Directing the Outcome of Deoxyribozyme Selections To Favor Native 3′−5′ RNA Ligation†

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ABSTRACT: Previous experiments have identified numerous RNA ligase deoxyribozymes, each of which can synthesize either 2′,5′-branched RNA, linear 2′−5′-linked RNA, or linear 3′−5′-linked RNA. These products may be formed by reaction of a 2′-hydroxyl or 3′-hydroxyl of one RNA substrate with the 5′-triphosphate of a second RNA substrate. Here the inherent propensities for nucleophilic reactivity of specific hydroxyl groups were assessed using RNA substrates related to the natural sequences of spliceosome substrates and group II introns. With the spliceosome substrates, nearly half of the selected deoxyribozymes mediate a ligation reaction involving the natural branch-point adenosine as the nucleophile. In contrast, mostly linear RNA is obtained with the group II intron substrates. Because the two sets of substrates differ at only three nucleotides, we conclude that the location of the newly created ligation junction in DNA-catalyzed branch formation depends sensitively on the RNA substrate sequences. During the experiment that led primarily to branched RNA, we abruptly altered the selection strategy to demand that the deoxyribozymes create linear 3′−5′ linkages by introducing an additional selection step involving the 3′−5′-selective 8−17 deoxyribozyme. Although no 3′−5′ linkages (≤1%) were detectable in the pool products at the point that the 3′−5′ selection pressure was applied, deoxyribozymes that specifically create 3′−5′ linkages quickly emerged within a few selection rounds. Our success in obtaining 3′−5′ linkages via this approach shows that the outcome of deoxyribozyme selection experiments can be dramatically redirected by strategic changes in the selection procedure, even at a late stage. These results relate to natural selection, in which abrupt environmental variation can provide a rapid change in selection pressure. Linear 3′−5′ RNA linkages are an important practical objective because the native backbone is desirable in site-specifically modified ribozymes assembled by ligation. Therefore, this new approach to obtain 3′−5′-selective RNA ligase deoxyribozymes is particularly important for ongoing selection efforts.

RNA ligation by reaction of a ribose hydroxyl group with a 5′-triphosphate can provide either 2′,5′-branched RNA, 2′−5′-linked linear RNA, or 3′−5′-linked linear RNA (Figure 1). In our previously described in vitro selection experiments to identify deoxyribozymes (*1*−*4*) that mediate such reactions, the RNA products have typically been 2′,5′-branched rather than linear (*5*, *6*). More recently, we found that changing the relative arrangement of the reacting functional groups to place the new junction within an RNA−DNA duplex strongly favors deoxyribozymes that create 3′−5′ linear RNA (*7*). While these other studies were in progress, we became interested in two related questions. First, is formation of 2′,5′-branched RNA inherently favored in certain RNA nucleotide sequence contexts, such as those similar to natural splicing-related RNAs? Second, can we successfully alter the outcomes of deoxyribozyme selection experiments by strategic changes in the procedure, even after the selection has already progressed to a significant extent?

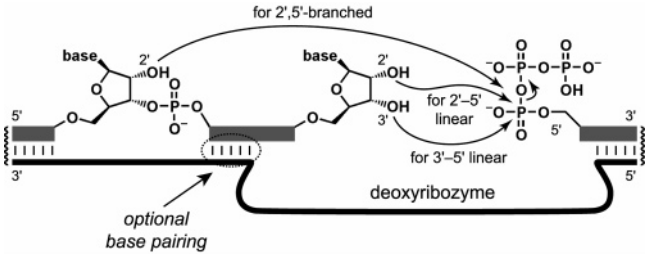

FIGURE 1: Branched and linear RNA synthesis by deoxyribozyme-catalyzed reaction of a hydroxyl group with a 5′-triphosphate. Three RNA isomers may be formed, depending on which hydroxyl group reacts with the 5′-triphosphate. For reaction of an internal 2′-hydroxyl group, the indicated optional base pairing may be absent (for example, see the reactions of Figure 2B).

To address these two questions, here we have performed new deoxyribozyme selections using RNA substrates that are derived from the natural sequences of branch-forming spliceosome substrates and group II intron RNAs. The results address the first question of linkage site preference by revealing a strong ligation-site dependence on the RNA substrate sequences, with a modest preference for the natural

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branch site in one of two tested cases. Partway through the latter selection, we altered the procedure specifically to favor the formation of 3′−5′ linkages by incorporating the previously identified 3′−5′-selective 8−17 deoxyribozyme (*8*, *9*) into the selection procedure. Our success in obtaining 3′−5′ linkages by this approach answers the second question (can selections be redirected at a late stage?) by showing that deoxyribozyme selections can be indeed substantially redirected in a rational way. Native 3′−5′ linkages are a significant practical goal in the context of preparing large site-specifically modified RNAs, and we anticipate that this practical approach to direct formation of 3′−5′ RNA linkages will be valuable in ongoing deoxyribozyme selection efforts.

