DNA Catalysis of a Normally Disfavored RNA Hydrolysis Reaction

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Oligonucleotides and in vitro selection

Oligonucleotides were prepared by solid-phase synthesis at Integrated DNA Technologies (Coralville, IA) and purified by denaturing 20% or 8% PAGE. The in vitro selection experiments were performed essentially as described previously,[1] using 200 pmol (~10^{14} molecules) in each initial round of selection where sequence space encompasses $4^{40} \approx 10^{24}$ possibilities (10^{-10} sampling). Reselection experiments were performed with 25% partially randomized pools, which were prepared using phosphoramidite mixtures as described previously.[2,3] When Zn^{2+} was used, it was added from a 10× stock solution of 10 mM $ZnCl_2$ in 20 mM HNO_3 and 200 mM HEPES, pH 7.5; this 10× stock was itself prepared from a solution of 100 mM $ZnCl_2$ in 200 mM HNO_3 and a solution of 1 M HEPES, pH 7.5.[4] Detailed procedures for all reactions steps are provided below. Oligonucleotide sequences were as follows (all written 5′ to 3′; uppercase = DNA; lowercase = RNA). Figure S1 depicts the detailed arrangement of oligonucleotide sequences used during in vitro selection.

Partially randomized 10MD5 pool for reselections: CGAAGCGCTAGAACATCGCTAGATAAGTGGGTGCGTTTGCTATAGCTGTCCCTCAAAGTACATGAGACTTAGCTGATCCTGATGG (underlined nucleotides were 25% randomized)

Random N_{40} pool for selection: CGAAGCGCTAGAACAT-N_{40}-AGTACATGAGACTTAGCTGATCCTGATGG

Forward PCR primer: CGAAGCGCTAGAACAT (5′-phosphorylated to enable ligation to 3′-end of substrate)

Reverse PCR primer: (AAC)$_4$XCCATCAGGATCAGCT (X = Glen Spacer 18 to stop Taq polymerase)

Single-rG DNA substrate for 10MD5 reselections: AAAGTCTCATGTACTTATATgTTCTAGCGCgga

Single-rG DNA substrate for deoxyribozyme assays: AAAGTCTCATGTACTTATATgTTCTAGCGCGGA

All-RNA substrate for 10MD5 reselection/N_{40} selection and deoxyribozyme assays:
aaagucucauguacuuauauguucuagcgcgga

Ligation splint (rounds 6+): ATGTTCTAGCGCTTCGTCCGCGCTAGAACATATAAGTACATGAGACTTT

Capture splint for 10MD5 reselection with single-rG DNA substrate, either without or with selection pressure: ATGTTCTAGCGCTTCGTCCGCGCTAGAACATATACTGCACGTCTCAGGGGCTGC

Capture oligonucleotide for use with above capture splint and T4 DNA ligase:
(AAC)$_{10}$AGCAGCCCCTGAGACGTGCAGTATATG

Capture splint for 10MD5 reselection with all-RNA substrate:
TTCGTCCGCGCTCATATACTGCACGTCTCAGGGGCTGC

Capture oligonucleotide for use with above splint and T4 RNA ligase:
(AAC)$_{10}$AGCAGCCCCTGAGACGTGCAGTATATGgga

Capture splint for N_{40} selection with all-RNA substrate:
TTCGTCCGCGCTAGAATACTGCACGTCTCAGGGGCTGC

Capture oligonucleotide for use with above splint and T4 RNA ligase:
(AAC)$_{10}$AGCAGCCCCTGAGACGTGCAGTAgga

Procedure for ligation step in round 1. A 20 μL sample containing 750 pmol of DNA pool and 1 nmol of substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 8 μL of 5× T4 RNA ligase buffer (250 mM Tris, pH 7.5, 50 mM DTT, 25 mM $MgCl_2$, and 0.25 mM ATP) and 2 μL of 10 U/μL T4 RNA ligase (Fermentas). The sample was incubated at 37 °C for 14 h and separated by 8% PAGE.

Procedure for ligation step in rounds 2–5. A 15.5 μL sample containing the PCR-amplified DNA pool (~5–10 pmol) and 100 pmol of substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 4 μL of 5× T4 RNA ligase buffer (250 mM Tris, pH 7.5, 50 mM DTT, 25 mM $MgCl_2$, and 0.25 mM ATP) and 0.5 μL of 10 U/μL T4 RNA ligase (Fermentas). The sample was incubated at 37 °C for 14 h and separated by 8% PAGE.

Procedure for ligation step in rounds 6+ (altered from rounds 2–5 to increase ligation yield). A 17 μL sample containing the PCR-amplified DNA pool (~5–10 pmol), 50 pmol of DNA splint, and 100 pmol of substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 2 μL of 10× T4 DNA ligase buffer (Fermentas: 400 mM Tris, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP) and 1 μL of 1 U/μL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 14 h and separated by 8% PAGE.

Procedure for selection step in round 1. Each selection experiment was initiated with 200 pmol of the ligated substrate-pool conjugate. A 20 μL sample containing 200 pmol of substrate-pool conjugate was

annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to 40 μL total volume containing 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 14 h. For reactions without selection pressure via a capture step, the sample was immediately separated on 8% PAGE. For reactions with selection pressure via a capture step, to the selection sample was added 60 μL of water, and the nucleic acids were precipitated by addition of 10 μL of 3 M NaCl and 330 μL of ethanol.

