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Supporting Information

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Combinatorial Mutation Interference Analysis Reveals Functional Nucleotides Required for DNA Catalysis**

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Materials

5'-*O*-DMT-2'-*O*-TOM-protected nucleoside 3'-β-cyanoethyl phosphoramidites of N^6-acetyladenosine, N^4-acetylcytidine, N^2-acetylguanosine and uridine were obtained from ChemGenes.
Standard 5'-*O*-DMT-protected 3'-β-cyanoethyl phosphoramidites of N^6-benzoyl-2'-deoxyadenosine, N^4-benzoyl-2'-deoxycytidine, N^2-(N,N-dimethylamino)methylene-2'-deoxyguanosine, and thymidine were from SAFC/Sigma-Aldrich. Benzylthiotetrazole (BTT) was from Carbosynth. Unmodified DNA oligonucleotides were obtained from Sigma-Aldrich. Ribonucleotide triphosphates (NTPs) for in vitro transcription reactions were purchased from Jena Bioscience. α-^{32}P-dATP (3000 Ci/mmol) and γ-^{32}P-ATP (3000 Ci/mmol) were from Perkin Elmer. T4 RNA ligase and T4 polynucleotide kinase (PNK) were from Fermentas, and Klenow fragment, 3'→5' exo$^-$, DNA polymerase I was purchased from Jena Bioscience.

Oligonucleotides

RNA substrates

7S11 L-RNA GGAAUACGACUCAC
7S11 R-RNA pppGGAAGAGAUGG
9DB1 L-RNA GGAAGUCUCAUGUACUA
9DB1 R-RNA pppGAUGUUCUAGCGCCGGA

Deoxyribozymes

7S11 CAACATCTC**CAGTGCAGGGC**GTGAG**GGCTCGG*TTCC***CGTATTCC
9DB1 CAAGGCGCTAGAACAT**GGATCATACGGTCGGAGGGGTTTGCCGTGAACATTCTTCA**AGTACATGAGACTTCC

The bold nucleotides represent the catalytic loop regions, and the non-bold nucleotides constitute the binding regions for RNA substrates. The bold italic nucleotides belong to loop B of 7S11 and are involved in formation of the paired region P4 with the R-RNA substrate (see Figure 1A in manuscript). The bold underlined nucleotides in 9DB1 are involved in predicted stems I and II (see Figure 1B in manuscript).

Template DNA oligonucleotides for 3'-^{32}P-labeling

for 7S11 GTGGAATACGGGAA
for 9DB1 GTGGAAGTCTCATGTACT

Solid-phase synthesis, deprotection and purification

All oligonucleotides were synthesized on polystyrene custom primer supports from GE Healthcare on a Pharmacia Gene Assembler Plus or on an ÄKTA Oligopilot 10 Plus, following slightly modified DNA standard methods. Detritylation was achieved with 3% (v/v) dichloroacetic acid in 1,2-dichloroethane for two minutes. For coupling, 75 µL of 0.1 M phosphoramidites in acetonitrile (see below for details on phosphoramidite mixtures for library synthesis) was activated with 250 µL of 0.25 M benzyl thiotetrazole (BTT) in acetonitrile; coupling time was 2 min. Capping was achieved with 1:1 (v/v) A/B for one minute (A, Ac_2O/sym-collidine/acetonitrile, 2:3:5, by vol.; B, 0.5 M 4-(dimethylamino)pyridine in acetonitrile). Oxidation was performed with 10 mM I_2 in acetonitrile/sym-collidine/water (10:1:5, v/v) for one minute. Phosphoramidite solutions, tetrazole solutions, and acetonitrile were dried over activated molecular sieves overnight. All sequences were synthesized trityl-off.

Deprotection and cleavage of ribonucleotide-containing deoxyribozyme libraries from solid support were achieved with concentrated (25%) ammonium hydroxide:ethanol 3:1 for 16 hours at 55°C. Unmodified RNA oligonucleotides were cleaved from support and deprotected with $MeNH_2$ in EtOH (8 M, 500 µL) and $MeNH_2$ in water (40%, 500 µL) for six hours at 37°C. In both cases, the solution was then evaporated to dryness. Removal of the 2'-*O*-silyl groups was afforded by treatment with tetrabutylammonium fluoride trihydrate (TBAF·$3H_2O$) in THF (1 M, 500 µL) for 5 hours at 40°C (deoxyribozyme libraries) or for 12 hours at room temperature (all-RNA oligonucleotides). The reaction was quenched by the addition of

Tris.HCl (1 M, pH 7.4, 500 µL). The volume of the solution was reduced to 0.5 mL and directly applied on a Sephadex G 10 column (3x5 ml HiTrap column, GE Healthcare) monitored by UV-detection at 280 nm. The product was eluted with water and evaporated to dryness.

RNA oligonucleotides with 5′-triphosphates were prepared by in vitro transcription with T7 RNA polymerase and a synthetic DNA template.[1] Transcription reactions with T7 RNA polymerase were performed using 1 µM reverse strand and 1 µM promoter strand (5′-CTGTAATACGACTCACTATA-3′) in 40 mM Tris-HCl, pH 8.0, 30 mM $MgCl_2$, 10 mM DTT, 4 mM each NTP, and 2 mM spermidine at 37 °C for 3–5 h. The reaction was quenched by the addition of 50 mM EDTA and the transcription product was isolated by denaturing PAGE.

