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Review

Deoxyribozymes: useful DNA catalysts *in vitro* and *in vivo*

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Abstract. Deoxyribozymes (DNA enzymes; DNAzymes) are catalytic DNA sequences. Using the technique of *in vitro* selection, individual deoxyribozymes have been identified that catalyze RNA cleavage, RNA ligation, and a growing range of other chemical reactions. DNA enzymes have been used *in vitro* for applications such as biochemical RNA manipulation and analytical assays for metal ions, small organic compounds, oligonucleotides, and proteins. Deoxyribozymes have also been utilized as *in vivo* therapeutic agents to destroy specific mRNA targets. Although many conceptual and practical challenges remain to be addressed, deoxyribozymes have substantial promise to contribute meaningfully for applications both *in vitro* and *in vivo*.

Keywords. Deoxyribozyme, DNA enzyme, DNAzyme, RNA cleavage, RNA ligation, *in vitro* selection, *in vivo* therapeutic agents.

Introduction

The discovery of natural RNA catalysts, or ribozymes, in the early 1980s revealed that nucleic acids participate in biology beyond merely storing and transferring genetic information [1, 2]. This breakthrough spurred the identification of artificial ribozymes using *in vitro* selection [3–5], a technique in which many random-sequence RNAs are tested to identify specific sequences that have desired catalytic activities [6]. A great variety of ribozymes have now been identified for catalysis of chemical reactions ranging from RNA cleavage, ligation, and polymerization to the Diels-Alder reaction of two small-molecule substrates [7]. Analogous catalysis by artificial DNA enzymes, or deoxyribozymes (DNAzymes), was first demonstrated experimentally in 1994 by Breaker and Joyce, who used *in vitro* selection to identify a specific DNA sequence that catalyzes Pb^{2+}-dependent cleavage of an RNA phosphodiester linkage [8]. Since that first report, hundreds of deoxyribozymes have been identified, and the range of reactions catalyzed by DNA and the number of applications demonstrated for deoxyribozymes has increased significantly [7, 9–11]. A review in this journal in 2002 described the field of deoxyribozymes at that time [12]. In the present review, we survey the field and specifically discuss the new advances both in fundamental research and in applications of deoxyribozymes to interesting problems in chemistry, biology, and beyond.

In vitro selection to identify deoxyribozymes

Because deoxyribozymes rely on DNA as both information carrier and catalyst during the *in vitro* selection procedure, a scheme to identify deoxyribozymes is conceptually rather simple (Fig. 1). The selection procedure begins with synthesis of a random sequence of DNA, termed the 'random pool', by automated solid-phase synthesis. The random region

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is typically 40–80 nucleotides in length, denoted N_{40}–N_{80}. The length of the random pool depends among other factors on the difficulty of the chemical reaction being catalyzed. The choice is ultimately subjective on the basis of current knowledge, and random regions from 20 to 228 nucleotides in length have been used. The random region is flanked by constant primer-binding regions, which are important to allow PCR amplification as described below. Once the random pool has been designed, an initial portion (typically 10^{14}–10^{15}) of pool molecules is incubated under conditions where catalytically active sequences undergo an appropriate chemical transformation and thereby become physically separable from the vastly larger portion of inactive pool sequences, which remain chemically unchanged. The basis for physical separation of active DNA sequences depends on the catalytic activity being sought. A good example is from the original deoxyribozyme selection for RNA cleavage [8], as illustrated in Figure 1. In this effort, a biotin moiety attached at the terminus of the pool becomes detached from the random region upon self-cleavage by an active sequence, thereby allowing the active sequences to flow through a streptavidin column without being retained. In contrast, the inactive sequences maintained their biotin groups and are retained on the streptavidin column. Once the active sequences have been separated, they are amplified by PCR, which is possible due to the constant primer-binding regions that flank each random region.

The end result of this single round of *in vitro* selection is a pool of DNA molecules that is enriched (relative to the originally random pool) in catalytically active sequences. The overall selection process is then iterated for typically 5–15 rounds until the activity of the pool as a whole is sufficiently high to warrant cloning, identification, and more detailed analysis of individual deoxyribozyme sequences. More than one round of selection is required because inactive sequences have a small but nonzero probability of surviving through any particular round of selection; many selection rounds are therefore needed to ensure that the surviving sequences are reproducibly competent at the desired catalytic activity. Although deoxyribozyme selection schemes (such as that depicted in Fig. 1) are indeed conceptually simple, in practice technical challenges often complicate the implementation.

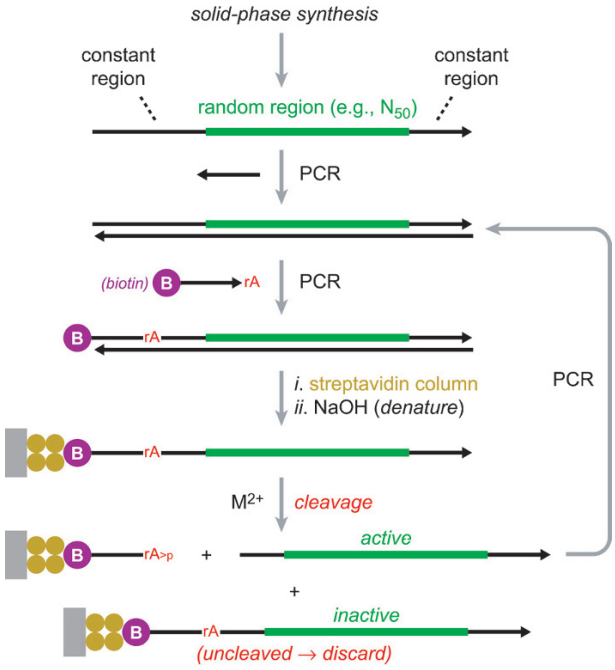

Figure 1. *In vitro* selection of deoxyribozymes, illustrated for selection of RNA-cleaving deoxyribozymes. In this example, the key selection step is based on the detachment of biotin from the DNA random region as a consequence of the RNA cleavage reaction. However, the key selection step can be based on any chemical modification that allows physical separation of catalytically active DNA sequences.

Reactions catalyzed by deoxyribozymes: DNA-catalyzed RNA cleavage

The earliest DNA-catalyzed reaction to be identified was RNA cleavage [8], and this is still the most commonly studied deoxyribozyme activity. Although a comprehensive discussion of DNA-catalyzed RNA cleavage is beyond the scope of this review [13], here we provide a very brief description of the accomplishments to date. Most selection procedures to identify RNA-cleaving deoxyribozymes have been performed with the key step based on biotin as shown in Figure 1. However, some efforts have instead used increased mobility on polyacrylamide gel electrophoresis (PAGE) as the basis for separating catalytically active DNA sequences [14]. The RNA cleavage chemistry has invariably relied on attack of an 2′-hydroxyl group on the adjacent phosphodiester linkage [15, 16], forming a 2′,3′-cyclic phosphate with displacement of a 5′-hydroxyl group (Fig. 2A). This is analogous to the mechanism of RNA cleavage by some but not all natural RNA-cleaving ribozymes, especially including the small and well-studied hammerhead, hairpin, and hepatitis delta virus ribozymes [17]. Some selection experiments have presented a long, continuous RNA stretch to the DNA random region (for which cleavage anywhere in the RNA stretch would correspond to an active deoxyribozyme), whereas other reports have presented only a single RNA nucleotide embedded within an otherwise-DNA context as the cleavage site.

