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An oligodeoxyribonucleotide that supports catalytic activity in the hammerhead ribozyme domain

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ABSTRACT

A study of the activity of deoxyribonucleotide-substituted analogs of the hammerhead domain of RNA catalysis has led to the design of a 14mer oligomer composed entirely of deoxyribonucleotides that promotes the cleavage of an RNA substrate. Characterization of this reaction with sequence variants and mixed DNA/RNA oligomers shows that, although the all-deoxyribonucleotide oligomer is less efficient in catalysis, the DNA/substrate complex shares many of the properties of the all-RNA hammerhead domain such as multiple turnover kinetics and dependence on Mg^{2+} concentration. On the other hand, the values of kinetic parameters distinguish the DNA oligomer from the all-RNA oligomer. In addition, an analog of the oligomer having a single ribonucleotide in a strongly conserved position of the hammerhead domain is associated with more efficient catalysis than the all-RNA oligomer.

INTRODUCTION

Biological catalysis was once thought to be a property of proteins alone. However, reports in 1982 (1) and 1983 (2) showed that some RNA molecules could also catalyze biological reactions. Given the structural similarity of RNA and DNA, namely the sugar-phosphate backbone and the attached nitrogen bases, it would seem that even though the 2′-hydroxyl group renders some distinctive properties to RNA, DNA might also display catalytic capabilities. The hammerhead domain of RNA catalysis originally discovered by Prody *et al.* (3) and the subsequent development of the *trans* reaction by Uhlenbeck (4) were key elements in our choice of this small domain as a model system for the evaluation of structural and functional differences between deoxy- and ribo-nucleotides in ribozymes. Studies in which deoxyribonucleotides were systematically substituted for ribonucleotides has allowed the determination of essential and important 2′-hydroxyls in this domain. Thus, the 2′-hydroxyl of C17 (Fig. 1A) is an absolute requirement for catalysis (5–7), as expected from its role as the nucleophile in the reaction. The absence of the 2′-hydroxyls at G5, G8 or A9 and A15.1 diminishes, but does not completely eliminate, catalytic activity (8,9). These data allowed us to construct a catalytically active oligomer, termed a nucleozyme, containing 31 deoxyribonucleotides and only four ribonucleotides (10). Therefore, an all-ribonucleotide backbone is not absolutely required for catalysis. We now extend these studies by demonstrating that an oligodeoxyribonucleotide corresponding to a segment of the RNA hammerhead structural domain is capable of supporting the cleavage of a phosphodiester bond in the presence of Mg^{2+}.

Previously, it had been shown that the hammerhead domain could be partitioned between the catalytic and the substrate moieties in different ways (4,11). In Figure 1B, one such partition is shown (12). In this arrangement only two of the influential 2′-hydroxyls, those of G12 (13) and A15.1 (10), are in the fragment defined as the ribozyme. Since it had been shown that these 2′-hydroxyls are not essential for catalysis (14), it seemed plausible that this fragment, made exclusively of deoxyribonucleotides, might also promote transphosphorylation. We therefore synthesized the appropriate molecules shown in Figure 1B and C and tested the ability of this modified domain to cleave the scissile phosphate bond.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

All RNA, mixed and DNA polymers were made using chemical procedures described previously (10). The oligonucleotides were purified on 15% polyacrylamide/7 M urea gels, the product bands were excised and eluted with water. After isolation, the polymers were desalted on Sephadex G-50 (Pharmacia) and lyophilized to dryness.

Radioisotopic labeling

T4 polynucleotide kinase was obtained from New England Biolabs. [γ-^{32}P]ATP (10 mCi/ml) was obtained from NEN Dupont. The substrate was 5′-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. A 5 µl reaction mixture containing

