the 5′-side of the catalytic domain was similar to that seen in the hammerhead ribozyme, which had an optimal length ratio (helix I/helix III) of 5/10 (Hendry and McCall, 1996). The benefit of a shorter helix I in the ribozyme was attributed to a possible decrease in counter productive interference between it and helix II. In the case of the DNA enzyme, which lacks the equivalent of a helix II, this effect is more likely related to the respective heteroduplex stability of each individual binding domain-substrate complex. In support of this argument, the hybridization free energy for the truncated 5′-arm of the c-*myc*-cleaving deoxyribozyme was found to be unusually low indicating that it was capable of generating more stability than expected for its size compared with the average duplex sequence. However, although this explains the fact that there was no reduction in rate compared with the longer symmetric counterpart, it does not really explain why there was an apparent rate enhancement in the shorter molecule. One possibility is that when this RNA substrate is bound with exceptionally high stability by the full-length deoxyribozyme, the reaction rate is inhibited by the rigidity of the enzyme-substrate complex. Another possibility is that the full-length deoxyribozyme is capable of forming an alternative secondary structure that is not catalytic.

In contrast to the observations in this system, truncation of the same arm from a deoxyribozyme targeting a different substrate derived from the HPV16 E6 translation initiation region, resulted in a decrease in reaction rate. In this case, the predicted hybridization free energy of the 5′-arm was substantially higher, which may have made it more sensitive to shortening than the previous example.

2. Sequence Specificity. With the potential to bind any RNA sequence and cleave purine-pyrimidine junctions, the 10-23 DNA enzyme has unprecedented target site flexibility. However, despite the enormous capacity to cleave different sequences, the actual substrate specificity of an individual deoxyribozyme with defined RNA binding domains, appears to be very high. This ability to discriminate is particularly important in biological applications where unwanted side reactions between the deoxyribozyme and some closely related or unrelated substrate could be very undesirable. The issue of specificity was central in a study by Taira and coworkers (Kuwabara et al., 1997) comparing the in vitro cleavage activity of deoxyribozymes and hammerhead ribozymes targeting RNA derived from the junction of the *bcr-abl* fusion. In this system, deoxyribozymes had the advantage for two reasons: one, they were able to get closer to the junction because of their superior target flexibility; and two, their activity was more easily perturbed by mismatch and hence were less reactive with the RNA sequence from the wild type *abl* gene. The difference between the ribozyme and deoxyribozyme in this study was attributed to the lower stability of the DNA-RNA heteroduplex compared with the RNA-RNA homoduplex (Ota et al., 1998). Indeed when the heteroduplex stability was increased by lengthening the RNA binding domains, the specificity of the *bcr-abl* (b2a2)-cleaving deoxyribozymes also decreased slightly (Warashina et al., 1999). To achieve the desired specificity in the *bcr-abl* (b3a2) target, Wu et al. (1999) used deoxyribozyme arm length asymmetry (6/12 bp), such that the short arm is less likely to allow cleavage of *bcr* transcripts. However, the key point is not so much the difference in duplex stability, but the sensitivity to this difference, which the deoxyribozyme displays because it maintains relatively high activity at comparatively low heteroduplex stability. In our experience the kinetic efficiency of any given 10-23 deoxyribozyme seems to approach a maximum when the heteroduplex stability is just greater than a minimum threshold level. When the stability falls below this threshold by the introduction of a binding domain mismatch or truncation, the catalytic activity of the molecule becomes severely impaired. The influence of single base mismatch was demonstrated empirically at different positions of the substrate by the introduction of point mutations (Santoro and Joyce, 1998). In this analysis, any mismatch with the substrate was detrimental to the catalytic efficiency, although the extent of this effect varied substantially from one position to the next and between different types of mispairing. We have also examined the specificity of the 10-23 deoxyribozyme by observing its ability to discriminate between sequences that differ by as little as a single nucleotide polymorphism (Cairns et al., 2000b). In this experiment, reactions between deoxyribozyme and matching substrate sequences (derived from a polymorphic site in the L1 gene of six different clinically relevant HPV types) were compared with reaction in the unmatched substrates. In each case only the perfectly matched type-specific deoxyribozymes were capable of achieving substantial cleavage of the corresponding substrate despite the similarity between the different sequences. In each of these studies the specificity of cleavage was examined with respect to binding domain-substrate interactions where some mismatches, particularly those producing "wobble" pair, can be tolerated (Santoro and Joyce, 1998; M. J. Cairns et al., 2000a). If however, the difference between the target and nontarget substrate lies at the cleavage site, such that the purine-pyrimidine (R-Y) becomes R-R, Y-Y, or Y-R, then the deoxyribozyme would have no activity on the nontarget substrate.

3. Biological Activity. The ability of the 10-23 deoxyribozyme to specifically cleave RNA with high efficiency under simulated physiological conditions has fueled expectation that this agent may have useful

biological application in a gene inactivation strategy. To explore this potential a number of groups (including our own) have attempted to examine the activity of deoxyribozymes in biological systems (summarized in Table 2). In our laboratory, we initiated experiments with deoxyribozymes targeting the viral sequences from the HPV16 E6 and E7 genes; and in smooth muscle cells (SMCs) with deoxyribozymes targeting the c-*myc* gene (Cairns et al., 1999; Sun et al., 1999). The biological activity of deoxyribozymes that cleave RNA derived from the *bcr-abl* fusion, *Egr-1*, *huntingtin*, the *env* gene of HIV and CCR5 chemokine receptor have also been examined (Dash et al., 1998; Goila and Banerjea, 1998; Santiago et al., 1999; Warashina et al., 1999; Wu et al., 1999; Zhang et al., 1999).

Deoxyribozymes targeting the translation initiation regions of c-*myc* and the HPV16 E6 were optimized in terms of RNA binding domain length through kinetic analysis of in vitro RNA cleavage reactions. To improve the stability of the deoxyribozyme oligonucleotide to serum and intracellular nucleases while maintaining catalytic activity, a single 3′-terminal nucleotide inversion modification was introduced to the oligonucleotides during synthesis by the formation of a 3′-3′-internucleotide linkage (Fig. 5A). After ensuring that the catalytic activity was preserved, the serum nuclease stability of the modified oligonucleotides was examined by incubation in 100% human serum. In this environment, oligonucleotides modified by 3′-inversion were found to have a half-life of 24 h at 37°C compared with 2 h with the unmodified counterpart (Fig. 5B). The modified anti-E6 deoxyribozymes were either cotransfected with an E6 gene expression vector into 3T3 cells with aid of the DOTAP transfection reagent. In the transient E6 expression system the anti-E6 deoxyribozyme was found to substantially reduce the level of E6 mRNA compared with a nonactive oligonucleotide with the same nucleotide composition (unpublished observation).

More compelling evidence of intracellular activity was found when proliferating rat smooth muscle cells were treated with anti-c-*myc* deoxyribozymes (Sun et al., 1999). A range of molecules targeting the translation initiation site were found to suppress SMC proliferation after serum stimulation. The molecular basis of this effect was supported by the level of both c-*myc* RNA in Northern blot analysis and metabolically labeled c-MYC protein from immunoprecipitation. The dose response of the lead molecule (Rs6) was very competitive with the antisense counterpart with an IC_{50} around 50 nM (Fig. 6). Surprisingly the extent of biological effect on SMC proliferation with different length and modification analogues followed the pattern observed in vitro with cleavage in both the full-length substrate and in a short synthetic substrate (Sun et al., 1999). This was exemplified by Rs6 (9/9-bp arms and 3′-inversion), which demonstrated outstanding activity in both the biological system and in vitro.

