

Deoxyribozymes that Synthesize Branched and Lariat RNA

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Figures in this Supporting information are prefixed by the letter X (e.g., Figure X1) to distinguish them from the manuscript Figures. All references cited by number are from the manuscript. See ref. 5 for experimental details of the selection procedure and kinetic analyses.

RNA and DNA sequences

The sequence of the left-hand RNA substrate L was 5'-UAAUACGACUCACUAUX-3', where X=A, C, dA, or dC, and the sites of branching are in boldface. 9F7 and 9F21 synthesize a branch at the boldface A, while 9F13 and 9F18 create a branch at the boldface U. This L substrate has a 5'-hydroxyl and an unmodified 2',3'-vicinal diol and was prepared by solid-phase synthesis. The sequence of the right-hand RNA substrate R was 5'-GGAAGGAGAUAGGCGACGG-3', with a 5'-triphosphate and a 2',3'-vicinal diol. This R substrate was prepared by in vitro T7 RNA polymerase transcription from a DNA oligonucleotides template. These are the same substrate sequences as in ref. 5, although the termini at the ligation junction are different. The substrate binding arms that base-pair with the deoxyribozymes are underlined.

As revealed by automated sequencing, the new deoxyribozyme sequences were as follows. Underlined nucleotides are the substrate binding arms, and boldface nucleotides are the deoxyribozyme region. A lowercase nucleotide in the binding arm indicates a non-Watson-Crick match for the RNA substrate, and a dash indicates a missing DNA nucleotide. We are currently investigating the roles and requirements for these unpaired and missing nucleotides, which are found in 9F7, 9F13, and 9F18 but not 9F21. Note that the 9F13 enzyme region is only 38 nt long, versus 40 nt for the other DNA enzymes. This is apparently due to *Taq* polymerase-associated deletions during selection (see ref. 5).

9F7: 5'-CCGTCGCCATCTCAATGAGGCTTGGCAGGGATTTAGTATTTTTAACACTCCCGGT-AGTCGTATTATCC-3'
 9F21: 5'-CCGTCGCCATCTCAATGATGCTTGACAGGGTCTATAGTTTCTATGTAGCCCGAGTGAGTCGTATTATCC-3'
 9F13: 5'-CCGTCGCCATCTCAGGATGTGGGGTTTTGCCCGAGGGTATGGCAGT--GGGGAGaGAGTCGTATTATCC-3'
 9F18: 5'-CCGTCGCCATCTCGGGATGTGGGGCGCCACCAAGTTAATGTTTGGTTTGGGGAGaGAGTCGTATTATCC-3'

For the experiments involving lariat formation, the sequence of the RNA pre-lariat substrate was 5'-GGAAGAGAUGCGACGGACACACACAACCCACCCCCAAAAACAACAAGGCCUACGAAAUACACACCAAAACCCCCACCCCACACCCCAACCAACACCCACAAAACCCCGGAUAAUACGACACUAUA-3'. The single-underlined nucleotides are the substrate binding arms; the two boldface nucleotides surround the cleavage site for a 10–23 deoxyribozyme; and the italicized nucleotides denote the binding arms for the 10–23 deoxyribozyme (see below). The poly(A/C) region sequences were chosen such that no strong secondary structure was present, as predicted by the mfold program (SantaLucia, J., Jr. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1460-1465; available online at www.bioinfo.rpi.edu/~zukerm). This was done such that secondary structure in the loop connecting the substrates would not interfere with lariat formation. Of course, many interesting lariats do have secondary structure in the loop, and we are currently investigating deoxyribozyme-mediated lariat formation in these cases. The double-underlined A near the 3'-end of the pre-lariat substrate was preemptively mutated from U in the original left-hand substrate to avoid potential secondary structure

formation as indicated was a possibility by mfold (the corresponding nucleotide in the 9F21 deoxyribozyme's binding arm was changed to A, to maintain base pairing). The pre-lariat substrate was prepared by in vitro T7 RNA polymerase transcription from a DNA template (itself prepared by PCR) with incorporation of α - 32 P-CTP. The 10–23 deoxyribozyme sequence was 5'-GGTGTG**ATTTCG**GGCTAGCTACAACGAAGGCCTTTGTT-3', where the enzyme region is boldface and the binding arms are italicized.