EXPERIMENTAL PROCEDURES

Selection Procedure, Cloning, and Surveying of New Deoxyribozymes. Sources of materials are described in detail in our previous report (*9*). The left-hand and right-hand RNA substrate sequences for the BX selection (yeast actin pre-mRNA spliceosomal substrate; refs *11* and *12*) and CA selection (ai5γ group II intron; refs *13* and *14*) are shown in Figure 2A. The general selection procedure was described previously (*9*), with the exception of the additional 8−17 cleavage step that is described below. After cloning, the activities of individual deoxyribozymes were surveyed as described (*9*), incorporating some specific modifications to the method (*15*). For the kinetic and ligation junction assays, the left-hand RNA substrate was 5′-^{32}P-radiolabeled. The kinetic and ligation junction assay methods have been reported in detail (*5*, *6*, *9*, *15*). The standard incubation conditions for the kinetic assays were 50 mM HEPES,[1] pH 7.5, 150 mM NaCl, 2 mM KCl, and 20 mM $MnCl_2$ at 37 °C. The RNase T1 assay is described below.

Additional 8−17 Selection Step To Direct Formation of 3′−5′ Linkages in the Ligated RNA Product (Figure 6A). The sequence of the 8−17 deoxyribozyme used for the strategy of Figure 6 was 5′-GCGCTAGAACATATCCGAGCCGGACGAGTTAGTACATGAGAC-3′, where the enzyme region is underlined. The product of the selection step (<10 pmol) and the 8−17 deoxyribozyme (200 pmol) were annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min in 12 μL volume containing 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA. The sample was brought to 20 μL total volume containing 40 mM Tris, 150 mM NaCl, and 60 mM $MgCl_2$. The sample was incubated at 37 °C for 1.5 h, and PAGE was performed as described (*9*). Only the band corresponding to cleavage by 8−17 was excised from the gel. For the control reactions of Figure 6B, the RNA−DNA chimera strand was obtained by T4 RNA ligase-mediated joining of a standard 3′−5′-linked RNA to the original DNA enzyme strand (N_{40} region). The control reactions used ∼0.5 pmol of the RNA−DNA chimera and 200 pmol of 8−17 deoxyribozyme.

RNase T1 Assay for G↓G Ligation Junctions (Figure 4B). The RNA ligation products from individual deoxyribozyme clones (prepared by ligation and DNase digestion as described in ref *9*; ∼30 fmol) were incubated at room temperature for 5 min in 10 μL of 50 mM Tris, pH 7.5, and 1 mM EDTA containing 1 unit of RNase T1 (Ambion). Approximately 150 fmol of the L RNA was used in a similar reaction to generate the T1 digestion ladder.

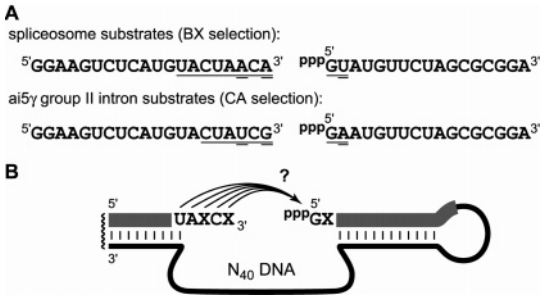

FIGURE 2: RNA substrates and the selection strategy. (A) The RNA substrate sequences used in the two new selections, which are designated BX and CA. The underlined nucleotides indicate the key regions of the substrates that are derived from natural intron RNA sequences (see text). Note that the two sets of substrates differ at only three nucleotide positions, which are double underlined. (B) The key step of the selection strategy in which a covalent linkage is made between the left-hand RNA substrate and the 5′-triphosphate of the right-hand RNA substrate (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 20 mM $MnCl_2$, 37 °C, 1 h).

RESULTS

Design of the New Selections. Many of our previous selection experiments resulted in deoxyribozymes that synthesize 2′,5′-branched linkages using the 5′-triphosphate RNA substrate combination shown in Figure 1 (*5*, *6*, *10*). Because these other selections were initially undertaken in part to explore the overall catalytic abilities of deoxyribozymes, they used essentially arbitrary RNA substrate sequences. As a consequence, their relationship to branch formation with biologically derived RNAs is unclear. Here we chose new substrates whose sequences near the ligation termini are based on the naturally occurring sequences of spliceosome substrates from the yeast actin pre-mRNA (*11*, *12*) and the ai5γ group II intron RNA (*13*, *14*) (Figure 2A). These sets of substrates differ at merely three nucleotide positions, which are double underlined in Figure 2A. RNA ligase deoxyribozymes that ligate these substrates were obtained via selection as described previously (*9*) using N_{40} random DNA regions. The selection strategy imposed no inherent bias toward any specific connectivity between the substrates (i.e., no bias toward any specific reaction among the possibilities of Figure 1). Instead, the strategy demanded only that the substrates somehow become covalently linked to survive each selection round (Figure 2B). To avoid substantial reorganization of deoxyribozyme−substrate interactions, as we have observed elsewhere due to Taq polymerase mutations (*10*), the DNA binding arms that surround the DNA enzyme region were regenerated in their entirety from PCR primers during each selection round, thereby preventing the accumulation of mutations (*10*). In each round, the key selection step of Figure 2B was performed for 1 h in 50 mM HEPES, pH 7.5, with 20 mM Mn^{2+} at 37 °C. These incubation conditions strongly supported high rates and yields of RNA ligation involving a 5′-triphosphate substrate in our previous studies (*5*, *6*).

