

Review

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

D. A. Baum and S. K. Silverman*

Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801 (USA), Fax +1 217 244 8024, e-mail: scott@scs.uiuc.edu

Received 16 January 2008; received after revision 20 February 2008; accepted 26 February 2008
Online First 29 March 2008

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 Pb²⁺-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

* Corresponding author.

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.

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

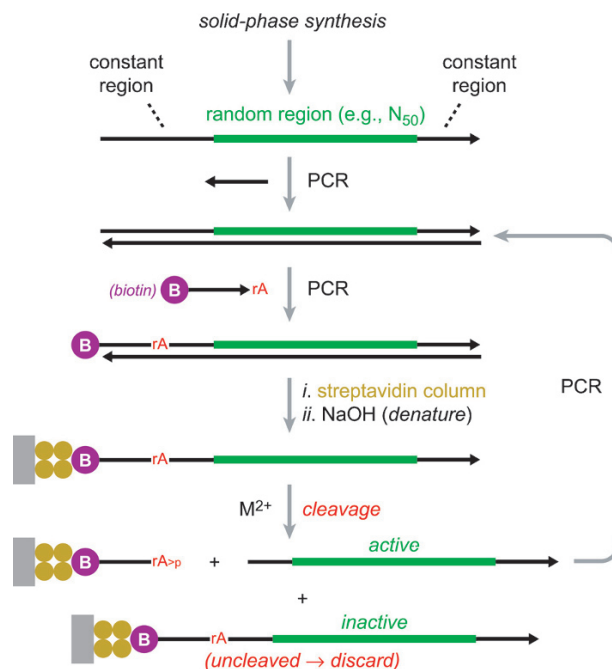


Figure 1. *In vitro* selection of deoxyribozymes, illustrated for selection of RNA-cleaving deoxyribozymes. In this example, the key selection step is based on the detachment of biotin from the DNA random region as a consequence of the RNA cleavage reaction. However, the key selection step can be based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

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

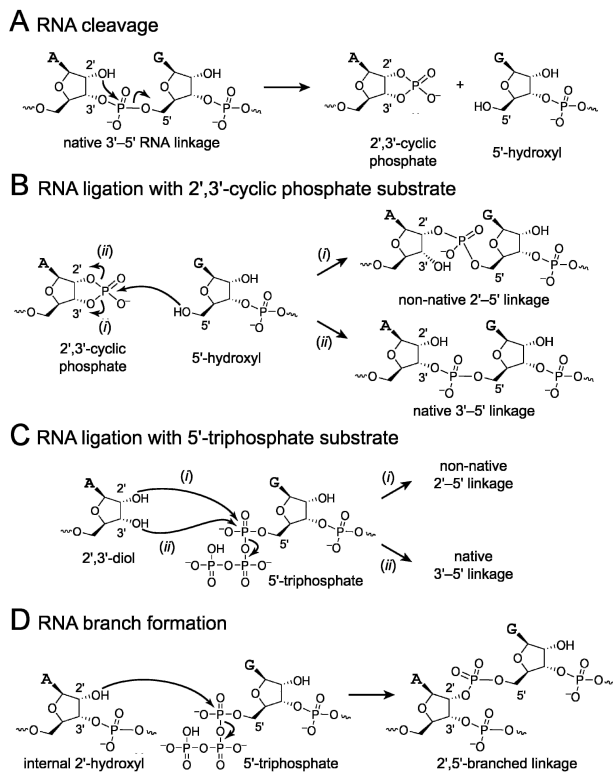


Figure 2. RNA cleavage and ligation reactions catalyzed by deoxyribozymes. (A) RNA cleavage by attack of a 2'-hydroxyl at the adjacent phosphodiester linkage. (B) RNA ligation by attack of a 5'-hydroxyl at a 2',3'-cyclic phosphate, which can form either a native 3'-5' linkage or a non-native 2'-5' linkage. (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-

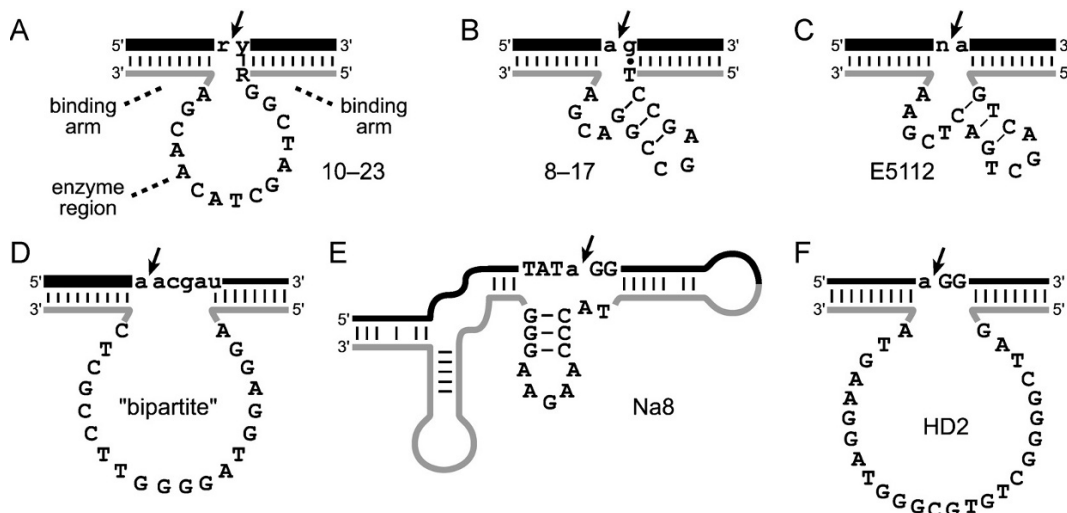


Figure 3. RNA-cleaving deoxyribozymes. Thick lines and lowercase letters denote RNA; thin lines and uppercase letters denote DNA (R = purine, Y = pyrimidine, and N = any nucleotide). The cleavage site is marked with an arrow. (A) 10-23 and (B) 8-17 deoxyribozymes [18]. Only the two R↓Y and A↓G nucleotides 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].

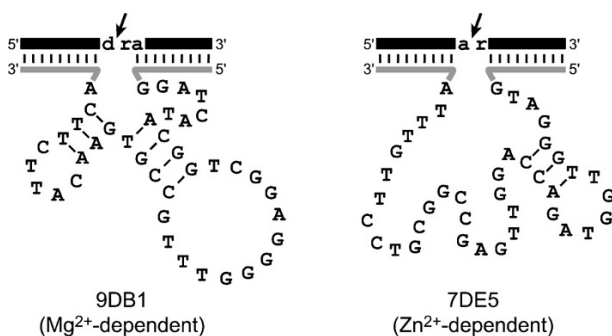


Figure 4. RNA-ligating deoxyribozymes that form native 3'-5' linkages [35]. 9DB1 requires Mg^{2+} and a D↓RA ligation junction (R = purine; D = any nucleotide except C); 7DE5 requires Zn^{2+} and an A↓R ligation junction.

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).

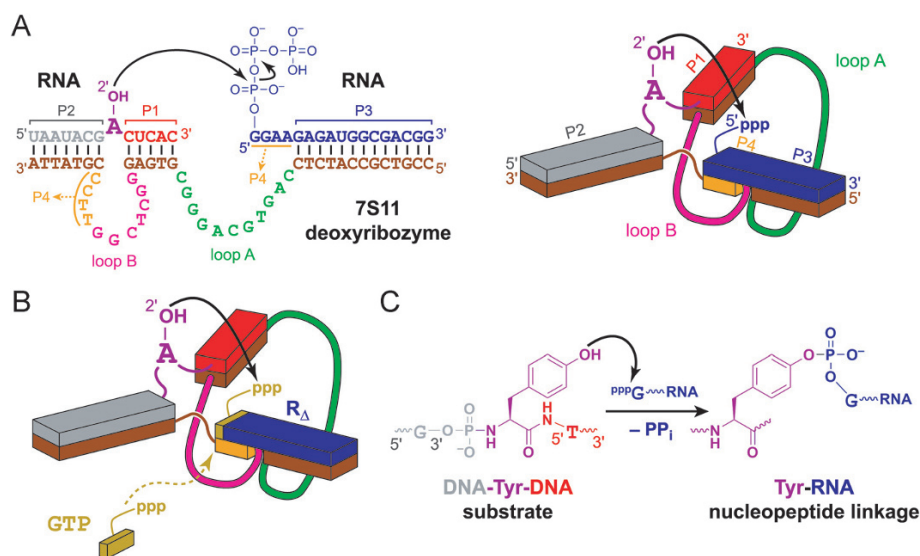


Figure 5. Deoxyribozymes that form three-helix-junction (3HJ) complexes with their substrates. (A) The 7S11 deoxyribozyme that forms 2',5'-branched RNA [41,42]. At left is the secondary structure depicting the four paired regions P1–P4; at right is a schematic three-dimensional model emphasizing the 3HJ structure. (B) A variant of 7S11, 10DM24 [43], engineered to accept GTP as a discrete small-molecule substrate [60]. R_A 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 $10^9 \text{ M}^{-1} \text{ min}^{-1}$) 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.