TABLE 2
Summary of deoxyribozyme gene suppression studies

Target	Cell	Arms	Modification	Delivery	Activity	Controls	Reference
HPV E6	3T3 (transient)	8/8	3′-3′ inversion	DOTAP	60% suppression E6 RNA	Scrambled	Unpublished
c-*myc*	SMC	7/7–9/9	3′-3′ inversion	DOTAP	80% suppression cell proliferation	Inactivated	Sun et al., 1999; Cairns et al., 1999
BCR-ABL	BV173	8/8–15/15	2′ *O*-methyl cap	Lipofectin	Apoptotic morphology	Inactivated & antisense	Warashina et al., 1999
BCR-ABL-luciferase	HeLa (transient)	8/8–15/15	2′ *O*-methyl cap	Lipofectin	99% suppression luciferase expression	Inactivated & antisense	Warashina et al., 1999
BCR-ABL	K562	12/6	Phosphorothioate 2-base cap	Cytofectin	40% protein suppression, 50% cell proliferation	Inactivated	Wu et al., 1999
BCR-ABL	CD34+ CML-bone marrow	12/6	Phosphorothioate 2-base cap	Cytofectin	53–80% suppression of growth in bcr-abl-positive CFU	Inactivated	Wu et al., 1999
HIV-1 *env*	HeLa (transient)	7/7	None	Lipofectin	50% fusion	Irrelevant & antisense	Dash et al., 1998
CCR5	HeLa (transient)	7/7	None	Lipofectin	50% fusion	Irrelevant	Goila and Banerjea, 1998
HIV-1 *env*	U87	7/7	None	Lipofectamine	77–81% suppression viral load (p24)	Inactivated	Zhang et al., 1999
huntingtin	HEK-293 (transient)	8/8	3′-3′ inversion	Lipofectamine	85% reduction in Huntingtin protein	Inactivated	Yen et al., 1999
NGFI-A	SMC	9/9	3′-3′ inversion	SuperFect	75% proliferation	Inactivated & antisense	Santiago et al., 1999

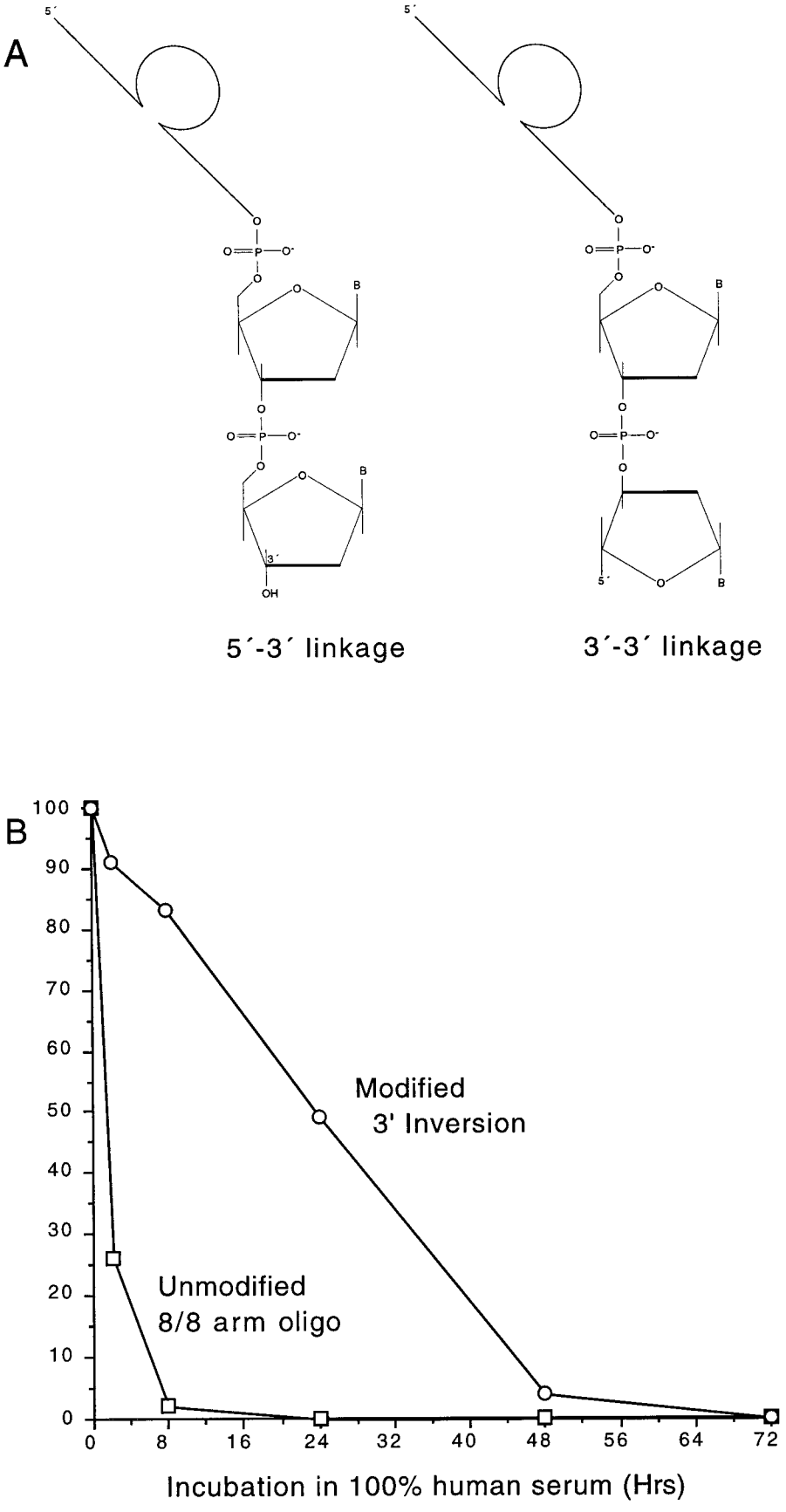

FIG. 5. The 3′-3′-internucleotide linkage and its effect on the serum nuclease stability of the deoxyribozyme oligonucleotide. Panel A contains the structure of a normal 5′-3′-internucleotide linkage and a 3′-3′-linkage inversion. Panel B shows the stability profile of an unmodified oligonucleotide and its 3′-3′-inversion modified counterpart in human serum.

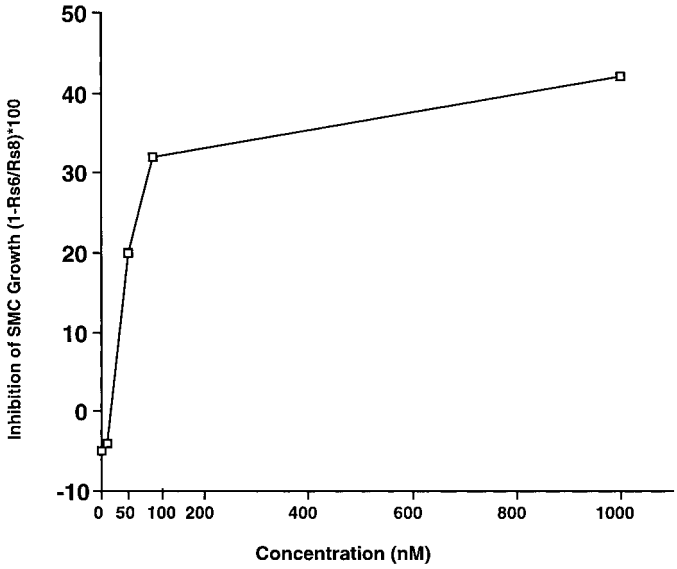

FIG. 6. Dose response for deoxyribozyme Rs6 mediated suppression of serum-stimulated SV40LT-SMC smooth muscle cell proliferation. Cell growth suppression was determined by cell counting and expressed as percentage of the inactivated control oligonucleotide.

