Multiple Occurrences of an Efficient Self-Phosphorylating Deoxyribozyme Motif†

Simon A. McManus and Yingfu Li*

Department of Biochemistry and Biomedical Sciences and Department of Chemistry, McMaster University, 1200 Main Street West, Hamilton, Canada L8N 3Z5

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ABSTRACT: The catalytic and structural characteristics of two new self-phosphorylating deoxyribozymes (referred to as deoxyribozyme kinases), denoted "Dk3" and "Dk4", are compared to those of Dk2, a previously reported deoxyribozyme kinase. All three deoxyribozymes not only utilize GTP as the source of activated phosphate and Mn(II) as the divalent metal cofactor but also share a common secondary structure with significant sequence variations. Multiple Watson−Crick helices are identified within the secondary structure, and these helical interactions confine three extremely conserved sequence elements of 8, 5, and 14 nucleotides in length, presumably for the formation of the catalytic core for GTP binding and the self-phosphorylating reaction. The locations of the conserved regions suggest that these three deoxyribozymes arose independently from in vitro selection. The existence of three sequence variants of the same deoxyribozyme from the same in vitro selection experiment implies that these catalytic DNAs may represent the simplest structural solution for the DNA self-phosphorylation reaction when GTP is used as the substrate.

DNA is inherently stable, and its propensity for double-helical structure formation makes it the ideal material for storage of genetic information. In single-stranded form, DNA behaves similarly to RNA and has the ability to perform functions such as ligand binding and catalysis. Although no naturally occurring enzymes made of DNA have been found to date, many catalytic DNA molecules (deoxyribozymes or DNAzymes) have been isolated from single-stranded random-sequence DNA libraries by in vitro selection (*1*−*4*). Deoxyribozymes are now known to catalyze an increasing number of reactions including RNA cleavage (*5*), DNA cleavage (*6*), DNA phosphorylation (*7*), DNA adenylation (*8*), DNA ligation (*9*), RNA ligation (*10*), RNA branching (*11*), depurination (*12*), thymine dimer repair (*13*), and porphyrin ring metalation (*14*). The existence of such a large array of deoxyribozymes implies that DNA is capable of folding into complex structures needed for catalysis.

Deoxyribozyme kinases are single-stranded DNA molecules capable of self-phosphorylation at their 5′ ends. The reaction scheme is illustrated in Figure 1A. These deoxyribozymes have previously been isolated in experiments designed to investigate catalytic DNA sequence diversity, metal ion cofactor specificity, and DNA's molecular discrimination ability toward small-molecule substrates (*7*, *15*). We are currently using this model system to examine the level of structural diversity and complexity capable by DNA catalysts. Specifically, we are studying the structures of several highly efficient deoxyribozyme kinase obtained from an in vitro selection experiment conducted under stringent reaction conditions. We have previously characterized two of these deoxyribozymes (named Dk1[1] and Dk2) and reported their probable secondary structures (*16*). After further analyzing the terminal population of this selection, we found two other molecules that are significantly different in primary sequences from Dk1 and Dk2. Interestingly, the results from the characterization and structural analysis to be described in this report show that these two additional deoxyribozymes share a common secondary structure with Dk2. This secondary structure contains multiple short helical regions and 28 absolutely conserved nucleotides. The multiple observation of this structural motif suggests that it may represent the simplest structural arrangement to perform DNA self-phosphorylating reaction under the in vitro selection conditions. These results were obtained through substrate and metal ion cofactor characterization, structural probing via base-pair alteration, and methylation interference to identify guanosine residues that are essential to deoxyribozyme activity.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Other Materials. DNA was prepared using standard phosphoramidite chemistry (Mobix Laboratory, McMaster University; Integrated DNA Technologies, Coralville, IA). Oligonucleotides were purified by 10% denaturing (8 M urea) PAGE. Purified oligonucleotides were

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* To whom correspondence should be addressed. E-mail: liying@mcmaster.ca. Tel: (905) 525-9140. Fax: (905) 522-9033.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; CIAP, calf intestine alkaline phosphatase; CTP, cytidine 5′-triphosphate; Dk1−4, deoxyribozyme kinase 1−4; dGTP, deoxyguanosine 5′-triphosphate; DMS, dimethyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5′-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ITP, inosine 5′-triphosphate; m(7)GTP, 7-methylguanosine 5′-triphosphate; N^7-dGTP, 7-deazaguanosine 5′-triphosphate; NTP, nucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; T4 PNK, T4 polynucleotide kinase; UTP, uridine 5′-triphosphate.

