# General Deoxyribozyme-Catalyzed Synthesis of Native 3′–5′ RNA Linkages

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General experimental considerations

DNA oligonucleotides were prepared at IDT (Coralville, IA). 5′-Triphosphorylated (5′-ppp) RNA oligonucleotides were prepared by in vitro transcription using T7 RNA polymerase and a synthetic double-stranded DNA template that was prepared by annealing two DNA oligonucleotides.[1] For transcripts beginning with 5′-pppA, appropriate modifications were made to the DNA template.[2] RNA oligonucleotides without a 5′-triphosphate were obtained by dephosphorylation of 5′-triphosphate RNAs with calf intestinal phosphatase (CIP). The left-hand (L) and right-hand (R) RNA substrate sequences used during the selection experiments (original sequences) were 5′-GGAAGUCUCAUGUACUA-3′ and 5′-GAUGUUCUAGCGCCGGA-3′. For all kinetic assays, the L substrate was 5′-$^{32}$P-radiolabeled with γ-$^{32}$P-ATP and T4 polynucleotide kinase (PNK). The ratio L:E:R was 1:5:15, with the concentration of E (deoxyribozyme) equal to ~0.5 μM. Values of $k_{obs}$ and final yield were obtained by fitting the yield versus time data directly to first-order kinetics, yield = $Y(1–e^{–kt})$, where $k = k_{obs}$ and $Y$ = final yield. The standard incubation conditions for the assays with $Mg^{2+}$ were 50 mM CHES, pH 9.0, containing 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$ at 37 °C. Alternatively, 50 mM HEPES, pH 7.5 was effective but with lower $k_{obs}$ (as reported in the manuscript), which was anticipated on the basis of the reduced pH value. The standard incubation conditions for the assays with $Zn^{2+}$ were 70 mM Tris, pH 7.5, containing 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$ at 23 °C.

The detailed procedure for the analytical-scale assays of Fig. 2 was as follows for the $Mg^{2+}$-dependent 9DB1 deoxyribozyme. A sample was prepared that contained 1 pmol of L substrate, 5 pmol of 9DB1 deoxyribozyme (E), and 15 pmol of R substrate (L:E:R = 1:5:15) in 7 μL of 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min. The volume was increased to 10 μL containing 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$. The 10-μL reaction solution was incubated at 37 °C. At appropriate timepoints, 1-μL aliquots were quenched onto 8 μL of stop solution (80% formamide, 1× TB [89 mM each Tris and boric acid, pH 8.3], 50 mM EDTA, and 0.025% each xylene cyanol and bromophenol blue). The substrates and products were separated using 20% PAGE and visualized by exposure to a PhosphorImager screen.

For the $Zn^{2+}$-dependent 7DE5 deoxyribozyme, a sample was prepared that contained 1 pmol of L substrate, 5 pmol of 7DE5 deoxyribozyme, and 15 pmol of R substrate in 7 μL of 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min. The volume was increased to 10 μL containing 70 mM Tris, pH 7.5, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$. The $Zn^{2+}$ was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM $HNO_3$, and 200 mM Tris, pH 7.5; this 10× stock solution was prepared by combining appropriate volumes of 1 M Tris, pH 7.5 and a 100× stock solution of 100 mM $ZnCl_2$ and 200 mM $HNO_3$. The 10-μL reaction solution was incubated at room temperature (23 °C). At appropriate timepoints, 1-μL aliquots were quenched onto 8 μL of stop solution (80% formamide, 1× TB [89 mM each Tris and boric acid, pH 8.3], 50 mM EDTA, and 0.025% each xylene cyanol and bromophenol blue). The substrates and products were separated using 20% PAGE and visualized by exposure to a PhosphorImager screen.

Deoxyribozyme selections and characterization of the newly formed RNA linkages

The selections were performed using the general approach described previously[3] in an RNA:DNA arrangement with one-nucleotide RNA overhangs at the ligation site (A↓G), as shown in Fig. 1. In the key selection step in which the two RNA substrates were joined, the DB ($Mg^{2+}$) and DE ($Zn^{2+}$) selections used incubation conditions listed above for 2 h. The percent RNA ligation activity by round is shown in Fig. S1A. The 8–17 deoxyribozyme cleavage step to enforce 3′–5′ selectivity (as described in ref. 4; deoxyribozyme sequence 5′-TCCGCGCTAGAACATTCCGAGCCGGACGAAGTACATGAGACTTCC-3′; enzyme region underlined) was included beginning with round 2 for the DB selection ($Mg^{2+}$) and round 5 for the DE selection ($Zn^{2+}$). The percent cleavage by 8–17 at each round of the selection is shown in Fig. S1B. At the conclusion of the selection process but before individual deoxyribozymes were cloned, each of the selection pools was assayed for RNA linkage using the 8–17 deoxyribozyme, which selectively cleaves 3′–5′ linkages.[3] These data indicated that both pools create >95% 3′–5′ RNA linkages (Fig. S1C). Consistent with this, both of the individual 9DB1 and 7DE5 ligation products were readily cleaved by the 8–17 deoxyribozyme (Fig. S1D), demonstrating that each deoxyribozyme indeed synthesizes 3′–5′ RNA linkages.