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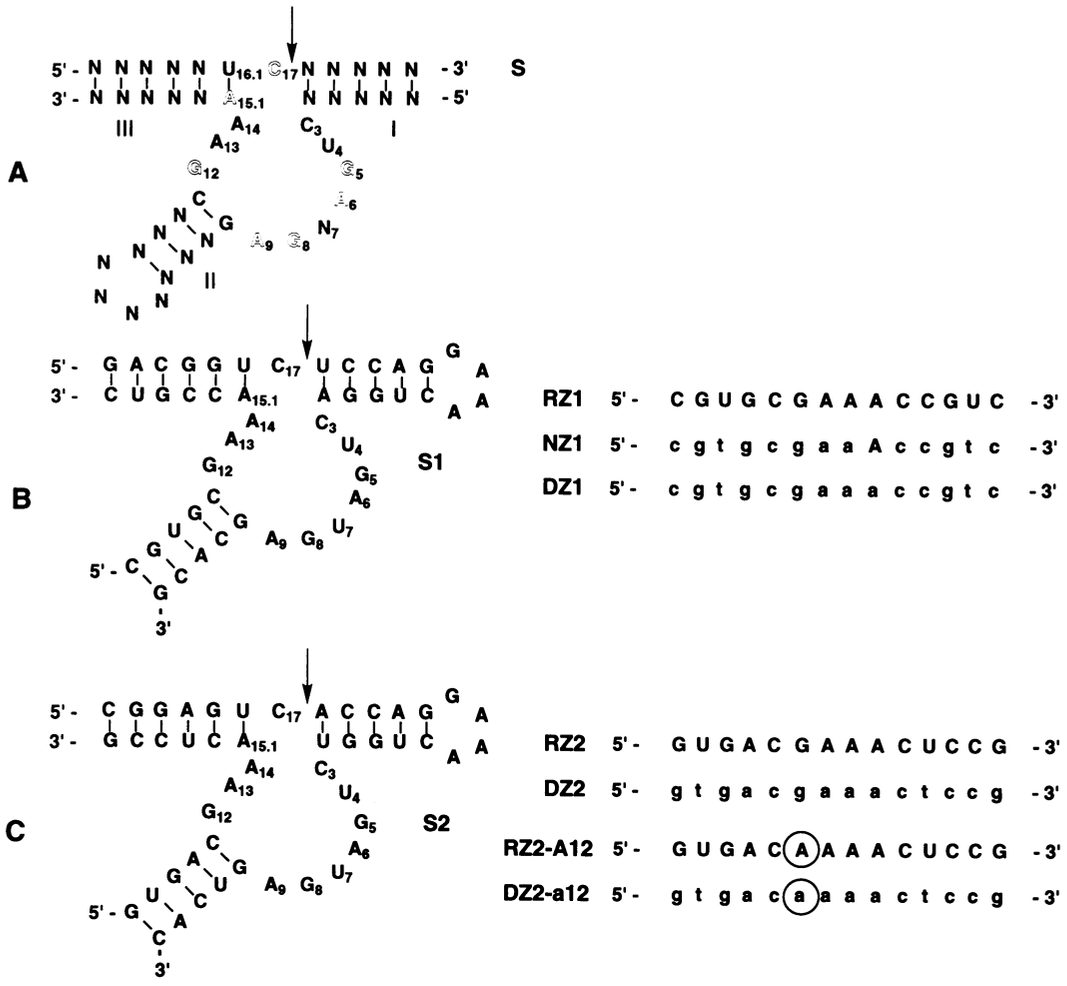

Figure 1. (**A**) The general structure of the RNA hammerhead domain. The arrow indicates the cleavage site in the substrate. Deoxyribonucleotides can be substituted at the positions indicated by black type with little or no effect on catalytic activity. Catalytic activity is more strongly affected if deoxyribonucleotides are substituted at positions in outlined characters. The 2′-hydroxyl of C17 in the substrate (S) is essential to catalysis, whereas the lack of 2′-hydroxyls at other outlined characters only diminishes the activity of the ribozyme. N represents any nucleotide; however those in the base paired regions must be complementary to the nucleotide across the helix. (**B**) An alternate structure of the hammerhead domain created by removal of the loop at the end of Stem II in (A) and construction of a loop to close Stem I. The sequences shown correspond to the S1, RZ1, NZ1 and DZ1 oligonucleotides described in the text. (**C**) An alternate sequence of the structure shown in (B). The sequences shown correspond to the S2, RZ2, DZ2, RZ2-A12 and DZ2-a12 oligonucleotides described in the text. Deoxyribonucleotides are in lower case, ribonucleotides in upper case and the G12→A12 mutation is circled. S, all-RNA substrate, RZ, all-RNA ribozyme, NZ, mixed polymer nucleozyme and DZ, all-DNA deoxyribozyme.

