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Ribozyme Inhibitors: Deoxyguanosine and Dideoxyguanosine Are Competitive Inhibitors of Self-Splicing of the *Tetrahymena* Ribosomal Ribonucleic Acid Precursor†

Brenda L. Bass‡ and Thomas R. Cech*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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ABSTRACT: The intervening sequence (IVS) of the *Tetrahymena* rRNA precursor catalyzes its own splicing. During splicing the 3′-hydroxyl of guanosine is ligated to the 5′ terminus of the IVS. One catalytic strategy of the IVS RNA is to specifically bind its guanosine substrate. Deoxyguanosine (dG) and dideoxyguanosine (ddG) are found to be competitive inhibitors of self-splicing. Comparison of the kinetic parameters (K_i = 1.1 mM for dG; K_i = 5.4 mM for ddG; K_m = 0.032 mM for guanosine) indicates that the ribose hydroxyls are necessary for optimal binding of guanosine to the RNA. dG is not a substrate for the reaction even at very high concentrations. Thus, in addition to aiding in binding, the 2′-hydroxyl is necessary for reaction of the 3′-hydroxyl. A second catalytic strategy of the IVS RNA is to enhance the reactivity of specific bonds. For example, the phosphodiester bond at the 3′ splice site is extremely labile to hydrolysis. We find that dG and ddG, as well as 2′-*O*-methylguanosine and 3′-*O*-methylguanosine, reduce hydrolysis at the 3′ splice site. These data are consistent with an RNA structure that brings the 5′ and 3′ splice sites proximal to the guanosine binding site.

The intervening sequence (IVS)[1] that interrupts the rRNA precursor (pre-rRNA) of *Tetrahymena thermophila* is a catalytic RNA molecule, or ribozyme (Kruger et al., 1982; Bass & Cech, 1984). The IVS RNA lowers the activation energy for a number of intramolecular RNA cleavage–ligation reactions, including its own excision from the pre-rRNA by self-splicing and its own cyclization, and it catalyzes the intermolecular cleavage–ligation of oligonucleotide substrates (Zaug & Cech, 1986).

The IVS RNA has many properties of an enzyme even when it acts intramolecularly. First, it is a highly structured molecule, and its activity is dependent on maintenance of its native structure (Cech et al., 1983; Price et al., 1985; Waring et al., 1985; Tanner & Cech, 1985; Burke et al., 1986). Second, the IVS RNA can enhance the reactivity of specific bonds (Zaug et al., 1984; Inoue et al., 1986). Finally, the IVS RNA has a specific interaction with its guanosine substrate (Bass & Cech, 1984). On the basis of kinetic and structure–reactivity data, this interaction has been interpreted as classic Michaelis–Menten binding of a substrate to an active site (Bass & Cech, 1984). Now we demonstrate that deoxyguanosine (dG) and dideoxyguanosine (ddG) are competitive inhibitors of the guanosine substrate, thereby fulfilling a major prediction of the model.

Experimental Procedures

Synthesis of Precursor rRNA. Plasmid pJK43-SP6 (Inoue et al., 1986) was cut with *Eco*RI. Transcription of this linearized template with SP6 RNA polymerase yields a 1340-base pre-rRNA: 303 bases of 5′ exon, 624 bases of 3′ exon, and the 413-base IVS. For each transcription reaction, plasmid DNA (1.5 μg) was mixed with 100 μL of 40 mM Tris-HCl,

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* Author to whom correspondence should be addressed.

‡ Present address: Fred Hutchinson Cancer Center, Seattle, WA 98104.

[1] Abbreviations: IVS, intervening sequence; ddG, 2′,3′-dideoxyguanosine; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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pH 7.5, 6 mM $MgCl_2$, 10 mM dithiothrietol, 4 mM spermidine, 0.5 mM ATP, UTP, GTP, and CTP, 0.25–0.5 mCi of [α-^{32}P]ATP, and 200 units of SP6 RNA polymerase. SP6 RNA polymerase was purified by the procedure of Butler and Chamberlin (1982). The mixture was incubated for 2 h at 30 °C and then stopped by adding 20 μL of 100 mM EDTA and 80 μL of gel sample buffer (0.20 g of urea in 200 μL of 0.025% bromophenol blue, 0.025% xylene cyanol, 0.5% NaDodSO$_4$, and 20% sucrose). The transcription products were separated by preparative gel electrophoresis on a 4% polyacrylamide–8 M urea gel (Cech et al., 1981).

