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Small, Highly Active DNAs That Hydrolyze DNA

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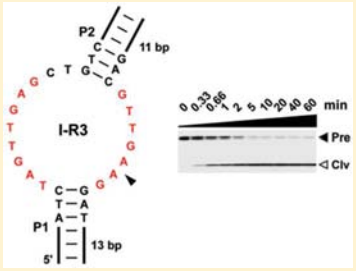
Supporting Information

ABSTRACT: DNA phosphoester bonds are exceedingly resistant to hydrolysis in the absence of chemical or enzymatic catalysts. This property is particularly important for organisms with large genomes, as resistance to hydrolytic degradation permits the long-term storage of genetic information. Here we report the creation and analysis of two classes of engineered deoxyribozymes that selectively and rapidly hydrolyze DNA. Members of class I deoxyribozymes carry a catalytic core composed of only 15 conserved nucleotides and attain an observed rate constant (k_{obs}) of ~1 min^{-1} when incubated near neutral pH in the presence of Zn^{2+}. Natural DNA sequences conforming to the class I consensus sequence and structure were found that undergo hydrolysis under selection conditions (2 mM Zn^{2+}, pH 7), which demonstrates that the inherent structure of certain DNA regions might promote catalytic reactions, leading to genomic instability.



INTRODUCTION

Uncatalyzed hydrolysis of phosphoester bonds is very unfavorable, and it is estimated that the half-life for the spontaneous hydrolysis of DNA phosphodiester bonds is measured in millions of years under physiological conditions.[1−3] Therefore, catalysts that promote DNA hydrolysis must achieve rate enhancements of more than 10^9-fold to be easily detected in the laboratory. Even larger rate enhancements are necessary for catalysts to match the speeds of natural DNase enzymes.

More than a decade ago, this laboratory[4,5] and others[6−9] conducted directed evolution experiments that could have given rise to self-hydrolyzing deoxyribozymes.[10−12] However, these experiments, and other unpublished efforts that were intentionally focused on isolating self-hydrolyzing DNAs, never yielded such molecules. The difficulty of isolating self-hydrolyzing DNAs is further highlighted by the fact that it has been relatively straightforward to create deoxyribozymes that promote DNA cleavage by oxidative[4] or depurination[8] mechanisms. This history suggests that structures required to promote DNA hydrolysis are scarcer in sequence space relative to DNAs that promote other reactions such as RNA cleavage.

Despite the catalytic challenge and the historical difficulty in isolating self-hydrolyzing DNAs, multiple deoxyribozymes that catalyze this reaction using Zn^{2+} and Mn^{2+} as cofactors were isolated recently.[13−15] These self-hydrolyzing deoxyribozymes were an unexpected outcome of a directed evolution experiment intended to yield DNAs that cleave peptide bonds. However, the resulting deoxyribozymes are able to cleave an all-DNA substrate with rate enhancements of at least 10^{12}, corresponding to observed rate constants (k_{obs}) of ~0.05 min^{-1}.

Similarly, Zn^{2+}- and Mn^{2+}-dependent self-hydrolyzing deoxyribozymes were isolated[16] by selecting for the cleavage of DNA sequences displayed in preorganized bulges that are formed when a substrate domain interacts with the deoxyribozyme. For example, deoxyribozymes selected to cleave within a six-nucleotide bulge exhibit rate constants of ~1 min^{-1}. Additional types of self-hydrolyzing deoxyribozymes that use only Zn^{2+} or lanthanide ions were also created,[17,18] and together, these findings prove that numerous highly active deoxyribozymes that catalyze DNA hydrolysis indeed can be isolated. However, the precise sequence at the cleavage site and its structural presentation are important factors for deoxyribozymes to attain these speeds. Also, the reaction conditions and available cofactors are likely to be critical for the successful outcome of directed evolution for this activity.

In the current study, we set out to isolate self-hydrolyzing deoxyribozymes using a selection method that permits DNA cleavage to occur at almost any location within each DNA construct, including the region that is initially randomized. This method is expected to permit the isolation of novel classes of highly active deoxyribozymes because numerous possible substrate sequences and architectures are simultaneously made available in the selection pool. We describe the implementation of this selection method to isolate two novel classes of Zn^{2+}-dependent deoxyribozymes that hydrolyze DNA with rate constants as high as 1 min^{-1}. The identification of small DNA-cleaving deoxyribozymes that function with high speed under mild reaction conditions should permit their use in various biotechnology applications. Furthermore, the discovery

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of these self-cleaving DNAs hints at possible sources of genomic instability.

RESULTS AND DISCUSSION

Method for the Directed Evolution of Self-Hydrolyzing Deoxyribozymes. Various selective amplification methods have been used to isolate many types of ribozymes and deoxyribozymes from random sequence populations.[19,20] Selection methods historically used to isolate deoxyribozymes that cleave RNA[6,7] or DNA[4] require that the self-cleavage event takes place within a short region of known nucleotide sequence located near the 5′ terminus of the selection construct. This was a necessary requirement because replication of the cleaved deoxyribozymes requires that the 3′ cleavage fragment retains the ability to serve as a template for PCR (or a similar DNA amplification method) to copy and regenerate the complete deoxyribozyme. In contrast, any deoxyribozyme that cleaves within the original random-sequence domain of the selection construct is lost to the population because it cannot be replicated.

One of the first selective amplification strategies employed to create novel RNA-cleaving ribozymes made use of a unique method wherein members of a population of circular RNAs become linearized by ribozyme-mediated chain cleavage.[21] These cleavage products were subsequently religated to create intact RNA templates for subsequent amplification. This strategy allows the RNAs to cleave at any position because the ligation step regenerates intact templates suitable for amplification.

