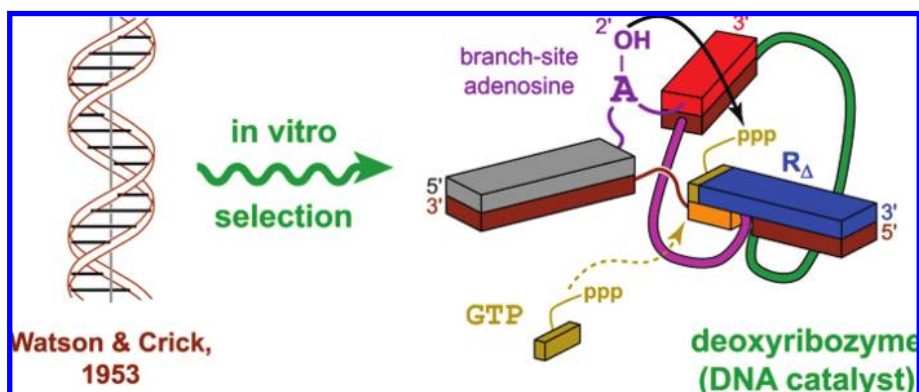# Deoxyribozymes: Selection Design and Serendipity in the Development of DNA Catalysts[†]

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CONSPECTUS

One of the chemist's key motivations is to explore the forefront of catalysis. In this Account, we describe our laboratory's efforts at one such forefront: the use of DNA as a catalyst.

Natural biological catalysts include both protein enzymes and RNA enzymes (ribozymes), whereas nature apparently uses DNA solely for genetic information storage. Nevertheless, the chemical similarities between RNA and DNA naturally lead to laboratory examination of DNA as a catalyst, especially because DNA is more stable than RNA and is less costly and easier to synthesize. Many catalytically active DNA sequences (deoxyribozymes, also called DNAzymes) have been identified in the laboratory by in vitro selection, in which many random DNA sequences are evaluated in parallel to find those rare sequences that have a desired functional ability. Since 2001, our research group has pursued new deoxyribozymes for various chemical reactions. We consider DNA simply as a large biopolymer that can adopt intricate three-dimensional structure and, in the presence of appropriate metal ions, generate the chemical complexity required to achieve catalysis.

Our initial efforts focused on deoxyribozymes that ligate two RNA substrates. In these studies, we used only substrates that are readily obtained biochemically. Highly active deoxyribozymes were identified, with emergent questions regarding chemical selectivity during RNA phosphodiester bond formation. Deoxyribozymes allow synthesis of interesting RNA products, such as branches and lariats, that are otherwise challenging to prepare. Our experiments have demonstrated that deoxyribozymes can have very high rate enhancements and chemical selectivities. We have also shown how the in vitro selection process itself can be directed toward desired goals, such as selective formation of native 3′−5′ RNA linkages. A final lesson is that unanticipated selection outcomes can be very interesting, highlighting the importance of allowing such opportunities in future experiments.

More recently, we have begun using nonoligonucleotide substrates in our efforts with deoxyribozymes. We have especially focused on developing DNA catalysts for reactions of small molecules or amino acid side chains. For example, new deoxyribozymes have the catalytic power to create a nucleopeptide linkage between a tyrosine or serine side chain and the 5′-terminus of an RNA strand. Although considerable further work remains to establish DNA as a practical catalyst for small molecules and full-length proteins, the progress to date is very promising. The many lessons learned during the experiments described in this Account will help us and others to realize the full catalytic power of DNA.

Introduction

Deoxyribozymes—also called DNA enzymes or DNAzymes—are DNA sequences that catalyze chemical reactions. Our laboratory's studies on deoxyribozymes were initiated in 2001, when we began in vitro selection experiments seeking ligation of two RNA substrates. Since then, we have published many deoxyribozyme manuscripts, most on RNA ligation[1−22] but some on ligation of non-RNA oligonucleotide substrates[23,24] and a growing number on reactions of substrates that are not oligonucleotides at all.[25−28] This Account describes the motivations and logic behind many of our previous efforts, as well as our ongoing work and likely future directions with deoxyribozymes.

Why Pursue DNA as an Artificial Catalyst?

Of the three natural biopolymers, DNA, RNA, and proteins, only the last two are known to have evolved directly for catalysis. Why, then, would anyone want to pursue DNA as an artificial catalyst? Instead, why not just look to RNA or proteins? The nucleic acids DNA and RNA together have key practical advantages relative to proteins, for example, regarding both the size of sequence space that must be searched (for an oligomer of length n, 20^n for proteins is much larger than 4^n for DNA/RNA) and the simplicity of oligonucleotide synthesis (which lacks the challenges inherent to protein expression). If the catalytic functions of DNA/RNA are useful, which is ultimately an empirical question, then the practical advantages of nucleic acids relative to proteins will allow more efficient and simpler identification and application of new catalysts. In the direct comparison of DNA to RNA, DNA is much more stable, less costly, and easier to synthesize. Of course, none of these advantages of DNA are important if the catalytic repertoire of DNA is restricted to uninteresting reactions. Therefore, in our efforts with deoxyribozymes, we have sought new catalytic activities, with an eye toward probing the limits of DNA catalysis.

The motivation to pursue deoxyribozymes can also be understood from a different direction, by considering the fundamental chemical requirements for building an effective biopolymeric catalyst. Nature has given us proteins, which adopt complex three-dimensional structures and position a variety of functional groups to enable catalytic mechanisms. Nature has also given us ribozymes, which can form intricate 3D structures despite having only four rather similar monomers. Judicious use of metal ions and acid/base interactions equips ribozymes with significant chemical tools for catalysis, even if ribozymes are not quantitatively as good as proteins (and the jury is still out on how good RNA can actually

be[29]). DNA is very similar to RNA in all of these aspects, and DNA additionally has all of the key advantages as mentioned above. Therefore, expanding the frontier of catalysis by studying deoxyribozymes is a potentially high-reward endeavor.

Why Pursue RNA Ligation as the Target Reaction?

The immediate practical motivation for our initial decision to pursue deoxyribozymes that ligate RNA was the desire for improved RNA ligation catalysts. Since 1992, the principal method for ligating two RNA substrates has been "splint ligation", which uses T4 DNA ligase to join two RNA substrates held together by a complementary DNA splint.[30,31] Although splint ligation has certainly been successful in many cases, thereby enabling synthesis of large RNAs that incorporate site-specific modifications for structure−function studies, the method has well-known idiosyncrasies. For example, the ligation efficiency for two particular RNA sequences (especially for large RNAs or those that have considerable secondary structure) is difficult to predict without simply preparing the two substrates and attempting the splint ligation. Preparing large RNA substrates for these tests is not always trivial. In 2001 at the outset of our efforts, we thought, "wouldn't it be great if the DNA splint can simultaneously be the ligation catalyst?" Perhaps using a deoxyribozyme to join two RNAs would be more effective than splint ligation, at least in some cases.