The most commonly studied RNA-cleaving deoxyribozymes are the 10–23 and 8–17 (Fig. 3A, B; see below), which were named for the round number and

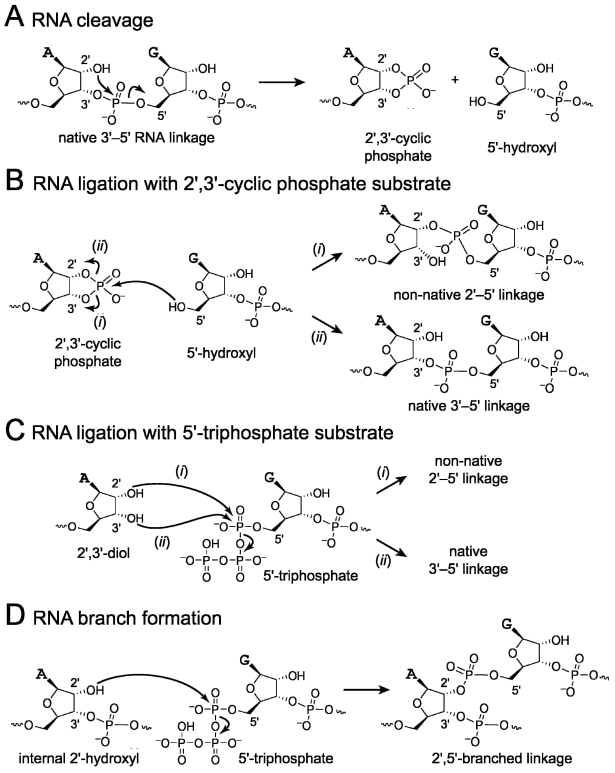

Figure 2. RNA cleavage and ligation reactions catalyzed by deoxyribozymes. (*A*) RNA cleavage by attack of a 2′-hydroxyl at the adjacent phosphodiester linkage. (*B*) RNA ligation by attack of a 5′-hydroxyl at a 2′,3′-cyclic phosphate, which can form either a native 3′–5′ linkage or a non-native 2′–5′ linkage. (*C*) RNA ligation by attack of a terminal 2′,3′-diol group at a 5′-triphosphate, which can form the same two linear linkages as in (*B*). (*D*) RNA ligation by attack of an internal 2′-hydroxyl group at a 5′-triphosphate, forming a 2′,5′-branched linkage.

individual clone number of the selection procedure in which they were identified [18]. Both of these deoxyribozymes require divalent metal ions such as Mg^{2+} for their activity. The 8–17 deoxyribozyme appears to be the simplest DNA motif capable of RNA cleavage [14], analogous to the hammerhead ribozyme as the simplest self-cleaving RNA motif [19], and 8–17 variants have been isolated independently on multiple occasions [14, 18, 20–22]. A collection of deoxyribozymes related to the 8–17 is capable of cleaving nearly any RNA-RNA dinucleotide sequence junction, with different 8–17-like variants used for particular sets of cleavage sites (e.g., Fig. 3C) [14]. RNA-cleaving deoxyribozymes other than the 10–23 and 8–17 have also been identified (e.g., Fig. 3D) [23, 24], including some DNA enzymes that do not require any divalent metal ion cofactor (e.g., Fig. 3E) [25, 26] and another that requires the amino acid histidine as an obligatory cofactor [27] (Fig. 3F).

DNA-catalyzed RNA ligation

Deoxyribozyme-catalyzed RNA ligation rather than cleavage has also been pursued, presaged by an early report of a deoxyribozyme that ligates two DNA substrates [28]. For RNA ligation, more than one combination of functional groups can be used. Experimental efforts to identify deoxyribozymes that ligate RNA began with a 2′,3′-cyclic phosphate RNA substrate, which can react with an RNA 5′-hydroxyl group to form either a native 3′–5′ linkage or a non-

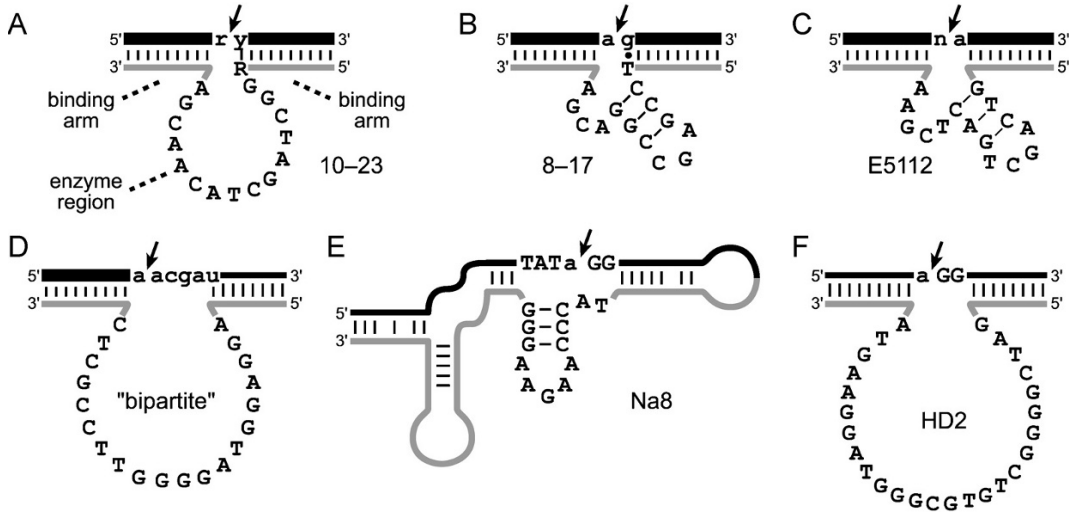

Figure 3. RNA-cleaving deoxyribozymes. Thick lines and lowercase letters denote RNA; thin lines and uppercase letters denote DNA (R = purine, Y = pyrimidine, and N = any nucleotide). The cleavage site is marked with an arrow. (*A*) 10–23 and (*B*) 8–17 deoxyribozymes [18]. Only the two R↓Y and A↓G nucleotides flanking the respective cleavage sites are required by each deoxyribozyme. (*C*) A representative 8–17 variant, E5112, for cleavage of N↓A junctions [14]. The reported variants can be used to cleave 14 out of the 16 possible dinucleotide junctions. (*D*) The 'bipartite deoxyribozyme', named as such because of the clustering of purine versus pyrimidine nucleotides in the enzyme region [23, 24]. (*E*) The Na8 deoxyribozyme, which does not require any divalent metal ion for its activity [25]. (*F*) The HD2 histidine-dependent deoxyribozyme [27].

native 2′–5′ linkage (Fig. 2B) [29]. For reasons not presently understood, DNA-catalyzed formation of the 2′–5′ linkage is favored in this reaction [29–31], although particular combinations of substrate and metal ion cofactor can lead to deoxyribozymes that create the 3′–5′ linkage [32]. It remains an ongoing challenge to develop a selection approach that can reliably lead to 3′–5′ linkages using 2′,3′-cyclic phosphate RNA substrates.

Unrelated RNA-ligating deoxyribozymes have been developed to function with a 5′-triphosphate RNA substrate. An unmodified 2′,3′-diol RNA can react essentially irreversibly with a 5′-triphosphate, displacing pyrophosphate as the leaving group (Fig. 2C). Successful creation of native 3′–5′ linkages has been achieved in this reaction by special approaches [33], such as imposition of a strict selection pressure that allows survival only of those deoxyribozymes that create the desired bond [34]. The selection pressure was based on the highly 3′–5′-selective cleavage of RNA by the 8–17 deoxyribozyme; unfortunately, the reversibility of the ligation reaction of Figure 2B thwarts any attempt to apply a similar strategy with the 2′,3′-cyclic phosphate substrate. One report described deoxyribozymes that use either Mg^{2+} or (separately) Zn^{2+} as a cofactor and create 3′–5′ linkages from a 5′-triphosphate substrate with useful rate, yield, and RNA substrate sequence generality (Fig. 4) [35]. Experiments are currently in progress to obtain a collection of deoxyribozymes that allow creation of all possible RNA ligation junction sequences [D. A. B., S. K. S., and co-workers, data not shown], in analogy to the available collection of RNA-cleaving deoxyribozymes as discussed above [14].

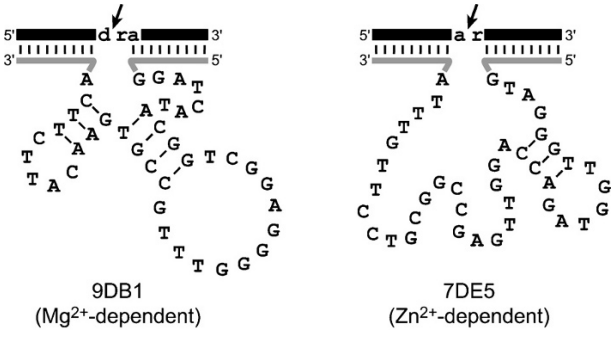

Figure 4. RNA-ligating deoxyribozymes that form native 3′–5′ linkages [35]. 9DB1 requires Mg^{2+} and a D↓RA ligation junction (R = purine; D = any nucleotide except C); 7DE5 requires Zn^{2+} and an A↓R ligation junction.

