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Deoxyribozymes: DNA catalysts for bioorganic chemistry†

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Deoxyribozymes are DNA molecules with catalytic activity. For historical and practical reasons, essentially all reported deoxyribozymes catalyze reactions of nucleic acid substrates, although this is probably not a fundamental limitation. *In vitro* selection strategies have been used to identify many deoxyribozymes that catalyze RNA cleavage, RNA and DNA ligation, and a variety of covalent modification reactions of nucleic acid substrates. Many deoxyribozymes are capable of catalysis with substantial rate enhancements reaching up to 10^{10}-fold over background, and their very high selectivities would often be difficult or impossible to achieve using traditional organic synthesis approaches. This report summarizes the current utility and potential future applications of deoxyribozymes from the bioorganic chemistry perspective.

Introduction: catalytic nucleic acids in nature and in the laboratory

Many organic chemists probably think of nucleic acids solely as carriers of genetic information, either permanently as genomic DNA or transiently as messenger RNA. Over 20 years ago, the catalytic roles of natural RNAs were discovered,[1,2] and today the central molecules of biochemistry – the ribosome for protein translation[3] and almost certainly the spliceosome for RNA splicing[4] – are known to be RNA catalysts (RNA enzymes, or ribozymes).[5] In addition, catalytic RNA likely played an important role in the natural evolution of life.[6] Nevertheless, relatively few studies focus on nucleic acids as catalysts for bioorganic chemistry. Upon considering the functional groups available to RNA and DNA for catalysis, this assessment initially seems reasonable. In a comparison between proteins and RNA, the former have a substantial diversity of 20 sidechains in addition to the hydrogen-bonding capability of the polyamide backbone, whereas RNA with just four similar monomers is restricted to hydrogen bonding, π-stacking, and metal-ion coordination for interactions with potential substrates (Fig. 1, left). DNA appears even less catalytically competent than RNA, in part because DNA lacks the 2′-hydroxyl group that can engage in hydrogen bonding as both donor and acceptor (Fig. 1, right). In addition, DNA naturally exists almost entirely in double-helical form for long-term information storage, whereas the structural variety and physical flexibility permitted by single-stranded conformations are probably required for catalysis. Indeed, no DNA enzymes are known in nature, and certainly too little is understood to design deoxyribozymes from first principles; steps in this direction are just being taken for proteins.[7] How can we possibly identify DNA sequences with catalytic activities and put them to practical laboratory use?

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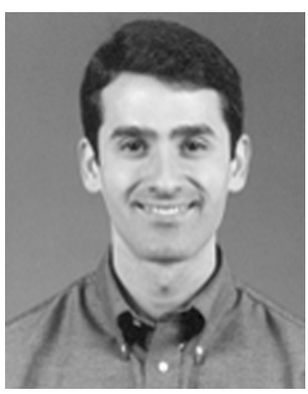

Scott K. Silverman

In vitro selection *versus* combinatorial chemistry

Regardless of their specific research interests, organic chemists are increasingly familiar with combinatorial chemistry, which is applied in areas as diverse as drug discovery,[8] sensor development,[9] and synthetic methodology.[10] The *screening* approach typically used in combinatorial chemistry – *i.e.* to synthesize many compounds and separately test each of them for activity – is impractical for identifying catalytic nucleic acids simply because useful library sizes are impossibly large. Instead, for nucleic acids a *selection* approach is essential. In selection, catalytically competent sequences survive a challenge of tunable difficulty that the vast majority of sequences cannot survive. Therefore, very large libraries ('sequence pools') may effectively be surveyed *via* iterated selection–amplification cycles.

Using *in vitro* selection, 10^{14} unique nucleotide sequences are routinely examined to find those with desired catalytic activities, and in some cases up to 10^{16} sequences have been surveyed in a single experiment.[11] The size of these sequence pools dwarfs that of most combinatorial chemistry libraries, where 10^6 compounds is the practical upper limit, and most combinatorial libraries are much smaller.[12] Of course, nucleic acid polymers have relatively limited structural variation compared with a typical combinatorial compound library, which is restricted in diversity primarily by the ingenuity of the chemist. Despite this limitation, many highly active artificial ribozymes have been identified through *in vitro* selection and improved through *in vitro* evolution.[13] The latter approach simply alternates selection with sequence diversification – *i.e.* mutagenesis – to

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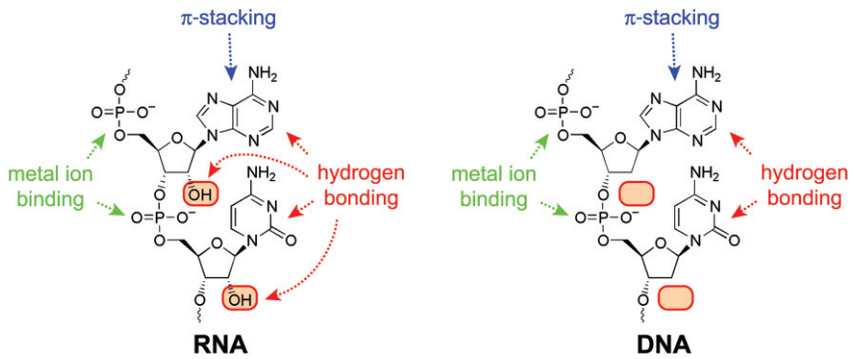