Procedure for selection step in rounds 2+. A 10 μL sample containing the ligated substrate-pool conjugate was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to 20 μL total volume containing 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 14 h. For reactions without selection pressure via a capture step, the sample was immediately separated on 8% PAGE. For reactions with selection pressure via a capture step, to the selection sample was added 80 μL of water, and the nucleic acids were precipitated by addition of 10 μL of 3 M NaCl and 330 μL of ethanol.

Procedure for capture step involving T4 DNA ligase in rounds 7+. A 17 μL sample containing the precipitated selection product, 50 pmol of capture splint, and 75 pmol of capture oligonucleotide was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 2 μL of 10× T4 DNA ligase buffer (Fermentas: 400 mM Tris, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP) and 1 μL of 1 U/μL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 14 h and separated by 8% PAGE.

Procedure for capture step involving T4 RNA ligase in round 1. A 31 μL sample containing the precipitated selection product, 300 pmol of capture splint, and 500 pmol of capture oligonucleotide was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 8 μL of 5× T4 RNA ligase buffer (250 mM Tris, pH 7.5, 50 mM DTT, 25 mM $MgCl_2$, and 0.25 mM ATP) and 1 μL of 10 U/μL T4 RNA ligase (Fermentas). The sample was incubated at 37 °C for 14 h and separated by 8% PAGE.

Procedure for capture step involving T4 RNA ligase in rounds 2+. A 15.5 μL sample containing the precipitated selection product, 50 pmol of capture splint, and 75 pmol of capture oligonucleotide was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 4 μL of 5× T4 RNA ligase buffer (250 mM Tris, pH 7.5, 50 mM DTT, 25 mM $MgCl_2$, and 0.25 mM ATP) and 0.5 μL of 10 U/μL T4 RNA ligase (Fermentas). The sample was incubated at 37 °C for 14 h and separated by 8% PAGE.

Procedure for PCR. In each selection round, two PCR reactions were performed, 10-cycle PCR followed by 30-cycle PCR. First, a 100 μL sample was prepared containing the PAGE-separated selection product, 200 pmol of forward primer, 50 pmol of reverse primer, 20 nmol of each dNTP, 10 μL of 10× Taq polymerase buffer (1× = 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100), and 1 μL of Taq polymerase (prepared in our laboratory). This sample was cycled 10 times according to the following PCR program: 94 °C for 2 min, 10× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Taq polymerase was removed by phenol/chloroform extraction. Second, a 50 μL sample was prepared containing 1 μL of the 10-cycle PCR product, 100 pmol of forward primer, 25 pmol of reverse primer, 10 nmol of each dNTP, 20 μCi of α-^{32}P-dCTP (800 Ci/mmol), 5 μL of 10× Taq polymerase buffer, and 0.5 μL of Taq polymerase. This sample was cycled 30 times according to the following PCR program: 94 °C for 2 min, 30× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Samples were separated by 8% PAGE.