With a 5′-triphosphate RNA substrate, an alternative RNA ligation reaction forms not linear RNA as in Figure 2C but 2′,5′-branched RNA via nucleophilic attack of a specific internal 2′-hydroxyl group at the 5′-triphosphate (Fig. 2D). In the same selection experiment it has been possible to identify multiple deoxyribozymes, some that create linear RNA and others that create branched RNA, because all of these products are formed via attack of a hydroxyl group at the 5′-triphosphate [34]. The first RNA ligase selection experiment with a 5′-triphosphate substrate led to formation of only branched RNA [36, 37]. Several of the new deoxyribozymes were additionally able to create lariat RNA, which is the subclass of branched RNAs that have a closed loop as formed in the natural pre-mRNA splicing process [38–40]. A subsequent selection effort also led to formation of branched RNA by the 7S11 deoxyribozyme [41], which forms a very interesting three-helix-junction architecture in combination with its two RNA substrates (Fig. 5A) [42]. 7S11 and its variants have been exploited both for additional selection experiments and for applications, as described below. A more sequence-tolerant variant of 7S11, 10DM24, was subsequently reported [43], and 10DM24 is currently the most useful deoxyribozyme for creating various sequences of 2′,5′-branched RNA. Other branch-forming deoxyribozymes have been identified [44], including one DNA enzyme that is especially proficient at creating lariat RNA [45].

DNA-catalyzed reactions other than RNA cleavage or ligation

In addition to RNA cleavage and ligation as described above, Table 1 shows that many other DNA-catalyzed reactions have been identified [7, 9, 10]. This tabulation surely does not represent the limit of what is possible, and more experiments are needed in this regard. For extending the capabilities of DNA as a catalyst with nucleic acid substrates, the general three-helix-junction platform of 7S11 and related deoxyribozymes has been used to explore new deoxyribozyme function. In one instance, the electrophilic 5′-triphosphate moiety and the adjacent guanosine nucleoside were disconnected as a unit from the remainder of the RNA substrate, leading to a deoxyribozyme that uses GTP as a discrete small-molecule substrate by associating with an engineered binding site (Fig. 5B) [60]. The GTP substrate was bound by Watson-Crick hydrogen bonds, as shown by switching of selectivity to favor ATP when the corresponding deoxyribozyme nucleotide was changed from C to T. In another example, new deoxyribozymes were identified that create tyrosine-RNA nucleopeptide linkages [49]. This was achieved by performing *in vitro* selection with a tyrosine in place of the branch-site ribonucleotide while retaining the three-helix junction arrangement (Fig. 5C).

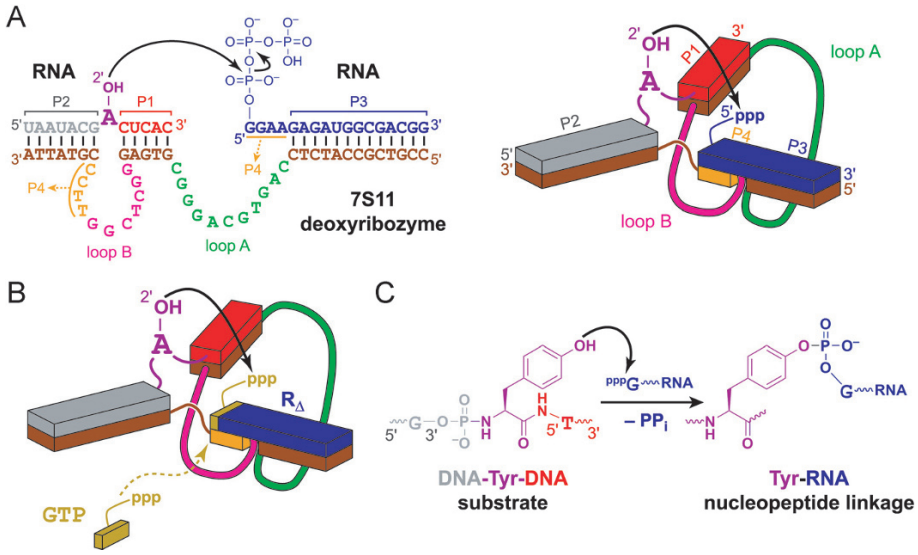

Figure 5. Deoxyribozymes that form three-helix-junction (3HJ) complexes with their substrates. (*A*) The 7S11 deoxyribozyme that forms 2′,5′-branched RNA [41,42]. At left is the secondary structure depicting the four paired regions P1–P4; at right is a schematic three-dimensional model emphasizing the 3HJ structure. (*B*) A variant of 7S11, 10DM24 [43], engineered to accept GTP as a discrete small-molecule substrate [60]. R_Δ is the obligatory oligonucleotide cofactor that, along with GTP, composes the nucleotides of the original 5′-triphosphate-RNA substrate from panel *A*. (*C*) Reaction catalyzed by the Tyr1 deoxyribozyme, which forms a tyrosine-RNA nucleopeptide linkage [49]. The substrate providing the nucleophile is a DNA-Tyr-DNA conjugate, in which the tyrosine replaces the branch-site adenosine of 7S11 (components are colored as in the other panels).

Table 1. Reactions catalyzed by deoxyribozymes.

Reaction catalyzed	Bond	# Random nt	Rate enhancement	M^{2+} requirement	References
RNA cleavage	O–P	50	$\sim 10^5$	Pb^{2+}	[8]
RNA cleavage	O–P	50	n.d.	Mg^{2+}	[18]
RNA cleavage	O–P	40	$\sim 10^8$	none	[25]
RNA ligation (3′–5′ and other)	O–P	40	2×10^4	Zn^{2+}	[32]
RNA ligation (3′–5′)	O–P	40	$\sim 10^4$	Mg^{2+}	[35]
RNA ligation (3′–5′)	O–P	40	$\sim 10^5$	Zn^{2+}	[35]
RNA ligation (branch formation)	O–P	40	5×10^6	Mn^{2+}	[36, 37]
RNA ligation (branch formation)	O–P	40	$\sim 10^5$	Mg^{2+}	[41, 42]
RNA ligation (lariat formation)	O–P	40	$\sim 10^5$	Mn^{2+}	[44, 45]
DNA phosphorylation	O–P	70	$\sim 10^9$	Mn^{2+}	[46]
DNA adenylation (capping)	O–P	70	2×10^{10}	$Mg^{2+} + Cu^{2+}$	[47]
DNA ligation	O–P	116	3×10^3	Cu^{2+} or Zn^{2+}	[28]
DNA ligation	O–P	150	$\sim 10^5$	Mn^{2+}	[48]
Nucleopeptide linkage formation	O–P	40	5×10^5	Mg^{2+} or Mn^{2+}	[49]
Oxidative DNA cleavage	C–O	50	$\sim 10^6$	Cu^{2+}	[50–52]
DNA depurination	C–N	85	9×10^5	Ca^{2+}	[53]
DNA depurination (IO_4^--dependent)	C–N	70	n.d.	none	[54]
Diels-Alder reaction	C–C	36	4×10^5	Ca^{2+}	[55]
Thymine dimer photoreversion	C–C	40	3×10^4	none	[56]
Phosphoramidate cleavage	N–P	72	$\sim 10^3$	Mg^{2+}	[57]
Porphyrin metalation	Cu–N	228	1×10^3	Cu^{2+} or Zn^{2+}	[58, 59]

This tabulation is representative, not comprehensive.
n.d., not determined.

