Capping DNA with DNA†

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ABSTRACT: Twelve classes of deoxyribozymes that promote an ATP-dependent "self-capping" reaction were isolated by in vitro selection from a random-sequence pool of DNA. Each deoxyribozyme catalyzes the transfer of the AMP moiety of ATP to its 5′-terminal phosphate group, thereby forming a 5′,5′-pyrophosphate linkage. An identical DNA adenylate structure is generated by the T4 DNA ligase during enzymatic DNA ligation. A 41-nucleotide class 1 deoxyribozyme requires Cu^{2+} as a cofactor and adopts a structure that recognizes both the adenine and triphosphate moieties of ATP or dATP. The catalytic efficiency for this DNA, measured at 10^4 M^{-1}•min^{-1} using either ATP or dATP as substrate, is similar to other catalytic nucleic acids that use small substrates. Chemical probing and site-directed mutagenesis implicate the formation of guanine quartets as critical components of the active structure. The observation of ATP-dependent "self-charging" by DNA suggests that DNA could be made to perform the reactions typically associated with DNA cloning, but without the assistance of protein enzymes.

Single-stranded DNA is capable of forming intricate tertiary structures that can bind various ligands and promote chemical transformations (*1*). Although catalytic DNAs have not been found in nature, new deoxyribozymes are being isolated from random-sequence pools using various in vitro selection protocols (*2*−*5*). The catalytic repertoire of DNA encompasses some of the same reactions that are catalyzed by ribozymes. Moreover, the catalytic efficiency exhibited by many deoxyribozymes is comparable to that of ribozymes with identical or related catalytic activities. Therefore, it is likely that DNA could be used as a versatile format in which to build new enzymes for both in vitro (*6*) and in vivo applications (*7, 8*).

Our research has primarily focused on the ability of DNA to promote self-processing reactions and to catalyze various modification reactions on separate nucleic acid substrates. These reactions are of particular interest to those who seek new means by which to manipulate nucleic acids. Moreover, self-processing reactions are catalyzed by most natural ribozymes and thus are the most logical types of reactions to examine for possible biological relevance. Several classes of self-processing deoxyribozymes already have been isolated from random-sequence pools of DNA using in vitro selection. For example, deoxyribozymes that exhibit oxidative cleavage of DNA (*9, 10*), joining of chemically activated DNAs (*11*), and DNA phosphorylation (*12*) have been isolated. In addition, cleavage of RNA by divalent metal-dependent, histidine-dependent, and cofactor-independent deoxyribozymes has recently been reported (*4*).

In a previous study (*12*), we created nearly 50 classes of deoxyribozymes capable of catalyzing DNA phosphorylation in the presence of ribo- or deoxyribonucleoside 5′-triphosphates (NTPs or dNTPs). These 'kinase' deoxyribozymes were made to mimic the catalytic function of the protein enzyme T4 polynucleotide kinase (T4 PNK), which transfers the γ-phosphate of ATP to the 5′-terminal oxygen of a substrate oligonucleotide. In this study, we set out to isolate deoxyribozymes that would mimic one of the two catalytic functions of another DNA processing enzyme—that of T4 DNA ligase.

T4 DNA ligase catalyzes the joining of two DNA molecules when the two are properly juxtaposed within a duplex (*13*). This enzyme achieves DNA ligation by promoting two consecutive DNA modification reactions (*14*). First, the enzyme catalyzes the formation of a 5′,5′-pyrophosphate linkage between the 5′-phosphate group of the DNA donor oligonucleotide and the α-phosphate group of ATP via a covalent AMP−enzyme intermediate. The resulting 5′,5′-pyrophosphate 'cap' on the DNA donor (5′,5′-AppDNA) is subsequently used by T4 DNA ligase as an activated substrate that is joined to an acceptor oligonucleotide when both donor and acceptor are properly aligned on a template. The phosphoanhydride linkage in the cap is split upon formation of the new 3′,5′-phosphodiester linkage between donor and acceptor, with concomitant release of 5′-AMP.

We speculated that the cap structure generated as a reaction intermediate by T4 DNA ligase could also be formed by a deoxyribozyme (Scheme 1). ATP-dependent "DNA kinase" deoxyribozymes have been isolated previously (*12*), demonstrating that DNA can specifically recognize ATP as a substrate for a phosphate transfer reaction. Moreover, in vitro selected ribozymes reported recently (*15*) exhibit cap-forming activities, thus providing precedence for ribozyme-catalyzed

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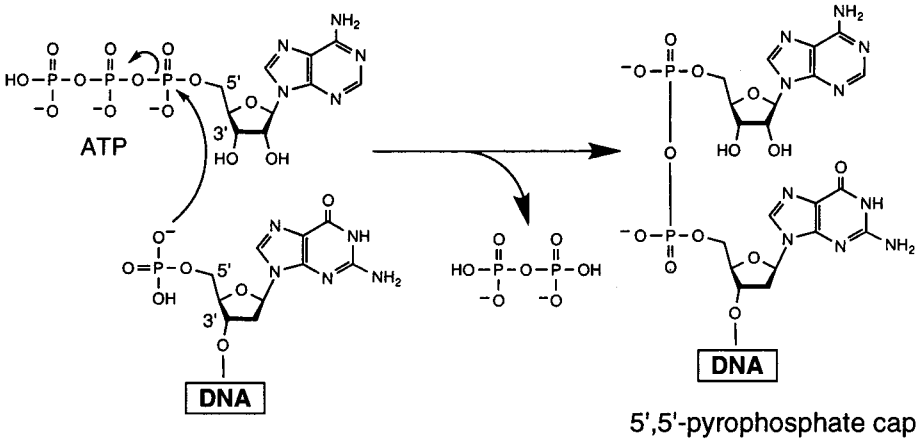
Scheme 1: Formation of a 5′,5′-Pyrophosphate Cap (5′,5′-AppDNA) by Attack of a DNA 5′-Phosphate Oxygen on the α-Phosphorus Center of ATP



phosphoanhydride exchange reactions. Therefore, we developed a new selection strategy to isolate ATP-dependent self-capping deoxyribozymes from a pool of random-sequence DNAs.

MATERIALS AND METHODS

Nucleotides and Oligonucleotides. Synthetic DNAs were prepared by standard automated synthesis (Keck Biotechnology Resource Laboratory, Yale University) and were purified by preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE) prior to use. Single-stranded DNAs were either internally ^{32}P-labled during synthesis by the polymerase chain reaction (PCR) using [α-^{32}P]dGTP, or labeled at the 5′ terminus using [γ-^{32}P]ATP and T4 polynucleotide kinase (T4 PNK). Standard ribo- and deoxyribonucleoside 5′-triphosphates (NTPs and dNTPs), [α-^{32}P]dGTP, [α-^{32}P]ATP, and [γ-^{32}P]ATP, were purchased from Amersham Pharmacia. ATP analogues were obtained from Sigma.

