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DNA Oligonucleotide 3′-Phosphorylation by a DNA Enzyme

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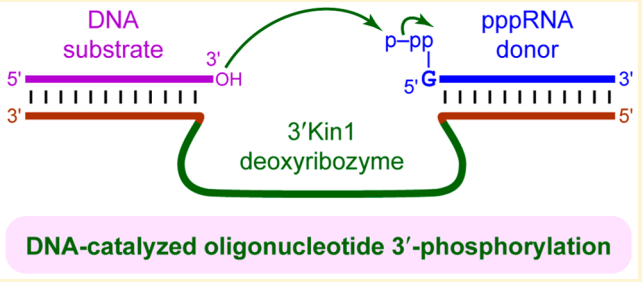
Supporting Information

ABSTRACT: T4 polynucleotide kinase is widely used for 5′-phosphorylation of DNA and RNA oligonucleotide termini, but no natural protein enzyme is capable of 3′-phosphorylation. Here, we report the in vitro selection of deoxyribozymes (DNA enzymes) capable of DNA oligonucleotide 3′-phosphorylation, using a 5′-triphosphorylated RNA transcript (pppRNA) as the phosphoryl donor. The basis of selection was the capture, during each selection round, of the 3′-phosphorylated DNA substrate terminus by 2-methylimidazole activation of the 3′-phosphate (forming 3′-MeImp) and subsequent splint ligation with a 5′-amino DNA oligonucleotide. Competing and precedented DNA-catalyzed reactions were DNA phosphodiester hydrolysis or deglycosylation, each also leading to a 3′-phosphate but at a different nucleotide position within the DNA substrate. One oligonucleotide 3′-kinase deoxyribozyme, obtained from an N_{40} random pool and named 3′Kin1, can 3′-phosphorylate nearly any DNA oligonucleotide substrate for which the 3′-terminus has the sequence motif 5′-NKR-3′, where N denotes any oligonucleotide sequence, K = T or G, and R = A or G. These results establish the viabilty of in vitro selection for identifying DNA enzymes that 3′-phosphorylate DNA oligonucleotides.



Oligonucleotide kinase activity is a broadly useful enzymatic function for biochemists. In vitro, the protein enzyme T4 polynucleotide kinase (T4 PNK) is commonly used to phosphorylate the 5′-terminus of DNA and RNA oligonucleotides.[1−5] However, nature has not evolved analogous protein enzymes for oligonucleotide 3′-phosphorylation. Using in vitro selection,[6−11] synthetic ribozymes have been identified that have oligonucleotide 5′-kinase or internal 2′-kinase activity,[12−20] and synthetic deoxyribozymes (DNA enzymes, DNA catalysts)[21−26] with 5′-kinase activity have also been found.[27−31] In this report, we describe a strategy for in vitro selection of deoxyribozymes that have DNA oligonucleotide 3′-kinase activity, where previously we reported DNA-catalyzed tyrosine kinase activity.[32,33] In many earlier selections for DNA-catalyzed RNA ligase activity,[23,34] the oligonucleotide 3′-terminus attacked the α-phosphate of 5′-triphosphorylated RNA (pppRNA). Here, the in vitro selection process was designed to provide DNA enzymes that catalyze attack of a DNA 3′-OH group at the γ-phosphate of pppRNA, creating a DNA 3′-phosphate rather than resulting in ligation. One new oligonucleotide kinase deoxyribozyme is useful for 3′-phosphorylation of a substantial subset of all possible DNA sequences. Our findings demonstrate that in vitro selection can be used to identify oligonucleotide 3′-kinase DNA enzymes.