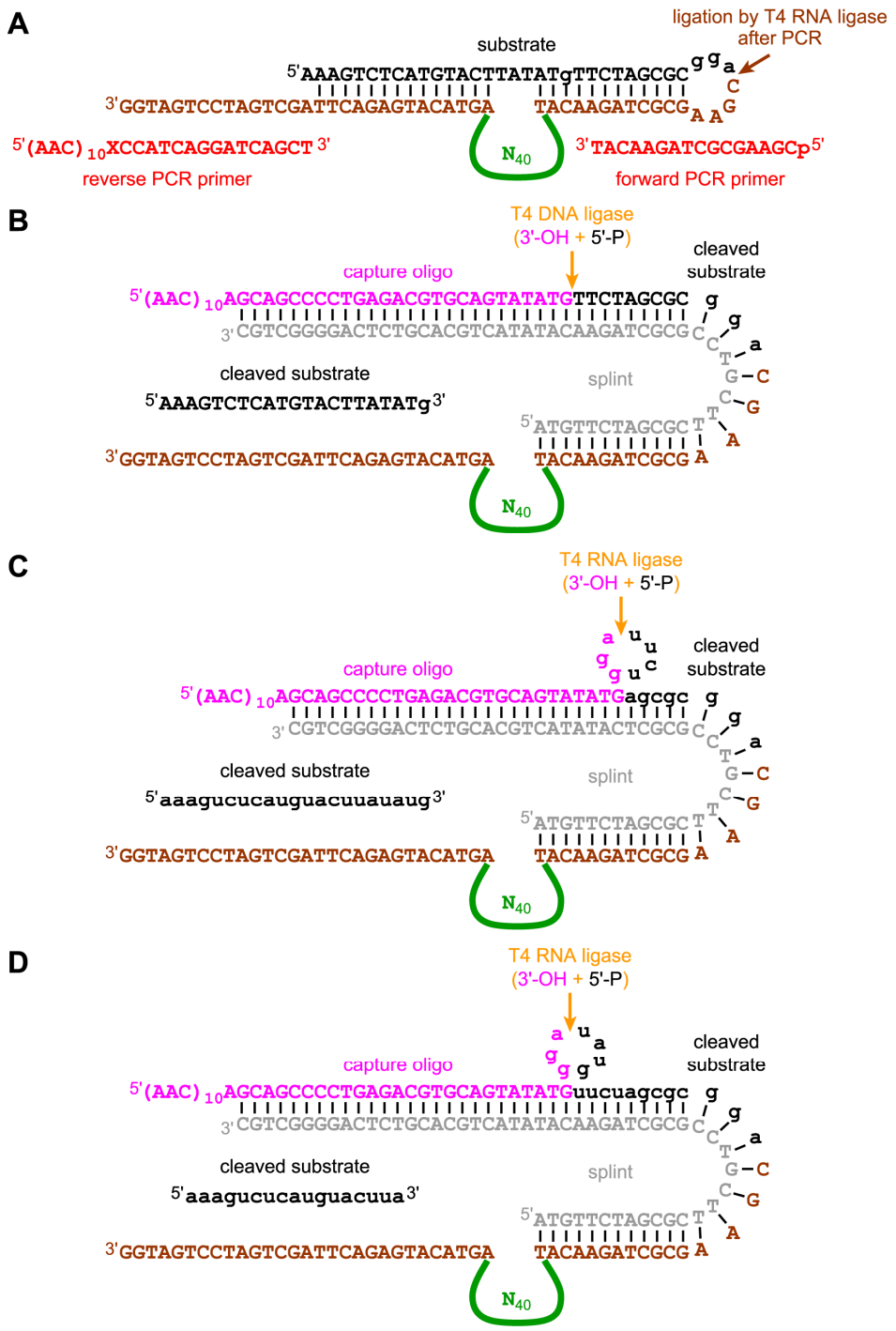

Figure S1. Details of oligonucleotide sequences used during in vitro selection. DNA nucleotides are uppercase; RNA nucleotides are lowercase. (A) Sequences of substrate and deoxyribozyme pool along with PCR primers, shown after ligation by T4 RNA ligase for the single-rG DNA substrate. The 3′-terminal ...gga ribonucleotides of the substrate are present solely to allow ligation of substrate to deoxyribozyme pool by T4 RNA ligase after PCR in each selection round. (B) Capture with T4 DNA ligase, as used in the 10MD5 reselection with the single-rG DNA substrate. (C) Capture with T4 RNA ligase, as used in the 10MD5 reselection with the all-RNA substrate. (D) Capture with T4 RNA ligase, as used in the N_{40} selection with the all-RNA substrate. Because T4 RNA ligase does not require a specific number of unpaired nucleotides in order to achieve ligation, the DNA-catalyzed RNA cleavage reactions in panels C and D could occur at other nearby nucleotides instead of the particular site illustrated (g^u for panel C; a^u for panel D), and indeed this occurred (see observed cleavage sites in Table S1).

Deoxyribozyme activity assay procedure

Deoxyribozyme activity assays were performed under single-turnover *in trans* conditions as described previously.[2] All deoxyribozymes for activity assays were 5′-CCGCGCTAGAACAT-N_{40}-AGTACATGAGACTT-3′, where N_{40} represents the specific 40 nucleotides of the originally random region as determined through the selection process. The substrate was 5′-^{32}P-radiolabeled using γ-^{32}P-ATP and T4 polynucleotide kinase. The final incubation conditions were 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C, with 10 nM substrate and 1 μM deoxyribozyme (1:100) in 20 μL total volume. Samples were separated by 20% PAGE and quantified with a PhosphorImager.

Initial determination of 10MD5 activity with DNA and RNA substrates

The 10MD5 deoxyribozyme was identified by in vitro selection with an all-DNA substrate. We evaluated 10MD5's activity using two modified substrates, either including a single ribonucleotide (rG) at the original ATG^T cleavage site or using an all-RNA substrate. The results (Figure S2) show that the single rG still permits 10MD5 activity, albeit with ca. 40-fold lower k_{obs}, but the all-RNA substrate does not support measurable activity by 10MD5 (<0.5% cleavage).

