

## Review

## Catalytic DNA: Scope, Applications, and Biochemistry of Deoxyribozymes

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The discovery of natural RNA enzymes (ribozymes) prompted the pursuit of artificial DNA enzymes (deoxyribozymes) by *in vitro* selection methods. A key motivation is the conceptual and practical advantages of DNA relative to proteins and RNA. Early studies focused on RNA-cleaving deoxyribozymes, and more recent experiments have expanded the breadth of catalytic DNA to many other reactions. Including modified nucleotides has the potential to widen the scope of DNA enzymes even further. Practical applications of deoxyribozymes include their use as sensors for metal ions and small molecules. Structural studies of deoxyribozymes are only now beginning; mechanistic experiments will surely follow. Following the first report 21 years ago, the field of deoxyribozymes has promise for both fundamental and applied advances in chemistry, biology, and other disciplines.

### Nucleic Acids as Catalysts

Nature has evolved a wide range of protein enzymes for biological catalysis. The notion that biomolecules other than proteins can be catalysts was largely disregarded until the early 1980s, when the enzymatic abilities of natural RNA catalysts (**ribozymes**; see [Glossary](#)) were discovered [1,2]. In modern biochemistry, catalysis by RNA appears restricted to RNA cleavage and ligation as well as to peptide bond formation in the ribosome, although a primordial RNA World with broader RNA-based catalysis may have existed [3,4]. The properties of self-cleaving ribozymes were recently reviewed in this journal [5]. With the introduction of ***in vitro* selection** methods [6–9], synthetic ribozymes can be identified that target particular substrates or even catalyze entire chemical reactions that are not biologically relevant. What about DNA as a catalyst?

The chemical structures of DNA and RNA are very similar, differing only by the respective absence and presence of ribose 2'-hydroxyl groups on every nucleotide monomer, as well as by including T and U nucleobases. However, nature uses DNA and RNA for markedly different purposes. DNA is the long-term repository of genetic information, whereas RNA is the transient genetic messenger and is also used for a myriad of other biological functions. As the genetic material, DNA is double-stranded at nearly all times. A DNA duplex is structurally regular, but enzymatic activity requires irregular 3D (tertiary) conformations that can bind substrates and position various functional groups for catalysis. Therefore, natural duplex DNA is unlikely to be catalytic.

Nevertheless, many experiments have shown that DNA can be a catalyst (**deoxyribozyme**). How can this be? The key is to consider single-stranded DNA. In its single-stranded form, DNA is similar to RNA with regard to many biochemical properties, particularly in the ability to fold into secondary structures and intricate 3D conformations. Indeed, synthetic RNA and DNA

### Trends

As a synthetic catalyst identified by *in vitro* selection, single-stranded DNA has many advantages over proteins and RNA. The growing reaction scope of deoxyribozymes is increasing our fundamental understanding of biocatalysis, enabling applications in chemistry and biology.

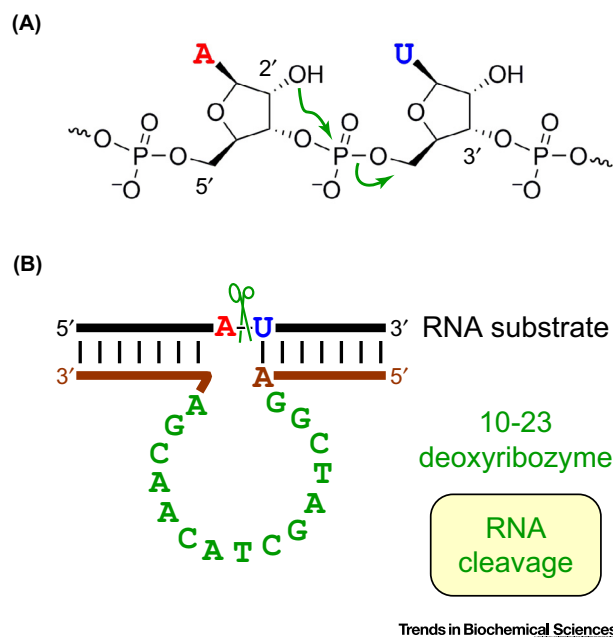
Including modified DNA nucleotides enhances the catalytic ability of deoxyribozymes. Several approaches for this purpose are being evaluated.

Investigators are pursuing practical applications for deoxyribozymes, such as *in vivo* mRNA cleavage and sensing of metal ions and small molecules.

High-resolution structural analysis of deoxyribozymes is a new field; the first crystal structure was recently reported. This structure provides an understanding of how DNA can be as catalytically competent as RNA. Structural information will enable mechanistic studies of deoxyribozymes.

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**Figure 1. DNA-Catalyzed RNA Cleavage.** (A) RNA cleavage reaction, in which the 2'-hydroxyl group attacks the adjacent phosphodiester bond. (B) The 10-23 deoxyribozyme that cleaves RNA [37]. Note the Watson–Crick base-pairing interactions between deoxyribozyme and RNA cleavage substrate.

**aptamers** that bind to a wide range of molecular targets have been identified by *in vitro* selection methods [10], and natural RNA riboswitches that bind metabolites are important regulators of gene expression [11–13]. Upon searching through initially random sequence populations by *in vitro* selection methods, synthetic single-stranded deoxyribozymes can readily be found. Fundamentally, protein, RNA, and DNA enzymes are similar in that each is a well-defined sequence of monomers (amino acids or nucleotides) that adopts a tertiary structure to catalyze a chemical reaction.

The first report of a deoxyribozyme was published in December 1994 by Breaker and Joyce [14]. Their deoxyribozyme cleaved at an RNA monomer within an oligonucleotide by modulating attack of the 2'-hydroxyl group at the adjacent phosphodiester linkage (Figure 1). The same reaction is catalyzed by protein enzymes such as RNase [15]. Since that time many groups have collectively reported hundreds and perhaps thousands of individual deoxyribozyme sequences, encompassing a broad and growing scope of catalytic activity. Several pertinent reviews have addressed the field of deoxyribozymes [16–23], including both general overviews and the relationship of DNA catalysis to other applications of DNA. This review describes the advantages of DNA as a catalyst and highlights recent advances in the scope, applications, and structural investigation of deoxyribozymes.