We employed a similar strategy involving a circular DNA population by using an ATP-dependent enzyme called CircLigase to recircularize DNA constructs that have undergone DNA hydrolysis (Figure 1). CircLigase requires 5′ phosphate and 3′ hydroxyl groups on its DNA ligation substrates.[22] Therefore, this method permits the isolation of deoxyribozymes that hydrolyze any 3′ phosphoester bond within the circular DNA. Given the chemical restrictions on CircLigase substrates, this method precludes the isolation of DNAs that cleave a 5′ phosphoester bond. DNA cleavage by oxidation or depurination mechanisms, which are known to be promoted by DNA structures,[4,8,23] would cause a loss of a nucleotide. Therefore, the emergence of deoxyribozymes with these mechanisms also is precluded because of this loss of genetic information.

To initiate selective amplification, we generated a starting pool of ~10^{14} linear single-stranded DNAs (Figure 1, stage i) of 145 nucleotides in length that carry a total of 100 random-sequence positions (see the Supporting Information for details on DNA constructs). These DNAs were circularized by CircLigase, and the products were purified by PAGE (stage ii). Pre-existing abasic sites resulting, for example, from depurination during chemical synthesis can undergo chain cleavage by β-elimination,[8,23,24] and will yield a high background signal upon deoxyribozyme selection. Therefore, DNAs with this damage were removed from the population by incubation with piperidine.[8] Intact circular DNA molecules were recovered by PAGE (stage iii) and subsequently incubated under our chosen reaction conditions for *in vitro* selection [selection buffer: 50 mM HEPES (pH 7.05 at 23 °C), 100 mM NaCl, 20 mM $MgCl_2$, and 2 mM $ZnCl_2$]. Mg^{2+} and Zn^{2+} ions, which are relatively abundant in cells, were added to possibly function as cofactors for catalysis.

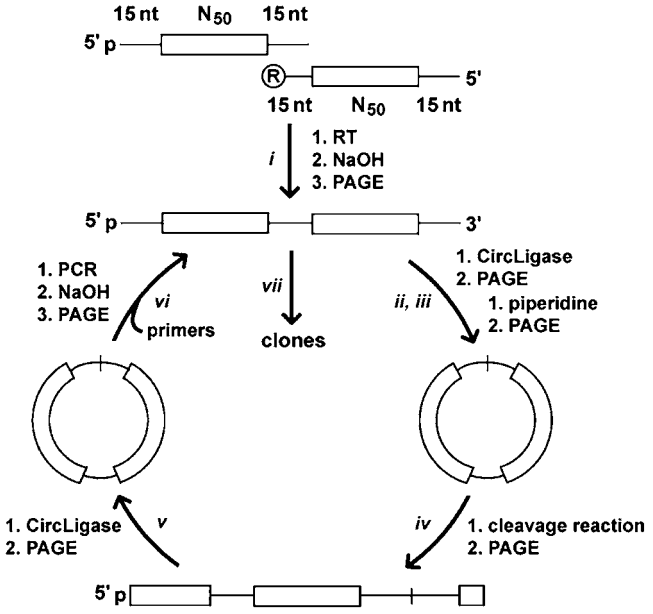

Figure 1. *In vitro* selection scheme for isolating self-hydrolyzing deoxyribozymes. Members of the initial 145 nt single-stranded DNA pool contain two 15 nt primer binding sites (5′ and 3′ termini) and two 50 nt random-sequence domains linked by a 15 nt bridge (middle). N designates random-sequence nucleotides, and R designates a ribonucleotide. Full-length DNA molecules were constructed (i) by reverse transcriptase (RT)-mediated primer extension of two synthetic 80 nt DNA populations, alkaline (NaOH)-mediated cleavage of the ribose-containing strand, and purification of the intact 145 nt single-stranded DNA by denaturing, 8% polyacrylamide gel electrophoresis (PAGE). RT was used for pool preparation due to convenience, and other DNA polymerases could also have been employed. The linear single-stranded DNAs were (ii) ligated to form DNA circles by using CircLigase, and the ligated products were isolated by using PAGE. To reduce background linearization of the pool by spontaneous cleavage of abasic sites, DNAs were (iii) treated with piperidine and the DNAs that remain intact were recovered by PAGE. Circular DNAs were then (iv) incubated in selection buffer. Cleaved DNAs were separated by PAGE and (v) religated with CircLigase. Circular DNAs were isolated by PAGE, (vi) amplified by PCR to generate double-stranded amplification products that were treated and separated as in step i to generate linear products. The DNAs from this stage were either (vii) cloned and sequenced for further analysis or subjected to additional rounds of selective amplification by repeating stages ii–vi. In step iv, only one possible DNA cleavage product is depicted, whereas cleavage could occur at any position.

Hydrolysis at any linkage yields linear DNAs of identical length and identical mobility upon separation by PAGE (stage iv). Linear DNAs isolated from the selection reaction were recircularized by using CircLigase, and the recircularized products were purified again by PAGE (stage v). These DNAs were then amplified by PCR to build the next linear single-stranded DNA pool (stage vi). This selective-amplification cycle was repeated until the pool was enriched for deoxyribozymes that efficiently cleave DNA.

Isolation and Analysis of Self-Hydrolyzing Deoxyribozymes. After 14 rounds of selective amplification, the DNA pool (G14) exhibited 13% linearization after 1 min of incubation (Figure S1, Supporting Information), indicating that some members of the population can undergo rapid self-cleavage. Analysis of 50 clones from the G14 pool revealed the existence of two common classes of deoxyribozymes termed

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class I and class II (Figure 2 and Figures S2 and S3, Supporting Information).

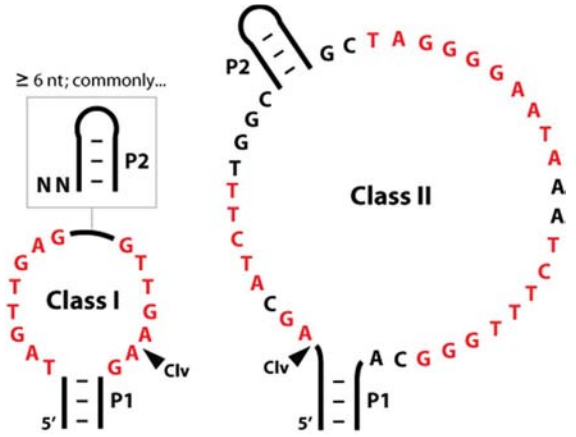

Figure 2. Consensus sequences and secondary structure models for class I and II deoxyribozymes. Red nucleotides identify positions that are present in the most numerous representatives from the original selection and from the reselections using mutagenized parent constructs. Nucleotides in black are present in the parent construct used for reselection, but these positions are not well conserved in the selected population. Arrows designate the site of DNA cleavage (Clv).

