A DNA enzyme that cleaves RNA

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Background: Several types of RNA enzymes (ribozymes) have been identified in biological systems and generated in the laboratory. Considering the variety of known RNA enzymes and the similarity of DNA and RNA, it is reasonable to imagine that DNA might be able to function as an enzyme as well. No such DNA enzyme has been found in nature, however. We set out to identifiy a metal-dependent DNA enzyme using *in vitro* selection methodology.

Results: Beginning with a population of 10^{14} DNAs containing 50 random nucleotides, we carried out five successive rounds of selective amplification, enriching for individuals that best promote the Pb^{2+}-dependent cleavage of a target ribonucleoside 3'-O-P bond embedded within an otherwise all-DNA sequence. By the fifth round, the population as a whole carried out this reaction at a rate of 0.2 min^{-1}. Based on the sequence of 20 individuals isolated from this population, we designed a simplified version of the catalytic domain that operates in an intermolecular context with a turnover rate of 1 min^{-1}. This rate is about 10^5-fold increased compared to the uncatalyzed reaction.

Conclusions: Using *in vitro* selection techniques, we obtained a DNA enzyme that catalyzes the Pb^{2+}-dependent cleavage of an RNA phosphoester in a reaction that proceeds with rapid turnover. The catalytic rate compares favorably to that of known RNA enzymes. We expect that other examples of DNA enzymes will soon be forthcoming.

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Introduction

It has been more than a decade since the discovery of catalytic RNA [1,2]. The list of known naturally-occurring ribozymes continues to grow (for reviews see [3–5]) and, in recent years, has been augmented by synthetic ribozymes obtained through *in vitro* evolution (for reviews see [6–8]). It is reasonable to assume that DNA can have catalytic activity as well, considering that it contains most of the same functional groups as RNA. But except for certain viral genomes and replication intermediates, nearly all of the DNA in biological organisms occurs as a complete duplex, precluding it from adopting complex secondary and tertiary structure. Thus it is not surprising that DNA enzymes have not been found in nature.

It is well known that single-stranded DNA can assume interesting tertiary structures. A tRNA and its DNA analog form very similar structures [9]. Furthermore, it has been possible to replace ribonucleotides with deoxyribonucleotides in as many as 31 of 35 positions of a hammerhead ribozyme and retain at least some catalytic activity [10–13].

In vitro selection techniques have been applied to large populations of random-sequence DNAs, leading to the recovery of specific DNA 'aptamers' that bind a target ligand with high affinity [14–16]. Recently, two groups carried out the first NMR structural determination of an aptamer, a 15-nucleotide DNA molecule that forms a G-quartet structure and binds the protein thrombin with high affinity [17,18]. These findings were corroborated by an X-ray crystallographic analysis [19].

DNA has the ability to bind a substrate molecule with high affinity and specificity, which is a prerequisite of a good enzyme. In addition, an enzyme must make use of well-positioned functional groups, either within itself or a cofactor, to promote a particular chemical transformation. Furthermore, the enzyme must remain unchanged over the course of the reaction and show catalytic turnover. Some would add the requirement that it be an informational macromolecule, composed of subunits whose specific ordering is responsible for catalytic activity. Although these criteria are open to debate on both semantic and chemical grounds, they serve to distinguish among the phenomena of chemical rate enhancement, which range from simple solvent effects to the ability of some biological enzymes to operate at the limit of substrate diffusion [20].

In the present study, we sought to develop a general method for rapidly obtaining DNA catalysts and DNA enzymes, starting from random sequences. As an initial target, we chose a reaction that we felt was well within the capability of DNA — the hydrolytic cleavage of an RNA phosphodiester, assisted by a divalent metal cofactor. This is the same reaction that is carried out by a variety of naturally-occurring RNA enzymes, including the hammerhead and hairpin motifs [21–23]. Pan and Uhlenbeck [24,25] have shown that, beginning with a randomized library of tRNA molecules, new

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ribozymes can be obtained that have Pb^{2+}-dependent, site-specific RNA phosphoesterase activity at neutral pH. This activity is analogous to the fortuitous self-cleavage reaction of yeast $tRNA^{Phe}$ [26], which depends on specific coordination of a Pb^{2+} ion at a defined site within the tRNA [27,28]. Similarly, we sought to develop DNAs that could carry out Pb^{2+}-dependent cleavage of a particular RNA phosphoester, initially presented within a short leader sequence attached to the 5' end of the DNA, and ultimately located within a separate molecule that can be cleaved in an intermolecular fashion with rapid catalytic turnover.

We made no assumptions about how the DNA would interact with the target phosphoester and surrounding nucleotides. We began with a pool of ~10^{14} DNAs containing a region of 50 random nucleotides and used *in vitro* selection to search for catalytically-active molecules. After five rounds of selection, carried out over four days, the population as a whole had attained the ability to cleave the target phosphoester in the presence of 1 mM Pb^{2+} at a rate of about 0.2 min^{-1}. This is ~10^4-fold faster than the spontaneous rate of cleavage under the same reaction conditions. Individuals were isolated from the population, sequenced, and assayed for catalytic activity. Based on this information, the reaction was converted to an intermolecular format, then simplified to allow site-specific cleavage of a 19-nucleotide substrate by a 38-nucleotide DNA enzyme, in a reaction that proceeds with a turnover rate of 1 min^{-1} at 23 °C and pH 7.0 in the presence of 1 mM PbOAc.

