CHAPTER FIVE

Use of Deoxyribozymes in RNA Research

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Abstract

Since their first identification by *in vitro* selection in 1994, deoxyribozymes have been developed to catalyze a variety of chemical reactions. The first DNA-catalyzed reaction was cleavage of a ribonucleotide linkage within an oligonucleotide substrate. In subsequent years, growing collections of deoxyribozymes have been developed for several reactions that have practical utility for RNA research. These deoxyribozymes are useful for site-specific RNA cleavage as well as ligation to form linear, branched, and lariat RNA products. An application related to RNA ligation is deoxyribozyme-catalyzed labeling of RNA (DECAL), which is used to attach a biophysical tag to a desired RNA sequence at a specific position. With current achievements and likely future developments, deoxyribozymes are a useful contributor to the toolbox of RNA research methods.

1. Introduction

Deoxyribozymes (also called DNA enzymes or DNAzymes) are specific sequences of DNA that have catalytic activity. All currently known deoxyribozymes have been identified by *in vitro* selection from large random-sequence DNA pools (Joyce, 2004; Silverman, 2009). The catalytic range of DNA encompasses both oligonucleotide and nonoligonucleotide substrates (Baum and Silverman, 2008; Silverman, 2008). This report focuses on deoxyribozymes that are useful for reactions of RNA substrates, especially to assist studies of RNA structure, folding, and catalysis.

For anyone who wishes to use a deoxyribozyme as a practical RNA cleavage or ligation catalyst by following the procedures described in this chapter, we recommend that a positive control experiment should be performed in parallel, using an RNA substrate that is known to be tolerated well by an analogous deoxyribozyme. For any nucleic acid enzyme that binds to its oligonucleotide substrate via extensive Watson–Crick interactions, it is impossible in practice to validate catalytic activity with all possible substrate sequences. Therefore, rates and yields with any particular substrate can vary from the values reported for other substrates. If the "experimental" sample fails to show the desired reactivity, then the results with the positive control will distinguish specific failure of the particular deoxyribozyme–substrate combination from a more general problem with the overall

application of deoxyribozymes (e.g., a problem with buffers, metal ions, oligonucleotide purification, and so on).

2. Deoxyribozymes for RNA Cleavage

The first deoxyribozyme was described in 1994 and catalyzes the Pb^{2+}-dependent cleavage of a DNA oligonucleotide strand at a particular embedded ribonucleotide (Breaker and Joyce, 1994). Since that first report, a large number of RNA-cleaving deoxyribozymes have been identified, including many that function well with oligonucleotide substrates that are made entirely out of RNA (Silverman, 2005). The cleavage reaction is shown in Fig. 5.1A and forms 2′,3′-cyclic phosphate and 5′-OH RNA termini, which are the same products as formed by RNase A and other ribonuclease protein enzymes. At present, nearly all possible sites within an arbitrarily chosen RNA target sequence can be cleaved by a deoxyribozyme, simply by choosing a suitable combination of catalytic region and Watson–Crick "binding arms" to interact with the RNA target (Fig. 5.1B). Essentially all of the useful RNA-cleaving deoxyribozymes require Mg^{2+} and/or Mn^{2+} for their catalytic activity. In addition to their preparative utility, RNA-cleaving deoxyribozymes can also be applied as analytical tools; for example, for mapping RNA branch sites (Pyle *et al.*, 2000) or for revealing the presence of chemical modifications on long RNAs (Silverman, 2004).

2.1. Deoxyribozymes available for different RNA cleavage-site sequences

Santoro and Joyce (1997) identified the 10–23 and 8–17 deoxyribozymes, which respectively cleave at R↓Y and A↓G dinucleotide junctions (↓ denotes the cleavage site; R, purine; Y, pyrimidine). Figure 5.1B depicts the sequences of the 10–23 and 8–17 deoxyribozymes, along with several other subsequently discovered variants of 8–17 that allow cleavage of other RNA linkages. Both 10–23 and 8–17 are active with 40–100 m*M* Mg^{2+} alone as the divalent metal ion cofactor, although in practice the cleavage rates are substantially higher if 10 m*M* Mn^{2+} is also included. Only if Mn^{2+}-induced RNA degradation is a concern (e.g., for a particularly long RNA substrate) should Mn^{2+} be omitted.

Li and coworkers have described numerous 8–17 variants that collectively enable cleavage of 14 out of the 16 possible RNA dinucleotide junctions. Their *in vitro* selection experiments were performed with substrates that contained only a single ribonucleotide linkage embedded within an otherwise all-DNA strand (i.e., DNA–rX–DNA). Therefore, separate

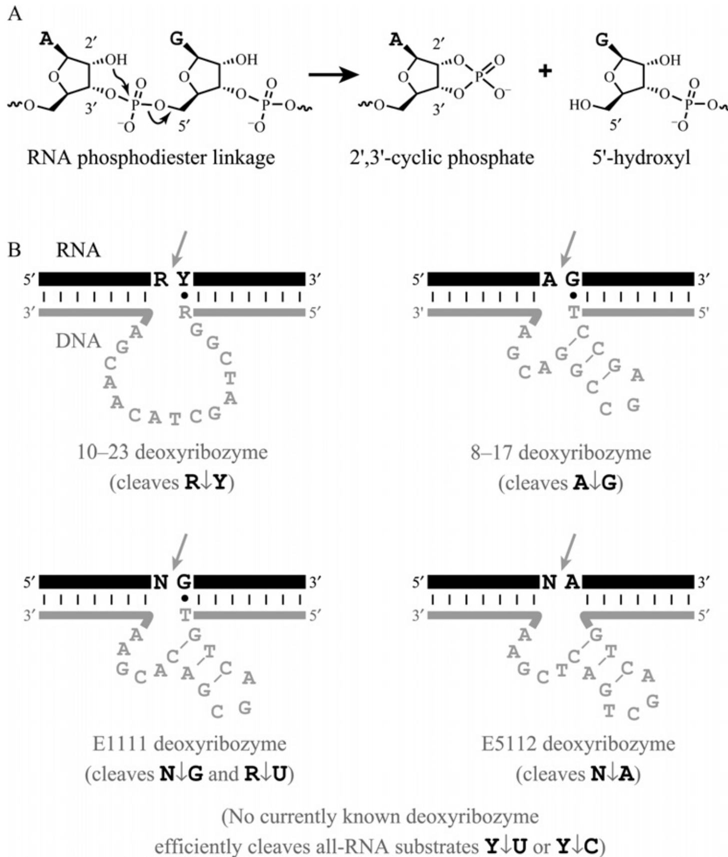

Figure 5.1 Deoxyribozyme-catalyzed RNA cleavage. (A) The cleavage reaction, which forms 2′,3′-cyclic phosphate and 5′-OH RNA termini. (B) Individual deoxyribozymes and their target sequences for efficient cleavage of all-RNA substrates. N, any nucleotide; R, purine; Y, pyrimidine. Outside of the explicitly indicated nucleotides, any RNA sequence is tolerated as long as Watson–Crick RNA:DNA covariation is maintained.

