J|A|C|S
ARTICLES
Published on Web 02/08/2003

Deoxyribozymes with 2′−5′ RNA Ligase Activity

Amber Flynn-Charlebois,† Yangming Wang,‡ Tracey K. Prior, Imran Rashid, Kelly A. Hoadley, Rebecca L. Coppins, Amanda C. Wolf, and Scott K. Silverman*

Contribution from the Department of Chemistry, University of Illinois at Urbana−Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

Received October 2, 2002; E-mail: scott@scs.uiuc.edu

Abstract: In vitro selection was used to identify deoxyribozymes that ligate two RNA substrates. In the ligation reaction, a 2′−5′ RNA phosphodiester linkage is created from a 2′,3′-cyclic phosphate and a 5′-hydroxyl group. The new Mg^{2+}-dependent deoxyribozymes provide 50−60% yield of ligated RNA in overnight incubations at pH 7.5 and 37 °C, and they afford 40−50% yield in 1 h at pH 9.0 and 37 °C. Various RNA substrate sequences may be joined by simple Watson−Crick covaration of the DNA binding arms that interact with the two RNA substrates. The current deoxyribozymes have some RNA substrate sequence requirements at the nucleotides immediately surrounding the ligation junction (either UAUA↓GGAA or UAUN↓GGAA, where the arrow denotes the ligation site and N equals any nucleotide). One of the new deoxyribozymes was used to prepare by ligation the *Tetrahymena* group I intron RNA P4−P6 domain, a representative structured RNA. Nondenaturing gel electrophoresis revealed that a 2′−5′ linkage between nucleotides A233 and G234 of P4−P6 does not disrupt its Mg^{2+}-dependent folding ($\Delta\Delta G^{\circ\prime} < 0.2$ kcal/mol). This demonstrates that a 2′−5′ linkage does not necessarily interfere with structure in a folded RNA. Therefore, these non-native linkages may be acceptable in modified RNAs when structure/function relationships are investigated. Deoxyribozymes that ligate RNA should be particularly useful for preparing site-specifically modified RNAs for studies of RNA structure, folding, and catalysis.

Site-specific modifications hold much promise for investigating RNA structure, folding, and catalysis (e.g., refs 1 and 2). Ribonucleosides with various modifications in the sugar or nucleobase are readily converted to phosphoramidites for solid-phase oligoribonucleotide synthesis.[3] However, RNAs >100 nucleotides in length are difficult or impossible to make directly in most cases, because of limitations inherent to solid-phase methodology. Therefore, preparing modified RNAs that comprise several hundred nucleotides (such as group I and group II introns,[4] RNase P,[5] or other catalytic RNAs) requires ligation of two or more RNA substrates, any or all of which may incorporate modifications.

An ideal RNA ligation technique would be simple, dependable, and generally applicable. A common method is splint ligation, in which a bridging DNA oligonucleotide (splint) holds together two RNA substrates for joining by T4 DNA ligase.[6,7] Variants of this procedure using T4 RNA ligase are also known.[8] Unfortunately, splint ligation mediated by T4 DNA ligase is well-known to be idiosyncratic, often inexplicably yielding little ligated product with particular RNA substrates (e.g., refs 1 and 9). Because preparation of these substrates is often laborious and almost always expensive, and because failed ligations are time-consuming and frustrating, alternative approaches to RNA ligation are highly desirable.

DNA is an inexpensive, stable, and reliable tool for molecular biology, and deoxyribozymes (DNA enzymes) have received significant attention in recent years.[10,11] Deoxyribozymes have been identified that ligate DNA[12] or cleave RNA,[13−15] but such

* To whom correspondence should be addressed: phone (217) 244-4489; fax (217) 244-8024.
† Present address: Department of Chemistry and Physics, William Paterson University of New Jersey, Wayne, NJ 07470.
‡ Department of Biochemistry, University of Illinois.

(1) Silverman, S. K.; Cech, T. R. *Biochemistry* **1999**, *38*, 8691−8702.
(2) (a) Herschlag, D.; Eckstein, F.; Cech, T. R. *Biochemistry* **1993**, *32*, 8299−8311. (b) Abramovitz, D. L.; Friedman, R. A.; Pyle, A. M. *Science* **1996**, *271*, 1410−1413. (c) Silverman, S. K.; Cech, T. R. *Biochemistry* **1999**, *38*, 14224−14237. (d) Silverman, S. K.; Deras, M. L.; Woodson, S. A.; Scaringe, S. A.; Cech, T. R. *Biochemistry* **2000**, *39*, 12465−12475. (e) Silverman, S. K.; Cech, T. R. *RNA* **2001**, *7*, 161−166. (f) Young, B. T.; Silverman, S. K. *Biochemistry* **2002**, *41*, 12271−12276.
(3) (a) Scaringe, S. A.; Wincott, F. E.; Caruthers, M. H. *J. Am. Chem. Soc.* **1998**, *120*, 11820−11821. (b) Earnshaw, D. J.; Gait, M. J. *Biopolymers* **1998**, *48*, 39−55. (c) Scaringe, S. A. *Methods Enzymol.* **2000**, *317*, 3−18.
(4) (a) Cech, T. R. *Annu. Rev. Biochem.* **1990**, *59*, 543−568. (b) Saldanha, R.; Mohr, G.; Belfort, M.; Lambowitz, A. M. *FASEB J.* **1993**, *7*, 15−24. (c) Michel, F.; Ferat, J. L. *Annu. Rev. Biochem.* **1995**, *64*, 435−461. (d) Fedorova, O.; Julie Su, L.; Pyle, A. M. *Methods* **2002**, *28*, 323−335.
(5) (a) Frank, D. N.; Pace, N. R. *Annu. Rev. Biochem.* **1998**, *67*, 153−180. (b) Kurz, J. C.; Fierke, C. A. *Curr. Opin. Chem. Biol.* **2000**, *4*, 553−558.
(6) Moore, M. J.; Query, C. C. In *RNA−Protein Interactions: A Practical Approach*; Smith, C. W. J., Ed.; Oxford University Press: Oxford, U.K., 1998; pp 75−108.
(7) Moore, M. J.; Sharp, P. A. *Science* **1992**, *256*, 992−997.
(8) (a) Bain, J. D.; Switzer, C. *Nucleic Acids Res.* **1992**, *20*, 4372. (b) Sherlin, L. D.; Bullock, T. L.; Nissan, T. A.; Perona, J. J.; Lariviere, F. J.; Uhlenbeck, O. C.; Scaringe, S. A. *RNA* **2001**, *7*, 1671−1678.
(9) (a) Han, H.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4955−4959. (b) Zahler, A. M.; Roth, M. B. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2642−2646. (c) Strobel, S. A.; Ortoleva-Donnelly, L. *Chem. Biol.* **1999**, *6*, 153−165.
(10) Breaker, R. R. *Science* **2000**, *290*, 2095−2096.
(11) Emilsson, G. M.; Breaker, R. R. *Cell. Mol. Life Sci.* **2002**, *59*, 596−607.
(12) Cuenoud, B.; Szostak, J. W. *Nature* **1995**, *375*, 611−614.
(13) Santoro, S. W.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262−4266.
(14) Santoro, S. W.; Joyce, G. F. *Biochemistry* **1998**, *37*, 13330−13342.

10.1021/ja028774y CCC: $25.00

catalysts are unknown for RNA ligation (although ribozymes that ligate RNA have been described[16]). The most useful DNA enzymes for RNA ligation would operate in a generalizable "binding arms" format, in which DNA binding arms that have Watson−Crick complementarity to any two RNA substrates flank the region of DNA that encompasses the catalytic activity. In this report, we have used in vitro selection to identify deoxyribozymes that ligate RNA in this general way with regioselective creation of 2′−5′ RNA linkages. The ability to create 2′−5′ RNA linkages will enable study of the roles of individual phosphodiester junctions in RNA structure and function. These linkages may also have value in biological assays where nuclease resistance conferred by a non-native 2′−5′ linkage is advantageous. Perhaps most significantly, these new deoxyribozymes allow practical ligation of RNA in a general way, and they should therefore be useful for synthesis of site-specifically modified RNAs.

Results

A New Selection Strategy Identifies Deoxyribozymes That Ligate RNA. A selection strategy was designed to identify deoxyribozymes that ligate RNA (Figure 1A). Each selection round comprised three steps A−C, with separation after each step by denaturing polyacrylamide gel electrophoresis (PAGE). Two specific RNA substrates of largely arbitrary sequence were maintained for all selection rounds; their successful ligation creates a new A↓G RNA junction. In step A of the first selection round, the right-hand RNA substrate was joined by T4 RNA ligase to the deoxyribozyme strand, which was prepared by solid-phase synthesis with an N_{40} random DNA region (N = any nucleotide A, G, T, or C with equal probability). Because known DNA enzymes that cleave RNA are ≤15 nucleotides long,[13,14] an N_{40} region was thought sufficient to provide RNA ligase deoxyribozymes. Four RNA nucleotides were left unpaired on either side of the ligation junction, in the anticipation that this may be necessary for ligation activity. In selection step B, a 5′-hydroxyl on the right-hand RNA substrate was joined with a 2′,3′-cyclic phosphate on the left-hand substrate, forming a new phosphodiester bond that may be either 2′−5′ or 3′−5′ (Figure 1B). If the bond is 3′−5′, then the ligation reaction is the exact reverse of the cleavage reaction catalyzed by protein and deoxyribozyme ribonucleases,[13−15] at least in terms of the reactants and products. This consideration offered precedent that the desired ligation reaction is achievable by a deoxyribozyme. Finally, in selection step C, the deoxyribozyme strand was regenerated by PCR with two appropriate DNA primers, one of which contained several PEG spacer units plus a short oligodeoxynucleotide tail that blocks extension by *Taq* polymerase.[17] This allowed separation by denaturing PAGE of the two single-stranded DNA products, only one of which was desired. The new deoxyribozyme strand, now enriched in DNA sequences competent for RNA ligation, was ligated to fresh right-hand RNA substrate with T4 RNA ligase as step A of the next selection round.

