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Nucleic Acids

DNAzyme-Catalyzed Site-Specific N-Acylation of DNA Oligonucleotide Nucleobases

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Abstract: We used in vitro selection to identify DNAzymes that acylate the exocyclic nucleobase amines of cytidine, guanosine, and adenosine in DNA oligonucleotides. The acyl donor was the 2,3,5,6-tetrafluorophenyl ester (TFPE) of a 5′-carboxyl oligonucleotide. Yields are as high as >95% in 6 h. Several of the N-acylation DNAzymes are catalytically active with RNA rather than DNA oligonucleotide substrates, and eight of nine DNAzymes for modifying C are site-specific (>95%) for one particular substrate nucleotide. These findings expand the catalytic ability of DNA to include site-specific N-acylation of oligonucleotide nucleobases. Future efforts will investigate the DNA and RNA substrate sequence generality of DNAzymes for oligonucleotide nucleobase N-acylation, toward a universal approach for site-specific oligonucleotide modification.

Introduction

Site-specifically modified oligonucleotides are valuable for biochemical and biophysical investigations.[1] One particularly interesting nucleobase modification is N-acylation of the nucleotides cytidine (C), guanosine (G), and adenosine (A). Natural N^4-acetyl-C (ac4C) influences RNA translation and other biologically relevant outcomes,[2] and N-acylation at any of the nucleobases can be used to introduce biophysical labels. For many kinds of modifications,[3] bottom-up solid-phase synthesis of modified oligonucleotides by incorporation of suitable nucleoside phosphoramidite monomers is frequently performed,[4] along with enzymatic ligation when long oligonucleotides are sought.[5] A chemoenzymatic ligation-based synthetic method based on nucleoside 3′,5′-bisphosphates is also possible.[6] For ligation-free modification of fully intact long oligonucleotides, top-down chemical methods can sometimes be used.[7] Top-down enzymatic modification of intact oligonucleotides is less commonly pursued,[8] despite the inherent simplicity and specificity when suitable enzymes can be identified.

DNAzymes are particular DNA sequences with catalytic activity,[9] identified by in vitro selection starting from random-sequence populations.[10] They are simple to use (e.g., for RNA cleavage[11]) and can be highly site selective. In this study, we sought to expand DNAzyme catalysis to include direct, top-down N-acylation of specific C, G, and A nucleobases in DNA oligonucleotide substrates. There are no reports of DNAzymes for nucleobase N-acylation; we previously found DNAzymes for guanosine N^2-alkylation,[12] and others have reported ribozymes for various nucleobase alkylations.[13] We considered our recent effort in which DNAzyme-catalyzed N-acylation of aliphatic amine nucleophiles (e.g., lysine) was achieved using phenyl ester (PE) and 4-fluorophenyl ester (4FPE) electrophiles, whereas the background reactivity of 2,3,5,6-tetrafluorophenyl ester (TFPE; Figure 1A) was too high to allow any rate enhancement.[14] Here, with the less reactive exocyclic aromatic amine nucleophiles N^4 of C, N^2 of G, and N^6 of A (Figure 1B), we surmised that the more reactive TFPE electrophile may be appropriate as the acyl donor. We therefore performed new in vitro selection experiments in

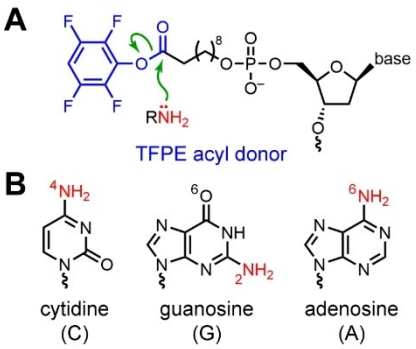

Figure 1. Electrophile and potential nucleophiles for DNAzyme-catalyzed nucleobase N-acylation. A) 2,3,5,6-Tetrafluorophenyl ester (TFPE), at the 5′-end of a DNA oligonucleotide acyl donor as the electrophile. B) Nucleobases of nucleotides C, G, and A, each of which has an exocyclic aromatic amine nucleophile: N^4 of C, N^2 of G, and N^6 of A.

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which DNA nucleobases have the amine nucleophiles and TFPE is the acyl donor.