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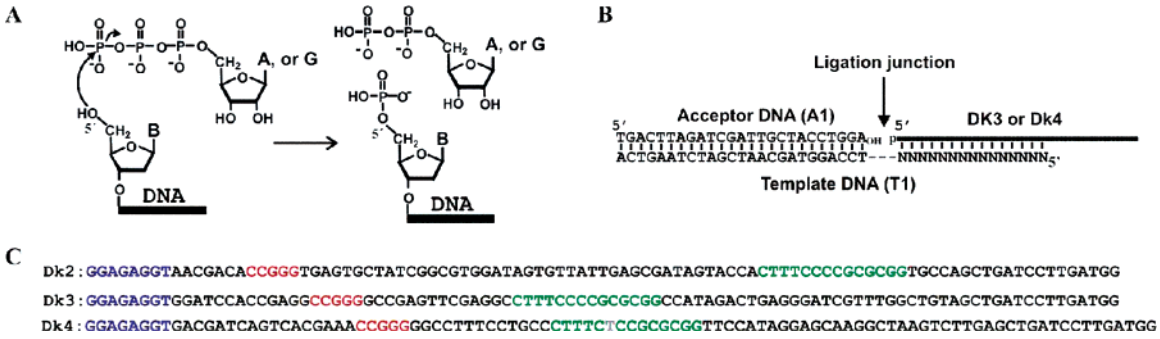

FIGURE 1: (A) Proposed reaction mechanism for DNA self-phosphorylation. Nucleophilic attack by the 5′-hydroxyl group on the γ-phosphate of ATP or GTP results in phosphate transfer to the 5′ end of the deoxyribozyme. (B) Indirect method for detection of phosphorylation. After phosphorylation, the DNAzyme is ligated to an acceptor DNA in the presence of T4 DNA ligase. Only deoxyribozymes that have reacted (therefore acquired a 5′-phosphate) will be able to ligate. The increased size of the catalytic DNA molecules allows easy identification and isolation by PAGE separation. N's in T1 refer to the antisense of the 5′ nucleotides of Dk2, Dk3, or Dk4, as shown in part C of this figure. (C) Sequences of Dk2, Dk3, and Dk4. Conserved domains are colored.

dissolved in water and their concentrations determined spectroscopically as previously described (*15*). T4 PNK, T4 DNA ligase, and CIAP were purchased from MBI Fermentas. [γ-^{32}P]ATP was purchased from GE Healthcare. All other chemicals were purchased from Sigma.

Catalytic Assays. Dk3 and Dk4 constructs used in secondary-structural studies were assembled by ligation of two oligonucleotides with the donor oligonucleotide labeled with a ^{32}P at its 5′ end. This procedure generated deoxyribozyme constructs internally labeled with ^{32}P to allow the detection of these molecules. Ligation was performed as follows. Donor DNA was first phosphorylated using PNK in the presence of [γ-^{32}P]ATP. After incubation for 30 min at 37 °C, 1 mM unlabeled ATP was added and the solution incubated for an additional 10 min to ensure complete phosphorylation. The solution was then incubated at 90 °C for 5 min to inactivate PNK. Template and acceptor oligonucleotides were then added directly to this solution, followed by T4 DNA ligase buffer and T4 DNA ligase. The solution was incubated at room temperature for 1 h for ligation. The mixture was then precipitated with ethanol and the ligated DNA isolated by 10% denaturing PAGE.