Representatives of these two classes carry distinct sequence and structural features, and truncated DNA constructs containing these features undergo site-specific self-cleavage at sites that are characteristic for their corresponding class (Figure 3A and B). For example, three representatives of class I deoxyribozymes share nucleotide sequences within two unpaired DNA segments that are flanked by either one or two base-paired substructures called P1 and P2 (Figure 3A). All three constructs undergo self-cleavage on a minutes time scale, and each representative precisely cleaves the 3′ phosphoester bond between the conserved dinucleotide sequence ApA.

The precise location of each DNA cleavage site was assigned by comparison with synthetic DNA markers separated by using denaturing PAGE. The mobility of each deoxyribozyme 3′ cleavage product matches that of the corresponding synthetic DNA carrying a 5′ phosphate group (Figures S3D and S4, Supporting Information). The chemical composition of the hydrolyzed DNA termini of class I representative 3 (I-R3) was further examined by monoisotopic (exact mass) spectrometry of PAGE-purified reaction products (Figure 4). Again, these results are consistent with hydrolysis of the ApA dinucleotide sequence to yield 3′ hydroxyl and 5′ phosphate termini on the 5′ and 3′ cleavage products, respectively.

Representatives of class I dominate the clones at G14 and are also the most common type of deoxyribozyme observed upon examining more than a million reads from high-throughput sequencing of the G9 population. Comparative sequence analysis revealed the existence of 15 highly conserved nucleotides in the bulge (or loop) region of class I deoxyribozymes (Figure 2). A 5′ ^{32}P-labeled sample of I-R3 cleaves to greater than 90% in 2 min when incubated in the selection buffer at 37 °C (Figure 3A). This corresponds to a k_{obs} value of ~1 min^{-1} or a half-life of ~40 s (Figure 3C and D). Incubation of representatives I-R1, I-R2, and I-R3 in reaction buffers containing different metal ions revealed that the class I deoxyribozymes are Zn^{2+} dependent. Mg^{2+} supports robust

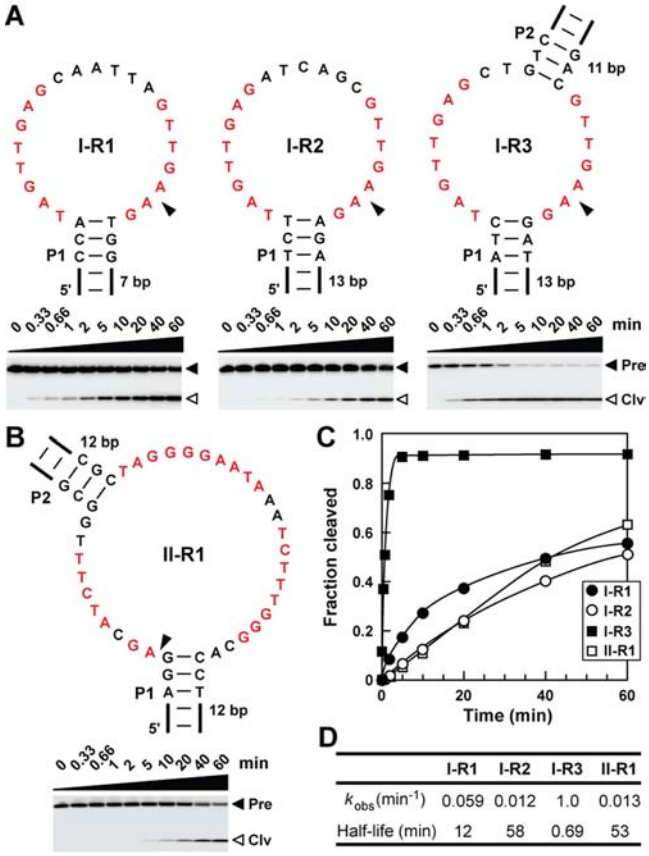

Figure 3. Sequences, deoxyribozyme cleavage assays, and kinetic parameters for representatives of class I and II deoxyribozymes. (A) Top: Representatives of class I self-cleaving deoxyribozymes. Red nucleotides are highly conserved, and arrowheads designate the cleavage site. Complete sequences are provided elsewhere (Figure S2, Supporting Information). Bottom: Trace amounts of DNAs undergoing hydrolysis were 5′ ^{32}P-labeled and incubated at 37 °C in the selection buffer for the times indicated and separated by denaturing 10% PAGE. Pre and Clv identify uncleaved precursor and 5′ cleavage fragments, respectively. (B) Representative of class II self-cleaving deoxyribozymes. Details are as described for part A. (C) Plot of the fraction of DNA cleaved versus the incubation time for class I and II representatives. Data was quantified on the basis of the PAGE gels in parts A and B. (D) The k_{obs} and half-life values for the class I and II constructs depicted in parts A and B.

cleavage activity of I-R1 but can be eliminated without loss in activity of I-R2 and I-R3 when Zn^{2+} is also present (Figure 5A).

Construct I-R3 is most active at Zn^{2+} cofactor concentrations near 1 mM (Figure 5B–E and Figure S5A, Supporting Information), and its activity drops sharply at cofactor concentrations even modestly above or below this optimum (Figure S5A, Supporting Information). I-R3 also has a pH optimum near 7.0, as self-cleavage activity drops dramatically with small variances from neutral pH (Figure S5B and C, Supporting Information). I-R3 exhibits improved activity at higher temperatures (Figure S5D and E, Supporting Information), and the DNA exhibits a k_{obs} value of 1.6 min^{-1} (half-life of 26 s) when incubated at 45 °C.