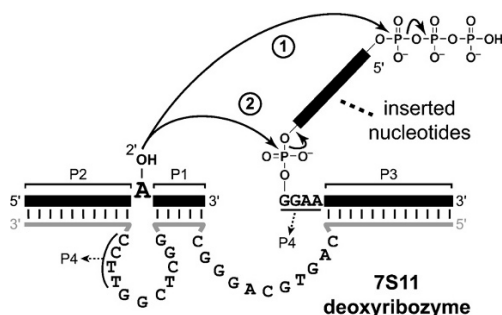


Figure 6. Two competing reactions of the three-helix-junction 7S11 deoxyribozyme when the reactive 5'-triphosphate is offset by from its original position due to insertion of one or more nucleotides [83]. Reaction 1 occurs by attack of the 2'-hydroxyl at the 5'-triphosphate, which is spatially offset 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 m⁵C 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'-³²P-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 m⁵C 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 Pb²⁺ and the uranyl cation, UO₂²⁺ [109–113]. For example, their UO₂²⁺ 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].

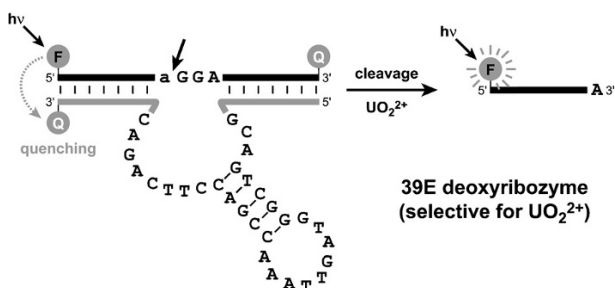


Figure 7. The 39E deoxyribozyme, which allows sensitive detection of the uranyl cation (UO_2^{2+}) by metal-dependent RNA cleavage [113]. The substrate strand is entirely DNA except for a single adenosine ribonucleotide at the cleavage site (lowercase a). The Black Hole Quencher (Q) on the 3'-end of 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 deoxyribo-

zymes 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 <i>in vivo</i> 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 <i>M. tuberculosis</i>	[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]
Penicillin-binding protein	[165]
PML/RAR α fusion gene of acute promyelocytic leukemia	[166]
Respiratory syncytial virus (RSV) nucleocapsid protein	[167]
SARS associated coronavirus 5'-untranslated region	[168]
Survivin	[169]
TGF- β 1	[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 Mg²⁺ *in vitro*, versus usually <1 mM Mg²⁺ *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* Mg²⁺ 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 Mg²⁺ 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

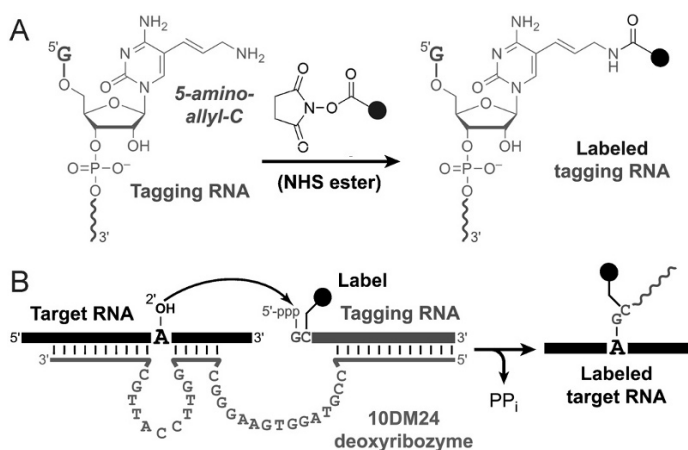


Figure 8. Deoxyribozyme-catalyzed labeling (DECAL) of RNA using the 10DM24 deoxyribozyme [200]. (A) Synthesis of the labeled tagging RNA by modification of the amino group of an *in vitro* transcript that has a single 5-aminoallyl-C residue. (B) Attachment of the tagging RNA to the target RNA using 10DM24.

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 $\alpha 5\gamma$ 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].

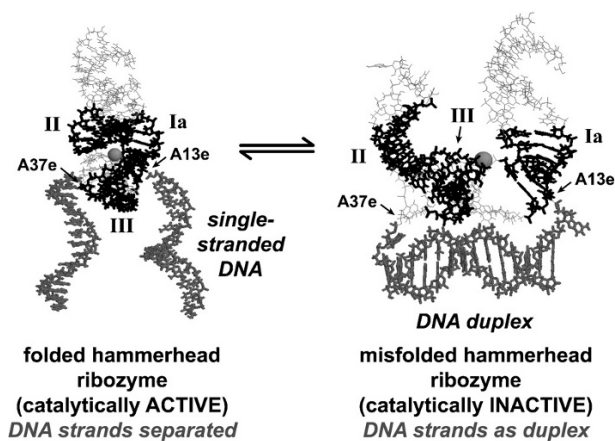


Figure 9. Control of hammerhead ribozyme catalysis by double-stranded DNA constraints, which were attached by a deoxyribozyme [205]. The 9FQ4 deoxyribozyme was used to attach DNA at both A13 and A37 of the enzyme (e) strand of the hammerhead ribozyme. When the two attached single-stranded DNAs are complementary, 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 (<i>in vitro</i> or <i>in vivo</i>)	<ul style="list-style-type: none"> · more stable chemically and biologically · less expensive · easier to synthesize 	<ul style="list-style-type: none"> · narrower range of reactions currently known
Protein enzymes (<i>in vitro</i> or <i>in vivo</i>)	<ul style="list-style-type: none"> · active under many conditions, including nonphysiological · readily modified for increased stability 	<ul style="list-style-type: none"> · 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 (<i>in vivo</i>)	<ul style="list-style-type: none"> · allow catalytic destruction of target RNA · have potential for higher target selectivity 	<ul style="list-style-type: none"> · more complex design due to sequence, structure, or cofactor requirements beyond base pairing
RNA interference via siRNA (<i>in vivo</i>)	<ul style="list-style-type: none"> · catalytically independent of cellular machinery · have potential for rational modulation of catalytic activity 	<ul style="list-style-type: none"> · 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 pur-

poses such as RNA manipulation and sensor applications; and evaluating the utility of deoxyribozymes *in vivo* for therapeutic RNA cleavage.

Acknowledgements. Research in the Silverman laboratory on deoxyribozymes is supported by the US National Institutes of Health, the US National Science Foundation, and the David and Lucile Packard Foundation. D. A. B. is the recipient of an NIH postdoctoral fellowship.