Mn²⁺-mediated formation of 2',5'-branched RNA by the new deoxyribozymes

In Figure 2 are reported data for Mn²⁺-mediated ligations that form 2',5'-branched RNA. Below in Figure X1 are shown complete data sets for all four new deoxyribozymes.

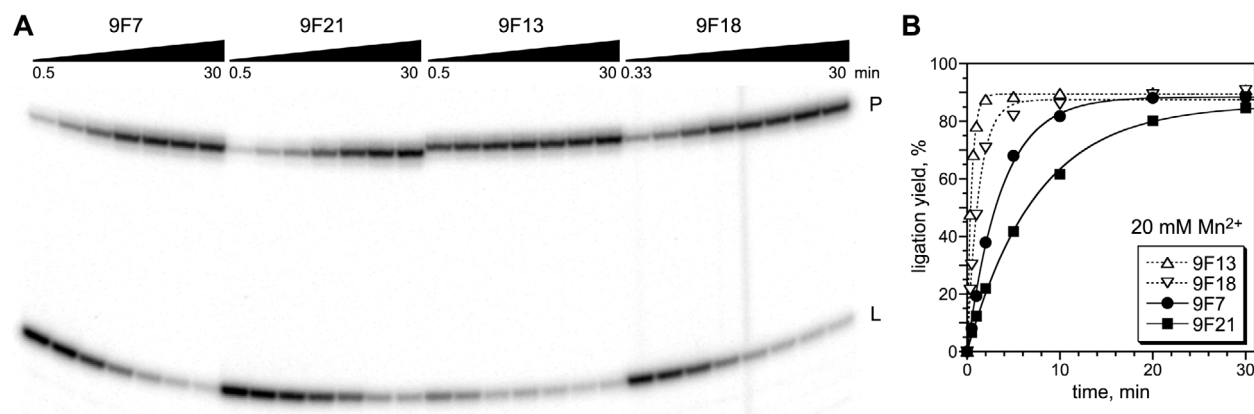


Figure X1. (A) 20% PAGE showing ligation to form branched RNA by the 9F7, 9F21, 9F13, and 9F18 deoxyribozymes (37 °C, 50 mM HEPES, pH 7.5, 20 mM Mn²⁺). Timepoints: 0.5, 1, 2, 5, 10, 20, and 30 min; there is an additional 20 s timepoint for 9F18 only. (B) Timecourse of branched RNA formation. k_{obs} , min⁻¹: 9F13, 2.2; 9F18, 0.81; 9F7, 0.27; 9F21, 0.13.

Mg²⁺-mediated formation of 2',5'-branched RNA by the new deoxyribozymes

The same branched linkages are also formed by each deoxyribozyme using Mg²⁺ instead of Mn²⁺ as the cofactor, with high yield but at a lower rate. Representative data at 80 mM Mg²⁺ is shown in Figure X2. A complete analysis of the metal dependence of the ligation reaction will be reported elsewhere.