The Distribution of RNA Linkage Sites Depends on the Substrate Sequences. The two selections using the spliceosome and group II intron substrate sequences were designated

[1] Abbreviations: k_{obs}, observed rate constant; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

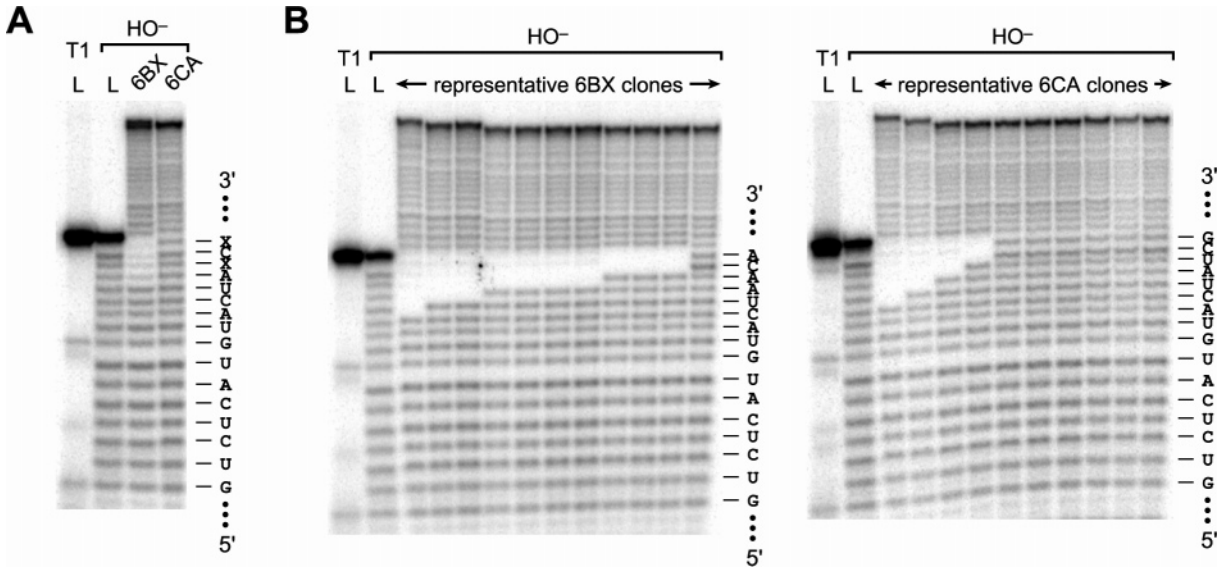

FIGURE 3: Partial alkaline hydrolysis assays to determine the branched or linear nature of the RNA linkages formed by the new deoxyribozymes. (A) Assays of the ligated RNA products from the uncloned round 6 BX and CA pools. T1 = RNase T1 digestion (G-specific) for ladder calibration. (B) Representative assays for individual 6BX and 6CA clones. The ladders reveal that each deoxyribozyme synthesizes only one linkage, and there is a significantly different distribution of linkages for the BX and CA selections. See Figure 5 for the quantitative distributions for all cloned selection rounds.

BX and CA according to our laboratory's nomenclature, as labeled in Figure 2A. After six selection rounds, the activities of the BX and CA deoxyribozyme pools each rose to ~39%, with no further increase upon continuing the selection rounds (data not shown). The distribution of RNA linkages formed by each pool of DNA enzymes was initially assessed with a partial alkaline hydrolysis assay of the ligated RNA substrates (Figure 3A). Upon such treatment, both 2′−5′ and 3′−5′ linear RNA lead to an approximately uniform ladder, whereas branched RNA leads to a ladder with a distinct gap whose size depends on the precise branch-site location. Despite the equivalent enzyme−substrate base-pairing interactions for the BX and CA selections (Figure 2B), assays of the two round 6 pools showed divergent outcomes. In particular, the BX selection appeared to contain deoxyribozymes that collectively create a mixture of linear and branched RNA, whereas the CA deoxyribozymes appeared to synthesize primarily linear RNA.