Results

In vitro selection scheme

We generated a starting pool of ~10^{14} single-stranded DNA molecules, all of which contain a 5' biotin moiety, followed successively by a fixed domain that includes a single ribonucleotide, a potential catalytic domain composed of 50 random deoxyribonucleotides, and a second fixed domain that lies at the 3' terminus (Fig. 1). The pool was constructed by a nested PCR (polymerase chain reaction) technique, beginning with synthetic DNA that contains 50 random nucleotides flanked by primer binding sites. The nested PCR primer was a 5'-biotinylated synthetic oligodeoxynucleotide with a 3'-terminal adenosine ribonucleotide. Ribonucleotide-terminated oligodeoxynucleotides efficiently prime template-directed elongation in the context of the PCR (L.E. Orgel, personal communication), in this case giving rise to an extension product that contains a single embedded ribonucleotide.

The PCR products were passed over a streptavidin affinity matrix, resulting in noncovalent attachment of the 5'-biotinylated strand of the duplex DNA. The nonbiotinylated strand was removed by brief washing with 0.2 N NaOH, and the bound strand was equilibrated in a buffer containing 0.5 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$, and 50 mM HEPES (pH 7.0) at 23 °C. Next, 1 mM PbOAc was provided in the same buffer, allowing Pb^{2+}-dependent cleavage to occur at the target phosphoester, thereby releasing a subset of the DNAs from the streptavidin matrix. In principle, an individual DNA might facilitate its own release by various means, such as disruption of the interaction between biotin and streptavidin or cleavage of one of the deoxyribonucleotide linkages. We felt that cleavage of the ribonucleoside 3'-O-P bond would be the most likely mechanism for release, based on the relative lability of this linkage, and that Pb^{2+}-dependent hydrolytic cleavage would allow release to occur most rapidly. In principle, however, the *in vitro* selection procedure should identify the most favorable release mechanism as well as those individuals best able to carry out that mechanism.

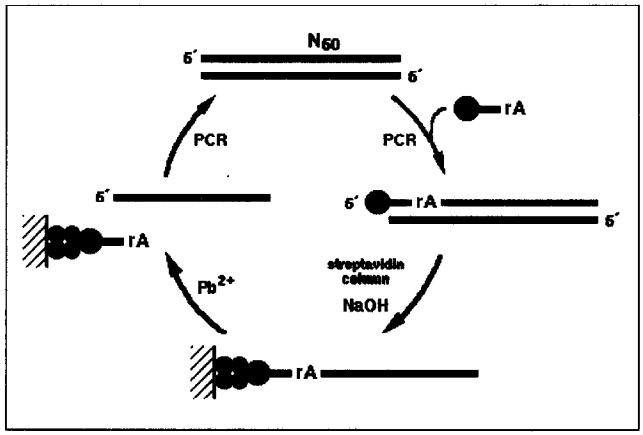

Fig. 1. Selective amplification scheme for isolation of DNAs that cleave a target RNA phosphoester. Double-stranded DNA that contains a stretch of 50 random nucleotides is amplified by PCR, employing a 5'-biotinylated (B) DNA primer that is terminated at the 3' end by an adenosine ribonucleotide (rA). This primer is extended by *Taq* polymerase to yield a DNA product that contains a single embedded ribonucleotide. The resulting double-stranded DNA is immobilized on a streptavidin matrix and the unbiotinylated DNA strand is removed by washing with 0.2 N NaOH. After re-equilibrating the column with a buffered solution, the column is washed with the same solution with added 1 mM PbOAc. DNAs that undergo Pb^{2+}-dependent self-cleavage are released from the column, collected in the eluate, and amplified by PCR. The PCR products are then used to initiate the next round of selective amplification.

DNA molecules released from the matrix upon addition of Pb^{2+} were collected in the eluate, concentrated by precipitation with ethanol, and subjected to nested PCR amplification. As in the construction of the starting pool of molecules, the first PCR amplification used primers that flank the random region and the second used a 5'-biotinylated primer that has a 3'-terminal riboadenylate, thereby reintroducing the target RNA phosphoester. The entire selective amplification procedure requires three to four hours to perform. The molecules are purified in three ways during each round of this procedure: first, after PCR amplification, by extracting twice with phenol and once with chloroform/isoamyl alcohol, then precipitating with ethanol; second, after attachment of the DNA to streptavidin, by washing away all the nonbiotinylated molecules under

strongly denaturing conditions; and third, after elution with Pb^{2+}, by precipitating with ethanol. There is no gel electrophoresis purification step, and thus no selection pressure constraining the molecules to a particular length.

