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In Vitro Evolution of an RNA-Cleaving DNA Enzyme into an RNA Ligase Switches the Selectivity from 3′–5′ to 2′–5′

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Abstract: Deoxyribozymes that ligate RNA expand the scope of nucleic acid catalysis and allow preparation of site-specifically modified RNAs. Previously, deoxyribozymes that join a 5′-hydroxyl and a 2′,3′-cyclic phosphate were identified by in vitro selection from random DNA pools. Here, the alternative strategy of in vitro evolution was used to transform the 8–17 deoxyribozyme that cleaves RNA into a family of DNA enzymes that ligate RNA. The parent 8–17 DNA enzyme cleaves native 3′–5′ phosphodiester linkages but not 2′–5′ bonds. Surprisingly, the new deoxyribozymes evolved from 8–17 create only 2′–5′ linkages. Thus, reversing the direction of the DNA-mediated process from ligation to cleavage also switches the selectivity in forming the new phosphodiester bond. The same change in selectivity was observed upon evolution of the 10–23 RNA-cleaving deoxyribozyme into an RNA ligase. The DNA enzymes previously isolated from random pools also create 2′–5′ linkages. Therefore, deoxyribozyme-mediated formation of a non-native 2′–5′ phosphodiester linkage from a 5′-hydroxyl and a 2′,3′-cyclic phosphate is strongly favored in many different contexts.

Many laboratories are interested in structural and functional studies of site-specifically modified RNAs.[1] Solid-phase synthesis is the most reliable means to incorporate functional group modifications and biophysical probes internally into RNA.[2] Because such approaches typically cannot synthesize long RNAs (>100-mers), combinations of oligonucleotides and transcripts must be ligated to provide larger modified RNAs. New methodology for RNA ligation is therefore crucial to enable detailed study of catalytically active intron ribozymes and other large RNAs, which comprise several hundred nucleotides.[3] There are few reagents currently available to perform RNA ligations. Typically, the protein enzymes T4 DNA ligase or T4 RNA ligase are used,[4–6] but problems with generality and yield are common, and alternative approaches are needed.

To address this issue, we have initiated efforts to identify deoxyribozymes (DNA enzymes)[7] with RNA ligase activity. Our initial experiments focused on in vitro selection of RNA ligase deoxyribozymes from random DNA pools.[8] The desired chemical reaction (Scheme 1) is that of a 5′-hydroxyl with a 2′,3′-cyclic phosphate (**1**), which can provide either the non-native 2′–5′ phosphodiester linkage (**2**) or the native 3′–5′ phosphodiester bond (**3**). For mechanistic reasons that are not yet known, the deoxyribozymes isolated from random DNA pools all create the non-native 2′–5′ linkage **2** with >95% selectivity over formation of **3**.[8] Although a non-native linkage **2** is often acceptable for RNA structure/function studies and may even be useful in some contexts, there remains the desire to identify

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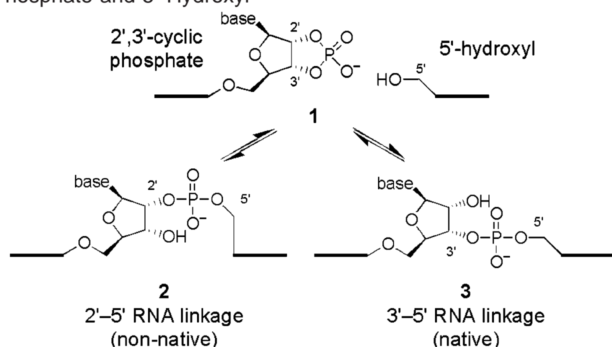
Scheme 1. Possible RNA Ligation Junctions from a 2′,3′-Cyclic Phosphate and 5′-Hydroxyl



deoxyribozymes that mediate formation of the native 3′−5′ linkage **3**.