Similar functional characteristics were found for representatives of the other major deoxyribozyme class identified from the selected pool. A representative of class II deoxyribozymes called II-R1 (Figure S3B, Supporting Information) carries 32 nucleotides within an unpaired bulge that is flanked by two

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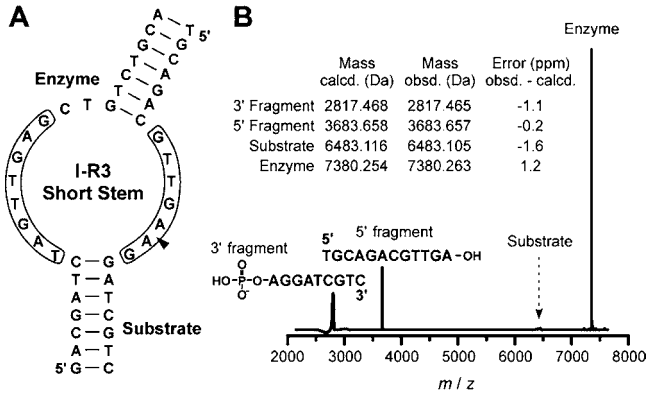

Figure 4. Monoisotopic (exact mass) spectroscopic determination of the cleavage products of deoxyribozyme I-R3. (A) Separate enzyme and substrate strands of a truncated construct used in the reaction, wherein the substrate strand undergoes hydrolysis. (B) Exact mass spectrum of the bimolecular I-R3 reaction mixture with annotation of the proposed hydrolysis product peaks. The calculated (calcd.) and observed (obsd.) mass and errors are presented for each DNA.

base-paired stems. A truncated bimolecular version of this DNA (Figure 3B) undergoes cleavage at a single site with a rate constant that is comparable to some class I representatives (Figure 3D). Moreover, like class I DNAs, II-R1 is strictly dependent on Zn^{2+} for its catalytic activity (Figure S6, Supporting Information), has narrow divalent metal (Figure S7A, Supporting Information) and pH (Figure S7B and C, Supporting Information) optima, and functions more than 50-fold faster at 45 versus 23 °C (Figure S7D and E, Supporting Information).

Importance of Conserved Structures and Sequences of Class I Deoxyribozymes. The consensus sequences and structural models for both deoxyribozyme classes indicate that the base-paired substructures might be generalizable, such that any DNA carrying the GTTGAAG consensus of the substrate domain could be cleaved by the appropriate enzyme sequence. This was assessed by conducting self-cleavage assays with several variant enzyme and substrate domains based on I-R3. Mutations that disrupt the base pairing in either of the two stems inactivate the deoxyribozyme, whereas compensatory mutations that restore base pairing also restore DNA cleavage activity (Figure S8, Supporting Information). These findings are consistent with our speculation that base pairing, rather than sequence identity, in the P1 and P2 stems is important for robust activity.

A bimolecular construct based on the I-R3 deoxyribozyme with shortened P1 and P2 stems (Figure 4) was prepared to assess whether the construct is capable of multiple turnover. The short stems are expected to facilitate the release of the cleavage products. Indeed, approximately 20 turnovers were observed after a 15 h incubation wherein a 500-fold excess of substrate was present (Figure S9, Supporting Information).

The high level of sequence conservation in the core of class I DNAs suggests that nucleotide changes here are likely to be deleterious to deoxyribozyme activity. One example of a critical position is at position 7, where a single point mutation (A7T) in the catalytic core completely inactivates I-R3 (Figure S8, Supporting Information). However, not all nucleotide changes in the core cause complete loss of activity. Numerous mutations are observed in sequence variants analyzed from the original population and from a reselected population, although the variants we examined all exhibit reduced rate constants.

The precise sequence at the site of cleavage has limited importance on the activity of class I deoxyribozymes (Figure S10, Supporting Information). When one of the two deoxyribonucleotide A (dA) residues that immediately flank the hydrolysis is deleted, the reaction progresses far more slowly, but substrate cleavage still occurs after the remaining dA residue. Moreover, changing the dA nucleotide immediately 5′ of the hydrolysis site to any of the other three deoxribose nucleotides (dG, dT, or dC), the deoxyribozyme retains nearly all activity. However, the bimolecular I-R3 complex is rendered inactive if either the enzyme or the substrate domain is prepared as an RNA polymer.

Using Deoxyribozymes to Selectively Cleave Large DNA Substrates. The ability to design variant class I deoxyribozymes to cleave certain DNA substrates was exploited to process the large single-stranded genome of bacteriophage M13. As noted above, deoxyribozyme I-R3 can tolerate single nucleotide changes to the nucleotide immediately 5′ of the hydrolysis site (Figure S10, Supporting Information), and therefore, only six specific nucleotides are required in the substrate domain. On average, these six nucleotides will occur once in every 4096 nucleotides. Three potential substrate sequences within the 7249 nt M13 genome (Figure 6A and Figures S1−S3, Supporting Information) were identified, and we designed three enzyme domains (E1−E3) that target these substrates.

Incubation of the M13 DNA with different combinations of the three enzyme domains and Zn^{2+} yields DNA fragments with the sizes expected for site-specific cleavage by the deoxyribozymes (Figure 6B). The selectivity and the efficiency by which these engineered deoxyribozymes cleave single-stranded DNA should permit their use as reagents for molecular biology protocols involving DNA processing.

Self-Cleavage Activity of Natural DNA Sequences. Given the small size and high DNA cleavage activity particularly of class I deoxyribozymes, we were interested in determining whether natural DNA sequences could be found that are similar to the class I consensus, and if so whether these sequences exhibit self-cleavage activity. The identities of approximately 15 nucleotides are conserved in the catalytic core of the class I deoxyribozymes (Figure 2). The probability of encountering a specific 15 nt sequence in a random-sequence DNA is 0.25,[15] or once in approximately 10^9 nucleotides.

