Zn^{2+}-Dependent Deoxyribozymes That Form Natural and Unnatural RNA Linkages†

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ABSTRACT: We report Zn^{2+}-dependent deoxyribozymes that ligate RNA. The DNA enzymes were identified by in vitro selection and ligate RNA with k_{obs} up to 0.5 min^{-1} at 1 mM Zn^{2+} and 23 °C, pH 7.9, which is substantially faster than our previously reported Mg^{2+}-dependent deoxyribozymes. Each new Zn^{2+}-dependent deoxyribozyme mediates the reaction of a specific nucleophile on one RNA substrate with a 2′,3′-cyclic phosphate on a second RNA substrate. Some of the Zn^{2+}-dependent deoxyribozymes create native 3′−5′ RNA linkages (with k_{obs} up to 0.02 min^{-1}), whereas all of our previous Mg^{2+}-dependent deoxyribozymes that use a 2′,3′-cyclic phosphate create non-native 2′−5′ RNA linkages. On this basis, Zn^{2+}-dependent deoxyribozymes have promise for synthesis of native 3′−5′-linked RNA using 2′,3′-cyclic phosphate RNA substrates, although these particular Zn^{2+}-dependent deoxyribozymes are likely not useful for this practical application. Some of the new Zn^{2+}-dependent deoxyribozymes instead create non-native 2′−5′ linkages, just like their Mg^{2+} counterparts. Unexpectedly, other Zn^{2+}-dependent deoxyribozymes synthesize one of three unnatural linkages that are formed upon the reaction of an RNA nucleophile other than a 5′-hydroxyl group. Two of these unnatural linkages are the 3′−2′ and 2′−2′ linear junctions created when the 2′-hydroxyl of the 5′-terminal guanosine of one RNA substrate attacks the 2′,3′-cyclic phosphate of the second RNA substrate. The third unnatural linkage is a branched RNA that results from attack of a specific internal 2′-hydroxyl of one RNA substrate at the 2′,3′-cyclic phosphate. When compared with the consistent creation of 2′−5′ linkages by Mg^{2+}-dependent ligation, formation of this variety of RNA ligation products by Zn^{2+}-dependent deoxyribozymes highlights the versatility of transition metals such as Zn^{2+} for mediating nucleic acid catalysis.

The discovery of naturally occurring ribozymes (RNA enzymes) demonstrated that proteins are not the only biomolecules that catalyze chemical reactions in nature (*1*−*4*). Although DNA has even fewer functional groups than RNA and although no naturally occurring deoxyribozymes (DNA enzymes) have been discovered, many deoxyribozymes have been identified by in vitro selection (*5*−*9*). The catalytic activities of known deoxyribozymes include RNA cleavage (*10*−*23*), DNA cleavage (*24*−*26*), DNA ligation (*27*, *28*), DNA phosphorylation (*29*−*31*), DNA capping (*32*), DNA deglycosylation (*33*), porphyrin metalation (*34*), and thymine dimer photoreversion (*35*). Deoxyribozymes are interesting both for their practical applications (*36*−*42*) and for their implications about the inherent catalytic capabilities of nucleic acids (*43*−*45*). Recently, we have reported many deoxyribozymes that ligate RNA (*46*−*56*). To date, all of our RNA ligase deoxyribozymes have been identified by in vitro selection with Mg^{2+} as the metal-ion cofactor. These DNA enzymes indeed require Mg^{2+} or Ca^{2+} for their activity, although Mn^{2+} is sometimes effective as well.

Many of the Mg^{2+}-dependent deoxyribozymes mediate RNA ligation via reaction of a 2′,3′-cyclic phosphate, which may be opened by an attacking nucleophile with cleavage of either the P−O$^{2'}$ bond or the P−O$^{3'}$ bond. If the nucleophilic group is a 5′-hydroxyl, then these two reaction pathways lead to the native 3′−5′ linkage or isomeric non-native 2′−5′ linkage, respectively (Figure 1). Of course, the attacking nucleophile is not necessarily a 5′-hydroxyl, but this functional group is typically the most nucleophilic of those present in RNA. Indeed, in our previous studies, all of the Mg^{2+}-dependent deoxyribozymes that use a 2′,3′-cyclic phosphate RNA substrate were found to create only non-native 2′−5′ linkages (path *ii* in Figure 1), although the selection strategy itself did not compel this high selectivity against 3′−5′ linkages (*46*−*50*). Therefore, understanding the distribution of ligation products upon DNA-catalyzed reaction of a 2′,3′-cyclic phosphate RNA substrate is of particular interest. In part, this interest is strengthened by the desire to use deoxyribozymes for the practical synthesis of native 3′−5′ RNA linkages, as a counterpart to other methods that are based on protein enzymes (*57*−*61*). Deoxyribozymes that synthesize native 3′−5′ RNA linkages will be valuable for preparation of large RNAs with site-specific modifications, among other likely applications.

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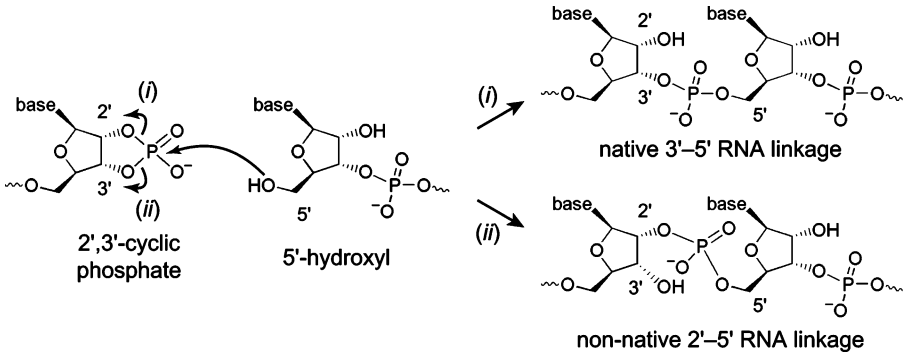

FIGURE 1: Two possible products from the ligation reaction of a 2′,3′-cyclic phosphate RNA substrate. Attack of the 5′-hydroxyl group of another RNA substrate leads to either a native 3′−5′ linkage or a non-native 2′−5′ linkage. When a nucleophile other than the 5′-hydroxyl group attacks the 2′,3′-cyclic phosphate, this forms an unnatural linkage that is neither 3′−5′ nor 2′−5′ (see the text).

Some deoxyribozymes specifically require transition metals instead of Mg^{2+} (*10*, *17*, *22*−*27*, *31*, *32*). In this study, we pursued RNA ligase deoxyribozymes that both use 2′,3′-cyclic phosphate RNA substrates and require Zn^{2+} instead of Mg^{2+}. Because each metal ion has its own ligand preferences, pK_a for bound water molecules, and other chemical properties, we anticipated that Zn^{2+} may mediate a different spectrum of chemical reactions than Mg^{2+}. Indeed, Zn^{2+}-dependent deoxyribozymes were collectively found to synthesize a variety of RNA linkages. This includes for the first time formation of the native 3′−5′ linkages that have been elusive using Mg^{2+} as the cofactor with cyclic phosphate substrates. Other newly identified Zn^{2+}-dependent deoxyribozymes synthesize non-native 2′−5′ linkages or unnatural linkages that are neither 3′−5′ nor 2′−5′. These results have both conceptual and practical implications for our ongoing efforts to understand and design metal-dependent nucleic acid catalysts.

EXPERIMENTAL PROCEDURES

Materials. DNA oligonucleotides were prepared at IDT (Coralville, IA). RNA oligonucleotides were prepared either by solid-phase synthesis (Dharmacon, Inc., Lafayette, CO, or HHMI−Keck Laboratory, Yale University, New Haven, CT) or by in vitro transcription using T7 RNA polymerase with an appropriate double-stranded DNA template (*62*, *63*). The terminal 2′,3′-cyclic phosphate of the left-hand RNA substrate was provided by cleaving a longer precursor RNA with an appropriate 10−23 deoxyribozyme (*14*), as described previously (*46*). All DNA and RNA oligonucleotides were purified by denaturing PAGE with running buffer 1× TBE [89 mM each tris(hydroxymethyl)aminomethane (Tris)[1] and boric acid and 2 mM ethylenediaminetetraacetic acid (EDTA) at pH 8.3] as described previously (*46*, *52*).

In Vitro Selection Procedure. We previously described the overall in vitro selection strategy (*46*, *47*). 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 2A). Immediately prior to this reaction, the right-hand RNA substrate was joined to the DNA using T4 RNA ligase, as denoted by the asterisk

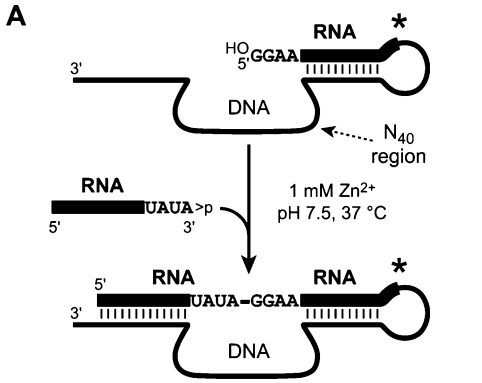

FIGURE 2: RNA ligation mediated by new Zn^{2+}-dependent deoxyribozymes. (A) Key Zn^{2+}-dependent step of each selection round. In the first round, the N_{40} deoxyribozyme region is fully random, whereas in subsequent rounds, this region is enriched in those 40-nucleotide DNA sequences that are competent for Zn^{2+}-dependent RNA ligation. During the selection procedure, the covalent connection (*) between the right-hand RNA substrate and the deoxyribozyme strand (as created by T4 RNA ligase) means that catalytically active DNA sequences attach the left-hand RNA substrate to themselves via the right-hand RNA substrate. Thus, the active DNA sequences are readily separable by PAGE; see our previous reports for details of the selection procedure (*46*, *47*). However, for application of the new deoxyribozymes, the covalent RNA−DNA connection is neither required nor present, and ligation occurs in a trimolecular format (see Figure 3A). (B) Nucleotide sequences of the new Zn^{2+}-dependent deoxyribozymes. The sequences correspond to the N_{40} enzyme region of the deoxyribozyme strand of (A); the DNA binding arm sequences are complementary to the RNA substrate sequences as listed in the the Experimental Procedures. The 12BB8 enzyme region is only 39 nucleotides long, reflecting a deletion during the selection process.

in Figure 2A (*46*, *47*). For the J selection, the incubation conditions during the key selection step were 70 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) at pH 7.5, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$ at 37 °C for 14 h. For the BB selection, the conditions were 70 mM Tris at pH 7.5, 150 mM NaCl, 2 mM KCl, and 1 mM

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; k_{obs}, observed rate constant; k_{bkgd}, background rate constant; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization.

