In vitro selection of self-cleaving DNAs

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Background: Ribozymes catalyze an important set of chemical transformations in metabolism, and 'engineered' ribozymes have been made that catalyze a variety of additional reactions. The possibility that catalytic DNAs or 'deoxyribozymes' can be made has only recently been addressed. Specifically, it is unclear whether the absence of the 2′ hydroxyl renders DNA incapable of exhibiting efficient enzyme-like activity, making it impossible to discover natural or create artificial DNA biocatalysts.

Results: We report the isolation by *in vitro* selection of two distinct classes of self-cleaving DNAs from a pool of random-sequence oligonucleotides. Individual catalysts from 'class I' require both Cu^{2+} and ascorbate to mediate oxidative self-cleavage. Individual catalysts from class II use Cu^{2+} as the sole cofactor. Further optimization of a class II individual by *in vitro* selection yielded new catalytic DNAs that facilitate Cu^{2+}-dependent self-cleavage with rate enhancements exceeding 1 000 000-fold relative to the uncatalyzed rate of DNA cleavage.

Conclusions: Despite the absence of 2′ hydroxyls, single-stranded DNA can adopt structures that promote divalent-metal-dependent self-cleavage via an oxidative mechanism. These results suggest that an efficient DNA enzyme might be made to cleave DNA in a biological context.

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Introduction

Biological catalysis is dominated by enzymes made of protein and, to a lesser extent, by enzymes made of RNA [1–4]. In contrast, DNA is used only as a medium for the storage of genetic information. DNA is considerably more resistant than RNA to degradation in aqueous solutions and hence is well-suited to serve as a reservoir for biological information. The relative instability of RNA is due to the ribose 2′ hydroxyl. In the presence of certain cationic metals or under alkaline conditions, this hydroxyl forms a 2′ oxyanion that acts as a nucleophile in a transesterification reaction, cleaving the RNA chain. RNA phosphodiesters cleave with a rate (k_{obs}) of ~10^{-7} min^{-1} under conditions of ionic strength and temperature that approximate that of a typical mammalian cell [5]. The hydrolytic cleavage of DNA has been assessed using the model compound dimethyl phosphate. Phosphate ester hydrolysis proceeds with an estimated rate of ~10^{-12} min^{-1} in aqueous solution [6], indicating a half life in excess of one million years for DNA phosphodiester hydrolysis.

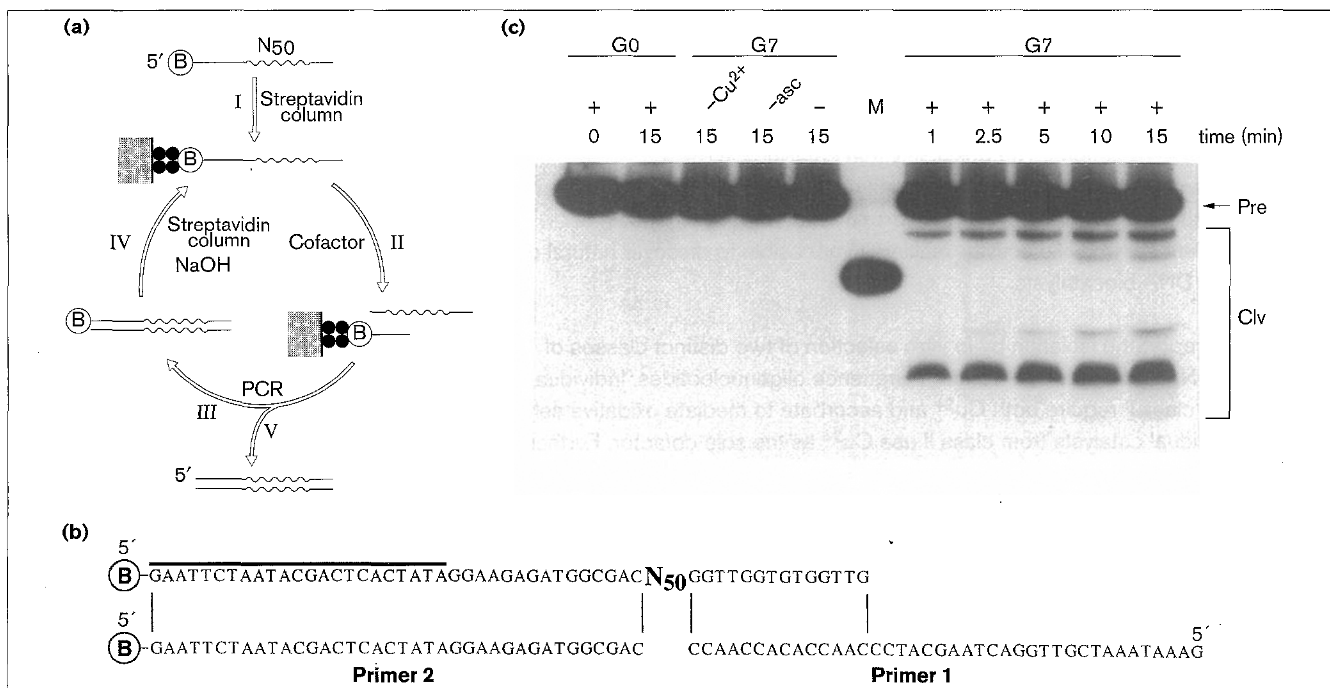
In biological polymers, primary hydroxyls can serve both as acceptors and donors in hydrogen-bonding interactions that facilitate tertiary structure formation. It is generally accepted that hydrogen-bonding contacts involving 2′ hydroxyl groups are essential for RNA's ability to form complex tertiary structure and for its catalytic activity [7]. But does the absence of the 2′ hydroxyl render DNA incapable of efficiently catalyzing various chemical reactions? *In vitro* selection for catalytic polynucleotides [8,9] has been used recently to create several classes of DNA enzymes, including DNAs that cleave RNA [5,10], ligate chemically-activated DNAs [11] and promote porphyrin metallation [12]. To further examine the catalytic potential of DNA, we have employed an *in vitro* selection protocol (Fig. 1a) to isolate DNA catalysts that promote their own cleavage.