We used a bioinformatics search algorithm[25] to identify natural DNA sequences deposited in the RefSeq database[26] and the Global Ocean Survey[27] that are similar to the sequence and secondary structure consensus of class I deoxyribozymes. A total of 28 examples of natural DNAs spanning 54 or fewer contiguous nucleotides were chosen for experimental analysis (Table S1, Supporting Information). These DNAs carry sequences similar to the core of class I deoxyribozymes, and have the base-pairing potential to form a P1 stem. When these DNAs were incubated at pH 7.4 with a Zn^{2+} concentration (50 μM) like that typically found in cells, none of the constructs exhibited detectable self-hydrolysis. However, six of these DNAs (Figure 7A, NS1−NS6) exhibit site-specific cleavage when incubated under our selection buffer conditions (Figure 7B).

Under these conditions, NS4 cleaved to the greatest extent (39.5%) after a six-day incubation, corresponding to a k_{obs} value of 4.8×10^{-5} min^{-1} and an estimated half-life of about 10 days

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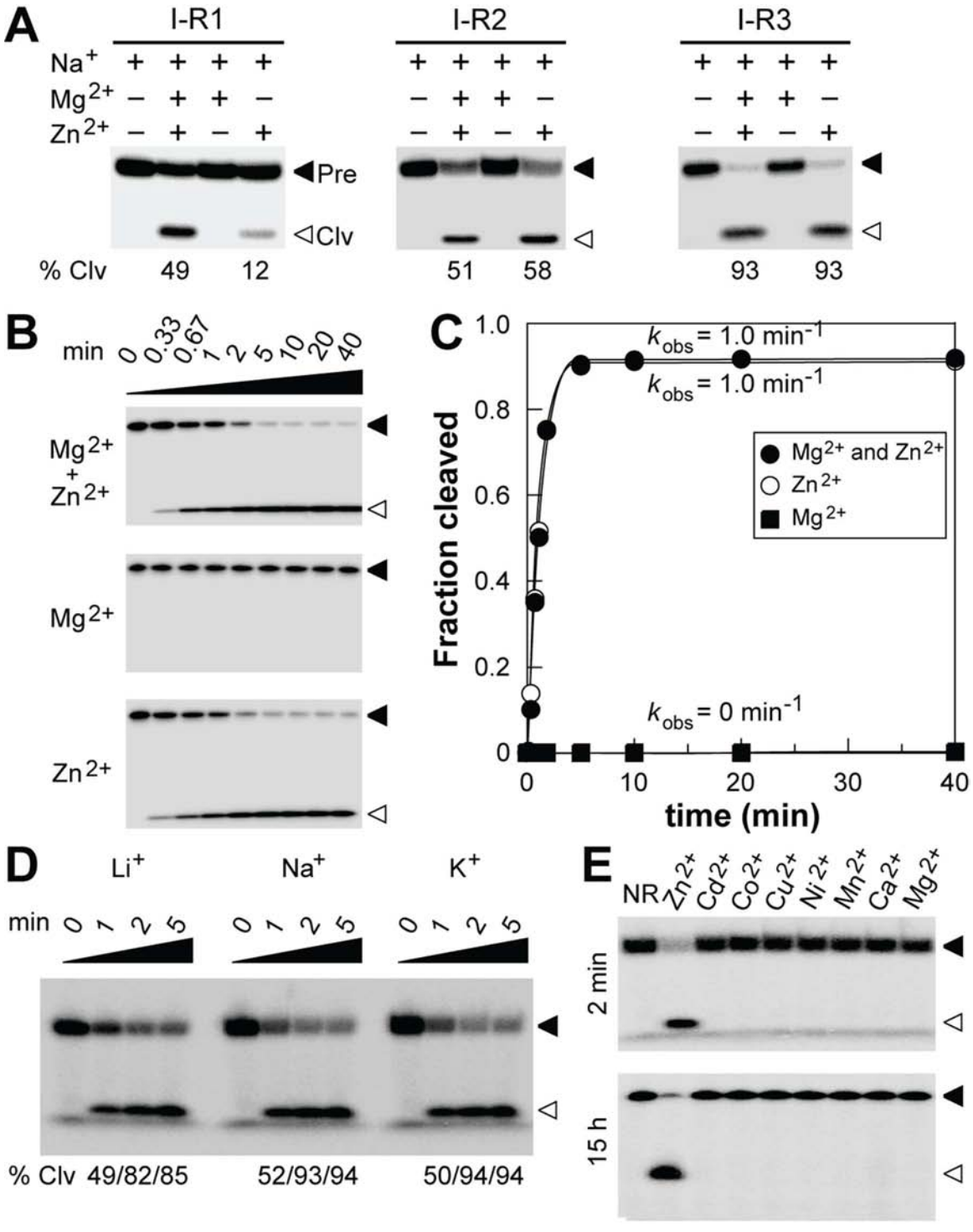

Figure 5. Metal ion dependence of class I deoxyribozymes. (A) Cleavage of 5′ ^{32}P-labeled I-R1, I-R2, and I-R3 in reaction buffers containing different combinations of Na^+ (100 mM), Mg^{2+} (20 mM), and Zn^{2+} (2 mM). The incubation times for I-R1, I-R2, and I-R3 are 60, 60, and 5 min, respectively. (B) Kinetic analysis of I-R3 in reaction buffers containing either Mg^{2+} (20 mM), Zn^{2+} (2 mM), or both. Cleavage products were separated by denaturing 10% PAGE, and yields were quantified. Filled and open arrowheads designate uncleaved and 5′ cleavage products, respectively. (C) Plot of the fraction of DNA cleaved versus the incubation time for I-R3. Values are derived from the PAGE band intensities in part B. (D) Evaluation of the effects of different monovalent ions on I-R3 function. Samples were incubated in buffers with Zn^{2+} (2 mM) and 100 mM of one of the three monovalent metal ions as indicated. Other details are as described in part B. (E) Analysis of the function of I-R3 with other divalent metal ions. Samples were incubated with 2 mM of the divalent cation for 2 min and 15 h as indicated. NR indicates no reaction, and other details are as described in part B. Note that only Zn^{2+} supports I-R3 deoxyribozyme activity.