In contrast to the situation with oligonucleotide substrates, utilization of DNA to catalyze reactions of small-molecule substrates has been rather limited. The deoxyribozymes that phosphorylate or adenylate DNA (Table 1) inherently use ATP as a substrate, and the 7S11 variant that was engineered to use GTP as a substrate is discussed above (Fig. 5B). Our research group has recently identified a deoxyribozyme that catalyzes the Diels-Alder reaction between anthracene and maleimide derivatives as two small-molecule substrates [55]; this is analogous to several reported Diels-Alder ribozymes [61, 62]. Other than these limited examples, deoxyribozymes that function with small-molecule substrates largely remain to be identified. The ability of DNA aptamers [63] to bind to a wide range of targets [64, 65] bodes well for these future efforts.

Structural and mechanistic characterization of deoxyribozymes

In general, considerable structural and mechanistic characterization of deoxyribozymes remains to be performed. The most attention in this regard has been provided to RNA-cleaving deoxyribozymes, in part because they have been studied the longest and in part because they are most commonly used in practical applications (see below). The observation of diffusion-controlled catalytic efficiency for the 10–23 deoxyribozyme (i.e., multiple turnover with k_{cat}/K_m in the range of 10^9 M^{-1} min^{-1}) demonstrates that mechanistic steps other than product release can be rate-limiting [18, 66]. Mutagenesis studies for both the 10–23 [67–70] and 8–17 [71, 72] deoxyribozymes have revealed specific information about nucleotide requirements, including in both cases the establishment of a conserved 'catalytic core'. In the absence of a functionally relevant X-ray crystal structure or NMR structure for any deoxyribozyme, RNA-cleaving or otherwise [73], several studies have focused on understanding the spatial relationship among the double-helical stem regions as a function of incubation conditions [74–76]. A recent study examined the 8–17 deoxyribozyme at the single-molecule level, finding intriguing evidence for a preformed metal binding site in the case of Pb^{2+} but not Mg^{2+} [77]. In principle, a sufficiently detailed analysis using techniques such as FRET and nondenaturing PAGE can allow very detailed understanding of deoxyribozyme structure in the same way that the naturally occurring VS ribozyme has been studied [78], but certainly high-resolution X-ray or NMR data on deoxyribozymes would be welcome.

Of the RNA-ligating deoxyribozymes, the class of three-helix-junction DNA enzymes exemplified by 7S11 (Fig. 5A) has been studied in the most biochemical detail. Because many natural ribozymes have multi-helix junctions [79], 7S11 offers the opportunity to study a specific nucleic acid junction in a well-defined model system. The first report of 7S11 noted that its use of a bulged adenosine as the branch-site nucleophile mimics the first step of natural pre-mRNA splicing [41]. The preference for adenosine over the other three nucleotides in the branch-forming reaction was subsequently confirmed in a more detailed set of selection experiments, indicating that there is some (as yet poorly understood) chemical preference for adenosine to be the branch-site nucleophile [43]. The transformation of a 7S11 variant to accept GTP as a small-molecule substrate as described above (Fig. 5B) [60] suggests that deoxyribozymes permit a useful degree of structural engineering, although the limits of this approach have barely been addressed. It is noteworthy that multiple turnover was unambiguously observed for the 7S11 variant that uses GTP as a small-molecule substrate. For all other RNA-ligating deoxyribozymes, multiple turnover has not been observed, which has been ascribed to product inhibition (i.e., tighter binding of the deoxyribozyme with the ligated product as compared with the unligated substrates) [29]. This is similar to the product inhibition found in RNA ligation by protein enzymes such as T4 DNA ligase, which are used in stoichiometric or greater quantities for practical RNA ligation due to their single-turnover behavior.

Other deoxyribozymes have been characterized biochemically. For example, a deoxyribozyme that photochemically cleaves thymine dimers was revealed to use a G-quadruplex as an 'antenna' [56, 80], and a self-phosphorylating deoxyribozyme was shown to adopt a pseudoknotted structure that also involves a G-quartet [81]. The RNA-cleaving deoxyribozymes have also been studied using nontraditional techniques. In particular, the 8–17 deoxyribozyme was examined using electron hole flow patterns, which provided new information about changes in global geometry and also new specific information about the environment of individual nucleotide residues [82].

In all of the reported DNA-catalyzed reactions, few instances have been found of any deoxyribozyme that forms two or more products in the same reaction. This is consistent with considering deoxyribozymes (and ribozymes) as having enzyme-like selectivities when making or breaking bonds. The few exceptions to this one-deoxyribozyme, one-product rule are illuminating. In the case of the three-helix-junction 7S11 deoxyribozyme that forms 2′,5′-branched RNA, cata-

lytic activity was maintained (but reduced) when the 5′-triphosphate moiety was offset from its normal location by inserting one or more nucleotides into the corresponding RNA substrate [83]. This observation suggests that this deoxyribozyme has some tolerance for changing the relative spatial relationship of the reacting nucleophile and electrophile. Furthermore, when the 5′-triphosphate was offset by insertion of exactly two RNA nucleotides into the substrate, a balance was created in which the deoxyribozyme equally well catalyzed two competing reactions (Fig. 6): (1) Attack of the branch-site adenosine 2′-hydroxyl at the 5′-triphosphate, offset spatially from its normal location by the two inserted nucleotides; and (2) attack of the adenosine 2′-hydroxyl at the original electrophilic phosphate center, which was at the original spatial position but now with a dinucleotide rather than pyrophosphate as leaving group (i.e., a poorer leaving group). A reasonable interpretation is that for 7S11, the 'spatial' and 'leaving group ability' factors are exactly balanced when two nucleotides are inserted at the 5′-triphosphate position, and both products are formed equally well. A second example of DNA enzymes creating more than one product in the same reaction is the group of RNA-cleaving deoxyribozymes that require Cu^{2+} and ascorbate (or Cu^{2+} alone) and function via an oxidative mechanism [50–52]. In this case, the 'region-specific' cleavage events [50] likely involve a reactive and diffusible intermediate that explains the relatively nonselective cleavage chemistry.

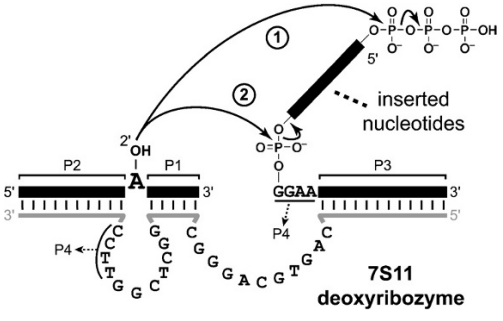

Figure 6. Two competing reactions of the three-helix-junction 7S11 deoxyribozyme when the reactive 5′-triphosphate is offset by from its original position due to insertion of one or more nucleotides [83]. Reaction 1 occurs by attack of the 2′-hydroxyl at the 5′-triphosphate, which is spatially offset from its original position but still with pyrophosphate as the leaving group. Reaction 2 occurs by attack of the 2′-hydroxyl at the original phosphate center, with an oligonucleotide as the poorer leaving group. When the insertion is exactly two nucleotides, the rates of reactions 1 and 2 are equivalent.

Comparison of deoxyribozymes to other biomolecular catalysts

Catalysis by deoxyribozymes may be compared to catalysis by ribozymes or protein enzymes. Nature has evolved certain RNA molecules as well as many proteins for catalytic function, whereas there are no known examples of natural catalytic DNA. When comparing DNA with RNA, the simple consideration that DNA has one less functional group (the 2′-hydroxyl) than does RNA at every nucleotide position has led to speculation that DNA should be catalytically inferior to RNA [84]. However, the available experimental evidence suggests that DNA and RNA actually have comparable catalytic efficiencies, at least where direct comparisons have been made [55]. The rate enhancements for deoxyribozymes as listed in Table 1 support this conclusion, because analogous rate enhancements for ribozymes are in the same range [7].

Some proteins have much larger rate enhancements than those shown for DNA in Table 1. Nevertheless, a detailed analysis of RNA-cleaving catalysts by Breaker and co-workers suggests that nucleic acid enzymes are probably capable of high, protein-like rate enhancements, if multiple catalytic strategies are used simultaneously [85, 86]. Ultimately, experiments will decide whether deoxyribozymes can achieve rate enhancements comparable in magnitude to those of the best protein enzymes. Because RNA and DNA catalysis is a relatively young field, it seems likely that future studies will reveal new examples of catalytic nucleic acids with higher rate enhancements than those already reported.