testing was required to assess the cleavage activities of the 8–17 variants with all-RNA substrates (Mi Zhang, D.A.B., and S.K.S., unpublished results). The two deoxyribozymes E1111 and E5112 depicted in Fig. 5.1B (Cruz *et al.*, 2004) cleave either N↓G or N↓A linkages, respectively, within all-RNA substrates. E1111 can also cleave R↓U RNA substrates. The Li laboratory reported additional 8–17 variants named 8–17NG and 8–17NA (Schlosser *et al.*, 2008a) that also cleave N↓G or N↓A linkages within all-RNA substrates. We are unaware of any reported deoxyribozymes that efficiently cleave all-RNA substrates at either Y↓U or Y↓C linkages.

Indeed, Y↓U linkages in any type of oligonucleotide have been refractory to efficient cleavage by any deoxyribozyme (Schlosser *et al.*, 2008b). Unfortunately, in our hands neither E2112 (Cruz *et al.*, 2004) nor 8–17NC (Schlosser *et al.*, 2008a) works well with an all-RNA N↓C substrate, although these deoxyribozymes were reported to cleave N↓C at a single embedded ribonucleotide.

In Section 2.2 are provided experimental procedures for using the RNA-cleaving deoxyribozymes shown in Fig. 5.1B. The analytical-scale procedure is appropriate for a picomole-scale amount of a 5′-^{32}P-radiolabeled RNA substrate. The preparative-scale procedure is appropriate for a nanomole-scale amount of an unradiolabeled RNA substrate. In all cases, we typically design the deoxyribozyme binding arms each to have at least 12 kcal/mol of DNA:RNA binding energy, as readily computed using published parameters (Sugimoto *et al.*, 1995). In practice, this is usually equivalent to binding arms with 10–14 bp, although the exact length depends on the nucleotide composition. It should be noted that multiple turnover is generally not required, and the deoxyribozyme is used in excess relative to the RNA substrate; typically the deoxyribozyme is less costly (in both time and money) than the RNA substrate. We recommend an analytical-scale check of the overall cleavage activity of a given deoxyribozyme–substrate combination before committing large amounts of RNA to the preparative-scale procedure.

For those RNA substrates that are very long or have intrinsically strong secondary structure, use of 2′-OMe or LNA binding arms has been successful for disrupting secondary structure within RNA targets (Schubert *et al.*, 2004; Silverman, 2005). However, because oligonucleotides with these chemical modifications are more expensive than unmodified DNA, it is reasonable to perform an initial check of whether the appropriate unmodified deoxyribozyme is capable of useful RNA cleavage. If not, then preparing and testing the chemically modified variant would be the next step. Alternatively, discrete "disruptor oligonucleotides" complementary to key regions of the target RNA secondary structure can be included so that the desired cleavage site is accessible to the deoxyribozyme. The design of such disruptor oligonucleotides is beyond the scope of this chapter; for an example that should assist design for any particular large RNA, see Wang and Silverman (2005b).

2.2. Experimental procedures

2.2.1. General annealing procedure

All procedures involving deoxyribozymes begin with an annealing step to assist proper binding interactions between the deoxyribozyme and the RNA substrate(s). The deoxyribozyme and RNA substrate(s) are mixed with 5 m*M* Tris, pH 7.5, 15 m*M* NaCl, and 0.1 m*M* EDTA (HEPES may

be used instead of Tris). The sample is annealed by heating at 95 °C for 3 min and cooling on ice for 5 min. The annealed sample is then used for subsequent reaction steps.

2.2.2. Analytical-scale RNA cleavage by a deoxyribozyme

In our experience, incubation conditions related to those reported by Cruz *et al.* (2004) provide optimal RNA cleavage activity for all 8–17 deoxyribozyme variants. Our standard incubation conditions (1×) for 8–17 cleavage include 50 m*M* Tris, pH 7.5, 10 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, and 150 m*M* NaCl at 37 °C. A typical incubation time ranges from 1 h to overnight, depending on which specific 8–17 variant and RNA substrate sequence are used. Suitable buffer compounds such as HEPES can be used in place of Tris. For RNA cleavage by the 10–23 deoxyribozyme, our standard incubation conditions are the same as for 8–17, except that including Mg^{2+} is unnecessary (although not deleterious).

Reagents

- 5′-^{32}P-Radiolabeled RNA substrate to be cleaved (prepared by reaction of the RNA with γ-^{32}P-ATP and T4 polynucleotide kinase; alternatively, 3′-^{32}P-radiolabeling with ^{32}P-pCp and T4 RNA ligase can be used)
- 8–17 Deoxyribozyme, designed with binding arms complementary to the RNA substrate as shown in Fig. 5.1B
- 10× annealing buffer (50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA)
- 10× cleavage buffer (500 m*M* Tris, pH 7.5, 1.5 *M* NaCl)
- 10× Mg^{2+}/Mn^{2+} mix (100 m*M* $MgCl_2$, 100 m*M* $MnCl_2$)—note that solutions containing Mn^{2+} should be stored at −20 °C to suppress oxidation of the metal ion

Procedure

The deoxyribozyme is used in excess to the RNA substrate. Commonly at least threefold excess of deoxyribozyme is used to ensure that all of the RNA substrate has the opportunity to be cleaved. Typical mole amounts are 0.1–1 pmol RNA substrate and 3 pmol deoxyribozyme in 10 μl final volume, providing final concentrations of 10–100 n*M* RNA substrate and 300 n*M* deoxyribozyme. The deoxyribozyme and RNA substrate are annealed as described in Section 2.2.1. The cleavage reaction is initiated by addition of 10× cleavage buffer followed by 10× Mg^{2+}/Mn^{2+} mix to give final incubation conditions of 50 m*M* Tris, pH 7.5, 10 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, and 150 m*M* NaCl. The sample is incubated at 37 °C for several hours. Aliquots are removed at appropriate times, quenched onto stop solution (80% aqueous formamide, 1× TB [89 m*M* each Tris and boric acid, pH 8.3], 50 m*M* EDTA, 0.25% each bromophenol blue and xylene cyanol), and analyzed by denaturing PAGE (e.g., 20%).