In Vitro Selection. Each round of selection was adapted from the general selection scheme depicted in Figure 1A. For the first round, 200 pmol of the initial DNA population (G0) was heated in 200 μL of deionized water at 90 °C for 1 min, cooled to room temperature, and combined with 250 μL of 2× selection buffer comprised of 100 mM HEPES (pH 7.0 at 23 °C), 800 mM NaCl, 200 mM KCl, 20 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM $MnCl_2$, and 100 μM $CuCl_2$. The selection reaction was initiated by the addition of 50 μL of 10 mM ATP. The mixture was incubated at 23 °C for 20 h and terminated by combining with an equal volume of 30 mM EDTA (pH 8.0). DNA was isolated from the reaction mixture by precipitation with ethanol. The pelleted DNA was separated from ATP by denaturing 10% PAGE and recovered by elution from the gel.

Recovered DNA was dissolved in 890 μL of 20 mM NaCl containing 1 μM template and 1.3 μM acceptor oligonucleotides (see Figure 1B). The mixture was incubated at 90 °C for 1 min, cooled to room temperature, and combined with 100 μL of 10× T4 DNA ligase buffer [500 mM Tris-HCl (pH 7.8 at 23 °C), 200 mM NaCl, 100 mM $MgCl_2$, 10 mg/mL BSA] and 10 μL of 400 units/μL T4 DNA ligase (NEB, Beverly, MA). Note that no ATP is included in the ligase reaction to favor the ligation of only those DNAs that acquire the appropriate 5′,5′-pyrophosphate cap. After incubation at 16 °C for 24 h, DNA was precipitated with ethanol, and the ligated molecules (approximately 123 nucleotides in length) were isolated by denaturing 10% PAGE. The DNA was eluted from the gel and amplified by PCR using 50 pmol each of primer 1 (5′-CCATCAGGATCAGCT) and primer 2 (5′-GAATTCTAATACGACTCACTATrA; rA represents a ribonucleotide) as reported previously (*16*). Amplified DNA was precipitated with ethanol, resuspended in 90 μL of 0.25 N NaOH, heated at 90 °C for 10 min to cleave the single RNA linkage (*17*−*19*), and neutralized with 10 μL of 3 M NaOAc (pH 5.2 at 23 °C). The resulting DNA products were precipitated with ethanol and separated by denaturing 10% PAGE. DNAs corresponding to the original pool size were recovered and dissolved in 87 μL of deionized water. The DNAs were phosphorylated by adding 10 μL of commercial 10× T4 polynucleotide kinase buffer (NEB) and 3 μL of 10 units/μL T4 PNK (NEB) and incubating at 37 °C for 1 h. The resulting DNA was recovered by precipitation with ethanol and used to initiate the next round of selection. Subsequent rounds of selection were performed at 1/10th scale of the initial reaction. For rounds 1 through 9, a reaction time of 20 h and an ATP concentration of 1 mM were used. Incubation times for T4 DNA ligase were shortened to 30 min at 23 °C after the first round of selection, as longer reaction times were deemed unnecessary. In addition, hypermutagenic PCR (*20*) was performed at rounds 10 and 15 to introduce additional sequence diversity. The DNA population at G22 was cloned by using the TOPO-TA cloning kit (Invitogen), and the plasmids from individual clones were sequenced using a ThermoSequenase kit (Amersham Pharmacia).

Catalytic Assays and Kinetic Analysis. To assess the catalytic activity of individual clones, internally ^{32}P-labeled DNAs (≈100 nM) were incubated at 23 °C under standard assay buffer conditions [50 mM HEPES (pH 7.0 at 23 °C), 400 mM NaCl, 10 mM $MgCl_2$, 10 μM $CuCl_2$] containing 5 μM ATP unless otherwise stated. Self-capping activity was assessed either by the same method of T4 DNA ligation and PAGE separation used for selection or by direct analysis using denaturing 15% PAGE after precipitation of the sample with ethanol (truncated DNAs only). Analysis of the resulting gels was achieved using a Molecular Dynamics PhosphorImager, and yields were quantified using ImageQuaNT software. Analysis of truncated versions of class 1 deoxyribozyme employed 5′-labeled constructs that were prepared

using T4 PNK and [γ-^{32}P]ATP. Initial rate constants (k_{obs}) for DNA capping reaction were determined by plotting the natural logarithm of the fraction of precursor converted to capped product versus time. The slope of the line that represents the negative k_{obs} of the reaction was determined by a least-squares fit to the data.

Chemical Probing with DMS. Random partial methylation of G residues within class 1 deoxyribozymes was conducted with 0.1 μM 5′ ^{32}P-labeled DNA in a reaction containing 0.2% dimethyl sulfate (DMS) in deionized H_2O that was incubated at 23 °C for 40 min. This treatment results in the methylation of the N7 positions of G residues of each DNA with approximately one modification per molecule. DNA was recovered after methylation by precipitation with ethanol. Methylation of DNA was conducted either with precursor DNA prior to the capping reaction or with capped DNA that was generated prior to methylation and purified by 15% PAGE. The methylated DNAs that retained capping activity were separated from unreacted DNAs by 15% PAGE. Both DNA preparations were subjected to methylation-dependent strand cleavage by heating to 90 °C for 30 min in 50 μL of water containing 10% piperidine. Cleavage reactions were lyophilized, and the resuspended DNA fragments were separated by 15% PAGE and imaged by autoradiography.

RESULTS AND DISCUSSION

*In Vitro Selection of Self-Capping Deoxyribozymes.*The phosphoanhydride exchange reaction depicted in Scheme 1 is expected to be the most likely route by which the desired capped structure can be formed. Specifically, a deoxyribozyme will use an oxygen on its own 5′-phosphate group to attack the phosphorus center of the α-phosphate of ATP. Considering that displacement of the pyrophosphate leaving group is very favorable, the reaction could proceed either via an S_N1 or an S_N2-like mechanism.

To isolate DNAs that form the desired 5′,5′-pyrophosphate cap in the presence of ATP, we employed the selection scheme shown in Figure 1A. This strategy exploits the fact that T4 DNA ligase forms a 5′,5′-AppDNA structure that is an essential intermediate of the enzymatic ligation of DNA. If T4 DNA ligase is not supplied with ATP, then only DNAs that carry a preformed 5′,5′-pyrophosphate cap are available for ligation. In our selection scheme, individual DNAs from the random-sequence pool are ligated to an acceptor oligonucleotide by T4 DNA ligase only if the molecules acquire the appropriate 5′,5′-pyrophosphate cap when prereacted in the presence of ATP. Therefore, self-capping DNAs are selectively ligated to an acceptor molecule by T4 DNA ligase, which facilitates their separation by PAGE and their selective amplification by PCR.