Santoro and Joyce previously used in vitro selection to discover Mg^{2+}-dependent deoxyribozymes that *cleave* RNA (Figure 1A).[9,10] Their 8−17 and 10−23 deoxyribozymes have been developed into general and reliable tools for cleaving RNA in vitro and in vivo.[11] (The names of these two DNA enzymes were derived from their identification as the 17th and 23rd clones during the 8th and 10th respective rounds of selection.) The 8−17 and 10−23 deoxyribozymes recognize RNA with Watson−Crick substrate binding arms (Figure 1A) and efficiently cleave the RNA to provide 2′,3′-cyclic phosphate and 5′-hydroxyl termini of the resulting fragments. This occurs if and only if the RNA cleavage substrate is linked 3′−5′ (but not 2′−5′) at the scissile bond.[8−10] That is, the 8−17 and 10−23 deoxyribozymes selectively perform the reaction **3** → **1** but not **2** → **1**.

The desired ligation reaction **1** → **3** is the reverse of the cleavage reaction **3** → **1** performed by the 8−17 and 10−23 deoxyribozymes, and we thought to capitalize on this preexisting 3′−5′ selectivity. However, these deoxyribozymes cannot be made to perform RNA ligation merely by changing environmental variables such as salt concentration, pH, or temperature (S.K.S., unpublished results and Y. Lu, UIUC, personal communication). Therefore, we adopted an evolutionary approach. Using the 8−17 and 10−23 RNA-cleaving DNA sequences as starting points, we report here the evolution of deoxyribozymes with RNA ligase activity. This overall approach is termed "evolution" rather than selection because the starting sequences were themselves the product of selection. Despite the strong 3′−5′ selectivity of the parent DNA enzymes, the deoxyribozymes that emerged from this evolutionary process ligate RNA with highly selective formation of 2′−5′ phosphodiester linkages (**1** → **2**).

Results

In Vitro Evolution of RNA Ligase Deoxyribozymes. Using solid-phase DNA synthesis, we randomized the 13-mer enzyme region of the 8−17 deoxyribozyme (Figure 1A) to the average extent of 2−3 nucleotide changes per sequence ("low" randomization) or, separately, 6−7 changes per sequence ("high" randomization). In both cases, seven rounds of selection for Mg^{2+}-dependent RNA ligation activity were then performed,

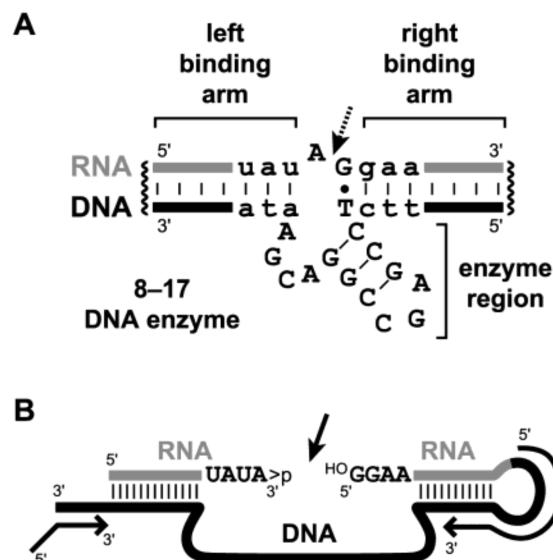


Figure 1. (A) Structure of the 8−17 RNA-cleaving DNA enzyme, bound to the two specific RNA substrates used here to evolve RNA ligase activity. Only part of each 17-mer RNA substrate is shown. The arrow indicates the cleavage site. Lowercase nucleotides may be changed as long as base pairing is retained. (B) The key step during each round of selection for deoxyribozymes that ligate RNA. See Experimental Section for a brief description and ref 8 for complete details. The bold arrowhead indicates the ligation site between a 2′,3′-cyclic phosphate (on the left-hand RNA substrate) and a 5′-hydroxyl (on the right-hand RNA substrate). The thin strands with arrowheads denote DNA primers for PCR amplification of the deoxyribozyme pool following the key selection step that joins the RNA substrates.

