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

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Assays for background RNA ligation rate

The rate constant of the Zn^{2+}-dependent background reaction of a 2',3'-cyclic phosphate with a 5'-hydroxyl was determined using control reactions as shown in Figure X1. In these assays, instead of a deoxyribozyme, a DNA splint was used that was either exactly complementary to the two RNA substrates (n = 0; the T_0 splint) or had n = 1–4 unpaired T nucleotides inserted between the Watson-Crick binding arms (the T_1–T_4 splints). We also tested the initial deoxyribozyme strand containing a random N_{40} sequence inserted between the binding arms. The incubation conditions were 70 mM Tris, pH 7.9, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$ at 23 °C. The k_{bkgd} values for the T_0, T_1, and T_2 splints are listed in the figure caption. Product bands were barely detectable for the T_3 and T_4 splints, and the k_{bkgd} values (derived from the slope of the yield versus time data) were not reproducibly different from zero. Not surprisingly, no product bands at all were detected for the N_{40} control reaction. The k_{bkgd} value for the exactly complementary T_0 splint is similar to that shown in Figure 6 of ref. 46, for which Mg^{2+} was used at pH 7.5 (HEPES) and 37 °C, instead of Zn^{2+} at pH 7.9 (Tris) and 23 °C as used here. A background assay in the complete absence of all divalent metal ions (1 mM EDTA) or in the absence of the DNA splint led to no detectable ligation activity (data not shown).

One feature to note in the gel images of Figure X1B is the band just above the unreacted left-hand substrate band. If the 2',3'-cyclic phosphate is opened by hydrolysis, the resulting 2'(3')-monophosphate should migrate slightly *faster* than the unreacted substrate L, which is inconsistent with the observed band just above L (slower). However, if this monophosphate were subsequently removed by hydrolysis even faster than the hydrolytic opening of the cyclic phosphate, then the resulting 2',3'-diol RNA should migrate slightly *slower* than L, as observed. We have no further information to support this specific assignment of the new band, which is also seen occasionally in our deoxyribozyme-mediated ligations (Figure X2 below, particularly the 37 °C 6J2 data in panel B). The presence of this band in the background assays is evidence against the possibility that the band originates as a secondary decay product from the ligated substrates in the presence of particular deoxyribozymes. Instead, it is almost certainly a side product formed directly from the unreacted left-hand substrate.

The ligated RNA products from the various background assays have been tested to determine the nature of the ligation junction (i.e., 3'–5', 2'–5', or an unnatural linkage). These data are shown in Figure 6 and demonstrate that each of the background reactions forms the non-native 2'–5' linkage.

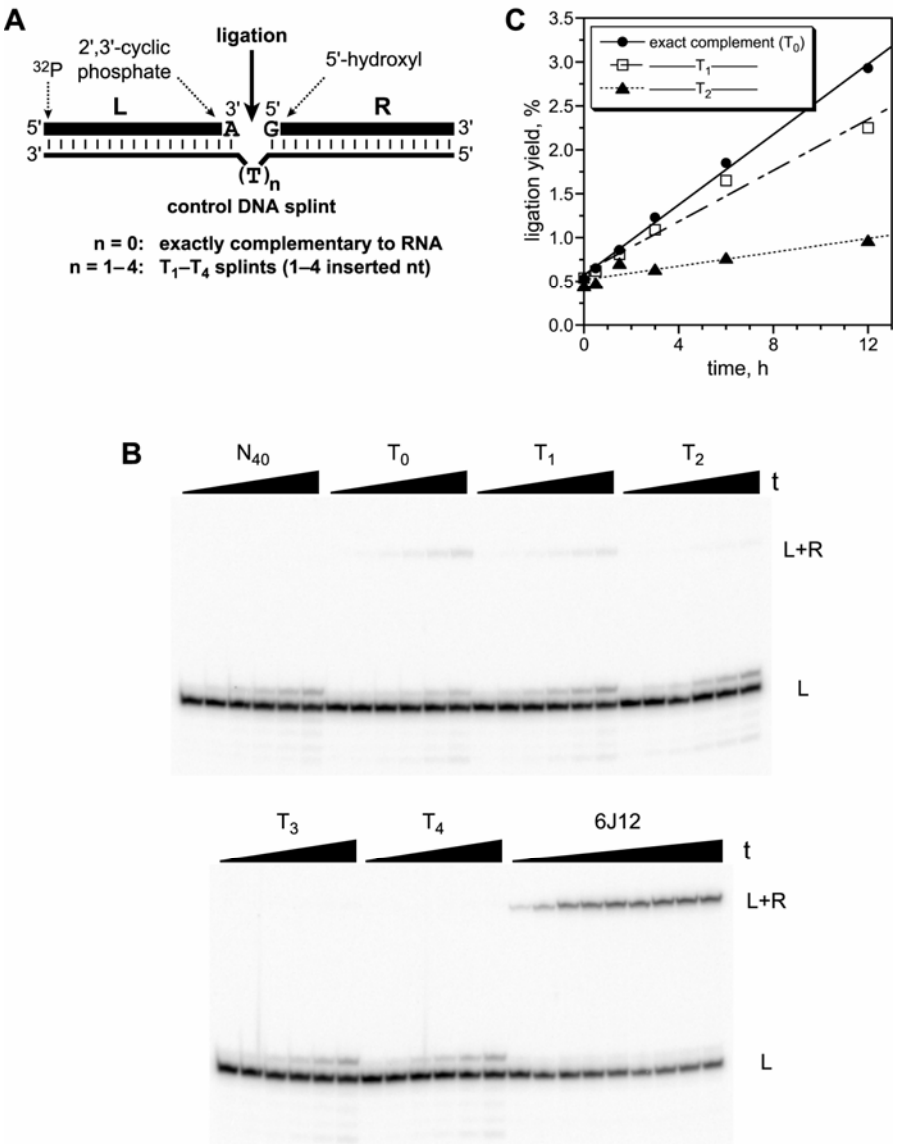

Figure X1. Background reaction assays. (A) Trimolecular assay format. This format is identical to that used for assaying ligation by the new deoxyribozymes (Figure 3A and Figure X2). The left-hand substrate was internally ^{32}P-radiolabeled and began with 5'-GGA; the right-hand substrate was prepared by solid-phase synthesis. (B) Gel images for the various background reactions. A positive control with 6J12 is also shown. Timepoints for control splints: 0, 0.5, 1.5, 3, 6, and 12 h. Timepoints for 6J12: 0.5, 1, 1.5, 2, 3.5, 6, 10, 15, and 20 min. (C) Kinetic data. The slopes of the linear fits were T_0, 0.0020 h^{-1}; T_1, 0.0015 h^{-1}, and T_2, 0.00039 h^{-1}. The detection limit for this experiment was ~0.5% (note the yield at t = 0).