Regardless of immediate practical utility, a second objective for pursuing DNA-catalyzed RNA ligation was simply to learn more about the catalytic properties of DNA, both in terms of basic selection principles and in terms of reactions that can be catalyzed. The first deoxyribozyme was reported in 1994 for cleavage of an RNA phosphodiester linkage embedded within a DNA oligonucleotide substrate.[32] Between 1994 and 2001, many additional RNA-cleaving deoxyribozymes were reported, all of which catalyze the same cleavage reaction (including within all-RNA substrates),[33] but DNA catalysis of other reactions was and still is rare.[34−37] As reported by others, representative examples of such reactions include DNA ligation,[38] porphyrin metalation,[39] DNA depurination,[40] and thymine dimer photoreversion.[41,42] Exploring new DNA catalysis is an important fundamental science objective. As explained below, catalysis of RNA ligation afforded the opportunity to study key aspects of DNA catalysis related to chemical selectivity that could not be explored with RNA cleavage. Our initial efforts with deoxyribozymes for RNA ligation have led more recently to exploring other DNA-catalyzed reactions, with several promising future directions.
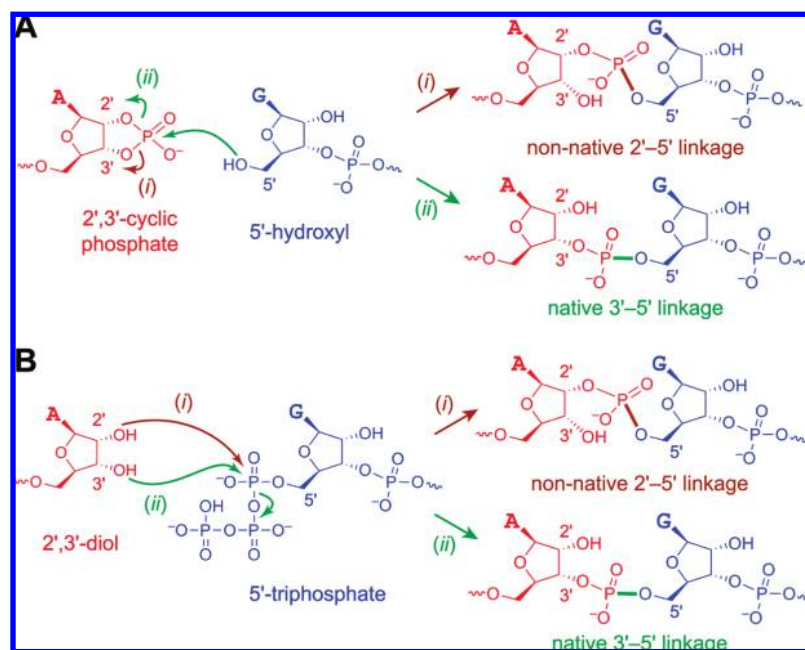
FIGURE 1. RNA substrate combinations for DNA-catalyzed RNA ligation: (A) 2′,3′-cyclic phosphate with 5′-hydroxyl; (B) 3′-hydroxyl with 5′-triphosphate.

Initial Plans to Identify Deoxyribozymes for RNA Ligation

In 2001, when we began pursuing RNA-ligating deoxyribozymes, two critical decisions were required. First, what RNA substrates would we use? Second, what in vitro selection strategy would we use?

RNA Substrate Combinations. For DNA-catalyzed RNA cleavage, the substrate is a standard RNA phosphodiester linkage, with attack of the cleavage-site 2′−OH at the adjacent phosphate group.[33,43] In contrast, for RNA ligation a variety of reaction chemistries can be envisioned, including many with non-natural functional groups. We restricted ourselves to functional group combinations that are readily obtained without undue effort, to ensure the widest utility for any resulting deoxyribozymes. In practice, this limited us to two substrate combinations (Figure 1): 2′,3′-cyclic phosphate with 5′-hydroxyl and 3′-hydroxyl with 5′-triphosphate. In both cases, this led directly to questions of chemical selectivity that must be addressed during the in vitro selection process.

In Vitro Selection Strategy. Inspired by previous approaches to RNA-cleaving deoxyribozymes, we devised the general in vitro selection strategy for RNA ligation depicted in Figure 2. To initiate the selection process, a "random pool" DNA oligonucleotide is prepared by solid-phase synthesis, incorporating an N_{40} region between two fixed sequences that serve as PCR primer binding sites. First, a portion of this pool (∼200 pmol = 10^{14} molecules) is covalently joined to one RNA substrate. Second, the other RNA substrate is introduced,
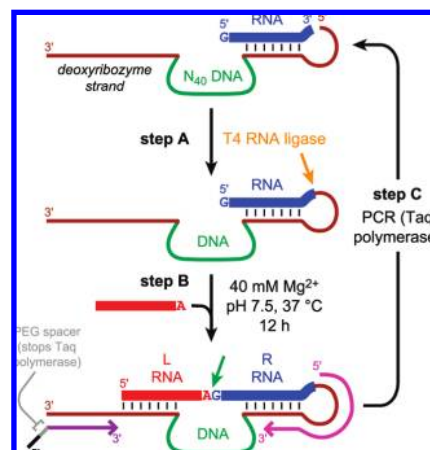

FIGURE 2. General in vitro selection strategy to identify deoxyribozymes that ligate RNA.[1] The indicated conditions in the key selection step B can be changed as desired.

and the collection of DNA sequences is allowed the opportunity to ligate the two RNA substrates. Catalytically active DNA sequences are separated by polyacrylamide gel electrophoresis (PAGE), because the entire RNA−DNA conjugate has grown larger by the size of the second RNA substrate. Finally, PCR is used to regenerate the DNA pool, now enriched in sequences that are capable of RNA ligation. The overall strategy is iterated for multiple (typically 5−15) selection rounds, enriching the population with DNA sequences that are reproducibly competent at RNA ligation. At the end of the selection process, individual deoxyribozyme sequences are identified by standard cloning methods, and their catalytic properties (product identity, rate, yield, selectivity, etc.) are examined.
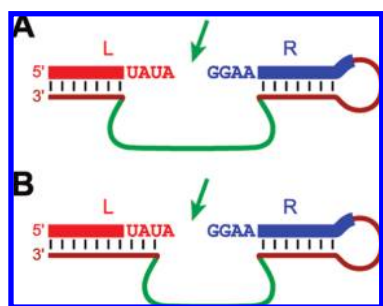
FIGURE 3. Arrangements of DNA and RNA for DNA-catalyzed RNA ligation. Base pairs in the binding arms are not depicted quantitatively: (A) detailed arrangement of the first RNA ligation experiments, using either substrate combination from Figure 1;[1,8] (B) subsequently used arrangement with the 5′-triphosphate substrate combination.[10]