Applications of deoxyribozymes in chemistry, biochemistry, and biology

A primary impetus for basic research into deoxyribozymes has been their downstream practical application. In particular, RNA-cleaving and RNA-ligating deoxyribozymes have been utilized in many ways, ranging from *in vitro* chemical and biochemical experiments to *in vivo* applications as therapeutics.

RNA-cleaving deoxyribozymes as *in vitro* biochemical and analytical tools

Recombinant DNA technology has been enabled by the availability of restriction enzymes, which allow selective cleavage of a wide range of double-stranded DNA target sequences. Development of equivalent reagents for single-stranded RNA would be very useful for *in vitro* biochemical manipulations of RNA, e.g., for generation of homogeneous termini

to enhance crystallization or NMR spectroscopy [87–89] and for mapping branch points or sites of cross-linking [90–93]. RNA-cleaving deoxyribozymes provide this set of tools for RNA biochemistry. The initial study of the 10–23 and 8–17 deoxyribozymes [18] has been expanded to provide a collection of 8–17 variants that together can be used to cleave nearly any RNA dinucleotide junction, as described above [14]. These variants have already found some direct applications [34, 94], and many additional examples are likely to follow. In particular, deoxyribozymes are an effective alternative to other methods for preparative RNA cleavage, such as use of RNase H and a DNA/2′-*O*-methyl oligonucleotide template [89, 95].

RNA-cleaving deoxyribozymes have been used as analytical tools for assessment of site-specific RNA modifications such as pseudouridylation, 2′-*O*-methylation, and m^5C formation [94, 96]. In one case, the presence of pseudouridine (Ψ) or 2′-*O*-methylribose in an RNA target directly blocked 10–23 or 8–17 deoxyribozyme cleavage activity [94]. In another report, the presence of pseudouridine was revealed by deoxyribozyme-catalyzed cleavage immediately 5′ of the RNA modification site, followed by 5′-^{32}P-radiolabeling of the cleavage fragment, digestion with nuclease P1 to monophosphorylated nucleotides, and separation of the 5′-pU and 5′-pΨ products by 2D TLC [96]. A similar strategy was employed for analysis of m^5C RNA modification. In related fashion, the 10–23 deoxyribozyme was applied for analysis of nucleotide mutations [97]. Such experiments can potentially benefit from subtle DNA nucleotide changes that increase the efficiency of 10–23-catalyzed RNA cleavage, such as substituting deoxyinosine for deoxyguanosine near the conserved enzyme region [70]. The experiments can also be improved by using locked nucleic acid (LNA) or 2′-*O*-methyl substitutions in the deoxyribozyme binding arms. This substantially increases the efficiency of DNA : RNA binding, especially for structured RNA targets [98–100].

The 10–23 deoxyribozyme is an integral part of the 'DzyNA-PCR' procedure for real-time DNA detection [101], which includes detection of specific DNA sequences in biologically derived samples. In DzyNA-PCR, the complement of the 10–23 sequence is included within one PCR primer, such that PCR amplification generates the functional sense-strand deoxyribozyme. In turn, this DNA enzyme cleaves a separately added reporter substrate that has an embedded ribonucleotide linkage flanked by fluorophore and quencher groups; the fluorescence signal increases in proportion to the amount of generated deoxyribozyme. Ribonucleases in biological samples might induce detectable reporter substrate cleavage independent of the deoxyribozyme. Therefore, application of DzyNA-PCR should be improved by the use of RNA-cleaving deoxyribozymes that cleave non-biological RNA linkages, which are readily incorporated into the synthetic reporter substrate. Examples of such linkages include a 3′–5′ linkage adjacent to an L-ribonucleotide (rather than a natural D-ribonucleotide) or a 2′–5′ linkage adjacent to a natural D-ribonucleotide [102].

The high selectivity of deoxyribozyme-catalyzed RNA cleavage allows for analysis of mixed sequence populations, where only the designated target is cleaved in the presence of competing RNAs. In one report, RNA-cleaving deoxyribozymes were used to analyze a mixed microbial community [103]. By targeting a species-specific region of 16S rRNA with 10–23, the relative abundance of a particular bacterial species was quantified in microbial mixtures used for wastewater treatment. The high abundance of 16S rRNA allowed for direct detection of deoxyribozyme cleavage products without PCR amplification, which made the assay both time- and cost-effective. This approach should allow for rapid identification of microbial species in many contexts.

Several RNA-cleaving deoxyribozymes have had their catalytic activities modulated photochemically. This has been accomplished irreversibly by photocleavage of a light-sensitive nucleobase protecting group [104, 105]. Alternatively, reversible photochemical control of RNA cleavage activity has been achieved by photoisomerization of an azobenzene group appended either to a deoxyribose [106] or to a non-nucleotidic backbone spacer [107, 108]. In principle, such photochemical modulation would allow spatial control of DNA-catalyzed RNA cleavage, e.g., within a living organism, if light can be delivered appropriately. The practical applications of photochemically modulated RNA-cleaving deoxyribozymes are largely still to be developed.

Regulated RNA-cleaving deoxyribozymes as sensors

One particularly interesting aspect of RNA-cleaving deoxyribozymes is the potential for strategic regulation of their catalysis by other compounds. When such regulation occurs via selective binding of ions or small molecules, RNA-cleaving deoxyribozymes can be used as sensors for these targets. As a representative example, work in the Lu laboratory has shown that deoxyribozymes can be the basis for sensing environmentally relevant ions such as Pb^{2+} and the uranyl cation, UO_2^{2+} [109–113]. For example, their UO_2^{2+} sensor functions by DNA-catalyzed RNA cleavage only in the presence of the metal ion, and this cleavage separates a fluorophore (fluorescein) from a quencher (Black Hole Quencher; Fig. 7).

Their deoxyribozyme-based system was capable of detecting UO_2^{2+} at 45 pM (11 parts per trillion), which is several orders of magnitude below the 130 nM toxic level as established by the Environmental Protection Agency [113]. Moreover, UO_2^{2+} was detected with at least millionfold selectivity over competing ions such as Th^{4+} in contaminated soil samples, indicating substantial practical utility outside the research laboratory. In addition to sensors for Pb^{2+} and UO_2^{2+}, deoxyribozyme sensors based on RNA cleavage have been created for Cu^{2+} [114] and Hg^{2+} [115].

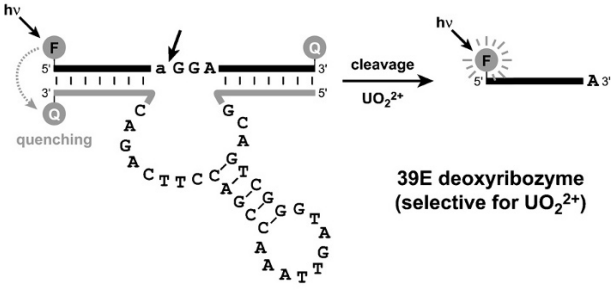

Figure 7. The 39E deoxyribozyme, which allows sensitive detection of the uranyl cation (UO_2^{2+}) by metal-dependent RNA cleavage [113]. The substrate strand is entirely DNA except for a single adenosine ribonucleotide at the cleavage site (lowercase a). The Black Hole Quencher (Q) on the 3′-end of the deoxyribozyme prevents fluorescence from fluorescein (F) until UO_2^{2+}-dependent DNA-catalyzed cleavage has occurred. The second quencher on the 3′-end of the substrate reduces the background fluorescence signal by suppressing fluorescence from any intact substrate strands that are not associated with the enzyme strand.

Related experiments in several laboratories have further explored the utility of RNA-cleaving deoxyribozymes as metal ion sensors. By placing a fluorophore-quencher pair directly on the RNA cleavage substrate from the outset of selection, more effective signaling deoxyribozymes have been identified [116]. Efforts have been made to entrap fluorescently signaling deoxyribozymes in sol-gel matrices, which may have certain benefits over solution-phase assays [117], and an electrochemical Pb^{2+} sensor based on metal-dependent RNA cleavage has been developed [118]. The fluorophore and quencher components of the general system have also been replaced with gold nanoparticles, which change color upon cleavage-induced disassembly, allowing simple colorimetric detection of metal ions [110, 119, 120]. This is important for field work, including home use, e.g., for detecting Pb^{2+} in paint [119]. Optimization of the gold nanoparticle approach has been pursued extensively for Pb^{2+} detection [121]. Metal-dependent gold nanoparticle assembly rather than disassembly using deoxyribozymes is advantageous [122], and considerable future work will likely focus on this approach.