The 1340-base precursor was cut out of the gel and eluted by shaking in 1 mL of 500 mM $NH_4C_2H_3O_2$, pH 6.8–7.0, 0.1 mM EDTA, and 0.1% NaDodSO$_4$ overnight at room temperature. The acrylamide gel was removed by sterile filtration and the supernatant further purified on a Sephadex G-50 column (in 0.01 M Tris, pH 7.5, 0.25 M $NaC_2H_3O_2$, 0.001 M EDTA, and 0.25% NaDodSO$_4$). The column-purified material was ethanol precipitated twice and stored in 0.01 M Tris-HCl, pH 7.5 at −20 °C, until needed for splicing reactions.

In Vitro Splicing of Pre-rRNA. The standard splicing buffer was 30 mM Tris-HCl, pH 7.5, 100 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, and ~100 pM precursor. Except where noted, splicing reactions were performed at 30 °C. Reactions were performed for various times in 10 μL of the standard splicing buffer with varying amounts of guanosine and/or inhibitor. Reactions were stopped by adding 2 μL of 100 mM EDTA and putting the mixture on ice. The products of splicing reactions were separated by electrophoresis on 4% polyacrylamide–8 M urea gels. Following electrophoresis the gel was soaked in running buffer to remove the urea, dried, and autoradiographed.

To quantitate the splicing products, the precursor and IVS bands were cut out of the dried gel and the ^{32}P radioactivity was determined by liquid scintillation counting in a toluene-based fluor. The rate of the splicing reaction, $v = d[IVS]/dt$, was calculated as previously described (Bass & Cech, 1984). All experiments included a $t = 0$ and a $t = \infty$ control (Bass & Cech, 1984).

HPLC Purification of Nucleosides and Nucleotides. Nucleosides and nucleotides were purified by reversed-phase HPLC using an Altex ultrasphere-ODS column. Nucleosides were purified by using a linear gradient from 0 to 20% acetonitrile in water in 60 min at a flow rate of 1 mL/min. Nucleotides were purified with the same procedure except a buffer of 0.1 M triethylammonium acetate, pH 7.2, replaced the water. All compounds, except unmodified guanosine and GMP, were HPLC-purified at least 3 times before use. Guanosine and GMP were used at micromolar concentrations, and contaminants were considered insignificant.

Results

dG and ddG Are Competitive Inhibitors. A number of guanosine analogues containing modifications in their ribose moieties were previously tested for activity as splicing inhibitors (Bass & Cech, 1984). In all cases no inhibition of splicing was observed at 30 °C with a 200–500-fold excess of analogue to guanosine substrate. In the experiments described here, screening for inhibitors was performed under conditions that were modified and considered more permissive for analogue binding and the detection of inhibition. One modification was to use molar ratios of 8000–32 000:1 (inhibitor:substrate), and the second was to perform intial screening at 42 °C. Under these modified conditions inhibition was observed with dG and ddG (Figure 1).[2] Increasing the amount of inhibitor decreased the amount of excised IVS and ligated exons. Deoxyguanosine was a better inhibitor than dideoxyguanosine; 2 mM dG showed significant inhibition, while comparable inhibition was not observed with ddG until a concentration >8 mM (Figure 1). The amount of circular IVS also decreased. This is the expected result since circular IVS is produced by the intramolecular cyclization of the excised linear IVS (Grabowski et al., 1981). 2′-*O*-Methylguanosine and 3′-*O*-methylguanosine did not inhibit IVS excision or exon ligation even at a concentration of 2 mM (8000-fold excess; data not shown). The limited solubility of the methylated compounds precluded testing higher concentrations. (The 5′-phosphorylated versions of these molecules are not commercially available.)