Results and Discussion

In a preliminary in vitro selection experiment, we sought DNAzymes that acylate the CCC 3′-overhang of a DNA oligonucleotide substrate, using a 5′-TFPE oligonucleotide as the acyl donor at pH≈6, and PAGE shift as the basis of selection (Figure 2A). Five DNAzymes were found (Figure S1), establishing that TFPE is a viable electrophile for this process. Therefore, we more systematically used the TFPE acyl donor in new selection experiments with each of C, G, and A nucleophiles as the trinucleotide 3′-overhang. Offering three of the same nucleobase allows us to learn about the site selectivity of the resulting DNAzymes. We established incubation conditions that include EDC and TFP at each of pH 6.5, 7.4, and 8.5 (a useful pH range for DNAzymes), enabling in situ TFPE formation from a 5′-CO_2H DNA oligonucleotide (Figure S2). We performed the in vitro selection experiments for all 3×3=9 combinations of trinucleotide 3′-overhang and pH. Each selection used an N_{40} DNA pool, with incubation at 37 °C for either 2 h or 16 h depending on the uncatalyzed splinted background reactivity with the relevant combination of 3′-overhang and pH (Figure 2B; Figure S3). We kept this background yield to <5 % so that sufficient enrichment of catalytically active DNA sequences could be achieved in each selection round, where 5 % background corresponds to a maximum per-round enrichment of 20-fold. At pH 6.5 and 7.4, a combination of 40 mM Mg^{2+}, 20 mM Mn^{2+}, and 1 mM Zn^{2+} was included, where each of these divalent metal ions has been a useful DNAzyme cofactor for a wide range of reactions.[9] At the higher pH of 8.5, only Mg^{2+} could be included because Mn^{2+} oxidizes and Zn^{2+} precipitates.

Within 5 to 8 rounds, all nine selection experiments led to substantial yield of the DNA pool above the corresponding splinted background level (Figure 3). Seven of these nine selection experiments also had substantial yield when the

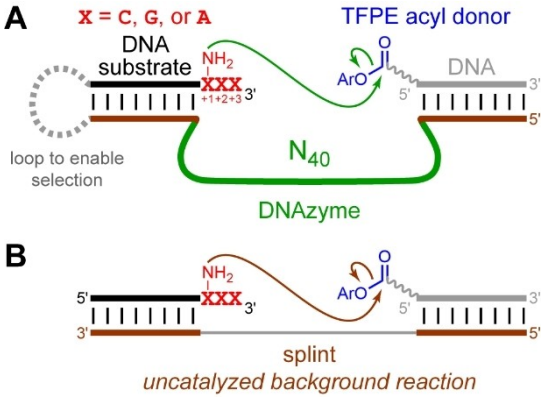

Figure 2. In vitro selection design for DNAzyme-catalyzed N-acylation of DNA oligonucleotide nucleobases. See Figure S4 for nucleotide details. A) The key enrichment step of each selection round (Ar=2,3,5,6-tetrafluorophenyl). Any of the three "X" nucleotides (here depicted as the first nucleotide, +1, in the 3′-overhang) may provide the amine nucleophile to allow the particular DNAzyme sequence to survive the selection round. The site selectivity of each DNAzyme is investigated after the selection process is completed. The 3′-OH at the end of the DNA substrate could also be a nucleophile, leading to unwanted O-acylation DNAzymes. For the TFPE electrophile (Figure 1A), a 5′-CO_2H DNA oligonucleotide is activated by including 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 2,3,5,6-tetrafluorophenol (TFP) throughout the enrichment step. The loop connecting the DNAzyme pool and the DNA substrate is required to enable selection, because attachment of the acyl donor oligonucleotide results in an upward PAGE shift only for catalytically active DNA sequences. B) The uncatalyzed splinted background reaction, in which the N_{40} nucleotides are absent. Nucleotide C+1 is depicted as providing the nucleophile, although any of C+1 to C+3 or the 3′-OH could react in this way.