40 pmol of substrate, 50 pmol of ATP, 10 mM $MgCl_2$, 20 mM Tris–HCl, pH 8 and 1 μg of BSA was incubated for 30 min at 37°C. The product was purified on 15% polyacrylamide/7 M urea gel and isolated as described above.

Cleavage reactions

Standard conditions for the cleavage reactions were as follows. In 8 μl of 50 mM Tris–HCl, 1 μM of the substrate and 0.1 or 1 μM of oligonucleotide catalyst were mixed at pH 7.5, heated to 70°C for 90 s and rapidly cooled on ice. The reactions were initiated by adding 2 μl of 50 mM $MgCl_2$ and incubated at 30°C. The reactions were stopped by adding 10 μl of formamide-dye mix and loaded onto a 15% polyacrylamide/7 M urea gel.

Kinetic analysis

Michaelis–Menten kinetic reactions were performed in 10 μl of 50 mM Tris–HCl pH 8.5 at 30°C with 50 mM $MgCl_2$ (optimized conditions). For RZ1 and NZ1, 10 nM of catalyst was used with eight substrate concentrations varying from 0.1 to 10 μM for RZ1 and 0.5 to 50 μM for NZ1. Concentrations of 100 or 500 nM of DZ1 catalyst were used with eight substrate concentrations varying from 0.5 to 200 μM. Aliquots of 1 μl were taken at appropriate times between 0 and 10 min for RZ1 and NZ1 and between 0 and 3 h for DZ1. The cleavage reactions samples were analyzed on 15% polyacrylamide/7 M urea gels and gel slices corresponding to substrate and product positions were excised from the gel and quantified by Cerenkov counting. Kinetic parameters were determined from Eadie–Hofstee plots using the Enzfitter program. All kinetic results are for duplicate observations. For magnesium curves, rate constants were measured using 100

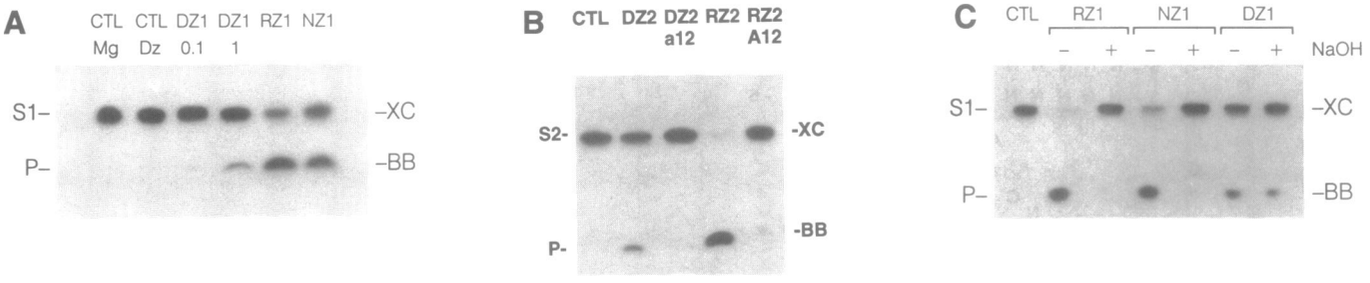

Figure 2. (**A**) Catalytic activity gel. Lanes DZ1, RZ1 and NZ1 represent the cleavage reactions of S1 (1.0 μM) with DZ1, RZ1 and NZ1, respectively, as described in Materials and Methods. In the lane labeled DZ1/0.1, one-tenth or 0.1 μM of DZ1 was used with the same quantity of substrate. Lane CTL/Mg corresponds to the reaction containing S1, Mg^{2+}, but no DZ1, whereas CTL/DZ1 contains S1, DZ1 but no Mg^{2+}. XC, xylene cyanol; BB, bromophenol blue; and P, product. (**B**) Test of A12 variants. The lanes DZ2 to RZ2-A12 represent the cleavage of labeled S2 with DZ2, DZ2-a12, RZ2 and RZ2-A12, respectively, under the same conditions as above except that the concentration of $MgCl_2$ was 25 mM and the pH was 8.5. Lane CTL represents the incubation of labeled S2 in the presence of 25 mM $MgCl_2$ only. (**C**) Catalytic activity after alkaline treatment of catalysts. Incubation of 100 pmol of RZ1, NZ1 and DZ1 was for 90 min at 85°C in 100 μl of 0.1 N NaOH. Lanes labeled RZ, NZ and DZ correspond to the cleavage reactions treated with (+) NaOH and (–) indicates the untreated controls. Lane CTL is labeled S1 incubated with $MgCl_2$.

nM of each catalyst and 50 nM of S1 in 50 mM Tris–HCl at 30°C and pH 7.5 using $MgCl_2$ concentrations varying from 1 to 500 mM.