Kinetic characterization of RNA ligation by the 6J deoxyribozymes

Using the methods of ref. 46, the ligation kinetics of the four 6J deoxyribozymes were determined under the standard conditions of 70 mM Tris, pH 7.9, 150 mM NaCl, 2 mM KCl and 1 mM $ZnCl_2$ at room temperature (23 °C), as shown in Figure X2A. The 6J2 deoxyribozyme provides higher yield at 37 °C (Figure X2B) than at 23 °C, although the yield is still low in the absolute sense. See Figure 3 for plots of the data. Because HEPES was used in the J selection procedure, we briefly examined ligation for 6J2 at 37 °C in HEPES instead of Tris buffer; the ligation yield did not improve (data not shown). The approximate [Zn^{2+}] optimum for 6J2 determined at 37 °C (data not shown) was indistinguishable from that of 6J12 (~1.0 mM; Figure 4B).

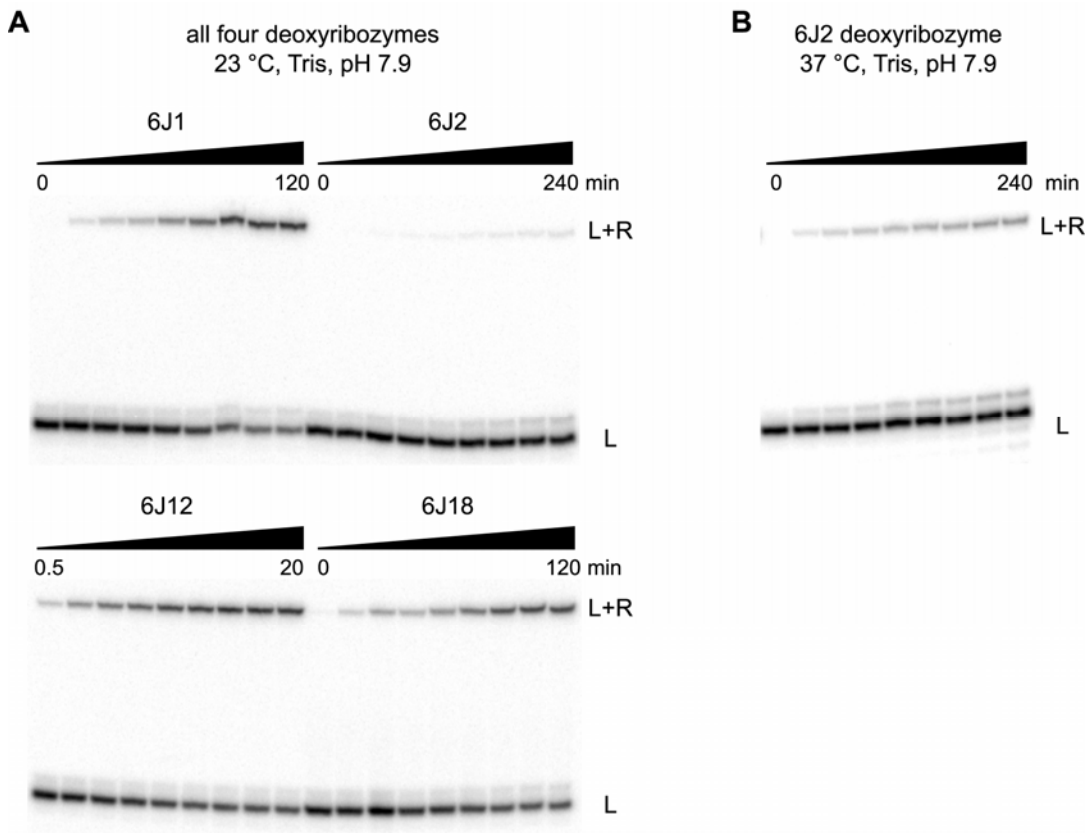

Figure X2. RNA ligation by the 6J deoxyribozymes in Tris, pH 7.9 buffer. (A) All four deoxyribozymes at room temperature (23 °C). (B) 6J2 at 37 °C. See Figure 3A for a visual depiction of the assay format. Plots of the 23 °C data for 6J12, 6J1, and 6J18 are shown in panels C and D of Figure 3, and k_{obs} values are in that figure caption. For 6J2, the yield at 240 min was only about 4% at 23 °C. A plot of the 6J2 data at 37 °C is in Figure 3D.

Assays of metal ion dependence for the 6J deoxyribozymes

The metal ion concentration dependence of 6J12 was determined comprehensively for Zn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Cu^{2+}, separately using either 10 µM, 100 µM, 1 mM, or 10 mM of each ion (Figure X3). Of these tested ions, only Zn^{2+} supported any detectable RNA ligation activity; see Figure 4B for the Zn^{2+} concentration dependence. The gel image shown in Figure 4C is from a subset of the assays shown in Figure X3.