All DNA and RNA oligonucleotides (prepared by solid-phase synthesis or in vitro transcription) were purified by denaturing PAGE on 10%, 15%, or 20% denaturing polyacrylamide gels containing 7 M urea. Gels were 0.7 mm thick, 20 x 30 cm, with 4 or 8 lanes. The gels were run in 1x TBE (89 mM each Tris and boric acid, pH 8.3, 2 mM EDTA), at 35 W. Oligonucleotides were visualized by UV shadowing and extracted by crush and soak into TEN (10 mM Tris.HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl) at 37°C for 2x 3-5h, followed by precipitation with ethanol at –80°C.

The purity of all RNA and DNA oligonucleotides was confirmed by denaturing anion-exchange HPLC on a Dionex DNAPac PA200 column: eluant A, 25 mM Tris.HCl, pH 8.0, 6 M urea; eluant B, 25 mM Tris.HCl, pH 8.0, 0.5 M $NaClO_4$, 6 M urea; detection at 260 nm; gradient, 45 minutes 0–60% B in A; 80°C.

Coupling efficiency of RNA phosphoramidites in presence of DNA phosphoramidites

To determine the coupling efficiency of ribonucleotide phosphoramidites in the presence of deoxribonucleotide phosphoramidites, we prepared 5-mer model oligonucleotides (ACGTT together with their rN-containing derivatives), in which one coupling step was performed with rN/dN mixtures (i.e., mixtures of ribonucleotide and deoxyribonucleotide containing the same nucleobase), containing 5%, 10%, 15%, 20%, or 25% of 2'-*O*-TOM-protected ribonucleotide phosphoramidites, at a final (total) phosphoramidite concentration of 100 mM (mixtures were prepared from 100 mM stock solutions of DNA and RNA phosphoramidite solutions in CH_3CN that have been dried over molecular sieves for 24 h). The coupling conditions were as described above. The oligonucleotides were cleaved from the solid support and phosphate backbone and nucleobases were deprotected by treatment with 500 µL of 25% NH_4OH and 500 µL of 40 % $MeNH_2$ in H_2O (20-30 min at room temperature and 20 min at 65°C). The mixtures of unmodified DNA pentamer and oligonucleotides containing one 2'-*O*-TOM-protected ribonucleotide were analyzed by denaturing anion exchange HPLC. The ratio of unmodified and 2'-*O*-TOM-rN-containing pentamer was determined by area integration (peak detection at 260 nm). Figure S1 shows the HPLC traces for oligonucleotides prepared with rG/dG, rA/dA, rC/dC and rU/dT mixtures containing 15% of 2'-*O*-TOM-ribonucleotide phosphoramidites. The experimentally determined value for ribonucleotide incorporation is indicated.

Similarly, the incorporation of ribonucleotides in the presence of each of the other three deoxyribonucleotides was analyzed for mixtures containing 10% or 15% ribonucleotide phosphoramidites. All individual datapoints are depicted in Figure S2. The average incorporation rates for 10% rN mixtures (from three individual experiments) are summarized in Table S1.

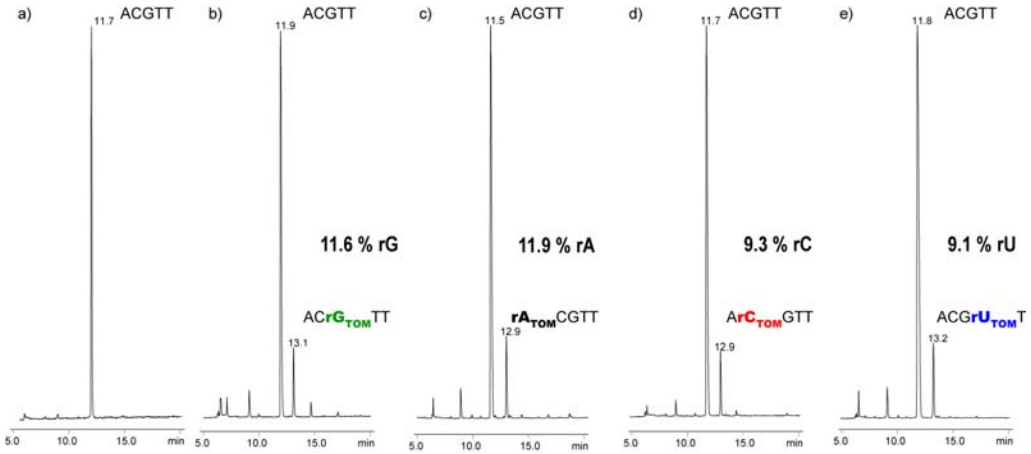

Figure S1. HPLC analysis of ribonucleotide-containing pentamer oligonucleotides. a) unmodified ACGTT. b) dG phosphoramidite was replaced by rG/dG = 15/85 mixture. c) dA phosphoramidite was replaced by rA/dA = 15/85 mixture. d) dC phosphoramidite was replaced by rC/dC = 15/85 mixture. e) dT phosphoramidite was replaced by rU/dT = 15/85 mixture. Dionex DNAPac PA200, 0 – 32% B in 24 min. Eluant A. 25 mM Tris.HCl, pH 8.0, 6 M urea; Eluant B. 25 mM Tris.HCl, pH 8.0, 0.5 M $NaClO_4$, 6 M urea; 80°C, detection at 260 nm.