2.2.3. Preparative-scale RNA cleavage by a deoxyribozyme

The preparative-scale RNA cleavage procedure is essentially the same as that used on the analytical scale, except the RNA substrate and deoxyribozyme concentrations are higher to avoid unreasonably large reaction volumes. Typically, 1 nmol of RNA substrate and 2 nmol of deoxyribozyme are used in a final volume of 100 μl (10 μM RNA substrate and 20 μM deoxyribozyme). The amount of deoxyribozyme may be decreased to as little as 1.05 equiv. of the RNA substrate, although usually the deoxyribozyme is less precious than the RNA substrate and therefore a larger excess can be used. After incubation, the nucleic acids are precipitated with ethanol and separated by PAGE. Care should be taken to ensure that the lengths of the desired RNA product and deoxyribozyme are sufficiently different to allow resolution by PAGE. If necessary, additional noncomplementary nucleotides may be included on either end of the deoxyribozyme to shift its PAGE mobility away from that of the desired RNA cleavage product. In addition, consideration should be given to the relative sizes of the two RNA cleavage products, which may have similar PAGE migration rates depending on location of the cleavage site. The deoxyribozyme may be isolated and reused in subsequent RNA cleavage experiments.

2.3. Further efforts needed to develop deoxyribozymes for RNA cleavage

With RNA-cleaving deoxyribozymes available as depicted in Fig. 5.1B, the remaining work needed to obtain a complete set of RNA-cleaving deoxyribozymes is to identify catalysts for efficient cleavage of Y↓U and Y↓C junctions in all-RNA substrates. In the absence of new experimental efforts in this direction, it is impossible to know whether such deoxyribozymes are chemically possible, but this seems likely, especially for Y↓C. Future studies could focus on evaluating metal ion cofactors other than Mg^{2+} and Mn^{2+}, which may allow higher cleavage rate constants even for difficult RNA cleavage sequences.

3. Deoxyribozymes for RNA Ligation: Synthesis of Linear RNA Products

The ability to ligate two RNA substrates enables studies of RNA structure, folding, and catalysis. For example, site-specific chemical modifications are often incorporated into large RNA molecules by initial chemical synthesis of a short RNA oligonucleotide bearing one or more modifications. This modified oligonucleotide is then ligated to one or more RNAs segments that are prepared by either chemical synthesis or

in vitro transcription. Many RNA ligation reactions have been achieved by enzymatic "splint ligation," in which a DNA oligonucleotide splint is used to hold together the two RNA substrates for joining by T4 DNA ligase (Moore and Query, 2000; Moore and Sharp, 1992). In some cases, T4 RNA ligase has instead been used, either in splinted fashion with unpaired RNA nucleotides at the ligation junction (Bain and Switzer, 1992; Stark *et al.*, 2006) or at an RNA secondary structure element where no external splint is necessary (Bruce and Uhlenbeck, 1978; Pan *et al.*, 1991). However, for some particular RNA substrates and ligation sites, an approach based on T4 DNA ligase or T4 RNA ligase does not work well empirically, and in general it is difficult to predict success or failure in advance of experiment. Therefore, alternative RNA ligation approaches would be valuable. Deoxyribozymes offer one such alternative strategy in which the ligation splint is also the ligation catalyst.

In 2003, we reported the first deoxyribozyme for RNA ligation (Flynn-Charlebois *et al.*, 2003), and our subsequent efforts have been reviewed (Silverman, 2008). This section provides information on deoxyribozymes that may be useful for RNA ligation with formation of linear products. For the ligation reaction, two combinations of RNA functional groups are possible (Fig. 5.2). First, joining a 2′,3′-cyclic phosphate with a 5′-OH group results in either a native 3′–5′ linkage or a nonnative 2′–5′ linkage (Fig. 5.2A); when the 3′–5′ linkage is formed, the reaction is the reverse of that shown in Fig. 5.1A. Second, ligating a 2′,3′-diol with a 5′-triphosphate also provides either a 3′–5′ or 2′–5′ linkage (Fig. 5.2B). The 2′,3′-cyclic phosphate RNA substrate can be prepared either using a deoxyribozyme as described in Section 2 or by other means, such as cleavage of a precursor RNA with a ribozyme (Ferré-D'Amaré and Doudna, 1996; Grosshans and Cech, 1991) or with RNase H and a helper oligonucleotide (Lapham and Crothers, 1996; Lapham *et al.*, 1997). The 5′-triphosphate substrate is generally prepared by *in vitro* transcription (Milligan and Uhlenbeck, 1989; Milligan *et al.*, 1987).

3.1. Deoxyribozymes available for 3′–5′ RNA ligation

A substantial thrust of our efforts with DNA-catalyzed RNA ligation has been to achieve synthesis of native 3′–5′ linkages. Some of our efforts have been described elsewhere (Silverman, 2008). Here, we focus upon the most useful final deoxyribozymes. The key considerations for a practical RNA ligation catalyst are the rate constant, yield, and substrate sequence requirements. Identifying deoxyribozymes with favorable properties in all three aspects has been challenging, but in several cases we have succeeded. In particular, by exerting a suitable selection pressure to enforce formation of 3′–5′ linkages (Wang and Silverman, 2005a), we found two specific deoxyribozymes that are useful for practical RNA ligation (Fig. 5.3) (Purtha *et al.*,