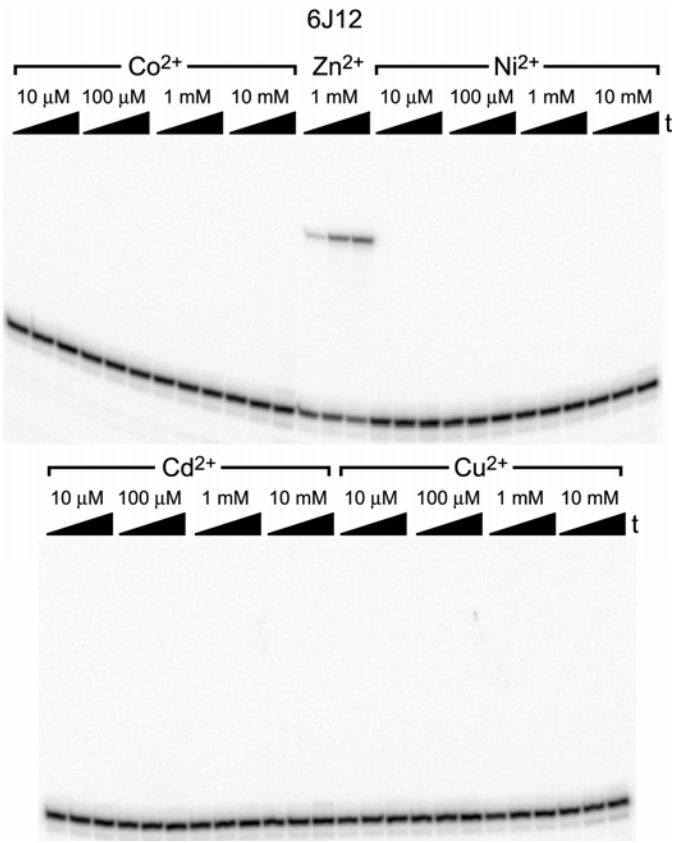

Figure X3. Assaying the metal ion dependence of the 6J12 deoxyribozyme. Reactions were conducted under the standard conditions of 70 mM Tris, pH 7.9, 150 mM NaCl, and 2 mM KCl at 23 °C, along with the indicated concentration of the divalent metal ion. Timepoints were taken at 0.5 min, 4 h, and 24 h for all samples except the Zn^{2+} positive control, for which timepoints were taken at 0.5, 2, and 10 min. The yield for the Zn^{2+} control reaction was 46% at 10 min.

The metal ion dependences of 6J1 and 6J18 were determined for the same set of ions (Figure X4). The ions Co^{2+} and Ni^{2+} were tested only at 100 μM and 1 mM; Cd^{2+} at 1 mM and 10 mM; and Cu^{2+} at 10 μM and 100 μM. As observed for 6J12, only Zn^{2+} was able to support RNA ligation activity for the 6J1 and 6J18 deoxyribozymes.

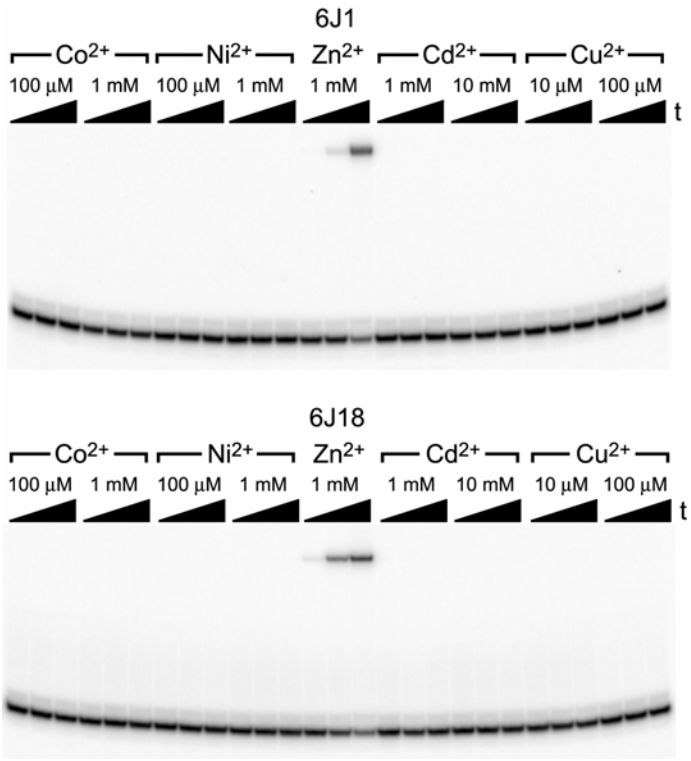

Figure X4. Assaying the metal ion dependences of the 6J1 and 6J18 deoxyribozymes. Reactions were conducted under the standard conditions of 70 mM Tris, pH 7.9, 150 mM NaCl, and 2 mM KCl at 23 °C, along with the indicated concentration of the divalent metal ion. Timepoints were taken at 0.5, 10, and 60 min for all samples. The yield for the Zn^{2+} control reaction was 50% at 60 min in both cases.

Assays of RNA substrate sequence dependence for the 6J deoxyribozymes

The RNA substrate sequence dependence of each of the four 6J deoxyribozymes was determined by varying the RNA substrates and, where appropriate, the deoxyribozyme binding arms. Ligation assays were performed on the various substrate/enzyme combinations. For all assays, the RNA substrate sequences are denoted in the format UAUA↓GGAA, where four nucleotides are shown on either side of the ligation site (arrowhead). In some cases, only a subset of these eight nucleotides is shown as appropriate, and in other cases, additional nucleotides are shown on the left or right side. Underlining is used to highlight the nucleotides of specific interest in a given experiment. Note that the eight UAUA↓GGAA nucleotides were not originally base-paired in the selection strategy (Figure 2A), so changes to these RNA nucleotides did not require any complementary changes in the DNA enzyme binding arms. However, changes to the RNA substrates outside of this ligation junction region prompted the corresponding Watson-Crick changes in the DNA binding arms.

We first determined the dependence of ligation activity on the nucleotides immediately adjacent to the ligation junction. The parent RNA substrate sequence is A↓G. For the left side, only the A↓G and G↓G combinations were tested, while on the right side, all four A↓X combinations were examined for each DNA enzyme (Figure X5). 6J2 ligates G↓G to a small extent, whereas none of the other three

deoxyribozymes shows any activity with this substrate combination. For the A↓X substrates, all four deoxyribozymes strictly require A↓G, except that 6J1 shows a small amount of ligation activity with A↓A.