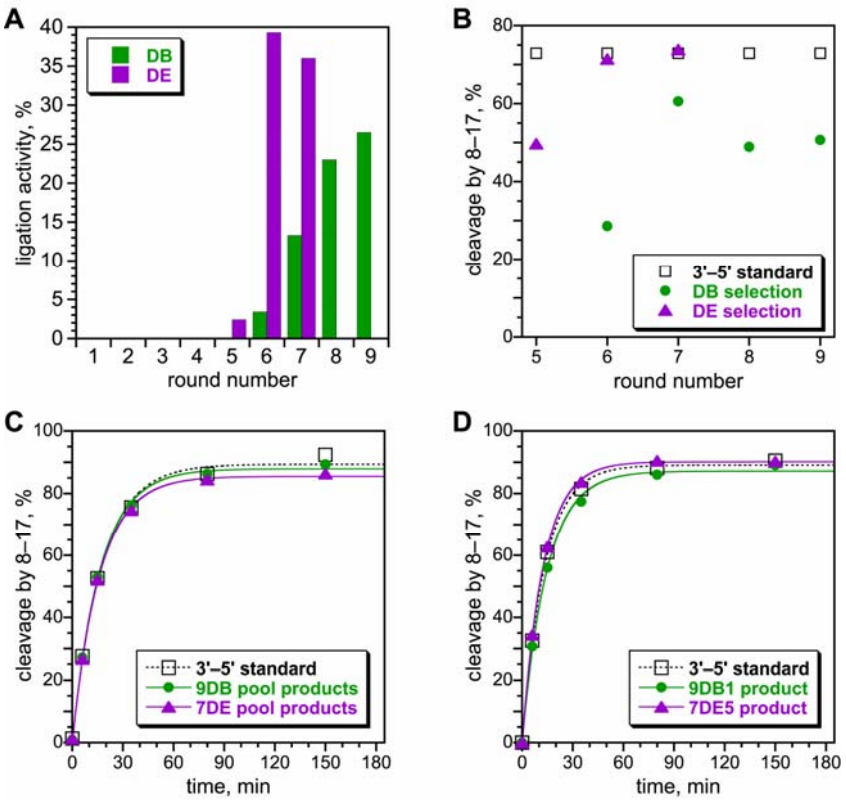


**Figure S1.** Deoxyribozyme selections and characterization of the new RNA linkages. (A) Ligation activities for the DB and DE selections by round. (B) Percent cleavage by the 3′–5′-selective 8–17 deoxyribozyme for the selection rounds in which this step was included (the value could of course be determined only for those rounds in which ligation activity was detectable). (C) Assays of the 9DB and 7DE uncloned pool products by 8–17 cleavage, demonstrating that they are predominantly (>95%) 3′–5′-linked. (D) Assays of the 9DB1 and 7DE5 ligation products by 8–17 cleavage, demonstrating that they are 3′–5′-linked.

Establishing the generality of 9DB1 and 7DE5 for ligating RNA substrate sequences

To determine the extent of generality of 9DB1 and 7DE5, we systematically tested various RNA substrate sequences. In the selection design, a single RNA nucleotide of each substrate was not Watson-Crick base-paired with DNA at the ligation site (A↓G), whereas all other RNA nucleotides were base-paired with DNA (Fig. 1). Experiments were performed for both 9DB1 (Fig. S2) and 7DE5 (Fig. S3) to determine their generality.

First (in the panels labeled A), the boundaries were established between RNA nucleotides with strict sequence requirements and the variable binding arms. For this purpose, the RNA substrate sequences were varied systematically relative to the original RNA sequences (A↔C; G↔U). Ligation assays were then performed using these variant RNA substrates and deoxyribozymes with the appropriate mutations that retain Watson-Crick RNA:DNA complementarity. For 9DB1 the nucleotide positions with requirements are X↓XX, and for 7DE5 the nucleotide positions with requirements are X↓X.

Second (in the panels labeled B), the precise requirements near the ligation site were established by testing single-nucleotide variants of the RNA substrates. The requirements are D↓RA for 9DB1, where D denotes A, G, or U (i.e., not C) and R denotes A or G. The requirements are A↓R for 7DE5. In both cases, pyrimidine nucleotides could not be tested at the ↓R positions because a 5′-triphosphate-pyrimidine cannot be introduced by in vitro transcription with T7 RNA polymerase, which was used to prepare the right-hand RNA substrates.

Third (in the panels labeled C), the variable binding arms were changed comprehensively to demonstrate their broad tolerance. The RNA substrate sequences were varied systematically relative to the original RNA sequences, as either "transitions" (A↔G, U↔C), "transversions of type 1" (A↔C; G↔U), or "transversions of type 2" (A↔U; G↔C). Our previous studies have used similar systematic changes to establish ligation generality for other deoxyribozymes.[5-7] In all cases, the effects on RNA ligation rate and yield were modest (<4-fold change in rate) despite the extensive changes to the substrate sequences (Fig. S2C and S3C).

Collectively, these data demonstrate that both 9DB1 and 7DE5 are very general for 3′–5′ RNA ligation with overall substrate sequence requirements of D↓RA and A↓R, respectively (Fig. S2D and S3D). All nucleotides not shown explicitly in these structures have no particular sequence requirement.