DNA is more susceptible to scission via depurination followed by β-elimination or via oxidative mechanisms than by hydrolysis [13]. To begin our comprehensive search for artificial DNA-cleaving DNA enzymes, we chose to screen for DNAs that facilitate self-cleavage by a redox-dependent mechanism. Cleavage of DNA by chelates of redox-active metals (e.g. Fe^{3+}, Cu^{2+}) in the presence of a reducing agent is expected to be a more facile alternative to DNA phosphoester hydrolysis due to the reactivity of hydroxyl radicals that are produced by reduction of H_2O_2 (i.e. by the Fenton reaction). Moreover, a variety of natural and artificial 'chemical nucleases' rely on similar cleavage mechanisms [14,15].

Results and discussion

Beginning with a pool of ~2×10^{13} random-sequence DNAs (Fig. 1b), we carried out eight rounds of selection [5,10] (see Materials and methods) for DNAs that self-cleave in the presence of $CuCl_2$ and ascorbate. The DNA pool that was isolated after seven rounds (G7 DNA) displays robust self-cleavage activity that requires both Cu^{2+} and ascorbate (Fig. 1c). Trace amounts of non-specific DNA cleavage can be detected with Cu^{2+} and ascorbate

Figure 1



In vitro selection of self-cleaving DNAs. **(a)** (I) A pool of 5′-biotinylated DNAs is immobilized on a streptavidin matrix, washed to remove unbound DNAs, then (II) eluted under the desired reaction conditions to separate self-cleaving DNAs from those that are inactive. (III) Selected DNAs are amplified by the polymerase chain reaction (PCR) and (IV) the selection round is completed by immobilizing the resulting double-stranded DNAs on new matrix followed by removal of the non-biotinylated strand by chemical denaturation. (V) The pool is prepared for further analysis by PCR amplification with non-biotinylated primers. Encircled B indicates 5′ biotin. **(b)** The construct used for the initial round of selection contains a domain of 50 random-sequence nucleotides (N_{50}) flanked by 38 and 14 nucleotides of defined sequence. DNAs used in subsequent rounds carry an additional 26 nucleotides, as defined by primer 1. Precursors that cleave within the overlined region retain sufficient 5′ primer binding site for amplification and are expected to be favored during selection. **(c)** Self-cleavage activity of the initial DNA pool (G0) and the pool isolated after seven rounds (G7) of selection. 5′ ^{32}P-labeled precursor DNA (Pre) was incubated in the presence (+) or absence (–) of 10 μM each of Cu^{2+} and ascorbate, or in the absence of Cu^{2+} or ascorbate (–Cu^{2+} and –asc, respectively) for various times as indicated. M is 5′ ^{32}P-labeled primer 3 and Clv identifies cleavage products.