MATERIALS AND METHODS

In Vitro Selection Procedure. See the text in the Supporting Information for detailed procedures and Figure S1 for nucleotide details. The 3′Kin1 deoxyribozyme sequence (72 nt) was 5′-*CCTTAAGACTGAATTC*GAAACGCA-CACGGAATCGCCAGCGAGCTATCGAAGCAGTG-TTAAGAAACGAGATAT-3′, where the pppRNA substrate binding arm is shown in italic font, the initially random (N_{40}) region is shown in boldface font, and the DNA substrate binding arm is underlined (the mutated nucleotide is also shown in italic font). The DNA substrate (20 nt) was 5′-GGAATATCTCGTTTCTTATA-3′. The pppRNA phosphoryl donor (17 nt) was 5′-GGAAUUCAGUCUUAAGG-3′; for the mutated sequence where the sole G is at the 5′-terminus, the second and third Gs were changed to A, and the fourth and fifth Gs were changed to C.

Single-Turnover Deoxyribozyme Assay Procedure. The DNA substrate was 5′-^{32}P-radiolabeled using γ-^{32}P-ATP and T4 polynucleotide kinase (Thermo Fisher). A 10 μL sample containing 0.25 pmol of 5′-^{32}P-radiolabeled DNA substrate, 10 pmol of deoxyribozyme, and 20 pmol of pppRNA phosphoryl donor was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The DNA-catalyzed phosphorylation reaction was initiated by bringing the sample to a total volume of 20 μL containing 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl (or other ion concentrations as appropriate). The Mn^{2+} was added from a 10× stock solution containing 200 mM

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$MnCl_2$. The Zn^{2+} was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM HNO_3, and 200 mM HEPES at pH 7.5; this stock solution was freshly prepared from a 100× stock of 100 mM $ZnCl_2$ in 200 mM HNO_3. The metal ions were added last to the final sample, which was divided into 2 μL aliquots that were all incubated at 37 °C. At appropriate time points, 2 μL aliquots were quenched with 4 μL stop solution (80% formamide, 1× TBE [89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3], 50 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were separated by 20% PAGE and quantified with a PhosphorImager. Values of k_{obs} were obtained by fitting the yield (Y) versus time data directly to first-order kinetics; i.e., $Y = Y_{max} \times (1 - e^{-kt})$ where $k = k_{obs}$ and Y_{max} is the final yield. Errors in k_{obs} values were calculated as the standard deviation from the indicated number of independent determinations. For Mn^{2+} K_d determination, initial-rate kinetics were determined as above, excluding Zn^{2+} and Mg^{2+}, and with the concentration of Mn^{2+} varied from 0.5 mM to 50 mM (t = 0−2 h). The k_{obs} value at each $[Mn^{2+}]$ was determined $n = 3$ times. Values were weighted as $1/\sigma^2$, where σ is the error from the linear fit. For determinations at low $[Mn^{2+}]$, where the yield was below the detection limit (~4% in 2 h, corresponding to $k_{obs} < 0.02$ h^{-1}), σ was assigned as 0.01 h^{-1}.

RESULTS

In Vitro Selection Strategy To Identify DNA Oligonucleotide 3′-Kinase Deoxyribozymes. The in vitro selection strategy is shown in Figure 1. A DNA oligonucleotide of arbitrary sequence was used as the 3′-phosphorylation substrate. A 5′-triphosphorylated RNA oligonucleotide (pppRNA; prepared by in vitro transcription using a DNA template and T7 RNA polymerase[35,36]) was used as the phosphoryl donor. Both the DNA substrate and RNA phosphoryl donor interact by Watson−Crick base pairs with fixed-sequence DNA segments that flank an N_{40}, N_{60}, or N_{80} random region (40, 60, or 80 random DNA nucleotides). Several different random region lengths were evaluated because, for both nucleic acid aptamers and catalysts, the outcome of in vitro selection can be strongly dependent on this variable.[37−41] In each selection round, the pool of initially random sequences was incubated under conditions anticipated to foster 3′-phosphorylation. These incubation conditions were 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C for 14 h. Each of Zn^{2+}, Mn^{2+}, and Mg^{2+} were included because they have served as useful metal ion cofactors for a variety of DNA enzymes, including with oligonucleotide substrates.[23,25] DNA sequences in the initially random pool that are capable of 3′-phosphorylating the substrate were captured via a two-step process in which the 3′-phosphate is first activated to its 2-methylimidazolide adduct (3′-MeImp). Subsequently, the 3′-MeImp terminus was joined with a 5′-amino-modified DNA oligonucleotide using a DNA splint, forming a phosphoramidate (P−N) linkage.[42−44] This bond formation leads to a shift on polyacrylamide gel elecrophoresis (PAGE), which enables separation of the catalytically active DNA sequences. These sequences were amplified by PCR, and the forward strand was ligated with the DNA substrate bearing a free 3′-OH and taken into the next selection round.