On the basis of our previous experiments, we expected that each deoxyribozyme creates one specific RNA ligation product, versus individual deoxyribozymes synthesizing a mixture of linkages. Assessing more quantitatively the product distribution required cloning and testing of individual deoxyribozymes, which was done as described previously (*5*, *6*, *9*, *15*). As expected, each tested DNA enzyme creates a single type of linkage (Figure 3B). The 3′−5′ and 2′−5′ isomers of linear RNA were distinguished for the appropriate 6BX deoxyribozymes, which create a linear A↓G junction, using an 8−17 deoxyribozyme (*8*) to cleave only 3′−5′ linkages (*9*), as shown in Figure 4A. These RNA isomers were also differentiated by incubation at pH 9.0 in the presence of Mg^{2+} and the exactly complementary DNA oligonucleotide; these conditions preferentially cleave 2′−5′ linkages (*9*). Alternatively, 3′−5′ and 2′−5′ linkages were distinguished for the 6CA deoxyribozymes, which create a G↓G junction, using RNase T1, which cleaves G↓N 3′−5′ linkages very rapidly but cuts the isomeric 2′−5′ linkages very slowly (Figure 4B). As expected, each 6BX or 6CA deoxyribozyme that creates linear RNA forms only a single isomer, either 2′−5′ or 3′−5′.

These experiments confirmed the pool assay results that the 6BX and 6CA deoxyribozymes collectively create significantly different distributions of linkages (Figure 5A). As a summary of the results for the individual 6BX and 6CA deoxyribozymes, the spliceosome substrates used in the BX selection led predominantly to deoxyribozymes that synthesize 2′,5′-branched RNA, with almost half of these branches formed using the adenosine nucleotide corresponding to that found naturally at the branch site. In sharp contrast, the group II intron substrates used in the CA selection led predominantly to linear RNA, with both 2′−5′ and 3′−5′ linkages represented strongly in the products.

Although the detailed kinetic activities of the new deoxyribozymes are not the focus of this report, we briefly characterized their ligation rates and yields. For over half of the individual deoxyribozymes, the ligation yields were 50−80% in 1 h at pH 7.5, 37 °C, and 20 mM Mn^{2+} (29 out of 44 6BX clones and 16 out of 28 6CA clones had these characteristics; data not shown). No systematic differences in activity were apparent among the deoxyribozymes that create the various kinds of linkages of Figure 1. Sequences and activities of individual deoxyribozymes are tabulated in the Supporting Information.

Redirecting the Outcome of Selection To Favor Linear 3′−5′ Linkages. During the selection process, it became apparent that a substantial portion of the CA deoxyribozymes are capable of synthesizing 3′−5′-linked RNA (Figure 5A), although our earlier independent efforts had yielded 3′−5′ products only upon careful redesign of the selection strategy (*7*). In contrast, of the three tested 6BX clones that create linear RNA (out of 44 6BX clones in total), all form 2′−5′ linkages, and the other 41 (out of 44) 6BX deoxyribozymes create 2′,5′-branched RNA. Even though an undetectable proportion (<1/44) of the BX pool at this point created linear 3′−5′ linkages, we sought to redirect the course of the selection toward this important practical goal. These experi-