RNA Ligation with 2′,3′-Cyclic Phosphate Substrates

Our initial effort used the RNA substrate combination shown in Figure 1A.[1] The cyclic phosphate substrates are themselves the products of RNA cleavage, either by ribonucleases such as RNase A or by deoxyribozymes. We used the well-known 10−23 deoxyribozyme[44] to prepare the RNA substrate that has a 2′,3′-cyclic phosphate. Then, as our first in vitro selection target, we sought RNA ligation using the selection strategy of Figure 2. Our initial design placed four unpaired RNA nucleotides of each substrate at the ligation junction, with all remaining nucleotides of each RNA substrate forming Watson−Crick base pairs with the deoxyribozyme "binding arms" (Figure 3A). For brevity, we designated the two RNA substrates as the "left-hand" and "right-hand" substrates, or L and R, as marked in Figure 3A. Four nucleotides were left unpaired on both L and R because active deoxyribozyme sequences might require non-Watson−Crick interactions with these nucleotides. Alternatively, the selection process could favor deoxyribozyme sequences that create canonical DNA:RNA base pairs.

The outcome of this initial selection effort was a set of Mg^{2+}-dependent deoxyribozymes that had two key features.[1] First, all new RNA linkages were non-native 2′−5′ rather than native 3′−5′, formed by opening of the cyclic phosphate with the 3′-oxygen rather than the 2′-oxygen serving as the leaving group. (Assignments of 2′−5′ rather than native 3′−5′ linkages were made by straightforward biochemical approaches, such as assaying the ligation products with a deoxyribozyme that is selective for cleaving 3′−5′ linkages and measuring the half-life for uncatalyzed cleavage when the RNA product is in a DNA:RNA duplex; the 2′−5′ linkages are much more labile in this context.) Second, each deoxyribozyme required several specific RNA nucleotide identities on

either side of the ligation junction. Our subsequent efforts to identify deoxyribozymes that function with the cyclic phosphate RNA substrate combination were designed to understand and ideally control the ligation junction connectivity and substrate sequence requirements.

In one set of efforts, we retained Mg^{2+} as the divalent metal ion cofactor and changed the selection design by reducing the random region length,[2,3] by reselecting initially identified sequences for improved catalysis,[4] or by changing the base-pairing status of the RNA nucleotides near the ligation site.[5] In all cases, our principal interest was the regioselectivity of the cyclic phosphate ring-opening reaction that forms either 2′−5′ or 3′−5′ linkages. Surprisingly, again only non-native 2′−5′ linkages were created. We do not yet have a mechanistic explanation for this uniform DNA-catalyzed synthesis of non-native linkages with Mg^{2+} and the cyclic phosphate RNA substrate combination.

In a second set of efforts, we performed selections using Zn^{2+} rather than Mg^{2+}. We were excited to find several deoxyribozymes that create native 3′−5′ linkages, along with deoxyribozymes that form a variety of other linkages.[6] Importantly, each new deoxyribozyme creates just one kind of linkage. We then modified the selection process (e.g., by changing the number of unpaired RNA nucleotides near the ligation junction) and found additional deoxyribozymes that also create native 3′−5′ linkages.[7] However, enough specific RNA nucleotides were required near the ligation junction that these deoxyribozymes would not be general for ligating a wide variety of RNA sequences. More work remains to solve this problem. In addition, we do not yet know the reason why changing the metal ion cofactor from Mg^{2+} to Zn^{2+} leads to different regioselectivities during the 2′,3′-cyclic phosphate opening reaction.

RNA Ligation with 5′-Triphosphate Substrates

Soon after we began efforts with the cyclic phosphate substrates, we also examined the 5′-triphosphate RNA substrate combination shown in Figure 1B. Initially, we used the same arrangement of RNA substrates and deoxyribozyme as in Figure 3A, merely replacing the functional groups on both substrates at the intended ligation site.[8,9] We originally expected that either the 2′-OH or 3′-OH of the L substrate would attack the 5′-triphosphate of the R substrate, forming a linear RNA linkage (either 2′−5′ or 3′−5′). However, the attacking nucleophile was actually a 2′-OH group from within L, leading to 2′,5′-branched RNA (Figure 4). For at least one of the resulting deoxyribozymes, L and R could be part of the same RNA
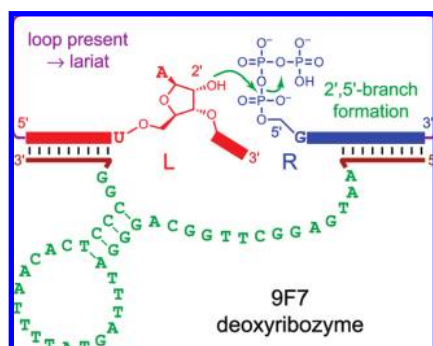
FIGURE 4. Formation of 2′,5′-branched RNA by attack of an internal 2′-OH group of L into the 5′-triphosphate of R.[8]

molecule, leading to a lariat topology in the RNA product (Figure 4; the lariat topology was demonstrated by a standard biochemical analysis in which random alkaline cleavage of the lariat leads to a characteristic pattern of gel bands). At this point, our efforts took two major directions.

Direction 1: Seeking Linear RNA Linkages. Initially we simply paired more nucleotides of L, seeking to favor reactivity of the terminal 2′,3′-diol by restricting the accessibility of the internal nucleotides (Figure 3B).[10] Instead, we identified the 7S11 deoxyribozyme, which catalyzes synthesis of 2′,5′-branched RNA by forming an intriguing three-helix-junction (3HJ) architecture with its RNA substrates (Figure 5A). Both 7S11 and the 3HJ platform have been exploited in many of our laboratory's subsequent experiments. Deoxyribozyme 7S11 was identified because the PCR primer used during each selection round allowed mutations due to Taq polymerase to accumulate within the DNA binding arm that binds to the L substrate. The emergent deoxyribozymes such as 7S11 no longer used this pre-existing binding arm at all, instead using nucleotides derived from the originally random region to interact with L. Therefore, in selection experiments performed after identifying 7S11, we used a longer PCR primer that includes all binding arm nucleotides. This suppresses Taq-derived mutations because the primer automatically restores any mutated nucleotides during a subsequent PCR cycle.

We also sought linear native 3′−5′ RNA linkages by moving the ligation site to within a DNA:RNA duplex region.[13] The rationale was that native 3′−5′ linkages are known to be more stable than non-native 2′−5′ linkages in this structural context; this relative stability should be felt to at least some extent in the ligation transition state, leading to selectivity. While this strategy proved successful and the resulting deoxyribozymes indeed created almost entirely 3′−5′ native linkages, too many specific RNA substrate nucleotides were required for practical utility.