The initial DNA population (G0) was comprised of approximately 10^{14} individual 100mers, each carrying a 70-nucleotide random-sequence domain flanked on each side by specific primer-binding sites (Figure 1B). Each DNA molecule in the population also carried a 5′-phosphate group. Initially, the 5′-phosphorylated population of DNAs was incubated with 1 mM ATP for 20 h, and the resulting reaction products were subjected to the selective-amplification process as detailed in Figure 1A. No significant capping activity was observed for the first several iterations of selective amplification as determined by the fraction of DNA pool that was ligated by T4 DNA ligase (Figure 1C). However, more than 5% of the population generated after six rounds of selection (G6) was joined to the acceptor oligonucleotide. By G9, nearly 15% of the DNA population was joined by T4 DNA ligase despite the absence of ATP in the ligase reaction.

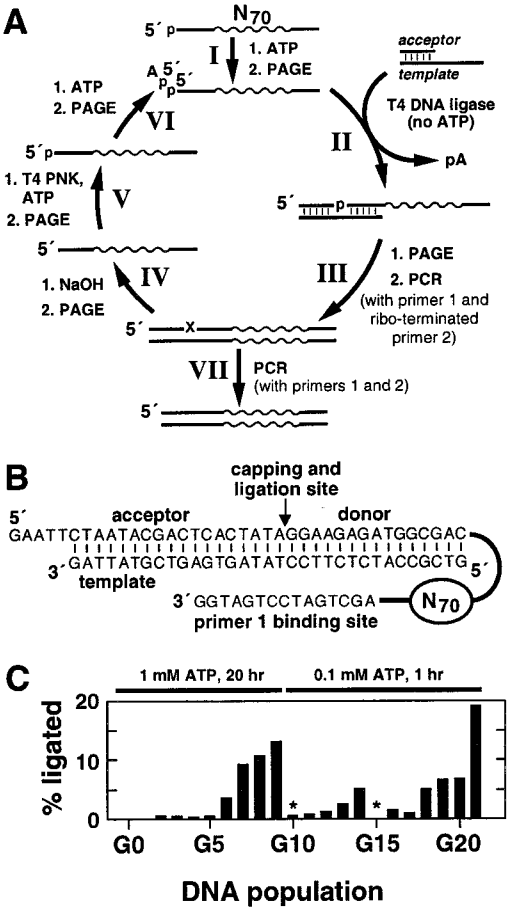


FIGURE 1: Selection of self-capping deoxyribozymes. (A) A pool of 10^{14} synthetic 100mer DNAs, each carrying a 70 nt random-sequence domain and a 5′-phosphate group, is (I) incubated in selection buffer containing ATP. After incubation, the DNAs are separated from ATP by PAGE, and the DNA recovered from the gel is (II) combined with excess acceptor and template oligonucleotides and treated with T4 DNA ligase in the absence of ATP. The ligated DNA (≈123 nt) is (III) isolated by PAGE, and the recovered DNAs are selectively amplified by PCR using primer 1 and a ribo-terminated primer 2. The resulting double-stranded PCR products are (IV) treated with NaOH to cleave the single RNA linkage, and the corresponding single-stranded DNAs (≈100 nt) are separated by PAGE. The recovered DNA is (V) phosphorylated with T4 PNK in the presence of ATP and purified by PAGE, and the resulting DNA population is (VI) used to initiate the next round of selection. DNAs from the final round of selection are (VII) PCR-amplified with primer 1 and an "all-DNA" version of primer 2 to facilitate cloning. N_{70} represents the random-sequence domain. (B) Sequences of the DNA population complexed with acceptor and template oligonucleotides. (C) Plot of the fraction of DNA population that is ligated during stage II for each round of selection. Asterisks identify populations G10 and G15 that were generated using hypermutagenic PCR.

To favor the isolation of DNAs with improved characteristics, we created more stringent selection conditions by reducing the concentration of ATP to 100 μM and by reducing the incubation time to 1 h. In addition, we subjected the DNA populations at G10 and G15 to a hypermutagenic version of PCR (*20*) to provide additional sequence diversity.

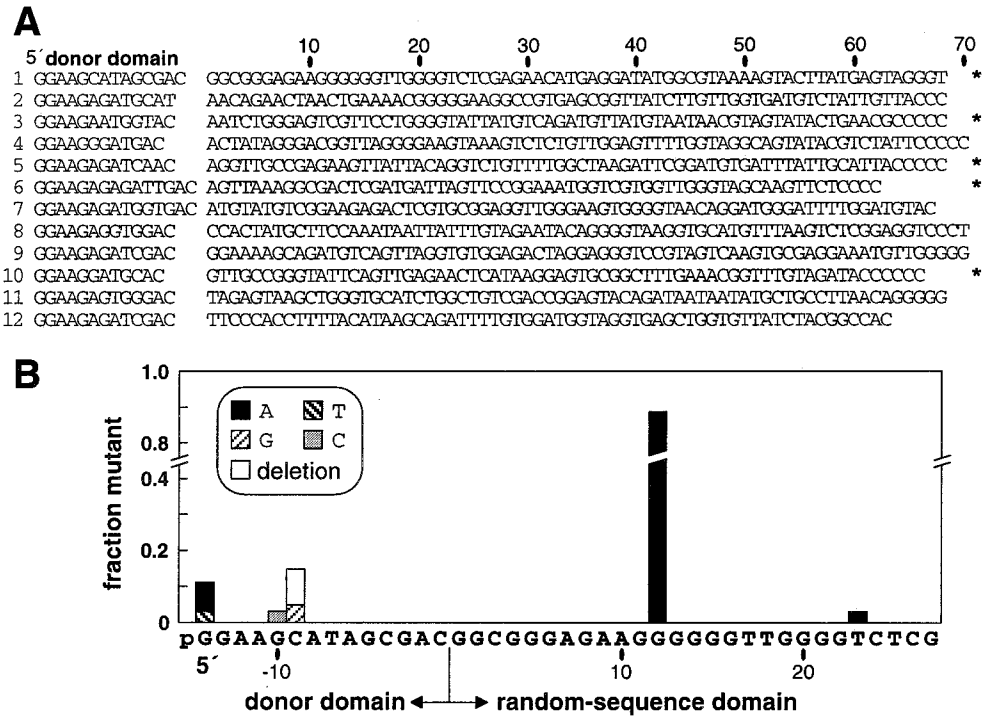


FIGURE 2: Classes of self-capping deoxyribozymes determined by sequence differences. (A) Sequence analysis of 40 individuals from G22 reveals 12 distinct classes of DNAs. Nucleotides corresponding to the original random-sequence domain are numbered. Asterisks indicate sequences that test positive for self-capping activity as determined by T4 DNA ligase activity. (B) Artificial phylogeny of the 5′ donor domain and the first 27 nucleotides of the original random-sequence domain. Bar heights reflect the fraction of the total number of variants examined that carry the mutation as indicated.