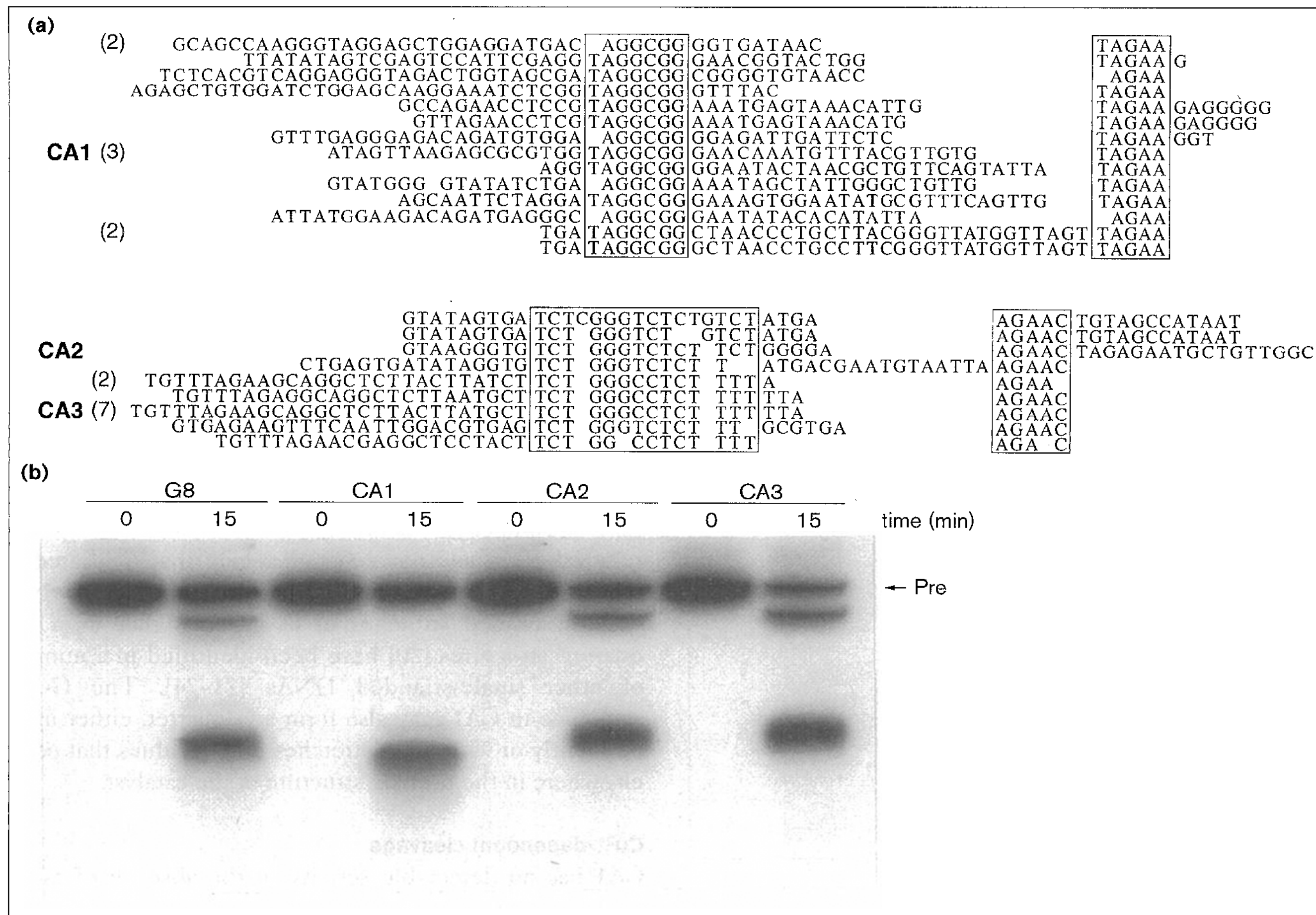
concentrations of 100 μM or above (data not shown), but no cleavage of random-sequence (G0) DNA was detected under the final selection conditions (10 μM of each cofactor). In contrast, incubation of G7 DNA yields a number of distinct DNA cleavage products, suggesting that the pool contains multiple classes of DNAs that promote self-cleavage at unique sites.

Two classes of self-cleaving DNA molecules

Sequence analysis of individual DNAs from G8 reveals a diverse set of catalysts that we divided into two groups (Fig. 2a) based on sequence similarities. Cleavage assays from three representative DNAs (CA1, CA2 and CA3) confirm that two distinct classes of catalysts have been isolated (Fig. 2b). We expected that the cleavage sites for the selected catalysts would reside exclusively within the first 23 nucleotides of the original construct (Fig. 1b). Cleavage in this region would result in release of the molecule from the solid matrix, yet the cleaved molecules would retain enough of the original primer-binding site to allow amplification by PCR. Cleavage elsewhere in a molecule would release a DNA fragment that has lost the 5′-terminal primer-binding site, and would be incapable of significant amplification during PCR. CA1 promotes DNA cleavage within this expected region. Surprisingly, however, both CA2 and CA3 cleave not only at the expected region near the 5′ terminus (Clv 1), but also at sites in a distal region (Clv 2) within the domain that was randomized in the original DNA pool. The Clv 1/Clv 2 product ratio of CA3 is ~2:1.