$ZnCl_2$ at 23 °C for 3 h. The change from HEPES to Tris was made because Zn^{2+} is stabilized in solution by Tris (*64*, *65*). For details on the selection procedure other than the key selection step shown in Figure 2A, including cloning of the deoxyribozymes, see our earlier papers (*46*, *47*, *49*). The sequence of the left-hand (L) RNA substrate used during the selection was 5′-UAAUACGACUCACUAUA-3′, with a 2′,3′-cyclic phosphate provided by deoxyribozyme-mediated cleavage of a longer RNA that was prepared by solid-phase synthesis (*46*). The sequence of the right-hand (R) RNA substrate was 5′-GGAAGAGAUGGCGACGG-3′, prepared by solid-phase synthesis. For the BB selection, the 5′-terminal nucleotide of the R substrate was 2′-deoxy-G. For the assays of Figure 12, a specific nucleotide of the R substrate was 2′-deoxy as noted in the figure. For some assays, the L substrate began with an additional 5′-GGA... and continued with the sequence given above. This alternative L substrate was prepared by in vitro transcription from a double-stranded DNA template using T7 RNA polymerase, and this transcription procedure necessitated the addition of the extra nucleotides. In the selection reactions and kinetic assays, the Zn^{2+} was added from a 10× stock in 20 mM nitric acid and 200 mM Tris at pH 7.9 (or other buffer as appropriate to match the incubation conditions). Note that this Zn^{2+} solution contributes 20 mM to the final buffer concentration of 70 mM. The 10× Zn^{2+} stock solution was itself prepared from a stock solution of 100 mM $ZnCl_2$ in 200 mM nitric acid and a solution of 0.5−1.0 M Tris or HEPES adjusted to the appropriate pH.

Kinetic Characterization of Deoxyribozymes. All of the ligation assays used the trimolecular format shown in Figure 3A. The ^{32}P-radiolabeled left-hand RNA substrate L was the limiting reagent relative to the right-hand substrate R and deoxyribozyme E (the ratio L/E/R was ∼1:3:6 to 1:10:30, with the concentration of E ≈ 0.5−3 μM). Increasing the concentration of E or R (or both) did not significantly change the observed kinetics or yields, indicating that the observed yields were not limited by availability of E or R. Because of concerns over Zn^{2+} precipitation, we used Tris buffer for most characterization assays, rather than HEPES (see the Results). See our earlier paper for a detailed description of the sample preparations and method of analysis (*46*). Values of the observed rate constant (k_{obs}) and final yield were obtained by fitting the yield versus time data directly to first-order kinetics; i.e., yield $= Y(1 - e^{-kt})$, where $k = k_{obs}$ and $Y =$ final yield.

Because of the sensitive dependence of 6J12 on pH and Zn^{2+} concentration (Figure 4), we recommend to anyone using the deoxyribozymes reported here that a sensible range of pH values and Zn^{2+} concentrations should be tested empirically. For example, it would be reasonable to test pH values between 7.5 and 8.1 and Zn^{2+} concentrations between 0.5 and 2.0 mM. This will ensure that subtle differences in sample preparation and incubation conditions (e.g., temperature) do not result in suboptimal activities.

For the deoxyribozyme-catalyzed cleavage experiments of Figures 7 and 10, a typical procedure for the preparation of the ligation product (i.e., the substrate for each cleavage reaction) was as follows. A combination of 5′-^{32}P-radiolabeled L substrate (5 pmol), deoxyribozyme (15 pmol), and R substrate (30 pmol) was annealed in 14 μL of annealing buffer (5 mM Tris at pH 7.5 or 7.9 as appropriate, with 15 mM NaCl and 0.1 mM EDTA) by heating at 95 °C for 3 min and cooling on ice for 5 min. To the annealed sample was added 4 μL of 5× selection buffer and 2 μL of 10× $ZnCl_2$ stock solution, resulting in final concentrations of 50 mM Tris at pH 7.5 or 7.9, 150 mM NaCl, 2 mM KCl, and 1.0 mM $ZnCl_2$. The sample was incubated at 23 or 37 °C for 20 min to 24 h, quenched with 40 μL of stop solution (80% aqueous formamide, 1× TB [89 mM each Tris and boric acid at pH 8.3], 50 mM EDTA, and 0.25% each of bromophenol blue and xylene cyanol), and purified by denaturing 20% PAGE. For the cleavage assays, this product was then incubated separately with a deoxyribozyme as described above for the ligation assays; the ratio of ligated product to deoxyribozyme was <1:3.

Partial Alkaline Hydrolysis Assays. For the alkaline hydrolysis assays (denoted AH) in Figures 6, 8, and 9, the 5′-^{32}P-radiolabeled ligation product from each deoxyribozyme (∼0.25 pmol) was incubated in 10 μL of 50 mM $NaHCO_3$ at pH 9.2 and 0.1 mM EDTA at 90 °C in a dry-block incubator in an 0.7-mL eppendorf tube for 10 min. The sample was placed on ice and quenched with 15 μL of stop solution (see above), and 4 μL of this quenched sample was loaded per gel lane. For the experiments in which quantitative $t_{1/2}$ values were determined (see the Supporting Information), 10-μL samples were instead prepared in 0.2-mL thin-walled PCR tubes and incubated in the block of an MJ Research PTC-200 thermal cycler at either 60 or 90 °C as appropriate. At each timepoint, a 1.2-μL aliquot was withdrawn and quenched into 8 μL of stop solution (see above); 4 μL of this quenched sample was loaded per gel lane.

Assays for 3′−5′ and 2′−5′ Linkages. The linkage assays of Figures 6 and 9 using either the 8−17 deoxyribozyme (for 3′−5′ linkages) or 100 mM Mg^{2+} at pH 9 with the exactly complementary DNA oligonucleotide (for 2′−5′ linkages) were performed as described previously (*46*). The ligation products used as substrates for these assays were 5′-^{32}P-radiolabeled, as for the cleavage assays described above.

Preparation of the 6J12 Ligation Product Trimer for Mass Spectrometry. The sequences of the L and R substrates with all nonessential G nucleotides removed were 5′-CAAAUAC-CACUCACUAGA-3′ and 5′-GGAAACCUCACAUCCCAC-3′, with the 2′,3′-cyclic phosphate of L provided by 10−23 deoxyribozyme cleavage of a ...UGGGUGCGA-3′ tail. The preparative ligation was performed using 82.5 nmol of L, 75 nmol of 6J12, and 82.5 nmol of R in 1000 μL of total volume with 1 mM $ZnCl_2$ for 1 h at 23 °C, with purification on 20% denaturing PAGE. The isolated yield was ∼17 nmol of ligated product. This product was digested with RNase T1 (Ambion, 3000 units) in 1000 μL containing 1× of the manufacturer's buffer for 50 min at 23 °C. After RNase T1 digestion, the desired A↓GG trimer was separated from the longer, flanking RNA sequences by anion-exchange (DEAE) chromatography (*66*, *67*). The column was prepared with 4 mL of DEAE Bio-Gel agarose (Bio-Rad catalog number 153-0740) packed into a 10-mL plastic pipet and washed successively with 6 mL of water, 6 mL of high-salt buffer (1 M triethylammonium acetate [TEAA] at pH 7.0), and 12 mL of low-salt buffer (10 mM TEAA at pH 7.0). The sample (1 mL from the RNase T1 digestion diluted to 5 mL with water) was loaded and eluted with a stepwise gradient from

10 mM to 1 M TEAA, collecting fractions of 1 mL volume. As detected by UV absorbance at 260 nm of undiluted aliquots from each fraction, the trimer eluted near 350 mM TEAA and the longer flanking substrates eluted above 750 mM TEAA. The fractions containing the trimer were combined and evaporated under vacuum (SpeedVac) to provide the sample for mass spectrometry. To calculate concentrations for preparing mass spectrometry samples, the recovery was assumed to be quantitative based on material input into the RNase T1 digestion reaction. For the DEAE column, the use of TEAA itself as the high-salt component of the elution buffer was essential. Although 10 mM TEAA plus increasing concentrations of NH_4Cl successfully led to elution of the trimer, this also led to a substantial amount of an unidentified white residue remaining after evaporation.

Mass Spectrometry of the 6J12 Ligation Product Trimer. Electrospray ionization (ESI) mass spectrometry was performed in negative ion mode on a Q-Tof Ultima instrument using a trimer sample dissolved at 4 μM in buffer containing 5 mM each of imidazole and piperidine in 1:1 isopropyl alcohol/water (*68*). The parent mass calculated for 5′-AGG-3′ with a 2′(3′)-monophosphate $[M-H]^-$ was 1036.15, and the parent mass found was 1036.2 ± 0.1. See the Supporting Information for tabulation of MS/MS fragmentation data. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed in negative ion mode (linear) on a Voyager SE-DTR instrument using a trimer sample dissolved at 10 μM in water. The parent mass calculated $[M-H]^{-1}$ was 1036, and the parent mass found was 1037 ± 1. Data were obtained at the UIUC School of Chemical Sciences mass spectrometry laboratory.

Regrettably, similar mass spectrometry assays could not be performed on the 6J2 or 12BB8 ligation products. For 6J2, the preparative-scale yields are too low to be practical. For 12BB8, the deoxyribozyme does not tolerate the sequence changes necessary to remove all extraneous G nucleotides from the substrates (data not shown), and cleavage at these other Gs would likely interfere with separation of desired product by anion-exchange chromatography.

RESULTS

In Vitro Selection of Zn^{2+}-Dependent Deoxyribozymes that Ligate RNA. The selection strategy to identify Zn^{2+}-dependent RNA ligase deoxyribozymes was analogous to that applied previously to obtain Mg^{2+}-dependent DNA enzymes (*46*, *47*). In the key selection step, two RNA substrates (designated the "left-hand" and "right-hand" substrates, which are abbreviated simply L and R) were joined in a deoxyribozyme-mediated reaction. As shown in Figure 2A, the RNA and DNA were arranged as described in our first RNA ligase selection effort (*46*). Starting with of an N_{40} DNA pool, selection rounds were performed with 1 mM Zn^{2+} at pH 7.5 and 37 °C as the incubation conditions during the key step in which the two RNA substrates are joined. In this study, two separate but related selection procedures were performed. In the first of these efforts, denoted "J" in accordance with our ongoing nomenclature, the right-hand RNA substrate had a 5′-terminal guanosine nucleotide. In the second effort, denoted "BB", the right-hand RNA substrate instead had a 5′-terminal 2′-deoxyguanosine nucleotide. The missing 2′-hydroxyl in the BB selection prevents this particular functional group from acting as the nucleophile to attack the 2′,3′-cyclic phosphate, which data presented below indicate occurred during the J selection.