Fig. 1 Chemical structures of RNA and DNA, noting the interactions that can contribute to catalysis. For brevity, only adenosine (A) and cytidine (C) nucleobases are shown.

explore more of 'sequence space', often *via* application of error-prone polymerase chain reaction (PCR). The issue of diversity can be important because even a typical, medium-sized 40-nucleotide random region encompasses $4^{40} = 10^{24}$ sequences, which is many orders of magnitude more than the 10^{14} to 10^{16} molecules of nucleic acid that can be examined in a single experiment. The length of the random region in a nucleic acid selection experiment can vary from 20 to >200 nucleotides. The specific length is chosen based on how chemically challenging the desired catalytic activity is thought to be, among other factors.[14]

The first examples of catalytic DNA: deoxyribozymes that cleave RNA

Deoxyribozymes – also called DNA enzymes, catalytic DNA, or (more awkwardly) DNAzymes – compose the subset of nucleic acid enzymes that are made entirely from DNA. The study of deoxyribozymes is only about a decade old. In 1994, Breaker and Joyce described the *in vitro* selection of a DNA enzyme that cleaves a specific RNA linkage within a nucleic acid strand.[15] Since then, many RNA-cleaving deoxyribozymes have been identified, some of which have been applied as practical metal sensors (*e.g.* for Pb^{2+})[16] or *in vivo* to degrade messenger RNAs, among other applications.[17] For RNA-cleaving deoxyribozymes, understanding both the process of their selection and the scope of their activity illuminates general principles that are relevant to essentially all known DNA enzymes.

All reported RNA-cleaving deoxyribozymes bind their RNA substrate *via* Watson–Crick base-pairing interactions, forming DNA:RNA duplex structures that extend from the site of reactivity (Fig. 2A). RNA cleavage occurs when the DNA facilitates attack of a specific 2′-hydroxyl group on the adjacent phosphodiester linkage, resulting in transesterification with cleavage of the RNA backbone (Fig. 2B). This reaction is the same as that promoted by most protein ribonucleases such as RNase A. The Watson–Crick binding mode between the deoxyribozyme and the RNA substrate has two important ramifications. First, because the DNA:RNA binding energy can be increased essentially without limit simply by lengthening the DNA binding arms to complement more of the RNA, deoxyribozymes can have very low K_M values (reflecting tight substrate binding) and consequently very high k_{cat}/K_M values that rival those of protein enzymes. For example, the Mg^{2+}-dependent '10–23 deoxyribozyme' shown in Fig. 2A can have k_{cat}/K_M of $>10^9$ M^{-1} min^{-1} under appropriate conditions, which exceeds the analogous value for RNase A by at least five-fold.[18,19] Second, because DNA:RNA hybrids adopt an A-form duplex structure regardless of the exact nucleotide sequence, many deoxyribozymes have wide generality for RNA substrate sequences, with only a limited requirement near the site of reactivity. The 10–23 deoxyribozyme requires only a purine nucleotide (adenosine A or guanosine G, abbreviated R) on the 5′-side of the cleavage site and a pyrimidine nucleotide (uridine U or cytidine C, abbreviated Y) on the 3′-side.

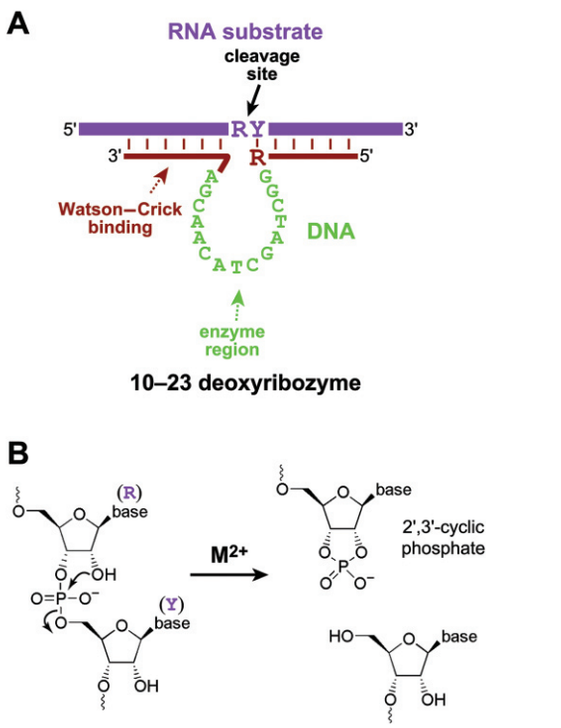

Fig. 2 RNA-cleaving deoxyribozymes. (A) The 10–23 deoxyribozyme,[18,19] showing its Watson–Crick mode of substrate binding and its generality for RNA substrate sequences. R = purine, Y = pyrimidine. (B). The chemical reaction of RNA cleavage. M^{2+} is a metal ion such as Mg^{2+}. This transesterification reaction is often inaccurately termed 'hydrolysis'; *e.g.* 'alkaline hydrolysis' is typically used to generate a regular cleavage ladder by random scission along an RNA strand under basic conditions. The specific sequence requirement for the 10–23 deoxyribozyme is shown.