### Advantages of Nucleic Acids over Proteins as Catalysts

As nucleic acids, DNA and RNA enjoy many inherent advantages as catalysts relative to proteins. Entirely new synthetic nucleic acid sequences with catalytic function can be identified from random sequence populations, without any known catalytic sequence as the starting point. By contrast, performing *in vitro* selection from random protein sequences is generally intractable. In the context of developing entirely new synthetic catalysts, many important differences can be identified between nucleic acids and proteins.

### Glossary

**Aptamer:** a DNA or RNA (or other oligonucleotide variant) sequence that binds to a molecular target.

Aptamers are identified by *in vitro* selection, which for aptamers is often called SELEX (systematic evolution of ligands by exponential enrichment), noting that the aptamer is the 'ligand' for the molecular target.

**Deoxyribozyme:** a particular DNA sequence that has catalytic activity, in analogy to a protein enzyme as a catalytic sequence of amino acids. Synonyms for 'deoxyribozyme' include DNA enzyme, DNzyme, DNA catalyst, and catalytic DNA. While deoxyribozymes are unknown in nature, synthetic catalytic DNA sequences can be identified by *in vitro* selection.

**Directed evolution:** the process by which a known enzyme sequence is improved with regard to some property, such as rate, yield, or substrate scope. The enzyme may be protein, DNA, or RNA. For protein enzymes, practical considerations dictate that only a very small subset of amino acids within the sequence may be varied; these residues are chosen on the basis of structural or mechanistic information. Alternatively, more randomly targeted approaches such as gene shuffling may be used. For DNA and RNA enzymes, by contrast, the entire nucleotide sequence is typically included in the directed evolution process.

***In vitro* selection:** the experimental process by which random sequences, such as those of DNA or RNA nucleotides, are examined in parallel (not one at a time) to identify those particular sequences that have a desired function such as binding or catalysis. This term is defined by analogy to natural selection, which is an undirected process. *In vitro* selection is conceptually distinct from screening, which is a one-at-a-time process.

**Ribozyme:** a particular RNA sequence that has catalytic activity (cf. deoxyribozyme). Both natural and synthetic ribozymes are known; the latter are identified by *in vitro* selection.

Sequence space of random DNA/RNA populations has size  $4^n$ , where  $n$  is the random region length in nucleotides. A typical  $N_{40}$  random pool (40 nt) comprises  $4^{40}$ – $10^{24}$  unique sequences. For physical reasons, an *in vitro* selection experiment usually begins with  $\sim 10^{14}$  actual sequences, allowing only  $\sim 10^{-10}$  coverage of  $N_{40}$  sequence space. Nevertheless, new DNA/RNA enzymes can be identified, especially noting degeneracy (i.e., some sequences are functionally equivalent) and that not all nucleotide positions will have constrained identities within a catalytic sequence. For proteins, sequence space is  $20^n$  when all 20 standard amino acids are included. Considering that protein enzymes are usually  $>100$  amino acids in length, this is  $>20^{100}$  or  $10^{130}$  sequences. **Directed evolution** of an existing protein enzyme sequence to improve rate or yield or to change substrate scope is often viable by screening variants that have mutations at a very small number of strategically chosen residues [24,25]. By contrast, identifying entirely new protein enzymes by *in vitro* selection from fully random amino acid sequences is generally unachievable, solely considering the numbers.

Moreover, most random protein sequences fail to adopt any particular tertiary structure owing to the cooperative nature of protein folding. Protein secondary structure elements such as  $\alpha$ -helices and  $\beta$ -sheets are almost always unstable by themselves [26], forming only in the context of higher-order tertiary structure. By contrast, DNA and RNA secondary structures such as stem-loops are very stable in the absence of tertiary structure, allowing higher-order structure to form in hierarchical fashion from secondary structure elements [27].

*De novo* computational design of new protein enzyme active sites has advanced, but the designed active sites are embedded into known protein structures and are optimized by directed evolution [28]; the entire enzyme is not designed from the ground up. Furthermore, the mechanism of such protein enzymes may not be as originally designed [29]. At present, and apparently for the foreseeable future, computationally designing any complete enzyme (protein or DNA/RNA) from scratch is not feasible.

Finally, all the necessary biochemical tools to identify new deoxyribozymes and ribozymes by *in vitro* selection are readily available. This particularly includes DNA polymerase, which allows a small number of DNA sequences to be amplified into a much larger number by PCR; for ribozymes, reverse transcriptase and RNA polymerase are also needed. By contrast, the methodology to perform *in vitro* selection from random protein sequences is generally unavailable [30]. The lack of any means to copy an arbitrary amino acid sequence provides a nearly insurmountable practical roadblock to *in vitro* selection of protein enzymes, regardless of all other considerations.

Among the nucleic acids themselves, essentially all practical considerations favor using DNA over RNA. Such considerations include chemical and enzymatic stability, cost, and ease of synthesis. Perhaps the sole practical scenario that favors RNA over DNA is *in vivo* applications, where intracellular production is straightforward to achieve for RNA but typically requires a delivery mechanism for DNA. However, this advantage of RNA over DNA is negated by the need to use chemically modified variants of RNA/DNA *in vivo* for various reasons such as stability.

### Reaction Scope of Catalysis by DNA

Since the initial report of an RNA-cleaving deoxyribozyme [14], many other catalytic activities of DNA have been reported. The scope of DNA catalysis is limited in part by the invention of suitable selection methodology for identifying catalytic sequences. In general, *in vitro* selection requires a way to separate active from inactive sequences without needing to screen each candidate sequence individually, as in separate wells of a plate or on separate beads. No one-at-a-time

screening method is viable with  $10^{14}$  candidate sequences. 'In vitro compartmentalization' (IVC) [31,32] has led to several ribozyme activities [33–36] and should be applicable to deoxyribozymes as well. Nevertheless, to date all deoxyribozymes have been identified by *in vitro* selection using either bead-binding or polyacrylamide gel electrophoresis (PAGE) shift as the separation basis. Other articles have covered various practical considerations of *in vitro* selection (e.g., [16]). This section assesses use of deoxyribozymes to catalyze reactions of oligonucleotide and non-oligonucleotide substrates, and it describes the inclusion of modified DNA nucleotides to expand DNA catalysis.