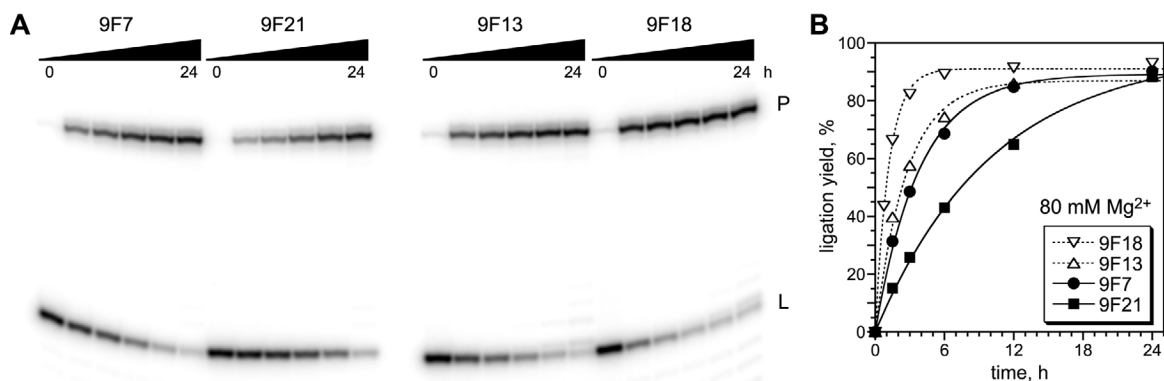


Figure X2. (A) 20% PAGE showing ligation to form branched RNA by the 9F7, 9F21, 9F13, and 9F18 deoxyribozymes (37 °C, 50 mM HEPES, pH 7.5, 80 mM Mg²⁺). Timepoints: 0, 1.5, 3, 6, 12, and 24 h. (B) Timecourse of branched RNA formation. k_{obs} , h⁻¹: 9F18, 0.85; 9F13, 0.37; 9F7, 0.26; 9F21, 0.10. The 9F18 data set includes a 45-min timepoint not shown on the gel image.

Background reaction rate and calculated rate enhancement with Mn^{2+} and with Mg^{2+}

The background reaction rate in 20 mM Mn^{2+} using an exactly complementary DNA splint was determined (Figure X3), giving a k_{bkgd} of ca. $4 \times 10^{-7} \text{ min}^{-1}$. For the fastest 9F13 deoxyribozyme ($k_{\text{obs}} = 2.2 \text{ min}^{-1}$), the calculated rate enhancement $k_{\text{obs}}/k_{\text{bkgd}}$ is 5×10^6 . For the 9F21 deoxyribozyme, which is the slowest of the four new DNA enzymes ($k_{\text{obs}} = 0.13 \text{ min}^{-1}$), the calculated rate enhancement $k_{\text{obs}}/k_{\text{bkgd}}$ is 3×10^5 . It should be noted that the RNA product of the background reaction is probably *not* any of the 2',5'-branched RNAs that are formed by deoxyribozyme-mediated ligation. The background product has not yet been analyzed in detail, which may prove difficult due to the very small amount of material formed. We expect that the background product will be linear—i.e., either 2'-5', 3'-5', or a mixture of these—but not 2',5'-branched. The background reaction rate specifically of an internal 2'-hydroxyl with the 5'-triphosphate would be quite difficult to measure directly, because the selectivity would be nearly impossible to enforce. If the rate of the branching background reaction is lower than the background rate to form linear 2'-5' or 3'-5' RNA, then the rate enhancements of our deoxyribozymes would be even higher than reported here, perhaps considerably so.

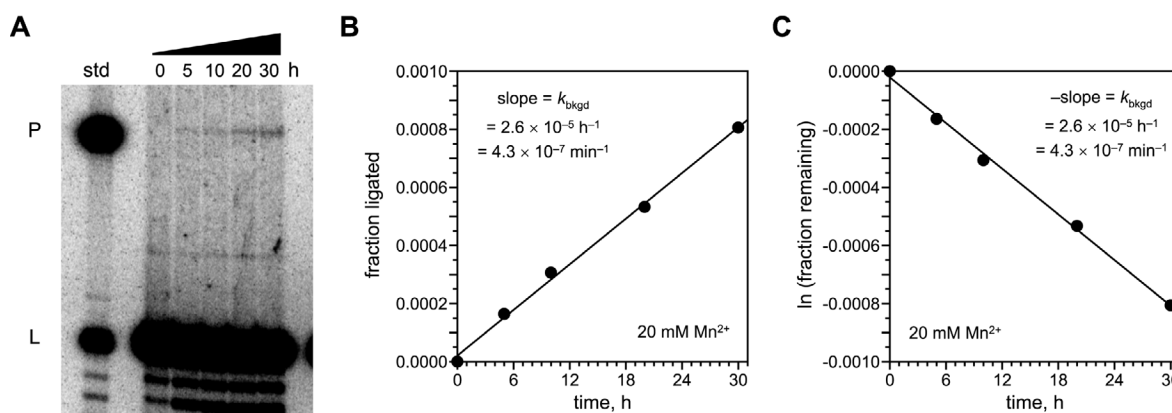


Figure X3. Determination of the background reaction rate in 20 mM Mn^{2+} (37 °C, 50 mM HEPES, pH 7.5). (A) 20% PAGE image. (B) and (C) Two alternative ways of plotting the data that give equivalent values of k_{bkgd} .