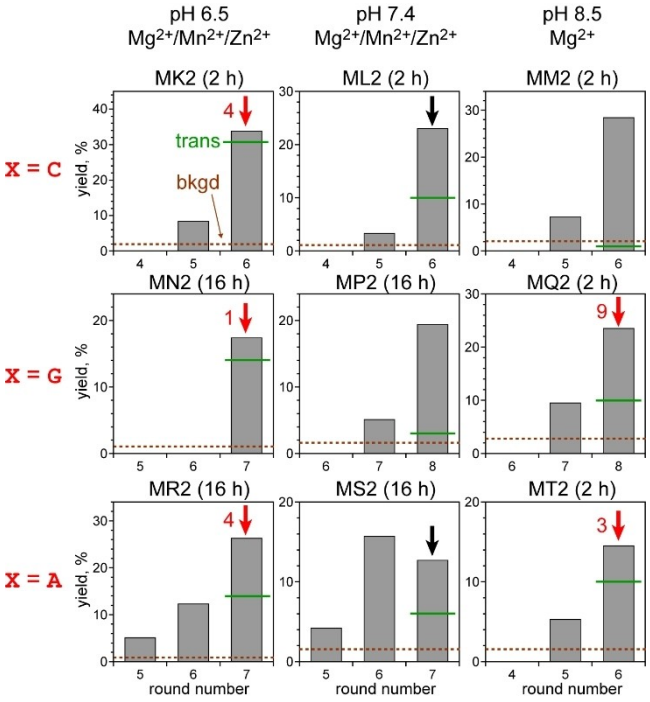

Figure 3. In vitro selection progressions. Each of the nine selections has an arbitrary alphanumeric designation, MK2 through MT2, depending on the combination of 3′-overhang sequence and incubation conditions during the enrichment step of selection (Figure 2A). Conditions were as follows, including 30 mM each EDC and TFP as well as 150 mM NaCl; see Figure S2 for detailed descriptions. pH 6.5: 105 mM HEPES, 40 mM $MgCl_2$, 20 mM $MnCl_2$, and 1 mM $ZnCl_2$. pH 7.4: 220 mM HEPES, 40 mM $MgCl_2$, 20 mM $MnCl_2$, and 1 mM $ZnCl_2$. pH 8.5: 240 mM CHES and 40 mM $MgCl_2$. With each plot is shown the incubation time at 37 °C during the enrichment step, the background yield under the same conditions (Figure S3), and the in trans assay yield (i.e., with gray loop of Figure 2A not intact) for the cloned round. Each plot shows the yields for the final three selection rounds. Arrows mark the seven cloned selection rounds; MM2 and MP2 were not cloned because the in trans yield was too low. Only the five selections MK2, MN2, MQ2, MR2, and MT2 led to individual active DNAzymes (red arrows, with the number of individual DNAzymes identified next to each arrow; Figure S5). Although ML2 and MS2 were cloned (black arrows), no individual active DNAzymes were identified. Including the preliminary selection designated JB2, 26 individual DNAzymes were identified in total.

DNA substrate was not covalently connected to the pool (in trans assay). Upon cloning, five of the seven selections, as marked with red arrows in Figure 3, led to a collective total of 21 individual DNAzymes, or 26 DNAzymes when including the five from the preliminary selection (Figure S5). None of the pH 7.4 selections led to DNAzymes, which may be related to the relatively poor observed stability of the TFPE at this intermediate pH value (Figure S2). With the CCC 3′-overhang, only the pH 6.5 selection (designated MK2 according to our lab's systematic alphanumeric nomenclature) provided DNAzymes. Both pH 6.5 and 8.5 selections with the GGG 3′-overhang (MN2 and MQ2) led to DNAzymes, as did both selections with the AAA 3′-overhang (MR2 and MT2). Each of the 26 individual DNAzymes was named according to its round number, selection designation, and clone number. For instance, the 6MK208 DNAzyme was found in round 6 of the MK2 selection as clone 08. An initial survey revealed acylation yields by individual DNAzymes as high as 97 % (Table S1). Our approach does not necessarily identify the optimal DNAzymes in the sampled sequence space.

Nine DNAzymes were identified with the CCC 3′-overhang substrate: five from the preliminary selection at pH ≈6, designated JB2, and four from the MK2 selection at pH 6.5. All nine DNAzymes were assayed with a DNA substrate that has a 2′,3′-dideoxy-C (ddC) terminus, which lacks the 3′-OH (Figure 4). Absence of the 3′-OH did not decrease any of the product yields, indicating that the substrate 3′-OH groups are not participating through O-acylation,[1b,15] and all products arise from C nucleobase N^4-acylation. Analogous assays with the ten G DNAzymes and seven A DNAzymes could not be performed because unlike for ddC, the ddG and ddA modifications are not readily introduced (the necessary solid supports are not commercially available).