For the J selection effort, six rounds of selection were performed, each with a 14-h incubation during the key step. In round 6, the ligation activity of the pool as a whole was ~21%, which is comparable to the activity of the pools used to clone our previous Mg^{2+}-dependent RNA ligase deoxyribozymes (*46*, *47*). For the BB selection, 12 rounds were performed, each with a 3-h incubation during the key step; the ligation activity was ~35% at round 12. Individual deoxyribozymes were cloned from the round 6 (J) or 12 (BB) pools and characterized for their ability to ligate RNA. On the basis of preliminary assessment of ligation activity, four (J) or six (BB) specific clones were sequenced and resynthesized as DNA oligonucleotides by standard solid-phase methods. These clones were named (for example) 6J12, where 6 denotes the cloned round, J designates the selection experiment, and 12 is the clone number. The sequences of the new 6J and 12BB deoxyribozymes are shown in Figure 2B. Consistent with the lack of sequence alignment among these deoxyribozymes, the mfold program (*69*, *70*) did not predict any common secondary structure (data not shown).

Characterization of Zn^{2+}-Dependent RNA Ligation Activity. The deoxyribozymes were assayed for RNA ligase activity in a trimolecular, catalytic format (Figure 3A). Others have observed that Zn^{2+} must be used with care at basic pH values because of precipitation of zinc oxides (for example, see ref *71*). Chelator compounds such as Tris and bis-Tris have been used to bind and stabilize transition metals such as Zn^{2+} in solution (*64*, *65*). We therefore performed the kinetic assays in Tris buffer, in which the results were reproducible (some difficulties with reproducibility were evident in HEPES buffer; data not shown). Representative kinetic data for the four 6J deoxyribozymes are shown in Figure 3, using standard incubation conditions of 70 mM Tris at pH 7.9 and 23 °C with 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$; the pH was chosen on the basis of experiments described below. Although the J pool was allotted 14 h for the reaction during each selection round (Figure 2A), several of the individual 6J deoxyribozymes require much less time for effective RNA ligation. The fastest 6J DNA enzyme, 6J12, has $k_{obs} = 0.5\ min^{-1}$ under the standard conditions; maximal ligation is observed in merely 5−10 min. Reactions at 37 °C generally provided no improvement in yield compared with 23 °C. However, 6J2 is higher yielding at 37 °C than at 23 °C (see the Supporting Information), although its ligation yield is modest at either temperature. For 6J12, variation of [NaCl] or [KCl] from 0 to 300 mM has very little effect on the ligation activity (data not shown).

Because 6J12 has a particularly high k_{obs} value, we focused substantial effort on its characterization. The pH dependence of 6J12 ligation activity was analyzed between pH 7.1 and 8.5 at 1 mM Zn^{2+} (Figure 4A). The ligation activity exhibited a sharp pH dependence, with maximal activity near pH 7.9. Because of the very sensitive pH dependence and the limitation on high pH as a result of zinc oxide precipitation, it was not feasible to obtain a quantitatively meaningful rate-versus-pH analysis. At pH 7.9, the Zn^{2+} concentration

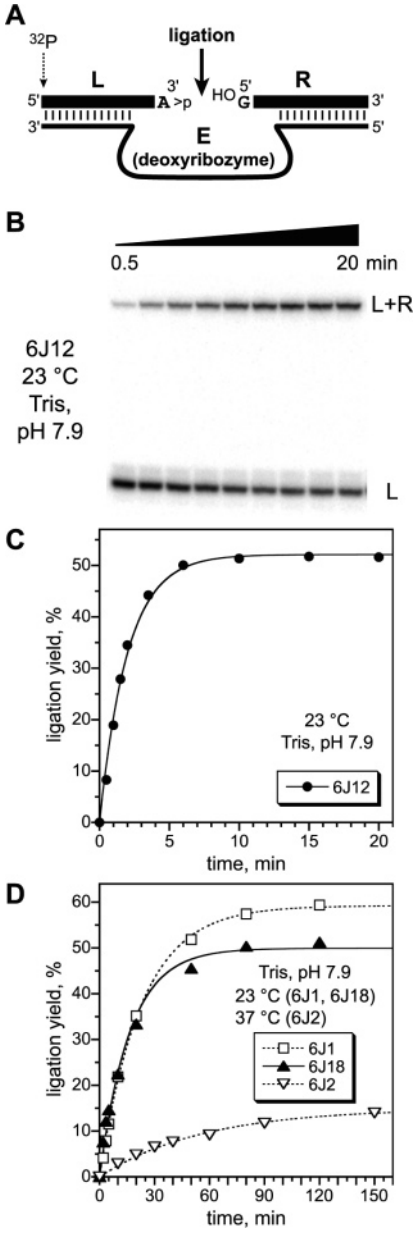

FIGURE 3: RNA ligation activity of the Zn^{2+}-dependent 6J deoxyribozymes. (A) Trimolecular format of the ligation assay. (B) Representative 20% PAGE image for RNA ligation as mediated by the 6J12 deoxyribozyme (70 mM Tris at pH 7.9, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$ at 23 °C). (C and D) Kinetic plots for 6J12, 6J1, 6J18, and 6J2 under the conditions of (B), except at 37 °C for 6J2. See Table 1 for k_{obs} values. For 6J2, an additional data point at 240 min falls directly on the fit line but was omitted for clarity. See the Supporting Information for full data sets for each of these deoxyribozymes.

dependence of 6J12 ligation activity was examined in detail. A sharp drop in 6J12 ligation yield was observed when the Zn^{2+} concentration was either raised or lowered from its optimal value of ~1.0 mM (Figure 4B). Finally, we established that the 6J deoxyribozymes are truly Zn^{2+}-dependent by assaying the ability of other metal ions to mediate RNA ligation. All four of the 6J deoxyribozymes were completely inactive when tested with Mg^{2+} instead of Zn^{2+} (<0.5% ligation in 24 h at 40 mM Mg^{2+}, pH 7.5, and 37 °C; data not shown). For 6J12, ligation assays with Ni^{2+}, Co^{2+}, Cd^{2+}, or Cu^{2+} at 10 μM, 100 μM, 1 mM, or 10 mM gave in each case no observable ligation activity even upon

Table 1: Summary of Ligation Rates and RNA Linkages Formed by the Zn^{2+}-Dependent Deoxyribozymes[a]

deoxyribozyme	k_{obs} (min^{-1})	RNA linkage[b]
6J12	0.50	linear 3′−2′ or 2′−2′
6J1	0.044	same as 6J12
6J18	0.061	same as 6J12
6J2[c]	0.018	linear 3′−2′ or 2′−2′ (whichever is not formed by 6J12)
12BB1	0.056/0.060[d]	linear 2′−5′ (non-native)
12BB2	0.077/0.060	linear 2′−5′ (non-native)
12BB5	0.020/0.013	linear 3′−5′ (native)
12BB12	0.011/0.0039	linear 3′−5′ (native)
12BB6	0.065/0.046	2′,3′-branch or 2′,2′-branch
12BB8	0.107/0.056	2′,3′-branch or 2′,2′-branch

[a] Incubation conditions were 70 mM Tris at pH 7.9 (for the 6J deoxyribozymes) or pH 7.5 (for the 12BB deoxyribozymes), 150 mM NaCl, and 2 mM KCl at 23 °C. See Figures 3 and 5 for representative kinetic data. [b] See Figures 1 and 11 for linkage structures, and see the text for an explanation. [c] The k_{obs} value for 6J2 was obtained at 37 °C instead of 23 °C. [d] The two k_{obs} values are given for ligation using right-hand RNA substrates with 5′-dG and 5′-G, respectively (see the text and Figure 5 for details).

extended incubation (Figure 4C and the Supporting Information). For 6J1 and 6J18, analogous assays also revealed no ligation activity for metal ions other than Zn^{2+}. Because of its relatively low ligation yield even under optimal incubation conditions with Zn^{2+}, 6J2 was not tested comprehensively with regard to its metal dependence.

Analogous kinetic studies were performed for the 12BB deoxyribozymes (Figure 5). These experiments were conducted under the BB selection conditions of pH 7.5, 1 mM Zn^{2+}, and 23 °C, and substantial ligation activity was observed in all cases. The effects of varying the pH and Zn^{2+} concentrations were not tested for these deoxyribozymes, although their inactivity with Mg^{2+} was verified (<0.5%; data not shown). For 12BB6 and 12BB8, the ligation rate and yield were equivalent for right-hand RNA substrates with either G or 2′-deoxy-G (dG) as the 5′-terminal nucleotide (Figure 5, filled versus open symbols). In contrast, for the other four 12BB deoxyribozymes, the ligation yield was higher for the R substrate with a 5′-terminal dG, which was the substrate used during the selection itself.

Background Reaction Assays. Before analyzing the structures of the RNA linkages created by the new deoxyribozymes, we examined the background ligation reactions that occur when the RNA substrates are annealed to an exactly complementary DNA splint instead of a deoxyribozyme. These data provide a baseline for understanding the inherent preferences in ligation reactions of 2′,3′-cyclic phosphates. At pH 7.9, 1 mM Zn^{2+}, and 23 °C, merely ~3% RNA ligation was observed in 12 h with the splint ($k_{bkgd} \approx 3 \times 10^{-5}$ min^{-1} under the standard incubation conditions; see the Supporting Information). This value for k_{bkgd} is similar to that observed with Mg^{2+} under similar conditions (*46*), whereas essentially no reaction is observed when divalent ions are omitted altogether (1 mM EDTA).