Before implementing the Figure 1 selection strategy, we attempted to optimize the capture step to reduce the survival of

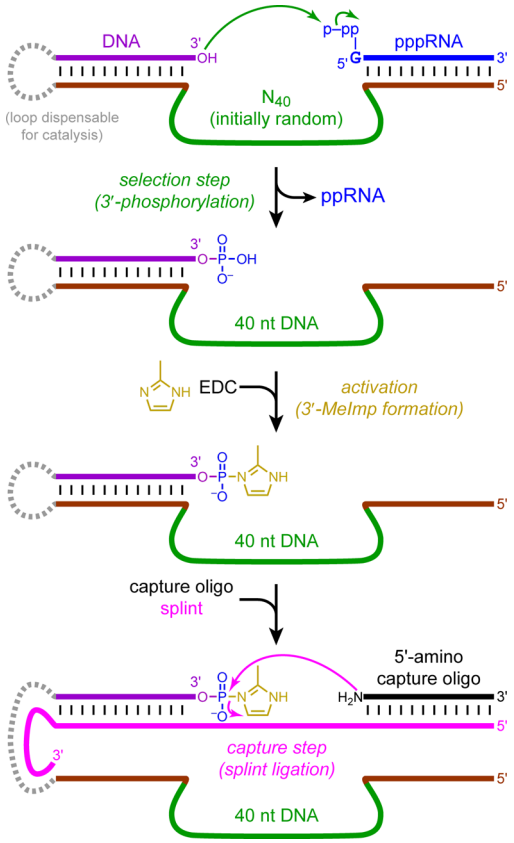

Figure 1. In vitro selection for DNA-catalyzed oligonucleotide 3′-phosphorylation. In practice, the activation and capture reactions were performed simultaneously (see the Supporting Information text for all procedures). See Figure S1 in the Supporting Information for nucleotide details.

DNA sequences that catalyze DNA hydrolysis or deglycosylation, rather than the intended 3′-phosphorylation (Figure S2 in the Supporting Information). Unrelated DNA enzymes are known to be capable of either hydrolyzing DNA with the formation of 3′-phosphate termini,[45] or deglycosylating DNA followed by elimination reactions that result in 3′-phosphate termini.[46,47] These unintended reactions lead to a DNA oligonucleotide product that is shorter than the product formed by direct 3′-phosphorylation, although the length difference can be as small as a single nucleotide. The optimization effort led to 72% capture of the intended 3′-phosphorylation product. However, we also observed 55% capture of the unintended product from either hydrolysis at, or deglycosylation of, one nucleotide to the 5′-side within the substrate (these two mechanisms cannot be distinguished solely on the basis of oligonucleotide product identity). Therefore, the per-round capture bias is 72%/55% = 1.3 in favor of intended rather than unintended deoxyribozymes. Despite this imperfect differentiation, which nevertheless should promote identification of the intended deoxyribozymes when iterated over many selection rounds, we performed the selection experiments to identify 3′-kinase deoxyribozymes. Note that the capture bias is not an enrichment factor between active and inactive DNA sequences; it is a bias between different types of active sequences. The enrichment factor itself is controlled primarily by the efficiencies of the DNA-catalyzed reactions, relative to efficiencies of uncatalyzed background reactions that allow the survival of random, catalytically inactive sequences.