The rates and yields of both the 9DB1 and 7DE5 deoxyribozymes (see text) are clearly of practical utility for RNA ligation. Moreover, the sequence requirements D↓RA and A↓R for 9DB1 and 7DE5 compare favorably with the requirements of the 10–23 and 8–17 deoxyribozymes,[10] which are widely used tools for RNA cleavage. The 10–23 and 8–17 deoxyribozymes require RNA substrates with the sequence motifs R↓Y and A↓G, respectively (note that these RNA-cleaving deoxyribozymes leave 2′,3′-cyclic phosphate and 5′-hydroxyl termini after cleavage). Quantitatively, if one assumes random-composition RNA and computes the probability of finding a specific combination of nucleotides at a particular site, R↓Y is expected with probability ½•½ = 1/4 and A↓G is expected with probability ¼•¼ = 1/16. In a similar computation, D↓RA is expected with probability ¾•½•¼ = 3/32 (i.e., more often than A↓G), and A↓R is expected with probability ¼•½ = 1/8. Thus both 9DB1 and 7DE5 should be immediately useful for RNA ligation roughly as often as the 10–23 and 8–17 deoxyribozymes are useful for RNA cleavage. It should also be noted that just as analogs of the 8–17 deoxyribozyme can be identified for other dinucleotide cleavage sites,[11] we anticipate that other $Mg^{2+}$- and $Zn^{2+}$-dependent RNA ligase deoxyribozymes can be identified that will create other RNA ligation junctions, and studies to identify such deoxyribozymes are now in progress.