Selection of catalytic DNA

We carried out five successive rounds of *in vitro* selection, progressively decreasing the reaction time following addition of Pb^{2+} to progressively increase the stringency of selection. During rounds 1 through 3 the reaction time was 1 h, during round 4 it was 20 min, and during round 5 it was 1 min. The starting pool of single-stranded DNAs, and the different populations of molecules obtained after each round of selection, were assayed for self-cleavage activity under conditions identical to those employed during *in vitro* selection (Fig. 2). For this assay, the molecules were prepared with a 5'-^{32}P rather than a 5'-biotin moiety, allowing detection of both the starting material and the 5' cleavage product. Following a 5-min incubation, there was no detectable activity in the initial pool (G0) or in the population obtained after the first and second rounds of selection. DNAs obtained after the third round (G3) exhibited a modest level of activity and this activity increased steadily, reaching ~50 % self-cleavage for the DNAs obtained after the fifth round of selection (G5). Cleavage is detected only at the target phosphoester, even after long incubation times. This activity is lost if Pb^{2+} is omitted from the reaction mixture.

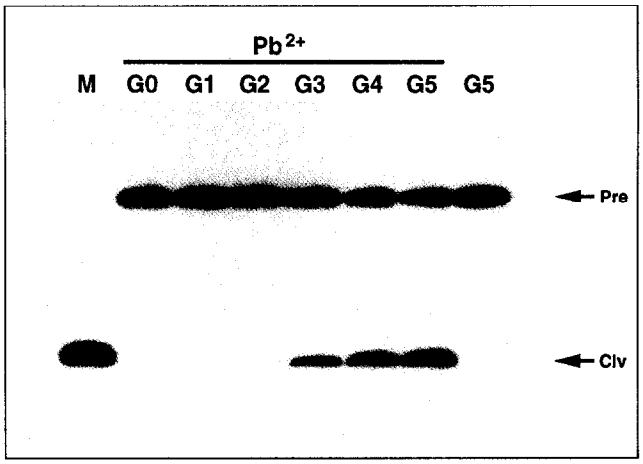

Fig. 2. Self-cleavage activity of the starting pool of DNA (G0) and populations obtained after the first through fifth rounds of selection (G1–G5). Reaction mixtures contained 0.5 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$, 50 mM HEPES (pH 7.0 at 23 °C), and 3 nM [5'-^{32}P]-labeled DNA, incubated at 23 °C for 5 min in either the presence or absence of 1 mM PbOAc. Reaction products were separated by electrophoresis in a denaturing 10 % polyacrylamide gel and visualized by autoradiography. Pre, 108-nucleotide precursor DNA; Clv, 28-nucleotide 5'-cleavage product; M, primer 3a, corresponding in length to the expected 5'-cleavage product.

We employed shotgun cloning techniques to isolate individuals from the G5 population and determined the complete nucleotide sequence of 20 of these subclones (Fig. 3). Of the 20 sequences, five were unique, two occurred twice, one occurred three times, and one occurred eight times. All of the individual variants share common sequence elements within the 50-nucleotide region that had been randomized in the starting pool of DNA. They all contain two presumed template regions, one with complementarity to a stretch of nucleotides that lies just upstream from the cleavage site and the other with complementarity to nucleotides that lie at least four nucleotides downstream. Between these two presumed template regions lies a variable domain of 1–11 nucleotides, followed by the fixed sequence 5'-AGCG-3', then a second variable domain of 3–8 nucleotides, and finally the fixed sequence 5'-CG-3' or 5'-CGA-3'. Nucleotides that lie outside of the two presumed template regions are highly variable in both sequence and length. In all of the sequenced subclones, the region corresponding to the 50 initially-randomized nucleotides retains its original length.

There is insufficient covariation among the sequenced subclones to construct a meaningful secondary structural model of the catalytic domain, other than to note that, like the hammerhead and hairpin ribozymes, it appears to contain a conserved core flanked by two substrate-binding regions that interact with the substrate through base-pairing interactions. Also like the hammerhead and hairpin ribozymes, the catalytic DNAs appear to require a short stretch of unpaired substrate nucleotides, in this case 5'-GGA-3', between the two regions that are involved in base pairing. It is interesting to note that each of the nine distinct variants exhibits a different pattern of presumed complementarity with the substrate domain. In some cases base pairing is contiguous, while in others it is interrupted by one or more noncomplementary pairs. The general tendency seems to be to form a tighter interaction with the nucleotides that lie upstream from the cleavage site than with those that lie downstream, but this conjecture must be substantiated by binding studies and site-directed mutagenesis analysis.

To gain further insight into the sequence requirements for catalytic function, we tested the self-cleavage activity of six of the nine variants, evaluated under the selection conditions. Not surprisingly, the sequence that occurred in eight of the 20 subclones proved to be the most reactive, with a first-order rate constant of 1.4 min^{-1}. All of the studied variants were active in the self-cleavage assay and all gave rise to a single 5'-labeled product, corresponding to cleavage at the target RNA phosphoester.

The dominant subclone was further analyzed under a variety of reaction conditions. Its self-cleavage activity is dependent on Pb^{2+}, but is unaffected if Mg^{2+} is omitted from the reaction mixture. There is also a requirement for a monovalent cation, which can be met by either Na^+ or K^+. The reaction rate increases linearly with increasing concentration of monovalent cation over the range of 0–1.0 M ($r = 0.998$). We have not yet evaluated other variables that might affect the reaction, such as pH, temperature, and the presence of other divalent metals.