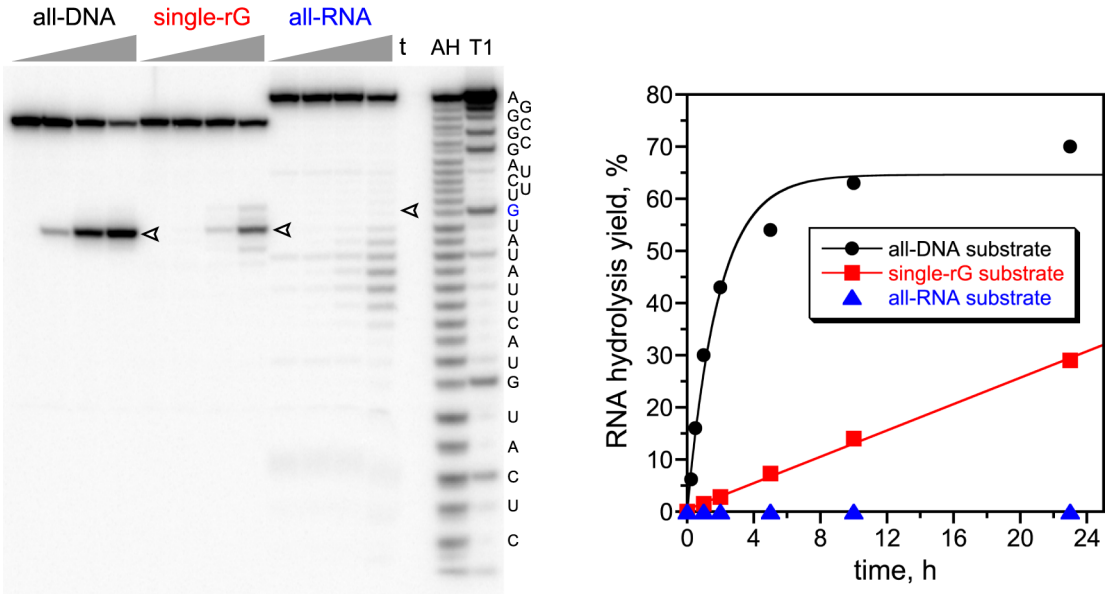

Figure S2. Determination of 10MD5 activity with DNA and RNA substrates. The PAGE image shows representative timepoints for each assay (t = 30 s, 15 min, 2 h, and 23 h). Also shown are two calibration lanes for the all-RNA substrate: "alkaline hydrolysis" (AH; 0.1 pmol of 5′-^{32}P-radiolabeled substrate in 9 μL of 50 mM sodium carbonate, pH 9.2, 1 mM EDTA, heated at 95 °C for 15 min) and RNase T1 digestion (T1; 0.1 pmol of substrate in 10 μL of 20 mM sodium citrate, pH 5.0, 1 mM EDTA, 7 M urea, 0.2 U RNase T1 [Ambion], incubated at room temperature for 15 min). The RNA substrate sequence is marked alongside the calibration lanes, where the blue G denotes the site of 10MD5 cleavage for its parent all-DNA substrate. The open arrowheads mark the positions of the observed cleavage products (all-DNA and single-rG substrates) or the expected position of the unobserved cleavage product (all-RNA substrate); the latter was assigned by comparison with the calibration lanes. Note the expected nonspecific RNA degradation observed in and near the short single-stranded region of the all-RNA substrate (i.e., where the substrate is not base-paired with the deoxyribozyme binding arms), several nucleotides to the 5′-side of the blue G. k_{obs} with all-DNA substrate, 0.53 h^{-1} (first-order kinetics fit). k_{obs} with single-rG substrate, 0.013 h^{-1} (linear fit).

Selection progressions

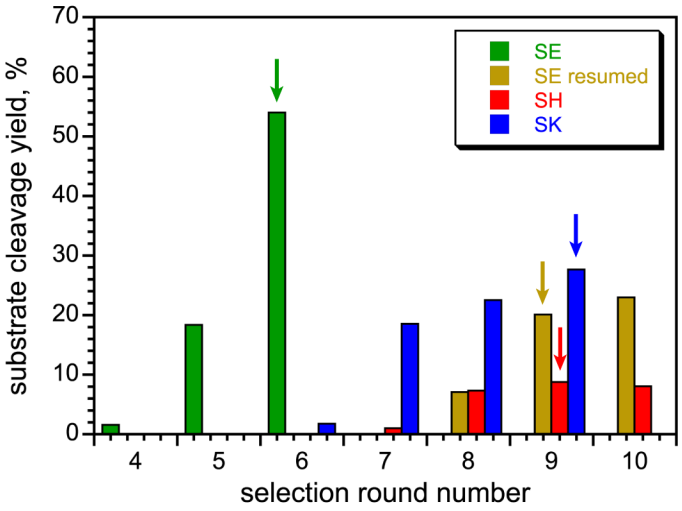

Figure S3. Progressions of the in vitro selection experiments in this study (substrate cleavage yield as a function of selection round number). Arrows mark selection rounds at which individual deoxyribozymes were cloned. All incubation times during selection were 14 h. The data labeled "SE" are for the selection experiment that led to the 6SE deoxyribozymes (cleavage by transesterification). The data labeled "SE resumed" are for the selection experiment that resumed at round 7 and continued for several additional rounds with the hydrolysis selection pressure, leading to the 9SE deoxyribozymes (cleavage by hydrolysis). The data labeled "SH" and "SK" are for the selection experiments that led respectively to the 9SH and 9SK deoxyribozymes (cleavage by hydrolysis).