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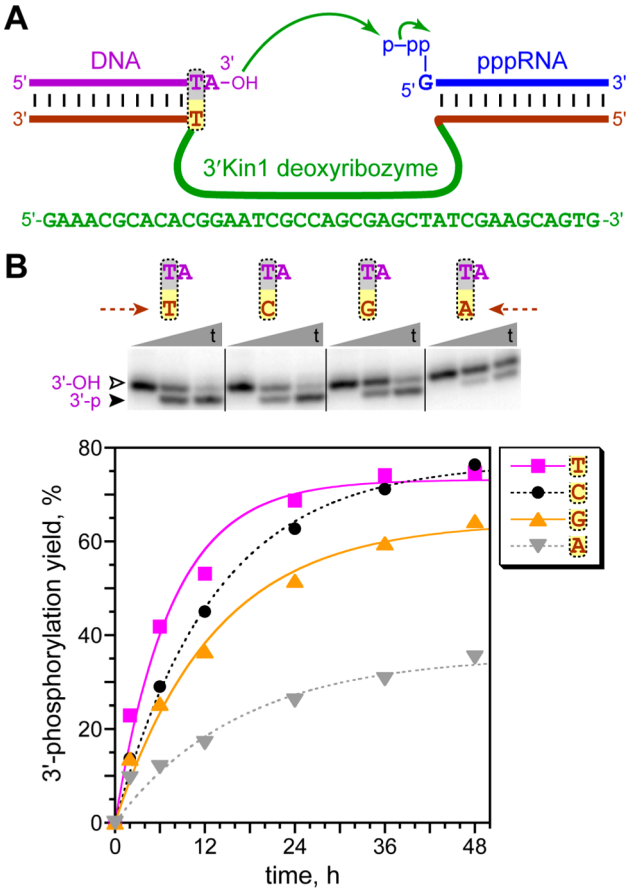

Figure 2. 3′Kin1 deoxyribozyme. (A) Sequence of the initially random region of 3′Kin1 and interaction of the deoxyribozyme with its DNA oligonucleotide substrate. (B) PAGE-shift assay of 3′Kin1, establishing optimality of a T/T mismatch with the substrate (in the gel image, representative time points are shown for t = 0, 12, 48 h). For the deoxyribozyme variant with T at the indicated position, single-turnover k_{obs} = 0.14 ± 0.01 h^{-1} (n = 5, mean ± standard deviation). Incubation conditions: 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C.