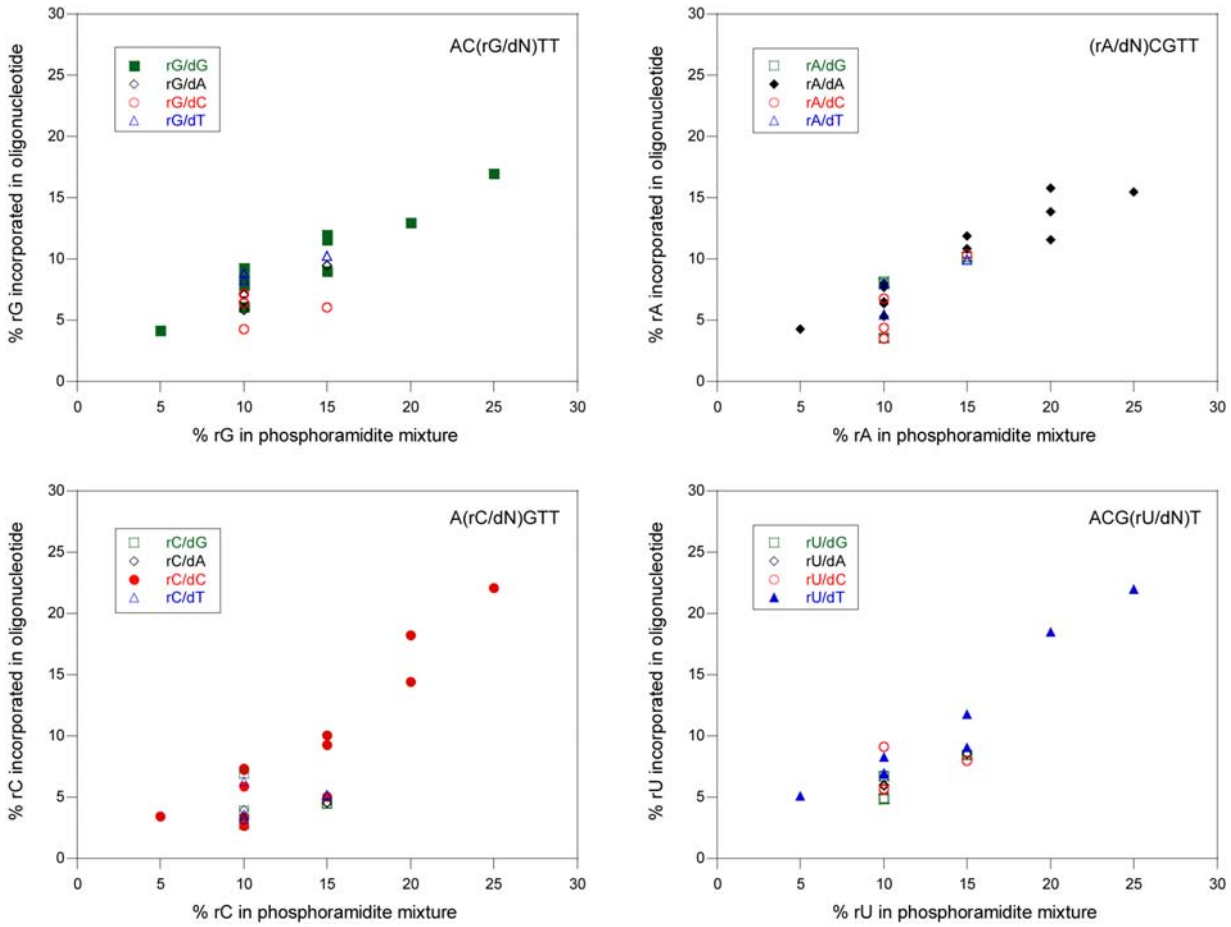

Figure S2. Ribonucleotide incorporation from rN/dN phosphoramidite mixtures, analyzed by anion exchange HPLC and area integration of peaks for unmodified DNA and 2'-*O*-TOM-protected ribonucleotide-containing pentamers. Filled datapoints correspond to rN/dN mixtures with the same nucleobase, whereas empty datapoints correspond to rN/dN mixtures with different nucleobases.

Table S1. Average ribonucleotide incorporation (in %) for phosphoramidite mixtures containing 10% rN.

	rG	**rA**	**rC**	**rU**
dG	7.9 (0.9)	6.6 (1.5)	4.5 (1.2)	5.5 (0.6)
dA	6.5 (0.4)	6.5 (0.7)	3.6 (0.4)	6.0 (0.2)
dC	6.0 (0.9)	5.0 (1.0)	4.0 (1.0)	6.8 (1.1)
dT	8.5 (0.3)	6.7 (1.3)	4.4 (1.0)	7.0 (0.2)
mean	**7.2** (0.6)	**6.2** (0.4)	**4.2** (0.2)	**6.3** (0.3)

The mean of three individual experiments and the standard error (in parentheses) are given for each mixture. The mean four each group of rN mixtures with all four standard DNA amidites is given in the last row. This number describes the average rN incorporation rate in the respective library, provided that all four nucleotides A, C, G, T occur with equal frequency in the DNA of interest.

Number of incorporated ribonucleotides in deoxyribozyme core

The number of nucleotides of the deoxyribozyme core that are subject to CoMA analysis determines the desired ribonucleotide incorporation rate in the mutant libraries.

The distribution of DNA molecules containing *i* number of mutations can be calculated by

$$P(i,n,rN_e) = (rN_e)^i \cdot (1-rN_e)^{(n-i)} \cdot C(n,i),$$

where *i* is the number of mutations per molecule, rN_e is the effective ribonucleotide incorporation rate, *n* is the number of coupling events for which rN/dN mixtures are used, and C(*n*,*i*) is the combinatorial function. For example, 40 times coupling (*n* = 40) of a rN/dN mixture that yields 5% actual ribonucleotide incorporation per coupling (rN_e = 0.05) will result in 13% unmodified DNA molecules (*i* = 0), 27% of single-mutants (*i* = 1), 28% of double-mutants (*i* = 2), 18% of triple-mutants (*i* = 3), and 14% of DNA molecules with more than 3 mutations. Figure S3 shows the distributions calculated for 7S11 and 9DB1 mutant libraries, for ribonucleotide incorporation rates of 2%, 5%, and 10%.