using 10 mM Mg^{2+} and a selection strategy described in detail elsewhere[8] and briefly here in the Experimental Section. The key step of each selection round is shown in Figure 1B. After round 7, both the "low" and the "high" randomization selections starting from the 8−17 sequence provided substantial ligation activity, with ~15% of the pool ligated (Figure 2).[12] Analogous selections starting from the RNA-cleaving 10−23 deoxyribozyme sequence[9,10] provided only a low level (<2%) of ligation activity, even after 10 selection rounds. For comparison, in the original selection of 8−17 and 10−23,[9] the cleavage activity was ~3−15% in round 10, and the subsequently identified DNA enzymes have high cleavage yields (>90%).

Cloning and Characterization of Individual Deoxyribozymes. Individual RNA ligase deoxyribozymes from the round 7 pools of the 8−17 selections were cloned and sequenced. Their RNA ligation properties were characterized at 37 °C and 40 mM Mg^{2+} (Figure 3). Of 10 examined clones from the "low" randomization pool, six with similar sequence and ligation rates are represented by clone 7P4 in Figure 3A. The remaining four clones were slower and were not pursued

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(12) The "activity" of a pool during a selection round differs from the chemical "yield" of an individual deoxyribozyme identified during the selection process. The activity refers to the fraction of deoxyribozyme molecules in the pool that successfully ligated the RNA substrates during the allotted incubation time (here, 12 h). Of course, each individual deoxyribozyme molecule has either 0% or 100% ligation yield during a selection round. In contrast, the yield of a deoxyribozyme may vary continuously between 0−100% and indicates the fraction of RNA substrates ligated by a given deoxyribozyme when many copies of the DNA are presented with the RNA substrates. The overall observed activity of the pool can be attained in many ways. For example, 10% activity could arise from 10% of the "winning" deoxyribozymes having 100% yield and the remaining having ~0% yield (yet still managing to proceed through the selection round under consideration). At the other extreme, 10% activity could be derived from every successful deoxyribozyme having ~10% yield. These possibilities cannot readily be distinguished without cloning and testing individual deoxyribozymes from the pool.

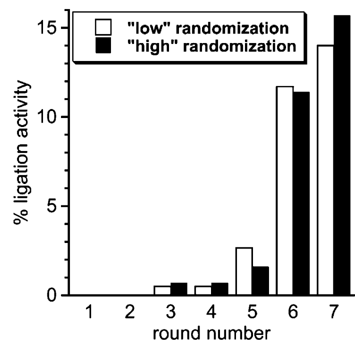

Figure 2. Selection progression for RNA ligase activity, starting with the partially randomized 8−17 RNA-cleaving DNA enzyme. Analogous selections starting with the 10−23 sequence provided <2% actvity by round 10. See ref 12 for a brief discussion of the distinction between activity and yield.