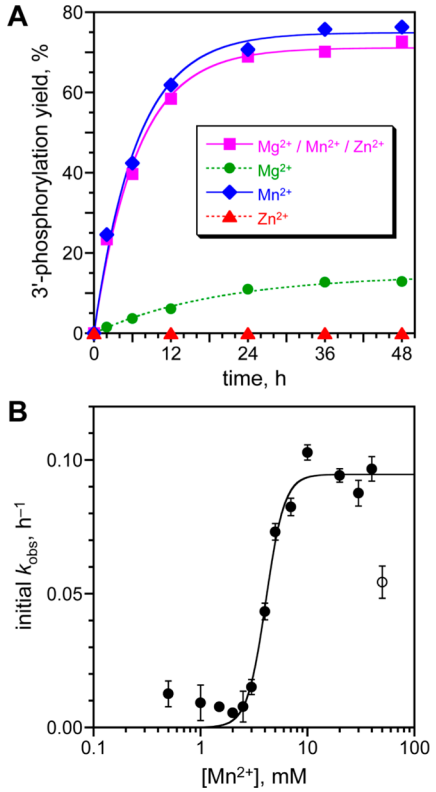

Figure 3. Metal ion dependence of 3′Kin1. (A) Assays with combinations of Zn^{2+}, Mn^{2+}, and Mg^{2+} (see comprehensive data in Figure S6). Incubation conditions as in Figure 2, with metal ions as indicated. (B) Determination of apparent K_d for Mn^{2+}. Each k_{obs} value was derived from initial-rate kinetics (0–2 h). The filled data points from n = 3 experiments, weighted according to $1/\sigma^2$, were fit to $k_{obs} = k_{max} \times ([Mn^{2+}]^n/([Mn^{2+}]^n + K_d^n)$, with fit values of K_d = 4.1 ± 0.2 mM, Hill coefficient n = 5.0 ± 1.0, and k_{max} = 0.095 ± 0.003 h^{-1}.

Identification and Initial Characterization of the 3′Kin1 Deoxyribozyme. Selections performed with N_{60} and N_{80} random regions were conducted through round 14, but no activity was observed, and these efforts were set aside. In contrast, the N_{40} selection experiment showed substantial activity at round 12 (see Figure S3 in the Supporting Information), and individual deoxyribozymes were cloned and characterized. One deoxyribozyme, designated 3′Kin1, was found (Figure 2A). The total length of 3′Kin1 is 72 nt, including both of the fixed-sequence segments that flank the initially random (N_{40}) region. Mfold[48] predicts numerous energetically similar secondary structures for this region (not shown). We also found four unrelated deoxyribozymes that appear to catalyze either DNA hydrolysis or deglycosylation (Figure S4 in the Supporting Information); these DNA enzymes were not characterized further.

The 3′Kin1 deoxyribozyme was found to 3′-phosphorylate the DNA substrate as intended. When the 5′-^{32}P-radiolabeled DNA substrate was provided to 3′Kin1, a PAGE shift consistent with 3′-phosphorylation was observed (Figure 2B). Phosphorylation was revealed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry of the PAGE-purified product (calcd. 6178.0, found 6177.3; Δ = −0.011%). Finally, 3′-phosphorylation was confirmed functionally by taking the 3′-phosphorylated oligonucleotide product through the two-step capture assay of 3′-MeImp formation and splinted reaction with 5′-amino oligonucleotide. Equivalent splinted reaction rate constants and yields were observed for the 3′Kin1 product and a 3′-phosphorylated oligonucleotide standard that was prepared separately by solid-phase synthesis (Figure S5 in the Supporting Information).

At the 3′-terminus of the DNA substrate, the penultimate nucleotide is positioned to form a Watson–Crick base pair with the first fixed-sequence nucleotide of the 3′Kin1 deoxyribozyme immediately to the 3′-side of its initially random region (see two encircled nucleotides in Figure 2A). In the cloned sequence of 3′Kin1, the first fixed-sequence nucleotide of the deoxyribozyme was found to be mutated from A to T, presumably due to a mutation introduced by Taq polymerase during the cloning process. In unrelated selections where a fixed-sequence mutation has been observed, restoration of the initially present nucleotide typically provides optimal catalytic activity. However, here we found that the mutation to T—which disrupts the putative T:A substrate:deoxyribozyme base pair, instead providing a T/T mismatch—was unexpectedly required for optimal activity (Figure 2B). With the fixed-sequence T mutation, the single-turnover rate constant k_{obs} of 3′Kin1 was 0.14 h^{-1} and 75% yield in 48 h. The divalent metal ion requirements of 3′Kin1 were surveyed (Figure 3 and Figure S6 in the Supporting Information). Mn^{2+} was necessary and

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Biochemistry 2016, 55, 2671–2676