The distribution of cleavage products between the two sites in CA3 is expected to result in a significant disadvantage during the selection process. About 35 % of CA3-like molecules cleave within the center of the molecule (and therefore are probably not amplified), while only about 65 % cleave at the expected site and can be perpetuated in the next round of selection via amplification by PCR. In contrast, 100 % of the catalysts that cleave exclusively in the primer-binding region can be amplified, giving individuals from class I an apparent selective advantage. However, CA3-like catalysts were found to persist in additional rounds of *in vitro* selection and actually come

Figure 2



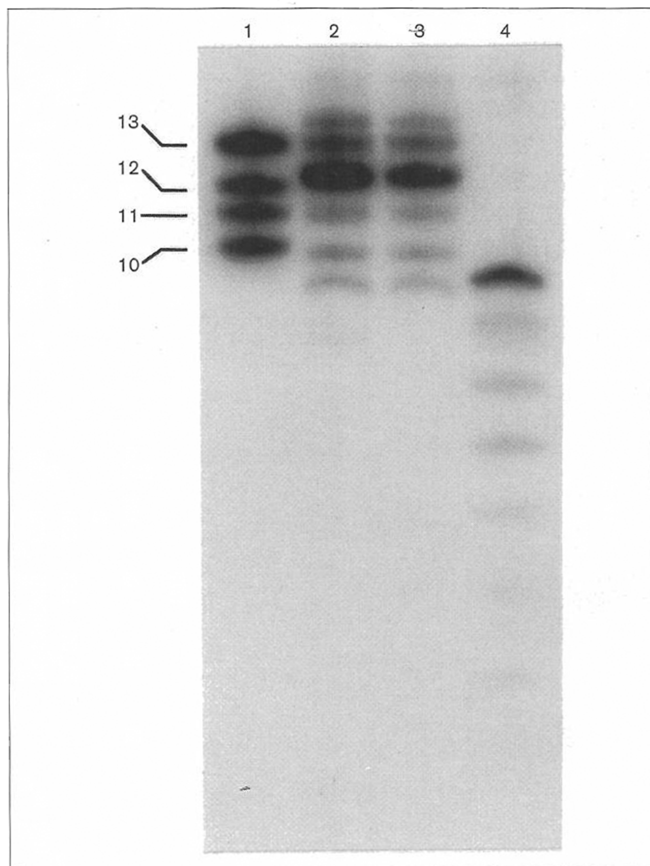
Sequence analysis and catalytic activity of individual G8 DNAs. **(a)** Alignment of 34 sequences reveal the presence of two major classes of molecules that are characterized by sets of common sequences (boxed nucleotides). DNA sequences that were encountered more than once are identified by noting the number of occurrences in parentheses. **(b)** Self-cleavage activity of ~5 nM 5′ ^{32}P-labeled precursor DNA from G8 DNA and from individuals CA1, CA2 and CA3 in the absence (–) or presence (+) of Cu^{2+} and ascorbate (10 μM each).

to dominate the population by generation 13. The success of these catalysts can be understood, in part, by examining the catalytic rates of CA1 and CA3. The cleavage rate (k_{obs}) of 0.018 min^{-1} was obtained for CA1 under the final selection conditions, while cleavage at Clv 1 of CA3 occurs with a k_{obs} of 0.14 min^{-1}. Despite a high frequency of cleavage in the distal region, class II catalysts more rapidly cleave at the correct site, giving CA3-like catalysts a distinct selective advantage over catalysts from class I.

Cleavage sites for both classes have been further localized by gel-mobility analysis of the 5′ ^{32}P-labeled self-cleavage products (Fig. 3). CA1 produces a major cleavage product with a gel mobility that corresponds to a 9-nucleotide fragment, and also yields a series of minor products that correspond to DNAs of 3–8 nucleotides. The cleavage site heterogeneity observed for CA1 is consistent with an oxidative cleavage mechanism that involves a diffusible hydroxyl radical. Typically, cleavage of nucleic acids by an oxidative cleaving agent occurs over a range of nucleotides, with a primary cleavage site flanked on each side by sites that are cleaved with decreasing frequency. It has been suggested that the frequency of DNA cleavage is proportional to the inverse of the distance that separates the target phosphoester linkage and the generation site of the hydroxyl radical [16]. The distribution of cleavage products formed by CA1 is, however, indicative of a unique active site that permits localized DNA cleavage to occur only at nucleotides that immediately flank the 5′ side of the major cleavage site.

Similarly, Clv 1 of CA3 consists of a series of products that range in mobility from 9 to 14 nucleotides, with the major product corresponding to a 12-nucleotide DNA (Fig. 3). The major product formed upon DNA scission at Clv 2 corresponds to 70 nucleotides, with minor products corresponding to DNAs of 66–69 nucleotides (data not shown). The most frequent site of cleavage at Clv 2 is located near position 34 (G) of the original random-sequence domain.

Figure 3



Cleavage site analysis of CA3 (lane 2), an optimized variant (variant 1, see Fig. 5b) of CA3 (lane 3) and CA1 (lane 4). DNA size markers (lane 1) are 5′ ^{32}P-labeled DNAs of 10–13 nucleotides as indicated. The nucleotide sequence of these markers correspond to the 5′ terminal constant region of the precursor.

