

SURVEY AND SUMMARY

In vitro selection, characterization, and application of deoxyribozymes that cleave RNA

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ABSTRACT

Over the last decade, many catalytically active DNA molecules (deoxyribozymes; DNA enzymes) have been identified by *in vitro* selection from random-sequence DNA pools. This article focuses on deoxyribozymes that cleave RNA substrates. The first DNA enzyme was reported in 1994 and cleaves an RNA linkage. Since that time, many other RNA-cleaving deoxyribozymes have been identified. Most but not all of these deoxyribozymes require a divalent metal ion cofactor such as Mg^{2+} to catalyze attack by a specific RNA 2'-hydroxyl group on the adjacent phosphodiester linkage, forming a 2',3'-cyclic phosphate and a 5'-hydroxyl group. Several deoxyribozymes that cleave RNA have utility for *in vitro* RNA biochemistry. Some DNA enzymes have been applied *in vivo* to degrade mRNAs, and others have been engineered into sensors. The practical impact of RNA-cleaving deoxyribozymes should continue to increase as additional applications are developed.

INTRODUCTION

Deoxyribozymes are DNA molecules with catalytic activity. Their RNA analogues, ribozymes, participate in fundamental reactions of modern biochemistry such as viral RNA self-processing (1), RNA splicing (2,3), and translation of RNA into protein (4,5). Ribozymes are widely hypothesized to have both carried information and performed catalysis during the primordial 'RNA World' (6,7). Unlike ribozymes, deoxyribozymes are not known to exist naturally. For either RNA or DNA, our chemical understanding is insufficient to allow prediction of a specific nucleotide sequence that will catalyze a desired reaction. Despite this lack of predictive ability (and, for DNA, without any clues from nature), artificial ribozymes

and deoxyribozymes can readily be identified through *in vitro* selection (8,9). In this approach, large random-sequence 'pools' of nucleic acids are iteratively examined until a small number of catalytically active sequences are obtained (10,11). These nucleic acid enzymes can be studied to determine their catalytic abilities, structures and mechanisms. In some cases, they can also be used for practical applications.

The known natural ribozymes all function with nucleic acid substrates, with the important exception of the ribosome that creates peptide bonds (1). In contrast, artificial ribozymes identified by *in vitro* selection catalyze a growing variety of chemical reactions (12–18). For deoxyribozymes, the substrates have almost always been nucleic acids themselves [with a few exceptions; (19,20)], and a number of recent reviews have addressed the scope of DNA's catalytic activity using such substrates (21–25). This article focuses specifically on deoxyribozymes that cleave RNA. Deoxyribozymes have also been termed DNA enzymes or catalytic DNA, and these terms are used interchangeably here. The contraction 'DNAzyme' will generally be avoided.

The first reported DNA enzyme was described by Breaker and Joyce (26) in December 1994 and cleaves a specific RNA linkage embedded within a longer nucleic acid strand. Many RNA-cleaving deoxyribozymes have been identified in the intervening decade (27–36). Some of these DNA enzymes have excellent catalytic rates, turnover numbers and abilities to cleave a wide range of RNA substrate sequences, and their practical utility has been exploited in several ways. The following sections describe how RNA-cleaving deoxyribozymes have been identified, characterized and applied in biochemistry and other areas.

***IN VITRO* SELECTION OF DEOXYRIBOZYMES THAT CLEAVE RNA, PART 1: THE FIRST EXPERIMENTS**

All known RNA-cleaving deoxyribozymes catalyze the chemically identical cleavage reaction: attack of an RNA

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2'-hydroxyl group on the adjacent phosphodiester linkage, which forms 2',3'-cyclic phosphate and 5'-hydroxyl RNA termini (Figure 1). This intramolecular cleavage reaction is also catalyzed by many protein ribonucleases such as RNase

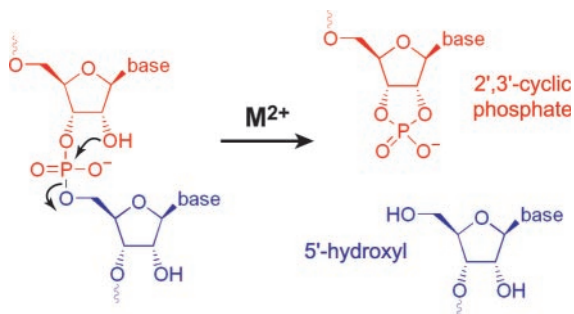


Figure 1. RNA cleavage with formation of 2',3'-cyclic phosphate and 5'-hydroxyl termini. This reaction can occur alone or with a catalyst such as a protein enzyme, ribozyme or deoxyribozyme. In most but not all cases, a divalent metal ion cofactor (M^{2+}) is required to achieve an appreciable reaction rate.

A, and the cleavage mechanism has been widely studied both in proteins and in small model systems (37). Formation of an RNA 2',3'-cyclic phosphate via intramolecular cleavage is facile even without catalysis; e.g. simply incubating an RNA strand under basic conditions leads to random scission along the entire length of the strand (38,39). Therefore, DNA does not have to work particularly hard to increase the rate of an already-favorable reaction. Nevertheless, the extremely high selectivity with which deoxyribozymes have been shown to cleave RNA substrates at particular locations is intriguing and useful.

In the earliest effort to identify catalytic DNA (26), five iterated selection rounds were used to identify a Pb^{2+} -dependent deoxyribozyme that cleaves RNA according to the reaction of Figure 1. The selection strategy used a nucleic acid substrate made entirely from DNA except with a single embedded ribonucleotide linkage, which directed cleavage to the sole RNA junction within the strand (Figure 2, strategy 1). For brevity, the substrate is termed here 'the RNA substrate', although only one linkage was in fact RNA; later experiments used substrates that had a longer stretch of RNA nucleotides