An *in vitro* selection approach to identify RNA-cleaving deoxyribozymes is shown in Fig. 3. Variations of this strategy are applied in most DNA enzyme selection experiments; the details depend largely on the particular reaction to be catalyzed. Key elements of the selection process generally include the following three steps. (1) Random-region DNA nucleotides are enzymatically attached to the RNA or DNA substrate of interest. (2) The key selection step is implemented, with provision for physical separation of 'winning' deoxyribozymes from those unable to catalyze the desired reaction. This separation is commonly based on a biotin–streptavidin interaction or on a physical size difference coupled with polyacrylamide gel electrophoresis (PAGE). For example, by cleaving the RNA substrate, the deoxyribozyme may remove a biotin tag that is attached at one end of the RNA:DNA hybrid, allowing the catalytically competent sequences to pass through a streptavidin column. Alternatively, the size difference due to loss of the RNA fragment may be exploited by PAGE to separate the active deoxyribozymes. (3) Finally, PCR amplification is used to generate the deoxyribozyme pool for the next selection round,

which is input into another step (1). Multiple selection rounds are always necessary because many DNA sequences that are not truly competent for catalysis nevertheless can survive a single selection round merely by chance, and the initial pool harbors many orders of magnitude more inactive DNA sequences than those with substantial catalytic activity. Iterating the selection for many rounds enriches the pool in the latter sequences. The number of required rounds can be as few as three or as many as dozens, depending on the reaction being catalyzed and many other variables (*e.g.* incubation time, temperature, pH, and concentrations of metal ions). At an appropriate point, the catalytically active DNA enzymes are cloned by standard methods and their sequences are identified. The DNA enzymes may then be prepared independently by solid-phase synthesis (SPS) and their catalytic competence verified.

By decreasing the time available for reaction in progressive selection rounds, the resulting DNA enzymes can be coaxed to have higher rate constants, assuming that this is chemically possible within the searched sequence space. In analogy to biological selection, this is colloquially termed 'increasing the selection pressure'. While increasing pressure is often used to select for higher rates, other enzyme characteristics cannot readily be forced in this manner. Two of the most important such characteristics are yield and turnover. For example, consider the issue of turnover in the context of the selection strategy shown in Fig. 3. Each candidate DNA enzyme molecule is covalently linked to a specific RNA substrate molecule. During each selection round, the specific substrate molecule is either cleaved or uncleaved (*i.e.* exactly 100% or 0% cleavage yield for that particular molecule), and no opportunity exists to select for DNA sequences that cleave many RNA strands in multiple-turnover fashion. The reason is that a strict correlation between each candidate DNA enzyme and its attached RNA substrate must be maintained for the information about active deoxyribozymes to be passed into the next selection round. In the case of RNA-cleaving deoxyribozymes, the emergent catalysts are typically efficient in terms of both yield and turnover, although this is not demanded by the selection procedure. This efficiency is rationalized by noting that the cleaved products bind less tightly to the enzyme than does the longer substrate (*i.e.* the substrate has the lower K_M value), thereby freeing the deoxyribozyme for another turnover cycle. However, turnover does not always occur for deoxyribozymes with other activities such as RNA ligation (see below).

A recent publication highlights the general scope of deoxyribozymes for RNA cleavage and also emphasizes another important practical characteristic of selection experiments. By using a systematic array of RNA substrates, Cruz *et al.* set out to identify a collection of deoxyribozymes that collectively cleave all $4 \times 4 = 16$ possible dinucleotide (N–N) RNA junctions.[20] They successfully identified deoxyribozymes that cleave 14 out of the 16 possible junctions, with only C–T and U–T linkages recalcitrant to cleavage. (The 3′-nucleotide is written as T rather than U because this nucleotide was DNA for technical reasons, but the cleavage reaction involves a 2′-hydroxyl nucleophile, as in the standard RNA cleavage reaction of Fig. 2B.) These findings underscore the broad utility of deoxyribozymes for reactions of nucleic acid substrates. Unexpectedly, all of the new deoxyribozymes share a clear structural relationship to the small RNA-cleaving '8–17 deoxyribozyme', which has merely a 13-nucleotide enzyme region and has been identified by multiple laboratories.[21] The repeated independent identification of 8–17 indicates that smaller motifs have a tremendous selective advantage and may come to dominate the outcome of selection, even if larger enzymes would also be functional. This conclusion has been reached for ribozymes as well.[22]

Deoxyribozymes that covalently modify nucleic acids

Since the first RNA-cleaving deoxyribozymes were reported, many reactions involving nucleic acid modification have been catalyzed by DNA.[21] Some of these involve changes in the phosphorylation status of an RNA or DNA strand, specifically DNA phosphorylation[23] and DNA adenylation (capping).[24] Other reactions include DNA deglycosylation,[25] porphyrin metalation,[26] thymine dimer photoreversion[27] and DNA cleavage.[28] The latter is a Cu^{2+}-mediated oxidative cleavage reaction that is mechanistically quite distinct from the reaction catalyzed by RNA-cleaving deoxyribozymes, because a DNA substrate lacks the 2′-hydroxyl group of RNA and thus cannot participate in the cleavage mechanism of Fig. 2B.