Oxidative cleavage of DNA can proceed by a variety of pathways, each of which produces distinct cleavage-product termini [17]. Therefore, conformation of these cleavage sites must now proceed by conducting a more detailed analysis of the chemical structures of the reaction products.

Secondary structure of CA1

To gain insight into the secondary structure of CA1, we produced an artificial phylogeny [18] of functional CA1 sequence variants for comparative sequence analysis [19]. We mutagenized the 50 nucleotides that correspond to the original random-sequence domain by preparing a synthetic DNA pool such that each wild type nucleotide occurs with a probability of 0.85 and each remaining nucleotide occurs with a probability of 0.05. The resulting pool was subjected to five additional rounds of selection for activity in the presence of 10 μM each of Cu^{2+} and ascorbate. Sequence alignment of 39 resulting clones (Fig. 4a) reveal two main regions (nucleotides 20–28 and 41–50) of strictly-conserved sequence interspersed with regions that tolerate variation. A total of 25 positions experienced two mutations or less. Other positions show sequence covariation, indicating that these nucleotides may make physical contact in the active conformation of the deoxyribozyme. For example, A32 and G40 frequently mutate to C or T, respectively. This suggests a preference for these bases to pair as C–G or A–T. Indeed, this inferred pairing occurs in a region (nucleotides 28–44) that has considerable base-pairing potential, consistent with the formation of a hairpin structure.

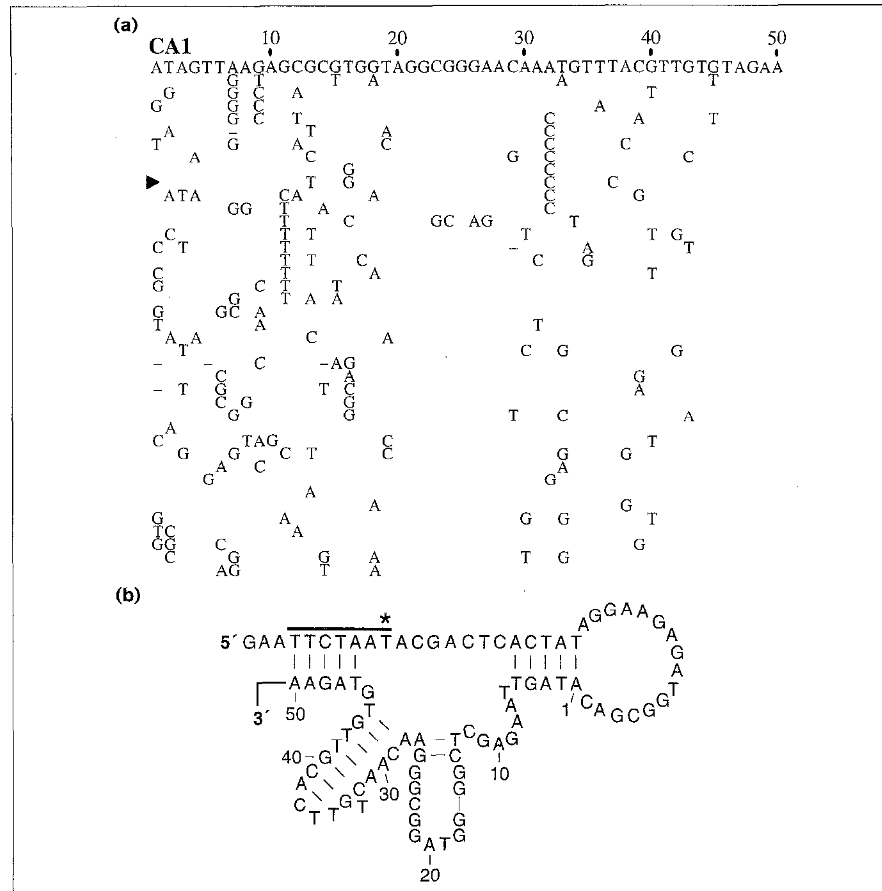
Using sequence data and truncation analyses, we have constructed a partial secondary-structure model for CA1 (Fig. 4b). Both the 5′- and 3′-terminal nucleotides show significant base-pairing potential with the substrate domain of the molecule. The putative hairpin domain described above (nucleotides 28–44) is flanked by the conserved 3′ terminus and by a highly-conserved region that is composed mainly of G residues. We find that removal of an additional G-rich region that is located in the 3′ primer binding site abolishes the catalytic activity of CA1. Extended stretches of G residues that form 'G-quartet' structures [20] have been identified in a number of other single-stranded DNAs [21–24]. The G-rich sequence in CA1 may also form a G-quartet, either independently or with other stretches of G residues that occur elsewhere in the primary structure of the catalyst.