### Deoxyribozymes for RNA Cleavage

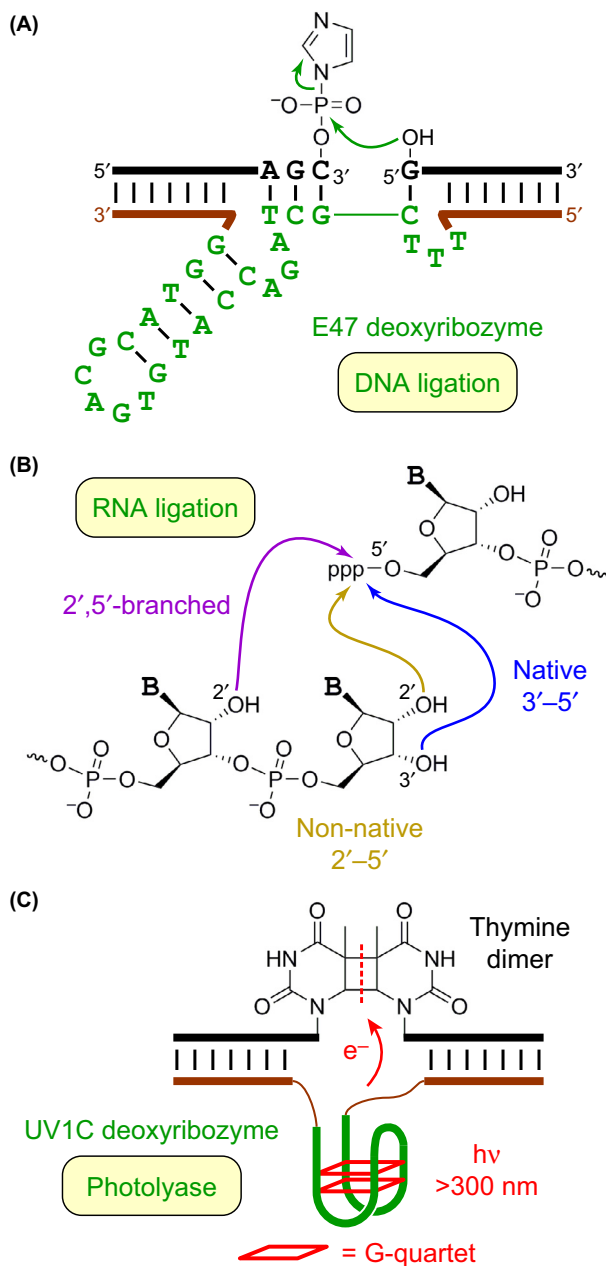
DNA-catalyzed RNA oligonucleotide cleavage by the mechanism of Figure 1 has been found in many independent experiments and is the most intensively studied of any DNA-catalyzed reaction. Soon after the initial report [14], several laboratories described additional RNA-cleaving deoxyribozymes. One motif, named 8-17, was identified by several different laboratories [37–40] and is likely to be the simplest possible catalytic DNA motif [41]. Indeed, the original RNA-cleaving deoxyribozyme [14] is likely an 8-17 motif. Owing to its simplicity, 8-17 and a different small deoxyribozyme named 10-23 that was identified at the same time [37] have frequently been exploited for a range of practical applications, as discussed more comprehensively in a later section.

### Deoxyribozymes for Other Reactions of Oligonucleotide Substrates

Beyond DNA-catalyzed RNA cleavage by the mechanism of Figure 1, many other reactions of oligonucleotide substrates have been catalyzed by deoxyribozymes. An early example was DNA ligation by  $Zn^{2+}/Cu^{2+}$ -dependent joining of 5'-hydroxyl and 3'-phosphorimidazolide groups (Figure 2A) [42]. Subsequent experiments led to a wide range of deoxyribozymes for RNA ligation using one or more divalent metal ions such as  $Mg^{2+}$ ,  $Mn^{2+}$ , and  $Zn^{2+}$  together with several combinations of functional groups. These deoxyribozymes catalyzed the formation of native 3'–5' linear RNA, non-native 2'–5' linear RNA, and 2',5'-branched RNA (including lariat RNA) [18,43] (Figure 2B). Some of the branch-forming deoxyribozymes were later developed for utility in site-specific RNA labeling [44,45].

Many different oligonucleotide cleavage reactions have been catalyzed by DNA. These reactions include  $Cu^{2+}$ -dependent oxidative cleavage [46–48],  $Cu^{2+}/Mn^{2+}$ -dependent oxidative nucleoside excision [49,50], DNA phosphodiester hydrolysis [51,52], RNA hydrolysis (involving a water molecule, a normally disfavored reaction relative to RNA cleavage by the Figure 1 mechanism) [53], and deglycosylation with subsequent backbone cleavage [54–56]. Oligonucleotide end modification has also been achieved by DNA 5'-phosphorylation (kinase activity) [57] and adenylation (capping) [58] followed by ligation [59]. However, different deoxyribozymes and reaction conditions are required for each member of this three-reaction series.

An unusual DNA-catalyzed photochemical reaction was achieved by Sen and coworkers, who identified the UV1C deoxyribozyme that induces photoreversion of thymine dimers (Figure 2C) [60–62]. Although photochemistry has not been broadly exploited in other DNA-catalyzed reactions, this report establishes the ability of DNA to use light in catalysis. The UV-absorbing nucleobases and higher-order G-quadruplex structures, which appear to be formed by UV1C, naturally support this photolyase ability. A pterin moiety, which is a guanine nucleobase analog, can replace any of six guanines in UV1C, allowing this deoxyribozyme to use violet light rather than UVB radiation and suggesting potential medical applications in photoreversion therapy [63]. The Sen laboratory also reported the Sero1C deoxyribozyme, which catalyzes thymine dimer photoreversion using serotonin as a discrete small-molecule cofactor [64]. In Sero1C the indole ring of serotonin (rather than a G-quadruplex of the deoxyribozyme itself) serves as an antenna to enable thymine dimer photocleavage.