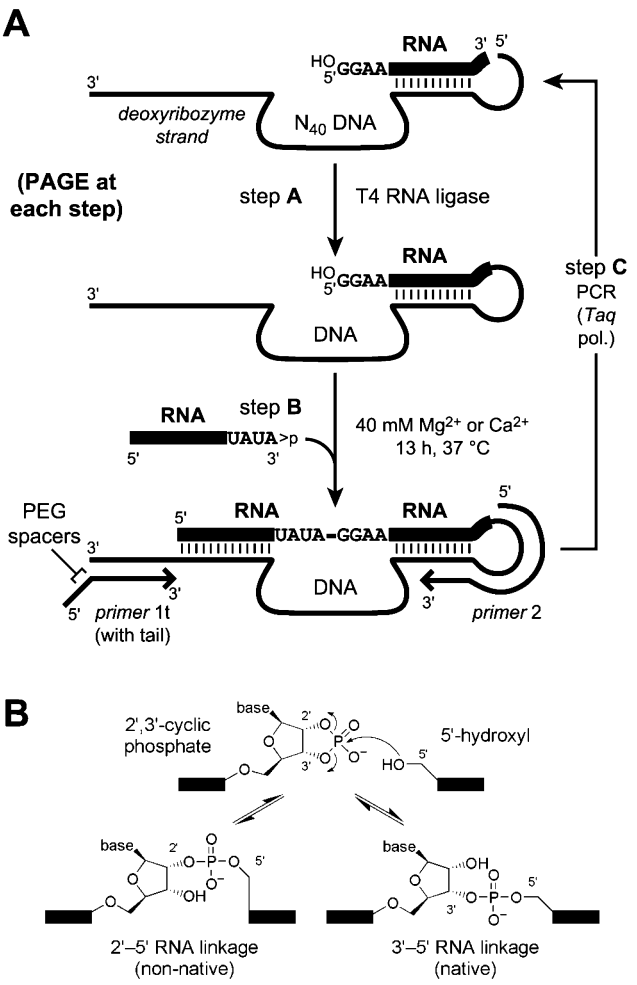

Figure 1. (A) Overview of the selection strategy for deoxyribozymes that ligate RNA. RNA is shown as a thick line and DNA as a thin line. See Experimental Section for details and exact nucleotide sequences. (B) Reaction scheme of a 2′,3′-cyclic phosphate with a 5′-hydroxyl to form either a 2′−5′ or a 3′−5′ phosphodiester linkage.

The permissive selection conditions in step B were 40 mM Mg^{2+} or Ca^{2+} for 13 h at 37 °C in a buffer containing 150 mM NaCl, 2 mM KCl, and 50 mM HEPES, pH 7.5. After nine selection rounds, the activities of both the Mg^{2+} and Ca^{2+} pools had risen considerably, such that at least 20% of each pool achieved ligation during step B of round 9 (Figure 2). As a control, we repeated the ninth Mg^{2+} round with either a 2′,3′-diol left-hand RNA substrate or a 5′-phosphate right-hand RNA substrate (or both). This led to no observable ligation activity in step B of the repeated round (data not shown). This suggested formation of the desired phosphodiester linkages in the original ninth selection round.

Determining the Identity of the Newly Created Ligation Junctions: 2′−5′ or 3′−5′? An inherent limitation of using a 2′,3′-cyclic phosphate as an RNA substrate is that the new phosphodiester linkages could be either 3′−5′ (native) or 2′−5′ (non-native), depending on which oxygen acts as the leaving group (Figure 1B). It is difficult to control directly which of the two isomers is formed in the reaction of any individual

(15) Pyle, A. M.; Chu, V. T.; Jankowsky, E.; Boudvillain, M. *Methods Enzymol.* **2000**, *317*, 140−146.
(16) (a) Bartel, D. P.; Szostak, J. W. *Science* **1993**, *261*, 1411−1418. (b) Ekland, E. H.; Szostak, J. W.; Bartel, D. P. *Science* **1995**, *269*, 364−370. (c) Hager, A. J.; Szostak, J. W. *Chem. Biol.* **1997**, *4*, 607−617. (d) Jaeger, L.; Wright, M. C.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14712−14717. (e) Robertson, M. P.; Ellington, A. D. *Nat. Biotechnol.* **1999**, *17*, 62−66. (f) Rogers, J.; Joyce, G. F. *Nature* **1999**, *402*, 323−325. (g) Robertson, M. P.; Hesselberth, J. R.; Ellington, A. D. *RNA* **2001**, *7*, 513−523. (h) Johnston, W. K.; Unrau, P. J.; Lawrence, M. S.; Glasner, M. E.; Bartel, D. P. *Science* **2001**, *292*, 1319−1325.
(17) (a) Williams, K. P.; Bartel, D. P. *Nucleic Acids Res.* **1995**, *23*, 4220−4221. (b) Sheppard, T. L.; Ordoukhanian, P.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7802−7807.

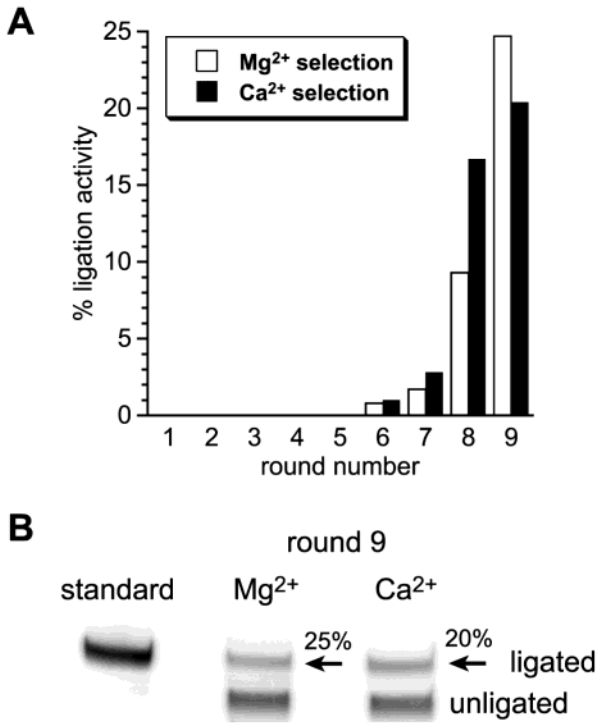

Figure 2. (A) Selection progression. The activities did not increase upon an additional selection round at 40 mM M^{2+} (M = Mg or Ca; data not shown). Also, several rounds of further selection at 10 mM M^{2+} (instead of 40 mM) led to a drop in activities to <4%; the products of these rounds were not pursued further. (B) Gel image from selection round 9, showing 20−25% ligation activity for the Mg^{2+} and Ca^{2+} pools.

deoxyribozyme molecule. Therefore, we anticipated that a mixture of the isomeric products could be formed. The two isomeric linkages were distinguished with a complementary pair of assays. To provide appropriate substrates for these assays, the products from selection step C (obtained from the round 9 Mg^{2+} and Ca^{2+} pools separately) were first 5′-^{32}P-radiolabeled and treated with DNase to remove the deoxyribozyme strand, leaving only the ligated RNA products. Then, to reveal any 3′−5′ linkages, these RNA products were incubated with the corresponding 8−17 RNA-cleaving deoxyribozyme,[13−15] which cleaves at the newly created A↓G junctions if and only if they are 3′−5′ (as demonstrated with control RNA substrates; Figure 3A). Similarly, any 2′−5′ linkages were revealed by Mg^{2+}-dependent alkaline hydrolysis in the presence of the DNA complement, which selectively cleaves an RNA 2′−5′ junction (Figure 3B).[18,19] The results showed that at least 95% of each pool's product was linked 2′−5′ (Figure 3C). Because the Mg^{2+} and Ca^{2+} selections each appeared to provide a similar overall level of ligation activity and because both selections provided only 2′−5′-linked RNA, the Ca^{2+} pools were not examined further in this study.

Cloning and Characterization of Individual Deoxyribozymes. Individual deoxyribozymes from the round 9 Mg^{2+} pool were cloned and tested for ligation activity in trans with the same two RNA substrates that were used during selection. That is, the right-hand RNA substrate was not connected to the deoxyribozyme, making the ligation reaction formally trimolecular with respect to the individual substrates and deoxyribozyme (Figure 4A). Under single-turnover conditions (see Experimental Section), 28 individual deoxyribozymes were found to ligate the RNA substrates with $k_{obs} = 0.08-0.7\ h^{-1}$ at 37 °C and pH 7.5, some with yields over 50% (Figure 4B,C). Appropriate background reaction assays are described below. The ligation activities were initially screened with individual deoxyribozymes prepared by PCR amplification from plasmid miniprep DNA. On the basis of these activities, 12 particular clones were chosen for sequencing. No clear consensus was observed; representative sequences are shown in Figure 5.