Based on the above experiments, we realized that we needed to impose a selection pressure specifically to enforce formation of linear native 3′−5′ linkages without simultaneously requiring any specific arrangement of deoxyribozyme and substrates. The appropriate selection pressure was imposed by using the RNA-cleaving 8−17 deoxyribozyme during each round.[11] The 8−17 cleaves only 3′−5′ but not 2′−5′ linkages. Therefore, only those deoxyribozyme sequences that created a 3′−5′ linkage during the selection round are allowed to survive, because only for those sequences will the attached RNA product be cleaved by 8−17 as the last step of the round.

Finally, combining all of these approaches, we found both Mg^{2+}- and Zn^{2+}-dependent deoxyribozymes that satisfy all key requirements for deoxyribozymes that ligate RNA (Figure 6).[12] The Mg^{2+}-dependent 9DB1 and Zn^{2+}-dependent 7DE5 deoxyribozymes each create native 3′−5′ RNA linkages with useful rate, yield, and substrate sequence generality.

Direction 2: Directly Seeking Branched RNA Linkages. We sought to exploit the ability of deoxyribozymes to create 2′,5′-branched and lariat RNA. The 7S11 deoxyribozyme requires base pairing with every nucleotide of the two RNA substrates except the branch-site nucleotide (Figure 5A). This suggested significant generality for many RNA substrate sequences, and indeed this was so.[14] Separately, one of the deoxyribozymes found during development of the 8−17 selection pressure (6CE8)[11] was shown to be useful for creating branched RNA with any branch-site nucleotide,[16] which is one of the only nucleotides whose identity is restricted for 7S11. We also showed that another deoxyribozyme (6BX22) can be used for one-step synthesis of lariat RNAs directly related to biological sequences,[17] and we developed a two-step ligation route to lariats that cannot be made efficiently in one step.[18]

We examined the origin of the branch-site adenosine preference of 7S11 by performing selection experiments using the 3HJ architecture where the branch-site nucleotide was U rather than A.[19] Although the DNA pool never had access to branch-site A during the selection process, the resulting deoxyribozymes still strongly preferred A over U. This suggested a chemical basis for the branch-site preference, although the detailed nature of this chemical basis remains to be understood. These experiments also led to 10DM24, a deoxyribozyme structurally related to 7S11 with slightly improved RNA sequence generality.
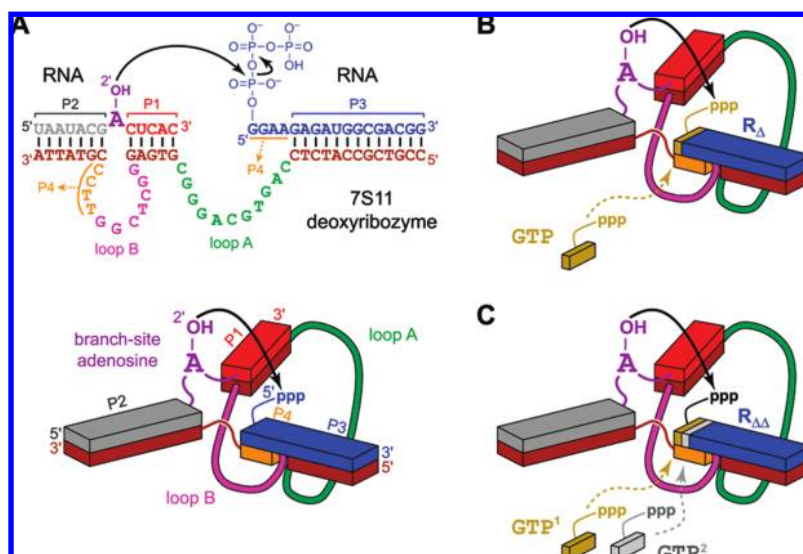
FIGURE 5. Deoxyribozymes that form a three-helix-junction (3HJ) architecture with their RNA substrates: (A) The 7S11 deoxyribozyme, which forms 2′,5′-branched RNA.[10,14] The related 10DM24 deoxyribozyme has a similar architecture.[19] (B) Conversion of 10DM24 to use GTP as a small-molecule substrate along with a shortened oligonucleotide cofactor (R_Δ).[25] (C) Use of two molecules of GTP as substrate and cofactor, along with an oligonucleotide cofactor ($R_{\Delta\Delta}$).
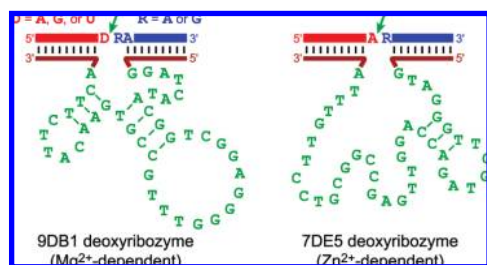

FIGURE 6. Two general deoxyribozymes for RNA ligation using the 5′-triphosphate substrate combination.[12]

Applications of Deoxyribozymes that Ligate RNA

We used branched RNAs formed by deoxyribozymes to enable biochemical studies that would otherwise have been essentially impossible.[20,22] These studies investigated biochemical pathways that are either known to involve branched RNA[20] or controversially proposed to do so[22] (in the latter case, our data support the conclusion that branched RNA is not actually involved). However, the number of such experiments that make use of synthetic branched RNA is smaller than we had originally hoped. Despite over two decades of literature statements that synthetic access to branched RNAs is a major roadblock to biochemical experiments, we have found that devising meaningful experiments using synthetic branches is actually rather difficult. Many biochemical processes related to branched RNA (such as pre-mRNA splicing) are highly orchestrated, and a separately synthesized branch
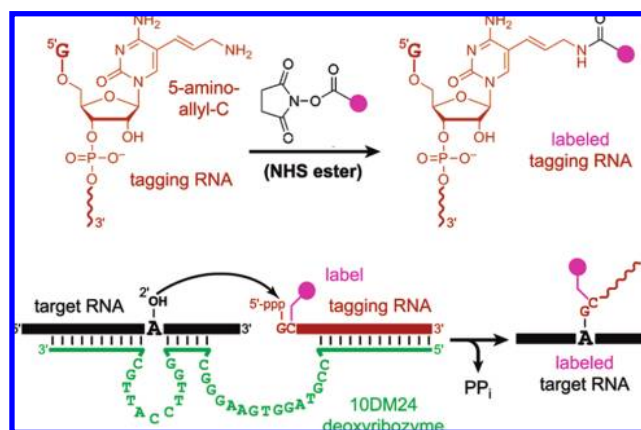

FIGURE 7. Strategy for deoxyribozyme-catalyzed labeling (DECAL) of an RNA target.[21] The labeled tagging RNA is prepared (top) and attached to the target RNA using the 10DM24 deoxyribozyme (bottom).

cannot be readily integrated into most of the relevant biochemical assays.