Two different groups have been involved in exploring the activity and specificity of deoxyribozymes in leukemic cells that contain the Philadelphia chromosome and express the *bcr-abl* fusion (Warashina et al., 1999; Wu et al., 1999). In both respects the deoxyribozymes compared favorably with previous work with hammerhead ribozymes and antisense oligonucleotides (Gewirtz et al., 1998). Intracellular activity of deoxyribozymes targeting the b2a2 splice junction was demonstrated with *bcr-abl* luciferase and *abl* luciferase reporter gene constructs in HeLa cells; and against endogenous *bcr-abl* and *abl* expressing BV173, H9 cells, respectively (Warashina et al., 1999). Similarly deoxyribozymes also showed activity against the *bcr-abl* (b3a2) expression in K562 cells and $CD34^+$ bone marrow cells from patients with chronic myeloid leukemia (Wu et al., 1999). The nuclease stability was enhanced in these molecules by capping the terminals with either two phosphorothioate linkages or by 2′-*O*-methyl modification of two terminal residues. Both of these modified deoxyribozymes were found to have sustained intracellular activity; however, only the 2′-*O*-methyl-modified molecules maintained specificity for the target *bcr-abl*. The phosphorothioate-modified deoxyribozymes (and inactivated controls), like their antisense counterpart, did not discriminate between the mutant target *bcr-abl* and the normal *abl* gene (Warashina et al., 1999). This loss of specificity with the phosphorothioate-modified deoxyribozymes is puzzling because this modification usually has the effect of destabilizing a DNA-RNA heteroduplex, which should if anything enhance cleavage specificity. One possibility is that RNase H activation at *abl* binding component of the deoxyribozyme is responsible for the nonspecific target destruction. This seems unlikely because, despite the lack of RNase H activity in the 2′-*O*-methyl-modified oligonucleotides (Inoue et al., 1987), the unmodified and inactivated versions of the same molecule would also be expected to be capable of eliciting a response. This effect on the ABL luciferase reporter could have been the result of some nonspecific phosphorothioate interaction. Loss of specificity in deoxyribozymes with phosphorothioate caps did not appear to be a problem on the b3a2 splice junction after truncation of the *bcr* binding arm to 6 bases (Wu et al., 1999). This group also examined the cleavage activity of the same 10-23 deoxyribozymes used by Warashina et al. (1999) against the b2ab splice junction and could not find cleavage. Alternatively, they used an 8-17 deoxyribozyme against this site (Wu et al., 1999). The difference in cleavage activity of this molecule experienced by the two groups may have been due to differences in the substrate lengths.

In another comparative study, Banerjea and coworkers have examined the activity of hammerhead ribozymes and the 10-23 deoxyribozyme in an HIV model system (Dash et al., 1998; Goila and Banerjea, 1998). In this model a luciferase reporter construct is used to indicate the extent of HIV-1 envelope-CD4-mediated cell fusion (Nussbaum et al., 1994). Deoxyribozymes, which cleave the HIV *env* transcript, and the CCR5 chemokine receptor were both found to be potent inhibitors of fusion between CD4 positive HeLa cells and HeLa cells expressing the HIV-1 envelope gene. In each case the deoxyribozyme performed better in terms of full-length RNA cleavage and intracellular activity, than the hammerhead ribozymes—although these were not targeting the same site. In more recent work, Zhang et al. (1999) directed deoxyribozymes to the conserved V3 loop of HIV *env*, suppressing both viral replication (viral load/p24 antigen) and virus infection (single cycle reporter system).

The activity of the 10-23 deoxyribozyme has also been investigated against the *huntingtin* mRNA (Yen et al., 1999). The mutant protein expressed from this transcript is thought to be the causative agent in Huntington's disease. The mutation responsible for this neurodegenerative disorder is derived from expansion of a CAG repeat, and although being an ideal target, was not susceptible to deoxyribonucleotide cleavage. However, despite the lack of activity in this area, deoxyribozymes specific for a number of other target sites were chosen on the basis of RNA secondary structure prediction. Two of these deoxyribozymes demonstrated substantial activity against the *huntingtin* transcript in vitro, and protein in HEK-293 cells cotransfected with the *huntingtin* gene. Interestingly, the intracellular activity of these two deoxyribozymes when used together (at half the active concentration) was synergistic such that the overall suppression gene expression was greater than when tested individually.

More recently a 10-23 deoxyribozyme targeting the transcription factor Egr-1 has been shown to inhibit

smooth muscle cell proliferation in culture and neointima formation in the rat carotid artery damaged by ligation injury or balloon angioplasty (Santiago et al., 1999). Suppression of Egr-1 was also monitored at the RNA and protein level in treated smooth muscle cells by Northern and Western blot analysis, respectively. This was the first evidence of deoxyribozyme efficacy in vivo, and furthermore the activity displayed by this anti-Egr-1 molecule could potentially find application in various forms of cardiovascular disease such as restenosis.

4. Target Site Selection. Underpinning the tremendous versatility of the 10-23 deoxyribozymes is its ability to bind a target RNA sequence via Watson-Crick base pair interactions. This means it has the potential to bind and cleave any RNA molecule both in vitro and in vivo. However, like other agents that function by hybridization with single-stranded RNA, the deoxyribozyme must compete with the targets own stable intramolecular base pairing, which forms its characteristic secondary structure. Fortunately the 10-23 deoxyribozyme cleavage sites are plentiful in most biological substrates and thus provide a host of opportunities to achieve maximum cleavage efficiency. Finding these sites in the target RNA that are amenable to efficient hybridization and cleavage is usually a difficult and time-consuming task (one neglected in most studies to date) involving empirical testing of many deoxyribozyme in long-folded RNA transcript in vitro. In an attempt to streamline this process, we developed a multiplex approach to target site selection, which allowed the simultaneous analysis of many different deoxyribozyme cleavage sites in a single reaction (Cairns et al., 1999). Using this strategy, the cleavage efficiency of 80 different deoxyribozymes targeting the entire E6 component of the full-length E6/E7 transcript from HPV16, was determined in a single experiment. Molecules with both efficient and inefficient RNA cleavage activity were then compared for their ability to inhibit E6 gene expression in a cell-free system. In this assay the most efficient cleavers were also the most efficient inhibitors of E6 gene expression. The deoxyribozyme target site selection strategy was also used to identify the most efficient cleavers of a full-length rat c-*myc* transcript. Again some of the more active molecules from 60 deoxyribozymes tested under multiplex conditions were compared with less active cleavers in their ability to suppress rat SMC proliferation. As c-*myc* gene expression is closely associated with proliferation in response to serum stimulation in this model, the level of post-treatment growth could be used as an indicator of anti-c-*myc* activity. Gene suppression in this system was found to correlate with deoxyribozyme activity in the multiplex cleavage assay, with the most efficient cleavers also having the greatest effect on SMC proliferation. The multiplex assay was found to be an effective screen for deoxyribozyme cleavage sites, because it could efficiently identify the molecules with high activity against long-folded substrate RNA. In addition to this application, the multiplex selection assay may also be useful for identifying target site accessibility for other RNA binding agents such as antisense oligonucleotides and ribozymes.