To provide focus while exploring the scope of the new RNA ligation activities, four individual clones (9A12, 9A5, 9A6, and 9A2) were chosen for more detailed study. It was first established that each of these deoxyribozymes retained RNA ligation activity when prepared independently by solid-phase synthesis, which is the form in which the DNA enzymes would most likely be used in practice. Indeed, comparable or better rates and yields were observed in all cases. By the assays of Figure 3, each of the four deoxyribozymes was shown unambiguously to provide 2′−5′-linked RNA product (see Supporting Information), consistent with the whole-pool assays. The same was true for the remaining eight sequenced deoxyribozymes. For each of these deoxyribozymes, when Mg^{2+} was omitted from the reaction, ligation activity was abolished (data not shown). The quantitative Mg^{2+} dependences and the abilities of other metal ions to support ligation activity are currently under investigation. It was also confirmed that each of the four new deoxyribozymes absolutely requires for ligation activity a 2′,3′-cyclic phosphate and a 5′-hydroxyl on the left-hand and right-hand RNA substrates, respectively (see Supporting Information). Multiple turnover was not detected, which is the expected result if the ligated RNA product binds relatively tightly to the DNA enzyme (i.e., product inhibition; see Supporting Information). A similar situation is found with T4 DNA ligase, which is used stoichiometrically in practical RNA ligations.[6]

The kinetic activities (k_{obs}) shown in Figure 4C were obtained at 37 °C and 50 mM HEPES, pH 7.5. We briefly examined the effects of changing the temperature and/or pH on the ligation reaction. Lowering the reaction temperature to 10 °C at pH 7.5 did not improve the yields and led to a modest reduction in observed rate (see Supporting Information for details of experiments at different temperatures; others have reported that lower temperature increases the ability of the hammerhead ribozyme to ligate rather than cleave RNA[20]). At 45 °C, k_{obs} for our deoxyribozymes increased slightly, but the yields dropped approximately 2-fold. Thus, changing the reaction temperature was not particularly useful. In contrast, k_{obs} depended substantially on pH; the k_{obs} values were 10−20 times higher at pH 9.0 than at pH 7.5. Specifically, at 37 °C and 50 mM CHES, pH 9.0, the fastest deoxyribozyme (9A5) ligated RNA with $k_{obs} = 0.18\ min^{-1}$, albeit with a 25% yield (Figure 4D). The other three deoxyribozymes of Figure 4D provided 40−50% yield of ligated RNA in ∼1 h under these conditions.

Comparison to Appropriate Background Ligation Reactions. The observed ligation rates should be compared to rates for appropriate control reactions to determine the rate enhancement by the new deoxyribozymes. In terms of mechanism, a

(18) Usher, D. A.; McHale, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1149−1153.
(19) Rohatgi, R.; Bartel, D. P.; Szostak, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 3340−3344.
(20) Stage-Zimmermann, T. K.; Uhlenbeck, O. C. *Nat. Struct. Biol.* **2001**, *8*, 863−867.

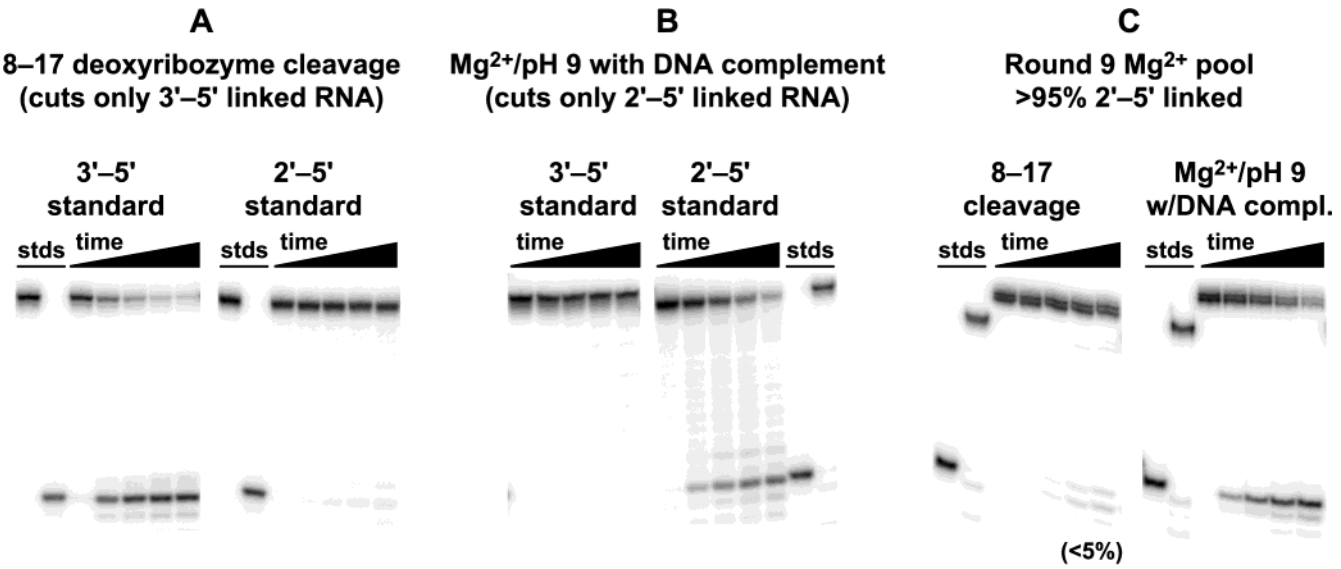

Figure 3. Determination of the new RNA linkages as 3′−5′ or 2′−5′. (A) Control experiments with the 8−17 deoxyribozyme that cleaves only 3′−5′ phosphodiester linkages. Time points are at 0 and 20 min and 1, 4, and 7 h. (B) Control experiments with hydrolysis at 100 mM Mg^{2+} and pH 9 in the presence of the DNA complement, which cleaves only 2′−5′ phosphodiester linkages. Time points are at 0, 2, 6, 13, and 24 h. (C) Assay of the RNA product from the round 9 Mg^{2+} pool by the two complementary methods, revealing that the pool is composed of >95% 2′−5′-linked RNA. The starting material for these assays is slightly larger than the standard because several DNA nucleotides at the 3′-end of the RNA are removed inefficiently by DNase (see Experimental Section).

deoxyribozyme could simply act as a splint to hold the 2′,3′-cyclic phosphate and 5′-hydroxyl close in space. Comparisons to control reactions allow us to determine the contribution of such proximity (chelate) effects[21] to the rate enhancement. Control experiments were performed by replacing the deoxyribozyme of Figure 4A with various short DNA oligonucleotide splints and repeating the ligation assays (Figure 6). At 37 °C and pH 7.5, using a DNA oligonucleotide splint that was exactly complementary to both RNA substrates gave only ca. 6% ligation in 24 h ($k_{obs} \approx 0.0024\ h^{-1}$). With this DNA splint, even though the 2′,3′-cyclic phosphate and 5′-hydroxyl are constrained to be extremely close together, the uncatalyzed ligation reaction is very slow. The 9A5 deoxyribozyme ($k_{obs} = 0.76\ h^{-1}$) has a 320-fold rate enhancement compared with this background reaction, and 9A2 has a 33-fold rate enhancement ($k_{obs} = 0.08\ h^{-1}$). The 9A5 and 9A2 deoxyribozymes are the fastest and slowest, respectively, of those shown in Figure 4C.

We also examined fully complementary DNA splints that additionally had 1−4 unpaired T nucleotides opposite the ligation site (the T_1 through T_4 splints). These unpaired nucleotides were located between the two binding arms of the DNA splint that interact with the RNA substrates in complete Watson−Crick fashion (see Figure 6A). The added unpaired nucleotides were expected to relax the spatial constraint between the reacting RNA functional groups and thus provide a more appropriate background reaction rate for the newly selected deoxyribozymes, which have many nucleotides between the Watson−Crick binding arm duplexes. Indeed, all four splints T_1 through T_4 showed a reduced ligation rate when compared with the exactly complementary splint (Figure 6). The T_3 and T_4 splints gave almost no observable product in 24 h ($k_{obs} < 0.0002\ h^{-1}$), which corresponds to rate enhancements of >3800-fold and >400-fold for 9A5 and 9A2, respectively. This lack of background reaction with the T_3 and T_4 splints is consistent with the observation that the original (N_{40}) deoxyribozyme strand shows no ligation activity during the early rounds of selection or when tested in trimolecular fashion. In the latter assays, the cleanest gel images (those with the longest exposure and least background noise) revealed no observable ligation product in 24 h with a detection limit of <0.1%, equivalent to $k_{obs} < 0.00004\ h^{-1}$. If this is used as the background reaction rate, the calculated rate enhancements for 9A5 and 9A2 are >19 000-fold and >2000-fold, respectively.

All of the control splints were also examined at 37 °C and pH 9.0 (see Supporting Information). After 90 min, which corresponds to the final time point of Figure 4D, only ca. 4% ligation was observed with the exactly complementary splint ($k_{obs} \approx 0.00045\ min^{-1}$). This leads to calculated rate enhancements relative to this splint of 400-fold for 9A5 and 51-fold for 9A2. These values are comparable to or better than the values obtained at pH 7.5. Rate enhancements at pH 9 determined for the other splints were also comparable to those at pH 7.5.

RNA Substrate Sequence Specificity of the New Deoxyribozymes. As shown in Figure 1A, at each round the selection strategy employed a constant set of four overhanging (non-base-paired) RNA nucleotides on either side of the ligation junction. For the four new deoxyribozymes, we determined whether any of these substrate nucleotides are required for ligation activity. This was done by testing RNA substrates in which single-nucleotide changes were made relative to the original substrates (see Supporting Information for details). By checking all possible nucleotides immediately on either side of the ligation junction, the 9A12, 9A5, and 9A6 deoxyribozymes were found strictly to require the particular nucleotides A↓G, while 9A2 permitted any nucleotide on the left side (N↓G, although purines worked better than pyrimidines at the N position by 3−6-fold in terms of yield). At the next three more remote positions on each side, the new deoxyribozymes generally required the specific nucleotides that were present in the original selection substrates (for practical reasons, only transitions and not

(21) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678−1683.