The background reaction rate in 80 mM Mg^{2+} using an exactly complementary DNA splint was found to be $k_{\text{bkgd}} \approx 10^{-5} \text{ h}^{-1}$ (data not shown). This value is comparable to the background rate in Mn^{2+} (see above) and is also comparable to the Mg^{2+} value of $2 \times 10^{-5} \text{ h}^{-1}$ reported in Rohatgi et al., *J. Am. Chem. Soc.* **1996**, *118*, 3332-3339 under slightly different incubation conditions. This value of k_{bkgd} leads to a calculated rate enhancement for Mg^{2+} of $k_{\text{obs}}/k_{\text{bkgd}}$ up to ca. 10^5 -fold, which is 1–2 orders of magnitude lower than the rate enhancement for Mn^{2+} .

Analysis of the Figure 4 experiment demonstrating the structure of the lariat RNA product

The lariat structure is as proposed in Figure 4. Gel images from the same experiment are shown below in Figure X4, with groups of lanes labeled for reference. Analysis of each portion of the gel image is given below. Incubation conditions for each of the assays are summarized after the analyses.

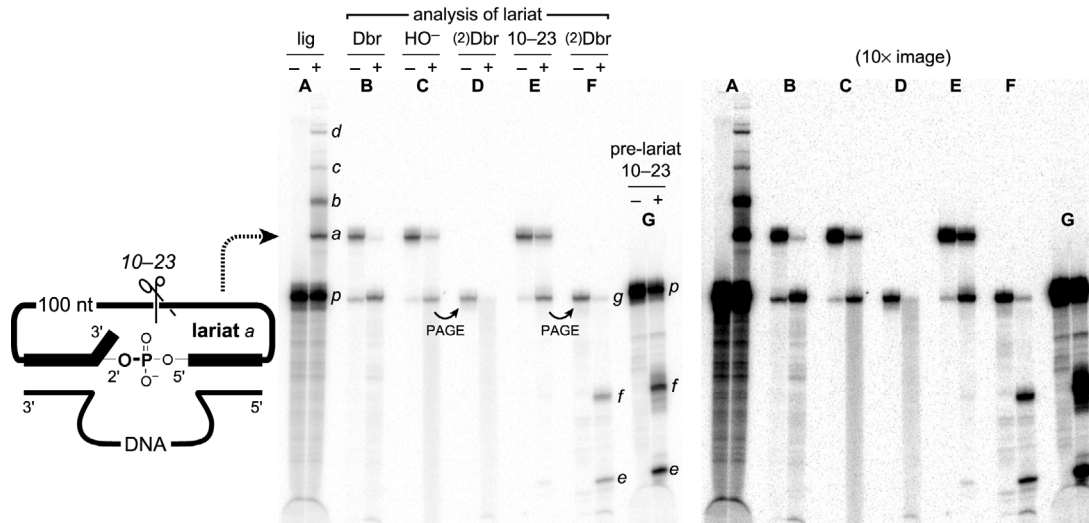


Figure X4. Comprehensive experiment demonstrating the lariat structure of the major ligation product *a*, which is obtained upon 9F21-mediated intramolecular ligation of the pre-lariat RNA *p*. The right-hand image is a 10× version of the left-hand image. The substrate for the ligation reaction was prepared by T7 RNA polymerase transcription with internal radiolabeling by incorporation of α -³²P-CTP.