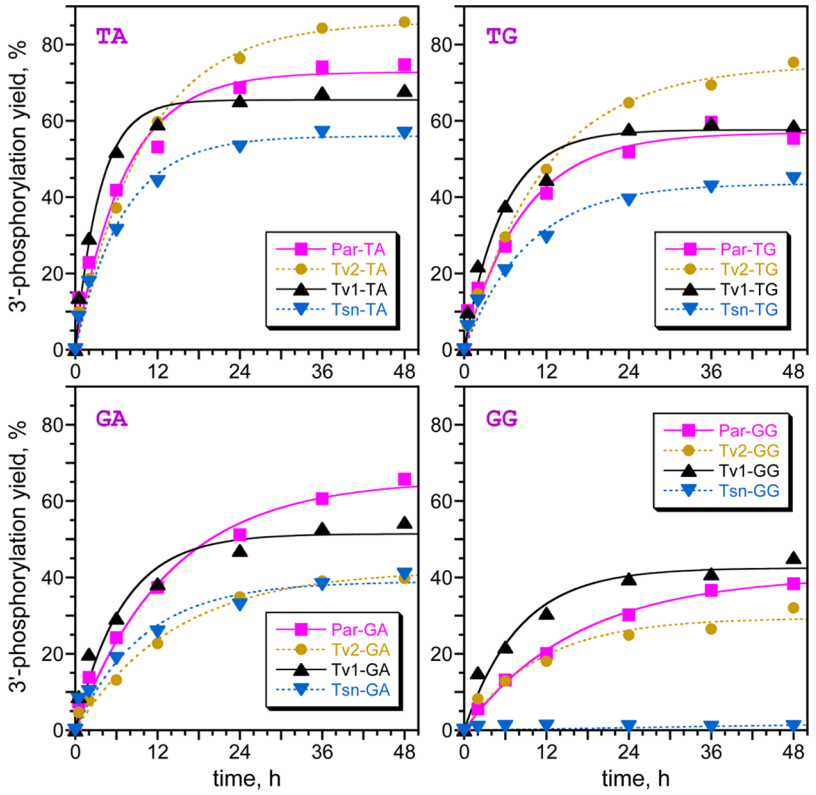

Figure 4. Determining substrate sequence scope of 3′Kin1. Incubation conditions as in Figure 2. Each plot shows 3′-phosphorylation assays for four DNA substrates, all of which end at the 3′-terminus with one of TA, TG, GA, or GG. In each set of four assays, "Par" denotes the parent DNA substrate sequence as used during selection, 5′-GGAATATCTCGTTTCTTATA-3′. "Tv2" denotes systematic transversions of the underlined nucleotides according to A↔T, G↔C; "Tv1" denotes transversions A↔C, G↔T; and "Tsn" denotes transitions A↔G, T↔C. The illustrated data are representative of two independent datasets.

sufficient for full activity, with an apparent K_d value of 4 mM and cooperative binding to more than one Mn^{2+} ion. Mg^{2+} alone supported modest activity with 3-fold lower k_{obs} and substantially reduced yield, whereas Zn^{2+} alone did not support catalysis. 3′Kin1 has greatly reduced activity (11% yield in 48 h) when the substrate is all DNA except for a 3′-terminal ribonucleotide, and 3′Kin1 has no activity at all when the entire substrate is RNA (see Figure S7 in the Supporting Information). When tested with GTP and an RNA oligonucleotide missing the 5′-terminal pppG nucleotide, 3′Kin1 had no activity (data not shown).

Substrate Sequence Scope of the 3′Kin1 Deoxyribozyme. A deoxyribozyme such as 3′Kin1 is most useful when it functions with a wide range of DNA oligonucleotides, covarying the binding arm to maintain Watson−Crick base pairing with the substrate. We assessed the substrate sequence scope of 3′Kin1 in a comprehensive series of experiments (Figure 4). The two nucleotides at the 3′-terminus of the DNA substrate may be any of TA, TG, GA, or GG. Substrates ending with TA are 3′-phosphorylated in 55−85% yield, those ending with TG or GA in 40−75% yield, and those ending with GG in 30−45% yield (the specific sequence combination ending with GGG could not be 3′-phosphorylated using 3′Kin1 with any of T, C, or G at the first nucleotide to the 3′-side of the initially random region). From these data, we conclude that 3′Kin1 has sequence requirement 5′-NKR-3′, where N denotes any oligonucleotide sequence up to the final two nucleotides, K = T or G, and R = A or G. Some additional substrates that do not fit the 5′-NKR-3′ motif were also detectably 3′-phosphorylated, but without further systematic pattern of success (see Table S1 in the Supporting Information).