Deoxyribozyme-based sensors have also been used for detection of small organic compounds rather than metal ions. For example, the 8–17 deoxyribozyme was rendered dependent on the small molecule adenosine, which binds to an engineered aptamer portion of the DNA and activates 8–17-catalyzed RNA cleavage, in turn leading to gold nanoparticle disassembly and a color change [123]. This study is closely related to experiments that use only DNA aptamers (not DNAzymes), which have enabled detection of compounds such as adenosine and cocaine [124]. Allosteric deoxyribozyme-based sensors for ATP have been created, based either on DNA ligation [125, 126] or RNA cleavage [127]. In the latter case, a short regulatory oligonucleotide was included; ATP binding to the DNA aptamer region displaced the oligonucleotide and allowed adoption of an active deoxyribozyme structure. This approach was derived from extensive studies in the Li laboratory on 'structure-switching signaling aptamers' [128–131], which allow straightforward generation of non-enzymatic signaling systems for a wide range of small-molecule targets. This approach is also related to the 'expansive regulation' approach of Sen and co-workers, who have used oligonucleotide effectors to regulate the catalysis of both ribozymes and deoxyribozymes [132–134]; others have exploited a similar approach [135]. RNA-cleaving deoxyribozymes can be used directly as oligonucleotide sensors via a 'catalytic molecular beacon' design [136] or via an approach involving a binary deoxyribozyme, in which the oligonucleotide target is the platform for assembling the functional DNA enzyme from two fragments [137]. An RNA-cleaving deoxyribozyme has also been used to detect streptavidin upon its binding to biotin covalently attached to the DNA near the active site, thereby inhibiting catalysis [138]. A general conclusion is that deoxyribozymes are well-behaved components of molecular-scale sensors, for which ingenuity and necessity appear to be the limiting design factors.

RNA-cleaving deoxyribozymes as *in vivo* therapeutic agents

The utility of RNA-cleaving deoxyribozymes extends beyond the *in vitro* applications described above. In particular, DNA enzymes that cleave RNA have been used widely to demonstrate that these abilities are maintained in cellular settings and constitute a viable therapeutic strategy. Nearly all of the *in vivo* efforts have utilized the 10–23 deoxyribozyme motif. Its simple cleavage-site requirement (needing only adjacent purine and pyrimidine nucleotides; Fig. 3A)

means that there are numerous potential target sites in any given RNA. Table 2 lists some of the reported *in vivo* deoxyribozyme targets for RNA cleavage.

Table 2. *In vivo* targets for deoxyribozyme-catalyzed RNA cleavage.

Target RNA for *in vivo* deoxyribozyme-catalyzed cleavage	References
Egr1 zinc finger transcription factor	[139–142]
Epstein-Barr virus latent membrane protein	[143]
Hepatitis B virus X protein	[144]
Hepatitis B virus HBs and HBe antigens	[145]
Hepatitis C virus core protein	[146]
HIV-1 Gag	[147–149]
HIV-1 Tat protein	[150,151]
HIV-1 5′-untranslated region	[152]
Human telomerase reverse transcriptase	[153]
Influenza virus A	[154]
Isocitrate lyase from *M. tuberculosis*	[155]
c-Jun leucine zipper transcription factor	[156,157]
β-Lactamase	[158,159]
12-Lipoxygenase	[160]
c-Myc proto-oncogene	[161–163]
Ornithine decarboxylase	[164]
Pencillin-binding protein	[165]
PML/RARα fusion gene of acute promyelocytic leukemia	[166]
Respiratory syncytial virus (RSV) nucleocapsid protein	[167]
SARS associated coronavirus 5′-untranslated region	[168]
Survivin	[169]
TGF-β1	[170]
Twist helix-loop-helix transcription factor	[171]
Vascular endothelial growth factor receptor 2 (VEGFR)	[172]

This tabulation is representative, not comprehensive. Additional examples and reviews are cited in ref. [13].

One application for RNA-cleaving deoxyribozymes is the targeting of antibiotic-resistant strains of common infectious agents such as *Staphylococcus aureus.* Antibiotic resistance typically occurs when the infectious agent obtains a plasmid or mutation that permits survival in the presence of an environmental challenge. Because treatment with various antibiotics can result in new antibiotic-resistant strains and even lead to multi-drug resistance, new therapeutic approaches are clearly needed [173, 174]. By using RNA-cleaving deoxyribozymes, mRNAs that encode the proteins responsible for antibiotic resistance can be destroyed. In studies with antibiotic-resistant *S. aureus*, mRNAs for β-lactamase [159] and penicillin-binding protein (PBP2) [165] were targeted for cleavage by 10–23. Intracellular introduction of the deoxyribozyme led to a decrease in the levels of the targeted mRNA as well as the number of colony-forming units (CFUs) in a dose-dependent manner, while also increasing the antibiotic sensitivity of the treated bacteria. Studies with ampicillin-resistant *Escherichia coli* demonstrated the increased effectiveness of using a di-DNAzyme, which is a single DNA oligonucleotide containing two 10–23 enzyme regions with binding arms directed to two separate target sites on a single transcript [158]. Whereas single deoxyribozymes showed high catalytic activity *in vitro*, the di-DNAzyme approach was even more effective *in vivo* and led to decreased bacterial growth in the presence of ampicillin.

Deoxyribozymes are also being developed as therapeutics for viral diseases such as HIV and hepatitis. Targeting different regions of the HIV genome with deoxyribozymes allows for inhibition at different stages of the HIV life cycle. Because the HIV-1 Gag region RNA sequence is highly conserved among various subtypes, deoxyribozymes identified to cleave this region are expected to be broadly applicable [148, 149]. Both 10–23 and 8–17 successfully cleaved the Gag transcript. Moreover, the activities were greatly enhanced by including additional oligonucleotides that base-pair with the RNA regions near the deoxyribozyme binding site [149]. This enhancement is likely due to disruption of local RNA structure by the additional oligonucleotides, which allow the deoxyribozyme to bind its target sequence more efficiently. Added oligonucleotides can also facilitate binding of deoxyribozymes to the RNA target through cooperative effects [175, 176].

Therapeutic targets for deoxyribozymes are not limited to viral RNAs. As seen in Table 2, deoxyribozymes have been used to cleave a variety of cancer-related gene products, with success both *in vitro* and in cell culture. Even more encouraging is that 10–23 deoxyribozymes targeting VEGFR [172], c-Jun [157], and Egr-1 [142] significantly reduced tumor sizes in relevant mouse models. These animal studies demonstrate that RNA-cleaving deoxyribozymes are viable *in vivo* therapeutic agents, although further work is needed to make a successful transition into the clinic. While the goal of nucleic acid-based therapeutics is usually destruction of the target RNA, deoxyribozymes and antisense oligonucleotides differ in their modes of action. Antisense oligonucleotides either physically block enzymes to prevent gene expression or possibly act in concert with RNase H to direct cleavage of the mRNA target [177]. In contrast, deoxyribozymes directly catalyze site-specific cleavage of the RNA phosphodiester backbone. Because this inherently requires binding of the deoxyribozyme

to the RNA target, differentiating cleavage from antisense effects is challenging. For deoxyribozymes targeting hepatitis B RNA, use of an antisense analogue (no enzyme region) as a control led to a much smaller reduction in gene expression (90 versus 30 % in 48 h), indicating that the effect with the active deoxyribozyme truly depends on catalysis and is not merely an antisense phenomenon [145]. Similarly, a deoxyribozyme targeting ornithine decarboxylase led to greater suppression of protein production than an inactive (mutant) deoxyribozyme as control, although the difference was relatively modest (75 versus 58 % in 24 h) [164]; this suggests that both antisense and catalytic mechanisms are operative in this case. However, when the HIV-1 leader region RNA was targeted with 10–23 that had LNA incorporated into the binding arms, superior reductions in RNA levels were observed with analogous LNA-containing antisense oligonucleotides [152]. These observations leave open the possibility that the deoxyribozyme effect in this case is due not to catalysis but instead to an antisense mechanism. Overall, caution is appropriate when interpreting the mechanism of a deoxyribozyme's effect in any particular *in vivo* experiment.