We assayed the small amount of Zn^{2+} background ligation product to determine the structure of its linkage. Using two previously described approaches (*46*), the background product was incubated either with the 8−17 deoxyribozyme (*14*) [which selectively cleaves native 3′−5′ linkages (*46*)] or at 100 mM Mg^{2+} and pH 9 with the exactly complementary DNA oligonucleotide [which selectively cleaves non-native

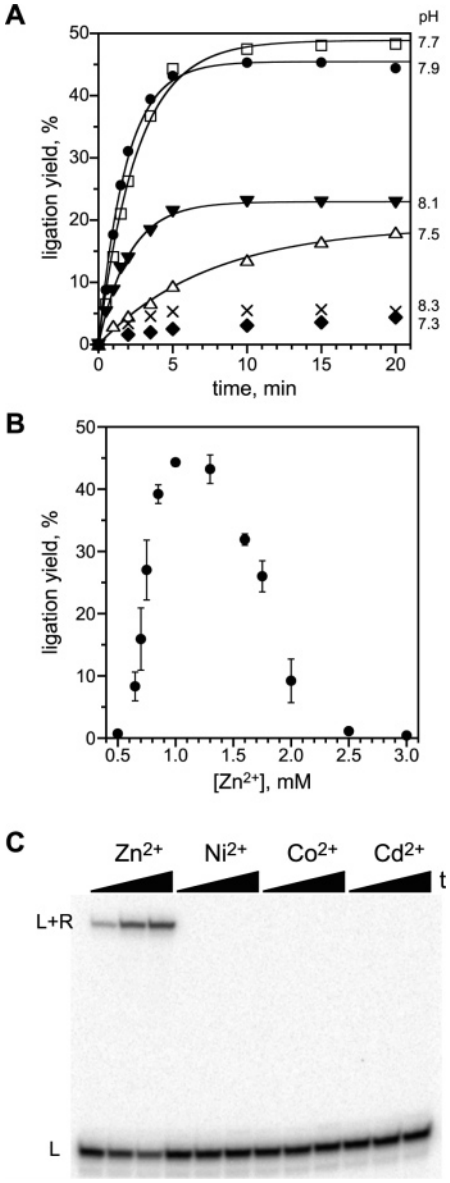

FIGURE 4: Dependence of 6J12 ligation activity on pH, Zn^{2+} concentration, and metal-ion identity. (A) pH dependence of 6J12 at 23 °C. The pH was maintained with 70 mM Tris at the indicated values; the other solution components were 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$. At pH 7.1 or 8.5, there was no detectable ligation product (<0.5% yield at 20 min). k_{obs} values (min^{-1}): pH 7.5, 0.13; pH 7.7, 0.39; pH 7.9, 0.54; and pH 8.1, 0.48. (B) [Zn^{2+}] dependence of 6J12 activity (70 mM Tris at pH 7.9, 150 mM NaCl, and 2 mM KCl at 23 °C). Yields are the average ± standard deviation of three determinations at each Zn^{2+} concentration. (C) Metal-ion dependence of 6J12 activity. The RNA ligation reaction was performed under standard conditions (70 mM Tris at pH 7.9, 150 mM NaCl, and 2 mM KCl at 23 °C) in the presence of either 1 mM $ZnCl_2$, $NiCl_2$, $CoCl_2$, or $CdCl_2$. For the Zn^{2+} assay, timepoints were taken at 0.5, 2, and 10 min; for the other assays, timepoints were taken at 0.5 min, 4 h, and 24 h. For a more comprehensive version of the experiment showing data for all of these ions and also for $CuCl_2$ from 10 μM to 10 mM, see the Supporting Information.

2′−5′ linkages (*46*, *72*)]. The results clearly reveal that the Zn^{2+} background product is a 2′−5′ phosphodiester linkage (parts A and B of Figure 6). The same outcome is also found upon testing the Mg^{2+} and Mn^{2+} background products, which suggests an inherent preference for forming 2′−5′ linkages upon uncatalyzed, templated reaction of a 5′-hydroxyl with a 2′,3′-cyclic phosphate. This preference is independent of which divalent metal ion is present, although the presence of a metal is required for observable reaction.

The identification of the Zn^{2+}, Mg^{2+}, and Mn^{2+} background products as 2′−5′ was further supported by partial alkaline hydrolysis and RNase T1 digestion assays (Figure 6C), which were later applied to the products of the new Zn^{2+}-dependent deoxyribozymes (see below). A conventional alkaline hydrolysis assay generates a regular "ladder" by random cleavage of RNA along its backbone. This occurs by attack of an individual 2′-hydroxyl on its adjacent phosphodiester linkage (technically, this is transesterification and not hydrolysis). An unbroken ladder for each background product confirmed its linearity, because a branched product would have a characteristic gap within the ladder (*51*, *73*). RNase T1 cleaves specifically after G nucleotides, providing bands that align with the appropriate counterparts in the alkaline hydrolysis ladder. Digestion of each background product provides the expected G-specific bands. However, the band corresponding to cleavage after G of A↓G (where the arrow denotes the ligation site) is very faint, as observed for RNase T1 digestion of the 2′−5′ standard RNA but not for the 3′−5′ standard RNA, which cleaves normally.

The Four 6J Deoxyribozymes Create Two Different RNA Linkages. In addition to the ligation kinetics and yield, the other major issue for the products from the new deoxyribozymes is of course their linkage. Ring-opening reactions of 2′,3′-cyclic phosphates are inherently reversible, because the 2′-hydroxyl or 3′-hydroxyl leaving group remains covalently attached after opening (Figure 1). To provide information on the structural relationships among the various ligation products, we first determined the extent to which each of the four 6J deoxyribozymes can cleave its own product and also the products from the other three deoxyribozymes. This experiment is important for determining the absolute number of different ligation products created by the collection of deoxyribozymes. The 5′-^{32}P-radiolabeled product from each of the four 6J deoxyribozymes was incubated separately with each of the four 6J deoxyribozymes under the standard incubation conditions used for ligation, and the cleavage progress for each of these $4 \times 4 = 16$ experiments was monitored (Figure 7). The 6J12 deoxyribozyme cleaved the ligation products from the 6J12, 6J1, and 6J18 deoxyribozymes with experimentally indistinguishable rates ($k_{obs} \approx 0.2$ min^{-1} and ~40% final yield in all three cases), whereas the 6J2 product was barely cleaved (≤5% yield). Similarly, the 6J1, 6J18, and 6J2 DNA enzymes each showed an unambiguous distinction between their cleavage abilities for the 6J12/6J1/6J18 products and the 6J2 product. The simplest explanation for these data is that the four 6J deoxyribozymes collectively create just two different RNA linkages. Specifically, 6J12, 6J1, and 6J18 all create the same linkage (hereafter termed merely the "6J12 product"), which is distinct from the 6J2 product. This conclusion greatly assists the subsequent experiments, because only two 6J ligation products instead of four need to be assayed in greater detail.

RNA Linkages Created by the 6J Deoxyribozymes Are Neither 3′−5′ Nor 2′−5′. Several bioichemical assays were used to provide more information on the two RNA linkages formed by the 6J deoxyribozymes. As shown in Figure 8, the 5′-^{32}P-radiolabeled 6J12 and 6J2 RNA ligation products

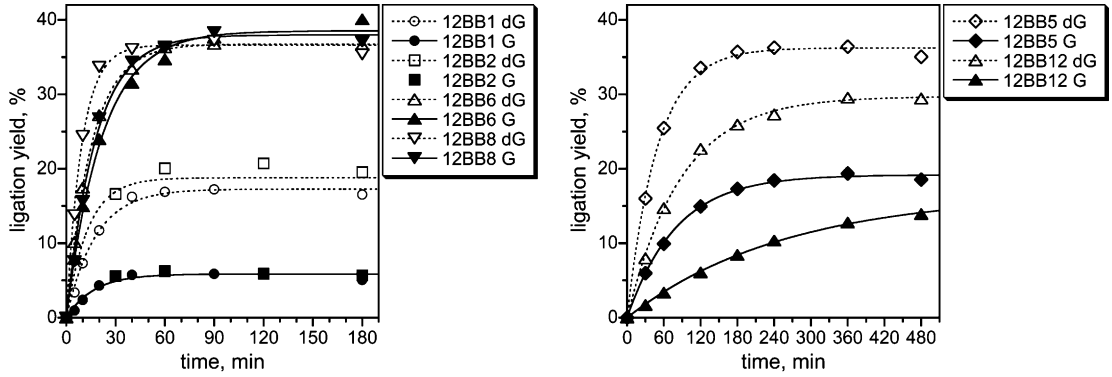

FIGURE 5: Ligation activities for individual 12BB deoxyribozymes. Incubation conditions were 70 mM Tris at pH 7.5, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$ at 23 °C. The 5′-terminal nucleotide of the right-hand RNA substrate was either G (filled symbols) or 2′-deoxy-G (dG; open symbols). See Table 1 for k_{obs} values.