# of clones						
substrate:	3'- CAGCGGTAGAGAAGG -					ATATCACTCAGCATAATCTTAAGCAGGG -5'
8	5'- TCACA CATCTCT	GAAGT	AGCG	CCGC	CG-	TAT-GTGA--CGCTA GGGGTTCGCCT -3'
3	5'- GGGGGGAACGCCGTAACAA G-CTCT	GAACT	AGCG	GTTG	CGA	TATAGT----CGTA -3'
2	5'- CGGGACTCCGTAGCCCA TTGCTTTTT--	GC	AGCG	TCAA	CGA	-ATAG-----CGTATTA -3'
2	5'- CCACCA TGTCTTCTC-	G	AGCCG	AAC	CG-	-ATAGTTA--CGTCAT ACCTCCCGTAT -3'
1	5'- GCCAGA TTGCTG-CT--	ACC	AGCG	GTA	CGA	AATAGTGAA--GTGTT CGTGACTAT -3'
1	5'- ATAG GCCATGCTTT	GGCT	AGCG	GCAC	CG-	TATAGTG-----TA CCTGCCCTTATCG -3'
1	5'- TCTGCTCTC CTCTATTCT-	AGCAGTGC	AGCG	AAATATGT	CGA	-ATAGT----CG-GT -3'
1	5'- TTGCCCAGCATA GTCGGCA-----	GACGTGGTGTT	AGCG	ACA	CG-	-ATAG-G-CCCG-GT -3'
1	5'- TTGCTAGCTCGGCTGA ACTTCT	GT	AGCG	CAAC	CGA	AATAGTGAG--GC-TTG A -3'

Fig. 3. Sequence alignment of individual variants isolated from the population after five rounds of selection. The fixed substrate domain is shown at the top, with the target riboadenylate shown in red. Substrate nucleotides that are commonly involved in presumed base-pairing interactions are indicated by a vertical bar. Sequences corresponding to the 50 initially-randomized nucleotides are aligned antiparallel to the substrate domain. All of the variants are 3'-terminated by the fixed sequence 5'-CGGTAAGCTTGGCAC-3' (not shown). Nucleotides within the initially-randomized region that are presumed to form base pairs with the substrate domain are shown in blue. The highly-conserved nucleotides within the putative catalytic domain are shown in green.

Conversion to an intermolecular format

Based on the variable pattern of presumed base-pairing interactions between the catalytic and substrate domains of the studied variants, we felt that it would be reasonably straightforward to convert the DNA-catalyzed reaction to an intermolecular format. In doing so, we wished to simplify the two substrate-binding regions of the catalyst so that each would form an uninterrupted stretch of 7–8 base pairs with the substrate. In addition, we wished to provide a minimal substrate, limited to the two base-pairing regions and the intervening sequence 5'-GGA-3' (Fig. 4a). In designing the catalytic domain, we relied heavily on the composition of the most reactive variant, truncating the originally randomized region by two nucleotides at the 5' end and 11 nucleotides at the 3' end. The 15 nucleotides that lie between the two template regions were left unchanged and a single adenosine nucleotide was inserted into the 3' template region to form a continuous stretch of nucleotides capable of forming base pairs with the substrate. The substrate was simplified to give the sequence 5'-TCACTATrA•GGAAGAGATGG-3', where the underlined nucleotides correspond to the two regions involved in base pairing with the catalyst.

The simplified reaction system, employing a 38-nucleotide oligomer composed entirely of deoxyribonucleotides as the catalyst, and a 19-nucleotide oligomer containing a single ribonucleotide embedded within an otherwise all-DNA sequence as the substrate, allows efficient DNA-catalyzed phosphoester cleavage with rapid turnover. Over a 90-min incubation in the presence of 0.01 μM catalyst and 1 μM substrate, 46 % of the substrate is cleaved, corresponding to 46 turnovers of the catalyst. We carried out a preliminary kinetic analysis of this reaction, evaluated under multiple-turnover conditions. The DNA catalyst exhibits Michaelis–Menten kinetics, with values for k_{cat} and K_m of 1 min^{-1} and 2 μM, respectively (Fig. 4b). The value for K_m is considerably greater than the expected dissociation constant between catalyst and substrate based on Watson–Crick interactions.

We incubated the substrate under identical reaction conditions, but in the absence of the catalyst, and obtained a value for k_{uncat} of about 10^{-5} min^{-1}. This is significantly slower than expected based on the literature value of