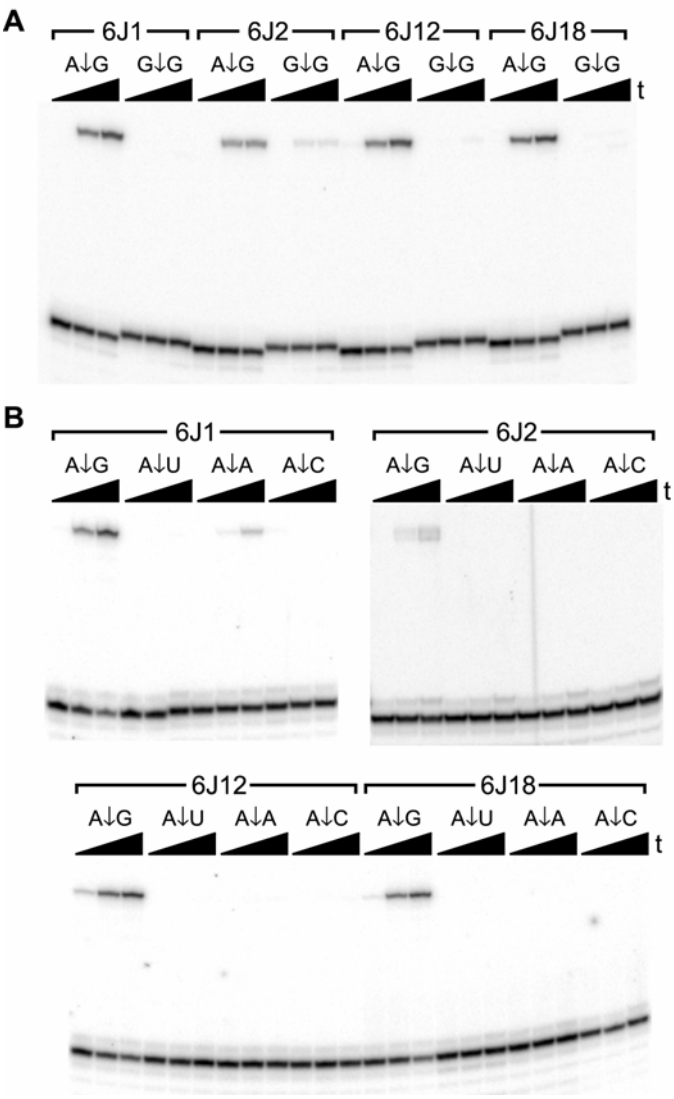

Figure X5. Dependence of RNA ligation activity on the nucleotides immediately surrounding the ligation junction. (A) Dependence on the single nucleotide to the left of the ligation junction. For all four deoxyribozymes, only the A↓G and G↓G substrate combinations were tested. The G↓G substrates were not detectably ligated by any of the deoxyribozymes except 6J2, for which A↓G gave 23% ligation and G↓G gave 4.0% ligation in this experiment. The left-hand substrates were 5'-^{32}P-radiolabeled and did not have the 5'-GGA; the right-hand substrate was prepared by solid-phase synthesis. Timepoints were as follows. 6J1 and 6J18: 0, 10 min, 1 h; 6J2: 0, 40 min, 1.5 h; 6J12: 20 s, 2 min, 10 min. Assays were in 70 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM KCl, and 1 mM $ZnCl_2$ at 37 °C. (B) Dependence on the nucleotide to the right of the ligation junction. For all four deoxyribozymes, all of the possible A↓X substrate combinations were tested. The A↓H (H≠G) substrates were not detectably ligated by any of the deoxyribozymes except 6J1, for which A↓G gave 55% ligation and A↓A gave 8.0% ligation in this experiment. The left-hand substrate was internally ^{32}P-radiolabeled and had the 5'-GGA; the right-hand substrates were prepared by solid-phase synthesis. Timepoints were as follows. 6J1 and 6J18: 0, 10 min, 1 h; 6J2: 0, 30 min, 2 h; 6J12: 20 s, 2 min, 10 min. Assays were in 70 mM Tris, pH 7.9 and 1 mM $ZnCl_2$ at 37 °C.

We then surveyed the dependence of ligation activity on RNA nucleotides more remote from the ligation junction. First, the binding arms were examined. In the initial assays, the new RNA sequences

were identical to those shown in Figure 7A of ref. 46. The original and new RNA sequences are as follows: left-hand original, 5'-GGAUAAUACGACUCACUAUA-3'; left-hand new, 5'-GGACCAAGUCCUAAGUCUAUA-3' (both left-hand RNAs have a 2',3'-cyclic phosphate); right-hand original, 5'-GGAAGAGAUGGCGACGG-3'; right-hand new, 5'-GGAAUUCUGUUGAUAUG-GAU-3'. The underlined nucleotides are identical between the original and new sequences, and almost every nucleotide outside of these regions is different. Thus the common sequence element for all of the substrate combinations in this survey is CUAUA↓GGAA. Deoxyribozymes were synthesized with complementary Watson-Crick binding arms. That is, every RNA nucleotide outside of the UAUA↓GGAA region was matched by its DNA complement.

The results of switching both binding arms simultaneously (from "original" to "new") are shown in Figure X6. For 6J2, all of the nucleotides outside of CUAUA↓GGAA may be changed without disrupting activity. However, for the other three deoxyribozymes, changing all of the flanking nucleotides has a substantial negative impact on ligation. For 6J12 only, we additionally examined the effect of changing only the left or right binding arm sequence, but not both at once (Figure X7A). The results indicate that there is some requirement further to the right of the ↓GGAA sequence, but not to the left of CUAUA↓. In addition, changes to any of the nucleotides ↓GGAA were not tolerated by 6J12 (Figure X7B). To identify the boundary of the required nucleotides on the right side of the ligation junction, we additionally tested the substrate sequences ↓GGAAG, ↓GGAAGA, ↓GGAAGAG, and ↓GGAAGAGA, where every nucleotide to the right of the listed sequence was different from the original. The results (Figure X7C) clearly indicate that only one additional nucleotide is required for 6J12 activity (↓GGAAG). Therefore, up to this point, the data show that 6J12 requires CUAUA↓GGAAG, where the underlined nucleotides are strictly required and the non-underlined ones are examined next.