Sequences of individual deoxyribozymes

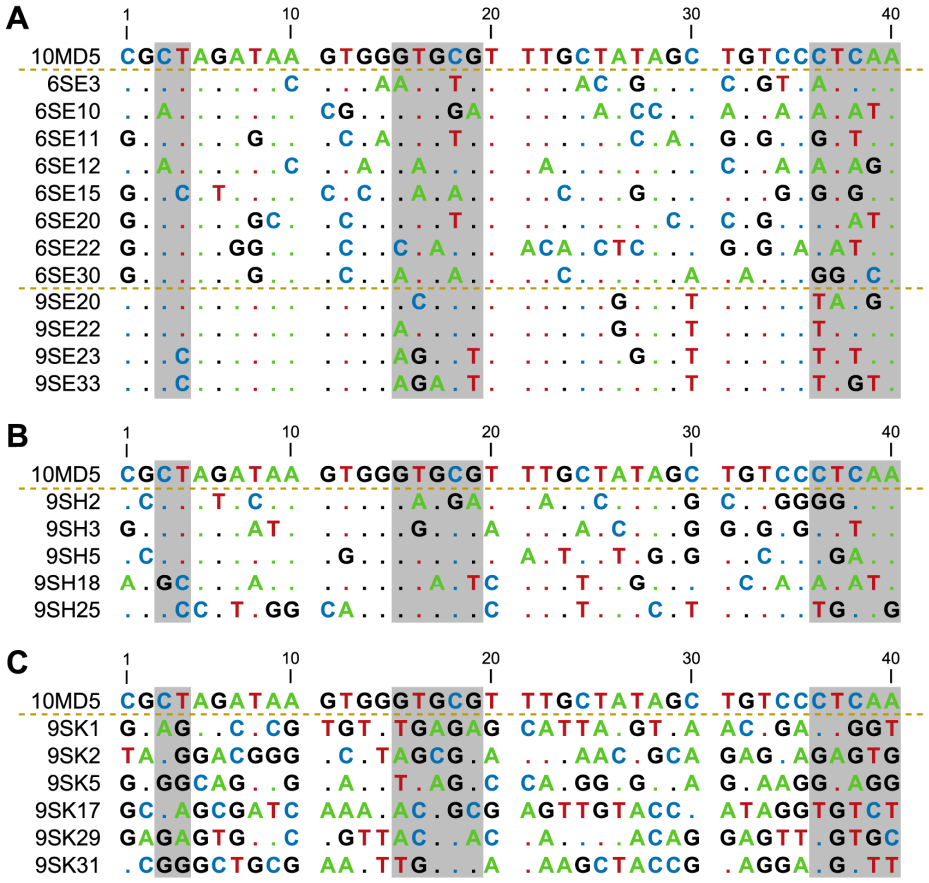

Figure S4. Deoxyribozyme sequence alignments. (A) Alignment of 10MD5 with deoxyribozymes from the reselection experiment using the rG substrate through round 6 without hydrolysis selection pressure (6SE deoxyribozymes) and through round 9 with three rounds of hydrolysis selection pressure (9SE deoxyribozymes). The twelve 10MD5 nucleotides highlighted in grey were found previously to be highly variable upon 10MD5 reselection for improved DNA hydrolysis activity, whereas the other 28 nucleotides were highly conserved.[2] On the basis of the illustrated sequence alignment, the 10MD5 C30T/C36T double mutant was tested for RNA cleavage activity but was found to be inactive. (B) Alignment of 10MD5 with deoxyribozymes from the reselection experiment using the all-RNA substrate through round 9 with the hydrolysis selection pressure for all nine rounds (9SH deoxyribozymes). Upon PAGE purification, the 9SH3 deoxyribozyme led reproducibly (in two independent syntheses) to a broad smear, likely due to poorly urea-denaturable internal structure, and therefore after solid-phase synthesis this deoxyribozyme was purified by phenol/chloroform extraction and ethanol precipitation. (C) Alignment of 10MD5 with deoxyribozymes from the N_{40} selection experiment using the all-RNA substrate through round 9 with the hydrolysis selection pressure for all nine rounds (9SK deoxyribozymes).

MALDI mass spectrometry for individual deoxyribozymes and cleavage site locations

deoxyribozyme	cleavage site[a]	mass L calcd.	mass L found	L error, % (found – calcd.)	mass R calcd.	mass R found	R error, % (found – calcd.)
6SE3 [b]	ACTTATATg^TT	6498.2	not obs.[b]	–	3662.4	3660.8	–0.04
		6516.2	6516.9	+0.01			
6SE10	ACTTATATg^TT	6498.2	not obs.[b]	–	3662.4	3663.6	+0.03
		6516.2	6518.4	+0.03			
6SE11	ACTTATATg^TT	6498.2	6499.6	+0.02	3662.4	3662.6	+0.01
		6516.2	6517.8	+0.02			
6SE12	ACTTATATg^TT	6498.2	6497.9	–0.005	3662.4	3662.1	-0.01
		6516.2	6515.9	–0.005			
6SE15	ACTTATATg^TT	6498.2	6500.0	+0.03	3662.4	3663.3	+0.02
		6516.2	6517.5	+0.02			
6SE20	ACTTATATg^TT	6498.2	6502.0	+0.06	3662.4	3664.2	+0.05
		6516.2	6519.6	+0.05			
6SE22	ACTTATATg^TT	6498.2	6499.1	+0.01	3662.4	3662.8	+0.01
		6516.2	6517.1	+0.01			
6SE30	ACTTATATg^TT	6498.2	6498.8	+0.01	3662.4	3662.6	+0.01
		6516.2	6516.9	+0.01			
9SE20 [c]	ACTTATATg^TT	6436.2	6436.3	+0.002	3742.4	3743.0	+0.02
9SE22	ACTTATATg^TT	6436.2	6434.7	+0.02	3742.4	3742.7	+0.01
9SE23	ACTTATATg^TT	6436.2	6441.3	+0.08	3742.4	3748.1	+0.15
9SE33	ACTTATATg^TT	6436.2	6438.3	+0.03	3742.4	3743.3	+0.02
9SH2 [d]	acuua^uauguu	5357.3	5360.5	+0.06	5179.1	5181.8	+0.05
9SH3	acuuaua^uguu	5992.7	5997.0	+0.07	4543.7	4546.4	+0.06
9SH5	acuua^uauguu	5357.3	5359.7	+0.04	5179.1	5181.3	+0.04
9SH18	acuuauaugu^u	6950.2	6954.0	+0.05	3586.2	3587.3	+0.03
9SH25	acu^uauauguu	4721.9	4725.5	+0.08	5814.5	5818.0	+0.06
9SK1 [e]	a^cuuauauguu	4110.5	4112.8	+0.06	6425.8	6429.4	+0.06
9SK2	acuu^auauguu	5028.0	5028.6	+0.01	5508.3	5509.3	+0.02
9SK5	acuua^uauguu	5357.3	5360.8	+0.07	5179.1	5180.8	+0.03
9SK17	acu^uauauguu	4721.9	4721.5	–0.01	5814.5	5814.4	–0.002
9SK29	acuu^auauguu	5028.0	5029.4	+0.03	5508.3	5509.9	+0.03
9SK31	acu^uauauguu	4721.9	4722.5	+0.01	5814.5	5815.6	+0.02