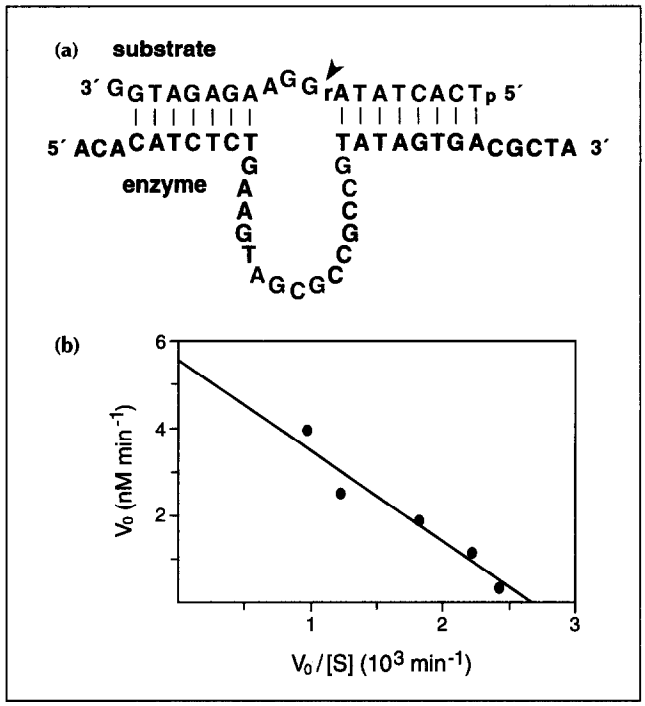

Fig. 4. DNA-catalyzed cleavage of an RNA phosphoester in an intermolecular reaction that proceeds with catalytic turnover. **(a)** Diagrammatic representation of the complex formed between the 19-nucleotide substrate and 38-nucleotide DNA enzyme. The substrate contains a single adenosine ribonucleotide (red), flanked by deoxyribonucleotides (blue). The synthetic DNA enzyme is based on a 38-nucleotide portion of the most frequently occurring variant shown in Fig. 3. Highly-conserved nucleotides located within the putative catalytic domain are shown in green. **(b)** Eadie–Hofstee plot used to determine K_m (negative slope) and V_{max} (y-intercept) for DNA-catalyzed cleavage of [5'-^{32}P]-labeled substrate under the conditions employed during *in vitro* selection. Initial rates of cleavage were determined for reactions involving 5 nM DNA enzyme and either 0.125, 0.5, 1, 2, or 4 μM substrate.

2×10^{-4} min^{-1} for hydrolysis of a typical RNA phosphoester in the presence of 10 mM $MgCl_2$ and 0.05 mM PbOAc at pH 7.0 and 25 °C [25]. We therefore prepared an all-RNA version of the substrate and incubated it in the presence of 10 mM $MgCl_2$ and 1 mM PbOAc at pH 7.0 and 23 °C. This provided a value for K_{uncat} of about 10^{-3} min^{-1} per nucleotide, averaged over the entire molecule. However, there was considerable heterogeneity in the susceptibility of various nucleotide positions to Pb^{2+}-dependent cleavage. The position corresponding to the target riboadenylate was cleaved about 10-fold slower than the average, at a rate of about 10^{-4} min^{-1}. Under the selection conditions, the uncatalyzed rate was about 2-fold slower, both on average and at the position corresponding to the target riboadenylate. Although the hydrolysis rate of a particular RNA phosphoester in the context of an all-RNA molecule may not be strictly comparable to the rate for the same bond in an otherwise all-DNA molecule, these results provide a possible explanation for the surprisingly slow rate of uncatalyzed Pb^{2+}-dependent cleavage of the target substrate. In the presence of the DNA catalyst, the all-RNA version of the substrate is somewhat protected from Pb^{2+}-dependent hydrolysis, with the position corresponding to the target riboadenylate being especially stable.

We presume that the phosphoester cleavage reaction proceeds via a hydrolytic mechanism involving attack by the ribonucleoside 2'-hydroxyl on the vicinal phosphate, generating a 5' product with a terminal 2'(3')-cyclic phosphate and 3' product with a terminal 5'-hydroxyl. In support of this mechanism, the 3'-cleavage product is efficiently phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP, consistent with the availability of a free 5'-hydroxyl (data not shown). Furthermore, the all-DNA analog of the substrate is not cleaved by the catalyst under our standard reaction conditions, as might be expected if the 2'-hydroxyl is involved in the reaction mechanism.

Discussion

After five rounds of *in vitro* selection, we obtained a population of single-stranded DNA molecules that catalyze efficient Pb^{2+}-dependent cleavage of a target RNA phosphoester. Based on the common features of representative individuals isolated from this population, we constructed a simplified version of both the catalytic and substrate domains, leading to a demonstration of rapid catalytic turnover in an intermolecular context. Thus the 38-nucleotide, catalytic domain provides an example of a DNA enzyme, or by analogy to ribozymes what might be termed a 'deoxyribozyme'. This molecule is an informational macromolecule capable of accelerating a chemical transformation in a reaction that proceeds with rapid turnover and obeys Michaelis–Menten kinetics. Considering how quickly it was obtained from a pool of random-sequence DNAs, we expect that other examples of synthetic DNA enzymes will appear in the near future.