The DNA at G21 exhibits significantly improved ligation characteristics, with nearly 20% of the population becoming ligated by T4 DNA ligase in the absence of ATP. Additional rounds of selection using more demanding selection conditions did not provide further improvement in the characteristics of the population.

The DNA population at G22 was cloned, and 40 individuals were sequenced. At least 12 distinct classes of DNAs were identified based on their unique sequence composition (Figure 2A). In each case, the impact that mutagenesis has had on the population is evident by the variable length of the original random-sequence domain and by the mutations that accumulated in the 5′ donor domain that was held constant in the design of the original construct. Five classes (1, 3, 5, 6, and 10) were arbitrarily chosen for preliminary examination, and all exhibit ATP-dependent self-capping activity as determined by the ligation−PAGE method employed for in vitro selection (data not shown).

Class 1 Self-Capping Deoxyribozyme. The dominant sequence among the cloned DNAs is class 1; therefore, we chose to examine this class of DNAs in greater detail. We conducted a reselection based on class 1 DNAs to further optimize its activity and to generate an artificial phylogeny of deoxyribozyme sequence variants. Mutations were introduced throughout the original random-sequence domain again by using hypermutagenic PCR. Numerous sequence variants that display activity were isolated after four rounds of selection, although no significant increase in catalytic activity above the original clone was observed. Upon examination of the variants, we observed that a high frequency of random mutations is tolerated by the DNA in the region spanning nucleotides 28−68 (Figure 2A) of the original random-sequence domain (data not shown). Almost without exception, however, mutations are not tolerated in the first 27 nucleotides (Figure 2B). The most frequent variation in this highly conserved domain is a G to A mutation at position 12. These results suggest that the highly conserved nucleotides 1−27 are critical for deoxyribozyme function while the remaining variable region might be superfluous for catalytic function.

Interestingly, mutations also have occurred in the donor domain for class 1 deoxyribozymes. This is somewhat unexpected for two reasons. First, this domain was not mutated in the initial random-sequence pool, nor was it intentionally subjected to mutation during the reselection. Second, the base pairing potential of the donor domain cannot be substantially altered during selection because it must be recognized by the template oligomer that is used during ligation by T4 DNA ligase. However, variant deoxyribozymes were found to carry up to four mutations compared to the original donor sequence (Figure 2B), indicating that at least a portion of this domain is not essential for efficient deoxyribozyme function.

A Minimized Class 1 Deoxyribozyme. We used the artificial phylogeny as a guide to design truncated deoxyribozymes in an effort to define the minimum contiguous class I DNA that retains full catalytic activity. Synthetic DNAs corresponding to truncations at nucleotides 24−28 were 5′-^{32}P-labeled and individually incubated under self-capping reaction conditions. PAGE separation of the self-capping reaction products prior to treatment with T4 DNA ligase reveals that certain DNAs yield a product that is indicative of the addition of a 5′,5′-pyrophosphate cap (Figure 3A). These results demonstrate that a DNA terminating at G27 encompasses the minimal essential structure for full activity, while shorter DNAs that terminate at C24, T25, or C26 do not exhibit

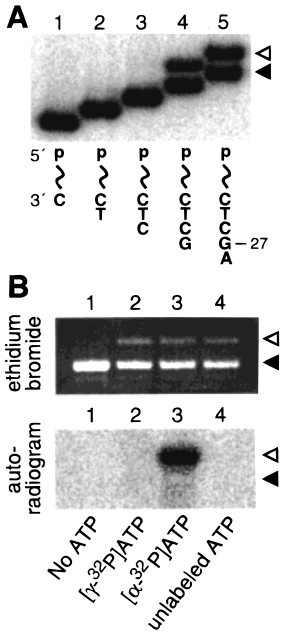


FIGURE 3: Minimal self-capping domain for class 1 deoxyribozymes. (A) Five synthetic oligonucleotides corresponding to truncated class 1 deoxyribozyme at C24, T25, C26, G27, and A28 (as indicated in lanes 1−5, respectively) were assayed for self-capping activity. Each oligonucleotide radiolabeled at the 5′ terminus with ^{32}P was incubated for 5 h under standard buffer conditions (see Materials and Methods). Reaction products were separated by denaturing 15% PAGE after precipitation with ethanol. The image presented was generated using a PhosphorImager. In lane 5, filled and open arrowheads identify precursor and capped DNAs, respectively. In the remaining lanes, precursor and capped molecules exhibit progressively greater gel mobility with decreasing chain length of the precursor DNA used. Only those DNAs that include G27 (lanes 4 and 5) retain catalytic activity. (B) Analysis of the cap structure generated by class 1 deoxyribozymes. Unlabeled 41mer deoxyribozymes that carry a 5′-phosphate were incubated for 5 h in standard reaction buffer containing no ATP (lane 1) or in 10 μM ATP (lanes 2−4) containing a trace amount of [γ-^{32}P]-ATP (lane 2), a trace amount of [α-^{32}P]ATP (lane 3), or no added ^{32}P-labeled ATP (lane 4). The reaction mixtures were analyzed on 15% PAGE after precipitation with ethanol. The top image is of the resulting PAGE gel upon staining with ethidium bromide and UV translumination while the bottom image is an autoradiograph of the same gel. Filled and open arrowheads identify the locations of precursor and capped DNAs, respectively, as visualized by ethidium bromide staining and by their relative mobility compared to marker dyes.

catalytic activity. Therefore, a total of 41 nucleotides from the 5′ terminus of the original class 1 sequence encompass the minimum contiguous deoxyribozyme domain.

Product Verification Using Class 1 Deoxyribozymes. The observation that T4 DNA ligase accepts deoxyribozymes as donors in a ligation reaction only upon preincubation with ATP is consistent with the view that deoxyribozymes generate the intended 5′,5′-pyrophosphate cap. We also observed (data not shown) that rapid dephosphorylation of the prereacted deoxyribozymes by alkaline phosphatase occurs only if the capped structure has been subjected to periodate oxidation followed by β-elimination (*21*). This treatment is expected to selectively remove the adenosine moiety from the pyrophosphate structure only if the 2′- and 3′-hydroxyl groups of the nucleoside are displayed at the terminus of the deoxyribozyme, thereby making the 5′-^{32}P moiety available for removal by phosphatase. The observation that periodate oxidation/β-elimination treatment is required for efficient phosphatase activity also is consistent with the deoxyribozyme-mediated formation of the predicted cap structure.