Table S1. MALDI mass spectrometry data for individual deoxyribozymes (all values are for $[M+H]^+$, in m/z for $z = 1$). Representative MALDI spectra are shown in Figures 2 and 3. L = left-hand cleavage product; R = right-hand cleavage product.

[a] DNA nucleotides are uppercase; RNA nucleotides are lowercase. For each deoxyribozyme, the observed cleavage site is marked with a caret (^). The full substrate sequence is 5′-AAAGTCTCATGTACTTATATgTTCTAGCGCGGA-3′ (6SE, 9SE deoxyribozymes) or the analogous 5′-aaagucucauguacuuauauguucuagcgcgga-3′ (9SH, 9SK deoxyribozymes), where the three underlined nucleotides are not base-paired with the deoxyribozyme binding arms (see Figure S1A).

[b] 6SE deoxyribozymes (all catalyze transesterification): 10MD5 reselection, rG substrate, without hydrolysis selection pressure for six rounds; see Figure 2B (*top*). For the L product, data is shown for both the primary product, 2′,3′-cyclic phosphate (L>p; first line for each deoxyribozyme) as well as the secondary product, 2′(3′)-monophosphate (Lp; second line for each deoxyribozyme). For 6SE3 and 6SE10, the primary product was not detected by mass spectrometry due to its subsequent opening to the secondary product, although PAGE directly demonstrates sole formation of the primary product (Figure S5).

[c] 9SE deoxyribozymes (all catalyze hydrolysis): 10MD5 reselection, rG substrate, without hydrolysis selection pressure for six rounds and then with hydrolysis selection pressure for three rounds; see Figure 2B (*bottom*).

[d] 9SH deoxyribozymes (all catalyze hydrolysis): 10MD5 reselection, all-RNA substrate, with hydrolysis selection pressure for all nine rounds; see Figure 3A.

[e] 9SK deoxyribozymes (all catalyze hydrolysis): N_{40} selection, all-RNA substrate, with hydrolysis selection pressure for all nine rounds; see Figure 3B.

Samples for mass spectrometry were prepared as follows. A 20 μL sample containing 1.2 nmol of substrate and 1.0 nmol of deoxyribozyme was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The cleavage reaction was initiated by addition of stock solutions to a final volume of 50 μL containing HEPES, pH 7.5, and metal ions as described above for in vitro selection. The sample was incubated at 37 °C for 14 h. The nucleic acids were precipitated by adding 50 μL of water, 10 μL of 3 M NaCl and 330 μL of ethanol and dissolved in 10 μL water. The sample was purified by 20% PAGE, excising a ~2 cm tall region of the gel where any cleavage products could reasonably have migrated. This was done to avoid biasing the mass spectrometry results towards the predominant (major) cleavage products, in case there were smaller amounts of less predominant (minor) products. The cleavage products were together extracted, precipitated with ethanol, and dissolved in 10 μL of water. The sample was desalted by C_{18} ZipTip and analyzed by MALDI mass spectrometry with 3-hydroxypicolinic acid as matrix. MALDI mass spectra were obtained in the mass spectrometry laboratory of the UIUC School of Chemical Sciences.

In all cases of transesterification (*T*), i.e., for all 6SE deoxyribozymes, the initially formed 2′,3′-cyclic phosphate on the left-hand (L) product was partially hydrolyzed to the 2′(3′)-monophosphate during the mass spectrometry experiment. In parallel, PAGE of the cleavage products verified that the *T* primary product is entirely the 2′,3′-cyclic phosphate, whereas the hydrolysis (*H*) primary product is entirely the 2′,3′-diol (Figure S4).