One means of 3′-^{32}P-radiolabeling using 3′Kin1 is to transfer the γ-^{32}P-phosphate group of a radiolabeled pppRNA substrate to the DNA 3′-OH group. For this purpose, which is chemically equivalent to the analogous reaction with non-radiolabeled substrate, the pppRNA must be ^{32}P-radiolabeled at its γ-phosphate. Because γ-^{32}P-GTP is commercially available, we assayed whether a pppRNA that lacks G at any nucleotide position except the first can be used by 3′Kin1. The original 17-mer pppRNA sequence used during the selection process has five G nucleotides, including 5′-pppGG at the first two positions. When a 17-mer 5′-pppGA transcript lacking all G nucleotides except the first was assayed with a deoxyribozyme that has Watson−Crick compensatory binding arm mutations, the 3′-phosphorylation activity was fully maintained (data not shown). Therefore, 3′Kin1 should be usable for 3′-^{32}P-radiolabeling by using the appropriate γ-^{32}P-pppRNA transcript.

DISCUSSION

In this study, we have demonstrated the viability of in vitro selection for identifying deoxyribozymes that 3′-phosphorylate DNA oligonucleotide substrates. Although the capture method is not strongly selective for the intended 3′-phosphorylated DNA terminus, one deoxyribozyme with 3′-kinase activity was identified. For this new deoxyribozyme, 3′Kin1, the same initially random (N_{40}) region sequence can be used with many different DNA substrate sequences by appropriate choice of the fixed-sequence segment on the 3′-side of the initially random

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Biochemistry 2016, 55, 2671−2676

region. At the first nucleotide position of the fixed-sequence segment, a T is favored, regardless of the DNA substrate sequence. At the remaining positions of the fixed-sequence segment, straightforward Watson−Crick base pairing should be included. With this arrangement, almost any DNA substrate that has sequence 5′-NKR-3′ can be 3′-phosphorylated, where N denotes any oligonucleotide sequence, K = T or G, and R = A or G. Certain other substrate sequences may also be 3′-phosphorylated.

Several future challenges must be addressed for DNA enzymes that 3′-phosphorylate oligonucleotide substrates. Such DNA enzymes may have the greatest practical utility for 3′-^{32}P-radiolabeling, which for DNA is presently accomplished using an α-^{32}P-dNTP and terminal deoxytransferase (TdT), but often with product heterogeneity because TdT can attach more than one successive ^{32}P-nucleotide to the 3′-terminus. A DNA enzyme for 3′-^{32}P-radiolabeling would preferably use commercially available γ-^{32}P-ATP or GTP as the phosphoryl donor with low K_m value, rather than the pppRNA required by 3′Kin1. Our ongoing efforts toward deoxyribozymes for peptide side chain phosphorylation support the expectation that such reactivity with a small-molecule phosphoryl donor such as ATP or GTP should be achievable.[32,33] 3′-Phosphorylation of RNA rather than DNA is also a valuable objective. For all of these applications, the most general substrate sequence scope is desired, either from an individual DNA enzyme or collectively from a set of DNA enzymes. Such generality has been achieved with RNA-cleaving deoxyribozymes[49−51] and RNA ligase deoxyribozymes[52,53] and seems likely for DNA-hydrolyzing deoxyribozymes as well,[45,54] suggesting the viability of general DNA-catalyzed oligonucleotide 3′-phosphorylation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00151.

Experimental details and additional data (PDF)

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Notes

The authors declare no competing financial interest.

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