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Dinucleotide Junction Cleavage Versatility of 8-17 Deoxyribozyme

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Summary

We conducted 16 parallel in vitro selection experiments to isolate catalytic DNAs from a common DNA library for the cleavage of all 16 possible dinucleotide junctions of RNA incorporated into a common DNA/RNA chimeric substrate sequence. We discovered hundreds of sequence variations of the 8-17 deoxyribozyme—an RNA-cleaving catalytic DNA motif previously reported—from nearly all 16 final pools. Sequence analyses identified four absolutely conserved nucleotides in 8-17. Five representative 8-17 variants were tested for substrate cleavage in *trans*, and together they were able to cleave 14 dinucleotide junctions. New 8-17 variants required Mn^{2+} to support their broad dinucleotide cleavage capabilities. We hypothesize that 8-17 has a tertiary structure composed of an enzymatic core executing catalysis and a structural facilitator providing structural fine tuning when different dinucleotide junctions are given as cleavage sites.

Introduction

In vitro selection techniques [1, 2] have been widely explored to isolate single-stranded DNA molecules with catalytic functions (denoted DNA enzymes, deoxyribozymes, or DNAzymes) from random-sequence DNA libraries [3–7]. Among all known catalytic DNA species, RNA-cleaving deoxyribozymes [8–16] are particularly desirable, as they have great potential to be used both in vivo to digest RNA molecules of biological importance and in vitro as biosensing tools [7, 17]. The first DNAzymes found to cleave an all-RNA substrate were 10-23 and 8-17, discovered by Santoro and Joyce [11]. 10-23, with a catalytic efficiency of $\sim 10^9\ M^{-1}min^{-1}$ [11, 18], has been used to inhibit gene expression effectively in vivo [17]. 10-23 has the ability to cleave any purine-pyrimidine junction (DNA or RNA sequence is written from 5′ to 3′ if not otherwise indicated), with robust activity for A-U and G-U sites, and significantly reduced activity for A-C and G-C sites [11, 18, 19]. 8-17 was initially shown to cleave an A-G junction [11] and was later demonstrated to cleave any N-G junction (N stands for all four standard ribonucleotides) [13]. Although 8-17 has not been shown to be as useful in vivo, it has been exploited for many innovative in vitro applications including functioning as a lead sensor [20] and a catalytic probe to construct logical gates for DNA-based computing [21].

Based on the findings with 10-23 and 8-17, we hypothesized that it should be possible to isolate new DNAzymes that could collectively cleave all possible ribodinucleotide junctions. Our motivation was to build a battery of RNA-cleaving DNAzymes to provide more choices for use either as diagnostic tools or gene therapeutics. We designed a method that allowed simultaneous selection of a large number of DNAzymes that could cleave at least one of the 16 possible dinucleotide junctions from a single DNA pool. Using this approach, we have identified many catalytic sequences that together can cleave all 16 possible dinucleotide junctions. Surprisingly, we discovered a large set of 8-17 variants that are together capable of cleaving wide-ranging dinucleotide junctions.

Results

Deriving Deoxyribozymes that Collectively Cleave All 16 Dinucleotide Junctions

We set out to explore in vitro selection techniques to create diverse DNA enzymes that together could cleave all 16 dinucleotide junctions of RNA. To make our experiment manageable, we used 16 analogous substrates, each containing a single ribonucleotide linkage and differing only at the dinucleotide junction to be cleaved (Figure 1). Each substrate was generated by joining a 15-nt ribo-terminated S1 to an 8-nt S2 over 33-nt T1 as the ligation template (Figure 1A). S1 has four variations at the terminal nucleotide (ribonucleotide A, C, G, or U), and S2 also has four variations at the first nucleotide (deoxyribonucleotide A, C, G, or T).

The initial pool contained approximately 10^{14} molecules and was produced by mixing equal amounts of six random-sequence synthetic DNA oligonucleotides (Libraries A–F, Figures 1B and 1C). An internal stem-loop (stem 3 in Figure 1C) was placed in the middle of the sequence in five of the six libraries (Libraries A–E). Our hope was to allow the selected deoxyribozymes an opportunity to recruit this motif as an essential structural element. Deoxyribozymes with such a structural feature are desirable because they could be conveniently designed into allosteric deoxyribozymes via the "communication module" strategy [22–24]. Since both 8-17 and 10-23 have a catalytic core under 20 nucleotides, we reasoned that a random region of ~20 nucleotides might be sufficient for the creation of a catalytic domain. The random nucleotides were arranged around the preordained stem-loop in several ways, as shown in Figure 1C, to allow more opportunities for potential small deoxyribozymes to arise during selection. Library F contained no predetermined secondary structures, and its inclusion served to ensure the selection of diverse deoxyribozymes if the five semirationally designed libraries failed.

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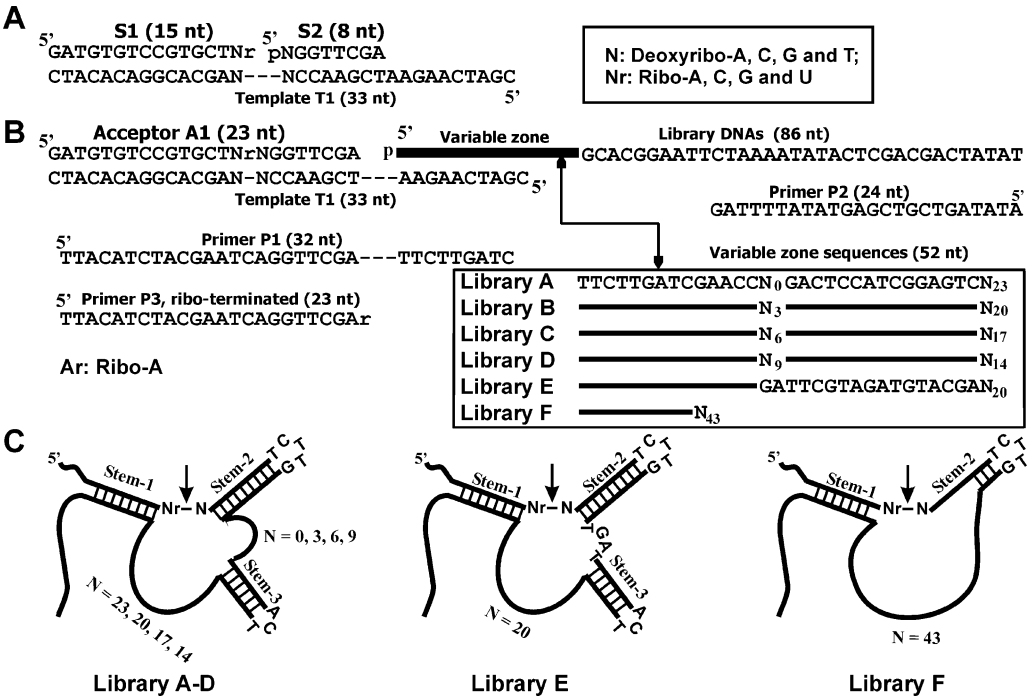


Figure 1. DNA Molecules Used for the Study

(A) The sequences of S1 and S2 for making substrate A1. T1 is used as a template for the DNA ligation reaction.