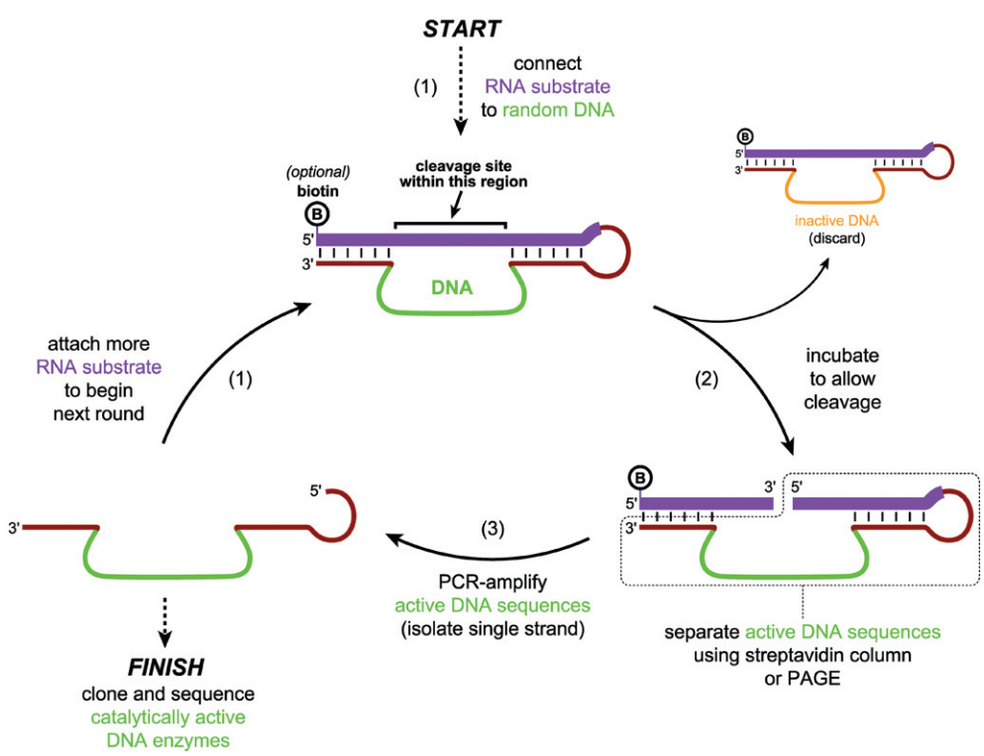

Fig. 3 A generic *in vitro* selection strategy for identifying RNA-cleaving deoxyribozymes. With suitable modifications, a similar approach can be applied to other DNA-catalyzed reactions (see text). Although the RNA and DNA are depicted as covalently connected for the purpose of selection, such attachment is not required for practical application of the deoxyribozymes (*e.g.* see Fig. 2A).

Deoxyribozymes that ligate DNA or RNA

A growing variety of deoxyribozymes ligate nucleic acid substrates. These reactions are distinguished from other DNA-catalyzed processes such as RNA cleavage in that molecules are joined rather than fragmented, and thus these deoxyribozymes are of particular interest to bioorganic chemists from the synthetic perspective. The first example of a ligase deoxyribozyme was reported soon after the initial description of an RNA-cleaving deoxyribozyme.[29] This DNA ligase deoxyribozyme joins two DNA substrates by reaction of a 5′-hydroxyl group with an activated 3′-phosphorimidazolide, and it requires either Zn^{2+} or Cu^{2+} for activity. More recently, a DNA enzyme was described that ligates DNA by reaction of a 3′-hydroxyl with a 5′-adenylated strand, which itself was created by a DNA-capping deoxyribozyme.[30] Both of these deoxyribozymes face hurdles to their practical application for joining of generic DNA substrates, either because the activated termini are not readily obtained[29] or because their sequence requirements substantially limit the substrates that may be used.[30]

My own laboratory has initiated significant efforts towards deoxyribozymes that ligate RNA. Our dual criteria are that the deoxyribozymes must have conceptual interest and, whenever possible, practical utility. These experiments use one of two chemical reactions, each of which requires a pair of RNA termini that are readily obtained using common biochemistry protocols (Fig. 4A).[31,32] Therefore, the deoxyribozymes that emerge from these selections should be useful for practical laboratory manipulations of RNA. The selection strategy is essentially identical to that shown in Fig. 3, except the desired deoxyribozymes are those that join two RNA substrates, rather than cleave a single RNA substrate.