Challenges for *in vivo* therapeutic applications of RNA-cleaving deoxyribozymes

Significant challenges remain for *in vivo* applications of deoxyribozymes that cleave RNA targets. For most deoxyribozymes, maximal activity is achieved *in vitro* using divalent metal ion concentrations that are much higher than the expected available concentrations within cells (e.g., 10–100 mM Mg^{2+} *in vitro*, versus usually <1 mM Mg^{2+} *in vivo* [178, 179]). As demonstrated in assays that targeted either the HIV-1 Gag region [148] or human telomerase reverse transcriptase [153], deoxyribozymes with lower *in vitro* Mg^{2+} requirements function more effectively *in vivo*. By altering *in vitro* selection conditions to decrease the metal ion requirements, new deoxyribozymes can likely be identified that will function optimally under physiologically relevant conditions. In the absence of such new selection experiments, the 10–23 deoxyribozyme (which was identified using 10 mM Mg^{2+} during selection [18]) has already proven useful under the lower metal concentrations present *in vivo* (Table 1).

As with other nucleic acid-based *in vivo* applications such as antisense, siRNA, and ribozymes [180, 181], deoxyribozymes also face considerable challenges related to delivery. Deoxyribozymes are at least dozens of nucleotides long and have a charged backbone; both of these characteristics inhibit the efficiency of cellular uptake [182]. Approaches to address this issue include electroporation [159, 165, 183]; single-stranded DNA expression vectors [184–186]; and complexing deoxyribozymes with dendrimers [187, 188]. Furthermore, for targeted delivery the free deoxyribozyme must be introduced directly to the area of interest, which is not always practical in clinical settings. To allow for more specific targeting, deoxyribozymes can be packaged within nanoparticles that are directed to particular cell types, including tumors, with the surface-attached transferrin glycoprotein [163]. By instead directly conjugating peptides to free deoxyribozymes, cellular uptake has been improved and intracellular localization of the deoxyribozyme can be controlled [189]. Further development of these approaches will improve the prospects of systemic delivery, which is desirable for practical therapeutic applications.

Another challenge for nucleic acid therapeutics is the issue of stability in the cellular environment. Whereas DNA is more stable than RNA in this context, the lifetime of an unmodified DNA oligonucleotide is still relatively short [162], which can limit the functional abilities of deoxyribozymes over relevant time periods. A variety of approaches have been used to improve the intracellular lifetime of DNA. Circular DNA enzymes as created by ligating two linear precursors have increased stability in serum [190], and circular deoxyribozymes can have activity in bacterial cells [186]. A simple chemical modification that greatly improves DNA stability is incorporation of a 3′–3′-inverted nucleotide [161, 162, 191]. Phosphorothioates [143, 162, 191], phosphoramidates [154], and LNA [140, 152, 191] have also been incorporated into the binding arms to prevent nuclease-catalyzed degradation and, in the case of LNA, to improve the specificity of RNA cleavage. LNA can additionally improve the activity of deoxyribozymes by enhancing target binding [98, 100, 192]. Because these chemical modifications are readily incorporated into DNA oligonucleotides during solid-phase synthesis, generating appropriately modified deoxyribozymes in large quantities is feasible for clinical applications. Even simple unmodified hairpins incorporated at each end of the deoxyribozyme appear to improve intracellular stability, so standard DNA itself may have therapeutic promise [193].

RNA-ligating deoxyribozymes to enable studies of RNA structure and function

Deoxyribozymes that create linear RNA offer an experimental alternative to protein-mediated ligation of two RNA substrates by use of either T4 DNA ligase and a bridging oligonucleotide 'splint' [194, 195] or T4 RNA ligase with or without a splint [196, 197]. The deoxyribozyme approach is particularly useful when the protein enzymes are found empirically to work

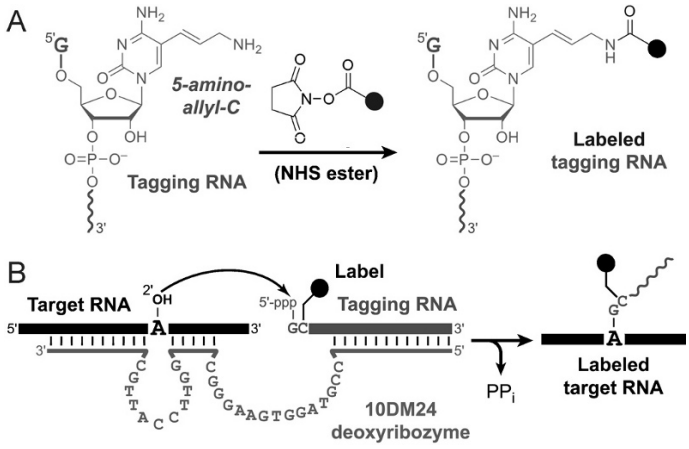

Figure 8. Deoxyribozyme-catalyzed labeling (DECAL) of RNA using the 10DM24 deoxyribozyme [200]. (*A*) Synthesis of the labeled tagging RNA by modification of the amino group of an *in vitro* transcript that has a single 5-aminoallyl-C residue. (*B*) Attachment of the tagging RNA to the target RNA using 10DM24.

poorly (for reasons that are not always apparent, but likely do not always derive from structure in the RNA target). Applications of RNA-ligating deoxyribozymes for preparing modified RNAs should be facilitated by the identification of deoxyribozymes that simultaneously achieve high rate, yield, and RNA substrate sequence generality, as described above (Fig. 4) [35].

Deoxyribozymes that synthesize 2′,5′-branched RNA are useful to explore biochemical phenomena in which branched RNAs are known or postulated to participate. In two specific cases, branch-forming deoxyribozymes have been used directly to elucidate features of biochemical pathways. First, the 7S11 deoxyribozyme was used to prepare branched versions of the ai5γ group II intron RNA that correspond to mis-splicing at the 5′-splice site [198]. Evaluation of the abilities of these branched RNAs to undergo the reverse of the first step of splicing provided evidence against the long-standing but previously untested hypothesis that first-step reversibility is a proofreading mechanism for 5′-splice site selection. The results also suggested that an alternative proofreading mechanism could allow generation of the correct ligated exons even after a mis-splicing event at the 5′-splice site.

Second, a deoxyribozyme that works well with any of the four standard RNA nucleotides at the branch site [44] was used to enable a direct test of the hypothesis that branched RNA is an obligatory intermediate in retrotransposition of the Ty1 element [199]. Demonstration that the proposed branched RNA intermediate is not a viable substrate for efficient read-through of the branch point by the Ty1 reverse transcriptase was interpreted as evidence against the viability of the proposed branched RNA as a natural Ty1 retrotransposition intermediate.

Deoxyribozymes that create branched RNA have also been used in two cases to enable studies for which the branch is not normally part of a biological system. First, the 10DM24 branch-forming deoxyribozyme was applied to enable deoxyribozyme-catalyzed labeling, or DECAL, of RNA with moieties such as fluorophores or biotin (Fig. 8) [200]. A key advantage of this labeling approach is that the target RNA is not assembled from fragments, but rather the intact RNA is directly functionalized. Therefore, the DECAL strategy may be particularly useful for large RNAs (or RNA-protein complexes) that are not amenable to fragment-based assembly approaches, either for synthetic reasons [201] or because reassembling a functional system that includes the modified synthetic RNA is not feasible. A disadvantage of the DECAL approach as currently implemented is that the modification (such as a fluorophore) is attached as part of a relatively long oligonucleotide chain, which might perturb the function of the resulting modified RNA. However, such perturbation may in fact be minimal in any given system, and this must be tested on an individual basis. It may be possible to combine the small-molecule substrate approach of Figure 5B with a chemical modification to enable a DECAL-related strategy in which only a single nucleotide is appended to the large RNA target, although this has not yet been accomplished.