(B) DNA molecules used for the construction of the six DNA libraries (Libraries A–F) and for PCR amplification. All six libraries had the same length but contained a variable region with sequence variations indicated in the box. A1, 16 ribonucleotide-containing substrates; T1, template for ligating A1 to the libraries; P1-3, primers for PCR. N_x represents the random-sequence domain (X is the number of random nucleotides).

(C) Secondary structures by design.

Selection Scheme, Reaction Time, and Metal Ion Cofactors

Catalytic DNAs were derived using the 8-step in vitro selection scheme shown in Figure 2A. In step I, the mixture of the 16 A1 substrates (each in equal amount) was ligated with the 86-nt DNA pool by T4 DNA ligase in the presence of the template T1. After purification by denaturing PAGE (step II), 109-nt single ribonucleotide-containing DNA molecules were allowed to cleave in the presence of divalent metal ions (step III). The reaction mixture was subjected to PAGE to isolate 94-nt cleavage fragments (step IV), which then were amplified by two consecutive PCR reactions (steps V and VI). The DNA product from the second PCR reaction was digested under alkaline conditions to regenerate single-stranded DNA molecules (step VII), which, after PAGE purification and DNA phosphorylation (step VIII), were used to initiate the next round of selection.

Mg^{2+} and Mn^{2+} were used as potential deoxyribozyme cofactors. Mn^{2+} was chosen for two considerations: (1) in a previous study, we found that Mn^{2+} was more capable than Mg^{2+} in promoting the selection of diverse deoxyribozymes [25], and (2) many existing deoxyribozymes are either highly specific for Mn^{2+} or have a significantly enhanced catalytic activity in the presence of Mn^{2+} [14, 16, 25, 26], suggesting that Mn^{2+} is a useful deoxyribozyme cofactor.

To derive diverse catalytic DNA motifs, we used an incubation time of 4 hr for RNA cleavage in every selection round. Since the catalytic DNAs were to be isolated on the basis of cleaving an attached substrate, the diversity of catalytic DNA sequences to be obtained should be proportional to the length of the incubation time. Deoxyribozymes capable of RNA cleavage in 4 hr were estimated to have a k_{obs} of $\sim 10^{-3}$ min^{-1}, affording a rate enhancement of at least 10^4-fold (the uncatalyzed RNA cleavage rate under our selection conditions was estimated to be $\sim 10^{-7}$ min^{-1} using the empirical formulas described in [27]).

16 Catalytic DNA Pools Derived by Parallel Selection

The deoxyribozyme selection was performed under the following solution conditions: 100 mM KCl, 400 mM NaCl, 50 mM HEPES (pH 7.0) at 23°C, 7.5 mM $MgCl_2$, and 7.5 mM $MnCl_2$. When the selection reached generation 6 (G6), 14% of the attached RNA substrates were cleaved (Figure 2B). In G7, we split the catalytic DNA population into four subpools, each of which was ligated to a group of four substrates containing ArN, CrN, GrN, or UrN sites. Significant activity was detected in all four G7 pools. In round 8, we split each pool further into four sub-subpools (denoted single-substrate pools), each including only one defined substrate. Each single-substrate pool showed significant cleavage in rounds 8, 9, or 10, indicating that we had succeeded in establishing deoxyribozymes for the cleavage of all 16 dinucleotide junctions.

We cloned and sequenced the final 16 single-substrate pools and found numerous deoxyribozymes in every pool. Table 1 lists the number of sequenced

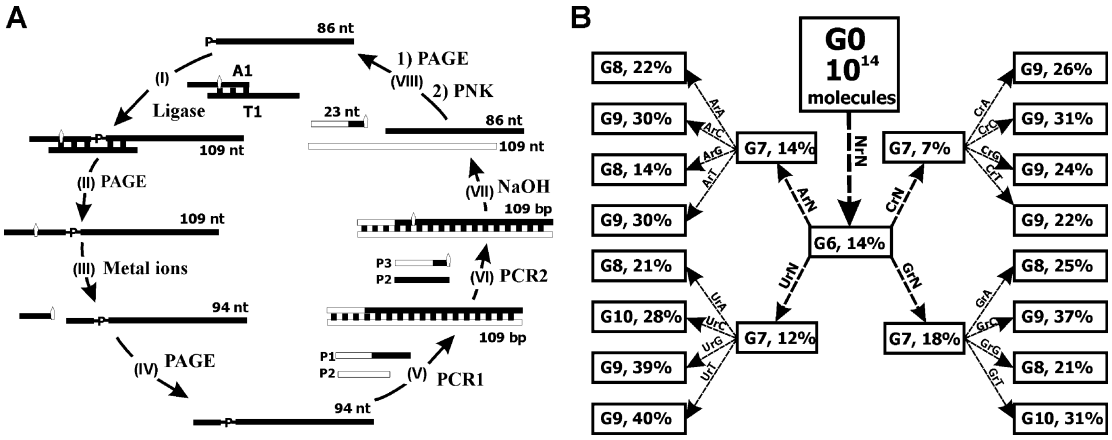


Figure 2. Selection of RNA-Cleaving Catalytic DNAs

(A) Selection scheme. Each selection cycle consists of steps I–VIII. I, 86-nt DNA L1 is ligated to acceptor DNA A1; II, ligated 109-nt DNA is isolated by PAGE; III, purified 109-nt DNA is incubated with divalent metal ions for RNA cleavage; IV, 94-nt cleavage fragment is isolated by PAGE; V, the recovered 94-nt DNA is amplified by PCR using primers P1 and P2; VI, 109-bp PCR product in step V is further amplified by PCR using primers P2 and P3 to introduce a ribonucleotide linkage embedded within DNA; VII, the resulting double-stranded DNAs are treated with NaOH to cleave the ribonucleotide linkage; VIII, the 86-nt cleavage fragments are purified by PAGE, phosphorylated at the 5′ end, and used to initiate the next round.