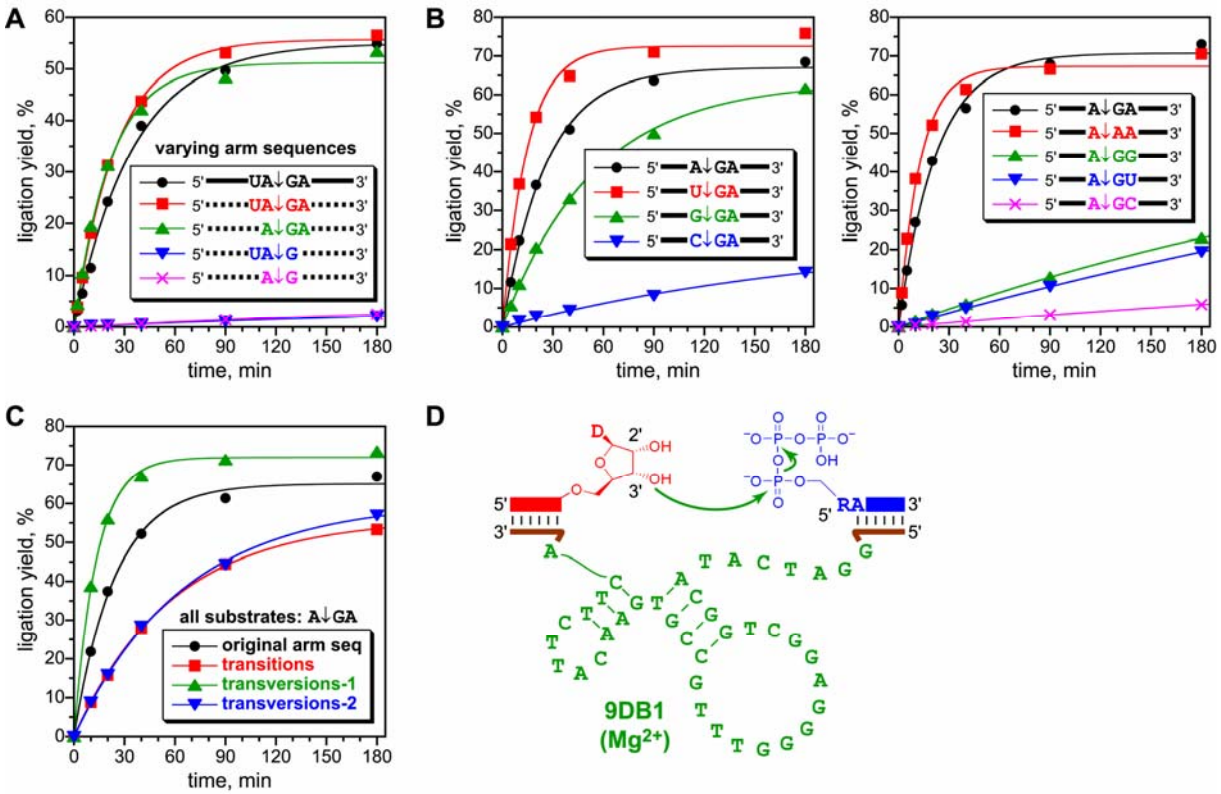


**Figure S2.** Establishing the generality of the $Mg^{2+}$-dependent 9DB1 deoxyribozymes for various RNA substrate sequences. (A) Experiments to establish the boundaries beyond which RNA substrate nucleotides may be varied (here, X↓XX). The variant RNA sequences (indicated by dashed bars) were changed from the original arm sequences (indicated by solid bars) by transversion-1 (A↔C, G↔U), with complementary changes in the DNA binding arms. (B) Experiments to establish the specific requirements for the N↓NN nucleotide positions (here, D↓RA). All RNA nucleotides in the arms were the original sequences (solid bars). The first set of experiments establishes that the requirement for the last nucleotide of the left-hand substrate is D↓, because only C↓ reacts poorly. Then, because ↓G<u>A</u> succeeds whereas ↓G<u>G</u>, ↓G<u>U</u>, and ↓G<u>C</u> fail, the requirement at the second position of the right-hand substrate is ↓X<u>A</u>. Finally, the comparison of ↓<u>G</u>A and ↓<u>A</u>A substrates establishes that both are succesful, so the requirement is ↓R (because ↓Y cannot be tested readily, ↓R is a conservative estimate). Combining all of these results, the required ligation motif is D↓RA. (C) Experiments to demonstrate full generality for changing the RNA substrate sequences away from the ligation junction. Except for the A↓GA nucleotides that were retained near the ligation junction, all RNA substrate nucleotides were changed systematically from the original arm sequences by transitions (A↔G, U↔C), transversion-1 (A↔C, G↔U), or transverison-2 (A↔U, G↔C), with complementary changes in the DNA binding arms. (C) The mfold-predicted secondary structure[8,9] of 9DB1 is shown interacting with its RNA substrates, and the D↓RA sequence requirements are illustrated explicitly.

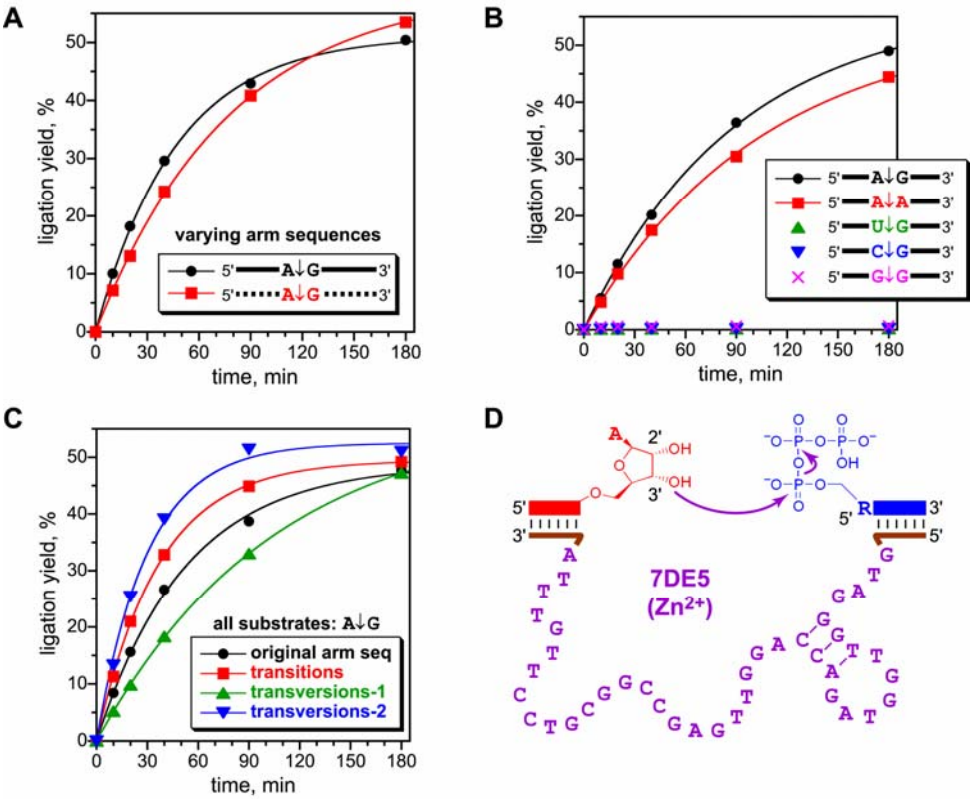


**Figure S3.** Establishing the generality of the $Zn^{2+}$-dependent 7DE5 deoxyribozymes for various RNA substrate sequences. (A) Experiments to establish the boundaries beyond which RNA substrate nucleotides may be varied (here, X↓X). The variant RNA sequences (indicated by dashed bars) were changed from the original arm sequences (indicated by solid bars) by transversion-1 (A↔C, G↔U), with complementary changes in the DNA binding arms. (B) Experiments to establish the specific requirements for the N↓N nucleotide positions (here, A↓R). All RNA nucleotides in the arms were the original sequences (solid bars). Because A↓G succeeds whereas U↓G, C↓G, and G↓G fail completely, the requirement for the last nucleotide of the left-hand substrate is A↓. Then, the results with A↓G and A↓A (which both work well) indicate that A↓R is the overall requirement. Again, this is a conservative estimate because of our inability to test ↓Y. (C) Experiments to demonstrate full generality for changing the RNA substrate sequences away from the ligation junction. Except for the A↓G nucleotides that were retained near the ligation junction, all RNA substrate nucleotides were changed systematically from the original arm sequences by transitions (A↔G, U↔C), transversion-1 (A↔C, G↔U), or transverison-2 (A↔U, G↔C), with complementary changes in the DNA binding arms. (C) The mfold-predicted secondary structure[8,9] of 7DE5 is shown interacting with its RNA substrates, and the A↓R sequence requirements are illustrated explicitly.