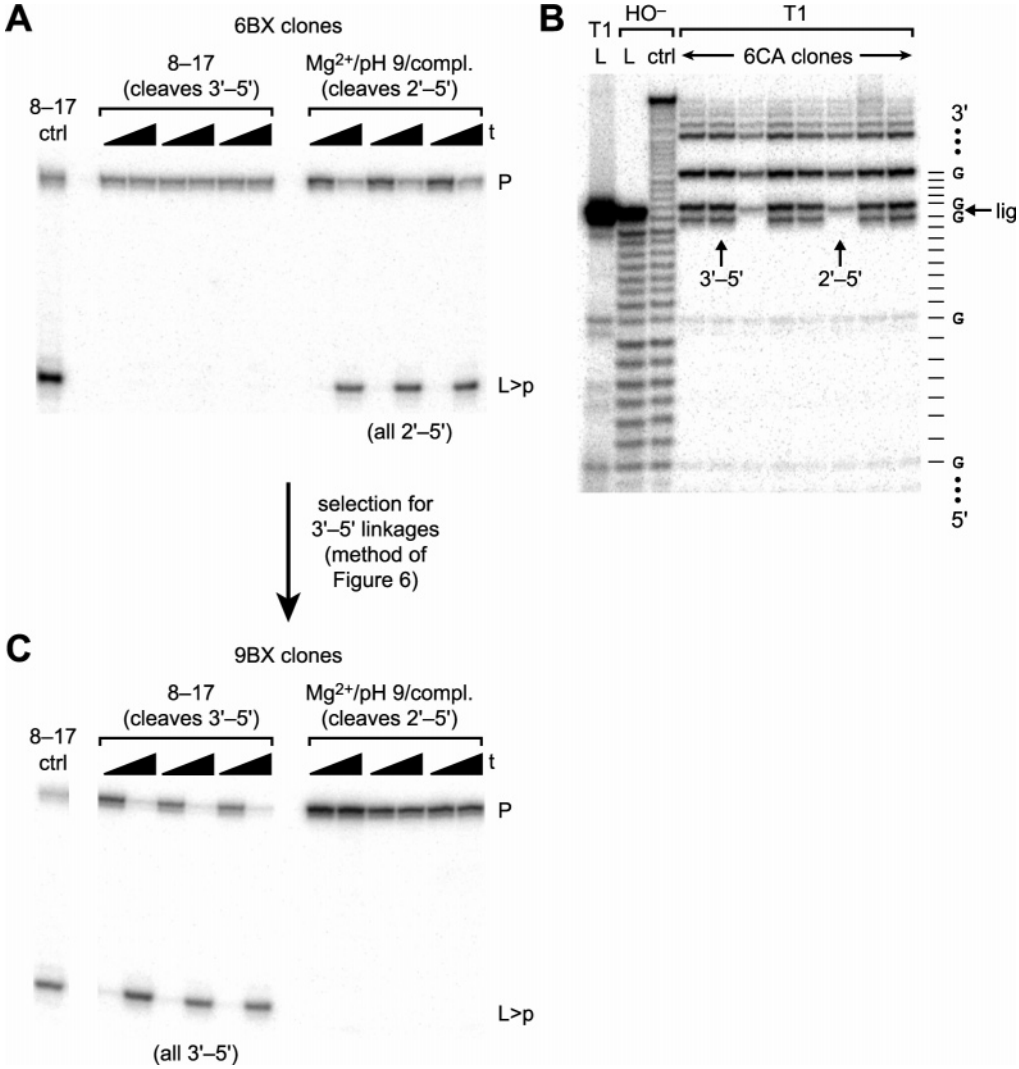

FIGURE 4: Assays to distinguish 3′−5′ from 2′−5′ linkages for the linear RNA products. (A) Distinguishing 3′−5′ from 2′−5′ linkages for the three 6BX deoxyribozymes that synthesize linear RNA. Two assays were used: 8−17 cleavage of 3′−5′ linkages and cleavage of 2′−5′ linkages in the presence of the DNA complement and 100 mM Mg^{2+} at pH 9 (*9*). P = ligated product; L = left-hand RNA substrate, here with a 2′,3′-cyclic phosphate due to the cleavage reaction; ctrl = linear 3′−5′-linked standard. (B) Distinguishing 3′−5′ from 2′−5′ linkages for representative 6CA deoxyribozymes that synthesize linear RNA. The assay used RNase T1, which has a greatly diminished cleavage rate for G nucleotides that are 2′−5′-linked to the adjacent residue on their 3′-side. HO^- denotes partial alkaline hydrolysis. For the six 6CA clones that form 2′−5′ linkages, this connectivity was verified by the 2′−5′-selective assay of panel A (data not shown). For all of the 6CA clones, partial alkaline hydrolysis as in Figure 3 gave a regular ladder (data not shown). (C) Distinguishing 3′−5′ from 2′−5′ linkages for representative 9BX deoxyribozymes that synthesize linear RNA, using the two assays of panel A. Of the 23 9BX clones analyzed in this manner, all create 3′−5′ linkages.

ments are also a test of how dramatically a selection effort can be redirected even at a late stage. To identify 3′−5′-selective deoxyribozymes, during each round an additional step was incorporated into the selection procedure, in which only linear 3′−5′-linked RNA would survive (Figure 6A). This step is based on cleavage of the new 3′−5′ linkage by an 8−17 deoxyribozyme (*8*), which is highly selective for 3′−5′-linked RNA over linear 2′−5′ RNA or 2′,5′-branched RNA (*9*) (note that 8−17 is the same DNA enzyme that was used above to distinguish 3′−5′ from 2′−5′ linkages among the ligation products). By retaining only the portion of the deoxyribozyme pool for which the ligated RNA substrates are cleaved by 8−17, this strategy was anticipated to enforce a stringent selection pressure in favor of deoxyribozymes with the desired 3′−5′ ligase activity.

When round 6 of the BX selection was repeated with inclusion of this additional 8−17 cleavage step, only about 5% of the repeated round 6 pool's selection products were cleaved by 8−17 in 1.5 h. This contrasts with ~70% 8−17 cleavage in a control reaction performed on authentic linear 3′−5′-linked RNA covalently attached to N_{40} random-pool DNA (Figure 6B) and >85% cleavage of 3′−5′ RNA that is not attached to DNA at all (data not shown). The incomplete 8−17 cleavage in the control reaction with attached DNA is likely due to intramolecular competition for the RNA sequences by the covalently attached DNA binding arms, which physically prevent 8−17 from binding (Figure 6A). Separately, we have noticed that some 2′,5′-branched RNAs related to the 3′−5′-linked sequence do cleave upon exposure to 8−17, albeit with substantially reduced rate relative to linear 3′−5′ RNA (data not shown). Therefore, the observed ~5% cleavage of the round 6 pool product represents an upper limit on its 3′−5′ content. Consistent with this, direct 8−17 assay of the ligated RNA prepared intermolecularly by the round 6 BX pool DNA (i.e., with the DNA not attached to the RNA) shows that only