(B) Selection progress. During the first six rounds of selection (G0 to G6), 16 A1 molecules carrying all 16 dinucleotide junctions (i.e., NrN) were used. G7 DNA was split into four pools for four parallel selections, each of which used four A1 molecules carrying ArN, CrN, GrN, and UrN sites, respectively. The four DNA pools derived from relevant G7 selections were split again into 16 pools where a single A1 was attached as the substrate. The percentage of RNA cleavage is indicated for the listed selection rounds. The reaction time for RNA cleavage was 4 hr.

clones, the number of unique sequences observed, and the "sequence diversity index" (an arbitrary parameter defined as the ratio between the number of unique sequences and the number of sequenced clones). In total, 283 clones were analyzed and 240 unique sequences were observed. Interestingly, none of the sequences resembled any of the five libraries with built-in secondary structures (Libraries A–E), suggesting that these libraries contained far fewer catalytic sequences than Library F, which was built with more random nucleotides.

8-17 Motifs Present in Most of Selected Pools

We first determined whether the small 8-17 motif emerged from our selection, particularly in the four NrG pools, since 8-17 was identified in several independent in vitro selection experiments [10, 11, 13, 28, 29] and was able to cleave any NrG junction under a proposed secondary structure setting [11]. Not unexpectedly, we observed extremely high frequencies of 8-17-containing sequences (sequences containing either original 8-17 motif or 8-17-like motifs, which will be collectively denoted 8-17 motif hereafter in this report) in the four NrG

Table 1. Sequencing Information

Selection Pool	Total Clone Sequenced	Unique Sequence Identified	Sequence Diversity Index	8-17-Containing Sequences	Percentage of 8-17
ArG	19	18	0.95	16	89
CrG	19	18	0.95	12	67
GrG	16	16	1.0	16	100
UrG	19	19	1.0	17	89
ArA	19	17	0.89	16	94
CrA	19	18	0.95	16	89
GrA	18	14	0.78	14	100
UrA	18	12	0.67	10	83
ArC	14	12	0.86	9	75
CrC	21	13	0.62	11	85
GrC	17	14	0.82	12	86
UrC	15	13	0.87	2	15
ArT	16	15	0.94	1	7
CrT	19	11	0.58	0	0
GrT	18	16	0.89	5	31
UrT	16	14	0.88	2	14
Total	283	240	0.85	159	66

Column 1 lists all the dinucleotide junctions; columns 2–4 indicate the number of clones sequenced, the number of unique sequences found, and the sequence diversity index (column 3/column 2). Column 5 lists the number of sequences that may contain 8-17 motifs, and the last column is the percentage of 8-17-containing sequences.

pools (see Figure S1 in the Supplemental Data available with this article online; non-8-17-containing sequences are given in Figure S2).

To our great surprise, many 8-17 motifs were also found in 11 out of the 12 remaining pools (Figure S1). 8-17-containing sequences were observed at a very high frequency (75%–100%) in all four NrA pools as well as ArC, CrC, and GrC pools. 8-17 motifs were also observed in UrC, ArT, GrT, and UrT pools, although at a much lower frequency (7%–31%). The only pool where the 8-17 motif was not observed was the CrT pool. Altogether, 159 sequences contain the core of the 8-17 motif, accounting for 66% of all the catalytic sequences identified in the 16 pools. Although the 8-17 motif was discovered in several previous studies [10, 11, 13, 28, 29], observation of a catalytic DNA motif at such high frequencies in so many catalytic DNA pools is truly unprecedented.

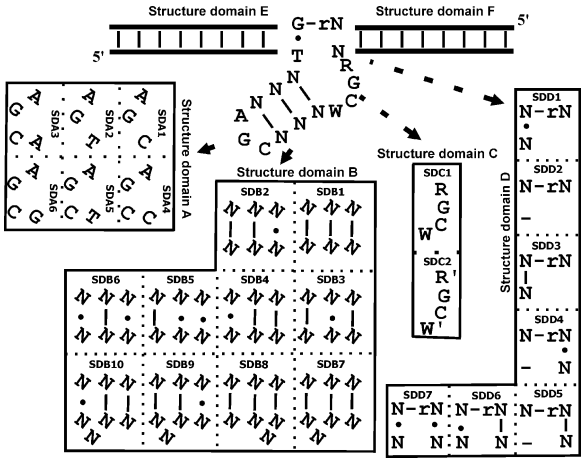


Figure 3. Structural Categorization

The proposed secondary structure for the original 8-17 deoxyribozyme is dissected into six secondary structure domains (denoted SDA to SDF), and individual boxes list the observed variations in SDA-SDD. Structural domain A (SDA) is a trinucleotide loop (triloop), SDB is a 3-bp stem, SDC is the single-stranded region opposite the cleavage site, SDD consists of three nucleotides, two on the substrate strand right at the cleavage junction (i.e., NrG) and one on the catalytic strand, and SDE and SDF are two substrate binding arms.

Categorizing Structural Variations of 8-17 Deoxyribozyme

Although it was to our advantage to study the remaining 87 sequences that did not appear to contain 8-17 nor other RNA-cleaving motifs found in previous studies (see Figure S2), we decided that further investigation was needed of the secondary structures of the 8-17 motif permutations we found. We wanted to confirm their abilities toward cleaving all 16 different dinucleotide junctions for three reasons. First, many of the new 8-17 motifs were considerably different from the original 8-17 deoxyribozymes because they contained previously undocumented mutations (see below). Since 8-17 is an extremely small DNA enzyme, such a high level of mutation raised concern as to whether each suspected 8-17 motif was indeed responsible for the observed cleavage activity. Second, 8-17-containing sequences were observed in nearly all 16 final pools, suggesting that 8-17 may have the ability to cleave a much broader range of RNA dinucleotide junctions than previously observed. Characterizing the relationship between the structural variations of 8-17 and its dinucleotide-cleaving ability would likely uncover important information for the understanding of this incredibly small yet catalytically efficient deoxyribozyme. Third, since suspected 8-17 motifs appeared in almost all final pools, any attempt to derive new RNA-cleaving DNA motifs by in vitro evolution may only lead to the reselection of efficient 8-17 variants. Therefore, an understanding of the dinucleotide junction susceptibility to 8-17 could facilitate our ultimate goal of deriving diverse RNA-cleaving catalytic DNA motifs.

We observed a large number of point mutations at various locations in the secondary structure originally proposed by Santoro and Joyce [11] (Figure 3). In order to characterize the structural variations, we arranged the secondary structure into six structural domains, as illustrated in Figure 3.