The 7S11 mutant libraries (containing 22 mutated positions) were synthesized with rN/dN mixtures containing 15% ribonucleotide phosphoramidites, which resulted in 5 – 10% rN incorporation per coupling. The 9DB1 libraries (containing 40 mutated positions) were synthesized with rN/dN mixtures containing 10% ribonucleotide phosphoramidites, which resulted in 4 – 7% rN incorporation per coupling.

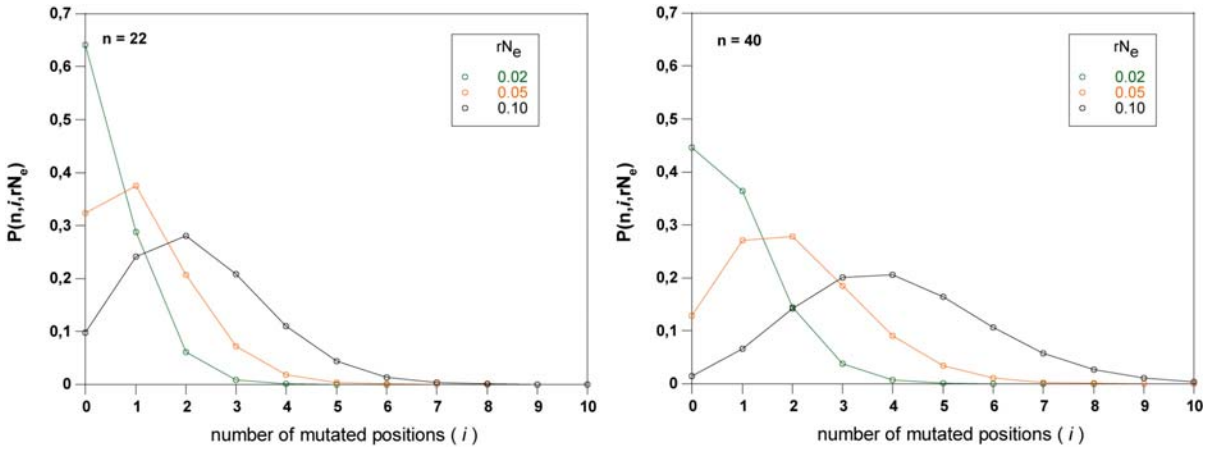

Figure S3. Distribution of number of library members containing *i* mutations. a) 7S11 mutant library with 22 loop positions, and b) 9DB1 mutant libraries with 40 positions. The calculated curves are shown for effective ribonucleotide incorporation rates of 2, 5, and 10% per coupling.

Enzymatic 5'-phosphorylation of deoxyribozyme libraries

The ribonucleotide-containing deoxyribozyme libraries (2 nmol) were incubated with 5 units of T4 PNK in the presence of 1 mM ATP, 1x PNK buffer (50 mM Tris.HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) in a final volume of 40 µL for 4 hours at 37°C. The enzyme was inactivated by denaturation at 70°C for 5 min. The 5'-phosphorylated products were isolated by precipitation with ethanol at –80°C, and then used for the ligation reactions without further purification.

Ligation of deoxyribozyme libraries to R-RNA

The 5'-phosphorylated deoxyribozyme library (2.0 nmol) and R-RNA (2.25 nmol) were annealed in a final volume of 23 µL containing 2.3 µL of 10x annealing buffer (50 mM Tris.HCl, pH 7.5, 150 mM NaCl, 1 mm EDTA) by heating to 95°C for 2 min and slow cooling to room temperature over a period of 30 minutes. The ligation reaction was initiated by addition of 3.0 µL 10x T4 RNA ligase buffer (500 mM Tris.HCl, pH 7.5, 100 mM DTT, 50 mM $MgCl_2$), 3.0 µL 10 mM ATP and 10 units T4 RNA ligase (1 µL of 10 u/µL). The reaction mixture was incubated for 6 h at 25°C, quenched by the addition of 30 µL stop solution (80% formamide, 1xTBE, 50 mM EDTA, 0.0025% each of bromophenol blue and xylene cyanol), and then separated by denaturing PAGE (15% polyacrylamide, 0.7 mm, 35 W, 1h50). The products were visualized and isolated as described above.

3'-^{32}P-labeling of DNA

The deoxyribozyme libraries and the ligated RNA-DNA conjugates (R-E) were 3'-^{32}P-radiolabeled by templated addition of α-^{32}P-dATP using Klenow DNA polymerase.[2] The DNA substrates (60 pmol) and splint oligonucleotides (180 pmol) were annealed in a volume of 10 µL by heating to 95°C for 2 minutes, followed by slow cooling over a period of 30 minutes. After the addition of α-^{32}P-dATP (10 µCi), 1.5 µL of 10x Klenow reaction buffer (100 mm Tris.HCl, pH 7.5, 50 mM $MgCl_2$, 75 mM DTT) and 5 u Klenow DNA polymerase, the reaction reaction volume was adjusted to 15 µL by addition of H_2O, and the mixture was incubated for 1.5 hours at 37°C. The 3'-^{32}P-labled products were isolated by denaturing PAGE (12%, 0.7 mm, 35W, 1h).

DNA-catalyzed RNA ligation and separation of active and inactive deoxyribozyme mutants

The 3'-^{32}P labelled products R-E were mixed with L-RNA (300 pmol) and annealed in the presence of 5 mM HEPES, pH 7.5, 15 mM NaCl, 0.1 mM EDTA by heating up to 95°C and slow cooling to 25°C over 30 minutes. The reaction was initiated by the addition of 10 µL 5x CHES ligation buffer (containing 250 mM CHES, pH 9.0, 750 mM NaCl, 10 mM KCl) and 5 µL of 400 mM $MgCl_2$ to a final volume of 50 µL. The 7S11 ligation reactions were incubated for 3 hours at 25°C, and the 9DB1 ligation reactions were incubated for 3 hours at 37°C. The active (ligated) deoxyribozyme mutants were separated from inactive derivatives (unligated) by denaturing PAGE (12%, 0.7 mm, 35W, 1h50 (7S11) or 2h20 (9DB1)). The average ligation yields are summarized in Table S2.