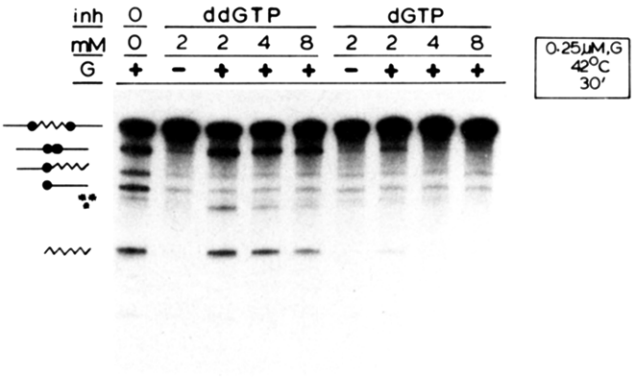

FIGURE 1: dG and ddG inhibit splicing. Pre-rRNA was incubated for 30 min at 42 °C in the standard splicing buffer. (+ lanes) Pre-rRNA incubated with 0.25 μM guanosine substrate in the presence or absence of an inhibitor. The triphosphorylated forms of the inhibitors were used. (− lanes) Incubation with inhibitor but no guanosine substrate; included to ensure that IVS excision did not occur with dGTP and ddGTP. RNA species are indicated by cartoons in which straight lines represent exons, wavy lines represent the IVS, and filled circles represent the splice sites. The first four RNA species are pre-rRNA, ligated exons, 5′ exon–IVS, and 3′ exon. Two circular IVS RNA species, • and **, are byproducts of the reaction (Zaug et al., 1983; Inoue et al., 1986).

Table I: Kinetic Parameters for Splicing in the Presence or Absence of Inhibitors[a]

inhibitor	substrate	K_m or K_m^{app} (μM)	k_{cat} (min^{-1})	K_i (mM)
	G	32 ± 8	0.9 ± 0.1	
ddG	G	44 ● 16	0.8 ± 0.1	5.4 ± 1.9
dGMP	G	92 ± 32	1.0 ± 0.2	1.1 ± 0.4

[a] K_m, K_m^{app} (2 mM inhibitor), and k_{cat} values for pre-rRNA self-splicing at 30 °C in the standard splicing buffer were obtained by a nonlinear least-squares fit to the Michaelis–Menten equation with an analysis program based on the Marquardt algorithm (Marquardt, 1963).

At high inhibitor to substrate ratios, inhibition by dG and ddG could also be detected at 30 °C. The velocity of the splicing reaction (v) was determined at this temperature for a number of different concentrations of guanosine (s) in the presence and absence of dG or ddG. The data were analyzed as described previously (Bass & Cech, 1984) and are presented as v vs. s plots in Figure 2A. The kinetic parameters are listed in Table I.

[2] In accordance with past work (Bass & Cech, 1984) the inhibition was independent of the number of phosphates on the 5′ terminus of the analogue (data not shown). In the text dG and ddG refer to the nucleosides as well as the phosphorylated derivatives. For many experiments the phosphorylated compound was used for its solubility properties. The actual compound used for each experiment is noted in the appropriate figure legend. In all experiments the substrate used was the nucleoside guanosine.