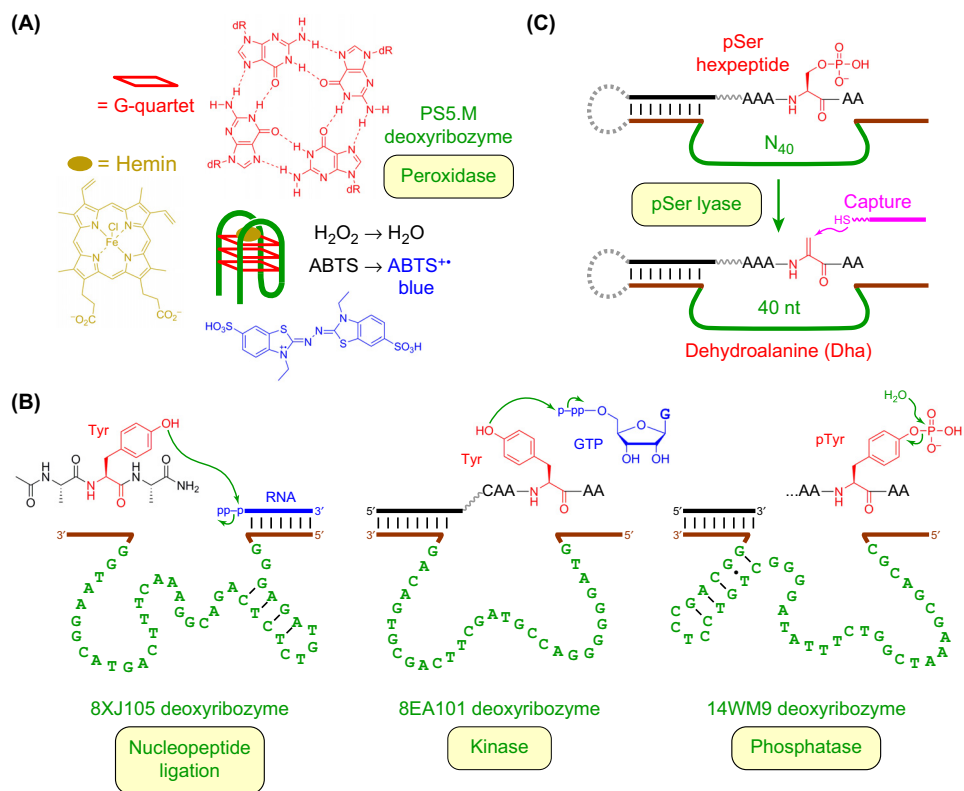


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**Figure 2. Reactions of Oligonucleotide Substrates Catalyzed by Deoxyribozymes.** (A) DNA ligation by the E47 deoxyribozyme [42]. (B) RNA ligation by reaction of a 3'- or 2'-hydroxyl with a 5'-triphosphate, leading to the formation of various linear and branched linkages [18,43]. RNA ligase deoxyribozymes can also join the Figure 1A products of DNA-catalyzed RNA cleavage, 5'-hydroxyl and 2',3'-cyclic phosphate. (C) Thymine dimer photoreversion by the UV1C deoxyribozyme (photolyase activity) [60].

### Deoxyribozymes for Reactions of Substrates Other than Oligonucleotides

Growing efforts are revealing the capacity of DNA for catalysis involving non-oligonucleotide substrates. The first example was DNA-catalyzed porphyrin metallation, in which the deoxyribozyme was identified, unusually, by *in vitro* selection for binding to a transition state analog [65]. Another example is peroxidase deoxyribozymes, which are important for practical sensor



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**Figure 3.** Reactions of Non-Oligonucleotide Substrates Catalyzed by Deoxyribozymes. (A) Peroxidase activity by a G-quadruplex deoxyribozyme [66,67]. (B) Examples of DNA-catalyzed peptide side chain and backbone modification [22,68–74]. (C) Capture step during *in vitro* selection for the identification of deoxyribozymes that form dehydroalanine (Dha) from phosphoserine (pSer) [74].

applications (see next section). Particular G-quadruplex DNA sequences can bind to the porphyrin hemin and oxidize small-molecule chromogenic substrates, leading to optical signals [66,67] (Figure 3A).

Silverman and coworkers have focused on identifying deoxyribozymes for modifying peptide side chains and backbones. Some of this work was recently reviewed [22]; selected highlights are provided here (Figure 3B). Deoxyribozymes have been found for conjugation of oligonucleotides to tyrosine [68–70], tyrosine phosphorylation [71,72], phosphotyrosine (pTyr) and phosphoserine (pSer) dephosphorylation [73], the formation of dehydroalanine (Dha) by elimination of phosphate from pSer [74], and lysine modification [75]. The case of tyrosine phosphorylation illustrates that DNA catalysts can distinguish among various peptide substrate sequences [72], which is important for sequence-specific modification. In most of these experiments, a crucial aspect was developing the necessary selection methodology, in particular designing a ‘capture step’ that within each selection round is highly selective for the intended reaction product and also sufficiently fast and high-yielding. As one example, identification of Dha-forming deoxyribozymes depends crucially upon a thiol-based capture by nucleophilic attack into the electrophilic Dha group, which leads to a PAGE shift solely for those initially very rare DNA sequences that catalyzed the formation of Dha (Figure 3C) [74].

During many reactions, a DNA catalyst must interact with a small-molecule substrate. For example, in DNA-catalyzed adenylation and phosphorylation of oligonucleotides [57,58], an

NTP is one substrate. Few purely small-molecule 'organic' reactions have been pursued with deoxyribozymes; one instance is the Diels–Alder reaction [76]. Whenever a small-molecule substrate is required, one could consider integrating aptamers with DNA catalysts. This approach was evaluated in two recent studies. Dokukin and Silverman found no practical value in providing an ATP aptamer adjacent to a random DNA pool when identifying new tyrosine kinase deoxyribozymes, although this study provided the first clear evidence of modularity in DNA catalysis [77]. Using a very different approach, Willner and coworkers improved catalysis of an existing peroxidase deoxyribozyme by appending a substrate (dopamine or arginine) aptamer [78], and they further refined their approach to improve the catalysis [79]. If broadly applicable, these results when considered together imply that finding new DNA catalysts that require small-molecule substrates may not be assisted by combining aptamers with random pools at the outset of selection, but some existing deoxyribozymes can be improved functionally by attaching aptamers.