All assembled constructs were treated with CIAP to remove any 5′-phosphate. DNA was heated to 90 °C for 1 min and cooled rapidly to room temperature to prevent structural folding. CIAP buffer (10×) and CIAP were added, and the solution was incubated at 37 °C for 1 h. CIAP was then removed with two rounds of equal-volume phenol/chloroform extractions.

For self-phosphorylation, reaction volumes were 10 μL. The phosphorylation trials were carried out as follows. DNA was dissolved in water at a concentration of 200 nM. Reaction buffer (2×) was added (800 mM NaCl, 200 mM KCl, 20 mM $MnCl_2$, 100 mM HEPES, pH 7.0 at 23 °C), and the solution was incubated for 5 min at room temperature. The reactions were initiated by the addition of a nucleoside 5′-triphosphate (such as GTP) to the final concentration of 1 mM. After incubation for a set length of time, the reactions were quenched with Na_2EDTA (at the final concentration of 15 mM) as well as 10 pmol of the template DNA and 15 pmol of the acceptor DNA. Solutions were immediately precipitated with ethanol. The DNA was resuspended and ligated using 10× T4 DNA ligase buffer and 5 units of T4 DNA ligase. The reactions were incubated at room temperature for 1 h. The solutions were precipitated with ethanol before being subjected to analysis by 10% denaturing PAGE.

The rate constants were determined using Graphpad Prism software by plotting percent ligation versus reaction time. All reported rates are the average of at least two independent time−course experiments with a variation of less than 20%.

For Dk3 and Dk4 mutants, sequence differences are as follows. Dk3: covaried P1, T_8 to A and A_{15} to T; mismatch P1, G_7 to C; covaried P2, G_{21} to C and C_{55} to G; mismatch P2, G_{54} to C; covaried P3, G_{27} to C and C_{40} to G, mismatch P3, G_{27} to C. Dk4: covaried P1, G_9 to C and C_{19} to G; mismatch P1, G_9 to C; covaried P2, A_{25} to T and T_{58} to A; mismatch P2, C_{26} to G; covaried P3, G_{31} to C and C_{43} to G; mismatch P3, G_{31} to C.

Methylation Interference Assays. Each reaction started with 200 pmol of DNA. For the control reactions, each relevant deoxyribozyme was incubated with 1 mM GTP, as described above, to initiate self-phosphorylation. After being quenched and precipitated, the DNA was dissolved in water and methylated by adding an equal volume of freshly made 0.4% DMS (v/v) and incubated at room temperature for 40 min. The DNA was recovered by precipitation with ethanol and two 70% ethanol washes. For the test reactions, the same procedure was followed except that the DMS treatment was performed before the self-phosphorylation reaction. The DNA from these reactions was then ligated to the acceptor DNA as described for the catalytic assays. The ligated DNA was isolated by 10% denaturing PAGE. The deoxyribozyme-acceptor chimeras were then treated with PNK and [γ-^{32}P]-ATP to label them at their 5′ ends. The reactions were precipitated with ethanol and purified by 10% denaturing PAGE. The DNA was then subjected to methylation-dependent cleavage by resuspending in 50 μL of 10% piperidine (v/v). After incubation for 30 min at 90 °C, the cleavage products were precipitated with ethanol and resolved by 10% denaturing PAGE.

RESULTS

Sequence Comparison of Dk2, Dk3, and Dk4. The sequences of Dk2, Dk3, and Dk4 are shown in Figure 1C. It should be noted that the DNA library used for the original in vitro selection experiment (*15*) contained the fixed sequence of 5′-**GGAAGAGAT**GGCGAC (the first nine nucleotides are in bold for comparison to the mutated