IV. Other Catalytic Nucleic Acids

Hammerhead and hairpin motifs have often found application in the design of *trans*-acting ribozymes. There are, however, several other naturally occurring ribozymes exhibiting useful activities, including those found within group I introns, ribonuclease P (RNase P) and a viroid-like human pathogen, the hepatitis delta virus (HDV).

A. Group I Introns

Introns are noncoding sequences that interrupt the coding sequences of most eukaryotic genes. These must be removed, or "spliced", after transcription to allow expression of functional mRNA, rRNA, or tRNA molecules. In the case of group I introns, excision is mediated by the autocatalytic activity of the intronic sequences themselves. There are several hundred examples of group I introns, including those found in plant and fungal mitochondria, bacteriophage, eubacteria, and chloroplast tRNA. Despite considerable variability in size and sequence, group I introns have phylogenetically conserved secondary structures (Michel and Westhof, 1990) and a common reaction mechanism. The first confirmation of self-splicing activity came from work on ribosomal RNA genes from *Tetrahymena thermophila* (Cech et al., 1981). In vivo, these reactions occur with the assistance of protein factors that, in some cases (Lambowitz and Perlman, 1990), are encoded within the intron itself. Interestingly, the *Neurospora* CYT-18 protein, which is known to interact with the *Neurospora* large mitochondrial ribosomal RNA and *ND1* group I introns, can substitute for an RNA domain in the *Tetrahymena* group I intron (Mohr et al., 1994). The *Neurospora* introns are able to form most of the RNA secondary structures required for activity but require the CYT-18 protein for in vitro and in vivo splicing activity. This convergence in function between RNA and protein factors perhaps signals a transition from the relatively simple self-splicing group I introns to the more complex splicing pathways seen in higher eukaryotes (Weiner, 1993).

The enzymatic activity of group 1 introns involves a two-step transesterification with a requirement for a divalent cation, such as magnesium, and a guanosine cofactor. The first step involves a nucleophilic attack of the guanosine cofactor 3′-hydroxyl group on the 5′-splice site forming a free 3′-hydroxyl on the 5′-exon. In a second step, this free hydroxyl group makes a nucleophilic attack on the 3′-splice site, releasing the intron as a circular molecule and leaving a ligated exon (Cech, 1990). Substrate specificity is determined by a sequence

within the intron, the internal guide sequence (IGS), and there is a requirement for a U at position −1, relative to the cleavage site, paired with a conserved G in the IGS (Cech et al., 1992). Site-specific mutagenesis of the IGS has shown that other RNA sequences can be targeted. Truncated versions of the *Tetrahymena* group I intron have been shown to catalyze many *trans* reactions in vitro including endonuclease, ligase, nucleotidyl transferase, phosphatase reactions with RNA (Cech, 1990), and, in some cases, DNA substrates (Herschlag and Cech, 1990; Robertson and Joyce, 1990). In vitro evolution methodologies have been employed to improve or alter the catalytic requirements of the ribozyme (Green et al., 1990; Green and Szostak, 1992; Beaudry and Joyce, 1992; Lehman and Joyce 1993).

The ability of group I ribozymes to perform *trans*-splicing reactions in vitro suggests the possibility of therapeutic modification of disease-relevant RNA targets in vivo. In a model system, truncated lacZ transcripts have been corrected in *Escherichia coli* (Sullenger and Cech 1994) and mammalian cells (Jones et al., 1996). Closer to a therapeutic application, the L-21 group I ribozyme has been used to correct mutant β^s-globin mRNA transcripts by *trans*-splicing with the γ-globin 3′-exon in sickle cell anemia-derived erythroid lineage precursor cells (Lan et al., 1998). Since the efficiency of the *trans*-splicing group I ribozymes can approach 25–50% of targeted transcripts (Jones and Sullenger, 1997), this approach has the potential to correct inherited and other diseases caused by expression of mutant mRNAs. Some questions remain regarding the specificity of the reaction as other mRNAs within the cell can be targeted, perhaps because the IGS recognition component is only 6 nucleotides in length (Jones et al., 1996) (see Fig. 7). RNA repair, using group I ribozymes or other methods (Kmiec, 1999; Puttaraju et al., 1999) has significant advantages over gene replacement strategies, and avoiding the need for regulated transgene expression.

B. RNase P

RNase P is a ribonucleoprotein involved in processing the 5′ termini of tRNA precursors during their maturation. RNA cleavage is via nucleophilic attack on the phosphodiester bond leaving a 5′-phosphate and 3′-hydroxyl at the cleavage site, and there is an absolute requirement for divalent metal ions. The *E. coli* RNase P comprises an RNA domain of ~400 nucleotides (the M1 subunit) and a protein component of 14,000 Da dubbed C5. In vitro, the M1 RNA component has been shown to possess intrinsic catalytic activity; in vivo, however, there appears to be a requirement for C5 (Guerrier-Takada et al., 1983). The RNase P protein component is believed to facilitate binding between M1 RNA enzyme and tRNA substrate by masking electrostatic repulsion between enzyme and substrate RNAs (Gardiner et al., 1985; Reich et al., 1988). Comparisons between RNase P enzymes from diverse organisms reveals substantial sequence variation; however, there are similar core sequences and secondary structures that likely represent the catalytic domains (Frank and Pace 1998). Eukaryotic RNase P complexes are less well characterized, seem to have a higher protein content, and the RNA component has no apparent in vitro enzymatic activity (Tanner, 1999).

There is no absolute dependence on conserved sequences at the tRNA-precursor cleavage site, the most important domains within the substrate consist of the aminoacyl acceptor stem and T-stem with conserved GUUC sequence (Kahle et al., 1990; Yuan and Altman, 1995). The ubiquitous nature of RNase P, the lack of a requirement for specific nucleotide sequences for cleavage, and the inherent efficiency of utilizing a cellular enzyme, have created interest in directing RNase P-mediated cleavage to therapeutic target mRNAs. Both *E. coli* and human RNase P were shown to cleave a target RNA that resembles a tRNA substrate in a bimolecular reaction (Yuan et al., 1992). The 3′-proximal sequence of the stem functions to identify the target and is considered to act as an external guide sequence (EGS) that can be targeted to essentially any target RNA. For example, an EGS based on the *E. coli* $tRNA^{tyr}$ and modified to hybridize to the bacterial chloramphenicol acetyltransferase mRNA sequence was capable of directing specific human RNase P-mediated cleavage of chloramphenicol acetyltransferase mRNA in vitro and in vivo (Yuan et al., 1992). A variation on this approach is to fuse an EGS to the bacterial M1 RNase P subunit itself, improving substrate binding and cleavage efficiency. A fusion between the M1 RNA and a sequence complementary to the herpes simplex virus I thymidine kinase (TK) mRNA was shown to efficiently cleave TK mRNA in vitro and reduce TK mRNA and protein levels by ~80% in transfected cells (Liu and Altman 1995). Direct expression of EGSs directed against a bacterial resistance gene (Guerrier-Takada et al.,1997) or essential viral genes (Plehn-Dujowich and Altman, 1998) has proven effective in functional cellular assays. Recently, deletion studies have defined that an EGS of only 30 nucleotides is sufficient for efficient cleavage in a biomolecular reaction in vitro (Werner et al., 1999). These results were generated using short, synthetic substrates, and it will be important to demonstrate similar findings when full-length mRNA transcripts are targeted either in vitro or in vivo. As with other approaches to gene suppression mentioned in this review, target selection, stability of synthetic molecules in biological fluids, and delivery of EGS, whether vector-based or as oligonucleotides, remain as challenges. Nevertheless, RNase P-mediated approaches to gene down-regulation offer considerable promise for genomic and therapeutic applications.