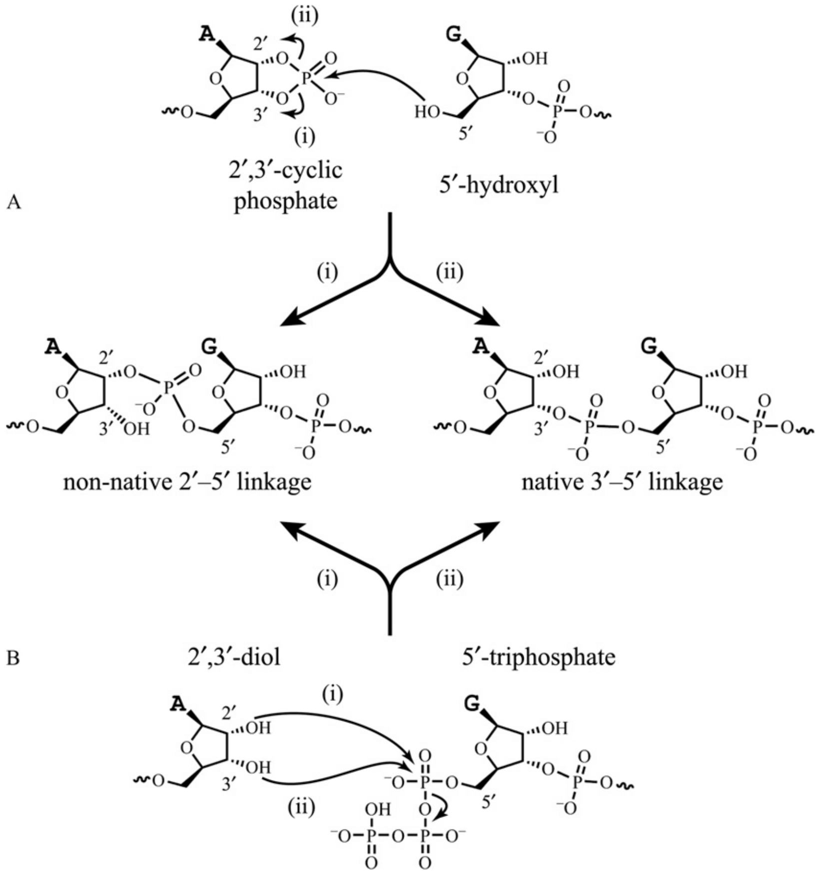

Figure 5.2 Reactions catalyzed by deoxyribozymes that ligate RNA to form linear products. (A) Reaction of a 2′,3′-cyclic phosphate with a 5′-OH group, leading to either a native 3′–5′ linkage or a nonnative 2′–5′ linkage. (B) Reaction of a 2′,3′-diol with a 5′-triphosphate, again leading to either a 3′–5′ or 2′–5′ linkage.

2005). The Mg^{2+}-dependent 9DB1 deoxyribozyme joins two RNA sequences that match the D↓RA motif (D = A, G, or U), whereas the Zn^{2+}-dependent 7DE5 deoxyribozyme requires A↓R. Both deoxyribozymes achieve their highest ligation yield within 2–4 h; the yield is 50–80% for 9DB1 and 40–50% for 7DE5. While variations in both rate and yield are observed for different RNA substrate sequences, our systematic surveys suggested that the indicated sequence requirements near the ligation site are the only ones.

In several other studies, we also found many deoxyribozymes that create native 3′–5′ RNA linkages, using either the 2′,3′-cyclic phosphate substrate combination of Fig. 5.2A (Kost *et al.*, 2008; Purtha *et al.*, 2005) or the

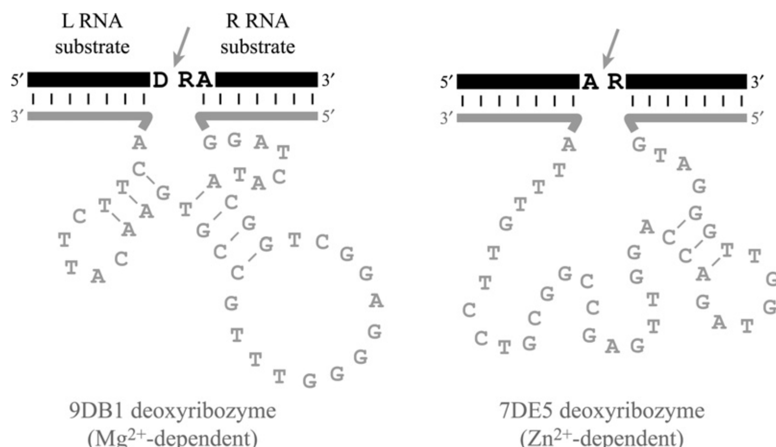

Figure 5.3 Individual deoxyribozymes for linear 3′–5′ RNA ligation. R, purine; D, one of A, G, or U. Outside of the explicitly indicated nucleotides, any sequence for either the left-hand (L) or right-hand (R) RNA substrate is tolerated as long as Watson–Crick RNA:DNA covariation is maintained.

5′-triphosphate substrate combination of Fig. 5.2B (Wang and Silverman, 2005a). In all such cases, however, we either know or suspect that these DNA enzymes require enough particular RNA nucleotides so that their sequence generality is poor. Such deoxyribozymes might be useful in practical RNA ligation, as long as the precise RNA sequence at the ligation site is not constrained.

3.2. Deoxyribozymes available for 2′–5′ RNA ligation

RNA oligonucleotides with an internal nonnative 2′–5′ linkage can be prepared by solid-phase synthesis using an appropriate ribonucleotide 2′-phosphoramidite, which is commercially available for the four standard RNA nucleotides. Alternatively, either of the DNA-catalyzed RNA ligation reactions of Fig. 5.2 can be used, even when one or both of the RNA substrates are much larger than can be synthesized by solid-phase methods. We have reported numerous deoxyribozymes that create 2′–5′ linkages via the reaction of Fig. 5.2A (Flynn-Charlebois *et al*., 2003; Hoadley *et al*., 2005; Kost *et al*., 2008; Semlow and Silverman, 2005). In all cases, there are likely to be substantial restrictions on the RNA sequences near the ligation site. Nevertheless, for some applications the mere presence of the 2′–5′ linkage in the RNA ligation product may be the most important consideration regardless of the precise sequence, and in such cases our deoxyribozymes provide a viable experimental approach. For the alternative ligation chemistry of Fig. 5.2B, again we have reported several deoxyribozymes that

form 2′–5′ linkages, albeit again with probable RNA sequence requirements (Wang and Silverman, 2005a).

3.3. Experimental procedures

The standard incubation conditions (1×) for 9DB1 ligation include 50 m*M* CHES, pH 9.0, 40 m*M* $MgCl_2$, 150 m*M* NaCl, and 2 m*M* KCl at 37 °C. The standard incubation conditions for 7DE5 ligation include 50 m*M* Tris, pH 7.5, 1 m*M* $ZnCl_2$, 150 m*M* NaCl, and 2 m*M* KCl at 37 °C. For both deoxyribozymes, the typical incubation time is 2–4 h. For 9DB1, the pH 9.0 buffer may be replaced with Tris at pH 7.5. The ligation rate will be much lower, but the reduced pH will result in less nonspecific RNA degradation, which may be important for especially long or sensitive RNA substrates.