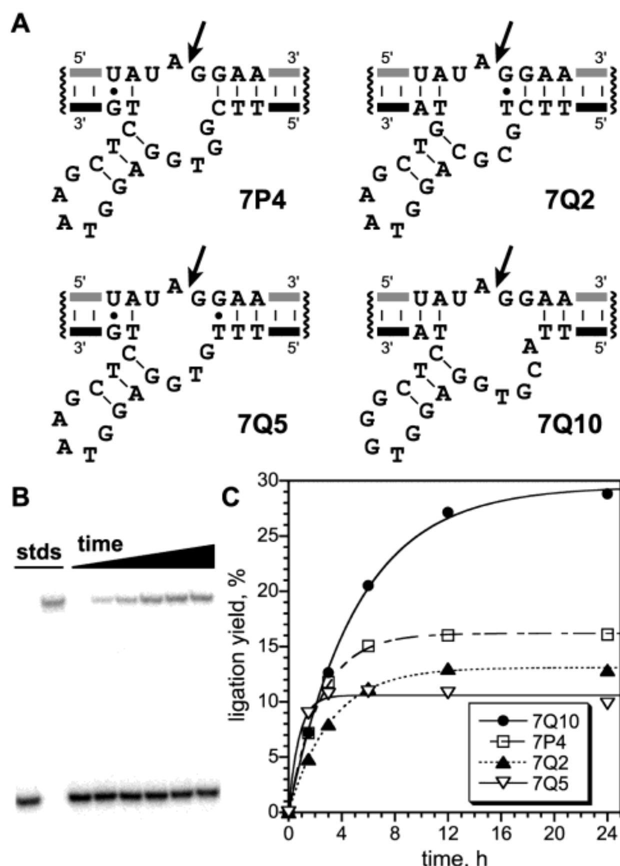

Figure 3. New deoxyribozymes that ligate RNA, evolved from the 8−17 RNA-cleaving DNA enzyme. (A) Sequences and predicted secondary structures of four new deoxyribozymes. 7Q5 is one nucleotide shorter than the other three DNA enzymes, presumably due to a deletion attributed to *Taq* polymerase during selection. The secondary structure predictions[15] were made using mfold, available online at www.bioinfo.rpi.edu/~zukerm. (B) Representative polyacrylamide gel image for a single-turnover ligation experiment using the 7Q10 deoxyribozyme (conditions: 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$, 37 °C). The left-hand RNA strand is 5′-^{32}P-radiolabeled and is the limiting reagent. (C) Kinetic data at 40 mM Mg^{2+}. From first-order exponential fits, k_{obs}, h^{-1}: 7Q10, 0.19; 7P4, 0.42; 7Q2, 0.32; 7Q5, 1.1. The value for 7Q5 was derived from an experiment in which additional early timepoints were collected.

further. Of five examined clones from the "high" randomization pool, a range of activities was observed, of which 7Q2, 7Q5, and 7Q10 are representative. The parent 8−17 deoxyribozyme itself cleaves RNA with $k_{obs} \approx 6\ h^{-1}$ (0.1 min^{-1}) under the pH 7.5 assay conditions and has little if any RNA ligase activity.

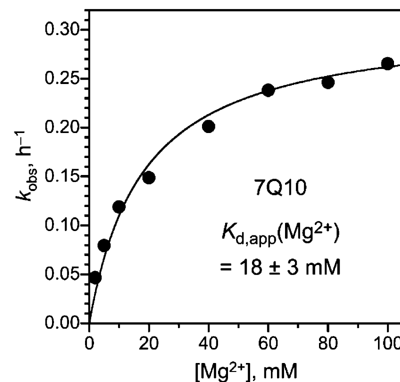

Figure 4. Determination of $K_{d,app}(Mg^{2+})$ for the 7Q10 deoxyribozyme. Assays were performed with $[Mg^{2+}]$ from 2 to 100 mM as in Figure 3. A data point at 200 mM Mg^{2+} is not shown but falls very close to the fit line (see Supporting Information).

In contrast, the newly selected deoxyribozymes have significant RNA ligase activity (Figure 3B), albeit with modest ligation yields (up to 30%). The single-turnover ligation rates are shown in Figure 3C; the k_{obs} values range from 0.2 to 1 h^{-1}. The new DNA enzymes require Mg^{2+} (Na^{+} and K^{+} are insufficient); the $K_{d,app}(Mg^{2+})$is 18 ± 3 mM for 7Q10 (Figure 4). Other divalent metal ions were tested with 7Q10 and 7P4. For both deoxyribozymes, Ca^{2+} and Mn^{2+} supported significant ligation activity, while Sr^{2+}, Zn^{2+}, Cu^{2+}, and the outer-sphere hexahydrated Mg^{2+} analogue $Co(NH_3)_6^{3+}$ did not (see Supporting Information). Other metals have not yet been tested.