Table S2. Yield of DNA-catalyzed RNA ligation by 7S11 and 9DB1 libraries

DNA library	7S11[a]	9DB1[b]
unmodified	60	50
rG	36	20
rA	38	27
rC	41	36
rU	35	27

ligation yield after 3h; [a] mean of four independent experiments, [b] two independent experiments

Alkaline hydrolysis

Aliquots (2 µL) of the active (L-R-E), inactive (R-E) and unligated DNAs (E) were mixed with 2 µL of 20 mM $NaOH_{aq}$ (final concentration 10 mM) or with 2 µL of water (for "–" lanes) and incubated for 10 min at 95°C. For best reproducibility and to avoid concentration changes during heating in standard reaction tubes, the hydrolysis reaction was performed in a PCR thermocycler with a heated lid. The reaction solution was mixed with loading dye (80% formamide, 1x TBE, 50 mM EDTA, 0.025% each bromophenol blue and xylene cyanol) (2 µL) and loaded directly on a denaturing sequencing gel (20%, 0.4 mm, 20x40cm, 35W, 1h50 (7S11) or 4h (9DB1)). Gels were dried at 80°C under vacuum for 30 min, and exposed to a Phosphor storage screen overnight.

Analysis and quantification of interference pattern

Analysis of CoMA data for deoxyribozymes was similar to described procedures for the analysis of NAIM data for RNA.[3] Individual band intensities were quantified using ImageQuant software (Molecular Dynamics). Volume and area analysis tools were both used and gave similar results (the reported interference values were obtained by area analysis). The band intensities were normalized for minor loading differences (we chose a position at which 2'-OH and nucleobase mutations did not have any effect on DNA activity, i.e. G17 for 7S11 and C36 for 9DB1). The interference values were calculated for every nucleotide position by dividing the band intensity in the unselected library by the band intensity in the active library fraction. Values that turned out larger than ten were set to 10.0. Values <2 (i.e., less than 50% change in intensity) were considered insignificant and colored green, values from 2 to 5 were termed small interference values and colored pink, and interference values >5 were termed strong and colored red. The interference values were then grouped according to the type of mutation at each position, i.e., 2'-OH introduction, transition, transversion-1, and transversion-2. The interference values for individual rN-containing libraries of 7S11 and 9DB1 are reported in Tables S3 and S4.

General description of kinetic assays

All trimolecular kinetic assays were performed similarly to previously described experiments.[4] The 5′-^{32}P-labeled L-RNA substrate (L for "left-hand substrate") was the limiting reagent relative to the deoxyribozyme (E) and the 5'-triphosphorylated R-RNA substrate. The ratio L:E:R was 1:10:20, with 1 µM deoxyribozyme. The RNA substrates and the deoxyribozyme were annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, 0.1 mM EDTA by heating at 95 °C for 3 min and slow cooling to room temperature over 30 min. The ligation reactions were performed in 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$. For 7S11, the reaction mixture was incubated at 25°C for up to 3 hours; for 9DB1, at 37 °C for up to 5 hours. At appropriate timepoints, aliquots were removed from the sample, quenched into stop solution (80% formamide, 1×TB [89 mM each Tris and boric acid, pH 8.3], and 50 mM EDTA containing 0.025% bromophenol blue and xylene cyanol) and stored on ice prior to analysis. Samples were separated by 20% denaturing PAGE at 35 W for 1.5 hours. Gels were dried at 80°C for 30 min, exposed to a Phosphor storage screen overnight, and imaged with a PhosphorImager. The data were fit to the equation yield = $Y\bullet(1-e^{-kt})$, where $k = k_{obs}$ and Y = final yield.

Demonstrating the concept of CoMA for rN-containing 7S11 deoxyribozymes

7S11 libraries were synthesized with mixtures of rG/dG, rC/dC, (rA/dA+ rU/dT), each in a rN/dN ratio of 15/85. (Variation of adenosine and thymidine positions together in one library is due to the low frequency of adenosine (2x) and thymidine (3x) nucleotides in the 7S11 loops). The alkaline hydrolysis pattern (Figure S4, left gel) reveals cleavage products only at the expected nucleotide positions and allows reading of the 7S11 sequence. The 7S11 ligation reaction was then conducted in the bimolecular format for each of the three libraries, and both ligated (L-R-E) and unligated (R-E) fractions were isolated and separately hydrolyzed (Figure S4, right gel). The appearance of all expected hydrolysis products in both fractions of each library indicates that the 2'-OH tag does not inhibit 7S11-catalzyed RNA ligation. This was also confirmed for an individual 7S11 derivative, in which dG at position 9 was replaced by rG. The trimolecular ligation kinetics is depicted in Figure S5.