(Figure 7C and D), which means that our optimal class I deoxyribozyme is more than 10 000-fold faster. Many of the natural sequences that are similar to the class I deoxyribozyme consensus carry mutations expected to reduce catalytic activity. For example, NS4 is likely to be less active because it carries a single nucleotide change in the enzyme core compared to the

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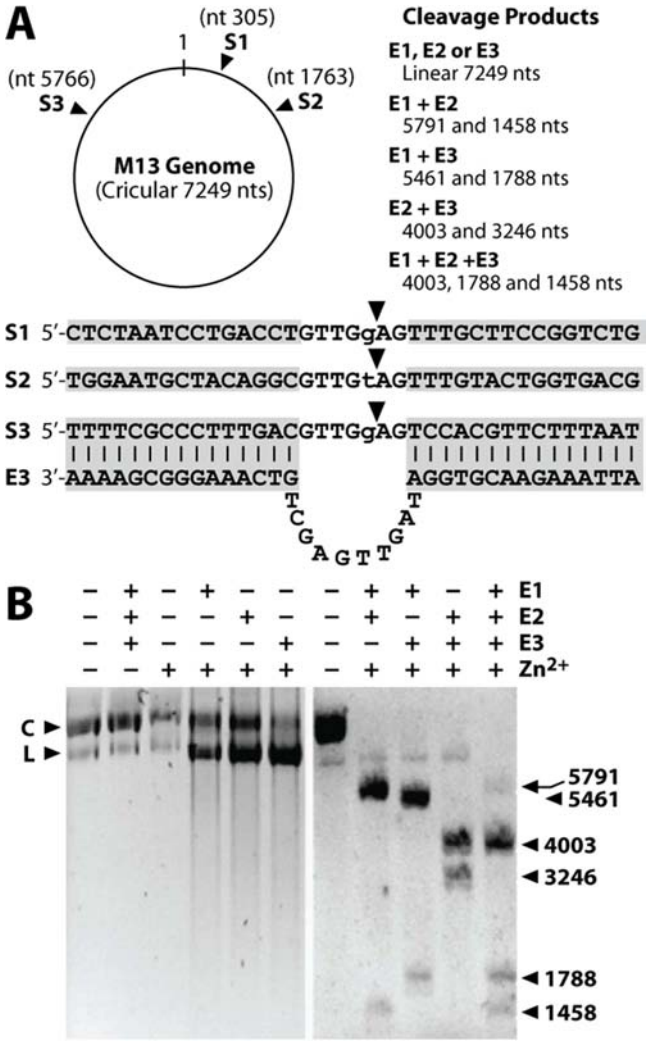

Figure 6. Cleavage of circular single-stranded bacteriophage M13 DNA with class I deoxyribozymes. (A) Target sites for cleavage of M13 (M13mp18) DNA by engineered deoxyribozymes. Potential substrates denoted S1, S2, and S3 within the M13 genome were chosen because they carry a sequence that is similar to the conserved nucleotides in the substrate domain of class I deoxyribozymes. Corresponding enzyme domains (E3−S3 complex is depicted) were engineered that carry the catalytic core sequence TAGTTGAGCT and that form 15 base pairs both upstream and downstream of the target substrate nucleotides. Arrowheads denote the site of cleavage, and gray shading identifies base-paired nucleotides. Lowercase letters identify nucleotides that vary from the substrate consensus sequence. (B) Cleaving M13 DNA by three class I deoxyribozymes. M13 DNA was incubated with various combinations of E1, E2, and E3 enzymes in selection buffer at 37 °C for 40 min, either with (+) or without (−) Zn^{2+}. Cleavage products were separated by denaturing (1.5 M (left) or 2.5 M (right) formaldehyde) 0.8% agarose gel electrophoresis, and the DNA products were visualized by staining with SYBR Gold. Bands corresponding to circular (C) and linear (L) M13 DNA are denoted, as are the bands corresponding to cleavage products generated on digestion with multiple deoxyribozymes. Note that some linear M13 DNA is present before exposure to deoxyribozymes due to random spontaneous cleavage of the circular DNA.

class I consensus, and it has a shorter P1 stem compared to fast class I constructs such as I-R3. Indeed, conversion of this variant sequence to match the consensus (construct NS4-gtoT) or to increase the length of P1 by the addition of five base pairs improves the rate constant for cleavage. These findings suggest that relatively modest mutations could occur that bring the natural DNAs closer in functional capability to engineered deoxyribozymes.

It is also possible that suboptimal class I DNA motifs could receive support from adjoining sequences and structures. A similar effect is seen with natural hammerhead ribozymes, which are enhanced in both speed and Mg^{2+} cofactor affinity by the formation of tertiary structures located adjacent to the catalytic core.[28] However, the deficiency in the function of NS4 was not overcome by the addition of 30 natural nucleotides on each end of the minimal construct to form construct NS4-Ext (Figure 7C and D). It remains possible that some flanking sequences or structures could improve the rate constant to yield natural examples of class I deoxyribozymes. Regardless, the measurable rate constants of the minimal natural DNA sequences examined in this study imply that under certain conditions DNA-catalyzed DNA hydrolysis could contribute to genomic instability.

■ CONCLUSIONS

Our results and other findings published recently demonstrate that there are likely to be many DNA sequences that can fold into structures that promote the hydrolysis of DNA by using metal ion cofactors. Both class I and class II deoxyribozymes described in this study are relatively simple in architecture, yet they promote DNA hydrolysis with rate enhancements in excess of 12 orders of magnitude over the uncatalyzed reaction. It is likely that other more rare classes also exist on our selected population that could be recovered for further analysis. Perhaps some of these DNAs will function with high speed under conditions that are more biologically relevant.

Even if there are no DNAs from the selected population that can function under cell-like conditions, the *in vitro* selection process used in this study could be employed to seek such deoxyribozymes by adjusting the selection conditions to more closely correspond to the conditions typical of cells. The addition of small organic compounds that could function as cofactors for DNA catalysis, as has been done previously with the amino acid histidine,[5] would further increase the chances that more sophisticated deoxyribozymes would be isolated that could function in cells.