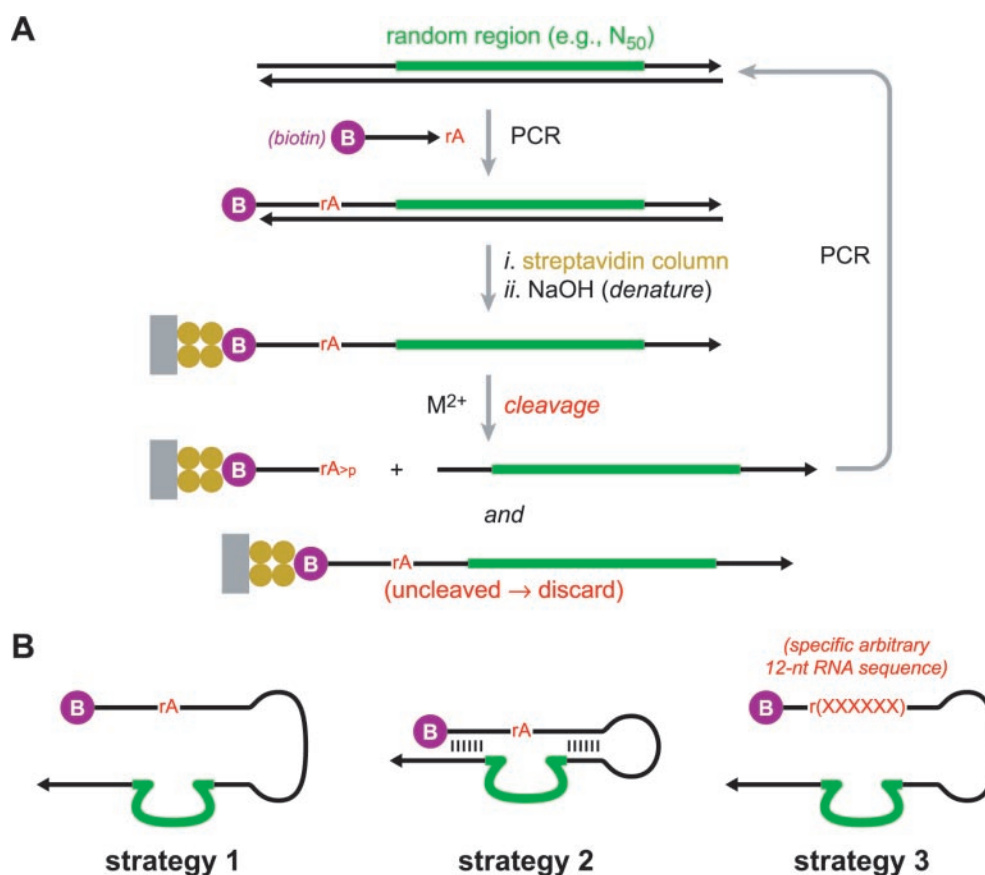


Figure 2. *In vitro* selection strategies for identifying RNA-cleaving deoxyribozymes. (A) The general four-step selection strategy, which involves iterated rounds of PCR to incorporate biotin; streptavidin chromatography to isolate the single-stranded nucleic acid; M^{2+} -catalyzed RNA cleavage; and PCR to regenerate the pool, which has been enriched in deoxyribozymes capable of cleaving RNA. (B) Simplified depictions of the three strategy variations used in all selection experiments for RNA-cleaving DNA enzymes reported to date. In strategy 1, a single ribonucleotide linkage (rA) is the cleavage target, and interactions between the substrate strand and the deoxyribozyme are not pre-programmed. This strategy was used for the first *in vitro* selection effort that identified catalytic DNA, which resulted in a Pb^{2+} -dependent RNA-cleaving deoxyribozyme (26), and it has been employed in other studies as well (28,33). In strategy 2, the single ribonucleotide cleavage site was placed between two Watson-Crick binding arms, as first reported by Breaker and Joyce (27) and used in several subsequent experiments (30,32,33,36,40). In strategy 3, a 12 nt stretch of RNA rather than a single ribonucleotide linkage was the cleavage target, giving rise to the 10–23 and 8–17 deoxyribozymes (29). The biotinylated RNA–DNA chimera was generated during the first step of each selection round by primer extension using reverse transcriptase instead of PCR (not depicted explicitly).