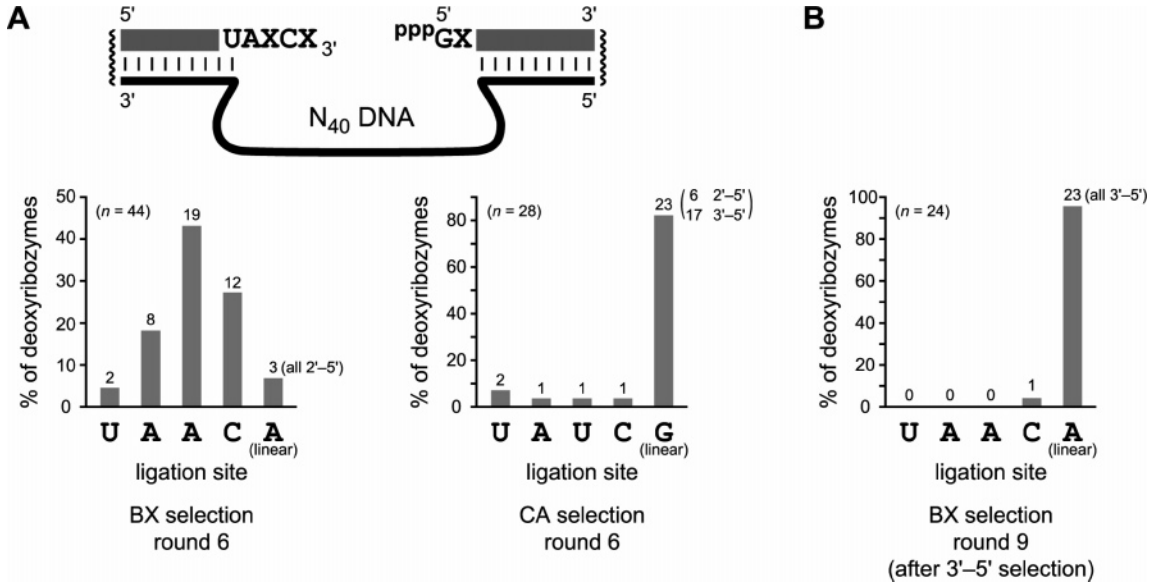

FIGURE 5: Distribution of linkages formed by the new deoxyribozymes. (A) Distributions within the BX and CA pools after round 6 (no 3′−5′ selection pressure applied). The bars show the percentage of tested clones that create the ligation junction at the indicated nucleotide. The number of individual clones with this activity is given above each bar (total number of tested clones $n = 44$ for BX and $n = 28$ for CA). (B) Distribution within the BX pool after round 9 ($n = 24$), where rounds 6−9 incorporated the 3′−5′-selective 8−17 cleavage step that is shown in Figure 6A.

≤1% of the linear RNA product is linked 3′−5′ (data not shown). This value of ≤1% 3′−5′ is consistent with the experimental observation mentioned above that 0 out of 44 individual 6BX clones create 3′−5′-linked RNA.

In four subsequent selection rounds, each of which incorporated the new 8−17 cleavage step, the fraction of the BX selection products cleaved by the 8−17 deoxyribozyme steadily increased, until the same fraction of the selection products was eventually observed to be cleaved as in the control reaction (Figure 6B). Therefore, although the original round 6 BX pool had very few, if any (≤1%), deoxyribozymes that create linear 3′−5′ linkages, the newly incorporated selection step dramatically redirected the outcome in favor of such linkages, and only a small number of rounds was required for this purpose. Individual 9BX deoxyribozymes were cloned, and the connectivities of their RNA ligation products were verified as for the 6BX and 6CA deoxyribozymes. Of 24 tested 9BX clones, all but one create linear 3′−5′ linkages (Figures 5B and 4C). This is quite unlike the initially examined 6BX clones, where 0 of 44 clones created 3′−5′ linkages. Thus the 3′−5′-selective strategy of Figure 6A was highly successful in redirecting the selection effort toward 3′−5′ linkages.

The kinetic activities of the 9BX clones were similar to or better than those obtained from round 6, before the 3′−5′ selection pressure was imposed (data not shown). DNA sequencing revealed many different 9BX sequences with little apparent homology (see Supporting Information). We have not yet analyzed these new 3′−5′-selective deoxyribozymes to determine the extent of their generality for joining various RNA substrate sequences, because this would require a very large number of time-consuming and material-intensive nucleotide covariation experiments to provide meaningful data for each deoxyribozyme. By analogy to our prior efforts (*6*), it seems unlikely that these new deoxyribozymes will be highly general for various RNA sequences, due to their use of single-stranded RNA overhanging regions (Figure 2B) that have typically fostered specific sequence requirements for the RNA substrates (e.g., ref *6*).