Mutations were observed in all six structural domains. Structural domain A (SDA) was originally reported to be an invariable AGC triloop [11, 13], but five variations were observed herein, and only A and G in the original triloop were absolutely conserved. SDB was reported to be a stem of three Watson-Crick base pairs, two of which had to be G-C pairs [11, 13]. However, we observed not only stems containing one or no G-C pairs but also less perfect stems with one mismatch pair (SDB2-4 and SDB9-10), two mismatch pairs (SDB5-6), and even a single-nucleotide bulge (SDB7-10). SDC, the single-stranded region opposite the cleavage site, was known to have the sequence WCGR (W = A or T, R = A or G or AA) [11, 13]; our sequence data confirmed the invariability of C and G but suggested more variations in W and R (denoted W′ and R′ herein).

In previous studies where 8-17 demonstrated an ability to cleave any NrG site, SDD must contain a G•T wobble and an unpaired nucleotide (the 5′ nucleotide of the cleavage site) [11, 13]. We observed six more variations, including two totally unpaired nucleotides at the cleavage site (SDD2) and several other Watson-Crick or wobble pairing patterns (SDD3-7). As for SDE and SDF, many non-Watson-Crick nucleotides were observed (Figure S1).

We then grouped all the observed options for structural domains A–D according to each cleavage site (Table 2). Two points merit special attention. (1) There are several structural domain options observed for most of the dinucleotide sites. For example, for the ArA site there are four options in SDA, three options in SDB, and two options in both SDC and SDD. (2) With the exception of GrT, it appeared that there were very limited structural domain options for NrT sites.

Synthetic DNAs Confirm Dinucleotide-Cleaving Versatility of 8-17 Deoxyribozyme

The above sequence analysis revealed a large array of new 8-17 motifs with a high degree of mutation at every position within the proposed 8-17 catalytic core except

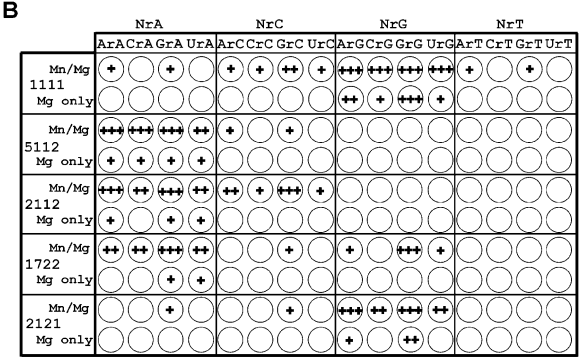
A

Name of synthetic DNAs	Corresponding structural domain E	D	B	A	B	C	F
E1111	GAATCGAACC	T	GTC	AGC	GAC	ACGAA	AGCACGGA
E5112	GAATCGAACC	-	GTC	AGCT	GAC	TCGAA	AGCACGGA
E2112	GAATCGAACT	-	GTC	AGT	GAC	TCGAA	AGCACGGA
E1722	GAATCGAACT	-	GCC	AGC	GCGC	GCGA	AGCACGGA
E2121	GAATCGAACC	T	CCC	AGT	GGG	GCGAG	GGCACGGA

Substrate sequence (written from 3' to 5')

CTTAGCTTGG-N-Nr----------TCGTGCCT

B



C

	ArA	CrA	GrA	UrA	ArC	CrC	GrC	UrC	ArG	CrG	GrG	UrG	ArT	CrT	GrT	UrT
1111 Mn/Mg	0.0003		0.0008		0.0005	<0.0001	0.0013	<0.0001	0.56	0.12	0.78	0.099	<0.0001		0.0004	
5112 Mn/Mg	0.14	0.044	0.12	0.012	0.0005		0.0006									
2112 Mn/Mg	0.057	0.016	0.026	0.007	0.021	0.0024	0.033	0.0010								
1722 Mn/Mg	0.0052	0.0044	0.015	0.0042			0.0001		0.0004		0.014	0.0001				
2121 Mn/Mg			0.0001				0.0002		0.039	0.0084	0.19	0.075				

Figure 4. Catalytic Activity of New 8-17 Motifs

(A) Sequences of five synthesized 8-17 variants. Each deoxyribozyme is named with four numerals, each corresponding to a specific option in structural domain A–D.

(B) Semiquantitative abilities of the DNA oligonucleotides toward cleaving all 16 dinucleotide junctions. Cleavage was allowed to proceed for 10 min, 60 min, or 240 min, and the relative activity of each deoxyribozyme toward each substrate (each circle) is indicated by the number of plus signs in each circle. +++, 10% cleavage or above was observed in 10 min reaction; ++, 10% cleavage or above was observed only in 60 min reaction but not in 10 min incubation; +, more than 3% cleavage was observed in 240 min reaction. Each reaction was carried out under two metal-ion conditions, denoted "Mn/Mg" (7.5 mM $MnCl_2$ and 7.5 mM $MgCl_2$) and "Mg only" (7.5 mM $MgCl_2$).

(C) Catalytic rate constants of the five deoxyribozymes. The k_{cat} values are expressed in min^{-1}. Each experiment was performed in duplicate (data variation was within 20%). The average values are listed.

for four nucleotides in the two single-stranded regions: A and G in structural domain A as well as C and G in structural domain D (Figure 3). However, since 8-17 is a small deoxyribozyme and has a catalytic core of less than 15 nucleotides, the probability of arbitrarily arranging a 15-nt DNA segment into 8-17-like secondary structures can be very high. It is possible that many of the 8-17 structural variations depicted in Figure 3 may not have any functional significance, while other sequence elements may be responsible for the RNA cleavage function.

We synthesized five short DNA oligonucleotides (Figure 4A) to test whether these new 8-17 motifs were, indeed, the catalytic element. The existence of 2–10 options in each structural domain made it impractical to test all combinations of the available structural domains listed in Figure 3. The five synthetic DNAs were designed to represent some of the combinations of most frequently occurring domains according to Table 2. For simplicity, each synthetic DNA was given a name beginning with "E" (stands for "enzyme") followed by four numerals indicative of a specific combination of four chosen variations in the order of SDA, SDB, SDC, and SDD. For example, E2121 is an 8-17 deoxyribozyme with the second option in both SDA and SDC and the first option in both SDB and SDD.