3.3.1. Analytical-scale RNA ligation by a deoxyribozyme

One of the two RNA substrates, either L or R (see Fig. 5.3), must be ^{32}P-radiolabeled. The L substrate may be 5′-^{32}P-radiolabeled by reaction with γ-^{32}P-ATP and T4 polynucleotide kinase, or the R substrate may be 3′-^{32}P-radiolabeled by reaction with ^{32}P-pCp and T4 RNA ligase. A typical incubation time for maximal ligation activity is 2–4 h, depending on the precise RNA substrate sequences that are used.

Reagents

- 5′-^{32}P-Radiolabeled L RNA substrate (or unradiolabeled)
- R RNA substrate (or 3′-^{32}P-radiolabeled)
- 9DB1 or 7DE5 deoxyribozyme, designed with binding arms complementary to the RNA substrates as shown in Fig. 5.3
- 10× annealing buffer (9DB1: 50 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA; 7DE5: 50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA)
- 10× ligation buffer (9DB1: 500 m*M* CHES, pH 9.0, 1.5 *M* NaCl, 20 m*M* KCl; 7DE5: 500 m*M* Tris, pH 7.5, 1.5 *M* NaCl, 20 m*M* KCl)
- 10× metal (9DB1: 400 m*M* $MgCl_2$; 7DE5: 10 m*M* $ZnCl_2$)—the 10× Zn^{2+} solution is prepared by diluting 1 volume of 100 m*M* $ZnCl_2$ in 200 m*M* HNO_3 with 2 volumes of 1 *M* Tris, pH 7.5, and 7 volumes of water

Procedure

The 5′-^{32}P-radiolabeled L RNA substrate (⋆L), deoxyribozyme (E), and R RNA substrate are used in approximate ratio $<$1:3:6. The key point is that ⋆L $<$ E $<$ R, so that all ⋆L is saturated with E and all E is saturated with R (if 3′-^{32}P-radiolabeled R is prepared, then ⋆R $<$ E $<$ L is used). Typical mole amounts are 0.1–1 pmol L, 3 pmol E, and 6 pmol R in 10 μl final volume, providing final concentrations of 10–100 n*M* L, 300 n*M* E, and 600 n*M* R. The deoxyribozyme and two RNA substrates are annealed

as described in Section 2.2.1. The ligation reaction is initiated by addition of 10× ligation buffer followed by 10× metal to give final incubation conditions of 50 m*M* CHES, pH 9.0, 40 m*M* $MgCl_2$, 150 m*M* NaCl, and 2 m*M* KCl (9DB1) or 50 m*M* Tris, pH 7.5, 1 m*M* $ZnCl_2$, 150 m*M* NaCl, and 2 m*M* KCl (7DE5). The sample is incubated at 37 °C for several hours. Aliquots are removed at appropriate times, quenched onto stop solution (80% aqueous formamide, 1× TB [89 m*M* each Tris and boric acid, pH 8.3], 50 m*M* EDTA, 0.25% each bromophenol blue and xylene cyanol), and analyzed by denaturing PAGE (e.g., 20%).

3.3.2. Preparative-scale RNA ligation by a deoxyribozyme

The preparative-scale RNA ligation procedure is essentially the same as that used on the analytical scale, except the concentrations of the two RNA substrates and deoxyribozyme are higher to avoid unreasonably large reaction volumes. Typically, 1.0 nmol of L, 1.1 nmol of E, and 1.2 nmol of R are used in a final volume of 100 μl (10 μ*M* L and slightly higher for each of E and R). After incubation, the nucleic acids are precipitated with ethanol and separated by PAGE. Care should be taken to ensure that the lengths of the desired RNA product and deoxyribozyme are sufficiently different to allow resolution by PAGE. If necessary, additional noncomplementary nucleotides may be included on either end of the deoxyribozyme to shift its PAGE mobility away from that of the desired RNA ligation product. The deoxyribozyme may be isolated and reused in subsequent RNA ligation experiments.

3.4. Further efforts needed to develop deoxyribozymes for linear RNA ligation

For 3′–5′ RNA ligation using the 5′-triphosphate substrate combination of Fig. 5.2B, our unpublished work (D. A. B. and S. K. S.) has shown that identifying deoxyribozymes with minimal RNA sequence requirements necessitates imposing a selection pressure directly for this generality. This goal can be approached by systematically changing the RNA substrate sequences in successive rounds of selection. Ongoing efforts are focused on implementing this selection pressure to identify a full set of general RNA-ligating deoxyribozymes. In addition, the requirement for a 5′-triphosphate on the R substrate means in practice that R must be an *in vitro* transcript. Although a method for appending a 5′-triphosphate to a synthetic RNA oligonucleotide via solid-phase synthesis has been reported (Paul *et al*., 2006), in our hands this approach is technically demanding and difficult to reproduce. Alternatively, an activated phosphorus at the RNA 5′-terminus can be provided by a 5′-adenylate, which can be installed onto readily obtained 5′-phosphorylated RNA using T4 RNA ligase (Silverman, 2004) or T4 DNA ligase (Wang and Silverman, 2006b). RNA-ligating deoxyribozymes that readily accept a 5′-adenylated R substrate still await development.

For 3′–5′ RNA ligation using the 2′,3′-cyclic phosphate substrate combination of Fig. 5.2A, a suitable combination of selection design aspects (including use of Zn^{2+} as the metal ion cofactor) leads to the desired native linkages (Kost *et al.*, 2008). However, as is the case for the 5′-triphosphate substrate combination, a selection pressure for sequence generality must again be imposed to identify useful deoxyribozymes.

For 2′–5′ RNA ligation using any substrate combination, no efforts have yet been made to identify deoxyribozymes that are sequence-general. Presumably the same approaches intended in this regard for 3′–5′ ligation would be applicable for 2′–5′ ligation as well.