To provide additional support for the DNA-mediated formation of a 5′,5′-pyrophosphate cap, we conducted an "α-^{32}P acquisition" analysis wherein different radiolabeled forms of ATP are used to assess the structure of the modification. Unlabeled 41mer class 1 deoxyribozymes corresponding to the parent sequence depicted in Figure 2B were independently incubated in the absence of ATP, or in the presence of [γ-^{32}P]ATP, [α-^{32}P]ATP, or unlabeled ATP (10 μM final concentration). Analysis of the DNA products by PAGE yields the expected capped DNA when any form of ATP is supplied, as visualized using ethidium bromide staining followed by UV translumination (Figure 3B). As expected, the absence of ATP precludes deoxyribozyme activity (Figure 3B, lane 1). However, only the product derived from the deoxyribozyme reaction conducted in the presence of [α-^{32}P]-ATP is visible by autoradiography of the same gel (Figure 3B, lane 3). This indicates that the DNA molecule incorporates the α-phosphate from ATP as a consequence of its catalytic activity. The acquisition of α-phosphate rather than γ-phosphate is consistent with the expected capping mechanism as depicted in Scheme 1.

The results described above, however, do not rule out the possibility that the deoxyribozyme acquires both the α- and β-phosphates of ATP by attacking the phosphate at the β position, with concomitant expulsion of the γ-phosphate. To examine this possibility, we tested the ATP analogues β,γ-methylene-ATP and α,β-methylene-ATP. Each carries a cleavage-resistant methylene linkage in place of the normal phosphoanhydride linkage. We found that while β,γ-methylene-ATP can be used by the deoxyribozyme, α,β-methylene-ATP cannot serve as a substrate (for further discussion, see below). This result is consistent with a mechanism whereby the phosphorus center of the α-phosphate group of ATP is attacked by an oxygen atom of the 5′-phosphate of the deoxyribozyme. This would result in release of pyrophosphate and formation of the expected 5′,5′-pyrophosphate cap.

Reaction Parameters for Optimal Deoxyribozyme Function. To establish the optimal reaction conditions for class I DNAs, we examined the influence of pH, ionic strength, and cofactor dependence of the 41mer deoxyribozyme. Although the selection reactions were conducted at pH 7.0, we find that the 41mer displays near full catalytic activity over a broad pH range with a maximum k_{obs} of 3×10^{-3} min^{-1} at pH 6.5 (Figure 4A). The deoxyribozyme requires both Cu^{2+} and Mg^{2+} as cofactors (Figure 4B,C), although it is not yet clear precisely what roles these divalent metals play in the self-capping reaction. Mg^{2+} can be replaced with Ca^{2+} with near equal efficiency (data not shown). Cu^{2+}, which binds to the deoxyribozyme with an apparent K_d of $\sim$3 μM, is known to serve as an essential cofactor for several other deoxyribozymes (*9*−*11*). However, it is important to note that Cu^{2+} becomes strongly inhibitory at concentrations above 20 μM, in accordance with its ability to interact nonspecifically with DNA and denature structures at concentrations in excess of 100 μM (*22*, *23*). The deoxyribozyme retains modest catalytic activity ($k_{obs} \sim 10^{-4}$−10^{-5} min^{-1}) under in vitro selection conditions that included 50 μM Cu^{2+}. As a result, the concentration of deoxyribozyme and its Cu^{2+}

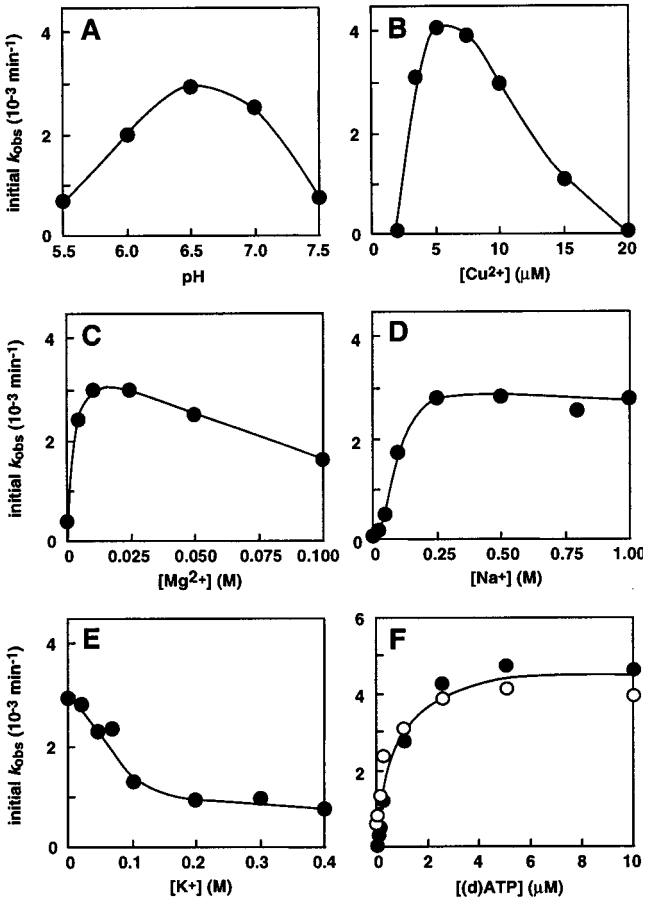


FIGURE 4: Kinetic analysis of class 1 self-capping deoxyribozymes. Rate constants were determined using a trace amount of 5′-^{32}P-labeled 41mer deoxyribozyme and 10 μM ATP. Plots A−F were established using assays conducted under standard buffer conditions (50 mM HEPES, pH 7.0 at 23 °C, 400 mM NaCl, 10 mM $MgCl_2$, 10 μM $CuCl_2$) with specified changes as defined in each case. The pH-dependent activity profile depicted in (A) was established by using 50 mM MES (pH 5.5, 6.0, and 6.5 at 23 °C) or 50 mM HEPES (pH 7.0 and 7.5 at 23 °C). Open and filled circles in plot F represent initial k_{obs} values established using ATP and dATP, respectively.