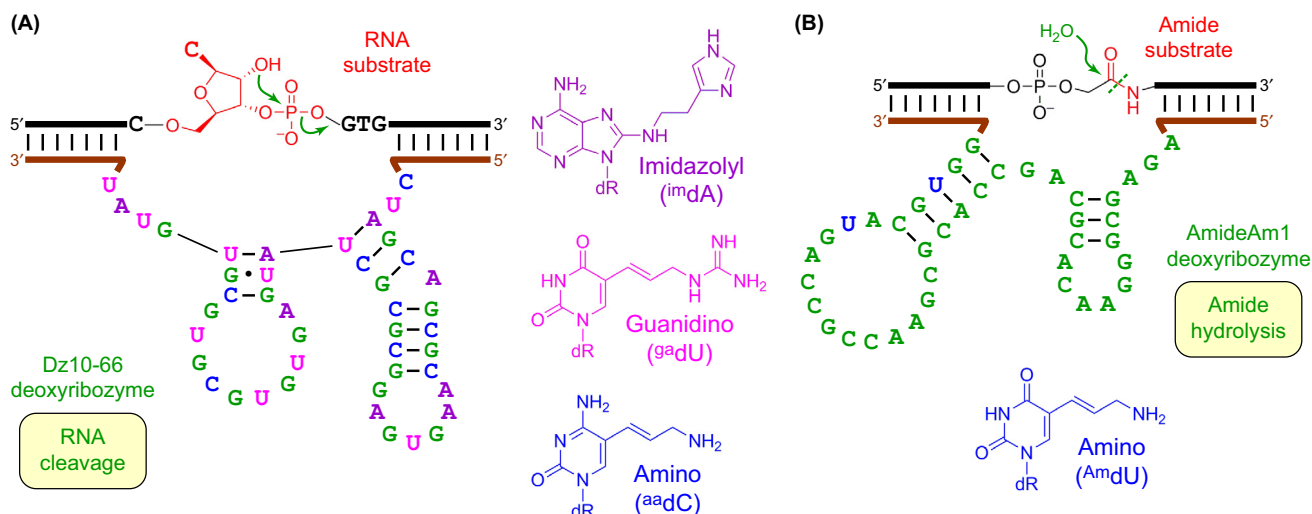
### Modified DNA Nucleotides and Other Approaches to Broaden the Scope of DNA Catalysis

Even using a suitable selection method, deoxyribozymes that have a particular catalytic activity are not always identified. In cases for which standard DNA that includes the usual A, G, T, and C nucleotides is incapable of the intended activity, incorporating suitably modified nucleotides may enable catalysis. Similarly, if standard DNA is found to be capable of only poor catalysis, modified nucleotides may improve important catalytic features such as rate, yield, or substrate scope (either by broadening or narrowing the scope as desired). It is important to clarify that nonstandard functional groups are not simply appended onto previously known, unmodified deoxyribozyme sequences, because there is no reason to expect that attaching such groups will convert a noncatalytic or poorly catalytic DNA sequence into a (more) functional version. Instead, modifications are included from the outset of *in vitro* selection, leading to modified DNA sequences that integrally make use of the nonstandard groups for catalysis.

The laboratories of Perrin [80] and others [81,82] have described modified RNA-cleaving deoxyribozymes, usually with the goal of reducing or obviating the divalent metal ion ( $M^{2+}$ ) requirement.  $M^{2+}$ -independent DNA-catalyzed RNA cleavage would be useful for *in vivo* mRNA cleavage (see next section). Later-generation deoxyribozymes incorporate three different types of modification (Figure 4A) and catalyze RNA cleavage in the complete absence of  $M^{2+}$  [83–85].

The Silverman laboratory recently reported the first example of modified deoxyribozymes to achieve hydrolysis of aliphatic amides (Figure 4B) [86]. Previously, unmodified DNA enzymes for hydrolysis of esters and aromatic amides were identified, but deoxyribozymes for aliphatic amide hydrolysis were not found [87]. Therefore, including modifications during the selection process enabled identification of the new DNA enzymes. One modified deoxyribozyme requires as few as one primary amino modification to achieve amide hydrolysis. Another modified deoxyribozyme was identified with primary hydroxyl groups in the form of 5-(hydroxymethyl)dU nucleotides, but unexpectedly, the same sequence in the form of entirely unmodified DNA retains substantial catalytic activity. An as-yet untested hypothesis is that an ordered water molecule can replace the 5-(hydroxymethyl) group for the unmodified DNA enzyme. These findings suggest both the utility and complexity of including modified DNA nucleotides during the identification of new deoxyribozymes.

Related to DNA catalysts with modified DNA nucleotides are 'XNAzymes', which are made from xeno-nucleic acids, XNAs [88]. XNAs have unnatural backbones such as those of threose nucleic acid (TNA), 2'-fluoro-arabinonucleic acid (FANA), and several others. The finding by Holliger and coworkers of catalysis by XNAzymes is exciting conceptually. This finding may also



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Figure 4. Modified DNA Nucleotides in Deoxyribozymes. (A) M<sup>2+</sup>-independent RNA-cleaving Dz10-66 deoxyribozyme, with three types of modified nucleotide [84]. The substrate has a single RNA nucleotide embedded within a DNA sequence. (B) AmideAm1 deoxyribozyme for amide bond hydrolysis, with amine-modified nucleotides [86].

have practical value that derives from the orthogonality of XNAs to natural biopolymers and the natural protein enzymes that interact with and degrade them.