Alkaline treatment of catalysts

Oligonucleotides (100 pmol) were incubated for 90 min at 85°C in 100 μl of 0.1 N NaOH. The reactions were then neutralized with acetic acid. The resulting solutions were desalted on Sephadex G-50 and one-quarter of the supernatants (corresponding to ~25 pmol of the original oligonucleotides) were evaporated to dryness. The resuspended catalysts were then incubated with 10 pmol of substrate under cleavage conditions described above.

RESULTS AND DISCUSSION

The catalytic activities for the all-RNA construct (RZ1), the predominantly DNA construct containing only one ribonucleotide at the critical position A15.1 (NZ1) as well as the all-DNA construct (DZ1) are shown in Figure 2A. Comparison of the two DZ1 lanes in the figure demonstrates that the yield of cleavage product (P) is dependent on the concentration of the deoxyribozyme. As indicated by the two controls, neither Mg^{2+} nor the oligodeoxyribonucleotide alone are able to support cleavage of the substrate, although Mg^{2+} ion does seem to promote some degradation of the substrate.

To test the sequence generality of the deoxyribozyme activity, a substrate (S2) was designed to be cleaved by its cognate DZ2 (Fig. 1C). The cleavage of S2 by DZ2 is shown in Figure 2B. Analogs of RZ2 and DZ2 containing A12 instead of G12 (RZ2-A12 and DZ2-a12) were inactive (Fig. 2B). Since the GAAA sequence is required for cleavage in the RNA hammerhead domain (15), this result suggests that the role of the catalytic unit in the case of the deoxyribozymes is to take part in the formation of the three-dimensional structure of the hammerhead domain in the same way as RNA.

Activity emanating from an RNA contamination of DZ1 was ruled out by treating RZ1, NZ1 and DZ1 with sodium hydroxide before incubation with the substrate. After 90 min of preincubation with 0.1 N NaOH, neither ribonucleotide-containing catalyst RZ1 nor NZ1 maintain activity higher than the background; only DZ1 is active (Fig. 2C).

The pH optima were determined to be 9 for RZ1 and NZ1 and 8.5 for DZ1. The optimal temperature was 50°C for RZ1 and 15°C for both NZ1 and DZ1. One factor contributing to this difference is likely the relative stability of the enzyme/substrate complexes: the DNA/RNA heteroduplexes present in the case of NZ1 and DZ1 are less stable at a high temperature than the RNA homoduplex present when RZ1 is used (16). This difference in optimal temperature may explain why catalytic activity was not observed in an oligodeoxyribonucleotide used as a control in a prior study on ribozymes (12).

It is well known in the hammerhead, as well as other catalytic RNAs, that divalent metal ions are essential for activity. Therefore, the cation activity spectrum was determined using $MgCl_2$, $MnCl_2$, $BaCl_2$, $CaCl_2$ and $SrCl_2$ at pH 7.5 and 8.0. Figure 3A shows that at pH 7.5, DZ1 is active only with $MgCl_2$ and $MnCl_2$. At pH 8.0 (Fig. 3B), the activity with $MgCl_2$ is higher than that at pH 7.5 whereas the reverse is the case with $MnCl_2$. This observation can be explained by the fact that $MnCl_2$ forms insoluble hydroxides at pH > 7 (17), thereby reducing the concentration of the metal ion. We also observed a slightly increased activity with $CaCl_2$ at the higher pH. These data are compatible with the behavior of the all-RNA hammerhead domain (18).