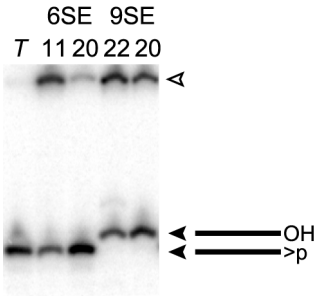

Figure S5. PAGE experiment to demonstrate that the primary product of transesterification (*T*) by the 6SE deoxyribozymes is the 2′,3′-cyclic phosphate on the left-hand (L) product. Open arrowhead denotes the substrate; filled arrowhead denotes the product. The 5′-^{32}P-radiolabeled DNA substrate was cleaved by the indicated 6SE or 9SE deoxyribozyme or by a 10-23 deoxyribozyme known to form the 2′,3′-cyclic phosphate (5′-TCCGCGCTAGAAG-GCTAGCTACAACGAATATAAGTACATGAGACTTT-3′; lane labeled *T*). Incubation conditions for the 10-23 deoxyribozyme assay: 10 pmol 10-23 deoxyribozyme, 35 mM HEPES, pH 7.5, 75 mM NaCl, 100 mM $MgCl_2$, and 100 mM $MnCl_2$ in 20 μL volume at 37 °C for 16 h. The 6SE products comigrate with the 10-23 product as the 2′,3′-cyclic phosphate, whereas the 9SE products migrate slightly more slowly as the 2′,3′-diol (no phosphate). All 6SE and 9SE deoxyribozymes had respective product migrations analogous to those illustrated here, by correlation of Figure 2B with the data shown in this figure. Especially note the absence of the faster-migrating 2′(3′)-monophosphate secondary product for the 6SE deoxyribozymes, demonstrating that the presence of this secondary product in the mass spectrum for each of these deoxyribozymes (Table S1) is merely an artifact of the mass spectrometry procedure.

Assignments of 5′-hydroxyl transesterification products and 5′-phosphate hydrolysis products—as concluded from the mass spectrometry data—were corroborated by a splint ligation assay (Figure S6). Right-hand cleavage products from each of the four representative 6SE30 (5′-hydroxyl) and 9SE20, 9SH3, and 9SK5 (5′-phosphate) deoxyribozymes were prepared using the appropriate 3′-^{32}P-pCp-radiolabeled selection substrate and deoxyribozyme followed by PAGE purification. The products were treated with T4 polynucleotide kinase (PNK) and ATP (6SE30, for 5′-phosphorylation) or calf intestinal alkaline phosphatase (CIAP; 9SE20, 9SH3, 9SK5, for 5′-dephosphorylation) and then subjected to splint ligation using the capture and splint oligonucleotides from the corresponding selection procedure. In all cases as expected, splint ligation was observed only when the 5′-terminus of the right-hand cleavage product was 5′-phosphorylated. This was the case for 6SE30 only when PNK was used (+ PNK) and for each of 9SE20, 9SH3, and 9SK5 only when CIAP was *not* used (– CIAP).

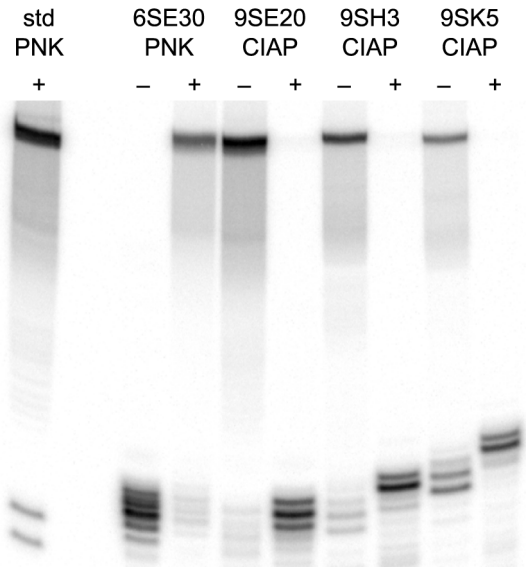

Figure S6. Splint ligation assay to corroborate 5′-hydroxyl transesterification products and 5′-phosphate hydrolysis products. Lane marked "std": 5′-hydroxyl standard oligonucleotide corresponding to the cleavage product for the SE selection, 5′-^{32}P-radiolabeled with T4 PNK and γ-^{32}P-ATP and subjected to splint ligation. In all cases, the heterogeneity in the unligated material was due to multiple additions of pC to the substrate in the 3′-^{32}P-radiolabeling step, where the pC arises from pCp by PNK-catalyzed 3′-dephosphorylation. Similar heterogeneity is not apparent for the ligation products due to their larger size.

Procedure for 3′-^{32}P-pCp-radiolabeling of cleavage substrate. 5′-^{32}P-Radiolabeled cytidine 3′,5′-bisphosphate (pCp) was prepared by incubating 20 pmol of cytidine 3′-monophosphate (Cp), 20 pmol of γ-^{32}P-ATP, and 5 U of T4 PNK (Fermentas) in 10 μL of 1× T4 PNK buffer (50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA) at 37 °C for 30 min. The PNK was inactivated by heating at 95 °C for 5 min and cooling on ice for 10 min. The resulting sample was assumed to contain 20 pmol of 5′-^{32}P-pCp. The 3′-^{32}P-radiolabeled substrate was then prepared by reaction of 10 pmol of unradiolabeled substrate with the sample of 20 pmol of 5′-^{32}P-pCp and 5 U of T4 RNA ligase (Fermentas) in 20 μL of 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10 mM DTT, and 50 μM ATP at 37 °C for 12 h, followed by 20% PAGE.

Procedure for deoxyribozyme-catalyzed substrate cleavage. The general assay procedure as described above was used. The final incubation conditions were 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C, with 20 nM substrate and 1 μM deoxyribozyme (1:50) in 20 μL total volume. The right-hand cleavage products were separated by 20% PAGE.