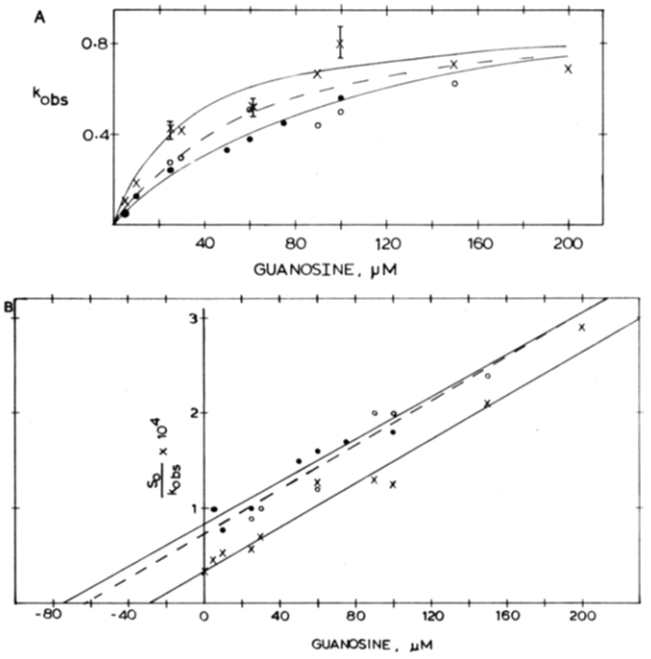

FIGURE 2: dG and ddG are competitive inhibitors. k_{obsd}, which is proportional to splicing velocity, was determined as previously described (Bass & Cech, 1984) for a number of different concentrations of guanosine in the presence or absence of an inhibitor. The data were analyzed graphically by (A) v vs. s plot and (B) s/v vs. s plot (Dixon & Webb, 1979). When two or more time courses were performed for a given substrate concentration, the data were averaged. The error bars in (A) indicate the range of values obtained. The slope of the s/v vs. s plot is inversely proportional to V_{max}, and the x-axis intercept is equal to $-K_m^{app}$. The graphical method in (B) is less accurate for determining kinetic parameters than the nonlinear least-squares fit to the hyperbolic (part A and Table I). However, it was considered useful for illustrating the type of inhibition that occurred. Solid line and ×, no inhibitor present; dashed line and ○, incubation in the presence of 2 mM ddG (nucleoside); solid line and ●, incubation in the presence of 2 mM dGMP.

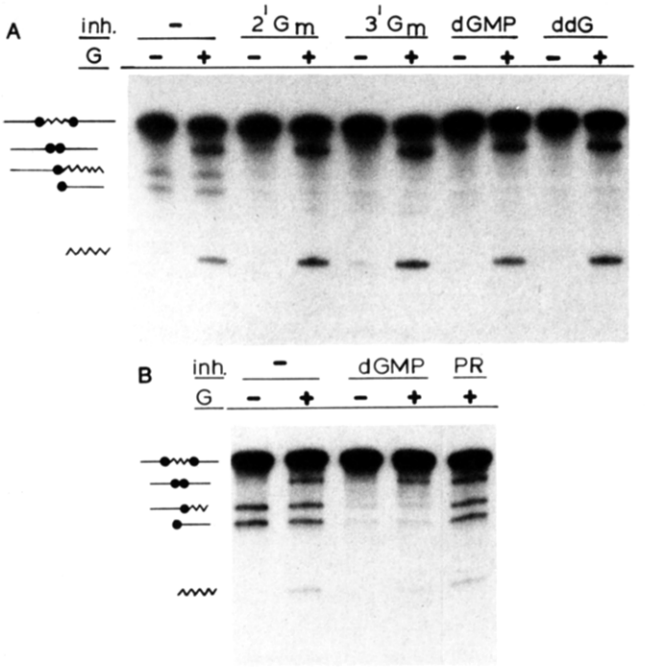

FIGURE 3: Protection of the pre-rRNA from hydrolysis. The splicing reactions were performed as in Figure 1. (A) Analogues used at a concentration of 1 mM. For 2′Gm, 3′Gm, and ddG the nucleoside was used; the deoxy compound used was dGMP. (B) dGMP used at a concentration of 4 mM. Purine ribonucleoside (unphosphorylated) was used at a concentration of 2 mM.

In the presence of dG or ddG splicing velocity (which is proportional to k_{obsd}) decreases. The degree of inhibition is less with higher concentrations of guanosine, suggesting that dG and ddG are inhibiting competitively. The kinetic data in the absence of inhibitor and in the presence of 2 mM dG or ddG are shown in a s/v vs. s plot in Figure 2B. The lines were determined by a least-squares analysis. The lines describing the splicing velocity in the presence of inhibitor are parallel to the line for the data of the uninhibited reaction, indicating that dG and ddG are true competitive inhibitors. They increase the apparent K_m but do not affect the maximum velocity of the reaction.