Upon base treatment (10 mM NaOH, pH 12.0, 55 °C), the products from all 26 DNAzymes were found to be hydrolyzed with $t_{1/2}$ 0.4–0.8 h, whereas an aliphatic amide-linked standard was uncleaved (<1 %), and an ester-linked standard was cleaved with 5- to 9-fold faster $t_{1/2}$ (Figure 5 and Table S2). These data are consistent with the conclusion from Figure 4 that the nine C DNAzymes all catalyze nucleobase N^4-C acylation, forming aromatic amides. These data also support the conclusion that the ten G and seven A DNAzymes catalyze nucleobase N^2-G acylation and N^6-A acylation, respectively. O^6-Acylation of G is inconsistent with these base treatment data, because such a product is expected to be even more strongly base-labile.[16]

Rate constants were determined for seven individual representative nucleobase N-acylation DNAzymes (Figure 6A). The rate constant (k_{obs}) and rate enhancement (k_{obs}/k_{bkgd}) values were as high as 0.79 h^{-1} and 300-fold for 8JB210 and 7MR202, respectively (Figure 6A). The products from the seven DNAzymes of Figure 6A were prepared with their DNA substrates on the 300 pmol scale and verified by MALDI-TOF mass spectrometry to have the masses expected for N-acylation (Table S3).

Considering the practical value of modifying RNA rather than DNA substrates, all 26 N-acylation DNAzymes were tested with an RNA substrate of the same sequence in place of DNA. Because the DNAzyme binding arm that interacts with the substrate is DNA, switching the substrate from DNA to RNA inherently changes the corresponding DNAzyme:substrate helix from B-form DNA:DNA to A-form DNA:RNA, while also introducing all of the substrate 2′-OH groups. Despite these substantial structural alterations, which could strongly influence DNAzyme function, nine of the 26 DNAzymes were found to tolerate the RNA substrate with >25 % yield in 48 h (Figure 6B). This set of nine DNAzymes includes one for C acylation (8JB210, which has ≈3-fold higher k_{obs} with RNA relative to DNA, and >95 % yield with both substrates), six for G acylation, and two for A acylation. Therefore, RNA nucleobase N-

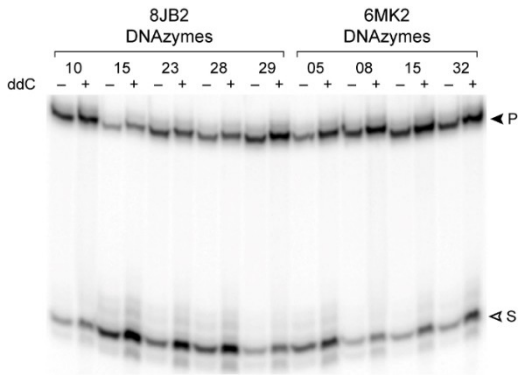

Figure 4. DNAzymes that N-acylate C nucleobases. Assays with DNA substrate that has CCC or CCddC 3′-overhang, to distinguish C nucleobase N^4-acylation from 3′-OH O-acylation (pH 6.5, 37 °C, 20 h). S = substrate; P = product. The observation of equivalent substrate reactivity with CCC or CCddC 3′-overhang is consistent with C nucleobase N^4-acylation but not 3′-OH O-acylation.

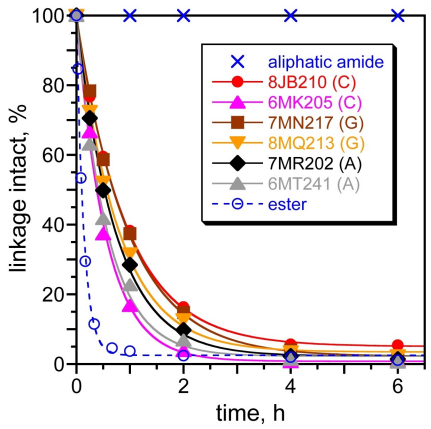

Figure 5. Product hydrolysis under alkaline conditions to establish amide formation by the DNAzymes. As shown here with data for each of six representative DNAzymes, base treatment (10 mM NaOH, pH 12.0, 55 °C) hydrolyzes all 26 products with $t_{1/2}$ 0.4–0.8 h, consistent with amide formation by nucleobase N-acylation. Half-lives for all 26 DNAzymes are in Table S2. The ester-linked standard product, which cleaved with $t_{1/2}$ 0.09 h, was made by splinted O-acylation reaction at pH 6.5 of a DNA substrate that has a 3′-rG terminus (see Figure S3).