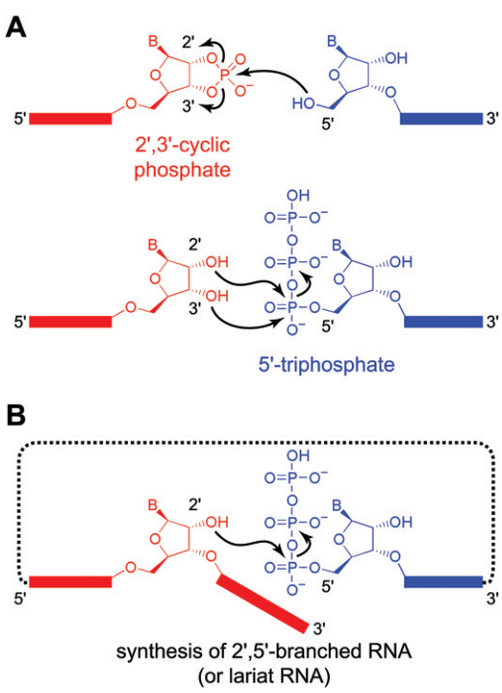

Fig. 4 Reactions catalyzed by RNA-ligating deoxyribozymes. (A) Two reactions that create linear RNA using readily available termini (B = nucleobase). In both cases, two product isomers are possible; controlling which isomer is formed is an active area of current investigation. (B) Reaction to synthesize 2′,5′-branched RNA. If the two RNA substrates are covalently connected by the dashed line, then the product is lariat RNA, which is topologically distinct from a 2′,5′-branch due to the closed loop. The RNA substrates shown in red and blue may have hundreds of chemically equivalent 2′-hydroxyl groups, but only one of these reacts as a nucleophile, without the use of any protecting groups. For the 9F7 and 7S11 deoxyribozymes that catalyze branched RNA formation, the DNA interacts largely in Watson–Crick fashion with various portions of the RNA regions that are denoted by the red and blue bars.[32,33,35,36]

The scope of reactivity that we have obtained to date is illustrated using two representative examples, those of the 9F7 and 7S11 deoxyribozymes. Both DNA enzymes synthesize 2′,5′-branched RNA by mediating the reaction of a specific internal 2′-hydroxyl group with a 5′-triphosphate (Fig. 4B).[32,33] They are also capable of synthesizing the topologically interesting lariat RNA, which is the natural form of branched RNA created during *in vivo* RNA splicing.[34] Detailed characterization indicates that 9F7 has rather stringent sequence requirements for its RNA substrates, which limits its general synthetic applicability.[35] In contrast, the 7S11 deoxyribozyme is broadly applicable for synthesis of biochemically relevant branches of varying sequence.[36] In addition, 7S11 itself is interesting as a nucleic acid catalyst from both the structural and evolutionary perspectives.[33,36]

One feature of the RNA ligase deoxyribozymes deserves special emphasis: their substantial site-selectivity. Each deoxyribozyme synthesizes just one branched product, not a mixture, and this product is formed without the use of any protecting groups, even when hundreds of equally reactive 2′-hydroxyl groups are found in the same substrate molecules. In sharp contrast, any approach to branched RNA based on traditional organic synthesis methodology would necessarily require intensive use of protecting groups. Such approaches have been applied in solid-phase synthesis, but they are laborious and often impose substantial requirements such that two of the polynucleotide 'arms' emerging from the branch site have identical sequences.[37] Furthermore, synthesis of lariat RNA has not been accomplished by solid-phase methods. Overall, the structural difficulty presented by the target of branched and lariat RNA suggests the synthetic application of an enzyme, and this is precisely what the RNA ligase deoxyribozymes deliver for this purpose. The high selectivities of many other deoxyribozymes can be described similarly. For example, the 10–23 RNA-cleaving deoxyribozyme (Fig. 2A) breaks just one bond within an RNA of essentially indefinite length, although every other internucleotide linkage is very similar chemically.

Deoxyribozyme catalytic parameters, mechanisms, and structures

One quantitative assessment of a deoxyribozyme's catalytic activity can be made by comparing its rate constant to that of an appropriate background reaction, *i.e.* determining its rate enhancement. For RNA ligation, the background reaction is reasonably taken as that mediated by a DNA 'splint' that has the DNA binding arms but entirely lacks the enzyme region or has random nucleotides in place of the enzyme region.[31] The background rate constant (k_{bkgd}) depends strongly on the reaction under consideration. For example, the two reactions of Fig. 4A differ in k_{bkgd} by approximately three orders of magnitude under comparable incubation conditions (reaction of the cyclic phosphate is faster). Upon dividing k_{obs} by the appropriate k_{bkgd}, the rate enhancement k_{obs}/k_{bkgd} may be computed. For 9F7, 7S11, and our other deoxyribozymes that create branched RNA, the rate enhancements are at least 10^6 to 10^7.[32,33] Rate enhancements for other deoxyribozymes are as high as 10^{10}.[21]

Little is known about the structures and mechanisms of any deoxyribozymes. Only one crystal structure of a deoxyribozyme (the 10–23) has been obtained, and the structure revealed a catalytically inactive 2:2 DNA:RNA stoichiometry.[38] Various deoxyribozymes have been characterized using standard biochemical approaches (*e.g.* refs. 19 and 35), but overall the field significantly lags behind analogous studies for ribozymes and protein enzymes. As more deoxyribozymes with interesting catalytic activities are discovered, the study of their mechanisms and structures will certainly grow in parallel. Despite our relative ignorance of deoxyribozyme mechanisms, one important consequence of the very high rate enhancements as

compared with a DNA splint is that a non-specific templating effect – *i.e.* effective molarity – cannot explain the observed reaction rates, as discussed below. Instead, some combination of transition-state stabilization and favoring of near-attack conformations[39] must be invoked to explain the catalytic prowess of deoxyribozymes.