- 1 Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E. and Cech, T. R. (1982) Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. *Cell* 31, 147–157.
- 2 Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. and Altman, S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* 35, 849–857.
- 3 Ellington, A. D. and Szostak, J. W. (1990) *In vitro* selection of RNA molecules that bind specific ligands. *Nature* 346, 818–822.
- 4 Tuerk, C. and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249, 505–510.
- 5 Robertson, D. L. and Joyce, G. F. (1990) Selection *in vitro* of an RNA enzyme that specifically cleaves single-stranded DNA. *Nature* 344, 467–468.
- 6 Joyce, G. F. (2004) Directed evolution of nucleic acid enzymes. *Annu. Rev. Biochem.* 73, 791–836.
- 7 Silverman, S. K. (2007) *In vitro* selection and application of nucleic acid enzymes (ribozymes and deoxyribozymes). *Wiley Encyclopedia of Chemical Biology*, in press.
- 8 Breaker, R. R. and Joyce, G. F. (1994) A DNA enzyme that cleaves RNA. *Chem. Biol.* 1, 223–229.
- 9 Achenbach, J. C., Chiuman, W., Cruz, R. P. and Li, Y. (2004) DNazymes: from creation *in vitro* to application *in vivo*. *Curr. Pharm. Biotechnol.* 5, 321–336.
- 10 Peracchi, A. (2005) DNA catalysis: potential, limitations, open questions. *ChemBioChem* 6, 1316–1322.
- 11 Höbartner, C. and Silverman, S. K. (2007) Recent advances in DNA catalysis. *Biopolymers* 87, 279–292.
- 12 Emilsson, G. M. and Breaker, R. R. (2002) Deoxyribozymes: new activities and new applications. *Cell. Mol. Life Sci.* 59, 596–607.
- 13 Silverman, S. K. (2005) *In vitro* selection, characterization, and application of deoxyribozymes that cleave RNA. *Nucleic Acids Res.* 33, 6151–6163.

- 14 Cruz, R. P. G., Withers, J. B. and Li, Y. (2004) Dinucleotide junction cleavage versatility of 8–17 deoxyribozyme. *Chem. Biol.* 11, 57–67.
- 15 Li, Y. and Breaker, R. R. (1999) Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2'-hydroxyl group. *J. Am. Chem. Soc.* 121, 5364–5372.
- 16 Soukup, G. A. and Breaker, R. R. (1999) Relationship between internucleotide linkage geometry and the stability of RNA. *RNA* 5, 1308–1325.
- 17 Doudna, J. A. and Cech, T. R. (2002) The chemical repertoire of natural ribozymes. *Nature* 418, 222–228.
- 18 Santoro, S. W. and Joyce, G. F. (1997) A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. USA* 94, 4262–4266.
- 19 Salehi-Ashtiani, K. and Szostak, J. W. (2001) *In vitro* evolution suggests multiple origins for the hammerhead ribozyme. *Nature* 414, 82–84.
- 20 Faulhammer, D. and Famulok, M. (1996) The Ca²⁺ ion as a cofactor for a novel RNA-cleaving deoxyribozyme. *Angew. Chem. Int. Ed. Engl.* 35, 2837–2841.
- 21 Li, J., Zheng, W., Kwon, A. H. and Lu, Y. (2000) *In vitro* selection and characterization of a highly efficient Zn(II)-dependent RNA-cleaving deoxyribozyme. *Nucleic Acids Res.* 28, 481–488.
- 22 Schlosser, K. and Li, Y. (2004) Tracing sequence diversity change of RNA-cleaving deoxyribozymes under increasing selection pressure during *in vitro* selection. *Biochemistry* 43, 9695–9707.
- 23 Feldman, A. R. and Sen, D. (2001) A new and efficient DNA enzyme for the sequence-specific cleavage of RNA. *J. Mol. Biol.* 313, 283–294.
- 24 Feldman, A. R., Leung, E. K., Bennet, A. J. and Sen, D. (2006) The RNA-cleaving bipartite DNAzyme is a distinctive metalloenzyme. *ChemBioChem* 7, 98–105.
- 25 Geyer, C. R. and Sen, D. (1997) Evidence for the metal-cofactor independence of an RNA phosphodiester-cleaving DNA enzyme. *Chem. Biol.* 4, 579–593.
- 26 Faulhammer, D. and Famulok, M. (1997) Characterization and divalent metal-ion dependence of *in vitro* selected deoxyribozymes which cleave DNA/RNA chimeric oligonucleotides. *J. Mol. Biol.* 269, 188–202.
- 27 Roth, A. and Breaker, R. R. (1998) An amino acid as a cofactor for a catalytic polynucleotide. *Proc. Natl. Acad. Sci. USA* 95, 6027–6031.
- 28 Cuenoud, B. and Szostak, J. W. (1995) A DNA metalloenzyme with DNA ligase activity. *Nature* 375, 611–614.
- 29 Flynn-Charlebois, A., Wang, Y., Prior, T. K., Rashid, I., Hoadley, K. A., Coppins, R. L., Wolf, A. C. and Silverman, S. K. (2003) Deoxyribozymes with 2'-5' RNA ligase activity. *J. Am. Chem. Soc.* 125, 2444–2454.
- 30 Flynn-Charlebois, A., Prior, T. K., Hoadley, K. A. and Silverman, S. K. (2003) *In vitro* evolution of an RNA-cleaving DNA enzyme into an RNA ligase switches the selectivity from 3'-5' to 2'-5'. *J. Am. Chem. Soc.* 125, 5346–5350.