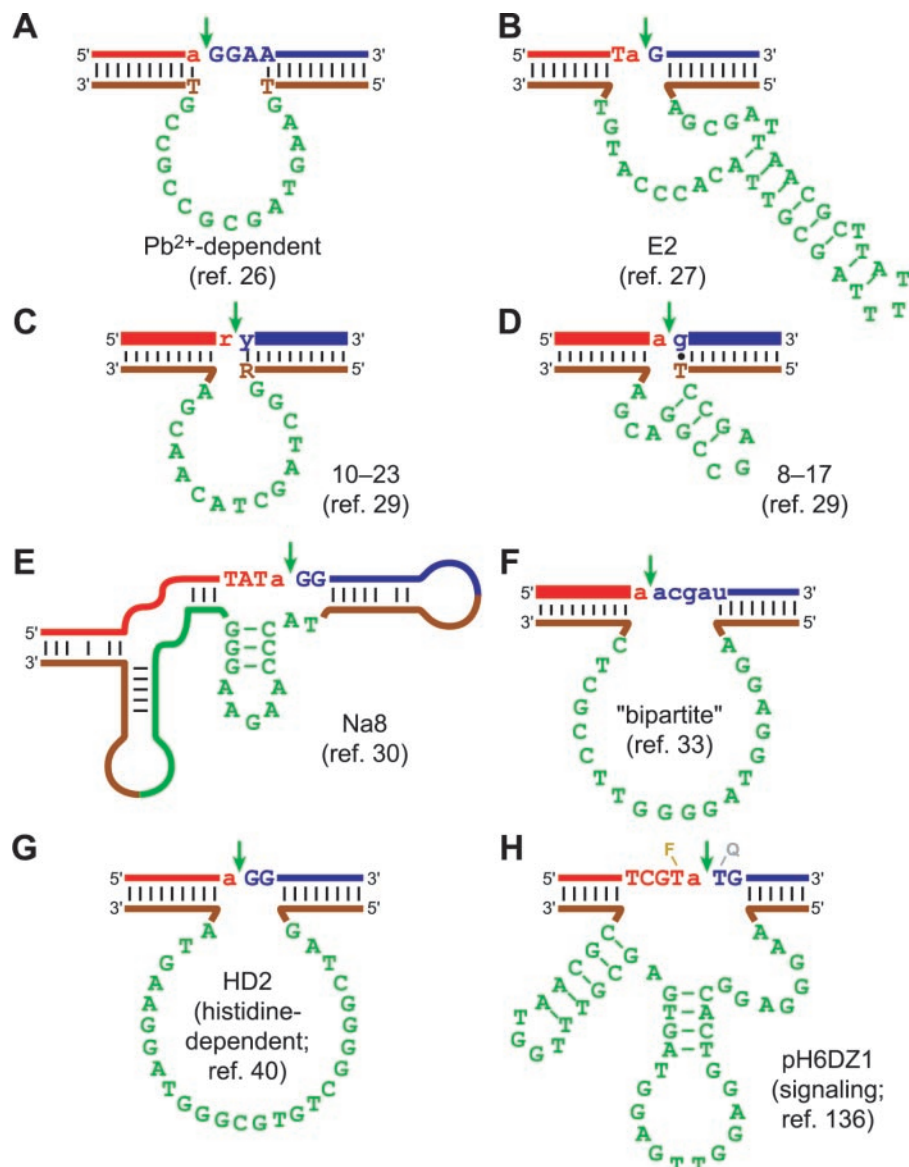


Figure 3. Gallery of RNA-cleaving deoxyribozymes identified by *in vitro* selection. Most of these deoxyribozymes function in the intermolecular format for *in trans* cleavage of an RNA linkage. Thick lines and lowercase letters denote RNA; thin lines and uppercase letters denote DNA, and the green arrow marks the cleavage site. The DNA enzyme regions are green; DNA nucleotides involved in Watson–Crick base pairing are brown; nucleotides of the substrate to the 5'-side of the cleavage site are red; and nucleotides to the 3'-side are blue. For clarity, not all nucleotides are shown explicitly, but this does not necessarily imply a lack of sequence requirements. In panel D, the 8–17 deoxyribozyme is shown with the RNA cleavage site requirement a|g. However, the closely related 17E deoxyribozyme (32) can cleave n|g (although a|g and g|g are preferred). Furthermore, a comprehensive study showed that 8–17 variants can be identified that collectively cleave almost any RNA dinucleotide linkage (36). In panel H, the letters F and Q denote a fluorophore and quencher that are separated upon cleavage by the signaling deoxyribozyme.

(see below). Because the RNA substrate during the selection process was 5'-biotinylated, successful RNA cleavage also separated the biotin tag from the 50 nt (N_{50}) DNA random region. This allowed the isolation of catalytically active DNA sequences by streptavidin chromatography, because the functional DNA sequences lost their biotin tag and were not retained on the column. As in essentially all selection efforts, the 'winning' deoxyribozyme sequences that survived a particular selection round were used to initiate another round of selection, and the entire process was iterated multiple times.

At the end of the selection procedure, when sufficiently high RNA cleavage activity was present in the pool as a whole, individual deoxyribozyme sequences were identified by standard cloning procedures. The new Pb^{2+} -dependent deoxyribozyme was found to interact with the RNA substrate using Watson–Crick binding arms on either side of the initially random enzyme region (sometimes called the catalytic core), as shown in Figure 3A. This modular arrangement spatially separates binding and catalytic functions and has subsequently been found for many of the deoxyribozymes that cleave RNA, as described below. As originally obtained, the

Pb²⁺-dependent deoxyribozyme cleaves near its own 5'-terminus (cleavage *in cis*). Because of the simple Watson-Crick nature of the deoxyribozyme-substrate interactions, engineering the Pb²⁺-dependent DNA enzyme to work intermolecularly (*in trans*) was achieved in straightforward fashion by removing the covalent loop that connects the RNA substrate with the DNA.

When the substrate for the Pb²⁺-dependent deoxyribozyme was replaced with an all-RNA analogue (i.e. not just a single RNA linkage at the cleavage site), catalytic activity was nearly abolished. This suggested that identification of deoxyribozymes for cleavage of all-RNA substrates—which would be of greater practical value—may require use of all-RNA substrates during the selection procedure itself. The Pb²⁺-dependent deoxyribozyme cleaved the lone RNA linkage *in trans* with $k_{\text{cat}} = 1 \text{ min}^{-1}$ and $K_m = 2 \text{ }\mu\text{M}$ at 1 mM Pb²⁺ (pH 7.0) and 23°C (with 0.5 M each NaCl and KCl present as well). From these values, $k_{\text{cat}}/K_m = 5 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$, and multiple-turnover was clearly evident; e.g. 46 turnovers were observed in 90 min. The uncatalyzed rate of RNA cleavage under the same conditions (i.e., spontaneous RNA cleavage in the absence of the deoxyribozyme) was $k_{\text{uncat}} = 10^{-5} \text{ min}^{-1}$, leading to a calculated rate enhancement $k_{\text{cat}}/k_{\text{uncat}}$ of 10^5 . This value is near that of the hammerhead ribozyme (41). Therefore, despite earlier speculation to the contrary (42), even this initial experimental study suggested that deoxyribozymes have no inherent functional limitations relative to their ribozyme cousins, despite the absence of 2'-hydroxyl groups in DNA.

Breaker and Joyce (27) immediately followed up their first study by identifying a Mg²⁺-dependent RNA-cleaving deoxyribozyme. In this second effort, an N₄₀ DNA region was explicitly positioned between two binding arm regions that interacted with their nucleic acid substrate by Watson-Crick base pairs (Figure 2, strategy 2). As in the first effort, a cleavage substrate with only a single ribonucleotide linkage was used. After six rounds of selection, an initial DNA enzyme clone had $k_{\text{obs}} = 0.002 \text{ min}^{-1}$ under single-turnover conditions at 1 mM Mg²⁺ (pH 7.0) and 23°C. Re-selection was performed by partially randomizing the enzyme region to the extent of 15% at each nucleotide and performing seven additional selection rounds (thereby technically making this an 'evolution' experiment, because evolution is selection coupled with introduction of variation after the start of the experiment). The overall effort led to the E2 deoxyribozyme (Figure 3B), which has $k_{\text{obs}} = 0.01 \text{ min}^{-1}$ at 1 mM Mg²⁺ and $k_{\text{obs}} = 0.08 \text{ min}^{-1}$ under saturating Mg²⁺ of >100 mM. As observed for the Pb²⁺-dependent deoxyribozyme, the rate enhancement $k_{\text{obs}}/k_{\text{uncat}}$ of E2 was about 10^5 , and an all-RNA substrate was not cleaved.

A third selection effort for RNA-cleaving deoxyribozymes was undertaken by Faulhammer and Famulok (28). These investigators originally sought a histidine-dependent deoxyribozyme and thus included only a small amount of Mg²⁺ (0.5 mM) in their experiments, which were performed using a selection strategy similar to that used in the initial Pb²⁺ effort (strategy 1 of Figure 2). Here, an N₇₄ region and 10 selection rounds were used, again with a single ribonucleotide linkage as the cleavage site. Surprisingly, the resulting Mg5 deoxyribozyme was histidine-independent and preferred Ca²⁺ over Mg²⁺ as the metal ion cofactor, although Ca²⁺ was never

present during the selection protocol. Indeed, Ca²⁺ was favored by an order of magnitude in rate over Mg²⁺, with k_{cat} for Ca²⁺ of 0.1 min^{-1} and $K_m = 6 \text{ }\mu\text{M}$ at 10 mM Ca²⁺ (pH 7.0) and 37°C ($k_{\text{cat}}/K_m = 1.6 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$). The rate enhancement $k_{\text{cat}}/k_{\text{uncat}}$ for Ca²⁺ was $\sim 10^4$ (43).