The Inhibitors Are Not Splicing Substrates. Splicing can be detected with as little as 0.01 μM guanosine (Cech et al., 1981). In our initial study (Bass & Cech, 1984) splicing was not observed at 30 °C when 1 mM dG or 1 mM ddG was substituted for guanosine. The oxygen involved in the covalent linkage to the 5′ end of the IVS is missing in ddG so this compound would not be expected to be a splicing substrate. The absence of splicing with dG was more surprising since the 3′-hydroxyl is present in this molecule. It was considered that splicing might be occurring very inefficiently with dG. To test this possibility, splicing reactions were performed in the presence of 2 mM dG or G under conditions most permissive for the reaction: 42 °C and 10 mM $MgCl_2$. At this temperature cyclization occurs so that three reaction products result: the linear IVS, the circle, and the oligonucleotide of length 15 (15-mer) released during cyclization. These products were observed in the guanosine reaction as expected (Grabowski et al., 1981; Zaug et al., 1983) and after 2 h to a very minor extent in the dG reaction. For both reactions the 15-mer, which has the nucleoside substrate ligated to its 5′ end, was gel-purified, labeled with polynucleotide kinase, and then digested with nuclease P1 to release the nucleoside 5′-monophosphate. The P1 cleavage products were analyzed by thin-layer chromatography (data not shown). A spot corresponding to [^{32}P]GMP was observed in the dG sample, but no [^{32}P]dGMP could be detected. Thus, any IVS excision observed must be due to small amounts of guanosine that remained after HPLC purification. The amount of contaminating guanosine present must be very low (<5 ppm), since splicing with dG is not observed under the conditions used for the inhibitor studies. The results of this experiment confirm that dG is not a splicing substrate under the reaction conditions tested.

Protection of the Pre-rRNA from Hydrolysis. Under normal splicing conditions the phosphodiester bond at the 3′ splice site is labile (Inoue et al., 1986). Incubation of pre-rRNA in splicing conditions yields, in addition to IVS and ligated exons, two hydrolysis products: a 3′ exon molecule and a molecule in which the 5′ exon is collinear with the IVS (5′ exon–IVS). In the course of our inhibitor studies we noticed that the two hydrolysis products were reduced in the presence of dG and ddG (Figures 1 and 3). Surprisingly, 2′Gm and 3′Gm, which do not inhibit splicing, also protected the pre-rRNA from hydrolysis (Figure 3A). Purine ribonucleoside was also tested (Figure 3B). This nucleoside lacks the functional groups on the six-membered ring that have been shown to be required for binding of guanosine to the ribozyme (Bass & Cech, 1984). As expected, this molecule does not inhibit splicing nor does it reduce hydrolysis at the 3′ splice site. The lack of inhibition and 3′ splice site hydrolysis with purine ribonucleoside confirm that dG and ddG are interacting specifically with the guanosine binding site and that inhibition is not due to a nonspecific effect of high concentrations of a nucleoside. Hydrolysis at the 3′ splice site is also reduced by

saturating concentrations of guanosine (data not shown). However, this result is difficult to interpret. At high concentrations of guanosine, pre-rRNA is converted to IVS very quickly; the decrease in hydrolysis products may be due to a depletion of pre-rRNA rather than a protection of the hydrolysis site by guanosine binding.