The lanes marked **A** show ligation of the pre-lariat RNA *p* by the 9F21 deoxyribozyme. The – lane is a control reaction with all components present except the deoxyribozyme; the + lane includes the deoxyribozyme. At least four products *a–d* are observed. Yields of *a–d* for this particular experiment (which are reproducible) were 15%, 10%, 1%, and 1%, respectively. Product *a* is assigned as the lariat, with analysis in the remainder of this section. Product *b* is assigned as the dimer, with analysis described fully in the next section. Products *c* and *d* have not been pursued in detail yet; preliminary evidence indicates that one may be the lariatized form of the dimer *b* (data not shown).

Lanes **B** show treatment of the lariat RNA product *a* with lariat debranching enzyme Dbr (a gift from B. Schwer and S. Schneider, Weill Medical College of Cornell University). As expected, in the + lane, a single band migrating at the position of the pre-lariat RNA *p* is formed. The – lane indicates a control incubation including all components except the Dbr (substituted with water). A trace amount of lower band migrating at the position of the debranched product is evident without Dbr. We attribute this trace material to unavoidable nonspecific hydrolysis of the long, unstructured RNA during the 2-h incubation period. Because the 3'-tail of the lariat is only two nucleotides long, nonspecific cleavage anywhere in the lariat is expected to give a product that migrates at the position of the debranched lariat, as observed. The experiment of lanes **F** (described below) demonstrates that Dbr does cleave at a specific place in the branched RNA product.

Lanes **C** show partial alkaline hydrolysis of the lariat RNA *a*. Because the 3'-tail of the lariat is only two nucleotides long, all products migrate at approximately the same position regardless of the site of cleavage, rather than “ladder” formation as usual for partial alkaline hydrolysis of a linear RNA. Note the small amount of nonspecific degradation in the – lane. Subsequent cleavage of the **C** product at a second site is expected to give a smear, as observed below the initial product.

Lanes **D** show Dbr treatment of the PAGE-purified product from **C**. Because the branch site in the alkaline hydrolysis product from **C** is distributed randomly at positions throughout the sequence, depending on the random site of cleavage in **C**, debranching of this product leads not to discrete product bands but instead to an undefined smear. This smear is particularly evident in **D** on the right-hand 10 \times image.

Lanes **E** show cleavage of the lariat RNA *a* by a 10–23 deoxyribozyme at a specific position in the loop (see sequences in first section of this Supporting Information). All product molecules *g* are identical in size, and they migrate at a position essentially equivalent to that of the linear pre-lariat RNA *p* because the 3'-tail is so short (note that *g* is isomeric with *p*; see also dimer analysis in the next section). Again, note the small amount of nonspecific degradation in the – lane, which represents incubation with all components except the 10–23 deoxyribozyme (substituted with water).

Lanes **F** show Dbr treatment of the PAGE-purified product *g* from **E**. Because the product from **E** is homogeneous, its debranching leads to two discrete product bands with migration rates consistent with their expected sizes of 55 nt (*e*) and 82 nt (*f*). This is in sharp contrast to the debranching products in **D**, which appear as a smear.

Finally, lanes **G** show cleavage of the pre-lariat RNA *p* with the 10–23 deoxyribozyme as a control reaction. As expected, the products migrate at the same positions as the Dbr cleavage products from **F**.

Incubation conditions for all experiments were as follows: **A**, 1 μ M pre-lariat RNA *p*, 1.2 μ M 9F21 deoxyribozyme, 50 mM HEPES, pH 7.5, 10 mM MnCl₂, 37 $^{\circ}$ C, 1.5 h. **B**, 0.1 μ M lariat RNA *a*, 25 ng/ μ L Dbr, 20 mM HEPES, pH 7.5, 0.5 mM MgCl₂, 125 mM KCl, 1 mM DTT, 30 $^{\circ}$ C, 2 h. **C**, 0.1 μ M lariat RNA *a*, 50 mM NaHCO₃, 0.1 mM EDTA, 90 $^{\circ}$ C, 10 min. The pH at room temperature of the 50 mM NaHCO₃ solution was \sim 9.2. **D**, product from **C** isolated by 8% PAGE and treated with Dbr as in **B**. **E**, 0.1 μ M lariat RNA *a*, 10 μ M 10–23 deoxyribozyme, 50 mM HEPES, pH 7.5, 2 mM MnCl₂, 37 $^{\circ}$ C, 1.5 h. **F**, product from **E** isolated by 8% PAGE and treated with Dbr as in **B**.