Procedure for PNK or CIAP treatment and splint ligation. Each of the four deoxyribozyme-catalyzed right-hand cleavage products was divided into two portions. One portion was used immediately in the splint ligation (capture) assay as described above. For 6SE30, the second portion was incubated with 10 nmol of ATP (1 mM final concentration) and 5 U of T4 PNK (Fermentas) in 10 μL of 1× T4 PNK buffer at 37 °C for 30 min, followed by heating at 95 °C for 5 min and cooling on ice for 10 min. For 9SE20, 9SH3, and 9SK5, the second portion was incubated with 1 U of CIAP (Fermentas) in 10 μL of 1× CIAP buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$) at 37 °C for 30 min, followed by heating at 95 °C for 5 min and cooling on ice for 10 min. Each of the four PNK-treated or CIAP-treated samples was then used in the splint ligation (capture) assay as described above.

k_{obs} values for deoxyribozyme kinetics assays

deoxyribozyme	k_{obs}, h^{-1}
6SE3	0.11
6SE10	1.38
6SE11	0.027 [a]
6SE12	0.10
6SE15	0.055
6SE20	0.53
6SE22	0.19
6SE30	0.38
9SE20	0.12
9SE22	0.018 [a]
9SE23	0.051
9SE33	0.071
9SH2	0.087
9SH3	0.12
9SH5	0.032 [a]
9SH18	0.022 [a]
9SH25	0.026 [a]
9SK1	0.82
9SK2	0.10
9SK5	1.19
9SK17	0.71
9SK29	0.15
9SK31	0.061

Table S2. k_{obs} values for deoxyribozymes as derived from kinetics plots in Figures 2B and 3. k_{obs} values are from first-order kinetic fits to $Y = Y_{max} \bullet (1 - e^{-kt})$ unless otherwise noted.
[a] k_{obs} values are from linear fits to the initial time points.

Explanation of the Figure 2C diagram

The diagram in Figure 2C is intended to assist visualization of the relationships among the four cloned selection rounds. Note that the diagram plots activation energy ($\Delta G^{\ddagger}$) versus number of mutations, unlike a conventional energy diagram of free energy (G) versus reaction coordinate. The various components of the diagram were created as follows.

The **x-axis** depicts the observed number of mutations (nucleotide differences) of each of the four sets of deoxyribozymes (6SE, 9SE, 9SH, 9SK) relative to the parent 10MD5 deoxyribozyme. As indicated in the main text, the numbers of such mutations were as follows:

- 6SE deoxyribozymes (transesterification, denoted *T*): 10–17 mutations; sequences are unrelated to 10MD5 because they evolved to catalyze an entirely different RNA cleavage mechanism.
- 9SE deoxyribozymes (hydrolysis, denoted *H*′): 4–9 mutations; sequences are closely related to 10MD5, because they evolved from 10MD5 to hydrolyze the substrate at the same nucleotide sequence junction.
- 9SH deoxyribozymes (hydrolysis, denoted *H*″): 10–14 mutations; sequences are unrelated to 10MD5, because they evolved from 10MD5 to hydrolyze the substrate at a different nucleotide sequence junction.
- 9SK deoxyribozymes (hydrolysis, denoted *H*‴): 26–36 mutations; sequences are unrelated to 10MD5, because they were identified from entirely random N_{40} sequences to hydrolyze the substrate at a different nucleotide sequence junction.

Because of the corresponding numbers of mutations, the diagram depicts *T* and *H*″ at a similar mutational distance from the parent 10MD5 deoxyribozyme (*H*), whereas *H*′ is shown nearer and *H*‴ is shown farther. Unsurprisingly, the 6SE (*T*) and 9SH (*H*″) deoxyribozymes are found at a similar mutational distances from 10MD5 (*H*), consistent with the fact that both sets of deoxyribozymes were evolved from the 25% randomized 10MD5 pool. That is, similarly distant regions of sequence space relative to 10MD5 were explored in these two reselection experiments.

The **y-axis** depicts each of *T*, *H*, *H*′, *H*″, and *H*‴ at vertical locations corresponding to the experimentally observed DNA-catalyzed cleavage rates, as assessed by the fastest deoxyribozyme in each set. Each of *T*, *H*, and *H*‴ are comparably fast (i.e., have relatively low activation energy); compare plots for 10MD5 $(H)^5$ with plots for 6SE (*T*) and 9SK (*H*‴) in Figure 2B (*top*) and Figure 3B, respectively. In contrast, *H*′ and *H*″ are rather slow (relatively high activation energy), as shown in the plots of Figure 2B (*bottom*) and Figure 3A, respectively.

The **lines** connecting *H* to each of *T*, *H*′, *H*″, and *H*‴ are drawn to suggest the difficulty of starting from 10MD5 (*H*) in N_{40} sequence space and reaching the final point by the corresponding experimental procedure, which was either reselection starting from 25% randomized 10MD5 (*T*, *H*′, *H*″) or an entirely new selection experiment starting from a fully random N_{40} pool (*H*‴):

- For each of 6SE (*T*), 9SH (*H*″), and 9SK (*H*‴), because the 6SE/9SH/9SK sequences are unrelated to 10MD5 (*H*), the intermediate sequences are unlikely to have catalytic activity of any kind, and the intermediate activation energies are therefore shown as relatively high.
- For 9SE (*H*′), because the 9SE sequences are closely related to 10MD5 (*H*), the activation energies for the intermediate sequences are shown as not very high.

References for Supporting Information

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