MALDI-MS data for the RNA oligonucleotide transcripts

As shown in Table X1, satisfactory MALDI-MS data was obtained for all RNA transcripts prepared using a synthetic DNA template and T7 RNA polymerase, as used in the experiments of Fig. S2 and Fig. S3.

| RNA sequence | calcd. | found |
|---|---|---|
| *left-hand (L) substrates* | | |
| 5′-GGAAGUCUCAUGUACUA-3′ | 5651.3 | 5650.5 |
| 5′-GGAcugagacgugcaUA-3′ | 5705.3 | 5706.5 |
| 5′-GGAgacucugcacgucA-3′ | 5681.3 | 5681.8 |
| 5′-GGAcugagacgugcagA-3′ | 5744.4 | 5742.5 |
| 5′-GGAucagaguacaugaA-3′ | 5714.3 | 5714.1 |
| 5′-GGAAGUCUCAUGUACUu-3′ | 5628.2 | 5628.9 |
| 5′-GGAAGUCUCAUGUACUg-3′ | 5667.3 | 5666.1 |
| 5′-GGAAGUCUCAUGUACUc-3′ | 5627.2 | 5627.4 |
| *right-hand (R) substrates* | | |
| 5′-GAUGUUCUAGCGCCGGA-3′ | 5682.3 | 5677.8 |
| 5′-GAcaccucgauauuaag-3′ | 5634.3 | 5636.4 |
| 5′-GAguggagcuauaauuc-3′ | 5691.3 | 5699.2 |
| 5′-GAacaagaucgcggccu-3′ | 5688.3 | 5689.0 |
| 5′-Ggcaccucgauauuaag-3′ | 5650.3 | 5649.2 |
| 5′-Gcguggagcuauaauuc-3′ | 5667.3 | 5668.5 |
| 5′-Guacaagaucgcggccu-3′ | 5665.3 | 5667.1 |
| 5′-aAUGUUCUAGCGCCGGA-3′ | 5666.3 | 5666.6 |
| 5′-GgUGUUCUAGCGCCGGA-3′ | 5698.3 | 5699.5 |
| 5′-GuUGUUCUAGCGCCGGA-3′ | 5659.2 | 5657.7 |
| 5′-GcUGUUCUAGCGCCGGA-3′ | 5658.2 | 5660.5 |

**Table S1.** MALDI-MS data for the RNA transcripts used in the experiments of Fig. S2 and Fig. S3. Sequences are colored as in these figures. Original RNA sequences are uppercase; modified blocks of nucleotides are lowercase; and individual modified nucleotides are lowercase and underlined. All transcripts were analyzed as their 5′-triphosphate form after transcription (the left-hand substrates were subsequently dephosphorylated and 5′-$^{32}$P-radiolabeled).

Application of 9DB1 and 7DE5 to synthesize P4-P6 and *xpt* G-riboswitch (Fig. 3A,C)

For preparation of P4-P6 by ligation using the 7DE5 deoxyribozyme (Fig. 3A), the left-hand (L) RNA substrate was prepared by solid-phase synthesis and 5′-$^{32}$P-radiolabeled with γ-$^{32}$P-ATP and PNK. The unradiolabeled right-hand (R) RNA substrate was prepared by T7 RNA polymerase transcription from a linearized plasmid template. The L substrate was nucleotides G102–A133 of the P4-P6 sequence.[12,13] The R substrate was nucleotides A134–A261 of the P4-P6 sequence. The 7DE5 deoxyribozyme was 5′-GTTTCCCCTGAGACTTGGTAGTAGGGTTGGTAGACCAGGTTGAGCCGGCGTCCTTGTTTAGAAC-GGCTGTTGACCCCTTT-3′, where the 40-nt enzyme region as shown in Fig. 1 is underlined. Relatively long (20-nt) binding arms were used on each side of the enzyme region to assist with disruption of the P4-P6 secondary structure and assure binding of the deoxyribozyme to the RNA substrates. The ligation assay was performed as described on page S2. Samples were analyzed using 6% PAGE. A full version of the gel image of Fig. 3A is shown in Fig. S4.