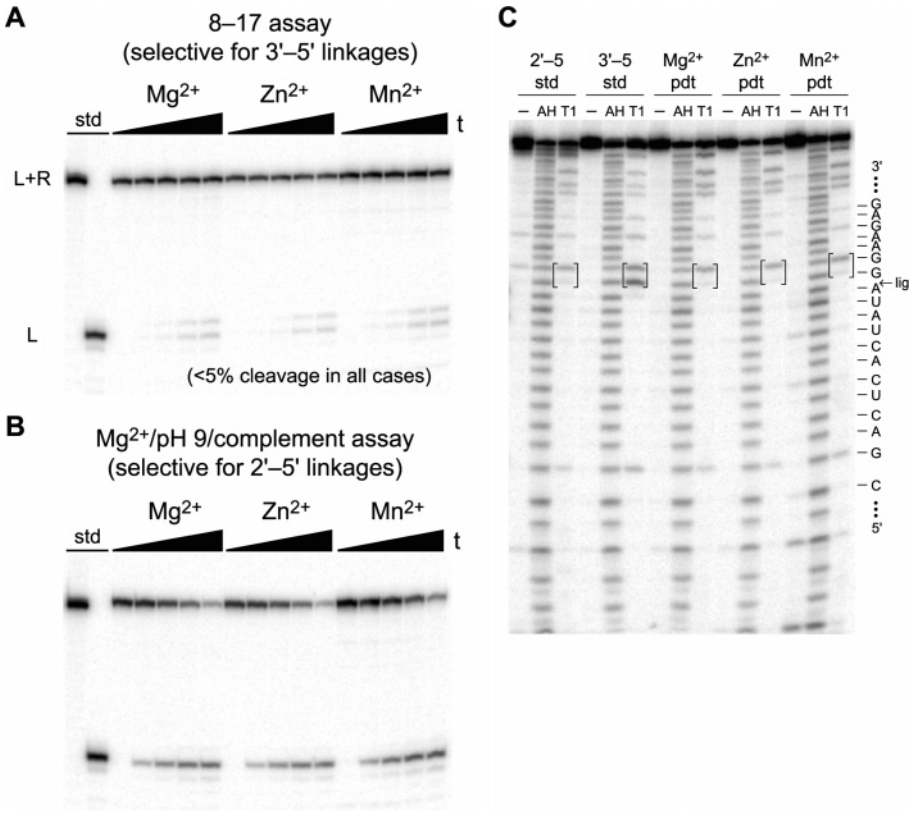

FIGURE 6: Analysis of the background ligation products formed with 1 mM Zn^{2+} (or 40 mM Mg^{2+} or Mn^{2+}) and an exactly complementary DNA splint instead of a deoxyribozyme. (A) Cleavage assay with the 8−17 deoxyribozyme, which selectively cleaves 3′−5′ linkages (t = 0−7 h). (B) Cleavage assay at 100 mM Mg^{2+} and pH 9.0 in the presence of the exactly complementary DNA oligonucleotide, which selectively cleaves 2′−5′ linkages (t = 0−7 h). See ref *46* for the assays of (B) and (C) performed on standard 3′−5′-linked and 2′−5′-linked RNAs as control experiments that validate this approach. (C) Partial alkaline hydrolysis (AH; 50 mM $NaHCO_3$ at pH 9.2 and 90 °C for 10 min) and RNase T1 digestion assays. The brackets mark key bands that distinguish 3′−5′ from 2′−5′ linkages in RNase T1 digestion. The lower of the two bracketed bands observed for the 3′−5′ RNA is present only in greatly reduced intensity for the 2′−5′ RNA. This is because RNase T1 has difficulty cleaving after the G of A↓GG when the indicated ligation junction is 2′−5′, although cleavage after the next G to the 3′ side is unaffected (upper bracketed band).

were analyzed using the partial alkaline hydrolysis and RNase T1 digestion assays that were previously applied to the background ligation products in Figure 6C. Upon alkaline hydrolysis, the 6J12 and 6J2 ligation products quickly revert to the left-hand substrate RNA as the clearly dominant band, instead of providing a nonspecific ladder (Figure 8; a secondary degradation ladder is apparent). This indicates that the ligation products are unusually base-labile at the newly formed bond. Complete reversion to the left-hand substrate occurs with $t_{1/2} \approx 2$ min at 90 °C (data not shown), which is orders of magnitude faster than formation of a nonspecific alkaline hydrolysis ladder (typically a 10-min incubation for "single-hit" conditions, which provide ≪50% cleavage). This indicates that the new bonds are neither native 3′−5′ nor non-native 2′−5′ linkages, because both of these natural linkages are relatively stable and contribute normally to a nonspecific alkaline hydrolysis ladder (Figure 6C). The complete absence of the RNase T1 band corresponding to cleavage after the G of A↓G also indicates that the 6J products are neither 3′−5′ nor 2′−5′, because these standard RNAs show this particular RNase T1 band with high and low relative intensity, respectively (Figure 6C).

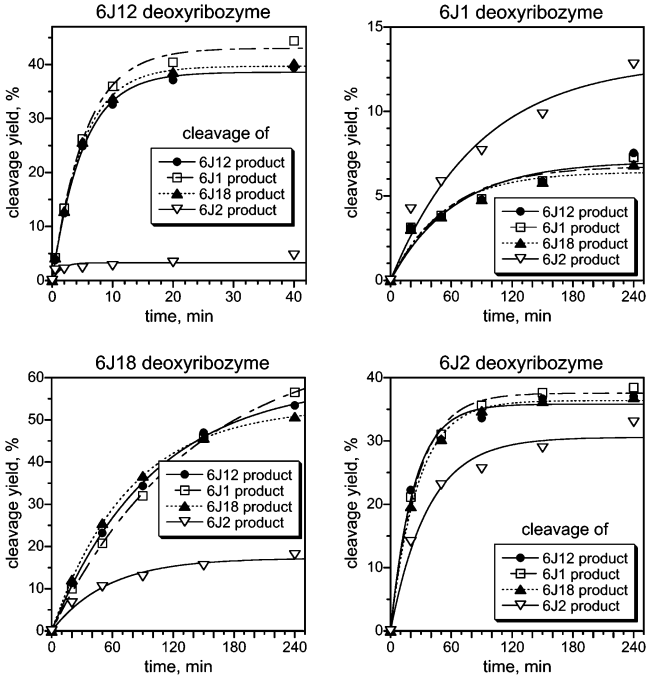

FIGURE 7: Cleavage of the 6J ligation products with the 6J deoxyribozymes. Each of the four 6J deoxyribozymes cleaves the 6J2 product (▽) with a different rate and yield than the products from the other three deoxyribozymes. These kinetic data separate the products into two distinct types, termed here the 6J12 product and 6J2 product.

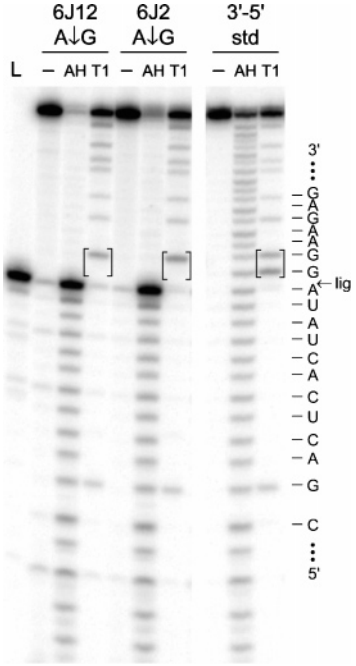

FIGURE 8: Partial alkaline hydrolysis and RNase T1 digestion assays of the ligated RNA products from the 6J12 and 6J2 deoxyribozymes. The ligation products were subjected either to no incubation (−), partial alkaline hydrolysis (AH), or treatment with RNase T1. L = left-hand RNA substrate standard with a 2′,3′-cyclic phosphate. The far right set of lanes are the assays performed on a 3′−5′-linked RNA standard. The sequence on the right side of the gel corresponds to the ligated RNAs with a 3′−5′ linkage. See Figure 6C for these assays performed on a 2′−5′-linked RNA standard. The brackets denote the key difference in the RNase T1 digestion pattern between the 6J products and the 3′−5′ standard, similar to the bands marked in Figure 6C.

Mass Spectrometric Analysis of the 6J12 Ligation Product. To provide a qualitatively different type of structural information, we pursued mass spectrometry analysis of the 6J12 ligation product. A trinucleotide fragment that contains the newly created linkage was excised from the long ligation product, taking advantage of the fact that RNase T1 does not cleave after the G of A↓G (Figure 8). To provide a suitable substrate for this RNase T1 cleavage reaction, appropriate RNA sequences were ligated by 6J12 to form the product 5′...G|A↓GG|A...3′, where the vertical bars denote incipient RNase T1 cleavage sites (i.e., 3′ of a G nucleotide) and the arrow denotes the deoxyribozyme ligation site. The RNA substrates for this experiment were carefully redesigned to remove all G residues except the three that surround the new linkage as written above; these sequence changes are fortunately permitted by the 6J12 deoxyribozyme (see the Supporting Information). RNase T1 digestion of the product then forms an A↓GG trimer plus two longer flanking sequences as a 17-mer and a 16-mer (see the Experimental Procedures). The small A↓GG trimer containing the new linkage was readily separated from these longer cleavage byproducts using DEAE anion-exchange chromatography (*66*, *67*), and the purified trimer was analyzed by ESI MS/MS and MALDI mass spectrometry.

Using MALDI, the parent mass for the 6J12 product was as expected for 5′-A↓GG>p-3′ in which the 2′,3′-cyclic phosphate (>p) has been hydrolyzed to the 2′(3′)-monophosphate, as often occurs with ribonucleases. The same parent mass that was expected for the A↓GG trimer was also observed using ESI−MS. As shown in detail in the Supporting Information, the observed ESI MS/MS fragments confirm that the linkage is between the terminal A↓G nucleotides and is not a branched linkage, as would be formed if an internal nucleotide of the right-hand substrate (i.e., not the 5′-terminal nucleotide) were to provide the nucleophile. Unfortunately, the 6J2 product could not be similarly analyzed because of low preparative-scale ligation yields. More detailed considerations of the possible unnatural linkages created by the 6J deoxyribozymes are deferred until after the 12BB deoxyribozyme products are described.

Various 12BB Deoxyribozymes Create Either 3′−5′ Linkages, 2′−5′ Linkages, or Unnatural Linkages. The 12BB ligation products were examined using the same array of biochemical assays that were applied above to the 6J and background ligation products (Figure 9). The data unambiguously reveal that the collection of 12BB deoxyribozymes forms three different products: the native 3′−5′ linkage (by 12BB5 and 12BB12), the non-native 2′−5′ linkage (by 12BB1 and 12BB2), and at least one unnatural linkage that is neither 3′−5′ nor 2′−5′ (by 12BB6 and 12BB8). Each of the latter two deoxyribozymes is observed to cleave both its own product and the other product with indistinguishable rate constants (see the Supporting Information), suggesting that these two products are identical. Therefore, only 12BB8 was considered further.