IN VITRO SELECTION OF DEOXYRIBOZYMES THAT CLEAVE RNA, PART 2: THE SECOND GENERATION OF EXPERIMENTS

Because all three of the initially identified deoxyribozymes could not cleave an all-RNA substrate, which was never available to the DNA during the selection procedure itself, it seemed opportune to perform a selection experiment in which the substrate had a long stretch of ribonucleotides as the cleavage target. In addition, although the rate enhancements over the uncatalyzed background cleavage rate were high in the initial investigations, catalytic parameters such as k_{cat}/K_m were suboptimal. Santoro and Joyce (29) addressed both of these issues in a landmark study that provided what has become the two most commonly used RNA-cleaving deoxyribozymes. Their experiment used a modified selection approach in which an all-RNA substrate strand was presented to an N₅₀ DNA pool, which could cleave anywhere along the 12 nt length of the RNA sequence (Figure 2, strategy 3).

After 8–10 rounds of selection, cleavage was observed at two favored sites within the 12 nt RNA region. The 8–17 and 10–23 deoxyribozymes (each of which were named for the round and clone number by which they were identified) were chosen for further study because they interacted with the RNA substrate using simple Watson-Crick binding arms, which would facilitate practical applications. Re-selections starting with the initially cloned sequences were used to provide information on the optimal catalytic cores (Figure 3C and D). For 10–23, $k_{\text{cat}} = 0.15 \text{ min}^{-1}$ and $K_m = 0.5 \text{ nM}$ under simulated physiological conditions of 2 mM Mg²⁺, 150 mM NaCl (pH 7.5), and 37°C ($k_{\text{cat}}/K_m = 3 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$). Because the apparent K_m for Mg²⁺ under these conditions was much higher ($\sim 180 \text{ mM}$), k_{cat} at 2 mM Mg²⁺ was clearly not maximal. Under more typical *in vitro* conditions of 50 mM Mg²⁺ (pH 8.0) and 37°C, $k_{\text{cat}} = 3 \text{ min}^{-1}$ and $K_m = 0.8 \text{ nM}$ ($k_{\text{cat}}/K_m = 4 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$). This value of k_{cat}/K_m is 1–2 orders of magnitude *higher* than for the naturally occurring hammerhead or hairpin RNA-cleaving ribozymes, further demonstrating that DNA has no inherent catalytic inferiority relative to RNA. As noted by the authors, 10–23's value of k_{cat} is much less favorable (by 10^4 -fold) than k_{cat} for a protein ribonuclease such as RNase A, but 10–23's value of K_m is much more favorable (by 10^5 -fold). Thus, by the k_{cat}/K_m criterion, deoxyribozymes are at least as good as—if not better than—protein enzymes at cleaving RNA. However, it is clear that a very favorable K_m for 10–23 and related deoxyribozymes can be obtained simply by increasing the binding arm lengths. This is because more Watson-Crick base pairs lead to tighter binding, and of course such a strategy is not available to a protein enzyme. It is important to note that the favorable deoxyribozyme K_m is eventually achieved at the expense of multiple turnover as the binding arm strengths continue to be increased, because product release soon becomes rate-limiting.

Three subsequent studies revealed remarkable convergence upon the catalytic core motif of the 8–17 deoxyribozyme across several selection experiments. In one investigation, Peracchi performed a comprehensive mutagenesis analysis of the 8–17 deoxyribozyme and found that most mutants—as well as the parent DNA enzyme itself—functioned with 10- to 20-fold higher k_{obs} in the presence of Ca^{2+} instead of Mg^{2+} (31). This was reminiscent of the Ca^{2+} -dependent Mg5 deoxyribozyme described above (28), and indeed the Mg5 deoxyribozyme was shown in this same study to incorporate the 8–17 motif within its conserved core. When several non-canonical interactions within the substrate binding arms of the originally identified Mg5 deoxyribozyme were converted to Watson–Crick base pairs, its catalytic activity was improved. These changes also conferred upon Mg5 the ability to cleave an all-RNA substrate, unlike the original Mg5 construct but similar to 8–17 itself. In essence, Mg5 is the 8–17 deoxyribozyme in masked form, although this relationship did not become clear until 8–17 itself was formally identified (29) and direct comparisons were made.

In a second investigation, Lu and coworkers (32) used selection strategy 2 of Figure 2 with the focused goal of finding DNA enzymes that operate with transition metal ion cofactors like Zn^{2+} rather than alkaline earth metals like Mg^{2+} or Ca^{2+} . They successfully identified the Zn^{2+} -dependent 17E deoxyribozyme, which—like the Mg5 deoxyribozyme—was found to have the 8–17 catalytic core. An explanation for the varying divalent metal ion dependencies of 8–17 and 17E was unclear on the basis of their data. Although 8–17 itself was reported to cleave only A↓G RNA linkages (29), 17E cleaves A↓G and G↓G equally well and about an order of magnitude faster than it cleaves either U↓G or C↓G.

Finally, in a comprehensive effort, Li and coworkers (36) identified a family of 8–17-like deoxyribozymes that collectively cleave almost any RNA dinucleotide junction. Their selection strategy was initially designed to identify entirely new RNA-cleaving deoxyribozymes using strategy 2 of Figure 2. However, closely related variants of the 8–17 catalytic motif rapidly came to dominate all of the pools. This was strong evidence that the 8–17 motif is the simplest solution for the RNA cleavage problem and therefore highly favored in DNA sequence space, just as the small hammerhead ribozyme motif is favored in RNA sequence space (44).

Considering the interest in the roles of metal ions in nucleic acid catalysis, Geyer and Sen (30) sought RNA-cleaving deoxyribozymes that functioned independently of divalent metal ions, M^{2+} . At the time of their study (1997), the ability of some natural ribozymes to function without M^{2+} was not yet appreciated (45–47), so their effort was particularly thought-provoking. Using strategy 2 of Figure 2, the self-cleaving Na8 deoxyribozyme (Figure 3E) was identified after 12 selections rounds, cloning and six additional selection rounds. The k_{obs} for the Na8 deoxyribozyme was relatively modest [0.007 min^{-1} at 0.5 M NaCl (pH 7.0) and 25 °C], but considering the estimated k_{uncat} of only $\sim 3 \times 10^{-10} \text{ min}^{-1}$ in the absence of M^{2+} , the rate enhancement $k_{\text{obs}}/k_{\text{uncat}}$ of 2×10^7 was remarkable. The M^{2+} -independence of the Na8 deoxyribozyme was convincingly demonstrated via a wide range of control measures and analytical assays. Adding common divalent metal ions such as Mg^{2+} , Ca^{2+} or Zn^{2+} had no effect upon activity. Furthermore, these M^{2+} were ineffective in the

absence of Na^+ , whereas monovalent ions other than Na^+ were also effective cofactors. Several of the RNA-cleaving deoxyribozymes from a separate effort by Faulhammer and Famulok (28) as mentioned above are clearly related to Na8 by sequence (30) and were shown to be metal-independent DNA enzymes as well (43).