- 31 Semlow, D. R. and Silverman, S. K. (2005) Parallel selections *in vitro* reveal a preference for 2'-5' RNA ligation by deoxyribozyme-mediated opening of a 2',3'-cyclic phosphate. *J. Mol. Evol.* 61, 207–215.
- 32 Hoadley, K. A., Purtha, W. E., Wolf, A. C., Flynn-Charlebois, A. and Silverman, S. K. (2005) Zn²⁺-dependent deoxyribozymes that form natural and unnatural RNA linkages. *Biochemistry* 44, 9217–9231.
- 33 Coppins, R. L. and Silverman, S. K. (2004) Rational modification of a selection strategy leads to deoxyribozymes that create native 3'-5' RNA linkages. *J. Am. Chem. Soc.* 126, 16426–16432.
- 34 Wang, Y. and Silverman, S. K. (2005) Directing the outcome of deoxyribozyme selections to favor native 3'-5' RNA ligation. *Biochemistry* 44, 3017–3023.
- 35 Purtha, W. E., Coppins, R. L., Smalley, M. K. and Silverman, S. K. (2005) General deoxyribozyme-catalyzed synthesis of native 3'-5' RNA linkages. *J. Am. Chem. Soc.* 127, 13124–13125.
- 36 Wang, Y. and Silverman, S. K. (2003) Deoxyribozymes that synthesize branched and lariat RNA. *J. Am. Chem. Soc.* 125, 6880–6881.
- 37 Wang, Y. and Silverman, S. K. (2003) Characterization of deoxyribozymes that synthesize branched RNA. *Biochemistry* 42, 15252–15263.
- 38 Domdey, H., Apostol, B., Lin, R. J., Newman, A., Brody, E. and Abelson, J. (1984) Lariat structures are *in vivo* intermediates in yeast pre-mRNA splicing. *Cell* 39, 611–621.
- 39 Padgett, R. A., Konarska, M. M., Grabowski, P. J., Hardy, S. F. and Sharp, P. A. (1984) Lariat RNA's as intermediates and products in the splicing of messenger RNA precursors. *Science* 225, 898–903.
- 40 Ruskin, B., Krainer, A. R., Maniatis, T. and Green, M. R. (1984) Excision of an intact intron as a novel lariat structure during pre-mRNA splicing *in vitro*. *Cell* 38, 317–331.
- 41 Coppins, R. L. and Silverman, S. K. (2004) A DNA enzyme that mimics the first step of RNA splicing. *Nat. Struct. Mol. Biol.* 11, 270–274.
- 42 Coppins, R. L. and Silverman, S. K. (2005) A deoxyribozyme that forms a three-helix-junction complex with its RNA substrates and has general RNA branch-forming activity. *J. Am. Chem. Soc.* 127, 2900–2907.
- 43 Zelin, E., Wang, Y. and Silverman, S. K. (2006) Adenosine is inherently favored as the branch-site RNA nucleotide in a structural context that resembles natural RNA splicing. *Biochemistry* 45, 2767–2771.
- 44 Pratico, E. D., Wang, Y. and Silverman, S. K. (2005) A deoxyribozyme that synthesizes 2',5'-branched RNA with any branch-site nucleotide. *Nucleic Acids Res.* 33, 3503–3512.
- 45 Wang, Y. and Silverman, S. K. (2005) Efficient one-step synthesis of biologically related lariat RNAs by a deoxyribozyme. *Angew. Chem. Int. Ed.* 44, 5863–5866.
- 46 Wang, W., Billen, L. P. and Li, Y. (2002) Sequence diversity, metal specificity, and catalytic proficiency of metal-dependent phosphorylating DNA enzymes. *Chem. Biol.* 9, 507–517.
- 47 Li, Y., Liu, Y. and Breaker, R. R. (2000) Capping DNA with DNA. *Biochemistry* 39, 3106–3114.
- 48 Sreedhara, A., Li, Y. and Breaker, R. R. (2004) Ligating DNA with DNA. *J. Am. Chem. Soc.* 126, 3454–3460.
- 49 Pradeepkumar, P. I., Höbartner, C., Baum, D. A. and Silverman, S. K. (2008) DNA-catalyzed formation of nucleopeptide linkages. *Angew. Chem. Int. Ed.* 47, 1753–1757.
- 50 Carmi, N., Shultz, L. A. and Breaker, R. R. (1996) *In vitro* selection of self-cleaving DNAs. *Chem. Biol.* 3, 1039–1046.
- 51 Carmi, N., Balkhi, S. R. and Breaker, R. R. (1998) Cleaving DNA with DNA. *Proc. Natl. Acad. Sci. USA* 95, 2233–2237.
- 52 Carmi, N. and Breaker, R. R. (2001) Characterization of a DNA-cleaving deoxyribozyme. *Bioorg. Med. Chem.* 9, 2589–2600.
- 53 Sheppard, T. L., Ordoukhanian, P. and Joyce, G. F. (2000) A DNA enzyme with *N*-glycosylase activity. *Proc. Natl. Acad. Sci. USA* 97, 7802–7807.
- 54 Höbartner, C., Pradeepkumar, P. I. and Silverman, S. K. (2007) Site-selective depurination by a periodate-dependent deoxyribozyme. *Chem. Commun.*, 2255–2257.
- 55 Chandra, M. and Silverman, S. K. (2008) DNA and RNA can be equally efficient catalysts for carbon-carbon bond formation. *J. Am. Chem. Soc.* 130, 2936–2937.
- 56 Chinnapen, D. J. and Sen, D. (2004) A deoxyribozyme that harnesses light to repair thymine dimers in DNA. *Proc. Natl. Acad. Sci. USA* 101, 65–69.
- 57 Burmeister, J., von Kiedrowski, G. and Ellington, A. D. (1997) Cofactor-assisted self-cleavage in DNA libraries with a 3'-5' phosphoramidate bond. *Angew. Chem. Int. Ed.* 36, 1321–1324.
- 58 Li, Y. and Sen, D. (1996) A catalytic DNA for porphyrin metallation. *Nat. Struct. Biol.* 3, 743–747.