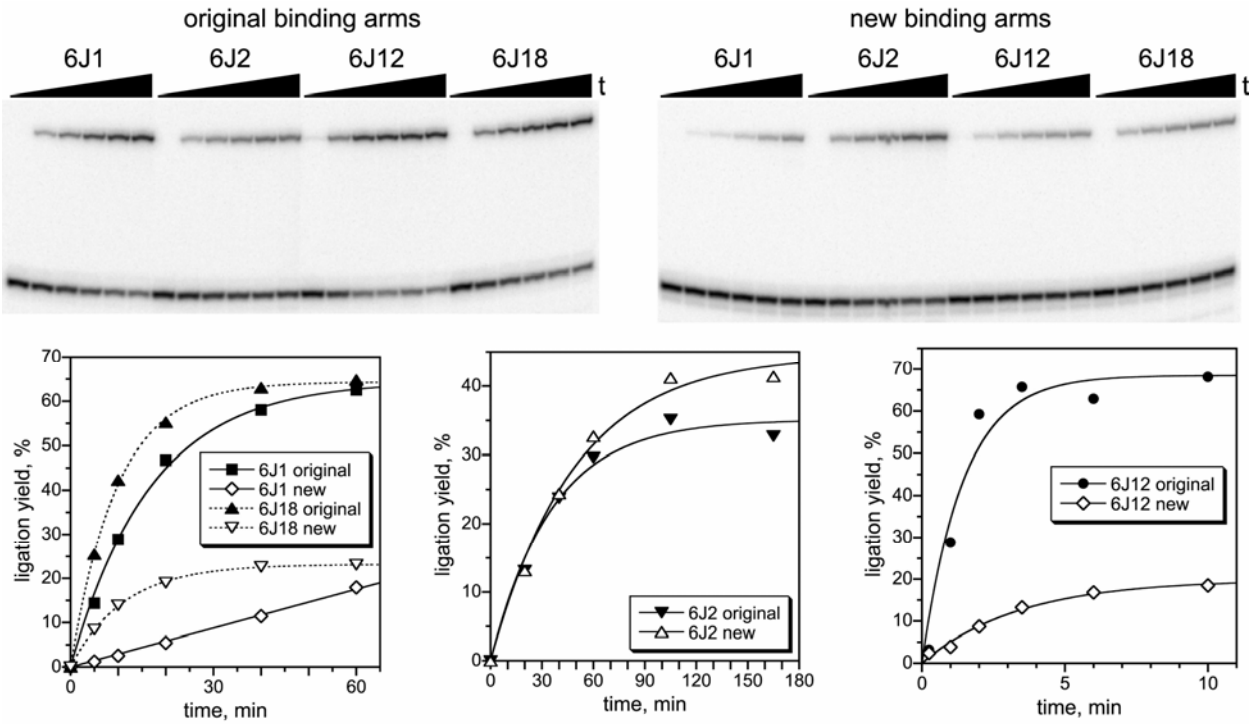

Figure X6. Dependence of RNA ligation on the binding arms. See text for "original" and "new" binding arm RNA sequences. The left-hand substrates were internally ^{32}P-radiolabeled and had the 5'-GGA; the right-hand substrates were prepared by in vitro transcription and dephosphorylation. Assays were in 70 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM

KCl, and 1 mM $ZnCl_2$ at 37 °C. For uncertain reasons, all of the ligation yields in this particular experiment were higher than those reported in the manuscript and in Figure X2 in Tris, pH 7.9 (e.g. 6J2, about two-fold higher here).

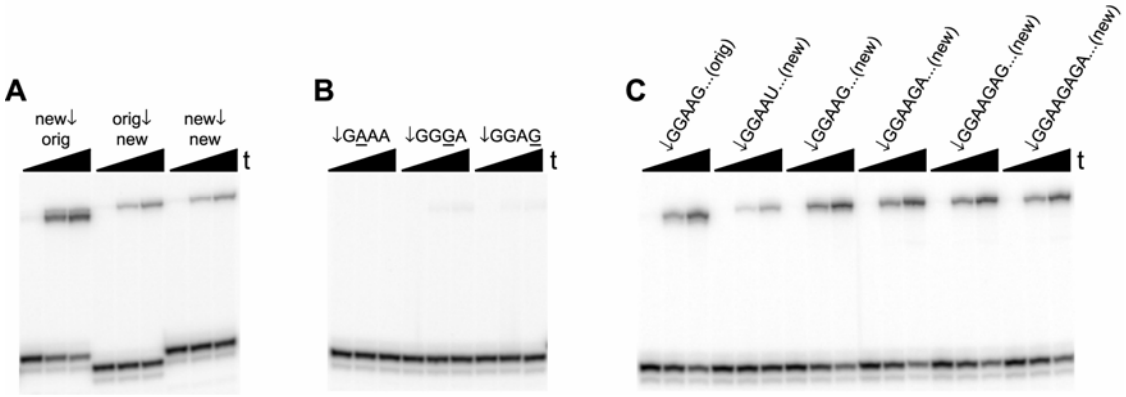

Figure X7. Investigation of the dependence of 6J12 RNA ligation on the substrate sequences. (A) Switching just one of the two binding arms. Yields at final timepoint: 62%, 22%, 19%. These data show that the left-hand binding arm may be changed orig→new without adversely affecting the yield, whereas the right-hand binding arm cannot be changed without affecting the yield. The positive control experiment (the orig↓orig ligation conducted in parallel) is shown in Figure X8B, where the yield is 66% at the final timepoint. (B) Changing nucleotides near the ligation site of the right-hand substrate. These data show that none of ↓GGAA may be changed via a transition (A↔G) without completely disrupting ligation activity. Yields at final timepoint: 0.4%, 1.2%, 1.0%. The positive control experiment is the same as in panel A. (C) Identifying the boundary in the right-hand substrate beyond which nucleotide changes are tolerated with no loss in ligation activity. These data identify that boundary as after the ↓GGAAG nucleotide. Yields at final timepoint: 49%, 13%, 57%, 58%, 55%, 53%. For all panels, the left-hand substrates were internally ^{32}P-radiolabeled and had the 5'-GGA; the right-hand substrates were prepared by in vitro transcription and dephosphorylation. Assays were in 70 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM KCl, and 1 mM $ZnCl_2$ at 37 °C. Timepoints were taken at 0, 2, and 10 min.