Discussion

A number of self-splicing RNAs (Kruger et al., 1982; Garriga & Lambowitz, 1984; Van der Horst & Tabak, 1985; Peebles et al., 1986; Van der Veen et al., 1986; Chu et al., 1986) and RNA enzymes (Guerrier-Takada et al., 1983; Zaug & Cech, 1986) have now been discovered, but little is known about the way these molecules interact with their substrates. It has been suggested that, when the substrate is a nucleic acid, Watson–Crick base-pairing interactions are utilized (Altman, 1984; Cech & Bass, 1986). In support of this idea we have previously shown that the functional groups on the six-membered ring of guanosine are necessary for optimal binding of the guanosine substrate to the ribozyme (Bass & Cech, 1984). The competitive inhibitor studies reported here indicate that, in addition, interactions with the 2′- and 3′-hydroxyls of the guanosine are important for binding. This result provides the first experimental evidence that catalytic RNAs utilize interactions in addition to Watson–Crick base pairing for binding their substrates.

Specifically, we have shown that dG and ddG are competitive inhibitors of the *Tetrahymena* pre-rRNA splicing reaction. The K_i of dG is 1.1 ± 0.4 mM and of ddG is 5.4 ± 1.9 mM. In contrast to a K_m value, a K_i value is a true dissociation constant (K_d) and can be used to calculate the free energy of inhibitor binding, $\Delta G = -RT \ln (1/K_d)$. Thus, the free energy for dG binding is −4.1 ± 1.5 kcal/mol and for ddG binding is −3.1 ± 1.1 kcal/mol. The Michaelis constant, defined as $K_m = (k_{-1} + k_2)/k_1$, is equal to K_d if $k_2 \ll k_{-1}$. On the basis of this assumption, the free energy for guanosine binding is −6.2 kcal/mol. Thus the difference in the binding free energy ($\Delta\Delta G$) between dG and G is 2.1 kcal/mol and between ddG and G is 3.1 kcal/mol. These differences can be interpreted in terms of hydrogen bonding. The energy contribution of a hydrogen bond (the difference in energy between a hydrogen bond to RNA and a hydrogen bond to solvent) can vary considerably depending on factors such as the length and linearity of the bond. An average value is around −1 kcal/mol (Freier et al., 1986; Fersht et al., 1985). The free energy differences we observe are consistent with the idea that a single hydrogen-bonding interaction is lost when guanosine is replaced by dG and two are lost when it is replaced by ddG. Alternatively, or in addition, other interactions such as van der Waals interactions could contribute to the free energy differences. Independent of the particular mechanism, it is clear that interactions with the 2′- and 3′-hydroxyls of the ribose sugar are important for binding. The interactions with the 2′-hydroxyl contribute about twice as much as those with the 3′-hydroxyl.

The analogues inosine and 2-aminopurine ribonucleoside are both splicing substrates (Bass & Cech, 1984). The high K_m values for these compounds relative to the guanosine substrate have been explained by a loss of two hydrogen bonds for each compound. The K_i value for dG indicates it has a higher affinity for the binding site than either of the two analogues. However, unlike inosine and 2-aminopurine ribonucleoside, dG is not a splicing substrate. This result indicates that the 2′-hydroxyl of the guanosine substrate, in addition to aiding in the binding to RNA, is in some way necessary for the reaction to occur. One possibility is that the 2′-hydroxyl acts as a general base and facilitates the deprotonation of the 3′-hydroxyl.

In many RNA cleavage reactions 2′-hydroxyls are involved in the formation of a cyclic 2′,3′-phosphate intermediate. These reactions yield products with 5′-hydroxyls and 2′- and 3′-phosphates. The products of *Tetrahymena* pre-rRNA splicing and the related reactions have 5′-phosphates and 3′-hydroxyls. Thus, it seems unlikely that the 2′-hydroxyl of guanosine is involved in the formation of a cyclic phosphate intermediate.

The kinetics of the splicing reaction in the absence of inhibitors has been previously determined for a 6400-base precursor synthesized in vitro in isolated *Tetrahymena* nuclei (Bass & Cech, 1984). For the purpose of this analysis, those experiments were repeated with a 1340-base precursor generated by SP6 RNA polymerase. While the K_m is within the stated uncertainty of the previously published value of 21 μM, the new k_{cat} value is almost 2-fold higher. The increase in the catalytic rate constant may be due to the different sizes of the precursors used in the two studies or to the different RNA isolation procedures employed.