- 59 Li, Y. and Sen, D. (1997) Toward an efficient DNAzyme. *Biochemistry* 36, 5589–5599.
- 60 Höbartner, C. and Silverman, S. K. (2007) Engineering a selective small-molecule substrate binding site into a deoxyribozyme. *Angew. Chem. Int. Ed.* 46, 7420–7424.
- 61 Tarasow, T. M., Tarasow, S. L. and Eaton, B. E. (1997) RNA-catalysed carbon-carbon bond formation. *Nature* 389, 54–57.
- 62 Seelig, B. and Jäschke, A. (1999) A small catalytic RNA motif with Diels-Alderase activity. *Chem. Biol.* 6, 167–176.
- 63 Bock, L. C., Griffin, L. C., Latham, J. A., Vermaas, E. H. and Toole, J. J. (1992) Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* 355, 564–566.
- 64 Hermann, T. and Patel, D. J. (2000) Adaptive recognition by nucleic acid aptamers. *Science* 287, 820–825.
- 65 Silverman, S. K. (2007) Artificial functional nucleic acids: aptamers, ribozymes and deoxyribozymes identified by *in vitro* selection. In: *Functional Nucleic Acids for Sensing and Other Analytical Applications*, Lu, Y. and Li, Y. (Eds.), Springer, New York.
- 66 Santoro, S. W. and Joyce, G. F. (1998) Mechanism and utility of an RNA-cleaving DNA enzyme. *Biochemistry* 37, 13330–13342.
- 67 Okumoto, Y. and Sugimoto, N. (2000) Effects of metal ions and catalytic loop sequences on the complex formation of a deoxyribozyme and its RNA substrate. *J. Inorg. Biochem.* 82, 189–195.
- 68 Zaborowska, Z., Furste, J. P., Erdmann, V. A. and Kurreck, J. (2002) Sequence requirements in the catalytic core of the '10-23' DNA enzyme. *J. Biol. Chem.* 277, 40617–40622.
- 69 Zaborowska, Z., Schubert, S., Kurreck, J. and Erdmann, V. A. (2005) Deletion analysis in the catalytic region of the 10-23 DNA enzyme. *FEBS Lett.* 579, 554–558.
- 70 Cairns, M. J., King, A. and Sun, L.-Q. (2003) Optimisation of the 10-23 DNAzyme-substrate pairing interactions enhanced RNA cleavage activity at purine-cytosine target sites. *Nucleic Acids Res.* 31, 2883–2889.
- 71 Peracchi, A. (2000) Preferential activation of the 8-17 deoxyribozyme by Ca²⁺ ions. Evidence for the identity of 8-17 with the catalytic domain of the Mg5 deoxyribozyme. *J. Biol. Chem.* 275, 11693–11697.
- 72 Peracchi, A., Bonaccio, M. and Clerici, M. (2005) A mutational analysis of the 8-17 deoxyribozyme core. *J. Mol. Biol.* 352, 783–794.
- 73 Nowakowski, J., Shim, P. J., Prasad, G. S., Stout, C. D. and Joyce, G. F. (1999) Crystal structure of an 82-nucleotide RNA-DNA complex formed by the 10-23 DNA enzyme. *Nat. Struct. Biol.* 6, 151–156.
- 74 Liu, J. and Lu, Y. (2002) FRET study of a trifluorophore-labeled DNAzyme. *J. Am. Chem. Soc.* 124, 15208–15216.
- 75 Kim, H. K., Liu, J., Li, J., Nagraj, N., Li, M., Pavot, C. M. and Lu, Y. (2007) Metal-dependent global folding and activity of the 8–17 DNAzyme studied by fluorescence resonance energy transfer. *J. Am. Chem. Soc.* 129, 6896–6902.
- 76 Lee, N. K., Koh, H. R., Han, K. Y. and Kim, S. K. (2007) Folding of 8-17 deoxyribozyme studied by three-color alternating-laser excitation of single molecules. *J. Am. Chem. Soc.* 129, 15526–15534.
- 77 Kim, H. K., Rasnik, I., Liu, J., Ha, T. and Lu, Y. (2007) Dissecting metal ion-dependent folding and catalysis of a single DNAzyme. *Nat. Chem. Biol.* 3, 763–768.
- 78 Lilley, D. M. (2004) The Varkud satellite ribozyme. *RNA* 10, 151–158.
- 79 Lilley, D. M. J. (2000) Structures of helical junctions in nucleic acids. *Q. Rev. Biophys.* 33, 109–159.
- 80 Chinnapen, D. J. and Sen, D. (2007) Towards elucidation of the mechanism of UV1C, a deoxyribozyme with photolyase activity. *J. Mol. Biol.* 365, 1326–1336.
- 81 McManus, S. A. and Li, Y. (2008) A deoxyribozyme with a novel guanine quartet-helix pseudoknot structure. *J. Mol. Biol.* 375, 960–968.
- 82 Leung, E. K. and Sen, D. (2007) Electron hole flow patterns through the RNA-cleaving 8-17 deoxyribozyme yield unusual information about its structure and folding. *Chem. Biol.* 14, 41–51.
- 83 Coppins, R. L. and Silverman, S. K. (2005) Mimicking the first step of RNA splicing: an artificial DNA enzyme can synthesize branched RNA using an oligonucleotide leaving group as a 5'-exon analogue. *Biochemistry* 44, 13439–13446.
- 84 Cech, T. R. (1987) The chemistry of self-splicing RNA and RNA enzymes. *Science* 236, 1532–1539.
- 85 Emilsson, G. M., Nakamura, S., Roth, A. and Breaker, R. R. (2003) Ribozyme speed limits. *RNA* 9, 907–918.
- 86 Breaker, R. R., Emilsson, G. M., Lazarev, D., Nakamura, S., Puskarz, I. J., Roth, A. and Sudarsan, N. (2003) A common speed limit for RNA-cleaving ribozymes and deoxyribozymes. *RNA* 9, 949–957.
- 87 Grosshans, C. A. and Cech, T. R. (1991) A hammerhead ribozyme allows synthesis of a new form of the *Tetrahymena* ribozyme homogeneous in length with a 3' end blocked for transesterification. *Nucleic Acids Res.* 19, 3875–3880.
- 88 Ferré-D'Amaré, A. R. and Doudna, J. A. (1996) Use of *cis*- and *trans*-ribozymes to remove 5' and 3' heterogeneities from milligrams of *in vitro* transcribed RNA. *Nucleic Acids Res.* 24, 977–978.
- 89 Lapham, J. and Crothers, D. M. (1996) RNase H cleavage for processing of *in vitro* transcribed RNA for NMR studies and RNA ligation. *RNA* 2, 289–296.
- 90 Pyle, A. M., Chu, V. T., Jankowsky, E. and Boudvillain, M. (2000) Using DNAzymes to cut, process, and map RNA molecules for structural studies or modification. *Methods Enzymol.* 317, 140–146.
- 91 Chu, V. T., Liu, Q., Podar, M., Perlman, P. S. and Pyle, A. M. (1998) More than one way to splice an RNA: branching without a bulge and splicing without branching in group II introns. *RNA* 4, 1186–1202.
- 92 Chu, V. T., Adamidi, C., Liu, Q., Perlman, P. S. and Pyle, A. M. (2001) Control of branch-site choice by a group II intron. *EMBO J.* 20, 6866–6876.
- 93 Hiley, S. L., Sood, V. D., Fan, J. and Collins, R. A. (2002) 4-thio-U cross-linking identifies the active site of the VS ribozyme. *EMBO J.* 21, 4691–4698.
- 94 Buchhaupt, M., Peifer, C. and Entian, K. D. (2007) Analysis of 2'-O-methylated nucleosides and pseudouridines in ribosomal RNAs using DNAzymes. *Anal. Biochem.* 361, 102–108.
- 95 Lapham, J., Yu, Y. T., Shu, M. D., Steitz, J. A. and Crothers, D. M. (1997) The position of site-directed cleavage of RNA using RNase H and 2'-O-methyl oligonucleotides is dependent on the enzyme source. *RNA* 3, 950–951.
- 96 Hengesbach, M., Meusburger, M., Lyko, F. and Helm, M. (2008) Use of DNAzymes for site-specific analysis of ribonucleotide modifications. *RNA* 14, 180–187.
- 97 Cairns, M. J., King, A. and Sun, L.-Q. (2000) Nucleic acid mutation analysis using catalytic DNA. *Nucleic Acids Res.* 28, E9.
- 98 Schubert, S., Furste, J. P., Werk, D., Grunert, H. P., Zeichhardt, H., Erdmann, V. A. and Kurreck, J. (2004) Gaining target access for deoxyribozymes. *J. Mol. Biol.* 339, 355–363.
- 99 Vester, B., Lundberg, L. B., Sorensen, M. D., Babu, B. R., Douthwaite, S. and Wengel, J. (2004) Improved RNA cleavage by LNAzyme derivatives of DNAzymes. *Biochem. Soc. Trans.* 32, 37–40.
- 100 Kierzek, E., Ciesielska, A., Pasternak, K., Mathews, D. H., Turner, D. H. and Kierzek, R. (2005) The influence of locked nucleic acid residues on the thermodynamic properties of 2'-O-methyl RNA/RNA heteroduplexes. *Nucleic Acids Res.* 33, 5082–5093.
- 101 Todd, A. V., Fuery, C. J., Impney, H. L., Applegate, T. L. and Houghton, M. A. (2000) DzyNA-PCR: use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format. *Clin. Chem.* 46, 625–630.