To address whether this overall redirection approach is generally applicable, we investigated an independent, parallel selection effort that was performed alongside the BX selection. This second effort, denoted BY, used a left-hand RNA substrate of sequence 5′-...UACU(dA)ACA-3′, where the dA indicates a single 2′-deoxy nucleotide in the otherwise all-RNA sequence used for BX. The BY selection was performed to favor branch formation at the ribo-A one nucleotide to the 3′-side of the dA.[2] Similar to the BX selection, the BY selection initially gave almost no 3′−5′ linkages. Therefore, the same redirection strategy as shown in Figure 6 was applied to BY, with substantially similar positive results (see Supporting Information). This establishes that our overall approach to redirect deoxyribozymes toward synthesis of 3′−5′ linkages is not idiosyncratic to any particular selection effort.

DISCUSSION

The Site of the New Linkage Depends on the RNA Substrate Sequences. At the outset of this study, we sought to determine how the RNA substrate sequences influence the ligation products that are created by in vitro selected deoxyribozymes. Surprisingly, the modest sequence differences between the two sets of RNA substrates tested here (Figure 2) led to a substantial difference in the distribution of linkages by the resulting deoxyribozymes (Figure 5A), as demonstrated by straightforward assays of the ligated RNA products (Figures 3 and 4). The explanation for the divergent linkage selectivities upon changing just three RNA substrate nucleotides is unclear. This relatively small number of

[2] The sequence fragment 5′-...UACUAAC...-3′ corresponds to the canonical branch site for yeast introns, with the underlined A as the branch site (*16*). We originally sought to distinguish the adjacent adenosines and specifically favor the A as the branch site.

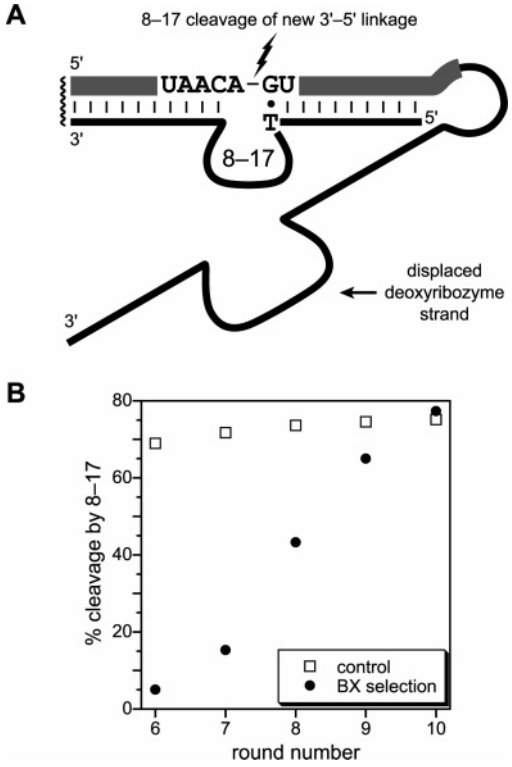

FIGURE 6: Modified strategy to select specifically for deoxyribozymes that create 3′−5′ linkages and not 2′−5′ linkages or 2′,5′-branched RNA. (A) The additional selection step that uses the 8−17 deoxyribozyme to cleave only the newly created linear 3′−5′ linkages. Note that for cleavage of the 3′−5′-linkage, 8−17 must intermolecularly displace the deoxyribozyme from the ligated RNA substrates to which it is covalently joined (see text). (B) Fraction of the pool's selection products cleaved by 8−17 during each of the five rounds in which this positive selection step was applied (round 6 repeated and then rounds 7−10). The control reaction was 8−17 cleavage of an RNA−DNA hybrid obtained by ligation of a standard 3′−5′-linked RNA to the original DNA enzyme strand (with a random N_{40} region). For each of the rounds 6−10, the overall pool ligation activity was unchanged at ∼45−50%. Note that the round 9 BX deoxyribozymes were cloned because essentially all of the corresponding deoxyribozymes create 3′−5′ linkages, as evidenced by the round 10 data.

experiments should not be used to draw broad conclusions about branch-site preferences, particularly because only one arrangement of the substrates and DNA enzyme was used in each case (i.e., the arrangement of Figure 2B). The data do strongly suggest that the selectivity for creating a specific linkage among the possibilities of Figure 1 depends sensitively on the RNA substrate sequences.