We also used deoxyribozymes to enable a new strategy for site-specific RNA labeling: DECAL, or deoxyribozyme-catalyzed labeling (Figure 7).[21] In DECAL, a specific 2′-OH nucleophile on the large "target" RNA attacks the 5′-triphosphate electrophile of a second "tagging" RNA, which carries a biophysical label (e.g., fluorescein or biotin) at its second nucleotide position. Using DECAL, we could readily place the fluorescence resonance energy transfer (FRET) dyes fluorescein and tetramethylrhodamine onto two specific nucleotides of the 160-

nucleotide P4—P6 RNA domain, thereby enabling a straightforward FRET folding experiment for this RNA.

General Themes from RNA-Ligating Deoxyribozymes

The several years since our first publication on RNA-ligating deoxyribozymes[1] have provided some perspective, which allows us to identify four general themes that have emerged from these efforts.

Theme 1: Catalytic Rate Enhancements Are Substantial. Many of our RNA-ligating deoxyribozymes achieve high rate enhancements, far beyond that of a simple templating effect. For example, the 9F13 deoxyribozyme that forms 2′,5′-branched RNA has a rate enhancement of nearly 10^7.[8] This is lower than that achievable by protein catalysts but in the range of both natural and artificial ribozymes.[45]

Theme 2: Deoxyribozymes Have Very High Chemical Selectivities. One of the most useful properties of a catalyst is high chemical selectivity, such as discrimination between two identical functional groups. Deoxyribozymes that form branched RNA again provide good examples, in that only one branch-site 2′-OH nucleophile is used although potentially hundreds of competing 2′-OH groups are available. For the 6BX22 deoxyribozyme that creates lariat RNA, site-selectivity in use of a single branch-site 2′-OH allows highly selective synthesis of lariats that have hundreds of nucleotides in the loop.[17] These products would be extremely challenging to make by any other means.

Theme 3: In Vitro Selection Allows for Significant Rational Intervention. Many of our experiments with RNA ligation have demonstrated the significant capability of an appropriate selection pressure to direct (or even redirect) the outcome of a selection experiment toward a desired catalytic goal. Probably the best example of this is the selection pressure applied by using the 8—17 deoxyribozyme to achieve 3′—5′ RNA ligation,[11] as described above.

Theme 4: Unanticipated Selection Outcomes Can Be Valuable. Identification of the 7S11 branch-forming deoxyribozyme is probably the best example of this final theme. Because the selection strategy permitted mutations to accumulate in the left-hand DNA binding arm, the 3HJ architecture of 7S11 emerged spontaneously. It is doubtful that we would have intentionally designed such an architecture, which we subsequently used to explore new DNA-catalyzed chemical reactions (see below).

Remaining Efforts Needed for DNA-Catalyzed RNA Ligation

Our efforts with DNA-catalyzed RNA ligation have left several unanswered questions. First, using the cyclic phosphate substrate combination of Figure 1A, can one find a general set of RNA-ligating deoxyribozymes for creation of native 3′-5′ linkages? So far, the Zn^{2+}-dependent deoxyribozymes have come close,[7] but their substrate sequence generality is poor.

Second, using the 5′-triphosphate substrate combination of Figure 1B, can one find a general set of deoxyribozymes for synthesis of linear native 3′—5′ linkages? Considerable unpublished work has shown that achieving this goal requires imposing a selection pressure directly for the desired generality. Furthermore, experiments have revealed how to implement this selection pressure by changing the RNA substrate sequences in successive rounds (D. A. Baum and S.K.S., unpublished results). What now remains is completing these relatively labor-intensive experiments. Then, these DNA catalysts will be available alongside splint ligation for preparation of large site-specifically modified RNAs, as envisioned at the outset of our work with deoxyribozymes.

Finally, although 7S11 and 10DM24 are quite general for branched RNA formation, catalysis is still suboptimal. In particular, these deoxyribozymes do not readily tolerate branch-site nucleotides other than adenosine, and they do not have the same level of catalytic activity with all tested RNA substrate sequences. Therefore, a final set of selection efforts is needed to identify the best possible deoxyribozymes for branched RNA formation. These experiments will also likely involve appropriate selection pressures to ensure substrate generality by the resulting deoxyribozymes.

Ligation with DNA (rather than RNA) Substrates

Follow-up efforts have focused on DNA rather than RNA as one or both of the ligation substrates. When the L substrate is DNA, the nucleotide with the 2′-OH nucleophile is still RNA. When the R substrate is DNA, the activated 5′-terminus is adenylated rather than triphosphorylated.[46] We have obtained deoxyribozymes that synthesize branched RNA—DNA conjugates (L RNA, R DNA)[23] as well as entirely branched DNA molecules (L and R both DNA).[24] As is the case for branched RNA formation, further experiments are needed to obtain the best DNA catalysts.

DNA Catalysis beyond Oligonucleotide Ligation

Our more recent efforts have turned to investigating DNA-catalyzed reactions other than oligonucleotide ligation.

Engineering a Selective Small-Molecule Binding Site. An important challenge for deoxyribozymes is use of small molecules rather than oligonucleotides as substrates. Because 7S11 and 10DM24 have a well-defined 3HJ architecture that spatially juxtaposes the nucleophilic 2'-OH and electrophilic 5'-triphosphate (Figure 5A), we were curious whether such a deoxyribozyme could be rationally engineered to use a mono-nucleotide 5'-triphosphate (NTP) as a small-molecule substrate. To explore this, we provided GTP as a substrate to 10DM24, along with an oligonucleotide that corresponds to the remaining portion of R (R_Δ; Figure 5B).[25] We were pleased to find that 10DM24 indeed retains catalytic activity, albeit with a relatively high concentration of GTP ($K_{d,app} > 1$ mM). We found that GTP can be replaced with ATP or its more strongly hydrogen-bonding analogue 2,6-diaminopurine ribonucleoside triphosphate (DTP), as long as Watson–Crick base pairing is maintained by changing the corresponding 10DM24 nucleotide from C to T. We also found that GTP can be simultaneously a cofactor and a substrate when two nucleotides are omitted from the R oligonucleotide ($R_{\Delta\Delta}$; Figure 5C). Because these efforts were enabled by the oligonucleotide nature of the original full-length R substrate, experiments to use non-NTP small-molecule substrates will need to involve more sophisticated design strategies.