Guanosine becomes covalently ligated to the 5′ end of the IVS, and thus the 5′ splice site must be near the guanosine binding site. Protection from 3′ splice site hydrolysis by dG and ddG indicates the 3′ splice site is also near the guanosine binding site and is consistent with a ribozyme structure that facilitates ligation by bringing the 5′ and 3′ splice sites together. In the absence of a nucleoside that can bind to the ribozyme, the ribozyme active site is presumably occupied by water. The hydrogen bonds on the RNA that interact with substrate are instead interacting with water. The reduction in 3′ splice site hydrolysis by splicing inhibitors is consistent with the simultaneous binding of the inhibitor and the displacement of water.

It was surprising to find that the 3′ splice site was protected from hydrolysis by 2′Gm and 3′Gm, which did not have detectable activity as competitive inhibitors. This result can perhaps be explained as follows. The binding of G, dG, ddG, and water to the ribozyme active site is mutually exclusive; binding of any of these four molecules eliminates the possibility of a second one binding until the first one leaves. At a concentration of 2 mM the methylated compounds bind too poorly to compete with guanosine binding. However, they do bind well enough to compete with the binding of water, and hydrolysis is reduced. In accordance with this interpretation we would predict that, if higher concentrations of the methylated compounds could be attained, splicing would be inhibited.

It is not surprising that 2′Gm and 3′Gm have such a low affinity for the binding site. A great deal of steric hindrance would occur when a methyl group was forced into a site meant to accommodate a hydrogen atom. It has been pointed out that two of the most important components of biological specificity are the avoidance of unsolvated charges and unfavorable steric interactions between enzyme and substrate (Fersht, 1984).

In summary, dG and ddG are true competitive inhibitors of the *Tetrahymena* splicing reaction. The work substantiates the existence of a guanosine binding site and indicates that the ribose hydroxyls are important for binding. In addition to aiding in binding, the 2′-hydroxyl is also necessary for reaction of the 3′-hydroxyl. This work demonstrates that ribozymes are amenable to the same methods of analysis as protein enzymes.

Registry No. dG, 961-07-9; ddG, 85326-06-3; 2′-*O*-methylguanosine, 2140-71-8; 3′-*O*-methylguanosine, 10300-27-3.

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The *Tetrahymena* Intervening Sequence Ribonucleic Acid Enzyme Is a Phosphotransferase and an Acid Phosphatase†

Arthur J. Zaug and Thomas R. Cech*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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ABSTRACT: A shortened form of the *Tetrahymena* intervening sequence (IVS) RNA acts as an enzyme, catalyzing nucleotidyl transfer and hydrolysis reactions with oligo(cytidylic acid) substrates [Zaug, A. J., & Cech, T. R. (1986) *Science (Washington, D.C.) 231*, 470–475]. These reactions involve phosphodiester substrates. We now show that the same enzyme has activity toward phosphate monoesters. The 3′-phosphate of C_5p or C_6p is transferred to the 3′-terminal guanosine of the enzyme. The pH dependence of the reaction (optimum at pH 5) indicates that the enzyme has activity toward the dianion and much greater activity toward the monoanion form of the 3′-phosphate of the substrate. Phosphorylation of the enzyme is reversible by C_5-OH and other oligo(pyrimidines) such as UCU-OH. Thus, the RNA enzyme acts as a phosphotransferase, transferring the 3′-terminal phosphate of C_5p to UCU-OH with multiple turnover. At pH 4 and 5, the phosphoenzyme undergoes slow hydrolysis to yield inorganic phosphate. Thus, the enzyme has acid phosphatase activity. The RNA enzyme dephosphorylates oligonucleotide substrates with high sequence specificity, which distinguishes it from known protein enzymes.