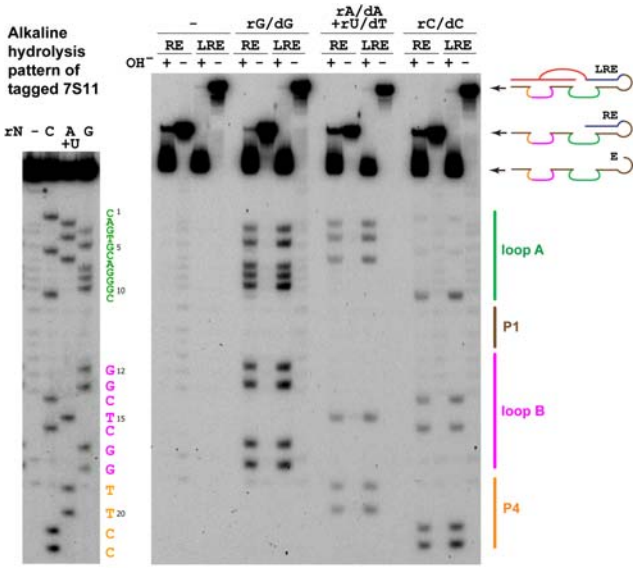

Figure S4. Demonstrating the concept of CoMA for 7S11. The appearance of all expected hydrolysis bands in both active and inactive fractions suggests that a 2'-OH group at any 7S11 loop nucleotide does not interfere with the DNA-catalyzed RNA ligation.

7S11 rG mutant for kinetic analysis in Figure S5 (rG bold, loops regions underlined):
7S11 rG: CAACATCTCCAGTGCAG**rG**GCGTGAGGGCTCGGTTCCCGTATTCC
LC-ESI-MS: mol.wt. found: 13516.2; calculated: 13516.6;

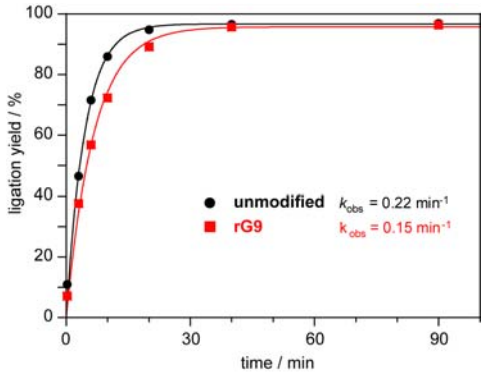

Figure S5. Trimolecular ligation kinetics of rG-containing 7S11 in comparison to unmodified 7S11 at 25°C in 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$.

Combinatorial mutation analysis of 7S11 deoxyribozyme

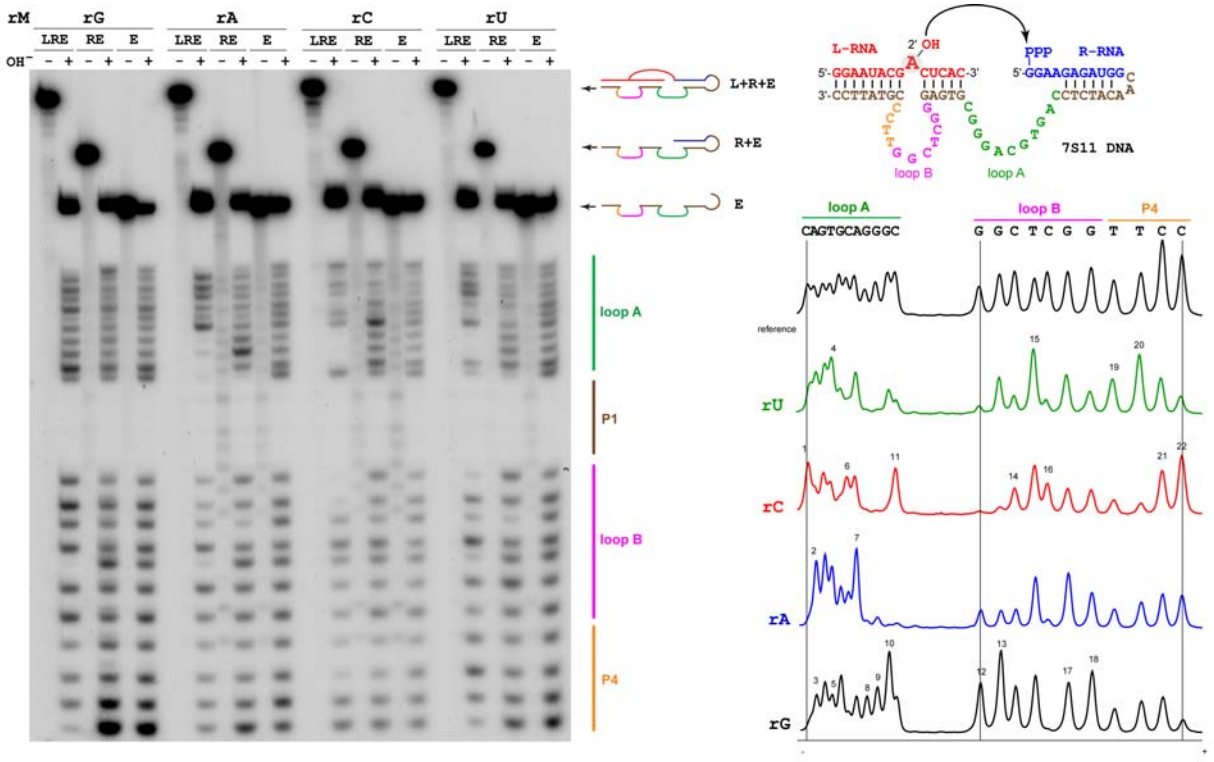

Figure S6. Full alkaline hydrolysis gel of 7S11 mutant libraries and line graphs of active fractions used for area analysis.