Diels–Alder Deoxyribozymes. Because DNA lacks the 2'-OH group of RNA on every nucleotide, DNA might be less catalytically active than RNA.[47] This concern is valid only to the extent that (i) DNA adopts a meaningfully smaller variety of tertiary structures than RNA, and (ii) DNA's catalytic mechanisms are impeded by the absence of 2'-OH groups. However, many studies with aptamers indicate that DNA has no systematic deficiency relative to RNA in target-binding ability.[48] To allow a direct catalytic comparison between DNA and RNA, we used in vitro selection to identify deoxyribozymes for the Diels–Alder reaction between anthracene and maleimide substrates (Figure 8).[28] The resulting deoxyribozymes were essentially equivalent to the previously found ribozymes[49] in terms of catalytic parameters (e.g., k_{cat}) and rate enhancement, suggesting that DNA and RNA are equally efficient catalysts for this C–C bond-forming reaction. Of course, it is inherently impossible to identify "all" nucleic acid enzymes from these relatively large sequence spaces; N_{40} random pools have $4^{40} \approx 10^{24}$ possible sequences, whereas 10^{14} molecules are used
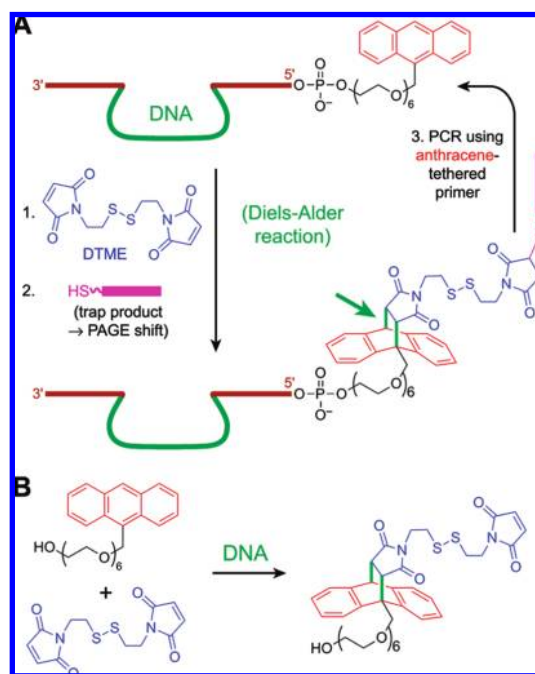

FIGURE 8. Deoxyribozymes that catalyze the Diels–Alder reaction: (A) in vitro selection strategy; (B) intermolecular catalysis of the reaction.[28]

to initiate the selection process. Nevertheless, when Diels–Alder catalysis was sought from DNA and independently from RNA, both kinds of nucleic acids provided sequences with comparable activities. Future efforts should explore a wider range of DNA-catalyzed chemical reactions, especially those that might more strongly benefit from a catalyst than the Diels–Alder reaction.

DNA-Hydrolyzing Deoxyribozymes. We have recently identified several deoxyribozymes that hydrolyze DNA phosphodiester linkages with high sequence selectivities.[50] Such deoxyribozymes are markedly distinct from other deoxyribozymes that oxidatively cleave DNA.[51] While it is too early to know how useful DNA-hydrolyzing deoxyribozymes will be, these catalysts have significant practical promise. Fulfilling this promise will require developing the ability to cleave many DNA sequences, especially in the context of double-stranded substrates such as plasmid DNA.

Seeking Protein Side Chains As Substrates. Finally, we have begun to explore DNA-catalyzed reactions of amino acid side chains.[26] We identified a deoxyribozyme, Tyr1, that creates a nucleopeptide linkage between a tyrosine side chain and the 5'-terminus of an RNA oligonucleotide (Figure 9).[27] Tyr1 adopts the 3HJ architecture, which positions the tyrosine -OH nucleophile close to the 5'-triphosphate electrophile. In a parallel set of selection experiments, neither serine nor lysine at the 3HJ intersection was successful as a nucleophile. However, we have recently shown that expanding the single
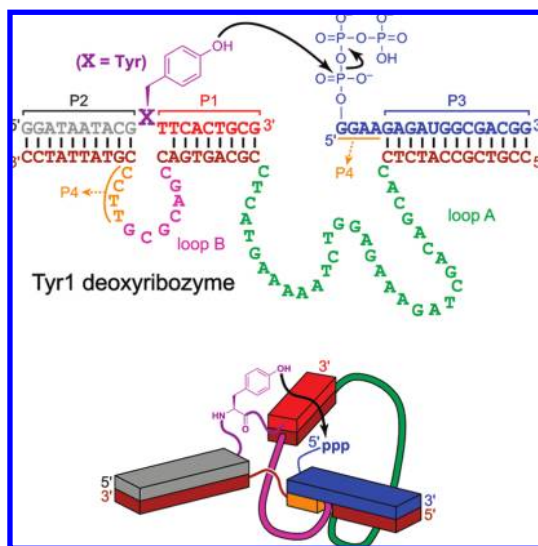
FIGURE 9. DNA-catalyzed nucleopeptide linkage formation between a tyrosine side chain and the 5′-triphosphate of an RNA oligonucleotide. The substrates are held by the Tyr1 deoxyribozyme in a 3HJ architecture (compare Figure 5).

amino acid placed at the 3HJ to a tripeptide does allow nucleopeptide formation involving a serine side chain (A. Sachdeva and S.K.S, unpublished results). We have also found that imposing the 3HJ architecture is not required to identify such deoxyribozymes (O. Wong and S.K.S., unpublished results). Clearly, considerable work remains to develop deoxyribozymes for catalysis of side chain reactivity, especially for the long-term goal of DNA-catalyzed reactions of full-length protein substrates with small-molecule electrophiles.

What's Next for Deoxyribozymes?

Now is an appropriate time to speculate on what the next period of deoxyribozyme research might reveal, both in our laboratory and elsewhere. I anticipate that exploration of DNA catalysis will be particularly interesting and useful in three related research areas:

Area 1: New DNA-Catalyzed Reactions. Deoxyribozymes may be most widely useful if they are not largely restricted to oligonucleotide substrates. Small-molecule substrates should be explored much more thoroughly. Site-selectivity in chemical reactivity of two or more functional groups on a small molecule should be evaluated, and a broad range of chemical reactions should be examined. A second class of interesting substrates is proteins. Our current efforts have examined "test" substrates that have only 1−3 amino acids, seeking to learn the capabilities of deoxyribozymes that function with amino acid side chains along with the basic rules for identification of these catalysts. We now need to bring in substrates that are free full-length proteins. For both small-mole-

cule and protein substrates, exploiting explicit aptamer domains to assist catalysis may be a useful approach.

Area 2: Investigation of Deoxyribozyme Structures and Mechanisms. The in vitro selection process demands catalytic activity but not any particular DNA structure or catalytic mechanism. Our understanding of deoxyribozyme structures and mechanisms lags substantially behind that for ribozymes and protein enzymes. Considering that no high-resolution structural information is available for any active deoxyribozyme,[52] investigating both structures and mechanisms of DNA catalysts should be a fruitful area for future studies.