RNA self-splicing exemplifies intramolecular catalysis in that specific cleavage–ligation reactions are accelerated many orders of magnitude beyond the basal chemical rate (Bass & Cech, 1984; Zaug et al., 1985). It has been useful to view the splice sites and cyclization sites as intramolecular substrates for an activity residing within the intervening sequence (IVS)[1] RNA (Zaug et al., 1984). This view was recently substantiated by the finding that the L – 19 IVS RNA, which is missing the 19 nucleotides that contain the cyclization sites, still retains RNA cleavage–ligation activity. When provided with oligonucleotide substrates, the L – 19 IVS RNA acts as an enzyme with nucleotidyltransferase [poly(C) polymerase] and phosphodiesterase (ribonuclease) activities (Zaug & Cech, 1986).

These two enzymatic activities of the L – 19 IVS RNA were predicted from the self-reactions of the IVS. That is, the nucleotidyltransferase activity employs the same reactions involved in IVS RNA cyclization and exon ligation, while the ribonuclease reaction is closely related to site-specific hydrolysis of the circular IVS RNA (Zaug & Cech, 1986). We now describe two additional activities of the L – 19 IVS RNA. It is a phosphotransferase with specificity for the 3′-terminal phosphate of oligo(cytidylic acid) and other oligo(pyrimidines). The phosphate is readily transferred to an oligo(pyrimidine) acceptor. At acidic pH, it is slowly transferred to water. Thus, the RNA has acid phosphatase activity.

Unlike the poly(C) polymerase and ribonuclease activities, the phosphotransferase activities were not anticipated. There are no 3′-phosphates generated during pre-rRNA self-splicing, so the requisite substrate is not even present during the biologically relevant reaction. Transphosphorylation is distinct from other L – 19 IVS RNA catalyzed reactions in that the substrate is a phosphate monoester rather than a diester. Thus, this finding extends the realm of RNA-catalyzed reactions.

Experimental Procedures

Enzyme Preparation. L – 19 IVS RNA was synthesized and purified essentially as described by Zaug and Cech (1986). In brief, RNA was transcribed from pSPTT1A3 with bacteriophage SP6 RNA polymerase in vitro and further incubated to promote self-splicing and cyclization of the IVS RNA. The RNA was subsequently incubated in $MgCl_2$ at pH 9.0 (site-specific hydrolysis conditions) to convert circular IVS RNA to L – 19 IVS RNA. The L – 19 IVS RNA was purified by polyacrylamide gel electrophoresis and Sephadex G-50 chromatography. Enzyme concentration was determined by spectrophotometry assuming a molar extinction coefficient ϵ_{260nm} of 3.26×10^6 M^{-1} cm^{-1}.

Preparation of Substrates. C_5p^*Cp and A_6p^*Cp were prepared from C_5-OH and A_6-OH, respectively, with T_4 RNA ligase (New England Nuclear), p*Cp, and ATP. Products were purified by 20% polyacrylamide–7 M urea gel electrophoresis and Sephadex G-25 chromatography. C_5p^* was prepared from C_5p^*Cp by treatment with calf intestinal phosphatase and β-elimination (Winter & Brownlee, 1978). Unlabeled C_5p was prepared in a similar manner with unlabeled pCp as donor in the ligase reaction. Concentration was determined by spectrophotometry using a molar extinction coefficient ϵ_{270nm} of 30×10^3 M^{-1} cm^{-1}.

Preparation of E-p.* Unlabeled L – 19 IVS RNA (16 pmol) was incubated with 5.2 pmol of C_5p^* in 50 mM NaOAc, pH 5.0, and 20 mM $MgCl_2$ at 42 °C for 10 min. The reaction was stopped by the addition of EDTA to 40 mM. The E-p* was purified from unreacted C_5p^* by column chromatography on Sephadex G-100–120, which was equilibrated in 0.01 M Tris-HCl, pH 7.5, 0.25 M NaCl, and 0.001 M EDTA. The

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* Author to whom correspondence should be addressed.

[1] Abbreviations: IVS, intervening sequence; L – 19 IVS RNA (read "L minus 19"), a 395-nucleotide linear RNA missing the first 19 nucleotides of the IVS; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; p*, ^{32}p within an oligonucleotide (for example, C_5p^*Cp is CpCpCpCpC[^{32}P]-pCp).

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