To examine more carefully the left-hand substrate requirement (where it was already established above in Figure X5 that the A↓ is strictly required), we tested changes in each of the four CUAUA↓ nucleotides. When CUAUA↓G was changed to GUAUA↓G (with the complementary G→C change in the deoxyribozyme), significant ligation activity was retained, although the yield decreased by a factor of two to three (Figure X8A). We have not tested the effect of changing the RNA nucleotide from C while retaining the DNA nucleotide as G (i.e, introducing a non-Watson-Crick interaction at that position). Changes to the nucleotides closer to the ligation junction CUAUA↓G were tolerated with no loss in activity (Figure X8B). Overall, the data establish that the left-hand RNA sequence requirement for 6J12 is merely A↓, with all other nucleotides to the left of the ligation junction variable without destroying ligation activity. Combining this finding with the experiments on the right-hand substrate, we conclude that the 6J12 ligation requirement for its RNA substrates is A↓GGAAG; all RNA substrate nucleotides outside of this region are changeable without completely disrupting the ligation activity. We subsequently showed that an A is tolerated in place of the fifth G on the right-hand substrate (A↓GGAAA; data not shown). This leads to the final required motif A↓GGAAR (R = purine) for 6J12.

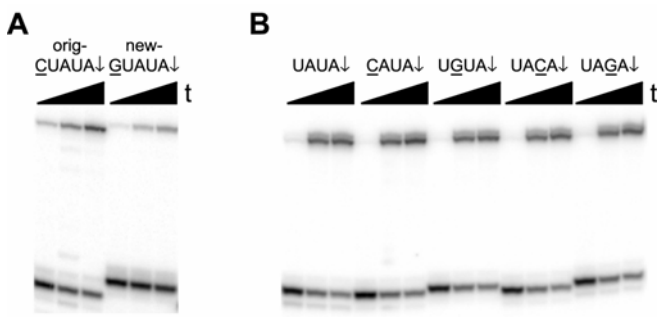

Figure X8. Examining the dependence of 6J12 RNA ligation on the left-hand substrate sequence. (A) Dependence on the nucleotide five to the left of the ligation junction (XUAUA↓). In Figure X7A it is shown that a left-hand substrate of the "new" sequence that terminates UAUA↓ provides equivalent ligation activity to a left-hand substrate of the "original" sequence that terminates UAUA↓. That is, outside of the CUAUA↓, orig→new changes to the nucleotides of the left-hand substrate do not affect ligation (Figure X7A). Here is tested orig-CUAUA↓G versus new-GUAUA↓, which have yields of 51 and 18% respectively at the final timepoint. Because the orig→new change alone is inconsequential, the CUAUA↓ to G change is solely responsible for a three-fold drop in ligation activity. The left-hand substrates were internally ^{32}P-radiolabeled and had the 5'-GGA; the right-hand substrates were prepared by solid-phase synthesis. Assays were in 70 mM Tris, pH 7.9, 150 mM NaCl, 1 mM KCl, and 1 mM $ZnCl_2$ at 23 °C. Timepoints were taken at 30 s, 2, and 10 min. (B) Dependence on the left-hand substrate nucleotides closer to the ligation junction. The results indicate that any of the UAUA↓ nucleotides may be changed as indicated without disrupting ligation activity. The left-hand substrates were internally ^{32}P-radiolabeled and had the 5'-GGA; the right-hand substrates were prepared by in vitro transcription and dephosphorylation. Assays were in 70 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM KCl, and 1 mM $ZnCl_2$ at 37 °C. Timepoints were taken at 20 s, 2, and 10 min. Yields at the final timepoint were each 64 to 66%.

A subset of the above experiments were performed for the 6J1, 6J18, and 6J2 deoxyribozymes, focusing solely on the UAUA↓GGAA nucleotides. For 6J1 and 6J18, changes to the left-hand substrate revealed that A↓ is the minimal requirement on the left side within these four nucleotides, while for 6J2, UA↓ is preferred but any of the tested XA↓ provide significant ligation (Figure X9). On the right side, 6J1 and 6J2 require ↓GGA, whereas 6J18 requires ↓GGAA (Figure X9). Based on these data, we conclude that the substrate sequence requirements within UAUA↓GGAA are as follows: 6J1, A↓GGA; 6J18, A↓GGAA; and 6J2, A↓GGA with UA↓GGA preferred. Based on the data in Figure X6, both 6J1 and 6J18 have additional substrate sequence requirements further away from the ligation junction that have not yet been identified. However, within the UAUA↓GGAA region immediately surrounding the ligation site, the requirements for these two DNA enzymes are as stated here. For 6J2, the data in Figure X6 in combination with the data in Figure X9 demonstrate that this deoxyribozyme has no substrate sequence requirements other than A↓GGA.