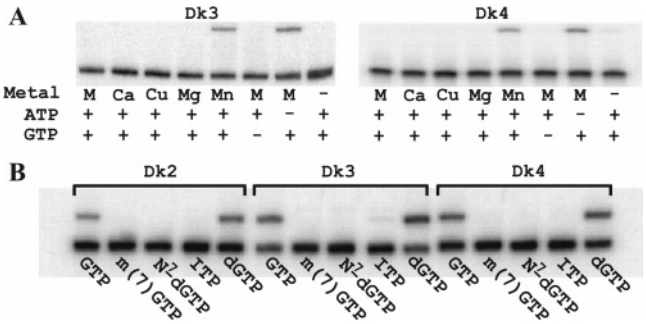

FIGURE 2: Characterization of Dk3 and Dk4. The upper bands in the gels represent reacted deoxyribozyme ligated to an acceptor DNA. The lower bands represent unreacted DNA. (A) Both deoxyribozymes were tested using different divalent metal ions and with ATP and GTP, as shown. M stands for a combination of Ca^{2+} (10 mM), Cu^{2+} (50 μM, plus 10 mM Mg^{2+}), Mg^{2+} (10 mM), and Mn^{2+} (10 mM). (B) Test of substrate specificity for Dk3 and Dk4. 1 mM respective substrate was used for each reaction. N^7-dGTP represents 7-deazaguanosine 5′-triphosphate and m(7)GTP represents 7-methylguanosine 5′-triphosphate.

sequence below). This sequence was designed to allow hybridization to the template DNA for the isolation of active sequences by the DNA ligase-mediated DNA ligation reaction (see Figure 1B). However, upon completion of the in vitro selection experiment, all three deoxyribozymes were found to have a common, but altered, eight-nucleotide (nt) sequence element at their 5′-ends, **GGAΔGAG*G*T** (blue letters in Figure 1C), with one deletion (indicated by Δ) and one base mutation (italicized G). In addition to this common motif, stretches of 5 and 14 nt (red and green letters, respectively, in Figure 1C) are found to be conserved in the interior of the deoxyribozyme sequences (with a one-base mutation in Dk4, which is shown in gray). It is noteworthy that these internal sequence motifs are found at different locations within the three deoxyribozymes and are sandwiched between different nucleotide content on either side in each deoxyribozyme. This, coupled with the selection strategy used, strongly suggests that the three deoxyribozymes arose independently from the original random-sequence DNA library and this point will be addressed in the Discussion section.

Characterization of Dk3 and Dk4. Dk3 and Dk4 were tested to see whether they shared the same substrate and metal ion cofactor requirements as Dk2, which was shown to use GTP as the source of phosphate and was dependent on Mn^{2+} for activity (*15*). Through the indirect ligation assay illustrated in Figure 1B, it is evident that both Dk3 and Dk4 indeed use GTP as a substrate and Mn^{2+} as a divalent metal ion cofactor (Figure 2A). These identical characteristics, along with the conserved sequence motifs, suggest that Dk2, Dk3, and Dk4 may use the same structural arrangement to bind GTP and perform catalysis.

To further characterize the substrate requirements for Dk2, Dk3, and Dk4, we conducted a specificity test using several GTP analogues (their chemical structures are given in Supporting Information, Figure 1). As shown in Figure 2B, Dk2, Dk3, and Dk4 can accept both GTP and dGTP as the substrate but not 7-methylguanosine 5′-triphosphate [m(7)-GTP] and 7-deazaguanosine 5′-triphosphate (N^7-dGTP). Dk3 exhibited very low activity (1% yield in 2 h) when inosine 5′-triphosphate (ITP) was tested as a substrate while Dk2 and Dk4 showed no activity toward ITP. These data indicate

