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

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Deoxyribozymes (DNA enzymes) are DNA catalysts for a variety of chemical reactions that typically involve nucleic acid substrates.[1] Our laboratory has focused on the in vitro selection of DNA enzymes for RNA ligation.[2] A highly challenging goal has been deoxyribozymes that synthesize native 3′−5′ RNA linkages rapidly and in high yield for a wide variety of RNA sequences, rather than for only a limited set of substrates.[2f] We recently described a selection strategy that favors native RNA ligation by incorporating a stringently 3′−5′-selective step into the selection rounds.[2h] We have now applied this strategy to identify two RNA ligase deoxyribozymes that rapidly form high yields of 3′−5′ linkages with modest sequence requirements for the two RNA substrates, thereby fulfilling all requirements for useful RNA ligase reagents. Because RNA ligation by protein enzymes[3] does not always provide acceptable yields,[4,5] the identification of general DNA enzymes for 3′−5′ RNA ligation enables alternative synthetic routes that will be useful for practical biochemistry.

Our recently described selection methodology[2a] was used to identify deoxyribozymes that join a 2′,3′-diol to a 5′-triphosphate (Figure 1). Previously, such ligations led to 2′,5′-branched RNA by reaction of an internal 2′-hydroxyl group,[2b,e] or they led to linear 3′−5′ RNA but with restrictive and impractical sequence requirements.[2f] Here, 3′−5′ selectivity during ligation was enforced by incorporating the RNA-cleaving 8−17 deoxyribozyme[6] into the selection procedure,[2h] starting at either round 2 (for selections using 40 mM Mg^{2+}) or round 5 (for selections using 1 mM Zn^{2+}). In both cases, >95% of the ligation products from each uncloned selection pool had 3′−5′ linkages (Figure S1). When the ligation activities had stopped increasing, individual deoxyribozymes were cloned. On the basis of a preliminary survey of activities, two clones, named 9DB1 (from round 9 of the Mg^{2+} selection) and 7DE5 (from round 7 of the Zn^{2+} selection) were examined further. By cleaving the ligation products from each of the two new deoxyribozymes with 8−17, which is highly selective for 3′−5′ RNA linkages,[2a] both 9DB1 and 7DE5 were verified to create 3′−5′ linkages (Figure S1).

The 9DB1 and 7DE5 deoxyribozymes ligate RNA under practical in vitro incubation conditions. As shown in Figure 2, 9DB1 provides 60−70% yield of ligated RNA with $k_{obs} \sim 0.04$ min^{-1} ($t_{1/2} \sim 15$ min) at 40 mM Mg^{2+}, pH 9.0, and 37 °C. Similarly, 7DE5 has 40−50% yield of ligated RNA with $k_{obs} \sim 0.02$ min^{-1} ($t_{1/2} \sim 30$ min) at 1 mM Zn^{2+}, pH 7.5, and 23 °C. The 9DB1 deoxyribozyme is also effective at pH 7.5, where k_{obs} is ~0.2 h^{-1} ($t_{1/2} \sim 4$ h; data not shown). Incubation at the lower pH value of 7.5 instead of 9.0 should be useful for synthesis of larger RNAs that may experience more nonspecific degradation during an overnight incubation period, particularly at higher pH.

In the selection design that led to 9DB1 and 7DE5, only one RNA nucleotide of each substrate was not base-paired with the DNA binding arms (the A↓G nucleotides that flank the ligation site; Figure 1). Experience from other selections suggested that base-paired RNA nucleotides are likely to tolerate simple RNA:DNA

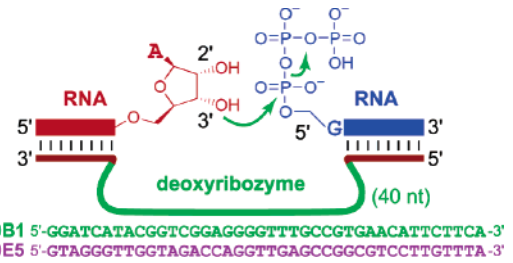

Figure 1. DNA-catalyzed 3′−5′ RNA ligation using 2′,3′-diol and 5′-triphosphate substrates. Shown are the sequences of the 40-nucleotide enzyme regions of the 9DB1 and 7DE5 deoxyribozymes.

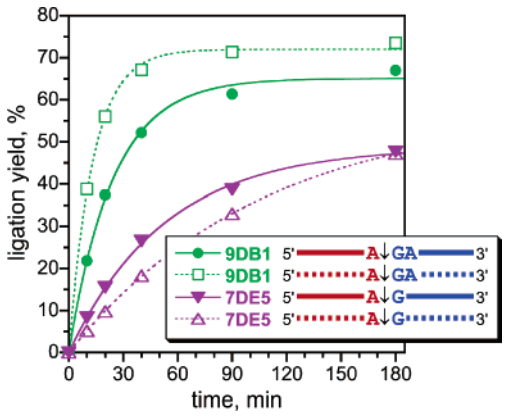

Figure 2. Kinetic assays for the 9DB1 (Mg^{2+}-dependent) and 7DE5 (Zn^{2+}-dependent) deoxyribozymes for 3′−5′ RNA ligation. Incubation conditions were 40 mM Mg^{2+}, pH 9.0, 37 °C and 1 mM Zn^{2+}, pH 7.5, 23 °C. The mutated RNA substrate sequences (dashed bars) differ from the original sequences (solid bars) at every nucleotide except those near the ligation site, as indicated in the legend. See Supporting Information for comprehensive generality assays. Values of k_{obs} for 9DB1: original substrates, 0.036 ± 0.006 min^{-1} ($n = 7$, mean ± standard deviation); mutant substrates, 0.064 ± 0.016 min^{-1} ($n = 3$). Values of k_{obs} for 7DE5: original substrates, 0.019 ± 0.005 min^{-1} ($n = 7$); mutant substrates, 0.012 ± 0.002 min^{-1} ($n = 3$).