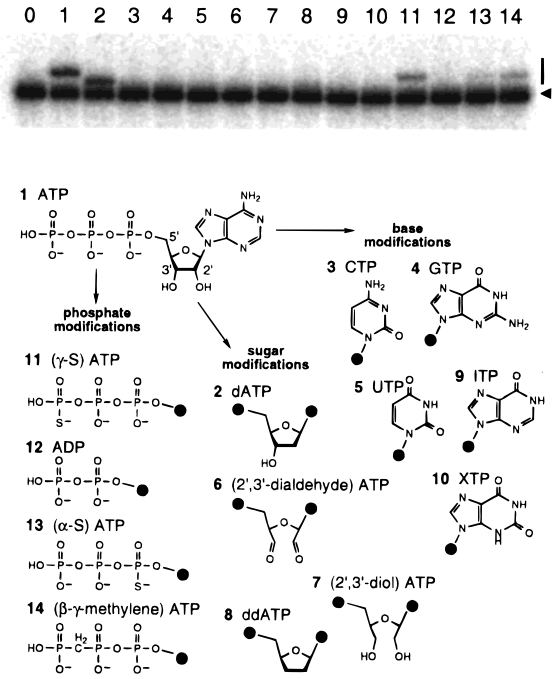


FIGURE 5: ATP analogues containing base modifications (compounds **3**−**5**, **9**, and **10**), sugar modifications (**2**, **6**, **7**, and **8**), or phosphate modifications (**11**−**14**) were examined for their ability to serve as substrate for class 1 deoxyribozymes. The 41mer deoxyribozyme (5′-^{32}P-labeled) was incubated in standard reaction buffer containing 10 μM of each ATP analogue at 23 °C for 5 h. Reaction products were separated by denaturing 15% PAGE and analyzed by PhosphorImager. The arrowhead identifies the precursor DNA, and the vertical bar marks the expected location of capped DNA products. Note that the capped product using dATP (lane 2) has a higher electrophoretic mobility than do those that carry the AMP cap. Interactions between borate (present in the PAGE buffer) and the *cis*-hydroxyl groups of the RNA cap are expected to reduce the mobility of the AMP-capped product (*38*). Therefore, the increased mobility observed with the dAMP-capped product is presumably due to the loss of the interactions between borate and the adjacent 2′- and 3′-hydroxyl groups that are otherwise located in the AMP cap.

cofactor must be tightly controlled in order to avoid deoxyribozyme inactivation.

Perhaps the most peculiar behavior of this deoxyribozyme is its requirement of Na^+ for full catalytic activity. A minimum of 0.25 M NaCl is required for the deoxyribozyme to reach maximum catalytic activity (Figure 4D), which might be expected if its role is exclusively to establish the ionic strength of the reaction mixture. However, K^+ fails to fully substitute for Na^+ in this capacity and even becomes somewhat inhibitory at concentrations above 0.1 M (Figure 4E). Likewise, neither Li^+ nor NH_4^+ can substitute for Na^+ in the reaction (data not shown). The inhibitory effect of K^+ indicates that this ion might selectively bind to the DNA and favor the formation of structures that compete with the active structure of the deoxyribozyme.

Substrate Recognition of ATP and Its Analogues. Class 1 deoxyribozymes exhibit classical Michaelis−Menten kinetics toward its ATP substrate, which has a K_M of ~500 nM (Figure 4F). Under saturating concentrations of ATP, the 41mer deoxyribozyme displays a k_{cat} of 4.8×10^{-3} min^{-1} and a k_{cat}/K_M of ~10^4 M^{-1} min^{-1} under optimal reaction conditions. Interestingly, the deoxyribozyme is equally efficient when dATP is substituted for ATP (Figure 4F). This indicates that the 2′-hydroxyl group of ATP does not contribute significantly to the binding and catalysis by class 1 DNAs.

To further establish the chemical groups of the substrate that are critical for molecular recognition and chemistry, we examined the substrate characteristics of a series of nucleoside triphosphates and ATP analogues (Figure 5). As expected, the catalytic activity of the 41mer can be observed upon incubation with either ATP or dATP (Figure 5, lanes 1 and 2, respectively). In contrast, the presence of an intact ribose ring and the 3′-hydroxyl group are critical for catalytic activity (lanes 6−8). In addition, the adenine base moiety of ATP carries key determinants of substrate specificity. This is evident by the fact that neither the three remaining NTPs nor the analogues ITP or XTP are accepted as substrate by the deoxyribozyme (lanes 3−5, 9, 10).

The activity of substrate analogues with changes to the triphosphate moiety of ATP reveals important characteristics of the adenylation reaction. First, the deoxyribozyme requires a triphosphate structure for catalysis, as adenosine 5′-diphosphate fails to serve as a substrate (Figure 5, lane 12). Second, the use of sulfur to replace single oxygen atoms on

the γ- and α-phosphate groups results in diminished catalytic activity (lanes 11 and 13, respectively). This suggests that these positions might form contacts with the deoxyribozyme that are critical for catalytic function. Sulfur substitution within the α-phosphate group also might reduce the rate of DNA capping by perturbing the electronic characteristics of the target phosphorus center. This might explain why the α-thiophosphate substitution has a greater impact on the catalytic rate than does the γ-thiophosphate substitution. Third, independent replacements of the two oxygen bridges that link the phosphates with methylene bridges have different outcomes. The deoxyribozyme exhibits reduced but measurable capping activity when supplied with the β,γ-methylene-ATP analogue (lane 14). However, no activity is detected when the deoxyribozyme is incubated with the α,β-methylene-ATP analogue (data not shown). The methylene bridges are expected to resist enzymatic cleavage by enzymes, made either of protein or of nucleic acid, that catalyze the cleavage of phosphoanhydride bonds. Therefore, the observation that the ATP analogue with the α,β-methylene bridge is not a substrate indicates that the phosphorus center at the α position is the target for the reaction and that the phosphoanhydride linkage between the α- and β-phosphates is cleaved. This finding lends further support for the mechanism depicted in Scheme 1.

Guanosines Serve a Dominant Role in Class 1 Tertiary Structure. The artificial phylogeny generated upon reselection of class 1 deoxyribozymes (Figure 2B) provides evidence that the nucleotide sequence of the catalytic domain cannot tolerate significant alteration. Unlike several other deoxyribozymes recently studied (*3*−*5*), the artificial phylogeny does not provide evidence of nucleotide covariations that would be indicative of unrestricted base pair interactions. Moreover, prediction of secondary structure folding using the Zuker DNA mfold program[1] identifies the potential for only a single 4 base pair element. The potential for other stem structures exists, but they include no more than 2 contiguous base pairs. However, we could not confirm any of seven potential stem elements using standard mutation and compensation−mutation analysis with synthetic DNAs (data not shown). Although important stem elements might have strict sequence requirements in order to support catalysis, these results and those of the artificial phylogeny are consistent with a tertiary structure for class 1 deoxyribozymes that is largely devoid of secondary structure elements.