We used a simple assay as shown in Figure 4B to obtain semiquantitative information about the dinucleotide susceptibility to each of the five deoxyribozymes. This simple assay was used because 16 different substrates and 5 different deoxyribozymes were involved. We first tested each DNA's ability toward cleaving each substrate in *trans* under the conditions used for in vitro selection (represented by "Mn/Mg" in Figure 4B). Three independent cleavage reactions were performed for each deoxyribozyme-substrate pair (deoxyribozyme/substrate = 50/1) with reaction times set at 10 min (+++, more than 10% cleavage in 10 min; k_{obs} of $\sim 10^{-2}$ min^{-1}, representing catalysis with high efficiency), at 60 min (++, more than 10% cleavage in 60 min; k_{obs} of $\sim 10^{-3}$ min^{-1}, medium efficiency), and at 240 min (+, more than 3% cleavage in 240 min; k_{obs} of $\sim 10^{-4}$ min^{-1}, low efficiency). These cleavage activities correspond to a rate enhancement of approximately 10^5-, 10^4-, 10^3-fold. Blank circles in Figure 4B indicate that no cleavage was observed at all three incubation times. Figure S3 lists the actual percentage of substrate cleavage from the most active reaction for each DNAzyme-substrate pair. (For example, if more than 10% cleavage was observed in the 10-min reaction, the data from both 60 min and 240 min reactions were not listed.)

The first synthetic DNA, E1111, has a sequence that is almost identical to that of the original 8-17 deoxyribozyme except that different substrate binding arms are used. As expected, E1111 exhibited strong activity with all four NrG sites (Figure 4B). It also registered low but detectable activities (+, 3%–33% cleavage in 240 min reaction; Figure S3) toward the substrates containing GrC, GrA, ArC, GrT, ArA, CrC, ArT, and UrC (arranged in the order of descending activity), but failed to cleave CrT- and UrT-containing substrates. These data not only support the previous finding that 8-17 can efficiently cleave any NrG site [13], but also indicate that the original 8-17 can cleave many other NrN sites with low efficiencies.

The second deoxyribozyme, E5112, exhibited very different cleavage-site selectivity. It had strong activity for ArA, CrA, and GrA, medium activity for UrA, and weak activity for ArC and GrC, but failed to register detectable activity for the remaining ten dinucleotide sites. Similar to E5112, the third deoxyribozyme, E2112, had medium-

Table 2. Observed Structural Variations from Each Dinucleotide Junction Selection

	Observed Structure Domain Options					
Cleave Site	SDA	SDB	SDC	SDD	W or W′	R or R′
ArA	1, 2, 3, 5	1, 2, 7	1, 2	2, 5	- A, C, G, T	-, A, AA, AG, AT
ArC	1, 4, 5	2, 6	1, 2	2, 3	-, A, T	A, AA, AG, TA
ArG	1, 2, 5, 6	1, 4, 7	1, 2	1, 2, 6	A, G, T, GT, TA	-, A, T, AA, AG, TA, TG
ArT	1	7	2	2	G	A
CrA	1, 2, 4, 5, 6	1, 3, 7	1, 2	2, 5	A, C, G, T, CG	-, A, AA, AC, AT, GA, TT
CrC	1, 3, 4, 5	2, 3, 5, 6	1, 2	2	-, A, C, G	AA, AC, CA
CrG	1, 2, 5	1, 7	1, 2	1, 2, 6	A, G, T	A, G, AA, CT, TA, TC, TT
CrT						
GrA	1, 2, 4, 5	1, 2, 7, 8	1, 2	2, 4	A, C, G, T	-, A, AA, AG
GrC	1, 2, 4, 5	1, 2, 6, 7	1, 2	2, 4	-, A, C, T	-, A, AA, AG
GrG	1, 2, 4, 5	1, 2, 3, 7, 10	1, 2	1, 2, 4, 7	-, A, G, T, CA	A, G, T, AA, AG, TG
GrT	1, 3, 5	1, 5, 7	1, 2	2, 4	A, AA	A, T
UrA	1, 2, 4, 5	1, 7, 9	1, 2	2, 4	A, C, G, T	-, A, T, AC, AT
UrC	2, 5	1, 3	2	2	C, G	A, AC
UrG	1, 2, 4, 5	1, 7	1, 2	1, 2, 6, 7	A, G, T	A, T, AT, TC, TT
UrT	2	1	2	4	G, AG	A, T

Each number in columns 2–5 indicates the corresponding variation of each structural domain listed in Figure 3. The last two columns list the observed nonconservative nucleotides in structure domain C. A dash indicates a nucleotide deletion.

to-strong activities for the four NrA sites and no detectable activity for NrG and NrT sites. However, this 8-17 variant had considerable ability to cleave the four NrC sites (high activity for GrC, medium activity for ArC, and low but detectable activity for CrC and UrC).

While E1111 and E5112 appeared to render efficient cleavage toward two mutually exclusive groups of dinucleotide sites (NrG by E1111 and NrA by E5112), the fourth synthetic DNA, E1722, was more degenerate toward the two groups. It had medium-to-strong activities for all four NrA sites and one of the NrG sites, GrG. It also showed a low activity for ArG, GrC, and UrG, but did not register detectable activity for the eight remaining sites. The fifth DNA oligonucleotide, E2121, behaved comparably to E1111, as it was able to cleave all four NrG sites with activities ranging from medium (for CrG and UrG) to strong (for ArG and GrG). It also showed very low activity for GrA and GrC, but failed to promote the cleavage of any of the ten remaining sites.

The above results demonstrate that many (if not all) of the suspected 8-17 motifs identified in this study are indeed responsible for the RNA cleavage activity. Since four of the five synthetic DNA oligonucleotides carry mutations that were not documented in previous studies, and all synthetic variants exhibited a strong cleavage activity toward at least two dinucleotide junctions, we can conclude that 8-17, despite its small size, is capable of cleaving a broad range of RNA dinucleotide junctions.

Each of the five synthetic deoxyribozymes, which carried a specific combination of structural domains as depicted in Figure 3, appeared to have the best ability to cleave the cluster of dinucleotide junctions with G or A as the 3′ nucleotide of the cleavage site. The four NrC sites were much less susceptible to 8-17, while the four NrT sites were almost inert to 8-17. Altogether, the five synthetic DNA enzymes demonstrated strong activity for 8 of the 16 dinucleotide junctions (all NrGs as well as ArA, CrA, GrA, and GrC), medium activity for two junctions (UrA and ArC), and low activity for four junctions (CrC, GrT, UrC, and ArT, in activity-descending order; see Figure S3). None of the synthetic DNAs showed a detectable activity toward the two remaining sites (CrT and UrT). It remains to be demonstrated whether 8-17 variants with other structural domain combinations could cleave the two remaining sites or have enhanced activities for the four less reactive cleavage sites.