In preliminary results, a substantial increase in ligation rates was observed at higher pH of 9.0. For example, $k_{obs} = 2.4\ h^{-1}$ for 7Q10, with no reduction in yield as compared to pH 7.5 (data not shown). This 13-fold increase in k_{obs} at pH 9.0 relative to that found at pH 7.5 brings the time scale of the ligation reaction into the 1 h range or better, as observed for our other RNA ligase deoxyribozymes.[8] In separate experiments, we examined ligation reactions at 10 °C and pH 7.5. No improvements in yield were observed; rather, both the rates and the yields dropped significantly (see Supporting Information). These experiments were prompted by the observation that some RNA enzymes that cleave and ligate nucleic acids have improved ligase activity relative to cleavage at temperatures lower than 37 °C.[13]

Selectivity of Phosphodiester Bond Formation. The 8−17 and 10−23 deoxyribozymes cleave 3′−5′ RNA phosphodiester bonds but not 2′−5′ linkages.[8−10] In the selections reported here for deoxyribozymes that ligate RNA, either a 2′−5′ linkage (**1** → **2**) or a 3′−5′ linkage (**1** → **3**) could be formed (Scheme 1). We assayed the ligated RNA products from the four representative new deoxyribozymes of Figure 3 to determine their selectivity for phosphodiester formation. Two complementary procedures were used (Figure 5).[8] First, the parent 8−17 deoxyribozyme itself was used as a test reagent to reveal 3′−5′ linkages in the ligated products. Second, Mg^{2+}-dependent hydrolysis at pH 9.0 preferentially cleaves a 2′−5′ linkage when the RNA is bound to its full DNA complement.[14] Surprisingly, although our new deoxyribozymes were evolved from the 8−17

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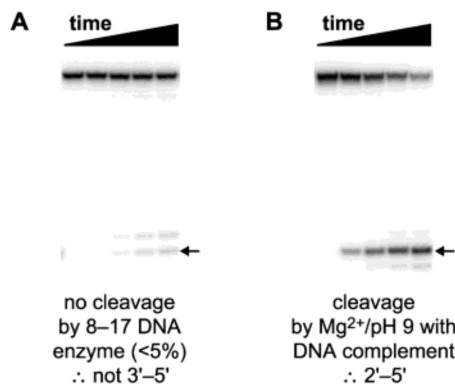

Figure 5. Characterizing the new phosphodiester linkages as 3′−5′ or 2′−5′. (A) The 8−17 DNA enzyme fails to cleave the new deoxyribozymes' ligation products, demonstrating that the products are *not* linked 3′−5′. Timepoints from 0 to 7 h; a control 3′−5′ linked RNA is cleaved fully within minutes. (B) Hydrolysis at 100 mM Mg^{2+} and pH 9 in the presence of the DNA complement cleaves the products, revealing a 2′−5′ linkage. Timepoints from 0 to 24 h. In both panels A and B, data for the 7Q10 ligation product are shown. The RNA product was 5′-^{32}P-radiolabeled, such that only the left-hand fragment is visible after cleavage. A standard for the cleaved RNA migrates at the position marked with an arrowhead. For similar assays with the other deoxyribozymes and for control experiments that validate the approach, see Supporting Information and also ref 8. Note that the cleavage reaction of panel B is technically not a "hydrolysis" but in fact a transesterification at a phosphodiester by a 2′-hydroxyl. However, this and related reactions are universally—if incorrectly—termed "hydrolysis" (e.g., ref 14 and the common method of "partial alkaline hydrolysis" to generate an RNA ladder).