The most striking feature of the class 1 DNA sequence is its base composition, wherein guanosine residues represent greater than 50% of the minimized 41mer deoxyribozyme (Figure 2B). Furthermore, the 22 G residues primarily reside in clusters containing from 2 to 6 repeats. Both the frequency and organization of G residues are suggestive of the formation of guanine quartets (*24*, *25*) in the active structure. Interestingly, dimethyl sulfate (DMS) methylation of the N7 positions at any of 14 different G residues within the original 41mer deoxyribozyme disrupts self-capping (Figure 6A, lanes 1 and 2). These results suggest that the N7 positions of each of these 14 residues form tertiary contacts that are critical for catalytic function. Moreover, the methylation-sensitive guanosines fall in a pattern that is again suggestive of the formation of as many as four successive, albeit incomplete, guanine quartets (Figure 6B).

One possible arrangement that places all methylation-sensitive guanosines into quartet structures is schematically depicted in Figure 6C. This model integrates nucleotides 13−22 (5′-GGGGTTGGGG) to form half of an element comprised of four tiers of guanine quartets. Interestingly, only six of the eight G residues in this region exhibit complete methylation sensitivity. Methylation at positions G13 and G22 (underlined) is not deleterious in the context of the original 41mer deoxyribozyme. Interestingly, introduction of an A to G mutation at position 7 (Figure 6B) results in only a minor (5-fold) reduction in self-capping activity, but this mutation specifically causes both G13 and G22 to become fully sensitive to methylation (Figure 6A, lane 3). This result would be expected if the exocyclic amine of the newly introduced guanosine at position 7 contacts the N7 position of the guanosine at position 22, and this in turn stabilizes the identical contacts between the guanosines at positions 22 and 13 (Figure 6D). These observations are consistent with a structural arrangement where the A (or G) at position 7 occupies one of the four positions of an incomplete guanine quartet with G22 and G13. If true, then nucleotides 4−7 (5′-GGG[A or G]) might form another portion of the proposed four-tiered stack of guanine quartets. The remaining guanosine residues that are methylation-sensitive (positions −14, −13, 1, and 2) could be used to fill the nucleotide positions remaining in each tier.

Although yet to be fully investigated, the structural model depicted in Figure 6C is consistent with all existing biochemical data. The stacked quartet has been modeled with a cross-strand configuration to reflect the apparent contacts made between positions 7 and 22, and between positions 22 and 13 (Figure 6A). Furthermore, the model places the site of deoxyribozyme action, the 5′-phosphate group, in a position to be encompassed by three putative single-stranded domains comprising the remainder of the minimized deoxyribozyme. Although guanine quartets are known to be stabilized by K^+ (*24*, *25*), perhaps the inhibitory effects observed with this monovalent ion (Figure 4E) are due to the stabilization of alternate guanine quartet arrangements that preclude catalytic activity. The exploitation of guanine quartets by class 1 DNAs would not be surprising, as these structures have been identified in a number of other deoxyribozymes (*4*). Guanine quartets, which also are believed to be the basic structural element of telomeric DNAs (*25*), are likely to be a common structural motif for the construction of stable DNA structures (*26*). It is important to note, however, that this deoxyribozyme is the only one of 12 classes of self-capping DNAs that has such extensive representation of guanosine residues (Figure 2A). Thus, it is evident that DNA can readily form catalytic structures without necessity for guanine quartets.

CONCLUSIONS

The Structural and Functional Diversity of Deoxyribozymes. In a previous report (*12*), we isolated nearly 50 different classes of deoxyribozymes that catalyze DNA phosphorylation. In the current study, at least 12 distinct sequence classes that promote ATP-dependent self-capping are present in the final DNA population selected. The

[1] The DNA mfold server can be accessed on the Internet (www.ibc.wustl.edu/~zuker/dna/form1/cgi).