The results obtained through the use of a small set of synthetic DNAs with a limited number of structural domain combinations are not sufficient to make conclusions on the variability of structural domains of 8-17 and its dinucleotide junction selectivity. However, we have observed that when the deoxyribozyme has a single T in SDD, it has robust activity toward all four NrG junctions but shows no activity, or significantly reduced activity, toward all other sites, consistent with previous observations [11, 13]; when the thymine residue is absent, the deoxyribozyme becomes active toward NrA and NrC sites but shows no activity, or reduced activity, toward NrG junctions. A full comprehension of the relationship between the structural variability and the cleavage site selectivity requires a systematic examination of the structural domain variations and is beyond the scope of the current study.

Since Mn^{2+} and Mg^{2+} were included in the selection buffer, and Mg^{2+} is a physiologically relevant metal ion, we sought to determine whether Mg^{2+} alone could support the cleavage activity of these deoxyribozymes. Therefore, we performed similar experiments using Mg^{2+} as the only divalent metal ion cofactor (Figure 4B, "Mg only"). Our data indicate that Mg^{2+} is a much less effective cofactor for the new 8-17 variants, as its use resulted in both a significantly reduced enzymatic activity and (perhaps as a consequence) a much narrower range of dinucleotide selectivity. Once again, our data suggest that Mn^{2+} can act as an effective metal ion cofactor for deoxyribozymes [16].

Rate Constants of New 8-17 Variants

Subsequently, we determined rate constants of the five deoxyribozymes in cleaving the NrN junctions identified

through the simple assay given in Figure 4B. This information should provide a more quantitative description on the catalytic proficiencies of these DNA enzymes. The rate constants were calculated from time-course studies of each deoxyribozyme-substrate pair under single-turnover conditions (deoxyribozyme/substrate = 500/1). The rate constants parallel the semiquantitative data given in Figure 4B, suggesting that our simplified assay is fairly accurate in gauging the relative activities of the five tested deoxyribozymes. It is noteworthy that E1111 cleaves its best substrate group (i.e., NrG) roughly one order of magnitude more efficiently than the other four variants cleave their most favorable substrate groups.

Discussion

Recurrence of 8-17

8-17 is one of the smallest nucleic acid enzymes ever known. It has been repeatedly identified from three independent in vitro selection experiments prior to our study [10, 11, 13, 29]. It surfaced again as the catalytic motif embedded in a huge number of catalytic DNA sequences isolated in the current study. It was speculated recently that several factors, including its small size, unique structural feature, and common selection strategy (i.e., all 8-17 variants were selected using the column-based strategy, which involves the immobilization of DNA library onto a solid support and the release of potential catalysts by elution with reaction buffers containing designated metal ion cofactors [10, 11, 13]), may be responsible for the repeated isolation of the 8-17 deoxyribozyme [30]. The factors that generally influence the recurrence of nucleic acid enzymes are well discussed by Lehman [31]. Since our study did not use the column-based selection strategy, we could rule out the selection method factor. We speculate that the most responsible factors might have been 8-17's small size, its sequence variability, and its catalytic fitness. Because of the extremely small size (a catalytic core of under 15 nt) and great sequence variability (only four absolutely conserved nucleotides), the 8-17 catalytic motif should occur at an extremely high frequency in any given DNA library. This high rate of occurrence in an initial pool gives 8-17 an unparalleled opportunity to outnumber other potential catalytic motifs that have a larger size and less tolerant sequence content during the entire process of in vitro selection. 8-17's catalytic fitness—including its large catalytic rate, its capability to function under various metal ion conditions, and its ability to cleave multiple dinucleotide junctions—makes it easy to survive the usual selection pressure imposed in most in vitro selection experiments (such as short incubation times or reduced metal ion concentrations). The diverse sequence variations seen with the new 8-17 motifs in this particular study were likely a result of three particular strategies employed in our efforts: the relatively long reaction time of 4 hr throughout all selection rounds, the use of a pool of 16 substrates containing all 16 possible dinucleotide junctions, and the parallel selection approach adopted after the establishment of a catalytic DNA population by the single selection approach. The long incubation time did not impose stringent selection pressure and permitted the selection of both fast deoxyribozymes and DNA catalysts with less optimal activities. The supply of 16 substrates to the original DNA pool may have allowed more deoxyribozymes an opportunity to emerge from the pool. The 16 parallel selection strategy employed by us toward the end of our selection effort favored the selection of deoxyribozymes with a distinct substrate preference that may not have been highly competitive if the single-stream selection was run from the beginning to the end.

Recurrence of other nucleic acid enzymes from in vitro selection has also been observed. For example, the hammerhead ribozyme not only has multiple natural origins [32–35], it has also been discovered three times by in vitro selection [36–38]. Similarly, common mutations that are crucial to enzymatic activity were observed in the class I ligase ribozyme variants derived from 13 independent evolution lineages [31]. These observations seem to suggest that recurrence of deoxyribozymes or ribozymes from in vitro selection may be a common phenomenon.

It is noteworthy that our study did not lead to the reisolation of 10-23, the other RNA-cleaving DNA enzyme found by Santoro and Joyce in the same study where 8-17 was discovered [11]. In the other two studies where 8-17 was reselected [10, 13], 10-23 was not reported either. The lack of recurrence of 10-23 is particularly puzzling considering that 10-23 is an extremely efficient deoxyribozyme and is about the same size as 8-17. One noticeable difference is that Santoro and Joyce used an all-RNA substrate for their selection [11], while the current study as well as the other two efforts used a single ribonucleotide-containing DNA substrate [10, 13]. Therefore, one possible explanation could be that 10-23 may have a particular penchant for an all-RNA substrate, while 8-17 has an equal ability to process both an all-RNA substrate and a DNA/RNA chimeric substrate.

Dinucleotide Junction Cleavage Versatility of 8-17

We were quite surprised to observe that 8-17 could cleave nearly all 16 types of dinucleotide junctions of RNA with rate enhancements ranging from approximately a thousand- to a million-fold. From a limited survey of 8-17 sequence variants using a synthetic DNA approach, we have already discovered that 8-17 variants can efficiently cleave more than half of all 16 dinucleotide junctions (k_{obs} of 0.01 min^{-1} or above). It is quite possible that 8-17 can efficiently cleave even more dinucleotide junctions when more variants are examined.

We were equally amazed by the observation that this small DNA enzyme can tolerate a very high degree of mutation within the catalytic core. The observed mutations are of three forms: point mutations, insertions, and deletions. The acceptance of so many forms of mutations may have worked as an added advantage, allowing 8-17 to compete successfully with other catalytic motifs during the selection process when different dinucleotide junctions were presented as the cleavage sites. A particular form of mutation may have been beneficial in providing a way to fine tune the enzyme structure so as to cleave a specific dinucleotide site (or a related group of dinucleotide sites).