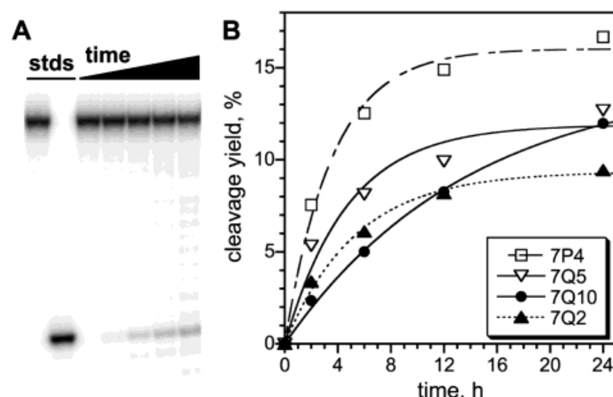

Figure 6. The new deoxyribozymes cleave 2′−5′ RNA linkages, although more slowly than they ligate RNA, and they do not cleave 3′−5′-linked RNA. (A) Representative gel image for a single-turnover cleavage experiment using the 7Q10 deoxyribozyme (conditions: 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$, 37 °C). The substrate has a single 2′−5′ RNA linkage at the scissile bond. (B) Kinetic data at 40 mM Mg^{2+}. k_{obs}, h^{-1}: 7P4, 0.28; 7Q5, 0.21; 7Q10, 0.07; 7Q2, 0.19. The data are plotted with the same symbols as in Figure 3C. From the data in both figures, the ratios k_{obs}(cleavage)/k_{obs}(ligation) are as follows: 7P4, 1.5; 7Q5, 5.2; 7Q10, 2.7; 7Q2, 1.7. RNA that is linked 3′−5′ rather than 2′−5′ was not detectably cleaved by any of the deoxyribozymes (<5%; see Supporting Information).

sequence that has very strong (>95%) 3′−5′ cleavage selectivity, all of the new deoxyribozymes ligate RNA to form solely 2′−5′ junctions. That is, using evolution to reverse the direction of the DNA-mediated reaction from cleavage to ligation has also switched the selectivity from 3′−5′ to 2′−5′. The RNA products from the uncloned round 7 "low" and "high" pools showed the same result (see Supporting Information), indicating that the individual deoxyribozymes of Figure 3 are representative of the selection products in general. The uncloned low-activity round 9 "low" and "high" randomization pools from the 10−23 selection also gave only 2′−5′ linked products, demonstrating that the observed selectivity switch upon evolution into an RNA ligase is not limited to the 8−17 deoxyribozyme.

Testing the Deoxyribozymes for RNA Cleavage Ability. Because the reaction of a 2′,3′-cyclic phosphate with a 5′-hydroxyl is reversible (Scheme 1), we considered the possibility that the rate of the cleavage reaction **2** → **1** may also be enhanced by the new deoxyribozymes. If so, this would contribute to their relatively low overall ligation yields. Indeed, as shown in Figure 6, all four of the new deoxyribozymes were able to cleave a 2′−5′ linkage (**2** → **1**), but they could not cleave a 3′−5′ linkage (**3** → **1**). The ratio of k_{obs}(ligation) to k_{obs}(cleavage) for 2′−5′ linkages ranges from 1.5 for 7P4 to 5.2 for 7Q5. The highest-yielding ligase from Figure 3, 7Q10, has a k_{obs}(ligation)/k_{obs}(cleavage) of 2.7.

Discussion

We recently described the selection of RNA ligase deoxyribozymes from random (N_{40}) DNA pools.[8] These deoxyribozymes join a 5′-hydroxyl with a 2′,3′-cyclic phosphate and provide 2′−5′ phosphodiester linkages with high selectivity (**1** → **2** with <5% of **3**). In those selection experiments, there was no basis for discrimination between formation of 2′−5′ and 3′−5′ linkages, either actively during the selection procedure or passively in the choice of the starting DNA pool. Native 3′−5′ linkages **3** are clearly a desirable preparative target, and it was thought that evolution of RNA ligase deoxyribozymes directly from DNA enzymes that selectively cleave 3′−5′-linked RNA would be more likely to provide 3′−5′ ligation selectivity. Surprisingly, the results reported here show that this expectation is unfulfilled for the 8−17 and 10−23 deoxyribozymes. Evolution of either the 8−17 or 10−23 deoxyribozyme into an RNA ligase provides deoxyribozymes that create 2′−5′ phosphodiester linkages with >95% selectivity over 3′−5′ linkages.