- 102 Ordoukhanian, P. and Joyce, G. F. (2002) RNA-cleaving DNA enzymes with altered regio- or enantioselectivity. *J. Am. Chem. Soc.* 124, 12499–12506.
- 103 Suenaga, H., Liu, R., Shiramasa, Y. and Kanagawa, T. (2005) Novel approach to quantitative detection of specific rRNA in a microbial community, using catalytic DNA. *Appl. Environ. Microbiol.* 71, 4879–4884.
- 104 Ting, R., Lermer, L. and Perrin, D. M. (2004) Triggering DNAszymes with light: a photoactive C8 thioether-linked adenosine. *J. Am. Chem. Soc.* 126, 12720–12721.
- 105 Lusic, H., Young, D. D., Lively, M. O. and Deiters, A. (2007) Photochemical DNA activation. *Org. Lett.* 9, 1903–1906.
- 106 Keiper, S. and Vyle, J. S. (2006) Reversible photocontrol of deoxyribozyme-catalyzed RNA cleavage under multiple-turnover conditions. *Angew. Chem. Int. Ed.* 45, 3306–3309.
- 107 Liu, Y. and Sen, D. (2004) Light-regulated catalysis by an RNA-cleaving deoxyribozyme. *J. Mol. Biol.* 341, 887–892.
- 108 Asanuma, H., Hayashi, H., Zhao, J., Liang, X., Yamazawa, A., Kuramochi, T., Matsunaga, D., Aiba, Y., Kashida, H. and Komiyama, M. (2006) Enhancement of RNA cleavage activity of 10-23 DNAzyme by covalently introduced intercalator. *Chem. Commun.*, 5062–5064.
- 109 Li, J., Zheng, W., Kwon, A. H. and Lu, Y. (2000) A highly sensitive and selective catalytic DNA biosensor for lead ions. *J. Am. Chem. Soc.* 122, 10466–10467.
- 110 Liu, J. and Lu, Y. (2003) A colorimetric lead biosensor using DNAzyme-directed assembly of gold nanoparticles. *J. Am. Chem. Soc.* 125, 6642–6643.
- 111 Liu, J. and Lu, Y. (2004) Accelerated color change of gold nanoparticles assembled by DNAzymes for simple and fast colorimetric Pb²⁺ detection. *J. Am. Chem. Soc.* 126, 12298–12305.
- 112 Chang, I. H., Tulock, J. J., Liu, J., Kim, W. S., Cannon, D. M., Jr., Lu, Y., Bohn, P. W., Sweedler, J. V. and Cropek, D. M. (2005) Miniaturized lead sensor based on lead-specific DNAzyme in a nanocapillary interconnected microfluidic device. *Environ. Sci. Technol.* 39, 3756–3761.
- 113 Liu, J., Brown, A. K., Meng, X., Cropek, D. M., Istok, J. D., Watson, D. B. and Lu, Y. (2007) A catalytic beacon sensor for uranium with parts-per-trillion sensitivity and millionfold selectivity. *Proc. Natl. Acad. Sci. USA* 104, 2056–2061.
- 114 Liu, J. and Lu, Y. (2007) A DNAzyme catalytic beacon sensor for paramagnetic Cu²⁺ ions in aqueous solution with high sensitivity and selectivity. *J. Am. Chem. Soc.* 129, 9838–9839.
- 115 Liu, J. and Lu, Y. (2007) Rational design of ‘turn-on’ allosteric DNAzyme catalytic beacons for aqueous mercury ions with ultrahigh sensitivity and selectivity. *Angew. Chem. Int. Ed.* 46, 7587–7590.
- 116 Mei, S. H., Liu, Z., Brennan, J. D. and Li, Y. (2003) An efficient RNA-cleaving DNA enzyme that synchronizes catalysis with fluorescence signaling. *J. Am. Chem. Soc.* 125, 412–420.
- 117 Shen, Y., Mackey, G., Rupcich, N., Gloster, D., Chiuman, W., Li, Y. and Brennan, J. D. (2007) Entrapment of fluorescence signaling DNA enzymes in sol-gel-derived materials for metal ion sensing. *Anal. Chem.* 79, 3494–3503.
- 118 Xiao, Y., Rowe, A. A. and Plaxco, K. W. (2007) Electrochemical detection of parts-per-billion lead via an electrode-bound DNAzyme assembly. *J. Am. Chem. Soc.* 129, 262–263.
- 119 Liu, J. and Lu, Y. (2004) Colorimetric biosensors based on DNAzyme-assembled gold nanoparticles. *J. Fluoresc.* 14, 343–354.
- 120 Liu, J. and Lu, Y. (2005) Stimuli-responsive disassembly of nanoparticle aggregates for light-up colorimetric sensing. *J. Am. Chem. Soc.* 127, 12677–12683.
- 121 Lu, Y. and Liu, J. (2006) Functional DNA nanotechnology: emerging applications of DNAzymes and aptamers. *Curr. Opin. Biotechnol.* 17, 580–588.
- 122 Liu, J. and Lu, Y. (2007) Colorimetric Cu²⁺ detection with a ligation DNAzyme and nanoparticles. *Chem. Commun.*, 4872–4874.
- 123 Liu, J. and Lu, Y. (2004) Adenosine-dependent assembly of aptazyme-functionalized gold nanoparticles and its application as a colorimetric biosensor. *Anal. Chem.* 76, 1627–1632.
- 124 Liu, J. and Lu, Y. (2006) Fast colorimetric sensing of adenosine and cocaine based on a general sensor design involving aptamers and nanoparticles. *Angew. Chem. Int. Ed.* 45, 90–94.
- 125 Levy, M. and Ellington, A. D. (2002) ATP-dependent allosteric DNA enzymes. *Chem. Biol.* 9, 417–426.
- 126 Cho, E. J., Yang, L., Levy, M. and Ellington, A. D. (2005) Using a deoxyribozyme ligase and rolling circle amplification to detect a non-nucleic acid analyte, ATP. *J. Am. Chem. Soc.* 127, 2022–2023.
- 127 Achenbach, J. C., Nutiu, R. and Li, Y. (2005) Structure-switching allosteric deoxyribozymes. *Anal. Chim. Acta* 534, 41–51.
- 128 Nutiu, R. and Li, Y. (2003) Structure-switching signaling aptamers. *J. Am. Chem. Soc.* 125, 4771–4778.
- 129 Nutiu, R. and Li, Y. (2004) Structure-switching signaling aptamers: transducing molecular recognition into fluorescence signaling. *Chem. Eur. J.* 10, 1868–1876.
- 130 Nutiu, R. and Li, Y. (2005) Aptamers with fluorescence-signaling properties. *Methods* 37, 16–25.
- 131 Navani, N. K. and Li, Y. (2006) Nucleic acid aptamers and enzymes as sensors. *Curr. Opin. Chem. Biol.* 10, 272–281.
- 132 Wang, D. Y. and Sen, D. (2001) A novel mode of regulation of an RNA-cleaving DNAzyme by effectors that bind to both enzyme and substrate. *J. Mol. Biol.* 310, 723–734.
- 133 Wang, D. Y., Lai, B. H. and Sen, D. (2002) A general strategy for effector-mediated control of RNA-cleaving ribozymes and DNA enzymes. *J. Mol. Biol.* 318, 33–43.
- 134 Wang, D. Y., Lai, B. H., Feldman, A. R. and Sen, D. (2002) A general approach for the use of oligonucleotide effectors to regulate the catalysis of RNA-cleaving ribozymes and DNAzymes. *Nucleic Acids Res.* 30, 1735–1742.
- 135 Sando, S., Sasaki, T., Kanatani, K. and Aoyama, Y. (2003) Amplified nucleic acid sensing using programmed self-cleaving DNAzyme. *J. Am. Chem. Soc.* 125, 15720–15721.
- 136 Stojanovic, M. N., de Prada, P. and Landry, D. W. (2001) Catalytic molecular beacons. *ChemBioChem* 2, 411–415.
- 137 Kolpashchikov, D. M. (2007) A binary deoxyribozyme for nucleic acid analysis. *ChemBioChem* 8, 2039–2042.
- 138 Stojanovic, M. N., de Prada, P. and Landry, D. W. (2000) Homogeneous assays based on deoxyribozyme catalysis. *Nucleic Acids Res.* 28, 2915–2918.
- 139 Santiago, F. S., Lowe, H. C., Kavurma, M. M., Chesterman, C. N., Baker, A., Atkins, D. G. and Khachigian, L. M. (1999) New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury. *Nat. Med.* 5, 1264–1269.
- 140 Fahmy, R. G. and Khachigian, L. M. (2004) Locked nucleic acid modified DNA enzymes targeting early growth response-1 inhibit human vascular smooth muscle cell growth. *Nucleic Acids Res.* 32, 2281–2285.
- 141 Bhindi, R., Khachigian, L. M. and Lowe, H. C. (2006) DNAzymes targeting the transcription factor Egr-1 reduce myocardial infarct size following ischemia-reperfusion in rats. *J. Thromb. Haemost.* 4, 1479–1483.
- 142 Mitchell, A., Dass, C. R., Sun, L.-Q. and Khachigian, L. M. (2004) Inhibition of human breast carcinoma proliferation, migration, chemoinvasion and solid tumour growth by DNAzymes targeting the zinc finger transcription factor EGR-1. *Nucleic Acids Res.* 32, 3065–3069.
- 143 Lu, Z. X., Ye, M., Yan, G. R., Li, Q., Tang, M., Lee, L. M., Sun, L. Q. and Cao, Y. (2005) Effect of EBV LMP1 targeted DNAzymes on cell proliferation and apoptosis. *Cancer Gene Ther.* 12, 647–654.
- 144 Hou, W., Ni, Q., Wo, J., Li, M., Liu, K., Chen, L., Hu, Z., Liu, R. and Hu, M. (2006) Inhibition of hepatitis B virus X gene expression by 10-23 DNAzymes. *Antiviral Res.* 72, 190–196.
- 145 Wo, J. E., Wu, X. L., Zhou, L. F., Yao, H. P., Chen, L. W. and Dennin, R. H. (2005) Effective inhibition of expression of