A Successful and Likely General Approach To Obtain 3′−5′-Selective RNA Ligase Deoxyribozymes. The most important practical finding from the present study is that native 3′−5′ linkages can be formed in a controlled fashion by the strategy of Figure 6. These results complement those obtained in a recent, independent selection effort from our laboratory (*7*). In this other effort, the ligation site was placed within an RNA−DNA duplex region that was offset by several base pairs from the DNA enzyme region. This placement was necessary for 3′−5′ selectivity but also appeared to induce specific RNA sequence requirements for several nucleotides on either side of the ligation site. Additionally, this placement means that other RNA−DNA arrangements that do not locate the ligation junction within an incipient duplex region will not necessarily have 3′−5′ selectivity. In contrast, the present method of directing 3′−5′ selectivity using the 8−17 deoxyribozyme during the selection procedure (Figure 6) is applicable to a wide range of individual selection strategies and RNA−DNA arrangements. We anticipate that the general strategy of Figure 6 should be applicable to enforce 3′−5′ selectivity on *any* deoxyribozymes that use the 5′-triphosphate substrate combination of Figure 1. The strategy should also be general for nearly any combination of RNA substrate sequences, in combination with deoxyribozymes related to 8−17 that collectively cleave almost any dinucleotide junction (*17*) or other nucleic acid enzymes. Indeed, our success with redirecting the BY selection effort (Supporting Information) as well as with several separate efforts currently underway in our laboratory (W. E. Purtha, R. L. Coppins, and S. K. S., data not shown) demonstrates that this generality can indeed be realized in practice.

We have performed many selections for deoxyribozymes that target a different RNA ligation reaction from that shown in Figure 1: the reaction between a 2′,3′-cyclic phosphate and a 5′-hydroxyl group (*9*, *15*, *18*, *19*). In such reactions as mediated by Mg^{2+}-dependent deoxyribozymes, the RNA product has been linked 2′−5′ rather than 3′−5′, for uncertain mechanistic reasons. When attempted, the strategy of Figure 6 has been thwarted in these cases, because the reversibility of the cyclic phosphate ligation reaction interferes (data not shown). That is, even when a large excess of the 8−17 deoxyribozyme is used in the reaction step represented in Figure 6A, some portion of the selected DNA enzyme region apparently binds intramolecularly to the ligated RNA substrates, displacing 8−17 from the RNA (this explanation was invoked above to explain the incomplete 8−17 cleavage for the control 3′−5′ RNA as shown by the data in Figure 6B). The facile reversibility of the DNA-mediated ligation for 2′,3′-cyclic phosphate RNA (*15*) then leads to cleavage of the just-ligated substrates regardless of whether they are 2′−5′ or 3′−5′, because each DNA enzyme can mediate the reverse reaction of the ligation products that it just created. However, if the deoxyribozyme used to cleave the 3′−5′ linkages were to function with a *different* metal ion than that required by the RNA ligase deoxyribozymes obtained through selection (e.g., Mg^{2+} versus Zn^{2+}), then our new redirection strategy should be applicable. In any case, the apparent irreversibility of the 5′-triphosphate ligation reaction avoids this pitfall entirely, thereby permitting the additional 8−17 cleavage step to select rigorously for 3′−5′-selective deoxyribozymes regardless of which metal ions are used in the various steps.

In What Circumstances Can Selections Be Redirected Successfully? A general issue for strategies in which selections are abruptly redirected is how far into the selection process such a change may be introduced. In nearly all reported selections for nucleic acid enzymes, the selection pressure is modulated smoothly, with relatively modest quantitative changes in each round. For example, the incubation time (*20*) or divalent ion concentration is typically lowered in the later rounds. In contrast, here we suddenly introduced a significant and qualitatively new hurdle at a late stage of the BX selection (Figure 6A). The abrupt requirement that the 8−17 deoxyribozyme must be able to cleave the ligation product introduces the strict demand that the newly created RNA junction is both linear and 3′−5′. This requirement was imposed despite the fact that the

deoxyribozyme pool had already passed through numerous rounds without this restriction and the selection was essentially "finished". At this point the pool contained very few deoxyribozymes that create linear RNA of any kind, and the linkages were predominantly (if not exclusively) 2′−5′ for those deoxyribozymes that do form linear RNA (Figure 5A). Nevertheless, the desired outcome of predominantly 3′−5′ RNA ligation was achieved by round 9, after just four rounds that incorporate the 3′−5′-selective step (Figure 6B).

From where did the 3′−5′-selective deoxyribozymes emerge? The ability to create 3′−5′ linkages may have been entirely absent from the BX pool when the additional 3′−5′-selective step was imposed starting with round 6. If so, then the additional 3′−5′-selective rounds would not have led to active deoxyribozymes unless mutations due to Taq polymerase permitted such activity to evolve under the stringent selection pressure. Further experiments would be required to determine if the emergent 3′−5′-ligase deoxyribozymes were present in very small amounts in the original round 6 pool or if they indeed evolved via mutation under the selection pressure. Regardless of which explanation is correct, the results are a direct experimental demonstration that deoxyribozyme selection outcomes can be dramatically redirected at a late stage. Although such changes may occur during selections without external guidance (*8*), here we have specifically redirected the outcome toward the desired goal of 3′−5′ linkage. In nature, rapid alterations in selection pressure often arise due to abrupt environmental changes, with dramatic consequences (e.g., ref *21*). The findings reported here indicate that, at the molecular level, deoxyribozymes can readily overcome such rapid swings in selection pressure.

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SUPPORTING INFORMATION AVAILABLE

Sequences and activities of deoxyribozymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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