The fact that the catalytic core and cleavage site nucleotides of the deoxyribozymes isolated in the current study emerged entirely from the random-sequence domain of the original constructs highlights the advantage of the selection process we employed. The two dominant classes of deoxyribozymes isolated in our study are distinct from the self-cleaving DNAs described in recent reports.[13−18] This might be due to the fact that emerging deoxyribozymes have a wider choice for cleavage sites and that our selection conditions are distinct from those used previously.

The small size and high speed of the class I representative I-R3 highlights two intriguing possibilities. First, it seems possible that short and efficient self-cleaving DNAs could be used as reagents for processing DNA substrates (Figure 6) or even integrated into larger DNA constructs for various biotechnology applications. As an example of the latter application, we have integrated a template for a class I into a single-stranded DNA template for rolling circle amplification. The repetitive product DNA generated by DNA polymerase using this template can undergo processing into monomeric DNA products when the sample is incubated under deoxyribozyme cleavage conditions.[29]

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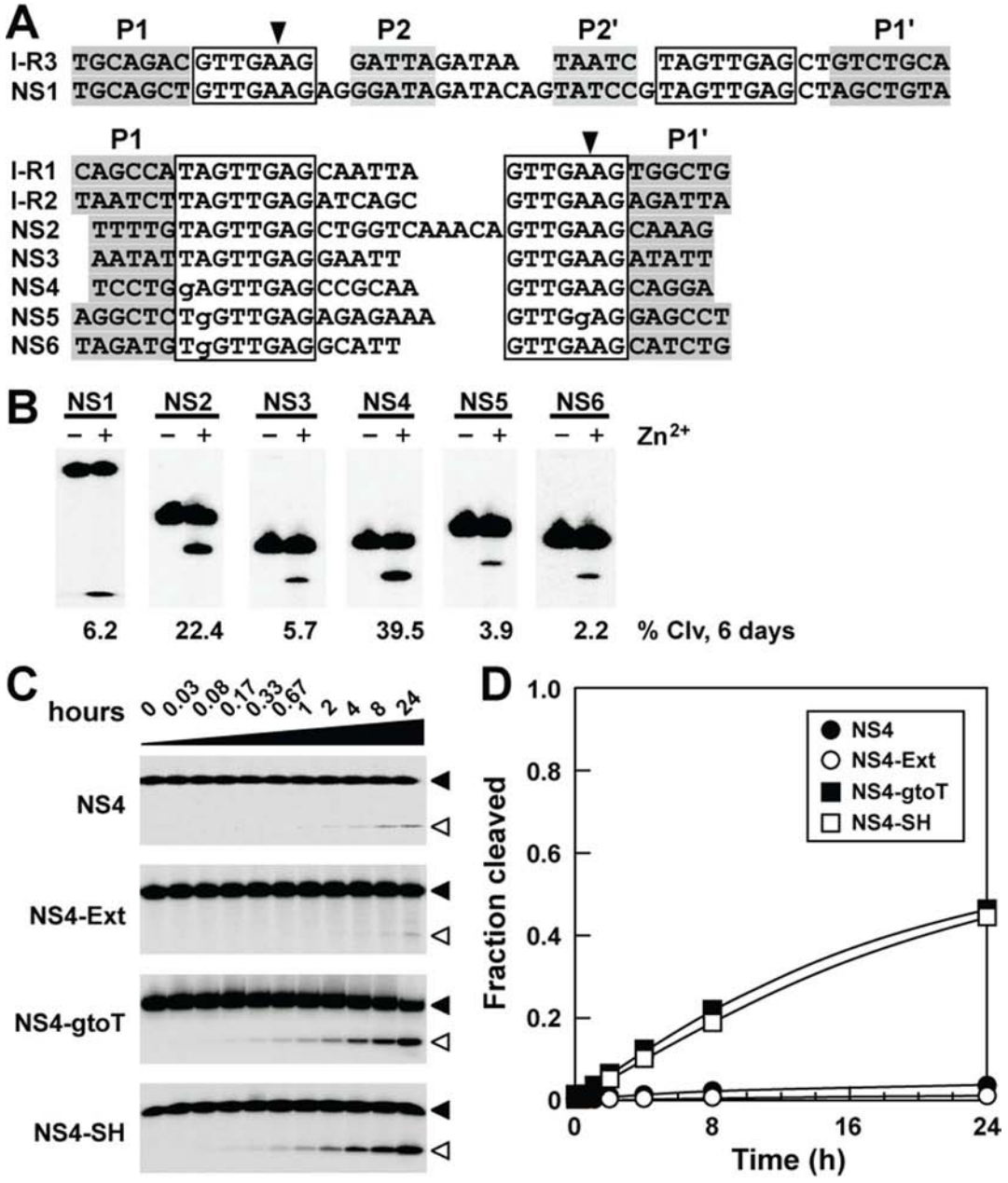

Figure 7. Instability of natural sequences that can conform to the concensus of the class I deoxyribozymes. (A) Alignment of natural DNA sequences to representatives of class I deoxyribozymes. Sequences corresponding to the conserved regions of class I deoxyribozymes are boxed. Other annotations are as described for Figure 6A. See the Supporting Information for the sources of the natural sequences (NS) 1–6. (B) Hydrolytic instability of natural DNA sequences. Trace amounts of each natural DNA (5′ ^{32}P-labeled) were incubated at 37 °C in reaction buffer without or with 2 mM Zn^{2+} for 6 days. Samples were subjected to denaturing 12% PAGE to separate 5′ cleaved products from precursor DNAs. (C) Kinetic analysis of construct NS4 and its derivatives. Annotations are as described for Figure 3A. NS4-Ext is an extended version of NS4 with 30 additional natural nucleotides at both the 5′ and 3′ ends. NS4-gtoT designates a point mutation that creates a perfect match for the conserved substrate region. NS4-SH is an extended NS4 with five base pairs added to the P1 stem. See the Supporting Information for additional details. (D) Plot of the fraction of DNA cleaved versus the incubation time for NS4 and its derivatives. Values were obtained from the data in part C.