4. Deoxyribozymes for RNA Ligation: Synthesis of Branched RNA Products

2′,5′-Branched RNA molecules are formed naturally during spliceosomal pre-mRNA processing (Wahl *et al.*, 2009) and during self-splicing by group II introns (Michel and Ferat, 1995). Although chemical (solid-phase) approaches to synthesis of branched RNA have been developed (Damha *et al.*, 1992), such methods are tedious and impractical for many biochemists. Alternatively, we have identified a variety of deoxyribozymes that create branched RNA by catalyzing attack of a 2′-OH group into a 5′-triphosphate (Fig. 5.4). Because these deoxyribozymes are found by *in vitro* selection, they are not constrained by the same substrate sequence requirements as the natural splicing enzymes, thereby offering the potential for synthesis of unnatural branched RNA molecules. This section describes the deoxyribozymes that have been used to synthesize 2′,5′-branched RNAs.

The biological pre-mRNA splicing intermediates are a specific subclass of branched RNAs termed "lariats." In a lariat RNA, the two RNA strands that emerge from the 5′- and 2′-oxygen atoms of the branch-site nucleotide are covalently connected (Fig. 5.4, dashed loop) (Domdey *et al.*, 1984; Padgett *et al.*, 1984; Rodriguez *et al.*, 1984; Ruskin *et al.*, 1984; Zeitlin and Efstratiadis, 1984). Lariats are even more challenging to synthesize than branches. Some of the deoxyribozymes developed for branched RNA synthesis can also be used for direct one-step lariat synthesis (Wang and Silverman, 2005b). Alternatively, a lariat may be formed in two steps, by closure of the loop of an initially synthesized branch (Wang and Silverman, 2006a). Although this section does not directly address lariat synthesis by either approach, the methodology to synthesize branches is directly useful for making lariats as well.

It should be noted that deoxyribozymes are capable of synthesizing branched nucleic acids in which one or both of the oligonucleotide strands are DNA rather than RNA (Mui and Silverman, 2008; Zelin and Silverman, 2007). (When the strand providing the 2′-OH nucleophile

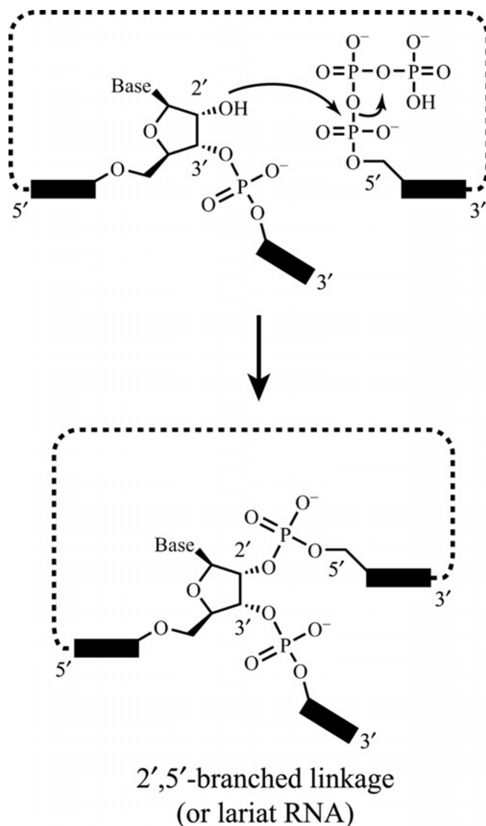

Figure 5.4 Synthesis of 2′,5′-branched and lariat RNA by reaction of a 2′-OH group with a 5′-triphosphate. The product is a lariat when the dashed loop is present connecting the two substrates.

is DNA, the particular nucleotide with the 2′-OH is still of course a ribonucleotide.) Such deoxyribozymes are found by *in vitro* selection approaches similar to those used in identification of DNA enzymes for branched RNA synthesis, and these deoxyribozymes are used to create branched nucleic acids via analogous procedures.

4.1. Deoxyribozymes available for 2′,5′-branched and lariat RNA synthesis

The selection strategies and outcomes for identifying branch-forming deoxyribozymes have been described in part elsewhere (Silverman, 2008). Here, the most useful DNA enzymes are briefly described. First, the Mg^{2+}-dependent 7S11 deoxyribozyme creates 2′,5′-branched RNA with a

preference for a branch-site adenosine nucleotide (Coppins and Silverman, 2005; Coppins and Silverman, 2004) (Fig. 5.5A). A branch-site guanosine is tolerated, albeit with about 50-fold lower ligation rate constant than with branch-site adenosine. The 5′-triphosphorylated RNA nucleotide that reacts with the 2′-OH can be either A or G. 5′-Adenylated C (Wang and Silverman, 2006b) is also accepted with lower rate and yield; 5′-adenylated U shows little reactivity. Other than these two nucleotides directly at the ligation junction, the two RNA substrates may have nearly any sequence while still allowing substantial ligation rate and yield. Because 7S11 works very quickly (k_{obs} of ~0.6 min^{-1}) with the RNA substrate sequences used during the selection process, even significant decreases in rate due to sequence changes still allow branch formation in preparatively useful fashion.

We subsequently identified a series of deoxyribozymes related to 7S11, of which 10DM24 (Fig. 5.5B) was examined in the most detail (Zelin *et al.*, 2006). 10DM24 is similar to 7S11 in its substrate acceptance, with slightly improved tolerance of different RNA sequences. We recommend that 10DM24 should be tested first for any new substrate combination. During