Another study from the Sen laboratory led to a DNA enzyme with no apparent relationship to the 8–17 deoxyribozyme (33). Using an N_{40} random region along with strategy 1 of Figure 2 for 12 rounds of selection with a single ribonucleotide in the substrate; cloning; and then seven rounds of re-selection with a substrate incorporating several ribonucleotides, the ‘bipartite DNAzyme’ was identified (Figure 3F). This deoxyribozyme also interacts with its RNA substrate using Watson–Crick binding arms, but it prefers several unpaired nucleotides near the cleavage site. Under multiple-turnover conditions with one particular substrate, $k_{\text{cat}} = 1 \text{ min}^{-1}$ and $K_{\text{m}} = 200 \text{ nM}$ [30 mM Mg^{2+} (pH 7.4), 37°C]. The value of $k_{\text{cat}}/K_{\text{m}} = 5 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ is only an order of magnitude lower than such values for natural ribozymes such as the hammerhead and hairpin ribozymes. An independent effort by Benner’s group led to the ‘614 deoxyribozyme’ that is Mg^{2+} -independent and also unrelated to 8–17 (48).

Protein enzymes commonly use organic cofactors for catalysis, so Roth and Breaker (40) sought to determine if RNA-cleaving deoxyribozymes could depend not on divalent metal ions but on a cofactor. Using strategy 2 of Figure 2 with an N_{40} region, and including EDTA to ensure the absence of free M^{2+} , they identified several histidine-dependent deoxyribozymes such as HD2 (Figure 3G). This process took 11 rounds of selection followed by five rounds of re-selection, and the resulting deoxyribozymes indeed require L-histidine as a cofactor with $K_{\text{d,app}}$ of $\sim 25 \text{ mM}$ or greater. The pH dependence of the reaction suggested a general base role for the imidazole moiety of histidine during catalysis. The HD2 deoxyribozyme requires K^+ as well as imidazole, suggesting the involvement of K^+ -dependent DNA structures such as G quartets.

TWO PRACTICAL CONSIDERATIONS FOR THE SELECTION EXPERIMENTS

In all of the above studies, streptavidin (or a related protein) and a biotinylated substrate were used as the physical basis of the selection effort. In principle, gel-based methods could instead be used to separate products on the basis of size changes after RNA cleavage, analogous to what has been done recently to identify deoxyribozymes that ligate RNA (49). Nevertheless, the biotin-based methods appear to be quite effective for identifying RNA-cleaving deoxyribozymes, so other methods have not been necessary in practice.

It may not be immediately obvious why all of these selection procedures need to be iterated for multiple rounds to identify catalytically active DNA sequences. If only particular DNA sequences are catalytically competent to cleave RNA, then why are they not identified in just a single round of selection? The resolution of this issue lies in recognizing that without exception, every DNA sequence has a nonzero probability of having the attached RNA substrate become cleaved during the allotted incubation time. For most DNA

sequences drawn from a random pool, this probability is quite low; the DNA sequence is essentially 'inactive', and RNA cleavage is not catalyzed by the attached DNA. However, there are a very large number of such catalytically incompetent DNA sequences in the initial pool. For a typical 200 pmol initial pool ($\sim 10^{14}$ molecules), even if a large number (e.g., 10^6) of the DNA sequences truly have substantial RNA cleavage activity, the vast majority of the pool ($10^{14} - 10^6 \approx 10^{14}$ molecules) consists of 'inactive' DNA sequences. Simply by chance, some of these nominally inactive DNA sequences will have their RNA substrate cleaved during any particular incubation period, and these inactive DNA sequences will thus survive the selection round. The fraction of the pool that contains truly catalytically competent DNA sequences increases during each round of the selection procedure; i.e., the pool becomes enriched in catalytically active sequences. However, multiple rounds are always needed in practice for this enrichment to reach the point where the truly active sequences constitute the dominant population. Experience suggests that 4–10 rounds are typically needed for unambiguous RNA cleavage activity to emerge from a random DNA pool of length N_{40} – N_{50} . Of course, the exact number of rounds to observe this emergence depends strongly on the details of the selection design.

CHARACTERIZATION OF DEOXYRIBOZYMES THAT CLEAVE RNA

The rate constants and catalytic efficiencies included within the above descriptions show that highly active RNA-cleaving deoxyribozymes can be obtained by *in vitro* selection. In terms of mechanism, diffusion-controlled catalytic efficiency k_{cat}/K_m in the range of $10^9 \text{ M}^{-1} \text{ min}^{-1}$ with considerable turnover (29,50) indicates that product release is not necessarily rate-limiting. Although RNA-cleaving deoxyribozymes are often well-behaved in terms of their kinetics, in at least one case the 10–23 deoxyribozyme was observed to have biphasic kinetics under single-turnover conditions (51), which suggests the potential for mechanistic complexity. In general, the mechanisms of RNA-cleaving deoxyribozymes are poorly understood, and this is an area deserving of experimental attention.

For many years, it was thought that all ribozymes are obligatory metalloenzymes; i.e., that they require metals with valence of two or more (52,53). However, a number of studies have shown that some natural ribozymes can function without divalent metal ions, although in such cases unusually high concentrations of monovalent metal ions (e.g. 4 M Li^+) are typically necessary (45–47). Similarly, some RNA-cleaving deoxyribozymes such as Na8 do not require divalent metal ion cofactors (30,43). Because the mechanisms of RNA-cleaving deoxyribozymes are poorly understood, the implications of divalent metal ion dependence (or independence) are unclear. Curiously, binding of Ca^{2+} to the Mg5 deoxyribozyme involves at least two cooperative binding events in the intramolecular form of cleavage, whereas Mg^{2+} binding does not involve cooperativity (43). For a shortened version of the 10–23 deoxyribozyme termed ML6, a specific nucleotide deletion switched the metal dependence of activity from favoring Mg^{2+} to favoring Ca^{2+} (54). Overall, the roles of divalent

metal ions in DNA-catalyzed RNA cleavage appear to be complicated.

The pH value is another experimental variable that can be manipulated to provide information on deoxyribozyme mechanisms. When they have been examined, most RNA-cleaving deoxyribozymes are found to have a log-linear dependence of rate on pH with slope near unity (32,50,55). This is consistent with the requirement for a single deprotonation event during the reaction, similar to many ribozymes in this regard (56–59). In contrast, the relative pH-independence of the Na8 deoxyribozyme and the 'bipartite DNAzyme' resembles that of the HDV ribozyme and suggests acid-base catalysis rather than metal-ion-assisted 2'-hydroxyl deprotonation (30,33). However, this type of mechanism has not yet been demonstrated experimentally for deoxyribozymes.