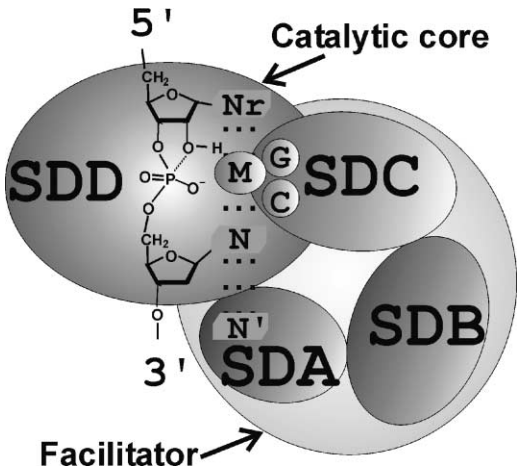


Figure 5. A Highly Hypothetical Structural Model for 8-17

The dashed lines indicate a proposed stacking interaction between a hypothetical triad Nr-N-N′. M represents a divalent metal ion.

A Catalytic Core and a Facilitator in the Tertiary Structure of 8-17?

Based on the preceding observations, we hypothesize that the catalytic element of the 8-17 motif (which does not include the two substrate binding arms) may have a tertiary structure composed of two interlinked structural domains, a catalytic core and a "facilitator" (Figure 5). The catalytic core provides the catalytic residue (a metal ion or a base with an altered pKa, as the base to deprotonate the 2′-OH group, for example) and a network of interactions to position the 2′-hydroxyl for the in-line attack on the nearby phosphate. The role of the facilitator is to provide an adjustable structural arrangement to maintain the integrity of the catalytic core. The catalytic core may consist of the dinucleotides at the cleavage site (Nr and N), the conserved C and G in SDC, and possibly a divalent metal ion (M) [39], while the facilitator is made of the remaining nucleotides in SDC and all nucleotides in SDA and SDB. Since A and G in SDA are also absolutely conserved, it is possible that these two nucleotides are either an important part of the catalytic core or act as the bridging unit between the catalytic core and the facilitator. We further speculate that a stacking interaction involving the two nucleotides at the cleavage site (Nr and N) and perhaps a purine elsewhere (such as A or G in SDA, designated as N′; the three nucleotides stack in the order of Nr-N-N′) may form the critical part of the interaction network.

Although purely speculative, this hypothesis could explain the key observations from our study. First, the catalytic core-facilitator hypothesis could help to explain why we have observed many mutations throughout the facilitator. That is, these mutations occurred because they are necessary for fine tuning the facilitator structure to support the catalytic core when different dinucleotide junctions are presented as the cleavage site. Second, our hypothesis on the existence of a stacking triad could help explain the two dinucleotide-susceptibility patterns: (1) NrG and NrA (N = G or A) are the most susceptible to 8-17, followed by NrC group, while NrT group is the least susceptible, and (2) within each dinucleotide-site group (such as NrG), ArN and GrN are always more reactive than CrN and UrN. Since purines tend to stack better than pyrimidines, the Nr-N-N′ triad with Nr and/or N being G or A produces a stronger stacking interaction than the triad where Nr and/or N is C or T. U (an analog of T) is known to produce negligible stacking [40]; this may explain why the NrT group cannot be efficiently cleaved by 8-17 since T occupies the central position of the Nr-N-N′ triad.

Implications of Discovery of New 8-17 Variants

The discovery of broad structural variability of the 8-17 deoxyribozyme and its ability to cleave wide-ranging dinucleotide junctions could have a few implications. First, these mutant deoxyribozymes could be useful for understanding the structural and mechanistic properties of this small catalytic DNA, particularly for structural studies by NMR and X-ray crystallography. Although many deoxyribozymes have been generated in the past ten years, there has been limited progress in tertiary structure determination of these single-stranded species [41]. The isolation of a large number of active mutants of the 8-17 deoxyribozyme may provide an enhanced opportunity for elucidating the tertiary structure of this DNA enzyme. Second, the existence of many efficient 8-17 variants coupled with their ability to cleave a broad range of dinucleotide junctions of RNA could facilitate the generation of a large number of allosteric deoxyribozymes. There has been ever-growing interest in the construction of allosteric nucleic acid enzymes, as they can be utilized as effective probes for many practical applications (such as biosensing) [22–24, 42–50]. The small size of 8-17 and its catalytic prowess along with its wide sequence variability and ability to cleave multiple dinucleotide sites should make this deoxyribozyme a highly useful catalyst for allosteric deoxyribozyme engineering and applications. Third, the understanding of the dinucleotide susceptibility of 8-17 can provide useful information in guiding the search for new RNA-cleaving motifs. RNA-cleaving DNA enzymes are highly desirable molecular tools. However, the search for new RNA-cleaving motifs can be impeded by the repeated appearance of 8-17 deoxyribozymes. The observation that certain dinucleotide linkages are not prone to cleavage by 8-17 should significantly facilitate efforts of searching for new RNA-cleaving motifs.

The original goal of this study was to derive diverse deoxyribozymes that together could cleave all 16 dinucleotide junctions of RNA. The observation of abundant 8-17 motifs in nearly all of our 16 selected pools and the characterization of new 8-17 variants have delayed our original plan. However, we obtained a large number of catalytic sequences that do not appear to contain 8-17 motifs, and these DNA molecules could form the basis for deriving new catalytic motifs for the cleavage of an even broader range of dinucleotide junctions.

Significance

RNA-cleaving deoxyribozymes are particularly desirable as they have great potential to be used both in vivo to digest RNA molecules of biological importance

and in vitro as biosensing tools. In this study, we adopted a new in vitro selection approach aimed at generating new catalytic DNAs for collectively cleaving all 16 possible dinucleotide junctions of RNA. The three key features of our approach were: (1) the use of an initial DNA pool combined from six different synthetic DNA libraries; (2) the use of 16 DNA/RNA chimeric substrates each containing a single ribonucleotide as the cleavage site and differing at the dinucleotide junction to be cleaved; and (3) a single stream of selection with the use of combined substrates to establish a catalytic DNA population, followed by four parallel selections each employing a group of four substrates, followed by 16 parallel selections each with a defined substrate. This effort eventually led to the isolation of a large number of DNA catalysts that are collectively capable of cleaving all possible RNA dinucleotide junctions. Surprisingly, most of the selected DNA pools were dominated by variants of the 8-17 deoxyribozyme, a small but efficient RNA-cleaving catalytic DNA motif previously discovered three times. We found that only four nucleotides with the ~15-nt catalytic core were absolutely conserved, suggesting that these nucleotides play crucial catalytic and/or structural roles for 8-17. Through the use of five synthetic deoxyribozymes, we revealed that 8-17 has the ability to cleave 14 out of 16 possible dinucleotide junctions in the presence of Mg^{2+} and Mn^{2+} as divalent metal ion cofactors. Our study indicates that 8-17, despite its miniature size, has a remarkable ability to accommodate nucleotide mutations within its catalytic core and to fine tune its structure when different dinucleotide junctions are presented as cleavage sites.