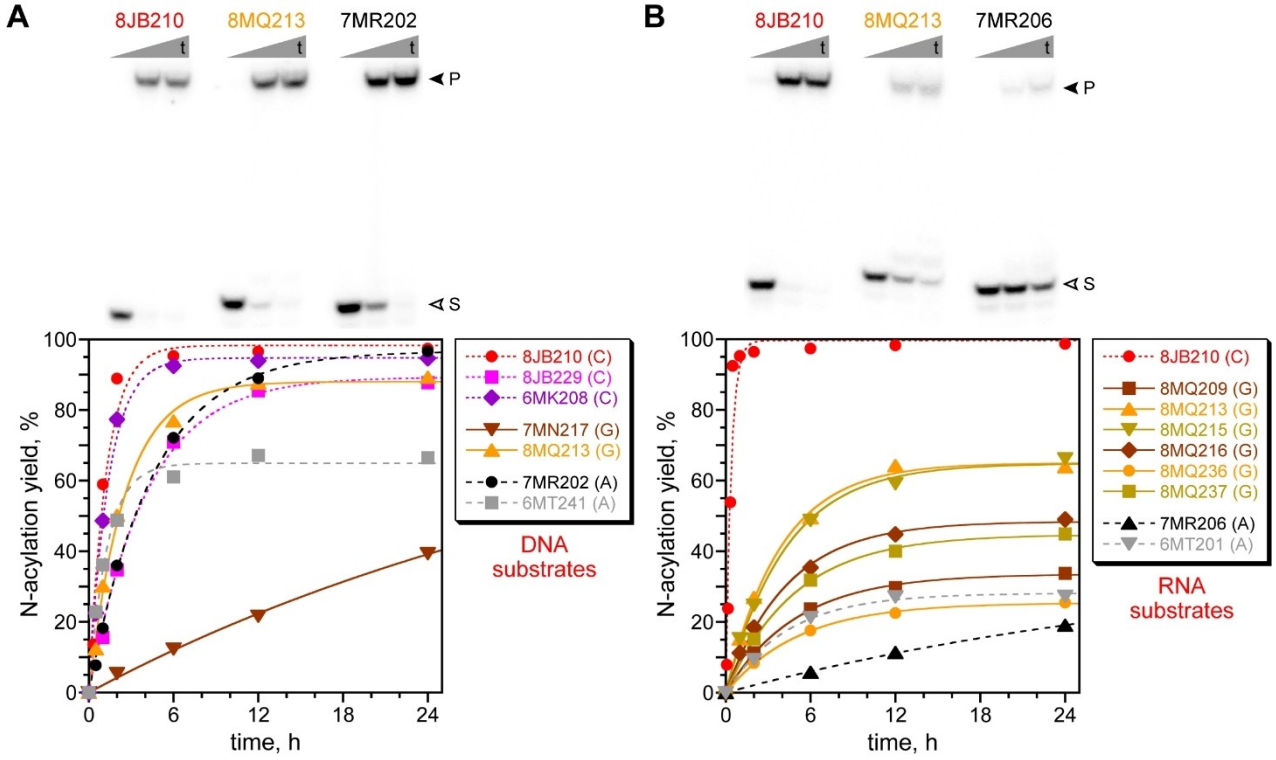

Figure 6. Representative PAGE data ($t = 30$ s, 6 h, 24 h) and single-turnover kinetic plots for nucleobase N-acylation DNAzymes with DNA and RNA oligonucleotide substrates. DNAzymes were incubated under the conditions of their identification, including the relevant divalent metal ions. 8JB210 was incubated under the pH 6.5 conditions used for 6MK208. A) Assays with DNA substrates. k_{obs} values (h^{-1}): 8JB210 0.79 ± 0.04 ($n = 3$), 8JB229 0.25, 6MK208 0.72, 7MN217 0.043, 8MQ213 0.39, 7MR202 0.22, 6MT241 0.77. k_{bkgd} values (Figure S3) were 4–7×10^{-3} h^{-1} (for DNAzymes identified using 2 h selection incubation, including 8JB210) and 5–7×10^{-4} h^{-1} (for DNAzymes identified using 16 h selection incubation). Rate enhancements (k_{obs}/k_{bkgd}): 8JB210 110, 8JB229 35, 6MK208 100, 7MN217 86, 8MQ213 65, 7MR202 300, 6MT241 180. B) Assays with RNA substrates. k_{obs} values (h^{-1}): 8JB210 2.7, 8MQ209 0.21, 8MQ213 0.28, 8MQ215 0.26, 8MQ216 0.24, 8MQ236 0.19, 8MQ237 0.23, 7MR206 0.028, 6MT201 0.29. The first four timepoints for 8JB210 are at 5, 10, 20, and 30 min.