Cu^{2+}-dependent cleavage

CA1 has no detectable activity in the absence of ascorbate, but, surprisingly, both the G8 population DNA and CA3 display significant cleavage when only Cu^{2+} is added (Fig. 5a). A $k_{obs} = 8 \times 10^{-4}$ min^{-1} for Clv 1 was measured for CA3 in the presence of 10 μM Cu^{2+} in the absence of ascorbate. We employed *in vitro* selection to isolate CA3 variants with enhanced Cu^{2+}-dependent activity. CA3 was mutagenized (see above) and subjected to five rounds of selection using 10 μM Cu^{2+} as the sole cofactor. Sequence alignment of 40 resulting clones (Fig. 5b) reveal a single region of highly-conserved sequence, spanning nucleotides 15–50 of the original random-sequence domain. The identities of 27 nucleotides within this region were so well conserved that they were found to vary in only 3 or fewer of the 40 individual sequences examined. The most notable exceptions to this sequence conservation are a T deletion between nucleotides 39 and 45, and a T to G mutation at nucleotide 28. In a related selection experiment, we have isolated active variants of CA3 in which nucleotides 1 through 20 of the original random-sequence domain have been deleted (S. Balkhi and R.R.B., unpublished data).

The catalytic activity of the reselected CA3 pool improved by nearly 100-fold, with variant DNAs 1, 2 and 3 (Fig. 5b) displaying k_{obs} values of 0.052 min^{-1}, 0.033 min^{-1} and 0.043 min^{-1}, respectively. The uncatalyzed rate of DNA cleavage

Figure 4



Sequence requirements and secondary-structure model of CA1. **(a)** An artificial phylogeny of CA1 variants. Numbered sequence is wild-type CA1 and nucleotides of variants that differ from this sequence are aligned below. A dash indicates a deleted nucleotide. **(b)** Partial secondary-structure model for a variant of CA1 (arrowhead). Numbered nucleotides are derived from the region that was randomized in the starting pool. The asterisk indicates the primary cleavage site and the bar defines the region that undergoes detectable cleavage. Not detailed are nucleotides within the 3′ primer binding site that are also required for catalytic activity.

in the presence of Cu^{2+} was assessed by incubating 5′ ^{32}P-labeled DNA oligomer (primer 3) under identical conditions. No Cu^{2+}-dependent cleavage of DNA was detected, even after a two-week incubation at 23 °C. We estimate the overall rate enhancement of the CA3 variants to be considerably greater than 10^6-fold compared to the uncatalyzed rate. Both CA3 and variant 1 probably use the same DNA cleavage mechanism, as evident by their similar catalytic cleavage patterns (Fig. 3). A synthetic 87-nucleotide version of variant 1 that lacks the 3′-terminal primer-binding site remains active (k_{obs} = 0.02 min^{-1} for Clv 1, 10 μM Cu^{2+}), while an inhibitory effect is observed with 100 μM Cu^{2+}. In addition, the self-cleavage activity of this truncated DNA has a pH optimum of 7.5, with no specific monovalent cation requirement. Sequential deletion of nucleotides from the 5′ terminus of this DNA results in a progressive reduction in catalytic activity, with a four-nucleotide deletion resulting in nearly complete loss of function.

Mechanism of cleavage

Our success in isolating a variety of self-cleaving DNAs with Cu^{2+}/ascorbate-dependence is consistent with an earlier report [25] of site-specific cleavage of a single-stranded DNA under similar conditions. These results confirm that DNA is indeed capable of forming a variety of structures that promote chemical transformations. In addition, the catalytic rates for both classes of self-cleaving DNAs compare favorably to those attained by other deoxyribozymes and by natural and artificial ribozymes. The finding that DNA is also able to perform self-cleavage with Cu^{2+} alone is unexpected, since the mechanism for the oxidative cleavage of DNA also requires a reducing agent such as ascorbate or a thiol compound [14,15].

A number of chemical nucleases have been prepared by others and examined for their potential as site-specific DNA-cleaving agents. For example, 1,10-phenanthroline and similar agents bind DNA, presumably via intercalation, and position copper ions near the ribose-phosphate backbone where formation of a reactive oxygen derivative favors cleavage of the DNA chain [15]. Alternatively, metal-binding ligands have been attached to oligonucleotide probes, to construct highly-specific DNA-cleaving agents that recognize DNA by triple-helix formation [26]. The catalytic DNAs described in this report probably replace the role of chemical nucleases by forming their own metal-binding pockets so as to promote region-specific self-cleavage. In fact, 1,10-phenanthroline

Figure 5

(a)

(b)