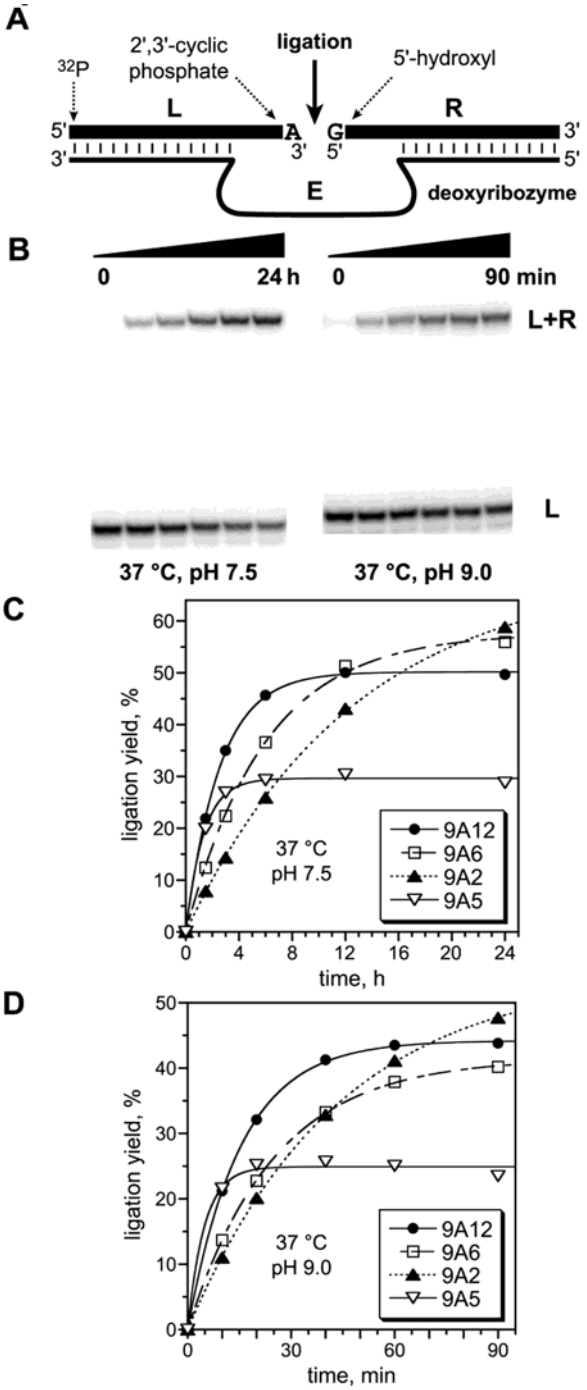

Figure 4. Representative kinetic assays for individual deoxyribozymes under single-turnover conditions ($[Mg^{2+}] = 40$ mM). (A) Intermolecular format for the assay. The 5′-^{32}P-radiolabeled left-hand RNA substrate L is the limiting reagent; typically L:E:R was 1:3:6 (see Experimental Section). Indistinguishable results were obtained when R instead of L was ^{32}P-radiolabeled and limiting. (B) Representative gel images for the 9A6 deoxyribozyme. Conditions: 37 °C; 50 mM HEPES, pH 7.5 (left) or 50 mM CHES, pH 9.0 (right). At pH 9.0, nonspecific degradation was often observed at time points ≥12 h (not shown). (C) Kinetic plots for new deoxyribozymes at 37 °C and pH 7.5. Values of k_{obs} (h^{-1}): 9A12, 0.39; 9A6, 0.17; 9A2, 0.08; 9A5, 0.76. Values of k_{obs} for the same combination of deoxyribozyme and substrate were reproducible to ±20%, and yields were reproducible to ±10%. The deoxyribozymes for this experiment were prepared by solid-phase synthesis, with enzyme region sequences as shown in Figure 5. A similar k_{obs} was found for 9A5 when more time points were taken between 0 and 3 h (data not shown). (D) Kinetic plots for new deoxyribozymes at 37 °C and pH 9.0. Values of k_{obs} (min^{-1}): 9A12, 0.065; 9A6, 0.040; 9A2, 0.023; 9A5, 0.18. A similar k_{obs} was found for 9A5 when more time points were taken between 0 and 10 min (data not shown).

9A12	5'-CTTTACGGTAGGGTGCCTGTGATAATGATCAGGGGACGGC-3'
9A5	5'-AAGGGGAGGGCCGATTGGCATTATCGGCGTCTTAGCTCTA-3'
9A6	5'-TAACCGTGGTTACCGTAAGCGCGGGGCTTATAGGGGATT-3'
9A2	5'-TGCGTTCTGATGAATTGCACATAGCTTATAGTTCCTTGCT-3'

Figure 5. Sequences of representative deoxyribozyme clones from the round 9 Mg^{2+} selection pool. These sequences correspond directly to the N_{40} region of the deoxyribozyme strand of Figure 1A, as determined by automated sequencing (see Experimental Section). Clone 9A6 is one nucleotide shorter than the others (39 versus 40 nucleotides), which we attribute to a deletion by *Taq* polymerase during an unidentified round of PCR during selection. Note the lack of any obvious consensus sequence.

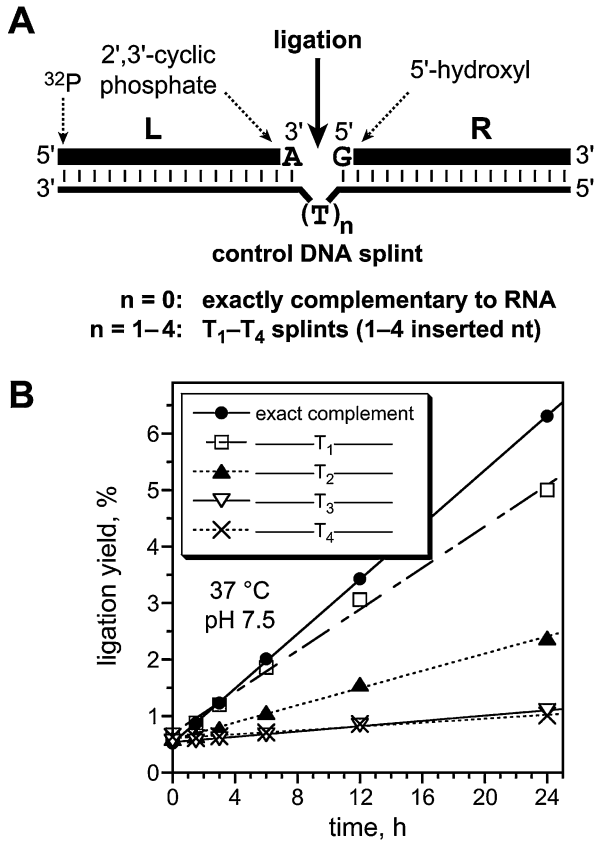

Figure 6. Control ligation assays with short complementary DNA splints, to characterize the background ligation reaction. The ligations were performed as in Figure 4 at 37 °C and pH 7.5, except a short DNA oligonucleotide splint was used instead of a deoxyribozyme. (A) Format of the reaction. The splint was either exactly complementary to the RNA substrates with no unpaired nucleotides (n = 0), or from 1 to 4 unpaired T nucleotides were inserted between the DNA binding arms that bind to the RNA substrates via Watson−Crick base pairs (n = 1−4; the T_1 through T_4 splints). (B) Results of the control assays. The detection limit for this experiment, which was representative of other experiments, was ∼0.5% (note the "yield" at $t = 0$), although in favorable instances the detection limit was as low as 0.1%. The slopes of the linear fits were (n = 0−4 inserted T nucleotides, top to bottom, h^{-1}) 0.0024, 0.0018, 0.00076, 0.00024, and 0.00017. For analogous assays at pH 9.0, see Supporting Information.

transversions were tested). In summary, the required RNA substrate sequences for 9A12, 9A5, and 9A6 are UAUA↓GGAA, while 9A2 requires UAUN↓GGAA. Some of the particular overhanging RNA nucleotides may be required for the catalytic mechanism. Alternatively, some of the nucleotides may be required merely for secondary structure formation—i.e., Watson−Crick base pairing—with some part of each DNA enzyme. If so, then strategic covariation of the appropriate (and as yet unidentified) part of the DNA sequence with the RNA substrates would allow more general RNA ligation, in analogy

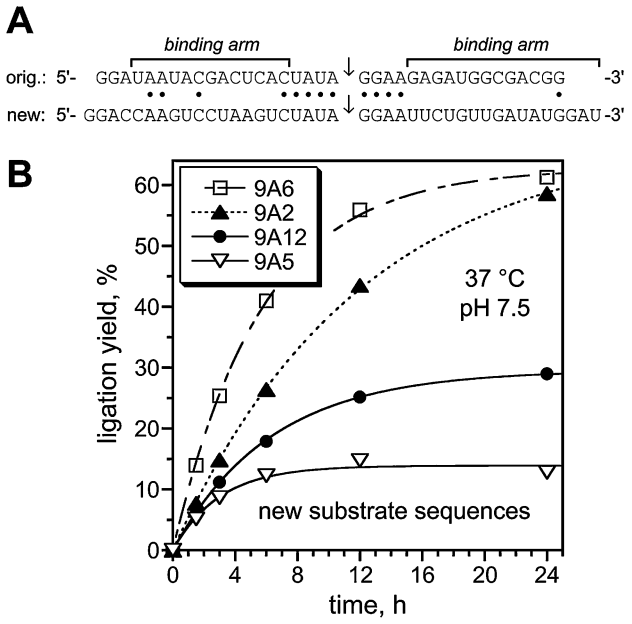

Figure 7. Assaying tolerance of the new deoxyribozymes to changes in the RNA substrate binding arms. (A) RNA sequences that were tested in ligation reactions. The "new" RNA sequences have binding arms largely unrelated to those of the "original" RNA sequences that were used in the selection procedure. During selection, the 5′-GGA of the original sequence was not present; these three nucleotides were included later to permit in vitro transcription of RNA substrates of varying sequence. (B) Results of the assay with the new RNA substrates. Compare with Figure 4C; the same symbols are used for each deoxyribozyme in both plots. Note in particular that the 9A6 and 9A2 deoxyribozymes ligate equally well both sets of substrates. Values of k_{obs} (h^{-}): 9A12, 0.16; 9A6, 0.18; 9A2, 0.08; 9A5, 0.33; see Supporting Information for full details. Control experiments with the "wrong" deoxyribozymes (those with binding arms not matching the RNA substrates) showed no ligation activity (data not shown).