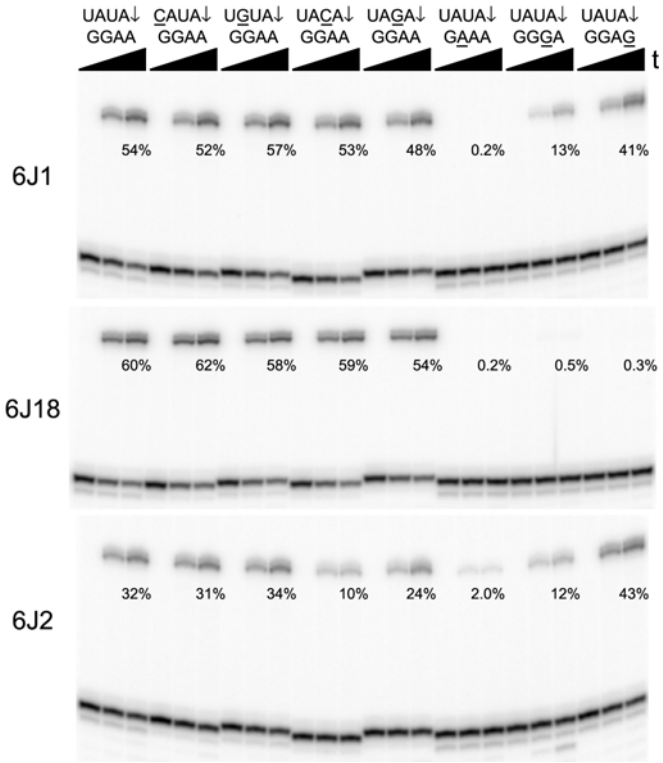

Figure X9. Assaying the dependence of 6J1, 6J18, and 6J2 RNA ligation on the RNA substrate sequences. The left-hand substrates were internally ^{32}P-radiolabeled and had the 5'-GGA; the right-hand substrates were prepared by in vitro transcription and dephosphorylation. Assays were in 70 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM KCl, and 1 mM $ZnCl_2$ at 37 °C. Timepoints for 6J1 and 6J18 were taken at 0, 20, and 60 min. Timepoints for 6J2 were taken at 0, 45, and 120 min. Yields at the final timepoint are shown near each product. Similar to the data shown in Figure X6, the 6J2 yields were higher here than compared to the yields reported in the manuscript and in Figure X2.

Dependence of 6J deoxyribozyme ligation activity on the RNA functional groups

For the 6J deoxyribozymes, we assayed the dependence of ligation activity on various functional groups of the RNA substrates (Figure X10). In particular, the 2',3'-cyclic phosphate of the left-hand RNA substrate was replaced with a 2',3'-diol; the 5'-hydroxyl of the right-hand RNA substrate was changed to a 5'-monophosphate; or both changes were made simultaneously. The results indicate that ligation activity strictly requires both the 2',3'-cyclic phosphate and the 5'-hydroxyl group for all four deoxyribozymes (Figure X10). Complete loss of ligation activity upon making a functional group alteration does not logically demand that the original functional group participates directly in the ligation reaction. For example, the altered functional group could instead chelate a critical metal ion, or the alteration could introduce steric perturbations. Indeed, data presented in the manuscript indicates that all four 6J deoxyribozymes do not use the 5'-hydroxyl group as the attacking nucleophile in the ligation reaction.

See Figure 12 for experiments with systematic 2'-deoxy substitutions of the R substrate for the 6J12, 6J2, and 12BB8 deoxyribozymes. As interpreted in the manuscript, these latter experiments are likely to reflect directly on the participation of the deleted functional group in ligation. This is because the removal of a 2'-hydroxyl (i.e., replacement of OH with H) is more subtle than a 5'-hydroxyl to 5'-monophosphate modification or a 2',3'-cyclic phosphate to 2',3'-diol modification.

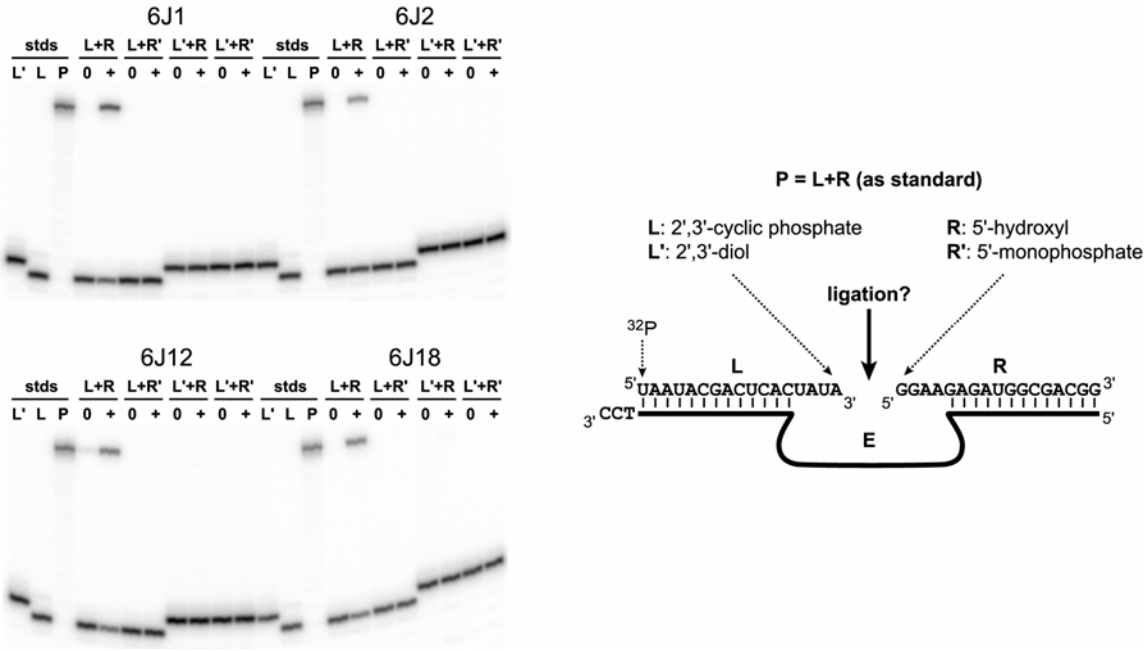

Figure X10. Testing the ligation dependence for the 6J deoxyribozymes on the functional groups of the two RNA substrates. Specifically, changes were made to the 2',3'-cyclic phosphate of L and the 5'-hydroxyl of R. The labels L and R denote RNA substrates with the termini used during selection (2',3'-cyclic phosphate and 5'-hydroxyl), whereas L' and R' denote the "wrong" termini. On the gels, "0" indicates a timepoint taken immediately after mixing, and "+" indicates a suitable reaction time (10 min for 6J12; 1 h for the other three deoxyribozymes). The concentration of deoxyribozyme was ~1.5 μM. The final incubation conditions were 70 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$ at 37 °C.