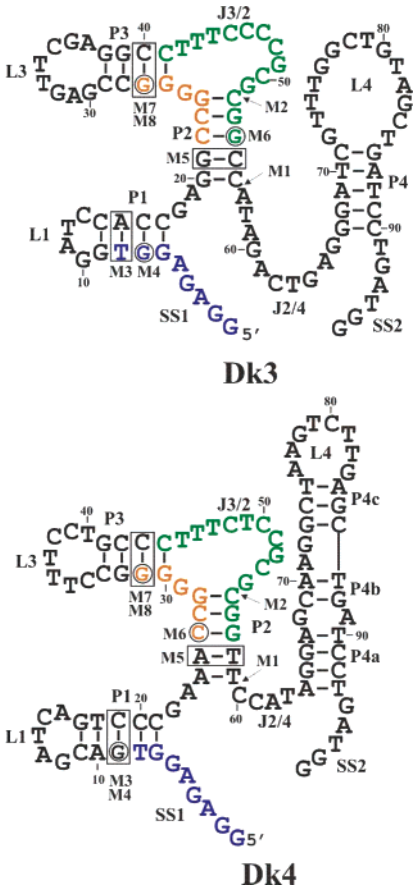

FIGURE 3: Secondary-structure prediction and confirmation for Dk3 and Dk4. Secondary structures of Dk3 and Dk4 predicted by the *mfold* program are shown separately. SS represents a single-stranded region, P a base-paired region, L a loop region, and J a junction between two base-paired regions. Lines with arrowheads indicate 3′-truncations up to that site. Rectangles around base pairs indicate sites where constructs were made with "flipped" base pairs (i.e., a C-G to a G-C). Circled bases were mutated to create a mismatch in the predicted stem. The exact changes are shown in Experimental Procedures. Reaction rates and percent yield for each mutant construct are shown in Table 1.

that all three deoxyribozymes recognize guanine highly specifically and the amine group at the C2 position on guanine is important for deoxyribozyme activity.

Secondary-Structure Study of Dk3 and Dk4. The secondary structures of Dk3 and Dk4 were analyzed using a variety of synthetic deoxyribozyme constructs and compared with the previously reported structure of Dk2 (*16*). Predicted structures for Dk3 and Dk4 using *mfold* software (*17*) are shown in Figure 3. The two structures are very similar, both containing four stem−loop regions, with the 14-nt internal conserved sequence acting as a junction between two of the stems. The secondary structures of Dk3 and Dk4 were confirmed and refined through the testing of mutant constructs with specific nucleotides mutated or removed. The key results are summarized in Table 1. First, truncations were made from the 3′ end to determine how many nucleotides could be removed without the loss of activity. For both Dk3 and Dk4, it was found that truncations could be made up to the end of the stem P2 (M1, Figure 3) without significant loss of activity. Further truncation resulted in inactive constructs (such as M2, Figure 3).

To test the three remaining predicted stems of both deoxyribozymes, constructs were made with different base

Table 1: Reaction Rates and Percent Yields of Truncated and Mutant Constructs Listed for Dk3 and Dk4[a]

	Dk3		Dk4	
	rate (min^{-1})	% yield	rate (min^{-1})	% yield
wild type	0.19	76	0.28	51
M1 (3′ truncation up to P2)	0.11	80	0.12	80
M2 (3′ truncation + half of P2)	no activity		no activity	
M3 (covaried base P1)	0.09	75	0.02	78
M4 (mismatched P1)	0.01	56	0.0058	26
M5 (covaried base P2)	0.03	35	0.018	10
M6 (mismatched P2)	0.002	5	no activity	
M7 (covaried P3)	0.06	69	0.014	64
M8 (mismatched P3)	0.002	30	0.012	62

[a] Abbreviations are in reference to the secondary structures shown in Figure 3.

pairs in each stem. As shown in Table 1, all covariation mutants for each stem were active for both Dk3 and Dk4, suggesting that these are indeed base-paired regions. Notably for both deoxyribozymes, the P2 stem shows a significant drop in activity and reaction yield when covariations are introduced. This suggests that the wild-type base pairs are preferred over different base-pair combinations. One possible explanation is that this region is involved in other tertiary interactions as well as being a stem. To further confirm these three stems, constructs were made in which mismatches were introduced into these stem regions. If these stems are present in the active structure and necessary for folding, then adding these mismatches should weaken the stem and reduce the catalytic activity. As shown in the table, both catalytic rate and reaction yield were significantly reduced or absent in all but one of the constructs tested, further confirming that these predicted base-pair regions are present and important in the active structure of Dk3 and Dk4. The only exception is P3 of Dk4, which showed catalytic rate and yield comparable to its corresponding covariation mutant. This could mean either that P3 is not necessary for proper folding of Dk4 or that a single mismatch is not enough to disrupt this stem.