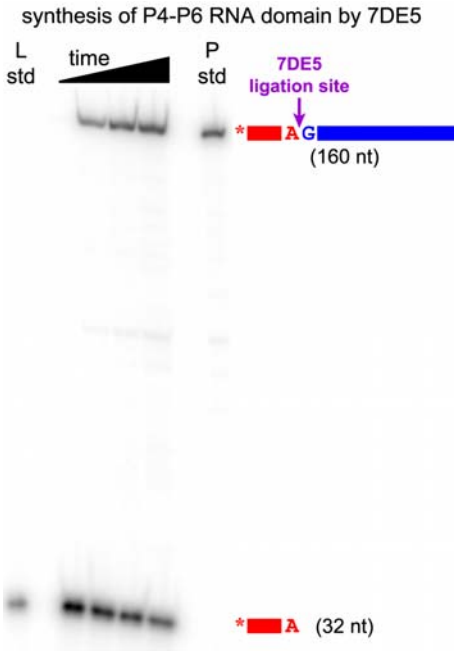


**Figure S4.** Full version of the gel image shown in Fig. 3A.

For preparation of the *xpt* G-riboswitch[14,15] using the 9DB1 and 7DE5 deoxyribozymes (Fig. 3C), two ligation sites were chosen within the 72-nt core of the RNA sequence, 5′-GGACACUCAUAUAAUC-GCGUG↓GAUAUGGCACGCAAGUUUCUACCGGGCACCGUA↓AAUGUCCGACUAUGGGUG-3′. This sequence has a leading 5′-GGA to permit transcription by T7 RNA polymerase followed by nucleotides C14–G82; the two ligation sites are underlined. For each ligation assay, the L substrate (21 or 54 nt) was prepared by T7 RNA polymerase transcription from a synthetic DNA template, dephosphorylated with CIP, and 5′-$^{32}$P-radiolabeled with γ-$^{32}$P-ATP and PNK. The unradiolabeled R substrate (51 or 18 nt) was prepared by transcription from a synthetic DNA template. The ligation assays were performed as follows, using the experimental procedures described on page S2. First, the 9DB1 deoxyribozyme (5′-GTAGAAACTTGCGTG-CCATATGGATCATACGGTCGGAGGGGTTTGCCGTGAACATTCTTCAACGCGATTATATGAGTG-3′, where the 40-nt enzyme region as shown in Fig. 1 is underlined) was used to create a linkage between G31 and G32 of the riboswitch (ligation site G↓GA, consistent with the D↓RA motif). Second, both 9DB1 (5′-CACCCATAGTCGGACATGGATCATACGGTCGGAGGGGTTTGCCGTGAACATTCTTCAACGGTGCCCGGTAGAAAC-3′) and

7DE5 (5′-CACCCATAGTCGGACATGTAGGGTTGGTAGACCAGGTTGAGCCGGCGTCCTTGTTTAACGGTGCCCGGTAGAAAC-3′) were separately used to create a linkage between A64 and A65 of the riboswitch (ligation site A↓AA, consistent with both the D↓RA and A↓R motifs). Samples were analyzed using 12% PAGE. A full version of the gel image of Fig. 3C is shown in Fig. S5.

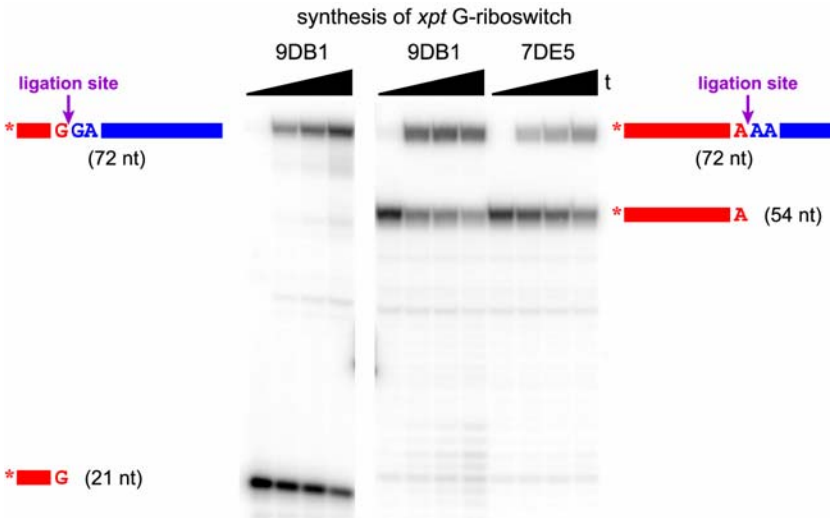


**Figure S5.** Full version of the gel image shown in Fig. 3C.

Nondenaturing (native) gel electrophoresis of P4-P6 (Fig. 3B)

The plasmids encoding wild-type P4-P6 (lanes labeled "wt" in Fig. 3B; P4-P6-wt, nt 102-261) and the unfolded control (lanes labeled "unf" in Fig. 3B; P4-P6-bp, nt 102-261 with nt 123-126 ACAG in the J5/5a joining region[12,16] replaced with UGU) were available from a previous study.[17] The unfolded control has several mutations in the J5/5a region that disrupt $Mg^{2+}$-dependent tertiary folding by introducing base pairs into a formerly flexible hinge region.[12,16,18] The P4-P6-wt and P4-P6-bp plasmids were linearized with EarI; RNA was transcribed using T7 RNA polymerase, dephosphorylated with CIP, and 5′-$^{32}$P-radiolabeled with γ-$^{32}$P-ATP and PNK.

The sample of P4-P6 prepared by 7DE5 ligation (Fig. 3A) was synthesized using the general procedure described on page S2 on slightly larger scale. A sample was prepared that contained 5 pmol of L substrate, 15 pmol of 7DE5 deoxyribozyme, and 30 pmol of R substrate in 14 µL of 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min. The volume was increased to 20 µL containing 70 mM Tris, pH 7.5, 150 mM NaCl, 2 mM KCl, and 1 mM $ZnCl_2$. The $Zn^{2+}$ was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM $HNO_3$, and 200 mM Tris, pH 7.5; this 10× stock solution was prepared by combining appropriate volumes of 1 M Tris, pH 7.5 and a 100× stock solution of 100 mM $ZnCl_2$ and 200 mM $HNO_3$. The 20-µL reaction solution was incubated at room temperature (23 °C). After 3.5 h, the sample was quenched with 30 µL of stop solution, and the ligation product was purified using 6% PAGE.