Second, the robust activity of the I-R3 hints at the possibility that natural variants of this deoxyribozyme class or others exist in the genomes of organisms. Although most cellular life forms maintain their genomic DNAs in the double-stranded form for the vast majority of time, the single-stranded form of genomic DNAs is present at least temporarily during the processes of replication, repair, and transcription. Given the small size and simplicity of class I self-hydrolyzing DNAs, natural DNAs that are similar in sequence and structure will be common. For example, the core of class I deoxyribozymes is similar in complexity to the core of hammerhead ribozymes, of which tens of thousands of examples have been discovered recently in organisms from all three domains of life.[30,31] Moreover, the hammerhead ribozyme is one of the most common self-cleaving RNA motifs recovered from directed evolution experiments.[32,33]

Natural class-I-like DNAs examined in this study are unusually unstable (Figure 7), albeit at conditions that are only close to those of the cellular environment. This observation demonstrates how close natural DNA sequences

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approximate the sequences, structures, and activities of the self-hydrolyzing deoxyribozymes isolated by directed evolution. Of course, low reaction rates, suboptimal reaction conditions, and scarcity of single-stranded genomic DNAs all would reduce the probability of DNA self-cleavage in cells. However, even exceedingly rare cleavage events could influence genomic arrangements over long evolutionary times.

Intriguingly, the recent observation[34] of mutation clustering at the site of cleavage by a natural self-depurinating DNA provides a possible precedence for the genetic effects of even infrequent deoxyribozyme action. Therefore, despite the suboptimal sequences and activities of the natural class I deoxribozyme variants, some organisms could suffer DNA damage due to the occasional formation of imperfect self-cleaving structures. Some cells naturally experience unusually high Zn^{2+} concentrations at times,[35] which could increase genomic instability by such deoxyribozymes. Perhaps, some living systems that require DNAs to be cleaved during their life cycle have evolved to take advantage of the inherent ability of certain DNA sequences to form high-speed self-hydrolyzing deoxyribozymes.

METHODS

DNA Oligonucleotides. DNAs were purchased from Integrated DNA Technologies (IDT) and purified by denaturing (8 M urea) 10 or 20% polyacrylamide gel electrophoresis (PAGE) before use. Details regarding the nucleotide sequences and the preparation of the pool for directed evolution are provided in the Supporting Information.

Directed Evolution. Approximately 100 pmol of DNA were used for the first round of selection, and subsequent rounds were conducted using between 20 and 40 pmol of DNA. DNAs were internally ^{32}P-labeled by adding a trace amount of α-^{32}P [dGTP] during primer extension or PCR amplification.

Pool DNAs were circularized by using CircLigase (Epicenter, see the Supporting Information for additional details) incubated at 60 °C for 1 h in a buffer containing 50 mM MOPS (pH 7.5 at 23 °C), 100 mM KCl, 2.5 mM $MnCl_2$, 0.05 mM ATP, and 1 mM DTT. Monomer circular DNAs were isolated by denaturing 8% PAGE, crush-soaked from the gel, and recovered by precipitation with ethanol. Circular DNAs were subjected to 0.1 M piperidine at 80 °C for 30 min to remove DNAs with abasic sites that might be generated during the chemical synthesis and preparation of the DNAs. Intact circular DNAs were recovered by PAGE as described above.

The initial pool was incubated in a reaction buffer containing 50 mM HEPES (pH 7.05 at 23 °C), 100 mM NaCl, 20 mM $MgCl_2$, and 2 mM $ZnCl_2$ at 37 °C for 3 h. Cleaved DNAs were separated by denaturing 8% PAGE as described above and ligated with CircLigase to regenerate circles. Circular DNAs were isolated by PAGE and amplified by PCR to generate double-stranded DNAs. The template strands were cleaved with alkali (0.25 M NaOH at 90 °C) for 5 min, and the intact strands were isolated by PAGE. Mutagenic PCR[36] was performed (G10−G11) to increase population diversity. Double-stranded DNA clones (TOPO TA Cloning Kit, Invitrogen) from the selection pool of G14 were individually sequenced, and the G9 pool was deep sequenced using next-generation sequencing technology by the W. M. Keck Biotechnology Resource Laboratory at Yale University.

Deoxyribozyme Cleavage Assays. Unimolecular deoxyribozyme constructs (5′ ^{32}P labeled) were allowed to fold in an x μL (x = 20−100) solution containing 50 mM HEPES (pH 7.05 at 23 °C), 100 mM NaCl after incubation at 90 °C for 2−3 min, followed by cooling on ice for 5 min, and then incubation at 37 °C. Cleavage reactions were initiated by adding another x μL of a solution containing 50 mM HEPES (pH 7.05 at 23 °C), 100 mM NaCl, 40 mM $MgCl_2$, and 4 mM $ZnCl_2$.

Bimolecular deoxyribozyme constructs (5′ ^{32}P-labeled substrate) for single-turnover reactions were conducted with 0.2 pmol of substrate and 10 pmol of enzyme in 30 μL as described above. For pH and metal ion dependence assays, these parameters were altered accordingly. At certain time points, 2 μL of the sample was removed and added to 8 μL of stop solution (90% formamide, 30 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were separated by denaturing 8 or 12% PAGE and visualized/quantified by PhosphorImager. Values for k_{obs} were established by using the following equation: Fraction cleaved = $FC_{max}(1 - e^{-kt})$, where $k = k_{obs}$ and FC_{max} = maximum of fraction cleaved. For multiple-turnover cleavage assays, the deoxyribozyme was present in an amount equal to 0.2−10% of the substrate (10 pmol), which was 5′ ^{32}P labeled. DNAs for mass spec analysis were prepared and analyzed as described in the Supporting Information.

Cleavage of circular M13mp18 DNA (NEB) was conducted in reactions containing 500 ng of M13 DNA, with class I deoxyribozymes E1−E3 in a 1:2 molar ratio. The reactions were conducted as described above for 40 min, and the products were separated using 1.5−2.5 M formaldehyde agarose gels to separate the cleaved fragments from the uncleaved precursor.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text (Materials and Methods; Table S1; Figures S1−S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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