We chose the Pb^{2+}-dependent cleavage of an RNA phosphoester as an initial target for DNA catalysis because it is a straightforward reaction that simply requires the proper positioning of a Pb^{2+}-coordinated hydroxyl to facilitate deprotonation of the 2' hydroxyl that lies adjacent to the cleavage site (for review see [29]). Pb^{2+} is known to coordinate to the N7 position of purines, the O6 position of guanine, the O4 position of uracil, and the N3 position of cytosine [30]. Thus, the differences in sugar composition and conformation of DNA compared to RNA seemed unlikely to prevent DNA from forming a well-defined Pb^{2+}-binding pocket. We chose a substrate that contains a single ribonucleotide within an otherwise all-DNA sequence because it provided a uniquely favored site for cleavage and ensured that any resulting catalytic activity would be attributable solely to DNA. Substrate recognition appears to depend on two regions of base-pairing interactions between the catalyst and substrate. The unpaired substrate nucleotides, 5'-GGA-3', that lie between these two regions may, however, be important in substrate recognition, metal coordination, or other aspects of catalytic function.

The DNA enzyme does not cleave an all-RNA substrate. We have not yet tested whether, through *in vitro* evolution, it could be made capable of doing so. We also wish to determine whether the proposed base-pairing interactions between enzyme and substrate can be generalized to any sequence. We view this Pb^{2+}-dependent deoxyribozyme as a model compound for exploring the structural and enzymatic properties of DNA. We believe that the methods employed in this study for the rapid development of DNA catalysts will have considerable generality, allowing us to use other cofactors to trigger the cleavage of a target linkage attached to a potential catalytic domain. It would be interesting, for example, to develop a Mg^{2+}-dependent DNA enzyme that specifically cleaves a target RNA under physiological conditions. Such a molecule would provide an alternative to traditional antisense and ribozyme approaches for the specific inactivation of target mRNAs.

The demonstration that DNA can have enzymatic activity raises the question of whether DNA enzymes exist in contemporary organisms or were important in the early history of life on earth. Most biological DNA exists in a fully double-stranded form, which would probably preclude it from carrying out complex catalytic tasks. It is not unreasonable, however, to wonder whether single-stranded DNA viruses that make use of an RNA primer to initiate replication might contain a structured motif that promotes hydrolytic cleavage of that primer. Regarding an ancient role for catalytic DNA, one could argue that, like RNA, DNA solves the 'chicken-and-egg problem' because it can act as both a genetic molecule and catalyst. One might even propose that there was a 'DNA world' — a genetic system based entirely on DNA. We believe that this is much less likely than the 'RNA world' hypothesis. The concept of an RNA world rests not only on the fact that RNA can serve as both gene and catalyst, but also on the observation that RNA has a primitive role in many of the most

highly conserved processes in biological organisms. It seems more likely to us that DNA was invented subsequent to RNA as a more stable repository of genetic information. Once DNA became trapped in the form of a complete duplex structure, which was selectively advantageous because it provided a means for mutational repair, the catalytic potential of DNA would have been stifled.

Significance

DNA now joins RNA and protein on the list of biological macromolecules that can exhibit enzymatic activity. The extent of DNA's catalytic abilities remains to be explored, but these explorations will be facilitated by *in vitro* selection methods such as those employed in this study. DNA enzymes offer at least two important advantages compared to other macromolecular catalysts. First, they are easy to prepare, in an era when most laboratories have access to an automated DNA synthesizer and the cost of DNA phosphoramidites has become quite modest. Second, they are very stable compounds, especially compared to RNA, facilitating their use in biophysical studies.

An intriguing possibility raised by our work is that DNA enzymes could be adapted to therapeutic applications that at present make use of antisense DNAs lacking RNA-cleavage activity. *In vitro* selection could be carried out with DNA analogs, including compounds that are nuclease-resistant, such as phosphorothioate-containing DNA, provided that these analogs can be prepared in the form of a deoxynucleoside 5'-triphosphate and are accepted as a substrate by a DNA-dependent DNA polymerase. Perhaps most importantly, DNA enzymes offer a new window on our understanding of the macromolecular basis of catalytic function. It may now be possible, for example, to carry out comparative analyses of protein-, RNA-, and DNA-based enzymes that catalyze the same chemical transformation.

Materials and methods

Oligonucleotides and oligonucleotide analogs

Synthetic DNAs and DNA analogs were purchased from Operon Technologies. The 19-nucleotide substrate, 5'-pTCACTATrAGGAAGAGATGG-3', was prepared by reverse transcriptase catalyzed extension of 5'-pTCACTATrA-3', as previously described [31], using the template 5'-CCAT-CTCTTCCTATAGTGAGTCCGGCTGCA-3'. Primer 3, 5'-GGGACGAATTCTAATACGACTCACTATrA-3', was either 5'-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (primer 3a) or 5'-thiophosphorylated with [γ-S]ATP and T4 polynucleotide kinase and subsequently biotinylated with N-iodoacetyl-N'-biotinylhexylenediamine (primer 3b).

DNA pool preparation

The starting pool of DNA was prepared by PCR using the synthetic oligomer 5'-GTGCCAAGCTTACCG-N_{50}-GTC-GCCATCTCTTCC-3', where N is an equimolar mixture of G, A, T and C. A 2-ml PCR, containing 500 pmoles of the randomized oligomer, 1000 pmoles primer 1 (5'-GTGC-CAAGCTTACCG-3'), 500 pmoles primer 2 (5'-CTGCAGAA-TTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3'), 500 pmoles primer 3b, 10 μCi [α-^{32}P]dATP, and 0.2 U μl^{-1} *Taq* DNA polymerase, was incubated in the presence of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3 at 23 °C), 0.01 % gelatin, and 0.2 mM of each dNTP for 1 min at 92 °C, 1 min at 50 °C, and 2 min at 72 °C, then 5 cycles of 1 min at 92 °C, 1 min at 50 °C, and 1 min at 72 °C. The resulting mixture was extracted twice with phenol and once with chloroform/isoamyl alcohol; DNA was isolated by precipitation with ethanol.