One approach to enhance DNA catalysis is including small-molecule cofactors. One early report showed that an RNA-cleaving deoxyribozyme could be dependent on the presence of histidine [89], and a later study used periodate in DNA-catalyzed depurination [55]. Beyond that, however, this aspect of DNA catalysis remains largely unexplored. In principle, small-molecule cofactors should be able to provide functional groups that would otherwise necessitate modified nucleotides; in that regard, cofactors could allow the use of standard unmodified DNA while simultaneously expanding the catalytic abilities of DNA. However, a drawback of obligatory cofactors is that the deoxyribozyme needs simultaneously to bind its substrate(s) and the cofactor as well as to perform catalysis, and such multitasking may be difficult.

### Are Deoxyribozymes 'Good' Catalysts?

Rate enhancements of deoxyribozymes can be large. For example, the background hydrolysis half-life for phosphoserine (at neutral pH, in the absence of metal ions, at ambient temperature) is  $\sim 10^{10}$  years, whereas DNA-catalyzed hydrolysis proceeds with half-life of  $\sim 1$  h [73]. Taking the ratio of these two half-lives leads to a calculated rate enhancement of  $10^{14}$ . Such calculations are complicated by the fact that divalent metal ions such as Zn<sup>2+</sup> are required for catalysis, and separating the contributions of DNA and metal ions is tricky. The safest conclusion may be that focusing on quantitative rate-enhancement values misses the point: the key observation is that DNA can perform catalysis in an interesting and potentially useful way. For ribozymes and deoxyribozymes alike, Breaker and coworkers posited that the maximum rate constants of  $\sim 1 \text{ min}^{-1}$  reflect suboptimal use of catalytic strategies; using all possible modes of catalysis would allow much greater rate constants [90,91]. Therefore, the primary limitation to deoxyribozyme catalysis is not theoretical but practical. Deoxyribozymes can in theory be outstandingly good catalysts – the important question is, how can they best be identified? Using appropriately designed 'capture steps', in some cases including modified DNA nucleotides, may enable finding deoxyribozymes that catalyze difficult or disfavored reactions.



### Applications of Deoxyribozymes

One major impetus for the development of new catalysts is their practical value. This section describes two specific applications for deoxyribozymes: *in vivo* mRNA degradation and sensors. Other applications such as site-specific RNA labeling [44,45], logic gates, computing circuits, and switches [92–94], and biofuel cell catalysis [95] are also being pursued. Many of the DNA-catalyzed reactions still under development are likely to enable an even broader range of practical applications.

### Deoxyribozymes for mRNA Degradation *In Vivo*

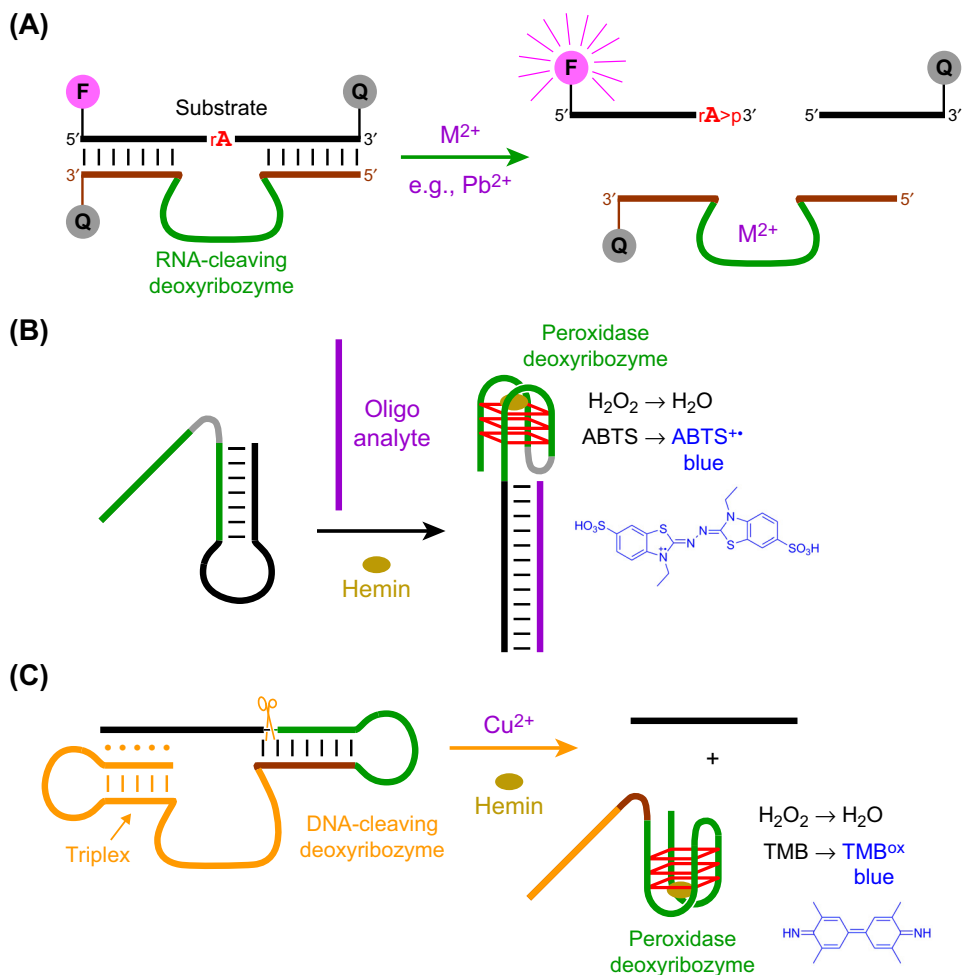
Intentionally degrading specific mRNAs is an important biological approach to control gene expression, both in the laboratory and in ongoing clinical trials with RNA interference (RNAi) [96]. RNA cleavage by an introduced deoxyribozyme has long been suggested as a means to accomplish mRNA degradation [37]. However, a major concern is whether any mRNA degradation is due to DNA-catalyzed cleavage or merely to an antisense effect [97]. Two articles in the medical literature reported using the 10-23 deoxyribozyme to treat cancer and asthma, respectively [98,99], although control experiments to distinguish DNA catalysis from antisense effects were not described. Because deoxyribozymes generally exhibit little or no catalysis at the free divalent metal ion concentrations inside cells, efforts with modified deoxyribozymes are intended to improve *in vivo* catalysis [80–85]. In one instance with otherwise unmodified DNA, introducing 2'-O-methyl substitutions to improve 10-23 deoxyribozyme stability was accompanied by data supporting *in vivo* mRNA degradation [100]. However, broadly compelling evidence for *in vivo* DNA-catalyzed mRNA degradation is currently unavailable.