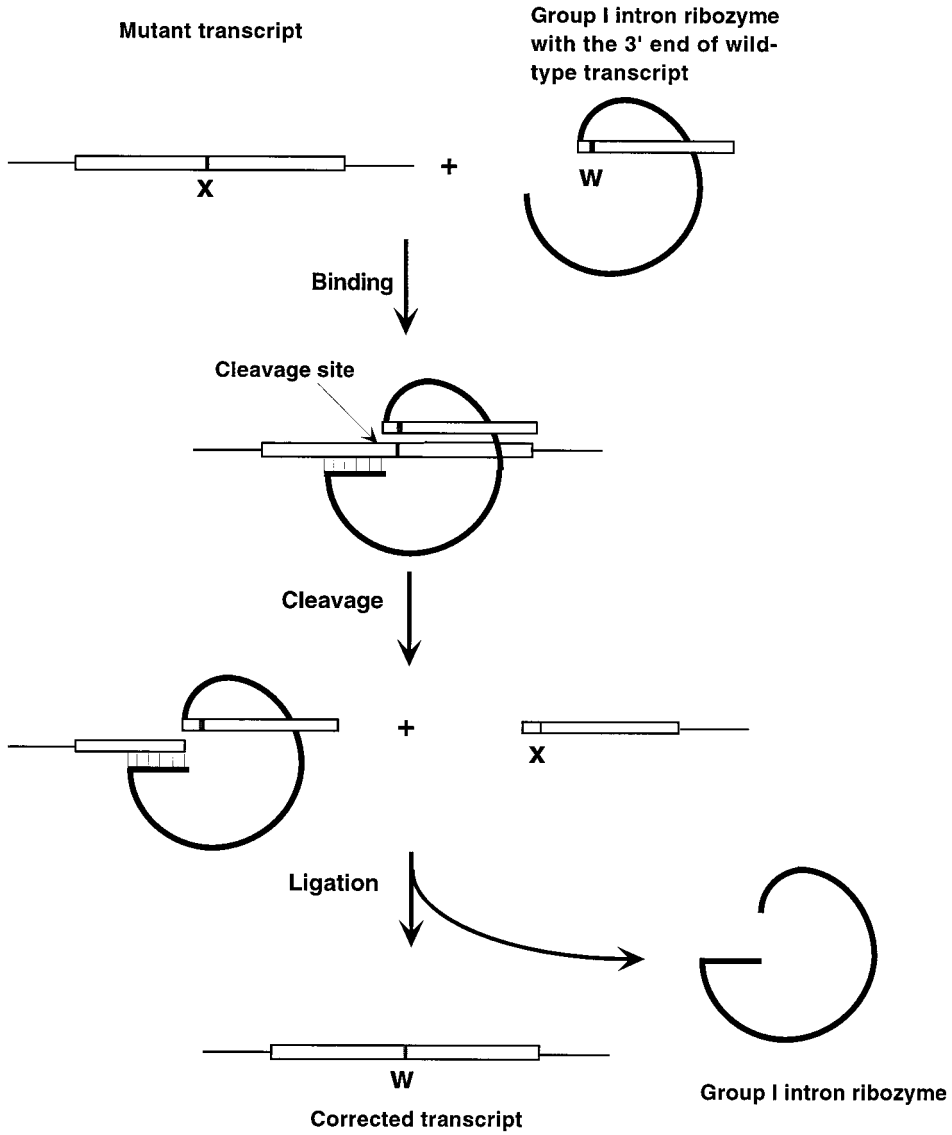

FIG. 7. Schematic description of RNA repair mechanism using a group I intron ribozymes (Sullenger and Cech, 1994). A ribozyme with the 3′-end of wild-type transcript was designed based on group I intron model. After cleavage, the ribozyme mediated a transfer of its 3′-exon tag to the 5′-cleavage product; thus RNA repair was accomplished.

C. Hepatitis Delta Ribozyme

The hepatitis delta virus virus (HDV) is a single-stranded circular RNA virus of approximately 1700 nucleotides, which causes significant pathology in man (Lai, 1995). HDV infection requires the presence of the hepatitis B virion either coincident with HDV infection or as a pre-existing infection. Hence, HDV is designated as a satellite virus of hepatitis B. HDV contains two ribozymes, both required for RNA replication (Macnaughton et al., 1993), one on the genomic, infectious RNA strand, the other in the complementary region of the antigenomic strand. The nucleotide sequence and four stem (P1–P4), pseudoknot secondary structure of the HDV ribozymes is different than other ribozymes described so far. The predicted secondary structure has been largely confirmed by X-ray crystallographic studies at a resolution of 2.3 A (Ferré-D'Amaré et al., 1998). The self-cleaving activity of HDV RNA is enhanced in the presence of denaturants (Rosenstein and Been, 1990), which suggests that the active structures are present in nascent transcripts, not the mature, folded, genomic RNA. Substrate recognition requires formation of the P1 stem, comprising a GU wobble pair plus six nucleotides hybridized to the target, with cleavage occurring just 5′ to the wobble base pair. The minimal ribozyme sequence is ~85 nucleotides, almost entirely 3′ with respect to the cleavage site, useful for the generation of discrete RNA transcript termini or processing ribozyme multimers to monomers in *cis*-reactions (Tanner, 1999). *Trans*-cleaving HDV ribozymes have been designed that are active against oligonucleotide substrates; these include a P4–2 ribozyme (Branch and Robertson, 1991; Perrotta et al., 1993; Wu et al., 1993), a P1 ribozyme (Perrotta and Been, 1993), a circular ribozyme (Perrotta et al., 1993;

Puttaraju et al., 1993), and a hybrid of both genomic and antigenomic HDV ribozymes (Been et al., 1992). However, the modest activity of *trans*-cleaving HDV ribozymes against long substrates (Roy et al., 1999a) limits their utility for therapeutic applications.