Correlating the Unnatural Products from the J and BB Selections: Formation of Three Different Unnatural RNA Linkages. The unnatural 6J12, 6J2, and 12BB8 products cleave rapidly and specifically under alkaline hydrolysis conditions (Figures 8 and 9). The rate constants for these reactions were determined quantitatively at lower temperature (60 °C) than used in standard alkaline hydrolysis conditions (90 °C), such that the $t_{1/2}$ values were larger and could

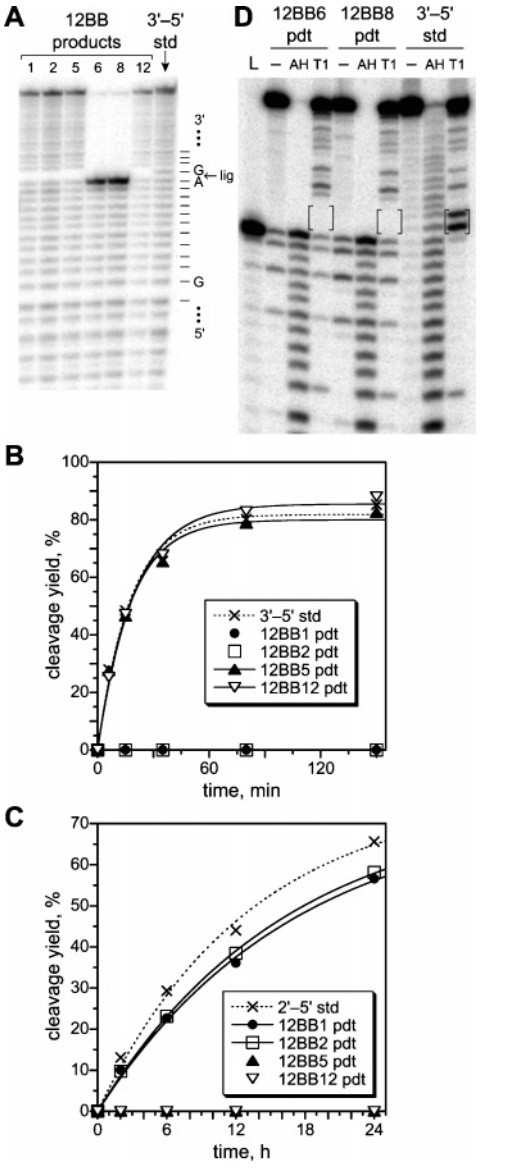

FIGURE 9: Linkage assays for 12BB ligation products. (A) Partial alkaline hydrolysis. For preparation of these products, the 5′-terminal nucleotide of the right-hand RNA substrate was 2′-deoxy-G (dG), as was the case during selection itself. Therefore, in each ladder, an expected 1-nucleotide gap is observed at the ligation site, which is denoted by the small arrowhead on the right side. As expected, analogous assays on the products prepared with a 5′-G substrate showed an unbroken ladder (data not shown). However, instead of a regular ladder, the 12BB6 and 12BB8 products show rapid reversion to the initial substrates, indicating that they are neither 3′−5′-linked nor 2′−5′-linked (see the text). (B) Cleavage assays with the 8−17 deoxyribozyme (3′−5′-selective; $t_{1/2}$ = 12−14 min), performed on the four 12BB products that showed a regular alkaline hydrolysis ladder and are therefore linear. Only the 12BB5 and 12BB12 products are 3′−5′-linked. (C) Cleavage assays at 100 mM Mg^{2+} and pH 9.0 in the presence of the exactly complementary DNA oligonucleotide (2′−5′-selective; $t_{1/2}$ = 9−12 h). Only the 12BB1 and 12BB2 products are 2′−5′-linked. (D) RNase T1 digestion of the 12BB6 and 12BB8 products. The brackets enclose the positions of two missing cleavage bands relative to the 3′−5′ standard (compare bracketed bands in Figure 6C and Figure 8). For preparation of all ligation products for (B)−(D), the 5′-terminal nucleotide of the right-hand RNA substrate was G.

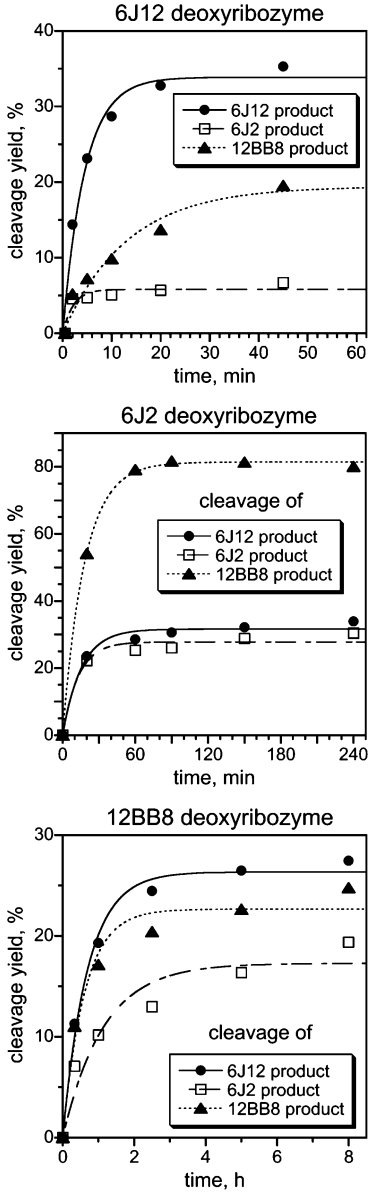

FIGURE 10: Cleavage assays to correlate the three 6J12, 6J2, and 12BB8 ligation products. Compare with Figure 7, which shows analogous cleavage assays among the four 6J deoxyribozymes and their products.

therefore be measured with greater accuracy. All of the linkages were cleaved with $t_{1/2} \approx 10$ min at 60 °C (see the Supporting Information), suggesting a similar structure. To correlate their structures more precisely, the unnatural 6J12, 6J2, and 12BB8 products were analyzed in cleavage assays with each of these three deoxyribozymes (Figure 10, constituting $3 \times 3 = 9$ experiments in all); this is similar to the assays for the four 6J deoxyribozymes and their products, as shown in Figure 7. The data in Figure 10 clearly indicate that the 12BB8 product is different than either the 6J12 product or the 6J2 product, and from Figure 7, the latter two products were already known to be different from each other. Therefore, all of the three 6J12, 6J2, and 12BB8 products are pairwise-distinct, and because none of them have 3′−5′ or 2′−5′ linkages, these three products represent three different unnatural RNA linkages.

Explicitly Testing the Possibility of Various Linear and Branched Linkages for the Deoxyribozymes that Form Unnatural Linkages. For the 6J12, 6J2, and 12BB8 deoxyribozymes that form different unnatural RNA linkages, a strong possibility is that one or more of these DNA enzymes uses a 2′-hydroxyl group from the right-hand (R) RNA

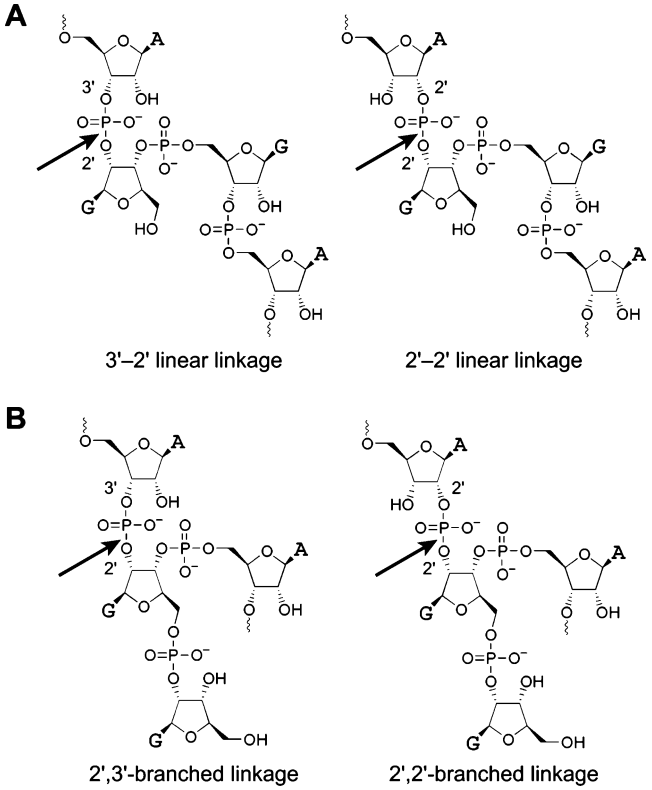

FIGURE 11: Possibilities for the unnatural RNA linkages formed by the 6J12, 6J2, and 12BB8 deoxyribozymes. (A) 3′−2′ and 2′−2′ linear linkages that would be formed by attack of the terminal guanosine 2′-hydroxyl of the right-hand RNA substrate (R) on the 2′,3′-cyclic phosphate of the left-hand RNA substrate (L), with the 2′- or 3′-oxygen of the cyclic phosphate as the leaving group, respectively. (B) The 2′,3′-branched and 2′,2′-branched linkages that would be formed by attack of an internal 2′-hydroxyl of R on the 2′,3′-cyclic phosphate of L, shown here using the 2′-hydroxyl group of the second nucleotide. Analogous branched products are possible using an internal 2′-hydroxyl group from further down the R substrate strand. All unnatural linkages are named systematically following the conventions for naturally occurring 3′−5′ and 2′−5′ linkages (i.e., native and non-native linear linkages) and for naturally occurring 2′,5′-branched linkages, which are distinct from the branches shown here (see the Discussion). In all structures, the 3′-terminal adenosine nucleotide of the L substrate (formerly bearing the 2′,3′-cyclic phosphate) and the first three nucleotides of the R substrate (5′-GGA...) are drawn explicitly. The newly formed bond is marked with an arrow; the oxygen of the guanosine 2′-hydroxyl nucleophile is adjacent to the arrowhead.

substrate as the nucleophile to attack the 2′,3′-cyclic phosphate of the left-hand (L) RNA substrate. If the nucleophile is the 2′-hydroxyl group of the 5′-terminal nucleotide of R, this would lead to a 3′−2′ or 2′−2′ linear linkage (Figure 11A), depending on which oxygen of the cyclic phosphate serves as the leaving group. Alternatively, if an internal 2′-hydroxyl of R is the nucleophile, then a 2′,3′-branched or 2′,2′-branched linkage would be formed (Figure 11B). Distinguishing these potential products by direct spectroscopic analysis is extremely challenging largely because of the small amount of material available. Fortunately, information on the linkages may be gleaned from simple functional assays in which individual 2′-hydroxyl groups of R are omitted one at a time and the deoxyribozyme ligation activities are assessed. The 3′−2′ and 2′−2′ linear linkages cannot be formed if the 2′-hydroxyl group of the terminal R nucleotide is missing. Conversely, the 2′,3′-branched or 2′,2′-branched linkages cannot be formed if the corresponding internal 2′-hydroxyl group of R is omitted.