Table S3. Interference values for 7S11.

		rG	rA	rC	rU
C	1	4.6	4.1	0.7	1.0
A	2	0.8	0.5	1.0	0.5
G	3	0.7	0.6	0.5	0.5
T	4	0.8	0.7	0.8	0.4
G	5	0.9	1.6	3.4	1.6
C	6	2.3	1.5	1.0	2.3
A	7	0.9	0.7	1.1	0.8
G	8	1.0	5.3	8.8	7.1
G	9	0.8	6.8	10.0	8.2
G	10	0.8	10.0	7.5	2.1
C	11	1.5	10.0	0.7	2.8
G	12	0.7	3.1	7.8	4.1
G	13	0.8	3.8	3.9	1.3
C	14	1.1	3.1	1.2	2.1
T	15	1.0	1.0	0.6	0.7
C	16	10.0	8.1	1.1	3.8
G	17	1.0	1.0	1.0	1.0
G	18	1.0	1.9	1.5	1.8
T	19	1.9	2.8	1.4	1.0
T	20	1.8	2.1	2.8	0.7
C	21	2.1	2.7	0.9	1.6
C	22	10.0	2.0	0.7	5.4

The interference values were calculated as described above. The average of 8 data sets from four different preparations of active library fractions is presented.

Analysis of ligation activity of selected 7S11 mutants

Based on CoMA data of 7S11, mutations at position 9 (in loop A) and position 16 (loop B) are predicted to render 7S11 inactive for catalysis of RNA ligation. The ligation activity of two 7S11 mutants was assayed in trimolecular format with R-RNA (GGAAGAGAUGGCGACGG) and L-RNA (GGAUAAUACGACUCAC) as substrates. The reactions were performed as described above, at pH 9.0 and 37°C, with 40 mM $MgCl_2$. Both mutants did not yield any detectable ligation product within 5 hours (Figure S7).

The 7S11 deoxyribozyme sequences used in this assay were:

7S11	CCGTCGCCATCTCCAGTGCAGGGCGTGAGGGCTCGGTTCCCGTATTATCC
7S11 G9A	CCGTCGCCATCTCCAGTGCAG**A**GCGTGAGGGCTCGGTTCCCGTATTATCC
7S11 C16G	CCGTCGCCATCTCCAGTGCAGGGCGTGAGGGCT**G**GGTTCCCGTATTATCC

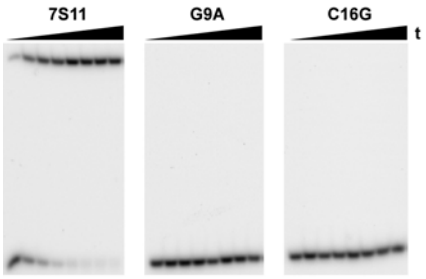

Figure S7. Testing the catalytic activity of 7S11 mutants G9A and C16G, at 37°C in 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$.

Combinatorial mutation analysis of 9DB1 deoxyribozyme

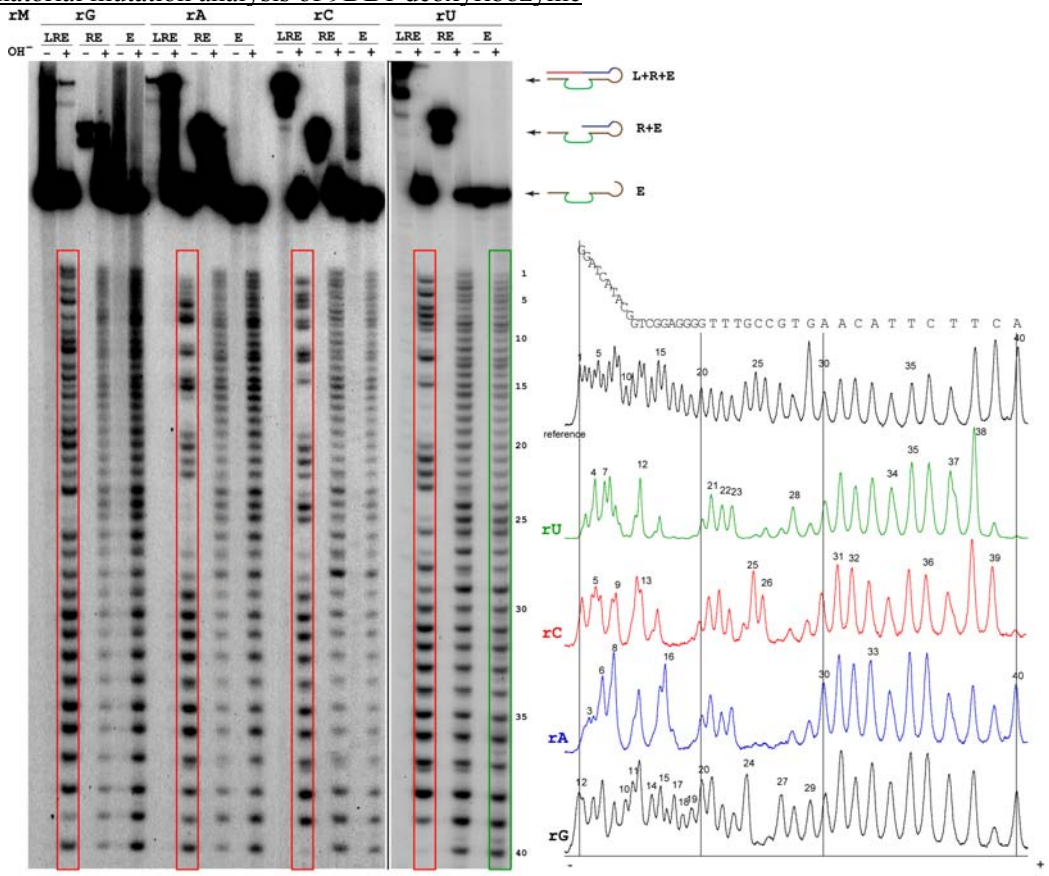

Figure S8. Full alkaline hydrolysis gel of 9DB1 and line graphs of active fractions used for area analysis.