The catalytic activities of the three enzymes were assayed at different concentrations of Mg^{2+} and these data are shown in Figure 4. The activity versus $MgCl_2$ concentration for DZ1 seems to reach a plateau near 100 mM $MgCl_2$, but thereafter the activity continues to increase showing no saturation until 500 mM $MgCl_2$ is reached. On the contrary, both RZ1 and NZ1 show saturation kinetics at ~50 mM $MgCl_2$.

Reaction kinetics were determined for RZ1, NZ1 and DZ1 and are shown in Table 1. The activity, k_{cat}/K_M, of DZ1 is lower than that of RZ1 by a factor of 340 at 30°C, 50 mM $MgCl_2$ and pH 8.5 (optimized conditions). The difference in activity between RZ1 and DZ1 is due in large part to the higher K_M of DZ1 (a factor of 42), whereas the k_{cat} of DZ1 is only 7-fold less than that of RZ1. The presence of a single ribonucleotide at position 15.1 of NZ1 has a dramatic effect on its activity: the K_M resembles that of RZ1, but surprisingly, the k_{cat} of NZ1 is higher than either that of RZ1 or DZ1 under these conditions. Under single turnover conditions, RZ1 is more active than NZ1 (see Figs 2A and 4), but under multiple turnover conditions, NZ1 is more active. These data can

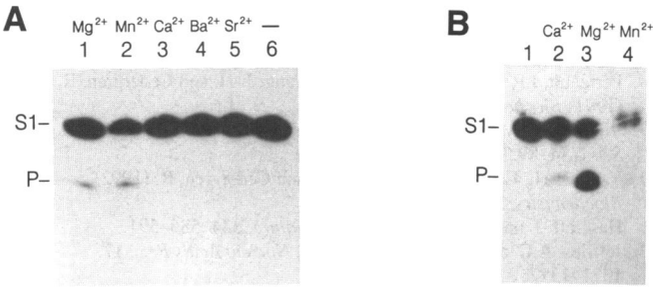

Figure 3. Cleavage activity of the DZ in the presence of divalent metal ions. (**A**) Cleavage at pH 7.5 under the conditions in Materials and Methods using DZ1 and S1 in the presence of 15 mM each of $MgCl_2$, $MnCl_2$, $CaCl_2$, $BaCl_2$ and $SrCl_2$, lanes 1–5, respectively. Lane 6 is a control incubation of S1 and DZ1 in the absence of metal ions. (**B**) Cleavage at pH 8.0 under the conditions of Figure 2A using DZ1 and S1 in the presence of 15 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$, lanes 2–4, respectively. Lane 1 is a control incubation in the absence of metal ions.

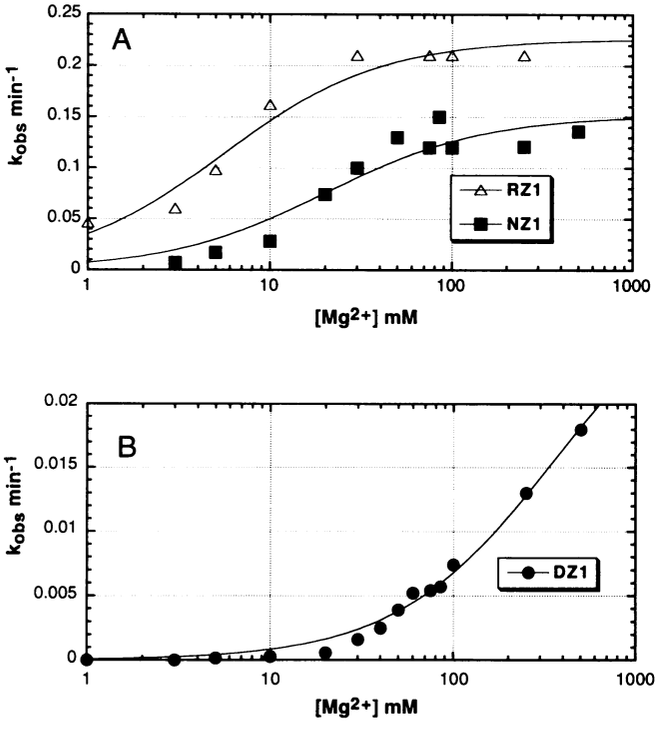

Figure 4. Dependence of the cleavage activity of RZ1, NZ1 and DZ1 on ($MgCl_2$). The rate constant (k_{obs}) was measured using 100 nM of each catalyst and 50 nM of S1 in 50 mM Tris–HCl at 30°C and pH 7.5 using $MgCl_2$ concentrations varying from 1 to 500 mM. (**A**) data from the RZ1 and NZ1; (**B**) data from DZ1. Symbols are: Δ, RZ1; ■, NZ1; and ●, DZ1.