- hepatitis B virus genes by DNazymes. *World J. Gastroenterol.* 11, 3504–3507.
- 146 Trepanier, J., Tanner, J. E., Momparler, R. L., Le, O. N., Alvarez, F. and Alfieri, C. (2006) Cleavage of intracellular hepatitis C RNA in the virus core protein coding region by deoxyribozymes. *J. Viral Hepat.* 13, 131–138.
- 147 Sriram, B. and Banerjee, A. C. (2000) *In vitro*-selected RNA cleaving DNA enzymes from a combinatorial library are potent inhibitors of HIV-1 gene expression. *Biochem. J.* 352, 667–673.
- 148 Dash, B. C. and Banerjee, A. C. (2004) Sequence-specific cleavage activities of DNA enzymes targeted against HIV-1 Gag and Nef regions. *Oligonucleotides* 14, 41–47.
- 149 Sood, V., Gupta, N., Bano, A. S. and Banerjee, A. C. (2007) DNA-enzyme-mediated cleavage of human immunodeficiency virus type 1 Gag RNA is significantly augmented by antisense-DNA molecules targeted to hybridize close to the cleavage site. *Oligonucleotides* 17, 113–121.
- 150 Unwalla, H., Chakraborti, S., Sood, V., Gupta, N. and Banerjee, A. C. (2006) Potent inhibition of HIV-1 gene expression and TAT-mediated apoptosis in human T cells by novel mono- and multitarget anti-TAT/Rev/Env ribozymes and a general purpose RNA-cleaving DNA-enzyme. *Antiviral Res.* 72, 134–144.
- 151 Sood, V., Unwalla, H., Gupta, N., Chakraborti, S. and Banerjee, A. C. (2007) Potent knock down of HIV-1 replication by targeting HIV-1 Tat/Rev RNA sequences synergistically with catalytic RNA and DNA. *AIDS* 21, 31–40.
- 152 Jakobsen, M. R., Haasnoot, J., Wengel, J., Berkhout, B. and Kijms, J. (2007) Efficient inhibition of HIV-1 expression by LNA modified antisense oligonucleotides and DNazymes targeted to functionally selected binding sites. *Retrovirology* 4, 29.
- 153 Yuan, B. F., Xue, Y., Luo, M., Hao, Y. H. and Tan, Z. (2007) Two DNazymes targeting the telomerase mRNA with large difference in Mg^{2+} concentration for maximal catalytic activity. *Int. J. Biochem. Cell Biol.* 39, 1119–1129.
- 154 Takahashi, H., Hamazaki, H., Habu, Y., Hayashi, M., Abe, T., Miyano-Kurosaki, N. and Takaku, H. (2004) A new modified DNA enzyme that targets influenza virus A mRNA inhibits viral infection in cultured cells. *FEBS Lett.* 560, 69–74.
- 155 Li, J., Zhu, D., Yi, Z., He, Y., Chun, Y., Liu, Y. and Li, N. (2005) DNazymes targeting the *icl* gene inhibit ICL expression and decrease *Mycobacterium tuberculosis* survival in macrophages. *Oligonucleotides* 15, 215–222.
- 156 Fahmy, R. G., Waldman, A., Zhang, G., Mitchell, A., Tedla, N., Cai, H., Gezy, C. R., Chesterman, C. N., Perry, M. and Khachigian, L. M. (2006) Suppression of vascular permeability and inflammation by targeting of the transcription factor c-Jun. *Nat. Biotechnol.* 24, 856–863.
- 157 Zhang, G., Dass, C. R., Sumithran, E., Di Girolamo, N., Sun, L. Q. and Khachigian, L. M. (2004) Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents. *J. Natl. Cancer Inst.* 96, 683–696.
- 158 Chen, F., Li, Z., Wang, R., Liu, B., Zeng, Z., Zhang, H. and Zhang, J. (2004) Inhibition of ampicillin-resistant bacteria by novel mono-DNazymes and di-DNAzyme targeted to β -lactamase mRNA. *Oligonucleotides* 14, 80–89.
- 159 Hou, Z., Meng, J. R., Zhao, J. R., Hu, B. Q., Liu, J., Yan, X. J., Jia, M. and Luo, X. X. (2007) Inhibition of β -lactamase-mediated oxacillin resistance in *Staphylococcus aureus* by a deoxyribozyme. *Acta Pharmacol. Sin.* 28, 1775–1782.
- 160 Liu, C., Cheng, R., Sun, L. Q. and Tien, P. (2001) Suppression of platelet-type 12-lipoxygenase activity in human erythroleukemia cells by an RNA-cleaving DNAzyme. *Biochem. Biophys. Res. Commun.* 284, 1077–1082.
- 161 Sun, L.-Q., Cairns, M. J., Gerlach, W. L., Witherington, C., Wang, L. and King, A. (1999) Suppression of smooth muscle cell proliferation by a *c-myc* RNA-cleaving deoxyribozyme. *J. Biol. Chem.* 274, 17236–17241.
- 162 Dass, C. R., Saravolac, E. G., Li, Y. and Sun, L.-Q. (2002) Cellular uptake, distribution, and stability of 10-23 deoxyribozymes. *Antisense Nucleic Acid Drug Dev.* 12, 289–299.
- 163 Pun, S. H., Tack, F., Belloq, N. C., Cheng, J., Grubbs, B. H., Jensen, G. S., Davis, M. E., Brewster, M., Janicot, M., Janssens, B. et al. (2004) Targeted delivery of RNA-cleaving DNA enzyme (DNAzyme) to tumor tissue by transferrin-modified, cyclodextrin-based particles. *Cancer Biol. Ther.* 3, 641–650.
- 164 Ackermann, J. M., Kanugula, S. and Pegg, A. E. (2005) DNAzyme-mediated silencing of ornithine decarboxylase. *Biochemistry* 44, 2143–2152.
- 165 Hou, Z., Meng, J. R., Niu, C., Wang, H. F., Liu, J., Hu, B. Q., Jia, M. and Luo, X. X. (2007) Restoration of antibiotic susceptibility in methicillin-resistant *Staphylococcus aureus* by targeting *mecR1* with a phosphorothioate deoxyribozyme. *Clin. Exp. Pharmacol. Physiol.* 34, 1160–1164.
- 166 Kabuli, M., Yin, J. A. and Tobal, K. (2004) Targeting PML/RAR α transcript with DNazymes results in reduction of proliferation and induction of apoptosis in APL cells. *Hematol. J.* 5, 426–433.
- 167 Zhou, J., Yang, X. Q., Xie, Y. Y., Zhao, X. D., Jiang, L. P., Wang, L. J. and Cui, Y. X. (2007) Inhibition of respiratory syncytial virus of subgroups A and B using deoxyribozyme DZ1133 in mice. *Virus Res.* 130, 241–248.
- 168 Wu, S., Xu, J., Liu, J., Yan, X., Zhu, X., Xiao, G., Sun, L. and Tien, P. (2007) An efficient RNA-cleaving DNA enzyme can specifically target the 5'-untranslated region of severe acute respiratory syndrome associated coronavirus (SARS-CoV). *J. Gene Med.* 9, 1080–1086.
- 169 Liang, Z., Wei, S., Guan, J., Luo, Y., Gao, J., Zhu, H., Wu, S. and Liu, T. (2005) DNAzyme-mediated cleavage of survivin mRNA and inhibition of the growth of PANC-1 cells. *J. Gastroenterol. Hepatol.* 20, 1595–1602.
- 170 Isaka, Y., Nakamura, H., Mizui, M., Takabatake, Y., Horio, M., Kawachi, H., Shimizu, F., Imai, E. and Hori, M. (2004) DNAzyme for TGF- β suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int.* 66, 586–590.
- 171 Hjiantonou, E., Iseki, S., Uney, J. B. and Phylactou, L. A. (2003) DNzyme-mediated cleavage of Twist transcripts and increase in cellular apoptosis. *Biochem. Biophys. Res. Commun.* 300, 178–181.
- 172 Zhang, L., Gasper, W. J., Stass, S. A., Ioffe, O. B., Davis, M. A. and Mixson, A. J. (2002) Angiogenic inhibition mediated by a DNAzyme that targets vascular endothelial growth factor receptor 2. *Cancer Res.* 62, 5463–5469.
- 173 Rice, L. B. (2006) Antimicrobial resistance in gram-positive bacteria. *Am. J. Infect. Control.* 34, S11–S19.
- 174 Gootz, T. D. (2006) The forgotten Gram-negative bacilli: what genetic determinants are telling us about the spread of antibiotic resistance. *Biochem. Pharmacol.* 71, 1073–1084.
- 175 Horn, S. and Schwenzer, B. (1999) Oligonucleotide facilitators enhance the catalytic activity of RNA-cleaving DNA enzymes. *Antisense Nucleic Acid Drug Dev.* 9, 465–472.
- 176 Fokina, A. A., Kuznetsova, M. A., Repkova, M. N. and Venyaminova, A. G. (2004) Two-component 10-23 DNA enzymes. *Nucleosides Nucleotides Nucleic Acids* 23, 1031–1035.
- 177 Corey, D. R. (2007) RNA learns from antisense. *Nat. Chem. Biol.* 3, 8–11.
- 178 Romani, A. and Scarpa, A. (1992) Regulation of cell magnesium. *Arch. Biochem. Biophys.* 298, 1–12.
- 179 Grubbs, R. D. (2002) Intracellular magnesium and magnesium buffering. *BioMetals* 15, 251–259.
- 180 Opalinska, J. B. and Gewirtz, A. M. (2002) Nucleic-acid therapeutics: basic principles and recent applications. *Nat. Rev. Drug Discov.* 1, 503–514.
- 181 Sioud, M. (2004) Ribozyme- and siRNA-mediated mRNA degradation: a general introduction. *Methods Mol. Biol.* 252, 1–8.