acylation by DNAzymes is feasible, even with DNAzymes originally identified by in vitro selection for DNA nucleobase acylation. The catalytic activities with RNA are likely to be improved by future reselection or new selection experiments that directly use RNA substrates.

Finally, the nucleotide attachment sites within the oligonucleotide substrates were determined. For the nine DNAzymes that use the CCC 3′-overhang DNA substrate, PAGE assay using a substrate that has an extended 3′-overhang CCCTTTTTT ($CCCT_6$) revealed products with three different migration positions (Figure 7A). Three of the DNAzymes (8JB210, 6MK208, and 6MK232) form the slowest-migrating product; five DNAzymes (8JB215, 8JB223, 8JB228, 8JB229, and 6MK205) create the fastest-migrating product; and one DNAzyme (6MK215) forms primarily the fastest-migrating product along with a lesser amount of a product with an intermediate migration position. We provisionally assigned the three products, from slowest (highest) to fastest (lowest) migration position, as arising from N^4-acylation at C + 1, C + 2, and C + 3 of the 3′-overhang (see Figure 2 for numbering; branched nucleic acids migrate more slowly on PAGE than isomeric linear nucleic acids). Of these nine DNAzymes, all but 6MK215 are > 95 % site selective, and 6MK215 has a product ratio of 3:1. Consistent with cytidine N^4-acylation rather than 3′-OH acylation, removing the hydroxyl nucleophile from the 3′-terminus by using the $CCCT_6$ddC substrate did not noticeably decrease the product yields (Figure S6). Unfortunately, analogous PAGE-shift experiments with the GGG and AAA substrates were thwarted by inactivity of the corresponding DNAzymes with $GGGT_6$ and $AAAT_6$, meaning that these other DNAzymes do not tolerate the extended 3′-overhang.

These provisional attachment site assignments for cytidine N^4-acylation were confirmed by base treatment of the 5′-^{32}P-radiolabeled RNA and DNA products. For the 8JB210 N-acylation product formed using the RNA substrate, a single digestion reaction was employed, as is used to characterize 2′-OH branched RNA (Figure 7B).[17] The base conditions, which included high Mg^{2+} concentration at relatively low pH (50 mM Tris, pH 8.0, 200 mM $MgCl_2$, 90 °C, 2 min), were optimized to favor phosphodiester cleavage by 2′-OH transesterification over amide cleavage (acyl hydrolysis), as is necessary to observe the cleavage ladder gap that reveals the nucleotide attachment site. To assess the attachment sites of the cytidine N^4-acylation products formed using all nine DNAzymes and the DNA substrate, we used a set of five individually ribo-substituted