Sequences and Secondary Structures of the New RNA Ligase Deoxyribozymes. Although we designed the "low" randomization pool to have DNA sequences with only 2−3 nucleotide changes on average relative to the parent 8−17 sequence of Figure 1A, the cloned deoxyribozymes were found to have many more differences (Figure 3A). This is possible because there were in fact many DNA molecules in the original pool with >3 changes relative to the parent sequence, as detailed in the Supporting Information. Apparently those molecules with only a small number of sequence changes ligate RNA relatively poorly and thus were out-competed during the selection procedure. Folding algorithms[15] (available online at www.bioinfo.rpi.edu/~zukerm) predict that the new RNA ligase deoxyribozymes have a three-base stem plus a five-base pentaloop, which roughly resembles the stem-triloop secondary structure of 8−17 (compare Figure 3A with Figure 1A). Among other differences, the putative stem is clearly positioned differently relative to the RNA substrates in the two types of deoxyribozyme. Nevertheless, the overall similarity is apparent. It is probably not a coincidence that this general 8−17-like motif has arisen in several independent selections for RNA cleavage activity[9,16] and now in a selection for RNA ligation activity. This repeated identification suggests that the general motif is particularly adept either structurally, functionally, or both for

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RNA cleavage/ligation reactions involving 2′,3′-cyclic phosphates and 5′-hydroxyls.

Because of the numerous base changes and overall apparent secondary structure differences, a simple relationship is difficult to discern between the parent 8−17 DNA enzyme and the new deoxyribozymes. As one consequence, our initial plans to study DNA sequences that are intermediate between 8−17 and the new deoxyribozymes are impractical. Nevertheless, some inferences may be drawn about the ligation mechanism of the new deoxyribozymes. Significantly, their ligation activity cannot reasonably be explained by a simple proximity effect[17] due to spatial juxtaposition of the 2′,3′-cyclic phosphate and 5′-hydroxyl. Control experiments in which the two RNA substrates were held together with an exactly complementary DNA oligonucleotide splint (lacking any enzyme region whatsoever) led to only minimal background ligation, with rate $k_{obs} = 0.0024$ h^{-1} at pH 7.5.[8] Use of an exactly complementary splint may significantly overestimate the proximity effect, because looped-out DNA (which permits structural flexibility) is absent; see ref 8 for a complete discussion of this issue. Using the data from the exactly complementary splint and therefore making a conservative estimate for the rate of the background reaction, we found that the rate enhancement due to the highest-yielding 7Q10 deoxyribozyme is at least 0.19/0.0024 = 79-fold. The rate enhancement is even higher for the other deoxyribozymes (e.g., 450-fold for 7Q5), although they have lower ligation yields.

Origin and Implications of 2′−5′ Selectivity. It is striking and unexpected that the new deoxyribozymes can both ligate and cleave RNA with 2′−5′ selectivity (Figures 3, 5, and 6). Native 3′−5′ linkages are neither formed nor cleaved within our detection limits (<5%). Therefore, the new deoxyribozymes must mediate access to a different transition state than does the highly 3′−5′-selective 8−17 DNA enzyme from which they were evolved. Each new deoxyribozyme apparently remains bound to the products of ligation after reaction, because multiple turnover was not observed (data not shown). Similarly, turnover was not observed for the RNA ligase deoxyribozymes identified from random pools.[8] On this basis, all of these reactions should perhaps be properly termed DNA-mediated RNA ligation rather than catalysis. However, we note that the protein *enzyme* T4 DNA ligase is very poor at turnover as well, and it is used stoichiometrically for preparative RNA ligation.[4]