The nondenaturing gel electrophoresis experiment of Fig. 3B were performed as described previously[18,19] with slight modifications. Such experiments have been used many times both by us[17-22] and by others[23,24] for assaying P4-P6 folding. Each 5′-$^{32}$P-radiolabeled RNA sample (~20–50 fmol) in 2 µL of water was mixed with 2 µL of 2× native gel loading buffer, which contained 2× TB, 10% glycerol, and 40 mM $MgCl_2$ (1× TB contains 89 mM each Tris and boric acid, pH 8.3). The sample was annealed by heating at 50 °C for 5 min and equilibrated at 35 °C for 10 min before loading into a gel

lane. The samples were electrophoresed at 200 V and 35 °C for 6 h on a 8% polyacrylamide gel containing 1× TB (made using a 40% 29:1 acrylamide:bis-acrylamide stock solution) and 20 mM $MgCl_2$, then dried at 60 °C for 20 min and exposed to a PhosphorImager screen.

Additional data to demonstrate 3′–5′ linkages in the P4-P6 RNA synthesized by 7DE5

The sample of P4-P6 prepared by 7DE5 ligation (Fig. 3A) was synthesized as described in the section above. In parallel, the wild-type P4-P6 RNA was obtained by transcription from a linearized plasmid template (see section on nondenaturing gel electrophoresis, above). The 8–17 deoxyribozyme was used to cleave both versions of P4-P6 between nucleotides A133 and G134 in side-by-side assays (Fig. S6). The 8–17 sequence was 5′-GTTTCCCCTGAGACTTGGTATCCGAGCCGGACGAGAACGGCTGTTGACCCC-TTT-3′ (the enzyme region is underlined, and the binding arms are complementary to P4-P6 nucleotides 113–132 and 135–154). The data show that the cleavage kinetics and the gel migration rates of the cleavage products are indistinguishable, confirming the 3′–5′ linkage created by 7DE5.

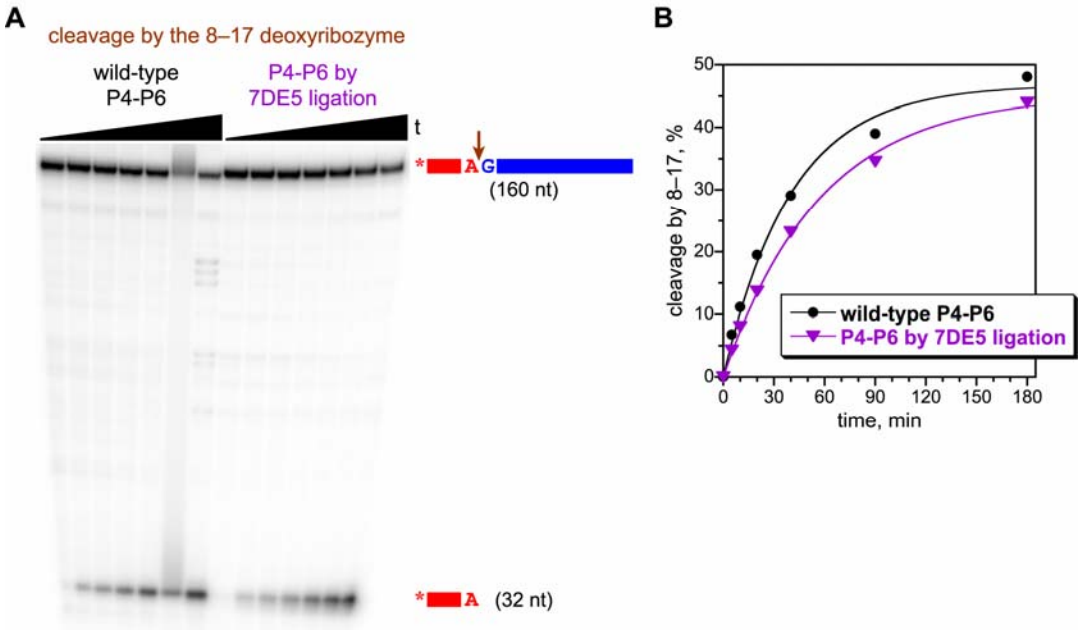


**Figure S6.** Using the 8–17 deoxyribozyme to cleave a specific 3′–5′ linkage within P4-P6. (A) Gel image of the 8–17 cleavage experiments performed on wild-type P4-P6 and also on P4-P6 synthesized by ligation using the 7DE5 deoxyribozyme. Note that the cleavage products migrate at indistinguishable positions. (B) Timecourses of the 8–17 cleavage assays. The ~50% cleavage is likely due to intramolecular secondary structure formation within P4-P6, which competes with intermolecular binding of the 8–17 deoxyribozyme.