Relationship of deoxyribozymes to DNA-templated synthesis and DNA display

The methods of DNA-templated synthesis and DNA display use DNA in the process of mediating chemical reactions. In DNA-templated synthesis,[40,41] two reactive functional groups are attached to separate nucleic acid strands and held together on a complementary DNA template in one of several geometries.[42] By capitalizing upon the effective molarity enforced by the DNA template, the reaction rate – not rate constant – is increased, and one or more reactions[43] of a wide range of types[44] may be orchestrated. In the context of catalysis, the role of the DNA in this process is passive; the DNA template increases the reaction rate solely *via* a non-specific effective molarity phenomenon. This is quite different from deoxyribozyme-catalyzed reactions, in which the DNA participates significantly beyond increasing the effective molarity. How do we know this, given that deoxyribozyme mechanisms are not known in detail? Structurally, the reactions of DNA-templated synthesis are analogous to the background splint-mediated reaction for most DNA enzymes, as described above, and the experimental data demonstrate that effective molarity is vastly inadequate to explain the large rate enhancements achieved by deoxyribozymes. The concepts of DNA-templated synthesis and of deoxyribozymes are fundamentally distinct and should not be confused.

Similarly, DNA display and deoxyribozymes are markedly different approaches to synthesis. DNA display is a recently introduced methodology in which an elegant oligonucleotide hybridization strategy facilitates DNA-programmed chemical synthesis.[45–47] This split-pool approach does not depend at all on effective molarity, but instead solves the problem of translating genetic information into small molecules by physical segregation according to DNA sequence (see refs. 45–47 for more information). As for DNA-templated synthesis, DNA plays no direct role in catalysis in DNA display, quite unlike the situation with deoxyribozymes.

Is the term 'catalyst' appropriate? A semantic issue

Is a DNA enzyme that suffers from product inhibition and therefore shows single-turnover behavior properly termed a 'catalyst'? Or is this a misuse of terminology? General chemistry textbooks define a *catalyst* as "a substance that takes part in a chemical reaction and speeds it up, but itself undergoes no permanent chemical change";[48] some advanced enzymology texts altogether avoid defining the term.[49,50] Using the general chemistry textbook definition, single-turnover deoxyribozymes are indeed catalysts, because they are not permanently changed during the reaction, and they can be re-used many times (this is routinely done in my own lab). In the literature and in common conversation, one occasionally finds reference to 'true catalysis', implying catalysis with multiple turnover, but there is apparently no justification for deeming only multiple-turnover catalysis to be 'true'. It should be noted that many naturally occurring 'enzymes' – comprising either proteins or nucleic acids – operate in single-turnover fashion, due either to chemical alteration upon catalysis (*e.g.* refs. 51 and 52) or simply to poor turnover. Enzymes limited in the latter fashion include the protein enzymes RNA and DNA ligase, which *in vitro* suffer from product inhibition and are used stoichiometrically for practical nucleic acid ligation.[53] A particularly important biological example is the spliceosome, which contains protein but almost certainly comprises catalytic RNA[4] and splices mRNA in single-turnover fashion *in vivo*.[54]

Why study catalytic DNA instead of catalytic RNA (*i.e.* deoxyribozymes instead of ribozymes)?

No DNA enzymes are known in nature (although it is an interesting but separate question if they are out there), and many *in vitro* experiments to identify nucleic acid enzymes have successfully used RNA. In this context, the question often arises: why study catalytic DNA instead of catalytic RNA? An initial consideration is that of functional activity: is DNA actually any less functional as a catalyst than RNA due to the missing 2′-hydroxyl groups (Fig. 1)? While this question cannot be answered for all possible reactions that one might wish to catalyze, the available evidence suggests that deoxyribozymes have no substantial functional handicap relative to their RNA counterparts. Specifically, DNA enzymes show no particular lack of quantitative catalytic ability for those reactions where both RNA and DNA catalysts have been identified. For example, consider the activity of the 10–23 deoxyribozyme for RNA cleavage, which meets or exceeds the activity of ribozymes for this purpose.

If DNA and RNA have similar catalytic potential, then practical concerns should be used to choose between them for particular applications. In this context, DNA has at least four advantages relative to RNA. (1) DNA costs significantly less than the analogous RNA when prepared by solid-phase synthesis. For example, from Integrated DNA Technologies a 1 μmol-scale preparation of a 40-mer DNA (several milligrams) currently costs $68, whereas a 1 μmol 40-mer RNA costs $480, *i.e.* a seven-fold higher cost for RNA. This does not account for the actual amount of pure nucleic acid typically obtained from SPS, which favors the DNA by a significant margin as well. (2) DNA can generally be made in longer sequence lengths than RNA and in higher purity by SPS. These facts reflect the need for 2′-protection of RNA during solid-phase synthesis, which also contributes to the cost difference. Alternatively, DNA can be generated in quantity by PCR, and simple methods exist for differentiating the single strands of the duplex PCR product by polyacrylamide gel or biotin/streptavidin-based approaches. (3) During the selection process itself, using DNA as both information carrier and catalyst obviates the need to transcribe and reverse-transcribe back and forth from DNA to RNA, which is required for ribozyme selections. Instead, catalytic DNA is directly amplified by PCR to generate DNA for the next round of selection [Fig. 3, step (3)]. (4) For real-life use of nucleic acid enzymes, DNA is preferred over RNA due to its relative chemical and biochemical stability. For example, environmentally ubiquitous ribonucleases are a considerable worry and recurrent problem when working with RNA *via* catalysis of the Fig. 2B cleavage reaction, but ribonucleases do not cleave DNA.