A common feature for all of the known RNA-cleaving deoxyribozymes is the unpaired nature of the ribonucleotide at the cleavage site. This unpaired nucleotide has plausibly been speculated to provide the structural flexibility necessary to allow an in-line attack conformation of the attacking 2'-hydroxyl nucleophile (39,43), although more direct experimental support for this hypothesis must be obtained. Several of the RNA-cleaving deoxyribozymes (e.g. Na8 and 'bipartite') prefer several unpaired nucleotides in the substrate near the cleavage site (Figure 3). Because the 10–23 and 8–17 deoxyribozymes need only one such unpaired nucleotide, this suggests different structural constraints upon the respective cleavage mechanisms. It may not be a coincidence that the Na8 deoxyribozyme and the 'bipartite DNAzyme' differ from 10–23 and 8–17 in both the nucleotide pairing near the cleavage site and the pH dependence of their reactivity.

For proteins and nucleic acids alike, mutagenesis is a common approach to explore structure-function studies. Mutational analysis of the enzyme region has been performed for several RNA-cleaving deoxyribozymes, including 10–23 (60) and 8–17 (31,61). Effects of mutations to the 10–23 binding arms near the cleavage site have also been examined, and rather subtle modifications were found to have a strong influence upon activity (62). Additional biophysical analysis of both folding and function has been performed for the 10–23 deoxyribozyme (63–65), the 8–17 deoxyribozyme (66–68) and the original Pb^{2+} -dependent deoxyribozyme (69).

From the structural biology viewpoint (i.e. X-ray crystallography and NMR spectroscopy), almost nothing is known about deoxyribozymes that cleave RNA. Only one attempt to crystallize a deoxyribozyme has been reported, using the 10–23 deoxyribozyme (70). However, the structure was not of the catalytically active RNA:DNA complex (71), so mechanistic information could not be inferred (the structure was instead an interesting 2:2 RNA:DNA complex that resembles a Holliday junction). Precise and mechanistically relevant high-resolution structural information on any RNA-cleaving deoxyribozyme will hopefully be obtained in the near future. These efforts should be particularly informative with regard to understanding the metal-ion dependence of RNA-cleaving deoxyribozymes. Likely targets for study are the 8–17 variants that prefer Mg^{2+} , Ca^{2+} or Zn^{2+} as well as the 8–17-related Na8 deoxyribozyme that is M^{2+} -independent, along with other deoxyribozymes that have curious metal dependencies such as the 'bipartite DNAzyme' (33).

APPLICATION OF DEOXYRIBOZYMES THAT CLEAVE RNA AS *IN VITRO* 'RESTRICTION ENZYMES' FOR RNA

A significant reason to study nucleic acid enzymes is their conceptual relevance for understanding catalysis by naturally occurring nucleic acids, and the efforts described above indicate the progress in this regard. Artificial ribozymes and deoxyribozymes may also be used for practical applications. Deoxyribozymes are being applied with ever-increasing frequency as straightforward laboratory reagents for cleavage of specific RNA sequences—essentially, as 'restriction enzymes' for RNA (72). The 10–23 and 8–17 deoxyribozymes are currently the workhorses for this purpose. The potential use of DNA for practical RNA cleavage was recognized in the initial report that described 10–23 and 8–17 (29), and other instances have since been reported. For example, RNA-cleaving deoxyribozymes have been used to assist with large-scale RNA preparation for structural biology experiments (73) and for small-scale RNA cleavage reactions (74–76). Other applications along these lines are noted in a later section.

The original 10–23 and 8–17 deoxyribozymes have substrate sequence requirements of R↓Y and A↓G, respectively, where R denotes a purine (A or G) and Y is a pyrimidine (U or C). Despite these limitations, 10–23 and 8–17 are quite useful in practice. The family of 8–17-like deoxyribozymes obtained by Li and coworkers (36) expands the range of RNA sequences that may be cleaved, thus providing a nearly universal means of cleaving RNA using catalytic DNA. With the 8–17 variants of Li and coworkers, most dinucleotide sequences N↓N may be cleaved *in vitro* with reasonable rate by choosing the appropriate deoxyribozyme; only the combinations Y↓C and N↓U are consistently poor substrates. In our laboratory, we routinely use their 8–17 variants to cleave RNA sequences for preparative purposes and also during *in vitro* selection procedures that require RNA cleavage (77,78) (S. K. Silverman and coworkers, unpublished data). In general, for *in vitro* applications in which an RNA substrate needs to be cleaved site-specifically, a deoxyribozyme such as 10–23 or 8–17 (or one of their variants) should be considered for this purpose.

For a deoxyribozyme to cleave its RNA target, binding between the DNA and RNA must occur. A proposed rule of thumb for designing the binding arms is that each arm should have a binding free energy (ΔG°) of at least 10–12 kcal/mol (72), as calculated on the basis of tabulated RNA:DNA interaction energies (79). This is a good guideline when the deoxyribozyme is used in excess of the cleavage substrate; i.e., under single-turnover conditions. In contrast, multiple-turnover conditions require that the deoxyribozyme dissociate from the cleavage products, which suggests the use of shorter binding arms. For *in vitro* applications, an excess of deoxyribozyme can generally be used, and greater binding affinity ensures maximal RNA cleavage activity. Another benefit of longer Watson–Crick RNA:DNA binding arms is that including additional DNA nucleotides can help to disrupt secondary structure near the cleavage site in the RNA target, when such secondary structure would otherwise inhibit binding of the DNA. Similarly, modifying the deoxyribozyme's binding arms by incorporating 2'-methoxy or locked nucleic acid (LNA) nucleotides can markedly improve cleavage

activity by increasing the binding affinity for the RNA target (80–82).

APPLICATION OF DEOXYRIBOZYMES THAT CLEAVE RNA FOR *IN VIVO* TARGETING OF mRNA

A logical extension of DNA's ability to cleave RNA *in vitro* is the application of RNA-cleaving deoxyribozymes for cleaving mRNAs *in vivo*. Several reviews have been published concerning *in vivo* mRNA cleavage by deoxyribozymes (83–85), some of which include extensive tables and literature references (86–89). A growing number of individual reports have described the *in vivo* application of RNA-cleaving deoxyribozymes to degrade mRNA in cell culture and sometimes even in animal models; representative examples are cited here (51,90–99). In general, RNA-cleaving deoxyribozymes should have an important place alongside other *in vivo* therapies that are based on nucleic acids, such as antisense oligonucleotides, RNA-cleaving ribozymes and siRNA (86,100–103).

Several issues that are not of concern for *in vitro* applications become essential to consider for *in vivo* studies. The deoxyribozyme must be delivered intracellularly to interact with its mRNA target. Single-stranded DNA expression vectors (104–109), electroporation (110), and modified delivery agents (111) have been used for this purpose. Once inside a cell, the DNA must remain stable on a relevant timescale. The use of modified LNA nucleotides, which enhances substrate binding affinity as noted above, additionally helps to increase specificity in targeting a desired mRNA among all of the other RNAs within a cell (112). To suppress nuclease degradation, covalent conjugation (113) or terminal modification of deoxyribozymes have been used (114,115). Sometimes more than one stabilization strategy has been applied simultaneously (116).