In vitro *selection*

The starting pool of DNA was resuspended in 500 μl of buffer A (1 M NaCl and 50 mM HEPES (pH 7.0 at 23 °C)) and was passed repeatedly over a streptavidin column (AffiniTip Strep 20, Genosys, The Woodlands, TX). The column was washed with five 100-μl volumes of buffer A, followed by five 100-μl volumes of 0.2 N NaOH, then equilibrated with five 100-μl volumes of buffer B (0.5 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$, and 50 mM HEPES (pH 7.0 at 23 °C)). The immobilized single-stranded DNA was eluted over the course of 1 h with three 20-μl volumes of buffer B with added 1 mM PbOAc. The entire immobilization and elution process was conducted at 23 °C. The eluate was collected in an equal volume of buffer C (50 mM HEPES (pH 7.0 at 23 °C) and 80 mM EDTA) and the DNA was precipitated with ethanol. The resulting DNA was amplified in a 100-μl PCR containing 20 pmoles primer 1, 20 pmoles primer 2, 0.05 U μl^{-1} *Taq* polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3 at 23 °C), 0.01 % gelatin, and 0.2 mM of each dNTP for 30 cycles of 10 sec at 92 °C, 30 sec at 50 °C, and 30 sec at 72 °C. The reaction products were extracted twice with phenol and once with chloroform/isoamyl alcohol, and the DNA was recovered by precipitation with ethanol. The amplified DNA (~4 pmoles) was added to a second, nested PCR containing 100 pmoles primer 1, 100 pmoles primer 3b, 20 μCi [α-^{32}P]dATP, and 0.1 U μl^{-1} *Taq* polymerase, in a total volume of 200 μl, then amplified for 10 cycles of 1 min at 92 °C, 1 min at 50 °C, and 1 min at 72 °C. The PCR products were once more extracted and precipitated, and the resulting DNA was resuspended in 50 μl buffer A, then used to begin the next round of selection.

The second and third rounds were carried out as above, except that the nested PCR at the end of the third round was performed in a 100-μl volume. During the fourth round, the elution time following addition of Pb^{2+} was reduced to 20 min (two 20-μl elution volumes) and only half of the recovered DNA was used in the first PCR, which involved only 15 temperature cycles. During the fifth round, the elution time was reduced to 1 min (two 20-μl elution volumes) and only one-fourth of the recovered DNA was used in the first PCR, which involved 15 temperature cycles. DNA obtained after the fifth round of selection was subcloned and sequenced, as described previously [32].

Kinetic analysis of catalytic DNAs

Populations of DNA and various individual subclones were prepared with a 5'-^{32}P label by asymmetric PCR in a 25-μl

reaction mixture containing 10 pmoles primer 3a, 0.5 pmoles input DNA, and 0.1 U μl^{-1} *Taq* polymerase, under conditions described above, for 10 cycles of 1 min at 92 °C, 1 min at 50 °C, and 1 min at 72 °C. The resulting [5'-^{32}P]-labeled amplification products were purified by electrophoresis in a 10 % polyacrylamide/8 M gel. Self-cleavage assays were carried out following preincubation of the DNA in buffer B for 10 min. Reactions were initiated by addition of PbOAc to 1 mM final concentration and were terminated by addition of an equal volume of buffer C. Reaction products were separated by electrophoresis in a 10 % polyacrylamide/8 M gel. Kinetic assays under multiple-turnover conditions were carried out in buffer B that included 50 μg ml^{-1} BSA to prevent adherence of material to the vessel walls. Substrate and enzyme molecules were preincubated separately for 5 min in reaction buffer that lacked Pb^{2+}, then combined, and the reaction was initiated by addition of PbOAc to a final concentration of 1 mM.