The new deoxyribozymes mediate both RNA ligation (Figure 3) and cleavage (Figure 6) with appreciable rates. This suggests a mechanism in which each DNA enzyme facilitates selective equilibration between the cleaved (**1**) and 2′−5′ ligated (**2**) RNA structures of Scheme 1 but cannot mediate interconversion of **1** and **3**. The single-turnover ligation rates (k_{obs}) are up to 5-fold higher than the corresponding cleavage rates, so it is reasonable to consider the new deoxyribozymes as RNA ligases. Because this ratio of k_{obs} values (ligation/cleavage) is always >1, the <50% ligation yields cannot be explained solely by equilibration between cleaved and ligated RNA substrates. A detailed mechanistic understanding of the new Mg^{2+}-dependent deoxyribozymes, including how they enforce selectivity in forming and breaking 2′−5′ phosphodiester linkages, requires further investigation.

Potential Utility of the New Deoxyribozymes. Straightforward selection efforts aimed at identifying RNA ligase deoxyribozymes that create a 3′−5′ phosphodiester linkage from a 2′,3′-cyclic phosphate appear thwarted, starting either from random DNA pools (ref 8) or from RNA-cleaving DNA enzyme sequences (this report). More complicated strategies can be envisioned, as can approaches that use alternative combinations of functional groups at the ligation junction. Efforts along these lines are currently in progress. Developing a mechanistic understanding of the observed selectivity for 2′−5′ phosphodiester formation will provide insight into the capabilities and requirements of nucleic acid catalysis. Despite the issues surrounding phosphodiester connectivity in the ligated products, the new deoxyribozymes will—after further characterization—likely prove useful for preparative RNA ligation, along with protein-mediated splint ligation.[4,5] The relatively modest ligation yields can likely be improved for these and other deoxyribozymes by continued selection efforts, and the non-native 2′−5′ linkages should be acceptable and even valuable in many contexts.[8] Ongoing development and optimization of the new DNA enzymes as RNA ligase reagents will require further experiments to establish the precise sequence requirements for the deoxyribozymes and for their RNA substrates. These studies are now underway.

Experimental Section

In Vitro Selection Procedure and Characterization of Deoxyribozymes. The general selection strategy has been described previously in great detail.[8] In the key selection step, two RNA substrates were ligated by a deoxyribozyme strand that holds the two RNA substrates together using Watson−Crick binding arms (Figure 1B). The incubation conditions during this step were 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, and 10 mM $MgCl_2$ at 37 °C for 12 h. Following the selection step, the successful deoxyribozymes were separated by PAGE (they were larger due to the attachment of the left-hand RNA substrate) and amplified by PCR, using the two DNA primers indicated in Figure 1B. One of the primers (left) has a nonamplifiable "tail" to permit PAGE isolation of the desired single DNA strand. Finally, the right-hand RNA substrate was attached using T4 RNA ligase, which provided the input material for the next key selection step. The same RNA substrates and DNA primers were used here as reported previously,[8] except that a partially randomized and shorter deoxyribozyme region was used in place of the N_{40} random pool for both the 8−17 and the 10−23 selections. For details on the design of the randomized DNA regions, see Supporting Information. Individual deoxyribozymes were cloned from the 8−17 round 7 selection pools as reported.[8]

Characterization of RNA Ligation Activity of the Deoxyribozymes. The ligation, cleavage, and 3′−5′/2′−5′ assays for individual deoxyribozymes were performed as reported.[8] The incubation conditions for the ligation and cleavage assays were the same as those listed above except with 40 mM $MgCl_2$ (note the $K_{d,app}$ for Mg^{2+} of ~20 mM found for 7Q10 in Figure 4).

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Supporting Information Available: Details of experiments not fully described in the text (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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