Due to the complexities of living cells, extrapolating efficacy from *in vitro* assays is sometimes misleading, and it can be frustrating to find experimentally that a deoxyribozyme known to work *in vitro* fails *in vivo*. One reason for such failure may be the fact that deoxyribozymes such as 10–23 and 8–17 were identified by *in vitro* selection using different RNA substrates than are actually targeted in a particular *in vivo* experiment. In such cases, it would be sensible to identify deoxyribozymes that specifically cleave precisely the desired target sequence; such an effort is justified if cleavage of the particular target is a sufficiently valuable objective. Towards this goal, as was done independently with ribozymes (117), selections were performed starting with a known 10–23 deoxyribozyme sequence and selecting for particularly accessible cleavage sites in a certain mRNA target, which were then successfully exploited *in vivo* using the newly identified deoxyribozymes (118).

Cleavage of an mRNA molecule can be viewed not only as a goal in itself, but also as the first step in a multi-step cleavage/ligation route for repairing aberrant mRNAs. Indeed, ribozyme-mediated RNA repair processes have been described (88,119–123). No deoxyribozymes have been reported for this purpose, but the possibility is intriguing, particularly because of the advantages of deoxyribozymes over ribozymes for mRNA cleavage applications in terms of cost and *in vivo* stability.

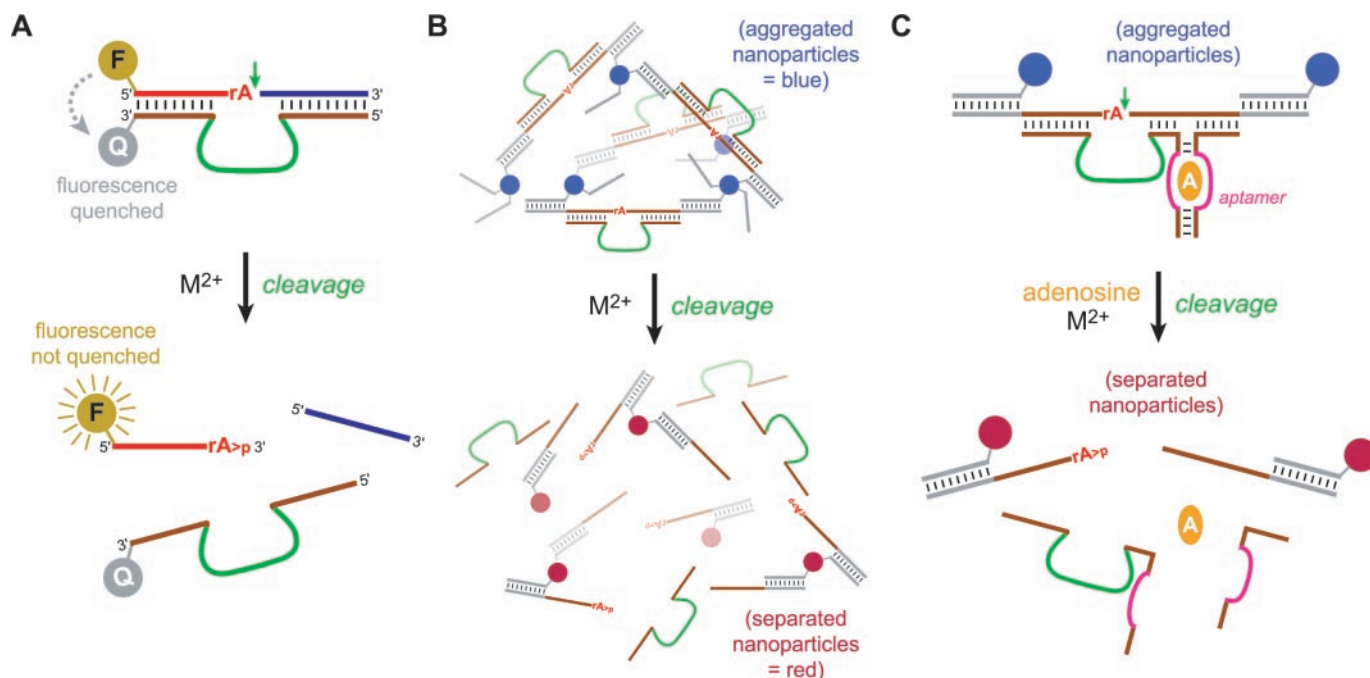


Figure 4. RNA-cleaving deoxyribozymes as sensors. (A) Transducing DNA-catalyzed RNA cleavage into a fluorescence signal by separation of a fluorophore and a quencher. These are denoted F and Q, respectively (e.g., TAMRA and Dabcyl, although other combinations may be used). The fluorophore and quencher may be placed in various places on the RNA substrate and deoxyribozyme, as long as RNA cleavage results in physical separation of F and Q (125). (B) Using gold nanoparticles to induce a colorimetric signal upon RNA cleavage, which separates the nucleic acid strands that hold the nanoparticles together in an aggregate. The RNA substrates are colored brown to avoid confusion with the aggregated (blue) and separated (red) gold nanoparticles. The gold nanoparticles are shown in a 'head-to-tail' orientation (128,129,134), but subsequent efforts showed that a 'tail-to-tail' orientation is optimal (126,135). (C) An allosteric adenosine sensor that was engineered by combining an aptamer with a deoxyribozyme (129). The deoxyribozyme binds well to the RNA substrate only when adenosine (orange) is bound with the aptamer (pink), whereupon RNA cleavage is transduced to a color change via separation of the gold nanoparticles.

APPLICATION OF DEOXYRIBOZYMES THAT CLEAVE RNA AS SENSORS

A sensor can be created by the combination of a molecular recognition event for a particular analyte with a signaling event. Nucleic acid enzymes are particularly amenable to sensor applications because their catalytic activities can be modulated by specific analytes and because these activities can be transduced into optically detected changes in several ways. The Lu research group interfaced a Pb^{2+} -dependent RNA-cleaving deoxyribozyme (32) with optical assays to create sensitive Pb^{2+} detectors. In these experiments, Pb^{2+} is a required cofactor for DNA-catalyzed RNA cleavage, so the sensor is directly activated by the metal ion analyte. As shown in Figure 4A, cleavage of an RNA substrate that is labeled with both a fluorophore and a quencher (e.g., TAMRA and Dabcyl) results in a fluorescence increase due to physical separation of the quencher from the fluorophore (124). Several improvements to this system were made (125,126), including the use of surface immobilization to improve the detection limit to 10 nM Pb^{2+} (127). Alternatively, gold nanoparticle technology was exploited to design colorimetric Pb^{2+} sensors, using the principle that DNA-catalyzed RNA cleavage disrupts gold nanoparticle aggregations, which changes the color from blue to red (Figure 4B) (128). The same group also designed an allosteric deoxyribozyme sensor for adenosine by integrating an adenosine aptamer unit into the complex between deoxyribozyme and RNA substrate (Figure 4C) (129). The latter approach is related to a sensing assay reported

by Sen and coworkers (130–132), in which binding of the oligonucleotide regulator was not dependent on binding of a separate analyte; thus their system directly sensed the oligonucleotide regulator itself. In general, such methods have great promise for development of sensors based on RNA-cleaving deoxyribozymes (22,133).