V. Pharmacology—The Key to Applications

A. Delivery of Oligonucleotides

The most challenging aspect of the use of catalytic nucleic acids in a pharmaceutical role is delivering these molecules to their site of action, the RNA target located within the cytoplasm or nucleus. Delivery requires that the oligonucleotide survive local or systemic administration long enough to bind to the target cells, cross the cytoplasmic membrane or become released from an endosomal/lysosomal vesicle, pass through the nuclear membrane (or cross the endoplasmic reticulum), and be able to functionally hybridize to the target RNA. This represents a long chain of events that are at best unlikely for a typical conventional drug let alone a large, labile, highly charged molecule (Liang et al., 1999). A range of viral vector constructs have been designed to express ribozymes endogenously within target, and potentially successful ex vivo applications have been described (Sun et al., 1995; Bramlage et al., 1998; Wang et al., 1998). The in vivo application DNAzymes and chemically synthesized ribozymes is presently restricted to exogenous delivery although relatively little work in this area has been described. Studies of exogenously delivered oligonucleotides have been dominated for several years in studies of antisense oligonucleotide therapy. The pharmacological issues critical to the delivery of catalytic DNA and RNA can be extrapolated from the wealth of data collected from studies of the pharmacology of antisense drugs (Crooke, 1998; Bennett, 1998; Juliano et al., 1999). By drawing on this wide body of data and including recent studies of ribozyme and DNAzyme delivery, it is possible to discuss how issues such as stability, toxicity, immunology, in vivo and cellular pharmacokinetics, and the employment of delivery agents are challenges common to all forms of oligonucleotide-based therapy.

B. Oligonucleotide Stability

As discussed in *Section III*, a wide range of chemical modifications have been employed to enhance the stability of synthetic ribozymes and DNAzymes to facilitate their in vivo application. For antisense oligonucleotides, the first generation phosphorothioate (PS) deoxyoligonucleotides have been studied extensively and are being evaluated in a number of clinical trials against a range of targets (Hogrefe, 1999; Persidis, 1999). However the stability offered by the PS backbone is only partial because oligonucleotides prepared by nonstero-controlled methods contain mixtures of the nuclease-susceptible configuration and the resistant configuration (Koziolkiewicz et al., 1997). Numerous additional modifications such as 2′-*O*-alkyl, 2′-C-allyl substitutions or, peptide nucleic acid, *p*-ethoxy, phosphorothioate, phosphotriester, morpholino, methylphosphonate linkages have long been used to enhance the biological stability of antisense drugs (Bennett, 1998; Stein, 1998). More recently antisense oligonucleotides containing 2′-modified bases or combinations of modifications to form mixed backbone oligonucleotides have been used in antiviral and antitumor applications in both parenteral and oral delivery models (Agrawal et al., 1997; Brown-Driver et al., 1999; Wang et al., 1999). To date reported studies of DNAzyme activity have been limited primarily to cell culture experiments, and only a small range of nucleic acid modifications have been employed. Comparison of the intracellular activity of phosphodiester (PO) versus PS or 2′-*O*-methyl modifications in the binding arms of the novel 10-23 DNAzyme has recently been reported by Warashina et al. (1999). DNAzymes containing either modification retained activity in mammalian cells longer than the unmodified PO form, with the 2′-*O*-methyl modification being most stable. A 33-mer DNAzyme protected with a 3′-3′-terminal linkage inversion (Fig. 5) was incubated with 100% human serum, and electrophoretic analysis revealed approximately 50% of the oligonucleotide was intact after 24 h of incubation (Sun et al., 1999). Using this modification, functional DNAzymes were stabilized in cell culture studies (Sun et al., 1999; Yen et al., 1999) and in an in vivo vascular injury model (Santiago et al., 1999).

A number of approaches have been explored to increase the stability of ribozymes such as chemical modifications, introduction of deoxyribonucleotide as chimeric RNA-DNA ribozymes and reduction of the helix II and its connecting loop of the hammerhead to form a minizyme (McCall et al., 1992; Yang et al., 1992; Hendry et al., 1995). In general, chemical modifications do not increase catalytic activity; in fact reductions are common. However, these drawbacks can be compensated by a significant increase in ribozyme stability and improved pharmacological properties of synthetic ribozymes. Improvements in the chemical synthesis of RNA have led to the ability to modify ribozymes (Scaringe et al., 1990; Wincott et al., 1995). Most of the modifications include replacement of the 2′-OH moieties with methyl allyl, amino, deoxy, fluoro, or *O*-methyl groups (Usman and Stinchcomb, 1996). Addition of a 3′-3′-linked, inverted T at the 3′-end of ribozyme protected the 3′ terminus of the ribozymes from exonuclease for at least 48 h in human serum (Ortigao et al., 1992; Beigelman et al., 1995; Sioud and Sørensen, 1998). In addition, the modification of the linkage from phosphates to phosphorothioates dramatically increased resistance to specific endonucleases in cells and from serum (Ruffner and Uhlenbeck, 1990). Combinations of multiple derivatives including a 3′-inversion and PS backbone in the binding arms and 2′-fluoro-2′-deoxy-

pyrimidine, 2′-*O*-methyl, or 2′-allyl nucleotides in various locations in the ribozyme sequence have been used to enhance their stability in cell culture (Jarvis et al., 1996a,b; Fell et al., 1997; Scherr et al., 1997; Prasmickaite et al., 1998; Bramlage et al.,1999) and in animal studies (Desjardins et al., 1996; Flory et al., 1996; Sandberg et al., 1999).

The enhanced stability of these synthetic ribozymes has been resolved using a range of analytical approaches. Using an internally radiolabeled, highly modified ribozyme, Sandberg et al. (1999) were able to demonstrate the detection of intact ribozyme by electrophoresis in mouse plasma up to 120 min after s.c. administration. Capillary gel electrophoresis was used to detect fluorescein-labeled 2′-*O*-methyl-modified ribozyme delivered to human THX melanoma cells in culture (Prasmickaite et al., 1998). Analysis of ribozyme from both intracellular extracts and growth medium revealed that the majority of degradation occurs in the intracellular compartment with 90% of the extracellular ribozyme remaining intact after 12 h of incubation. The stability of a fluorescent ribozyme (stabilized with 2′-*O*-allyl-modified bases and a 3′-T inversion) was followed after i.v. injection in a rat by analyzing plasma samples with a sequencing gel scanner (Desjardins et al., 1996). Direct electrophoresis of samples of plasma and urine revealed that the large majority of the ribozyme remained intact beyond 24 h in vivo.

C. Biodistribution and Pharmacokinetics

Extensive information about the circulation lifetime and biodistribution of i.v. administered naked phosphorothioate or mixed backbone oligonucleotides has been reported in several species including mouse (Phillips et al., 1997; Zhao et al., 1998), rat (Zhang et al., 1996; Bijsterbosch et al., 1997; Graham et al., 1998), monkey (Henry et al., 1999) and human (Glover et al., 1997; Henry et al., 1999; Séréni et al., 1999). Naked PS oligonucleotides are rapidly removed from the plasma and assuming a two-compartment model both the distribution phase and the elimination phase have been described for PS oligonucleotides, and mixed backbone oligonucleotides including ribozymes (Zhang et al., 1995; Desjardins et al., 1996; Phillips et al., 1997). A large proportion of the oligonucleotide in the plasma is protein-bound, and this effect may prevent renal excretion (Sawai et al., 1996; Raynaud et al., 1997; Crooke, 1998). Distribution half-lives ($t_{1/2\alpha}$) for PS oligonucleotides are typically short ranging from 19 min (0.6 mg/kg) in mice to 23 min (1 mg/kg) in rats (Phillips et al., 1997; Bijsterbosch et al., 1997). Using a one-compartment model Glover et al. (1997) measured the plasma half-life of PS antisense in humans to be 53 min (2 mg/kg). A similar result was obtained (68 min, 2 mg/kg) in humans using a first order plasma elimination half-life (Séréni et al., 1999). In these same studies the elimination half-life ($t_{1/2\beta}$) of PS antisense, where measured, typically extends for several hours. The rate of administration varies widely between studies and ultimately affects the maximum plasma concentration (C_{max}). At 2 mg/kg for example, the C_{max} for ISIS 2302 is approximately 8 μg/ml in humans when administered over 2 h but was as high as 40 μg/ml as a bolus injection in monkeys (Crooke, 1998). Prolonged s.c. oligonucleotide administration decreased the rates of both metabolism and elimination from the plasma although bioavailability may be affected (Raynaud et al., 1997). The distribution half-life for 2′-*O*-allyl modified ribozyme (1.25 mg/kg) was approximately 12 min in rat (Desjardins et al., 1996). Elimination half-lives of approximately 30 min were reported for multiply modified ribozymes administered as bolus injection (30 mg/kg) by either the i.v., i.p., or s.c. route (Sandberg et al., 1999).