Mass spectrometry data for the 6J12 ligation product

The ESI mass spectrum of the 6J12 ligation product trimer shows a parent mass at *m/z* 1036, which matches that expected for 5'-AGG-3' with its 2',3'-cyclic phosphate hydrolyzed to a 2'(3')-mono-phosphate (Figure X11). The MS/MS spectrum shows numerous fragments, nearly all of which are readily assigned to cleavage at specific locations within the 2'–2'-linked trimer as one possible product.

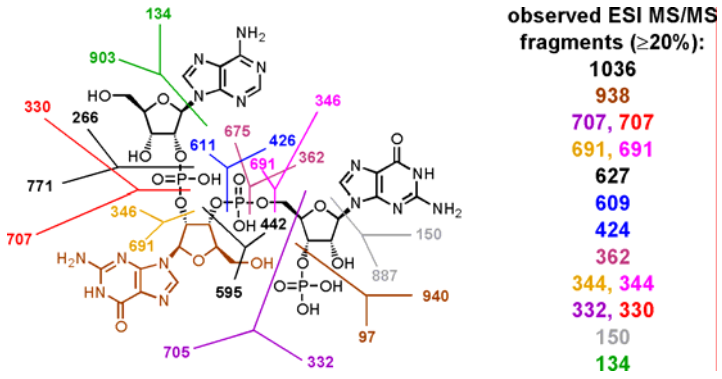

Figure X11. ESI MS/MS data for the 6J12 ligation product trimer. Fragmentation possibilities are superimposed on the 2'–2'-linked trimer structure, which is one of the two structural possibilities (see text and Figure 11). The isomeric 3'–2'-linked trimer structure gives identical fragmentation possibilities. Fragmentations shown in black on the structure were not observed but are shown for completeness. Of all observed peaks as tabulated on the right, only the peak at *m/z* 627 is not readily assignable by inspection of the structure; its origin is not assigned. Peaks were observed as follows, listed as *m/z* (relative intensity) and giving only the tallest peak of each cluster for those peaks with intensity ≥20%. With collision energy 0 V: 1036 (100), 707 (62). With collision energy 30 V: 1036 (93), 938 (66), 707 (100), 627 (31), 609 (32), 424 (40), 362 (32), 344 (34), 328 (21). At even higher collision energy 40 V, large peaks (50–60%) at *m/z* 150 and 134 are evident that correspond to fragmentation leading to the individual guanine and adenine nucleobases.

Cleavage of the 12BB6 and 12BB8 ligation products by 12BB6 and 12BB8

In analogy to the assays of Figure 7, the 12BB6 and 12BB8 deoxyribozymes were each used to cleave the 12BB6 and 12BB8 ligation products (2 × 2 = 4 assays). The results shown in Figure X12 strongly suggest that the two deoxyribozymes create identical ligation products.

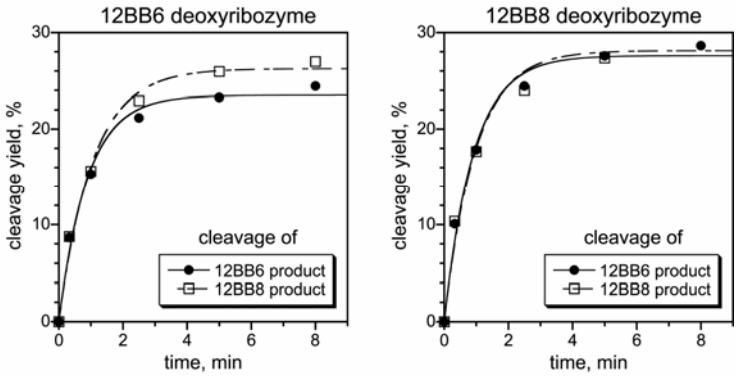

Figure X12. Cleavage of the 12BB6 and 12BB8 ligation products with the 12BB6 and 12BB8 deoxyribozymes. In both cases, the indicated deoxyribozyme cleaves the two products with experimentally indistinguishable rates. k_{obs} values: 12BB6, 1.12 ± 0.12 min^{-1} and 0.95 ± 0.10 min^{-1}; 12BB8, 1.10 ± 0.13 and 1.03 ± 0.17 min^{-1}. All values are best fit ± std. dev. from the exponential curve fit.

Quantitative determination of the alkaline hydrolysis $t_{1/2}$ values

The unnatural RNA ligation products from the 6J12, 6J2, and 12BB8 deoxyribozymes were subjected to partial alkaline hydrolysis at 60 °C to provide a quantitative estimate of their $t_{1/2}$ values. The data shown in Figure X13 reveal that all of the $t_{1/2}$ values are between 8 and 13 min. As noted in the manuscript, our best estimate for $t_{1/2}$ at 90 °C (which is a more conventional temperature for partial alkaline hydrolysis assays) is ~2 min under otherwise-similar incubation conditions.

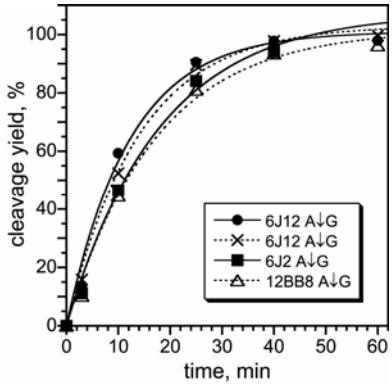

Figure X13. Cleavage of the deoxyribozyme ligation products under partial alkaline hydrolysis conditions at 60 °C (50 mM $NaHCO_3$, pH 9.2). Under these conditions, each product reverts quickly to the original left-hand RNA substrate, as shown in Figures 8 and 9. Samples were incubated in a PCR machine rather than a water bath or dry block for optimal temperature control. For this experiment, the 6J12 product was tested twice, with similar results. $t_{1/2}$ values, top to bottom in order of the figure legend (min): 8.4, 9.6, 12.4, 11.8. In each case, the standard deviation for $t_{1/2}$ as determined from the curve fit parameters was 10–20%.