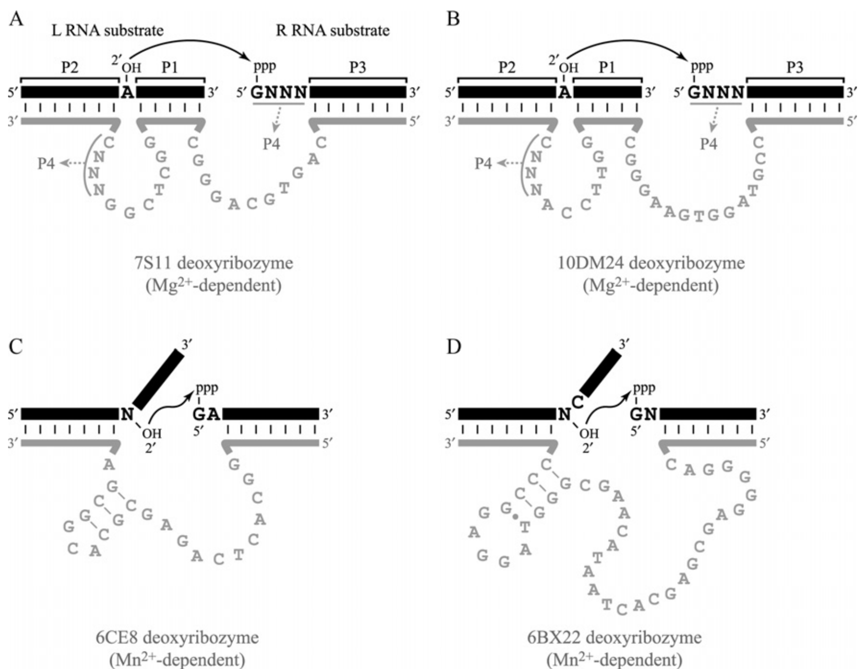

Figure 5.5 Individual deoxyribozymes for branched RNA synthesis. (A) 7S11 deoxyribozyme. Note the four Watson–Crick paired regions denoted P1–P4. (B) 10DM24 deoxyribozyme. (C) 6CE8 deoxyribozyme. (D) 6BX22 deoxyribozyme.

the same selection experiment, many other deoxyribozymes were identified, some of which have substantial ligation yields with branch-site C or U. Therefore, for synthesizing branched RNA with a pyrimidine branch-site nucleotide, one of these 10DM24-related deoxyribozymes may be useful.

Several other deoxyribozymes are also useful for branched RNA synthesis. The Mn^{2+}-dependent 6CE8 deoxyribozyme accepts any branch-site nucleotide, although it requires the sequence 5′-GAR for the RNA substrate that reacts with the branch-site 2′-OH group (Fig. 5.5C) (Pratico *et al.*, 2005). The Mn^{2+}-dependent 6BX22 deoxyribozyme also works with any branch-site nucleotide, although A is best (Fig. 5.5D) (Wang and Silverman, 2005b). A feature of 6BX22 is that it also works especially well to form lariat RNAs.

4.2. Experimental procedures

The analytical- and preparative-scale branch-forming procedures are essentially equivalent to those used for linear RNA ligation (Section 3.3). Appropriate pH and metal ion are used (7S11 and 10DM24: CHES, pH 9.0, with 40 m*M* Mg^{2+}, or HEPES, pH 7.5, with lower rate; 6CE8 and 6BX22: Tris, pH 7.5, with 20 m*M* Mn^{2+}).

4.3. Further efforts needed to develop deoxyribozymes for branched RNA synthesis

As described in Section 4.1 and depicted in Fig. 5.5, the currently available branch-forming deoxyribozymes each have modest but nonzero restrictions on the RNA substrate sequences that can be used. It should be possible to identify new branch-forming deoxyribozymes that have even more permissive sequence requirements. Selection experiments to identify such deoxyribozymes will likely use approaches analogous to those mentioned in Section 3.4 for linear RNA ligation.

5. Deoxyribozyme-Catalyzed Labeling (DECAL) of RNA

One specific application of branch-forming deoxyribozymes is named DECAL, which stands for deoxyribozyme-catalyzed labeling of RNA (Baum and Silverman, 2007). This section describes the DECAL approach and its implementation.

5.1. Overview of DECAL approach

In DECAL, the 10DM24 deoxyribozyme that forms branched RNA is exploited to place a biophysical tag (label) such as a fluorophore or biotin onto a target RNA of interest (Fig. 5.6). To accomplish this, a "tagging RNA" is created in two steps by first using T7 RNA polymerase to transcribe a short RNA oligonucleotide that has a 5-aminoallyl-C nucleotide at its second position. The primary amino group on the aminoallyl-RNA is then chemically modified with the desired biophysical tag; for example, by using an appropriate *N*-hydroxysuccinimidyl ester (Fig. 5.6A). The target RNA is then covalently modified with the tagging RNA using the 10DM24 deoxyribozyme (Fig. 5.6B). This section describes the methods for preparing the labeled tagging RNA (Section 5.2) as well as for attaching the tagging RNA to the target RNA (Section 5.3).

5.2. Experimental procedures for preparing the labeled tagging RNA

The two steps of the preparative procedure are each straightforward.

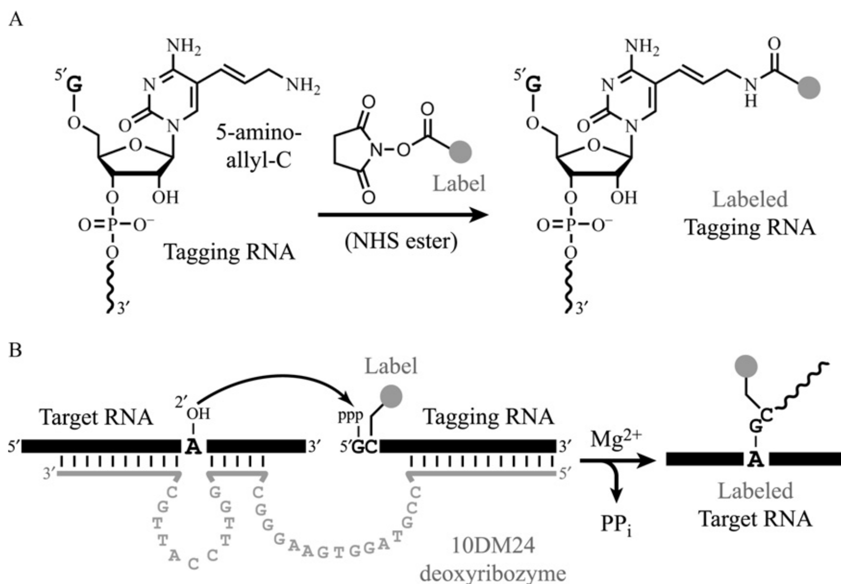

Figure 5.6 Deoxyribozyme-catalyzed labeling (DECAL) of RNA. (A) Synthesis of the tagging RNA. The unlabeled tagging RNA is prepared by *in vitro* transcription using commercially available 5-aminoallyl-CTP and T7 RNA polymerase. The sequence is designed such that the modified C is the only such nucleotide in the entire 19 nt sequence. (B) Attachment of the tagging RNA to the target RNA by the 10DM24 deoxyribozyme.