Experimental Procedures

Materials and Common Procedures

Standard oligonucleotides were prepared by automated DNA synthesis using cyanoethylphosphoramidite chemistry (Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University). Random-sequence DNA libraries were synthesized using an equimolar mixture of the four standard phosphoramidites. DNA oligonucleotides were purified by 10% preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE), and their concentrations were determined by spectroscopic methods.

The TOM protective group on the 2′-hydroxyl group of the RNA linkage was removed by incubation with 150 μl of 1M tetrabutylammonium fluoride (TBAF) in THF with shaking at 60°C for 20 hr, followed by the addition of 250 μl of 100 mM Tris (pH 8.3) and further incubation with shaking for 30 min at 37°C. The DNA was recovered using ethanol precipitation, dissolved in water containing 0.01% SDS, and the tetrabutylammonium salt was removed by centrifugation using a spin column (Nanosep 3K Omega, Pall Corp., Ann Arbor, Michigan).

Nucleoside 5′-triphosphates, [γ-^{32}P]ATP, and [α-^{32}P]dGTP were purchased from Amersham Pharmacia. *Taq* DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase (PNK) were purchased from MBI Fermentas. All chemical reagents were purchased from Sigma.

In Vitro Selection Procedures

3000 pmol of 86-nt libraries A–F (500 pmol each; all DNA sequences are given in Figure 1) was used for the first selection round (G0). The DNA library was mixed in an equimolar ratio with template T1 and acceptor A1 (the 16 different substrates were used at equimolar concentrations; all sequences are shown in Figure 1B), heated to 90°C for 30 s, cooled to room temperature, and combined with a 10× ligase buffer and T4 DNA ligase. The ligation mixture contained 50 mM Tris-HCl (pH 7.8 at 23°C), 40 mM NaCl, 10 mM $MgCl_2$, 1 mg/ml BSA, 0.5 mM ATP, and 0.1 U (Weiss) μl^{-1} T4 DNA ligase. The solution was incubated at 23°C for 1 hr, and the ligated 109-nt DNA was purified by 10% denaturing PAGE. The ligated DNA molecules were incubated at room temperature in the selection buffer (100 mM KCl, 400 mM NaCl, 50 mM HEPES [pH 7.0] at 23°C, 7.5 mM $MgCl_2$, 7.5 mM $MnCl_2$) for 4 hr. Because these selections target all 16 RNA dinucleotide junctions, we refer to these as NrN selections. The reaction was quenched with EDTA (1.5× molar concentration of divalent metals). The cleaved products (94 nt) were separated from the uncleaved precursor (109 nt) by denaturing PAGE. For the first round of selection, 1 pmol of a 94-nt synthetic DNA was added into the reaction mixture to assist the identification of cleaved DNA band and to increase the recovery yield of potential DNA catalysts (the doped molecules were made of the same 62-nt library but had a different sequence at 3′ end, and therefore they could not be amplified during PCR). The 94-nt cleaved products were amplified by two polymerase chain reactions (PCR). The first PCR used the primer set P1 and P2, while the second PCR used P2 and P3; their relationships are shown in Figure 1B. The reaction mixture also included 30 μCi of [α-^{32}P]dGTP for DNA labeling. Since P3 is a ribo-terminated primer, treatment of the second PCR products with NaOH following a protocol described previously [25] cleaved the embedded ribonucleotide and released the catalytic 86-nt fragment, which was purified by PAGE. The recovered DNA molecules were incubated with 10 units of PNK at 37°C for 1 hr for DNA phosphorylation in a 100 μl reaction mixture containing 50 mM Tris-HCl (pH 7.8 at 23°C), 40 mM NaCl, 10 mM $MgCl_2$, 1 mg/ml BSA, and 0.5 mM ATP. The 5′-phosphorylated DNA (denoted G1) was used for the second round of selection using the same procedure described for the first round of selection. The entire selection process was performed as diagrammed in Figure 2B. At G7, the pool was split into four subpools, each ligated with a group of four substrates (i.e., the mixed substrates containing ArN, CrN, GrN, or UrN as dinucleotide junctions) at equimolar concentrations. At G8, each subpool was further divided into four more pools, each then presented with a single substrate (i.e., a substrate containing a defined dinucleotide junction such as ArA, ArC, ArG, ArT, etc.).

Cloning and Sequencing of Selected Deoxyribozymes

DNA sequences from the final rounds of selection were amplified by PCR and cloned into a vector by the TA cloning method. The plasmids containing individual catalysts were prepared using a Qiagen MiniPrep Kit. DNA sequencing was performed on an LCQ2000 capillary DNA sequencer (Beckman-Coulter) following the procedures recommended by the manufacturer.

Kinetic Analyses

A typical reaction involved the following steps: (1) heat denaturation of deoxyribozyme-substrate pair in water for 30 s at 90°C, (2) incubation for RNA cleavage at room temperature in a reaction buffer for a designated time, (3) addition of EDTA to 30 mM to stop the reaction, (4) separation of cleavage products by denaturing 10% PAGE, and (5) quantitation using a PhosphoImager (Molecular Dynamics) and ImageQuant software. For deriving the catalytic rate constants, aliquots of an RNA cleavage reaction solution were collected at different reaction time points that were all under 20% completion, and the rate constant for the reaction was determined by plotting the natural logarithm of the fraction of DNA that remained unreacted versus the reaction time. The negative slope of the line produced by a least-squares fit to the data was taken as the rate constant.

Supplemental Data

The following information is available online at http://www.chembiol.com/cgi/content/full/11/1/57/DC1: (1) the sequences of all 8-17 variants identified from the 16 selected pools listed in Figure 2B, (2) the non-8-17 sequences from the same 16 pools, and (3) semiquantitative abilities of the five synthetic 8-17 variants listed in Figure 4 toward cleaving all 16 dinucleotide junctions.

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