As a final advantage, one may anticipate that for any reaction of RNA substrates, a relatively inexpensive and readily synthesized DNA enzyme will rarely if ever be the most precious component, whereas this may be untrue for a ribozyme. Thus the issue of single *versus* multiple turnover for DNA enzymes is typically rendered irrelevant in practice, because using a stoichiometric amount of deoxyribozyme is not prohibitive.

Extending chemical functionality with modified DNA nucleotides

For both ribozymes and deoxyribozymes, the repertoire of functional groups may be extended using chemically modified nucleotides. The overall topic has been reviewed recently,[55] so the discussion here is brief. The modified nucleotides typically bear functional groups such as metal chelators (*e.g.* pyridine or imidazole), and they are incorporated using mutant DNA polymerases that tolerate functionalized nucleotide triphosphates (NTPs) during PCR. The new functionality is typically introduced at every occurrence of a particular nucleotide, or alternatively at some fraction of a particular

nucleotide's sites within the DNA (by mixing a modified NTP with the unmodified NTP in a fixed ratio). However, it is impossible to introduce modified nucleotides site-specifically into DNA using a polymerase in this manner. The extent to which particular catalytic activities require extended chemical functionality is unclear; this is likely to be an area of active experimentation in the future. A practical disadvantage of using DNA with extended chemical functionality is that many specific modifications are not available commercially within DNA oligonucleotides, or if they are, they are prohibitively expensive when incorporated at many positions. Of course, this situation could change if a particular modification proves to be useful in practice.

Prospects for DNA-catalyzed reactions beyond phosphodiester chemistry

The logical and physical separation of essentially all reported deoxyribozymes into an enzyme region flanked by Watson–Crick binding regions (Fig. 2A) is very effective for reactions of nucleic acid substrates, which bind to the deoxyribozymes by base pairing. However, when contemplating future applications of deoxyribozymes to non-nucleic acid substrates, the use of base pairing to provide the binding affinity is problematic. If the substrate of interest is not a nucleic acid, then two reasonable choices can be described. (1) The substrate could be conjugated to a nucleic acid solely for binding to the DNA enzyme *via* conventional Watson–Crick base pairs. This substrate–nucleic acid linkage could include a cleavable moiety to allow removal of the nucleic acid after catalysis. (2) The DNA could bind the substrate using interactions other than base pairs. For example, selections could be performed directly to identify DNA sequences that simultaneously bind substrates and catalyze their reaction. Alternatively, independently generated DNA aptamers – *i.e.* DNA molecules that bind targets[56] – could be exploited as the source of binding energy. In the long term, one can envision creating modular DNA catalysts by systematically combining DNA aptamer and enzyme domains, where the former bind the substrates and the latter perform catalysis. Although it is impossible to know without experimentation if any of these plans are actually achievable, past successes with deoxyribozymes provide substantial encouragement that the attempts will be worthwhile.