Experiments demonstrating the structure of the dimer product

The dimer structure *b* is shown in Figure X5. Experiments analogous to those shown in Figure X4 for the lariat *a* were performed as illustrated. The Dbr (H) and alkaline hydrolysis (J) assays gave straightforward results: debranching to re-form the pre-lariat RNA *p* in the former case, and random cleavage to give a nonspecific smear in the latter case. The 10–23 deoxyribozyme cleavage experiment (K) was conducted under conditions where not every 10–23 site was cleaved (i.e., partial cleavage). With two 10–23 sites in the dimer, there are thus five expected products (box in Figure X5). All of the expected products are observed. Incubation conditions were as described in the previous section.

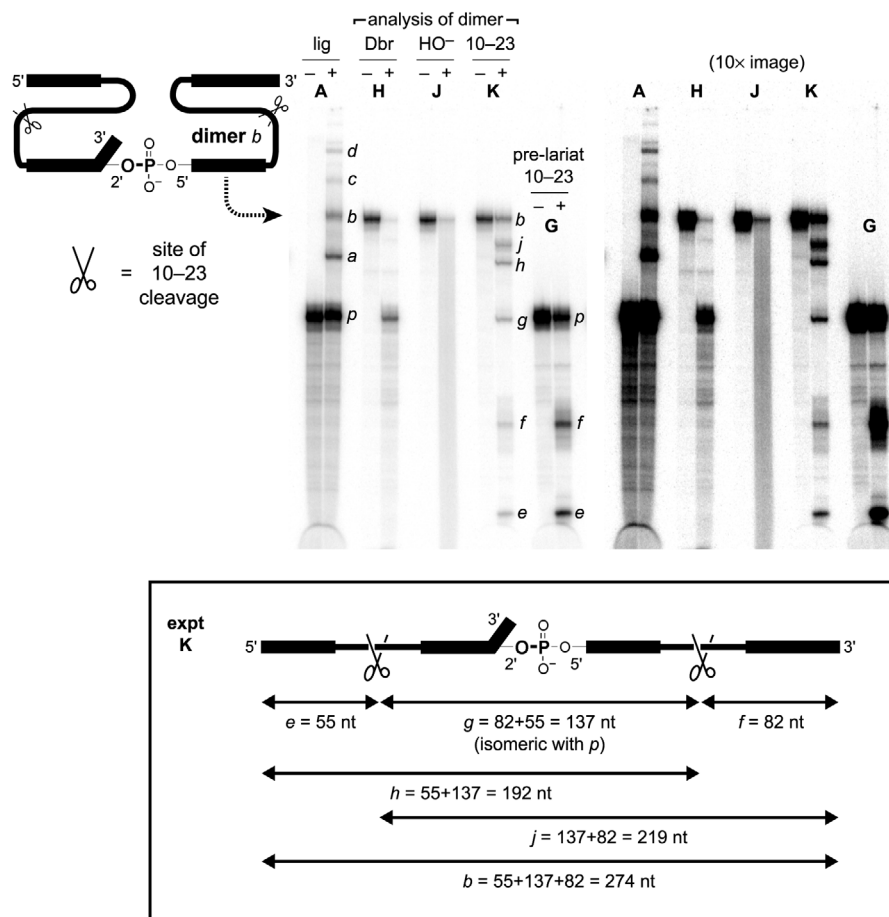


Figure X5. Comprehensive experiment demonstrating the dimer structure of *b*, which is formed as a second product in the 9F21-mediated ligation reaction of the pre-lariat RNA *p*. The right-hand image is a 10× version of the left-hand image. The box shows a detailed analysis of the 10–23 cleavage experiment in the lanes marked K.