Area 3: Applications of Deoxyribozymes. Finally, one of the key motivations to pursue new catalysts is to enable applications that require such catalysts. Further development of deoxyribozymes will likely be guided by the need for certain kinds of catalysis. For example, our ongoing work with DNA-catalyzed DNA hydrolysis is promising because developing new catalysts for this reaction is challenging, and DNA as a catalyst interacts quite naturally and sequence-specifically with DNA as a substrate. When considering other substrates such as small molecules and proteins, we hope that deoxyribozymes can be applied for difficult chemical transformations such as selective amino acid side chain modification.

In summary, our investigations of deoxyribozymes since 2001 have generated scientific excitement on several fronts. Our efforts began with DNA catalysis using RNA oligonucleotide substrates. Of course, DNA can interact well with a wide variety of substrates, including small-molecule compounds and proteins. We have adopted the broader view of DNA as an informational biopolymer that, like proteins and RNA, has latent catalytic abilities that can be coaxed with many substrates via appropriate in vitro selection designs and pressures, along with occasional assistance from serendipity. One of the reasons this research field is so exciting is the opportunity to discover new kinds of chemistry. It should be very interesting to see how the scope of DNA-catalyzed chemistry continues to expand in future experiments.

BIOGRAPHICAL INFORMATION

Scott K. Silverman was born in 1972 and raised in Los Angeles, California. He received his B.S. degree in chemistry from UCLA in 1991, working with Christopher Foote on photooxygenation mechanisms. He was an NSF and ACS Organic Chemistry predoctoral fellow with Dennis Dougherty at Caltech, studying high-spin organic polyradicals and molecular neurobiology and graduating with a Ph.D. in chemistry in 1997. After postdoctoral research on RNA biochemistry as a Helen Hay Whitney Foundation and American Cancer Society fellow with Thomas Cech at the University of Colorado at Boulder, he joined the University of Illinois at Urbana−Champaign in 2000, where he is currently Associate Professor of Chemistry, Biochemistry, and Biophysics. His laboratory studies nucleic acid structure, folding, and catalysis, especially the identification and application of deoxyribozymes.

FOOTNOTES

[†]This paper is dedicated to the memory of Christopher Foote, my undergraduate mentor at UCLA and senior editor of this journal from 1995 until his death in 2005.

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REFERENCES

1 Flynn-Charlebois, A.; Wang, Y.; Prior, T. K.; Rashid, I.; Hoadley, K. A.; Coppins, R. L.; Wolf, A. C.; Silverman, S. K. Deoxyribozymes with 2′−5′ RNA Ligase Activity. *J. Am. Chem. Soc.* **2003**, *125*, 2444–2454.

2 Flynn-Charlebois, A.; Prior, T. K.; Hoadley, K. A.; Silverman, S. K. 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.* **2003**, *125*, 5346–5350.

3 Ricca, B. L.; Wolf, A. C.; Silverman, S. K. Optimization and Generality of a Small Deoxyribozyme that Ligates RNA. *J. Mol. Biol.* **2003**, *330*, 1015–1025.

4 Prior, T. K.; Semlow, D. R.; Flynn-Charlebois, A.; Rashid, I.; Silverman, S. K. Structure-Function Correlations Derived from Faster Variants of a RNA Ligase Deoxyribozyme. *Nucleic Acids Res.* **2004**, *32*, 1075–1082.

5 Semlow, D. R.; Silverman, S. K. 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.* **2005**, *61*, 207–215.

6 Hoadley, K. A.; Purtha, W. E.; Wolf, A. C.; Flynn-Charlebois, A.; Silverman, S. K. Zn^{2+}-Dependent Deoxyribozymes That Form Natural and Unnatural RNA Linkages. *Biochemistry* **2005**, *44*, 9217–9231.

7 Kost, D. M.; Gerdt, J. P.; Pradeepkumar, P. I.; Silverman, S. K. Controlling Regioselectivity and Site-Selectivity in RNA Ligation by Zn^{2+}-Dependent Deoxyribozymes That Use 2′,3′-Cyclic Phosphate RNA Substrates. *Org. Biomol. Chem.* **2008**, *6*, 4391–4398.

8 Wang, Y.; Silverman, S. K. Deoxyribozymes That Synthesize Branched and Lariat RNA. *J. Am. Chem. Soc.* **2003**, *125*, 6880–6881.

9 Wang, Y.; Silverman, S. K. Characterization of Deoxyribozymes That Synthesize Branched RNA. *Biochemistry* **2003**, *42*, 15252–15263.

10 Coppins, R. L.; Silverman, S. K. A DNA Enzyme That Mimics the First Step of RNA Splicing. *Nat. Struct. Mol. Biol.* **2004**, *11*, 270–274.

11 Wang, Y.; Silverman, S. K. Directing the Outcome of Deoxyribozyme Selections To Favor Native 3′−5′ RNA Ligation. *Biochemistry* **2005**, *44*, 3017–3023.

12 Purtha, W. E.; Coppins, R. L.; Smalley, M. K.; Silverman, S. K. General Deoxyribozyme-Catalyzed Synthesis of Native 3′−5′ RNA Linkages. *J. Am. Chem. Soc.* **2005**, *127*, 13124–13125.

13 Coppins, R. L.; Silverman, S. K. Rational Modification of a Selection Strategy Leads to Deoxyribozymes That Create Native 3′−5′ RNA Linkages. *J. Am. Chem. Soc.* **2004**, *126*, 16426–16432.

14 Coppins, R. L.; Silverman, S. K. A Deoxyribozyme That Forms a Three-Helix-Junction Complex with Its RNA Substrates and Has General RNA Branch-Forming Activity. *J. Am. Chem. Soc.* **2005**, *127*, 2900–2907.

15 Coppins, R. L.; Silverman, S. K. 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* **2005**, *44*, 13439–13446.

16 Pratico, E. D.; Wang, Y.; Silverman, S. K. A Deoxyribozyme That Synthesizes 2′,5′-Branched RNA with Any Branch-Site Nucleotide. *Nucleic Acids Res.* **2005**, *33*, 3503–3512.

17 Wang, Y.; Silverman, S. K. Efficient One-Step Synthesis of Biologically Related Lariat RNAs by a Deoxyribozyme. *Angew. Chem., Int. Ed.* **2005**, *44*, 5863–5866.

18 Wang, Y.; Silverman, S. K. A General Two-Step Strategy to Synthesize Lariat RNAs. *RNA* **2006**, *12*, 313–321.

19 Zelin, E.; Wang, Y.; Silverman, S. K. Adenosine Is Inherently Favored as the Branch-Site RNA Nucleotide in a Structural Context That Resembles Natural RNA Splicing. *Biochemistry* **2006**, *45*, 2767–2771.

20 Wang, Y.; Silverman, S. K. Experimental Tests of Two Proofreading Mechanisms for 5′-Splice Site Selection. *ACS Chem. Biol.* **2006**, *1*, 316–324.