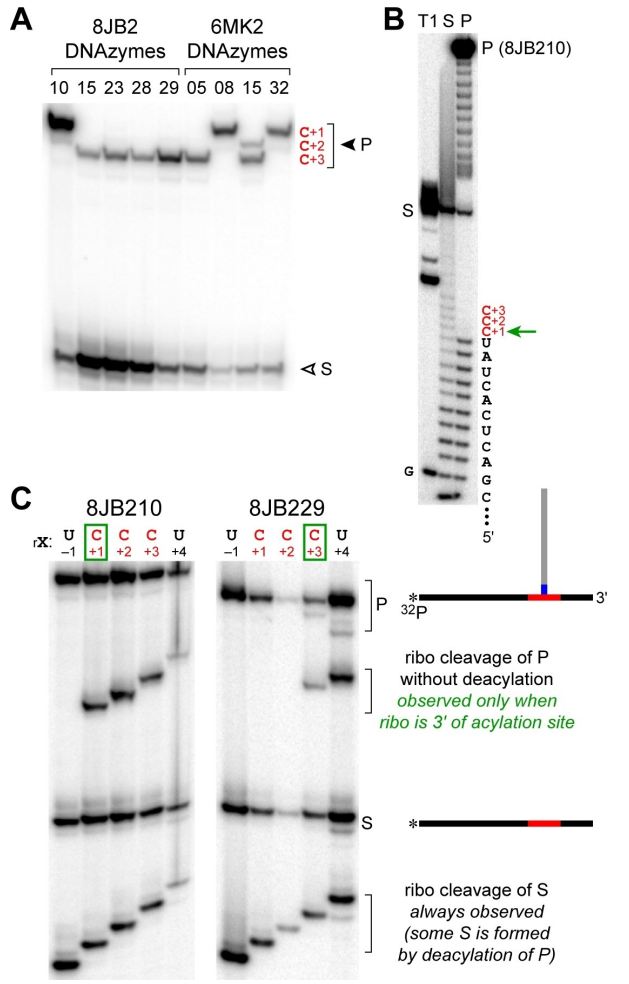

Figure 7. Determining nucleotide attachment points in the cytidine N^4-acylation products. A) Three different product migration positions are observed for the 8JB2 and 6MK2 DNAzyme products made from the $CCCT_6$ 3′-overhang DNA substrate. S = substrate; P = product. These migration positions correlate directly to modification at C + 1, C + 2, or C + 3. B) The 8JB210 product made from an RNA substrate is modified at nucleotide C + 1. The product (P) was prepared from the 5′-^{32}P-radiolabeled RNA substrate with 3′-CCC overhang extended by 11 nt as described in the Supporting Information (S). T1 = RNase T1 digestion (cleavage at G only) of S; S = base treatment of S; P = base treatment of P. (C) Assays with base treatment of five individually ribo-substituted $CCCT_6$ 3′-overhang DNA products, using 8JB210 (N^4-acylation at C + 1) and 8JB229 (N^4-acylation at C + 3) as representative examples. See Figure S8 for additional data. Because partial deacylation of P provides S, all five bands for ribo cleavage of S are observed regardless of the acylation site. In contrast, for any P that has not been deacylated, the ribo cleavage band is observed only when the ribo site is 3′ of the acylation site, which enables the acylation site assignment.

versions of the substrate. The results with base treatment of the products formed from the ribo-substituted DNA substrates for all nine DNAzymes (Figure 7C and Figure S8; optimized conditions of 50 mM CHES, pH 9.0, 600 mM $MgCl_2$, 90 °C, 10 min) confirmed the assignments made on the basis of the $CCCT_6$ product migration analysis (Figure 7A) and, for 8JB210, base treatment of its RNA product (Figure 7B).

Mfold analysis[18] of all 26 of the N-acylation DNAzymes led to numerous predicted secondary structures (Table S4 and Figure S9), for which the large number of experiments necessary to distinguish the possibilities have not been performed. We have not pursued three-dimensional structural information for any of these DNAzymes, considering the challenges associated with obtaining such data.[19]

Our previously reported RNA ligase DNAzymes often have minimal binding arm and 3′-overhang sequence requirements for their oligonucleotide substrates.[20] Also, they often tolerate long 3′-extensions on their substrates, meaning that they can modify these substrates at internal nucleotides rather than only on nucleotides at or near the 3′-end. We anticipate that future comprehensive experiments will reveal the same features for many of the nucleobase N-acylation DNAzymes. For instance, all DNAzymes that N^4-acylate C nucleobases tolerate substrate 3′-extensions (Figure 7).

Conclusion

In summary, using in vitro selection we have expanded DNAzyme catalysis to include N-acylation of C, G, and A nucleobases in DNA oligonucleotide substrates, with yields as high as > 95 % in 6 h. Eight of nine C DNAzymes were site-specific (> 95 %) for one particular nucleotide of the DNA substrate. Several of the new DNAzymes work well with the analogous RNA substrate, even though RNA was not used during their selection process. In future experiments, we intend that directly using RNA substrates and small-molecule acyl donors during in vitro selection will broaden and improve N-acylation DNAzyme catalysis. These efforts will move us further toward our goal of identifying DNAzymes for practical site-specific nucleobase N-acylation of DNA and RNA oligonucleotide substrates.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Deoxyribozymes · Nucleic Acids · Oligonucleotides · Ribozymes · in Vitro Selection

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