In all species tested, oligonucleotides including ribozymes are delivered to a wide range of tissues, although accumulation is primarily in the kidneys and the organs of the reticuloendothelial system, the liver, spleen, and bone marrow (Desjardins et al., 1996; Zhao et al., 1998). During the distribution phase, the largest proportion of oligonucleotides are deposited in the liver whereas the accumulation in the kidney is highest in proportion to its weight (Bijsterbosch et al., 1997; Zhao et al., 1998). Using cell fractionation techniques, Bijsterbosch et al. (1997) demonstrated that accumulation in the liver is primarily in endothelial and Kupffer cells. Recent studies suggest that distinct scavenger receptor types mediate the uptake of oligonucleotide in kidney and liver cells (Sawai et al., 1996; Biessen et al., 1998). It has been argued that reports of the urine being a major route of elimination (Zhang et al., 1996) may be an artifact of the indirect radiolabel detection methodologies used to measure urinary oligonucleotides (Crooke, 1998). Capillary electrophoresis is widely considered a more rigorous detection method allowing for detection of both intact oligonucleotide and its metabolites (Leeds et al., 1996). Using this technique has demonstrated that intact phosphorothioate oligonucleotide is undetectable in urine (Glover et al., 1997). In comparison to inulin, urinary excretion was demonstrated to be greatly restricted in an ex vivo model and renal accumulation of PS oligonucleotides was ascribed to both tubular reabsorption and uptake from the capillary side (Sawai et al., 1996). Studies of multiply modified (including PS backbone) ribozyme revealed the presence of intact, internally radiolabeled oligonucleotide in the urine of mice after administration of high doses (30 mg/kg) (Sandberg et al., 1999). It may be possible that saturation of plasma protein binding resulted in detectable levels of oligonucleotide in the urine (Crooke, 1998). In comparison, however, a high proportion of degradation products was observed in the urine of rats administered a 2′-*O*-allyl-modified ribozyme (Desjardins et al., 1996).

Studies of locally administered naked oligonucleotide have indicated long-term persistence and stability in

some models. Capillary electrophoretic analysis of oligonucleotide directly injected into the eyes of rabbits (Leeds et al., 1997) and monkeys (Leeds et al., 1998) revealed that intact oligonucleotide could be detected in the vitreous and retina for up to 14 days. Beyond 7 days, the majority of the residual drug exists as chain-shortened oligonucleotide in both the vitreous and retina thus demonstrating that extensive metabolism occurs in these compartments. After intra-articular administration in a rabbit arthritis model, a small percentage of modified ribozyme could be recovered in the synovial tissue (Flory et al., 1996). Over half the radiolabel detected at 4 h (100% intact) was retained in the synovial tissue after 3 days (72% intact).

D. Toxicity and Immune Modulation

A number of recent studies in human and animal models have confirmed that both systemic and regional administration of PS oligonucleotides was well tolerated and that any observed toxicities were unrelated to the antisense target (Monteith and Levin, 1999). Low toxicity profiles were noted for i.v. injected anti-intercellular adhesion molecule (ICAM) or for s.c. administered anti-BCL-2 in humans (Glover et al., 1997; Webb et al., 1997; Henry et al., 1999). Acute, reversible toxic effects tended to occur where the highest concentration of oligonucleotides had accumulated (Henry et al., 1999). Inflammation at sites of prolonged s.c. delivery (Webb et al., 1997) and bolus intravitreal administration have been reported in both rabbit and primate models (Leeds et al., 1997, 1998). Rodents are more sensitive to immune stimulation than primates and at high oligonucleotide doses mononuclear cell infiltration in the liver, spleen, and kidneys, splenomegaly, lymphoid hyperplasia, and Kupffer cell hypertrophy were commonly reported (Crooke, 1998; Henry et al., 1999). In primates, elevated doses of PS oligonucleotides resulted in increased granulation in proximal tubular epithelial cells and immunohistochemistry revealed that oligonucleotides had accumulated in these granules (Henry et al., 1999; Monteith et al., 1999). High doses of PS oligonucleotides administered rapidly as a bolus i.v. injection (20 mg/kg—10 min infusion) in monkeys resulted in transient, sometimes fatal decreases in blood pressure. The cause of this effect may have been related to effects of compliment activation and release of vasoactive substances (Galbraith et al., 1994). In humans, where PS oligonucleotides were typically administered at lower doses, no significant hemodynamic changes have been detected (Glover et al., 1997; Séréni et al., 1999). Complement activation via the alternative pathway has been detected in primates at high PS oligonucleotide doses (Galbraith et al., 1994; Henry et al., 1999).

Kreig et al. (1995) demonstrated that like bacterial DNA the unmethylated DNA of synthetic oligodeoxynucleotides has mitogenic activity suggesting an additional potential adjuvant role for synthetic oligonucleotides. Further studies of role of CpG motif in complement activation in mice revealed that this activity appears to be both sequence and backbone dependent (Boggs et al., 1997). PS backbones were typically less stimulatory than PO backbones of the same sequence. The stimulatory effect of the CpG on cytosine production could be eliminated by modifying all the cytosines in the oligonucleotide at the 5′-position of the cytosine ring (Boggs et al., 1997). An anticoagulant effect of oligonucleotides has been reported in a number of studies. Upon i.v. injection, a transient increase in blood clotting times (partial thromboplastin time or [aPTT]) has been observed in humans and primate models (Henry et al., 1997, 1999; Séréni et al., 1999). The peak anticoagulant effect appears to be related to oligonucleotide concentration and the [aPTT] recovers quickly after completion of the infusion (Glover et al., 1997; Séréni et al., 1999).