In addition to the *mfold*-predicted stems, we found a potential stem between the 5′ region and the large central conserved sequence element in both Dk3 and Dk4 (shown by thin dotted lines in Figure 6). Several constructs were designed to test this possibility, and the data (Supporting Information, Figure 2) suggest that this helical region may not exist or that the base content is essential to catalysis and could not be altered.

Structural Comparison of Dk2, Dk3, and Dk4. After revealing that Dk3 and Dk4 contained a common structural arrangement, we reexamined the previously reported structural data for Dk2 to look for structural similarities. We found that, indeed, the secondary structure of Dk2 is quite similar to those of Dk3 and Dk4. As shown in Figure 4, Dk2 contains two stems (corresponding to P2 and P3 in Dk3 and Dk4) flanking the conserved 14-nt sequence motif. In fact, when comparing the structures, we were able to identify a 3-bp extension of the central stem in Dk2 (P′ in Figure 4) that was not identified previously. This stem was confirmed through identification of covarying mutations present in the previously reported Dk2 reselection data (*16*). The confirmed stem P1 in Dk3 and Dk4 is absent in Dk2.

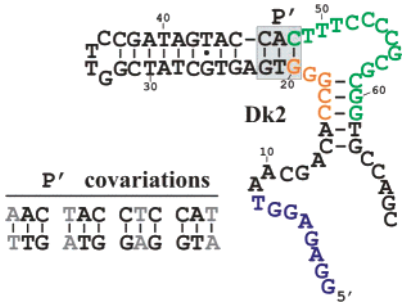

FIGURE 4: Secondary-structural model of Dk2. The newly observed stem addition P′ is shown in a gray box. Observed covariations of newly discovered P′ are shown with gray letters at the bottom left of this figure.

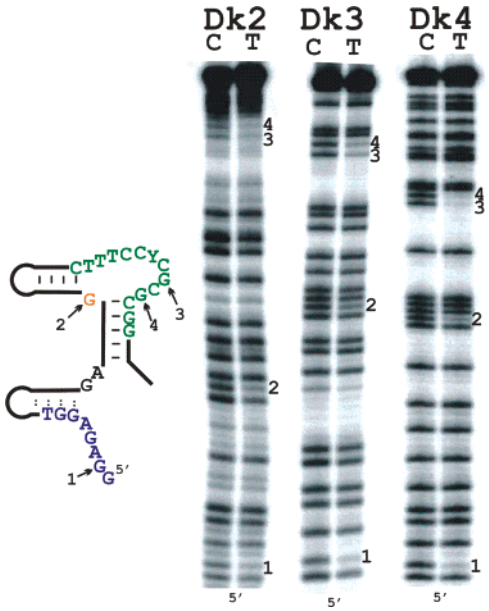

FIGURE 5: Methylation interference of Dk2, Dk3, and Dk4. "C" represents control samples, and "T" represents test samples. A common model for the three deoxyribozymes is shown on the left of the figure with conserved residues shown. Y represents a C in Dk2 and Dk3 and a T in Dk4. Dotted lines indicate base pairs present in Dk3 and Dk4 but not in Dk2. The numbers 1−4 represent guanines that show significant interference and are labeled on the gels for Dk2, Dk3, and Dk4 as well as in the corresponding model.

Methylation Interference Studies. Methylation interference assays have previously been used with several deoxyribozymes as a method of identifying guanine residues that are involved in tertiary interactions (*7*, *8*, *18*−*21*). The method used on deoxyribozyme kinases has been previously described in detail (*16*). Briefly, dimethyl sulfate (DMS) is used to methylate guanine residues specifically at their N7 position. These molecules are screened for activity, cleaved by piperidine at methylated guanine sites, and resolved by denaturing PAGE. This procedure can reveal sites where methylation interferes with deoxyribozyme activity.