- 182 Patil, S. D., Rhodes, D. G. and Burgess, D. J. (2005) DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS J.* 7, E61–E77.
- 183 Nunamaker, E. A., Zhang, H. Y., Shirasawa, Y., Benoit, J. N. and Dean, D. A. (2003) Electroporation-mediated delivery of catalytic oligodeoxynucleotides for manipulation of vascular gene expression. *Am. J. Physiol. Heart Circ. Physiol.* 285, H2240–H2247.
- 184 Datta, H. J. and Glazer, P. M. (2001) Intracellular generation of single-stranded DNA for chromosomal triplex formation and induced recombination. *Nucleic Acids Res.* 29, 5140–5147.
- 185 Tan, X.-X., Rose, K., Margolin, W. and Chen, Y. (2004) DNA enzyme generated by a novel single-stranded DNA expression vector inhibits expression of the essential bacterial cell division gene *ftsZ*. *Biochemistry* 43, 1111–1117.
- 186 Chen, F., Wang, R., Li, Z., Liu, B., Wang, X., Sun, Y., Hao, D. and Zhang, J. (2004) A novel replicating circular DNAzyme. *Nucleic Acids Res.* 32, 2336–2341.
- 187 Tack, F., Bakker, A., Maes, S., Dekeyser, N., Bruining, M., Elissen-Roman, C., Janicot, M., Brewster, M., Janssen, H. M., De Waal, B. F. et al. (2006) Modified poly(propylene imine) dendrimers as effective transfection agents for catalytic DNA enzymes (DNAzymes). *J. Drug Target.* 14, 69–86.
- 188 Tack, F., Bakker, A., Maes, S., Dekeyser, N., Bruining, M., Elissen-Roman, C., Janicot, M., Janssen, H. M., De Waal, B. F., Fransen, P. M. et al. (2006) Dendrimeric poly(propylene-imines) as effective delivery agents for DNAzymes: toxicity, *in vitro* transfection and *in vivo* delivery. *J. Control. Release* 116, e26–e28.
- 189 Kubo, T., Takamori, K., Kanno, K., Rumiana, B., Ohba, H., Matsukisano, M., Akebiyama, Y. and Fujii, M. (2005) Efficient cleavage of RNA, enhanced cellular uptake, and controlled intracellular localization of conjugate DNAzymes. *Bioorg. Med. Chem. Lett.* 15, 167–170.
- 190 Seifert, G., Taube, T., Paal, K., von Einsiedel, H. G., Wellmann, S., Henze, G., Seeger, K., Schroff, M. and Wittig, B. (2006) Stability and catalytic activity of novel circular DNAzymes. *Nucleosides Nucleotides Nucleic Acids* 25, 785–793.
- 191 Schubert, S., Gul, D. C., Grunert, H. P., Zeichhardt, H., Erdmann, V. A. and Kurreck, J. (2003) RNA cleaving ‘10-23’ DNAzymes with enhanced stability and activity. *Nucleic Acids Res.* 31, 5982–5992.
- 192 Vester, B., Hansen, L. H., Lundberg, L. B., Babu, B. R., Sorensen, M. D., Wengel, J. and Douthwaite, S. (2006) Locked nucleoside analogues expand the potential of DNAzymes to cleave structured RNA targets. *BMC Mol. Biol.* 7, 19.
- 193 Abdelgany, A., Wood, M. and Beeson, D. (2007) Hairpin DNAzymes: a new tool for efficient cellular gene silencing. *J. Gene Med.* 9, 727–738.
- 194 Moore, M. J. and Sharp, P. A. (1992) Site-specific modification of pre-mRNA: the 2'-hydroxyl groups at the splice site. *Science* 256, 992–997.
- 195 Moore, M. J. and Query, C. C. (1998) Use of site-specifically modified RNAs constructed by RNA ligation. In: *RNA-Protein Interactions: A Practical Approach*, pp. 75–108, Smith, C. W. J. (ed.), Oxford University Press, Oxford.
- 196 Bain, J. D. and Switzer, C. (1992) Regioselective ligation of oligoribonucleotides using DNA splints. *Nucleic Acids Res.* 20, 4372.
- 197 Stark, M. R., Pleiss, J. A., Deras, M., Scaringe, S. A. and Rader, S. D. (2006) An RNA ligase-mediated method for the efficient creation of large, synthetic RNAs. *RNA* 12, 2014–2019.
- 198 Wang, Y. and Silverman, S. K. (2006) Experimental tests of two proofreading mechanisms for 5'-splice site selection. *ACS Chem. Biol.* 1, 316–324.
- 199 Pratico, E. D. and Silverman, S. K. (2007) Ty1 reverse transcriptase does not read through the proposed 2',5'-branched retrotransposition intermediate *in vitro*. *RNA* 13, 1528–1536.
- 200 Baum, D. A. and Silverman, S. K. (2007) Deoxyribozyme-catalyzed labeling of RNA. *Angew. Chem. Int. Ed.* 46, 3502–3504.
- 201 Chow, C. S., Mahto, S. K. and Lamichhane, T. N. (2008) Combined approaches to site-specific modification of RNA. *ACS Chem. Biol.* 3, 30–37.
- 202 Miduturu, C. V. and Silverman, S. K. (2005) DNA constraints allow rational control of macromolecular conformation. *J. Am. Chem. Soc.* 127, 10144–10145.
- 203 Miduturu, C. V. and Silverman, S. K. (2006) Modulation of DNA constraints on macromolecular folding. *Angew. Chem. Int. Ed.* 45, 1918–1921.
- 204 Miduturu, C. V. and Silverman, S. K. (2006) Synthesis and application of a 5'-aldehyde phosphoramidite for covalent attachment of DNA to biomolecules. *J. Org. Chem.* 71, 5774–5777.
- 205 Zelin, E. and Silverman, S. K. (2007) Allosteric control of ribozyme catalysis using DNA constraints. *ChemBioChem* 8, 1907–1911.
- 206 Chen, Y., Wang, M. and Mao, C. (2004) An autonomous DNA nanomotor powered by a DNA enzyme. *Angew. Chem. Int. Ed.* 43, 3554–3557.
- 207 Tian, Y., He, Y., Chen, Y., Yin, P. and Mao, C. (2005) A DNAzyme that walks processively and autonomously along a one-dimensional track. *Angew. Chem. Int. Ed.* 44, 4355–4358.
- 208 Stojanovic, M. N., Mitchell, T. E. and Stefanovic, D. (2002) Deoxyribozyme-based logic gates. *J. Am. Chem. Soc.* 124, 3555–3561.
- 209 Stojanovic, M. N. and Stefanovic, D. (2003) Deoxyribozyme-based half-adder. *J. Am. Chem. Soc.* 125, 6673–6676.
- 210 Lederman, H., Macdonald, J., Stefanovic, D. and Stojanovic, M. N. (2006) Deoxyribozyme-based three-input logic gates and construction of a molecular full adder. *Biochemistry* 45, 1194–1199.
- 211 Stojanovic, M. N. and Stefanovic, D. (2003) A deoxyribozyme-based molecular automaton. *Nat. Biotechnol.* 21, 1069–1074.
- 212 Macdonald, J., Li, Y., Sutovic, M., Lederman, H., Pendri, K., Lu, W., Andrews, B. L., Stefanovic, D. and Stojanovic, M. N. (2006) Medium scale integration of molecular logic gates in an automaton. *Nano Lett.* 6, 2598–2603.
- 213 Stojanovic, M. N., Semova, S., Kolpashchikov, D., Macdonald, J., Morgan, C. and Stefanovic, D. (2005) Deoxyribozyme-based ligase logic gates and their initial circuits. *J. Am. Chem. Soc.* 127, 6914–6915.
- 214 Chen, X., Wang, Y., Liu, Q., Zhang, Z., Fan, C. and He, L. (2006) Construction of molecular logic gates with a DNA-cleaving deoxyribozyme. *Angew. Chem. Int. Ed.* 45, 1759–1762.
- 215 Travascio, P., Li, Y. and Sen, D. (1998) DNA-enhanced peroxidase activity of a DNA-aptamer-hemin complex. *Chem. Biol.* 5, 505–517.
- 216 Travascio, P., Bennet, A. J., Wang, D. Y. and Sen, D. (1999) A ribozyme and a catalytic DNA with peroxidase activity: active sites versus cofactor-binding sites. *Chem. Biol.* 6, 779–787.
- 217 Travascio, P., Witting, P. K., Mauk, A. G. and Sen, D. (2001) The peroxidase activity of a hemin-DNA oligonucleotide complex: free radical damage to specific guanine bases of the DNA. *J. Am. Chem. Soc.* 123, 1337–1348.
- 218 Cheglakov, Z., Weizmann, Y., Beissenhertz, M. K. and Willner, I. (2006) Ultrasensitive detection of DNA by the PCR-induced generation of DNAzymes: the DNAzyme primer approach. *Chem. Commun.*, 3205–3207.
- 219 Cheglakov, Z., Weizmann, Y., Basnar, B. and Willner, I. (2007) Diagnosing viruses by the rolling circle amplified synthesis of DNAzymes. *Org. Biomol. Chem.* 5, 223–225.
- 220 Tian, Y., He, Y. and Mao, C. (2006) Cascade signal amplification for DNA detection. *ChemBioChem* 7, 1862–1864.
- 221 Li, D., Shlyahovsky, B., Elbaz, J. and Willner, I. (2007) Amplified analysis of low-molecular-weight substrates or proteins by the self-assembly of DNAzyme-aptamer conjugates. *J. Am. Chem. Soc.* 129, 5804–5805.