Watson−Crick covariation without demanding particular RNA bases at the paired positions.[2a,c,d,g] Indeed, both 9DB1 and 7DE5 permit almost any changes to their RNA substrates away from the ligation site (Figure 2). Comprehensive assays revealed that 9DB1 requires only D↓RA (D = A, G, or U; R = A or G), and 7DE5 needs only A↓R (Figures S2 and S3).[7] For comparison, these practical D↓RA and A↓R sequence requirements are each less restrictive than that of the 8−17 deoxyribozyme (A↓G), which along with related DNA enzymes, such as 10−23 (R↓Y; Y = U or C), is widely used as a general RNA-cleaving biochemical tool.[8]

To demonstrate the utility of the new deoxyribozymes for synthesis of biologically derived RNAs, we used 7DE5 to prepare the *Tetrahymena* group I intron P4−P6 domain, a representative and often-studied RNA.[5,9,10] As shown in Figure 3A, synthesis of P4−P6 by 7DE5 was readily achieved in good yield, even though P4−P6 is completely unrelated to the short RNA substrates that

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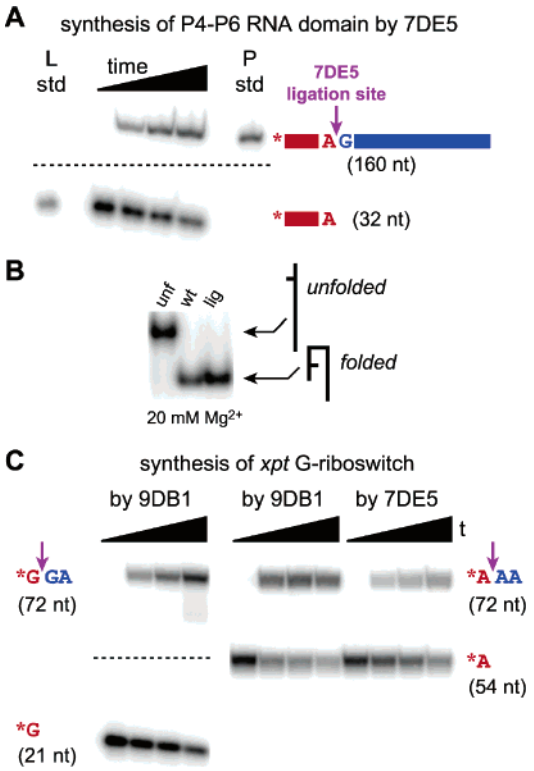

Figure 3. Application of the 9DB1 and 7DE5 deoxyribozymes to prepare large RNAs. (A) Ligation assay with 7DE5 for the 160-nucleotide P4−P6 RNA. The 32-nt left-hand RNA substrate was 5′-^{32}P-radiolabeled. The 128-nt right-hand RNA substrate was unradiolabeled. P denotes a product standard of wild-type P4−P6 that was prepared independently by transcription. Timepoints were taken at $t = 0.5$, 10, 40, and 180 min (full 6% PAGE image is shown in Supporting Information). The ligation yield at the final timepoint was 65%. (B) Functional assay of the ligated P4−P6 RNA. Mg^{2+}-dependent nondenaturing (native) PAGE[5,10] demonstrates that P4−P6 synthesized by 7DE5 folds equivalently to wild-type P4−P6. Unf, unfolded control mutant of P4−P6; wt, wild-type P4−P6; lig, P4−P6 by 7DE5 ligation. See Supporting Information for details. (C) Ligation assays for the 72-nt *xpt* G-riboswitch with 9DB1 and 7DE5 ($t = 0.5$, 30, 60, and 180 min; full 12% PAGE image is shown in Supporting Information). Ligation yields at the final timepoints were 55, 68, and 42%.

were used during the selection procedure that led to the identification of 7DE5. The P4−P6 synthesized by 7DE5 was shown conclusively to have a native 3′−5′ linkage at the ligation junction created by the deoxyribozyme (Figures S6 and S7), and the synthetic P4−P6 folds like wild-type P4−P6 as assayed by nondenaturing PAGE (Figure 3B).[5,10] To further demonstrate ligation generality, we used both 9DB1 and 7DE5 to prepare the 72-nucleotide core of the *xpt* G-riboswitch[11] (Figure 3C). In addition to showing the generality of the new deoxyribozymes, these results demonstrate a simple approach to synthesize the riboswitch with modifications for structure−function studies, as has been achieved for P4−P6.[5,10]

In summary, we have identified the 9DB1 and 7DE5 deoxyribozymes that rapidly create native 3′−5′ RNA linkages in useful yield and in a sequence-general fashion. These deoxyribozymes should be of immediate practical utility alongside the familiar but imperfect protein-mediated splint ligation methodology.[3] In addition, 9DB1 and 7DE5 are conceptually interesting in the context of their 3′−5′ selectivities, ligation mechanisms, and three-dimensional structures, all of which will be investigated in other studies. Recently, extensive selection experiments provided a large family of DNA enzymes similar to 8−17 that collectively cleave almost any RNA dinucleotide junction.[12] A similar effort for RNA ligation using a comprehensive set of RNA substrate sequences and the approach described here is anticipated to lead to an analogous family of 3′−5′ RNA ligase deoxyribozymes. Such experiments are in progress in our laboratory.

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Supporting Information Available: Selection details; experiments demonstrating the generality of 9DB1 and 7DE5 for RNA substrate sequences; MALDI-MS data for oligonucleotide transcripts; procedures for P4−P6 and riboswitch ligation reactions; nondenaturing PAGE details; and P4−P6 linkage assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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