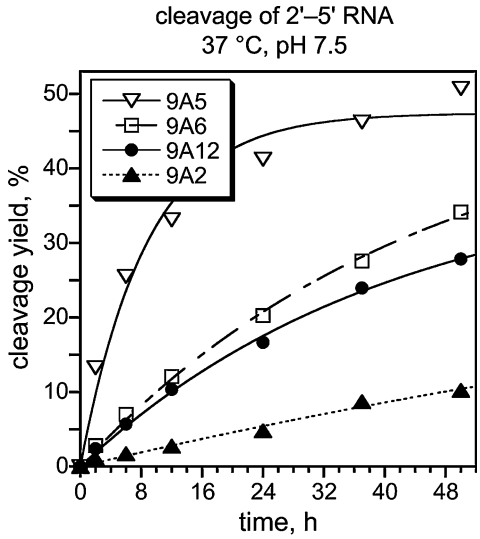

Figure 8. RNA cleavage experiments for the new deoxyribozymes. This figure shows cleavage of 2′−5′-linked RNA at the A↓G site marked "ligation" in Figure 4A. Compare these results with Figure 4C, which shows ligation under the same conditions. The same symbols are used for each deoxyribozyme in both plots; note the different *x*-axes. The cleavage k_{obs} for 9A5 was 0.11 h^{-1}, versus 0.76 h^{-1} for ligation under the same conditions (Figure 4C). The values of k_{obs} for cleavage for the other deoxyribozymes were much lower; note that the curves do not turn over even at >48 h. Linear fits to the initial data points gave the following k_{obs} values (h^{-1}): 9A12, 0.009; 9A6, 0.012; 9A2, 0.003. RNA that is linked 3′−5′ rather than 2′−5′ was not detectably cleaved by any of the deoxyribozymes (<5%; data not shown).

to the situation with RNA-cleaving deoxyribozymes.[13−15] We are currently dissecting these possibilities experimentally.

Ideally, the new deoxyribozymes would be able to ligate a wide range of RNA substrates by straightforward Watson−Crick covariation of the deoxyribozyme's substrate binding arms to match any desired RNA substrate sequences. For the four new deoxyribozymes, this ability was tested with the "new" RNA substrates of Figure 7A. The new substrate sequences—largely unrelated to those used during the original selection itself—were derived from the structured P4−P6 RNA domain, as described below. In this initial screen of generality, the four nucleotides on either side of the ligation site were retained from the original substrates (UAUA↓GGAA), to allow focus on the binding arms. The assays showed that all four deoxyribozymes are capable of ligating RNA substrates that have binding arm sequences largely unrelated to those used during the selection (Figure 7B). The 9A6 and 9A2 deoxyribozymes ligated the new substrates equally well as the original substrates in terms of rate and yield, while the 9A12 and 9A5 each showed only about a 2-fold drop in both rate and yield when the substrates were changed. This successful ligation with different binding arms clearly illustrates the generality of deoxyribozymes as RNA ligase reagents.

Assays for RNA Cleavage versus RNA Ligation Activity. To allow insight into the catalytic mechanisms, the four new deoxyribozymes were assayed to determine if they can cleave rather than ligate both 3′−5′-linked and 2′−5′-linked RNA. In an intermolecular format analogous to that shown in Figure 4A, a 34-mer RNA substrate corresponding to the ligated RNA product (i.e., L + R) was incubated with each deoxyribozyme. This substrates was obtained by solid-phase synthesis with either a 3′−5′ or a 2′−5′ linkage corresponding to the A↓G junction that is created during ligation. The 3′−5′ linkage was not detectably cleaved (<5%) by any of the new DNA enzymes. In contrast, the 2′−5′ linkage was cleaved, but with a wide range of rates and yields (Figure 8). The 9A5 cleavage yield of 40−50% is higher than its ligation yield of 30% under comparable conditions, although k_{obs} for ligation is significantly larger than k_{obs} for cleavage by a factor of 7. The other three deoxyribozymes all ligate 2′−5′-linked RNA much better than they cleave, in terms of both rate and yield (compare Figures 8 and 4C). The ratio of k_{obs}(ligation)/k_{obs}(cleavage) is 43, 14, and 27 for 9A12, 9A6, and 9A2, respectively.

Reselections to Improve the Ligation Activity. The initial deoxyribozyme pool contained 200 pmol = 10^{14} different DNA molecules, whereas there are $4^{40} = 10^{24}$ possible 40-mer DNA sequences. Because this allows only 10^{-10} coverage of sequence space, the initial selection efforts likely did not provide directly the most active possible deoxyribozymes. Therefore, we subjected some of the new deoxyribozymes to reselection for improved activity. Deoxyribozyme strands (Figure 1) were prepared with the sequence of an original clone but with a 25% probability at each nucleotide position of having one of the other three nucleotides (with an equal chance for each of the other three). These partially randomized deoxyribozyme strands were carried through the selection procedure for 7−8 rounds, with the final selection rounds performed by allowing as little as 20 min to 1 h for the DNA-mediated RNA ligation (step B of Figure 1). Individual deoxyribozymes were then cloned and their ligation kinetics were assayed as described above (Figure 4). In preliminary results, starting with the partially randomized 9A2 sequence, several clones were identified with significantly

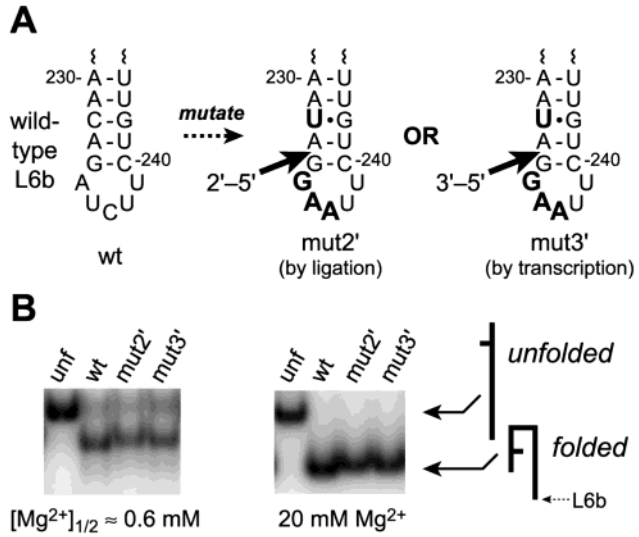

Figure 9. Application of the new deoxyribozymes to prepare structured RNAs by ligation. (A) RNA sequence near the L6b loop of P4−P6, as ligated by the 9A6 deoxyribozyme. The ligation site is at the arrowhead. For details of the preparative ligation, see Supporting Information. (B) Mg^{2+}-dependent nondenaturing PAGE[1] demonstrates that P4−P6 with a single 2′−5′ linkage at the new A↓G junction between nucleotides 233 and 234 folds like wild-type P4−P6. Samples: unf, unfolded control mutant of P4−P6; wt, wild-type P4−P6; mut2′, P4−P6 with the nucleotide changes of panel A and a 2′−5′ A↓G junction (prepared by deoxyribozyme-mediated ligation); mut3′, P4−P6 with the same nucleotide changes and a 3′−5′ A↓G junction (prepared by in vitro transcription). The wt, mut2′, and mut3′ RNAs each require similar Mg^{2+} concentrations for tertiary folding ($[Mg^{2+}]_{1/2} \approx$ 0.6 mM in Tris−borate, pH 8.3, at 35 °C; $\Delta\Delta G^{\circ\prime} < 0.2$ kcal/mol for comparing either mut2′ or mut3′ with wt).[1]

greater ligation rates than the relatively slow parent 9A2 deoxyribozyme ($k_{obs} = 0.08\ h^{-1}$). The fastest of the reselected 9A2 deoxyribozymes currently in hand is 7Z48, which has $k_{obs} = 2.9\ h^{-1}$ and 30% yield at 37 °C and pH 7.5 ($k_{obs} = 0.52\ min^{-1}$ and 32% yield at 37 °C and pH 9.0; data not shown). This is a 36-fold increase in k_{obs} at pH 7.5 when compared with the parent 9A2. We are currently working to characterize fully several of the more active reselected deoxyribozymes, and a complete report will be made elsewhere.

Application of the New Deoxyribozymes to Prepare and Study a Structured RNA: the P4−P6 RNA Domain. One significant potential use of deoxyribozymes that create 2′−5′ RNA linkages is to join RNA substrates in regions where the specific backbone connectivity is unimportant. In these cases, 2′−5′-linked RNA is functionally indistinguishable from native RNA, and the new deoxyribozymes reported here could find immediate use in practical RNA ligations. To demonstrate the experimental utility of deoxyribozymes for this purpose, the 9A6 deoxyribozyme was used to ligate near the L6b loop of the 160-nucleotide P4−P6 domain of the *Tetrahymena* group I intron RNA,[22] the partial sequence of which is shown in Figure 9A. Four nucleotide changes as indicated (in boldface type) were made relative to wild-type P4−P6 to accommodate the substrate sequence requirements of our first generation of RNA ligase deoxyribozymes.