21 Baum, D. A.; Silverman, S. K. Deoxyribozyme-Catalyzed Labeling of RNA. *Angew. Chem., Int. Ed.* **2007**, *46*, 3502–3504.

22 Pratico, E. D.; Silverman, S. K. Ty1 Reverse Transcriptase Does Not Read through the Proposed 2′,5′-Branched Retrotransposition Intermediate in Vitro. *RNA* **2007**, *13*, 1528–1536.

23 Zelin, E.; Silverman, S. K. Allosteric Control of Ribozyme Catalysis Using DNA Constraints. *ChemBioChem* **2007**, *8*, 1907–1911.

24 Mui, T. P.; Silverman, S. K. Convergent and General One-Step DNA-Catalyzed Synthesis of Multiply Branched DNA. *Org. Lett.* **2008**, *10*, 4417–4420.

25 Höbartner, C.; Silverman, S. K. Engineering a Selective Small-Molecule Substrate Binding Site into a Deoxyribozyme. *Angew. Chem., Int. Ed.* **2007**, *46*, 7420–7424.

26 Höbartner, C.; Pradeepkumar, P. I.; Silverman, S. K. Site-Selective Depurination by a Periodate-Dependent Deoxyribozyme. *Chem. Commun.* **2007**, 2255–2257.

27 Pradeepkumar, P. I.; Höbartner, C.; Baum, D. A.; Silverman, S. K. DNA-Catalyzed Formation of Nucleopeptide Linkages. *Angew. Chem., Int. Ed.* **2008**, *47*, 1753–1757.

28 Chandra, M.; Silverman, S. K. DNA and RNA Can Be Equally Efficient Catalysts for Carbon−Carbon Bond Formation. *J. Am. Chem. Soc.* **2008**, *130*, 2936–2937.

29 Emilsson, G. M.; Nakamura, S.; Roth, A.; Breaker, R. R. Ribozyme Speed Limits. *RNA* **2003**, *9*, 907–918.

30 Moore, M. J.; Sharp, P. A. Site-Specific Modification of Pre-mRNA: The 2′-Hydroxyl Groups at the Splice Site. *Science* **1992**, *256*, 992–997.

31 Moore, M. J.; Query, C. C. Joining of RNAs by Splinted Ligation. *Methods Enzymol.* **2000**, *317*, 109–123.

32 Breaker, R. R.; Joyce, G. F. A DNA Enzyme That Cleaves RNA. *Chem. Biol.* **1994**, *1*, 223–229.

33 Silverman, S. K. In Vitro Selection, Characterization, And Application of Deoxyribozymes That Cleave RNA. *Nucleic Acids Res.* **2005**, *33*, 6151–6163.

34 Achenbach, J. C.; Chiuman, W.; Cruz, R. P.; Li, Y. DNAzymes: From Creation in Vitro to Application in Vivo. *Curr. Pharm. Biotechnol.* **2004**, *5*, 321–336.

35 Peracchi, A. DNA Catalysis: Potential, Limitations, Open Questions. *ChemBioChem* **2005**, *6*, 1316–1322.

36 Silverman, S. K. Catalytic DNA (Deoxyribozymes) for Synthetic Applications—Current Abilities and Future Prospects. *Chem. Commun.* **2008**, 3467–3485.

37 Schlosser, K.; Li, Y. Biologically Inspired Synthetic Enzymes Made from DNA. *Chem. Biol.* **2009**, *16*, 311–322.

38 Cuenoud, B.; Szostak, J. W. A DNA Metalloenzyme with DNA Ligase Activity. *Nature* **1995**, *375*, 611–614.

39 Li, Y.; Sen, D. A Catalytic DNA for Porphyrin Metallation. *Nat. Struct. Biol*. **1996**, *3*, 743–747.

40 Sheppard, T. L.; Ordoukhanian, P.; Joyce, G. F. A DNA Enzyme with *N*-Glycosylase Activity. *Proc. Natl. Acad. Sci. U.S.A*. **2000**, *97*, 7802–7807.

41 Chinnapen, D. J.; Sen, D. A Deoxyribozyme That Harnesses Light to Repair Thymine Dimers in DNA. *Proc. Natl. Acad. Sci. U.S.A*. **2004**, *101*, 65–69.

42 Thorne, R. E.; Chinnapen, D. J.; Sekhon, G. S.; Sen, D. A Deoxyribozyme, Sero1C, Uses Light and Serotonin To Repair Diverse Pyrimidine Dimers in DNA. *J. Mol. Biol*. **2009**, *388*, 21–29.

43 Soukup, G. A.; Breaker, R. R. Relationship between Internucleotide Linkage Geometry and the Stability of RNA. *RNA* **1999**, *5*, 1308–1325.

44 Santoro, S. W.; Joyce, G. F. A General Purpose RNA-Cleaving DNA Enzyme. *Proc. Natl. Acad. Sci. U.S.A*. **1997**, *94*, 4262–4266.

45 Breaker, R. R.; Emilsson, G. M.; Lazarev, D.; Nakamura, S.; Puskarz, I. J.; Roth, A.; Sudarsan, N. A Common Speed Limit for RNA-Cleaving Ribozymes and Deoxyribozymes. *RNA* **2003**, *9*, 949–957.

46 Patel, M. P.; Baum, D. A.; Silverman, S. K. Improvement of DNA Adenylation Using T4 DNA Ligase with a Template Strand and a Strategically Mismatched Acceptor Strand. *Bioorg. Chem*. **2008**, *36*, 46–56.

47 Cech, T. R. The Chemistry of Self-Splicing RNA and RNA Enzymes. *Science* **1987**, *236*, 1532–1539.

48 Silverman, S. K. Artificial Functional Nucleic Acids: Aptamers, Ribozymes, and Deoxyribozymes Identified by In Vitro Selection. In *Functional Nucleic Acids for Analytical Applications*; Li, Y., Lu, Y., Eds.; Springer Science + Business Media, LLC: New York, 2009; pp 47−108.

49 Seelig, B.; Jäschke, A. A Small Catalytic RNA Motif with Diels-Alderase Activity. *Chem. Biol*. **1999**, *6*, 167–176.

50 Chandra, M.; Sachdeva, A.; Silverman, S. K. DNA-Catalyzed Sequence-Specific Hydrolysis of DNA. *Nat. Chem. Biol*. **2009**, *5*, in press.

51 Carmi, N.; Breaker, R. R. Characterization of a DNA-Cleaving Deoxyribozyme. *Bioorg. Med. Chem*. **2001**, *9*, 2589–2600.

52 Nowakowski, J.; Shim, P. J.; Prasad, G. S.; Stout, C. D.; Joyce, G. F. Crystal Structure of an 82-Nucleotide RNA-DNA Complex Formed by the 10−23 DNA Enzyme. *Nat. Struct. Biol*. **1999**, *6*, 151–156.