5.2.1. Transcription of the unlabeled tagging RNA

The transcription reaction is performed using T7 RNA polymerase according to the general protocol of Milligan *et al.* (1987), using a DNA template strand that incorporates 2′-OMe modifications at each of the first two nucleotides at the 5′-end to suppress formation of $n + 1$ transcription products (Kao *et al.*, 1999). The sequence of the 19-mer unlabeled tagging RNA is 5′-GC^{aa}AAGAGAUGGUGAUGGGA-3′, where C^{aa} denotes 5-aminoallyl-C.

Reagents

- Double-stranded DNA template comprising T7 RNA polymerase promoter sequence (5′-ACGCACGCTG*TAATACGACTCACTATA*-3′; promoter italicized) and reverse complement of the tagging RNA (5′-**UCCCATCACCATCTCTTGC**TATAGTGAGTCGTATTAC AGCGTGCGT-3′; boldface portion complementary to tagging RNA sequence; two underlined nucleotides 2′-OMe)
- Standard buffers and reagents (1 *M* Tris, pH 8.0, 1 *M* $MgCl_2$, 250 m*M* DTT, and 100 m*M* spermidine)
- 100 m*M* each ATP, GTP, and UTP
- 5-(Aminoallyl)cytidine 5′-triphosphate (5-aminoallyl-CTP; TriLink Biotechnologies or ChemCyte)
- T7 RNA polymerase (prepared by expression of a His_6-tagged construct, or purchased from a commercial supplier)

Procedure

The double-stranded DNA template is prepared by combining the two component strands in 5 m*M* Tris, pH 8.0, 15 m*M* NaCl, and 0.1 m*M* EDTA; the sample is annealed by heating at 95 °C for 3 min and cooling on ice for 5 min. The annealed DNA template is added to a transcription reaction, with final concentrations of 40 m*M* Tris, pH 8.0, 30 m*M* $MgCl_2$, 10 m*M* DTT, 2 m*M* spermidine, 4 m*M* each ATP, GTP, and UTP, 2 m*M* 5-aminoallyl-CTP, and T7 RNA polymerase (e.g., 1/20 by volume of a suitably diluted prepared stock). The transcription sample is incubated at 37 °C for 5 h and quenched by addition of sufficient 0.5 *M* EDTA, pH 8.0 to chelate all of the Mg^{2+}. The crude transcript is precipitated with ethanol and purified by 20% denaturing PAGE. For a 100 μl transcription reaction volume containing 100 pmol of each DNA template strand, a typical yield of purified unlabeled tagging RNA is 1.4–3.0 nmol.

Concentrations of 5-aminoallyl-CTP lower than 2 m*M* lead to reduced yield of the unlabeled tagging RNA transcript. In contrast, increasing the concentration above 2 m*M* does not improve the yield. If 2′-OMe groups are not included at the first two 5′-nucleotides of the reverse complement strand of the DNA template, then the yield of the correct length of this particular RNA transcript is decreased considerably.

5.2.2. Labeling of the tagging RNA

The optimal reaction conditions for derivatizing the tagging RNA with a biophysical label depend on the NHS ester used (Fig. 5.6A). Derivatizations with NHS-biotin or NHS-TAMRA can be performed under the same conditions; derivatization with NHS-fluorescein was optimal with different conditions.

Reagents

- 1 *M* sodium phosphate, pH 8.0 (for biotin or TAMRA), or 0.5 *M* $NaHCO_3$, pH 9.0 (for fluorescein)
- 5 m*M* EDTA, pH 8.0
- NHS ester reagent in DMSO (50 m*M* for biotin or TAMRA; 70 m*M* for fluorescein)

Procedure

Related procedures are used for all labeling reagents. For biotin or TAMRA, 1 nmol of the unlabeled tagging RNA is incubated in 200 μl with final concentrations of 5 μM RNA, 100 m*M* sodium phosphate, pH 8.0, 0.2 m*M* EDTA, 5 m*M* NHS ester, and 50% (v/v) DMSO. For fluorescein, 1 nmol of the unlabeled tagging RNA is incubated in 100 μl with final concentrations of 10 μM RNA, 70 m*M* $NaHCO_3$, pH 9.0, 0.2 m*M* EDTA, 21 m*M* NHS ester, and 30% (v/v) DMSO. The sample is incubated at 37 °C for 24 h (biotin) or 3 h (TAMRA). For labeling with fluorescein, in our experience the reaction was preparatively useful only using the conditions described here; 3 h incubation leads to lower labeling yield but minimal RNA degradation, whereas 12 h incubation leads to higher labeling yield at the expense of modest RNA degradation. The labeled tagging RNA is precipitated with ethanol and purified by 20% denaturing PAGE. A typical yield of purified labeled tagging RNA after gel extraction (Wang and Silverman, 2003) and precipitation is 270 pmol (biotin), 150 pmol (TAMRA), or 200 pmol (fluorescein). Some inefficiency is observed in the extraction process for the labeled RNAs, especially with the TAMRA label, leading to reduced preparative yields as compared with analytical-scale yield estimates.

5.3. Experimental procedures for DECAL using the labeled tagging RNA

The analytical- and preparative-scale DECAL procedures are essentially equivalent to those used for linear RNA ligation by the 9DB1 deoxyribozyme (Section 3.3) as well as for branched RNA formation. If degradation of a large target RNA is a concern, then the CHES, pH 9.0, 40 m*M* Mg^{2+} conditions may be replaced with HEPES, pH 7.5, 40 m*M* Mg^{2+}; a longer incubation time will be required.

If desired, 11 out of 19 nucleotides may be cleaved from the 3′-end of the tagging RNA after its attachment to the target RNA. This may be accomplished using a suitable 10–23 deoxyribozyme according to the procedures in Section 2.2 (Baum and Silverman, 2007).

5.4. Further efforts needed to develop deoxyribozyme-catalyzed labeling (DECAL) of RNA

Because DECAL depends upon branched RNA formation as catalyzed by 10DM24, this approach faces similar challenges related to undesired RNA target sequence requirements as indicated in Section 4.3 for conventional branched RNA synthesis. The presence of the biophysical label on the tagging RNA also presents a challenge; depending on the nature of this label, the catalytic ability of the deoxyribozyme may be affected. Therefore, selection experiments in which the label is specifically present on the selection substrate may be needed to obtain fully functional deoxyribozymes. The current approach uses a tagging RNA that incorporates 5-aminoallyl-C. We anticipate that analogous selection efforts should enable the use of 5-aminoallyl-U, which is more widely available as its NTP.

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