The results of the methylation interference experiments for Dk2, Dk3, and Dk4 are shown in Figure 5. It should be noted that methylation interference analysis has been performed previously for Dk2 (*16*) but was redone here for direct comparison with Dk3 and Dk4, as a different labeling procedure was used previously. The interference profiles for all three deoxyribozymes show a similar pattern with four guanines exhibiting obvious interference when methylated in both cases. In the context of the common secondary-

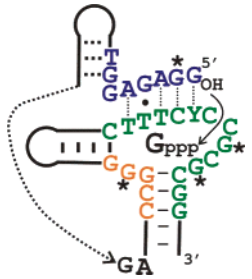

FIGURE 6: Common secondary-structural model for Dk2, Dk3, and Dk4. Thick dotted lines represent base pairs present in Dk3 and Dk4 but not in Dk2. Thin dotted lines (and a dot) represent suspected base pairs that could not be confirmed by mutational analysis. Conserved nucleotides in Dk2, Dk3, and Dk4 are shown in color as per their alignment in Figure 1. Y represents a cytosine in Dk2 and Dk3 and a thymine in Dk4. Asterisks represent guanines that showed methylation interference for all three deoxyribozymes. The dotted arrow indicates the chain direction at that location. The substrate GTP is shown as "Gppp".

structure model, these guanines are conserved in all three deoxyribozymes, with one guanine near the 5′ end, one guanine located between the two conserved stems, and two guanines within the large internal conserved sequence motif. These guanines likely play important catalytic or structural roles in the active structure of these deoxyribozymes.

DISCUSSION

In this study we have characterized two new deoxyribozyme kinases and compared them to a previously reported deoxyribozyme. We found that they all contained three common sequence motifs as well as identical substrate and divalent metal ion cofactor requirements. Using all of the structural data obtained for the three deoxyribozymes, we can construct a common secondary-structural model as shown in Figure 6. This model is complex and unique in comparison to other characterized kinase deoxyribozymes (*15*, *16*). The common structural features are the two helices flanking the 14-nucleotide conserved sequence. An additional stem was found near the 5′ end of Dk3 and Dk4 but not Dk2.

As shown in the model, it is likely that the 5′ region interacts in some way with the conserved 14-nucleotide internal sequence, simply based on the observation that these two regions are the only two large conserved sequence domains in the deoxyribozymes that are not implicated in helical interactions. A putative 5-bp stem is proposed as a possible way for these two single-stranded motifs to interact with each other. Although this helical interaction could not be proven using covariation mutational testing (Supporting Information, Figure 2), it is possible that there are helical interactions present, but the identity of these base pairs cannot be changed as the concerned bases may be involved in additional interactions with other residues. This proposed helix could also explain the C to T difference in the internal conserved region between Dk2 (as well as Dk3) and Dk4 (C53/C46 in Dk2 and Dk3, T49 in Dk4), as both C and T residues could bind with a guanine in a Watson−Crick or wobble base pair, respectively.

Another possible scenario for the variance at this specific base site is that this residue may be involved in base-pair-like interaction with the GTP substrate and, when mutated to T, can still form a wobble pair with GTP. If this is the case, it is possible that this mutation might broaden the substrate specificity of the deoxyribozyme to include ATP as a substrate (as the mutated thymine might be able to form a base pair with ATP). We can rule out this possibility because Dk4 contains a T at this site and can only use GTP but not ATP as a substrate (Figure 2A). By the same thinking, we noted that another cytosine (C54 in Dk2) in this region was shown to mutate to a thymine during the Dk2 reselection experiment (*16*). To investigate the substrate specificity of this mutation, we tested a construct of Dk3 containing this mutation (C47 to T in Dk3). As shown in Supporting Information, Figure 3, the mutated deoxyribozyme did not show altered specificity, indicating that this cytosine site is not involved in simple base-pair interaction with the substrate.

Even though we were not able to identify specific molecular interactions involved in these two highly conserved regions, it is highly likely that two important motifs are responsible for the binding of the GTP substrate and the catalysis of the phosphate transfer and may use molecular interactions beyond simple Watson−Crick base pairings. Hopefully, future developments in the high-resolution study of deoxyribozymes (which has so far remained elusive) will shed light on the nature of these unknown interactions.