CA3

TGTTTAGAAGCAGGCTCTTACTTATGCTTCTGGGCCTCTTTTTTAAGAAC

A Cu^{2+}-dependent self-cleaving DNA. **(a)** Cleavage assay of G8 DNA, CA1, CA3 and the optimized population of CA3 variants that was isolated after mutagenesis followed by five additional rounds of selection. **(b)** Sequence alignment of the mutagenized domain of individual CA3 variants that have been optimized for catalytic function with Cu^{2+}. Numbered sequence is wild-type CA3 and nucleotides of variants that differ from this sequence are aligned below. A dash indicates a deleted nucleotide. 5′- and 3′-terminal primer sequences are not shown. Arrowheads identify CA3 variants 1–3 as denoted. An asterisk and a bar indicate the major and minor Clv 2 cleavage sites, respectively.

actually inhibits the catalytic function of a synthetic class II DNA. The optimal Cu^{2+} concentration for the 87-nucleotide DNA is ~10 μM; catalytic activity is significantly lower at either 1 or 100 μM Cu^{2+}. The inhibitory effect of 1,10-phenanthroline might be due to the reduction in concentration of free Cu^{2+} upon formation of Cu^{2+}–phenanthroline complexes.

Several different mechanisms for the oxidative cleavage of class II DNAs seem possible. For example, the class II DNAs may simply scavenge for trace amounts of copper and reducing agents that are present in the reaction buffer. Alternatively, these DNA molecules might make use of an internal chemical moiety as the initial electron donor. In each example, the catalytic DNAs could still cleave by an oxidative mechanism, but would at least appear to be independent of the need for an external source of reducing agent. The importance of H_2O_2 in oxidative processes can be examined with catalase, an enzyme that efficiently promotes the dismutation of H_2O_2 molecules to yield water and molecular oxygen. The catalytic activity of a representative DNA from class II is completely inhibited by catalase, consistent with the notion that H_2O_2 is a necessary intermediate in an oxidative pathway for DNA cleavage (data not shown). The catalytic rate of CA3 variants is greatly increased when incubated in the presence of added H_2O_2. For example, the 87-nucleotide DNA can be made to cleave

quantitatively at Clv 1 (k_{obs} = 1.5 min^{-1}) in the presence of 10 μM Cu^{2+} and 35 mM H_2O_2.

We have yet to determine whether the H_2O_2 required by the catalyst is derived in trace amounts from water, or whether the DNA can itself produce H_2O_2 in the absence of a reducing agent. Preincubation of separate solutions (both containing catalase) of catalytic DNA in reaction buffer (minus Cu^{2+}) and of aqueous Cu^{2+}, followed by thermal denaturation of the catalase, results in full self-cleavage activity when the two solutions are mixed, suggesting that contaminating H_2O_2 may not be important for catalysis. We also find that self-cleavage of the 87-nucleotide variant reaches a combined maximum (Clv 1 + Clv 2) of ~70 %, regardless of the concentration of catalytic DNA present in the reaction, again suggesting that trace contaminants are not important for catalysis. Similarly, preincubation of a reaction mixture with excess unlabeled catalyst (1 μM) followed by the addition of a trace amount of identical 5′ ^{32}P-labeled catalysts produces normal yields of labeled DNA-cleavage products. Finally, addition of fresh reaction buffer to a previously-incubated reaction mixture does not promote further DNA cleavage, as might be expected if limiting amounts of reducing agent were responsible for activity.

Certain constructs of the self-splicing ribozyme of *Tetrahymena* have been shown to catalyze the cleavage of DNA via a transesterification mechanism [27,28], and the ribozyme from RNase P has been found to cleave DNA by hydrolysis [29]. Such ribozymes might also be made to serve as therapeutic DNA-cleaving agents, analogous to the function of RNA-cleaving 'catalytic antisense' ribozymes [30]. The secondary-structure model of CA1 (Fig. 4b) includes stretches of predicted base pairing both 5′ and 3′ to the primary cleavage site, suggesting that 'substrate' and 'enzyme' domains can be separated. Likewise, preliminary analysis of class II molecules reveals similar base complementation. We expect that both class I and class II DNAs can be engineered to create catalytic DNAs that specifically cleave DNA substrates with multiple turn-over kinetics. It might also be practical to engineer highly stable DNA enzymes, with user-defined substrate specificity, that cleave DNA *in vivo*.

Significance

We have isolated two distinct classes of DNAs that promote their own cleavage. One class requires only copper as a cofactor and catalyzes the oxidative cleavage of DNA with a rate in excess of 1 000 000-fold faster than background. Extensive regions of both classes of self-cleaving DNAs are important for the formation of catalytic structures, as suggested by sequence conservation found with selected individuals. These results support the view that DNA, despite the absence of ribose 2′-hydroxyl groups, has considerable potential to adopt higher-ordered structures with functions that are similar to ribozymes.