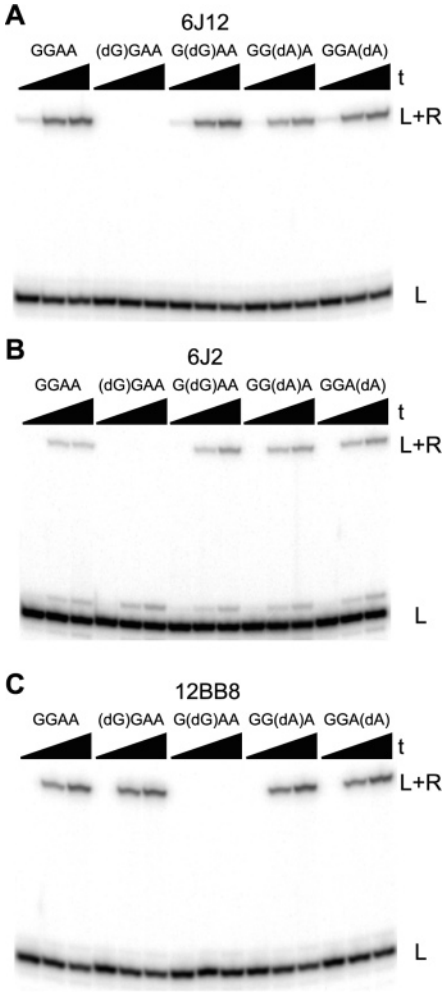

FIGURE 12: Experimentally testing the removal of individual 2′-hydroxyl group nucleophiles from the R substrate (one 2′-hydroxyl at a time) with the 6J12, 6J2, and 12BB8 deoxyribozymes. The four 5′-terminal nucleotides of the R substrate used in each kinetic assay are shown (t = 0.5, 2, and 10 min for 6J12; 0.5, 30, and 120 min for 6J2; and 0.5, 15, and 90 min for 12BB8), where (dG) or (dA) denotes the single 2′-deoxy-G or 2′-deoxy-A in each substrate as appropriate. No activity (<0.5%) is detected with the (dG)GAA substrate for 6J12 and 6J2, whereas no activity is detected with the G(dG)AA substrate for 12BB8.

Using four separate R substrates, each with a single 2′-deoxy substitution at one of the first four nucleotide positions (5′-GGAA...), these key experiments were performed (Figure 12). The clear outcome very strongly suggests the identities of the three unnatural linkages from the 6J12, 6J2, and 12BB8 deoxyribozymes. Both 6J12 and 6J2 fail completely with a 5′-terminal 2′-deoxyguanosine R substrate 5′-(dG)-GAA, but they retain substantial activity when the 2′-deoxy substitution is at any of the three internal nucleotides. The simplest explanation is that 6J12 and 6J2 create the 3′−2′ and 2′−2′ linear linkages, in one of the two possible combinations (which deoxyribozyme creates which of the isomeric linkages cannot be assigned from these data). In sharp contrast, 12BB8 retains activity with a 5′-terminal 2′-deoxyguanosine R substrate; therefore, it does not create either the 3′−2′ or 2′−2′ linear linkage. However, 12BB8 fails completely when the 2′-deoxy substitution is made at the next nucleotide position 5′-G(dG)AA...; activity is

retained when either of the next two nucleotides (adenosines) is 2′-deoxy. The simplest explanation is that 12BB8 creates either the 2′,3′-branch or 2′,2′-branch that is formed upon attack of the 2′-hydroxyl of the second guanosine nucleotide of R at the 2′,3′-cyclic phosphate of L.

DISCUSSION

We previously reported the in vitro selection of deoxyribozymes that use Mg^{2+} as the divalent metal-ion cofactor to ligate the RNA substrates of Figure 1 (*46*−*50*). For unclear mechanistic reasons, all of these deoxyribozymes selectively mediate formation of 2′−5′ linkages from a 5′-hydroxyl and a 2′,3′-cyclic phosphate. Notably, native 3′−5′ linkages are not formed, and neither are various unnatural linkages that would require the reaction of alternative nucleophiles such as the 2′-hydroxyl on the 5′-terminal nucleotide of the right-hand RNA substrate. Others have successfully used transition metals such as Zn^{2+} in selections for nucleic acid enzymes that have various catalytic activities, including RNA cleavage (*10*, *17*, *22*−*27*, *31*, *32*). Here, we sought to determine the effects of using Zn^{2+} instead of Mg^{2+} in selections for DNA enzymes that ligate RNA. The results show dramatic effects on both the rates and the ligation junction selectivities. Notably, for the first time, we have found deoxyribozymes that catalyze formation of native 3′−5′ RNA linkages from 2′,3′-cyclic phosphate substrates (although the particular deoxyribozymes that we have identified are likely not useful in a practical sense for 3′−5′ RNA ligation). We have also found that Zn^{2+} enables formation of many ligation products, in contrast to Mg^{2+} that has provided only 2′−5′ linkages, and that Zn^{2+} enables very rapid RNA ligation. An important general conclusion from our data is that the identity of the metal-ion cofactor plays a substantial role in DNA-catalyzed reactions of RNA substrates.

Using Zn^{2+} as the Cofactor instead of Mg^{2+}: Effects on RNA Ligation Activity. In the J selection, the only critical parameter that was changed relative to the selection in our original paper (*46*) was the divalent metal ion. Using 1 mM Zn^{2+} in place of 40 mM Mg^{2+} (Figure 2) had dramatic effects on the outcome of selection. One clear effect is that some of the resulting Zn^{2+}-dependent deoxyribozymes are at least an order of magnitude faster than their Mg^{2+} counterparts, with k_{obs} up to 0.5 min^{-1} at 23 °C and pH 7.9 for 6J12 (Figure 3). The 6J deoxyribozymes are highly sensitive to both pH and the Zn^{2+} concentration (at least for 6J12), and they are truly Zn^{2+}-dependent (Figure 4). For the BB selection, the 2′-hydroxyl was absent from the 5′-terminal guanosine of the right-hand RNA substrate. This selection also led to deoxyribozymes with substantial ligation activity (Figure 5), including one rather fast DNA enzyme (12BB8) with $k_{obs} \approx$ 0.1 min^{-1} at 23 °C and pH 7.9.

For the 6J12 deoxyribozyme, the high k_{obs} of 0.5 min^{-1} and the background rate data (Figure 6) lead to a calculated rate enhancement k_{obs}/k_{bkgd} of ∼17 000 (i.e., on the order of 10^4). This calculation assumes that the exactly complementary splint provides an appropriate background reaction with which to calculate the rate enhancement. However, using this splint may in fact overestimate the background rate, because the deoxyribozyme does not necessarily hold the substrates in the same relative spatial orientation as the splint. Thus, the actual rate enhancements may be even higher (see ref *46* for a more complete discussion of this issue). Considering the relatively high background rate for the reaction of a cyclic phosphate, these rate enhancements are considerable. In addition, k_{obs} values of ∼0.5 min^{-1} approach the recently postulated "speed limit" for naturally occurring nucleic acid enzymes (*74*, *75*), suggesting that Zn^{2+} permits deoxyribozyme reaction rates that are fast on an absolute scale.

Remarkably, the Zn^{2+}-dependent RNA ligase deoxyribozymes such as 6J12 are quite fast even without reselection to improve their activities. In comparison, our analogous selections using Mg^{2+} initially provided deoxyribozymes with rates only on the order of 0.2 h^{-1} at pH 7.5 and 40 mM Mg^{2+} (*46*), and reselections were necessary to improve the rates into the 0.5 min^{-1} range of 6J12 (*49*). The k_{obs} of 0.5 min^{-1} ($t_{1/2}$ = 1.4 min) for 6J12 is much higher than demanded by the J selection strategy, in which 14 h was allotted during the key selection step. This observation provides a counterpoint to the common and otherwise generally reasonable assertion that selections provide minimally competent solutions to the chemical problem that is posed (*76*). On the other hand, it is intriguing that the sharply optimum Zn^{2+} concentration of ∼1.0 mM for 6J12 (Figure 4B) is the same concentration that was used during the selection procedure itself. Without repeating the entire selection effort many times using different Zn^{2+} concentrations, it is impossible to know more generally if the Zn^{2+} optimum will always correlate directly with the Zn^{2+} concentration used during selection. For 6J12, the substantial drop in yield upon raising the Zn^{2+} concentration above its optimum suggests inhibitory metal-binding sites, but more experiments are needed to test this hypothesis.

Synthesis of Native 3′−5′ RNA Linkages by Zn^{2+}-Dependent Deoxyribozymes. The formation of native 3′−5′ linkages by the 12BB5 and 12BB12 deoxyribozymes (Figure 9) is the first example of deoxyribozymes that create 3′−5′ RNA linkages by reaction of a 2′,3′-cyclic phosphate. The use of Zn^{2+} was key to this outcome, because all previous Mg^{2+}-dependent deoxyribozymes that ligate RNA by reaction at a cyclic phosphate form 2′−5′ linkages (*46*−*50*). Others have described a Zn^{2+}-dependent deoxyribozyme that cleaves 3′−5′ linkages (with formation of a cyclic phosphate), but no ligation activity was detected (*17*). Using an entirely different ligation reaction, that of a terminal 3′-hydroxyl with a 5′-triphosphate, we have recently identified Mg^{2+}-dependent deoxyribozymes that specifically synthesize 3′−5′ RNA linkages (*54*) and have separately outlined a general approach to force selections involving this other chemical reaction to provide 3′−5′ linkages (*56*). However, it should be emphasized that there is no necessary relationship between deoxyribozymes that catalyze these two entirely different chemical reactions. It is important for practical reasons to develop RNA ligation approaches that make use of 2′,3′-cyclic phosphate substrates, because these substrates are readily available from both intra- and intermolecular RNA-processing reactions that use ribozymes, deoxyribozymes, or protein enzymes (*77*−*81*). Therefore, our present results with Zn^{2+}-dependent deoxyribozymes and cyclic phosphate substrates are a substantial advance toward practical formation of 3′−5′ RNA linkages.

Although 12BB5 and 12BB12 indeed create native 3′−5′ linkages using a 2′,3′-cyclic phosphate, these specific deoxy-

ribozymes are probably not optimal for general synthesis of 3′−5′-linked RNA. One concern is that these specific deoxyribozymes prefer a 5′-terminal dG instead of a G nucleotide on their right-hand RNA substrate (Figure 5). A second issue is that the ligation yields are not very high, particularly with a 5′-terminal G (Figure 5). A third issue is that these DNA enzymes are likely to have particular sequence requirements for their RNA substrates, although this has not been tested comprehensively. For other deoxyribozymes that were selected using a strategy where single-stranded RNA overhangs were present in the RNA substrates, we have generally observed substantial sequence requirements for the substrate nucleotides near the ligation junction (e.g., refs *46* and *52*). In ongoing efforts, we are using Zn^{2+} as the cofactor in new deoxyribozyme selections aimed specifically at preparatively useful synthesis of 3′−5′ RNA linkages from 2′,3′-cyclic phosphate substrates with tolerance of any RNA substrate sequences.