As mentioned earlier, it is our belief that these three deoxyribozymes containing a similar secondary structure and multiple conserved regions arose independently from the initial DNA library and are not descendants from a common sequence. The main piece of evidence for this is the fact that the internal conserved domains are located at different locations within the deoxyribozymes. Since the in vitro selection strategy used to select these deoxyribozymes included a PAGE-based size-selection step designed to isolate catalysts having a length of ∼100 nt and all selected deoxyribozymes indeed contain 98−100 nt (*15*), it is unlikely that polymerase pausing or jumping could be responsible for the observation of these sequence motifs at different locations as these events would yield sequences of variable lengths that would be lost during the size-exclusion step. The most likely explanation for the repeated observation of these conserved sequence motifs is that they were repeatedly sampled in the initial library or were introduced independently through mutations during PCR.

This type of independent isolation of catalytic motifs by nucleic acids has been observed previously. The first reported case is the hammerhead ribozyme motif. The hammerhead ribozyme is a small naturally occurring ribozyme that has been found in a wide range of organisms (*22*−*25*). Several groups have isolated this motif repeatedly during independent in vitro selection experiments designed to isolate RNA-cleaving ribozymes (*26*−*28*). This is in contrast to other naturally occurring RNA-cleaving ribozymes such as the hairpin ribozyme (*29*−*31*), *Neurospora* VS motif (*32*), and the hepatitis delta ribozyme (*33*), which have not been observed from in vitro selection experiments for RNA-cleaving ribozymes. Another well-documented case is a small RNA-cleaving deoxyribozyme known as "8-17", which has also been isolated several times from independent in vitro selection experiments. 8-17 was initially isolated during an in vitro selection for DNA molecules that can cleave an all-RNA substrate (*34*). The same catalytic motif has since been identified by several other groups in independent in vitro

selection experiments (*35*−*39*). As is seen with the common motif in Dk2, Dk3, and Dk4, many of these in vitro selection experiments led to the isolation of many different 8-17 variants that appear to have evolved independently. Also, there are many other efficient deoxyribozymes that cleave RNA, such as 10-23 (*34*), the bipartite deoxyribozyme (*40*), and the E2 deoxyribozyme (*41*), that have not shown repeated occurrences during in vitro selection experiments. It has been suggested that the recurrence of the 8-17 deoxyribozyme indicates that it is the simplest structural solution to the RNA-cleavage reaction for DNA catalysts (*28*, *38*).

Similar to 8-17, multiple occurrences of the common deoxyribozyme kinase seem to suggest that it could be the simplest design for the DNA self-phosphorylation reaction. In comparison to the 8-17 motif, however, the kinase motif has some important distinctions. While the common 8-17 secondary structure contains only four absolutely conserved nucleotides and a three-base-pair stem (*38*), the common kinase motifs share 28 conserved nucleotides (colored nucleotides in Figure 6) and two stems containing at least nine base pairs. The need for more conserved bases and structural elements may be due to the increased complexity of phosphorylation as opposed to RNA cleavage, as phosphorylation requires the binding of an exogenous small-molecule substrate as well as catalysis. The other difference is that while 8-17 has been found to be the dominant deoxyribozyme in several in vitro selection experiments, this kinase motif was found to be less abundant than a faster deoxyribozyme (Dk1) in the original in vitro selection experiment (*15*). This observation can be rationalized by the fact that Dk1 requires ATP for catalysis instead of GTP. Since both of these substrates were provided during the in vitro selection process, Dk2 (as well as Dk3 and Dk4) did not have to compete with Dk1 because they use different substrates. In fact, the deoxyribozymes containing this common kinase motif were found to be the only catalysts that are dependent on GTP in the selected deoxyribozyme pool, providing more evidence that this structural arrangement is likely the simplest arrangement to carry out the phosphorylation reaction using GTP as a substrate.

SUPPORTING INFORMATION AVAILABLE

Chemical structures of nucleoside 5′-triphosphates examined as potential substrates of Dk2, Dk3, and Dk4 (Figure 1), results from testing the putative helix between the 5′ region and the internal conserved sequence element in Dk3 and Dk4 (Figure 2), and results of the Dk3 C47T mutant substrate specificity test (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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