### Deoxyribozymes as Sensors

Two major classes of deoxyribozymes, RNA-cleaving and peroxidases, have been used to create many sensors. RNA-cleaving deoxyribozymes generally require specific metal ions, which enables the development of metal ion sensors (Figure 5A). Much of the work in this area has been performed by Lu and coworkers [101], who in 2000 reported a Zn<sup>2+</sup>-dependent RNA-cleaving deoxyribozyme [102] and more recently described fluorescence detection of UO<sub>2</sub><sup>+</sup> (uranyl) and Na<sup>+</sup> in living cells [103,104]. Others reported detection of Hg<sup>2+</sup> using deoxyribozymes that have modified DNA nucleotides [105]. In general, the metal ion selectivity of RNA-cleaving deoxyribozymes can be coupled with numerous detection platforms to allow sensitive and specific responses to particular metal ions in a variety of contexts [21,106]. For example, Xiang and Lu used a personal glucose meter to create a portable Pb<sup>2+</sup> sensor device; after Pb<sup>2+</sup>-induced RNA cleavage, a series of strand exchange events enables invertase-catalyzed conversion of sucrose to glucose, which the meter detects [107].

RNA-cleaving deoxyribozymes have also been applied to sense biomacromolecules. Willner and coworkers achieved sensitive detection of DNA itself via the autonomous assembly of polymeric DNA nanowires built from deoxyribozyme subunits [108]. An interesting recent application of RNA-cleaving deoxyribozymes by Li and coworkers is the sensing of specific bacterial species. Using deoxyribozymes whose activity depends on the presence of the crude extracellular mixture (CEM) of a particular pathogenic strain of *Clostridium difficile*, fluorogenic responses to that strain were generated [109]. This approach does not require identifying the CEM component responsible for activating the deoxyribozyme, although for *C. difficile* the authors did determine that a truncated transcription factor in the pathogenic strain activates the deoxyribozyme.

Peroxidases are the second class of deoxyribozymes upon which many sensors are designed. Detecting a variety of molecular targets is achieved by using those targets to regulate the G-quadruplex structure of the peroxidase deoxyribozyme. For example, an oligonucleotide analyte is detected when the target strand liberates the DNA nucleotides that constitute the



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**Figure 5. Sensor Applications of Deoxyribozymes.** (A) RNA-cleaving deoxyribozymes activated by specific metal ions ( $M^{2+}$ ). In the illustrated example,  $M^{2+}$ -dependent RNA cleavage separates a fluorophore (F) from its quenchers (Q), leading to a fluorescence signal [101]. (B) Oligonucleotide detection by a G-quadruplex peroxidase deoxyribozyme that functions by strand displacement [110]. In the presence of the oligonucleotide analyte and the porphyrin hemin, the deoxyribozyme uses  $H_2O_2$  to oxidize the small-molecule substrate ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], forming a colored product. (C)  $Cu^{2+}$  detection by a G-quadruplex peroxidase deoxyribozyme, triggered by  $Cu^{2+}$ -dependent DNA-catalyzed RNA cleavage [112]. Release of the DNA fragment allows G-quadruplex formation, and the peroxidase deoxyribozyme then uses  $H_2O_2$  to oxidize the small-molecule substrate TMB (3,3',5,5'-tetramethylbenzidine), forming a colored product.

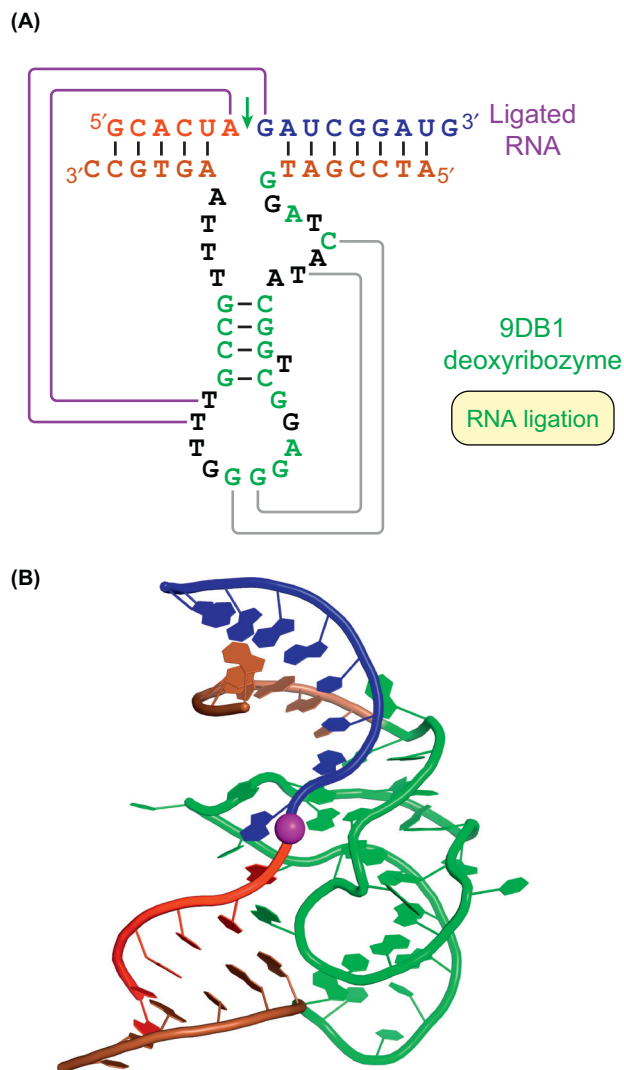
G-quadruplex (Figure 5B) [110], or when oligonucleotide binding triggers formation of deoxyribozyme-containing nanowires [111]. Several groups have created peroxidase deoxyribozyme sensors for metal ions and small organic compounds [101]. In one interesting example that exploits both RNA cleavage and peroxidase activity, Tan and coworkers detected  $Cu^{2+}$  via DNA-catalyzed RNA cleavage that enables proper folding of a peroxidase deoxyribozyme, thereby leading to a colorimetric signal (Figure 5C) [112].