The effects on Mg^{2+}-dependent P4−P6-folding of the new 2′−5′ linkage plus the four nucleotide changes were not known in advance. Nondenaturing polyacrylamide gel electrophoresis[1] showed that Mg^{2+}-dependent P4−P6 folding is essentially unaffected by these alterations (Figure 9B). This formally demonstrates that 2′−5′ linkages are tolerable in at least some contexts for preparing modified RNAs without loss of structural integrity. Specifically, the right-hand fragment of P4−P6 in this successful ligation is a 28-mer that is readily obtained with various chemical modifications by solid-phase RNA synthesis.[23]

Discussion

In vitro selection has been used to identify RNA and DNA sequences that bind to small-molecule targets (i.e., aptamers[24]) and also to discover sequences that catalyze chemical reactions. In studies of ribozymes, the activities investigated are often of significant theoretical interest, as in explorations of the "RNA world".[25] Many catalytic activities being pursued with deoxyribozymes have practical utility: e.g., deoxyribozymes that cleave RNA,[13−15,26] lead-sensing DNA enzymes,[27] or deoxyribozymes that are therapeutic agents.[11,28] For studies of RNA structure, folding, and catalysis, there is a clear need for new RNA ligation approaches. This is necessary because solid-phase synthesis—which permits site-specific incorporation of modified nucleotides—has an inherent size limitation, and protein-mediated RNA ligation is often suboptimal. Here we report the first deoxyribozymes that ligate RNA. These reagents are of theoretical interest because they expand the catalytic repertoire of nucleic acid enzymes, and they are of practical interest for their potential utility in preparing site-specifically modified RNAs.

Applications of 2′−5′-Linked RNAs That Are Created by the New DNA Enzymes. The ligated RNA products created by our new deoxyribozymes are joined by 2′−5′ phosphodiester linkages (Figure 3). Such 2′−5′-linked RNAs are of interest for at least three reasons. First, RNA substrates may be joined in unstructured regions (e.g., in or near loops) to provide site-specifically modified RNAs that are difficult or impossible to obtain by other methods. In these cases, the non-native linkage is not expected to perturb the RNA structure or function, a proposition that is testable experimentally in most cases (via preparation of wild-type RNA sequence by ligation and comparison to authentic wild-type RNA). Second, RNAs may be connected via a single, specific 2′−5′ linkage, to evaluate the role of that linkage in a structural or functional context. Third, RNA substrates may be joined through a nuclease-resistant 2′−5′ linkage to prevent degradation during an in vitro diagnostic assay or an in vivo therapeutic application. The data reported here (Figure 9) directly demonstrate the first two of these applications of 2′−5′ RNA linkages. The successful ligation to form P4−P6 without structural disruption due to the new 2′−5′ junction clearly illustrates that the specific backbone connectivity (i.e., 3′−5′ versus 2′−5′) at the tested linkage near the L6b loop is dispensable for P4−P6 RNA structure.

(22) Cate, J. H.; Gooding, A. R.; Podell, E.; Zhou, K.; Golden, B. L.; Kundrot, C. E.; Cech, T. R.; Doudna, J. A. *Science* **1996**, *273*, 1678−1685.
(23) Golden, B. L.; Gooding, A. R.; Podell, E. R.; Cech, T. R. *RNA* **1996**, *2*, 1295−1305.
(24) (a) Jayasena, S. D. *Clin. Chem.* **1999**, *45*, 1628−1650. (b) Hermann, T.; Patel, D. J. *Science* **2000**, *287*, 820−825. (c) Famulok, M.; Mayer, G.; Blind, M. *Acc. Chem. Res.* **2000**, *33*, 591−599.
(25) Joyce, G. F. *Nature* **2002**, *418*, 214−221.
(26) Joyce, G. F. *Methods Enzymol.* **2001**, *341*, 503−517.
(27) Li, J.; Zheng, W.; Kwon, A. H.; Lu, Y. *J. Am. Chem. Soc.* **2000**, *122*, 10466−10467.
(28) (a) Wu, Y.; Yu, L.; McMahon, R.; Rossi, J. J.; Forman, S. J.; Snyder, D. S. *Hum. Gene Ther.* **1999**, *10*, 2847−2857. (b) Santiago, F. S.; Khachigian, L. M. *J. Mol. Med.* **2001**, *79*, 695−706.

Practical RNA Ligation with the Newly Identified Deoxyribozymes. With appropriate RNA substrates, the new deoxyribozymes provide ligated RNA with useful rates and yields (Figure 4). Values for k_{obs} of ~0.2 h^{-1} at 37 °C and pH 7.5 (Figure 4C) are equivalent to maximal reaction in ~12 h, a condition achieved by several of the new DNA enzymes (e.g., 9A12 and 9A6). At pH 9.0, the new deoxyribozymes provide 40−50% yield of ligated RNA in about 1 h (k_{obs} ≈0.02−0.07 min^{-1}; Figure 4D), further demonstrating their utility for practical RNA ligation. The faster rate at higher pH may be counterbalanced by greater concern for nonspecific degradation, particularly for very large RNA substrates. This issue is being addressed in ongoing evaluations of the deoxyribozymes as RNA ligation reagents.

Although the observed ligation rates are slower than those found for most larger ribozymes that ligate RNA,[16] the deoxyribozymes have several significant advantages: they are more stable than RNA, they are much less expensive to synthesize, and they operate in the convenient and general intermolecular binding-arms format. The preliminary results with reselections indicate that we can expect orders of magnitude increases in the already-useful ligation rates over the deoxyribozymes that are characterized here. If the RNA substrate sequences that base-pair to the DNA binding arms are changed, then the deoxyribozymes retain ligation activity as long as Watson−Crick compensatory changes are made in the DNA binding arms (Figure 7). This is obviously of great importance for the general application of the deoxyribozymes. With fixed RNA substrates, shorter DNA binding arms are detrimental to both rate and yield (data not shown). This is expected if the affinity of the deoxyribozyme for the RNA substrates falls below a certain threshold. In general, we have had good success by choosing the DNA binding arm sequences such that the strength of each DNA−RNA interaction is $\Delta G^\circ \geq 12$ kcal/mol, as computed from published parameters.[29]

The broadest application of deoxyribozymes that ligate RNA would have minimal RNA substrate sequence constraints near the ligation junction. However, the deoxyribozymes currently in hand require either UAUA↓GGAA (for 9A12, 9A5, and 9A6) or UAUN↓GGAA (for 9A2, where N = purine is preferred). It is likely that some or all of these requirements originate in interactions between these specific nucleotides and those found within the deoxyribozyme itself. This possibly is currently being addressed experimentally, and we are hopeful that ongoing studies will find optimal deoxyribozyme motifs that reduce or eliminate these RNA substrate sequence requirements surrounding the ligation junction. Note that the current sequence requirements derive directly from the (largely arbitrary) choices of RNA substrate sequences that were used during the selection procedure. Therefore, in the worst case that the deoxyribozymes reported here cannot be made more general, repeating the selection with different RNA substrates would likely provide deoxyribozymes that require different nucleotides surrounding the ligation junction.

Mechanistic Considerations of the New Deoxyribozymes, Including 2′−5′ Regioselectivity. Protein enzymes that ligate nucleic acids require cofactors such as ATP, and they typically involve enzyme-bound intermediates.[6,7,30] Although the new deoxyribozymes require no cofactors other than Mg^{2+}, we do not yet know the mechanism(s) that they use to achieve RNA ligation. A detailed mechanistic understanding would be assisted by structural data from X-ray crystallography or NMR spectroscopy, although if the hammerhead ribozyme is any guide, even that may be insufficient to settle the issue.[31] Some important insights may be gleaned with data currently available. The control experiments with short complementary DNA splints (Figure 6) strongly suggest that proximity (chelate) effects[21] are *not* responsible for the majority of the ligation rate enhancement, because this enhancement is 320-fold for the fast 9A5 deoxyribozyme, using the shortest, exactly complementary DNA splint as the reference. It may be argued that this shortest possible DNA splint overestimates the appropriate background rate for the deoxyribozymes, because the splint enforces "too much" spatial constraint on the RNA substrates when compared with the deoxyribozymes. Instead, the T_3 and T_4 splints or the N_{40} parent deoxyribozyme strand may be a more appropriate reference. The rate enhancement for 9A5 compared with the latter splints is $>10^3$, clearly indicating that proximity effects are insufficient to explain the ligation activity. An even better background control reaction would use a deoxyribozyme mutated strategically to remove its catalytic competence, while still allowing it to bind the RNA substrates. This inactive deoxyribozyme would nevertheless enforce a spatial constraint on the reacting functional groups that is nearly identical to that enforced by the active DNA enzyme. The ratio of ligation rates of the active and inactive deoxyribozymes would correspond directly to the deoxyribozyme's rate enhancement, with little issue as to the appropriateness of the background reaction. We anticipate that such mutants will be identified as we further explore the deoxyribozymes reported here.