Table S4. Interference values for 9DB1

		rG	rA	rC	rU
1	G	1.6	9.8	5.7	3.7
2	G	2.0	3.9	1.5	1.0
3	A	4.6	2.8	4.6	2.0
4	T	2.2	1.9	1.6	0.9
5	C	5.4	2.2	1.5	3.6
6	A	1.6	1.2	1.5	0.8
7	T	8.1	1.4	7.2	0.7
8	A	3.4	1.4	2.4	1.3
9	C	5.0	5.6	1.6	1.7
10	G	2.1	10.0	10.0	4.5
11	G	1.9	4.2	4.4	3.0
12	T	1.4	1.5	1.3	0.8
13	C	5.9	7.8	1.6	7.9
14	G	1.9	5.8	5.9	4.3
15	G	1.9	1.8	2.5	1.5
16	A	3.5	1.5	10.0	10.0
17	G	2.5	4.4	10.0	7.3
18	G	3.4	10.0	10.0	10.0
19	G	2.2	10.0	8.4	7.5
20	G	1.6	2.3	2.5	1.7

		rG	rA	rC	rU
21	T	1.4	1.1	1.1	0.8
22	T	2.1	1.8	1.0	0.9
23	T	2.0	1.5	1.4	1.0
24	G	1.8	10.0	3.6	10.0
25	C	10.0	10.0	1.2	8.3
26	C	7.2	10.0	1.5	2.1
27	G	2.0	10.0	10.0	4.2
28	T	1.5	2.4	2.9	1.0
29	G	1.4	2.9	4.4	2.9
30	A	1.1	0.8	0.9	1.2
31	A	1.0	0.9	1.0	0.9
32	C	0.9	0.8	1.0	1.1
33	A	1.1	0.9	1.0	1.0
34	T	1.2	0.8	1.0	0.9
35	T	1.1	0.8	0.8	0.9
36	C	1.0	1.0	1.0	1.0
37	T	1.2	0.9	1.0	1.0
38	T	1.4	1.2	1.0	1.1
39	C	5.0	2.1	1.5	3.2
40	A	2.2	1.3	9.7	10.0

The interference values were calculated as described above. The average of 5 data sets from two different preparations of active library fractions is presented.

Analysis of ligation activity of selected 9DB1 mutants

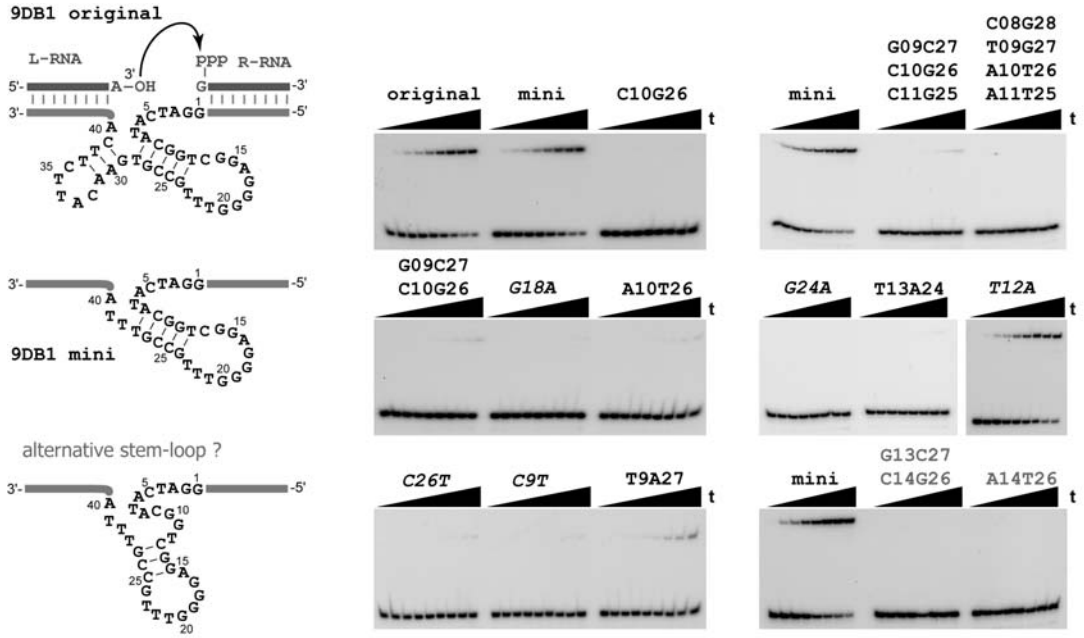

Figure S9. Testing the catalytic activity of 9DB1 derivatives at 37°C in 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$. Single mutants are labeled in italics, with the original nucleotide named first, followed by the position and the new nucleotide. For base-pair mutants, only the new nucleotides (plus their position) in the mutant are named.

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References for Supporting Information

[1] J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **1987**, *15*, 8783.

[2] Z. Huang, J. W. Szostak, *Nucleic Acids Res.* **1996**, *24*, 4360.

[3] a) J. C. Cochrane, S. A. Strobel, *Curr. Protoc. Nucleic Acid Chem.* **2004**, Unit 6.9; b) C. Waldsich, *Nat. Protoc.* **2008**, *3*, 811.

[4] a) A. Flynn-Charlebois, Y. Wang, T. K. Prior, I. Rashid, K. A. Hoadley, R. L. Coppins, A. C. Wolf, S. K. Silverman, *J. Am. Chem. Soc.* **2003**, *125*, 2444; b) C. Höbartner, S. K. Silverman, *Angew. Chem. Int. Ed.* **2007**, *46*, 7420.