Second, deoxyribozymes have been used for attachment of DNA to RNA, which allows implementing double-stranded DNA constraints for control of RNA folding and catalysis. Attachment of two single-stranded DNAs to a structured RNA has been shown to allow rational control of RNA conformation; when the attached DNA strands are complementary, formation of the DNA duplex can be incompatible with the RNA folded structure, thereby inducing the RNA to misfold [202, 203]. In the initial studies, attachment of DNA to RNA was achieved by a semisynthetic approach in which a chemically modified 5′-aldehyde-DNA was joined with 2′-

amino-RNA by reductive amination [204]. As an alternative approach, a deoxyribozyme was identified that attaches DNA directly to an RNA 2′-hydroxyl group, and this enabled predictable regulation of hammerhead ribozyme catalysis (Fig. 9) [205]. We have more recently shown that double-stranded DNA constraints attached by deoxyribozymes can be used for controlling catalysis by the larger multi-domain group I intron ribozyme [E. Zelin and S. K. S., unpublished observation].

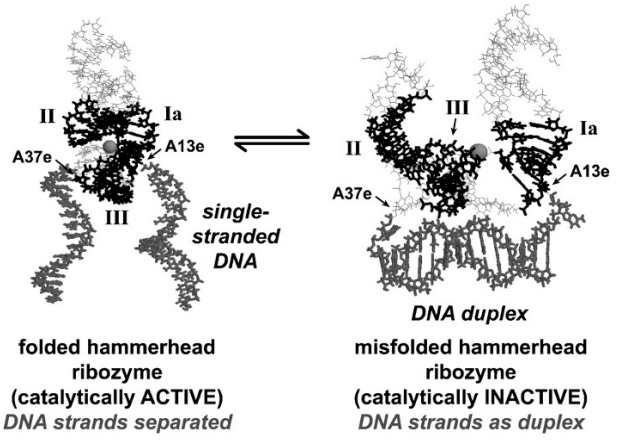

Figure 9. Control of hammerhead ribozyme catalysis by double-stranded DNA constraints, which were attached by a deoxyribozyme [205]. The 9FQ4 deoxyribozyme was used to attach DNA at both A13 and A37 of the enzyme (e) strand of the hammerhead ribozyme. When the two attached single-stranded DNAs are complementary, the active ribozyme structure (left) is misfolded upon formation of the DNA duplex (right). Stems Ia, II, and III are labeled; the active-site 2′-OH group is marked with a sphere.

Additional applications of deoxyribozymes

In several cases, deoxyribozymes have been used in applications other than those described above. RNA-cleaving deoxyribozymes have been exploited as integral components of DNA nanotechnology, e.g., a 'nanomotor' whose motion is controlled by DNA-catalyzed RNA cleavage [206] or an RNA-cleaving DNA enzyme that walks down a nucleic acid track [207]. RNA-cleaving deoxyribozymes have also been used in proof-of-principle computing applications. Stojanovic and co-workers have created Boolean logic gates [208–210], enabling the creation of 'molecular automata' that play tic-tac-toe perfectly against a human opponent [211, 212]. Logic gates have also been made using deoxyribozymes that ligate DNA [213] or cleave DNA [214].

Deoxyribozymes with activities other than RNA cleavage or ligation have been used sparingly in practical applications. One particular set of applications is built on a peroxidase deoxyribozyme, which uses H_2O_2 to oxidize a small organic substrate [215–217]. As depicted in detail in a recent review [11], Willner and co-workers have used this peroxidase deoxyribozyme to enable colorimetric and chemiluminescent readouts for quantification of a DNA target, by either a PCR-based method [218] or an approach based on isothermic amplification and rolling-circle amplification [219]; the latter approach was described independently by Mao and co-workers [220]. Willner's group has also used the peroxidase deoxyribozyme for detecting small molecules and protein targets (AMP and lysozyme) via an 'aptasensor' strategy, in which interaction of the target with its aptamer frees the deoxyribozyme to adopt its active structure [221]. The latter approach is closely related to the 'structure-switching allosteric deoxyribozyme' strategy reported by Li and co-workers for detection of ATP [127].

Comparison of deoxyribozymes with other biotechnology tools

For all of the practical applications described above, both *in vitro* and *in vivo*, one may consider the general advantages and disadvantages of deoxyribozymes as compared with other experimental options for achieving similar objectives. Table 3 provides a summary of these advantages and disadvantages for deoxyribozymes relative to four other common biotechnology tools.

For *in vitro* applications, DNA is inherently more stable both chemically and biologically than RNA and protein, and this stability can be further enhanced via chemical modifications. Such modifications are readily incorporated, and both modified and unmodified DNA can be produced in large quantities for practical applications. However, modifications may reduce the catalytic efficiency of the deoxyribozyme, particularly if introduced only after the deoxyribozyme is identified by *in vitro* selection. Deoxyribozymes as well as ribozymes can function optimally under highly non-physiological conditions; the same is generally not true for proteins. This tolerance makes nucleic acids, particularly deoxyribozymes, especially well suited for *in vitro* sensor applications. Many proteins have greater rate enhancements than are currently known for deoxyribozymes and ribozymes, but the maximum rate enhancements for the catalytic nucleic acids have probably not yet been achieved (as discussed above). Deoxyribozymes also have potential advantages for *in vivo* therapeutic applications via mRNA degradation. The relatively high stability of deoxyribozymes favors their utility *in vivo*. However, such applications have not been explored to the same extent as for other nucleic acid-based approaches such as ribozymes,

Table 3. Advantages and disadvantages of deoxyribozymes compared with other biotechnology tools

Alternative tool	Advantages of deoxyribozymes relative to alternative tool	Disadvantages of deoxyribozymes relative to alternative tool
Ribozymes (*in vitro* or *in vivo*)	· more stable chemically and biologically · less expensive · easier to synthesize	· narrower range of reactions currently known
Protein enzymes (*in vitro* or *in vivo*)	· active under many conditions, including nonphysiological · readily modified for increased stability	· narrower range of reactions currently known · lower rate of enhancements currently known (although theory suggests higher values are possible) · modifications to increase stability may decrease activity
Antisense oligonucleotides (*in vivo*)	· allow catalytic destruction of target RNA · have potential for higher target selectivity	· more complex design due to sequence, structure, or cofactor requirements beyond base pairing
RNA interference via siRNA (*in vivo*)	· catalytically independent of cellular machinery · have potential for rational modulation of catalytic activity	· difficult to engineer for activity under physiological conditions (e.g., low divalent metal ion concentrations)

antisense oligonucleotides, and RNA interference (RNAi). Antisense oligonucleotides rely solely on Watson-Crick base pairs to recognize their mRNA targets. In contrast, because deoxyribozymes (as well as ribozymes and RNAi) inherently use catalysis to destroy the target mRNA, they are relatively efficient and can also be more selective. These features come at the cost of requiring proper tertiary folding of the associated enzymes in addition to secondary structure formation. This folding may be difficult for deoxyribozymes under physiological conditions that typically include low divalent metal ion (Mg^{2+}) concentrations. The sequence requirements of deoxyribozymes and ribozymes do place restrictions on potential mRNA target sites, unlike the situation for antisense oligonucleotides and RNAi. However, unlike RNAi, catalytic nucleic acids do not rely upon the cellular machinery and therefore may have their catalytic activities modulated in rational fashion.

Concluding remarks: future directions for deoxyribozymes

Over the past three decades, our rather tidy view of nucleic acids – DNA for long-term information storage and RNA for short-term information transfer – has been transformed by the discoveries of ribozymes, aptamers, RNAi, and other phenomena. The 1994 announcement of artificial deoxyribozymes as catalytically active DNA molecules further blurred the formerly sharp functional distinction between DNA and RNA. The advances described in this review illustrate the growing scope of DNA-catalyzed reactions and their applications in chemistry, biochemistry, and biology. Likely future directions include defining more clearly the catalytic abilities of DNA; continuing to apply deoxyribozymes *in vitro* for practical purposes such as RNA manipulation and sensor applications; and evaluating the utility of deoxyribozymes *in vivo* for therapeutic RNA cleavage.

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