E. Delivery Agents and Cellular Uptake

With relatively short circulation lifetimes and inefficient uptake into cells, the use of carrier systems to increase the stability, circulation lifetime, and cellular uptake of nucleic acid drugs have long been in use (Liang et al., 1999). Cationic lipids such as DOTAP and Lipofectin are incorporated in liposomes, and under physiological conditions form aggregates or complexes with negatively charged oligonucleotides. Complex formation is driven by electrostatic interactions between the charges on the surface of the liposome and the nucleic acid, and these interactions force a condensation of the charged complex. Cationic lipid “transfection” reagents are used extensively to enhance intracellular delivery in functional assays of antisense (Juliano et al., 1999), ribozymes (Bramlage et al., 1998) and DNAzymes (Sioud and Sørenson, 1998; Sun et al., 1999; Warashina et al., 1999). In addition to lipids, a range of cationic reagents including polylysine (Stewart et al., 1996), helical peptides (Wyman et al., 1997), porphyrin derivatives (Benimetskaya et al., 1998), and polymeric dendrimers (DeLong et al., 1997) have been described, which form complexes with oligonucleotides and mediate cellular uptake in cell culture. When added to cells, “naked” fluorescent oligonucleotides tend to be concentrated into small punctate cytoplasmic structures (endosomes or lysosomes), and fluorescence is not observed in the nucleus (Bennett et al., 1992; Wyman et al., 1997). When complexed with cationic lipids (or other agents), increased levels of cellular fluorescence are observed. Within a few hours of incubation, the fluorescent oligonucleotide is located predominantly in the nucleus whereas the cationic lipids remain in the cytoplasm or associated with the cytoplasmic membrane (Bennett et al., 1992; Zelphati and Szoka, 1996; Marcusson et al., 1998). Mechanistic studies have suggested that the anionic lipids of the cytoplasmic membrane or vesicles participate in the release of the oligonucleotides from the complexes (Zelphati and Szoka, 1996). It appears

that the observation of release from the endosomal/lysosomal compartment may be cell specific. Using the cationic lipid DOTAP as a delivery agent, ribozymes (37-mer) were primarily associated with punctate structures in the cytoplasm of cultured THX cells after incubation up to 18 h without significant delivery to the nucleus (Prasmickaite et al., 1998). In this study complexing with DOTAP also increased the amount of intact ribozyme detected in cell extracts. In an in vivo tumor model, DOTAP-mediated uptake of a protein kinase Cα-specific ribozyme (45-mer) directly injected into a glioma tumor effectively blocked its growth (Sioud and Sørensen, 1998). However the presence of cationic lipids may result in nonspecific "efficacious" effects, and results must be interpreted with care. When comparing endogenous viral delivery of an anti-tumor necrosis factor-α ribozyme with exogenous DOTAP-mediated delivery in the presence of the cationic lipid, an irrelevant control ribozyme was equally as effective as both the exogenously delivered ribozyme or virally delivered ribozyme at reducing tumor necrosis factor-α protein levels). A similar nonspecific ribozyme/lipid effect has been previously reported (Freedland et al., 1996; Sioud and Sørensen, 1998).

Although cationic agents may be effective at mediating oligonucleotide uptake in cells in culture or when delivered locally in vivo, their use as systemic delivery agents is limited due to the size and high surface charge of the lipid complexes. Intravenous delivery of oligonucleotides complexed with DC-Chol/DOPE in mice leads to a rapid deposition in the capillary beds of the lung followed by release into the plasma and ultimate clearance into the spleen and liver (Litzinger et al., 1996). Cationic lipid containing liposomes have been reported to enhance the degree of oligonucleotide tissue uptake and serum stability (Gokhale et al., 1999) yet there is ultimately little alteration in the biodistribution profile when compared with free oligonucleotide. In addition, the condensation that occurs during complex formation is progressive, and within hours may result in the precipitation of large aggregates that are not suitable for i.v. delivery. Thus, although cationic complexing agents are effective at stimulating intracellular uptake and nuclear delivery of oligonucleotides, their use is limited by their inherent instability, poor biodistribution, and cytotoxicity (Hope et al., 1998). New applications of cationic reagents, however, point to more refined delivery systems. Novel use of cationic polyspermine in a block copolymer with polyoxyethylene that condenses oligonucleotide into small, stable, nontoxic self-assembling particles has recently been described (Roy et al., 1999b). These particles enhanced the uptake of antisense fibronectin oligonucleotide into the nuclei of retinal vascular cells when injected into the rat vitreous. Concomitant with enhanced uptake was a large decrease in both fibronectin mRNA and protein levels that persisted up to 6 days postinjection. In a novel in vivo application, a block copolymer pluronic gel (P127) used to immobilize and deliver a cationic reagent complexed anti-*Egr*-1 DNAzyme suspension around a carotid artery in a vascular injury model system used demonstrate inhibition of smooth muscle cell proliferation (Santiago et al., 1999).

The use of passive liposome encapsulation of antisense oligonucleotides has recently been reported (Yu et al., 1999; Klimuk et al., 2000). Encapsulation in "stealth" liposomes greatly increased the plasma lifetime in primates to approximately 58 h and resulted in complete protection of the oligonucleotide after 60 h in the circulation (Yu et al., 1999). Intravenous administration of passively liposome-encapsulated anti-ICAM antisense greatly increased the anti-inflammatory activity of this oligonucleotide in a rabbit ear inflammation model (Klimuk et al., 2000). Recent developments in formulation technology have resulted in methods for encapsulating high concentrations of oligonucleotides into small stabilized antisense-lipid particles or SALP (Webb et al., 1999; Semple et al., 2000). The use of an ionizable lipid allows active encapsulation of the oligonucleotide during formulation and results in a low surface charge at physiological pH. Specialized polyethylene glycol-ceramide lipids greatly extend the oligonucleotide circulation lifetime of the SALP by preventing opsonization of the particle surface. Complete encapsulation protects the oligonucleotides from in vivo degradation thus increasing their serum stability. Recent studies have demonstrated that systemically administered SALP can extravasate into tumors and sites of inflammation allowing "passive targeting", which results in a 10-fold increase in accumulation in these tissues versus nonencapsulated oligonucleotides (Webb et al., 1999; Semple et al., 2000). These developments suggest that it may be possible to use such carrier systems to alter the pharmacodynamics of catalytic nucleic acids significantly enough to expand their therapeutic potential.

VI. Conclusions

Catalytic nucleic acid, although lacking some of the functional groups and kinetic efficiency of protein-based enzymes, has a distinct advantage in reactions with nucleic acid substrates because of the high affinity between complementary sequences. This capacity for highly flexible binding and discrimination of nucleic acid substrates by virtue of Watson-Crick interactions enables these catalysts to facilitate reactions in biological systems with very high precision. Since the discovery of catalytic RNA, this capability led by RNA-cleaving ribozymes has rapidly found applications in research, biomedicine, and agriculture. Recently a number of clinical trials have also seen this gene inactivation technology used directly in humans. As these ribozymes can be encoded and transcribed from DNA, much of this devel-

opment has been complemented by advances in vector designs used in other forms of gene therapy. Indeed the *trans*-splicing activity of group I introns has also been explored as a means to effect gene correction. However, in addition to the gene expression-based approach to ribozyme delivery, there has been substantial progress in synthesis and delivery ribozyme oligonucleotides. Perhaps the most exciting development in regard to oligonucleotide-based catalysts has been the arrival of RNA-cleaving DNA enzymes or deoxyribozymes. These molecules exemplified by the 10-23 deoxyribozyme essentially combine the benefits of highly sequence-specific ribonuclease-independent RNA destruction, with the relatively robust constitution used in oligodeoxyribonucleotide-based antisense reagents. Perhaps the major obstacle to the further development of these technologies as gene suppression agents is the difficulty involved in effective cellular delivery and target colocalization. These challenges are being met with a multidisciplinary approach with the hope that a greater understanding of each facet of this problem will enable a more optimal utilization of this technology. As the focus of this research becomes more therapy oriented, with the use of clinically relevant animal models, future impact of catalytic nucleic acid-based therapies should become clearer. However, despite the foreseeable difficulties, we are optimistic that the full potential of these approaches can be achieved in a therapeutic context.

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