The observation that the new deoxyribozymes mediate RNA cleavage (Figure 8) in addition to ligation (Figure 4) is consistent with stabilization of the transition state for phosphodiester exchange, which increases the rate of both ligation and cleavage. In this context, in ligation experiments starting with the separate RNA substrates, we observe—in part—the approach to equilibrium. However, this cannot be a complete explanation, because the ratio of k_{obs}(ligation) to k_{obs}(cleavage) is ≥7 for all of the deoxyribozymes; e.g., this ratio is 43 for 9A12. Therefore, the new deoxyribozymes are truly "RNA ligases", in that they favor ligation over cleavage both thermodynamically and kinetically. Because multiple turnover was not observed, the ligated RNA products apparently remain bound to the deoxyribozyme. Product inhibition is also observed for protein enzymes that ligate RNA, such as T4 DNA ligase.[6] Because our assay does not require that the ligated RNA product is released from the deoxyribozyme, interactions between the RNA and the deoxyribozyme could affect the ligation thermodynamics or kinetics (or both). In the hammerhead and hairpin ribozymes, interactions between the ribozyme and RNA substrate contribute to the position of equilibrium between cleaved and ligated RNA.[32] It is likely that such considerations are important for the deoxy-

(29) Sugimoto, N.; Nakano, S.; Katoh, M.; Matsumura, A.; Nakamuta, H.; Ohmichi, T.; Yoneyama, M.; Sasaki, M. *Biochemistry* **1995**, *34*, 11211−11216.

(30) Lehman, I. R. *Science* **1974**, *186*, 790−797.

(31) (a) Wang, S.; Karbstein, K.; Peracchi, A.; Beigelman, L.; Herschlag, D. *Biochemistry* **1999**, *38*, 14363−14378. (b) Murray, J. B.; Scott, W. G. *J. Mol. Biol.* **2000**, *296*, 33−41.

(32) (a) Hertel, K. J.; Herschlag, D.; Uhlenbeck, O. C. *Biochemistry* **1994**, *33*, 3374−3385. (b) Fedor, M. J. *Biochemistry* **1999**, *38*, 11040−11050. (c) Fedor, M. J. *J. Mol. Biol.* **2000**, *297*, 269−291.

ribozymes described here, although detailed deconvolution of these effects will require significant further effort.

It is interesting that no consensus emerged from the limited number of deoxyribozyme clones that have been sequenced (Figure 5). Selections for other nucleic acid enzymes often provide sequences that clearly segregate into "families", but this may require compilation of many more sequences than we currently have in hand. Our data suggest that there are many ways for deoxyribozymes to enhance RNA ligation, at least at the level of primary DNA sequence. This is consistent with previous studies, in which (for example) at least 12 different classes of RNA-cleaving ribozymes were isolated from random RNA pools,[33] and 62 different RNA-cleaving DNA enzymes did not segregate into obvious sequence families.[13] The DNA sequences obtained so far bear no evident relationship to those of other deoxyribozymes, including the 10−23 or 8−17 DNA enzymes that cleave RNA.[13,14] More experimental characterization of individual deoxyribozymes, including more sequence analysis, will help to identify the mechanistic origin of their ligation activity.

A salient feature of the new deoxyribozymes is that they regioselectively create 2′−5′ phosphodiester linkages but not 3′−5′ linkages (Figure 3). The same strong preference is observed in the low-yielding cleavage reaction (Figure 8). Therefore, the new deoxyribozymes clearly mediate access to a transition state for ligation or cleavage that involves only the 3′-hydroxyl departing from or attacking the phosphorus center, respectively. This regioselectivity is surprising because we originally expected a mixture of 2′−5′- and 3′−5′-linked RNA from ligation (Figure 1B); we had further hoped that some DNA enzymes would be selective for one linkage and some for the other. However, the experimental data indicate otherwise. Any particular mechanistic explanation would be speculative at this point, but the absence of detectable 3′−5′-linked RNA product suggests a mechanistic imperative during the reaction, perhaps due to an in-line attack requirement at phosphorus. Others have observed a strong propensity for 2′−5′ phosphodiester formation in most (but not all[19]) nonenzymatic template-directed polymerizations of activated monoribonucleotides.[34] However, the relationship of these reactions to deoxyribozyme-mediated RNA ligation is uncertain, and we are wary of making unwarranted mechanistic comparisons.

Efforts toward Deoxyribozyme-Mediated 3′−5′ RNA Ligation. Clearly, deoxyribozymes that create native 3′−5′ phosphodiester linkages (rather than the 2′−5′ linkages obtained here) would be particularly valuable for synthesizing site-specifically modified RNAs. More complicated approaches to obtaining such deoxyribozymes were considered in the framework of the present selection strategy. We recognized from the outset that a mixture of 2′−5′- and 3′−5′-linked RNA was probable (Figure 1B). Extensive efforts were thus made to use a positive selection step to obtain ligases that are specifically 3′−5′. This was done by cleaving each selection round's ligated product—i.e., the product of step B of Figure 1—with an appropriate 8−17 deoxyribozyme. This DNA enzyme is selective for 3′−5′ linkages (Figure 3A) and should cleave at the newly created A↓G junction if and only if it is 3′−5′. However, because the ligation reaction is reversible (Figure 1B), we always obtained much more cleavage (up to 50% in some cases) than should have been possible if only <5% of the selected pool had 3′−5′-linked RNA (data not shown). Apparently the intramolecular binding of the deoxyribozyme to the newly ligated RNA product cannot be sufficiently outcompeted by intermolecular binding of the 8−17 DNA enzyme, even when >1000-fold excess of the 8−17 strand is added. Indeed, analysis of the products of these positive selection procedures indicated that >95% of the RNA was linked 2′−5′, no different than when the positive selection step was omitted. To date, we have not established a successful positive selection approach specifically to isolate 3′−5′ ligases in the context of this selection strategy.

We also considered the option of applying a negative selection step, in which any newly formed 2′−5′ linkages would be destroyed before the selection round is completed. However, it is unclear how to do this efficiently enough to overcome the observed strong 2′−5′ preference, whatever its mechanistic origin. There are apparently no known 2′−5′-selective phosphodiesterase protein enzymes that could be used. The selective cleavage of 2′−5′-linked RNA in the presence of the DNA complement at high pH and [Mg^{2+}] (Figure 3B) is slow ($t_{1/2} \approx$ 11 h; ref 19 and Figure 3B) and also relatively inefficient, with the extent of reaction at 24 h usually <75%. Intramolecular competition from the deoxyribozyme would also inhibit binding of the complement and provide a nonselective cleavage reaction, as described above for positive selection. Given the overwhelming preponderance during selection of 2′−5′ linkages (>95%; Figure 3C), it seemed very unlikely that a negative selection approach would provide enough selective pressure against 2′−5′ linkages, so it was not attempted.

More qualitative changes to the selection design to achieve 3′−5′ ligation are also possible. First, if the new phosphodiester linkage were formed using a nucleophile on the left-hand RNA substrate (e.g., by attack of a 3′-hydroxyl on a 5′-triphosphate of the right-hand RNA substrate), then the structural ambiguity during bond formation (Figure 1B) could potentially be resolved. Selections employing such alternative ligation reactions are in progress (Y.W., R.L.C., and S.K.S., unpublished results). Second, the known deoxyribozymes that cleave RNA do so selectively for 3′−5′ linkages in favor of 2′−5′ linkages (refs 13 and 14 and Figure 3). In principle, these RNA-cleaving deoxyribozymes could be evolved into RNA ligases, rather than selecting for ligation activity from completely random DNA pools as described here. Our experimental efforts along these lines are reported elsewhere (A.F.-C., K.A.H., and S.K.S., manuscript in preparation).

Conclusions

An in vitro selection strategy has identified deoxyribozymes that ligate RNA with formation of 2′−5′ phosphodiester linkages. The new deoxyribozymes provide preparatively useful amounts of ligated RNA under practical conditions (e.g., 40−50% yield in 1 h at 37 °C and pH 9.0). Currently, sequence restrictions for the RNA substrates encompass several nucleotides surrounding the ligation site (UAUA↓GGAA or UAUN↓GGAA), but more remote nucleotides may be changed freely. One of the new deoxyribozymes has been applied to study a structured RNA, the P4−P6 domain of the *Tetrahymena*

(33) Tang, J.; Breaker, R. R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5784−5789.
(34) (a) Orgel, L. E.; Lohrmann, R. *Acc. Chem. Res.* **1974**, *7*, 368−377. (b) Sawai, H. J. *J. Am. Chem. Soc.* **1976**, *98*, 7037−7039. (c) Lohrmann, R.; Orgel, L. E. *Tetrahedron* **1978**, *34*, 853−855.

group I intron RNA. In addition to providing interesting new examples of nucleic acid catalysts, deoxyribozymes that ligate RNA are anticipated to be a valuable practical complement to protein-mediated splint ligation for preparation of site-specifically modified RNAs.

Experimental Section

Nucleic Acid Substrates, Primers, and Standards. In the selection strategy (Figure 1A), the sequence of the left-hand RNA bearing the 2′,3′-cyclic phosphate was 5′-UAAUACGACUCACUAUA-3′. The sequence of the right-hand RNA bearing a 5′-hydroxyl was 5′-GGAAGAGAUGGCGACGG-3′. The 83-mer deoxyribozyme strand was 5′-CGAAGTCGCCATCTC-N_{40}-GTGAGTCGTATTAAGCTGATC-CTGATGG-3′, where the underlined regions are complementary to the right- and left-hand RNA substrates, respectively, and N denotes an equal probability of incorporating any of the four deoxynucleotides, corrected for unequal coupling efficiencies of the four phosphoramidites. The deoxyribozyme strand was prepared at the UIUC Biotechnology Center. The DNA primer 1t that is complementary to the 3′-end of the deoxyribozyme strand (where t indicates the PEG-containing "tail" through which *Taq* polymerase cannot read) was prepared at the UIUC Biotechnology Center with sequence 5′-$(AAC)_4X_2$CCATCAGGA-TCAGCT-3′, where X denotes the Glen Spacer 9 (PEG) monomer. The DNA primer 1 [same sequence as primer 1t, but without the tail; i.e., not including the $(AAC)_4X_2$ sequence] was prepared at IDT. The DNA primer 2 complementary to the 5′-end of the deoxyribozyme strand with sequence 5′-CGAAGTCGCCATCTC-3′ was prepared at IDT. Phosphorylated primer 2 was prepared at the UIUC Biotechnology Center with incorporation of the 5′-phosphate during solid-phase synthesis. The 17-mer right-hand RNA substrate was prepared by solid-phase synthesis at Dharmacon, Inc. (Lafeyette, CO). The 17-mer left-hand RNA substrate was prepared by 10−23 deoxyribozyme cleavage[13] of a 26-mer precursor RNA (sequence as above, plus ...UGGGUGCGA-3′, obtained from Dharmacon). The 34-mer RNA standards corresponding to the ligated left-hand plus right-hand RNAs were prepared at Dharmacon (3′−5′) or at Xeragon (Germantown, MD; 2′−5′). All DNA and RNA oligonucleotides were purified by 8%, 12%, or 20% denaturing PAGE as appropriate, with 1× TB (89 mM each Tris and boric acid, pH 8.3) as the running buffer.