In separate efforts, Li and coworkers used *in vitro* selection to identify fluorescently signaling RNA-cleaving deoxyribozymes (34), a collection of which has very broad specificities for pH and metal ions (35). A key element of these studies was that the fluorophore and quencher combination were present throughout each selection effort, rather than being grafted onto a functional deoxyribozyme at the end of the selection process. Therefore, the desired fluorescence signaling activity was directly identified. The Li group has recently characterized one of their signaling deoxyribozymes, pH6DZ1, finding that it has a rather complex secondary structure when compared with 10–23 and 8–17 (Figure 3H) (136). Although not technically RNA-cleaving deoxyribozymes, an entire collection of 'structure-switching signaling aptamers' has also been created by the same research group (137). In these designs, an interaction between a nucleic acid and an analyte controls a fluorescence signal without any catalytic events. It is likely that the principles from these experiments can be adapted for regulation of deoxyribozyme catalysis. Indeed, the first report of a signaling deoxyribozyme also described creation of an ATP-dependent allosteric sensor (34), and another effort along these lines was recently reported (138).

Other researchers have used RNA-cleaving deoxyribozymes as sensors in other ways. For example, Stojanovic and coworkers (139) have created fluorescent oligonucleotide sensors and also detected protein–ligand interactions by tethering biotin to a deoxyribozyme’s RNA substrate and modulating RNA cleavage activity with streptavidin. In general, combining the principles of deoxyribozyme catalysis with various types of sensor assays appears to be a fruitful approach for practical sensor development.

OTHER APPLICATIONS OF DEOXYRIBOZYMES THAT CLEAVE RNA

RNA-cleaving deoxyribozymes have been applied for purposes beyond those mentioned above. For example, such deoxyribozymes have been used for detecting *in vitro*-generated RNA modifications (140); for mapping sites of *in vitro* RNA crosslinking (141); for mapping RNA branch points (72,142,143); for probing higher-order RNA structure (144); for quantifying nucleic acid production during PCR amplification (145); for analyzing nucleic acid sequence mutations (146); for detecting specific microbial rRNAs (147); for constructing logical computation circuits (148–151); and for manipulating a DNA-based nanodevice (152). A deoxyribozyme has been identified that cleaves unnatural RNA linkages, which may be useful for certain biotechnology applications (153). It is likely that additional interesting applications of RNA-cleaving deoxyribozymes will be developed in the future.

RNA-CLEAVING DEOXYRIBOZYMES WITH NONSTANDARD NUCLEOTIDES

RNA-cleaving deoxyribozymes with protein-like functional groups have been selected from random-sequence DNA pools that were prepared incorporating one or more unnatural nucleotides (154–157). These unnatural nucleotides incorporate functionality such as imidazole and primary amino groups, which are not found among the natural DNA nucleobases. In some cases, use of such unnatural nucleotides requires a technical advance in the selection methodology; e.g., more tolerant DNA polymerases need to be identified (158). A complete discussion of the catalytic possibilities that are enabled by inclusion of unnatural nucleotides is beyond the scope of this review. Nevertheless, it is clear that in principle, including unnatural functional groups allows chemical possibilities beyond those provided by standard DNA alone. Of course, for practical applications it must be determined empirically whether or not these new chemical possibilities justify the synthetic effort necessary to use nonstandard DNA nucleotides in identifying new deoxyribozymes.

Nonstandard functional groups within nucleic acid enzymes are not limited to those such as imidazole with special acid-base properties. For example, at least two research groups have described photochemically controlled deoxyribozymes (159,160). This shows that unnatural nucleotides can be incorporated into DNA enzymes not for their direct catalytic contributions but because they can modulate deoxyribozyme function. Unnatural nucleotides can also be used to stabilize the deoxyribozymes against nuclease degradation (161), which is important for *in vivo* applications.

DEOXYRIBOZYMES THAT LIGATE RNA

RNA ligation by a deoxyribozyme is conceptually the ‘opposite’ of RNA cleavage, because two RNA strands are joined instead of cleaved. If the appropriate functional groups are involved, then RNA ligation by a deoxyribozyme can be the precise mechanistic reverse of cleavage as well. Our own research group has focused strongly on identifying deoxyribozymes that ligate RNA. In contrast to the uniform reaction pathway of RNA cleavage (Figure 1), DNA-catalyzed RNA ligation can occur in many ways, depending on the identities of the substrates and the pathways by which they can react. Not surprisingly, a wider variety of chemical reactions has been observed for DNA-catalyzed RNA ligation when compared with RNA cleavage [see (78,162,163) and references therein]. These RNA ligation experiments offer insight into the workings of nature’s nucleic acid catalysts such as the ribosome (5) and spliceosome (2,3), which also modulate bond-forming reactions. Furthermore, some RNA-ligating deoxyribozymes provide routes to important biomolecular targets such as lariat RNAs that would be challenging or impossible to prepare by other means (163). Therefore, RNA-ligating deoxyribozymes offer practical utility as well as conceptual insights. Applications of deoxyribozymes that ligate RNA will certainly increase in parallel with the applications of deoxyribozymes that cleave RNA.

PERSPECTIVE

In only a little over a decade since their initial identification, artificial RNA-cleaving deoxyribozymes have grown from a laboratory curiosity into widely appreciated research tools both *in vitro* and *in vivo*. Fundamental investigations of these deoxyribozymes should continue to offer substantial insight into how catalysis can be achieved with nucleic acid enzymes. For example, the identification of histidine-dependent deoxyribozymes (40) prompts a more general question: what are the limits and implications of DNA and RNA catalysis that is mediated by organic cofactors? Equally important are the opportunities enabled by combining DNA-catalyzed RNA cleavage with practical applications. Such applications currently range from *in vitro* preparative reactions to *in vivo* mRNA degradation to realistic sensor systems. In all of these areas, improvements can be expected. For example, a future direction likely lies in using array technologies to exploit RNA-cleaving deoxyribozyme sensors to the greatest possible extent, much as ribozyme arrays are currently being explored (164). One may anticipate that microfluidic and nanofluidic technologies (165) will also be integrated into devices for applications that depend upon RNA-cleaving deoxyribozymes.

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