be compared to the 10-fold increased activity observed in hammerhead domains where deoxyribonucleotides are present in the hybridizing arms as in NZ1 and DZ1 (19,20). A 2 h incubation of 50 μM RNA substrate (S1) with 100 nM DZ1 and 50 mM $MgCl_2$ at pH 8.5 yields 40 substrate turnovers (8% cleavage).

Table 1. Kinetic parameters of RZ1, NZ1 and DZ1

Catalyst	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M min^{-1} μM^{-1}
RZ1	3.4	0.5	6.8
NZ1	13	1.4	9.3
DZ1	0.5	21	0.02

Reactions were performed as in Materials and Methods.

Although many enzymes acting on macromolecular substrates have very demanding substrate requirements (21), the partition of the various elements of the hammerhead domain used in this study puts considerable constraints on the substrate structure, thereby raising the question of how many genes code for mRNAs that might be cleaved by a deoxyribozyme. This point is particularly important since the stability of deoxyribonucleotides as well as modified nucleotide-substituted ribozymes should be greater *in vivo* than that of all-ribonucleotide molecules, and thus could be advantageous for nucleic acid-based therapeutics. To investigate this issue, we have systematically searched the Genbank database for the motifs required in a potential substrate. The program used, RNAMOT (22), permits the use of secondary and tertiary structural elements as well as primary sequence in the search routine. Matches in the database required the presence of the sequence 5′-SUH-stem-loop structure-CUGANGA-3′ where S is G or C, H is A, U or C and N is any nucleotide. This motif corresponds to the region starting at GUC17-Helix I and finishing at the end of Helix II in Figure 1B. We found this motif in 3112 genes representing 2.4% of the genes in the Genbank database allowing for a stem-loop structure composed of a duplex of 6 base-pairs and any loop length. Using a duplex of 4 base-pairs 13 392 or 10.2% of the genes have the required pattern.

The work that we report is consistent with the notion of a catalytic activity associated with a oligodeoxyribonucleotide, since it supports substrate turnover, follows Michaelis–Menten kinetics and is not consumed in the reaction as required by definition. However, this definition must be carefully considered since part of the catalytic domain is furnished by the substrate. This case is not unique since there are many similar examples from the hammerhead domain (4,14), Group I (23) and Group II introns (24), where small catalytic units require a part of the substrate for catalysis. Also, a recent paper reporting the *in vitro* selection of a DNA enzyme that cleaves RNA makes use of a lead cofactor, a substrate 2′-hydroxyl nucleophile and a particular substrate sequence for its activity (25). Our report shows that even a known naturally occurring RNA catalytic domain can be an active catalyst with an oligodeoxyribonucleotide fragment. These observations raise issues regarding the significance of terminology when the substrate structure is important in catalysis (26).

The finding of a simple DNA molecule associated with catalytic activity lends itself to speculation on whether catalytic DNA could exist in nature and further, whether deoxyribonucleotides could have been implicated in the emergence of life as in the RNA World Theory. If such a hypothesis were possible, the deoxyribonucleotide oligomer would most likely be single-stranded, since functional groups are less accessible and much conformational flexibility is eliminated in the double-stranded form of DNA.

Concerning the fundamental question that we set out to investigate several years ago on the similarity of single-stranded DNA and RNA structures (27), it may now be said that the

structural differences are minimal, at least in some cases. Therefore the preferences for 2′- or 3′-endo ribose conformations shown in double-stranded forms of DNA and RNA, respectively (28), either do not apply or are insignificant in single-stranded regions of these molecules. A preliminary conclusion based on these data would be that the conformation of a given nucleotide is determined more by its environment than by its sugar component. Alternatively, the identity of the polymer does have a considerable effect on the binding of divalent cations, but without detailed side-by-side structural analysis of a DNA and the corresponding catalytic RNA molecule, it is difficult to interpret the kinetic differences in terms of a particular structure or lack of it.

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