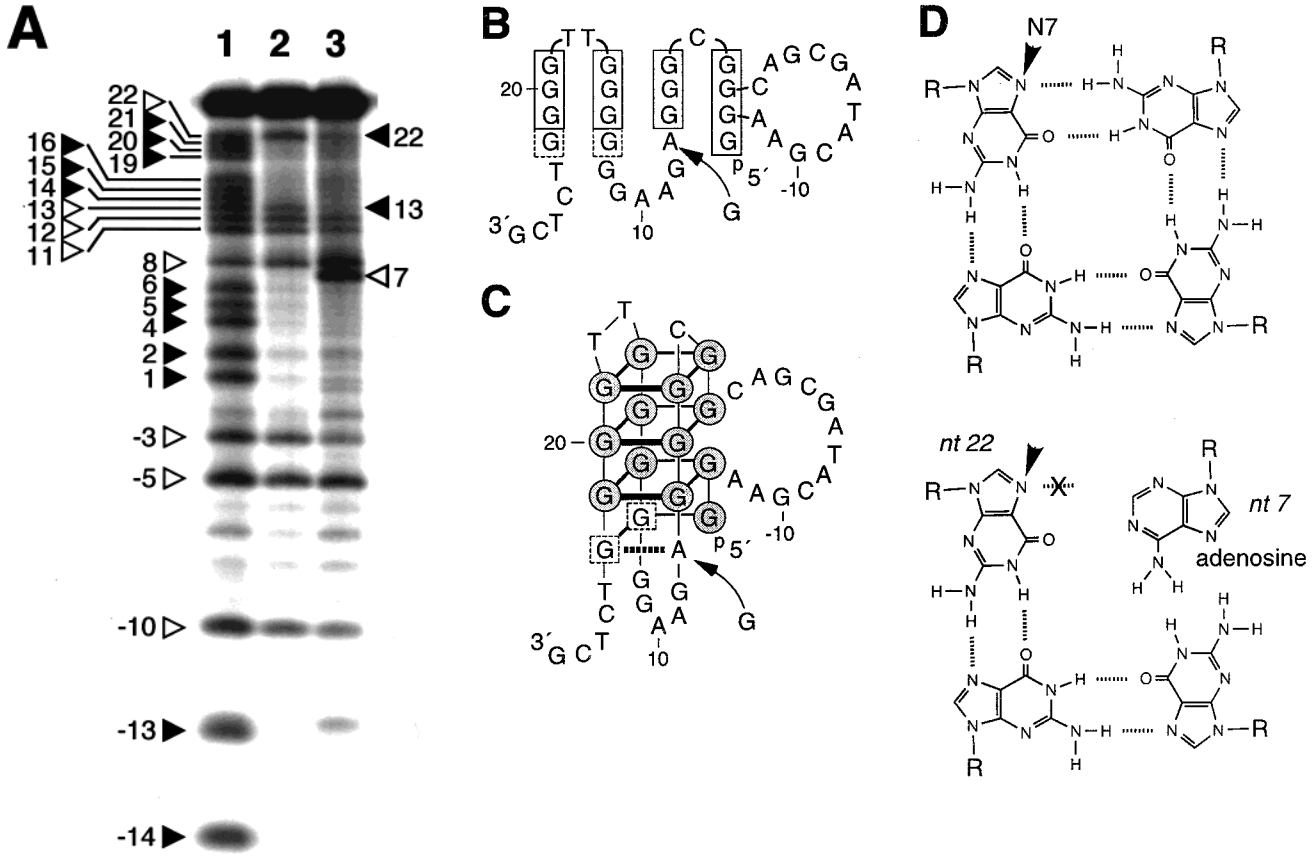


FIGURE 6: Preliminary structural model for the class 1 self-capping deoxyribozyme. (A) Chemical probing (*39*) of the original (Figure 2B) 41mer deoxyribozyme (lanes 1 and 2) versus the variant 41mer containing a single A to G mutation at nucleotide 7 (lane 3). DNAs with specific methylation patterns are rendered inactive by treatment of the two precursor DNAs with DMS. Guanosine nucleotides that tolerate methylation at the N7 position without losing catalytic activity are revealed by comparing the piperidine-induced cleavage pattern of capped DNAs that are modified either after (lane 1) or before (lanes 2 and 3) incubation with ATP. Bands that no longer appear in lanes 2 and 3 compared to lane 1 identify guanosine residues whose N7 positions are critical for deoxyribozyme function (filled arrowheads), while bands that remain identify guanosine residues whose N7 positions are not critical for deoxyribozyme function (open arrowheads). Similar notations on the right identify changes between the original and variant 41mer deoxyribozymes. Note that a band corresponding to the additional G residue in the variant DNA appears in lane 3 (nucleotide 7). (B) Depicted is the distribution of the sensitive guanosine residues in the original and variant 41mer deoxyribozymes. Dashed boxes identify nucleotides that are methylation-sensitive only in the variant deoxyribozyme. The arrow identifies the G to A mutation at nucleotide 7 of the variant DNA. (C) One possible structural model that is consistent with the chemical probing data includes guanine quartet structures. The N7 positions of the encircled nucleotides are critical for deoxyribozyme activity. The dashed boxes identify the guanosine residues at position 22 and 13 that become methylation-sensitive upon mutation of nucleotide 7 from A to G as depicted by the arrow. Note that no interference is observed upon methylation of the N7 group of guanosine at nucleotide 7 in the variant 41mer (A, lane 3). (D) Schematic representation of the base−base interactions of (top) a guanine quartet and (bottom) the possible interaction between nucleotides 7 and 22 in class 1 self-capping DNAs. The X represents the absence of methylation sensitivity at the N7 of position 22, possibly due to the absence of hydrogen bonding to the nucleotide at position 7 when A is present. Similarly, N7 methylation at position 22 is disruptive when position 7 is mutated to G.

propensity for DNA to provide multiple structural answers to solve specific chemical tasks is also evident from a number of other studies with deoxyribozymes (*9*, *16*, *19*, *27*−*30*), and similar observations have been made with DNA aptamers (*2*, *31*). For example, the ATP-dependent class 1 deoxyribozyme described herein is different in nucleotide sequence and structure to that of an ATP-binding DNA aptamer isolated previously (*32*), despite the fact that both are rich in guanosine residues. Taken together, these findings suggest that DNA has sufficient structural versatility to form various unique binding sites or active sites for specific functional challenges.

Catalytic Performance of the Capping Deoxyribozyme. The minimized class 1 deoxyribozyme achieves a modest catalytic rate constant (k_{cat}) of $\sim 5 \times 10^{-3}$ min^{-1}, but exhibits a high affinity for the external ATP substrate ($K_M = 0.5\ \mu$M) (Figure 4F). As a result, the catalytic efficiency (k_{cat}/K_M) of this deoxyribozyme approaches 10^4 M^{-1} min^{-1}. This catalytic efficiency is comparable to many of the natural ribozymes and other selected ribozymes and deoxyribozymes that use external substrates of similar size and nature. For example, k_{cat}/K_M values range from 10^3 to 10^4 M^{-1} min^{-1} for nucleic acid enzymes such as the *Tetrahymena* self-splicing intron (*33*), a self-phosphorylating ribozyme (*34*), a self-capping ribozyme (*35*), and a self-phosphorylating deoxyribozyme (*12*). Unfortunately, it is difficult to experimentally determine the rate constant for the uncatalyzed capping of DNA by ATP due to its slow rate. However, the overall rate enhancement achieved by the class 1 deoxyribozyme can be estimated by comparing its rate constant to that of the uncatalyzed rate of ATP hydrolysis, as has been done for the self-phosphorylating ribozymes and deoxyribozymes described previously (*12*, *34*). Hydrolysis of ATP has a reported second-order rate constant of $\sim 5 \times 10^{-7}$ M^{-1} min^{-1} in the presence of Mg^{2+} (*36*). Therefore, the rate enhancement $(k_{cat}/K_M)/k_{hydrolysis}$ for the class 1 self-capping deoxyri-

bozyme is $\sim 2 \times 10^{10}$, indicating that this deoxyribozyme transfers AMP from ATP to its 5′-phosphate group more than 10 billion times faster than the corresponding spontaneous hydrolysis of ATP.

Prospects for Deoxyribozymes with Related Catalytic Functions. It had previously been established that deoxyribozymes can utilize an activated substrate such as ATP to form a phosphoester bond to DNA at the expense of a phosphoanhydride bond (*12*). In this study, we identified deoxyribozymes that catalyze a phosphoanhydride exchange reaction between ATP and a phosphorylated DNA. With consideration of the catalytic activities of these deoxyribozymes, we expect that the catalytic challenge of forming a new 3′,5′-phosphodiester linkage by cleavage of a 5′,5′-pyrophosphate cap should be within reach of DNA. This latter reaction replicates the second step of DNA ligation that is catalyzed by such enzymes as T4 DNA ligase. Already, ligase ribozymes that perform the related ligase reaction with RNA substrates have been isolated (*37*), thus providing a precedence in nucleic acids for this catalytic activity. If each of these deoxyribozymes can be made to work in concert, then DNA could catalyze the coupling of DNA without the aid of protein enzymes such as polynucleotide kinase and DNA ligase, which have long been indispensable for the efficient enzymatic coupling of nucleic acids.

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