Additionally, we examined cleavage of both P4-P6 samples at 100 mM $Mg^{2+}$ and pH 9.0 when bound with complementary DNA. These conditions are known to induce preferential cleavage of 2′–5′ RNA linkages.[3,25] To ensure that the region of RNA encompassing the A133–G134 RNA linkage was bound with complementary DNA, we added three 51-mer DNA oligonucleotides to the sample of P4-P6. These three nucleotides were complementary to nt 104–154, 149–199, and 211–261 of P4-P6. As a negative control, we used wild-type P4-P6 as described above. As an imperfect (but still relevant) positive control for the overall ability of the assay to reveal the presence of a 2′–5′ linkage, we used a version of P4-P6 that has a single 2′–5′ linkage between nt A233 and G234 in the L6b loop, as prepared by the 9A6 deoxyribozyme.[3] The same three 51-mer complementary DNA oligonucleotides were used

with this positive control RNA. Both the negative and positive control RNAs behaved as expected (Fig. S7). In a parallel assay, P4-P6 prepared by 7DE5 ligation showed no cleavage (<0.5% in 24 h under the conditions described in ref. 3). Therefore, the P4-P6 RNA synthesized by the 7DE5 deoxyribozyme does not have an internal 2′–5′ linkage. This experiment is insufficient by itself to establish that the 7DE5-synthesized P4-P6 has a 3′–5′ linkage at the 7DE5 ligation site. However, this experiment strongly suggests that the synthetic P4-P6 does *not* have a 2′–5′ linkage at this site, and the experiment of Fig. S6 establishes conclusively that the synthetic P4-P6 has a 3′–5′ linkage at this site.

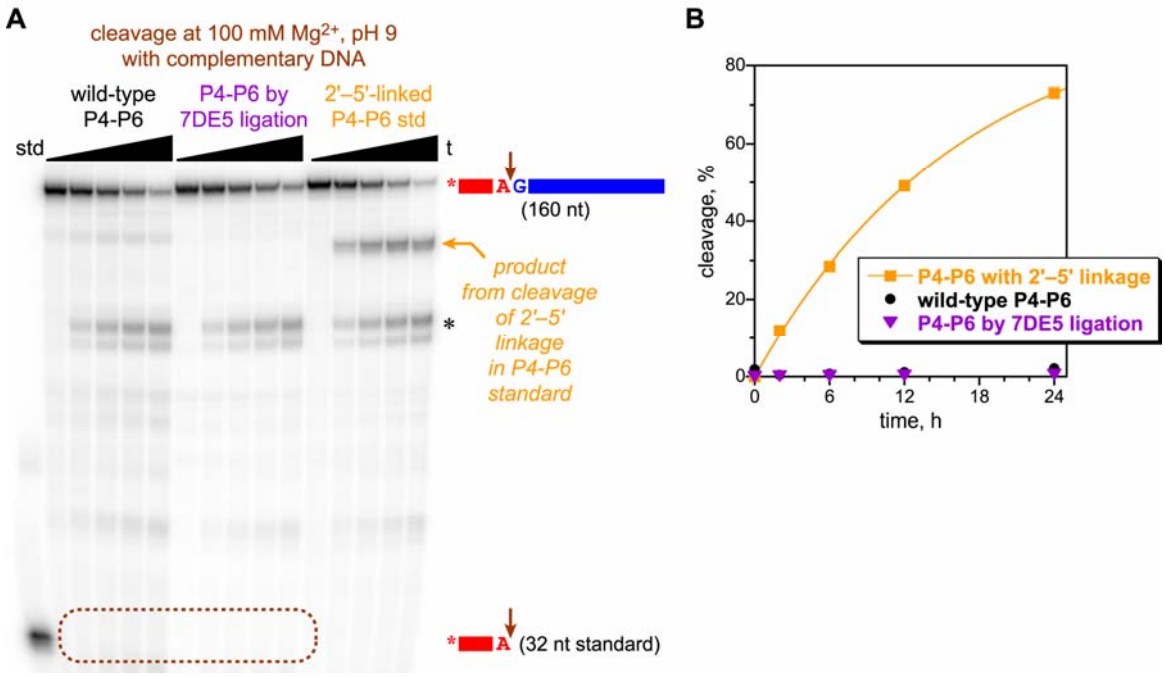


**Figure S7.** Cleavage at 100 mM $Mg^{2+}$ and pH 9.0 in the presence of compelementary DNA to show the absence of a 2′–5′ linkage within the synthetic P4-P6. In each assay, 150 fmol of 5′-$^{32}$P-radiolabeled P4-P6 RNA was incubated with 50 pmol of each of the three 51-mer DNA oligonucleotides described in the text along with 12.5 pmol of 5′-phosphorylated carrier RNA of unrelated sequence ($t$ = 0, 2, 6, 12, and 24 h). The carrier RNA was the 51-mer R substrate for the *xpt* G-riboswitch. The assays followed the approach described in ref. 3. (A) Gel image showing that the 2′–5′-linked P4-P6 standard cleaves readily under these conditions (orange arrow). In contrast, neither wild-type P4-P6 nor the P4-P6 prepared by 7DE5 ligation are cleaved detectably (<0.5%), which would lead to a band migrating at the same position of the standard (dotted brown box). All three P4-P6 samples show a generic band ascribed to nonspecific cleavage within the P5abc region (marked with an asterisk). (B) Timecourse of the cleavage reaction. The $t_{1/2}$ value for the 2′–5′-linked P4-P6 is ~11 h, as found in ref. 3.

## References for Supporting Information


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