Identifying the Unnatural RNA Linkages: Structural Considerations. Unexpectedly, many of the deoxyribozymes from the J and BB selections are found to create unnatural RNA linkages that are neither 3′−5′ nor 2′−5′. Specifically, the 6J12 and 6J2 deoxyribozymes form two different unnatural linkages, and 12BB8 creates a third unnatural linkage (Figures 7−10). For each of 6J12, 6J2, and 12BB8, whatever the identity of the attacking nucleophile into the 2′,3′-cyclic phosphate, two isomers of the products are possible, depending on whether the 2′- or 3′-oxygen of the cyclic phosphate serves as the leaving group. For a 5′-hydroxyl nucleophile, these products are the standard 3′−5′ and 2′−5′ linkages respectively, but neither of these linkages is formed by 6J12, 6J2, and 12BB8.

Solely on the basis of the chemical reactivity of the substrate (i.e., before considering the stability of the possible ligation products), possible nucleophiles from the 5′-terminal nucleotide of the right-hand substrate (other than the 5′-hydroxyl group itself) are the 2′-hydroxyl group (*51*, *53*), the guanosine O^6 atom (*82*), the guanosine N^7 atom (*83*, *84*), and the guanosine N^2 atom (*85*−*87*). Further along the right-hand substrate strand, each internal 2′-hydroxyl group is also a potential nucleophile, as are specific atoms of the internal nucleobases. Considering the chemical stability of the possible ligation products helps to rule out some of the products that would involve these nucleophiles. The ligation products from attack of N^7 at the 2′,3′-cyclic phosphate would almost certainly be unstable because of intramolecular displacement at P by the neighboring 2′- or 3′-hydroxyl group. Experimentally, none of the three unnatural ligation products is reactive upon $NaBH_4$/aniline treatment that is known (*84*) to cleave N^7-alkylated guanosines (data not shown), which is direct evidence against guanosine N^7 as the nucleophile. Similarly, putative linkages formed between O^6G and P would almost certainly be inherently unstable (e.g., ref *88*); therefore, such products are also unlikely. In contrast to N^7 and O^6G, the possibility of the guanosine N^2 amino group acting as a nucleophile cannot be discounted out of hand, because this functional group is known to be nucleophilic in several natural contexts (*85*−*87*). However, several guanosine N^2-phosphoramidate derivatives have been synthesized and are stable under highly basic conditions (*89*). This argues strongly against guanosine N^2 as the nucleophile for the 6J12, 6J2, and 12BB8 deoxyribozymes, because their ligation products are highly base-labile (Figures 8 and 9A). In view of these considerations, the most likely candidates for the three unnatural linkages are the structures shown in Figure 11, each of which arises from attack of a terminal or internal 2′-hydroxyl group of the right-hand RNA substrate at the 2′,3′-cyclic phosphate of the left-hand RNA substrate.

Identifying the Unnatural RNA Linkages: Experimental Evidence. The most compelling evidence for assigning the structures of the three unnatural RNA linkages formed by 6J12, 6J2, and 12BB8 derives from the functional assays that use 2′-deoxy substitutions in the R substrate (Figure 12). The complete lack of ligation activity upon deleting a single 2′-hydroxyl group is most simply explained by assigning that particular 2′-hydroxyl group as the nucleophile in the ligation reaction. This leads directly to the conclusion that 6J12 and 6J2 create the 3′−2′ and 2′−2′ linear linkages in one of the two combinations (Figure 11A), whereas 12BB8 creates either the 2′,3′-branch or 2′,2′-branch (Figure 11B).

The assigned unnatural linkages are corroborated by the RNase T1 digestion assays. The single "missing" RNase T1 bands in Figure 8 are consistent with the postulated unnatural linear structures for the 6J12 and 6J2 products. In these products, only the 5′-terminal guanosine has its 2′-hydroxyl group occupied in a covalent bond, such that RNase T1 cannot catalyze RNA cleavage at that particular nucleotide. Similarly, the two missing RNase T1 bands in Figure 9D are consistent with the postulated unnatural branched structure for the 12BB8 product. Here, the internal guanosine 2′-hydroxyl group is occupied in a covalent bond, and RNase T1 cleavage of the single-nucleotide 5′-guanosine tail of the branch does not cut the RNA strand into two large fragments; therefore, two RNase T1 digestion bands are missing.

The mass spectrometry data support the assignment of the 6J12 product as either the 3′−2′ or 2′−2′ linear linkage and not the 2′,3′-branched or 2′,2′-branched linkage. Both of the linear unnatural linkages of Figure 11A should give rise to the observed 5′-A↓GG-3′ trimer after RNase T1 digestion of the ligated product, whereas neither of the branched linkages of Figure 11B could lead to such a trimer. Because the trimer is in fact observed by mass spectrometry, the two branched linkages are strictly excluded as the 6J12 product.

Formation and Stability of Various "Branched RNA" Linkages. The 2′,3′-branched linkage shown on the left of Figure 11B is structurally equivalent to the branch that Tuschl et al. proposed is formed by a selected ribozyme (*90*). However, their very inefficient ribozyme (for which the reaction rate is barely above the background RNA degradation rate) is specific for a particular combination of RNA substrate sequences. Furthermore, their ribozyme becomes covalently incorporated into the ligation product, and its site of branch formation could not be mapped precisely. Similar to our finding, their nonstandard junction is very base-sensitive, with a reported alkaline hydrolysis $t_{1/2} \approx 1$ min under slightly different incubation conditions than those used here, for which $t_{1/2} \approx 2$ min at 90 °C. Another report on structurally related 3′−3′ linkages was recently published (*91*), and the kinetic data for base lability are quantitatively consistent with our observations. In all of these cases, the increased base lability of the unnatural linkage relative to a natural 3′−5′ or 2′−5′ linkage apparently derives from the weaker basicity and therefore greater leaving group ability of a ribose 2′(3′)-hydroxyl group as compared with a 5′-

hydroxyl group. The finding that each of our three experimentally observed unnatural linkages cleaves with comparable $t_{1/2}$ under alkaline hydrolysis conditions (~10 min at 60 °C; see the Supporting Information) supports their assignments as one of the structures of Figure 11, each of which should be comparably base-labile.

In nature, "branched RNAs" are formed during RNA splicing reactions. More specifically, these splicing products are 2′,5′-branched RNAs, for which an internal 2′-hydroxyl group of one RNA strand is connected via a phosphodiester linkage to the terminal 5′-oxygen of a second RNA strand (*92*, *93*). However, each of the unnatural linkages shown in Figure 11 is expected to have a substantially higher base lability (i.e., lower alkaline hydrolysis $t_{1/2}$ value) than a naturally occurring 2′,5′-branch. In each of the structures shown in Figure 11, a free 2′-hydroxyl or 3′-hydroxyl group is located adjacent to the new phosphodiester bond, rendering this bond unstable under basic conditions by intramolecular attack at P by the free 2′- or 3′-hydroxyl group. In contrast, a 2′,5′-branch has no free hydroxyl group adjacent to the branch site and therefore one expects even *less* base lability when compared with a standard RNA phosphodiester linkage. Indeed, the locations of 2′,5′-branch sites may readily be mapped by nonspecific partial alkaline hydrolysis of the branched RNAs in which they are embedded, because the 2′,5′-branched linkage is very stable under basic conditions (*51*, *73*). In contrast, the substantial base lability of our new unnatural linkages (Figures 8 and 9) directly prevents mapping of their branch sites by partial alkaline hydrolysis. Fortunately, the functional assays of Figure 12 allow this mapping via a qualitatively different type of experiment, as described above.

Deoxyribozymes Have the Ability To Change Reaction Pathways. The background ligation products are formed when an exactly complementary DNA splint instead of a deoxyribozyme is bound to both RNA substrates. Assays of the background ligation products formed with any of Zn^{2+}, Mg^{2+}, or Mn^{2+} clearly show that these products are linked 2′−5′ (Figure 6). Therefore, each of our Zn^{2+}-dependent deoxyribozymes that creates a linkage other than 2′−5′ (i.e., every deoxyribozyme described in this paper except 12BB1 and 12BB2) creates a "disfavored reaction product". These findings underscore the conclusion that deoxyribozymes can be proficient at changing the course of reaction pathways, rather than merely increasing the rates of reactions that would have happened in the absence of the DNA enzymes. A clear direction for future investigation is to understand the detailed structural and mechanistic basis by which Zn^{2+}-dependent deoxyribozymes catalyze specific RNA ligation reactions.

CONCLUSIONS

The finding that Zn^{2+}-dependent deoxyribozymes ligate RNA rapidly when compared with their Mg^{2+}-mediated counterparts demonstrates that transition metals can be particularly useful contributors to reactions catalyzed by nucleic acid enzymes. In this study, we have for the first time identified deoxyribozymes (12BB5 and 12BB12) that create native 3′−5′ RNA linkages from 2′,3′-cyclic phosphate RNA substrates, although these particular deoxyribozymes will probably not be useful in a practical sense. The use of Zn^{2+} instead of Mg^{2+} in this selection effort is clearly key to obtaining 3′−5′ linkages, because all of our previous efforts with Mg^{2+} have led only to non-native 2′−5′ linkages. On the basis of these results and because deoxyribozymes that use transition-metal-ion cofactors (rather than Mg^{2+} or Ca^{2+}) are often highly metal-specific (*31*), we conclude that expanded efforts are warranted to identify transition-metal-mediated deoxyribozymes and ribozymes for reactions of nucleic acids and other substrates. The discovery that Zn^{2+}-dependent deoxyribozymes catalyze a wide variety of ligation reactions that form either natural or unnatural RNA linkages indicates that Zn^{2+} is a particularly versatile cofactor for nucleic acid catalysis.

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SUPPORTING INFORMATION AVAILABLE

Assays for background RNA ligation rate. Kinetic characterization of RNA ligation by the 6J deoxyribozymes. Assays of metal-ion dependence and RNA substrate sequence dependence for the 6J deoxyribozymes. Dependence of 6J deoxyribozyme ligation activity on the RNA functional groups. Mass spectrometry data for the 6J12 ligation product. Cleavage of the 12BB6 and 12BB8 ligation products by 12BB6 and 12BB8. Quantitative determination of the alkaline hydrolysis $t_{1/2}$ values. This material is available free of charge via the Internet at http://pubs.acs.org.

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