Selection Strategy. The selection strategy is shown in Figure 1A. Details of the selection procedure were as follows:

Step A. The initial selection pool was prepared by ligation of 1 nmol (excess) of right-hand 17-mer RNA substrate (R) to 200 pmol of phosphorylated deoxyribozyme strand (∼12.5 pmol of which was 5′-^{32}P-radiolabeled; remainder cold-phosphorylated) with 20 units of T4 RNA ligase in a 40-μL reaction (typically >80% yield; purification by 8% denaturing PAGE). In subsequent selection rounds, 40−80 pmol of R was ligated to the selected deoxyribozyme pool with 10 units of T4 RNA ligase in a 20-μL reaction in the same manner.

Step B. For the first selection round, 400 pmol of 17-mer left-hand RNA substrate (L, with 2′,3′-cyclic phosphate) was incubated with ∼200 pmol of ligated right-hand substrate (R + deoxyribozyme strand) in 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$ at 37 °C for 13 h in a 40-μL reaction. The reaction was prepared as follows. The nucleic acids were annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min in a 30 μL volume containing 4 mM HEPES, pH 7.5, and 0.1 mM EDTA. Then, all components except Mg^{2+} were added to 38 μL and the sample was equilibrated at 37 °C for 2 min. Finally, $MgCl_2$ was added to 40 mM from a 10× stock and the sample was fully immersed in a 37 °C water bath. For the Ca^{2+} selections, $CaCl_2$ replaced $MgCl_2$. Successfully ligated products were separated by 8% denaturing PAGE, using as a standard the ligation product prepared by ligating a 34-mer (L + R) RNA to the phosphorylated deoxyribozyme strand with T4 RNA ligase. In subsequent selection rounds, 40−80 pmol of L was used in a 20-μL reaction in the same manner.

Step C. PCR amplification of the deoxyribozyme strand was performed in two steps. First, 10 cycles of PCR were performed on the selection products in a 100-μL reaction with 50 pmol of primer 1t, 100−200 pmol of phosphorylated primer 2, 3 mM $MgCl_2$, 0.2 mM each dNTP, 5 units of *Taq* polymerase (PCR conditions: 94 °C for 1 min, then cycles of 94 °C for 30 s, 47 °C for 30 s, and 72 °C for 20 s). Second, 30 cycles of PCR were performed on 0.5−1 μL of the 10-cycle PCR product in a 50-μL reaction with [α-^{32}P]dCTP, 25 pmol of primer 1t, 50−100 pmol of phosphorylated primer 2, 3 mM $MgCl_2$, 0.2 mM each dNTP, and 2.5 units of *Taq* polymerase as above. Products were separated by 8% denaturing PAGE and the lower band (originating from primer 2) was used as the input material for a new step A of the next selection round.

Cloning of Individual Deoxyribozymes. After nine rounds of selection, the 30-cycle PCR was performed as in step C above, with the following changes: only 1 μL of a 1/100 to 1/20 000 dilution of the 10-cycle PCR was used (the exact dilution was optimized for each cloning); the [α-^{32}P]dCTP was omitted; primer 1 was without the tail; and primer 2 was not phosphorylated. The resulting 100-bp double-stranded DNA product with single adenosine overhangs (added by *Taq* polymerase) was isolated by 2% agarose gel (QIAquick extraction, Qiagen). Individual deoxyribozymes were cloned by use of an Invitrogen TOPO TA kit. Miniprep DNA (Qiagen) was prepared and the presence of the expected deoxyribozyme insert was verified by *Eco*RI digestion and 2% agarose gel electrophoresis. Automated sequencing was performed at the UIUC Biotechnology Center. Deoxyribozymes for initial kinetic studies were prepared by PCR (YieldAce polymerase, Stratagene) directly from the miniprep DNA and quantified by radiolabeling with [γ-^{32}P]ATP and T4 polynucleotide kinase (PNK) in comparison with a concentration series of a standard oligonucleotide. For further kinetic studies, deoxyribozyme oligonucleotides of appropriate sequence were prepared by solid-phase synthesis (IDT).

RNA Ligation Assays with the New Deoxyribozymes. RNA ligation experiments with individual deoxyribozymes were performed in an intermolecular format, using the left-hand and right-hand RNA substrates (L and R) plus a deoxyribozyme (E). The [5′-^{32}P]L substrate was prepared by radiolabeling a 26-mer L precursor RNA with [γ-^{32}P]-ATP and T4 PNK and then cleaving with a 10−23 deoxyribozyme. Ligation experiments were performed with L:E:R ratios of (<1):3:6, typically (<5):15:30 pmol in 10-μL reactions. We routinely compared such experiments to those performed at ratios of 1:3:12 and 1:6:12, to verify that L was saturated with both E and R. Under these conditions, the observed single-turnover rates were not limited by the availability of E and R. It was also verified that L:E:R ratios of 6:3:(<1) gave similar results as experiments with L:E:R (<1):3:6. That is, it did not matter which RNA strand was radiolabeled and limiting. The 10-μL ligation reactions were prepared with 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$ as follows. The RNA substrates and deoxyribozyme were mixed in a volume of 7 μL containing 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min. Then, all components except Mg^{2+} were added to 9 μL and the sample was equilibrated at 37 °C for 2 min. Finally, $MgCl_2$ was added from a 10× stock solution and the sample was fully immersed in a 37 °C water bath. Aliquots of 1−2.5 μL were withdrawn at appropriate time points and quenched onto a solution of 80% formamide, 1× TB, and 50 mM EDTA containing 0.25% each bromophenol blue and xylene cyanol. The quenched aliquots were electrophoresed on 20% PAGE and imaged on a PhosphorImager.

Control Experiments to Assess Background Reaction Rate. These were performed in the same manner as the RNA ligation assays described above, except an appropriate DNA splint was used in place of a deoxyribozyme. For the exactly complementary DNA splint, the same DNA oligonucleotide as for the Mg^{2+}/pH 9 2′−5′ linkage assay (Figure 3B) was used.

RNA Cleavage Assays. These were performed in a similar manner as the RNA ligation assays. The 5′-^{32}P-labeled 34-mer RNA substrate strand was prepared by solid-phase synthesis and contained either a 3′−5′ or a 2′−5′ linkage at the site corresponding to the A↓G ligation junction. The cleavage assays were performed with an RNA substrate: deoxyribozyme ratio of 1:3, typically 5:15 pmol in 10-μL reactions.

Linkage Assays (3′−5′ or 2′−5′). As described in the Results, 3′−5′ and 2′−5′-linked ligated RNA were distinguished with two complementary assays (Figure 3). The 8−17 deoxyribozyme cleaves only 3′−5′-linked RNA, while hydrolysis at 100 mM Mg^{2+} and pH 9 in the presence of the DNA complement selectively cleaves 2′−5′-linked RNA. These assays were applied to the uncloned selection products from the round 9 DNA pool (product of step B of Figure 1A). Because both assays required hybridization of either an 8−17 deoxyribozyme or a DNA complement to the RNA, it was necessary to remove the intramolecularly competing deoxyribozyme strand before proceeding. This was done by 5′-^{32}P-radiolabeling (10 pmol of [γ-^{32}P]-ATP, 8 units of PNK, 37 °C, 15 min) and treatment with DNase (Promega; 0.4 unit, 37 °C, 45 min), followed by 20% PAGE. The DNase does not remove the last few DNA nucleotides 3′ of the junction with RNA, and as a result the ligated RNA product is slightly longer than the standard (L + R) RNA and has a ragged 3′-end, as seen in Figure 3C. To assay the RNA products from individual deoxyribozymes, ligated RNA was formed via the intermolecular reaction of Figure 4A and purified by 20% PAGE, then tested as for the uncloned pools (see Supporting Information for gel images). The 8−17 assay employed the radiolabeled RNA strand (<5 pmol) and 100 pmol of 8−17 deoxyribozyme, along with 5 pmol of unlabeled carrier RNA to prevent degradation of the small amount of labeled RNA. The Mg^{2+}/pH 9 hydrolysis assay used similar amounts of material; the full DNA complement was used in place of the 8−17 DNA enzyme.

Nondenaturing Gel Electrophoresis Experiments. These were performed as described previously.[1] For further details, see Supporting Information.

Acknowledgment. Supported by the Burroughs Wellcome Fund (New Investigator Award in the Basic Pharmacological Sciences to S.K.S.) and the UIUC Department of Chemistry. We are grateful to Terry Sheppard (Northwestern) and Ronald Breaker (Yale) for initial advice on the selection design. We thank Paul Hergenrother (UIUC) and members of the Silverman lab for discussions.

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.

JA028774Y