### Structural and Mechanistic Studies of Deoxyribozymes

#### Finally, the First Deoxyribozyme Crystal Structure

Although deoxyribozymes have been known for over 20 years, until very recently no high-resolution structure was available [113]. In early 2016 a research team led by Höbartner and

Pena reported the first deoxyribozyme X-ray crystal structure [114], that of the RNA-ligating 9DB1 deoxyribozyme [115]. This structure reveals several important facets of catalysis by DNA, including the fundamental confirmation that deoxyribozymes can have well-defined tertiary structures that are responsible for their catalysis. The intricate 3D structure of 9DB1 complexed with its RNA ligation product is a double pseudoknot in which two adjacent T residues interact with the A-G RNA nucleotides directly at the ligation junction (Figure 6). Recognizing these interactions, which were not evident from biochemical experiments, enabled re-engineering of the deoxyribozyme to broaden its RNA substrate scope by straightforward Watson–Crick



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**Figure 6. The First Deoxyribozyme Crystal Structure [114].** (A) Double pseudoknot secondary structure of the minimized 9DB1 deoxyribozyme that ligates RNA, bound to its ligation product. Red and blue denote the two halves of the RNA product, corresponding to the two RNA substrates. The deoxyribozyme binding arms are brown; black and green nucleotides are nonconserved and conserved core nucleotides, respectively. Pseudoknot interactions are purple and grey lines. Two adjacent T residues of the deoxyribozyme interact with the A and G nucleotides in the RNA product at the ligation junction (purple lines), enabling general RNA ligation by covariation of these nucleotides. (B) 3D structure, with the same colors as panel A except the entire core of the deoxyribozyme is green. The ligation site is marked with a purple sphere. Nucleobases are shown explicitly; the remainder of each nucleotide is shown in ribbon form. Image courtesy of C. Höbartner (PDB: 5ckk).

covariation. The structure also revealed contacts that explain why the newly formed phosphodiester bond is 3'–5' (native) instead of 2'–5'. This outcome was demanded by the selection strategy that led to the identification of 9DB1, but the underlying structural explanation was unknown.

Initially all ribozymes were thought to require divalent metal ions as catalytic cofactors [116], but both natural and synthetic ribozymes have been found that lack this requirement [117,118]. The 9DB1 deoxyribozyme requires either  $Mg^{2+}$  or  $Mn^{2+}$  for catalysis [115], but its crystal structure reveals no divalent metal ion at the active site. Instead, an internucleotide phosphodiester oxygen appears to make a critical contact. Therefore, this first deoxyribozyme crystal structure supports the conclusion that DNA enzymes are not obligatory metalloenzymes with regard to the catalytic role of divalent metal ions [89,104,119,120].

Another notable finding from the new structure is an important insight into the relationship between DNA and RNA enzymes. The lack of 2'-hydroxyl groups in DNA is expected to reduce its functional capacity relative to RNA, all other things being equal, but the magnitude of this effect is unknown. DNA and RNA aptamers in general have very similar distributions of binding constants ( $K_d$  values) [10], and a deoxyribozyme for a Diels–Alder reaction was found to have similar catalytic parameters as an independently obtained ribozyme [76]. The new structure reveals that the 9DB1 deoxyribozyme has a much broader range of sugar–phosphate backbone conformations than is found in natural and synthetic ribozymes. Therefore, the missing 2'-hydroxyl groups of DNA, while removing one source of functional interactions, in parallel expand the conformational variability of DNA relative to RNA. These counteracting effects appear to leave DNA at least as catalytically competent as RNA. Indeed, the greater backbone freedom for single-stranded DNA as observed in the 9DB1 crystal structure may enable DNA catalysis in ways not possible for RNA.

What does the future hold for high-resolution structural studies of deoxyribozymes? Now that the first crystal structure has been reported [114], surely others will follow. Höbartner's elegant combinatorial minimization methods, which were applied to 9DB1 before its crystallization [121], will likely be invaluable for enabling the crystallization of unrelated deoxyribozymes. In parallel, NMR studies of deoxyribozymes are surely possible, although none have yet been reported.

#### Mechanistic Studies of Deoxyribozymes

Investigations of biochemical mechanisms are often intimately linked to structural studies. For ribozymes, meaningful mechanistic insights have derived from biochemical experiments that were enabled by high-resolution structures (e.g., [122]). Considering the dearth of deoxyribozyme structures, it is unsurprising that few substantial mechanistic reports on DNA enzymes have yet appeared. For example, many studies have addressed the functional roles of metal ions and have investigated kinetics, but not to the extent where we truly understand mechanisms of DNA catalysis. With the first deoxyribozyme crystal structure now reported [114], and hopefully more on the way, the prospects are strong for understanding the detailed mechanisms by which DNA enzymes catalyze many reactions. A reasonable expectation is that deoxyribozymes and ribozymes will share common contributions to catalysis, such as acid–base chemistry involving the nucleobases [123].

#### Concluding Remarks

This review has described the many conceptual and practical advantages of DNA as a catalyst relative to proteins and RNA. From the relatively modest first report in 1994, deoxyribozymes have grown to encompass a broad range of catalytic abilities, including with the assistance of modified DNA nucleotide components. The first experiments to understand key aspects of deoxyribozyme structure and mechanism are currently emerging. Many questions are still

#### Outstanding Questions

What are the limits on the scope of reaction catalysis by DNA?

How important are modified nucleotides or other nonstandard features to enable catalysis by DNA?

For what practical applications (established or new) will deoxyribozymes have the greatest utility?

What are the structures of deoxyribozymes? In this regard, how are deoxyribozymes similar to, or different from, ribozymes and protein enzymes?

What are the mechanisms by which deoxyribozymes perform catalysis?

unanswered about the capabilities and applications of deoxyribozymes (see Outstanding Questions). The advantages of DNA as a synthetic catalyst provide strong encouragement for the continued development of deoxyribozymes.

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