Self-cleaving DNAs with catalytic activity that is similar to that of the DNAs described in this report could conceivably serve a biological function in the processing of single-stranded DNAs; the four natural self-cleaving ribozymes have a similar role in processing pathogenic and other multimeric single-stranded RNAs [2]. Whether DNA enzymes exist in nature or not, we believe that it may now be possible to create a wide range of engineered DNA enzymes for application as highly-stable artificial biocatalysts.

Materials and methods

Oligonucleotides

All synthetic DNAs were prepared by automated chemical synthesis (Keck Biotechnology Resource Laboratory, Yale University). The starting pool is composed of DNAs that carry a 5′-terminal biotin moiety and a central domain of 50 random-sequence nucleotides. Primer 3 is an analog of primer 1 (Fig. 1b) that contains a 3′-terminal ribonucleoside. Primer 4 is the nonbiotinylated version of primer 2 (Fig. 1b). Primer 5 is the 5′-biotinylated form of primer 1.

In vitro *selection*

A total of 40 pmoles of pool DNA in 40 μl buffer A (50 mM HEPES, pH 7.0 at 23 °C, 0.5 M NaCl, 0.5 M KCl) was loaded on two streptavidin-matrix columns (Affinitip Strep20, Genosys Biotechnologies) and incubated for ~5 min. Unbound DNAs were subsequently removed from each column by pre-elution with 500 μl of buffer A, then by 500 μl 0.2 N NaOH, and the resulting matrix-bound DNAs were equilibrated with 500 μl buffer A. Catalytic DNAs were eluted with three successive 20-μl aliquots of buffer B (buffer A, 100 μM $CuCl_2$, 100 μM ascorbate) for rounds 1–3, or buffer C (buffer A, 10 μM $CuCl_2$, 10 μM ascorbate) for rounds 4–8. Eluate from each column was combined with 120 μl 4 mM EDTA and 40 pmoles each of primers 1 and 2. Selected DNAs and added primers were recovered by precipitation with ethanol and amplified by PCR in a 200-μl reaction containing 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 each dNTP for 25 cycles of 10 s at 92 °C, 10 s at 50 °C and 30 s at 72 °C. The 5′-terminal region of each cleaved DNA, including the biotin moiety, was reintroduced at this stage. Subsequent rounds were performed by immobilizing 20 pmoles of pool DNA on a single streptavidin column and selected DNAs were amplified in a 100 μl reaction for 10–20 temperature cycles. Steps II–IV (Fig. 1) were repeated until the population displayed the desired catalytic activity, at which time the pool was PCR-amplified with primers 1 and 3, cloned (Original TA Cloning Kit, Invitrogen) and sequenced (Sequenase 2.0 DNA Sequencing Kit, U.S. Biochemicals). Reselections with CA1 and CA3 were initiated with 20 pmoles synthetic DNA. This is expected to offer near comprehensive representation of all sequence variants with seven or fewer mutations relative to wild type.

Catalytic assays

5′ ^{32}P-labeled precursor DNA was prepared by PCR-amplifying double-stranded DNA populations or plasmid DNA using 5′-^{32}P-labeled primer 4 and either primer 5 or primer 3. The antisense strand is removed either by binding the biotinylated strand to a streptavidin matrix (primer 5) or by alkaline cleavage of the RNA phosphodiester-containing strand, followed by PAGE purification (primer 3). DNA self-cleavage assays (~5 nM 5′ ^{32}P-labeled precursor DNA) were conducted at 23 °C in buffer A, with cofactors added as detailed for each experiment. For both *in vitro* selection and for assays, reaction buffers that contained ascorbate were prepared just prior to use. Self-cleavage assays conducted with catalase (bovine liver, Sigma) contained 50 mM HEPES

(pH 7.0 at 23°C), 50 mM NaCl, 10 μM $CuCl_2$, and 0.5 U μl^{-1} catalase, and were incubated at room temperature for 20 min. Catalase activity was destroyed by heating at 90°C for 5 min. Products were separated by denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE) using a 10% gel and visualized by autoradiography or visualized and quantitated by PhosphorImager (Molecular Dynamics).

Cleavage product analysis

Primary cleavage sites for CA1 and CA3 were identified by incubating 5′ ^{32}P-labeled precursor DNA in buffer C and examining the gel mobility of the 5′-terminal cleavage fragments by denaturing 20% PAGE as compared to a series of 5′ ^{32}P-labeled synthetic DNAs that correspond in sequence to the 5′ terminus of the precursor DNAs. Products resulting from scission at Clv 2 were analyzed by denaturing 6% PAGE.

Kinetic analysis

Catalytic rates were obtained by plotting the fraction of precursor DNA cleaved versus time and establishing the slope of the curve that represents the initial velocity of the reaction as determined by a least-squares fit to the data. Kinetic assays were conducted in buffer C or in buffer A plus 10 μM $CuCl_2$ as indicated for each experiment. Rates obtained from replicate experiments differed by less than two fold and the values reported are averages of at least two analyses.

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