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References

1 K. Kruger, P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gottschling and T. R. Cech, *Cell*, 1982, **31**, 147–157.
2 C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace and S. Altman, *Cell*, 1983, **35**, 849–857.
3 T. R. Cech, *Science*, 2000, **289**, 878–879.
4 C. A. Collins and C. Guthrie, *Nat. Struct. Biol.*, 2000, **7**, 850–854.
5 J. A. Doudna and T. R. Cech, *Nature*, 2002, **418**, 222–228.
6 *The RNA World*, 2nd edn., R. F. Gesteland, T. R. Cech and J. F. Atkins eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999.
7 M. A. Dwyer, L. L. Looger and H. W. Hellinga, *Science*, 2004, **304**, 1967–1971.
8 H. M. Geysen, F. Schoenen, D. Wagner and R. Wagner, *Nat. Rev. Drug Discov.*, 2003, **2**, 222–230.
9 S. C. McCleskey, M. J. Griffin, S. E. Schneider, J. T. McDevitt and E. V. Anslyn, *J. Am. Chem. Soc.*, 2003, **125**, 1114–1115.
10 M. B. Francis, T. F. Jamison and E. N. Jacobsen, *Curr. Opin. Chem. Biol.*, 1998, **2**, 422–428.
11 L. Jaeger, M. C. Wright and G. F. Joyce, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 14 712–14 717.
12 R. E. Dolle, *J. Comb. Chem.*, 2003, **5**, 693–753.
13 G. F. Joyce, *Annu. Rev. Biochem.*, 2004, **73**, 791–836.
14 P. C. Sabeti, P. J. Unrau and D. P. Bartel, *Chem. Biol.*, 1997, **4**, 767–774.
15 R. R. Breaker and G. F. Joyce, *Chem. Biol.*, 1994, **1**, 223–229.
16 J. Liu and Y. Lu, *J. Am. Chem. Soc.*, 2003, **125**, 6642–6643.
17 C. R. Dass, *Trends Pharmacol. Sci.*, 2004, **25**, 395–397.
18 S. W. Santoro and G. F. Joyce, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 4262–4266.
19 S. W. Santoro and G. F. Joyce, *Biochemistry*, 1998, **37**, 13 330–13 342.
20 R. P. G. Cruz, J. B. Withers and Y. Li, *Chem. Biol.*, 2004, **11**, 57–67.
21 G. M. Emilsson and R. R. Breaker, *Cell. Mol. Life Sci.*, 2002, **59**, 596–607.
22 K. Salehi-Ashtiani and J. W. Szostak, *Nature*, 2001, **414**, 82–84.
23 Y. Li and R. R. Breaker, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 2746–2751.
24 Y. Li, Y. Liu and R. R. Breaker, *Biochemistry*, 2000, **39**, 3106–3114.
25 T. L. Sheppard, P. Ordoukhanian and G. F. Joyce, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 7802–7807.
26 Y. Li and D. Sen, *Nat. Struct. Biol.*, 1996, **3**, 743–747.
27 D. J. Chinnapen and D. Sen, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 65–69.
28 N. Carmi, L. A. Shultz and R. R. Breaker, *Chem. Biol.*, 1996, **3**, 1039–1046.
29 B. Cuenoud and J. W. Szostak, *Nature*, 1995, **375**, 611–614.
30 A. Sreedhara, Y. Li and R. R. Breaker, *J. Am. Chem. Soc.*, 2004, **126**, 3454–3460.
31 A. Flynn-Charlebois, Y. Wang, T. K. Prior, I. Rashid, K. A. Hoadley, R. L. Coppins, A. C. Wolf and S. K. Silverman, *J. Am. Chem. Soc.*, 2003, **125**, 2444–2454.
32 Y. Wang and S. K. Silverman, *J. Am. Chem. Soc.*, 2003, **125**, 6880–6881.
33 R. L. Coppins and S. K. Silverman, *Nat. Struct. Mol. Biol.*, 2004, **11**, 270–274.
34 C. L. Peebles, P. S. Perlman, K. L. Mecklenburg, M. L. Petrillo, J. H. Tabor, K. A. Jarrell and H. L. Cheng, *Cell*, 1986, **44**, 213–223.
35 Y. Wang and S. K. Silverman, *Biochemistry*, 2003, **42**, 15 252–15 263.
36 R. L. Coppins and S. K. Silverman, 2004, submitted for publication.
37 M. J. Damha, K. Ganeshan, R. H. Hudson and S. V. Zabarylo, *Nucleic Acids Res.*, 1992, **20**, 6565–6573.
38 J. Nowakowski, P. J. Shim, G. S. Prasad, C. D. Stout and G. F. Joyce, *Nat. Struct. Biol.*, 1999, **6**, 151–156.
39 S. Hur and T. C. Bruice, *J. Am. Chem. Soc.*, 2003, **125**, 1472–1473.
40 J. T. Goodwin and D. G. Lynn, *J. Am. Chem. Soc.*, 1992, **114**, 9197–9198.
41 Z. J. Gartner and D. R. Liu, *J. Am. Chem. Soc.*, 2001, **123**, 6961–6963.
42 Z. J. Gartner, M. W. Kanan and D. R. Liu, *J. Am. Chem. Soc.*, 2002, **124**, 10 304–10 306.
43 X. Li, Z. J. Gartner, B. N. Tse and D. R. Liu, *J. Am. Chem. Soc.*, 2004, **126**, 5090–5092.
44 Z. J. Gartner, M. W. Kanan and D. R. Liu, *Angew. Chem., Int. Ed.*, 2002, **41**, 1796–1800.
45 D. R. Halpin and P. B. Harbury, *PLoS Biol.*, 2004, **2**, E173.
46 D. R. Halpin and P. B. Harbury, *PLoS Biol.*, 2004, **2**, E174.
47 D. R. Halpin, J. A. Lee, S. J. Wrenn and P. B. Harbury, *PLoS Biol.*, 2004, **2**, E175.
48 D. W. Oxtoby, N. H. Nachtrieb and W. A. Freeman, *Chemistry: Science of Change*, 2nd edn., Saunders College Publishing, Philadelphia, 1994, p. 588.
49 W. P. Jencks, *Catalysis in Chemistry and Enzymology*, Dover Publications, Inc., New York, 1969.
50 R. B. Silverman, *The Organic Chemistry of Enzyme-Catalyzed Reactions*, Academic Press, San Diego, 2002.
51 A. Juillerat, T. Gronemeyer, A. Keppler, S. Gendreizig, H. Pick, H. Vogel and K. Johnsson, *Chem. Biol.*, 2003, **10**, 313–317.
52 F. Berkovitch, Y. Nicolet, J. T. Wan, J. T. Jarrett and C. L. Drennan, *Science*, 2004, **303**, 76–79.
53 M. J. Moore and C. C. Query, in *RNA–Protein Interactions: A Practical Approach*, C. W. J. Smith, ed., Oxford University Press, Oxford, 1998, pp. 75–108.
54 S. W. Stevens, D. E. Ryan, H. Y. Ge, R. E. Moore, M. K. Young, T. D. Lee and J. Abelson, *Mol. Cell*, 2002, **9**, 31–44.
55 J. A. Bittker, K. J. Phillips and D. R. Liu, *Curr. Opin. Chem. Biol.*, 2002, **6**, 367–374.
56 M. Famulok, G. Mayer and M. Blind, *Acc. Chem. Res.*, 2000, **33**, 591–599.