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References

1. Kruger, K., Grabowski, P.J., Zaug, A.J., Sands, J., Gottschling, D.E. & Cech, T.R. (1982). Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence. *Cell* **31**, 147–157.
2. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* **35**, 849–857.
3. Cech, T.R. (1993). Structure and mechanism of the large catalytic RNAs: group I and group II introns and ribonuclease P. In *The RNA World.* (Gesteland, R.F. & Atkins, J.F., eds), pp. 239–269, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
4. Pyle, A.M. (1993). Ribozymes: a distinct class of metalloenzymes. *Science* **261**, 709–714.
5. Symons, R.H. (1994). Ribozymes. *Curr. Opin. Struct. Biol.* **4**, 322–330.
6. Joyce, G.F. (1994). *In vitro* evolution of nucleic acids. *Curr. Opin. Struct. Biol.* **4**, 331–336.
7. Breaker, R.R. & Joyce, G.F. (1994). Inventing and improving ribozyme function: rational design versus iterative selection methods. *Trends Biotechnol.* **12**, 268–275.
8. Chapman, K.B. & Szostak, J.W. (1994). *In vitro* selection of catalytic RNAs. *Curr. Opin. Struct. Biol.* **4**, 618–622.
9. Paquette, J., Nicoghosian, K., Qui, G.-A., Beauchemin, N. & Cedergren, R. (1990). The conformation of single-stranded nucleic acids: tDNA versus tRNA. *Eur. J. Biochem.* **189**, 259–265.
10. Perreault, J.-P., Wu, T., Cousineau, B., Ogilvie, K.K. & Cedergren, R. (1990). Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity. *Nature* **344**, 565–567.
11. Williams, D.M., Pieken, W.A. & Eckstein, F. (1992). Function of specific 2'-hydroxyl groups of guanosines in a hammerhead ribozyme probed by 2' modifications. *Proc. Natl. Acad. Sci. USA* **89**, 918–921.
12. Yang, J.-H., Usman, N., Chartrand, P. & Cedergren, R. (1992). Minumum ribonucleotide requirement for catalysis by the RNA hammerhead domain. *Biochemistry* **31**, 5005–5009.
13. Chartrand, P., Harvey, S.C., Ferbeyre, G., Usman, N. & Cedergren, R. An oligodeoxynucleotide with catalytic properties. In *RNA Processing.* p77 Proceedings of the RNA Society, Madison, Wisconsin.
14. Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H. & Toole, J.J. (1992). Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* **355**, 564–566.
15. Ellington, A.D. & Szostak, J.W. (1992). Selection *in vitro* of single-stranded DNA molecules that fold into specific ligand-binding structures. *Nature* **355**, 850–852.
16. Wyatt, J.R., *et al.*, & Ecker, D.J. (1994). Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion. *Proc. Natl. Acad. Sci. USA* **91**, 1356–1360
17. Wang, K.Y., McCardy, S., Shea, R.G., Swaminathan, S. & Bolton, P.H. (1993). A DNA aptamer which binds to and inhibits thrombin exhibits a new structural motif for DNA. *Biochemistry* **32**, 1899–1904.
18. Macaya, R.F., Schultz, P., Smith, F.W., Roe, J.A. & Feigon, J. (1993). Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc. Natl. Acad. Sci. USA* **90**, 3745–3749.
19. Padmanabhan, K., Padmanabhan, K.P., Ferrara, J.D., Sadler, J.E. & Tulinsky, A. (1993). The structure of α-thrombin inhibited by a 15-mer single-stranded DNA aptamer. *J. Biol. Chem.* **268**, 17651–17654.
20. Albery, W.J. & Knowles, J.R. (1976). Evolution of enzyme function and the development of catalytic efficiency. *Biochemistry* **15**, 5631–5640.
21. Forster, A.C. & Symons, R.H. (1987). Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* **49**, 211–220.
22. Uhlenbeck, O.C. (1987). A small catalytic oligoribonucleotide. *Nature* **328**, 596–600.
23. Hampel, A. & Tritz, R. (1989). RNA catalytic properties of the minimum (-)sTRSV sequence. *Biochemistry* **28**, 4929–4933.
24. Pan, T. & Uhlenbeck, O.C. (1992). *In vitro* selection of RNAs that undergo autolytic cleavage with Pb^{2+}. *Biochemistry* **31**, 3887–3895.
25. Pan, T. & Uhlenbeck, O.C. (1992). A small metalloribozyme with a two-step mechanism. *Nature* **358**, 560–563.
26. Dirheimer, G., *et al.*, & Werner, C. (1972). Primary structure of transfer RNA. *Biochimie* **54**, 127–144.
27. Rubin, J.R. & Sundaralingam, M. (1983). Lead ion binding and RNA chain hydrolysis in phenylalanine tRNA. *J. Biomol. Struct. Dyn.* **1**, 639–646.
28. Brown, R.S., Dewan, J.C. & Klug, A. (1985). Crystallographic and biochemical investigation of the lead(II)-catalyzed hydrolysis of yeast phenylalanine tRNA. *Biochemistry* **24**, 4785–4801.
29. Pan, T., Long, D.M. & Uhlenbeck, O.C. (1993). Divalent metal ions in RNA folding and catalysis. In *The RNA World.* (Gesteland R.F. & Atkins J.F., eds), pp. 271–302, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
30. Brown, R.S., Hingerty, B.E., Dewan, J.C. & Klug, A. (1983). Pb(II)-catalyzed cleavage of the sugar-phosphate backbone of yeast $tRNA^{Phe}$ — implications for lead toxicity and self-splicing RNA. *Nature* **303**, 543–546.
31. Breaker, R.R., Banerji, A. & Joyce, G.F. (1994). Continuous *in vitro* evolution of bacteriophage RNA polymerase promoters. *Biochemistry* **33**, 11980–11986.
32. Tsang, J. & Joyce, G.F. (1994). Evolutionary optimization of the catalytic properties of a DNA-cleaving ribozyme. *Biochemistry* **33**, 5966–5973.

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